THE EFFECTS OF GAMMA-RADIATION

ON DNA

By

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ABSTRACT

In this study gamma-radiation-induced DNA strand breaks have
been investigated using two systems; a plasmid-based assay, and
a whole nuclei-, or whole cell-based, alkaline filter elution
assay.

Addition of alkali metal halides to DNA irradiated under frozen
aqueous conditions were observed to have an effect on the
radiosensitivity of the DNA. This effect, which was not observed
with DNA irradiated under fluid aqueous conditions, would appear
to be due to two components; a physical component, and a
chemical component which is dependent on the anions used.
Addition of alkali metal halides appears to increase the volume
of the hydrating layer of water which is formed around the DNA
when it is frozen. This appears to increase the target volume
when it is irradiated, with an observed increase in damage
caused by $H_2O \cdot^+$ and non-hydrated electrons from the ionisation
of the hydration water. The chemical component, which may be a
protective or a sensitising effect, is dependent on the anion
in the system.

The scavenging of electrons produced from the direct action of
gamma radiation on DNA has been demonstrated using the
intercalator, mitozantrone. This was demonstrated using plasmid
DNA irradiated under frozen aqueous solutions, and compared with
the e.s.r. spectroscopy results obtained by my coworkers.

Finally, the protection of DNA by free-thiols has been
investigated. Under indirect, dilute aqueous conditions, the
amount of protection to plasmid DNA was observed to increase
with increasing positive charge on the thiols. Under direct,
frozen aqueous conditions, the radiosensitivity of plasmid DNA
by the compounds used was less clear cut. Possible reasons for
this contrast are discussed. The effect of a novel aminothiol
with a +3 positive charge on the amount of damage to tissue
culture cells was also investigated.

ACKNOWLEDGEMENTS

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INTRODUCTION

Public awareness and concern about radiation and its effects has increased over the past few years. Events such as the failure of the number 4 reactor at Chernobyl on 26th April 1986, problems with radioactive waste disposal, and concern over levels of radon gas in some areas, have made radiation headline news.

Radiation is a problem that organisms have had to learn to deal with. Some 87% of the radiation that we receive is from the breakdown of natural radionucleotides, such as potassium-40 and radon, and from cosmic rays [1]. Artificial radiation counts for approximately 13% of the radiation we absorb, this coming mainly from medical sources, such as x-ray machines and, to a minor extent, nuclear fallout, occupational sources, and nuclear discharges.

The critical site of action of radiation on the cell has been studied by many investigators, the consensus opinion is that it is the DNA that is the most important molecule to be attacked by radiation. Evidence for this has been reviewed by Elkind [2]. This includes the observation that DNA binding agents, such as dactinomycin, enhance radiation-induced killing, and the finding that DNA repair mutants in Escherichia coli (E.coli)have enhanced sensitivity to radiation. Similarly patients with ataxia telangiectasia, who have an increased sensitivity to ionising radiation, have been shown to have a defective DNA repair system [3,4]. Radiation is also well known to cause genetic mutations which can be inherited, a fact that also points to DNA being a major site of damage.

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The Structure of DNA

DNA, deoxyribonucleic acid, is made up of repeat subunits
called nucleotides, which consist of a 2'-deoxyribose sugar
bonded to a phosphate group and a purine or pyrimidine base.
The four most commonly occurring bases are two purines,
adenine (A) and guanine (G), and two pyrimidines, thymine (T)
and cytosine (C). These subunits are held together by a
phosphodiester link between the 3'-hydroxyl group of the sugar
of one unit to the 5' hydroxyl group of the next, Figure 1.1.
In the classic DNA structure proposed by Watson and Crick,
summarised by Crick [5], two helical polynucleotide chains are
coiled around a common axis, with the chains running in
opposite directions, Figure 1.2. The chains are held together
by hydrogen bonding between the bases, adenine to thymine, and
guanine to cytosine, which are situated on the inside of the
helix, with the sugar-phosphate backbone on the outside. In
the most common form of DNA, B-DNA, the helix is right handed,
with a diameter of 23.3Å [6,7]. The planes of the bases are
perpendicular to the helix axis, separated from adjacent bases
by 3.4Å along the axis, and are related by a rotation of 36°.
This means that the structure repeats after 10 residues, that
is at intervals of 34Å. A glance at the structure of the DNA
helix shows that there are two grooves around the molecules;
the major groove which is 12Å wide, and the minor groove which
is 6Å wide.

Among other forms of DNA that have been studied, two
deserve mentioning; A-DNA and Z-DNA. A-DNA is formed when the
relative humidity of the DNA is dropped below 75%. This also
has a right handed helix, but is wider and shorter than the

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a)

H   H····O   CH₃

N—H

Sugar   Adenine   Thymine

b)

H

Sugar   Guanine   Cytosine   Sugar

H

c)
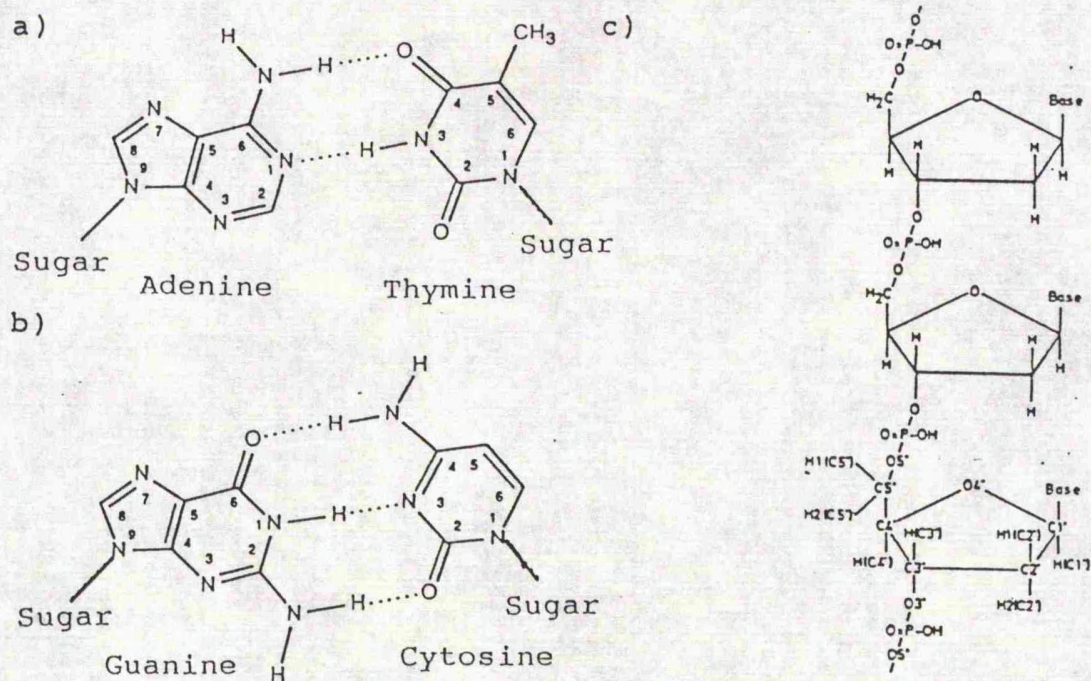
Base

Base

Base

Figure 1.1: Deoxyribonucleic acid. a) A model of a adenine-thymine base pair. b) A model of a guanine-cytosine base pair. c) The sugar-phosphate backbone.

sugar-phosphate backbone

5'

5'

3'

base

hydrogen bond

3'

1 helical turn = 3.4 nm

minor groove   major groove
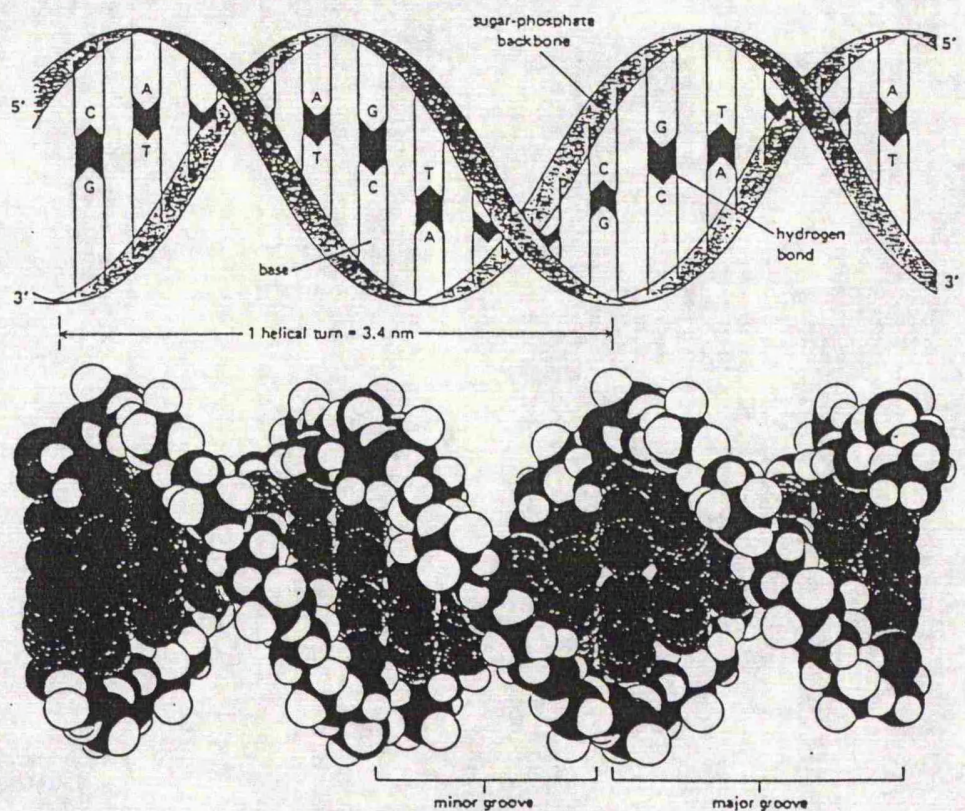
Figure 1.2: The B-DNA helix.

B-DNA helix, 25.5Å in diameter, and 24.6Å per turn. The bases
in A-DNA are tilted with respect to the helix, the form
resulting from puckering of the ribose. In A-DNA the phosphate
groups bind fewer solvating water molecules, so that
dehydration favours this form of DNA [8].

Z-DNA is formed in guanine- and cytosine- (GC-) rich
regions, and has been found in low concentrations within cells
where it may have a control function. The helix in Z-DNA is
left-handed, with the sugar-phosphate backbone "zigzagging"
down the helix. It shows only one helical groove, and has 12
base pairs (bp) per turn.

DNA packing in cells

The genome of E.coli contains about 4,000 kb (kilobases,
thousand base pairs) in a single chromosome, and the human
genome contains over 2,900,000 kb in 46 chromosomes, all of
which have to be packaged into cells. B-DNA is fairly
flexible, and can have twists added to, or unwound from, part
of the linear helix. This makes the helix coil up on itself
and make it more tightly packed, a process called
supercoiling. Twists in the helix are added or removed by
enzymes called topoisomerases. DNA gyrase from E.coli, which
is the most well characterised of these enzymes, utilises the
free energy of ATP (adenosine triphosphate) to unwind the DNA
duplex to induce so called negative supercoiling into the DNA.

DNA isolated under certain conditions from eukaryotes
appears under the electron microscope as beads on a string.
The beads, or nucleosomes, have the dimensions 110x110x55Å,
and consist of DNA wrapped around a core histonuclear

proteins, linked together by linker DNA, Figure 1.3. This DNA complex is called chromatin. The core consists of 140bp of DNA bound to an octamer consisting of two of each of histones H2A, H2B, H3, and H4, the DNA forming 1.75 turns of a left handed supercoil with a pitch of 28Å [6,9,10]. Prokaryotes do not have histones, but other proteins are thought to be involved in DNA packing.

Nucleosomes in eukaryotes are further packed into a helical solenoid with 6 nucleosomes per turn and a 30nm diameter [11]. This is called the "30nm fibre", the nucleosomes in the fibre being held together by histone H1. Various ideas have been voiced as to how the 30nm fibre is packed into the nucleus, most of these involve further looping of the fibre, with the loops held together by nonhistonuclear proteins [12]. These looped domains are thought to be structured still further by attachment to proteins that form the lamina of the nuclear envelope [13,14].

This association of DNA with histones and other proteins in the cell must be taken into account when comparing radiation damage to DNA in vitro with damage to DNA in vivo.

The Interaction of Radiation with DNA

The initial interaction of radiation with DNA has been reviewed by various authors including Upton [15], Ward [16], and in a much simplified form in the NRPB publication "Living With Radiation" [1].

Ionising radiations include electromagnetic radiations, x-rays and γ-rays, and particulate radiations such as electrons, protons, neutrons, α-particles, and heavy nuclei.

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Figure 1.3: The Nucleosome.

a) Schematic diagram of a region of chromatin containing a nucleosome.
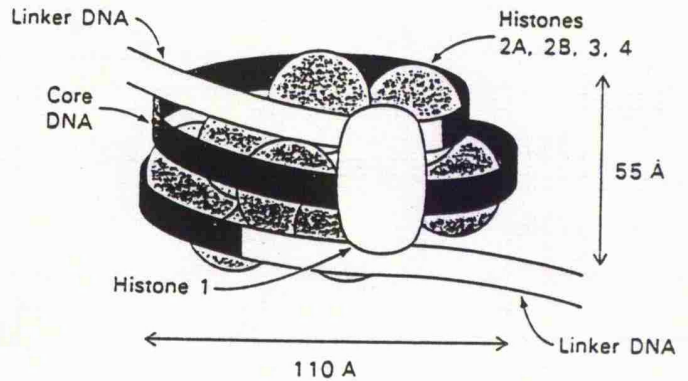
b) Model of a nucleosome core showing the DNA wound in a left-handed super-helix around a histone octamer. The locations of the four kinds of histone subunits are marked.
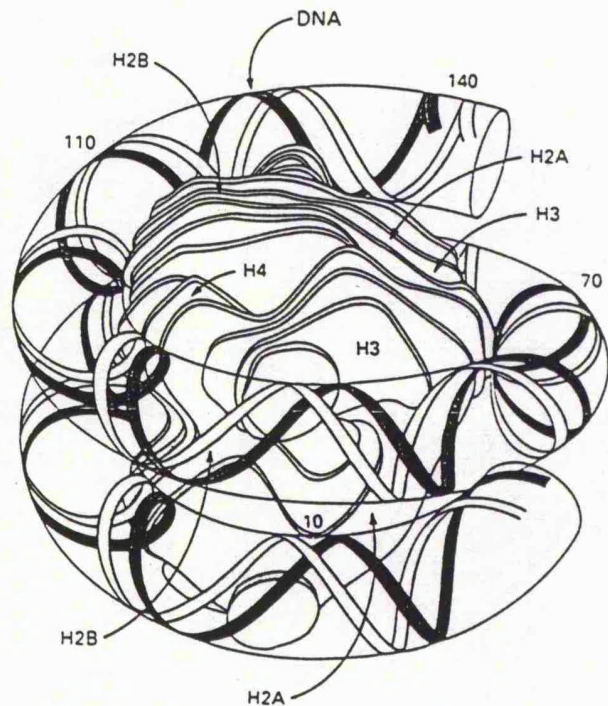
Both types are thought to cause biological effects by ionising important constituents of the cells in its path. The rate of energy deposition, or Linear Energy Transfer (L.E.T.), varies between different types of radiation. Low LET radiations, such as x-rays or gamma-rays, produce relatively few ionisations on their path through the cell, whereas a high LET, such as α-particles, will produce a high density of ionisations along its path [17].

Gamma-rays are at the high frequency end of the electromagnetic spectrum, and have a wavelength of between 10^{-17} and 10^{-8} metres. This overlaps with the waveband of x-rays. Gamma-ray photons are of high energy, and knock electrons out of their molecular orbitals when they interact with a molecule. These fast electrons, known as secondary radiation, in turn lose energy by interacting with other molecules to form secondary ionisations and excitations. The resulting molecular species with unpaired electrons are generally known as free radicals. These tend to react further until a chemically stable product is obtained by radical-radical recombination.

The ionising events from radiation have been observed to be nonhomogeneous through the medium through which the radiation was passing [18]. The radiation appears to form spurs, blobs, or short tracks, which are classified according to their appearance; a spur, for instance, is about 10Å in diameter, and contains 2-3 ion pairs. These primary and secondary ionising events take place some 10^{-18} to 10^{-12} seconds after the photon has hit, and free radical reactions are generally finished within a second [19].

When discussing radiation damage to DNA, or any other

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molecule, one has to address the possibility that damage may have occurred by two possible effects; by the direct effect, where, simply, damage is from a primary ionising event that took place on the same molecule, or by the indirect effect, where damage comes from radicals formed in the surrounding media, which in the case of fluid aqueous solutions are from water radiolysis products.

Indirect Damage to DNA

A great deal of work has been done in dilute aqueous solution. This has been done, partially for ease of experimentation, but also with the reasoning that since water constitutes about 75% of the mass of the cell, then about three quarters of ionising events in the cell will occur in the water. The radicals produced may then diffuse to attack the DNA.

The radiolysis products of water are fairly well understood. Fast electrons generated from irradiated water generate the cation, $H_2O^{\cdot+}$, (1).

$$H_2O \longrightarrow H_2O^{\cdot+} + e^- \qquad (1)$$
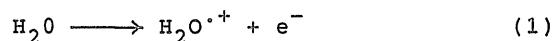
The electron released becomes solvated by other water molecules, designated e_{aq}^-, whilst the cation rapidly reacts, within 10^{-14} seconds, with other water molecules to form hydroxyl radicals, $\cdot OH$, and hydronium ions, H_3O^+, (2).

$$H_2O^{\cdot+} + H_2O \longrightarrow \cdot OH + H_3O^+ \qquad (2)$$

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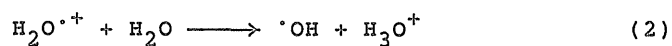
Hydroxyl radicals may also be formed by the absorption of a γ-ray photon by a water molecule. The excited water molecule then undergoes homolytic fission to form ˙OH radicals and hydrogen atoms, H˙, (3).

$$H_2O \longrightarrow H_2O^* \longrightarrow H^˙ + ˙OH \qquad (3)$$
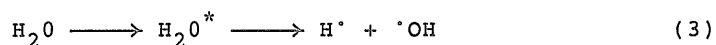
Solvated electrons can react with hydronium ions to form hydrogen atoms and water, (4).

$$e^-_{aq} + H_3O^+ \longrightarrow H^˙ + H_2O \qquad (4)$$
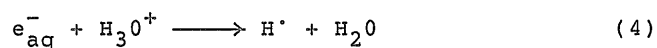
In addition to these reactions, hydroxyl radicals can react together to form hydrogen peroxide, (5), and hydrogen atoms can react to form hydrogen molecules, (6).

$$˙OH + ˙OH \longrightarrow H_2O_2 \qquad (5)$$

$$H^˙ + H^˙ \longrightarrow H_2 \qquad (6)$$

If oxygen is available in the solution it will react rapidly with solvated electrons to form the radical anion ˙O_2^-, (7), and with hydrogen atoms to form $HO_2^˙$ radicals, (8).

$$O_2 + e^-_{aq} \longrightarrow ˙O_2^- \qquad (7)$$

$$O_2 + H^˙ \longrightarrow HO_2^˙ \qquad (8)$$
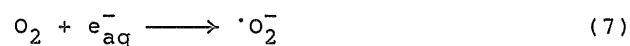
The $HO_2^˙$ formed in reaction 8 decomposes to form H^+ and ˙O_2^- in a reversible reaction with a pk of 4.9 [20]. The superoxide

radicals formed have been shown to react with hydroxyl radicals, reaction 9 [21].

$$\cdot O_2^- + \cdot OH \longrightarrow O_2 + OH^- \qquad (9)$$
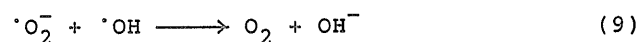
The G values, the number of molecules formed per 100eV of absorbed energy, for water radiolysis products are shown in table 1.

PRODUCT	G-VALUE
$\cdot OH$	2.7
e_{aq}^-	2.7
$H^+(H_3O^+)$	2.7
$H\cdot$	0.55
H_2	0.45
H_2O_2	0.70

Table 1.1: Yields of products formed by the action of sparsely ionising radiations on water. [20]

DNA within cells is associated with proteins and has been shown to produce a heterogeneous mix of single strand breaks (ssb), double strand breaks (dsb), DNA protein cross-links, and base damage; so when looking at the mechanisms for DNA base damage it has proved easier to look at model systems. These include bacteriophages, such as φx174 or PM2, naked calf thymus DNA, mononucleotides, and free bases. Base damage has been followed spectroscopically and conductometrically, and radiolysis products have been identified by variously using paper chromatography, High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Gas-liquid chromatography-mass spectroscopy, and NMR spectroscopy. High

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specificity biological systems such as enzymes and monoclonal antibodies have also been used to probe for damage in DNA. The methods used to investigate strand breaks are describe in detail in Chapter 2.

The effects of individual components can be studied by adding various compounds to react with or scavenge various radiolysis products. Nitrous oxide, for example, will react with solvated electrons to form hydroxyl radicals and hydroxide ions, reaction 10 [22].

$$e_{aq}^- + N_2O \longrightarrow N_2 + {}^\cdot OH + OH^- \qquad (10)$$
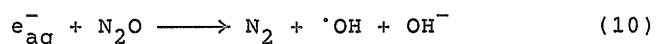
Alternatively carbon dioxide can be added to react with solvated electrons to form CO_2^-, a less reactive anion [23]. Alcohols, such as tertiary-butanol or propanol, have been the most commonly used substance to scavenge hydroxyl radicals.

The presence or absence of oxygen in a system must be considered since it has been shown to be a radiation sensitiser of whole cells [24]. Molecular oxygen in a system will scavenge solvated electrons and hydrogen atoms, the superoxide radical formed though is thought not to be important in causing radiation damage in cells [25]. More importantly though, molecular oxygen will peroxidise radicals formed on the DNA by radiation, which will alter the way that stable damage is formed.

Whilst it is generally believed that double strand breaks are important, van der Schans and coworkers [26] make the claim that when the bacteriophage PM2 was irradiated in dilute aqueous solution under oxic conditions, 87% of the

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inactivation was due to base damage, compared with 8.5% due to single strand breaks (ssb), and 4.5% due to double strand breaks (dsb). This implying that base damage is important. Since then a lot of work has been done in identifying the base damage products involved in the inactivation of DNA. This work has been extensively reviewed by several authors [20,27-31].

Pulse radiolysis of nucleotides under anoxic conditions has been shown to produce several long lived radicals whose fate can depend on many factors, including buffer used and contaminants. These long-lived radicals have been shown to react rapidly with oxygen to form peroxide radicals. It is thought that this peroxidation of secondary radicals may form products which are harder to repair, and so account for the observed radiosensitisation of cells when irradiated under oxic conditions [24]. Most researchers have reported little or no increase in the amount of damage observed in DNA when it is irradiated under oxygen. Siddiqi and Bothe [32], for example, irradiated calf thymus DNA under dilute aqueous conditions and produced a G value for single strand breaks formed under nitrogen $55nmol.J^{-1}$, and under oxygen a value of $54nmol.J^{-1}$.

Molecular oxygen will scavenge e^-_{aq} and $H^.$. Solvated electrons have been observed to react with bases at a diffusion controlled rate, but such electrons on bases are apparently transferred efficiently to oxygen. Hydrogen atoms, which are electron affinic have been shown to add to the 5,6 carbon-carbon double bonds of the pyrimidines to produce a small proportion of the damage caused to DNA. The consensus opinion of researchers in the field is that hydroxyl radicals produce most of the damage to DNA under conditions where indirect damage predominates.

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Pulse radiolysis studies of using thymine have shown that hydroxyl radicals will add to the 5,6 double bond, and will also abstract a hydrogen from the methyl group, with about 60% adding at C-5, about 30% at C-6, and about 10% abstracting hydrogen atoms from the methyl group. Paper chromatography has shown of the radiolysis products from thymine have shown that at least 21 products are formed. Some of the products formed are shown in Figure 1.4.

When free thymine, thymidine, or TMP are irradiated, the major radiolysis product that is formed is thymine glycol, 5,6-dihydro-5,6-dihydroxythymine. This is formed when oxygen reacts with the pyrimidine radical to form the corresponding 5(6)-dihydroxy-(6)5-peroxy radical. This may be reduced to form a hydroperoxide which can be further reduced to form thymine glycol.

In di, tri, and polynucleotides 5-hydroxy-6-hydrothymine, which is formed by hydrogen abstraction from an adjacent sugar by the thymine OH-adduct, is the more common product. Studies of DNA irradiated in vivo have proved that it is difficult to detect thymine glycol, but, by using monoclonal antibodies, Leadon [33] has shown that they are efficiently repaired. DNA glycosylases that can repair thymine glycol have been isolated from E.coli (exonuclease III), calf thymus, and HeLa cells. The finding that thymine glycol constitute a replicative block in vitro [34,35] may be a reason for cells having an efficient repair system for this product.

Cytosine forms products similar to those formed by thymine, the 5,6 double bond being the major site of ·OH attack. When DNA is irradiated under oxic dilute aqueous conditions free cytosine is released by cleavage of the
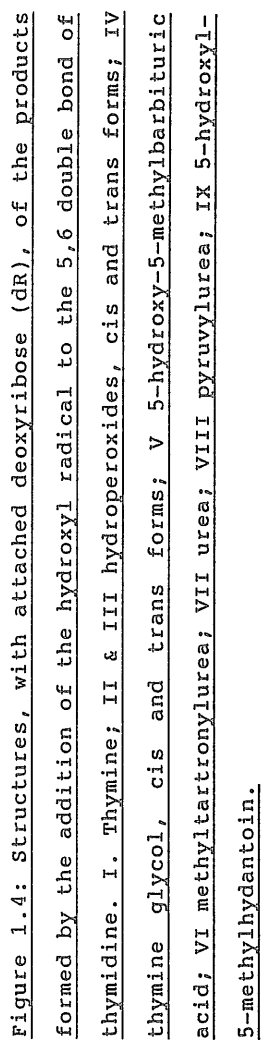
Figure 1.4: Structures, with attached deoxyribose (dR), of the products formed by the addition of the hydroxyl radical to the 5,6 double bond of thymidine. I. Thymine; II & III hydroperoxides, cis and trans forms; IV thymine glycol, cis and trans forms; V 5-hydroxy-5-methylbarbituric acid; VI methyltartronylurea; VII urea; VIII pyruvylurea; IX 5-hydroxyl-5-methylhydantoin.

N-glycosydic linkage with sugar, with trans-5,6-dihydroxy-5,6-dihydrouracil being formed. The trans-5,6-dihydroxy-5,6-dihydrouracil has been implicated in mutagenesis as causing C-T transitions [36]. Repair enzymes that are capable of repairing cytosine lesions have been reported in both prokaryotic and eukaryotic cells.

Less work has been undertaken on the purines. Pulse radiolysis studies have identified various carbon- and nitrogen-centred radicals. Some of the reactions that have been identified for deoxyadenosine are shown in Figure 1.5. Guanine has had less work done on it, but appears to correspond with the reactions observed for adenine.

Between 5% and 10% of hydroxyl radicals attack the sugar moiety in free nucleotides. In double stranded DNA this increases to about 20% of the total hydroxyl radical damage to DNA [37]. This is thought to be due to steric factors from the formation of the double helix protecting the bases and exposing the sugar phosphate backbone, though all sites of attack are still available. The formation of the double helix also leads to a reduced rate of ˙OH attack with the DNA, possibly due to the hydroxyl radical having to diffuse further, on average, before colliding with DNA. The distribution of sites of hydrogen abstraction by ˙OH is thought to be random. The radicals formed by ˙OH attack on the sugar may lead to strand breaks or base loss. Sites where there has been base loss are known as apurinic/apyrimidinic (AP) sites, and have the characteristic that they are susceptible to breakage when incubated in alkali [38]; hence their alternative name, alkali-labile sites.

AP sites have been observed to form by hydroxyl radical
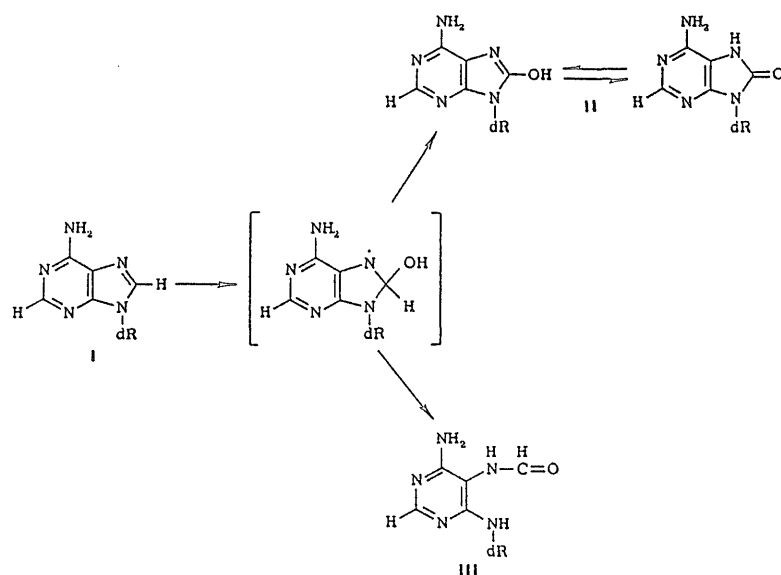
Figure 1.5: Products identified after the addition of hydroxyl radicals to deoxyadenosine. Structure I deoxyadenosine; II the tautomers 8-hydroxydeoxyadenosine and 7,8-dihydro-8-oxo-deoxyadenosine; III 4,6-diamino-5-formamidopyrimidine.
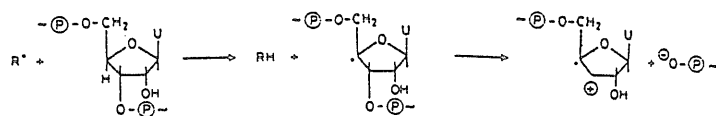
Figure 1.6: Hydrogen abstraction by 6-yl or 5-yl radicals in poly(U) leading to strand breakage. The base radicals abstract from C-2' or C-4' on the ribose, and single strand break formation occurs via heterolytic splitting of the sugar-phosphate bond in the β-position.

attack on C-1', C-4' [31], and C-2' [39] on the deoxyribose moiety leaving various residues attached. AP sites can also be formed when base modification, from for example $^{\cdot}$OH attack, weakens the N-glycosyl bond which makes it susceptible to spontaneous hydrolysis. Repair of damaged bases by DNA glycosylases may also leave AP sites. Unrepaired AP sites are thought to be potentially mutagenic or lethal; Sagher and Strauss [40], for example, using M13 DNA in an in vitro system have shown that T4 polymerase is stopped at the AP site and inserts a guanine or, more usually, an adenine nucleotide opposite it. AP endonucleases have been identified from both prokaryotes and eukaryotes, and have been shown to remove the sugar-phosphate moiety remaining at the AP site. More controversially, a "DNA-purine insertase" has apparently been isolated from humans which inserts a purine to replace an apurinic site [41].

Schulte-Frohlinde and coworkers [42-46] have investigated strand breakage in polyuridylic acid, poly(U). This homopolyribonucleotide possesses a randomly coiled conformation, is single stranded, and does not show any base stacking at room temperature. In dilute aqueous solutions some 20% of hydroxyl radicals will react with the ribose moieties of poly(U) [45]. Since about 40% of hydroxyl radicals that react with poly(U) lead to strand breaks (G_{ssb}=2.3), sugar radicals are not the only source of strand break formation. Some of the base radicals formed are thought to undergo H-abstraction from an adjacent sugar. Two chemical pathways have been discussed in the literature; the C-4' mechanism, and the C-2' mechanism. Under anoxic conditions the 6-yl or 5-yl radicals abstract a hydrogen atom from C-2' or C-4' and ssb

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formation occurs via heterolytic splitting of the
sugar-phosphate bond in β-position, Figure 1.6 [47].

The C-4' pathway under oxygen is more complex. The C-5 or
C-6 peroxy radical produced under oxic conditions attacks the
adjacent sugar by H-abstraction from the C-4' position. The
sugar radical is itself peroxidised and converted via a
tetroxide intermediate into an oxyl radical which then
undergoes cleavage of the C-3'/C-4' bond. After further
peroxyl formation, hydrolysis of the labile sugar-phosphate
bond finally leads to strand breakage, Figure 1.7.

Hydrogen-abstraction from the C-2' position is thought to
occur before oxygen addition takes place. After deprotonation
of the OH group at C-2' the β-phosphoric ester group is
released, resulting in breakage of the sugar-phosphate
backbone. This mechanism does not occur in DNA because of the
lack of the 2'-OH group.

Studies using polyadenylic acid, poly(A) [48], and have
shown that strand breakage occurs via the C-4' mechanism.
Similar studies on anoxic single-standed DNA [49] using
conductivity methods to study ssb have indicated that the C-4'
mechanism is also valid in this system.

Some questions still remain as to the role of the C-4'
mechanism in double-stranded DNA, since although strand break
efficiency for poly(U) and poly(dA) is relatively high, it is
low for poly(dU) and poly(A) [50]. This raises the question of
conformational requirements for radical transfer reactions in
polynucleotides. Computer modelling studies on the relatively
rigid structure of B-form DNA have shown that the base
radicals formed by ˙OH attack may be too far away from the
C-4' for it to be a contender for abstraction [51].
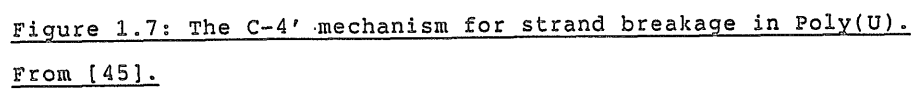
Figure 1.7: The C-4' mechanism for strand breakage in Poly(U).
From [45].

The formation of double strand breaks (dsb) in DNA irradiated in aqueous solutions has been the subject of much debate. A double strand break is formed when two single strand breaks on opposite strands coincide within ca. 16 base pairs of each other [52]. If the two ssb were formed independently by two separate radicals then dsb formation should be second order with respect to dose. A linear quadratic dose dependence for dsb induction was found however by Freifelder and Trumbo [52], and van der Schans and coworkers [26]. More recently Blok and Loman [53] found a first order dependency on dose using double stranded ϕx174 DNA. Using lower doses Siddiqi and Bothe [32] showed that dsb formation initially is first order, with the expected second order formation of dsb superimposed at higher doses. The linear dose dependency is interpreted as the dsb being formed by one single event.

Ward [54] has suggested that spur effects, where greater than one radical is produced in a spur per hit, may be responsible. This allows the possibility that both strands may be damaged by one hit. This can be used to explain the damage in cells where the DNA is surrounded by a high concentration of organic material which scavenges most radicals, such that the concentration of indirect damage is low. In N_2O saturated aqueous solutions with relatively low scavenger concentrations, spur reactions are unlikely to contribute to DNA damage [55].

Another mechanism that has been suggested involves radical transfer from one strand to another (interchain H-transfer). A DNA radical is produced by $^\cdot$OH attack on one strand, during this process a sugar radical will be formed which has a long lifetime and will disappear only by a

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bimolecular reaction with another radical. The transferred radical will lead to strand break formation and will result in two overlapping ssb [55]. The draw back of this mechanism is the long distances over which the H-atom has to transfer from the second chain which may place energetic constraints on the mechanism.

Direct Damage

Direct damage to DNA occurs when ionising radiation is deposited "directly" onto the DNA molecule without the mediation of an extrinsic radical or other clearly mobile species. When studying the direct damage mechanism it has proved important to prevent the radicals that cause the indirect effect reacting with the DNA. This was originally carried out by using high concentrations of scavenging molecules, but calculations have been done that show that using radical scavengers does not reproduce the conditions required for the direct damage mechanism to be accurately observed [56]. Using "dry" DNA films, where the only the solvating water of the DNA remains, has proved to be one way of limiting damage by water radiolysis products. Freezing the DNA containing solutions has also proved effective.

When DNA in solution is frozen, ice crystals are nucleated and attract and bind most of the water molecules in the system, leaving a layer of structured solvating water around the DNA. This is known as the frozen aqueous, or solid state, system. Sanner [57] observed that the indirect effect of hydroxyl radical attack on bacterial cells could be removed by irradiating them below -15°C. The radioprotective action of

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ethanol, a hydroxyl radical scavenger, was observed to disappear below -15°C, whilst the general radiosensitivity of the cells reduced between 0°C and -15°C.

Boon and coworkers [58] also found that when supercoiled pBR322 plasmid was irradiated at different temperatures there was a large increase in damage when the plasmid was irradiated above the melting temperature of the solution, Figure 1.8. Electron spin resonance spectroscopy (e.s.r.) showed that when DNA was irradiated at 77K (-196°C) ·OH formed in the ice phase of the system. These were lost on annealing to ca. 130K, before the system melted, and reacted to form hydrogen peroxide. This is not thought to attack the DNA [59].

Electron spin resonance spectroscopy, e.s.r., is used to detect paramagnetic molecules, that is molecules having one or more unpaired electrons. Hence it is extremely useful in the detection of radicals. In e.s.r. the sample is subjected to a magnetic field which causes an alignment of any unpaired electrons. The application of a microwave field causes the electrons to flip, or resonate, between two energy levels, giving a characteristic spectra for different radicals [60]. The primary radicals formed by radiation can be stabilised by drying a sample or freezing to 4K, for example, using liquid helium, or more conveniently to 77K using liquid nitrogen.

Initial e.s.r. studies showed that the first detectable radicals to be formed in DNA are guanine cations, $G^{·+}$, and thymine anions, $T^{·-}$, Figure 1.9 [61-63]. Other potential sites for electron loss are the phosphate moieties and the other bases, thymine, adenine, and cytosine, but e.s.r. has failed to detect any radicals associated with electron loss from these sites [59,64]. The sites of electron loss are sometimes

17
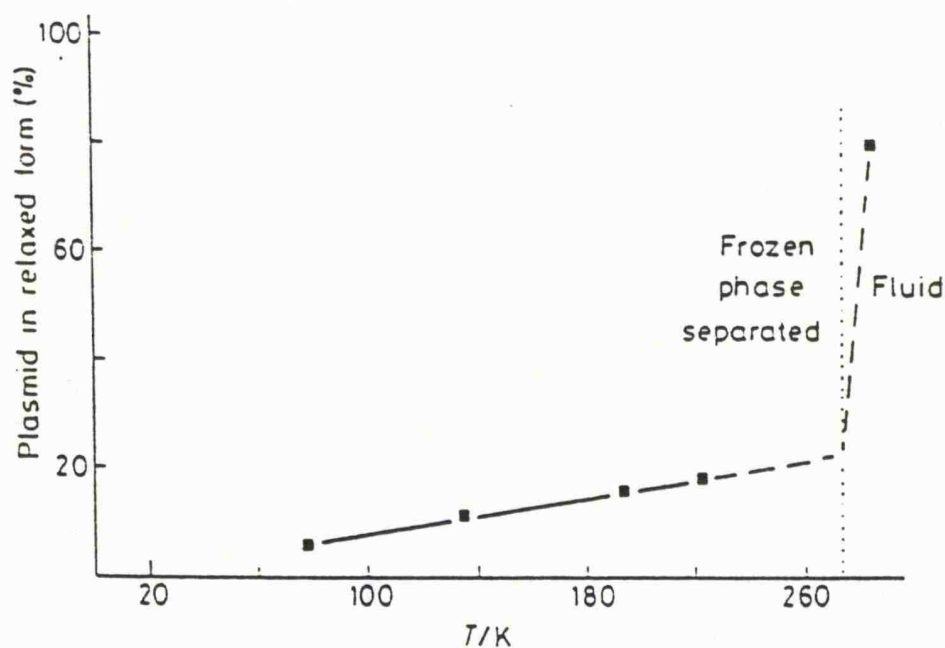
Figure 1.8: The effect of temperature and phase on γ-radiation induced damage to pBR322 plasmid DNA.
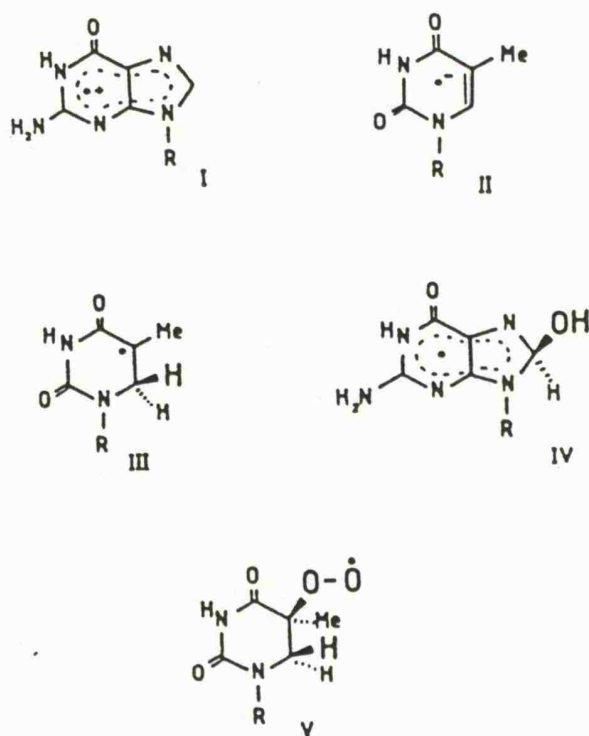
Figure 1.9: Structures of the base centred radicals identified by E.S.R. on direct irradiation of DNA.; I G$^{\cdot+}$; II T$^{\cdot-}$; III TH$^{\cdot}$; IV GOH$^{\cdot}$; V THO$_2{}^{\cdot}$.

referred to in the literature as "holes". The thymine anion, $T^{\cdot-}$, has been observed to form from electrons ejected by radiation being trapped at thymine. Cytosine and thymine probably have similar electron affinities since competition studies in our laboratories show equal electron capture. Of the evidence for and against $T^{\cdot-}$ being the major centre in DNA, the most compelling is the extensive formation of $TH^{\cdot}$ radicals on annealing, with no sign of $CH^{\cdot}$ radicals.

Electrons can also be gained by "dry-charge" electron transfer from $H_2O^{\cdot+}$ that forms in the structured water that surrounds the DNA. This is considered to be a direct effect since its products are typical of direct energy i.e. $G^{\cdot+}$ and $T^{\cdot-}$ [62]. Hydroxyl radicals formed in this solvating layer are thought not normally to be important [65]. The effects of additives on the size of the solvating layer in frozen aqueous systems and its importance in the radiation chemistry of the system is discussed in Chapter 3.

The yields of $G^{\cdot+}$ and $T^{\cdot-}$ are approximately equal, though there is some evidence that the precise ratios may vary somewhat between different types of DNA [63]. Two major mechanisms have been proposed to account for the transfer of charge so that it becomes localised on thymine or guanine. It may occur via stacked bases, the base stacking providing a good overlap of π-orbitals and permitting intramolecular energy and charge transfer to occur [62]. It has also been proposed that electrons may transfer through the structured solvating water surrounding the DNA [66]. An attempt to intercept these electrons using a compound with a high electron affinity, mitozantrone, is discussed in Chapter 4.

The thymine anion $T^{\cdot-}$ protonates at C-6 on annealing to

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ca. 130K to form the 5,6-dihydro-5-thymyl radical, $\cdot$TH. Under oxygen $\cdot$TH rapidly reacts with oxygen to form peroxyl radicals $RO_2\cdot$ [58,62]. $G^{\cdot+}$ is thought to convert via the loss of a proton from nitrogen to form a neutral radical, $GN\cdot$ [67]. $G^{\cdot+}$, or $GN\cdot$, may hydroxylate to form $GOH\cdot$ radicals which have been detected under alkaline conditions [59]. Under oxygen $GOH\cdot$ forms a peroxyl radical, $RO_2\cdot$. Addition of oxygen to N-3 is unlikely and it is more likely to add to C-5 instead [51].

Using supercoiled pBR322 plasmid irradiated in a frozen aqueous system at 77K it has been shown that, on warming, some of these radicals eventually form strand breaks. Estimation of G-values for radical and strand break formation suggest that approximately 35% - 45% of these radicals form strand breaks [59]. It is believed that under the conditions used strand breaks must arise from H-atom abstraction by base radicals from neighbouring sugar residues. Sugar radicals are not usually detected by e.s.r.. It is thought that the reason for this is that the rate of decay of these radicals is faster than the rate of their formation. Schulte Frohlinde [47] used laser photoionisation to eject electrons from the bases in poly(U) to produce cations analogous to $G^{\cdot+}$. He showed that the kinetics of strand break formation were very similar to those for strand break formation by $\cdot$OH, and concluded that strand break formation occurred via a common pathway analogous to the C-4' mechanism for indirect damage.

Analysis of the end groups of DNA strand breaks irradiated in anoxic dilute aqueous solution have shown that the 5' termini of strand breaks are phosphate groups, and the 3' termini phosphate or phosphoglycolate groups in approximately equal proportions [68,69]. When "dry" DNA is

irradiated at room temperature, or DNA is irradiated in a frozen aqueous system, 5′ phosphate termini are observed and almost exclusively 3′ phosphate termini. This indicates that there are differences in the pathways for strand break formation in DNA irradiated under indirect or direct conditions [70].

Computer modelling has shown that for B-form DNA ·TH is the most likely to abstract a H-atom from C-1′ or C-2′ of the deoxyribose 5′ to the radical bearing nucleotide to form 5,6 dihydrothymidine, TH_2 [51]. The peroxyl radical of ·TH is also thought to abstract from the same positions. It is unlikely that $G^{·+}$ (GN·) can do this. However ·GOH or $G-O_2^·$ are thought to abstract from C-1′, C-2′, or C-5′ on the deoxyribose 5′ to the sugar radical. The peroxyl radicals formed under oxygen from ·TH and GN· probably convert to single strand breaks with a higher efficiency, leading to the modest increase in the number of strand breaks that has been observed [58].

Using the supercoiled plasmid assay described in depth in Chapter 2 it was observed that the yield of dsb were several times too great to be accounted for by coincidence of two independent ssb [58]. If both $G^{·+}$ and $T^{·-}$ are precursors of strand breaks, and these two centres are initially formed close together from a single event, but on opposite strands, and provided it is postulated that electron and hole migration through stacked bases is not excessive, dsb would be comparatively frequent. Close radical ions of opposite charge are expected to return due to large coulombic forces; some charge recombination does take place leading to slight thermoluminescence on warming. It is thought that hydrogen

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bonding relaxations in the DNA helix will deepen the trap

forces and help to prevent electron return. If the distance ,

between $G^{\cdot+}$ and $T^{\cdot-}$ on opposite strands is ca. up to 30Å along

the helix, it is thought that they are likely to produce a dsb

[64].

The Biological Significance of DNA Strand Breaks

Immediate cell death from radiation is not thought to be

as much of a problem to an organism as mutagenic effects which

have the potential to kill the whole organism at a later

stage. Cell lethality or survival is relatively easy to study

compared with mutagenesis which occurs on a molecular level.

Much of the research done on radiation damage to whole cells

has therefore concentrated on cell death.

Both the indirect mechanism and the direct mechanism

cause the formation of DNA single strand breaks (ssb) and

double strand breaks (dsb). Several authors have studied the

effects of ssb and dsb on the biological activity of

bacteriophages such as φx174 and PM2 [26,53], and on plasmids

such as pBR322 [55]. In single-stranded phages ssb are lethal,

but in double-stranded phages ssb are generally considered to

be relatively harmless since the number of ssb is usually far

larger than the number of lethal events. This also holds true

for ssb in cellular systems; randomly distributed ssb in

mammalian cells, for instance, have hardly any effect on cell

killing [71].

Single strand breaks have been shown to be relatively

easy to repair. Up to 40% of ssb have been shown to be

repaired in vitro by polynucleotide ligase [72]. Up to 80%

ligation has been achieved by using exonuclease III to remove nucleotides from the 3' terminal, DNA polymerase I to fill in the gap, and polynucleotide ligase to religate the strands [73]. The quick repair of ssb has been demonstrated in mammalian cells [17,74].

In whole cells it has been demonstrated that dsb may be formed by the excision of damaged bases that are opposite each other, or opposite a ssb, by repair enzymes, in addition to those formed initially by the radiation [75-77]. Double strand breaks in phage or bacterial systems have been shown to be almost invariably lethal. But when pBR322 was irradiated in aqueous solution and was expressed in E.coli its survival did not correlate with dsb since the number of dsb formed is too low [55]. This may have been due to the limited time available for repair in E.coli not being long enough for the repair of every potentially repairable lethal damage.

Frankenberg and coworkers [78-80] using yeast mutants deficient in dsb repair concluded that 1-2 dsb constitute a potentially lethal lesion. Several authors including Radford [81] have found linear correlation between the levels of dsb and cell killing. Similarly Blöcher [82], using Ehrlich ascite tumour cells, concluded that residual dsb are a major cause of the loss of reproductive capacity in these cells on irradiation with α-particles.

The correlation between cell killing and the number of dsb has been investigated in radiosensitive and radioresistant human carcinoma cell lines [83]. The more radiosensitive strains showed more dsb for a given radiation dose than more resistant strains, and were shown to repair dsb at a reduced rate. This led to the conclusion that dsb appear to be a

factor in determining radiosensitivity at doses relevant to survival, that is, up to 10Gy. Conversely work comparing radioresistant TN-368 Lepidopteran cells with Chinese Hamster Ovary cells showed no differences in dsb repair capacity [84]. The lack of significant differences in the rate of repair of ssb and dsb in proliferating cells compared with more radiosensitive quiescent murine tumour cells have also shown that survival does not necessarily correlate with the rates of ssb and dsb repair [85].

When the survival of haploid and diploid fungal and invertebrate cells was investigated it was found that haploid cells were very sensitive to radiation [86]. This was consistent with the idea that there are similar mechanisms of repair in different organisms requiring duplicated chromosomes, this probably being some form of recombinational system.

Recombinational repair permits the recovery of coding information lost as a result of the injury. It involves the donation of an intact DNA strand into the damaged duplex from a homologous sister or daughter strand. More recently researchers have found that the recombinational genes, including RecA, are required for the repair of at least some of the dsb found in bacterial cells [87-89]. Using neutral elution it has been shown that, in both prokaryotic and eukaryotic cells, there are two rejoining processes in cells; a fast one with a half life of about 8 minutes involving DNA ligase in E.coli, and a slow half life lasting several hours which in E.coli involves recombinational processes [90].

Regel and Kampf [91,92] concluded from these studies that cell inactivation was not simply due to the number of dsb in a

cell, but rather their cooperation within a membrane associated superstructure unit, or MASSU, attached to the nuclear membrane. They argue that the differences in cell survival observed for different phases of cell growth is due to the alignment of both sister MASSU's during mitosis which allows recombination to take place, thus allowing survival of at least one of the growing sister cells.

Iliakis and Okayusu [93] noticed that much of the previous work stresses the importance of DNA dsb in cell killing, but correlate cell killing with the fraction of dsb, that remain unrepaired. They studied cell survival during different stages of the cell life cycle, and used additives to alter the response of cells to radiation. They proposed that the processes by which dsb are fixed may alter at different stages of the cell life cycle and lead to the different radiosensitivities observed. The fixation processes may include physical strand break separation due to chromatin reorganisation, or cellular metabolic processes not related to the repair process itself.

In addition to cell lethality it has been noted that many unrelated tumour-promoting agents have in common the ability to induce strand breaks [94]. It has been postulated that strand break damage, or the misrepair of strand breaks may be important in allowing a cell to express a malignant phenotype.

It is concluded that although the DNA strand breaks, especially double strand breaks are not the absolutely lethal lesions that some studies initially suggested, they still have the potential to be lethal to the cell, and are still a valid form of damage to assay when studying radiation damage to DNA.

DNA-Protein Interactions With Radiation

Electron spin resonance studies of both reconstituted histone-DNA complexes and natural chromatin extracted from cell nuclei have shown that, under direct conditions, histones act as radiosensitisers, and electrons are transferred from histones to the DNA. This produces an increase in the concentration of $T^{\cdot -}$, and may lead to an increase damage to the DNA [64].

Under indirect conditions, _in vitro_, hydroxyl radicals have been observed to initiate the formation of DNA-protein crosslinks (DPC) between DNA and surrounding proteins. In nucleohistone the core histones have been shown to predominate in the formation of DPC [95]. Gajewski and coworkers [96] have shown that by comparing thymine-amino acid crosslinks with DNA-protein crosslinks by using gas chromatography and mass spectroscopy, the amino acids glycine, valine, alanine, leucine, isoleucine, and threonine predominate in the forming of DPC.

It has been shown by using bovine serum albumin with DNA under aqueous conditions that oxygen inhibits the formation of DPC [97]. Meyn and coworkers [98] also found an oxygen effect when comparing the alkaline filter elution of DNA from oxic and hypoxic irradiated cells, with and without treatment with proteinase K to remove DPC that slow down DNA elution from the filter.

The significance of DPC to cells has yet to be fully studied. It has been shown that there is a background level of some 6000 DPC per cell in V79 cells, which may be related to the anchorage of chromosomal loops to the nuclear protein

matrix [99]. The formation of DPC in   cells has been
shown to be dependent on the concentration of cellular thiols,
such as glutathione, which scavenge free radicals, and oxygen.
Although fewer DPC are formed on irradiation than ssb, they
are repaired at a slower rate than ssb, and may block the
normal function of the nuclear matrix by inhibiting
replication and transcription.

The Relative Contribution Of Direct And Indirect Damage In

Cells

The contribution of water radiolysis products to
radiation damage in cells has been the subject of much debate.
Early studies of bacterial cells irradiated with hydroxyl
radical scavengers showed that protection against ssb
formation was a linear function of the rate constants of
hydroxyl radicals with the scavenger [47]. There proved to be
some drawbacks to these experiments, for instance a cell
containing, say 10% alcohol, may have a different repair
capacity than with no alcohol. More recent studies using
E.coli B/r showed only a relatively small effect of hydroxyl
radical scavengers under nitrogen, although under air some had
a larger protective effect; this leading the authors to
conclude that the effects may not be simply due to hydroxyl
radical removal [100].

Other groups have attempted to use N_2O to increase the
number of hydroxyl radicals in cells. Attempts with bacterial
cells appeared to show some increase in damage, apparently due
to hydroxyl radicals, but it was found that compounds in the
buffer used, N_2O, NaCl, O_2, and phosphate, on irradiation

produced compounds that are toxic to the cells [101]. Antoku
[22] using mammalian cells found an apparent increase in ssb
with N_2O when compared with cells irradiated under nitrogen,
but found that preheating the cells to above 50°C was required
to either "permeabilise" the cells to the N_2O, or to denature
a putative enzyme responsible for its decomposition. More
recent studies using mammalian cells irradiated under N_2O or
CO_2, but without any preheating, did not find any change in
cell survival [23].

The amount of water in the cell nucleus is relatively
small, and is has been argued that since DNA only accounts for
a small amount of the total mass of the nucleus, and hydroxyl
radicals are extremely reactive, they are more likely to react
with proteins and other molecules that surround the DNA, than
the DNA itself [59,64].

The increased probability of forming a double strand
break by direct damage, either initially, or after enzymatic
breakage of lesions on opposite strands [77] has been put
forward as a reason why direct damage could be of important in
the killing of cells [64]. The first order dependence of
survival on dose if repair is totally or partially absent has
been used to back up this claim.

Summary

During the course of this thesis I describe the use of
two systems for looking at radiation-induced strand breaks in
DNA. The first technique uses naked plasmid DNA, and has been
used in dilute aqueous systems, both in the fluid and solid
state. The second technique, alkaline filter elution, uses

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whole nuclei or cells to investigate ssb formation.

The effect that additives have on the structured solvating layer of DNA, and its effect on radiation damage in the direct system, has been investigated using the plasmid assay. Scavenging of electrons ejected from DNA by radiation is demonstrated by the use of the intercalator, mitozantrone.

Finally the effects of adding aminothiols, including the well known radioprotectors cysteamine and WR-1065 are investigated in both the fluid and solid state using the plasmid-based assay, and in whole cells using alkaline filter elution to measure damage.

CHAPTER TWO

MATERIALS AND METHODS

INTRODUCTION

Numerous techniques have been developed over the years to study strand breakage in DNA. Much of this work has been done on naked DNA or other model compounds. Bothe and coworkers [49] have utilised the observation that strand breaks in, for example, polyuridylic acid (Poly U) or single stranded DNA cause an increase in the conductivity of the solution. This is due to the release of counterions from around the region of each strand break.

Siddiqi and Bothe [32] have used low angle laser light scattering from a He/Ne laser at an angle of 3° to 7° to study stand breaks. The refractive index and the amount of scattering of a solution of Poly U or DNA alter according to the average molecular weight of the polynucleotide.

Many groups have studied strand breakage in bacteriophage or plasmids. Single-stranded φx174, for example, is usually circular and linearises when a single strand break (ssb) occurs; circular and linear phage can be separated out using a sucrose density gradient [102]. Several bacteriophage, such as PM2, and plasmids, such as pBR322, exist as supercoiled covalently closed circular (ccc) DNA. A single strand break (ssb) in ccc, or Form I, DNA will allow the DNA to relax, generating open circular, or Form II, DNA, Figure 2.1. Double strand breaks (dsb) allow the DNA to linearise to form linear, or Form III, DNA. The three forms of bacteriophage or plasmid DNA can ,be separated out by virtue of their differing

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superhelical plasmid
(form I)

double strand-break   single strand-break

linear plasmid
(form III)

open circular
nicked plasmid
(form II)

etc.
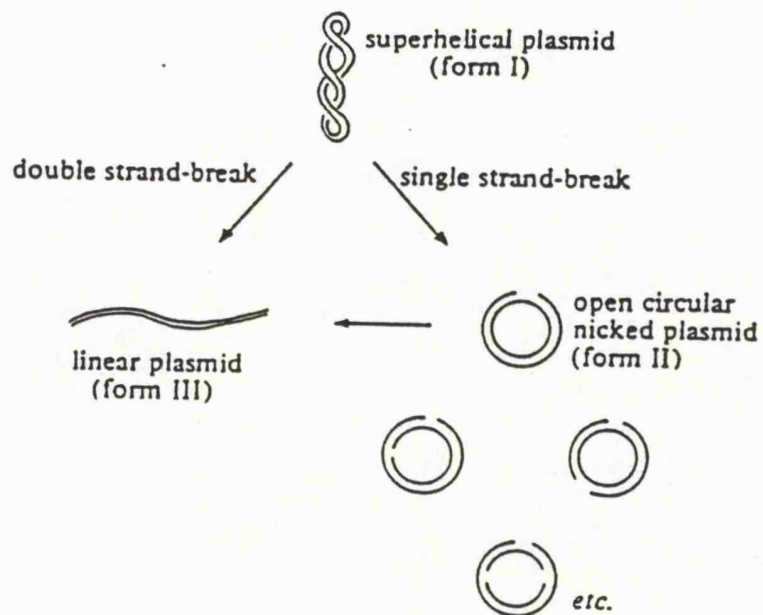
Figure 2.1: Method used for the analysis of radiation-induced strand breaks in DNA.

densities, or by their ability to pass through an agarose gel.

Van der Schans and coworkers [26] separated out PM2 DNA
using a 5%-20% w/v sucrose gradient. The Form I DNA bands at a
higher density than the Form II and the Form III DNA.

I have studied strand breaks in pBR322 using the method
of Boon and coworkers [58]. This uses agarose gel electro-
phoresis to separate the three forms of plasmid. The plasmid
is attracted to the anode, and is separated out by its ability
to move through pores in the agarose gel [103]. The DNA is
then visualised by staining with ethidium bromide and
illuminating with an ultra-violet light box. The DNA is then
quantified by scanning a negative photograph of the DNA with a
laser densitometer, figure 2.2.

This technique has been adapted to investigate DNA repair
in mammalian cells [104]. Simian Virus 40, SV40, possesses a
minichromosome structure within cells. When it is irradiated
in situ within the cell , the repair enzymes will repair it as
they would the cell's own DNA. By isolating nuclei at
different times after irradiation, extracting the virus, and
separating Form I, Form II, and Form III DNA by agarose gel
electrophoresis, and hybridising with ^{32}P-labelled SV40, DNA
repair has been demonstrated.

Alkaline gel electrophoresis has been used to study
damage from ssb and alkali labile sites in small amounts of
mammalian cells, such as skin biopsies, after irradiation or
chemical treatment [105]. Double stranded DNA is denatured in
alkali, undergoes alkaline agarose electrophoresis, is stained
in ethidium bromide, and the lengths of the DNA are compared
against standards of known length. This technique, the authors
claim, only requires 30-50ng of DNA, compared with ca. 1000ng
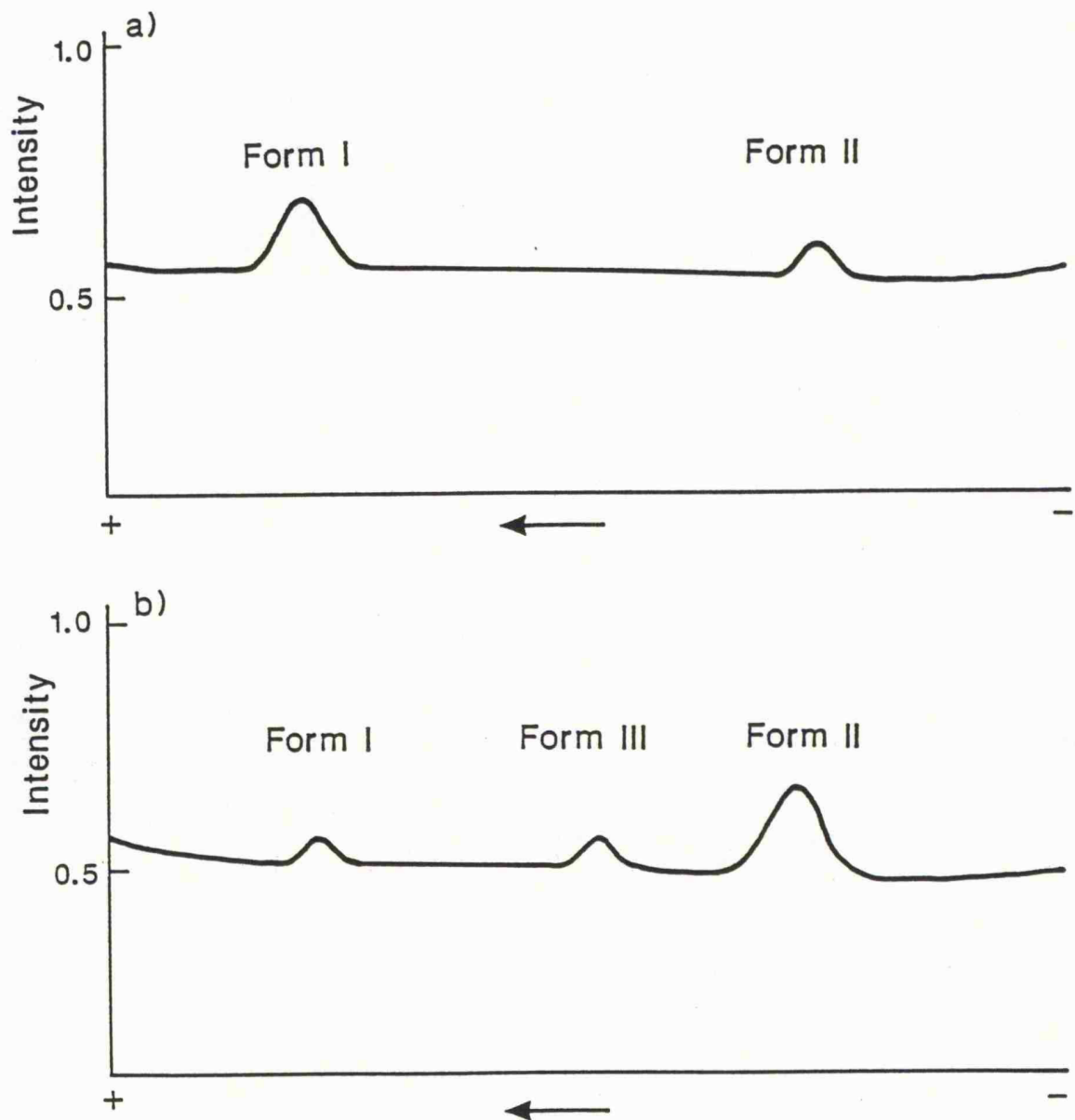
Figure 2.2: Fitted laser densitometer profile of pBR322 plasmid DNA separated by agarose gel electrophoresis under the conditions discussed in the materials and methods section. a) No irradiation. b) Irradiated with 12.0 kGY γ-radiation under direct conditions at 77K.

for alkaline filter elution, and can detect approximately 3 breaks per 10^9 Da.

Conventional agarose gel electrophoresis is limited to DNA below 0.75Mbp. Larger DNA molecules orient along the direction of the electric field and move equally, irrespective of their molecular weight. DNA of this length can only move through gel pores in agarose by being elongated and moving through a chain of gel pores. By using two alternating homogeneous electric fields at an angle of 120°, a technique known as Clamped Homogeneous Electric Field, CHEF, electrophoresis, it has been shown that it is possible to separate double stranded DNA up to 10Mbp long. Blöcher and coworkers [106] have recently used this technique to investigate dsb in Chinese Hamster Ovary cells, and have found that it can resolve damage to that caused by 1Gy.

A more traditional method for studying strand breakage in whole cells is the use of sucrose density gradients. Cells are usually lysed onto a 5%-20% sucrose density gradient, and spun in an ultracentrifuge. A neutral pH is used to study dsb, while a pH of over 12 is generally used to study ssb and breaks from alkali labile sites. A disadvantage of this technique is that a fairly high dose of radiation has to be used to produce detectable damage [91].

The rate at which alkali causes the unwinding of double-stranded DNA to form single-stranded DNA has been shown to increase with decreasing DNA chain length for a fixed concentration of DNA [107]. Unwinding starts from the break in the DNA, the greater the number of breaks in a DNA, the faster it unwinds. Ahnstrom and Erikson [108] have adapted this phenomenon to study strand breakage in DNA.

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In the alkaline unwinding technique alkali is added to DNA from cells, releasing it from cell membranes and other macromolecules, and inducing strand separation. After a set amount of time the alkali is neutralised and the DNA sonicated to prevent renaturation. Single- and double-stranded DNA are then separated out by hydroxylapatite chromatography. Recently van der Schans and coworkers [109] have increased the sensitivity of this technique by replacing the hydroxylapatite chromatography with an enzyme-linked immunosorbent assay, ELISA, based on an anti-single-stranded DNA antibody to detect single-stranded DNA.

A technique to detect strand breaks which is proving increasingly popular is filter elution. This has been developed by Köhn and coworkers [110-115], and is based on the separation of DNA fragments by the ease with which they pass through a filter. The greater the length of a DNA fragment, the longer the time, on average, that it takes to pass through the filter. Radiation induces strand breaks in the DNA, which in turn result in an increased rate of elution of DNA through the filter. Köhn originally developed the technique to study ssb, using alkali to denature the DNA and elute it through the filter. The technique is sensitive enough to detect 1 ssb per $2-3 \times 10^9$ Da, or 1 per 10^4 Kb.

Whole cells which have been irradiated or chemically treated are deposited onto filters prior to lysis with a detergent containing solution; lysing the cells in situ on the filters means that the minimum amount of shearing is done to the DNA. The DNA can be treated at this stage with a proteinase to remove any protein which has crosslinked to the DNA and may impede the DNA elution by adsorbing onto the

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filter. The DNA is then washed with an EDTA containing solution to remove the majority of cell debris, RNA, and proteins. Alkali is then added, and the DNA is eluted through the filter. The preferred alkali to use is tetrapropylammonium hydroxide because DNA has a lower transition pH (pH 11.3-11.9) for denaturation to single stranded DNA in it than in sodium hydroxide (pH 11.9-12.5). This allows a lower pH to be used for elution, reducing problems associated with strand breaks from alkali labile sites. A measurement of the number of alkali labile sites can be obtained by comparing the rates of elution at , for example, pH 12.1 and pH 12.6.

Filter elution has been used to study dsb. Originally DNA was eluted by some authors at a pH 9.6, but this pH was observed to cause an increase in the number of apparent dsb observed in irradiated DNA from E.coli. It has been recommended that DNA be eluted at pH 7.0 instead [116].

Much of the work on filter elution is carried out with DNA labelled with [^{14}C]-thymidine. The amount of DNA in each of the aliquots of eluted DNA is calculated by using a scintillation counter and compared with the activity remaining on the filter. The precision of elution assays can be increased with the use of internal standard cells labelled with [^{3}H]-thymidine which have been irradiated with x-rays at a dose of 1.5Gy or 3Gy; this dose of x-rays results in a near linear elution curve for DNA on a semilog plot of the log of the cells retained on the filter against elution time or volume. Results can then be plotted as the proportion of [^{14}C]-DNA retained on the filter against the proportion of [^{3}H]-DNA retained on the filter.

Radioactive labelling requires the use of proliferating

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cells to take up the label. The use of a fluorescent dye to quantify the DNA from filter elution allows the researcher to use quiescent cells, cells irradiated in vivo, or simply to use filter elution where facilities for the handling of , for example [^{14}C]-thymidine, are not available. DABA, 3,5-diaminobenzoic acid, has been used to quantify DNA from filter elution [117, 118]. DABA has the disadvantages that the DNA has to be precipitated, incubated with DABA, and have perchloric or hydrochloric acid added to it, before the fluorescence can be recorded. A more successful fluorochrome to use has proved to be Hoechst 33258.

Hoechst 33258 is also known by the names 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride pentahydrate, or more simply as bisbenzimide, figure 2.3. It is a nonintercalating DNA binding agent which has antihelminthic activity, but no antitumour activity [119]. Cesarone and coworkers [120] demonstrated the use of Hoechst 33258 to quantify sub-microgram quantities of DNA, with a view to possibly using the technique for filter elution. They found that the dye binds best to AT-rich regions, and the fluorescence of the dye is enhanced by at least 50 fold on binding to DNA. The fluorescence is relatively sensitive to pH, but is most stable over the range pH 6.5 - pH 7.5. The assay was found to be linear with respect to DNA concentration, was reproducible, and was rapid and simple in that long incubation with dye is not necessary and caustic solutions are not used. Stout and Becker [121] further investigated the use of Hoechst 33258, and found that single-stranded DNA produces a fluorescence yield of half that of double-stranded DNA. The alkaline eluting solution

・ 3HCl

・ 5H$_2$O
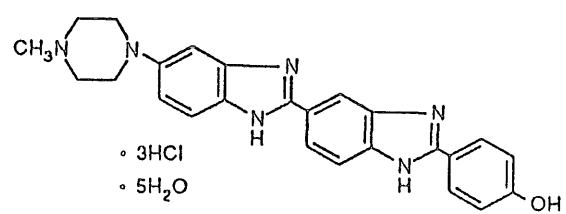
Figure 2.3: The structure of Hoechst 33258.

containing eluted DNA was also shown to require buffering with sodium phosphate to ca. pH 6.9 for optimum sensitivity with Hoechst 33258.

The use of Hoechst 33258 with filter elution has been directly compared with the assay using radioactively labelled cells, and has been shown to give identical DNA elution profiles [122, 123]. The ease with which the dye can be used has meant that the technique lends itself to automation. Sterzel and coworkers [124] have demonstrated a system in which the eluted DNA is neutralised with buffer, and the dye added. More recently it has been demonstrated that the buffer and dye can be added together without any adverse effects on DNA fluorescence [125]. Dr. Yvette Goward and myself have used an adaptation of these techniques to develop a semiautomated technique for studying single strand breaks in whole cells using alkaline filter elution combined with Hoechst 33258 to quantify the DNA eluted, figure 2.4.
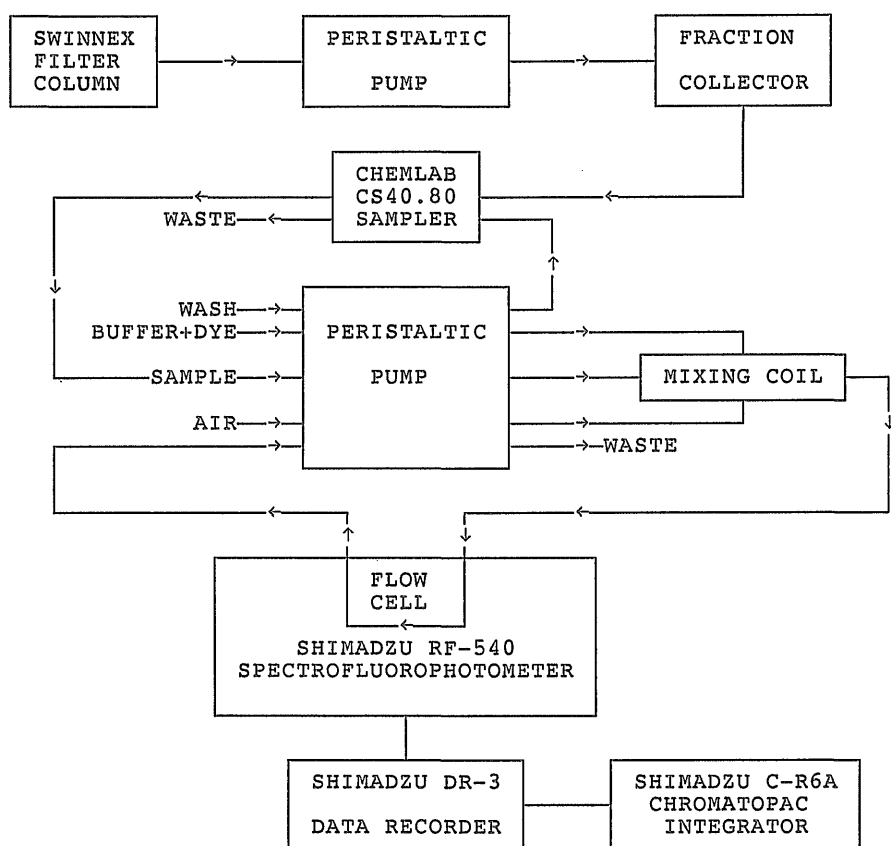
Figure 2.4: Flow diagram to show the layout of the equipment used for alkaline filter elution.

MATERIALS AND METHODS

The following buffers, media, and solutions were used extensively throughout this work. The composition of other solutions are described in the relevant protocols. All buffers and media were made up in water that had been passed through a Fisons cartridge deioniser or a still, followed by a Millipore "MilliQ" cartridge system, and a Millipore $0.2\mu m$ filter. Solutions were sterilised by autoclaving at 2 atmospheres for 10 minutes.

BUFFERS

Tris-EDTA (TE)

Tris.HCl pH 7.4   0.01M

Na_4EDTA   1mM

This was autoclaved ready for use.

Tris-Borate-EDTA (TBE)

10x stock

Tris.HCl   0.89M

Boric acid   0.89M

Na_4EDTA   250mM

This was prepared as a 10x stock solution, autoclaved, and diluted with distilled water when required.

Gel Loading Buffer

6x stock

0.25% w/v bromophenol blue

0.25% w/v xylene cyanol

30% v/v glycerol in water

This was stored at 4'C

MEDIA

L-Broth

Bactotryptone   10g/l

Yeast extract   5g/l

NaCl   5g/l

D-Glucose   1g/l

For L-agar, the glucose was omitted and 16g/l agarose was
added. Antibiotics were added as required after autoclaving;
typically 50μg/ml of ampicillin and/or tetracycline for pBR322
containing bacteria.

"Super-Broth"

For 400ml:-

    Bactotryptone   5g
    Yeast extract   10g
    Glycerol   2ml
    H_2O 360ml

This was autoclaved and allowed to cool before adding
40ml of autoclaved phosphate buffer.

Phosphate buffer

    K_2HPO_4 125g/l
    KH_2PO_4 38g/l

    Antibiotic was added as required; typically 50μg/ml of
ampicillin for pBR322 containing bacteria.

Antibiotics

Ampicillin

    This was held as a stock solution of 10 mg/ml in water,
was filter sterilised, and stored at -20°C.

<u>Tetracycline</u>

This was stored as a stock solution of 12.5 mg/ml in 50% ethanol at -20°C.

<u>PLASMID PREPARATION SOLUTIONS</u>

<u>Solution I (Lysis Buffer)</u>

    10x stock
    Tris.HCl pH 8.0   250mM
    Na$_4$EDTA   100mM
    D-glucose   500mM

This was prepared as a 10x stock solution, and was filter sterilised, since the glucose was found to caramelise if the solution was sterilised by autoclaving.

<u>Solution II (Lysis Solution)</u>

    Sodium dodecyl
            sulphate (SDS)   1% w/v
    Sodium hydroxide   250mM

Lysis solution was made up freshly from 10% w/v stock SDS solution, and autoclaved 5M sodium hydroxide solution.

Solution III

This was freshly made up from stock solutions. The volumes given are to make up 300ml of solution III.

Potassium
    Acetate (5M)   180ml
Glacial
    Acetic Acid   34.5ml
H_2O 85.5ml

ALKALINE FILTER ELUTION

Saline-Sodium-Citrate Solution

NaCl   0.14M
Na_3Citrate   15mM
Tris-Cl pH7.5   10mM

This was autoclaved, and PMSF, phenylmethylsulphonyl fluoride was added to a final concentration of 0.25mM; the PMSF being held as a stock solution of 0.2M in ethanol. PMSF is a potent protease inhibitor, and is used to inhibit degradation of the chick erythrocyte nuclei.

Phosphate-Buffered Saline (PBS)

$$10x \text{ Stock}$$

KCl	0.3% w/v
NaCl	10% w/v
Na_2HPO_4	1.4% w/v
KH_2PO_4	0.3% w/v

This was prepared as a 10x concentration stock solution in millipore water. The pH was adjusted to pH 7.2 with HCl, and it was refrigerated until required.

EDTA Wash Solution

Na_4EDTA pH 10.0   0.02M

Single-Strand Lysis Solution

NaCl	2M
Na_4EDTA pH 10	0.04M
Sarkocyl NL-30[*]	0.2%

Proteinase K (B.D.H. Ltd.) was added just before use to a concentration of 0.2mg/ml.

[*]Sodium dodecylsarkosine (Ciba-Geigy Ltd.)

Single-Strand Eluting Solution

EDTA	0.02M
Pr_4NOH	0.1M(2%)

5.85g of acid form EDTA was dissolved in 80ml out of 100ml 20%, or 1M, tetrapropylammonium hydroxide, Pr_4NOH, (batch 36199 888, Flucka Chemie AG.). This was made to 800ml with millipore water, and the pH was checked. The remaining Pr_4NOH was used to titrate the solution to pH 12.2. The solution was made up to 1litre, the pH was rechecked, and it was then filtered through Whatman no.1 filter paper. It was found that it was not advisable to keep the solution for much longer than a week, since the solution readily absorbed carbon dioxide from the air, making the pH become more acidic.

Standard Saline Citrate (SSC)

10xStock

NaCl	1.54M
Na_3citrate	0.15M

The pH was adjusted to pH 7.0 with HCl.

Phosphate For Neutralisation

Stock solution

KH_2PO_4	0.2M

Stock Hoechst 33258 Dye

Hoechst 33258 (Aldrich Ltd) was dissolved in millipore water to a concentration of 0.1 mg/ml. The concentration was checked by reading the absorbance of the solution at 338nm. The extinction coefficient of Hoechst 33258 at 338nm being $4.2 \times 10^4 M^{-1}$. The concentration of the dye was then adjusted to give a final concentration for the stock solution of $2.33 \times 10^{-4} M$.

Dye-Buffer Solution

This was freshly made up on the day of use.

Stock dye   200μl

10x SSC   20ml

0.2M KH_2PO_4 60ml

Made up to 300ml with water.

METHODS

Plasmid Preparation

The plasmid used throughout this work was pBR322, a 4362 base pair, <u>Escherichia coli</u> (<u>E.coli</u>) plasmid [126]. pBR322 contains the genes coding for resistance to the antibiotics ampicillin and tetracycline, bacteria containing the plasmid could therefore be selected for by growing on a medium containing one or more of the antibiotics.

pBR322 was maintained in <u>E.coli</u> strain JA221, a <u>RecA</u>⁻ strain of bacteria. The defect in the <u>RecA</u> gene, part of the

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bacterium's DNA damage repair system involved in recombination, minimises the formation of high molecular weight plasmid multimers. The strain was maintained in 1ml aliquots of 0.85ml of a 10ml L-broth overnight culture plus 0.15ml glycerol, stored at -20°C or -70°C. Bacteria were recovered by scratching the surface of the frozen culture with a sterile loop or wire.

Plasmid was prepared using a modification of the Birnboim and Doly alkaline lysis method which utilises the finding that at a pH of between 12.0 and 12.5 chromosomal DNA, but not covalently closed circular plasmid DNA, denatures, so enabling it to be selectively precipitated out [127]. JA221 cells containing the plasmid were streaked out onto L-agar plates containing 50μg/ml ampicillin and 12.5 μg/ml tetracycline. The plates were incubated overnight at 37°C. Individual colonies were selected and picked out into 10ml L-broth containing the same concentrations of antibiotic as before. These were then incubated overnight to provide starter cultures.

400 ml of "superbroth" plus ampicillin (50μg/ml) in 2l Nalgene flasks were inoculated with 2ml of the starter cultures and shaken for 48-58 hours at 37°C. Plasmid was prepared from the cultures as follows. The volumes given are for a bacterial pellet derived from 400ml of culture.

The cultures were decanted into 500ml Beckman polycarbonate bottles with screw lids and inserts, placed in a Beckman JA10 rotor, and centrifuged at 6000 rpm for 10 minutes at 4°C in a Beckman J-21B centrifuge. The resulting pellets were resuspended in 48ml of Solution I on ice. 12ml of freshly made Solution I containing 10mg/ml lysozyme was then added, and this was left to stand at room temperature for 10 minutes.

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The DNA was denatured by thoroughly mixing in 120ml of freshly made solution II. This was then allowed to stand on ice for 10 minutes. 60ml of precooled solution III was then mixed in, and was allowed to stand on ice for 15 minutes. This was centrifuged in the JA10 rotor at 8000 rpm and 4°C for 5 minutes.

The plasmid containing supernatant was removed, placed in a clean polycarbonate bottle and 150ml propan-2-ol was mixed into it. This was then spun in the JA10 rotor at 8000 rpm at 20°C for 5 minutes. The supernatant was then removed, and aspirated to leave the pellet as free from supernatant as possible. The pellet was then redissolved in 2-4ml of TE buffer.

Plasmid Purification

In order to separate supercoiled (CCC) DNA from open circular and chromosomal DNA, it was necessary to purify the plasmid DNA by passing it through a caesium chloride gradient. Plasmid dissolved in TE buffer was weighed, and to it was added caesium chloride at 1.019g/g, and 0.11ml/g ethidium bromide (stored as a stock solution of 10mg/ml ethidium bromide in water). The refractive index was then taken, and was adjusted to 1.3860 with saturated caesium chloride solution or distilled water. This was then put into a 38ml polyallomer tube, and topped up with TE buffer containing 1.019g/g caesium chloride and 0.11ml/g ethidium bromide. The tube was heat sealed and put in a Beckman Vti 50 rotor on a Beckman L5-65 ultracentrifuge. It was then spun at 48000 rpm for 18 hours.

The resultant ethidium stained bands were visualised under long-wave ultra-violet light. Usually two bands were seen; the upper one consisting of residual chromosomal DNA and non-supercoiled plasmid and the lower one consisting of supercoiled plasmid. The lower band was taken off using a syringe and needle pushed through the side of the tube. Depending on its volume it was either put in a 38ml polyallomer tube and placed in a Vti 50 rotor, or it was put into a 5.1ml polyallomer tube and placed into a Vti 65.2 rotor. In both cases the tubes were topped up with TE-CsCl-ethidium, sealed, and respun at 48000 rpm for 18 hours.

The supercoiled plasmid containing band was taken off for butanol extraction to remove the ethidium bromide, followed by ethanol precipitation. (see later sections)

Butanol Extraction

Butanol extraction was carried to remove ethidium bromide from the solution of DNA-CsCl-ethidium removed from caesium chloride gradients. The solution to be extracted was gently shaken with an equal volume of water-saturated butan-1-ol in a sterile Corex tube. The two layers were allowed to separate out, and the upper organic layer containing the ethidium was removed and discarded. This was repeated until the upper layer no longer appeared pink in colour. The DNA containing solution was then extracted twice with water saturated ether to remove any residual butanol. The residual ether was removed by allowing it to evaporate.

An equal volume of water was then added to the remaining

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aqueous phase, and an equal volume of propan-2-ol was added to give a final proportion of 50% propan-2-ol. This was left for two hours at room temperature. The DNA was then spun for 30 minutes at 7500 rpm in a swing out rotor on a Sorval RC5B centrifuge. The resulting DNA pellet was resuspended in 0.3M sodium acetate ready for ethanol precipitating.

Butanol Concentration of DNA

Butanol can be used to concentrate a solution of DNA. This was often done during or after butanol extraction where the DNA might become diluted during the course of it.

An equal volume of butanol was added to the DNA solution, gently mixed, and left to separate. The organic layer was taken off and discarded. This was repeated, with care taken not to precipitate out any salts, until the desired volume was reached. The DNA containing solution was then extracted twice with water saturated ether to remove the butanol. The ether was then evaporated off.

Removal of RNA

Any RNA remaining after the DNA had ben purified on a caesium chloride density gradient was removed by using pancreatic RNase A. The RNase was prepared by dissolving it to a concentration of 10mg/ml in 10mM Tris.HCl pH7.5 and 15mM NaCl. This stock solution was then heated to $100^{\circ}C$ for 15 minutes to deactivate any DNases present. The stock solution

was then dispensed into aliquots and stored at -20°C.

DNase free RNase was added to the DNA containing solution to a final concentration of 10μg/ml and was allowed to incubate at 37°C for 1 hour. The DNA was then phenol extracted to remove the RNase, and ethanol precipitated. (See later sections)

Phenol/Chloroform Extraction

Phenol/chloroform extraction was used to remove proteins from the plasmid. Phenol was redistilled and equilibrated with TE buffer pH 8.0, prior to adding 8-hydroxyquinoline to a final concentration of 0.1%. This was then stored at 4°C. The chloroform was used as a mixture of chloroform and isoamyl alcohol in the ratio of 24:1 chloroform:isoamyl alcohol.

An equal volume of phenol was shaken with the sample and centrifuged at full speed in a microfuge for 15 seconds. The upper aqueous layer containing the DNA was then carefully removed. DNA recovery was enhanced by back-extracting the remaining phenol layer with an equal volume of TE buffer, mixing, centrifuging, and carefully removing the aqueous layer, before combining it with the previous layer. This procedure was then repeated with an equal volume of 1:1 mixture of phenol and chloroform added instead of the phenol.

An equal volume of chloroform was finally added to the separated DNA containing aqueous layer, mixed, centrifuged, and the aqueous layer removed. The DNA was then precipitated out by ethanol precipitation. (see later section)

Ethanol Precipitation

DNA was made up to 0.3M with respect to sodium acetate, if not already in 0.3M sodium acetate, by the addition of a 1/10 volume of 3M sodium acetate pH 5.2. 3 Volumes of ice cold 100% ethanol were then added, and the sample was chilled to $-20^{o}C$ for between 20 minutes and 60 minutes (for DNA less than 1Kb long, or when it is in small amounts ca. <100ng/ml). For larger samples the DNA was spun in a sterile Corex tube in a swing out rotor on a Sorval RC5B centrifuge at 7500 rpm for 30 minutes, at $4^{o}C$. Smaller samples of DNA were spun out in a sterile Eppendorf tube in a Microfuge in a cold room for approximately 20 minutes. 1ml of ice cold 70% ethanol was added to the DNA pellet to help remove unwanted salts, and the DNA was recentrifuged as before.

The pellet was dried in a lyophiliser for 20 minutes before redissolving in TE buffer by allowing to stand at room temperature for several hours.

Calculation of DNA Concentration

$995\mu l$ of TE buffer was pipetted into a quartz cuvette with a pathlength of 1cm. This was placed into a spectro-photometer, and was used as a reference base line at 260nm. The optical density (O.D) of the TE buffer at 280nm was recorded. $5\mu l$ of DNA solution was then added, covered with paraffin film and mixed by inverting to give a 200 fold dilution to the DNA solution. The optical densities at 260nm and 280nm were then recorded, the $O.D._{280}$ being adjusted to take into account absorbance by the TE buffer.

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The ratio of the optical densities at the different wave lengths was used to give an indication of the purity of the sample. An $O.D._{260}:O.D._{280}$ ratio of 1.8 indicated pure DNA, while an $O.D._{260}:O.D._{280}$ would have a ratio of 2.0 indicated that the sample was pure RNA.

By extrapolating from measured values, it is known that DNA at a concentration of 1mg/ml has an optical density at 260nm of 20 optical density units. Hence the DNA concentration, in mg/ml, could be calculated according to the equation:

$$\text{DNA CONCENTRATION} = \frac{\text{Optical Density (260nm)}}{\text{Extinction Coefficient} \times \text{Dilution Factor}}$$

$$= \frac{\text{Optical Density (260nm)}}{200 \times 0.05} \ \text{mg/ml.}$$

Irradiation of Plasmid Samples

Samples were irradiated in a 'Vickrad' ^{60}Co gamma-ray source. Plasmid DNA was irradiated at a final concentration of 0.05mg/ml. Samples to be irradiated at room temperature were placed in sterile glass vials with push on plastic tops. Samples that were to be irradiated at 77K were pipetted into the end of a 5mm diameter, 3mm bore, glass tubing and frozen in liquid nitrogen. The ice pellet formed was then pushed out of the tube into sterile glass screw topped bottles and irradiated under liquid nitrogen. Typical sample size was 20µl, and all irradiations were carried out under ambient atmosphere unless otherwise stated.

Agarose Gel Electrophoresis

Agarose gels were made up by melting agarose (from Miles Laboratories) with 1X TBE to the required percentage, typically 1.4% w/v, and pouring on to a perspex plate to set. Each gel had slots, typically capable of holding ca.25μl of sample, formed in it by the addition of a moulding comb to the molten agarose until it had set. Prior to loading, each 20μl sample had 5μl of gel loading buffer added to it.

Gels were run in 1X TBE overnight at 20 Volts, 50mAmps, and were then stained by soaking in 2.5μg/ml ethidium bromide for 1 hour. The gels were then destained, to remove excess ethidium, by soaking in 1l of TBE buffer for 30 minutes. The DNA could then be visualised by placing on a short-wave ultra-violet light box, and photographed on to Kodak T-MAX professional film through a sharp cut out orange glass, using an exposure of 15 seconds at f4.5.

DNA containing intercalating drugs was run on TBE agarose gels containing 0.4μg/ml of the drug to help prevent the drug dissociating during the running of the gel, and hence smearing of the DNA bands. Gels containing ethidium bromide did not require staining and were destained in TBE for 2 hours. Those containing other intercalating drugs had to be stained in 2.5μg/ml ethidium bromide in water for ca. 3 days to allow the ethidium to compete with the drug and stain the DNA. This did not produce any significant diffusion of the DNA bands, and the gel was destained in TBE buffer for 2 hours before photographing. This differs from the method of B.W.Wren (1985)who extracted the intercalator with CsCl-saturated isopropanol before dialysing to remove the CsCl. This was not

done as it was found that it lead to a loss of a large amount
of DNA, and would therefore be wasteful.

Quantitation of DNA Strand Breaks

Each track on the photographic negative was scanned at
632.8nm from a Helium-Neon laser in an LKB Ultroscan laser
densitometer, linked to an Apple II microcomputer using LKB
2190-001 Gel Scan interface and software. This enabled the
absorption curve of the gel to be stored on disk.

The Gel Scan programme was used to calculate peak size
from the stored absorption curves. The programme does this by
fitting a Gaussian curve to the peaks and then integrating it.
The programme then produces a print out of peak size in
arbitrary units. The linear range of the densitometer tracings
was determined by measuring the density changes of the band
corresponding to form I (ccc) DNA with dilutions of the DNA
preparation.

The supercoiled form of pBR322 intercalates 20% less
ethidium bromide per molecule than nicked and linear forms, so
to account for this the values for supercoiled DNA had to be
multiplied by 1.22 [129].

A background level of 10%-20% nicked plasmid DNA was
unavoidably present in the stock plasmid used, due to shearing
of the supercoiled DNA during its purification from the
caesium chloride gradient. This had to be taken into account
when calculating the amount of nicked plasmid formed due to
γ-radiation induced single strand breakage of the supercoiled
plasmid. Hence the following equation was used:

$$NICKED_{corr.} = \frac{NICKED_{irr'd} - NICKED_{cont.}}{100 - NICKED_{cont.}} \times 100$$

WHERE:

   $NICKED_{corr.}$ = Percentage of nicked plasmid formed by γ-radiation.

   $NICKED_{irr'd}$ = Total percentage of nicked plasmid in the irradiated sample.

   $NICKED_{cont.}$ = Percentage of nicked DNA in the non-irradiated plasmid.

It was not thought necessary to make any correction to the amount of linear plasmid produced since linear plasmid was never observed in the stock solution of plasmid, and it was thought that supercoiled plasmid and nicked plasmid would register double strand breaks almost equally.

Additive effect ratio (a.e.r.)

If the $\log_{10}$ of the amount of Form I plasmid DNA is plotted against radiation dose for an experiment, and the slope calculated, it can be compared with the amount of damage done to a control experiment containing no additive. The additive effect ratio, a.e.r., is defined as the ratio of the slope of the semilogarithmic plot for the experiment in the presence of additive, to the slope of the semilogarithmic plot for the control experiment. An a.e.r. of greater than 1 corresponds to an enhancement of the radiation-dependent damage to the DNA due to the additive, i.e.: sensitisation,

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while an a.e.r. of less than 1 corresponds to a reduction in damage, i.e.: protection.

ALKALINE FILTER ELUTION

Alkaline filter elution was used to investigate strand breakage in mammalian cells. A modification of Kohn was used to measure damage in denatured DNA at pH 12.1-12.3 [112]. At this pH the DNA is denatured, and the damage observed is essentially single-strand breaks and some breaks at alkali-labile sites.

Preparation of Chicken Erythrocytes

Fresh chicken blood was collected from Poultry Processors Ltd, Leicester. 1 Litre of blood was collected in 200ml autoclaved 3.4% w/v sodium citrate to which chloramphenicol at 0.1% in ethanol had been freshly added. This was then stored on ice.

On return to the laboratory the blood was filtered through two layers of muslin to remove clots. The filtrate was then made up to 2 litres with Saline-Sodium-Citrate Solution, divided into four Beckman polyallomer centrifuge tubes, and centrifuged at 3000 rpm for 3 minutes in a Beckman JA 10 rotor on a Beckman J-21B centrifuge. The supernatant and buff coloured white cell layer were then aspirated off, and the remaining red blood cells were pooled into two tubes. They were then resuspended in Saline-Sodium-Citrate Solution, respun, aspirated and washed again. The cells were finally resuspended in Saline-Sodium-Citrate Solution and stored in

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10ml aliquots at -70°C.

Cells were allowed to thaw for about 45 minutes before use. 2×10^6 cells were used for each filter.

Tissue Culture Cell Growth and Irradiation

Mouse myeloma J588L cells were obtained from Dr.Tim Harrison, Department of Biochemistry, Leicester University. Chinese hamster fibroblast-like V79-379A lung cells were obtained from Dr. Kevin Prise at the Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex. These were grown in tissue culture by Dr. Tim Harrison and Miss Sarah Munsen of the Department of Biochemistry, Leicester University. Cells were grown in Dulbecco's Modification of Eagles Medium, with 10% horse serum, penicillin, and streptomycin added.

Mouse myeloma J588L cells were grown in suspension. V79 cells were grown on plates, and trypsinised to release them into suspension prior to use. Exponentially growing cells were spun down and resuspended in fresh growth medium and had their density adjusted so that 2×10^6 cells, containing ca. $12\mu g$ DNA in total, were present per aliquot in sterile plastic 2ml screw cap tubes. Cells were then incubated, where necessary, at 37'C with drug for typically 45 minutes. Each tube was then cooled on ice for 10 minutes.

Irradiation was carried out using a "Vickrad" ^{60}Co gamma-ray source. The radiation dose rate to each sample was decreased by encasing each tube in a precooled, cylindrical lead block with an average thickness of 35mm around the tube. Precooling of the lead block to 0°C was considered essential so as to ensure that as little as possible DNA repair took

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place after irradiation.

Filter Elution

A flow-diagram summarising the filter elution system used
is shown in figure 2.4.

Filter elution of the DNA took place using the "Swinnex
funnel" type arrangement [112]. The barrel of a 50ml
concentric luerslip syringe was connected, via a three-way
stopcock, to a 47mm "Swinnex" filter holder (Millipore (U.K.)
Ltd., Harrow, Middx.), containing a 2.0 μm polycarbonate
filter (Sterilin Ltd., Hounslow, Middx.). Another three-way
stopcock was connected to the underside of the filter holder
via a male luer connector. The stopcocks were used to control
the entry and exit of fluids to and from the syringe barrel
and the filter holder.

The filter assembly was precharged at least 1 hour before
use with ca. 25 ml ice-cold phosphate-buffered saline (PBS).
This was done by filling a 50ml syringe, connected to a
three-way stopcock, and attaching it to the underside of the
filter assembly. Holding the assembly vertically, PBS was
gradually passed through, ca. 5ml at a time, from the
underside. The filter assembly was continually tapped to
dislodge any bubbles that were present; this was found to be
very important since bubbles within the filter holder have
been found to cause shearing of the DNA [112]. When 20ml of
PBS was present in the syringe barrel, the stopcocks were
closed, and the assembly refrigerated until required. The
volume of the "dead space" between the end of the syringe
barrel and the filter itself was noted to be 3ml.

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Irradiated cells were added to the PBS in the filter assembly and were allowed to run through the filter holder under gravity. Before the level of the PBS had reached the bottom of the syringe barrel, 10ml of ice-cold PBS was added to wash the cells. This was repeated and, as the meniscus of the final wash reached the junction of the syringe barrel with the stopcock, the flow through the filter assembly was halted. 5ml of lysis solution was then added, and was allowed to flow through until the menicus reached the junction. This was allowed to stand at room temperature for 60 minutes. The lysis solution contained sodium dodecylsarkosine instead of SDS because SDS has been implicated in producing a high background fluorescence with Hoechst 33258 [130].

The lysed cells were washed by allowing 2 aliquots of 5ml of ice-cold EDTA wash solution to drip through. The bottom of the assembly was then connected to 0.89mm plastic peristaltic tubing, via a 19 gauge "Sabre" needle which had had the sharp tip removed (Richardsons Ltd, Leicester); "Sabre" needles being used because they do not contain aluminium which reacts with the caustic eluting solution. The tubing was connected to a peristaltic pump that had been made in the department, which contained a slow speed gearbox, and was run to give 0.035ml/min. with 0.89ml tubing. The tubing then passed to a 10 x 10 slot fraction collector, designed by Dr. Yvette Goward and built in the department's workshops.

Once the assembly was connected to the peristaltic pump and fraction collector, 40ml of single strand eluting solution was added to the syringe barrel, and the stopcocks were opened. The fraction collector was set to change at 90 minute intervals. Fractions were collected in 4ml plastic sample

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cups, and the system was designed in such a way as to switch off the peristaltic pump, which was slaved to it, after 10 samples had been taken. The system was then switched on and left overnight.

DNA Quantitation

The DNA in each aliquot was quantitised by using a fluorochrome, Hoechst 33258, 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride pentahydrate, bisbenzimide, figure 2.3. Cesarone and coworkers [120] showed that the dye could be used quantitatively to measure DNA concentrations in neutral solution. For alkaline elution it is therefore necessary to buffer the dye in order to lower the pH [121]. Detailed comparisons between the radioisotope assay using [^{14}C]thymidine-labelled cells [112], 1981), and the Hoechst 33458 fluorometric assay, have been published [122, 123], and have shown that the fluorometric assay produces comparable results to those for the radioisotope assay.

The volume of eluted solution in each of the sampling cups was noted and each cup was placed on to a Chemlab CS40.80 sampler, set on 55 second sample and 65 second wash, along with cups containing known concentrations (upto 5μg/ml) of calf thymus DNA that had been denatured by autoclaving in Pr$_4$NOH at 2 atm for 1 minute and rapidly cooling. Each group of samples was separated by 3 cups containing eluting solution, to aid identification. An aliquot of each sample was removed by the sampler, passed through a Chemlab CPP15 peristaltic pump to a glass mixing coil where prebuffered

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Hoechst 33258 and air were added and mixed to a final dye concentration of 5×10^{-8}M. This concentration of dye is 1/10 the concentration used by for example Sterzel [124], but was found by Goward [131] to produce a linear DNA dose response without the hysteresis associated with the higher concentration of dye. The air bubbles prevent carry-over of sample or wash

The dye-DNA solution was then passed through a debubbler to remove the air bubbles, and through a quartz flow cell in a Shimadzu RF-540 spectrofluorophotometer. This was set by the Shimadzu DR-3 data recorder attached to it, to excite the dye at 350nm, and to record the fluorescent emission at 475nm. The amount of fluorescence observed for each aliquot was calculated by a Shimadzu C-R6A Chromatopac integrator attached to the spectrofluorophotometer.

To find out the amount of DNA retained on the filters the filter holders were removed from the syringe barrel and upper taps, inverted over a 25ml universal bottle, and the peristaltic pump was reversed to push all of the fluid in the tubing and filter holder out. The tubing was then disconnected and 1-2ml of eluting solution was back flushed through the bottom tap and connector. The tap and connector were then discarded to be washed. Each filter holder was carefully unscrewed and, using a pair of forceps, the filter was folded and placed into the universal bottle. Both halves of the filter holder were carefully washed with eluting solution, and drained into the universal bottle. The filters in the bottles were cut into several pieces using a pair of scissors, and heated to 2 atm in a pressure cooker. When they had cooled they were vigorously shaken, and the volume of eluting

solution in each bottle was noted. A sample from each bottle was then assayed for DNA content.

Calculation of DNA Strand Scission Factor

The samples containing calf thymus DNA at known concentrations were used to produce a calibration curve of DNA concentration against fluorescence. Linear regression analysis was then carried out using the "Minitab" facility (Minitab Inc.) on the University's DEC Vax mainframe computer.

By using the calibration curve it was possible to calculate the amount of DNA in each aliquot of filtrate, and the total amount of DNA on each filter prior to elution. The data was then expressed as the proportion of DNA retained on the filter relative to the volume eluted. The rate at which the results could be calculated was accelerated by the use of a program kindly written by Mr. B.W. Taylor. The results were then plotted out using the "SAS" graph plotting facility on the mainframe computer. (SAS Institute Inc., Cary, N.C.)

The relative strand scission factor (SSF) was calculated using the equation:

$$SSF = -\log(F_x/F_o)$$

Where:

F_x = the fraction of DNA retained on the filter after 20ml of elution of the irradiated sample.

F_o = the fraction of DNA retained on the filter after 20ml of elution in the control sample.

The volume at which the SSF was determined was decided because it is approximately at the midpoint of the elution. 20ml of elution includes the 3ml of EDTA wash in the "dead space" that has to flow through the filter before the alkali does. Therefore the SSF is measured after a total of 20ml of fluid is eluted, 17ml of which is alkali. The choice of 20ml is somewhat arbitrary; however it does minimise the influence of the dead volume effect in the first fraction.

MISCELLANEOUS

RADIATION DOSE RATE

The rate at which the "Vickrad" ^{60}Co source produced gamma-rays on any particular day could be calculated by using the following equation:

$$D_t = D_o e^{-\lambda t}$$

Where:

D_t = The dose rate on a particular day.

D_o = The dose rate when the source was calibrated.

λ = Decay constant.

t = The time in days since the source was calibrated.

The source used was calibrated on 19/12/68 when it gave out 3.17×10^6 rad/hr (3.17×10^4 Gy/hr). The half life for ^{60}Co is 1910.26 days, with the decay constant related by the equation:

$$t_{1/2} = 0.693/\lambda$$

Therefore: $\lambda = 3.298 \times 10^{-4} day^{-1}$

Hence on 1/4/87, for example, the dose rate for the ^{60}Co gamma-radiation source was 0.353 Mrads/hr.

Fricke Dosimeter

The Fricke dosimeter was used to calibrate the lead block used to reduce the rate of irradiation of tissue culture cells for alkaline filter elution. The Fricke dosimeter is one of the most widely used chemical dosimeters, consisting of an air saturated solution of ferrous ammonium sulphate in sulphuric acid [132]. The reaction mechanism is shown below.

$$H_2O + \gamma \longrightarrow H^{\cdot} + {}^{\cdot}OH \qquad (2.1)$$

$${}^{\cdot}OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^{-} \qquad (2.2)$$

$$H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot} \qquad (2.3)$$

$$H^{+} + HO_2^{\cdot} + Fe^{2+} \longrightarrow Fe^{3+} + H_2O_2 \qquad (2.4)$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe(OH)^{2+} + {}^{\cdot}OH \qquad (2.5)$$
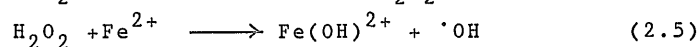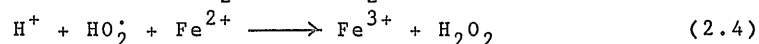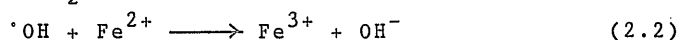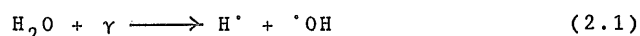
The hydroxyl radicals formed in (2.5) react in (2.2). Thus, for every water molecule decomposed, four ferrous ions are oxidised to ferric ions.

The ferrous ions become oxidised to ferric on irradiation with a G-value that is constant for a given type of radiation whatever the ferrous concentration, dose-rate, and irradiation temperature (within wide limits). The G-value the number of ferric ions formed per 100eV of absorbed energy, for ^{60}Co γ-radiation has been determined as 15.5, with the optimum

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range for calibration being between 4Gy and 400Gy.

The solution used for the Fricke dosimeter is shown below:

$Fe(NH_4)_2(SO_4)_3 \cdot 6H_2O$ 1mM

NaCl   1mM

H_2SO_4 0.4M

This was made up in air saturated "millipore" water. The chloride ions were added to inhibit the oxidation of ferrous ions by organic impurities.

The amount of ferric ions formed was found by spectrophotometric determination at 304nm, using an unirradiated solution as a blank. The molar extinction coefficient for ferric ions at 25°C is $2205M^{-1}cm^{-1}$, this having a temperature coefficient of 0.69% $°C^{-1}$. This compares with the extinction coefficient for ferrous ions, which is $1M^{-1}cm^{-1}$. The radiation dose rate is given by the equation given in the book by Spinks and Wood [132].

$$D = 2.76 \times 10^4 \times \Delta A/l \text{ rads}$$
$$= 2.76 \times 10^2 \times \Delta A/l \text{ Gy}$$

Where:

ΔA = Change in the absorption of the solution at 304nm.

l = Path length.

The measured dose rate was confirmed by the use of a thermoluminescence dosimeter (T.L.D.) from Dr. Alan Edwards of the N.R.P.B., and showed that the lead block allows about 13%

of the radiation from the ^{60}Co source through to the sample.

Chromous Chloride Oxygen Scrubber

Compressed nitrogen gas from commercial sources usually contains trace amounts of oxygen. It was therefore necessary to remove the oxygen from any nitrogen that was used for purging oxygen from samples.

Chromic acid was prepared by diluting 900 cm^3 of concentrated HCl (specific gravity 1.18) to 5dm^3 with distilled water, and adding 500g of CrCl$_3$. This was left to stir for 30 minutes.

Amalgamated zinc was prepared by washing 500g of granulated zinc with 65cm^3 concentrated HNO$_3$ and 13.5g Hg(NO$_3$)$_2$ in 1dm^3 of solution. This was allowed to soak for 20 minutes with occasional stirring. The amalgamated zinc was then thoroughly washed with distilled water.

1 dm^3 of chromic acid was added to 500g of amalgamated zinc in a stoppered flask, and was allowed to stand for 48 hours until the solution turned from green to blue.

Nitrogen was passed through two flasks of chromous chloride to remove the oxygen, and a flask of dilute sodium hydroxide solution, followed by a flask of sterile water, to remove any acid that may escape from the flasks. Bubbling through this system also ensured that the nitrogen used was water saturated.

Free-Thiol Estimation

Each of the aminothiols used for this thesis were tested for the concentration of free-thiol that they contained. This ensured that the same concentration of free-thiol containing monomer was used for each aminothiol. The free-thiol was estimated by using 5,5'-dithiobis-(2-nitrobenzoic acid), alternatively known as DTNB, 3-carboxy-nitrophenyl disulphide, or Ellman's Reagent, which reacts with free-thiols to produce a highly coloured anion which absorbs strongly at 412nm. [133,134].

1M stock solutions of each thiol were freshly made up in deoxygenated TE buffer, and a stock solution of DTNB was made up to 8mg/ml (2mM) in TE buffer. The reaction mixture typically consisted of DTNB (0.4mg/ml final), aminothiol (0.05mM final), and TE buffer. This was allowed to incubate at room temperature for 5 minutes to allow the colour to fully develop.

The absorption at 412nm was measured using a Shimadzu recording UV160 UV-Visible spectrometer. The free thiol content was calculated using the following equation:

$$C = AD/e$$

Where:

e = absorption coefficient (13600/M/cm)

D = dilution factor

C = free-thiol concentration

A = absorption

All of the thiols used were stored at under nitrogen at

-20'C in order to prevent oxidation of the free thiols. Stock
solutions were made up in deoxygenated TE buffer immediately
before to use.

RESULTS AND DISCUSSION

PLASMID ASSAY

Direct Conditions

Damage by water radiolysis products has been shown to be
removed by freezing aqueous solutions of plasmid DNA in liquid
nitrogen, and irradiating in the solid, or frozen aqueous,
state, figure 1.8 [58]. The gamma-radiation induced strand
breakage of plasmid DNA under direct conditions and ambient
oxygen is shown in figure 2.5 and table 2.1. The results
obtained are similar to those shown in the paper by Boon and
coworkers [58].

Despite numerous attempts to replicate the modest
increase in damage in oxic versus anoxic plasmid DNA seen by
Wren [58, 128], I found no significant difference between
oxygenated and deoxygenated plasmid on irradiation. These
attempts included gently purging the plasmid-containing
solution with chromous chloride-deoxygenated nitrogen for upto
an hour, which only resulted an increase in the amount of
sheared Form II plasmid, and irradiating the plasmid in TE
buffer which has been boiled and allowed to cool under a
nitrogen atmosphere. With hindsight all attempts involved the
use of sterile plastic Eppendorf tubes, and these may have
held large quantities of oxygen which may have been slowly
released into solution, as was reported by Chapman and
coworkers [135] who investigated the release of oxygen into
solution by several common types of plastic. Unfortunately
Wren [128] does not give any details on how he degassed his
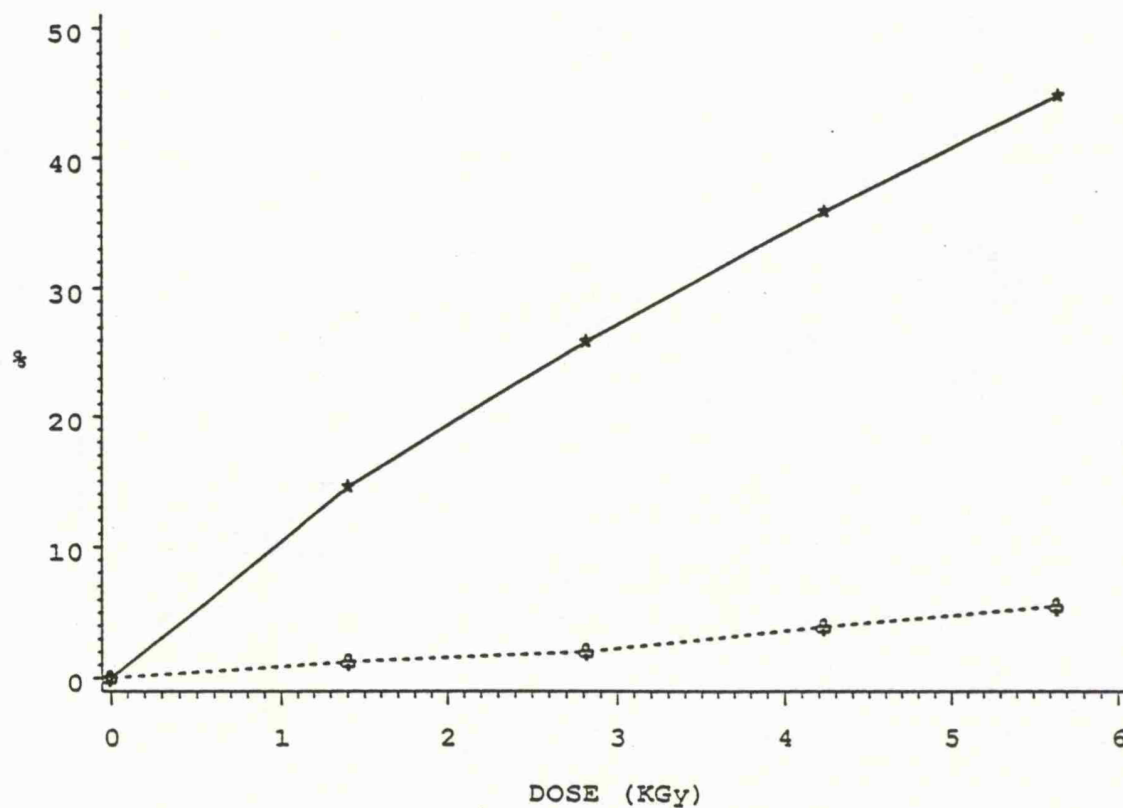
Figure 2.5: The effect of γ-radiation on Form I pBR322 plasmid DNA irradiated at 77K under direct conditions and ambient oxygen concentration. Form II DNA (★—★—★); Form III DNA (✥-✥-✥).

RADIATION DOSE (KGY)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}^{*}$	MEAN	S.D.$_{Mean}^{*}$
0.00	0.00	0.00	0.00	0.00
1.41	1.24	0.32	14.62	4.97
2.82	2.02	0.70	25.87	3.86
4.23	3.93	0.54	35.81	0.81
5.64	5.50	0.62	44.74	1.33

*Mean of four separate results.

Table 2.1: The effect of γ-radiation on Form I pBR322 plasmid DNA irradiated under ambient oxygen concentration and direct conditions at 77K.

RADIATION DOSE (GY)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}^{*}$	MEAN	S.D.$_{Mean}^{*}$
0.0	0.00	0.00	0.00	0.00
30.0	0.00	0.00	17.25	1.25
60.0	0.00	0.00	33.11	9.65
90.0	1.82	0.72	49.28	4.90
120.0	3.21	1.41	61.37	1.92
240.0	8.14	1.80	79.05	2.10

*Mean of four separate results.

Table 2.2: The effect of γ-radiation on Form I pBR322 plasmid DNA irradiated under ambient oxygen concentration in dilute aqueous solution at room temperature.

plasmid containing solutions. Any oxygen which was remaining in solution is thought to have been excluded out of the water-ice on freezing, into the phase containing the DNA, thus concentrating any remaining oxygen around the DNA. Because of the lack of a detectable oxygen-effect, and for ease of manipulation it was therefore decided to do all of the remaining plasmid assays under conditions of ambient oxygen concentration.

When irradiating DNA in the frozen aqueous system there is the possibility that damage to the DNA may occur on thawing due to a Fenton-type reaction with contaminating metal ions and hydrogen peroxide which is formed by hydroxyl radicals in the ice-phase.

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + {}^{\cdot}OH + OH^- \qquad (2.6)$$
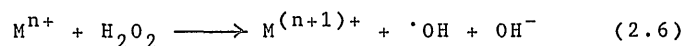
To test for this, plasmid was incubated for an hour at room temperature with 4mM hydrogen peroxide, a concentration which is about that which is formed on irradiating at the higher doses used. No damage to the plasmid DNA was observed, and it is concluded that hydrogen peroxide formed in the ice-phase during irradiation does not play a role in the radiation-induced strand breakage of plasmid DNA irradiated under direct conditions.

Indirect

The plasmid DNA throughout this work was irradiated in TE buffer containing 10mM Tris.HCl and 1mM Na_4EDTA, both of which have been shown to be hydroxyl radical scavengers. Gutterage

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[135] has shown that 11.1mM Tris (figure 2.6) protects deoxyribose from degradation by hydroxyl radicals by as much as 94%. Similarly Blazek and Peak [136], using a plasmid based assay, have shown that EDTA (figure 2.7) is also a good hydroxyl radical scavenger.

TE buffer was used to keep the irradiation conditions consistant with those used by previous members of the group [128, 138]. It was also used because the EDTA prevents degradation of the plasmid by chelating divalent metal ions which are required as cofactors by nucleases, so inhibiting their activity.

The effect of irradiating supercoiled pBR322 plasmid DNA in dilute aqueous solution with ^{60}Co γ-rays is shown in figure 2.8 and table 2.2. Dsb are not detected until after ca. 60Gy, which is consistent with the two hit hypothesis for dsb formation by two overlapping ssb, though very low concentrations of linear DNA, below ca. 0.02μg, proved hard to detect using the laser densitometer.

ALKALINE FILTER ELUTION

Some of the work reported here was done in conjunction with Dr. Yvette Goward.

Chick Erythrocytes

Attempts to use erythrocytes from the chick, Gallus domesticus, were made because the chick blood is readily available from abattoirs, chick erythrocyte nuclei were already used within the group to study radiation damage to

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$$CH_2OH$$
$$|$$
$$HOH_2C-C-NH_2 \cdot HCl$$
$$|$$
$$CH_2OH$$
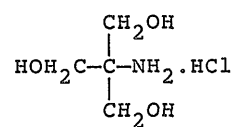
Figure 2.6: The Structure of Tris, 2-amino-2-(hydroxymethyl)
1,3-propanediol.

$$HO_2CCH_2 \diagdown \qquad \diagup CH_2CO_2H$$
$$NCH_2CH_2N$$
$$HO_2CCH_2 \diagup \qquad \diagdown CH_2CO_2H$$
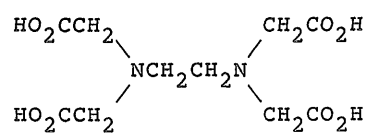
Figure 2.7: The structure of EDTA, ethylenediaminetetraacetic
acid.
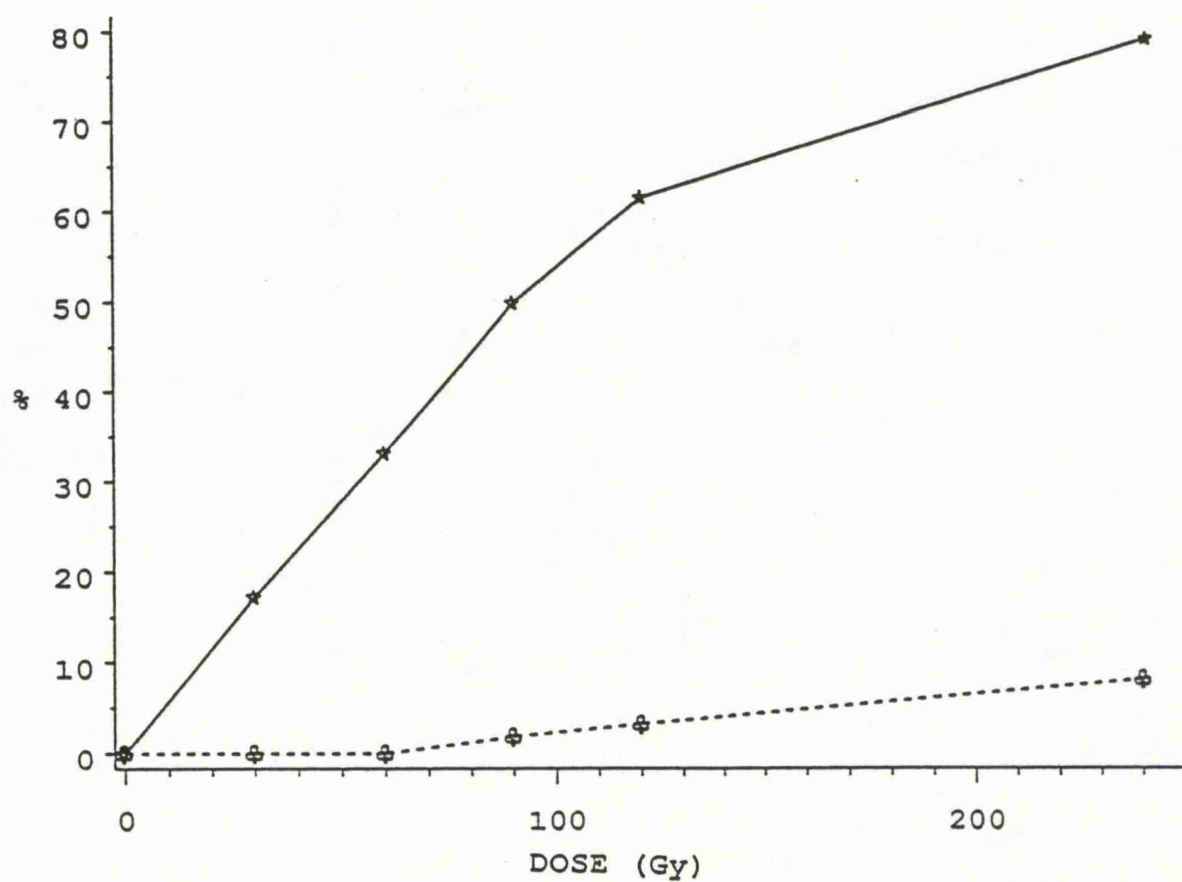
Figure 2.8: The effect of γ-radiation on Form I pBR322 plasmid DNA irradiated under ambient oxygen concentration in dilute aqueous solution at room temperature. Form II DNA (★—★—★). Form III DNA (⊕--⊕--⊕).

nuclei and chromatin by electron spin resonance spectroscopy, and it meant that tissue culture did not have to be used.

Figure 2.9 and table 2.3 shows that DNA from thawed cells is rapidly eluted from the filter. This may be due to shearing of DNA due to freezing of the cells, or due to the karyotype of <u>Gallus domesticus</u>.

When the thawed cells were looked at under a light microscope the cells appeared to be whole with little or no damage due to freezing. Obtaining fresh chicken blood on a regular basis would have been impractical since permission from the meat inspector had to be obtained on each occasion. Swenberg and coworkers [139] appear to have used V79 cells which had been frozen under liquid nitrogen in growth media for alkaline filter elution without undue damage to the cell's DNA.

Karyotype studies on <u>Gallus domesticus</u> have shown that its cells contain at least 39 pairs of chromosomes, over 20 of which are very small "minichromosomes" which are hard to distinguish under the light microscope [140]. It is thought that these "minichromosomes" may be small enough to account for at least some of the fast elution of DNA from the filter.

Mouse Myeloma J588L and Chinese Hamster V79 Cells

The rapid elution of DNA from chick erythrocytes prompted the use of tissue culture cells. Mouse Myeloma J588L cells were grown by Dr. T. Harrison and Miss S. Munsen on a regular basis as part of their on going research program. They kindly allowed Dr. Goward and myself to use them for our initial investigations. Chinese hamster fibroblast-like V79-379A lung
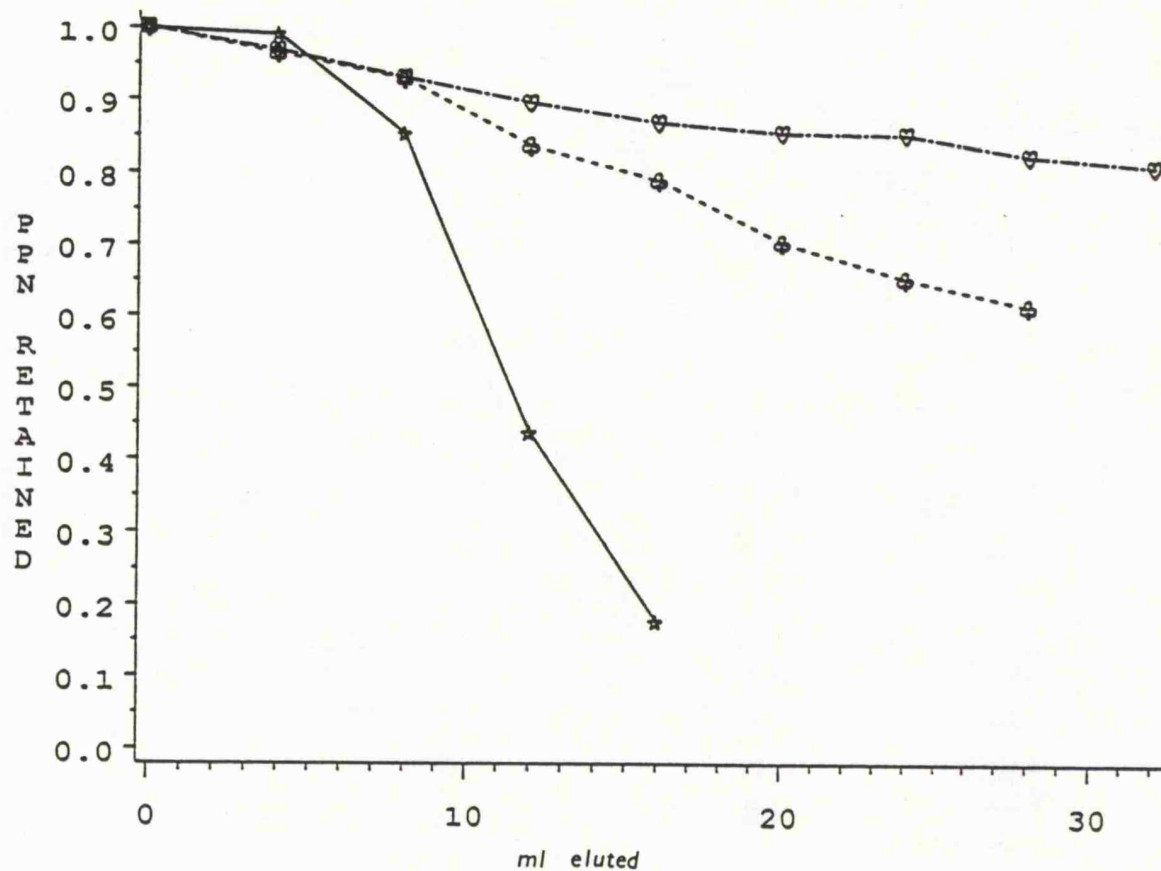
Figure 2.9: Alkaline filter elution profiles for non-irradiated Chick Erythrocytes (★—★—★), Mouse J588L cells (✿-✿-✿), and Chinese Hamster V79 cells (▽·-▽—▽).

| VOLUME ELUTED (ML) | PROPORTION OF DNA RETAINED ON FILTER | | | | | |
| | ERYTHROCYTES | | J588L CELLS | | V79 CELLS | |
	MEAN	S.D.[*]	MEAN	S.D[*]	MEAN	S.D.[*]
0	1.000	0.000	1.000	0.000	1.000	0.000
4	0.990	0.000	0.963	0.029	0.986	0.024
8	0.851	0.095	0.928	0.019	0.930	0.026
12	0.435	0.120	0.835	0.072	0.894	0.039
16	0.175	0.092	0.768	0.115	0.866	0.047
20			0.700	0.112	0.852	0.056
24			0.649	0.132	0.849	0.056
28			0.610	0.154	0.820	0.084
32					0.808	0.096

[*] Standard deviation of the mean for two aliquots of cells from the same batch on each of two separate days.

Table 2.3: The alkaline filter elution of nonirradiated chick erythrocyte, mouse J588L, and chinese hamster V79 cells.

cells were obtained from Dr. Kevin Prise of the Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex.

Nonirradiated J588L cells eluted through filters at a much slower rate than chicken erythrocyte cells, though with some variation on a day to day basis, figure 2.4, table 2.3. It is thought that this may have been due to the cells being grown at too high a concentration before being irradiated, and therefore not growing at a true exponential rate.

V79 cells proved to produce less day to day variation, figure 2.9, table 2.3. This may in part be due to the cells being grown at a lower concentration on flat plates, and possibly due to more practice resulting in less, and more consistent, time between when the cells leave the incubator to when they are lysed on the filter.

A semilog plot of the proportion of DNA retained on the filter against the volume of alkali eluted is shown in figure 2.10, table 2.4. It is interesting to see lack of increase in the rate of elution with volume which Kohn [112], for example, assumed to be from the breaking of alkali-labile sites. At first it was thought that this may have been due to a miscalibration of the ^{60}Co-source resulting in too low a dose being given, but repeated calibration by Fricke dosimetry and using a thermoluminescent dosimeter showed that the calibration of the source was correct.

A graph showing the single strand scission factors, SSF, observed for ^{60}Co γ-radiation on J588L and V79 cells is shown in figure 2.11, table 2.5. This shows that V79 cells appear to be less sensitive to radiation-induced ssb than J588L cells.

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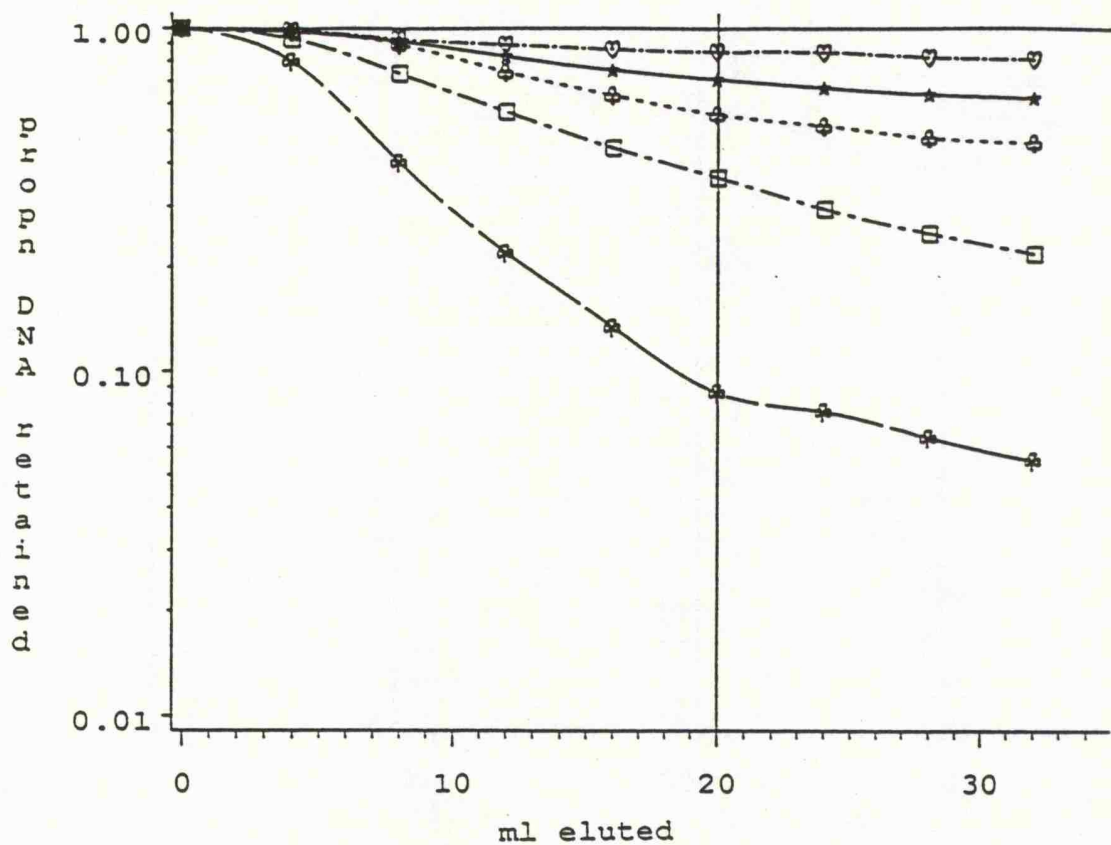
Figure 2.10: Alkaline filter elution profiles for Chinese Hamster V79 cells irradiated under ambient oxygen concentration. Non-irradiated (▽--▽—▽). 2.3 Gy (★—★—★). 4.6 Gy (✦-✦-✦). 9.2 Gy (▤—▢—▢). 18.4 Gy (✦—✦—✦).

VOLUME ELUTED (ml)	CONTROL		2.3 GY		4.6 GY		9.2 GY		18.4 GY	
	MEAN	S.D.*	MEAN	S.D.*	MEAN	S.D.*	MEAN	S.D.*	MEAN	S.D.*
0	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000
4	0.986	0.012	0.972	0.024	0.973	0.012	0.928	0.033	0.796	0.069
8	0.930	0.026	0.915	0.051	0.894	0.023	0.736	0.037	0.401	0.088
12	0.894	0.039	0.825	0.061	0.743	0.069	0.568	0.025	0.218	0.070
16	0.866	0.047	0.753	0.065	0.636	0.082	0.445	0.019	0.133	0.068
20	0.852	0.056	0.707	0.060	0.558	0.074	0.363	0.047	0.086	0.067
24	0.849	0.056	0.668	0.065	0.516	0.079	0.293	0.064	0.076	0.069
28	0.820	0.084	0.638	0.073	0.475	0.072	0.249	0.083	0.064	0.065
32	0.808	0.096	0.623	0.072	0.460	0.087	0.218	0.079	0.055	0.063

PROPORTION OF DNA RETAINED ON THE FILTER

* Standard deviation of the mean of two aliquots of cells from the same batch on two separate days

Table 2.4: Alkaline filter elution of V79 cells irradiated under ambient oxygen concentration with gamma-radiation.
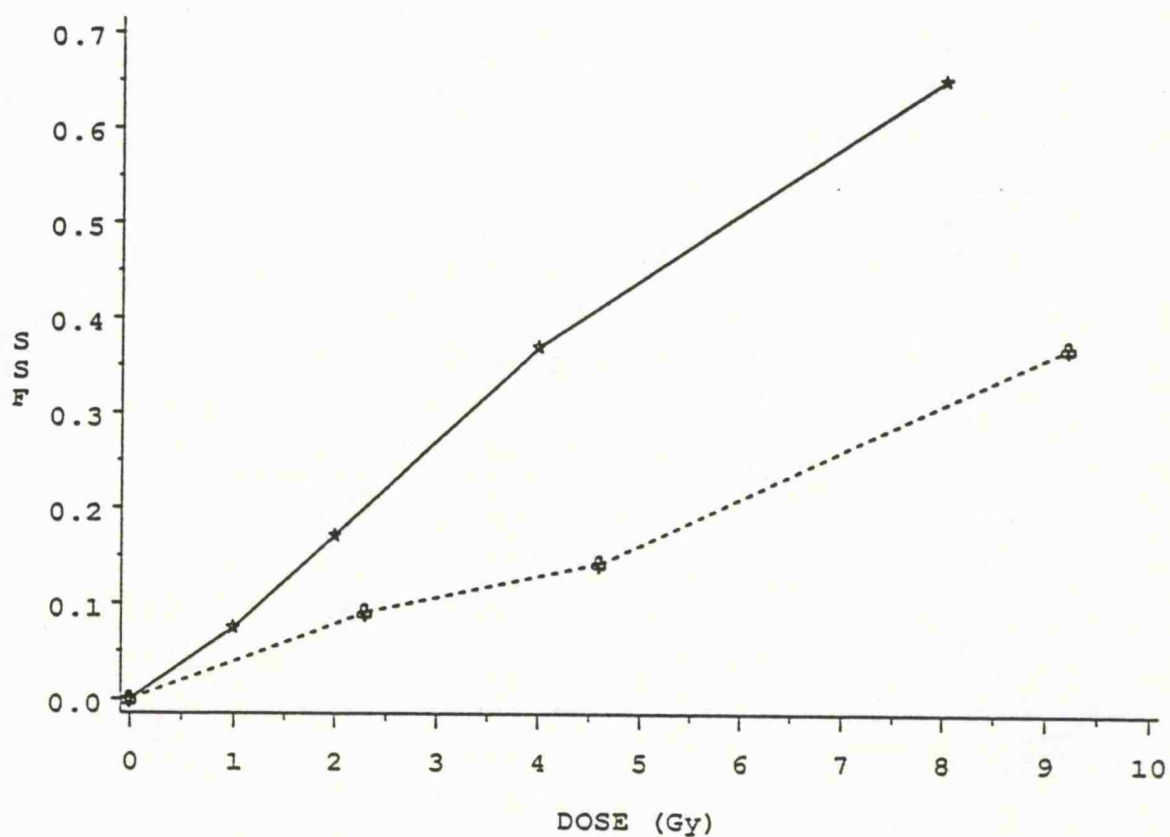
Figure 2.11: Strand Scission Factors for Mouse J588L cells (★—★—★) and Chinese Hamster V79 cells (⊕-⊕-⊕) γ-irradiated under ambient oxygen concentration at 0°C.

RADIATION DOSE (GY)	SSF	
	MEAN	S.D.$_{\text{Mean}}$*
0	0.000	0.000
1	0.075	0.006
2	0.172	0.020
4	0.369	0.185
8	0.650	0.212

* S.D.$_{\text{Mean}}$ for two aliquots of cells

from the same batch irradiated on each

of two days.

Table 2.5: Strand scission factors for mouse J588L cells
irradiated under ambient oxygen concentration with
γ-radiation.

RADIATION DOSE (GY)	SSF	
	MEAN	S.D.$_{\text{Mean}}$*
0.0	0.000	0.000
2.3	0.091	0.015
4.6	0.142	0.061
9.2	0.368	0.025
18.4	1.073	0.341

* S.D.$_{\text{Mean}}$ for two aliquots of cells

from the same batch irradiated on each

of three days.

Table 2.6: Strand scission factors for Chinese Hamster
V79 cells irradiated under ambient oxygen concentration
with γ-radiation.

CHAPTER 3

THE EFFECT OF ALKALI METAL HALIDES

INTRODUCTION

The negative charge on the sugar-phosphate backbone of DNA is neutralised by a shell of counterions of approximately 20Å [141], the sodium anion, Na^+, being the predominant counterion. Varying counterion concentration can alter the behaviour of the DNA. Increasing the concentration of sodium ions, for example, will increase the thermal stability of the DNA, resulting in a higher denaturation temperature, or Tm. This is due to the counterions shielding the negatively charged phosphate groups, whose mutual repulsion may provide some of the energy required to break the hydrogen bonds holding the DNA together [142].

Increasing salt concentration has been shown to decrease the amount of supercoiling in PM2 and superhelical λ DNA for the range 0.01M to 1M [143, 144]. Increasing the concentration of salt or a polar solvent decreases the amount of water around the DNA, and hydration of the DNA becomes more economical [145]. This drives the transition of B-form DNA, where the free oxygen atoms on the phosphate groups are 6.6Å apart and are individually hydrated, to A-form, where they are 5.3Å apart, or Z-form, where the oxygen atoms are 4.4Å apart; in the later two forms the oxygen atoms are bridged by water molecules, drawing the phosphate groups together.

Pulse radiolysis studies of aqueous sodium chloride solutions has identified the chloride radical anion, $Cl_2^{\cdot-}$, as a product [146]. This has been shown to have a relatively high

yield in acidic solutions, but a low yield at neutral pH.
Jayson and coworkers [147] proposed the following scheme for
the production of Cl_2^- in aqueous solutions of sodium
chloride:

$$^\cdot OH + Cl^- \rightleftharpoons ClOH^{\cdot -} \qquad (3.1)$$

$$ClOH^{\cdot -} + H^+ \longrightarrow Cl^\cdot + H_2O \qquad (3.2)$$

$$Cl^\cdot + Cl^- \longrightarrow Cl_2^- \qquad (3.3)$$

The reaction:

$$Cl^- + ClOH^{\cdot -} \longrightarrow Cl_2^- + OH- \qquad (3.4)$$
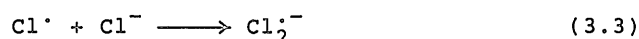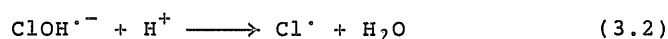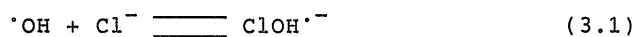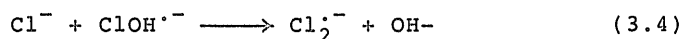
is another possible process.

Wold and coworkers [148] observed a five-fold increase in
the yield of Cl_2^- on addition of phosphate buffer; Na_2HPO_4
.$2H_2O$, KH_2PO_4, in equal proportions at pH 6.8. This, they
suggested, was due to a proton transfer from $H_2PO_4^-$ to $ClO_4^{\cdot -}$,
the rate of which is about one hundred fold lower than the
reaction between protons and $ClOH^{\cdot -}$.

Ginns and Symons [149] studied the formation of the
products from ^{60}Co γ-radiation on glassy, frozen aqueous,
solutions of alkali metal halides using E.S.R. spectroscopy.
They also identified Cl_2^- as a product, which they thought was
produced by the following reaction:

$$Hal^- + \gamma \longrightarrow Hal^\cdot + e^- \qquad (3.5)$$

$$Hal^- + Hal^\cdot \longrightarrow Hal_2^- \qquad (3.6)$$
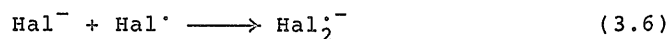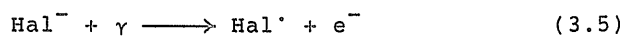
They also identified the hydrogen atom alkali-metal adduct
$MH^{\cdot +}$ and the radical ion $ClOH^{\cdot -}$:

$$M^+ + H^\cdot \longrightarrow MH^{\cdot +} \qquad\qquad (3.7)$$
(or possibly $M^+ + H_2O + e^- \longrightarrow MH^{\cdot +} + OH^-$)
$$^\cdot OH + Hal^- \longrightarrow HalOH^{\cdot -} \qquad\qquad (3.8)$$
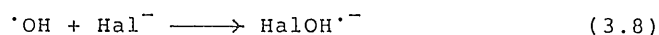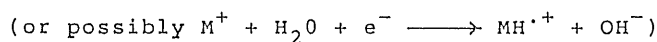
The formation of $MH^{\cdot +}$ was not thought to be of kinetic significance because of the weakness of the interaction.

Sodium iodide has been shown to reduce the total radical yield in DNA which has been irradiated under direct conditions at 77K [150]. It was found that, using a constant concentration of sodium iodide, but varying the DNA concentration, up to a 70% reduction in the amount of $G^{\cdot +}$ was observed. The iodide ion, I^-, reduced the yield of $G^{\cdot +}$ by adding an electron to it.

$$I^- + G^{\cdot +} \longrightarrow G + I^\cdot \qquad\qquad (3.9)$$
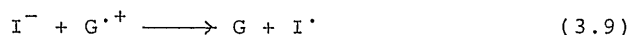
The iodine atom produced will react with iodide ions to form $I_2^{\cdot -}$ donate an electron to $G^{\cdot +}$.

$$I^\cdot + I^- \longrightarrow I_2^{\cdot -} \qquad\qquad (3.10)$$
$$I_2^{\cdot -} + G^{\cdot +} \longrightarrow I_2 + G \qquad\qquad (3.11)$$
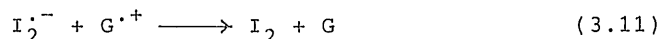
Studies of chromophore destruction by radiation in dilute aqueous solution have shown that at neutral pH DNA shows some protection by chloride ions [151]. This is thought to be due to the chloride ions changing the decay path of DNA radicals formed from hydroxyl radical attack, and not due to $Cl_2^{\cdot -}$ which is formed only in low concentrations at neutral pH. At acid pH, however, degradation on free pyrimidine nucleotides and nucleosides was observed due to $Cl_2^{\cdot -}$ attack. No such damage

was reported for the purine nucleotides or nucleosides, or for deoxyribose compounds. Ward and Mora-Arellano [152] more recently found, using pulse radiolysis, that $Br_2^{\cdot-}$ does not react with DNA in neutral, aqueous, solution.

The alkali metal halides have been observed to enhance the killing of yeast and bacteria by radiation [153]. Neither Kada and coworkers [154], using potassium iodide and transforming DNA from Bacillus subtilis irradiated in vivo and in vitro, or Shin-Chen [155], studying DNA from E.coli irradiated with sodium chloride, found any increase in DNA strand breakage. If anything there was a slight protection by potassium iodide in vitro. Both groups concluded that sensitisation was due to the action of either $Hal_2^{\cdot-}$ or $HalOH^{\cdot-}$ on other cell components, such as enzymes, or due to osmotic effects on the cell.

Raaphorst and coworkers [156-160] using Chinese Hamster V79 cells found that cell survival varied with the molarity of the salt solution, though with some variation between cation and anion types. By studying the amount of structured solvating water relative to free water by proton-spin lattice relaxation, they concluded that the salt effect on the survival of cells was due to changes in osmolality of the media. Hypotonic media results in the amount of water in the cell increasing, resulting in an increase in the amount of hydroxyl radical damage. Hypertonic media dehydrates the cell, condensing the nucleus, resulting in an increase in the probability of forming DNA-DNA or DNA-protein crosslinks, and an increase in chromosome aberrations.

In this chapter I demonstrate a sensitisation effect of alkali metal halides on the radiosensitivity of DNA irradiated

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in the frozen aqueous system. This increase in radiosensitivity of DNA is thought to be due to a physical effect on the structure of the water surrounding the DNA, combined with chemical effects caused by radicals formed by the halides used. This has resulted in an increase of our understanding of the structure and mechanisms involved in the frozen aqueous system.

METHODS

Plasmid DNA was used at a concentration of 0.05mg/ml, 0.076mM with respect to base pairs, as described in Chapter 2. Alkali metal halides, unless otherwise stated, were "analytical reagent" grade. Stock solutions were made up in TE buffer and sterilised by autoclaving.

Alkali metal halides were not observed to alter the staining of plasmid DNA by ethidium bromide, so dialysis of DNA prior to agarose gel electrophoresis was not required.

RESULTS

The effect of sodium chloride under direct conditions

The effect of adding "AnalaR" (99.9%) sodium chloride (BDH Ltd, Poole, Dorset), on the γ-radiation-induced breakage of Form I pBR322 DNA under direct conditions at 77K and under ambient oxygen concentrations is shown in Figure 3.1, Table 3.1. The level of single strand breaks, ssb, shown by the formation of Form II, or open circular DNA, is shown to increase with sodium chloride concentration. The level of

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Figure 3.1: The effect of sodium chloride on the γ-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under direct conditions and ambient oxygen concentration, with a dose of 2400 Gy. Two grades of sodium chloride were used; "AnalaR" 99.9% NaCl Form II DNA (★—★—★), Form III DNA (▽--▽--▽), and "Puratronic" 99.999% NaCl (Form II DNA (♦-♦-♦), Form III DNA (▭-▭-▭).

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	4.83	3.65	21.87	7.61
12.5	5.31	4.00	20.60	5.94
25.0	6.87	6.03	23.36	5.41
37.5	7.46	5.90	27.01	8.40
50.0	9.00	4.24	27.57	6.92
125.0	10.08	3.90	32.49	7.90
250.0	10.93	4.30	37.52	9.81
500.0	13.57	3.95	57.20	8.38

*Standard deviation of the mean of three separate results.

Table 3.1: The effect of "AnalaR" sodium chloride on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	3.05	4.02	19.17	5.91
12.5	5.22	3.97	23.43	6.11
25.0	5.50	4.25	27.17	5.46
37.5	5.65	4.20	28.10	5.95
50.0	6.19	5.23	28.83	6.35
250.0	7.90	4.90	45.75	7.84
500.0	10.56	3.98	58.87	8.94

*Standard deviation of the mean of two separate results.

Table 3.2: The effect of "puratronic" sodium chloride on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.

double strand breaks, dsb, shown by the formation of Form III, or linear, plasmid DNA, is shown to increase with sodium chloride concentration, the faster initial rate of increase slowing at ca. 50mM sodium chloride concentration. Two control experiments were devised to test the possibility that contaminants in the sodium chloride used were responsible for the apparent rise in radiation damage to the DNA.

In the first experiment plasmid DNA was incubated with 4mM hydrogen peroxide for 1.5 hours. This tested for the possibility that any contaminating metal ions could have reacted with hydrogen peroxide, produced by irradiating the frozen aqueous DNA, to produce damage, via a Fenton type reaction, by hydroxyl radicals.

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + {}^{\cdot}OH + OH^{-} \quad (3.12)$$
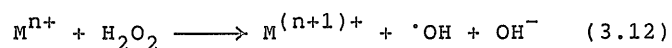
No increase in damage to the plasmid DNA was observed.

In the second experiment "puratronic" (99.999%) sodium chloride from Ventron GMBH was used instead of the "AnalaR" grade sodium chloride. The "pure" sodium chloride gave similar results to the "AnalaR" grade sodium chloride, Figure 3.1, Table 3.2, and it was concluded that the effect seen was due to the sodium chloride.

The sodium chloride effect for direct conditions was also tested using "puratronic" sodium chloride at a fixed concentration with varying radiation dose, compared with DNA without sodium chloride, Figure 3.2, Table 3.3. The results show that the increase in damage caused by sodium chloride holds true for different radiation doses.

Electron spin resonance spectroscopy, E.S.R., of calf

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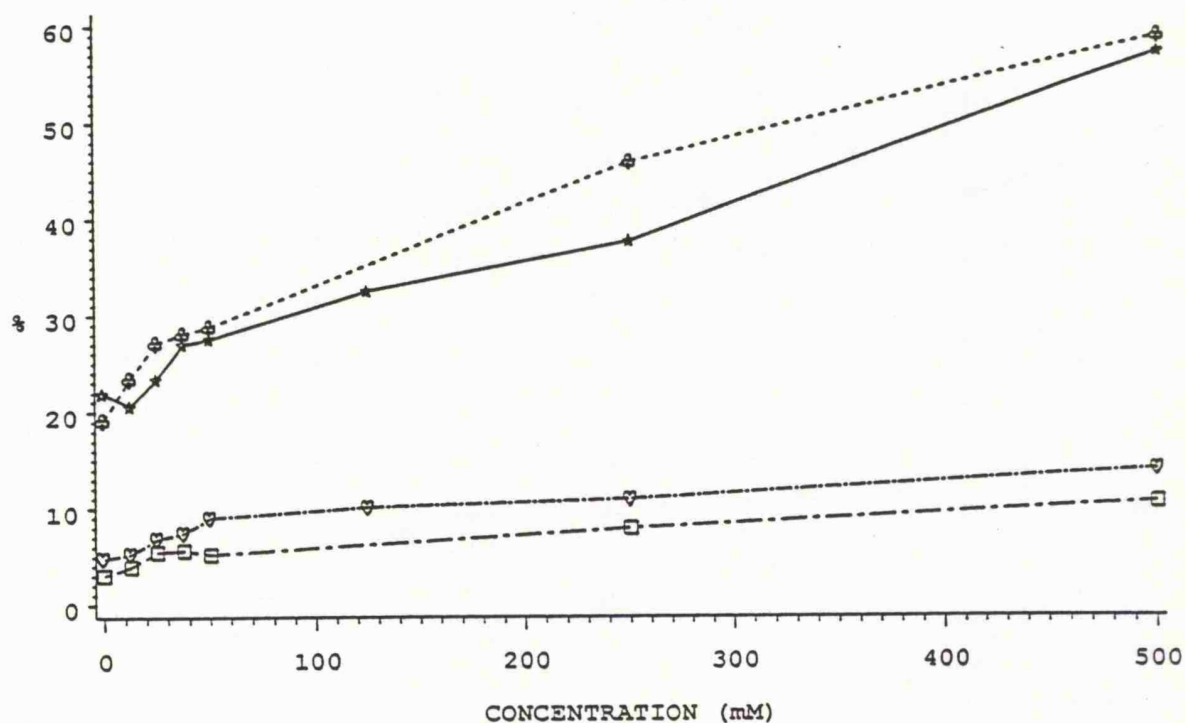
Figure 3.2: The effect of sodium chloride on the γ−radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under direct conditions and ambient oxygen concentration, with a dose of 2400 Gy. DNA only: Form II DNA (✶–✶–✶), Form III DNA (▽··▽–▽). DNA + 100mM NaCl: Form II DNA (✧–✧–✧), Form III DNA (▱–▱–▱).

RADIATION DOSE (kGY)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{\text{Mean}}$*	MEAN	S.D.$_{\text{Mean}}$*
0.00	0.00	0.00	0.00	0.00
1.41	1.80	0.32	22.54	4.97
2.82	3.56	0.72	33.72	3.86
4.23	5.30	0.54	44.57	0.71
5.64	7.68	0.62	48.64	1.33

*Standard deviation of the mean of three separate results.

Table 3.3a: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K under ambient oxygen concentration.

RADIATION DOSE (kGY)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{\text{Mean}}$*	MEAN	S.D.$_{\text{Mean}}$*
0.00	0.00	0.00	0.00	0.00
1.41	5.94	2.01	24.49	1.99
2.82	6.19	2.43	41.19	4.79
4.23	9.05	3.97	48.74	3.48
5.64	13.09	6.52	56.11	5.81

*Standard deviation of the mean of three separate results.

Table 3.3b: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated with 100mM sodium chloride at 77K under ambient oxygen concentration.

thymus DNA with sodium chloride was undertaken by Mr. A. S. Davies [161]. The E.S.R. data showed an increase in the total amount of free radicals produced by radiation with sodium chloride, though the effect was not noticable until ca. 500mM had been added. This was thought to be due to the relatively lower sensitivity of the E.S.R. technique, and due to DNA being used at 50mg/ml, compared with 50μg/ml of plasmid DNA, resulting in less sodium chloride, relative to the amount of DNA, being in solution. At concentrations above ca. 1.0M $Cl_2^{\cdot-}$ could be seen.

The effect of sodium chloride under indirect conditions.

Form I plasmid DNA was irradiated at room temperature with upto 250mM "AnalaR" sodium chloride with a radiation dose of upto 240Gy. No increase or decrease in the amount of radiation damage to DNA was observed for any of the concentrations of sodium chloride used. This compares with the results of Ward and Kuo [151], who studied chromophore destruction of DNA irradiated at room temperature, and found that 100mM sodium chloride had a slight protecting effect.

The lack of a sensitising effect by salt irradiated under indirect conditions shows that the effect is one that is associated with irradiating under direct conditions, that is freezing the DNA-containing solution and irradiating at 77K.

The effect of potassium and lithium cations.

The effect of potassium and lithium chlorides compared with "AnalaR" sodium chloride on the radiation-induced strand

breakage of Form I plasmid DNA under direct conditions is shown in figure 3.3, tables 3.1, 3.4 and 3.5.

The graph shows that potassium and lithium cations produce similar results for the enhanced formation of ssb. Sodium and potassium chlorides show similar curves for the production of dsb, but lithium chloride appears to produce less of an increase in dsb at lower concentrations, the reason for this being uncertain.

It is concluded from these results that the alkali metal cation has little effect on, at least, the observed increase in ssb.

The effect of different anions

The effect of adding sodium chloride, bromide, iodide, and perchlorate on the radiation-induced strand breakage of Form I plasmid DNA under direct conditions is shown in figure 3.4, tables 3.1, 3.6, 3.7, and 3.8.

The graph shows that sodium chloride and sodium iodide exhibit a slight initial decrease in the number of ssb, followed by a slight rise above ca. 50mM. This initial decrease would be consistant with the repair of $G^{\cdot +}$ by sodium iodide reported by Bartlett [150], reactions 3.9 – 3.11. Neither sodium bromide or sodium iodide show any significant rise or fall in the number of dsb formed with increasing salt concentration.

Sodium perchlorate, $NaClO_4$, was used because the perchlorate ion was thought less likely to produce reactive radicals on irradiation. The perchlorate ion has a relatively low electron affinity, and a relatively high ionisation

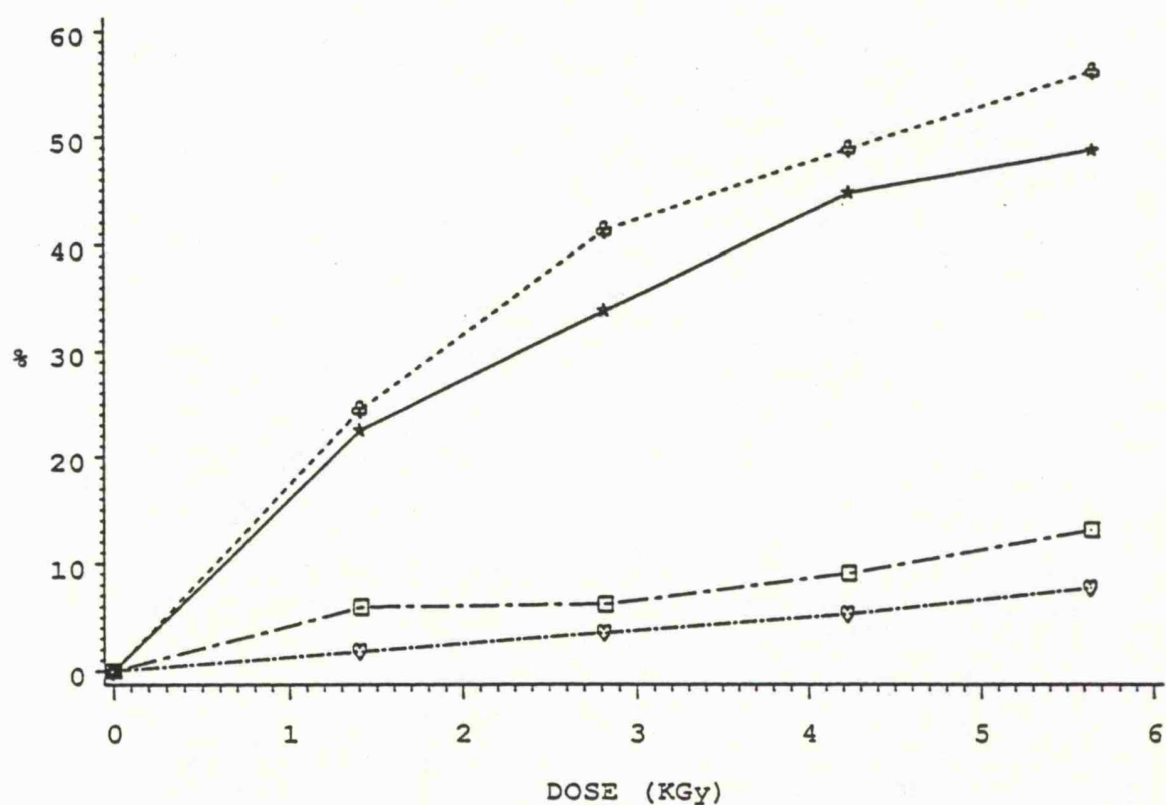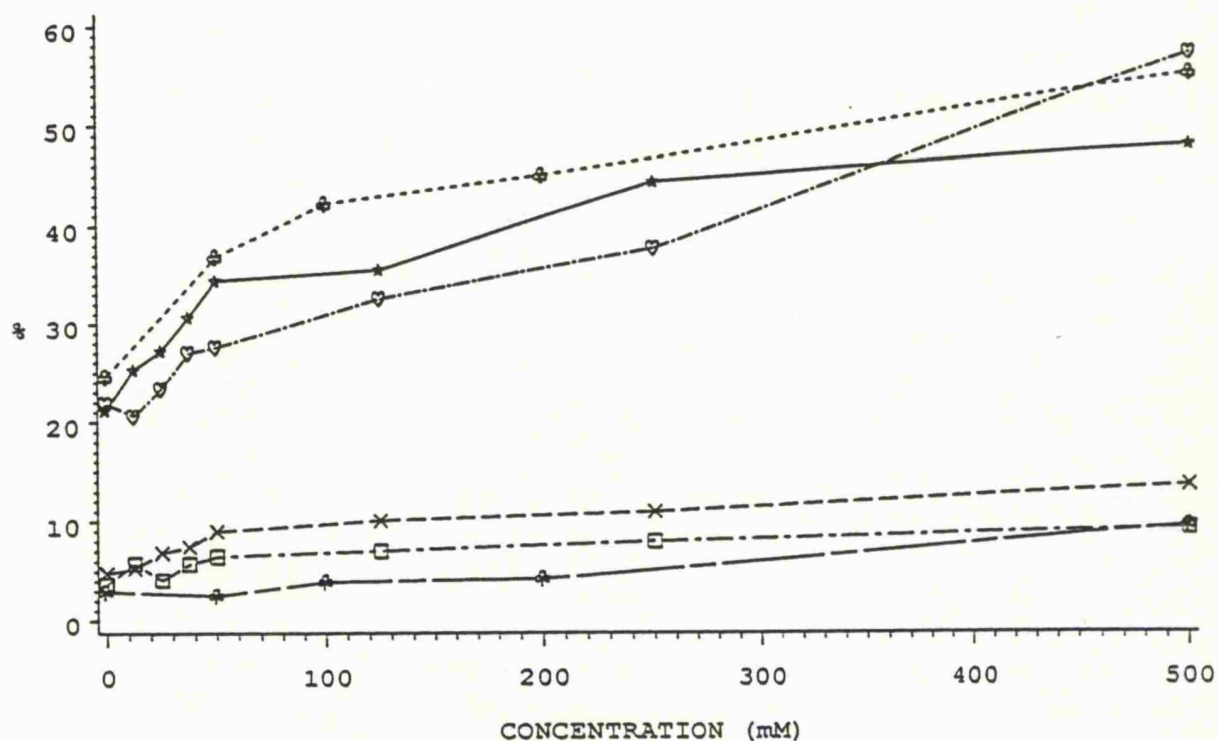Figure 3.3: The effect of sodium, potassium, and lithium chlorides on the γ-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under direct conditions and ambient oxygen concentration, with a dose of 2400 Gy. NaCl: Form II DNA (▽--▽--▽), Form III DNA (✳-✳-✳). KCl: Form II DNA (★-★-★), Form III DNA (▱-▱-▱). LiCl: Form II DNA (◌-◌-◌), Form III DNA (◌-◌-◌).

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	3.59	3.12	21.25	4.37
12.5	5.78	4.75	25.31	3.89
25.0	4.12	3.21	27.23	5.21
37.5	5.74	3.61	30.69	5.24
50.0	6.48	4.11	34.40	6.10
125.0	7.04	5.34	35.43	6.43
250.0	7.95	5.19	44.22	6.78
500.0	9.29	4.75	48.00	6.61

*Standard deviation of the mean of three separate results.

Table 3.4: The effect of potassium chloride on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	2.93	1.20	24.63	2.41
50.0	2.51	0.58	36.84	2.89
100.0	3.93	1.17	42.13	4.04
200.0	4.21	1.77	44.89	5.29
500.0	9.53	3.56	55.16	5.25

*Standard deviation of the mean of three separate results.

Table 3.5: The effect of lithium chloride on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.
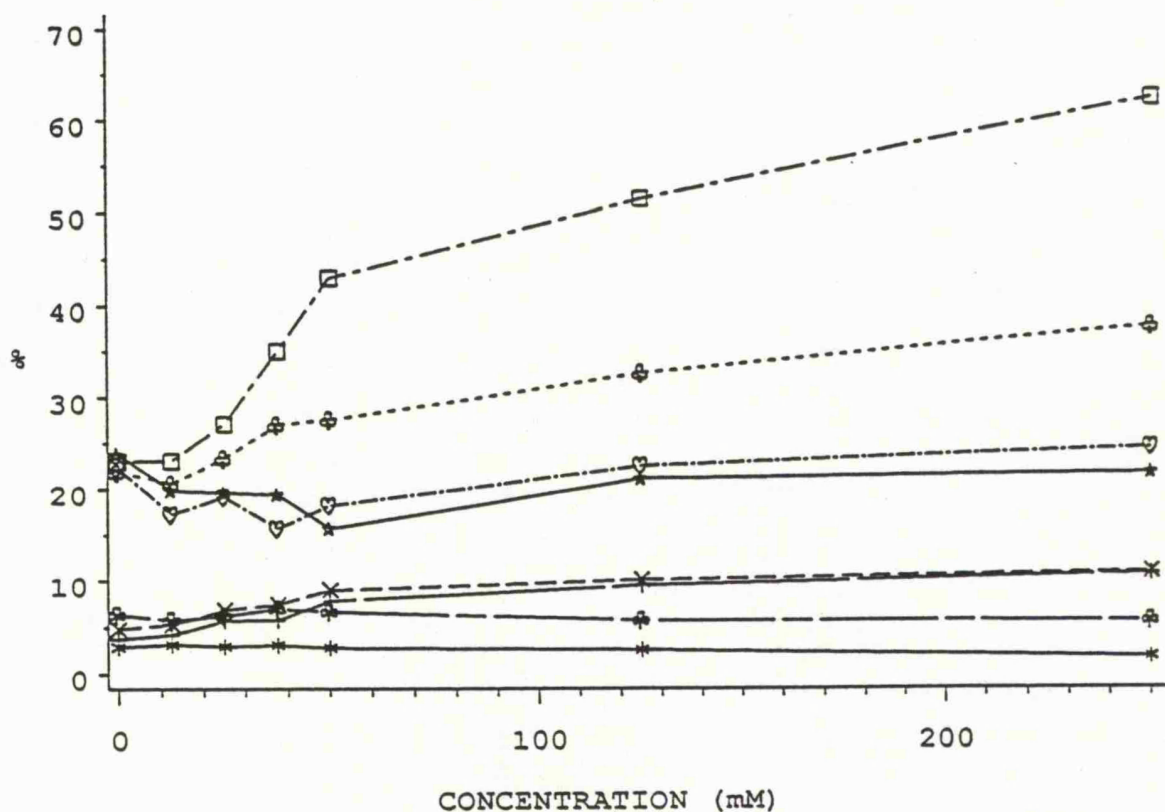
Figure 3.4: The effect of sodium chloride, bromide, iodide, and perchlorate on the γ-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under direct conditions and ambient oxygen concentration, with a dose of 2400 Gy. NaCl: Form II DNA ($\oplus$-$\oplus$-$\oplus$), Form III DNA ($\times$-$\times$-$\times$). NaBr: Form II DNA ($\star$-$\star$-$\star$), Form III DNA ($\oplus$-$\oplus$-$\oplus$). NaI: Form II DNA ($\triangledown$--$\triangledown$-$\triangledown$), Form III DNA ($\ast$-$\ast$-$\ast$). NaClO$_4$: Form II DNA ($\boxminus$-$\boxminus$-$\boxminus$), Form III DNA ($+$-$+$-$+$).

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	6.31	0.81	23.80	3.25
12.5	5.74	0.75	19.83	2.41
25.0	6.22	1.21	19.65	2.03
37.5	6.97	1.13	19.40	3.10
50.0	6.64	0.90	15.73	4.95
125.0	5.71	0.85	21.07	1.67
250.0	5.63	0.48	21.63	2.50

*Standard deviation of the mean of three separate results.

Table 3.6: The effect of sodium bromide on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	2.92	0.71	22.23	3.45
12.5	3.17	1.41	17.25	0.38
25.0	2.96	1.11	19.16	0.61
37.5	3.11	1.08	15.72	0.34
50.0	2.82	1.01	18.22	2.98
125.0	2.58	1.22	22.47	2.71
250.0	1.75	1.43	24.39	5.48

*Standard deviation of the mean of three separate results.

Table 3.7: The effect of sodium iodide on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.0	3.77	0.98	23.05	0.72
12.5	4.19	1.20	23.09	0.72
25.0	5.71	1.54	27.13	0.84
37.5	5.79	1.21	35.00	4.17
50.0	7.83	1.02	42.87	1.92
125.0	9.49	2.38	51.22	0.28
250.0	10.72	2.18	62.30	1.79
500.0	16.53	2.90	63.49	3.01

*Standard deviation of the mean of three separate results.

Table 3.8: The effect of sodium perchlorate on the gamma-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated at 77K, under ambient oxygen concentration, with a dose of 2400Gy.

potential. A possible reaction scheme for electron addition
is:

$$ClO_4^- + e^- \longrightarrow ClO_4^{\cdot 2-} \qquad\qquad (3.13)$$

$$ClO_4^{\cdot 2-} \longrightarrow ClO_3^- + O^{\cdot -} \qquad\qquad (3.14)$$

$$\text{(chlorate)}$$

$$O^{\cdot -} + H^+ \longrightarrow {}^{\cdot}OH \qquad\qquad (3.15)$$
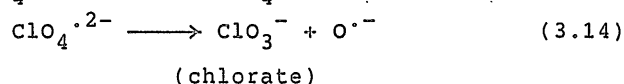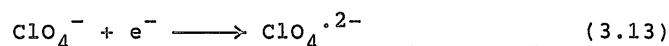
Electron loss would produce the following radicals:

$$ClO_4 \longrightarrow ClO_4^{\cdot} + e^- \qquad\qquad (3.16)$$

$$ClO_4^{\cdot} \longrightarrow {}^{\cdot}ClO_2 + O_2 \qquad\qquad (3.17)$$
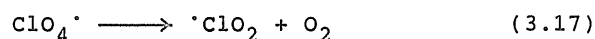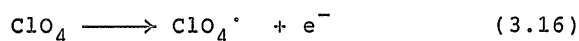
The ${}^{\cdot}ClO_2$ formed in reaction 3.17 is known to be a relatively
stable radical. Sodium perchlorate produced a greater rise in
ssb than sodium chloride, though the rise in dsb appear to be
about the same as for sodium chloride.

E.S.R. by Mr. A. S. Davies (1990) showed an approximately
equal rise for each of the salts in the amount of $T^{\cdot -}$ formed
with increasing salt concentration. The amount of increase in
$G^{\cdot +}$ varied for the different anions used. Sodium perchlorate
showed a large increase in the amount of $G^{\cdot +}$ with increasing
concentration, and radicals associated with the perchlorate
ions were not observed. Sodium chloride also showed an
increase in the amount of $G^{\cdot +}$, though not as great as that
seen for perchlorate, and $Cl_2^{\cdot -}$ was seen. Sodium bromide and
sodium iodide showed little increase in the amount of $G^{\cdot +}$
formed with increasing concentration, but $Br_2^{\cdot -}$ and $I_2^{\cdot -}$ were
detected.

Saturation of the "salt" effect

Close examination of Form II DNA curves for, for example, sodium perchlorate, figure 3.4, show that after ca. 50mM the increase in ssb tails off. This could be due to multiple ssb being registered in DNA which has already got a ssb in it. Form II DNA with two or more ssb will remain circular unless two ssb coincide within ca. 16bp to form a dsb; see, for example, figure 2.8, the effect of radiation on Form I plasmid DNA irradiated under indirect conditions.

If the tailing off of the increase in damage by a set increase in concentration was just due to multiple hits on Form II DNA, a semilog$_{10}$ plot of the disappearance of Form I DNA as a function of concentration will produce a straight line. Figure 3.5, table 3.11, shows a semilog$_{10}$ plot for sodium perchlorate. It can be seen that the graph is not linear. Above 50mM there appears to be a decrease in the effectiveness of the salt to produce an increase to the observed damage.

The effect of salt on the hydration of frozen aqueous DNA.

During the freezing of a solution the growing ice tends to reject the solute molecules which tend to form aggregates of very high local concentration in the interstices of the ice crystals. Water in the ice phase can be detected by infrared spectroscopy. The solute phase will contain DNA and its solvating, or hydrating, layer of water, plus any other compounds with their solvating water.

Low temperature infrared spectroscopy was undertaken by

CONCENTRATION (mM)	PERCENTAGE FORM I REMAINING	LOG FORM I REMAINING
0.0	73.18	1.864
12.5	72.72	1.862
25.0	67.16	1.827
37.5	59.21	1.772
50.0	49.30	1.693
125.0	39.29	1.594
250.0	26.98	1.431
500.0	19.98	1.301

Table 3.9: The effect of sodium perchlorate on the amount of Form I pBR322 plasmid DNA remaining after irradiation with γ-radiation. Plasmid DNA was irradiated with a dose 2400Gy under direct conditions at 77K under ambient oxygen concentration.
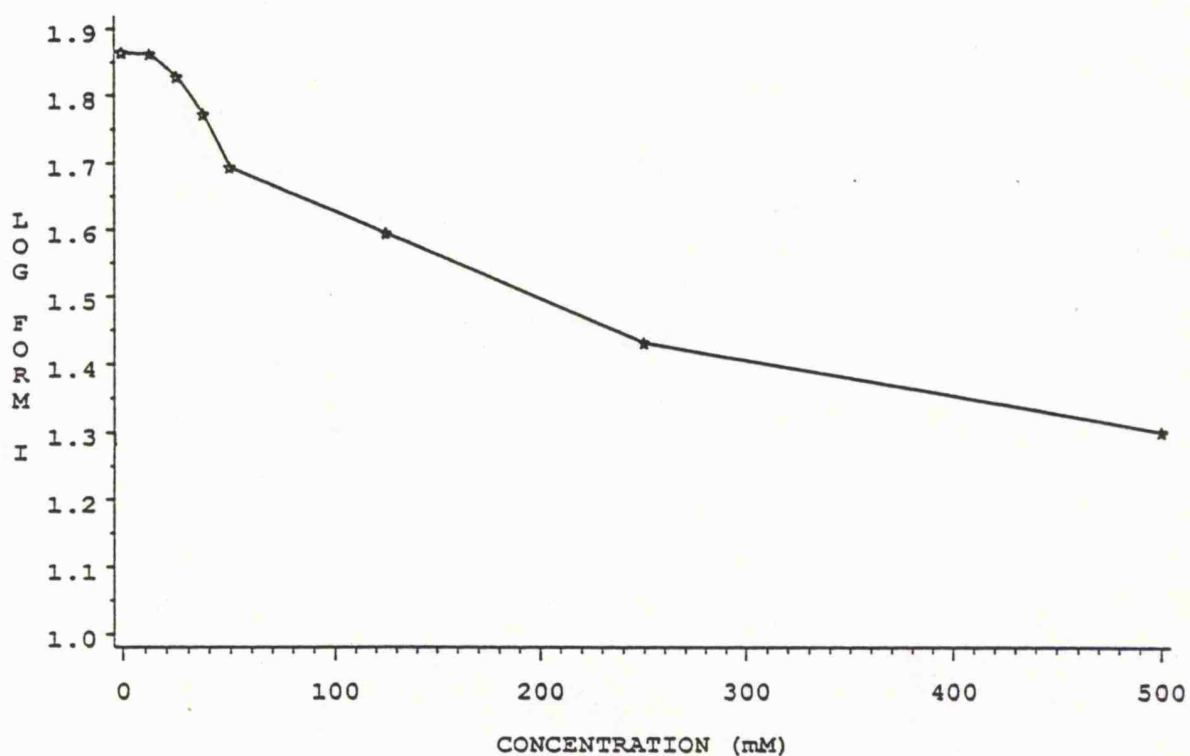
Figure 3.5: Semilogarithmic plot of the proportion of Form I pBR322 plasmid DNA remaining after irradiation with a dose of 2400 Gy at 77K, under ambient oxygen concentration, with varying concentration of sodium perchlorate.

Mr. C. M. Blackburn [162] using calf thymus DNA. Initial
results showed that there was a decrease in the amount of ice
seen with increasing salt concentration. This implied that
there was more water associated with the solvation of the salt
ions which were to be found in the DNA phase on freezing.
Above 500mM a third phase of salt hydrate of, so far, unknown
composition was seen to form, which was separate from the DNA
phase and the ice phase, and showed that at high
concentrations some of the salt ions excluded out into a
separate phase to the DNA. Damage to this phase by radiation
was detected by E.S.R.

DISCUSSION

These results show that adding sodium chloride to DNA and
irradiating it in the frozen aqueous system produces an
increase in damage. Two effects appear to be involved in
producing this radiosensitisation; firstly a physical effect
caused by the addition of the alkali metal halide, which
causes a sensitisation of the DNA, and secondly a chemical
effect which varies according to the anions used.

It is not thought that a decrease in supercoiling,
observed to occur with increasing salt concentration [143,
144], is enough to account for the radiosensitisation of DNA
by sodium chloride. Although the unwinding of the supercoiled
plasmid would, if anything increase the target area for
radiation attack, this effect would not be seen with the
relaxed calf thymus DNA used for the E.S.R. experiments. A
more plausible explanation for the increase in radiation
damage involves the increase in the amount of solvating water

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surrounding the DNA on addition of sodium chloride to the frozen aqueous system.

Gregoli and coworkers [62] found that the yield of DNA free radicals found with freeze-dried DNA irradiated at 77K was approximately half of that of frozen aqueous DNA irradiated at 77K. This was concluded to be due to the ionisation of hydration water and transfer to the DNA of the resulting dry, non-hydrated, charges.

$$H_2O + \gamma \longrightarrow H_2O^{\cdot +} + e^- \qquad (3.18)$$

$$H_2O^{\cdot +} + H_2O \longrightarrow H_2O + H_2O^{\cdot +} \qquad (3.19)$$

$$H_2O^{\cdot +} + H_2O \longrightarrow {\cdot}OH + H_3O^+ \qquad (3.20)$$
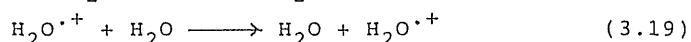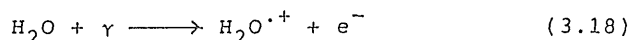
It is well established that the rate of reaction 3.19 is ca. ten times faster than reaction 3.20, and hence electron transfer, rather than proton transfer, is most likely to occur.

The degradation pathway for damage produced in this way is indistinguishable from initial damage produced on the DNA itself, since they both produce $G^{\cdot +}$ and $T^{\cdot -}$. With regard to radiation effects due to attack on the DNA itself and on the hydration layer, hydrated DNA could be considered as a DNA molecule with a cross section twice as large and absorbing twice as much radiation energy, for any given dose, as anhydrous DNA.

Initial low temperature infrared spectroscopy results have shown that there is a large fall in the amount of ice as the concentration of sodium chloride increases, this being constant with a greater proportion of water existing as hydrating water in the DNA phase. This is due to sodium and

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chloride ions being excluded into the DNA phase along with their associated hydrating water. The increase in the volume of the hydrating water effectively means that there is an increase in the DNA target volume, with the observed increase in damage being due to damage caused by $H_2O^{\cdot +}$ and non-hydrated electrons from the ionisation of the hydration water.

The rate of increase in damage caused by the addition of sodium chloride or sodium perchlorate is observed to reduce after ca. 50mM. This is consistant with the exclusion of the salt into the separate salt hydrate phase at higher concentrations of salt. This could also be due, in part, to the volume of the hydration water surrounding the DNA increasing to such a large size such that dry charges from the ionisation of the hydrating water at points further away from the DNA cannot diffuse far enough to attack the DNA.

The rapid increase in double strand breaks observed for sodium chloride, potassium chloride, and sodium perchlorate, can be accounted for using radiation damage from the hydrating water. They are formed by ionisation events which are close enough to the DNA to produce $G^{\cdot +}$ and $T^{\cdot -}$ radicals on the DNA which are close enough together to produce two overlapping ssb on opposite strands. Ionisation events which occur further away from the DNA are more likely to attack the DNA at well separated points and produce two separate ssb. The slight continuing rise in dsb production after 50mM is due to the formation of two overlapping ssb from two separate ionisation events.

There is considerable difference between the amount of damage caused in the presence of different anions. E.S.R. has shown that this is due to differences in the ability of the

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anions to prevent the formation of $G^{\cdot +}$. All of the anions could potentially react with $H_2O^{\cdot +}$ and reduce the amount of $G^{\cdot +}$ formed by it. In the case of the halides:

$$Hal^- + H_2O^{\cdot +} \longrightarrow H_2O + Hal^{\cdot} \qquad (3.21)$$
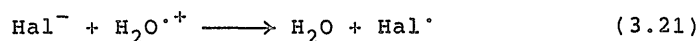
Alternatively the electron produced by the formation of $Hal^{\cdot}$ from Hal^- by gamma radiation, reaction 3.5, could react with $H_2O^{\cdot +}$ to form H_2O. In both of these cases $Hal_2^{\cdot -}$ will be formed by reaction of $Hal^{\cdot}$ with Hal^-, reaction 3.6. When DNA was irradiated with sodium, potassium, and lithium chlorides, $Cl_2^{\cdot -}$ was observed by E.S.R., as with sodium bromide was $Br_2^{\cdot -}$, and with sodium iodide, $I_2^{\cdot -}$, observed. Perchlorate is not thought to be a scavenger of holes because the expected radicals, were not observed by E.S.R. spectroscopy.

It was observed that the chloride ion allowed a greater sensitisation than bromide or iodide ions. This could be due to two factors. Firstly $Cl_2^{\cdot -}$ could attack the DNA and produce more ssb. The following reaction is thought to be possible:

$$Cl_2^{\cdot -} + G \longrightarrow 2Cl^- + G^{\cdot +} \qquad (3.22)$$
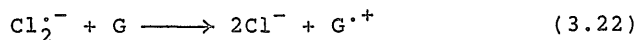
It could also produce damage which would not necessarily be detected by E.S.R. spectroscopy because the $Cl_2^{\cdot -}$ could potentially attack the DNA bases or the sugar and would not necessarily produce a stable radical.

Secondly the bromide and iodide ions could react with $H_2O^{\cdot +}$, reaction 3.21, and/or $G^{\cdot +}$, reactions 3.9-3.11, so reducing the observed level of $G^{\cdot +}$ and hence ssb. Net scavenging of $G^{\cdot +}$ would lead to the slight decrease in ssb

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observed for the lower concentrations of bromide and iodide ions. In the case of bromide and iodide ions it is thought that the hole scavenging effect roughly balances the sensitisation effect of the increased volume of the hydrating water, so resulting in little change in the numbers of ssb seen.

In conclusion, the addition of alkali metal halides to DNA has two effects on its radiosensitivity in the frozen aqueous system; the first is a sensitising effect due to the increased volume of hydrating water surrounding the DNA caused by the addition of the alkali metal halide. The second, protecting, effect is in competition with the first effect, is dependent on anion type, and involves the scavenging of $H_2O^{\cdot+}$ and $G^{\cdot+}$. These findings are important because they show that when using the frozen aqueous system to study radiation damage the effect of the additive on the amount of hydration water surrounding the DNA, or any other compound, must be taken into account.

CHAPTER 4

THE EFFECT OF INTERCALATORS

INTRODUCTION

Ethidium bromide, daunorubicin and its derivative doxorubicin (adriamycin), and mitozantrone (Figure 4.1) belong to a group of compounds which interact with DNA, and are loosely called DNA intercalators.

Interest in intercalators began because many of them have medicinal and mutagenic activity. Acridines, such as proflavine, 2:8-diaminoacridine sulphate, figure 4.1a, for example,were known to retard bacterial growth and induce mutations, and ethidium bromide was used for its antitrypanosomal activity. These compounds were observed to have planar aromatic ring(s) and cationic groups, and there was a growing realisation that DNA was important in their activity.

Much of the early work on drug-DNA binding was undertaken by Peacocke and coworkers [172, 173] on proflavine-DNA binding. They used equilibrium dialysis, partition analysis into aqueous or organic phases, and spectroscopy where they observed that binding produces changes in the visible and fluorescent spectra observed. Two type of binding were found: Type I, which involves the interaction of the drug with DNA in the grooves of the helix, and Type II, which is electrostatic between the cationic groups on the drug molecule and the polyanions of the sugar-phosphate backbone of the DNA. Type II binding was found to be sensitive to ionic strength changes and was found to be

a)
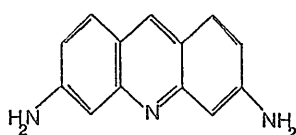
b)
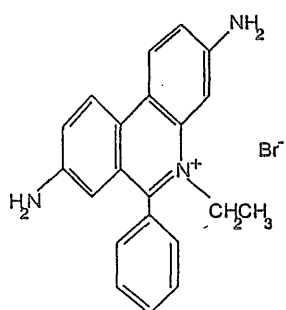
c)
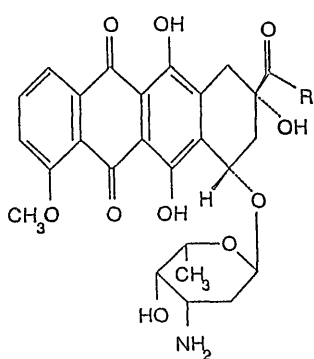
d)
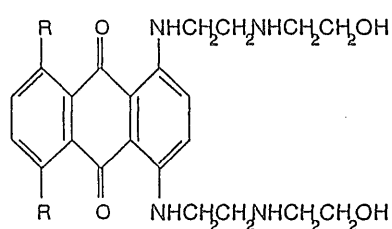
Figure 4.1: The structures of some intercalators.
a) Proflavine. b) Ethidium bromide. c) R=CH₃ Daunorubicin
(daunomycin); R=CH₂OH Doxorubicin (adriamycin). d) R=H
Ametantrone; R=OH Mitozantrone.

relatively unimportant at physiological conditions. The flat ring was implicated in Type I binding, since it was found that hydrogenation of the ring resulted in less binding taking place. This work has been continued by several research groups, much of which is reviewed in the articles by Neidle [174], Wilson and Jones [175], and Saenger [176].

The binding of proflavine to DNA results in enhanced DNA viscosity and a decrease in the sedimentation coefficient of the DNA. X-ray diffraction of DNA-proflavine complexes show that the 3.4Å meridional spots are kept, but the layer line pattern, the strong meridian reflections caused by base pair stacking, of B-DNA is lost. It was concluded from these observations and model building that the rod-like structure, hydrogen bonding, and covalent structure of B-DNA is retained. The DNA length is increased by 3.4Å to create space for each proflavine molecule, unwinding of the DNA helix being required to make space for the acridine ring to intercalate, the long axis of the ring being parallel to the adjacent base pair.

Later viscosometric measurements showed that the length increase varies from intercalator to intercalator. Using superhelical covalently closed circular (ccc) DNA it has been possible to calculate the unwinding angle of each intercalator. If right handed superhelical ccc DNA with a known number of turns is used, addition of intercalator unwinds the superhelix until it forms an open circle. Addition of more intercalator results in a left handed superhelix being induced into the DNA. The alteration of the superhelix can be followed by a change in the sedimentation coefficient of the ccc DNA. Using this

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technique it has been shown that ethidium bromide unwinds the DNA helix by $26°$, acridines by $17°-20°$, most intercalators by ca. $18°$, and daunorubicin and doxorubicin by $10°-12°$. The variation between unwinding angles appears to be due to variations in the size of the rings and their constituents, and in the specific hydrogen bonding of some compounds such as the anthracyclines, daunorubicin and doxorubicin.

Model building and x-ray diffraction studies of intercalators with oligonucleotides have shown the unwinding of the helix may take place over several nucleotides either side of the intercalator site. Intercalator binding alters the backbone tortional angles of the DNA helix, and also has been observed to alter the puckering of the sugar moiety. This distortion of the surrounding helix may lead to the prevention of other molecules intercalating next to the already intercalated molecule, so called "nearest-neighbour exclusion". Intercalation may also be directly prevented by side groups on the intercalated molecule blocking the sites either side of it.

Binding in the major or minor grooves of the DNA helix has been determined by either altering the side groups of the intercalator and observing the effects on binding, or by using different types of DNA with the major or minor grooves occluded by chemical moieties. Lown and coworkers [177], for example, compared the binding of intercalators to calf thymus DNA, T4 phage DNA, or anthramycin treated DNA. T4 phage DNA has its major groove occluded by α-glucosylated cytosine residues. Anthramycin-treated DNA was used in these studies because the drug is known to bind in the minor groove of the DNA.

The binding of intercalators has more recently been followed by sodium-23 NMR [178]. Addition of ethidium bromide to DNA in solution results in a decrease in the ^{23}Na line width, due to Na^+ release from the DNA backbone. The charge on the intercalator neutralises the anionic charge on the DNA, and the phosphate-phosphate distance increases on intercalation, leading to a lower local charge density, resulting in the release of sodium counterions.

Ethidium bromide, figure 4.1b, is a phenanthridine analogue which has been used for many years in the treatment of trypanosomiasis, and more recently as a stain for nucleic acids. Ethidium bromide is selectively toxic against trypanosomes which would appear to be based on the differing membrane permeability of mammalian and trypanosome cells to ethidium bromide. Trypanosomes take up ethidium bromide more rapidly than mammalian cells, and thus exhibit greater cytotoxicity than mammalian cells. The drug inhibits nucleic acid synthesis in vivo and in vitro, which implies that DNA is a target for the action of the drug. Considerable evidence has built up over recent years that mitochondrial DNA, which exists as closed circular DNA, is the in vivo target of ethidium bromide.

Binding of ethidium bromide to DNA induces a metachromic shift in the maximum emission wavelength of the drug from 480nm to 520nm. Both types of binding have been observed; Type II binding, which is not important at physiological salt concentrations, and intercalative Type I binding, which has been observed to involve several stages. The amino groups on the molecule are important, and have been observed to be required

for maximal binding to DNA, and for maximal biological activity. Removal of the phenyl group, results in a lower binding constant and biological activity. It also produces a more planar molecule which may have a higher tendency to stack with other drug molecules on the surface of the DNA. The quaternary group has little or no effect on the binding or activity of the drug molecule.

Models of ethidium bromide bound to DNA show the bulky phenyl and ethyl groups protruding from the helix. The amino groups on the drug molecule are thought to be involved in forming hydrogen bonds with phosphate oxygen atoms on the DNA backbone, which help to stabilise drug-DNA complex.

Daunorubicin (daunomycin) and its 14-hydroxy derivative, doxorubicin (adriamycin), figure 4.1d, are highly toxic anthracycline antibiotics which were originally isolated from Streptomyces peucetius [179]. Daunorubicin has been used in the treatment of acute lymphocytic and myelogenous leukaemias. Doxorubicin has a broad spectrum of activity, which includes a variety of solid tumours. They have the disadvantage that they are both highly cardiotoxic.

The drugs have been observed to inhibit cell growth in a number of normal and neoplastic cell lines, and have the characteristic that they cause cell damage, especially to the nucleus. Both DNA and RNA synthesis are inhibited, but not protein synthesis, the inhibition being independent of the concentration of polymerase, implying that the DNA template is involved.

A wide spectrum of binding studies have been undertaken, using a variety of conditions and methodology. The protonated amino group aids stability of the drug-DNA complex in interacting with the DNA's sugar-phosphate backbone. The C-14 hydroxyl group of doxorubicin may additionally stabilise binding to DNA by hydrogen bonding to phosphate groups at the intercalation sites, resulting in the higher binding constant reported by some authors. Removal of the sugar group has been reported to lower the affinity of daunomycin for DNA [180].

Both drugs have a complex chemistry when used in the cells, discussed later, which is thought to be involved in cytotoxicity, and in particular in the high level of cardiotoxicity observed with the drug. This level of cardiotoxicity has limited their use in the treatment of tumours, and has resulted in other, less toxic, compounds being investigated. Mitozantrone is one of these compounds. Mitozantrone is also known by various other names: mitoxantrone, Novantrone, dihydroxyanthracenedione, DHAQ, 1,4-dihydroxy-5,8-bis(2-[(2-hydroxyethyl)amino]ethyl)-9,10-anthracenedione dihydrochloride. Its development and properties have been reviewed by Todd and coauthors [181], White [182] and Smith [183].

During the mid-1970's the Medical Research Division of American Cyanamid Co. began a search for compounds with structural features that were predicted to favour intercalation with DNA. During the late 1930's a number of blue anthracenedione dyes were manufactured for the clothing industry. These had a planar structure, and tests showed that

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one of these possessed cytotoxic properties. Further research resulted in the production of two analogues; ametantrone, and its 5,8-dihydroxy analogue mitozantrone. Of these, mitozantrone was shown to be the most effective antitumour compound.

Mitozantrone has been shown to be active against a wide variety of tumour types, both _in vivo_ and _in vitro_, including leukaemias and solid tumours. For example, Drewinko and coworkers [184] using a colon carcinoma cell line, LoVo, and Cavan and coworkers [185] using a human tumour cloning system, have shown that mitozantrone is more cytotoxic than doxorubicin. It has been shown using the T47 human breast tumour cell line that mitozantrone depresses the rate of [3H]-thymidine and [3H]-uridine incorporation. It also leads to alterations in chromatin structure, nucleolar disintegration, and an increase in the density of the mitochondrial matrix [186]. Mitozantrone has a great advantage over the anthracycline antibiotics in that it lacks the cumulative cardiotoxic liability associated with them, whilst at the same time being more potent as an antitumour agent.

The mechanism of action of mitozantrone is unclear, but intercalation appears to be important [177]. Some reports indicate that it shows a GC-binding preference [187, 188]. [1]H and ^{31}P one and two dimensional nmr have been undertaken with mitozantrone bound to selected oligodeoxyribonucleotides [187]. The side chain of mitozantrone is observed to be in the major groove of the DNA, with the central amino group and the terminal hydroxyl group interacting with neighbouring bases to the intercalation site and phosphate groups on the backbone. The

compound unwinds DNA by 17° upon intercalation. Studies by Lown and coworkers using T4 phage DNA and anthramycin-treated DNA, indicate that at least part of the mitozantrone molecule extends into the minor groove.

Lown and coworkers [188] have studied mitozantrone binding to pBR322 plasmid DNA using electron microscopy. Form III, linear DNA was seen to increase in length on addition of mitozantrone, due to intercalation by the drug molecule. The authors failed to observe any change in length with Form I supercoiled DNA and mitozantrone. This was thought to be due to the length increase on intercalation being offset by an increase in the supercoiling of the DNA, and compacting of the DNA.

The induction of supercoiling was confirmed by using relaxed PM2 DNA which had been enzymically cut and then religated to form covalently closed open circle of DNA. Binding of the drug to the PM2 DNA was observed to induce supercoiling into the circle of DNA. At high concentrations mitozantrone was observed to induce crosslinking between PM2 molecules to form a lace-like network of DNA. Studies using mitozantrone analogues showed that the formation of crosslinks involved the side chains of the molecule.

The binding of mitozantrone to the DNA helix or other cellular components may be involved in cytotoxicity. Some intercalators have been observed to stabilise DNA-topoisomerase complexes [189]. Alternatively the drug may bind to components of the cell or mitochondrial membrane, and disrupt their function [190]. Other researchers, however, have concentrated

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on the redox behaviour of anthracenediones and anthracyclines to explain their cytotoxicity.

All antitumour quinones can undergo reversible enzymatic reduction and oxidation, reviewed by Gianni [180] and Powis [191]. Both one electron reduction to form semiquinone, Figure 4.2, and two electron reduction to form the less reactive hydroquinone have been observed. Researchers have used several different enzyme systems to encourage the reduction of quinones. These include rat or human liver microsomes with NAD(P)H [192, 193], a xanthine/xanthine oxidase system [194], ferridoxine reductase [195], NADPH-cytochrome c ferridoxine oxidoreductase [196], and NADPH-cytochrome P-450 reductase [197, 198].

Several groups have noted that anthracenediones have a lower cytotoxicity than anthracyclines. Basra and coworkers [192, 193] studied the formation of semiquinone radicals by ESR, using liver microsomes and NADPH to oxidise the quinones under anaerobic conditions. They found that the amount of semiquinone produced at equilibrium was about 2000 times greater with doxorubicin than mitozantrone. Other researchers using NADH-cytochrome P450 reductase to reduce quinones, have produced similar results [197]. These differences may be due to differences in the ability of the enzymes to react bind with the compounds, and/or due to different one electron reduction potentials.

Pulse radiolysis has been used to calculate the one electron reduction potentials for a variety of compounds at pH 7 [199]. The values they found for mitozantrone was -527mV, compared with -328mV for doxorubicin. These results indicate

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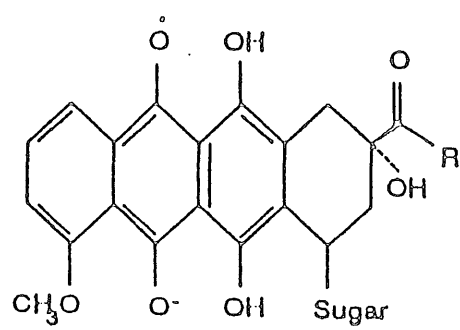
Figure 4.2: The anthracycline semiquinone radical.

differences in the ability of the enzymes to react with the compounds would appear to be responsible for the observed difference in the ability to form semiquinone. At least two groups have shown that binding the drug to DNA inhibits the reduction of the drug by enzymatic systems [196, 200].

Under aerobic conditions anthracycline and anthracenedione semiquinone radicals will react with oxygen to form superoxide radicals. The one electron reduction potential for oxygen is -155mV which means that electrons are readily transferred between mitozantrone or doxorubicin semiquinones to oxygen to form superoxide radicals ($O_2^{\cdot-}$), the mitozantrone semiquinone radical being the most efficient as transferring an electron to oxygen. Using human liver microsomes and NADPH under aerobic conditions, Basra and coworkers [193] found that doxorubicin produced a relatively large increase in the amount of superoxide radical observed by ESR, when compared with mitozantrone under the same conditions. This compares with the similar results obtained for the production of semiquinone under anaerobic conditions, and shows that the limiting factor for the production of superoxide free radicals is the rate of production of the semiquinone radical. Under aqueous conditions and neutral pH, about 10% of the superoxide radicals will be protonated, and can react with another superoxide radical to form hydrogen peroxide.

$$HO_2^{\cdot} + O_2^{\cdot-} \rightarrow HO_2^- + O_2 \rightarrow O_2 + H_2O_2 + OH^- \quad (4.1)$$
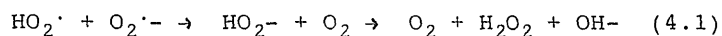
In the presence of transition metal ions the hydrogen peroxide can be decomposed to form hydroxyl radicals in a Fenton type reaction.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + \cdot OH + OH^- \qquad (4.2)$$
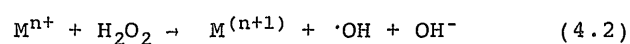
There is some evidence to suggest that doxorubicin may form a complex with iron ions and catalyse the breakdown of hydrogen peroxide [195, 196, 201]. Reaction 4.3 shows the scheme proposed by Bates and Winterbourn [201].

Xanthine
+
Xanthine oxidase   (4.3)

$O_2 \cdot^-$ $\underset{\xleftarrow{}}{\xrightarrow{\text{doxorubicin}}}$ $dox \cdot^-$

M^{n+}

H_2O_2 H_2O_2

Dismutation

$\cdot OH$

deoxyribose

TBA-reactive chromogen
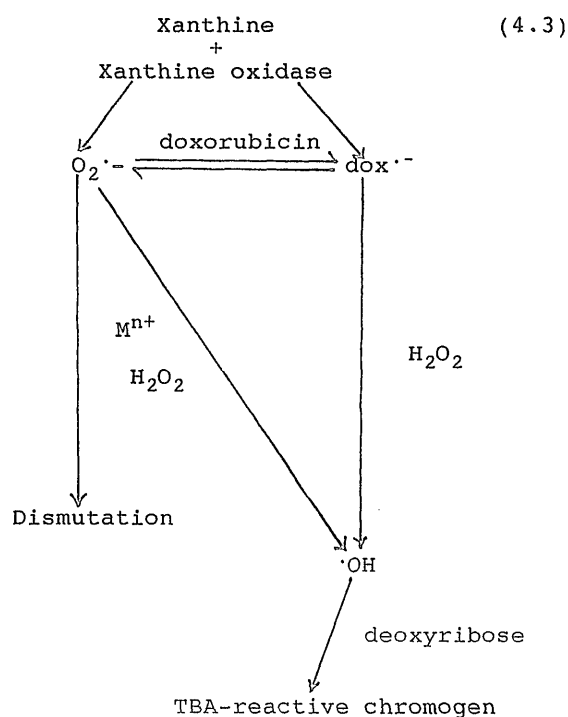
Doxorubicin has been shown to react with hydrogen peroxide, in the presence of xanthine and xanthine oxidase, to breakdown deoxyribose to produce a thiobarbituric acid chromogen [201]. The exact mechanism for the reaction of doxorubicin with hydrogen peroxide is still uncertain, but would appear to involve either the semiquinone radical bound to iron, or just the semiquinone radical itself. The species which reacts with the deoxyribose is thought to be the hydroxyl radical, or a "pseudo-hydroxyl radical" with a similar reactivity [196]. The species breaks down cytochrome c and reacts with methional to form ethylene with a reactivity that is similar to that expected for hydroxyl radicals [194]. The decomposition of hydrogen peroxide is inhibited by hydroxyl radical scavengers, and high concentrations of oxygen. The oxygen apparently competes with hydrogen peroxide for semiquinone radicals to form superoxide radicals. Superoxide radicals can themselves reduce transition metal ions (from $M^{(n+1)}$ to M^{n+}) to allow Fenton reactions to take place.

The differences in the amount of free radicals produced by doxorubicin and mitozantrone would appear to account for at least some of the differences in cytotoxicity between the two compounds. Some researchers, however, still believe that metabolically generated mitozantrone free radicals might participate in antitumour and/or cardiotoxic activity by mechanisms not involving superoxide radical formation [193].

The high amount of cardiotoxicity observed with anticancer quinones as a whole can also be at least partially accounted for by considering the free radical chemistry involved. Doxorubicin

and daunorubicin semiquinone radicals have been observed to form glutathione radicals, the glutathione removing the toxic semiquinone radicals. Cardiac tissue is known to have a low concentration of glutathione peroxidase which is involved in the recycling of glutathione to allow it to react with more drug or other radicals. Cardiac mitochondria also contain as little as 1% of the catalase and superoxide dismutase found in other cell types. Hence the tissue is more prone to free radical damage [190].

The intercalative binding, together with negative one electron reduction potentials of some intercalators, has meant that they are ideal candidates as scavengers of free radicals resulting from the interaction of ionising radiation with DNA.

METHODS

Ethidium bromide, and doxorubicin hydrochloride were obtained from Sigma Chemical Co. Ltd. Mitozantrone was generously supplied as the soluble dihydrogenchloride salt by Dr. L. Patterson from the Department of Pharmacology, Leicester Polytechnic. All stock solutions were made up in sterile TE buffer in sterile Eppendorfs or glassware. pBR322 plasmid DNA was used at a concentration of 0.05mg/ml, 0.076mM with respect to base pairs, as described in Chapter 2.

Some studies, such as that by Carmichel and Riesz [202], have indicated that mitozantrone and doxorubicin may be susceptible to photoexcitation by light with a wavelength of about 313nm. These photoexcited drug molecules may then be able to react with oxygen to produce superoxide radicals. To prevent the possibility of this occurring, all solutions containing the drug were shielded from light as much as possible, including the running of agarose gels containing DNA with drug, which were run in the dark.

DNA and drug were incubated together for 20 minutes at room temperature to allow time for the drug to intercalate into the DNA. Incubation times of above this did not produce any detectable difference in results.

Irradiation was carried out under ambient oxygen concentration and the resulting strand breaks quantified using the techniques described in Chapter 2.

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RESULTS

Controls

Control samples of DNA with increasing concentrations of
mitozantrone without irradiation were run on agarose gels and
stained and photographed as described in Chapter 2. DNA was run
on 1.4% w/v agarose in TBE with 0.4µg drug/ml agarose, and
stained for ca. 3 days in 2.5µg/ml ethidium bromide, before
destaining and photographing.

When plasmid DNA was incubated with concentrations of
mitozantrone of between 0 and 0.1mM (ca. 1.32 mitozantrone
molecules per DNA base pair), without irradiating the DNA, the
amount of DNA that was stained and photographed was constant for
both Form I and Form II plasmid DNA, and the total amount of DNA
loaded. Staining reached a maximum intensity after ca. 2 days,
but was allowed to proceed for about a further day to ensure
full staining. Staining for this amount of time resulted in a
small amount of diffusion of the bands, but they were still
fully resolvable by the Gelscan program attached to the laser
densitometer which was used to quantify the DNA. This shows that
incubating plasmid DNA with mitozantrone, under the conditions
described, does not produce any detectable damage. This compares
with the work of Wren [128] who found a slight increase in
damage when pBR322 DNA was incubated with 1mM mitozantrone,
extracted with caesium chloride saturated isopropanol, and run
on an agarose gel.

It was noted that drug concentrations of above 0.1mM produced smearing of the DNA bands which by a concentration of 1mM (ca. 13.2 mitozantrone molecules per DNA base pair) made the bands unresolvable by the Gelscan program. This may have been due to the mitozantrone side chains interacting with neighbouring plasmid molecules to form the intermolecular crosslinks observed by Lown and coworkers [188] at higher concentrations of drug (upto 14 molecules of mitozantrone per base pair DNA) using pBR322 DNA and electron microscopy. McClymont [203] found that at a drug to base pair ratio of 1:7.5 with calf thymus DNA at a concentration of 1mg/ml, the DNA precipitated as a fibrous coloured DNA-drug complex. At such high concentrations of drug the cross linking could also be due to the drug molecules stacking, and the stacks of drug molecules forming the inter-DNA chain crosslinks.

A heterogenous mixture of linked plasmid molecules would be expected to move through an agarose gel at different rates under the influence of the electric field during electrophoresis, and produce a diffuse band. This would be also be dependent on a number of other factors such as the net charge on the DNA molecule due to the binding of the positively charged drug molecule to the DNA, and whether or not mitozantrone molecules dissociated from the DNA molecules during the course of the gel electrophoresis.

It was noted that freezing the DNA/drug complex in liquid nitrogen, followed by thawing, resulted in a reduction in the concentration at which the DNA bands could be resolved by a factor of about 10. At mitozantrone concentrations above 0.01mM

the DNA bands became increasingly difficult to resolve, and by
a concentration of 0.1mM the DNA was completely unresolvable.
Freezing a drug + DNA solution will result in the drug and DNA
being excluded into a separate phase from the bulk of the water
surrounding the drug and DNA. This will result in a higher local
concentration of drug and DNA, and result in more drug binding
to the DNA than that of DNA incubated with the drug at room
temperature.

Intercalation results in the unwinding of the DNA helix,
so it was expected that the Form I, supercoiled, DNA helix would
unwind and the position of the Form I band with respect to Form
II DNA would alter. It was, however, noted that increasing the
concentration of the drug did not alter the relative positions
of either Form I or Form II plasmid DNA on the gel. This could
indicate that intercalation of mitozantrone was hindered by
supercoiling. Lown and coworkers [188] showed that mitozantrone
produced no apparent increase in molecular length in supercoiled
pBR322 plasmid DNA, as measured by electron microscopy, though
a length increase was observed with other intercalators, such
as bisantrene. Studies with nicked open-circular, and
toposisomerase-relaxed PM2 DNA showed that mitozantrone does
produce a length increase, and will induce supercoiling in
topoisomerase-relaxed PM2 DNA. Nicked Form II open circular
pBR322 DNA, and linear Form III pBR322 DNA, also showed an
increase in length with mitozantrone. They concluded that it is
most likely that mitozantrone does intercalate into supercoiled
DNA but, in so doing, it increases the amount of supercoiling

and compacting of the DNA. In terms of over-all measured length, one effect is offset by the other.

With hindsight, intercalation may have been possibly tested spectrometrically. Blake and Peacocke [173] noted that the absorbtion spectrum for intercalators alter when the drug molecule intercalates into the hydrophobic environment of the DNA helix. It should be possible to measure the difference in spectra between unbound drug and intercalated drug, the effect of any residual Form II plasmid DNA being accounted for by comparing with the spectra for plasmid DNA which has been topoisomerase relaxed.

With regards to agarose gel electrophoresis of plasmid DNA other factors probably also play a role. The number of charged mitozantrone molecules which will intercalate into Form I DNA will probably be less than number that will intercalate into Form II or Form III DNA, as is the case for ethidium bromide staining of plasmid DNA [129], owing to stearic hindrance. This will result in the different forms of the DNA having different charges due to the positive charge of the mitozantrone molecule at neutral pH. Intercalation may well alter the flexibility of the DNA, as well as the length and compactness, and alter the ability of it to pass through the pores in the agarose gel. These factors will all contribute to the electrophoretic mobility of the different plasmid forms. Over-all though, because of the study by Lown and coworkers [188], and the fact that ethidium bromide intercalates into supercoiled pBR322 DNA, the assumption has been made that intercalation does occur in the system used here.

The effect of hydrogen peroxide

Hydrogen peroxide is formed on irradiating water with ionising radiation. To ensure that mitozantrone in its non-radical form, or any metal contaminants associated with the batch used, did not react with the hydrogen peroxide produced during irradiation (as suggested by Gutteridge [195], Youngman [196], and Bates and Winterbourne [201]), it was decided to perform a control experiment.

Plasmid DNA was incubated with 4mM hydrogen peroxide in the dark at room temperature for a period of upto 1.5 hours with mitozantrone at a concentration of 0.01mM. No increase in DNA damage was detected, and it is concluded that in the system used, mitozantrone, in its non-free radical form, does not react with hydrogen peroxide to produce a product which is capable of inducing strand breaks in DNA.

The effect of mitozantrone under direct conditions.

Mitozantrone was irradiated at different concentrations with pBR322 DNA under ambient oxygen concentration at 77k with a dose of 3500Gy. The results are shown in Table 4.1 and Figure 4.3. The results show that with increasing concentration of mitozantrone there is a decrease in the number of double strand breaks observed, and a slight increase in the number of single strand breaks.

The effect was tested to see whether it was radiation dose dependent, as well as drug dose dependent. Aliquots of pBR322 DNA plus or minus 0.01mM mitozantrone was irradiated with

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CONCENTRATION	PERCENTAGE FORM III		PERCENTAGE FORM II	
(mM)	MEAN	S.D.$_{Mean}$ *	MEAN	S.D.$_{Mean}$ *
DNA ONLY	4.76	0.60	31.30	4.30
0.00001	3.97	3.23	28.63	7.50
0.0001	3.62	1.20	36.01	1.28
0.001	2.31	0.91	38.01	4.41
0.01	0.81	0.82	40.30	3.91

* Standard deviation of the mean of four separate results.

Table 4.1: The effect of mitozantrone on the gamma-radiation induced breakage of Form I pBR322 plasmid DNA. Plasmid DNA was irradiated at 77K under ambient oxygen concentration with a dose of 3500Gy.
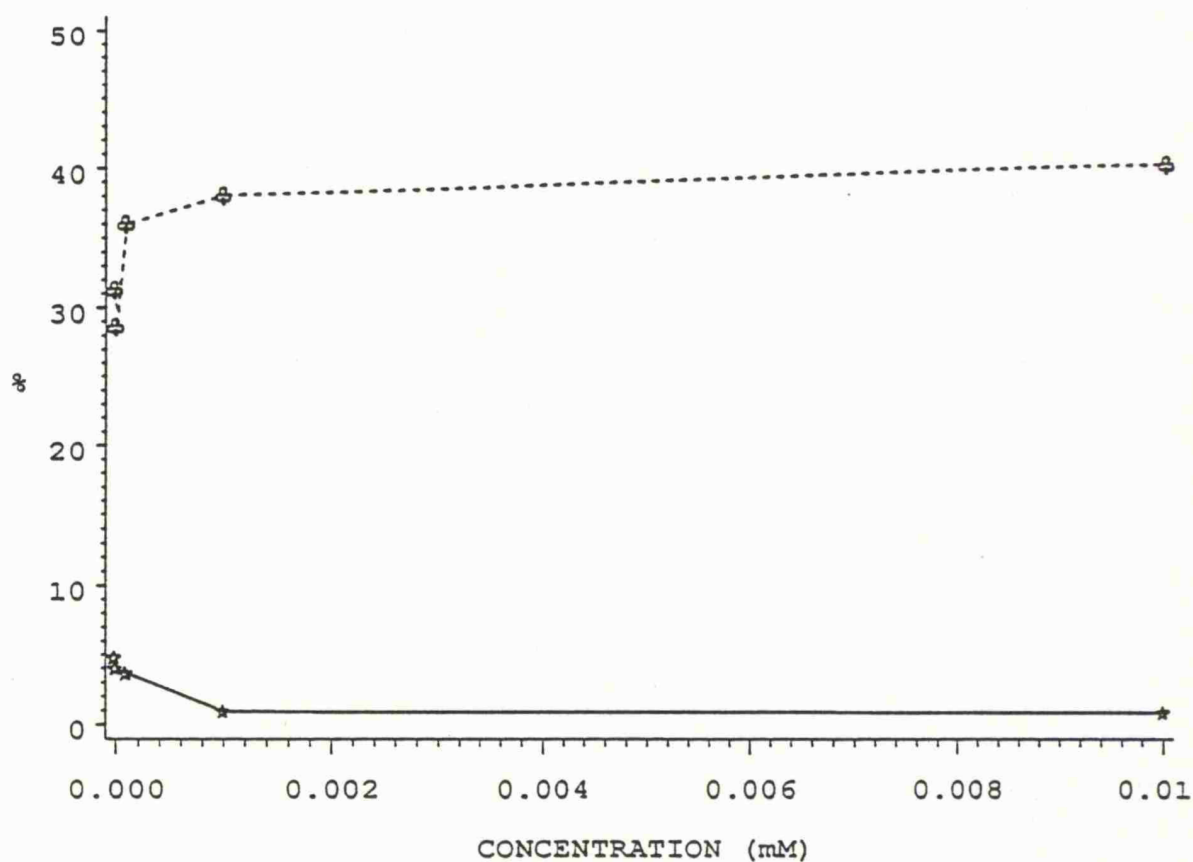
Figure 4.3: The effect of mitozantrone on the γ−radiation induced breakage of Form I pBR322 DNA. Plasmid was irradiated at 77K, under direct conditions and under ambient oxygen concentration, with a dose of 3500 Gy. Form II DNA (⊕-⊕-⊕). Form III DNA (★—★—★).

different radiation doses under ambient oxygen concentration. The results are shown in Table 4.2 and Figure 4.4. The results show that 0.01mM mitozantrone reduces the number of double strand breaks observed for each radiation dose, but increases the number of single strand breaks observed.

Wren [128], used pBR322 irradiated at 77k under ambient oxygen concentration with 1mM mitozantrone. He reported that at a concentration of 1mM mitozantrone is a potent sensitiser of single strand break damage, though he fails to mention double strand break damage. This sensitising effect was reduced by deoxygenating the DNA-containing solution, implying that the sensitising effect is an oxygen-related effect. Attempts by myself to repeat the deoxygenated result of Wren failed to find any decrease in the amount of radiation damage between "oxygenated" and "deoxygenated" solutions. This could be due to problems in removing oxygen from the solutions used, as discussed in Chapter 2.

E.S.R. studies have been undertaken by Wren and McClymont using calf thymus DNA [128, 203, 204]. Wren [128] showed that mitozantrone had no effect on the levels of $G^{\cdot+}$ observed in DNA irradiated under ambient oxygen concentration, but by a drug to base pair ratio of 1:75 all traces of $T^{\cdot-}$ were removed.

McClymont [203, 204] used mitozantrone in oxygenated, ambient, and "deoxygenated" DNA. He interestingly notes that however stringently the solutions used were degassed, even with "deoxygenated" solutions there were still $ROO^{\cdot}$ signals detectable from the irradiated DNA, indicating that the effects of oxygen are hard to remove from the systems used. He found

Figure 4.4: The effect of 0.01mM mitozantrone on the γ-radiation induced breakage of Form I pBR322 DNA irradiated under direct conditions at 77K and ambient oxygen concentration. DNA only: Form II DNA (★—★—★), Form III DNA (▽--▽—▽). DNA + 0.01mM mitozantrone: Form II DNA (⊕-⊕-⊕), Form III DNA (⊟—⊟—⊟).

RADIATION DOSE (kGY)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{\text{Mean}}$*	MEAN	S.D.$_{\text{Mean}}$*
0.00	0.00	0.00	0.00	0.00
1.75	1.75	0.24	17.25	2.11
3.50	3.01	0.39	30.81	4.92
5.25	5.21	0.91	43.75	2.15

*Standard deviation of the mean of two separate results.

Table 4.2a: The gamma-radiation induced breakage of Form I pBR322 plasmid DNA. Plasmid DNA was irradiated at 77K under ambient oxygen concentration.

RADIATION DOSE (kGY)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{\text{Mean}}$*	MEAN	S.D.$_{\text{Mean}}$*
0.00	0.00	0.00	0.00	0.00
1.75	0.14	0.22	22.52	3.42
3.50	0.72	0.34	43.42	5.22
5.25	1.00	0.23	54.03	4.71

*Standard deviation of the mean of two separate results.

Table 4.2b: The gamma-radiation induced breakage of Form I pBR322 plasmid DNA irradiated with 0.01mM mitozantrone. Plasmid DNA was irradiated at 77K under ambient oxygen concentration.

that with neither oxygenated, ambient, or deoxygenated, DNA
irradiated with mitozantrone was there any alteration in the
amount of $G^{\cdot+}$ formed, within the error of measurement by E.S.R..
The amount of $T^{\cdot-}$, and hence $TH^{\cdot}$, formed was reduced at a
concentration of about 1 drug molecule for every 110 base pairs
of DNA, about 50% of the original concentration of $T^{\cdot-}$ was seen.
A mitozantrone free radical ($Int^{\cdot-}$), thought to be that of the
semiquinone radical, was also seen. Some of the mitozantrone
free radical was observed to decay rapidly on warming the DNA
to 190K when oxygen was present. The remaining drug signal fell
off rapidly between 250K and 260K.

McClymont, using mitozantrone without DNA, found that the
radical anion of the drug was easy to form by irradiating the
drug in solution at 77K with gamma-radiation. The radical cation
was, however, hard to form, even using organic solvents commonly
used in radical cation work, such as freon
(fluorotrichloromethane) [58]. Thus implying that electron loss
from the mitozantrone molecule is unlikely to be involved.

The effect of ethidium bromide or doxorubicin under direct conditions.

Neither ethidium bromide or doxorubicin, up to a
concentration of 0.01mM, had any effect on the amount of
radiation damage observed with pBR322 DNA when irradiated at 77K
under ambient oxygen concentration. As with mitozantrone, DNA

incubated with the drugs stained linearly for the concentrations used.

The effect of mitozantrone under indirect conditions.

Mitozantrone was irradiated at a concentration of upto 0.1mM with pBR322 DNA under ambient oxygen concentration at room temperature with a dose of 100Gy. The results are shown in Table 4.3 and Figure 4.5. The results show that mitozantrone has a protective effect when irradiated with DNA at room temperature. The amount of protection, relative to concentration, plateauing with drug concentration.

DISCUSSION

The decrease in double strand breaks observed with mitozantrone on irradiating DNA under direct conditions is consistent with the scavenging of, or the prevention of the formation, of one or both of the primary radicals, $G^{\cdot+}$ and $T^{\cdot-}$. Wren [128] and McClymont [203, 204] found that the level of $G^{\cdot+}$ was unchanged, but the amount of $T^{\cdot-}$ and $TH^{\cdot}$ detected was decreased. This implies that the electrons produced by the interaction of gamma-radiation with the DNA are being scavenged by the mitozantrone molecules before they are stably trapped at thymine moieties. The original positive "holes", resulting from the initial point of electron loss, are still able to migrate
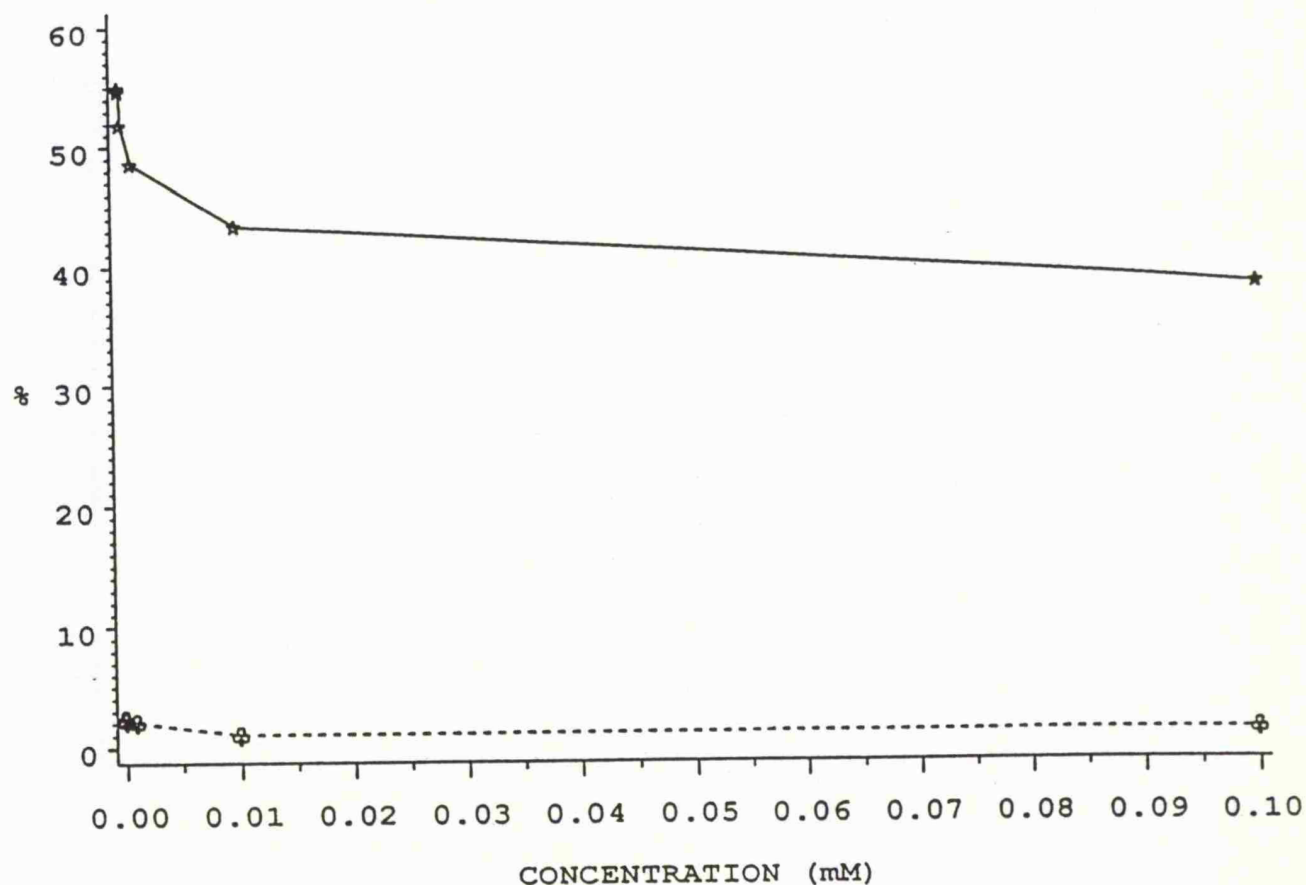
Figure 4.5: The effect of mitozantrone on the γ-radiation induced breakage of Form I pBR322 plasmid DNA. Plasmid DNA was irradiated at room temperature under ambient oxygen concentration. Form II DNA with a dose of 100Gy. (★—★—★), Form III DNA (⊕-⊕-⊕).

CONCENTRATION (mM)	PERCENTAGE FORM III		PERCENTAGE FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00000	2.34	0.62	54.96	5.49
0.00001	2.42	0.89	54.71	5.90
0.0001	2.23	0.92	51.87	6.13
0.001	2.10	0.30	48.59	1.89
0.01	1.01	0.49	43.38	5.44
0.1	1.23	0.39	38.33	4.88

*Standard deviation of the mean of three separate results.
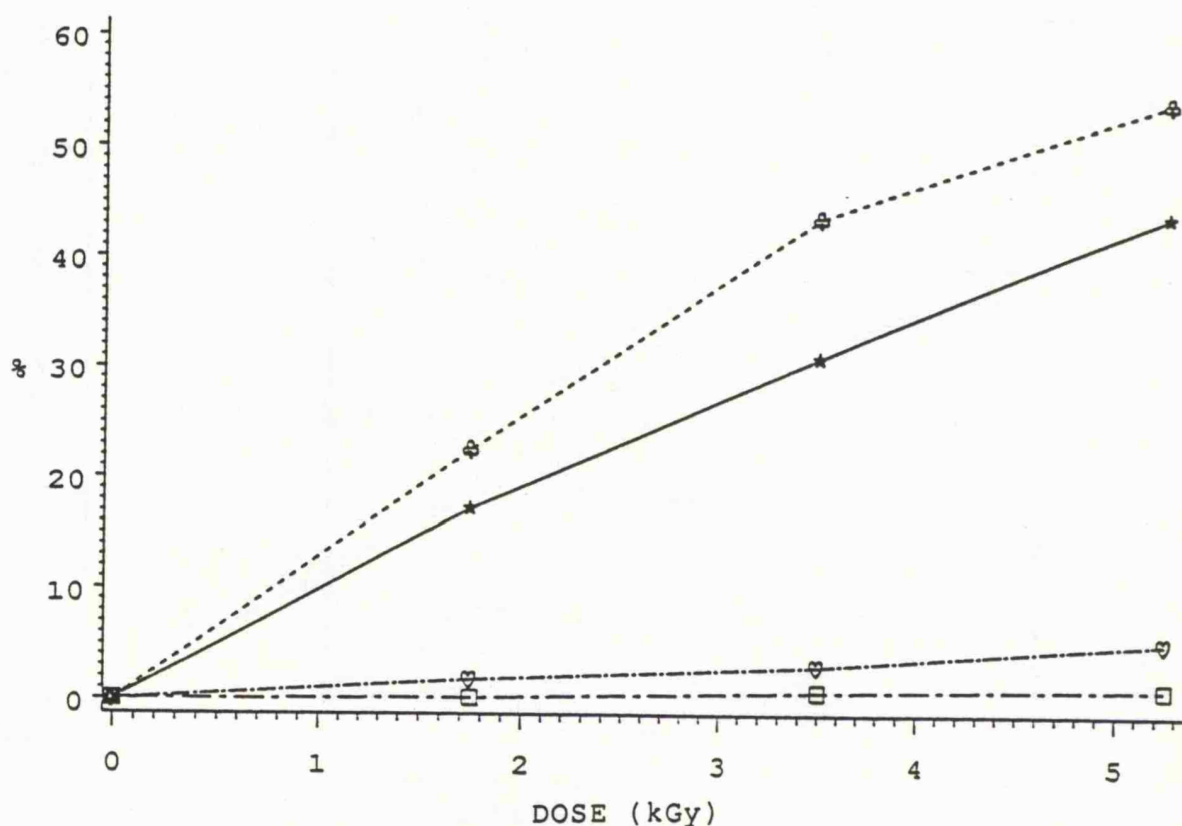

Table 4.3: The effect of mitozantrone on the γ-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated under indirect conditions at room temperature, under ambient oxygen concentrationwith a dose of 100Gy.

until they are trapped and stabilised at guanine residues. The scavenged electrons are originally trapped on the mitozantrone molecules, and have been detected ($Int^{\cdot-}$), and tentatively identified as forming semiquinone radicals. Mitozantrone is shown to be very efficient at scavenging electrons since the level at which a decrease in double strand breaks is detected is as low as a ratio of drug to base pair of 1:76. E.S.R. also shows that the scavenging is very efficient, with the amount of $T^{\cdot-}$ reported by McClymont [203, 204] to be reduced to 50% of the original levels by a drug to base pair ratio of ca. 1:110, and by Wren [128] to be reduced to zero by a ratio of 1:75.

If all of the $T^{\cdot-}$ is scavenged, a 50% reduction in the amount of single strand breaks would be expected, assuming that $G^{\cdot+}$ and $T^{\cdot-}$ are the only precursors of strand breaks in the system used. The concentrations of mitozantrone involved are too low for a target volume effect, similar to that observed for sodium chloride (Chapter 3), to be involved. The increase is more likely to involve the drug itself, and in particular the semiquinone radical, since electron loss from mitozantrone itself is unlikely.

McClymont [203] observed that the E.S.R. signal which was assigned to the mitozantrone semiquinone radical was rapidly oxidised on annealing to 190K in the present of oxygen. This infers that the mitozantrone free radical reacts with oxygen. Any remaining signal was rapidly lost between 240K and 260K. This second phase of signal loss was thought to be due to either; a second influx of oxygen (unlikely since the system was still frozen), release of the drug from the DNA hydration shell

due to unfavourable binding of the semiquinone, or local relaxations enabling neighbouring bases, especially thymine, to take the electron from the intercalator, protonate, and decompose to form strand breaks. The latter transfer of electrons from the drug molecules to the bases would be too rapid at this temperature to be captured by E.S.R..

Semiquinones react with oxygen to form superoxide radicals [180, 191]:-

$$Int^{\cdot -} + O_2 \rightarrow Int + O_2^{\cdot -} \qquad (4.4)$$
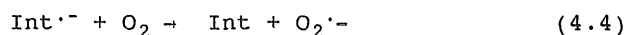
Oxygen will be present within the DNA helix itself, so the intercalator may not have to be released from the DNA to react with it. At neutral pH the superoxide radical can protonate to form a protonated superoxide radical:-

$$H^+ + O_2^{\cdot -} \rightleftharpoons HO_2^{\cdot} \qquad (4.5)$$

The protonated superoxide radical is energetic enough to react with DNA and form strand breaks, or to form hydrogen peroxide (reaction 4.1). At neutral pH the equilibrium lies over to the left of the equation, hence the amount of protonated superoxide radical is small.

The superoxide radical could reduce transition metal ions, which could then react with hydrogen peroxide produced from the radiolysis of water, to form hydroxyl radicals. Hydrogen peroxide can also be split into hydroxyl radicals by reacting directly with the semiquinone radical. The control experiment

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in which DNA was incubated with 4mM hydrogen peroxide shows that mitozantrone, under the conditions used, is unlikely to react directly the hydrogen peroxide produced by water radiolysis.

The semiquinone radical could abstract a proton from a base on the DNA, and the resulting base radical could then form a strand break. This, and the reaction of the semiquinone radical with hydrogen peroxide, are thought to be unimportant, owing to the apparent oxygen dependency of the sensitising effect, as observed by Wren [128].

Any damage to DNA which occurs due to oxygen is unlikely to be seen by E.S.R. under the conditions used by Wren or McClymont. The DNA containing the semiquinone radical has to be annealed to 190K before the intercalator signal is seen to decay. At this temperature, any superoxide or hydroxyl radicals are likely to react too rapidly to be seen by E.S.R.. The only way that these radicals might be detected is by spin trapping.

Doxorubicin is well known to form semiquinone radicals, so the lack of an effect was especially surprising. Perhaps freezing the doxorubicin-containing solution results in the drug being occluded from the DNA helix. Otherwise the intercalated doxorubicin molecule may trap electrons from the action of radiation on the DNA, to form a semiquinone radical, but on annealing the radical reacts with the DNA to form a strand break.

The rate at which a doxorubicin semiquinone radical could react with oxygen to form a superoxide radical, or with hydrogen peroxide, is likely to be lower than that for mitozantrone

because it has been shown to have a more positive one electron reduction potential (mitozantrone = -527mV, doxorubicin = -328mV, oxygen = -155mV [199]). However if doxorubicin semiquinone radicals were to be formed, and they then went on to react with compounds other than DNA, a reduction in at least double strand breaks would be expected to be seen.

E.S.R. of DNA irradiated with doxorubicin has yet to be undertaken. Should it be undertaken, it should be able to resolve whether or not doxorubicin does form a semiquinone radical and whether it reacts with oxygen in a similar fashion to mitozantrone.

The protective effect observed with mitozantrone irradiated with DNA under indirect conditions is likely to be due to hydroxyl radical scavenging. The protective effect plateaus with increasing drug dose. This is indicative of mitozantrone scavenging hydroxyl radicals which are formed in the water surrounding the DNA.

E.S.R. STUDIES

The E.S.R. results show that mitozantrone will efficiently trap electrons, resulting from the direct action of gamma-radiation, moving on the DNA helix. As shown above, the efficiency of the drug is such that 1 drug molecule per 760 base pairs can produce a detectable reduction in the number of double strand breaks. This agrees reasonably with the E.S.R. results which show that 50% scavenging of TH$^{\cdot -}$ occurs at ca. one intercalator molecule per 110 base pairs. This means that

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electrons traverse on average ca. 200 bases before becoming trapped. This shows that electrons which result from the direct action of gamma-radiation on DNA are relatively free to move.

The free electrons are thought to migrate through the overlapping π-electrons of the DNA bases. On reaching thymine, there would appear to be a relatively low but finite probability of capture, so that many thymine bases are traversed before capture ensues. This shows that there would appear to be competition between a mode where the electron is captured, and a mode where the electron is allowed to transfer. The differences in modes may be due to a shape change, or due to partial or complete proton migration with one of the hydrogen bond linking thymine to adenine.

The E.S.R. results as they stand cannot, unfortunately, explain the increase in single strand breaks observed using the plasmid-based assay, for reasons discussed above. It is hoped that further studies, using for example spin trapping, may be able to clarify this apparent discrepancy in results.

CHAPTER 5

THE EFFECT OF NET CHARGE ON THE

EFFICACY OF THIOLS

INTRODUCTION

Thiols, and particularly aminothiols, are among the most effective radioprotectors found to date, both *in vivo* and *in vitro*. Patt and coworkers reported in 1949 [205] that the naturally occurring amino acid, cysteine, protects rats against killing by x-irradiation. The disulphide of cysteine, cystine, did not have an effect. Further work during the 1950's showed that cysteamine (β-mercaptoethylamine, MEA) was also an effective protector against x-irradiation in systems as diverse as mice, insects, bacteria and barley [206]. Cysteamine is a component of coenzyme A, but no decrease in CoA levels were found after irradiation, implying that MEA was not incorporated into CoA, but was instead directly involved in radioprotection. Studies showed that the amino group and the free-thiol group were required for maximal protection. Since then researchers have produced numerous aminothiol radioprotectors with varying radioprotecting capacity. They have also found that cells contain a naturally occurring thiol, glutathione, γ-L-glutamyl-L-cysteinylglycine, GSH. Cells deficient in this compound have been found to have increased radiosensitivity [207-209]. The thiols used within the current study are shown in Figure 5.1.

MERCAPTOETHANOL $HOCH_2CH_2SH$

CYSTEAMINE $NH_3^+CH_2CH_2SH$ $.Cl^-$

WR-1065 $NH_3^+CH_2CH_2CH_2NH_2^+CH_2CH_2SH$ $.2Cl^-$

RW-222 $NH_3^+CH_2CH_2CH_2NH^+(CH_3)CH_2CH_2CH_2NH_2^+CH_2CH_2SH$ $.3Cl^-$

NORSPERMIDINE $NH_3^+CH_2CH_2CH_2NH^+(CH_3)CH_2CH_2CH_2NH_2^+CH_2CH_3$ $.3Cl^-$

Figure 5.1: The compounds used within this study.

Thiols undergo a variety of reactions with free radicals. Hydroxyl radicals can abstract hydrogen atoms from target molecules [207, 210, 211]:

$$XH + \cdot OH \rightarrow X\cdot + H_2O \qquad (5.1)$$
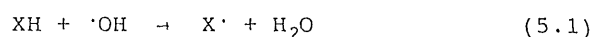
Free-thiols (RSH) can react with the target free radical and undergo hydrogen transfer (metathesis reaction) with it. For example, the repair of thymine radicals produced by hydroxyl radicals by cysteine, and the repair of sugar radicals from dCMP irradiated in solution with γ-radiation, by cysteamine, have been observed [210-212]:

$$X\cdot + RSH \rightarrow XH + RS\cdot \qquad (5.2)$$
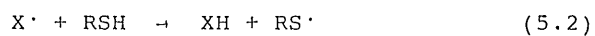
Alternatively the hydroxyl radical may add to the target molecule and the free-thiol may then react with the radical to form a product which may still be lethal [207,213]:

$$X + \cdot OH \rightarrow XOH\cdot \qquad (5.3)$$
$$XOH\cdot + RSH \rightarrow XOH_2 + RS\cdot \qquad (5.4)$$
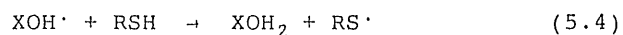
Free-thiols can also scavenge hydroxyl radicals:

$$RSH + \cdot OH \rightarrow RS\cdot + H_2O \qquad (5.5)$$

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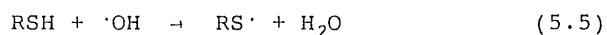
Radicals formed by hydrogen addition can react with free-thiol to form a dihydro-derivative which may still be a lethal product:

$$XH\cdot + RSH \rightarrow XH_2 + RS\cdot \qquad (5.6)$$
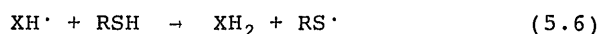
The hydrogen atom comes from the S-H bond which is substantially weaker than C-H bonds. The thiyl radical produced on reacting with hydroxyl radicals or with the target radical may react with other free-thiol to produce a radical complex:

$$RS\cdot + RSH \rightarrow RSSR\cdot^- + H^+ \qquad (5.7)$$
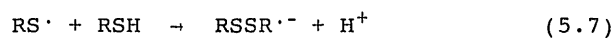
Oxygen inhibits repair by competing with free-thiol for target free radicals. For example:

$$X\cdot + O_2 \rightarrow XO_2\cdot \rightarrow \rightarrow damage \qquad (5.8)$$
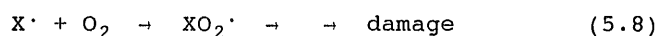
Oxygen will also react with thiyl radicals in a reaction which is complex in nature, but probably involves initial addition [214]:

$$RS\cdot + O_2 \rightarrow RSOO\cdot \qquad (5.9)$$

Free-thiol will itself react with oxygen and remove it from a system, to form hydrogen peroxide. The hydrogen peroxide produced may form hydroxyl radicals, a reaction which requires transition metal ions in a Fenton-type reaction [215-218]:

$$2RSH + O_2 \rightarrow RSSR + H_2O_2 \qquad\qquad (5.10)$$

$$H_2O_2 + M^{n+} \rightarrow M^{(n+1)+} + \cdot OH + OH^- \qquad (5.11)$$
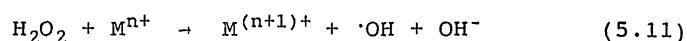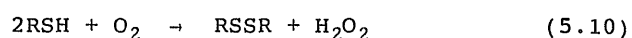
The disulphide can react with target radicals [220]:

$$RSSR + X\cdot \rightarrow XSR + RS\cdot \qquad\qquad (5.12)$$

Disulphide will also react with hydroxyl radicals, but the exact mechanism is still uncertain [68].

In summary, there are three basic mechanisms by which aminothiols may protect a compound. These are; the scavenging of water radiolysis products, hydrogen donation, and the induction of hypoxia in a system.

Symons and coworkers [221] studied the effect of low concentrations of water soluble thiols on the amount of direct damage to DNA by γ-radiation. Glutathione, cysteine, cysteamine and dithiothreitol (DTT) were irradiated at 77K with calf thymus DNA, and the radicals produced were recorded using E.S.R.. DNA strand breaks were studied using the pBR322 plasmid assay used in the current study. Under anoxic conditions there was no change in the initial yield of $G^{\cdot+}$ and $T^{\cdot-}$ as judged by E.S.R.. On annealing to ca. 200K the number of DNA radicals were observed to decrease, with a concomitant growth of an E.S.R. signal characteristic of the $RS-S^{\cdot}R^-$ radical anion. The radical anion did not appear to be able to react with DNA. At a ratio of about 1 thiol molecule to 25 base pairs about 50% of DNA radicals were lost. Strand break analysis with 5mM and 40mM

cysteamine under anoxic conditions showed marked reductions in both single strand breaks and double strand breaks. It was concluded that under anoxic conditions free-thiols react with DNA radicals by hydrogen atom donation to form thiyl radicals. The thiyl radicals then rapidly react with free-thiol to form the radical anion observed.

The results observed under oxic conditions were less definitive. Free-thiol was observed to react with DNA peroxy radicals resulting in a loss of DNA radicals at ca. 200K. The thiyl radicals produced are able to react with oxygen to form $RSO_2\cdot$ (Reaction 5.9) which is probably able to react with DNA. Strand break studies showed that the amount of protection observed under oxic conditions compared with anoxic conditions was reduced at a concentration of cysteamine of 5mM, but at 40mM was largely unaffected by the presence of oxygen. The reaction scheme proposed is shown in figure 2.

Hydrogen atom donation by free-thiol to the base peroxy radicals initially occurs to give the thiyl radicals. These react with oxygen to form $RSO_2\cdot$ rather than $RS\text{-}S\cdot R^-$. As the concentration of free-thiol is increased pathway (b) begins to dominate, oxygen is consumed, and DNA is protected. Reaction to give the $RS\text{-}S\cdot R^-$, pathway (a), will also occur as the concentration of free-thiol increases. At low concentrations the thiol is effectively consumed. Pathway (c), in which the $RSO_2\cdot$ radical abstracts a proton from a base which then reacts with oxygen, accounts for the attenuation of the protection of free-thiols at low concentrations.

DNA + γ ———→R'·
                    |O$_2$
                    ↓
          R'O$_2$·———→DAMAGE
                    |RSH
                    ↓   O$_2$ (c) R"H
      R'O$_2$H + RS·————————→RSO$_2$·————————————→RSO$_2$H + R"·
              (a)|RSH   (b)|RSH |O$_2$
                 ↓   ↓   ↓
            RS–S·R⁻   RS· + RSO$_2$H   R"O$_2$·
                                                     |
                                                     ↓
                                                  DAMAGE
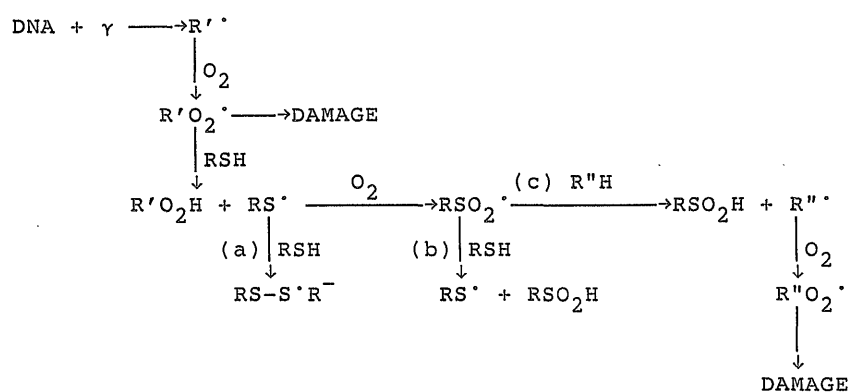
Where:

R'·, R"·= Deprotonated base

R'O$_2$·, R"O$_2$·= Base peroxy radical

RSH = Thiol

Figure 5.2: Scheme proposed for the effect of oxygen on
radioprotection by thiols. (From 22*l*)

Under anoxic conditions TH· is thought to react with free-thiol to give TH_2. The effect of free-thiol on $G^{·+}$ is less clear, but thought to include donation of a hydrogen atom.

The interpretation of the above results does not take into account any "salt-type" effect, of the type discussed in Chapter 3, on adding increasing concentrations of free-thiol. Changing the concentration of cysteamine from 5mM to 40mM with the strand break assay will almost certainly have a sensitising effect, based on the "target size" of the DNA and its hydration shell. This would mean that pathways (a) and (b) may be of more importance than originally thought in the paper by Symons et al. [221].

Much of the early work on radioprotection by thiols concentrated on the naturally occurring thiols, such as cysteamine and cysteine. During 1959, however, the United States Army initiated its Antiradiation Drug Development Program which resulted in the eventual testing of over 4000 potential radioprotective compounds [222]. This work was declassified in 1965 and terminated in 1988. The most significant development was the synthesis and early testing of phosphothioate compounds, the best of which proved to be the compound designated with the Walter Reed number WR-2721, and its free-thiol derivative WR-1065.

WR-2721, S-2-(3-aminopropylamino)ethyl phosphothioic acid, is dephosphorylated in cells by phosphatases to produce WR-1065, S-[(aminopropyl)amino]ethanethiol. Phosphorylation of the free-thiol results in a lower toxicity of the drug. The phosphothioate derivative of cysteamine, WR-638, has been shown

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to have a higher LD_{50} than cysteamine, and hence is able to be used at a higher dose [223].

There have been numerous studies on the effects of aminothiols on single strand breaks (ssb), double strand breaks (dsb) and cell killing in mammalian systems. Much of this work has been under taken by Murray, Meyn and coworkers using various aminothiol derivatives including cysteamine, WR-2721, WR-1065, and the N-methyl derivative of WR-1065, WR-255591, and its phosphothioate derivative WR-3689. They used alkaline filter elution and neutral filter elution to measure ssb and dsb in jejunum cells from mice killed immediately after irradiation [224, 225], and Chinese Hamster Ovary (CHO) cells [215, 226-229]. The aminothiols used were observed to decrease the levels of ssb and dsb observed to form on irradiation. Additionally sister chromatid exchanges in bone marrow cells irradiated <u>in</u> <u>vivo</u> with cysteamine, and chromosome aberrations in mice with WR-2721, which are indicative of double strand breaks, have been observed by other groups to be prevented [230, 231].

Murray and coworkers used a clonogenic assay to study cell killing with CHO cells, and compared the protection for ssb in jejunum cells versus survival in mice. They found that the protection factor observed for ssb and dsb was lower than that for cell survival [225-228, 232]. Though ssb are not necessarily lethal, the effects of the drug on the levels of ssb would expect to mirror the levels of protection of lethal damage if the drug protected all forms of damage equally. Dsb induction usually is measured at a dose range of about 20 to 90 Gy. More recent work found that if dsb levels were calculated using cells

irradiated at the same radiation dose as that used in the survival studies, 3 to 30 Gy, the protection factors obtained were closer. This appears to imply that the higher doses conventionally used to measure dsb do not necessarily extrapolate to lower doses. At the higher doses the chemical repair and fixation processes by, for example, exogenous and endogenous thiols, may become saturated. Enhanced oxidation of the aminothiol or generation of $RS^{\cdot}/RSO_2^{\cdot}$ after higher γ-ray doses may also be contributory factors.

Radford [233] found that cysteamine differentially protected against the induction of lesions in mammalian cells in the order: dsb > ssb $\geq$ DNA-protein crosslinks > γ-endonuclease sensitive sites. This fits with the finding by Murray and coworkers that WR-1065 and WR-255591 produced higher protection factors for DSB than for SSB [227, 228].

The aminothiols would appear to alter the spectrum of damage produced by the action of radiation on DNA. Schulte-Frohlinde [234, 235] reports that different repair mutants of E. coli are irradiated, the differences in protection factor obtained with cysteamine cannot be explained by simple radical scavenging. It was concluded that hydrogen donation does not necessarily lead to the original DNA structure, but one which is more easily repaired. This is borne out by mutagenesis studies on the hypoxanthine-guanine phophoribose transferase (HGPRT) locus in V79 cells [222, 236-238].

WR-1065 at a concentration of 4mM will reduce the number of HGPRT mutants by a factor of 5 when present during irradiation. It will also reduce the number of mutants by a

factor of 2 if added immediately after irradiation [222], though it was noted that WR-1065 had to be present during irradiation to prevent cell killing [238]. The amount of protection against mutation observed was similar whether the cells were irradiated under hypoxic or aerated conditions. This implies that protection by WR-1065 is not simply due to its ability to scavenge radiation-induced free radicals, but rather that it may also mediate these effects through the scavenging of metabolically produced free radicals. The actual spectrum of mutations, with cysteamine, with respect to point mutations, gene deletions, or partial deletions, was noted to change [237]. This implies that cysteamine may selectively protect against large scale molecular changes.

Part of the action of aminothiols could be the activation of DNA repair processes. Cysteamine and WR1065 have been reported as inhibitors of ssb repair [215, 225, 232]. WR-1065 has no effect on the rate of dsb repair [225]. Cysteamine has also been reported as an inhibitor of normal DNA synthesis [210, 215], and to delay cell cycle progression [239]. Delay of cell cycle progression would allow repair of damage to take place before replication, and hence damage fixation and mutation, to take place. This is called the biochemical shock theory. The aminothiols will also probably protect the repair enzymes themselves, resulting in there being more likelihood of damage being repaired quickly.

Aminothiols have been observed to have an effect on other aspects of cellular metabolism. Incubating cysteamine with rat or other mammalian cells, for example, has been observed to

result in altered NADH/NAD$^+$ levels, an increase in the levels of exogenous serotonin, an efficient radioprotector, and increased levels of endogenous thiols [210]. Cysteamine and other thiols, such as aminoethylisothiourea (AET), have been observed to produce an increase in the concentration of cyclic AMP (cAMP), a secondary messenger molecule of cells [240]. The rate of cAMP-dependent protein phosphorylation has also been observed to increase. cAMP is involved in the control of several metabolic processes, including the biosynthesis of nucleic acids and proteins, and thiol disulphide and carbohydrate metabolism.

The most protection seen in E.coli with cysteamine [241, 242], and metabolising barley seeds [243] and mouse skin by WR-2721 [244] has been found to be under oxic rather than anoxic conditions. The amount of protection by WR-2721 has been shown to be dependent on the amount of oxygen in the system. Protection in skin and lung tissues is most efficient at intermediate levels of oxygen, and falls off in fully oxic and anoxic conditions [245]. Durand [246] and Purdie [247] postulated that WR-2721 at least partially protects cells _in vivo_ by oxygen depletion.

Purdie and coworkers [247] used an isothermal micro-calorimeter with cultured mammalian cells. When WR-2721 was added there was heat produced as the WR-2721 was rapidly dephosphorylated to produce WR-1065, which in turn reacted with oxygen to form disulphide. Using a biological oxygen monitor it was seen that WR-1065 and cysteamine rapidly consumed all oxygen in a culture medium, with 10mM WR-1065, within 2 minutes. With the rapid consumption of oxygen, it was concluded that free-

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thiols do not coexist for long with oxygen in cells. At modest doses of aminothiol, the free-thiol will be rapidly consumed in the presence of oxygen.

The oxygen tension in a cell is important with respect to the amount of damage expected from radiation. The rate at which DNA radicals react with oxygen is about two hundred times greater than the rate at which they react with free-thiol [207]. At high oxygen concentrations oxygen may react with free-thiol and still be at a high enough concentration to dominate in any reactions with DNA radicals. In hypoxic cells there will be few peroxy radicals formed, and hence the DNA will be easier to repair. Within any cell, however, there will be a critical oxygen tension where the addition of free-thiol will remove sufficient oxygen for there to be an effect on cell survival [245, 248]. Microelectrode studies have shown that the amount of oxygen is heterogenous in every tissue studied, presumably reflecting the distance of cells from the nearest capillary, and varies from tissue to tissue. Within tumours the amount of oxygen depends on the size and the amount of vascularisation of the tumour. Large tumours with poor blood supply tend to have low concentrations of oxygen, and may therefore be protected to a lesser extent by aminothiols than normal tissue.

The amount of radiation protection given be aminothiols varies from tissue to tissue, and tumour to tumour. Several factors could account for this: oxygen concentration, as discussed above, vascularisation, differing abilities of cells to uptake the drug, and differing phosphatase activity to remove the terminal phosphate from phosphothioates [222, 228, 249-251].

Aminothiols have been observed, for example, to afford little protection to the central nervous system due to the problem of getting the drug across the blood/brain barrier. Larger tumours with poor blood supply tend to be less well protected than those with a better blood supply.

The active uptake of the drug will be factor in the amount of protection observed. If a liver cube is incubated at 4°C with WR-2721 only diffusion of the drug into the cells is seen. If the liver is incubated with WR-2721 at 37°C, the drug is actively concentrated in cells to a concentration above the level in the medium. MCa-11 tumours, however, do not actively uptake WR-2721 [250].

The method of uptake of WR-2721 by liver is uncertain, since it is not inhibited by KCN, sodium azide, or oubain, and therefore does not appear to be a true active system [250]. A wide range of mammalian cells have been shown to possess polyamine uptake systems [252]. The exact reason for possessing these systems is uncertain since enzymes for the production of polyamine are ubiquitous. Polyamines, such as putrescine, spermine and spermidine are involved in numerous functions including the stabilising of nucleic acids, the stimulation of synthesis of certain proteins, and the modulation of protein kinases and phosphatases [253]. Difluoromethylornithine (DFMO) blocks polyamine synthesis, and results in an increase in the rate of polyamine uptake. There would appear to be multiple systems involved in polyamine uptake, some of which are Na^+-dependent, which are controlled by different hormonal and environmental factors. Cohen and coworkers [254] looked at the

effect of various compounds on the uptake of putrescine or spermidine by Ehrlich ascite tumour cells. These included the compound RW-222, N^1-mercaptoethyl-N^4-methylnorspermidine, Figure 5.1, which was shown to inhibit spermidine uptake, and was therefore concluded to be uptaken by cells. The active uptake of at least some of the aminothiols would appear to involve polyamine transport systems.

It has been noted that there are differences between the amount of radioprotection observed by different compounds. Transforming DNA from Bacillus subtilis, for example, was γ-irradiated under oxic and anoxic conditions with various compounds, including the naturally occurring cellular thiol glutathione (GSH) [211]. The amount of damage observed was GSH > dithiothreitol $\approx$ cysteine > cysteamine. GSH has a net charge of -1, dithiothreitol and cysteine have no net charge, and cysteamine a net charge of +1.

Zheng and coworkers [255] measured free base release from calf thymus DNA in γ-irradiated, aerated, solution. They compared the amount of damage from mercaptosuccinic acid (with a net charge of -2), GSH (-1), dithiothreitol (0), mercaptoethanol (0), cysteamine (+1), and WR-1065 (+2). The amount of damage observed for free-thiols without any net charge was equal to that expected for free-thiols. The order of damage, based on net charge of the compounds used was:

$$-2 > -1 > 0 > +1 > +2$$

Studies have shown that the rate at which thiols react with hydroxyl radicals is not charge dependent, and is essentially

diffusion controlled, the rate being around 1×10^{10} $M^{-1}sec^{-1}$
[211].

Manning [256, 257] developed theoretical analysis of the
behaviour of counterions in the vicinity of cylindrical
polyelectrolyte molecules. A fraction of counterions condense
in a delocalised manner around the polyelectrolyte, the degree
of condensation being dependent on chain length. This accounts
for the increase in conductivity associated with the formation
of strand breaks, which are attributed to the release of
counterions. Monte Carlo simulations for cylindrical
polyelectrolytes with parameters appropriate to DNA have shown
that monovalent counterions can be condensed to concentrations
at the surface of the polyanion of greater than 1M. This is two
orders of magnitude greater than that found at 10nm or greater
from the surface of the polyanion.

Smoluk and coworkers [269] studied the binding of thiols
to sonicated calf thymus DNA. DNA was incubated with thiol in
Tris buffer. The DNA was then concentrated using a Centricon
spin filter, and the amount of thiol in the DNA retentate was
compared with that in the DNA-free filtrate. The ratio of thiol
in the retentate to that in the filtrate was calculated. A ratio
below unity showed that the thiol was being repelled from the
DNA. A figure above unity showed that the thiol is attracted to
the DNA. Binding was shown to be in the following order: WR-1065
(+2) > cysteamine (+1) > mercaptoethanol (0) > mercaptoethane-
sulphonic acid (-1). Mercaptoethanol produced a ratio of
approximately unity, indicating that it did not bind to DNA. The

relationship between the net charge on the thiol and activity is known as the counterion condensation theory.

The rate of chemical repair of pBR322 DNA has been shown to be charge dependent [259]. Plasmid was placed as a thin layer onto filter disks, equilibrated with thiol at pH 7, and irradiated with a 5 ns pulse of electrons under hypoxic conditions. After a preset time delay oxygen was added to make the system oxic. The amount of Form I DNA which changed into Form II DNA was scored by HPLC, and the data plotted as percentage Form I versus time delay for the oxygen addition. The differences in the rates of repair is consistent with perturbations in the local concentration of thiols around the DNA. The rates of repair observed were consistent with the counterion condensation theory:

WR-1065 > cysteamine > 2-mercaptoethanol > GSH

The aim of the work discussed in the current chapter is to test the counterion condensation theory using thiols with a range of net charges from zero to +3 using the pBR322 plasmid strand break assay. The effect of the novel compound RW-222 on ssb, as measured by alkaline filter elution, is also demonstrated.

<u>METHODS</u>

Figure 1 shows the compounds used throughout the current set of experiments. At first sight it would appear that ethanethiol (CH_3CH_2SH) would have been a logical choice of thiol without any net charge to use. Ethanethiol has a strong stench, so for "environmental" reasons 2-mercaptoethanol which has a "slightly more pleasant stench" was used.

Mercaptoethanol and cysteamine (β-mercaptoethylamine) were obtained from Sigma Chemical Co. Ltd. WR-1065 (S-[(aminopropyl)-amino]ethanethiol), RW-222 (N^1-mercaptoethyl-N^4-methyl-norspermidine) and N^4-methylnorspermidine (3,3'diamino-N-methyl-dipropylamine, called for simplicity in the current work norspermidine), were synthesised by Dr. Richard Wheelhouse [260]. All compounds were stored under nitrogen, and, with the exception of mercaptoethanol which is liquid at room temperature, were stored at $-20°C$ to prevent oxidation of the compound.

All of the compounds were tested for the amount of free-thiol that they contained using Ellmans reagent, as described in Chapter 2. This proved to be important, since the amount of free-thiol available varied from batch to batch. The proportions of free-thiol in the batches of thiol used are shown in Table 5.1. The amount of thiol was adjusted accordingly to ensure that the same amount of free-thiol was used was constant from compound to compound. All concentrations quoted throughout this study are therefore for the amount of <u>free-thiol</u>.

COMPOUND	PROPORTION FREE-THIOL
MERCAPTOETHANOL	0.851
CYSTEAMINE	0.825
WR-1065	0.781
RW-222	0.810

Table 5.1: The proportion of free-thiol in the aminothiols used. The amount of free thiol was calculated using Ellmans reagent as described in Chapter 2.

Mercaptoethanol for use in the plasmid assay was made up as a stock solution of 60mM in deoxygenated TE buffer and stored at 77K. This was used up within 48 hours. All other compounds were freshly made up as stock solutions of 60mM in deoxygenated TE buffer immediately prior to use.

Plasmid DNA was used at a concentration of 0.05mg/ml, 0.076mM with respect to base pairs, as described in Chapter 2. Irradiations were carried out under ambient oxygen concentration at either 77K or room temperature. Any increase in the amount of damage due the drug itself was taken into account by comparing the results obtained on irradiation with a non-irradiated control sample, as discussed in Chapter 2.

WR-1065 or RW-222 were made up in prewarmed growth medium and incubated at 37°C with V79 cells for 45 minutes prior to irradiation. This compares with a 30 minute incubation which Murray and coworkers used for WR-1065 [227]. The longer incubation time was used because, at the time that the experiments were undertaken, it was uncertain how quickly the larger RW-222 molecule would be uptaken. Irradiation and alkaline filter elution were carried out as described in Chapter 2.

RESULTS

PLASMID ASSAY

Controls

The total amount of DNA observed on staining with ethidium
bromide remained constant for the concentrations drug used
(between 0 and 10mM). This would appear to show that the
addition of the drug did not effect the amount of fluorescence
observed on staining with ethidium bromide.

Stewart [261] reports that the binding of polyamines
reduces the amount of fluorescence observed upon the binding of
ethidium bromide to DNA. However, in my system, the volume of
the plasmid-drug containing solution was typically 20μl, which
was loaded onto an agarose gel in ca. 1l TBE buffer. After
undergoing electrophoresis, the agarose gels were stained in ca.
1l in 2.5μg/ml ethidium bromide. Hence the final concentration
of the drug left next to the DNA, even with the binding of the
drugs to DNA, would have been negligible, when compared with the
concentration of ethidium bromide used for staining.

The effect of incubating the thiols and norspermidine at
room temperature for 40 minutes under ambient oxygen
concentration with pBR322 DNA is shown in Table 5.2 and Figure
5.3. The results show that incubating the drugs with plasmid DNA
under ambient oxygen concentration damages the DNA. The amount
of damage observed on incubating with mercaptoethanol,
cysteamine and WR-1065 increases with increasing concentration

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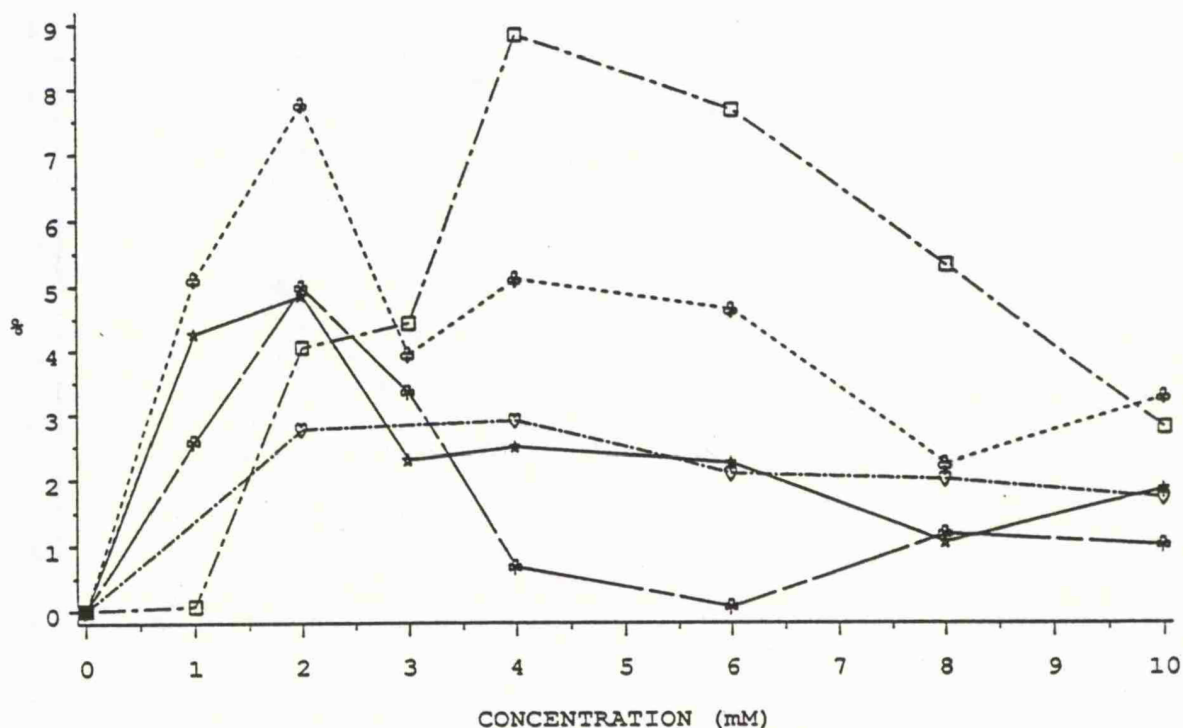
Figure 5.3: The production of Form II pBR322 plasmid DNA from Form I plasmid DNA by mercaptoethanol (⊕-⊕-⊕), cysteamine (★-★-★), WR-1065 (⊕-⊕-⊕), RW-222 (⊟-⊟-⊟), and norspermidine (▽··▽-▽). The compounds were incubated with DNA for 40 minutes, under ambient oxygen concentration, at room temperature, under fluorescent light.

CONC. (mM)	MERCAPTOETHANOL		CYSTEAMINE		WR-1065		RW-222		NORSPERMIDINE	
	MEAN	S.D.*	MEAN	S.D.*	MEAN	S.D.*	MEAN	S.D.*	MEAN	S.D.*
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	5.11	0.94	4.26	2.90	2.57	5.41	0.07	0.09	2.76	0.77
2	7.75	3.84	4.85	2.60	4.97	3.56	4.04	1.31		
3	3.93	0.48	2.29	2.75	3.34	3.18	4.41	2.10		
4	5.09	3.30	2.48	3.65	0.67	4.21	8.82	3.41	2.89	0.95
6	4.61	2.15	2.24	3.33	0.05	2.24	7.65	0.86	2.08	1.35
8	2.22	2.22	1.04	2.79	1.17	2.00	5.29	1.68	1.99	1.97
10	3.26	3.26	1.85	1.49	1.00	2.44	2.78	0.33	1.71	1.64

* standard deviation of the mean of three separate results.

Table 5.2: The formation of Form II pBR322 DNA by incubating with mercaptoethanol, cysteamine, WR-1065, RW-222, and norspermidine, at room temperature and under ambient oxygen concentration, for 40 minutes. The DNA was incubated and underwent agarose gel electrophoresis under fluorescent light in the laboratory.

to peak at ca. 2mM, and then decreases with increasing concentration. RW-222 has a similar shape, except that the peak amount of damage occurs at ca. 4mM, and the decrease after 4mM would appear to be the most marked of the free-thiols used.

Cysteamine has been noted to be toxic to tissue culture cells at low concentrations (0.2mM - 5mM), but at higher concentrations of above 5mM the toxicity is reduced [226, 236]. No toxic effect was observed with cystamine. Human leucocytes were incubated with cysteamine at 37°C for 40 minutes by Kanabus-Kaminska and coworkers [217], and the level of strand breaks were measured by alkaline unwinding. The amount of damage was observed to increase upto a maximum of 1-2mM, and then decrease. The damage was observed to be prevented by the addition of catalase. The use of a Clark electrode to monitor the concentration of oxygen in the system revealed that oxygen was consumed on the addition of cysteamine.

The authors of these papers concluded that the initial toxic effect was probably due to the oxidation of free-thiol to produce hydrogen peroxide and disulphide (Reaction 5.10), though the precise reaction is uncertain. As the concentration of free-thiol increases, hydrogen peroxide is scavenged by the free-thiol, and probably by the disulphide produced on oxidation, hence the amount of damage was observed to decrease. The same reaction scheme would account for the peak in the amount of damage observed with the thiols used in the current investigation, and the subsequent tailing off in the amount of damage observed. Addition of catalase, which is known to inhibit

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the reaction [216, 217].Unfortunately this is clouded by the results obtained by incubating plasmid DNA with norspermidine.

When pBR322 was incubated with norspermidine a modest increase in damage was observed between 0 and 2mM. The extent of the damage was less than that seen for the thiols under the same conditions, and lacks the rapid tailing off in the amount of damage seen. The total amount of DNA observed did not vary with the amount of norspermidine in the system, hence the effect would not appear to be an artifact of using ethidium bromide to stain the DNA. One possibility for the result is that the norspermidine is reacting with the DNA.

Saito and coworkers [262-265] have reported that primary alkylamines exhibit extraordinary high reactivity towards thymidine in DNA, especially above neutral pH. If, for example, spermidine is incubated with DNA under a germicidal lamp emitting a peak wavelength at about 254nm and then undergoes mild heating, below the melting temperature of the DNA, the DNA exhibits thymidine specific cleavage. In the system currently investigated, the plasmid DNA was incubated under the fluorescent lamps of the laboratory which emit a broad spectrum of light, including ultra-violet light. This may have resulted in primary amine of the norspermidine reacting with the DNA. Form I supercoiled plasmid DNA is under torsional stress, which is probably increased as the plasmid undergoes agarose gel electrophoresis, which may have been enough to break the DNA without the mild heating reported by Saito and coworkers. The aminothiols used may also have reacted with the DNA in the same way. Had time been available this could have been tested by

carrying out all manipulations as far as possible in darkness. The use of WR-255591, where the primary amine of WR-1065 has a methyl group added, and comparing it with WR-1065, would also test this. The scheme would not of course account for the damage observed to occur with mercaptoethanol, which lacks an amine group.

The effect of thiols on radiation-induced damage to plasmid DNA irradiated under direct conditions.

Plasmid DNA was irradiated under direct conditions at 77K with 6mM thiol or norspermidine. The results are shown in Table 5.3(a-f), and in Figure 5.4. Figure 5.5 shows a semilogarithmic plot of the amount of Form I plasmid DNA remaining after irradiation with 6mM drug, and Table 5.4 shows the linear regression analysis of the plot, and the additive effect ratio (A.E.R.) calculated from the slope. An A.E.R. of less than unity shows that a compound has protected the plasmid DNA against radiation-induced strand breaks. An A.E.R. of greater than unity shows that a compound has sensitised the plasmid DNA.

The A.E.R. shows that the amount of damage observed in plasmid DNA irradiated with 6mM drug is: RW-222 > norspermidine > DNA only > cysteamine > WR-1065 > mercaptoethanol. The protection of plasmid DNA by free-thiols was expected, since it was shown by Symons and coworkers [221] that 5mM and 40mM cysteamine protect plasmid DNA irradiated under similar direct conditions. The result showing that 6mM cysteamine does indeed protect plasmid DNA would appear to fit with those results, and
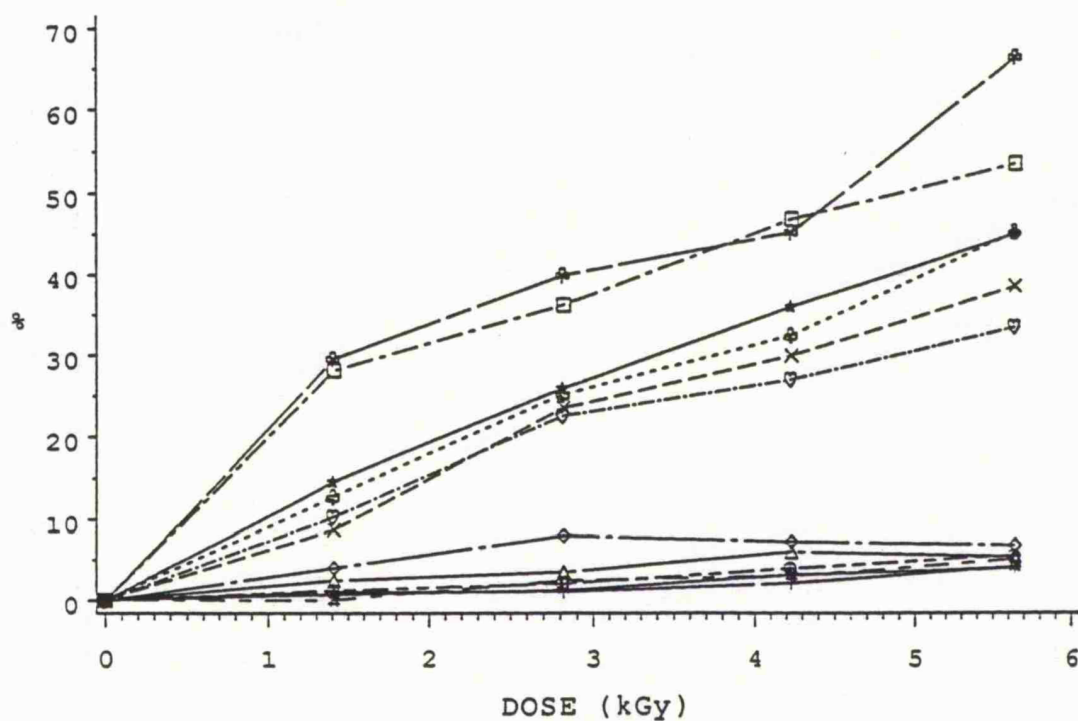
Figure 5.4: The effect of aminothiols of differing charge on the γ-radiation induced breakage of Form I pBR322 DNA under direct conditions at 77K. Plasmid DNA was irradiated 6mM of each compound under ambient oxygen concentration. DNA only: Form II DNA (✦–✦–✦), Form III DNA (⊖–⊖–⊖). Mercaptoethanol: Form II DNA (▽··▽–▽), Form III DNA (+–+–+). Cysteamine: Form II DNA (⊕–⊕–⊕), Form III DNA (✳–✳–✳). WR-1065: Form II DNA (✶–✕–✕), Form III DNA (✳–✳–✳). RW-222: Form II DNA (✢–✢–✢), Form III DNA (△–△–△). Norspermidine: Form II DNA (▱–▱–▱), Form III DNA (◇–◇–◇).

RADIATION DOSE (KGY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00	0.00	0.00	0.00	0.00
1.41	1.24	0.32	14.62	4.97
2.82	2.02	0.70	25.87	3.86
4.23	3.93	0.54	35.81	0.71
5.54	5.50	0.62	44.74	1.33

*Standard deviation of the mean of four separate results.

Table 5.3a: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K under ambient oxygen concentration.

RADIATION DOSE (KGY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00	0.00	0.00	0.00	0.00
1.41	1.03	0.38	10.34	6.29
2.82	0.99	0.86	22.50	7.29
4.23	2.09	0.10	26.88	7.25
5.54	4.09	0.04	33.23	1.56

*Standard deviation of the mean of two separate results.

Table 5.3b: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K with 6mM mercaptoethanol under ambient oxygen concentration.

RADIATION DOSE (KGY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00	0.00	0.00	0.00	0.00
1.41	0.66	0.41	12.83	3.18
2.82	1.21	0.59	25.14	0.48
4.23	3.09	1.44	32.29	7.48
5.54	3.98	1.03	44.98	2.97

*Standard deviation of the mean of two separate results.

Table 5.3c: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K with 6mM cysteamine under ambient oxygen concentration.

RADIATION DOSE (KGY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00	0.00	0.00	0.00	0.00
1.41	0.00	0.00	8.76	1.15
2.82	2.40	2.63	23.44	2.27
4.23	3.01	0.07	29.80	7.64
5.54	5.03	1.88	38.26	9.25

*Standard deviation of the mean of two separate results.

Table 5.3d: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K with 6mM WR-1065 under ambient oxygen concentration.

RADIATION DOSE (KGY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00	0.00	0.00	0.00	0.00
1.41	2.42	0.43	29.53	5.30
2.82	3.46	1.36	39.70	4.33
4.23	5.99	1.53	44.96	3.04
5.54	5.35	1.02	66.15	4.65

*Standard deviation of the mean of two separate results.

Table 5.3e: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K with 6mM RW-222 under ambient oxygen concentration.

RADIATION DOSE (KGY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$*	MEAN	S.D.$_{Mean}$*
0.00	0.00	0.00	0.00	0.00
1.41	3.98	1.59	28.10	7.43
2.82	7.97	2.51	36.06	4.91
4.23	7.24	3.25	46.56	10.93
5.54	6.74	0.21	53.27	8.65

*Standard deviation of the mean of two separate results.

Table 5.3f: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at 77K with 6mM norspermidine under ambient oxygen concentration.
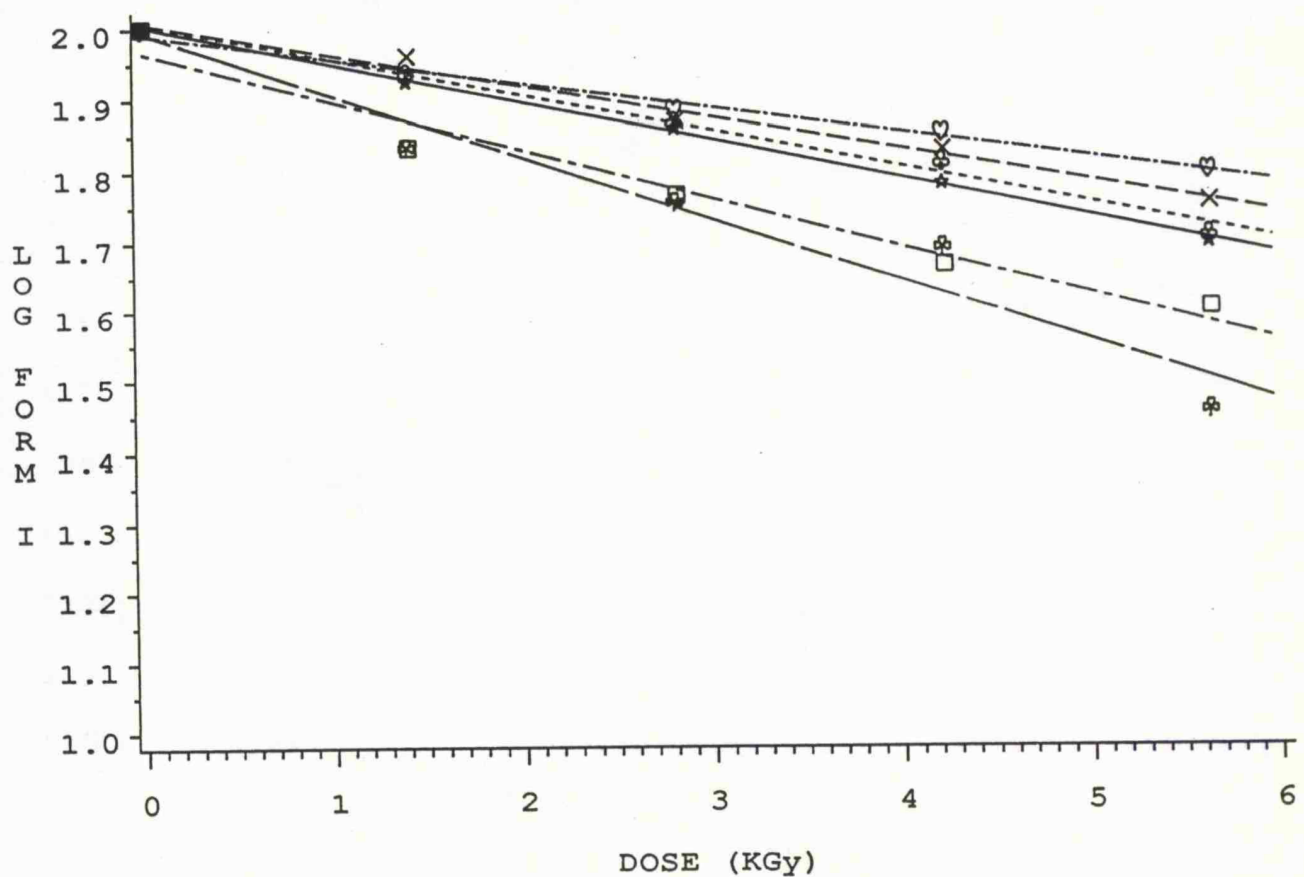
Figure 5.5: Semilog. plot of the γ-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated under direct conditions at 77K, under ambient oxygen concentration, with 6mM of each compound. DNA only (★—★—★), mercaptoethanol (♡··♡—♡), cysteamine (♣-♣-♣), WR-1065 (✕—✕—✕), RW-222 (✚—✚—✚), norspermidine (▭-▭-▭).

COMPOUND (6 mM)	SLOPE[1]	S.D.*	CONSTANT	S.D.*	A.E.R.
DNA ONLY	5.33	0.14	2.002	0.004	–
MERCAPTOETHANOL	3.46	0.23	1.990	0.008	0.649
CYSTEAMINE	5.04	0.29	2.000	0.010	0.946
WR-1065	4.55	0.28	2.007	0.010	0.854
RW-222	8.88	1.23	2.000	0.042	1.666
NORSPERMIDINE	6.83	0.73	1.970	0.025	1.281

[1]$-Log_{10}$ % Form I DNA Gy^{-1} x 10^{-5}. *Standard deviation.

Table 5.4: Linear regression analysis of a graph of the a
semilogarithmic plot of the logarithm of the percentage of
Form I pBR322 DNA remaining against radiation dose. Plasmid
DNA was irradiated at 77K under ambient oxygen
concentration.

show that DNA is protected as a result of the reactions discussed in above and shown in Figure 5.2. The sensitisation of DNA by RW-222 and norspermidine, and the lack of a net charge related effect presents some problems. Indeed, the observation that changing one of the primary amine groups for a free-thiol group results in an apparent increase in sensitisation is problematical.

More work needs to be undertaken to explain this phenomenon. Had time been available, a study of the effect of drug concentration on DNA sensitisation would have hopefully shed more light on any "salt effect" type sensitisation which was taking place.

The effect of thiols on radiation-induced damage to plasmid DNA irradiated under indirect conditions.

Plasmid DNA was irradiated under indirect conditions at room temperature under ambient oxygen concentration with 6mM thiol or norspermidine. The results are shown in Table 5.5(a-f) and Figure 5.6. A semilogarithmic plot of the amount of Form I plasmid DNA remaining against radiation dose is shown in Figure 5.7. The linear regression analysis of this plot, and the A.E.R. calculated from it are shown in Table 5.6.

The results show that the amount of protection observed with the compounds is in the following order: RW-222 ≥ WR-1065 > cysteamine > mercaptoethanol > norspermidine. It is noted that the resolution between the results obtained for the individual thiols is poor. A concentration of 6mM was originally chosen
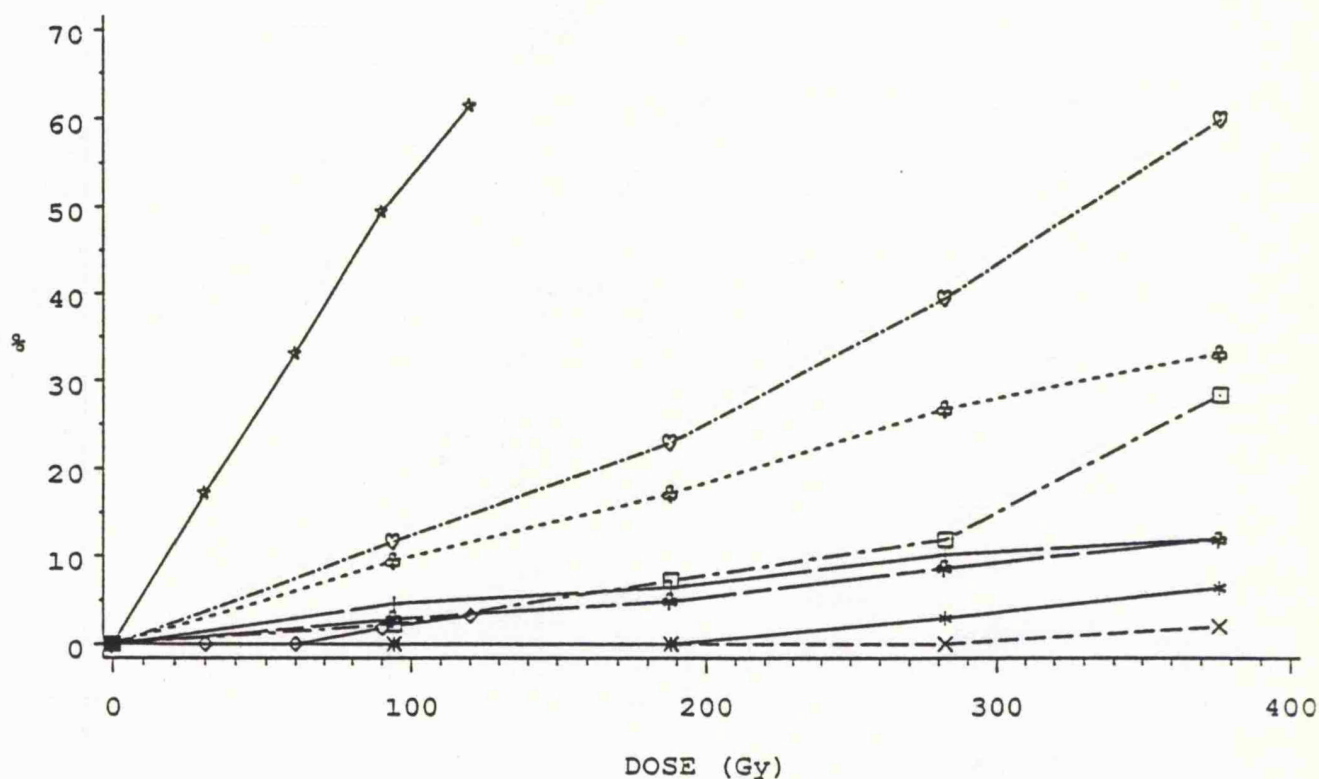
Figure 5.6: The effect of aminothiols of differing charge on the γ-radiation induced breakage of Form I pBR322 DNA under indirect conditions at room temperature. Plasmid DNA was irradiated 6mM of each compound under ambient oxygen concentration. DNA only: Form II DNA (★—★—★), Form III DNA (◇—◇—◇). Mercaptoethanol: Form II DNA (⊕-⊕-⊕), Form III DNA (✕-✕-✕). Cysteamine: Form II DNA (⊟-⊟-⊟). WR-1065: Form II DNA (┼—┼—┼). RW-222: Form II DNA (⊕-⊕-⊕). Norspermidine: Form II DNA (▽--▽--▽), Form III DNA (✳—✳—✳).

RADIATION DOSE (GY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$ *	MEAN	S.D.$_{Mean}$ *
0	0.00	0.00	0.00	0.00
30	0.00	0.00	17.25	1.25
60	0.00	0.00	33.12	9.05
90	1.82	0.72	49.28	4.90
120	3.21	1.41	61.37	1.92

*Standard deviation of the mean of four separate results.

Table 5.5a: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at room temperature under ambient oxygen concentration.

RADIATION DOSE (GY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$ *	MEAN	S.D.$_{Mean}$ *
0	0.00	0.00	0.00	0.00
94	0.00	0.00	9.48	0.40
188	0.00	0.00	17.19	1.79
262	0.00	0.00	26.71	4.18
376	1.98	0.30	33.02	11.14

*Standard deviation of the mean of three separate results.

Table 5.6b: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at room temperature with 6mM Mercaptoethanol under ambient oxygen concentration.

RADIATION DOSE (GY)	FORM III		FORM II	
	MEAN	S.D.$_{Mean}$ *	MEAN	S.D.$_{Mean}$ *
0	0.00	0.00	0.00	0.00
94	0.00	0.00	2.26	0.25
188	0.00	0.00	7.23	4.00
262	0.00	0.00	11.92	3.10
376	0.00	0.00	28.27	8.08

*Standard deviation of the mean of three separate results.

Table 5.5c: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at room temperature with 6mM cysteamine under ambient oxygen concentration.

RADIATION DOSE (GY)	FORM III		FORM II	
	MEAN	S.D.Mean*	MEAN	S.D.Mean*
0	0.00	0.00	0.00	0.00
94	0.00	0.00	4.61	1.12
188	0.00	0.00	6.42	1.17
262	0.00	0.00	10.15	2.18
376	0.00	0.00	12.01	4.90

*Standard deviation of the mean of three separate results.

Table 5.5d: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at room temperature with 6mM WR-1065 under ambient oxygen concentration.

RADIATION DOSE (GY)	FORM III		FORM II	
	MEAN	S.D.Mean*	MEAN	S.D.Mean*
0	0.00	0.00	0.00	0.00
94	0.00	0.00	2.86	0.79
188	0.00	0.00	4.87	3.22
262	0.00	0.00	8.63	4.27
376	0.00	0.00	11.89	2.21

*Standard deviation of the mean of three separate results.

Table 5.5e: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at room temperature with 6mM RW-222 under ambient oxygen concentration.

RADIATION DOSE (GY)	FORM III		FORM II	
	MEAN	S.D.Mean*	MEAN	S.D.Mean*
0	0.00	0.00	0.00	0.00
94	0.00	0.00	11.70	9.17
188	0.00	0.00	22.92	3.39
262	2.95	3.10	39.26	6.36
376	6.36	5.52	59.84	5.15

*Standard deviation of the mean of three separate results.

Table 5.5f: The gamma-radiation induced strand breakage of Form I pBR322 DNA irradiated at room temperature with 6mM norspermidine under ambient oxygen concentration.
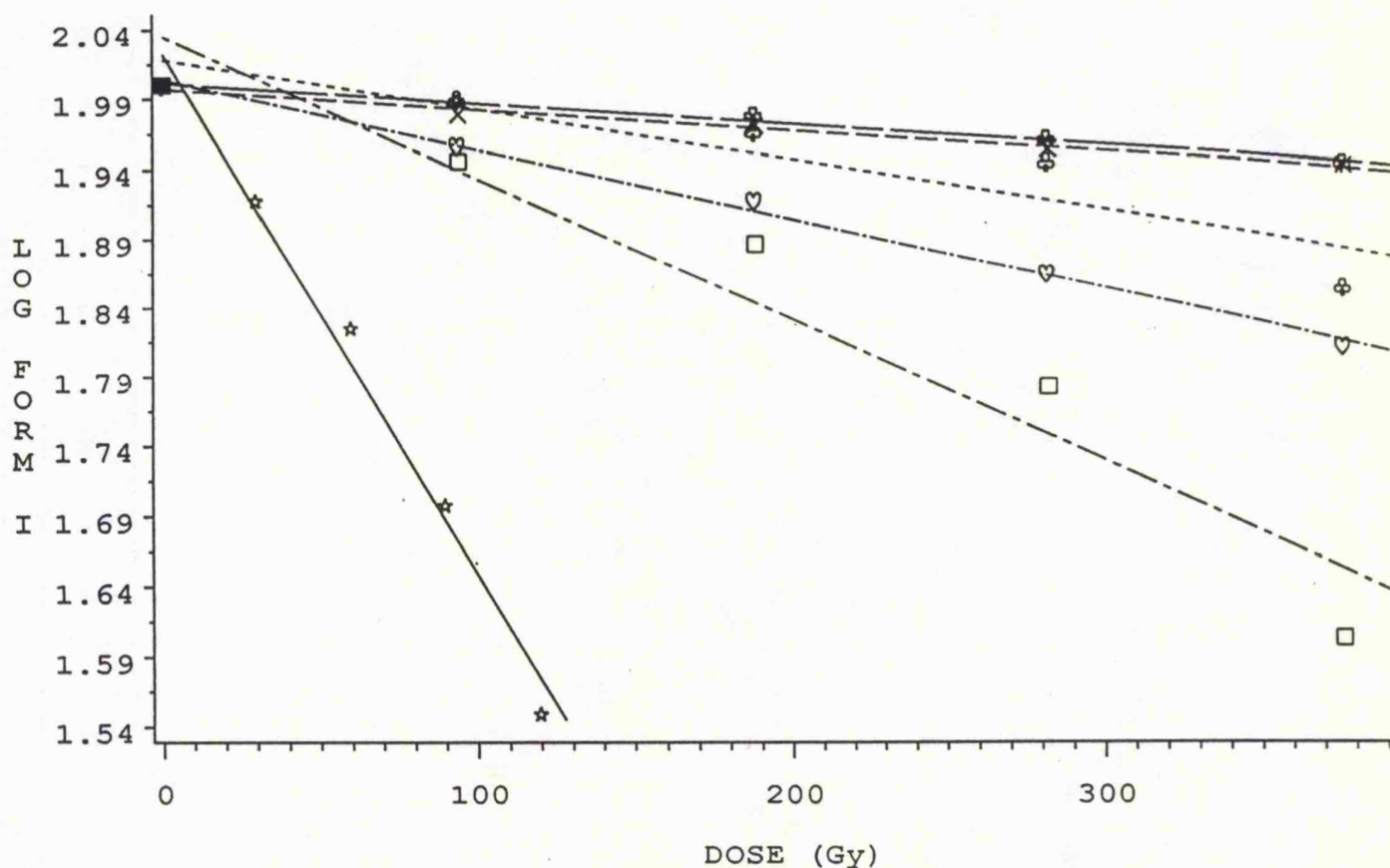
Figure 5.7: Semilogarithmic plot of the γ-radiation induced breakage of Form I pBR322 DNA. Plasmid DNA was irradiated under indirect conditions at room temperature, under ambient oxygen concentration, with 6mM of each compound. Mercaptoethanol (♡--♡—♡), cysteamine (✿-✿-✿), WR-1065 (✕—✕—✳), RW-222 (✤-✤-✤), norspermidine (▱-▱—▱), DNA only (★—★—★).

COMPOUND (6 mM)	SLOPE[1]	S.D.*	CONSTANT	S.D.*	A.E.R
DNA ONLY	37.71	2.64	2.022	0.004	–
MERCAPTOETHANOL	4.96	0.18	2.004	0.005	0.132
CYSTEAMINE	3.54	0.90	2.018	0.021	0.094
WR-1065	1.46	0.19	1.977	0.001	0.038
RW-222	1.45	0.09	2.001	0.002	0.038
NORSPERMIDINE	10.16	1.59	2.035	0.037	0.270

[1]-Log_{10} % Form I DNA Gy^{-1} x 10^{-4}.   *Standard deviation.

Table 5.6: Linear regression analysis of a graph of the a semilogarithmic plot of the logarithm of the percentage of Form I pBR322 DNA remaining against radiation dose. Plasmid DNA was irradiated at room temperature under ambient oxygen concentration.

because at this concentration protection would be expected to be observed with cysteamine when plasmid DNA was irradiated under direct conditions, as reported by Symons and coworkers [221]. This means that the ratio of drug : base pairs is ca. 80:1. This is a high ratio, but it was hoped that it would enable an excess of free-thiol to be left within the system to undergo radical repair reactions, after some of the free-thiol had been converted to disulphide by reacting with oxygen. It unfortunately probably partially saturated any counterion condensation effect that was likely to be seen. With hindsight, lowering the concentration of drug would have probably increased the resolution between the different drugs.

The results show that polyamines, such as norspermidine, are capable of protecting DNA by scavenging water radiolysis products, such as hydroxyl radicals. The addition of a free-thiol group to the polyamine chain, to form RW-222, considerably increases the amount of protection seen.

The order in which the thiols protected is in keeping with the counterion condensation theory:

RW-222(+3) ≥ WR-1065(+2) > cysteamine(+1) > mercaptoethanol(0)

Besley [266] used sodium-23 N.M.R. to study the binding of aminothiols to DNA. ^{23}Na N.M.R. looks at the ^{23}Na line width of a DNA containing system [267]. This can be related to the amount of Na^+ associated with the DNA. On addition of an aminothiol to the DNA sodium ions are released, resulting in a broadening of the line width. Besley [266] found that the amount of Na^+

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released increased with increasing positive charge RW-222 (+3) > WR-1065 (+2) > cysteamine (+1), implying that the amount of binding of aminothiols to DNA increases with increasing positive charge.

Wardman [268] used acridine orange quenching, where the amount of fluorescence observed when acridine orange is bound to DNA, is quenched by added aminothiols, to study the binding, the greater the quenching, the greater the amount of binding. It was found that the amount of quenching observed was in the order: RW-222 > WR-1065 > cysteamine > mercaptoethanol. The effect of the third positive charge on the amount of binding was especially marked, and would appear to maximise the amount of binding of aminothiol ligands.

If a lower concentration of thiol had been used to increase the resolution between the different drugs, the difference between WR-1065 and RW-222 should have been clearer, and have provided clearer evidence of the effect of net charge on the amount of radioprotection observed on aminothiols.

Alkaline Filter Elution

The effect of WR-1065 and RW-222 on the induction of single strand breaks in V79 cells

The effect of 6mM WR-1065 and RW-222 on the formation of single strand breaks in V79 cells is shown in Table 5.7, and Figure 5.8.

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Figure 5.8: The γ-radiation induced breakage of Chinese Hamster V79 cells DNA, as measured by alkaline filter elution. Cells were incubated with 6mM of compound at 37°C for 45 minutes, and irradiated under ambient oxygen concentration. No drug (★—★—★). WR-1065 (♥··♥—♥). RW-222 (�ᵇ-♁-♁).

DOSE (Gy)	STRAND SCISSION FACTOR					
	DNA ONLY		WR-1065		RW-222	
	MEAN	$SD_{MEAN}{}^1$	MEAN	$SD_{MEAN}{}^2$	MEAN	$SD_{MEAN}{}^2$
CONTROL	(0.903)	(0.025)	(0.604)	(0.068)	(0.689)	(0.026)
2.3	0.091	0.015	0.071	0.030	0.125	0.021
4.6	0.142	0.061	0.189	0.081	0.217	0.048
9.2	0.368	0.025	0.369	0.130	0.289	0.028
18.4	1.073	0.341	0.585	0.215	0.572	0.159

[1]Standard deviation of the mean of two aliquots of cells on each of three days.

[2]Standard deviation of the mean of two aliquots of cells on each of two days.

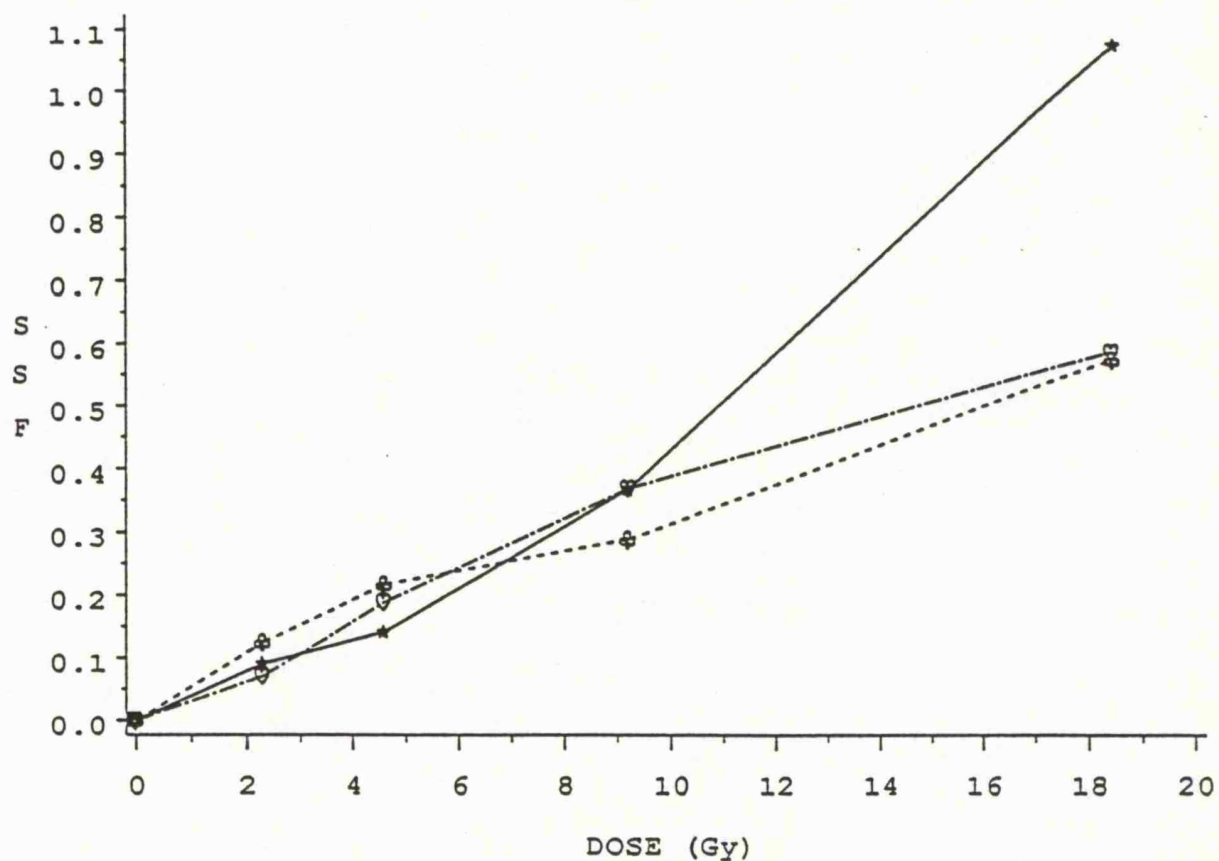Table 5.7: The effect of WR-1065 and RW-222 on the γ-radiation induced breakage of Chinese Hamster V79 cells DNA, as measured by alkaline filter elution. Cells were incubated with 6mM of the compound for 45 minutes at 37°C, and were irradiated under ambient oxygen concentration. The figures given for the controls are the proportion of DNA retained on each filter after 20 ml of elution for each compound.

The figures given for the amount of DNA retained on each filter after 20ml of elution, when compared with cells incubated without drug under the same conditions, show that incubating Chinese Hamster V79 cells with WR-1065 or RW-222 results in the production of DNA strand breaks. This may be due to oxidation of the drug occurring with the net production of disulphide and damaging hydrogen peroxide [215, 217, 236]. The consumption of oxygen by WR-1065 and cysteamine is known to occur very rapidly within cells, within 2 minutes of addition of 10mM WR-1065 [247].

The increase in ssb in the control cells would appear to show that WR-1065 and RW-222 are able to reach the nucleus of the cells in the time allowed for incubation with the drugs prior to irradiation. Cohen [254] has shown, by looking at the effect of the addition of RW-222 on spermidine uptake in Ehrlich ascites tumour cells, that RW-222 is uptaken by the cells. Wardman [104] used acridine orange quenching, to investigate whether the WR-1065 or RW-222 can cross into the nucleus of V79 cells. He found that some RW-222 and WR-1065 do indeed find their way into the nucleus of cells.

A study of the Strand Scission Factors obtained shows that radiation doses of between 0 and 10 Gy no definitive conclusion can be obtained as to whether the compounds protect or sensitise the DNA in V79 cells. The standard deviations are too large to form enable a clear picture to be formed. Above 10Gy WR-1065 and RW-222 would appear to protect the DNA in V79 cells, though the order in which they do so is unclear.

Had more time been available it is hoped that a combination of more practice with the technique of alkaline filter elution, and more sets of data, would reduce the amount standard deviation between results.

Murray and coworkers [227] using WR-1065 with Chinese Hamster Ovary cells, and alkaline filter elution. They showed that the amount of protection observed increased with the concentration of WR-1065 upto a concentration of 6mM, where a protection factor of ca. 1.8 was observed. Above 6mM the amount of protection decreased.

Wardman [268] has initially investigated effect of 4mM cysteamine, WR-1065, and RW-222 on cell survival using x-radiation and exponentially growing V79 cells. It was found that cysteamine and WR-1065 protected approximately equally, but RW-222 protected to a lesser extent. These results are a little disappointing, since it was expected that, if the counterion condensation theory was to follow to whole cell systems, the RW-222 should protect better than WR-1065 and cysteamine. Some of the protective effect observed with the compounds on cell survival could be due to extracellular protection of the membrane of the cells, or due to protection of cellular components other than DNA. The amount of free-thiol in the growth medium before and after irradiation was measured using Ellman's reagent. The observed protection was more closely related to the amount of free-thiol in the media during irradiation. Hence radiolysis products from the extracellular media may be involved in the inhibition of cell growth by radiation in the system used. A more thorough investigation

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using a range of concentrations and systems, including the use of more penetrating γ-radiation will hopefully show that RW-222 does indeed protect DNA to a greater extent than thiols with lower net charges.

DISCUSSION

The results show that incubating Form I plasmid DNA with thiols or norspermidine at room temperature will produce damage to the DNA. This may be due to the oxidation of the free-thiol to produce hydrogen peroxide and disulphide, a form of damage which is reduced at higher concentrations of thiol due to scavenging of the hydrogen peroxide by the drug molecules. A second process would appear to be at work in producing the reduced levels of damage seen with norspermidine, and probably produces some of the damage seen with the thiols. The process possibly involves the scheme suggested by Saito and coworkers [34-37] involving the reaction of the primary amine group with thymidine under ultra-violet light. This could be tested by incubating the compounds in darkness, or using WR-255591 and comparing it with WR-1065.

The radioprotection studies of plasmid DNA irradiated under direct conditions were inconclusive. The amount of damage observed was not charge related. The use of 6mM cysteamine confirmed that the compound protects DNA under direct conditions, as do mercaptoethanol and WR-1065. RW-222 and norspermidine, however, appear to sensitise DNA. This could possibly due to a "salt-type effect" where the compound increases the effect target size, as discussed in Chapter 3, and may involve the radiolysis products of the chloride counterions of the drug. These results demonstrate the importance fully understanding the frozen aqueous system with respect to target size and radiation chemistry. Studies on the effect of varying

the concentration of drug would hopefully reveal more about the system.

The radioprotection studies on the effect of the thiols and norspermidine on indirect damage to DNA were successful, in that they demonstrated the expected charge dependent effect on the amount of radiation protection observed. The resolution of the results could have been improved by lowering the concentration on the drug used. The results also showed the importance of the free-thiol group in the amount of radiation protection observed.

Finally, the use of alkaline filter elution disappointingly failed to conclusively demonstrate an protection by WR-1065 or RW-222 at low radiation doses. At higher doses, above 10 Gy, some protection was observed, though no significant difference between the compounds was observed.

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