THE EFFECTS OF GAMMA RADIATION on DNA

A Thesis Submitted for the Degree of DOCTOR OF PHILOSOPHY in The Department of Chemistry of the University of Leicester

Marion Carol Sweeney 1986
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**************************
ABBREVIATIONS

A  adenine
AMPS ammonium persulphate
AP apurinic (apyrimidinic)
ATP adenosine triphosphate
bp base pairs
BSA bovine serum albumin
C  cytosine
Ci  curies
dATP deoxy-adenosine-5'-triphosphate
dCTP deoxy-cytidine-5'-triphosphate
dGTP deoxy-guanosine-5'-triphosphate
dTTP deoxy-thymidine-5'-triphosphate
ddATP 2',3'-dideoxy-adenosine-5'-triphosphate
ddCTP 2',3'-dideoxy-cytidine-5'-triphosphate
ddGTP 2',3'-dideoxy-guanosine-5'-triphosphate
ddTTP 2',3'-dideoxy-thymidine-5'-triphosphate
DMF dimethylformamide
DNA deoxyribonucleic acid
DNAse deoxyribonuclease
DSB double strand break
DTT dithiothreitol
EDTA ethylene diamine tetraacetate
EtBr ethidium bromide
G  guanine
IPTG isopropyl B-D-thiogalactoside
KF Klenow fragment of DNA polymerase I
K-MES potassium 2-(N-morpholino) ethane sulphonic acid
KP potassium phosphate buffer
MCE mercaptoethylamine
O.D. optical density at x nm
P  phosphate group
PCA perchloric acid
PEG polyethylene glycol
PG phosphoglycolate group
psi pounds per square inch
RNA ribonucleic acid
RNAse ribonuclease
rpm revolutions per minute
SDS  sodium dodecyl sulphate
SSB  single strand break
T    thymine
TBE  tris borate EDTA buffer
TE   tris EDTA buffer
TEMED  N,N,N',N'-tetraethylenediamine
TFB  transformation buffer
TM   tris HCl buffer containing magnesium
Tris tris (hydroxymethyl)-aminomethane
U    uracil
UV   ultraviolet
X-gal 5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside
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CHAPTER

one
CHAPTER 1

INTRODUCTION

1.1 Mechanisms of Ionising Radiation Damage to DNA.

Gamma rays are at the high frequency end of the electromagnetic spectrum, and have a wavelength of between $10 \times 10^{-14}$ and $13 \times 10^{-14}$ metres. The wavelength range overlaps that of X-rays. (Between $7 \times 10^{-14}$ and $11 \times 10^{-14}$ metres.) Both gamma and X-rays are types of ionising radiation. Unlike visible light of a specific wavelength or ultraviolet light, ionising radiation is not selectively absorbed by certain types of molecules. The effects upon matter arise as the result of interaction of fast charged particles with atomic electrons. A stream of high energy photons passes through the molecule, allowing individual photons to knock atomic electrons out of their orbitals. Much of the kinetic energy from the photons is transferred to the ejected electrons, which in turn lose kinetic energy by interacting with other electrons in atomic orbitals. This usually transfers enough energy to ionise the molecule, thus forming radical species. These primary ionisation events occur very rapidly: within $10^{-6}$ to $10^{-15}$ seconds after the target has been 'hit'. Secondary ionisations will arise if the newly ejected electron has sufficient energy. Thus in aqueous solutions (and tissues), radiolysis products are not evenly distributed, but are formed along the track of the ionising particle. These clusters of ionisation events are termed
spurs and blobs.

Primary ionisations do not necessarily lead to detectable damage, since damaged molecules or structures may be repaired or replaced. The primary lethal target for ionising radiation within bacterial and mammalian cells has always been thought to be DNA. However, the importance of other cellular components, particularly as potential radioprotectors or radiosensitisers of DNA cannot be ignored. The energy deposited in any cellular molecular species is roughly proportional to the fraction of that species by mass, hence DNA is implicated as the primary lethal target partly due to its large molecular size. In addition, it is well known that maintenance of the integrity of the genome is of fundamental importance to the cell, due to the uniqueness of the information it contains. In the review 'DNA Damage and Cell Killing' (Elkind 1985) several pieces of experimental evidence are cited which support the hypothesis that DNA is indeed the primary lethal target. These include the observation that many DNA repair mutants in E.coli have an enhanced sensitivity to ionising radiation. In mammalian cells, studies of radiation-induced cell killing have shown that specific DNA binding agents such as the intercalator dactinomycin, added either pre- or post-irradiation enhance cell killing.

In aqueous solutions, most of the DNA damage is due to indirect effects resulting from the radiolysis of water. (Ward, 1985) Fast electrons generated in irradiated water lead to $H_2O^+$ ion formation. These decompose very fast (within around $10^{-14}$ seconds), to generate $H_3O^+$ and $OH^-$. As stated previously, clusters of ionisations and excitations
are produced along the track of a fast particle; resulting in a relatively high local concentration of $H_2O^+$ and $OH^-$. This allows the formation of $H_2O_2$ and $H_2$ by the following reactions:

\[
OH^- + OH^- \rightarrow H_2O_2 \\
e^- + H_3O^+ \rightarrow H^+ + H_2O \\
2e^- + 2H_2O \rightarrow H_2 + 2OH^- \\
e^- + H^+ + H_2O \rightarrow H_2 + OH^- \\
H^+ + H^- \rightarrow H_2
\]

In addition, if oxygen is present:

\[
O_2 + H^+ \rightarrow HO_2^- \\
O_2 + e^- + H_2O \rightarrow 'O_2^-
\]

The evidence suggests that $OH^-$ radicals are the most important radiolysis product in terms of causing DNA damage. For instance, addition of hydroxyl radical scavengers during irradiation of cells gives significant protection against strand breakage and cell death. Furthermore, there is a correlation between the level of radioprotection achieved and the efficiency of the $OH^-$ scavenger. However, there is some evidence that $H^-$ radicals are also effective in causing DNA damage under certain conditions (such as anoxia). (Nabben et al, 1984)

In samples irradiated in frozen aqueous solution or dry as films, DNA damage due to diffusing radicals is largely prevented (see below), and most damage is the result of radicals formed on the DNA molecule itself. This is often referred to as the 'direct' damage mechanism. One of the most
straightforward techniques used to study the initial radicals formed under direct irradiation conditions is electron spin resonance (ESR) spectroscopy. This detects paramagnetic molecules, that is, molecules having one or more unpaired electrons. In ESR spectroscopy, the sample is subjected to a magnetic field, which causes an alignment of the unpaired electrons. A microwave field is then applied, which causes 'resonance' of the electrons, that is, a flipping of the spin between two energy levels. The resonance of different radicals gives rise to characteristic spectra. The primary radicals are stabilised by drying the sample or freezing it at a very low temperature. This is conveniently obtained by suspending in liquid nitrogen. (77 Kelvin) During freezing, ice excludes the solute molecules which form aggregates of very high local concentration between the ice crystals. This leads to the formation of a well organised microcrystalline structure, which in DNA is aided by base pairing and vertical base stacking. Base stacking is the interaction of the polar bonds of one base with the polarisable ring of an adjacent base. These interactions allow intermolecular charge migration to occur along the axis of the DNA molecule. Frozen DNA solutions are therefore phase separated, and although hydroxyl radicals are formed in the ice phase, they recombine to form stable species before interaction with the DNA is possible. The hydration shell around the DNA molecule remains in the DNA phase, so that some interaction between radicals formed in the hydration layer and DNA is possible. One consequence of phase separation is the much greater dose required to produce the same level of damage in DNA irradiated frozen at 77K as in

-4-
DNA irradiated in aqueous solution at RT. This is due to the much smaller effective target size at 77K. The magnitude of this effect has been determined by irradiating supercoiled plasmid DNA with a dose of 10KRad, at a range of temperatures between 77K and RT then quantifying the conversion to nicked molecules by gel electrophoresis. (Boon et al, 1984) The results demonstrate a gradual increase in the number of strand breaks on increasing the temperature up to the softening point of ice, where a dramatic rise in the number of strand breaks occurs due to the onset of OH⁻ attack. This is illustrated in figure 1.0. It is very difficult to remove all of the hydration water by drying the DNA sample, therefore interaction with the hydration water is still possible. This is borne out by the observation that the same dose of radiation on dry and frozen samples gives rise to approximately the same level of DNA damage, suggesting that their target sizes are the same. (See Chapters 5 & 6)

Several studies have been carried out to determine the ESR spectra of dry DNA and DNA in frozen solution. (Grasslund, 1971; and Gregoli 1971) The major findings are summarised as follows. Spectra consistent with a guanine cation, G⁺, and a thymine anion, T⁻ are always seen in approximately equal yield. This is interpreted to be the result of positive hole and electron migration along the DNA molecule, which finally becomes localised at guanine and thymine. The reason for trapping out at these particular bases is not clear, particularly since cytosine has a higher electron affinity than thymine. To determine the ESR spectrum of DNA in frozen solution, concentrated DNA solutions (typically 50mg/ml) are irradiated at 77K. The initial spectrum is obtained at 77K.
FIG 1.0  Temperature/Phase Effect

Dose: ca 10 K rad
Conc: 80 µg/ml.
Atmos: Ambient

% Plasmid in Relaxed Form

Temp. K

Frozen Phase Separated Fluid
then the solution is allowed to slowly warm up. ESR reveals features due to OH· radicals formed in the water phase, in addition to DNA radicals. On warming, OH· radicals are lost with no alteration to the DNA radical signals, indicating that the OH· radicals are phase separated. (Boon et al, 1984, 1985; Cullis et al, 1985.) The OH· radicals are lost between 110K and 130K, leaving equal yields of ·G+ and ·T-.

In the absence of oxygen, ·T- becomes protonated to form 'TH, whereas ·G+ apparently undergoes deprotonation at the 3'-N position to form GN'. (Hüttermann et al, in press.) In the presence of oxygen, ·T- and ·G+ become oxygenated, ·T- to \( \text{TH-O}_2^- \) and ·G+ to \( \text{RO}_2^- \), where R is a neutral adduct of G. The primary radical products are illustrated in figure 1.1. The radical composition was found to be unaffected by using DNA with different AT:GC ratios, and essentially the same pattern was found for denatured DNA. (Gregoli et al, 1979)

The subsequent steps leading to the formation of stable damage products are not fully understood, since the intermediate products cannot be detected. One way to probe these pathways is to irradiate DNA in the presence of additives likely to alter radical yields. Any change in radical yield as detected by ESR spectroscopy can then be compared with the alteration in radiation sensitivity, which can be measured by a variety of techniques.
Figure 1.1
Structures of the DNA bases and the primary base radicals resulting from irradiation under direct conditions. (a) adenine, (b) cytosine, (c) guanine, (d) thymine, (e) 'G', (f) 'T-', (g) TH'.
1.2 Stable DNA Damage Products.

Primary and secondary ionisations lead to irreversible changes in molecular conformation by about $10^{-12}$ seconds after the initial ionisation. At this point, chemical reactions occur to 'neutralise' the radical species, forming stable damage products. Free radical reactions are largely complete, and stable damage products formed by between 0.001 and 1.0 seconds (at room temperature). These have been extensively studied and reviewed in detail. (Ward, 1981; Hutchinson, 1985; and in 'Effects of Ionising Radiation on DNA' Eds. Hütterman et al, 1978). The types of lesions observed are double and single strand breaks, base removal, base alteration, cross-linkage between DNA strands and denaturation. If proteins are present, then DNA-protein cross-links can occur.

Strand breaks are the most widely studied DNA lesion. There are a variety of techniques available for quantifying both single and double strand breaks. The most widely used are sedimentation through sucrose gradients, filter elution (Kohn, 1981) and gel electrophoresis. These methods and others are described in detail in 'DNA Repair- a Laboratory Manual of Research Procedures' Eds. Friedberg & Hanawalt (1981), see also Chapter 3. Sedimentation through sucrose gradients and filter elution are techniques suitable for the analysis of very large DNA molecules, for instance total DNA of irradiated cells. Gel electrophoresis is the method of choice for analysis of strand breaks in small circular DNA molecules such as plasmids and bacteriophages. This method is described
in greater detail in Chapter 2, section 1.2. Alkali treatment of irradiated DNA reveals the presence of alkali-labile sites or 'latent' strand breaks; these are various types of base and sugar damage, which are not only alkali-labile, but may slowly convert to strand breaks over several hours post irradiation (at RT). Thus quantifying strand breaks under alkaline conditions gives a value which includes base and sugar damage as well as double and single strand breaks.

It has been suggested that strand breaks are one of the most serious lesions in terms of impairing DNA function. Strand breaks have been reported to lead to loss of transcriptional activity in vitro (Hagen et al, 1970) and loss of transforming ability, (Thorsett & Hutchinson, 1981). However, it has been shown by many workers that strand breaks are rapidly repaired by both bacterial and mammalian cells. (eg. McGrath & Williams, 1966 and Hutchinson, 1979 in Proceedings of the 6th. Congress of Radiation Research, Eds. Okada et al.) See also section 1.4.

The study of altered sugars and bases has been hampered due to the large number of products, each one present in only a very a small amount. The following paragraphs describe the major radiolysis products of deoxyribose sugars and bases, arising from irradiation under conditions where oxygen is present. A different spectrum of products is formed in anoxic conditions, but these are not considered here due to the limited relevance of such studies to the in vivo situation. Much of the work on the characterisation of stable radiation products has been carried out under indirect irradiation conditions, partly for traditional reasons, and partly due to the greater complexity of the radiolysis products and the
Figure 1.2
Sugar products from DNA irradiated in dilute oxygenated solution. Structures I to V are those produced by abstraction of an H atom from one of the five O atoms, followed by the addition of O$_2$. All except IV have been identified chemically. Structures XII, XIV and XVII are apurinic/apyrimidinic sites, which are alkali labile as shown. (From Hutchinson, 1985)
very low yields of the products under direct irradiation conditions.

In aqueous solution, hydroxyl radicals attack deoxyribose sugars, by way of hydrogen atom abstraction. One way of studying the radiolysis products of sugars is the reduction of irradiated sugars with sodium borate followed by reaction with trimethyl silane. This gives rise to ethers which are volatile and can be identified by gas chromatography. Hydrogen atom abstraction occurs primarily at the C-4' and C-5' positions. (Figure 1.2, IV and V) Both of these products can give rise to DNA strand breaks, although strand breaks from the C-4' radicals are now thought to be the most likely. (Bothe et al., 1984; see also Chapter 6) Hydrogen atom abstraction on the C-3' position would lead to the formation of product III shown in fig. 1.2, however, such structures have not been detected. Hydrogen atom abstraction at the C-1' or the C-2' positions gives rise to alkali labile bonds. (Fig 1.2, I and II) and apurinic and apyrimidinic sites (which are also alkali labile), arise from depurination or depyrimidation. (Fig. 1.2, XVII) Damage to the sugar can lead to the release of undamaged bases, sometimes with sugar fragments still attached. Some of these are released almost immediately after irradiation, whereas others are slowly released over a period of several hours.

The irradiation products of bases in aqueous solution at RT has been studied for isolated bases, nucleosides and in DNA. In aqueous solution, the free bases are more radiosensitive than they are within DNA. All sites of OH' attack on free bases which lead to identifiable products are accessible in double helical DNA. (Hutchinson, 1985) The yields for loss of
intact bases are usually measured by the loss of UV absorption, the O.D. being the sum of the increase due to breakage of H-bonding between the bases, and the decrease due to the saturation of double bonds. The G values for the chemical change in free bases irradiated in dilute, oxygenated solution are given in the table below:

<table>
<thead>
<tr>
<th>BASE</th>
<th>ASSAY</th>
<th>G</th>
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<tr>
<td>Thymine</td>
<td>Loss of UV absorption</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; &quot;</td>
<td>2.6</td>
</tr>
<tr>
<td>Cytosine</td>
<td>Loss of UV absorption</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; &quot;</td>
<td>2.5</td>
</tr>
<tr>
<td>Adenine</td>
<td>Colometric test for A</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Loss of UV absorption</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; &quot;</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; &quot;</td>
<td>0.65</td>
</tr>
<tr>
<td>Guanine</td>
<td>Loss of UV absorption</td>
<td>0.55</td>
</tr>
</tbody>
</table>

From Hutchinson, 1985.

G = The number of events (molecules formed or destroyed) per 100eV of absorbed energy.

This shows that for a given dose, bases are released in the decreasing order T=C, A, G. The radiolysis of bases can be studied by a number of techniques in addition to the loss of UV absorption. For instance, base destruction can be measured by formic or perchloric acid hydrolysis of irradiated DNA, carried out at 175°C. The liberated bases are separated by chromatography, and the number of intact bases determined by UV absorption of the eluates. However, several products of base damage are themselves acid labile, making the acid hydrolysis technique unsuitable for the study of some types of base damage. This problem is avoided by the use of enzymatic
Figure 1.3

The products formed by the addition of OH\(^-\) to the 5,6 double bond of thymidine. I, thymine; II and III, cis and trans hydroperoxides; IV, thymine glycol; V, 5-hydroxy-5-methylbarbituric acid; VI, methyltartronoylurea; VII, urea; VIII, pyruvylurea; IX, 5-hydroxy-5-methylhydantoin.

(From Hutchinson, 1985)
techniques one of which is described in the following paragraph.

Over thirty products arise from the irradiation of thymine, and these are almost all separable by thin layer chromatography (TLC). Semi-stable hydroperoxides are produced, as revealed by spraying the TLC plate with potassium iodide. The major pathway is addition of OH\(^-\) to the 5,6 double bond, leading to thymine glycol formation (fig. 1.3, IV) and to a lesser extent, 5-hydroxy-5-methyl hydantoin. (Fig 1.3, IX) Radioimmunoassay has been used for the quantification of thymine glycols. (West et al, 1982). Breimer and Lindahl (1984) have described the use of a damage specific E.coli endonuclease to release damaged thymine bases from irradiated DNA. The enzyme used had rather a broad specificity, catalysing the release of ring-saturated and ring-fragmented thymines from double stranded DNA. The released products were analysed by high pressure liquid chromatography (HPLC). The main products from DNA irradiated under indirect conditions were cis- and trans- thymine glycols and 5-hydroxy-5-methylhydantoin.

Hydroxyl radicals preferentially attack the 5,6 double bond in cytosine. The major products are 5-hydroxycytosine and isodialuric acid. (Fig. 1.4, IV & XI) Hydroperoxides with the proposed structures shown in fig 1.4, II and III have short half lives (less than an hour at RT) so have not yet been detected. About half of the products have lost the C-4' amino group, and subsequently give rise to uracil-related products similar to those found for the non-deaminated cytosines.

The purines are less well studied than the pyrimidines. The major product from adenine is the 8-hydroxyadenine derivative
Figure 1.4
Structures of the products formed after the addition of OH' to the 5,6 double bond of deoxycytidine in the presence of oxygen. I, deoxycytidine; II and III, presumed hydroperoxides; IV, 5-hydroxydeoxycytidine; V, 4-amino-1-deoxyriboyl-5-hydroxy-2-oxo-3-imadazoline; VI, trans 1-carbamoyl-3-deoxyriboyl-4,5-dihydroxy-2-oxoimidazoline; VII, N-formyl-N'-glyoxylurea; VIII, 5-hydroxyhydantoin; IX, 5,6 dihydro-5,6 dihydroxydeoxyuridine; X, 5-hydroxydeoxyuridine (isobarbituric acid); XI, 5,6-dihydro-5-hydroxy-5-oxodeoxyuridine (isodialuric acid).
(From Hutchinson, 1985)
Figure 1.5
Products identified after the addition of OH' to deoxyadenosine. Structure I, deoxyadenosine; II, the tautomers 8-hydroxydeoxyadenosine and 7,8-dihydro-8-oxodeoxyadenosine; III, 4,5-diamino-5-formamidopyrimidine.
(From Hutchinson, 1985)
fig 1.5), which may further react to give additional products. Ring cleavage of adenine giving rise to formamidopyrimidine derivatives has been shown to occur. (Fig 1.5, III) Guanine is the least well studied of the bases, and to date no studies on the radiolysis of guanine in the presence of oxygen have been carried out. In anoxic conditions; 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-hydroxyguanine have been identified. (Equivalent to III and II in fig. 1.5)

As stated above, very few studies on product analysis of DNA irradiated under direct conditions have been attempted. Most of these to date have been carried out by Cadet et al. The products resulting from irradiation of adenosine and thymidine at 77K have been partially characterised, using ESR spectroscopy to identify the radicals, in conjunction with separation of the products using HPLC, TLC and silica gel columns (Cadet et al, 1983). The main products resulting from the irradiation of deoxyadenosine at 77K are 8-hydroxy-2'-deoxyadenosine and 5,8-cyclo-2-deoxyadenosine. The former results from a base-centred radical, and the latter from the C-5' sugar radical. Two major radiolysis products from thymidine are the diastereoisomers of 5,6-dihydrothymidine. These arise from the protonation of the pyrimidine radical anion to form the 5,6-dihydrothymid-5-yl radical, which undergoes H-atom transfer thus producing the diastereoisomers. The pyrimidine radical cation on the other hand, can either be hydrated to form the 6-hydroxy-5,6-dihydrothymid-5-yl radical which may in turn lead to the formation of thymine hydrates; or deprotonated to form the 5-methyl-2'-deoxyuridyl radical. The latter is converted to
stable monomeric or dimeric products of 5-hydroxymethyl-2'-deoxyuridine. The relative biological importance of the various base and sugar lesions formed under direct or indirect irradiation conditions is not known.
1.3 Radiation Damage in the cell

The arrangement of DNA in vivo must be considered when studying the effects of radiation on living cells; and when extrapolating findings in vitro to cellular systems. Both eukaryotic and prokaryotic cells have their DNA organised in chromatin. This consists of DNA in a highly condensed form in association with proteins. In eukaryotes the proteins are divided into two types, the histone and the non-histone proteins. The histones are a highly conserved group of proteins of which there are five types: H1, H2A, H2B, H3, and H4. The non-histone proteins are a very diverse group comprising all the DNA binding proteins. The DNA is packaged in units called nucleosomes, which consist of around 200 base pairs of DNA associated with a histone octamer. This is made up of pairs of all the histones except H1. Unfolding the higher orders of structure shows that the nucleosomes have the appearance of beads on a string when visualised by electron microscopy. Each 'bead' is separated from the next by 60 base pairs of 'linker' DNA. The remainder of the DNA associated with the nucleosome is coiled around the outside of the histone octamer. (This is always 146 base pairs) The basic form of chromatin packing is the 30nm fibre, seen when cells are gently lysed onto an electron microscopy grid. There are at least two models for the packing of the nucleosomes into the 30nm fibre, it is beyond the scope of this summary to describe them here. H1 is involved in this packaging, by forming attachments to the linker DNA of one bead and the histone core of adjacent nucleosomes, pulling them together in a regular fashion. ('The Molecular Biology of the Cell'}
The organisation of bacterial chromatin is less well understood, and has been recently discussed by D. Lilley (1986). Structural DNA binding proteins, histone-like units (HU) have been shown to be present in stoichiometric quantities, and are thought to have a histone-like role. The DNA becomes more tightly wound when complexed to HU, with one turn of the superhelix to three or four HU monomers.

The question arises as to the relative importance of the direct and indirect mechanisms on DNA irradiated in vivo. Generally speaking, cells contain over 80% water, but this is largely contained within the cytoplasm. DNA within the nucleus however, is very densely packed as described above, and hence is highly concentrated. Direct mechanisms are therefore likely to play an important role. Hydroxyl radicals are likely to be formed in the cytoplasm but are unlikely to be able to cross the nuclear membrane. Hydroxyl radicals may, however, be formed to some extent within the nucleus itself.

It has been reported that the indirect mechanism is the most important route to DNA damage in vivo. (Ward, 1981) However a recent report by Pohlit and Drenkard (1985) suggests that only the direct mechanism is important. They irradiated mammalian cells in suspension, saturated with N\textsubscript{2}O or CO\textsubscript{2}. Under CO\textsubscript{2}, the production of hydroxyl radicals is reduced by a factor of two compared with the situation under N\textsubscript{2}O. The cell survival was not affected by the gas conditions, suggesting that hydroxyl radicals were not the main cause of cell death. The relative contributions of the direct and indirect mechanisms to DNA damage in vivo is therefore not certain, but it seems likely that both have a role to play.
1.4 DNA Repair Mechanisms

Alterations to the DNA molecule will result in cell death if not rapidly repaired. Not only must the coding properties of the DNA be maintained, but in addition, the integrity of the molecule must be preserved in order to allow efficient replication and transcription. A great variety of enzymic processes operate in both prokaryotic and eukaryotic cells to correct lesions in DNA induced either spontaneously or by external DNA damaging agents. These processes often result in 'error-free' repair of the DNA, but mutations do arise in response to all DNA damaging agents. These can occur by one of two routes. Mutagenesis can arise directly if the DNA is damaged in such a way that the coding properties of the base are altered, allowing an incorrect base to be inserted opposite the altered one. (For example, alkylating agents give rise to 0-6-methylguanine residues which have altered pairing properties.) It is possible that inversions, deletions or translocations may arise as the result of double strand breaks being incorrectly/inefficiently repaired. In addition, there are known to be a number of inducible 'error-prone' pathways, the best known being the SOS system of E.coli; which whilst enhancing DNA repair, also gives rise to an increased frequency of mutagenesis.

There is an enormous variety of DNA damaging agents; these are almost invariably also mutagens and carcinogens. Repair mechanisms allow cells to tolerate the presence of DNA damaging agents up to a certain level, after which the repair systems become swamped, and cell death results. The following discussion outlines the major mechanism for DNA repair in
cells, with particular reference to those pathways which operate on gamma radiation-induced lesions. DNA repair mechanisms are described in detail in the following: 'UCLA Symposia on Molecular and Cellular Biology' New Series Vol II. Eds Friedberg and Bridges (1983); Walker (1984); Kenyon (1983); Lindal (1982) and Hanawalt et al (1979).

There are several strategies for the removal of DNA damage. Damage can be reversed or removed by various enzymes without the breakage of a phosphodiester bond; such as the removal of pyrimidine dimers from UV irradiated DNA by photoreactivating enzyme. Double stranded DNA is a substrate for excision repair, which involves incision of the damaged strand near the lesion, followed by excision of the damaged region and resynthesis using the opposite strand as the template. This mechanism operates on a variety of types of DNA damage often involving an initial incision event that is specific for a particular type of damage. It is possible that in the cell, the replication fork may encounter a damaged site before it has been repaired. It is generally accepted that many of these lesions can be by-passed by the polymerase, giving rise to gaps in daughter strands. These gaps can be dealt with by post replication repair systems, using mechanisms involving strand exchange. If not subsequently repaired, the original damaged site will become diluted out as the cell divides.

Missing, incorrect or altered bases are all found in gamma-irradiated DNA. Missing bases are usually repaired by the excision repair pathway. However, there does appear to be an enzyme activity in human fibroblasts which can directly insert the appropriate base into an apurinic (AP) site. More usually, there is an initial step by an apurinic site
specific endonuclease. A variety of these have been isolated from both bacterial and mammalian cells. They fall into two classes: those which initially incise on the 3' side of the lesion (class I enzymes), and those which initially incise on the 5' side. (Class II enzymes.) The major apurinic endonuclease in *E. coli* is exonuclease III, which is a class II enzyme. *E. coli* endonuclease III is another apurinic endonuclease, and also has an associated glycosylase which removes ring-opened thymine products. At least two other *E. coli* apurinic endonucleases have been identified. Mammalian AP endonucleases are predominantly type II enzymes, and at present none with an associated exonuclease activity has been found.

There is a wide variety of damage-specific endonucleases, isolated from a variety of sources. Schön-Bopp et al. (1977) described extracts from *Micrococcus luteus* which showed nicking activity against UV and gamma radiation-treated DNA. These activities could be separated out from an AP specific endonuclease activity. Some mammalian enzymes similar to *E. coli* endonuclease III have been isolated (from human, bovine and rodent cells) which have activities against damage induced by oxidising agents, ionising radiation and UV. In addition, Doetsch et al. (1986) have recently described a similar enzyme from calf thymus. This was shown to have activities against UV and osmium tetroxide-damaged DNA; and modified pyrimidines arising from ionising radiation and H2O2. The best-characterised of the damage-specific endonucleases is the UVR ABC endonuclease from *E. coli*. This is required for the initial incision/excision events in the repair of 'bulky' base damage, such as pyrimidine dimers and
adducts caused by psoralen plus light and N-acetoxy-acetylanilofluorine. Thus although originally characterised as a UV repair enzyme, it has rather a broad specificity. The adduct is excised from the DNA between breaks made 7 bases from the 5' side and 3-4 bases from the 3' side of the lesion.

The incision/excision steps of excision repair lead to single-stranded gaps in the DNA which must be filled in. This could be accomplished by any of the known E.coli DNA polymerases. In addition polymerases I and III could perform coupled resynthesis and excision, since they also have a 3' to 5' exonuclease activity. Most repair patches in E.coli are around 20 to 30 nucleotides in length.

The majority of studies on excision repair in mammalian cells have focused on skin fibroblast cell lines derived from patients with the condition Xeroderma pigmentosum; which renders them abnormally sensitive to UV light. This enhanced sensitivity is due to deficiencies in one or more of the DNA repair pathways. These cells show deficiencies in pyrimidine dimer excision and repair replication. In addition to enhanced UV sensitivity they are also sensitive to a variety of chemical agents which produce bulky adducts. They can, however, efficiently repair strand breaks. Most of these cell lines appear to have a deficiency in the initial incision step of excision repair. Studies on these cell lines have indicated that excision repair proceeds in a similar manner to that in E.coli, that is, an initial incision event mediated by one enzyme followed by excision and resynthesis. Whether excision and resynthesis are coupled as suggested for E.coli is not certain, although there is some evidence to
suggest that this is the case. Mammalian systems apparently have two pathways for repair synthesis, short patch repair (3-4 nucleotides) and long patch repair. (Patches of around 35 nucleotides, but may be as long as 120 nucleotides.) Short patch repair operates on the major damage products of ionising radiation (including strand breaks) and alkylating agents. Long patch repair operates on lesions which distort the DNA helix. ('UV-like' agents)

Another major group of enzymes which repair base damage are the DNA glycosylases. These cleave base:sugar bonds, acting only on damaged nucleotides. There is a wide variety of DNA glycosylases, each one having a narrow substrate specificity. Glycosylases are present in both bacterial and mammalian cells, where their main functions are the removal of alkylated, deaminated bases and pyrimidine dimers. The currently known glycosylases of particular importance in the repair of ionising radiation-induced damage are urea-DNA-glycosylase and thymine glycol DNA-glycosylase. The former removes urea residues generated as the remnants of pyrimidines by cleaving the urea:deoxyribose bond, and the latter catalyses the release of thymine glycols (5',6'-dihydroxydihydrothymine residues).

Recently an enzyme from E.coli has been described which directly repairs ring opened purines. (Chetsanga & Grigoran, 1985) Ionising radiation leads to the damage of imidazole rings of purines, resulting in formamido-pyrimidine products. These act as replication blocks, and hence need to be repaired. In theory, excision repair could operate on these lesions if an appropriate endonuclease exists. However, the identification of this enzyme activity means that the
ruptured imidazole rings can be reclosed in situ, restoring the C-8 to N-9 linkage. This enzyme has been named purine imidazole ring cyclase.

Ionising radiation can lead to the formation of interstrand cross links. These are likely to act as complete blocks to DNA replication, and furthermore, neither strand can act as a template for error free synthesis. However, both bacterial and mammalian cells have been shown to repair this type of damage. The mechanism appears to be excision repair followed by strand exchange. One possible mechanism requires the UVR AB product for the initial incision step, followed by excision involving the 5' to 3' exonuclease of DNA polymerase I.

Single and double strand breaks induced by ionising radiation are both repairable in cells. (McGrath & Williams, 1966; Hutchinson, 1979) This was demonstrated by a post irradiation increase in molecular weight of DNA from cells allowed to incubate after irradiation. The DNA was analysed on alkaline and neutral sucrose gradients. In addition, cells given a dose of radiation which was fractionated, allowing the cells to incubate in between, gave rise to a higher average molecular weight of the DNA than cells given a single dose of the same magnitude.

Although the repair of single strand breaks is efficient and rapid in cells, the repair of double strand breaks is more difficult since the continuity of the molecule has been lost. Bryant (1985) has shown that mammalian cells can repair staggered breaks much more efficiently than blunt ended breaks when these are caused by restriction enzymes. In addition, it was shown that restriction enzymes which produce
blunt ends give rise to chromosome aberrations similar to those seen in cells treated with ionising radiation. There is a correlation between chromosome aberrations and the degree of cell killing, suggesting that these are a major cause of the loss of reproductive capacity in irradiated mammalian cells.

Sargentini and Smith (1986) have recently described strand break repair pathways in E.coli. Two groups of processes appear to be involved in the repair of single strand breaks in E.coli; type II repair and type III repair. Type II repair is a growth medium-independent process which can occur in cells held in buffer. It is dependent on the PolA gene, and is a fast process, with a half time of 1 to 2 minutes at room temperature. Type III repair is both growth medium and RecA dependent. It is a slow process, taking 40 to 60 minutes at 37°C. The repair of double strand breaks was shown to be similar to type III repair of single strand breaks. After irradiation, cells were held in buffer to allow type II repair to occur, then the number of strand breaks was measured. It was found that the number of single strand breaks was now equal to twice the number of double strand breaks, suggesting that type III repair is essentially the repair of double strand breaks. Furthermore, RecA- cells show no detectable repair of double strand breaks. Since an active RecA gene is required, double strand break repair is thought to involve a recombinational event. In addition to this, it has been observed that E.coli cells grown under conditions where they have only one genome equivalent per cell cannot repair double strand breaks. (Normally, there are 3-5 genomes per cell) Repair is also prevented in E.coli and yeasts if
RNA synthesis is blocked, suggesting the involvement of an inducible system.

So far, nothing has been said of the underlying molecular mechanisms responsible for the repair of strand breaks in cells. The repair mechanism will depend on the end groups of radiation-induced strand breaks, and in the case of double strand breaks whether the breaks are blunt ended or staggered. The end groups on gamma radiation-induced strand breaks and possible repair mechanisms are discussed further in Chapter 6.

In addition to the repair processes described above, there are also inducible repair systems, the most widely studied being the SOS system of *E. coli*. (See Walker, 1984) A whole series of genes are induced in response to DNA damaging agents which partially degrade the DNA and block DNA replication, such as UV and gamma radiation, aflatoxin B1 and methyl methanesulphonate. (MMS) It is now known that a number of genes are under control of the RecA/LexA regulatory system, and are induced in response to DNA damage. In the uninduced state, the LexA protein acts as a repressor to the SOS genes, although they may be transcribed at low levels even when repressed. DNA damage acts as an inducing signal, which activates a protease activity of the RecA protein. Activated RecA protein (RecA*) now cleaves the LexA protein, rendering it unable to continue as a repressor. This allows the transcription of the repressed genes at greatly increased levels. As the level of the inducer decreases, RecA* loses its protease activity. LexA protein once more accumulates, and is able to repress the SOS genes. (Including itself) This leads to a return to the non-induced state. The nature of the
inducing signal is unclear but it is presumably a DNA degradation product, possibly a released oligonucleotide or the appearance of single-stranded DNA. It is now known that at least 20 genes are repressed by LexA, and derepressed in response to DNA damage. The role of many of these is not understood, but some are known to be involved in the inhibition of cell division, DNA repair and mutagenesis.

The induction of repair and mutagenesis by the SOS system has been known ever since the observation that the survival of UV-irradiated phage lambda is enhanced if the phage are transfected into UV-irradiated (SOS induced) E.coli, as opposed to unirradiated cells. Similarly, whilst UV treatment of lambda gave rise to only a very low frequency of mutagenesis in unirradiated cells, pre-irradiation of cells gave a marked increase in phage mutagenesis. These phenomena were termed Weigle reactivation and Weigle mutagenesis. UV-induced mutagenesis of the E.coli chromosome has also been shown to be RecA/LexA dependent. Most mutations arising after treatment with SOS inducing agents are 'targetted', that is, at the site of the initial lesion. However, there is at a lower frequency, 'untargetted' mutagenesis. It is not certain which lesions will subsequently give rise to mutagenesis. The molecular mechanism for SOS repair is not yet understood, but is thought to involve a new or altered polymerase which inserts an incorrect nucleotide. Yeasts and mammalian cells both have inducible repair systems; and in mammalian cells, phenomena analogous to Weigle reactivation and mutagenesis have been described with some viruses, (eg. Herpes).

It is clear from the above discussion that both prokaryotic and eukaryotic cells need to utilise a number of different
repair pathways in order to repair all of the lesions induced by ionising radiation. That cells do repair ionising radiation induced damage was shown initially by the observation that in tumor therapy, multifractionation of dose increases the dose required to kill a tumor. (Elkind, 1985) This also applies to the survival of irradiated cells in culture. (Elkind, 1984) In the sublethal range, DNA structure can become completely restored; repair has even been observed after lethal radiation doses. Strand breaks, alkali labile lesions and base damage can all be repaired in cells, invoking many of the pathways described above. (Hagen et al, 1980)

The hypothesis that strand-breaks, in particular, double-strand breaks are a major cause of cell killing and mutagenesis in gamma-irradiated cells is supported by a number of studies on mutants defective in strand-break repair. In the yeast Saccharomyces cerevisiae, for instance, two mutants in double-strand break repair have been isolated, Rad52 and Rad54.3. The latter is a temperature-sensitive mutant which has a normal sensitivity to X-rays at 23 C, but is hypersensitive to X-rays at 36 C. Since mutants in both of these genes are no more X-ray sensitive than either mutant alone, this suggests that both of their gene products are involved in the same DNA repair pathway. In both mutants, there is a 1:1 relationship between X-ray-induced lethal events per cell, and the number of unrepaired double-strand breaks. (Rudd & Mortimer, 1982)

A number of mutants have been isolated from cultured hamster ovary cells which are hypersensitive to a variety of agents which produce DNA strand-breaks. Out of these, some
show normal single-strand break repair, but are deficient in double-strand break rejoining. The mutants xrs5 and xrs7, for instance have been shown to repair only a small proportion of double-strand breaks (12% and 25% respectively), whereas wild-type cells repair 72% of double-strand breaks. (Kemp et al, 1984) Another mutant, EM9, is hypersensitive to alkylating agents and X-rays (Thompson et al, 1982). These agents give rise to a large number of strand breaks, directly in the case of X-rays, but presumably due to an intermediate step in DNA repair in the case of alkylating agents. EM9 was shown to have little or no capacity to repair strand-breaks. The mutant also has an unusually high frequency of sister chromatid-exchanges (SCF's), which may be responsible for the chromosome aberrations often seen in irradiated cells, and which are thought to be a major cause of the loss of reproductive capacity.

Further light has been thrown on the role of strand breaks as potentially lethal lesions by studies on cells from patients with the disease Ataxia-telangiectasia (AT). (Cox et al, 1986) The main symptoms of this condition are immunodeficiency and the loss of neurones. The cells show hypersensitivity to ionising radiation and demonstrate a high degree of chromosomal aberrations. Although the cells are hypersensitive to agents which cause strand-breaks, they exhibit a normal sensitivity to UV radiation, suggesting that they have a defect in strand-break repair. Although it has been shown that AT and normal cells have an identical capacity for the repair of single and double-strand breaks, it has recently been demonstrated that AT cell lines repair strand-breaks with a greatly decreased fidelity. (Cox et al,
The basis for this may be the overproduction of exonucleases which degrade double-strand breaks, or deficiency in factors which normally protect the termini of strand-breaks from exonuclease attack. However, increased exonuclease activity in AT cell lines has not been demonstrated. It has therefore been proposed that there is a 'disequilibrium' between correct rejoicing and exonuclease digestion (Debenham et al, in press).

Mutants in *E.coli* cells with deficiencies in strand break repair have also been isolated. The RorA mutants for instance, show an enhanced sensitivity to X-rays, but like the AT cell lines have a normal sensitivity to UV radiation and show increased levels of DNA breakdown. (Glickman et al, 1971) *E.coli* mutants such as RecN, which are deficient in conjugal recombination are also deficient in the repair of damaged DNA, and are hypersensitive to agents which give rise to double-strand breaks. This implicates recombinational processes in the repair of double-strand breaks. Postreplication repair of UV-induced lesions, however is unaffected, such as with the Rad52 mutants of yeast (Picksley et al, 1984).
1.5 Radiation-Induced Mutagenesis

Cell death and mutagenesis are known to be consequences of gamma irradiation. The preceding sections have described the types of DNA damage caused by gamma irradiation, and possible mechanisms for their repair. This section discusses mechanisms of radiation-induced mutagenesis; in particular, which types of lesions are 'pre-mutagenic'? Studies in systems which lack both excision and recombinational repair show that SOS mutagenesis gives rise to mutations at the site of the lesion (targetted) and elsewhere (untargetted).

The best studied DNA-damaging agent is probably UV light. Like gamma radiation UV radiation is mutagenic, but studies on potential mutagenic lesions are easier since fewer types of damage are formed, and all of these are base lesions. The major mutagenic UV-induced lesions are thought to be 6,4 photoproducts, rather than pyrimidine dimers although the latter are the predominant type of damage. (Haseltine, 1983 in 'UCLA Symposia on Molecular and Cellular Biology' Vol II, Eds. Friedberg & Bridges.)
This was revealed by the study of a large number of point mutations in the LacI gene of E.coli. Most of the base changes were found to be due to C to T transitions, even though UV specific endonucleases which incise only at pyrimidine dimers act mainly at TT sites. No mutagenesis hotspots were found at TT sequences. 6,4 photoproducts are known to be alkali labile, and studies of the positions of alkali labile sites after UV irradiation were found to correlate with mutagenesis hotspots. Thus 6,4 photoproducts appear to be the main pre-mutagenic lesion in UV irradiated (SOS induced) cells.

Brandenberger et al (1981) determined the DNA sequences of 174 bacteriophage M13 revertants isolated from amber mutants grown in UV irradiated and unirradiated E.coli. No obvious specificity of mutations for pyrimidine dimers was observed, which correlates with the study described above. In addition, it was observed that unless the host cells were irradiated, no increase of mutagenesis over the spontaneous frequency was observed, suggesting that UV photoproducts are only indirectly mutagenic. The same study on gamma-irradiated M13 showed that whilst mutagenesis was enhanced even in unirradiated cells, it was increased to a much greater extent if the host cells were irradiated. This indicates that there is a contribution both from direct and indirect (SOS mediated) mutagenesis. Gamma radiation-induced mutations, like those arising from UV treatment gave a wide variety of base changes with no clear specificity.

The above study concentrated on only a very small target area of DNA, making mutational hotspots almost impossible to observe. A recent study investigated gamma-induced
mutagenesis of the M13 mp10 Lac gene, in which the phage were
grown in unirradiated cells. (Ayakii et al, 1986) This gene is
not essential for growth, but mutants can be easily
identified by their white or pale blue phenotype on indicator
plates. (Wild type phage give bright blue plaques.) 116
mutants with white or pale blue plaques were picked and
sequenced directly. Single base changes accounted for 87% of
all mutants. One base addition and one non tandem double base
substitution were found. The other mutations were presumably
outside the sequenced region. Alterations from C's accounted
for 70% of all base changes, with the change from C to T
being the most frequent, occurring in 60% of cases. (That is,
A was preferentially inserted.) The other bases were inserted
in order of decreasing preference; G, T, C. Three mutagenesis
hotspots were noted, two of these were in the same position
as known UV mutagenesis hotspots, and one of these was also a
known depurination hotspot. The predominance of the C to T
change could be as the result of the gamma radiation-induced
alteration of cytosine to its derivatives, such as 5,6
dihydroxy-5,6-dihydro uracil. This pairs with A rather than
G, resulting in a C to T transition. Some of the mutations
may be the result of abasic sites, which are known to be
products of gamma radiation. It has been shown in vitro that
A's are preferentially inserted opposite these, but that
subsequent polymerisation does not occur. (Sager & Strauss;
1983,1985) If abasic sites are absolute blocks to DNA
synthesis then this makes them unlikely candidates for
mutagenesis. Studies on the spectrum of gamma radiation-
induced terminating mutations in the LacI gene of E.coli obtained
from SOS - induced cells revealed similar results. The
predominant base changes were G:C to A:T transitions, and the
distribution of mutations was fairly random, although hotspots
were observed. (Glickman et al., 1980; Kato et al., 1985.)
However, studies on mutagenesis patterns of gamma irradiated
M13 DNA in SOS induced E.coli cells are required for a true
comparison of direct and SOS induced mutagenesis.

However, Kunkel (1984) has shown that if apurinic sites
are artificially induced in M13 single stranded DNA, mutants
in the Lac gene do arise, whether the AP phage are grown in
irradiated or unirradiated E.coli cells. Furthermore, the
mutations were shown to result from AP sites rather than
other lesions, since treating the DNA with AP-specific
endonucleases or alkali (which causes the fragmentation of
single-stranded DNA at AP sites) virtually abolished
mutagenesis. There was a low frequency of mutagenesis if
phage were grown in unirradiated cells, only twice that of
the spontaneous frequency, whereas in SOS-induced cells the
frequency was 15 times the spontaneous frequency. The most
likely explanation is that polymerisation can proceed at low
frequency through AP sites, giving rise to the mutations
which were not the result of the SOS system. This low
incidence of readthrough would probably not have been picked
up in the experiments by Sager and Strauss (1983, 1985). Most
mutations observed were single base substitutions, and the
distribution of these was not random, hotspots tended to
occur within regions which had a high pyrimidine content.
Possibly AP sites form more readily where there is a high
proportion of pyrimidines.

In conclusion, there is clear evidence that gamma radiation
gives rise to mutations both directly and via the SOS system.
Double strand breaks, bases with altered pairing properties and possibly abasic sites are all likely to be directly mutagenic. These lesions may also give rise to mutations via SOS-like systems. Lesions which partially or totally block DNA replication, such as thymine glycols (Clark & Beardsly, 1985) and abasic sites may give rise to mutations via indirect, SOS-like mechanisms. The underlying mechanism for targeted and untargeted SOS mutagenesis is still not understood.
1.6 The Aims and Scope of this Work

This study has concentrated on the direct irradiation mechanism, since this is less well studied than the indirect mechanism, particularly at the biochemical level. As described above, the direct mechanism may be important in vivo. Other members of the group are studying the initial radicals formed on irradiation of frozen aqueous DNA, and the work described in this thesis investigates the stable DNA damage products formed on irradiation under direct irradiation conditions. In the long term it is hoped that the comparison of these types of studies will provide a greater understanding of the chemical and biochemical processes involved in the conversion of the primary radicals to DNA damage products which lead to cell death and mutagenesis.

In this work, stable DNA damage products have been detected and quantified in plasmid and bacteriophage DNA, and the relationship between loss of infectivity and strand breaks established. The site-specificity of gamma radiation-induced strand breaks and lesions which block DNA polymerase activity has been investigated. In addition, the end groups on radiation-induced strand breaks have been studied. In most of these experiments, samples have also been irradiated under indirect radiation conditions, to allow the similarities and differences between the two mechanisms to be discussed.
CHAPTER
two
CHAPTER 2

MATERIALS AND METHODS

(1) BUFFERS AND MEDIA

The following buffers and media were used extensively throughout this work. The composition of other buffers used is described in the relevant protocols. All buffers and media were made up in distilled water, and were sterilised by autoclaving (15psi for 15 minutes), except for the restriction endonuclease buffers which were made up from sterile components. Buffers described elsewhere were made up from sterile components unless stated otherwise.

1.1. Buffers.

1X Tris-EDTA buffer (TE):
0.01M Tris-HCl pH 7.4
1mM EDTA

10X Tris borate EDTA buffer (10X TBE):
0.89M Tris-HCl
0.89M Boric acid
25mM EDTA
pH to 8.3 with acetic acid
10x Tris magnesium buffer (TM):
0.1M Tris-HCl pH 7.5
50mM MgCl₂

**Restriction endonuclease buffers**

With all restriction enzymes, a general buffer of the appropriate salt concentration was used; with the exception of *SmaI*; which has a requirement for potassium ions.

10x Low salt buffer:
10mM Tris-HCl pH 7.5
10mM MgCl₂
1mM DTT

10x Medium salt buffer:
50mM NaCl
10mM Tris-HCl pH 7.5
10mM MgCl₂
1mM DTT

10x High salt buffer:
100mM NaCl
50mM Tris-HCl pH 7.5
10mM MgCl₂
1mM DTT

10x *Smal* buffer:
20mM KCl
10mM Tris-HCl pH 8.0
10mM MgCl₂
1mM DTT
1.2 Media

Luria broth and agar (L-broth and L-agar):
10g Bactotryptone
5g Yeast extract
5g NaCl
1g Glucose (L-broth only)
Made up to 1l with water
pH to 7.0 with NaOH

For L-agar: 16g agar added per litre.

2×TY broth:  
16g Bactotryptone
10g Yeast extract
5g NaCl
Made up to 1l with water

H-plates:  
10g Bactotryptone
8g NaCl
12g Agar
Made up to 1l with water

H-top agar:
10g Bactotryptone
8g NaCl
8g Agar
Made up to 1l with water

M9 Salts:  
6g Na$_2$HPO$_4$
3g KH$_2$PO$_4$
1g NH$_4$Cl
0.5g NaCl
Stored at 4°C.

Glucose/Minimal plates:
1l M9 Salts
15g Agar
1ml MgSO$_4$ (1M)
1ml CaCl$_2$ (0.1M)
2g D-Glucose
Note that throughout this work, centrifugation in a microfuge was always carried out at the high speed setting, that is, 10,500 xg to 11,500 xg.

2.1 Phenol/Chloroform extraction.

Phenol extraction was used to remove proteins from nucleic acid preparations. This is carried out more effectively by also using a second solvent, chloroform, as described below.

All phenol used was redistilled and equilibrated with TE buffer pH 8.0. 8-Hydroxyquinoline was added to a final concentration of 0.1%, prior to storage at -20°C. This helps to prevent oxidation of the phenol.

An equal volume of phenol was thoroughly shaken with the aqueous phase of the sample, then centrifuged for 1 minute in a microfuge to facilitate separation of the two phases. The upper (aqueous) phase was carefully removed and re-extracted if desired. The phenol layer can be back extracted with an equal volume of TE to improve recovery of DNA.

The chloroform used was a mixture of chloroform and isoamyl alcohol in the ratio 24:1. After phenol extraction, the aqueous phase was shaken with an equal volume of a 1:1 mixture of phenol and chloroform. The layers were separated by centrifugation as above, and the upper aqueous phase retained. The aqueous phase was then subjected to a further extraction with chloroform alone. The aqueous phase was then ether extracted prior to ethanol precipitation.
2.2 Ether extraction

Extraction against an equal volume of ether was used to remove phenolic compounds. This was done either after a chloroform extraction, or where phenol extractions only were carried out. An equal volume of ether was shaken with the sample, then left for a few moments for the layers to settle out. Ether forms the top layer, and was removed with a Gilson pipette. The remainder was blown off. Two ether extractions were usually performed.

2.3 Ethanol precipitation

A 1/10 volume of 3M NaAc pH 5.5 was added to the DNA solution, followed by 2-3 volumes of cold (-20°C.) 100% ethanol. The two phases were gently mixed together, and the sample cooled to -70°C in an ethanol/dry ice bath. The sample was left for at least 10 minutes to allow the precipitate to form. The precipitate was recovered by centrifugation in a microfuge for 10 minutes. The ethanol was removed, and the pellet washed with 1ml 70% cold ethanol. After a brief centrifugation, the ethanol was removed, and the DNA dried in a vacuum desiccator. The pellet was resuspended in a known volume of TE buffer.

If desired, the DNA concentration was estimated by measuring the O.D at 260nm. 1 O.D unit is equivalent to 50μg/ml of DNA.(40μg/ml for single-stranded DNA) The O.D 260:280 ratio gives a measure of the purity of the DNA with respect to proteins. A ratio of 2:1 indicates that the DNA is relatively protein-free.
2.4 Sephadex G50 and G25 Spun Column Chromatography

This technique was used to remove high molecular weight DNA from smaller molecules, particularly unincorporated $^{32}$P αdATP and $^{32}$P γATP from labelling reactions. The method was also useful for desalting samples.

As the sample is spun through the column, low molecular weight materials become stuck within the pores of the beads, whilst high molecular weight materials pass through. G25 has a smaller pore size than G50, hence this was the choice for purifying DNA of less than 100 base pairs.

A 1ml sterile plastic syringe was plugged with glass wool and placed in a 10ml conical based tube over a capless Eppendorf tube. Sterile G50 or G25 (pre-equilibrated with TE buffer) was poured into the syringe, and the column centrifuged for 4 minutes at 3000rpm (100xg) in a bench top centrifuge. The column packs down during centrifugation, and was topped up with more G50/G25 and re-centrifuged until a column volume of 0.9ml was reached. The column was then washed three or four times with the desired buffer, or more usually, water. For each wash, the column was centrifuged for exactly 4 minutes at 3000rpm.

The sample was applied to the column in a volume of 200μl, and centrifuged at 3000rpm for 4 minutes. The sample was then collected from the Eppendorf tube, and ethanol precipitated or lyophilised prior to resuspending in the desired volume of water or TE.
(3) SILICONISATION AND STERILISATION

All plasticware used was sterilised by autoclaving at 15 psi for 15 minutes. For some experiments, Eppendorf tubes were siliconised by washing in 'Repelcote' followed by drying on a tissue paper, then autoclaving. Repelcote is a solution of 27 dimethylchlorosilane in chloroform. Glassware was sterilised by baking in an oven at 300°C overnight. Corex tubes were siliconised prior to baking by rinsing out with Repelcote (ensuring that all inside surfaces were covered), then draining off the excess onto a tissue in a fume hood.

(4) IRRADIATION

Irradiations were carried out using a 'Vikrad' $^{60}$Co source; which delivers gamma rays at a dose rate of 0.6 MRad/hour. All samples were irradiated in sterile glass tubes of 5mm diameter, 3mm bore, which were sealed at one end. For irradiations at 77K, the tubes were placed in glass screw cap vials, and irradiated in a Dewar vessel containing liquid nitrogen. Irradiations at 202K were carried out in a chloroform / dry ice bath in the same type of glass vessels as used at 77K. For irradiations at room temperature, the glass tubes were placed in glass vials with push on plastic caps. All irradiations were carried out under conditions of ambient atmosphere unless otherwise stated.
(5) PLASMID PREPARATION

The plasmid used throughout this work was pBR322. (Bolivar et al., 1977) This is a multi-copy Escherichia coli (E.coli) plasmid, which has resistance genes for the antibiotics ampicillin and tetracycline. It is therefore selectable, and due to its high copy number, is relatively easy to purify in milligram quantities. It is 4362 base pairs in size, and has a molecular weight of $2.88 \times 10^6$ daltons. Its complete nucleotide sequence is known. (Sutcliffe, 1979)

pBR322 was maintained in E.coli strain SK 1571, which is a RecA derivative of K12. It is necessary to use a RecA (recombination deficient) host in order to minimise the formation of high molecular weight plasmid multimers.

Plasmid was prepared using the alkaline lysis method. (Birnboim and Doly, 1979) SK 1571 cells containing the plasmid were grown up on Luria agar plates containing ampicillin (50 μg/ml), and tetracycline (15 μg/ml in ethanol). The plates were incubated overnight at 37°C. Individual colonies were selected and picked into 10ml Luria broth (L-broth). These were grown up overnight at 37°C to provide starter cultures.

250 to 500 ml of L-broth in two litre flasks were inoculated with 2ml of the starter culture, and shaken overnight at 37°C. Plasmid was prepared from the cultures as follows. The volumes given are for a bacterial pellet derived from a 250ml culture. Note that all stages were carried out on ice unless otherwise stated.

The cultures were centrifuged at 5000 rpm (4000xg) for 10 minutes at 4°C. The resultant pellets were resuspended in 8ml lysis buffer containing 2mg/ml lysozyme, added just before
Lysis buffer: 25mM Tris-HCl pH 8.0
10mM EDTA
50mM D-Glucose

DNA was denatured with 12ml. of a solution containing 10% SDS and 2M NaOH, mixing for 10 minutes. Neutralisation was achieved by addition of 12ml. 3M KAc (pH 5.0), mixing for a further 10 minutes. This caused chromosomal DNA to precipitate out, forming an insoluble clot. The plasmid remained in solution, and was removed in the supernatant following centrifugation of the chromosomal material at 8000 rpm (10000xg) for 30 minutes at 4°C. Plasmid was precipitated out with an equal volume of isopropanol, and centrifuged at 8000rpm (10,000xg) for 30 minutes to form a pellet. Note that this stage was carried out at room temperature rather than on ice in order to avoid the precipitation of salts.

5.1 Purification of plasmid DNA.

The plasmid pellet was dried until it showed a cracked appearance, and resuspended in TE buffer. Supercoiled plasmid DNA was purified from open circular plasmid and chromosomal DNA by centrifugation through a caesium chloride gradient. CsCl was added until a refractive index of 1.396 to 1.398 was reached (1.12g/ml), and ethidium bromide was added to a final concentration of 0.2mg/ml. The DNA/CsCl solutions were transferred to 10ml. polycarbonate centrifuge tubes, and
topped up with light paraffin oil. The tubes were carefully balanced and sealed with aluminium screw caps. Centrifugation was carried out at 40,000 rpm (14,000-16,000xg) for 48 hours at 20°C in a 10x10 titanium rotor.

The resultant ethidium-stained bands were visualised under short wave ultra-violet light. Usually two bands were observed; the upper band consisting of residual chromosomal material and non-superciled plasmid; the lower band consisting of supercoiled plasmid. The lower band was removed, and extracted several times against an equal volume of CsCl-saturated isopropanol to remove ethidium bromide. Caesium chloride was removed by extensive dialysis against TE buffer. (4 changes of 2 litres)

Residual RNA was removed by treatment with RNAseA from bovine pancreas. The RNAse was purchased from Sigma Chemical Co., and was rendered DNAse free by boiling for ten minutes. Finally, the plasmid was phenol-extracted two or three times to remove residual proteins, ether-extracted twice, then ethanol-precipitated. (See section (2))

(6) BACTERIOPHAGE M13 PREPARATION

Bacteriophage M13 strains were developed as cloning vectors by J. Messing. Many of the techniques used in M13 work are described in his article in 'Methods in Enzymology' volume 101. (1983)

M13 is a male specific bacteriophage; entering the E.coli cell via the bacterial pilus. These structures are only present in 'male' strains of bacteria, that is, those
harbouring the F (or fertility) plasmid. One such strain is JM101, which was used throughout this work for propagation of M13. Without the F plasmid, JM101 cannot grow on minimal medium. Therefore stocks of JM101 were maintained on minimal plates (or minimal stab cultures for long term storage) in order to maintain selection for the F plasmid, which is otherwise rapidly lost. Note that all incubations were carried out at 37°C.

6.1 Infection and growth.

JM101 cells from a minimal plate were used to inoculate 10ml. of TY broth, and grown up overnight to give a starter culture. This was used to inoculate a further 10ml of 2xTY, (to give an O.D$_{550}$ of 0.02 to 0.04) along with 50-100μl of phage stock. After 6 to 8 hours growth, this was used to inoculate a larger volume of TY broth, typically 200ml. This was shaken overnight.

The cultures were centrifuged for 5 minutes in a microfuge, or for 10 minutes at 6000rpm (5000xg) in an MSE centrifuge, to produce a cell pellet and a supernatant enriched in phage particles. The centrifugation was repeated to ensure that all bacterial cells were removed. Phage was precipitated out by incubating for 20 minutes at RT with a 1/5 volume of a solution containing 20% polyethylene glycol (PEG) and 2.5M NaCl. The phage precipitate was recovered by centrifugation, and the PEG/NaCl removed with a Gilson pipette followed by blotting of excess liquid with a tissue. The phage pellet was resuspended in either 2xTY broth or TE buffer, and stored frozen at -20°C.
7. M13 DNA PREPARATION

7.1 Template DNA Preparation.

M13 infected cultures of JM101 were used to generate a phage pellet as described above. The pellet was resuspended in TE buffer and phenol-extracted two or three times to remove phage coat proteins. This was followed by a phenol/chloroform extraction, a chloroform extraction then two ether extractions. (See section (2)) Finally the DNA was ethanol-precipitated and resuspended in TE buffer, to a concentration of 200mg/ml. Template DNA was stored at -20°C.

7.2 Replicative Form (RF) Preparation.

The replicative form of bacteriophage M13 can be prepared by the 'mini-prep' method described below, or by the alkaline lysis method, described in section (5).

For highly purified RF DNA for use as a vector, the alkaline lysis method was followed exactly as described, using M13 infected JM101. For small quantities of less pure RF DNA; such as that used for the cutting out of a cloned fragment for re-cloning; the mini-prep method was used.

7.2.1 RF Preparation by the 'Mini-Prep' Method.

A 1ml overnight culture of a phage infected JM101 culture was centrifuged in a microfuge for 5 minutes. The supernatant was removed and re-centrifuged; and the bacterial pellets
discarded. 10μl of 10mg/ml lysozyme in lysis buffer (Section 1) was added to the supernatant, and left to stand for 10 minutes at room temperature. The mixture was then heated at 100°C for 45 seconds, and centrifuged for 10 minutes. The resultant gelatinous precipitate was removed with a toothpick. This contains proteins and high molecular weight DNA. 200μl of cold isopropanol was added to the supernatant, and frozen at -70°C. This precipitated out RF DNA, which could then be recovered by centrifugation for 10 minutes in a microfuge.

The resultant pellet was resuspended in 100μl TE buffer, and the aqueous phase phenol–extracted several times to remove residual proteins. The aqueous phase of the RF DNA was ether–extracted twice, then ethanol–precipitated. The purified RF pellet was resuspended in 40ml TE buffer. Its concentration was estimated by running aliquots on an agarose gel against standards of known concentration, and comparing the relative intensities of ethidium bromide fluorescence under UV light. See section (9).

(8) TRANSFORMATION

8.1 Calcium chloride method.

(Mandel & Higa, 1970)

This method was used in all the 'biological activity' assays. JM101 cells were grown until an optical density (550nm.) of 0.30 to 0.35 was reached; and harvested by centrifugation at 3000 rpm (100xg) for 5 minutes in an MSE
bench centrifuge. The cell pellet was resuspended in 1/2 volume of 50mM CaCl$_2$ which had been pre-chilled on ice. The cells were centrifuged a second time as above, then resuspended in 1/10 volume of the CaCl$_2$ solution. The cells were then competent and could be kept on ice for up to twelve hours without losing transformational efficiency.

300µl aliquots of the competent cells were transferred to 1ml. Eppendorf tubes on ice. DNA was added to the tubes in a volume of up to 10µl. Tubes were incubated for 40 minutes (still on ice); then heat shocked at 42°C. for 3 minutes. The transformed cell mix was then ready to plate out. (See section 8.3)

8.2 Hanahan method. (Hanahan, 1983)

This method gives a significantly higher transformation frequency than the CaCl$_2$ method, and is therefore useful when the amount of DNA available is limited. This method was used for transforming cells with ligation mixtures in the generation of M13 clones with homopolymer tracts.

Transformation buffer (TFB):

10mM K-MES (pH 6.2)
100mM KCl
45mM MnCl$_2$·4H$_2$O
10mM CaCl$_2$·2H$_2$O
3mM Hexamine cobalt (III) chloride
1M K-MFS was adjusted to pH 6.2 using KOH, filter-sterilized (0.22μm filter) and stored at -20°C.

TFB was also filter-sterilized, then stored at 4°C.

2.25M Dithiothreitol was made up in 40mM potassium acetate (pH 6.0), filter sterilized and stored at -20°C.

Cells were harvested at an optical density (550nm) of 0.30 to 0.35 as for the CaCl₂ method. The cells were resuspended in 1/3 volume of TFB by gentle vortexing then left on ice for 10 to 15 minutes. The cells were centrifuged a second time, then resuspended in 1/12.5 volume TFB. Fresh dimethyl formamide (DMF) was added to 3.5%. The mixture was swirled, and left on ice for 5 minutes. DTT was added to 75mM, swirled and left on ice for 10 minutes. Another portion of DMF was added to 3.5%, and the mixture left for a further 5 minutes on ice. 210μl aliquots were added to chilled eppendorf tubes, and DNA added in a volume of less than or equal to 10μl. The tubes were swirled, then incubated on ice for 30 minutes. Heat shock was carried out for 90 seconds at 42°C. The transformed cell mix was then plated out. (See below)

8.3 Plating out

To each tube of heat-shocked cells was added 3ml top agar, 40μl 100mM IPTG, and 40μl 2% X-Gal (Made up in DMF)

Top agar is described in section 1.2. The tubes were rolled to mix and poured onto H-agar plates. Plates were inverted and incubated overnight.
(9) AGAROSE GEL ELECTROPHORESIS

Agarose was from Miles Laboratories. Ethidium bromide was from Sigma Chemical Co. Agarose gels were made up to the required percentage in 1x TBE, and run in the same buffer. Slab gels were poured onto perspex plates and run in the horizontal position. Prior to loading, approximately 10μl of agarose beads was added to each 20μl sample.

For 20ml Agarose beads, 18ml TE Buffer, 2ml 100% Glycerol, 2mg Bromophenol Blue and 40mg agarose were mixed together and heated in a microwave oven (high power for 4 minutes) to dissolve the agarose, then cooled to set. When set the beads were forced through a syringe to form a fine slurry.

Small gels (8cm) were usually run in buffer containing 2.5μg/ml EtBr, but large 25cm gels (all those used in the strand break studies) were stained after running. These gels were soaked for 30 minutes in 2.5μg/ml EtBr, then destained by soaking for 30 minutes in 2l. of water. After staining, gels were visualised under UV light and photographed using Polaroid Land Film.
10.1 Preparation and running of gels.

Acrylamide and bis-acrylamide were purchased from Serva Chemical Co. TFMED (N,N,N',N'-Tetrathylenediamine) was purchased from Bio-Rad Laboratories. Ammonium persulphate was from Sigma Chemical Co.

A stock solution of 38% acrylamide, 2% bisacrylamide was made up in distilled water and deionised by stirring for one hour with 5g Amberlite monobed resin MB1 for each 300ml of acrylamide solution. Amberlite was removed by suction filtration through a Whatman glass fibre filter paper. The solution was stored at 4°C in the dark.

Denaturing polyacrylamide gels were made up to the required percentage in 1× TBF buffer, (See appendix) with 42% urea. Gels were normally made up to a volume of 40ml. The urea was dissolved by stirring prior to the addition of 300μl AMPS and 40-60μl TEMED, which catalyse the polymerisation reaction. Samples were mixed with 2-3μl formamide dyes (section 8), and heated at 80°C for 10 minutes prior to loading.

Nondenaturing gels were made up as described above, with the absence of urea. Samples were dissolved in 2-4μl of loading buffer, which was a 0.25% solution of bromophenol blue and xylene cyanol in 40%(W/V) sucrose.

Gels were poured between two glass plates separated by 0.3mm width spacers. Sharkstooth combs were used throughout. Gels were run in the vertical position at high voltage (800-1700 volts).
10.2 Autoradiography.

Preparative gels were covered with a sheet of Saran Wrap and exposed to X-ray film at room temperature. Analytical gels < 20% acrylamide were soaked in 10% methanol/10% acetic acid for 10 to 30 minutes. This removes urea, and 'fixes' the gel to prevent band migration. The gels were then dried down onto Whatman 3MM paper using a Biorad gel drier. The drying procedure improves resolution of the bands. Gels were then exposed to film at room temperature, or at -70°C with a phosphotungstate screen.

20% gels could not be dried down, so these were always exposed at -70°C to minimise band migration. Fuji R-X X-Ray film was used throughout.

(11) UNIT DEFINITIONS OF ENZYMES

The enzymes used throughout this work have the following unit definitions as analysed by the Company from which they were purchased.

Alkaline phosphatase: 1 unit catalyses the hydrolysis of 1μmole of p-nitrophenyl phosphate/min. at 37°C pH 10.5.

DNA Ligase: 0.006 'Weiss' units ligate 1μg of lambda HindIII fragments to an extent of 50%.

DNaseI: 1 unit produces an A_{260} change of 0.001/min/ml at pH 4.6 at 25°C. 400-600 units/mg of protein.
Klenow fragment of DNA polymerase I: 1 unit catalyses the incorporation of 10nmol total deoxyribonucleotide into acid soluble product in 30 mins at 37°C.

Polynucleotide kinase: 1 unit catalyses the transfer of 1nmol of phosphate from ATP to polynucleotide in 30 mins. at 37°C.

Restriction endonucleases: 1 unit is defined as the amount required to restrict 1µg of lambda DNA to completion in 60 mins. at optimum temperature.

RNAaseA: 1 unit produces acid soluble oligonucleotides equivalent to an \( A_{260} \) change of 1.0 in 30 mins. at 37°C. 50-75 units/mg of protein.

(12) QUANTITATION OF DNA STRAND BREAKS

Plasmid (pBR322) DNA was prepared as described in section (5) above, and irradiated for various lengths of time at a concentration of approximately 100µg/ml. Following irradiation, 1µg (10µl) aliquots were mixed with 10µl of agarose beads and loaded onto a 25cm long 0.65% agarose gel, made up in TBE buffer as described in section (9). The gels were run overnight at 40 Volts, 50mAmps, this effectivley separated out the three forms of plasmid DNA. The gels were then stained with ethidium bromide and photographed as described.
The negatives of the gels were scanned at 410nm using a Schimadzu UV240 spectrophotometer, and the resulting peak areas estimated by weighing. This gives a value (in arbitrary units) of the relative amount of DNA in each band on the gel. The supercoiled form intercalates 30% less ethidium bromide per molecule than the nicked and linear forms, so to account for this the values for supercoiled DNA must be multiplied by a factor of 1.43. (Determined by Lloyd et al, 1978) Strand breaks in M13 phage and template DNA were quantified exactly as for plasmid DNA with the following alterations. Phage particles were mixed with a 1/10 volume 2% SDS to remove coat proteins prior to loading on the gel. (Usually a volume of 20μl was loaded per well.) Template DNA was irradiated at a concentration of 200μg/ml, and 1μg (5μl) loaded per well. Gels were run at 200 volts for about 10 minutes, then the voltage was decreased to 40 volts for the rest of the run. This appeared to give better separation of circular DNA from linear DNA.
MEASUREMENT OF DNA/PROTEIN RATIO IN BACTERIOPHAGE M13

2×10ml cultures of M13 infected JM101 were used to produce cell-free phage-rich supernatants as described in section (7). These were PEG precipitated and combined together. The precipitate was then split into two equal portions prior to centrifugation, to produce two identical phage pellets. The resultant pellets were washed briefly with TE buffer. The pellets were treated as follows:

(a) Resuspended in 2ml of 0.5M perchloric acid (PCA) for the diphenylamine DNA assay.

(b) Resuspended in 1ml TE buffer for Lowry protein assay.

13.1 Lowry Protein Assay

(Lowry et al., 1951)

The following solutions were required.

Solution A:

0.2% Na₂CO₃
0.4% NaOH
0.16% Na/K Tartrate
1.0% SDS

Solution B was a 0.4% solution of CuSO₄, and solution C was made up by mixing 100ml solution A with 100μl of solution B. Dilutions of the phage solution in TE buffer were added to Eppendorf tubes. The volume was made up to 200μl with water. 1ml of solution C was added to each, and the tubes were
incubated for 15 minutes at room temperature. Following incubation, 100µl of Folins reagent was added (1:1 with water). The tubes were mixed well and incubated for 45 minutes (at room temperature) for the colour to develop. The optical density of the solutions was read at 660 nm. A standard curve was constructed with 5-50µg of BSA. (Bovine serum albumin.)

13.2 Diphenylamine assay for DNA content. (Burton, 1956)

Diphenylamine reagent:
3.2g Diphenylamine
80ml Acetic acid
8ml 12M PCA
4ml Acetaldehyde solution*

* 100µl acetaldehyde, 50ml H₂O

The phage pellet in 0.5M perchloric acid (PCA) was heated at 70°C for 10 minutes in order to hydrolyse nucleic acids. The solution was centrifuged at 3000rpm (100×g) for 10 minutes in order to remove debris. The supernatant was retained; the pellet resuspended in 2ml PCA and the hydrolysis step repeated. The debris was removed by a second centrifugation, and the supernatants pooled.

0.6ml of the diphenylamine reagent was added to 0.5ml of supernatant, then incubated overnight at 30°C. The optical density was measured at 595nm. A standard curve was constructed with 1-20µg of deoxyribose. Solutions were made up to 0.5ml with water, 0.6ml diphenylamine reagent was added and treated as above. To convert O.D at 595nm to DNA content,
the appropriate value was read off the standard curve and multiplied by 4.21. This factor is to account for the difference in O.D between deoxyribose and DNA solutions of the same concentration before adding the reagent.

(15) REPARATION OF THE KLENOW FRAGMENT OF DNA POLYMERASE I

(Joyce & Grindley, 1983)

A deletion of the PolI gene which encodes the Klenow Fragment of DNA polymerase I has been cloned to give pCJ155, which also has the ampicillin resistance gene. The Klenow fragment gene is under the control of the lambda promoter PL. This promoter is temperature sensitive, therefore the gene will not be expressed at 30°C, but will be rapidly induced at 42°C. The strain was grown at 30°C until induction was required. The strain of E.coli housing this plasmid was made available by Dr.L.Eperon; and the following preparative procedure was carried out with her help.

14.1 Growth and Harvesting of Cells.

Individual colonies were grown up on ampicillin containing plates (100µg/ml), and used to inoculate a starter culture. (2xTY broth containing 100µg/ml ampicillin.) 10ml of starter culture was used to inoculate each 600ml culture in a 2l flask. The cultures were grown for about 6 hours until an optical density of approximately 0.8 at 660nm was reached. The cells were then ready for the induction stage.

For induction, a further 1/3rd volume of TY broth pre-heated to 90°C was added to the flasks, and incubation
continued for a further 2 hours at 42°C. This allows time for the Klenow fragment to be produced in large quantities. The cells were harvested by centrifugation at 6000rpm (5000xg) for 10 minutes. The resultant cell pellets were frozen at -70°C overnight.

Induction was checked on a 0.3mm thick SDS/polyacrylamide gel. The Klenow fragment has a similar mobility to BSA, which was used as a marker. The presence of an abundant protein at around the correct position indicated that induction had been successful.

14.2 Cell Lysis.

Cells were thawed in lysis buffer. This was a solution of 50mM Tris-HCl, 2mM EDTA, 1mM dithiothreitol, 20nM phenylmethylsulphonyl fluoride (made up as a stock in ethanol) and 2mg/ml lysozyme. The latter two ingredients were added just before use.

4ml of lysis buffer was added per gramme of cells. Lumps were removed by gently pipetting up and down, and the cells left on ice for 15 mins. The cells were then broken open by sonication. This was achieved by several bursts of 10 seconds at low power and an amplitude of two. The cells were cooled on ice between bursts. A crude supernatant was obtained by centrifugation at 10,000rpm (12,000xg) for 10 minutes.

14.3 Ammonium Sulphate Fractionation.

Ammonium sulphate was slowly added to 60% saturation. (36.1g per 100ml extract) This was left stirring for 1 hour in the
cold room. The precipitated proteins were removed by centrifugation at 17,000 rpm (34,000xg) for 10 minutes. The supernatant was retained and ammonium sulphate added to an 85% saturation. (An additional 16.4g per 100ml.) After stirring for a further 1 hour at 0°C the solution was given a further centrifugation as above. This time the pellet was retained, and redissolved in 10mM potassium phosphate, pH 7.0, 1mM DTT. (KP buffer) Approximately 1ml per 3g of cell pellet was used. The resuspended proteins were dialysed against 500ml KP buffer for 2 hours, with one change.

14.4 BioRex 70 Chromatography.

The resin was titrated to pH 7.0, and was equilibrated with KP buffer. The column used was 1.5x25cm in size. It was washed prior to use with KP buffer. The sample was loaded and washed through the column for several hours with KP buffer.

The protein was eluted with a 0 to 0.5M NaCl gradient, over about 5 hours. 1ml fractions were collected and examined by SDS gel electrophoresis. (Section 11.6) The gel showed a single band at around the correct position, in fractions 60 to 75. The protein concentration was estimated by measuring the O.D at 280nm, which confirmed the electrophoresis results. (See figure 2.1) These fractions were concentrated in a pre-cooled 'Amicon' spin concentrator, by centrifugation at 5000rpm (4000xg) for 15 minutes at 4°C.

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Preparation of kinesin fragments: Fractionation through Bio-Rex column. 0.02 M measurements of various fractions were taken. The salt concentration at which each fraction eluted from the column is also shown.
Figure 2.2
Preparation of Klenow Fragment: Fractionation through Sephacryl column. The O.D. profile of fractions eluting from the column is shown.
14.5 Sephracyl S-200 Chromatography.

A 1cm x 29cm column which had been equilibrated overnight with KP buffer was used. The sample was loaded and washed into the column with 3cm KP buffer, measured as column distance. The proteins were slowly eluted, and collected in 1ml fractions. The fractions were examined for protein content by measuring the O.D at 280nm. The peak was found in fractions 10 to 17. (See figure 2.2) These were retained and concentrated in an 'Amicon' spin concentrator as before.

The final product was stored at -20°C in Klenow diluent, a solution of 50% glycerol containing 50mM KP buffer at pH 7.0 and 0.5mM DTT. The product was assayed for activity and contaminating nucleases by Dr.I.Eperon. The final concentration of the polymerase was estimated to be 5 units/μl. (The same as commercially available enzyme, see section (11)).

14.6 SDS-Polyacrylamide Gel Electrophoresis.

A stock solution of 40% acrylamide was made up as previously described. (Section 10.1)

Stacking gel:
2.5ml 4x Stacking buffer
1.25ml Acrylamide
6.35ml Water
200μl 10% AMPS
25μl TEMED

Separating gel (10%):
5ml 4x Separating buffer
5ml 40% Acrylamide
10ml Water
150μl AMPS
60μl TEMED

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The cleaned gel plates were taped together, separated by a 0.3mm spacer. The separating gel was poured to within 4cm of the top of the plates, and carefully covered with a layer of isopropanol to give a straight gel front. After leaving for 10 minutes to set, the solvent was removed and the stacking gel poured. The gel was allowed to set for 30 minutes before using.

**Buffers**

<table>
<thead>
<tr>
<th>4x Stacking Buffer:</th>
<th>4x Separating buffer:</th>
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</thead>
<tbody>
<tr>
<td>0.25M Tris base</td>
<td>1.5M Tris base</td>
</tr>
<tr>
<td>0.2% SDS</td>
<td>0.4% SDS</td>
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<td>pH to 6.8</td>
<td>pH to 8.8</td>
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<table>
<thead>
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<th>2x Sample buffer:</th>
<th>10x Running buffer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4% Bromophenol blue</td>
<td>0.2M Glycine</td>
</tr>
<tr>
<td>0.4% Xylene cyanol</td>
<td>0.25M Tris base</td>
</tr>
<tr>
<td>0.2M B-Mercaptoethanol</td>
<td>1.0% SDS</td>
</tr>
<tr>
<td>2% SDS</td>
<td></td>
</tr>
<tr>
<td>100mM Tris-HCl pH 8.5</td>
<td></td>
</tr>
</tbody>
</table>

5μl of dyes were added to each sample prior to loading.

**Staining protein gels**

The gels were stained for 30 minutes in a solution of 0.1% coumassie blue in 50% methanol and 10% acetic acid. Gels were destained for 30 to 60 minutes in 5% methanol / 10% acetic acid.
DNA SEQUENCING BY THE DIDEOXY CHAIN-TERMINATING METHOD

F. Sanger et al. (1977)

Deoxy and dideoxy nucleotide triphosphates were purchased from Sigma Chemical Co. $\alpha^3$P-dATP; specific activity 3000Ci/mmol was from Amersham International. The Klenow fragment (KF) of DNA polymerase I was purified in this laboratory (section 14) or purchased from P-L Biochemicals.

Stock solutions of the deoxy nucleotide triphosphates were made up to 0.1M in TE buffer, and dideoxy nucleotide triphosphates were made up to 10mM, also in TE buffer. Both solutions were stored at -20°C.

The following working solutions were made up:

(i) N* mixes

<table>
<thead>
<tr>
<th>C'</th>
<th>A'</th>
<th>G'</th>
<th>T'</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCTP (µl)</td>
<td>10</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>dGTP (µl)</td>
<td>200</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>dTTP (µl)</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

(ii) Dideoxy solutions

- ddCTP: 0.08mM
- ddATP: 0.06mM
- ddGTP: 0.16mM
- ddTTP: 0.50mM
Formamide Dyes

900µl Deionised Formamide
100µl EDTA (0.5M)
0.3mg Xylene Cyanol
0.3mg Bromophenol Blue

All were stored at -20°C.

For a complete sequence (ie. A,T,G and C tracks); 2µl 10×TM, 0.5µl Primer (2.5ng/µl), 2.5µl Template DNA (200µg/ml) and 5µl Water were mixed together. Template DNA and primer were annealed by heating at 70°C for 10 minutes, then slowly cooling to room temperature. The mixture was then split into 4×2µl aliquots. To each was added 1µl ddNTP, 1µl N’, 1µl Klenow Polymerase (0.25units) and 1µl α32PdATP (1µCi), where N is A,T,G or C. The mixture was incubated for 20 minutes at room temperature, unless otherwise stated. 2µl chase mix was then added to each tube. This consists of a mixture of all four dNTP’s each at a concentration of 0.5mM. The mixture was then incubated for a further 20 minutes at RT. Following incubation, each sample was divided into 2×4µl aliquots. One was frozen and retained for future use, to the other was added 2µl of formamide dyes, and the sample was denatured by heating at 80°C for 10 minutes. (With tubes open to allow the formamide concentration to increase.) The tubes were then plunged onto ice, and the samples loaded immediately onto a denaturing polyacrylamide gel of the required percentage. (Section 10)
16 CONSTRUCTION OF M13 CLONES WITH HOMOPOLYMER TRACTS.

16.1 Vector DNA

M13 mp11 replicative form (RF) DNA was purchased from BRL. M13 mp8 RF was prepared in this laboratory by the alkaline lysis method. (See sections 5 and 7.2) Vector DNA was cut with the appropriate restriction enzymes, and 0.5 units of calf intestinal phosphatase added 20 minutes before the end of the digestion. The reactions were terminated by phenol extraction of the aqueous phase 3 to 4 times, followed by ether extraction and ethanol precipitation. Treating with phosphatase removes 5'-phosphate groups and hence prevents self ligation of the vector.

16.2 Ligation Reaction.

T4 DNA ligase rejoins blunt or sticky ended DNA molecules. The enzyme joins a 3' hydroxyl group to a 5' phosphate group, resulting in the formation of a phosphodiester bond. Magnesium ions and ATP are required as cofactors in the reaction. The ligase used in these experiments was purchased from Amersham International.
The general procedure used was as follows.

<table>
<thead>
<tr>
<th>Reaction mixture:</th>
<th>10x Ligase buffer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA insert (100ng) 5μl</td>
<td>0.5M Tris-HCl pH 7.4</td>
</tr>
<tr>
<td>DNA vector (20ng) 2μl</td>
<td>0.1M MgCl₂</td>
</tr>
<tr>
<td>10x ligase buffer 1μl</td>
<td>1mg/ml BSA</td>
</tr>
<tr>
<td>ATP (10mM) 1μl</td>
<td></td>
</tr>
<tr>
<td>DTT (50mM) 1μl</td>
<td></td>
</tr>
<tr>
<td>T4 DNA ligase 1μl *</td>
<td></td>
</tr>
</tbody>
</table>

* The enzyme stock was diluted so that it was always added in a volume of 1μl. For sticky ended ligation 0.1 units were used, and for blunt ended ligation, 0.4 units. (See activity definition in section 11) Note that the ligation of sticky ends is much more efficient than that of blunt ends.

Sticky ends were incubated for 4 to 12 hours at 14°C.
Blunt ends were incubated overnight at 4°C. In order to check the efficiency of ligation, a ligation reaction was carried out with cut non-phosphatased vector in the absence of insert DNA.

16.3 Preparation of insert DNA and selection of recombinants.

16.3.1 Adenine and Thymine

Poly dAdT was purchased from PI-Biochemicals. This was supplied as double stranded DNA many kilobases in length. To produce fragments of a suitable size, the enzyme DNaseI was used. This enzyme is a magnesium-dependent endonuclease which acts upon single or double-stranded DNA, giving rise to nicks.
in the phosphodiester backbone. If the substrate is double-stranded, (as in this case) DNaseI will produce fragments which have single-stranded 'tails' of various lengths.

The following procedure was used to generate blunt ended fragments within the approximate size range 20 to 500 base pairs. 4μg of poly dAdT was incubated with 100ng of DNaseI in 1xTM buffer for 10 minutes at room temperature; to produce fragments of the desired size range. This was shown by agarose gel electrophoresis in parallel with a HaeIII digest of pBR322 as size markers.

The DNaseI reaction was stopped by the addition of 1μl of 0.5M EDTA. The fragments were purified by phenol extraction followed by ethanol precipitation. The fragments were resuspended in TE buffer; and rendered blunt ended by treatment with the Klenow fragment of DNA polymerase I (5units/μg DNA) in the presence of dATP and dTTP. (5μl of 0.5mM solutions/μg of DNA) Since this enzyme is a 5' to 3' polymerase, only fragments with 3' recessed termini will be substrates for the polymerase activity. However, the Klenow fragment also has a 3' to 5' exonuclease, which will remove 3' overhangs.

Cloning of poly dAdT fragments into M13

The blunt ended poly dAdT fragments produced as described above were inserted into the SmaI site of M13 mp8 by blunt-ended ligation. The ligated mixtures were used to transform JM101 by the Hanahan method. (Section 8.2)

White plaques were picked into 2ml 2xTY broth, to which 20μl of an overnight culture of JM101 was added. Following an
8 hour incubation (shaking at 37°C), template DNAs were prepared as described (section 7.1) and analysed by the dideoxy sequencing method. (Section 15) This enabled identification of a clone containing a run of 51 adenines in the template strand. (See figure 2.3) No clones with a run of thymines were identified. This was overcome by using the 'clone turn around' procedure. This relies on the use of an M13 vector with its polylinker region in the opposite orientation to the vector containing the required insert. The insert is cut out of the original vector with appropriate restriction enzymes, and reinserted into the new vector which has been cut with the same enzymes.

In this case, RF DNA from the clone with a run of 51x A's was prepared by the mini-prep method (section 7.21) and the insert cut out using EcoRI and HindIII. This was then inserted into EcoRI/HindIII cut M13 mp11 by sticky-ended ligation. The ligated mixture was transformed into JM101; then recombinants were selected and screened as described. This enabled the identification of a clone with a run of 51 thymines in its template strand. (see figure 2.3)

16.32 Cytosine and Guanine

Poly dGdC was purchased from PL Biochemicals, but in contrast with the poly dAdT, proved refractory to DNAsel digestion. An alternative strategy was therefore employed.

A pBR322 derived vector with a poly dGdC run of 14 base pairs was obtained as a gift from Dr. A. Greenland. (Leicester Biocentre) The plasmid was cleaved with EcoRI and PstI to
generate several fragments, one of which contained the homopolymer run.

The desired fragment was inserted into the *EcoRI/PstI* cut vectors M13 mp8 and mp11 by sticky ended ligation. This was able to insert in only one orientation. Therefore two vectors with the polylinker region in opposite orientations were used, to allow the production of two clones; one with a run of guanines in the template strand, and the other with a run of cytosines. (Illustrated in figure 2.3)

The ligated mixture was used to transform JM101 by the Hanahan method. White plaques were picked into 2ml of 2xTY broth the following day, to which were added 20μl aliquots of an overnight culture of JM101. The cultures were incubated for 8 hours, shaking at 37°C. DNA templates were then prepared from the cultures as described in section 7.1. Templates were analysed for the presence of a homopolymer tract by the dideoxy sequencing method. (section 15)
17.1 Labelling Oligomer at the 5' End.

The oligonucleotide used throughout this work is the 'universal primer', which has the sequence given below:

3' -TGACCGGCAGCAAAATG-5'

Other primers used for annealing to the homopolymer clones are shown in figure 2.3.

12.5µl oligonucleotide primer (2.5ng/µl) was incubated for 45 minutes at 37°C with 50µCi γ<sup>32</sup>PATP and 10 units of T<sub>4</sub> polynucleotide kinase in 1x TM buffer. (Total volume 50µl.) If the labelled oligomer was for use as a primer for M13 templates, 1µl aliquots were removed and treated as described below.(17.2) If the oligomer was required for the study of the end groups on gamma radiation-induced strand breaks, then the reaction mixture was dried down and resuspended in 5µl formamide dyes, heated for 10 minutes at 80°C, then loaded onto a 20% polyacrylamide gel. (Non-denaturing) The gel was run at approximately 1700 volts until the bromophenol blue dye had reached half way down the gel. The band of labelled oligomer was localised by autoradiography (-5 mins exposure) and cut out. The gel slice was placed into 200µl of water, and allowed to elute overnight at 37°C. The supernatant was removed, and purified by centrifugation through a G-25 Sephadex column. (See section 2) The purified oligomer was then dried down, and resuspended in the desired volume of water. Typically, specific activities of 500,000cpm/µg of DNA were obtained. (Determined by scintillation counting.)

The use of DNA fragments of defined sequence to study DNA
damage has been described by W.A.Haseltine et al (1980).

17.2 Priming and Polymerising M13 Templates.

1μl aliquots of 5’ end-labelled oligonucleotide primer, prepared as described above, were mixed with 2μl of 100μg/ml M13 template DNA in 5xTM buffer. Templates were irradiated for various times prior to annealing, or left unirradiated as a control. Primer and template were annealed by heating at 70°C for 5 minutes, then allowing to cool to room temperature. To each annealed mix, 3μl of a dNTP mix (dATP, dTTP, dGTP and dCTP all at 0.5mM) and 0.5 units of Klenow fragment were added. The reactions were incubated overnight at room temperature. (The optimum conditions for complete polymerisation were determined by varying the time allowed for the reaction, and the amount of enzyme available.) The following morning, each sample was split into 2x3.5μl aliquots. One was retained for future use, and to the other was added 3μl of formamide dyes. The samples were denatured by heating at 80°C for 10 minutes, then plunged onto ice and immediately loaded onto a 6% polyacrylamide sequencing gel. The gels were run at approximately 1200 volts, until the bromophenol blue dye had reached the bottom of the gel. Gels were dried down and exposed to X-ray film as described in section (10).
pBR322 was prepared as described in section (5). Typically, 10\(\mu\)g of pBR322 (200\(\mu\)g/ml) was cleaved by incubating with 10 units of BamHI and 10 units of HindIII in 1\(\times\) medium salt buffer. Incubations were carried out for 3-4 hours at 37°C. This gives rise to a 4016 and a 346 base pair fragment. The cleaved DNA was purified by phenol extraction followed by ethanol precipitation. Labelling was carried out by incubating the cut fragments for 1 hour at room temperature with 50\(\mu\)Ci \(^{32}\)PdATP and 5 units of the Klenow fragment in 1\(\times\)TM buffer. The mixture was then lyophilised to dryness and resuspended in 5\(\mu\)l of sucrose-based loading dyes. The labelled fragments were then loaded onto a 4\% non denaturing polyacrylamide gel, and ran at 1200 volts until the bromophenol blue dye had reached the bottom of the gel. The fragments were localised by autoradiography (~1 hour exposure) and the 346 bp fragment excised. The fragment was eluted into water as described above (Section 17.1), and purified by centrifugation through a G-50 Sephadex column followed by ethanol precipitation and resuspension in the desired volume of water. Typically, specific activities of 20,000cpm/\(\mu\)g of DNA were achieved.
19.1 Chemical Cleavage at G and A.

A. Maxam and W. Gilbert (1977) developed a method whereby DNA fragments labelled at one end could be sequenced, by using chemical reagents which cleave the molecule at specific bases. In this work, end-labelled DNA molecules were subjected to the reaction which cleaves at guanine and at cytosine; to provide markers for the analysis of end groups and site specificity in gamma radiation-induced strand breaks. DNA fragments were labelled with $^{32}$P at the 3' and 5' ends as described in sections (17) and (18). The G and A cleavage reaction was performed on these substrates as follows. The labelled DNA was desalted by Sephadex G50 or G25 spun column chromatography. The DNA was lyophilised to dryness and resuspended in the desired volume of water, typically 50μl. Aliquots were taken out and made up to 20μl with water. Approximately 0.6ng of the 17 base oligonucleotide; or 1μg of the 346 base pair restriction fragment was used per reaction.

To each aliquot was added 2μl of 1.0M piperidine formate (pH 2.0), which was made up by diluting 98μl piperidine and 40μl formic acid in 862μl water. After thorough mixing, oligomers were incubated for 22 minutes at 37°C, and restriction fragments for 1 hour at RT. The samples were then lyophilised to dryness, resuspended in 20μl of water, and lyophilised a second time. Samples were resuspended in 100μl 1.0M piperidine formate (freshly diluted) made up as described above. The Eppendorf tubes were tightly closed, and
placed in a water bath at 90°C for 30 minutes. The tubes were weighted down with a lead pot in order to prevent the caps from popping, since piperidine is very volatile. Following incubation, the samples were lyophilised and washed twice with 10μl of water, lyophilising after each wash. The resultant dry sample was stored at -20°C until required. Prior to loading on a gel, samples were dissolved in 2μl formamide dyes, and denatured by heating at 80°C for 10 minutes.

Note that due to the higher temperature required to cleave oligomers, non-specific cleavage reactions occur, particularly at cytosine. This must be taken into account when reading the sequence.

19.2 DNAse I Digest.

DNAse I was used to digest end labelled DNA to act as markers in the studies on sites and structure of gamma radiation induced strand breaks. DNAse I cleaves the DNA molecule, giving a 3' hydroxyl end group and a 5' phosphate end group. Aliquots of labelled DNA (1μg of restriction fragment or 0.6ng of oligomer) in 1xTM were incubated for 30 minutes at room temperature with DNAse I. The reaction was stopped by lyophilising the mixture, the dried pellet was then resuspended in 2μl of formamide dyes and denatured, prior to loading on Sequencing gels.
19.3 Polynucleotide Kinase Exchange Reaction.

Polynucleotide kinase is usually used to catalyse the transfer of the gamma phosphate on ATP to a 3'OH terminus on DNA. However, in the absence of ATP, the 'exchange' reaction occurs, in which the enzyme catalyses the removal of 3' phosphate groups. To investigate whether irradiated DNA was a substrate for this reaction, aliquots of approximately 12ng of 5' end labelled irradiated oligomer were run on a 20% sequencing gel as described in section (10) and the bands localised by autoradiography. The bands of interest were excised from the gel and eluted into water as described in section 17.1. The fragments were purified by centrifugation through a G-25 Sephadex column, lyophilised to dryness and resuspended in 20µl of water. 1x10µl aliquot was left untreated as a control, and the other was treated as described below.

The fragments were incubated with 10 units of polynucleotide kinase for 3 hours at 37°C in a buffer containing 10mM Tris-HCl pH 6.5, 1mM MgCl₂, and 0.5mM mercaptoethanol. The reaction mixes were lyophilised, and resuspended in 2µl of formamide dyes prior to denaturation and loading onto a 20% polyacrylamide sequencing gel. Gels were run at approximately 1700v until the bromophenpl blue dye was halfway down the gel. The gels were exposed to X-ray film for 2 to 8 days.
CHAPTER
three
CHAPTER 3

THE EFFECT OF HYDROGEN PEROXIDE AND MERCAPOETHYLAMINE ON GAMMA RADIATION-INDUCED STRAND BREAKS

3.1 INTRODUCTION

3.11 Quantitation of DNA Strand Breaks

Many studies on the quantitation of gamma radiation-induced damage have focused on the use of DNA strand break measurements as an index of DNA damage. Base damage is much more difficult to quantify due to the very large number of different types of base damage occurring, each contributing to the total amount of base damage by a relatively small proportion. Virtually every type of base damage requires a different assay system, and at present, assays have only been developed for some of the more predominant types of base damage, such as thymine glycols (West et al, 1982). Strand breaks, on the other hand are probably present in much greater proportion than any of the individual base damages, although not necessarily in greater proportion than base damages as a whole. Although strand breaks have been shown to be repairable in the cell (see Chapter 1), double strand breaks are thought to be one of the most serious DNA lesions in terms of causing cell death. Double strand breaks cause loss of the integrity of the DNA molecule, so that even if the breaks are rejoined, the correct ends may not be selected. Bryant (1985) has shown that blunt-ended double
strand breaks in genomic DNA in vivo generated by restriction enzymes give rise to chromosome aberrations similar to those induced by ionising radiation. In addition, there appears to be a correlation between chromosome aberrations and cell killing.

There are several assays available for the quantitation of DNA single and double strand breaks, (See 'DNA Repair- a Laboratory Manual of Research Procedures' Eds. Friedberg & Hanawalt, 1981). One of the earliest methods used was sedimentation through neutral or alkaline sucrose gradients, as described by McGrath and Williams (1966). This method is of use for the measurement of strand breaks in DNA of high molecular weight, for instance total cellular DNA. To measure strand breaks in cells, irradiated cells are gently lysed onto the sucrose gradient in an ultracentrifuge tube, and centrifuged through the gradient at high speed. The DNA fragments sediment according to molecular weight, the larger fragments sedimenting more rapidly, hence ending up towards the bottom of the tube. Fractions are collected from the bottom of the tube and analysed for DNA content, either by optical density measurements or scintillation counting if the DNA has been labelled. Labelling is achieved by growing the cells for one or two generation times in the presence of $^3$H or $^{14}$C thymidine. The sedimentation profile of the DNA is used to calculate the number of strand breaks, by utilising the relationship between the molecular weight of the DNA and the distance of sedimentation. Sedimentation through neutral sucrose gradients allows the quantitation of double strand breaks, whereas sedimentation through alkaline sucrose gradients denatures the DNA, allowing the quantitation of
single and double strand breaks together. This latter measurement also includes single strand breaks induced at alkali-labile sites, for instance, apurinic/apyrimidinic sites. Crine and Verly (1976) have developed a method whereby these types of breaks can be distinguished from those resulting directly from irradiation. Two aliquots of the DNA sample to be analysed are denatured, one with formamide and the other with NaOH. NaOH treatment breaks a phosphodiester bond at each alkali labile site, whereas formamide leaves these sites intact. The NaOH treated sample is neutralised, then both samples are sedimented through neutral sucrose gradients. The difference in the number of breaks calculated for these two samples will be equivalent to the number of alkali-labile sites.

A more recent technique for the quantitation of single and double strand breaks in cellular DNA is filter elution. (Kohn, 1983) This method has the advantage that in addition to strand breaks and alkali-labile sites, DNA:protein cross links can be quantified. This method involves the use of filters with a 2µm pore size to discriminate between molecules of different size. Cells are lysed directly onto the filter and washed through by a continuous flow of buffer. RNA and proteins are largely washed through before the appearance of the first DNA fraction. The DNA retained on the filter is treated with proteinase K to digest bound proteins, if strand breaks only are to be determined (once RNA and cytoplasmic proteins have been washed through the filter). Protein absorption to the filter is minimised by lysing the cells with a solution containing 2% SDS, and using a polycarbonate filter. If DNA:protein cross links are to be
determined, however, the cells are lysed with a solution containing 0.2% sodium dodecyl sarcosine and 2M NaCl onto a PVC filter, and not treated with proteases. These procedures maximise protein absorption to the filter. Small DNA fragments pass through the filter rapidly, but the passage of longer fragments is impeded by the filter, these therefore have a longer retention time. The average time for a DNA molecule to pass through the filter is proportional to its length. Fractions emerging from the filter are collected and assayed for DNA content by the same methods as used for sucrose gradients (see above). Filter elution is a very sensitive technique, enabling the detection of one DNA strand break per $10^7$ nucleotides.

The techniques described above are of use for the determination of strand breaks in DNA of high molecular weight. However, it is often of use to be able to quantify strand breaks in small defined DNA molecules, treated in vitro with DNA damaging agents. Techniques have been developed which utilise small circular supercoiled DNA molecules as the substrate. This method involves the conversion of the supercoiled form to open circular and linear forms by single and double strand breaks, and the separation of these by agarose gel electrophoresis. Before the technique can be described in detail, it is necessary to discuss DNA supercoiling. ("Genes" by B. Lewin, Pub. John Wiley & Sons, 1983.)

In double-stranded DNA, the two polynucleotide chains are antiparallel (run in opposite directions) and are twisted about one another to form a right handed double helix, which has 10 bp per turn. The helix has a major groove (22Å) in
diameter) and a minor groove (12Å). This structure is known as B-DNA, and is thought to be the predominant form in vivo. In circular DNA, the two strands of the double helix run continuously about one another, and in chromatin, there are large loops of DNA held together at the base which effectively have no free ends. (These regions are known as domains.) In both of these cases, the lack of free ends allows supercoiling. This can be envisaged as a twisting of the DNA about its own axis. In naturally occurring DNA, the twisting is always in the opposite direction to the turns of the double helix, and is hence termed negative supercoiling. A typical level of supercoiling in vivo is one negative turn per 200 bp. Supercoils are introduced by enzymes known as topoisomerases, such as bacterial DNA gyrase. This enzyme is thought to act by holding a positive supercoil in place in the DNA, then inverting the sign of the supercoil by introducing a break in one of the double strands, passing it through the other strand and resealing the break. (See figure 3.1) Thus if the strands of the double helix are then denatured, two interlocking rings are formed since the two strands cannot completely separate unless a nick is introduced in one strand.

The introduction of a single strand nick in a supercoiled circular molecule (known as form I) immediately relaxes the supercoils, giving rise to nicked open circular (form II) DNA. A double strand break in the supercoiled form leads to the production of linear (form III) DNA. This also arises as the result of a second single strand break in open circular form opposite to the first. (See figure 3.2) Although all these three forms are of the same molecular weight, they can
be separated by agarose gel electrophoresis due to their different conformations.

Figure 3.1: Proposed mechanism for the action of bacterial DNA gyrase; the introduction of a negative supercoil by the inversion of a positive supercoil.

The supercoiled form is the most 'compact' of the three, and hence has the fastest mobility. Open circular form is the least compact, and has the slowest mobility. The linear form has a mobility somewhere between that of the other two. (See figure 3.3 panel A.) The supercoiled DNA is treated with the DNA damaging agent of interest, and the samples run out on agarose gels to separate the three forms. The gels are then stained with ethidium bromide, and photographed under UV light. The different forms of the DNA give rise to three separate bands, the fluorescence in each band being proportional to the amount of DNA present. This is with the exception of the supercoiled form which takes up less ethidium bromide than the relaxed forms due to topological constraints. It has been calculated that for bacteriophage PM2 DNA, the supercoiled form takes up EtBr with 70% the efficiency of non supercoiled forms. (Lloyd et al, 1978) This factor must therefore be taken into account when calculating
Analysis of γ Radiation Induced Single and Double Strand Breaks Using Plasmid DNA.

Superhelical Plasmid
FORM I

Double Strand Break

Single Strand Break

Linear Plasmid
FORM III

Open Circular "Nicked" Plasmid
FORM II

etc.
Figure 3.3

Agarose gel electrophoresis of irradiated DNAs. The direction of DNA migration in all of the gels is from the top to the bottom of the page.

Panel (A) is plasmid pBR322 DNA (100μg/ml) irradiated at 77 K in the presence and absence of 40mM mercaptoethylamine, oxic and anoxic conditions. The faint band nearest the wells (top of the page) is chromosomal DNA. The next down is form II DNA, and the band nearest the bottom of the gel is form I DNA. The band with intermediate mobility is form III DNA. (Not present in all tracks.) Tracks 1, 6, 16 are unirradiated. Tracks 2, 7, 12 & 17 given 0.9 MRad. Tracks 3, 8, 13 & 18 given 0.6 MRad. Tracks 4, 9, 14 given 0.9 MRad. Tracks 5, 10, 15 & 20 given 1.2 MRad. Tracks 1-10 irradiated under oxygenated conditions, tracks 11-20, irradiated under deoxygenated conditions. Tracks 1-10 given 0.9 MRad, tracks 11-20, given 1.2 MRad.

Panel (B) M13 bacteriophage particles irradiated at 77 K buffer, and treated with SDS prior to loading onto the gel. The band towards the top of the gel is circular M13 DNA, and the band below it is linear M13 DNA. Tracks 1 & 2, unirradiated. Tracks 3 & 4, 0.3 MRad; tracks 5 & 6, 0.6 MRad; tracks 7 & 8, 1.2 MRad; tracks 9 & 10, 1.8 MRad; tracks 11 & 12, 1.8 MRad.

Panel (C) is M13 single stranded DNA irradiated at RT concentration of 200μg/ml. The positions of the bands are the same in panel (A). Tracks 1 & 2, unirradiated. Tracks 3 & 4, 0.1 MRad; tracks 5 & 6, 0.2 MRad; tracks 7 & 8, 0.3 MRad.
the relative amounts of DNA present in each band. In order to quantify the amount of DNA present in each band, the gels or negatives of the gels are subjected to densitometric scanning. Molecules with multiple double strand breaks form a heterogeneous population of molecular weights, and therefore give rise to a 'smear' on the gel. This technique can also be applied to the measurement of strand breaks in circular single-stranded DNA molecules. The single-stranded circle is converted to a linear molecule by one single strand break. The circular and linear forms can be separated out by agarose gel electrophoresis, and the strand breaks quantified as described above for double-stranded DNA.

The relaxation of supercoils has been used to determine strand breaks introduced by bleomycin (Lloyd et al., 1978) and bleomycin analogues (Heary et al., 1981). Malvy (1984) has described a modification of the method to determine the strand breaks induced by various intercalators. In this case the intercalator must be removed by extraction with isobutanol prior to gel electrophoresis, to prevent interference with the ethidium bromide staining. Strong and Crooke (1978) have described a modification of the method to determine strand breaks induced by the antitumor antibiotic tumblesomycin. Supercoiled PM2 DNA samples were treated with the drug, then placed in denaturing buffer containing 0.15M NaOH and 22μg/ml EtBr. Under these conditions, the EtBr binds primarily by intercalation, and was shown to have negligible binding to single-stranded DNA. On denaturation, supercoiled DNA retains some of its native conformation, and does bind EtBr, whereas the non-supercoiled forms do not. The amount of supercoiled DNA remaining after treatment with the drug can
therefore be measured as the fluorescence of the sample above background.

A recent publication (Frisch et al., 1985) has demonstrated the use of an SV40 viral probe for the assay of DNA strand breaks applicable to mammalian cells. SV40 is a small circular DNA virus which has its DNA complexed with host histones, and hence has a genome analogous to eukaryotic chromatin. The viruses were irradiated, and then infected into host cells. Irradiation apparently had a negligible effect on viral uptake by the cells. The cells were allowed to incubate for various times, then the cellular DNA (which also contains progeny viral DNA molecules) was extracted and run out on agarose gels. The DNA was transferred from the gels to nitrocellulose filters which were probed with ³²P labelled SV40 DNA. The viral bands were localised by autoradiography, and strand breaks quantified by densitometric scanning. This method is likely to prove of use in the study of DNA repair by mammalian cells.

Our group has used the conversion of supercoiled pBR322 DNA to open circular and linear forms for the quantitation of gamma radiation-induced strand breaks in the presence of various additives. (Boon et al., 1984, 1985; Cullis et al., 1985). The strand break assay was carried out exactly as described in Chapter 2, section (12). It has been established that under the conditions used, the reproducibility of the method is ±0.74% for single strand breaks, but less accurate for double strand breaks which make up only a very small percentage of the total. (Wren, 1985). In the study described here, the plasmid based assay has been used to quantify the gamma radiation-induced strand breaks in the presence of
hydrogen peroxide, a radiosensitiser; and mercaptoethylamine (MCE), a radioprotector.

3.12 DNA Damage by Active Oxygen Species

During gamma irradiation of aqueous systems, most of the energy is deposited in the water, since this forms a much greater target area than the solutes dissolved in it. The radiolysis of water leads to the formation of a number of products: $H_2O_2$, $H_2$, $H^\cdot$, $OH^\cdot$ and solvated electrons. In the presence of oxygen, solvated electrons and oxygen react to form $HO_2^\cdot$ and $O_2^-$. The latter is the superoxide anion, which can react further to give $OH^\cdot$ via $H_2O_2$. The reactions leading to the formation of these are described in greater detail in Chapter 1, section 1.1. The hydroxyl radical, $OH^\cdot$ is considered to be the main species responsible for DNA damage resulting from the gamma irradiation of dilute aqueous solutions of DNA under oxic conditions at RT. In fact under these conditions, diffusing $OH^\cdot$ radicals are thought to account for 80-90% of the DNA damage. (Ward, 1975; see also Chapter 1)

The reduced oxygen products $H_2O_2$, $O_2^\cdot$, $HO_2^\cdot$ and $OH^\cdot$ are not unique to irradiated systems, but are common products of the reduction of oxygen in biological systems. For instance, the superoxide anion is produced during the oxidation of substrates such as ferrodoxins and haemoglobins, and also by the action of some oxidative enzymes. $H_2O_2$, $O_2^\cdot$ and $OH^\cdot$ have all been implicated as causes of cell death, mutagenesis, ageing, carcinogenesis and wasting diseases such as arthritis. (Mello-Filho et al., 1984). $H_2O_2$ is found at a
concentration of between $10^{-8}$ and $10^{-9}$ M in fibroblast cell lines, and is cytotoxic at $10^{-5}$ M. From the above discussion it is clear that activated oxygen species are a severe problem for all metabolising oxidative cells. It is therefore of no surprise to find cellular enzymes concerned with the removal of such potentially damaging species. The superoxide anion is eliminated by a family of enzymes known as the superoxide dismutases (SODs), which catalyse the following reaction:

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Although this reaction will in fact occur spontaneously at neutral pH, the spontaneous reaction is around $10^{10}$ times slower than the enzymic one due to the low steady state concentration of $\cdot O_2^-$ in the cell. It is essential for the cell to also remove $H_2O_2$, since this can react to produce hydroxyl radicals, which are highly reactive species. $H_2O_2$ is scavenged in the cell by the enzyme catalase:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

As stated above, $H_2O_2$ can lead to $OH^-$ radical formation. This reaction is catalysed by transition metal ions, in particular, iron (the 'Fenton' reaction).

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^- + Fe^{3+}$$

Therefore the superoxide dismutases and catalase are essential in the prevention of cell damage by active oxygen.
species, in particular, OH'. There have been suggestions, however, that a very low level of OH' may be important in the induction of mutations, and hence genetic variability in oxidatively metabolising organisms. (Brawn & Fridovich, 1980)

It is clear from the above discussion that both superoxide dismutases and catalase should protect DNA against the indirect effects of ionising radiation, by scavenging the potentially damaging radiolysis products of water. Indeed, several authors have shown that this is the case. Van Hemmen and Meuling (1975) demonstrated that SOD and catalase protect the phage ϕX174 from inactivation by the indirect action of gamma rays in phosphate–buffered formate solution. It has been reported that SOD has a radioprotective effect against strand breaks induced in calf thymus DNA irradiated in aqueous solution. (Zalesna et al, 1980) In addition, it was shown that boiled (hence inactive) SOD did not give radioprotection, indicating that the radioprotective ability is a consequence of enzymic activity, presumably acting to remove \( \cdot O_2^- \) from the system. Brawn and Fridovich (1980, 1981) demonstrated that the ColE-1 plasmid developed nicks when exposed to a flux of \( \cdot O_2^- \) and \( H_2O_2 \) (induced enzymically by the xanthine oxidase reaction). The nicking was found to be virtually eliminated by SOD or catalase. Since either enzyme provided complete protection, this suggested that both \( \cdot O_2^- \) and \( H_2O_2 \) were required for nicking.
These can react together to generate $\cdot OH^-$, in a two step reaction catalysed by transition metal ions (the Haber and Weiss reactions):

$$\text{Fe}^{3+} + \cdot O_2^- \rightarrow \text{Fe}^{2+} + O_2 \quad \text{(i)}$$

$$\text{Fe}^{2+} + H_2O_2 \rightarrow \text{Fe}^{3+} + \cdot OH^- \quad \text{(ii)}$$

Since this reaction depends on the presence of transition metal ions, it is expected that chelating agents will effectively 'mop' these up, and hence protect the DNA against damage. However, McCord and Day (1978) demonstrated that the chelating agent EDTA did not prevent the reaction between $\cdot O_2^-$ and $H_2O_2$, although the reaction was prevented by another chelator, DETAPAC. (Diethylene triamine pentaacetic acid.) Fridovich and Braun showed that DETAPAC also had a protective effect in their system. $H_2O_2$ is known to have a damaging effect on cells, and many studies showing this have been carried out. For instance, single strand breaks have been shown to be produced in the DNA of human fibroblast cells by $H_2O_2$, although it does not induce strand breaks in purified fibroblast DNA. (Brawn & Fridovich, 1980) This suggests the involvement of a cellular cofactor, and from the above discussion, it seems likely that this is an intracellular metal ion, probably $\text{Fe}^{3+}$. Indeed, the chelating agent 1,10-phenanthroline was able to protect cells against $H_2O_2$ induced strand breaks, (Brawn & Fridovich, 1980). Meneghini and Hoffmann (1980) working on fibroblast nuclei and Lesko et al. (1980) working on phage T7 DNA both came to the
conclusion that DNA damage induced by $H_2O_2$ occurs via a
reaction with transition metal ions giving rise to the highly
reactive species $OH^-$. These studies have been carried further
by Hoffmann et al. (1984) who demonstrated a strong
correlation between cell killing and $OH^-$ induced strand break
formation.

This study focuses on the effect of exogenously added $H_2O_2$
to the level of gamma radiation-induced strand breaks in
pBR322 DNA irradiated in frozen aqueous solution. As
described above and in Chapter 1, the radiolysis products of
water give rise to the majority of gamma radiation-induced
damage in dilute aqueous solutions. In frozen solutions,
however, the DNA and water form a phase-separated system with
only the hydration water partitioning out with the DNA phase.
It has been discussed in previous work of the group that
compounds added to the DNA solution prior to freezing
partition out largely in the DNA phase. (Roon et al., 1985;
Cullis et al., 1985). Therefore if $H_2O_2$ is added prior to
freezing, it is expected to remain in close proximity to the
DNA during irradiation, undergoing radiolytic conversion to
$OH^-$. The $OH^-$ radicals are likely to attack the DNA as occurs
in irradiated dilute aqueous solutions, hence $H_2O_2$ is
expected to act as a radiosensitizer. During these
experiments, steps were taken to ensure that Fenton-type
reactions to yield $OH^-$ via transition metal ion catalysed
reactions were not occurring. (These are discussed later.)

Another purpose of this study was to investigate the
observation by H. Loman (personal communication) that during
irradiation at 77K, if a DNA sample is removed from the
irradiation chamber and warmed to room temperature, followed
by re-freezing and continuing the irradiation, a greater level of damage is seen than in a sample irradiated for the same dose but not warmed to RT. In the light of the above discussion, it is possible that this phenomenon is due to the production of $H_2O_2$ in the water phase during irradiation, which is released on warming the solution up. On re-freezing, this is now expected to partition out in the DNA phase; thus leading to radiosensitisation. Loman's observation was repeatable in this study, and it was also demonstrated that subjecting a DNA sample to a number of freeze-thaw cycles does not damage the DNA in the absence of irradiation. The experiments with $H_2O_2$ demonstrated that in frozen aqueous solution, $H_2O_2$ acts as a radiosensitiser.

3.13 Radioprotection by Thiols

In aqueous systems, there are basically two mechanisms of radioprotection, scavenging of diffusing free radicals (for instance by ethanol and ethylene glycol which are OH' radical scavengers), and 'chemical repair' on target molecules. The thiols (compounds with an -SH group) are perhaps the most extensively studied of the radioprotectors; these act both as OH' scavengers and chemical repair agents. Many thiols occur in cells and may therefore act as 'natural' radioprotectors, particularly if bound to DNA. (Copeland, 1978) A major mechanism for OH' attack is the abstraction of hydrogen atoms from the DNA molecule:

$$RH \rightarrow R^- + H'$$
Such types of damage may be chemically repaired by H atom donation:

\[ R^- + R'SH \rightarrow R'S^- + RH \]

If such repair processes do not occur, the reaction proceeds to yield stable DNA damage products, which can then only be repaired by enzymic pathways.

Radioprotection by thiols has been shown to be in competition with oxygen. Hydrogen atom donation from -SH compounds to radiolytically produced organic radicals has been demonstrated by pulse radiolysis in a purely chemical system, and this reaction has been shown to be in competition with oxygen. (Adams et al, 1966) Many studies in biological systems have also demonstrated this phenomenon. For instance, the protection by a range of thiols against the radiation induced inactivation of transforming DNA (Zalesna et al, 1980), and the protection by glutathione and cysteine against strand breaks induced in calf thymus DNA by gamma rays (Held, 1984). Radioprotection by thiols is therefore more effective in anoxic than in oxic systems; presumably the reaction of oxygen with DNA radicals is faster than the reaction of -SH compounds with DNA radicals.

Most studies on the protective effects of thiols have focused on dilute aqueous solutions. The study described here investigates the effect of the thiol mercaptoethylamine (cysteamine, \( HSCH_2CH_2NH_2 \)) on radiation induced strand breaks in oxic and anoxic solution at 77K. From previous studies we know that strand breaks occur under both oxic and anoxic irradiation conditions at 77K. (Boon et al, 1984) The DNA
radicals which arise under these conditions are thought to react largely via intramolecular H-atom abstraction reactions on adjacent deoxyribose moieties, thus leading to strand breaks (among other types of damage). Strand breaks may arise via a β-elimination mechanism similar to that proposed to occur in dilute aqueous solution. (Bothe et al, 1984; see also Chapter 6) It is therefore to be expected that thiols will act as radioprotectors against direct as well as indirect irradiation damage. The study described here reveals that 40mM mercaptoethyamine (MCE) protects against radiation-induced strand breaks under oxic and anoxic conditions to about the same extent. At 5mM MCE, however, competition between oxygen and the thiol was observed.
3.2 RESULTS

3.21 The Effect of Hydrogen Peroxide on Radiation Induced Strand Breaks

(i) The Effect of $\text{H}_2\text{O}_2$ concentration.

pBR322 DNA was irradiated at a concentration of approximately 100µg/ml at 77K, and given a total dose of 0.65 MRad (ambient atmosphere). Hydrogen peroxide was included in the samples during irradiation at a range of concentrations between $4\times10^{-5}$ mM and 4mM. Samples were also irradiated for the same dose in the absence of $\text{H}_2\text{O}_2$. The irradiated samples (and unirradiated controls) were analysed by agarose gel electrophoresis, to separate the supercoiled (form I), open circular (form II) and linear (form III) plasmid molecules. The amount of DNA present in each band on the gel was quantified as described in Chapter 1, section (12). The percentage of supercoiled DNA was calculated, and the average of the duplicates plotted against $\text{H}_2\text{O}_2$ concentration. (See figure 3.4) The data are shown in Table I below.
Figure 3.4

The effect of hydrogen peroxide concentration on the $\log_{10} \%$ form I (supercoiled) DNA remaining following an irradiation dose of 0.65 MRad at 77K.
TABLE I: The Effect of H$_2$O$_2$ concentration on Radiation-Induced Strand Breaks.

<table>
<thead>
<tr>
<th>[H$_2$O$_2$] mM (0.65 MRad)</th>
<th>ARBITRARY UNITS</th>
<th>Form I</th>
<th>Form II</th>
<th>Form III</th>
<th>Log$_{10}$ % Form I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td></td>
<td>27</td>
<td>266</td>
<td>58</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>337</td>
<td>58</td>
<td>0.95</td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td>36</td>
<td>320</td>
<td>83</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34</td>
<td>316</td>
<td>58</td>
<td>1.06</td>
</tr>
<tr>
<td>4x10^{-2}</td>
<td></td>
<td>46</td>
<td>376</td>
<td>52</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>370</td>
<td>42</td>
<td>1.32</td>
</tr>
<tr>
<td>4x10^{-3}</td>
<td></td>
<td>140</td>
<td>366</td>
<td>41</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>227</td>
<td>30</td>
<td>1.55</td>
</tr>
<tr>
<td>4x10^{-4}</td>
<td></td>
<td>112</td>
<td>336</td>
<td>32</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164</td>
<td>280</td>
<td>34</td>
<td>1.63</td>
</tr>
<tr>
<td>4x10^{-5}</td>
<td></td>
<td>91</td>
<td>206</td>
<td>31</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>124</td>
<td>282</td>
<td>36</td>
<td>1.55</td>
</tr>
<tr>
<td>0.0</td>
<td></td>
<td>132</td>
<td>302</td>
<td>38</td>
<td>1.55</td>
</tr>
<tr>
<td>Unirradiated, no H$_2$O$_2$</td>
<td>500</td>
<td>180</td>
<td>0</td>
<td></td>
<td>1.90</td>
</tr>
<tr>
<td>Unirradiated, 4mM H$_2$O$_2$</td>
<td>168</td>
<td>64</td>
<td>0</td>
<td></td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>1119</td>
<td>518</td>
<td>0</td>
<td></td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>219</td>
<td>40</td>
<td>0</td>
<td></td>
<td>1.96</td>
</tr>
</tbody>
</table>

(ii) The Effect of 4mM H$_2$O$_2$ at a Range of Radiation Doses.

The radiosensitising effect of H$_2$O$_2$ on gamma radiation-induced strand breaks appears to reach a plateau at a concentration of around 4mM. This concentration was therefore chosen to investigate the dose-response effect. Samples of plasmid DNA were irradiated in frozen aqueous solution at 77K (ambient atmosphere) in the presence and absence of 4mM H$_2$O$_2$. 

-89-
or left unirradiated as controls. Details of experimental protocol and calculation of the data are as described in Chapter 2, section (12). The data are shown in tables II and III below. See also Figure 3.5.

**TABLE II:** Irradiation at 77K in the presence of 4mM H$_2$O$_2$.

<table>
<thead>
<tr>
<th>DOSE, MRAD</th>
<th>% Form II</th>
<th>% FORM III</th>
<th>LOG$_{10}$ % FORM I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.5</td>
<td>0</td>
<td>1.88</td>
</tr>
<tr>
<td>08.8</td>
<td>0</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>18.8</td>
<td>0</td>
<td>1.91</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>43.5</td>
<td>3.2</td>
<td>1.73</td>
</tr>
<tr>
<td>43.4</td>
<td>5.2</td>
<td>1.71</td>
<td></td>
</tr>
<tr>
<td>46.7</td>
<td>3.5</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>50.6</td>
<td>5.4</td>
<td>1.64</td>
</tr>
<tr>
<td>50.3</td>
<td>6.9</td>
<td>1.63</td>
<td></td>
</tr>
<tr>
<td>53.8</td>
<td>4.8</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>72.4</td>
<td>13.0</td>
<td>1.64</td>
</tr>
<tr>
<td>61.5</td>
<td>12.0</td>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td>78.7</td>
<td>10.3</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>71.9</td>
<td>18.1</td>
<td>1.00</td>
</tr>
<tr>
<td>66.0</td>
<td>21.7</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>76.7</td>
<td>15.6</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>77.6</td>
<td>18.6</td>
<td>0.59</td>
</tr>
<tr>
<td>56.0</td>
<td>28.5</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>74.8</td>
<td>21.5</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>
TABLE III: Irradiation at 77K in the absence of H$_2$O$_2$.

<table>
<thead>
<tr>
<th>DOSE MRAD</th>
<th>%FORM II</th>
<th>%FORM III</th>
<th>LOG$_{10}$ % FORM I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.1</td>
<td>0</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>21.0</td>
<td>0</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>16.1</td>
<td>0</td>
<td>1.92</td>
</tr>
<tr>
<td>0.1</td>
<td>29.5</td>
<td>0</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>31.5</td>
<td>4.7</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>39.5</td>
<td>0</td>
<td>1.78</td>
</tr>
<tr>
<td>0.2</td>
<td>41.4</td>
<td>0</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>25.6</td>
<td>6.0</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>46.6</td>
<td>2.8</td>
<td>1.70</td>
</tr>
<tr>
<td>0.3</td>
<td>44.6</td>
<td>3.0</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>39.8</td>
<td>6.4</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>47.7</td>
<td>3.6</td>
<td>1.69</td>
</tr>
<tr>
<td>0.6</td>
<td>45.9</td>
<td>7.6</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>45.8</td>
<td>7.3</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>59.6</td>
<td>4.4</td>
<td>1.57</td>
</tr>
<tr>
<td>0.9</td>
<td>56.6</td>
<td>8.7</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>46.1</td>
<td>8.9</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>61.6</td>
<td>9.1</td>
<td>1.47</td>
</tr>
</tbody>
</table>

In addition to plotting the percentages of form II and form III DNA against dose in figure 3.5, the figures for Log$_{10}$ % form I DNA against dose were used to calculate the best straight line for Log$_{10}$ % form I DNA against dose, by the method of least squares fit. In the absence of H$_2$O$_2$, the calculated line has a slope of -0.335 and passes through the y-axis at 1.885 Log$_{10}$ % form I DNA. In the presence of H$_2$O$_2$, the line has a slope of -1.462, and passes through the y-axis at 1.855 Log$_{10}$ % form I DNA. By comparing the calculated slopes of these two lines, it was estimated that 4mM H$_2$O$_2$ increases the rate of disappearance of form I DNA about 4 fold.
Figure 3.5
The effect of 4mM hydrogen peroxide on the % of form II (nicked) and form III (linear) plasmid DNA at a range of irradiation doses at 77K. △, ●: no H₂O₂. □, ■: 4mM H₂O₂. Open symbols, form III DNA; filled symbols, form II DNA. (Conditions of ambient atmosphere.)
(iii) The Effect of the Addition of $H_2O_2$ After Irradiation.

Samples were irradiated at 77K for 0.6 MRad in the presence or absence of 4mM $H_2O_2$. The effect on DNA strand breaks of adding $H_2O_2$ after irradiation was analysed. Quantitation of SBs was carried out as described in Chapter 2, section (12). Each experiment was carried out in triplicate, the averages of the triplicates are shown in table IV below:

<table>
<thead>
<tr>
<th>$H_2O_2$</th>
<th>IRRADIATION</th>
<th>% FORM I</th>
<th>% FORM II</th>
<th>% FORM III</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>NONE</td>
<td>91.3</td>
<td>8.6</td>
<td>0</td>
</tr>
<tr>
<td>4mM</td>
<td>NONE</td>
<td>89.2</td>
<td>10.8</td>
<td>0</td>
</tr>
<tr>
<td>NONE</td>
<td>0.6MRad</td>
<td>50.1</td>
<td>48.2</td>
<td>1.8</td>
</tr>
<tr>
<td>4mM during</td>
<td>0.6MRad</td>
<td>31.3</td>
<td>64.1</td>
<td>4.6</td>
</tr>
<tr>
<td>4mM after</td>
<td>0.6MRad</td>
<td>49.8</td>
<td>48.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

(iv) The Effect of Dose Fractionation on Radiation-Induced Strand Breaks.

All samples were irradiated at 77K, with a total dose of 0.3 MRad. Samples were removed from the chamber at various times during irradiation and allowed to warm to RT before re-freezing and continuing the irradiation. The data are shown in Table V below, and the $\log_{10} %$ form I DNA shown plotted against the number of freeze-thaw cycles in figure 3.6.
Figure 3.6
The effect of dose fractionation during irradiation on the 
$\log_{10}$ % form I plasmid DNA. Samples were removed from the 
chamber during irradiation, and allowed to warm to room 
temperature before re-freezing and continuing the 
irradiation. All samples were given a total dose of 0.3MRad. 
(Conditions of ambient atmosphere.)
### TABLE V.

<table>
<thead>
<tr>
<th>No of freeze-thaw cycles</th>
<th>Form I</th>
<th>Forms II + III</th>
<th>( \log_{10} % ) Form I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>190</td>
<td>208</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>196</td>
<td>196</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>365</td>
<td>378</td>
<td>1.76</td>
</tr>
<tr>
<td>1</td>
<td>192</td>
<td>251</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>164</td>
<td>228</td>
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<tr>
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<td>254</td>
<td>354</td>
<td>1.71</td>
</tr>
<tr>
<td>2</td>
<td>174</td>
<td>246</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>157</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>193</td>
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<td>1.63</td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>237</td>
<td>1.63</td>
</tr>
<tr>
<td>4</td>
<td>160</td>
<td>322</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>200</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>211</td>
<td>351</td>
<td>1.66</td>
</tr>
</tbody>
</table>

(v) The Effect of Freeze-Thaw Cycling After Irradiation.

A sample of pBR322 DNA was irradiated at 77K with a dose of 3.6 MRad to produce a population containing approximately 85% form II DNA and 15% form III DNA. The irradiated sample was divided into a number of aliquots, and subjected to a number of freeze-thaw cycles. The relative proportions of each form of DNA was quantified after treatment. The data are shown in Table VII below.
TABLE VII.

<table>
<thead>
<tr>
<th>No. of freeze-thaw cycles</th>
<th>% Form II</th>
<th>% Form III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>87.6</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>83.6</td>
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<td>16.7</td>
</tr>
<tr>
<td></td>
<td>86.4</td>
<td>13.6</td>
</tr>
<tr>
<td>4</td>
<td>84.2</td>
<td>15.8</td>
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<td></td>
<td>85.3</td>
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<tr>
<td>6</td>
<td>87.5</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>87.1</td>
<td>12.9</td>
</tr>
</tbody>
</table>

3.22 The Effect of Mercaptoethylamine on Gamma Radiation-Induced Strand Breaks

The experiments described in this section were carried out in collaboration with G.D.D Jones. Samples of plasmid DNA at a concentration of 100μg/ml in the presence and absence of mercaptoethylamine (MCE) were purged with oxygen or nitrogen for 30 minutes prior to irradiation. Experiments were carried out in the presence (and absence) of 40mM or 5mM MCE. Quantitation of strand breaks was carried out as described in Chapter 2, section (12), and the data are shown in tables VII and VIII below. The average of duplicates is given. The percentage of forms II and III DNA against radiation dose is plotted in figures 3.7a and 3.8a. The $\log_{10}$ % of form I DNA is plotted against dose in figures 3.7b and 3.8b.
TABLE VII: The effect of 40mM MCE on radiation-induced strand breaks.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>DOSE MRAD</th>
<th>% FORM II</th>
<th>% FORM III</th>
<th>$\text{LOG}_{10} % \text{ FORM I}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MCE</td>
<td>0</td>
<td>12.1</td>
<td>0</td>
<td>1.94</td>
</tr>
<tr>
<td>+$N_2$</td>
<td>0.3</td>
<td>39.9</td>
<td>3.5</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>56.5</td>
<td>3.5</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>67.1</td>
<td>3.8</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>66.8</td>
<td>6.9</td>
<td>1.41</td>
</tr>
<tr>
<td>40mM MCF</td>
<td>0</td>
<td>16.8</td>
<td>0</td>
<td>1.92</td>
</tr>
<tr>
<td>+$N_2$</td>
<td>0.6</td>
<td>36.2</td>
<td>2.9</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>44.6</td>
<td>1.8</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>40.9</td>
<td>1.7</td>
<td>1.75</td>
</tr>
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<td>No MCE</td>
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<td>12.0</td>
<td>0</td>
<td>1.94</td>
</tr>
<tr>
<td>+$O_2$</td>
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<td>44.7</td>
<td>2.2</td>
<td>1.72</td>
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<td>0.9</td>
<td>62.8</td>
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</tr>
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<td>1.2</td>
<td>60.9</td>
<td>8.9</td>
<td>1.48</td>
</tr>
<tr>
<td>40mM MCF</td>
<td>0</td>
<td>14.5</td>
<td>0</td>
<td>1.93</td>
</tr>
<tr>
<td>+$O_2$</td>
<td>0.3</td>
<td>28.9</td>
<td>1.6</td>
<td>1.84</td>
</tr>
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<td></td>
<td>0.6</td>
<td>33.5</td>
<td>1.1</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>42.8</td>
<td>2.1</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>49.7</td>
<td>1.7</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Figure 3.7a
The effect of 40mM MCE on z form II and form III plasmid DNA irradiated under oxic and anoxic conditions at 77K.
• purged with N₂ prior to irradiation. △, △ purged with O₂ prior to irradiation. ○, △ no MCE. •, △ 40mM MCE.
Figure 3.7b
The effect of 40mM MCE on $\log_{10}$ % form I plasmid DNA irradiated under oxic and anoxic conditions at 77K.
### TABLE VIII: The effect of 5mM MCF on radiation-induced strand breaks.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>DOSE MRAD</th>
<th>% FORM II</th>
<th>% FORM III</th>
<th>$\log_{10} %$ FORM I</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MCE</td>
<td>0</td>
<td>15.0</td>
<td>0</td>
<td>1.93</td>
</tr>
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</tr>
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<td>0.9</td>
<td>50.2</td>
<td>4.8</td>
<td>1.65</td>
</tr>
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<td>1.2</td>
<td>55.1</td>
<td>5.0</td>
<td>1.60</td>
</tr>
<tr>
<td>5mM MCE</td>
<td>0</td>
<td>14.7</td>
<td>1.3</td>
<td>1.92</td>
</tr>
<tr>
<td>+$N_2$</td>
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<td>34.4</td>
<td>1.2</td>
<td>1.81</td>
</tr>
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<td></td>
<td>0.6</td>
<td>34.9</td>
<td>2.1</td>
<td>1.80</td>
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<td>0.9</td>
<td>43.6</td>
<td>2.5</td>
<td>1.73</td>
</tr>
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<td>1.2</td>
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<td>No MCF</td>
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<td>14.7</td>
<td>0</td>
<td>1.93</td>
</tr>
<tr>
<td>+$O_2$</td>
<td>0.3</td>
<td>40.1</td>
<td>2.1</td>
<td>1.76</td>
</tr>
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<td></td>
<td>0.6</td>
<td>43.2</td>
<td>4.5</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>50.8</td>
<td>8.4</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>53.9</td>
<td>8.8</td>
<td>1.57</td>
</tr>
<tr>
<td>5mM MCE</td>
<td>0</td>
<td>19.7</td>
<td>0</td>
<td>1.90</td>
</tr>
<tr>
<td>+$O_2$</td>
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<td>35.5</td>
<td>0.6</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>42.9</td>
<td>2.3</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>48.9</td>
<td>2.0</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>53.7</td>
<td>3.6</td>
<td>1.63</td>
</tr>
</tbody>
</table>
Figure 3.8a
The effect of 5mM MCE on Z form II and form III plasmid DNA irradiated under oxic and anoxic conditions at 77 K. o, purged with N₂ prior to irradiation, Δ, Δ, purged with O₂ prior to irradiation, o, Δ no MCE, ●, ▲ 5mM MCE.
The effect of 5mM MCE on $\log_{10} \%$ form I plasmid DNA irradiated at 77K under oxic and anoxic conditions.
3.3 DISCUSSION

3.31 The Effects of Hydrogen Peroxide and Fractionated Dose on Radiation-Induced Strand Breaks.

ESR spectroscopy of calf thymus DNA irradiated at 77K under conditions of ambient atmosphere, in the presence and absence of \( \text{H}_2\text{O}_2 \) was carried out by G.D.D Jones and J.D McClymont. As described in Chapter 1, in the absence of additives, there are three primary radicals detected by ESR spectroscopy of DNA irradiated at 77K: \( \cdot\text{G}^+ \), \( \cdot\text{T}^- \) in the DNA phase and \( \cdot\text{OH}^- \) in the ice crystallites. In the presence of \( \text{H}_2\text{O}_2 \), \( \cdot\text{HO}_2^- \) radicals were additionally detected. As the hydrogen peroxide concentration was increased, the concentration of the DNA radicals was seen to fall, concurrent with the appearance of new features, designated "X". Little change in the spectra over those obtained in the absence of additives was observed between 0.04mM and 0.4mM \( \text{H}_2\text{O}_2 \), but between 4 and 40mM \( \text{H}_2\text{O}_2 \) several changes were apparent. There was a substantial lowering of the temperature at which DNA radicals were lost, in the absence of additives, these were lost by around 255K, but in the presence of \( \text{H}_2\text{O}_2 \), DNA radicals were lost between 190 and 210K. In the presence of \( \text{H}_2\text{O}_2 \), little or no formation of \( \cdot\text{TH}^+ \) was observed. By 165K, the predominant features were "X" and \( \cdot\text{HO}_2^- \) radicals, these are not detected in the absence of additives.

In this and previous studies (Boon et al, 1984, 1985; Cullis et al, 1985) it was noted that ESR spectroscopy reveals that hydroxyl radicals in the ice crystallites are lost on annealing without change in the DNA radicals.
indicating that they react to form non-radical species without coming into contact with the DNA. The contribution of OH' to DNA damage under direct irradiation conditions is therefore negligible. The ESR data support the assumption that on freezing of DNA solutions at 77K with exogenously added $H_2O_2$, the $H_2O_2$ partitions into the DNA phase. The evidence for this is the appearance of spectral features for HO' which resemble those obtained in aqueous glassy systems rather than those obtained in dilute aqueous solution.

Since the presence of $H_2O_2$ during irradiation was seen to diminish both 'T- and TH', this suggests that $H_2O_2$ must be acting as an electron trap:

$$H_2O_2 + e^- \rightarrow OH^- + OH^-$$

Although OH' features are expected to be produced, they were not detected, presumably due to masking by the OH' features from ice. The OH' radicals in ice disappear by around 130K, but OH' radicals are still not detectable in the DNA phase, suggesting that by this temperature they must have reacted with the DNA. This is supported by the appearance of "X" features, which are assumed to be radicals formed from H-atom abstraction reactions at various positions in the deoxyribose sugars. (See Cullis et al, 1985.)

The ESR data suggest that OH' radicals are formed from $H_2O_2$ in the vicinity of the DNA, and these subsequently go on to attack the sugars. Hydrogen atom abstraction reactions lead to various types of DNA damage including DNA strand breaks. (See Chapter 1) Therefore an increase in DNA strand breaks when $H_2O_2$ is included during irradiation is expected. This
was indeed observed, both single and double strand breaks increased as the \( \text{H}_2\text{O}_2 \) concentration was increased up to around 4mM. (See figure 3.4) No effect was seen below about 4x10^-4 mM. The increase in the percentage of forms II and III DNA irradiated in the presence of 4mM \( \text{H}_2\text{O}_2 \) over a range of doses was compared with irradiation in the absence of \( \text{H}_2\text{O}_2 \). At all doses, radiosensitisation by \( \text{H}_2\text{O}_2 \) was observed. (See figure 3.5) The data for \( \log_{10} \) form I were used to calculate the slope of the line for \( \log_{10} \) form I DNA against dose in the presence and absence of \( \text{H}_2\text{O}_2 \). This showed that form I DNA is lost four times more rapidly in the presence of 4mM \( \text{H}_2\text{O}_2 \) than in its absence.

As discussed in previous work of the group (Boon et al. 1984), the yield of double strand breaks resulting from the irradiation of plasmid DNA at 77K is much greater than expected from the coincidence of two single strand breaks on opposite strands. This suggests that 'G+' and 'T-' (which are formed as the result of a single ionisation event) must tend to become trapped fairly close together, and furthermore, both must be potential precursors of strand breaks. Charge migration is therefore not thought to be extensive, this is discussed further in Chapter 5. In the presence of \( \text{H}_2\text{O}_2 \), double strand breaks are increased still further, presumably because \( \text{OH}^- \) and \( \text{HO}_2^- \) are initially trapped close to the DNA molecule, and therefore have a greater chance of becoming trapped in pairs, compared to the situation in the fluid system.

As described in 3.12 above, transition metal ions react with \( \text{H}_2\text{O}_2 \) to produce \( \text{OH}^- \) radicals. It is possible that transition metal ions are present as contaminants in the
plasmid preparation, in particular, iron. The plasmid DNA was kept throughout these studies in TE buffer, which contains 1mM EDTA. However, it has been shown that EDTA-transition metal ion complexes are capable of catalysing the conversion of $H_2O_2$ to $OH^-$. (McCord & Day, 1978) To determine whether such reactions were occurring in these experiments, plasmid DNA was incubated without irradiation in the presence and absence of $H_2O_2$. No increase in DNA strand breaks in the presence of $H_2O_2$ was observed. However, it is possible that irradiation alters any contaminating metal ions in such a way that they are now able to take part in a Fenton type reaction, for instance, by alteration of the oxidation level. (eg. $Fe^{3+}$ to $Fe^{2+}$.) Therefore samples irradiated in the absence of $H_2O_2$ were incubated with $H_2O_2$ prior to carrying out the strand break analysis. No enhancement of strand breaks was apparent by carrying out a post-irradiation incubation with $H_2O_2$. Therefore in these studies, enhancement of DNA strand breaks by $H_2O_2$ was shown to be a solely radiation dependent phenomenon.

These experiments confirmed the observation by Loman that a sample irradiated at 77K for a fractionated dose, with warming to room temperature in between gave a greater level of strand breaks than a sample irradiated for the same total dose without fractionation. (See figure 3.6) In addition, it was shown that this was a result of irradiation rather than 'mechanical' damage due to repeated freeze-thawing, by carrying out a number of freeze thaw cycles in the absence of irradiation, in this case no enhancement of strand breaks was observed. (See table VI) These results strongly suggest that this phenomenon is due to the formation of $H_2O_2$ in the ice.
phase during irradiation, which on annealing to RT and re-freezing partitions into the DNA phase, where it acts as a radiosensitiser via the mechanisms postulated above.

3.2 The Effect of Mercaptoethylamine on Radiation-Induced Strand Breaks.

ESR experiments were carried out by G.D.D Jones and J. L. ea. Concentrations of MCE from 0.1 to 10mM were found not to alter the yields of \( ^{1} \text{G}^{+} \) or \( ^{1} \text{T}^{-} \) in irradiated frozen aqueous DNA, whereas concentrations from 40 to 140mM gave a small decrease in the yield of \( ^{1} \text{T}^{-} \). This was thought to be due to electron capture by a contaminating disulphide which was either present in the commercial preparation of MCF, or was formed during incubation of MCE with DNA. Under bothoxic and anoxic conditions in the presence of various thiols, DNA radicals were lost at temperatures well below those normally observed in the absence of additive. This loss was concomitant with the rise of signals for sulphur-centred radicals. Under anoxic conditions, quenching of DNA radicals occurred at concentrations as low as 0.5mM thiol, but under oxic conditions, between 5 and 10mM thiol was required for quenching. The quenching of DNA radicals by MCE is reflected in its radioprotective effects with respect to DNA strand breaks. (Figures 3.7 & 3.8)

Possible mechanisms of radioprotection by thiols are H-atom donation by RSH, and electron transfer from RS\(^{-}\). Although RS\(^{-}\) has a lower ionisation potential than RSH, the mechanism of radioprotection is not thought to arise through electron transfer (for instance to \( ^{1} \text{G}^{+} \), restoring G), since the amount
of RS⁻ available at neutral pH is is very small. In addition, it was found that methyl-S-cysteamine, which is likely to be a better electron donor than mercaptoethylamine, but which lacks an -SH group did not quench DNA radicals. (The effect of methyl-S-cysteamine on radiation-induced DNA strand breaks was not determined.)

Underoxic conditions, the main sulphur radical detected by ESR is RSO₂⁻, which arises from the addition of molecular oxygen to the thyl radical, RS'. The latter arise as the result of H-atom donation from RSH (for example, H₂NCH₂CH₂SH) to base radicals. The RSO₂⁻ radicals can further react via H-atom abstraction on the DNA to give RSO₂²⁻ and R'⁻. The latter may become oxygenated to form base peroxy radicals. In this way DNA damage is enhanced. (See scheme 3.9 below.) Alternatively, RSO₂⁻ may react with RSH, giving rise to RSO₂²⁻, and regenerating RS'. RS⁻ can then be oxidised once again to form RSO₂⁻, this cycle of events will proceed until all of the oxygen is used up. Hence there is a competition between the reaction of RSO₂⁻ with DNA (leading ultimately to DNA damage), and reaction of RSO₂⁻ with RSH to regenerate RS'. Thus as the concentration of thiol (RSH) is increased, regeneration of RS' (by the reaction of RSO₂⁻ with RSH) will predominate over H-atom abstraction by RSO₂⁻.
Figure 3.9: Scheme proposed for the effect of oxygen on radioprotection by thiols. (See text)

Under anoxic conditions, the TH radical is quenched by thiols to give TH₂ (reduced thymine), rather than T. Indeed, this is a product detectable by chemical analysis of DNA irradiated under direct conditions. (Cadet et al, 1983)
fate of \(^{1}G^+\) is uncertain, but if, as suggested, \(^{1}G^+\) decays by the loss of a proton to give \(GN^-\) (Huttermann et al., in press), H-atom donation by thiols would restore the guanine base.

The strand break data support these conclusions. MCE was found to act as a radioprotector both at 5mM and 40mM. However, at 40mM, MCE gave rise to approximately the same level of radioprotection under both oxic and anoxic conditions, in spite of the differences in ESR spectra under these conditions. (See figure 3.7b) However, on lowering the concentration of MCE to 5mM, although the level of radioprotection was reduced compared with 40mM MCE, it was apparent that protection was greater under anoxic than under oxic conditions. (See figure 3.8b) This supports scheme 3.9 above. It is therefore proposed that under oxic conditions and relatively low RSH concentrations (5mM), radioprotection occurs via H-atom donation to base radicals. However, oxygenation of the resultant thyllyl radicals gives rise to \(RSO_{2}^-\), which gives rise to DNA damage through H-atom abstraction. If the concentration of RSH is raised to 40mM, reaction of \(RSO_{2}^-\) with RSH to regenerate thyllyl radicals predominates over H-atom abstraction by \(RSO_{2}^-\) on DNA, thus increasing the overall level of radioprotection.
CHAPTER

fovr
CHAPTER 4

GAMMA RADIATION-INDUCED DAMAGE IN BACTERIOPHAGE M13

4.1 INTRODUCTION

Bacteriophage M13 is a single stranded filamentous coliphage with a circular DNA genome of about 6400 bases. The protein coat of the virion contains only two different proteins, the major coat protein of which there are around 2000 copies, and the minor coat protein of which there are only four copies present. The latter are required for adsorption to the host. The genome contains at least eight genes, and more than 90% of the genome is coding sequence. The biology of the filamentous phages is discussed in detail in 'The Single-Stranded DNA Phages' Eds. Denhart et al. (1979).

M13 phage particles enter the E.coli cell via adsorption to the bacterial pilus, a structure only present in 'male' bacteria, that is, those harbouring the F (fertility) plasmid. As well as coding for functions that give rise to pilus formation, the F plasmid contains the pro gene, which allows the host cells to grow on minimal medium. If this selection pressure is not maintained, the F plasmid, which is present as a single copy, is rapidly lost, thus E.coli cells must be maintained on minimal medium if they are to be infected by M13.

The phage particles adsorb to the F-pilus, and as they enter the cell are stripped of their coat proteins in the cell membrane. (See figure 4.1) The single-stranded DNA then
Fig 4.1. The life cycle of bacteriophage M13
acts as a template for the synthesis of the double-stranded form of the phage, the replicative form (RF). The viral single-stranded DNA is always the (+) strand, therefore synthesis of the complementary (-) strand is required as a template for mRNA synthesis as well as the production of progeny virus DNA. Once the RF molecule has been synthesised, phage proteins, including the gene II product accumulate. This protein induces nicking of the (+) strand of the RF. The free 3'-OH of the RF is extended by DNA polymerase using the (-) strand as the template, while the 5' end is displaced. After the (+) strand synthesis has migrated around the entire (-) strand, the gene II product cleaves the (+) strand again, thus separating the new (+) strand from the parental one. The new (+) strand is then converted to RF form. When 100 to 200 copies of the RF have accumulated in this way, the gene V protein reaches high levels, and interferes with (-) strand synthesis by binding selectively to the (+) strands. This allows the accumulation of (+) strands. These migrate to the periplasmic space where the gene V proteins are removed and replaced with phage coat proteins. The mature phage particles are then released without breakdown of the bacterial cell wall. M13 forms turbid plaques on bacterial lawns, since infected cells have a significantly slower growth rate than uninfected cells.

Several features of the life cycle of M13 make it ideal for use as a cloning vector. The single and double stranded forms can both be prepared from infected cells with relative ease, and both can be introduced into E.coli cells by transformation. The single-stranded form can also be introduced by infection with phage particles. The double
stranded form can be handled and maintained as a plasmid if desired. M13 has been developed into a cloning vector (Messing, 1983) by the introduction of a section of DNA into the genome, which contains a number of unique restriction enzyme sites, binding sites for unique oligonucleotide primers and the β-galactosidase gene. (The region containing the unique restriction enzyme sites is known as the polylinker; this varies between different M13 'strains'.)

The β-galactosidase gene forms the basis for the selection system for phage with DNA fragments inserted into the polylinker region. In the absence of cloned fragments, the β-galactosidase gene is intact, and in the presence of the inducer IPTG (isopropyl-β-D-thiogalactoside) is transcribed. The β-galactosidase enzyme hydrolyses the substrate, X-Gal, producing a blue dye. If fragments have been inserted into the polylinker region, the β-galactosidase gene is rendered inactive. Thus if a lawn of M13 infected bacteria is provided with IPTG and X-Gal, 'wild type' phage give rise to blue plaques, and recombinant phage to white plaques. The presence of a unique oligonucleotide primer binding site to the 3' side of the cloned fragments facilitates the production of a primed template for dideoxy sequencing or the production of labelled probes. The complete nucleotide sequence for the M13 vector, mp18 has recently been published. (Yanisch-Perron et al.)

The above considerations make M13 an ideal system for the study of the contribution of various types of radiation-induced damage to loss of infectivity. Both the single and the double-stranded forms can be investigated and the effect of irradiation on the phage particle compared with the effect
on purified single-stranded DNA. This study has concentrated exclusively on the single-stranded form, since this minimises DNA repair by the host cells. Single-stranded DNA is not a substrate for excision repair due to the requirement for a template strand. Experiments on the single-stranded phages φX174 and S13 show that there is no host cell reactivation of phage particles unless the host cells have been pre-irradiated. Reactivation of irradiated phage particles requires an active RecA gene and de novo protein synthesis, that is, the involvement of an inducible repair system. (See Chapter 1, section 1.4) In addition, there is little evidence of repair through genetic exchange between two or more damaged phages, and importantly, it has been shown that a single strand break causes inactivation of these single-stranded phages. ("The Single-Stranded DNA Phages" Eds. Denhart et al, 1978.)

M13 is a useful system for the study of radiation induced mutagenesis, mutants in the β-galactosidase gene being clearly identifiable by the production of white plaques. (Ayaki et al, 1986) Most lesions in M13 are lethal or 'silent', since if irradiated phage are introduced into unirradiated host cells, the mutation frequency is low; only 5 times greater than the spontaneous frequency in the LacI gene (Ayaki et al, 1986), and 4 times the spontaneous frequency in reversion of amber mutants (Brandenberger et al, 1981).

The aim of this study was to investigate the contribution to loss of infectivity (or transformation frequency) in M13 phage particles and purified M13 single-stranded DNA. The vector mp8 was used throughout. The effect of irradiating in
2xTY broth and TF buffer was investigated, and direct versus indirect irradiation conditions compared. Strand breaks were quantified by measuring the conversion of circular to linear DNA by agarose gel electrophoresis as described in Chapter 2, section 12; and Chapter 3. In addition, the strand breaks were visualised, but not quantified using electron microscopy by P. McTurk (Dept. of Biochemistry). The infectivity of phage particles was measured as the number of plaque forming units (PFU) per ml. of phage suspension, and the transformation frequency as the PFU per ng. of single-stranded DNA. The ratio of DNA:protein in the phage particles was also determined.
4.2 RESULTS

4.21 DNA:Protein Ratio in M13 Phage Particles.

Phage pellets were prepared from 2x10ml cultures, resuspended in TF buffer and PFG precipitated as described in Chapter 2, sections (6) and (13). The precipitates were combined, mixed, then split into two identical aliquots. The phage pellets were recovered by centrifugation. One pellet was used for the Lowry protein assay, and the other for the diphenylamine assay for DNA content. The assays are described in Chapter 2, section (13).

(i) Lowry Protein Assay.

The $O.D_{660}$ measurements for BSA were used to construct a standard curve, see figure 4.2. 2x200\(\mu\)l of phage suspension each gave $O.D_{660}$ readings of 0.415, and 1x100\(\mu\)l aliquot gave an $O.D_{660}$ of 0.335. Reading from the standard curve, this gives an average value of 13.0mg protein per 200\(\mu\)l phage suspension. The phage pellet was resuspended in a volume of 500\(\mu\)l, therefore 32.4mg protein were present in the phage pellet.

(ii) Diphenylamine Assay.

The $O.D_{595}$ measurements for deoxyribose were used to construct a standard curve, see figure 4.3. 1x100\(\mu\)l gave an $O.D_{595}$ of 0.135, and 1x300\(\mu\)l gave an $O.D_{595}$ of 0.245. Reading from the standard curve and multiplying by the correction...
Figure 4.2
Lowry protein assay: bovine serum albumin (BSA) standard curve. $O.D_{660}$ nm against mg BSA.
Figure 4.3
Diphenylamine assay for DNA content: Deoxyribose standard curve. 0.0_{595} nm against mg deoxyribose.
factor, 4.21 gives values of 134.7μg/ml and 120.7μg/ml phage suspension respectively. Since the pellet was resuspended in a volume of 1ml, this gives an average of 127.7μg DNA present in the pellet.

(iii) DNA:protein Ratio.

In each phage pellet there is approximately 32.4mg protein and 127.7μg (0.1277mg) DNA. Therefore there are 253mg protein for every 1mg of DNA.

4.22 Irradiation of M13 Phage and M13 Single-Stranded DNA.

Phage particles and single-stranded (ss) DNA were precipitated to form pellets. These were resuspended in 2×TY broth or TE buffer, in the case of ss DNA, at a concentration of 200μg/ml. The samples were irradiated at RT or frozen and irradiated at 77K. In all cases, irradiations were carried out under conditions of ambient atmosphere.

Irradiated samples were split and used either in the infectivity assays or analysed for strand breaks by agarose gel electrophoresis. Phage were mixed with 1/10 volume 2% SDS prior to loading on the gel in order to remove coat proteins. The appearance of agarose gels of ss DNA and phage are shown in figure 3.3, panels (B) and (C). Single-stranded DNA was used to transform E.coli JM101 cells by the CaCl2 method (See Chapter 2, section 8.1.), and the phage particles used to infect exponentially growing JM101 cells. (Chapter 2, section 6)
Representative sets of data from each experiment are shown. All the data were collected and used to calculate the slopes of the graphs by the method of least squares fit. Calculating the ratios of the slopes for strand breaks versus infectivity assays gives a value for the proportion of lethal damage due to strand breaks, assuming that all strand breaks are 'lethal.'

(i) M13 ss DNA Irradiated at 77K in TF Buffer or 2xTY Broth.

Table I: Log$_{10}$ transformation frequency (pfu/ng) against irradiation dose. The data are represented graphically in figure 4.4.

<table>
<thead>
<tr>
<th>DOSE MRAD</th>
<th>LOG$_{10}$ pfu/ng</th>
<th>IRRADIATION MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.20</td>
<td>TF buffer</td>
</tr>
<tr>
<td></td>
<td>2.35</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>1.8</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.15</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>-0.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.75</td>
<td></td>
</tr>
</tbody>
</table>

......continued overleaf
Table II: Log$_{10}$ % circular DNA against irradiation dose. The average of duplicates is shown. See also figure 4.5.

<table>
<thead>
<tr>
<th>DOSE MRAD</th>
<th>LOG$_{10}$ % CIRCULAR</th>
<th>IRRADIATION MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.75</td>
<td>TY broth</td>
</tr>
<tr>
<td></td>
<td>1.81</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.6</td>
<td>1.51</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>1.42</td>
<td>&quot;</td>
</tr>
<tr>
<td>1.2</td>
<td>1.20</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>1.15</td>
<td>&quot;</td>
</tr>
<tr>
<td>1.8</td>
<td>0.90</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>&quot;</td>
</tr>
<tr>
<td>2.4</td>
<td>0.05</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>&quot;</td>
</tr>
<tr>
<td>3.6</td>
<td>-0.45</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>-0.60</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

See also figure 4.12, which shows electron micrographs of irradiated and unirradiated M13 single-stranded DNA. In the unirradiated sample, most of the DNA molecules are intact circles. After a dose of 0.6 MRad (at 77K), both linear and circular molecules are seen. After a dose of 2.4 MRad (at
Figure 4.4
The effect of irradiation at 77K on the transformation frequency of M13 single-stranded DNA, in 2xTY broth or TE buffer. ▲ TE buffer. ■ 2xTY broth. (Conditions of ambient atmosphere.)
Figure 4.5
The effect of irradiation at 77K on M13 single-stranded DNA in 2xTY broth (■) and TE buffer (▲). % circular DNA remaining against irradiation dose. (Conditions of ambient atmosphere.)
77K), few intact circles are present, and many small fragments can be seen. Electron microscopy was carried out by P. McTurk in the Department of Biochemistry.

(ii) M13 phage particles irradiated at 77K in TE buffer and TY broth.

Table III: Log$_{10}$ infectivity (pfu/ml) against irradiation dose. See also figure 4.6.

<table>
<thead>
<tr>
<th>DOSE MRAD</th>
<th>LOG$_{10}$ pfu/ml</th>
<th>IRRADIATION MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.85</td>
<td>TE buffer</td>
</tr>
<tr>
<td></td>
<td>12.75</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.6</td>
<td>12.27</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>12.35</td>
<td>&quot;</td>
</tr>
<tr>
<td>1.2</td>
<td>11.50</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>11.60</td>
<td>&quot;</td>
</tr>
<tr>
<td>1.8</td>
<td>11.08</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>11.16</td>
<td>&quot;</td>
</tr>
<tr>
<td>2.4</td>
<td>11.00</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>10.90</td>
<td>&quot;</td>
</tr>
<tr>
<td>3.6</td>
<td>10.20</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>10.18</td>
<td>&quot;</td>
</tr>
<tr>
<td>0</td>
<td>14.05</td>
<td>TY broth</td>
</tr>
<tr>
<td>0.6</td>
<td>13.20</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>13.15</td>
<td>&quot;</td>
</tr>
<tr>
<td>1.2</td>
<td>12.60</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>12.70</td>
<td>&quot;</td>
</tr>
<tr>
<td>1.8</td>
<td>12.25</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>12.30</td>
<td>&quot;</td>
</tr>
<tr>
<td>2.4</td>
<td>11.95</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>11.90</td>
<td>&quot;</td>
</tr>
<tr>
<td>3.6</td>
<td>11.10</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>11.30</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Figure 4.6
The effect of irradiation at 77K on the infectivity of M13 phage particles, in 2xTY broth (■) or TE buffer (▲).
(Conditions of ambient atmosphere.)
Figure 4.7
The effect of irradiation at 77K on M13 phage particles in 2xTY broth (■) and TE buffer (▲). % circular DNA remaining against irradiation dose. (Conditions of ambient atmosphere.)
Table IV: Log10 % circular DNA against irradiation dose. The average of duplicates is shown.

<table>
<thead>
<tr>
<th>DOSE MRAD</th>
<th>LOG10 % CIRCULAR</th>
<th>IRRADIATION MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.950</td>
<td>TF buffer</td>
</tr>
<tr>
<td>0.3</td>
<td>1.805</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>1.760</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>1.660</td>
<td></td>
</tr>
<tr>
<td>1.8</td>
<td>1.648</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.965</td>
<td>TY broth</td>
</tr>
<tr>
<td>0.3</td>
<td>1.835</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>1.775</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>1.710</td>
<td></td>
</tr>
<tr>
<td>1.8</td>
<td>1.665</td>
<td></td>
</tr>
</tbody>
</table>

(iii) M13 single-stranded DNA irradiated at RT in TE buffer.

Table V: Log10 infectivity (pfu/ng) against irradiation dose. See also figure 4.8.

<table>
<thead>
<tr>
<th>DOSE KRAD</th>
<th>LOG10 pfu/ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>3.67</td>
</tr>
<tr>
<td>5.1</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>3.53</td>
</tr>
<tr>
<td>10.2</td>
<td>2.61</td>
</tr>
<tr>
<td></td>
<td>2.65</td>
</tr>
<tr>
<td>15.3</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>2.63</td>
</tr>
<tr>
<td>20.4</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>1.70</td>
</tr>
</tbody>
</table>
Figure 4.8
The effect of irradiation at RT (ambient atmosphere) on the transformation frequency of M13 single-stranded DNA in TE buffer.
Figure 4.9
The effect of irradiation at RT (ambient atmosphere) on M13 single-stranded DNA in TE buffer. % circular DNA remaining against irradiation dose.
Table VI: \( \log_{10} \% \) circular DNA against irradiation dose. The average of duplicates is shown. See also figure 4.9.

<table>
<thead>
<tr>
<th>DOSE (KRAD)</th>
<th>( \log_{10} % ) CIRCULAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.910</td>
</tr>
<tr>
<td>3.3</td>
<td>1.765</td>
</tr>
<tr>
<td>6.7</td>
<td>1.640</td>
</tr>
<tr>
<td>10.0</td>
<td>1.565</td>
</tr>
<tr>
<td>13.3</td>
<td>1.400</td>
</tr>
<tr>
<td>15.0</td>
<td>1.360</td>
</tr>
</tbody>
</table>

(iv) M13 phage irradiated at RT in TE buffer.

Table VII: \( \log_{10} \) infectivity (pfu/ml) against irradiation dose. See also figure 4.10.

<table>
<thead>
<tr>
<th>DOSE (KRAD)</th>
<th>( \log_{10} ) pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.79</td>
</tr>
<tr>
<td>10</td>
<td>17.85</td>
</tr>
<tr>
<td>40</td>
<td>16.66</td>
</tr>
<tr>
<td>60</td>
<td>15.52</td>
</tr>
<tr>
<td>80</td>
<td>14.53</td>
</tr>
</tbody>
</table>
Figure 4.10
The effect of irradiation at RT (ambient atmosphere) on the infectivity of M13 phage particles in TE buffer.
Table VIII: $\log_{10}$ % circular DNA against irradiation dose. The average of duplicates is shown. See also figure 4.11.

<table>
<thead>
<tr>
<th>DOSE KRAD</th>
<th>$\log_{10}$ % CIRCULAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.943</td>
</tr>
<tr>
<td>50</td>
<td>1.886</td>
</tr>
<tr>
<td>80</td>
<td>1.890</td>
</tr>
<tr>
<td>100</td>
<td>1.877</td>
</tr>
<tr>
<td>140</td>
<td>1.849</td>
</tr>
<tr>
<td>200</td>
<td>1.798</td>
</tr>
</tbody>
</table>

(v) M13 phage irradiated at RT in TE buffer followed by extraction of single-stranded DNA.

M13 phage particles were irradiated for various doses in TE buffer at RT. After irradiation, the samples were split into two aliquots. One was used to determine the strand breaks and infectivity of the phage particles, and from the other, single-stranded DNA was extracted prior to analysis for strand breaks and transformation frequency. Extraction of DNA from phage is described in Chapter 2, section 7.

Table IX: $\log_{10}$ infectivity (pfu/ml) of M13 phage irradiated in TE buffer at RT, and $\log_{10}$ % transformation frequency (pfu/ng) of DNA extracted from irradiated phage. The average of duplicates is shown.

<table>
<thead>
<tr>
<th>DOSE KRAD</th>
<th>$\log_{10}$ pfu/ml PHAGE</th>
<th>$\log_{10}$ pfu/ng SS DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.16</td>
<td>2.10</td>
</tr>
<tr>
<td>100</td>
<td>12.98</td>
<td>1.68</td>
</tr>
<tr>
<td>200</td>
<td>12.46</td>
<td>1.24</td>
</tr>
</tbody>
</table>
The effect of irradiation at RT (ambient atmosphere) on M13 phage particles in TE buffer. % circular DNA remaining against irradiation dose.

Graphs were not plotted. Instead, the data were used to calculate the slopes of the best straight line fitting the data by the method of least squares. The best straight lines are given below:

- EXPERIMENT
- SLOPE MRIAD = 1

This shows that whilst the number of strand breaks decreases at about the same rate in the two samples, the phage particles lose infectivity about three times faster than the loss of infectivity in extracted DNA.
Table X: $\log_{10}$ % circular DNA remaining in M13 phage particles irradiated in TE buffer at RT, and in single-stranded DNA extracted from irradiated phage.

<table>
<thead>
<tr>
<th>DOSE (KRAD)</th>
<th>( \log_{10} % \text{ CIRCULAR DNA PHAGE} )</th>
<th>( \log_{10} % \text{ CIRCULAR DNA SS DNA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.94</td>
<td>1.91</td>
</tr>
<tr>
<td>100</td>
<td>1.86</td>
<td>1.83</td>
</tr>
<tr>
<td>200</td>
<td>1.77</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Graphs were not plotted, instead the data were used to calculate the slopes of the best straight lines fitting the data by the method of least squares fit. The best straight lines are given below:

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>SLOPE MRAD(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>phage infectivity</td>
<td>$11 \log_{10}$ pfu/ml</td>
</tr>
<tr>
<td>phage strand breaks</td>
<td>$0.5 \log_{10}$ % circular</td>
</tr>
<tr>
<td>SS DNA infectivity</td>
<td>$4.0 \log_{10}$ pfu/ng</td>
</tr>
<tr>
<td>SS DNA strand breaks</td>
<td>$0.6 \log_{10}$ % circular</td>
</tr>
</tbody>
</table>

This shows that whilst the number of strand breaks increases at about the same rate in the two samples, the phage particles lose infectivity about three times faster than the loss of infectivity in extracted SS DNA.
(vi) Calculation of the slopes of the graphs.

The data in parts (i) to (iv) were used to calculate the slopes of the best straight lines through the data points by the method of least squares fit, to allow a comparison of the data. Where an experiment has been carried out more than once, the averages slope is given. The ratios of infectivity: strand breaks were then calculated.

Key to table: (a) Irradiation temperature, 77K or RT (room temperature); (b) M13 phage particles (phage) or single stranded M13 DNA (SS); (c) Irradiation medium; TE buffer or 2×TY broth; (d) Parameter measured, infectivity or strand breaks (SB'S).
Table XI: Calculated slopes of graphs.

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>SLOPE MRAD^(-1)</th>
<th>AVERAGE SLOPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) (b) (c) (d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>77K PHAGE TE INFECTIVITY</td>
<td>0.270</td>
<td>0.270</td>
</tr>
<tr>
<td>&quot; &quot; TY &quot;</td>
<td>0.272</td>
<td>0.284</td>
</tr>
<tr>
<td>&quot; &quot; PHAGE TE &quot;</td>
<td>0.296</td>
<td></td>
</tr>
<tr>
<td>&quot; SS PHAGE TE TY &quot;</td>
<td>0.291</td>
<td>0.295</td>
</tr>
<tr>
<td>&quot; &quot; SS &quot; TY &quot;</td>
<td>0.299</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; SS TY TE &quot;</td>
<td>0.243</td>
<td>0.244</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; &quot;</td>
<td>0.245</td>
<td></td>
</tr>
<tr>
<td>&quot; PHAGE TE SB'S</td>
<td>0.057</td>
<td>0.057</td>
</tr>
<tr>
<td>&quot; &quot; TY &quot;</td>
<td>0.053</td>
<td>0.054</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>&quot; SS TE &quot;</td>
<td>0.047</td>
<td>0.045</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>&quot; PHAGE SS INFECTIVITY TY &quot;</td>
<td>0.041</td>
<td>0.047</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>0.052</td>
<td></td>
</tr>
<tr>
<td>RT PHAGE TE INFECTIVITY</td>
<td>9.94</td>
<td>9.94</td>
</tr>
<tr>
<td>&quot; SS &quot; &quot;</td>
<td>38.88</td>
<td>38.88</td>
</tr>
<tr>
<td>&quot; PHAGE &quot; SB'S</td>
<td>0.216</td>
<td>0.216</td>
</tr>
<tr>
<td>&quot; SS &quot; &quot;</td>
<td>11.88</td>
<td>11.88</td>
</tr>
</tbody>
</table>
Table XII: Ratios of infectivity: strand breaks.

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>INFECTIVITY / STRAND BREAKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
</tr>
<tr>
<td>77K PHAGE</td>
<td>TE</td>
</tr>
<tr>
<td>&quot; SS</td>
<td>TF</td>
</tr>
<tr>
<td>&quot; PHAGE</td>
<td>TY</td>
</tr>
<tr>
<td>&quot; SS</td>
<td>TY</td>
</tr>
<tr>
<td>RT PHAGE</td>
<td>TE</td>
</tr>
<tr>
<td>&quot; SS</td>
<td>TF</td>
</tr>
</tbody>
</table>

Table XIII: Ratios of slopes at RT: slopes at 77K.

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>SLOPE AT RT / SLOPE AT 77K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
</tr>
<tr>
<td>PHAGE INFECTIVITY</td>
<td>36.8</td>
</tr>
<tr>
<td>&quot; SS</td>
<td>131.8</td>
</tr>
<tr>
<td>PHAGE STRAND BREAKS</td>
<td>3.79</td>
</tr>
<tr>
<td>&quot; SS</td>
<td>264.0</td>
</tr>
</tbody>
</table>
Figure 4.12

Electron micrographs of irradiated and unirradiated single stranded M13 DNA. (a) Unirradiated, (b) 0.6 MRad at 77K, (c) 2.4 MRad at 77K.

4.23 Summary of Results

The results described above can be summarised as follows.

(i) The DNA: protein ratio in bacteriophage M13 is 253mg protein per 1mg of DNA.

(ii) Irradiating at 77K in TF buffer or in 2xTY broth gives similar results. That is, infectivity and % circular DNA decrease at the same rate regardless of the irradiation medium.

(iii) In phage particles and single-stranded DNA irradiated at 77K, strand breaks account for 1/5 to 1/6 of the lethal hits.

(iv) In single-stranded DNA irradiated at RT, strand breaks accounted for approximately 1/3 of the lethal hits, but in phage particles irradiated at RT, strand breaks accounted for only 1/46 of the lethal hits.

(v) In samples irradiated at 77K, loss of infectivity and generation of strand breaks occurred at about the same rate in both phage particles and single-stranded DNA.

(vi) In samples irradiated at RT, the single stranded DNA was much more radiosensitive than phage particles. Single-stranded DNA was only about 4 times more sensitive than phage with respect to infectivity, but 55 times more sensitive with respect to strand breaks.

(vii) The procedure employed to extract DNA from phage particles did not give rise to strand breaks.

(viii) DNA extracted from irradiated phage contained the same level of strand breaks as DNA within the phage particle which was not subjected to the extraction procedure. However, the
infectivity of the intact phage decreased around 3 times faster with increasing irradiation dose than the infectivity of the DNA extracted from irradiated phage.

(ix) The decrease in infectivity of phage particles is about 37 times faster in samples irradiated at RT than in samples irradiated at 77K. The decrease is 132 times faster in single-stranded DNA.

(x) The increase in strand breaks is about 4 times faster in phage particles irradiated at RT than in samples irradiated at 77K. The increase is 254 times faster in single-stranded DNA.
4.3 DISCUSSION

There are several types of radiation induced damage which may cause loss of infectivity of phage particles; these are strand breaks, base damage, cross links (DNA-DNA and DNA-protein) and damage to the protein coat. In the case of purified bacteriophage DNA, all of these except protein damage may cause loss of biological activity. The contribution of these various types of damage to lethality in gamma and X-ray - irradiated phage and DNA has been investigated by several groups. Some of the earlier studies are reviewed by Blok and Loman (1973). Several different phages have been studied, in particular, the T phages, phage PM2 and φX174. Apparently no studies of this type have been carried out on M13, though φX174 is similar, both being single stranded filamentous DNA phages.

In a series of experiments, Blok (1967) compared the number of strand breaks (by analytical ultracentrifugation) with biological activity on E.coli spheroplasts in φX174 single stranded DNA irradiated at RT (ambient atmosphere) in phosphate buffer. On average, 50% of the lethal hits were shown to be due to strand breaks. Swinehart and Cerruti (1975) investigated the formation of ring saturated 5,6-dihydroxy dihydrothymine residues in φX174 single stranded DNA and phage particles irradiated under indirect conditions. The phage DNA was labelled with methyl $^3\text{H}$ thymidine in vivo. DNA damage in irradiated samples was assessed by an alkaline degradation assay (to quantify ring saturated thymines) and the release of $^3\text{H}$ in $\text{H}_2\text{O}$. The results showed that the release of $^3\text{H}$ $\text{H}_2\text{O}$ was suppressed in the phage
about 9 times over the level of release found for single-stranded DNA. However, the yield of 5,6-dihydroxy-dihydrothymine products was apparently unaffected. This may be due to reaction of thymines with the protein coat in a way that protects the 5,6 double bond. Although a linear relationship was demonstrated between the formation of thymine ring-saturated products, release of $^3\text{H}\text{H}_2\text{O}$ and survival, no direct evidence was provided that these are lethal lesions. In fact at the dose giving a survival of 37% of the plaque forming ability (equivalent to an average of one lethal hit per genome), 200 ring-saturated thymine products were found to be present. Therefore these are not necessarily lethal, or alternatively, are repairable in vivo.

Several studies have focused on the double-stranded DNA phage PM2, or the double stranded RF form of ϕX174 DNA. These are likely to be able to tolerate a larger number of "hits" than single stranded DNA's since the presence of a complementary strand allows excision repair by the host cells. Lafluer et al. (1979) subjected ϕX174 RF DNA irradiated under oxic conditions at RT to alkali treatment prior to the determination of strand breaks (by sucrose gradient sedimentation) and biological activity. Alkali treatment doubled the yield of breaks, but increased the biological activity nearly to the level of the unirradiated sample. This suggests that virtually all of the lethal nucleotide damage is converted to single strand breaks by alkali, and that most of the strand breaks are not lethal. Wallace and Moran (1985) irradiated the double-stranded phage PM2 under anoxic conditions at RT. The phage were irradiated
in the presence of the radical scavenger citrate to minimise
damage to the protein coat by diffusing free radicals.
Chemical analysis revealed that 1.06 alkali-labile sites, 0.4
thymine ring saturation products, 2.09 single strand breaks
and 0.11 double strand breaks were present per lethal hit. By
chemically introducing thymine glycols and apurinic (alkali-
labile) sites into PM2 DNA and measuring the remaining
biological activity, it was found that 7 or 8 of either of
these was present per lethal hit. From these figures and the
inactivation efficiencies calculated for single and double
strand breaks from van der Schans et al (1975, see below) it
was estimated that alkali labile sites and ring saturation
products account for 15-20% of phage inactivation, double-
strand breaks to about 11% and single strand breaks to about
4%, the other inactivations being caused by unidentified
types of damage.

Using PM2 double stranded DNA irradiated at RT under oxic
conditions, van der Schans et al (1975) analysed the
contribution of various types of damage to loss of
infectivity by separating out supercoiled (form I) phage DNA
from the relaxed forms (II and III) by filtration, then
determining the biological activity of the separated
fractions as well as the total sample. Strand breaks were
determined by sucrose gradient sedimentation. The results are
given in the table below, and show the predominance of the
contribution of nucleotide damage to inactivation.
<table>
<thead>
<tr>
<th>DAMAGE TYPE</th>
<th>% CONTRIBUTION</th>
<th>% INACTIVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide damage</td>
<td>85±10%</td>
<td>54±23%</td>
</tr>
<tr>
<td>Double strand break</td>
<td>3±0.2%</td>
<td>100%</td>
</tr>
<tr>
<td>Single strand break</td>
<td>12±10%</td>
<td>10±15%</td>
</tr>
</tbody>
</table>

Although double strand breaks form only a small fraction of the total inactivation, they are a serious lesion in terms of causing loss of infectivity, since each one constitutes a lethal event. Similar experiments have been carried out on PM2 DNA irradiated at 77K, that is, direct irradiation conditions. The results from these are summarised in the table below. (van der Schans & Bleichrodt, 1974)

<table>
<thead>
<tr>
<th>DAMAGE TYPE</th>
<th>% CONTRIBUTION</th>
<th>% INACTIVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide damage</td>
<td>86±12%</td>
<td>13±3%</td>
</tr>
<tr>
<td>Double strand break</td>
<td>8.1±0.6%</td>
<td>100%</td>
</tr>
<tr>
<td>Single strand break</td>
<td>6.0±12%</td>
<td>2.0±4%</td>
</tr>
</tbody>
</table>

The above tables show that the contribution of various types of damage to inactivation is similar under direct and indirect irradiation conditions, in spite of the quite different mechanisms operating under these conditions. One difference which may be significant is the lower inactivation efficiency of nucleotide damage and single strand breaks under direct irradiation conditions. This is likely to be due
to the different nature of these types of damage under direct and indirect irradiation conditions, presumably under direct conditions, these are more easily by-passed during DNA replication, or are more easily repaired by host cell repair mechanisms.

The results described here for irradiation of M13 single-stranded DNA under direct and indirect irradiation conditions agree with the general conclusions discussed above, that nucleotide damage is the major cause of loss of biological activity. In M13 DNA irradiated at RT, single strand breaks accounted for about 33% of the lethal hits, and in M13 DNA irradiated at 77K, they accounted for 17% to 20% of lethal hits. The results of van der Schans' group (described above) concluded an even higher contribution of nucleotide damage to lethality in PM2 DNA under both direct and indirect irradiation conditions. This is presumably because PM2 has a double-stranded genome, therefore single strand breaks are not always lethal, and double strand breaks (although always lethal), occur with relatively low frequency. A greater contribution of strand breaks to lethality (around 15%) was observed by Wallace and Moran (1985), in PM2 phage particles irradiated at RT in the presence of the radical scavenger, citrate. In single-stranded phage DNA irradiated under indirect conditions, Blok (1967) concluded that strand breaks were responsible for 50% of the lethal hits, significantly higher than in this study. This may be due to the different assay procedures employed or differences between ϕX174 and M13 DNA. The irradiation medium (TE buffer or 2xTY broth) had no effect on strand break formation or loss of infectivity.
An interesting observation from these studies is that under direct irradiation conditions, strand breaks contribute to lethality to a similar extent in phage particles and purified single-stranded phage DNA. Furthermore, the loss of biological activity occurs at about the same rate in phage and single-stranded DNA. This means that the protein coat is not having a significant effect on the radiosensitivity of the phage with respect to either of these two parameters, even though protein forms a far greater proportion of the phage particle by mass than DNA. Thus either damage to the protein is minimal, or does not contribute significantly to loss of infectivity. Under indirect irradiation conditions, however, single-stranded DNA is much more radiosensitive than the phage, around 4 times more sensitive with respect to biological activity, but 55 times more sensitive with respect to strand break formation. This strongly suggests that the protein coat protects the DNA from diffusing free radicals (though not direct ionisation events), but is a target for radiation damage itself. Protein coat damage presumably prevents adsorption of the phage to host cells, or injection of the DNA. (Perhaps due to DNA-protein cross links.) Further evidence for lethal protein damage was obtained by extracting DNA from irradiated phage, and comparing the strand breaks and infectivity with phage particles. The level of strand breaks was about the same in both samples, which shows that the DNA extraction procedure did not induce strand breaks. However, the infectivity of the phage particles decreased about 3 times faster than infectivity of the extracted DNA. The greater loss of infectivity in the phage must be due to protein damage. Swinehart and Cerruti (1975)
also demonstrated protection of phage DNA from indirect irradiation damage by the protein coat.

Studies on the double-stranded T4 bacteriophage irradiated under anoxic conditions at RT, in the presence of the radical scavenger histidine, revealed the presence of DNA-protein (and DNA-DNA) cross links, these may therefore contribute to inactivation. (Ronne et al, 1970) Other studies showed that in this phage, irradiated under identical conditions, around 5% of inactivated phages had lost the ability to inject DNA into the host cells. Of these, about half had ruptured protein coats, and the rest had damage in the 'tail' proteins, which are responsible for adsorption to host cells. (Coquerelle & Hagen, 1972)

The G value for strand break formation (number of strand breaks per 100eV) in plasmid DNA irradiated at 77K is approximately 0.55, whereas the G value for 'G+ / 'T- formation is about 1.5. This suggests that 37% of these radicals go on to form strand breaks. From the results discussed here for single-stranded M13 DNA irradiated at 77K, 17% to 20% of lethal hits are due to strand breaks. These values are remarkably similar considering the different methods used to determine these figures, and the fact that plasmid DNA is double-stranded, whereas the M13 used was single-stranded. The difference between the G value for strand breaks and 'G+ / 'T- formation can be accounted for by reaction of these radicals to form nucleotide damage.

The M13 assay system for DNA damage investigated here is likely to prove of use in extending the groups' studies on the effect of additives on the course of radiation damage to DNA. Unlike the plasmid based assay (see Chapter 3), the
relative effects of additives on strand breaks and other types of damage could be determined, and the influence of the protein coat could be investigated.
CHAPTER
five
CHAPTER 5

SITE SPECIFICITY OF GAMMA RADIATION-INDUCED DAMAGE

5.1 INTRODUCTION

Previous chapters have discussed the detection and quantitation of gamma radiation-induced damage; and some of the differences between direct and indirect radiation damage. This chapter describes investigations into the site specificity of gamma radiation-induced damage. Two types of assay are available for the detection of sites of action of DNA damaging agents. Both are based on DNA sequencing techniques.

The first is based on the Maxam-Gilbert sequencing technique, (Maxam & Gilbert, 1977) and involves the end labelling of single or double-stranded DNA fragments, usually with $^{32}$P. This method is discussed in detail by Haseltine et al (1980). DNA fragments labelled at one end are treated with the DNA damaging agent of interest. If strand breaks arise, a population of end-labelled molecules of various lengths is generated. These are separated out by polyacrylamide gel electrophoresis, which enables the separation of fragments differing in size by as little as one nucleotide. The positions of the strand breaks are analysed by running the treated fragments in parallel with markers, such as specific chemical cleavage reactions on the restriction fragment of interest. This method can also be used to detect damage sites which are converted to strand breaks by subsequent treatment.
For instance, treatment with alkali prior to gel analysis may reveal the presence of alkali-labile sites.

The second method is based upon the dideoxy sequencing technique of Sanger et al (1977). To utilise the Sanger method for this purpose, single-stranded template DNA is treated with the DNA damaging agent then annealed to an oligonucleotide primer labelled at the 5' end with $^{32}$P. The primer is then extended by a DNA polymerase, and the labelled polymerisation products analysed on sequencing gels in parallel with the dideoxy sequencing reactions. This method has an advantage over the 'Maxam-Gilbert' based technique, which is restricted to the detection of directly or indirectly induced strand breaks, in that sites of 'biologically important' damage are detected. The polymerisation reaction will stop at every strand break, and also at various base types of base damage, for instance, pyrimidine dimers. (Doetsch et al, 1985.) The three possible scenarios are illustrated in figure 5.0. DNA templates treated with a DNA damaging agent, in this case gamma irradiation, may contain (a) strand breaks only, (b) other types of damage which block DNA polymerase, (c) both (a) and (b). Subsequent analysis of the radiolabelled synthesised strand on polyacrylamide gels reveals the positions of the premature termination sites, but the limitation of the method is that it is not possible to distinguish whether products have arisen from situations (a), (b) or (c). Some types of base damage may not block DNA polymerisation, these will not be detected in this assay. However, if by-pass of the lesions is possible, a correct nucleotide may be selected, resulting in the production of 'wild-type' progeny phage, or an
Scheme representing the possible events on irradiation of M13 template DNA. (a) Strand breaks only. (b) Base damage which blocks DNA polymerase only. (c) Base damage and strand interruption.
incorrect nucleotide may be selected, which may or may not have lethal or mutagenic consequences. Therefore lesions which cannot be by-passed by the polymerase (that is, those detected by this assay) are likely to be the most serious in terms of giving rise to lethality.

In a recent review, (Wilkins, 1984) the application of these techniques to the study of various DNA damaging agents is discussed. Many of these agents interact with DNA in a sequence-specific manner. A large number of chemical agents such as neocarzinostatin and bleomycin cause DNA strand breaks. Analysis by the 'Maxam-Gilbert' type technique has revealed that the strand breaks are often not randomly distributed, but tend to occur at specific sequences. Neocarzinostatin for instance breaks DNA predominantly at T's and to a lesser extent A's in a reaction dependent on the presence of a reducing agent. Bleomycin, on the other hand cleaves at GC, GT and to a lesser extent AT sequences; however, in the presence of ferrous ions, additional breaks occur at TT, TA and AT sequences. The basis of these specificities is thought to involve the sequence specific binding of these drugs and the subsequent production of locally high concentrations of radicals which give rise to strand breaks. Other DNA damaging agents which bind covalently but don't give rise to strand breaks can be analysed by 'nick-translation' assay. Double-stranded DNA nicked at a specific site in one strand is treated with the DNA damaging agent of interest. DNA polymerase I can extend from the nick in a 5' to 3' direction, whilst digesting the old strand (in the 3' to 5' direction). The polymerase will stop at any sites on the template strand which act as blocks.
to DNA synthesis. This technique depends upon the ability of the 3' to 5' exonuclease to completely digest the damaged strand. Actinomycin D is an intercalator that binds unusually slowly to DNA. This binding blocks DNA polymerase action in a nick translation assay, specifically at GC sequences. Distamycin C on the other hand, binds in the minor groove of the DNA molecule, preferentially at AT sequences. (Wilkins, 1982) The sites of binding of these types of agents can also be determined by methidium-EDTA (MPE-EDTA) 'footprinting'. (van Dyke et al, 1982) Methidium-EDTA is an intercalator which when complexed with Fe(II), and in the presence of molecular oxygen generates a hydroxyl radical close to the site of intercalation which induces a series of reactions leading to a strand break. Intercalation of MPE-EDTA is non site specific; so the binding of other agents which block intercalation can be identified as the sites protected from MPE-EDTA-Fe(II) induced cleavage. The traditional footprinting technique involves the inhibition of DNaseI induced cleavage by molecules bound covalently to DNA. This has been of use for detecting the binding sites of DNA binding proteins, but has not been shown to be useful for detecting the binding sites of small molecules, which include most of the chemotherapeutic agents. (van Dyke et al, 1982)

DNA damaging agents which give rise to covalent adducts can only be analysed by the Maxam-Gilbert type techniques if there is a method available for cleaving the DNA at the site of the adducts. For instance, alkylating agents such as the nitrogen mustards can be cleaved at the sites of adduct formation by heating at neutral pH followed by exposure to hot alkali. Most studies on the sites of covalent adducts,
However, have utilised the Sanger sequencing-based method. The psoralens, for example, form mono or diadducts on single stranded DNA (or single-stranded regions of double-stranded DNA), upon illumination with 365nm light. Piette and Hearst (1985) showed that these adducts are blocks to DNA polymerase activity, the polymerase stopping predominantly one base before thymine residues. A variety of DNA polymerases stop at the N-(guan-8-yl)-2-aminofluorine adducts formed from N-acetylaminofluorene (AAF). E.coli polymerase I and T4 DNA polymerase stop one base before the adducts; but AMV reverse transcriptase inserts a base opposite the adduct. This enzyme lacks the 3' to 5' exonuclease activity present both in polymerase I and T4 DNA polymerase, which may be responsible for removing any nucleotide inserted opposite the adduct. However, this is not the case for UV-induced base alterations, which cause DNA polymerases to stop one base before the damage, regardless of the presence or absence of the 3' to 5' exonuclease activity. (Moore & Strauss, 1979; Moore et al, 1981)

Studies on UV-irradiated DNA have shown that both pyrimidine dimers and 6-4 photoproducts (see structures in Chapter 1, section 1.5) act as blocks for E.coli DNA polymerase I. (Doetsch et al, 1985) Most studies of this type have been carried out on single-stranded DNA, but double-stranded DNA can be studied by the nick-translation assay described above. An alternative approach to the study of distribution of lesions equally applicable to both single and double-stranded DNA is exonuclease digestion by the 3' to 5' exonuclease of T4 DNA polymerase. This digests single and double-stranded DNA, unlike exonuclease III which digests
only double-stranded DNA. The digestion of double-stranded DNA is inefficient, however, due to the 5' to 3' polymerase activity of the enzyme. This technique has been used by Doetsch et al (1985) on UV irradiated DNA. Irradiated DNA were treated with Micrococcus luteus UV-specific endonuclease which cleaves between pyrimidine dimers, or with hot alkali which cleaves the glycosidic bond at the 3' side of 6-4 photoproducts. Untreated irradiated DNA was run on sequencing gels in parallel with UV-irradiated DNA given one of the above treatments. This showed that the formation of pyrimidine dimers and 6-4 photoproducts occurs at about the same rate in both single and double-stranded DNA. These lesions both act as blocks to T4 exonuclease digestion, and furthermore, their kinetics of formation suggest that no other unidentified photoproducts act as blocks to digestion.

Gamma radiation gives rise to a variety of types of DNA damage including strand breaks and base damage. The sites of strand breaks can be studied by the 'Maxam-Gilbert' based technique, or a wider spectrum of lesions by the Sanger sequencing based method or exonuclease digestion. The indirect damage mechanism is expected to result in non sequence-specific DNA damage. Damage under these conditions is largely due to the action of diffusing hydroxyl radicals. (Ward, 1977) OH radicals have been shown to give rise to DNA strand breaks in a non-sequence-specific manner, by exposing end-labelled DNA to ferrous sulphate. This catalyses the formation of hydroxyl radicals from molecular oxygen, which give rise to DNA strand breaks at every possible nucleotide position with equal frequency. (Henner et al, 1983) These authors have also shown that single strand breaks arising
from irradiation under indirect conditions are produced in a
non sequence specific manner.

In DNA irradiated as dry films or in frozen aqueous
solution, the direct damage mechanism predominates. As stated
in Chapter 1 (Section 1.1) under these conditions, the damage
is thought to largely result from primary radicals formed
specifically on thymine and guanine. ('G' and 'T-') It is
therefore of interest to know whether this initial
specificity is reflected in the specificity of the stable DNA
damage products. To date no studies to investigate this have
been published.

In this chapter, the sites of gamma radiation-induced
damage has been studied by two methods. Sites of gamma
radiation-induced strand breaks were analysed in the 346 base
pair end-labelled BamHI/HindIII fragment from pBR322 . No site
specificity was observed under conditions of direct or
indirect action. Damage sites which act as blocks to DNA
synthesis were analysed on irradiated single-stranded M13mp8
template DNA . Again, little or no site specificity was
observed in samples irradiated under either condition. Since
direct radiation damage is thought to result from radicals
centered on G and T, it was hoped that damage sites would be
observed primarily at these bases. However, subsequent charge
migration even through only one or two bases would tend to
obscure any initial specificity. To see if this was the case,
four M13 clones were constructed, each having a homopolymer
tract of one of the four bases. The DNA polymerase stop sites
were determined as for the M13mp8 templates. The results
revealed that DNA damage was reduced within a run of adenines
or guanines compared with the flanking sequences, but
remained at about the same level as the flanking sequences within a run of thymines or cytosines. This applied to template DNA irradiated under either direct or indirect irradiation conditions.

It was observed that the Klenow fragment of DNA polymerase I has preferential 'pause' sites on template DNA under limiting conditions. These sites had no obvious relationship to primary or secondary structure. Polymerisation was able to go to completion given sufficient time and amount of enzyme.

5.2 RESULTS

5.2.1 Ability of the Klenow Fragment to Copy Round an Undamaged Template.

Polymerisation of primed M13 templates under conditions of limited time or amount of enzyme gave rise to a number of discrete bands on polyacrylamide gels. (See figure 5.4 and tracks 14-17 on figure 5.6) These are polymerisation products which have not gone to completion, and are presumably the result of the polymerase pausing or dissociating at preferential sites. The banding pattern observed was identical regardless of the source of the enzyme (prepared as in chapter 2, section (14) or purchased from Pharmacia) or the batch of template used. Given sufficient time and amount of enzyme, the reaction proceeded to completion. The positions of the bands were mapped with respect to the DNA sequence. These are shown in figure 5.1.

There are three potential hairpin loops in the region of the template analysed. (Figure 5.2) Although several of the
Figure 5.1

Klenow stop sites in M13 mp8 template DNA.

The major Klenow pause sites on irradiated (↑) and unirradiated (•) M13mp8 template are shown. Pause sites on unirradiated templates were present where either time allowed for the reaction or Klenow concentration were limiting. The irradiation pause sites shown were present under conditions of both direct and indirect action, with the following minor exceptions. Pause sites in brackets [↑] were not present in samples irradiated as dry films. Pause sites in brackets {↑} were present only in the dry samples, with the exception of the central thymine residue. (See text for details.) Sequences underlined are those forming the stems of potential stem-loop structures. (See figure 5.2)

3' *** * * *** *******
GACGTTCCAGCTGCGAGGCCCCTTAAGCATTAGTACCAGTATCGAACAAGGGACACACTT
↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑

***** * * ***** ****** * * * * *** **
TAACATAGGGCAGTGTATAAGGTGGATTGATGCTGGCCTTCGTATTTCACTTCTCGGACC
↑↑↑↑↑↑↑↑↑↑↑↑↑↑↑↑↑

***** * * ***** * * * * * * * * * * * * ****
CCACGGATTACCTACACTCGATGGATGTTAATTAACGCAACGCAGTGACGGGGCAGAGT
↑↑

***** * * **** * * *
CAGCCTTTGGAGACACGTCGACGCAGTAATTACTTACG 5'
**Figure 5.2**

**Potential hairpin loops in M13 mp8.**

[1] 3'  

\[\text{TTTCC} \quad \text{AAAGG} \quad \text{GCT}\]

\text{Size of loop: 5 bases}

[2] 3'  

\[\text{TGTGTGAAATTGT} \quad \text{ACACAC} \quad \text{TTAACA} \quad \text{CTGC}\]

\text{Size of loop: 9 bases}

[3] 3'  

\[\text{AGTGTGAG} \quad \text{TAATG} \quad \text{ATTAC} \quad \text{ACTCAATC}\]

\text{Size of loop: 16 bases}

Sequences in bold type represent loops.
Figure 5.3

Sequences of the M13 clones with homopolymer tracts.


EcoRI 5'x1'A's BamHI
TTACGAATTC CCCAAA....AAAAGGGGATCCGACCTGCAGCCAAGCTTGGC AACTGGGCGTCGTTTTACA
TGACCGGCAGCAAAATG

direction of DNA synthesis


BamHI 5'x1'T's EcoRI
GCCAAGCTTTGCTGACGGGATCCCTTTTT....TTTGGGAATTCACTGGCCGTCGTTTTACACA
TGACCGGCAGCAAAATG


PstI 14xG's
GGCTGCAGGG....GGGAATCTCCAAGAAAAAAAAGAGGATAAATGGCTTCATCCATTCTCTCATCGCGG
TAGGTAAGAGAGTAGG

269 bases EcoRI
CTG....TGAATTCACT 3'

[4] C-RUN: M13mp8 with 348 base pair FcoRI/PstI fragment containing 14 C's inserted into the EcoRI/PstI sites.

EcoRI 324 bases 14xC's PstI
TTACGAATTCAGC....AGATTTCC....CCCTGCAAGCCAAAGCTTGGC AACTGGGCGTCGTTTTACAACCTGC
TGACCTGG 3'

Sequences in bold type represent insert DNA.
Klenow pause sites on gamma irradiated M13mp8 template DNA. Approximately 0.2µg DNA loaded per track. Samples ran on a sequencing gel. Tracks 1-13 were run for 4 hours at 1200V; tracks 14-23 for 2 hours at 1200V. Tracks 1-4 and 14-17 are dideoxy sequencing reactions, A,T,G,C respectively. Tracks 18, primers extended with 0.5 units Klenow polymerase. Tracks 6 & 19, 0.05 units; 7 & 20, 0.005 units. Tracks 21, 0.5 units commercially available Klenow, tracks 9 & 10, 0.05 units, 10 & 23, 0.005 units. Tracks 11, 12 and 13 were primers extended with 0.5 units of Klenow, track 11, 30 seconds incubation, track 12, 10 seconds incubation and track 13, 60 seconds incubation. Reactions were carried out using Klenow prepared in the Dept. of Biochemistry (0.5 units), incubated overnight at room temperature unless otherwise stated.
Klenow 'pause' sites do occur within these sequences, the proportion of pause sites within these loops is not significantly greater or less than that outside these potential loops. The pause sites also share no obvious consensus sequences that would suggest primary structure as the cause of premature 'termination'. The percentages of each base flanking the pause sites and at these sites was calculated, along with the percentage base composition of the whole of the region sequenced. The mean percentage of each base is clearly 25%, and the standard deviation from the mean, calculated from the % base composition is ±2.10%. The standard deviation is calculated according to the following formula:

$$SD = \sqrt{\frac{\sum X^2 - (\sum X)^2}{N-1}}$$

SD = standard deviation, X = value of variable, N = number of variables.

The values for polymerase pause sites at each base are shown in bold type if they differ by more than 1.96x standard deviations from the mean. That is, 25 plus or minus 4.2 (20.8 to 29.2). In a 'normal' distribution, 95% of values are expected to fall within ±1.96x standard deviations from the mean, therefore the figures marked in bold type represent values that have a less than or equal to 5% probability of having arisen by chance. Thus, there appears to be a preference for cytosine 3' to the pause site and adenines and cytosines at the pause sites themselves, as shown in the
The 3' end-labelled 346 bp BamHI/HindIII fragment from pBR322 was prepared as described in Ch. 2, Section 18. Aliquots were irradiated at a concentration of approximately 100μg/ml both in dilute aqueous solution, and frozen solution at 77K. The irradiated samples were run on 8% denaturing polyacrylamide gels, in parallel with the G + A chemical cleavage reaction and an unirradiated control. (See figure 5.5.) In the irradiated samples, a band was present at every possible position in the sequencing ladder, and all bands were of approximately equal intensity. There is therefore no sequence specificity for gamma radiation-induced strand breaks, either under direct or indirect irradiation conditions. Some degradation was apparent in the unirradiated sample, possibly due to autoradiolysis, but this was quite small compared with the degradation in the irradiated samples. The radiation products comigrate with the products of the G + A chemical cleavage reaction, which are known to have 5' phosphate termini. This suggests that the irradiation products also have phosphate groups at their 5' termini. The
3' end–labelled 346 bp BamHI/HindIII fragment of pBR322
Approximately 20,000 cpm (1µg) loaded per track. Samples were run on an 8% sequencing gel. Track 1 is unirradiated DNA. Track 6 is a G+A chemical cleavage reaction. Tracks 2, 3, & 4 are samples irradiated in aqueous solution at RT, 0.3 and 1 MRad respectively. Tracks 4 & 5 are samples irradiated at 77K, 6 and 12 MRad respectively.
end groups on gamma radiation induced strand breaks are discussed in detail in Chapter 6.

5.23 Klenow Stop Sites on Gamma Irradiated M13mp8 Template DNA

M13mp8 templates were irradiated in dilute aqueous solution at room temperature (indirect action), frozen aqueous solution at 77K or as dry films, (direct action). The aqueous solutions were at a concentration of 200μg/ml. The irradiated templates were annealed to the universal sequencing primer, and the primer extended as described in Chapter 2 section (17). The products of the reactions were run out on 6% polyacrylamide sequencing gels, and the bands localised by autoradiography. Exposures of 1 to 7 days at -70°C were usually sufficient. (See figure 5.6.) Given a sufficient dose of radiation, bands were present at every possible position in the sequencing ladder. This shows that DNA damage is occurring at every nucleotide in the DNA molecule.

Some of the bands in the ladder appeared to be noticeably stronger than the majority. The positions of these with respect to DNA sequence was mapped, and these are shown in figure 5.1. The percentage of each base at these strong stop sites and the bases at their flanking positions is shown in the table below. The figures were compared with the percentage base composition, and values differing by greater than 1.96x standard deviations from the mean are shown in bold type. See section 5.31 for explanation of calculations. This shows that there was no preference for any base 3' to the stop site (the base first encountered by the polymerase).
Figure 5.6

Gamma-irradiated M13mp8 template DNA ran on a 6% sequencing gel. Approximately 0.2μg DNA loaded per track. Tracks 1-4, dideoxy sequencing reactions, A,T,G,C respectively. Tracks 2, unirradiated template. Tracks 5-8, irradiated in aqueous solution at RT, 0.05 MRad, 0.3 MRad and 0.6 MRad respectively. Tracks 9 & 10, irradiated at 77K, 3.6 MRad and 0.3 MRad respectively. Tracks 13 & 14, irradiated dry at RT, 3.6 MRad and 14.4 MRad respectively. Tracks 14 unirradiated template under conditions of limited polymerase activity. Tracks 14 & 16, 0.05 units and tracks 15 & 17, 0.005 units.
but there does appear to be a preference for thymine bases at the stop site and 5' to it.

<table>
<thead>
<tr>
<th>BASE</th>
<th>A</th>
<th>T</th>
<th>G</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' to stop site</td>
<td>22.4</td>
<td>22.4</td>
<td>26.6</td>
<td></td>
</tr>
<tr>
<td>Stop site</td>
<td>34.7</td>
<td>18.4</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td>5' to stop site</td>
<td>37.0</td>
<td>30.4</td>
<td>15.2</td>
<td></td>
</tr>
</tbody>
</table>

The strong stop sites on gamma-irradiated templates do not correspond to the preferential 'pause' sites on unirradiated templates. (See tracks 14-17 figure 5.6) Like the pause sites, however, the gamma radiation-induced strong stop sites are neither depleted or enhanced within the potential hairpin loops.

5.24 Gamma-Irradiated Templates Containing Homopolymer Tracts.

M13 templates containing runs of each of the four bases were constructed as described in Chapter 2 section (16). The sequences of these in the regions of the homopolymer tracts, and the primers used on each are shown in figure 5.3. Clone A has a run of 51x adenines, clone T has a run of 51x thymines, clone G has a run of 14x guanines and clone C has a run of 14x cytosines. The templates were irradiated, annealed to the appropriate primer, and the primers extended as described. (See 5.23) One problem encountered with clones A and T was that the polymerase had difficulty reading through these long runs during dideoxy sequencing, giving rise to bands in all tracks at some positions. This is a frequently encountered problem, and can sometimes be overcome by carrying out the sequencing reactions at an elevated temperature, since the
problem is thought to be due to non covalent interactions between adjacent bases. However, in this case, incubating at 37°C rather than at room temperature did not solve the problem. Occasionally, good sequences were obtained, which allowed the length of the tracts to be accurately determined. In the absence of dideoxy nucleotides, polymerisation was able to go virtually to completion (tracks 1 and 13 figure 5.7a), therefore difficulty of readthrough was assumed not to be a problem in the irradiated samples.

Gamma radiation-induced damage occurred to a lesser extent within a run of adenines (figures 5.7a+b) or guanines (figures 5.9a+b) compared with the flanking sequences, the regions of reduced damage corresponding exactly to the positions of the homopolymer runs. Damage did occur within these regions, and given a sufficiently large dose, the 'protection' effect within the polypurine tracts was abolished. The level of DNA damage within the tracts of thymines (figures 5.7a, 5.8a and 5.8b) and cytosines (figures 5.10a+b), however, was approximately the same as in the flanking sequences.
Figure 5.7a

M13 clones A and T: Tracks 1-12, clone A, tracks 13-24, clone T. Tracks 1 & 13, unirradiated template. Tracks 2-4 and 14-16, irradiated in aqueous solution at RT, 0.05, 0.3 and 0.6 MRad respectively. Tracks 5-6 and 17-18, irradiated dry at RT, 7.2 Mrad and 3.6 Mrad respectively. Tracks 7-8 and 19-20, irradiated at 77K, 3.2 Mrad and 7.2 MRad respectively.
Figure 5.7b

Enlargement of figure 5.7a, tracks 1–12.
M13 clone T. Tracks 1-5, dideoxy sequencing reactions on clone T: T,A,A,G,C respectively. Tracks 14-17, dideoxy sequencing reactions on M13mp11; A,T,G,C respectively. Track 6, unirradiated sample. Tracks 7-9, irradiated in aqueous solution at RT, 0.3, 0.6 and 0.9 MRad respectively. Track 11, irradiated dry at RT, 6 MRad. Tracks 11-13, irradiated at 77K, 1.8, 6.0 and 12.0 MRad respectively.
Figure 5.8b

Enlargement of figure 5.8a. Tracks 1-16 equivalent to track 2-17 in fig. 5.8a.
Figure 5.9a

M13 clone G. track 1, unirradiated sample. Tracks 2 & 3, dideoxy sequencing reactions on clone G, G and C reactions respectively. Tracks 2-4, irradiated in aqueous solution at RT, 0.3, 0.6 and 0.9 MRad respectively. Tracks 5 & 6, irradiated dry at RT, 3 and 6 MRad. Tracks 7 & 8, irradiated at 77K, 3 and 6 MRad.
Figure 5.9b

Enlargement of figure 5.9a. Tracks as in fig. 5.9a.
Figure 5.10 panel A

M13 clone C. Track 1, unirradiated sample. Tracks 8-11, dideoxy sequencing reactions on clone G, G and C reactions respectively. Tracks 2-4, irradiated in aqueous solution at RT, 0.3, 0.6 and 0.9 MRad respectively. Track 5 irradiated dry at RT, 6 MRad. Tracks 6 & 7, irradiated at 77K, 3 and 6 MRad.

Figure 5.10 panel B

M13 clone C. Track 1, unirradiated sample. Tracks 9 & 11, dideoxy sequencing reactions on clone G, G and C reactions respectively. Tracks 2-4, irradiated in aqueous solution at RT, 0.3, 0.6 and 0.9 MRad respectively. Tracks 5 & 8, irradiated dry at RT, 3 and 6 MRad. Tracks 7 & 8, irradiated at 77K, 3 and 6 MRad.
Figure 5.10b
Enlargement of 5.10a panel A.
5.3 DISCUSSION

5.31 Klenow 'Pause' Sites on Unirradiated Template DNA

The observation that the Klenow polymerase has preferred arrest sites on DNA templates is consistent with the findings of Hillebrandt and Beattie (1985). They demonstrated that the rate of incorporation of nucleotides appeared to vary greatly along the template with a variety of DNA polymerases. (T4 DNA polymerase, AMV reverse transcriptase and the Klenow fragment of DNA polymerase I.) Allowing polymerisation to proceed for a limited time gave rise to preferred 'pause' sites for all the polymerases, although these sites were different for all of the enzymes investigated. These sites can also be interpreted as preferential dissociation sites of the polymerases. All of the polymerases paused at the base of potential stem-loop structures, but about half of the pause sites appeared unrelated to secondary structure. The Klenow fragment was noted to 'pause' throughout regions of secondary structure, perhaps indicating a slow rate of incorporation in these regions. The results described in section 5.21 for the Klenow fragment under conditions of limited time or enzyme agree with these findings. In addition, it was shown that the pause sites were unaffected by altering the batch of template used or the source of the enzyme. The results from these studies suggest that the DNA sequence is important in determining the positions of these pause or dissociation sites. However, no obvious consensus sequences for pause sites could be deduced. It was noted that there was a slight tendency for cytosine residues 3' to these sites, and adenine
and cytosine residues at the sites themselves. The important sequences for pause or dissociation sites are likely to be those which alter the conformation of the DNA in that region.

5.22 Sequence Specificity of Gamma Radiation-Induced Damage on 'Native' DNA

The Klenow polymerase was able to pass through all of the potential pause sites given sufficient time and amount of enzyme. The irradiation studies were therefore carried out under the optimum conditions for complete polymerisation. (0.5 units of Klenow polymerase, incubated overnight at room temperature.) Template DNA was irradiated under conditions of direct and indirect action. Under all irradiation conditions used, a band was present at every possible position in the sequencing ladder, indicating that there is no strong preference for gamma-induced damage occurring at specific sequences. It was also observed that there was no site specificity for gamma radiation-induced strand breaks in an end-labelled restriction fragment, irradiated under direct or indirect conditions. These results agree with the results of Henner et al. (1982), who demonstrated that under conditions of indirect action, gamma radiation-induced strand breaks occurred at all possible positions in the DNA sequence, with approximately equal frequency. However, some bands observed following the polymerisation of irradiated templates were noticeably stronger than the majority. The positions of these bands were mapped, and although they differed from the positions of the pause sites on unirradiated templates, like these they did not tend to correspond to regions of potential
secondary structure. Sequence analysis revealed that there was a preference for the base at the DNA damage site to be thymine, and the base 5' to it to be thymine or cytosine.

This technique for looking at the sites of DNA damage on M13 template DNA reveals all lesions that cannot be read through by the polymerase. These must include strand breaks, and may also include base damage. Some types of base damage have been shown to act as replicative blocks in vitro. For instance, thymine glycols and urea residues chemically produced on template DNA's. (Ide et al. 1985; Clark & Beardsley, 1985.) These workers found that all terminations occurred opposite a putative thymine glycol sites, and at one nucleotide before urea sites. Apurinic/apyrimidinic sites have also been shown to block polymerisation. (Sagar & Strauss, 1983 & 1985). At some sites, termination occurred one nucleotide before the abasic site, but at others, termination was directly opposite the site. It is not clear why polymerisation is unable to proceed further, having inserted a nucleotide opposite the lesion, but this may be due to the lack of base pairing.

Abasic sites are implicated as 'premutagenic' lesions even in the absence of the induction of the SOS repair system. (Kunkel, 1984) This suggests that there may be readthrough of these sites at low frequency in vivo. Sites which block DNA polymerase may also act as premutagenic lesions via the SOS system. Induction of the SOS system may allow polymerisation through these lesions, leading to mutagenesis via decreased fidelity of DNA synthesis. Possible mechanisms of gamma radiation -induced mutagenesis are discussed in Chapter 1 section 1.5.
Abasic sites and thymine glycols are both known to be present in gamma-irradiated DNA. In Chapter 4 it was shown that on irradiation of single-stranded M13 DNA, about 30% of the lethal lesions are due to strand breaks under indirect irradiation conditions, and 17-20% under direct conditions. Therefore base lesions are the predominant types of damage giving rise to lethality in M13. As discussed in section 5.1, the major lethal lesions are likely to be those which arrest DNA synthesis. Since Henner et al. (1982) demonstrated the uniformity of strand breakage under indirect irradiation conditions, and the results of similar experiments described above have revealed no sequence specificity for strand breaks under indirect or direct irradiation conditions, the predominant polymerase stop sites may be due to base damage or removal rather than strand breaks.

The radiosensitivity of bases in aqueous solution has been shown to decrease in the order T, C = A, G. (‘The Effects of Ionising Radiation on DNA’ Eds. Huttermann et al., 1978) This fits in with the observation that there is a preference towards thymine at the site of irradiation induced polymerase stop sites. However, bearing in mind the observation discussed above that the polymerase often stops one base before putative thymine urea residues and abasic sites, it would be expected that a bias towards thymine would be observed 3' to the major pause sites. This was not observed, but it should be noted that the relative radiosensitivities of the various bases in solution does not necessarily reflect the relative radiosensitivities of the bases when within the DNA molecule.
A report by Ahnstrom (1985) on the irradiation of lambda DNA states that on alkali treatment of DNA irradiated under indirect conditions, strand breaks arise at each of the bases in the order G, C, A, T, (G being the most frequent site of strand breakage), although no site specificity was observed in the absence of alkali treatment. Alkali treatment gives rise to strand breaks at abasic sites (and possibly at other types of damaged bases), which suggests that the specificity observed reflects the relative radiosensitivities of the bases within DNA. Ahnstrom's results suggest thymine to be the least radiosensitive base, whereas the results in this chapter implicate thymine as the most sensitive. A report by Duplaa and Teoule (1985) also concludes that there is specificity of gamma radiation induced alkali labile lesions. They used as their substrate restriction fragments end labelled with $^{32}$P. The labelled fragments were irradiated, then treated with 1M piperidine under conditions which apparently did not cleave unirradiated DNA. The products were separated on polyacrylamide sequencing gels, and the bands localised by autoradiography. In irradiated fragments which were not alkali treated, no site specificity for strand breaks was observed. However, the piperidine-treated samples showed that alkali labile sites occured preferentially at T, followed by G, A and C in decreasing order. This is quite different from Ahnstrom's results, this may be due to the different alkali treatments used, the different treatments possibly cleaving the DNA with different efficiencies at various damage sites. Another report has demonstrated that enzyme activities from Micrococcus luteus preferentially nick irradiated DNA at T's and C's. (Hentosh et al, 1985) The
M. luteus extract comprises at least five separate endonucleases, including activity against abasic DNA. It is not clear whether the observed preference for nicking at T and C reflects a greater level of damage at these bases, or to a property of the enzyme(s). Taking the evidence described, there appears to be no gross sequence specificity for gamma radiation-induced strand breaks under conditions of either direct or indirect action, but there may be some specificity of base damage. The proportions of each base seen to be damaged depends on the assay employed.

5.3.3 Sequence Specificity of Gamma Radiation-Induced Damage on DNA's with Homopolymer Tracts

The studies on the irradiation of M13 clones with homopolymer tracts showed that gamma radiation-induced damage is reduced with respect to the flanking sequences within a run of purine bases, but is more or less unaltered within a pyrimidine run. The region of reduced damage in the purine tracts corresponded exactly to the positions of the poly A and poly G tracts. The results were the same for samples irradiated under conditions of direct and indirect action. Previous findings of our group suggest that under direct irradiation conditions, the primary radical centres on G and T become trapped quite close together, and the charges do not subsequently migrate extensively. (Cullis & Symons, 1985) Therefore it was expected that DNA damage would be enhanced in poly G and poly T tracts, assuming that both 'G' and 'T' give rise to DNA damage. In a 'random' sequence, any specificity for damage at G's and T's may well not be seen.
since charge migration even through one or two bases would tend to obscure the pattern.

The primary radicals 'G' and 'T' must initially be formed close together, providing that electron and hole migration are not extensive, since a single ionisation event will clearly result in electron ejection and positive hole formation at the same site. It has been shown that under direct radiation conditions, the yield of double strand breaks is much greater than expected if they are formed as the result of two independent single strand breaks on opposite strands. (Boon et al, 1984) This suggests that double strand breaks can arise as the result of a single ionisation event, so it has been suggested that both 'G' and 'T' can lead to the formation of a single strand break, and that the observed high proportion of double strand breaks will result if these two centres become trapped close together, and on opposite strands. It is usually assumed that a double strand break constitutes two single strand breaks separated on opposite strands by a distance of less than about 16 base pairs. (Friefelder & Trumbo, 1966) The radical pairs must be sufficiently separated to prevent electron return. However, there is evidence to suggest that extensive charge migration is possible. This includes the thermoluminesence of DNA films after gamma irradiation, which must be the return of weakly trapped electrons to cations.

Since there was a reduction of damage in polypurine tracts under conditions of both direct and indirect irradiation, this cannot be explained by radical formation on specific bases, since the indirect damage mechanism is due primarily to hydroxyl radical attack. It therefore appears that radical
reactions in general occur less frequently in polypurine tracts than in the flanking sequences. One explanation is that the DNA conformation within such regions hinders radical reactions. Polypurine tracts in single-stranded DNA are base stacked, making the molecule rather more 'compact' within these regions. ("The Effects of Ionising Radiation on DNA." Eds. Hutterman et al, 1978) This may reduce the access of diffusing hydroxyl radicals to the bases in these regions. The rate of hydroxyl radical attack is lower on bases stacked in double helical DNA than in single stranded DNA's, perhaps because base stacking reduces the number of sites which are accessible to hydroxyl radicals. (Hutchinson, 1985) In the case of direct damage, the base stacking interactions (which facilitate charge migration) may tend to conduct radical centres away from the polypurine regions.

As enhanced damage within the poly G and poly T tracts was not observed, there are three possible explanations. Firstly, the primary radicals 'G' and 'T-' may not be responsible for giving rise to stable DNA damage products. This possibility is discounted since there is a certain amount of circumstantial evidence to implicate 'G' and 'T-' as the sole damage precursors. (Roon et al, 1984, 1985; Cullis et al, 1985) Secondly, there may be further charge migration after the formation of 'G' and 'T-' through quite large distances (tens of base pairs) obscuring any initial specificity. However, the migration would really have to be quite large to account for the lack of any enhancement of damage even in the centre of a run of 51 thymines. As discussed above, there is evidence to suggest that charge migration is not extensive. This leaves the third possibility that primary radicals on
bases other than G and T are formed within homopolymer tracts. Some evidence that this may be the case comes from ESR studies on the irradiation of nucleotides and nucleotide mixtures at 77K. (Gregori et al, 1970) Radicals are formed on each of the nucleotides when these are irradiated individually, giving rise to characteristic spectra. If pyrimidines and purines are both present, charge migration occurs. For instance, in an equimolar mixture of adenosine and thymidine, a complete charge transfer from A to T is observed. In freeze-dried nucleotide mixtures, base stacking occurs, as in double helical DNA. If the proportions of each nucleotide are the same in the mixture as in DNA, identical spectra are observed. Therefore the extent of charge migration from base to base not only depends on base stacking, but also the nucleotide composition. In a homopolymer run transfer of positive charges to G and negative charges to T may not be complete because of the distances involved. However, this suggestion only explains the occurrence of DNA damage in runs of C and A, and not the lack of enhanced damage in poly G and poly T tracts. The correct explanation for these results may be that there is enhanced charge migration out of the polypurine tracts, coupled with the formation of other primary radicals in the absence of G and T.
In conclusion, this study has demonstrated that there is little or no site specificity for gamma radiation-induced strand breaks, but an apparent preference for T at the site of damage which blocks DNA polymerase activity, and T and C 5' to the damaged site. In single-stranded DNA, the level of damage is reduced within a run of purines but unaffected in a run of pyrimidines. In addition, it has been noted that the Klenow fragment of DNA polymerase I has preferred 'pause' or 'dissociation' sites on DNA templates, but complete polymerisation can be achieved given sufficient time and amount of enzyme.

The work discussed in this chapter could be usefully extended, and some of the hypotheses tested by the following experiments. It would be possible to test the hypothesis that base stacking is responsible for the reduced level of DNA damage occurring within polypurine tracts by irradiating under conditions where base stacking is prevented, for instance, at an elevated temperature, (For example, 50-60°C). If base stacking is the cause of 'protection' of DNA within polypurine tracts, then irradiating under denaturing conditions should wholly or partly abolish this effect. It would also be useful to investigate the pattern of radiation-induced damage on DNA templates with adjacent purine and pyrimidine tracts, particularly if these allow the formation of a stable hairpin loop. If the level of DNA damage in the purine tract is still lower than that in the flanking sequences, then this suggests that charge transfer between DNA strands is not occurring. However, if base pairing to a
polypyrmidine strand partly or completely eliminates the 'protection' effect within a purine tract, then presumably charge migration between DNA strands is occurring.

The pattern of gamma radiation-induced damage could be studied in double-stranded DNA by 'nick translation' assay and T4 3' to 5' exonuclease assay. (As described in the introduction to this chapter.) The nick translation assay would enable the DNA polymerase stop sites on double-stranded DNA to be mapped and compared with the results discussed here for single-stranded DNA. The T4 exonuclease assay would allow the positions of the lesions which prevent 3' to 5' exonuclease digestion, on both single and double-stranded DNA. It seems likely that radiation-induced damage in double-stranded DNA is non-sequence-specific as for single-stranded DNA. This has already been shown to be the case for strand breaks. In the light of the work of Ahnstrom and Duplaa & Teoule (see section 5.32), it would be useful to investigate the site specificity of alkali labile lesions using some of these techniques.

The possibility that the strong bands observed on the analysis of gamma-irradiated DNA templates are the result of base damage or removal has been discussed above. This hypothesis could be tested by the purification of irradiated template DNAs which are still full length (that is, have no strand breaks) on a non denaturing polyacrylamide gel prior to annealing to a template and polymerising. Following polymerisation under the usual conditions, the samples would then be run on a denaturing polyacrylamide gel. This would focus on polymerase termination sites arising from base and sugar modification, and would not include termination sites
at strand breaks.
CHAPTER
six
CHAPTER 6

THE TERMINI OF GAMMA RADIATION-INDUCED STRAND BREAKS

6.1 INTRODUCTION

Strand breaks are perhaps the most widely studied of the lesions produced in DNA by ionising radiation. This is partly due to the relative ease with which both single and double strand breaks can be detected and quantified, as discussed in Chapter 3; and partly because these are considered to be one of the most serious DNA lesions in vivo in terms of leading to cell death. However, single and double strand breaks have both been shown to be rapidly repaired in the cell. (McGrath & Williams, 1966; Hutchinson, 1979) Some possible mechanisms for the repair of strand breaks have been described in Chapter 1 section 1.4. It is clear from this that the molecular mechanisms leading to the repair of DNA strand breaks are rather poorly understood. To gain insight into how such mechanisms may operate, the chemical nature of gamma radiation-induced strand breaks must be fully characterised. A detailed knowledge of the end groups on these strand breaks is therefore of great use in proposing repair pathways, and in addition, provides us with an insight into the mechanisms leading to strand break formation in irradiated DNA.

To this end, several studies into the nature of the end groups on gamma-irradiated DNA have been carried out, these are reviewed in detail by Von Sonntag et al, 1981. Enzymatic analysis has been used to study the end groups on gamma radiation-induced strand breaks, the enzymes used and the end
groups they detect are summarised in the table below.

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>REACTION</th>
<th>END GROUP DETECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polynucleotide kinase (PNK)</td>
<td>Transfers phosphate from ATP to 5'-OH end groups.</td>
<td>5'-OH</td>
</tr>
<tr>
<td>PNK exchange reaction</td>
<td>Removes 3'-phosphate groups in the absence of ATP.</td>
<td>3'-phosphate</td>
</tr>
<tr>
<td>Phosphatase combined with PNK</td>
<td>Dephosphorylation of 5' phosphate groups to 5'-OH groups to allow phosphorylation by PNK.</td>
<td>5'-phosphate</td>
</tr>
<tr>
<td>Phosphatase activity of exonuclease III</td>
<td>Dephosphorylates 3' phosphate end groups.</td>
<td>3'-phosphate</td>
</tr>
<tr>
<td>Terminal nucleotidyl transferase</td>
<td>Polymerisation on 3'-OH end groups.</td>
<td>3'-OH</td>
</tr>
<tr>
<td>Polynucleotide ligase</td>
<td>Joins 3'-OH end groups with 5'-phosphate end groups.</td>
<td>3'-OH and 5'-phosphate</td>
</tr>
<tr>
<td>DNA polymerase I (E.coli)</td>
<td>Polymerisation on 3'-OH end groups.</td>
<td>3'-OH in intact double helix</td>
</tr>
</tbody>
</table>

Table modified from von Sonntag et al., 1981.

In studies on DNA irradiated under indirect conditions, Bopp and Hagen (1970) showed that most of the 5' end groups on irradiated DNA are phosphate (P) groups, by incubating irradiated DNA with PNK in the presence of γ³²P ATP. Only a small proportion of label was transferred to the DNA, indicating that very few 5'-OH groups were present. However, incorporation of label into DNA almost reached completion if the DNA was first treated with phosphatase, which removes 5'-phosphate groups, leaving a free 5'-OH group. Bopp et al. (1973) demonstrated the release ³²P from 3' end-labelled
irradiated DNA by exonuclease III action, enabling the  
calculation that around 20% of the 3' ends were phosphate  
groups. In other experiments, irradiated DNA was incubated  
with DNA ligase, this demonstrated that 30-40% of the strand  
breaks could be directly rejoined, that is, 30-40% of strand  
breaks had a 3'-OH and a 5'-phosphate terminus.  
(Mitzel-Landbeck et al, 1973)  

In a series of papers, Henner and coworkers (Henner et al,  
1982a, 1982b, 1983) have shown that under indirect  
irradiation conditions, all of the strand breaks have a 5'  
phosphate terminus, and the 3' termini are either phosphate  
(P), or phosphoglycolate (PG) groups, in approximately equal  
proportions. This was shown by irradiating 3' or 5' end  
labelled DNA, then running the products out on denaturing  
polyacrylamide gels in parallel with standards having known  
termi. The distance of migration of products on  
polyacrylamide gels is a function of their charge:mass ratio.  
Therefore products with the same number of nucleotides may  
not migrate at the same position in the gel if they posess  
different end groups, and hence different charge:mass ratios.  
This is particularly true for fragments of relatively low  
molecular weight, where the differences in charge:mass ratio  
will be greater. Thus by comparing the mobilities of the  
irradiation products with known standards, Henners' group  
were able to tentativley identify the 3' and 5' termini. DNA  
labelled at the 3' end and then irradiated, comigrated with  
products known to terminate in 5'-phosphate groups. With DNA  
labelled at the 5' end, two bands were present at each  
nucleotide position in the ladder, indicating two different  
end groups. The slower migrating of the two bands comigrated
with products known to have 3' phosphate termini, but the band with faster mobility migrated neither with these nor with products known to terminate in 3'-OH groups. The identity of the 5' end group was confirmed by the action of alkaline phosphatase, which removes 5'-P groups; and the identity of one of the 3' end groups by the action of PNK, which removes 3' phosphate groups. The dephosphorylated products were detected by their altered mobilities on polyacrylamide gels. The other 3' end group was identified by the purification of single nucleotide fragments from irradiated polyG DNA from gels, followed by thin layer chromatography (TLC) in parallel with chemically synthesised 3',5'-dGDP and 3',5'-dGTP-glycolate. The gamma radiation produced fragments were found to co-migrate with authentic dGTP-glycolate, indicating that these fragments have 3' phosphoglycolate termini. These findings suggest that none of the radiation induced strand breaks are directly ligatable by DNA ligase. This contradicts some of the results described above, but this may be due to the different gaseous conditions employed, the experiments in which -OH groups were detected were carried out under anoxic conditions. (See Discussion.)

The studies discussed in the above two paragraphs have only analysed strand breaks resulting from the indirect radiation mechanism, that is, hydroxyl radical attack. Some studies on DNA irradiated in cells, where some direct attack is likely have been carried out. These studies have made the assumption that cellular repair can be largely prevented by irradiating the cells at 0°C and lysing within a few seconds after irradiation. PNK and phosphatase action on DNA isolated from
irradiated cells has shown that 10-15% of 5' termini are OH groups, and 20-40% are phosphate groups. However, in DNA irradiated dry in vitro, about 25% of 5' end groups were shown to be phosphate groups, and no 5'-OH groups were detected. (Coquerelle et al, 1973) The 3' end groups on DNA irradiated in vivo were analysed by DNA polymerase I and terminal deoxynucleotidyl transferase (TdT). (Lennartz et al, 1973) TdT adds dNTP's to free 3'-OH groups on single-stranded DNA or DNA with a protruding 3' end, to produce a single stranded 'tail'. (TdT will also extend blunt-ended DNA or DNA with a recessed 3' terminus, but only in the presence of Co^{2+}, which was not present in these studies.) DNA polymerase I requires a free 3'-OH group and was found to have no activity on irradiated double-stranded DNA, perhaps due to a distortion of the double helix, or the requirement for the opposing terminus to be a substrate for the 3' to 5' exonuclease activity of the polymerase. TdT, however, was able to act on 70% of strand breaks, indicating that 70% of strand breaks have a 3'-OH group. These results differ from those obtained from DNA irradiated in vitro in dilute aqueous solution in that many more 3' and 5'-OH groups were detected in DNA irradiated in vivo or dry.

Apparentley, no studies have been carried out on the end groups on strand breaks resulting from irradiation of aqueous solutions at 77K. This chapter describes end group analysis on DNA labelled at the 3' or 5' ends irradiated in vitro under conditions of direct action (frozen aqueous solution at 77K) and indirect action (aqueous solution at RT), in both cases in ambient atmosphere. The products were analysed by polyacrylamide gel electrophoresis, in experiments similar to
The results for samples irradiated in aqueous solution agreed with the findings of Henner et al. That is, a 5'-P terminus and 3'-P and 3'-PG termini, the latter in approximately equal proportions. Samples irradiated under direct conditions (frozen at 77K, 202K or dry), however, were found to have predominantly phosphate termini at both the 3' and the 5' ends. The 3' phosphoglycolate terminus constituted only a very small proportion of the end groups under direct conditions. In addition, samples were irradiated at 77K in the presence of hydrogen peroxide. \( \text{H}_2\text{O}_2 \) is a radiosensitiser, which is suggested to give rise to enhanced DNA damage via hydroxyl radical attack. (See Chapter 3.)
6.2 RESULTS

6.21 The 5' Termini of Strand Breaks

The 346 bp BamHI/HindIII fragment from pBR322 was labelled at the 3' end as described in Chapter 2, section (18). Aliquots of labelled DNA were irradiated in dilute aqueous solution at RT and frozen solution at 77K. (See Chapter 5, figure 5.5) In the irradiated samples, a band was present at every possible position in the sequencing ladder, and all the bands were of approximately equal intensity. (For a further discussion of the site specificity of gamma radiation-induced strand breaks see Chapter 5.) The irradiation products comigrate with products of the G + A chemical cleavage reaction, which have 3'-P and 5'-P termini. The chemical cleavage reaction involves an opening of the purine ring at the C-7 position adjacent to the glycosidic bond by 1M piperidine, followed by displacement of the ring opened product from the sugar, and heat-induced cleavage of the phosphodiester backbone to leave a phosphate group at both the 3' and the 5' termini.

These results suggest that the 5' termini of strand breaks induced under conditions of direct and indirect action are phosphate groups.
6.22 The 3' Termi ni of Strand Breaks

The 'universal' M13 oligonucleotide primer (17 bases) was labelled at the 5' end with $^{32}$P (as described in Chapter 2 section 17), and irradiated in dilute aqueous solution at RT, or frozen solution at 77K. The markers used were the G + A chemical cleavage reaction, which gives rise to 3' and 5'-P termini (see section 6.21), and DNAseI digestion which gives rise to a 3'-OH and a 5'-P group. In all cases, irradiation leads to strand breakage at every possible nucleotide with approximately equal frequency, as expected from the results discussed in Chapter 5. In the samples irradiated under indirect conditions (aqueous at RT), doublet bands were present at each site. (See figures 6.1,6.2) This is consistent with the results of Henner et al, who demonstrated the presence of these doublet bands in single and double stranded DNA irradiated under indirect conditions. These doublets indicate that there are two possible positions of cleavage of the sugar-phosphate backbone at each nucleotide. None of the bands comigrated with DNAseI digestion products, but the slower migrating of the bands comigrated with products of the G + A reaction. As discussed in (6.1), subsequent enzymic and chromatographic analysis by Henner's group showed that the slower migrating band in the doublet was a 3'-P group, and the faster migrating band a 3' PG group.

The samples irradiated frozen at 77K or as dry films gave only one major band at each site, which comigrated with products known to terminate in a 3'-P group. (Figure 6.1, track 4 and figure 6.2 tracks 3-5) This suggests that the strand breaks resulting from irradiation under direct
Figure 6.1.

5' end-labelled oligonucleotide ran on a 20% sequencing gel. Approximately 500,000cpm (1ng) DNA loaded per track. Track 1, unirradiated DNA. Track 2, G + A chemical cleavage reaction. Track 5, DNAseI digest. Tracks 3 & 4, irradiated samples. Track 3, 0.6 MRad aqueous solution at RT; track 4, 6MRad TMM.
Figure 6.2
5' end-labelled oligonucleotide ran on a 20% sequencing gel. Approximately 500,000 cpm (1ng) DNA loaded per track. Track 1, unirradiated DNA. Tracks 2 & 8, irradiated in aqueous solution, 0.6MRad. Tracks 3 & 4, irradiated dry at RT, 6 and 3MRad respectively. Track 5, irradiated at 77K, 6MRad. Tracks 6 & 7, irradiated at 202K, 6 and 3MRad respectively.
Figure 6.3

5' end-labelled oligonucleotide ran on a 20% sequencing gel. Approximately 500,000 cpm (1ng) DNA loaded per track. Tracks 1 & 2, unirradiated. Tracks 3 & 4, irradiated in aqueous solution at RT, 0.6MRad. Tracks 5 & 6, irradiated at 77 K, 6MRad. Track 7, DNAseI digest. Tracks 2, 4 & 6, incubated or irradiated in the presence of 4mM H₂O₂.
conditions have predominantly P groups at the 3' termini. Faint bands corresponding to the positions of the 3'-PG termini were seen. Densitometric scanning of the autoradiographs revealed that an average of 86±14% of the 3' termini under direct irradiation conditions were P groups. Under indirect irradiation conditions, an average of 69±18% of 3' termini were P groups, and 31±18% were PG groups.

The identity of the end groups was confirmed by running irradiated samples on a 20% denaturing polyacrylamide gel, visualising the bands by autoradiography, excising doublet bands from a sample irradiated in aqueous solution at RT, and the corresponding singlet from a sample irradiated at 77K. The DNA fragments were eluted into water and purified on a Sephadex G-25 column. (See Chapter 2, section 19) The resultant samples were split into two aliquots, one was left untreated as a control, and the other was used as a substrate for the PNK exchange reaction (described in section 6.1), which removes 3'-P groups. Following incubation, the treated and untreated samples were run on a 20% denaturing polyacrylamide gel, and autoradiographed. The results are shown in figures 6.4 and 6.5. The figures are essentially the same except that figure 6.5 includes a G + A chemical cleavage reaction as markers. The results from this are quite clear; in the sample irradiated under indirect conditions, the faster moving band of the doublet is unaffected by PNK action, but the slower moving band has its mobility reduced still further by the PNK reaction, as it is converted to a hydroxyl group. This confirms the results of Henner et al, (1982,1983) who in addition went on to identify the slower moving band as a 3'-PG terminus. The band from the samples
irradiated under direct conditions behaved in the same way as
the slower migrating band of the 'indirect' irradiation
sample, that is, its' mobility was further reduced by PNK
action. Therefore fragments generated through damage by the
direct action of ionising radiation terminate in a
3'-phosphate group.

In order to demonstrate that the mechanism leading to
strand break formation is the same over a wide temperature
range below the softening point, samples were irradiated at
202K in a dry ice/ chloroform bath in addition to 77K.
Samples were also irradiated at room temperature as dry
films. Gel analysis revealed that in all these cases, the
pattern of banding was the same, that is, little or no
production of strand breaks with 3'-PG termini. (See figure
6.2) Samples were also irradiated in the presence of 4mM
hydrogen peroxide. (See figure 6.3) At this concentration,
H$_2$O$_2$ had no effect on unirradiated DNA. (Tracks 1 & 2) As
expected, H$_2$O$_2$ acted as a radiosensitiser on samples
irradiated under direct and indirect conditions. (Tracks 3-6)
On visual inspection of the autoradiographs, DNA degradation
is greater in the presence of H$_2$O$_2$ than in its absence, for
samples given the same dose of irradiation. However, H$_2$O$_2$
does not appear to alter the banding pattern in either case.
Samples irradiated in aqueous solution at RT with H$_2$O$_2$ have
doublet bands at each site, and samples irradiated at 77K
with H$_2$O$_2$ only have singlet bands.
Figure 6.4
Irradiation products (singlet from 77K irradiation, doublet from RT irradiation) eluted from gel and run on a second Zyen sequencing gel. Tracks 1 & 3, irradiated 6MRad at 77K. Tracks 2 & 4, irradiated 0.6MRad in aqueous solution at RT. Tracks 1 & 4, treated with PNK (exchange reaction).
Figure 6.5

Irradiation products (singlet from 77K irradiation, doublet from RT irradiation) eluted from gel and run on a second DNA sequencing gel. Track 1, G + A reaction. Tracks 4 & 5 irradiated 6MRad at 77K. Tracks 2 & 3, irradiated 0.6MRad in aqueous solution at RT. Tracks 3 & 5, treated with MEl (exchange reaction).
6.3 DISCUSSION

Gamma radiation-induced strand breaks were found to occur at all possible positions in the nucleotide chain with approximately equal frequency, under all of the irradiation conditions employed. For a fuller discussion of site specificity of radiation-induced damage, see Chapter 5.

The series of experiments described above confirmed the results of Henner et al. (1982, 1983), which unequivocally demonstrated that under indirect irradiation conditions, the 5' termini of gamma radiation-induced strand breaks are phosphate groups, and the 3' termini phosphate or phosphoglycolate groups in approximately equal proportions. Importantly, it has been demonstrated that under direct radiation conditions, the mechanism of reaction leading to strand breaks gives rise almost exclusively to 3' and 5' phosphate termini. Other workers report the presence of a significant proportion of 3'-OH termini under indirect irradiation conditions, as discussed in the introduction. (Jacobs et al., 1972; Mitzel-Landbeck et al., 1970) This may be because these workers carried out the irradiations under anoxic conditions, whereas in this study and the work of Henner's group, samples were irradiated under conditions of ambient atmosphere. It is not surprising that differences in the end groups on radiation-induced strand breaks should arise under oxic and anoxic conditions, since in anoxic conditions, aqueous electrons and hydrogen atoms are considered to make a significant contribution to DNA damage, whereas these are of minor importance in oxic conditions. (Nabben et al., 1984) Furthermore, there is a preponderance of
long lived free radicals in anoxic conditions, which in oxic conditions would be removed by reaction with oxygen. (Hutchinson, 1985) DNA irradiated in cells was shown to have 3' and 5'-OH termini on a fairly large proportion of the total strand breaks. (lennartz et al, 1973; Coquerelle et al, 1973.) It is supposed that in vivo both direct and indirect damage mechanisms operate, this suggests that OH termini may result from direct and / or indirect mechanisms. However, no OH termini were detected in this study or in the work of Henner's group. In a recent paper, Bases et al. (1986) have isolated a specific repetitive DNA sequence from irradiated mammalian cells, end-labelled these, and analysed them on acrylamide gels. These results showed that if the DNA is labelled at the 5' end, doublet bands of approximately equal intensity are seen, which comigrate with those arising from irradiation of this DNA in aqueous solution at RT in vitro. The end groups have not yet been formally characterised, but if they prove to be 3'-P and 3'-PG groups, then this suggests that the indirect damage mechanism may predominate in vivo. This is perhaps contrary to what we would expect. (See Chapter 1, section 1.3) Although some of the reports described above appear to present conflicting data, it may not be valid to compare in vivo to in vitro results due to the large number of additional factors involved when irradiating in vivo.

Since under direct or indirect irradiation conditions phosphate (or phosphoglycolate) groups are present at the 3' termini and P groups at the 5' termini, the mechanism of strand breakage must involve the release of a base and an altered sugar, resulting in a gap on the irradiated molecule.
Figure 6.6
The structure of gamma radiation-induced strand breaks. (A) 3' and 5' phosphate termini, (B) 3' phosphoglycolate and 5' phosphate termini. (From Henner et al, 1983)
Released altered bases and sugars have been detected in irradiated DNA solutions. (Ward, 1975) Cellular repair of radiation-induced strand breaks must therefore involve several enzymic steps. The first step is likely to be the removal of the 3'-P and 3'-PG groups by phosphatase action, to give a 3'-OH group, which is required for the initiation of DNA polymerase. This could be accomplished by exonuclease III action, which removes 3'-P (and PG) groups prior to the sequential removal of nucleotides. Henner et al. (1983) have shown that E.coli exonuclease III can remove 3'-P and 3'-PG end groups in vitro. A polymerase step is then required to fill in the gap in the 3' to 5' direction, followed lastly by sealing of the gap by DNA ligase.

The nature of the end groups on strand breaks in DNA irradiated under indirect conditions is consistent with the mechanism for OH• radical attack proposed by Bothe et al. (1983). This mechanism proposes that the 3'-P and 3'-PG groups can both be formed via a common C-4 sugar radical. (The 4' position is suggested to be the favoured position of OH• attack on the sugar.) Two subsequent β-elimination reactions lead to the formation of strand breaks with both 3' and 5'-P groups, by steps shown in figure 6.7. Alternatively, the formation of tetroxide intermediates has been proposed, which gives rise to strand breaks with 5'-P and 3'-PG termini. (Figure 6.7)

Irradiation under direct irradiation conditions gives rise to strand breaks with predominantly 3' and 5'-P termini. There was some evidence of 3'-PG formation, but this represented only a small proportion of the total strand breaks compared with the much larger proportion arising under
indirect irradiation conditions. Some faint bands at other positions in the gel were seen, these did not comigrate with products terminating in P, PG or OH groups, and may represent molecules with a damaged sugar at the 3’ terminus. The primary radicals formed under direct irradiation conditions undergo a series of reactions leading to the DNA damage products finally observed. In the presence of oxygen (as in the work discussed here) ‘G+ and ‘T- give rise predominantly to base peroxy radicals, RO₂⁻. The yield of strand breaks under these conditions suggests that RO₂⁻ radicals are precursors of strand breaks. (Boon et al., 1984) It has been suggested that these react by intramolecular hydrogen atom abstraction from the sugar. The site for this H-atom abstraction has not been identified, but the C-4’ position (which is the favoured position of H-atom abstraction by hydroxyl radicals), is shown by the analysis of three dimensional models to be poorly positioned for H-atom abstraction by base peroxy radicals. Other positions, the C-2’ for instance, are more favourably placed for possible H-atom abstraction by base radicals.

In spite of this, plausible mechanisms can be proposed leading to the production of C-4 sugar radicals from base radicals which would lead to the end groups detected in this work. (See figure 6.8) As for the indirect mechanism, two subsequent β-elimination steps would give rise to strand breaks with 3’ and 5’-P termini. The tetroxide intermediate presumably would not form under frozen or dry conditions due to the limited availability of oxygen and restricted mobility of the radicals. This may account for the loss of the 3’-PG end group under direct irradiation conditions. Another
Figure 6.7

A mechanism for the production of strand breaks resulting from hydroxyl radical attack. (Irradiation under indirect conditions.)
(1) $\beta$ elimination

(11) $+O_2$

(continued)

3' or 5' PHOSPHATE
$\sim P - O - CH_2$

$\sim P - O - CH_2$

$\sim P - O - CH_2$

$\sim P - O - CH_2$

$\sim P - O - CH_2$

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Figure 6.8

A mechanism for the production of strand breaks from primary base radicals, via a C-4' sugar radical. (Irradiation under direct conditions).


GUANINE (G) → (G⁺ 17G Singlet) + e⁻ → RO₂?

THYMINE (T) + e⁻ → (T⁻ 23G Doublet) → ('TH Octet) → RO₂ (TO₂⁻)

(continued)
possibility why 3'-PG groups do not result from irradiation under direct conditions could be that the reaction pathways leading to the 3'-P and 3'-PG end groups have different activation energies. Whilst the rates of reaction are approximately the same at RT, the rate of the reaction leading to 3'-PG formation may decrease much more rapidly as the temperature is lowered than the rate of the reaction to form 3'-P termini. This would mean that at 77K, the rate of reaction leading to 3'-PG formation is so much slower than the alternative pathway, that strand breaks with 3'-PG termini comprise only a very minor fraction of the total. To test this hypothesis, samples were irradiated at an intermediate temperature, but below the softening point. (202K) Under these conditions, the proportion of strand breaks with 3'-PG groups was the same as at 77K, indicating that different rates of reaction were not a factor in the lack of 3'-PG group formation at 77K. Furthermore, irradiating samples dry at RT showed about the same proportion of strand breaks with 3'-PG groups as observed at 77K. Therefore these films of DNA are considered to be similar to the frozen aqueous system.

Since the C-4' of the sugar does not appear to be in a favourable position for H-atom abstraction by base radicals, it is possible that under direct irradiation conditions, strand breaks are formed via a completely different pathway to the indirect mechanism, that is, one which does not involve the C-4' sugar radical intermediate. Hydrogen atom abstraction at an alternative position in the sugar may lead (by unknown mechanisms) to strand breaks with exclusively 3' and 5'-P termini. Another possibility is that attack on the
sugar by base radicals may lead to 3'-P termini, whereas attack on the sugar by OH· radicals may lead to 3'-PG termini. Under indirect conditions, OH· radicals attack both the sugar and the base. Therefore 3'-PG termini may result from the direct attack by OH· on the sugar, and 3'-P termini from attack of OH· generated base radicals on the sugar. Since under direct irradiation conditions, attack on the sugar is always via a base radical, strand breaks with exclusively 3'-P termini would be produced.

The addition of H₂O₂ during irradiation gave rise to increased DNA degradation over the same dose in the absence of H₂O₂, whereas in the absence of irradiation, no effect was observed. This was expected since H₂O₂ is a known radiosensitiser. (See Chapter 3) The mechanism of radiosensitisation involves the radiolysis of H₂O₂ to produce hydroxyl radicals. This is one of the steps involved in the production of hydroxyl radicals from the radiolysis of water. (See Chapter 1, section 1.1) Therefore by including H₂O₂ during irradiation of frozen aqueous solution, the strand breaks should arise partly from OH· radical attack. This means that strand breaks terminating in 3'-PG as well as 3'-P groups should be observed. However, the proportion of strand breaks terminating in 3'-PG groups was apparently unchanged by the inclusion of 4mM H₂O₂ during irradiation. As discussed in Chapter 3, 4mM H₂O₂ increases the number of DNA strand breaks arising from irradiation in frozen aqueous solution by around 25%. This will give rise to an increase in the fraction of strand breaks terminating in 3'-PG groups of only 12.5%, and it may not be possible to detect this relatively small change. Another possibility is that the different
environments of the OH· radicals at 77K and RT may effect the reaction mechanism. At 77K, the OH· radicals are produced in a frozen matrix close to the DNA molecule, whereas at RT, OH· are produced in the bulk water and can diffuse freely through the medium. Perhaps it is only this latter environment which allows the mechanism leading to strand breaks with 3'-PG termini.

6.4 CONCLUSION

In conclusion, the work presented in this chapter allows a comparison of the end groups on gamma radiation-induced strand breaks under conditions of direct and indirect action. Under indirect irradiation conditions, the 5' termini are P groups and the 3' termini P or PG groups, in roughly equal proportions. These end groups are consistent with OH· radical attack on the C-4 position on the sugar, allowing either β-elimination or the formation of tetroxide intermediates. Under direct irradiation conditions, both of the termini were found to be P groups. This allows the proposal of a mechanism involving the production of base peroxy radicals from primary base radicals, which cause H atom abstraction on the sugar. If attack by base radicals is at the C-4' position, strand breaks may subsequently arise from β-elimination reactions. The formation of tetroxide intermediates may be prevented due limitation of oxygen availability and radical mobility. Since topological considerations suggest that attack on the C-4' position by base peroxy radicals is unlikely, H-atom abstraction at an alternative position may give rise to a pathway which results in strand breaks with exclusively 3'-P
(and 5'-P) termini. The nature of gamma radiation-produced strand breaks (in particular, the presence of a gap in the DNA chain) has important implications for cellular DNA repair.
SUMMARY
SUMMARY

The work presented in this thesis is an attempt to improve the understanding of molecular mechanisms of radiation damage to DNA irradiated in vitro, and to gain some insight into the distribution of various types of damage with respect to DNA sequence. The contribution of the different types of damage to loss of viability in bacteriophage is also discussed. The work concentrates largely on direct irradiation damage, that is, damage resulting from radicals formed on the DNA molecule itself or very close to it. The direct mechanism predominates in samples irradiated in the dry state or as frozen aqueous solutions. However, several experiments were also carried out under indirect irradiation conditions, in order to compare and contrast the two damage mechanisms. The indirect mechanism predominates in samples irradiated in dilute aqueous solution, under these conditions, DNA damage is largely the result of diffusing hydroxyl radicals formed from the radiolysis of water. (Ward, 1977)

Under direct irradiation conditions, direct ionisation of the DNA molecule gives rise to an electron gain and an electron loss centre for each ionisation event. Electron spin resonance spectroscopy reveals that these charges become trapped out specifically at guanine to form a guanine cation, 'G+', and thymine to form a thymine anion, 'T-'. The only other primary radicals detected are hydroxyl radicals, OH', which are not thought to contribute to DNA damage under these conditions, since they are lost without alteration to the DNA radicals. (Cullis & Symons, 1986) Since under conditions where 'G+' and 'T-' are the only primary radicals detectable,
DNA strand breaks arise, this strongly suggests that \( \cdot G^+ \) and \( \cdot T^- \) are the sole precursors of strand breaks under direct irradiation conditions. Furthermore, since the G value for \( \cdot G^+ \) and \( \cdot T^- \) formation is about 1.5, and the G value for strand break formation for plasmid DNA irradiated at 77K is about 0.55, the difference can be accounted for by the production of other types of DNA damage from these primary radicals.

Our group have investigated the effect of the addition of various chemicals to DNA during irradiation on the ESR spectra and the yield of strand breaks. From the above discussion, it is clear that additives which alter the yield of \( \cdot G^+ \) and \( \cdot T^- \) are expected to also alter the yield of strand breaks. The studies completed so far have all implicated \( \cdot G^+ \) and \( \cdot T^- \) as the sole precursors of strand breaks. In addition, it has been possible to draw conclusions about the mechanisms of radioprotection or radiosensitisation by a variety of additives.

In this thesis, hydrogen peroxide was shown to increase the yield of DNA strand breaks in plasmid DNA irradiated at 77K. The ESR data suggested that this radiosensitisation was the result of hydroxyl radicals arising from the radiolysis of hydrogen peroxide which partitioned into the DNA phase on freezing. A radiosensitisation was also achieved by repeatedly thawing and re-freezing a DNA sample during irradiation. This was thought to arise through hydrogen peroxide formed from the radiolysis of water coming close to the DNA on re-freezing.

The thiol mercaptoethylamine was shown to act as a radioprotector with respect to strand breaks induced under
direct irradiation conditions, and the protection was found to be in competition with oxygen. Sulphydryl-containing compounds have long been known to protect against hydroxyl radical-induced DNA damage, and this protection is also known to be in competition with oxygen. Combining the FSR and the strand break data obtained in the presence of mercaptoethylamine under direct irradiation conditions, we have been able to propose mechanisms for the radioprotective effects.

Experiments were carried out to determine the contribution of strand breaks to loss of infectivity in the single-stranded bacteriophage M13. A comparison was made between the irradiation of intact phage particles versus purified phage DNA, and between direct and indirect irradiation conditions. In phage particles and in purified phage DNA irradiated under direct conditions (77K), strand breaks were found to account for 1/5 to 1/6 of the lethal hits. Loss of infectivity and strand break formation occurred at about the same rate in these samples, suggesting that damage to the protein coat (if it occurred) had minimal effect on either of these parameters; the protein coat appearing to act neither as a radioprotector or as a radiosensitiser of the phage DNA. In phage DNA irradiated under indirect (dilute aqueous) conditions, strand breaks accounted for 1/3 of the lethal hits. However, in phage particles irradiated under these conditions, strand breaks accounted for only 1/46 of the lethal hits. Purified DNA was much more radiosensitive than the phage particles, 55 times more sensitive with respect to strand breaks, but only 4 times as sensitive with respect to infectivity. This suggests that some of the loss
of infectivity in this case is due to damage to the protein coat. This was confirmed by determining the infectivity and strand breaks in DNA extracted from irradiated phage particles. It was found that the level of strand breaks was the same in the extracted DNA as in the phage particles, but that the infectivity of the extracted DNA was about three times greater than the infectivity of the phage particles. It was also noted in these studies that the dose required to give a certain level of DNA damage is at least an order of magnitude greater in samples irradiated under direct conditions than in those irradiated under indirect conditions. These studies could be extended by carrying out similar assays in the presence of various additives. It would be interesting to see whether additives alter the yield of DNA damage in phage particles via charge transfer across the protein coat. It would also be possible to ascertain whether additives alter the ratio of strand breaks to infectivity concomittantly with alterations in the yield of \('G^+\) and \('T^-\). From this it may be possible to draw further conclusions about the mechanisms of damage production from these radicals.

Since under direct irradiation conditions, \('G^+\) and \('T^-\) are thought to be the sole percursors of DNA damage, it is predicted that stable DNA damage products will arise predominantly at G and T residues, providing that charge migration is not extensive. Evidence that this is the case is provided by the analysis of strand breaks in plasmid DNA irradiated under direct conditions. A much greater proportion of double-strand breaks is observed than expected from the co-incidence of two randomly distributed single-strand
breaks, suggesting that the electron-gain and electron-loss centres tend to become trapped close together, and that both give rise to strand breaks. (Boon et al, 1984) To counter this, however, the thermoluminesence of irradiated DNA suggests that charge migration may be extensive.

This thesis describes investigations into the sites of gamma radiation-induced damage under direct and indirect conditions. The sites of gamma radiation-induced strand-breaks were localised by analysing irradiated end-labelled restriction fragments on polyacrylamide sequencing gels. Sites of damage which act as blocks to DNA polymerase were studied by the extension of an end-labelled primer on M13 template DNA. The products from these reactions were also analysed on polyacrylamide sequencing gels. The results revealed no preferred sites for strand breakage under direct or indirect irradiation conditions. The analysis of damage sites which block DNA polymerase activity revealed a preference for thymine at the damage site, and thymine or cytosine immediately 5’ to the damage site in samples irradiated under both direct and indirect conditions. Little or no specificity of indirect irradiation damage was expected, since it has been shown that OH- radicals formed by the reaction between ferrous sulphate and molecular oxygen give rise to DNA strand breaks at every possible base with approximately equal frequency. (Henner et al, 1983)

The results presented here suggest that the specificity for DNA polymerase arrest sites at particular bases is due to base damage rather than strand breaks, since these were shown to arise at random. Since the same specificity was observed in samples irradiated under direct and indirect conditions.
this suggests a generally increased sensitivity to radical attack at some bases rather than the specific formation of radicals on guanine and thymine. It would be possible to confirm that the specificity observed was due to base damage rather than strand breaks by the purification of irradiated DNA with no strand breaks prior to gel analysis.

These studies were carried further by investigating the pattern of irradiation damage in M13 clones with tracts of one of the four bases, since in a 'random' sequence any specificity for enhanced damage at guanines and thymines may not be seen if charge migration occurs through only one or two bases. It was found that for samples irradiated under both direct and indirect conditions, radiation-induced damage was reduced with respect to the flanking sequences in a run of purine bases, but was more or less unaltered within a polypyrimidine tract. These results suggest that radical reactions in general occur less frequently in a polypurine tract, possibly due to base-stacking interactions in these regions. Since the homopolymer tracts were quite large (approximately 50 bases for A and T), charge migration would have to be extensive to account for no enhanced damage within a run of thymines. If, however, charge migration is not extensive, the occurrence of damage in poly C and poly A tracts in sample irradiated under direct conditions may be explained by the possibility that radicals are formed on bases other than G and T within the homopolymer tracts. DNA damage may be depleted in the polypurine tracts due to enhanced charge migration out of the region aided by base-stacking. The problem of the extent of charge migration could be resolved by examining the size distribution of
radiation-produced fragments, for instance by electron microscopy. If charge migration is minimal, a preponderance of small fragments would be expected.

The end groups on gamma radiation-induced strand breaks were investigated, to enable insights into the possible mechanisms for strand break formation. In addition, the nature of the end groups has important implications for DNA repair. End groups on 5' or 3' end labelled DNA fragments were analysed by comparing the mobilities of the irradiated fragments on sequencing gels with standards having known termini. Enzymic analysis was also used to unequivocally identify some of the end groups. It was found that in samples irradiated under indirect conditions, the 5' termini were all phosphate groups, and the 3' termini phosphate or phosphoglycolate groups, in approximately equal proportions. Under direct irradiation conditions, however, both the 3' and the 5' termini were phosphate groups. No phosphoglycolate end groups were observed in samples irradiated dry, frozen at 77K or at 202K.

The end groups detected under indirect conditions are consistent with hydroxyl radical attack on the C-4' position of the sugar, allowing β-elimination (which would give rise to phosphate groups at both termini) or the formation of tetroxide intermediates (which would give rise to a 5' phosphate and a 3' phosphoglycolate group). The position of the 4' carbon on the sugar makes it unlikely to be the site of H-atom abstraction caused by base radicals in the direct mechanism, therefore strand breaks may be formed via a completely different pathway under these conditions. Tetroxide intermediates are unlikely to be formed under...
direct irradiation conditions due to the limited availability of oxygen. The proposal of mechanisms for the production of strand breaks under direct irradiation conditions is not really possible until the site of H-atom abstraction on the sugar is known. This could be probed by the synthesis of DNA containing sugar residues labelled with tritium at the various possible positions, and looking for the formation of tritiated water during irradiation.

The work presented in this thesis describes the quantitation of some gamma radiation-induced damage products, investigations into the site-specificity of of radiation-induced damage and the analysis of end groups on radiation-produced strand-breaks. The direct and indirect damage mechanisms have been compared. It is not certain which damage mechanism predominates in vivo, and in any case it is very difficult to extrapolate any of these results to cellular systems due to the many additional factors involved in cells, in particular, the organisation of DNA into chromatin. One simple model to the in vivo situation is the virus SV40, which is essentially a circular DNA molecule complexed with core particles. It would be possible to extend the damage quantitation asays to this system. Site specificity and end groups could analysed on end-labelled DNA fragments complexed with just one core particle.
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