An Investigation of DNA Damage Induced by Oxidative Stress in Neuronal Cells

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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ABSTRACT

This project aimed to investigate the hypothesis that oxidative DNA damage has a role in neuronal dysfunction. Reactive oxygen species (ROS) are known to damage important cellular macromolecules, including DNA. They have been implicated in many pathological conditions, including common neurodegenerative disorders, but the mechanisms of cellular dysfunction and death involved remain unclear. Oxidative DNA damage was therefore studied in an in vitro model system and pathological tissue. A novel, sensitive assay based on the fortuitous finding that avidin binds directly to the DNA lesion 8-oxodeoxyguanosine (8-oxodG) was used in these investigations. Pre-lethal DNA damage was assessed in terminally differentiated human neuroblastoma IMR32 cultures after exposure to various forms of oxidative stress (hydrogen peroxide, UVA irradiation and paraquat). Avidin binding was significantly increased in these cells, indicating the presence of oxidative DNA damage; repairable and non-repairable damage were both detected. Binding was decreased by pre-incubation with the antioxidant α-tocopherol or iron chelator desferrioxamine, demonstrating the involvement of ROS in the mechanism of DNA damage induced during oxidative stress. Avidin binding was also assessed visually in cells exposed to hydrogen peroxide via fluorescent microscopy, and was shown to be located primarily within the nucleus. Once again, levels of damage were decreased after incubation with α-tocopherol. Finally preliminary studies were carried out to assess levels of oxidatively-damaged DNA in spinal cord sections from patients suffering from MND compared to age-matched control subjects. Binding was once again detected directly in situ using immunofluorescence microscopy. The novel methodology used allows the demonstration of oxidative DNA damage directly in situ, something not previously possible, but is only semi-quantitative, and more work is needed to assess the precise site and mechanism of avidin binding. However, the direct demonstration of damage may help elucidate the mechanisms involved in cellular dysfunction produced by oxidative stress, and so might provide a greater understanding of the causes of the most common neurodegenerative diseases.
This thesis is dedicated to my mother, without whom I would never have managed to get this far. Thanks Mum.
Acknowledgements

The production of this thesis would not have been possible without the time and help of several people at various points during my PhD.

First of all, special thanks to my supervisor, Dr Siân Thomas, for all her help, encouragement, patience, friendship and proof-reading throughout the practical work and thesis preparation, and to fellow student and co-worker Tracey Benn for her advice, support, friendship and computing facilities!

Thanks also to the following: my colleagues Rakhee Patel and Jennie Clark, who both worked with me on the avidin project; Dr Roger Pamphlett, who allowed me to work for 6 weeks in the Department of Pathology at the University of Sydney, and to everyone in the department for making me feel so welcome; Steve Kum Chew, who’s workload must have doubled during my stay in Sydney when he helped me prepare all the sections I needed; Dr Fiona Manning, who was my contact at Biotrin International, and made sure I received the reagents I needed for the avidin assay; Ariane De Luca, for the cerebellar granule cell samples; Dr Karl Frei, for the cerebrospinal fluid samples; Dr David Shuker, for supervising the final year of my practical work at Leicester.

Finally I must thank my family - my Mum and my sisters Nik, Kate and Sianne - for all their support over these past 4 years. And last, but not least, thank you Matt for keeping me sane and making sure I actually finished what I started when it seemed the end was never in sight – can I have a break now???

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<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
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<tr>
<td>AFU</td>
<td>Arbitrary Fluorescent Units</td>
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>AM</td>
<td>Aged Mice</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One Way Analysis of Variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
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<tr>
<td>bp</td>
<td>Base Pairs</td>
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<tr>
<td>BrdU</td>
<td>5'-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>Copper ions (oxidised)</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
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<tr>
<td>ChA</td>
<td>Choline Acetyl Transferase</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
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<tr>
<td>COMT</td>
<td>Catechol-o-Methyl Transferase</td>
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<tr>
<td>COO⁻</td>
<td>Carboxyl group</td>
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<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<td>dG</td>
<td>deoxyguanosine</td>
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<td>DIV</td>
<td>Days <em>In Vitro</em></td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<tr>
<td>EAA</td>
<td>Excitatory Amino Acids</td>
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<tr>
<td>EBSS</td>
<td>Earle’s Balanced Salt Solution</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Animal Cell Cultures</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>FALS</td>
<td>Familial Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>Iron ions (Reduced)</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>Iron ions (Oxidised)</td>
</tr>
<tr>
<td>Fe=O²⁺</td>
<td>Ferryl Iron</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>GAh</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
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<tr>
<td>GPₓ</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>Bicarbonate Anion</td>
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<td>HEC</td>
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<td>HO₂⁻</td>
<td>Hydroperoxyl Radical</td>
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<td>H₂O₂</td>
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<td>H₂SO₄</td>
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<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<td>HIFCS</td>
<td>Heat Inactivated Foetal Calf Serum</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
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<tr>
<td>HVA</td>
<td>Homovanillic Acid</td>
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<td>IEG</td>
<td>Immediate-Early Genes</td>
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<td>IMS</td>
<td>Industrial Methylated Spirit</td>
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<td>JNK</td>
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<td>K⁺</td>
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<td>L-O₂⁻</td>
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<tr>
<td>L-O₂H</td>
<td>Lipid Hydroperoxide</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>MND</td>
<td>Motor Neuron Disease</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<td>MTT</td>
<td>3-(4,5)-dimethyl-thiazol-2-yl-2,5-diphenyl tetrazolium bromide</td>
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MPTP  Methylphenyltetrahydropyridinium
MPP*  Methylphenylpyridinium
NADH  Nicotinamide Adenine Dinucleotide (reduced form)
NADPH  Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NaOH  Sodium Hydroxide
nDNA  Nuclear DNA
NEAA  Non-Essential Amino Acids
NGS  Normal Goat Serum
NO  Nitric Oxide
NOx  Nitrogen Oxides
NOS  Nitric Oxide Synthetase
NTA  Nitrilotriacetic Acid
O21  Singlet Oxygen
O2-  Superoxide Radical
O22  Peroxide Ion
OH  Hydroxyl Radical
ONOO-  Peroxynitrite
ONOOH  Nitroxyl Radical
8-oxoG  8-oxoguanine
8-oxodG  8-oxodeoxyguanosine
OPD  o-Phenylenediaminedihydrochloride
p38/RK  p38/Reactive Kinases
PBS  Phosphate Buffered Saline
PD  Parkinson’s Disease
PFA  Paraformaldehyde
PHF  Paired Helical Filaments
PMD  Post-Mortem Delay
RER  Rough Endoplasmic Reticulum
ROS  Reactive Oxygen Species
rpm  Revolutions Per Minute
RT  Room Temperature
SALS  Sporadic Amyotrophic Lateral Sclerosis
SAPK  Stress-Activated Protein Kinases
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<td>Single-stranded DNA</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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<td>SOD</td>
<td>Superoxide Dismutase</td>
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<td>TH</td>
<td>Tyrosine Hydroxylase</td>
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<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
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<td>UV</td>
<td>Ultra-Violet</td>
</tr>
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<td>Young Mice</td>
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CHAPTER 1

INTRODUCTION
1.1 HISTORICAL PREAMBLE

This project is based on the fortuitous finding that avidin, a molecule commonly used in systems designed to detect binding of biotinylated antibodies, is itself able to bind directly to ROS-induced damage to DNA. The initial aim was to characterise binding of a primary, polyclonal antibody (Ab) to oxidatively-damaged DNA (Herbert et al., 1994), Ab529, in cells exposed to oxidative stress. Preliminary experiments carried out during an MSc practical class involved an assessment of antibody binding in fibroblast cultures exposed to UVA light, a known method of ROS-production. The detection system entailed the use of an avidin conjugated to the fluorescent dye fluorescein isothiocyanate (FITC) for visualisation of binding using fluorescence microscopy. During the course of these studies fluorescence localised to the nuclei of UVA-irradiated cells was observed in supposedly negative control wells containing avidin-FITC in the absence of either primary or secondary antibody (Figure 1.1). No binding was seen in sham-irradiated control cultures. These results strongly implied that avidin itself was capable of binding directly to damaged cells.

Preliminary inhibition experiments were also performed during later work for an MSc thesis, to investigate possible epitopes for Ab529. The antibody was incubated together with increasing concentrations of single-stranded DNA (ssDNA), before addition to cultures exposed to UVA light and subsequent assessment of binding. Avidin-FITC was once again chosen to detect antibody binding, this time using a fluorescent plate reader. Antibody binding to cells was expected to gradually decrease in the presence of higher concentrations of DNA if the epitope was ssDNA, and to remain constant if it was not. In fact the results obtained showed levels of fluorescence to increase with DNA concentration in a dose-dependent manner. It was suggested that the antibody was binding to the ssDNA itself, rather than damaged DNA in the irradiated cultures. When these results were looked at together with those obtained during the practical class, it seemed feasible to hypothesise that avidin itself binds directly to oxidatively modified DNA.

This thesis investigated the hypothesis that nuclear DNA damage is involved in neuronal cellular dysfunction and cell death initiated by oxidative stress. The idea that avidin is able to detect oxidatively-damaged DNA itself was therefore studied further, with a view to developing an assay able to detect ROS-induced DNA damage directly in neuronal cells exposed to oxidative stress.
Figure 1.1 Avidin was seen to bind to the nucleus of UVA irradiated cells. No binding was observed in sham-irradiated control cells (A). Nuclear binding of avidin-FITC was detected in the absence of primary antibody (B).
1.2 OXYGEN TOXICITY AND NEURODEGENERATIVE DISEASE

Oxygen is essential for the survival of all aerobes, but paradoxically it can also be toxic in these same organisms (Halliwell and Gutteridge, 1989a). It is now widely accepted that this toxicity is due to various reactive oxygen species (ROS), formed during processes occurring both extra- and intra-cellularly. These include superoxide (O$_2^-$), the hydroxyl radical ('OH), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (O$_2^1$). The former two are free radicals, as is oxygen itself, whilst H$_2$O$_2$ and O$_2^1$ are not radicals in themselves, but play an important role in the formation and propagation of these species. Oxygen free radicals may be defined as 'free radicals where redox reactions occur at oxygen atom centres' (Borg, 1993). In cells this encompasses alkoxyl and peroxyl radicals, especially those of lipids, in addition to the superoxide and hydroxyl species mentioned earlier. ROS, on the other hand, include hydrogen peroxide, formed during the partial reduction of oxygen, and singlet oxygen, which is usually a product of a photochemical reaction. Care should therefore be taken when using terminology associated with oxygen toxicity, because free radicals and ROS are not synonymous. ROS do have important biological roles, some of these are positive, such as their involvement in the phagocytic killing of bacteria by macrophages, neutrophils, and microglial cells of the brain (Babior, 1978a,b), whilst others are more detrimental to the cell, and ROS have been implicated in many pathological disease states (Halliwell and Gutteridge, 1989a). In order to study the mechanisms of ROS in cell death, it is necessary to have appropriate means of identifying pre-lethal markers of DNA damage. The aim of this project was to attempt to identify oxidatively modified DNA lesions in neuronal cells exposed to oxidative stress, both in \textit{in vitro} model systems and in pathological tissue.

1.2.1 WHAT IS A FREE RADICAL?

A free radical may be defined as 'any species capable of independent existence with one or more unpaired electrons' (Halliwell and Gutteridge, 1989a). This broad description includes oxygen itself. Molecules consist of atomic nuclei surrounded by electrons. These electrons occupy energy orbitals centred around the nuclei. Only two electrons can occupy any one atomic orbital, and the rules of quantum mechanics, which determine the electronic configuration of an atom, state that they must have opposite spins. These are referred to as paired electrons, represented as $\uparrow \downarrow$. Electrons of like spin
locate themselves as far away from each other as possible. This is one of the most basic rules of quantum mechanics, known as the Pauli exclusion principle. Molecules are most stable when their orbitals are full, therefore free radicals are often highly reactive, reacting with molecules in the immediate vicinity. These processes may lead to the production of new radical species, another common property of free radicals being their ability to propagate chain reactions. The life-span of an individual free radical is therefore generally very short (Pryor, 1986), although their effects may be more long-term as they have the potential to damage important macromolecules within cells such as lipid membranes, proteins and DNA. There are however several examples of unreactive, chemically stable radical species which are biologically important (Borg, 1993). Vitamin E, for example, interacts with lipid peroxyl radicals produced during lipid peroxidation; the chromanoxyl free radicals produced are relatively stable, and therefore will not propagate any chain reactions. This stability is responsible for the antioxidant effect of the tocopherols.

1.2.2 ROS AND NEURODEGENERATION

ROS have been implicated in many different disease states, ranging from cancer to arthritis. It is likely that oxidative stress is a secondary feature of most human diseases, with tissue damage leading to increased ROS formation. The important question therefore, is not whether ROS are formed, but rather if the levels produced are capable of causing damage which is clinically significant. In order to assess whether ROS-mediated damage has a mechanistic role in any disease state, several factors must be satisfied (Halliwell, 1992). It must be shown that:

- ROS are formed at the site of injury.
- the time-course of their formation is such that they are able to participate in the disease pathology.
- the removal of ROS, or the prevention of their formation, decreases the damage observed.
- in experiments, direct application of ROS at in vivo levels produces most or all of the injury seen in the disease.

Demonstrating all four of these factors is very difficult. For instance, the failure of antioxidant therapy, such as α-tocopherol supplementation, in disease states where oxidative stress is thought to be important clinically may not necessarily mean that ROS
are not involved in the disease mechanism. It may instead be due to the fact that the antioxidant did not enter the correct cells, or that lipids were not a target for damage by the ROS produced. If on the other hand an antioxidant appears to improve a patient's condition, it must be shown that this is due to the removal of ROS, and is not a separate beneficial action of the antioxidant alone. In spite of these difficulties, research suggests that these molecules are involved in the development of pathological diseases, including neurodegenerative conditions (Coyle and Puttfarcken, 1993; Olanow, 1993; Simonian and Coyle, 1996), as well as non-pathological conditions such as the normal ageing process (Nohl, 1993; Zhang et al, 1993; Barja et al, 1994). It is widely believed that the mechanisms involved in neurodegeneration are very complex, with both genetic and external factors playing a role (Strange, 1993), but so far the aetiology remains unclear. The number of people suffering from some form of neurodegenerative disease is growing all the time as the elderly population in the Western world increases, and the putative role of ROS in these conditions is therefore under intense investigation.

The central nervous system (CNS) is particularly susceptible to damage by ROS for several reasons (Olanow, 1992). Firstly, there are high levels of polyunsaturated lipids in the neuronal membranes which are vulnerable to ROS attack, and the brain contains low levels of protective antioxidant molecules. High levels of free iron and the reducing agent ascorbic acid are present, both of which are believed to be important in the formation of ROS. Finally, the synthesis and degradation pathways of neurotransmitters such as the catecholamines and dopamine are also sources of ROS. These factors all help to create an environment which favours ROS production, especially as the brain utilises a proportionately large amount of oxygen in relation to its size. The unique function of neurons, together with the fact that they are not renewable, means damage affecting their function will have severe consequences. ROS have been implicated in the common neurodegenerative disorders Parkinson's disease (PD) (reviewed by Adams and Odunze, 1991), Alzheimer's disease (AD) (reviewed by Markesbery, 1997) and motor neuron disease (MND) (Olanow, 1993; Kisby et al, 1997), as well as the neuropsychiatric disorder schizophrenia (Cadet and Kahler, 1994). Although many factors can initiate oxidative stress in neurons, it has been hypothesised that overactivation of glutamate and related excitatory amino acid (EAA) receptors may be involved in many cases (Figure 1.2) (Coyle and Puttfarcken, 1993; Dawson and Dawson, 1996).
Figure 1.2 Proposed route of OH production in cells caused by a rise in cytosolic Ca²⁺ due to an increase in excitatory amino acids (EAA). Ca²⁺ may activate: 1. A nitric oxide synthetase (NOS), which forms nitric oxide (NO⁻) from arginine and 2. A protease which converts xanthine dehydrogenase to xanthine oxidase, which in turn forms O₂⁻ from xanthine. NO⁻ and O₂⁻ may then interact to form peroxynitrite (ONOO⁻), which is protonated to form the nitrosyl radical (ONOOH). This then decomposes to form 'OH. It has been demonstrated that both O₂⁻ and NO⁻ are important in models of excitotoxicity, as tissue damage can be blocked by (a) NOS inhibitors which block NO⁻ formation; (b) reduced haemoglobin which clears NO⁻; (c) xanthine oxidase inhibitors which block O₂⁻ formation; and (d) superoxide dismutase (SOD) which clears O₂⁻. (Taken from Olanow, 1993)
1.2.2.1 PARKINSON’S DISEASE - AN EXAMPLE OF ROS-INDUCED NEURODEGENERATION?

The common neurodegenerative disorder Parkinson’s disease is widely studied, and a good example of a condition of the CNS in which ROS are strongly implicated. This disease is a slowly progressive neurodegenerative disorder affecting around 5-10% of the population over 65 (Strange, 1992). It is characterised by tremor, rigidity and disturbances of movement, with few or no cognitive changes in the majority of cases. The neuropathological changes are very distinct, with degeneration of dopaminergic neurons in the substantia nigra (pars compacta), leading to a marked decrease in the catecholamine dopamine within this region and those connected to it, being the most obvious on examination of cell loss in a Parkinson’s brain. The presence of intraneurial inclusions known as Lewy bodies present in the substantia nigra and the locus coeruleus are highly characteristic of this disease (Forno, 1986). It is not yet clear why these pathological changes occur.

Several lines of evidence point to the involvement of ROS (reviewed by Adams and Odunze, 1991; Jenner et al, 1992; Olanow, 1992): increased levels of iron, some of which is in the reactive form, are present in the substantia nigra of some PD patients (Dexter et al, 1992; Riederer et al, 1992), lipid peroxidation is increased in the parkinsonian brain (Dexter et al, 1989), and levels of glutathione, involved in the detoxification of hydrogen peroxide, are decreased, whilst an increase in SOD-like activity was observed in parkinsonian brains (Marttila et al, 1988). The latter may occur in response to the production of the superoxide radical. It is not yet clear whether it is the cytosolic Cu,Zn SOD or the Mn form, found in the mitochondria, which are increased. Levels of other protective antioxidants and enzymic systems appear to be mostly unchanged.

Iron is needed in dopaminergic neurons because one of the enzymes involved in dopamine synthesis, tyrosine hydroxylase, is an iron-containing enzyme. Increased iron in the substantia nigra may be due to a compensatory mechanism reflecting the low dopamine levels in PD, producing an increase in tyrosine hydroxylase activity, and therefore increased dopamine turnover, in the remaining neurons. The presence of excess reactive iron promotes the formation of ROS in the presence of hydrogen peroxide and a reducing agent. Hydrogen peroxide is continually produced during the catabolism of dopamine in the mitochondria, either by autooxidation or via the enzyme monoamine oxidase. Cellular damage is usually prevented by the action of the enzymes catalase or
glutathione. The brain however contains very low levels of catalase, whilst glutathione appears to be concentrated in glial cells, rather than neurons. Cell death will inevitably occur, and this in turn may exacerbate the damage produced; compensatory mechanisms within the remaining neurons are likely to mean turnover of dopamine is increased, with a resultant increase in $\text{H}_2\text{O}_2$ (Coyle and Puttfarcken, 1993; Olanow, 1993). The dopaminergic system would therefore seem to be particularly sensitive to oxidative stress, and there is a large amount of research investigating the role ROS play in Parkinson's disease.

1.2.2.2 ROS-INDUCED DNA DAMAGE MAY BE IMPORTANT IN NEURODEGENERATION

It is difficult to assess whether ROS have a causal role in the development of neurodegenerative diseases, or whether they are part of a final common pathway of cell damage triggered by other factors. Whatever part they play, an understanding of the mechanisms involved is important for the future understanding of these various disease states. In order to do this the effects of ROS on different cell macromolecules need to be investigated at a cellular level. DNA is known to be a major target for ROS within the cell (Imlay and Linn, 1988; Halliwell and Aruoma, 1991), and increased levels of oxidatively-modified DNA have been observed in brain tissue from patients with common neurodegenerative disorders (Sanchez-Ramos et al, 1994; Alam et al, 1997; Lyras et al, 1997). This evidence suggests a role for DNA damage in diseases such as PD, AD and MND. For this reason many researchers are attempting to understand the mechanisms underlying ROS-mediated DNA damage in the brain. In order to fully appreciate these mechanisms, a description of the different species involved is needed, along with an understanding of how they are produced and the methods cells use to restrict and control their levels.
1.3 REACTIVE OXYGEN SPECIES

Oxygen first appeared in the Earth’s atmosphere in significant amounts about 2x10⁹ years ago. This was probably due to the evolution of photosynthetic organisms, in which oxygen was formed as a by-product of their energy cycle. An ozone layer almost certainly developed in the upper atmosphere around the same time, leading to the absorption of damaging ultraviolet rays. These two factors were responsible for the development of aerobic organisms which were able to utilise molecular oxygen as the terminal oxidant in respiration (Fridovich, 1978; Halliwell and Gutteridge, 1989a). The success of this evolutionary process is demonstrated by the complexity and variety of aerobes today. This is due to the large energetic advantages gained over previous anaerobic pathways, along with the development of multiple defence mechanisms needed to cope with the toxicity of oxygen. As mentioned previously, the detrimental effects of oxygen are now known to be caused by the formation of reactive oxygen species (ROS), which include both free radical and non-radical molecules. The most important of these are singlet oxygen, superoxide, hydrogen peroxide, the hydroxyl radical and lipid peroxides.

1.3.1 OXYGEN IS A FREE RADICAL

Oxygen in its ground state has two unpaired electrons, each located in a different π* antibonding orbital. These electrons have parallel spins (i.e. they have the same spin quantum number), and it is this which greatly restricts the reactivity of oxygen with non-radical species. For oxygen to accept electrons from another atom/molecule both must be of parallel spin to move into the unoccupied orbital spaces, according to Pauli’s principle. This is highly unlikely; therefore oxygen reacts readily with radicals, accepting electrons one at a time, and very slowly with most non-radicals. Aerobic organisms have taken advantage of this fact and utilise oxygen as a terminal oxidant or electron acceptor in the respiratory chain. Energetic advantages are gained over fermentation and other respiratory pathways, however a consequence of this is the formation of ROS as by-products. One electron reduction of O₂ involves the production of biologically important species:

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

(1)
Levels of these molecules must be strictly controlled because they have the ability to interact with cellular macromolecules in a detrimental way.

1.3.3 SINGLET OXYGEN ($O_2^\cdot$)

This is formed when an input of energy excites one of the unpaired electrons, allowing it to move into the same orbital as the other and thus alleviating the spin restriction. Two singlet states of oxygen exist, the $^1\Delta gO_2$ state and the $^1\Sigma g^+O_2$ state, both of which are more reactive than oxygen. The latter however is very energetic, decaying quickly to the former. For this reason only the $^1\Delta gO_2$ moiety is thought of as biologically significant, and references to ‘singlet oxygen’ are commonly used to refer to this state alone. Singlet oxygen is not a free radical in itself; there are no unpaired electrons. It does have a greater oxidising ability than oxygen though, and may be formed during some radical reactions and trigger the formation of others. $O_2^\cdot$ is able to interact with several biological molecules including proteins, $\alpha$-tocopherol and DNA (Halliwell and Gutteridge, 1989a), and may be produced in excess in some disease states such as the porphyrias, caused by defects in the metabolism of haem and other porphyrins (Halliwell and Gutteridge, 1989b). It is commonly formed in vivo during photosensitization reactions, for instance via chloroplasts, riboflavin and retinal when illuminated with the appropriate wavelength of light (Halliwell and Gutteridge, 1984).

1.3.3 SUPEROXIDE RADICAL ($O_2^-$)

The superoxide radical is formed when a single electron is accepted by ground state $O_2$, entering one of the $\pi^*$ antibonding orbitals. $O_2^-$ therefore has one unpaired electron. This molecule is formed in almost all aerobic cells (Fridovich, 1975, 1978), important sources being the ‘respiratory burst’ of phagocytic cells (Babior, 1978a,b) and as a side-product of oxidative phosphorylation in the mitochondria. It is also produced enzymatically via cytoplasmic enzymes such as cytochrome P450 and xanthine oxidase, and directly via autooxidation reactions in the mitochondria (Coyle and Puttfarcken, 1993). $O_2^-$ is a relatively mild reactant compared to other radical species. However, in solution it exists in equilibrium with the hydroperoxyl radical ($HO_2^-$):

$$O_2^- + H^+ \rightleftharpoons HO_2^-$$

At physiological pH around 99% is $O_2^-$, but as the pH falls $HO_2^-$ becomes a more predominant species. $HO_2^-$ is much more reactive than $O_2^-$, and more lipid soluble.
Therefore under acidic conditions the superoxide radical becomes very reactive, and in the non-polar environment of the membrane formation of HO$_2^-$ is favoured. Superoxide undergoes a spontaneous dismutation reaction, which may be summarised as:

$$2O_2^– + 2H^+ \rightarrow H_2O_2 + O_2$$ (3)

It should be noted that the dismutation rate of HO$_2^-$ is faster than that of O$_2^–$. Most cells, however, possess an enzyme, superoxide dismutase (SOD), which causes this reaction to proceed at a much faster rate. The discovery of this specific enzyme led to the ‘superoxide theory of oxygen toxicity’ (Halliwell and Gutteridge, 1989a), and suggestions that this species plays an important role in ROS damage to cells.

1.3.4 HYDROGEN PEROXIDE (H$_2$O$_2$)

Addition of two electrons to molecular O$_2$ results in the formation of the peroxide ion, O$_2^{2–}$. The oxygen-oxygen bond is relatively weak in this non-radical species and at physiological pH it is immediately protonated, producing H$_2$O$_2$. Another major source of H$_2$O$_2$ is the dismutation of the superoxide radical via SOD, as mentioned previously. The rate of this reaction is quicker at acidic pH values. H$_2$O$_2$ itself is not a radical, and is a relatively stable oxidant. However, it does play an important role in the formation of the highly reactive 'OH *in vivo*.

1.3.5 HYDROXYL RADICAL (OH$^–$)

The hydroxyl radical is one of the most reactive ROS formed in cells, with a half-life of ~10$^{-9}$s (Pryor, 1986). This means it will interact immediately with the nearest biological molecule. OH$^–$ is formed in several ways. It may be produced via homolytic fission of the relatively weak O-O bond in H$_2$O$_2$:

$$H_2O_2 \xrightarrow{\text{energy}} 2^{'}OH$$ (4)

The required energy may be in the form of heat or ionizing radiation. These species are also formed when the O-H bonds of water are broken. Hydroxyl radicals are produced during aerobic respiration too, as previously shown. Another potentially important source was described by Fenton (Fenton, 1894), then later elaborated on by Haber and Weiss (Haber and Weiss, 1934), during investigations surrounding the role played by
transition metals in reduction of $\text{H}_2\text{O}_2$. 'OH is a major product of this interaction, which is often summarised in the following way:

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

(O$_2^-$ reduces the iron salt)

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

(FENTON REACTION)

Net reaction:

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

(HABER-WEISS REACTION - Metal catalysed)

A metal catalyst is essential for reaction (7) to occur; without which the rate constant is very close to zero (Halliwell and Gutteridge, 1984b). This catalyst may be iron, and much research has been carried out on the role it appears to play in free radical production in vivo (Halliwell and Gutteridge, 1984b; Larramendy et al., 1987), however, other metal ions such as copper and selenium might act as catalysts in some instances. The interaction of endogenously formed nitric oxide (NO'), an important and widespread cytokine, with O$_2^-$ (reviewed by Olanow, 1993) may also generate hydroxyl radicals. In neurons, for example, the radicals involved may be produced via the action of excitatory amino acids such as glutamate, as mentioned previously (Figure 1.2). However, it is not yet clear how relevant this is in causing tissue injury (Borg, 1993). It is important to note that any damage induced by this radical will only be significant if it is produced in the immediate vicinity of a sensitive biological macromolecule. These site-specific reactions, as they are known, are often characterised by the sites of the metal catalysts involved.

1.3.6 METALS AND FREE RADICAL PRODUCTION

The fact that metal ions appear to be important in the production of the hydroxyl radical has focused research on iron and copper as potential catalysts of the Haber-Weiss reaction in vivo (reviewed by Halliwell and Gutteridge, 1990). These transition metals are often found at the active site of oxidases and oxygenases because they can donate and accept single electrons easily, allowing oxygen to overcome its spin restriction (Hill, 1981). Their availability for the production of \('\text{OH}\) has been investigated thoroughly however. Particular interest has been paid to the role of iron in neurodegenerative
disorders (Gutteridge, 1992). This is because high levels of this metal have been observed in regions of the brain, specifically the globus pallidus and substantia nigra (Dexter et al., 1992; Riederer et al., 1992). These are the regions most affected during Parkinson's disease, suggesting that iron-mediated radical production may have a role in this disorder.

Iron enters the body in the oxidised Fe$^{3+}$ form, and must be solubilised, then reduced to the Fe$^{2+}$ state in order to enter the body through the gut wall. HCl in the stomach achieves solubilisation, whilst dietary Vitamin C reduces some iron to the Fe$^{2+}$ state, thus aiding absorption. The metal then enters the circulation bound to the carrier protein transferrin, a glycoprotein with 2 sites able to bind Fe$^{3+}$ very tightly with the help of the bicarbonate anion (HCO$_3^-$). The levels of free iron in the body are strictly controlled. About two thirds of the body's iron-burden circulates in the plasma as a constituent of haemoglobin, with smaller amounts being bound to myoglobin, various enzymes and transferrin. Under normal circumstances transferrin is only 30% loaded with iron, therefore there are virtually no free iron salts present in the plasma. The rest is stored intracellularly, mainly in complexes with the storage proteins ferritin and haemosiderin, but also as simple complexes with ATP, GTP and citrates. Again, ferritin is very rarely saturated with iron in vivo, therefore limiting a potentially dangerous accumulation of iron inside the cell. It is the current view that iron bound to transferrin is taken up by cells via pinocytosis. The contents of the resulting vacuole are then acidified and free iron released to form the complexes mentioned above. This small pool of non-protein bound iron is available for the synthesis of iron proteins. The iron-free transferrin is ejected from the cell.

Virtually all iron is thought to be in the Fe$^{3+}$ form, which is unable to catalyse the Haber-Weiss reaction described earlier. Iron may be removed from ferritin and other chelates within the cell as Fe$^{2+}$ if there is a decrease in pH and/or via the action of reducing agents such as the superoxide radical. Thus a local increase of O$_2^-$ and/or increase in metabolic acidosis could result in significant levels of free Fe$^{2+}$ for site-specific redox reactions. The small pool of non-protein bound iron may also be responsible for radical production in "Fenton-like" reactions, where 'OH is produced via action of iron chelated to ligands such as DNA:

$$\text{Fe}^{2+}\cdot\text{ligand} + \text{H}_2\text{O}_2 + \text{H}^+ \longrightarrow \text{Fe}^{3+} + \text{HO}^+ + \text{H}_2\text{O} \quad (8)$$
It has been shown that available iron is present at \( \mu M \) levels in the human synovial fluid (taken from rheumatoid patients) and cerebrospinal fluid (Gutteridge et al., 1981; Gutteridge, 1982), as measured by the bleomycin assay. Thus it appears that iron is available in these fluids at sub\( \mu M \) - \( \mu M \) concentrations to interact with \( H_2O_2 \).

\[
\begin{align*}
    L-Fe^{3+} + O_2^- & \rightarrow L-Fe^{2+} + O_2 \quad (9) \\
    L-Fe^{2+} + H_2O_2 & \rightarrow L-Fe^{3+} + \cdot OH + OH^- \quad (10) \\
    & \rightarrow L-Fe^{IV}=O^{2+} + H_2O \quad (11) \\
    & \rightarrow L-Fe^{2+/3+}-H_2O_2 \text{ complexes} \quad (12)
\end{align*}
\]

Figure 1.3. Possible steps in the production of ferryl iron (Step 11) and iron-peroxy compounds (Step 12). Iron is bound to cellular constituents, represented by L-.

It should be mentioned that some researchers have stated there are other iron species involved in oxidative damage to cells (Bielski, 1992; Halliwell and Gutteridge, 1984; Borg, 1993); ferryl iron (\( Fe=O^{2+} \)) and perhaps an iron-peroxy complex, as yet unidentified, are suggested. Probable pathways of formation have been suggested (Figure 1.3). It is still not clear what role, if any, these species have in biological damage, and at the moment it is still thought that the effects of Fenton-like reactions \textit{in vivo} are almost certainly caused by production of excess \( \cdot OH \). More work is therefore needed to find the exact nature of Fenton-reactive iron \textit{in vivo}.

Copper would appear to be a less likely candidate for involvement in the production of hydroxyl radicals at first glance. In plasma this metal is tightly bound to the protein caeruloplasmin or albumin and amino acids such as histidine, and is not as readily released from these complexes as iron. It is possible, however, that in the presence of a reducing agent such as superoxide copper ions may be able to generate \( OH^- \) which can interact with the binding molecule. In this way, site-specific damage to structures such as DNA may be induced (Borg, 1993; Halliwell and Aruoma, 1991).

\textbf{1.3.7 SOURCES OF ROS IN THE CELL}

Cells constantly produce partially reduced forms of oxygen during many different processes. Sometimes this is deliberate, but at other times the species may be incidental...
to the reaction occurring. ROS may be generated as a response to internal or external events in a cell’s environment.

**ENDOGENOUS SOURCES.**

ROS are formed via several biochemical reactions occurring within the cell (Figure 1.4). Important sources are produced as follows:

- During normal aerobic respiration $O_2$ undergoes a 4 electron reduction to $H_2O$, resulting in the formation of ATP which is vital for metabolic reactions. This occurs in sequential steps within the mitochondria, and is catalysed very efficiently by respiratory enzymes such as cytochrome oxidase, found attached to the mitochondrial inner membrane:

$$
e^- \rightarrow 2H^+ \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow 2OH^- \rightarrow H_2O$$

Inevitably some ROS are released as by-products of respiration/metabolism. In rat liver cells, approximately $10^{12}$ $O_2$ molecules are processed each day in this way, with around a 2% leakage of activated species, equivalent to approximately $2 \times 10^{10}$ $O_2^-$ and $H_2O_2$ molecules per day (Chance *et al*, 1979). It is likely that these levels are similar in human cells.

- Microbial killing by phagocytic cells involves an oxidative burst, in which nitric oxide (NO), $O_2^-$, $H_2O_2$ and $OCl^-$ are produced.

- Peroxisomes degrade fatty acids and other molecules, producing $H_2O_2$ in the process. Under certain circumstances some may escape these organelles and enter other compartments within the cell (Kasai *et al*, 1989).

- Cytochrome P450 enzymes within cells produce ROS during detoxifying reactions. Other oxygen requiring enzymes are also sources of ROS.

**EXOGENOUS SOURCES**

There are many ways in which an organism’s environment may potentially result in an increase in ROS. One major source is smoking, during which high levels of nitrogen oxides ($NO_x$) are produced. Another important one is radiation, most notably certain wavelengths present in sunlight. Other sources include various chemicals, certain therapeutic drugs, pesticides and pollutants.
Figure 1.4. Mechanism by which reactive oxygen species are continuously formed within a living cell. (Taken from Machlin and Bendich, 1987)
1.4 BIOLOGICAL EFFECTS OF ROS

There are many potential targets for ROS in cells, the most important of which are lipid membranes, the sulphydryl bonds of proteins and the nucleotides of DNA. Damage to any of these may be involved in the development of different pathological conditions, including neurodegenerative diseases. The mechanisms involved in these processes are therefore of great interest, because an understanding of these may lead to the causes of these disorders, and potential new therapies.

1.4.1 DAMAGE TO PROTEINS

The action of ROS on proteins may effect a cell in several ways; essential enzymes may be inactivated, membrane transport proteins be disrupted and the integrity and metabolism of structural proteins may be altered. Further metabolic stress is placed upon the cell as energy is utilised during the disposal of these damaged macromolecules. Sulphydryl bonds are particularly sensitive to ROS attack, leaving sulphhydryl enzymes especially vulnerable to damage as proteins become cross-linked by the formation of disulphide bonds. Work carried out on cellular proteins in solution (Wolff and Dean, 1986) and mitochondrial proteins (Dean and Pollak, 1985) have shown that they are more susceptible to enzymatic proteolysis after ROS attack. This suggests that conformational changes caused by ROS attack may enhance their susceptibility to the action of certain proteinases. Kainic acid and MPP⁺, both inducers of oxidative stress, have also been shown to increase intracellular protein degradation (Thomas and Anderton, 1991), and it was suggested that this altered protein metabolism may be useful in assessing the sublethal toxicity of certain compounds. Finally, the formation of peroxides in some amino acids/proteins exposed to ROS have been detected (Gebicki and Gebicki, 1993), leading to the suggestion that damaged proteins themselves could become secondary toxic molecules which cause further disruption within the cell by depleting the antioxidant defence available. Another important field of study involves phosphorylated proteins, as tyrosine residues critical for their correct activity are highly susceptible to attack by ROS. Damage to this type of protein may cause serious disruption of the cytoskeleton and signalling pathways within the cell.

1.4.2 DAMAGE TO LIPID MEMBRANES

Lipid membranes of the cell are a major potential target of ROS, and damage to
these macromolecules will clearly have deleterious consequences. The whole membrane need not be affected; localised damage over a small area can have severe consequences by increasing membrane fluidity or altering the alignment of cell receptors within the membrane. Radical species initiate chain reactions, which can be divided into 3 stages; initiation, chain propagation and termination (reviewed by Halliwell and Gutteridge, 1984; Braughler and Hall, 1989; Borg, 1993). Iron may also be involved in secondary reactions.

**INITIATION**

Initiation of lipid peroxidation occurs when membranes are attacked by any species reactive enough to abstract a hydrogen atom. This type of abstraction reaction is aided by the presence of unsaturated C-C bonds, and the greater the number of unsaturated bonds in a membrane, the higher the susceptibility to peroxidation by ROS. Not a lot is known about the type of molecules involved in initiation reactions. If the 'OH radical is used as an example of the reactive species involved, then the reaction may be represented as follows:

\[
L-H + \cdot OH \rightarrow L' + H_2O \tag{13}
\]

Singlet oxygen has been suggested as another possible initiator (Halliwell and Gutteridge, 1984), as has UV irradiation (Borg, 1993). Although superoxide is not reactive enough itself to abstract a hydrogen atom, it is thought that the HO₂⁻ molecule favoured within the polar environment of the membrane is able to attack fatty acids directly (Bielski et al., 1983). Whichever species is involved, a chain reaction has been started. This may now be propagated.

**CHAIN PROPAGATION**

After initiation the fatty acids involved have been left with an unpaired electron, as the hydrogen removed has only one electron. The carbon radical formed (L') tends to be stabilised by molecular rearrangement, producing a conjugated diene which may then react with oxygen to produce a hydroperoxy radical (L-O₂⁻) as follows:

\[
L' + O_2 \rightarrow L-O_2^- \tag{14}
\]

This molecule is able to abstract hydrogen from other lipid molecules. The abstracted hydrogen combines with the hydroperoxy radical to produce a lipid hydroperoxide (L-O₂H):
\[ \text{L-O}_2^+ + \text{H}^+ \rightarrow \text{L-O}_2\text{H} \]  (15)

Reaction (15) is very slow in chemical terms (\( \geq 10^s \)) (Borg, 1993), and the lipids themselves are able to move through the membrane in the plane of the bilayer. It is therefore possible for damage to be found at sites distant from the initiation site. Lipid radicals continue to interact with other lipids in the membrane, and in this way a whole range of reactions are started by the initiation step.

Metal ions such as iron are also intimately involved in lipid peroxidation. Their major role is catalysing the decomposition of the hydroperoxides formed during propagation reactions (Halliwell and Gutteridge, 1984; Braughler and Hall, 1989; Borg, 1993). Iron is available for this in the form of simple complexes with cellular constituents such as ADP, or via ferritin, as discussed earlier. It has been suggested that there may be a high enough background level of Lipid-O_2H, formed by direct action of singlet oxygen or some species like it, for these iron-dependent reactions to occur in the absence of other radical initiation steps (Halliwell and Gutteridge, 1984). Alkoxyl (LO.) and peroxyl (LO_2^+) radicals are produced as follows:

\[ \text{LO}_2\text{H} + \text{Fe}^{2+} \rightarrow \text{LO}' + \text{OH}' + \text{Fe}^{3+} \]  (16)

\[ \text{LO}_2\text{H} + \text{Fe}^{3+} \rightarrow \text{LO}_2' + \text{Fe}^{2+} \]  (17)

Both these products may take place in further chain reactions, although alkoxyl radicals are more reactive than peroxyl ones. These species may also be important in mediating damage to other cellular macromolecules such as DNA (Yang and Schaich, 1996).

**CHAIN TERMINATION**

Reactions between radical and non-radical species could continue until either lipid substrate or available O_2 supplies are exhausted. Chain termination reactions, in which 2 radical species interact to form a non-radical molecule, stop the peroxidation process. These include:

\[ 2 \text{L}^\cdot \rightarrow \text{L-L} \]  (18)

\[ 2 \text{L-O}^\cdot \rightarrow \text{LOOL} \]  (19)

\[ 2 \text{LO}_2^\cdot \rightarrow \text{LOOL} + \text{O}_2 \]  (20)

The chain-breaking antioxidant molecule \( \alpha \)-tocopherol is also important in these termination reactions. This lipid soluble molecule is found in membranes, and is able to
interact with radical species to form a relatively stable chromanoxyl radical, which is more likely to become involved in termination reactions rather than propagation because of its low reactivity.

1.4.3 DAMAGE TO DNA

DNA is a major target for ROS within the cell (Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Floyd and Carney, 1992; Ames et al., 1995). It has been shown that DNA exposed to ROS becomes more antigenic (Alam et al., 1993; Blount et al., 1989, 1990), a fact which may partly explain the presence of autoantibodies to native DNA in the serum of patients suffering from systemic lupus erythematosis (SLE), a chronic inflammatory disorder in which ROS levels are constantly elevated. This suggests that DNA damage produced in cells undergoing oxidative stress is biologically relevant. A wide range of products are formed, including single and double strand breaks, covalent crosslinks with proteins such as histones and sugar and base modification (Imlay and Linn, 1988). The presence of these abnormalities may interrupt the normal flow of genetic information and could therefore be potentially mutagenic. Highly efficient repair systems for various lesions do exist, and these usually have very broad substrate specificity (Demple and Levin, 1991). Again this shows the biological importance of the damage produced. It may be hypothesised that DNA damage is involved in cellular dysfunction and death in various neurodegenerative disorders, and in fact increased levels of DNA lesions have been observed in brain sections from patients suffering from these disorders (Sanchez-Ramos et al., 1994; Alam et al., 1997; Lyras et al., 1997). The mechanisms involved in this process are therefore of great interest.

1.4.3.1 MECHANISMS OF DAMAGE

Not all ROS appear to be directly involved in the production of DNA lesions. Hydrogen peroxide and superoxide, for instance, are not thought to attack DNA directly (Aruoma et al., 1989a). These species are strongly implicated in the mechanisms involved, however; damage is often inhibited by the addition of catalase or SOD when cells are incubated with H$_2$O$_2$ or superoxide-producing agents, if the molecule enters the cells (Halliwell and Aruoma, 1991).

It has been postulated that the hydroxyl radical, produced via the Fenton-type reactions described previously, is a major inducer of this damage (Mello Filho et al., 1984; Dizdaroglu et al., 1991; Meneghini, 1997). Due to its short life-span, this species
must be produced in close proximity to DNA in order to attack the molecule. Metal ions
are known to be closely associated with DNA (Bryan et al., 1981; Henle and Linn, 1997),
but it is also possible that metal ions may be released from intracellular stores as a result
of oxidative stress, then bind to DNA (Halliwell, 1987). These metal ions may act as
catalytic sites, with superoxide acting as a reducing agent. Relatively stable hydrogen
peroxide produced in the cytoplasm or extracellularly, may diffuse in through membranes
to reach the nuclear or mitochondrial DNA for the reaction to proceed. Many studies
have shown that H₂O₂, in the presence of either ferric or cupric ions, is capable of
inducing a pattern of DNA base damage suggestive of the action of OH radicals (Mello
Filho et al., 1984; Aruoma et al., 1989b; Aruoma et al., 1991; Dizdaroglu et al., 1991;
Drouin et al., 1996).

Although most research has focused on the involvement of hydroxyl radicals in
DNA damage induced by oxidative stress, it should be recognised that other species may
be involved. These include singlet oxygen and hydroperoxides, the products of lipid
peroxidation (Sies, 1993; Yang and Schaich, 1996). These could be just as important as
OH, because damage is produced in the absence of any metal catalyst, but are less
studied.

1.4.3.2 BASE DAMAGE

Research has focused on damaged base products induced by ROS partly because of
their mutagenic potential, but also because they may be useful biomarkers of oxidative
damage. Different lesions are produced by different inducers of oxidative stress. Singlet
oxygen, produced primarily when photosynthesisers such as flavins and NADH are
illuminated in the presence of oxygen, attacks guanine bases in preference to any other,
whilst the hydroxyl radical produces more extensive damage to a wide range of bases
(Epe et al., 1993a,b; Halliwell and Aruoma, 1991). Major products formed during
hydroxyl attack on DNA include 8-oxoguanine and 8-oxoadenine (Halliwell and
Aruoma, 1991). Methods able to quantify levels of the damaged base 8-oxoguanine
(8-oxoG), or the corresponding nucleoside 8-oxodeoxyguanosine (8-oxodG) (base +
deoxyribose sugar group), have been developed in recent years. This has led to the use
of this lesion as a biomarker of oxidative damage (Halliwell and Dizdaroglu, 1992;
Shigenaga et al., 1994).
14.3.3 8-OXODEOXYGUANOSINE (8-OXOdG)

The 8-oxodG base lesion was discovered by Kasai et al in 1984, during studies involving a model for cooked foods (reviewed by Kasai and Nishumura, 1991). It appears to be responsible for loss of base pairing specificity, misreading of adjacent pyrimidine bases or incorrect base insertion opposite the oxidative lesion. The major substitution observed is guanine → thymine, and this was shown to have mutagenic potential (Cheng et al, 1992). The existence of specific repair pathways within cells for this lesion is further confirmation of its biological importance (Bessho et al, 1993; Tchou and Grollman, 1993). This information, together with the development of a sensitive detection method for 8-oxodG using HPLC with electrochemical detection (HPLC-EC) (Floyd et al, 1986), has increased the popularity of this base as a biomarker for oxidative stress, although the reliability of the results obtained in various results has been questioned (Collins et al, 1996).

There have been many studies involving the measurement of 8-oxoG/8-oxodG in DNA and cells using high performance liquid chromatography (HPLC) with electrochemical detection (Halliwell and Dizdaroglu, 1992; Kaur and Halliwell, 1996; Kennedy et al, 1997). More recently gas chromatography-mass spectrometry (GC-MS) methods have been developed to give a specific profile of hydroxyl radical damage (Hamberg and Zhang, 1995; Teixeira et al, 1995). Although GC-MS is more sensitive method of detection than HPLC the equipment needed is very expensive, and so the latter is still favoured. A direct comparison of the results obtained using these different methodologies provides very different values for background levels of oxidation however (Ravanat et al, 1995; Collins et al, 1996), and the usefulness of 8-oxodG as a marker of oxidative DNA damage has been questioned because of this (Collins et al, 1996; Te Koppele et al, 1996). This is thought to be due to the artefactual production of 8-oxodG during steps taken to isolate and purify DNA prior to analysis (Ravanat et al, 1995; Collins et al, 1996; Douki et al, 1996). Research into better methods of isolation and purification has therefore been carried out with some success (Douki et al, Herbert et al, 1996, Nakajima et al, 1996), and more reliable methods will hopefully follow.

HPLC and GC-MS are not the only quantitative methods available to measure oxidatively-modified bases. Capillary electrophoresis (CE) methods are in development, but lack concentration sensitivity to date (Poon et al, 1995). Other current detection methods include $^{32}$P post-labelling techniques (Carmichael et al, 1992; Bykov et al,
1995; Devanaboyina and Gupta, 1996) and antibody assays (Musarrat and Wani, 1994; Yin et al, 1995). These still require the isolation of DNA or chromatin from cells, however, with the associated problems. Direct in situ demonstration of oxidative damage to DNA would be advantageous, since this would decrease the number of steps involved, and therefore the experimental error and artefacts present with other techniques. This type of study has been described by Yarborough et al (1996) using a monoclonal antiserum said to recognise 8-oxodG with avidin conjugated to horseradish peroxidase in the detection system, but does not appear to have been continued after this initial stage. There is no recent literature describing direct detection of the oxidised base lesion 8-oxodG in either cells or pathological tissue.
1.5 PROTECTIVE MECHANISMS WITHIN THE CELL

As ROS are constantly being produced the cell has developed mechanisms to restrict and control their levels. Compartmentation of ROS-producing systems separates radicals from the rest of the cell (in organelles such as mitochondria, lysosomes and peroxisomes). In addition, specialist enzyme systems and small molecules with antioxidant properties have also evolved (Figure 1.5). A delicate balance exists between free radical generation and the protective systems available to deal with them. When the latter are overwhelmed by a sudden increase in ROS levels in the cell, oxidative stress occurs with subsequent tissue damage.

1.5.1 ENZYMIC DEFENCE SYSTEMS

Ubiquitous antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) which inactivate ROS as they are produced. These are well studied, and their importance emphasised as lower enzyme activity is observed in some tumour cells. For instance, a higher incidence of abnormal DNA lesions has been linked with low GPx, SOD and CAT activities in cancerous lung tissue (Jaruga et al., 1994). It remains to be seen if the low levels of antioxidant enzymes seen have a causal role in carcinogenesis or are merely a result of the disease. Enzyme systems have also developed to repair lesions caused by oxygen radicals in DNA, restricting possible mutagenic and carcinogenic effects (Demple and Levin, 1991).

1.5.2 ANTIOXIDANT MOLECULES

In addition to these enzyme systems antioxidant molecules are also present. Many of these are essential nutrients i.e. cannot be synthesised by the body and so must be absorbed from the diet, or have essential nutrients as a component of their molecule.

1.5.2.1 VITAMIN E

α-tocopherol, the most effective isomer of vitamin E, is a potent chain-breaking antioxidant situated within lipid membranes of the cell. It scavenges ROS at these sites, terminating free radical chain reactions and therefore suppressing lipid peroxidation reactions. In vitro studies have shown this molecule to inhibit oxidation of phosphatidylcholine liposomes by free radical chain mechanisms (Niki, 1987b). Once stores had been depleted oxidation took place at the same rate as that without the antioxidant, the length of inhibition observed being directly proportional to its
Figure 1.5 Antioxidant defences developed by the cell to protect against ROS, which are continuously produced. (Taken from Machlin and Bendich, 1987)
concentration. It also appeared to be very mobile within the lipid bilayer (Niki, 1987a). This is an important property because the ratio of vitamin E to lipids in biological membranes is very low (usually between 1-100). Other important antioxidant molecules include lipid-soluble β-carotene and water soluble vitamin C. There may be interplay between all these molecules as studies have shown synergistic action between lipid soluble vitamin E and the water soluble radical scavenger vitamin C (Niki, 1987a). This is thought to be due to the fact that the tocopheroxyl radical formed on reaction with a free radical can be reduced by vitamin C back to a non-radical vitamin E molecule. Vitamin E levels will only start to fall when all the vitamin C has been utilised. Vitamin E may itself preserve β-carotene by protecting conjugated double bonds present within the latter. However, these facts still have to be confirmed in vivo.

Several studies have shown that antioxidants such as α-tocopherol may be able to protect cells from oxidative stress in certain pathological conditions. Diets supplemented with vitamins E, C and A improved the immune responses of people exposed to high levels of free radicals in the environment and also increased the activation of cells involved in tumour immunity in the elderly (Bendich, 1993). In addition to this, a lower risk of colon cancer has been associated with high levels of these vitamins in the diet, whilst people with low circulating levels of vitamin E and β-carotene have a significantly greater risk of lung cancer (note that these molecules are found at lower concentrations in smokers when compared to non-smokers) (Machlin and Bendich, 1987). This agrees with evidence that the levels of circulating antioxidant nutrients depend on the level of intake in the diet; people with diets that are low in these molecules may have increased health problems associated with free radical damage, as their body’s defences are lowered. A vitamin E deficient diet also affects the brain, altering levels of the neurotransmitter glutamate in the substantia nigra (Steffen et al, 1994). This suggests that antioxidant levels may have a role in neurodegenerative disease. The evidence all points to a protective action of α-tocopherol in pathological processes. The ability of this compound to block the effects of free radical stress was investigated in this study.

1.5.2.2 Desferrioxamine

Since the recognition of the role iron plays in free radical formation there has been an increased interest in the use of iron chelators as protective antioxidant molecules. One of the first used was desferrioxamine or desferal. This molecule is produced by Streptomyces pilosus and consists of a chain of three hydroxamic acids each ending in a
free amino group. These arms are able to wrap tightly around the Fe$^{3+}$ nucleus with high affinity. It is known to enter several tissues with subsequent catabolism. Desferrioxamine has been used in the treatment of patients with thalassaemia since the early 1960's. These people must have regular blood-transfusions to survive and as a consequence become overloaded with iron (each unit of blood contains about 0.2 g of iron). This build-up has many pathological side-effects in which the action of free radicals are strongly implicated; lipid peroxidation and the formation of OH$^-$ via the Haber-Weiss reaction are the most important lines of evidence here. Administration of desferrioxamine binds excess iron, inhibiting the formation of OH$^-$. The stable ferrioxamine complex formed is unable to penetrate cells due to a change in configuration of the molecule, and is therefore rapidly excreted, mainly in the urine, but also through the bile. Thus the iron is removed from the body. Studies are currently underway using this compound to try and protect against post-ischaemic reperfusion injury (Hershko, 1992), whilst topical application of desferrioxamine to skin sections has been seen to reduce UV induced damage (Jurkiewicz and Buettner, 1994). The ability of this molecule to decrease free radical damage was also looked at during this investigation.
1.6 AIMS

**HYPOTHESIS**: Nuclear DNA damage is involved in cellular dysfunction and cell death initiated by oxidative stress.

Changes within the brain are very difficult to study directly, therefore the establishment of biologically relevant *in vitro* model systems to investigate these neurological processes is very desirable. This thesis aimed to assess damage in neurons exposed to oxidative stress in an *in vitro* model system, and also pathological tissue from patients with neurodegenerative disease. IMR32 neuroblastoma cells were chosen as a model system for *in vivo* events occurring in the brain after ROS damage. These cells have been shown to express mature neuronal markers such as neurotransmitter synthesis and storage and ion channels (Gotti *et al.*, 1987), and also mature cytoskeletal markers when differentiated with 5′bromodeoxyuridine (BrdU) (Mortimore, 1996). They have also been used successfully in previous studies of ROS-induced damage (Thomas and Anderton, 1991; Johnston, 1994).

Several parameters have been used previously to assess ROS-induced damage to cell macromolecules, including levels of lipid peroxidation, damage to proteins and DNA damage. DNA base damage was chosen as a marker of oxidative damage in this study. Much work has gone into identifying the altered base products produced on exposure to ROS. Specifically the lesion 8-oxoguanosine, known to be a major mutagenic lesion, is considered to be an acceptable biomarker of ROS damage. Most of the methods used to detect 8-oxodG involve isolation of DNA from cells followed by separation of bases by chromatography before detection. Sensitive assays involve radioisotopes. A method able to detect such lesions directly in both *in vitro* cultures and pathological tissue would be of great benefit to this field of study. This project was instrumental in the development of a novel affinity technique (patented) able to recognise the damaged base product 8-oxodG, using avidin conjugated to both colorimetric and fluorometric detection molecules. The ability of this assay to successfully recognise damaged DNA in cultured cells exposed to a variety of ROS-generating systems, and pathological tissue from patients with neurodegenerative diseases in which ROS have been strongly implicated, was of great interest.

To complement *in vitro* studies some *ex vivo* work was carried out. Diagnosis of certain diseases can be very difficult, as the range of symptoms presented by the patient...
may be representative of several states. The problems are heightened in diseases involving the brain and CNS. For instance Alzheimer's may only be confirmed after post-mortem by the presence of neurofibrillary tangles and senile plaques. In these cases investigators are looking for further biomarkers specific to certain disease states. Where ROS are almost certainly involved at some point it may be worth looking for damaged base products of DNA. It can be hypothesised that excised products of DNA repair may be released from neurons into their surroundings, and thus DNA fragments may be present in biological fluids, such as cerebrospinal fluid (CSF). It would be expected that DNA repair would be increased in cells undergoing oxidative stress, and therefore levels of excised DNA bases would be raised. DNA could provide an early ex vivo biomarker of neuronal damage. Therefore clinical samples in which ROS-mediated damage was suspected were analysed, looking specifically for DNA fragments using a capillary electrophoresis technique developed within the Division of Chemical Pathology.

With these points in mind, the aims of this project were as follows:

1. IMR32 neuroblastoma cultures differentiated with BrdU were chosen as an appropriate in vitro system in which to investigate oxidative DNA damage in the brain, because it has been demonstrated previously that they can be differentiated terminally and have mature neuronal markers. Further studies were performed to look at the way in which the morphology of these cultures develops during differentiation with respect to time. The pre-lethal effects of a variety of ROS-generating systems were then studied, and preliminary dose-response studies were carried out. In this way, the level of insult required to initiate pre-lethal damage was established.

2. In order to assess ROS-induced DNA damage, a novel, sensitive assay, based on the apparent ability of avidin to bind to oxidatively-modified DNA, was developed. Experiments were carried out using naked DNA and cultured cells. Hydrogen peroxide was the chosen method of inducing oxidative stress in differentiated IMR32 cells.

3. The levels of DNA damage in relation to cell metabolism and death in vitro in differentiated IMR32 cultures exposed to different ROS-generating systems, hydrogen peroxide, UVA irradiation and paraquat, were assessed. Experiments aimed to
investigate the consequences of DNA damage, the temporal relationship between DNA damage and cell death, and the mechanisms involved in this system.

4. Current methodologies do not allow the direct demonstration of DNA damage *in situ*. In this study, oxidative DNA lesions were visualised directly in both cultured cells and pathological brain tissue, using a novel assay involving avidin conjugated to a fluorescent label. Results were assessed using fluorescence microscopy. Initial experiments investigated the localisation of avidin binding in differentiated IMR32 cultures exposed to hydrogen peroxide. The potential antioxidant effect of α-tocopherol was also studied in these cells. Preliminary experiments were then carried out to assess the binding of avidin to motor neurons in mouse spinal cord, and human spinal cord from patients suffering from motor neuron disease and age-matched controls. This was to establish whether oxidatively-modified DNA could be detected in pathological tissue sections. The methodology used may be of value for the *in vivo* study of ROS-mediated damage, because it allows the demonstration of cells specifically affected during oxidative stress, rather than more general measurements of damage.

5. The medium from cultured cells exposed to oxidative stress, and clinical cerebrospinal fluid (CSF) samples taken from patients with disease states in which ROS damage is known to occur, was investigated for evidence of DNA fragments. It was hypothesised that these lesions may be released from cells undergoing DNA repair, or from apoptotic cells. A novel method of detection using capillary electrophoresis was used.
CHAPTER 2

METHODOLOGY:
CELL CULTURE
2.1 INTRODUCTION

The brain consists basically of 2 cell types; neurones, which are involved in the transfer and processing of information, and glial cells, which play a maintenance role (Strange, 1992). Such apparent simplicity belies the actual complexity of the organ. Both neurones and glial cells can be divided into subsets of cells, each with highly specialised functions, which are able to interact successfully to regulate a wide array of intricate functions. This complexity, along with its poor accessibility \textit{in vivo}, make the brain one of the hardest organs to study.

In order to investigate the fundamental mechanisms involved in the function of the central nervous system, tissue culture techniques, using both neuronal and glial cells, have been developed. These \textit{in vitro} methods of experimentation involve growing cells outside a living organism, within carefully-controlled laboratory conditions. Tissue culture methodology has enabled scientists to investigate how damage is induced in specific populations of cells. This type of mechanistic study becomes important when the consequences of cellular dysfunction are not seen clinically until a large amount of cell death has occurred, as in neurodegenerative disorders (Strange, 1993). Exposure to a toxin may lead to early, pre-lethal events within cells, which are not always detected using conventional endpoints, such as changes in morphology. This is especially true of the nervous tissue, where cells have the capacity to compensate for large losses without any outward sign of dysfunction. The use of \textit{in vitro} model systems has become increasingly important in this type of mechanistic study, in fields such as neurotoxicity (Atterwill and Walum, 1989; Veronesi, 1992). The mechanisms involved both in normal brain function and in various neurological disorders, may be addressed more readily via tissue culture techniques, and there is great interest in the development of neuronal model systems.

2.1.1 \textit{IN VITRO} NEURONAL STUDIES

Several forms of tissue culture have been used to examine the function of the nervous system \textit{in vitro}. Primary cultures are obtained from cells maintained outside an organism for more than 24 hours. When small pieces or slices of tissue are removed and kept in this way the cultures are referred to as organotypic or explant cultures. These are the most complex form of culture, and therefore are the closest representation of the
tissue from which they were derived *in situ*. They are, however, technically the most difficult cultures to maintain and have a finite life-span.

The next level of complexity is obtained when brain tissue is dissociated into single cells before being placed in a culture vessel to develop a primary cell line. Cell lines are formed when cultures are subcultured from one vessel to another for several generations. Primary cell lines are initially diploid in character and may correlate well with cells found *in vivo*, developing and maturing in a similar manner. It is possible that the cultures obtained will react to their environment much as neurones in the brain. Once again, however, they only have a limited life-span, and animals must be used to gain the tissue initially.

In some cells the genome may be altered such that they can proliferate indefinitely. These established cell lines are effectively 'immortal'. They often come from tumours, which are spontaneous, or induced by viral or chemical means. It is also possible for normal cells to spontaneously transform, or for researchers to create their own cell line by virally or chemically transforming them. Established cell lines commonly have an unstable genome and are usually non-diploid. They also tend to be less differentiated than primary cultures. In spite of this there is a wide literature base in their use. Cells representative of both neurones and glia are available from several different species, including humans, and this form of tissue culture is the most commonly used during *in vitro* studies of neuronal cells.

Tissue culture techniques have several advantages over whole animal studies. Firstly, whole cells are used in an environment that can be carefully controlled. Special culture media is supplemented with the correct nutrients and growth factors for the specific cell type being used, allowing maximal cell growth and proliferation. In this way large numbers of cells may be obtained. The cells under investigation can also be identified and characterised. Thus the properties and actions of one type of cell can be analysed. The use of cell cultures also means fewer animals need to be used; tissue culture experiments are much more simple than *in vivo* models, and are therefore more desirable. Finally, use of human-derived cells addresses the issue of cross-species extrapolation. Tissue culture techniques do have their limitations however. Cells are not in their natural environment; they often exist in a 2-dimensional form with altered cell-cell and cell-matrix contacts. This has led to concerns that their appearance and
metabolism may be altered, leading to questionable results. Cell lines arising from tumours may not be representative of normal cells from the same tissue. Genetically unstable cells may also change in character after several passages. However, if care is taken in experimental design and results are not over-interpreted, these problems may be kept to a minimum. The complexity of the central nervous system cannot be reproduced in vitro and tissue culture will never completely replace in vivo work, but when this technique is used in conjunction with others available it may provide an insight into the mechanisms involved in neurodegeneration.

11.2 NEUROBLASTOMA CELL LINES

Several cell lines have been established from tissue taken from neuroblastoma, a common childhood tumour which may originate from any dividing nerve cell (Prasad, 1975). These cultures are relatively undifferentiated. A more mature neuronal morphology may be observed if differentiation is induced under suitable conditions, however (reviewed by Prasad, 1975). Neuroblastoma cell lines may therefore be used as in vitro model systems to investigate neurones and their actions (Clementi et al., 1987; Thomas and Anderton, 1991; Veronesi, 1992).

2.1.2 IMR32 CELL LINE

The IMR32 cell line, established by Tumilowicz in 1970, is a human-derived neuroblastoma line. It is a continuous, hyperdiploid human cell line derived from tissue removed during an exploratory operation to investigate an abdominal mass, later diagnosed as neuroblastoma (Tumilowicz et al., 1970). Two morphologically distinct cells of common clonal origin were identified; the major cell type is a small 'teardrop-shaped' neuroblast which grows densely and can form focal accumulations, whilst the minor cell type is a larger fibroblast cell found in much lower numbers. The latter appears to be more adherent and residual cells are often seen in flasks after subculture.

IMR32 cells can be induced to differentiate by the addition of certain chemicals; the morphology of the culture thus becomes more neuronal in character. Compounds which have been used for this purpose include vasoactive intestinal peptide (Pence and Shorter, 1990), nerve growth factor (Reynolds and Perez-Polo, 1981), $N^6-O^2$-dibutyryl cyclic adenosine 3'-5' monophosphate (Bt$_2$cAMP) (Gotti et al., 1987; Gupta et al., 1985) and 5'-bromo-2'-deoxyuridine (Prasad et al., 1973; Gotti et al., 1987; Gupta et al., 1985; Thomas and Anderton, 1991). Gupta et al. found cells in cultures differentiated with
BrdU/mitomycin produced long neurites which could form contacts with neighbouring cells, and resembled normal neurones. The neurites had several growth cones and sometimes showed beaded varicosities along their entire length. Gotti et al agreed with these observations, noting that this cell line developed a large network of neurites which connected clumps of cells together. The neurites were described as being long, thin and regular, and some swellings were seen along their length. In all studies the optimal differentiation time occurred after at least 10 days of differentiation.

This cell line has also been characterised biochemically when differentiated (Prasad et al, 1973; Gupta et al, 1985; Clementi et al, 1986; Gotti et al, 1987; Elliott et al, 1992). Prasad et al found evidence of tyrosine hydroxylase (TH) and catechol-o-methyl transferase (COMT) in cells differentiated for 10 days with BrdU when compared to undifferentiated cultures, whilst levels of choline acetyl transferase (ChA) decreased by 50%. TH and COMT are both associated with the metabolism of the neurotransmitter dopamine, whilst ChA is involved in the synthesis of another neurotransmitter, acetylcholine. Further studies have confirmed the presence of acetylcholine and dopamine, and have also detected low levels of norepinephrine and high levels of serotonin in BrdU differentiated cells (Gotti et al, 1987; Gupta et al, 1985). Thus IMR32 cells seem capable of synthesising more than one class of neurotransmitter. Gotti et al also showed an increase in the level of muscarinic receptors and voltage-dependent Na⁺ channels, as well as higher TH activity in their investigation. More recently a subclass of 5-HT receptor has been characterised in IMR32 cells, which corresponds with the presence of serotonin (O'Dorisio et al, 1994). Thus it can be said that differentiated IMR32 cells display biochemical and morphological features common to normal neurones.

The use of IMR32 and other human neuronal cell lines in studies of neurodegeneration is not common. This is mainly because it is difficult to successfully maintain a mature neuronal morphology in cells over a prolonged period of time. Not only is the life-span of the culture limited, but the differentiating agent must remain in the medium throughout any experiments, and may therefore interfere with results obtained. In spite of this, differentiated IMR32 cultures have been used as model systems in some neurotoxicity studies (Clementi et al, 1987; Thomas and Anderton,
1991; Thomas et al, 1991) and investigations into epitopes associated with the development of Alzheimer's disease (Guy et al, 1991; Ko et al, 1990). In experiments carried out by Thomas and Anderton (1991) and Thomas et al (1991) a method of differentiation was used in which the chemical BrdU was absent from media during experiments without any effect on cell morphology. BrdU has been used to induce differentiation of IMR32 cells, and the long-term survival of these cultures in a mature neuronal state was not dependent on the presence of the chemical; cells were maintained successfully for up to 6 weeks in MEM supplemented with 5%(v/v) HIFCS and 1%(v/v) NEAA only (Mortimore, 1996). It was suggested that they may provide a good in vitro model for the study of neuronal properties.

2.1.3 AIMS

This project initially aimed to establish the levels of ROS which lead to pre-lethal DNA damage in an IMR32 model system. IMR32 cultures were terminally differentiated with BrdU during these experiments. The normal morphology of cells was first assessed over an 8 week differentiation period, to see how closely it related to that of primary neuronal cultures, which are generally accepted to be the closest in vitro representation of neurons in vivo. Cell morphology was then studied after exposure to either hydrogen peroxide, paraquat and UVA light, all of which induce oxidative stress. The metabolism of these cultures was investigated in parallel with this, in order to establish whether changes in cellular function were observed in the absence of any noticeable morphological change. In this way a dose-range was determined for each method of ROS-induction, in which pre-lethal DNA damage could be studied further.
2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

All tissue culture biochemicals and media were obtained from Gibco BRL (Paisley, UK), except the heat inactivated foetal calf serum (HIFCS) and non-essential amino acids (NEAA) which were from Sigma (Poole, UK). Tissue culture plastics were from Becton Dickinson Labware (Plymouth, UK). Hydrogen peroxide and paraquat were also from Sigma (Poole, UK), while Hanks Balanced Salt Solution (with calcium and magnesium) (HBSS) was from Gibco BRL (Paisley, UK).

2.2.2 CELL LINES

The IMR32 neuroblastoma cell line (passage number 68) was obtained from the European Collection of Animal Cell Cultures (ECACC) (Porton Down, Salisbury, UK).

The 3T3 mouse Swiss Albino embryo fibroblast cell line (arbitrary passage number 10) was provided by Sue Davies, Division of Chemical Pathology, Glenfield Hospital. The source and passage number were unknown.

2.2.3 METHODS

2.2.3.1 MEDIA PREPARATION

The 3T3 cell line was routinely maintained in Dulbecco’s Modified Eagle medium (DMEM) containing 10% (v/v) HIFCS and 2mM Glutamax I Supplement.

The IMR32 cell line was routinely maintained in growth medium consisting of α-minimal essential medium (α-MEM) supplemented with 10% (v/v) HIFCS and 1% (v/v) NEAA. This cell line was chemically differentiated with 5’-bromodeoxyuridine (BrdU) before all experiments, as described by Hartley et al. (1997), in order to obtain a more mature neuronal morphology in cultures. Differentiation medium consisted of α-MEM supplemented with 5% (v/v) HIFCS, 1% (v/v) NEAA and 1x10⁻⁵ M BrdU.

Media was made up immediately prior to use at all times.

2.2.3.2 CELL LINE MAINTENANCE

Stocks of original cultures, certified mycoplasma-free, were stored in liquid nitrogen shortly after the initial batch of cells were received. Both cell lines were maintained in a humidified atmosphere of 95% air, 5% CO₂ at 37°C in 75cm² flasks. To ensure cells were free of any microbial contamination which would interfere with
experimental results, cells were regularly examined microscopically. This is because bacterial and fungal infections may not be obvious to the naked eye in their early stages. Antibiotics were left out of the medium, because their presence may mask the presence of infectious agents. Mycoplasma infection is more difficult to detect as these organisms cannot be seen with the naked eye, and samples were tested periodically to ensure cultures remained infection-free. General measures were used to ensure minimal contamination; cells were subject to strictly sterile conditions when thawed for use, and they were not passaged more than 10 times before reverting to frozen stock. Limited passage also keeps genetic instability to a minimum.

Cultures were kept at sub-confluent levels in the logarithmic phase of growth. Confluence was judged microscopically via the number of gaps in the cell monolayer. It is important to harvest cells before confluence is reached (no gaps between cells) as the morphology, growth and biochemical characteristics of established cell lines have been shown to change after this stage (Todaro and Green, 1963). All cells were harvested approximately 2 times each week. Routine passage was carried out at a ratio of 1:5 into 75cm$^2$ flasks.

IMR32 cells were detached for passage by shaking into a small volume of fresh growth medium, then resuspended by gentle trituration through an 18 gauge needle. 20 to 30 mls of this cell suspension was added to each flask and these were placed in an incubator at 37°C. The final cell density was approximately $5 \times 10^4$ cells per ml media.

3T3 cells were harvested as follows: media was poured from the flasks and the cell monolayer was washed briefly with Earle's balanced salt solution (EBSS) (without calcium and magnesium), before incubation with 0.01mg/ml trypsin (0.1mg/ml stock solution diluted with phosphate buffered saline (PBS)) for 2-3 minutes at 37°C. Cells were rinsed out of the flask with a small volume of fresh growth medium (serum stops the action of trypsin) and collected by centrifugation at 1000g for 5 minutes at 4°C. They were then resuspended by gentle trituration through an 18 gauge needle in a small volume of growth media and added to 75cm$^2$ flasks at the correct cell density, as described previously.

Cells to be frozen were detached from the flasks as previously described, then resuspended in the appropriate growth medium containing 10% (v/v) dimethylsulphoxide
(DMSO) as a cryoprotective agent, at a cell density of $2 \times 10^6$ cells per ml medium. One ml volumes of this cell suspension were placed in plastic cryogenic vials and cooled to $-70\degree C$ at a rate of $1\degree C$ per minute. After 24 hours these frozen cells were transferred to liquid nitrogen for storage.

Cells were recovered from frozen stocks after rapid thawing by immersion of the vial in sterile water at $37\degree C$. The cell suspension was diluted with an equal volume of fresh growth medium, and the cells collected by centrifugation at 1000g for 5 minutes at $4\degree C$. Medium containing DMSO was poured off gently and cells were resuspended as previously described in a final volume of 25-30mls in a 75cm$^2$ flask. This was placed at $37\degree C$ and maintained as usual.

12.3.3 PREPARATION OF CELLS FOR EXPERIMENTS

3T3 cells were harvested as previously described and resuspended in a small volume of growth medium. Cell numbers were estimated using a haemocytometer and the cell density corrected to $5 \times 10^4$ cells/ml, before plating out into Nunc 96-well plates (200μl/well). Cultures were kept overnight at $37\degree C$ before use. Cells must not be kept any longer than this before an experiment to ensure they are not overgrown.

IMR32 cells were harvested as previously described, then resuspended in a small volume of differentiation media containing 10μM BrdU. BrdU initiates differentiation by inhibition of DNA synthesis; cells do not divide and exhibit a more flattened morphology, whilst neurites become extended (Schubert and Jacobs, 1970). Cell numbers were estimated as before and the cell suspension was made up to a density of $2 \times 10^5$ cells/ml. Cells were plated out into Falcon 96-well plates (200μl/well) or 8-well Labtek slides (400μl/well) and left to differentiate.

22.3.4 EXPOSURE OF DIFFERENTIATED CELLS TO HYDROGEN PEROXIDE

Differentiated IMR32 cells in 96-well plates were exposed to $H_2O_2$ as follows. Medium was carefully removed using an aspirator and replaced with pre-warmed Hank’s balanced salt solution (with calcium and magnesium), with or without various concentrations of $H_2O_2$ (200μl/well). These solutions were freshly prepared, immediately before use. Cells were left at $37\degree C$ for 1 hour, then the medium was removed carefully using an aspirator. Fresh medium ($\alpha$-MEM supplemented with 5%(v/v) HIFCS and
1%(v/v) NEAA) was added and cultures left for 1 hour or 24 hours at 37°C before photographs were taken using Ilford FP4 Plus 125 black and white film, with automatic exposure times. Cell viability was also assessed at these times, using the MTT assay.

2.2.3.5 EXPOSURE OF DIFFERENTIATED CELLS TO UVA IRRADIATION

A Blak-Ray UV lamp, Model UVL-56 with a glass filter (to eliminate any residual UVB light present) was used to irradiate cultures; $\lambda = 366$ nm, 750 W/cm$^2$ at 15 cm. The UV lamp was obtained from Knight Optical Technologies (Leatherhead, UK), as was the Optical Radiometer used to measure the UV dose received by the cells across the plate.

Differentiated IMR32 cells in 96-well plates were irradiated with UVA light at room temperature from above in a 50μl volume of differentiation media. The rest of the medium was removed very carefully with a multistepper pipette. Before each irradiation the intensity of UV light was checked with an Optical Radiometer. The dose received by the cells was estimated using the following equation:

$$\text{Maximum Intensity (mWcm}^{-2}) \times \text{Time (s)} = \text{UV dose (mJcm}^{-2})$$

Cells were irradiated from 15cm at a dose of 128mJcm$^{-2}$. Control wells were sham irradiated i.e. wells were covered in foil during the irradiation of the plate. After exposure to UVA, fresh medium ($\alpha$-MEM supplemented with 5%(v/v) HIFCS and 1%(v/v) NEAA) was added (150μl/well) and cells left at 37°C for either 1 hour or 24 hours before photographs were taken as above.

Initially experiments were carried out with the light source 5cm from the cells (h=5cm), to decrease irradiation times. However, variation in intensity of UVA across the exposed plate was seen at this height. Irradiation times were calculated according to maximum intensity readings, and radiometer readings confirmed that cells around the edge of the plate received a lower dose from those nearer the centre (Figure 2.1). This 'edge effect' was seen to decrease considerably when the height of the lamp from the plate was increased to 15cm, and this resulted in longer irradiation times. At the doses used in this study cells were not adversely affected by the longer irradiation time, however other cell lines are more resistant to UVA damage and must be irradiated for hours rather than minutes, leading to damage via dehydration effects rather than ROS. It was also observed that the intensity of the UV light decreased over a period of weeks,
Figure 2.1. Intensity of UVA light across the lamp. A. h=5cm; maximum dose received by the cells is $0.8 \text{ mWcm}^{-2} \times \text{Time (s)}$, and cells around the edge of the plate appear to receive as little as half the maximum dose. B. h=15cm; maximum dose received by cells is $0.29 \text{ mWcm}^{-2} \times \text{Time (s)}$, and the intensity across the plate does not vary so much.
demonstrated in this project as longer irradiation times were used in later work when the intensity fell from 0.24 mWcm\(^{-2}\) to 0.2 mWcm\(^{-2}\). This may decrease reproducibility of results. Finally, it is difficult to measure the maximum light intensity accurately between experiments. All these factors increase the error observed between experiments, therefore this method of UVA irradiation may not be a good system for ROS production in general.

2.2.3.6 EXPOSURE OF DIFFERENTIATED CELLS TO PARAQUAT

Differentiated IMR32 cells in 96-well plates were exposed to paraquat as follows. Medium was carefully removed using an aspirator and replaced with various concentrations of paraquat (200\(\mu\)l/well), made up in HBSS (with calcium and magnesium) Solutions were all prepared immediately before use. Cells were incubated for 1 hour at 37°C, before removal of the toxin and replacement with fresh media (\(\alpha\)-MEM with 5%(v/v) HIFCS and 1%(v/v) NEAA) (200\(\mu\)l/well). Cultures were left for 1 hour or 24 hours at 37°C before photographs were taken as above.

2.2.3.7 LIMITATIONS OF NEURONAL CULTURES

There were some limitations related to the use of IMR32 cultures in this study. It was very important to take great care when manipulating the cells once they were differentiated, as they were not found to be strongly adherent. Any loss of cells from plates due to rough handling during experimental procedures would likely lead to a large variation in results on data analysis. This could make any trends difficult to determine with any certainty. The environment of the incubators in which cultures were maintained during differentiation also had to be monitored carefully. It was found that this cell line was sensitive to the anti-microbial agent Roccal. Cells in the outer wells of the plates, which were more exposed to the external environment, did not grow or differentiate properly, became very rounded and died quickly. When Roccal was substituted with copper sulphate cultures differentiated as normal.

2.2.3.8 MTT ASSAY

This assay measures the metabolism of the tetrazolium salt 3-(4,5)-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT), which is taken up into the cells and reduced by dehydrogenase enzymes. The formazan product cannot pass across the
plasma membrane, and so accumulates within the cell. It is liberated by solubilisation of the cells and can be directly quantified using simple colorimetric techniques. Thus the viability of the cells present can be assessed by the degree of MTT metabolism.

One hour before each time point 20µl MTT (5mg/ml in 0.01M PBS) was added to each well (final concentration 0.5mg/ml), and cells incubated at 37°C for 1 hour. A medium blank in wells containing no cells was also set up. The medium was then removed carefully with an aspirator and 100µl isopropanol added. Plates were left shaking gently for 30 minutes, or until the formazan product was completely solubilised. Absorbance values were read on an Anthos reader 2001 (Labtech) using measuring and reference filters of 550nm and 620nm respectively.

2.2.3.9 STATISTICS

Results were analysed using the Statgraphics computer programme (Manugistics Inc., Maryland, USA). Data were checked for normal or non-parametric distribution using probability plots before analysis. The Students t-test (at a 95% confidence level) was used to compare two sample means, in this case MTT metabolism in the presence of hydrogen peroxide was compared to metabolism observed in the absence of the toxin.
2.3 RESULTS

This study used the IMR32 neuroblastoma cell line as a model neuronal cell system to investigate ROS-mediated DNA damage. Cultures were chemically differentiated with BrdU as described by Thomas and Anderson (1991), producing cells which were more neuronal in character. The morphology of cultures was assessed over a 2 month period as they matured.

Undifferentiated IMR32 cells had flattened cell bodies with small neurites extending at various places (Figure 2.2). Intracellular organelles were visible as dark cytoplasmic inclusions. There was no clear difference between the morphology of fibroblast-like or neuroblast-like cell types, recognised in this cell line by Tumilowicz (Tumilowicz et al., 1970). Some lighter, rounded cell bodies were observed, which were probably dividing cells, although some may have been dead cells which had become detached from the culture flask. The decision to sub-culture cells was made microscopically by eye, when cells covered a large percentage of the flask surface, with small surrounding gaps (Figure 2.2A). Cells were liable to overgrow and die if the time between sub-culture was misjudged. Spaces between cells were filled completely and individual cell bodies were packed tightly against each other (Figure 2.3). Neuritic extensions were not visible. Many round, light-coloured cells were observed. These were dead or dying cells which had become detached from the flask, or newly-divided cells with no room for attachment. Lots of rounded cell bodies were observed floating in the media. More cells died and detached as nutrients essential for growth and survival ran out in the media. Surviving cells were unlikely to be typical of this cell line, because their growth and biochemical nature can change under these conditions (Todaro and Green, 1963). They were therefore not used in experiments.

On addition of BrdU the morphology of IMR32 cells slowly started to change, becoming more neuronal in nature. During the early stages of differentiation (5 days) cells began to move together, producing small, 3-dimensional clumps (Figure 2.4). Neuritic extensions started to increase in number and length, especially around the newly-forming cell clusters. Some cells within these clusters were observed to be rounded. This occurred as they detached from the surface of the flask and adhered to other cells within the clump. Thus, small, 3-dimensional, sphere-like structures started to form. These remained attached to the flask surface via adherent cells towards the bottom of the sphere. Some minor-type fibroblast-like cells were apparent at this time (Figure 2.4, arrow).
These were larger than the neuroblasts and lacked neurites. They were observed in the spaces forming between cells as neuroblast-like cells migrated towards each other.

After 12 days of differentiation larger cell clumps had formed (Figure 2.5). More rounded cells were observed on the outer surface of these clusters, as they attached to other cells rather than the culture flask. Intracellular organelles were no longer visible in cells in the clusters. These clusters were uneven in size and shape at this point, and there were still neuroblast-like cells in the spaces around them. The neurites had increased in length, especially those extending from the cell clumps, and a network of thin neuritic extensions had begun to form, interconnecting the cell bundles. Fibroblast-like cells could still be seen.

The differentiation process continued over a period of 3-4 weeks. After 22 days the cells had formed very definite 3-dimensional spheres (Figure 2.6). These were more regular in size and were rounded in shape. Most of the neuroblasts had migrated towards each other, and cell bodies were seen travelling along neurites (Figure 2.6A). An underlying layer of fibroblast-like cells could now be observed, although they were low in number. The neuritic network had become more extensive, interconnecting cell clusters in the same vicinity. Some neurites had also become thicker than others. This morphology remained unchanged after 29 days of differentiation (Figure 2.7). At this stage cell clusters were still prominent, and the neuritic network very dense. However, cells were more easily dislodged after 1 month. Cell bodies were no longer seen migrating along neurites after this time either. After 2 months of differentiation the cultures had started to disintegrate (Figure 2.8). The cell clusters appeared slightly darker, clumps were large and uneven and did not appear healthy. It is likely that cells near the core of these clusters were necrotic as nutrients would be scarce at this time point, and any available would be likely to be utilised by cells nearer the surface of the clumps in closer contact with the medium. Some interconnecting neurites were still present, but these were very thin and delicate-looking. Most of the neuritic network was gone. There was no underlying cell layer after this time. The cultures were very easily dislodged and no longer useful for experimental purposes.

IMR32 cultures differentiated for 2-4 weeks were to be used in an investigation of oxidative stress in neurones. Cells were exposed to a variety of ROS-generating systems; hydrogen peroxide, paraquat and UVA irradiation. The morphology of the cells after treatment was then investigated microscopically, to see whether any changes could be detected 1 hour or 24 hours post-exposure in fresh media. Control cultures in the same
plate were exposed to buffer only or sham irradiated, depending on the type of oxidative stress involved. There was no change in morphology in any of these control cultures at either time point investigated (Figure 2.9, Figure 2.10). No difference was observed 1 hour after exposure of cells to $10^{-6}$M $H_2O_2$ (Figure 2.11A) when compared to control cells exposed to buffer only (Figure 2.9A). Cells exposed to $10^{-3}$M $H_2O_2$, however, appeared more rounded, with fewer neurites (Figure 2.11B), and some cells were lost from the plate after treatment. 24 hours post-treatment cultures exposed to $10^{-6}$M $H_2O_2$ (Figure 2.12A) were still no different morphologically from control cultures (Figure 2.9B). Cultures treated with $10^{-3}$M $H_2O_2$, however, were dead or dying; the cell density had greatly decreased, cell bodies were rounded, and very few neurites were observed (Figure 2.12B).

The morphology of cells also remained unchanged 1 hour after exposure to either $10^{-6}$M or $10^{-3}$M of the herbicide paraquat (Figure 2.13A, 2.13B). Cells may have been slightly more rounded after exposure to $10^{-3}$M of the compound, but this was not highly noticeable (Figure 2.13B). 24 hours after exposure to $10^{-6}$M paraquat the morphology was still normal (Figure 2.14A), but some cell death was evident in cultures treated with the higher concentration of the compound; cells were definitely more rounded, and the number of neurites had greatly decreased (Figure 2.14B).

1 hour after irradiation with 128mJcm$^{-2}$ UVA light cultures showed no changes in morphology when compared to sham irradiated cells; cell clusters were still evident and the network of neurites remained intact (Figure 2.15A). There was no sign of cell death. This was also true 24 hours after exposure (Figure 2.15B).

The only outward sign of cell damage was observed after exposure of cultures to high concentrations of either hydrogen peroxide or paraquat. The metabolism of cells was therefore investigated using the MTT assay, to see if the oxidative stress induced was having a sub-lethal effect. $H_2O_2$ was used as cells seemed more sensitive to this toxin. Metabolism was raised above that of control cultures 1 hour after exposure to a $10^{-6}$M concentrations of $H_2O_2$, and greatly decreased after exposure to $10^{-3}$M $H_2O_2$ (Figure 2.16). This decrease corresponded with slight changes in morphology and loss of cells observed under the microscope (Figure 2.11B). 24 hours post-treatment the metabolism had returned to control levels after exposure to $10^{-6}$M. After treatment with $10^{-3}$M there was a large decrease in metabolism (Figure 2.16), which corresponded with the complete disruption in morphology seen at the later time point (Figure 2.13B).
Figure 2.2 Undifferentiated IMR32 cells plated out 48 hours previously. (A) Magnification x200 (B) Magnification x320. Cells have flattened morphology and small, extending neurites. These cells have reached the correct cell density for sub-culture.
Figure 2.3 Undifferentiated IMR32 cells 4 days after subculture. (A) Magnification x200 (B) Magnification x320. Cells have become highly confluent, with no gaps being apparent between cell bodies. Neurites cannot be seen. The growth and biochemical nature of these cultures may change under these conditions.
Figure 2.4  IMR32 cells 5 days after differentiation in medium containing $2 \times 10^{-5}$M 5-bromo-2-deoxyuridine (BrdU). (A) Magnification x200 (B) Magnification x320. Cells have begun to clump together and develop longer neuritic extensions.
Figure 2.5 IMR32 cells 12 days after differentiation with $2\times10^{-5}$M BrdU. (A) Magnification x200 (B) Magnification x320. More cells have migrated to form larger clusters, and neurites are becoming more apparent. Some neurites are linking groups of cells.
Figure 2.6 IMR32 cells 22 days after differentiation with $2 \times 10^5 \text{M BrdU}$. (A) Magnification x200 (B) Magnification x320. Most cells are now part of well-defined cell clusters. Cell bodies can be observed migrating along neurites towards these clusters (arrow). A large network of interconnecting neurites has developed by this time.
Figure 2.7 IMR32 cells 29 days after differentiation with $2 \times 10^{-5}$M BrdU. (A) Magnification x200 (B) Magnification x320. Cell clusters remain prominent, and the neuritic network is still dense. At this stage cells are more easily dislodged than previously.
Figure 2.8 IMR32 cells 2 months after differentiation with $2 \times 10^{-5}$ M BrdU. (A) Magnification x200 (B) Magnification x320. Cells in clusters are beginning to die, and the neuritic network is disintegrating. Cells are very easily dislodged.
Figure 2.9 Morphology of control cultures for experiments where differentiated IMR32 cells were exposed to either hydrogen peroxide or paraquat. These cells were treated with HBSS only for 1 hour at 37°C, whilst other cells were exposed to various concentrations of toxin. Fresh media was then added, and cultures were left for 1 hour (A) or 24 hours (B) at 37°C before photographs were taken. Magnification x320.
Figure 2.10 Morphology of control cultures for experiments where differentiated IMR32 cells were irradiated with UVA light. Cells in media were irradiated from above at room temperature, then fresh media was added and cultures were left for 1 hour (A) or 24 hours (B) at 37°C before photographs were taken. Control cells were sham irradiated. Magnification x320.
Figure 2.11 Differentiated IMR32 cells 1 hour after exposure to (A) $10^{-6}$M or (B) $10^{-3}$M hydrogen peroxide. Cells were treated for 1 hour at 37°C, then fresh media was added and cultures were left for 1 hour at 37°C before photographs were taken. Magnification x320.
Figure 2.12 Differentiated IMR32 cells 24 hours after exposure to (A) $10^{-7}$M or (B) $10^{-3}$M hydrogen peroxide. Cells were treated for 1 hour at 37°C, then fresh media was added and cultures were left for 24 hours at 37°C before photographs were taken. Magnification x320.
Figure 2.13 Differentiated IMR32 cells 1 hour after exposure to (A) $10^{-6}$M or (B) $10^{-3}$M paraquat. Cells were treated for 1 hour at 37°C, then fresh media was added and cultures were left for 1 hour at 37°C before photographs were taken. Magnification x320.
Figure 2.14 Differentiated IMR32 cells 24 hours after exposure to (A) $10^6$M or (B) $10^7$M paraquat Cells were treated for 1 hour at 37°C, then fresh media was added and cultures were left for 24 hours at 37°C before photographs were taken. Magnification x320.
Figure 2.15 Differentiated IMR32 cells 1 hour (A) or 24 hours (B) after exposure to 128mJcm$^{-2}$ UVA light. Cells in media were irradiated from above at room temperature, then fresh media was added and cultures were left for 1 hour or 24 hours at 37°C before photographs were taken. Magnification x320.
A comparison of the metabolism of differentiated IMR32 cells
1 hour and 24 hours after exposure to hydrogen peroxide

Figure 2.16 Viability of IMR32 cells 1 hour and 24 hours after exposure to various concentrations of \( \text{H}_2\text{O}_2 \), as measured by the MTT assay. Differentiated cells were incubated with \( \text{H}_2\text{O}_2 \) for 1 hour at 37°C. This was replaced by fresh media and cells were left at 37°C for 1 hour or 24 hours before the cell viability was assessed. Values are the means ± standard error of the mean (SEM), where \( n \geq 6 \) wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using Student’s t-test procedures. The data is representative of at least 3 experiments.
2.4 DISCUSSION

Oxidative stress has been implicated in the major neurodegenerative diseases (Olanow, 1993), therefore a greater understanding of the mechanisms involved in neuronal dysfunction would be of interest. The aim of this project was to investigate pre-lethal damage to DNA in neuronal cells exposed to oxidative stress. Differentiated IMR32 cells were used as the in vitro model during these studies. The IMR32 cell line is a human-derived neuroblastoma line (Tumilowicz, 1970) which may be terminally differentiated with BrdU to give a mature neuronal morphology (reviewed by Prasad, 1975). They are known to express mature neuronal markers such as neurotransmitter synthesis and storage, receptors, ion channels (Gotti et al, 1987) and mature cytoskeletal elements (Johnston, 1995; Hartley et al, 1997), and have previously been used in in vitro studies with some success (Thomas and Anderton, 1991; Johnston, 1995).

Previous studies have not extensively examined the morphology of differentiating cells over a prolonged period of time. Cells chemically differentiated with BrdU, with slight modifications from the method described by Thomas and Anderton (1991), were photographed at various times over a 2 month interval, and changes in cell distribution, shape and neurite development noted. Cells grew well under the conditions described, without infection in the absence of antibiotics. Subculture was carried out during the exponential phase of growth at all times every 2-3 days, and care was taken not to let cells overgrow at any stage. This ensured that cultures used during different experiments had a very similar genetic identity over the prolonged period of this study, and that there were no microbial contaminants.

The morphology of undifferentiated IMR32 cultures (p71) was very similar to the description first given by Tumilowicz (1970), although the neuroblast-like cells and fibroblasts could not be easily distinguished at this stage. Continuous cell lines are known to exhibit slight changes in character as they are passaged because of their inherent genetic instability. This may explain the apparent slight difference in appearance of IMR32 cells in this study (used between passages 68-78) and the original cells. Cultures have been described as having a homogenous morphology by other groups (Clementi et al, 1987; ), suggesting that the minor fibroblast cell types make up a very small percentage of the total cell population. On addition of BrdU the cells gradually changed, becoming more neuronal in morphology as they matured, a feature which has been described by others (Thomas and Anderton, 1991; Johnston, 1995).
Cells began migrating towards each other to form small, spherical structures within 5 days of differentiation. The differentiation process continued over the next 8 weeks, along with the development of an interconnecting neuritic network. After approximately 12 days the morphology of cultures closely resembled primary mixed cultures of rat embryonic mid-brain, as recorded by Johnston (1995). It is known that there are increases in certain neurotransmitter and receptor levels in cells at this stage of differentiation (Gotti et al., 1987), and some mature cytoskeletal elements have also been shown to exist (Mortimore, 1996). These cultures were therefore thought suitable for use in experiments 2-3 weeks post-differentiation. Microscopic examination showed the morphology of differentiating cultures to remain stable for up to 4 weeks, after which time they began to disintegrate. The neuritic network becoming sparser and cell bundles much less adhesive to the plates. Darkened areas were also observed on the outside of the spheres, suggestive of necrosis. It is likely that cells near the core are dead, whilst those on the surface remain alive. This has been shown to be true in differentiated SH-SY5Y neuroblastoma cultures (Hartley et al., 1997). Cell death was most likely due to a lack of necessary nutrients in the medium, which was not changed during the 8 week period in order not to disturb the cultures. If fresh media were added weekly the cells might remain viable for longer than 4 weeks.

The similarity between differentiated IMR32 cells and primary cultures, along with the 3-dimensional character of the cell clusters, suggests IMR32 cells may provide a useful in vitro representation of neurons in vivo state. Continuous cell lines are more convenient to work with than primary cultures, and therefore more desirable. Differentiated neuroblastoma cell lines have also previously been used as a model neuronal system in studies of oxidative stress induced by methylphenyl-tetrahydropyridinium (MPTP), kainic acid, aluminium, ascorbate/iron mixture or acrylamide (Thomas and Anderton, 1991; Johnston et al., 1996; Cookson et al., 1996; Mortimore, 1996). These factors led to the decision to use differentiated IMR32 cells in experiments designed to investigate the effects of oxidative stress.

Oxidative stress was induced using a variety of methods, each producing ROS via a different mechanism and at different sites within the cultures; cells were exposed to H₂O₂ (which diffuses in through cell membranes to initiate chain reactions within the cell), UVA light (which passes straight into cells to initiate intracellular reactions) or the herbicide paraquat (which is actively taken into cells from the surrounding medium via
specific receptors, and then undergoes redox cycling to produce superoxide cytoplasmically). The morphology of cells is also important when studying pre-lethal events; results are only of interest when cultures show very little or no sign of cell loss. Cell morphology was therefore photographed 1 hour and 24 hours after treatment. This helped to clarify how quickly each insult damaged cells and whether any changes in morphology observed were reversible, suggesting repair of damage, or led to cell death. The greatest response was seen with hydrogen peroxide; cell morphology was altered slightly 1 hour after exposure to $10^{-3}\text{M}$, and uniform cell death observed after 24 hours. Paraquat produced noticeable changes in morphology 24 hours after exposure to the same concentration, while UVA had no effect at all. Thus the effects of oxidative stress on these cells was only apparent to the naked eye after exposure to relatively high levels of toxins.

The end-points investigated in studies mentioned previously were all pre-lethal markers of toxicity, including protein degradation (Thomas and Anderton, 1991), changes in neurofilament proteins (Cookson et al, 1996; Mortimore, 1996), and changes in cellular metabolism (Johnston et al, 1993). It is likely that macromolecules were damaged in IMR32 cultures after exposure to levels of oxidative stress which did not elicit any morphological changes. This suggests that conventional light microscopic studies of cell morphology are a poor indicator of toxicity. To investigate this further cells were treated with $\text{H}_2\text{O}_2$, and their metabolism was assessed using the MTT assay. In this study, MTT metabolism was increased above that of control cells 1 hour after exposure to $10^{-6}\text{M}$ toxin, whilst the cell morphology remained unchanged. The uncoupling of oxidative phosphorylation would result in increases in MTT metabolism. Patriarca and Maresca (1990) have demonstrated that heat shock in Saccharomyces cerevisae induces uncoupling of phosphorylation. It is therefore possible that the increases in MTT metabolism observed are due to the induction of a stress response within IMR32 cultures. Increases in cellular metabolism may also be indicative of the need for extra energy to repair damaged DNA and destroy abnormal proteins. In cells exposed to $10^{-3}\text{M}$ $\text{H}_2\text{O}_2$, however, a large decrease in metabolism was observed after 1 hour, which corresponded with the slight changes in morphology and cell death observed microscopically. These results imply that pre-lethal changes within these cultures have led to impaired function. 24 hours later the metabolism of cells exposed to the lower concentration of $\text{H}_2\text{O}_2$ had fallen back to control levels once more. This
may indicate that any damage has been repaired and the cultures have recovered from the insult, or that some cell death has occurred, with the remaining cells still metabolising at a high rate. The first explanation is more likely because no cell death was observed under the microscope, and the morphology appeared intact. 24 hours after exposure to 10^{-3} M H_2O_2 an even greater drop in metabolism was seen, once again corresponding to the extensive cell death observed under these conditions. Thus cultures were shown to be responding to oxidative stress before any outward changes in cell morphology or cell death. Differentiated IMR32 cells appear to be sensitive to ROS attack, and are therefore appropriate for use as a model system in investigations into pre-lethal effects of oxidative stress.

There are further lines of investigation which could be pursued in order to characterise the morphology of IMR32 cultures during differentiation. It would be interesting to investigate whether the cultures were terminally differentiated, as occurs \textit{in vivo} in the brain. Terminal differentiation implies that a cell has reached a mature phenotype and cannot progress further (Freshney, 1987). In neurons this phase is irreversible, and once a cell reaches this state it cannot divide further. The simplest method that could be used to investigate this involves cell counts at various points during differentiation. This would be very difficult with this type of culture, however, because of the formation of cell clusters, and would not give accurate results at later differentiation stages, because the numbers of cells still dividing may be very low at this time. Protein assays could also be carried out, but once again these would only give a general idea of terminal differentiation, and would not be able to detect single cells dividing after 4 weeks differentiation. More accurate would be thymidine uptake assays, where actively dividing cells incorporate radiolabelled thymidine into their DNA which may be quantified using liquid scintillation, or proliferation index assays, such as the one used by Pence and Shorter (1990), where a monoclonal antibody, Ki 67, is used to detect a nuclear antigen present in actively proliferating cells. The detection of BrdU incorporation into cellular DNA has been used in the latter type of assay, but this was not possible with IMR32 cells as this compound was used as a differentiation agent. Alternatively, images of living cells could be collected using confocal microscopy. Cell viability may be assessed by their retention of a compound such as the fluorogenic dye calcein-AM, as described by Hoyt \textit{et al} (1997). This dye is a substrate for intracellular esterases, and will only be cleaved to form a fluorescent product in living cells. This
type of experiment is relatively easy to perform and it would be possible to detect small numbers of viable cells, although the equipment needed is very complex.

The morphological investigations carried out during this study were very basic. Different techniques could be used to investigate differentiation characteristics of the cultures, and thus their maturity, in more depth. This would also allow for easier an comparison between the IMR32 cell line and other human neuroblastoma cell lines, such as the SH-SY5Y line, or primary neuronal cultures. The easiest method of doing this would be to record simple measurements of neurite length and cell cluster diameter at specific time points, as carried out by Reynolds and Perez-Polo (1981) and Pence and Shorter (1990). This can easily be done at light microscopy level, and requires no additional equipment or methodology. More accurate methods of assessing cell differentiation involve the measurement of specific characteristics such as cytoskeletal development or levels of a particular neurotransmitter. Levels of different cytoskeletal proteins may be estimated in situ using antibodies conjugated to fluorescent of colorimetric markers. Binding of these antibodies can then be assessed using light microscopy (Johnston, 1995; Hartley et al, 1997; Mortimore, 1996) or Western blotting techniques (Hartley et al, 1997; Mortimore, 1996). Once again these techniques are relatively easy to carry out, although slightly more specialised equipment is needed. Neurotransmitter levels, and those of the enzymes involved in their synthesis, may be measured in differentiating cells using specific assays (Prasad et al, 1973; Gupta et al, 1985), or high performance liquid chromatography (HPLC) techniques after sonification of cells (Gupta et al, 1985). These measurements may give a good measurement of the neuronal maturity of the cultures, but are slightly more complex than others mentioned previously. Finally, ultrastructural studies, such as those carried out by Gupta et al (1985) and Gotti et al (1987) involving scanning and transmission microscopy, can give an idea of the structural maturity of cellular organelles. Gupta et al, for example, have shown that differentiated IMR32 cells contain well-developed organelles and many neurosecretory granules not present in undifferentiated cultures. These methods are very specialised however, and would therefore be more difficult to perform. It would be of interest to investigate the differentiation of IMR32 cultures using some of the techniques described, and to compare the results with parallel studies of chick or rat primary neural cultures, in order to further assess the suitability of this cell line as an in
vitro model of neurons in vivo. However, in this study these cultures were being used as a tool to study DNA damage in neurons after oxidative stress.

IMR32 cultures differentiated with BrdU were chosen as a model system in which to investigate the mechanisms of DNA damage induced in neuronal cells exposed to increased levels of ROS. It is thought that in vitro model systems such as these may play an important role in studies aimed to provide a greater understanding of the processes involved in neurodegenerative disease states, where oxidative stress is strongly implicated in neuronal dysfunction (Olanow, 1993). Differentiated IMR32 cells have been shown to exhibit a mature neuronal morphology, very similar to rat primary neural cultures, after 12 days. These cultures were stable for up to 4 weeks. The morphology was only altered after exposure to high concentrations of H$_2$O$_2$; paraquat, UVA and lower concentrations of H$_2$O$_2$ did not have any effect on culture morphology. Changes in MTT metabolism after exposure to lower concentrations of H$_2$O$_2$ did show this cell line was sensitive to the actions of ROS, however. DNA is very sensitive to oxidative attack (Imlay and Linn, 1988), and damage may result in the inefficient transcription of gene products vital for normal cellular function. Differentiated IMR32 cells were therefore used as a model system to investigate DNA damage after exposure of cultures to different forms of oxidative stress.
3.1 INTRODUCTION

DNA is very susceptible to damage by ROS, and lesions such as 8-oxodG are now widely accepted biomarkers of oxidative damage within cells (Halliwell and Dizdaroglu, 1992; Shigenaga et al, 1994). Measurement of this modified base has previously involved the prior isolation, and in some cases derivitisation, of DNA from cells, with resulting artefactual damage. A method able to directly demonstrate 8-oxodG within cells would be of great importance; it would allow researchers to identify specific cells affected within pathological tissue, and would also provide a simple method for investigating the mechanisms involved in cellular dysfunction induced by oxidative stress in in vitro model systems. Immunofluorescence experiments designed to investigate binding of a new primary antibody, raised against oxidatively modified DNA (Herbert et al, 1994), to UVA irradiated cells resulted in the hypothesis that the molecule avidin was able to bind directly to oxidatively-modified DNA. This led to a more comprehensive study of the avidin and its natural ligand biotin, and ultimately the development of a novel assay able to measure oxidative DNA damage directly in cells.

3.1.1 BINDING OF AVIDIN TO DAMAGED DNA

Initially structural similarities between biotin and damaged DNA products were studied, and a literature review carried out for avidin, its properties and structure. Early attempts at isolating this glycoprotein resulted in the production of a complex between avidin and nucleic acid, although this does not appear to have been investigated further (Fraenkel-Conrat et al, 1952). ‘Non-specific’ binding of avidin to the nucleus of cells has also been reported. Heggeness (1977) found that fluorescently-conjugated avidin bound to condensed chromatin of W138 cells, but not uncondensed chromatin. Wood and Warnke (1981) and Duhamel and Johnson (1985) have also reported specific nuclear staining with avidin conjugates. In the latter study this was prevented by blocking with non-fat dry milk. In each case the mechanism of binding was not considered. Commercial kits are available, in which cells are pre-treated with excess avidin and biotin in order to eliminate this complication by blocking endogenous binding sites. The possibility that avidin may be binding specifically to the nucleic acid of cells has not been studied to date.
CHAPTER 3

METHODOLOGY:
DEVELOPMENT OF DNA DAMAGE ASSAY
The presence of avidin was first detected when a nutritional deficiency was observed in rats whose sole source of protein was dried egg whites (Boas, 1927). Further investigation led to the identification of its natural ligand biotin, and partial purification of avidin itself (reviewed by György, 1954). Studies have since shown avidin to be a natural component of egg-white, where it makes up just 0.05% of the total protein content. It is not found in all species; avidin activity has been isolated from chicken eggs, and is produced in the oviducts of birds, amphibians and reptiles. Avidin was shown to be synthesised in the epithelial goblet cells of the oviduct in response to the hormone progesterone (O'Malley et al., 1969), but the biological function of this molecule remains unknown. The most plausible explanation is some kind of antibacterial action. Circumstantial evidence for this is based on (i) the fact that egg white contains several known antibacterial molecules, and (ii) the discovery of a bacterial analogue, streptavidin, in culture filtrates of Streptomyces avidinii, a species known to be versatile in the production of antibiotic systems. A role for avidin in reproduction, or as a remedial protein, has also been suggested (Elo and Korpela, 1984), but these proposals still await confirmation.

The interaction between avidin and biotin is very strong (affinity constant, $K_a=10^{15} \text{M}^{-1}$) (Bayer and Wilchek, 1990), with a slow off-rate of dissociation. This is demonstrated by the fact that avidin can inhibit the growth of microorganisms needing biotin concentrations of just $10^{-10}\text{M}$ to $10^{-11}\text{M}$ to survive (Hertz, 1946). The affinity observed is higher than that usually observed between antibodies and their epitopes; for example, antibodies raised against biotin have affinity constants orders of magnitude lower ($K_a=10^{-9}\text{M}$) (Dakshinamurti and Rector, 1990). It is this remarkably strong interaction which has led to the development of many new techniques involving the avidin-biotin complex, including affinity chromatography, affinity cytochemistry, immunoassays and drug delivery (reviewed by Wilchek and Bayer, 1988). It is used primarily as a secondary detection or amplification system, where a primary or secondary antibody, or sometimes target molecule, has been biotinylated. Avidin may be conjugated to a variety of labels, including gold, peroxidase, radioactivity and fluorescence, to visualise binding. To date, avidin has not been specifically used in the direct detection of molecules other than biotin within cells.
3.1.3 STRUCTURE OF AVIDIN

The unusually strong association between avidin and biotin prompted investigations into the structure of purified avidin. Initial studies were hampered by the difficulties involved in isolation of pure avidin from egg-white, where it is present at very low levels. Fraenkel-Conrat et al (1952a,b) attempted to develop an easier method of purification, and produced three forms of avidin from dried egg white; a basic glycoprotein (designated avidin A, corresponding to fully active avidin), and complexes of this with an acidic glycoprotein (avidin XA) or low molecular weight fragments of DNA (avidin NA). The binding between avidin and nucleic acid was observed to be very stable, and it was suggested that this complex was one of the natural physiological states of avidin within egg white. These studies also provided evidence that the nucleic acid involved was a desoxypentose, and stated that two molecules of biotin were bound by each avidin molecule. In spite of this early work, the nature of the binding of avidin to DNA does not seem to have been investigated further. New methods of isolation have been developed, however. Pure crystals have now been produced, and their general properties and structure analysed.

Avidin is a basic glycoprotein (M_r = 62,400), stable over a wide temperature and pH range, and resistant to the actions of proteolytic enzymes. Structural investigations and molecular modelling techniques have shown the molecule to consist mainly of β-strands and bends (Green, 1975; Honzatko and Williams, 1982; Pugliese et al, 1993). The active molecule consists of four identical monomers, which assemble as a tetrameric structure with twofold symmetry. Each monomer is organised in an 8-stranded, antiparallel β-barrel (Pugliese et al, 1993), and possesses one biotin-binding site. These sites are arranged in pairs on opposite faces of the molecule. Studies on avidin polymers have confirmed this structure (Green, 1975; 1990), and shown the binding sites to be located in a depression in the protein surface (Green, 1990). The overall stability of the molecule is greatly enhanced by binding of the ligand biotin.

3.1.4 BIOTIN BINDING SITE

Biotin is a small, water-soluble vitamin, which functions as a coenzyme in several carboxylase enzymes e.g. pyruvate carboxylase. It is covalently bound to these proteins at a lysine residue, and acts as a carrier molecule for the carboxyl group (COO') between substrates on 2 different active sites of the enzyme. While there are several natural
biotin-binding proteins in addition to avidin, none appear to exhibit such a strong affinity for the molecule. Investigations into the biotin binding site, and the reasons for the strength of its interaction with avidin, have therefore been carried out (Green, 1975; Pugliese et al, 1993).

Biotin is 70% hydrophobic in nature, and consists of an imidazolidone ring and ureido group fused together in a cis configuration, with a valeric acid side chain on the C\textsubscript{2} of the former, also cis in relation to the imidazolidone ring. Binding to avidin is of a non-covalent nature, and all the biotin molecule appears to interact to some degree with the binding site, which is located in a deep pocket within each avidin monomer (Green, 1975). The orientation of the biotin molecule when bound is roughly perpendicular to the axis of the β-barrel within this cleft, with the valeric acid side-chain running in a shallow channel near the surface of the molecule (Pugliese et al, 1993). The imidazolidone ring is located deep within the binding site (Pugliese et al, 1993), and appears to be a very important factor in the strength of interaction; when it is broken or modified the dissociation constant increases (Green, 1975). Chemical modifications to other parts of the biotin molecule do not greatly affect its affinity for avidin. Investigations involving analogue binding, chemical modification studies and spectroscopic evidence have all pointed to the direct involvement of several tryptophan residues in biotin binding (Green, 1975). It is thought that strong hydrogen bonds form between functional groups of the ring and ureido group and the binding site. Although a wide range of biotin-related compounds bind to this site, the avidin-biotin interaction can be said to be very specific because related compounds do not bind significantly at the 1-10 mM level.

3.1.5 STREPTAVIDIN

This protein was discovered and isolated in culture filtrates of several species of Streptomyces (Tausig and Wolf, 1964; Chaiet et al, 1964). Streptavidin, as it was named, also consists of 4 identical monomers, and has a molecular weight of 60,000. It is identical to avidin at 33% of its residues, including the critical tryptophan residues involved in the biotin-binding site, and has the same antiparallel β-barrel organisation. Studies on polymers led to the conclusion that the openings of the binding clefts of streptavidin are closer together than those of avidin, although the structure of subunits and binding sites are very similar (Green, 1990). Streptavidin is different from avidin in
two major ways however; it is an acidic protein, and has no carbohydrate moiety. In
despite of the differences observed, the binding affinity of streptavidin with biotin is similar
to that of avidin, although the rate of dissociation is much higher, and this bacterial
protein may be substituted for avidin in the many visualisation techniques available.

3.1.6 AIMS

This led to an investigation of potential structural similarities between biotin, the
natural ligand for avidin, and oxidatively-modified DNA. It was observed that whilst the
purine base guanine is structurally very different from the biotin molecule, the keto forms
of 8-oxodeoxyguanine and 8-oxodeoxyguanosine (Aida and Nishimura, 1987), both
damage products of oxidative attack on DNA, have a surprisingly similar structure to
biotin. Therefore experiments were set up to examine the ability of avidin, streptavidin
and anti-biotin monoclonal antibodies to bind to oxidatively modified DNA. Binding of
these molecules to naked DNA, cultured cells and pathological tissue was assessed,
where increased levels of damaged base products, including 8-oxodeoxyguanosine, were
known to be present. Using these results, an assay was developed to detect increases in
levels of damaged bases in cultured neuronal cells systems and isolated DNA after
exposure to oxidative stress. This was extended to pathological brain tissue. Both
colorimetric and fluorescent end-points were utilised. Binding was detected directly in
cells, and did not involve isolation of DNA with the subsequent potential artefactual
damage.
3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

All biochemicals were from Sigma (Poole, UK), except for the following: normal goat serum (NGS) from Dako (High Wycombe, UK); methanol and ethanol from Fisons (Loughborough, UK). Components for the colorimetric determination of avidin binding were provided as a research evaluation kit by Biotrin International (Dublin, Eire). 8-Oxodeoxyguanosine was a kind gift from Dr. K. Herbert (Leicester University), and was prepared according to the Udenfriend system (Kasai and Nishimura, 1984) and its purity checked by electrospray mass spectrometry.

Experiments were initially carried out with an antibody raised against DNA, damaged by UVC and UVA irradiation (Ab529), using a detection system based on a biotinylated secondary antibody to which avidin-FITC would bind. Results obtained in cells after exposure to UVA showed nuclear binding of avidin-FITC in control wells containing no antibody. No binding was seen in sham irradiated cells (Figure 1.1). It was hypothesised that avidin-FITC may bind directly to oxidatively modified DNA, and appropriate investigations were made. All washing steps were carried out using a Tris buffer supplied by Biotrin International unless stated otherwise.

3.2.2 METHODS

3.2.2.1 FLUORESCENT BINDING ASSAY

An assay was developed in which the ability of avidin and a monoclonal antibody to bind to the oxidatively-modified base 8-oxodeoxyguanosine (8-oxodG) could be assessed in a semi-quantitative manner. These results were compared to those of parallel studies in which deoxyguanosine (dG), the non-modified analogue of 8-oxodG, was used instead. The methodology was based on that of the immunocytochemistry assay used during the initial primary antibody experiments described in the introduction. The assay involved assessing how sensitive avidin, or the anti-biotin monoclonal, were at detecting various concentrations of 8-oxodG or dG immobilised on a substratum of fixed cellular material. The mechanism of binding of the bases to the substratum was not known, but this layer was necessary because neither base bound to untreated plates alone. It was assumed that base binding was uniform across the plate. The cell type or morphology did not matter; 3T3 fibroblasts, undifferentiated and differentiated IMR32 cultures were all used with
success. 3T3 cells were chosen for these experiments because they were easy and quick to grow, and they became firmly attached to the plates, ensuring a uniform covering in all wells.

3T3 cells were plated out in 96-well plates as described previously (Section 2.2.3.3). Untreated cultures were fixed and permeabilised with 4% (w/v) paraformaldehyde (PFA) (made up from frozen 40%(w/v) stock immediately prior to use in 0.01M PBS, pH 7.4) for 30 minutes at room temperature, then dehydrated in 99% (v/v) ice-cold methanol at -20°C, and left until needed. Plates were used within 2 days. These cells provide a substrate for the attachment of 8-oxodeoxyguanosine (8-oxodG) or deoxyguanosine (dG), and subsequent binding assays. Before use, cells were rehydrated in 0.01M PBS for 5 minutes at room temperature. Non-specific binding sites were blocked by incubation with 10% (v/v) normal goat serum (NGS) for 1 hour at room temperature, then cells washed 3x with 0.01M PBS. Concentrations of normal (1mM stock) or oxidised base (50mM stock), diluted in ultrapure H2O (18MΩ resistance) immediately prior to use, were added to wells and incubated for 1 hour at room temperature. Cells were washed 3x with 0.01M PBS before addition of avidin-FITC (1:100 dilution in PBS) for 1 hour at room temperature in the dark. Cells were washed 3x with 0.01M PBS and levels of avidin binding assessed using a Wellfluor Microplate fluorescence reader (Denley, Billinghurst, UK), excitation 485nm and emission 530nm. PBS was removed before this reading was taken.

Binding of a FITC-conjugated anti-biotin monoclonal antibody (1:80 dilution in PBS containing 0.2%(v/v) NGS) was also assessed in this system. Methods were as for avidin-FITC assay, except washes were carried out with PBS containing 0.2% (v/v) NGS.

3.12.2 THE EFFECT OF ALKALI TREATMENT ON AVIDIN BINDING

Cells damaged by reactive oxygen species were exposed to alkaline conditions known to cause degradation of the imidazolidone group of 8-oxodG (Torres et al, 1996), and the effects on avidin binding were assessed. IMR32 cells were exposed to various concentrations of hydrogen peroxide, as described in Section 2.2.3.4, then left in fresh media for 1 hour. Cells were fixed and permeabilised with 2%(w/v) PFA, 0.5%(v/v) glutaraldehyde for 15 minutes on ice, then dehydrated through graded alcohol (1-2 minutes in 70%(v/v) industrial methylated spirits (IMS), 95%(v/v) IMS) and left in
99% (v/v) IMS overnight at -20°C. Cultures were rehydrated through graded alcohol (1-2 minutes in 95% (v/v) IMS, 70% (v/v) IMS), then left in wash concentrate for 10 minutes with gentle agitation before treatment with alkali. Cells were incubated with wash solution at either pH 7.0 or pH 12.0 (corrected with NaOH) for 30 minutes at 37°C in a humidified chamber, then washed briefly before blocking with wash solution containing 10% (v/v) NGS for 1 hour at room temperature. After washing 3x, avidin-HRP (2µg/ml in wash concentrate, 50µl/well) was added and plates were left overnight at 4°C with gentle agitation. Cells were washed 5x, before the degree of avidin binding was assessed using the substrate o-phenylenediaminedihydrochloride (OPD) (see Table 3.1). Absorbence values were determined using an Anthos 2001 plate reader.

3.2.2.3 DEVELOPMENT OF A DNA DAMAGE ASSAY.

This assay was developed from the immunocytochemistry assay used with a primary antibody, therefore the same protocol was followed with avidin initially. Procedures were gradually optimised during investigations on IMR32 cells in which oxidative damage was induced using hydrogen peroxide (H₂O₂), as described in Section 2.2.3.4.

3.2.2.3.1 INITIAL ASSAY PROTOCOL

Cells were prefixed and permeabilised by addition of 2% (w/v) paraformaldehyde (made up immediately prior to use in 0.01M PBS from frozen 40% (w/v) stock, pH 7.4) for 30 minutes at room temperature, followed by ice-cold methanol for at least 30 minutes at -20°C. Plates could be left at this point if wrapped in Nescofilm to prevent them drying out. Cells were rehydrated in 0.01M PBS for 10 minutes, then non-specific sites were blocked with PBS containing 10% (v/v) normal goat serum (NGS) for 1 hour at room temperature. The blocking solution was washed off with PBS (3x) and DNA damage determined by addition of FITC-conjugated avidin (1:100 dilution made up in PBS immediately prior to use) for 1 hour at room temperature in the dark. Cells were washed 3x with 0.01M PBS before a quantitative assessment of fluorescence levels using a Wellfluor Microplate fluorescence reader (Denley, Billinghamurst, UK); measuring and reference filters were 485nm and 530nm respectively. PBS was removed before reading.

3.2.2.3.2 CHANGES IN FIXATION PROCEDURE

The optimum fixation method using PFA and methanol was established. A 4% (w/v) stock solution of PFA was made up weekly and stored at 4°C. After this time
any remaining was disposed of. Immediately prior to fixation a solution of PFA and glutaraldehyde (GAh) was made up, to give a final concentration of 2%(w/v) PFA, 0.5%(v/v) GAh when added to the cells. Cells were prefixed with 2%(w/v) PFA, 0.5%(v/v) GAh for 15 minutes on ice, then washed briefly before dehydration through graded alcohol (1-2 minutes in 70%(v/v), 95%(v/v) IMS on ice, then at least 30 minutes in 99%(v/v) IMS on ice). If cells were to be left overnight in 99%(v/v) IMS, plates were wrapped in Nescofilm and placed at -20°C. Cells were rehydrated through graded alcohol (1-2 minutes in 95%(v/v), 70%(v/v) IMS), then left in wash buffer for 10 minutes with gentle agitation. This protocol was used in the final assay, with one slight modification; cells were not incubated with 95%(v/v) IMS at any point.

3.2.2.3 DETECTION OF AVIDIN BINDING

A fluorescent end-point detection system was used initially, because studies involving the primary antibody, on which this assay was based, utilised avidin conjugated to the fluorophore FITC. The fluorescent plate reader on which data were read did not give reproducible results, however. This type of plate reader is also not always readily available in standard laboratories. It was therefore decided to use a colorimetric end-point.

Damaged cells were fixed and permeabilised, then non-specific sites blocked as described above. Avidin conjugated to horseradish peroxidase (HRP) (2µg/ml made up in wash buffer) was used to detect damaged base products rather than avidin-FITC, and was added to cultures for 1 hour at 37°C in a humidified chamber. Cells were washed 5x before visualisation of avidin binding. Two different substrates were used, o-phenylenediaminedihydrochloride (OPD) and 3,3',5,5'-tetramethylbenzidine (TMB), each with slightly different methodology (Table 3.1). The end product was determined spectrophotometrically using an Anthos 2001 plate reader. TMB was the substrate of choice in future experiments. Thus both colorimetric and fluorometric end-points were appropriate in this assay.

3.2.2.4 AVIDIN DILUTIONS

The lowest concentration of avidin conjugate needed to accurately assess changes in DNA damage was investigated. Differentiated IMR32 cells were exposed to a concentration range of hydrogen peroxide in which changes in cellular metabolism were known to occur (Section 2.2.3.4). It was assumed that pre-lethal events such as DNA damage would also occur within this concentration range.
o-phenylenediamine-dihydrochloride (OPD)

0.5 mg/ml OPD dissolved in 
0.05M phosphate-citrate buffer 
(pH 5.0) containing 0.03% (w/v) 
sodium perborate rather than
H₂O₂. Solutions must be made up 
immediately prior to use.

Add substrate solution to wells 
(50µl/well) and leave for 10
minutes in the dark at room
temperature, or until a colour 
develops. Stop reaction by addition 
of 2M H₂SO₄ (25µl/well). The
product is determined
spectrophotometrically, excitation
492nm, emission 620nm.

3,3',5,5'-tetramethyl-
benzidine (TMB)

Provided by Biotrin International
in ready to use form.

Add substrate solution to wells 
(50µl/well) and leave for 20
minutes in the dark at room
temperature. Stop reaction by 
addition of 0.5M H₂SO₄
(50µl/well), and agitate gently for
60 seconds. The product is
determined spectrophotometrically, excitation
450nm, emission 620nm.

Table 3.1 A comparison of the methodology involved using 2 different substrates in the detection of avidin-HRP binding to IMR32 cells exposed to reactive oxygen species.

Cells were fixed as described above after cells had been placed in fresh media and left for 1 hour at 37°C. After rehydration, non-specific sites were blocked by incubation with wash solution containing 10%(v/v) NGS for 1 hour at 37°C, then cells washed 3x. Cultures were then incubated with various concentrations of avidin-HRP, ranging from 0.1µg/ml to 2µg/ml for 1 hour at 37°C in a humidity chamber. After washing 5x, avidin binding was visualised using the substrate TMB (Table 3.1), and the end-product determined spectrophotometrically at 450nm, emission 620nm. MTT assays, as described in Section 2.2.3.8, were carried out on cells treated in parallel, to assess the viability of cultures after exposure to oxidative stress. This was to ensure that any changes detected were pre-lethal. It was decided that a concentration of 1µg/ml would be used in the final assay protocol.

3.2.2.3.5 BLOCKING AGENTS

The most effective blocking agent with respect to background binding was assessed. Untreated differentiated IMR32 cells were fixed and permeabilised with 2%(w/v) PFA, 0.5%(v/v) GAh, then graded alcohol as described above in Section 3.2.2.3.2. Non-specific sites were blocked with wash solution containing either 10%(v/v) NGS, 10%(v/v) HIFCS or 1%(w/v) gelatin for 1 hour at 37°C in a humidified chamber.
Control wells were treated in parallel with wash concentrate only. Blank wells (no cells) were treated with 10%(v/v) NGS. Wells were washed 3x, before addition of avidin-HRP (1µg/ml in wash solution, 50µl/well) and incubated overnight at 4°C with gentle agitation. Levels of binding were detected using TMB as a substrate. TMB solution was added to wells and left for 20 minutes in the dark at room temperature. The reaction was stopped by addition of an equal volume of 0.5M H₂SO₄, and agitating gently for 60 seconds. The product was determined spectrophotometrically, excitation 450nm, emission 620nm. Freshly prepared normal goat serum was considered to be the most effective blocking agent in this assay.

The minimum dilution of NGS needed to effectively lower the background levels of binding was investigated. Avidin-HRP binding to untreated IMR32 cells was assessed as described immediately above with the following exception: Non-specific sites were blocked with wash concentrate containing either 1%(v/v), 5%(v/v), 10%(v/v) or 15%(v/v) NGS for 1 hour at 37°C in a humidified chamber. It was decided that a dilution of 10%(v/v) NGS would be used in the final assay protocol.

Finally, the optimum blocking conditions were investigated in this system. Avidin-HRP binding to untreated IMR32 cells was once again assessed as described immediately above, with the following alterations to the blocking step. Cells were incubated with 10%(v/v) NGS for either 1 hour or 2 hours at room temperature with gentle agitation or 1 hour or 2 hours at 37°C in a humidified chamber. The best blocking conditions were shown to be incubation with 10%(v/v) NGS for 1 hour at 37°C, and this was used in the final assay protocol.

3.2.2.3.6 THE EFFECTS OF FIXATION TIME IN ALCOHOL

The effects of leaving prefixed cells in 99%(v/v) IMS overnight at -20°C on avidin binding was assessed in IMR32 cells exposed to hydrogen peroxide. Cultures were fixed with 2%(w/v) PFA, 0.5%(v/v) GAh for 15 minutes on ice, washed briefly, then dehydrated in 70%(v/v) IMS for 1-2 minutes before addition of 99%(v/v) IMS. This was left for either 30 minutes on ice or overnight at -20°C. Cells were then rehydrated in 70%(v/v) IMS for 1-2 minutes and left in wash concentrate for 10 minutes with gentle agitation, before blocking with 10%(v/v) NGS for 1 hour at 37°C. Avidin-HRP (1µg/ml in wash solution, 50µl/well) was added after cells were washed 3x, and plates left overnight at 4°C. Levels of binding were assessed colorimetrically after washing, using
the substrate TMB, as described above. Levels of avidin binding were higher after incubation in 99% (v/v) IMS for 30 minutes on ice, when compared to cells left overnight at 4°C, therefore this incubation time was used in the final assay protocol.

3.2.3.7 THE EFFECTS OF INCUBATION TIME WITH AVIDIN CONJUGATE ON BINDING LEVELS

The effects of incubation time in avidin-conjugate was investigated in untreated IMR32 cells, to see if more accurate results were obtained when cells were left in the conjugate solution overnight, rather than 1 hour. Cultures were fixed with 2% (w/v) PFA, 0.5% (v/v) GAh, washed briefly, then dehydrated in 70% (v/v) IMS for 1-2 minutes and 99% (v/v) IMS for 30 minutes on ice. After rehydration in 70% (v/v) IMS (1-2 minutes) and wash solution (10 minutes at room temperature with gentle agitation) non-specific sites were blocked by incubation with wash solution containing 10% (v/v) NGS for 1 hour at 37°C. Cells were washed 3x, then incubated with avidin-HRP (1 μg/ml in wash solution, 50 μl/well) for 1 hour at 37°C in a humidified chamber or overnight at 4°C. Cells were washed 5x and levels of binding visualised spectrophotometrically using the substrate TMB, as described above. It was decided that cells would be incubated with avidin conjugates overnight at 4°C in the final assay procedure.

3.2.3.8 FINAL ASSAY PROTOCOL

Damaged cells were prefixed and permeabilised by addition of 2% (w/v) PFA (made up immediately prior to use in wash solution from 4% (w/v) stock stored at 4°C, pH 7.4), 0.5% (v/v) GAh for 15 minutes on ice, then dehydrated in 70% (v/v) IMS (1-2 minutes, on ice) and 99% (v/v) IMS (30 minutes on ice). Cells were rehydrated in wash solution for 10 minutes with gentle agitation, then non-specific sites were blocked with wash solution containing 10% (v/v) normal goat serum (NGS) for 1 hour at 37°C. The blocking solution was washed off (3x) and DNA damage determined by addition of FITC-conjugated avidin (1:200 dilution made up in wash solution immediately prior to use) or avidin-HRP (1 μg/ml in wash solution, 50 μl/well) overnight at 4°C in the dark. Cells were washed 5x before assessment of avidin binding. Fluorescence levels were determined using a Wellfluor Microplate fluorescence reader after removal of wash solution from the wells (Denley, Billinghurst, UK) (measuring and reference filters were 485 nm and 530 nm respectively). Avidin-HRP binding was visualised using the substrate TMB, and absorbence values determined spectrophotometrically at 450 nm, emission 620 nm.
3.2.2.4 STATISTICAL ANALYSIS

Data were analysed using the Statgraphics computer programme (Manugistics Inc., Maryland, USA). Data were checked for normal or non-parametric distribution using probability plots before analysis. One-way analysis of variance (ANOVA) and Scheffe multiple range tests (at a 95% confidence level) were used to assess effects on normally distributed data. This procedure looks at the effect of one qualitative factor (such as concentration of base attached to plate) on one response (e.g. binding of avidin). Multiple range tests investigate the differences between the group containing the control and other test means. The Students t-test (at a 95% confidence level) was used to compare two sample means, where appropriate.
3.3 RESULTS

Avidin-FITC was first seen to bind directly to the nucleus in IMR32 cells irradiated with UVA light, in which ROS-mediated damage was expected (Figure 1.1). This discovery was made during experiments designed to investigate binding of an antibody, raised against oxidatively-modified DNA, to UVA-irradiated fibroblast cells. Fluorescence was observed localised in the nucleus of irradiated control cells in the absence of primary antibody. No binding was observed in the nuclei of sham irradiated cells. This led to a comparison of the structures of biotin and oxidatively-modified DNA bases, to ascertain whether avidin might bind directly to these products.

3.3.1 STRUCTURES OF 8-OXODEOXYGUANOSINE, 8-OXOGUANINE, GUANINE AND BIOTIN

It was observed that the optimised structures of the 6,8-diketo tautomer of either 8-oxoguanine (8oxoG) or 8-oxodeoxyguanosine (8-oxodG) are very similar to biotin (Figure 3.1). These molecules all possess an imidazolidone group. The 2 rings of the biotin molecule lie in a cis-configuration, with a valeric acid side-chain attached to the C2 of the ureido group, also lying cis with respect to the imidazolidone ring (Green, 1975). 8-oxoG and 8-oxodG also consist of two rings fused in the cis-configuration. Whilst neither possess an alkyl side-chain the deoxyribose sugar group of 8-oxodG could lie in a similar position to the acidic side-chain of biotin. Although this sugar moiety is attached to the N3 of the imidazolidone ring, its 2 oxygen groups may be in similar positions to the two oxygens of the valeric acid in biotin (Figure 3.2). This apparent structural similarity was enough to warrant an investigation into the hypothesis that avidin binds to 8-oxodG, and that this was the reason for results observed in UVA irradiated cells.

3.3.2 FLUORESCENT BINDING ASSAY

Initial studies investigated the sensitivity of avidin binding to various concentrations of 8-oxodG. These results were compared to those obtained using deoxyguanosine (dG). These bases did not bind to untreated culture plates (data not shown), but were apparently immobilised on a substratum of fixed 3T3 fibroblast cells, although the mechanism of this binding is not known. Avidin conjugated to the fluorescent label FITC bound in a significant manner to 8-oxodG (p<0.001) (Figure 3.3). Maximum binding occurred to 10^4M 8-oxodG, with levels remaining similar after this point. Binding of avidin to 10^5M 8-oxodG was statistically significantly greater than binding in the
binding in the presence of $10^{-5}$M dG on the same plate ($p=0.04$) (Figure 3.4). Levels of binding to both dG and 8-oxodG were significantly higher than to cells alone (data not shown).

Binding of avidin to a greater concentration range of both 8-oxodG ($10^{-16}$M-$10^{-9}$M) and dG ($10^{-12}$M-$10^{-6}$M) was then investigated. Significant levels of binding were observed to $10^{-15}$M 8-oxodG, with maximum binding occurring at $10^{-11}$M ($p=0.057$) (Figure 3.5). Levels remained constant after this point. However, binding to dG increased slowly in a dose-dependent manner, reaching a maximum around $10^{-5}$M ($p=0.013$), although there was one seemingly aberrant point at $10^{-10}$M. The level of dG binding appeared to be 6 magnitudes lower than 8-oxodG, but avidin binding was significantly increased in the presence of both moieties. There was no statistical difference between 0% levels or 100% levels of binding for bases in these experiments.

A FITC-conjugated monoclonal antibody raised against biotin had a lower affinity for 8-oxodG and dG when compared to avidin-FITC. A dose-dependant increase was detected up to a maximum of $10^{-9}$M for both 8-oxodG ($p=0.046$) and dG ($p=0.20$), after which a decrease in binding was observed (Figure 3.6). The level of binding was not as high as that of avidin, over the same concentration range of either base product. For a direct comparison to be made between the antibody and avidin, data for the former were expressed as a percentage of maximum binding of avidin-FITC from the data set shown in Figure 3.5.

### 3.3.3 THE EFFECT OF ALKALI TREATMENT ON AVIDIN BINDING

It is known that 8-oxodG is unstable under basic conditions, and alkali treatment of oligonucleotides containing this oxidised base causes them to decompose (Torres et al., 1996). It would therefore be expected that exposure of oxidatively damaged cells to alkaline conditions would decrease avidin binding, due to the destruction of the proposed 'epitope' for this molecule. IMR32 cells exposed to hydrogen peroxide were treated with alkali before incubation with avidin, and the level of binding observed compared with cells left at neutral pH (Figure 3.7). In cultures which were left at pH 7.0 there was increased binding of avidin after exposure to $10^{-9}$M and $10^{-6}$M hydrogen peroxide compared to the control. When cultures were exposed to an alkaline environment (pH 12.0), however, no increase in binding was observed, suggesting that the binding site of avidin has been destroyed in these cells.
3.3.4 FURTHER EVIDENCE

Further evidence supporting the hypothesis that avidin binds directly to oxidatively modified DNA has been obtained using cell systems, DNA bound to ELISA-type plates and pathological tissue (Table 3.2) (Struthers et al, 1997; Appendix 1). Experiments were carried out on DNA treated with methylene blue, which is known to cause oxidative base damage, specifically 8-oxodG, and the results obtained compared with those for normal DNA. Avidin binding was greater to methylene blue-treated single-stranded (ss) DNA and double-stranded (ds) DNA compared to normal ssDNA or ds DNA respectively, although levels of binding were higher in both normal and treated ssDNA than dsDNA. Fixation with 4%(w/v) paraformaldehyde, however, removed this difference. Binding of the avidin conjugate could be blocked by incubation with specific competitors, including 8-oxodG and biotin. The efficacy of inhibition was 8-oxodG > biotin > 8-oxoguanine > guanosine > guanine. Experiments carried out on IMR32 cells grown in Labtek slides showed biotin and 8-oxodG to block binding of fluorescently labelled avidin after exposure to hydrogen peroxide. Guanine did not inhibit this binding. Avidin binding to post-mortem sections of neural pathological tissue taken from patients suffering from neurodegenerative disease, in which oxidative damage is suggested to occur, was much higher than that observed in age-matched control samples.

These results all strongly support the hypothesis that avidin is able to bind directly to the oxidatively-modified base 8-oxodeoxyguanosine. A simple assay allowing detection of this base lesion in cell systems and pathological tissue was therefore optimised.

3.3.5 DEVELOPMENT OF A DNA DAMAGE ASSAY

The initial protocol followed was the same as the immunocytochemistry assay used with the antibody under investigation when avidin was first observed to bind directly to fixed cells. Several changes were made to this procedure in the attempt to standardise the assay and decrease variability.

3.3.5.1 DETECTION OF AVIDIN BINDING

The initial protocol used FITC-conjugated avidin as a fluorometric end-point to assess levels of binding. Although this is a very sensitive detection method, the equipment involved in determining fluorescence levels in cells grown in 96-well plates is not widely available. Results obtained were also not found to be very reproducible due
to the limitations of the machine available. A colorimetric end point was therefore investigated. Binding of avidin conjugated to the enzyme horseradish peroxidase (HRP) was detected in IMR32 cells exposed to hydrogen peroxide, using the substrates o-phenylenediamine (OPD) or 3,3',5,5'-tetramethylbenzadine (TMB) (Figure 3.8). These are metabolised by the HRP attached to the avidin, causing a colour change which can be measured simply using a spectrophotometer. A dose-dependent increase in avidin binding was observed using both end-points, with no significant differences between results. It was therefore decided that TMB would be used in future experiments for convenience; it is provided in a ready to use form and is easy to dispose of, unlike OPD which is highly toxic.

3.3.5.2 AVIDIN DILUTIONS

The concentration of avidin-HRP used was initially 2μg/ml. On addition of TMB in these experiments a suitable colour change was observed within 10 minutes. The recommended incubation time for this substrate is 15 to 30 minutes, suggesting that the conjugate could be diluted further. An experiment was therefore carried out to assess the lowest concentration of avidin-HRP able to detect increases in damaged base products in IMR32 cells exposed to hydrogen peroxide (Figure 3.9). The greatest change in absorbance between control cells and treated cells occurred when 1μg/ml avidin-HRP was used in the assay. Avidin binding was significantly higher in cells treated with both concentrations of hydrogen peroxide when compared to untreated control cultures (p<0.001). There was also increased binding after exposure to 10^{-6}M H_{2}O_{2}, as opposed to 10^{-9}M H_{2}O_{2}. The same pattern of binding was observed when 2μg/ml avidin-HRP was used, but changes in absorbance measured in control and treated cultures were not as great. Increased binding of avidin-HRP was also observed when 0.25μg/ml was used, but this was not significant. There was no significant change in binding at a concentration of 0.1μg/ml, with results showing no consistent pattern, and larger variation. It was therefore decided to use a concentration of 1μg/ml in further experiments, as this detects damage in treated cells with the least variability.

3.3.5.3 BLOCKING AGENTS AND CONDITIONS

The tissue culture plates used in these experiments are specially coated with proteins for the attachment of adherent cell lines. The avidin conjugate appears to bind strongly to this coating in a non-specific manner. It was therefore necessary to block these binding sites, as background binding of the conjugate was very high. Several
potential blocking agents were investigated, and results compared to wells in which buffer only was added. This experiment was carried out on untreated cells. 10%(v/v) normal goat serum (NGS) was shown to give the lowest background level of binding, while the other agents used appeared to increase binding of the conjugate to the plate (Figure 3.10). A comparison was made between background levels observed after blocking with freshly prepared NGS or NGS stored at 4°C for 1 week or less. Freshly prepared NGS was shown to give the best results (experiment carried out by Siân Thomas). NGS prepared immediately before use was therefore the blocking agent of choice in the final assay protocol.

The lowest dilution of blocking agent needed to give a consistently low background of binding was then investigated. Cells were incubated with decreasing dilutions of NGS under the same conditions, and the results compared with background levels observed after incubation in wash solution only (Figure 3.11). Once again untreated cells were used, and all NGS was prepared immediately before use. 1%(v/v) NGS did not lower the background binding appreciably, whilst 5%(v/v) NGS decreased the absorbance from approximately 0.9 units to 0.7 units. 10%(v/v) NGS decreased this further to approximately 0.6 units. This was not reduced further by increasing the dilution of NGS to 15%(v/v). It was therefore decided to use freshly prepared 10%(v/v) NGS as the blocking agent in the final assay procedure.

The optimum conditions for blocking were then considered. Cells were blocked at room temperature or 37°C for either 1 hour or 2 hours (Figure 3.12). Absorbance values were higher when blocking was carried out at room temperature for either time point. Incubation for 1 hour at room temperature did not greatly decrease non-specific binding, although incubation for 2 hours at this temperature did lower the mean absorbance from approximately 0.85 units to 0.65 units. The background binding was reduced further to 0.60 units after incubation for 1 hour at 37°C, with a slight decrease when the incubation time was raised to 2 hours. From these results it was decided that blocking for 1 hour at 37°C would be sufficient for use in the final assay.

The final procedure for blocking non-specific binding sites involved incubation with 10%(v/v) NGS, freshly prepared immediately before use, for 1 hour at 37°C.
3.3.5.4 THE EFFECTS OF FIXATION TIME IN ALCOHOL

In the initial assay protocol cells were often left in 99%(v/v) IMS overnight at -20°C, before rehydration and blocking steps. It is known that an incubation time of 30 minutes on ice is sufficient for fixation. The effects of leaving prefixed cells for longer than this was therefore assessed in IMR32 cells exposed to hydrogen peroxide. Avidin binding was observed to be lower in both treated and untreated cells left overnight (Figure 3.13). It was therefore decided to use an incubation time of 30 minutes on ice for this step in the final assay protocol.

3.3.5.5 THE EFFECTS OF INCUBATION TIME WITH AVIDIN CONJUGATE ON BINDING LEVELS

The incubation time with avidin-HRP initially varied between experiments, with cells being left in the conjugate for either 1 hour at 37°C or overnight at 4°C. Incubation for 1 hour at room temperature is not as effective as 1 hour at 37°C (data not shown). Any differences in binding levels under these different incubation conditions were therefore compared. Avidin binding was observed to be higher after incubation overnight at 4°C, with a mean absorbance of 0.2 units (data not shown). The mean absorbance was less than half this when incubation was for 1 hour at 37°C. It was decided that cells should be incubated in avidin-HRP overnight at 4°C in the final assay.

These results have led to the development of an assay capable of detecting increased levels of 8-oxodeoxyguanosine in cultured cells damaged by hydrogen peroxide.
Figure 3.1 Line diagrams showing the chemical structures of the most common tautomeric form of guanine, the 6,8-diketo tautomers of 8-oxoguanine and 8-oxodeoxyguanosine compared to biotin, the natural ligand for avidin.
Figure 3.2 Ball and stick diagrams showing the structures of the most common tautomeric form of guanine, the 6,8-diketo tautomers of 8-oxoguanine and 8-oxodeoxyguanosine compared to biotin, the natural ligand for avidin. This shows how oxygen groups of the sugar in 8-oxodeoxyguanosine may lie in similar positions to those of the valeric acid group of biotin.
guanine

6,8-diketo tautomer of 8-hydroxyguanine

biotin

6,8-diketo tautomer of 8-hydroxyguanosine
Avidin affinity for 8-oxodeoxyguanosine (at concentrations of $10^{-5}$-$10^{-11}$M)

![Graph showing the affinity of FITC-conjugated avidin for 8-oxodeoxyguanosine.](image)

**Figure 3.3** The affinity of FITC-conjugated avidin for the oxidatively modified base 8-oxodeoxyguanosine was assessed by the level of binding to increasing concentrations of base bound to a substratum of prefixed cells. Results were collected using a fluorescent plate reader (excitation 550nm, emission 620nm). Data was expressed as Mean Arbitrary Fluorescent Units (AFU) vs Concentration of Base (M). All values are mean± standard error of the mean (SEM), where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values, using one-way ANOVA procedures with Sheffe multiple range tests. This data is representative of at least 5 different experiments.
A comparison of avidin-FITC binding in the presence of $10^{-4}$M 8-oxodG or $10^{-4}$M dG

![Bar chart showing avidin binding to 8-oxodeoxyguanosine (8-oxodG) and its unmodified deoxynucleotide 8-deoxyguanine (dG). Data points are means AFU ± SEM, where n = 8 wells. Data was analysed using the students 2-tailed t-test. * shows a significant difference from the dG value (p = 0.04). This data is representative of at least 5 different experiments.]()
A comparison of avidin affinity for 8-oxodeoxyguanine (10^{-9}-10^{-10}M) and its unmodified deoxynucleotide analogue deoxyguanine (10^{-6}-10^{-7}M)

![Graph showing the affinity of avidin-FITC for 8-oxodeoxyguanosine or deoxyguanine](image)

Figure 3.5 The affinity of avidin-FITC for 8-oxodeoxyguanosine or deoxyguanine was compared by assessing the level of binding to different concentrations of base bound to a substratum of cells. Data are expressed as a percentage difference between the maximum and minimum levels of binding for either 8oxodG or dG. There was no statistical difference between maximum or minimum values for binding to either base. Values are means ± SEM, where n = 8 wells. * shows points which are significantly different at the 95% confidence level from control levels in that data set, using ANOVA procedures and Sheffe multiple range tests. Deoxyguanosine data are offset for clarity. This data is representative of at least 3 different experiments.
Affinity of an anti-biotin antibody for 8-oxodeoxyguanosine (10^{-11}-10^{-4}M) or its unmodified deoxynucleotide deoxyguanine (10^{-11}-10^{-3}M)

Figure 3.6 The affinity of a FITC-conjugated monoclonal antibody to biotin for 8-oxodeoxyguanosine or deoxyguanine was compared by assessing the level of binding to different concentrations of base bound to a substratum of cells. Data are expressed as a percentage difference between the maximum and minimum levels of binding for either 8oxodG or dG. There was no statistical difference between maximum or minimum values for binding to either base. Values are means±SEM, where n = 8 wells. * shows points which are significantly different at the 95% confidence level from control levels in that data set, using ANOVA procedures and Sheffe multiple range tests. DG data are offset. This data is representative of at least 3 different experiments.
An investigation into the effects of pH on avidin binding carried out on IMR32 cells fixed 1 hour after exposure to hydrogen peroxide

Figure 3.7 IMR32 cells were exposed to hydrogen peroxide for 1 hour, then left in fresh media for a further hour before fixing. After fixation cells were incubated in wash buffer at either pH7.0 or pH12.0 (corrected with 1M NaOH) for 30 mins at room temperature, before blocking and incubation with avidin-HRP. Levels of avidin were assessed colorimetrically using TMB. The data are means ± SEM, where n = 8 wells. This data is representative of at least 3 different experiments.
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<td><strong>To investigate the specificity of avidin for oxidatively modified DNA</strong></td>
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Table 3.2 Other evidence supporting the hypothesis that avidin binds directly to oxidatively modified DNA. These experiments were carried out by Rahkee Patel, Jennie Clark and Siân Thomas, in conjunction with work presented in this thesis (Struthers et al., 1997).
Results of avidin assay carried out on IMR32 cells fixed 1 hour after exposure to various concentrations of hydrogen peroxide - Comparison of TMB and OPD end points

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A comparison of avidin assay results in IMR32 cells exposed to hydrogen peroxide using different dilutions of conjugate.

Figure 3.9 The optimum concentration of avidin-HRP needed to detect abnormal base lesions was assessed in IMR32 cells exposed to $10^{-9}$M or $10^{-6}$M hydrogen peroxide. A concentration of 1µg/ml was shown to give the clearest results. Data points are the mean absorbance ± SEM, where $n = 8$ wells. * shows points which are significantly different at the 95% confidence level from control levels in that data set, using ANOVA procedures and Sheffe multiple range tests.
The effects of different blocking agents on avidin binding to IMR32 cells exposed to HBSS only

**Figure 3.10** The effectiveness of different blocking agents in the avidin assay were assessed in untreated cells, using an avidin-HRP detection system. Levels of binding were quantified using TMB as a substrate. Data points are the mean absorbance ±SEM, where n = 16 wells.
Graph showing the effectiveness of different dilutions of normal goat serum as a blocking agent in the avidin assay

![Bar chart showing mean absorbance](image)

**Figure 3.11** The effectiveness of different dilutions of normal goat serum as a blocking agent in the avidin assay were assessed in untreated cells, using an avidin-HRP detection system. Levels of binding were quantified using TMB as a substrate. Data points are the mean absorbance ±SEM, where n = 16 wells.
Graph showing the effectiveness of 10% (v/v) normal goat serum as a blocking agent under different incubation conditions in the avidin assay.

**Figure 3.12** The optimum blocking conditions were investigated in untreated IMR32 cells using 10% (v/v) normal goat serum. Different incubation temperatures and times were assessed for their ability to decrease background binding in the avidin assay. Avidin-HRP was used, with TMB as a substrate. Absorbance values are means ± SEM, where n = 16 wells.
An investigation into the effects of fixation time in 99% (v/v) IMS on avidin-HRP binding levels:
A = fixation over 30 mins, B = fixation overnight

**Figure 3.13** The effects of fixation time in 99% (v/v) IMS was investigated in IMR32 cells exposed to 10^{-6}M H_2O_2 and untreated cells. Cultures were left in 99% (v/v) IMS for 30 minutes on ice or overnight at -20°C before incubation with avidin-HRP and detection of binding using TMB as a substrate. Absorbance values are means ± SEM, where n = 16 wells.
3.4 DISCUSSION

The modified base 8-oxoguanine is thought to be most stable in its 6,8-diketo form (Aida and Nishimura, 1987). This molecule consists of 2 fused rings, and has an imidazolidone group identical to that of biotin. It is this part of biotin which appears to be largely responsible for its high affinity with avidin, with the interaction between the N-1 moiety and protein being particularly strong (Green, 1975). In DNA the base is attached to a deoxyribose sugar via the N-3 of the imidazolidone residue. This bulky substituent could lie in a similar position to the valeric acid alkyl chain found on the C-2 of the ureido group in biotin, which also contributes to the binding of biotin and avidin. Therefore the structure of the modified deoxynucleoside 8-oxodeoxyguanosine (8-oxodG) was also investigated; 8-oxodG and biotin are both 2-ring structures, possessing identical imidazolidone groups, with bulky substituents attached in similar positions, although the aromatic ring of 8-oxodG, or that of 8-oxoG, is not the same as the ureido group of biotin. These compounds clearly possess certain similarities, and a rigorous structural investigation of the binding between protein and base, using molecular modelling techniques, is currently underway. The second pyrimidine base, adenine, also consists of 2 rings, one of which is an imidazolidone group. This suggests that avidin will bind 8-oxoadenine and 8-oxodeoxyadenosine in addition to 8-oxodG and 8-oxoG. Although this could make the technique less specific it may increase the strength of the signal, as two base products would be detected instead of one. The validity of the method would not be affected because levels of 8-oxodeoxyguanosine are taken as an indication of total DNA damage. This investigation of the nature of avidin binding was restricted to 8-oxodG, which is thought to be a major oxidative lesion (Floyd et al, 1986; Ames, 1989).

Studies carried out by Jennie Clark investigated avidin binding to DNA attached to 96-well plates using an ELISA-type assay (Struthers et al, 1998). The fact that avidin bound to untreated DNA is perhaps not surprising; commercial DNA is processed extensively, and levels of 8-oxoG have been shown to be 0.4nmol/mg DNA or 3.2 mol/10^5 mol guanine in calf thymus, using HPLC (Ames et al, 1995). These experiments showed a pre-requisite for the detection of oxidatively-modified bases in intact DNA with avidin appeared to be single-strandedness in the region of interest. Binding to unfixed ssDNA was higher than to dsDNA, whether it was untreated or treated with methylene blue. When the assay was carried out after fixation of the DNA with paraformaldehyde, however, this difference in binding was not observed. It is known that paraformaldehyde
forms crosslinks between protein molecules, and this is what preserves the architecture of the cell so well. Another effect of the fixation process is denaturation. The extent of this is thought to be relatively low in cells exposed to paraformaldehyde compared to other fixatives, but results show that fixation of DNA resulted in increased binding of avidin. Although the avidin molecule is relatively small, it is highly unlikely that it could bind damaged bases within the intact, ds helix of DNA. This suggests that the lesions detected lie in regions of single-strandedness which are caused partly by exposure to oxidative stress, and partly by processes which occur during the fixation procedure. It would be difficult to further denature proteins surrounding DNA in cells and tissue, using methods such as heat, without introducing artefactual damage, and thus decreasing the value of the assay, but it may be of interest to investigate fixation methods further, to see whether increased exposure of avidin to the sites of damage can be achieved.

Inhibition studies were also carried out on naked DNA attached to 96-well plates and in cultured IMR32 cells, both of which were exposed to ROS-generating systems known to induce oxidatively-modified DNA (Struthers et al, 1997). Levels of avidin binding to methylene blue treated DNA were much greater than to normal DNA. This binding was decreased greatly by co-incubation of avidin with biotin, 8-oxodG and 8-oxoG, although guanine and guanosine had a smaller inhibitory effect. Other experiments showed avidin binding to be inhibited in hydrogen peroxide-treated IMR32 cells, when incubated with either 8-oxodG and biotin, but not guanine. This was demonstrated visually using fluorescence microscopy. These results showed that avidin binding is specific for DNA, and that the biotin binding site is directly involved, since binding to DNA is blocked by biotin. The fact that 8-oxodG could also block binding suggested that this lesion could be the epitope for avidin.

The affinity of avidin for various concentrations of 8-oxodG was investigated, to assess the sensitivity of binding. A layer of 3T3 fibroblast cells, fixed and permeabilised, was used as a substratum to which the different concentrations of modified nucleoside bound. Cells were untreated, and their density across the plate was constant. This fibroblast cell line was used because cells were easy to grow, and were ready to use the day after they were plated out. Differentiated and undifferentiated IMR32 cells have also been used as a substratum however, showing that the type of cell is not important. Avidin, conjugated to a fluorescent marker, was then incubated with bound base and levels of binding assessed using a fluorescent plate reader. Binding of avidin to oxidised
base was only observed in the presence of an underlying cellular layer, and when assays were carried out on blank plates very little fluorescence was seen. The mechanism of attachment of base to substratum is not known; it is purely phenomenological. Binding is non-specific, and the most likely explanation is of an electrostatic attraction between the fixed cells and base.

A comparison of avidin binding to various concentrations of 8-oxodG or the unmodified deoxynucleoside dG showed the protein to have a much higher affinity for the former, although some binding to dG was seen at high concentrations. Significant binding was observed to $10^{-15}$M 8-oxodG. Assuming 100% binding of the modified base to the substratum, this suggests this technique may be a very sensitive method for assessing oxidatively-damaged DNA, with a theoretical detection limit of approximately $10^4$ molecules of 8-oxodG in $10^6$ cells (1 base product per 100 cells) (see Appendix 2 for calculation). An anti-biotin antibody had a much weaker affinity for 8-oxodG than avidin; levels of binding were less than 50% lower, and a maximum was observed at base concentrations of $10^{-9}$M, several magnitudes lower than avidin. This is not unexpected, as the affinity of monoclonal antibodies for biotin are known to be much lower than that of avidin (Dakshinamurti and Rector, 1990). The exact epitope for this antibody is not known. It is possible it may not bind to biotin in the same manner as avidin, and would therefore not be expected to have the same affinity for the molecule. The decrease in binding observed at higher concentrations could be due to steric hindrance, as antibodies are much larger than avidin.

In these experiments avidin was observed to bind significantly to dG, although its affinity appeared to be approximately 6 magnitudes lower than 8-oxodG. The anti-biotin monoclonal also bound to dG, as well as the oxidatively modified base, and as mentioned previously, guanine and guanosine were shown to inhibit avidin binding to oxidatively damaged DNA. It is known that commercial preparations of deoxyguanosine contain a small amount of 8-oxodeoxyguanosine, at the level of 1 residue per 137,440 dG residues (Park, 1992). This may vary in different batches of stock dG, with the ratio being on average 1 residue per 100,000 (Ames, 1989), although values may be as high as 1 residue per 20,000. This would clarify why some binding of avidin to dG is observed in these experiments. It would also explain why the maximum level of binding to dG at $10^{-5}$M was comparable to $10^{-11}$M for 8-oxodG.
This type of assay was only carried out using immobilised dG or 8-oxodG, not other nucleotides, although a similar pattern of non-specific binding would be expected for each base. It would perhaps be of interest to substitute biotin for the oxidatively modified bases, allowing a direct comparison. The main disadvantage of this method was that attachment of the base to the cellular substratum could not be quantified across the plate. The patterns of avidin binding observed in these experiments were somewhat 'saw-toothed' in nature, although the trends were clear. It is possible this was caused by irregular binding of bases in wells. Covalent attachment of bases to the plate would ensure uniform coverage, but certain aspects, such as the correct orientation of the molecule, would be difficult to achieve satisfactorily. A better solution would be to alter the assay slightly by immobilising the avidin itself, using streptavidin-coated plates or magnetic streptavidin-coated beads, rather than the base. This would almost certainly produce more accurate results with fewer inconsistencies.

Further experiments were carried out in whole cells to help establish the epitope of avidin in biological systems. Cultured neuronal cells were exposed to hydrogen peroxide, an established method of inducing oxidatively-damaged DNA. Avidin binding was observed to increase in an insult dependent manner (Struthers et al., 1998). When cells were exposed to alkali after hydrogen peroxide treatment this binding was decreased to the level observed in untreated control cultures. Alkaline conditions are known to destroy the imidazolidone group (Torres et al., 1996), which is only present in the oxidised form of the nucleoside. This strongly suggests that avidin binding involves the imidazolidone group of 8-oxodG, and observed binding to dG is due to the presence of small amounts of the oxidised nucleoside.

There is evidence for the direct involvement of ROS in neurodegenerative diseases such as MND and Parkinson’s Disease (Coyle and Puttfarcken, 1993). As DNA is very sensitive to ROS attack, the ability of avidin to bind to human pathological brain tissue was also investigated (Struthers et al., 1998). Sections were taken from the motor cortex of patients who had died of motor neuron disease (MND), and avidin binding was higher in these when compared to levels observed in sections taken from control cases.

It is of interest that avidin binding in the absence of primary antibody has been demonstrated previously in pathological brain tissue (Cullen, 1994). Avidin was used to detect neurofibrillary tangles, senile plaques and neuropil threads in sections from patients
with Alzheimer’s disease. Avidin appeared to bind directly to these pathological inclusions. Their structure, however, has not been shown to contain DNA to date, although they are heterogeneous in nature. The mechanism of avidin binding was not determined in this study. Other evidence for avidin binding specifically to cellular DNA is circumstantial. Avidin binding has been observed in the nucleus of cells (Heggeness, 1977; Duhamel and Johnson, 1985), and it has been suggested that avidin binds to necrotic cells (Wood and Warnke, 1981). Special blocking kits are available commercially, where samples are pre-treated with avidin and biotin to pre-block 'non-specific' sites (Duhamel and Johnson, 1990). A systematic investigation of this phenomenon does not seem to have been carried out however. Non-specific binding of avidin observed is often thought to be due to the presence of endogenous biotin within the cell. Biotin is primarily found within carboxylase enzymes, such as pyruvate carboxylase, where it acts as a carrier molecule for activated CO₂. These enzymes are located within the mitochondria. This interpretation of results does not easily account for the nuclear binding observed. Neither does it explain the fact that avidin binding was greatly increased after exposure to oxidative stress, whilst negative controls, exposed to buffer only, showed low levels of binding. There is no evidence to suggest that levels of carboxylase enzymes increase following exposure to H₂O₂, and so a different explanation must be sought, especially as avidin binding was inhibited by the presence of 8-oxodG but not dG.

Together, the experimental evidence described all suggests that avidin binds to 8-oxodG and biotin with similar affinity, and that this binding involves the biotin-binding site of avidin. Preliminary experiments have used this property to demonstrate DNA damage in naked DNA, cultured neuronal cells, and pathological tissue. This type of direct demonstration of damaged is very desirable, as methods to date involve isolation of DNA from cells and tissue; resulting artefactual damage may greatly influence the results obtained using these techniques. The method utilising avidin was therefore developed further, with each step being optimised to give the best results.

The initial assay was based on an immunocytochemistry protocol involving a primary antibody, with avidin-FITC acting as part of the detection system. This was used for direct visualisation of binding in cells grown on slides. When this methodology was transferred to cells grown in 96-well plates, results were not always reproducible however. To obtain the best results from this technique the assay needed to be adapted
and optimised for use in 96-well plates. Differentiated IMR32 cells exposed to hydrogen peroxide were used as a model system during assay development; this method was known to produce oxidatively-damaged DNA, and avidin had been shown to bind in a dose-dependent manner to the 8-oxodG lesion using it.

A fluorometric end-point had been used previously, with avidin being conjugated to FITC. This is useful when binding is to be visualised directly in cells, but not very practical for an ELISA-type assay, as fluorescent plate readers are expensive and not widely available. A colorimetric end-point would be of much more value when investigating avidin binding in cells grown in 96-well plates, and would allow semi-quantitative results to be obtained with ease. Two end-points were studied, OPD and TMB. These are substrates for horse-radish peroxidase which may be conjugated to avidin. Both had the same sensitivity of detection. Slightly higher errors were obtained when OPD was used. This compound is highly toxic, and must be made up from stock reagents each time the assay is carried out. TMB is in a ready-to-use format, which is easier and also decreases the potential for practical errors, and is not as toxic as OPD, making it safer to use. TMB was therefore the colorimetric end-point of choice for the DNA damage assay.

It was also important to establish the correct concentration of avidin-conjugate to use in experiments. An avidin-FITC concentration of 5μg/ml was used when the initial observation of binding in UVA irradiated cells was made, because the molecule was acting as part of an amplification step in the visualisation method. A much lower concentration of conjugate should still be able to detect damaged DNA, because of the high affinity and specificity of the interaction. If binding were only observed with a high concentration of avidin, then a high level of non-specific binding would be suspected. The clearest detection of DNA lesions was obtained using a concentration of 1μg/ml avidin-HRP. This was much lower than the original concentrations, and shows the technique to be a very specific and sensitive method for investigating levels of DNA damage. This concentration should be appropriate for both colorimetric and fluorometric end-points.

Blocking of non-specific binding sites was important when this method was carried out on cells grown in 96-well plates, as the avidin appeared to bind strongly to the treated plastic. This non-specific binding was virtually eliminated by incubation with normal goat
serum before addition of the conjugate. The mechanism of binding is again unknown, but it was assumed that part of the avidin-HRP molecule was sticking to the wells. The appropriate controls should therefore be carried out, to ensure non-specific binding to blank wells is very low. It should also be noted that different blocking agents may be needed for different systems; when studies were carried out on DNA using Millipore Multiscreen filtration plates 1%(w/v) gelatin was found to be the most appropriate for blocking non-specific sites.

Further changes made were relatively minor. The most appropriate dilution of the blocking agent, and incubation conditions for this, fixatives and avidin conjugate were decided. The final assay procedure gave much more reproducible results and smaller errors. The reproducibility could be improved further, however, by optimising the fixation procedures. A paraformaldehyde/glutaraldehyde solution was used because this is a well-established procedure in cytochemical experiments. Fixation is necessary for avidin binding to occur, and, as mentioned previously, this may be because it produces regions of ssDNA to which the probe can bind. It is possible that a different fixation technique would allow even greater access to the damaged bases, thus increasing the signal detected. Another aspect which could be improved is the incubation times at each step, because there was not time to properly evaluate these in the course of this study. Improving these aspects of the assay could improve reproducibility and decrease errors still further.

Exposure to hydrogen peroxide is a well-established method of inducing oxidative stress, and was therefore chosen for use in the development of this assay. Other groups have shown increased levels of oxidative DNA damage in naked DNA or cultured cells treated with H$_2$O$_2$ using HPLC (Blakely et al, 1990; Rosen et al, 1996), alkaline elution methods (Giandomenico et al, 1997) and in situ nick translation (Hoyt et al, 1997). The increased avidin binding observed in differentiated IMR32 cells after treatment with H$_2$O$_2$ was presumed to be due increased levels of oxidatively-modified bases. It is important, however, to confirm this fact using another method. DNA isolated from cells treated with a concentration of H$_2$O$_2$ known to produce increased binding of avidin should therefore be analysed using HPLC, and the results compared with those obtained from the DNA of control cells not exposed to oxidative stress. Levels of 8-oxodG would be expected to be higher in treated cells treated with H$_2$O$_2$. This should be done in the near future.
A limitation of this assay is that it is semi-quantitative, unlike techniques such as HPLC and GC-MS which are commonly used to assess oxidatively-damaged base products. An attempt was made to quantify the levels of avidin bound to 8-oxodG using a method modified from a novel protein assay (Mortimore, 1996), and oligomers containing a known number of 8-oxodG residues. Dilutions ranging from 1μg/μl to 10⁻¹⁰μg/μl were prepared and attached to filter paper by baking. This paper was then incubated with avidin-peroxidase and binding was visualised using DAB substrate. Unfortunately no binding was detected. It is most likely that the DNA was not securely attached to the filter paper, and was washed away during subsequent steps. It would be of interest to develop this method further with modifications designed to attach the oligomers to the filter paper more firmly. It may also be possible to modify this type of experiment for use with the Millipore Multiscreen 96-well filtration plates utilised during ELISA investigations of avidin binding to naked DNA. The base of these plates consist of a 0.45μm hydrophilic, low-protein binding, Durapore membrane, to which DNA attaches very strongly. Time did not allow for these studies to be carried out during the course of this thesis.

An assay utilising the ability of avidin to bind to the oxidatively-modified base 8-oxodG was therefore developed. This was capable of detecting DNA lesions directly in cells, and binding was demonstrated in cultures exposed to H₂O₂, a compound known to produce 8-oxodG (Aruoma et al, 1989; Dizdaroglu et al, 1991; Rosen et al, 1996). Further work needed to be carried out to investigate levels of damage induced on exposure to lower concentrations of H₂O₂, and so discover the sensitivity of the assay, to confirm that the avidin binding was dependent on ROS, and also to study the ability of avidin to recognise DNA damage induced by ROS-generating systems.
CHAPTER 4

DETECTION OF DNA DAMAGE IN CELLS EXPOSED TO DIFFERENT ROS-GENERATING SYSTEMS
4.1 INTRODUCTION

A novel assay was used to investigate whether it was possible to quantitatively assess oxidative DNA damage induced by a variety of ROS-generating systems. Cells were exposed to a wide range of H$_2$O$_2$ concentrations, and either UVA light or paraquat, both known to damage DNA indirectly via ROS. Avidin binding was then assessed. UVA has been used as a method of ROS production with this cell line in a previous study (Struthers et al, 1995). The results obtained would help establish whether the assay developed was capable of detecting 8-oxodG in more than one model of oxidative stress.

4.1.1 GENERATION OF ROS VIA HYDROGEN PEROXIDE (H$_2$O$_2$)

Hydrogen peroxide is routinely produced in mammalian cells during several processes, including normal aerobic respiration (Halliwell and Gutteridge, 1989). Normal levels are usually low (approx. 10$^{-8}$ M) (Boveris and Chance, 1973), but these levels, along with those of other ROS, may be raised during pathological conditions such as neurodegeneration, when the delicate balance between ROS production and cellular protection is disrupted and a state of oxidative stress ensues (Olanow, 1993).

H$_2$O$_2$ is an uncharged molecule, has a low molecular weight, and is also relatively stable (Pryor, 1986). It is believed that these properties allow H$_2$O$_2$ to diffuse from the site of production through cell membranes (Frimer et al, 1983). H$_2$O$_2$ itself is not thought to interact with DNA directly however (Aruoma et al, 1989b). It can be hypothesised that hydrogen peroxide added extracellularly to medium diffuses in through the cell plasma-membrane, but does not damage DNA directly; rather a set of a chain reactions is started as the molecule interacts with macromolecules such as lipid membranes, culminating in oxidative stress of the cell and DNA damage via species such as lipid peroxides and the hydroxyl radical. Current research has suggested that $'\text{OH}$ radicals are the main source of the damage observed (Aruoma et al, 1989b; Aruoma et al, 1991; Dizdaroglu et al, 1991). These may be produced during the interaction of H$_2$O$_2$ and metal ions such as Fe$^{3+}$ and Cu$^{2+}$, in the presence of a reducing agent such as the superoxide radical, via Fenton chemistry, as mentioned in Chapter 1 (Henle and Linn, 1997). Metal ions are known to be incorporated into the DNA helix, making site-specific damage to DNA components, including bases, very likely (Mello Filho et al, 1984; Drouin et al, 1996; Henle and Linn, 1997). The potential role of lipid peroxides in oxidative DNA damage remains to be elucidated at this time.
Hydrogen peroxide is a commonly used method of inducing oxidative stress in cell cultures, and is known to generate both strand breaks and oxidative lesions in DNA (Mello Filho et al., 1984; Hinshaw et al., 1993; Hoyt et al., 1997; Gardner et al., 1997). Lesions produced are known to include 8-oxodeoxyguanosine and 8-oxoguanine (Blakely et al., 1990; Rosen et al., 1996; Kennedy et al., 1997), and H$_2$O$_2$ was therefore used as a method of generating ROS in differentiated IMR32 cultures.

4.1.2 GENERATION OF ROS VIA UVA LIGHT

The UV component of sunlight may be divided into 2 distinct regions; UVA (320-380 nm, near UV) and UVB (290-320 nm, mid-UV). It is widely thought that the most damaging part of the spectrum in relation to skin carcinoma is UVB, as these wavelengths are directly absorbed by DNA. UVB is known to be responsible for many mutagenic lesions, the major one being cyclobutane pyrimidine lesions. This direct action of UVB, involving hydrogen abstraction of electron transfer, is known as a Type I reaction. It is now known that UVA light is able to penetrate deeply into the skin, and also has the potential to damage DNA in the presence of oxygen via oxidative stress (reviewed by Tyrrell, 1991). Human exposure to UVA appears to be increasing, partly due to the depletion of the ozone layer, and partly due lifestyle changes which involve longer exposure times in the sun coupled with the widespread use of UVB-absorbing sunscreen, and the use of modern tanning equipment. The recognition of the strong link between UV light and the incidence of skin cancer in recent years has led to a considerable amount of effort to elucidate the mechanisms of damage involved. Most of these studies using have involved skin fibroblasts as an in vitro model (Horio and Okamoto, 1987; Jurkiewicz and Buettner, 1994).

It is known that molecular oxygen is involved in UVA-induced damage (Tyrrell, 1991). UVA light entering cells may be absorbed by cellular compounds such as tryptophan, riboflavin, NADH and NADPH, which act as photosensitisers to generate H$_2$O$_2$ and superoxide intracellularly (Andley and Clark, 1989). Cellular membranes also contain many photosensitisers, such as cytochrome oxidase and other molecules involved in electron transport, which may contribute to lipid peroxidation events. In addition, singlet oxygen may be produced by the interaction of UVA light with chromophores such as flavins, quinones and porphyrins, via a Type II photodynamic process. Thus cells enter a state of oxidative stress, with the associated damage to cellular macromolecules, including DNA. It has been shown that the action of singlet oxygen and photosensitizers
in the presence of UVA generate predominantly base modifications, as opposed to single-strand breaks and sites of base loss (Müller et al, 1990; Epe et al, 1993). The predominant cellular defence against UVA appears to involve antioxidant pathways, rather than the multiple repair pathways involved in eliminating damage produced via UVB radiation (Tyrrell, 1991). This further emphasises the induction of oxidative stress as a consequence of UVA irradiation. Although UV exposure is not relevant to neurological systems this study used UVA as a method of intracellular ROS-production.

4.1.3 GENERATION OF ROS VIA PARAQUAT

Paraquat is a common herbicide, which has been involved in many human fatalities (Timbrell, 1990). This is usually due to deliberate oral ingestion, occasionally accidental, rather than the contamination of foodstuffs. The target organ of this compound is the lung, which selectively accumulates paraquat, and retains it even once plasma levels have fallen. This is because of the similarity in structure between paraquat and polyamines such as spermine, spermidine and putrescine, which have selective active transport systems in the lung (Timbrell, 1990). Most studies involving paraquat have therefore involved lung cells (Ali et al, 1996; Kawaguchi et al, 1996; Fabisiak et al, 1997). Work on tissue slices, however, show this toxin is actively taken up by brain cells (Smith and Wyatt, 1981). It should therefore be a viable method of inducing oxidative stress in neuronal cultures.

The toxicity of paraquat is due to the enzymatic production of its free radical form via a redox cycling process (Figure 4.1); superoxide is formed as a side-product, whilst the paraquat cation is regenerated and NADPH levels depleted. Superoxide can then initiate the formation of other ROS, such as hydrogen peroxide and the hydroxyl radical, all of which may damage cell macromolecules, including DNA.

Polyamine receptors exist in brain tissue, although neurones do not appear to accumulate paraquat (Timbrell, 1990). This may be due to the protective action of the blood-brain barrier, which prevents many potentially harmful molecules from entering the brain. It is likely, however, that the compound would be taken up by neuronal cells in vitro via dopamine uptake receptors. Paraquat has been shown to mediate DNA damage within rat lung microsomes (Ali et al, 1996). It will also induce the DNA repair enzyme 8-hydroxyguanine endonuclease (Kim et al, 1996), suggesting that the superoxide produced is involved in the production of the oxidative base lesion 8-oxoguanine.
Figure 4.1 Diagram showing the proposed mechanism of paraquat toxicity. SOD = superoxide dismutase. (Taken from Timbrell, 1995).
4.1.4 AIMS

The ability of avidin to detect 8-oxodG lesions in differentiated IMR32 cells, produced via these three different mechanisms was investigated. Avidin has already been shown to bind to DNA damaged by hydrogen peroxide during the development of the assay (Chapter 3). In the experiments involved, significant binding was observed over the concentration range $10^{-9}$M to $10^{-6}$M H$_2$O$_2$, with cell death occurring at concentrations greater than this. In order to establish a complete dose-response curve, it was necessary to expose cells to much lower concentrations of the toxin. The viability of cells was assessed in parallel cultures using the MTT assay, whilst protein assays were carried out to check that the cell density was even across the plate.

To further establish whether the damage detected was mediated by ROS, cells were preincubated with the antioxidant $\alpha$-tocopherol or the iron chelator desferrioxamine immediately prior to insult; decreases in avidin binding would be expected to be observed in the presence of these molecules if the DNA lesions were produced indirectly via the action of ROS. The morphology of cells was investigated after exposure to $\alpha$-tocopherol or ethanol, and at the end of each assay procedure.
4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

All biochemicals used were from Sigma (Poole, UK), except for the following: normal goat serum (NGS) from Dako (High Wycombe, UK); methanol, ethanol and glacial acetic acid from Fisons (Loughborough, UK). Components for the determination of avidin binding were provided as a research evaluation kit by Biotrin International (Dublin, Eire).

4.2.2 METHODS

Three methods of inducing different types of oxidative stress were investigated in a neuroblastoma model system; UVA irradiation, exposure to hydrogen peroxide and exposure to the herbicide paraquat. Experiments were carried out after 2-4 weeks of differentiation. The morphology of the cells was checked before, during and after each experiment. The extent of DNA damage was assessed using the novel assay developed, based on the binding of avidin to the oxidatively modified base 8-oxoguanine. The viability of the cells after insult and recovery were assessed by measuring the intracellular metabolism of the tetrazolium salt MTT. This is a standard colorimetric assay. Cell density across the plate was estimated using the Kenacid Blue protein assay. Cells were preincubated with the antioxidant α-tocopherol or iron chelator desferrioxamine before exposure to oxidative stress, to see whether levels of DNA damage were decreased, and therefore were due to the action of ROS.

4.2.2.1 PREINCUBATION OF CELLS WITH ANTIOXIDANT/IRON CHELATOR

Cells were pre-loaded with the lipid-soluble free radical scavenger α-tocopherol or iron chelator desferrioxamine in order to investigate their possible protective role during ROS damage. α-Tocopherol has previously been shown to enter IMR32 cells at concentrations which are protective (Thomas and Anderton, 1991). Desferrioxamine may potentially decrease damage by ROS produced via Fenton-like reactions.

α-Tocopherol and desferrioxamine (1000mM stock made up in absolute ethanol and HBSS respectively, immediately prior to use) were added to fresh medium (α-MEM supplemented with 5% (v/v) HIFCS and 1% (v/v) NEAA). Half the existing medium on cells was replaced with medium plus antioxidant or iron chelator, to a final concentration of 200μM, 24 hours prior to irradiation (Figure 4.2). Medium containing an equal
Figure 4.2 Pattern of dosing of IMR32 cells in 96-well plates with α-tocopherol or desferrioxamine and their respective carriers, ethanol and HBSS. Both compounds were added to a final concentration of 200μM.
volume of vehicle (absolute ethanol or HBSS) was added to control cells on the same plate. Cultures were incubated at 37°C overnight. After each manipulation, cell morphology was assessed using light microscopy.

4.2.2.2 EXPOSURE OF CELLS TO HYDROGEN PEROXIDE

Differentiated IMR32 cells were exposed to H₂O₂ as described in Section 2.2.3.4. Fresh medium (α-MEM supplemented with 5%(v/v) HIFCS and 1%(v/v) NEAA) was added and cultures left for various times at 37°C before assessment of cell death, using the MTT assay, and detection of DNA damage as described in Section 3.2.2.3.8.

4.2.2.3 EXPOSURE OF CELLS TO UVA IRRADIATION

Initial experiments were carried out on cells after UV-irradiation. UVA light was used as a ‘clean’ source of intracellular ROS generation for radical-mediated damage. A Blak-Ray UV lamp, Model UVL-56 with a glass filter, described in Section 2.2.3.5, was used.

Differentiated IMR32 cells were exposed to UVA light in the presence and absence of either α-tocopherol or desferrioxamine as described in Section 4.2.2.1. Cultures were initially irradiated at room temperature from above in a small volume of pre-warmed Hank’s Balanced Salt Solution (HBSS), after removal of media and washing with the buffer. Cells became easily detached under these conditions, leading to very uneven cell density in wells, and subsequently large standard errors. Irradiation after careful removal of media, with no washing of cultures was not any better. Irradiation in 50μl medium gave much better results. The rest of the medium was removed very carefully with a multistepper pipette. Before each irradiation the intensity of UV light was checked with an Optical Radiometer and the dose calculated, as described in Section 2.2.3.5. Cells were irradiated from 15cm at doses ranging from approximately 5-140 mJcm⁻² (Table 4.1). Control wells were sham irradiated. Exposed cells received cumulative doses, as this decreased the period of time they were not in media. After exposure to UVA, fresh medium (α-MEM supplemented with 5%(v/v) HIFCS and 1%(v/v) NEAA) was added (150μl/well) and cells left at 37°C for either 1 hour or 24 hours. Cell death was then assessed using the MTT assay, and avidin binding measured using a fluorometric end-point, as described in Section 3.2.2.3.8.
Table 4.1 Irradiation times and corresponding dose of UVA light received by cells. 8 wells were exposed to each dose. 8 control wells were sham irradiated. Light sources was 15 cm from cells. Doses are calculated from the average radiometer reading taken over the experiments carried out, and are therefore only approximate values.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Dose (mJcm$^{-2}$)</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>4.6</td>
</tr>
<tr>
<td>40</td>
<td>9.2</td>
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<tr>
<td>80</td>
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<td>160</td>
<td>36.8</td>
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<tr>
<td>320</td>
<td>73.6</td>
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<tr>
<td>640</td>
<td>147.2</td>
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</tbody>
</table>
4.2.2.4 EXPOSURE OF CELLS TO PARAQUAT

Differentiated IMR32 cells were exposed to paraquat as described in Section 2.2.3.6. Cultures were left for various times at 37°C before assessment of cell death, using the MTT assay, and detection of DNA damage as described in Section 3.2.2.3.8.

4.2.2.5 MTT ASSAY

This was carried out as described previously in Section 2.2.3.8.

4.2.2.6 DNA DAMAGE ASSAY

Assays were carried out using a fluorometric or colorimetric end-point, as described previously in Section 3.2.2.3.8.

4.2.2.7 PROTEIN ASSAY

Kenacid blue protein assays were carried out on selected plates after MTT assays or DNA damage assays to assess whether cell density was consistent across the plate. These plates could be wrapped in Nescofilm to prevent evaporation of contents, then stored at 4°C for up to 2 weeks before the protein assay was carried out.

The Kenacid blue stain (88%(v/v) dye stock, 12%(v/v) glacial acetic acid) was prepared from a dye stock solution (0.05% (w/v) Commassie Brilliant Blue R-250, 28%(v/v) ethanol, 72%(v/v) distilled H$_2$O, stored at room temperature) immediately before use. Solutions in the wells were tipped out carefully and Kenacid Blue stain was added (150µl/well). Plates were left for 2 hours, shaking gently. After this time cells were washed with washing solution (5%(v/v) glacial acetic acid, 10%(v/v) ethanol, 85%(v/v) distilled H$_2$O) (200µl/well), 2x for 1-2 min, 1x for 20 minutes, shaking gently. Desorbing solution (1M potassium acetate in 70%(v/v) ethanol, 30%(v/v) distilled H$_2$O) was added (150µl/well), and plates left for 20 minutes with rapid agitation, until the dye had gone completely into solution. Absorbance values were read on an Anthos 2001 plate reader at 405nm, reference 550nm.

4.2.2.8 STATISTICS

Data was analysed using the Statgraphics computer programme (Manugistics Inc., Maryland, USA). One-way and multifactor analysis of variance (ANOVA) were carried out and the conservative Scheffé multiple range test (at a 95% confidence level) used. The one-way analysis of variance procedure looks at the effect of one qualitative factor (in this study dose of UV light/toxin) on one response (here either metabolism of MTT or
binding of avidin). Multiple range tests investigate the significance of differences between means. Points which are not significantly different from each other are placed together in a statistically homogenous group. For a dose to give a significant effect it must be significantly different from the statistically homogenous group containing the control. The two-way analysis of variance procedure analyses the effect of two qualitative factors (here UV dose/toxin concentration and treatment with antioxidant/iron chelator) on one response (again metabolism of MTT or binding of avidin). All data was checked for a normal distribution before applying parametric analyses.
4.3 RESULTS

Oxidative stress was induced in differentiated IMR32 cultures using different methods, and the avidin assay developed previously (Chapter 3) was used to detect modified DNA base lesions at various times post-exposure. The viability of the cells was investigated in parallel to confirm that pre-lethal damage was being assessed. The morphology of the cultures was checked using light microscopy at all stages.

Some experiments involved the preincubation of cells with the antioxidant \( \alpha \)-tocopherol or iron chelator desferrioxamine prior to exposure to toxin or irradiation with UV light. Cell morphology was investigated before treatment with any ROS-generating system to ensure that these compounds, or their carriers, had no adverse effect themselves. This did not appear to change after preincubation with \( \alpha \)-tocopherol or its carrier ethanol (Figure 4.3); cell clusters were still evident and the neuritic network was undisturbed. Preincubation with 500\( \mu \)M of the iron chelator, however, caused the cells to become rounded and die (pictures not shown). This may have been caused by the compound making iron within the media unavailable to the cells; these ions are utilised during cell attachment, and in their absence adherent cultures, such as IMR32, may become detached and die. The concentration was therefore decreased to 200\( \mu \)M, and no changes in cell morphology were subsequently detected. Preincubation with an equivalent volume of the carrier HBSS had no deleterious effect.

The morphology of cultures was also investigated after assays carried out to assess levels of oxidative DNA damage, cell viability, and the protein content in each well, to judge whether these processes disrupted the cells in any way. Morphology was intact after the fixation and permeabilisation step of the DNA damage assay (Figure 4.4A). This was also true after incubation with MTT, used to investigate the viability of cells. The insoluble formazan product of MTT metabolism was observed within the cells as a diffuse, dark substance (Figure 4.4B), although the precise location of the dye could not be determined. Photographs taken after the Kenacid blue protein assay showed the morphology of cells which had previously undergone the MTT assay to be slightly disturbed; neurites were still present, but looked fragile, whilst the cell bodies appeared more rounded than previously (Figure 4.5A). Further investigation showed this to be a reaction of the cells to the isopropanol used to solubilise the formazan product. In spite of their appearance, the cell density across the plate remained relatively constant, with
the exception of a few wells where cells were lost completely. This was not true in cultures which had previously undergone the DNA damage assay, whose morphology remained intact (Figure 4.5B).

4.3.1 EXPOSURE OF CELLS TO HYDROGEN PEROXIDE

IMR32 cells were exposed to a wide range of H$_2$O$_2$ concentrations ($10^{-18}$M to $10^{-6}$M), and oxidative base lesions were assessed 1 hour after treatment. Results over this range were obtained from 2 separate plates, with overlapping data points being included i.e. plate 1 was exposed to $10^{-18}$M to $10^{-11}$ M H$_2$O$_2$, whilst plate 2 was exposed to $10^{-13}$M to $10^{-6}$M H$_2$O$_2$. A steady, dose-dependent increase was observed, to a maximum at $10^{-10}$M H$_2$O$_2$, after which levels remained similar ($p<0.0001$) (Figure 4.6). Significant levels of DNA damage were detected after exposure to concentrations as low as $10^{-17}$M H$_2$O$_2$. Cells were preincubated with the antioxidant α-tocopherol before exposure to $10^{-18}$M to $10^{-6}$M H$_2$O$_2$, the lowest concentrations investigated previously. DNA damage was this time assessed 2 hours after treatment. Once again, significant increases in DNA damage were detected over this concentration range ($p=0.0048$) (Figure 4.7A). No avidin binding was observed after preincubation with α-tocopherol ($p=0.4124$). Thus treatment with this antioxidant significantly lowered DNA damage produced by incubation of this model system with hydrogen peroxide ($p=0.0087$) (Figure 4.7A). A slight increase in cell metabolism was observed in the absence of the antioxidant ($p=0.0206$), which was not apparent in the presence of α-tocopherol (Figure 4.7B). In this experiment, a decrease in MTT metabolism was seen at $10^{-12}$M H$_2$O$_2$, the highest dose used.

A time-course experiment was carried out to investigate avidin binding to oxidatively modified DNA up to 48 hours after exposure to toxin. The concentration range $10^{-10}$M to $10^{-3}$M H$_2$O$_2$ was used. The metabolism of MTT in cells treated with $10^{-4}$M and $10^{-3}$M H$_2$O$_2$ fell below that of untreated cells at all time points investigated; DNA damage decreased in parallel, and was not shown at these points. 1 hour post-treatment, significant increases in avidin binding were detected after exposure to $10^{-10}$M to $10^{-5}$M H$_2$O$_2$ ($p=0.0008$), with levels falling after this point in correspondence with lowered cell metabolism (Figure 4.8A). MTT metabolism in cells was also increased from $10^{-10}$M to $10^{-6}$M H$_2$O$_2$, after which it fell in a dose-dependent manner (Figure 4.8B). This decrease was significantly lower than control values at $10^{-4}$M and $10^{-3}$M.
4 hours after treatment no significant DNA damage was detected (p=0.4762), although avidin binding appeared slightly increased at all concentrations plotted (Figure 4.9A). MTT metabolism was also raised slightly up to 10^{-7} \text{M} \text{H}_2\text{O}_2, decreasing in a dose-dependent manner after this point (p<0.0001) (Figure 4.9B). 18 hours after exposure to the toxin DNA damage was only detected at 10^{-6} \text{M} (p<0.0001) (Figure 4.10A). Levels were equivalent to untreated cells at all other concentrations of \text{H}_2\text{O}_2. The metabolism of MTT was still significantly increased at the lower concentrations of toxin used (p<0.0001), falling once again after this point (Figure 4.10B). By this time all the cells exposed to the highest concentration of \text{H}_2\text{O}_2, 10^{-3}\text{M}, and most of those expose to 10^{-4}\text{M}, had been lost from the plate completely. This was confirmed when the wells were investigated under a light microscope. 24 hours post-exposure levels of avidin binding remained constant up to 10^{-6}\text{M}, where it was significantly increased (p<0.0001) (Figure 4.11A). Cell metabolism was also constant up to 10^{-6}\text{M} \text{H}_2\text{O}_2, after which it decreased in a dose-dependent manner (p<0.0001) (Figure 4.11B). This pattern was repeated for both DNA damage and MTT metabolism in cells 48 hours after treatment, although levels of avidin binding were raised slightly at 10^{-6}\text{M} \text{H}_2\text{O}_2 as well (p<0.0001 for both assays) (Figure 4.12).

Avidin was therefore shown to detect levels of DNA damage after exposure to remarkably low concentrations of hydrogen peroxide. It was now of interest to investigate whether lesions produced via an alternative method could also be detected. Assays were therefore carried out after differentiated IMR32 cells were exposed to either UVA light or paraquat.

4.3.2 EXPOSURE OF CELLS TO UVA LIGHT

IMR32 cells were exposed to various doses of UVA light to generate ROS. Levels of 8-oxodG, as well as the viability of the cells, was then assessed either 1 hour or 24 hours after irradiation. Doses ranged from 16\text{mJcm}^{-2} to approximately 160\text{mJcm}^{-2}.

Avidin bound significantly to IMR32 cells both 1 hour (p=0.0008) and 24 hours (p=0.0005) after UVA irradiation (Figure 4.13), although the pattern of binding was different. 1 hour post-irradiation fluorescence levels increased slowly after the initial UVA dose, and appeared to plateau off at the highest two doses used. 24 hours later however, a large increase in binding was observed at the lowest doses of UVA, which
began to decrease at the highest doses. The greatest level of binding was detected at the later time point. The metabolism of the cells remained unchanged 1 hour after irradiation (data not shown), but was raised after 24 hours, although not significantly so (p=0.5717) (Figure 4.14B). The increase observed was dose-dependent, levelling off at the highest doses of UVA.

Preincubation of cells with α-tocopherol significantly reduced the level of avidin binding 24 hours after low doses of UVA irradiation (p=0.0075), and prevented the drop in avidin binding levels observed at the highest doses of UVA (Figure 4.14A). The presence of this antioxidant also appeared to prevent the increase in cell metabolism observed after 24 hours; levels of MTT metabolism were only slightly increased at all doses except 32mJcm\(^{-2}\), where it was raised (Figure 4.14B). Preincubation of cells with α-tocopherol did not appear to have an effect on avidin binding 1 hour after UVA irradiation (data not shown).

Preincubation with 200μM desferrioxamine, an iron chelator, also appeared to decrease DNA damage in cells 24 hours after exposure to UVA light (20-160mJcm\(^{-2}\)). A highly significant dose-dependent increase in avidin binding was seen in the absence of desferrioxamine, to a maximum at 30mJcm\(^{-2}\) UVA (p=0.0003) (Figure 4.15). Levels did not reach a plateau. Avidin binding was not significantly increased in the presence of 200μM desferrioxamine (p=0.1134). These increases in DNA damage corresponded to a slight increase in MTT metabolism, which was not significant for the control (p=0.066), at the same time point (data not shown). In this case levels observed in the presence of desferrioxamine were slightly higher than the control values, significantly so at 10 and 20 mJcm\(^{-2}\) UVA (p<0.001).

### 4.3.3 EXPOSURE OF CELLS TO PARAQUAT

A third method of ROS-generation involved incubation of IMR32 cells with the herbicide paraquat. DNA damage and cell metabolism were once again assessed 1 hour or 24 hours after exposure to the compound.

1 hour after treatment there was significant binding of avidin, indicative of oxidative DNA damage (p=0.0308) (Figure 4.16A). Binding was not dose-dependent; a maximum was reached at 10\(^{-9}\)M, with levels remaining similar after this point. There was no noticeable decrease in binding at the higher doses of paraquat, unlike H\(_2\)O\(_2\).
Metabolism of MTT was approximately the same in treated and untreated cells until $10^{-6}$M paraquat, when a significant increase was observed ($p=0.0366$) (Figure 4.16B). At 24 hours post-exposure avidin binding was not dose-related, although there may have been a slight overall increase which was not significant ($p=0.1260$) (Figure 4.17A). Errors were large however, and it could not be stated with any certainty whether DNA damage was still raised, or had been repaired. The metabolism of the cells appeared raised overall, especially at the higher concentrations of paraquat (Figure 4.17B). Once again this was not a significant increase, merely an observed trend ($p=0.1657$). Levels of metabolism did not fall below those seen in untreated cells, and there were no indications of cell death at the highest concentration of paraquat used. This was very different from results observed after exposure to H$_2$O$_2$.

4.3.4 PROTEIN ASSAYS

The Kenacid Blue protein assay was carried out on some plates after the results of DNA damage or MTT assays had been obtained, in order to assess the cell density across the plate. Results obtained for plates used in 24 hour time points showed a steady protein level across the plate up to $10^{-4}$M H$_2$O$_2$, where the absorbance dropped sharply (Figure 4.18). This corresponds to an even cell density up to the point where cell death is observed. These results were also obtained in plates at other time points. In plates examined 1 hour after exposure, however, protein levels were constant across the plate, with no decrease at higher concentrations corresponding to the drop in MTT metabolism (data not shown).
Figure 4.3 Photographs showing the morphology of cultures after preincubation for 24 hours with α-tocopherol (A) or the carrier ethanol (B). Cells appear healthy, and no difference can be observed between either set of cells. The morphology resembles that of cultures in media with no additions. Magnification x320.
Figure 4.4 Photographs taken of IMR32 cells after (A) an MTT assay and (B) fixation with 2%(w/v) paraformaldehyde, 0.5%(v/v) glutaraldehyde, followed by IMS. In the former, the blue formazan product of MTT metabolism is observed within cell bodies. The morphology of cultures is unchanged after both treatments. Magnification x320.
Figure 4.5 Photographs taken of IMR32 cells after the Kenacid blue protein assay. This was carried out on cells after (A) the MTT and (B) the DNA damage assays. The morphology of the cells has remained virtually intact throughout all the processing of both assays. Magnification x320.
Results of an avidin assay carried out on differentiated IMR32 cells 1 hour after exposure to various concentrations of hydrogen peroxide ($10^{-6}$ to $10^{-18}$ M)

![Graph showing avidin binding to IMR32 cells exposed to a wide H$_2$O$_2$ concentration range ($10^{-18}$ M to $10^{-6}$ M). Differentiated cells were incubated with the toxin for 1 hour at 37°C, then left in fresh medium for 1 hour at 37°C. Levels of avidin binding were assessed spectrophotometrically using the substrate TMB. Values are the means ± standard error of the mean (SEM), where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests. Results shown are from cells exposed to low ($10^{-18}$ M to $10^{-11}$ M) and high ($10^{-13}$ M to $10^{-6}$ M) concentrations of H$_2$O$_2$ on 2 separate plates treated in parallel. The data is representative of at least 2 experiments.](image-url)
A Results of an avidin assay carried out on cells fixed 2 hours after initial exposure to various concentrations of hydrogen peroxide ($10^{12}$M to $10^{18}$M) in the presence and absence of α-tocopherol

![Graph A](image)

B Results of an MTT assay carried out 2 hours after initial exposure of IMR32 cells to various concentrations of hydrogen peroxide ($10^{12}$M to $10^{18}$M) in the presence and absence of α-tocopherol

![Graph B](image)

Figure 4.7 An assessment of the protective action of α-tocopherol on oxidative DNA damage after exposure to low levels of H$_2$O$_2$ ($10^{12}$M to $10^{18}$M). Cells were preincubated with α-tocopherol (200mM) for 24 hours at 37°C prior to exposure to toxin. Avidin binding was measured colorimetrically in cells fixed 2 hours post-treatment (A). Cell viability was assessed using the MTT assay (B). Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
A Results of an avidin assay carried out 1 hour after removal of hydrogen peroxide from IMR32 cells

Figure 4.8 Avidin binding (A) and cell viability (B) in IMR32 cells 1 hour after exposure to various concentrations of H$_2$O$_2$. Differentiated cells were incubated with various concentrations of H$_2$O$_2$ for 1 hour at 37°C. This was replaced by fresh medium and cells were left at 37°C for 1 hour before processing. The levels of DNA damage were estimated using avidin-HRP (A), whilst the viability of the cells was assessed using the MTT assay (B). Values are the means ± standard error of the mean (SEM), where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests. The data is representative of at least 3 experiments.
A Results of an avidin assay carried out 4 hours after removal of hydrogen peroxide from IMR32 cells

![Avidin binding graph]

B Results of an MTT assay carried out 4 hours after removal of hydrogen peroxide from IMR32 cells

![MTT assay graph]

Figure 4.9 Avidin binding (A) and cell viability (B) in IMR32 cells 4 hours after exposure to various concentrations of H₂O₂. Avidin-HRP binding was detected spectrophotometrically using the substrate TMB. Cell viability was assessed using the standard colorimetric MTT assay. Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
A Results of an avidin assay carried out 18 hours after removal of hydrogen peroxide from IMR32 cells

![Avidin binding graph]

B Results of an MTT assay carried out 18 hours after removal of hydrogen peroxide from IMR32 cells

![Cell viability graph]

Figure 4.10 Avidin binding (A) and cell viability (B) in IMR32 cells 18 hours after exposure to various concentrations of H₂O₂. Avidin-HRP binding was detected spectrophotometrically using the substrate TMB. Cell viability was assessed using the standard colorimetric MTT assay. Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
A  Results of an avidin assay carried out 24 hours after removal of hydrogen peroxide from IMR32 cells

B  Results of an MTT assay carried out 24 hours after removal of hydrogen peroxide from IMR32 cells

Figure 4.11 Avidin binding (A) and cell viability (B) in IMR32 cells 24 hours after exposure to various concentrations of H₂O₂. Avidin-HRP binding was detected spectrophotometrically using the substrate TMB. Cell viability was assessed using the standard colorimetric MTT assay. Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
A Results of an avidin assay carried out 48 hours after removal of hydrogen peroxide from IMR32 cells

![Avidin binding graph]

B Results of an MTT assay carried out 48 hours after removal of hydrogen peroxide from IMR32 cells

![Cell viability graph]

Figure 4.12 Avidin binding (A) and cell viability (B) in IMR32 cells 48 hours after exposure to various concentrations of H$_2$O$_2$. Avidin-HRP binding was detected spectrophotometrically using the substrate TMB. Cell viability was assessed using the standard colorimetric MTT assay. Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
A comparison of results for immunofluorescence assays carried out on IMR32 cells fixed 1 hour or 24 hours after UVA irradiation

Figure 4.13 DNA damage observed in IMR32 cells 1 hour and 24 hours after irradiation with UVA light (0-140 mJcm\(^{-2}\)). Cultures were irradiated from above in a small volume of medium, then fresh medium added and cells left at 37°C for either 1 hour or 24 hours. They were then fixed and a DNA damage assay carried out. Values are means ± SEM, where \( n \geq 6 \) wells. * signifies points which are significantly different at the 95% confidence level from control values in the same group, using ANOVA procedures and Sheffe multiple range tests. The data is representative of 3 experiments.
Results of immunofluorescence assay carried out on IMR32 cells fixed 24 hours after UVA irradiation in the presence and absence of α-tocopherol

Results of MTT assay on IMR32 cells 24 hours after UVA irradiation in the presence and absence of α-tocopherol

Figure 4.14 An assessment of the protective action of α-tocopherol on oxidative DNA damage after irradiation with UVA light. Cells were preincubated with α-tocopherol (200μM) for 24 hours at 37°C prior to irradiation from above in medium. 24 hours post-irradiation avidin binding was measured fluorometrically, using a FITC label (A). Cell viability was assessed using the MTT assay (B). Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set. using ANOVA procedures with Sheffe multiple range tests.
A Results of immunofluorescence assay carried out on IMR32 cells fixed 24 hours after UVA irradiation in the presence and absence of α-tocopherol

![Immunofluorescence assay graph](image)

B Results of MTT assay on IMR32 cells 24 hours after UVA irradiation in the presence and absence of α-tocopherol

![MTT assay graph](image)

Figure 4.14 An assessment of the protective action of α-tocopherol on oxidative DNA damage after irradiation with UVA light. Cells were preincubated with α-tocopherol (200μM) for 24 hours at 37°C prior to irradiation from above in medium. 24 hours post-irradiation avidin binding was measured fluorometrically, using a FITC label (A). Cell viability was assessed using the MTT assay (B). Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
Results of an immunofluorescence assay carried out on IMR32 cells 1 hour after irradiation with UVA light in the presence and absence of desferrioxamine.

Figure 4.15 An assessment of the protective action of desferrioxamine on oxidative DNA damage after irradiation with UVA light. Cells were preincubated with desferrioxamine (200μM) for 24 hours at 37°C prior to irradiation from above in medium. 24 hours post-irradiation avidin binding was measured fluorometrically, using a FITC label (A). Cell viability was assessed using the MTT assay (B). Values are the means ± SEM, where n=8 wells. * signifies points which are significantly different at the 95% confidence level from control values in the same data set, using ANOVA procedures with Sheffe multiple range tests.
A Results of avidin assay carried out on IMR32 cells fixed 1 hour after exposure to various concentrations of paraquat

B Results of MTT assay carried out 1 hour after exposure of IMR32 cells to various concentrations of paraquat

Figure 4.16 DNA damage (A) and cell viability (B) in IMR32 cells 1 hour after exposure to paraquat. Avidin binding was detected colorimetrically using the substrate OPD. Cell viability was assessed using the MTT assay. Values are the means ± SEM, where n=8 wells.
A Results of avidin assay carried out on IMR32 cells fixed 24 hours after exposure to various concentrations of paraquat.

B Results of MTT assay carried out 24 hours after exposure of IMR32 cells to various concentrations of paraquat.

Figure 4.17 DNA damage (A) and cell viability (B) in IMR32 cells 24 hours after exposure to paraquat. Avidin binding was detected colorimetrically using the substrate OPD. Cell viability was assessed using the MTT assay. Values are the means ± SEM, where n=8 wells.
A Results of a protein assay carried out on cells after an avidin assay (24hrs after exposure to hydrogen peroxide)

![Graph A](image1.png)

B Results of a protein assay carried out on cells after an MTT assay (24hrs after exposure to hydrogen peroxide)

![Graph B](image2.png)

Figure 4.18 Results of the Kenacid blue protein assay, carried out on cells after the DNA damage assay (A) or MTT assay (B). This was done to estimate cell density after treatments across the plate. Values are the means ± SEM.
4.4 DISCUSSION

This study involved the investigation of ROS-induced DNA damage in neuronal cultures in vitro. Damage was induced via exposure to H\textsubscript{2}O\textsubscript{2}, UVA light or paraquat, and levels of DNA lesions were assessed using the novel assay developed previously (Chapter 3, Struthers et al, 1998). Repairable and non-repairable damage were both detected, and the involvement of lipid peroxides were strongly implicated in the mechanisms of this damage. The fact that oxidatively-modified DNA was detected in all systems investigated show that different methods of ROS induction have similar effects on cellular macromolecules in this neuronal model system. It is therefore possible that oxidative DNA damage observed in neurodegenerative disease is the product of different aetiological agents, with ROS being involved in a final general pathway of neuronal death.

In order to understand the mechanisms of neurodegenerative diseases, events occurring prior to cell death need to be defined. Pre-lethal markers of cell death are therefore of great interest. In this study, the pre-lethal effects of ROS on DNA were investigated. Initial experiments were carried out using cultures exposed to H\textsubscript{2}O\textsubscript{2}, because this was the method of ROS-induction used during development of the novel assay. Cell viability was studied in parallel with DNA damage, to confirm that lesions were being assessed in viable cultures. The MTT assay was used, as described by Mossman in 1983, because it is a more sensitive indicator of cell damage than the more traditional lactate dehydrogenase assay. MTT assays are commonly used as an indicator of cell proliferation, and in the past it has been generally assumed that MTT is reduced within the mitochondria. Evidence has since suggested that most of the cellular reduction of MTT occurs extramitochondrially (Berridge and Tan, 1993), therefore results should be interpreted with great care. Differentiated IMR32 cultures do not actively divide, therefore the assay was used to assess general metabolism and cell death, rather than mitochondrial activity in particular. Previous studies have shown increased metabolism of MTT within differentiated IMR32 cultures immediately after oxidative stress to correspond with cell damage at later time points (Johnston, 1994). Increases in MTT metabolism within cells may be explained in several ways. It may sustain an energy-requiring stress response, where new proteins are synthesised to metabolise macromolecules damaged by H\textsubscript{2}O\textsubscript{2}. The repair of damaged DNA by specific enzymes
also requires an increase in cellular energy production. Finally, damage to the mitochondrial membrane may result in an uncontrolled metabolism of MTT, as energy production is uncoupled from substrate availability. The latter explanation is not likely if most MTT reduction takes place outside of the mitochondria, however. Decreases in MTT metabolism are indicative of cell death in this model system, something which may be confirmed microscopically.

Hydrogen peroxide has been used to induce oxidative stress in both non-neuronal (murine fibroblasts, rat liver epithelial cells, Chinese hamster ovary cells) and neuronal (PC12 pheochromocytoma cells, primary rat forebrain neurons) cell lines. The level of \( \text{H}_2\text{O}_2 \) capable of inducing damage to cellular macromolecules and cell death in these studies varied. In PC12 cultures, \( 100\mu\text{M} \text{H}_2\text{O}_2 \) produced microtubule depolymerization over a 3-4 hour time course, with 40% cell death after 6 hours (Hinshaw et al., 1993). Primary rat forebrain cultures were more sensitive to this toxin; exposure of cells to \( 10\mu\text{M} \text{H}_2\text{O}_2 \) for 30 minutes resulted in almost total cell death 20 hours later, whilst significant levels of DNA strand breaks were detected both 12 and 20 hours after exposure to \( 25\mu\text{M} \text{H}_2\text{O}_2 \) (Hoyt et al., 1997). Non-neuronal cells also showed signs of damage on exposure to \( \text{H}_2\text{O}_2 \); 8-oxodG levels increased 42% over that of the control in rat liver epithelial cells, after incubation with \( 5\text{mM} \text{H}_2\text{O}_2 \) for 20 minutes (Rosen et al., 1996), and single strand breaks were increased in Chinese hamster ovary cells after exposure to \( 100\mu\text{M} \text{H}_2\text{O}_2 \) for 5 minutes, with approximately 20% cell death after incubation with the same concentration of toxin for 1 hour (Cantoni et al., 1989).

In this study, DNA damage was only assessed in IMR32 cultures exposed to \( \text{H}_2\text{O}_2 \) concentrations lower than \( 10^{-5}\text{M} \), because viability assays showed significant cell death as a result of higher levels of oxidative stress. The disruption in morphology, and scale of cell loss, observed during this early cell death suggest it was necrotic in nature. \( \text{H}_2\text{O}_2 \) is also known to induce apoptotic cell death (Gardner et al., 1997; Hoyt et al., 1997), a more ordered pathway of destruction. Both these mechanisms of cell death may occur some time after the initial insult, if cultures are unable to repair the damage induced. Experiments were therefore carried out to study DNA damage and cell viability over a period of 48 hours after exposure to \( \text{H}_2\text{O}_2 \). Results obtained suggest that there may be three scenarios of damage produced by \( \text{H}_2\text{O}_2 \). Firstly there is immediate cell death seen within 1 hour of exposure to high levels of stress, and DNA damage was not assessed in
this case, because events were obviously not pre-lethal. Secondly, there is non-repairable DNA damage; significantly raised levels of 8-oxodG were detected up to, and including, 48 hours after insult, whilst cells were viable and maintained a normal morphology. It is this type of lesion which is the most dangerous, with subsequent abnormal cell function and eventually death at a much later time point. Finally, there is the repairable damage seen after exposure to low concentrations of toxin. In this case base lesions appeared to have been repaired, or at least greatly decreased, and cultures were healthy 48 hours after insult. It would be of interest to follow the viability of the cells over a longer time period in order to assess whether cell death occurs after both permanent and repairable damage. If cell death is observed the mechanism involved, apoptotic or necrotic, should be investigated. This type of long-term study is complicated by the fact that differentiated IMR32 cultures have a finite life-span however.

8-OxodG levels were shown to be significantly raised in cultures exposed to concentrations of H$_2$O$_2$ as low as 10$^{-17}$M. This is equivalent to 1 molecule of H$_2$O$_2$ for approximately every 70 cells (see Appendix 3 for calculation). It seems difficult to believe that such low numbers of H$_2$O$_2$ are capable of damaging cellular macromolecules, although these results did appear to be reproducible. One potential explanation is that the H$_2$O$_2$ reacts with the cell membrane, initiating a chain reaction, and resulting in sufficient levels of ROS to induce oxidative DNA damage. It would be of interest to see whether proteins and lipids are oxidatively modified in this cell line after exposure to such low levels of H$_2$O$_2$. This may help clarify whether the results observed were real, or a result of experimental or assay error. The damage induced was repaired within 24 hours, and the cells appeared to recover. Although any lesions produced under these conditions would not be biologically relevant, if true, these results show the extreme sensitivity of differentiated IMR32 cells to H$_2$O$_2$ (damage was not detected after exposure to such low levels of other ROS-generating systems), along with the sensitivity of the assay used to detect the damage. These surprising results certainly warrant further investigation.

Although most research seems to have focused on the ability of hydroxyl radicals to interact directly with DNA, producing base lesions, lipid peroxides are also thought to play a role (Yang and Schaich, 1996). In order to investigate the hypothesis that 8-oxodG lesions were induced by the actions of lipid peroxides, cells were preincubated
with the lipid-soluble, chain-breaking antioxidant \( \alpha \)-tocopherol before exposure to \( \text{H}_2\text{O}_2 \), and subsequent assessment of DNA damage. This would also help to clarify whether the DNA damage observed was specifically due to the action of ROS. \( \alpha \)-Tocopherol has previously been shown to accumulate within cell membranes (Thomas, 1986; Thomas and Anderton, 1991), and to have a protective effect in IMR32 cells after toxic insult (Thomas and Anderton, 1991). The morphology of the differentiated cultures had been effected by the carrier ethanol in previous studies (Struthers, 1994), but microscopic examination showed no change in cells at the volumes used in these experiments. The fact that levels of DNA damage were decreased in the presence of \( \alpha \)-tocopherol showed that ROS were involved in the mechanism of damage. It also strongly suggested that lipid peroxidases are able to diffuse into the nucleus and interact with the DNA; if they were not involved to some extent, then \( \alpha \)-tocopherol, which protects against lipid peroxidation, would have no effect on the levels of 8-oxodG produced.

It may be hypothesised that oxidative DNA damage is induced by lipid peroxides produced as \( \text{H}_2\text{O}_2 \) interacts with the lipid membranes of different cellular compartments during the diffusion process. Lipid peroxides, like \( \text{H}_2\text{O}_2 \), are relatively stable (Pryor, 1986) and therefore may interact with macromolecules some distance from their site of production within the cell. For this reason, they could be said to be the most dangerous species produced during oxidative stress. The hydroxyl radical, on the other hand, has such a short half-life that it interacts with the nearest available macromolecule. Oxidising lipids are known to damage DNA (Vaca et al, 1988a; 1988b), although the mechanisms involved are poorly understood compared to those for hydroxyl radicals. Both \( \text{H}_2\text{O}_2 \) and lipid peroxides are able to react with the metal ions associated with nuclear DNA to produce hydroxyl or alkoxy radicals respectively, as follows:

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{Fe}^{2+} (\text{Cu}^+) & \rightarrow \cdot \text{OH} + \cdot \text{OH}^- + \text{Fe}^{3+} (\text{Cu}^2+) \\
\text{ROOH} + \text{Fe}^{2+} (\text{Cu}^+) & \rightarrow \text{RO}^- + \cdot \text{OH}^- + \text{Fe}^{3+} (\text{Cu}^2+)
\end{align*}
\]

These radicals are highly damaging species, and are known be capable of damaging DNA (Fraga and Tappei, 1988; Dizdaroglu et al, 1991; Yang and Schaich, 1996). In this way it may be possible for extracellular events, such as an increase in levels of \( \text{H}_2\text{O}_2 \), to induce a series of chain reactions intracellularly, resulting in oxidative DNA damage.
The ability of avidin to recognise oxidatively-modified DNA in IMR32 cells exposed to different ROS-generating systems was studied. The methods of choice involved exposure to either UVA light or the herbicide paraquat. DNA damage and cell viability were assessed in parallel cultures 1 hour and 24 hours after treatment. These time points were chosen in order to investigate both repairable and non-repairable damage to DNA.

UVA irradiation is a method of inducing ROS both intracellularly and extracellularly. The results obtained here confirm that 8-oxodG is a product of UVA irradiation, as seen previously (Rosen et al., 1996). The fact that damage was more pronounced 24 hours after insult suggest that chain reactions, which propagate over a period of time, are important in the mechanisms of UVA-induced DNA damage. It also appears that repair mechanisms are not as efficient after this form of damage, compared to H$_2$O$_2$. It is possible that the UVA light has damaged repair enzymes, antioxidant enzymes such as catalase, or both types of molecules, as suggested by Tyrrell (1991). It may also be hypothesised that lesions such as pyrimidine dimers, which are produced to a small extent after exposure to shorter wavelengths of UVA (Ahmed et al., 1993; Peak and Peak, 1989), can block the access of repair proteins to the base lesions. The pattern of damage is different from that observed with H$_2$O$_2$, and this could be due to the fact that alternative attacking species are responsible, or that different levels of ROS are produced within the cell. It is not surprising that UVA resulted in higher levels of damaged DNA than H$_2$O$_2$, indicative of higher levels of oxidative stress, because ROS may be formed intracellularly as well as extracellularly. They therefore do not need to diffuse in from the exterior before initiating chain reactions and producing cellular damage.

The mechanism of DNA damage via UVA irradiation was investigated using the antioxidant α-tocopherol once again. In addition, the effect of preincubation with the iron chelator desferrioxamine on levels of DNA damage was investigated. Desferrioxamine would be expected to decrease levels of free iron available, and thus decrease ROS production via the Haber-Weiss reaction. Unlike α-tocopherol the ability of desferrioxamine to enter these cultures is not known, although this compound has been shown to enter other cell lines, including liver cells (Hershko, 1992). Measurements therefore need to be carried out to assess the concentration of the iron.
chelator within the neuronal cell line used in this study after pre-incubation. If the DNA
damage observed is caused by ROS, then the presence of one or both of these molecules
would be expected to have a protective effect.

It was presumed that desferrioxamine did in fact enter cells because preincubation
with this molecule resulted in decreased levels of avidin binding. This also suggests that
ROS produced via the iron-catalysed Fenton reaction are partly responsible for the DNA
damage seen. Cell metabolism appeared to be greater in cells preincubated with
desferrioxamine when compared to control cultures. Although the reason for this
remains unclear, it may be that desferrioxamine enters the cells via an active transport
mechanism, and that metabolism is increased to provide the energy needed. The levels of
DNA damage and cell metabolism should be followed at later time points, as a potential
consequence of sub-lethal damage is the permanent loss of normal function, causing cell
death some time after the original insult.

α-Tocopherol was observed to decrease DNA damage once again, although it did
not completely prevent avidin binding. Levels of oxidative lesions in protected cells
increased at a slower rate than in control cells, suggesting that damage was produced at
a slower rate in these cultures due to decreased levels of destructive ROS. These results
all suggest that lipid peroxides were involved in damaging reactions, along with other
species of ROS. It is likely that the hydroxyl radical is involved in site-specific damage
as described previously. The apparent decreases in levels of oxidative DNA lesions
observed in control cultures exposed to high doses of UVA are very likely due to cell
death. Even though there is no corresponding drop in metabolism at this time point, cells
may have been lost leaving the remaining ones metabolising at a very high rate. The
DNA damage observed in cells preincubated with α-tocopherol was detected in the
absence of an overall increase in metabolism and once again it would be of interest to
investigate the level of avidin binding and metabolism of the cells at later time points, to
see if damage is repaired or if it is an early indicator of cell death.

Preliminary studies were carried out using paraquat to generate ROS. This toxin
has been shown to induce DNA damage via ROS, and is known to be actively taken up
by brain (Smith and Wyatt, 1981). This toxin did not induce cell death at any dose used,
unlike H₂O₂, where cell death was very obvious within 1 hour. It is very likely that much
lower concentrations of paraquat actually enter the cell, because it must rely on active
transport into the cells, unlike H$_2$O$_2$ which is able diffuse in easily. Intracellular levels of both H$_2$O$_2$ and paraquat should be measured to investigate this further. It would also be interesting to measure levels of both compounds in the extracellular medium at various time points during the hour-long incubation, to see whether this time could be shortened. It is possible that hydrogen peroxide could react with other components in the medium, making it unavailable to the cells as soon as 15 minutes after initial exposure (Giandomenico et al., 1997), although the stability of paraquat in vitro has not been assessed to date.

These initial experiments using paraquat need to be repeated to fully validate the results obtained, as they were only carried out in duplicate, and errors were somewhat large. It is also important to carry out a time course experiment with this toxin, to see whether damage is repaired, or if cells die at a later time point. This would be of interest in cultures exposed to UVA irradiation as well. The mechanism of cell damage in cultures exposed to paraquat remains to be fully elucidated. It is thought to cause toxicity via its free radical form, which is involved in redox cycling in the presence of NADPH and oxygen, as described previously. Superoxide radicals are produced, and these may act as a reducing agent during site-specific production of hydroxyl radicals on DNA in the vicinity of chelated metal ions via Fenton chemistry. They may also be involved in the production of hydrogen peroxide within the cell. The fact that the paraquat radical species is relatively stable means it can diffuse some distance before it reacts with macromolecules within the cell, and it is likely to reach the nucleus to take part in this type of chain reaction. In order to further investigate the mechanism of paraquat toxicity within this model system, cultures should be preincubated with antioxidants such as α-tocopherol and iron chelators such as desferrioxamine, as in the studies involving hydrogen peroxide and UVA irradiation. It would also be of interest to pre-incubate with the enzyme superoxide dismutase (SOD), which inactivates the superoxide radical. In this way the involvement of ROS, and superoxide radicals in particular, in DNA damage could be assessed.

These results can be used as the basis for several further lines of investigation. It is important to investigate levels of 8-oxodG in cultures exposed to each method of ROS-production using a technique other than the avidin assay. This is to confirm that the lesion is actually produced in each case, and also to assess the efficiency of the method
for measuring lesions. HPLC is a very common method of quantifying levels of oxidative base lesions (Floyd et al, 1986; Kaur and Halliwell, 1996; Herbert et al, 1996), and would be a simple way of confirming increased levels of 8-oxodG after exposure to various ROS-generating systems. Differentiated cultures should be treated with levels of 

H$_2$O$_2$, UVA or paraquat thought to produce both repairable and non-repairable DNA damage, then the DNA isolated and analysed after various recovery times. It may also be possible to measure DNA repair products, such as 8-oxodG, in the medium of the cells at each time point by analysing samples using HPLC; cells in which lesions have been repaired may have higher levels of lesions than cells where the damage remains, although it is not known whether these products are released from the cell or not however. The efficiency of avidin binding to modified base in solution would also be relatively easy to assess using either HPLC or magnetic streptavidin-coated beads. This would likely bear little resemblance to efficiency levels of binding to oxidative DNA damage within cells or tissue, however, as avidin would not be in direct contact with the lesion, and DNA cannot be isolated without artefactual damage which would interfere with results. Therefore this assay remains semi-quantitative, able to assess changes in levels of 8-oxodG after exposure to oxidative stress relative to values in control cultures.

In order to clarify the mechanism of damage for each ROS-generating method DNA damage could be assessed after pre-incubation of cultures with various protective molecules. These may include enzymes such as catalase and glutathione, which inactivate H$_2$O$_2$, superoxide dismutase, which confers protection from damage produced by superoxide radicals. It would also be of interest to compare the protective action of tocopherol in the presence and absence of Vitamin C, as these two antioxidant molecules are meant to act synergistically together to decrease lipid peroxidation (Niki, 1987a). The action of the iron chelator desferrioxamine should also be investigated further in each of the systems studied, and results compared with those obtained in the presence of a copper chelator such as nitrilotriacetic acid (NTA). In this way the relative importance of iron and copper ions in the formation of oxidatively-damage bases could be assessed in this model system.

It would also be of interest to carry out these experiments on other neuronal cells, as the DNA damage assay developed is expected to provide a method general for all cells. The neuroblastoma cell line SH-SY5Y can be differentiated in a similar manner to
IMR32 cells, using retinoic acid as the differentiating agent rather than bromodeoxyuridine. These cultures survive for more than 1 month and are more adherent than IMR32, making them easier to work with and useful for longer-term studies. They could not be used during this project because infection-free stocks were not available. The use of a mouse neuroblastoma culture, such as the C1300 cell line, in experiments would provide a cross-species comparison, whilst work on primary cell lines such as chick micromass cultures would also be useful for comparison, as these are meant to provide a better representation of the in vivo state of neurons than continuous cell lines. It would also be of interest to use other methods of generating ROS, such as glutamate, which has been implicated in the development of various neurodegenerative disorders (Coyle and Puttfarcken, 1993).

Finally the mechanism of cell death, whether apoptotic or necrotic, should be investigated. Apoptosis is a controlled form of cell death characterised by cell shrinkage, 'blebbing' of the membrane and the breakdown of nuclear DNA in a regular manner by endonucleases whilst organelles and proteins remain intact. Finally the cells breaks up into a series of apoptotic bodies which are removed by phagocytosis via surrounding macrophages/cells. Necrosis, on the other hand, is characterised by cell swelling, the breakdown of organelles and the random degradation of DNA, with the cell eventually bursting and releasing the contents to the surroundings. Thus apoptosis causes much less disruption to the environment than necrosis. It can be hypothesised that a cell may undergo either apoptosis or necrosis depending on the mechanism and level of cell damage, and scientists are interested in understanding the mechanisms of both forms of cell death. Evidence of apoptosis or necrosis could be assessed morphologically and using assays of DNA fragmentation. Light and electron microscopy may be used to look for the classical features of apoptosis described above. Detection of in situ DNA fragmentation can be assessed using the Apoptag kit (Oncor, Gaithersburg, MD), whilst degradation of DNA into oligonucleosome-length fragments can be detected in genomic DNA analysed by agarose gel electrophoresis in parallel cultures. Changes in gene products associated with apoptotic mechanisms, such as Bcl-2, c-Jun and c-Fos can also be investigated using immunocytochemical techniques and Western blotting. It would be of interest to see whether the oxidative DNA damage detected using the avidin assay developed is a predictor of apoptosis.
Overall increased levels of the oxidative lesion 8-oxodG have been demonstrated in cultures exposed to three different ROS-generating systems; \( \text{H}_2\text{O}_2 \), UVA irradiation and paraquat. Cell density was constant across the 96-well plates used, and cell morphology was intact after both DNA damage and MTT assays. Any difference in absorbance observed must therefore have been due to changes in levels of DNA damage and MTT metabolism respectively. The antioxidant \( \alpha \)-tocopherol decreased levels of 8-oxodG produced by exposure to \( \text{H}_2\text{O}_2 \) or UVA, confirming the involvement of ROS, including lipid peroxides, in nuclear DNA damage. Preincubation with the iron chelator desferrioxamine also decreased avidin binding after exposure to UVA light, showing iron ions to be involved in the production of DNA damage in this system.

A hypothesis can be formed, based around the belief that oxidative stress is a final common pathway for cell death, and that DNA damage is a generic marker of cellular dysfunction produced via these mechanisms (see Appendix 4). Various aetiologial agents may trigger an imbalance in the pro-oxidant/anti-oxidant status of the cells, either by decreasing the antioxidant capacity or increasing production of ROS. The excess ROS, which may include some, or all, of hydrogen peroxide, superoxide, singlet oxygen, hydroxyl radicals and lipid peroxides, become involved in self-perpetuating chain reaction systems, with resulting oxidative damage to important cellular macromolecules, including nuclear and mitochondrial DNA. Oxidatively-modified DNA may result in the incorrect transcription of genes, which in turn may lead to the production of aberrant proteins. These might be structural proteins, such as those involved in cytoskeletal formation, leading to abnormalities in the formation, structure and function of the neuronal cytoskeleton and subsequent cell death. If these proteins were involved in cellular metabolism, then consequences could be excessive metabolism with a corresponding large increase in ROS production, or energy depletion leading to a slight, chronic depolarisation of the membrane, activation of glutamate receptors, and a subsequent increase in cytosolic calcium. The latter is also capable of initiating abnormalities in cytoskeletal structure and function, as well as apoptosis. Thus DNA damage produced as a result of oxidative stress may (i) result in cell death via necrosis or apoptosis, (ii) remain as a fixed mutation within the cell or (iii) be completely repaired. Permanent lesions have the potential to cause the greatest long-term problems, although cell death would have serious consequences if a large number of neurons were involved.
CHAPTER 5

DETECTION OF DNA DAMAGE DIRECTLY \textit{IN SITU}
5.1 INTRODUCTION

It has been suggested that oxidative damage to DNA is important in the development of many common neurodegenerative disorders (Sanchez-Ramos et al, 1994; Alam et al, 1997; Fitzmaurice et al, 1996; Lyras et al, 1997), as well as the ageing process (Nohl, 1993). Although *in vitro* studies enable scientists to investigate the mechanistic role of ROS-mediated damage to neuronal DNA, the direct visualisation of oxidative DNA lesions are more important when studying pathological tissue from patients with neurodegenerative disease. This would enable accurate assessments of the brain regions most affected by ROS in different disorders, and would also allow detection of specific cells with high levels of damage. Previous studies of oxidative DNA damage in neurodegeneration have utilised HPLC or GC-MS methodology to investigate levels of damaged DNA (Sanchez-Ramos et al, 1994; Alam et al, 1996; Fitzmaurice et al, 1996; Lyras et al, 1997). These techniques involve prior isolation and derivitisation of DNA, with subsequent artefactual damage as described in Chapter 1, and there have been suggestions that the results obtained may not be accurate (Collins et al, 1996). Antibodies raised against ROS-mediated DNA damage have been used to investigate levels of 8-oxodG in human cells (Musarrat and Wani, 1994; Yin et al, 1995), but these usually involve isolation of DNA prior to the assay. A study by Yarborough et al (1996), however, has described the direct visualisation of 8-oxodG in cultured cells and rat liver sections using a monoclonal antiserum. In spite of this, methods involving antibody detection of oxidative DNA lesions do not appear to have been used in the study of brain tissue from patients with neurodegenerative disease. Evidence of ROS damage in the ageing process and neurodegeneration were therefore studied using the avidin assay. DNA damage has been shown directly in differentiated IMR32 cells exposed to H$_2$O$_2$, and in a preliminary examination of tissue from patients with motor neuron disease, using this method (Struthers et al, 1998).

5.1.1 THE INVOLVEMENT OF ROS IN AGEING

The ‘free radical theory of ageing’ was first proposed in 1956 (Harman, 1956), long before the existence of ROS and their effects could be shown experimentally. It was suggested that the ageing process was a result of random deleterious effects of free radicals on tissues. Although it remains difficult to establish whether ROS are actually a cause or a consequence of cell death and ageing (Nohl, 1993), there is increasing
evidence to support this hypothesis. For example, age-related increases in hydroxyl radical stress has been observed in regions of gerbil brain (Zhang et al, 1993). In addition oxidative DNA damage has been related to the metabolic rate of species (Adelman et al, 1988), and it has been suggested that species with a high maximum life span produce ROS at a lower rate than those with a shorter life-span (Barja et al, 1994). Barja et al proposed that ROS production near critical DNA targets was an important factor in determining the longevity of a species. Separate studies have shown a correlation between age and levels of the base lesion 8-oxodG in nuclear and mitochondrial DNA (Richter et al, 1988; Fraga et al, 1990; Mecocci et al, 1993; Kaneko et al, 1996), in agreement with the idea that oxidative DNA damage is instrumental in ageing. In all these studies, however, DNA has had to be isolated from the tissue before analysis of base lesions. A direct demonstration of levels of 8-oxodG in neurons of young and old animals would therefore be of great interest.

5.1.2 MOTOR NEURON DISEASE (MND) AND ROS

There are many different human motor neuron diseases. The most serious of these is amyotrophic lateral sclerosis (ALS), which effects approximately 0.05% of the population (Coyle and Puttfarcken, 1993). Symptoms of this disease generally appear in mid-life, and are characterised by a rapidly progressive paralysis caused by the degeneration of the lower motor neurons in spinal cord and the upper motor neurons in the cerebral cortex. Death from respiratory paralysis usually occurs within 3-5 years, although the time scale does vary. A pathological examination of brain tissue from patients suffering from this neurodegenerative disorder shows selective shrinkage and loss of the motor neurons. This eventually encompasses cells in the spinal cord, brain stem and motor cortex (Brown, 1997). Abnormal cytoskeletal structures are also often observed in affected cells; axons contain ‘spheroid’ swellings loaded with structurally normal neurofilaments (Carpenter, 1968), and filamentous accumulations may be observed in the cell body, which may contain phosphorylated neurofilaments (Munoz et al, 1988) and ubiquitin (Leigh et al, 1988).

Approximately 10% of ALS cases are familial (FALS). This form of the disease has been associated with mutations in the SOD1 gene which codes for cytosolic Cu,Zn SOD (Rosen et al, 1993), an enzyme important for the conversion of superoxide radicals to hydrogen peroxide. The latter is converted in turn to water by catalase or glutathione
peroxidase. SOD is also an important buffer for Cu and Zn within the cell, and the enzymes catalytic activity is dependent on Cu (Brown, 1997). If mutated SOD were involved in the development of FALS it would strongly implicate ROS in the aetiology of this disease. The presence of increased levels of protein carbonyls in patients with ALS compared to controls also strongly suggests a role for oxidative stress (Shaw et al., 1995).

Mutations have been detected in approximately 20% of FALS cases (Bredeson et al., 1996; Brown, 1997). Most of these appear to effect the structural backbone of the enzyme rather than the active site (Deng et al., 1993). It is now widely believed that degeneration of motor neurons is caused by some gain of function of the mutant SOD enzyme rather that a loss of activity (Bredesen et al., 1996; Simonian and Coyle, 1996; Brown, 1997), although the exact mechanism involved is not yet known. Decreases in SOD activity have been observed in erythrocyte lysates (Deng et al., 1993) and post-mortem brain tissue (Bowling et al., 1993), but research has suggested this is due to the decrease in enzyme concentration, and specific activity of the enzyme could not be related to disease severity (Bowling et al., 1995). Transgenic mice which overexpress mutant human Cu,Zn SOD have been shown to develop a paralytic disease with pathology primarily affecting motor neurons of the spinal cord and brainstem, which is very similar to ALS (Dal Canto and Gurney, 1995). This evidence strongly favours the gain of function hypothesis.

Several potential mechanisms of toxicity have been suggested. It may be that mutations decrease the strength of SOD binding to Cu, leading to an increase in free metal able to take part in the Haber-Weiss reaction (Brown, 1995). Another possible mechanism suggests that mutated SOD interacts more readily with peroxynitrate, with subsequent nitration of tyrosine residues of essential proteins (Beckman et al., 1993). It has also been suggested that the toxicity of mutant Cu,Zn SOD is related more to abnormal structural properties of the protein, such as folding or solubility, leading to precipitation of the protein within the cell (Bredeson et al., 1996). Whatever the involvement of SOD in FALS turns out to be, it is likely that both FALS and sporadic ALS (SALS) share a common pathological mechanism encompassing the action of ROS because these diseases are clinically indistinguishable from one another.
5.1.2.1 DNA DAMAGE IN MOTOR NEURON DISEASE

It has been proposed that a deficiency in DNA repair is responsible for the aetiology of ALS (Bradley and Krasin, 1982). This hypothesis states that:

"ALS is due to a deficiency in normal DNA repair mechanisms with resultant accumulation of damaged DNA, which causes abnormal transcription of RNA and, thus, translation of abnormal proteins or absence of synthesis of specific proteins (Bradley and Krasin, 1982)."

Deficiencies detected in the apurinic/apyrimidinic endonuclease (APE) protein, which is important in base-excision repair, are further evidence for this hypothesis (Kisby et al, 1997). Increased levels of oxidative base lesions would be expected in cells deficient in DNA repair, and it increased levels of 8-oxodG have in fact been detected in ALS spinal cord tissue (Fitzmaurice et al, 1996). Increased oxidative DNA damage has also been observed in the motor neurons of mice exposed to inorganic mercury (Pamphlett et al, 1998), a heavy metal implicated in the pathology of motor neuron disease (Clarkson et al, 1988). Studies have been hampered by the inability to detect DNA lesions directly in pathological tissue however.

5.1.3 AIMS

Experiments were carried out to establish levels of oxidative DNA damage directly in spinal cord tissue from aged mice and patients suffering from motor neuron disease. Avidin was conjugated to the fluorescent label FITC for these studies, and levels of binding assessed using fluorescence microscopy.

Initially a study of binding was assessed in in vitro neuronal cultures exposed to ROS-generating systems in the presence and absence of the antioxidant α-tocopherol. These cells were exposed to concentrations of H₂O₂ known to induce both low and high levels of oxidatively-modified DNA. Images were then captured on photographic film and avidin binding assessed subjectively by eye. Controls were carried out to investigate autofluorescence of the cells and non-specific binding of avidin itself. Decreased binding was observed after preincubation of cultures with α-tocopherol, suggesting that the DNA damage detected was due to the action of ROS. These experiments were carried out within the Department of Pathology at the University of Leicester, UK.
Preliminary experiments were then carried out to study the binding of fluorescently-conjugated avidin to motor neuron cells within the spinal cord of both mice and human tissue. Initial studies investigated the effect of different fixation protocols on immunofluorescence results. This was done because formalin is known to be autofluorescent, and it was thought this may produce a high background. It was also important to establish whether the heavy processing involved in paraffin embedding of tissue would effect the final results, because almost all tissue available from patients with neurodegenerative disease is treated in this way. Both mouse and human tissue was used in these investigations, and fluorescence was assessed both subjectively and quantitatively. The hypothesis that oxidative DNA damage is raised in the brains of old subjects compared to younger ones was then investigated. Levels of avidin binding were assessed in the motor neurons in cervical spinal cord from young mice (7 weeks) compared to aged mice (>18 months). Finally, levels of oxidative DNA damage were assessed in motor neurons of patients who had suffered from clinically diagnosed MND and compared to results obtained from age-matched control patients who had shown no sign of neurodegenerative disorders at the time of death. This was to investigate the hypothesis that DNA damage produced during oxidative stress is raised in patients suffering from neurodegenerative disorders. These experiments were carried out within the Department of Pathology at the University of Sydney, Australia.
5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

Components for the fluorometric determination of avidin binding in cultured cells were provided as a research evaluation kit by Biotrin International (Dublin, Eire). Chemicals used during the detection of avidin binding in brain tissue were obtained from the following sources: Histoclear and xylene from Fronine (Riverstone, NSW, Australia); ethanol from CSR Distilleries (Yarraville, Victoria, Australia); glutaraldehyde and gelatin from Ajax Chemicals (Sydney, Australia); Trizma R base and avidin-FITC from Sigma (USA); Entellin, paraformaldehyde and isopentane from BDH Chemicals (Kilsyth, Victoria, Australia).

5.2.2 ANIMALS

Male BALB/c mice were obtained from the Animal Resources Centre (Perth, Australia). Female BALB/c mice were obtained from the Gore Hill Research Lab (St. Leonards, Sydney, Australia). Mice were kept 4 to a cage and given routine food and water.

5.2.3 HUMAN BRAIN TISSUE

The brain and spinal cord were obtained at autopsy from 3 patients (male and female, aged 69 to 74 years) who died from clinically diagnosed motor neuron disease, and from control patients who died from causes other than neurological or psychiatric disorders. Post-mortem times ranged from 4 to 72 hours. These details are shown in Table 5.1.

5.2.4 METHODS

The extent of oxidatively-damaged DNA was investigated directly in situ in cultured IMR32 neuroblastoma cells, mouse and human spinal cord and human brain tissue, using a fluorescent end-point to detect avidin binding. Oxidative stress was induced in cultured cells by exposure to hydrogen peroxide before levels of avidin binding were assessed. In brain tissue it is thought that DNA damage is caused in vivo by ROS produced during processes such as aerobic respiration, as described previously. Increased levels of ROS are thought to be present in neurodegenerative disorders, and this may result in increased levels of oxidative base lesions. This was therefore investigated. The effect of different fixation procedures on results was assessed in both mouse and human neuronal tissue.
Table 5.1 Information on age and post-mortem delay for control and MND paired samples used during this study. Avidin binding was compared in the motor neurons of age-matched sections in order to assess whether increased levels of oxidative DNA damage were present in patients suffering from neurodegenerative disease compared to those showing no sign of this type of disorder on death.

<table>
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<th>AGE</th>
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<tr>
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<td>21 hours</td>
</tr>
<tr>
<td><strong>MND 1</strong></td>
<td>74 years</td>
<td>14 hours</td>
</tr>
<tr>
<td><strong>CONTROL 2</strong></td>
<td>77 years</td>
<td>19 hours</td>
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<tr>
<td><strong>MND 2</strong></td>
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<tr>
<td><strong>CONTROL 3</strong></td>
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<td>10 hours</td>
</tr>
<tr>
<td><strong>MND 3</strong></td>
<td>69 years</td>
<td>4 hours</td>
</tr>
</tbody>
</table>
Avidin binding was also compared in sections of fresh frozen spinal cord from aged mice and young mice, and sections from paraffin-embedded human spinal cord of pairs of age-matched motor neuron disease patients and control patients.

5.2.4.1 IN SITU DETECTION OF OXIDATIVE DNA DAMAGE IN CULTURED CELLS

Differentiated IMR32 cells in 8-well Labtek slides were exposed to $10^{-9}$M, $10^{-6}$M and $10^{-3}$M H$_2$O$_2$, as described in Section 2.2.3.4. Cells were left in fresh media to recover for 1 hour before fixation and detection of avidin binding using a fluorescent end-point as described in Section 3.2.2.3.8. Fluorescence was visualised using an Olympus fluorescence microscope, and photographs taken showing avidin binding on Kodak Extrachrome P1600x film.

5.2.4.2 FIXATION OF NEURONAL TISSUE

Mice were given a lethal dose (0.3 ml) of Pentothal anaesthetic, and cervical or lumbar spinal cord was dissected out. Some small pieces of tissue (<1 cm diameter) were placed in Tissue-Tek embedding fluid (OCT compound) on a piece of cork on metal chucks, then snap frozen immediately by immersion in isopentanol cooled in liquid nitrogen. They were then left at -20°C for 1-2 hours before being transferred to -70°C for storage until needed. Other pieces of tissue were left in 10%(v/v) formaldehyde for 2 hours or 24 hours at room temperature before being snap frozen as above, or left in 0.01M phosphate buffer at 4°C until being embedded in paraffin in a Tissue Tek III Paraffin Processor. The latter tissue was dehydrated gradually through graded alcohol solutions, then xylene over 48 hours at room temperature, before addition of paraffin at 60°C over 4 x 6 hour cycles. Blocks were then stored at room temperature until needed.

Most blocks of human tissue were obtained from the brain bank at the Department of Pathology, University of Sydney. This was collected at post-mortem and placed in 10%(v/v) formalin until paraffin-embedding as above, then stored at room temperature. Some medulla and motor cortex brain tissue was snap frozen as above after being left in 10%(v/v) formalin for 5 days. These tissue blocks were larger than the mouse spinal cord, and were therefore placed in 30%(w/v) sucrose in 10%(v/v) buffered formalin solution for 3 days before freezing to cryoprotect against artefactual damage.
IN SITU DETECTION OF OXIDATIVE DNA DAMAGE IN MOUSE AND HUMAN SPINAL CORD AND HUMAN BRAIN TISSUE

12μm sections were cut from frozen tissue at -18°C on a cryostat (Bright model OTF), placed on clean, gelatinised slides, and allowed to air dry for 1 hour. Fresh, unfixed sections were either used immediately, or individually wrapped in foil and stored at -70°C until needed, whilst formalin-fixed sections were stored at room temperature in slide boxes. Either 7μm or 12μm sections were cut from paraffin embedded tissue. These were allowed to expand at 45°C in a water bath, then place on clean gelatinised slides and left to air dry for 1 hour. Sections were stored at room temperature in slide boxes.

Frozen, unfixed sections was fixed and permeabilised by incubation with 4%(w/v) paraformaldehyde, 0.5%(v/v) glutaraldehyde for 15 minutes on ice, washed briefly in 0.1M Tris Buffer, then dehydrated through graded alcohols (2 minutes each in 70%(v/v), 95%(v/v) ethanol, absolute alcohol at room temperature). Tissue was then rehydrated through graded alcohols (2 mins each in 95%(v/v) ethanol, 70%(v/v) ethanol at room temperature), washed briefly, then left in fresh 0.1M Tris Buffer for at least 10 minutes. Avidin-FITC (1:500 dilution made up in Tris Buffer) was then added to sections (200μl or 500μl, depending on section size), and slides placed in a humidity chamber at 4°C overnight. Sections were then washed 5 times with Tris Buffer, excess liquid removed, and coverslips mounted using anti-fade Entellan mountant. Slides were wrapped in foil and kept at 4°C until examination for fluorescence. This was carried out within 24 hours of staining.

Formalin-fixed frozen sections were treated as unfixed frozen sections without incubation with paraformaldehyde and glutaraldehyde solution.

Paraffin sections were first dewaxed by incubation with xylene (2x2 minutes) and Histoclear (2x2 minutes), before rehydration through graded alcohols and exposure to avidin-FITC as above.

CAPTURING IMAGES OF CELLS AND QUANTITATION OF FLUORESCENCE

Images of cell bodies of large motor neurons in the anterolateral part of the anterior horn in mouse and human spinal cord, and the hypoglossal nucleus in the medulla of human brain were collected directly onto computer. Cells were viewed using an Olympus BX60 fluorescence microscope with a narrow band FITC filter; excitation
485nm, emission 505nm (Omega Optical, Vermont, USA). Photographs were then taken with a Quantix slow-scan camera (air-cooled to -25°C) (Photometrics, Arizona, USA), and images captured directly onto computer using V for Windows software (Digital Optics Ltd, Auckland, New Zealand). These were transferred into the NIH Image 1.6 programme on a Macintosh Quadra 800 computer. The images obtained were of a higher quality than those acquired in preliminary experiments, which were photographed onto black and white film and then scanned into the NIH Image 1.6 program with a Microtek Scanmaker 600ZS scanner (Pamphlett et al, 1998). The new methodology also meant there was less likelihood of different exposure times and handling procedures affecting the final result. This should therefore be a more accurate method of quantifying DNA damage directly in cells.

Fluorescence levels in motor neurons of the right and left anterior horns of mouse spinal cord were analysed. The perikaryon was outlined using the freehand tool in the NIH programme, and the mean fluorescence obtained by the programme in greyscale units from 0 (black) to 255 (white). The unstained nucleus was excluded from these measurements. The mean background fluorescence of an area adjacent to the neuron was also measured, then subtracted from the original figure to give a corrected perikaryol reading. Images were edited in the Adobe Photoshop 4.0.1 programme, then transferred to the Adobe Pagemaker 6.0 programme before printing. The colour scale was inverted. Fluorescence levels in human motor neurons could not be quantified because of high autofluorescence levels, which varied from cell to cell. These images were printed out as above.
5.3 RESULTS

Avidin binding was assessed in *in vitro* neuronal cultures, mouse cervical spinal cord sections and pathological human spinal cord sections. Experiments were carried out to assess the effects of different fixation procedures on levels of binding, to show background levels of DNA lesions, to compare levels of DNA damage in tissue from young mice as opposed to aged mice, and to investigate oxidatively-modified DNA in motor neurons from patients with neurodegenerative disease compared to age-matched controls.

5.3.1 AVIDIN BINDING TO IN VITRO CULTURES

Initial experiments aimed to visualise avidin binding in differentiated IMR32 neuroblastoma cells exposed to oxidative stress. DNA damage assays were carried out on these cultures 1 hour after incubation with various concentrations of hydrogen peroxide. Control cultures were exposed to the carrier buffer HBSS only. Avidin conjugated to the fluorescent dye FITC was used, and the level of binding was assessed with fluorescence microscopy. Low levels of fluorescence were observed in cells incubated with HBSS only (Figure 5.1A), with increasing levels being detected in cultures exposed to $10^{-9}$M and $10^{-6}$M hydrogen peroxide respectively (Figures 5.1B, 5.1C). Avidin binding appeared to be localised within the nucleus of the cells, although some diffuse binding was observed throughout the cytoplasm. There was no binding within the neurites. Levels of fluorescence were then investigated in cells exposed to $10^{-6}$M H$_2$O$_2$ in the presence and absence of $\alpha$-tocopherol; cultures were preincubated with this chain-breaking antioxidant, or the carrier ethanol, 24 hours prior to treatment. Binding was clearly decreased in cultures preincubated with $\alpha$-tocopherol, compared to those exposed to ethanol only (Figure 5.2). Once again, binding was localised within the nuclei of the cells, with some diffuse patches throughout the cytoplasm. Avidin binding was therefore demonstrated *in situ* in cultured cells exposed to oxidative stress, with increasing levels being observed in cells treated with higher concentrations of H$_2$O$_2$. The ability of this assay to detect DNA lesions in brain tissue thought to have been exposed to raised levels of ROS was investigated. Levels of DNA damage were assessed in both mouse and human tissue using FITC-conjugated avidin once again.
5.3.2 AVIDIN BINDING TO MOUSE SPINAL CORD AND HUMAN BRAIN TISSUE

Mouse cervical spinal cord sections were used to investigate the effect of different fixation procedures on avidin binding, and during experiments to study levels of oxidative lesions in aged animals as opposed to young animals. Binding within the motor neurons of the anterior dorsal horn (Figure 5.3) was of interest, because these are affected during the neurodegenerative disorder motor neuron disease. These neurons are large, pyramidal cells easily distinguishable among other neuronal and glial cells (Figure 5.3B). Initial experiments carried out on human tissue investigated avidin binding within the motor neurons of the hypoglossal nucleus of the medulla (Figure 5.4) after different fixation procedures. These cells can also be easily distinguished from others in the vicinity, as they have large, irregular cell bodies and clearly visible axons (Figure 5.5B). Lipofuscin was evident as a browny-yellow area within cells visualised using an H&E stain (Figure 5.5B, arrow).

5.3.2.1 THE EFFECT OF FIXATION METHODOLOGY ON AVIDIN BINDING

The first experiments carried out investigated the effect of different fixation procedures on levels of fluorescence detected within motor neurons of both mouse and human tissue. Cervical spinal cord sections were taken from healthy mice, and medulla slices from the brains of patients without neurodegenerative disease, for this study of background binding. Sections were fixed as shown in Table 5.2. Avidin conjugated to the fluorescent label FITC was detected using fluorescence microscopy, with images being captured directly onto computer for later quantitative analysis. Avidin binding was observed in both neurons and glial cells throughout each section studied, although more fluorescence was generally observed in neurons. The motor neurons were easily distinguished from other cells in each section studied. The colour of the images was inverted in each case, and fluorescence therefore appeared as darker areas.

5.3.2.1.1 BINDING TO MOTOR NEURONS WITHIN MOUSE CERVICAL SPINAL CORD

Sections taken from tissue which had been flash-frozen immediately on dissection were not always intact, and the cells observed under the microscope appeared slightly 'blurred'. Fluorescence was obvious within the motor neurons however, with higher levels being seen in the cytoplasm rather than the nucleus (Figure 5.6A). Non-specific binding of avidin was not apparent. Images of control tissue showed no autofluorescence within these cells (Figure 5.6B), and this was true in each subsequent case. The images
of cells collected from tissue which had been formalin fixed, then frozen (Figure 5.7) or paraffin embedded (Figure 5.8) were more sharply defined than for fresh tissue. Once again avidin was concentrated within the cytoplasm of the cells rather than the nucleus, to which there was clearly little binding (Figure 5.7; Figure 5.8). No obvious difference in levels of binding could be seen by eye when comparing results involving tissue frozen immediately, or fixed in formalin for 2 hours before freezing or paraffin embedding (Figure 5.6A; Figure 5.7A; Figure 5.8A). Results from tissue which had been formalin fixed for 24 hours before further processing, however, did appear to show increased levels of fluorescence in neurons (Figure 5.7B; Figure 5.8B).

Quantitation of fluorescence within the cytoplasm of motor neurons was carried out using the NIH Image 1.6 programme, in order to assess more accurately any differences in avidin binding to alternatively fixed tissue. Results showed levels of fluorescence were lowest in cells assessed within fresh, frozen tissue and highest in tissue fixed in formalin then frozen (Figure 5.9). Intermediate levels of binding were observed in sections taken from paraffin embedded tissue. Fluorescence was higher in cells that had been fixed for 24 hours in formalin, then processed further, when compared to cells processed in the same way after 2 hours formalin fixation. Levels of binding were significantly raised in cells fixed 24 hours in formalin before flash freezing when compared to results for fresh frozen tissue (p=0.0003) and paraffin-embedded tissue (2 hours formalin, p=0.005; 24 hours formalin, p=0.0492). No significant difference was observed between results for fresh, frozen tissue and paraffin embedded tissue.

5.3.2.1.2 BINDING TO MOTOR NEURONS WITHIN HUMAN MEDULLA

Fluorescence observed within motor neurons of the hypoglossus nucleus in human medulla sections was not obviously different in tissue fixed in formalin for 5 days before freezing (Figure 5.10A) or embedded in paraffin immediately on dissection (Figure 5.10B). Once again binding was concentrated within the cellular cytoplasm, with the nucleus being clearly non-fluorescent. A granular pattern of staining was evident within these cells however, rather than the more uniform fluorescence observed in mouse spinal cord tissue. Lipofuscin was very autofluorescent, and was clearly defined within the each cell (Figure 5.10A, arrow). This meant quantitative measurements could not be taken using human tissue. Control tissue, incubated with Tris buffer in the absence of avidin-FITC, clearly showed the autofluorescent lipofuscin component of each cell
(Figure 5.11). There was no granular pattern of fluorescence present in these sections however, and the outline of the cells was not visible. All negative controls for human sections were like this.

5.3.2.2 DNA DAMAGE IN TISSUE FROM HEALTHY YOUNG MICE VS HEALTHY AGED MICE

In order to try and show an increase in oxidative DNA damage with age, avidin binding was assessed in the motor neurons of cervical spinal cord of young mice (7 weeks) (Figure 5.12), and the results were compared with those obtained using tissue from aged mice (>18 months) (Figure 5.13). Fresh tissue frozen immediately after dissection was used for this study, as this had previously been shown to give the lowest background levels of avidin binding. Fluorescence was once again clearly concentrated within the cytoplasm, and increased levels did appear to be present within neurons of the aged mice (Figure 5.13) compared to those in sections from young mice (Figure 5.12). In one case increased binding was also apparent in glial cells as well as neurons (Figure 5.13B).

Levels of avidin binding within these cells were quantified using NIH Image 1.6 once again. Results showed fluorescence to be slightly lower in motor neurons of young mice compared to aged mice (Figure 5.14), and this was significant in some cases (Table 5.3).

5.3.2.3 AVIDIN BINDING IN SPINAL CORD SECTIONS FROM PATIENTS WITH NEURODEGENERATIVE DISEASE AND AGE-MATCHED CONTROLS

Experiments were finally carried out to investigate levels of oxidative DNA damage in tissue from patients with motor neuron disease compared to age-matched controls who showed no signs of neurodegenerative disease. Cervical/lumbar spinal cord paraffin sections were used in these studies. The post-mortem delays (PMD) for MND tissue and age-matched controls chosen were as close as possible. Details of the patients chosen are shown in Table 5.1.

Pathological changes within the spinal cord were evident in MND patients compared to control; there was loss of grey and white matter in the latter, and both ventral and dorsal horns are obviously shrunken (Figure 5.15). The large, lower motor neurons investigated were situated within the anterolateral area of the dorsal horn (Figure 5.15, arrows). Motor neurons were readily visible throughout the dorsal horn of control
tissue, but none were obvious in spinal cord sections taken from most MND patients (Figure 5.16). Some MND sections did contain several visible motor neurons, and these appeared normal under the microscope. An examination of the patients history in these cases, however, showed them to be suffering symptoms to the upper motor limbs rather than the lower. In control sections the nucleus within many motor neurons was visible, and lipofuscin was evident as very dense, dark patches by the stain used (Figure 5.17A, arrow). The few motor neurons visible in MND sections at high power were shrunken, very darkly stained, and had no obvious nucleus (Figure 5.17B).

Very preliminary experiments assessed binding of avidin-FITC to the large motor neurons of the ventral horn in sections taken from 3 MND patients, and compared these results to those obtained from sections taken from age-matched controls. This was done subjectively using fluorescence microscopy, as quantitative studies were made difficult by the level of autofluorescent lipofuscin within the tissue. In one case it is possible that more fluorescence was observed within MND motor neurons (Figure 5.18B) compared to control cells (Figure 5.18A). In the other 2 cases it could not be said whether higher levels of avidin binding was present in MND motor neurons compared to controls (Figure 5.19), although the 'grainy' pattern of fluorescence was once again present in cells from both sections. Binding was again very obviously cytoplasmic, with the nucleus appearing completely clear of fluorescence. There was more fluorescence in neurons compared to glial cells, as seen in mouse spinal cord tissue. Lipofuscin was highly fluorescent within the cells and could clearly be distinguished from avidin binding. This was confirmed looking at sections under UV light in which autofluorescence is visible, but not FITC (data not shown). Control sections incubated with buffer rather than avidin-FITC also showed lipofuscin to be the only autofluorescent component visible within both control and MND sections (Figure 5.20).
Figure 5.1 The location of avidin binding in IMR32 cells 1 hour after exposure to HBSS in the absence of (A) or presence of $10^6$M (B) or $10^7$M hydrogen peroxide (C) (Magnification x320).
Figure 5.2 Inhibition of avidin binding by pre-incubation of IMR32 cells with the antioxidant α-tocopherol. Cells were exposed to 10 M H₂O₂ in HBSS in the absence (A) and presence (B) of α-tocopherol, left for 1 hour to recover at 37°C, then fixed with 2%(w/v) paraformaldehyde, 0.5%(v/v) glutaraldehyde before the DNA damage assay was carried out and photomicrographs taken (Magnification x320).
Figure 5.3  Mouse cervical spinal cord showing the location of motor neurons in the anterior dorsal horn (arrows). (Nissl stain (A) x100 (B) x 200).
Figure 5.4 Human medulla showing the location of motor neurons in the hypoglossal nucleus (arrows) (Haematoxylin and eosin (H&E) x10).
Figure 5.5 Human medulla showing the location of motor neurones in the hypoglossal nucleus of a control patient (arrows). Lipofuscin is evident in these cells (arrow, section B) (H&E (A) x100 (B) x400).
### Table 5.2 Different treatments used to fix mouse cervical spinal cord sections and human medulla sections. The effect of these on avidin binding was assessed in tissue taken either from healthy mice and patients not suffering from neurodegenerative disorders.

<table>
<thead>
<tr>
<th>SECTION</th>
<th>FIXATION TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MOUSE CERVICAL SPINAL CORD</strong></td>
<td></td>
</tr>
<tr>
<td>(i) Fresh tissue, flash-frozen immediately in isopentane cooled in liquid</td>
<td></td>
</tr>
<tr>
<td>(ii) Tissue fixed in formalin for 2 hours, then flash frozen as above.</td>
<td></td>
</tr>
<tr>
<td>(iii) Tissue fixed in formalin for 24 hours, then flash frozen as above.</td>
<td></td>
</tr>
<tr>
<td>(iv) Tissue fixed in formalin for 2 hours, then processed and paraffin embedded</td>
<td></td>
</tr>
<tr>
<td>(v) Tissue fixed in formalin for 2 hours, then processed and paraffin embedded</td>
<td></td>
</tr>
<tr>
<td><strong>HUMAN MEDULLA</strong></td>
<td></td>
</tr>
<tr>
<td>(i) Tissue fixed in formalin for 5 days, then flash frozen as above.</td>
<td></td>
</tr>
<tr>
<td>(ii) Tissue processed and paraffin embedded immediately on dissection.</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.6 (A) Avidin binding in motor neurons of mouse cervical spinal cord. Tissue was frozen immediately after dissection, with no fixation. Colour is inverted, with dark areas representing fluorescence. Binding is observed mainly in the cytoplasm, with the nucleus appearing as a pale oval within the cell (arrow). (B) Control tissue incubated with Tris buffer only. (Magnification x400).
Figure 5.7  Avidin binding in motor neurons of mouse cervical spinal cord. Tissue was (A) fixed in formalin for 2 hours before freezing, or (B) fixed in formalin for 24 hours before freezing. (Colour inverted, x400).
Figure 5.8  Avidin binding in motor neurons of mouse cervical spinal cord. Tissue was (A) fixed in formalin for 2 hours before paraffin embedding, or (B) fixed in formalin for 24 hours before paraffin embedding. (Colour inverted, x400).
An investigation into the effect of different fixation procedures on retina binding in ocular surgery of mammalian retinal degeneration and repair.

A

B
An investigation into the effect of different fixation procedures on avidin binding in motor neurons of mouse spinal cord using a fluorescence end-point

**Figure 5.9** Results of quantification of fluorescence within motor neurons of mouse spinal cord. The effects of different fixation procedures on avidin binding within these cells. Values are the means ± standard deviation, where n ≥ 7 readings. * signifies points which are significantly different at the 95% confidence level from fluorescence observed in unfixed, frozen tissue, using ANOVA procedures with Scheffe multiple range tests.
Figure 5.10  Avidin binding in motor neurons of the hypoglossal nucleus in human medulla. Tissue was (A) fixed in formalin for 5 days before freezing, or (B) processed and embedded in paraffin immediately after dissection from brain. Lipofuscin is apparent as very dark areas within the neuron (arrow). (Colour inverted, x400).
Figure 5.11 Control tissue for human medulla. Tissue was (A) fixed in formalin for 5 days before freezing, or (B) processed and embedded in paraffin immediately. This was incubated with Tris buffer rather than avidin-FITC during the assay. Autofluorescence observed here within the motor neurons was due to their lipofuscin content. (Colour inverted, x400).
Figure 5.12 Avidin binding in motor neurons of cervical spinal cord taken from healthy young mice (7 weeks). Tissue was frozen immediately after dissection. (Colour inverted x400).
Figure 5.13  Avidin binding in motor neurons of cervical spinal cord taken from healthy aged mice (18 months). Tissue was frozen immediately after dissection. (Colour inverted, x400).
An investigation into levels of avidin binding in spinal cord motor neurons of young mice (7 weeks) compared to aged mice (18 months)

Figure 5.14 Results of quantification of fluorescence within motor neurons of spinal cord from healthy young (7 weeks) and aged (18 months) mice. Values are the means ± standard deviation, where n ≥ 20 readings. * signifies points which are significantly different at the 95% confidence level from fluorescence observed in young mouse 1, using ANOVA procedures with Scheffe multiple range tests.
Table 5.3 Results of quantitative analysis of fluorescence within motor neurons of cervical spinal cord from young mice (YM) (7 weeks) and aged mice (AM) (> 18 months). Mean values were calculated from readings of at least 20 cells. Statistical analysis was carried out using ANOVA procedures with Scheffe multiple range tests. If p < 0.05, then the result was deemed statistically significant.

<table>
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<th>MOUSE AGE</th>
<th>MEAN FLUORESCENCE</th>
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<td></td>
<td></td>
<td>vs OM 1</td>
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<td>p = 0.043 (S)</td>
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<tr>
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<td>vs OM 2</td>
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Figure 5.15 Human lumbar spinal cord comparing the overall architecture of lumbar spinal cord from a control patient showing no sign of neurodegenerative disease (A) with that of an MND patient (B). The location of motor neurons within the anterolateral region of the ventral horn is also shown in each section (arrows). Both patients were 69 years of age, and PMD was 10 hours for control and 4 hours for MND. (Cresyl violet, x10).
Figure 5.16  Human lumbar spinal cord showing the distribution of motor neurons within the anterior dorsal horn of a control patient (A) and an MND patient (B). Both patients were 69 years of age, and PMD was 10 hours for control and 4 hours for MND. The ventral horn is shrunken in the MND section, and no obvious motor neurons are visible in this case (B). (Cresyl violet, x100).
Figure 5.17 A comparison of motor neurons in a control patient (A), and an MND patient (B). MND neurons are shrunken with no visible nucleus. Lipofuscin is observed as dark, dense areas within the neuron (arrow). (Cresyl violet, x200).
Figure 5.18 Avidin binding in motor neurons of human cervical cord in (A) a control patient, and (B) an MND patient. Patients were both 74 years old. Post mortem delay was 21 hours for control tissue and 14 hours for MND tissue. (Colour inverted, x400).
Figure 5.19 Avidin binding in motor neurons of human cervical cord in (A) a control patient, and (B) an MND patient. Patients were both 77 years old. Post mortem delay was 19 hours for control tissue and 15 hours for MND tissue. (Colour inverted, x400.)
Figure 5.20 Control sections incubated with buffer in the absence of avidin-FITC, showing autofluorescence of lipofuscin within the cells of motor neurons from a control (A) and MND (B) patients. (Colour inverted, x400) (H&E (A) x100 (B) x400).
**A**

These experiments aimed to determine whether oxidative DNA damage directly induced in both cultured cells and human and mouse models could occur. Results were obtained using transfection methodology. DNA damage was not previously observed directly in neurons, although Yamamoto et al. (1992) have described the lipid peroxidation of mitochondria using a mono-iodo substrate. This lesion was detected in cultured hippocampal neurons treated with \( \text{H}_2\text{O}_2 \) in vitro and in vivo experiments. Results from these experiments and data from other laboratories contribute to the theory that oxidative DNA damage is a major cause of cellular damage in Alzheimer's disease. A similar effect in the absence of a primary antibody has also been described in human brain sections (Cullen, 1994), although the mechanism of binding remains undetermined.

**B**

All experiments were carried out using UVA (340 nm) light-emitted in both nuclear and mitochondrial regions for oxidative damage to DNA. Levels of binding were greater in both cases, and these results are consistent with the hypothesis that oxidative DNA damage is induced by UVA light. In order to investigate the role of oxidative DNA damage in the progression of oxidative stress and its implications for the lipid-oxidized and other cells, additional experiments were conducted to determine the effect of oxidized DNA damage induced by oxidative stress on the oxidative stress response. The results also showed that the exposure to oxidative DNA damage induced by UVA light is effective in the presence of ultraviolet-induced DNA damage. These results are consistent with the hypothesis that oxidative DNA damage is a major cause of cellular damage in Alzheimer's disease.
5.4 DISCUSSION

These experiments aimed to demonstrate oxidative DNA damage directly in situ in both cultured cells and human and mouse spinal cord sections. Results were visualised using fluorescence microscopy. DNA damage has not previously been shown directly in neurons, although Yarborough et al (1996) have described the direct visualisation of 8-oxodG using a monoclonal antiserum. This lesion was detected in cultured hepatocytes treated with H$_2$O$_2$ or aflatoxin B$_1$, cryostat liver sections of rats treated with aflatoxin B$_1$, and human oral mucosal cells from smokers and non-smokers. Specific nuclear antibody binding was detected in each case, with increased levels of binding being observed in cells exposed to oxidative stress. It should be noted, however, that avidin conjugated to horseradish peroxidase was used as the detection system, and no mention of negative controls in the absence of the antibody are mentioned. The evidence presented in this thesis that avidin is able to bind directly to 8-oxodG (Struthers et al, 1998), suggest that the binding observed by Yarborough et al may have been due to the detection system used rather than the monoclonal antibody. The direct visualisation of pathological features of Alzheimer’s disease using avidin in the absence of a primary antibody has also been described in human brain sections (Cullen, 1994), although the mechanism of binding remained undetermined.

All experiments using differentiated IMR32 neuroblastoma cells showed clear, nuclear localisation of avidin-FITC after exposure to H$_2$O$_2$. Levels of binding were greater in cells exposed to higher concentrations of the toxin. This correlates well with the hypothesis that avidin is binding to the lesion 8-oxodG, produced by H$_2$O$_2$. In order to investigate mechanism of ROS action in these cells levels of oxidative DNA lesions were directly visualised after incubation with H$_2$O$_2$ in the presence and absence of the lipid-soluble molecule α-tocopherol. Nuclear binding of avidin was obviously decreased in cultures preincubated with this chain-breaking antioxidant. Previous studies had also shown this molecule to protect against oxidative DNA damage induced by both H$_2$O$_2$ and UVA irradiation (see Chapter 4). These results provide more evidence for the involvement of lipid peroxidases in nuclear DNA damage; if these molecules were not involved then α-tocopherol, which protects against lipid peroxidation, is unlikely to have any effect on the levels of 8-oxodeoxyguanosine produced.
Further experiments should be carried out to try and clarify the contribution of lipid-phase radicals to DNA damage in this model system. Oxidised DNA lesions may be assessed in the presence of other lipid soluble radical scavengers, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) used in studies of lipid peroxide induced DNA damage in plasmids (Yang and Schaich, 1996), and the results compared to those obtained in the presence of cytosolic antioxidants such as glutathione. The effect of metal chelators (e.g. desferrioxamine) on oxidised base levels in this model system would also be of interest, in order to investigate the role of metal ions in the damage observed. In this way the importance of lipid peroxidation reactions on DNA damage could be properly assessed.

It would also be of interest to use more sophisticated microscopy techniques, such as electron microscopy and confocal microscopy, to visualise binding of fluorescently-labelled avidin to 8-oxodG under greater resolution. In this way the exact site of damage could be studied. It is very likely that mitochondrial DNA (mtDNA) is affected, as well as the nuclear DNA visualised so clearly in these cells. It has been hypothesised that the accumulation of mutations in the mtDNA is important in the development of degenerative diseases and also ageing (Linnane et al, 1989). This theory is based on the close proximity of mtDNA to relatively high levels of ROS, the lack of histones which protect the DNA, the poor repair capacity of this genome and its high rate of replication, all of which lead to a high level of mutations. Levels of mitochondrial damage have been shown to be more extensive than nuclear DNA in human fibroblast cells (Yakes and Van Houten, 1997), whilst levels of oxidative damage were higher in mtDNA compared to nDNA in the brain tissue from patients with Alzheimer’s disease (Mecocci et al, 1994). In view of these facts, it should be determined whether oxidised base damage occurs earlier, and at higher levels, in mtDNA compared to nDNA.

DNA damage was also visualised directly in brain tissue. This is of importance when investigating post-mortem tissue from patients with neurodegenerative disease, where levels of ROS are believed to be raised (Olanow, 1993). Increased levels of 8-oxodG have been observed in brain tissue from patients with AD compared to controls by some investigators (Mecocci et al, 1994; Lyras et al, 1997), but not others (Te Koppele et al, 1996). This variability may be a consequence of the techniques used, in which DNA must first be isolated from cells or tissue before analysis, with the consequent
risk of artefactual damage. These techniques also do not allow detection of damage in specific neurons within a certain brain area. Preliminary work using avidin conjugates for the direct detection of 8-oxodG have shown binding to neural post-mortem tissue from individuals with neurodegenerative disease in excess of that observed in age-matched controls (Struthers et al, 1998). Increased levels of this oxidative base lesion have also been observed in the motor neurons of mice exposed to mercury compared to those not exposed (Pamphlett et al, 1998). This heavy metal has been implicated in the development of MND (Fitzmaurice et al, 1996). These lines of research were therefore investigated further.

Initial experiments were carried out to investigate the effects of different fixation procedures on binding of avidin. It was thought that fresh tissue, frozen immediately after dissection, would contain lower background levels of DNA damage, as opposed to tissue which was formalin fixed or embedded in paraffin, mainly because of a lack of exposure to chemicals or changes in temperature. Formalin is also autofluorescent, and could interfere with the results obtained. The results obtained during fixation experiments suggested that accurate data could be obtained from processed brain tissue taken from patients with MND and controls, because levels of damaged DNA in paraffin sections were not significantly different from fresh, frozen tissue. Formalin did appear to produce higher background levels of fluorescence, as predicted. This is likely to be due to the autofluorescent qualities of the fixative enhancing fluorescence of FITC. Therefore whilst fresh, frozen tissue was preferred during investigations of damage in motor neurons of mouse spinal cord, paraffin embedded tissue was preferred to formalin-fixed tissue during experiments using human tissue. It should be noted that all the binding observed was cytoplasmic, with the nucleus appearing as a clear, circular space within the cell.

Problems arose when trying to measure fluorescence in motor neurons in human tissue because of the strong autofluorescence of lipofuscin within these cells. Lipofuscin is created when a breakdown product of lipid peroxidation, malondialdehyde (MDA), reacts with thiol groups of protein amines to form a polymerised fluorescent compound (Pierrefiche and Laborit, 1995). This compound is a cumulative histological marker of ageing, and cells from both aged controls and MND patients contained variable amounts, making an accurate assessment of fluorescence very difficult. For this reason no quantitative data was obtained from human tissue. Once again, avidin binding was
evident in the cytoplasm of human motor neuron cell, and was granular in nature, but did not appear to be associated with lipofuscin. This type of binding was reminiscent of Nissl staining, in which the rough endoplasmic reticulum (RER) is visualised (Hartmann and Davidson, 1982). It is possible that avidin is binding to 8-oxodG lesions within ribosomal RNA present in the ribosomes which 'coat' the RER. DNA damage may also be observed within the mitochondrial genome, which is very susceptible to the actions of ROS (Linnane et al., 1989). Finally, it may be hypothesised that DNA lesions clipped out of the nuclear genome via repair enzymes and expelled into the cytoplasm bind non-specifically to cellular macromolecules, and are therefore available for avidin binding. This could also be the mechanism of avidin binding to pathological inclusions in brain tissue from AD patients during experiments carried out by Cullen (1994). Another explanation is that avidin is binding to endogenous biotin, found as part of carboxylase enzymes within the mitochondria, as mentioned previously (Section 3.4). More detailed microscopy studies could help to clarify the actual site of binding, and whether nucleic acids or carboxylase enzymes are involved. The lack of damage observed in the nucleus of both mouse and human motor neurons remains unexplained to date. It is surprising because nucleur binding of avidin has been observed previously in cells of the human cortex. It may be that avidin is not entering the nucleus under the experimental conditions used, or that nuclear oxidative lesions are low due to the action of repair systems. If the latter is the case, then levels of excised base products would be expected to be relatively high within the cellular cytoplasm. Further experiments are needed to investigate the pattern of binding further.

Very preliminary studies were then carried out to assess levels of oxidative DNA damage in spinal cord motor neurons of young mice compared to aged mice, and the same cells in patients with motor neurons compared to age-matched controls. Fresh, frozen mouse tissue was used, whilst sections of human tissue was taken from paraffin blocks. Initial results seemed to show an increase in DNA lesions in aged mice compared to young mice, both visually and quantitatively. Data was only obtained from sections taken from 2 young mice and 2 old mice, although the mean of >25 measurements was taken for each animal. Care should therefore be taken when interpreting the information obtained. The levels of binding observed in these animals were relatively high, considering they represented a background reading of oxidative DNA damage in control
populations. It is likely that methodological modifications could help decrease this reading, making comparisons with damage in motor neurons of mice exposed to mercury easier to visualise. Many more results need to be obtained for a real picture of age-related base damage in mice to be achieved. Experiments may also be carried out using human tissue to investigate whether age-related increases in DNA damage can be demonstrated using this technique.

Investigations of DNA damage in MND tissue was compared to age-matched controls to ensure that any age-related lesions did not interfere with results. Differences in post-mortem delay were also kept to a minimum. There was some evidence of increased avidin binding in one case out of the three investigated, although binding was restricted to the cytoplasm once again. 'Nissl-like' staining was also observed. Lipofuscin was evident again, therefore no quantitative results could be made. A proper comparison could be carried out if a non-fluorescent end-point such as horseradish peroxidase was used, and this should be done in further studies. It should be noted that previous work investigating levels of oxidatively-modified DNA in MND brain tissue has involved the use of avidin-HRP rather than avidin-FITC (data not shown). In this case nuclear staining was clearly evident, indicative of damage to nuclear DNA. This clearly needs to be investigated further. Another problem experienced was a variation in cell size between motor neurons from control cases and those from MND cases. MND neurons were very shrunken, and fluorescence may appear very intense as it is contained in a smaller area than in control cells, leading to a false impression that DNA damage is actually increased in MND cases. This could be investigated by measuring the mean area of cells in paired samples using the NIH Image program and comparing results for MND samples with those for control samples. Further investigations could involve electron microscopy studies to analyse levels and precise sites of oxidative DNA damage in individual neurons. It would also be of interest to investigate correlations between levels of 8-oxodG and the severity of the disease. In this preliminary study a small number of cases were examined, and therefore no clear conclusions can be made from the results obtained. As before, many more sections need to be investigated in order to obtain results of any significance.

The pattern of avidin binding was very different in tissue sections compared to the cell line in this study. In the former, binding was almost always cytoplasmic in nature,
whereas in IMR32 cells avidin was clearly located in the nucleus. These results were surprising, because nuclear binding of avidin has been previously observed in pathological tissue sections (data not shown). There are several possible explanation for these results, including binding of avidin to endogenous biotin, mitochondrial DNA, RNA or DNA repair products which have been retained in the cytoplasm, all mentioned previously. It is also possible that the methodology is at fault in some way, and it should be carefully checked and compared with methodologies of experiments where nuclear binding has been observed. It would also be more helpful if more controls were included. Levels of fluorescence in the absence of avidin-FITC were assessed in both cells and tissue sections. Experiments should also be carried out to investigate binding of unconjugated FITC, to ensure that the binding in these experiments is due to avidin alone. Avidin binding could also be viewed after preincubation with either biotin or 8-oxodG, to look at the specificity of the interaction.

In conclusion, although the initial results of these preliminary studies are interesting, and suggest a lot, much more work needs to be carried out before they can be confirmed.
CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS
AND FUTURE WORK
6.1 GENERAL DISCUSSION

This thesis aimed to investigate the hypothesis that nuclear DNA damage initiated by oxidative stress is an early biomarker of neuronal cellular dysfunction and death. The unique function of the neurones, together with the fact that they are non-renewable, means damage affecting their function will have severe consequences. ROS have been implicated in the pathology of the normal ageing process (Nohl, 1993; Baija et al, 1994) and many different disease states, including common neurodegenerative disorders (Coyle and Puttfarcken, 1993; Olanow, 1993; Simonian and Coyle, 1996). It is possible that these species are part of a final common pathway of cell death, and that oxidative damage to cellular DNA has an important role to play, although the mechanisms involved remain unclear.

DNA is very sensitive to attack by ROS (Imlay and Linn, 1988; Ames, 1995), and high levels of damage may be of biological importance. The DNA sites of gene products which are heavily transcribed, such as for proteins of the cytoskeleton, may be more sensitive to damage by ROS than others. Inaccurate transcription could result in the production of inactive or faulty proteins. Therefore it is possible that DNA damage could have the potential to indirectly disrupt vital macromolecules such as the neuronal cytoskeleton (see Appendix 4 for hypothetical chain of events). Changes in the structure of the neuronal cytoskeleton are a common feature of many neurodegenerative diseases, and neuronal inclusions observed in brain tissue from patients who have died from these disorders have been found to contain abnormal cytoskeletal elements (Carpenter, 1968; Goldman et al, 1983; Lee et al, 1991; Doering, 1994). The consequences of abnormal deposition of cytoskeletal elements in these diseases remain unclear, but it is likely that the cell stability is decreased, along with disruption to essential processes such as axonal transport. The specificity of different inclusion bodies in different disorders implies other more specific cellular responses than DNA damage. Different aetiological agents, such as neurotoxins, genetic factors and viruses, may act as an initiator of oxidative stress, and it is very likely that more than one cause is responsible for the development of these diseases. The nature of cell death seen in different neurodegenerative diseases may be dictated by the causal agent, with the action of ROS forming a final common pathway of damage. Understanding the mechanisms involved in these processes may lead to novel therapeutic approaches in these diseases. This thesis aimed to study the mechanisms
involved in DNA damage induced by oxidative stress, both in neuronal cells \textit{in vitro} and pathological tissue. In order to achieve this, a suitable \textit{in vitro} model system and a method for detecting oxidative DNA lesions needed to be established.

Differentiated IMR32 neuroblastoma cultures were chosen for these experiments, because they are known to express mature neuronal biochemical (Gotti \textit{et al}, 1987) and cytoskeletal (Johnston, 1995; Mortimore, 1996) markers. Differentiating cultures were investigated microscopically over a period of 2 months, and were found to develop a morphology very similar to that of primary rat brain cultures (Johnston, 1995), which are generally thought to provide the closest cell culture representation of neurons \textit{in vivo}. These results suggest that differentiated IMR32 cells may be used as a simple \textit{in vitro} model of mature neurons \textit{in vivo}. Successful preliminary studies were also carried out to assess the dose range of oxidative stress needed to investigate pre-lethal events within the cell, with the results once again being visualised using microscopy.

It is hypothesised that levels of oxidative DNA damage may provide an early biomarker of pre-lethal neuronal damage. An assay capable of measuring these DNA lesions was therefore needed. Results of experiments carried out to investigate binding of an antibody raised against oxidative DNA damage led to the serendipitous finding that avidin itself binds to the lesion 8-oxodG. This lesion is a widely-accepted biomarker of oxidative damage (Halliwell and Aruoma, 1991; Shigenaga \textit{et al}, 1994). An assay was therefore developed which was able to assess levels of 8-oxodG directly \textit{in situ}, using avidin conjugated to either colorimetric or fluorometric markers. Previous methodologies used to quantify levels of oxidative DNA damage have involved prior isolation of DNA from cells and derivitisation of bases, and the resultant artefactual damage introduced has led to very variable results. The avidin method can demonstrate damage directly in cells, and should therefore give more accurate results. To date the technique is only semi-quantitative, allowing relative levels of DNA damage to be assessed rather than absolute values. It is still able to provide important information on the mechanisms of ROS damage to DNA in both \textit{in vitro} systems and pathological tissue however, something which has not been possible previously. Although this assay was developed to study the role of ROS in neurodegenerative disorders, the scope for its use is very wide because of the huge range of pathological processes in which oxidative stress is implicated.
In vitro experiments were designed to investigate levels of DNA damage in differentiated IMR32 cultures exposed to different ROS-generating systems, using the avidin assay developed. Increased levels of 8-oxodG were detected after oxidative stress, and this lesion was shown to be a pre-lethal marker of cell death. Repairable damage was detected after exposure to very low levels of oxidative stress, with permanent damage being observed at higher levels. Although repairable damage is not biologically relevant with respect to the development of neurodegenerative disorders, it raises the possibility that excised repair products, including 8-oxodG, may be released from cells into the surroundings, providing an easily measurable biomarker of oxidative stress. It can also be hypothesised that when these products are released from the nucleus they 'stick' to macromolecules within the cytoplasm, interfering with their normal function. This could be a potential source of cellular dysfunction in neurodegenerative disease, and could provide an explanation for the mechanism of avidin binding in the absence of any antibody to abnormal inclusions in Alzheimer's tissue observed by Cullen (1994). A more detailed study of the precise site of avidin binding within cells should help resolve this.

Another factor studied using the in vitro model system was the effect of preincubation with the antioxidant α-tocopherol or iron chelator desferrioxamine. ROS-mediated DNA damage was shown to be decreased in the presence of α-tocopherol, which is a lipid-soluble molecule known to accumulate within cell membranes and protect the cell against the actions of radicals produced during lipid peroxidation. Although lipid hydroxyl and alkoxyl molecules have previously been shown to induce DNA damage (Yang and Schaich, 1996), most research has focused on the role of hydroxyl radicals in this process (Mello Filho et al, 1984; Dizdaroglu et al, 1991; Meneghini, 1997). Lipid peroxides could be important DNA-damaging agents under conditions of oxidative stress, because of their relative stability compared to other radicals (Pryor, 1986), and this hypothesis has been strengthened by the results obtained in this thesis. The role of the metal-catalysed Haber-Weiss reaction in oxidative DNA damage was also investigated, by assessing levels of 8-oxodG in cultures after preincubation with desferrioxamine. Some protection was afforded against UVA irradiation, but damage was not decreased when cells were exposed to \( \text{H}_2\text{O}_2 \). The reasons for this are not clear. It may be that copper ions are more important catalysts of damage than iron, and experiments with a copper chelator rather than an iron chelator should help elucidate this. It is also possible that the concentration of desferrioxamine within the cultures is not high enough to have any
effect. Whilst α-tocopherol is known to accumulate within cell membranes of differentiated IMR32 cultures (Thomas, 1986; Thomas and Anderton, 1991), desferrioxamine has not been shown to enter these cells. The fact that some protective effect has been observed suggests that sufficient levels of desferrioxamine are entering these cultures in some cases. Intracellular concentrations of this iron chelator should therefore be checked.

The observation of increased levels of 8-oxodG \textit{in vitro} led to an investigation of \textit{in vivo} situations during studies of oxidative DNA damage in pathological tissue and biological fluids. Experiments were designed to investigate whether an age-related increase in 8-oxodG could be detected in mouse spinal cord tissue, because of the existing evidence for age-dependent changes in oxidative DNA damage (Richter \textit{et al}., 1988; Nohl, 1993; Barja \textit{et al}., 1994). Levels of this base lesion were also investigated in tissue from patients with MND, in which the damaging actions of ROS are strongly implicated, and compared to those in age-matched controls. Results were not conclusive because of the small number of samples used, but they did suggest that 8-oxodG may be increased in motor neurons of aged mice compared to young mice, and MND patients compared to controls. It is important that these experiments are repeated using many more samples in order to obtain meaningful results, because previous studies have used methodologies which involved prior isolation of DNA (Sanchez-Ramos \textit{et al}., 1994; Lyras \textit{et al}., 1997); increases in oxidative DNA damage observed in tissue from patients with neurodegenerative disease may not have provided an accurate representation of true \textit{in vivo} levels, due to artefactual damage. The avidin methodology involves no prior manipulation of DNA, and also allows the precise location of damage to be ascertained.

It was surprising that avidin binding was detected in the cytoplasm of motor neurons and not the nucleus. It seems most likely that avidin is not entering the nucleus under the conditions used for some reason, because a background level of 8-oxodG would be expected. It may be hypothesised that binding in the cytoplasm is due to damaged RNA in the ribosomes, and lesions in mitochondrial DNA. It is also possible that DNA repair products released from the nucleus have attached to cellular macromolecules such as the endoplasmic reticulum, and are therefore available for avidin binding. More detailed microscopy studies should help resolve this. The problems associated with the use of a fluorescent detection system in tissue, particularly of human
origin, suggest that an colorimetric label, such as horseradish peroxidase, would be more suitable for these studies.

The fact that repair was known to occur in cells exposed to low levels of ROS (see Chapter 4), led to the hypothesis that DNA repair lesions may be released into biological fluids such as the CSF during oxidative stress, and could be measured using techniques such as CE. Experiments, which were once again preliminary, demonstrated the presence of DNA both in the extracellular medium of cells undergoing developmental apoptosis and CSF taken from patients with disease states in which ROS are implicated. DNA fragments have not been previously detected in CSF. It would be of interest to correlate levels of DNA detected in each sample with either the level of oxidative stress experienced by cultures in vitro, or the severity of disease in the case of CSF samples. It is possible that this type of measurement could give the earliest indication of oxidative stress in the brain, as levels of repair are being assessed rather than levels of cell death.

6.2 CONCLUSIONS

Several conclusions can be made from the results of work carried out in this thesis:

• IMR32 cells chemically differentiated with 5′-bromo-2-deoxyuridine for 2-4 weeks provide a stable neuronal model system with a mature morphology, in which the mechanisms of oxidative damage to DNA can be investigated. The morphology of these cultures remains unchanged when exposed to non-lethal levels of oxidative, which can induce increases in cellular metabolism suggestive of pre-lethal cell damage.

• Levels of ROS-induced DNA damage can be demonstrated directly in situ, using a novel methodology based on the ability of avidin to bind to the oxidative base lesion 8-oxodG. Semi-quantitative results and direct microscopic visualisation can be obtained using both colorimetric and fluorometric end-points.

• Increased levels of 8-oxodG can be detected in mature neuronal cultures exposed to various forms of oxidative stress in vitro. Damage induced by low levels of ROS is repairable, whilst lesions appear more permanent in cells exposed to higher levels of oxidative stress. Increased DNA damage is predictive of cell death in cultures exposed to the highest levels of stress.

• Pre-incubation of IMR32 cultures with the lipid-soluble antioxidant α-tocopherol can decrease levels of 8-oxodG induced by H$_2$O$_2$ or UVA. This is strong evidence for the
involvement of lipid peroxides in the production of nuclear DNA damage. The iron chelator desferrioxamine also prevented UVA-induced DNA damage in these cultures, showing the importance of the Haber-Weiss reaction in ROS-mediated reactions.

- Preliminary experiments suggest that levels of 8-oxodG may be raised in the motor neurons of aged mice, compared to young mice, and patients with MND as opposed to age-matched controls. Lesions were detected directly in situ in spinal cord sections using a fluorometric end-point. These results may help determine the importance of DNA damage in the development of neurodegenerative diseases in relation to other parameters of cell dysfunction observed.

- DNA can be detected in the extracellular medium of cells undergoing developmental apoptosis and biological, cell-free CSF samples taken from patients with disease states in which apoptosis is thought to be ongoing. It is possible that this is a measure of DNA repair, as repair products of oxidative damage may be released from cells. DNA in extracellular fluids could provide a quantifiable biomarker of neurodegenerative disease, as levels may be raised due to increased damage and subsequent repair. This could allow damage to be detected in vivo before the presentation of symptoms in these patients.

6.3 FUTURE WORK

The results obtained in this thesis lead to many further potential lines of investigation. One of the most important is the elucidation of the precise site of DNA damage, both in vitro and in pathological tissue. It is likely that mitochondrial DNA is a major site of damage, and it has been shown that mitochondrial DNA has higher levels of oxidative lesions than nuclear DNA (Richter et al., 1988). Mutations have also been found in the mitochondrial genome of brain tissue from patients with neurodegenerative disease (Ikebe et al., 1995; Bowling and Beal, 1995). It has been hypothesised that ROS-mediated DNA damage may have a central role in the ageing process, especially in the mitochondria (Adelman et al., 1988; Barja et al., 1994), and increased levels of 8-oxodG have also been detected in mitochondrial DNA in brain tissue from patients with Alzheimer’s disease compared to controls (Mecocci et al., 1994). Electron microscopy using avidin-gold labelling should allow the precise site of damage to be determined. Levels of mitochondrial and nuclear DNA damage could therefore be compared directly.

Microscopy techniques could also be used to analyse levels and sites in individual neurones of oxidative DNA damage in pathological tissue from patients with common
neurodegenerative disorders as compared to age-matched controls. *In situ* detection allows the precise location of cells with oxidative DNA damage to be determined. Preliminary investigations were carried out using samples from 3 MND patients and controls, but these were not able to yield conclusive results. Correlations between levels of 8-oxodG and the severity of disease could be investigated. For instance it would be of interest to compare levels of oxidative DNA damage with the distribution pattern of amyloid deposits and neurofibrillary changes described by Braak and Braak (1991) at different stages of Alzheimer's disease. Cells surrounding amyloid plaques could also be assessed for oxidative DNA lesions, as it has been suggested that the β-amyloid protein may exert toxicity by the production of free radicals. These experiments would enable the relationship between DNA damage and other pathological changes to be determined at the level of individual neurones.

Another area of interest is the temporal relationship between DNA damage and other indications of cellular dysfunction, such as altered cytoskeletal elements. The mechanisms involved in the development of neuronal inclusion bodies in neurodegenerative disorders are under intense investigation, because of their apparent importance in the neurodegenerative process. Studies have implicated a family of proteins known as the stress-activated protein kinases (SAPKs), also known as c-Jun N-terminal kinases (JNKs), in the development of paired helical filaments (PHF) in AD. As levels of these proteins will be raised in affected neurones before obvious development of PHF, the SAPKs may provide an early marker of cellular dysfunction in certain neurodegenerative diseases. However, their precise involvement in the development of these disorders remains unclear. The temporal relationship between oxidative DNA damage and SAPK activation is not known. Therefore, it would be of interest to establish which parameter provides the earliest indicator of cellular dysfunction. It may be hypothesised that DNA damage precedes SAPK activation, and a causal relationship should be considered.

The consequences of the sub-lethal changes may eventually lead to the initiation of apoptotic cell death. There is increasing evidence for a role of apoptosis in AD (reviewed by Cotman and Anderson, 1995). The Aβ peptide, a major protein in senile plaques, has been shown to cause neuronal cell death via apoptosis *in vivo* (LaFerla *et al*, 1995), and the products of immediate early genes (IEG) c-jun and c-fos associated with apoptosis...
have been observed in model systems and in pathological tissue from AD (Anderson et al., 1995; Anderson et al., 1996). SAPK/JNK is known to increase synthesis of c-Jun, therefore this may be another way in which the SAPK may be involved in the development of AD. Oxidative stress is known to induce apoptotic cell death in neurones both in vitro and in vivo (Whittemore et al., 1994; Mukherjee et al., 1995). As ROS have been implicated in many common neurodegenerative diseases it would be of interest to see whether this mechanism is involved in the cell loss observed. Therefore it would be of interest to investigate the temporal relationship between DNA damage, SAPK and the mechanism of cell death in cell cultures exposed to ROS, and to ascertain if DNA damage can be detected at lower levels of oxidative stress than is required to activate SAPK/JNK or p38/reactive kinases (p38/RK; a related group of SAPKs). Markers of apoptotic cell death in pathological tissue from patients who have died from neurodegenerative disease could be investigated as well.

It is also important to continue work investigating levels of excised DNA lesions in the media of cultures undergoing oxidative stress and in samples of biological fluid such as CSF, as this could lead to the discovery of markers of neurodegenerative disorders. The CE methodology used appeared to be sensitive and reproducible enough to obtain accurate results, and would therefore be useful in further experiments. Many more samples need to be analysed in order to build up a clear picture of DNA damage and repair in both in vitro model systems and clinical samples. CSF samples should then be analysed from patients with neurodegenerative disease, and it would be of interest to compare these results with those for parallel blood samples taken from these patients. The use of other techniques, such as flow cytometry, could also provide useful data in the analysis of these samples. It may eventually be possible to correlate levels of DNA repair lesions with severity of the disease. This in turn could lead to a method of assessing the effects of therapy for neurodegenerative disorders.

In summary the relationship between oxidative DNA damage and other markers of oxidative stress may be determined in experimental systems and neuropathological specimens sensitively and at a single cell level. This will enable a better assessment of the importance of oxidative damage to cellular pathology in neurodegenerative disease to be made.
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APPENDICES
APPENDIX 1

SPECIAL NOTE

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Direct Detection of 8-Oxodeoxyguanosine and 8-Oxoguanine by Avidin and Its Analogues

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8-Oxodeoxyguanosine is present in DNA from many tissues. The direct demonstration of 8-oxodeoxyguanosine as a potential biomarker of oxidative DNA damage has implications for the study of mutagenesis, carcinogenesis, and free radical toxicity. Avidin is shown here to bind with high specificity to this potentially mutagenic oxidized nucleoside, 8-oxodeoxyguanosine, and to the oxidatively modified base, 8-oxoguanine. The serendipitous finding that avidin bound to the nuclei of UVA-irradiated cells has led to the development of a technique which allows detection of the damage product in a manner analogous to that of immunological techniques. The technique has been shown to be applicable to isolated DNA and to DNA in fixed cellular material and postmortem tissue. Statistically different levels of damage can be demonstrated in both isolated DNA and cultured cells exposed to free radical generating systems using a 96-well plate-based methodology. The sensitivity of this method allows the detection of $10^{-19}$ mol of 8-oxodeoxyguanosine. This novel usage of avidin conjugates applies also to its bacterial analogue, streptavidin, and to a lesser extent to the monoclonal antibody to biotin (the ligand bound by the parent compound). This finding has tremendous potential as a simple method analogous to immunotechniques for the direct detection of 8-oxodeoxyguanosine. From structural considerations we speculate that avidin would also bind to 8-oxodeoxyadenosine.

Key Words: DNA; 8-oxodeoxyguanosine; free radicals; oxidation, avidin; detection.

The 8-oxoguanine adduct is used as a sensitive marker of DNA base damage caused by oxygen free radicals (1) although it is only 1 of at least 20 products formed (2). 8-Oxoguanine is formed in DNA treated with a variety of reducing agents (3) and oxygen radical generating systems (4) and is reported to cause mutations through its ability to form hydrogen bonds with bases other than cytosine (5, 6). In addition to having an early role in the pathway toward mutagenesis and carcinogenesis, this lesion may also be pathologically and diagnostically important in a number of conditions in which oxygen free radicals possess genotoxic potential; these include autoimmune inflammatory diseases, neurodegenerative diseases, atherosclerosis, ischemia/reperfusion injury, stroke, and chronic respiratory disease.

The widespread use of 8-oxoguanine as an important marker of oxidative damage to DNA followed the description of its analysis as the deoxynucleoside (8-oxodeoxyguanosine) in DNA digests by high-performance liquid chromatography (HPLC) with electrochemical detection (7). In this procedure DNA is first extracted from cells and tissues and then enzymatically digested to yield free deoxynucleosides which are separated by reversed-phase HPLC; 8-oxodeoxyguanosine is measured sensitively by electrochemical detection. In the same HPLC run native deoxyguanosine can be measured by UV absorbance in order to provide a reference. Alternatively, gas chromatography–mass spectrometry (GC–MS) methods have been used to quantitate 8-oxoguanine as the free base (8). These techniques (9), however, often give disparate results. In general, levels of 8-oxoguanine are higher when determined by the GC–MS technique. In freshly isolated cells, values of between 2- and 11-fold higher have been reported by GC–MS. A number of methodological factors may account for such discrepancies including incomplete enzymatic hydrolysis in the presence of oxidatively modified DNA, suboptimal enzymatic hydrolysis of DNA, and the balance between incomplete depurination versus
formation of artefacts by formic acid hydrolysis of DNA and trimethylsilylation when applying the GC–MS preparation steps. A recently described HPLC analysis of the 8-oxoguanine base may aid in accounting for this methodological variability (10). Antibody techniques for the identification of thymine glycol, 8-oxoguanine, and pyrimidine dimers have been used (11–13) although it would appear that it is necessary to extract the DNA prior to assessment of damage with the possible generation of artefactual DNA oxidation (14). A number of other approaches to the determination of 8-oxoguanine in DNA have been described. \(^{32}\)P-postlabeling procedures are well established in the literature (15–17); these methods provide the potential for very sensitive detection, although the techniques are very time-consuming and cumbersome, involving DNA purification, digestion with enzymes, enrichment of the 8-oxodeoxynucleotide, addition of a radiolabeled phosphate group, and thin-layer chromatography. The time-span of each batch of analyses is in the order of days. Capillary electrophoretic determinations of 8-oxodeoxyguanosine and 8-oxoguanine (18, 19) have been described. To date, these techniques lack concentration sensitivity due to inherent lack of sensitivity of UV absorbance measurements. The direct in situ demonstration of oxidative damage to DNA is therefore very desirable.

Avidin is a naturally occurring factor found in egg white, which has a remarkable affinity for the vitamin biotin. Avidin is a stable tetramer with twofold symmetry containing four biotin binding sites arranged in two clusters on opposing faces of the molecule. The major distinguishing feature of this interaction is the extraordinarily high affinity \((K_a = 10^{15} \text{ M}^{-1})\) between avidin and biotin (the bacterial analogue, streptavidin from \textit{Streptomyces avidinii}, has a similar binding affinity) (20). Binding of this affinity is generally restricted to liganded metal ions either as partial covalent bonds or as chelates. A characteristic of this degree of binding is the slow off-rate of dissociation, although the rate of dissociation is much higher for streptavidin than it is for avidin. This high affinity of binding is greater than that normally seen between antibodies and their epitopes; specifically, antibodies raised against biotin have affinities orders of magnitude lower than avidin for biotin (21).

Avidin is widely employed as a tool in both research and technology primarily as a secondary means of detection and amplification. It is frequently used in immunoassays where either a primary antibody is directly biotinylated or a secondary biotinylated antibody is employed. It is also used where a target molecule, such as a nucleic acid base, is chemically modified by biotinyllation to aid in its visualization. In order to eliminate the complication of “nonspecific” binding in these techniques the material under study is sometimes pretreated with both avidin and biotin to block endogenous binding sites. It has been reported previously that avidin binds to necrotic cells (22). Avidin is not currently used for the direct detection of biomolecules other than biotin.

The biotin molecule is largely hydrophobic and consists of an imidazolidone ring and an ureido group. It would appear that the whole molecule interacts with the avidin binding site. It has been proposed that the imidazolidone group is buried in the binding site with the carboxyl group interacting with a critical tryptophan residue and hydrogen bonds forming between the other functional groups of the ring (23). Hydrogen bonds are also thought to form between the binding site and the ureido group. Although a wide range of compounds that are analogues of small fragments of the biotin molecule will bind to avidin, the binding appears to be of relatively high specificity since related compounds do not bind significantly at the 1–10 mM level (23).

As a consequence of the phenomenological observation that avidin conjugates bind directly, and apparently specifically, to the nuclei of oxidatively damaged cells and tissue, the structural similarities between components of DNA and biotin were considered. The purine base guanine bears little structural relation to the biotin molecule; however, the keto forms of 8-oxodeoxyguanine and 8-oxodeoxyguanosine (24), damage products of oxidative DNA damage, are remarkably similar structurally to biotin. Therefore, the ability of avidin, streptavidin, and antibodies to biotin to bind to 8-oxodeoxyguanosine in a variety of model systems was investigated.

The ability of avidin (and its analogues) to bind to oxidatively modified DNA was investigated using a variety of different model systems in which it is known or suspected that levels of 8-oxodeoxyguanine are elevated. These model systems included isolated DNA, cultured cells, and pathological tissue. A variety of approaches were also employed to investigate the specificity of binding.

**EXPERIMENTAL PROCEDURES**

**Materials**

IMR 32 neuroblastoma cells were from the European Collection of Animal Cell Cultures (ECACC) (Porton Down, Salisbury). Monoclonal goat anti-biotin–FITC\(^{2}\)

Abbreviations used: FITCS, fluorescein isothiocyanate; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; αMEM, alpha minimal essential medium; NEAA, nonessential amino acids; MTT, 3-(4,5)-dimethyl-thiazol-2-yl-2,5-diphenyl tetrazolium bromide; FBA, fluorescence binding assay; HBSS, Hanks’ buffered salt solution; NGS, normal goat serum; TMB, 3,3’,5,5’-tetramethylbenzidine; MND, motor neuron disease.
SPECIAL NOTE

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anjugate antibody (Clone No. BN-34) was from Sigma (Poole, UK). Avidin–FITC conjugate was also from Sigma (A 2050). Components for the spectrophotometric determination of avidin binding were a research valuation kit from Biotrin International (Dublin, Ireland) (25). 8-Oxodeoxyguanosine was a kind gift from R. K. Herbert (Leicester University) and was prepared according to the Udenfriend system (26); its purity was checked by electrospray mass spectrometry. All other chemicals were of the highest possible purity and were from Sigma.

Methods

Preparation of oxidized DNA. Treatment of DNA with methylene blue leads almost exclusively to the formation of deoxyguanosine residues to 8-oxodeoxyguanosine. DNA (0.5 mg/ml in water) was incubated in the presence of methylene blue (20 μg/ml final concentration in 0.1 M tris, pH 8.5) in a petri dish on ice held from a white light source by 0.5 cm water in a upturned petri dish lid (light source to DNA distance as 3 cm). Irradiation was for 3 h at which time solid xanthium chloride was added to a final concentration of 1:500 directly into solutions of the potential competitors and added directly to the DNA preparations. The potential competitors were prepared in PBS immediately prior to use and pH corrected to 7.4.

Cell culture. The IMR 32 (passage number 66) cell line was obtained from ECACC. The cells were maintained in a humidified atmosphere of 95% air, 5% CO2 at 37°C. The IMR 32 cell line was routinely maintained in alpha-minimal essential medium (αMEM) with 10% (v/v) HIFCS and 1% (v/v) nonessential amino acids (NEAA). No antibiotics were used at any time. Cells were chemically differentiated before all experiments. Cells were plated out in 96-well plates for quantification of DNA damage by fluorimetry and measurement of 3-(4,5)-dimethyl-thiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) metabolism. IMR 32 cells were plated out in 96-well plates at 2 × 10⁵ cells per well in 200 μl of medium (αMEM with 5% (v/v) HIFCS and 1% (v/v) nonessential amino acids) with 5'-bromodeoxyuridine (1 × 10⁻⁵ M) and allowed to differentiate for various time periods (27). For fluorescence cytochemistry cells were plated out in 8-chamber plastic LabTek slides at 2 × 10⁶ cells per well in 300 μl of medium (αMEM with 5% (v/v) HIFCS and 1% (v/v) nonessential amino acids) with 5′-bromodeoxyuridine (1 × 10⁻⁵ M).

Fluorescent binding assay (FBA) for 8-oxodeoxyguanosine. Cultures of IMR 32 cells were grown in 96-well plates as described above and allowed to differentiate. Untreated cultures were fixed and permeabilized as above to provide a substrate to which 8-oxodeoxyguanosine would bind, thus permitting FBAs to be performed. Various concentrations of either the normal or the oxidized base were incubated on these plates (100 μl/well) for 1 h. The wells were then washed 3 times with PBS before visualizing bound base with either avidin-conjugated FITC (1:200 in PBS for 1 h) or with a FITC-conjugated anti-biotin monoclonal antibody (1:80 in PBS for 1 h). The level of binding was quantified with a fluorescence plate reader (Denley, Billingshurst, UK), 485 nm excitation and 535 nm emission. The absolute level of binding of 8-oxodeoxyguanosine and deoxyguanosine to the substratum was not determined using an alternative technique.

Hydrogen peroxide treatment. Differentiated IMR 32 cultures were exposed to hydrogen peroxide as fol-
freshly prepared hydrogen peroxide. The cultures were incubated with hydrogen peroxide for 1 h after which the HBSS was replaced with fresh media (αMEM with 5% (v/v) HIFCS and 1% (v/v) nonessential amino acids) and cultures were allowed to recover for 24 h under standard incubation conditions before cell death was assessed using the MTT assay and binding of avidin assessed by fluorescence analysis.

**MTT assay.** The reduction of MTT by mitochondrial succinate dehydrogenase (28) is a standard colorimetric, cytotoxicity assay. One hour before each time point, 20 μl of MTT (5 mg/ml in PBS) was added to each well and cell cultures were incubated for 1 h at 37°C. The medium was carefully aspirated and 100 μl of isopropanol was added to each well to solubilize the formazan product deposited within the viable cells. Absorbance was read at 550 nm on a scanning multwell spectrophotometer after agitating the plates for 5 min to ensure complete dissolution of the formazan product.

**Fluorescent assessment of avidin binding.** Cells were prefixed by the addition of 2% (w/v) paraformaldehyde (in PBS at pH 7.4) for 15 min after washing the cells with PBS. Cells were washed in warm PBS. Cultures were fixed and permeabilized with ice-cold methanol for 15 min, rehydrated in PBS before blocking with PBS containing 10% (w/v) normal goat serum (NGS). The blocking solution was washed off with PBS containing 0.2% (w/v) NGS. DNA damage was visualized with avidin-conjugated FITCS (1:200 in PBS for 1 h) or with a FITCS-conjugated anti-biotin monoclonal antibody (1:50 in PBS for 1 h) either for fluorescence microscopy or for quantitative assessment with a fluorescence plate reader (Denley, Billinghamurst, UK).

**Inhibition of avidin binding.** The ability of modified DNA bases and biotin to prevent binding of avidin to hydrogen peroxide-treated cells was investigated using IMR 32 cells grown in LabTek slides. Media were carefully aspirated and prewarmed HBSS was added to each well (300 μl) with or without 100 μM freshly prepared hydrogen peroxide. The cultures were incubated with hydrogen peroxide for 1 h after which the cells were fixed as above. Avidin–FITCS solutions with or without competitors present were prepared as follows: 100 μM solutions of biotin, guanine, and 8-oxodeoxyguanosine were prepared in PBS (pH 7.4) immediately prior to use. Avidin–FITCS (Sigma) was added (1:100) to the potential competitors and preincubated in the dark at room temperature for 1 h. The control, avidin–FITCS, in the absence of competitor, was treated in parallel. The solutions were centrifuged at 10,000 g in a microfuge for 5 min (room temperature) and the supernatant was used for binding experiments. Fixed cells were incubated with the solutions for 1 h in the dark at room temperature. The slides were washed three times in PBS before coverslips were mounted on the slides using Vectorshield (Vector Laboratories, Peterborough, UK), a glycerol-based antibleaching mountant.

**Alkali treatment.** In order to further investigate the specificity of avidin for the oxidised base, hydrogen peroxide-treated cultures were exposed to conditions known to lead to degradation of the imidazolone group (29). Cultures were exposed to hydrogen peroxide as above, for 1 h. Postexposure the hydrogen peroxide was replaced with complete medium for 1 h after which time the medium was removed and the cultures were washed carefully. Cells were prefixed by the addition of 2% (w/v) paraformaldehyde (in PBS at pH 7.4) for 15 min after washing the cells with PBS. Cells were washed in warm PBS. Cultures were fixed and permeabilized with ice-cold methanol overnight then rehydrated in PBS before treating with alkali. The cultures were incubated in wash solution (PBS/Tween, Biotrin) at either pH 7.0 or pH 12.0 (corrected with NaOH) for 1 h at room temperature. The cultures were washed three times in wash solution and then blocked with PBS containing 5% (w/v) NGS. The blocking solution was removed and wells were washed 3 times with wash solution. Avidin–HRP was added to the wells (2 mg/ml in PBS, 50 μl/well) and incubated at 37°C for 1 h in a humidified chamber. Detection of bound peroxidase-labeled avidin was with 3,3′,5,5′ tetramethylbenzidine (TMB) (Biotrin) according to manufacturer’s instructions. Following washing with wash solution 3 times, detection of bound peroxidase-labeled avidin was performed using 50 μl/well of TMB substrate (Biotrin); the reaction was stopped after 10 min at room temperature using 2 M H2 SO4 100 μl/well) and the product was determined spectrophotometrically at 450 nm using an Anthos 2001 plate reader.

**Pathological sections.** Flash-frozen tissue sections were thawed at room temperature for 15 min then fixed by the addition of 4% (w/v) paraformaldehyde (in PBS at pH 7.4) with 0.5% glutaraldehyde on ice for 5 min, and washed in PBS then saline. The samples were dehydrated through alcohol, then endogenous peroxidases blocked by incubation in methanol with 0.3% hydrogen peroxide for 15 min. The samples were rehydrated and washed in PBS. Avidin binding was visualized with avidin-conjugated FITCS (1:200 in PBS) for 1 h. Sections were washed extensively before coverslips were mounted on the sections using Vectorshield (Vector Laboratories).

**Statistical analysis.** Data were analyzed using the Statgraphics V.5.0 program (STSC Inc., U.S.A.). Data
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Avidin binding to UVA-treated cells. Avidin-FITC bound to the nucleus of IMR 32 cells irradiated with UVA for 10 min at room temperature. Cells were fixed and permeabilized immediately after irradiation. Antibody binding (not shown here) was visualized with avidin-FITC. There was no binding to sham-irradiated controls (A); however, even in the absence of any primary antibody avidin bound to nuclear material in irradiated cells (B).

RESULTS

The initial observation that avidin conjugates bound to DNA in the absence of any antibody was in UVA-treated cells. While investigating the potential of an antibody to UV-mediated DNA damage to bind to UVA-treated cells, in which it was anticipated that there would be free radical-mediated damage (including to DNA), binding of avidin–FITC to nuclei of the cells was observed (Fig. 1B), with no binding to nuclei of sham-irradiated cells (Fig. 1A).

This observation lead to an investigation of the structural similarities of oxidatively modified DNA bases with that of the natural ligand, biotin. Although there is little similarity between the unmodified base guanine and biotin, the 6,8-diketo tautomers of both 8-hydroxyguanine and 8-oxodeoxyguanosine share structural similarity with biotin (Fig. 2). The 6,8-diketo tautomers of both bases possess an imidazolone group in common with biotin, although the deoxyribose group is attached to N3 of the imidazolone of 8-oxodeoxyguanosine. Biotin possesses a valeric acid group on the C5 of the ureido group. The two rings of biotin are fused in the cis-configuration and the valeric acid side chain is cis with respect to the imidazolone ring (30). There appeared to be sufficient structural similarity to investigate the hypothesis that avidin binds to 8-oxodeoxyguanosine and that this was responsible for the phenomenological observations.

Treatment of DNA with methylene blue leads to oxidative base damage, specifically generating 8-oxodeoxyguanosine. The affinity of avidin for methylene blue-damaged DNA was investigated using an ELISA-type assay. The level of binding of avidin to both single-stranded and double-stranded methylene blue-treated DNA was greater than that seen to normal DNA (Fig. 3A). The level of binding was considerably higher to both normal and methylene blue-treated single-stranded DNA than to double-stranded DNA. Fixation of the DNA with parafomaldehyde (4% w/v), increased the level of binding of avidin to double-stranded DNA such that there was no longer a significant difference in the level of binding to normal single-stranded and double-stranded DNA (Fig. 3B). The difference in binding of avidin to methylene blue-treated DNA when compared to that to normal DNA was highly significant. Competition experiments showed that coincubation with 8-oxodeoxyguanosine (10 μM) produced the greatest inhibition of avidin binding to double-stranded DNA.
The specificity of avidin for oxidatively modified DNA. Both single-stranded and double-stranded DNA were treated with methylene blue to generate oxidatively modified base damage. The methylene blue and unmodified DNA (100 ng/ml) were bound to ELISA plates (A) (n = 8) without paraformaldehyde fixation or to MultiScreen filtration plates after fixation with 4% (w/v) paraformaldehyde (B) (n = 8) and the level of binding of horseradish peroxidase-conjugated avidin was assessed spectrophotometrically. *Significant at 95% level, **significant at 99% level with respect to appropriate normal DNA; n = 8 except for 8-oxodeoxyguanosine and 8-oxoguanine, where n = 4.

The sensitivity of avidin as a detection system for 8-oxodeoxyguanosine was investigated using different concentrations of 8-oxodeoxyguanosine or deoxyguanosine bound to a substrate of fixed cellular material and compared to that seen with a monoclonal antibody to biotin. Since 8-oxodeoxyguanosine does not bind to untreated plates, using a substrate of fixed cells was found to be a convenient way in which to immobilize the DNA bases. Avidin binding to 8-oxodeoxyguanosine was significantly different from background levels at $10^{-15}$ M and reached a maximum at $10^{-10}$ M; the decrease at higher levels is not statistically relevant and is not sustained at even higher concentrations (the approximate concentration of avidin was $10^{-8}$ M) (Fig. 4A). In contrast, binding to deoxyguanosine increased with increasing concentration, reaching a maximum at $10^{-6}$ M deoxyguanosine (Fig. 4A). The 0 and 100% values obtained between experiments were not statistically different. The monoclonal antibody to biotin did not appear to have the same degree of specificity as avidin, binding to both 8-oxodeoxyguanosine and deoxyguanosine with a maximum at $10^{-9}$ M (Fig. 4B). The level of binding of the antibody was considerably lower than that of avidin at the same concentration of deoxynucleoside in the same experiment.

DNA damage in cultures exposed to hydrogen peroxide were quantified by measuring the amount of avidin-FITC binding to fixed cells using a 96-well plate fluorescence plate reader (Denley). Exposure of IMR 32 cultures (differentiated for 24 h) to hydrogen peroxide for 1 h led to a highly significant increase in binding of avidin–FITCS with respect to hydrogen peroxide concentration ($p = 0.0026$ using ANOVA). With 10 nM hydrogen peroxide (Fig. 5A) the level of avidin binding was statistically different from that of the group that contained the control. An inherent level of fluorescence observed with control cultures is attributable to a low level of inherent binding of the avidin–FITCS, observable by microscopic analysis, and to autofluorescence of the cellular material. The level of binding plateaued at 100 nM hydrogen peroxide. In contrast, overt cell death 24 h after the initial exposure was seen only at concentrations of hydrogen peroxide of 100 μM and greater (Fig. 5B). The binding of the avidin–FITCS was located primarily in the nucleus of cultures (Fig. 6B) whereas no nuclear binding was observable in cells not treated with hydrogen peroxide (Fig. 6A).

Inhibition studies were carried out in order to determine whether the avidin–FITCS binding could be pre-
Binding of avidin to 8-oxodeoxyguanosine and its unmodified deoxynucleoside analogue. The binding affinity of FITC-conjugated avidin for 8-oxodeoxyguanosine and deoxyguanine (A) were assessed by the level of binding seen to different concentrations of the base bound to a substratum of fixed cells using a fluorescence plate reader (excitation 485 nm, emission 535 nm). Data are expressed as a percentage of the maximum level of binding (A). There was no statistical difference between the maximum and minimum values for avidin binding to 8-OHdG or dG in this experiment. The binding of a FITC-conjugated monoclonal antibody to biotin for 8-oxodeoxyguanosine and deoxyguanine (B) was compared to that of avidin by the level of binding to different concentrations of the base bound to a substratum of fixed cells. Data are expressed as a percentage of the maximum binding seen with avidin-FITC. Values are means ± standard error of the mean (SE) where n = 8. The data presented are of a representative experiment.

Alkaline treatment of DNA containing 8-oxodeoxyguanosine has been shown to lead to modification of the imidazolidine ring; therefore, treatment of cultures with alkali should prevent binding of avidin if the "epitope" for avidin binding to 8-oxodeoxyguanosine is specific for the imidazolidine structure. IMR 32 cells were exposed to hydrogen peroxide as above; however, in this case the cultures were treated with alkali prior to

FIG. 5. Avidin binding to IMR 32 cells treated with hydrogen peroxide. After exposure to various concentrations of hydrogen peroxide in HBSS for 1 h, fresh complete medium was added to IMR 32 neuroblastoma cultures and the cultures were incubated for 24 h before assessing the level of binding of avidin using FITC-conjugated avidin (A) using a fluorescence plate reader (excitation 485 nm, emission 535 nm). Cytotoxicity was assessed using the MTT assay (B). Values are means ± standard error of the mean (SE) where n = 8. *Significantly different at the 95% confidence level from that group containing the control value using ANOVA procedures and Scheffe multiple-range tests. The data presented are of a representative experiment.
FIG. 6. Inhibition of avidin binding in IMR 32 cells treated with hydrogen peroxide. The location of the binding of avidin immediately after exposure of IMR 32 cells to HBSS in the absence of (A) or with (B) hydrogen peroxide (100 μM) for 1 h is shown in photomicrographs (×200) of the cells. The ability of biotin (C), 8-oxodeoxyguanosine (D), and guanine (E) (all at 100 μM) to inhibit avidin binding was also investigated.

incubation with avidin and the level of binding was compared with that seen in cultures not treated with alkali (Fig. 7). Cultures incubated in parallel at pH 7.0 showed an increase in the level of binding of avidin with increasing concentration of hydrogen peroxide as shown for a similar experiment in Fig. 5A. However, treatment of the cultures at pH 12.0 abolished any concentration-related increase in avidin binding, implying that the treatment with high pH had indeed destroyed the epitope for avidin binding.
16. Effect of alkali treatment on avidin binding. After exposure to various concentrations of hydrogen peroxide in HBSS for 1 h, fresh complete medium was added to IMR 32 neuroblastoma cultures and the cultures were incubated for 1 h before fixing the cultures. After fixation the cultures were incubated in wash solution at either pH 7.0 or pH 12.0 for 1 h at room temperature prior to assessing the level of avidin using horseradish peroxide-conjugated avidin as a detection system. Avidin binding was quantified colorimetrically using TMB as the substrate. The data presented are of a representative experiment.

The binding of avidin–FITC to postmortem sections of neural tissue from patients who died of neurodegenerative disease was compared to that of age-matched controls (Figs. 8A–8D). Although there was evidence of some binding in the aged controls (Figs. 8A and 8D) the level of binding was much higher in those of the MND (Figs. 8B and 8C) patients.

DISCUSSION

As a result of the phenomenological observation that avidin–FITC conjugate bound to the nuclei of free-radical-damaged cells, the structural similarity between biotin, avidin’s natural ligand, and oxidatively modified DNA bases was considered. It has been proposed that the most stable form of 8-oxoguanine is the 6,8-diketo form (32). The imidazolidone group of biotin identical to that of the 6-8 diketo tautomeric form of 8-oxoguanine; however, in DNA it is presumed that the oxidized base is bound to the sugar moiety via a nitroso residue on the imidazolidone ring. Although both groups of biotin interact strongly with the avidin binding site, the interaction of the N-1’ is particularly strong (23). The valeric acid group on the C-2 group of the ureido group contributes to the binding of biotin to avidin, and although neither 8-oxoguanine or 8-oxodeoxyguanosine possess a comparable alkyl chain, 8-oxodeoxyguanosine does possess a bulky substituent in a comparable position. The possession of a ring structure attached to the imidazolidone group also contributes to the binding of biotin; although both 8-oxoguanine and 8-oxodeoxyguanosine possess an aromatic ring structure attached to the imidazolidone group, it is not the same as the ureido group of biotin. The three-dimensional structure of the 8-oxodeoxyguanosine is likely to be modified in intact DNA, both by its interaction with adjacent and opposite bases and by its interaction with the DNA backbone. Clearly there are some close simi-
larities between the structures of these compounds, and a rigorous investigation of the nature of binding of these oxidized DNA bases to the binding site of avidin is underway; however, this is beyond the scope of this paper.

The binding of avidin to oxidatively modified guanine and deoxyguanosine has been considered in this study since 8-oxodeoxyguanosine is considered to be one of the most abundant oxidative lesions (33). From structural considerations it is likely that avidin will bind to 8-oxoadenine and 8-oxodeoxyadenosine since these compounds also possess the imidazolidone group. Although binding additionally to 8-oxodeoxyadenosine effectively reduces the specificity of the technique, it may provide a stronger signal, since two damage products would be identified and the validity of 8-oxodeoxyguanosine as an indicator of oxidative DNA damage is dependent on its being a biomarker of total DNA damage.

It would seem likely that a prerequisite of binding to modified DNA residues in intact DNA is that either the bases are in existing regions of single strandedness, or the DNA is rendered single stranded by the fixation technique, such as with paraformaldehyde. Although avidin is considerably smaller than an antibody, it would seem unlikely that it would have access to even damaged bases within intact double-stranded DNA. This is confirmed by our finding that the extent of binding of avidin to unfixed DNA is greater to single-stranded DNA whether it is untreated or methylene blue-treated. Although the level of binding of avidin to methylene blue-treated DNA is considerably greater than that to normal DNA, there is binding to untreated DNA. This may not be surprising since commercial DNA is processed extensively; even within the experimental procedures employed here the DNA is not protected from oxidative damage. The level of 8-oxoguanine has been determined in commercial calf thymus using HPLC; the levels were 0.4 nmol/mg DNA or 3.2 mol/10^5 mol guanine (34).

The binding of avidin to free radical-damaged DNA is not dependent on the conjugate or the end-point method of detection. The inhibition studies also suggest that binding of avidin to DNA is specific and dependent upon the binding site since biotin inhibits binding of avidin. Since 8-oxodeoxyguanosine can also block binding this suggests that this may be the epitope for avidin. Therefore, these data strongly suggest that the binding is mediated by the binding site of avidin.

Guanine and guanosine also appear to inhibit avidin binding, but at high concentrations. It has been shown previously (31) that a commercial preparation of deoxyguanosine contained 8-oxodeoxyguanosine at the level of 1 residue per 137,440 dG residues, this value clearly will vary from batch to batch; the authors (personal communication) suggest that the ratio is on average 1 residue per 100,000 and may be as high as 1:20,000 in some batches. This would clearly explain our finding that the absolute level of binding of avidin to deoxyguanosine at 10^{-4} M is comparable to that of avidin to 10^{-10} M 8-oxodeoxyguanosine. To address this question, an alternative approach was to treat 8-oxodeoxyguanosine with alkali which leads to destruction of the imidazolidone ring (only present in the oxidized form of the nucleoside); we have shown here that alkali-treatment leads to a loss of binding of avidin to the hydrogen peroxide-treated cells. Therefore this would suggest that binding of avidin involves the imidazolidone group of 8-oxodeoxyguanosine.

The antibody to biotin seems less discriminatory for the oxidized status of the DNA bases; however, the exact epitope recognized by this antibody is not known.

The sensitivity of avidin as a detection system for 8-oxodeoxyguanosine was also estimated. Assuming 100% binding of 8-oxodeoxyguanosine to the substrate, this suggests that the theoretical (and conservative) sensitivity of avidin binding to oxidized DNA allows detection of approximately 10^4 molecules of 8-oxodeoxyguanosine in 10^6 cells (1 base product per 100 cells).

The structure recognized by avidin on the DNA appears to be generated by an oxidative free radical mechanism. The level of binding of avidin to cells increases in an insult-dependent manner, whether this is mediated by incubation with hydrogen peroxide, an established means of generating oxidatively mediated DNA damage, including the generation of 8-oxodeoxyguanosine, or a variety of other free-radical-generating systems (unpublished observations). For the sake of consistency, data are presented only on the binding of avidin to free-radical-damaged IMR 32 neuroblastoma cells; however, we have also seen similar binding with other cell lines, including other neuroblastoma cell lines, 3T3 fibroblast cells, and a human-derived keratinocyte cell line.

The ability of avidin to bind to pathological tissues sections was also investigated. There is evidence for the direct involvement of reactive oxygen-mediated damage in a number of neurodegenerative disorders including motor neuron disease (MND) and Parkinson's disease (35). The finding of mutations in the cytoplasmic superoxide dismutase in familial MND (36) indicates that free-radical-induced damage is important in the pathogenesis of the familial form of MND, and probably also in the pathogenesis of sporadic MND. DNA is a prime target for damage by free radicals and reactive oxygen species; however, DNA damage in neural tissue does not appear to have been researched to any significant extent, presumably as a result of the concentration insensitivity of current methods and the lack of available tissue (37). Human pathological tissue
was utilized in order to establish that this technique was also applicable to human tissue as well as in in vitro systems. We have shown here that it can be applied to flash-frozen tissue and have also successfully investigated formaldehyde-fixed tissue (data not shown). However, the location of affected cells and the mechanisms by which DNA is damaged in aging and in neurodegeneration are beyond the scope of this present study and will be discussed elsewhere.

It is of interest to note that avidin binding in the absence of any primary antibody has previously been demonstrated (38) in tissue from Alzheimer’s disease patients. In this case the binding appeared to be to the neurofibrillary tangles, the characteristic pathological features of this disease. These structures are heterogeneous in nature, although they have not previously been shown to contain DNA. The mechanism of binding was not addressed in this study.

Nonspecific binding of avidin in histochemical procedures is well established, to the extent that modified avidin (with the carbohydrate moieties removed) is sometimes employed to reduce this nonspecific contribution. Blocking kits are also available whereby prior to addition of avidin the samples are pretreated with both avidin and biotin to preblock nonspecific sites. There is anecdotal evidence that researchers using avidin–biotin technology on tissues that have been subject to some form of free radical stress have changed their detection strategy because of nonspecific binding to the nucleus. It has also been suggested that avidin binds to necrotic cells (22). Therefore, although there is some suggestion in the literature that there is binding of avidin to cells, it has not apparently been systematically investigated previously with respect to binding to oxidatively modified DNA.

Avidin conjugates appear to have similar affinities for 8-oxodeoxyguanosine, 8-oxoguanine, and biotin. Avidin can be used to demonstrate damage to DNA both in fixed cellular material from in vitro culture experiments and in fixed pathological sections. Although binding of avidin to DNA has been discussed here primarily with respect to nuclear DNA, it is anticipated that it is equally likely to bind to DNA of mitochondria which have been shown to possess limited repair and are considerably more susceptible to oxidative damage than nuclear DNA (39). The application of this technique potentially covers a wide range of usages. DNA damage is currently being researched intensely and the methodology used is both expensive and time-consuming. Although 8-oxodeoxyguanosine is only one of a range of oxidative DNA damage products, it has been widely used as a biomarker of oxidative DNA damage. The approach described in this paper is based on the identification of the modified base directly in pathological specimens without the prerequisite for extraction or purification prior to identification which has so often been found to lead to artefactually high levels. The methodology is likely to have wide application in studies of DNA damage in cell culture and in pathological specimens. Identification of DNA damage is becoming increasingly important in medical diagnostics, pathology, and occupational health. The method may also have potential for toxicological screening in both the pharmaceutical and chemical industries for compounds with potential genotoxicity.

ACKNOWLEDGMENTS

This work was supported in part by Biotrin International. Brain sections were a kind gift from the Parkinson Disease Society Brain Bank. The authors thank David Shuker (Medical Research Council, Leicester) for his helpful comments on the manuscript.

REFERENCES

DIRECT DETECTION OF 8-OXODEOXYGUANOSINE BY AVIDIN

APPENDIX 2

SENSITIVITY OF AVIDIN DETECTION

The lowest concentration of 8-oxodG detected by avidin was $10^{-15}$ M

$10^{-15}$ moles of 8-oxodG in 1000ml solution at this concentration

∴ $10^{-18}$ moles of 8-oxodG in 1ml

100μl of 8-oxodG was added per well

∴ $10^{-19}$ moles of 8-oxodG per well

Multiply by Avogadro’s number to find the number of molecules per well:

$$= 10^4 \text{ or } 10{,}000 \text{ molecules of 8-oxodG per well}$$

Significant binding of avidin was observed when $10^4$ molecules of 8-oxodG were present

When IMR32 cells are plated out at a cell density of $2 \times 10^5$ cells/ml, there are $10^6$ cells in each well.

Therefore, theoretically, the avidin could be said to be able to detect $10^4$ molecules of 8-oxodG in $10^6$ cells i.e. 1 damaged base product per 100 cells, assuming there is 100% binding of avidin. In reality this is highly unlikely. The level of 8-oxodG in cultures exposed to oxidative stress will almost certainly be much higher than suggested using this type of calculation.
APPENDIX 3

AVOGADRO'S LAW states that:

1 mole of any compound contains $6.023 \times 10^{23}$ molecules (Avogadro's number)

A 1M H$_2$O$_2$ solution consists of 1 mole H$_2$O$_2$ in 1000mls buffer.

A $10^{-17}$M H$_2$O$_2$ solution consists of $10^{-17}$ mole H$_2$O$_2$ in 1000mls buffer.

$10^{-17}$ mole H$_2$O$_2$ contains $6.023 \times 10^{23} \times 10^{-17}$ molecules

= $6.023 \times 10^6$ molecules

There are $6.023 \times 10^6$ molecules H$_2$O$_2$ in 1000mls buffer

= $6.023 \times 10^6$ molecules H$_2$O$_2$ in 1ml = 1000µl

= 6023 molecules H$_2$O$_2$ in 1000µl

100µl H$_2$O$_2$ was added to each well

There are $6023 \times 100$ molecules of H$_2$O$_2$ in 100µl solution.

= 602 molecules H$_2$O$_2$

There are therefore approximately 600 molecules H$_2$O$_2$ in 100µl of a $10^{-17}$M solution, as added to each well of a 96-well plate.

Each well of the 96-well plate contains 200ml of IMR32 cells at a density of $2 \times 10^5$ cells/ml

Cell number per well = $2 \times 10^5 \times 200$

= 40 000 cells

Therefore, 600 molecules of H$_2$O$_2$ were added to wells containing 40 000 IMR32 cells

i.e. there was 1 molecule of H$_2$O$_2$ for every 67 cells.
APPENDIX 4

HYPOTHESIS: Oxidative damage to nuclear and mitochondrial DNA in neurons results in abnormalities in the cytoskeleton and subsequent cell death in common neurodegenerative disorders
APPENDIX 5

FURTHER PUBLICATIONS


Immunodetection of UV-induced DNA damage in a neuronal cell line

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Reactive oxygen species (ROS) are involved in several pathological processes, including brain tissue damage. Their possible involvement, whether acute as in stroke, or chronically as in neurodegenerative disease, is of great interest due to the large number of people suffering from these conditions. There is therefore a need to develop in vitro model systems to study the mechanisms involved in ROS damage to neuronal cells. In this investigation IMR32 neuroblastoma cells were irradiated with ultra-violet (UV) light, a known generator of ROS. UVA and UVB light exert their effects in different ways; the former acts via damage secondary to ROS generation, whilst UVB damages DNA directly. In this study the effects of both forms of irradiation on cell cultures were investigated for the purpose of comparison. A novel antibody raised against DNA damaged by ROS was used. This has already been found to detect UV-induced DNA damage in in vivo models [1]. To establish whether DNA damage was produced by secondary ROS or a direct result of UV irradiation, studies were carried out on cells preincubated in the presence and absence of α-tocopherol or desferrioxamine. α-tocopherol, an isomer of Vitamin E, is a lipid-soluble molecule able to terminate free radical chain reactions at cell membranes and so suppress lipid peroxidation. Desferrioxamine is an iron chelator, and would theoretically decrease damage caused by free radicals produced via the Haber-Weiss reaction, catalysed by reduced iron. Therefore preincubation with these compounds may restrict UV irradiation damage to IMR32 cells mediated by free radicals.

Differentiated IMR32 cells were pre-incubated with 200μM of either α-tocopherol or desferrioxamine for 24 hours at 37°C, washed with HBSS and irradiated from above with UVA (13.6-109.1 mJ/cm²) or UVB (0.5-2.0 mJ/cm²) light. Cells were either fixed immediately or 24 hours later. For the latter time point fresh media was added after irradiation and cells kept at 37°C until needed. Cells were fixed and permeabilised by addition of 2% (v/v) paraformaldehyde (pH 7.4) for 1 hour, followed by ice-cold methanol overnight at -20°C. They were subsequently rehydrated with 0.01M PBS, and non-specific binding sites blocked with 10% (v/v) normal goat serum (NGS)/PBS for 1 hour before incubation with primary antibody (1:5000 dilution (v/v) of 0.2% NGS/PBS) overnight at 4°C. Cells were washed and the secondary antibody (goat anti-rabbit IgG biotin conjugate; 1:200 dilution (v/v) in 0.2% NGS/PBS) added for 1 hour, then avidin-fluorescein isothiocyanate (FITC) conjugate (1:100 dilution (v/v) in PBS) for 1 hour in the dark, to detect antibody binding. Cells were washed before fluorescence levels were determined using a Denley Wellflour Microplate fluorescence reader (measuring and reference filters 485nm and 530nm respectively).

A dose-related increase in antibody binding was observed in cells fixed immediately after irradiation with both UVA and UVB light. Preincubation with α-tocopherol reduced the level of this binding after UVA irradiation only, at the highest 2 doses. 24 hours after UVA irradiation the increased binding detected was

<table>
<thead>
<tr>
<th>UVB dose (mJ/cm²)</th>
<th>Control</th>
<th>α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>393.0 ± 7.8</td>
<td>404.0 ± 6.6</td>
</tr>
<tr>
<td>0.5</td>
<td>359.0 ± 12.5</td>
<td>381.8 ± 13.0</td>
</tr>
<tr>
<td>1.0</td>
<td>300.1 ± 5.6</td>
<td>329.3 ± 8.4</td>
</tr>
<tr>
<td>2.0</td>
<td>296.5 ± 9.6</td>
<td>280.8 ± 5.6</td>
</tr>
</tbody>
</table>

Table 1. Immunofluorescence assay on IMR32 cells 24 hours after UVA irradiation

Antibody binding was detected in cultures preincubated with 200μM α-tocopherol or the appropriate vehicle. Values represent the mean ± S.E.M. for 8 wells.

Table 2. Immunofluorescence assay on IMR32 cells 24 hours after UVB irradiation

Antibody binding was detected in cultures preincubated with 200μM α-tocopherol or the appropriate vehicle. Values represent the mean ± S.E.M. for 8 wells.

Even higher, and was once again noticeably decreased in cells preincubated with α-tocopherol at the highest 3 doses (Table 1). Preincubation with desferrioxamine also decreased antibody binding at the highest 2 doses. A dose-related decrease in binding occurred 24 hours after UVB irradiation, corresponding to the death of cells observed by microscopy. Preincubation with α-tocopherol did not alter this in any way (Table 2). This was also true of desferrioxamine.

The fact that antibody binding was greater 24 hours after UVA irradiation than immediately after irradiation is consistent with the hypothesis that UVA-induced damage is caused by secondary ROS generation. This evidence is supported by the demonstration that preincubation with α-tocopherol and desferrioxamine decreased binding of the antibody. It is concluded that ROS generation was involved in the damage detected, with involvement of ROS generation via the Haber-Weiss reaction. α-tocopherol has been shown previously to incorporate into cell membranes and to have a protective role in IMR32 cells after toxic insult [2]. Although it is not known what concentration of desferrioxamine was achieved intracellularly, the fact that this compound had a protective effect after preincubation implies that it does enter the cell. IMR32 cells were much more sensitive to UVB light, indicated by the fact that cell death was apparent 24 hours after irradiation.

Preincubation of cells with either α-tocopherol or desferrioxamine did not protect against UVB damage. Therefore it can be concluded that UVB light has a direct rather than secondary effect on the DNA.


Abbreviations used: FITC, avidin-fluorescein isothiocyanate; NGS, normal goat serum; ROS, reactive oxygen species; UV, ultra-violet.
DETECTION OF 8-OXODEOXYGUANOSINE IN A NEURONAL CELL LINE

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It has been suggested that reactive oxygen species (ROS) may play a role in the development of certain neurodegenerative disorders in which oxidant stress is known to occur1. Important sites of damage include DNA, with the modified base product 8-oxodeoxyguanosine (8-oxodG) being an accepted biomarker of oxidative damage. This study used a novel ELISA-type assay to investigate the formation of 8-oxodG in a neuronal cell line after exposure to UVA light, which is known to produce this base lesion indirectly via ROS2. Differentiated IMR32 neuroblastoma cells were irradiated from above with UVA (16-128 mJcm⁻²). 8-oxodG levels were then assessed 1 hour and 24 hours after irradiation, using the novel system developed within this laboratory (patented)3. A significant dose-related increase in binding was detected at both time points (P < 0.001 for both), with the greatest level of binding being seen at the later time point (Table 1). After preincubation of the cultures with the antioxidant α-tocopherol (200μM) binding was significantly decreased after 24 hours (P=0.008) (Table 1), but not after 1 hour.

Table 1. Avidin binding in UVA irradiated cells 1 hour and 24 hours after insult

<table>
<thead>
<tr>
<th>UVA Dose (mJcm⁻²)</th>
<th>1 hour (Control)</th>
<th>24 hours (Control)</th>
<th>24 hours (+ α-tocopherol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>16.0</td>
<td>1.01 ± 0.04</td>
<td>1.69 ± 0.15</td>
<td>1.27 ± 0.07</td>
</tr>
<tr>
<td>32.0</td>
<td>1.16 ± 0.06</td>
<td>1.70 ± 0.13</td>
<td>1.37 ± 0.09</td>
</tr>
<tr>
<td>64.0</td>
<td>1.25 ± 0.07</td>
<td>1.58 ± 0.10</td>
<td>1.61 ± 0.05</td>
</tr>
<tr>
<td>128.0</td>
<td>1.25 ± 0.04</td>
<td>1.33 ± 0.09</td>
<td>1.57 ± 0.07</td>
</tr>
</tbody>
</table>

It can be concluded therefore that this system has been used to detect 8-oxodG lesions produced via ROS after UVA irradiation, and this damage is restricted by the presence of the antioxidant α-tocopherol.

Acknowledgement: This work was supported by Biotrin International (Dublin).

1 Olanow, C.W. (1993) TINS 16(11), 439-444
3 International Patent Application No. 9510954.2 “Detection of DNA Damage”