

AN ANALYSIS OF 8-OXODEOXYGUANOSINE AS A MARKER OF
OXIDATIVE STRESS: INVOLVEMENT IN UV-MEDIATED
DNA DAMAGE IN CELLS

by

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Abstract

Oxidative DNA damage is thought to play a role in the aetiology of ageing and a number of diseases including cancer, chronic inflammation, ischemia, degenerative arterial and autoimmune diseases. 8-oxodeoxyguanosine (8-oxodG), an oxidative DNA adduct, has gained much popularity as a biomarker of damage to DNA. In this thesis the reliability of 8-oxodG as a marker for oxidative stress and its involvement in ultraviolet (UV)-mediated DNA damage in cells was investigated. Because of our concern and those expressed in the literature over the possible induction of artefactual 8-oxodG during phenol extraction procedures, a comparative study on DNA extraction methods was undertaken. It was found that phenol isolation of DNA yielded higher levels of 8-oxodG when directly compared to pronase E isolation, irrespective of the model of oxidative stress used to treat DNA or cells. Furthermore results from peripheral blood mononuclear cells from patients under oxidative stress (systemic lupus erythematosus and rheumatoid arthritis) showed a high degree of variability leading to inconclusive results. This led to investigation of alternative methods of analysis of the 8-oxodG lesion. 8-oxoguanine is measured conventionally as the deoxynucleoside by high performance liquid chromatography with electrochemical detection (HPLC-ECD) or as the free base by gas chromatography combined with mass spectrometry. A 'hybrid' analysis of the 8-oxoG base by HPLC was established. The new procedure combined formic acid hydrolysis of DNA with guanase treatment of the resultant base mixture. Guanase was found to specifically degrade guanine thus allowing reversed-phase HPLC quantitation of 8-oxoG. This procedure avoided problems with enzymes used to degrade DNA to deoxynucleosides which may lack specificity toward oxidatively damaged DNA. A reliable method to measure oxidative DNA damage, including 8-oxodG, at ultra-low levels, without artefactual generation of 8-oxoG which is suggested to be unavoidable when extracting and/or derivatising DNA, is clearly of great importance. Therefore, as a model, a polyclonal antibody to UV-induced DNA damage was developed and utilised in flow cytometric and immunocytochemical techniques to detect UV-mediated DNA damage in cells. Ultraviolet radiation has been reported to produce a number of potentially mutagenic photoproducts and has consequently been implicated in skin tumourigenesis. The polyclonal antibody utilised was successful at detecting UVB-induced DNA damage and UVA-induced single strand breaks. The levels of DNA damage and p53 expression following UVA irradiation of keratinocytes were found to be cell-cycle and dose dependent. UVA was found to cause a dose-dependent increase in 8-oxoG formation also as determined by HPLC. Therefore it is likely that oxidative DNA damage has a role in UVA-induced DNA damage in cells. It is concluded that the use of specific antibodies may represent an accurate and reliable measure of oxidative lesions directly in cellular DNA as an alternative to procedures that require DNA extraction.

To my mother Monica Finnegan Sr. and to the memory of my late beloved father

James Finnegan. Their love has always been inspirational.

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Abbreviations

Full name	Abbreviation
Absorbance units	A.U.'s
<i>Ad libitum</i>	AL
Arbitrary fluorescence units	afu
Benzo(a)pyrene	BaP
Capillary electrophoresis	CE
Choline-deficient L-amino acid defined diet	CDAA
50% Cytotoxicity	IC ₅₀
Deoxyguanosine	dG
Deoxyribonucleic acid	DNA
2,6 Diamine-4-hydroxy-5-formamidopyrimidine	FapyGua
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
Dulbecco's modified eagles medium	DMEM
Electrochemical detection	ECD
Enzyme linked immunosorbent assay	ELISA
Fluorescein isothiocyanate	FITC
Formamidopyrimidine-DNA glycosylase	fpg protein
Gas chromatography / mass spectrometry	GC/MS
Glutathione	GSH
Hanks balanced salt solution	HBSS
High performance liquid chromatography	HPLC

Minimal erythemat dose	MED
Normal goat serum	NGS
8-Oxo-2'-deoxyguanosine	8-oxodG
8-Oxoguanine	8-oxoG
Peripheral blood mononuclear cells	PBMC
Peroxisomal proliferators	PP
Phorbolmyristate-13-acetate	PMA
Phosphate buffered saline	PBS
Polymorphonuclear leukocytes	PMNs
Reactive oxygen species	ROS
Rheumatoid arthritis	RA
Selected ion monitoring	SIM
Standard deviation	SD
Standard error of the mean	SEM
Superoxide dismutase	SOD
Systemic lupus erythematosus	SLE
Thymine glycol	Tg
(-)-Trans-7-8, dihydroxy-7,8-dihydrobenzo(a)pyrene	(-)-BP-7,8-diol
Ultraviolet	UV
Ultraviolet modified DNA	UV-DNA

Chapter 1

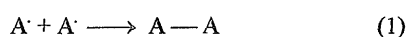
Introduction

1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) is the term applied to a range of oxygen containing radical and non-radical species such as singlet oxygen ($^1\text{O}_2$), hydroxyl radical ($\cdot\text{OH}$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Oxygen as it occurs naturally has two unpaired electrons each located in a different orbital ($\text{O}_2 (\uparrow)(\uparrow)$). The dictates of quantum mechanics lead to a spin restriction that hinders the divalent reduction of O_2 and favours a univalent pathway (Fridovich, 1978).

1.1.1 What is a free radical ?

A free radical can be simply defined as any species capable of independent existence that contains one or more unpaired electrons (an unpaired electron being one that is alone in an orbital). Because electrons are more stable when paired together in an orbital (each electron having equal but opposite spin $\uparrow\downarrow$), radicals are in general more reactive than non-radical species. Radicals can react with other molecules in a number of ways and there is considerable variation in their reactivity. Thus, if two radicals meet, they can combine their unpaired electrons (symbolized by \cdot) and join to form a covalent bond (a shared pair of electrons)(equation 1).



A radical might donate its unpaired electron to another molecule, or it might take an electron from another molecule in order to pair. However, if a radical gives one electron to,

or takes one electron from, a non-radical, the latter becomes a radical. Thus, a feature of free radical reactions is that they tend to proceed as chain reactions: one radical begets another and so on.

1.1.2 Singlet oxygen

A reactive form of oxygen, known as singlet oxygen ($^1\text{O}_2$), can be generated by an input of energy (Sies and Menck, 1992). In this form the spin restriction is removed as the electrons occupy one orbital and have opposite spin ($\uparrow\downarrow$). Singlet oxygen is most often generated in the laboratory by photosensitization reactions. If certain molecules are illuminated with light of a given wavelength the electrons absorb it and the energy raises the molecule into an 'excited state'. The excitation energy can then be transferred onto an adjacent oxygen molecule, converting it to the singlet state (photoexcitation) whilst the photosensitizer molecule returns to ground state, e.g. methylene blue (Devasagayam, 1991).

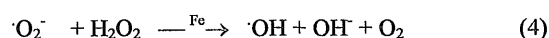
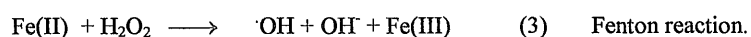
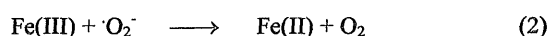
Singlet oxygen can interact with other molecules in essentially two ways: it can either combine chemically with them, or else it can transfer its excitation energy to them, returning to the ground state while the molecule enters an excited state. This chemical reactivity is the basis of biological damage inflicted by singlet oxygen e.g. additions to olefins (Wasserman and Murray, 1979) and oxidations of sulphides and phenols (Sies, 1993).

1.1.3 Superoxide radical

The superoxide radical (O_2^-) is formed during biological reduction, for example, as a by-product of mitochondrial electron transport, although the predominant responsible reaction can be neither specified nor its extent precisely quantitated in any particular cell (Ames and Shigenaga, 1993). The predominant reaction of superoxide under physiological conditions is spontaneous dismutation to hydrogen peroxide and oxygen; this reaction can be catalysed by superoxide dismutases (SOD)(McCord and Fridovich, 1969). Thus systems producing superoxide usually produce hydrogen peroxide. Hydrogen peroxide is a weak oxidising agent, but because of its lipid solubility can readily traverse biological membranes and transfer oxidising potential to cellular targets distant from its site of generation (Frimer *et al.*, 1983). The inherently low reactivity of superoxide and hydrogen peroxide has led to the widely held view that these species could partly exert their toxicity via formation of more reactive species such as hydroxyl radicals.

1.1.4 Hydroxyl radical

Hydroxyl radicals are generated from H_2O_2 and O_2^- in a series of steps referred to as the iron catalysed Haber-Weiss reaction (equation 4). In this reaction Fe(III) is reduced by O_2^- to Fe(II)(equation 2) which in turn reduces H_2O_2 to OH^\cdot (equation 3), a very potent oxidant. This latter reaction is often called the Fenton reaction. Hydroxyl radicals react at diffusion controlled rates with virtually all biomolecules (Anabar and Neta, 1967).



(Fridovich, 1978, Frenkel, 1992)

Although iron is shown in the above reactions, other transition metals can substitute e.g. Cu(I) may reduce hydrogen peroxide in a reaction analogous to the Fenton reaction. Alternatively, hydrogen peroxide could oxidise Cu(II) to a highly oxidising Cu(III) species. Superoxide is acting as the reductant in equation 2, but could be substituted by other reducing species such as ascorbate, thiols or hydro/semiquinones.

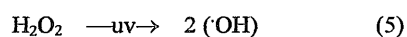
In biological systems transition metal ions are more likely to be complexed with biomolecules and therefore equations 2 and 3 are less likely to occur in bulk phase solution. Thus the concept has developed of a localised Fenton reaction producing hydroxyl radicals and/or hypervalent transition metal-oxygen complexes at sites sequestered from the medium in close proximity to the target (Samuni *et al.*, 1981; Chevion, 1988). Therefore the status of a biomolecule as a target for oxidative attack could be due to the presence of redox active transition metal ion(s) (Evans *et al.*, in press).

1.1.5 Hydrogen peroxide

The weak oxidising agent, hydrogen peroxide inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. For example, glyceraldehyde-3-phosphate dehydrogenase, an enzyme of the glycolytic pathway, is inactivated by H_2O_2 in this way

(Halliwell and Gutteridge, 1989). Thus exposure of cells to large doses of H_2O_2 can lead to ATP depletion by inhibition of glycolysis (Halliwell and Gutteridge, 1989). Hydrogen peroxide can cross membranes rapidly whereas O_2^- usually cannot (Meneghini and Martins, 1993). Once inside the cell, H_2O_2 can probably react with Fe(II) , and possibly Cu(I) , ions to form the hydroxyl radical and this may be the origin of many of its toxic effects.

Hydrogen peroxide also enhances the damaging effects of near-ultraviolet radiation on bacteria and viruses. The damage seen in the presence of both agents is usually much greater than that done by each agent alone (a synergistic effect)(Epe, 1993). It is possible that the UV light can cause homolytic fission of the hydrogen peroxide and hence increase the production of $\cdot\text{OH}$:



The damage appears to affect DNA especially, causing single-strand breaks and DNA-protein cross links.

Reactive oxygen species have a wide range of reactivities and low reactivity can be associated with the ability to transfer damaging potential to sites distant from the site of generation (Evans *et al.*, 1995). The ROS superoxide and hydrogen peroxide can be readily converted into more reactive species (hydroxyl radicals) by interaction with other reactive oxygen species and/or transition metals.

1.2 Oxidative DNA damage

Considerable interest has arisen in recent years in the formation and consequences of oxidative damage to DNA and its possible role in disease processes (Halliwell and Gutteridge, 1988). This derives largely from the perception that the use of oxygen by aerobic organisms is accompanied by the formation of reactive by-products (Demple and Harrison, 1994). Many toxic agents, e.g. paraquat, also generate intracellular oxygen radicals which leads to DNA damage (Demple and Harrison, 1994). Oxidative damage refers to the damage formed by these reactive oxygen species. It is also possible that ROS, for example singlet oxygen first react with cell membrane components leading to lipid peroxidation, the products of which in turn might react with DNA (Vaca *et al.*, 1988).

Oxidative DNA damage constitutes probably the most varied class of DNA damage: nearly 100 different free radical damage products have been identified (Dizdaroglu, 1992; Demple, 1994). Much of the current knowledge about such damage derives from studies of the radiation chemistry of DNA and nucleotides (Dizdaroglu, 1991). The identified oxidation products include both primary damage, which is often unstable, and the breakdown products of this damage arising from hydrolysis and rearrangement reactions (Lindahl, 1993). Thymine glycol (Tg), 8-oxoguanine (8-oxoG) and 2,6-diamine-4-hydroxy-5-formamidopyrimidine (FapyGua) are examples of these free radical damaged DNA products (Figure 1.1). 8-oxoguanine has become the species of greatest current interest, as the assay developed to measure this lesion is relatively simple. 8-Oxoguanine is discussed in detail in sections 1.3.1 to 1.3.10.

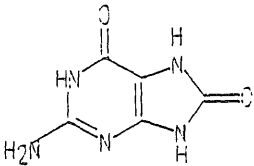
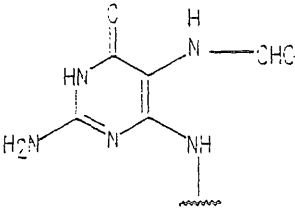
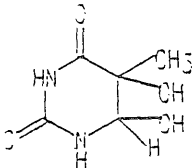
DAMAGE	FORMATION	PROPERTIES
<p>8-Oxoguanine</p> 	<p>$\cdot\text{OH}$; ionizing radiation; singlet oxygen</p>	<p>Miscoding; mutagenic</p>
<p>Formamidopyrimidine</p> 	<p>$\cdot\text{OH}$; ionizing radiation</p>	<p>Replicative block</p>
<p>Thymine glycol</p> 	<p>$\cdot\text{OH}$; ionizing radiation; ultraviolet radiation</p>	<p>Replicative block</p>

Figure 1.1: Representative oxidative base lesions.

1.3 Formation and role of 8-oxodG

Deoxyguanosine (dG) residues in DNA are hydroxylated at the C-8 position both *in vitro* and *in vivo* to produce 8-oxodeoxyguanosine (8-oxodG) (Figure 1.2). 8-Oxoguanine (8-oxoG) describes the purine base guanine in which the H atom at position 8 is replaced by an OH group. It can be released from DNA by acid hydrolysis (Dizdaroglu, 1992). If enzyme hydrolysis is used instead, 8-oxoG may be released still attached to the 2-deoxyribose sugar. This product is called 8-oxo-2'-deoxyguanosine (8-oxodG)(Halliwell and Dizdaroglu, 1992).

8-oxodG is one of the DNA-damaged products formed by various agents that produce oxygen radicals such as reductants-O₂, metal ions-O₂, polyphenol-H₂O₂-Fe³⁺, asbestos-H₂O₂ or ionizing radiation (reviewed in Kuchino *et al.*, 1987) (Figure 1.2). A sensitive quantitative method for 8-oxodG has been developed by Floyd *et al.*, (1986b) using high performance liquid chromatography (HPLC) in combination with electrochemical detection (ECD). Thus 8-oxodG has become a widely used marker for oxidative damage to DNA. The role that 8-oxodG may play in the mechanism of chemical carcinogenesis, ageing and disease processes has been investigated utilising many different model systems. Presented below (sections 1.3.2 - 1.3.10) are some examples of the work that has been performed utilising the 8-oxodG adduct as an indicator of oxidative damage both *in vitro* and *in vivo*.

Deoxyguanosine

8-Oxodeoxyguanosine

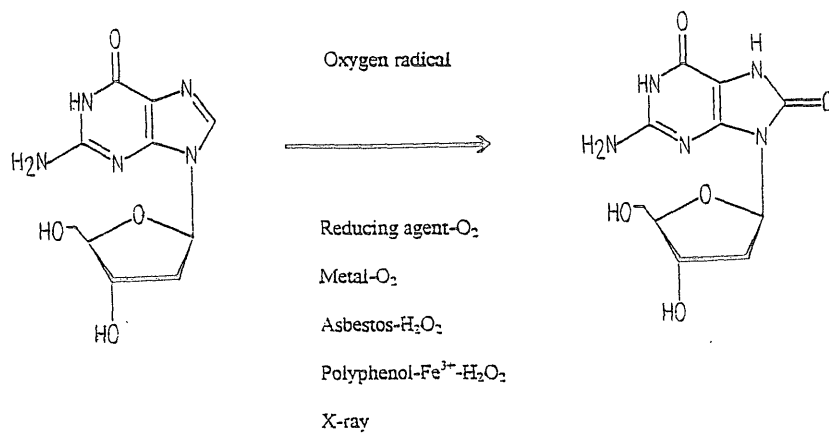


Figure 1.2: Formation of 8-oxodeoxyguanosine by various agents that cause oxidative DNA damage (adapted from Kasai *et al*, 1984).

1.3.1 Effect of 8-oxoG on replication of DNA

8-oxoG in DNA has significant miscoding potential *in vitro*: Replicative DNA polymerases of both bacteria and mammalian cells insert dAMP preferentially opposite 8-oxoG in the template, whereas polymerases associated with repair insert dCMP (the “correct” nucleotide) preferentially (Shibutani *et al.*, 1991; Moriya and Grollman, 1993). This *in vitro* miscoding specificity is reflected in results of transfection studies with single-stranded DNA vectors containing a single 8-oxoG residue, which gave rise to G-T transversions at a frequency of ~1% after replication in *E. coli* (Wood *et al.*, 1990).

1.3.2 Renal carcinogenesis and 8-oxodG

Kasai *et al.*, (1987) observed a significant increase of 8-oxodG in male Fischer 344 rat kidney DNA following oral administration of a renal carcinogen, potassium bromate. Sai *et al.*, (1994) observed that 8-oxodG formation occurred after incubation of rat renal nuclei and rat renal proximal tubules with a lipid peroxidising system, autooxidized methyl linolenate, or potassium bromate. Umemura *et al.*, (1995) found that 8-oxodG levels in the kidneys of female F344 rats were significantly elevated with doses of potassium bromate of 200mg/kg (0.83 (SD=0.15) moles 8-oxodG/10⁵ moles dG) and 400mg/kg (1.54 (SD=0.15) moles 8-oxodG/10⁵ moles dG) in comparison to untreated rats (0.37 (SD=0.1) moles 8-oxodG/10⁵ moles dG). Ballmaier and Epe, (1995) suggested that for potassium bromate, the mechanism of 8-oxodG formation involves bromine radicals rather than hydroxyl radicals or singlet oxygen. The DNA damage profile observed in experiments in cultured mammalian cells and in a cell-free system involving potassium bromate with various scavengers (catalase, superoxide dismutase, desferoxamine, azide, tert-butanol) and D₂O as

solvent excluded the involvement of hydroxyl radicals and singlet oxygen in the damage production, but were consistent with a radical mechanism involving bromine radicals (Ballmaier and Epe, 1995).

1.3.3 Hepatic damage and 8-oxodG

Shen *et al.*, (1995) observed a time- and dose-dependent increase in 8-oxodG in rat hepatic DNA after a single intraperitoneal injection of the liver carcinogen aflatoxin B1, rising from a control background level of 1.5 (SD=0.3) moles 8-oxodG/10⁵ moles dG to 4.25 (SD=0.25) moles 8-oxodG/10⁵ moles dG at 100µg/100g body weight. Denda *et al.*, 1994, found that the anti-inflammatory drug acetylsalicylic acid (ASA) prevented liver cirrhosis and carcinogenesis in F344 rats as well as reducing the generation of 8-oxodG and thiobarbituric acid-reactive substances (an indicator of lipid peroxidation) caused by feeding the rats a choline-deficient, L-amino acid-defined (CDAA) diet. The data presented by Denda *et al.*, (1994) demonstrated that ASA can prevent the pathogenesis of both cirrhosis and hepatocarcinogenesis caused by a CDAA diet, which is partly associated with the prevention of ROS production and prostaglandin inhibition. Shimoda *et al.*, (1994) found that 8-oxodG content in human livers from patients with chronic hepatitis was significantly higher than the 8-oxodG content in normal livers. Thus, chronic inflammation in the liver produces oxidative DNA damage, which may increase the risk of genomic alterations causing hepatocarcinogenesis. Wang *et al.*, (1995) investigated oxidative modification of DNA bases in rat liver and lung during chemical carcinogenesis. Liver tumours were induced by 2-fluorenylacetamide or N-nitroso-N-2-fluorenylacetamide and lung tumours were induced by sodium nitrite plus trimethylamine. Elevated amounts of modified DNA

bases were found in most of the DNA samples isolated from these cancerous tissues when compared to controls. The modified DNA bases were identified and quantified by gas chromatography/mass spectrometry with selected-ion monitoring. These compounds were characterized as 5-hydroxyuracil, Tg, 8-hydroxyadenine, and 8-oxoG.

1.3.4 Ageing and oxidative DNA damage

Fraga *et al.*, (1990) found that levels of 8-oxodG in DNA increased with age in liver, kidney, and intestine but remained unchanged in brain and testes of F344 rats. Hayakawa *et al.*, (1991a) found that age-associated accumulation of 8-oxodG does occur in human mitochondrial DNA in the muscle of diaphragm. Sohal *et al.*, (1994) investigated the effect of age and caloric restriction on DNA oxidative damage (8-oxodG) in different tissues of C57 BL/6 mice. Results showed that dietary restricted (DR) mice kept on a 60% caloric intake as compared to the *ad libitum*-fed mice showed a lower concentration of 8-oxodG content in all tissues. The DR-related amelioration of oxidative DNA damage was greater in the post-mitotic tissues (skeletal muscle, brain and heart) compared to those undergoing slow mitosis (liver and kidney). These results support the hypothesis that oxidative damage to long-lived post-mitotic cells may be a key factor in the ageing process (Harman, 1992).

1.3.5 Benzo(a)pyrene and oxidative DNA damage

Mauthe *et al.*, (1995) observed that early passage Syrian hamster embryo and human mammary carcinoma cell line MCF-7 cultures treated with 1-5µg/ml of benzo(a)pyrene (BaP) for 24 hours and then exposed to fluorescent light for 4 hours contained 3-fold (30

moles 8-oxodG/10⁵ moles dG)(1µg/ml) and 8- to 10- fold (80-100 moles 8-oxodG/10⁵ moles dG)(5µg/ml) higher 8-oxodG levels than either those not exposed to light or not treated with BaP. These findings suggest that oxidative damage of DNA could be involved in tumour induction by BaP in tissues, such as skin, in which exposure to BaP can occur in the presence of light.

Mehrotra *et al.*, (1994) found that human polymorphonuclear leukocytes (PMNs) previously treated with 12-O-tetradecanoyl phorbolmyristate-13-acetate (PMA) to initiate the oxidative burst activate (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene ((-)-BP-7,8-diol) to DNA-binding intermediates. Addition of nitrite, the major aqueous dissolution product of NO₂, stimulated the formation of (+)-anti-BPDE and subsequent binding to both nuclear DNA in PMNs (about 2-fold) and to DNA present outside the cells (2- to 4-fold). Preliminary experiments suggested that nitrite stimulates the metabolism of (-)-BP-7,8-diol by direct interaction with myeloperoxidase and hydrogen peroxide (Mehrotra *et al.*, 1994). Measurement of hydroxyl radical-induced DNA damage by estimating the formation of 8-oxodG in resting PMNs revealed low amounts of adducts (1 adduct/10⁶ dG to 1 adduct/10⁵ dG) as measured by HPLC-ECD. Pretreating the cells with PMA or PMA in conjunction with nitrite had no significant effect on 8-oxodG adduct formation.

1.3.6 Benzene and oxidative DNA damage

Subrahmanyam *et al.*, (1991) found that exposure of human promyelocytic leukemia (HL-60) cells to the benzene metabolites, hydroquinone and 1,2,4-benzenetriol resulted in increased steady-state levels of 8-oxodG in DNA. Kolachana *et al.*, (1993) observed an increase in 8-oxodG in the bone marrow of C57BL/6 xC3H F1 mice *in vivo* 1 hour after benzene administration. A dose of 200mg/kg benzene produced a 5-fold increase in the 8-oxodG level from a control level of 3 moles 8-oxodG/10⁵ moles dG to 19 moles 8-oxodG/10⁵ moles dG.

1.3.7 Biomonitoring and oxidative DNA damage

Oxidative DNA products are eliminated by repair enzymes (excision enzymes and glycosylases) and appear as free base products (Tg and 8-oxoG) or as nucleoside derivatives (thymidine glycol and 8-oxodG) (Richter *et al.*, 1988; Bergtold *et al.*, 1988; Fraga *et al.*, 1990; Simic and Bergtold, 1991). Once released from DNA, they are taken up into the circulation and excreted in urine (Simic, 1992). Despite their specificity, the free-base products (Tg and 8-oxoG) do not qualify as true biomarkers because they may be absorbed from dietary sources through the digestive system (Arouma and Halliwell, 1995). The nucleosides thymidine glycol and 8-oxodG, however are not absorbed through the digestive system (Cathcart *et al.*, 1984) and do qualify as biomarkers (Simic, 1992). Urinary biomarkers of oxidative DNA base damage were introduced by Ames and co-workers as a novel approach to non-invasive assessment of *in vivo* genetic damage (Cathcart *et al.*, 1984; Ames, 1989a). However 8-oxodGTP may be a source of 8-oxodG

excreted in the urine. Tagesson *et al.*, (1993) found increased urinary excretion of 8-oxodG in asbestos workers, rubber workers and azo-dye workers when compared with controls. Lagario *et al.*, (1994) carried out a biomonitoring study on petrol pump attendants which showed a dose-response effect between personal exposure to benzene and urinary 8-oxodG concentrations. Measurement using 8-oxodG is therefore viewed as a very good index of *in vivo* oxidative stress.

1.3.8 Smoking and oxidative DNA damage

Loft *et al.*, (1992) found the mean 8-oxodG excretion in humans was 252 (SD=103) pmol/kg body weight/24 hours with a range from 78 to 527 (n=83). The 8-oxodG excretion in 30 smokers was 320 (SD=84) pmol/kg/24 hours compared with 213 (SD=84) pmol/kg/24 hours in 53 non-smokers. The results suggest that smoking increases oxidative DNA damage by ~50%. Fraga *et al.*, (1991) found that dietary ascorbic acid protects human sperm from endogenous oxidative DNA damage that could affect sperm quality and increase the risk of genetic defects. This is particularly important in populations with low ascorbic acid such as smokers. Susuki *et al.*, (1995) observed that the level of 8-oxoG in rat urine increased by a factor of 2 to 4 after an intraperitoneal administration of 2-nitropropane (25mg/kg), paraquat (11.3mg/kg) or hydroquinone (11mg/kg). On the other hand, the urine of smokers and persons exposed to air polluted with car exhaust also contained 1.9 and 3.8 fold more 8-oxoG, respectively than that of control non-smokers (Susuki *et al.*, 1995). These results indicated that the amount of 8-oxoG in urine is a useful marker for monitoring the level of *in vivo* oxidative stress. However, when measuring the base 8-oxoG in urine dietary effects must always be taken into consideration, the rat studies were controlled for diet but the human studies were not. However, Susuki *et al.*, (1995)

noted that the large individual variation of urinary 8-oxoG in humans was relatively comparable to that in rats, regardless of diet.

1.3.9 Metals and oxidative DNA damage

Toyokuni and Sagripanti (1994) implanted male Wistar rats with osmotic mini pumps that continuously administered saline, CuCl_2 or a copper chelate, cupric nitriloacetate (Cu-NTA), at a rate of 4mg copper/kg body wt/day. This experimental design maintained serum copper concentrations at a level of 30-70% (CuCl_2) or 100-120% (Cu-NTA) higher than in untreated controls. At different times postimplantation the level of 8-oxodG was measured in DNA of kidney, liver and tissue surrounding the pump implant. Hepatic and renal levels of 8-oxodG in CuCl_2 or Cu-NTA treated animals were significantly higher than in control animals. To place these levels of copper in context, the amount of copper administered in these experiments could be released in the uterus of women after a few months of continued use of intrauterine contraceptive devices (Toyokuni and Sagripanti, 1994). Stohs and Bagchi (1995) state that recent studies have shown that metals including iron, copper, chromium, and vanadium undergo redox cycling, while cadmium, mercury, and nickel as well as lead, deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of ROS such as superoxide ion, hydrogen peroxide and hydroxyl radicals. These ROS are known to cause oxidative DNA damage, which was measured as an increase in the levels of 8-oxodG in DNA and cells (Arouma and Halliwell, 1995). George *et al.*, (1987) state that Cu plays a key role in determining the structure and density of histone free DNA almost certainly by acting as a link between DNA and specific structural proteins. Therefore the presence of copper in the DNA structure may be a vital specific target for oxidative DNA damage. Recently, Spear and Aust (1995) conclude that glutathione (GSH)

can catalyze the *in vitro* hydroxylation of dG when the ratio of GSH to copper is low, however, when the ratio is high, GSH is an effective antioxidant.

1.3.10 Peroxisome proliferators and oxidative DNA damage

Kasai *et al.*, (1989) found that administration of ciprofibrate, which is among the most potent and efficacious of the peroxisome proliferators (PP), in diet at a concentration of 0.025% for 16, 28 and 40 weeks resulted in significant increases in the levels of 8-oxodG in the liver DNA of male F344 rats when compared to controls. Cattley and Glover (1993) found absence of quantitative relationships between dietary exposure of PP (WY-14,643, DEHP (di(2-ethylhexyl)phthalate), clofibric acid, phenobarbital) and induced carcinogenicity and also oxidative DNA base damage (as 8-oxodG). There was also a failure to localize this oxidative damage to nuclear DNA. They suggest two possible conclusions: (1) quantitation of 8-oxodG does not accurately reflect the potential of peroxisomal H₂O₂-dependent DNA damage and carcinogenicity of PP in rodents (liver of male F344 rats); (2) other hepatic responses may be more critical features of the mechanism of PP carcinogenicity. Chengyu *et al.*, (1994) observed that both ciprofibrate (0.01%) in the diet, and perfluorodecanoic acid (PFDA) at 10mg/kg (injected every 14 days) induced oxidative damage in the form of 8-oxodG in liver isolated from rats. The inhibition of peroxisomal beta-oxidation by PFDA does not affect the development of 8-oxodG. This adds to the controversy in the literature (Elliot and Elcombe, 1987; Chengyu *et al.*, 1994) whether or not the carcinogenicity observed with peroxisome proliferators is due to oxidative DNA damage or some other processes (Cattley and Glover, 1993).

1.4 Cellular defence mechanisms against DNA damage

Oxidants are produced as by-products of mitochondrial electron transport, various oxygen utilising enzyme systems, peroxisomes and other processes associated with normal aerobic metabolism, as well as lipid peroxidation (Dempfle and Harrison, 1994). Many enzymatic and non-enzymatic defence mechanisms within the organism have evolved to limit the levels of reactive oxygen species and the damage they induce. Among the defences are superoxide dismutase, catalase and glutathione peroxidase (Fridovich, 1978; Floyd and Carney, 1992) as well as the antioxidants β -carotene, tocopherols and vitamin C (Ames, 1988). Because of the finite time between generation of oxidants and their destruction by a defence mechanism, steady-state levels of oxidants can exist for sufficient time to produce damage to cellular macromolecules. For nuclear DNA, however, the mammalian cell has three more levels of defence. First DNA is compartmentalised away from mitochondria and peroxisomes where most oxidants are probably generated. Second, most nonreplicating nuclear DNA is surrounded by histones and polyamines, (Basu *et al.*, 1993) which may protect against oxidants. Finally most of the types of DNA damage produced can be repaired by efficient enzyme systems (Dempfle and Levin, 1992). The net result of this multilevel defence is that nuclear DNA is very well, but not completely, protected from oxidants (Ames, 1988). Richter *et al.*, (1988) found that mitochondrial DNA (mtDNA) was much more oxidatively damaged than nuclear DNA, although the damage of nuclear DNA also appeared very high. 8-OxodG was present at a level of 1 per 130,000 bases in nuclear DNA and 1 per 8000 bases in mtDNA (Richter *et al.*, 1988).

Higuchi and Linn (1995) used a purification scheme which maximized the yield of all forms of HeLa cell mtDNA and minimized damage to DNA during its isolation. Treatment of

intact mitochondria with DNase I removed nuclear DNA. The avoidance of phenol and the isolation by CsCl density gradients in the absence of ethidium bromide and subsequent detection by Southern Hybridisation dot-blots minimized DNA damage. Four different mtDNA forms free of apparent nuclear DNA were obtained: closed circular, open circular, linear and a large multimer complex which was characterized by agarose gel electrophoresis and electron microscopy. Using this procedure, mtDNA was obtained from both whole cells or intact mitochondria treated with H₂O₂. Significant fragmentation was observed after treatment at 37°C but not at 0°C, and more damage was observed when treating whole cells than isolated mitochondria. They found on assessment of damage caused by H₂O₂ treatment of HeLa mitochondria or intact cells that the levels of 8-oxodG in all cases were very low (less than 1 mole 8-oxodG/10⁵ mole dG).

1.4.1 Heat shock protein 70 and oxidative DNA damage

Abe *et al.*, (1995) have observed that pre-existing heat shock protein 70 (hsp70) translocated into the nucleus of human amniotic cultured cells (WISH) treated with 1mM H₂O₂. This was most marked 30 min after exposure. The content of 8-oxodG in nuclear DNA increased during the early period of exposure to H₂O₂, that is, it peaked at 1 hour in the cells treated with 1mM H₂O₂ and then declined but not to control levels within 4 hours. These results suggest that pre-existing hsp70 protein translocated into the nucleus to either protect chromatin DNA from further damage or to facilitate the repair of DNA damage in some unknown way. A further line of defence against tumour formation induced by cellular oxidative damage is provided by the protein p53.

1.4.2 The tumour suppressor gene p53

The proliferation of normal cells is thought to be regulated by growth-promoting proto-oncogenes, counterbalanced by growth-constraining tumour suppressor genes (Weinberg, 1991). Mutations that potentiate the activities of proto-oncogenes create the oncogenes that force the growth of tumour cells. Conversely, genetic lesions that inactivate suppressor genes liberate the cell from the constraints imposed by these genes, yielding the unconstrained growth of the cancer cell (Weinberg, 1991). Progression of many tumours to full malignancy requires both types of changes in the tumour cell genome (Weinberg, 1991). The p53 gene is a tumour suppressor gene (Liu *et al.*, 1994).

The p53 gene is located on the short arm of chromosome 17 and codes for a 53000 Dalton nuclear phosphoprotein. The protein which can be found in the nucleus at low concentrations has a cell cycle control function during the transition from the G₁-to the S-phase (Smith *et al.*, 1994). After genotoxic stress, p53 functions as a transcription factor and transactivates effector genes such as GADD45 and p21^{Waf1/Cip1}, although both these genes can be induced by other pathways (Kastan *et al.*, 1991; Michieli *et al.*, 1994). p21^{Waf1/Cip1} inhibits the kinase activity of multiple cyclin-dependent kinase complexes, which may be one mechanism by which it suppresses cellular growth (El-Deiry *et al.*, 1994), and it inhibits the ability of proliferating cell nuclear antigen to activate polymerase δ for viral DNA replication *in vitro* (Smith *et al.*, 1994). Halting at this G₁ checkpoint allows the cell time to repair the damage before continuing to divide. Liu *et al.*, (1994) demonstrated that the induction of p53 protein in mouse keratinocytes following UVB irradiation occurred post-transcriptionally, and was due to a significant increase in p53 protein half-life. It has been found that p53 may indirectly stimulate DNA repair through turning on the GADD45

(growth-arresting-and-DNA-damage-inducible) gene and may directly stimulate repair by binding to ERCC3, one of several excision repair molecules that together recognize and remove damaged segments from DNA (Marx, 1994a). If the damage is too great the increased p53 expression results in apoptosis (Canman *et al.*, 1995).

The tumour suppressor p53 can be inactivated by allelic loss and mutation (Baker *et al.*, 1989) and by interaction with viral oncoproteins, including SV40T antigen (Lane and Crawford, 1979; Levine *et al.*, 1991). In general the expressed quantity of the wild type p53 protein is very low and hardly detectable by standard immunohistochemical techniques under physiological conditions in undamaged cells. In comparison with the wild type, the mutated protein has a prolonged half life and thus accumulates in the cell (Muller and Wiethage, 1995). The detection of p53 by immunohistochemical techniques in atypical (preneoplastic or tumorigenic) cells is a distinct indication of a mutated protein (Muller and Wiethage, 1995).

1.5 Mechanisms of DNA repair

Cellular DNA may be damaged by a variety of chemical and physical agents and so all cells possess mechanisms for repair. Mutations are produced by mistakes in base pairing, covalent modification of bases and the deletion and insertion of bases. The process of DNA biosynthesis, although highly precise, is intrinsically imperfect. Relatively common DNA biosynthetic errors include insertion of an incorrect base; for example, T opposite G, or the addition of an extra nucleotide or two, resulting in unpaired bases within the helix. It is the job of one of the major pathways, the cellular mismatch repair system to recognize such mispairs and to eliminate biosynthetic mistakes from newly synthesised DNA strands

(Modrich, 1994). Hereditary nonpolyposis colon cancer which afflicts perhaps one person in 200 in the United States, has been reported to have a defect in its mismatch repair mechanism (Modrich, 1994) which leads to the development of the disease.

Human cells manifest apparently well co-ordinated responses to DNA damaging agents. Excision repair relies on the redundant information in the duplex to remove a damaged base or nucleotide, for example 8-oxoG or a thymine dimer, and replace it with a normal base by using the complementary strand as a template. In base excision repair the removal of the lesion occurs in two steps: First, the damaged base is released by a DNA glycosylase and then the abasic sugar (AP site) is excised by AP endonucleases. In nucleotide excision repair, an enzyme system hydrolyses two phosphodiester bonds, one on either side of the lesion, to generate an oligonucleotide carrying the damage. The excised oligonucleotide is released from the duplex, and the resulting gap is then filled in and ligated to complete the repair reaction (Sancar, 1994). For example, pyrimidine dimers formed by the action of ultraviolet light are excised by the uvrABC excinuclease, an enzyme that removes a 12-nucleotide region containing the dimer (Stryer, 1988). Xeroderma pigmentosum, a genetically transmitted disease, is caused by defective repair of DNA; patients with this disease have an increased risk of skin cancer some 1000 to 2000 times greater than normal (Marx, 1994).

1.5.1 Repair of 8-oxoG

E. coli fpg is a 31-kDa enzyme, purified originally as having a DNA glycosylase activity that releases fragmented purine lesions (formamidopyrimidines, or FAPy; Figure 1.1) from methylated, alkali-treated DNA. FAPy lesions strongly block DNA synthesis *in vitro*, and so have cytotoxic potential, although their mutagenic effect could be limited (Lindahl, 1993). Thus, the significance of an enzyme specific for FAPy lesions was open to debate. However, fpg was found to act as an efficient 8-oxoG glycosylase (Tchou *et al.*, 1991), which accounted for an activity previously termed 8-hydroxyguanine endonuclease (Chung *et al.*, 1991). Hence, fpg removes purines with either ruptured (FAPy) or intact (but oxidised) imidazole rings (8-oxoG). The base is excreted from the cell and finally into the urine (Arouma and Halliwell, 1995). The ease with which 8-oxoG is formed by numerous oxidising agents (Kasai and Nishimura, 1991) suggests that 8-oxoG could make a significant contribution to mutagenesis and genetic instability in some circumstances. The biological importance of 8-oxoG removal by fpg protein came suddenly into focus with the realisation that the *mutM* gene (Cabrera *et al.*, 1988) of *E. coli* is identical to *Fpg* (Michaels *et al.*, 1991). Strains with *mutM* mutations have a ~5 fold increase in the spontaneous rate of GC-TA transversions (Cabrera *et al.*, 1988).

1.6 Oxidative DNA damage in systemic lupus erythematosus and rheumatoid arthritis

ROS have been proposed to contribute to certain chronic inflammatory disorders (Lunec *et al.*, 1985). Systemic lupus erythematosus (SLE) is a chronic inflammatory connective tissue disorder which affects many organs of the body and is characterised by the presence of

antibodies against nuclear components (anti-nuclear antibodies) particularly against dsDNA (Blount *et al.*, 1990). Patients with SLE have diverse clinical manifestations, including arthritis, vasculitis, skin rashes and renal complications, and the disease is notable for the wide variety of autoantibodies found in the serum; anti-double-stranded DNA antibodies being the most specific serological finding in SLE (Lunec *et al.*, 1994). A result of the autoimmunity is the formation of pathological circulating immune complexes in SLE (Bruneau and Benveniste, 1979). The reaction between DNA and anti-DNA antibodies and subsequent immune complex formation has been extensively studied in order to obtain information about the aetiology and pathogenesis of SLE (Blount *et al.*, 1990; Lunec *et al.*, 1994). The marker used as an indicator of oxidative DNA damage in these studies was 8-oxodG.

Immune dysfunction is also a characteristic of rheumatoid arthritis (RA). Patients with RA have an increased risk of sepsis and death due to infection (Prior *et al.*, 1984). Decreased responses of both T and B lymphocytes to mitogens and incomplete progression towards lymphocyte activation have been described in patients with RA (Felder *et al.*, 1985; Pitzalis *et al.*, 1987). Some of these changes could be explained by metabolic changes in lymphocytes (Bhusate *et al.*, 1992). Immune dysfunction may be linked with lymphocyte DNA metabolism (Carson *et al.*, 1986). In particular, DNA damage may impair lymphocyte function and induce increased cell turnover; such changes are of relevance to the pathogenesis of rheumatoid arthritis (Bhusate *et al.*, 1992).

During inflammatory states, such as SLE and RA, activated neutrophils release toxic oxygen species into the extracellular environment. Out of all the ROS generated during the oxidative burst of polymorphonuclear neutrophils (PMN's) only H₂O₂ can readily cross

plasma and nuclear membranes and reach DNA (Frimer, 1983; Frenkel, 1992). As discussed earlier (section 1.1.5) it is H_2O_2 by virtue of being neutral and quite unreactive in the absence of reduced transition metal ions, that reaches the nucleus where it can cause site specific damage. The mechanism for site specific damage is thought to involve iron ions bound to the phosphate groups of nucleic acids, or copper ions bound to proteins, which can reduce incoming H_2O_2 to $\cdot OH$ or other oxygen radical species and that this second generation of ROS is responsible for the oxidation of bases in DNA (Frenkel, 1992).

Circulating lymphocytes from patients with inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus have a significantly higher level of genomic damage and are more sensitive to killing by genotoxic agents than similar cells from healthy donors or patients with non-immune conditions (Harris *et al.*, 1994). Harris *et al.*, (1994) investigated the susceptibility of lymphocytes to oxidative stress, this included the measurement of 8-oxodG in cellular DNA. The levels were found to be significantly higher in the DNA of lymphocytes from donors with various inflammatory diseases associated with autoimmunity compared to healthy donors.

In many SLE patients, the development of cutaneous lesions and systemic symptoms are provoked by sunlight (Tan and Stoughton, 1969). The incidence of photosensitivity has generally been reported to be between 40 and 60%, though this figure varies depending upon the population of SLE patients examined (Rosenstein *et al.*, 1992). Cutaneous lesions have also been elicited by exposure to UV radiation under controlled laboratory conditions (Rosenstein *et al.*, 1992). Recent investigations indicate that the UV-induced transposition of the small ribonucleoprotein SSA/Ro to the plasma membrane of epidermal keratinocytes may be an important component of the cutaneous response to solar ultraviolet radiation in

SLE patients who express anti-SSA/Ro autoantibodies (Furukawa *et al.*, 1990). Casciola-Rosen *et al.*, (1994) demonstrated that the autoantigens targeted in SLE are clustered in two populations of surface structures on UV-induced apoptotic keratinocytes. The population of smaller blebs contains fragmented endoplasmic reticulum and ribosomes, as well as the ribonucleoprotein, Ro. The larger apoptotic bodies (blebs) contain nucleosomal DNA, Ro, La, and the small nuclear ribonucleoproteins. These autoantigen clusters have in common their proximity to the endoplasmic reticulum and nuclear membranes, sites of increased generation of reactive oxygen species in apoptotic cells (Casciola-Rosen *et al.*, 1994). Oxidative modification at these sites may be a mechanism that unites this diverse group of molecules together as autoantigens (Casciola-Rosen *et al.*, 1994)

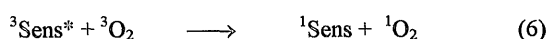
1.7 Direct and indirect UV damage to DNA

The ultraviolet component of sunlight incident on the earth's surface is usually divided into two wavelength regions which are termed UVA (320-400 nm) and UVB (290-320 nm). UVC (200-290) the third component of the solar UV spectrum is effectively absorbed in the earth's atmosphere. Experiments using UVC (particularly 254 nm) have been important in characterizing UV-induced DNA damage. UVC damages DNA via direct photon energy to give rise to pyrimidine photoproducts, breaks and DNA protein crosslinks (Peak *et al.*, 1989). The carcinogenic effects of UVC and UVB photons have been known for decades (Blum, 1959), and light-induced lesions in DNA are believed to be the initiating events. This is consistent with the high probability that UVB photons will be absorbed by DNA (Setlow, 1974), resulting in the formation of photoproducts, especially pyrimidine dimers and adducts. UVA photons, long considered biologically harmless, have recently been shown to be weakly carcinogenic in experiments in which shorter-wavelength (UVB and

UVC) photons were carefully eliminated (Sternberg and van der Leun, 1990). Because of the low absorptivity of DNA in this wavelength region, it is highly likely that photons of wavelengths longer than 320nm are absorbed by non-DNA chromophores that participate in UVA-induced carcinogenesis (Peak *et al.*, 1993).

The association between sun exposure and skin cancer has long been recognized. UV-radiation induced skin cancers are predominantly basal and squamous cell carcinomas arising from keratinocytes (Scotto and Fraumeni, 1982), but also malignant melanomas arising from melanocytes may be UV induced (Kopf *et al.*, 1984). Although the exact mechanism of UV carcinogenesis is not clear, much work has concentrated on UV-induced damage to DNA. Upon excitation by visible light or near-UV radiation many photosensitizers induce oxidative DNA damage, either indirectly via singlet oxygen (type II reaction) or directly via hydrogen abstraction or electron transfer (type I)(Sies and Menck, 1992). 8-oxodG is predominantly the product of $\cdot\text{OH}$ attack on dG (Floyd *et al.*, 1986) via the Haber-Weiss (or superoxide-driven Fenton) reaction (see section 1.1.4). UV light can drive this reaction by generating superoxide anion through the mediation of cellular sensitizers.

Endogenous photosensitizers, on irradiation with visible or near-UV light, enter an excited state (sens^*). The mechanism of energy transfer allows the photosensitizer to transfer energy to O_2 which enters an excited state while the photosensitizer returns to its ground state (equation 6 and 7).



In an aqueous medium saturated with air reaction 6 and 7 often compete successfully with type I reactions (Epe, 1993). As the energy of singlet oxygen is only 22 kcal/mol higher than that of ground state oxygen even sensitizers absorbing in the red range of the visible spectrum can supply enough energy to generate singlet oxygen (equation 6). The generation of superoxide (equation 7) requires an oxidizable sensitizer. Therefore, the observed photochemical effects of solar radiation on cellular target molecules are probably mediated by endogenous photosensitizers including NADPH, flavins and porphyrins (Cadet *et al.*, 1992) (Figure 1.3).

The spectrum of DNA modifications generated by these reactions is very different from that induced by hydroxyl radicals (Epe *et al.*, 1993). Hydroxyl radicals, for example generated by ionizing radiation or by superoxide in the presence of Fe(III)-EDTA, induce approximately equal amounts of base modifications (Arouma *et al.*, 1989), single strand breaks and sites of base loss (AP sites). However, both singlet oxygen and several photosensitizers in the presence of light generate predominantly base modifications (Epe *et al.*, 1993). Some (or all) of the base modifications are recognized by the repair endonuclease formamidopyrimidine-DNA glycosylase (fpg protein). At least some of the fpg-sensitive base modifications have been identified as 8-oxoguanine (Epe *et al.*, 1993).

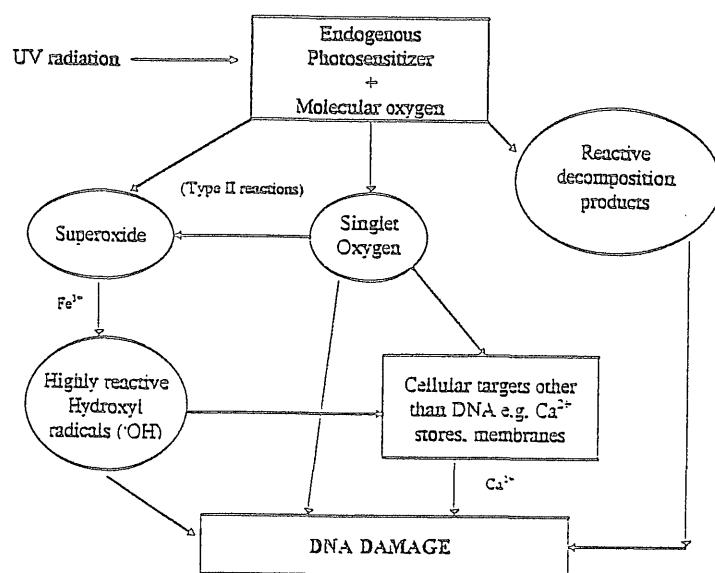
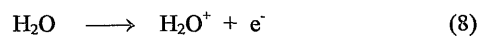


Figure 1.3: Possible mechanisms of indirect DNA damage in a cellular environment by excited photosensitizers (e.g. NADPH, flavins, porphyrins)(adapted from Epe 1993).

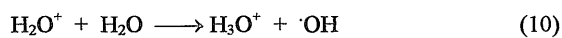
Yamamoto *et al.*, (1992) described the formation of 8-oxodG in cellular DNA by photo-irradiation of cultured mouse lymphoma cells (L5178Y) in the presence of riboflavin (0.2, 2, or 20µg/ml). Subsequently Bessho *et al.*, (1993) described the formation of 8-oxodG in cellular DNA of mouse FM3A cells treated with visible light and riboflavin (160µM). However, Fischer-Nielsen *et al.*, (1992) demonstrated that at low doses glutathione, ascorbate and 5-aminosalicylic acid considerably reduced 8-oxodG formation in UV treated calf thymus DNA while in the presence of iron (FeCl₂/H₂O₂ system) 8-oxodG formation was accelerated (except for GSH at pH 7.4). Subsequently Fischer-Nielsen *et al.*, (1993) showed that both ascorbate and 5-aminosalicylic acid on preincubation with V79 Chinese hamster cells diminished the 8-oxodG formation induced by light from a sun lamp emitting light in the UVC (<290 nm), the UVB (290-320 nm), the UVA (320-400 nm) and the near-visible/visible light spectrum. Gilchrest *et al.*, (1994) concludes that both ageing and photoageing alter the expression of selected genes (c-fos, SPR2 and IL-1ra) that are implicated in growth, differentiation, immunomodulation, and UV response in human epidermis. This may explain, in part, the predisposition to photocarcinogenesis in chronically sun-exposed skin of older individuals.

1.3 Gamma irradiation of aqueous solutions containing DNA

The two major processes of the interaction of ionizing radiation (usually a ^{60}Co source emitting γ -radiation) with matter are ionization and electronic excitation. In liquid water these reactions are described by equations 8 and 9.



The water radical cation is a strong acid and rapidly loses a proton to the surrounding water molecules (equation 10).



The electron becomes solvated within a very short time (equation 11).



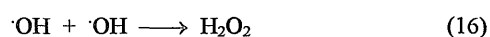
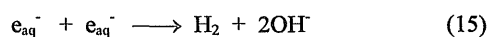
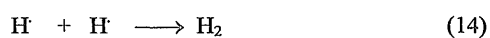
The excited water molecules formed in the second primary process (equation 9) can break up into H-atoms and OH radicals (equation 12).



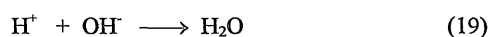
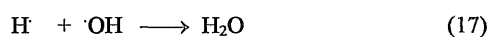
These species either react with one another within the immediate vicinity (Jonah and Miller, 1977) or diffuse into the bulk solution. Hence by the reaction of the solvated electrons with protons, further H-atoms are produced (equation 13).



Reactions 14-16 yield the molecular products H_2 and H_2O_2 .



A considerable proportion of the radicals formed in the spur are reconverted to water (equation 17 and 18), and protons and hydroxide ions eventually neutralize one another (equation 19).



All these processes are practically diffusion controlled (von Sonntag, 1987). A considerable number of radicals escape the spur (Buxton, 1968) and these account for the damage to DNA when it is irradiated with γ -irradiation using a ^{60}Co source. On irradiation of cells,

radical scavengers within the cell may reduce the amount of damage caused. 8-oxoG has been measured as a marker of this oxidative DNA damage in cells (Dizdaroglu, 1991).

1.9 Controversy surrounding measurement of 8-oxoguanine by high performance liquid chromatography with electrochemical detection (HPLC-ECD) versus measurement by gas chromatography/mass spectrometry (GC/MS)

Nucleosides and bases are readily separated by reversed-phase HPLC (Perrett, 1987). A number of purine nucleosides and bases are readily electro-oxidisable, the ease of oxidation being dependent upon the number of substituted OH groups in the molecule (Winyard *et al.*, 1990). By electrochemical detection the sensitivity of detection for 8-oxodG is about one-thousand fold enhanced over optical detection (Fraga *et al.*, 1990). The sensitivity and specificity of HPLC-ECD for 8-oxodG has allowed this technique to be widely used as an assay that measures oxidative DNA base damage (section 2.7). These measurements of 8-oxodG have produced evidence that oxidative damage to DNA does occur in isolated cells and in whole organisms, although care must be used in interpreting the data. There is controversy in the literature over the artefactual oxidative damage caused in isolating the DNA from these cells and tissues and what the control baseline levels of 8-oxodG are (Claycamp 1992; Birnboim 1994). Attack of the $\cdot\text{OH}$ radical upon guanine can lead to formation of 8-oxoG by oxidation of the C8 position to the $\cdot\text{OH}$ -adduct radical, but this radical can lead to other products such as FapyGua (Dizdaroglu, 1991). For example, iron ion-dependent systems generating $\cdot\text{OH}$ cause substantial formation of FapyGua as well as 8-oxodG in DNA, (Arouma *et al.*, 1989; Dizdaroglu *et al.*, 1991a) whereas systems containing copper ions and H_2O_2 greatly favour 8-oxodG over FapyGua (Arouma *et al.*,

1991; Dizdaroglu *et al* 1990 and 1991a). Therefore, HPLC-based analysis of 8-oxodG as a method of measuring oxidative DNA damage, despite its undoubted value, has intrinsic limitations.

1.9.1 Discussion of GC/MS sensitivity for the analysis of oxidatively-modified DNA bases

Characterization of various types of damage to DNA by oxygen-derived species can be achieved by the technique of gas chromatography/mass spectrometry (GC/MS), which may be applied to DNA itself or to DNA-protein complexes such as chromatin. The DNA or chromatin are hydrolyzed usually by formic acid and the products converted to volatile derivatives, which are separated by gas chromatography and conclusively identified by the structural evidence provided by a mass spectrometer (Dizdaroglu, 1992). Higher sensitivity and selectivity of detection can be achieved by operating the mass spectrometer in the selected ion monitoring (SIM) mode. In SIM, the mass spectrometer is set to monitor an ion derived by fragmentation of a particular product during the time at which this product emerges from the GC column. The lowest background level of a base modification in DNA, or in chromatin, that is measurable by currently-used GC/MS-SIM techniques corresponds to 1 modified base in approximately 10^6 bases (Dizdaroglu, 1991). This makes the technique broadly comparable in sensitivity to measurement of 8-oxodG by HPLC with electrochemical detection (Halliwell and Dizdaroglu, 1992). The exact sensitivity achieved is affected by, among other factors, the GC/MS instrument used and the type of column (Arouma and Halliwell, 1995). In most cases, it is not the absolute sensitivity of the technique that matters, but the "background" levels of base modification in untreated DNA, or DNA from "unstressed" cells or tissues. To date, fewer studies on DNA freshly-isolated

from cells and tissues have been conducted using GC/MS-SIM than with HPLC, but the figures available show around 40 8-oxoG per 10^6 DNA bases, about 2- to 11- fold greater than the figures recorded by HPLC (Halliwell and Dizdaroglu, 1992).

1.9.2 Possible explanations of the discrepancy between GC/MS and HPLC-ECD

Modifications of DNA bases affect cell metabolism and may be related to carcinogenesis (Cerutti *et al.*, 1989; Ames, 1989b; Richter, 1988) so it is important to understand this apparent discrepancy. Before discussing potential explanations in detail it is worth pointing out that isolation of DNA from cells may introduce some oxidative modification, particularly if phenol-based methods are used, since oxidising phenols produce a wide range of reactive radicals (Halliwell and Gutteridge, 1989). This is one of the reasons why the more "gentle" extraction of chromatin for analysis may be preferable (Dizdaroglu, 1991; Gajewski *et al.*, 1990; Dizdaroglu *et al.*, 1991b). This technique is milder than those used for DNA extraction and may minimize the loss of extensively fragmented DNA and of DNA that has become cross-linked to protein as a result of oxidative damage (Dizdaroglu, 1994). HPLC analysis relies upon extraction of DNA from cells and complete enzymic hydrolysis before quantitative measurement of 8-oxodG can be achieved.

There are several explanations for the discrepancy between the GC/MS and HPLC-ECD techniques. First, extraction of DNA that has undergone extensive modification and fragmentation may be impaired, because of the easy loss of small DNA fragments (Dizdaroglu, 1991; Floyd *et al.*, 1986) and cross-linking of the DNA bases to amino acid residues in nuclear proteins (Dizdaroglu, 1991). Although this artefact could lead to an underestimate of DNA damage in heavily-stressed cells, it would not be expected to affect

measurement of the low levels of DNA base damage in cells not deliberately subjected to oxidative stress.

Second, the efficiency of exonucleases and endonucleases in hydrolysing DNA is greatly affected by modification of the bases (Breimer, 1990; Dizdaroglu *et al.*, 1978). For example, Maccubbin *et al.*, (1991) showed that 8-oxoG severely inhibits digestion of dinucleotides by phosphodiesterases. Thus it is not always certain that modified bases are completely hydrolyzed from DNA, especially when published hydrolysis techniques are transplanted from one laboratory to another and not re-validated.

Third, the HPLC technique as usually used measures 8-oxodG, and not 8-oxoG (Devasagayam *et al.*, 1991). Frenkel *et al.*, 1991, showed that an acidic pH (frequently used for nuclease P1 digestion) can promote hydrolysis of 8-oxodG to 8-oxoG, causing a loss of HPLC-detectable material. Extensive free radical damage might also lead to release of modified guanine from the DNA backbone to leave an abasic site (Halliwell and Dizdaroglu, 1992). Frenkel *et al.*, (1991) considered these artefacts and, using a more-complex HPLC technique than is commonly employed, they found that DNA extracted from murine epidermal cells contains baseline 8-oxodG levels of at least 30 per 10^5 DNA bases, closer to the values measured by GC/MS than to those measured by conventional HPLC assays (Halliwell and Dizdaroglu, 1992).

Do derivatization and hydrolysis cause artefactual formation of 8-oxoG? Data from Nackerdian *et al.*, (1992) suggest that 8-oxoG is not formed by the hydrolysis procedures used. It is not known whether it is formed during the derivatization procedure. In conclusion we do not know the “baseline” level of products of DNA damage by oxygen-

derived species *in vivo*, since different measurement techniques give different results. Greater attention must be given to resolving these methodological questions before the widespread adoption of such methods as a true index of oxidative DNA damage.

1.10 Alternative/complementary methods of analysis of oxidative DNA damage

1.10.1 Potential of immunoaffinity isolation for detection and quantitation of 8-oxodG and 8-oxoG

Immunoassays have been developed for the quantitative analysis of specific types of DNA damage products involving bulky alkyl and polycyclic aromatic derivatives (Srickland and Boyle, 1984). A similar approach employing polyclonal and monoclonal antibodies raised against oxidized bases of DNA has been used to develop highly sensitive assays that detect the γ -irradiation-induced oxidative adducts 8-oxoadenine (West *et al.*, 1982) and thymidine glycol (Leadon and Hanawalt, 1983; Rajagopalan *et al.*, 1984). The successful detection and quantitation of specific oxidation products of DNA led Degan *et al.*, (1991) to raise polyclonal antibodies to 8-oxodG and 8-oxoG. Using these polyclonal antibodies immunoaffinity columns were prepared which allowed the rapid isolation of 8-oxodG and 8-oxoG from urine. However these polyclonals had a decreased affinity for 8-oxodG in DNA and this limited their usefulness for quantitating endogenous levels of oxidative damage in intact DNA. Mussart and Wani, (1994) have reported the development of a polyclonal antibody that is capable of detecting 8-oxodG in oxidized DNA using immunoslot blot assays. Recently Yin *et al.*, (1995) have developed an immunoaffinity chromatography-monoclonal antibody-based enzyme linked immunosorbent assay (ELISA). This assay was compared directly with the HPLC-EC method for monitoring 8-oxodG in human DNA

samples. Due possibly to a certain amount of crossreactivity with other oxidised bases or to oligonucleotides detected by the ELISA, but not the HPLC, the absolute values of 8-oxodG were approximately six-fold higher in the ELISA (1.3-7.8 moles 8-oxodG/ 10^5 moles dG) than determined by HPLC. It would appear that the best approach may be a combination of an immunoaffinity assay which would concentrate the amount of 8-oxodG in a sample with subsequent analysis via HPLC-EC for the detection and quantitation of 8-oxodG and 8-oxoG.

1.10.2 New alternative methods for detection of 8-oxoG in DNA

^{32}P -postlabelling is a technique which has been developed for the detection of DNA constituents which have been chemically altered by reaction with genotoxins. The ^{32}P -postlabelling assay may be performed on virtually any biological sample from which DNA can be extracted and involves the enzymatic radiolabelling of non-radioactive nucleotides. The basic protocol entails the enzymatic hydrolysis of DNA to deoxyribonucleoside 3'-monophosphates with micrococcal nuclease and calf spleen phosphodiesterase. The dNps are then radioactively labelled via T4 polynucleotide kinase catalyse [^{32}P]-phosphate transfer from [$\gamma^{32}\text{P}$]-adenosine triphosphate to yield deoxyribonucleoside-3'-5'-bisphosphates (Jones and Parry, 1992). Any damaged or adducted deoxyribonucleoside-3'-5'-bisphosphates present in the sample are then purified and separated from their normal (undamaged) counterparts. This is generally achieved using multidimensional anion exchange thin layer chromatography on polyethyleneimine-cellulose plates (Jones and Parry, 1992).

Capillary electrophoresis (CE) has been shown to be a powerful analytical procedure, due to the high resolution, rapidity of analysis and the very small volumes that can be injected.

Different modes of CE exist and even uncharged species can be analysed by a technique known as micellar electrokinetic capillary chromatography. Recently this procedure has been used for the detection of 8-oxodG in DNA damaged *in vitro* (Guarnieri *et al.*, 1994). One disadvantage of this method was poor sensitivity, which did not allow the extremely low concentrations of oxidised nucleosides generated by endogenous sources of free radicals to be determined. However this technique can be suitably employed to determine 8-oxodG and other adducts of DNA produced in *in vitro* pro-oxidant systems (Guarnieri *et al.*, 1994). Herbert *et al.*, 1995 (personal communication) advocates CE as an enrichment step for postlabelling.

1.10.3 Development of an antibody to UV-induced DNA damage as an alternative to using chromatographic techniques

Conventionally DNA is thought to be relatively non-immunogenic. Nevertheless, some thirty years ago Plescia *et al.*, (1964) described how the immunogenicity of DNA could be induced by complexing with methylated bovine serum albumin. Subsequently, antibodies to UV irradiated DNA were facilitated using this technique (Levine *et al.*, 1966). In a series of studies Tan showed that such anti-UV DNA antisera were able to detect UV irradiated nuclei in tissue sections using immunofluorescence (Tan *et al.*, 1969). Previous studies (Wakizaka *et al.*, 1979a and 1979b) used mainly UV irradiation within the UVC region of the spectrum (190-280nm). Whilst initiating immunogenicity in DNA these wavelengths of UV light appear to have little direct relevance to studies of UV-induced changes to biological systems on earth. Most of the UV energy from the sun reaching the earth's surface is UVA (320-400 nm) with a smaller but very significant proportion (~5%) of UVB radiation (280-320 nm). For the purpose of measuring DNA damage without the need for

processing and manipulation of normal and pathological material, a polyclonal antiserum which has specificity for UV damaged DNA has been developed in the Division of Chemical Pathology, University of Leicester (Herbert *et al.*, 1994). The full characterisation of this antibody and a report on its ability to detect sequence-specific modifications induced by UV irradiation of DNA has been described previously (Herbert *et al.*, 1994).

1.10.4 Potential problems and pitfalls associated with immunocytochemistry

Immunocytochemistry is the identification of a tissue constituent *in situ* by means of a specific antigen-antibody reaction, tagged by a microscopically visible label. Provided that a suitable antibody can be produced and the antigen preserved, there is no limit to the substances that may be localised in this way (Van Noorden, 1985). A compromise between good tissue preservation and antigen availability has to be reached for each antigen. Every antiserum must be tested on a known positive control to find the 'correct' working dilution for the staining method to be used. The specific antibody should not stain inappropriate tissues, and on appropriate preparations no reaction should be seen if any of the steps of the reaction are omitted, or if an inappropriate antibody or non-immune serum (at the same dilution) is substituted for the primary antibody. If staining occurs under these circumstances a cause for the 'non-specific background' should be sought. Some tissues, particularly in the form of cryostat sections or whole-cell preparations, have receptors for the Fc portion of the immunoglobulins, resulting in the antibody becoming attached to non-antigenic sites. Usually this can be avoided by a preliminary blocking of the receptors with normal serum from the species donating the second antibody. Fc-binding sites will be covered by immunoglobulin from the normal serum and will not be available for attachment of the primary antibody. The second antibody will not identify these immunoglobulins as

they will be from the same species. Hydrophobic and electrostatic forces can bind immunoglobulin to tissue. These types of truly non-specific binding can be prevented by diluting the primary (and secondary) antisera as far as possible, or by increasing the ionic strength of the washing buffers or the antibody diluent. As well as above-mentioned causes for binding of antibody to tissue, endogenous tissue components may be intrinsically fluorescent, e.g. elastic fibres, or have fluorescence induced by formaldehyde. Since polyclonal antibodies are heterogeneous, antisera may contain antibodies to tissue constituents, to the coupler used in immunisation, and to natural hazards previously encountered by the immunised animal. In general, if the antibody is of high titre and a sensitive method is used, these can be removed by using high dilution of the antibody.

1.10.5 Utilising immunocytochemistry labelling techniques to detect levels of a protein or a DNA damaged product by the flow cytometry method

Flow cytometry is a technique which allows measurements to be made on single particles or cells as they flow in a stream through a detection point, transected by a laser beam. Information is derived on the light scatter characteristics of the cell and the fluorescent intensity of its label (Creamer, 1992). If a molecule absorbs energy from light of a certain wavelength it will move up to a higher 'excited' state. On returning to its original state, energy is emitted in the form of light, i.e. fluorescence. Many such molecules or 'fluorochromes' exist, each with their own optimal excitation and emission wavelengths. An ideal fluorochrome should be capable of absorbing and yielding large amounts of light energy, should be easily linked to another protein (antibodies and ligands) and should be stable and non-toxic. Fluorescein, in its isothiocyanate form (FITC) is widely used. Its absorption maximum (495nm) is close to the wavelength of an argon ion laser (488nm) and

it emits light strongly at 520nm (green). Conjugation of fluorescent dyes to ligands and to polyclonal and monoclonal antibodies has enabled the density and distribution of cell surface and cytoplasmic determinants and receptors to be studied (Carter *et al.*, 1988). While most of the information acquired by flow cytometry can be obtained by other means, for example fluorescence microscopy, flow cytometry has the benefit of speed: up to 32,000 measurements may be made every second. As well as speed of measurement, the most important feature of flow cytometric analysis is that each particle is measured individually rather than as a population. The computer software with which the fluorescence activated cell sorter are equipped permits rapid integration, calculation and display of results.

Sample preparation for flow cytometry: The aim of sample preparation is to produce a suspension of single particles, stained in a specific way, which will pass through the system without disrupting the smooth flow of fluid or blocking tubes or orifices (Carter *et al.*, 1988). Normally, cells are stained by incubation, in appropriate conditions, with a fluorescent dye or fluorescent-conjugated antibody or ligand. For accurate interpretation of results, it is important that the staining is specific for and proportional to the feature to be measured. Measurement of DNA using flow cytometry utilizes fluorescent dyes which bind specifically to nucleic acids. Propidium iodide binds to DNA though will not pass through an intact cell membrane: it can therefore be used to discriminate dead and live populations (Creamer, 1992). Because the DNA content of cells varies predictably throughout the cell cycle quantitation of DNA can give information on the percentage of cells in any part of the cell cycle. The problems of sample preparation and staining are easily appraised by the use of suitable controls designed to measure the specificity and accuracy of the measurements.

1.10.6 The single cell microgel electrophoresis assay (comet assay) as an alternative technique to examine DNA damage and repair

The comet assay is a sensitive and rapid method for DNA strand break detection in individual cells (Singh *et al.*, 1990). In order to execute the assay, a suspension of individual cells must be prepared. Cells are generally suspended in low melt point agarose at a final concentration of 0.5-1.0% at 35-45°C and cast on a fully frosted slide. The gels are allowed to solidify briefly. Following preparation the embedded cells are lysed by gently immersing the slides in a lysis buffer. Alkaline lysis solution is used for single strand break detection. Prior to electrophoresis, the slides are equilibrated in alkaline electrophoresis solution. The desired voltage and time of electrophoresis will be related to the levels of DNA damage expressed in the cells and the salt concentration of the running buffer. Following electrophoresis, slides are washed and stained with a fluorescent DNA binding stain for image analysis. Östling and Johanson, (1984) observed that the extent of DNA liberated from the head of the comet during electrophoresis was a function of the dose of irradiation. The methods of comet image analysis reported are as varied as the applications for which the comet assay has been used (Fairbairn *et al.*, 1995). Since comets are formed upon the principle of releasing damaged DNA from the core of the nucleus with electrophoresis, several different attempts have been made to evaluate and quantify comet formation patterns. An increasingly popular method of comet evaluation is an endpoint referred to as the 'tail moment', which is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). With comet image analysis systems that calculate tail moment, it is also possible to determine the total comet

fluorescence which has been equated with DNA content and thus with cell cycle position (Collins, 1992). Bivariate analysis of DNA damage and DNA content, accommodates the application of more elaborate statistical methods which aid in further defining subtle changes in response to various treatments in cells within the cell cycle (Fairbairn *et al.*, 1995).

1.10.7 Applications of the comet assay for measurement of UV-induced DNA damage

Agents such as UV radiation that produce DNA lesions which do not form strand breaks directly can be examined using the single cell gel assay (Collins, 1992). Rather than detecting strand breaks produced by the irradiation, it is possible to detect strand breaks produced by the cell as it attempts to repair the lesion (Collins, 1992). Intermediate strand breaks result from the incision step of excision repair. Detectable strand break levels are representative of the equilibrium between the initial incision and rejoining steps. Damaged cells allowed no repair time demonstrate no detectable tail formation, much like negative control cells in other exposure systems. With repair time, comets appear and disappear in accordance with the formation of strand breaks and ligation corresponding to incision and rejoining. DNA repair of UV radiation generated damage as measured by the comet assay has kinetics consistent with excisable damage (Collins, 1992). Oxidative and other DNA damage induced endogenously can be readily studied using experimental variations of the comet assay because of its sensitivity. Theoretically, virtually any type of conceivable DNA lesion could act as a determinable quantity provided that the lesion could be converted physically or enzymatically to a strand break for measurement.

Objective and aims

The major objective of this thesis was to evaluate 8-oxodG as a reliable marker for oxidative stress and to investigate its role in UV-mediated DNA damage in cells. In order to achieve this objective the following experimental aims were defined:

1. To establish a robust HPLC assay for 8-oxodG in DNA and subsequently to apply this assay to the accurate and precise quantitation of absolute levels in DNA.
2. To study the effect of different models of oxidative stress on the levels of 8-oxodG measured in DNA following extraction from cells and to investigate the effects of type of DNA extraction on the levels of 8-oxodG measured in cells.
3. To validate this assay by stressing of SLE and RA peripheral blood mononuclear cells with ROS. SLE and RA patient cells were used because it has been established that there is a high baseline level of 8-oxodG in these diseases (Bashir *et al.*, 1993), probably due to an increased sensitivity to oxidative stress (Harris *et al.*, 1994).
4. To investigate the use of an antibody raised to UV-damaged DNA in the detection of UVA-induced DNA damage in a human keratinocyte cell line; and relate to measurement of 8-oxoguanine as a marker of oxidative DNA damage in keratinocyte DNA. Subsequently to relate degree of immunodetection of UVA-induced DNA damage including 8-oxoguanine or its repair (single strand breaks) with levels of the protein p53 and stage of cell cycle in a keratinocyte cell line.

Chapter 2

Materials and Methods

2.1 Materials

All general laboratory chemicals were supplied by Sigma Chemical Company (Poole, Dorset) apart from those listed below.

2.1.1 Analyses of DNA hydrolysates.

HPLC system 1 was set up at Glenfield General Hospital. On relocation to the Centre for Mechanisms of Human Toxicity system 2 was set up.

2.1.1.1 HPLC System 1

Analyses of DNA hydrolysates was performed by reversed-phase HPLC using a Gilson Model 302 pump (Anachem, Luton, U.K.), a Rheodyne 7125 valve injector (Cotati, California), fitted with a 50µl loop, an LC-4B amperometric detector (Biotech, Luton, U.K) and a Gilson UV detector operated at 254nm. The stainless steel columns used were (i) a guard column Co Pell ODS (50 x 4.6 mm id)(Technicol Ltd, Stockport, U.K.) with a Spherisorb 5µm ODS-2 column (250 x 4.6 mm id)(Technicol) or (ii) a Hypersil 3µm ODS column (100 x 4.6 mm id)(Shandon Scientific Ltd., Cheshire, U.K.). HPLC methanol was obtained from Fisons plc., Loughborough, or Rathburn Chemicals Ltd., Walkerburn, U.K. The samples were analysed at a sensitivity of 0.01 A.U.'s for UV and a range of 2nA for ECD. In samples where 8-oxodG was detected alone the peak areas were compared with the peak area of the 8-oxodG standard.

2.1.1.2 HPLC System 2

Analyses of DNA hydrolysates were also performed by reversed-phase HPLC using a Beckman autosampler 507, Diode Array Detector Module 168, Programmable Solvent Module 126, an Analog Interface Module 406, Beckman System Gold software (all; Beckman Instruments, High Wycombe, U.K.) and an EG&G electrochemical detector Model 400 (Princeton Applied Research, Princeton, USA). A stainless steel column containing 3 μ m ODS Hypersil was used (Shandon Scientific Ltd., Runcorn, U.K.).

2.1.2 Isolation of PBMC from whole blood and extraction of DNA from PBMC

Tri-sodium citrate (Fisons plc., Loughborough, U.K.) used at a concentration of 3.4% w/v was added to whole blood to prevent clotting. Ethylene diaminetetraacetic acid (EDTA) (BDH Laboratory Supplies, Poole, U.K.) was used to prevent clotting also. Phenol (washed with Tris buffer until pH of the phenolic phase was >7.8) (Fisons plc.) and chloroform:isoamyl alcohol (BDH Laboratory Supplies) were used in the organic isolation of DNA. Absolute ethanol (Leicester University Chemical Stores) was used for precipitation of DNA. The enzymes Pronase E (protease, from *Streptomyces griseus*, Type XXV, 3.8 units/mg solid) and Proteinase K (protease from *Tritirachium album*, Type XI, 11.8 units/mg protein) were obtained from Sigma, Poole, U.K.

2.1.2.1 Enzymes for DNA hydrolysis

RNase (EC 3.1.27.5 from bovine pancreas, Type XII-A, 100Kunits/mg protein), DNase I (EC 3.1.21.1, from bovine pancreas, Type IV, 1750Kunits/mg protein), endonuclease (EC 3.1.30.1 from *Neurospora crassa*, 238 units/mg protein), nuclease, micrococcal (Micrococcal endonuclease; EC 3.1.31.1 from *Staphylococcus aureus*, Foggi strain, 195 units/mg protein), alkaline phosphatase (EC 3.1.3.1 from *Escheria coli*, Type III-S, 10.5 units/mg protein) and phosphodiesterase (EC 3.1.4.1 from *Crotalus atrox* venom, Type VII, 0.18 units/mg protein) were used in the digestion of DNA to its deoxynucleosides. Hydrochloric acid (HCl)(BDH laboratory Supplies, Poole, U.K.) was used to alter the pH during hydrolysis.

2.1.3 Tissue Culture

All cells cultured in Sanyo incubators in a 5% CO₂ atmosphere.

2.1.3.1 Media constituents for peripheral blood mononuclear cells (PBMC)

Medium I consisted of RPMI 1640 (Flow Laboratories, Irvine, U.K.; catalogue number 041-02404) with 10% heat inactivated foetal calf serum (Advanced Protein Products Ltd., Brierley, U.K.), L-glutamine (2mM) (ICN Biomedicals, High Wycombe, U.K.) and Penicillin-G-streptomycin solution (penicillin 100U/ml, streptomycin 100µg/ml)(Flow Laboratories, Irvine, U.K.).

2.1.3.2 Culture Medium for RHT keratinocytes.

The RHT cells were a gift from Professor Irene Leigh, Department of Dermatology, London Hospital Medical College.

Medium RM+ consisted of DMEM to Ham's F12 at a ratio of 3:1, 10% Heat inactivated foetal calf serum(HiFCS)(Advanced Protein Products Ltd., Brierley, U.K.) plus mitogens (see appendix I for composition). Dulbecco's modified eagles medium (DMEM) without sodium pyruvate with 4500g/l glucose containing L-glutamax (catalogue number 32430-027) was obtained from Life Technologies Ltd., Paisley, U.K. Ham's Nutrient Mixture F-12 (Ham's F-12) with 1.242 mg/l phenol red and 1.176g/l sodium bicarbonate without L-glutamine (catalogue number 1-755-14) was obtained from Imperial Laboratories Ltd., Andover, U.K.

Medium RM- contained DMEM and Ham's F-12 at a ratio of 3:1 without HiFCS and mitogens.

2.1.3.3 Culture Medium for Human acute lymphoblastoid cells (CCRF-HSB-2).

The source of the CCRF-HSB-2 cells was ICN Biomedicals, High Wycombe, U.K. Medium II consisted of RPMI 1640 (Flow Laboratories, Irvine, U.K; catalogue number 041-02404) with 10% Heat inactivated foetal calf serum (Advanced Protein Products Ltd., Brierley, U.K.) and L-glutamine (2mM)(ICN Biomedicals, High Wycombe, U.K.). The CCRF-HSB-2 cells were maintained at a concentration of $5-8 \times 10^5$ cells/ml.

2.1.3.4 Culture medium for human promyelocytic leukaemic cell lines (HL60)

The source of the HL60 cell line was the European collection of animal cell cultures (ECACC), Porton Down, U.K. Medium III consisted of RPMI 1640 (Flow Laboratories, Irvine, U.K.; catalogue number 041-02404), 10% Heat inactivated foetal calf serum (Advanced Protein Products Ltd., Brierley, U.K.) and L-glutamine (2mM)(ICN Biomedicals, High Wycombe, U.K.). Cells were passaged at $1-3 \times 10^5$ /ml twice weekly and maintained at a low cell density(i.e. $<8 \times 10^5$ cells/ml).

2.1.4 Sterile disposables

Ninety-six well plates, Nuclon twenty-four well plates, Vented tissue culture flasks (t25cm², t80cm², t125cm²) and petri dishes (85 x 15mm) were obtained from (Nunc™) Life Technologies, Paisley, U.K. Polystyrene, round bottom tubes (12 x 75mm)(Falcon 2052) and ninety-six well plates (Falcon 3072) were obtained from (Falcon™) Becton Dickinson Labware, Lincoln Park, USA. Petri dishes (35 x 10mm) were purchased from Becton Dickinson Labware, Plymouth, U.K.

2.1.5 Materials used in antibody staining of cultured cells

AMCA streptavidin was purchased from Vector laboratories, Peterborough, U.K. Biotinylated goat anti-rabbit IgG (whole molecule) antibody (B-8895) was obtained from Molecular Probes Inc., Eugene, USA. Dried skimmed milk was a product from Tesco Stores Ltd., Cheshunt, U.K. The monoclonal mouse anti-human p53 protein (DAKO, DO-

7), fluoresceinated goat anti-rabbit IgG (whole molecule) antibody (F8646), fluoresceinated goat anti-mouse IgG (Fc-specific) antibody (F-0511) and peroxidase labelled streptavidin/biotin (DAKO, K-377) were all products purchased from Sigma, Poole, U.K.

2.1.6 Materials used in 8-oxoguanine assay

The 2-amino-6,8-dihydroxypurine (8-oxoguanine) chemical was obtained from Aldrich-Chemie, Poole, U.K. The enzyme guanase (guanine deaminase, guanine aminohydrolase)-rabbit liver, E.C.3.5.4.3; 0.06-0.2 units/mg protein) was purchased from ICN, Thame, U.K.

2.1.7 Equipment

The Shimadzu UV-160A UV visible recording spectrophotometer and the Shimadzu RF-5001 PC spectrofluorometer were both purchased from Shimadzu, Duisburg, Germany. The fluorescence spectrophotometer (204-A) and Xe source white light (150 Xe power supply) were obtained from Perkin Elmer, Beaconsfield, U.K. The UVA-model UVL-56, Blak-Ray lamp (long wave UV-366nm), the optical radiometer and the UVB-model UVM-57, Chromato-Vue lamp (302nm) were purchased from Knight Optical Technologies, Leatherhead, U.K. The microplate fluorescence reader was supplied by Denley Wellfluor, Denley instruments Ltd., Billingham, U.K. The Walker Class II Microbiological safety cabinets were supplied by Walker Safety Cabinets Ltd., Derbyshire, U.K. The light phase microscope (standard 20) was purchased from Zeiss, Hanover, Germany. The inverted microscope (Nikon TMS) was obtained from Nikon, Japan. The Colourmorph (Software)

was purchased from Perceptive Instruments, Halstead, U.K. A Savant Speedvac was purchased from Savant Instruments, Hicksville, U.S.A.

2.2 Isolation of Peripheral Blood Mononuclear cells (PBMC) from Whole Blood

Human blood (10ml) was collected into tubes containing 3.4% tri-sodium citrate (1ml/9ml blood) and mixed by gentle inversion. An equal volume of RPMI 1640 was added and mixed by gentle inversion. Centrifuge tubes were prepared by addition of Histopaque 1077 (lymphocyte separation medium) (3ml) which was at room temperature. The diluted blood was gently layered onto the Histopaque using a sterile pasteur pipette and the interface was kept as discrete as possible. The tubes were centrifuged at 400g for 30 min at room temperature. The upper plasma/Histopaque layer was removed by pipette to within 1 cm of the mononuclear cell layer. Mononuclear cells were carefully collected, with as little agitation as possible. The volume was made up to 10ml with RPMI-1640, and centrifuged at 200g for 10 min at room temperature. The supernatant was then decanted and the pellet resuspended in the residual volume by gentle tapping, with addition of a small volume of RPMI 1640 (1-2ml). All cells were pooled into one tube and mixed gently but thoroughly. A small aliquot (20 μ l) was removed for counting see Section 2.3. The tubes were centrifuged at 200g for 10 min at room temperature. Cells were resuspended by tapping and a sufficient volume of RPMI 1640 added to give a cell concentration of 1×10^6 cells/ml.

2.3 Enumeration of cells

An aliquot of cell suspension (typically 20 μ l) was mixed with an equal volume of Trypan Blue (0.4% w/v in 0.8% w/v sodium chloride, 0.06% w/v dipotassium hydrogen phosphate). The haemocytometer was prepared by moistening the edges of the counting chamber with a damp tissue, the coverslip was then placed by sliding it on to produce Newton's rings. The cell/dye suspension (~20 μ l) was then applied by touching the edge of

the coverslip with the tip of the pipette and slowly filling the chamber. The cells were counted in all squares under a $\times 40$ objective. Viable cells were colourless, non viable cells had taken up the Trypan Blue and stained a blue colour.

Total cell numbers were determined as follows using a factor (10^4) derived from the volume of the counting chamber:-

Viable cells per ml = viable cells counted \times dilution factor $\times 10^4$.

2.4 Isolation of peripheral blood mononuclear cell DNA

DNA was isolated from peripheral blood mononuclear cells using either (i) Pronase E extraction (based on Kendall *et al.*, 1991) or (ii) Phenol extraction method (based on Winyard *et al.*, 1990).

2.4.1 Pronase E extraction

PBMC were separated by centrifugation at 300g for 10 min at room temperature. Supernatants were collected and processed as in section 2.5.1. The cells were resuspended in 3.5ml ice-cold buffer 1 (5mM trisodium citrate, 20mM sodium chloride, pH 6.5) with vigorous mixing. Pronase E (2mg/ 10^7 cells) in buffer 1 (0.5ml) was added to each tube followed by 4ml buffer 2 (20mM Tris, 20mM EDTA, 1.5% w/v sarkosyl, pH 8.5). The tubes were vortexed during each addition and incubated overnight at 45°C in a water-bath. The contents of the tubes were transferred into 50ml centrifuge tubes. Buffer 3 (10mM Tris-HCl, 10mM EDTA, pH 7.5; 4ml) and 7.5M ammonium acetate (1ml) were added to

each tube and mixed by inversion. DNA was precipitated by the addition of approximately 30ml absolute ethanol with mixing by inversion until precipitation was seen.

Samples were then centrifuged at 500g for 10 min, and the DNA washed in 70% (v/v) ethanol for 30 min, 100% ethanol for 10 min, 70% (v/v) ethanol for 10 min, with centrifugation at 500g for 10 min between each step if necessary. The samples were then air dried for 5 min and resuspended by mixing overnight in buffer 3 (1ml) at 4°C. To determine the amount of DNA in solution and the DNA/protein ratio, the absorbance of the DNA solution was measured at 260nm and at 280nm against buffer 3 and the ratio $A_{260/280}$ calculated. An absorbance of 1.0 at 260nm implied that there was 50µg/ml of DNA present and it was important that the DNA/protein ratio was at least 1.8 to ensure a high purity isolation.

2.4.2 Phenol extraction

Following treatments the cells were separated by centrifugation at 300g for 10 min at room temperature. The supernatants were collected and processed as in section 2.5.1. A further aliquot of RPMI 1640 (10ml) was added to each tube. The cells were centrifuged at 1500g for 20 min at 4°C and the pellets containing nuclei were dispersed in 0.1ml 10mM Tris-HCl, 1mM EDTA, pH 8.0 (TE buffer) and 1ml proteinase K solution (proteinase K: 100µg/ml, 5mM EDTA and 0.5% w/v sarkosyl, pH 8.0) and incubated at 50°C for 2 hours. DNA was extracted with an equal volume of phenol and subsequent phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and chloroform:isoamyl alcohol (24:1 v/v)(x2) extractions, the aqueous layer contained the DNA at each step. Nucleic acids were precipitated by addition

of 0.3M sodium acetate (1/10 volume) and absolute ethanol (2.5 volumes) and left at -20°C for at least 3 hours. The DNA and RNA were pelleted by centrifugation and dried under nitrogen gas to remove ethanol residues. The pellet was dissolved in RNase A solution (RNase 100µg/ml in 50mM Tris-HCl, 10mM EDTA and 10mM NaCl, pH 8.0) and incubated at 37°C for 2 hours. DNA was extracted with phenol:chloroform:isoamyl alcohol and subsequently with chloroform:isoamyl alcohol (x2) and precipitated with ethanol and sodium acetate as before. DNA was dried as before, dissolved in buffer 3 (see Section 2.4.1) and stored at -20°C.

2.4.3 Enzymatic hydrolysis of DNA

Magnesium chloride was added to a final concentration of 10mM to DNA samples in buffer 3, followed by heating in eppendorf tubes for 3 min in a boiling water bath. Tubes were then rapidly cooled in ice subsequent to incubation at 37°C overnight with DNase I (0.1mg/mg DNA) and endonuclease (0.14 units/4µg DNA). The digests were brought to pH 8.0 with Tris base (1mM), and alkaline phosphatase (1 unit/mg DNA) and phosphodiesterase (0.04 units/mg DNA) added followed by overnight incubation at 37°C. The pH was adjusted to 7.0 with 1N HCl and the samples stored at -20°C prior to analysis by high performance liquid chromatography with electrochemical detection (HPLC-ECD) (see section 2.7).

2.5 Protocol for *in vitro* experiments using PBMC

PBMC were isolated from peripheral blood of patients and healthy controls as described in section 2.2. Cells were resuspended to 1×10^6 /ml and cultured in 24 well plates in a volume of 2 ml medium I (see section 2.1.3.1). At various intervals cell viability was determined using trypan blue exclusion (section 2.3).

The cells were separated by centrifugation at 300g for 10 min at room temperature and supernatants collected and resuspended in ice-cold buffer 1 (section 2.4.1) or TE buffer as appropriate for pronase E or phenol extraction of DNA respectively. DNA was isolated from cells as described in section 2.4.1 or as in section 2.4.2 and digested as in section 2.4.3 before analysis by HPLC/ECD (section 2.7).

2.5.1 Processing of cell supernatants

Supernatants were collected after centrifugation and two volumes of ice-cold ethanol added. Any protein present was allowed to precipitate at -20°C for 24 hours. The supernatant was then aspirated and any particulate matter removed by centrifugation at -4°C for 10 min in an eppendorf centrifuge ($\times 1500\text{g}$). The supernatant was dried down using a Gyrovap and precipitates reconstituted in 1 ml sterile milli Q grade water.

2.6 Preparation of 8-oxodeoxyguanosine using the Udenfriend system

The 8-oxodeoxyguanosine (8-oxodG) standard was synthesised by a modified version (see section 3.1) of the Udenfriend system (Kasai *et al*, 1984). Deoxyguanosine (0.25g) was dissolved in 0.13M sodium phosphate buffer (pH6.8) in a 1 litre Erlenmyer flask. Ascorbic acid 0.1M (35ml), 0.1M EDTA (16.25ml), 0.1M FeSO₄ (3.25ml), were then added successively. Oxygen gas was bubbled continuously through the solution which was incubated at 37°C in a water bath for three hours in the dark. The solution was adjusted to pH 3.7 with 1 N HCl, charcoal powder (2.5g) was then added with stirring. The charcoal was packed in a silica glass column (1m x 2cm diameter). The column was washed with milli Q grade H₂O (125ml), and eluted with aqueous acetone (1:1,v/v; 125ml). The eluate was evaporated to dryness (a rotary evaporator with a water bath at 70°C was employed for this purpose) and the residue (8 ml) fractionated by HPLC.

The injection volume was 500µl and a total of sixteen injections were made. The small, UV peak eluting just after the main peak of deoxyguanosine (dG) was collected (in 1ml fractions in eppendorfs) and subsequently evaporated to dryness using the rotary evaporator and a boiling water-bath. The residue was resuspended in 5% aqueous methanol (3 ml) and reinjected onto the HPLC column. Fractions were collected as before, then combined and divided into aliquots of 1 ml and placed in eppendorf tubes. These fractions were freeze-dried and stored at -20°C. A sample was sent to Dr. Peter Farmer, MRC Toxicology Unit and its identity confirmed by fast atom bombardment mass spectrometry. (See section 3.2).

2.7 HPLC analysis of deoxyguanosine and 8-oxodeoxyguanosine

Analyses of DNA hydrolysates (section 2.4.3) were performed by reversed-phase HPLC with UV and ECD. The mobile phase was 50mM sodium acetate and 1mM EDTA containing 10%, 5%, or 2% methanol at pH 5.1, vacuum filtered and sonicated to degas and used at a flow rate of 0.8ml/min. The amperometric detector used a glassy carbon electrode at a potential of +0.6V, providing selectivity for 8-oxodG, measured against an Ag/AgCl/3M NaCl reference electrode. Typically 50µl samples were injected. Identification of deoxyguanosine was by co-injection with authentic standard. Quantitation of deoxyguanosine was by peak height measurement. 8-oxodG was identified by co-injection with a known standard and plotting a voltammogram ranging from 0.4V to 1V (See Figure 2.1). Quantitation of 8-oxodG was by comparison of peak heights with peak heights of known standards (Section 2.7.1).

2.7.1 Quantitation of HPLC analyses

A known concentration of the dG and 8-oxodG standards were injected onto the column. The area of each peak was measured. The amount of dG and 8-oxodG were calculated per mm. Thus the amounts in each sample were calculated by multiplying the peak area by the known value per mm of dG (µmoles) and 8-oxodG (nmoles). 8-oxodG in DNA hydrolysates was calculated as follows; mol 8-oxodG/10⁵ mol dG.

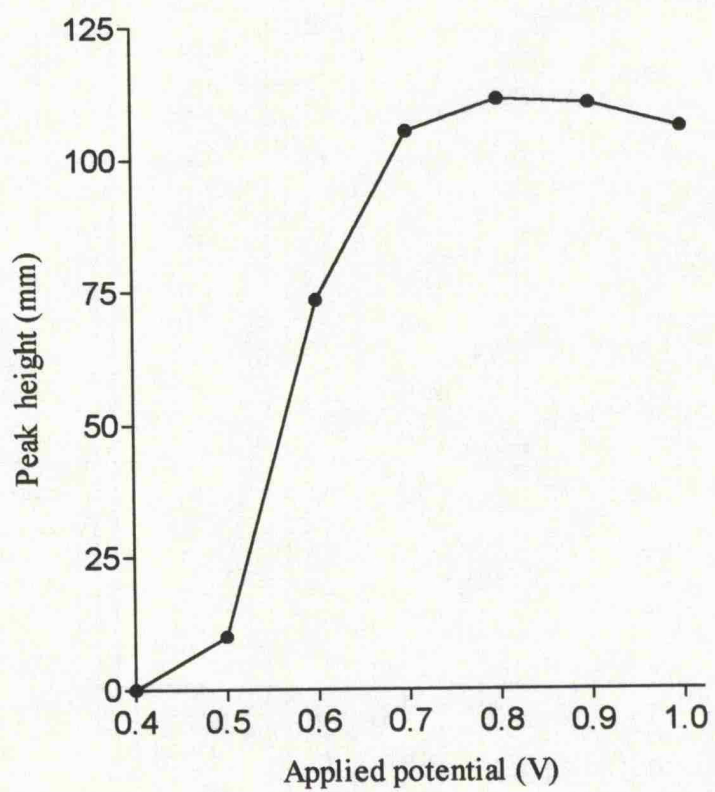


Figure 2.1: Effect of applied potential (vs Ag/AgCl reference) on peak height on HPLC of a 500nM 8-oxodG standard.

2.8 Formic acid hydrolysis and HPLC analysis of 8-oxoguanine

Due to the discrepancies reported in the literature between the levels of 8-oxoG measured using HPLC/ECD and GC/MS (Halliwell and Dizdaroglu, 1992), an assay was developed (section 6.0) combining formic acid hydrolysis, used for the preparation of samples for GC/MS, with HPLC/ECD. Nackerdian *et al*, (1992) found that formic acid hydrolysis at a concentration of 60% did not cause artefactual formation of 8-oxoG and allowed complete release of the lesion from DNA. An aliquot of DNA (100µg) was treated with 0.5ml of formic acid (60% v/v in water) in evacuated and sealed tubes at 140°C for 30min. The sample was then freeze-dried using a Savant speedvac. The DNA was then reconstituted in 200µl (or 500µl for DNA extracted from RHT keratinocytes) ultra pure water and divided into two aliquots. Tris base (1mM pH 8.0) was added dropwise to give a pH of 8.0 (~5 µl). To one of the samples 1µl guanase (neat; 1.265 units/ml) was then added and the mixture incubated for 1 hour at 37°C. An injection of each sample, with and without enzyme, was made on the HPLC. Analyses of DNA hydrolysates were performed by reversed-phase HPLC using the system 2 described in section 2.1.1.2. The eluant was 40mM potassium phosphate, 1mM EDTA at pH 5.0 containing 1% methanol (MeOH). The flow rate was 1ml/min. The HPLC column was washed once a week with 100% ultra pure water for 10min followed by 100% MeOH for 30min followed up by water for a further 10min, this kept the system free from residue left on the column. The amperometric detector used a glassy carbon electrode at a potential of +0.6V, measured against an Ag/AgCl/3M NaCl reference electrode, providing selectivity for 8-oxoguanine (8-oxoG). Typically 50µl samples were injected. Identification of guanine was by co-injection with authentic standard. 8-oxoguanine was identified by co-injection with known standards and plotting a

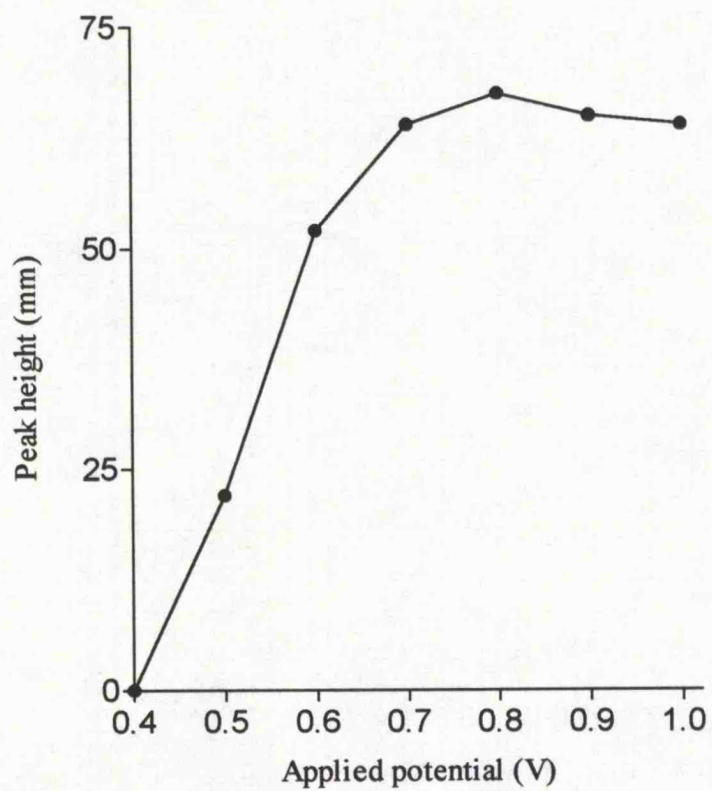


Figure 2.2: Effect of applied potential (vs Ag/AgCl reference) on peak height on HPLC of a 2 μ M 8-oxoguanine standard.

voltammogram ranging from 0.4V to 1V (See Figure 2.2). Quantitation was by determination of peak height and relation to peak height of known standards.

2.9 RHT cell culture

Human immortalised RHT keratinocytes were grown to confluence in RM+ medium (section 2.1.3.2) and routinely passaged every 3-4 days. At various passages, an aliquot (1×10^6 cells/ml) was suspended in FCS/RM+/dimethyl sulfoxide (50/40/10; v/v/v). These stocks were then frozen and stored in liquid nitrogen

2.9.1 MTT assay

The reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase (Mossman, 1983) forms the basis of a standard colorimetric cytotoxicity assay. The MTT is taken up into cells and reduced in a mitochondria-dependent reaction to yield a formazan product. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. On solubilisation of the cells, the product is liberated and can readily be detected and quantified by a simple colorimetric method i.e. a spectrophotometric plate reader. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability and/or cell number. The assay has therefore been adapted for use with cultures of exponentially growing cells. Measurement of their ability to reduce MTT to the formazan product after exposure to test compounds, compared to the control situation, enables the relative toxicity of test chemicals or toxic

insults to be assessed. This assay of mitochondrial activity was used as a measure of cell viability (Mossman, 1983). RHT cells were plated at a concentration of 2×10^5 cells/ml in 96-well plates (200 μ l/well). The cells were allowed to grow for up to 18 hours in RM+ medium. The media was aspirated and wells rinsed with Hanks Balanced Salt Solution (HBSS). Selected wells were then irradiated with either UVB light (3.3-85.7 mJ/cm²) or UVA light (1-5 J/cm²). Medium RM+ was then replaced into the wells. Cell toxicity was determined at 1 hour and 24 hours post irradiation. MTT (5 mg/ml)(10 μ l) was added to media in wells 1 hour prior to the end of incubation. Media containing MTT was aspirated and 100 μ l isopropanol added. Once the formazan crystals had dissolved (5-15 min) the absorbance was read at 550nm using 620nm as the reference wavelength.

2.9.2 UV irradiation of RHT cells on coverslips

Glass coverslips were placed in 85x15mm deep petri dishes and RHT cells seeded at a concentration of 3×10^5 /ml (10ml per dish) and left overnight at 37°C. The medium was removed and the cells rinsed twice with HBSS prior to irradiation with UVB at 3.3-85.8 mJ/cm². Cells were then fixed with 2ml/dish of ice cold methanol:acetone (1:1) for a minimum of 10 min at 4°C. The MeOH:acetone was then aspirated and the coverslips left to dry and stored at -20°C. Cells were rehydrated with 0.1M phosphate buffered saline (PBS) and blocked for 1 hour with 10% normal goat serum (NGS) in PBS at room temperature. The coverslips were washed x3 with PBS and primary antibody 529 applied at 1:5000 dilution (in 0.1% NGS in PBS) and left at 4°C overnight. The coverslips were washed x3 with PBS and the biotinylated goat anti-rabbit secondary antibody applied (1:200 in 0.1% NGS in PBS) for 1 hour at room temperature. After coverslips were washed x3

with PBS, avidin linked fluorescein isothiocyanate (FITC)(1:100 in 0.1% NGS in PBS) was applied for 1 hour at room temperature in the dark. Coverslips were finally rinsed x3 with PBS, and mounted using Vector shield on slides. The cells were then examined under a fluorescence microscope using a filter for FITC.

2.9.3 Treatment of RHT cells by UV radiation in 96 well plates and immunostaining

Cells were plated out in a 96 well plate at a concentration of 2×10^5 /ml, 200 μ l/well and left overnight at 37°C in an incubator containing 5% CO₂. The plates were rinsed twice with HBSS and irradiated with UVA at 0, 1, 2, 3, 4, 5J/cm². After 1, 24 and 48 hours post irradiation the medium was removed from the plates and the cells were rinsed with HBSS. The cells were then fixed with 25 μ l/well of ice cold methanol:acetone (1:1) for a minimum of 10 min at 4°C. The MeOH:acetone was then aspirated and the plates were then left to dry and stored at -20°C. Cells were rehydrated with 0.1M PBS and blocked for 1 hour with 10% normal goat serum (NGS) in PBS at room temperature. The plates were washed x3 with PBS and primary antibody 529 applied at 1:5000 (in 0.1% NGS in PBS) and left at 4°C overnight. The plates were washed x3 with PBS and the FITC labelled goat anti-rabbit secondary antibody was applied (1:100 in 0.1% NGS in PBS) for 1 hour at room temperature in the dark. Plates were finally rinsed x3 with PBS and read on a Denley Wellfluor microplate fluorescence reader at an excitation wavelength of 485nm and an emission wavelength of 530nm.

2.9.4 UV treatment of RHT cells prior to immunostaining for flow cytometry

The RHT cells were seeded in 35x10mm petri dishes at 2×10^6 cells per dish and left overnight until they were 60-80% confluent by eye. Cell synchronisation was achieved after culturing in RM- (DMEM:Hams F12; 3:1)(section 2.1.3.2) for 48 hours (serum deprivation see section 8.3). Complete medium was reintroduced to cells to induce growth. They were irradiated (0.5 and 1 J/cm² UVA) after rinsing with HBSS at 0, 1, 2, 3, and 24 hours post reintroduction of RM+ (DMEM:Hams F12, 10% HiFCS and 1% mitogens)(section 2.1.3.2). The cells were then incubated with 2ml of 0.02% (w/v) EDTA in PBS at 37°C for 30 min and followed by addition of 200µl of x10 concentrated Tris-EDTA buffer for 10 min at 37°C. A threefold volume of 2% foetal calf serum in PBS was subsequently added, prior to aspiration of the cells through a 25µm gauge needle to obtain a single cell suspension. The cells were pelleted at 400g for 10 minutes at 4°C, before cell counting by microscopy and fixation by the vigorous addition of (1:1) methanol:acetone and incubation at 4°C for at least ten minutes.

2.9.5 Immunostaining prior to flow cytometry

For immunofluorescence staining, the fixed cells were harvested by centrifugation before resuspension in 250µl of incubation buffer A (0.01M PBS, 20mM EDTA, 0.5% Tween 20, 10% normal goat serum) to give a final cell concentration of 1×10^6 cells/ml. The primary antibodies; IgG fraction of anti-UV DNA antiserum 529 and monoclonal mouse anti-human p53 protein were added at a final concentration of 1 in 100 in 0.1% NGS in PBS to 10^6 cells (total volume per 500µl) and incubated overnight at 4°C. The cells were then washed twice

with 5ml 20mM EDTA in 0.01M PBS (PBS/EDTA), before resuspension in 250µl incubation buffer A. Either goat anti-rabbit IgG-FITC labelled (whole molecule), for antibody 529, or goat anti-mouse IgG-FITC labelled, Fc-specific, for anti-p53, was used as a secondary antibody at 1 in 80 or 1 in 160 dilution in 0.1% NGS in PBS respectively. Following incubation for one hour on ice, the cells were washed twice with PBS/EDTA and finally resuspended in 800µl sterile PBS. To this was added 100µl of propidium iodide solution (100µg/ml in sterile water) and 100µl of RNase solution (1mg/ml in sterile water) followed by incubation for 15 min at 37°C prior to analysis. The samples were kept on ice during flow cytometric analysis.

2.9.6 Flow Cytometric Analysis

The purpose of these experiments was to relate the amount of antibody 529 binding to the levels of p53 in UVA irradiated keratinocytes and also to determine if the stage of the cell cycle was important. Quantitation of DNA damage and the amount of p53 protein present, as determined by the amount of antibody binding, was performed using the Becton Dickinson FACScan flow cytometer. Cells with increased intensity of green fluorescence (FITC), compared to controls, due to greater antibody binding, were judged to be UVA-affected. The nuclear counterstain, propidium iodide, enabled cell cycle analyses to be performed in tandem.

2.10 Exposure of DNA to white light/methylene blue

Exposure of DNA to white light/methylene blue has been reported in the literature to cause predominantly 8-oxodG formation (Floyd *et al*, 1989). The treated DNA was to be used as a substrate in the development of a nuclei assay to measure the repair of 8-oxodG. Calf thymus DNA (0.5mg/ml in water, final concentration) was incubated in the presence of methylene blue (20µg/ml final concentration in 0.1M Tris, pH 8.5) in a petri dish, on ice, shielded from a white light source by 0.5cm of water in an up-turned petri dish lid (light to DNA approx. 3cm). Irradiation was for 3 hours at which time solid sodium chloride was added to a concentration of 1M and DNA precipitated with ethanol on ice. The DNA was removed by pipette and dissolved in ultra-pure water. This procedure was repeated twice further to remove traces of methylene blue. The white light/methylene blue treated DNA was then stored at -80°C prior to use.

2.11 Extraction of nuclei from CCRF-HSB-2 cells

Cells were harvested from CCRF-HSB-2 suspension culture, rinsed with PBS at pH 7.4, and pelleted at 160g for 3 min at room temperature. The pellets were resuspended in 10mM Tris-HCl, 1mM CaCl₂, pH 7.0 (Tris/CaCl₂) at a concentration of 2×10^7 cells/ml, then incubated on ice for 10 min. During this time the cells became swollen as was observed by light microscopy. Cells were lysed mechanically using 21 strokes of a Dounce homogeniser (pestle B). The lysate was layered over 3ml of 1.7M sucrose in Tris/CaCl₂ and centrifuged at 4°C for 60 min. at 650g. Viability of nuclei and purity of preparation was examined utilising the nuclear stain propidium iodide at the outset of each experiment (see section

5.6.4). The pellet was resuspended in 100µl reaction mixture A (50mM phosphate buffer, pH 7.4, 100mM KCl plus 50µg of DNA treated with white light/methylene blue) at concentration equivalent to 1×10^7 nuclei/ml. The mixture was then incubated at 37°C for 20 min. The reaction was stopped by the addition of 250µl (-20°C) ice cold ethanol, and the resultant mixture kept at -20°C for 30 min. The samples were then centrifuged at 1500g for 15 min at 4°C in an eppendorf centrifuge. The precipitated DNA and the supernatant fraction were separated and dried under vacuum at room temperature. The supernatants were reconstituted in ultra pure water prior to injection onto the HPLC to examine levels of 8-oxoguanine that were released into the medium from the nuclei.

2.12 The single cell microgel electrophoresis assay (comet assay)

RHT cells were treated with UVA at 0, 0.5, or 1 J/cm² and harvested immediately (see section 2.9.4) or 5 hours post treatment. A layer of normal melting point molten agarose (150µl; 0.5% w/v in 0.01M PBS, 37°C) was spread on a frosted slide using a coverslip, and placed at 4°C for 10 min to cool. Low melting point agarose (100µl/10⁵ cells; 0.7% w/v in 0.01M PBS; LMP, 37°C) was added to the RHT cells and this mixture was then spread evenly over the slide using the coverslip and again allowed to cool. A final layer of low melting point agarose was applied, the coverslip replaced, and the agarose allowed to set for 5 min at 4°C. The coverslips were removed and the slides were then left in lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 1% w/v N-lauryl sarcosinate, pH to 10.0 with NaOH, 1% Triton-X-100 and 10% dimethyl sulfoxide) in the dark at 4°C for 1 hour. The slides were subsequently placed into an electrophoresis tank, which was then filled with electrophoresis buffer (0.3M NaOH, 1mM EDTA), and left for 20 min to allow unwinding

of DNA. Electrophoresis was performed at 25 volts for 20 min. Once the slides were removed from the tank, (~3ml) neutralisation buffer (0.3M Tris-HCl, pH 7.5) was added dropwise and left for 5 min. Any excess buffer was removed and this procedure was repeated twice. Finally, 50µl ethidium bromide (0.002% w/v in H₂O) was added to each slide followed by a fresh coverslip. The slides were stored for up to 72 hours at 4°C in a dark moist chamber prior to examination under a fluorescence microscope using the green filter. The comets fluoresced red and their characteristics were measured using the Colourmorph comet software (section 2.1.7); fifty cells were counted per slide. The statistical analysis of these samples was carried out using a combination of the Microsoft Excel and Statgraphics software.

2.13 Antibody purification on protein A columns (low salt)

The pH of the serum containing the antibody was adjusted to 8.0 by addition of 1/10 volume of 1.0M Tris (pH 8.0). The antibody solution was then passed through a protein A bead column. The beads were washed with 10 column volumes of 100mM Tris (pH 8.0), followed by 10 column washes of 10mM Tris (pH 8.0), (both solutions were refrigerated at 4°C prior to use). The column was eluted finally with 100mM glycine (pH 3.0). This buffer was added stepwise, approximately 500µl per sample. The eluate was collected in 1.5ml conical tubes which contained 50µl of 1M Tris (pH 8.0). Each tube was mixed gently to bring the pH back to neutral. The IgG fraction was identified by SDS PAGE (0.001% w/v). This reducing gel containing mercaptoethanol, cleaves disulphide bonds. The gel was stained with 1% Coomassie Brilliant Blue and the IgG was seen as two bands, one at 50 kD and one at 25 kD. The molecular weight of IgG is ~150 kD therefore the two light chains

were contained at the 25 kD band and the two heavy chains were contained at the 50 kD band. The IgG fraction of the antibody was collected and stored at -80°C. The amount of protein per ml of IgG fraction was 0.8mg/ml as measured on a spectrophotometer at A_{280} , using an absorbance of 1.0=1.0mg/ml.

2.14 Enzyme linked immunosorbent assay (ELISA)

DNA extracted from treated and untreated (control) human PBMC using pronase E (section 2.4.1) and phenol (section 2.4.2) isolation, was bound to ELISA plates at 50µg /ml in PBS (50µl/well). All incubations were at 37°C for 1 hour in a humidified chamber. Following three washes in PBS, a 4% (w/v in PBS) dried skimmed milk powder solution (200µl/well; milk/PBS) was used to block free sites. Serum from rabbit 529 was diluted, typically 1 in 5000, in milk/PBS and 50µl added to plates washed with PBS. After incubation and subsequent washing of wells three times with PBS containing Tween 20 (0.05% v/v PBS/Tween), peroxidase-conjugated goat-anti-rabbit immunoglobulins (IgA, IgG and IgM classes) was used at a dilution of 1 in 2000 (in milk/PBS; 50µl/well) to detect antibodies bound to DNA. Following further washing with PBS/Tween detection of bound peroxidase-labelled antibody was performed using 50µl/well of o-phenylenediamine (0.5mg/ml in 0.05M phosphate-citrate, pH 5.0, and containing 0.03% w/v sodium perborate as a substitute for H₂O₂) as substrate; the reaction was stopped after 15 min at room temperature using 2M H₂SO₄ (25µl/well) and the product determined spectrophotometrically at 492nm.

Chapter 3

Validation of 8-oxodG assay

3.1 Synthesis of 8-oxodeoxyguanosine

8-Oxodeoxyguanosine (8-oxodG), an oxidative DNA adduct has gained much popularity as a biomarker of damage to DNA. HPLC combined with electrochemical detection provides a selective and sensitive method of measuring 8-oxodG. This assay was developed to measure oxidative damage to DNA as outlined in section 2.7. The establishment of the 8-oxodG assay depended on synthesis of the 8-oxodG standard. 8-OxodG was synthesised according to a slightly modified version of the Udenfriend system (see section 2.6)(Kasai *et al.*, 1984). Kasai *et al.*, (1984) used aqueous acetone to elute compounds from the charcoal column. The aqueous acetone was then simply evaporated and the residue collected and injected onto the HPLC system. In this study, at first an ethyl acetate extraction was carried out in a separating funnel (ethyl acetate:aqueous acetone (5:1)). Ethyl acetate (2.5ml) and 0.5ml of the aqueous acetone eluent were placed in an inert plastic tube, vortexed and centrifuged at 400g for 5min. It was found that there was a loss of 8-oxodG and 8-oxoguanine (8-oxoG) in polypropylene tubes with a rounded bottom, possibly due to it being adsorbed onto the surface. Polypropylene tubes with a conical shaped bottom (Sarsdedt, 12ml) did not display the same characteristics and were used in all subsequent experiments. The top layer was aspirated and evaporated to dryness with N₂ gas. This residue was reconstituted in 0.1ml of ultra-pure water containing 15% methanol (v/v). This was injected onto the HPLC column (2x50µl injection) and analysed using UV and EC detection (HPLC mobile phase: 50mM sodium acetate, 1mM EDTA, 5% methanol, pH 5.1). An injection of 1 in 10 dilution of aqueous acetone solution in 15% aqueous methanol was also made. When the peak heights were compared the percentage recovery of 8-oxodG in the ethyl acetate separation was 7% (Figures 3.1 and 3.2).

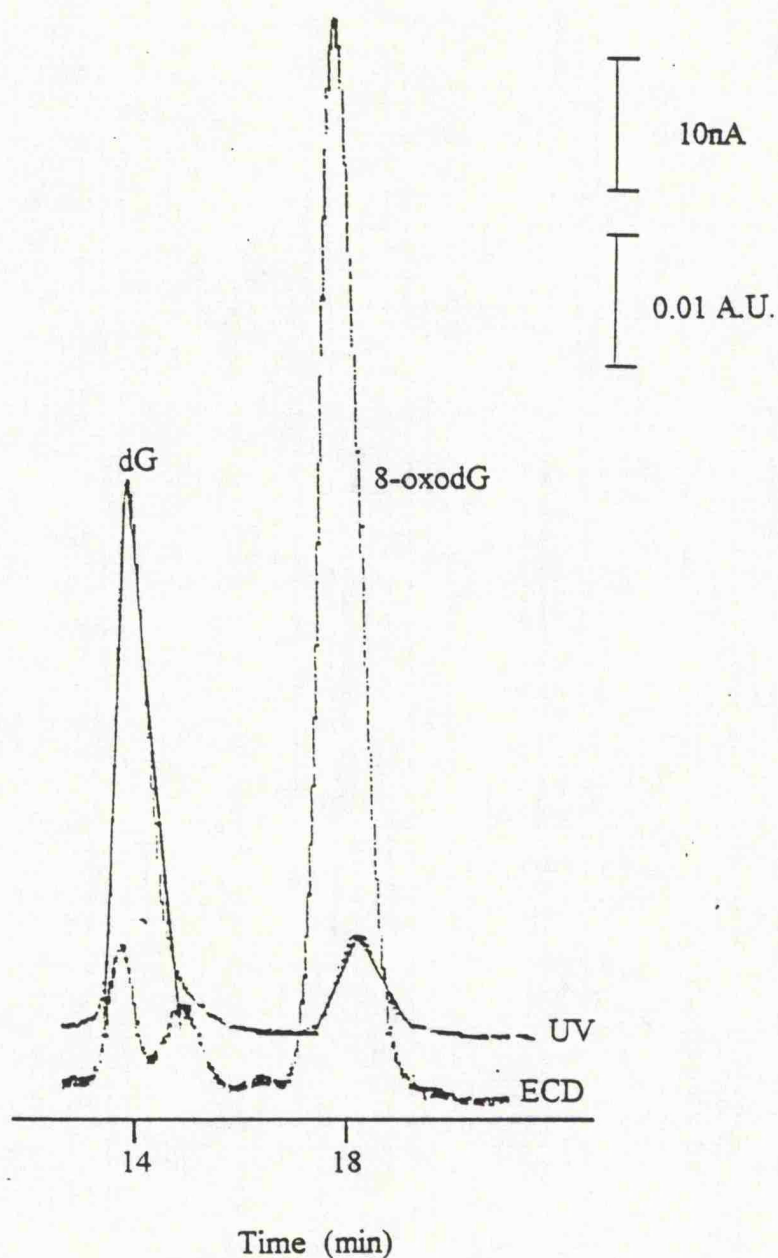


Figure 3.1: Representative HPLC of ethyl acetate extract from aqueous acetone containing 8-oxodG. Electrochemical detection at an applied potential of +0.6V. UV detection in series was at 254 nm. The flow rate was 0.8ml per minute, the mobile phase was as described in section 2.7.

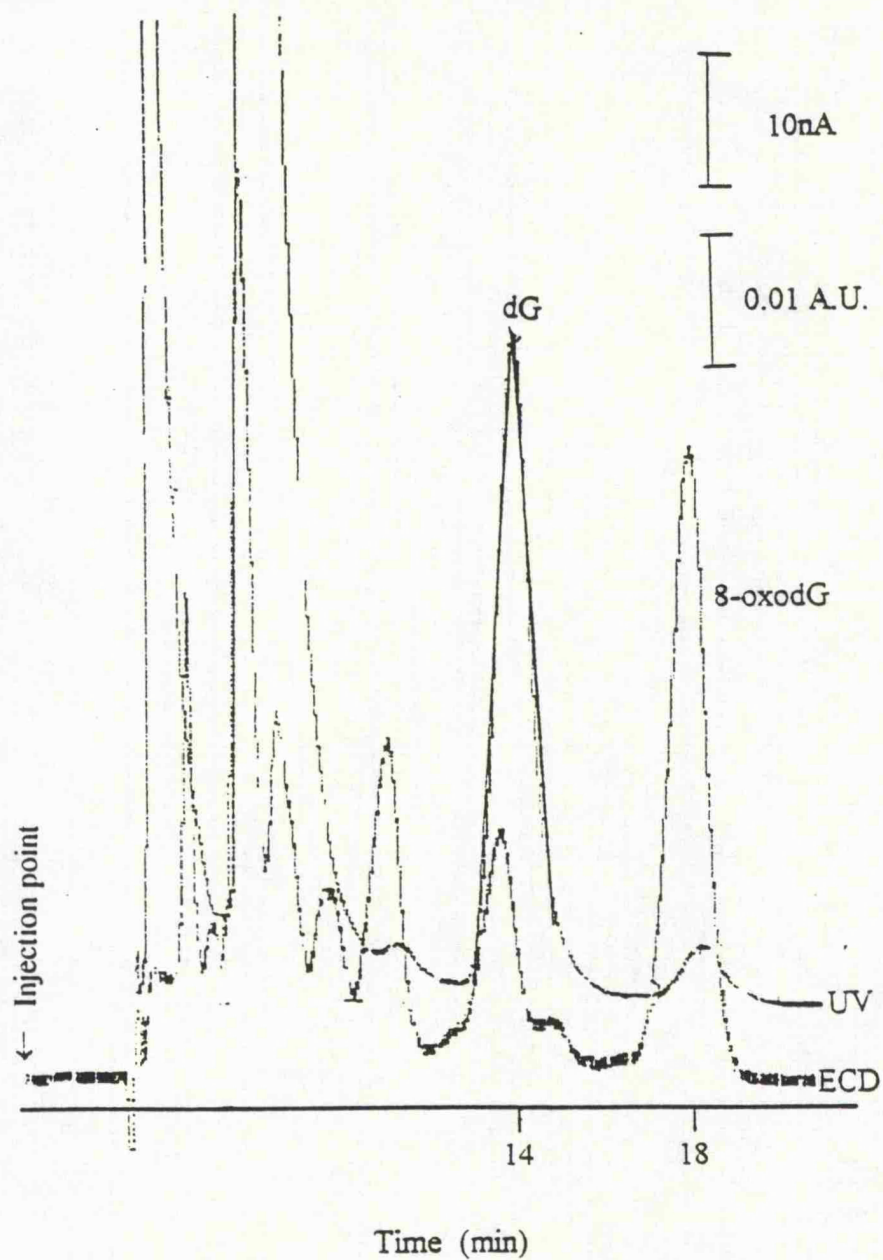


Figure 3.2: Representative HPLC of aqueous acetone containing 8-oxodG. Electrochemical detection at an applied potential of +0.6V. UV detection in series was at 254nm. The flow rate was 0.8ml per minute, the mobile phase was as described in section 2.7.

Two further ethyl acetate extractions of the same aqueous acetone aliquot were carried out to increase the percentage recovery to 12%. It was decided that the aqueous acetone should be evaporated down using a rotary evaporator and a water bath at 70°C and the residue reconstituted in a minimum volume of ultra-pure water; the final volume was 8.0 ml. This was then injected onto the HPLC according to the method outlined above.

3.1.1 HPLC separation of deoxyguanosine and 8-oxodeoxyguanosine

It has been previously reported that the oxidative damage that white light/methylene blue inflicts on DNA is specific to deoxyguanosine (Fraga *et al.*, 1990) and that 8-oxodG is the major product formed (Floyd *et al.*, 1986b). This information was utilised so that the separation between deoxyguanosine (dG) and 8-oxodeoxyguanosine could be elucidated prior to fractionation of synthetic mixtures of the two compounds, on the HPLC, to ensure that the correct peak was collected. Deoxyguanosine (2.5mM in sodium phosphate buffer, pH 6.8) was incubated with methylene blue (200µM). One sample was placed in the fluorescence spectrophotometer (Perkin Elmer, 204A) and irradiated with Xe source white light (Perkin Elmer, 150W Xe power supply). The control sample containing dG and methylene blue was left in the dark. At each sample time 5µl of the mixture was injected directly onto the reversed phase HPLC column. [Mobile phase; 50mM sodium acetate, 1mM EDTA, 5% methanol, pH 5.1: UV 254nm and 0.1 A.U's, ECD 1.0V and 50nA.] 8-oxodG is an electrochemically active compound. To investigate the retention time of 8-oxodG we exploited this property and used 1.0V to get the maximum response from the compound. See chromatogram Figure 3.3. This showed that the retention time of 8-oxodG was 18min compared to 14min for dG.

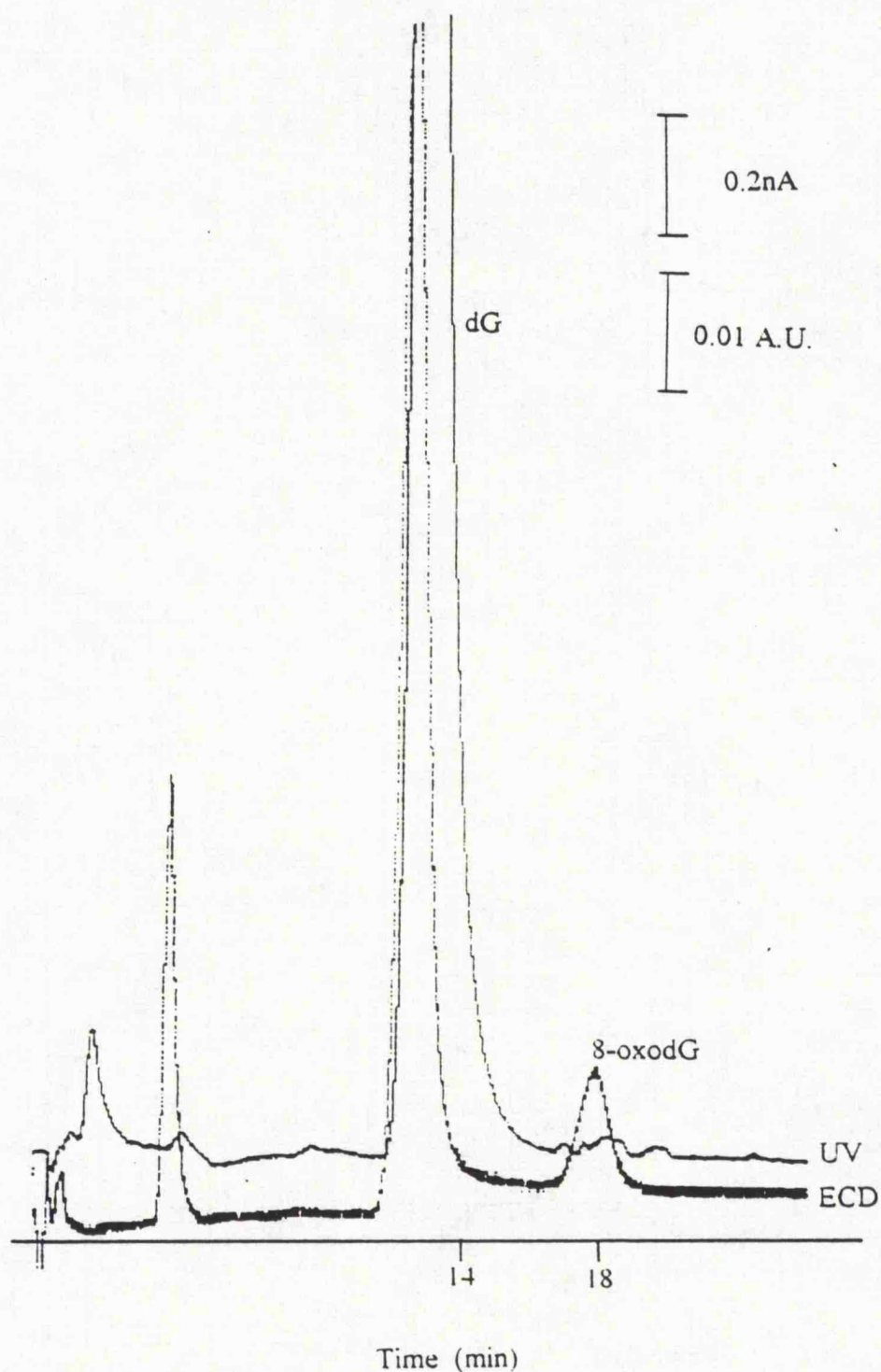


Figure 3.3: Representative HPLC of the result of incubating deoxyguanosine with methylene blue/white light for 2 hours on ice. Electrochemical detection at an applied potential of +1.0V. UV detection in series was at 254nm. The flow rate was 0.8ml per minute, the mobile phase was as described in section 2.7

The limit of detection of 8-oxodG for the assay was 250 femtomoles on column. The response was found to be linear over a range of concentrations from 25nM to 500nM. The coefficient of variance for 10 manual injections was found to be 5.2%. A voltammogram of 8-oxodG produced the profile seen in Figure 2.1 section 2.7.

A sample of the 8-oxodG standard was sent to us courtesy of Dr. Steve Faux from the University of Birmingham. The retention time of this authentic 8-oxodG standard was used to confirm that the peak which was being collected was the correct one.

3.2 Confirmation of 8-oxodG structure

UV spectral analysis was carried out using a Shimadzu UV-160A spectrophotometer at a scan speed of 120nm/min from 190 to 400nm, see Figure 3.4. This compared to the published UV spectrum and ϵ_{max} for 8-oxodeoxyguanosine and was found to be identical (Culp *et al.*, 1989). Therefore using $\epsilon_{290} = 9.7\text{mM}^{-1}$ (Culp *et al.*, 1989), the concentration of 8-oxodeoxyguanosine was calculated to be 57 μM . On injection of the synthesised standard onto the HPLC-UV-ECD system no other peaks were observed apart from the 8-oxodG peak.

An eppendorf containing approximately 32 μg was sent to Dr. Peter Farmer in the MRC Toxicology Unit, Carshalton, Surrey, for confirmation of 8-oxodG by fast atom bombardment mass spectrometry. See Figure 3.5.

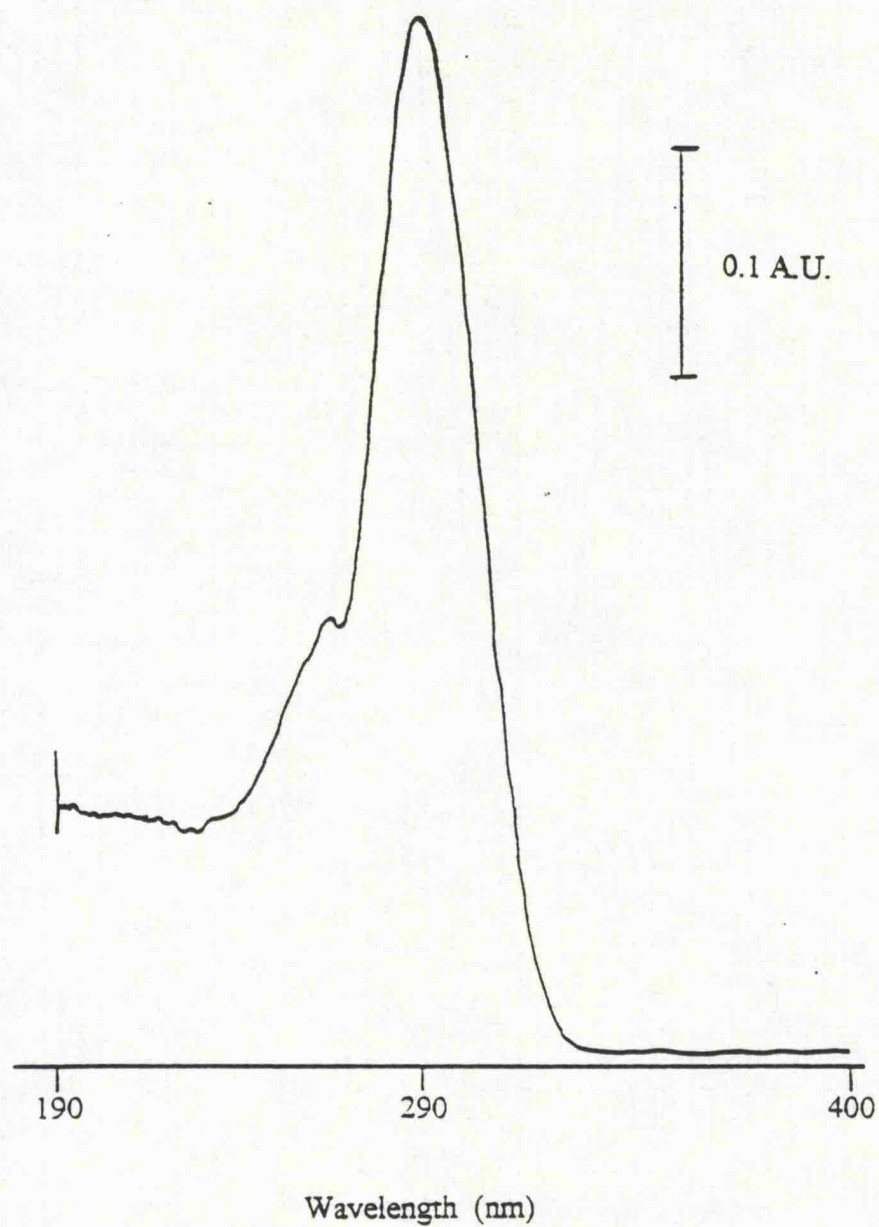


Figure 3.4: Representative UV spectrophotometric scan of 8-oxodG showing ϵ_{max} absorbance at 290nm.

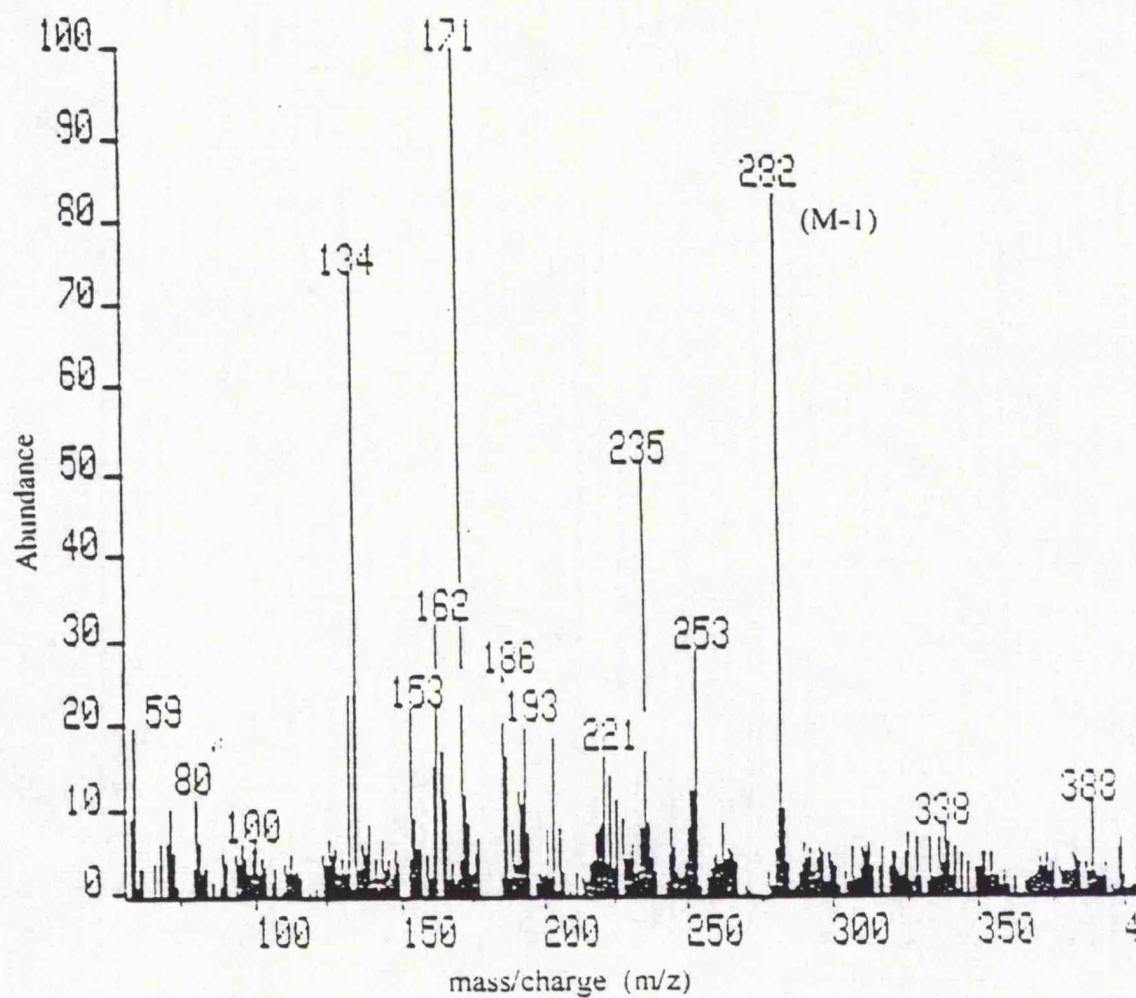


Figure 3.5: Representative mass spectrum of 8-oxodG, showing the M-H ion at 282 mass/charge ratio and a contaminant ion at 171.

3.2.1 Interpretation of mass spectra

Mass spectrometry is concerned with the electron ionisation and subsequent fragmentation of molecules, also with the determination of the mass to charge ratios (m/z) and the relative abundance of the ions which are produced. Functional groups in the molecule direct the fragmentation pattern in such a way that, knowing the fragmentation pattern, a plausible structure of the original molecule can be suggested. In addition, the technique allows the molecular weight to be determined and this is probably the most important piece of information available from a mass spectrum (Ardrey, 1985).

In the synthesis of 8-oxodG the conditions were kept at a pH of approximately 7.0 in keeping with the physiological pH at which 8-oxodG is formed *in vivo*. Therefore, the tautomeric structure of the 8-oxodG form was assumed to be the 6,8 diketo form (Culp *et al.*, 1989). This therefore helped to explain the fragmentation pattern of the 8-oxodG in the mass spectrum obtained. The molecular ion was found to be at 282 which was 8-oxodG -H ion but another strong ion was observed at 171. The identity of the ion at 171 was unknown. However, the 171 ion did not interfere with the chromatography of the 8-oxodG.

3.3 Development of mobile phase

When developing the method for separation of deoxyguanosine (dG) and 8-oxodeoxyguanosine (8-oxodG) from other nucleic acid-derived nucleosides, methylene blue

incubated with dG and exposed to white light was the simplest system to use as outlined in section 3.1.1.

The objective of the assay was to have as short a run time as possible with a baseline separation of the compounds of interest. Different mobile phases were made up (i) 12.5mM citric acid, 25mM sodium acetate, 30mM sodium hydroxide, 10mM acetic acid, pH 5.1 plus 10% methanol (Floyd *et al.*, 1986) (ii) 50mM sodium acetate, 1mM EDTA, pH 5.1 plus 10% methanol. Their ability to separate the compounds of interest was compared (see Figures 3.6a and 3.6b). The most effective mobile phase was found to be the one containing 50mM sodium acetate and 1mM EDTA as it yielded the best peak shape. The percentage of methanol affected the retention time of the compounds eluting from the column. Mobile phases containing 15%, 10%, 8%, and 5% were used to determine the retention time and separation of the deoxynucleoside standards dG, T, dC, dA and 8-oxodG from one another. The 50mM sodium acetate, 1mM EDTA, pH 5.1 mobile phase containing 5% methanol was found to be effective for baseline separation in digested DNA extracted from cells. The pH of the mobile phase found to give the sharpest peak shape was pH 5.1. The pH of the mobile phase (between 5 and 6) did not appear to have any effect on separation (see Figures 3.7a and 3.7b).

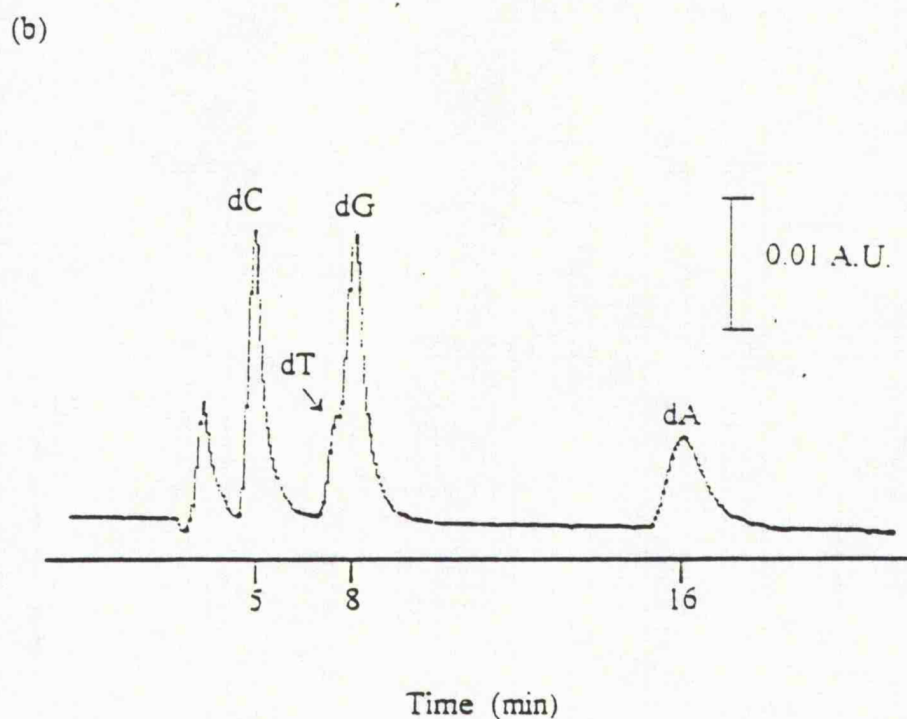
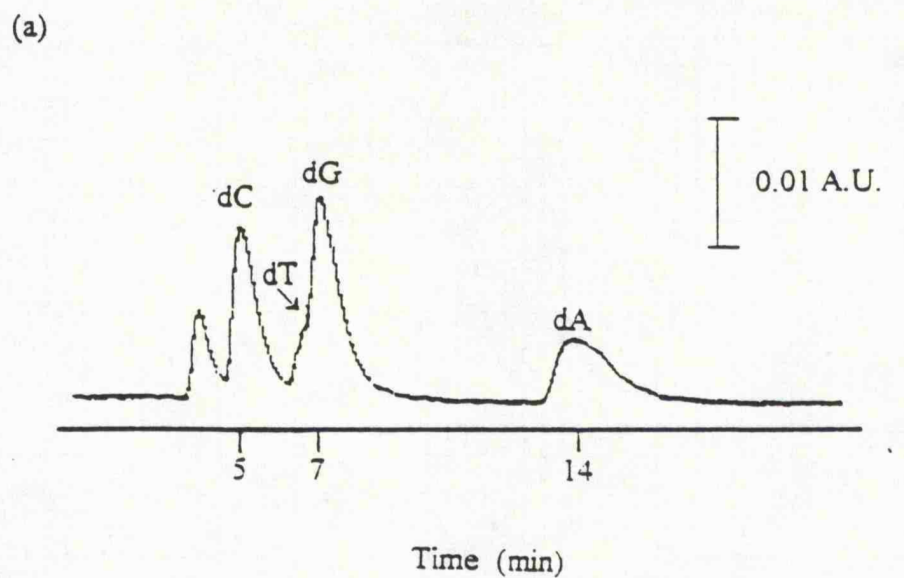


Figure 3.6: Representative HPLC of four deoxynucleoside standards. Figure 3.6a was run using mobile phase (i) in section 3.3. Figure 3.6b was run using mobile phase (ii) in section 3.3. The flow rate was 0.8ml per minute. UV detection was at 254nm.

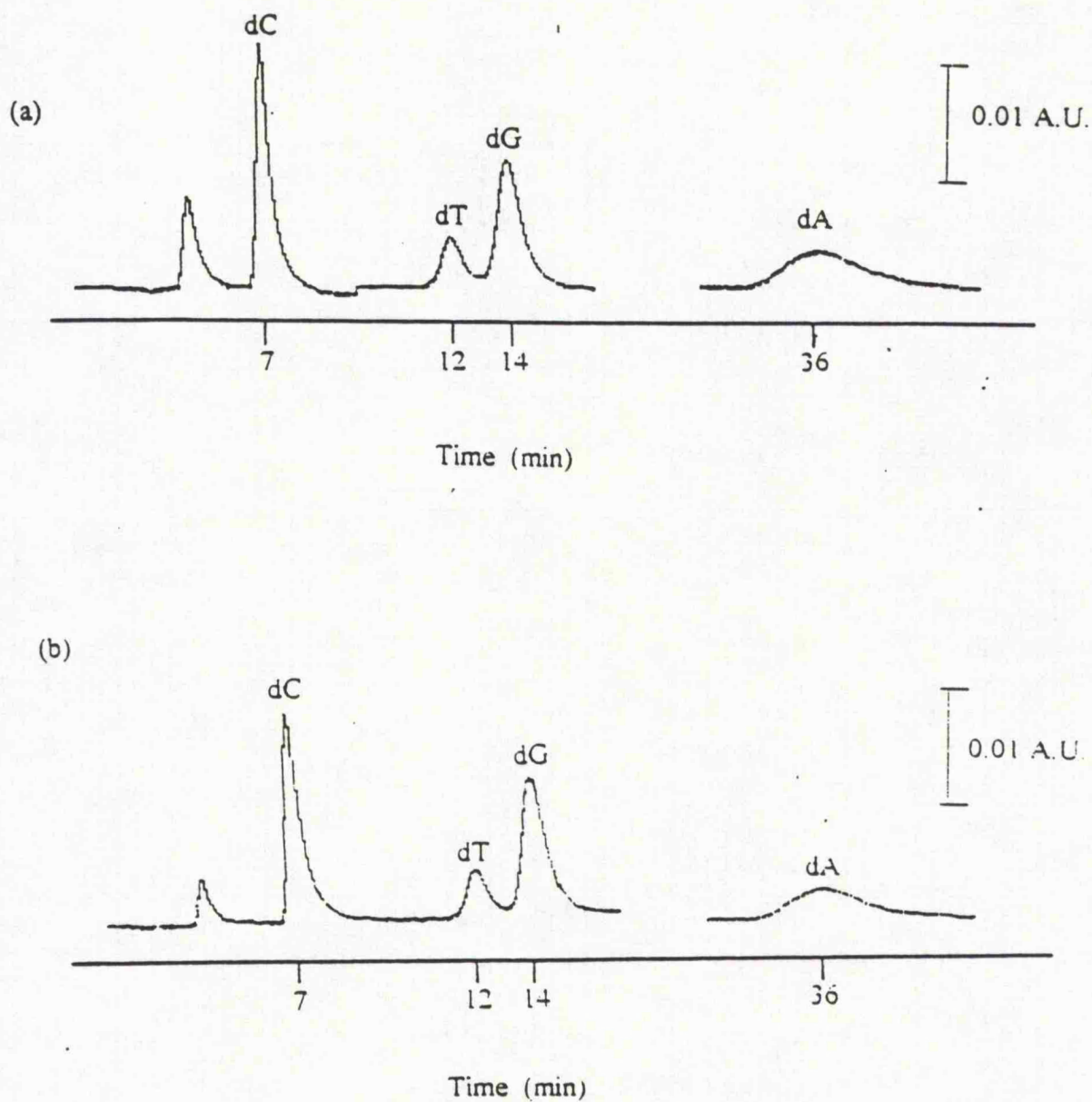


Figure 3.7: Representative HPLC of four deoxynucleoside standards. The mobile phase was 50mM sodium acetate and 1mM EDTA containing 5% methanol at a pH of 6.0 in (a) and pH 5.1 in (b). The flow rate was 0.8ml per minute. UV detection was at 254nm.

3.3.1 Development of mobile phase for cell supernatants

On investigation of 8-oxodG levels in supernatants of PBMC it was found that an unknown compound 'Ax' co-eluted with the 8-oxodG (Figure 3.8). Because of this a mobile phase containing 2% methanol was used in later separations as it provided separation of these peaks.

The k' values for deoxynucleosides are shown in Table 3.1, under the conditions described in section 3.3 and using the formula in appendix II.

Table 3.1: Capacity factors for deoxynucleosides.

Deoxynucleoside	dC	dT	dG	dA
k' value	2.5	5	6	17.5

3.4 Isolation of PBMC from whole blood

The isolation of PBMC was carried out according to section 2.2 (Boyum, 1968). An alternative method described by Kendall *et al.*, (1992) was also carried out for comparison. Whole blood (100ml), from a normal human volunteer, was collected in 25ml aliquots into four 50ml tubes containing 2.5ml sodium citrate (3.4% w/v) mixed and centrifuged at 1300g at room temperature for 30min. The buffy coat was collected from the plasma/packed red cell interface using a pasteur pipette and transferred to four 50ml centrifuge tubes on ice. Approximately 50ml of 155mM ammonium chloride (NH_4Cl) in RPMI 1640 was added to lyse residual red blood cells during incubation for 30min at 37°C.

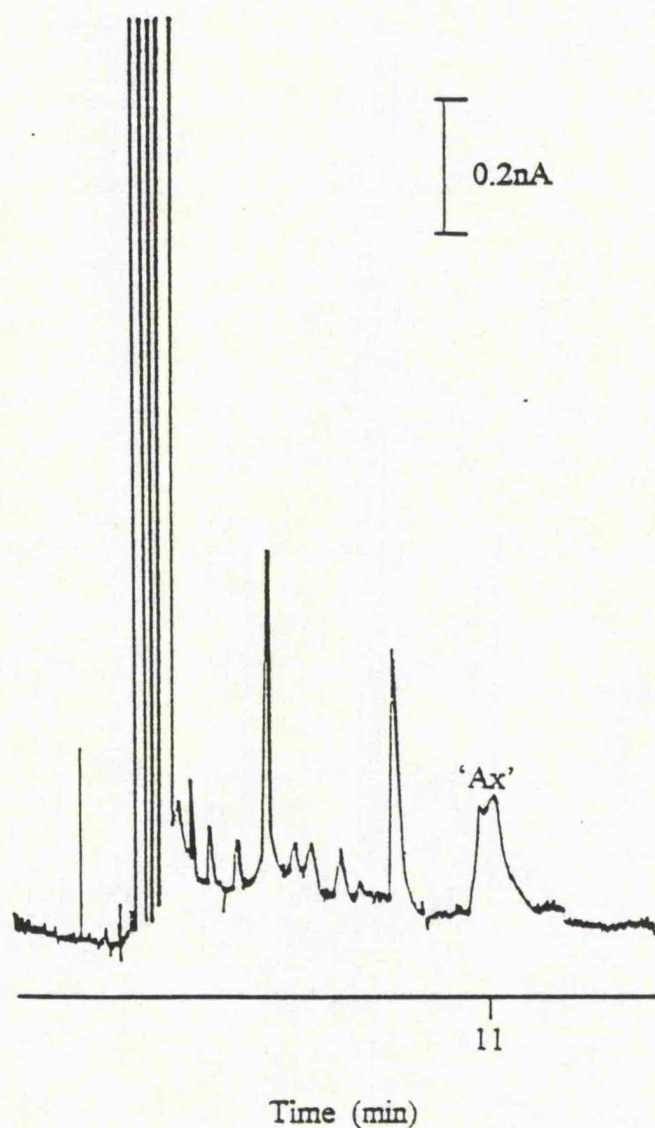


Figure 3.8: Representative HPLC of supernatant from untreated PBMC from an SLE patient. The mobile phase was 50mM sodium acetate and 1mM EDTA containing 5% methanol at a pH of 5.1. The flow rate was 0.8 ml per minute. Electrochemical detection was at 0.6V. The 'Ax' peak co-eluted with the 8-oxodG peak.

The samples were then pelleted at 500g for 5min, many red cells remained, so samples were incubated for a further 10min in RPMI, NH_4Cl , then centrifuged at 500g for 5min. However too many red blood cells remained. All samples were then combined and centrifuged at 1300g for 15min at room temperature. Distilled water was added for 30 seconds followed by x2 PBS and centrifugation at 500g for 5min. Nevertheless, a large number of red blood cells remained. Finally RPMI containing 155mM NH_4Cl was added for a further 5min incubation on ice and then centrifuged at 500g for 5min. However the samples still remained contaminated with red blood cells. Therefore the original method chosen was used routinely as it proved to be more effective, reliable and simple.

3.4.1 Isolation of DNA from cells

Methods utilising phenol are commonly used to isolate DNA from cells, see section 2.4.2. However, it had been reported in the literature that phenol may sensitise the DNA to further oxidative damage (Claycamp, 1992). Therefore, it was decided to try an alternative DNA isolation method, the pronase E method, see section 2.4.1. From initial results using PBMC, see section 4.4, it was realised that this was possibly a less harsh method of isolation of the DNA. This lead to a direct comparison between the two methods. To do this the experimental design had to be rigorous, therefore we chose to treat the cells with different types of insults to see if the mechanism of damage had different effects on the recovery of the DNA, the level of damage and the type of damage. See results section 4.4.

Chapter 4

**Comparative study of pronase E and phenol
extraction of DNA prior to analysis of 8-oxodG**

4.1 Introduction

Oxidative damage is thought to play a role in the aetiology of ageing and a host of diseases including cancer, chronic inflammation, ischemia, degenerative arterial and autoimmune diseases (Richter *et al.*, 1988). Oxygen radicals are formed in cells not only as the result of exogenous chemical treatment or radiation but also by intra-cellular metabolism (Jones *et al.*, 1990). Oxygen radical activity is difficult to measure *in vivo* because of the inherent reactivity of these species. 8-Oxodeoxyguanosine (8-oxodG), an oxidative DNA adduct, has gained much popularity as a biomarker of damage to DNA as it is relatively easy to measure using HPLC and electrochemical detection (Birnboim *et al.*, 1994). Hydrogen peroxide (H₂O₂), ultraviolet light, and ionising radiation induce the formation of diverse types of DNA lesions including 8-oxodG (Dizdaroglu, 1985; Blakely *et al.*, 1990; Allan *et al.*, 1988; Beehler *et al.*, 1992).

High performance liquid chromatography combined with electrochemical detection (HPLC-ECD) provides a selective and sensitive method of measuring 8-oxodG at the deoxynucleoside level (Kasai *et al.*, 1986, Shigenaga *et al.*, 1994). Gas chromatography combined with mass spectrometry (GC-MS) provides a sensitive method for measuring 8-oxodG at the base level (Dizdaroglu, 1986 and 1994). Both the deoxynucleoside and base derivatives are measured in DNA extracted from cells. The measurement of trace substances in any experimental system includes the risk of measuring contaminants and/or artificially induced concentrations of the monitored compound; this may be even more of a problem for markers of oxidative damage. Claycamp's (1992), study on the phenol sensitisation of DNA to subsequent oxidative damage in 8-oxodG assays, demonstrated the profound problems that are faced in measuring the basal level of the 8-oxodG lesion in

DNA that is extracted using organic solvents. In the following study we compared pronase E isolation of DNA (Kendall *et al.*, 1991), with phenol isolation (Winyard *et al.*, 1990). In order to determine which method was the most efficient and then use it with confidence routinely, we measured the ability of different models of oxidative damage to induce the 8-oxodG adduct in DNA using UV-irradiation, γ -irradiation and hydrogen peroxide treatment of cells.

4.2 Aim

To examine and compare the efficacy of the pronase E isolation of DNA with the phenol isolation method with respect to (a) amount of DNA (b) levels of 8-oxodG detected.

4.3 Method

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood according to the method outlined in section 2.2, and treated using three different exposure protocols to induce oxidative stress. Firstly, the mononuclear cells were treated on ice, to reduce DNA repair, with H_2O_2 (400 μ M) for 15min. Secondly, mononuclear cells were exposed on ice at 6cm distance from a combined UVA and UVC source, wavelengths 366 and 254nm, (Andermann and Co. Ltd.) for 30min. (8.49J/cm², 5.25J/cm² respectively). Thirdly, mononuclear cells were exposed on ice to a Vickrad ⁶⁰Co source, and treated with gamma irradiation at a rate of 0.48 Gy/sec (0Gy, 20Gy, 200Gy). Mononuclear cell DNA was extracted using the pronase E method of Kendall *et al.*, (1991) outlined in section 2.4.1, or the phenol method based on a procedure described by Winyard *et al.*, (1990) outlined in section 2.4.2. Quantitation of DNA was by absorbance at 260nm and purity

checked using A_{260}/A_{280} ratios. Calf thymus DNA (1mg/ml) and isolated DNA were enzymatically digested to the deoxynucleoside level (Faux *et al.*, 1992); see section 2.4.3. Hydrolysates were analysed immediately or stored at -20°C prior to analysis. The method for reversed-phase HPLC of the DNA deoxynucleosides was modified from the procedure of Floyd *et al.*, (1986b) as outlined in section 2.7. Statistical analyses were performed using the Mann Whitney U test using the Statgraphics software package.

4.4 Results

Using PBMC from normal donors a minimum of three separate incubations followed by pronase E and phenol DNA isolation were carried out for 8-oxodG analysis. Although the mean levels of DNA extracted using phenol were consistently higher, these were not statistically different from levels for pronase E extraction (using Mann Whitney U test)(see Table 4.1). The results show that lower levels of 8-oxodG were observed in both PBMC (Figure 4.1) and naked calf thymus DNA (Figure 4.2) when DNA was isolated using the pronase E method compared to the phenol method. This was true whether comparing control samples or samples treated with H_2O_2 (Figure 4.1 and 4.2). However, it is important to note that the minimum number of cells required for DNA isolation using the pronase E method was 3×10^6 cells whereas phenol can extract DNA from 2×10^6 cells. In the experiments where the cells were treated with hydrogen peroxide in the presence of foetal calf serum (FCS) a response to treatment with H_2O_2 was observed ($p < 0.05$) in that the 8-oxodG levels were increased from 8.5 (SD=5.5) compared to 23.6 (SD=21.2) moles 8-oxodG/ 10^5 moles dG but only for phenol extraction of DNA (Figure 4.1).

Table 4.1: Comparison of the levels of dG isolated using either pronase E or phenol as an indicator of the amount of DNA extracted. Volume of each isolate was 50µl. Values represent the mean, values in brackets represent the standard deviation (SD).

Treatment of PBMC	Concentration of dG isolated (µM)	
	Pronase E	Phenol
⁶⁰ Co 0Gy	84.7 (40.3)	90.9 (29.3)
20Gy	59.9 (28.2)	77.7 (84.9)
200Gy	89.7 (44.5)	80 (50.8)
H ₂ O ₂ Control (+FCS)	41.8 (44.6)	76.5 (30.3)
Control (RPMI)	70.8 (53.9)	81.8 (40.5)
400µM (+FCS)	44.1 (39.5)	61.9 (29.7)
400µM (RPMI)	77 (54.3)	79.2 (37.9)
UV Control	258.3 (171.4)	186.9 (90.8)
+UV	113.2 (72.4)	154.4 (95.5)

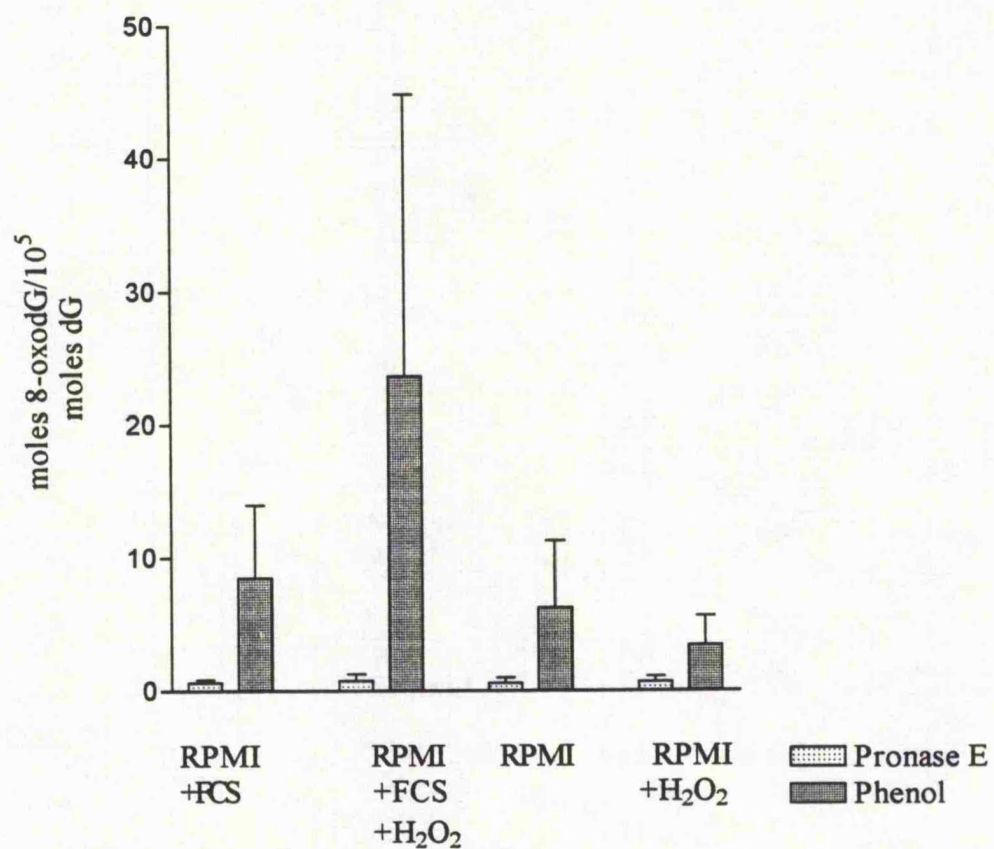


Figure 4.1: H₂O₂ (400μM) treatment of healthy volunteer PBMC for 15min on ice with or without foetal calf serum (FCS). Direct comparison between pronase E and phenol isolation. Each value represents the mean +SD for three separate experiments.

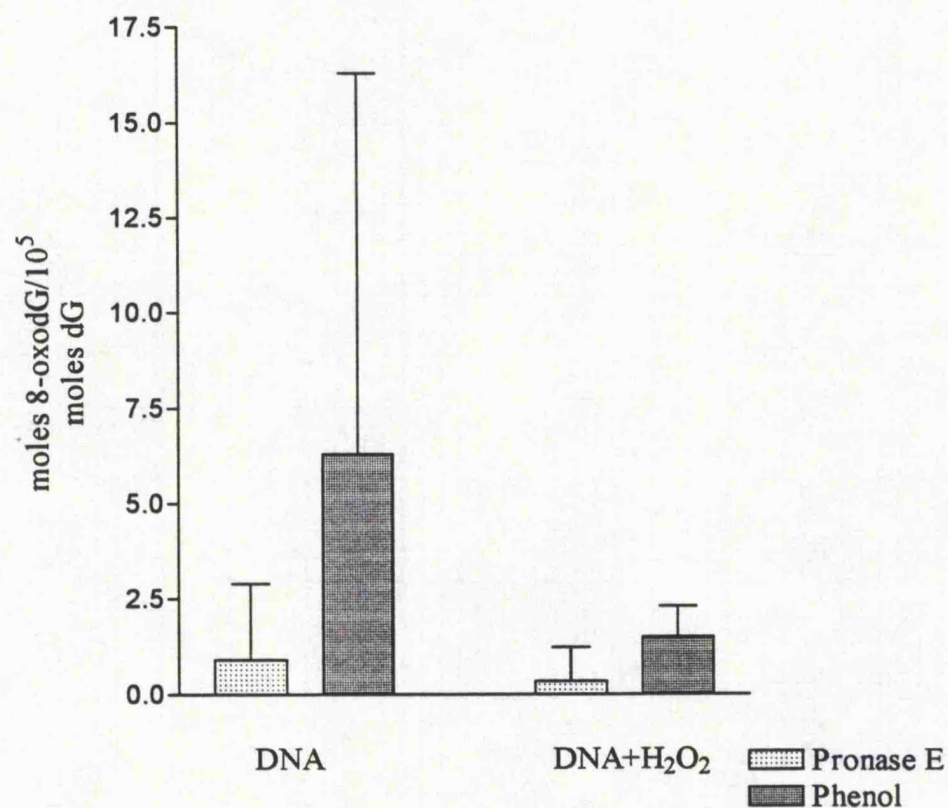


Figure 4.2: H₂O₂ (400μM) treatment of calf thymus DNA for 15min on ice, direct comparison between pronase E and phenol isolation. Each value represents the mean +SD for three separate experiments.

Similarly for γ -irradiated cells the levels of 8-oxodG observed using pronase E extraction were lower than the levels observed using the phenol method ($p < 0.05$) (Figures 4.3 and 4.4). A dose response was suggested using phenol isolation from γ -irradiated PBMC 0-200Gy (Figure 4.3); the level of 8-oxodG increased from the control value of 2.0 (SD=2.4) to 10.0 (SD=8.8) at 20Gy and to 16.5 (SD=2.1) moles 8-oxodG/ 10^5 moles dG at 200Gy (Figure 4.3). For the pronase extraction method the amount of 8-oxodG increased from 0.2 (SD=0.5) at 0Gy to 0.8 (SD=1.9) at 20Gy; but a decrease was observed at 200Gy, 0.1 (SD=0.3) moles 8-oxodG/ 10^5 moles dG.

Higher levels of 8-oxodG were observed on extraction of naked calf thymus DNA using the phenol method ($p < 0.05$) but no increase in 8-oxodG proportional to irradiation dose was seen (Figure 4.4). The data was more variable for phenol treatment, for example at 0Gy the mean level of 8-oxodG/ 10^5 dG was 3.2 (SD=8.2); at 20Gy and 200Gy the corresponding values were 3.1 (SD=7.2) and 1.4 (SD=0.6) respectively. For pronase isolation the levels of 8-oxodG did not vary with dose of γ -irradiation.

There was no significant difference in the levels of 8-oxodG detected using pronase E or phenol extraction of DNA from PBMC whether treated or not with ultraviolet light (Figure 4.5). Similarly for 'extraction' of UV treated or untreated naked calf thymus DNA no significant difference was observed between pronase E and phenol isolation of DNA (Figure 4.6). The mean value for phenol-treated, UV-irradiated DNA 5.6 (SD=6.9) moles 8-oxodG/ 10^5 moles dG was higher than that of pronase E-extracted DNA 2.3 (SD=4.0) although this did not reach statistical significance (Figure 4.6).

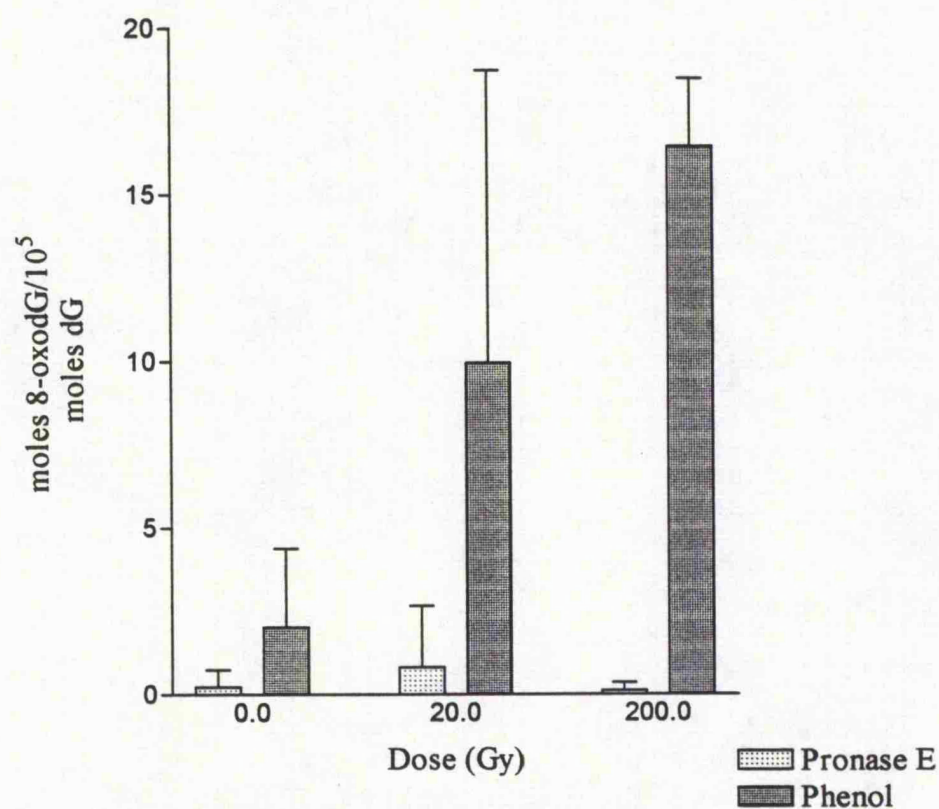


Figure 4.3: γ -irradiation of healthy volunteer PBMC, direct comparison between pronase E and phenol isolation. Each value represents the mean +SD for three separate experiments.

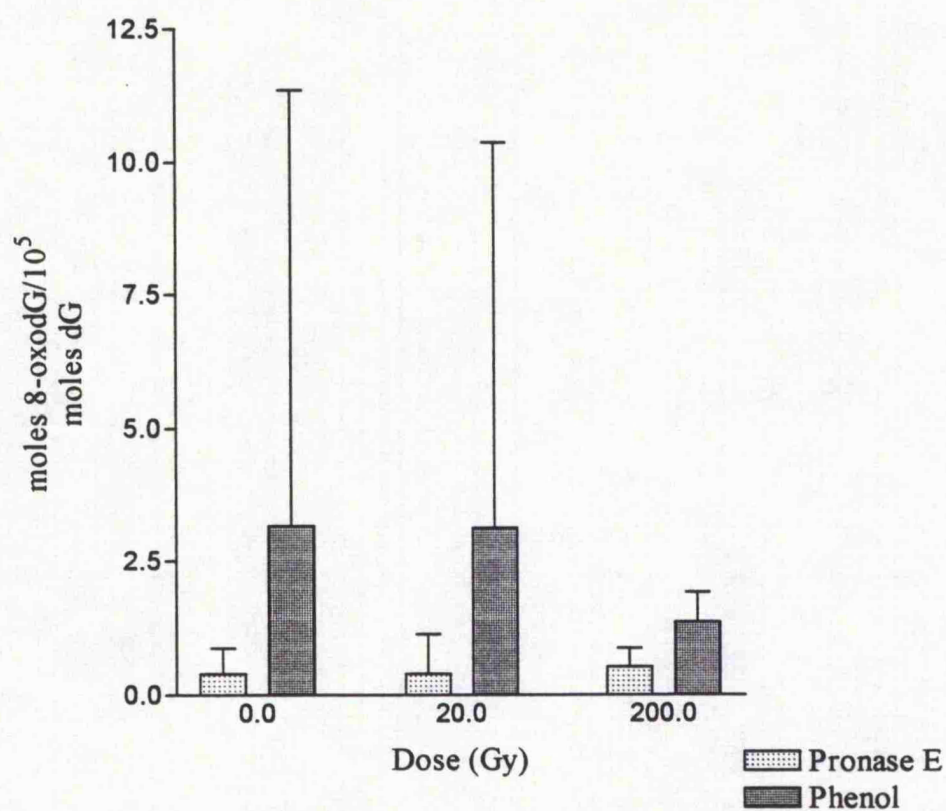


Figure 4.4: γ -irradiation of calf thymus DNA, direct comparison between pronase E and phenol isolation. Each value represents the mean +SD for three separate experiments.

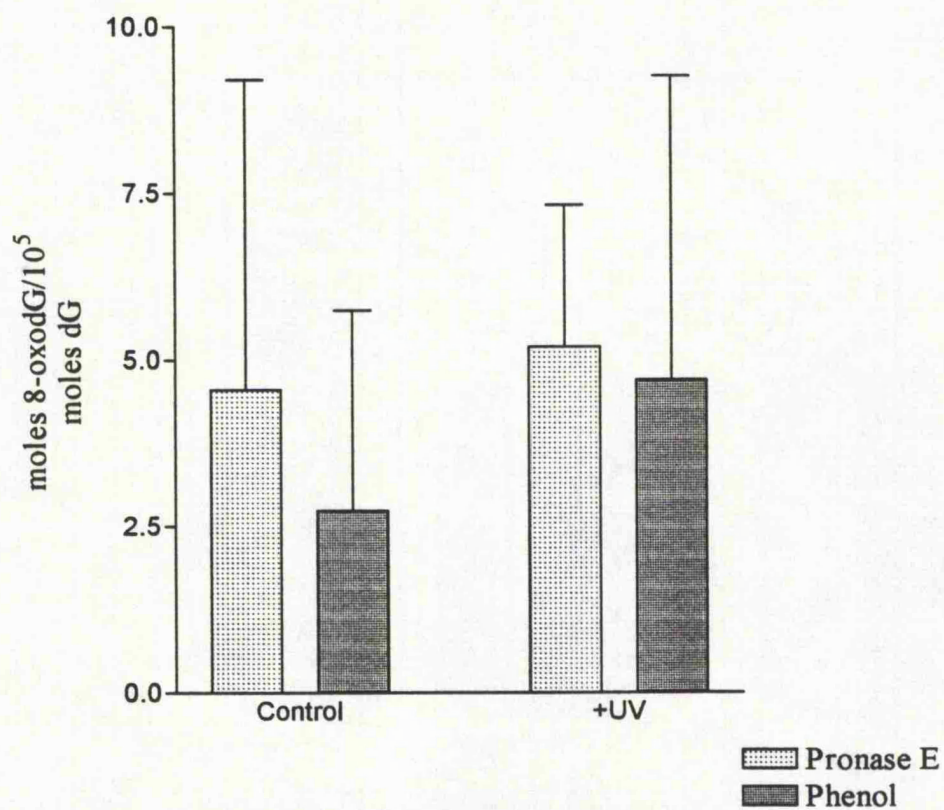


Figure 4.5: Combined UVC/UVA treatment of healthy volunteer PBMC for 30min on ice, direct comparison between pronase E and phenol isolation. Each value represents the mean +SD of three separate experiments.

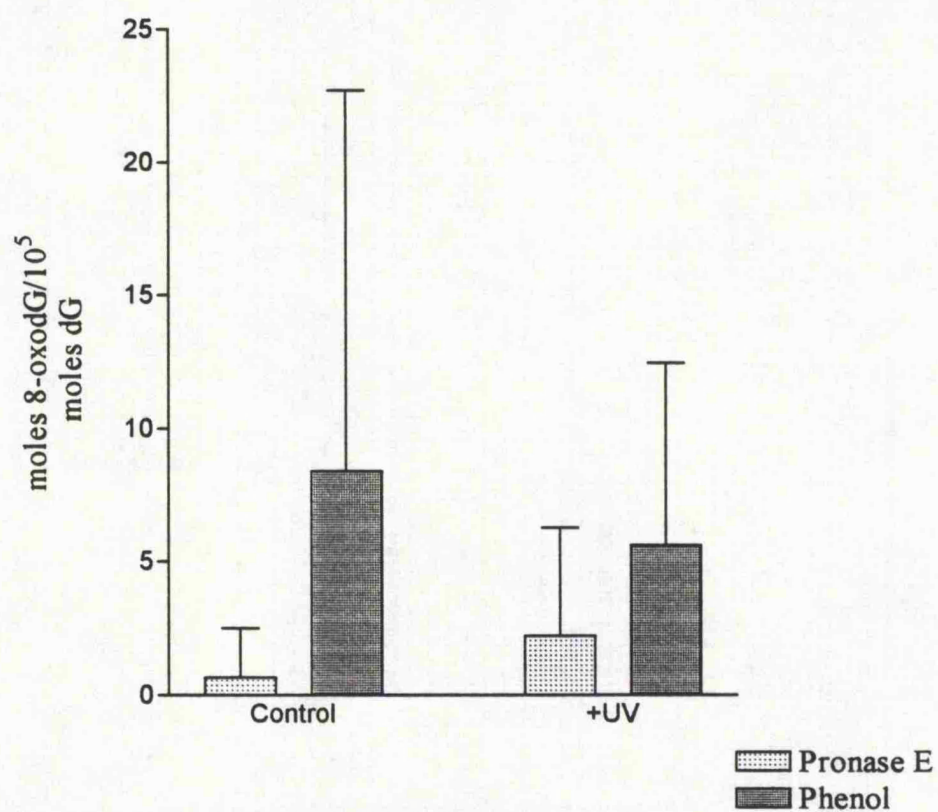


Figure 4.6: Combined UVC/UVA treatment of calf thymus DNA for 30min on ice, direct comparison between pronase E and phenol isolation. Each value represents the mean +SD for three separate experiments.

4.5 Discussion

This comparative methodological study was undertaken to determine whether phenol sensitises DNA to further oxidative damage. The level of 8-oxodG was taken as a suitable marker of oxidative DNA damage. It is important to ensure that the levels of a marker being quantitated are not artificially high due to artefactual formation. Whilst both methods yielded comparable amounts of DNA from both native and oxidatively stressed PBMC, some important and significant differences in the levels of 8-oxodG were subsequently observed. In the case of H_2O_2 and γ -irradiation the molar ratio of 8-oxodG/dG was between twofold and twentyfold higher for DNA extracted using the phenol technique when compared to the pronase E method. Greater differences were observed when the cells had been previously exposed to oxidative stress. This may be accounted for by either the preferential extraction of altered/damaged DNA or alternatively, by the subsequent oxidation of labile sites generated in the DNA during extraction. This latter hypothesis is supported by the work of Claycamp (1992) and questions the validity of using phenol extraction for oxidatively stressed cells (Claycamp, 1992). In addition, Harris *et al.*, (1994) demonstrated that unless precautions such as using freshly made phenol reagent are taken, the levels of 8-oxodG are generally higher in DNA from cells that have been exposed to an oxidative stress (Harris *et al.*, 1994). However, since there is not a linear relationship between sensitisation during extraction and initial oxidative damage, the use of phenol extraction techniques for the study of *in vivo* oxidation of DNA is inappropriate.

The toxicity of H_2O_2 suggests that it readily diffuses through cell membranes. Frimer demonstrated via a chromogenic peroxidase reaction that H_2O_2 can cross the lipid bilayer membrane without affecting the cell wall's integrity (Frimer *et al.*, 1983). Apart from

H₂O₂'s inherent toxicity, in this study, the presence of foetal calf serum potentiated the capacity of H₂O₂ to cause oxidative DNA damage. L-histidine, glucose, and iron in extracellular milieu have been reported to potentiate H₂O₂ damage in mammalian and bacterial cells (Brandi *et al.*, 1992). These components were present in the complete medium (+FCS) used in this study in which the cells were exposed to H₂O₂. Therefore this may account for the dose response observed with PBMC treated with H₂O₂ (Figure 5.1). To investigate this, incubation of the PBMC with an iron chelator such as desferrioxamine, or with a lower concentration of glucose, or less L-histidine, prior to treatment with H₂O₂ may reduce the effects of H₂O₂. Human PMNs produced 9.4 (SD=0.8) nmol of H₂O₂/10⁶ cells during 50 min of exposure to 2.0mg/ml phorbol-12-acetate-13-myristate (PMA) (Dizdaroglu *et al.*, 1993). These workers demonstrated that exposure to activated polymorphonuclear leukocytes (PMNs) caused DNA base modification in target cells *in vitro* typical of those induced by hydroxyl radical attack (Dizdaroglu *et al.*, 1993).

After phenol extraction, but irrespective of the 'oxidative stress' technique employed the levels of 8-oxodG were always greater in DNA isolated from oxidatively stressed PBMC than in re-extracted oxidatively stressed DNA. This may possibly be due to the presence of other sensitising agents within cells, that generate labile sites which are susceptible to subsequent phenol oxidation. Since H₂O₂ had no effect on 8-oxodG levels in naked DNA under these conditions, this suggests that for PBMC, direct radical attack on DNA is only responsible for a small part of the damage incurred.

We have shown that pronase E is less likely to induce further oxidative DNA damage during extraction. These data are in agreement with a study published during preparation of this thesis (Adachi *et al.*, 1995) where the pronase E procedure was compared to the phenol

method. The pronase E method resulted in lower levels of 8-oxodG than the phenol method in analyses of both rat liver and calf thymus DNA (Adachi *et al.*, 1995). Commercial DNA extraction kits are abundantly available that do not require the use of phenol although many contain other 'harsh' chemicals; these are often expensive. It is therefore important to choose a method which is both inexpensive and yields consistent results.

Using pronase E extraction indicated the limitations of the HPLC-EC detection of 8-oxodG as the levels in untreated PBMC and naked DNA were frequently at or below the limit of detection (1.1×10^{-5} nmoles 8-oxodG/ μg DNA). Birnboim *et al.*, (1994) recently stated that one might expect lower values of 8-oxodG in DNA from untreated cells (0.006-0.014 8-oxodG/ 10^5 dG) in comparison to those previously reported (0.5-2.0 8-oxodG/ 10^5 dG) (Floyd, 1990). However these values were calculated from DNA alkylation adducts and at present must only be used as an indication of the levels to be expected in cells. Birnboim *et al.*, (1994) repeated an earlier study by Floyd *et al.*, (1986a) where 8-oxodG in DNA isolated from human granulocytes stimulated with tetradecanoylphorbolacetate (TPA) yielded for example 43.0 moles 8-oxodG/ 10^5 moles dG. On repeating the experiments without using phenol in the extraction of DNA the levels of 8-oxodG were found to be no greater than controls at ~ 7.0 moles 8-oxodG/ 10^5 moles dG. This agrees with the data presented in this thesis.

The control values reported in this study ranged from 0.2 - 23.6 moles 8-oxodG/ 10^5 dG, which compares to 0.3 - 22.0 moles 8-oxodG/ 10^5 moles dG in the literature (Halliwell and Dizdaroglu, 1992). It is clear that using GC-MS may eliminate the need to use phenol and thereby bypasses the DNA extraction difficulties as after acid hydrolysis of the chromatin,

one measures the base (0.9-9.7, 8-oxoguanine/ 10^5 DNA bases)(Olinski *et al.*, 1992). With measurement by GC-MS one must also be aware of the possibility of artificially elevated levels of 8-oxodG as formic acid hydrolysis operates at high temperatures (140°C). However, Dizdaroglu and Halliwell report no such formation of 8-oxoG from G under hydrolysis conditions (Halliwell and Dizdaroglu, 1992). This lead us to develop a technique which would allow us to compare both formic acid hydrolysis with enzymatic hydrolysis of DNA prior to HPLC analysis, and in the future, GC-MS with HPLC. This procedure is addressed in chapter 6.

Chapter 5

**Analysis of 8-oxodG lesions in peripheral blood
mononuclear cells from patients with pathology
associated with oxidative stress**

5.1 Introduction

Reactive oxygen species (ROS) have been proposed to contribute to ageing, cancer, ischemic heart disease, cataract formation and certain chronic inflammatory disorders (Richter *et al.*, 1988). Systemic lupus erythematosus (SLE) is a chronic inflammatory connective tissue disorder which affects many organs of the body and is characterised by the presence of antibodies against nuclear components (anti-nuclear antibodies), particularly against double stranded DNA (Blount *et al.*, 1990; Swaak *et al.*, 1985). The reaction between DNA and anti-DNA antibodies and subsequent pathological immune complex formation has been extensively studied (Bruneau *et al.*, 1979; Blount *et al.*, 1990; Swaak *et al.*, 1985) in order to obtain information about the aetiology and pathogenesis of SLE. Immune dysfunction is also a characteristic of rheumatoid arthritis (RA) (Emery *et al.*, 1984). Immune dysfunction may be linked with lymphocyte DNA metabolism. In particular, DNA damage may impair lymphocyte function and induce increased cell turnover (Carson *et al.*, 1986); such changes are of relevance to the pathogenesis of rheumatoid arthritis (Bhusate *et al.*, 1992; Diamond *et al.*, 1992). During inflammatory disease states, such as SLE and RA, activated neutrophils release toxic oxygen species into the extracellular environment. Out of all the ROS generated during the oxidative burst of polymorphonuclear neutrophils (PMN) only hydrogen peroxide (H_2O_2) is believed to readily cross plasma and nuclear membranes and reach DNA (Frenkel, 1992). It is the properties of H_2O_2 , namely of being neutral and quite unreactive in the absence of reduced transition metal ions, that allow it to reach the nucleus where it can cause site specific damage (Frenkel, 1992). The mechanism for site specific damage is thought to involve iron ions bound to the phosphate groups of nucleic acids, or copper ions bound to proteins, which

can reduce incoming H_2O_2 to hydroxyl radical ($\cdot\text{OH}$) or other oxygen radical species and that this secondary generation of ROS is responsible for oxidation of bases in DNA (Frenkel, 1992).

5.2 Aims

To measure absolute levels of 8-oxodG in DNA of oxidatively stressed peripheral blood mononuclear cells (PBMC) and tissue culture supernatants, from SLE patients. A further aim was to investigate the level of repair of 8-oxodG in this system.

5.3 Methods

The following sets of experiments were carried out under controlled conditions using aseptic techniques. Once the PBMC were isolated as described in section 2.2, they were incubated with or without H_2O_2 , at 37°C and 5% CO_2 . Pronase E was the method of DNA isolation used (see section 2.4.1). All of the DNA samples were enzymatically digested to deoxynucleosides as described in section 2.4.3. Samples (50 μl) were then injected onto the HPLC column and analysed for 8-oxodG by electrochemical and UV detection, see section 2.7.

5.4 Results

5.4.1 Minimum cell number required to produce quantifiable DNA and 8-oxodG using pronase E isolation

Initial experiments were carried out using whole blood taken from healthy controls. Cell viability was monitored throughout all the experiments using the trypan blue exclusion assay (see section 2.3). In untreated PBMC, the cell viability was found to be greater than ninety-five percent (Figure 5.1 and Figure 5.2).

Initially the PBMC were isolated and incubated at a concentration of 1×10^6 cells per ml and 2 ml per well of a 24 well tissue culture plate. As cells were untreated, it was possible to pool the cells and examine the minimum number of cells required for the isolation of DNA. The minimum number of cells examined was 2×10^6 , from which a quantifiable amount of DNA was obtained; that is 10 μg (SEM=5) DNA/ 10^6 cells. However, the lowest level of control DNA that yielded an 8-oxodG peak when analysed on the HPLC-ECD system was an injection of 28 μg of DNA which yielded 250 femtomoles on column. This led to the use of a minimum number of 3×10^6 cells per experimental time point.

5.4.2 The effect of H_2O_2 on the viability of healthy volunteer PBMC

The effect of increasing concentrations of H_2O_2 on PBMC viability and the level of oxidative damage and repair in DNA was also investigated. The levels of 8-oxodG released

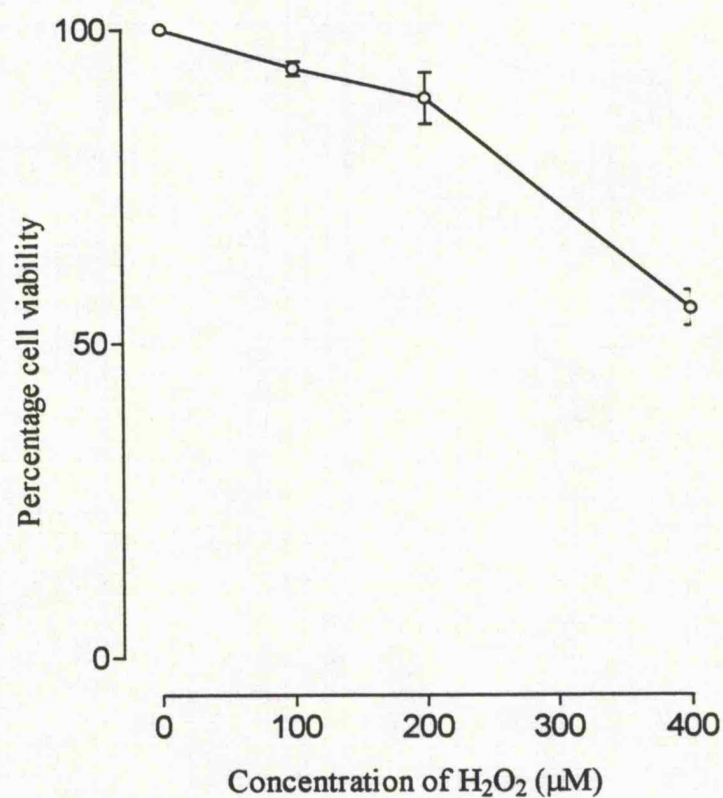


Figure 5.1: Effect of H_2O_2 on the viability of peripheral blood mononuclear cells from healthy volunteers at various concentrations of H_2O_2 , 24 hours post treatment. The percentage cell viability at time 0 was ~100% at all concentrations of H_2O_2 . Each point represents the mean of three experiments. Error bars are SEM.

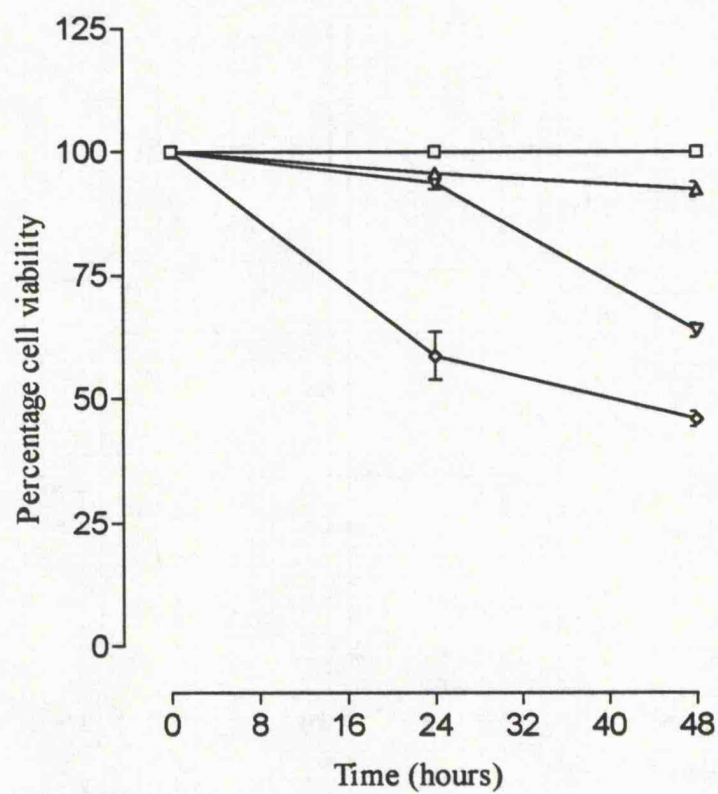


Figure 5.2: Viability of healthy volunteer peripheral blood mononuclear cells at 0, 24, and 48 hours post treatment with 0, 100, 200 and 400 μM H_2O_2 . Each point represents the mean of two experiments.

—□— 0
 —△— 100 μM
 —▽— 200 μM
 —◇— 400 μM

into the supernatant from the PBMC were examined as an indicator of DNA repair (see section 5.4.3). The experimental conditions were the same as described above see section 5.3. H_2O_2 was used at 100, 200 and 400 μM and PBMC viability was determined 24 and 48 hours later.

The first set of experiments examined the effect of H_2O_2 on cell viability at time zero and 24 hours later using PBMC from 3 healthy volunteers (Figure 5.1). At 0, 100, 200 and 400 μM H_2O_2 the cell viability was 100% at time zero. The PBMC viabilities 24 hours post treatment with 0, 100, 200 and 400 μM H_2O_2 were 100%, 93.7% (SEM=1.2), 89.0% (SEM=4) and 55.5% (SEM=2.9) respectively, therefore cell viability decreased with increasing concentration of H_2O_2 . A set of experiments to examine the cell viability of PBMC from two healthy volunteers at both 24 and 48 hours post H_2O_2 treatment was conducted. At 0, 100, 200 and 400 μM H_2O_2 the cell viability was 100% at time zero. At 24 hours post treatment with 0, 100, 200 and 400 μM H_2O_2 the cell viabilities were 100%, 95.6% (range 95.2-96.0), 93.8% (range 92.5-95.0), 58.7% (range 53.8-63.8). After 48 hours post treatment with 0, 100, 200 and 400 μM H_2O_2 the cell viabilities were 100%, 92.5% (range 90.9-94.0), 63.9% (range 62.5-65.2), 45.9% (range 44.4-47.4) (Figure 5.2). H_2O_2 at a concentration of 100 μM was found to cause the least cell death (~9%) when compared to untreated samples (Figure 5.1 and Figure 5.2). Among all PBMC cells treated with H_2O_2 (100 μM) the cell viability was greater than 90%.

5.4.3 The effect of H₂O₂ on cell viability and levels of 8-oxodG in PBMC from patients with systemic lupus erythematosus and rheumatoid arthritis

Using the conditions established for healthy PBMC, similar experiments were carried out on patient samples. The patient blood samples were provided by the Rheumatology Department at the University of Birmingham. The experiments were carried out without any knowledge concerning the patient diagnosis i.e. whether it was a rheumatoid, systemic lupus, or a normal control sample which was being processed. The patient PBMC were isolated as described in section 2.2 and then incubated for a period of 24 hours with H₂O₂ (0, 100 and 200µM). The pronase E DNA isolation procedure was used (see section 2.4.1). In total there were six patient samples; in some cases it was not possible to treat the PBMC with H₂O₂ as cell numbers were too low (<3x10⁶ per 10ml blood) . However, where the cell numbers were high (>6x10⁶ per 10ml blood) the PBMC were treated with H₂O₂ (100µM and 200µM)(3x10⁶ cells per dose point) for a 24 hour period.

At time zero the cell viability for patient 2 (SLE), patient 3 (RA), patient 4 (RA) and patient 6 (normal) at 0, 100 and 200µM H₂O₂ was 100%. At the 24 hour time point the cell viabilities for patient 2 (SLE) at 0, 100 and 200µM H₂O₂ were 97.5% (range 95.0-100.0), 93.4% (range 92.4-94.4) and 73.2% (range 71.4-75.0) respectively (see Figure 5.3). However, at the 24 hour time point the cell viabilities for patient 3 (RA) at 0, 100 and 200µM H₂O₂ were 96.4% (range 92.8-100.0), 95.8% (range 91.5-100.0), 58.4% (range 50.0-66.7) respectively (Figure 5.3). In contrast, at the 24 hour time point the cell

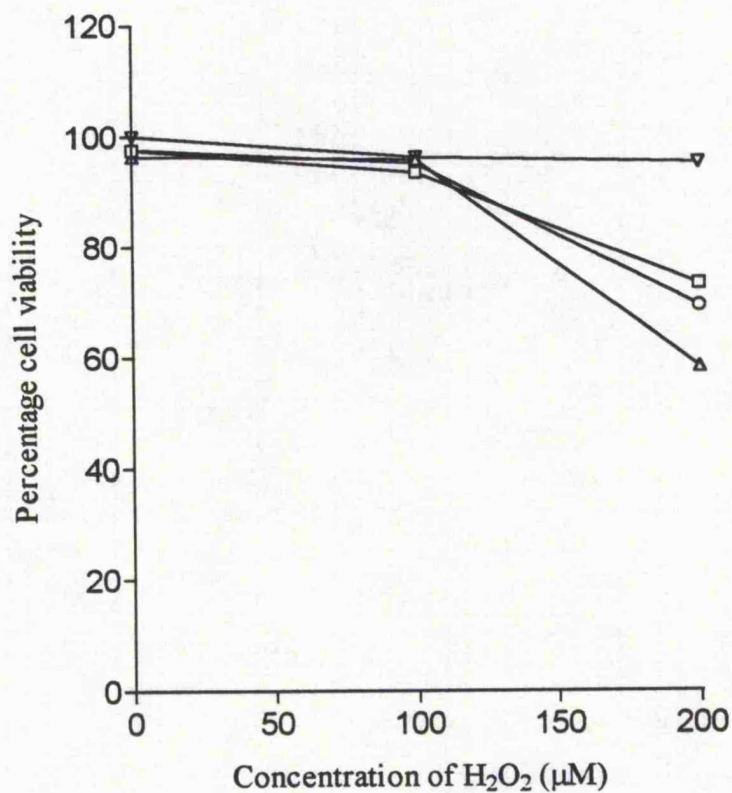


Figure 5.3: Effect of H_2O_2 concentrations on the percentage cell viability of patients peripheral blood mononuclear cells 24 hours post treatment with H_2O_2 . Each point represents the mean of two experiments.

- Patient 2 (SLE)
- ▲ Patient 3 (RA)
- ▼ Patient 4 (RA)
- Patient 6 (Normal)

viabilities for patient 4 (RA) at 0, 100 and 200 μ M H₂O₂ were 100%, 96.2% (range 96.0-96.3) and 95.2% (range 94.5-95.8) respectively (see Figure 5.3). Finally for patient 6 (normal) at the 24 hour time point the cell viabilities at 0, 100 and 200 μ M H₂O₂ were 97.6% (range 97.6-97.7), 95.1 (range 94.5-95.6) and 69.3 (range 66.7-71.8) respectively (see Figure 5.3). The cell viability at 0 and 100 μ M H₂O₂ in all patient and normal groups remained above 92% at 24 hours post treatment. However, on treatment with 200 μ M H₂O₂ the cell viability ranged between 58.4% for patient 3 (RA), 69.3% for patient 6 (normal), 73.2% for patient 2 (SLE) and in contrast to all the other patients the cell viability for patient 4 (RA) was 95.2%.

A low cell yield was obtained on isolation of PBMC from patient 5 (control), although a relatively high level of 8-oxodG (5.8nM) was found in the supernatant from PBMC of this patient (Figure 5.4). Also in patient 5 (control), 8-oxodG was observed in the cell extract at 0.4 mole/10⁵ moles dG (Figure 5.5). In the other supernatant samples where 8-oxodG was present at detectable levels, the level of 8-oxodG appeared to have increased with increasing concentration of H₂O₂; for patient 2 (SLE) from 0.4 to 1.1 nM and for patient 4 (RA) from 0.6 to 1.0 nM at 100 and 200 μ M H₂O₂ respectively. For patient 2 and patient 4 the levels of 8-oxodG in the supernatants of untreated cells were below the limit of detection.

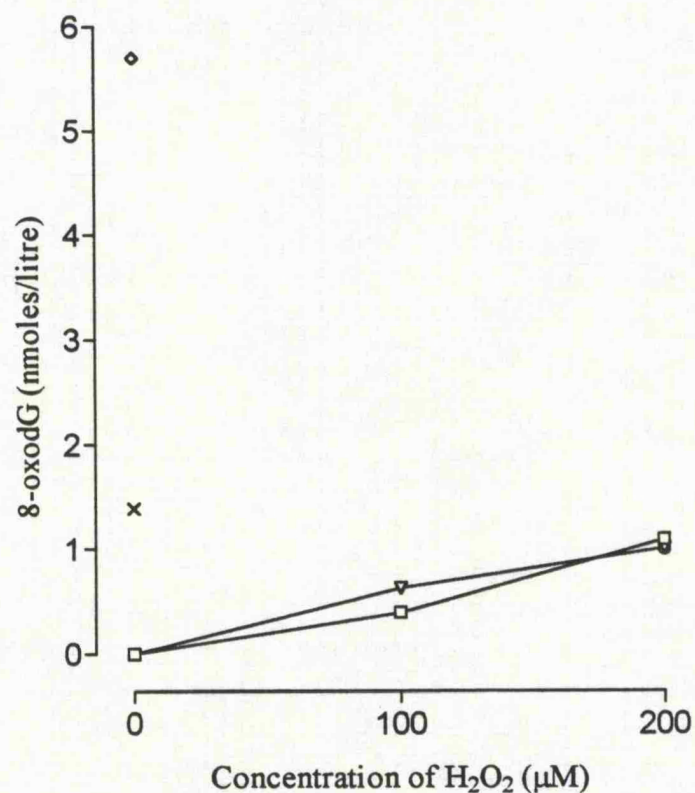


Figure 5.4: Effect of H₂O₂ on the levels of 8-oxodG observed in the supernatant of patients peripheral blood mononuclear cells treated with increasing concentrations of H₂O₂. Each point represents the mean of two experiments.

- × Patient 1 (SLE)
- Patient 2 (SLE)
- ▽— Patient 4 (RA)
- ◇ Patient 5 (Normal)
- Patient 6 (Normal)

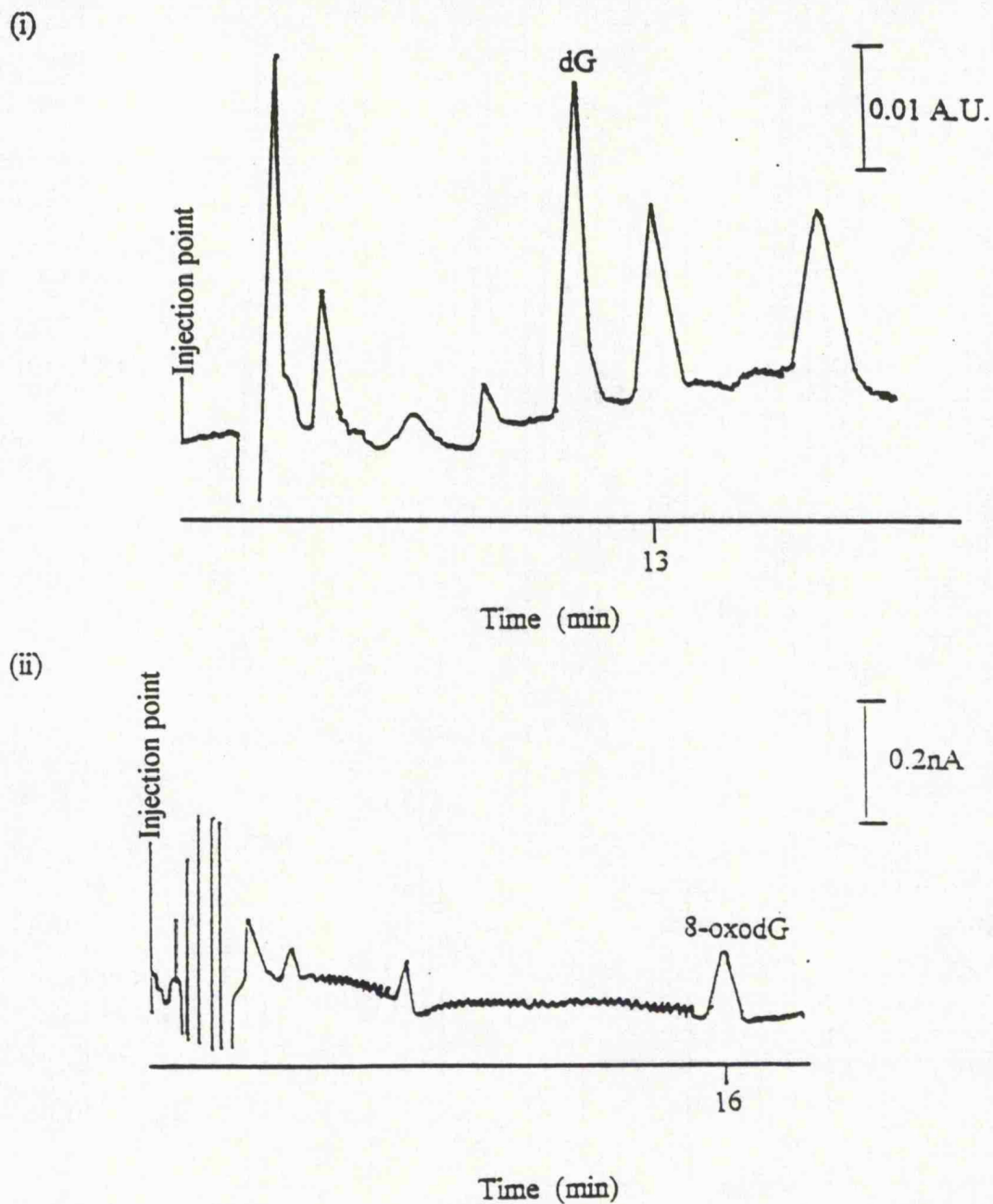


Figure 5.5: Representative HPLC of DNA extracted from peripheral blood mononuclear cells (patient 5-healthy volunteer) treated with $100\mu\text{M}$ H_2O_2 . (i) UV trace at 254nm (ii) Electrochemical trace at an applied potential of 0.6V. The flow rate was at 0.8ml/min and mobile phase as described in section 2.7.

5.4.4 Investigation of the origin of the 'Ax' peak in supernatants

Initially in the experiments examining the level of 8-oxodG in the supernatants of PBMC cultures, an unidentified peak 'Ax' co-eluted with the 8-oxodG peak. These experiments were carried out using 5% methanol (v/v). The peaks were separated on reduction of the methanol to 2% (v/v). The Ax peak was reduced in height as the concentration of H_2O_2 increased; this was true at both 24 and 48 hours (Figure 5.6).

Due to the fact that initially a peak ('Ax') was observed that co-eluted with 8-oxodG it was necessary to carry out an experiment on complete RPMI-1640 medium in the absence of cells. An experiment was carried out on RPMI-1640 in the presence or absence of heat inactivated foetal calf serum (10%). This was to ascertain if there was any 8-oxodG present in the medium that would lead to anomalous results and whether the Ax peak was formed by the action of H_2O_2 on a component of the medium. The medium was treated with H_2O_2 (0, 100 and 200 μM) for periods of 24 and 48 hours. These were subsequently processed as supernatants (see section 2.5.1). There were no detectable levels of 8-oxodG or of the Ax peak, suggesting Ax was cell-derived and that H_2O_2 influences its levels. Recently Shigenaga, (1994), reported the appearance of a peak which co-eluted with 8-oxodG in supernatant from cells analysed using HPLC-ECD, the identity of which is unknown.

5.4.5 The effect of H_2O_2 on the levels of 8-oxodG in the PBMC of SLE and controls

The preliminary experiments detailed in 5.4.1 to 5.4.4 allowed further experiments with SLE and normal PBMC to investigate if there were any differences between their ability to

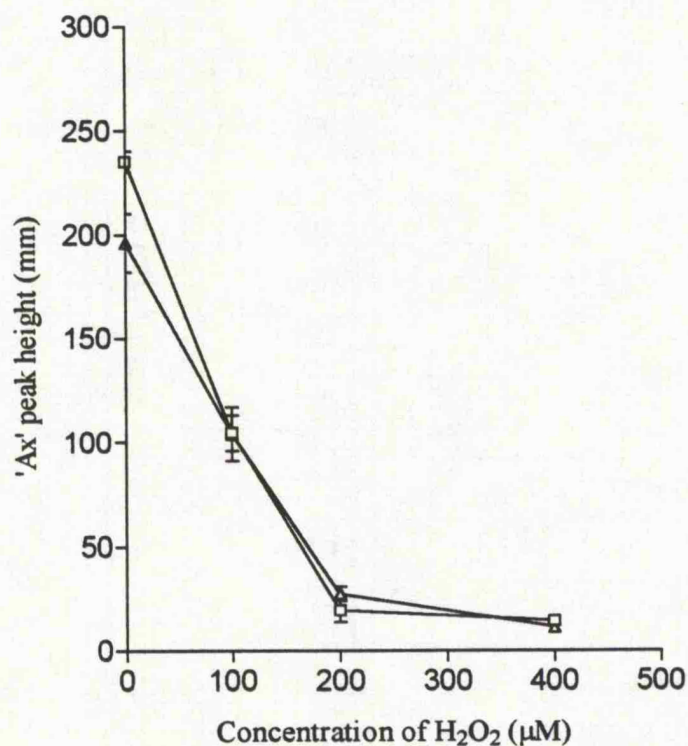


Figure 5.6: Determination of 'Ax' peak in the supernatant of peripheral blood mononuclear cells treated with increasing concentrations of H₂O₂. This graph represents the mean of two experiments on cells obtained from the blood of healthy volunteers.

—□— 24 hours
—▲— 48 hours

deal with oxidative stress. To prevent the necessity of pooling patient PBMC, which may have affected the results, the amount of blood from which the PBMC were isolated was increased from 20ml to 100ml. In total ten paired samples were examined, that is samples from a healthy volunteer and an SLE patient were treated and processed concomitantly for each experiment. The PBMC were treated with H_2O_2 (100 μM) over a 24 hour period, samples were taken at 0, 1, 2, 4, 6, and 24 hours. Previously in our laboratory the PBMC of SLE patients had been examined over a period from one day up to a week (Blount *et al.*, 1991). We wanted to investigate what happened at earlier time points to the levels of 8-oxodG in the PBMC of SLE patients and controls treated with H_2O_2 . The pronase E method of DNA isolation yielded lower levels of 8-oxodG than those observed when the phenol method of isolation was used (see section 4.0 for full details). This led to the problem of particular samples having levels of 8-oxodG below the limit of detection of the assay. The levels of 8-oxodG had invariably fallen in the DNA of PBMC from the SLE patients for example for patient 7, when examined initially at time 0 (0.26 moles/ 10^5 moles dG), and subsequently at 2 hours post treatment (0.09 moles/ 10^5 moles dG)(Table 5.1). Similarly for patient 8, values decreased from 0.04moles/ 10^5 moles dG at zero time to below the limit of detection at 4 hours post treatment. A similar finding was observed for patient 9, from 0.26 moles/ 10^5 moles dG at zero time to below the limit of detection at 6 hours post treatment with 100 μM H_2O_2 (Table 5.1). In controls the level of 8-oxodG in the isolated DNA had fallen to below the limit of detection, when examined initially at time 0 and at 6 (from 0.09 moles/ 10^5 moles dG) and 24 (from 0.38 moles/ 10^5 moles dG) hours post treatment with 100 μM H_2O_2 (Table 5.1).

Table 5.1 Levels of 8-oxodG in DNA isolated from PBMC of sex matched SLE patients and controls, measured at time 0 and various times post treatment, at 37°C with H₂O₂ (100µM).

Patient No.	H ₂ O ₂ exposure (hours)	8-oxodG in DNA of PBMC (moles / 10 ⁵ moles dG)			
		Experiment 1		Experiment 2	
		Control	Patient	Control	Patient
Pair 1, 6	0	<	0.09	<	<
	1	<	0.09	<	<
Pair 2, 7	0	-	-	<	0.26
	2	-	-	<	0.09
Pair 3, 8	0	<	0.13	-	0.04
	4	<	0.11	0.08	<
Pair 4, 9	0	<	<	0.09	0.26
	6	<	<	<	<
Pair 5, 10	0	0.38	<		
	24	<	<		

The "<" symbol indicates that the level of 8-oxodG was below the limit of detection of the assay for that particular sample. The "-" symbol indicates there was no DNA observed when sample was injected onto the HPLC. For each incubation time point with 100µM H₂O₂ at 37°C two separate experiments were carried out. DNA was isolated using the pronase E method. Samples from patient 10 were accidentally contaminated.

In the spent media of PBMC from SLE patients the levels of 8-oxodG had decreased at 1 (3.5nM) and 6 (5.8nM) hours post treatment but increased at 4 (5.4nM) hours when compared with the levels of 8-oxodG in these samples at time 0; 4.1nM, 6.5nM and below the limit of detection respectively. In the spent media of the normal PBMC the levels of 8-oxodG had increased at 1 (6.2nM) and 4 (3.1nM) hours and decreased at 6 (5.4nM) hours post treatment with 100 μ M H₂O₂ (See Table 5.2), when compared with the levels in these samples at time 0; 5.2nM, 2.5nM and 6.5nM respectively. Therefore, it was not possible to show a consistent increase or decrease in the levels of 8-oxodG measured in supernatant from control or patient PBMC.

Table 5.2 Levels of 8-oxodG in the supernatant of PBMC of sex matched SLE patients and controls, measured at time 0 and at various times post treatment, at 37°C with H₂O₂ (100µM).

Patient No.	H ₂ O ₂ exposure (hours)	8-oxodG in supernatant from PBMC (nmoles / litre)			
		Experiment 1		Experiment 2	
		Control	Patient	Control	Patient
Pair 1, 6	0	<	<	5.2	4.1
	1	<	<	6.2	3.5
Pair 2, 7	0	<	<	<	<
	2	<	<	<	<
Pair 3, 8	0	<	<	2.5	<
	4	<	<	3.1	5.4
Pair 4, 9	0	<	<	6.5	6.5
	6	<	<	5.4	5.8

The “<” symbol indicates that the level of 8-oxodG was below the limit of detection of the assay for that particular sample. For each incubation time point with 100µM H₂O₂ at 37°C two separate paired experiments were carried out.

5.5 Discussion

Blount *et al.*, (1991) proposed that due to a defect in the repair of oxidatively damaged DNA, patients with SLE develop a high titre of antinuclear antibodies. Anti-DNA antibodies represent a significant autospecificity in SLE because they are essentially diagnostic of the disease and they contribute to renal pathology (Diamond *et al.*, 1992). We set out to investigate whether SLE patients were capable of repairing the 8-oxodG lesion in DNA. The 8-oxodG lesion if left unrepaired may illicit an antibody response. Lunec *et al.*, (1994) showed that the level of 8-oxodG excreted in the urine was much lower in SLE than RA or control patients. Blount *et al.*, (1991) showed that a high level of 8-oxodG was present in the immune complexes isolated from SLE patients. These results would indicate that there may possibly be a defect in the ability of cells from SLE patients to repair the 8-oxodG lesion.

To set up the assay, initial experiments were carried out using healthy volunteer PBMC. In contrast to the level of H_2O_2 (200 μM) used in the experiments by Blount *et al.*, (1991) we found it necessary to reduce the level of H_2O_2 to 100 μM . Cell viability for both untreated patients and controls remained above 90% for the duration of the experiments. This prevented an increase in 8-oxodG due to cell death, which may have given rise to anomalous results. We found that the level of cell viability in controls, RA and SLE patients decreased with increasing concentrations of H_2O_2 (section 5.4.2 and 5.4.3). On incubation of PBMC from SLE and healthy volunteers with H_2O_2 (100 μM) for periods up to 24 hours the levels of 8-oxodG reduced with time in the DNA (section 5.4.5). These data suggested that the PBMC from SLE patients were capable of reducing the level of

8-oxodG in their DNA, that is, of repairing DNA lesions. The data from measurement of 8-oxodG in supernatant did not provide such an obvious trend as the levels of 8-oxodG did not always increase on incubation with H_2O_2 , for example at 1 and 6 hours they decreased (Table 5.2). This makes overall trends in the experiments more difficult to interpret. It remains to be investigated whether the rate of repair of the 8-oxodG lesion is different in SLE when compared with healthy volunteers. If the rate of repair is slower or the mechanism of repair is different this may allow the longer lived oxidatively damaged DNA to become either antigenic or mutagenic.

5.6 Measurement of repair of 8-oxoG in isolated nuclei

5.6.1 Introduction

An important question is whether or not 8-oxodG in DNA is repaired. If 8-oxodG is really harmful to living organisms, the cells should have a mechanism for its removal, as is the case for other types of DNA modification. In fact it was observed previously that the amount of 8-oxodG produced in rat liver DNA of X-ray irradiated mice decreased with time after irradiation, (Kasai *et al.*, 1986), suggesting the presence of a repair process acting on 8-oxodG in mouse liver. An endonuclease has been identified in *E. coli* that repairs 8-oxodG from DNA, (Chung *et al.*, 1991a). Chung has reported the presence in human polymorphonuclear neutrophils (PMN) of an endonuclease that removes 8-oxodG in the same way as the *E.coli* enzyme by specifically cleaving one strand of the DNA at two sites before and after the position of the 8-oxodG residue (Chung *et al.*, 1991b). Recent results from several laboratories together indicate the fundamental importance of the repair of 8-oxoguanine. It was reported that translesion synthesis past 8-oxoguanine in a gapped duplex vector yielded G to T transversions as the main detectable mutations both *in vitro* and *in vivo* (Demple, 1994). Moreover, the 8-oxodG nuclease identified by Nishimura's group is evidently identical with a FAPy glycosylase, fpg (Tchou *et al.*, 1991). The biological importance of 8-oxoG removal by fpg protein came into focus with the realization that the *mutM* gene of *E. coli* is identical to the *fpg* gene (Michaels *et al.*, 1991). The *mutM* mutants have a strongly elevated spontaneous rate of G to T transversions, which suggests that 8-oxodG is formed at a significant level by endogenous processes and that the fpg/mutM protein is the prime enzyme for its repair (Demple, 1994).

5.6.2 Aim

To set up an assay to measure the repair of the DNA adduct 8-oxoG in vitro. The basis of this assay was to incubate isolated nuclei with a substrate containing 8-oxodG.

5.6.3 Outline of assay

Cells were harvested from a CCRF-HSB-2 suspension culture and the nuclei isolated according to the method outlined in section 2.11. The nuclei at an equivalent concentration of 1×10^7 cells were incubated at 37°C with white light/methylene blue treated DNA (50µg) substrate (see section 2.10 and 2.11) for 20mins. The reaction was stopped by the addition of two and a half volumes of (-20°C) ice cold ethanol. The precipitated DNA and the supernatant fraction were separated and dried under vacuum at room temperature. The supernatants were then reconstituted in ultra pure water prior to analysis on the HPLC to examine the levels of 8-oxodG (see section 2.7) and 8-oxoG (see section 2.8) that were released into the supernatant.

5.6.4 Methods

The method developed for the isolation of nuclei from CCRF-HSB-2 cells was based on the method of Bellard (1989, (see section 2.12)). Once the nuclei were isolated a sample was taken and stained with propidium iodide (700µl PBS, 200µl PI (50µg/ml), 100µl nuclei). The nuclei fluoresced a pink/orange colour when examined under the fluorescence microscope (excitation wavelength 498nm). If dead cells, ghost cells and cellular debris were present in the nuclei preparation they would be observed at this stage under light

microscopy. Therefore the quality of the nuclei isolation was observed at this point. It was found that only 60% of the preparation was isolated nuclei and that some cellular debris still remained. The preparation was measured on the FacScan. The experiments were carried out according to the method described in section 2.11. The HPLC conditions were according to sections 2.7 and 2.8 for 8-oxodeoxyguanosine and 8-oxoguanine respectively.

5.6.5 Results

5.6.5.1 Incubation of CCRF-HSB-2 nuclei with white light/methylene blue treated DNA

In these preliminary experiments nuclei isolated from CCRF-HSB-2 cells were incubated with white light/methylene blue treated DNA to ascertain if the repair products of the 8-oxodG lesion could be measured. In the processed supernatant a peak of electrochemical activity which eluted at the same retention time as 8-oxoG was observed (Figure 5.7). The electrochemical detector was set at a potential of 0.6V versus a Ag/AgCl reference electrode. However, guanine and hypoxanthine have a similar retention time to 8-oxoG (Dr. M.D. Evans, personal communication). At this applied potential guanine is not very electrochemically active; peaks are not seen unless it is present in large quantities.

The supernatant was also examined for levels of 8-oxodG using the method described in section 2.11 and 2.7. On examination of the chromatogram in Figure 5.8 a peak which eluted slightly earlier than the 8-oxodG standard was observed. As was observed with the supernatant from cells (section 5.4.4) a large electrochemically active peak eluted prior to it. The controls used in these experiments consisted of (i) nuclei incubated with PBS alone

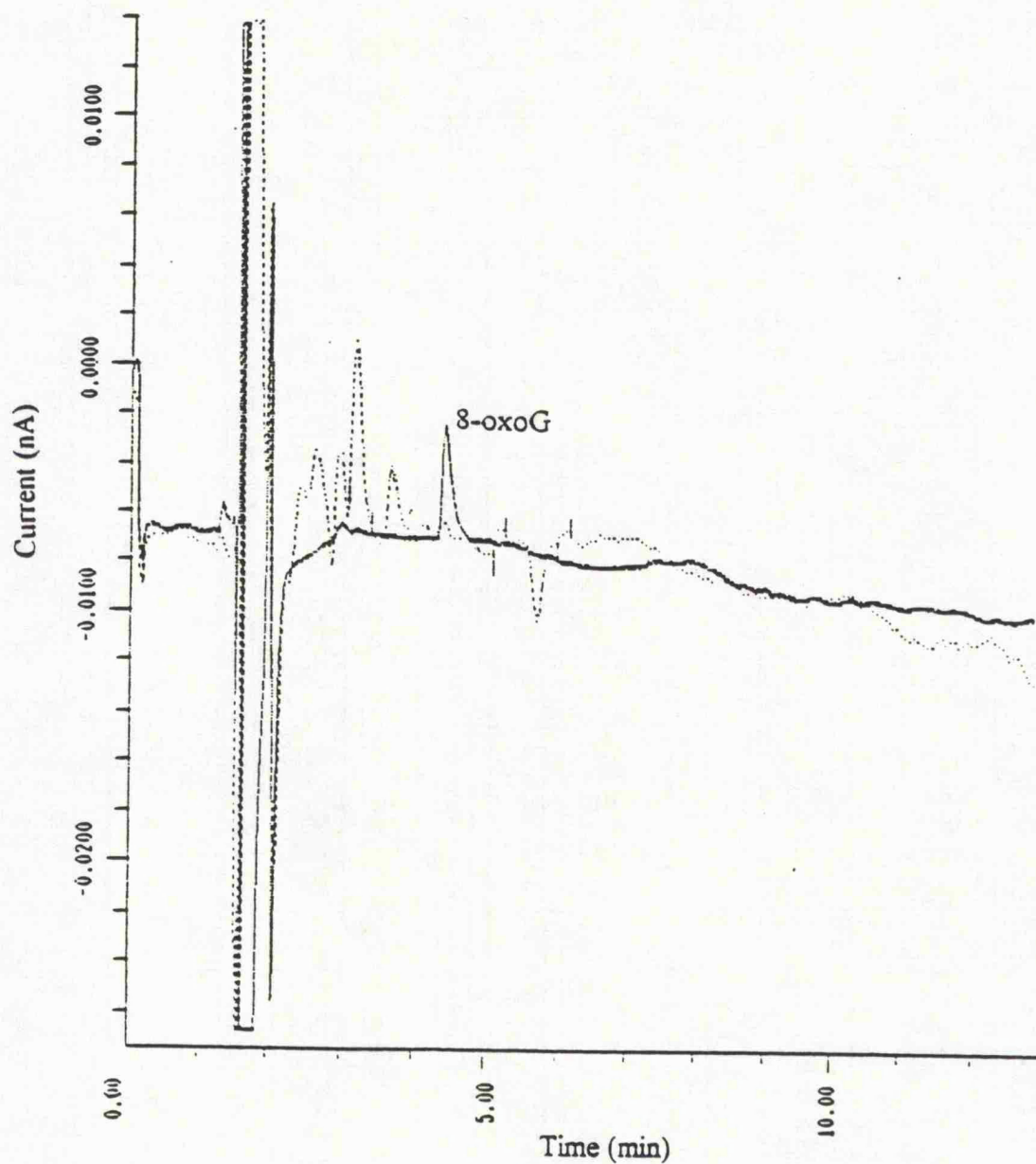


Figure 5.7: Representative HPLC of bases from supernatant from CCRF-HSB-2 nuclei incubated with white light/methylene blue treated DNA (50 μ g)(---). The 8-oxoG standard is shown for comparison (—). Electrochemical trace at an applied potential of 0.6V. The flow rate was at 1.0 ml/min. The mobile phase was as described in section 2.8.

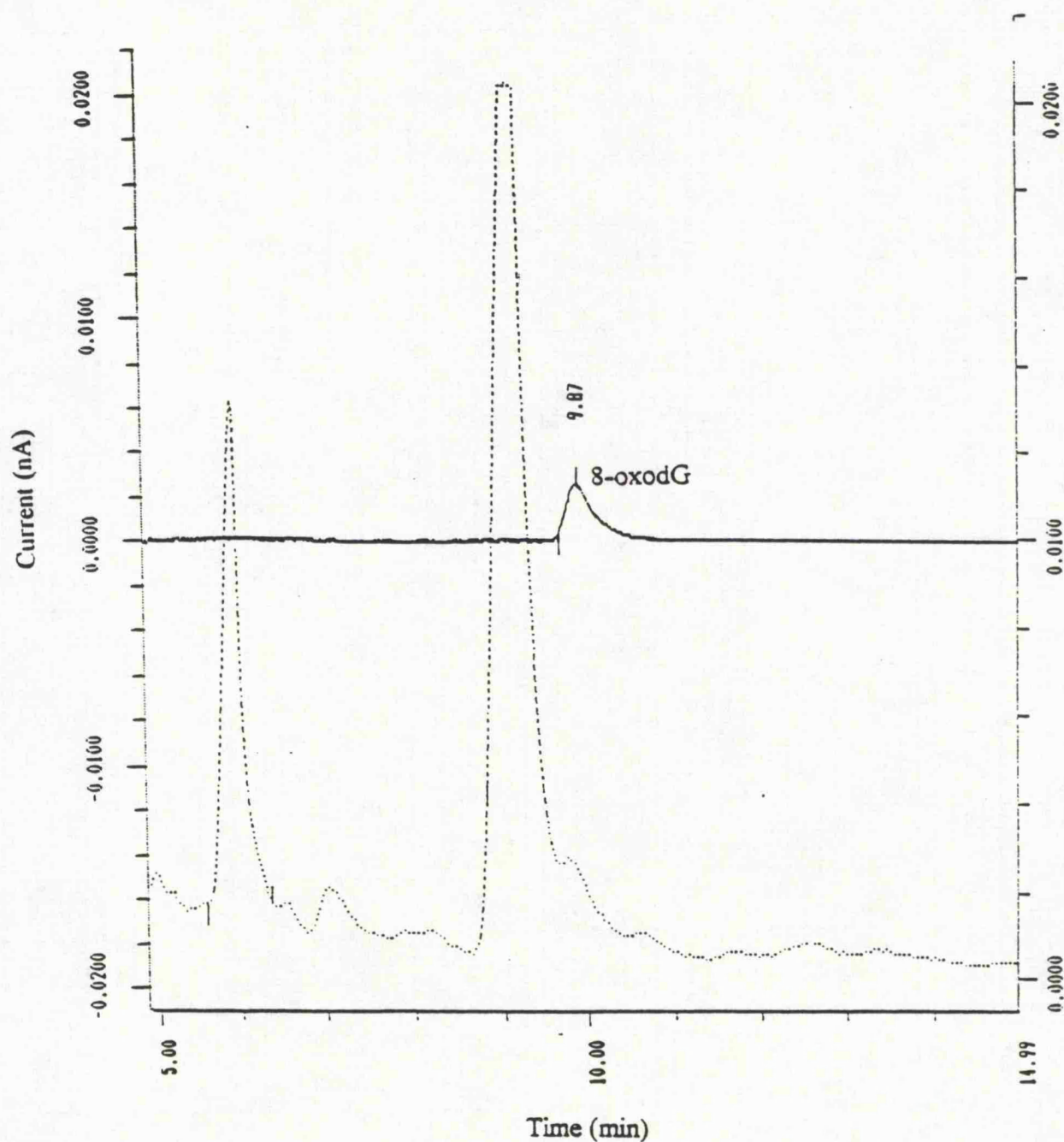


Figure 5.8: Representative HPLC of deoxynucleosides from supernatant from CCRF-HSB-2 nuclei incubated with white light/methylene blue treated DNA (50 μ g)(---). The 8-oxodG standard is shown for comparison (—). Electrochemical trace at an applied potential of 0.6V. The flow rate was at 1.0 ml/min. The mobile phase was as described in section 2.7.

without white light/methylene blue/DNA and (ii) white light/methylene blue/DNA without nuclei. In the processed supernatants of these controls the levels of 8-oxoG and 8-oxodG were below the limit of detection of the assays.

5.6.6 Discussion

The rationale behind the development of a nuclei repair assay was that since the majority of DNA in the cell is located in the nucleus the repair protein for the 8-oxodG lesion would be located here also. The problem encountered using this particular system was that the actual level of 8-oxodG in the substrate presented to the nuclei was unknown and perhaps if all the lesions were not repaired that the levels of 8-oxodG and 8-oxoG would be below the limit of detection of the assays used. Hence an attempt was made at inducing the enzyme responsible for the repair of the 8-oxodG lesion by pre-treatment of the CCRF-HSB-2 cells with H_2O_2 . However, various aspects may have been improved to increase the sensitivity of the assay. An increase in the incubation time with the substrate may have allowed repair to take place and hence elevated the levels of 8-oxodG and 8-oxoG measured in the supernatant. Reconstitution of the supernatant in a smaller volume of ultra pure water may have concentrated the 8-oxodG and 8-oxoG, allowing HPLC detection. An increase in the number of nuclei or in the concentration of substrate may have increased the likelihood of observing an 8-oxodG or 8-oxoG peak. Further validation work was needed on this assay. The purpose of developing this assay was to study the rate of repair in nuclei isolated from cells. The assay would on complete validation have proved useful in examining the rate of SLE lymphocyte repair in comparison to controls. The synthesis of a defined substrate containing a known amount of the 8-oxodG lesion would be ideal as a basis for the assay. This would allow direct quantitation of the repair rates of healthy and diseased cells, for

example in patients with autoimmune disease. In the disease SLE, the symptoms are exacerbated by exposure to UV light (Beighlie *et al.*, 1975). Increased sensitivity of SLE cells to reactive oxygen species-producing systems, particularly UV light has been reported previously (Compton *et al.*, 1984, Golan *et al.*, 1984). In cells from patients with the human inherited disease xeroderma pigmentosum, incision at pyrimidine dimers is defective (Sato *et al.*, 1993). An assay capable of measuring the repair rates in PBMC of healthy and SLE volunteers may prove a useful tool for investigating the reported susceptibility of SLE patients to oxidative DNA damage.

Chapter 6

Novel HPLC assay for the determination of 8-oxoG lesions in DNA

6.1 Introduction to 8-oxoguanine assay

Oxidative DNA damage produced by free radicals or other DNA-damaging agents has been implicated to play a role in mutagenesis, carcinogenesis and ageing (Halliwell *et al.*, 1989). An oxidative DNA adduct 8-oxoguanine (8-oxoG) is measured conventionally as the deoxynucleoside by high performance liquid chromatography (HPLC-ECD) (Floyd *et al.*, 1986b), or as the free base by gas chromatography combined with mass spectrometry (Dizdaroglu, 1994). A 'hybrid' analysis of the 8-oxoG base by HPLC was developed (see section 6.2) in order to account for reported differences between the two more established techniques. It was found that guanase degrades guanine and not 8-oxoG, allowing reversed-phase HPLC analysis of 8-oxoG in acid hydrolysates of DNA without interference from guanine (Herbert *et al.*, 1994).

6.2 Development of 8-oxoguanine assay

The measurement of the 8-oxodeoxyguanosine lesion may also be carried out using gas chromatography combined with mass spectrometry. 8-Oxodeoxyguanosine is measured on a GC-MS system in the base form as 8-oxoguanine (8-oxoG) following derivatisation to its volatile trimethylsilyl compound. It had been established previously that the enzyme guanase converted the base guanine to xanthine (see Figure 6.1) and proved not to affect the concentration of the 8-oxoG (Figure 6.2) (Herbert *et al.*, 1994). When this enzymatic digestion was combined with formic acid hydrolysis it provided a way in which a direct comparison between the HPLC-ECD methods for 8-oxodG and 8-oxoG measurement of 8-

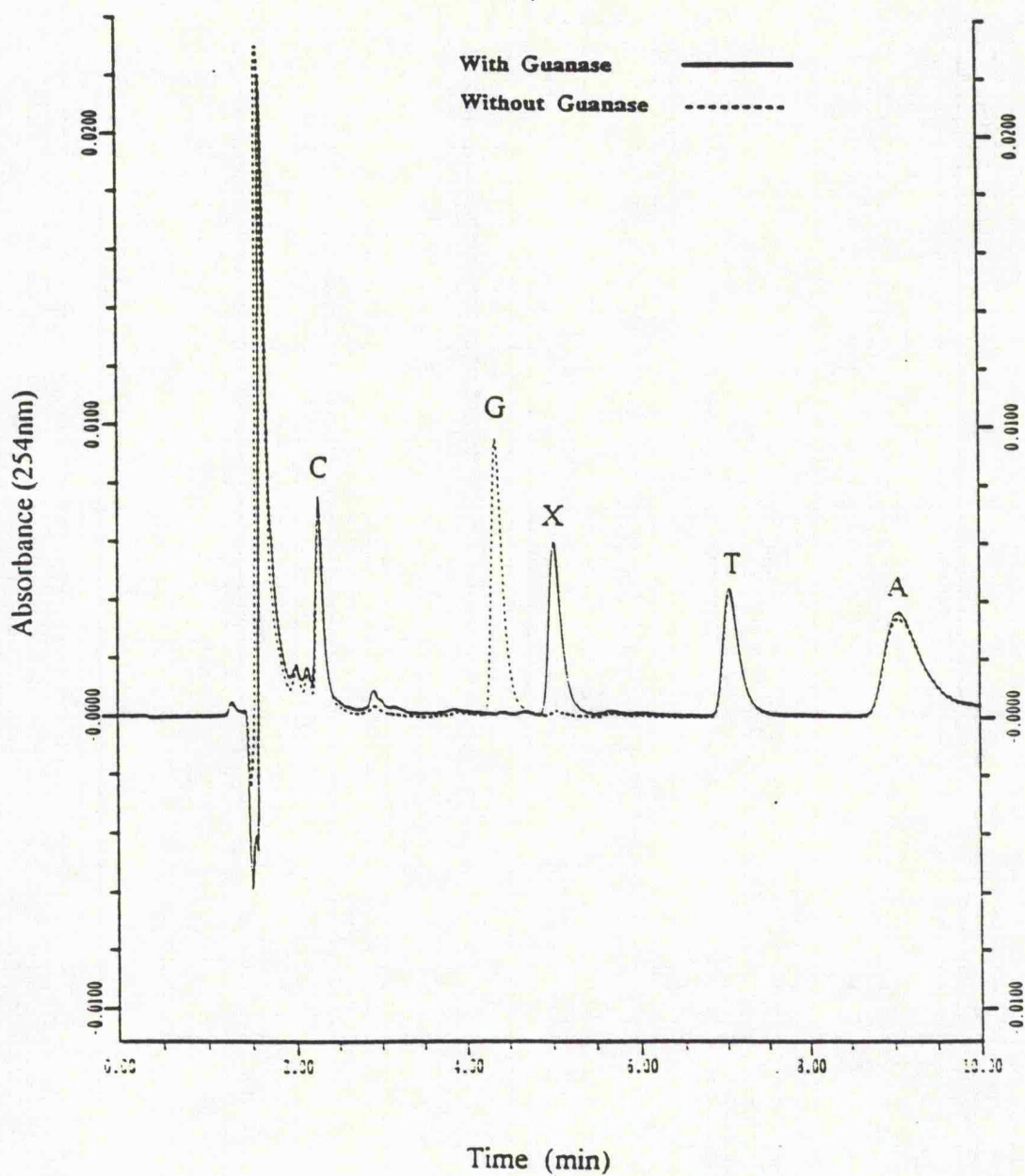


Figure 6.1: Representative HPLC of the effect of guanase on bases derived from a formic acid hydrolysate of calf thymus DNA. Samples were analysed by HPLC with UV detection prior to (---) and following (—) guanase treatment as described in section 2.8. C, cytosine; G, guanine; X, xanthine; T, thymine; A, adenine.

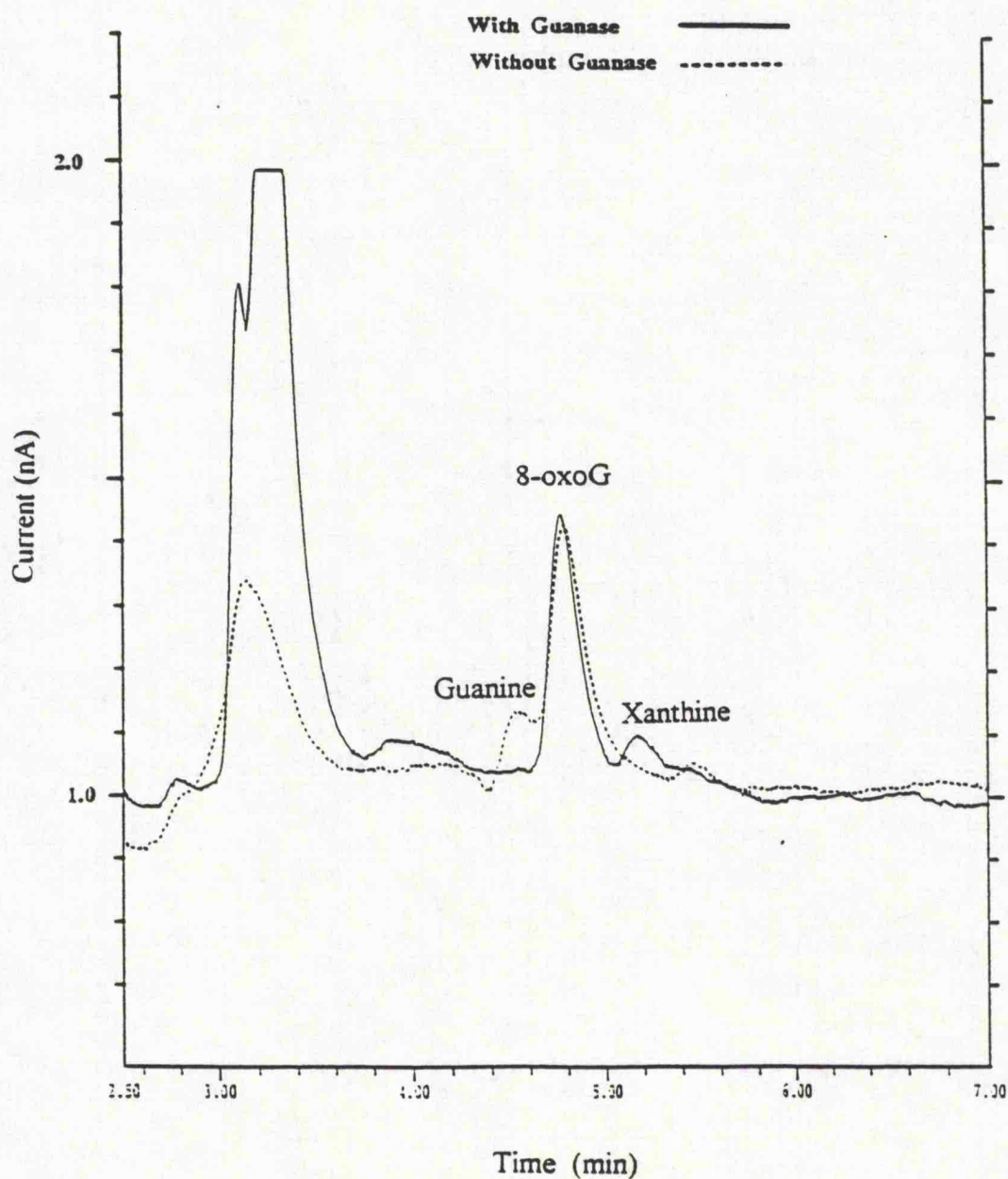


Figure 6.2: Representative HPLC of the detection of 8-oxoguanine in gamma-irradiated (400Gy) calf thymus DNA. Samples were analysed by HPLC with electrochemical detection prior to (---) and following (—) guanine treatment as described in section 2.8.

oxoG could be carried out. See results section 6.4. The conditions for formic acid hydrolysis were adapted from Dizdaroglu *et al.*, 1992.

Initially the mobile phase was that of the 8-oxodG assay (section 2.7). However, the retention time of 8-oxoG was very short when either 5% or 2% methanol was used and the percentage used finally was 1% methanol. The mobile phase was changed from the 50mM sodium acetate, 1mM EDTA, pH 5.1 to a mobile phase containing 40mM potassium phosphate, 1mM EDTA, pH 5.0 as this gave better peak shapes.

6.3 Aim

To develop an assay to measure the 8-oxoguanine lesion using HPLC combined with formic acid hydrolysis and to compare this assay with the 8-oxodG assay using calf thymus γ -irradiated DNA as a model.

6.4 Methods

Calf thymus DNA at a concentration of 1mg/ml was dissolved in ultra-pure water and irradiated using a ^{60}Co γ -source at a dose of 400Gy. The unirradiated control DNA and irradiated samples were then divided into two aliquots. The first aliquot was dried down under nitrogen, prior to hydrolysis with formic acid 60% (v/v) at 140°C for 45min (see section 2.8). This was then reconstituted in ultra pure water (250 μ l), and the pH adjusted to 8.0 with 1M Tris-EDTA (pH 8.0) prior to incubation with guanase for 1 hour at 37°C. The samples were then neutralised with 1N HCl and then analysed by HPLC-ECD as

outlined in section 2.8. The second aliquot was enzymatically hydrolysed to the deoxynucleoside level using a procedure described by Faux *et al.*, (1992) and described in section 2.4.3 and analysed on the HPLC-ECD as described in section 2.7. 8-oxoG was analysed using 40mM potassium phosphate, pH 5.0, containing 1mM EDTA and 1% v/v methanol in the mobile phase, at a flow rate of 1ml/min. The deoxynucleoside, 8-oxodeoxyguanosine (8-oxodG), was analysed using 50mM sodium acetate, pH 5.1, containing 1mM EDTA and 5% (v/v) methanol at a flow rate of 1.0 ml/min. The method was developed as outlined in section 6.2.

6.5 Results

The levels of 8-oxoG and 8-oxodG in DNA increased on treatment of the DNA with gamma irradiation (see Table 6.2). Using the guanase assay the level obtained from calf-thymus DNA irradiated with 400Gy was 5.0 (SD=1.20) nmol/mg DNA and 1.6 (SD=0.30) nmol/mg DNA for enzymic hydrolysis determination. The limit of detection of the guanase assay was 500 femtomoles on column and for the 8-oxodG assay was 250 femtomoles on column.

6.6 Discussion

The reported background levels of 8-oxoG obtained from commercially available calf-thymus DNA, measured using GC-MS, are 0.5-1.0 nmol/mg DNA (Halliwell *et al.*, 1992); the values obtained using the enzymatic hydrolysis were within this range at 0.7 (SD=0.12) nmol/mg DNA. Using the guanase method the values were much lower at 0.04 nmol/mg

Table 6.2: Direct comparison of 8-oxoguanine in gamma-irradiated DNA determined as the base and the deoxynucleoside (n=4).

Treatment	Enzymatic Hydrolysis (nmol 8-oxodG/mg DNA)	Guanase Assay (nmol 8-oxoG/mg DNA)
Non-irradiated sample (0 Gy)	0.70 (SD=0.12)	0.04
Irradiated sample (400 Gy)	1.60 (SD=0.30)	5.00 (SD=1.20)

DNA in control DNA. In this preliminary study, on direct comparison of the levels of 8-oxoG with 8-oxodG there appeared to be a difference between the two assays. On irradiation of DNA, the guanase assay yields higher levels of the oxidative DNA adduct 8-oxoG. This increase in the DNA adduct may be due to the use of the formic acid hydrolysis which may cause artificially high levels to be formed or it may be that the enzymatic hydrolysis had not reached completion; in the latter case the amount of 8-oxodG would be underestimated. However Kasai *et al.*, (1989) assessed that for every hour incubation with alkaline phosphatase there was an extra 0.3 residues of 8-oxodG/10⁵ dG formed. The new procedure combines formic acid hydrolysis with an enzyme which is specific for guanine and avoids problems with nucleolytic enzymes which may lack specificity toward oxidatively damaged DNA. The 'hybrid' guanase assay can be used in place of both the 8-oxodG assay on the HPLC-ECD and the 8-oxoG assay on the GC-MS. The guanase assay is cheaper and faster than the other assays for measurement of the oxidative DNA adduct 8-oxoG; there is only one enzyme step required and the HPLC run time is less than 10min. Using γ -irradiation (400Gy) as a model of oxidative DNA damage showed that 8-oxoG could be detected in the DNA treated using the guanase assay and that it was a more sensitive index than using the deoxynucleoside assay. This assay has been established within the Division of Chemical Pathology at the University of Leicester and is being used to further compare the reported differences between the HPLC-ECD and GC-MS techniques for measuring 8-oxoguanine.

Chapter 7

**Investigation of UVA and UVB induced DNA
damage in a keratinocyte cell line and comparison
of levels of 8-oxoG with levels of UVA induced
DNA damage**

7.1 Introduction

Experiments using short-wave UVC (particularly 254nm) have been important in characterising UV-induced DNA damages (Beehler *et al.*, 1992). UVC damages DNA via direct photon energy to give rise to pyrimidine photoproducts, breaks and protein crosslinks (Peak *et al.*, 1987). However, these wavelengths are not present in the solar irradiation that reaches the earth's surface. The terrestrial solar spectrum consists of UVB (290-320nm) and UVA (320-400nm). UVB is the wavelength range of light that is thought to damage DNA by both direct and indirect mechanisms, suggesting it may serve as both an initiator and a promoter of cancer (Peak *et al.*, 1989). Although UVA lacks direct DNA-damaging activity, it has been suggested to generate oxygen free radicals in the presence of photosensitizer and thereby indirectly damage DNA (Peak *et al.*, 1987).

UV-radiation-induced skin cancers are predominantly basal and squamous cell carcinomas arising from keratinocytes, but also malignant melanomas arising from melanocytes (Beehler *et al.*, 1992). Although the exact mechanism of UV carcinogenesis is not clear, much work has concentrated on UV-induced damage to DNA. An important and specific means of analysis of UV-DNA utilises antibodies raised to UV-modified DNA (UV-DNA), which recognise adducts such as thymine dimers and the 6-4 photoproduct within the DNA chain (Levine *et al.*, 1966, Mori *et al.*, 1991) and can be applied to DNA in cells and tissues.

For the purpose of measuring DNA damage without the need for processing and manipulation of normal and pathological material, a polyclonal antiserum which has specificity for UV damaged DNA has been developed in our laboratory. The full characterisation of this antibody and a report on its ability to detect sequence-specific

modifications induced by UV irradiation of DNA has been described previously (Herbert *et al.*, 1994). However, the specificity of the polyclonal antisera at lower dilutions than 1 in 5000 has not been fully described and may depend upon conformational changes caused by pyrimidine dimerisation, (Wakizaka *et al.*, 1979a), in particular the antibody may detect single-stranded DNA (ssDNA).

7.2 Aim

To investigate the use of antibody 529 to detect UVB and UVA damage in a human SV40T transformed keratinocyte cell line (RHT cells), as a model system for immunodetection of DNA damage *in situ*.

7.3 Rationale for development of RHT cell system as a model for examining

UV damaged DNA

Preliminary experiments with UV irradiation and a transformed cell line (CCRF-HSB2 cells) led to the discussion of the relevance of particular cell types and the types of damage applied. The part of the human body that has the greatest exposure to UV irradiation is the skin, hence the subsequent utilisation of a keratinocyte cell line (RHT cells).

Initially RHT cells were cultured on slides and coverslips which had been treated overnight with chromic acid and rinsed with water prior to being autoclaved. Eventually the cells were cultured on plastic chamber slides, as more sample points could be taken from a single

slide; each slide contained eight chambers. The cells adhered and grew equally well on both the glass and plastic surfaces.

7.3.1 Immunocytochemistry protocol

The antibody 529 was developed in the Division of Chemical Pathology at the University of Leicester. It was characterised and found to react with UVC (Herbert *et al.*, 1994) and tentatively reported as reacting with UVA (Thomas *et al.*, 1994) damage to which it had been raised. The tissue work that was carried out previously in this laboratory utilising the antibody involved fixation of the tissue by paraformaldehyde or fixation of frozen tissue with methanol. Both of these fixation protocols were investigated for the keratinocyte cell line.

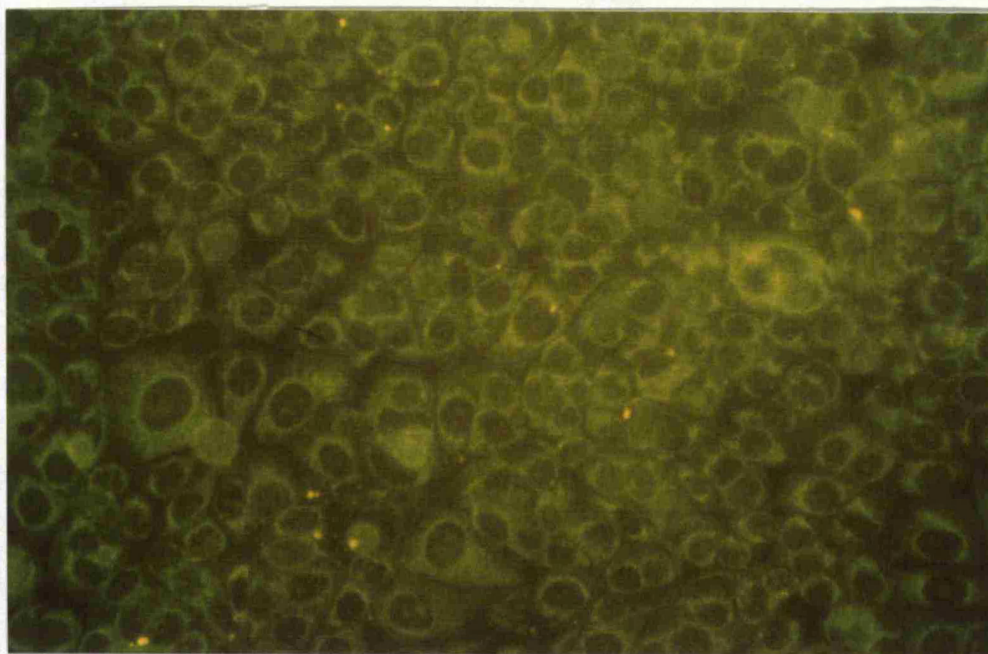
The RHT cells were initially fixed with 2% (v/v) paraformaldehyde for 1 hour at room temperature followed by three PBS rinses and then placed in ice cold methanol (-20°C) for an overnight incubation at 4°C. The slides were then rinsed three times with PBS before being blocked for 1 hour at room temperature with 10% normal goat serum (NGS) in PBS. Following three further rinses with PBS the primary antibody 529 (anti-UV DNA damage antibody) was applied at a concentration of 1:2500 in 1% (w/v) milk in PBS and left in a moist chamber at 4°C overnight. Following three rinses with PBS the samples were incubated with goat-anti-rabbit secondary antibody 1:200 in 2% (v/v) NGS in PBS for 1 hour. Subsequently the samples were rinsed three times with PBS and stained with a streptavidin FITC label at 1:100 in PBS for 1 hour at room temperature in the dark. The coverslips were then washed copiously with PBS and rinsed finally with distilled water prior

to being mounted using Vectorshield and then examined under the fluorescence microscope. This method yielded samples that had a uniformly very high background fluorescence over the surface of the slide. This led to further investigation into the methods of fixation. A shorter period of 15min incubation with paraformaldehyde to a final concentration of 2% was examined along with a shorter period of fixation with methanol (1 hour). However these modifications still presented samples that had higher background fluorescence, particularly in the perinuclear area of the cell (Figure 7.1).

It was necessary to use solvents which would allow the antibody access to the nucleus, therefore a combination of ice-cold methanol:acetone (1:1) for 10min at 4°C was finally used to fix samples. The background staining was reduced so the differences in antibody binding were more easily observed between cells that had been treated with UVB irradiation and those which were unirradiated. Figure 7.2 (photographs Figure 7.2 were taken by Miss S. Davies, Division of Chemical Pathology, University of Leicester) clearly showed the reduction in the background levels of fluorescence and how the antibody reacts to UVB damaged cells, the increased staining being most obvious in the nucleus.

It was noted that slides processed without primary antibody had a high background autofluorescence throughout the cell. The reasons were possibly either that the secondary biotin labelled antibody bound to the dried skimmed milk or the streptavidin-FITC bound to the milk, as the level of background fluorescence increased with increasing concentrations of dried skimmed milk. The initial diluents of the primary antibody 529 were made up in 1% dried skimmed milk in PBS, this was found to give high fluorescence. Dilutions of 2, 1, 0.5%, 0.1% NGS were investigated in place of the dried skimmed milk. NGS at a dilution of 0.1% was found to have a lower level of background fluorescence and was thus chosen

a)



b)

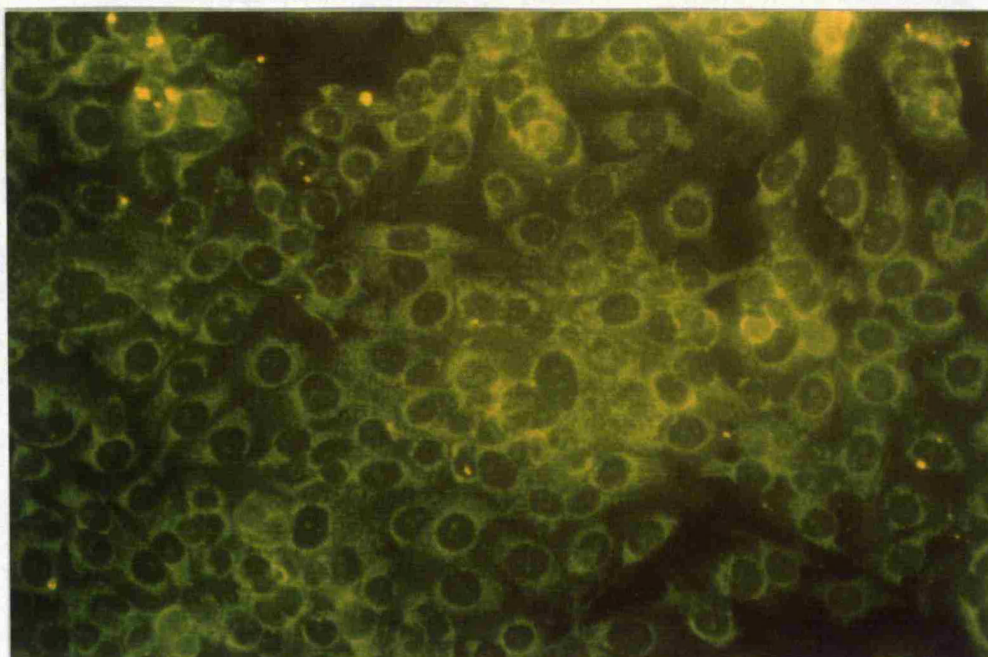
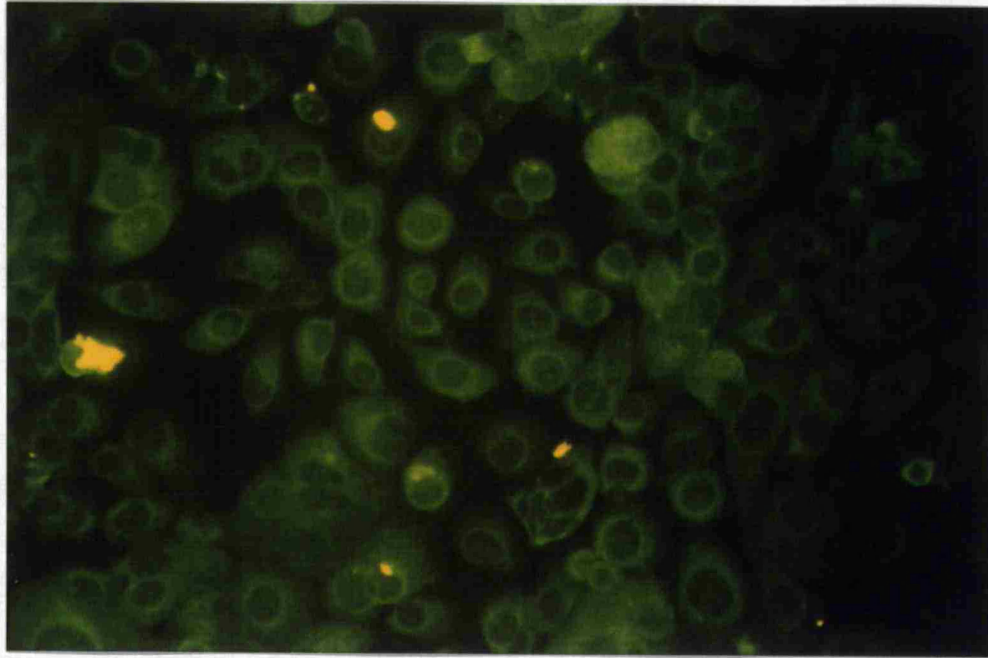


Figure 7.1: RHT keratinocytes (low magnification (x20)) following treatment with UVB (a) 0 mJ/cm² and (b) 36mJ/cm². Cells fixed immediately post-irradiation with paraformaldehyde (4%) and treated with antibody 529 and FITC labelled (see section 2.9.2). The cells have a uniformly high level of background staining, making differentiation between (a) and (b) difficult.

a)



b)

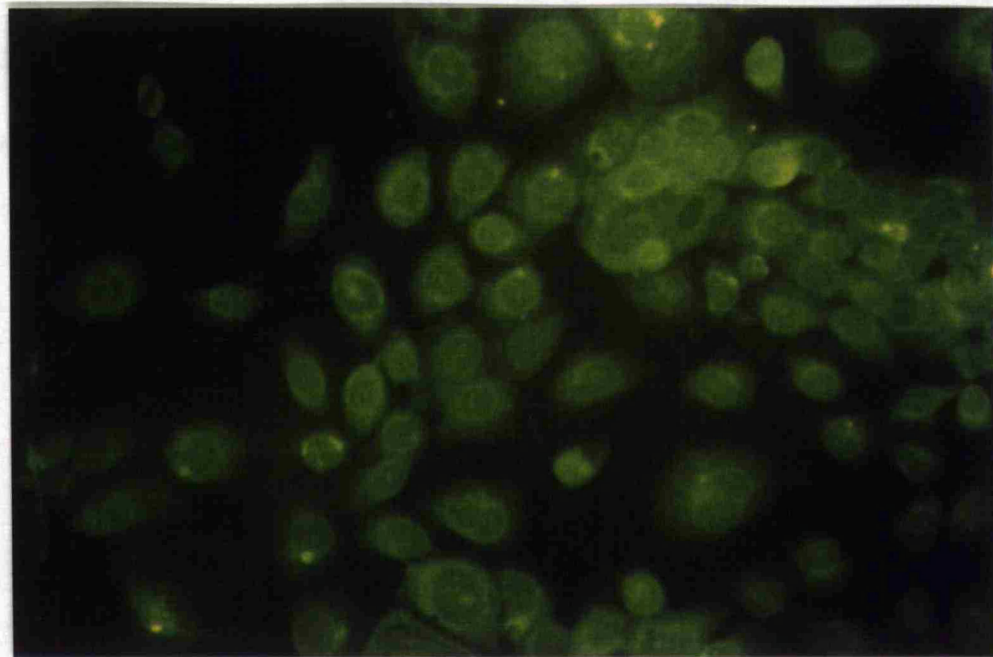


Figure 7.2: RHT keratinocytes (low magnification (x20)) following treatment with UVB (a) 0 mJ/cm² and (b) 36mJ/cm². Cells fixed immediately post-irradiation with methanol:acetone (1:1) and treated with antibody 529 and FITC labelled (see section 2.9.2). The cells have increased nuclear staining post UVB-irradiation.

as the carrier for the primary and secondary antibodies. Secondary antibody may bind non-specifically to Fc receptors. Therefore NGS was used as it blocks non-specific binding by saturating the receptors.

7.3.2 Peroxidase protocol

Cells treated with UVB and control cells were fixed with methanol:acetone and rehydrated with PBS. The coverslips were immersed in 0.3% (v/v) hydrogen peroxide in ultra-pure water (to eliminate endogenous peroxidase) for 15min at room temperature, then washed and equilibrated in PBS for 3min. All samples were blocked with 10% (v/v) NGS in PBS for 1 hour. Primary antibody 529 at a dilution of 1:2500 v/v in 2% NGS in PBS was applied and incubated overnight at 4°C. Samples were washed x3 with PBS and incubated with biotinylated anti-rabbit IgG at a 1:200 v/v dilution in 2% (v/v) NGS at 37°C for 45min. The samples were then washed x3 with PBS and covered with peroxidase streptavidin/biotin complex, which was made up with 1ml PBS, 1µl peroxidase-streptavidin plus 1µl biotin, and incubated for 30min at room temperature (the concentrations of the peroxidase-streptavidin complex and biotin had been adjusted by DAKO to give optimal response when mixed as recommended K377 section 2.1.5). The coverslips were then washed x2 with PBS and drained to remove excess buffer and covered with freshly prepared 3,3-diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide substrate. This substrate was prepared by combining 0.5ml of DAB (10mg/ml) plus 9.5ml PBS, filtering through a Whatman filter and activated with 0.1ml of 3% hydrogen peroxide v/v immediately prior to use. The sections were incubated for 5-15min in a covered chamber

and finally washed with tap water. The coverslips were then mounted onto a slide with a drop of glycerol and examined under a light microscope. See photographs Figure 7.3.

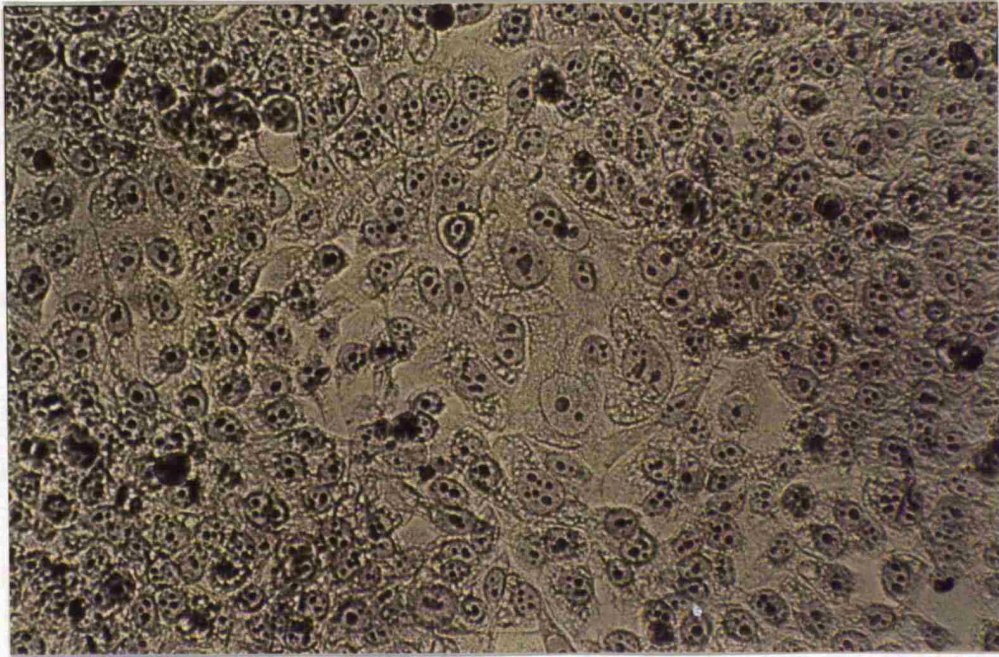
In the control (Figure 7.3a) the controls were only positive for haematoxylin; following UVB (Figure 7.3b) treatment the nucleus stained brown. The UVB dose was 0.9 MED a biologically relevant dose.

Protease treatment of the RHT cells post fixation was also examined. The coverslips were washed with PBS and equilibrated at 37°C for 5mins. The PBS was aspirated off and the coverslips were covered with 1ml of protease solution (0.05% w/v protease in PBS, pH 7.4) for 10min at 37°C. This proved to be too harsh a treatment as all the cells were washed off the coverslip when the protease was rinsed with PBS prior to being stained.

An AMCA labelled secondary anti-rabbit IgG antibody at a concentration of 1:100 was used instead of the FITC labelled secondary antibody but high background fluorescence was observed with this fluorescent marker and no differences between UVB treated (48mJ/cm²) and untreated cells were noted.

The antibody titre was also altered to compensate for the polyspecificity of the antibody. A concentration of antibody which was selective for the damage that was under investigation had to be used; too high a titre and not all the damaged sites would be picked up, too low a titre and non specific binding could occur. Dilutions such as 1:100, 1:500, 1:1000, 1:2500, 1:5000, were all examined and the final concentration selected was 1:5000. This provided the best staining pattern for RHT cells that were treated with UVB irradiation (0-48mJ/cm²).

a)



b)

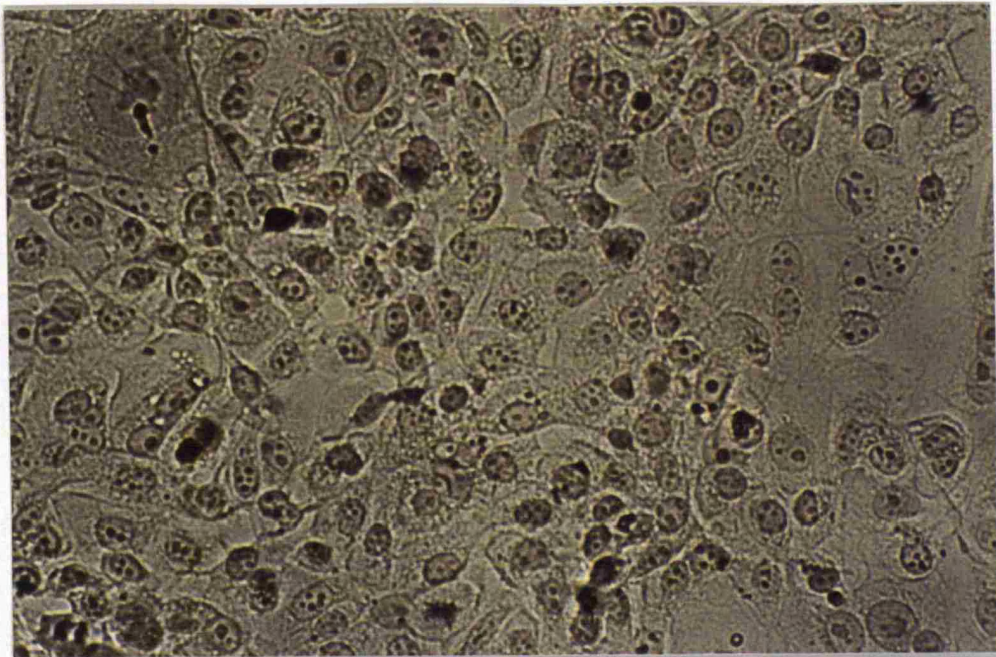


Figure 7.3: RHT keratinocytes (low magnification (x20)) following treatment with UVB (a) 0 mJ/cm² and (b) 36mJ/cm². Cells fixed immediately post-irradiation with methanol:acetone (1:1) and treated with antibody 529 and peroxidase labelled (see section 7.3.2). The cells were counterstained with haematoxylin and have increased nuclear staining post UVB-irradiation.

7.3.3 Immunostaining of RHT cells cultured in 96 well plates

The use of 96 well plates was developed as soon as the final method of fixation with methanol:acetone was decided upon. This provided a scaling up of the number of doses and time points that could be looked at within one experiment. The use of a fluorescence plate reader allowed this technique to be developed directly in plates. It was possible to measure quantitatively as opposed to qualitatively the increased antibody binding in relation to the dose of UV irradiation applied to the RHT cells (see section 7.5).

7.4 Methods

The RHT cells were grown up in media described in section 2.1.3.2 and routinely passaged every 3 days. The cells were grown on slides, coverslips, in 96-well plates and in petri-dishes (see sections 2.9.2, 2.9.3, 2.9.4). Prior to each experiment the cells were washed with Hank's balanced salt solution to remove any excess media. On irradiation the cells were generally fixed immediately (see section 2.9.2) apart from the parallel experiments carried out with the MTT cytotoxicity assay (1, 24, 48 hours)(see section 2.9.1) and for the flow cytometry work as the cells had to be put into a single cell suspension prior to fixation (~50min)(see section 2.9.4). The UVB irradiations were always carried out at room temperature and at a distance of 5cm from the cell layer and ranged from 3.3-85.5mJ/cm². The UVA irradiations were also carried out at room temperature and always carried out at a distance of 4cm from the cell layer and ranged from 1-5 J/cm². The statistical test used was the two sample t-test on the Statgraphics software.

7.5 Results

7.5.1 Qualitative observation of the effects of UVB irradiation on RHT cells examined using fluorescence microscopy

As outlined in the introduction (section 7.1) the polyclonal antibody 529 has been partially characterised; therefore UVB was the initial UV region investigated. UVB has been reported to induce thymine dimers in DNA and the antibody appears to have a propensity toward UV-irradiated poly T (Herbert *et al.*, 1994) in particular adjacent thymines and a third pyrimidine. Therefore the antibody would appear to be specific for classical UV damage. Initial experiments were carried out with UVB irradiation of cells on slides (see photographs section 7.3 and methods section 2.9.2) and an increased amount of binding was seen with increasing dose of UVB over a range of 3.3-85.5mJ/cm², which corresponds to biologically relevant doses of human exposure to sunlight.

7.5.2 Measurement of the effects of UVB irradiation on RHT cells using the antibody 529 fluorescence assay in parallel with the MTT assay

Parallel experiments were carried out to examine the effects of UVB irradiation (16.2, 32.5, 48.8mJ/cm²) on the level of antibody 529 binding and the level of mitochondrial activity, as an index of cell viability, according to the methods described in section 2.9.3 and 2.9.1 respectively.

One hour after irradiation with UVB, an initial decrease in mitochondrial activity was observed at 16.2 and 32.5mJ/cm² to 88 and 84% respectively in comparison to control (100%) (p<0.01)(Figure 7.4). However, an increase in activity was seen at 48.8mJ/cm² to 124% (p<0.01)(Figure 7.4). The antibody binding increased slightly at 16.2 and 32.5mJ/cm² to 106 and 105% respectively with a significant increase at 48.8mJ/cm² to 116% in comparison to control (100%) (p<0.01) when analysed immediately post irradiation (Figure 7.5).

Subsequently the RHT cells were irradiated at 16.2, 32.5 and 48.8mJ/cm² and incubated for 24 hours; the MTT and fluorescence assays were then carried out. The mitochondrial activity was reduced at all dose levels in comparison to control (unirradiated) (Figure 7.6). The mitochondrial activity of cells treated with 16.2 mJ/cm² (65%) (p<0.01 compared with control) was significantly lower than that of the cells treated with 32.5 mJ/cm² UVB (86%) (p<0.05) but similar to that for the 48.8mJ/cm² treated cells (59%)(p<0.01) (Figure 7.6). The corresponding antibody binding 24 hours post UVB irradiation had decreased significantly and in a dose-dependent manner at 16.2, 32.5, 48.8 mJ/cm² UVB to 88, 75 and 74% of control respectively (Figure 7.7)(p<0.05).

To examine the inter-experimental and processing variability for antibody binding two separate experiments were carried out on two separate microtitre culture plates. There was a dose-dependent increase in antibody staining response with 0-48.8 mJ/cm², the antibody binding increased with increasing dose of UVB to 109, 116 and 122% respectively (p<0.05) for all doses compared to control (Figure 7.8). Furthermore the two dose response curves were very similar indicating good reproducibility.

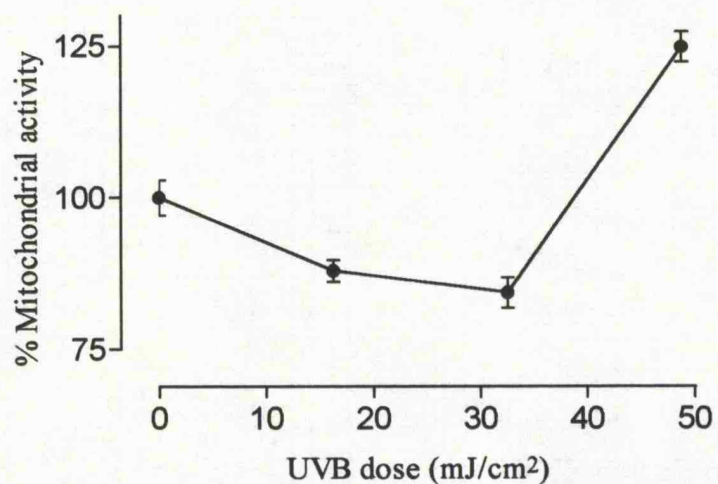


Figure 7.4: MTT assay of UVB treated RHT cells at 1 hour post treatment. Values represent mean and SEM (n=16).

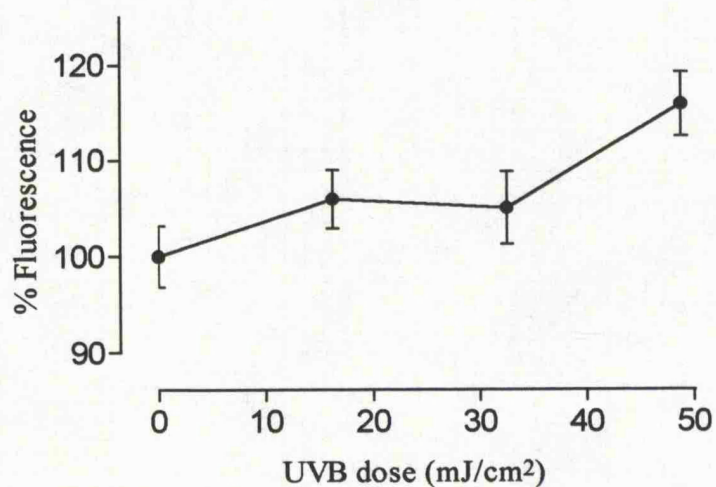


Figure 7.5: Antibody 529 binding to RHT cells at zero time post UVB irradiation. Values represent mean and SEM (n=16).

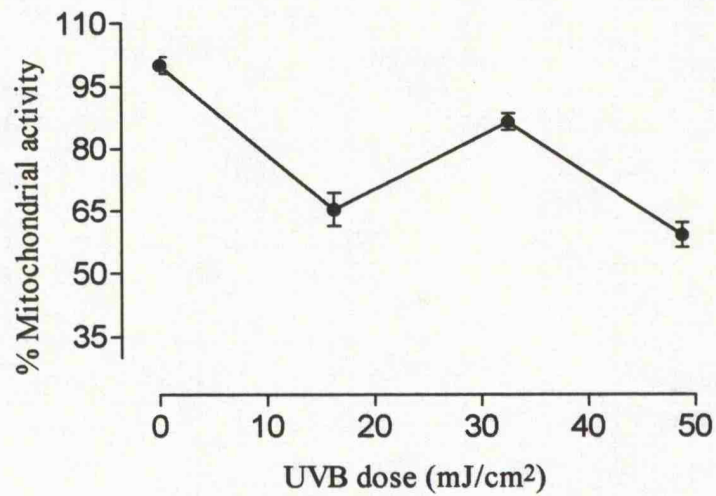


Figure 7.6: MTT assay of UVB treated RHT cells at 24 hours post irradiation. Values represent mean and SEM (n=16).

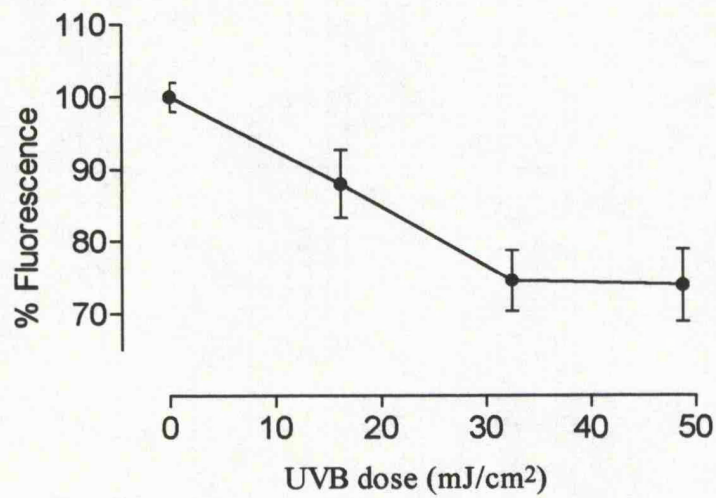


Figure 7.7: Antibody 529 binding to RHT cells at 24 hours post UVB irradiation. Values represent mean and SEM (n=16).

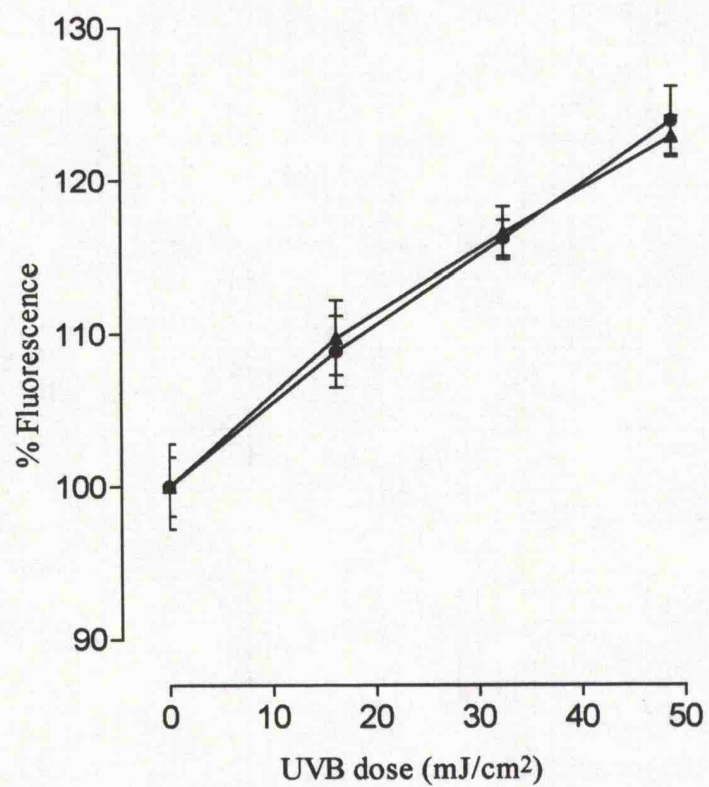


Figure 7.8: Antibody 529 binding to RHT cells at zero time post UVB irradiation in two separate experiments. Values represent the mean and SEM.

7.5.3 Measurement of the effects of UVA irradiation on RHT cells using the antibody 529 fluorescence assay in parallel with the MTT assay

Previous experiments carried out in the laboratory on differentiated IMR-32 cells, a neuronal cell line, had suggested that antibody 529 could detect DNA damage caused by UVA irradiation (Thomas *et al.*, 1994). On pre-incubation of these cells with α -tocopherol (200 μ M)(lipid soluble antioxidant) and desferrioxamine (200 μ M)(iron chelator) for a period of 24 hours the level of antibody 529 binding to UVA treated cells was reduced (Thomas *et al.*, 1994). This implied that the antibody 529 was capable of detecting oxidant induced damage as it had been reported in the literature that UVA causes DNA damage normally associated with oxygen free radicals (Tyrrell, 1992).

Following UVA treatment of RHT cells, an MTT assay was performed and concomitant antibody binding was determined at 1, 24 and 48 hours post irradiation. At 1 hour post treatment the mitochondrial activity showed a UV-dose-dependent decrease from 1-5 J/cm² ($p < 0.01$ compared to control) (Figure 7.9). The mean mitochondrial activity decreased from 96% at 1 J/cm² to 41% at 5 J/cm² (Figure 7.9). Conversely, the antibody binding at 1 hour post irradiation showed a UV-dose-dependent increase from 3-5 J/cm² ($p < 0.05$ compared to control) (Figure 7.10). Antibody binding increased from 110% at 1 J/cm² to 140% at 5 J/cm² (Figure 7.10). At 24 hours post irradiation the mitochondrial activity at all doses was below control level with 0>1>2>3 J/cm² at 100, 98, 92, and 89% respectively. The doses at 2 and 3 J/cm² were significantly lower than control ($p < 0.05$ for both). However at 4 and 5 J/cm² the levels of mitochondrial activity (95% and 93% respectively) were not significantly different from the control (Figure 7.11). Antibody binding at 24

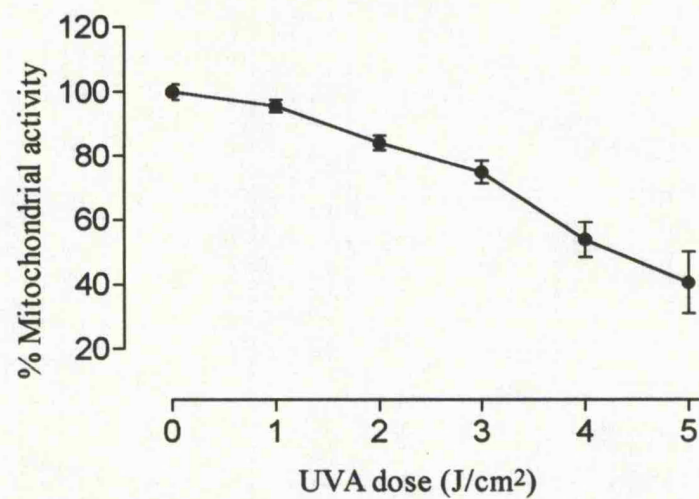


Figure 7.9: MTT assay of UVA treated RHT cells 1 hour post treatment. Values represent mean and SEM (n=16).

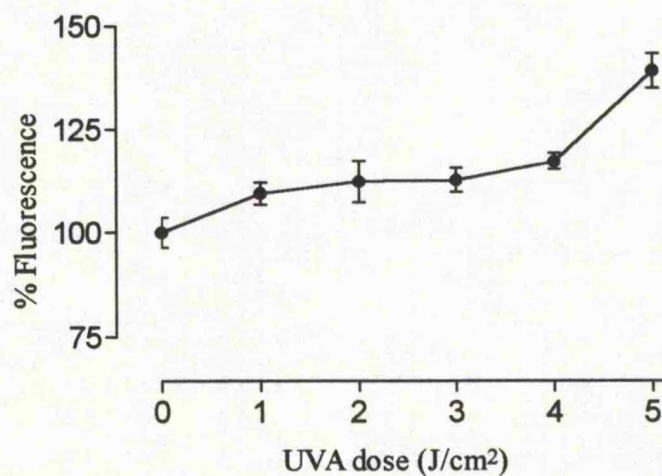


Figure 7.10: Antibody 529 binding to UVA treated RHT cells 1 hour post irradiation. Values represent mean and SEM (n=16).

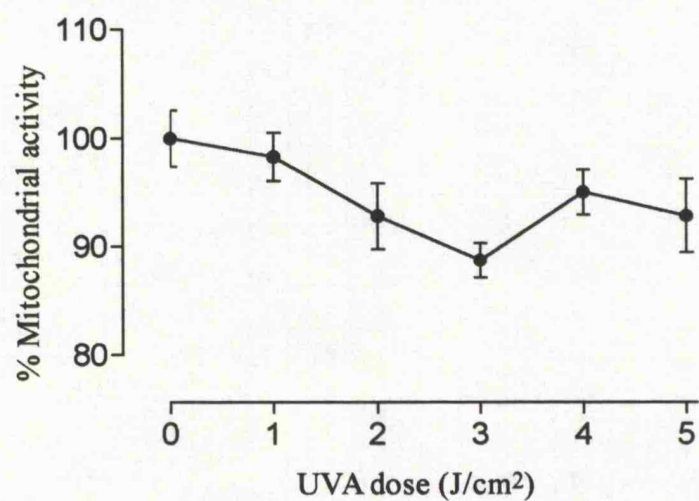


Figure 7.11: MTT assay of UVA treated RHT cells 24 hours post irradiation. Values represent mean and SEM (n=16).

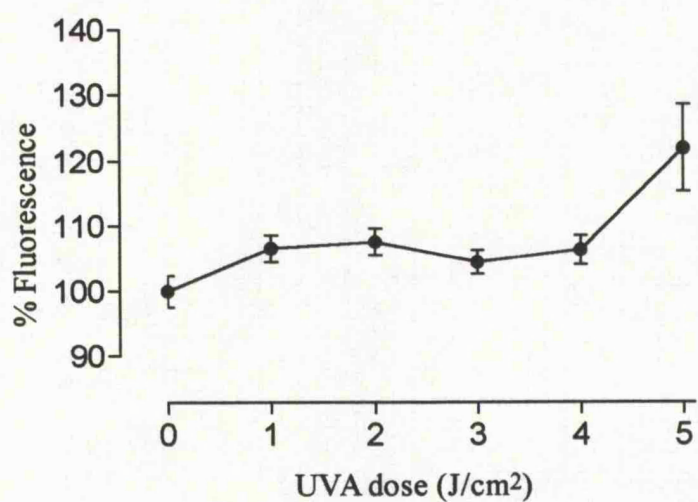


Figure 7.12: Antibody 529 binding to UVA treated RHT cells 24 hours post irradiation. Values represent mean and SEM (n=16).

hours post UVA irradiation was slightly higher than control levels with $0 < 1 < 2 < 3 < 4 \text{ J/cm}^2$ at 100, 107, 108, 105, and 106% respectively, with 122% at 5 J/cm^2 in comparison to control (Figure 7.12).

At 48 hours post UVA irradiation the mitochondrial activity of the RHT cells at all doses was below 80% of control levels (Figure 7.13) ($p < 0.01$ for all doses). There was a significant difference between 1 J/cm^2 at 68% and all the higher doses ($p < 0.05$). However there was no significant difference between 2, 3, 4, 5 J/cm^2 , where the levels of mitochondrial activity were 75, 75, 76 and 78% of control respectively (Figure 7.13). Antibody binding at 48 hours post irradiation was significantly higher than control ($p < 0.05$ for all doses) but there were no significant differences between the different UVA doses; at 1, 2, 3, 4, 5 J/cm^2 the levels were 108, 109, 109, 113 and 109% respectively (Figure 7.14).

7.6 Discussion

These experiments were carried out to ascertain the ability of an antibody raised to UV damaged DNA to detect UVB and UVA-induced DNA damage in a human keratinocyte cell line (RHT cells). The keratinocytes were treated with either UVB or UVA and the percentage of antibody 529 binding, as an indicator of DNA damage, and the percentage mitochondrial activity using the MTT assay, as an indicator of cell viability, were measured. The RHT cells are a transformed cell line and therefore are not terminally differentiated. This complicates the interpretation of the MTT assay at longer periods post irradiation, as the cells are still dividing. It may appear that the cells have recovered when in fact they have divided to fill the space vacated by dead cells. The MTT assay is limited as a

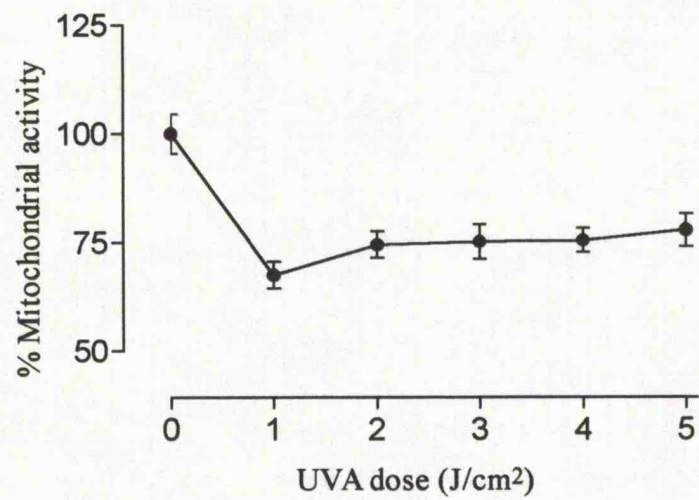


Figure 7.13: MTT assay of UVA treated RHT cells at 48 hours post irradiation. Values represent mean and SEM (n=16).

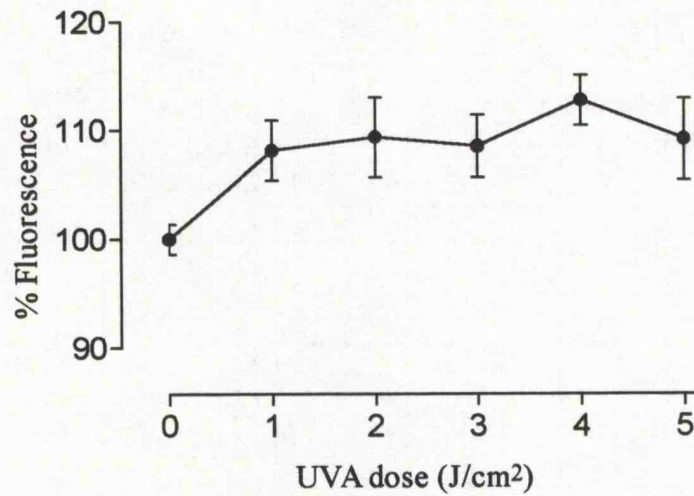


Figure 7.14: Antibody 529 binding to UVA treated RHT cells 48 hours post irradiation (n=16).

cytotoxicity assay as it compares the level of mitochondrial activity of treated cells with control levels. When this assay was applied to a continuously dividing cell line, as opposed to terminally differentiated cells, the cells may have remained static but may not in fact have died. Therefore the use of the MTT assay in a transformed cell line requires close monitoring of cell numbers which can be achieved by performing a protein assay at all dose points (Berridge and Tan, 1993).

UVB damage was assessed initially as an indicator of the selectivity and sensitivity of the antibody, and to allow a fixation and staining protocol to be developed for the RHT keratinocyte cell line. The UVB (290-320nm) portion of the solar spectrum possesses the highest activity for the induction of skin cancer and has the capacity to stimulate epidermal proliferation (Shah *et al.*, 1993). UVB is expected to affect membranes, proteins and nucleic acids. For example it inhibits epidermal growth factor binding to its surface receptor suggesting a membrane effect (Matsui *et al.*, 1989). It induces also the photodimerization of neighbouring pyrimidines in DNA via electronic excitation as well as base damage and DNA strand breakage by oxidative processes (Hariharan *et al.*, 1977; Hirschi *et al.*, 1981; Niggli *et al.*, 1983). Therefore, the overall biological consequences of UVB are expected to result from superimposition of its effects on multiple cellular targets. In the set of experiments carried out using UVB, it was found that at 16.2 and 32.5mJ/cm² the mitochondrial activity had decreased at 1 hour post irradiation but had increased at 48.8mJ/cm² (7.5.2). The minimal erythral dose (MED) of UVB irradiation is 30-40mJ/cm² (Parrish, 1983). The MED is defined as the UV dose that causes skin reddening up to 24 hours in the irradiated area of the skin (Parrish, 1983). In experiments with the RHT cells the initial dose that caused 58% cytotoxicity (IC₅₀) for UVB was 48.8mJ/cm²,

which is approximately 1 MED and therefore a biologically relevant dose. The high dose of 48.8 mJ/cm^2 caused an initial increase at 1 hour post irradiation in mitochondrial activity which was considerably reduced at 24 hours post irradiation in comparison to control, indicating cell death. The data appeared to show an initial respiratory burst as the RHT cells respond to the insult, but at 24 hours it appears that the damage was too great for the cells defence capacity. The binding of the antibody 529 at the highest dose (48.8 mJ/cm^2) therefore corresponded to the stimulation of mitochondrial activity at 1 hour post irradiation, suggesting a relationship between induction of thymine dimerisation in DNA and the stress response of the cells. However 24 hours later apparent loss of cell viability at all doses resulted in dose-dependent loss of antibody 529 binding; at this time point lowering of antibody binding resulted from the effects of DNA repair and loss of dead cells from the wells.

UVA damage to DNA has been reported to be caused by oxygen free radicals (Miyachi *et al.*, 1983). The data presented in this system on UVA damage implied the antibody 529 is capable of detecting oxidatively damaged DNA. This was also demonstrated previously in IMR-32 cells (Thomas *et al.*, 1994), however the epitopes responsible have not yet been identified. The RHT cells showed a decrease in mitochondrial activity with increasing dose of UVA from $1\text{-}5 \text{ J/cm}^2$, concomitantly there was a dose-dependent increase in antibody binding at one hour post irradiation. This decrease in mitochondrial activity and increase in antibody 529 binding, in comparison to controls, was maintained at 24 and 48 hours post UVA irradiation. Initially it is likely that the MTT and antibody changes measured are cytotoxicity due to UV-induced DNA damage. In the later time points the DNA damage may be due to the process of cell death. The minimal erythral dose for UVA is 80 J/cm^2 .

In our experiments with the RHT cells the IC_{50} for cytotoxicity by UVA was 4.3 J/cm^2 . Therefore the RHT cells appeared to be more susceptible to UVA than to UVB when compared on the basis of minimal erythema dose.

7.7 Effects of UVA irradiation on RHT cells: the level of antibody 529 binding, mitochondrial activity and the level of oxidative damage in DNA

In order to investigate the specificity of antibody 529, the levels of antibody binding to cells *in situ* were correlated with the levels of a specific marker of oxidative DNA damage, 8-oxoguanine (Arouma and Halliwell, 1995), in the DNA isolated from RHT cells treated with UVA. UVA irradiation has been shown to cause oxidative damage to DNA in cells through indirect mechanisms involving sensitizers such as porphyrins, NADPH and flavins present in the cell (Cadet *et al.*, 1992 see section 1.7 for a review). Certain human diseases are either initiated or aggravated by exposure to sunlight. Systemic lupus erythematosus is an example, and this disease is characterised by the presence of serum antibodies to DNA (Tan and Stoughton, 1969) which react particularly well with DNA damaged by ROS (Blount *et al.*, 1990). The antibody 529 had been shown in earlier experiments (section 7.5.3), to be an indicator of UVA initiated damage in keratinocytes. Therefore levels of 8-oxoG were compared with antibody 529 binding to compare the efficacy of the HPLC guanine assay with the antibody 529 immunoassay.

7.7.1 Aim

The aim of these experiments was to relate the level of antibody 529 binding with the level of 8-oxoguanine in DNA from RHT keratinocytes treated with increasing levels of UVA irradiation. This would validate the use of antibody 529 in detection of oxidative damage to DNA in cells, *in situ*.

7.7.2 Methods

The RHT cells were grown up in media described in section 2.1.3.2 and routinely passaged every 3 days. The cells were grown in 96-well plates and petri-dishes (see section 2.9.3 and 2.9.4). Prior to each experiment the cells were washed with HBSS to remove any excess media. Following irradiation in 96-well plates, the cells were replenished with complete media for 1 hour so that the fluorescence assay was comparable with the MTT cytotoxicity assay (2.9.1). Media was then aspirated off and the plates washed with HBSS prior to fixation with MeOH:Acetone (see section 2.9.3). These experiments were carried out in parallel with the MTT cytotoxicity assay (see section 2.9.1). For the cells that would undergo the pronase E DNA isolation and the guanase assay (see sections 2.4.1 and 2.8) the cells were removed from the dishes by the addition of 0.02% EDTA in PBS for 40 mins. Each petri-dish contained approximately 5×10^6 cells and 6 dishes were pooled per dose point (total 30×10^6 cells). The cells were rinsed with PBS and centrifuged at 400g for 10 mins at 4°C. The DNA was isolated from the RHT cells using the pronase E method of isolation with the inclusion of an RNase step (see section 2.4.1). The DNA was then hydrolysed using formic acid (see section 2.8) followed by guanase digestion of an aliquot

(250 μ l). The DNA hydrolysates, guanase treated and untreated were analysed using the HPLC-ECD method described in section 2.8. Four separate experiments were conducted. Parallel experiments were carried out to examine the effects of UVA irradiation (0-3J/cm²) on the level of antibody 529 binding, the level of mitochondrial activity and the level of 8-oxoG formation according to the methods described in 2.9.3, 2.9.1 and 2.8 respectively. With respect to the antibody 529 fluorescence assay, a directly labelled secondary antibody (goat anti-rabbit FITC) was utilised in these experiments. A two step procedure utilising a biotinylated secondary antibody, detected by avidin-FITC was previously used. However there was some concern about the high background binding in these experiments.

7.7.3 Results

At 1 hour post treatment, the mitochondrial activity was unaffected at a dose of 1J/cm² 101% (SEM=6.5)(Figure 7.15). At 2J/cm² the mitochondrial activity had decreased slightly but not significantly to 96.5% (SEM=5.7). However at 3J/cm² the mitochondrial activity had decreased (but not significantly) from a control value of 100% (SEM=9.4) to 84.7% (SEM=4.5)(Figure 7.15).

The antibody 529 binding at 1 hour post-irradiation showed no difference in binding irrespective of dose (0, 1, 2 and 3J/cm²)(Figure 7.16). The antibody binding in comparison to control was 99.1% (range=95 - 103), 100.6% (range=97 - 104), 97.5% (range=94 - 101) and 98.8 a.f.u (range=94 - 104) for 1, 2 and 3 J/cm² respectively (Figure 7.16). Thus from this data in contrast to that presented in section 7.5.3 it appears that antibody 529 does not

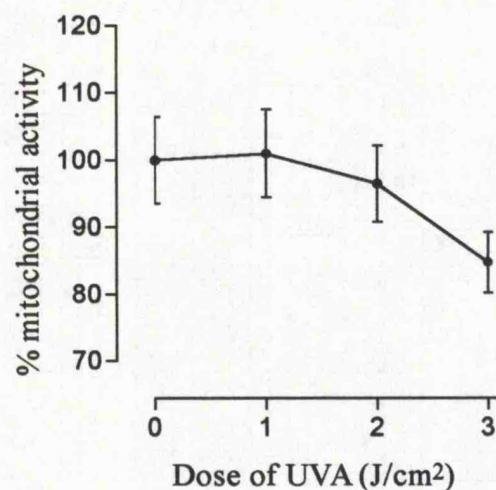


Figure 7.15: Effect of increasing doses of UVA irradiation on the percentage mitochondrial activity of RHT cells 1 hour post treatment (n=4).

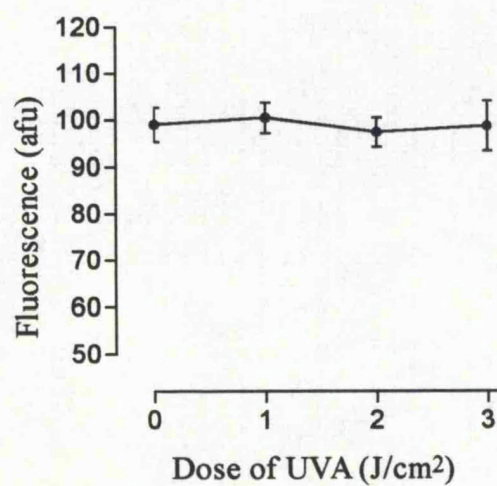


Figure 7.16: Effect of increasing doses of UVA irradiation on the level of antibody 529 binding to RHT cells 1 hour post treatment. Each value represents the mean with range (n=2).

detect UVA damaged DNA in keratinocytes; the difference being the methodology used to detect antibody binding.

Using the guanase assay, an increase in the levels of 8-oxoG was observed from a control value of 0.041 (SEM=0.005) nmoles 8-oxoG/mg DNA to 0.095 (SEM=0.007) nmoles 8-oxoG/mg DNA at 1J/cm² UVA irradiation ($p<0.01$)(Figure 7.17) to 0.12 (SEM=0.26) nmoles 8-oxoG/mg DNA at 2J/cm²(Figure 7.17). At 3J/cm² of UVA irradiation the level had increased to 0.133 (SEM=0.029) nmoles 8-oxoG/mg DNA (Figure 7.17). Thus over the dose range of 1-2 J/cm², an increasing trend in the level of 8-oxoG in DNA isolated from RHT keratinocytes was shown prior to an increase in cell death determined using the MTT assay.

7.7.4 Discussion

This set of experiments was designed to determine if there was a relationship between the level of antibody 529 binding and oxidative DNA damage measured using the guanase assay, with increasing doses of UVA radiation. It was found that at a 1 in 5000 dilution and using a directly labelled secondary antibody that no detectable change in antibody 529 binding was observed over a range from 0-3J/cm². The minimal erythral dose that causes skin reddening in normal skin 24 hours post UVA irradiation is approximately 80J/cm². The dose used here ranged from a minimum of 1J/cm² (1/80 of an MED) to a maximum of 3J/cm² (1/27 of an MED). In the earlier experiments outlined in section 7.5.3 the IC₅₀ for cytotoxicity for UVA was 4.3J/cm². It was decided to use sublethal doses in this set of experiments. This would allow a direct correlation between damage and dose of UVA

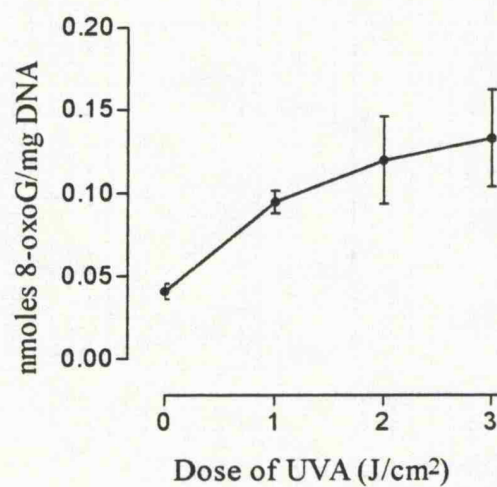
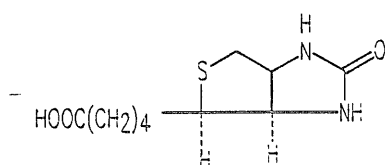


Figure 7.17: Effect of increasing doses of UVA irradiation on the level of 8-oxoguanine in DNA isolated from RHT keratinocytes. Each value represents the mean and SEM (n=3).

without the added complexities of taking cell death into consideration. From the earlier work carried out by Herbert *et al.*, (1994) on the characterisation of 529 it was shown that the main epitope formed by UV irradiation was adjacent thymines and either a 3' or a 5' pyrimidine. In a competition ELISA carried out by Miss Nalini Mistry, of the Division of Chemical Pathology, it was found that the antibody bound to DNA which had been subjected to UVA and UVC irradiation to the same extent as to UVC alone. Furthermore the binding to DNA treated with UVA appeared the same as to native DNA. On a comparison between native and UVA irradiated, single stranded and double stranded DNA it was found that the antibody 529 bound to single stranded DNA greater than to double stranded DNA. This was to be expected since the antigen used to raise the antibodies was UV-single-stranded DNA (Herbert *et al.*, 1994). In the literature it has been suggested that an antibody to single stranded DNA may be used as an indicator of DNA damage (Van der Schans *et al.*, 1989). Therefore the antibody 529 may be utilised to detect single stranded DNA in the nuclei of cells in addition to damage caused by UVB irradiation. In the earlier set of experiments, see section 7.5.3, at the same dilution of primary antibody 529, but utilising a two step secondary antibody amplification, there was an apparent dose response for UVA from 2-5J/cm². This is a subject of further investigations in the Division of Chemical Pathology at the University of Leicester. It has been suggested that the avidin component recognises 8-oxoG in the DNA (patent applied for); this is proposed to be due to the similarity in structures of biotin and 8-oxoG (see structures Figure 7.18).

An increasing trend was seen on measurement of 8-oxoG levels in the DNA isolated from RHT cells treated with increasing doses of UVA (see Figure 7.17). At 0, 1, 2 and 3J/cm² the levels were 13, 30, 37 and 41 residues of 8-oxoG per 10⁶ DNA bases. The background

Biotin



8-Oxoguanine

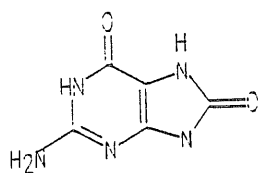


Figure 7.18: Structure of biotin and 8-oxoguanine.

level observed was closer to the baseline levels observed for 8-oxodG as determined by HPLC than for the background levels observed for GC/MS (Halliwell, 1993). For baseline 8-oxodG levels in rat kidney DNA ~6-12 8-oxodG per 10^6 bases was measured by Fraga *et al.*, (1990) using HPLC. Using GC/MS the baseline levels of 8-oxoG measured in chromatin isolated from murine hybridoma cells were 35-40 8-oxoG per 10^6 bases (Dizdaroglu *et al.*, 1991b). In a review by Arouma and Halliwell, (1995) it is discussed that formic acid hydrolysis has been shown not to cause artefactual damage to DNA but little is known about the derivatisation procedure and what effect it may have on causing artefactual damage to DNA prior to measurement via GC/MS. DNA isolated from cells was analysed for 8-oxoG using the HPLC assay described in chapter 6.0: such experiments had not been reported previously in the literature. It would appear that UVA causes the increased formation of the 8-oxoG lesion when treated with levels of UVA that did not appear to cause any cell death as indicated by the MTT assay, that is $1-2\text{J}/\text{cm}^2$. Thus from these experiments it was found that the antibody 529, at the dilution used, did not detect UVA damage in RHT cells, although UVA irradiation caused increasing levels of 8-oxoG in a dose dependent manner in DNA isolated from RHT cells. As far as the literature is concerned this is the first time that an increasing trend in the level of 8-oxoG in DNA isolated from keratinocytes treated with UVA alone has been observed. In order to investigate the effect of UVA further, alterations in the amount of p53 present in RHT cells relative to the amount of UV exposure and damage induced were examined. To elucidate further the utility of antibody 529 as a marker of DNA damage, perhaps by an increase in the amount of single stranded DNA or by thymine dimer formation by UVA, it was used in tandem with an antibody to p53. These experiments are described in chapter 8.0.

Chapter 8

**Investigation of the relationship between UVA
irradiation and levels and type of DNA damage
and levels of the p53 protein in keratinocytes**

3.1 Measurement of the effects of UVA irradiation of RHT cells on the levels of p53 protein and antibody 529 binding using flow cytometry

The p53 tumour suppressor protein is thought to play a major role in the defence of the cell against agents that damage DNA. The p53 tumour suppressor protein is a nuclear phosphoprotein that is activated in response to a variety of DNA damaging agents (Milne *et al.*, 1995). Activation of p53 leads to cell growth arrest at the G₁/S boundary or the induction of apoptosis, thereby preventing the proliferation of genetically damaged cells. Loss of p53 suppressor function through mutation is a common event in the development of a wide variety of human cancers and may contribute to an increase in the number of genetic abnormalities (Milne *et al.*, 1995). In addition to blocking cell cycle progression and helping trigger programmed cell death, p53 may directly and indirectly stimulate the DNA repair machinery of the cell (Marx, 1994). In its mutated form p53 has been found in 50% of all human cancers and ~60% of all skin cancers. The antibody utilised in these experiments was to both the mutated and wild type p53. The IgG portion of the 529 antibody was prepared by N. Mistry, Division of Chemical Pathology, University of Leicester (see section 2.13) and was utilised in all the flow cytometry experiments. In this form and at a relatively high concentration (0.8mg/ml IgG) the antibody would be expected to bind to single-stranded regions of native DNA. In cells these single-stranded regions could be induced by single strand breaks (Timmerman *et al.*, 1995).

Flow cytometry is a technique for making rapid measurements on particles or cells as they flow in a fluid stream one by one through a sensing point. The important feature of flow cytometric analysis is that measurements are made separately on each particle within the

suspension in turn and not just as average values for the whole population. The cellular parameters i.e. the antibody binding to the nucleus and the cell cycle analysis are carried out using fluorescent markers. The primary antibody was detected using a fluoresceinated secondary antibody, and the fluorescent dye propidium iodide was used to detect nuclear material. These were measured using separate lasers, this allowed the simultaneous analysis of the cell cycle and the level of antibody binding. To allow a minimisation of the variables within and between experiments due to different numbers of cells being in different stages of the cell cycle, the cells were synchronised using 48 hour serum deprivation.

3.2 Aim

To investigate the relationship between DNA damage, detected immunocytochemically, and the level of p53 protein in a human keratinocyte cell line (RHT) exposed to UVA irradiation.

3.3 Flow cytometry

In developing an experimental system for looking at the stages in the cell cycle of RHT cells, many important factors had to be dealt with. The RHT cell line grows as an adherent cell line, therefore to make it into a single cell suspension it was necessary to put the cells through a rigorous and lengthy harvesting protocol as outlined in section 2.9.4. As a result time zero was at plus 50min.

3.3.1 Reduction of s-phase in RHT cells

In order to examine the various stages in the cell cycle it was important that the cells were synchronised. Initial experiments to reduce the level of cells that were dividing (in s-phase) using hydroxyurea and serum deprivation were carried out. The results of these showed that hydroxyurea did not reduce the level of s-phase in the cell sufficiently (see Table 8.1). Following 1.5 hours incubation with hydroxyurea, the s-phase was 17.3% and the percentage of cells in control in s-phase was 17.4% (Table 8.1). At 3.5 and 6.5 hours incubation with hydroxyurea the s-phase was reduced to 15.4% and 16.6% respectively. After 8.5 hours incubation with hydroxyurea the number of cells in s-phase had reduced to 15.7% which was not significantly less than control levels. As the experiments being developed involved the irradiation of cells with UV it was noted from the literature that hydroxyurea forms a complex with DNA and is sensitive to UV irradiation. Therefore, due to the inability of the hydroxyurea to greatly reduce the levels of s-phase at the concentration of $7.5 \times 10^{-3}M$ in RHT cells and in the light of our future experiments to examine DNA damage involving UV irradiation, it was decided that serum deprivation experiments should be carried out.

Serum deprivation of RHT cells involved using medium without foetal calf serum and nitogens, as normally used for the culturing of these cells. Serum deprivation was carried out for a 24 hour period, the cells were harvested as a single cell suspension (section 2.9.4), stained and then stained with propidium iodide and examined on the FacScan (section 2.9.5 and 2.9.6). Serum deprivation for 24, 25, 26 and 27 hours was examined. It was found that the 27 hour period of serum deprivation reduced the percentage of cells in the s-phase

the greatest extent, from a control level of 11.3% to 5.4% (Table 8.2). The serum deprivation experiments were lengthened to 48 hours which resulted in reduction of the s-phase level in the cells to below ~3% when compared to a normal level of 19% and a level of ~6% at 24 hours serum deprivation see results section 8.5 and tables 8.1 and 8.2.

8.3.2 Optimisation of antibody titre for flow cytometry

The optimum antibody titres for 529, p53 and secondary antibodies were defined using the flow cytometer prior to experimentation. The appropriate controls were also carried out: cells with or without antibody and cells with or without UV irradiation. The antibody titre used for p53 was as recommended by DAKO for immunocytochemistry (1/100). The difficulty in choosing the correct dilution for 529 arose from the fact that it was a polyclonal antibody, therefore as the titre was reduced more non-specific binding was observed. According to Carter (1988), the recommended level of a monoclonal antibody for flow cytometry work was 1.0 µg protein/10⁶ cells. This dilution however, was found to be suitable for the polyclonal 529 antibody due to the fact that on UVA irradiation of RHT cells an increase in 529 binding was observed (see section 8.4). The dilution for the secondary antibodies which were a goat anti-rabbit IgG-FITC labelled (whole molecule) for antibody 529 and a goat anti-mouse IgG-FITC labelled, Fc-specific, for anti-p53 were as recommended by the manufacturers for immunocytochemistry, (1/80) and (1/160) respectively.

Table 8.2:

This table shows the percentage of RHT cells in various stages of the cell cycle. The cells were either incubated with complete medium, RM+ which contained 1% mitogens and 10% heat inactivated foetal calf serum and DMEM:Ham's F12 (3:1), or with RM- medium, containing DMEM:Ham's F12 (3:1) alone. The cells were harvested as a single cell suspension and analysed after treatment with the nuclear stain propidium iodide on the FacScan.

Treatment time (hours)		% RHT cells			
RM-	RM+	Sub G ₀ -G ₁	G ₀ -G ₁	S-phase	G ₂
27	-	6.1	75.7	5.4	7.9
24	3	6.3	72.9	6.7	9.1
25	2	7.2	72.5	7.0	9.0
26	1	7.0	72.7	6.4	8.5
-	27	11.7	62.6	11.3	10.7

3.4 Methods

Human, SV40-transformed, keratinocytes (RHT cells) were cultured in media according to section 2.1.1. The cells were synchronised by serum deprivation for 48 hours prior to irradiation with UVA at 0.5 or 1J/cm² (section 2.9.4). The cells were harvested as a single cell suspension and fixed with MeOH: acetone (section 2.9.4). Cells were then probed with the 529 antibody to UV-damaged DNA (rabbit polyclonal, IgG fraction) and mouse monoclonal anti-p53 protein antibody (clone DO-7, which detects both wild type and mutated forms of the protein) using fluorescein-labelled secondary antibodies (section 2.9.5). Propidium iodide was used to detect nuclear material which indicated stage of cell cycle. Quantitation of antibody binding was performed by a Becton Dickinson FACScan flow cytometer.

3.5 Results

To examine the effects of UVA on RHT cells, two doses were chosen, 0.5 and 1J/cm², that would not cause significant cell death according to data presented in section 7.5. Figures 8.1 and 8.2 show the levels of 529 and p53 binding, respectively, at the UVA dose of 0.5J/cm² at 0, 1, 2, 3 and 24 hours post reintroduction of complete medium. Fluorescence was expressed as a percentage of the control fluorescence at zero J/cm². On irradiation at 1 and 2 hours post reintroduction of complete medium, the binding had decreased from a control value of 112% (SEM=21) to 96% (SEM=17) and 88% (SEM=16) for 529 and from a control value of 100% (33) to 92% (SEM=10) and 86% (SEM=10) for p53. Levels of 529 and p53 binding were maximal at 3 hours post reintroduction of complete medium,

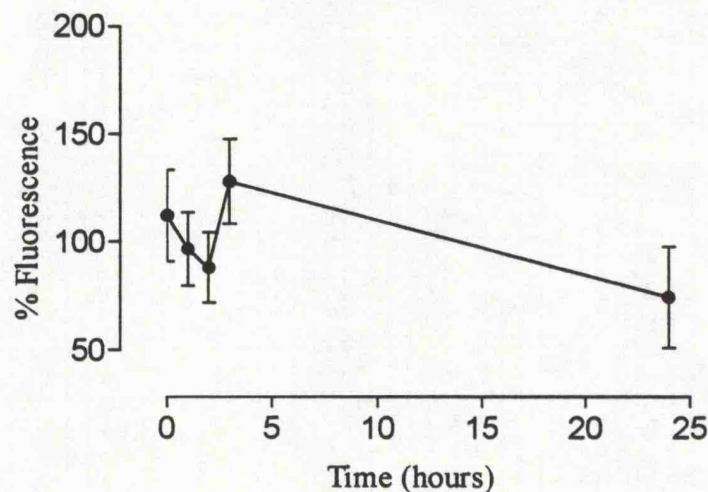


Figure 8.1: This graph represents the percentage antibody 529 binding to RHT cells treated with 0.5J of UVA irradiation at various times post reintroduction of serum. Values represent the mean and SEM for five separate experiments and are normalized to the amount of fluorescence in untreated cells at each time point.

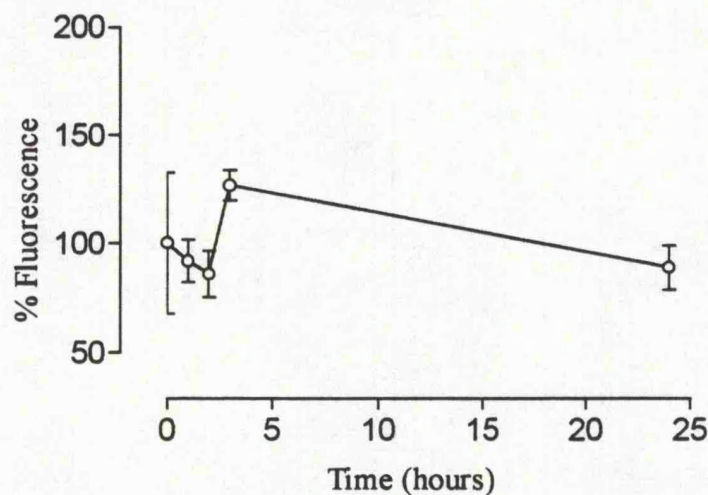


Figure 8.2: This graph represents the percentage antibody p53 binding to RHT cells treated with 0.5J of UVA irradiation at various times post reintroduction of serum. Values represent the mean and SEM for four separate experiments and are normalized to the amount of fluorescence in untreated cells at each time point.

128% (SEM=19), 127% (SEM=7) respectively; although this did not reach statistical significance for either antibody. On irradiation with 0.5J/cm² UVA 24 hours after reintroduction of complete medium, 529 binding had decreased to 75% (SEM=23) from a control value of 112% (SEM=21) and p53 binding had decreased to 89% (SEM=10) from 100% (SEM=33).

Figures 8.3 and 8.4 show the levels of 529 and p53 binding, respectively, at the UVA dose of 1J/cm² post reintroduction of complete media. On irradiation with 1J/cm² at 1 and 2 hours post reintroduction of complete medium the binding had decreased from a control value of 105% (SEM=10) to 96% (SEM=29) and 77% (SEM=17) respectively for antibody 529. In a similar manner to 0.5J/cm² UVA, levels of 529 and p53 binding were maximal at 3 hours post reintroduction of complete medium, at 127% (SEM=34) and 119% (SEM=18) respectively, although this did not reach statistical significance. Another smaller increase for p53 binding occurred one hour after reintroduction of medium and irradiation at 1J/cm² (92%, SEM=6) compared to zero irradiation which was 74% (SEM=23). However this decreased at 2 hours to 69% (SEM=13). On irradiation with 1J/cm² 24 hours after reintroduction of complete medium 529 binding had decreased to 89% (SEM=26) from control value of 105% (SEM=10); p53 binding had increased to 100% (SEM=20) from 74% (SEM=23). The overall trends therefore were for greater binding of p53 and 529 on irradiation three hours post reintroduction of complete medium. These data show that levels of p53 and 529 binding correlate and vary according to the time at which irradiation occurs following introduction of complete medium.

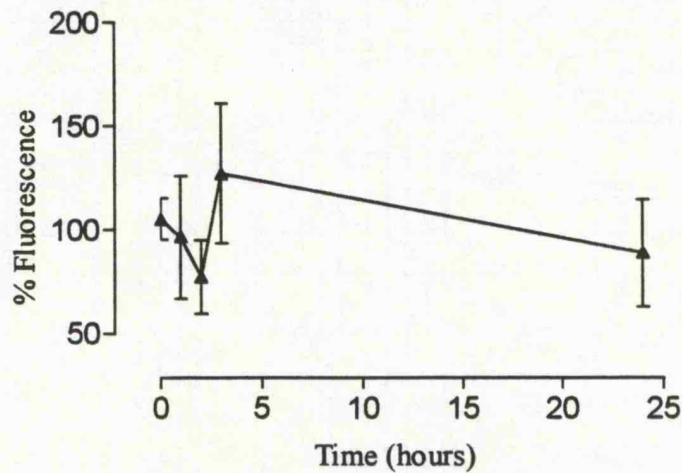


Figure 8.3: This graph represents the percentage antibody 529 binding to RHT cells treated with 1J of UVA irradiation at various times post reintroduction of serum. Values represent the mean and SEM for three separate experiments and are normalized to the amount of fluorescence in untreated cells at each time point.

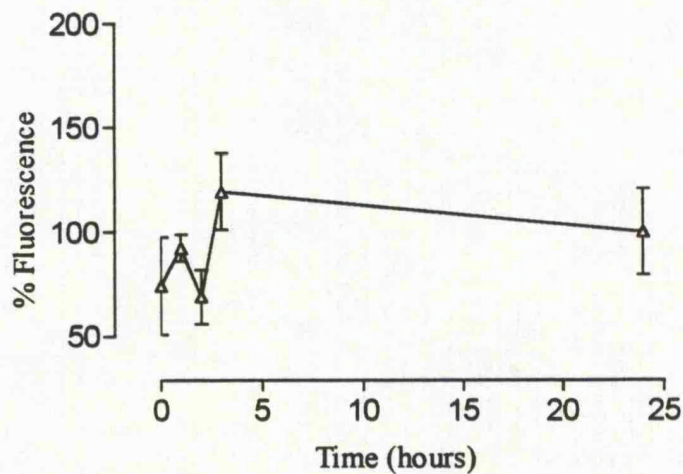


Figure 8.4: This graph represents the percentage antibody p53 binding to RHT cells treated with 1J of UVA irradiation at various times post reintroduction of serum. Values represent the mean and SEM for three separate experiments and are normalized to the amount of fluorescence in untreated cells at each time point.

The effect of UVA on the number of cells in the G_0/G_1 phase of the cell cycle was also determined (Figure 8.5). UVA had no effect on the number of cells in G_0/G_1 . However, the number of cells in G_0/G_1 varied with the time post reintroduction of medium (Figure 8.5). At time zero the percentage of cells in G_0/G_1 at 0, 0.5 and $1\text{J}/\text{cm}^2$ respectively was 59.8% (SEM=2.6), 62.5% (SEM=1.8) and 62.6% (SEM=1.5); there was no significant difference between the irradiated and non-irradiated samples. At one hour post reintroduction of complete medium the percentage of cells in G_0/G_1 at 0, 0.5 and $1\text{J}/\text{cm}^2$ was 63.7% (SEM=1), 63.4% (SEM=1) and 62.0% (SEM=0.7) respectively. There was no significant difference between the $0\text{J}/\text{cm}^2$ control and the UVA doses of 0.5 and $1\text{J}/\text{cm}^2$ at this one hour time point. At two hours post reintroduction of complete medium the percentage of cells in G_0/G_1 at 0, 0.5 and $1\text{J}/\text{cm}^2$ respectively was 62.1% (SEM=1.1), 63.3% (SEM=1.1) and 61.1% (SEM=1.8). There was no significant difference between the $0\text{J}/\text{cm}^2$ control and the UVA doses of 0.5 and $1\text{J}/\text{cm}^2$ at this two hour time point.

Using a two sample t-test it was noted that at three hours post reintroduction of complete medium the percentage of cells in G_0/G_1 at the 0J UVA dose point, (66.2%, SEM=0.7) was significantly higher than at the 0 ($p<0.01$), 1 ($p<0.01$) and 2 hour ($p<0.01$) time points. For the $1\text{J}/\text{cm}^2$ dose point at 3 hours the number of cells in G_0/G_1 was significantly higher, 67.1% (SEM=0.6) than 0 ($p<0.05$), 1 ($p<0.05$) and 2 ($p<0.01$) hours. However, the 0.5J dose point at 3 hours post reintroduction of RHT the number of cells in G_0/G_1 , (63.6% SEM=0.8) was not significantly higher than at 0, 1, and 2 hours but there was a trend for increasing numbers of cells in this stage of the cell cycle (Figure 8.5).

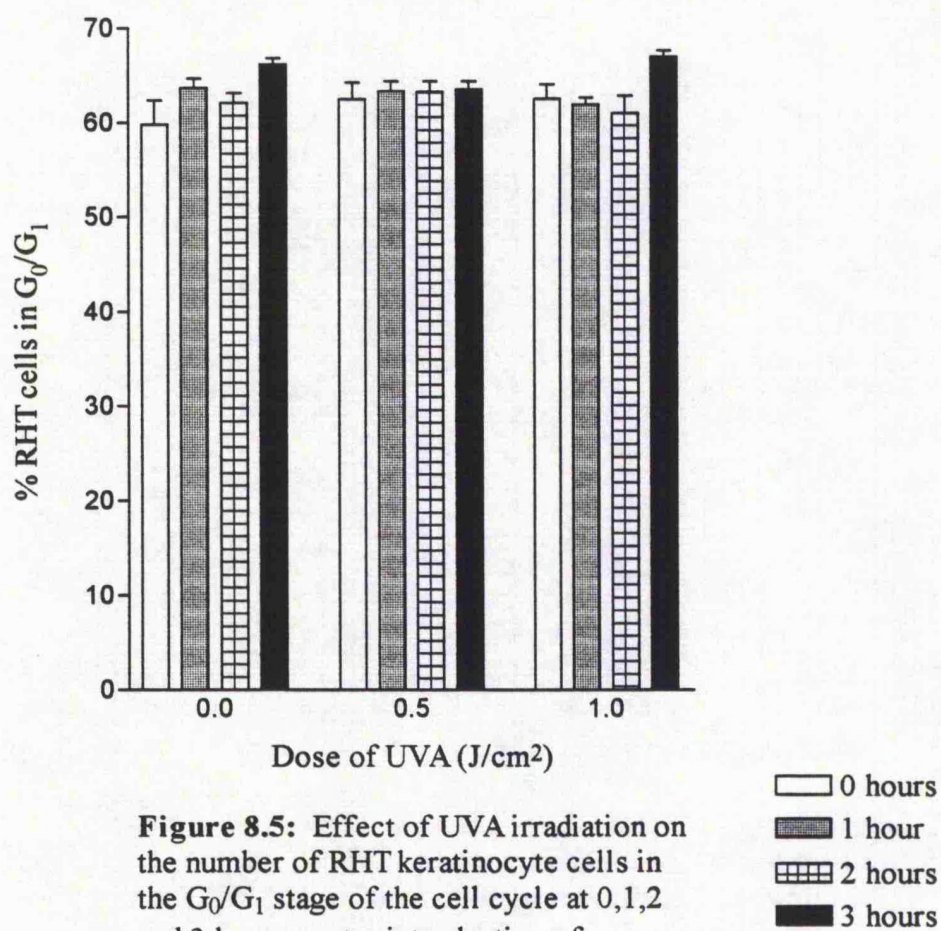


Figure 8.5: Effect of UVA irradiation on the number of RHT keratinocyte cells in the G_0/G_1 stage of the cell cycle at 0,1,2 and 3 hours post reintroduction of complete medium. Each value represents the mean and SEM (n=4).

3.6 Discussion

A direct correlation between UV-induced DNA damage and p53 induction has been shown previously by Nelson *et al.*, (1994) for UVB. The results presented in this thesis suggest the increase in p53 and the increase in antibody 529 binding may appear to be related to the stage of the cell cycle. There does not appear to be a significant increase in the levels of either antibody 529 or p53 binding when the keratinocytes were irradiated at 0.5 or 1 J/cm², compared to control. Maximal levels of damage and p53 protein occur on irradiation at three hours post reintroduction of complete media. An increasing trend in the number of cells in G₀/G₁ occurs in both irradiated and non-irradiated cells, therefore the increase in G₀/G₁ is not due to UV irradiation and an arrest of the cell cycle by UV induced p53. Due to the purified IgG fraction of 529 that was used in these experiments at 10µg/10⁶ cells there may be some anti-ssDNA binding due to a concentration effect. Therefore it may be possible that on irradiation with UVA there is an increase in the level of single stranded DNA. Recently an anti-ssDNA antibody has been used to detect DNA damage caused by ionizing radiation (Timmerman *et al.*, 1995). The cell cycle stage at which cells are irradiated would appear to be very important in the interpretation of increases of 529 binding and p53 protein. Cells that are exposed to a genotoxic insult are likely to halt the cells in G₀/G₁ until the damage is repaired, thus preventing a genetic lesion that may lead to carcinogenesis if passed onto a daughter cell.

3.7 The single cell microgel electrophoresis assay (comet assay)

It is not known to what extent the effects of solar irradiation are mediated by thymine glycols, pyrimidine hydrates, purine or purine-pyrimidine moieties, DNA single-strand breaks or DNA-to-protein cross-links, but all occur at frequencies much lower than pyrimidine dimers (Arlett *et al.*, 1993). Free radical damage is known to cause the formation of single strand breaks in cells (Arouma and Halliwell, 1995) and the comet assay is a measure of these.

3.7.1 Aim

To examine the ability of UVA to cause single-strand breaks in RHT keratinocytes in order to help explain the antigenicity of nuclear material for antibody 529.

3.7.2 Methods

RHT cells were grown to 60-80% confluence in 35x10mm petri dishes. The cells were irradiated with 0, 0.5 or 1 J/cm² and removed immediately or 5 hours later. The cells were harvested as a single cell suspension (section 2.9.4) and 1x10⁵ cells were used for each point. Details of the single cell microgel electrophoresis assay are outlined in section 2.12.

Briefly, cells were embedded in soft agar on frosted microscope slides, which were then placed in a high-salt lysis mixture and transferred to alkaline buffer, after which an electric current (25 volts for 20min) was applied. Under these conditions the cell contents are removed, leaving behind the nuclei. If the DNA contains a strand break (alkali-labile site) it streams out toward the anode in the form of a comet tail when viewed by fluorescence microscopy after staining with ethidium bromide. Undamaged DNA remains within the nucleus. Slides were stained with ethidium bromide, and the tail moment of 50 comets/dose point were determined using the Colourmorph software. The tail moment is a measure of the tail length and intensity and is more commonly used as an indicator for comet analysis.

8.7.3 Results

The arbitrary fluorescence values given by the Colourmorph software to the various tail moments were compared using the Statgraphics statistical software two sample t-test analysis. At $0\text{J}/\text{cm}^2$ at zero time, (which was approximately 50min post irradiation as the cells had to be harvested as a single cell suspension) the mean was 0.39 arbitrary fluorescence units (a.f.u.) (SEM=0.07) and was lower than at $0.5\text{J}/\text{cm}^2$ and $1\text{J}/\text{cm}^2$ which were 1.7 a.f.u. (SEM=0.19) and 0.79 a.f.u. (SEM=0.12) respectively (Figure 8.6). Both of the UVA dose points were significantly different from control ($p<0.05$). At 5 hours post irradiation with $0.5\text{J}/\text{cm}^2$ the RHT cells had a tail moment of 1.3 a.f.u. (SEM=0.28), this value was decreased slightly but not significantly from the $0.5\text{J}/\text{cm}^2$ at zero time (see Figure 8.6).

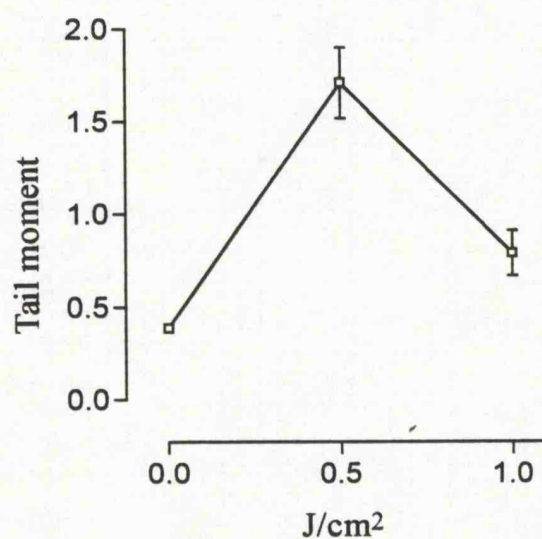


Figure 8.6: Effect of UVA irradiation on DNA strand breaks in RHT cells. Breaks were determined immediately after irradiation (time zero=50min) and values represent mean tail moment and SEM for 50 comets for each dose.

3.7.4 Discussion

The comet assay is used as a measure of single strand breaks, the larger the tail moment the more strand breaks that have occurred. This experiment was designed to investigate if UVA caused the formation of single strand breaks in RHT keratinocytes which could explain the binding of antibody 529 to such cell nuclei. Wavelengths in the UVA range cause various types of DNA damage, including cyclobutane-type pyrimidine dimers, strand breaks and DNA-protein crosslinks (Tyrell, 1991). Agents such as UV, which induce bulky adducts requiring processing by the excision repair pathway, can be studied using single cell gel electrophoresis (comet assay), with comets reflecting the strand breaks produced as part of the excision repair process (Ross *et al.*, 1995). Peak *et al.*, (1991) suggest that different photons excite different sensitisers, depending upon the absorbance characteristics of the sensitiser molecule. Thus single strand breaks may be formed in cells on irradiation with UVA as a result of photosensitisers interacting with the DNA or as a result of the repair process.

The RHT keratinocytes in this experiment were harvested as a single cell suspension (time zero=50min). Therefore it would appear that UVA causes an increase in single strand breaks and by comparison with the data obtained in section 7.5 perhaps the antibody 529 can be used to detect an increase in the level of ssDNA resulting from such breaks. Recently, an anti-ssDNA antibody has been utilised by Timmerman *et al.*, (1995) to detect increased levels of DNA damage due to ionizing radiation. The most abundant lesions induced in DNA by ionizing radiation are the single-strand breaks (Timmerman *et al.*, 1995). Further experiments would need to be carried out to show that between zero time

and 5 hours that repair had occurred. Utilising the comet assay in tandem with flow cytometric analysis showed that antibody 529 can be used as an indicator of DNA damage possibly by detecting an increase in ssDNA.

Chapter 9

General Discussion

General Discussion

ROS are generated both physiologically and pathologically in mammals and induce many kinds of cellular damage (Floyd *et al.*, 1990), including DNA damage. Among ROS-induced forms of DNA damage, 8-oxodG is indicative of oxidative DNA damage (Takeuchi *et al.*, 1994). The overall objective of this thesis was to investigate 8-oxodG as a reliable marker for oxidative DNA damage in disease processes and to investigate models of UVA damage.

The first stage of these investigations was to establish an assay for 8-oxodG. Since GC-MS facilities were not available in the Division of Chemical Pathology in the early part of this study, HPLC was the method of choice. HPLC with ECD has been used widely in the literature (for a review see introduction section 1.3 to 1.4) and many of the concepts of the importance of 8-oxodG in pathology are based on this assay (originally described by Floyd, 1986b). In order to establish an HPLC-ECD assay for 8-oxodG the standard compound was synthesised according to the Udenfriend system described by Kasai *et al.*, (1984). The identity of 8-oxodG was confirmed by UV spectral analysis (Culp *et al.*, 1989) and by mass spectrometry (courtesy of Dr. Peter Farmer, MRC, Leicester). During the course of setting up the HPLC-ECD assay to measure 8-oxodG a report by Claycamp (1992) highlighted the concerns of some workers in the field by questioning the suitability of the widely used phenol extraction method, for the isolation of DNA. Claycamp (1992) reported that phenol extraction sensitised the DNA to further oxidative DNA damage. An alternative method of DNA extraction, the pronase E method (Kendall *et al.*, 1991), was investigated in this thesis.

A comparative methodological study of DNA extraction by pronase E versus phenol was undertaken to clarify the suitability of each procedure in the analysis of DNA damage (Finnegan *et al.*, 1995; Appendix III). Three different modes of induction of oxidative DNA damage in PBMC and to naked calf thymus DNA were used; hydrogen peroxide, gamma irradiation and a combination of UVC and UVA irradiation (see section 4.0). In these experiments the levels of 8-oxodG in DNA isolated using the phenol method were consistently higher than when using the pronase E method of isolation. Also during the course of this thesis Harris *et al.*, (1994) demonstrated that unless precautions such as using freshly distilled phenol reagent are taken, the levels of 8-oxodG are generally higher in DNA from cells that have been exposed to an oxidative stress. However, because there is an absence of a linear relationship between sensitisation during extraction and initial oxidative damage, the use of phenol extraction techniques for the study of *in vivo* oxidation of DNA is inappropriate. A review of the literature since 1992 suggests that most workers that investigate oxidative DNA damage are avoiding the use of phenol-based extraction procedures. There are now many commercial DNA extraction kits available which do not contain phenol.

There are two main methods of analysis for 8-oxodG lesions reported in the literature presently. HPLC-ECD of the deoxynucleoside adduct (Floyd *et al.*, 1986b) is the most commonly used method of analysis followed by the GC-MS method for the base (Dizdaroglu, 1991). On comparison of the reported levels of 8-oxodG in the literature, GC-MS yields consistently higher background levels than the HPLC-ECD method (Halliwell and Arouma, 1995). DNA is either digested to deoxynucleosides using enzymatic hydrolysis or hydrolysed to the base using formic acid. The discrepancy between the levels

of 8-oxoG obtained using formic acid hydrolysis for GC/MS and the levels of 8-oxodG observed using enzymatic hydrolysis for HPLC-ECD is of some concern and is not yet resolved. A very recent report (Hamberg and Zhang, 1995) demonstrating a derivatisation method that gave levels of 8-oxoG in calf thymus DNA which were lower than those recently found by GC/MS but similar to those determined by HPLC-ECD, has provided a further link in resolving the discrepancy between 8-oxoG levels measured using GC/MS or HPLC-ECD. As an alternative to the 8-oxodG assay a novel assay was established during the course of this study, involving the use of the enzyme guanase which allowed 8-oxoG, released by formic acid hydrolysis of DNA, to be measured by HPLC-ECD (see section 6.0)(Herbert *et al.*, 1994). A direct comparison between the two HPLC-ECD methods was conducted using γ -irradiation of calf thymus DNA as a model of oxidative DNA damage. For the irradiated sample (400Gy) the 8-oxoG level was higher than the 8-oxodG level. This increase in the DNA adduct may be due to artificially high levels formed as a result of formic acid hydrolysis or it may be that the enzymatic hydrolysis had not reached completion. Subsequently the assay was applied to cells. For investigating UVA induced DNA damage in cells, a human keratinocyte cell line was chosen as an appropriate model. DNA was isolated using the pronase E method of extraction from UVA irradiated human keratinocytes. The DNA was then hydrolysed with formic acid and analysed using the guanase assay on the HPLC. An increasing trend of 8-oxoG was observed, that is, there was an increase in the level of 8-oxoG concomitantly with an increase in the UVA dose (see section 7.7). This is the first report of the use of the guanase assay in the analysis of DNA from mammalian cells. The assay was developed to combine the optimum hydrolysis method with HPLC-ECD detection. Furthermore derivatisation is not necessary, eliminating a potential source of artefactual 8-oxoG generation (Hamberg and Zhang,

1995). This assay has potential as it is fast, efficient and cheap when compared to either the 8-oxodG assay or the GC-MS assay. Further applications to cellular systems and comparison with existing analyses are underway in the Division of Chemical Pathology at Leicester University.

The main drawback of both GC/MS and HPLC-ECD analysis of 8-oxodG is that it is necessary to isolate the DNA, which may introduce some oxidative modifications. The samples are then hydrolysed during which time there is further potential for artefactual oxidative damage to occur. Immunoassays have been developed by some workers for the quantitative analysis of specific types of DNA damage products involving bulky alkyl and polycyclic aromatic derivatives (Srickland and Boyle, 1984). A similar approach employing polyclonal and monoclonal antibodies raised against oxidised bases of DNA has been used to develop highly sensitive assays that detect the γ -irradiation-induced oxidative adducts, 8-oxoadenine (West *et al.*, 1982) thymidine glycol (Leadon and Hanawalt, 1983; Rajagopalan *et al.*, 1984). The goal of successful detection and quantitation of specific oxidation products of DNA lead Degan *et al.*, (1991) to raise polyclonal antibodies to 8-oxodG. Using these polyclonal antibodies immunoaffinity columns were prepared which allowed the rapid isolation of 8-oxodG and 8-oxoG from urine. However these polyclonals had a decreased affinity for 8-oxodG in nucleic acids and this limited their usefulness for quantitating endogenous levels of oxidative damage in intact DNA. Mussart and Wani (1994) have reported the development of a polyclonal antibody that is capable of detecting 8-oxodG in oxidised DNA using immunoslot blot assays. Recently Yin *et al.*, (1995) have developed an immunoaffinity chromatography-monoclonal antibody-based enzyme linked immunosorbent assay (ELISA). This assay was compared directly with the HPLC method for monitoring 8-oxodG in human DNA samples. Due possibly to oligonucleotides

detected by the ELISA but not the HPLC method or crossreactivity with other damaged bases present in the immunoaffinity purified material, the absolute values of 8-oxodG were approximately six-fold higher in the ELISA than determined by HPLC (Yin *et al.*, 1995). Despite this drawback a monoclonal antibody is perhaps appropriate to develop as it has increased selectivity and sensitivity in addition to providing a continuous source of antibody. Since polyclonal antibodies are heterogeneous, antisera may contain antibodies to tissue constituents, to the coupler used in immunisation, and to environmental antigens previously encountered by the immunised animal. However, if the antibody is of high titre and a sensitive method is used, these factors can be removed by using high dilution of the antibody. The disadvantage of the crossreactivity of a monoclonal or a polyclonal antibody may be used to advantage as broad-spectrum antibodies may be used as the basis for immunoaffinity chromatography, which is a very valuable purification step for adducts before their analysis (Farmer, 1994). Currently in the Division of Chemical Pathology at Leicester University antibodies to various types of DNA damage are being developed.

As an alternative to isolating DNA, an immunocytochemical assay involving the use of an antibody raised to UV-damaged DNA was utilised in a model system: UVB induced DNA damage to keratinocytes *in situ* (see section 7.0). UVB damage was assessed initially as an indicator of the selectivity and sensitivity of the antibody, and to allow a fixation and staining protocol to be developed for the RHT keratinocyte cell line. This was achieved successfully. The immunocytochemical assay was then utilised to investigate UVA-induced DNA damage. The aim was to relate the amount of fluorescence staining to the level of an oxidative DNA marker, 8-oxoG, in DNA isolated from cells. A dose response was observed on measurement of 8-oxoG in the DNA isolated from RHT cells treated with

increasing doses of UVA (see section 7.7). However no detectable change in the UV-antibody binding was observed with increasing UVA dose (see section 7.7).

A protocol to relate the degree of immunodetection of UVA-induced DNA damage with levels of the protein p53 and stage of cell cycle in the RHT keratinocyte cell line was also established. In order to accomplish this, the stage of the cell cycle at which UVA irradiation took place was controlled by utilising synchronised cell populations. The results discussed in section 8.0 show that there was a maximal level of damage (as indicated by UV-antibody binding) and p53 protein on irradiation with UVA at three hours post reintroduction of complete cell culture medium. An increasing trend in the number of cells in G₀/G₁ phase of the cell cycle in both irradiated and non-irradiated cells also occurred at this three hour time point (section 8.0). Few investigations have employed both UVA irradiated keratinocytes to study p53 induction, which may be relevant to UV-induced skin cancer and UV-sensitivity in SLE patients. Increased p53 levels in transformed cells, due to binding to oncoprotein of DNA tumour viruses or mutant p53, result from a stabilised protein with loss of function of wild-type activity (Weinberg *et al.*, 1995). Gujuluva *et al.*, 1994, reported that cells with a mutant p53 gene fail to arrest cell cycle when exposed to DNA-damaging agents. Excision repair activity has been observed to vary with the metabolic state of the cell and therefore the timing of damage induction could have important consequences to the mutagenic and carcinogenic process (Schrader *et al.*, 1992). For example, a variety of techniques have been used to demonstrate that excision-repair activity is low in quiescent PBLs but can be stimulated several-fold when the cells are induced to move from G₀ into a cycling state by exposure to a lectin (Schrader *et al.*, 1992). The results discussed in section 8.0 show that the stage of cell cycle has an affect on the

antigenicity of DNA and the levels of p53 in cells treated with UVA. This may be due to a conformational change in the DNA as the cells halt in G₀/G₁ prior to entering s-phase of the cell cycle. The DNA may be in a less condensed form and therefore more susceptible to UVA induced damage. Although the upstream mechanism by which the cell senses the UVA damage and signals to p53 is unclear, the result is to stabilise p53 by a post-translational mechanism (Kastan *et al.*, 1991)

The discrepancy between the data obtained using the flow cytometer and that obtained by the fluorescence plate reader for immunodetection of UVA-induced DNA damage may be due to the purified IgG fraction of 529 that was used for the flow cytometry experiments. There may have been some anti-ssDNA binding due to the lower dilution when compared to the fluorescence assay. Recently an anti-ssDNA antibody has been used to detect DNA damage in single cells caused by ionizing radiation (Timmerman *et al.*, 1995).

One of the aims of this thesis was to measure the level of 8-oxodG in the DNA isolated from SLE and RA patient PBMC. During inflammatory disease states, such as SLE and RA, activated neutrophils release toxic oxygen species into the extracellular environment. Blount *et al.*, (1991) proposed that due to a defect in the repair of oxidatively damaged DNA, patients with SLE develop a high titre of antinuclear antibodies. A series of experiments was designed to investigate the levels of oxidative damage in the DNA of peripheral blood mononuclear cells isolated from whole blood taken from healthy volunteers, RA and SLE patients. The levels of 8-oxodG in the pronase E isolated DNA were found to be generally at or below the limit of detection for the assay, in normal, RA and SLE cell extracts (see section 5.0). There was no detectable difference in the levels of 8-oxodG in the DNA isolated from SLE or healthy volunteer PBMC in contrast to results

presented by Winyard *et al.*, 1989. In corroboration with results presented by Blount *et al.*, (1991) there was no difference between these patient groups in their ability to excise and excrete 8-oxodG into the cell culture medium.

Rosenstein *et al.*, 1992, have found that SLE cells are able to repair normally several types of DNA lesions. However, UV-sensitive SLE skin fibroblast cell strains were unable to maintain a normal level of DNA-protein crosslinks or DNA single-strand breaks following exposure to simulated sunlight (Rosenstein *et al.*, 1992). Arlett *et al.*, 1993, investigated the hypersensitivity of human lymphocytes to UV-B and solar irradiation. These authors suggest that although UVB is attenuated by passage into the living portion of the epidermis, the hypersensitivity of extracapillary lymphocytes could render them susceptible to damage. Under *in vivo* conditions any enhanced, but as yet undemonstrated, hypersensitivity to UVA might become more important, since these longer wavelengths are able to penetrate the skin more efficiently than UVB (Arlett *et al.*, 1993). It has been found in previous work by Rosenstein *et al.*, 1985, that UVA wavelengths produce damages that are eliminated primarily by a short-patch repair mechanism. Zamansky *et al.*, (1985) and Blount *et al.*, (1989) have hypothesised that persistent sites of DNA damage in patients with SLE may result in enhanced levels of anti-DNA antibodies and pathogenic immune complexes containing DNA. Mice immunised with UV-irradiated DNA develop immune complex deposits at the dermal-epidermal junction upon subsequent exposure to UV radiation (Natali and Tan, 1973). Pre-immunised animals also develop glomerular deposits of immune complexes upon administration of irradiated DNA (Natali and Tan, 1972). Thus it appears possible that exposure to sunlight may result in the formation of structural alterations that enhance the antigenicity of DNA (Rosenstein *et al.*, 1992). An inability to

properly repair these lesions may result in prolonged presence of antigenic DNA, higher levels of immune complexes, and greater tissue damage in SLE patients. Thus an antibody raised to UV-induced DNA damage may be a useful tool in discerning the mechanism of autoantibody formation in SLE patients. Use of this antibody in an immunoaffinity purification step, could allow further investigation of the levels of UV damaged DNA in supernatant and cells to be carried out. Perhaps the pathway of extrusion of the damaged DNA from the cell and its subsequent role as an antigen may be studied. This antibody raised to UV-induced DNA damage may also be used when investigating the mechanism of human skin carcinogenesis. This is being performed by other workers in the Division of Chemical Pathology at Leicester University.

Conclusions

A robust HPLC assay for 8-oxodG was established. Subsequently in a comparative study of pronase E and phenol extraction of DNA prior to analysis of 8-oxodG it was shown that pronase E is less likely to induce further oxidative DNA damage during extraction, irrespective of the model of oxidative stress used. Utilising pronase E isolation of DNA from the PBMC of SLE and RA patients the levels of 8-oxodG were found to be at or below the limit of detection.

A novel HPLC assay for the determination of 8-oxoG in DNA was developed and using γ -irradiation (400Gy) as a model of oxidative DNA damage, was shown to be a more sensitive index than using the deoxynucleoside assay (HPLC of 8-oxodG). UVA irradiation caused increasing levels of 8-oxoG in a dose dependent manner in DNA isolated from RHT cells.

A polyclonal antiserum which has specificity for UV damaged DNA was used successfully to detect UVB induced DNA damage in RHT keratinocytes *in situ*.

Utilising the polyclonal antibody, single strand breaks were detected in UVA -irradiated RHT cells concomitantly with increased p53 protein levels. This increase in levels of p53 and DNA damage on UVA irradiation was most pronounced when the cell numbers in the G_0/G_1 stage of the cell cycle had increased.

Future Work

A direct comparison between the established GC-MS technique for 8-oxoG, the novel HPLC assay developed in this thesis and the HPLC analysis of 8-oxodG would help address some of the discrepancies between the levels of 8-oxoG measured using GC-MS and 8-oxodG levels measured using the deoxynucleoside assay. Further applications of the 8-oxoG assay to cellular systems and comparison with existing analyses are underway in the Division of Chemical Pathology.

The polyclonal antibody raised against UV-DNA may be utilised to investigate UV sensitivity in SLE and the mechanism of UV induced skin carcinogenesis. Immunocytochemical work on skin tissue sections and on keratinocyte cell lines are planned in the Division of Chemical Pathology.

A direct comparison between the levels of UV-induced DNA damage and the levels of p53 in UVB irradiated keratinocytes would further elucidate the mechanism of UV-induced damage in cells.

Because of the problems associated with isolation and hydrolysis of cellular DNA leading to generation of artifacts, as highlighted in this thesis, in situ techniques for determination of oxidative DNA damage would be preferable. The development of antibodies to DNA damage is ongoing in the Division of Chemical Pathology. These antibodies will have a wide applicability to both direct immunocytochemical work and for the development of immunoaffinity columns as an enrichment step prior to chromatographic analyses.

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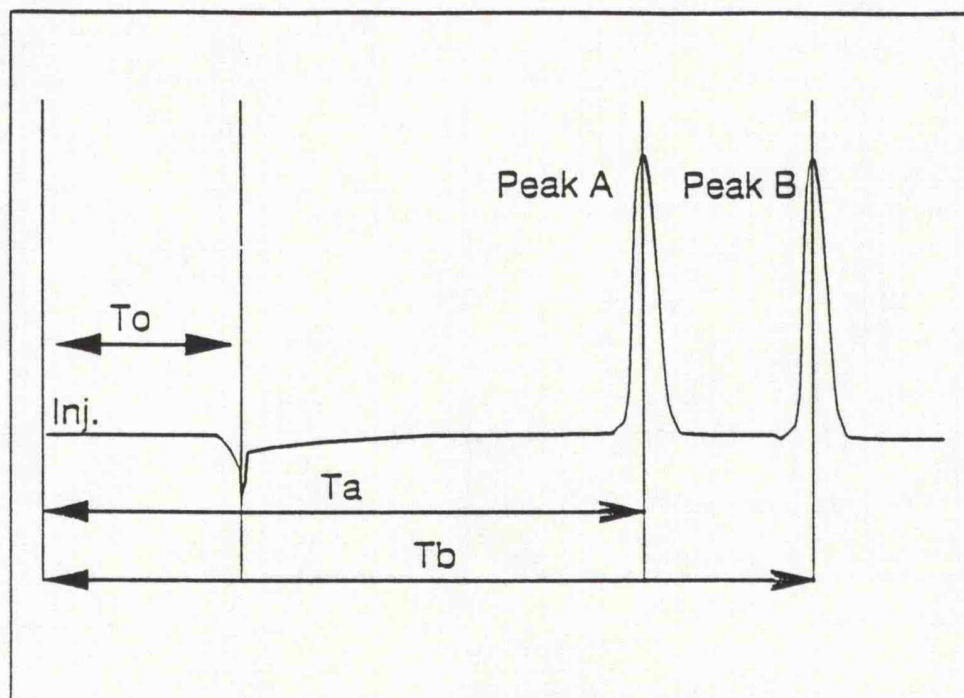
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Appendix I Composition of mitogens in RM+ medium

Hydrocortisone	0.41µg/ml
Choleratoxin	1×10^{-10} M
Transferrin	51µg/ml
Lyothyronine	2×10^{-11} M
Adenine	1.8×10^{-4} M
Insulin	51µg/ml
Epidermal growth factor	10ng/ml

Appendix II Calculations for capacity factors (k')



$$k' \text{ (Peak A)} = \frac{T_a - T_0}{T_0}$$

$$k' \text{ (Peak B)} = \frac{T_b - T_0}{T_0}$$

Appendix III Publications arising from this thesis

Cooke M.S., Finnegan M.T., Herbert K.E. and Lunec J. (1995) Cell cycle and dose dependence of DNA damage and p53 expression following UVA irradiation. *Biochem. Soc. Trans.*, 23, 3, 481S.

Finnegan M.T., Herbert K.E., Evans M.D. and Lunec J. (1995) Phenol isolation of DNA yields higher levels of 8-oxodeoxyguanosine compared to pronase E isolation. *Biochem. Soc. Trans.*, 23, 3, 430S.

Finnegan M.T., Herbert K.E., Evans M.D., Farooq P., Podmore I.D. and Lunec J. (1995) Development of an assay to measure 8-oxoguanine using HPLC with electrochemical detection. *Biochem. Soc. Trans.*, 23, 3, 431S.

Finnegan M.T.V., Herbert K.E., Evans M.D., Griffiths H.R. and Lunec J. (In press) Evidence for sensitisation of DNA to oxidative damage during isolation. *Free Rad. Biol. Med.*

Herbert K.E., Evans M.D., Finnegan M.T.V., Farooq S., Mistry N., Podmore I.D., Farmer P. and Lunec J. (In press) A novel HPLC procedure for the analysis of 8-oxoguanine in DNA. *Free Rad. Biol. and Med.*

Cell cycle and dose-dependence of DNA damage and p53 expression following UVA irradiation.

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Ultraviolet radiation (UV) has been reported to produce a number of potentially mutagenic DNA photoproducts and has consequently been implicated in skin tumorigenesis [1,2]. UV irradiation may also result in the activation of transduction pathways and the induction of specific genes [3]. One such gene product is p53, a vital regulator of a G₁ cell cycle checkpoint and of apoptosis following DNA damage, although the relationship between damage and elevation of p53 protein levels is unclear [4,5]. The presence of thymine dimers and altered p53 expression has been investigated in fixed tissue sections as a biomarker of UV exposure and DNA damage [6]. Here we describe preliminary results relating p53 levels with UVA-induced DNA damage. The critical role of p53 means that impairment of this system may have serious consequences for the cell.

Cultured, human, SV40-transformed, keratinocytes were synchronised by serum deprivation and UVA irradiated. Cells were then probed with an antibody to UV-damaged DNA ("529"; [7]) and monoclonal anti-p53 protein antibody (clone DO-7, which detects both wild type and mutated form of the protein), using fluorescein-labelled secondary antibodies. Quantitation of antibody binding was performed by a Becton Dickinson FACScan flow cytometer.

Tables one and two show the levels of p53 and 529 binding, respectively, in synchronised human, SV40-transformed, keratinocytes treated with 0.5 and 1J/cm² UVA. Fluorescence was expressed as a percentage of the control fluorescence at zero J/cm² UVA. Levels of p53 protein were maximal when irradiation was carried out three hours after reintroduction of medium; although this did not reach statistical significance, this was consistent for both doses. Another, smaller peak occurred following irradiation one hour after medium reintroduction, in this case the peak was higher for the 1J/cm² dose. A similar relationship was obtained for the levels of antibody 529 binding in human, SV40-transformed keratinocytes treated with UVA. Once again a trend for greater binding was seen at three hours post irradiation. These data show that levels of p53 and 529 binding correlate and vary according to the time at which irradiation occurs following introduction of medium.

A relationship has been demonstrated between UV-induced DNA damage and p53 induction [5], and our results support this. We suggest that antibody 529 detects damage produced by UVA, and that as a consequence of this levels of p53 protein alter. As yet the epitope(s) induced by UVA, responsible for antibody 529 binding, is unknown. The amount of damage depended on the length of time following reintroduction of media at which the cells were irradiated.

Table 1. Levels of p53 protein in synchronised human SV40-transformed keratinocytes following irradiation after reintroduction of complete medium. Values are means of n=3 experiments (standard error of mean).

Time post reintroduction of complete medium (hr)	UVA dose (J/cm ²)	
	0.5	1
0	74 (26)	74 (23)
1	87 (12)	92 (6)
2	81 (13)	69 (13)
3	130 (9)	119 (18)
24	92 (14)	100 (21)

Table 2. Levels of antibody 529 binding in synchronised human SV40-transformed keratinocytes following irradiation after reintroduction of complete medium. Values are means of n=3 experiments (standard error of mean).

Time post reintroduction of complete medium (hr)	UVA dose (J/cm ²)	
	0.5	1
0	114 (37)	105 (10)
1	93 (31)	97 (30)
2	67 (15)	77 (18)
3	140 (33)	127 (34)
24	62 (34)	89 (26)

Maximal levels of antibody 529 binding and p53 protein occurred after irradiation around three hours after reintroduction of media, the cause of this is unclear but would seem to involve the stage in the cell cycle. Further to cell cycle analysis, the elevated levels at three hours may be more easily explained. More detailed analysis around the 2-3 hour period would benefit our understanding of the processes involved following UVA irradiation.

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Phenol isolation of DNA yields higher levels of 8-oxodeoxyguanosine compared to pronase E isolation.

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Oxidative damage is thought to play a role in the aetiology of aging and a number of diseases including cancer, chronic inflammation, ischemia, degenerative arterial and autoimmune diseases [1]. 8-oxodeoxyguanosine (8-oxodG), an oxidative DNA adduct, has gained much popularity as a biomarker of damage to DNA. HPLC combined with electrochemical detection provides a selective and sensitive method of measuring 8-oxodG [2]. It has been reported that phenol extraction of DNA may cause sensitization of DNA to subsequent oxidative damage and result in higher 8-oxodG levels [3]. In this study we compare phenol isolation with pronase E isolation of DNA [4].

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Histopaque 1077 and treated using two different exposure protocols to induce oxidative stress. First, the mononuclear cells were treated on ice, to reduce DNA repair, with H_2O_2 (400 μ M) for 15 min. Second, PBMC were exposed on ice to a ^{60}Co source, and treated with gamma irradiation at a rate of 0.48 Gy/sec (0 Gy, 20 Gy, 200 Gy). Mononuclear cell DNA was extracted using the pronase E method of Kendall et al. [4] or the phenol method based on a procedure described by Winyard et al. [5]. DNA was enzymatically digested to the deoxynucleoside level [6]; micrococcal nuclease (0.14 units/4 μ g DNA) was used in place of *N.crassa* endonuclease. The method for reversed-phase HPLC of the DNA deoxynucleosides was modified from the procedure of Floyd et al. [7].

The levels of DNA extracted using either method were not significantly different (data not shown). The results in Figure 1 show that lower levels of 8-oxodG were observed in H_2O_2 treated and untreated PBMC and naked calf thymus DNA when DNA was isolated using the pronase E method compared to the phenol method. In the experiments where the cells were treated with hydrogen peroxide in the presence of foetal calf serum (FCS) a response to treatment with H_2O_2 was observed ($p < 0.05$) in that 8-oxodG levels were increased but only for phenol extraction of DNA (Figure 1). Similarly for γ -irradiated cells the levels of 8-oxodG observed using pronase E extraction were lower than the levels observed using the phenol method ($p < 0.05$) (Figure 2). A dose response was suggested using phenol isolation from γ -irradiated PBMC 0-200 Gy (Figure 2). Higher levels of 8-oxodG were observed on extraction of naked calf thymus DNA using the phenol method ($p < 0.05$) but no increase in 8-oxodG proportional to increased irradiation was seen for phenol extracts of DNA.

The importance of establishing a reliable method to measure

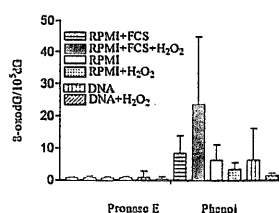


Figure 1: This graph shows the levels of 8-oxodG in PBMC or naked DNA treated with 400 μ M hydrogen peroxide for 15 min. on ice. Each value represents the mean \pm SEM for three separate experiments.

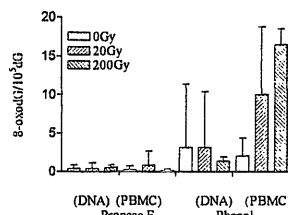


Figure 2: γ -irradiation of PBMC or DNA, direct comparison between pronase E and phenol isolation. Each value represents the mean \pm SEM for three separate experiments.

8-oxodG at ultra-low levels is clear. We are currently using selective and sensitive antibodies to specific DNA damage products and immunocytochemistry to detect oxidative products of DNA damage in cells. This would appear to be a promising approach, because these methods limit the potential for artefactual production of DNA damage caused by manipulations inherent in other methods. In conclusion, phenol isolation of DNA appeared to cause artificially high levels of the oxidative DNA adduct 8-oxodG. Lower levels of 8-oxodG were detected using pronase E extraction of DNA from PBMC or naked calf thymus DNA solutions, but such levels were very often at or near the limit of detection of the assay.

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Abbreviations used: 8-oxodG, 8-oxodeoxyguanosine; HPLC, high performance liquid chromatography; PBMC, peripheral blood mononuclear cells; FCS, foetal calf serum; H_2O_2 , hydrogen peroxide; Gy, Gray; *N.crassa*, neurospora crassa.

Development of an assay to measure 8-oxoguanine using HPLC with electrochemical detection.

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Oxidative DNA damage produced by free radicals or other DNA-damaging agents has been implicated to play a role in mutagenesis, carcinogenesis and aging [1]. An oxidative DNA adduct 8-oxoguanine (8-oxoG) is measured conventionally as the deoxynucleoside by high performance liquid chromatography with electrochemical detection (HPLC-ECD) [2] or as the free base by gas chromatography combined with mass spectrometry [3]. We have established a 'hybrid' analysis of the 8-oxoG base by HPLC in order to account for reported differences between the two more established techniques. We found that guanase degrades guanine and not 8-oxoG, allowing HPLC analysis of 8-oxoG in acid hydrolysates of DNA without interference from guanine.[4].

Calf thymus DNA was dissolved in water at a concentration of 1mg/ml and irradiated using a ⁶⁰Co gamma source at a dose of 400Gy. The unirradiated control and irradiated sample were then divided into two aliquots. The first aliquot was dried down under nitrogen prior to formic acid hydrolysis (60%) v/v at 140°C for 45min. This was then reconstituted in ultra pure water, and the pH adjusted to 8.0 prior to incubation with guanase for 1 hour at 37°C. The samples were then neutralised and analysed by HPLC-ECD. The second aliquot was enzymatically digested to the deoxynucleoside level using a procedure described by Faux et al [5] and analysed on the HPLC-ECD. HPLC-ECD was performed on a 3µm ODS-Hypersil column (150 x 4.6mm i.d.). 8-oxoG was analysed using 40mM potassium phosphate, pH 5.0, containing 1mM EDTA and 1% v/v methanol in the mobile phase, at a flow rate of 1ml/min. The deoxynucleoside, 8-oxodeoxyguanosine (8-oxodG), was analysed using 50mM sodium acetate, pH 5.1 containing 1mM EDTA and 5% v/v methanol at a flow rate of 1ml/min.

The experiment was used as a model to assess the efficacy of the 'hybrid' assay using a direct comparison between measurement of the oxidative DNA adduct 8-oxoG as the base or as the deoxynucleoside, 8-oxodG. The results are shown in table 1. The levels of 8-oxoG and 8-oxodG in DNA increased on treatment of the DNA with gamma irradiation. The reported background levels of 8-oxoG obtained from commercially available calf-thymus DNA, measured using GC-MS, are 0.5-1nmol/mg DNA [6]; the values obtained using the enzymatic hydrolysis were within this range at 0.7nmol/mg DNA.

Abbreviations used: HPLC-ECD, high performance liquid chromatography with electrochemical detection; 8-oxoG, 8-oxoguanine; 8-oxodG, 8-oxodeoxyguanosine; EDTA, ethylenediaminetetra-acetic acid; GC-MS, gas chromatography combined with mass spectrometry.

Table 1: Direct comparison of 8-oxoguanine in DNA determined as the base and the deoxynucleoside.

	Enzymatic hydrolysis (nmol 8-oxodG/mg DNA)	Guanase assay (nmol 8- oxoG/mg DNA)
Non- irradiated sample (0Gy)	0.7	Below the limit of detection.
Irradiated sample (400Gy)	1.6	5.0

Using the guanase assay the level obtained from calf-thymus DNA irradiated with 400Gy was 5.0nmol/mg DNA. The limit of detection of the guanase assay was 500 femtomoles and for the 8-oxodG assay was 250 femtomoles.

In this preliminary study, on direct comparison of the levels of 8-oxoG with 8-oxodG there appears to be a difference between the two assays. The guanase assay yields higher levels of the oxidative DNA adduct 8-oxoG. This increase in the DNA adduct may be due to the use of formic acid hydrolysis which may cause artificially high levels to be formed or it may be that the enzymatic hydrolysis had not reached completion. The new procedure combines formic acid hydrolysis with an enzyme which is specific for guanine and avoids problems with enzymes which may lack specificity toward oxidatively damaged DNA. The 'hybrid' guanase assay can be used in place of both the 8-oxodG assay on the HPLC-ECD and the 8-oxoG assay on the GC-MS. The guanase assay is cheaper and faster than the other assays for measurement of the oxidative DNA adduct 8-oxoG; there is only one enzyme required and the run time is less than 10 minutes. Using gamma irradiation (400Gy) as a model we were able to detect 8-oxoG in DNA. This assay is now established within our laboratory and is being used to compare the reported differences between the HPLC-ECD and GC-MS techniques for measuring 8-oxoguanine.

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Brief Communication

EVIDENCE FOR SENSITISATION OF DNA TO OXIDATIVE DAMAGE DURING ISOLATION

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Abstract—The oxidative base lesion 8-oxo-deoxyguanosine (8-oxo-dG) has been identified in DNA isolated from normal tissue and may occur at elevated levels during disease. However, the use of phenol during DNA extraction may artificially elevate the detected levels of this lesion. Herein, we have performed a comparative methodological study using both pronase E and phenol extraction techniques; native or oxidatively stressed DNA was isolated to determine the validity of each extraction technique for the subsequent determination of 8-oxo-dG. Whilst the yields of DNA were comparable, after pronase E extraction there was no detectable induction of 8-oxo-dG in reextracted naked DNA or peripheral blood mononuclear cell DNA that had been oxidatively stressed. However, phenol extraction enhanced the basal levels of 8-oxo-dG detected, and also induced a significant increase in levels of the modified base after exposure to oxidative stress. The latter was dependent on the presence of foetal calf serum in the extracellular medium. We have confirmed that phenol extraction sensitises native DNA to subsequent oxidative damage. In addition, this work shows that the extent of sensitisation occurring during phenol extraction varies with the degree of oxidative damage already incurred and infers that labile guanine sites generated during oxidative stress may be detected as 8-oxo-dG residues after phenol extraction.

Keywords—Oxidative DNA damage, 8-Oxo-dG, Phenol extraction, Pronase E extraction, DNA sensitisation, Free radicals

INTRODUCTION

There is substantial evidence to suggest that oxidative damage to DNA occurs *in vivo* during periods of oxidative stress.^{1–3} This is manifested by an increase in strand breaks, by the generation of DNA crosslinks and by the presence of damaged base products.^{4–6} Of the many oxidised products generated, 8-oxo-deoxyguanosine (8-oxodG)⁷ has been adopted as a highly sensitive and specific marker of oxidative DNA damage that can be measured at the femtomole level by high performance liquid chromatography combined with electrochemical detection (HPLC-ECD).⁸ Elevated levels of 8-oxo-dG have been reported to occur in chronic inflammation and autoimmune disease.⁹ This has led to the hypothesis that oxidative DNA damage may be important in the aetiology and/or progression of these diseases. However, the significance of these findings must be questioned in the light of the inherent variability in the endogenous level of 8-oxo-dG (0.3–

10 moles/10⁵ moles dG)¹⁰ and because the measurement of such trace levels may be subject to artefactual increases incurred both in the preparative and analytical stages.

Claycamp et al. have reported artificially raised levels of 8-oxo-dG in DNA isolated using phenol extraction;¹¹ however, more recently Harris et al. have disputed this finding.¹² Herein, we have investigated the hypothesis that phenol may sensitise the DNA to further oxidative damage *in vitro*, thereby leading to overestimation of the degree of damage incurred *in situ*. In order to determine a reliable method, we have compared levels of 8-oxo-dG present in DNA extracted from both native cells and oxidatively treated cells using either a conventional organic phenol procedure¹³ or by pronase E digestion.¹⁴

MATERIALS AND METHODS

Unless otherwise stated, all materials were of Analar grade and supplied by the Sigma Chemical Company, (Poole, Dorset). Also from Sigma were; calf thymus DNA, proteinase K, pronase E from *Streptomyces griseus*, DNase I Type IV from bovine pancreas, mi-

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crococcal nuclease from *Staphylococcus aureus*, alkaline phosphatase Type III-S, phosphodiesterase I Type VII from *Crotalus atrox* venom, endonuclease from *Neurospora crassa*, and RNase was Type IIa from bovine pancreas. Ethanol was from Leicester University Chemical stores. Trisodium citrate and phenol were from Fisons, (Loughborough, Leics). Liquefied phenol (also from Fisons) was washed in Tris buffer to equilibration at pH > 7.6. Tissue culture supplies were from Flow Laboratories (ICN Biomedicals, Oxon, UK). Chloroform/isoamyl alcohol (24:1) was from BDH, Poole, Dorset.

Isolation of peripheral blood mononuclear cells (PBMC) from whole blood

Peripheral blood (100 ml) was taken into heparin and mixed with an equivolume of RPMI 1640. Diluted blood (7 ml per tube) was gently layered onto 3 ml Histopaque 1077 and centrifuged at $400 \times g$ for 30 min at room temperature. Following centrifugation the mononuclear cell layer was carefully collected and washed in RPMI 1640. After centrifugation at $200 \times g$ for 10 min at 4°C, the pellets of PBMC were washed as before. The pellets were finally resuspended to give a concentration of 2×10^6 cells/ml RPMI containing 10% foetal calf serum (FCS) unless otherwise stated.

Reactive oxygen species (ROS) treatment of naked calf thymus DNA and PBMC

Calf thymus DNA (1 mg/ml PBS) and PBMC were exposed to ROS generated by two mechanisms;

1. with hydrogen peroxide, H_2O_2 (400 μ M) for 15 min on ice to minimise DNA repair capacity
2. by gamma irradiation at a dose rate of 0.48 Gy/s (0 Gy, 20 Gy, 200 Gy) using a Vickrad ^{60}Co source. In aerated solution, a mixture of the superoxide anion radical and hydroxyl radical are formed.

Isolation of DNA from PBMC

DNA was isolated from paired cell populations in parallel by using either the pronase E method,¹⁴ or the phenol method described by Winyard *et al.*¹³

Pronase E method. PBMC (5 ml of 2×10^6 cells/ml) were centrifuged at $300 \times g$ for 10 min at room temperature. The cells were resuspended in 3.5 ml of ice cold buffer 1 (5 mM trisodium citrate, 20 mM sodium chloride, pH 6.5) with vigorous mixing. To DNA samples and washed PBMC, Pronase E (0.5 ml of 4 mg/ml in buffer 1) was added followed by 4 ml

of buffer 2 (20 mM Tris, 20 mM EDTA, 1.5% sarkosyl, pH 8.5). The tubes were vortexed during each addition and incubated overnight at 45°C. Buffer 3 (4 ml of 10 mM Tris-HCl, 10 mM EDTA, pH 7.5) and 7.5 M ammonium acetate (1 ml) were added to each tube and mixed by inversion. DNA was precipitated by the addition of ethanol (approximately 30 ml) with mixing by inversion until precipitation was seen. Samples were then extracted by centrifugation at $500 \times g$ for 10 min. This was followed by three further washes in 70% ethanol (v/v) for 30 min, 100% ethanol for 10 min, and finally 70% ethanol (v/v) for 10 min. The samples were then air dried for 5 min and resuspended in 1 ml of buffer 3 at 4°C.

Phenol extraction. PBMC were pelleted by centrifugation at $1500 \times g$ for 20 min. The nuclei-containing pellets were dispersed in 0.1 ml TE buffer (100 mM Tris-HCl and 1 mM EDTA, pH 8) and to both calf thymus DNA samples and resuspended nuclei, 1 ml proteinase K buffer (100 μ g/ml proteinase K, 5 mM EDTA and 0.5% sarkosyl, pH 8) was added. Tubes were then incubated at 50°C for 2 h. DNA was extracted with an equal volume of phenol. Subsequently the DNA was further extracted using phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and 2 \times chloroform:isoamyl alcohol (24:1 v/v). Nucleic acids were precipitated by the addition of ethanol (2.5 volumes) and 0.3 M sodium acetate (0.1 volumes) and left at -20°C for at least 3 h. The DNA and RNA were pelleted by centrifugation and dried under nitrogen. The pellet was dissolved in 1 ml RNase A buffer (RNase 100 μ g/ml, 50 mM Tris-HCl, 10 mM EDTA, and 10 mM NaCl pH 8) and incubated for 37°C for 2 h (this stage was omitted for calf thymus DNA). DNA was then extracted first with phenol chloroform isoamyl alcohol, then with chloroform isoamyl alcohol ($\times 2$) and subsequently precipitated with ethanol and sodium acetate as before, prior to air drying.

DNA hydrolysis

Prior to analysis, quantitation of DNA was achieved by A_{260nm}/A_{280nm} and purity checked using A_{260nm}/A_{280nm} ratios. DNA was enzymatically digested to the deoxynucleoside level according to the method of Beland,¹⁵ except that micrococcal nuclease (0.14 U/4 μ g DNA in Buffer 3) was used in place of *Neurospora crassa* endonuclease. Hydrolysates were stored at -20°C prior to analysis, and analysed within 2 weeks.

HPLC analysis of deoxyguanosine and 8-oxodeoxyguanosine

The method for the reversed phase HPLC of DNA nucleosides was modified from the method of Floyd

et al.^{8,10} 8-Oxodeoxyguanosine was determined by using an EG and G model 400 amperometric electrochemical detector with a glassy carbon working electrode. This was operated at an applied potential of +0.6 V versus silver/silver chloride reference electrode and typically at 1 nA full scale deflection. A ODS-hypersil 3 μ M column (100 mm \times 4.6 mm ID) was used to separate samples (50 μ l) at a flow rate of 1 ml/min. The mobile phase was 40 mM sodium acetate buffer pH 5.1, containing 1 mM EDTA, and 5% methanol. Deoxyguanosine (dG) was determined by ultraviolet detection at 254 nm. This enabled the results to be expressed as moles of 8-oxo-dG per mole of dG in the hydrolysate. Standard 8-oxo-dG was synthesised using the Udenfriend system,⁷ and the structure was confirmed by fast atom bombardment mass spectrometry (courtesy of Dr. Peter Farmer, MRC Toxicology Unit, Leicester). The detection limit of the method was 250 femtomoles of 8-oxo-dG at a signal to noise ratio of 3.

Statistics

Statistical analyses were performed using the Mann-Whitney U test using the Statgraphics software package. Each experiment was repeated three times prior to statistical analysis.

RESULTS

To determine the efficiencies of DNA extraction from PBMC, the relative yields were calculated after using both phenol and pronase E methods concurrently. Table 1 shows that there was no significant difference between these methods in the level of dG extracted from PBMC. In addition, these results confirm that the preexposure of PBMC to oxidant stress does not significantly alter the subsequent yield of DNA extracted by either technique.

The exposure of naked calf thymus DNA to 400 μ M H₂O₂ (15 min on ice) did not cause any significant increase in the basal level of 8-oxo-dG present, when DNA was extracted using the pronase E method (Fig. 1). However, after paired DNA samples were concurrently extracted by the phenol technique, elevated levels of 8-oxo-dG in both the control and the treated DNA were observed, when compared to the pronase E method. The effect was significant ($p < 0.05$) for the control DNA sample, but not for treated DNA. The treatment of DNA with H₂O₂ on ice had no significant effect on 8-oxo-dG/dG ratio when compared with the untreated control, independent of the extraction method used.

The exposure of PBMC to 400 μ M H₂O₂ for 15 min on ice had no significant effect on the levels of

Table 1. DNA Yield From PBMC Extracted Using Either Phenol or Pronase E

Treatment of PBMC	Method of DNA Isolation	
	Pronase E DNA Yield (μ M dG)	Phenol DNA Yield (μ M dG)
⁶⁰ Co 0 Gy	84.73 \pm 40.3	90.9 \pm 29.25
⁶⁰ Co 20 Gy	59.05 \pm 28.2	77.7 \pm 84.97
⁶⁰ Co 200 Gy	89.7 \pm 44.52	80 \pm 50.83
0 μ M H ₂ O ₂ (RPMI/FCS)	41.77 \pm 44.59	76.5 \pm 30.3
0 μ M H ₂ O ₂ (RPMI)	70.78 \pm 53.88	81.8 \pm 40.5
400 μ M H ₂ O ₂ (RPMI/FCS)	44.1 \pm 39.49	61.9 \pm 29.7
400 μ M H ₂ O ₂ (RPMI)	77 \pm 54.29	79.2 \pm 37.85

This table shows the yield of DNA extracted from PBMC using either the pronase E method or the phenol method. DNA was extracted from 10⁷ cells as described in the Materials and Methods section, and the results are expressed as the means \pm SEMs of three separate experiments.

8-oxo-dG detected by HPLC-ECD following pronase E extraction; Figure 1 illustrates that in the order of 1 mole 8-oxo-dG / 10⁵dG is present in both treated and untreated samples. However, after paired samples were analysed for 8-oxo-dG content following phenol extraction of DNA from treated PBMC, the levels of 8-oxo-dG were significantly raised ($p < 0.01$) when compared with untreated control. In addition, the level of 8-oxo-dG / dG in control PBMC was significantly higher ($p < 0.05$) when measured in DNA isolated by phenol extraction (8.5 \pm 5.5 mole 8-oxo-dG / 10⁵dG), when compared with that isolated by the pronase E technique (1 \pm 0.35 mole 8-oxo-dG / 10⁵dG).

To identify further the effects of extracellular medium on the reported levels of 8-oxo-dG, PBMC were exposed to H₂O₂ in the presence or absence of FCS. Following pronase E extraction, Figure 2 illustrates that the level of 8-oxo-dG / dG remained at basal levels in both the treated and untreated PBMC. However, following phenol extraction, the 8-oxo-dG / dG ratio was significantly higher in both the treated cells and untreated controls compared with the levels observed following pronase E extraction ($p < 0.01$). H₂O₂ treatment in the presence of FCS significantly increased the level of 8-oxo-dG present in PBMC DNA from 8.5 \pm 5.5 mole 8-oxo-dG / 10⁵mole dG to 23.7 \pm 20.4 mole 8-oxo-dG / 10⁵mole dG ($p < 0.05$), when DNA was isolated using the phenol procedure. However, H₂O₂ treatment in the absence of FCS had no effect on the level of 8-oxo-dG / dG. The 8-oxo-dG level in cells treated in the absence of FCS was significantly lower than the amount of 8-oxo-dG / dG found in PBMC exposed to H₂O₂ in complete medium ($p < 0.05$).

The two techniques for isolating DNA were also compared using DNA and cells treated with a defined

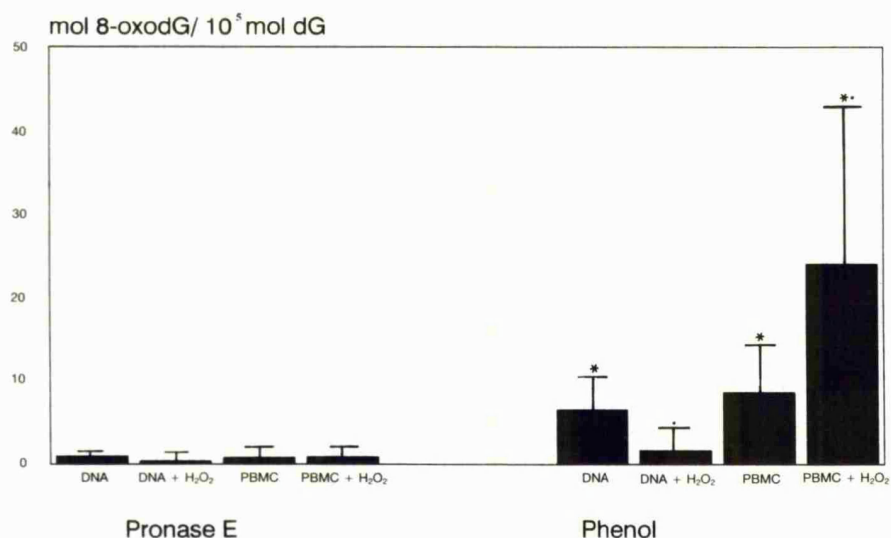


Fig. 1. Fresh peripheral blood mononuclear cells or naked DNA were exposed to 400 μ M H₂O₂ for 15 min on ice. Paired samples were then subsequently extracted using the pronase E method or the phenol technique. The amount of 8-oxo-dG detected following HPLC-EC is expressed as a ratio against the total amount of dG present. The results are expressed as the means + SEMs of triplicate experiments where * = $p < 0.01$ versus paired samples extracted using the pronase E technique and † = $p < 0.05$ versus the untreated control extracted using phenol.

oxidative stress generated by gamma radiation. Doses of up to 200 Gy did not cause any significant increase in 8-oxo-dG levels above basal in irradiated DNA that was reextracted using the pronase E method (see Fig.

3). However, following phenol extraction, levels were higher in irradiated DNA when compared to the levels observed following pronase E extraction ($p < 0.05$). There was no significant change in the level of 8-oxo-

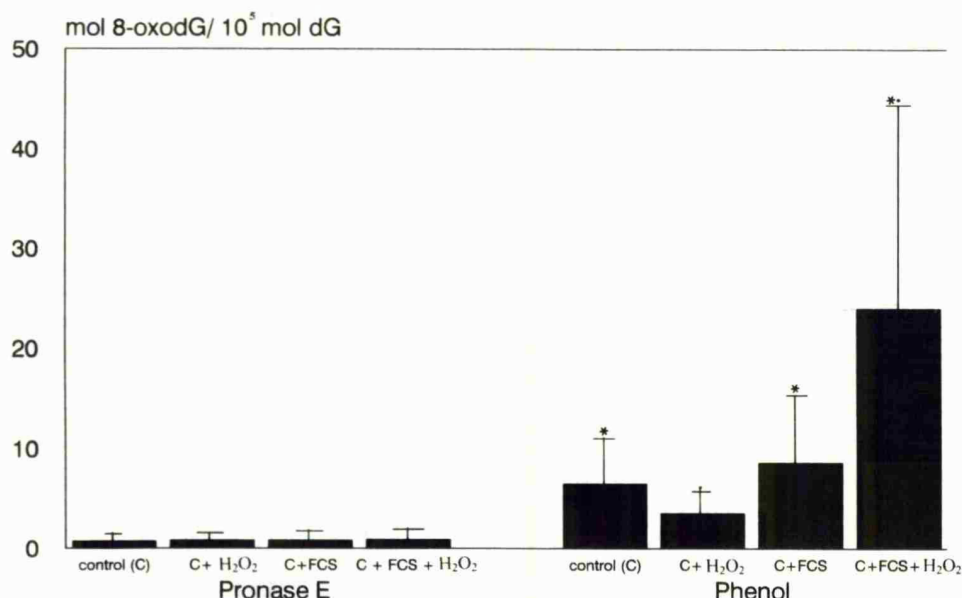


Fig. 2. Fresh peripheral blood mononuclear cells in the presence or absence of 10% FCS were exposed to 400 μ M H₂O₂ for 15 min on ice. Paired samples were then subsequently extracted using the pronase E method or the phenol technique. The amount of 8-oxo-dG detected following HPLC-EC is expressed as a ratio against the total amount of dG present. The results are expressed as the means + SEMs of triplicate experiments where * = $p < 0.01$ versus paired samples extracted using the pronase E technique and † = $p < 0.05$ versus the untreated control extracted using phenol.

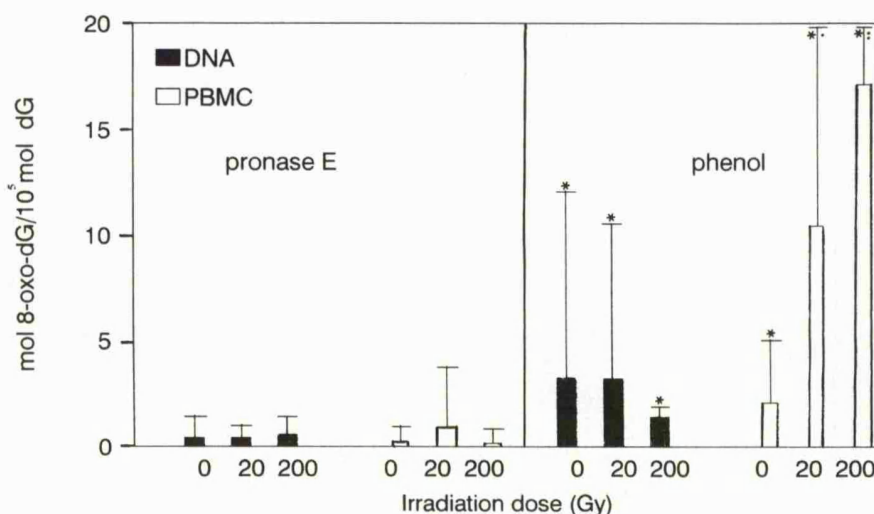


Fig. 3. Fresh peripheral blood mononuclear cells or naked DNA were exposed to steady state gamma radiolysis from a Vickrad Cobalt source for up to 7 min on ice at a dose rate of 0.48 Gy/s to give final doses of 0, 20, and 200 Gy. Paired samples were then subsequently extracted using the pronase E method or the phenol technique. The amount of 8-oxo-dG detected following HPLC-EC is expressed as a ratio against the total amount of dG present. The results are expressed as the means + SEMs of triplicate experiments where * = $p < 0.01$ versus paired samples extracted using the pronase E technique; * = $p < 0.05$ and ** = $p < 0.01$ versus the untreated control extracted using phenol.

dG with increasing dose following irradiation of DNA, with the pronase E method of extraction.

The exposure of PBMC to gamma irradiation up to a dose of 200 Gy did not have any significant effect on the mole 8-oxo-dG/mole dG in DNA isolated using the pronase E method (see Fig. 3). However, when DNA was extracted using phenol from irradiated PBMC, a significant and dose-dependent increase in 8-oxo-dG was observed ($p < 0.05$). Furthermore, for each irradiation dose, the levels of 8-oxo-dG were always significantly higher in phenol extracted DNA than in DNA isolated using the pronase E method ($p < 0.05$).

DISCUSSION

This comparative methodological study of DNA extraction by pronase E and phenol has been undertaken to clarify their suitability in the analysis of oxidative DNA damage. Whilst both methods yielded comparable amounts of DNA from both native and oxidatively stressed PBMC, some important and significant differences in the levels of 8-oxo-dG were subsequently observed. In all cases, the molar ratio of 8-oxo-dG / dG was between two-fold and twenty-fold higher for DNA extracted using the phenol technique when compared to the pronase E method. Greater differences were observed when the cells had been previously exposed to oxidative stress. This may be accounted for by either the preferential extraction of altered/dam-

aged DNA or alternatively, by the subsequent oxidation by labile sites generated in the DNA during extraction. This latter hypothesis is supported by the work of Claycamp et al.¹¹ and questions the validity of using phenol extraction for oxidatively stressed cells. In addition, Harris et al.¹² have demonstrated that unless precautions such as using freshly made phenol reagent are taken, the levels of 8-oxo-dG are generally higher in DNA from cells that have been exposed to an oxidative stress. However, because there is not a linear relationship between sensitisation during extraction and initial oxidative damage, the use of phenol extraction techniques for the study of in vivo oxidation of DNA is inappropriate.

After phenol extraction, but irrespective of the "oxidative stress" technique employed, the levels of 8-oxo-dG were always greater in DNA isolated from oxidatively stressed PBMC than in reextracted oxidatively stressed DNA. This may possibly be due to the presence of other sensitising agents within cells that generate labile sites that are susceptible to subsequent phenol oxidation. Because H₂O₂ had no effect on 8-oxo-dG levels in naked DNA under these conditions, this suggests that for PBMC, direct radical attack on DNA is only responsible for a small part of the damage incurred. Further investigation showed that following phenol extraction, significant induction of 8-oxo-dG was only observed after H₂O₂ treatment in the presence of FCS, again indicating that under these conditions, the effects of extracellular H₂O₂ alone on the induction

of 8-oxo-dG in PBMC is limited. The presence of *L*-Histidine and glucose in the extracellular milieu have been reported to potentiate H₂O₂ damage in mammalian and bacterial cells.¹⁶ These components were present in the medium in which the cells were exposed to H₂O₂ and may therefore have contributed to the generation of damaged sites within DNA, which following phenol extraction appeared as elevated levels of 8-oxo-dG.

This work has established that pronase E extraction does not induce any significant further oxidative DNA damage during isolation, and by this method there is no evidence for the induction of 8-oxo-dG within DNA after exposure to oxidative stress under the HPLC conditions employed. However, the basal levels of 8-oxo-dG measured were within the range reported by Floyd *et al.*¹⁷ for calf thymus DNA and were at the limit of detection by HPLC-ECD. A recent report by Adachi *et al.*¹⁸ has described an improved analytical method for 8-hydroxydeoxyguanosine in nuclear DNA, with the detection limit being improved one order of magnitude. Under these conditions 8-oxo-dG can be detected after nuclear DNA extraction by pronase E, indicating that pronase E does not select against the isolation of damaged DNA. The use of chromatin extracts eliminates the problems encountered during the isolation method.¹⁹ We are currently developing a technique, the guanase method, which combines these optimal parameters by hydrolysing chromatin and using HPLC detection.

The importance of establishing a reliable method for the detection of 8-oxo-dG at very low levels is clear. We are also currently undertaking studies using selective and specific antibodies to oxidative DNA damage products.²⁰ This eliminates the requirement for DNA extraction from cells because the antibody can be applied to fixed whole cells. Therefore, this appears to be a novel method for identification and quantification of 8-oxo-dG without the potential for selective isolation of certain DNA molecules (whether native or modified) and with limited potential for artefactual induction of DNA damage.

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Brief Communication

A Novel HPLC Procedure for the Analysis of 8-Oxoguanine in DNA

Cap 5

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Abstract—The chromatographic quantitation of 8-oxoguanine adducts in DNA is widespread in the literature, although results obtained by HPLC of 8-oxodeoxyguanosine do not always agree with levels determined by GC-MS. To help explain this discrepancy, here we describe a novel procedure for the analysis of 8-oxoguanine adducts in DNA. Although it proved difficult to directly quantitate 8-oxoguanine in the presence of high levels of endogenous guanine using conventional reversed-phase HPLC, a simple preincubation of DNA acid hydrolysates with guanase allowed such analyses. The assay relied on our observation that 8-oxoguanine was not a substrate for guanase, and on sensitive electrochemical detection. The limit of detection for 8-oxoguanine was 5 nM or 250 fmol on column. Using this procedure, the background level of 8-oxoguanine in commercially available calf thymus DNA was 0.4 nmol/mg DNA or 3.2 mol/10⁹ mol guanine.

Keywords—DNA damage, Free radicals, High-performance liquid chromatography, Guanine, Guanase, 8-Oxoguanine

INTRODUCTION

The 8-oxoguanine adduct is frequently used as a marker of DNA-base damage caused by oxygen free radicals,¹ although it is only 1 of at least 20 products formed.^{2,3} 8-Oxoguanine is formed in DNA treated with a variety of reducing agents⁴ and oxygen radical-generating systems^{2,5} and is reported to cause mutations through its ability to form hydrogen bonds with bases other than cytosine.^{6,7} In addition to this possible role in mutation and carcinogenesis,⁸ this lesion may also be important in the pathology of a number of inflammatory conditions in humans for example rheumatoid arthritis and systemic lupus erythematosus,^{9,10} where oxygen free radicals produced during inflammation have genotoxic potential.

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 HPLC with electrochemical detection.¹¹ In this procedure, DNA is first enzymatically digested to yield deoxynucleosides, which are separated by reversed-phase HPLC; 8-oxodeoxyguanosine can be determined sensitively by electrochemical detection.¹² In the same HPLC run, native deoxyguanosine can be measured by UV absorbance to provide a reference. Alternatively, GC-MS methods have been used to quantitate 8-oxoguanine, as the base,¹³ but the number of research groups reporting such data has been far fewer than for HPLC, perhaps because the equipment is more expensive and technically demanding to use than HPLC. Nevertheless, the two techniques have been compared¹⁴ and results obtained do not concur. For example, in commercial calf thymus DNA, a range of 8–302 8-oxodeoxyguanosine residues/10⁶ bases has been quoted using the HPLC method¹⁵ compared to 159–318 residues/10⁶ bases by GC-MS;^{2,14} in general, levels of 8-oxoG were higher when determined by the latter technique, and even in freshly isolated cells 2–11-fold higher values have

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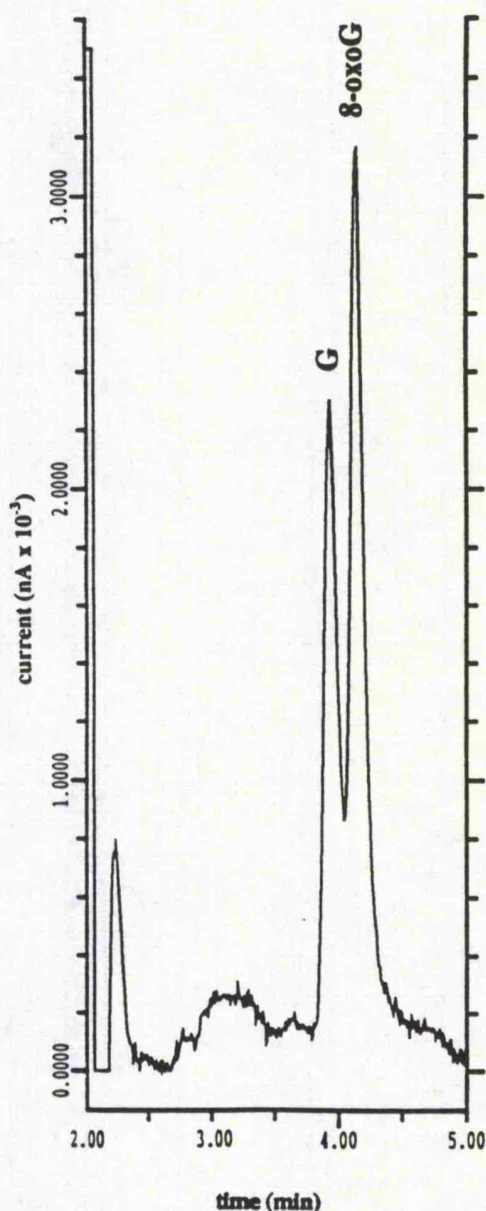


Fig. 1. Reversed-phase HPLC analysis, using electrochemical detection at +600 mV, of a solution containing 500 nM guanine (G) and 40 nM 8-oxoguanine (8-oxoG). Chromatographic conditions were as described in Materials and Methods except the mobile phase was 50 mM sodium acetate, 1 mM EDTA, pH 5.1 containing 2% methanol.

been reported by GC-MS. A number of factors may account for such discrepancies, including incomplete enzymatic hydrolysis in the presence of oxidatively modified DNA, suboptimal enzymatic hydrolysis, and the balance between incomplete depurination vs. formation of artefacts by formic acid hydrolysis of DNA. To attempt to account for some of these methodologi-

cal variables we now describe an analysis of 8-oxoguanine by HPLC.

MATERIALS AND METHODS

Materials

Guanase (EC 3.5.4.3) from rabbit liver was obtained from ICN Biomedicals Ltd (Thame, UK). Calf thymus DNA was from Calbiochem Novabiochem UK Ltd (Nottingham, UK). 2-Amino-6,8-dihydroxypurine (8-oxoguanine) was from Aldrich (Gillingham, UK). HPLC grade methanol was obtained from Fisons Ltd (Loughborough, UK). All other chemicals were of the highest grade available and obtained from Sigma (Poole, UK). The HPLC consisted of Beckman 126 solvent module (Beckman Instruments UK Ltd, High Wycombe, UK), model 507 autosampler, a stainless steel column (150 × 4.6 mm i.d.) packed with 3 µm ODS-Hypersil (Shandon, Runcorn, UK), a model 168 diode array detector, and an EG & G Instruments

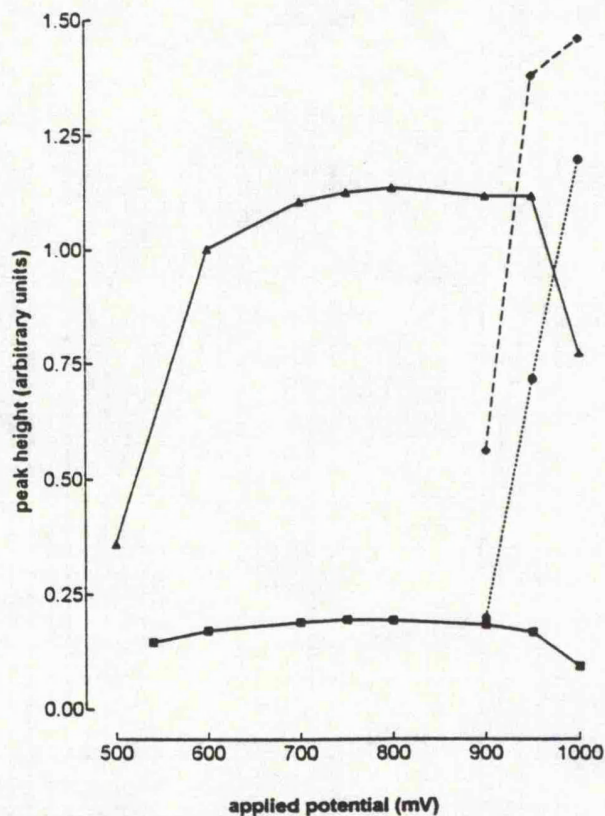


Fig. 2. Voltammograms for uric acid (Δ), guanine (◆), 8-oxoguanine (■) and xanthine (●). Compounds were analyzed using the HPLC procedure described in Materials and Methods and peak heights determined for amperometric responses at applied potentials ranging from +500 to +1000 mV.

model 400 electrochemical detector. The HPLC was controlled and data analyzed using Beckman System Gold Software.

nase digestions; this was performed on selected peaks over the range 200–350 nm.

RESULTS AND DISCUSSION

Reversed-phase HPLC determination of 8-oxoguanine in the presence of excess guanine

Near baseline separation was achieved for the four native DNA bases and 8-oxoguanine using a 3 μ m ODS-Hypersil column (150 \times 4.6 mm i.d.) packed in house. However, when using a 10⁵-fold molar excess of guanine, as to be expected from a 'real' DNA sample, the 8-oxoguanine peak was not resolved from the guanine peak (Fig. 1). This was a problem, because guanine is also electrochemically active and in excess interfered in the analysis of 8-oxoguanine (Fig. 2). Potentials of 580, 560, and 540 mV were also used with guanine at only a 50-fold molar excess in an attempt to eliminate such interference; this was reduced but not eliminated (data not shown). Increasing the column length, and modification of pH, ionic strength and organic modifier content of the mobile phase did not effect the separation (data not shown). Therefore, using this chromatographic system we considered that analysis of 8-oxoguanine in DNA in the presence of guanine was not feasible.

One possible solution to this analysis was to remove the excess guanine without affecting 8-oxoguanine. A series of experiments was performed to characterize the

Formic acid hydrolysis

Acid hydrolysis using formic acid cleaves the glycosidic bonds between the deoxyribose moieties and the bases in DNA, releasing both modified and unmodified bases. Samples of calf thymus DNA were hydrolyzed according to the optimized methodology of Dizdaro-glu.¹³ Aliquots (100 μ g) of DNA were treated with 0.5 ml formic acid (60% v/v) at 140°C for 30 min in evacuated and sealed tubes. Samples were dried under vacuum in a Savant SpeedVac [Life Sciences International (UK) Ltd, Basingstoke, UK]. Additional drying was performed under a stream of nitrogen gas prior to reconstitution with purified water (200–250 μ l).

Removal of guanine

Undiluted (1.265 units/ml) guanase was added to solutions of bases to 1.5 mU activity/0.5 ml of 5 μ M guanine, or to reconstituted DNA hydrolysates to the level of 1.5 mU/40 μ g DNA. Incubation was for 1 h at 37°C. Samples were then diluted by a factor of 10 prior to analysis by HPLC.

HPLC Conditions. DNA bases were separated by reversed-phase HPLC using a 3 μ m ODS-Hypersil (150 \times 4.6 mm i.d.) column. Elution was performed isocratically at 1 ml/min with a mobile phase of 40 mM KH₂PO₄, 1 mM EDTA, pH 5.0 containing 1% (v/v) methanol. Samples (50 μ l) were injected by autosampler. Detection of native DNA bases was by UV absorbance at 254 nm and of 8-oxoguanine by electrochemical detection using a glassy carbon working electrode at a potential of +600 mV vs. a Ag/AgCl reference electrode. Both EG & G model 400 and BAS LC-4B (Biotech, Luton, UK) amperometric detectors were used successfully to quantitate 8-oxoguanine; coulometric detection would also be applicable. Peaks were quantified by reference to peak areas obtained from authentic standards. Diode array detection was used to assign probable peak identities following gua-

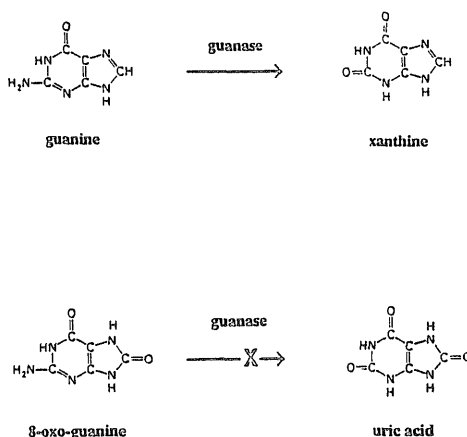


Fig. 3. Reaction scheme for the oxidative deamination of guanine by guanase. The formation of uric acid from 8-oxoguanine was not detected by HPLC with electrochemical detection.

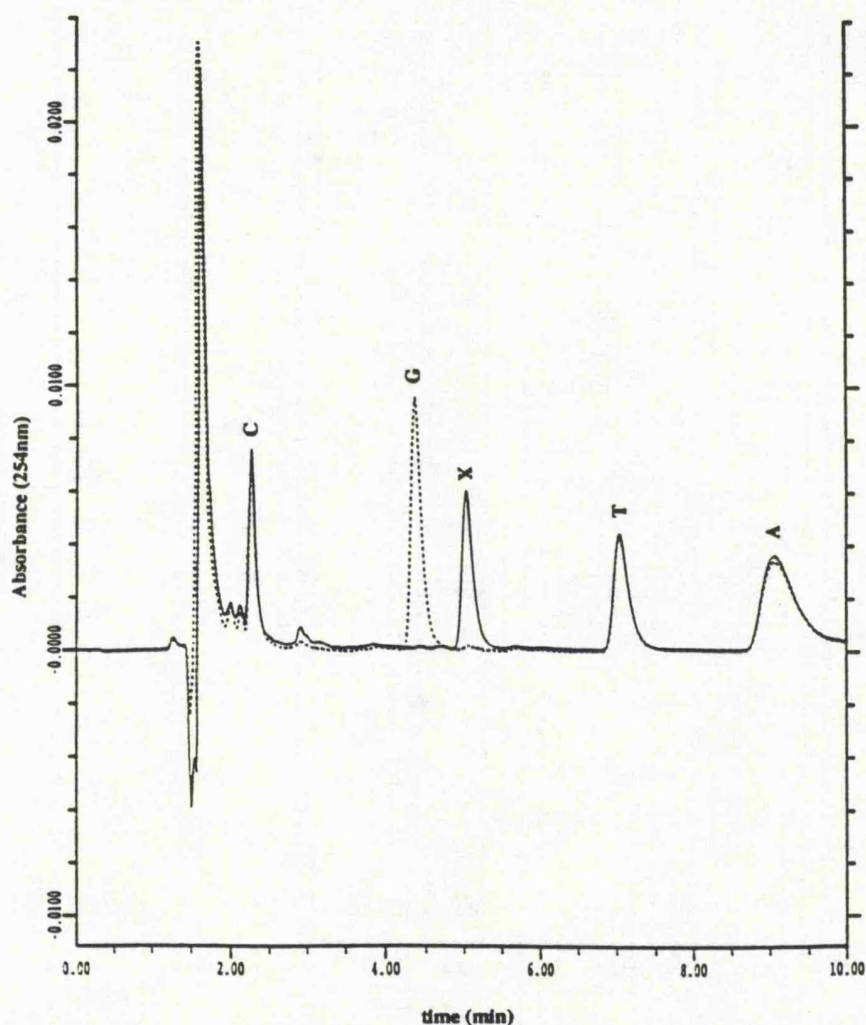


Fig. 4. Effect of guanase on bases derived from a formic acid hydrolysate of calf thymus DNA. Samples were analyzed by HPLC with UV detection prior to (---) and following (—) guanase treatment as described in Materials and Methods. C, cytosine; G, guanine; X, xanthine; T, thymine; A, adenine.

reaction catalyzed by guanase (guanine deaminase; guanine aminohydrolase; EC 3.5.4.3). HPLC with electrochemical and UV detection was used to monitor reaction mixtures. Diode-array detection confirmed that xanthine, the product of the deamination of guanine, in fact, was produced by guanase; the spectrum of a xanthine standard was superimposable upon that of the guanase product peak, which eluted at a similar retention time to the standard (data not shown). Furthermore, under identical enzymatic conditions using electrochemical and UV detection we could not detect the formation from 8-oxoguanine of uric acid, the hypothetical product of deamination of 8-oxoguanine (Fig. 3); the voltammogram for uric acid is shown in Fig. 2, and the limit of detection for uric acid was <2 nM at 600 mV, confirming the lack of

synthesis by guanase. In all these experiments with guanase, the HPLC peak corresponding to guanine was abolished, whereas that of 8-oxoguanine was not affected (recovery was $> 95\%$). Furthermore, using electrochemical detection, the 8-oxoguanine peak was fully resolved from the xanthine peak in contrast to the poor separation between 8-oxoguanine and guanine. No interfering peaks were observed in control chromatograms where guanase was incubated in the absence of purines and an aliquot analyzed using electrochemical detection (data not shown). We conclude that 8-oxoguanine is not a substrate for guanase. To our knowledge, these observations have not been reported before. Depending on the type of modification at the C8 position, purine derivatives may or may not be substrates for guanase. For example, 8-azagua-

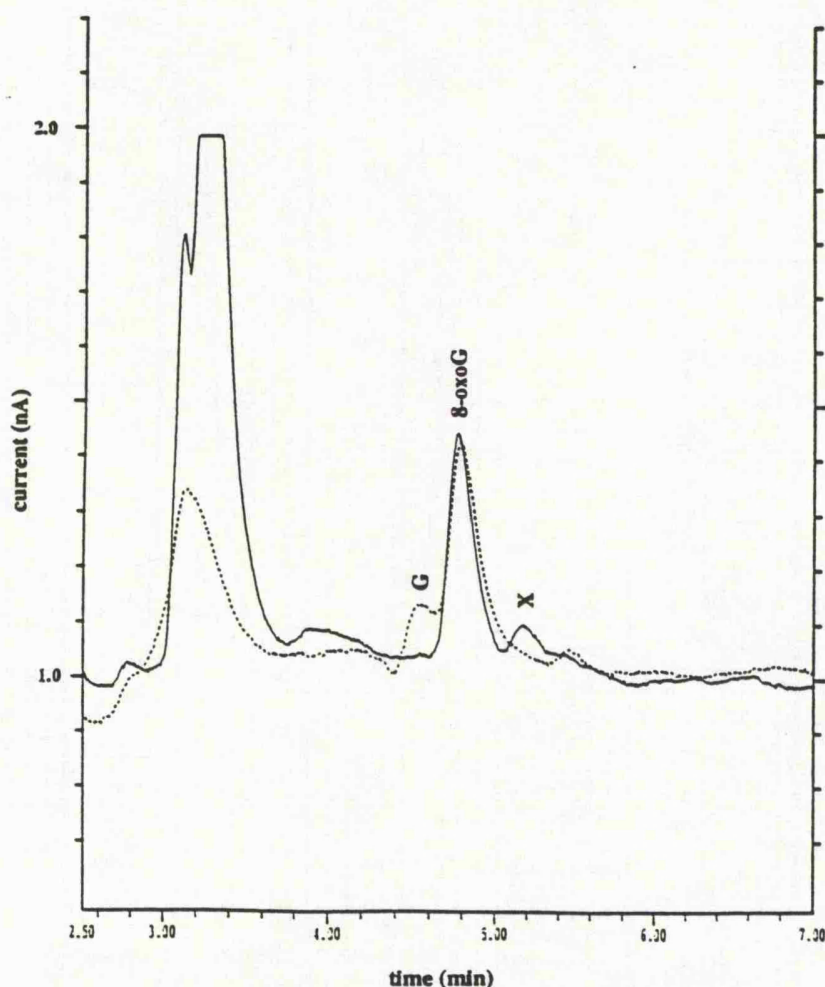


Fig. 5. Detection of 8-oxoguanine in gamma-irradiated (400 Gy) calf thymus DNA. Samples of formic acid hydrolyzed DNA were analyzed by HPLC with electrochemical detection before (—) and after (---) guanase treatment.

nine¹⁶ and substituted 8-azaguanines¹⁷ are substrates for guanase but 8-aminoguanine is neither a substrate nor does it inhibit the deamination of guanine.¹⁸ Our observations suggest 8-oxoguanine is rather like 8-aminoguanine in this respect.

In order to account for any possible conversion of, or inhibition by, the other DNA bases or other products of acid hydrolysis, the guanase reaction was carried out on a sample of formic acid-hydrolyzed DNA. Using HPLC with detection at 254 nm (Fig. 4), guanine was completely converted to xanthine by guanase treatment, whereas cytosine, thymine, and adenine were unchanged, assuming that a product(s) was not formed with identical capacity factor(s) and molar extinction coefficient(s) as the parent base(s).

The formic acid hydrolysis conditions for release of 8-oxoguanine, its stability under such conditions and

the possibility of its artefactual formation by acid treatment of DNA have been investigated previously.^{14,19} It was concluded that formic acid at a concentration of 60% (v/v) was optimal for hydrolysis; the hydrolysis conditions described in the Materials and Methods section are those described by these authors.

Given the success, the simplicity, and low cost of guanase, further validation of the HPLC analysis was performed. Using a series of 8-oxoguanine standards a calibration curve was established that was linear over the range 5–500 nM, with a limit of detection of 5 nM or 250 fmol on column (S/N = 3).

Background levels of 8-oxoguanine in commercial calf thymus DNA

Having established the HPLC determination, the application of the method to commercial calf thymus

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DNA (Calbiochem) was investigated. The mean level of 8-oxoguanine detected in five samples of DNA was 0.38 nmol/mg DNA (or 3.2 mol/10⁵ mol guanine). This mean value was similar to literature values, determined by GC-MS, using commercial calf thymus DNA;¹⁴ the reported range for such DNA is 0.50–1.0 nmol 8-oxoguanine/mg DNA. Using gamma irradiation (392 Gy) of DNA as a model, 8-oxoguanine was detected in formic acid hydrolysates of DNA using this procedure (Fig. 5) at a level of 4.77 (SEM 0.54) nmol/mg DNA. This value is of the same order as levels of 8-oxoguanine quoted in the literature using GC-MS.²⁰ Further work will compare 8-oxoguanine levels in DNA obtained using this method to those obtained by the HPLC analysis of 8-oxodeoxyguanosine and by GC-MS of 8-oxoguanine. This will help to resolve differences that may be due to oxidation artifacts and/or problems with enzymatic hydrolysis of damaged DNA. Some of these discrepancies may only be resolved completely by the establishment of an accurate standard DNA or oligonucleotide that contains a known amount of 8-oxoguanine. The procedure we have described is also applicable to the determination of the amounts of 8-oxoguanine produced in culture supernatants as a result of DNA repair in cells.

In many laboratories the level of 8-oxoguanine in DNA is expressed in relation to the number of guanines present. If the level of guanine is required, then the sample can be divided following formic acid hydrolysis of the DNA. One sample is analyzed directly by HPLC with UV detection (8-oxoG does not interfere due to its low level); the other sample can be processed using guanase as described. Alternatively, we suggest that xanthine may be used as a measure of the amount of guanine present; this can be determined using HPLC/electrochemical detection in the same run as used to quantitate 8-oxoguanine.

A number of other approaches to the determination of 8-oxoguanine in DNA have been described. ³²P-postlabeling procedures are described in the literature;^{21,22} these methods provide the potential for very sensitive detection, although the techniques are very time consuming and cumbersome. Capillary electrophoretic determinations of 8-oxodeoxyguanosine²³ and 8-oxoguanine²⁴ have been described. To date, these techniques lack concentration sensitivity due to the inherent lack of sensitivity of UV absorbance measurements. However, sufficient sensitivity may be achieved using analyses based on fluorescent postlabelling, which has been reported for both HPLC²⁵ and capillary electrophoresis with laser-induced fluorescence detection.²⁶

In conclusion, we have established an HPLC assay

for 8-oxoguanine that has proven applicable to the analysis of oxidized DNA. The method is simple, rapid, sensitive, and reproducible and, unlike GC-MS, derivatization is not required, although a simple guanase treatment step is necessary. The analysis time is shorter than by GC-MS and by the HPLC determination of 8-oxodeoxyguanosine. Although more samples can be run in parallel with the latter enzymatic hydrolysis procedure, this is merely a function of the number of hydrolysis tubes and the facilities available for their handling in the case of the guanase/HPLC assay.

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ABBREVIATIONS

EDTA—ethylenediaminetetraacetic acid
 GC-MS—gas chromatography-mass spectrometry
 HPLC—high-performance liquid chromatography
 ODS—octadecylsilyl
 UV—ultraviolet