DAMAGE TO DNA BY REACTIVE OXYGEN SPECIES:

RELEVANCE TO THE PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

by

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Synopsis

The purpose of this work was to study the effects of reactive oxygen species (ROS) on DNA and to investigate the relevance of ROS-induced DNA damage in systemic lupus erythematosus (SLE).

Using model systems of ROS generation, it was found that DNA was damaged by ROS at all levels of its structure, causing strand breaks, base modifications and conformational changes. Hydrogen peroxide, a ROS generated during inflammation in vivo, produced a characteristic type of site-specific damage dependent on the DNA-bound metal ion catalysis of its degradation.

8-hydroxydeoxyguanosine (8OHDG), a modified DNA base, was used as a marker of oxidative damage to investigate the role of DNA damage in the aetiopathogenesis of SLE.

Excretion of this adduct was detected in normal urine and is believed to arise from normal oxidative metabolic processes. In patients with active rheumatoid arthritis, this level of 8OHDG excretion was significantly elevated. In contrast, in SLE patients with inflammatory activity, 8OHDG was undetectable in the urine.

Investigation of the mechanism responsible for this showed that SLE cells had aberrant removal of 8OHDG from DNA following oxidative stress in vitro compared to normal cells, and that ROS-denatured DNA accumulated in circulating immune complexes associated with the disease.

SLE is also characterised by circulating anti-DNA antibodies. These antibodies were found to bind better to ROS-DNA than to native double-stranded DNA. Furthermore, ROS-DNA was able to stimulate lymphocytes to produce anti-DNA antibodies.

The pattern of DNA damage seen in SLE patients was typical of that induced by hydrogen peroxide in vitro. This suggests that inflammation generates ROS which cause DNA damage. As a result of defective repair within cells, ROS-DNA is released into the circulation following cell death which can form complexes with anti-DNA antibodies. In addition, the ROS-DNA can stimulate further anti-DNA antibody production by acting directly on cells thus perpetuating the disease process and contributing to immune complex deposition, a deleterious manifestation of the disease process.
To my Family
Acknowledgements

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Finally I am very grateful to Dr Gareth Bowen and Dr George Kitas for their critical appraisal of this thesis.
**List of Abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Ascorbic Acid</td>
</tr>
<tr>
<td>ADPRT</td>
<td>ADP Ribosyl Transferase</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti-Nuclear Antibody</td>
</tr>
<tr>
<td>8AZA</td>
<td>8-Azaadenine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BSTFA</td>
<td>Bis(trimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>C</td>
<td>Complement</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic Granulomatous Disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>dA</td>
<td>2'-deoxyadenosine</td>
</tr>
<tr>
<td>dG</td>
<td>2'-deoxyguanosine</td>
</tr>
<tr>
<td>dC</td>
<td>2'-deoxycytosine</td>
</tr>
<tr>
<td>dT</td>
<td>2'-deoxythymidine</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorter</td>
</tr>
<tr>
<td>FADU</td>
<td>Fluorometric Analysis of DNA Unwinding</td>
</tr>
<tr>
<td>FAPY Adenine</td>
<td>4,6-diamino-5-formamidopyrimidine</td>
</tr>
<tr>
<td>FAPY Guanine</td>
<td>2,6-diamino-4-hydroxy-5-formamidopyrimidine</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas Chromatography with Mass Spectroscopy</td>
</tr>
<tr>
<td>GPS</td>
<td>Glutamine, Penicillin, Streptomycin</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HIFCS</td>
<td>Heat Inactivated Foetal Calf Serum</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HPLC/ECD</td>
<td>High Performance Liquid Chromatography with Electrochemical detection</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MCA</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MP</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>8OHA</td>
<td>8-hydroxyadenine</td>
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<tr>
<td>8OHGD</td>
<td>8-hydroxydeoxyguanosine</td>
</tr>
<tr>
<td>8OHG</td>
<td>8-hydroxyguanine</td>
</tr>
<tr>
<td>OPD</td>
<td>Orthophenylene Diamine</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline with 0.05% Tween 20</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Leucocyte</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acids</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RMRF</td>
<td>Relative Molar Response Factor</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected Ion Monitoring</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SPA</td>
<td>Staphylococcus Aureus Protein A</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric Acid</td>
</tr>
<tr>
<td>TBE</td>
<td>89mM Tris Borate, pH 8.0</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris, 1mM EDTA</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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</table>
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GENERAL INTRODUCTION
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1.1.1: Reactive Oxygen Species

Reactive oxygen species (ROS) are molecules derived from oxygen by univalent reduction reactions. They are formed during normal oxidative metabolism and have a high chemical reactivity.

Oxygen itself is essential for the maintenance of aerobic metabolism and comprises the terminal electron acceptor in mitochondrial respiration. In this metabolic process, water and carbon dioxide are formed from the tetravalent reduction of oxygen by the efficient mitochondrial enzyme cytochrome oxidase, with the concomitant production of ATP. However, oxygen can also react with single electrons that may leak from the respiratory chain and undergo univalent reduction by virtue of the electronic configuration conferred on the oxygen molecule. This produces superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^-$) from the 1-, 2- and 3-electron reductions of oxygen respectively. It has been estimated that during normal metabolism 5% of oxygen is converted to O$_2^-$ by this alternative reduction mechanism (Fridovich, 1978; Fridovich, 1984).

Most biological molecules have paired electrons in their outer shell ($\uparrow \downarrow$) and react with each other in thermodynamically favourable reactions. However, oxygen is a diradical with two unpaired electrons with unidirectional spin in its outer shell ($\uparrow O_2 \downarrow$), one of which must undergo spin inversion before it can react with molecules with paired electrons. The length of time for spin inversion to occur is long compared with molecular interactions and therefore oxygen will react more readily with other molecules with parallel spin electrons or with free radicals rather than undergoing spin inversion.

A free radical is an atom or molecule that has one or more unpaired electrons in its outer orbital. Free radicals can have cationic (H$_2$O$^+$), anionic (O$_2^-$) or neutral characteristics (O$_2$) and are extremely reactive, with rate constants of the order of $10^5 - 10^{10}$ M$^{-1}$ s$^{-1}$ (Wardman, 1978; Halliwell and Gutteridge, 1985).
Free radicals may be generated either from the unimolecular homolytic fission of a covalent bond between two atoms as shown below:

\[ \begin{array}{c}
1 \quad X-Y \\
\rightarrow \quad X^- + Y^-
\end{array} \]

or from oxidation or reduction reactions in which electrons are transferred directly from one redox intermediate to another, producing bound or free radicals.

The first mechanism, requiring bond scission, generates two neutral species and can be induced by high energy radiation if the energy input is greater than the bond dissociation energy. Ultraviolet (UV) irradiation of H\textsubscript{2}O\textsubscript{2} producing two molecules of OH\textsuperscript{-} provides a typical example of this (see section 2.4.2).

The second mechanism is frequently mediated by transition metal ions, many of which have unpaired electrons in their outer orbital. This allows them to accept and donate single electrons and they are found at the active site of many enzymes catalysing redox reactions (Hill, 1981).

1.1.2: Reactions of ROS

1.1.2.1: Superoxide Radical Anion

The acceptance of electrons by oxygen, one at a time, overcomes the spin restriction of the native molecule and produces O\textsubscript{2}^- as the first electron product of O\textsuperscript{2-}. This anion has only one unpaired electron (\(\uparrow \downarrow \text{O}_2\uparrow\)) and is an energetically stable intermediate in the reduction of oxygen. As a result of this, chemical and physicochemical studies have suggested that it is not a very reactive radical, causing damage only indirectly by giving rise to more powerful oxidants (Bielski and Richter, 1977; Sawyer and Valentine, 1981) such as OH\textsuperscript{-}. However, this view is not held by Fridovich (1986), who documents that O\textsubscript{2}^- finds critical targets in living cells, inactivating catalase and glutathione peroxidase, and mobilising iron from ferritin (section 1.1.3).

At physiological pH, O\textsubscript{2}^- is a relatively strong reducing agent, weak oxidising agent and nucleophile. These features facilitate the reaction between two molecules of O\textsubscript{2}^- in a dismutation reaction, one molecule being reduced, the other oxidised, to form H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2}:

\[ 2 \quad O_2^- + O_2^- \rightarrow H_2O_2 + O_2 \]
The reaction proceeds most rapidly at acidic pH, whereby $O_2^-$ becomes protonated to form the perhydroxyl radical, $HO_2^-$:

$$3 \text{HO}_2^- \rightarrow \text{H}^+ + \text{O}_2^-$$

The pKa for the equilibrium of reaction 3 is $\approx 4.8$ (Bielski and Allen, 1977).

At physiological pH the overall rate of dismutation is relatively slow ($\approx 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). This allows $O_2^-$ to react not only with itself but also with other biological material.

One important competing reaction of $O_2^-$ is its reaction with trivalent ferric ions of the metalloprotein ferritin, to release the divalent ferrous form (Biemond et al., 1984; Biemond et al., 1988). Ferrous ions play an integral part in the generation of further ROS from $O_2^-$ as described in section 1.1.2.2.

The reactions of $O_2^-$ need not be confined to the cell in which the anion is produced. The protonated form of $O_2^-$ ($HO_2^-$), is lipophilic and can diffuse through the lipid bilayer. It is also more reactive than $O_2^-$ and will attack the fatty acid components of membranes (Bielski, Arudi and Sutherland, 1983). In addition to this, $O_2^-$ may be transported across the cell membrane via an anion channel although the only example of this is in the red cell membrane (Lynch and Fridovich, 1978). With its relatively long half life this may allow $O_2^-$ to diffuse away from its site of production and extend the area of ROS-induced damage.

The dismutation of $O_2^-$ can also be catalysed by superoxide dismutase (SOD) (McCord and Fridovich, 1969). The SODs are a family of metalloenzymes that is widely distributed in mammalian tissue and increases the rate of dismutation of $O_2^-$ from $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ to $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.

1.1.2.2: Hydrogen Peroxide

Dismutation of $O_2^-$, occurring either spontaneously or catalysed by SOD, results in the generation of $H_2O_2$. This is a two electron reduction product of oxygen but has no unpaired electrons ($H^+ + O_2 + \downarrow H$): it is not a free radical and is more appropriately referred to as a reactive oxygen species.

The mechanism of $H_2O_2$ reduction to produce further reactive metabolites of oxygen has been the subject of many studies and is still controversial. Shortly after the discovery of SOD, $O_2^-$ was
suggested to react with $\text{H}_2\text{O}_2$ via the Haber-Weiss reaction to produce $\text{OH}^-$ (Beauchamp and Fridovich, 1970):

$$4 \text{O}_2^{-} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^-$$

Although this is thermodynamically feasible (Koppenol, 1976), $\text{O}_2^{-}$ dismutates faster than formation of $\text{OH}^-$ in this reaction (Halliwell, 1976; Weinstein and Bielski, 1979). It has now been shown that the rate constant for the Haber-Weiss reaction is less than $10^{-8}$ $\text{M}^{-1}$ $\text{s}^{-1}$ (Rigo and Stevanato, 1977) and cannot occur at the low steady-state concentrations of $\text{O}_2^{-}$ and $\text{H}_2\text{O}_2$ found in biological systems (Halliwell, 1976). The stoichiometry of the Haber-Weiss reaction can be achieved in the presence of catalytic metal ions (McCord and Day, 1978; Halliwell, 1978a; Halliwell, 1978b):

$$5 \text{Fe(III)} + \text{O}_2^{-} \rightarrow \text{Fe(II)} + \text{O}_2$$

$$6 \text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \text{OH}^-$$

Reaction 6 is referred to as the Fenton reaction. It does not require high concentrations of Fe(II) and proceeds at a rate of $1-10^4$ $\text{M}^{-1}$ $\text{s}^{-1}$ (Simic, Bergtold and Karam, 1989). The role of Fe(II) is to catalyse the homolytic fission of $\text{H}_2\text{O}_2$, and the sum of reactions 5 and 6 gives rise to an iron-catalysed Haber-Weiss reaction. The role of $\text{O}_2^{-}$ would appear to be to reduce Fe(III) to its catalytic form of Fe(II). However, if alternative reducing agents, such as ascorbic acid (AA), are available to react with Fe(III), $\text{H}_2\text{O}_2$ can give rise to $\text{OH}^-$ in a reaction which cannot be inhibited by SOD (Winterbourn, 1981; Rowley and Halliwell, 1983a). Despite this, the $\text{O}_2^{-}$ catalysed release of Fe(II) from iron complexes is important, because physiological iron complexes such as peroxidases and ferritin cannot catalyse the Haber-Weiss reaction (Halliwell, 1978a).

In vitro, Fe-EDTA can provide catalytic iron in its complexed form. It is, therefore, frequently used to study the reaction of iron in Fenton chemistry since it provides a soluble redox active form of iron (Walling, Kurz and Schugger, 1970; McCord and Day, 1978). Using this model system, Gutteridge (1990) has recently suggested that whilst classical Fenton chemistry describes $\text{H}_2\text{O}_2$ as both a reductant (7) and oxidant (8) of iron salts and their complexes:

$$7 \text{H}_2\text{O}_2 + \text{Fe}^{3+} \rightarrow \text{HO}_2^{-} + \text{H}^{+} + \text{Fe}^{2+}$$

$$8 \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^{-} + \text{OH}^{-} + \text{Fe}^{3+}$$

iron bound to EDTA is unlikely to be reduced by $\text{H}_2\text{O}_2$ (Gutteridge, Maidt and Poyer, 1990); it is $\text{O}_2^{-}$ that predominates in the reduction of ferric-EDTA and other iron complexes to the ferrous state (reaction 5), providing $\text{H}_2\text{O}_2$, essential for $\text{OH}^-$ formation (as above).
1.1.2.3: Hydroxyl Radical

In the iron catalysed Haber-Weiss reaction (reactions 5 and 6), $H_2O_2$ ultimately gives rise to the hydroxyl radical ($OH^\cdot$). The identification of the radical species as $OH^\cdot$ has been the subject of controversy, predominantly because $OH^\cdot$, once generated, will react immediately with surrounding biomolecules at a diffusion-controlled rate within a few atomic collisions of where it is formed (Anbar and Neta, 1967). It is therefore difficult to detect and measure directly. Alternatives to $OH^\cdot$ have been suggested as major contributors to oxidative damage in biological systems. These include the ferryl radical ($FeO^{2+}$), in which iron has an oxidation number of four and the ROS does not dissociate from it (Walling, 1982; Rush and Koppenol, 1986). However, whilst the ferryl radical may be the reactive species at the active sites of horseradish peroxidase compounds I and II (Dunford, 1982) and is important in haemoglobin / myoglobin reactions (Harel and Kanner, 1988; Peterson, Symons and Taiwo, 1989), there is no clear evidence for its involvement in other systems.

Further evidence against the production of $OH^\cdot$ is the apparent inability of $OH^\cdot$ scavengers to protect in the Fenton reaction (Borg and Schaich, 1984; Czapski, 1984). This may be explained by production of an alternative radical species such as ferryl ($FeO^{2+}$) (Winterbourn, 1987), perferryl ($FeO^+$) or a crypto $OH^\cdot$ radical (Youngman, 1984) in which the radical species does not dissociate from the catalytic metal ion producing it. However, if the Fenton reaction is catalysed by a bound metal ion, the $OH^\cdot$ produced may react preferentially with the biomolecule at the metal binding site in a site-specific manner, rather than with the scavenger molecule. Further support for $OH^\cdot$ as the product of the Fenton reaction comes from recent work of Gutteridge (1990). This work shows that for an iron catalysed reaction, only when an iron complex or iron ion reacting with $H_2O_2$ does not release $OH^\cdot$ into free solution, or cause site-specific damage, does the formation and oxidative damage by species other than $OH^\cdot$ become more important in biological situations (Gutteridge, 1990). With this in mind $OH^\cdot$ will be described as the main product of the Fenton reaction.

$OH^\cdot$ is a highly reactive radical species with a half-life of $10^4$ seconds (Roots and Okada, 1975) and has been shown to diffuse only 1.5 nm before reacting with a surrounding molecule. This distance was determined by measuring the rates of reaction of $OH^\cdot$ with DNA and increasing concentrations of scavenger in a system where it was assumed that all $OH^\cdot$ could be scavenged (Ward, Blakely and Joner, 1985). It is thus the diffusion potential of $H_2O_2$ and $O_2^-$ that will limit the area damaged by $OH^\cdot$. 

6
H\(_2\)O\(_2\), O\(_2\)\(^-\) and OH\(^-\) arise from successive one electron reductions of oxygen. An alternative way to increase the reactivity of oxygen is to move one of the unpaired electrons in a way that alleviates the spin restriction. This requires an input of energy and generates singlet oxygen (\(\text{\textit{t}} \downarrow \downarrow \text{O}_2\)) referred to as \(1^1\text{O}_2\). This has no unpaired electrons and is not a radical. \(1^1\text{O}_2\) is formed when photosensitive compounds are illuminated in the presence of oxygen eg. retinal pigments in the eye (Kirschfeld, 1982). There is however no conclusive evidence for the formation of \(1^1\text{O}_2\) in \(\text{O}_2\)\(^-\)-generating systems or by phagocytic cells (Arudi, Bielski and Allen, 1984; Halliwell and Gutteridge, 1985).

1.1.2.5: Hypochlorous Acid

Hypochlorous acid (HOCl) is not a radical species and is formed when myeloperoxidase (MP), the most abundant neutrophil protein, catalyses the conversion of H\(_2\)O\(_2\) and Cl\(^-\) to HOCl (Harrison and Schultz, 1976). Although HOCl is potently microbicidal and cytotoxic, it is unclear how efficiently it is formed within the phagosome and how much it contributes to bacterial killing and other inflammatory reactions of neutrophils.

MP reacts with H\(_2\)O\(_2\):

\[
\begin{align*}
9 & \quad \text{MP}^{3+} + \text{H}_2\text{O}_2 & \rightarrow & \text{MP}^{3+}\text{H}_2\text{O}_2 \\
10 & \quad \text{MP}^{3+}\text{H}_2\text{O}_2 + \text{Cl}^- & \rightarrow & \text{MP}^{3+} + \text{HOCl} + \text{OH}^- \\
11 & \quad \text{MP}^{3+} + \text{O}_2\text{O}^- & \rightarrow & \text{MP}^{2+}\text{O}_2
\end{align*}
\]

and O\(_2\)\(^-\):

Acting as a combined SOD/catalase enzyme (see section 1.1.6).

Although the rate of reaction of MP\(^{3+}\) with O\(_2\)\(^-\) is ten times slower than that with H\(_2\)O\(_2\), there is evidence that O\(_2\)\(^-\) enhances the activity of myeloperoxidase and optimises production of HOCl by stimulated neutrophils (Kettle et al., 1988). This implies that O\(_2\)\(^-\) may potentiate inflammatory tissue damage by enhancing the production of HOCl (Kettle and Winterbourn, 1988) and that the anti-inflammatory effect of SOD (Niwa et al., 1985) may, in part, be due to the inhibition of this reaction. In view of this Kettle and Winterbourn (1990) have suggested that HOCl, rather than OH\(^-\) may be the principle agent responsible for tissue damage in inflammation.

1.1.3: Availability of Catalytic Iron

It has been demonstrated that production of OH\(^-\) through the Haber-Weiss reaction (reactions 5 and 6)
can be effectively catalysed with ferrous ion, Fe(II) (section 1.1.2.2). The majority of iron stored in the body is complexed to haemoglobin and myoglobin (Harrison and Hoare, 1980); a small amount is found in iron-containing enzymes and the transport protein transferrin (Aisen and Listowsky, 1980), and the rest is complexed to the intracellular storage proteins ferritin (Harrison, 1977) and haemosiderin (Ozaki, Kawabata and Awai, 1988). This leaves little free iron to drive the Haber-Weiss reaction.

In spite of this, $O_2\cdot^-$ can reduce ferritin-bound iron to stimulate the formation of $OH\cdot$ from $O_2\cdot^-$ and $H_2O_2$ (Bannister, Bannister and Thornally, 1984). This is not true for release of iron from lactoferrin and transferrin, which are not thought to be major physiological catalysts of $OH\cdot$ production (Halliwell and Gutteridge, 1985). In view of the reactivity of $OH\cdot$, even the ferritin catalysed production of $OH\cdot$ is only likely to cause extensive tissue damage close to ferritin deposits.

There is also a small transit pool of low molecular weight iron chelates present in cells. These are either attached to phosphate esters eg. ATP (Floyd, 1983), polar heads of membrane lipids (Halliwell and Gutteridge, 1984), proteins (Gutteridge and Stocks, 1981) and DNA (Floyd, 1981; Gutteridge and Toeg, 1982), or move as an Fe-citrate complex between transferrin, the cell cytoplasm, mitochondria and ferritin. This can provide iron to catalyse the Fenton reaction (Halliwell and Gutteridge, 1989 pp 22-85) but as part of the defence against ROS-induced damage, is kept to a minimum. Where the iron is bound to a particular region of a biomolecule, the generation of $OH\cdot$ will be targeted to that residue, rendering the molecule susceptible to site-specific damage. This occurs with iron bound to DNA and is discussed further in section 1.1.5.4.

1.1.4: Production of ROS in vivo

In the preceding sections, the production of ROS has been described in mechanistic terms; the following sections describe the biochemical reactions that generate these products in vivo. Particular attention will be paid to mechanisms relating to inflammation.

1.1.4.1: Arachidonic Acid Metabolism

When phagocytic cells are activated, phospholipase $A_2$ cleaves enzymatically arachidonic acid from membrane phospholipids allowing it to be subsequently oxidised by either of two metabolic processes: dioxygenation catalysed by the haem enzyme cyclooxygenase, to produce prostaglandin metabolites, or
mono-oxygenation catalysed by the non-haem iron enzyme lipoxygenase to produce mono-hydroxy-eicosa-tetraenoic acids (HETES) and hydroperoxy-eicosa-tetraenoic acids (HPETES). Both pathways give rise to free radical intermediates, one of which, possibly OH\(^{-}\), may oxidatively deactivate cyclooxygenase, thereby modulating the inflammatory process by negative feedback (Ogino et al., 1978).

1.1.4.2: Peroxisomes

These detoxifying organelles contain many oxidases (eg. glycolate oxidase, D-amino acid oxidase, fatty acyl oxidase), all of which produce \( \text{H}_2\text{O}_2 \). Of this, 40-80% is destroyed by intracellular catalase, and the remaining 20-60% diffuses easily through the peroxisome membrane and accounts for 35% of \( \text{H}_2\text{O}_2 \) produced in the liver (Boveris, Oshino and Chance, 1972).

1.1.4.3: Endoplasmic Reticulum and Nuclear Membranes

Cytochrome P450/P450 reductase and cytochrome b\(_5\) reductase are present in the endoplasmic reticulum and nuclear membranes and catalyse desaturation, demethylation and hydroxylation reactions of endogenous substrates or foreign substances, such as drugs. They normally require NADPH and NADH as electron donors but may become uncoupled during the catalytic cycle to produce \( \text{H}_2\text{O}_2 \). The mechanism for this uncoupling is not understood (Freeman and Crapo, 1982).

1.1.4.4: Mitochondria

In aerobic conditions, mitochondria are responsible for production of the major intracellular sources of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (Loschen et al., 1974). The 4 electron reduction of \( \text{O}_2 \) to \( \text{H}_2\text{O} \) on the inner mitochondrial membrane, through the respiratory assemblies does not generate ROS. However, when the electron transport chain is highly reduced and the respiratory rate is dependent on ADP availability (eg. in ischaemic tissue) then leakage of electrons occurs to produce \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (Boveris and Chance, 1973).

1.1.4.5: Autoxidation of Small Molecules

Small molecules such as hydroquinones, catecholamines, reduced ferredoxins and thiols autoxidise to produce \( \text{O}_2^- \). They can also reduce chelated iron to catalyse the Fenton reaction eg. the NADPH-supported enzymatic reduction of molecular oxygen by ferredoxin-ferredoxin: NADP\(^+\) oxidoreductase
produces free OH\textsuperscript{-} (Morehouse and Mason, 1988).

The reaction of haemoglobin with low concentrations of H\textsubscript{2}O\textsubscript{2} also gives rise to ROS but it is generally accepted that the less reactive ferryl radical (FeO\textsuperscript{2+}) and not OH\textsuperscript{-} is the main oxidant in this system (Puppo and Halliwell, 1988).

This autoxidation process is also responsible for the toxicity of some drugs, including paracetamol and metronidazole (Clark, Cowden and Hunt, 1985), and for the anti-tumour activity of bleomycin (Lown and Sim, 1977). Drugs that contain the hydralazine moiety, eg. hydralazine and isoniazid can also be enzymatically activated to form free radical derivatives (Sinha, 1983); those derived from hydralazine metabolism having been shown to cause DNA strand breaks (Sinha and Patterson, 1983).

1.1.4.6: Cytosolic Sources

Many cytosolic oxidases and oxygenases have transition metal ions at their active site and generate free radicals during redox cycling. Free radicals can also be produced when the metabolic status of a tissue changes, influencing an enzyme substrate. This is illustrated by xanthine oxidase, a cytosolic enzyme normally present in its dehydrogenase form which uses NAD\textsuperscript{+} as its electron acceptor and converts xanthine or hypoxanthine to uric acid:

\[
\begin{align*}
12 & \quad \text{Hypoxanthine} + H_2O + NAD^+ \rightarrow Xanthine + H^+ + NADH \\
13 & \quad Xanthine + H_2O + NAD^+ \rightarrow \text{Uric acid} + H^+ + NADH
\end{align*}
\]

However, during ischaemia, reversible sulphhydryl oxidation or irreversible proteolytic modification converts the enzyme to an oxidase form in a Ca\textsuperscript{2+}-dependent process (Della Corte and Stirpe, 1972). In the reperfusion stage, following ischaemia, the oxidase uses molecular oxygen (O\textsubscript{2}) as its electron acceptor to break down the accumulated xanthine. This produces O\textsubscript{2}\textsuperscript{-}:

\[
\begin{align*}
14 & \quad \text{Xanthine} + H_2O + O_2 \rightarrow \text{Uric acid} + 2O_2^- + 2H^+
\end{align*}
\]

This exchange of electron acceptors with subsequent generation of O\textsubscript{2}\textsuperscript{-} has been suggested to be important in ischaemia/reperfusion injury (McCord, 1985) (section 1.1.7).

1.1.4.7: Plasma Membranes

The plasma membrane is a primary target of oxygen radicals owing to its vulnerable interfacial position between intra- and extra-cellular sources of radicals and because of its high content of peroxidisable
lipids and proteins (see sections 1.1.5.1 and 1.1.5.2). However, one of these proteins, the NADPH oxidase enzyme, is a source of ROS believed to be important in the defence of phagocytes against invading pathogens (Babior, Kipnes and Curnutte, 1973).

The NADPH oxidase consists of an electron transport chain containing cytochrome b$_{245}$ (Segal and Jones, 1978) (which becomes reduced on stimulation of neutrophils), and a 44-45 KD flavoprotein which has non-covalently bound FAD as a prosthetic group (Cross and Jones, 1982). Cytochrome b$_{245}$ is an unusual cytochrome with a very negative redox potential and is localised both in the plasma membrane and in the membrane of the specific secretory granules that fuse with the phagocytic vesicle soon after ingestion of bacteria (Michell, 1983).

Before phagocytosis can occur, the invading pathogen must be coated with host-derived proteins. Once in the vesicle, bacteria are destroyed by exposure to proteases released from cytoplasmic granules and from exposure to ROS generated by the phagocyte (Babior, 1978).

The generation of ROS by phagocytes (neutrophils, sensitised monocytes, macrophages and eosinophils) is achieved by a respiratory burst. During this, oxygen uptake is increased and the hexose monophosphate shunt metabolises glucose to produce NADPH. Membrane bound NADPH oxidase is activated to produce $O_2^{-}$ and $H_2O_2$ using NADPH as an electron donor and regenerating NADP$^+$ for further glucose oxidation (Segal, 1984).

Release of ROS plays a fundamental role in microbial killing and is triggered by perturbations in the phagocyte plasma membrane mediated by the Fc region of immunoglobulins (Ig) within immune complexes (Weiss and Ward, 1982; Ryan et al., 1990), complement (C) component C5a (Goldstein et al., 1975), aggregated IgG (Messner and Jelinek, 1970), the cytokines interleukin (IL) 1 and 6 and tumour necrosis factor alpha (TNF$\alpha$) (Ward, Warren and Johnson, 1988).

Root and Metcalfe (1977), have suggested that 80% of the $O_2^{-}$ generated is converted to $H_2O_2$ by dismutation. This can then react to generate the more reactive OH$^{-}$, but may also diffuse into the surrounding environment before doing so.
Chronic granulomatous disease (CGD), a condition inherited as an autosomal recessive or X-linked trait, provides strong evidence for the direct role of oxygen radicals in bacteriocidal and cytotoxic activities of phagocytic cells in vivo. The genetic lesion in CGD leads to a failure to phosphorylate the 44-45 KD flavoprotein (Segal et al., 1985) or a lack of the cytochrome b$_{245}$ (Segal et al., 1983). As a result, neutrophils fail to have a respiratory burst following bacterial stimulation and patients suffer from recurrent bacterial infections (Johnston and Newman, 1977).

1.1.4.8: Ultra-Violet Irradiation

UV radiation, with a wavelength of 260nm, generates ROS and is strongly absorbed by DNA bases (Deering, 1962). This can cause the formation of hydroxylated residues such as thymine glycol (Leadon, 1987; Leadon, 1990) or the photochemical fusion of two adjacent pyrimidines into non-pairing dimeric structures (Hart, Hall and Daniel, 1978) which have the potential to cause mutations (Lehmann et al., 1977). The DNA of an epithelial skin cell exposed to normal daylight would acquire thousands of dimers and base adducts per day were these not removed by repair enzymes (D’Ambrosio et al., 1981; Freeman et al., 1987).

Xeroderma pigmentosum, a human skin disease, is caused by a genetic defect in the enzymes that remove dimers and other UV-induced lesions (Cleaver, 1968) (see section 1.1.5.4.7). Some patients with systemic lupus erythematosus (SLE) also show enhanced sensitivity to UV light demonstrated by a characteristic erythematosus butterfly rash on the face, or by a more generalised maculo-papular rash on all light-exposed skin (Kochevar, 1985). However, the genetic and/or biochemical lesion responsible for this response remains to be identified (see sections 1.2.2.1 and 1.2.6).

These observations suggest that it is as important to repair ROS-induced lesions as it is to restrict their formation.

1.1.5: Reaction of ROS with Biomolecules

Reactions of ROS with biomolecules are generally of three types: a) abstraction of a hydrogen or halide ion, b) addition to unsaturated bonds (eg. purine and pyrimidine bases and unsaturated lipids) or c) electron transfer which may result in production of a secondary radical.
1.1.5.1: ROS and Lipids

Polyunsaturated fatty acids (PUFA) of membrane lipids are particularly susceptible to oxidative damage because their double bonds are usually unconjugated and separated by methylene groups. This leads to a lower bond dissociation energy of the methylene C-H bonds and makes the hydrogen more susceptible to abstraction by any species that has sufficient reactivity to cleave this bond.

The process of lipid peroxidation can be divided into three phases: initiation, propagation and termination (Aust and Svingen, 1982). Initiation involves abstraction of a hydrogen atom resulting in formation of a carbon radical.

\[
\text{Initiation: } \text{LH} + \text{Fe(III)} \rightarrow \text{L}^\cdot + \text{Fe(II)} + \text{H}^+ 
\]

In PUFA this tends to be stabilised by a molecular rearrangement producing a conjugated diene which reacts rapidly with oxygen to give a hydroperoxy radical.

\[
\text{Propagation: } \text{L}^\cdot + \text{O}_2 \rightarrow \text{LOO}^\cdot \\
\text{LOO}^\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}^\cdot
\]

Propagation of lipid peroxidation may also arise from \text{LOO}^\cdot reacting with another divinyl methane of the same molecule or a different molecule to generate \text{LOOH} and \text{L}^\cdot.

Alternatively transition metal ions can cleave \text{LOOH} to reactive \text{LOO}^\cdot; this can then react with LH, thereby exacerbating the reaction by increasing the rate of decomposition of hydroperoxide into the chain initiators.

Once initial hydrogen abstraction has occurred, lipid peroxidation is essentially autocatalytic (Logani and Davies, 1980) and continues until an antioxidant intervenes eg. vitamin E, or the substrate has been used up, ie. no further radical species remain to react (Slater, 1984).

\[
\text{Termination: } \text{L}^\cdot + \text{L}^\cdot \rightarrow \text{LL} \\
\text{L}^\cdot + \text{LOO}^\cdot \rightarrow \text{LOOL} \\
\text{LOO}^\cdot + \text{LOO}^\cdot \rightarrow \text{LOOL} + \text{O}_2
\]

Lipid peroxidation can be initiated by \text{OH}^\cdot, alkoxy radicals (RO^\cdot) and peroxo radicals (ROO^\cdot) but not \text{H}_2\text{O}_2 or \text{O}_2^\cdot (reviewed by Halliwell and Gutteridge, 1984). The protonated form of \text{O}_2^\cdot, \text{HO}_2^\cdot, whose formation is favoured by the environment of non polar membrane interiors, may also attack fatty acids
directly (Bielski, Arudi and Sutherland, 1983). The role of OH- as an initiator has been questioned due to poor inhibition by OH- scavengers. However, it is possible that either membrane-bound metal ions generate site-specific radicals inaccessible to scavengers (Czapski, 1984), or that only very small amounts of OH- are needed to initiate lipid peroxidation before other radicals predominate.

Lipid peroxidation may cause decreased fluidity and membrane potential, increased permeability to ions or in the case of extensive damage, membrane rupture (Fong et al., 1973). Released lipid peroxides may be cytotoxic, block macrophage action, inhibit protein synthesis, inactivate enzymes and kill bacteria (Slater, 1984). They can also cause site-specific cleavage of double stranded DNA (dsDNA) (Inouye, 1984), and production of clastogenic material which reacts with DNA to form chromosome aberrations and sister chromatid exchanges (Khan and Emerit, 1985). Recently, evidence for a role of circulating lipid hydroperoxides in atherogenesis has been suggested (Carew, Schwenke and Steinberg, 1987). The process of lipid peroxidation increases both the uptake of low density lipoproteins by macrophages, and deposition of these lipid laden foam cells in the arterial intima (Frei, Stocker and Ames, 1988). Mechanisms that act to degrade lipid hydroperoxides in human plasma could therefore be helpful in the prevention of atherosclerosis.

1.1.5.2: ROS and Proteins

Proteins are major components of both the intracellular and extracellular matrix and are important targets for ROS. All levels of the protein structure may be attacked by ROS resulting in fragmentation, aggregation, cross-linking and modification of individual amino acid residues.

Cysteine, and the aromatic amino acids tryptophan and tyrosine, with their unsaturated structures, are the most sensitive to oxidative attack but other amino acid residues may be altered (Adams et al., 1972). For example, histidine residues are often located at metal binding sites of proteins, the metal ions (copper in particular) catalysing the production of OH- (Kleinveld et al., 1989).

The biological consequences of amino acid modification are illustrated by α-1 antitrypsin. Oxidation of a critical methionine residue modifies the enzymic activity and biological function of this protein (Matheson, Wong and Travis, 1979; Gee, Kittridge and Willson, 1985). Proline is also susceptible to attack by ROS and causes fragmentation of proteins such as collagen, following spontaneous hydrolysis.
of these residues after OH- attack (Wolff, Garner and Dean, 1986).

Aggregation of proteins following ROS attack has been shown in the acute phase protein caeruloplasmin (Winyard et al., 1984), and IgG (Griffiths et al., 1988a), and is due partly to disulphide bond rearrangements and partly to uncharacterised amino acid interactions. Histidine (Verweij, Dubbelman and Van Steveninck, 1981), lysine and tyrosine have all been suggested to contribute to protein cross-linking.

Oxidation of proteins may cause gross conformational changes and may also affect their hydrophobicity. In addition, many proteins are rendered more susceptible to enzymic hydrolysis (Davies and Lin, 1988) and this is likely to be due to structural deformation facilitating access of degradative proteases.

1.1.5.3: ROS and Carbohydrates

Simple monosaccharides can autoxidise under physiological conditions to produce O$_2$' (Wolff, Crabbe and Thorvalley, 1984) and ROS can react with carbohydrate polymers such as hyaluronic acid to cause fragmentation (McCord, 1974; Halliwell, 1978b).

Irradiation of carbohydrates (Bucknall et al., 1984) or exposure to Fenton systems (Gutteridge, 1981) liberates carbonyl compounds from opening of the ring structure which can react with thiobarbituric acid (TBA). Deoxyribose, the sugar in the repeating unit of the DNA backbone, reacts with OH-, and if treated under acidic conditions produces malondialdehyde, a mutagenic product which may be detected by its ability to react with TBA to form a pink chromogen (Gutteridge, 1981; Gutteridge 1987). The anti-tumour antibiotic, bleomycin, also attacks deoxyribose through production of a reactive oxygen intermediate (Wu, Kozarich and Stubbe, 1983) and both these processes result in DNA strand breakage.

The distribution of sugars within a cell, as part of the DNA molecule or as integral components of glycoproteins as well as free sugar monomers or polymers is such, that damage to carbohydrate residues affects not one but many cellular biomolecules.
1.1.5.4: ROS and DNA

1.1.5.4.1: General Aspects

Modifications to DNA by ROS are particularly important in cell development because DNA contains the genetic material which encodes for constitutive proteins. Any ROS-induced modifications not deleted from the genetic material may become incorporated into germ-line genes and cause mutation, which in turn may form proteins with abnormal functions.

DNA is made up of deoxyribonucleotides linked together by phosphodiester bridges. The base moieties form an inner core of the molecule surrounded by phosphate and deoxyribose units. Damage to DNA by ROS can occur: a) directly as a result of chemical reaction of exogenous or endogenous agents or their metabolites with DNA (eg. production of OH· from radiation-induced ionisation of water molecules - section 2.4.1) or b) indirectly by secondary intermediates upon interaction with non-DNA targets such as interactions of lipid peroxides with DNA (section 1.1.5.1).

Much of the characterisation of DNA damage by ROS has been done by radiation biologists and although the doses used do not usually reflect those that are physiologically relevant, identification of ROS-induced products by these methods may be used to identify evidence of ROS-induced damage in disease situations by modification of sensitivity levels.

1.1.5.4.2: DNA Strand Breaks

Irradiation of DNA in aqueous solution causes single-strand breaks (Rhaese and Freese, 1968), double-strand breaks, base liberation (Rhaese and Freese, 1968), base damage (Massie, Samis and Baird, 1972) and intra- and inter-strand cross-links (Lesko, Drocourt and Yang, 1982), all mediated by the actions of ROS.

These changes are mediated predominantly by ionising radiation producing OH· in solution: this reacts directly with DNA by hydrogen abstraction from deoxyribose to form a carbon centred radical and a direct strand break. Alternatively, OH· can react by addition to the pyrimidine bases forming a base adduct which may also result in a strand break. Damage formed by ionising radiation is likely to be randomly distributed throughout the DNA structure (Ward, 1981).
Reaction of $H_2O_2$ with DNA can also produce DNA damage. This reaction requires metal ion catalysis. If the metal ions are bound to DNA, the $OH^-$ produced will only diffuse a maximum of 1.5 nm before reacting (Ward, Blakely and Joner, 1985). This is less than the DNA helix diameter and therefore, the majority of $OH^-$ produced will react with the DNA in a site-specific manner.

Iron binds firmly to DNA (Mello Filho, Hoffmann and Meneghini, 1984) and copper has an important role in stabilising the association between DNA supercoils and the non-histone nuclear matrix (Minchenkova and Ivanov, 1967). Several classes of proteins involved in DNA binding or gene regulation also contain metal-binding domains (Berg, 1986). $H_2O_2$ can react with these metal ions to generate $OH^-$ (Mello Filho, Hoffmann and Meneghini, 1984) or can reduce the metal ions back to their original state (Halliwell and Gutteridge, 1984). The replenished metal ions can then generate further radical species, including $O_2^-$ and the perhydroxy radical which also plays a role in DNA nicking.

In the case of Cu$^+$ complexing with DNA, the interaction is so strong ($Ka = 10^9 M^{-1}$) that $OH^-$, generated in a Fenton reaction at the copper binding site (possibly G-C base pair regions), reacts site-specifically with the DNA, in a reaction not significantly affected by the presence of $OH^-$ scavengers (Stoewe and Prütz, 1987; Dizdaroglu, Aruoma and Halliwell, 1990).

1.1.5.4.3: Base Damage

Reaction of ROS with DNA, as well as producing strand breaks, can also cause base changes and base release. Deoxyguanosine is particularly susceptible to attack by several radical producing drugs. These include bleomycin (Halliwell and Gutteridge, 1989 pp 416-508), and cis-platinum which forms an intra-strand cross-link between N-7 atoms of adjacent guanosine residues (Pinto and Lippard, 1985). Regions of polydeoxyguanosines have also been shown to exert some specificity to the production of $OH^-$ by $H_2O_2$ and metal ions (Sagripanti and Kraemer, 1989). This has been attributed to the electronegativity of deoxyguanosine (Mattes, Hartley and Kohn, 1986) and its ability to bind metal ions (Zimmer and Venner, 1970).

One important oxidative product of deoxyguanosine is 8-hydroxydeoxyguanosine (8OHGDG), formed by the addition of $OH^-$ to C-8 of the native base (Kasai and Nishimura, 1984a). Other important oxidative products of DNA bases include 5-hydroxymethyluracil (Teebor, Frenkel and Goldstein, 1984; Lewis
and Adams, 1985), thymine dimers (Freeman et al., 1987), thymine glycol (Leadon, 1987; Leadon 1990), 8-hydroxyadenine (8OHA) (Hissung et al., 1981) and 5-hydroxymethylcytosine (Childs, Ellison and Pilon, 1983).

1.1.5.4.4: DNA Cross-links
A further type of DNA damage is the production of cross-links. These can be between DNA and protein such as the linkages of thymine and lysine induced by OH· (Dizdaroglu and Gajewski, 1989). Lysine constitutes a high proportion of the amino acids in histones and helps binding to chromatin. Cross-links can also be formed between lipid peroxidation products and DNA (Fujimoto, Neff and Frankel, 1984). The nuclear membrane is particularly susceptible since lipid peroxidation products react predominantly within the membrane structure. DNA may act to quench the peroxidation reaction by reacting with peroxy radicals which in turn generate guanyl radicals, resulting in site-specific cleavage of DNA at guanine residues (Inouye, 1984). In addition, reaction of malondialdehyde, a lipid peroxidation product, with DNA causes inter-strand cross-links with guanine, cytosine and adenine (Brooks and Klamerth, 1968) and DNA-protein cross-links (Summerfield and Tappel, 1984).

1.1.5.4.5: DNA Repair
Although ROS are capable of inducing all these modifications studying the rates of reaction with any one species, Massie, Samis and Baird (1972) showed that damage to DNA is inflicted as: base destruction > single strand breaks > double strand breaks > cross-links. This is related to the half-lives of the damage: thymine glycol has a half-life of 7 minutes (Mattern, Hariharan and Cerutti, 1975) when compared to 4 minutes for a single strand break (Ward, Blakely and Moberly, 1983) and it is thus more likely that a second radical species produced in the vicinity of the first will react with the site of base damage.

Any change in DNA induced by ROS has the potential to be mutagenic if it is not correctly repaired. The ability of cells to recover from ROS-induced damage to DNA has provided information regarding the lethality of certain DNA lesions. Singly damaged sites are unlikely to affect the cell if efficient repair can take place (Cantoni, Murray and Meyn, 1986). With the undamaged strand of the double-stranded DNA acting as a template if base insertion is needed, the fidelity of the genetic code can be maintained. However, lesions present on both DNA strands in a local region cause more problems for
cellular repair. In addition, all double strand breaks are not equally lethal. Those with constituent single strand breaks directly opposite are more lethal than those which are offset (Ward, 1988).

A key enzyme in the repair of DNA strand breaks is ADP ribosyl transferase (ADPRT) (Purnell, Stone and Whish, 1980). Strand breaks are a cofactor for ADPRT (Benjamin and Gill, 1980) which has a role in excision repair, especially at the ligation step. ADPRT uses NAD$^+$ to catalyse the repair of strand breaks. If there are sufficient strand breaks to deplete the cell of its supplies of NAD$^+$ (which at the same time depletes ATP stores), the cell dies (Schraufstätter et al., 1985; Schraufstätter et al., 1986).

Modified bases are removed from DNA by repair enzymes if they are recognised by the cell as foreign. Specific glycosylases have been identified which remove 5-hydroxymethyluracil (Hollstein et al., 1984; Boorstein, Levy and Teebor, 1987) and 5-hydroxymethylcytosine (Cannon, Cummings and Teebor, 1988) from DNA.

Any damage to DNA induced by ROS is strongly influenced by the surrounding environment. Irradiation of DNA initially produces thymine $T^-$ and guanine $G^+$ radicals which are the precursors to single and double strand breaks. However, localised protein, contained in complexed histones, can increase the production of $T^-$ by electron transfer and thus enhances the production of strand breaks (Cullis et al., 1987).

1.1.5.4.6: Cellular DNA Damage

Cellular DNA is packaged into the nucleus and is separated from extracellularly produced ROS by plasma and nuclear membranes. $H_2O_2$ is lipophilic and can diffuse through lipid bilayers and can react with DNA (Allan et al., 1988). However, it can also react with the membrane components to produce secondary products. This mechanism is proposed to explain the generation of clastogenic factors (Emerit and Michelson, 1980). Clastogenic factors are produced during chronic inflammation and are also observed as an indirect effect of ionising radiation (Hallowell and Littlefield, 1968). They are characterised as low molecular weight peroxidation products ($<10,000$ Da) and their appearance in cells correlates with increased DNA damage (Emerit and Cerutti, 1982). Several lipid peroxidation products have been characterised and malondialdehyde is known to be both clastogenic and mutagenic (Petkau, 1980).
DNA from normal cells incubated with clastogenic factors in vitro undergoes chromosome breakage and rearrangements, such as sister chromatid exchanges (Emerit and Michelson, 1980); this transferable property of clastogenic factors rules out a short lived radical species and may contribute to both the persistence of DNA damaging agents and to the distance over which the damage is done. Inhibition of arachidonic acid metabolism reduces the effects of clastogenic factors and is further evidence for the involvement of lipid peroxidation products in DNA damage (Emerit, Levy and Cerutti, 1983).

1.1.5.4.7: DNA Damage in Disease

The importance of DNA damage and more importantly its repair is illustrated in several disease states. Patients with xeroderma pigmentosum have increased sensitivity to UV light correlated with an inability to excise UV-induced DNA damage or to seal daughter strand gaps left after DNA synthesis on a damaged template (Cleaver, 1968). Cockayne syndrome is also characterised by severe photosensitivity as well as skeletal and retinal abnormalities; patients have an increased sensitivity to UV irradiation and increased levels of sister chromatid exchanges (Guzzetta, 1972). The defect identified in these patients is not an inability to remove pyrimidine dimers but an inability to repair transcriptionally active DNA at a rate faster than other DNA (Venema et al., 1990). Bloom's syndrome is associated with a high incidence of cancer (Bloom, 1966; German and Passarge, 1989) and cells show an elevated frequency of sister chromatid exchanges (Latt et al., 1983) and genomic instability (German, 1983). An elevated level of $O_2^-$ has been shown to produce increased DNA damage and lipid peroxidation, and this increased oxidative stress is a likely explanation for the cancer prone phenotype of Bloom's syndrome (Emerit and Cerutti, 1981).

Fanconi's anaemia is another disease associated with an increased rate of spontaneous mutation arising from chromosomal instability and may be due to a defect in the DNA repair enzyme DNA ligase (Hirsch-Kauffmann et al., 1978; Schroeder, 1982). However, Porfirio et al., (1989) have shown that desferrioxamine can exert a partial correction of chromosome instability which suggests that free radical formation and processing in the cells are crucial events in the development of Fanconi's anaemia.

Repair of DNA damage has also been investigated in autoimmune diseases which appear to associate with a higher incidence of malignancy than normal age and sex matched controls (Louie and Schwartz, 1978). In SLE it was found that there is defective repair of the adduct $O^6$-methylguanine (Harris et al.,
1982), a potent premutagenic DNA base, resulting in its accumulation in lymphocytes. This defective repair mechanism could account for the associated malignancy in this disease (see section 4.2.1).

1.1.6: Defence Against ROS-Induced Damage

Several defence mechanisms have evolved to protect the cell against ROS produced during normal oxidative metabolism. These mechanisms rely on either enzymatic breakdown of the reactive species or non-specific scavenging to limit their deleterious effects.

The ability of SOD to degrade $O_2^-$ enzymatically has been described in section 1.1.2.1, and this enzyme is very effective at physiological pH. This is exemplified by the maintenance of intracellular $O_2^-$ concentrations at $10^{-11} \text{ M}$ (Freeman and Crapo, 1982). Two different SOD enzymes are found in the cell: the cytosolic enzyme has binding sites for copper and zinc (CuZn SOD) whilst the mitochondrial enzyme contains manganese (Mn SOD). Extracellular concentrations of SOD are very low (McCord, 1974) but it has been suggested that extracellularly generated $O_2^-$ may enter cells via an anion channel and become inactivated intracellularly (Lynch and Fridovich, 1978). Red blood cells, which contain $10^5 \text{ M}$ SOD, may act in this way to limit the damage caused by ROS released by activated neutrophils, plasma having little SOD activity (McCord, 1974). Whilst SOD acts to protect the cell against the actions of $O_2^-$, SOD itself becomes inactivated by the product of its reaction, $H_2O_2$ (Blech and Borders, 1983). Oxidative inactivation of SOD by $H_2O_2$ generates a modified protein, which is recognised and selectively degraded by an intracellular proteolytic pathway, and free OH-, which may account for the biological damage associated with elevated intracellular SOD activity (Yim, Chock and Stadtman, 1990). The mechanism of inactivation comprises oxidative modification of a histidine residue important in copper binding (Salo et al., 1990).

SOD is constitutively expressed in leukocytes and other tissues and levels vary little with increasing age (Niwa, et al., 1989). However, the significant induction of SOD activity by agents increasing intracellular $O_2^-$ concentrations is reduced in the elderly and may play a role in development of malignancy (Niwa, Ishimoto and Kanoh, 1990).

Catalase, a ferrirprotoporphyrin enzyme catalyses the breakdown of $H_2O_2$. This enzyme is only effective at removing $H_2O_2$ when its levels are elevated by endogenous or exogenous metabolites. Removal of
low concentrations of $\text{H}_2\text{O}_2$ produced during normal metabolism, and maintenance of an intracellular concentration of $\text{H}_2\text{O}_2$ of $10^{-6}$-$10^{-9}$M (Boveris and Chance, 1973) is regulated by glutathione peroxidase. This enzyme is found in both the cytoplasm and mitochondria (Freeman and Crapo, 1982) whilst catalase is found predominantly in peroxisomes (Boveris and Chance, 1973). As for SOD, extracellular levels of catalase are very low.

Glutathione peroxidase also catalyses the degradation of lipid hydroperoxides and DNA hydroperoxides:

\begin{align*}
15 & \quad 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \\
16 & \quad 2\text{GSH} + \text{LOOH} \rightarrow \text{GSSG} + \text{LOH} + \text{H}_2\text{O}
\end{align*}

Glutathione peroxidase is found in low concentration in the nucleus. In this organelle glutathione transferases may be important alternatives in the degradation of organic hydroperoxides (Ketterer and Meyer, 1989), having been shown to reduce the level of peroxidised lipids in vitro (Tan et al., 1984) and peroxidised DNA in the nucleus (Tan et al., 1988). Glutathione (GSH) is the most important non-protein thiol in mammalian cells and becomes oxidised to replenish SH groups on functional proteins that have themselves become oxidised. Glutathione reductase converts oxidised glutathione (GSSG) back to its reduced form at the expense of NADPH. Thus, glutathione peroxidase activity shifts the major, soluble redox active cofactors from a predominantly reduced state to a predominantly oxidised state, GSH to GSSG and NAD(P)H to NAD(P)$^+$. An increase in GSSG is also paralleled by an increase in oxidised protein-bound thiols, largely because GSSG can react with protein-bound thiols to form glutathione mixed disulphides (Farber, Kyle and Coleman, 1990). These changes in both soluble and protein-bound thiols are accompanied by a release into the cytosol of sequestered stores of calcium (Bellomo, Thor and Orrenius, 1984) which may also cause oxidation of pyrimidine nucleotides and changes in cell regulation thus mediating cell injury associated with acute oxidative stress (Bellomo and Orrenius, 1985).

The high reactivity of OH$^-$ precludes enzymic removal; prevention of damage by OH$^-$ occurs by scavenging the radical itself. Thiols of high (albumin) and low (cysteine) molecular weight scavenge OH$^-$. Albumin also binds Cu$^{2+}$ and decreases the formation of OH$^-$ in free solution at the expense of ROS damage to the protein site-specifically (Rowley and Halliwell, 1983b; Marx and Chevion, 1985). Thiol compounds also have an important protective role as donators of H$^-$. When reacting with DNA, OH$^-$ reacts with both sugar and base molecules, the reaction with the sugar involving hydrogen
abstraction. Donation of H· from a thiol results in true regeneration of the original molecule. However, the reaction of OH· with DNA bases is predominantly by addition, so H· donation by a thiol does not restore the original base (Ward, 1983). Since it is damage to the sugar rather than damage to a base that is most likely to result in the formation of a strand break (Ward, 1975), thiols appear to protect DNA predominantly from strand breaks.

Ascorbic acid scavenges both OH· and O$_2^·$ when it is present at high concentrations (> 0.5mM) forming an ascorbate free radical which disproportionates to form AA and dehydroascorbic acid (Rose, 1990). AA scavenges O$_2^·$ at 1/3 the rate of SOD (Blake et al., 1984). However, AA can also act as a pro-oxidant when present at concentrations < 20μM (Johnson, 1979) reducing iron to the ferrous form for participation in the Fenton reaction.

Caeruloplasmin can also scavenge O$_2^·$ but is only 1/3000 as effective as SOD (Goldstein et al., 1979). It is a major serum anti-oxidant and as an acute phase protein its concentration is increased during inflammation when there is an increase in radical production. It is also important for its ability to oxidise Fe$^{2+}$ to Fe$^{3+}$ reducing the availability of iron to participate in the Fenton reaction and favouring the storage of Fe$^{3+}$ by ferritin (Gutteridge and Stocks, 1981).

Uric acid, the end product of purine metabolism in mammals, is also a free-radical scavenger and important biological antioxidant. It has high reactivity with ROS and exists in high concentrations in biological fluids. Further, it can also complex iron and therefore inhibit the Fenton reaction (reaction 6) (Hochstein, Hatch and Sevanian, 1984).

Vitamin E is the major lipid soluble anti-oxidant reacting with O$_2^·$, OH· and lipid peroxides (Packer, Slater and Willson, 1979). As a membrane soluble vitamin it can inhibit lipid peroxidation by scavenging peroxy and alkoxy radicals so breaking the chain reaction (Redpath and Willson, 1973).

These reactions aim to minimise the damage caused by ROS under normal metabolic conditions. However, during periods of increased oxidative stress or when defence mechanisms are impaired these processes may become overwhelmed and damage to cellular molecules will occur.
1.1.7: ROS and Tissue Damage

Most if not all tissues may suffer damage from ischaemia/reperfusion events. During ischaemia (ie. poor oxygenation of tissue) ATP levels fall, xanthine accumulates and calcium activates the protease, calpain, to cleave a bond in the enzyme xanthine dehydrogenase forming xanthine oxidase (McCord, 1985). Upon reperfusion of this ischaemic tissue, damage occurs when xanthine oxidase degrades the accumulated xanthine thus producing high levels of $\text{O}_2^\cdot$$. The levels of $\text{O}_2^\cdot$ produced are sufficient to inactivate enzymes and following injury to the brain for example, glutamine synthetase is inactivated causing increased levels of L-glutamate, which may be important in the inflicted neurotoxicity following ischaemia/reperfusion events (Oliver et al., 1990).

Inflammation is also associated with considerable tissue injury. Phagocytic cells can be stimulated by bacterial or immunological stimuli, and the NADPH oxidase activated, to produce ROS (see section 1.1.4.7). Bacterial stimulation of macrophages releases IL1 and TNF, cytokines that act on many cells and have a variety of pro-inflammatory effects (Ward, Warren and Johnson, 1988). For example IL1 and TNF both directly initiate oxidant production by phagocytes and prime macrophages (Warren et al., 1988) and neutrophils (Berkow et al., 1987) for enhanced $\text{O}_2^\cdot$ responses in the presence of IgG immune complexes. They also cause non-cytotoxic modification of endothelial cells on contact, enhancing the susceptibility of these cells to ROS-mediated damage (Pober et al., 1986).

Immune complexes also stimulate neutrophils to release ROS and proteases in a complement mediated process (Weiss and Ward, 1982; Ryan et al., 1990). Deposition of immune complexes causes complement activation and the generation of C5a, a chemotactic factor needed for recruitment of neutrophils from the vascular compartment. ROS released from activated neutrophils subsequently causes the release of epithelial and endothelial cells from basement membranes which contributes to tissue injury (Ward PA et al., 1987). For example, in nephritis, immune complex-mediated tissue injury causes release of these cells from the glomerular basement membrane which results in permeability defects in the kidney (Harlan et al., 1981).

During the inflammatory response many cell types can contribute to tissue injury and platelets accumulating in the vascular lumen also accentuate the response of neutrophils to produce $\text{O}_2^\cdot$ (Ward PA et al., 1987).
The effects of tissue damage may, however, result in cells increasing their defence against oxidative stress (Brown et al., 1990). Pretreatment of cells with IL1 can induce mRNA for SOD (Wong and Goeddel, 1988), and TNF primes granulocytes, enhancing $O_2^-$ released by a second stimulus (Berkow et al., 1987) such that an early oxidant stress may decrease the effects of a subsequent oxidant insult by increasing the levels of endogenous antioxidants and perhaps influencing other defence mechanisms also.

ROS have not only been associated with tissue injury but also in the pathogenesis of certain disease processes. A well studied example is the chronic inflammatory disorder, rheumatoid arthritis (RA).

ROS, thought to originate from activated phagocytes in the inflamed joint, can cause destruction of cartilage (Dean, Roberts and Forni, 1984), decrease the de novo synthesis of cartilage-proteoglycan (Schalkwijk et al., 1985), peroxidise cell membranes (Lunec et al., 1981), depolymerise hyaluronic acid (Greenwald and Moy, 1980) and attack proteins (Blake et al., 1986). Protein modification by ROS in RA may have particular relevance since most patients possess serum antibodies directed against their own IgG (known as rheumatoid factors). It has been suggested that the IgG has been altered, possibly by ROS, thus acquiring antigenicity (Lunec, Griffiths and Brailsford, 1988).

In vitro, ROS can modify IgG, inducing fluorescent changes (Wickens et al., 1983), an increased thiol group content (Kleinveld et al., 1986), and changes in constituent amino acids (Griffiths et al., 1988b). Further, ROS-altered IgG has been shown to have an increased binding to serum rheumatoid factors (Lunec et al., 1986; Griffiths and Lunec, 1988; Swaak et al., 1989).

Although the role of ROS in the pathogenesis of RA is not definitive, the tissue damage caused by increased ROS production highlights the need to control carefully reactions involving or producing ROS.
CHAPTER 1.2: SYSTEMIC LUPUS ERYTHEMATOSUS

1.2.1: General Aspects

Systemic lupus erythematosus (SLE) is a chronic inflammatory connective tissue disorder which affects many organs of the body and is characterised by the presence of antibodies against nuclear components (anti-nuclear antibodies - ANAs), particularly against dsDNA.

It affects approximately 0.1% of the population and is more common in coloured races, with an overall prevalence of approximately 1 in 700 women increasing to 1 in 250 amongst black women in the USA. It is about nine times more common in young women than men with a peak incidence between 20 and 40 years of age.

SLE is extremely variable in its clinical manifestations, many of which appear to be due to the consequences of a widespread vasculitis affecting capillaries, arterioles and venules. There are general features such as fever and malaise which are very common, particularly during exacerbations of the disease. Rashes occur in \( \approx 80\% \) of patients, the characteristic erythematosus butterfly rash being evident in \( \approx 50\% \). Skin lesions have a predilection for sun exposed areas and there is marked photosensitivity in one third of patients. Polyarthralgia is one of the commonest features occurring in \( \approx 90\% \) of patients and is frequently the first manifestation of the disease. Although the joints are painful, radiological erosions typical of RA are rarely seen. An important manifestation of SLE is nephritis, which occurs in \( \approx 50\% \) of patients and contributes to most of the deaths due to SLE (Dubois et al., 1974; Rosner et al., 1982). Mild to moderate proteinuria is the most common presenting feature of lupus nephritis and is usually associated with serological abnormalities indicative of immune complex formation such as low serum complement levels. The glomerular damage typical of SLE is associated with granular deposits of immunoglobulin and complement which accumulate in the capillary walls. Other manifestations of this multisystem disease include involvement of the lungs (50% - pleural effusion, pneumonitis), the heart (40% - pericarditis, myocarditis, endocarditis), the central nervous system (60%) and the blood (75% - anaemia, leucopenia and thrombocytopenia).

The systemic nature of SLE was first described by Kaposi in 1872 and the disease was thought of as a frequently fulminating, progressive and fatal condition. Today, sensitive serological techniques allow...
diagnosis of milder forms of the disease displaying a broader clinical spectrum than was once thought.

In most cases the disease pursues a chronic unpredictable course in which episodes of activity are interrupted by periods of clinical remission (Dieppe et al., 1985).

1.2.2: Aetiology

The aetiology of SLE is unknown. It is thought that a combination of environmental, genetic and immunological factors are involved predisposing to polyclonal B cell activation, production of anti-nuclear and other autoantibodies and impaired T cell regulation.

1.2.2.1: Environmental Factors

Environmental factors which contribute to the development of SLE include infection, exposure to sunlight and drugs.

The hypothesis that SLE may be due to a defective host response to an exogenous infectious agent (eg. viral) has been suggested. The mechanism of action of viruses may involve: selective inactivation of suppressor lymphocytes thereby allowing proliferation of autoreactive B cell clones, selective infection of autoreactive B cells thereby stimulating their continued proliferation or transformation of B cells already reacting with an unrelated antigen (Denman, 1981). In addition, retroviruses have been implicated in mouse models of lupus (Mountz and Steinberg, 1989). However, there is no conclusive evidence for persistent viral infection, and attempts to isolate type C virus from human SLE tissues have been unsuccessful.

Despite this, a correlation between autoimmune diseases and the expression of cellular oncogenes (Mountz et al., 1984) and retroviral genes (Krieg, Khan and Steinberg, 1988) has been shown but it is again not clear whether abnormal expression of these genes leads to autoimmune disease or predisposes to it.

SLE is exacerbated by exposure to sunlight in 30% of cases (reviewed by Kochevar, 1985). UV light causes specific denaturation of DNA with the formation of antigenic thymine dimers, the implications of which are discussed in section 1.2.6.

Certain drugs, notably hydralazine and procainamide can cause drug-induced SLE (Hess, 1988). Drug
induced lupus occurs more readily in slow acetylators, and hydralazine-induced lupus shows a close association with the major histocompatibility complex (MHC)-related antigen, HLA DR4 (see section 1.2.2.2). It is more common in the elderly and the clinical picture is very similar to idiopathic SLE in this age group, with manifestations involving predominantly the joints, heart and lungs but rarely renal complications. ANAs are present in high titres and react predominantly with histones. Anti-dsDNA antibodies common in idiopathic SLE are rarely seen in drug-induced lupus and serum complement levels usually remain normal. In addition, withdrawal of the drug usually results in rapid recovery although ANAs may persist for several months (Dieppe et al., 1985).

1.2.2.2: Genetic Factors

About 10% of patients with SLE have one or more first or second degree relatives with the same disease but data from studies on identical twins shows that only 15% of monozygotic twins are concordant for SLE (Fessel, 1988). Susceptibility to SLE is determined in part by genes located within the MHC and associations between disease and polymorphic markers of HLA DR and C-4 loci in particular have been shown.

In Caucasians there is an increased frequency of SLE patients possessing either HLA DR2 or HLA DR3 compared to healthy controls (Reinertsen et al., 1978). There is also an association between SLE and a deficiency of complement components suggesting that the complement deficiency state might predispose to SLE (reviewed by Steinberg and Klinman, 1988).

C4a binds to immune complexes (Law, Dodds and Porter, 1984) to prevent their precipitation in membranes and the deficiency of C4a found in 11% of SLE patients may interfere with normal clearance of immune complexes and their solubilisation (Schifferli et al., 1986). Further abnormalities in immune complex clearance arise from a deficiency of the CR1 receptor (Theofilopoulos and Dixon, 1981) found on erythrocytes that binds C3b and C4b. CR1 binds to immune complexes consisting of antigen-antibody-complement and transports them to the reticuloendothelial system of the liver and spleen where they are eliminated. Decreased levels of CR1 receptor have been found to correlate closely with disease activity (Ross et al., 1985).

A lupus-like disease also occurs in 40% of patients with an inherited homozygous deficiency of the
complement component C2 (Fessel, 1988). The disease has a lower incidence of renal complications and a low prevalence of antibodies to native dsDNA.

However, despite the close association of complement defects with SLE, most individuals with complement deficiencies do not develop SLE. This suggests that complement deficiencies are likely to be just one aspect of a multifactorial disorder.

The production of particular autoantibodies in SLE has also been suggested to be associated with MHC antigens. Autoantibodies to native DNA, especially those found in SLE patients with renal involvement, have been suggested to be associated with DR2 and DQw1 (Alvarellos et al., 1983), or DQw6 (Fronek et al., 1988), but this has been disputed by other investigators (Reveille et al., 1989). The role of the MHC in the autoantibody response in SLE is still unclear.

1.2.2.3: Immunological Factors

Patients with SLE have several abnormalities of the immune system. It is thought that some are inherent to the disease process whilst others may occur as a consequence of the disease and therefore are found especially or entirely during periods of disease exacerbations.

The overproduction of antibodies reactive with nuclear determinants (e.g. DNA), cytoplasmic proteins and cell membrane molecules is characteristic of SLE (Stollar, 1981). These autoantibodies are involved in several pathogenic processes including the development of glomerulonephritis and vasculitis. During periods of active disease, patients have increased numbers of activated B cells (Tan et al., 1980) resulting in increased immunoglobulin secretion (Blaese, Grayson and Steinberg, 1980).

Not all patients develop high levels of the same autoantibodies; some have increased anti-DNA antibodies, others increased anti-cardiolipin antibodies while still others develop a generalised hypergammaglobulinaemia without apparent antigenic bias (Harley and Gaither, 1988). The hypergammaglobulinaemia seen in SLE is characterised predominantly by antibodies of the IgG class (Talal, 1976).

The increase in activated B cells in SLE is accompanied by an absolute reduction in the number of
circulating T cells (Messner, Lindström and Williams, 1973). This may be partly due to the presence of anti-lymphocyte antibodies, including autoantibodies directed against various T cell subsets (Litvin, Cohen and Winfield, 1983). There appears to be a specific reduction in the numbers and activity of suppressor T cells which is suggested to be an important mechanism underlying B-cell hyperactivity and the production of multiple autoantibodies (Talal, 1976). However, suppressor cell defects are not found in all patients (Theofilopoulos and Dixon, 1985) whilst they are found in some clinically healthy family members of SLE patients (Miller and Schwartz, 1979). This suggests that suppressor T cell abnormalities are not sufficient to induce SLE.

Helper T cells and T helper cell-derived lymphokines also appear to play a central role in the pathogenesis of SLE possibly through their regulatory role on B cell function (Wofsy, 1986). In mice models of the disease, treatment with antibodies to helper T cells can reverse established renal disease and extend survival (Wofsy and Seaman, 1987).

1.2.3: Autoantibodies

Whilst the diagnosis of SLE is associated with a variety of autoantibodies, the connection between these and the clinical manifestations of the disease remains unclear. For example, anti-dsDNA antibodies are highly specific for SLE and are detected at a high frequency (75-95%) in untreated patients with active disease (Levine and Stollar, 1968). They are generally claimed to correlate with disease activity, especially in patients with renal involvement (Stollar, 1981). This may associate with their ability to form immune complexes with DNA which play an important role in the pathogenesis of lupus nephritis (see section 1.2.5).

The detection of IgG anticardiolipin antibodies also seems to be associated with particular disease manifestations, particularly thrombosis, recurrent foetal loss and thrombocytopaenia (Harris et al., 1986). Patients with these antibodies tend to have a mild form of SLE. They do not have high levels of circulating anti-dsDNA antibodies or the associated renal complications (Ishii et al., 1990).

Antibodies to histone proteins are also found in patients with SLE and drug-induced lupus, and have been shown to correlate with severity of disease, particularly lupus nephritis (Kohda et al., 1989; Gompertz, Isenberg and Turner, 1990).
In addition to anti-dsDNA, anti-histone and anti-cardiolipin antibodies other antibodies commonly found in SLE patients bind single stranded DNA (ssDNA), RNA, other nuclear antigens such as Sm, RNP, and the non-histone chromatin proteins, phospholipids, cell surface antigens and cytoskeletal proteins.

1.2.4: Anti-DNA Antibodies

Antibodies to DNA are the best studied of all the autoantibodies found in SLE (Casperson and Voss, 1983; Stollar and Schwartz, 1986; Isenberg and Shoenfeld, 1987). Anti-DNA antibodies are generally classified as either specific for ssDNA, reactive with both ssDNA and dsDNA or specific for dsDNA (Arana and Seligmann, 1967; Stollar, 1979). Anti-dsDNA antibodies are a characteristic feature of SLE whilst anti-ssDNA antibodies are found in many rheumatic disorders (Harley and Gaither, 1988).

Whilst anti-dsDNA antibodies are known to participate in immune complex formation, their relevance to the clinical expression of SLE is perplexing because of the clinical diversity among patients who share identical autoantibody profiles.

Antibodies reacting with native dsDNA recognise specific determinants on the deoxyribose-phosphate backbone not expressed on ssDNA (Steinman, Deesomchok and Spiera, 1976; Stollar and Papalian, 1980); the native helical DNA structure has its bases inaccessibly directed inward, surrounded by this protective deoxyribose-phosphate backbone. The repeating units of nucleosides and the regular turns of the helix increase the binding avidity of the antibodies due to the repeating nature of the antigenic determinants (Eilat, 1986).

Antibodies recognising purine or pyrimidine bases, or base sequences react only with denatured DNA (Stollar, 1973). This is because the anti-DNA antibodies can only react with DNA when the base-paired double helical structure has been disrupted by heat or by chemical denaturants which expose the base residues.

Antibodies which react with helical regions of ssDNA as well as dsDNA determinants (Stollar and Papalian, 1980) also show remarkable cross-reactivity with molecules other than polynucleotides, presumably by virtue of the antigenic determinant being expressed on the sugar-phosphate backbone.
The phospholipid cardiolipin, has a backbone containing phosphate esters, separated by three carbon atoms, that may mimic the DNA backbone (Schwartz and Stollar, 1985). It has been suggested that several other compounds such as vimentin (Geisler and Weber, 1982) and platelets (Shoenfeld et al., 1983) can function as antigenic targets for anti-DNA antibodies, their structures containing repeating negatively charged groups.

Antibodies to DNA found in the circulation are, themselves, very heterogeneous in their immunoglobulin class, complement fixing ability, functional activity and the tendency to bind bivalently to one antigen molecule (Sontheimer and Gilliam, 1978). They also appear to differ in their pathogenicity (Hahn, 1982). High levels of IgG antibodies are more specific for SLE and are more closely related to renal complications than IgM antibodies (Rothfield and Stollar, 1967). Antibodies of the IgA class may also associate with active nephritis (Gripenberg and Helve, 1986). Antibodies reacting with dsDNA are thought to be more pathogenic than those reacting with ssDNA (common in other autoimmune diseases). This is based on the observations that there is a relative specificity for anti-dsDNA antibodies in SLE nephritis (Stollar, 1981), a slow dissociation rate between anti-DNA antibodies and dsDNA (Taylor et al., 1979) and a preferential reaction of IgG anti-DNA antibodies, eluted from glomeruli of SLE patients, with dsDNA (Miniter, Stollar and Agnello, 1979).

The mechanism underlying the production of antibodies reactive with DNA is unclear. One theory proposes that autoantibody production in SLE is induced and perpetuated by immunisation with an autoantigen or an antigen cross-reactive with self (Eilat, 1985). DNA is inherently a very poor immunogen in its native state although it becomes more immunogenic after modification (Schwartz and Stollar, 1985). Nucleic acids altered during cell disintegration or by chemical or physical factors may induce an antigenic response. However, it has not been possible to induce SLE-like disease in animal models by immunisation with native DNA (Madaio et al., 1984). Furthermore, whilst modified DNA is more immunogenic, the altered nucleic acids tend only to induce antibodies that are highly specific for the particular modification rather than antibodies with polyreactivity to native dsDNA (Stollar, 1975).
Tan and Stoughton (1969) were able to show that native DNA irradiated with UV light was immunogenic and although producing antibodies primarily against the modified UV-irradiated DNA, low concentrations of antibodies with specificity for native DNA could be induced.
In view of the little evidence supporting DNA as the eliciting antigen in the production of anti-DNA antibodies (Schwartz and Stollar, 1985; Isenberg and Shoenfeld, 1987), attention has been diverted to the possibility that other factors are responsible for driving their production. Diamond and Scharff (1984) showed that a mutation in the V_H gene of the myeloma protein S107 (glutamine 35 replaced by alanine), led to a loss of the antibody's phosphorylcholine-binding activity and a gain in its ability to bind to DNA and cardiolipin, and have suggested that autoantibodies may arise by mutation of genes that encode antibodies against bacterial antigens. Alternatively, Naparstek et al., (1986) hypothesised that autoantibodies characteristic of SLE might be a subset of antibodies encoded by unmutated germ-line genes.

Another theory proposes that autoantibodies in SLE arise from polyclonal B cell activation (Klinman and Steinberg, 1987). Epitopes on non-nucleic acid structures can activate a large family of B cells, some of which form antibodies that can cross-react with nucleic acids. It is thought that the disordered immune regulation described previously (section 1.2.2.3) allows the spontaneous activation of B cells.

Although the mechanism for the production of autoantibodies remains to be elucidated, it is now thought that both antigen-driven and polyclonal B cell activation mechanisms contribute to anti-DNA antibody production in SLE. Low affinity IgM autoantibodies appear to arise as a consequence of polyclonal B cell activation (Levinson et al., 1987; Sanz and Capra, 1988) whilst high affinity IgG autoantibodies appear to be antigen-driven (Nakamura et al., 1988; Shlomchik et al., 1990).

However, for DNA to participate in an antigen-driven response the DNA must be not only immunogenic in nature but must also be available as an antigen for presentation to antibody producing cells.

1.2.5: Immune Complexes

Circulating immune complexes represent a normal and effective immunological effector mechanism for antigenic clearance (Contreras et al., 1982). Complement facilitates the solubilisation of these complexes by contributing the fragments C3b and C3d to the complex. These bind to receptors on phagocytic cells and facilitate antigen degradation.

There is abundant evidence that circulating DNA/anti-DNA antibody complexes are detectable in the
serum or plasma of patients with SLE (Bruneau and Benveniste, 1979; Adu, Dobson and Williams, 1981), and in those with associated nephritis DNA has been found in the diseased glomeruli (Koffler, Schur and Kunkel, 1967). IgG antibodies to DNA have also been eluted from kidneys of patients with SLE implying that DNA/anti-DNA complexes are present in these organs (Winfield, Faiferman and Koffler, 1977).

Attempts to isolate DNA from circulating immune complexes have led to differences in the sizes of fragments obtained. Adu, Dobson and Williams (1981) showed that both DNA and anti-DNA antibodies could be found in some but not all immune complexes in patients with SLE nephritis and demonstrated the ability of these complexes to activate complement in vitro. Activation of complement in vivo by such immune complexes may contribute to the tissue damage seen in SLE (Adu and Williams, 1984). Bruneau and Benveniste (1979) characterised the DNA isolated from immune complexes as being of low molecular weight and the corresponding antibodies of the IgG isotype were specific for native DNA.

Ikebe, Gupta and Tan (1983) also isolated dsDNA from immune complexes of four different sizes in both normal and SLE sera, the larger fragments of 150-240 bp and 370-470 bp most notable in the SLE patients. Sano and Morimoto (1981) confirmed the presence of low molecular weight fragments but Contreras et al., (1987) showed that DNA of 17-20 kb could be detected in immune complexes isolated by cryoprecipitation. The DNA was found to have significant homology with human genomic DNA, supporting the work of Steinman (1984), and suggesting that the circulating DNA is derived from, and composed of, sequences repeated in the human genome. The view that plasma derived DNA has a cellular origin was confirmed by Rumore and Steinman (1990) who characterised the DNA as largely double-stranded but with single-stranded regions containing nucleotide base sequences, present in the human genome and similar to oligonucleosomes, the structural units of chromatin. Further characterisation of the DNA by these workers showed that it formed a series of multimeric complexes, at least a portion of which are bound noncovalently to histone and thus protected from nuclease degradation.

One explanation for the differences in size of DNA isolated from the serum or plasma of SLE patients is that the smaller fragments of DNA isolated may in fact be degradation products of an immunogenic form of DNA generated during clearance of the molecule.
Injection of experimental animals with high molecular weight DNA leads to its rapid elimination from the circulation either via liver-mediated clearance of ssDNA or via degradation by serum and/or tissue nucleases (Emlen and Mannik, 1984). Persistence of DNA in the circulation of patients with SLE may arise because the DNA is predominantly complexed to antibodies and associated DNA binding proteins, and thus protected from the action of nucleases such as DNase. Emlen, Ansari and Burdick (1984) demonstrated protection of DNA from DNase digestion but found that the DNA was most effectively protected by bivalent F(ab')\textsubscript{2} fragments and was only 35-45 bp in size. These small lattice immune complexes would be unlikely to fix complement or deposit in tissues (Haakenstad and Mannik, 1974). However, experiments were not done in antibody excess, which would lead to larger, more pathogenically relevant immune complexes in vivo. The size and strandedness of the circulating DNA does alter the clearance kinetics and mechanisms (Emlen and Mannik, 1984). dsDNA remains in the circulation longer than ssDNA and its clearance is therefore more susceptible to variations in serum nuclease levels and has an increased chance of binding to circulating antibodies and specific tissues such as the glomerular basement membrane (Izui, Lambert and Miescher, 1976). The competition of anti-DNA antibodies with nucleases may inhibit enzymic digestion of the DNA and cause its persistence in the circulation. In addition it has been shown that there is decreased activity of circulating nucleases in SLE patients which would also contribute to a prolonged presence of DNA, particularly dsDNA in the circulation (Frost and Lachmann, 1968; Chitrabamrung, Rubin and Tan, 1981). The amount of DNA in the plasma has been positively correlated with disease activity, especially vasculitic symptoms and has an elevated level of nicks, hairpins, dG-dC content (61% compared with 38% in bulk human DNA), and other uncommon structures that may serve as targets for antibodies in SLE serum (Krapf et al., 1989).

The interactions between anti-DNA antibodies and the glomerular basement membrane may be mediated by charge. Antibodies which bind to the kidney have a high proportion of positively charged amino acids at physiological pH suggesting the presence of subpopulations of anti-dsDNA antibodies which preferentially bind to glomeruli at negatively charged sites in the basement membrane (Dang and Harbeck, 1984). Therefore, the deposition of immune complexes in the kidney may occur by trapping of circulating DNA / anti-DNA immune complexes or the initial binding of free DNA to the renal glomerulus followed by subsequent interaction with anti-DNA antibodies in situ (Izui, Lambert and Miescher, 1976).
Histones also have a high affinity for the glomerular basement membrane, related to charge (pI > 8.5) (Schmiedeke et al., 1989), and Brinkman et al., (1990) have suggested that anti-DNA antibodies might bind to DNA linked to histone proteins in the circulation to form immune complexes. These may then adhere to the glomerular basement membrane through the favourable charge interaction of histones with heparan sulphate (Brinkman et al., 1990).

A further source of DNA may arise from the polyclonal activation of B cells; human peripheral blood lymphocytes can both excrete DNA (Distelhorst, Cramer and Roger, 1978) and have DNA associated with their cell surface (Moyer, 1979). Thus, the circulating source of DNA may be from dying cells or be produced by activated lymphocytes. DNA has been shown to be associated with a cell surface receptor, the role of which is to scavenge and degrade exogenous DNA, possibly constituting a nucleotide salvage pathway (Bennett, Gabor and Merritt, 1985). There is defective binding of DNA to peripheral blood mononuclear cells from patients with SLE and autoantibodies to this receptor, which may also contribute to the elevated levels of circulating DNA in SLE (Hefeneider et al., 1990).

1.2.6: SLE and ROS

Although there is an increasing amount of evidence to suggest that ROS produced during cell metabolism (e.g. during phagocytosis), may play an important role in the pathogenesis of RA (see section 1.1.7), there has been little extrapolation of these findings to the investigation of the role of ROS in other chronic inflammatory disorders.

Several experimental findings, however, do suggest that ROS are involved in SLE. Immunisation of female rats with a DNA molecule modified by the O$_2^\cdot$ generating system, xanthine/xanthine oxidase elicited the production of antibodies that could react with both native and the modified DNA whilst native dsDNA did not induce the production of anti-DNA antibodies (Jansson, 1985). This suggests that ROS modification of DNA renders it more antigenic than the native form.

If ROS modification of DNA is important in altering the antigenic state of the molecule SLE patients are likely to be more susceptible to these effects for several reasons. It has already been discussed that DNA entering the circulation of SLE patients persists longer than in normal controls (see section 1.2.5). Furthermore, Harris et al., (1982) have shown that DNA damage in SLE is not repaired as efficiently as
in normals. This results in accumulating concentrations of altered base residues such as O\textsuperscript{6}-methylguanine thus exacerbating any effects that may be due to the formation of a ROS-denatured DNA.

Further evidence for involvement of ROS in SLE is based on the ability of hydralazine, a drug forming free radical derivatives, to induce a lupus-like syndrome (section 1.1.4.5 and 1.2.2.1) and the role of UV light exacerbating the disease manifestations. An altered form of DNA containing thymine dimers is formed following exposure of the molecule to UV light (Deering, 1962) and rabbits immunised with UV-irradiated DNA produce antibodies against this denatured DNA (Natali and Tan, 1971). Its antigenic determinant is the conformational distortion produced by the formation of the dimer residues. Sensitivity to UV is a prominent feature of SLE and Golan and Borel (1984) showed an increased sensitivity to near UV in the NZB/NZW mouse model of SLE. In this mouse model there is defective endonuclease activity and inefficient repair of the damaged DNA. Studies in SLE patients by Beighlie and Teplitz (1975) have demonstrated a much lower excision repair in lymphocytes from SLE patients compared to normals whilst D'Ambrosio et al., (1983) found levels of repair of UV-induced damage to be only similar or slightly lower in skin from patients with SLE compared to normal skin. Although the evidence is conflicting, it again appears that a ROS-modified DNA persists longer in the cells of SLE patients than in the cells of normals.

Emerit and Michelson (1980) have proposed that the photosensitivity to UV seen in SLE is manifested not by a repair enzyme deficiency but by a clastogenic factor formed from the action of free radicals on lipids. SLE serum contains this chromosome-breaking agent of low molecular weight which produces chromosomal breakage and rearrangement of DNA (Emerit et al., 1980). The breakage factor is released from SLE lymphocytes and the aberration rate induced by this clastogenic agent is reduced to normal values if SOD is added to the culture medium. Emerit and Michelson (1981) have also suggested that the clastogenic factor is itself photosensitive and reacts with UV light to produce O\textsubscript{2}\textsuperscript{•−}, which can then react with more clastogenic factor and DNA, causing excessive damage.

Whichever mechanism, if either, is responsible for the UV photosensitivity, the data from both sets of experiments suggest that there is increased susceptibility to ROS, produced by UV light. In addition to this, the chronic inflammatory component of SLE will generate greater concentrations of ROS during neutrophil activation than during normal oxidative metabolism, and result in tissue damage.
Furthermore, a factor present in the serum of SLE patients causes neutrophils to generate significantly increased levels of ROS which are cytotoxic to endothelial cells (Hashimoto, Ziff & Hurd, 1982). This enhanced damage to endothelial cells, which may result in vascular injury, is inhibited by SOD and catalase (Niwa et al., 1985).

Increased ROS production may be involved in the pathogenesis of SLE. ROS-denatured DNA is thought to be more antigenic than native DNA and since DNA is a component of the circulating immune complexes in SLE sera, the exposure of this modified antigen may be relevant to the process of anti-DNA antibody production.

The general aim of this thesis was to investigate the potential role of ROS-damaged DNA in the aetiopathogenesis of SLE.

The following specific questions were addressed:

Can damage to DNA by biologically relevant levels of ROS provide characteristic, measurable products in vivo?

Can this damage be ascribed to a specific radical species?

Can ROS-induced DNA products be detected in human biological material, and thus used as an indicator of both excreted and accumulated damage?

Can ROS-DNA act as a mediator of anti-DNA antibody production?
SECTION 2:

MATERIALS AND METHODS
CHAPTER 2.1: REAGENTS AND SUPPLIERS

2.1.1: Enzymes

Phosphodiesterase 1 (EC 3.1.4.1): from crotalus atrox venom with a specific activity of 0.13 units/mg,
Endonuclease (EC 3.1.30.1): from Neurospora crassa with a specific activity of 420 units/mg,
Alkaline phosphatase (EC 3.1.3.1): from Escherichia coli with a specific activity of 2.5 units/mg,
Superoxide dismutase (EC 1.15.1.1): from bovine erythrocytes with a specific activity of 3,000 units/mg,
Catalase (EC 1.11.1.6): from bovine liver with a specific activity of 11,000 units/mg,
S1 nuclease (EC 3.1.30.1): from Aspergillus oryzae with a specific activity of 31,250 units/mg,
Proteinase K (EC 3.4.21.14): from Tritirachium album with a specific activity of 10.7 units/mg,
Deoxyribonuclease 1 - DNase (EC 1.11.1.7): from bovine pancreas,
Ribonuclease A - RNase (EC 3.1.27.5),
were all supplied by Sigma Chemical Company; Poole, Dorset, UK.

To inactivate any contaminating DNase in the RNase preparation, RNase at a concentration of 10mg/ml in 10mM Tris-HCl (pH 7.5) and 15mM NaCl was heated to 100°C for 15 minutes and allowed to cool slowly to room temperature. The enzyme was then aliquoted at 1mg/ml and stored at -20°C.

2.1.2: Immunochemicals

Goat anti-human IgG (gamma chain specific), goat anti-human IgM (μ chain specific) and goat anti-human IgA (α chain specific) were from Sigma.

Peroxidase conjugated goat anti-mouse polyvalent immunoglobulin and peroxidase conjugated goat anti-human IgG (gamma chain specific), goat anti-human IgM (μ chain specific) and goat anti-human IgA (α chain specific) were also from Sigma.

IgA from human colostrum and polyclonal human IgG were from Sigma, and polyclonal human IgM was from Calbiochem, Cambridge, UK.
Monoclonal antibodies were a gift from Professor Norman Staines; Kings College, London. The antigenic determinants recognised by each of the antibodies listed below, and their optimum working dilutions are shown in table 3.5 (chapter 3):

<table>
<thead>
<tr>
<th>Group</th>
<th>Monoclonal Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>402, 410</td>
</tr>
<tr>
<td>2</td>
<td>212, 405</td>
</tr>
<tr>
<td>3</td>
<td>152</td>
</tr>
<tr>
<td>4</td>
<td>233, 228</td>
</tr>
<tr>
<td>5</td>
<td>223</td>
</tr>
</tbody>
</table>

Autoantibody control sera, positive and negative for the presence of anti-DNA antibodies were from Sigma.

2.1.3: Disposables

Ninety-six well Nunc-Immuno plates were from Gibco; Denmark, 24 well sterile plates and 6cm petri dishes were from Sterilin; Hounslow, UK; all other tissue culture disposables were from Flow Laboratories; Irvine, Scotland. Three ml glass disposable tubes were from Laboratory Sales (UK) Ltd., Rochdale; acrylic cuvettes were from Sarstedt Ltd.; Leicester, UK and polytetrafluoroethylene capped hypovials were from Pierce Chemical Co.; Rockford Illinois, USA.

2.1.4: HPLC Apparatus

For reverse phase chromatography a 25cm C-18 ODS 5μm column with a guard column of 10cm was used, both from Technicol; Stockport, Cheshire, UK.

SEP-PAK C-18 1ml cartridges were obtained from Waters (Millipore); Watford, UK.

2.1.5: Miscellaneous

Ficoll-Paque for isolation of PBMC was from Pharmacia; Uppsala, Sweden, Ficoll-Hypaque mono-poly resolving medium with a density of 1.114 ± 0.002 for isolation of polymorphonuclear leucocytes was from Flow.

Desferal (referred to in the text as desferrioxamine) was a gift from Ciba; Horsham, Sussex, UK.
Poly-L-lysine hydrobromide was from British Drug Houses (BDH) Ltd.; Dorset, UK.

Hoechst 33258 dye was from Hoechst; Frankfurt, West Germany.

Isoamyl alcohol was from Fisons Scientific Equipment, Loughborough, Leicestershire, UK.

RPMI 1640, foetal calf serum and L-glutamine were from Flow.

Penicillin was from Glaxo; Greenford, Middx., UK and Streptomycin was from Evans Med.; Greenford, Middx., UK.

8-hydroxyguanine (8OHG) was from Chemical Dynamics Corp.; South Plainfield, NJ, USA.

DNA from human placenta, agarose, sodium dodecyl sulphate (SDS), hydrogen peroxide (30% solution with stabilisers), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane, bovine serum albumin (BSA), cytochrome C from horse heart, thiourea, mannitol, orthophenylenediamine (OPD) 10mg tablets, trypan blue, ethidium bromide, phenol, protein A crude cell suspension from Staphylococcus Aureus (SPA), sigmacote, 2'deoxy D-ribose, 2'deoxyribonucleosides and 2'deoxy 5'nucleotides, 8-azaadenine (8AZA), 5-hydroxyuracil and 4,6-diamino-5-formamidopyrimidine (FAPY Adenine) were of the highest grade available and obtained from Sigma.

Solvents used for HPLC buffers were of Spectrosol grade and, with all other general laboratory reagents (of Analar grade) were from BDH.

2.1.6: Buffers

The principal buffers used include: 10mM Tris, 1mM EDTA (pH 8.0) (TE), 89mM Tris Borate (pH 8.0) (TBE), phosphate buffered saline (0.01M; pH 7.4) (PBS) and complete culture medium: RPMI 1640 containing 10% heat inactivated foetal calf serum - HIFCS, penicillin (5 units), streptomycin (5 units) and L-glutamine (0.15mg) - GPS

Wherever possible, buffers were made up in chelex treated water and autoclaved prior to use.
2.1.7: Clinical Samples

Samples used throughout this study were obtained from patients with:

RA (classical or definite - according to American Rheumatism Association criteria)

SLE (satisfying the revised criteria for the diagnosis of SLE - Tan et al., (1982), and receiving conventional non-steroidal anti-inflammatory drugs and steroids)

scleroderma and primary Raynauds as diagnosed by standard clinical and serological criteria attending rheumatology clinics at the Rheumatism Research Wing, University of Birmingham.

Young normal control samples were obtained from healthy laboratory staff and elderly normal control samples were from informed volunteers attending out-patient clinics at the Queen Elizabeth Hospital, Birmingham.

CHAPTER 2.2: EQUIPMENT

For all HPLC analysis a model 510 solvent delivery system and WISP 710B autosampler were connected to a 490 multiwavelength detector (all from Waters) and an LC-4B amperometric electrochemical detector from Biotech Instruments Ltd., Luton, Beds, UK. Data collection was facilitated using two Waters 740 data modules.

Analysis of samples by gas-chromatography mass spectroscopy (GCMS) employed a mass selective detector, controlled by a computer work station, interfaced to a gas chromatograph (all instruments from Hewlett Packard Co., Bracknell, Berkshire, UK, with model numbers S970B, S970C and S890A respectively).

A Model LS-5 Scanning Fluorescence Spectrophotometer from Perkin-Elmer Ltd.; Beaconsfield, Bucks., UK, was used for determination of DNA concentration using Hoechst 33258 dye and also for the fluorometric analysis of DNA unwinding (FADU) assay.

The mini-gel apparatus was from BioRad Laboratories Ltd.; Hemel Hemstead, Herts., UK.
A cobalt 60 source in the Department of Radiation Physics, University of Birmingham, was used for gamma radiolysis experiments.
The UV lamp was from Andermann and Company Ltd.; Surrey, UK and emitted light at wavelengths of 366nm and 254nm corresponding to average light intensities of $17\mu W/cm^2$ and $10.5\mu W/cm^2$ respectively at 1m, according to the manufacturers specification.

Enzyme linked immunosorbent assay (ELISA) plates were washed using a Wellwash 4 platewasher from Denley Instruments Ltd.; Billingshurst, Surrey, UK and read on a Titertek Multiscan spectrophotometer, from Flow.

A Mistral 3000 refrigerated centrifuge (MSE Scientific Instruments; Crawley, Sussex, UK) was used for cell isolation procedures and a microfuge from Beckman; High Wycombe, Bucks., UK was used in DNA preparation.

For maintenance of lymphocytes in culture a Leec MkII Proportional Temperature Controllers incubator from Leec; UK was used and sterile separation of cells was done in a Gelaire BSB4 hood from Flow.

The Fluorescence Activated Cell Sorter - FACS 440 was from Becton Dickinson, Cowley, Oxford, UK.

CHAPTER 2.3: GENERAL METHODS

The work described in this thesis uses a variety of methods. Some were developed specifically for the purpose; others were from existing methods with minor modifications for performing particular experiments. Consequently, several specific techniques are described in separate "methods sections" in the first relevant chapter in which they are used. The methodology described below reflects basic experimental techniques common to the thesis as a whole.

2.3.1: DNA Processing Techniques

2.3.1.1: Isolation of Genomic DNA from Cells

Isolation of DNA from cells was based on the method described by Maniatis, Frisch and Sambrook (1982), pp 280-281 and pp 458-462. Cells were washed twice in ice-cold PBS by centrifugation at 300 x g for 10 minutes at 4°C and resuspended in fresh buffer. After the final wash the cell pellet was resuspended at a concentration of $10^8$ cells/ml in ice-cold TE. To this was added 10 volumes of 0.5M EDTA (pH 8.0), 100µg/ml proteinase K, and 0.5% Sarcosyl as a detergent. The suspension of lysed cells was incubated at 50°C for 3 hours with periodic swirling.
To extract the DNA, an equal volume of phenol was added to the viscous solution and the two mixed until an emulsion formed. After centrifuging for ≈15 seconds in an Eppendorf centrifuge at room temperature, and good separation of the two layers, the upper aqueous phase was transferred to a fresh polypropylene tube. The aqueous phase was extracted a second time with phenol, then incubated with 100μg/ml of DNase-free RNase at 37°C for 3 hours. Further extraction, as for the phenol, was performed with: firstly, an equal volume of a 1:1 mixture of phenol and chloroform, and secondly, an equal volume of chloroform. DNA was recovered by precipitation with two volumes of ice-cold ethanol and incubation at -20°C for up to 24 hours. After centrifugation of the DNA at 0°C for 10 minutes in an Eppendorf centrifuge, the ethanol supernatant was discarded and remaining traces of supernatant were removed by vacuum desiccation. Finally, the DNA was dissolved in the minimum volume of TE.

2.3.1.2: Determination of DNA Concentration

DNA concentration was accurately determined fluorimetrically using the DNA binding dye Hoechst 33258. The assay was based on that described by Labarca and Paigen (1980), showing no significant interference by RNA and protein in the range used. The binding of Hoechst 33258 to DNA was linear over the range of concentrations used in this thesis and is shown in the calibration curve constructed using human placental DNA from 0-100μg/ml (Figure 2.1a) and 0-1000μg/ml (Figure 2.1b).

To determine DNA concentration, Hoechst 33258 was diluted to 1μg/ml in a buffer consisting of 0.05M sodium phosphate, 2M NaCl and 1mM EDTA, pH 7.4. Two microlitres of human placental DNA of known concentration diluted in TE (for the calibration curve) or 2μl of extracted DNA, also in TE, were added to 2mls of dye-containing buffer and the fluorescence measured at excitation 356nm and emission 458nm using acrylic cuvettes.

2.3.1.3: Agarose Gel Electrophoresis

Agarose gel electrophoresis of DNA was used in conjunction with the data obtained from the fluorescence assay described in 2.3.1.2 to determine the purity of isolated DNA and also to estimate its size. Using a mini-gel apparatus 0.2g agarose was dissolved in 20mls TBE to give a gel concentration of 1%. Ethidium bromide was added to a final concentration of 0.75μg/ml. Twenty microlitres of DNA mixed with 5μl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% w/v
Figure 2.1

Fluorometric determination of DNA concentration by Hoechst dye binding. The calibration curve shows the linear increase in fluorescence from Hoechst dye binding to a) 0-100μg/ml and b) 0-1000μg/ml human placental DNA obtained from the means ± 1SD of three separate experiments.
sucrose in water) was loaded onto the gel and electrophoresis was carried out at 70V for 30 minutes in TBE. DNA bands were identified using UV-illumination and the gels photographed.

2.3.1.4: Enzymic Digestion of DNA

Digestion of DNA to its nucleoside components was carried out according to the method of Beland, Dooley and Casciano (1979). DNA, dissolved in TE, pH 7.4 at a concentration of ≈1mg/ml was made 10mM in magnesium chloride, heated for 3 minutes in boiling water and rapidly cooled with ice. DNase 1 (0.1mg/mg DNA) and endonuclease (6 units/mg DNA) were added and the solution incubated overnight at 37°C. The pH was then adjusted to 8.0 using 1M Tris base, and the enzymes phosphodiesterase 1 (0.04 units/mg DNA) and alkaline phosphatase (1 unit/mg DNA) were added. The incubation was continued overnight at 37°C, after which the pH was re-adjusted to 7.0 with 1M HCl and the solution stored at -20°C prior to analysis. This method was found to give the highest yield of 80HDG from DNA when compared to other digestion procedures described in Floyd et al., (1990).

2.3.1.5: DNA Preparations

All glass and plastic surfaces used for storage or incubation of DNA samples were coated with Sigmacote to prevent sticking of the DNA to the tube surfaces. All surfaces were briefly immersed in Sigmacote, drained and incubated at 37°C for 1 hour to complete evaporation of the solution, prior to use in DNA experiments.

Phenol: Highly purified phenol was purchased from Sigma and used without further distillation. The phenol was melted at 68°C and 8-hydroxyquinolone was added to a final concentration of 1%. The phenol was then extracted several times with an equal volume of buffer; 1.0M Tris, pH 8.0, followed by 0.1M Tris, pH 8.0 until the pH of the aqueous phase was >7.6. The phenol was stored under the final equilibration buffer at 4°C using the hydroxyquinolone dye as an indicator of oxidation.

Chloroform: A mixture of chloroform and isoamyl alcohol (24:1 v/v) was prepared and stored in a closed bottle at room temperature. This solution is referred to as "chloroform".
2.3.2: ELISA Assays

2.3.2.1: Direct Binding ELISA

In the direct binding ELISA, the interaction between an immobilised ligand and its antibody was measured colorimetrically. All incubations were for 1 hour at 37°C in a humidified environment, volumes pipetted into wells were consistently 50μl and between each stage in the assay the plates were washed 3 times in PBS containing 0.05% tween 20 (PBST).

Ligand was coated onto 96 well plates in 0.05M carbonate/bicarbonate buffer (pH 9.6) to achieve maximum immobilisation to the positively charged plastic plates. BSA (1%) was used to block remaining sites on the plate thus avoiding non-specific binding of the antibody to the plate. Antibody diluted in PBST was then added in triplicate leaving the first row of each plate blank to control for any interaction between the conjugated antibody and the coating ligand. Determination of antibody, complexed to the bound ligand, was achieved using peroxidase-conjugated antibodies directed against the primary antibody. The reaction of bound peroxidase with H₂O₂ and OPD at room temperature produced a characteristic yellow colour. The reaction was stopped with 4M H₂SO₄ and the optical density (OD) read at 492nm on a Titertek Multiscan.

2.3.2.2: Competition ELISA

In a direct binding ELISA, immobilisation of ligand to the plate may sterically hinder interaction of the complexing antibody with certain epitopes on the ligand. To overcome this problem a competition ELISA can be used. In this assay, increasing concentrations of ligand are available in free solution to compete with immobilised ligand for the specific antibody. In a similar way, a comparison of affinity of an antibody for two different ligands can be determined using this assay (sections 3.6 and 5.1).

The direct binding assays used in this thesis were found not to give significantly different results from those of a competition ELISA (section 5.2.3.2) and therefore the direct binding assays alone were used.

2.3.3: Cell Isolation Techniques

2.3.3.1: Isolation of Peripheral Blood Mononuclear Cells (PBMC) (after Boyum, 1968).

Whole venous blood was collected into plastic universals containing 100μl of preservative free heparin, then diluted to 50% with RPMI 1640. Diluted blood (7.5mls) was layered gently onto 2.5mls of Ficoll-
Paque, in sterile 10ml round bottomed disposable plastic tubes. Tubes were centrifuged at 300 x g for 30 minutes. Mononuclear cells collected at the interface were removed using a pasteur pipette and washed three times in RPMI 1640, by centrifugation at 300 x g for 10 minutes, in 10ml V-bottomed plastic tubes. Cells were pelleted and resuspended in the appropriate medium required for culture or further processing. Mononuclear cell preparations were consistently greater than 97% viable and contained 10-20% monocytes.

2.3.3.2: Isolation of Polymorphonuclear Leucocytes (PMN)

Whole venous blood was collected into EDTA and layered onto Mono-Poly Resolving Medium. Separation and washing of cells was as described for mononuclear cells in section 2.3.3.1.

2.3.3.3: Viable Cell Counts

Cells were resuspended to 5 x 10⁶ per ml. The cell suspension was diluted 1:1 with 1% trypan blue in PBS and incubated at room temperature for 5 minutes before counting. Dead cells were stained dark blue. Both unstained (viable) and stained (dead) cells were enumerated separately to obtain a percentage as well as absolute viable cell counts.

CHAPTER 2.4: GENERATION OF REACTIVE OXYGEN SPECIES

The role of ROS in normal metabolism has been discussed at length in chapter 1, and in particular the role of ROS in inflammation. To characterise the modifications caused by ROS-mediated damage to DNA in an in vivo situation it is first necessary to establish the changes occurring, and to develop and/or evaluate reliable assays to determine these end points.

Investigation of the effects of ROS on DNA in an in vitro situation was simplified by using several model systems to generate ROS. These models, and the ROS they produce, have been well characterised: they also have the advantage of being chemically manipulated to produce one species predominantly as illustrated in the following sections.

2.4.1: Gamma Radiolysis

Exposure of aqueous solutions to high energy ionising radiation, from a Cobalt 60 gamma source,
ionises the water molecules as described in the equation:

\[
2\text{H}_2\text{O} \rightarrow \text{OH}^- + \text{H}_3\text{O}^+ + e^- \quad \text{eqn 1}
\]

The yields (G values: number of molecules formed per 100 eV of energy absorbed) are: \( e^- \), 2.7; \( \text{OH}^- \), 2.7; \( \text{H} \), 0.5 and \( \text{H}_3\text{O}^+ \), 2.76 (Singh & Singh, 1982).

The reducing aqueous electron and oxidising free radical will react further with themselves or other solutes and in the presence of oxygen, the additional reactive species formed include peroxo radicals (\( \text{RO}_2^- \)), superoxide anions (\( \text{O}_2^- \)) and singlet oxygen (Singh & Singh, 1982).

The mechanism of pure superoxide radical generation in an oxygenated solution of 100mM formate has been described by Bielski (1978) as shown below:

\[
\begin{align*}
2\text{H}_2\text{O} & \rightarrow \text{OH}^- + \text{H}_3\text{O}^+ + e^- \\
e^- & + \text{O}_2 \rightarrow \text{O}_2^- \\
\text{H}_3\text{O}^+ & + \text{O}_2 \rightarrow \text{HO}_2^- + \text{H}_2\text{O} \\
\text{HO}_2^- & \rightarrow \text{H}^+ + \text{O}_2^- \\
\text{OH}^- & + \text{HCO}_3^- \rightarrow \text{CO}_3^- + \text{H}_2\text{O} \\
\text{CO}_3^- & + \text{O}_2 \rightarrow \text{O}_2^- + \text{CO}_2
\end{align*}
\]

To produce peroxo radicals in solution, a UV absorbing compound (eg. 10mM phenylalanine) is added to scavenge both \( \text{OH}^- \) and \( \text{O}_2^- \) producing \( \text{R(OH)}^- \) and \( \text{R(OH)}\text{O}_2^- \) respectively as shown below (Gee Kittridge and Willson, 1985; Willson, 1985):

\[
\begin{align*}
\text{OH}^- & + \text{R} \rightarrow \text{R(OH)}^- \\
\text{R(OH)}^- & + \text{O}_2^- \rightarrow \text{R(OH)}\text{O}_2^-
\end{align*}
\]

2.4.1.1: Gamma irradiation of DNA

DNA at 0.5mg/ml in 40mM sodium phosphate buffer, pH 7.4, was irradiated in glass tubes in the presence or absence of either 100mM formate or 10mM phenylalanine. A dose of up to 1000 Gray (Gy) was given at a dose rate of \( \approx 10 \) Gy per minute; tubes were shaken at regular intervals to reoxygenate the solutions.
2.4.1.2: Gamma irradiation of 2'deoxyguanosine

2'deoxyguanosine (dG) at 2.5mM in 40mM sodium phosphate buffer, pH 7.4, was irradiated as for DNA.

2.4.1.3: Gamma irradiation of RPMI-1640

RPMI-1640 was irradiated as for DNA. Formate and phenylalanine were found to reduce cell viability above that found in control cells and therefore were not included in these experiments.

2.4.1.4: Gamma irradiation of PBMC

For some experiments cells were irradiated directly. PBMC at a concentration of 2 x 10^6 cells/ml in an aerated solution of 2mls RPMI-1640 were irradiated in sterile glass tubes at 0°C at a dose of ≈25 Gy per minute up to 200 Gy.

2.4.2: UV Radiation

It has been established that the UV region most potent in its effects on living cells is near 260nm (Deering, 1962). DNA absorbs most strongly in this region, the UV light being predominantly absorbed by the bases. Cytosine and thymine, the pyrimidines, are far more sensitive to the effects of UV than the purines, adenine and guanine. A characteristic change induced by UV light is the photochemical fusion of two adjacent pyrimidines into non-pairing structures like the cyclobutane-containing thymine dimer (Deering, 1962).

UV light can photoionise water molecules to produce a source of protons and hydroxyl radicals and in aerated solutions, hydrogen atoms can react with oxygen to produce OH⁻ (McCord and Fridovich, 1973):

\[ \text{H}^- + \text{O}_2 \rightarrow \text{HO}_2^- \rightarrow \text{H}^+ + \text{O}_2^- \]
\[ \text{H}^- + \text{HO}_2^- \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{UV} \rightarrow 2\text{OH}^- \]

The subsequent action of UV light on pure H₂O₂ is one of the most efficient ways of producing OH⁻ in solution.
2.4.2.1: UV irradiation of DNA

DNA at 0.5mg/ml in PBS was irradiated in the presence or absence of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ in a 6cm petri dish at a distance of 6cm from the source. Irradiations were done on ice to minimise evaporation and aliquots were removed at 15 minute intervals.

2.4.2.2: UV irradiation of 2'deoxyguanosine

dG at 2.5mM in 0.1M sodium phosphate buffer, pH 6.8 was irradiated in the presence or absence of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ as for DNA.

2.4.2.3: UV irradiation of RPMI 1640

For exposure of PBMC to UV irradiation, RPMI 1640 was irradiated as for DNA, sterile filtered through a 0.2μm syringe, and 0.9ml added to the lymphocytes resuspended in 0.1ml of HIFCS containing 10μl GPS to give a final concentration of 2 x 10$^6$ cells/ml.

2.4.2.4: UV irradiation of PBMC

PBMC were irradiated on ice in a sterile plastic petri dish as for DNA at a concentration of 2 x 10$^6$ cells/ml for a maximum of 60 minutes. Aliquots were removed every 15 minutes.

2.4.3: Fenton Reaction

In the presence of a reducing agent and a transition metal ion catalyst [such as Fe(II), Cu(I) and Co, and the more recently described Cr(VI) (Shi and Dalai, 1990)], H$_2$O$_2$ reacts to produce OH· (the Fenton reaction). With Fe$^{2+}$ as a metal ion catalyst the reaction proceeds:

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

Traces of Fe$^{3+}$ can react further with H$_2$O$_2$:

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{O}_2^- + 2\text{H}^-
\]

to replenish Fe$^{2+}$ in the absence of a reducing agent (Halliwell and Gutteridge, 1984).

Although the availability of metal ions in biological fluids to catalyse the Fenton reaction has been questioned (section 1.1.3) there is substantial evidence that H$_2$O$_2$ induces DNA damage by interacting with DNA-bound iron to generate OH· radicals (Floyd, 1981; Mello-Filho, Hoffmann and Meneghini, 1984).
2.4.3.1: \( \text{H}_2\text{O}_2 \) and DNA

DNA at 0.5mg/ml in PBS was incubated at 37°C for 1 hour with increasing concentrations of \( \text{H}_2\text{O}_2 \) up to 200\( \mu \)M, in the presence of 10mM ascorbic acid (AA) as a reducing agent. All reagents were made up in chelex-treated water and DNA-bound metal ions alone were available to catalyse the Fenton reaction.

2.4.3.2: \( \text{H}_2\text{O}_2 \) and 2' deoxyguanosine

Incubation of dG with \( \text{H}_2\text{O}_2 \) was used as model system to generate 80HDG; levels of \( \text{H}_2\text{O}_2 \) were not required to be physiological and in this system 2.5mM dG was incubated with 50mM \( \text{H}_2\text{O}_2 \) in 0.1M sodium phosphate buffer, pH 6.8.

2.4.3.3: \( \text{H}_2\text{O}_2 \) and RPMI-1640

Increasing concentrations of \( \text{H}_2\text{O}_2 \) up to 200\( \mu \)M were made up in RPMI-1640 and added to HIFCS and GPS as for UV irradiation of RPMI-1640 (section 2.4.2.3) to give a final cell concentration of 2 x 10⁶ cells/ml.

2.4.4: Activation of PMN

PMN release ROS following activation of the membrane bound NADPH oxidase. In an in vivo situation activation at an inflammatory site is caused by stimuli such as immune complexes, complement or bacteria, but in vitro the stimulation of PMN can be mimicked using the tumour promoter, phorbol myristate acetate (PMA). To determine the effect of PMA-derived ROS on DNA the cellular DNA of PMN was studied directly, rather than adding further DNA to the system since it would not have been possible to differentiate between the cellular and naked DNA.

Isolated PMNs were incubated at a concentration of 2 x 10⁶ cells/ml at 37°C for 15 minutes prior to activation with PMA at 10ng/ml in RPMI 1640 for 30 minutes at 37°C. DNA was then isolated from stimulated or non-stimulated PMNs, incubated as for the stimulated cells but with the omission of PMA, as described in section 2.3.1.1.

Superoxide radical generation was measured spectrophotometrically by the SOD-inhibitable reduction of 160\( \mu \)M cytochrome C at 550nm over a 30 minute period at 37°C. Superoxide release was calculated in
nmoles/10⁶ cells using the molar extinction coefficient of 21 x 10³ M⁻¹ cm⁻¹ for cytochrome C.

All reagents were diluted in RPMI 1640 (without phenol red, to prevent interference in the colorimetric assay) to maintain an isotonic solution.

CHAPTER 2.5: SCAVENGERS OF REACTIVE OXYGEN SPECIES

As described in section 2.4, the use of model systems to generate ROS predominantly results in the production of more than one reactive molecule. Identification of the ROS responsible for causing degradative changes to DNA in any one system can be facilitated by the use of antioxidants (table 2.1) but conclusive evidence cannot be drawn from this alone. Spin-trapping of radicals, and electron paramagnetic resonance patterns are more informative of the presence of radical species (Floyd, Lewis and Wong 1984).

CHAPTER 2.6: HPLC ASSAY FOR 8OH DG

2.6.1: Introduction

8OH DG is a product of hydroxyl radical damage to dG, either as a free base or derived from DNA. It was first described in two separate publications using GCMS (Dizdaroglu, 1985a) and HPLC (Kasai & Nishimura, 1984a). The use of HPLC to measure this altered DNA base has been pursued by several laboratories (Floyd et al., 1986b; Schraufstätter et al., 1988). The assay is ~1000 fold more sensitive when compared with previous HPLC assays measuring the altered bases, thymine glycol and 5-hydroxymethyluracil, the increase in sensitivity being achieved using electrochemical detection (ECD) (Floyd et al., 1986b). Previous assays relied on the UV absorbance of the modified bases but by virtue of its low redox potential 8OH DG not only responds to electrochemical oxidation but responds in a region where the native bases do not interfere.

2.6.2: Methods

2.6.2.1: Separation of 8OH DG and 2′deoxyguanosine by HPLC/ECD

Measurement of 8OH DG in native or ROS-denatured DNA required enzymic digestion of the DNA (as described in section 2.3.1.4) prior to analysis of the samples by HPLC/ECD. Samples of dG and ROS-denatured dG were analysed without further treatment.
<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Final Concentration</th>
<th>Species Scavenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>27,000 units/ml</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>SOD</td>
<td>3,000 units/ml</td>
<td>O$_2$.-</td>
</tr>
<tr>
<td>Thiourea</td>
<td>50mM</td>
<td>OH-. and metal chelator</td>
</tr>
<tr>
<td>Mannitol</td>
<td>50mM</td>
<td>OH-.</td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>0.5mM</td>
<td>Chelates iron</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50mM</td>
<td>OH-.</td>
</tr>
<tr>
<td>Cysteine</td>
<td>50mM</td>
<td>General scavenger of ROS</td>
</tr>
</tbody>
</table>

Table 2.1

Characteristics of the scavengers used in in vitro experiments to determine the individual effects of ROS on DNA. The final concentration and species scavenged by each of the reagents is shown. SOD = Superoxide Dismutase, ROS = reactive oxygen species.
Ten microlitres of sample was injected onto a reverse phase 25cm C-18 ODS 5μm column. Separations were performed at a flow rate of 0.8ml/min with a mobile phase of 30mM sodium hydroxide, 10mM acetic acid, 41mM sodium acetate and 12.5mM citric acid, pH 5.1 containing 10% methanol (from Kasai et al., 1986). The eluent was monitored simultaneously by absorption spectroscopy at 254nm and by ECD at 600mV oxidation as compared with the Ag/AgCl reference electrode.

Figure 2.2 shows a typical chromatogram of all four DNA bases measured by absorbance at 254nm. Figure 2.3 illustrates the electrochemical and UV response of 8OHDG showing the significant enhancement in sensitivity from the ECD signal. Guanine (derived from RNA) in its native and ROS-denatured form did not give an electrochemical response at 600mV which eliminates the possibility that the 8OHDG measured could be contaminated by RNA-derived guanine adducts.

The assay was standardised using dG, 2′deoxyadenosine (dA) and 8OHDG and the presence of these compounds in DNA digests and other biological samples was confirmed by spiking the peaks with authentic compound. Further identification of the 8OHDG peak was by GCMS (section 4.4) and by cyclic voltametry.

Cyclic voltammetry can be used to determine the purity of a peak, each compound having a different curve, the shape of which is altered by contamination from a co-eluting product. The electrochemical response of pure 8OHDG was monitored between 100mV and 800mV oxidation as compared to the Ag/AgCl reference electrode and figure 2.4 shows the curve obtained plotting the percentage maximum peak height against mV (ie the hydrodynamic voltammogram). The maximum peak height was at 600mV, the detection setting for the assay.

To confirm the identity of peaks co-eluting with 8OHDG in later chapters a comparison of the voltammogram curve with the standard curve was made.

The coefficient of variation for the assay based on ten separate injections of 8OHDG was 4.7% and the detection limit of the assay was 0.1 picomoles 8OHDG.
Representative chromatogram of the UV absorbance at 254nm of a DNA digest showing all four native DNA bases dG (deoxyguanosine), dA (deoxyadenosine), dT (deoxythymidine) and dC (deoxycytosine).
Figure 2.3

Representative chromatogram of the electrochemical response at 600mV oxidation (broken line) and the UV response at 254nm (continuous line) of 8OHDG showing the significant enhancement in sensitivity from the ECD signal.
Figure 2.4

The hydrodynamic voltammogram of 8-hydroxydeoxyguanosine (8OHDG) as compared to the Ag/AgCl reference electrode compiled from the means ± 1SD of readings obtained in three separate experiments. The 8OHDG used in these experiments was supplied by Dr H Kasai.
2.6.2.2: ROS-Mediated Production of 8OHDG

8OHDG cannot be bought commercially at present. It can be synthesised using the Udenfriend system (Kasai & Nishimura, 1984a) and this procedure was used to prepare 8OHDG on a large scale from dG for use in quantitative determination by HPLC/ECD.

For the synthesis, 1g of dG was dissolved in 780mls of 0.13M sodium phosphate buffer, pH 6.8, and 140mls of 0.1M AA, 65mls of 0.1M EDTA and 13mls of 0.1M Fe(NH₄)₂(SO₄)₂ were added successively. The solution was incubated at 37°C in the dark whilst constantly bubbling pure oxygen through it. After 3 hours, the solution was adjusted to pH 3.7 with 1M HCl, and 10g charcoal powder was added with stirring. The charcoal was recovered by filtration and packed in a column (2 x 13cm). The column was washed with 500mls water and then the compounds of interest were eluted with 500mls of aqueous acetone (1:1, v/v). The eluate was evaporated to dryness and the residue was fractionated by HPLC.

To separate 8OHDG from the unmodified base, the eluate was injected onto the 25cm C-18 ODS 5μm column and eluted with 10% aqueous methanol. The small peak eluted just after the main peak of dG was collected and evaporated to dryness. The residue was crystallised from water.

8OHDG was stored at a concentration of 1mg/ml at -20°C and was found to be stable for the duration of storage (3 months maximum). This is in agreement with data from Floyd et al., (1990) who found that optimum storage of 8OHDG was at neutral pH. The concentration of 8OHDG was determined by UV absorbance at 245nm using the extinction coefficient of 12,300 as stated by Kasai & Nishimura (1984a). A comparison with a sample of 8OHDG kindly donated by Dr Kasai, and analysis of the standard by GCMS (section 4.4.3) confirmed the authenticity of the prepared product.

CHAPTER 2.7: STATISTICAL ANALYSIS

Statistical analysis was performed using the Students "t" test, for parametrically distributed results, which were expressed as means and standard deviations in the figures or tables.

Groups of biological data were analysed using non-parametric statistics (Mann-Whitney U test) and are presented as medians and ranges, or scatter plot diagrams.
SECTION 3:

MOLECULAR CHARACTERISATION OF ROS-INDUCED DAMAGE TO DNA IN VITRO
CHAPTER 3:

The DNA molecule consists of a backbone of deoxyribose moieties linked together by phosphodiester bonds. Attached to each deoxyribose is one of the four heterocyclic bases, guanine, adenine, thymine or cytosine. It is the DNA bases that carry the genetic information whilst the sugar and phosphate groups play a structural role.

DNA exists as a helix consisting of two of these backbone structures running in opposite directions, the structure being stabilised by hydrogen bonds between complementary bases. Adenine pairs with thymine and guanine pairs with cytosine. The diameter of the DNA helix is 2nm and adjacent bases are separated by 0.34nm, the helical structure repeating itself after 10 residues on each chain.

The amount of DNA in the 46 chromosomes of a normal human cell is $\approx 6 \times 10^{12}$g which would stretch a distance of 2 metres if it was not packaged into nucleosomes. These structures are disk-like with a thickness of 5.7nm and a radius of 5.5nm with two coils of DNA wrapped around each circumference, 2.7nm apart. The nucleosome core is made up of histone proteins, but is not tightly packed and contains hydration (bound) water.

The effects of ionising radiation on DNA have been extensively studied, and are justified by the central role DNA plays in cell development. As a major cellular target for ROS-induced damage (Ward, 1985; Hagen, 1986), cell death, mutagenesis and transformation may all be caused by damage to this molecule.

Ionising radiation can cause damage to DNA directly by energy deposition in the molecule or indirectly by ionisation of other molecules that then attack DNA (Ward, 1985). The major source of indirect damage is the ionisation of water, which produces ROS. The water molecules bound to DNA are particularly important because they allow production of radical species in close proximity to the DNA. DNA bound metal ions, which form an integral part of the DNA molecule (Mello Filho, Hoffmann and Meneghini, 1984) and act to stabilise the DNA structure (Minchenkova and Ivanov, 1967) are also important in site-directing reactions of ROS to cause damage (see section 1.1.5.4).
High energy irradiation of DNA in aqueous solution produces ROS which cause damage at all levels of the structure; single-strand breaks (Rhaese and Freese, 1968), double-strand breaks, base liberation (Rhaese and Freese, 1968), base damage (Massie, Samis and Baird, 1972) and intra- and inter-strand cross-links (Lesko, Drocourt and Yang, 1982). These changes may have important biological implications if they become incorporated into the germ-line (section 1.1.5.4).

Experiments in this chapter have utilised model systems to generate ROS, the aim being to characterise the changes to DNA induced by ROS at the molecular level; specifically, damage to bases and the ribose moiety as well as structural changes to the overall molecule by analysing strand breaks and damage to specific epitopes.
CHAPTER 3.1: BASE DAMAGE

3.1.1: Introduction

ROS are difficult to measure in vivo since they are present in low concentrations and have very short half-lives ($t_{1/2}$ for OH$^·$ is $\approx 10^9$ seconds - Roots and Okada, 1975). However, the detection of specific products generated by ROS, reacting with for example, DNA, help to affirm their presence in vivo and also demonstrate the ability of certain ROS to diffuse into the nucleus before reacting.

The inherent problem in the detection of ROS-damaged DNA is the measurement of very low levels of adducts in the presence of normal bases, the ratio of damaged : normal bases being typically 1 : $10^6$ (Park, Cundy and Ames, 1989). Selectivity, to distinguish clearly between the unmodified and modified base residue, and sensitivity are, therefore, essential in the determination of DNA base adducts.

DNA bases react with OH$^·$ produced by water radiolysis (Ward, 1981) at almost diffusion-controlled rates ($4 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$). The major reaction of OH$^·$ is with double bonds of molecules (positions 5,6 for pyrimidines and positions 4,5 for purines). Reaction of OH$^·$ with thymine is almost exclusively with the 5,6 double bond and there is a clear preference of reaction of OH$^·$ with thymine over cytosine > adenine > guanine (Hüttermann et al., 1978).

The products most well characterised following reaction of OH$^·$ with DNA include 5-hydroxymethyluracil, 8-hydroxyadenine, 4,6-diamino-5-formamidopyrimidine (FAPY adenine), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPY Guanine) and thymine glycol (Jackson et al., 1989).

Thymine glycol is one of the most studied of the oxidised DNA bases and has been measured by HPLC (Frenkel, Goldstein and Teebor, 1981), and by enzyme immunoassays using both polyclonal (Rajagopalan et al., 1984) and monoclonal antibodies (Leadon, 1987). More recently, two groups using different methodology described a novel base adduct, 8OH DG. Kasai and Nishimura (1984a) developed an HPLC procedure using UV detection to analyse the oxidatively modified DNA base, whereas Dizdaroglu (1985a) developed an assay to measure a range of oxidised DNA bases including 8OH DG, by GCMS. Floyd et al., (1986b) improved the sensitivity of the HPLC assay by three orders of
magnitude (to femtomolar levels of determination) by incorporating electrochemical detection (ECD) into the assay.

Many base adducts are electrochemically active whilst normal bases are not. The only exception is guanine, which has an ECD response at an oxidation potential of >0.8V versus Ag/AgCl reference electrode. This phenomenon serves to increase the selectivity of the HPLC/ECD assay for monitoring altered DNA bases (Park, Cundy and Ames, 1989).

There are many examples of the production of 80HDG during oxidative stress in vivo and in vitro: 80HDG has been detected in DNA following its exposure to X-rays (Kasai, Tanooka and Nishimura, 1984), gamma radiation (Dizdaroglu, 1985a), asbestos and H₂O₂ (Kasai and Nishimura, 1984b), UV and H₂O₂ (Floyd et al., 1988) and autoxidising aminophenols and polyphenols (Kasai and Nishimura, 1984c). Using whole cell systems, 80HDG has been isolated from the DNA of leucocytes stimulated with tetradecylphorbolacetate (Floyd et al., 1986a) and HeLa cells after irradiation in culture (Kasai et al., 1986), while in an in vivo model it was isolated from liver extracts of mice following total body irradiation (Kasai et al., 1986). A recent review has suggested that 80HDG is the most suitable marker for measuring the steady state level of oxidative damage in normal tissues (Aruoma, Halliwell and Dizdaroglu, 1989). Its credibility as a marker of oxidative damage has been enhanced by the observations that a) the levels of 80HDG and thymine glycol in cellular DNA and urine are of the same order (Simic, Bergtold and Karam, 1989) and b) mitochondrial DNA, which has immense oxygen metabolism, relatively inefficient DNA repair and an absence of histones has 80HDG levels of 1/8,000 bases compared with 1/130,000 bases in nuclear DNA (Richter, Park and Ames, 1988). Furthermore, when lipid peroxidation is increased in mitochondrial membranes, there is a marked increase in the 80HDG content of the DNA suggesting a relationship between two markers of oxidative stress (Hruszkewycz and Bergtold, 1990).

Successful validation of assays measuring 80HDG by several groups (Floyd et al., 1986b; Aruoma, Halliwell and Dizdaroglu, 1989) has confirmed its reliability as a measure of oxidative damage in DNA. Experiments in this section have used model systems to generate ROS, and have measured 80HDG, produced either from dG in free solution or from dG as an integral part of the DNA molecule, to
Figure 3.1

Irradiation of 2.5mM dG in the absence (figure 3.1a) or presence (figure 3.1b) of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ to produce 8OHdG. In the absence of Fe(II) no significant 8OHdG production was detected but addition of Fe(II) significantly enhanced the conversion of dG to 8OHdG. Results are means ± 1SD of five separate experiments.
To investigate the radical species responsible for this increase, irradiation of dG was repeated at the maximum dose of 500 Gy (which produced the maximum yield of 8OHG) in the presence of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ to facilitate 8OHG production, and various scavengers of ROS. Table 3.1 shows the effects of these scavengers on the conversion of dG to 8OHG. In the presence of 100mM formate (producing pure O$_2^\cdot$ - section 2.4.1) there was complete inhibition of 8OHG production at low doses of irradiation and at 500 Gy the level of 8OHG detected was not significantly elevated above background.

However, in the presence of 10mM phenylalanine, to generate peroxy radical species, there was a significant increase in the amount of 8OHG produced at every dose studied ($p<0.001$). Thiourea, a hydroxyl radical scavenger, also reduced the formation of 8OHG to background levels as did 10mM ethanol.

Thiourea may inhibit the production of 8OHG by scavenging the OH$^\cdot$ produced in solution by gamma radiation and preventing it from reacting with dG. This is supported by strong evidence from other workers that the production of 8OHG is mediated by OH$^\cdot$ (discussed in section 1.1.5.4 and in the introduction to this chapter). Thiourea is also a metal ion chelator. Although the ion predominantly chelated by this molecule is copper, at mM concentrations of iron it is possible that thiourea was also able to chelate some of the iron ions, clearly essential for efficient conversion of dG to 8OHG.

To further examine the role of Fe(II) in the production of 8OHG, dG was irradiated in the presence of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 1mM, 5mM or 10mM desferrioxamine, a true iron chelator which binds Fe(III) to form a complex with high stability ($K_a = 10^{31}$) (Lederman et al., 1984). The results show that at each of the concentrations studied the production of 8OHG was enhanced (figure 3.2).

3.1.3.2: Gamma Irradiation of DNA

Irradiation of 0.5mg/ml DNA in aerated 40mM sodium phosphate buffer resulted in a dose-dependent increase in the amount of 8OHG incorporated into the DNA; this was not significantly affected by the addition of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ (figure 3.3).

Irradiation in the presence of 100mM formate significantly inhibited the conversion of dG to 8OHG.
### Table 3.1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dG</th>
<th>DNA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98.2±1.79</td>
<td>97.4±1.34</td>
<td></td>
</tr>
<tr>
<td>100 mM Formate</td>
<td>3.8±0.7</td>
<td>5.1±0.7</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>10 mM Phenylalanine</td>
<td>129.2±1.92</td>
<td>104±2.77</td>
<td>NS</td>
</tr>
<tr>
<td>50 mM Thiourea</td>
<td>7.54±0.38</td>
<td>13.12±1.51</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>10 mM Ethanol</td>
<td>7.06±0.57</td>
<td>8.26±0.78</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>100 μg/ml SOD</td>
<td>88.52±3.47</td>
<td>95.4±2.07</td>
<td>NS</td>
</tr>
<tr>
<td>1 mM Desferrioxamine</td>
<td>187±6.96</td>
<td>173.4±5.5</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Effect of ROS scavengers and the iron chelator desferrioxamine on the conversion of dG to 8OHdG induced by irradiation of 2.5mM dG or 0.5mg/ml DNA with 500 Gy in the presence of 1.3mM Fe(NH₄)₂(SO₄)₂. The results are means ± 1SD of three separate experiments with significance values obtained by paired t-test.
Figure 3.2

Effect of desferrioxamine (des) on irradiation of dG in the presence of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$. Results are the means ± 1SD of five separate experiments.
Figure 3.3

Dose-dependent increase in 8OHDG production following irradiation of DNA with increasing doses of gamma radiation. Results are shown for DNA in the presence and absence of 1.3mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and represent means ± 1SD of five separate experiments.
(p < 0.001) as did both the hydroxyl radical scavengers, thiourea and ethanol. The effect of phenylalanine enhancing the production of 8OHdG was not as marked for DNA as for dG: although a slight increase in conversion of DNA-derived dG to 8OHdG was noted, this was not significant. The presence of SOD caused no significant decrease in the production of 8OHdG whilst 1mM desferrioxamine enhanced the reaction by 73% (p < 0.001) - table 3.1.

3.1.3.3: UV Irradiation of 2'deoxyguanosine

Irradiation of dG as described in section 2.4.2.2 produced 8OHdG in a dose-dependent manner over a period of 60 minutes. Addition of Fe(II) promoted the conversion still further (figure 3.4a). Inhibition of this reaction was studied at 60 minutes UV irradiation of dG in the absence of iron to identify the ROS involved. H₂O₂ is an intermediate in the production of OH⁻ from UV (section 2.4.2) and this is reflected in the 79% inhibition of production of 8OHdG caused by catalase. 91% inhibition of 8OHdG production was also caused by thiourea, whilst desferrioxamine promoted the reaction by 59%. SOD did not cause any significant change in 8OHdG generation (table 3.2).

3.1.3.4: UV Irradiation of DNA

Irradiation of DNA as in section 2.4.2.1 for a maximum of 60 minutes also produced 8OHdG (figure 3.4b). The reaction was not significantly increased in the presence of Fe(II) and was inhibited by 90% in the presence of thiourea and 81% in the presence of catalase. The levels of 8OHdG were unaffected by SOD, whilst desferrioxamine caused an enhancement of 19% (table 3.2). The concentration of adduct increased with the dose of UV irradiation. Although the absolute levels of 8OHdG produced were the same as those produced by gamma irradiation, when compared in terms of radicals produced (500 Gy producing 200μM OH⁻, 60 minutes UV producing 200μM H₂O₂, which theoretically can produce 400μM OH⁻ from homolytic fission - section 2.4.2) gamma irradiation appears to be more effective in the conversion of dG to 8OHdG.

3.1.3.5: H₂O₂ and 2'deoxyguanosine

The reaction of H₂O₂ with dG, in the presence of a reducing agent forms the basis of the Udenfriend assay which is described in section 2.6.2.2. This system is very effective in the production of 8OHdG, converting ≈ 1 in 10⁴ dG residues into 8OHdG, and was used to assess the potential of a range of scavengers to inhibit the reaction. The results are shown in table 3.3. Mannitol and thiourea, both
A. UV Irradiation of dG

B. UV Irradiation of DNA

Figure 3.4

Effect of UV irradiation on dG (figure 3.4a) or DNA (figure 3.4b) in the absence or presence of 1.3mM Fe(NH₄)₂(SO₄)₂. Levels of 8OHDG produced represent means ± 1SD of five separate experiments.
### Table 3.2

Effect of ROS scavengers and the iron chelator desferrioxamine on the conversion of dG to 8OHDG by exposure of 2.5mM dG or 0.5mg/ml DNA to 60 minutes of UV radiation. The results are means ± 1SD of three separate experiments with significance values obtained by paired t-test.

<table>
<thead>
<tr>
<th></th>
<th>dG</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>98±1.5</td>
<td>98±2.1</td>
</tr>
<tr>
<td>50 mM Thiourea</td>
<td>9.7±2.4 p&lt;0.001</td>
<td>10.8±2.6 p&lt;0.001</td>
</tr>
<tr>
<td>0.5 mM Desferrioxamine</td>
<td>159±2.9 p&lt;0.001</td>
<td>119±2.3 p&lt;0.001</td>
</tr>
<tr>
<td>500 μg/ml Catalase</td>
<td>21±1.6 p&lt;0.001</td>
<td>19±1.6 p&lt;0.001</td>
</tr>
<tr>
<td>100 μg/ml SOD</td>
<td>94±1.2 NS</td>
<td>96±1.8 NS</td>
</tr>
<tr>
<td>Treatment</td>
<td>% 8OHdG generated compared to control</td>
<td>Significance (t-test)</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>--------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>2.5mM dG + 50mM H$_2$O$_2$</td>
<td>100±1.79</td>
<td></td>
</tr>
<tr>
<td>50mM Mannitol</td>
<td>74±2.30</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>50mM Thiourea</td>
<td>63±1.92</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>100µg/ml SOD</td>
<td>134±2.39</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>1mM Desferrioxamine</td>
<td>154±3.19</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>50mM Cysteine</td>
<td>0.4±0.3</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3.3

Effect of ROS scavengers and the iron chelator desferrioxamine on the conversion of dG to 8OHdG by exposure of 2.5mM dG to 50mM H$_2$O$_2$ in the Udenfriend reaction. Results are means ± 1SD of three separate experiments and significance values were obtained by paired t-test.
hydroxyl radical scavengers, inhibited the conversion of dG to 80HDG by 26\% and 37\% respectively. Cysteine, a general scavenger of all oxygen radicals, was most effective in inhibiting the reaction with no significant production of 80HDG above control values. Desferrioxamine caused a significant increase in generation of 80HDG (54\% - p<0.001) as did SOD (34\% - p<0.001).

3.1.3.6: $H_2O_2$ and DNA

Hydrogen peroxide, in the presence of a reducing agent, 10mM AA, reacted in the absence of any added metal ions to produce 80HDG within the DNA backbone in a dose-dependent manner, the maximum concentration of $H_2O_2$ used being 200$\mu$M (figure 3.5a). The reaction was enhanced by addition of Fe(II) to the DNA at concentrations up to 4mM, and plateaued with concentrations up to 8mM; beyond this the addition of Fe(II) inhibited the production of 80HDG (table 3.4). In the absence of $H_2O_2$ there was no significant reaction of either AA or Fe(II) with DNA in terms of 80HDG production; however, $H_2O_2$ alone induced significant production of 80HDG (p<0.01) (figure 3.5b).

3.1.4: Discussion

In each of the model systems studied, there was no significant production of 80HDG in the control samples of DNA. This suggests that the preparations of DNA used did not undergo significant oxidative damage either in their commercial processing or during the extraction procedures described in section 2.3.1.4.

Steady-state gamma radiolysis of dG produced 80HDG only after addition of Fe(II) to the solution. However, incubation of dG with Fe(II) alone did induce formation of 80HDG (figure 3.1). This may be accounted for by auto-oxidation of Fe(II) to Fe(III) producing ROS (Halliwell and Gutteridge, 1985). Conversion of dG to 80HDG was inhibited by formate and thiourea, suggesting that $O_2^\cdot$ does not directly contribute to the formation of the adduct, 80HDG (table 3.1). There is evidence that the conversion of 80HDG to dG is mediated by OH\cdot in many but not all situations (Floyd et al., 1989; Kohda et al., 1987) and gamma irradiation is known to produce OH\cdot free in solution (section 2.4.1). The poor yield of 80HDG from dG after gamma radiation in the absence of Fe(II) suggests that exposure of dG to OH\cdot does not proceed favourably unless metal ions are present. The conversion of dG to 80HDG was further enhanced in the presence of phenylalanine. Peroxyl radicals have a half-life of $\approx$5-10 times longer than OH\cdot (von Sonntag, 1988) and probably enhance 80HDG production by
Figure 3.5

Figure 3.5a shows the effect of $\text{H}_2\text{O}_2$ in the presence of a reducing agent (AA) on 8OHGD formation in DNA. Figure 3.5b shows the contributions of AA, Fe(II) and $\text{H}_2\text{O}_2$ alone, or in combination, to 8OHGD formation in DNA. Results shown are means ± 1SD of three separate experiments.
<table>
<thead>
<tr>
<th>Concentration Fe(II) mM</th>
<th>Arbitrary Units of 8OHGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.4±1.2</td>
</tr>
<tr>
<td>1</td>
<td>15.1±2.1</td>
</tr>
<tr>
<td>2</td>
<td>18.0±2.2</td>
</tr>
<tr>
<td>4</td>
<td>20.0±1.7</td>
</tr>
<tr>
<td>8</td>
<td>16.1±1.9</td>
</tr>
<tr>
<td>16</td>
<td>7.3±1.3</td>
</tr>
<tr>
<td>32</td>
<td>1.1±0.9</td>
</tr>
<tr>
<td>64</td>
<td>0.5±0.2</td>
</tr>
</tbody>
</table>

Table 3.4

Effect of increasing concentrations of Fe(II) - Fe(NH₄)₂(SO₄)ₓ on 8OHGD formation from 0.5mg/ml DNA incubated with 200μM H₂O₂ in the presence of 10mM AA. Results are means ± 1SD of three separate experiments.
increasing the time period over which conversion can take place. Peroxyl radicals are also more lipophilic than OH⁻ (von Sonntag, 1988) and may be able to diffuse closer to the dG molecules that are themselves poorly soluble in water.

The ability of desferrioxamine to enhance the production of 8OHdG was surprising, but may be explained by its chemical structure: desferrioxamine is a hydroxylamine compound containing three ionised hydroxamic acid groups (-NOH). On irradiation, a nitroxide free radical is formed (DFNO⁻) which is sufficiently long lived to be observed by electron spin resonance (Davies et al., 1987; Willson, 1988). It is possible that this radical may also participate in the production of 8OHdG and by virtue of its greater stability, will continue to react with dG after all the OH⁻ has been consumed. The reaction of H₂O₂ with dG was also enhanced by desferrioxamine, suggesting that H₂O₂-treated desferrioxamine may also generate a denaturing species, possibly the nitroxide radical (table 3.3). Alternatively, the enhancement of the reaction may result from acceleration of the conversion of Fe(II) to Fe(III), generating ROS, catalysed by the removal of Fe(III) by chelation, which would otherwise drive the equilibrium reaction back in the direction of H₂O₂ and Fe(II) formation (reaction 6, section 1.1.2.2).

Gamma irradiation of DNA produced 8OHdG in a dose-dependent manner by a mechanism independent of exogenous Fe(II). The reaction was inhibited by thiourea, ethanol and formate (table 3.1). The enhancement of 8OHdG production by phenylalanine was evident but not as marked as for dG in solution. Although irradiation of phenylalanine produces the peroxy radical which is more lipophilic than OH⁻ and may be more able to diffuse into the groove region of the DNA molecule where the base residues are situated, the poor enhancement of the reaction suggests that the peroxy radical cannot react with the DNA base, dG, any more effectively than OH⁻, despite its longer half-life and different chemical characteristics. Alternatively it may be that peroxy radicals react preferentially with DNA components other than dG, to form damage products undetected in this system.

The mechanism of formation of 8OHdG may differ between dG in solution and dG as an integral part of the DNA molecule because Fe(II) only serves to promote the production of 8OHdG in the case of free dG. In DNA, addition of OH⁻ to dG occurs in the absence of exogenous Fe(II). This is probably because stability is conferred on radical intermediates in the formation of 8OHdG by interactions with neighbouring base residues, metal ions, and nuclear proteins and histones (Cullis et al., 1987). dG in
solution lacks these groups to stabilise radical intermediates: this may explain the requirement of Fe(II), needed to lower the activation energy for product formation and to facilitate 8OHDG production. It is also likely that DNA-bound metal ions act as catalysts of 8OHDG production, as Fe(II) in solution does when reacting with dG.

The different mechanisms of 8OHDG production in dG and DNA suggest also that 8OHDG is not produced by a direct energy effect of gamma radiation because this would be less likely to be affected by conformational restraints of the individual bases either in free solution or incorporated into DNA.

UV irradiation of dG and DNA produced similar results to those induced by gamma radiation although there was less dG converted to 8OHDG per mole of radical produced, with each dose compared for UV versus gamma radiation. 500 Gy produces 200µM OH⁻ in solution, compared with UV producing 200µM H₂O₂ which theoretically can give rise to 400µM OH⁻. The lower rate of conversion in DNA for UV versus gamma radiation (1 in 10⁴ bases for UV compared to 1.5 in 10⁴ bases for gamma) probably reflects incomplete conversion of H₂O₂ to OH⁻.

The increased conversion of dG to 8OHDG in the presence of Fe(II) may again be explained in terms of stability constants, but UV light also generates H₂O₂ (section 2.4.2) which, in the presence of catalytic metal ions, can react in a Fenton reaction to produce OH⁻ (section 2.4.3). Thus, UV light has the potential to generate OH⁻, both from the energetic splitting of H₂O₂, and its metal ion catalysed decomposition, which produces OH⁻ site-specifically. The role of H₂O₂ in the production of 8OHDG is confirmed by the ability of catalase to inhibit the reaction effectively.

Incubation of dG with H₂O₂ produced 8OHDG in the absence of Fe(II) when the reducing compound, AA was present. AA has metal ions associated with it which probably react in a Fenton reaction to produce OH⁻ (Gutteridge and Wilkins, 1982). Although most of these would preferentially react with AA, because of the short distance over which OH⁻ diffuses once formed, some might react with dG. In support of this, the reaction was further enhanced in the presence of Fe(II). SOD was found to increase the production of 8OHDG from 3 in 10³ to 4 in 10³ dGs. This finding may be partly explained if the catalytic role of SOD is considered: the production of H₂O₂ is from the slow spontaneous or faster SOD-catalysed dismutation of two molecules of O₂⁻ in an equilibrium reaction. The enhancement of this
reaction in the presence of SOD ensures that the equilibrium lies in favour of \( \text{H}_2\text{O}_2 \) reacting with Fe(II) to produce further OH\(^-\) rather than reacting to produce O\(_2\)\(^-\).

Inhibition of the reaction of dG with \( \text{H}_2\text{O}_2 \) by thiourea was not as marked as for inhibition of the gamma radiation induced 8OHdG. This probably reflects the site-specific generation of OH\(^-\) by \( \text{H}_2\text{O}_2 \), and is supported by mannitol providing even less inhibition, itself reacting with OH\(^-\) at a rate \( \approx \) 4 times slower than thiourea. The importance of 8OHdG as an oxidative product of DNA is shown by the significant inhibition of the reaction by cysteine, a scavenger of all reactive oxygen products.

The reaction of DNA with \( \text{H}_2\text{O}_2 \) to generate 8OHdG was again enhanced by the presence of Fe(II) in a dose-dependent manner up to a concentration of \( \approx \) 8mM (table 3.4). Further increases in concentration of Fe(II) in the medium caused a reduction in the levels of 8OHdG produced. This suggests that \( \text{H}_2\text{O}_2 \) may be rate-limiting such that there is no further \( \text{H}_2\text{O}_2 \) to react with the added Fe(II). However, since redox cycling of metal ions can also generate radicals (Halliwell and Gutteridge, 1985) it is likely that a fraction of the 8OHdG formed after apparent saturation of the system is from iron-mediated reactions only. Since Fe(II) would not normally inhibit this reaction it seems likely that the reduced levels of formation of 8OHdG seen at high iron concentrations reflects a saturation of the solution with iron such that it successfully competes with the DNA for any OH\(^-\) produced and in this way acts as a scavenger, preventing OH\(^-\) from reacting with DNA.

In summary, there is a clear requirement for metal ions to catalyse the reaction of OH\(^-\) with free dG, and to a lesser extent with dG in DNA, to produce 8OHdG. This is probably explained by the presence of DNA-bound metal ions, which in turn will direct the production of OH\(^-\) to localised regions of the DNA backbone. The difference in scavenging ability of thiourea, for example, also highlights the different mechanisms whereby OH\(^-\) is produced. Thiourea can scavenge OH\(^-\) produced by gamma irradiation of aqueous solution more effectively than OH\(^-\) produced from \( \text{H}_2\text{O}_2 \), gamma radiation forming OH\(^-\) in solution whereas \( \text{H}_2\text{O}_2 \) reacts with DNA-bound metal ions, forming OH\(^-\) in close proximity to DNA where the scavenger has only limited access.
CHAPTER 3.2: DAMAGE TO DEOXYRIBOSE

3.2.1: Introduction
The sugar-phosphate backbone is the region of the DNA molecule exposed to the surrounding aqueous environment. It is therefore a primary target for attack by cellular and exogenous genotoxins including ROS.

In vitro it has been shown that high energy radiation of aqueous solutions and the oxidation of metal ions both produce OH· that can react with carbohydrates, such as deoxyribose, to form a TBA reactive product (Scherz, 1968; Halliwell and Gutteridge, 1981). The TBA reaction has been used extensively to detect oxidative damage to lipids: during lipid peroxidation the peroxides break down to form carbonyls, which form a characteristic chromogen with 2 molecules of TBA (Sinnhuber, Yu and Yu, 1958).

Malondialdehyde (MDA) is one such carbonyl produced during lipid peroxidation measured by the TBA assay; it has been suggested that free MDA is a minor component of oxidised lipid, and that most of the MDA is derived from peroxidic precursors which decompose to MDA during the acid heating stage of the assay (Gutteridge, 1981). In a similar way, Halliwell and Gutteridge (1981) have demonstrated that deoxyribose reacts in the presence of Fe(II) to form a TBA-reactive compound in a hydroxyl-radical mediated reaction.

However, reaction of deoxyribose within the DNA molecule has not previously been investigated for ROS generated in the model systems described in section 3.2.2. Since deoxyribose degradation is known to be associated with the formation of single strand breaks, the TBA assay was used to investigate the effects of ROS on deoxyribose, as an integral part of the DNA molecule rather than as an isolated sugar, thereby characterising damage to the DNA backbone.

3.2.2: Methods
The TBA reactivity of deoxyribose as a component of the DNA backbone was investigated using an assay based on that described by Halliwell and Gutteridge (1981). The reaction mixture contained Fe(NH₄)₂(SO₄)₂ at a final concentration of 4mM, and human dsDNA at 50µg/ml in a final volume of 1ml PBS, pH 7.4.
The reagents were incubated at 37°C for one hour after which 1ml of 1% TBA and 1ml of 2.8% trichloroacetic acid were added to all the samples prior to a second incubation at 100°C for 10 minutes. The samples were left to cool and the chromogen was measured fluorimetrically at excitation 532nm and emission 553nm using excitation and emission slit widths of 10nm.

All reagents were made up daily for the assay.

3.2.3: Results

3.2.3.1: Gamma Irradiation of DNA

DNA (0.5mg/ml in 40mM sodium phosphate buffer) was denatured by gamma radiation as described in section 2.4.1.1, at increasing doses from 0 to 1000 Gy. The DNA was diluted to 50μg/ml in PBS for use in the TBA assay.

Native dsDNA showed no significant reactivity with the TBA reagent. However, following exposure to irradiation there was an increase in TBA reactivity, which was linear up to ≈750 Gy. Higher doses of radiation did not cause any further significant increase (figure 3.6a).

3.2.3.2: UV Irradiation of DNA

DNA irradiated by UV as described in section 2.4.2.1 was assessed for TBA reactivity at 50μg/ml. The DNA was irradiated for a maximum of 60 minutes, with aliquots removed at 15 minute intervals. During this time there was no significant increase in the TBA reactivity above the background level (figure 3.6b).

3.2.3.3: H₂O₂ and DNA

DNA was denatured with H₂O₂ and AA as described in section 2.4.3.1 using doubling dilutions of H₂O₂ from 220μM to 3.44μM; TBA reactivity of 50μg/ml DNA was assayed.

It was found that production of the chromogen increased exponentially following exposure of DNA to H₂O₂ (figure 3.6c).

For comparison of results on the basis of moles of radicals produced, data is shown up to 220μM H₂O₂ only.

83
Figure 3.6

The formation of TBA reactive products following exposure of DNA to gamma radiation (3.6a), UV radiation (3.6b) and H$_2$O$_2$ (3.6c). Figure 3.6c shows the effects of serial two-fold dilutions of H$_2$O$_2$ causing an exponential increase in the TBA reactive products. Results shown in each graph are means ± 1SD of three separate experiments.
3.2.4: Discussion

Intact deoxyribose does not react with TBA to give a coloured chromogen; it must first be degraded by Fe(II) to generate a reactive carbonyl. Native dsDNA at concentrations of 0-100 μg/ml when incubated with Fe(II) and TBA reagents did not give a positive reaction. This supports data from Gutteridge, Beard and Quinlan (1985) who showed that only after addition of bleomycin to iron and DNA (thus forming ROS), did a positive TBA reaction occur. This suggests that deoxyribose, when incorporated into the DNA backbone, cannot react with Fe(II) to form a TBA reactive carbonyl.

Nonetheless, ROS-denatured DNA does produce a TBA-reactive compound, probably because deoxyribose is exposed and can react with Fe(II). It cannot be determined from these experiments whether free deoxyribose is released from DNA before reacting with Fe(II) or whether relaxation of the DNA structure by strand breakage is sufficient to allow the TBA reaction to proceed.

The damage caused by gamma radiation reached a plateau at ≈750 Gy whereas damage induced by H₂O₂ continued to rise dose-dependently beyond 220 μM. This phenomenon may reflect positioning of metal ions along the DNA backbone which direct the production of OH⁻ to sugar residues when reacting with H₂O₂. By increasing the concentration of H₂O₂, more metal ions can be reduced back to their reactive state as H₂O₂ can act as a reductant and react with Fe(III) to regenerate Fe(II) (reaction 7 - section 1.1.2.2).

Gamma radiation produces OH⁻ free in solution (section 2.4.1). Whilst increasing doses may produce more OH⁻ (1000 Gy producing ≈400 μM OH⁻), there is no targeting of these radicals to the sugar-phosphate backbone specifically. OH⁻ will react preferentially with base residues, rather than with the sugar-phosphate backbone to produce a single strand break. Therefore, it is possible that initially OH⁻ react with the sugar residues to cause strand breaks, base residues being unaccessible. However, this will cause a relaxation in the conformation of the DNA molecule due to a reduction in base stacking interactions thus rendering OH⁻ accessible to the base residues. OH⁻ will then react preferentially with the bases (Prigodich and Martin, 1990) rather than the sugar-phosphate backbone and therefore, any further DNA damage caused will not be detectable in the TBA assay.

UV light showed no significant ability to produce a TBA reactive product from DNA. Whilst UV light
is predominantly absorbed by the DNA bases (Deering, 1962), it is known to cause strand breaks by cleaving the sugar-phosphate backbone (Kaufmann and Briley, 1987). Therefore, it seems likely that UV light does affect deoxyribose. It is, however, possible that the magnitude of damage caused to deoxyribose during strand break production cannot be detected by the TBA assay system used.
CHAPTER 3.3: ANALYSIS OF DNA STRAND BREAKS

3.3.1: Introduction

There are at least two postulated mechanisms by which DNA strand breakage can occur: via the cleavage of the phosphodiester linkages in one of the polynucleotide chains (a mechanism used by DNA repair enzymes), or via destruction of the deoxyribose ring.

The presence of a single-strand break in DNA causes localised denaturation of the molecule and increases the probability of further attack at this site by nucleases, and potentially ROS, due to the loss of the protective effect of base-stacking interactions. The single-strand break is one of the major types of lesions produced by reaction of OH- with DNA but is not usually lethal (Ward, 1985); it is double-strand breaks which are thought to be the lesions responsible for causing cell death following oxidative damage (section 1.1.5.4) (Frankenberg et al., 1981).

Since low levels of base damage have been previously difficult to measure by physical or chemical means, (Paterson, 1978) strand break assays, by virtue of their greater sensitivity, have been widely employed by radiation biologists, to characterise DNA damage. The two techniques commonly used are alkaline filter elution and alkali unwinding. In alkaline solution, hydrogen bonds stabilising the DNA α helix are broken and the two strands unwind. The rate of unwinding is accelerated in the presence of strand breaks (Ahnström and Erixon, 1973). In such alkaline conditions the types of lesions measured include actual strand breaks and alkali-labile bonds; the latter can be further divided into base deletions, apurinic/apyrimidinic sites, neutral labile sites and alkali-labile sites. Therefore both assays are measuring the sum of the yields of a variety of distinct chemical lesions.

The principle of the alkaline elution assay is to measure strand breaks based on the molecular weight dependent retention of the molecule on a 2μm pore size polyvinylchloride filter. The initial DNA elution rate and the logarithm of the unaffected DNA fraction are both proportional to the amount of DNA damage (Blakely, Ward and Joner, 1982). However, the assay requires radiolabelling of cellular DNA prior to analysis, which is not appropriate for non-dividing cells and is very time-consuming.

The alternative alkaline-unwinding assay (FADU) uses the fluorescent dye, ethidium bromide, as a direct probe of DNA structure (Birnboim and Jevcak, 1981). Ethidium bromide will selectively bind to
double-stranded DNA in the presence of single-stranded DNA, when short duplex regions in single-
stranded DNA are destabilised by alkali. Conditions of the assay show fluorescent enhancement
preferentially with dsDNA with little interference from RNA, ssDNA or other cellular molecules
(Birnboim and Jevcak, 1981).

The FADU assay was used to analyse directly strand breaks induced by different ROS interacting with
the DNA molecule, to compare the damage caused by each of the ROS-generating systems, and to
investigate the effects of the strand breaks produced on cell viability.

3.3.2: Methods

The principle of the FADU assay is to calculate the percentage double stranded DNA in a sample, based
on the difference in fluorescence between a sample containing total dsDNA and a sample containing
negligible dsDNA.

The contribution to fluorescence by components other than dsDNA is estimated from a blank sample (B)
in which the cell extract is sonicated and then treated with alkali under conditions which cause complete
unwinding of low molecular weight dsDNA.

A second sample measures total fluorescence (T). The difference provides an estimate of the amount of
double-stranded DNA in the extracts. The strand breaks induced by a DNA damaging agent in a
particular sample (P) are measured as the fluorescence of the sample less the fluorescence of the blank
(P-B) and are expressed as a percentage of the total double-stranded DNA (T-B).

The double-stranded DNA remaining (D) is calculated as:

\[
D = \frac{(P-B)}{(T-B)} \times 100
\]

To determine the strand breaks induced by ROS in the DNA of PBMC, the cells were isolated from
whole blood as described in section 2.3.3.1 and resuspended to a final concentration of 5 x 10⁶ cells/ml
in 0.25M meso-inositol-10mM sodium phosphate-1mM MgCl₂ (pH 7.2). Aliquots of this suspension
(0.2ml) were distributed in quadruplicate to disposable glass tubes, designated T, B, or P. 0.2ml 9M
urea-10mM NaOH-2.5mM cyclohexanediaminetetraacetate-0.1 % SDS (A) was added to each tube,
which were then incubated at 0°C for 10 minutes to allow cell lysis and chromatin disruption to occur.
All tubes were then transferred to an area of subdued lighting for completion of the assay. To tubes P and B, 0.2ml of 0.45 volumes A in 0.2M NaOH was added gently without mixing and the tubes were incubated for a further 30 minutes at 0°C allowing alkali diffusion into the lysed cell suspension. The contents of tubes designated B were then sonicated for 1-2 seconds to cause rapid denaturation of the DNA in alkali, and both tubes P and B were incubated at 15°C for 15 minutes. Denaturation was stopped by chilling the tubes to 0°C and adding 0.4ml 1M glucose-14mM mercaptoethanol. After mixing, the tubes were vortexed, diluted with 1.5mls 6.7μg/ml ethidium bromide-13.3mM NaOH and the fluorescence read immediately at excitation 520nm, emission 590nm. A 10nm slit band width was used for both wavelengths.

The T tubes differ from the P tubes in that the neutralising solution (1M glucose-14mM mercaptoethanol) is added before the alkaline solution (0.45 volumes A in 0.2M NaOH) so that the DNA is never exposed to a denaturing pH. The percentage D values were calculated as described above for damage to DNA induced by ROS.

3.3.3: Results

3.3.3.1: Gamma Irradiation of PBMC

Isolated cellular DNA (section 3.3.2) was exposed to gamma radiation from 0 to 200 Gy at a dose of 25 Gy/minute at 0°C, and was analysed for the presence of DNA strand breaks and for cell viability (section 2.3.3.3). An initial lag in the development of strand breaks was seen, followed by a rapid decrease at doses greater than 100 Gy (figure 3.7a). Parallel investigation of viability showed that the greatest reduction in cell viability occurred before the rapid decrease in dsDNA (figure 3.8a).

The formation of strand breaks was inhibited by irradiating cells in the presence of 50mM thiourea (p<0.01) and 10mM ethanol (p<0.01) (figure 3.9). Catalase, desferrioxamine and SOD did not inhibit the formation of strand breaks in this system.

3.3.3.2: UV Irradiation of PBMC

PBMC were irradiated on ice for a maximum of 60 minutes to produce DNA strand breaks. An initial lag in the onset of strand breaks was followed by a linear decrease in dsDNA plateauing after 30
Figure 3.7

Effect of increasing exposure of DNA from PBMC to gamma radiation (3.7a), UV radiation (3.7b) and \( \text{H}_2\text{O}_2 \) (3.7c). Results show the % dsDNA remaining with increasing doses of ROS, as means ± 1SD of three separate experiments.
Decreases in cell viability induced by exposure of PBMC to increasing doses of the ROS-generating systems: gamma radiation (3.8a), UV radiation (3.8b) and $H_2O_2$ (3.8c). Results are means ± 1SD of three separate experiments.
Figure 3.9

Effect of desferrioxamine (desfer - 0.5mM), catalase (27,000 units/ml), thiourea (50mM), ethanol (10mM) and SOD (3,000 units/ml) on the % dsDNA remaining after exposure to 200 Gy. The control value (0 Gy) and the %dsDNA remaining after exposure to 200 Gy in the absence of any inhibitors are shown for comparison. Results are means ± 1SD of three separate experiments.
minutes UV irradiation with \( \approx 20\% \) dsDNA remaining (figure 3.7b). Viability studies showed that the decrease in dsDNA corresponded well with cell death (figure 3.8b).

The presence of catalase and thiourea inhibited the reaction significantly, (\( p < 0.001 \) and \( p < 0.01 \) respectively) (figure 3.9). Ethanol also inhibited the formation of strand breaks by 41\% (\( p < 0.01 \)) but irradiation in the presence of desferrioxamine and SOD had no significant effect on the level of DNA damage caused as measured by the FADU assay.

### 3.3.3.3: \( \text{H}_2\text{O}_2 \) and PBMC

Incubation of PBMC with increasing concentrations of \( \text{H}_2\text{O}_2 \) from 0 to 200\( \mu \text{M} \) at 37\(^\circ\)C for 30 minutes induced strand breaks in the DNA, (figure 3.7c) and a decrease in cell viability (figure 3.8c). The percentage dsDNA remaining did not decrease significantly beyond 75\( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) at which point 21\% of cells were still viable.

Catalase inhibited the reaction by 93\% (\( p < 0.001 \)), thiourea by 54\% (\( p < 0.01 \)) and desferrioxamine by 66\% (\( p < 0.01 \)) (figure 3.9). Ethanol inhibited the formation of strand breaks by 43\% (\( p < 0.05 \)) but SOD was once more ineffective in preventing strand break damage to DNA.

### 3.3.4: Discussion

The FADU assay measures strand breaks in DNA by the intercalation of the fluorescent dye, ethidium bromide, into the intact structure. The results demonstrate that ROS generated by each of the model systems produces strand breaks.

The incorporation of scavengers into the experimental ROS generating systems shows that \( \text{O}_2^- \) is not responsible for the DNA strand breaks, SOD being unable to inhibit the production of strand breaks. Since all systems generate \( \text{OH}^- \), the results also show that gamma radiation produces \( \text{OH}^- \) that can be scavenged more effectively than \( \text{OH}^- \) generated by UV or \( \text{H}_2\text{O}_2 \), since thiourea and ethanol inhibited the formation of strand breaks to a greater extent in the radiolysis system. This almost certainly reflects the potential for gamma radiation to form \( \text{OH}^- \) free in solution by the energetic cleavage of water molecules (section 2.4.1). These radicals are accessible to scavengers, whilst UV, and \( \text{H}_2\text{O}_2 \) especially are more dependent on DNA-bound metal ions to form \( \text{OH}^- \) (sections 2.4.2 and 2.4.3). These radicals react site-
specifically and therefore, preferentially, with DNA rather than the scavenger molecules.

Inhibition by desferrioxamine in the H$_2$O$_2$ model, infers that DNA bound metal ions are involved in the generation of strand breaks. In the UV model, H$_2$O$_2$ can be split into OH· by homolytic cleavage, as well as reacting with any DNA bound metal ions: as a result, OH· formation is not so dependent on metal ions. Inhibition of strand break formation with desferrioxamine was not as marked in the UV as in the H$_2$O$_2$ system, and although it caused a slight increase in the level of dsDNA remaining this was not significant.

The different mechanisms of OH· generation also appeared to affect the targeting of the DNA damage: after exposure to gamma radiation, in contrast to the iron chelator desferrioxamine, OH· scavengers were effective in the prevention of strand break formation. This may be partly a result of the generation of OH· independently of metal ion catalysis and partly due to the generation of strand-breaking nitroxide radicals (Davies et al., 1987; Willson, 1988) following irradiation of the desferrioxamine molecule.

Comparing the three models of ROS generation, there is always an initial lag before the percentage dsDNA decrease to ≈20% dsDNA. This level is reached following irradiation of cells with 200 Gy (equivalent to formation of 80μM OH·), or by incubating cells with ≈75μM H$_2$O$_2$, which can give rise to the same concentration of OH·. For UV, at 30 minutes irradiation the concentration of OH· in solution can approach 200μM if there is complete conversion of H$_2$O$_2$ to OH· by homolytic fission. However, the results suggest that OH· levels reached by UV in solution only approach 100μM if strand breaks are formed by the same mechanism in each of the systems studied. This may represent either incomplete homolysis of H$_2$O$_2$ or reaction of two molecules of OH· together, to drive the equilibrium reaction back in the direction of H$_2$O$_2$ formation.

The initial lag period before the rapid decrease in dsDNA in each of the systems (figures 3.7a, 3.7b and 3.7c) were also seen in studies by Ward (1975). This probably represents the formation of initial strand breaks which do not change the conformation of the DNA molecule and therefore do not allow penetration of the dye into the structure. Once a critical number of strand breaks has been produced, unwinding is accelerated (figure 3.7).
Whilst the three systems damage cellular DNA to the same extent in terms of percentage strand breaks formed, analysis of the cell viabilities suggests that strand breaks induced by gamma radiation are potentially more lethal to the cell than those produced in the other two systems. Gamma radiation produces spurs in which radicals are highly concentrated and can react to produce multiple damaged sites, i.e., many radical attacks in close proximity to each other (Ward, 1985). These have been shown to be more lethal than singly damaged sites (Ward, 1988). Since $\text{H}_2\text{O}_2$ reacts with bound metal ions, it will produce OH$^-$ at these discrete sites along the DNA backbone. From these results obtained in this section, it appears that this distribution of damage is more easily tolerated by the cell than the multiple site damage caused by gamma radiation.
CHAPTER 3.4: ANALYSIS OF DNA STRAND BREAKS IN NUCLEOIDS

3.4.1: Introduction

When cells are lysed in the presence of non-ionic detergents and high salt concentrations, structures resembling nuclei are released (Cook and Brazell, 1976). These are referred to as nucleoids and contain nearly all nuclear DNA and RNA but are depleted of histones and lack most chromatin proteins (Cook, Brazell and Jost, 1976).

The DNA packaged in nucleoids is superhelical and about $10^4$ single strand breaks are required to destroy the DNA supercoils (Cook and Brazell, 1975).

The conformation of populations of nucleoids has been measured by sedimentation in sucrose gradients, any single strand breaks present, allowing the DNA to unwind and sediment less rapidly than a population of intact nucleoids (Pitout et al., 1982). This principle has been applied by Milner, Vaughan and Clark (1987), to determine damage to nucleoids using a flow cytometer. Extracted nucleoids stained with the fluorescent dye, ethidium bromide, are individually exposed to laser light within the flow cytometer. The intensity of scattered and fluorescent light from each are expressed graphically as a frequency histogram.

Allan et al., (1988) have successfully demonstrated the use of this technique to analyse ROS damage to nucleoids isolated from PBMC. In the following section damage to DNA nucleoids has been studied more extensively in an attempt to identify the precise ROS involved and their mechanism of action.

3.4.2: Methods

PBMC were isolated from whole blood as described in section 2.3.3.1 and exposed to ROS (see sections 2.4.1.3, 2.4.2.3 and 2.4.3.3). Irradiation of RPMI-1640, or supplementation of the medium with \( \text{H}_2\text{O}_2 \), provided a highly reproducible oxidant environment in which to culture cells. The cells were resuspended immediately after recovery from the culture medium, at a concentration of $1 \times 10^6$ cells/ml in 1ml lysis buffer containing 2M NaCl, 10mM Tris (hydroxymethyl)methylamine and 10mM EDTA. The cell suspension was stored on ice for 30 minutes and then analysed by FACS according to the method of Milner, Vaughan and Clark (1987).
Briefly, each sample was stained with 50μg/ml ethidium bromide and analysed 90 seconds later, approximately 45 seconds after dye-substrate equilibrium had been reached. Nucleoids were drawn into a flow chamber and ejected singly through a 60 μm diameter orifice in a stream of PBS at a rate of 400/second. Ten microns below the point of injection the sample stream was intercepted by an argon 488nm laser beam, and light scattered from the nucleoids was collected by a photomultiplier array and single semiconductor detector. Forward scattered laser light was detected by the semiconductor sensor lying opposite the light source. The intensity of scattered light from each nucleoid was recorded as a digital signal and stored by the computer as a data point in a frequency histogram (10,000 nucleoid events were recorded for each sample).

3.4.3: Results

3.4.3.1: Gamma Irradiation of RPMI-1640

PBMC were incubated in irradiated RPMI-1640 (0-1000 Gy), for 24 hours prior to analysis of the cell derived nucleoids by FACS.

The expansion of PBMC-derived nucleoids following exposure to ROS generated by gamma irradiation, was not linear; there was only a significant difference between the unirradiated control sample and the PBMCs exposed to RPMI-1640 which had received a dose of 1000 Gy (figure 3.10).

To investigate the effects of ROS scavengers and the iron chelator desferrioxamine, these reagents were added to the RPMI-1640 prior to its irradiation. 50mM thiourea and 10mM ethanol were able to protect the cells against the damage caused by a dose of 1000 Gy by 50% (p<0.01) and 62% (p<0.01) respectively; desferrioxamine in contrast, offered no significant protection (figure 3.10).

3.4.3.2: UV Irradiation of RPMI-1640

RPMI exposed to UV light for a maximum of 60 minutes was then incubated with PBMC for 24 hours prior to FACS analysis. The effect of ROS scavengers and the iron chelator desferrioxamine was investigated by UV irradiation of the culture medium in the presence of catalase (27,000 units/ml), thiourea (50mM) or desferrioxamine (0.5mM).
Figure 3.10

Effect of gamma radiation on nucleoid expansion and the protective nature of thiourea (50mM), ethanol (10mM) and desferrioxamine (0.5mM). The nucleoids were isolated from PBMC incubated for 24 hours in complete culture medium (section 2.1.6) which had been exposed to gamma radiation prior to use (section 3.4.3.1). Results are means ± 1SD of the median channel numbers of three separate experiments.
Following exposure to UV radiation, a dose-dependent increase in the median channel number was observed (figure 3.11) representing an increase in the size of the nucleoid population.

Catalase was an effective inhibitor of the damage, reducing nucleoid expansion by 40% (p < 0.01) in samples exposed to 60 minutes UV. Thiourea, an OH· scavenger and metal ion chelator, also offered significant protection against ROS-mediated damage (53% - p < 0.01). Again, desferrioxamine, showed no significant inhibition of the effect of UV light on nucleoid populations.

3.4.3: H₂O₂ and RPMI-1640
The effect of addition of H₂O₂ to PBMC from 0 to 200μM for 24 hours on the cell-derived nucleoids is shown in figure 3.12. Concentrations of up to 50μM appeared to be tolerated by the cells; at 100μM concentration there was a significant increase in median channel number indicative of nucleoid expansion, with a further increase after exposure to 200μM H₂O₂. The effect of ROS scavengers and the iron chelator desferrioxamine was investigated by adding these reagents to the medium with H₂O₂.

As for gamma radiation, thiourea was an effective inhibitor of the production of DNA strand breaks induced by 200μM H₂O₂ (54% - p < 0.01). In this case, desferrioxamine also inhibited nucleoid expansion (31% - p < 0.01).

3.4.4: Discussion
This technique uses FACS to measure differences in light scatter between a control population of nucleoids and a population of nucleoids exposed to ROS generating systems. An increase in light scatter represents a relaxation of the DNA structure and this has been postulated to be caused by strand breaks nicking the superhelical structure (Milner, Vaughan and Clark, 1987). Alternatively, a diminished pattern of light scatter represents a decrease in size of the nucleoid population and is caused by increased association or cross-linking of the DNA within the superhelix.

Gamma irradiation of RPMI-1640, and its subsequent addition to cells, caused a significant increase in the size of the nucleoids analysed only after exposure to a dose of 1000 Gy. Irradiation of the medium produces OH· but these species have a short half-life and would not be predicted to remain in the medium. Instead, the radical species most likely to predominate is the longer-lived peroxy radical,
Figure 3.11

Effect of UV radiation alone and in the presence of catalase (27,000 units/ml), thiourea (50mM) and desferrioxamine (0.5mM) on PBMC nucleoid expansion. The nucleoids were isolated from PBMC previously incubated for 24 hours in complete culture medium (section 2.1.6) which had been exposed to UV radiation prior to use (section 3.4.3.2). Results are means ± 1SD of median channel numbers obtained in three separate experiments.
Figure 3.12

Effect of H$_2$O$_2$ alone and in the presence of thiourea (50mM), desferrioxamine (0.5mM) and mannitol (50mM) on PBMC nucleoid expansion. The nucleoids were isolated from PBMC previously incubated for 24 hours in complete culture medium (section 2.1.6) to which 200µM H$_2$O$_2$ had been added. Results are the means ± 1SD of median channel numbers from three separate experiments.
formed from the interaction of ROS with lipids and aromatic amino acids present in the culture medium. The results would suggest that a high dose of gamma radiation (1000 Gy), is required to produce sufficient concentrations of the damaging agent to cause strand breaks that induce nucleoid expansion. Both thiourea and ethanol were able to offer protection against nucleoid-damage. Both are OH· scavengers and their presence in solution consumes free OH·, thereby preventing the formation of any longer-lived radical species such as the peroxy radical. Desferrioxamine can chelate iron to prevent Fenton-generated OH· but this is not the major mechanism for the production of OH· in a gamma irradiated system. Desferrioxamine can also react with OH· (K ≈ 10^{10} M^{-1} s^{-1}) (Hoe, Rowley and Halliwell, 1982; Halliwell and Gutteridge, 1989, pp 188-276) but as described in section 3.1.4 upon irradiation it can also form a long lived nitroxide free radical. Therefore, the lack of protection by desferrioxamine against damage is most likely explained by its inability to prevent the formation of a longer-lived radical species which could cause DNA damage in this system.

In a similar study of the effects of gamma radiation on nucleoid expansion, Allan et al., (1988) found that preincubation of PBMC with desferrioxamine prior to direct irradiation of the cells was also unable to prevent ROS-induced damage to DNA. This might also be explained by formation of the nitroxide radical species and an inability of desferrioxamine to scavenge OH· as effectively as thiourea or ethanol.

UV irradiation of medium caused a dose-dependent increase in nucleoid expansion. The effect was inhibitable by catalase and thiourea; desferrioxamine also reduced nucleoid expansion but this was not significant. This suggests that $H_2O_2$, produced in solution by UV light, may react with DNA-bound metal ions to produce OH·, which damage the DNA in a site-specific manner. However, the predominant OH· production is from direct homolytic fission of $H_2O_2$.

Similar results were obtained for cells cultured in medium containing $H_2O_2$. Whilst low concentrations of $H_2O_2$ appeared to be tolerated well by the cells, significant damage was detected in samples incubated with concentrations of $H_2O_2$ exceeding 100µM. At concentrations lower than this, the strand breaks randomly induced along the DNA backbone may be present in such insufficient numbers and so spatially distributed that they do not cause a significant relaxing of the superhelical structure.

At 37°C cells also have the capacity to repair single strand breaks (Ward JF et al., 1987). DNA
damage has been shown to stimulate the nuclear enzyme, poly-ADP-ribose polymerase (Schraufstätter et al., 1986), which is associated with DNA repair and catalyses the conversion of NAD to nicotinamide and protein-bound poly-ADP-ribose. This process depletes the cell of NAD and subsequently of ATP, such that excessive repair of single strand breaks leads to cell death, not as a direct result of the presence of strand breaks but from depletion of cellular metabolites. This process may, however, be an inbuilt suicide mechanism for the cell. As described in section 1.1.5.4, strand breaks opposite each other may be incorrectly repaired, and as the concentration of strand breaks increases this event will become more frequent. The energy depletion caused by large numbers of strand breaks prevents repair of these strand breaks so ensuring that mis-paired strands are not replicated. This mechanism acts to maintain the fidelity of the genetic code.

It is possible that the lack of nucleoid expansion induced by low concentrations of H₂O₂ represents a tolerance to damage by repair of strand breaks and that expanded nucleoids reflect DNA derived from cells of low viability no longer able to repair ROS-induced DNA damage.
CHAPTER 3.5: CIRCULAR DICHROISM

3.5.1: Introduction

Circular dichroism (CD) is a type of electronic absorption spectroscopy that uses circularly polarised light. It is sensitive to the conformation of a molecule and can be applied to randomly orientated molecules in solution (Johnson, 1978). To exhibit a CD spectrum a molecule must be assymetric. Since most biological molecules are assymetric, CD spectroscopy has been used extensively to investigate the conformation of proteins (Cassim and Yang, 1970), nucleic acids (Lewis and Johnson, 1974) and sugars (Nelson and Johnson, 1976).

CD spectra are sensitive to secondary structure in biopolymers. In native dsDNA the differences in base composition and sequence are not sufficient to lead to noticeable differences in the spectra in the UV region (400-200nm); however, base-base interactions and the relative orientation of the bases can be detected in the vacuum UV region (200-100nm) (Brahms and Mommaerts, 1964; Johnson, 1978).

The CD spectra of native dsDNA typically consists of two parts, one positive with a maximum at \( \approx 273 \text{nm} \) and one negative with a minimum at \( \approx 243 \text{nm} \). At about 252nm the CD is zero and changes sign (Brahms and Mommaerts, 1964). In general, the CD spectra of DNA molecules are of low intensity but this does not represent a lack of helical organisation in solution (Luzzati, Nicolaëff and Masson, 1961). It is thought that within the DNA double helix, with two anti-parallel strands, the chromophores within each strand may lead to CD contributions of opposite signs and the resulting rotational strengths are therefore subtractive (Josse, Kaiser and Kornberg, 1961; Brahms and Mommaerts, 1964).

The experimental evidence indicating that the CD bands of nucleic acids are related to the helical conformation, is supported by the fact that disordered forms show no such bands. An increase in temperature inducing DNA melting causes a decrease of the CD bands, corresponding to a loss of secondary structure (Brahms, 1965).

In this section, the effects of ROS on the gross physical structure of the DNA molecule were investigated using this technique.
3.5.2: Methods

CD spectra were recorded on a JASCO J-40CS CD spectrophotometer. The concentration of DNA was 100\(\mu\)g/ml in PBS. The optical pathlength used was 1cm. Absorption spectra were measured prior to CD measurement on a Cary 17 spectrophotometer.

3.5.3: Results

3.5.3.1: Gamma Irradiation of DNA

DNA was denatured as in section 2.4.1.1 from 0 to 1000 Gy. The CD spectrum obtained for native dsDNA showed the typical minimum and maximum at \(\approx 245\)nm and \(\approx 275\)nm respectively. Irradiation of DNA at doses of 200 Gy and 500 Gy showed no significant difference in the CD spectra when compared to the control. However, the trace for DNA exposed to 1000 Gy showed a significant reduction in both the minimum and maximum peaks which are characteristic of the helical conformation of DNA (figure 3.13). The CD spectra obtained following irradiation in the presence of 100mM formate or 10mM phenylalanine were not significantly different from that obtained for the native dsDNA.

3.5.3.2: UV Irradiation of DNA

DNA was denatured as in section 2.4.2.1 at 15 minute intervals for a maximum of 60 minutes. Again no dose-dependent decrease in the minimum and maximum at \(\approx 245\)nm and \(\approx 275\)nm was observed, with exposure up to 30 minutes. At 45 minutes exposure, however, a sudden shift occurred in the spectrum; there was no further detectable change in the DNA structure at 60 minutes (figure 3.14). The decrease in both the minimum and maximum peaks by UV irradiation was of a similar nature but of lower magnitude to that observed after gamma irradiation. This suggests less damage to the DNA molecule occurred causing a smaller change in conformation.

The most obvious change to the DNA following UV irradiation was detected in the 220nm region. There was a dose-dependent shift towards the region of far-UV noticeable at 15 minutes increasing further with time. There was also a slight increase in intensity of this peak (figure 3.14).

3.5.4: Discussion

The decrease in both minimum and maximum peaks after exposure to gamma irradiation (at a dose of
Figure 3.13

Effect of gamma radiation on the CD spectrum of DNA. The continuous line represents native dsDNA. The broken line represents dsDNA exposed to a dose of 1000 Gy. Doses of 500 Gy and less had spectra not significantly different from that of the native dsDNA. Results are averaged curves from three separate samples exposed to the same doses of radiation in different experiments.
Figure 3.14

Effect of UV radiation on the CD spectrum of DNA.

- = 0 minutes UV
- - - = 15 minutes UV
- - - = 30 minutes UV
------ = 45 minutes UV

Changes were maximum at 45 minutes with the curve for 60 minutes exposure not significantly different from that of 45 minutes. At 15 and 30 minutes the traces were only different in the 200-220nm region. Results are averaged curves from three separate samples exposed to the same dose of radiation in different experiments.
1000 Gy) suggests that the integrity of the DNA structure is altered upon exposure to ROS (section 3.5.1). The lack of dose-response to gamma radiation implies that the strand breaks induced by exposure to low doses of irradiation are not able to cause gross structural damage to the DNA, until they are present in large numbers. The ability of formate or phenylalanine to prevent changes to the CD spectra occurring, suggests that OH\(^-\) alone was responsible for the changes induced by exposure to gamma radiation.

UV irradiation of an aqueous solution produces 200\(\mu\)M \(\text{H}_2\text{O}_2\) over a period of 60 minutes, the majority of this having been formed after 30 minutes. This has the potential to produce 400\(\mu\)M OH\(^-\) in solution. The changes in CD spectra following UV denaturation of DNA reflect this conversion of water molecules to ROS, with the shift in spectra maximised at 45 minutes.

Gamma radiation, however, produces OH\(^-\) dose-dependently, a dose of 1000 Gy forming 400\(\mu\)M OH\(^-\) in aqueous solution. The CD spectra for gamma irradiation of DNA showed a greater conformational change as a result of reaction of a greater concentration of OH\(^-\) with the DNA.

In both cases the changes in maximum and minimum peaks were not dose-responsive but occurred after a finite amount of damage. This probably reflects the initial protective effect of the hydrogen bonded \(\alpha\) helix which can tolerate a number of single strand breaks before the secondary structure undergoes conformational changes. The ability of UV derived OH\(^-\) to cause such changes at a lower concentration, may reflect the site-specific production of OH\(^-\) as a result of the DNA-bound metal ion reduction of \(\text{H}_2\text{O}_2\) rather than the complete random generation of the molecule in free solution.

The change in the CD spectra at \(\approx 220\)nm reflects a change in base interactions (Johnson, 1978). It is known that UV light causes production of thymine dimers in DNA (Cerutti, 1976). These dimers are cross-linked residues of thymine which can cross-link either the same or the opposite DNA strand. Cross-linking will exert a new stability on the DNA molecule which may account for the minor change in the maximum and minimum peaks \(\approx 275\)nm and \(\approx -245\)nm. The production of thymine dimers following exposure to UV was suggested by the appearance on the absorption spectra of a compound absorbing at 310nm, characteristic of the dimer residue, which increased with UV dose. Thymine
dimers are bulky residues and it is this parameter that may influence the CD spectra as demonstrated by the shift in the peak maximum from 219nm to 212nm.

Analysis of CD spectra has been used to determine the secondary structure of nucleic acids, chromatins, nucleoids, and different forms of DNA and RNA which all give characteristic spectra (Isenberg and Johnson, 1971; Rill and Van Holde, 1973). CD has probably been applied most successfully to the determination of unknown conformations using previously characterised data. It is clear that the changes induced by ROS do not alter the secondary conformation of DNA to an extent that is easily detected by CD. Therefore, other methods of structural and chemical determination would seem to be more suitable for analysis of ROS-induced damage to DNA.
CHAPTER 3.6: MONOCLONAL ANTIBODY BINDING TO DNA

3.6.1: Introduction

In man, the spontaneous production of autoantibodies reactive with DNA and other intracellular and cell surface antigens is characteristic of SLE and has been addressed in detail in section 1.2.4. In view of the poor immunogenicity of native dsDNA the availability of a mouse model of SLE from which monoclonal antibodies (MCA) can be produced is particularly useful.

Autoantibodies from mice with lupus-like disease have been well characterised, and a library of MCA reactive with DNA have been derived from two strains, (NZB x NZW)F1 and MRL/lpr/lpr (Morgan et al., 1985a). These have similar characteristics to those produced by Hahn et al (1980) and Jacob and Tron (1982).

The MCA can be classified on immunological criteria into five groups (Table 3.5). Of these, DNA reactive MCA which show cross-reactivity with cardiolipin and other ligands are predominantly members of groups II and III, the common property being recognition of different determinants expressed on both ssDNA and dsDNA. dsDNA specific antibodies (group I) do not react primarily with phosphodiester configurations and MCA reactive with base-dependent determinants do not bind cardiolipin.

A panel of these MCA was used to probe the structure and antigenicity of dsDNA following attack by ROS, to determine the changes induced by each of the model systems.

3.6.2: Methods

Changes to dsDNA induced by ROS were analysed by MCA in a competition ELISA, the principle of which is described in section 2.3.2.2. Volumes pipetted into wells were consistently 50μl and between each stage in the assay the plates were washed three times with PBST. Nunc 96 well plates were pre-sensitised with poly-L-lysine (25μg/ml in PBS) for 18 hours at 4°C. Native dsDNA was then added at 20μg/ml in PBS for 1 hour at 37°C. Any remaining binding sites were blocked with 100μg/ml albumin for 1 hour at 37°C to prevent binding of the competing DNA to the poly-L-lysine as well as the MCAs themselves. The competing antigen, ROS-denatured DNA, diluted in PBS and titrated at doubling dilutions across the ELISA plate from 1000μg/ml to 2μg/ml final concentration, was incubated at 4°C.
for 2 hours before an equal volume of MCA (at its optimum working concentration - Table 3.5) was added to the wells thus diluting the competing antigen by a factor of 2. The incubation was continued for a further 2 hours at 4°C to allow the MCA to bind to the immobilised DNA and the DNA free in solution. The plate was then washed and peroxidase-conjugated goat anti-mouse polyvalent immunoglobulins diluted 1/350 in PBS were added to label the MCA bound to the immobilised antigen. The reaction between \( \text{H}_2\text{O}_2 \) and OPD at pH 5 was measured spectrophotometrically at 492nm.

3.6.3: Results

DNA, denatured by ROS, as described in sections 2.4.1.1, 2.4.2.1, 2.4.3.1 and 2.4.4 was used as a competing antigen against native dsDNA and probed in a competition ELISA with MCAs of the five distinct groups whose antigenic specificities are described in table 3.5. The optimum working concentration for each antibody is also shown in table 3.5.

3.6.3.1: Group 1

MCAs 402 and 410 bind to the conformational determinants on dsDNA only. When DNA was exposed to 1000 Gy from a Co\(^{60}\) gamma source, the resulting antigen showed an increased binding affinity to both MCAs, represented by a negative shift in the binding curve by 2.5-fold and 8-fold for MCAs 410 and 402 respectively (figure 3.15).

UV radiation for 60 minutes, which theoretically generates the same amount of OH\(-\) as 1000 Gy from gamma radiation, resulted in a smaller increase in binding affinity to the denatured antigen as demonstrated by the smaller changes in the ID50, with no apparent change in the number of available binding sites. This was not true of DNA exposed to \( \text{H}_2\text{O}_2 \). Although DNA incubated with \( \text{H}_2\text{O}_2 \) only showed a slight increase in the number of binding sites which was further increased by incubation of DNA with the same concentration of \( \text{H}_2\text{O}_2 \) in the presence of AA, this was not significant. The major change induced by \( \text{H}_2\text{O}_2 \) and AA was a two-fold increase of the binding affinity of MCA 410 to the conformational determinants on dsDNA. The cross-over of the two curves suggests that on \( \text{H}_2\text{O}_2 \) denaturation there was a change in nature of the antigenic determinants, the MCAs possibly recognising a new epitope.
<table>
<thead>
<tr>
<th>Taxonomic Group</th>
<th>MCA number</th>
<th>Working Dilution</th>
<th>Antigenic Determinant recognised</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>402, 410</td>
<td>1/5120</td>
<td>Conformational determinants on dsDNA only - recognising groove region.</td>
</tr>
<tr>
<td>2</td>
<td>212, 405</td>
<td>1/160, 1/320</td>
<td>Conformational determinants on sugar - phosphate backbone expressed weakly on dsDNA.</td>
</tr>
<tr>
<td>3</td>
<td>152</td>
<td>1/640</td>
<td>Determinants on ssDNA expressed weakly on dsDNA</td>
</tr>
<tr>
<td>4</td>
<td>233, 228</td>
<td>1/640</td>
<td>Base-dependent determinants on ssDNA</td>
</tr>
<tr>
<td>5</td>
<td>223</td>
<td>1/640</td>
<td>Determinants on ssDNA exclusively.</td>
</tr>
</tbody>
</table>

Table 3.5

Classification of DNA-reactive murine monoclonal antibodies (MCA) showing the antigenic determinant and working dilution of each MCA used in all experiments.
Figure 3.15

Exposure of DNA to ROS from gamma radiation, UV radiation and H$_2$O$_2$ and subsequent effects on monoclonal antibody binding to DNA. The figure shows % binding of MCA from group 1 (table 3.5) to DNA immobilised on the ELISA plate, with serial two-fold dilutions of the competing antigen (Nat-DNA or ROS-DNA). For gamma radiation the ROS-DNA was exposed to 1000 Gy; for UV radiation the ROS-DNA was exposed to UV light for 60 minutes and for H$_2$O$_2$ the effects of 200μM H$_2$O$_2$ alone and in the presence of 10mM AA were studied. Results are means of three separate experiments.
3.6.3.2: Group 2

MCAs 212 and 405 bind to the sugar-phosphate backbone of both dsDNA and ssDNA.

Both gamma and UV radiation of DNA caused an increase in affinity of the binding interaction between the MCAs and ROS-denatured DNA (figure 3.16). Gamma radiation caused a two-fold greater shift in the binding curves when compared to UV, reflecting possibly the lower concentration of radicals generated by the latter.

Incubation of DNA with H₂O₂ and AA caused a significant positive shift in the titration curve (p<0.01) increasing the ID50 by a factor of ≈3. This represents a decrease in binding affinity. The intersection of the two titration curves suggests that a modification of the antigenic determinant recognised was also caused by exposure of DNA to ROS.

3.6.3.3: Group 3

MCA 152 recognises determinants on ssDNA that are expressed weakly on dsDNA.

Gamma and UV radiation of DNA induced an increase in binding affinity for this MCA which binds very poorly to native dsDNA. Gamma radiation caused a 6-fold reduction in ID50 compared to a 2-fold reduction for UV (figure 3.17).

H₂O₂ and AA reacted with DNA to cause a slight, but not significant increase in the binding of this antibody to DNA. This reflects an increase in availability of the ssDNA determinants recognised by MCA 152.

3.6.3.4: Group 4

MCAs 233 and 228 recognise base residues on single-stranded regions of DNA.

Both MCAs showed poor reactivity with native dsDNA but were able to bind more effectively to a ROS-denatured antigen. Exposure of DNA to gamma and UV irradiation and to H₂O₂ caused an increase in binding affinity of these MCAs to base residues. The ID50 was reduced significantly by 0.5
Figure 3.16

Exposure of DNA to ROS from gamma radiation, UV radiation and H$_2$O$_2$ and subsequent effects on monoclonal antibody binding to DNA. The figure shows % binding of MCA from group 2 (table 3.5) to DNA immobilised on the ELISA plate, with serial two-fold dilutions of the competing antigen (Nat-DNA or ROS-DNA). For gamma radiation the ROS-DNA was exposed to 1000 Gy; for UV radiation the ROS-DNA was exposed to UV light for 60 minutes and for H$_2$O$_2$ the effects of 200µM H$_2$O$_2$ alone and in the presence of 10mM AA were studied. Results are means of three separate experiments.
Figure 3.17

Exposure of DNA to ROS from gamma radiation, UV radiation and $H_2O_2$ and subsequent effects on monoclonal antibody binding to DNA. The figure shows % binding of MCA from group 3 (table 3.5) to DNA immobilised on the ELISA plate, with serial two-fold dilutions of the competing antigen (Nat-DNA or ROS-DNA). For gamma radiation the ROS-DNA was exposed to 1000 Gy; for UV radiation the ROS-DNA was exposed to UV light for 60 minutes and for $H_2O_2$ the effects of 200μM $H_2O_2$ alone and in the presence of 10mM AA were studied. Results are means of three separate experiments.
(p < 0.01), 2 (p < 0.01) and 5-fold (p < 0.001) for \( \text{H}_2\text{O}_2 \), UV radiation and gamma radiation respectively (figure 3.18).

The nature of the curves obtained for the MCA 233, suggests that the change in affinity induced by exposure of DNA to gamma and UV radiation was not shown as clearly for DNA incubated with \( \text{H}_2\text{O}_2 \). In this case the nature of the antigenic determinant appears to have been altered as shown by a change in shape of the titration curve.

3.6.3.5: Group 5

MCA 223 binds exclusively to ssDNA and indeed showed poor reactivity with the native molecule. UV irradiation of DNA did not change significantly the ID50 of >1000\( \mu \)g/ml for this MCA and there was no change in affinity of binding. Gamma radiation, producing \( \approx \) twice the concentration of \( \text{OH}^- \) in solution than UV caused a significant increase in binding affinity to regions of single stranded DNA, reducing the ID50 from >1000\( \mu \)g/ml to 251\( \mu \)g/ml. The change induced by \( \text{H}_2\text{O}_2 \) also showed a trend towards production of regions of single stranded DNA but this was not significant (figure 3.19).

3.6.3.6: Effects of Scavengers on MCA Binding

The scavengers behaved similarly for the five groups of MCA studied but differently between the models of ROS generation. Thiourea and ethanol inhibited gamma-induced ROS production such that no significant difference was observed between Nat-DNA and ROS-DNA for any of the MCAs; mannitol offered 74 ± 6% (p = 0.01) inhibition whilst desferrioxamine and SOD had no effect on the titration curves.

In the case of UV-induced damage, thiourea and ethanol were again effective inhibitors reducing the maximum difference between the two curves by 81 ± 3% (p < 0.001) and 75 ± 4% respectively (p < 0.001). Mannitol and desferrioxamine also inhibited the ROS-induced changes by 42 ± 4% and 49 ± 5% respectively, whilst SOD was ineffective as an inhibitor.

None of the inhibitors offered significant protection for damage induced by reaction of \( \text{H}_2\text{O}_2 \) only. However, for \( \text{H}_2\text{O}_2 \) and AA reacting together, desferrioxamine and thiourea significantly inhibited the
Figure 3.18

Exposure of DNA to ROS from gamma radiation, UV radiation and H₂O₂, and subsequent effects on monoclonal antibody binding to DNA. The figure shows % binding of MCA from group 4 (table 3.5) to DNA immobilised on the ELISA plate, with serial two-fold dilutions of the competing antigen (Nat-DNA or ROS-DNA). For gamma radiation the ROS-DNA was exposed to 1000 Gy; for UV radiation the ROS-DNA was exposed to UV light for 60 minutes and for H₂O₂ the effects of 200μM H₂O₂ alone and in the presence of 10mM AA were studied. Results are means of three separate experiments.
Figure 3.19

Exposure of DNA to ROS from gamma radiation, UV radiation and $\text{H}_2\text{O}_2$ and subsequent effects on monoclonal antibody binding to DNA. The figure shows % binding of MCA from group 5 (table 3.5) to DNA immobilised on the ELISA plate, with serial two-fold dilutions of the competing antigen (Nat-DNA or ROS-DNA). For gamma radiation the ROS-DNA was exposed to 1000 Gy; for UV radiation the ROS-DNA was exposed to UV light for 60 minutes and for $\text{H}_2\text{O}_2$ the effects of 200$\mu$M $\text{H}_2\text{O}_2$ alone and in the presence of 10mM AA were studied. Results are means of three separate experiments.
reactions by 52±3% and 34±2% respectively. SOD, mannitol and ethanol did not cause a significant change in the ROS-DNA titration curve.

3.6.3.7: DNA Denatured by Neutrophil-Derived ROS

DNA isolated from stimulated and resting neutrophils was also used as the competing antigen in the ELISA assay. Using the reduction of cytochrome c as an indicator of the $O_2^\cdot$ produced (section 2.4.4), activated neutrophils generated 200µM / 10^6 cells / hour of which at least 80% is known to dismutate to form $H_2O_2$ (Root and Metcalfe, 1977).

Results of DNA isolated from resting lymphocytes showed no significant difference from native dsDNA, and DNA from activated cells produced titration curves that were superimposable on the curves obtained from DNA incubated with $H_2O_2$ in the presence of the reducing agent, AA (figures 3.15-19).

3.6.4: Discussion

The monoclonal antibodies used in these studies have been well characterised by Staines, Thompson and Morgan (1986). They show that ROS generated in different systems have diverse effects on the DNA macromolecule. The changes induced by UV and gamma irradiation do not always reflect the changes induced by exposure to $H_2O_2$/AA. Gamma radiation can produce OH· free in solution following ionisation of water molecules surrounding DNA, and UV can form OH· in solution from the energetic dissociation of $H_2O_2$. Owing to their high reactivity these OH· can only react with DNA if produced in close proximity to the macromolecule. Scavengers can compete with the DNA for reaction with OH· as illustrated by the ability of ethanol and thiourea to protect against damage. SOD catalyses the dismutation of $O_2^\cdot$ to $H_2O_2$ and this might be predicted to accelerate the production of OH·, although no enhancement of DNA damage was seen.

The production of OH· by 1000 Gy is 400µM compared with 200µM $H_2O_2$ by 60 minutes exposure to UV light that can also potentially give rise to 400µM OH·. The greater effect of damage seen in the DNA exposed to ionising radiation probably arises from the distribution of OH· in solution: gamma radiation produces OH· in spurs (see section 3.3.4) and in aqueous solution the concentration of OH· within a spur is ≈0.5M (Ward, 1988). Reaction of OH· at several sites on DNA in close proximity to
each other enhances the damage done to a localised area. UV light does not produce OH$^-$ in this way but from the breakdown of $H_2O_2$ which has a more even distribution in solution.

In the absence of metal ions in solution (following chelex treatment of all reagents), $H_2O_2$ can react only with DNA-bound metal ions to generate OH$^-$. This model gives rise to site-specific DNA damage by virtue of the diffusion limited potential of OH$^-$ to react within 1.5nm of its site of generation (Ward JF et al., 1987).

Damage caused by this site-specific interaction of OH$^-$ with DNA produced a pattern of change in DNA atypical of that produced by the two models of high energy radiation. The changes involved, affected the affinity of the binding reaction more commonly than the number of available binding sites.

The changes seen in ROS-denatured DNA suggest that a relaxation of the dsDNA helix occurs. MCAs 402 and 410 indicated an increase in binding affinity to conformational determinants on dsDNA following exposure of DNA to UV and gamma radiation, suggesting that these determinants are partially shielded by the native conformation. Exposure to $H_2O_2$ may also increase availability of previously shielded epitopes to the MCA since it appears that new epitopes are recognised following site-specific damage to the DNA molecule as catalysed by DNA-bound metal ions.

MCAs 152 and 223 suggest that the denaturation of DNA by ROS does not reflect damage induced by heating. Both these MCAs recognise determinants on ssDNA. $H_2O_2$ could not significantly induce changes to dsDNA that could be recognised by these antibodies, indicating that large regions of ssDNA were not formed. However, binding of MCAs 233 and 228 to base residues after ROS denaturation by gamma and UV radiation suggest that the damage induced does expose the usually protected base residues. The affinity change suggests that epitopes recognised by the MCAs may be altered increasing the affinity of the interaction post-irradiation. Again, $H_2O_2$ modification appeared to expose new epitopes; these may be oxidised derivatives of the native bases such as 8OHGDG, shown to be formed in such ROS-mediated reactions in section 3.1.

The results of experiments using DNA derived from activated neutrophils suggest that reaction of DNA with $H_2O_2$ in the presence of a reducing agent provides a reliable model system for ROS generated
during inflammation. It further shows that excessive ROS production such as that generated during the respiratory burst of neutrophils is detrimental to the cell, causing severe DNA damage.

The protection offered by the scavengers was most effective in the models of radiation-generated ROS. Thiourea, an OH· scavenger and a metal ion chelator, inhibited the effects of H₂O₂ most effectively but did not cause significant reduction of the reaction of DNA with H₂O₂ alone. Thiourea seemed to play a more significant role in the prevention of reduction of the DNA-bound metal ions, thus diminishing perpetuation of the denaturing Fenton reaction. The poor protection seen with mannitol and ethanol may reflect an inability of the scavengers to gain access to the OH· before it reacted with DNA, since the OH· is generated on the DNA backbone directly at the metal ion binding sites. Also, thiourea is 4.7 times more effective at scavenging OH· than mannitol (Halliwell and Gutteridge, 1989 pp 22-85).

Desferrioxamine did not significantly inhibit the gamma irradiation-induced changes; this may again reflect the interaction of ROS with desferrioxamine itself, which appears to affect the characteristic inhibition seen by the native molecule (Halliwell and Gutteridge, 1989 pp 188-276).

The monoclonal antibodies derived from mouse models of SLE bind to DNA and, by virtue of their classification, can provide information concerning the structure of the molecule. It has been demonstrated that different methods of ROS production give rise to distinct and characteristic changes to DNA which can be used further to probe DNA that is suspected to have been exposed to ROS.
CHAPTER 3.7: SUMMARY

The work described in this chapter utilised several techniques to measure the effects of ROS on the biomolecule DNA.

The conclusions drawn from the results obtained are as follows:

The hydroxyl radical appears to be an important damaging species in all ROS-generating systems used. However, the scavenging potential of agents such as thiourea and ethanol in these systems differs. The damage induced by gamma radiation is inhibited by these scavengers much more effectively than damage induced by UV or H$_2$O$_2$. This most probably reflects the metal ion dependency of OH$^-$ formation in the latter two systems, particularly H$_2$O$_2$.

As all reagents were chelex-treated, metal ions were restricted to the DNA itself. The ability of scavengers to inhibit at all in this situation probably reflects the type of metals bound to DNA. Whilst copper ions have a very strong association with DNA and produce OH$^-$ that are not significantly scavenged (Stoewe and Prütz, 1987; Dizdaroglu, Aruoma and Halliwell, 1990), the association between iron and DNA is weaker, allowing some ions to be chelated.

This may be important in biological systems. During inflammation neutrophils produce O$_2^-$, 80% of which dismutates to H$_2$O$_2$ (Root and Metcalfe, 1977). This then reacts to produce OH$^-$. The natural scavengers of OH$^-$ such as ascorbate and uric acid (section 1.1.6) may be unable to prevent ROS-induced damage in this case since they are inaccessible to site-specifically generated OH$^-$. They may, however, be more able to prevent ROS from UV light (producing some OH$^-$ in solution) reacting with DNA.

In addition to damage caused by OH$^-$, other radical species, postulated to be peroxy radicals, appeared also to be important in these systems. Peroxy radicals have extended half-lives compared to OH$^-$ and may be very relevant in vivo, since they are formed from reaction of OH$^-$ with lipids and amino acids. They may be formed within the nuclear membrane as radicals diffuse towards DNA: whilst this membrane may provide a protective barrier, it may also produce these long-lived radical products that can damage DNA.
The scavengers studied behaved similarly in each of the ROS-generating systems differing only in the extent of protection they afforded, with the exception of the chelator, desferrioxamine.

This molecule chelates iron and can also scavenge OH⁻. In experiments where desferrioxamine was added to cell preparations, it protected DNA damage from H₂O₂, where metal ions are involved, but was unable to protect DNA from the effects of UV or gamma radiation. In contrast, addition of desferrioxamine directly to dG or DNA consistently resulted in increased damage by all three ROS-generating systems.

There is evidence for the generation of a long-lived nitroxide radical following gamma irradiation of desferrioxamine (Davies et al., 1987; Willson, 1988) and this may account for the increased DNA damage, the nitroxide radical increasing the time of radical exposure. Since UV and H₂O₂ also generate similar radicals it is likely that desferrioxamine can be altered by these agents also. However, the results suggest that the generation of nitroxide radicals in a whole cell system is in some way prevented, since the enhancement of DNA damage was not seen in these situations. The prevention of nitroxide radical formation may occur if desferrioxamine complexes with cellular protein, or binds more efficiently to DNA. It is also possible that nitroxide radicals are produced, but are scavenged by cellular components.

This highlights the caution that must be used when interpreting data using scavengers to implicate radical species and reiterates the need for several scavengers of any one radical, such as thiourea, ethanol and mannitol for OH⁻, to be used.

Although each of the three model systems generating ROS consistently damaged DNA by the same mechanisms as determined by scavenger studies, the measurable end points of DNA damage varied both in their sensitivities and specificities.

Using CD to monitor structural changes to DNA induced by ROS, it was demonstrated that the technique detected changes in UV-damaged DNA more easily than DNA damaged by exposure to gamma radiation, UV light producing bulky adduct residues. The CD spectra gave a crude estimation of alterations to DNA and was only able to reflect modifications to DNA that resulted in extreme
structural changes. It would clearly not be appropriate for measuring low levels of damage that might persist in the germ-line and have biological relevance.

Changes in DNA structure caused by levels of ROS found in vivo (ie. 200μM H₂O₂) were more successfully determined using a panel of murine monoclonal antibodies. Although the method is not particularly sensitive, using several antibodies with different binding specificities a detailed picture of the DNA damage could be obtained. In later chapters, where DNA was isolated from biological material in sufficient quantity, this method was chosen to assess DNA structural changes in different disease pathology.

Two assays were used to measure strand breaks in DNA. Neither assay allowed differentiation of double strand breaks from single strand breaks, both estimating strand breakage on the basis of ethidium bromide dye intercalation into the DNA structure. Strand breaks produced by very low levels of ROS were detected by both assays; combined with viability data, this provided an index of lethality. In later chapters the nucleoid assay was used in conjunction with other assays to give an estimation of the initial damage to DNA caused by exposure to ROS and to quantify this damage. It was chosen due to its better reproducibility and simplicity compared with the FADU assay.

The TBA assay was not used further. Although damage to deoxyribose could be detected, the assay was not sensitive enough to detect changes in all systems. Furthermore, there is evidence that products measured in this assay may be generated during the assay itself, so it is not considered to be a definitive measure of ROS damage.

The assay used predominantly to assess oxidative damage to DNA in the following chapters is measurement of 8OHGD by HPLC/ECD. There is considerable evidence to support 8OHGD as a reliable indicator of OH⁻-induced damage to DNA (see section 3.1) and this is supported by the findings in this chapter. The assay itself is quick to perform and very sensitive, detecting levels of 8OHGD down to 0.1 picomoles. Specificity can be achieved using ECD, since most bases do not give a signal at the applied potential (600mV). It is therefore ideal for measurement of DNA damage and has been applied here to the analysis of ROS-induced DNA damage produced during inflammation.

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SECTION 4:
ROS DAMAGE TO DNA IN INFLAMMATORY DISEASE STATES
The general in vivo defence mechanisms that limit the concentrations of ROS, and the damage they induce to biological material have been described in sections 1.1.5, 1.1.6 and 1.1.7. A further specific mechanism for maintenance of the integrity of nuclear DNA in mammalian cells is its compartmentalisation away from peroxisomes and mitochondria, where most radicals are likely to be generated (sections 1.1.4.2 and 1.1.4.4) (Richter, Park and Ames, 1988).

Also, most non-replicating nuclear DNA is surrounded by a protective barrier of histones and polyamines. In spite of this, DNA is still susceptible to spontaneous damage such as attack by endogenous and exogenous agents and ionising radiation, all of which can cause strand breakage and modification of base residues (Ward, 1985; Hagen, 1986). To avoid the detrimental biological consequences of this, most of the types of DNA damage can be removed by efficient repair systems (Lindahl, 1982).

Most cells contain a few enzymes that specifically reverse DNA damage. Examples include photolyase, which reverses the photochemical fusion of adjacent pyrimidine bases into pyrimidine dimers caused by UV (Haseltine, 1983); and O6-methylguanine methyl transferase, which enzymatically removes methyl groups from the highly mutagenic base adduct as well as from the phosphodiester backbone (Cairns et al., 1981). However, the predominant mechanism of DNA repair is excision repair (Lindahl, 1982). This involves removal of a single base or a segment of DNA producing a single strand break or a gap in the DNA helix: from this gap, DNA polymerase can initiate synthesis of a new strand using the complementary strand as a template. Alternatively, glycosylases act by removing the damaged base from the sugar residue leaving the backbone intact and creating a hole (an AP site - apurinic or apyrimidinic) (Coulondre et al., 1978). This hole is recognised by an AP endonuclease which cleaves the backbone upstream from the site of damage and DNA polymerase again initiates synthesis of the DNA.

Once removed from DNA, the damaged DNA bases may, if released as deoxynucleotides, lose a phosphate to become deoxynucleosides, which are not metabolised further (Ames, 1988). The low
molecular weight of these residues allows them to pass easily through the filtration system of the glomerular capillaries and be excreted in the urine (section 4.1.2.4).

Measurement of DNA bases recovered in the urine can be used as an indicator of the level of DNA damage and assays have been described for detection of thymine glycol (Cathcart et al., 1984; Adelman, Saul and Ames, 1988) and 8OHDG (Shigenaga, Gimeno and Ames, 1989; Fraga et al., 1990).

Other workers have measured DNA base adducts in tissues rather than urine because considerable preparation of urine is needed before analysis (Floyd et al., 1986a; Fraga et al., 1990). Also, many products excreted in urine are electrochemically active and present in concentrations many times greater than DNA bases. DNA isolated from tissues can be easily purified and since most DNA bases do not give an electrochemical signal there is less interference from other products (section 3.1.1). However, measurement of DNA damage, in tissues only, does not give an indication of excreted damaged DNA bases, only the accumulation in the individual cells.

Several preparatory procedures have been described to facilitate analysis of DNA-derived compounds in urine by HPLC/ECD (Cundy, Kohen and Ames, 1988; Shigenaga, Gimeno and Ames, 1989) and with the use of cyclic voltametry as described in section 2.6.2.1 to determine peak purity, urinary levels of DNA bases can be accurately measured.

The methods for detection of damage to DNA have been described in chapter 3. In this section they have been used to assess DNA damage caused by the inflammatory process. The specific aims were to investigate and monitor 8OHDG as a marker of oxidative damage to DNA occurring in the inflammatory diseases SLE and RA, and in healthy controls.

8OHDG was measured in preference to other DNA base adducts, since, as described in section 3.1.1 it is a reliable indicator of ROS-mediated damage to DNA that can be sensitively detected using HPLC with ECD (Floyd, 1990). However, in view of the difficulties of measuring urine-derived compounds by HPLC/ECD, a further confirmatory technique was used to detect 8OHDG. This was GCMS which detects compounds on the basis of molecular weight, each compound giving a signal, characteristic of that compound and distinct from any other signal.
CHAPTER 4.1: MEASUREMENT OF 8OHGD IN URINE

4.1.1: Introduction

RA is a chronic inflammatory disorder with the inflammation located predominantly in the joints. The characteristic swelling, pain and loss of function of the joint are mediated by neutrophils accumulating in the synovial fluid, producing levels of $O_2^{-}$ at a rate exceeding 100nM/min. $O_2^{-}$, and its derivatives (section 1.1.2) can then react with surrounding biomolecules to cause tissue damage (section 1.1.7).

Previous work has shown that IgG denatured by ROS in vitro, closely resembles IgG which has been isolated from the serum and synovial fluid of patients with RA (Griffiths and Lunec, 1991). It has also been shown that ROS-denatured IgG binds better than native IgG to serum rheumatoid factor (Lunec et al., 1986; Swaak et al., 1989), suggesting that the altered antigenicity of the ROS-denatured IgG molecule may play a role in the production of anti-IgG autoantibodies in RA (Griffiths and Lunec, 1988; Kleinveld et al., 1989).

Damage to DNA from cells in the rheumatoid synovium has not been characterised; evidence relating to tissue damage and studies related to DNA damage in isolated neutrophils however, suggest that DNA damage does occur during periods of oxidative stress (Floyd et al., 1986a).

In SLE, another chronic inflammatory disorder, antibodies that react with DNA are found in the serum (Stollar, 1981). The potential role of a ROS-denatured DNA molecule in the pathogenesis of SLE has been tentatively suggested by Halliwell (1982), Emerit and Michelson (1981), and Johnson and Ward (1982).

8OHGD, excreted in the urine of SLE and RA patients and controls, was measured as a marker of oxidative stress to further investigate the role of a ROS-denatured DNA in inflammation in general and more specifically in SLE.

Measurement of 8OHGD in urine was used as an indicator of the levels of altered DNA base removed from cellular DNA following oxidative insult, and as a marker of the ability of the patients to cope with any DNA damage caused during periods of disease activity.
4.1.2: Methods

4.1.2.1: Clinical Samples

Early morning urine samples were collected from 33 SLE patients with normal renal function (29 female, 2 male, age range 20-64, median = 39), 10 RA patients (9 female, 1 male, age range 57-80, median = 67) and two groups of 10 normal controls: young normal controls (8 female, 2 male, age range 20-47, median = 26) not differing significantly in age or sex from the SLE group, and elderly normal controls (9 female, 1 male, age range 62-82, median = 70) not differing significantly in age or sex from the RA patients. Samples were stored at -20°C prior to analysis.

RA patients were prospectively selected to have an active inflammatory status based on an elevated level of the acute phase protein, C-reactive protein (CRP). In this group CRP values ranged from 42-123 mg/l, median = 64 mg/l (normal range < 6 mg/l).

The 33 SLE patients studied had a range of inflammatory disease activity as determined by levels of erythrocyte sedimentation rate (ESR) (5-92 mm/hour, median = 26 mm/hour - normal range < 20 mm/hour).

Two different indicators of inflammatory status were used because CRP is not a good indicator of inflammatory activity in SLE, whilst disease-associated anaemia can affect ESR but not CRP values in RA.

4.1.2.2: Preparation of Urine Samples for Analysis by HPLC/ECD

Urine contains many electrochemically active compounds in high concentrations, whilst products of DNA damage are present at concentrations in the picomolar range. Therefore, before analysis by HPLC/ECD it was necessary to remove selectively these compounds, which would otherwise mask the presence of the DNA base.

This was done using cartridges of reverse phase material. The selective precolumn separation of 8OHDG from other urinary components was achieved using increasing concentrations of aqueous methanol as described below.
Urine (500μl) was added to a 1ml C-18 SEP-PAK column preconditioned with 10mls methanol and 10mls water. The eluant was discarded and the column washed with 5% aqueous methanol. The fraction containing 8OHGD in highest concentration was then eluted with 15% aqueous methanol. The eluant was evaporated to dryness and the sample resuspended in 200μl mobile phase (section 2.6.2.1).

The percentage recovery of 8OHGD, based on calculations using a urine sample spiked with a known concentration of authentic standard, was 59±6%. The value obtained is similar to the values of 55-65% for purification of thymine glycol and thymidine glycol from urine (Cathcart et al., 1984) and compares favourably to the value of ≈35% for extraction of 8OHGD from urine (Shigenaga, Gimeno and Ames, 1989).

The percentage recovery reflects the inability to recover 8OHGD from residual volumes remaining on the column at each stage of the extraction, and the particularly low recovery of 35% obtained by Shigenaga, Gimeno and Ames (1989), reflects an increased number of column extractions compared to the method used here.

4.1.2.3: HPLC Analysis

10μl of the final fraction of the urine sample was analysed by HPLC/ECD as described in section 2.6.2.1.

Results are expressed as picomoles 8OHGD excreted, determined by comparison with a known concentration of standard 8OHGD, per μmol creatinine.

4.1.2.4: Correction of Results for Renal Function

Plasma creatinine passes freely through the glomerular filter and is neither absorbed nor excreted by the tubules. Therefore, the quantity excreted in the urine is identical with the amount filtered by the glomeruli. However, damage to the kidneys can cause a decrease in the glomerular filtration rate which subsequently causes a rise in serum creatinine as less of the molecule is excreted.

Measurement of creatinine was used in this study as an indicator of urine concentration in patients selected to have normal renal function, since in patients with abnormal renal function creatinine
Creatinine was measured by a commercial kit (Sigma) using a colorimetric assay based on the reaction of creatinine with picric acid, under alkaline conditions, to produce a yellow-orange complex (Heinegard and Tiderström, 1973).

For measurement of 8OHHDG in urine results were standardised for the concentration of urine by determination of creatinine concentration. Early morning urine samples were used in the analysis because of the diurnal variation in excretion, and these samples are, in general, more concentrated thus increasing the levels available for detection.

4.1.3: Results

The results of measurement of 8OHHDG in patients with SLE, RA and the two control groups are shown in Figure 4.1 and table 4.1. Levels of 8OHHDG were undetectable in all SLE patients studied ie levels were < 0.1 picomole 8OHHDG / µm creatinine. The normal ranges established from the control groups were 1.0-5.2 picomoles 8OHHDG / µm creatinine (median = 3.0 picomoles 8OHHDG / µm creatinine) for the young normal controls, and 0.75-3.9 picomoles 8OHHDG / µm creatinine (median = 2.5 picomoles 8OHHDG / µm creatinine) for the elderly normal controls. The difference between these two control groups was not significant.

In RA, the levels of 8OHHDG excreted were 25-32 picomoles 8OHHDG / µm creatinine (median = 29.5 picomoles / µm creatinine) and were significantly different from the values obtained for the elderly controls (p < 0.001).

There was no significant difference between voltammograms (section 2.6.2.1) of the authentic 8OHHDG standard and peaks co-eluting with authentic 8OHHDG in either the RA or control group (figure 4.2).

4.1.4: Discussion

The results obtained for the normal controls in this study are of the same order of magnitude as those described by Cundy, Kohen and Ames (1988). There was no significant difference in the levels of 8OHHDG excreted by the young and elderly normal controls, although a slight decrease is noticeable in
Figure 4.1

Log plot of levels of 8OHDG found in early morning urine samples of SLE patients (n=33), young normal controls (n = 10), RA patients (n = 10) and elderly controls (n = 10). The figure shows the median and ranges for each of the groups, all the results for the SLE group being below the detection limit of the assay. Each point plotted represents the mean of triplicate urine samples analysed for each person studied.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>age range</th>
<th>median</th>
<th>pmoles 8OHDG/μM creatinine</th>
</tr>
</thead>
<tbody>
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<td><strong>SLE</strong></td>
<td>33</td>
<td>20-64</td>
<td>39</td>
<td>&lt;0.1</td>
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<tr>
<td><strong>Control (I)</strong></td>
<td>10</td>
<td>20-47</td>
<td>26.5</td>
<td>1.0-5.2</td>
</tr>
<tr>
<td><strong>RA</strong></td>
<td>10</td>
<td>57-80</td>
<td>67.5</td>
<td>25-32</td>
</tr>
<tr>
<td><strong>Control (II)</strong></td>
<td>10</td>
<td>62-72</td>
<td>70</td>
<td>0.75-3.9</td>
</tr>
</tbody>
</table>

Table 4.1

Measurement of 8OHDG in urine from patients with SLE and RA and in two control groups aged and sex matched for SLE (I) and RA (II) respectively. Results are expressed as medians and range of pmoles 8OHDG / μM creatinine. Each value represents the mean of results obtained from triplicate urine samples.
Figure 4.2

A representative hydrodynamic voltammogram of 8-hydroxydeoxyguanosine (8OH DG) peaks from normal and RA urine samples (continuous line) compared to the Ag/AgCl reference electrode, and compiled from the means ± 1SD of readings obtained in three separate experiments. The 8OH DG used as the reference in these experiments (broken line) was supplied by Dr H Kasai.
the figures obtained for the elderly group. Fraga et al., (1990), have shown that in rats there is a significant decrease in the level of excreted 8OHDG with age but a significant increase in the levels of 8OHDG found in DNA in some but not all tissues. Although this decrease in the rate of excretion has not been confirmed in humans it is thought that ROS damage to DNA could contribute to the aging process (Ames, 1983; Cutler, 1984). This is believed to be due to a slow loss of DNA nuclease activity but may also be due to an increase in the rate of oxidative DNA damage.

In RA, there was a significant increase in the levels of 8OHDG excreted, compared to normal. There are at least two explanations for this. The RA patients studied were selected for their active disease based on serological measurements of CRP. In an active inflammatory state production of IL1, IL6 and TNFα can cause pyrexia which in turn can increase the basal metabolic rate of the patients. This alone could be responsible for the elevation of DNA damage products: previous work has shown a positive correlation between metabolic rate and excreted DNA base adducts for a number of altered DNA bases including 8OHDG and thymine glycol (Adelman, Saul and Ames, 1988; Bergtold et al., 1988; Shigenaga, Gimeno and Ames, 1989). There is also an inverse correlation between metabolic rate and life span suggesting that oxidative damage causes irreversible DNA damage over a period of time that results in an increased risk of malignancy towards the end of the life span and finally death (Ames, 1988). This finding may be relevant to patients with RA since they have a greater incidence of certain malignancies compared to controls (Miller, 1967; Louie and Schwartz, 1978).

A second possible reason for the observed increase in excretion of 8OHDG in RA patients with active disease is increased oxidative damage to DNA caused by an exacerbated inflammatory response. This damage is predominantly in the joint, where the synovial fluid is heavily infiltrated by neutrophils, and deposits of iron in the synovial lining can catalyse ROS production (reviewed by Blake, Allen & Lunec, 1987).

Purity of the 8OHDG peaks were confirmed by voltammograms. This eliminates the possibility of drugs or other compounds interfering in the assay and giving false positive results.
The group of patients with SLE had a range of ESR values (5-92 mm/hour - median = 26 mm/hour), some indicative of high inflammatory activity and others not significantly raised above the normal range (< 20 mm/hour).

Extrapolating from the results for the RA patients, it would be expected that if similar mechanisms of 8OHDDG excretion exist in SLE, there would be increased levels of 8OHDDG in the urine of SLE patients with active disease and high ESR values, and lower values in patients with inactive disease and normal ESR values. However, all SLE patients studied had undetectable levels of 8OHDDG in the urine.

This is unlikely to be due to inadequate sensitivity of the assay since normal controls with no inflammatory activity had measurable levels of 8OHDDG. It cannot be explained by abnormal renal function and clearance either, since all patients were specifically selected to have normal renal function.

In view of this, possible explanations for the lack of excretion of 8OHDDG in urine of SLE patients included an inability of SLE cells to repair 8OHDDG, or accumulation of damaged DNA in circulating immune complexes which are known to occur in this disease.
CHAPTER 4.2: 8OHGD IN DNA AND SUPERNATANTS OF SLE AND NORMAL CELLS

4.2.1: Introduction

The mechanisms for removal of damaged DNA bases from the intact strand have been described in section 4.1.1. This involves excision of the DNA base adduct from the cell and its removal from the body in urine. The apparatus for repair is contained within the cell and cells in culture have been used as a model for the assessment of DNA repair. Hariharan and Cerutti (1974), demonstrated successfully the removal of damaged thymine residues from an ROS-damaged synthetic polymer of adenine and thymine using crude extracts of Escherichia coli.

In this study, whole cells were exposed to \( \text{H}_2\text{O}_2 \). Cellular DNA and supernatants of these cells were collected over a period of time, and assessed for 8OHGD content.

Cells used were PBMC which can be easily separated from other blood components to produce a healthy population of cells (section 2.3.3.1). In SLE and RA there is immunological dysfunction and an abnormal production of autoantibodies from lymphocytes results. These patients are also more susceptible to malignancy, particularly those affecting the immune system ie. lymphomas and leukaemias (Miller, 1967; Louie and Schwartz, 1978; Green, Dawson and Walker, 1978). Both these disease associations could arise from cellular dysfunction following DNA damage at sublethal levels.

More recent evidence suggests that susceptibility to malignancy may be more related to therapy than to an immune dysfunction (Green et al., 1986).

PBMC were incubated with physiological concentrations of \( \text{H}_2\text{O}_2 \) to determine the effects of ROS on SLE PBMC DNA, and to compare these effects with DNA from PBMC of normal controls.

4.2.2: Methods

4.2.2.1: Clinical samples

PBMC were isolated from peripheral blood of 23 SLE patients (22 female, 1 male, age range 21-56, median = 33) and 20 normal controls (19 female, 1 male, age range 22-51, median = 34) as described in section 2.3.3.1. Cells were resuspended to \( 1 \times 10^6 / \text{ml} \) and cultured in 24 well plates in a volume of 2mls complete culture medium (see section 2.1.6).
4.2.2.2: FACS Analysis

FACS analysis of cell nucleoids was shown in section 3.4.4 to be an indicator of ROS-mediated damage and was used to measure any differences between SLE and normal cells following the initial ROS insult.

SLE and normal PBMC isolated as above were incubated with 200μM H₂O₂ for 24 hours. The cells were then washed twice in PBS, resuspended to a concentration of 1 x 10⁶ / ml in lysis buffer and analysed by FACS as in section 3.4.2.

4.2.2.3: Cell Incubation

Cells from each individual were incubated in the presence or absence of 200μM H₂O₂ for 7 days. At intervals during this period cell viability was determined as in section 2.3.3.3 and cells and supernatants collected.

The cells were separated by centrifugation at 300 x g for 10 minutes at 4°C, washed twice in PBS and resuspended to 10⁸ / ml in ice-cold TE. DNA was isolated from cells as described in section 2.3.1.1 and digested as in section 2.3.1.4 before analysis by HPLC/ECD.

Supernatants were collected after centrifugation and 2 volumes of ice-cold ethanol was added to each sample. Any DNA was allowed to precipitate at -20°C for 24 hours and pelleted by centrifugation at 0°C for 10 minutes in an Eppendorff centrifuge. The remaining supernatant was freeze-dried and resuspended in 1ml sterile water. Ethanol precipitation of the supernatants was used to remove large fragments of DNA arising from cell death so that the only detectable 8OHDG was from individual bases or small fragments of DNA arising from excision repair.

4.2.2.4: HPLC/ECD Analysis of DNA and Supernatants

10μl of digested DNA or resuspended supernatant was analysed by HPLC/ECD as described in section 2.6.2.1.

4.2.3: Results

4.2.3.1: FACS Analysis

Incubation of PBMC from SLE and normal patients with 200μM H₂O₂ caused a significant increase in
nucleoid size, p < 0.05 for both groups. There was, however, no significant difference between the untreated SLE and normal PBMC, or between the two groups incubated with H$_2$O$_2$ for 24 hours (figure 4.3).

4.2.3.2: Cell Viability

No significant difference was found between the loss of viability for SLE and normal cells incubated in the absence of H$_2$O$_2$. In the presence of H$_2$O$_2$ a significant decrease in viability was found both in SLE (p < 0.001) and normal (p < 0.001) cells when compared with the untreated cells between days 2 and 7 (figure 4.4a and figure 4.4b respectively).

SLE cells (figure 4.4a) showed a significantly greater reduction in viability compared to normal cells (figure 4.4b) under H$_2$O$_2$-induced oxidant stress (p < 0.05). This was evident between days 2 and 6.

4.2.3.3: 80HDG in Cell Supernatants

80HDG was measured in the supernatants collected from cells incubated with and without H$_2$O$_2$. There was no detectable 80HDG in the supernatants from normal or SLE PBMC incubated in the absence of H$_2$O$_2$.

However, 80HDG was detectable in the supernatants of both SLE and normal PBMC incubated with H$_2$O$_2$ (figure 4.5). Levels of 80HDG in normal cell supernatants reached a maximum at day 2 and slowly decreased after this. Such an initial increase in the 80HDG levels was not observed in SLE supernatants, but a very gradual increase was seen over the 7 day period (figure 4.5).

4.2.3.4: 80HDG in Cellular DNA

DNA isolated from the cultured cells was digested and analysed by HPLC/ECD for 80HDG content. The results are expressed as pmoles 80HDG / µg DNA (1µg DNA was extracted from $\approx 10^6$ cells).

Cells incubated in the absence of H$_2$O$_2$ had detectable levels of 80HDG in the DNA after 4 days. For normal cells, levels of 80HDG then plateaued at a constant level for the remainder of the incubation period. In contrast, the level of 80HDG in SLE cells continued to rise gradually, and appeared to be still increasing at day 7 (figure 4.6a).
Figure 4.3

Effect of exposure of PBMC to 200µM \( \text{H}_2\text{O}_2 \) on nucleoid expansion. Results are shown for PBMC from SLE patients (n = 23) and normal controls (n = 20) incubated in the presence or absence of \( \text{H}_2\text{O}_2 \). Results represent the mean ± 1SD for the median channel numbers of each sample.
Figure 4.4

Decrease in cell viability of SLE (4.4a) and normal (4.4b) PBMC incubated in the presence or absence of 200μM H₂O₂. Results show the means ± 1SD of three separate experiments.
Figure 4.5

Picomoles of 8OHDG excreted into the supernatants of SLE and normal PBMC incubated with H$_2$O$_2$ for a period of seven days. Results are expressed as picomoles of 8OHDG / 10$^6$ cells and represent the mean ± 1SD of three separate experiments.
Figure 4.6

Picomoles of 8OH DG in cellular DNA from SLE and normal PBMC incubated in the absence (4.6a) or presence (4.6b) of 200µM H₂O₂ for a maximum of seven days. Results are expressed as picomoles 8OH DG / 10^6 cells from which 1µg DNA was obtained and represent the mean ± 1SD of three separate experiments.
DNA isolated from cells incubated in the presence of $H_2O_2$ had the highest level of 8OHDG at day 1 in both the SLE and the control group. In normal cells, this level decreased rapidly and reached a minimum at day 6. This was not significantly different from the level at the equivalent time point in the untreated cells. The profile of 8OHDG as a component of DNA in SLE cells differed from that of the normal cells: whilst a similar peak of 8OHDG production was achieved at day 1, the rapid return to baseline was not seen; there was a more gradual reduction in the level of 8OHDG (figure 4.6b). The differences in the two curves at days 2, 4, and 6 were significant at $p<0.01$.

4.2.4: Discussion
The viabilities of the normal and SLE PBMC cultured without $H_2O_2$ were not significantly different during the seven day incubation period. Since the blood was separated into components by Ficoll on the basis of density (Boyum, 1968), the layer containing mononuclear cells is likely only to contain healthy cells; any abnormal cells probably sedimenting at a greater rate. On this basis, SLE cells would be expected to have similar properties to those of normal mononuclear cells; this might explain the similar viabilities of the two groups of cells in an unstressed environment.

Exposure of both SLE and normal cells to $H_2O_2$ led to a significant reduction of their viabilities. On day 1 of the experiments the viabilities of the $H_2O_2$-treated cells for the SLE and normal group were not significantly different by conventional counting. However, from day 2 onwards there was a significantly greater decrease ($p<0.05$) in the viabilities of SLE cells compared to normal cells. These results suggest that SLE cells are more susceptible than normal to $H_2O_2$ as a ROS-generating system.

Increased sensitivity of SLE cells to ROS-producing systems such as UV light has been reported previously (Compton, Steinberg and Sano, 1984; Golan and Borel, 1984): the mechanisms involved have been suggested to include accumulation of DNA-damage products and an increased susceptibility of the DNA to degradation.

To investigate any difference in the induction of DNA damage, FACS analysis was used to monitor changes in nucleoid expansion as a marker of structural changes in DNA. On day 1 of $H_2O_2$ exposure there was no significant difference in the size of the nucleoids from SLE or normal cells. This corresponded well with viability data from day 1 which showed no significant difference between SLE
and normal cells at the same time point. It is suggested that ROS, produced from $H_2O_2$ reacting in a DNA-bound metal ion catalysed reaction to produce $OH^-$, damage DNA from both SLE and normal cells by the same mechanism. From these results it appears that DNA of SLE cells is not more susceptible to ROS-mediated damage than normal. The decreased viability of SLE cells therefore is due to an event following the initial induction of damage.

Supernatants of SLE and normal PBMC cultured with or without $H_2O_2$ were collected for analysis of 8OHGD. They were mixed with ethanol to remove any large fragments of DNA arising from cell lysis; thus, any 8OHGD in the remaining fraction was assumed to arise from excision repair of DNA. Supernatants of untreated cells did not contain detectable levels of 8OHGD. Although 8OHGD is known to be produced during normal metabolism, this occurs only at a low rate of conversion (1 in $10^5$ dGs - < 0.03 pmoles/μg DNA, Floyd et al., 1986a) and when excreted into the culture medium it was probably diluted to a level undetected in the system.

Supernatants from $H_2O_2$-exposed cells contained 8OHGD, whether these were normal or SLE derived. In the normal supernatants, there was a rapid excision of 8OHGD from DNA increasing until day 2, after which the level declined slowly until at day 7 there was < 1 picomole 8OHGD / 2 x 10^6 cells. The maximum excretion of 8OHGD at day 2 follows the damage induced by ROS seen both by FACS analysis and by the maximum conversion of dG to 8OHGD in cellular DNA on day 1 (figure 4.6b). The $H_2O_2$ added to the cells acts initially to produce DNA damage but by reacting becomes degraded. Any intracellular catalase would also contribute to the degradation of $H_2O_2$ to $H_2O$. Therefore the decrease in 8OHGD after 2 days was probably due to removal of the ROS.

SLE supernatants showed a different pattern of 8OHGD excretion. There was a slow increase of 8OHGD which persisted beyond day 7. Data from the FACS experiments indicated that an equivalent amount of DNA damage had been caused by $H_2O_2$ to SLE cells and this was confirmed by the incorporation of 8OHGD in cellular DNA not differing significantly between the SLE and normal cells. However, once formed, the 8OHGD in SLE cells was clearly not repaired as in the normal cells.

The 8OHGD content of DNA from the SLE and normal cells also showed a different pattern of distribution. DNA from untreated normal cells contained detectable levels of 8OHGD at day 3, and this
level then remained constant. On day 4, 36% of the cells were dead and the build up of 8OHDG probably reflects decreasing exonuclease activity of the remaining viable cells. Untreated SLE cells also had levels of 8OHDG detectable from day 4 but the level continued to rise suggesting that 8OHDG was accumulating. This could be because of an inability to excise 8OHDG from the DNA, which would in turn contribute to the number of cells dying by decreasing the integrity of the DNA.

Both normal and SLE cells exposed to $H_2O_2$ showed a rapid conversion of dG to 8OHDG at day 1 suggesting that the two cell populations were equally susceptible to damage. However, whilst normal cells showed a rapid removal of the damaged mutagenic base from the DNA paralleled by an increase of the base in the supernatant, SLE cells showed a considerable decrease in this rate of removal.

The results obtained suggest that SLE cells are not abnormally sensitive to the initial effects of ROS-induced damage, ie the formation of the oxidative products, but that they are unable to mount an adequate response to repair this damage. This seems to involve an impaired repair system for 8OHDG in SLE. As a result the cells accumulate the altered base adduct which is mutagenic, causes mispairing of DNA (Kuchino et al., 1987; Shibutani, Takeshita and Grollman, 1991), and contributes to cell death. This would account for the decrease in viability of SLE cells following exposure to $H_2O_2$ as well as UV and other ROS-generating systems when compared with normal.
4.3.1: Introduction

Circulating immune complexes represent a normal and effective immunological effector mechanism for the clearance of antigens (Contreras et al., 1982), complement aiding this process by inducing their solubilisation. However, immune complex deposition may also play an important pathogenic role in diseases such as SLE as described more fully in section 1.2.5.

As shown in section 4.1.3, inflammation in RA is associated with elevated levels of the excreted DNA damage product 8OH DG in urine. This does not appear to be the case in inflammation associated with SLE.

Preceding repair, damaged DNA bases may be excised. In this case only a few nucleotides are linked, which are not modified further and appear in the urine. However, extensive DNA damage results in cell death due to depletion of essential energy sources such as ATP and NAD (Schraufstatter et al., 1985; Schraufstätter et al., 1986). The energy sources are cofactors for the repair of DNA strand breaks. If sufficient DNA damage occurs to deplete the cell of ATP and NAD during repair, cell death occurs.

Therefore, beyond a critical level of DNA damage, the DNA repair process causes cell death and lysis, releasing DNA of high molecular weight (Schraufstätter et al., 1986). This may be a mechanism to eliminate cells that have undergone extensive DNA damage.

DNA is known to be found in circulating immune complexes in SLE (Bruneau and Benveniste, 1979; Adu, Dobson and Williams, 1981; Contreras et al., 1987) and may be derived from cells following lysis. The persistence in the circulation may then result from a failure to clear the immune complexes containing the bound DNA effectively.

The hypothesis that a ROS-denatured DNA molecule may persist in the circulation of SLE patients and play a role in the pathogenesis of the disease was investigated by isolating DNA from immune complexes from sera of SLE patients and analysing this DNA for 8OH DG as a marker of oxidative damage.
Immune complexes were also isolated from a group of normal controls to determine if DNA could be detected. This was investigated to see if immune complexes are normally involved in the clearance of cellular DNA.

Immune complexes were also isolated from a group of RA patients as an inflammatory control group to investigate the effects of increased cellular DNA damage on immune complex clearance of antigen.

4.3.2: Methods

4.3.2.1: Clinical Samples

Plasma was collected as described in section 4.3.2.2 from 29 SLE patients, 14 with normal renal function (12 female, 2 male, age range 25-51, median = 31), and 15 with abnormal renal function (15 female, age range 27-66, median = 37). Also from 10 RA patients (9 female, 1 male, age range 57-80, median = 67) and two groups of 10 normal controls (8 female, 2 male, age range 20-47, median = 26.5) and (9 female, 1 male, age range 62-82, median = 70) not differing significantly in age or sex from the groups of SLE and RA patients respectively.

4.3.2.2: Isolation of Immune Complexes from Sera

Venous blood was collected into heparin and centrifuged for 30 minutes at 1500 x g. The temperature of the blood was maintained at 37°C immediately after collection and throughout the duration of the centrifugation procedure. Plasma (10mls) was collected from the blood sample and incubated at 4°C for 7 days to induce cryoprecipitation. 0.01% sodium azide was added to the samples as a preservative and antioxidant.

After incubation, the 10ml aliquots were centrifuged at 800 x g for 30 minutes at 4°C; the supernatant was discarded and 1ml of cold PBS added; after 1 hour at 4°C the tubes were centrifuged at 800 x g for 30 minutes at 4°C; the supernatant was discarded and the pellet washed three times with 10mls PBS (800 x g, 30 minutes, 4°C). Finally 1ml PBS was added to the samples which were stored at 4°C. The samples were incubated at 37°C for 1 hour to allow resolubilisation immediately prior to analysis.

4.3.2.3: Isolation of DNA from Immune Complexes

Proteinase K (100μg/ml) and 1% SDS in a 1ml volume of 20mM EDTA, pH 8.0, were added to the 1ml
volume of resolubilised immune complex and the mixture was incubated at 65°C for 30 minutes (from Contreras et al., 1987). The protein was removed by extracting three times with 1/2 volume of phenol and 1/2 volume of chloroform (Maniatis, Fritsch and Sambrook, 1982, pp 458-462). To precipitate the DNA, 2 volumes of cold ethanol in the presence of 0.3M sodium acetate were added to the aqueous phase which was then left at -20°C for 24 hours. The DNA was washed with 70% ethanol, lyophilised and resuspended in 100μl TE.

4.3.2.4: Analysis of DNA Isolated from Serum Immune Complexes by HPLC/ECD

An aliquot of the DNA isolated from immune complexes was digested to individual nucleosides as described in section 2.3.1.4. Ten microlitres of the digested DNA was then injected onto the HPLC column and 8OHGD detected as described in section 2.6.2.1.

A further aliquot was used to determine the amount of DNA in the sample as described in section 2.3.1.2.

Results are expressed as moles 8OHGD per μg DNA, the volume of sera from which the DNA was obtained being the same in each case.

4.3.2.5: Analysis of DNA Isolated from Serum Immune Complexes by Agarose Gel Electrophoresis

An aliquot of DNA was loaded onto an agarose gel as described in section 2.3.1.3 for analysis of size. This was determined by its ability to move through the gel at an applied voltage, and by comparison of the bands obtained with markers of known molecular weight (from lambda DNA cut with the restriction enzymes, EcoRI and Hind III).

4.3.3: Results

Cryoprecipitates were isolated from the plasma of 27/29 SLE patients studied, 10/10 RA patients and 3/20 normal controls.

The amount of DNA isolated from each of these cryoprecipitates, from 10mls plasma was measured. There was no significant difference between the amount of DNA isolated from the groups of SLE patients with (100-270μg) and without (105-248μg) renal disease. There was, however, significantly
less DNA isolated from the cryoprecipitates of normals (2-7μg - p < 0.001) and RA patients (2-8μg - p < 0.001).

Figure 4.7 shows the concentration of 8OHDG, corrected for the amount of DNA isolated from the immune complex for each of the patient groups. There was no detectable 8OHDG in the DNA isolated from the RA or normal cryoprecipitates (ie < 0.1 picomole 8OHDG / μg DNA) and when compared with the SLE group with normal (2.3-9.25nM / μg DNA) or abnormal (1.35-12.7nM / μg DNA) renal function this reduction was significant for both groups at the p<0.001 level.

A comparison of the two groups of SLE patients (with or without associated renal complications) showed that as for the amount of isolated DNA, there was also no significant difference between the amount of 8OHDG isolated from DNA of these immune complexes.

Analysis of the molecular weight of DNA isolated from the immune complexes showed that the DNA was consistently ≈ 20 kilobases based on a comparison with the gel markers (figure 4.8). There was no difference in the size of the DNA obtained from patients with or without renal disease, or from patients with RA or the normal controls (figure 4.9).

4.3.4: Discussion

Immune complexes play a role in the clearance of antigens but are quickly solubilised by complement. There is evidence of defective solubilisation and clearance of immune complexes from the circulation in SLE (Baatrup et al., 1983). This is reinforced by the results obtained in this study. 27 of the 29 patients investigated had cryoprecipitable circulating immune complexes.

RA is also an autoimmune disease associated with circulating immune complexes, consisting predominantly of IgG and autoantibodies reactive with IgG. These may arise from oversecretion of immunoglobulins capable of associating to form complexes or from an interaction between autoantibody and antigen (Nydeggar and Lambert, 1982).

All 10 patients with RA also had immune complexes in their plasma. However, cryoprecipitable material was isolated only from three of the normal controls, two from the elderly group and 1 from the
Figure 4.7

Log plot of nmoles of 8OHGD / μg DNA detected in DNA isolated from SLE and RA patients and normal controls. Levels of 8OHGD in the RA and control groups were below the detection limits of the assay. The median and ranges of values for the SLE group, subdivided into patients with or without normal renal function are shown.
Figure 4.8

A typical gel of DNA isolated from an SLE immune complex showing in lane 2 the predominant band of DNA corresponding to \( \approx 20 \) Kbases from comparison with the molecular weight markers of lambda DNA cut with the restriction enzymes, EcoRI and HindIII which give the molecular weight fragments shown.
A typical gel of DNA isolated from immune complexes of SLE, RA and control sera. Lane 1 represents DNA from sera of an RA patient, lanes 2-4 DNA from sera of SLE patients, and lanes 5-7 DNA from normal controls. Although the DNA recovered varies in amount, there is no significant difference in size.
group age-matched for SLE patients. This supports evidence for an imbalance in the immunological network and reduced clearance of circulating immune complexes in SLE and RA.

The results obtained from analysis of the cryoprecipitates for DNA content suggest that autoantibodies play a major role in the formation of immune complexes in both SLE and RA and the constituents found therein.

However, the mechanisms for immune complex formation may differ in the two diseases. In SLE, antibodies to double and single stranded DNA are found in the serum (Stollar, 1981). DNA is not usually found in the circulation and anti-DNA antibodies with the potential to bind to DNA, released from cells for example, may inhibit the action of nucleases which would normally degrade the released DNA.

Once formed, these complexes may, due to the presence of tissue surface antigens also in the complex, become deposited in tissues. Complexes containing DNA and anti-DNA antibodies have been isolated from glomerular basement membranes (section 1.2.5).

In RA, the complexed antigen is IgG, itself an antibody which is commonly found in the circulation. The antibodies to IgG are of the IgM class, predominantly, and IgM can form large lattice complexes as a result of its pentameric valency (Pope, Teller and Mannik, 1974). Therefore, in RA, immune complexes may form more readily due to the overproduction of immunoglobulins and the favourable binding properties of IgM.

It is suggested that although immune complexes may be formed in normals to permit antigenic clearance (Contreras et al., 1982), and in RA because of an immunological imbalance (Gronowicz and Coutinho, 1976; Shlomick, Nemaze and Sato, 1986), DNA does not naturally become sequestered into these complexes because it is degraded by nucleases. Only when the DNA is complexed to an anti-DNA antibody, which protects against the degradative effects of nucleases, does DNA become incorporated into cryoprecipitable complexes and persist in the circulation of SLE patients.

The lack of a significant difference between DNA found in circulating immune complexes from patients
with and without normal renal function suggests that a finite amount of complexes containing DNA may circulate with no detrimental effect to the kidney (as demonstrated by the presence of DNA/anti-DNA antibody complexes in the circulation of patients with normal renal function). However, once there is deposition of these complexes in the kidney an equilibrium may exist between DNA containing complexes in the serum and those bound to the tissue such that deposition of complexes in tissues does not deplete the presence of complexes in the circulation completely.

In RA, although DNA damage does occur, the DNA does not become trapped in a complex as shown in the results (section 4.3.3). The lack of DNA in RA complexes may infer that the anti-DNA antibodies sometimes associated with RA, with specificities to predominantly ssDNA, are of low affinity with low dissociation constants, thereby enabling the nucleases to degrade the DNA more easily than the DNA bound by high affinity antibodies in SLE.

The size and conformation of the isolated DNA will be discussed more fully in section 5.1 but from these results it is clear that the size of the DNA fragments isolated from the complexes are consistently \( \geq 20 \) kilobases. From this it is suggested that this DNA is from cells following lysis that accompanies cell death rather than from active excision of DNA from the intact genome. The concentration of 8OHGD in this DNA suggests that ROS-mediated damage in SLE cells produces 8OHGD but that this lesion is not effectively repaired, contributing to or resulting in cell death.

The DNA isolated from immune complexes of RA patients and normal controls did not contain detectable levels of 8OHGD. It is known that 8OHGD is formed as a result of normal oxidative metabolism (Richter, Park and Ames, 1988) as well as during periods of oxidative stress such as inflammation (Floyd et al., 1986a). The lack of detection of the altered base in these samples provides further evidence that an effective repair mechanism exists for repair of this potentially mutagenic base. This repair process, by producing single bases or small fragments of DNA too small to be antigenic, but removing the lesions from cellular DNA, prevents anti-DNA antibody formation and releases these damaged bases into the circulation for final excretion in urine.
CHAPTER 4.4: GAS-CHROMATOGRAPHY MASS SPECTROSCOPY

4.4.1: Introduction

Analysis of compounds by HPLC/ECD relies on the identification of an unknown by comparing its elution time with that of a pure standard and "spiking" the sample containing the unknown with this standard. This gives rise to the possibility that a compound with the same retention time but a different chemical composition could produce a false positive result in a chemical assay. To overcome this, if the compound is UV absorbing a comparison of the maxima and minima absorption values can be made and in the case of a compound detected by ECD a voltammogram can be compiled from the response of the compound at increasing voltages (see section 2.6.2.2). In both cases different compounds are unlikely to give the same response but to identify the exact chemical composition of a molecule a technique such as mass spectroscopy must be applied.

Gas-chromatography combined with mass-spectroscopy has been used successfully to analyse radiation damage to the DNA sugar moiety (Dizdaroglu, von Sonntag and Schulte-Frohlinde, 1975) and more recently applied to the detection of damage to DNA bases (Dizdaroglu, 1985a; Dizdaroglu, 1985b; Dizdaroglu and Bergtold, 1986). GCMS can detect damage to DNA bases at levels that are biologically relevant (Dizdaroglu, 1986a; Dizdaroglu, 1986b; Dizdaroglu and Bergtold, 1986) ie levels from which the cells can recover. It also has the advantage over many other analytical procedures for the analysis of base damage to DNA, that products of the four bases can be well separated from one another and also from the four intact bases.

Before analysis by GCMS, because of their low volatility, bases must be derivatised to make them suitable for analysis.

The process of gas chromatography involves the physical separation of a moving gas phase by adsorption onto a stationary phase consisting of an inert solid such as silica gel coated with a non volatile liquid (silicone oil), on the inner surface of a long heated capillary tube. A constantly flowing stream of an inert gas such as helium keeps the volatile compounds moving through the column. Separation of the vaporised DNA bases is dependent on the differing affinities of the compounds of the gas mixture for the stationary phase. Gases which are strongly attracted to the stationary phase move
through the column at a slower rate and emerge later than those that are less attracted.

As the individual products emerge from the column, they are detected by mass-spectroscopy. This involves bombardment of the molecule with high energy electrons, causing it to fragment into ions characteristic of that compound. The ions of most importance in this study are the intact molecular ion (M$^+$), and M-15$^+$, typical of trimethylsilylated derivatives arising from loss of a methyl radical from M$^+$ (Dizdaroglu, 1989).

Sensitivities of as low as 10 femtomoles per injection have been achieved by incorporating selected ion monitoring (SIM) into the GCMS assay (Dizdaroglu and Bergtold, 1986). In the SIM mode the mass spectrometer is set to measure only those ions that are characteristic of a specific compound in a complex mixture. This is more sensitive than total ion monitoring because it permits the mass analyser to dwell longer on, and monitor only specific masses.

When signals of specifically monitored ions, with their typical abundances relative to one another are detected, a positive identification is assumed.

GCMS/SIM was used to measure 8OHGD in urine and cryoprecipitate DNA to confirm the identity of the products that had been detected by HPLC/ECD.

4.4.2: Methods

For analysis of DNA isolated from immune complexes (section 4.3.2.3), the DNA was ethanol precipitated as in section 2.3.1.1, and freeze dried. 2.5 nmoles 8AZA were added before the modified DNA bases were cleaved from the polynucleotide chain prior to analysis. This was achieved by hydrolysis of the DNA samples with 1ml of formic acid (88%) in evacuated and sealed tubes at 150°C for 30 minutes.

After hydrolysis, samples were lyophilised, and derivatised by trimethylsilylation with 0.25ml of a 1.5:1 mixture of BSTFA, containing 1% trimethylchlorosilane and acetonitrile, in polytetrafluoroethylene capped hypovials, by heating at 130°C for 30 minutes. The derivitisation was to enable the samples to vaporise in the injection port of the GCMS apparatus.
Hydrolysed and derivatised DNA samples were then analysed by GCMS/SIM using a mass selective detector controlled by a computer work station and interfaced to a gas chromatograph. The injection port and the ion source were kept at 250°C. The GCMS interface was maintained at 270°C. Separations were carried out on a fused silica capillary column (12.5m x 0.32mm i.d) coated with crosslinked 5% phenylmethyl silicone gum phase (film thickness, 0.17μM). The column was programmed from 120°C to 250°C at 8°C/min, after 2 minutes at 120°C. Helium was used as the carrier gas at an inlet pressure of 10 KPa. Samples were injected using the splitless mode. Mass spectra were obtained at 70 eV.

When used for calibration of the GCMS system, standard individual DNA bases were analysed as for DNA omitting the hydrolysis step. When used as internal standards, the bases were added prior to any preparation of the DNA samples and were therefore hydrolysed.

DNA bases, excised from the polynucleotide chain, are excreted in the urine in monomeric form. Therefore, urine samples, were prepared as in section 4.1.2.2, and freeze dried. 8AZA (2.5 nmoles) was added to each urine sample and the mixture freeze dried again. The samples were then analysed without undergoing acidic hydrolysis, by GCMS as described for DNA.

The injection volume for all standards and samples was 1μl.

4.4.3: Results:

Quantitative analysis of base damage to DNA is dependent on the selection of appropriate internal standards, and calibration of the mass spectrometer for its response to standard quantities of the internal standards and the compounds of interest.

8AZA was used as an internal standard because it is structurally similar, differing from the normal purine residue only by substitution of an N atom at C-8. (8-azaguanine has very low solubility in aqueous solution and was therefore unsuitable). 8AZA also eluted close to the compound of interest but was well separated from all the components in the mixture and was found to be stable during preparation of the samples.
8AZA (2.5 nmoles) was added to all samples before hydrolysis and derivitisation (10 pmoles per injection).

A calibration curve for the response of the mass spectrometer to known quantities of both 8OHG and 8AZA was obtained by plotting the ratio of the areas under the curve (ion current ratio) for 8OHG:8AZA against the ratio of the amount of 8OHG:8AZA (Figure 4.10). The slope of the line (3.6 in this case) represents the relative molar response factor (RMRF) for 8OHG. Values of 8OHG in samples were calculated using the formula:

\[
RMRF = \frac{\text{amount of standard (8AZA)} \times \text{peak area of analyte (8OHG)}}{\text{amount of analyte (8OHG)} \times \text{peak area of standard (8AZA)}}
\]

To determine retention times of the DNA base adducts, the following standards were injected onto the column;

- 8AZA - retention time 8.979 ± 0.071 mins (mean ± 1SD)
- 8OHG - retention time 13.99 ± 0.065 mins (mean ± 1SD)
- FAPY adenine - retention time 9.582 ± 0.577 mins (mean ± 1SD)
- 5-hydroxyuracil - retention time 5.979 ± 0.478 mins (mean ± 1SD)

The characteristic M⁺ and M-15⁺ ions for each of these compounds are shown in table 4.2 and the mass spectrum for each of these compounds is shown in figure 4.11.

Using these times the mass spectrometer was set up to monitor each ion for a specified period only (SIM mode).

Five samples of DNA, isolated from cryoprecipitates of SLE patients, were analysed by GCMS/SIM quantitatively for 8OHG and qualitatively for other base adducts. The concentrations of 8OHG found in the samples, corrected for the amount of DNA isolated from the cryoprecipitates (section 4.3.2.3) are shown in table 4.3 and compared with the values obtained by HPLC/ECD. The spectra obtained for one of the cryoprecipitates illustrating the bases, 8OHG (1) and 8AZA (2) is shown in figure 4.12. The other numbered peaks represent FAPY adenine (3), 8OHA (4), 5-hydroxycytosine (5) and 5-hydroxyuracil (6).

Figure 4.13 shows no detectable levels of 8OHG in the sample analysed. This sample was obtained
Figure 4.10

Calibration plot of the trimethylsilyl derivatives of 80HG relative to 8AZA. The slope of the line represents the relative molar response factor (RMRF) for 80HG and each point represents the mean ± 1SD of three experiments. Data were fitted by least squares linear regression analysis.
<table>
<thead>
<tr>
<th>Compound</th>
<th>(M$^+$)</th>
<th>(M-15$^+$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8AZA</td>
<td>280</td>
<td>265</td>
</tr>
<tr>
<td>8OHG</td>
<td>455</td>
<td>440</td>
</tr>
<tr>
<td>8OHA</td>
<td>367</td>
<td>352</td>
</tr>
<tr>
<td>FAPY Adenine</td>
<td>369</td>
<td>354</td>
</tr>
<tr>
<td>5-hydroxyuracil</td>
<td>344</td>
<td>329</td>
</tr>
<tr>
<td>5-hydroxycytosine</td>
<td>343</td>
<td>328</td>
</tr>
</tbody>
</table>

Table 4.2

Molecular weights of the intact molecular ion (M$^+$) and M-15$^+$ (typical of trimethylsilylated derivatives arising from loss of a methyl radical from M$^+$) for the oxidised products of DNA base residues analysed in this assay.
Figure 4.11

Total-ion chromatograms and mass spectra of the standard peaks of A) FAPY Adenine, B) 8AZA, C) 5-hydroxyuracil and D) 8OHG are shown. The characteristic $M^+$ and $M-15^+$ ions for each of the compounds are listed in table 4.2.
<table>
<thead>
<tr>
<th>Cryoprecipitate</th>
<th>80HDG (nmoles) GCMS</th>
<th>80HDG (nmoles) HPLC/ECD</th>
<th>DNA (μg)</th>
<th>nmoles 80HDG/μg DNA GCMS</th>
<th>nmoles 80HDG/μg DNA HPLC/ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>274±22</td>
<td>171±19</td>
<td>127±4</td>
<td>2.16±0.25</td>
<td>1.35±0.04</td>
</tr>
<tr>
<td>2</td>
<td>1219±47</td>
<td>1458±41</td>
<td>182±3</td>
<td>6.7±0.37</td>
<td>8.01±0.14</td>
</tr>
<tr>
<td>3</td>
<td>472±19</td>
<td>373±23</td>
<td>135±6</td>
<td>3.5±0.31</td>
<td>2.76±0.07</td>
</tr>
<tr>
<td>4</td>
<td>767±46</td>
<td>784±25</td>
<td>118±7</td>
<td>6.5±0.82</td>
<td>6.74±0.15</td>
</tr>
<tr>
<td>5</td>
<td>535±30</td>
<td>579±27</td>
<td>104±5</td>
<td>5.1±0.04</td>
<td>5.57±0.17</td>
</tr>
</tbody>
</table>

Table 4.3

Comparison of values of 80HDG found in five cryoprecipitate samples analysed by GCMS and HPLC/ECD. Results (means ± 1SD) are expressed as nmoles 80HDG / μg DNA of three separate experiments by both GCMS and HPLC/ECD.
Figure 4.12

Selected-ion chromatogram of trimethylsilylated DNA base products found in DNA isolated from a circulating immune complex of a patient with SLE. Peak identification is:
1. 8OHG 2. 8AZA 3. FAPY Adenine 4. 8OHA 5. 5-hydroxycytosine 6. 5-hydroxyuracil.
Figure 4.13

Selected-ion chromatogram of trimethylsilylated DNA base products found in DNA isolated from a circulating immune complex from a normal control. The mass spectra shows the presence of the internal standard (8AZA) in the sample but there was no detectable level of 8OHG.
Eight urine samples were also analysed by GCMS. Figure 4.14 shows a total ion chromatogram for one of the control urine samples and figure 4.15 shows the same sample analysed using selected ion monitoring, demonstrating the overall reduction in size of the peaks in figure 4.15 but amplification of the relevant peaks by SIM. It is still noticeable that more compounds are detected in the urine samples in this mode than for the DNA preparations.

The values obtained for each of the samples are shown in table 4.4.

4.4.4: Discussion

The levels of 80HG detected in DNA from the cryoprecipitates of five SLE patients were not significantly different from the values obtained by HPLC/ECD (table 4.3). There was also no detected 80HG in the sample isolated from plasma of a normal control even though the sensitivity of the technique was \(10^3\) times greater.

The results for the urine samples from normal controls were again found not to be significantly different from the results obtained by HPLC/ECD. However, for the urine samples from SLE patients, whilst 80HDG was undetectable by HPLC/ECD, it was positively identified in the urine by GCMS/SIM.

GCMS has been used with a confirmatory application and the results obtained for the DNA from SLE and normal cryoprecipitates, and for the urines from normal controls were of the same order as those obtained by HPLC/ECD. This confirmed the presence of this altered DNA base in the samples analysed. The detection of the two characteristic ions \((M^+\) and \(M-15^+)\) in equal amounts confirms the purity of the peak by GCMS and the fact that there was no significant difference between these and the values measured by HPLC/ECD strongly suggests that the peaks measured by the latter were not contaminated with other co-eluting compounds.

The detection of 80HG at concentrations of the same order as those detected by HPLC/ECD also suggests that there is no significant contribution to the 80HG detected by GCMS from RNA. The HPLC method measures 80HDG with the deoxyribose sugar still attached to the modified base as a
Figure 4.14

Total-ion chromatogram of a urine sample from one of the normal controls. There is a high relative abundance of three peaks which reduces the peaks of interest to undetectable levels.
Figure 4.15

Selected-ion chromatogram of the same urine sample analysed in figure 4.14 from a normal control. The chromatogram shows peaks of abundances of several magnitudes lower than those in figure 4.14 but allows detection of compounds present in low concentrations such as 8OHG. The mass spectra shows identification of 8OHG in this sample.
<table>
<thead>
<tr>
<th>Urine</th>
<th>age</th>
<th>sex</th>
<th>Urine Creatinine (mM/l)</th>
<th>8OHGDG/μM Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>F</td>
<td>7.3±0.04</td>
<td>2.5 pmole</td>
</tr>
<tr>
<td>Control</td>
<td>22</td>
<td>M</td>
<td>13.4±0.03</td>
<td>6.2 pmole</td>
</tr>
<tr>
<td>SLE</td>
<td>22</td>
<td>F</td>
<td>6.6±0.05</td>
<td>20 fmole</td>
</tr>
<tr>
<td>SLE</td>
<td>27</td>
<td>F</td>
<td>3.9±0.04</td>
<td>10 fmole</td>
</tr>
<tr>
<td>SLE</td>
<td>28</td>
<td>M</td>
<td>5.9±0.10</td>
<td>12 fmole</td>
</tr>
<tr>
<td>SLE</td>
<td>32</td>
<td>F</td>
<td>12.2±0.02</td>
<td>6.6 fmole</td>
</tr>
<tr>
<td>SLE</td>
<td>25</td>
<td>F</td>
<td>6.9±0.04</td>
<td>4.5 fmole</td>
</tr>
<tr>
<td>SLE</td>
<td>29</td>
<td>F</td>
<td>5.6±0.11</td>
<td>9.2 fmole</td>
</tr>
</tbody>
</table>

Table 4.4

Values of 8OHGDG analysed by GCMS/SIM in two control and six SLE urine samples expressed as pmoles or fmoles 8OHGDG / μM creatinine. Results are means ± 1SD of three separate analyses of each sample.
label that the base has been derived from DNA. It has also been shown that 8OHG is not detected under the conditions of this assay (section 2.6.2.1) and therefore is unable to contribute to the level of 8OHDG detected. However, the acidic hydrolysis removes the sugar residue from the DNA base and the remaining, measurable 8OHG by GCMS could theoretically arise from either DNA or RNA.

Detection of 8OHG in the urine of SLE patients at levels of femtomolar/µM creatinine may be explained by a decreased excretion of the damaged DNA base, 8OHDG that was not detectable by HPLC/ECD. However, it may also reflect detection of 8OHG derived from RNA, at levels that did not significantly affect measurement of picomoles of 8OHDG derived from DNA.

Damage to DNA is repaired by excision of the base from the DNA strand and the base is then excreted in urine. The lack of 8OHDG in the urine of SLE patients may arise from a defective repair mechanism for 8OHDG. There is known to be defective repair of the base, O°-methylguanine in DNA of patients with SLE and other autoimmune diseases (Harris et al., 1982). This base is premutagenic and its accumulation in the cell is potentially lethal. 8OHDG has been shown to have mutagenic properties (Kuchino et al., 1987; Shibutani, Takeshita and Grollman, 1991) and similarly would be detrimental to the cell if not effectively repaired. The considerably reduced excretion of 8OHDG seen in SLE patients does suggest that there is poor excision of the DNA adduct. The concentration of 8OHDG in the DNA isolated from the circulating immune complexes also could account for the lack of the adduct in the urine. However, the DNA found in the immune complexes was ≈ 20 kilobases in size which is much larger than the fragments removed from DNA as a result of excision repair. This suggests that the DNA in the immune complexes is derived from cells, following death and subsequent lysis. The presence of 8OHDG in this DNA suggests that in SLE, 8OHDG is not easily repaired and its accumulation may be responsible for cell death. In addition, as a result of the inflammatory response, it is likely that ROS generated by neutrophils and macrophages will be increased especially in localised areas of inflammation, and that this would contribute to an increased conversion of dG to 8OHDG.

Evidence to support the role of neutrophil-derived ROS in the DNA damaging process is documented by Jackson et al., (1989) who showed that the DNA base products formed from ROS generated by gamma irradiation differ significantly from those formed in reactions of DNA with neutrophil-derived ROS. 8OHA, which was the predominant altered DNA base found in the cryoprecipitate samples analysed, is
found in low yield in gamma irradiated DNA but is the major product of neutrophil-derived damage and so further supports the hypothesis that DNA bound to cryoprecipitates has been exposed to ROS.

Finally, in section 3.6 it was shown that DNA derived from activated neutrophils, when probed with the panel of MCAs, had a similar binding profile to DNA denatured with $\text{H}_2\text{O}_2$ rather than DNA denatured with gamma or UV radiation. This provides further evidence that DNA damage may arise from a neutrophil-mediated inflammatory reaction.
SECTION 5:

ANTIGENIC PROPERTIES OF ROS-DNA
CHAPTER 5:

Antibodies to native dsDNA represent a characteristic serological finding in patients with SLE, and are of important diagnostic and clinical value. They are thought to be highly specific for this disease, particularly when renal complications are present (Aitcheson and Tan, 1982), but they also reflect disease activity (Isenberg et al., 1986) and associate with hypocomplementaemia.

DNA/anti-DNA complexes (Adu, Dobson and Williams, 1981) in SLE sera may also play a role in the pathogenesis of the disease, particularly when they are deposited in tissues, such as the glomerular basement membrane (Bruneau and Benveniste, 1979). However, the significance of circulating DNA/anti-DNA complexes remains uncertain.

The effects of ROS on the physicochemical properties of DNA have been discussed in chapter 3. It has also been described how neutrophil-derived $\text{H}_2\text{O}_2$ might damage DNA by virtue of its ability to cross cellular and nuclear membranes (section 1.1.5.4). This process would be detrimental to any cell, but more so in SLE, since cells from SLE patients appear to have a reduced capacity for repair of the ROS-induced lesion 8OHDG (section 4.2). As a result of this, and possibly other mechanisms of DNA repair processing which may be impaired (Harris et al., 1982), large fragments of DNA, containing ROS-induced products, persist in the circulation of these patients.

Work predominantly on UV-irradiated DNA has led to the general belief that denaturation of DNA increases its antigenicity (Tan and Stoughton, 1969) and there is compelling evidence to suggest that ROS-denatured DNA molecules induce an increased immune response on injection into animals compared with native DNA (Natali and Tan, 1971; Jansson, 1985).

The following sections were aimed to investigate: a) the role of ROS-DNA as a potential antigen and immunogen for anti-DNA antibody production in SLE, and b) the possible use of ROS-DNA as a substrate in the development of a sensitive ELISA for measurement of anti-DNA antibodies.
CHAPTER 5.1: CHARACTERISATION OF DNA IN SLE SERUM

5.1.1: Introduction

The evidence for the presence of DNA in circulating immune complexes in SLE sera has been presented in section 1.2.5 and the isolation of DNA from these complexes is described in section 4.3.2.3. Since DNA is usually packaged into the cell nucleus and quickly degraded by nucleases on entering the circulation, the ability of DNA to act as an antigen against which antibodies can be raised has been questioned (Schwartz and Stollar, 1985; Isenberg and Shoenfeld, 1987). The presence of immune complex-associated DNA in the circulation is possibly the only source of available antigen against which anti-DNA antibodies can be raised, if DNA is the true immunogen at all (Schwartz and Stollar, 1985).

The ability of ROS modification to alter the antigenicity of DNA has also been discussed (section 1.2.6). In animal models, ROS-denatured DNA has elicited the production of anti-DNA antibodies with affinities predominantly for the altered DNA, but also some cross-reactivity with native dsDNA (Tan and Stoughton, 1969; Jansson, 1985).

In the following section, the conformation and chemical characteristics of the DNA isolated from the sera of SLE patients were studied, using the panel of MCAs described in section 3.6.

5.1.2: Methods

5.1.2.1: Clinical Samples

5 SLE patients having concentrations of cryoprecipitable DNA of >200μg/ml were selected from the group described in section 4.3.3. Plasma (50mls) was then collected from each of these patients over a period of time. The extracted DNA was pooled, and its final concentration was measured as in section 2.3.1.2. DNA was resuspended in PBS at a concentration of 1000μg/ml.

Similarly, DNA extracted from cryoprecipitates of 2 normal controls and 3 RA patients was collected and pooled from a total plasma volume of 200mls. The DNA concentration was again measured and the samples finally resuspended at a concentration of 100μg/ml.
5.1.2.2: ELISA Assay

The ELISA assay for determination of monoclonal antibody binding to plasma cryoprecipitate DNA compared with native dsDNA was as described in section 3.6.2. For DNA isolated from SLE plasma the competing antigen was titrated in PBST from 1000µg/ml in doubling dilutions across the plate. For DNA isolated from the sera of RA patients and the normal controls the competing antigen was titrated from 100µg/ml again in doubling dilutions across the plate.

The cryoprecipitated DNA was probed with three different MCAs: 212 - recognising the sugar-phosphate backbone on ss- and dsDNA, 228 - recognising bases on ssDNA only, and 402 - recognising conformational determinants on dsDNA (table 3.5).

5.1.3: Results

Figure 5.1 shows the titration curves for the three MCAs binding to commercially prepared native DNA, and DNA isolated from the cryoprecipitates of each of the normal controls and RA patients. For each MCA there was no significant difference between the curves.

Figure 5.2 shows the titration curves for the same MCAs binding to native dsDNA and DNA isolated from SLE cryoprecipitates.

For MCA 212, there is a significant positive shift in the binding curve signifying a decrease in affinity of the available binding sites on SLE-derived DNA compared to Nat-DNA, and the cross over of the curves suggests a change in the binding avidity of the MCA to the SLE-derived DNA.

MCA 228 shows an increased binding affinity to the base determinants of SLE-derived DNA compared to native dsDNA as illustrated by the positive shift in the titration curve.

The curve for MCA 402 binding to SLE-derived DNA shows an increased affinity as well as an increase in the number of available binding sites for dsDNA conformational determinants compared to native dsDNA.

The differences in each of the binding curves for the three MCAs were significant at the p<0.01 level.
Figure 5.1

% Binding of MCAs 212, 228 and 402 to serial two-fold dilutions of native dsDNA or DNA isolated from circulating immune complexes of normal controls (N) and RA patients (RA). Points plotted are means of triplicate samples.
Figure 5.2

% Binding of MCAs 212, 228 and 402 to serial two-fold dilutions of native dsDNA or DNA isolated from circulating immune complexes of 5 patients with SLE. The points plotted are the means of the values obtained for the 5 patients, samples for each patient being assayed in triplicate.
5.1.4: Discussion

The binding specificities of monoclonal antibodies derived from mouse models of SLE have been characterised and it has been shown that mouse monoclonal DNA-binding antibodies are very similar to those found in humans with SLE (Morgan et al., 1985b). Also the diversity of specificity of DNA-reactive antibodies is small (Morgan et al., 1985b).

In this section, MCAs were used to show that there is a difference between DNA isolated from patients with SLE, and the control and RA groups on the basis of differences in MCA binding to the DNA samples. This supports data from studies of 8OHDG analysis by HPLC/ECD and GCMS which showed evidence of ROS-induced damage to cryoprecipitate DNA only in patients with SLE (sections 4.3 and 4.4).

The presence of 8OHDG in the SLE cryoprecipitate DNA is an indicator that the DNA has been exposed to ROS, and particularly to OH−-mediated reactions (see sections 1.1.5.4 and 3.1). However, in chapter 3 the pattern of damage inflicted on DNA has been shown to differ between ROS-inducing systems detected by the differing binding capacities of MCAs which recognise a variety of epitopes (table 3.5). This seems to relate to the distribution of metal ions along the DNA backbone catalysing site-specific OH• in H₂O₂-producing systems, and random generation of OH• by the gamma radiation-mediated homolytic fission of water molecules. The characteristic pattern of DNA damage detected by MCA binding was used to compare any changes in the cryoprecipitate DNA with DNA exposed to the ROS-generating systems (section 3.6.3).

There was no significant difference between DNA isolated from cryoprecipitates of normal controls or RA patients and native dsDNA when probed with the MCAs. This suggests that the DNA in these two groups is predominantly native dsDNA. The size of the isolated DNA (≈20 kilobases - section 4.3.3) suggests that this DNA is most likely derived from a lysed, dying cell. Its presence as part of a circulating immune complex may represent a normal mechanism for the removal of circulating antigens, as mentioned in sections 1.2.5 and 4.3.1. There was also significantly less DNA found in the immune complexes of these two groups, compared to the SLE patients and this may result from quicker and more effective clearance of DNA. This is supported by evidence that DNA persists longer in the
circulation of patients with SLE (Frost and Lachmann, 1968; Chitrabamrung, Rubin and Tan, 1981), and also by the difference in urinary excretion patterns for the altered DNA base, 8OHdG (section 4.1).

The results from MCA binding studies suggest that the DNA from the SLE complexes is different from native dsDNA. There was an increase both in number and affinity for dsDNA conformational determinants. Also, a reduction in binding affinity and a change in the nature of the determinants expressed on the sugar-phosphate backbone, as well as an increase in binding affinity to base residues.

These appear to be similar to induced by H$_2$O$_2$ reacting with DNA and do not reflect the damage induced by ionising radiation (section 3.6.3). This indicates that DNA found in circulating SLE immune complexes has been exposed to a ROS-generating system that produces a pattern of damage typical of exposure to H$_2$O$_2$. It was shown in section 3.6.3.7 that neutrophil-derived ROS produced a pattern of damage typical of that produced by H$_2$O$_2$ reacting with DNA-bound metal ions. Therefore, the damage to DNA found in SLE sera may have been mediated by H$_2$O$_2$, derived from O$_2^-$ produced from activation of the NADPH oxidase of activated neutrophils and macrophages during inflammation.

The data from the studies using GCMS to monitor base damage also support neutrophil-derived ROS (ie. O$_2^-$ reacting with metal ions to form H$_2$O$_2$ and OH$^-$ - section 1.1.4.7) as the damaging agents in the modification of SLE cryoprecipitate DNA (section 4.4.4) (Jackson et al., 1989).

The small distance over which OH$^-$ can diffuse (Ward, Blakey and Joner, 1985) limits its reactivity to the region in close proximity to the DNA-bound metal ions. Damage to specific bases in preference to others may reflect the positioning of metal ions along the backbone, for example regions of polydeoxyguanosine with a high electronegativity are favourable positions for metals to bind (section 1.1.5.4).

The results suggest that the DNA bound to circulating SLE immune complexes has been exposed to ROS and that these ROS are produced during the respiratory burst of phagocytic cells.

SLE cells do not appear to excise 8OHdG from their DNA as effectively as normal cells (section 4.2); this would account for the fact that DNA derived from dying SLE cells contains increased levels of
damaged-DNA products. However, it cannot be excluded that the DNA persisting in the circulation of SLE patients (Frost and Lachmann, 1968; Chitrabamrung, Rubin and Tan, 1981) becomes damaged by ROS produced extracellularly after it enters the circulation since serum has little SOD or catalase activity (section 1.1.6).

Regardless of the mechanism responsible, the presence of an altered form of DNA in the circulation may be involved either in the production of antibodies to DNA, or it may affect the affinity and/or avidity of binding and thus the pathogenicity of existing antibodies.
5.2.1: Introduction

H₂O₂ is sufficiently lipophilic to permeate both cellular and nuclear membranes (Halliwell and Gutteridge, 1985) and has been shown to be capable of reacting with DNA to induce conformational changes (Allan et al., 1988), base damage (Massie, Samis and Baird, 1972) and double- and single-strand breaks (Rhaese and Freese, 1968; Ward JF et al., 1987).

This modification is possibly important in SLE since a distinct population of anti-DNA antibodies recognise determinants on denatured forms of DNA (Stollar, 1973) which may be exposed when dsDNA is damaged by ROS. Antibodies from SLE sera have been characterised as binding to:

1. Purine or pyrimidine bases (Stollar and Levine, 1963), which are exposed only in ssDNA and denatured dsDNA.
2. The linear sugar-phosphate backbone conformation on dsDNA. These antibodies may also recognise helical regions of ssDNA (Stollar, 1981).
3. Conformational determinants on the dsDNA helix (Steinman, Deesomchok and Spiera, 1976). These antibodies are specific for dsDNA.

Using an antibody reference serum (sero-positive for anti-dsDNA antibodies), the role of DNA denatured by ROS (ROS-DNA) as a potential antigen for anti-DNA antibody production was investigated by measuring binding of SLE anti-DNA antibodies to ROS-DNA compared with binding to native dsDNA (Nat-DNA).

5.2.2: Methods

5.2.2.1: Preparation of ELISA Antigens

5.2.2.1.1: Native DNA (Nat-DNA)

Double-stranded human DNA was dissolved in chelex-treated PBS at 2.5mg/ml. Dilution to 0.5mg/ml with 0.1M acetate, pH 4.8, containing 0.05M zinc chloride and subsequent incubation with S1 nuclease (100 units/mg DNA) at 45°C for 1 hour digested any contaminating ssDNA. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation as described in section 2.3.1.1 and the final
A260nm/A280nm of the preparation was greater than 1.85, indicating that the preparation was effectively free from contaminating protein.

Single-stranded DNA was prepared by immersing a sample of this stock solution in a boiling water bath for 15 minutes then rapidly cooling on ice.

dsDNA and ssDNA were diluted to 100μg/ml in 0.05M carbonate buffer, pH 9.6, for coating to the ELISA plates.

5.2.2.1.2: ROS-Denatured DNA (ROS-DNA)

dsDNA and ssDNA at 0.5mg/ml in PBS prepared fresh from the stock solution were incubated in separate experiments with 200μM H₂O₂ and 10mM AA at 37°C for 1 hour. All preparations of ROS-DNA were made immediately prior to use in the ELISA assay.

5.2.2.2: Scavenger and Chelator Studies

The following free radical scavengers were included in the preparation of ROS-DNA at the concentrations stated in table 2.1, to define the nature of the ROS responsible for changes induced in human DNA by H₂O₂ and AA: cysteine, histidine, SOD, mannitol and thiourea, and the iron chelator, desferrioxamine.

5.2.2.3: ELISA Assay

The ELISA assay was based on that described by Stokes, Cordwell and Thompson (1982) and was performed as described in section 2.3.2.1. ROS-DNA and Nat-DNA were bound to microtitre plates in carbonate buffer. Binding of anti-DNA antibodies in the seropositive antibody control sample to DNA was monitored using peroxidase-conjugated goat anti-human IgG, IgA and IgM antibodies at a working dilution of 1/1000 in PBST.

The coefficient of variation for intra- and inter-batch analysis for five separate preparations of ROS-DNA from the same stock solution, using the seropositive anti-DNA antibody control as a source of anti-DNA antibodies, was 3.06% and 6.8% respectively (table 5.1). For Nat-DNA the intra-batch variation was 2.7% and the inter-batch variation was 7.7%.
### Table 5.1

Multiple analysis of serum samples to determine the interbatch and intrabatch variation of the ROS-DNA ELISA. The individual OD values are shown from which were calculated the mean, standard deviation (SD) and coefficient of variation (CV).

<table>
<thead>
<tr>
<th>Interbatch Variation</th>
<th>Intrabatch Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.406</td>
<td>1.050</td>
</tr>
<tr>
<td>0.442</td>
<td>1.090</td>
</tr>
<tr>
<td>0.497</td>
<td>1.063</td>
</tr>
<tr>
<td>0.495</td>
<td>1.101</td>
</tr>
<tr>
<td>0.408</td>
<td>1.070</td>
</tr>
<tr>
<td>0.466</td>
<td>1.133</td>
</tr>
<tr>
<td>0.466</td>
<td>1.144</td>
</tr>
<tr>
<td>0.455</td>
<td>1.089</td>
</tr>
<tr>
<td>0.448</td>
<td>1.076</td>
</tr>
<tr>
<td>0.476</td>
<td>1.052</td>
</tr>
<tr>
<td>1.045</td>
<td>1.118</td>
</tr>
<tr>
<td>1.107</td>
<td>1.040</td>
</tr>
<tr>
<td>1.115</td>
<td>1.043</td>
</tr>
<tr>
<td>1.110</td>
<td>1.110</td>
</tr>
</tbody>
</table>

Mean = 0.456 = 1.085  
SD = 0.031 = 0.033  
CV = 6.8% = 3.06%
5.2.3: Results

5.2.3.1: Optimisation of Coating Antigen Concentration

ELISA assays require efficient immobilisation of the coating antigen onto the plate and there are several techniques described to achieve this for DNA (Zouali and Stollar, 1986; Eaton, Schneider and Schur, 1983). Coating DNA in carbonate buffer and coating DNA in PBS with a precoating of poly-L-lysine were initially investigated. Carbonate buffer was chosen as a means of immobilising the antigen in these experiments in preference to precoating the plates with poly-L-lysine since there was no significant difference in the DNA binding values and this reduced the number of steps in each assay.

At pH 9.6, DNA dissolved in carbonate buffer, is positively charged, and is therefore attracted to the negatively charged polystyrene ELISA plates.

To optimise the concentration of DNA required to give maximal coating of the ELISA wells, Nat-DNA and ROS-DNA were diluted from 500μg/ml to 1μg/ml in carbonate buffer, and the amount of anti-DNA antibody binding determined by optical density. This was performed for each DNA concentration and measured using a seropositive anti-DNA antibody control (reconstituted in 0.5ml PBS and used neat as recommended) and peroxidase-conjugated pooled anti-sera to IgG, IgA and IgM.

Table 5.2 shows the optical densities obtained for Nat-DNA and ROS-DNA. There was a dose-dependent increase in the amount of anti-DNA antibody bound from 1-100μg/ml DNA after which the values plateaued and the difference in optical density for DNA concentrations of 100μg/ml and 200μg/ml was not significant. Therefore, the coating concentration of DNA used in further experiments was chosen as 100μg/ml.

5.2.3.2: Direct and Competition ELISAs for Anti-DNA Antibody Determination

The immobilisation of DNA onto an ELISA plate may shield epitopes on the DNA molecule and so reduce the number and variety of interactions between antibody and antigen.

To establish the difference in binding of anti-DNA antibodies to immobilised DNA compared to DNA free in solution, a direct binding ELISA was compared with a competition ELISA.
### Table 5.2

Binding of Nat-DNA and ROS-DNA to ELISA plates. The binding of Nat-DNA and ROS-DNA diluted in carbonate buffer, pH 9.6, over a range of concentrations, was measured using a seropositive anti-DNA antibody control and peroxidase-conjugated goat anti-human, IgG, IgA and IgM.
The results of anti-DNA antibody binding to increasing concentrations of DNA in a direct binding ELISA are shown in table 5.2.

For the competition ELISA, DNA was coated at 100μg/ml as for the direct binding assay and the protocol described in section 2.3.2.2 was followed. Nat-DNA or ROS-DNA was then titrated from 1000μg/ml in doubling dilutions across the plate and incubated at 37°C for 1 hour. A seropositive anti-DNA antibody control was then added in equal volume to all wells and further incubated for 1 hour at 37°C. The ELISA was then completed by addition of labelled anti-sera as in section 2.3.2.1. Results for anti-DNA antibody binding to the immobilised antigen in the presence of increasing concentration of competing antigen are shown in table 5.3.

There is no significant difference between the binding of the antibody to the DNA either free in solution or when bound to the solid matrix. Therefore, a direct binding ELISA was used for further analysis of the interaction between anti-DNA antibodies and DNA.

5.2.3.3: Anti-DNA Antibody Binding to ROS-DNA

The effect of H₂O₂ and AA on the binding of seropositive anti-DNA antibody control serum to dsDNA and ssDNA is shown in figure 5.3. There is a dose-dependent increase in anti-DNA antibody binding to both dsDNA and ssDNA with increasing dose of H₂O₂ for an AA concentration of 10mM. There was no further increase in antibody binding beyond 200μM H₂O₂ and this concentration was used for denaturation of DNA by ROS (section 5.2.2.1.2).

The contribution to the increase in antibody binding by 200μM H₂O₂ and 10mM AA alone, and in combination is shown in figure 5.4. There was no difference in binding as a result of incubating DNA with AA only, and although a slight increase was noted for incubation with H₂O₂ alone, this was not significant. However, there was a significant increase in binding induced by H₂O₂ in the presence of AA, the mean optical density increasing from 0.51 to 0.93 for dsDNA (p<0.001) and from 0.53 to 0.99 for ssDNA (p<0.001).
<table>
<thead>
<tr>
<th>Concentration Competing DNA (µg/ml)</th>
<th>OD Nat-DNA</th>
<th>OD ROS-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.306±0.034</td>
<td>1.475±0.054</td>
</tr>
<tr>
<td>1</td>
<td>1.214±0.023</td>
<td>1.423±0.032</td>
</tr>
<tr>
<td>2</td>
<td>1.179±0.034</td>
<td>1.411±0.033</td>
</tr>
<tr>
<td>4</td>
<td>1.045±0.045</td>
<td>1.390±0.033</td>
</tr>
<tr>
<td>8</td>
<td>0.949±0.055</td>
<td>1.109±0.012</td>
</tr>
<tr>
<td>16</td>
<td>0.826±0.054</td>
<td>0.977±0.022</td>
</tr>
<tr>
<td>32</td>
<td>0.731±0.032</td>
<td>0.819±0.044</td>
</tr>
<tr>
<td>65</td>
<td>0.525±0.034</td>
<td>0.673±0.033</td>
</tr>
<tr>
<td>125</td>
<td>0.371±0.012</td>
<td>0.526±0.022</td>
</tr>
<tr>
<td>250</td>
<td>0.306±0.032</td>
<td>0.407±0.021</td>
</tr>
<tr>
<td>500</td>
<td>0.244±0.012</td>
<td>0.323±0.011</td>
</tr>
<tr>
<td>1000</td>
<td>0.216±0.023</td>
<td>0.278±0.011</td>
</tr>
</tbody>
</table>

Table 5.3

Comparison ELISA to compare binding of SLE serum antibodies to Nat-DNA and ROS-DNA in free solution. The binding of SLE anti-DNA antibodies to Nat-DNA and ROS-DNA was measured by binding Nat-DNA to the plate in carbonate buffer at 100 µg/ml. Titrations of Nat-DNA and ROS-DNA in doubling dilutions across the plate were used as competing antigens. Seropositive and seronegative controls diluted 1/50 were added in equal volume to the DNA solutions and after incubation (37°C/1 hour), anti-DNA antibody binding to DNA on the solid phase was measured by peroxidase-conjugated goat anti-human IgG, IgA and IgM in total.
Dose-dependent increases in anti-DNA antibody binding to single- and double-stranded DNA. Anti-DNA antibody binding to double (•) and single (●) stranded DNA, both incubated in the presence of 10mM AA, was measured as a function of increasing H$_2$O$_2$ concentration.
Figure 5.4

Effect of $\text{H}_2\text{O}_2$ and AA on anti-DNA antibody binding. Double (■) and single (⊗) stranded DNA were incubated with 10mM AA, 200$\mu$M $\text{H}_2\text{O}_2$ and PBS. Results are means ± 1SD of four separate experiments.
The seronegative control serum (reconstituted in 1ml PBS and used neat as recommended) showed no detectable binding, as determined by optical density values not significantly different from those of background, to either Nat-DNA or ROS-DNA.

5.2.3.4: Inhibition of Anti-DNA Antibody Binding to ROS-DNA by Scavengers

The effect of various ROS scavengers on the induction of antigenic changes to both dsDNA and ssDNA is shown in figure 5.5. Thiourea, a hydroxyl radical scavenger and Cu^{2+} chelator, caused inhibition in serum binding of 71% (p<0.001) and 62% (p<0.001) for ssDNA and dsDNA respectively, when compared to ROS-DNA. Similarly, the iron chelator desferrioxamine inhibited autoantibody binding by 69% (p<0.001) for ssDNA and 78% (p<0.01) for dsDNA. Mannitol, histidine, cysteine and SOD did not offer significant protection against ROS-induced damage.

5.2.3.5: Detection of ROS-Induced Changes to DNA by Individual Isotypes

The differential binding of the major isotypes of anti-DNA antibodies to ROS-denatured DNA was measured by ELISA using anti-serum to individual isotypes as peroxidase conjugate rather than pooled anti-IgG, -IgA and -IgM. The IgG, IgA and IgM antibody isotypes all individually detected the increase in seropositive serum binding to ROS-denatured dsDNA and ssDNA compared to the control, at a significance level of p < 0.01 (figure 5.6). The inhibition induced by the presence of desferrioxamine and thiourea, however, varied between the isotypes. For example, desferrioxamine inhibited binding of serum anti-DNA antibodies to dsDNA by 20, 10 and 8% for IgG, IgA and IgM respectively, and thiourea similarly inhibited by 14% for IgG, 10% for IgA and 3% for IgM. The decreases in absorbance for the IgM isotype were not significant for all inhibitors used; a significant decrease in anti-DNA antibody binding to DNA was detected by the IgG isotype for dsDNA incubated with desferrioxamine (p<0.01) and thiourea (p<0.01). Binding of the IgA isotype to both dsDNA and ssDNA was also significantly inhibited in the presence of thiourea (p<0.001) and desferrioxamine (p<0.001). Although IgG and IgA isotypes showed a similar response, the actual OD measurements for the IgA isotype were ≈4-times smaller than those for IgG.

5.2.4: Discussion

The results show that anti-DNA antibodies, such as those found typically in SLE, have a greater capacity to bind to ROS-DNA than to Nat-DNA. This denaturation, mediated by H_{2}O_{2}, is highly
Figure 5.5

Effect of radical scavengers on anti-DNA antibody binding. Results are means ± 1SD of four experiments showing the inhibition by 0.5mM desferrioxamine and 50mM thiourea of the ROS-induced increase in anti-DNA antibody binding to double (■) and single (●) stranded DNA.
Figure 5.6

Differential binding of anti-DNA isotypes against ROS-DNA. Binding of anti-IgG, IgM and IgA isotypes to double (○) and single (□) stranded DNA.
dependent on metal ions and a reducing agent. There is evidence to suggest that metal ions, notably iron and copper, are bound to DNA (Mello Filho and Meneghini, 1984; Goldstein and Czapski, 1986; Ward, Blakely and Joner, 1985) and stabilise the association between DNA supercoils and the non-histone matrix (Dijkwel and Wenink, 1986).

The reaction between $H_2O_2$ and metal ions is described in section 1.1.2.2 and the chelation of all assay reagents necessitates that the metal ions catalysing the production of OH$^-$ are bound to DNA. The results show that $H_2O_2$ alone caused an increase in anti-DNA antibody binding and this is probably due to the reaction of $H_2O_2$ with the reduced forms of metal ions bound to DNA. These become oxidised to produce OH$^-$ and then need to be reduced to further catalyse the production of OH$^-$. Addition of AA to the reaction mixture caused a significant increase in the binding of anti-DNA antibodies to DNA and demonstrates the need for rejuvenation of metal ions to perpetuate the damaging Fenton reaction.

In sections 3.1 and 3.2 it was shown that concentrations of up to 200$\mu$M $H_2O_2$ caused an increase in DNA damage detected by the formation of 8OHDG and TBA-reactive products respectively. Therefore, the reduction in linearity of polyclonal antibody binding to ROS-DNA seen beyond 50$\mu$M $H_2O_2$ may reflect either that further damage done to DNA does not cause additional changes in DNA conformation that are detected by polyclonal antibodies, or that continued alteration of sites damaged by concentrations of $H_2O_2$ greater than 50$\mu$M does not affect antibody binding further.

Inhibition of ROS-induced damage was effected by thiourea and desferrioxamine. Thiourea is a hydroxyl radical scavenger and can scavenge metal ions, particularly copper, whilst desferrioxamine is an iron ion chelator. The hydroxyl radical is highly reactive (see section 1.1.2.3) and can diffuse only 1.5nm before reacting (Ward, Blakely and Joner, 1985). OH$^-$ produced by DNA-bound metal ions reacting with $H_2O_2$ are more likely to react in close proximity to DNA than with a scavenger molecule in solution. This is reflected by the inability of mannitol, a hydroxyl radical scavenger to inhibit the reaction. Although the relative rate constant for scavenging OH$^-$ is 4.7 times greater for thiourea than mannitol (Halliwell and Gutteridge, 1989 pp 22-85), and thiourea may be more able to diffuse closer to DNA by virtue of its greater lipophilic nature, thiourea is most probably effective at inhibiting ROS-induced damage by binding to and chelating DNA-bound metal ions and preventing their participation.
in the Fenton reaction (reaction 6 - section 1.1.2.2). This is supported by the ability of desferrioxamine to effectively inhibit the ROS-induced changes to DNA as well.

The predominant isotype of anti-DNA antibodies is shown in figure 5.6 to be IgG. However, both IgG and IgA isotypes were sensitive to ROS-induced changes. There are many reports of the pathogenicity of anti-DNA antibodies (reviewed by Hahn, 1982) and positive associations between certain isotypes and disease manifestations. For example IgA anti-DNA antibodies have been shown to occur in patients with active nephritis (Gripenberg and Helve, 1986) and renal disease and disease activity in general are more closely related to high levels of circulating levels of IgG anti-DNA antibodies than to IgM antibodies (Rothfield and Stollar, 1967; Sontheimer and Gilliam, 1978). The IgG and IgA isotypes may recognise new epitopes exposed on ROS-denatured DNA following a change in conformation of the molecule. Results from chapter 3 show, using monoclonal antibodies (section 3.6) and strand break analysis (sections 3.3 and 3.4) that there is relaxation of the DNA molecule following damage and this may allow antibodies accessibility to previously hidden regions of DNA. This may have a role in the disease process, particularly in the formation of immune complexes with circulating anti-DNA antibodies reacting effectively with ROS-denatured DNA, released from cells.
CHAPTER 5.3: ROS-DNA AS AN ANTIGEN FOR ANTI-DNA ANTIBODY MEASUREMENTS

5.3.1: Introduction

Although little is understood of the role of anti-DNA antibodies in the aetiopathogenesis of SLE (section 1.2.4) the measurement of anti-DNA antibodies, to dsDNA in particular, is nevertheless used as a diagnostic marker for this disease (Aitcheson and Tan, 1982).

Several other inflammatory conditions, however, including RA, are associated with circulating antibodies to ssDNA and less commonly to dsDNA (Bell, Talal and Schur, 1975). Therefore, results obtained from anti-DNA antibody assays must be interpreted with caution (Swaak et al., 1981; Swaak and Smeenk, 1985; Eilat, 1986) and must be considered in the light of clinical observations.

At present, quantitative assays to determine circulating serum anti-DNA antibody levels rely on a radioimmunoassay (RIA) using radio-isotope-labelled DNA which, after complexing to antibody, is precipitated with ammonium sulphate or a co-precipitating antibody (Lewis, Stollar and Goldberg, 1973; Stokes, Cordwell and Thompson, 1982). RIA techniques show low binding to circulating antibodies in control sera, but patients with RA, Sjögren's syndrome and mixed connective tissue disease have levels that extend into the region shared by mildly active SLE patients (Notman, Kurata and Tan, 1975).

In section 5.1 the nature of circulating DNA has been described showing that the DNA has been damaged by ROS; the pattern of damage reflects that inflicted by \( \text{H}_2\text{O}_2 \) which is produced during the inflammatory exacerbations of the disease. Although this DNA is complexed to anti-DNA antibodies, a single antibody combining site is large enough only to encompass both backbone regions of dsDNA over an extent of 2-3 base pairs (Stollar, 1973; Stollar, 1975), and there may be large regions of DNA unshielded by antibody protein. Therefore, it may be possible for ROS-DNA associated with immune complexes to act as an antigen for production of anti-DNA antibodies. This DNA may also be able to act as an immunogen before interacting with antibodies since the kinetics of this reaction are unknown.

The results in section 5.2 show that a control anti-DNA antibody positive serum sample had an increased binding to ROS-DNA when compared to Nat-DNA. In the following sections further serum samples from SLE patients and from patients with other connective tissue diseases have been
investigated using the ELISA assay to compare the binding specificities of serum anti-DNA antibodies for Nat-DNA and ROS-DNA. A comparison of the results obtained by ELISA with those obtained by the conventional RIA currently used to measure serum anti-DNA antibodies was also undertaken, to determine whether ROS-modification of the dsDNA antigen could discriminate further between SLE, RA, and other connective tissue disorders.

5.3.2: Methods

5.3.2.1: Clinical samples

Forty-one patients (38 female, 3 male) with SLE, aged 22-55 years (median = 35) were studied. SLE patients were divided into an inactive group on the basis of having a stable clinical state, no new symptoms and low ESRs (15 female, age range 25-55, median = 32), or an active group with high ESRs, and new clinical symptoms in at least one major organ (15 female, age range 23-55, median = 30).

They were also sub-divided into a dsDNA positive group, if they had ever had a positive result by RIA (24 female, 3 male, age range 22-55, median = 35), and into a dsDNA negative group if they had never had a positive result in this assay (14 female, age range 25-55, median = 37).

All SLE patients studied had a positive ANA result.

Twenty patients (15 female, 5 male) with classical or definite RA, aged 22-78 (median = 55), were used as a control connective tissue disease group. These were sub-divided as seropositive or seronegative for rheumatoid factor. All but one of these patients were ANA negative and dsDNA negative by RIA. The one ANA positive RA patient had a dsDNA binding titre of 20% by RIA.

A third disease control group were patients with connective tissue disease other than RA or SLE, who were ANA positive or ANA negative, and dsDNA negative by RIA. 23 patients had scleroderma and the 4 remaining patients had primary Raynauds. The diagnosis of these patients was based on standard clinical and serological criteria for these diseases.
19 of these were ANA positive (17 female, 2 male, aged 30-79, median = 50) and 8 ANA negative (4 male, 4 female, aged 34-57, median = 44).

Disease groups were compared with thirty-four healthy controls, (26 female, 8 male, aged 18-73, median = 44) who were not significantly different for age and sex.

To obtain sera from these patients, blood samples were allowed to clot at room temperature and the serum separated by centrifugation at 1000 x g for 10 minutes prior to storage at -20°C.

5.3.2.2: ELISA Assay

The ELISA assays using Nat-DNA and ROS-DNA as antigen were performed as described in section 5.2.2.3. Sera from patients and controls were diluted 1/100 in PBST and plated in triplicate. Standard seropositive and seronegative samples (prepared as in sections 5.2.3.1 and 5.2.3.3) were included on each plate.

5.3.2.3: Radioimmunoassay

All sera from patients and control groups analysed for anti-dsDNA antibodies by the ELISA assay described in section 5.3.2.2 were also analysed by the routine conventional RIA at the Regional Department of Immunology, East Birmingham Hospital, using the method described by Stokes, Cordwell and Thompson (1982).

5.3.3: Results

Initial experiments investigated the sensitivity and specificity of the ROS-DNA ELISA compared with those of a Nat-DNA ELISA and the conventional RIA, in discriminating patients with SLE from those with RA, and from normal controls.

In the Nat-DNA ELISA, antibody binding to dsDNA clearly differed between the SLE and RA groups (figure 5.7). Binding of serum antibodies to native dsDNA is measured as the OD at 492nm produced from peroxidase-conjugated antibodies binding to IgG, IgA and IgM autoantibodies in total in the sera.

In SLE, antibody binding was significantly higher (median - 0.714 and range - 0.5-0.95) than RA (median - 0.483 and range - 0.33-0.62) (p<0.001). However, there was a considerable overlap...
Figure 5.7

Anti-DNA antibody binding to Nat-DNA in patients with SLE and RA. Each point represents the OD at 492nm produced from the peroxidase-conjugated antibodies binding to total IgG, IgA and IgM autoantibodies in the sera.
between the two groups, with 50% of RA patients having values above the lowest level of the range obtained for SLE.

When Nat-DNA was substituted by ROS-DNA as the coating antigen for the ELISA, a different pattern of antibody binding was observed (figure 5.8a). In SLE, denaturation of dsDNA with ROS induced a significant increase in serum antibody binding, the median and range increasing from 0.50 and 0.33-0.79 for Nat-DNA to 0.64 and 0.37-0.94 for ROS-DNA (p<0.01). Of the 15 patients' sera studied, only one sample showed decreased binding to ROS-DNA when compared to Nat-DNA and in another serum sample binding remained unchanged (figure 5.8a).

In contrast, only 53% of RA sera showed increased binding to ROS-DNA, 26% showed a decrease, and 21% remained unchanged. The median and range values for the binding of antibodies to Nat-DNA and ROS-DNA for the RA group were not significantly different from each other (figure 5.8b).

The levels of anti-DNA antibody binding in the ROS-DNA ELISA assay for SLE and RA patients, and a group of normal controls age and sex matched for the SLE patients are shown in figure 5.9. Values obtained for SLE were significantly higher than those of either the RA or control groups (p<0.001 in both cases). In contrast to figure 5.7, taking a cut-off point of 0.25 above which all the SLE patients are placed, only 1 (5%) of the RA patients had levels of serum anti-DNA antibody binding in this region. This was the only RA patient who had a positive ANA result and positive dsDNA binding by RIA.

The binding of anti-DNA antibodies to ROS-DNA was further investigated by division of the SLE group into dsDNA positive or dsDNA negative patients as determined by the RIA. The percentage dsDNA binding of anti-dsDNA antibodies to calf thymus DNA in the RIA is shown in figure 5.10. All controls were negative for dsDNA binding by this assay, the cut-off point being 10% binding. Only one RA patient had a titre greater than this (20%). Of the SLE patients in the dsDNA positive group 8 out of 27 (29.6%) were dsDNA negative by the RIA at the time of sampling.

Using a cut-off point of 0.25 in the ROS-DNA ELISA, all controls and all but one RA patient were negative. The ANA positive, dsDNA positive RA patient (by RIA) was also positive in the ROS-DNA ELISA (OD = 0.27). For the dsDNA negative group DNA binding values were significantly lower.
Figure 5.8

Optical density values obtained from the binding of sera from 15 SLE patients and 15 RA patients to Nat-DNA and ROS-DNA. Lines have been drawn between the ODs of both antigens for each individual patient to illustrate the pattern of change.
Figure 5.9

Anti-ROS-denatured activity of IgG, IgM and IgA antibodies in sera from three groups: patients with SLE, patients with RA and a group of controls age matched for the SLE patients. Results show the medians and ranges of each of the groups, each point representing analysis of the sample in triplicate.
Figure 5.10

Percentage binding of serum anti-DNA antibodies to radiolabelled DNA in a radioimmunoassay with a cut-off point of 10%. According to this, patients with SLE were divided into anti-dsDNA positive or negative. All controls and all but one RA patient were anti-dsDNA negative.
than those of the dsDNA positive group (p<0.01) but were still significantly higher from those of either the RA (p<0.001) or control group (p<0.001) (figure 5.11).

More importantly, in contrast to the RIA, the ROS-DNA ELISA identified all SLE patients as positive for anti-DNA antibodies, with values clearly distinguishable from either the RA or normal control groups.

To assess the ability of the assay to reflect disease activity, 30 SLE patients were prospectively selected to have active or inactive disease and a comparison of the two groups was made by RIA and ROS-DNA ELISA.

The results obtained by RIA (figure 5.13) for the two groups of SLE patients, 20 RA patients subdivided as seropositive or seronegative, and 20 controls, 10 age and sex matched for RA and 10 age and sex matched for SLE show that all the controls were negative, having a dsDNA binding of <10%, and the RA patient with a positive titre (see figure 5.10) was classified as seropositive for rheumatoid factor. This patient also had a consistently high dsDNA binding as measured by ELISA (figures 5.11 and 5.12). All other RA patients had negative titres.

The SLE patients with active disease had a range of dsDNA binding titres with 2 of the 15 patients (13%) having a negative result in this assay and all the patients with clinically inactive disease having DNA binding titres below 10%.

Figure 5.12 shows the results obtained by ROS-DNA ELISA. There was no significant difference between the normal control group and either of the RA groups or between the two RA groups. However, all three groups had significantly lower antibody binding than either active SLE (p<0.001), or inactive SLE (p<0.001). In addition, within the total SLE group, patients with active disease had significantly higher antibody binding than those with inactive disease (p<0.001) (figure 5.12). Although there was overlap between these two SLE sub-groups, a cut-off point of 0.25 discriminated successfully >95% of patients with active or inactive disease.
Figure 5.11

Anti-DNA antibody binding to ROS-DNA by ELISA as a reflection of disease activity as defined by the presence or absence of dsDNA antibodies in the RIA. The results show medians and ranges of dsDNA negative and dsDNA positive SLE patients as well as RA patients and controls.
ROS-DNA ELISA to determine the differential binding of anti-DNA antibodies in serum from: patients with active and inactive SLE, patients with seropositive and seronegative RA and controls age and sex-matched for SLE (*) and RA (■) respectively. Each point represents the mean of triplicate serum samples per patient, and shows the median and ranges for each of the groups.
Figure 5.13

Analysis of anti-DNA antibody binding determined by RIA in SLE patients classified as having either active disease with new clinical symptoms in a major organ, or inactive disease with a stable clinical state and no new symptoms. All SLE patients with inactive disease had a negative titre by this assay as did 26% of the patients with clinically active disease. All the controls were negative and one RA patient had a positive titre.
To assess the specificity of the ROS-DNA ELISA assay further, two additional groups of patients were studied: a group of ANA positive, dsDNA negative patients, with a diagnosis other than SLE, and a group of ANA negative patients with a diagnosis other than SLE or RA. These were compared with the SLE, RA and control groups mentioned above.

The results are shown in figure 5.14. Values obtained for anti-DNA antibody binding in the ANA positive group were significantly lower than those of either the dsDNA negative SLE group (p<0.01) or dsDNA positive SLE group (p < 0.001). Also, values for the ANA negative group were significantly higher than RA values (p<0.01). However, the distribution of DNA binding values clearly shows that there is no single cut-off point between these groups.

5.3.4: Discussion

Anti-dsDNA antibodies are highly specific for SLE and have been detected at high frequency in untreated patients with active disease (Tan, 1982). Several, but not all reports, have also identified a correlation between levels of anti-dsDNA antibodies and disease activity (Isenberg et al., 1986), especially in patients with renal involvement (Davis, Percy and Russell, 1977).

Many assays exist for measuring anti-DNA antibodies (Isenberg et al., 1987). This reflects problems of sensitivity and specificity in identifying particular disease groups with antibodies to dsDNA but not ssDNA or other anti-nuclear antibodies (ANAs).

Detection of anti-dsDNA antibodies is usually by RIA. This assay tends to detect high avidity anti-dsDNA antibodies which are thought to be associated with renal complications in SLE (Feltkamp, Aarden and Swaak, 1979). Alternatively, ELISA assays have been described which measure low avidity antibodies (Emlen, Pisetsky and Taylor, 1986). These require immobilisation of the DNA onto plastic and whilst ssDNA binds easily, dsDNA binds only poorly (Engvall, 1976). Several different methods have been used to enhance coating of DNA onto ELISA plates. These include irradiation of the plastic with UV light (Zouali and Stollar, 1986), precoating of the plates with a positively charged molecule such as protamine sulphate or poly-L-lysine to which the negatively charged DNA can more effectively (Eaton, Schnneider and Schur, 1983), or diluting the DNA in a positively charged coating buffer (eg. carbonate buffer, pH 9.6) which allows it to bind effectively to the negatively charged plastic.
Figure 5.14

Measurement of anti-DNA antibody binding to ROS-DNA by ELISA in sera of patients with: ANA positive SLE, ANA negative RA, and ANA positive or negative patients with connective tissue disorders other than SLE or RA (mostly scleroderma). Results show the medians and ranges of each of the groups, each point representing analysis of serum samples in triplicate.
Both ELISA and RIA require removal of contaminating ssDNA from the antigen preparation, particularly since other connective tissue disorders are more commonly associated with antibodies to ssDNA (Notman, Kurata and Tan, 1975). To eliminate this problem, closed circular DNA from plasmids is used in some assays (Emlen, Jarusiripipat and Burdick, 1990) and the results in general appear to be more specific for SLE patients.

Results from section 5.2 show that a control anti-DNA antibody positive serum sample had increased binding to ROS-denatured DNA when compared to Nat-DNA. Based on this property, sera from a large group of patients with SLE and other connective tissue disorders were analysed for binding to ROS-DNA, in order to investigate the sensitivity, specificity and possible clinical value of a ROS-DNA ELISA.

Using Nat-DNA as antigen in the ELISA assay there was a lack of discrimination between SLE and RA patients with 50% of the RA patients having DNA binding values within the SLE range. This is unlikely to be due to reactivity with ssDNA, since the dsDNA preparation was treated with S1 nuclease prior to coating of the plates.

To investigate the binding of anti-DNA antibodies to ROS-DNA compared with Nat-DNA, sera from SLE and RA patients were initially studied. Whilst antibody binding increased significantly after ROS-denaturation of DNA in SLE, it did not show a consistent change in RA. This suggests that ROS-DNA may detect antibodies in SLE sera undetected by Nat-DNA and not present in RA.

The ROS-DNA ELISA was then used to measure anti-DNA antibody binding to SLE and RA patients and controls. There was a greater discrimination between the SLE and RA groups in this assay (figure 5.9) compared to the Nat-DNA ELISA (figure 5.7). Both groups had levels of DNA binding significantly higher than the young normal controls, which were matched for age with SLE but not with RA. Investigation of DNA binding in young and elderly controls (figure 5.12) showed that levels increase with age; this is consistent with findings of other workers (Manoussakis et al., 1987). When the normal range of anti-DNA antibody binding was defined according to both young and elderly controls, all RA patients (with the exception of one who was dsDNA positive by RIA) were within the normal range.
There was a significant difference in the DNA binding values obtained after division of the SLE group, either by disease activity or by the presence or absence of dsDNA antibodies in the sera by RIA. Both the active SLE group and the dsDNA positive group had higher levels of anti-DNA antibody binding in the ROS-DNA ELISA than the inactive and dsDNA negative groups. This supports the existing evidence (Aitcheson and Tan, 1982; Isenberg et al., 1986) that the presence of dsDNA antibodies in the serum correlates with disease activity.

Both in the ROS-DNA and Nat-DNA ELISAs, all SLE patients, including those classified as dsDNA negative by RIA, were positive for dsDNA binding. There was a significantly higher sensitivity of the ROS-DNA ELISA for detection of SLE patients compared to the RIA and an absolute specificity of discrimination between patients with SLE and those with RA. The only exception to this was an RA patient with a positive dsDNA binding titre by RIA who was also positive by ROS-DNA ELISA.

The increased sensitivity of the ROS-DNA ELISA assay may relate to the introduction of ROS-DNA as antigen. It is possible that this assay detects low affinity anti-dsDNA antibodies that exist in the serum of SLE patients with mild or inactive disease and are not detected by RIA.

In the ROS-DNA ELISA, taking a cut-off point of 0.25, all the SLE patients gave a positive result with the RA and controls lying below this. One RA patient consistently gave a positive result both by RIA and ELISA and will be monitored closely for manifestations of SLE. A prospective study of more than four hundred non-SLE patients with anti-dsDNA antibodies showed that =85% of these developed SLE within five years (Swaak and Smeenk, 1985).

In the initial experiments, all but one RA patient and all normal controls were ANA negative whilst all SLE patients were ANA positive. Therefore to examine the specificity of the ROS-DNA ELISA further, it was necessary to determine its ability to differentiate between ANA positive and dsDNA positive patients. For this, a group of patients with other connective tissue disorders, predominantly scleroderma and primary Raynauds were studied and divided into ANA positive or ANA negative groups.
In the ANA negative group, anti-DNA antibody binding in the ROS-DNA ELISA was only slightly higher than in the ANA positive dsDNA negative, non-SLE group. Although DNA binding values were significantly lower than those of the dsDNA negative SLE group, they overlapped considerably with DNA binding values of both the dsDNA positive and negative SLE groups. This suggests that the ROS-DNA ELISA is less specific than the RIA in detecting ANA positive patients without the clinical diagnosis of SLE. This may be explained in several ways.

The low levels of serum anti-DNA antibodies for the control groups and the RA group show that the values obtained do not merely reflect the non-specific binding of a serum component to the coating antigen. The values are also unlikely to reflect binding to either protein or ssDNA since the DNA preparation was protein-free and treated with S1 nuclease to remove any regions of ssDNA from it. Lack of binding to ssDNA is also supported by the low titres in the RA group which frequently have antibodies to ssDNA (Aitcheson et al., 1980).

This suggests that the assay measures a specific interaction between antibodies in the serum of ANA positive patients and the ROS-DNA antigen, whether these patients have SLE or not.

It is well documented that ELISA assays measure both low affinity antibodies that are not readily detected by RIA, as well as high affinity antibodies measured in both assays (Emlen, Pisetsky and Taylor, 1986). The positivity of the sera of SLE patients having negative dsDNA titres by RIA may reflect detection of low affinity anti-DNA antibodies in these patients by ELISA. The ELISA may also detect such antibodies in the ANA positive patients, again undetected by RIA.

Whilst this explains why low affinity antibodies may not be detected by RIA, this does not identify their antigen of recognition. Some of the ANA have been characterised and two of these, Ro and La, are peptides with associated RNA (Stephano, 1984; Gaither et al., 1987). From the results examining the antigenicity of ROS-DNA it is possible that low molecular weight fragments of DNA, with or without associated protein, could act as nuclear antigens. Compton, Steinberg and Sano (1984) showed that there is an increased accumulation of degraded fragments of nuclear DNA in SLE lymphocytes which they suggest may arise from excision repair of DNA. This would be consistent with the findings reported here regarding increased modification of base residues induced by ROS increasing DNA
damage that is then abnormally processed leading to its accumulation in cells (section 4.2).

If ROS-DNA is an antigen for low affinity antibodies found in all ANA positive patients, the gradation of DNA binding for the SLE and ANA positive patients could represent differences in the levels of antibodies truly reactive with ROS-DNA or alternatively, it could represent the presence of antibodies reactive with different antigens but cross-reactive with ROS-DNA in some or all of the patients studied.

High affinity antibodies are most commonly associated with immune complexes (Winfield, Faiferman and Koffler, 1977) and low affinity antibodies may be less relevant to the pathogenesis of SLE. This is supported by their presence in ANA positive patients who do not develop SLE. However, the assay using ROS-DNA as antigen does have the capacity to reflect disease activity (figures 5.11 and 5.12) possibly because it detects high affinity antibodies associated with disease activity as well as low affinity antibodies. Therefore, the ROS-DNA ELISA may be useful for measuring disease fluctuations in the long-term monitoring of SLE patients.

It is possible that ROS-DNA may elicit anti-DNA antibody production in patients with connective tissue disease. However, from studies of the pathogenicity of anti-DNA antibodies it would appear that the progression of the immune repertoire to produce high affinity antibodies to either Nat-DNA or ROS-DNA or both is most relevant to the development of the severe disease manifestations of SLE such as glomerulonephritis. This is supported by isolation of high affinity anti-DNA antibodies from glomeruli of SLE patients with active kidney disease (Winfield, Faiferman and Koffler, 1977). Mechanisms that could be responsible for anti-DNA antibody production in inflammatory conditions are outlined in chapter 6.
CHAPTER 5.4: STIMULATION OF PBMC WITH ROS-DNA

5.4.1: Introduction

The mechanism of anti-DNA antibody production in SLE is unknown and may involve the stimulation of autoreactive B cell clones, may result from production of antibodies to DNA, or may be the consequence of a circulating antigenic molecule producing antibodies cross-reactive with DNA (Isenberg and Shoenfeld, 1987).

Native DNA is a poor immunogen (Madaio et al., 1984); ROS-denaturation however, increases its binding to anti-DNA antibodies (sections 5.2 and 5.3) and elicits anti-DNA antibody production in animal models (Tan and Stoughton, 1969; Jansson, 1985).

In 1985, Mumford et al., investigated the ability of normal lymphocytes to produce autoantibodies. Following polyclonal activation with pokeweed mitogen, normal lymphocytes were stimulated to secrete autoantibodies of several antigenic specificities, rather than the restricted specificities seen in the various autoimmune diseases. This suggests that autoreactive B cell clones are not deleted but are more likely to be selectively recruited in autoimmune diseases.

Braude et al., (1988) showed that SLE lymphocytes in culture can spontaneously secrete anti-DNA antibodies, the levels of which can be increased by stimulation of the cells with DNA. Since ROS-DNA appears to be a better antigen for SLE anti-dsDNA antibodies (section 5.3) and has been shown to exist in the circulation associated with immune complexes (section 4.3), the ability of ROS-DNA to stimulate the production of anti-dsDNA antibodies from isolated PBMC was investigated in both SLE patients and in a group of normal controls in order to determine the importance of antigen driven anti-DNA antibody production versus polyclonal B-cell activation.

5.4.2: Methods

5.4.2.1: Clinical Samples

Twenty SLE patients (19 female, 1 male, age range 22-55, median = 33), five disease controls with RA (5 female, age range 35-60, median = 41), and five normal controls (4 female, 1 male, age range 22-51, median = 27) were studied.
5.4.2.2: Cell Stimulation

PBMC were isolated from peripheral blood on Ficoll gradients as in section 2.3.3.1 and resuspended in RPMI-1640 to $1 \times 10^6$ cells/ml. Cells were plated in 2ml volumes in 24 well plates and cultured in a humidified atmosphere with 5% CO$_2$ at 37°C. To measure the spontaneous production of anti-DNA antibodies cells were cultured for 8 days and the supernatants harvested by centrifuging the samples at 300 x g. To measure antigen-induced anti-DNA antibody production cells were incubated with Nat-DNA or ROS-DNA at 20μg/ml for 6 days; they were then washed thoroughly (5 washes in RPMI-1640, 300 x g, 5 minutes) to remove the DNA, replated, and cultured for a further 6 days before harvesting the supernatants as above.

Cells were also stimulated with the polyclonal B cell activator SPA at a concentration of 100μg/ml for 8 days after which the supernatants were harvested.

The final culturing conditions stated were obtained from time-course experiments. Day 7 was found to be the period of maximum immunoglobulin production and cells were harvested on day 8. For cells cultured with DNA, exposure of 6 days was used as described by Braude et al., (1988). After washing, immunoglobulin production peaked at days 4 and 5 and cells were harvested on day 6. Other mitogens such as pokeweed mitogen and lipopolysaccharide were also used to stimulate the B cells but were not as effective as SPA.

The supernatants were analysed for the presence of immunoglobulins and anti-DNA antibodies.

5.4.2.3: ELISA Assay for Immunoglobulin Determination

Immunoglobulin levels in tissue culture supernatants were measured by ELISA as described in section 2.3.2.1 using goat anti-human IgG, IgA and IgM as coating antigens at a dilution of 1μg/ml in 0.05M carbonate buffer, pH 9.6. Supernatants were added neat or at doubling dilutions in PBST and the immunoglobulins detected using peroxidase-conjugated goat anti-human IgG, IgA or IgM (all at a working dilution of 1/1000 in PBST).

5.4.2.4: ELISA Assay for Anti-dsDNA Antibodies

Measurement of anti-DNA antibodies in cell supernatants was as described in section 5.2.2.3 using
ROS-DNA as antigen (section 5.2.2.1.2) and adding the supernatants neat onto the plate. Measurement of serum anti-DNA antibodies was also as in section 5.2.2.3 using a dilution of 1/100 for all samples.

5.4.3: Results

The predominant isotype of anti-DNA antibodies in SLE is IgG and it is antibodies of this class that have also been isolated from the glomerular basement membrane of SLE kidneys (Hahn, 1982).

Levels of IgG were measured in SLE, RA and control cells after culture of the PBMC in complete medium (section 2.1.6) for 8 days in the absence of any stimulating agents.

Fourteen SLE and 2 RA patients had levels of spontaneous IgG production of $>1\mu g/ml$ per $10^8$ cells; this was used as the cut-off point in the assay for a positive response to cell stimulation. None of the normal control PMBC had detectable levels of spontaneous immunoglobulin production.

The 14 SLE and 2 RA patients with spontaneously produced immunoglobulins were stimulated with SPA, Nat-DNA and ROS-DNA and the levels of IgG and anti-DNA antibodies were measured. The normal PBMC were also stimulated since spontaneous immunoglobulin production is not typical of these cells as it is in the two autoimmune conditions, SLE and RA.

For the control PBMC levels of IgG in the culture supernatants were detectable following stimulation of the cells with all three agents when compared to the negligible levels in supernatants of unstimulated cells. There was however, no significant difference between the different stimuli (table 5.4). There was also a significant increase in the levels of anti-DNA antibodies detected by the ROS-DNA ELISA following stimulation not only by ROS-DNA but by Nat-DNA and SPA (table 5.4). Measurement of serum anti-DNA antibodies showed that both these controls had DNA binding values (figure 5.15) below the cut-off point of the ROS-DNA ELISA (section 5.3.3)

Results for the RA patients showed that the spontaneous level of immunoglobulin secretion was not significantly affected by any of the stimuli, and in none of the samples was any positive level of anti-DNA antibodies detected. There was again no detectable anti-DNA antibody binding to ROS-DNA in the sera from these patients (figure 5.15).
Table 5.4

Concentrations of IgG (μg/ml) and anti-DNA antibodies (OD at 492nm) in the supernatants of PBMC stimulated with SPA, Nat-DNA and ROS-DNA, from two normal controls.
Figure 5.15

ROS-DNA ELISA of serum anti-DNA antibody binding of the 14 SLE patients and the 2 RA patients with spontaneous anti-DNA antibody production by PBMC in culture and the two normal controls with stimulated anti-DNA antibody production (see section 5.4.3).
Of the 14 SLE patients in which spontaneous immunoglobulin production was observed, all had positive OD values for anti-DNA antibodies in the serum (figure 5.15). Of the 6 patients in which this could not be detected 5 had never had detectable levels of dsDNA binding in the routine RIA (section 5.3.2.1). They had positive DNA binding values by the ROS-DNA ELISA but these were significantly lower (median = 0.64 compared with 0.93 p<0.01) than for the other 14 patients in which immunoglobulin production was detectable.

Measurement of anti-DNA antibodies in SLE supernatants showed that the level of anti-DNA antibodies was increased significantly in 6 patients by stimulation with ROS-DNA, in 4 of these 6 patients with Nat-DNA and in only 1 of these 6 patients with SPA. Figure 5.16 shows the means ± ISD for the 6 SLE patients responsive to stimulation and the remaining 8 patients with levels of anti-DNA antibody production in the stimulated cultures not significantly different from the original level of spontaneous anti-DNA antibody production.

The fluctuations in IgG immunoglobulin levels (figure 5.17) did not always reflect the changes seen in anti-DNA antibody production and in 7 patients significant changes in immunoglobulin production were not accompanied by significant increases in anti-DNA antibody production, 13 out of 14 patients having increased levels of IgG in response to stimulation (figure 5.17).

5.4.4: Discussion

The results obtained for both the SLE and RA patients support the evidence that B cells from these patients spontaneously produce immunoglobulins in vitro. It cannot be determined if these cells were previously activated in vivo. However, the results of the stimulation experiments with RA PBMC also support the results of Mumford et al., (1985) and show that whilst patients with the autoimmune disease RA produce autoantibodies (to IgG in this case) they cannot be stimulated to produce other autoantibodies such as anti-DNA antibodies. This is clearly not the case for PBMC isolated from normal controls. The normals had no spontaneous production of IgG immunoglobulin or anti-DNA antibodies but were stimulated with a polyclonal B cell stimulator (SPA) and ROS- and Nat-DNA to produce levels of the same order as levels produced by SLE PBMC.
Figure 5.16

Spontaneous and SPA, Nat-DNA and ROS-DNA induced secretion of anti-DNA antibodies by PBMC from patients with SLE. Results shown are means ± 1SD of 6 patients whose cells after stimulation produced levels of anti-DNA antibodies above those secreted spontaneously (responsive) and of 8 patients whose cells did not respond to stimulation (non-responsive).
Figure 5.17

Optical densities representing levels of secreted IgG in μg/ml from PBMC of SLE patients stimulated with SPA, Nat-DNA or ROS-DNA as a comparison of the spontaneously produced levels of IgG. The results represent the means ± 1SD of 13 patients responsive to stimulation (responsive) and of the 1 remaining patient where the level of spontaneous IgG production was not significantly increased by cell stimulation (non-responsive).
Not all normal controls responded to the stimulation (this may reflect levels of immunoglobulin produced that were below the sensitivity of the assay) but this may also reflect differing susceptibilities of individuals to production of autoantibodies. The cause of immune dysfunction in SLE is unknown but there is a genetic predisposition to the disease (see section 1.2.2.2). The normals responsive to stimulation in this study may have genetic factors that favour disease susceptibility.

Stimulation of SLE PBMC by Nat-DNA and ROS-DNA to produce immunoglobulins of which anti-DNA antibodies are clearly a component, may be important in the pathogenesis of disease. The results show that ROS-DNA was most effective as a stimulating agent in this group of patients and may therefore influence the production of anti-DNA antibodies in the circulation which once produced appear to have increased binding to this altered form of DNA compared to Nat-DNA.

The results suggest that in the group of SLE patients studied the levels of immunoglobulins and anti-DNA antibodies produced are affected both by the B cell hyperactivity and by cell stimulation from circulating antigens. Although the initial antigen responsible for anti-DNA antibody production, if it exists at all, is unknown, ROS-DNA by stimulating anti-DNA antibody production can perpetuate the response and increase the circulating anti-DNA antibody concentration. This in turn may lead to increased formation of immune complexes since the antibodies have been shown to have increased binding to the ROS-DNA, which may result in further deposition of complexes in tissues. This is a manifestation of the disease that can cause renal dysfunction and is associated with high avidity IgG antibodies bound to DNA.

Therefore, ROS-DNA may be involved in the perpetuation of anti-DNA antibody production by stimulating PBMC to produce antibodies which then bind to further molecules of ROS-DNA.
GENERAL DISCUSSION
The aims of this thesis were:

a) to study the effects of reactive oxygen species (ROS) on the biomolecule DNA, and
b) to investigate the relevance of ROS-induced DNA damage in the inflammatory autoimmune condition, systemic lupus erythematosus (SLE), which is associated with the presence of anti-DNA antibodies.

Effects of ROS on DNA:
Initial experiments were designed to characterise extensively ROS-damaged DNA, using model systems to control generation of ROS, and specific methods to address the nature of damage to this biomolecule. The results of these experiments have been summarised in section 3.7. They show that ROS can cause damage to DNA at all levels of its structure, from individual bases and ribose moiety to the sugar-phosphate backbone. Damage to the sugar-phosphate backbone may cause strand breaks. It also relaxes the conformation of the molecule by reducing base stacking interactions, thus increasing the accessibility of ROS to internal regions of the structure where the bases are situated. Further conformational studies using circular dichroism and monoclonal antibodies supported this mechanism, showing that such damage may cause considerable distortion of the macromolecule, probably by relaxing its structure as stabilising hydrogen bonds become broken.

Measurement of ROS-induced DNA damage:
To assess the actual occurrence of ROS-mediated DNA damage and its relevance in vivo, a reliable assay was required. This was of particular importance since radical species, mainly OH-, are inherently difficult to detect and measure directly because of their high reactivity with all biological molecules. Further studies presented in sections 2.6 and 3.1 led to the development of a reliable assay for measurement of the ROS-induced DNA component 8-hydroxydeoxyguanosine (8OHDG). In vitro, 8OHDG was shown to be generated in a hydroxyl radical-mediated reaction, and to be a reliable and stable marker of oxidative damage to DNA. More importantly, it was shown to be detected with sensitivities relevant to in vivo biological situations. This suggested that 8OHDG in biological material could be used as a marker of oxidative DNA damage in vivo.

Role of oxidative DNA damage in the aetiopathogenesis of SLE:
ROS have been implicated in several disease processes. In this thesis work was restricted to the role of
ROS in the aetiopathogenesis of SLE. This autoimmune disease is associated with inflammation and also with the presence of autoantibodies to DNA. The possibility arose therefore that:

a) ROS-mediated pathogenic mechanisms may be involved in SLE and

b) that ROS-induced changes to DNA may render it immunogenic and either initiate or facilitate the production of anti-DNA antibodies.

**Measurement of DNA damage in vivo:**

Damaged DNA bases are removed from DNA by excision repair mechanisms and released from the cell as a single base or as a small DNA fragment (Lindahl, 1982). These may then either react with enzymes which salvage the phosphate residue or are removed from the body unaltered and excreted in urine (Ames, 1988). This suggests that measurement of an adduct such as 80HDG in the urine, reflects the formation and subsequent repair of 80HDG and thus oxidative damage to DNA in vivo. The technique developed is quite advantageous, since it is non-invasive and with the use of an automated analytical system such as HPLC/ECD 80HDG can be detected sensitively, specifically and quickly in many samples. Furthermore, its sensitivity may be increased by the nature of urine formation itself, since DNA lesions are removed from all the cells of the body and concentrated in the relatively small volume of urine excreted. Although the method may be subject to artifacts such as adducts derived from DNA of ingested food material, this problem may to a great extent be overcome by collection of early morning samples which minimise contamination from such sources.

**Inflammation-induced oxidative damage to DNA in vivo:**

Studies in chapter 4 showed that rheumatoid arthritis (RA), a typical example of an autoimmune condition associated with active inflammation, is associated with significantly increased rates of 80HDG excretion compared with normal controls. Based on this, it is suggested that oxidative stress in inflammatory conditions causes increased DNA damage. When such damage is efficiently repaired by excision enzymes, as may be the case in RA, increased levels of 80HDG removed from cells are excreted in the urine, thus maintaining normal cellular function. This may reflect an enhancement of normal DNA repair processes to compensate for increased levels of damage.

In SLE, cells are likely to be subjected to oxidative stress of similar magnitude to that observed in RA. Furthermore, they appear to be as susceptible to ROS-induced damage as normal cells (chapter 4).
What then is the explanation for the greatly reduced levels of 80HDG found in the urine of patients with SLE?

Studies in chapter 4 suggest that this may be due to:

(a) Aberrant removal of 80HDG, due to defective DNA repair.
(b) Accumulation of 80HDG-containing, damaged DNA in immune complexes.

Defective DNA repair in SLE cells:

Compared with normal, SLE cells appear to have a reduced ability to remove damaged DNA bases such as 80HDG. These accumulate in the cell and therefore may not be excreted in large quantities in the urine. This deficiency appears to manifest itself in vitro as an initial lack of excision repair immediately following the oxidative insult, after which surviving cells have repair kinetics not significantly different from those of normal cells. Excessive DNA damage is known to cause energy depletion, resulting in cell death and lysis rather than repair of the lesions (Schraufstätter et al., 1986). This could act as a protective mechanism against the consequences of DNA damage, since it may prevent replication of severely altered DNA which may form non-functional proteins. However, it may also result in the release of DNA in the circulation: the consequences of this in the aetiopathogenesis of SLE are discussed later.

In addition to this, in situations of lower oxidant stress, deficient repair may have further serious pathogenic implications. 80HDG is a mutagenic base adduct causing mis-pairing at its site of incorporation (Kuchino et al., 1987; Shibutani, Takeshita and Grollman, 1991). Since this adduct does not appear to be adequately repaired in SLE, the mutagenic residues may be incorporated into the DNA and expressed. This may provide an explanation for the increased risk of malignancy in patients with SLE (Miller, 1967; Louie and Schwartz, 1978; Green, Dawson and Walker, 1978). Such a mechanism may be particularly relevant to patients with mildly active disease, where cells exposed to low level oxidative stress, may incur DNA damage not resulting in cell death. This could be investigated in epidemiological studies of SLE patients with consistently low disease activity or patients in long periods of clinical remission.

Rheumatoid arthritis is also associated with an increased risk of malignancy (Miller, 1967; Louie and
Schwartz, 1978), which, however, does not appear to be due to an intrinsic defect of DNA repair mechanisms. Oxidative damage has been suggested to play a role in the aging process and the increased risk of malignancy towards the end of the life span (Ames, 1983; Cutler, 1984). This is believed to be related to the constant damage to cellular components that becomes less effectively repaired with age, resulting in eventual malfunctioning of the cell. It is possible that inflammation in RA, through increased ROS production, may cause an accelerated aging, such that malignancy appears to be more common. This could apply to other chronic inflammatory conditions as well.

Role of circulating ROS-damaged DNA in SLE:

(a) Formation of circulating immune complexes:
Following cell lysis, damaged DNA may be released into the circulation and become bound by anti-DNA antibodies which bind native dsDNA, often with high affinity (Aarden, De Groot and Lakmaker, 1976; Riley, Addis and Taylor, 1980). Evidence that such a mechanism may operate in SLE arises both from the detection of large DNA fragments in SLE sera (chapter 4.3.3), and from the analysis of DNA isolated from circulating immune complexes. Using both base residue modification and monoclonal antibody binding, it was shown that DNA from SLE circulating immune complexes displayed similar characteristics to DNA exposed to ROS in vitro (chapter 3.1 and 3.6). This suggests that it had been exposed to ROS and was considerably oxidised in vivo.

DNA fragments trapped in circulating immune complexes may not be degraded by serum nucleases, a process suggested to be impaired in SLE (Frost and Lachmann, 1968; Chitrabamrung, Rubin and Tan, 1981). This may be a further explanation for the very low levels of adduct observed in urine from patients with SLE. It may also have important pathogenic implications, through the consequent deposition of immune complexes in tissues, such as glomeruli, and the resultant tissue damage (discussed in detail in chapter 1, section 1.2.5).

(b) Production of anti-DNA antibodies:
It is still highly controversial whether production of anti-DNA antibodies is due to antigen (DNA) driven responses or due to polyclonal B cell activation. This has been discussed in detail in chapter 1, section 1.2.4. Recent evidence tends to suggest that both mechanisms may contribute (Casali and Notkins, 1989; Casali et al., 1989). There is, however, some evidence that ROS-altered DNA is more
immunogenic than native dsDNA (Natali and Tan, 1971), and that antibodies elicited by ROS-DNA may cross-react with native dsDNA (Tan and Stoughton, 1969; Jansson, 1985). In view of this, three lines of evidence from the work described in this thesis suggest that ROS-DNA may be involved in anti-DNA antibody production in SLE:

Firstly, and possibly most directly, free ROS-DNA and to a lesser extent native dsDNA were shown to be able to initiate production of anti-DNA antibodies from normal peripheral blood mononuclear cells (PBMC) in vitro, and to enhance their production from already spontaneously anti-DNA producing SLE cells.

Secondly, this potentially immunogenic, ROS-DNA was shown to be present in the circulation of patients with SLE. It exists mainly as part of immune complexes, but theoretically it should be available in free (antigenic) form after its release from cells and before it combines with antibody. Even in complexed form, antigenic regions may still be exposed, in which case immune complexes themselves may elicit anti-DNA antibody production. Inadequate degradation of released ROS-DNA by serum nucleases, formation of immune complexes and impaired clearance of these complexes by the reticuloendothelial system in SLE, may all contribute to the persistence of DNA in the circulation, and thus initiate or potentiate the production of anti-DNA antibodies.

Lastly, anti-DNA antibodies from SLE sera were shown to bind better to ROS-DNA than native dsDNA, possibly indicating higher specificity or affinity. This may be of pathogenic significance, since high affinity antibodies appear to be particularly important in immune complex formation and deposition (Rothfield and Stollar, 1967). Low affinity antibodies on the other hand, may be equally important: these may dissociate from DNA more readily (Riley, Addis and Taylor, 1980), thus releasing free DNA to elicit or enhance further anti-DNA antibody production. Indeed, work in chapter 3.6 of this thesis showed that ROS may induce subtle changes to the DNA molecule which may affect the affinity of binding interactions between ROS-DNA and anti-DNA antibodies.

Clinical applications of the reactivity of anti-DNA antibodies with ROS-DNA: Measurement of auto-antibodies in patients with connective tissue disorders is widely used to aid the diagnosis of particular conditions, but its value is limited by the lack of absolute specificity of any one
autoantibody for a particular disorder. This is certainly true of anti-dsDNA antibodies and SLE. Whilst they are present in approximately 70% of SLE patients and appear to correlate with disease activity, they can also be found in other connective tissue disorders.

Serum anti-DNA antibodies from SLE patients showed increased reactivity with ROS-DNA when compared to native dsDNA (chapter 5.2 and 5.3). This suggested that an assay based on this property of ROS-DNA may be advantageous in terms of specificity and sensitivity compared with currently available, conventional anti-DNA antibody assays. An ELISA was therefore developed, to measure binding of serum anti-DNA antibodies to ROS-modified DNA.

Initial experiments using SLE, ANA negative RA and normal sera suggested that this ELISA detected anti-DNA antibodies with much higher sensitivity than the conventional RIA. All SLE patients studied, even those who were anti-dsDNA negative by RIA, had clearly detectable antibodies by ROS-DNA ELISA; the levels correlated well with disease activity. The increased sensitivity is most likely due to detection of both high and low affinity antibodies by ELISA. In contrast, RIA may detect only high affinity antibodies, the antibodies of lower affinity not forming stable precipitable antibody-antigen complexes (Casperson and Voss, 1980). This remarkable increase in sensitivity of detection, did not appear to be at the expense of specificity; SLE patients had clearly higher levels of anti-DNA antibodies than either patients with RA or normal controls.

Further analysis of the disease specificity of this assay was then attempted, by analysing an ANA positive group of patients, mostly with scleroderma. In this case, almost all of these patients were identified as anti-DNA antibody positive by the ROS-DNA ELISA, whilst they were negative by RIA. This obviously limits significantly the diagnostic value of this assay for SLE. It is however appropriate to speculate on the explanation and significance of this finding. These results are definitely not due to reactivity with either ssDNA or protein antigens (e.g. histones), since these were extracted prior to preparation of the ROS-DNA antigen. It is possible, that as in anti-dsDNA negative (by RIA) SLE, the antibodies detected in the ANA positive group of patients, are low affinity anti-DNA antibodies; these would not have been detected by RIA. More importantly, this would indicate that ROS-DNA is a nuclear antigen, detected in the initial ANA-screening method but not by RIA. To support this there is evidence to suggest that low molecular weight fragments of DNA are detected in the nuclei of cells.
during normal metabolism (Compton, Steinberg and Sano, 1984) and that there is an increased number of fragments produced in SLE cells (Sano et al., 1983; Compton, Steinberg and Sano, 1984). This in itself suggests that fragmentation of DNA by ROS is a normal metabolic process and antibodies to ROS-DNA may act to clear the damaged molecules. In addition, ultraviolet radiation of keratinocytes has been shown to induce antigens on the surface of the cell that were recognised by ANAs (Furukawa et al., 1990). This suggests that ROS, produced during inflammation or periods of UV exposure, may also alter cell surface and internal components to increase ANA binding and this is supported by recent results of Sequi, Leigh and Isenberg (1991).

Increased damage to DNA in SLE and other inflammatory conditions may up-regulate the production of ANA and anti-DNA antibodies thus altering the fine balance of the immune system and producing excess numbers of antibodies with the potential to react with Nat- and ROS-DNA.

Figure 6.1 shows a schematic representation of how phagocyte-derived ROS may cause DNA damage. Initial activation of the phagocyte by bacterial stimuli or immune complexes generates $\text{O}_2^-$ which dismutates to produce $\text{H}_2\text{O}_2$ (section 1.1.2.1). This can then diffuse into cells (Allan et al., 1988) causing lipid peroxidation upon interaction with the cell membrane (section 1.1.5.1). The products of lipid peroxidation themselves can interact with DNA to cause damage directly (Inouye, 1984), and can form clastogenic factors which extend the time and area over which ROS damage is inflicted (section 1.1.5.4.6). Emerit et al., (1980) have demonstrated increased levels of this clastogenic factor in SLE serum and this provides indirect evidence for the increased production and/or inadequate defences against ROS in SLE. In addition to lipid peroxidation, ROS will attack other cellular biomolecules, including proteins (section 1.1.5.2), carbohydrates (1.1.5.3) and DNA (1.1.5.4). Damage to DNA is particularly important since this molecule is central to cell differentiation and expression of constitutive proteins.

Since phagocytosis is a normal metabolic process, cellular defence mechanisms exist to degrade ROS with no significant detrimental effects to the cell (section 1.1.6). However, in chronic inflammatory diseases it can be envisaged that increased stimulation of phagocytes may initially increase damage to all cellular components, including DNA, thus exerting pressure on defence mechanisms to maintain normal
Figure 6.1

Hypothesis: schematic representation of the role of inflammation-derived ROS in DNA damage and the pathogenic consequences of this.
cellular function. This may result in an increase in DNA excision repair activity, concomitantly increasing levels of oxidised products in extracellular fluids. These become concentrated, and are thus, measurable in urine.

This adaptation of cells to deal with increased oxidative stress, may be most relevant either to moderate inflammatory activity where levels of ROS do not increase dramatically, or to a gradual increase in ROS whereby the cell can adapt more efficiently by up-regulating defence mechanisms (as described in Brown et al., 1990).

In cases where levels of ROS rise suddenly, defence mechanisms may become over-whelmed resulting in inefficient repair of DNA. If the damage is sub-lethal, the cell will survive but may incorporate mutations into its genome (ROS may represent initiating or promoting factors in the multi-stage theory of mutagenesis). Support for this hypothesis is a notably increased risk of malignancy in chronic inflammatory conditions (Miller, 1967; Louie and Schwartz, 1978; Green, Dawson and Walker, 1978).

As an alternative, damage to DNA may generate a product irreparable by the cell (Schraufstätter et al., 1986). Cell death, and thus degradation of modified DNA would appear to be advantageous to maintenance of normal cell development. However, abnormalities of the immune system in chronic inflammatory disorders, SLE in particular, further complicate maintenance of normal cell metabolism.

SLE serum contains antibodies to DNA which combine with DNA, released from cells, to form immune complexes (see section 1.2.4 and 1.2.5). If DNA damage is increased, levels of DNA excretion are also increased which in turn can form immune complexes.

The role of released cellular DNA in the initiation of anti-DNA antibodies is unclear although the greater the damage to the DNA, the more immunogenic it is (Tan and Stoughton, 1969; Jansson, 1985). However, DNA/anti-DNA antibody immune complexes exacerbate the inflammatory response of SLE by contributing to further stimulation of phagocytic cells (Weiss and Ward, 1982; Ryan et al., 1990). This results in a positive feedback mechanism for ROS generation. Immune complexes also contribute to SLE disease manifestations by their deposition in tissues (section 1.2.5) which may also be a result of initial ROS production.
Future Work

a) Evaluation of DNA repair in RA PBMC

8OHDG was excreted in significantly higher quantities in the urine of RA patients compared to control samples. This was thought to result from efficient repair of increased levels of ROS-induced DNA damage produced during inflammation.

Preliminary results from several patients with active RA suggest this to be the case; repair of 8OHDG in oxidant-stressed RA PBMC did not differ significantly from PBMC of normal controls. However, further investigation of a larger number of RA patients is needed to confirm this finding.

b) Immunoassays for detection of 8OHDG

The measurement of 8OHDG in urine has been shown to be a reliable indicator of DNA damage in vivo but there are problems of peak identification using HPLC/ECD as discussed in the introduction to chapter 4. To improve analysis of 8OHDG in urine, an immunoassay using polyclonal antisera or monoclonal antibodies directed against 8OHDG could be developed. This may have the sensitivity of the HPLC/ECD assay, and would diminish any doubts about specificity for 8OHDG detection.

There are several references to both polyclonal and monoclonal antibodies raised against 8OHDG although none have been used diagnostically (Kasai and Nishimura, 1986; Fraga et al., 1990).

For the production of antibodies, rabbits could be immunised with dG reacted with any of the model systems described in section 3.1 (using this information to maximise the yield obtained) and conjugated to albumin. It would then be necessary to screen and remove from the antisera antibodies reactive with dG and other ROS-induced products as well as the carrier molecule using well-established experimental procedures.

An antibody to 8OHDG produced in this way could also be used as a fluorescent stain to detect 8OHDG in tissues. This would require invasive procedures to obtain DNA but could be useful as a marker of accumulating DNA damage, potentially important in malignancy. For example, cigarette smoke can damage DNA to produce 8OHDG (Leanderson and Tagesson, 1990) and there is a positive correlation between the number of aromatic hydrocarbon-DNA adducts in the lungs of smokers and the number of...
cigarettes smoked (Phillips et al., 1988). Analysis of 8OHGD in lung tissue of smokers may enable the prediction of susceptibility to malignancy as a function of unrepaird DNA damage.

c): ROS-DNA as an antigen

Results from the ROS-DNA ELISA have shown that sera from ANA positive patients react with ROS-DNA to give a positive result. The possibility that ROS-DNA is a nuclear antigen recognised in the ANA test could be clarified by extraction of nuclear antigens from cells (by isolation of nuclei from other cellular material), selective depletion of known antigens with antibodies to these, such as anti-Ro and anti-La antibodies found commonly in patients with scleroderma, and analysis of the remaining ethanol precipitable fraction with monoclonal antibody screening (shown to give a highly definitive pattern of ROS-damaged DNA).

ROS-DNA as antigen has also been shown to detect low affinity antibodies present in the serum of SLE patients with mildly active and inactive disease as well as high affinity antibodies more characteristic of active disease states. The ROS-DNA ELISA assay may therefore have potential for use in longitudinal studies to monitor any fluctuations of disease activity. This could determine if the assay could be used to predict periods of disease flair or remission.

d): ROS-DNA as an immunogen

ROS-DNA and Nat-DNA stimulated normal and SLE PBMC in culture, to produce anti-DNA antibodies. Since anti-DNA antibodies are not a characteristic component of serum, this suggests that DNA released from cells during normal metabolism is quickly cleared from the circulation and does not induce anti-DNA antibody formation. However, it also suggests that when DNA excretion is increased, such as in active chronic hepatitis (Davis and Read, 1975), clearance mechanisms may be temporarily over-whelmed because anti-DNA antibodies do become detectable in serum from these patients. However, the rise in anti-DNA antibodies appears to be only transient. In SLE, regulation of anti-DNA antibody production clearly differs since the levels may and often do persist in the absence of an active inflammatory response. This may be due to a constant inability to clear DNA effectively or an overproduction of antibodies from polyclonal B cell activation. Both mechanisms however, would contribute to the formation and persistence of circulating immune complexes (section 1.2.5).
Circulating immune complexes may have a role in the production of anti-DNA antibodies. This could be investigated in a similar way to analysis of DNA-stimulated anti-DNA antibody production from SLE PBMC. This would require culturing of PBMC with immune complexes and measuring anti-DNA antibody production as in section 5.4.

Further, the epitopes and associated idiotypes of anti-DNA antibodies produced by ROS-DNA and Nat-DNA could be studied with available anti-idiotypic monoclonal antibodies. This would enable the pathogenicity of DNA-induced (antigen-driven) antibodies and any immune complex-induced antibodies to be determined. This may increase the understanding of the role of these antibodies and their initiating antigen in the manifestations of SLE.

e): Relevance of inflammatory status to DNA damage

The effects of ROS on DNA were effectively inhibited with scavengers of OH-, and metal ion chelators in particular, even for DNA-bound metal ion site-directed damage. Therefore, it is possible that in inflammatory conditions where ROS generation is increased, such treatment with antioxidants could be therapeutically beneficial.

This could be investigated in RA patients treated for active disease and followed over the months of treatment. Urinary 8OHDG measured in these patients would determine whether or not, on suppression of the active inflammatory response, levels of 8OHDG returned to normal with the corresponding decrease in inflammatory activity. Although treatment for RA does not involve conventional antioxidants, widely used drugs act to reduce the levels of neutrophil- and macrophage-derived ROS, thus acting indirectly as antioxidants (Forrest and Brooks, 1988).
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