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Oxidative sensitivity of alternative functions of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase

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OXIDATIVE SENSITIVITY OF ALTERNATIVE FUNCTIONS OF THE GLYCOLYTIC ENZYME, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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DECLARATION

This thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the requirements for the degree of Master of Science (Honours). The material presented in this thesis is the result of my own unaided work and has not previously been submitted at another university or institution.

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1st March, 1995

ABSTRACT

The glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is susceptible to inflammatory oxidants such as hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2). Evidence [1] suggests that the monomeric form of GAPDH exhibits uracil-DNA glycosylase (UDG) activity. As oxidative damage has often been implicated as a possible cause of carcinogenesis, the sensitivity of UDG to inflammatory oxidants was investigated.

An assay, modified from Sirover (1979) [2], was developed which successfully quantitated the UDG activity of both bacterial UDG and UDG extracted from a human cell line, HCT 116. UDG activity in these cells was proportional to cell number, with an upper limit of 2.5×10^5 cells, possessing 3033U UDG/mg protein. Aliquots containing 2.5×10^5 cells were exposed to the inflammatory oxidants, OCl^- and H_2O_2. Exposure of HCT 116 cells to OCl^- up to a concentration of 5×10^{-4}M resulted in no change in UDG activity released from these cells. However, exposure to OCl^- at concentrations higher than 5×10^{-4}M resulted in complete loss of cellular UDG activity. In contrast, UDG activity was not inhibited at all in HCT 116 cells exposed to H_2O_2 up to a concentration of 10^{-2}M. Bacterial UDG, however, was not inhibited by either OCl^- up to a concentration of 10^{-3}M, or H_2O_2 up to a concentration of 10^{-2}M. As bacterial UDG displays different properties to human UDG, it may not be used as a model to investigate the mode of inactivation of human UDG by OCl^-.

GAPDH activity was reduced by 50% when HCT 116 cells were exposed to OCl^- at concentrations between $10^{-3}M$ and $2 \times 10^{-3}M$ and H_2O_2 at concentrations of $5 \times 10^{-4}M$. These results illustrate that both UDG and GAPDH are susceptible to oxidation by OCl^-, but only GAPDH was inhibited by H_2O_2.

Commercially prepared, tetrameric GAPDH did not exhibit any UDG activity, even at high concentrations. Likewise, the monomeric form of GAPDH, prepared by reconstituting native GAPDH in 10% SDS, did not exhibit any UDG activity. These results, in conjunction with the oxidation studies, suggest that UDG and GAPDH may be two separate enzymes, displaying very different activities and properties. Nevertheless, oxidative stress within the nucleus may inhibit key DNA repair enzymes and/or directly damage DNA, which may result in mutation and ultimately, carcinogenesis.

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ABBREVIATIONS

API	-	α_1-Protease Inhibitor
ATP	-	Adenosine Triphosphate
Cys_{149}	-	Cysteine Residue Number 149
1,3-DPG	-	1,3-diphosphoglycerate
DNA	-	Deoxyribonucleic Acid
DTT	-	Dithiothreitol
FBS	-	Foetal Bovine Serum
Fmlp	-	Formyl Methionyl Leucyl Phenylalanine
G-3-P	-	Glyceraldehyde-3-phosphate
GAG	-	Glycosaminoglycan
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
GPx	-	Glutathione Peroxidase
GR	-	Glutathione Reductase
GSH	-	Reduced Glutathione
GSSH	-	Oxidised Glutathione
[^{3}H]dUTP	-	Tritiated Deoxyuracil
H_2O_2	-	Hydrogen Peroxide
HCT 116	-	Human Colon Cancer Cell Line
$HO^{\bullet}$	-	Hydroxyl Radical
MCD	-	Monochlorodimedone
MPO	-	Myeloperoxidase
NAD^+	-	Oxidised Nicotinamide Adenosine Dinucleotide
NADH	-	Reduced Nicotinamide Adenosine Dinucleotide
$NO^{\bullet}$	-	Nitric Oxide
$O_2^{\bullet-}$	-	Superoxide Radical
8-OH-dG	-	8-Hydroxy-2'-Deoxyguanosine
OCl^-	-	Hypochlorite

PBS	-	Phosphate Buffered Saline
P_i	-	Organic Phosphate
PMN	-	Polymorphonuclear Granulocytes
PUFA	-	Polyunsaturated Fatty Acid
R-5-P	-	Ribose-5-phosphate
RNA	-	Ribonucleic Acid
RNS	-	Reactive Nitrogen Species
ROI	-	Reactive Oxygen Intermediates
ROS	-	Reactive Oxygen Species
UDG	-	Uracil-DNA Glycosylase

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1. GENERAL INTRODUCTION

1.1 FREE RADICALS INVOLVED WITH INFLAMMATION

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are an important component of host defence. Unfortunately, the potent reactivity of ROS and RNS may sometimes be misdirected, and is therefore often responsible for detrimental pathological changes in cells and tissues, which can then lead onto a number of diseases. In order to combat debilitating diseases affiliated with the inflammatory response, the etiology of these diseases in relation to ROS and RNS chemistry must first be understood.

It has long been established that the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is susceptible to inflammatory oxidants such as hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2). Evidence suggests that GAPDH also possesses many alternative functions, including DNA repair activity. Meyer-Siegler *et al.* (1991) [1] proposed that the monomeric form of GAPDH exhibits uracil-DNA glycosylase (UDG) activity. Oxidative damage has often been implicated as a possible cause of carcinogenesis, and may therefore be linked with inhibition of DNA repair enzymes such as UDG.

1.1.1 Reactive Oxygen Species (ROS) And Reactive Nitrogen Species (RNS)

1.1.1.1 Introduction

Reactive oxygen and nitrogen intermediates include species such as hydrogen peroxide, singlet oxygen and the free radicals [3,4]. ROS and RNS are created when the covalent bond between two atoms is broken, with one electron from each of the shared pair remaining with each of the atoms, a

process known as homolytic fission. The energy required to dissociate the covalent bond can be provided by electromagnetic radiation, heat, or other means [5]. The electron orbitals of most chemical substances contain paired electrons with opposite spins [6]. Molecules or fragments of molecules capable of independent existence, containing at least one unpaired electron in their outermost orbital, are known as free radicals [5]. The natural tendency of single electrons, however, is towards pairing, which induces the unpaired electrons to bond with each other. This also causes the species to be attracted slightly to a magnetic field (i.e. paramagnetic) [7]. Therefore, most free radicals are extremely reactive and, as a result, short-lived [6].

The ions of all the metals in the first row of the d-block in the Periodic Table contain unpaired electrons, with the sole exception of zinc, and therefore qualify as radicals [7]. They are also known as transition elements and their most important feature in relation to radical chemistry is their variable valency, which allows them to undergo changes in oxidation state. The most prominent transition element is iron, which may exist in three oxidative states, iron (II), iron (III) and less commonly, iron (IV). Iron (III), or ferric iron, is generally the most stable of the three states, whereas iron (II) salts (involving ferrous iron) are weakly reducing (i.e. are electron donors). Ferryl compounds in contrast, are powerful oxidising agents, that is, absorb electrons from the molecule they oxidise. Thus, iron (II) can be oxidised to the iron (III) state while oxygen dissolved in these iron containing solutions is reduced to the superoxide radical $O_2^{\bullet -}$ [5].

Intermediate Complexes

1.1.1.2 Chemical Reactions Of ROS And RNS

Molecular oxygen (O_2) may be regarded as a biradical and is a relatively weak oxidant owing to its free electrons with parallel spins. Reactive intermediates of oxygen, however, are generated by excitation or reduction [6]. In the course of the complete tetravalent (four-electron) reduction of oxygen, water is formed. If, in contrast, it undergoes sequential univalent (one-electron) reduction, which is more likely to occur *in vivo*, reactive intermediates such as the superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$), are formed [8].

The generation of ROS and RNS is widespread in biological materials and oxygen-derived and nitrogen-derived radicals include species such as those listed in Table 1.1 (references 9-53). ROS and RNS are generated during enzymatic processes (for example, xanthine oxidase), in subcellular organelles (such as mitochondria and chloroplasts), or during cell functions (such as phagocytosis) [6].

Table 1.1: *Summary Of ROS and RNS Properties.*

Nomenclature And Chemical Formula	Source	Relative Reactivity	Comments	Reference
Superoxide $O_2^{\bullet-}$	Irradiated aqueous solutions: $e_{aq}^- + O_2 \rightarrow O_2^{\bullet-}$ Biological molecules such as: glyceraldehyde; the reduced forms of riboflavin and its derivatives FMN and FAD; adrenalin; and thiol compounds such as cysteine. The electron transport chains of mitochondria and endoplasmic reticulum.	Relatively limited, with the radical diffusing significant distances before reacting with a target.	$O_2^{\bullet-}$ oxidises transition metal complexes and organic substrates. Reactions include: spontaneous dismutation; the Haber-Weiss reaction; protonation; and the formation of $^1O_2$.	5, 6, 9, 10, 11, 12, 13, 14, 15, 16.
Hydrogen peroxide H_2O_2	Dismutation of superoxide by the bivalent reduction of oxygen catalysed, for example, by glucose oxidase and other methods such as catabolism of polyamines by amine oxidases. Spontaneous dismutation reaction: $HO_2^{\bullet} + O_2^{\bullet-} + H^+ \xrightarrow{SOD} H_2O_2 + O_2$	Small, uncharged and relatively stable molecule, which can be directly responsible for cytotoxic effects. Its ability to interact with organic molecules is restricted.	Host damage due to H_2O_2 has been found to result from decomposition due to the relatively weak O-O bond. H_2O_2 can rapidly react with transitional metal ions, notably Fe and Cu, and their complexes, in a process known as the Fenton reaction.	5, 6, 12, 15, 16, 17, 18, 19, 20.
Hypochlorite OCl^-	Production by myeloperoxidase (MPO) during phagocytosis: $Cl^- + H_2O_2 \xrightarrow{MPO} OCl^- + H_2O$	OCl^- is highly reactive.	Neutrophil granule derived MPO catalyses a 2 electron oxidation of chloride by H_2O_2 to generate OCl^-.	21, 22.

Hydroxyl radical **HO•**	Homolytic fission: $H_2O_2 \xrightarrow{\text{energy}} 2HO^{\bullet}$ The Fenton reaction: $O_2^{\bullet-} + M^{n+1} \rightarrow M^n + O_2$ $M^n + H_2O_2 \rightarrow M^{n+1} + HO^{\bullet} + OH^-$ $O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + OH^-$ where M^n is the reduced form of the metal and M^{n+1} is the oxidised form of the metal.	$HO^{\bullet}$ is extremely reactive and immediately attacks molecules such as phospholipids, proteins, polysaccharides, nucleotides, organic acids and other macromolecules in their vicinity.	Physiologically, the Haber-Weiss reaction occurs at very slow rates. The interaction between $O_2^{\bullet-}$ and H_2O_2 can, however, be effectively accelerated by metal catalysts such as Fe. Therefore, $HO^{\bullet}$ is generated in different parts of the cell whenever metal complexes that promote $HO^{\bullet}$ formation, are available.	5, 6, 11, 13, 17, 19, 23, 24, 25, 26, 27.
Chloramine NH_2Cl	OCl^- can chlorinate endogenously produced NH_3, from the decarboxylation of amino acids or the high urease activity of *Helicobacter pylori*, to form NH_2Cl.	NH_2Cl is exceptionally reactive and toxic because of its high lipophilic property and low molecular weight, making it extremely short-lived *in vivo*.	Extracellular amines such as taurine and methionine immediately react with NH_2Cl to yield less toxic N-chloramines.	28, 29.
Alkoxy radical **RO•** **Peroxy radical** **ROO•**	Produced by the reaction between alkyl radicals formed during lipid peroxidation and molecular oxygen.	Readily abstracts hydrogen from lipids, proteins, antioxidants or nucleic acids.	$ROO^{\bullet}$ is responsible for the continual regeneration of radicals and concomitant reduction in lipid chain length during lipid peroxidation.	5, 17.
Organic hydroperoxide ROOH	Produced by the abstraction of hydrogen by $ROO^{\bullet}$ from nearby molecules such as proteins, antioxidants or nucleic acids.	ROOH compounds are transiently stable, decomposing to form numerous low molecular weight substances, including hydroxyalkenals.	Reactions are catalysed by transition metals, quinones, or metal containing compounds such as cytochrome P_{450}.	5, 30, 31.

Singlet molecular oxygen $^1O_2$	Production by the transfer of one of the unpaired electrons of molecular oxygen to a higher energy orbital via energy absorption and spin inversion. Modified Haber-Weiss reaction: $O_2^{\bullet-} + H_2O_2 \rightarrow {}^1O_2 + HO^{\bullet} + OH^-$ In the presence of hypochlorite: $OCl^- + H_2O_2 \rightarrow {}^1O_2 + Cl^- + H_2O$	High reactivity towards molecules that contain regions of high electron density, such as carbon-carbon double bonds.	The combination of H_2O_2 and OCl^- is biologically relevant since OCl^- can be formed by the enzyme MPO during phagocytosis.	9, 17, 32, 33, 34.
Nitric oxide $NO^{\bullet}$	$NO^{\bullet}$ is synthesised in an NADPH-dependent process by the enzyme nitric oxide synthase, which incorporates molecular oxygen into the terminal nitrogen in the guanidino group of L-arginine, forming nitric oxide and citrulline.	$NO^{\bullet}$ can diffuse across cell membranes quite freely, thereby producing cytotoxic effects such as the inhibition of both the Krebs cycle enzyme aconitase, and the mitochondrial electron transport chain complexes I and II. $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet-}$ to form $ONOO^-$, thereby providing a chemical barrier to cytotoxic free radicals.	$NO^{\bullet}$, or endothelium-derived relaxing factor, and $NO_2^{\bullet}$ contain unpaired electrons in their outer orbitals. The predominant forms of $NO^{\bullet}$ in (human) plasma are S-nitrosothiols (RS-NO, where RS is a thiyl group), mostly nitrosoproteins. $NO^{\bullet}$ readily complexes with haem metalloproteins such as haemoglobin and interferes with DNA synthesis through inhibition of ribonucleotide reductase.	35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52.
Peroxynitrite $ONOO^-$	Production by $NO^{\bullet}$: $ON^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^-$	Unstable.	$ONOO^-$ may be cytotoxic by itself or decompose to $HO^{\bullet}$ and $NO_2^{\bullet}$: $ONOO^- + H^+ \rightarrow ONOOH$ $ONOOH \rightarrow HO^{\bullet} + NO_2^{\bullet}$	49, 50, 51, 52.
Perhydroxy radical $HO_2^{\bullet-}$	Protonation: $H^+ + O_2^{\bullet-} \rightarrow HO_2^{\bullet-}$	A more powerful reducing agent and oxidant than $O_2^{\bullet-}$.	Not much $HO_2^{\bullet-}$ present at physiological pH due to the acidic requirements for the protonation reaction.	5, 6, 53.

1.1.2 Respiratory Burst Of Phagocytes

One of the principal mechanisms of host tissue damage, or microbicidal activity, of activated neutrophils is generation of reactive metabolites from atmospheric oxygen [54]. Although neutrophil metabolism is based largely on glycolysis for ATP production, an increase in oxygen consumption can be shown during phagocytosis, and is known as the "respiratory burst" (Figure 1.1) [55].

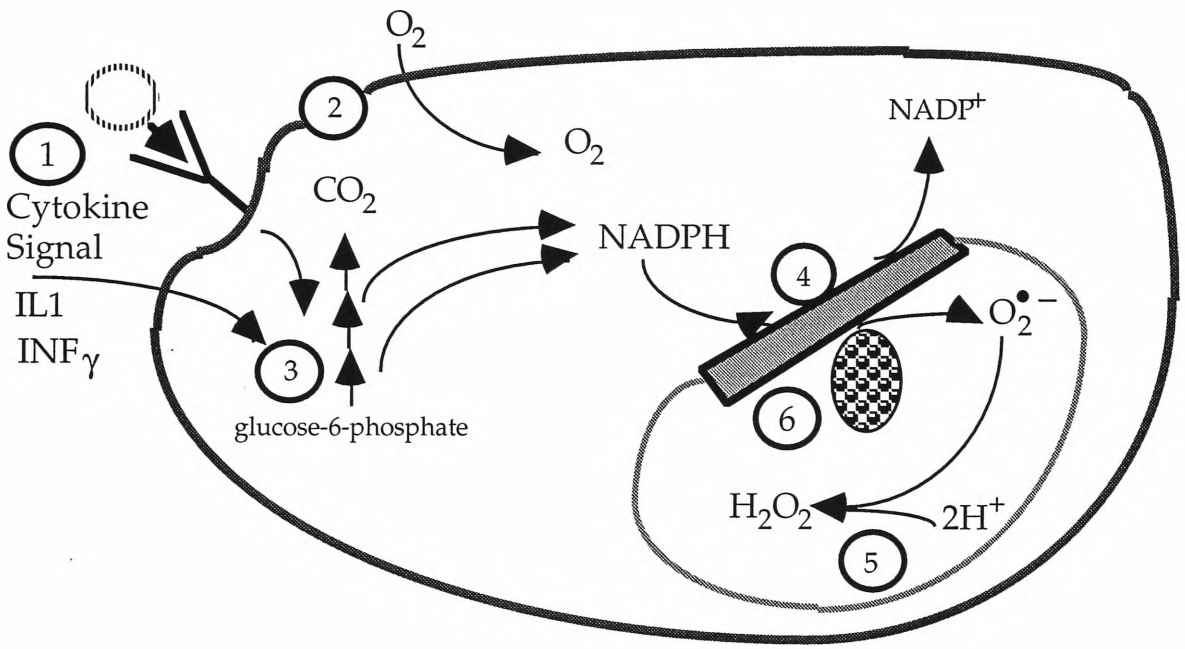


Figure 1.1: *Schematic Representation Of The Events Of The Respiratory Burst Of Phagocytic Cells [Adapted from 56].*

Stimulation by a cytokine signal or immunological contact (1) results in the uptake of molecular oxygen (2) and the production of $O_2^{\bullet -}$ and H_2O_2 (5). This series of reactions involves assembly of an NADPH oxidase complex (4) from components present in the membrane, cytosol and granules of the resting cell [54]. NADPH, found in the cytosol, is generated through the hexose monophosphate pathway, when glucose-6-phosphate (G-3-P) is

oxidised to ribose-5-phosphate (R-5-P) (3), as outlined in the following equation [57]:

$$\text{G-6-P} + 2\text{NADP}^{+} + \text{H}_2\text{O} \longrightarrow \text{R-5-P} + 2\text{NADPH} + 2\text{H}^{+} + \text{CO}_2$$

As the foreign particle (6) is being phagocytosed, the NADPH substrate binds to the Fe^{2+} containing receptor in the NADPH oxidase complex, located on the cell membrane (4), releasing free hydrogen ions. The electron transporting mechanism of this system contains a unique, iron containing cytochrome *b* [58] with a very low mid-point potential which, at -245mV, is capable of directly reducing oxygen to $O_2^{\bullet -}$. It is located in the plasma membrane, and in neutrophils it is also distributed in the specific granules and then translocated to the plasma membrane. The β-subunit of cytochrome *b* is the FAD-containing flavoprotein dehydrogenase [59, 60], which possibly also involves a quinone [58]. The electron-transporting apparatus of this NADPH oxidase is therefore entirely contained within a membrane-bound flavocytochrome.

The NADPH oxidase complex thus catalyses the one-electron reduction of atmospheric oxygen (2) to form $O_2^{\bullet -}$ in the lipid bilayer [61], in the region of the plasma membrane in direct proximity with the particle. The reaction between NADPH and oxygen is shown in the equation [28]:

$$\text{NADPH} + 2\text{O}_2 \longrightarrow \text{NADP}^{+} + \text{H}^{+} + 2\text{O}_2^{\bullet -}$$

The lag period provides the time required for closure of the vacuole, thereby limiting the release of the reduced oxygen species to the exterior [60].

Following this respiratory burst, $O_2^{\bullet -}$ dismutates either spontaneously or as catalysed by superoxide dismutase to form H_2O_2 [62, 63]. Neutrophil granule derived MPO then catalyses the oxidation of chloride (and other ubiquitous halide ions) by H_2O_2 to generate highly reactive hypohalite ions such as hypochlorite (OCl^-), which are potent microbicidal agents [21, 22].

Phagocytosis and the production of reactive species therefore provides an extremely complex, but highly effective, method of host defence against invading particles. However, this mechanism also has the potential to cause extensive host tissue damage.

1.1.3 Antioxidants And Scavengers Of ROS And RNS

A number of cellular antioxidant defences have evolved due to the potential damage that can be engendered by ROIs produced as a result of normal metabolic processes. These include the transfer of sensitive material to compartmentalised sites where they are protected from sites of ROS and RNS generation, for example, DNA in the nucleus, where there is little oxidative metabolism and where, under normal circumstances, the local ROS and RNS concentration is low. In addition, peroxidation chain reactions are terminated by chain-breaking antioxidants, including water-soluble glutathione, ascorbate and urate and lipid-soluble vitamin E, ubiquinone and β-carotene [64], with vitamin E (α-tocopherol) being the most potent antioxidant found in biomembranes [65].

Other reagents that function as antioxidants include the amino acids methionine, taurine and glycine, which act as scavengers of OCl^- by generating chloramines [34, 66], carotenoids which quench $^1O_2$ [67], and alcohols such as ethanol [26] and mannitol, which act against $HO^{\bullet}$ [68].

Antioxidant protection is also accomplished through the use of enzymes such as superoxide dismutase and catalase [20]. The enzyme superoxide dismutase, converts $O_2^{\bullet -}$ to H_2O_2 [69]. It is present in the cytosol as a copper (Cu)- and zinc (Zn)-containing enzyme [70], while in the mitochondria, contains manganese (Mn) [8, 55]. Peroxidases and catalases, however, are ferric iron-porphyrin-containing enzymes [64] that catalyse reactions in which H_2O_2 is the electron acceptor [16, 27]. In the glutathione oxidation-reduction cycle, the selenium (Se) containing glutathione peroxidase (GPx) [64] utilises reduced glutathione (GSH) to reduce H_2O_2, or other peroxides, to nontoxic forms. The oxidised glutathione (GSSG) resulting from this reaction is then reduced by glutathione reductase (GR) [8, 71]:

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Regeneration of GSH from GSSG by glutathione reductase requires NADPH as the electron donor species [8]. Therefore, this reaction acts as a competitor against the respiratory burst of phagocytes and thus as a negative feedback system when there is an over-production of H_2O_2. All these endogenous natural antioxidants contribute to the elimination of extra- and intracellularly produced oxygen radicals and provide a defence against oxygen-dependent injury [72].

1.2 INFLAMMATION

1.2.1 General Inflammatory Response

> *"Inflammation is the process by which cells and exudate accumulate in an irritated tissue and tend to protect it from further injury" [73].*

When host tissue is invaded by a foreign entity or is subjected to trauma, the immune system immediately mounts a response. This inflammatory response is complex and is mediated by a variety of signalling molecules produced locally by mast cells, platelets, nerve endings, and leukocytes as well as by the activation of complement. These molecules are then responsible for the dilation of vascular tissue to allow immune cells, particularly neutrophils and monocytes, to migrate into the damaged tissue where they are required. Other immune components then become involved, such as antibodies, prostaglandins, leukotrienes, lymphokines, monokines and interferons [74], in an attempt to eliminate the foreign particle. The phagocytic potential of inflammatory cells need not, however, be restricted to the attempted removal of the irritant alone, but also functions as the mechanism, in part, for the removal of debris produced by the inflammatory response.

1.2.1.1 Tissue Macrophages

Macrophage effector functions include the expression of cell surface proteins, intracellular enzyme activity, and the secretory capacity of the cell. Primarily, receptors such as the Fc receptor for Igs and CR1 for activated C3 fragments of complement, are necessary for binding to opsonised antigens such as microorganisms or tumour cells, and subsequent phagocytosis [33], while others such as the mannosyl-fucosyl receptors (MFR) can bind to non-

encapsulated microorganisms carrying these surface sugars. Macrophages also possess azurophilic granules and many cytoplasmic lysosomes, which contain several acid hydrolases and peroxidase [75], although, not MPO [69], which play an important role in the killing repertoire of these cells. Also produced by monocytes and macrophages are complement components, prostaglandins, interferons and monokines such as interleukin (IL) 1 and tumour necrosis factor (TNF) [75].

1.2.1.2 Polymorphonuclear Granulocytes

Polymorphonuclear granulocytes (PMN) may be observed in a variety of pathological conditions as disparate as arthritis, ischaemia and cancer, but are essential in host defence against bacterial infection. Neutrophils circulate in the peripheral blood, migrate to sites of acute inflammation and ingest foreign particles or microorganisms. They also possess enzyme-containing cytoplasmic granules which may be discharged during the inflammatory response. The killing of bacteria involves both oxygen-dependent and oxygen-independent processes, which probably also participate in damage to host tissue [54].

Neutrophil responses are initiated by the binding of the agonist to its receptor and the subsequent interaction of the agonist-receptor complex with a GTP-binding protein. Additional events that occur in the course of interaction of chemotactic factors with leukocytes include rapid membrane depolarisation, activation of cell membrane-associated Na^+K^+ATPase, transmembrane fluxes of cations (Na^+, K^+, Ca^{2+}), increased cell volume, activation of the hexose monophosphate shunt and the subsequent generation of oxidants [76]. The transient rise in cytosolic free calcium [77] and the activation of protein kinase C are events stimulated by phospholipase C mediated hydrolysis of phosphatidyl inositol to yield inositol phosphates and diacylglycerol. This process is believed to be

responsible for the production of energy required for the respiratory burst in the phagocytes [6]. Extending pseudopodia form a tight cleft between the PMN and target cell linked via FcRs and adhesion molecules, and subsequently engulf the target cell. Adhesion molecules do not activate a metabolic response or degranulation, but the FcR occupation is linked to the generation of intracellular 'second messengers' which translate the receptor-ligand interaction into a cellular response, involving both activation of the oxidative burst and degranulation [72]. Almost all proteolytic enzymes and other biologically active proteins released by neutrophils at sites of inflammation are derived from cytoplasmic storage granules formed during the development of these cells in the marrow. Neutrophils also have the ability to discharge granule contents either internally (into a phagocytic vacuole) or externally in response to appropriate secretory stimuli [54]. There are two principal, functionally distinct types of neutrophil granules: primary and secondary (also called "azurophilic" and "specific," respectively).

Primary granules are formed early in neutrophil development and have a complete enzymatic armament including MPO, neutral serine proteases such as elastase, and essentially all the lysosomal acid hydrolases found in neutrophils [72]. Several other effector molecules which possess cytotoxic properties include a group of small cysteine-rich cationic peptides known as defensins, which lyse human target cells and act synergistically with H_2O_2, resulting in enhanced cytolysis [78]. The normal function of these granules appears to be associated largely with lysosome-like, intracellular digestive processes [72]. The granule contents are maintained in an inactive state at a pH of about 5.0, but the pumping of millimolar concentrations of electrons, unaccompanied by protons, into the vacuole results in the consumption of hydrogen ions within the lumen, thus increasing the pH. The neutral proteases are activated when exposed to the relatively alkaline environment

within the vacuole [60]. Of note is the presence of high concentrations of β-glucuronidase, which is of specific importance because of the abundance of glucuronic acid in certain bacterial capsules [54]. In general, primary granules fuse with phagosomes and do not release their contents to the extracellular milieu except after stimulation with high concentrations of chemotactic secretagogues [58], or unless they are damaged to the point of cytolysis [72].

MPO is an iron-containing haem protein [22], constituting between 1 and 5 percent of the dry weight of neutrophils, or 0.5-1.0μg MPO/10^7 cells, and is responsible for the peroxidase activity characteristic of primary granules. In the presence of H_2O_2 and halide, MPO produces OCl^- and chloramines (in the presence of amino acids) which has been shown to be a potent mechanism for halogenating and destroying target cells [58]. Thus, granule constituents participate in both oxygen-dependent (for example, MPO) and oxygen-independent (for example, defensins) microbicidal systems.

In essence, the neutrophil responds to perturbation of its membrane or to binding at a surface receptor by initiating a change in subcellular ion distribution and by events which may include directed migration, assembly and activation of an NADPH oxidase complex, and mobilisation of granule contents, ultimately resulting in the dissolution of foreign particles.

The discovery that phagocytes involved in an inflammatory response produce large quantities of the oxygen radical $O_2^{\bullet -}$, as well as other nonradical reactive oxygen species such as H_2O_2, suggest the possibility that inflammation and mutagenesis might be related. Reactive oxygen species generated by inflammatory phagocytes may therefore injure target cells in a manner which contributes to carcinogenesis [69].

1.3 MECHANISMS OF HOST TISSUE DAMAGE AS A RESULT OF INFLAMMATION

1.3.1 General Pathology And Disease

Human neutrophils provide an effective host defence against bacterial and fungal infection, but they are also important in the pathogenesis of tissue damage in certain noninfectious diseases. The connection between neutrophil accumulation and joint destruction has been established in diseases such as gout and autoimmune arthritis. Neutrophils have also been linked with the pathogenesis of inflammatory bowel diseases [79] and play a part in enhancing tissue injury in myocardial infarction. Studies have demonstrated that mammalian mitochondria, like bacteria, produce formyl methionyl leucyl phenylalanine (Fmlp) [80]. This observation raises the possibility that the release of such mitochondrial contents in the presence of tissue injury or anoxia may provide another mechanism for recruiting neutrophils to non-infectious inflammatory sites [58]. Thus, inflammatory cells play an important role in mediating tissue injury, often resulting in disease.

1.3.2 Extracellular Targets

ROS such as $O_2^{\bullet -}$ and H_2O_2 are fairly long-lived, relatively non-reactive and can penetrate cell membranes [60] and travel within the extracellular spaces. Materials contained in these extracellular spaces include the body fluids and the framework or matrix in which many cells are embedded. Connective tissue is particularly rich in extracellular materials [81], therefore, continued attack on extracellular targets by ROS may cause connective tissue damage. They may also activate latent collagenase, inactivate protease inhibitors [62], and directly damage extracellular matrix proteins [61]. Till and others (1991) [82] have also revealed through morphological studies that damage of lung

alveolar capillary endothelial cells, in areas of intimate contact with oxidant producing neutrophils, display destruction of vascular basement membrane, fibrin deposition, interstitial and intra-alveolar oedema and intra-alveolar haemorrhage. It is however, only speculated that this destruction is caused by inflammatory oxidants. Therefore, ROS which are originally generated to destroy harmful foreign entities, may ultimately become harmful themselves and injure host tissue.

1.3.2.1 Extracellular Matrix Targets

Inflammatory neutrophils are recognised to play a key role in mediating the dissolution and remodelling of connective tissues at sites of inflammation [83]. The release of lysosomal constituents, such as elastase, collagenases and lactoferrin, is associated with the exocytosis of cytoplasmic granules of phagocytes [84, 85]. PMN elastase is responsible for the degradation of the core proteins of cartilage proteoglycans into fragments which are too small to be entrapped by the collagen meshwork, therefore diffusing out, resulting in proteoglycan depletion [86]. Similarly, collagenases cleave peptide bonds located in the helical regions of collagen, thereby disrupting the fibrous mesh [57]. Lactoferrin in contrast, sequesters iron [33] and therefore has been implicated as an iron donor for the catalysis of $HO^{\bullet}$ in the Fenton reaction. Halliwell and others (1988) [87] however, dispute this proposal, stating that lactoferrin "retains its iron-binding capacity at sites of inflammation".

One important means of inactivating elastase is through the acute phase serpin, α_1-protease inhibitor (API). Oxidation of a methionine residue (met_{358}) at the reactive centre of API, mediated by free radicals released from neutrophils, inactivates the protease inhibitor in the microenvironment of the neutrophils which potentially allows proteases released from these cells to damage adjacent connective tissue structures more readily [39, 85, 88].

Triggered neutrophils also use chlorinated oxidants to inactivate other antiproteases such as plasminogen activator inhibitor (PAI-1) [89].

$O_2^{\bullet-}$ and $HO^{\bullet}$-generating systems have the ability to damage extracellular matrix components such as collagen, elastin, hyaluronic acid and proteoglycans [90]. A basement membrane glycoprotein, fibronectin, was found to be susceptible to exposure to H_2O_2 [91], while sugars including glucose, mannitol and deoxy sugars react readily with $HO^{\bullet}$. Hyaluronic acid, which forms the central axis of proteoglycans, contains a repeating disaccharide unit of glucuronic acid and N-acetyl-glucosamine which upon exposure to free radical systems, fragments, leading to destabilisation of connective tissue [39]. Bates *et al.* (1985) [92] found that exposure of articular cartilage to H_2O_2 *in vitro*, inhibited proteoglycan synthesis. Katrantzis *et al.* (1991) [90] have shown that degradation of proteoglycan aggregates, due to the cleavage of the proteoglycan core protein in or near the hyaluronic acid binding region, occurs following exposure to OCl^-. However, they did not produce any evidence which suggested that OCl^- was responsible for cleavage of the glycosaminoglycan (GAG) chains. Although OCl^- oxidises and chlorinates a number of amino acids, the cleavage of the proteoglycan core protein may possibly have resulted from the production of $HO^{\bullet}$ during chlorination. Thus, extracellular matrix components of cartilage are all susceptible to damage by oxygen-derived reactive species, directly or indirectly.

1.3.3 Intracellular Targets

ROS and RNS are responsible for a variety of diverse intracellular aberrations. Of the various oxidants generated by leukocytes during the inflammatory response, H_2O_2 is the most likely oxidant to penetrate cells and reach the various cellular targets, including cell membranes, DNA,

proteins, calcium reservoirs and mitochondria. These aberrations may consequently lead to cellular dysfunction and often cell death.

1.3.3.1 Lipid Peroxidation And Membrane Damage

The oxidative decomposition of polyunsaturated lipids, referred to as lipid peroxidation, primarily involves free radical mechanisms [93, 94]. Lipid peroxidation of a membrane not only damages the membrane structure and function by degrading the highly unsaturated fatty acids such as $C_{20:4}$ and $C_{22:6}$, and thereby affecting protein-lipid interactions, but also forms breakdown products that can lead to membrane damage and disturbances elsewhere [94]. Products of lipid peroxidation include:

- lipid hydroperoxides [95]
- hydroxy fatty acids
- epoxy-fatty acids [96]
- alkanals (aldehydes)
- alkenals, including 4-hydroxy-nonenal [30]
- ketones
- alkanes [59].

Lipid peroxidation is a complex process initiated by abstraction of an electron by a reactive free radical such as $HO^{\bullet}$ or $NO^{\bullet}$, from the allylic position of polyunsaturated fatty acids (PUFAs) [5, 17]. Abstraction ruptures the covalent binding between a carbon and a hydrogen atom in the α-methylene ($-CH_2-$) group, leaving behind an unpaired electron on the carbon. The carbon radical (also known as an alkyl radical $R^{\bullet}$) thus generated tends to be stabilised by a molecular rearrangement to produce a conjugated diene [5] - an organic compound containing two carbon to carbon double bonds [97]. The alkyl radical then easily reacts with an oxygen molecule to give a peroxy radical ($ROO^{\bullet}$). $ROO^{\bullet}$ may then abstract hydrogen from another lipid

molecule, creating a new alkyl radical which in turn reacts with another oxygen molecule and so, once the process is initiated, it tends to proceed with the continual regeneration of a radical while reducing the lipid chain length [5]. This is the propagation stage of lipid peroxidation [17].

$ROO^{\bullet}$ may also abstract hydrogen from other nearby molecules such as proteins, antioxidants or nucleic acids, thereby producing hydroperoxides (ROOH) [5]. ROOH decomposes to form low molecular weight substances which act as modulators or damaging factors for cell biochemistry and function, such as blocking macrophage action and inhibiting protein synthesis due to structural modifications in ribosomes, particularly by hydroxy-*trans*-nonenal [98]. Furthermore, phospholipid antibodies may be induced as a result of free radical damage [39].

Lipoxygenase enzymes are also responsible for conjugating fatty acid hydroperoxides from the oxygenation of PUFAs. For instance, arachidonic acid is converted to leukotrienes which may ultimately cause changes in chemotaxis, provoking vascular damage and increased capillary permeability. Likewise, cyclooxygenase converts arachidonic acid into unstable endoperoxides which are responsible for platelet aggregation [99]. Enzymes that require $-NH_2$ or -SH groups for their activity, such as glucose-6-phosphatase and adenylate cyclase, are also usually inhibited during lipid peroxidation, particularly by aldehydes and 4-hydroxy-nonenal. The Na^+K^+ATPase enzymes, involved in maintenance of correct ion balance within cells, are also inactivated, as are surface receptor molecules that allow cells to respond to hormones. In addition, aldehydes such as malonaldehyde, can attack amino groups on the protein molecule to form both intramolecular cross-links and also cross-links between different protein molecules. This leads to a loss of membrane integrity which may

have marked effects on membrane fluidity [100, 101] and may be responsible for the inappropriate movement of calcium ions [5].

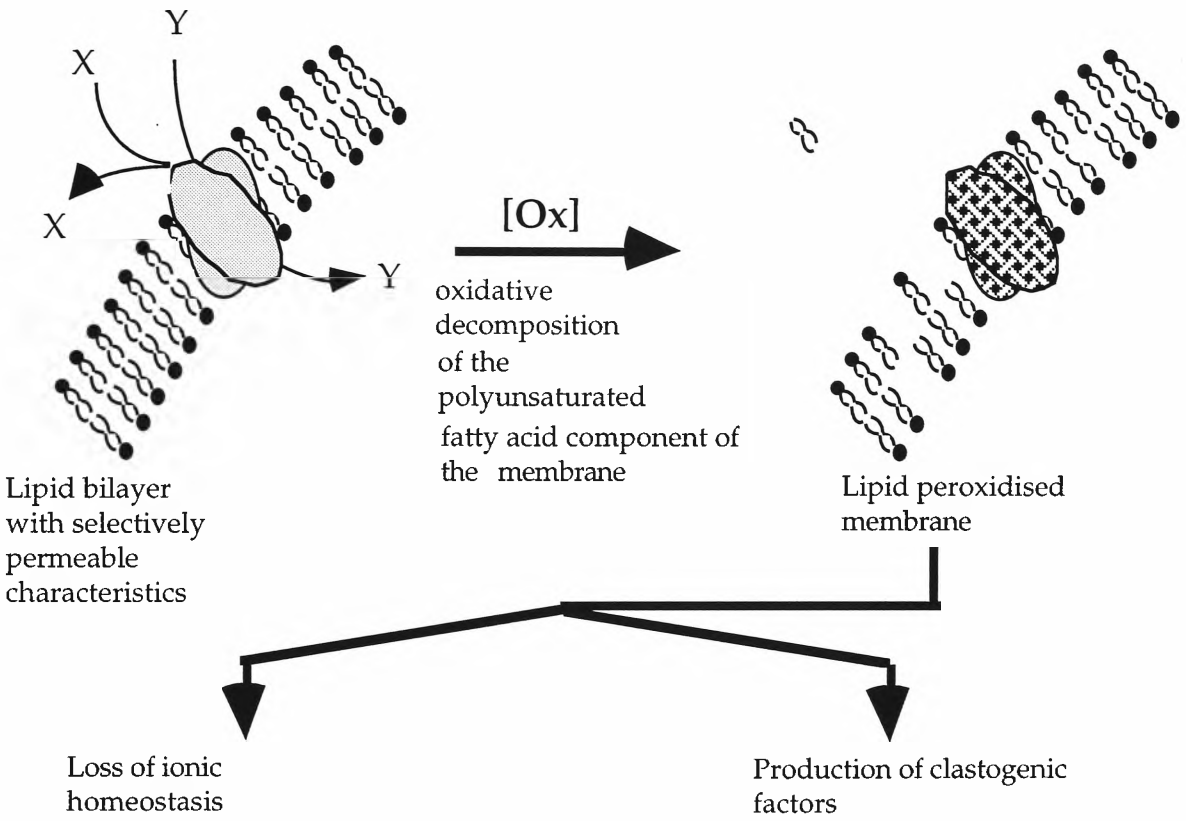


Figure 1.2 *Lipid Peroxidation And The Effects On Membrane Integrity [Adapted from 56].*

1.3.3.2 Oxidative Damage To Nucleic Acids

Activated neutrophils are often associated with alterations in the structure and function of nucleic acids in neighbouring nonphagocytic cells, including strand breaks, base modifications, mutations, sister chromatid exchanges and other chromosomal aberrations. Furthermore, the diffusible products of lipid peroxidation are also able to cause extensive DNA damage [8, 102].

The predominant extracellular oxidant responsible for neutrophil-induced DNA damage in target cells appears to be H_2O_2 [103]. This long-lived ROS readily diffuses into cells, as does $O_2^{\bullet -}$. In contrast, $HO^{\bullet}$ is extremely reactive and short-lived [4] (diffuses approximately 6nm from site of production) [12], requiring regeneration at the DNA target site [102].

The toxic effect of H_2O_2 on DNA is enhanced in the presence of transition-metal ions, and certain metal-ion chelators [102], such as L-histidine (the strongest metal ion chelator of all the amino acids) [104]. This suggests that intracellular metal-ion-catalysed production of $HO^{\bullet}$ by the Fenton reaction may be responsible for H_2O_2 toxicity [12]. Thus, DNA is not attacked by $O_2^{\bullet-}$ *per se*, instead, $O_2^{\bullet-}$ donates an electron to form the ferrous species required in $HO^{\bullet}$ formation from H_2O_2 [26, 27, 103, 105, 106]. Likewise, $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ to give the strongly oxidising $ONOO^-$ [107].

H_2O_2 is the dominant oxidant leading to DNA strand breaks in intact cells and purified DNA [108, 109], specifically fragmenting deoxyribose 3' termini, although $O_2^{\bullet-}$ is also capable of strand scission [110]. This effect is considerably enhanced in the presence of iron ions, inducing strand breaks at an average of 55% of sites. OCl^- in contrast, only produces strand breaks at about 5% of sites, while the corresponding figure for the combination of H_2O_2 and OCl^- (to generate $^1O_2$) is 20% [66, 111, 112, 113].

The reaction of $HO^{\bullet}$ with aromatic ring structures, such as purine and pyrimidine bases present in DNA and RNA, proceeds by its addition to the double bonds of DNA bases [114]. Furthermore, $HO^{\bullet}$ is capable of rapidly reacting with nucleic acids through the abstraction of hydrogen atoms from saturated carbon atoms. Therefore, all four bases and also the sugar moiety of DNA, are subject to attack by $HO^{\bullet}$. Nitrosylation of nucleic acids by $NO^{\bullet}$ and $ONOO^-$ causes rupture of DNA strands [107], by directly oxidising deoxyribose [49]. $NO^{\bullet}$ is also responsible for catalysing the oxidative deamination of cytosine (refer to section 1.4.2.1) and adenine to uracil and hypoxanthine, respectively [115, 116, 117, 118].

ROS also possess the ability to activate gene transcription. The immediate early oncogenes *c-fos, c-myc, c-jun, EGR1* and β-actin encode transcription factors which participate in the induction of cell growth, differentiation and

development and are induced rapidly by ionising radiation and H_2O_2 [119, 120, 121, 122, 123, 124, 125], possibly through the induction of DNA strand breaks. In carcinogenesis and possibly inflammatory disease, overproduction of radicals may cause excessive proliferation of tissue due to activation of these oncogenes. Free radicals have been implicated also in the activation of the transcription factor, NF-κB, which is an important transcription factor in inflammatory systems as it controls the transcription of a number of cytokine genes including IL2 and TNF-α, as well as the IL2 receptor gene and MHC Class I genes [124, 126].

Amongst the major irreversible modifications of DNA bases, is the production of thymine glycol [127], 5-hydroxymethyluracil [69] and 8-hydroxy-2'-deoxyguanosine (8-OH-dG), which is formed by $^1O_2$ attack at the C-8 position of the guanine base [128]. Boiteux *et al.* (1992) [129] suggested that the formation of 8-OH-dG might be explained by an initial electron transfer from the guanine ring to $^1O_2$, resulting in the generation of a guanine radical cation and $O_2^{\bullet -}$. 8-OH-dG also base-pairs with dA in DNA, thus contributing to spontaneous mutagenesis by favouring the G:C to T:A transversion [130]. These DNA products are eventually eliminated by repair enzymes, including excision enzymes and glycosylases [131]. DNA damage may however, occasionally escape repair, or its repair may be incorrect. In these instances, unrepaired or mis-repaired DNA damage could have deleterious consequences. Thus, neutrophil-induced DNA base damage could underlie gene modifications that may ultimately promote cellular transformation.

1.3.3.3 Oxidative Damage To Proteins

Radical production may result in protein damage and therefore directly influence protein activity, cytolysis and cell functioning and thus exacerbate the damaging effects of free radicals. ROS and RNS can readily modify

amino acid residues of proteins which results in: (a) cleavage and fragmentation [132, 133] (b) crosslinking and aggregation; (c) changes in conformation and (d) loss of function of target proteins [134, 135]. Oxidative damage to proteins is therefore inherently problematic due to the high concentration of proteins in cells [114].

Proteins which have been exposed to oxidants such as the $HO^{\bullet}$, exhibit altered primary structure (and consequently distortions in secondary and tertiary structure) and increased proteolytic susceptibility [136]. Increased susceptibility to proteolysis correlates with increased denaturation of native proteins which may expose previously buried hydrophobic residues that mark a protein for degradation [137]. In contrast, Khan *et al.* (1989) [138] describe a situation where fragmentation is associated with the spontaneous hydrolysis of proline residues and a concomitant increase in carboxylate functional groups. This results in a stoichiometric increase in glutamate concentration, which could result in unfolding of the protein due to the change in size and charge of the altered amino acid and consequently, a shift in the isoelectric point. Fragmentation of oxidised proteins may also result in a decrease in molecular weight due to the scission of the peptide backbone, generating random peptide fragments [137].

Oxidation in biological systems, frequently leads to cross-linking of proteins [133]. Catalano *et al.* (1989) [139] have established that the amino acid in the oxidised protein that carries the unpaired electron density from the free radical is an aromatic residue. This is consistent with the propensity of $HO^{\bullet}$ to react with aromatic rings [114]. Therefore, oxidation of a tryptophan [140], histidine (for example, in glutamine synthetase and pyruvate kinase) or phenylalanine (~4% of all amino acids) yields a radical that is eventually transferred to a tyrosine residue [114, 141]. This tyrosine residue then cross-links with other tyrosine residues, forming di-tyrosine functional groups,

thus covalently modifying the proteins and ultimately altering the structure causing inactivation of enzymes [68, 141].

Oxidative damage to protein thiols (-SH) is an important cause of alterations in enzymatic activity of carrier or receptor functions [8]. As an example, OCl^- oxidises plasma membrane sulphydryl groups and disturbs various protein functions such as inactivation of glucose and amino acid transporters and loss of potassium ion (K^+) pumping capacity [66]. Thiol groups show different levels of reactivity according to their location within a protein, with thiol groups exposed on the protein surface usually being more reactive [142]. During oxidative stress in the cell, the cysteine residues may become blocked due to formation of disulphide bonds [143], resulting in changes in the native conformation of the protein [144].

Protein S-thiolation, formation of a mixed disulphide of protein reactive sulphydryls and low molecular weight thiols such as glutathione (GSH) [145, 146], cysteine and cysteamine, plays an important role during oxidative stress [147, 148, 149]. This can have deleterious consequences for enzymes such as GAPDH [150], carbonic anhydrase III [151], cytoplasmic creatine kinase [152] and papain [153]. The nature of the products formed after H_2O_2 oxidation of protein thiols are frequently assumed to be disulphides, although, this is rarely the case. Instead, stabilised sulphenic acids may be formed upon reversible SH-oxidation by peroxides [154]. Several proteins and peptides are affected by oxidation of one of their methionine residues. Among these are lysozyme, ribonuclease, α-I-proteinase inhibitor, adrenocorticotropic hormone, Fmlp and complement factor C5a [59]. An even more potent oxidant however, is the $ONOO^-$, which oxidises sulphydryls about 103 times faster than does H_2O_2 [50].

Zinc is also frequently coordinated with the sulphur atom of one or more cysteine residues in metalloproteins such as metallothionein, hormone

receptors and transcription factors [155]. Fliss and Ménard (1992) [156], have evidence which suggests that $O_2^{\bullet -}$ and OCl^- are responsible for the intracellular mobilisation of complexed zinc. This results in the inactivation of zinc-requiring metalloproteins and a number of cellular processes such as calcium regulation, immune function and gene expression as a consequence of aberrant regulatory effects of the mobilised zinc [155].

1.3.3.4 Alterations In Calcium Homeostasis

Polla *et al.* (1990) [157] have established that oxidant cytotoxicity is associated with, and potentiated by, a massive increase in cytosolic calcium (Ca^{2+}). This increase in cellular calcium may initiate a cascade of events leading to cell death, among which are:

- activation of phospholipase A_2
- subsequent breakdown of cell membranes
- ATP depletion
- uncoupling of oxidative phosphorylation in mitochondria
- activation of neutral proteases
- release of potentially damaging lysosomal acid hydrolases [158, 159].

The low concentration of calcium in the cytosol (0.1μM), compared with high extracellular calcium concentrations (>1mM) [74] is maintained by active transport and compartmentation processes. Mitochondrial calcium homeostasis is regulated by a cyclic mechanism where calcium uptake requires the coupling of calcium transport with the hydrolysis of ATP, against an electrochemical gradient. Net calcium uptake also requires the presence of an intact permeability barrier to calcium provided by membrane phospholipids [160]. Calcium release which is mediated by a Ca^{2+}/H^+ antiporter, appears to be regulated by the redox level of intramitochondrial pyridine nucleotides, although membrane bound protein thiols are also

important in modulating mitochondrial calcium fluxes. The active transport of calcium through the endoplasmic reticulum and plasma membrane is mediated by Ca^{2+}-stimulated, Mg^{2+}-dependent ATPases which are critically dependant upon reduced sulphydryl groups for activity [161].

Oxidant damage to cells is rapidly followed by depolarisation of organelle membranes, resulting in the release of calcium from these organelles and thereby increasing cytosolic calcium concentrations. These organelles have therefore also lost their capacity to remove calcium from the cytosol [162]. The decreased uptake of calcium could result either from the direct inactivation of Ca^{2+}-Mg^{2+}-ATPase by the oxidants or from the reduced membrane fluidity, which in turn can inhibit Ca^{2+}-Mg^{2+}-ATPase activity [163].

A complex link also exists between intracellular thiol status and calcium homeostasis, since sulphydryl groups lead to a perturbation of intracellular calcium levels when oxidised [4]. This is illustrated by the rapid depletion of intracellular glutathione levels, loss of protein thiols and marked inactivation of the thiol-dependent enzyme GAPDH, that precedes the gradual elevation of cytosolic calcium ions [164], when exposed to oxidants such as H_2O_2 [66, 165, 166].

1.3.3.5 Alterations In Energy Homeostasis

Starke and Farber (1985) [105] have proposed, that disruption of energy metabolism by oxidative stress, may be one direct cause of cell death or at least contribute to it by limiting vital repair processes. The concept that oxidant damage to DNA initiates ADP ribosylation, which depletes the cellular NAD(H) pool and subsequently disrupts metabolic activity, supports this statement.

The consequence of DNA strand breakage due to oxidative stress, is the activation of poly (ADP-ribose) synthetase, an enzyme that polymerises ADP-ribose residues from nicotinamide-adenine dinucleotide (NAD) [167]. These polymeric ADP-ribose units are then covalently attached by adenosine diphosphate ribosyltransferase [168] to the ribosyltransferase itself (auto-ADP-ribosylation), histones or other nuclear proteins at arginine or cysteine residues. Mono-ADP-ribosylation involves the transfer of a single ADP-ribose group to the acceptor protein, whereas poly-ADP-ribosylation involves the transfer of many ADP-ribose residues. ADP-ribosylation is thought to be associated with depletions of NAD and adenosine triphosphate (ATP) [169, 170], and rises in intracellular calcium concentration [26, 171]. These events occur concurrently with an increased demand for NADPH as GSH is oxidised by the action of glutathione peroxidase catalytically degrading H_2O_2 [172]. Competitive consumption of reducing equivalents can therefore interfere with important metabolic functions and divert glucose from other pathways by inducing the monophosphate shunt [173]. Decreased glycolytic pathway activity due to inhibition of GAPDH activity [154], and/or loss of the mitochondrial membrane potential [162, 174], and therefore reduced ATP levels, can down regulate vital cellular processes like protein and DNA synthesis. Maintenance of cytoskeletal architecture is also compromised, with the appearance of cell rounding and membrane blebbing associated with reorganisation of filamentous actin [175], which may lead to cell injury and malignant transformation of cells [103]. Thus, reactions involving ROS and RNS, contribute to the overall metabolic perturbations that result in tissue damage and disease.

1.4 GAPDH AS A SENSITIVE TARGET OF OXIDATIVE STRESS

1.4.1 General Properties Of GAPDH

GAPDH (D-glyceraldehyde 3-phosphate:NAD^+oxidoreductase (phosphorylating); EC 1.2.1.12) is a hydrophilic, and therefore, water-soluble, protein [176]. It is present in very large amounts in the cell, and in muscle makes up for about 7% of the soluble protein [177]. Ercolani *et al.* (1988) [178] have cloned a functional 12 kilobase pair human genomic GAPDH gene consisting of 9 exons and 8 introns with eukaryotic signals necessary for the transcription and translation of GAPDH mRNA. Nasrin and co-workers (1990) [179] have also identified an insulin-responsive *cis*-acting element in the gene for GAPDH, capable of conferring insulin inducibility to a marker gene.

1.4.1.1 Enzyme Structure

GAPDH is a key enzyme in glycolysis and exists at equilibrium as a mixture of tetramers (75 x 70 x 80Å), dimers and monomers [180], with the tetrameric form being responsible for the glycolytic activity of GAPDH. The GAPDH tetramer is composed of 4 identical 37kD subunits, each containing 4 cysteine residues [181]. Also essential for enzymatic activity are four equivalent and independent exposed thiol sites per tetrameric enzyme molecule [182]. These thiols are located at $cysteine_{149}$ (cys_{149}) [150] of each subunit and are tightly bound to the coenzyme, NAD. Yang and Deal (1969) [183] have found, that the two nucleotide moieties in NAD have almost totally separate functions. The adenine nucleotide moiety is mainly responsible for binding, while position 4 on the pyridine ring of the nicotinamide nucleotide moiety, is responsible for catalysis.

1.4.1.2 Glycolytic Biochemistry Of GAPDH

GAPDH catalyses the reaction of glyceraldehyde-3-phosphate (G-3-P) with NAD^+ and phosphate ion (P_i), giving rise to 1,3-diphosphoglycerate (1,3-DPG) and NADH via a two-step reaction, namely an oxidation/reduction and a group transfer step with an acyl-enzyme thioester as an intermediate [184, 185].

$$\text{G-3-P} + \text{NAD}^+ + \text{P}_i \xleftrightarrow{\text{GAPDH}} \text{1,3-DPG} + \text{NADH} + \text{H}^+$$

An energy-rich phosphate compound is thus generated, when NAD^+ is concomitantly reduced to NADH. The active enzyme is then regenerated when the bound NADH is oxidised by a free NAD^+ in solution [57].

1.4.2 Alternative Functions Of GAPDH

GAPDH is recognised as a multifunctional protein, belonging to a large multiple gene family containing 150 or more GAPDH-like sequences [178], many of which are processed pseudogenes [186]. The human genome however, encodes a single functional GAPDH gene, as illustrated in transfected cells [178]. The pseudogenes may therefore represent genes which produce proteins which are structurally similar to GAPDH, but which may be functionally distinct. Allen *et al.* (1987) [187] however, suggest the possibility that the variants represent post-translationally modified forms of a single gene product, perhaps through conversion of glutamines and asparagines to their respective deaminated forms. Table 1.2 (references 188-195) summarises some of the alternative functions of GAPDH.

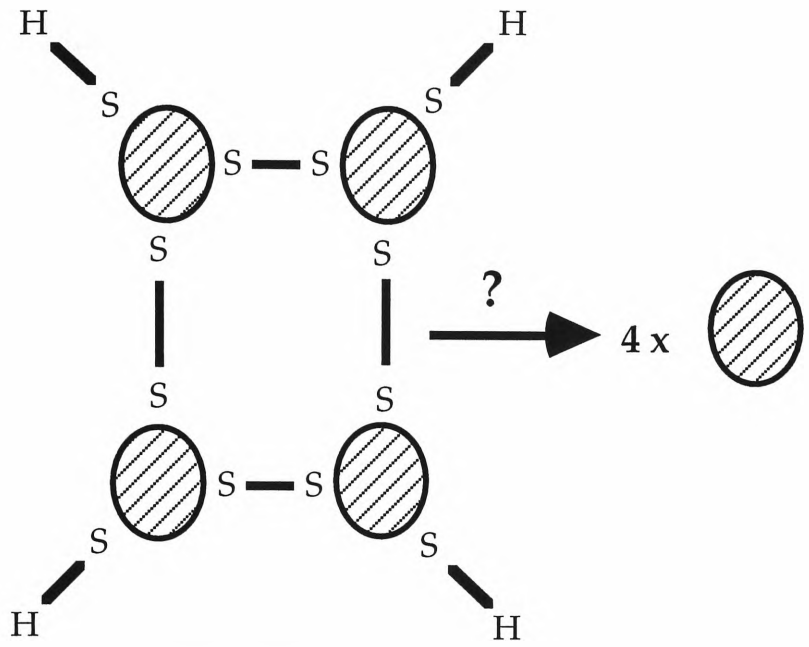
Table 1.2: *Summary Of Alternative Functions Of GAPDH.*

Alternative Functions Of GAPDH	Comments	Reference
Binding of GAPDH to plasma membranes	GAPDH binds to the cytoplasmic pole of band 3 in erythrocyte cell membranes. Both the binding and the inhibition of enzymatic activity can be reversed with media of high ionic strength or low pH.	176, 187, 188.
GAPDH as a structural protein	GAPDH is located inside the cell in association with microtubules, microtubule bundles and rabbit skeletal muscle triad junctions.	176, 189, 190, 191, 192, 193.
GAPDH as a DNA binding protein	GAPDH has an affinity for single-stranded DNA but not for double-stranded DNA. However, the binding of GAPDH to DNA is strongly inhibited by NAD. GAPDH has been implicated as a transfer RNA (tRNA) binding protein, participating in RNA export. NAD disrupts complex formation between tRNA and GAPDH. GAPDH has been associated with chromatin concomitant with the transition to the postmitotic state in the nuclei of cerebral cortex and cerebellar neuron cells.	177, 194, 195.

1.4.2.1 GAPDH As A DNA Repair Enzyme

Human cells contain two major excision-repair pathways to remove critical lesions from DNA. The nucleotide excision pathway excises bulky DNA adducts, while the base excision pathway removes alkylated bases and alterations due to spontaneous DNA damage [196. 197, 198]. Uracil, usually confined to RNA, can be formed in DNA as a result of utilisation of 5' dUTP during DNA synthesis [199, 200] or by deamination of existing cytosine residues by nitric oxide [115, 117]. Uracil derived from cytosine deamination can be easily distinguished from uracil deriving from dUTP misincorporation as uracil pairs with guanine, leading to a mismatched base-pair (U/G), whereas the misincorporated uracil, sterically similar to thymine, perfectly matches with adenine on the complementary strand (U/A). DNA glycosylases are responsible for removing these modified bases from DNA by hydrolysis of the N-glycosylic bond between the base and the deoxyribose, producing an apyrimidinic site, which is a substrate for apurinic/apyrimidinic endonuclease (AP endonuclease). Repair is completed by exonucleolytic digestion of the DNA segment, resynthesis by a polymerase and closure by a polynucleotide ligase [197, 198, 201]. In the absence of DNA repair, deamination of cytosine would be highly mutagenic in proliferating cells, as it induces the C/G → T/A transition, although the correction of U/G and U/A base-pairs is also necessary in resting cells.

During characterisation of a plasmid containing a functional cDNA for uracil-DNA glycosylase (UDG), Meyer-Siegler and co-workers (1991) [1] found that the cDNA sequence was completely homologous (>99%) with the nucleic acid and amino acid sequences for the 37kD subunit of GAPDH. The monomeric subunit of GAPDH was assayed for, and subsequently found to possess, UDG activity.



Glyceraldehyde-3-Phosphate Dehydrogenase (4 x 37kD)

Uracil-DNA Glycosylase (37kD)

Figure 1.3: *Schematic Representation Of The Suggestion By Meyer-Siegler et al. (1991) [1], That The Monomeric Form Of The Glycolytic Enzyme, GAPDH, Is The DNA Repair Enzyme, UDG [Adapted from 56].*

Aasland *et al.* (1990) [202] have detected a human placental UDG gene on chromosome 12, which corresponds with the location of the GAPDH gene. Muller and Caradonna (1991) [203] however, have isolated a human T cell UDG gene, from chromosome 5. A plausible explanation might be that the two UDG enzymes correspond to different subcellular forms of the enzyme, as at least two different forms of UDG, nuclear [204] and mitochondrial [204, 205], have been purified from eukaryotes.

GAPDH is therefore directly involved in a variety of cell functions ostensibly unrelated to each other, with its ability to function in DNA repair being of particular interest.

1.4.3 Oxidant Sensitivity Of GAPDH

1.4.3.1 S-Thiolation Of GAPDH

GAPDH activity is extremely sensitive to oxidative stress [206], due to the highly reactive thiol at cys_{149}. The amino acid sequence around the catalytic cysteine residue is: Ser-Asn-Ala-Ser-Cys*-Thr-Thr-Asn-Cys-Leu-Ala-Pro, with the catalytically active cysteine residue marked with an asterisk [207]. A unique feature of this sequence is the presence of a second cysteine residue four amino acids removed on the carboxyl side of the catalytically active cysteine residue. It has been suggested therefore, that the reversible inactivation of GAPDH by oxidative reagents is due to the formation of an intra-chain disulphide bond between the catalytically active cysteine residue and this second cysteine, Cys_{153} [150, 207]. However, the sulphydryl groups other than the ones required for catalysis, are buried in the three-dimensional structure of the enzyme, preventing the immediate formation of a disulphide bond. Little and O'Brien (1969) [154] have evidence which suggests that peroxides oxidise the catalytically active SH-groups of GAPDH to sulphenic acids which then undergo further oxidation either by dissolved oxygen or excess H_2O_2 to sulphonic acids.

1.4.3.2 S-Nitrosylation And ADP-Ribosylation Of GAPDH

An important action of $NO^{\bullet}$ on cells is the S-nitrosylation and subsequent inactivation of GAPDH. The binding of $NO^{\bullet}$ to reduced thiol groups on GAPDH could change or alter the equilibrium mixture of monomers, dimers and tetramers and in that way possibly modulate the different activities of GAPDH [46].

GAPDH also undergoes endogenous ADP-ribosylation in response to oxidation with $NO^{\bullet}$ and S-nitrosylation [45, 182, 208]. Mono-ADP-ribosylation of GAPDH occurs when the ADP-ribose moiety is cleaved to the active cysteine residue [209]. In contrast, auto-ADP-ribosylation of GAPDH involves a nonenzymatic post-translational modification of the protein, and direct binding of the ADP-ribose moiety with NAD, the coenzyme [208]. Either way, GAPDH activity is inhibited, diminishing the amount of ATP formed from glycolysis, consequently decreasing the flow of substrates to the electron transport chain [36].

1.5 AIMS OF THESIS

It is clear that oxidants released during inflammation cause injury to tissues. Since elucidation of the chemical nature of neutrophil-induced damage may enhance our understanding of the mechanisms underlying tissue damage and carcinogenesis associated with inflammation, the present study was undertaken to investigate whether inflammatory oxidants damage human GAPDH and its recently ascribed activity of a UDG.

Therefore, the principal aims of this study were:

(i) To develop a functional and reproducible UDG assay

(ii) To confirm whether human GAPDH possesses UDG activity

(iii) To extract UDG and GAPDH activities from human cell lines

(iv) To examine the effects of inflammatory oxidants such as hydrogen peroxide and hypochlorite on UDG activity

(v) To compare the effects of (iv) with those seen for GAPDH under the same conditions

2. MATERIALS AND METHODS

2.1 MATERIALS AND EQUIPMENT

2.1.1 General Chemicals

All buffers and solutions were prepared with autoclaved Milli-Q water (Millipore, England). General chemicals such as: Tris hydrochloride (Tris-HCl), Magnesium chloride ($MgCl_2$), bovine serum albumin (BSA), sodium chloride (NaCl), phenol, chloroform, ethanol, isopropanol, ammonium acetate, ethylene diaminetetraacetic acid (EDTA), sodium acetate, L-cysteine, magnesium sulphate ($MgSO_4$), glycerol and Triton X-100 were all reagent grade or better. Chemicals required for tissue culture such as: potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), glucose and sodium bicarbonate ($NaHCO_3$) were all tissue culture grade or better.

2.1.2 Chemicals And Equipment For The UDG Assay

Materials used in the preparation of a UDG substrate were attained from various companies and are listed with their corresponding supplier: Poly(dA)·$(dT)_{12}$, salmon sperm DNA, deoxy-uracil triphosphate (dUTP), dithiothreitol (DTT) and dialysis tubing (benzoylated, cellulose tubing) (Sigma Chemical Co., St Louis, MO, USA); tritiated dUTP ([^{3}H]dUTP) (Amersham, Australia); Polymerase I and herring sperm DNA (Boehringer Mannheim, Germany); the SpeedVac concentrator (Savant, Farmingdale, NY, USA); Nucleic Acid Purification (NAP-10) Columns (Pharmacia, Sweden); Bio-Spin Chromatography Columns (P6) (BioRad, Australia); and the Nick Translation Kit (Promega, Madison, WI, USA). *E. coli* Uracil-DNA Glycosylase (UDG) was supplied by Boehringer Mannheim, Germany. The scintillation cocktail was purchased from Packard Instrument Company Inc.,

Downers Grove, Ill, USA, while an LKB liquid scintillation counter (Linbrook International, NSW, Australia) was used to quantitate tritiated samples.

2.1.3 Chemicals And Equipment For The GAPDH Assay

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay components, such as: triethanolamine buffer, 3-phosphoglyceric acid (3-PGA), adenosine 5'-triphosphate (ATP), 3-Phosphoglyceric phosphokinase (3-PGK), reduced β-nicotinamide adenine dinucleotide (β-NADH) and GAPDH were supplied by Sigma Chemical Co., St Louis, MO, USA. A Pharmacia LKB-Ultrospec III spectrophotometer was used to quantitate GAPDH activity as well as hypochlorite anion and hydrogen peroxide concentration of stock solutions.

2.1.4 Chemicals And Equipment For Cell Culture

HCT 116 cells were supplied by American Type Culture Collection (ATCC), Rockville, MD, USA and were maintained at 37°C in RPMI 1640 media and foetal bovine serum (FBS) supplied by CSL, Australia. Cells were removed from tissue culture flasks (Crown Scientific, Australia) in a Class II tissue culture hood (Email, Australia), using trypsin/EDTA (Cytosystems, Castle Hill, NSW, Australia). Solutions requiring filter sterilisation were filtered with prefilters, 0.45μm and 0.22μm filters from Millipore, England. A Branson Ultrasonics Corporation sonicator (Danbury, CT, USA) was used to lyse cells during enzyme extraction.

2.1.5 Chemicals And Equipment For The Standard Protein Assay

A modified Lowry protein assay was conducted with a DC Protein Assay Kit and a 3550 Microplate Reader purchased from BioRad, Australia. ELISA microtitre plates were supplied by Crown Scientific, Australia.

2.1.6 Chemicals Used For The Oxidation Of Enzymes

The hypochlorite anion was supplied as sodium hypochlorite (NaOCl) by BDH Chemicals, Poole, England, while hydrogen peroxide was purchased from Merck, Damstadt, Germany. Monochlorodimedone (MCD) and trypan blue were supplied by Sigma Chemical Co., St Louis, USA, while phosphate buffered saline (PBS) was provided by Oxoid, Basingstoke, Hampshire, England.

2.2 PREPARATION OF GENERAL LABORATORY EQUIPMENT

2.2.1 General Glassware And Plasticware

Glassware was washed once with tap water and detergent, then rinsed with deionised water and again with glass distilled water. All bottles used for storage of buffers, media and other solutions, pipette tips and glass pipettes, including Pasteur pipettes, were sterilised in an autoclave for 20min at 121°C. Plastic tubes on the other hand, were either purchased sterile or were sterilised by microwaving on high for 20min.

2.2.2 Thin Bore Pipettes

Thin bore Pasteur pipettes were used for more efficient removal of supernatants where the remaining pellet was to be used for further experimental work, for example, ethanol precipitated [^{3}H]dUTP-labelled DNA pellets, and cells following oxidation. They were prepared by heating the thin barrel of the pipette in a naked blue flame and drawing the barrel out with a pair of tweezers. The thin bore pipettes were then rendered DNase free by autoclaving.

2.3 PREPARATION OF SUBSTRATE FOR THE UDG ASSAY

In order to measure UDG activity, a UDG substrate must initially be prepared. Several procedures were attempted in order to incorporate uracil into an oligonucleotide. Of these, the best was found to be the nick translation method which obtained the substrate with the highest level of incorporated [^{3}H]dUTP.

2.3.1 Incorporation Of Uracil Into A Poly(dA)·(dT)$_{12}$ Oligonucleotide

Poly(dA)·(dT)$_{12}$ was labelled with tritiated deoxyuracil ([^{3}H]dUTP) in a reaction catalysed by polymerase I, according to the method of Sirover (1979) [2]. Polymerase I catalysed the incorporation of [^{3}H]dUTP into Poly(dA)·(dT)$_{12}$ in the reaction buffer: 50mM Tris-HCl (pH 8.0)/5mM $MgCl_2$/50µM dUTP/2µg/µl BSA/0.5µCi/50µl [^{3}H]dUTP/1µg/50µl poly(dA)·(dT)$_{12}$/3U/50µl polymerase I. Following incubation at 37°C for 30min, the mixture was extracted with phenol and chloroform (Appendix A), then dialysed against 1M NaCl/50mM Tris-HCl (pH 7.6) and then 50mM Tris-HCl (pH 7.6) (Appendix A). This substrate, however, was not labelled adequately as indicated by the low number of scintillation counts. The substrate was therefore concentrated using various methods such as ethanol and isopropanol precipitation and oligonucleotide cleaning columns, which also aided in removal of contaminating [^{3}H]dUTP from the solution. Smaller volumes of substrate could then be added for UDG assays.

2.3.1.1 Ethanol/Isopropanol Precipitation Of UDG Substrate

The [^{3}H]dUTP-labelled poly(dA)·(dT)$_{12}$ substrate was precipitated in a solution containing 1.25M ammonium acetate, 62.5µg/ml herring sperm DNA and 2.5 volumes of 100% ice-cold ethanol or 1 volume isopropanol. The substrate was dissolved in TE buffer (10mM Tris-HCl/1mM EDTA; pH

8.0) to a final volume of 100µl. A number of the resuspended pellets were also heated to 37°C for 1h, as gentle heating aids in dissolving DNA. 200µl samples of the supernatants and the entire resuspended oligonucleotide pellet from all samples was then measured for radioactivity.

2.3.1.2 Use Of A NAP-10 Column For Removing Contaminating Free-[^{3}H]dUTP From The Labelled Oligonucleotide

The Sephadex G-25 NAP-10 column separates oligonucleotides with a size greater than 10 bases from smaller oligonucleotides, including free bases. Samples were eluted from the column with TE buffer and measured for radioactivity. The fraction recording the highest amount of radioactivity was then appropriate to use in a UDG assay.

2.3.1.3 Use Of A Bio-Spin Chromatography Column (P6) For Removing Contaminating Free-[^{3}H]dUTP From The Labelled Oligonucleotide

Fresh substrate was made with a new supply of polymerase and was incubated for 1h rather than 30min. dATP was also added to the reaction at a final concentration of 18.2µM, to bind to the dTTP tail of the oligonucleotide. The substrate was subsequently passed through a Bio-Spin Chromatography Column (P6) which functions by retaining free nucleotides within the column, while eluting the oligonucleotide from the column through centrifugal forces.

2.3.2 Nick Translation Procedure

The nick translation system provides an efficient method for the incorporation of labelled deoxynucleotide triphosphates into duplex DNA. DNase I creates free 3'-hydroxyl ends (nicks) within the unlabelled DNA, while DNA polymerase I catalyses the addition of a nucleotide residue to the

3'-hydroxyl terminus of the nick. At the same time, the 5' → 3' exonuclease activity of this latter enzyme removes the nucleotide from the 5'-phosphoryl terminus of the nick. The new nucleotide is incorporated at the position where the original nucleotide was excised, and the nick is thus shifted along one nucleotide at a time in a 3' direction. This 3' shift of the nick results in the removal of the pre-existing nucleotides, with the sequential addition of new deoxynucleotides, including radioactively labelled nucleotides, to the DNA [210].

The Promega Nick Translation Kit was utilised to label salmon sperm DNA with [^{3}H]dUTP as follows: 12 aliquots of 3µl of [^{3}H]dUTP (1mCi/ml) and 6 aliquots of 4µl of [^{3}H]dUTP were dried in a SpeedVac, in separate tubes, until the ethanol storage solution was completely evaporated. All tubes were then stored on ice until use. The following reagents (Appendix B) were then added to each of the 6 tubes containing 4µl of dried [^{3}H]dUTP:

dNTP mix (dATP, dCTP, dGTP)	60µM
Nick translation 10x buffer	1x
Salmon sperm DNA	1µg
DNA polymerase I/DNase I mix	5U DNA polymerase I/1ng DNase I
Sterile water (from kit)	make up to total volume of 50µl

The tubes were mixed well, but gently, centrifuged for 5s and incubated at 15°C for 1h. The tubes were then pulse centrifuged and their contents transferred to each of 6 tubes containing 3µl of dried [^{3}H]dUTP. Another 5µl of DNA polymerase I/DNase I mix was added to each of the 6 tubes, mixed well, spun briefly and then incubated again at 15°C for 1h. This process was then repeated with the final 6 tubes each containing 3µl of dried [^{3}H]dUTP. Following the final incubation, 5µl of stop buffer (0.25M EDTA, pH 8.0) was added to each of the 6 reaction tubes, mixed and centrifuged for 5s. The nick

translated salmon sperm DNA was then cleaned of contaminating free-[^{3}H]dUTP by ethanol precipitation with 3M sodium acetate and carrier DNA.

2.3.3 Methods For Removing Contaminating Free-[^{3}H]dUTP From The Labelled UDG Substrate

Various methods of removing the contaminating free-[^{3}H]dUTP were investigated. All experiments were conducted in triplicate and the average cpm values compared. The method of removing contaminating free-[^{3}H]dUTP from the labelled substrate which was used in subsequent preparations of labelled substrate is described in section 2.3.3.1.

2.3.3.1 Protocol Used For Subsequent Removal Of Contaminating Free-[^{3}H]dUTP From Nick Translated Substrate

The ethanol precipitation protocol which attained the labelled substrate with the highest radioactive counts and the least background counts due to contaminating free-[^{3}H]dUTP was as follows: 100µl of herring sperm DNA (1mg/ml) was added to the 65µl of nick translated salmon sperm DNA, followed by 20µl of 3M sodium acetate (pH 7.0) (Appendix B) and 1ml of ice-cold ethanol (100%). The solution was mixed by inversion and stored at -70°C for at least 2h, usually overnight. The mixture was then centrifuged at 12,500 x g at 4°C, for 20min. The supernatant was removed and the pellet washed in 100µl of 70% ethanol and spun at 12,500 x g for 2min at room temperature (RT). The supernatant was again removed and the pellet dried in the SpeedVac, then resuspended in 300µl of 50mM Tris-HCl. Following reconstitution at RT for at least 1h, 30µl of 3M sodium acetate and 1ml of 100% ethanol (-20°C) was added and the process repeated. Altogether, the [^{3}H]dUTP-labelled DNA substrate was ethanol precipitated 3 times and finally resuspended in 500µl of 50mM Tris-HCl. 10µl of this solution was required as a substrate in the UDG assay.

2.4 ENZYME ASSAYS

2.4.1 The UDG Assay

The UDG assay involves the quantitation of excised [^{3}H]dUTP from a labelled substrate, which is directly related to enzyme activity. The UDG assay was conducted at 4°C as follows: 50µl of UDG assay buffer (50mM Tris-HCl, pH 8.0/20mM EDTA/10mM dithiothreitol/400µg/ml BSA) was combined with 10µl of nick translated [^{3}H]dUTP-labelled salmon sperm DNA and the UDG enzyme, up to a maximum volume of 10µl. The total assay volume of 100µl was made up with 50mM Tris-HCl. Following incubation at 37°C for 1h, the reaction was terminated by immersion in an ice-water bath. The remaining UDG substrate was precipitated by the addition to each tube of 300µl of 100% ethanol (-20°C), 20µl 3M sodium acetate and 100µl of herring sperm DNA (1mg/ml). All assayed tubes were stored at -70°C for at least 2h (usually overnight). The assayed tubes were centrifuged at 12,500 x g for 20min at 4°C and a 200µl aliquot of the ethanol-soluble supernatant removed and its radioactivity determined by scintillation counting.

2.4.2 The GAPDH Assay

GAPDH activity was measured by a continuous spectrophotometric assay based upon the 3-PGK catalysed, ATP-dependent, phosphorylation of 3-PGA, to produce the substrate for the reaction, 1,3-bisphosphoglycerate (1,3-BPG). 1,3-BPG was then reduced with β-NADH to glyceraldehyde-3-phosphate (G-3-P) by GAPDH. GAPDH activity was quantitated in quartz cuvettes, with the following reagents:

Table 2.1: *Reagents Used In The GAPDH Assay.*

Reagent	Final Concentration In Cuvette
100mM Triethanolamine buffer (pH 7.6)	80mM
100mM 3-PGA	6.7mM
200mM L-Cysteine (prepare fresh)	3.3mM
100mM $MgSO_4$	1.7mM
34mM ATP (prepare fresh)	1.1mM
100U/ml 3-PGK	2.5U/1.5ml
7mM β-NADH (prepare fresh)	0.12mM

The reaction was initiated by the addition of the enzyme, GAPDH, appropriately diluted in 100mM triethanolamine buffer (pH 7.6). The rate of disappearance of β-NADH was monitored spectrophotometrically for 5min at a wavelength of 340nm, using an OmniScribe chart recorder attached to a Pharmacia LKB-Ultrospec III spectrophotometer. One unit of GAPDH was defined as the amount of enzyme required to catalyse the reduction of 1μmole of 3-PGA to G-3-P per minute at pH 7.6 at 25°C.

2.5 CELL CULTURE AND PROCESSING

HCT 116 cells, a colon cancer cell line, were maintained in RPMI 1640 media (Appendix C) containing 10% heat-inactivated foetal bovine serum (FBS) (Appendix C). Cells were incubated at 37°C in an atmosphere containing 5% CO_2, with confluency being obtained at approximately 10^6 cells/cm.

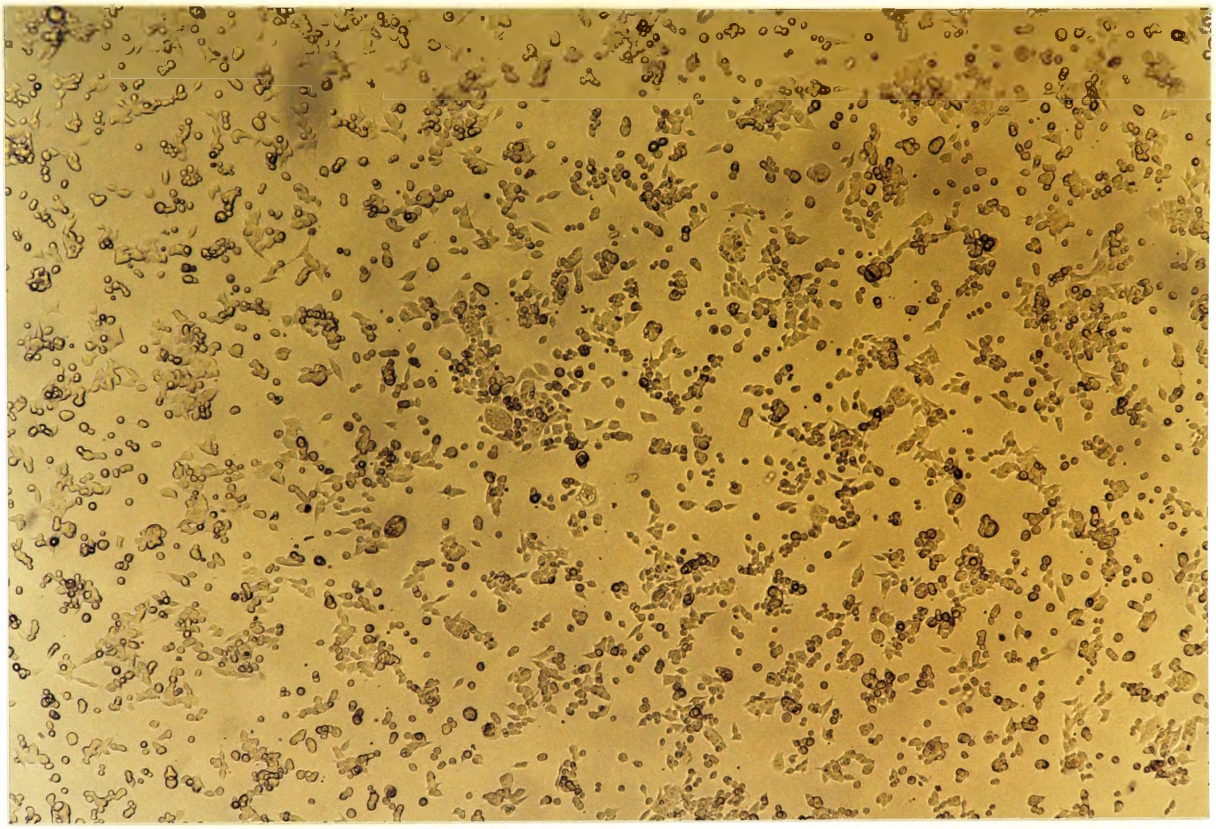


Figure 2.1: *HCT 116 Cells In Culture, 35-40% Confluent (x4).*

Cells required for enzyme extraction were washed in the flask with Ca^{2+}, Mg^{2+} and phenol red-free Hank's Balanced Salt Solution (HBSS) (Appendix C) and then removed with trypsin/EDTA. The cells were then washed twice and resuspended in HBSS.

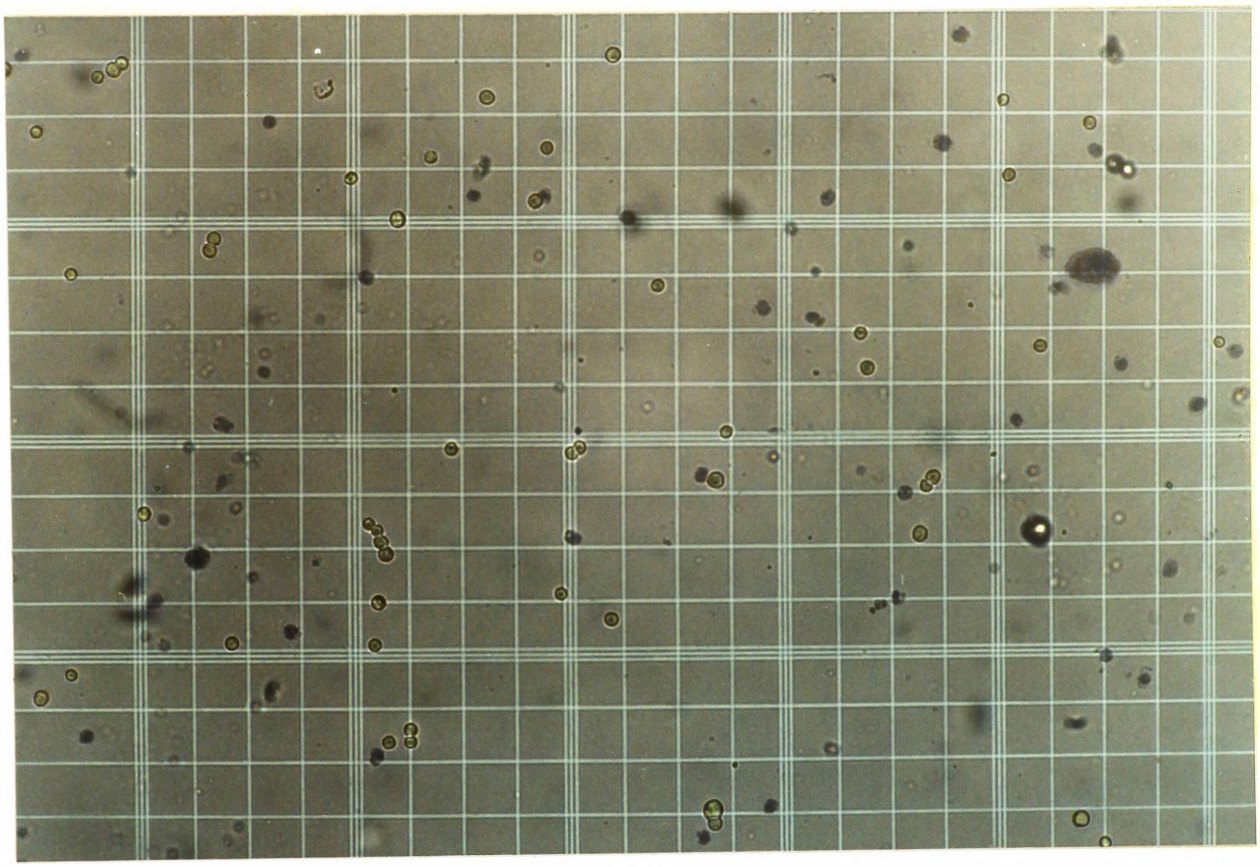


Figure 2.2: *HCT 116 Cells In A Neubauer Haemocytometer (x10).*

Cell number was determined using a Neubauer haemocytometer as described by Dacie and Lewis (1984) [211].

Aliquoted cells, from 10^4 to 2.5 x 10^6 cells, in a total volume of 1ml HBSS, were centrifuged at 560 x g for 5min at RT. The supernatant was removed and the cell pellet resuspended in 100µl of appropriate cell lysis buffer and stored overnight at -20°C. Lysates used for UDG assays were resuspended in 20mM Tris-HCl, pH 8.0/10mM $MgCl_2$/20% glycerol/1mM DTT/0.5% Triton X-100, while extracts for GAPDH assays were resuspended in 100mM triethanolamine buffer/20% glycerol/10mM $MgCl_2$. Lysing of the cell suspensions was initiated by freeze/thawing 3 times in liquid nitrogen and completed by sonication. Cells were sonicated for 20s with a constant duty cycle and an output of 35%. Cell lysates were spun at 2,500 x g for 10min at 4°C and the supernatant collected.

2.5.1 Standard Protein Assay

A standard protein curve was constructed using the modified Lowry protein assay method, using a BioRad DC Protein Assay Kit. Standard protein solutions with concentrations between 0 and 1.2mg/ml protein (Appendix D) were prepared in PVC ELISA microtitre plates with bovine serum albumin (BSA) and assayed according to the following protocol: 5µl samples of the standards (in cell lysis buffer containing Triton X-100) and subsequent protein samples, were added to 25µl of Reagent A + S (alkaline copper tartrate; Appendix D). 100µl of Reagent B (Folin Reagent) was then added following which, all solutions were agitated gently. Another 100µl of Reagent B was then added and again gently agitated. The standards and samples were then all incubated at RT for 15min. The absorbance at 595nm was subsequently read for each sample on the ELISA plate reader.

2.6 STUDIES INVOLVING OXIDANTS

2.6.1 The Hypochlorite Anion (OCl^-)

Stock solutions of hypochlorite anion were kept at 4°C and protected from light until required. The concentration of OCl^- in the stock solution was determined by monitoring the conversion of monochlorodimedone (MCD) to dichlorodimedone with the concomitant loss of absorbance at 290nm (ε_{290} = 17,700$M^{-1}cm^{-1}$) [212]. A stock solution in PBS (Appendix C) of 6mM MCD was further diluted in PBS to give a final MCD working solution of 50μM. To initiate the reaction, freshly prepared, serially diluted OCl^- samples (50μl) (kept on ice), were added to 1.45ml of MCD working solution in quartz UV cuvettes, with the final concentration of OCl^- in the 1.5ml assay volume not exceeding the concentration of MCD (50μM). The absorbance at 290nm was then measured using a Pharmacia LKB-Ultrospec III spectrophotometer after a 5min incubation period at 25°C.

2.6.2 Hydrogen Peroxide (H_2O_2)

An ε_{240} of 46.6$M^{-1}cm^{-1}$ was used to determine the stock concentration of H_2O_2. Working solutions were prepared by diluting stock H_2O_2, 1:100, in Milli-Q water. Freshly prepared H_2O_2 working solutions (50μl) were then added to 1.45ml of Milli-Q water in a quartz UV cuvette and the absorbance measured at 240nm.

2.6.3 Exposure Of Bacterial UDG To Oxidants

E. coli UDG (1U/μl) was initially diluted 1:10 in 50mM Tris-HCl (pH 8.0). The diluted bacterial UDG was then exposed to final OCl^- concentrations of between 4 x 10^{-5}M and 10^{-3}M, or H_2O_2 concentrations of between 10^{-5}M and

10^{-2}M, on ice for 30min. 10µl aliquots of oxidised bacterial UDG were then used immediately in a UDG assay.

2.6.4 Exposure Of HCT 116 Cells To Oxidants

HCT 116 cells were prepared from tissue culture flasks as outlined in section 2.5. Experiments were conducted in quadruplicate, with one tube being used for cell viability counts following incubation. Cells were aliquoted at 2.5 x 10^5 cells/tube and exposed to final OCl^- concentrations of between 10^{-5}M and 10^{-2}M, or H_2O_2 concentrations of between 10^{-6}M and 10^{-2}M. Following incubation at 37°C for 30min, the cells were centrifuged at 560 x g, the supernatant removed and the remaining cell pellet resuspended in the appropriate cell lysis buffer. Cells were lysed as outlined in section 2.5 and the supernatant collected. Cell extracts prepared for GAPDH assays were used immediately, whereas cell extracts used in UDG assays were stored at -20°C until use.

2.6.4.1 Determination Of Cell Viability

Trypan blue was purchased as a sterile, ready to use solution. The cell sample was mixed with trypan blue in a 1:1 ratio in an Eppendorf tube and counted on a haemocytometer. Dead cells take up the dye and appear blue.

3. RESULTS

3.1 PREPARATION OF A UDG SUBSTRATE

3.1.1 Incorporation Of Uracil Into A Poly(dA)·$(dT)_{12}$ Oligonucleotide

Polymerase I was used to label a poly(dA)·$(dT)_{12}$ oligonucleotide with [^{3}H]dUTP. The labelled oligonucleotide was then phenol/chloroform extracted and dialysed. All the dialysis solutions recorded high radioactivity counts indicating that contaminating free-[^{3}H]dUTP had not been adequately removed from the labelled substrate at the completion of dialysis.

3.1.1.1 Removal Of Contaminating Free-[^{3}H]dUTP From The Poly(dA)·$(dT)_{12}$ Oligonucleotide

Contaminating free-[^{3}H]dUTP was removed from the labelled substrate by treating aliquots of the [^{3}H]dUTP-labelled poly(dA)·$(dT)_{12}$ oligonucleotide with the following procedures:

(a) Ethanol precipitation with 5M ammonium acetate

(b) Isopropanol precipitation with 5M ammonium acetate

(c) Passage through a NAP-10 column

(d) Passage through a Bio-Spin Chromatography Column (P6)

The supernatants from the alcohol precipitations recorded counts of up to 712 ± 51 and 180 ± 14 for the ethanol and isopropanol samples respectively. The resuspended pellets recorded very low counts of 12 ± 0 and 20 ± 0 for the ethanol and isopropanol samples respectively.

The radioactive fraction from the NAP-10 column was used to assay the activity in bacterial UDG at concentrations of 10^{-1} to 10^{-5}U UDG. It was expected that UDG activity would increase with enzyme concentration,

however all assay tubes recorded counts no higher than the background. This indicated that the substrate was inadequate to use in a UDG assay.

The eluant (UDG substrate) from the P6 column recorded low counts (4,200cpm for the entire substrate sample) and did not appear to have labelled well as the P6 column remained very radioactive following elution of the oligonucleotide (a small sample of the column packing material resulted in radioactive counts of 934cpm). A UDG assay was conducted using the newly prepared UDG substrate, but recorded similar results to the assay using the NAP-10 prepared substrate. It was therefore concluded that the poly(dA)·$(dT)_{12}$ oligonucleotide had not incorporated the [^{3}H]dUTP label.

3.1.2 Nick Translation And [^{3}H]dUTP-Labelling Of The UDG Substrate

Salmon sperm DNA was nick translated with [^{3}H]dUTP and ethanol precipitated twice. A 5µl aliquot (of a total of 50µl) was then measured on the scintillation counter and recorded a high reading of 32,393cpm, indicating that the salmon sperm DNA had been labelled with [^{3}H]dUTP.

3.1.2.1 Amount Of Stock [^{3}H]dUTP Required For Labelling

Two identical nick translation reactions were conducted except that one reaction used 5µl of stock [^{3}H]dUTP (Reaction 1) while the other used 15µl of stock [^{3}H]dUTP (Reaction 2). An aliquot of each of the two samples of UDG substrate was found to be relatively similar with regard to radioactive content (#1: 120,397cpm; #2: 148,000cpm) implying that using an increased concentration of stock [^{3}H]dUTP did not substantially increase the amount of [^{3}H]dUTP incorporated into the DNA. A 10µl aliquot of stock [^{3}H]dUTP was therefore used in subsequent nick translation reactions.

3.1.2.2 Removing Contaminating Free-[^{3}H]dUTP From The Nick Translated [^{3}H]dUTP-Labelled UDG Substrate

Oligonucleotides, including DNA, are insoluble in alcohols and precipitation is enhanced with various salts [210]. A number of methods were undertaken to establish the most successful method of removing contaminating free [^{3}H]dUTP from the [^{3}H]dUTP-labelled salmon sperm DNA.

(a) Ethanol precipitation with 3M sodium acetate

(b) Bio-spin chromatography column (P6)

(c) Ethanol precipitation with 3M sodium acetate and carrier DNA

(d) Ethanol precipitation with 50mM magnesium chloride

(e) Ethanol precipitation with 2M sodium chloride

(f) Ethanol precipitation with 10% trichloroacetic acid

A 5µl sample of the labelled substrate was cleaned of contaminating free-[^{3}H]dUTP either by ethanol precipitation with 3M sodium acetate or through a P6 column. The P6 column was set up as outlined in section 2.3.1.3, however the column storage buffer was replaced with 50mM Tris-HCl. 5µl of ethanol precipitated substrate was added to 95µl 50mM Tris-HCl and loaded onto the column. The entire eluant was counted, therefore the total counts recorded were for the original 5µl sample.

Table 3.1: *Comparison Of Radioactive Content Of Labelled Substrate.*

Method	cpm/µl
Ethanol precipitation	3,239
P6 column	2,352

The lower final cpm recorded by the P6 column cleaned substrate indicates that a greater amount of free-[^{3}H]dUTP has been removed from the substrate solution. The total radioactive content of the substrate solution however has been lowered following interaction with the P6 column, compared with ethanol precipitation.

A 5µl aliquot of original nick translated substrate was again centrifuged using a P6 column, and then ethanol precipitated, with both the supernatant and the resuspended pellet being measured for radioactivity.

Table 3.2: *Comparison Between Ethanol Precipitation And The P6 Column In Removing Contaminating Free-[^{3}H]dUTP From The Poly(dA)·(dT)$_{12}$ Oligonucleotide.*

Method	cpm/Supernatant Sample
P6 column + 1 x ethanol precipitation	2,616
2 x ethanol precipitation	319

The supernatant from the substrate which was ethanol precipitated twice (2 x ethanol precipitation), recorded comparatively lower counts, and therefore less contaminating free-[^{3}H]dUTP in the substrate containing solution, than the P6 column sample (which was ethanol precipitated once). This indicates that the P6 column was not efficient in removing the contaminating free-[^{3}H]dUTP from the solution containing the substrate.

The following experiments were conducted in order to improve the substrate cleaning technique and thereby remove as much of the contaminating free-[^{3}H]dUTP as possible from the solution containing the substrate.

Table 3.3: *Efficiency Of Ethanol Precipitation With And Without Carrier DNA In The Removal Of Contaminating Free-[^{3}H]dUTP From The Poly(dA)·(dT)$_{12}$ Oligonucleotide.*

Method (Ethanol Precipitation Using 3M Sodium Acetate)	cpm/50µl Resuspended Substrate	cpm/Total Aliquot Of First Supernatant	cpm/Total Aliquot Of Second Supernatant
Without carrier DNA	16,873 ± 7,930	2,547	387
With carrier DNA	75,549 ± 4,533	7,825	3,012

These results suggest that the UDG substrate is cleaned more efficiently with carrier DNA than by ethanol precipitation alone. The higher scintillation counts recorded from the supernatants from the precipitations with carrier DNA indicate that more contaminating free-[^{3}H]dUTP is removed from the substrate solution. The different total activities of the labelled DNA present indicates the inability to recover the same amount of total added DNA without carrier DNA, suggesting that the DNA must be adhering to the tube. In the case of added carrier DNA, less labelled DNA adheres to the tube and therefore the total recovered DNA (supernatant plus pellet) is greater. This justifies the use of added carrier in future preparations.

Table 3.4: *Comparison Of Efficiency Of Various Methods For The Removal Of Contaminating Free-[^{3}H]dUTP From The Poly(dA)·(dT)$_{12}$ Oligonucleotide.*

Method Of Substrate Cleaning	cpm/50µl Resuspended Substrate	cpm/Total Aliquot Of First Supernatant	cpm/Total Aliquot Of Second Supernatant
Ethanol precipitation using 3M sodium acetate and carrier DNA	75,549	7,825	3,012
Ethanol precipitation using 50mM $MgCl_2$	65,844 ± 16,461	4,373	155
Ethanol precipitation using 2M NaCl	62,160 ± 1,243	19,915	1,179
Ethanol precipitation using 10% TCA	16,037	27,582	9,733

Of the methods employed, ethanol precipitation with 2M NaCl produced the most reproducible levels of recovery of radioactivity (SD = 1,243cpm). However, the most efficient method for removing unincorporated [^{3}H]dUTP from the solution containing the [^{3}H]dUTP-labelled UDG substrate was ethanol precipitation with carrier DNA and 3M sodium acetate. This was indicated by the high level of radioactivity in the first supernatant and the large decrease in radioactivity in the second

supernatant (the actual values in the table are not additive because they refer to aliquots). In addition, the substrate with the highest level of radioactivity following cleaning was also important, which was seen with ethanol precipitation with carrier DNA and 3M sodium acetate.

3.1.2.3 Number Of Precipitations Required For The Removal Of Contaminating Free-[^{3}H]dUTP From The Nick Translated [^{3}H]dUTP-Labelled UDG Substrate

Adequate removal of unincorporated [^{3}H]dUTP from UDG substrate samples generally required 3 ethanol precipitations of each sample. The radioactivity recorded in the supernatants removed at each stage however, were found to be quite high. A new experiment was therefore set up where ethanol precipitation was carried out 6 times, to establish whether increased washing resulted in a cleaner substrate.

A UDG substrate was ethanol precipitated 6 times (samples 1 - 6 in Figure 3.1) with 3M sodium acetate and carrier DNA and the radioactivity of each supernatant counted. Only a 200µl sample of each supernatant was counted, however, the values observed on the graph have been corrected and represent the activity of the entire supernatant sample. Sample "a" represents the total radioactivity recorded in the UDG substrate before cleaning. Sample "b" represents the UDG substrate following 3 washes, while sample "c" is the UDG substrate following the 6th wash.

These results indicate that a high percentage of the [^{3}H]dUTP is not incorporated into the UDG substrate, but is removed in the supernatants when the substrate is ethanol precipitated. It appears that 3 ethanol precipitations of the UDG substrate is adequate for the removal of unincorporated [^{3}H]dUTP from the solution. Further washes tend to only decrease the total radioactivity of the UDG substrate, which may result from

fragmentation of the labelled DNA. This may be confirmed by running a SDS-PAGE gel of the labelled fragment.

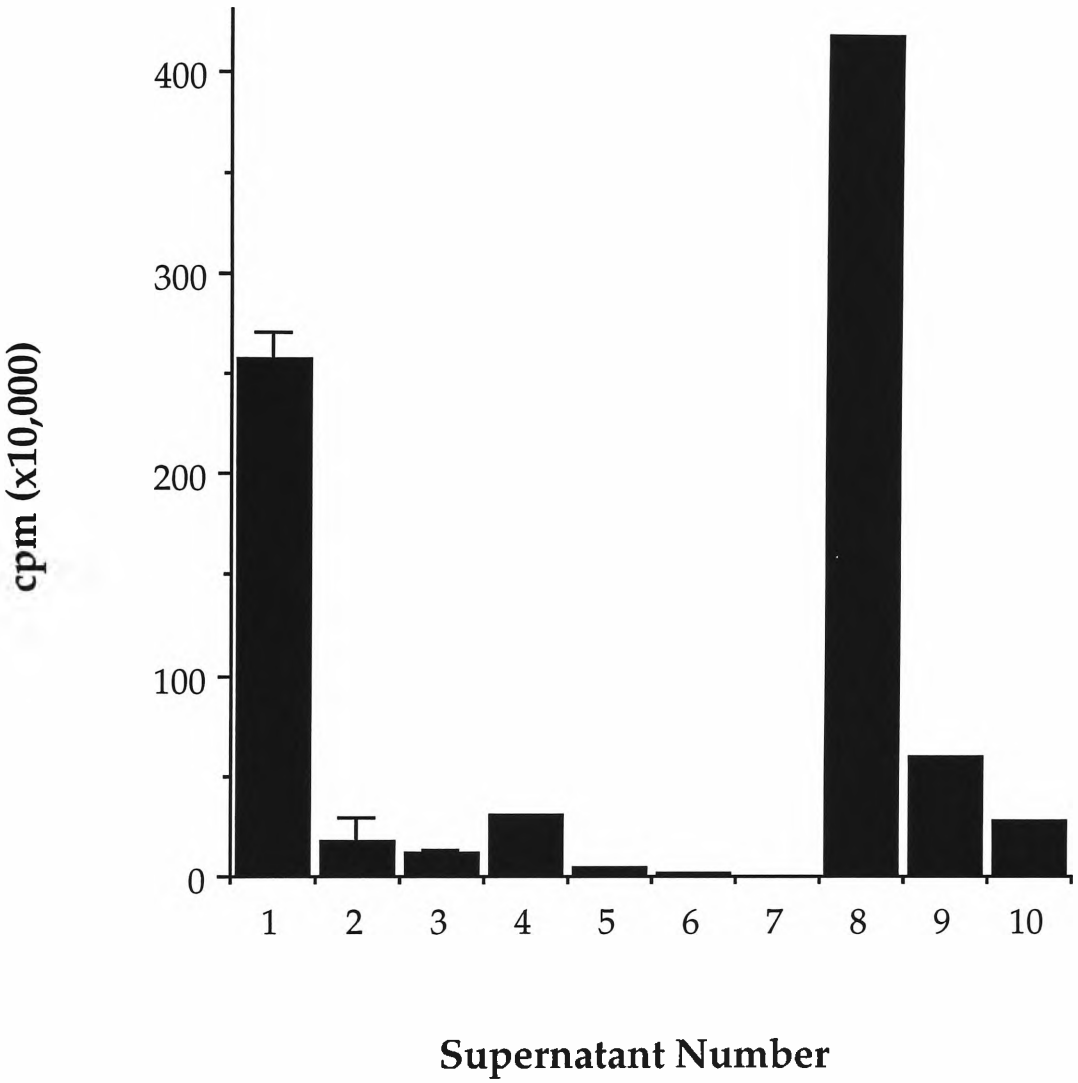


Figure 3.1: *Radioactivity Of Supernatants And UDG Substrate Following Ethanol Precipitation.*

3.2 THE UDG ASSAY

The UDG assay used in this study was based upon excision of dUTP from DNA. A UDG substrate was synthesised by incorporating [^{3}H]dUTP into a DNA template. Under suitable conditions of buffer and temperature, UDG was able to excise the [^{3}H]dUTP from this substrate. Liberated [^{3}H]dUTP was separated from labelled substrate by ethanol precipitation as described above, and the radioactivity measured by scintillation counting.

3.2.1 Standard UDG Curve

The standard UDG assay was modified from Sirover (1979) [2]. In this assay, *E. coli* UDG (diluted 1:10 in 50mM Tris-HCl, pH 8.0) was employed to produce a standard curve. The UDG enzyme was added to the assay mixture just prior to incubation. Enzyme activities were assayed in triplicate and the mean value calculated. UDG activity was expressed as a percentage of the upper limit of the assay (100% activity), which was 1.0U for the standard UDG curve.

The UDG assay contained increasing amounts of UDG and resulted in a proportional increase in activity. The R^2 value for the standard UDG curve was 0.979, indicating that activity was linearly related to enzyme concentration in the assay. When the raw data (all 15 points) was plotted, the R^2 value equalled 0.962, indicating that the mean value of the triplicate samples reflected the true measured values. Percentage standard errors (%SE) were calculated and did not exceed 6%.

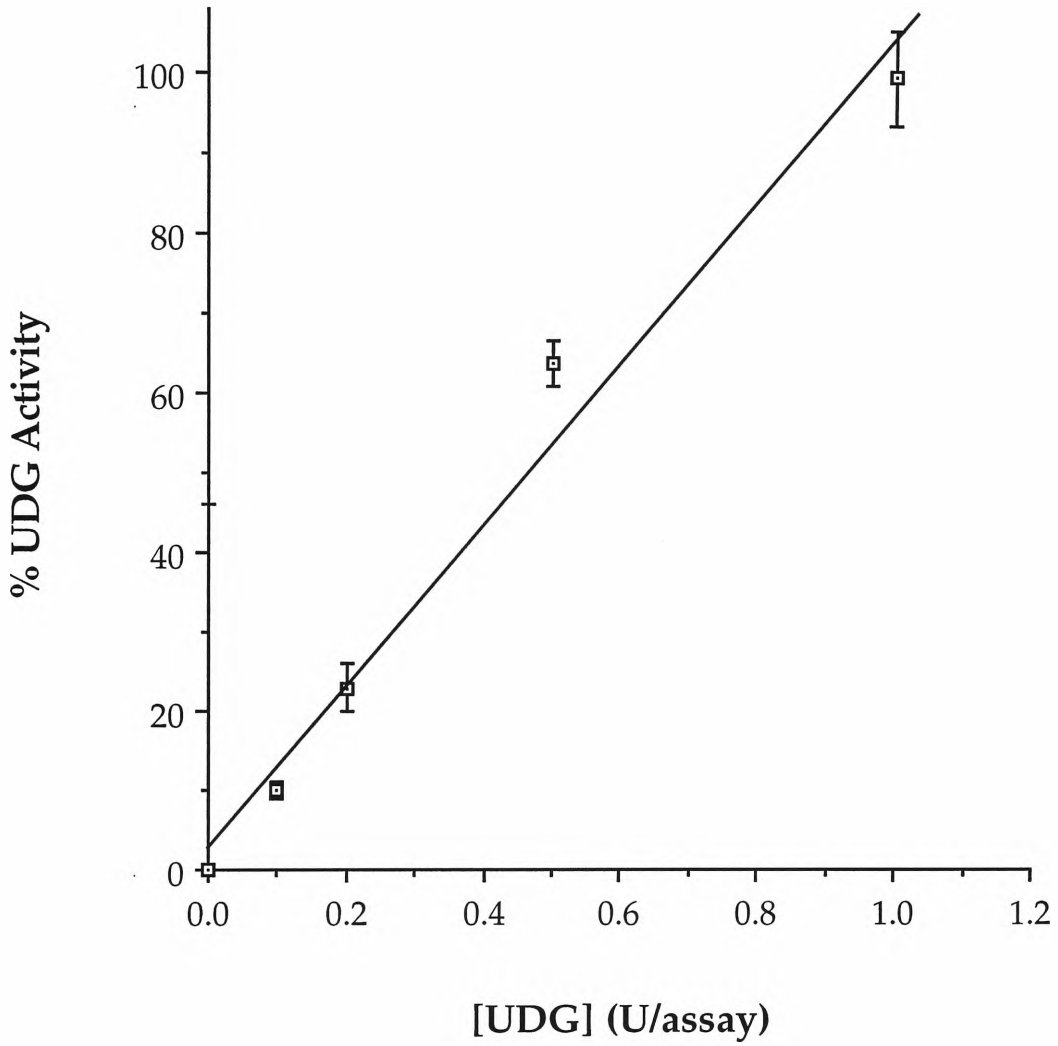


Figure 3.2: *Standard UDG Curve.*

3.2.2 Limitations Of The UDG Assay

The UDG assay contains a fixed concentration of substrate. Therefore, enzyme activity is limited and cannot increase with increases in UDG concentration under conditions when all the [^{3}H]dUTP substrate is metabolised. Fixed assay concentrations of substrate place limits on enzyme concentration and incubation time, therefore conditions were established under which to conduct all subsequent assays.

3.2.2.1 Limitations Of Enzyme Concentration On UDG Activity Within The Assay

A standard assay using enzyme amounts of 0, 0.2, 0.5, 0.75, 1.0 or 2.0U/assay was set up in triplicate and the mean values calculated. Enzyme activity was plotted as a percentage of the activity obtained for the 2U value of enzyme (100% activity).

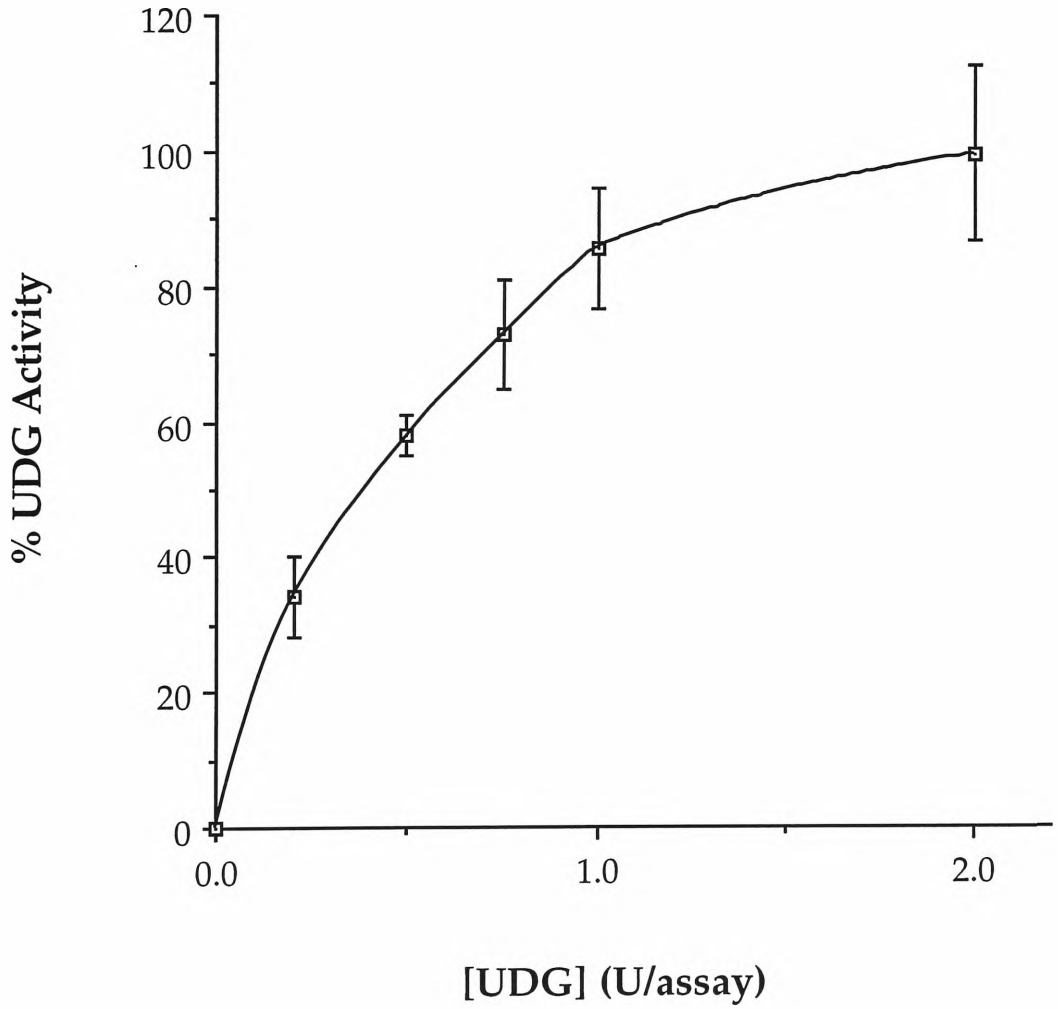


Figure 3.3: *Enzyme Concentration Limit For The UDG Assay.*

The R^2 value for the graph between 0 and 1.0U was 0.945, which was considered to be linear, that is, enzyme concentration was directly proportional to activity. The R^2 value between 0 and 2U however, was 0.789, that is, linearity had declined. UDG activity reached a plateau after an enzyme amount of 1U/100µl assay was used, indicating the upper limit of the standard UDG assay was 1.0U. %SE were below 10% up to and including 1.0U, however, the 2U assays had a %SE of 13%, indicating that assays were becoming less reproducible at the higher enzyme concentration.

3.2.2.2 Time Limits For The UDG Assay

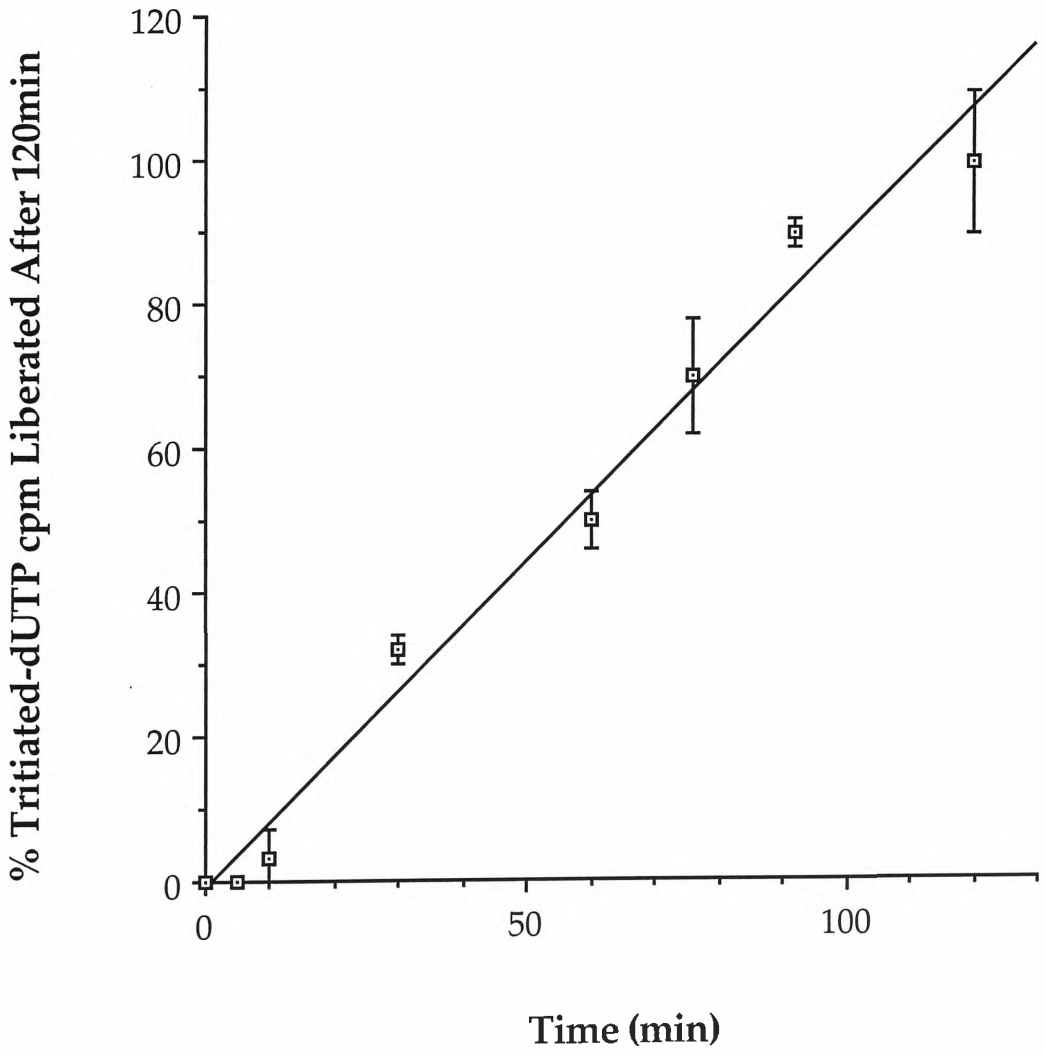


Figure 3.4: *Time Course For UDG Assay.*

Assay substrate may also become depleted prior to the completion of the assay, therefore, a standard time course assay must be conducted. 0.5U of UDG/100µl assay was assayed for varying amounts of time between 0 and 120min. Means (± %SE) were plotted as a percentage of the activity recorded at 120min (100% activity).

[^{3}H]dUTP release was linear with time for a minimum of 120min (R^2 = 0.982). This indicated that there was enough substrate within the 100µl of assay solution for the UDG enzyme to maintain total activity. Standard UDG assays were incubated for 60min, which was within the linear section of the time graph, validating the length of time that UDG assays were conducted. %SE were at or below 10% with a %SE for 60min of 4%.

3.2.2.3 Effect Of 0°C Incubation On The UDG Reaction

The efficiency of the mechanism used to stop the UDG assay following incubation was investigated. The assay was conducted in triplicate, using 0U and 2 x 0.5U of UDG. One of the assays containing 0.5U was not incubated, but stopped immediately following the addition of enzyme. The other 2 samples (0U and 5U) were incubated at 37°C. All assays were stopped in the same manner, that is, placed on ice and had 100% ethanol and 3M sodium acetate added to them. UDG activity was measured as previously outlined (section 2.4.1). The incubated sample containing 0.5U was considered as having 100% activity and measurements were plotted with %SE.

The tubes containing 0.5U of UDG, which were not assayed at 37°C did not display any activity, indicating that the method for inhibiting UDG activity at the completion of the assay was successful and thereby did not create false positive results.

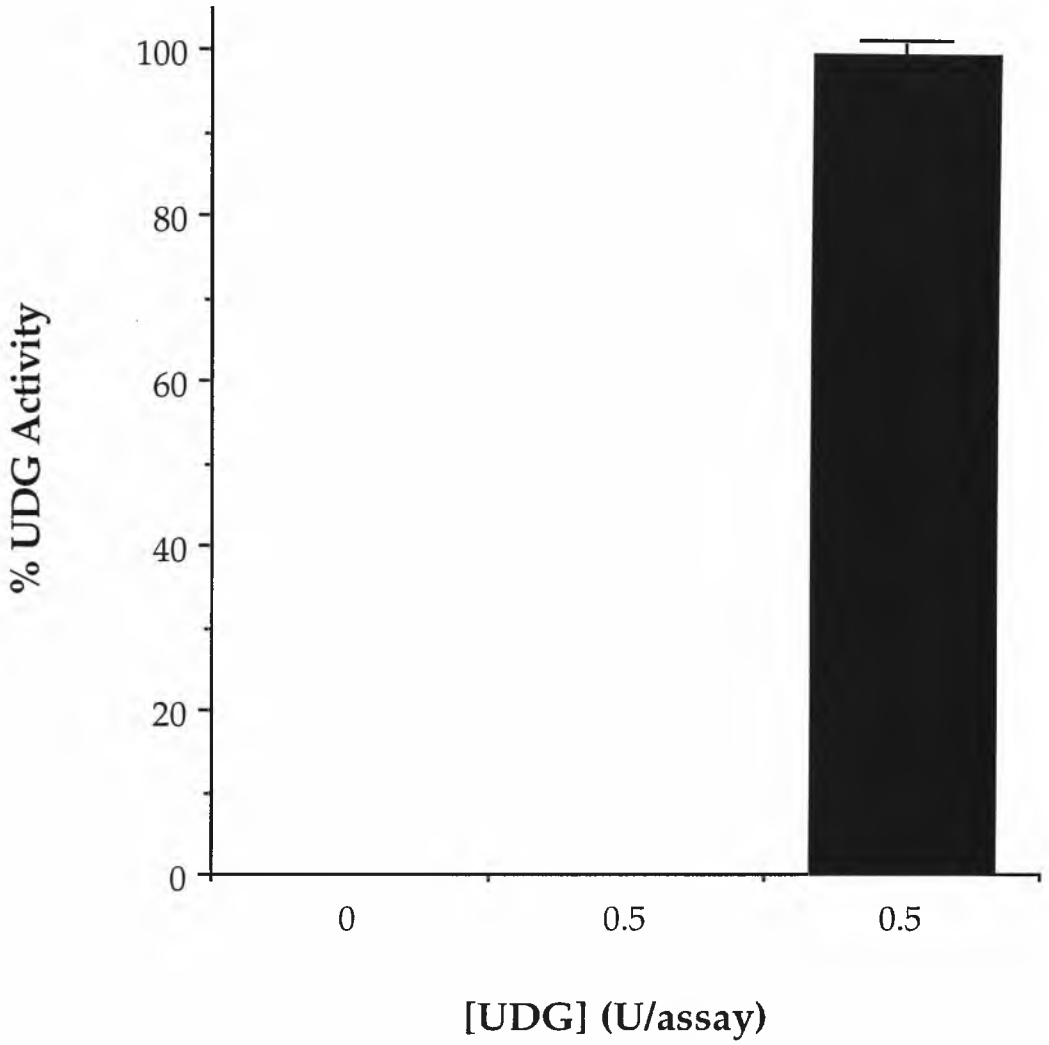


Figure 3.5: *Effect Of 0°C Incubation On The UDG Reaction.*

3.2.2.4 Thermolability Of Bacterial UDG

UDG assays were performed at the physiological temperature of 37°C. However, UDG assays were also conducted, to investigate the effects of extreme heat on the enzyme's thermolability. In this experiment, 0.25U of UDG which had been heated to 100°C on a heating block for 10min, was assayed. Data is presented as percentages of the 1.0U value (100% activity), with %SE less than 6%.

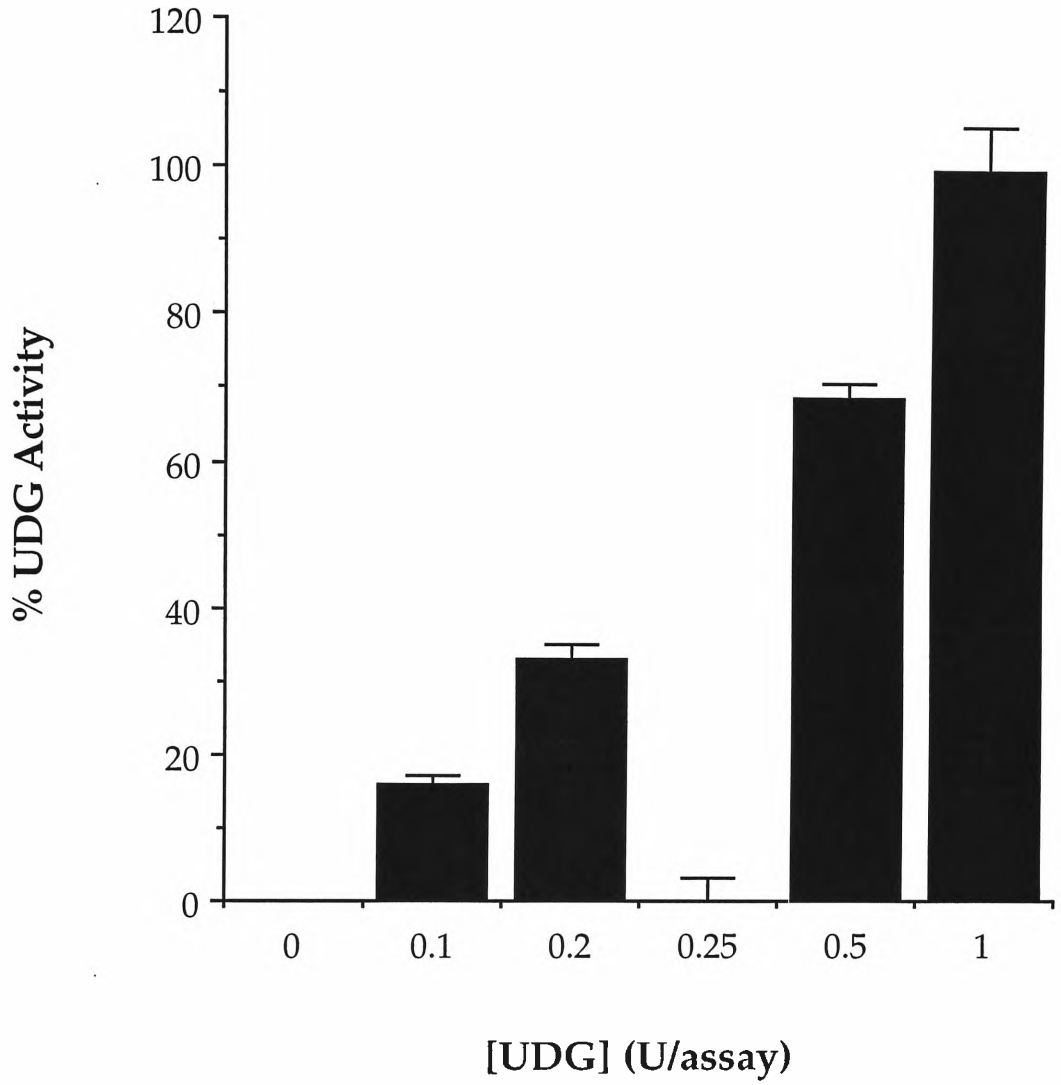


Figure 3.6: *Thermolability Of Bacterial UDG.*

The standard curve recorded for this experiment had an R^2 value of 0.958, indicating linearity and therefore proportionality between enzyme concentration and recorded activity. When 0.25U of boiled UDG sample was analysed, no activity was found. Loss of activity was due to heat denaturing of the UDG enzyme.

3.2.2.5 Stability Of The UDG Substrate

In order to establish whether storage time of the UDG substrate had any effect on the authenticity of the UDG assay, *E. coli* UDG was assayed using two UDG substrates that were both prepared by the nick translation method and varied only in age. The first UDG substrate had been freshly prepared and stored at -20°C for only 14 days, while the second UDG substrate had been stored at -20°C for approximately 5 months. 0, 0.5 and 1.0U of *E. coli* UDG were assayed under standard conditions so that the two different UDG substrates could be compared. Means (± %SE) were plotted as a percentage of the activity recorded for 1U (100% activity) of UDG enzyme.

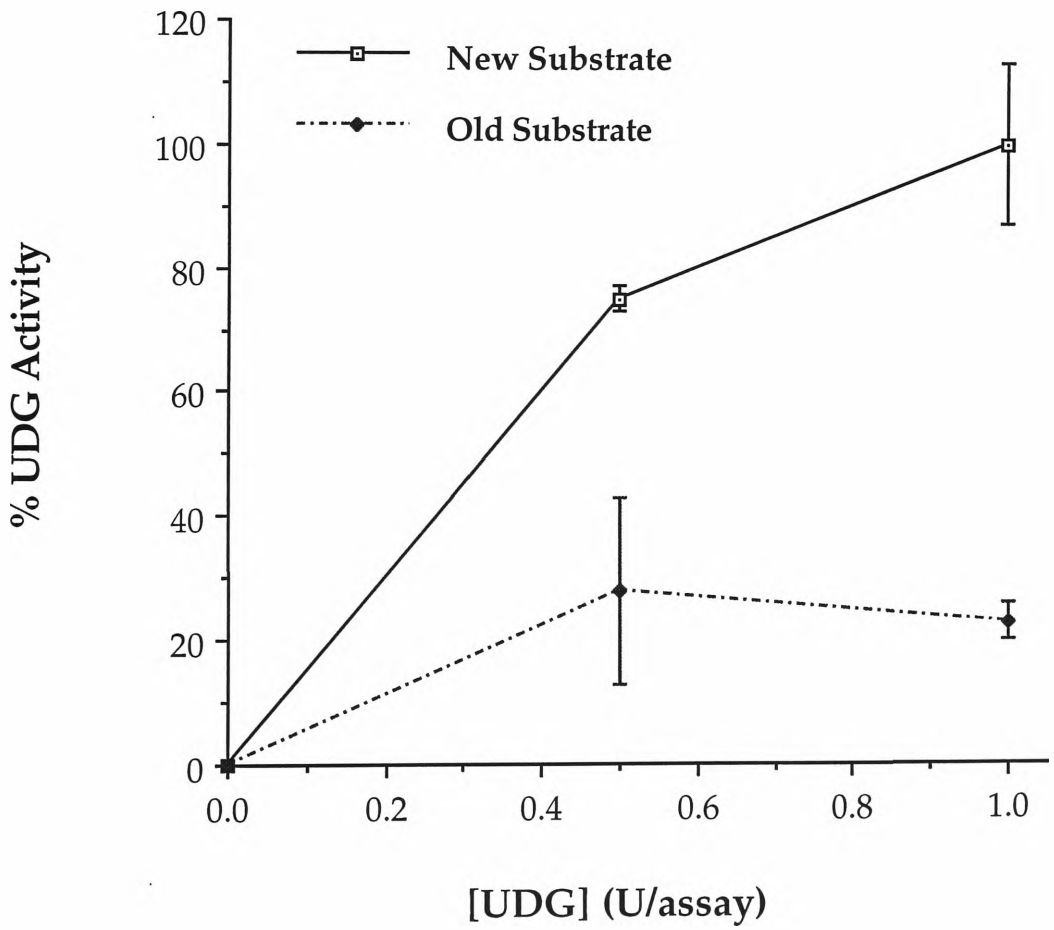


Figure 3.7: *Comparison Of The Efficiency Of The UDG Substrates With Varying Storage Time.*

The two different substrates were analysed by measuring UDG activity and comparing the linearity of the standard curves produced by the two assays. The new substrate produced a curve with an R^2 value of 0.923, while the old substrate produced a curve with an R^2 value of 0.593. The assay containing the older substrate had lost the proportionality between enzyme concentration and recorded activity.

3.2.3 UDG Activity Of Commercial GAPDH Preparations

The suggestion by Meyer-Siegler and co-workers (1991) [1] that GAPDH exhibited UDG activity was investigated in the following experiments.

3.2.3.1 UDG Activity Of Native GAPDH

1.9mg of solid GAPDH (E.C. 1.2.1.12, 60U/mg solid) was diluted into 500µl of 50mM Tris-HCl, pH 8.0/10% glycerol and used in a UDG assay. Data is presented as cpm with SE, as percentage activity would be misleading.

Standard UDG assays recorded radioactivity levels between approximately 300 and 3100cpm for UDG amounts of 0.1 and 1.0U/100µl assay (these values are corrected for background). The highest value obtained by GAPDH in the UDG assay was for the 33.4µg sample which was only 134cpm, which is much less than the recorded value for 0.1U of UDG. The SE of the GAPDH samples were also very large, indicating that the means were not truly indicative of the true values obtained. These results suggest that native GAPDH had very little, if any, UDG activity.

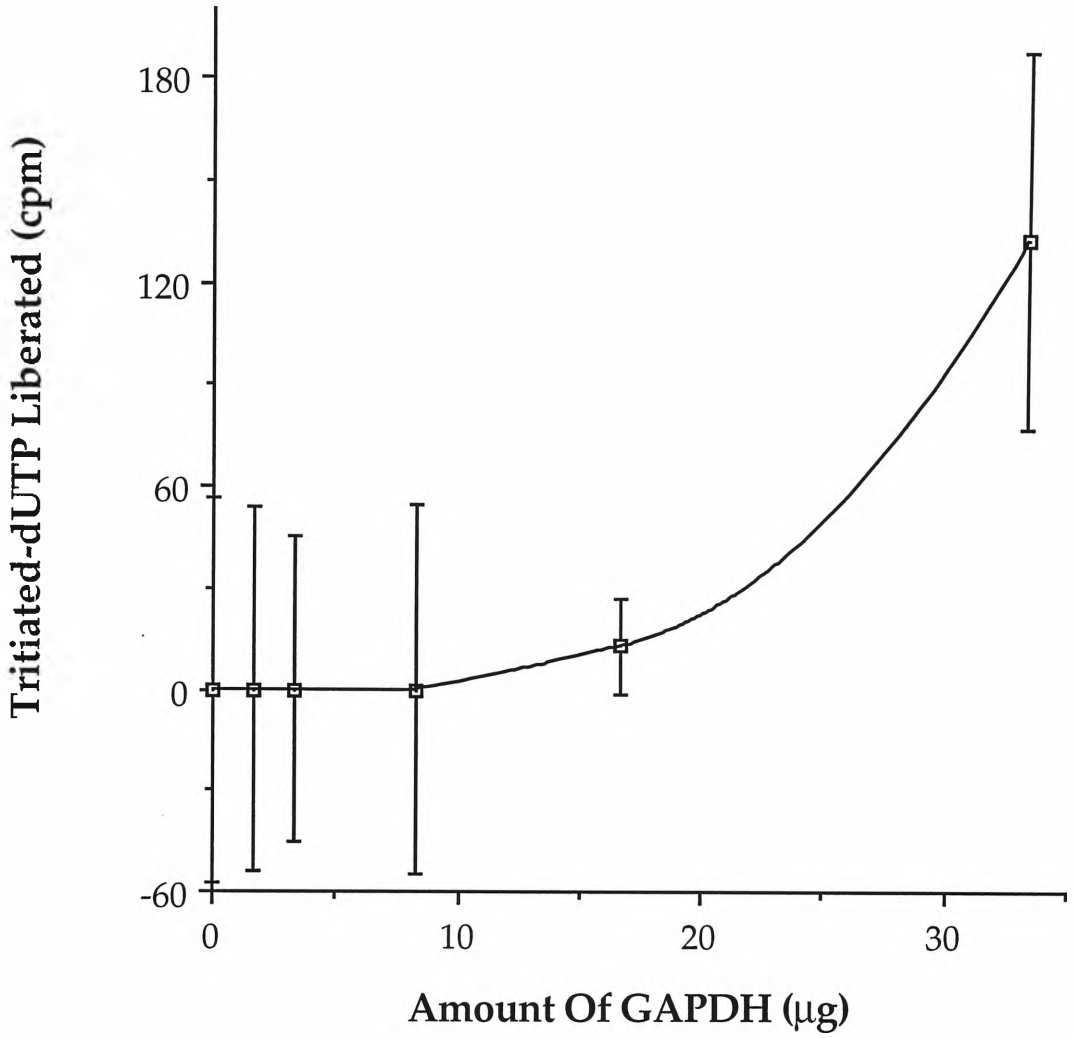


Figure 3.8: *UDG Activity Of GAPDH.*

3.2.3.2 UDG Activity Of SDS Treated GAPDH

The UDG activity of SDS denatured GAPDH was also investigated, when 1.9mg of commercial GAPDH was reconstituted into 100µl of 10% SDS. The GAPDH samples contained 19µg, 95µg or 190µg/100µl assay. Standard UDG samples containing 0, 0.5 and 1.0U of UDG were also assayed for comparison with SDS treated GAPDH samples. The GAPDH samples reconstituted in 10% SDS did not display any UDG activity. This suggested that the monomer of GAPDH did not contain UDG activity after SDS treatment.

3.3 EXTRACTION OF CELLULAR ENZYME

UDG and GAPDH were extracted from the human cell line, HCT 116 (colon cancer cell line). The enzyme activity from the cells then had to be standardised within the assay before oxidative studies could commence.

3.3.1 Cell Lysis And Enzyme Extraction

In order to successfully extract active enzymes from HCT 116 cells, plasma and intracellular membranes must be completely lysed and the enzyme stored in a buffer containing glycerol, to inhibit freezing at -20°C. Cell lysis may be monitored by observing the cells under the microscope following treatment.

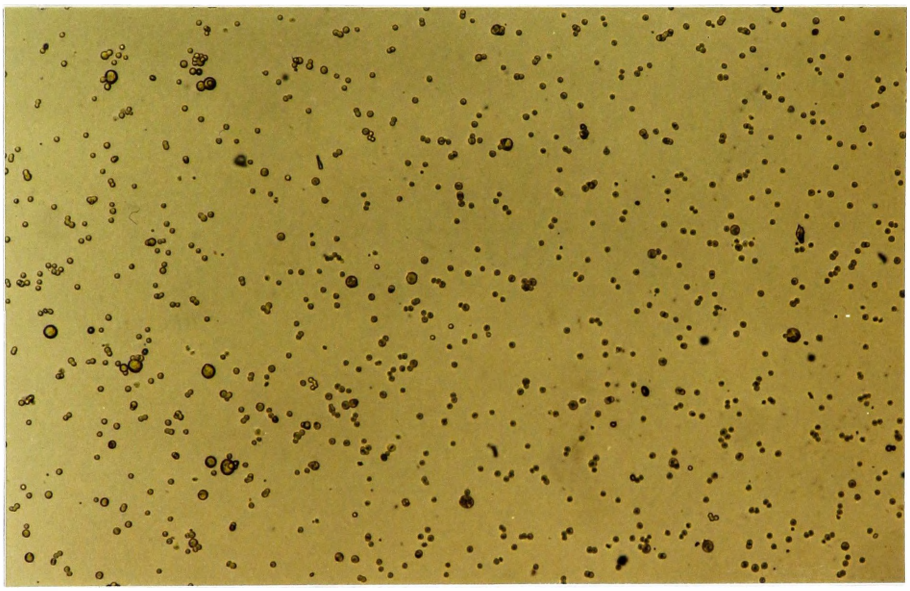


Figure 3.9: *Whole HCT 116 Cells Before Lysis Treatment (x4).*

Following treatment such as freeze/thawing, sonication and exposure to detergents such as Triton X-100, cells must be compared with intact cells (Figure 3.9) in order to establish whether they have actually been lysed or not. Before lysing treatment, HCT 116 cells appear round and there is no evidence of debris.

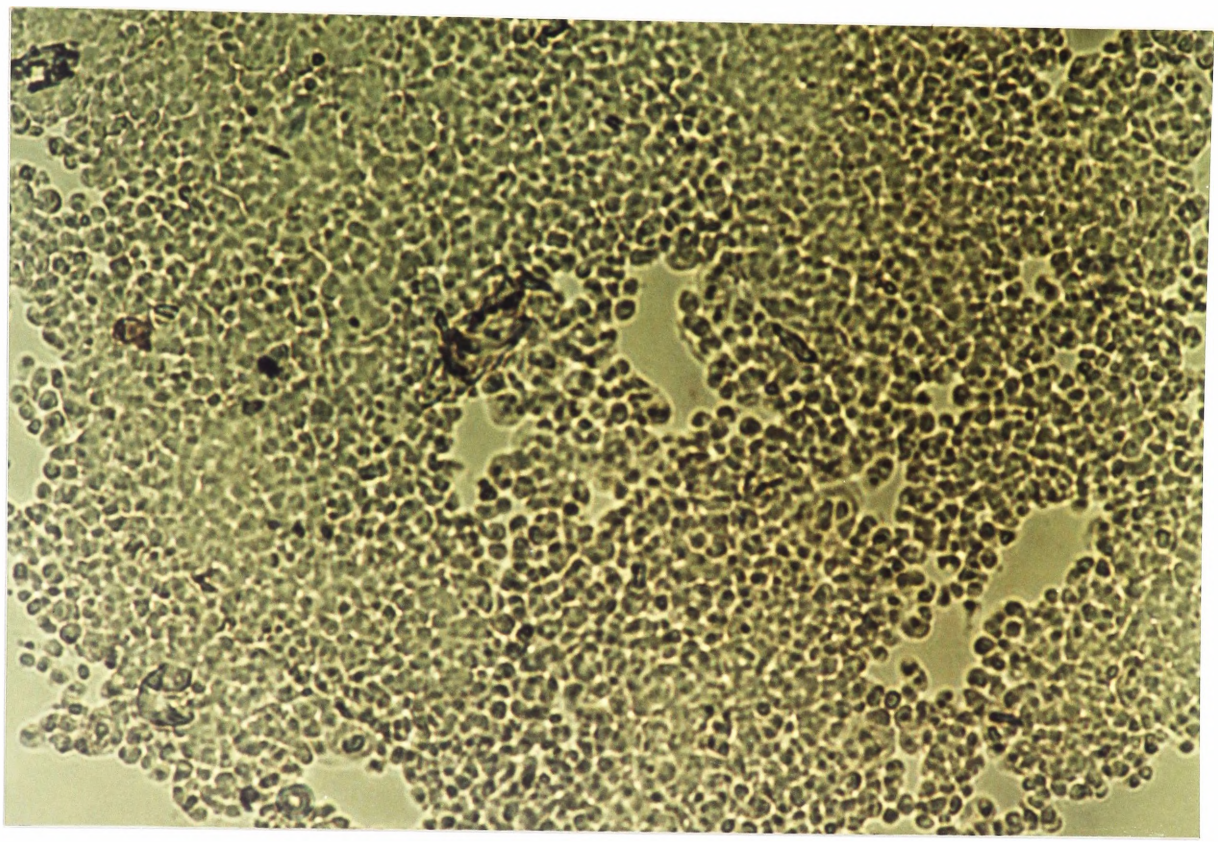


Figure 3.10: *HCT 116 Cells Following Freeze/Thawing Treatment (x20).*

Initially, cells resuspended in lysis buffer were freeze/thawed, in order for lysis to occur. The cells clumped however, and remained intact, as observed in Figure 3.10. Cells were then sonicated in a 240V sonicating bath (filled with ice water). These cells were then spun down, the supernatant collected and the pellet resuspended in lysis buffer and both assayed for UDG activity. Both the supernatant and the resuspended pellet displayed UDG activity. The fact that the resuspended pellet contained UDG activity indicated that the cells were incompletely lysed. When a Branson sonication probe was used instead, UDG activity increased, but was still not proportional to cell number.

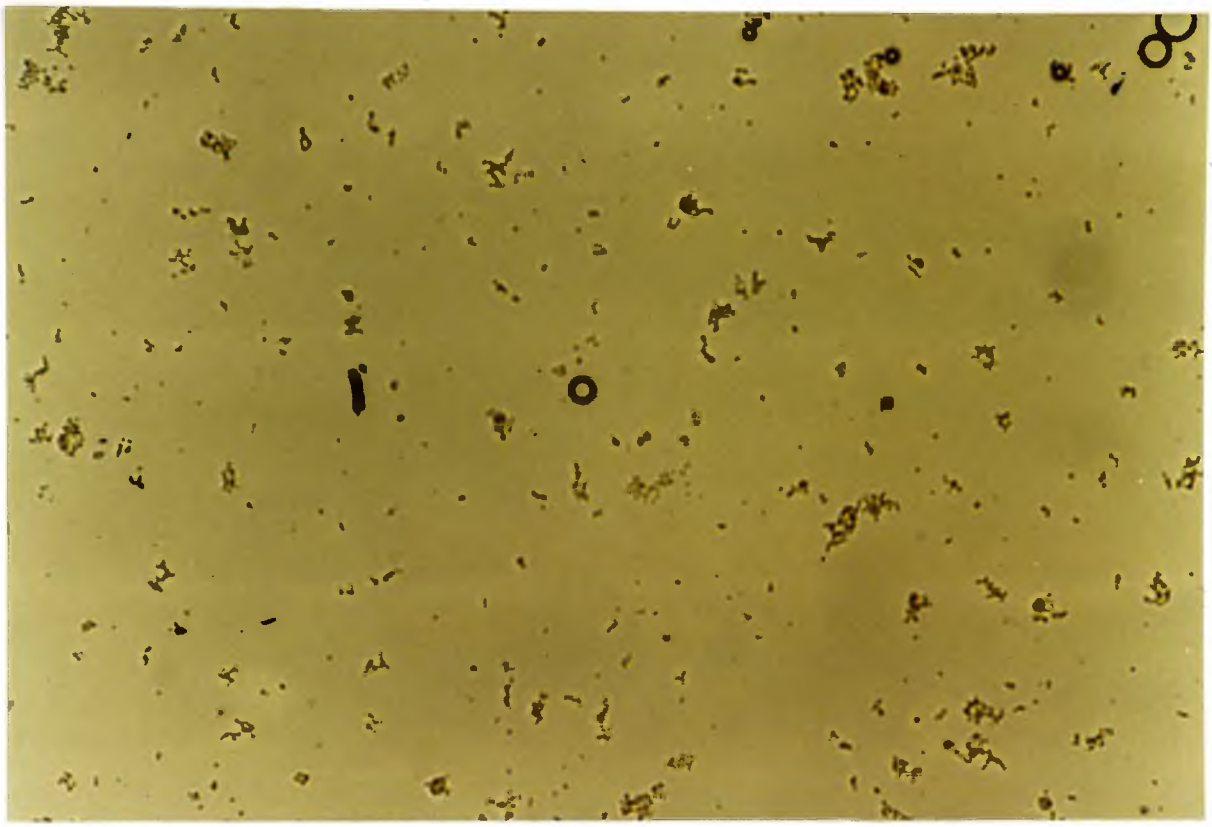


Figure 3.11: *HCT 116 Cells Following Resuspension In Cell Lysis Buffer And Triton X-100 (x4).*

Fresh cells were removed from culture and resuspended in lysis buffer containing 0.5% Triton X-100. Cells were now less clumped, which was more appropriate for sonicating, as more cell membrane area was exposed to the sonication waves.

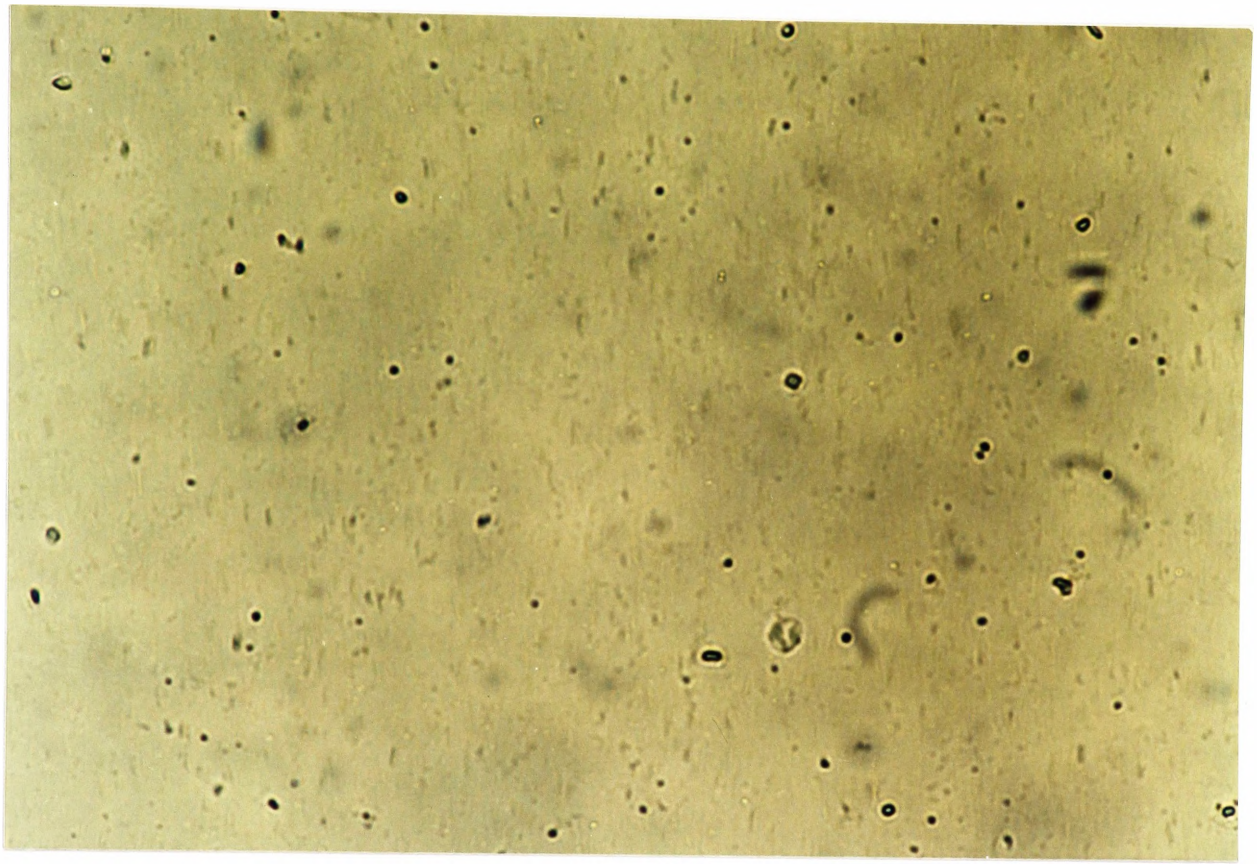


Figure 3.12: *HCT 116 Cells Following Sonication (x20).*

Cells were resuspended in lysis buffer containing 0.5% Triton X-100, freeze/thawed and sonicated with a probe for 20s. Cells now appeared broken and substantial amounts of debris was present on the microscope slide as illustrated in Figure 3.12. The cell debris was then removed by centrifugation and the supernatants assayed for UDG activity. These results illustrate that sonication was imperative for cell lysis and maximising UDG recovery from HCT 116 cells.

3.3.2 Standard Assays With Cellular UDG

The supernatant of cell lysates was assayed according to cell number and standardised against a standard curve using commercial *E. coli* UDG.

3.3.2.1 UDG Activity Versus Cell Number

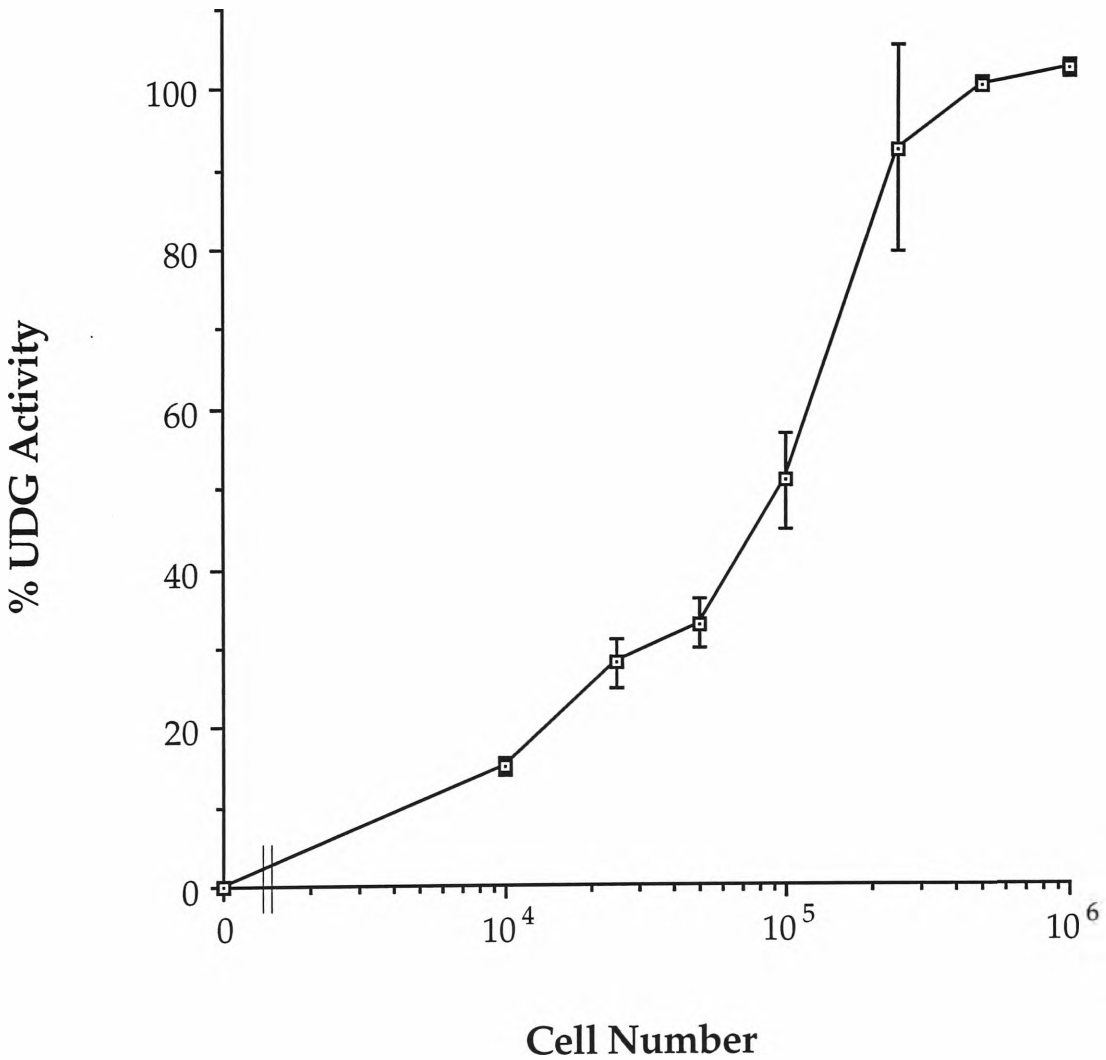


Figure 3.13: *Relationship Between Cell Number And UDG Activity.*

Cell lysates from 10^4 to 2.5 x 10^6 cells were assayed and plotted against their respective percentage activity.

The highest percentage activity (100% activity) of cell lysates was taken from the largest number of cells (2.5 x 10^6 cells). The curve was proportional between percentage activity and cell number until approximately 2.5 x 10^5 cells, where the graph reached a plateau, indicating the upper limit of the assay. 2.5 x 10^5 cells thus contained enough UDG to be easily quantitated and was therefore the number of cells that was used in the oxidative studies.

3.3.2.2 Soluble Protein Assay

A protein assay was conducted using the modified Lowry protein assay method. This assay was used to estimate the amount of total cellular protein. The protein standard curve had an R^2 value of 0.999. The total protein content of the 2.5 x 10^5 cells was then calculated to be 0.303mg ± 0.01SD protein/ml.

3.3.2.3 UDG Content Of Cell Extracts

A 1µl aliquot (⊗) of the lysate from 2.5 x 10^5 cells was assayed and compared with commercial *E. coli* UDG for total UDG activity. The 1µl sample of cellular UDG contained the equivalent of 98% (± 3%) of the activity of 1U of UDG in the standard UDG curve. The total 100µl of cell lysate containing 2.5 x 10^5 cells was then calculated to contain 91U of UDG activity. The 100µl samples of 2.5 x 10^5 cells therefore contained 91U of UDG/0.03mg protein, or 3033U/mg protein.

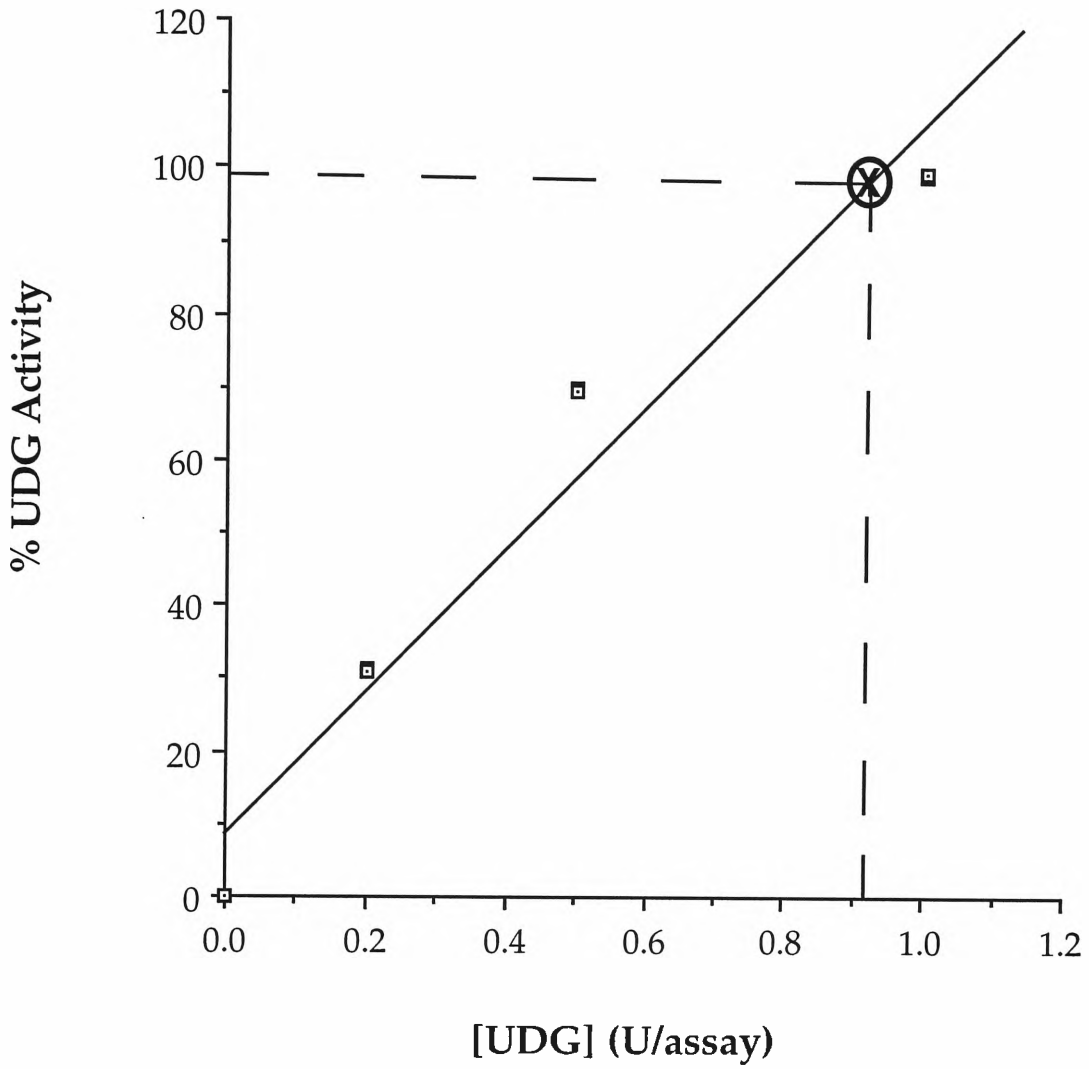


Figure 3.14: *Total UDG Activity Of Cell Extracts.*

3.4 OXIDATION OF ENZYMES

Once a standard UDG curve had been established, alterations to total UDG enzyme could be quantitated. Bacterial UDG and HCT 116 cells were both exposed to OCl^- or H_2O_2 and the UDG and GAPDH assayed for activity. A student *t*-test was carried out between the control which had not been exposed to oxidants in each case with a $p < 0.05$ being considered significant (Appendix F).

3.4.1 Oxidation Of Bacterial UDG

3.4.1.1 Exposure Of Bacterial UDG To Hypochlorite (OCl^-)

E. coli UDG was exposed to OCl^- at concentrations of 4×10^{-5}M to 10^{-3}M. The control contained bacterial UDG that was not exposed. Data is presented as percentage activity of the unoxidised control.

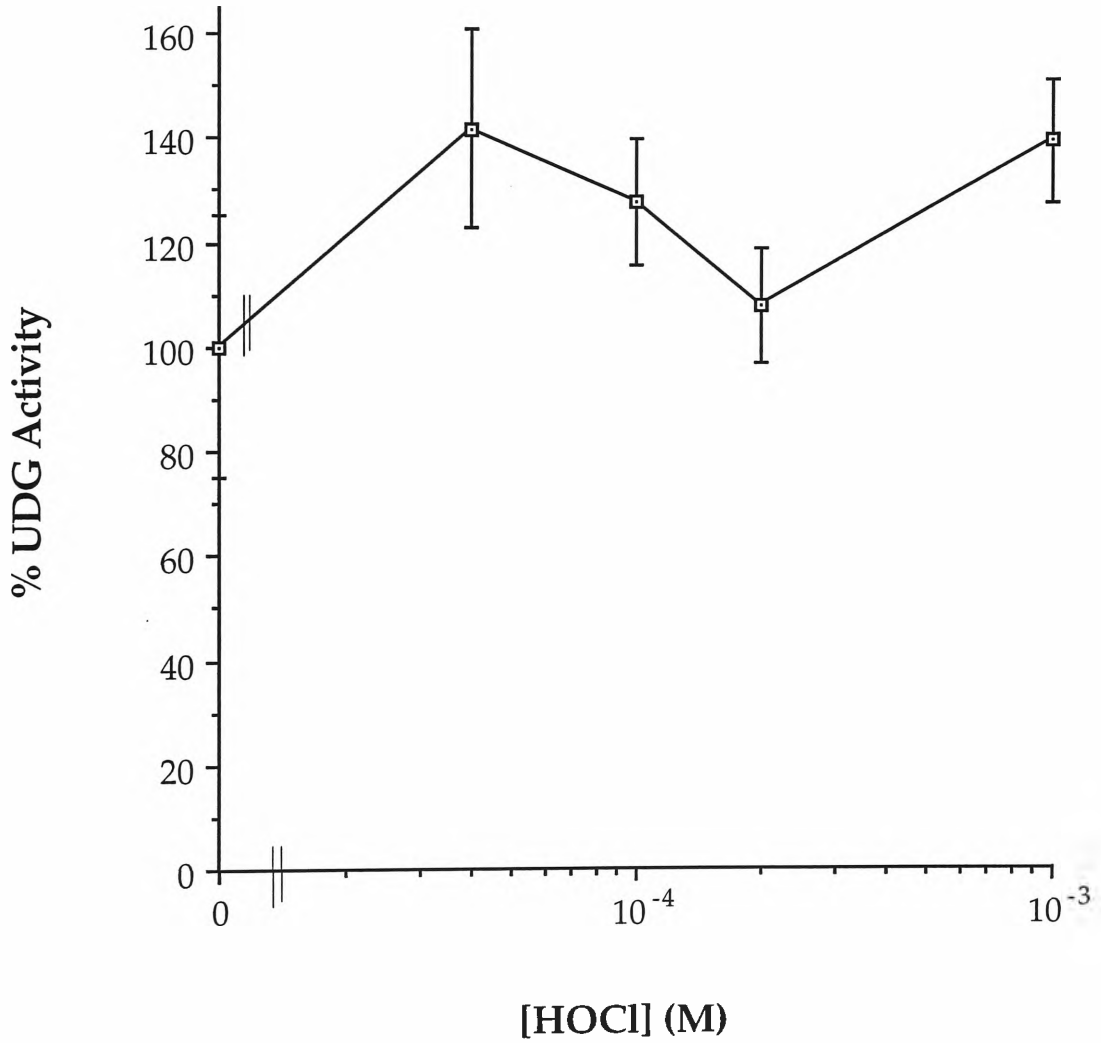


Figure 3.15: *Effect Of OCl^- On Bacterial UDG.*

All of the oxidised samples recorded slightly more activity than the unoxidised sample. All student *t*-tests resulted in $p > 0.20$ and therefore

were not considered to be significantly different from each other. The combined results indicated that bacterial UDG was not inhibited by OCl^- up to a concentration of 10^{-3}M.

3.4.1.2 Exposure Of Bacterial UDG To Hydrogen Peroxide (H_2O_2)

E. coli UDG was exposed to H_2O_2 at concentrations of 10^{-5} to 10^{-2}M. Data is presented as percentage activity of the unoxidised control.

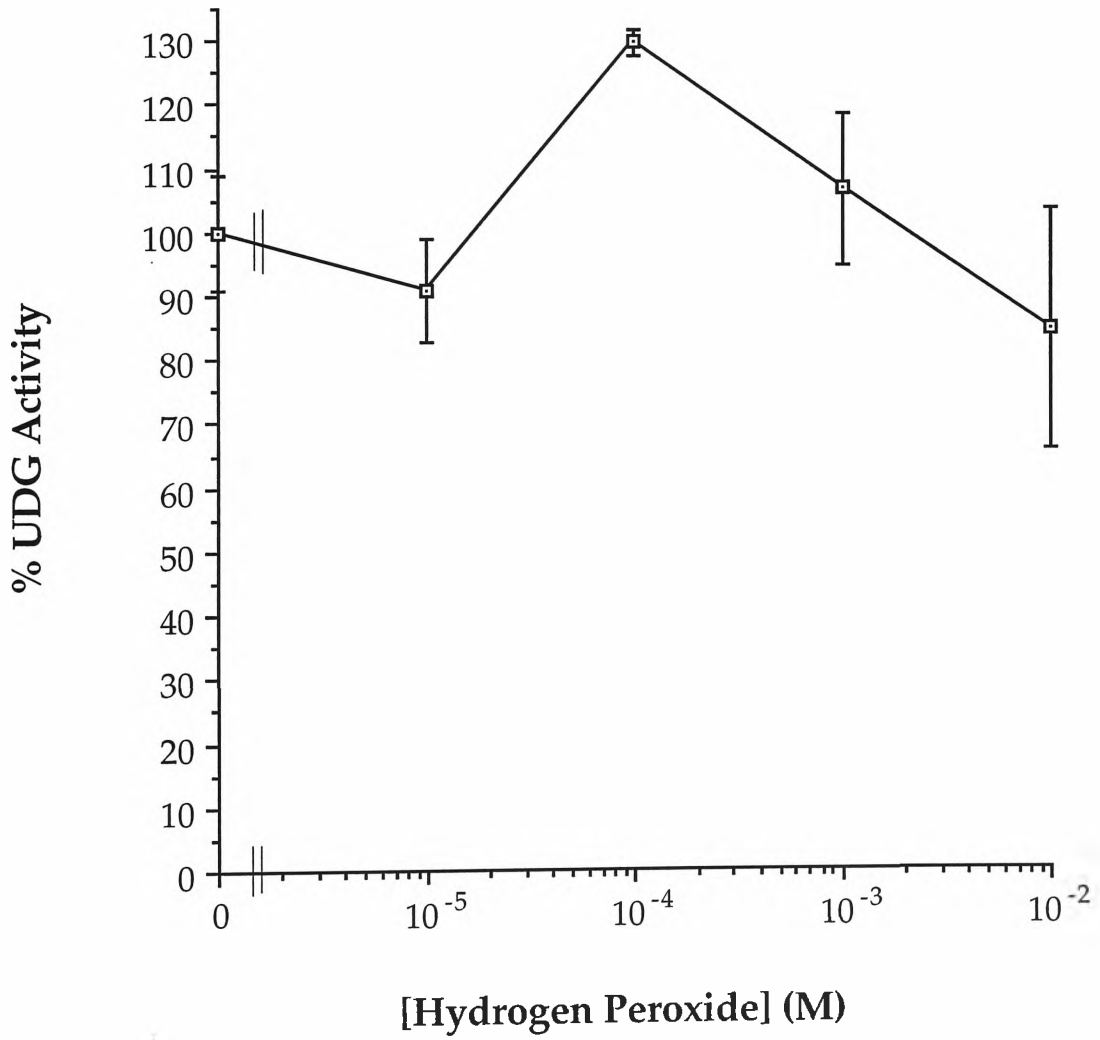


Figure 3.16: *Effect Of H_2O_2 On Bacterial UDG.*

Although the UDG activity obtained after treatment with 10^{-5}M and 10^{-2}M H_2O_2 were slightly lower than control, the other two samples were slightly higher than the control.

Only the sample for 10^{-4}M H_2O_2 obtained a $p < 0.05$, indicating that it was significantly different than the unoxidised sample. This value however, was higher than the control and therefore, was not inhibited by H_2O_2. All other student *t*-tests resulted in $p > 0.20$ and therefore were not considered to be significantly different from each other. Overall the results indicated that bacterial UDG was not inhibited by H_2O_2 up to concentrations of 10^{-2}M.

3.4.2 Oxidation Of Cellular Enzymes

HCT 116 cells were exposed to inflammatory oxidants such as OCl^- or H_2O_2. Cells were aliquoted into tubes at a concentration of 2.5×10^5 cells/tube. Each concentration of oxidant was placed into four tubes containing cells (that is, each oxidant was set up in quadruplet). The extracts from each of three of the four tubes were then assayed three times. The fourth tube was used for viability tests. This entire process was carried out a minimum of three times to authenticate results.

3.4.2.1 UDG Activity In Cells Exposed To The Inflammatory Oxidants, OCl^- And H_2O_2

HCT 116 cells were exposed to OCl^- at concentrations of 10^{-5}M to 10^{-2}M. Cellular extracts were assayed for UDG activity. Controls consisted of UDG-containing extracts that had not been oxidised. Data is presented as percentages of UDG activity from the cells not exposed to oxidants.

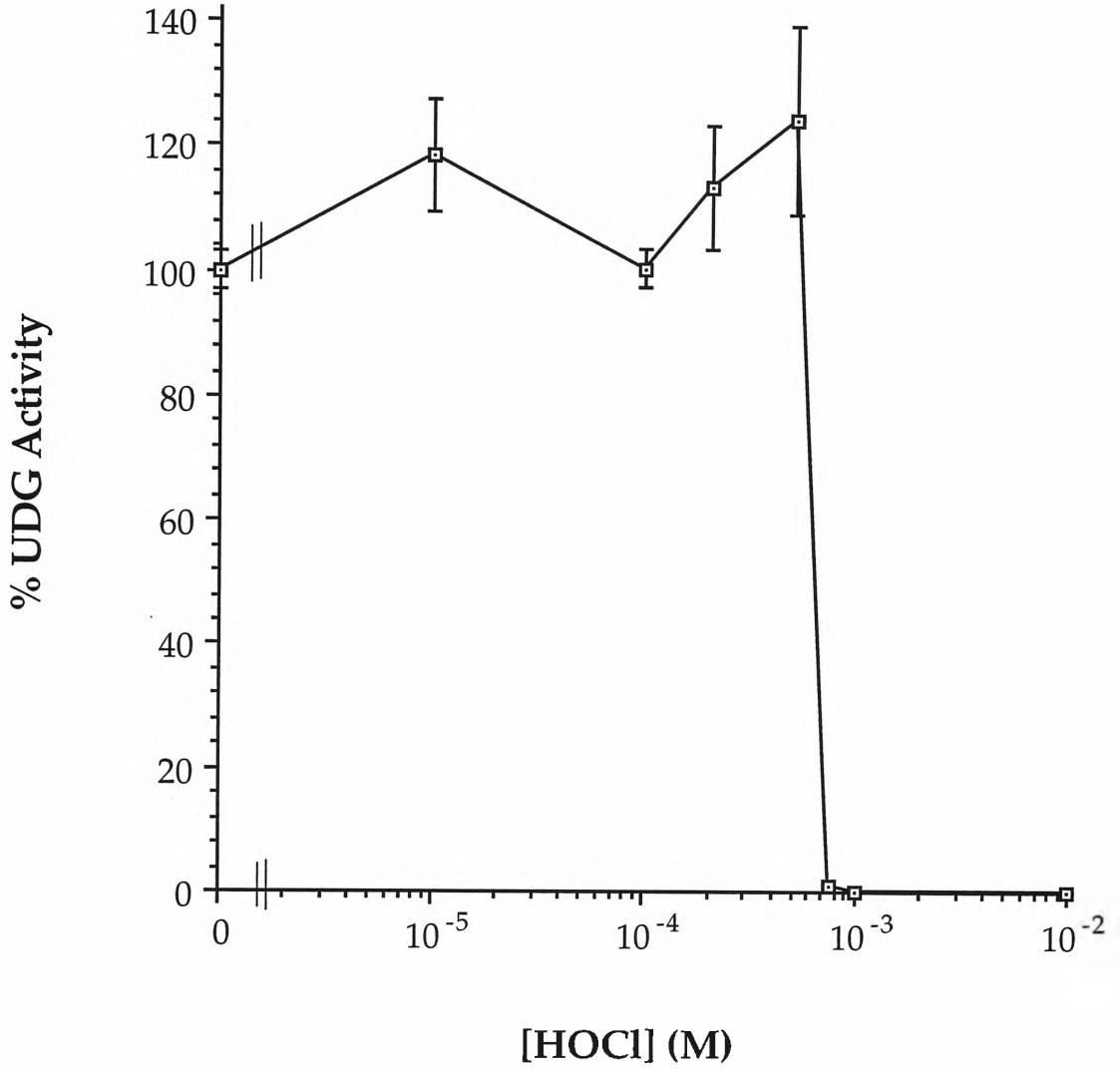


Figure 3.17: *UDG Activity Following Treatment Of HCT 116 Cells With OCl^-.*

Exposure of HCT 116 cells to OCl^- at 0M to 5 x 10^{-4}M resulted in no change in UDG activity released from these cells ($p > 0.20$). However exposure to OCl^- at concentrations higher than 5 x 10^{-4}M resulted in complete loss of cellular UDG activity ($p < 0.001$; Appendix F). There appears to be a virtually "all or none" response, with respect to UDG activity when cells are exposed to OCl^-.

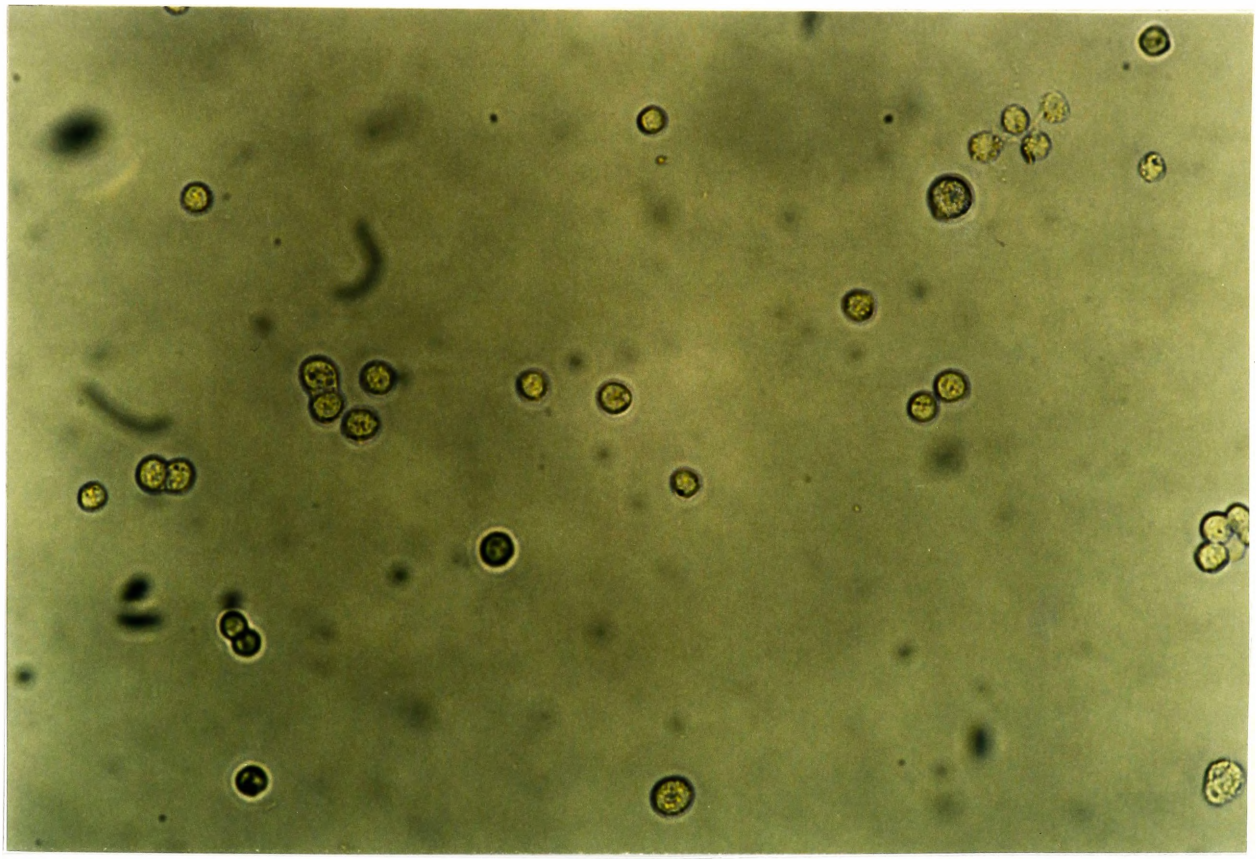


Figure 3.18: *Trypan Blue Stained HCT 116 Cells Following Incubation With $10^{-3}M$ OCl^- (x20).*

In order to exclude cell loss following oxidation as a reason for decreased levels of UDG activity, cells were stained with trypan blue and viewed under the microscope for viability.

The cells appeared whole and still excluded the blue dye. It appeared as though the cells had remained intact following treatment with high concentrations of OCl^-.

The viability test indicated that the minimum viability was 84% for the cells following treatment with OCl^-.

HCT 116 cells were exposed to H_2O_2 at concentrations of 10^{-7}M to 10^{-2}M and cellular extracts assayed for UDG activity. Controls consisted of UDG-containing extracts that had not been oxidised. Data is presented as percentages of the cells not exposed to oxidants.

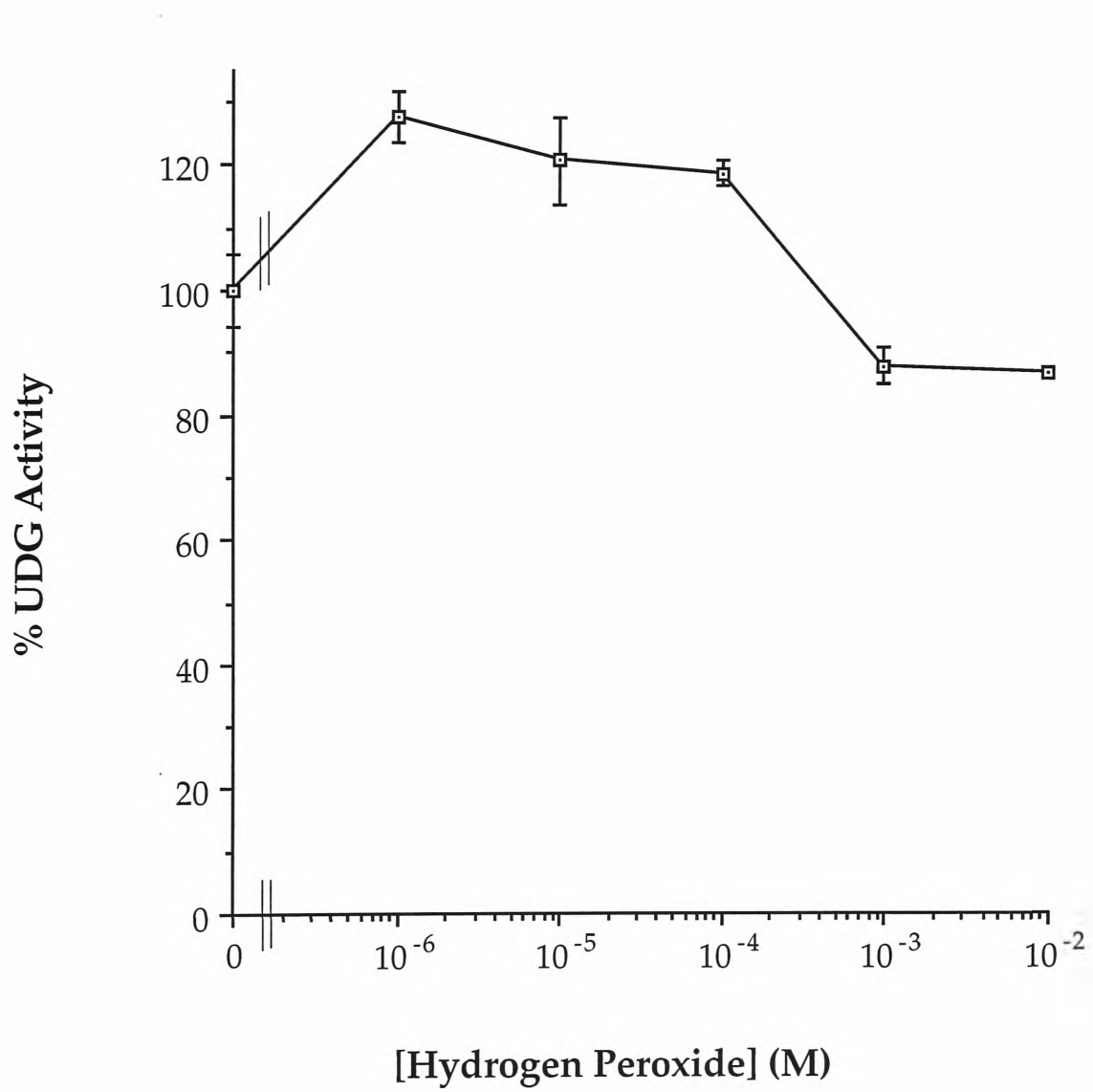


Figure 3.19: *UDG Activity Following Treatment Of HCT 116 Cells With H_2O_2.*

Cells exposed to H_2O_2 concentrations of 10^{-6}M to 10^{-4}M recorded levels of UDG activity similar to that of unoxidised controls (10^{-6}M, $p < 0.10$; all other samples $p > 0.20$).

Samples exposed to H_2O_2 concentrations of 10^{-3}M to 10^{-2}M however, recorded slightly lower levels of UDG activity, but were not significantly different from the control ($p < 0.20$). It may therefore be concluded, that UDG activity was not inhibited in HCT 116 cells that had been exposed to H_2O_2 up to a concentration of 10^{-2}M.

Viability tests indicated that cells exposed to H_2O_2 remained intact, with viability of 83% or greater.

3.4.2.2 GAPDH Activity In Cells Exposed To The Inflammatory Oxidants, OCl^- And H_2O_2

The GAPDH assay is spectophotometrically based and conforms with the Beer-Lambert Law: $A = \varepsilon bc$, where A is the absorbance of the species, ε is a constant, specific for that species (eg. ε_{340} for NADH = $6.22 \times 10^3 M^{-1}cm^{-1}$), b is the length of the light path (1cm) and c is the concentration of the species in the solution.

The standard GAPDH curve had an R^2 value of 0.981, which was considered linear. This indicated that GAPDH activity increased proportionally with an increase in concentration. The procedure used for this assay was therefore acceptable and may be used to monitor the activity of GAPDH that had been altered, for example, by exposure to various oxidants.

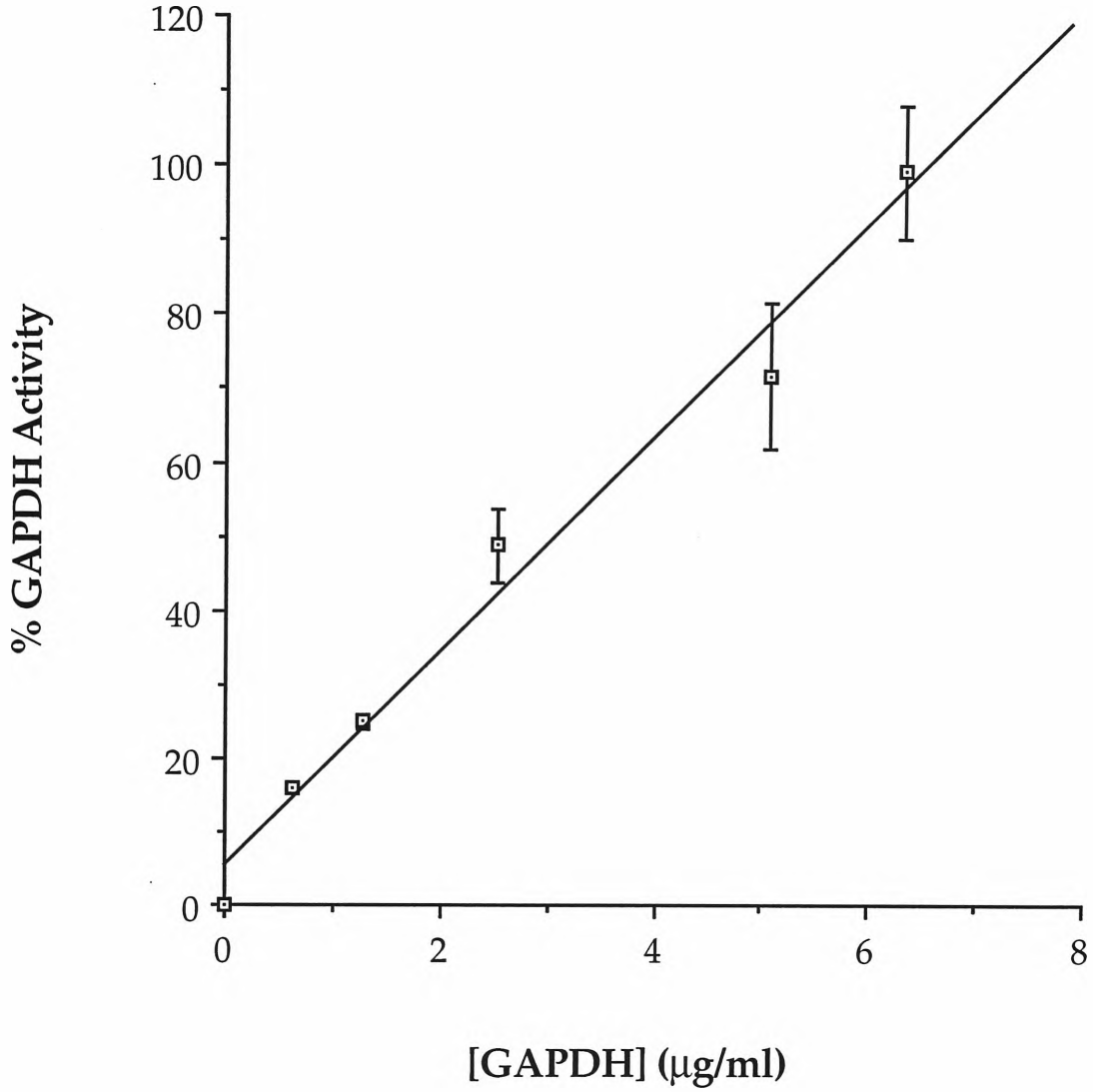


Figure 3.20: *Standard GAPDH Assay.*

HCT 116 cells were exposed to the inflammatory oxidants, OCl^- and H_2O_2, at concentrations of between 10^{-7}M and 10^{-2}M. 50% Inhibition of GAPDH activity was observed between OCl^- concentrations of 10^{-3}M and 2×10^{-3}M. H_2O_2 inhibited GAPDH activity by 50% at a concentration of 5×10^{-4}M.

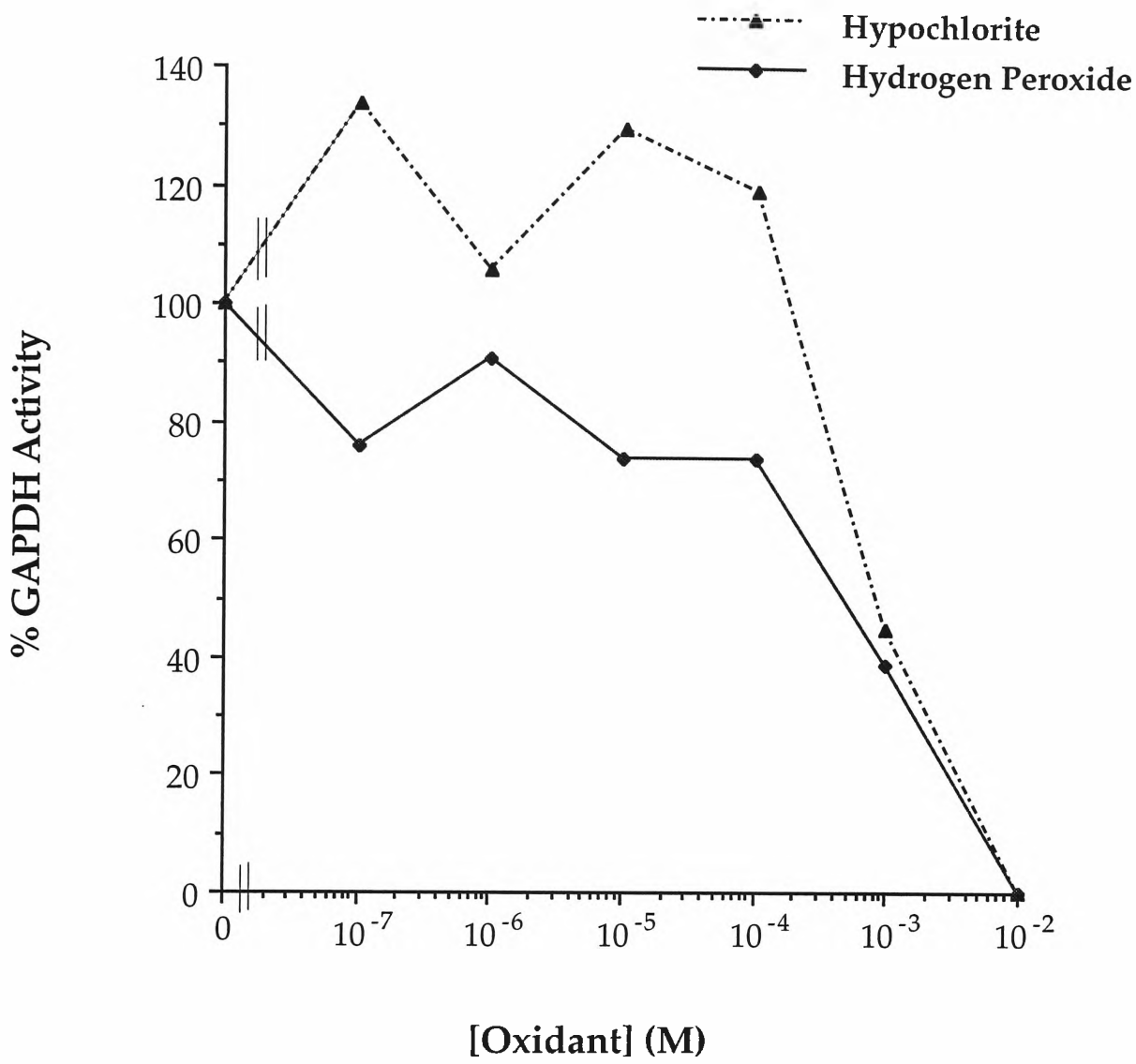


Figure 3.21: *Comparison Of GAPDH Activity Following Exposure Of HCT 116 Cells To OCl^- And H_2O_2.*

Commercially prepared GAPDH was oxidised in this laboratory by Mike Smith (1994) [56]. Native GAPDH activity was inhibited by 50% with concentrations of OCl^- at 5×10^{-6}M and H_2O_2 at 8×10^{-7}M.

4 DISCUSSION

Reactive oxygen species produced by phagocytic cells can cause cell injury not only to natural target cells, like microorganisms or tumour cells, but also to surrounding tissue cells, especially when exposed to a long-standing stimulation as seen in areas of chronic inflammation [1]. It has therefore been suggested that inflammatory oxidants play a role in carcinogenesis [213, 214]. In view of the possible impact of these reactive oxygen species on carcinogenesis *in vivo*, the effects of inflammatory oxidants on DNA repair systems must be analysed. It has been suggested by Meyer-Siegler *et al.* (1991) [1] that UDG, a DNA repair enzyme, is a monomer of the oxidant sensitive glycolytic protein, GAPDH [154, 215]. These inhibitory effects of inflammatory oxidants on GAPDH may therefore also apply to UDG, resulting in misincorporated uracil residues in the DNA, which could lead to mutations and ultimately, carcinogenesis.

4.1 DEVELOPMENT OF A UDG ASSAY

In order to evaluate the effects of inflammatory oxidants on enzymes such as UDG, a reproducible assay needed to be developed to quantitate enzyme activity. For this purpose, it was necessary to establish:

(i) the overall stoichiometry of the reaction catalysed;

(ii) whether the enzyme required cofactors such as coenzymes or metal ions;

(iii) its dependence on substrate and cofactor concentrations;

(iv) its optimum pH;

(v) a temperature zone in which it is stable and has high activity; and

(vi) a simple analytical procedure for determining the disappearance of substrate or the appearance of the reaction products [216].

Enzymes are generally assayed in test systems in which the pH is optimum and the substrate concentration is above the saturating level. That is, there is always enough substrate present in the assay to bind with the enzyme. Under these conditions the initial reaction rate is proportional to enzyme concentration alone. In the case of enzymes requiring cofactors, such as coenzymes or metal ions, the cofactors must also be added in concentrations that exceed saturation so that the true rate-limiting factor in the system is the enzyme concentration. Usually, measurement of the rate of formation of the reaction product is more accurate than measurement of the disappearance of the substrate, since the substrate must be present at relatively high concentration in order to preserve saturation. The reaction product(s) may then be measured by specific chemical or spectrophotometric methods [216].

UDG hydrolyses uracil-glycosidic bonds at dUTP sites in single-stranded and double-stranded DNA, excising uracil and creating alkali sensitive abasic sites in the DNA [217]. UDG is specific for deoxyribose, but not ribose, as found in RNA [218], and does not show any activity with free dUMP or the four common nucleotide residues in DNA. The minimum size recognised as a substrate is an oligonucleotide made up of only 4 uracil bases, that is, $(pU)_4$ [131]. UDG does not have a cofactor dependence or any metal ion requirements and is therefore fully active in the presence of EDTA [217]. Boehringer Mannheim Biochemica recommend that UDG be assayed at a pH of 8.0 and a temperature of 37°C. UDG activity is then quantitated as the release of ethanol-soluble radioactivity ([^{3}H]dUTP) from a polynucleotide substrate.

4.1.1 Preparation Of A UDG Substrate

Initially, DNA polymerase I was used to incorporate [^{3}H]dUTP into a poly(dA)·$(dT)_{12}$ template, using a method adapted from Sirover (1979) [2].

DNA polymerase I catalyses the polymerisation of deoxynucleoside 5'-triphosphates onto the 3'-hydroxy terminus of a primer in the direction of a single stranded DNA template [210]. It was expected that the [^{3}H]dUTP would base pair with the dATP residues in the poly(dA)·$(dT)_{12}$ template. After several unsuccessful attempts at incorporating [^{3}H]dUTP into the oligonucleotide, even with the use of fresh DNA polymerase, it was decided that the poly(dA)·$(dT)_{12}$ template was annealing with itself, and therefore unable to incorporate the labelled nucleotide.

Salmon sperm DNA was nick translated with [^{3}H]dUTP, which produced a substrate with high specific activity. Generally, nick translation reactions are only incubated for 1 hour, as longer incubation times tend to slightly reduce the overall length of the labelled fragment. Longer incubation times, however, also result in greater incorporation of label, which is a priority for the UDG assay substrate [210]. UDG assays rely on measuring the excised [^{3}H]dUTP released into solution following incubation with the UDG enzyme. Therefore, contaminating free-[^{3}H]dUTP must be removed from the substrate solution. Various methods were employed to achieve this. 1µg of salmon sperm DNA is a minute quantity to precipitate. Therefore, 100µl of herring sperm DNA (1mg/ml) was added to the solution as a carrier to aid precipitation. This was extremely successful for the ethanol precipitation of the labelled oligonucleotide. Various salts were also examined for their precipitation qualities, including 2M NaCl, which Sirover (1979) [2] used to precipitate the substrate following incubation in the assay. The number of ethanol precipitations required to remove the maximum amount of free-[^{3}H]dUTP from the substrate solution, while retaining its integrity, was also investigated. It was established that 3 precipitation washes with ethanol, carrier DNA and 3M sodium acetate resulted in the best quality substrate (refer to Figure 3.1).

4.1.2 The UDG Assay

The curve of the standard UDG assay had an R^2 value of 0.979, demonstrating that enzyme activity was directly proportional to enzyme concentration up to a concentration of 1U/100µl assay (Figure 3.2). Concentrations of UDG higher than 1U/100µl assay resulted in a plateau effect, indicating the upper limits of the assay, as proportionality was lost (Figure 3.3). UDG was also assayed with respect to time (R^2 = 0.982). The UDG assay was incubated for 60min, which was in the linear section of Figure 3.4, indicating that the rate of the reaction was not limited by substrate concentration during the course of the assay.

Null activity was recorded for the UDG assay, in which enzyme was not incubated for any substantial time with substrate, but stopped immediately following the addition of all components in the assay (refer to Figure 3.5). Enzymes are generally not active at low temperatures, therefore, placing the assay tubes on ice immediately following incubation, inactivates the enzyme. Addition of ice-cold 100% ethanol precipitates the oligonucleotide so that the UDG cannot access the substrate, thereby, also inactivating the assay. UDG is also inhibited in solutions of high ionic strength [219], which opposes the opening of the double helix necessary for the formation of the enzyme-substrate complex [220]. Therefore, addition of 3M sodium acetate following incubation not only precipitates the substrate but also interferes with UDG-substrate binding, necessary for catalysis.

4.1.3 Extraction Of UDG And GAPDH From A Human Cell Line

Evidence suggests that eukaryotic cells enhance base excision repair enzymes, including UDG, during transition from the quiescent to the proliferative state (that is, during DNA synthesis) [221, 222]. This is because the increased level of deoxynucleotides and of DNA polymerase will tend to

favour the incorporation of uracil into DNA [223]. The production of GAPDH is also dependent on the state of growth of cells, and is synthesised in large quantities in growing cells, with synthesis decreasing considerably in dense cultures [177]. The cell line, HCT 116, was used as a source of human UDG and GAPDH. These cells are cancerous and are therefore in a constant state of rapid proliferation. Consequently, the two enzymes should be in abundance at all times in non-confluent cultures.

Cells which were used for the production of UDG, were lysed in a buffer containing Triton X-100, a nonionic detergent, which is responsible for the loss of membrane integrity [176]. The buffer also contained dithiothreitol, a water-soluble reagent for preserving monothiols completely in the reduced state and reducing disulphides quantitatively to dithiols [224]. It is important to maintain thiols in the reduced state, as oxidised thiols may interfere with the catalytic activity of a number of enzymes, for example, GAPDH and creatine kinase [152]. Cells used for GAPDH experiments, however, were not lysed with Triton X-100, as Caswell and Corbett (1985) [176] found that this detergent was a very potent inhibitor of GAPDH activity, probably due to the contaminating oxidising compounds (for example, peroxides) that it contains [225]. Lysis of all cells was accomplished by freeze/thawing followed by sonication, refer to Figure 3.12.

Figure 3.13 illustrates the same principles as Figure 3.2. That is, radioactivity was released from a [^{3}H]dUTP-containing substrate in a manner proportional to the amount of UDG. In this example, the different amounts of UDG corresponded with the different numbers of cells. Therefore, as cell numbers increased, so did total enzyme activity. The upper limit of this assay was 2.5 x 10^5 cells which should correspond with an enzyme concentration of above 1U/100µl assay. Figure 3.14 illustrates that this has occurred, as 1µl of cell extract from 2.5 x 10^5 cells corresponded with

0.91U/100µl assay. 10µl of cell extract from 2.5 x 10^5 cells was used in the standard curve for increasing cell numbers, which corresponds to 9.1U/100µl assay, which corresponds to the plateau section of the standard UDG curve and is therefore within the upper limits of the assay. From this information, we may extrapolate that 2.5 x 10^5 cells contains 91U of UDG (as cells were reconstituted to a total volume of 100µl). The total protein content of 2.5 x 10^5 cells was calculated to be 0.303mg/ml (Section 3.3.2.2). Therefore, 2.5 x 10^5 cells contain 91U/0.03mg, or 3033U/mg protein.

4.1.4 Limitations Of The UDG Assay

In this particular UDG assay, Figure 3.3 illustrates that activity reaches an upper limit at about 1U/100µl assay. Figure 3.13 displays a similar pattern, with 2.5 x 10^5 cells at the upper limit of the assay. Therefore, aliquots of 2.5 x 10^5 cells were treated with various concentrations of oxidant to observe any inhibitory effects resulting in a measurable decrease in activity.

One negative aspect of this assay is sample number per assay. It was discovered that measured cpm varied between assays for equivalent samples, although the overall trend remained the same. For example:

Table 4.1: *Comparison Of Overall Trend Between Various Standard UDG Assays.*

Background (cpm)	0.5U UDG (cpm)	1.0U UDG (cpm)	R^2 Value
499	2,492	3,607	0.974
630	5,967	8,490	0.959
1,226	2,175	2,845	0.990

Although the slope of the graph varied considerably, the R^2 values were all close to 1.0, indicating that enzyme concentration was proportional to enzyme activity in each case. Therefore, enzyme activity was plotted as a percentage of the maximum value in the standard curve or as a percentage of the sample not treated with oxidants. This variability appeared to correlate with centrifugation following storage at -70°C to precipitate the substrate at the completion of the assay. Assays were therefore conducted on 18 samples at a time as the microcentrifuge only held 18 tubes at one time (5 different samples assayed in triplicate, with a negative control in triplicate).

The integrity of the substrate was investigated and found to lose stability over a period of time. Figure 3.7 illustrates the fact that proportionality between enzyme concentration and enzyme activity is decreased when assays are conducted with a substrate which has degraded with time. Therefore, standard UDG assays should be conducted regularly to examine the integrity of the substrate.

4.2 UDG Activity Of GAPDH

To investigate the proposal that GAPDH contained UDG activity, native GAPDH was reconstituted to a concentration of 3.8mg/ml, and immediately assayed for UDG activity (refer to Figure 3.8). The highest concentration of GAPDH in the UDG assay was 33.44µg/100µl assay and recorded a low count of 134cpm on the scintillation counter (background corrected). In comparison, 1U of UDG had an average value of about 2,500cpm and seldom recorded values less then 1,300cpm (background corrected). The standard errors in Figure 3.8 are also quite large, implying that the measurement was not very consistent. The activity measured for 33.4µg of GAPDH is, however, significantly different ($t(4) = 2.9$, $p < 0.05$) from the negative control. From these results, it appears that native GAPDH does not contain UDG activity.

Native GAPDH is separated into its monomeric subunits when electrophoresed on an SDS-PAGE gel (results not shown) [226]. Birkett (1973) [150] has also stated that GAPDH is denatured with SDS. Therefore, native GAPDH was reconstituted in 10% SDS and assayed for UDG activity. The highest concentration of SDS treated GAPDH (0.19mg/100µl assay) did not have any UDG activity compared with the positive UDG controls. Therefore, other methods of dissociation of the tetrameric GAPDH into its monomeric subunits should perhaps be investigated. Yang and Deal (1969) [227] have shown that yeast GAPDH is slowly dissociated into monomers and dimers by ATP, while Constantinides and Deal (1969) [180] have evidence that adenine nucleotides have the ability to inactivate and dissociate rabbit muscle GAPDH rapidly and extensively, with ATP having the fastest rate of inactivation, although the monomers and dimers produced by ATP are somewhat unfolded. Rabbit muscle GAPDH also dissociates into dimers at 0°C with ammonium sulphate and monomers with KCl [180], however, UDG is inactivated by high ionic solutions [219], particularly KCl [228].

In contrast to the results obtained in this thesis, Meyer-Siegler *et al.* (1991) [1] and Mansur *et al.* (1993) [229] have, however, assayed a 37kD subunit of commercial GAPDH, electro-eluted from an SDS-PAGE gel, and found that it possessed UDG activity. The reasons for this discrepancy are unknown. This thesis illustrated in sections 3.2.3.1 and 3.2.3.2 that native GAPDH does not possess UDG activity, even when treated with SDS to imitate the activity of UDG extracted from an SDS-PAGE gel. The work of Meyer-Siegler *et al.* (1991) [1] should be repeated to substantiate their work, as the work in this thesis was repeated at least 3 times, with all results indicating that GAPDH whether native or SDS treated, does not possess UDG activity.

4.3 THE GAPDH ASSAY

The standard GAPDH assay resulted in a curve with an R^2 value of 0.981, indicating that enzyme concentration was proportional to enzyme activity (refer to Figure 3.20). The addition of cysteine to the reaction solution is imperative in maintaining reactive site thiols in their reduced form for catalysis [185].

4.4 EFFECTS OF INFLAMMATORY OXIDANTS ON UDG AND GAPDH

4.4.1 Oxidant Sensitivity Of UDG

Figure 3.17 illustrates that the UDG extracted from oxidised cells maintained complete activity when exposed to OCl^- concentrations up to 5×10^{-4}M, while only 1% of activity was seen at 7.5×10^{-4}M and activity was completely inhibited at 10^{-3}M and 10^{-2}M. In contrast, UDG activity, following exposure of cells to H_2O_2 up to a concentration of 10^{-2}M, did not display any significant inhibition of activity (refer to Figure 3.19). To date there does not appear to be any literature evidence with which to compare these findings.

This phenomenon could well be attributed to the different sites of attack on the cell of these two oxidants. Protein sulphydryls are particularly sensitive to oxidation by OCl^- [230], whereas H_2O_2 might be more generally responsible for lipid peroxidation and DNA strand breakage [69, 103]. Fliss (1988) [231] also found that OCl^- was much more effective than H_2O_2 in oxidising proteins from rat heart and lung. Figure 3.6 also illustrates that UDG is heat-inactivated and thereby loses activity due to conformational changes. OCl^- may also be responsible for conformational changes, by producing disulphide bridges within the enzyme. H_2O_2, although capable of oxidising -SH groups, does not cause general -SH oxidation [230] This is

probably due to the high cellular levels of glutathione, which readily reduces H_2O_2 to nontoxic forms and keeps sulphydryls reduced [64]. H_2O_2 is capable of freely diffusing across cell membranes and subsequently into the nucleus, whereas OCl^- is more reactive and therefore does not generally migrate as far. For example, Weitzman and Gordon (1990) [69] found that micromolar concentrations of H_2O_2 induced DNA strand breaks in target cells, whereas OCl^- generally did not [230]. Cochrane (1991) [66] also found that H_2O_2, in the presence of iron ions, induced strand breaks in DNA at an average of 55% of sites, while OCl^- was only responsible for 5% of sites. The DNA damage is not produced by H_2O_2 *per se*, but by hydroxyl radicals formed when H_2O_2 forms hydrogen bonds with DNA and reacts with chromatin-bound Fe^{2+} [232, 233]. The slight increase in UDG activity observed when cells were exposed to low concentrations of H_2O_2 (Figure 3.19) may be due to the requirement of DNA repair processes as a response to oxidative damage on the DNA.

To examine whether oxidation of susceptible thiols with OCl^- has occurred, cells should be exposed to competitive thiol containing compounds such as the amino acids cysteine and methionine before oxidation with OCl^-. The addition of the amino acids should be made prior to oxidation to avoid the production of disulphide bridges by OCl^-. These two amino acids would protect thiols in the enzyme from oxidants.

E. coli UDG, used as a standard in the UDG assay, was not inhibited by either OCl^- (Figure 3.15) or H_2O_2 (Figure 3.16) and therefore can not be used as a model to investigate the inactivation of human UDG by inflammatory oxidants. Human UDG is generally compartmentalised within the nucleus or mitochondria [234]. The membranes of these organelles therefore partially protect human UDG from oxidation from OCl^-. Bacterial UDG however, does not possess this protection and may therefore have adapted

other forms of protection from oxidation by OCl^-. Indeed, Muller and Caradonna (1991) [203] and Varshney *et al.* (1988) [235] have found that the amino acid sequence of *E. coli* UDG does not possess any homology with the human UDG which they isolated.

4.4.2 Oxidant Sensitivity Of GAPDH

HCT 116 cells were exposed to inflammatory oxidants, GAPDH extracted and analysed for activity. Preliminary data (Figure 3.21) suggests that GAPDH extracted from oxidised cells was inhibited by 50% at a concentration of 5 x 10^{-4}M with H_2O_2 (or 2 x 10^{-2}M at 98%) and between 10^{-3}M and 2 x 10^{-3}M with OCl^-. This is in accordance with results from Brodie and Reed (1990) [153], who found that cellular GAPDH activity decreased by 98% when exposed to H_2O_2 at a concentration of 6 x 10^{-2}M. Schraufstätter *et al.* (1990) [230] however, found that GAPDH from cells exposed to OCl^- at a concentration of 4 x 10^{-5}M was inactivated by 50%. This discrepancy may be accounted for by the low amount of GAPDH activity recorded in the non-oxidised control cells, which resulted in GAPDH activity of more than 100% for OCl^- concentrations from 10^{-7}M to 10^{-4}M.

Figure 3.21 illustrates that GAPDH retained 100% activity until cells were exposed to approximately 10^{-5}M OCl^-. GAPDH activity then decreased rather slowly at first, until 10^{-4}M, then rapidly until complete inactivation at 10^{-2}M. This pattern corresponds with the concentration of OCl^- responsible for cell lysis, 7.5 x 10^{-6}M, found by Schraufstätter *et al.* (1990) [230]. However, cells exposed to H_2O_2 immediately displayed a gradual decrease in GAPDH activity with the lowest concentration of H_2O_2 (10^{-7}M). This is consistent with the relative diffusibility of H_2O_2 and OCl^-, that is, H_2O_2 diffuses through cell membranes readily, thereby having access to intracellular targets such as GAPDH and nucleic acids, whereas OCl^- is highly reactive

[236] and tends to oxidise membrane proteins and therefore does not migrate great distances.

The effect of 5 x 10^{-3}M H_2O_2 on the K_m and V_{max} of other enzymes of the glycolytic pathway has been analysed, and it was found that only GAPDH was affected [230]. No such specificity was seen with OCl^- [230, 236], which rather indiscriminately oxidised -SHs. It has been suggested by Little and O'Brien (1969) [154] however, that H_2O_2 oxidises the catalytically active SH-groups of GAPDH (cys_{149}) to sulphenic acids which then undergo further oxidation either by dissolved oxygen or excess H_2O_2 to sulphonic acids. Inhibition of cellular GAPDH by H_2O_2 may also be due to ADP-ribosylation and consequent decrease of available NAD [45, 182, 208].

Native, commercial GAPDH is inactivated by lower concentrations of H_2O_2 (50% at 8 x 10^{-7}M) [56] and OCl^- (50% at 5 x 10^{-6}M) [56]. Schraufstätter *et al.* (1990) [230] also found that GAPDH activity from lysed cells that had been exposed to OCl^-, was inhibited at a lower concentration (90% at 10^{-5}M). These findings indicate that the cytoplasmic location protects GAPDH from inactivation in intact cells to a considerable degree, but not completely. The irreversible inactivation of oxidised GAPDH may also be a consequence of proteolysis. Patnode *et al.* (1976) [206] and Prinsze *et al.* (1990) [237] have shown that GAPDH with blocked thiol groups undergoes a conformational change and is therefore more susceptible to digestion by the proteolytic enzymes, trypsin and chymotrypsin, whereas the native enzyme is resistant to proteolysis. Intracellularly, GAPDH with oxidised sulphydryl groups may be susceptible to fragmentation by lysosomal proteases, thereby accounting for the observed decreases in specific activity.

4.5 CONCLUSIONS

The development of an assay, modified from Sirover (1979) [2], for the quantitation of UDG activity has promoted the investigation of UDG activity from a variety of sources and under various conditions. Although GAPDH does not appear to exhibit UDG activity in either its tetrameric or monomeric forms as suggested by Meyer-Siegler *et al.* (1991) [1], the effects of inflammatory oxidants on UDG were still investigated. Both UDG and GAPDH were susceptible to the inflammatory oxidant, OCl^-, which specifically oxidises thiols. H_2O_2, in contrast, only inhibited GAPDH activity, as a consequence of sulphenic and sulphonic acid production rather than disulphide bridges. The inhibitory effect illustrated with H_2O_2 on GAPDH activity is also believed to be a consequence of ADP-ribosylation subsequent to DNA damage by the oxidant. Not only is the process of ADP-ribosylation responsible for the inhibition of GAPDH through depletion of NAD molecules available for GAPDH catalysis, but also for direct binding of polymerised ADP-ribose moieties to the active site of GAPDH. GAPDH is also more susceptible to proteolysis following oxidative stress. Inhibition of GAPDH activity by inflammatory oxidants such as OCl^- and H_2O_2 may therefore result in decreased cellular ATP levels which may ultimately lead to cell death. Likewise, the occurrence of uracil in DNA may be a direct consequence of inactivation of UDG by OCl^- which may possibly lead to mutation and ultimately carcinogenesis.

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APPENDIX A

Phenol/Chloroform Extraction Of Labelled Oligonucleotide

Phenol/chloroform extraction was undertaken in the fume hood on a portable tray used for radioactive spillage. TE buffered phenol (0.5 - 1ml) was added to the 600µl [^{3}H]dUTP-labelled oligonucleotide sample and mixed by inversion. The sample was then centrifuged for 15min at 12,000 x g and the aqueous (upper) layer placed into a fresh Eppendorf tube. Extraction with phenol was repeated until the supernatant was clear (twice was usually adequate). Chloroform (0.5 - 1ml) was then added to the supernatant, mixed by inversion and centrifuge for 1 - 2min at 12,000 x g. The aqueous layer was then transferred to a fresh tube, ready for dialysis.

Dialysis Of Labelled Oligonucleotide

Preparation Of Dialysis Tubing

Dialysis tubing was boiled in glass distilled water with EDTA (~0.2g) for 10min. It was then rinsed in glass distilled water and stored at 4°C in 70% ethanol. Dialysis tubing should be handled with gloves and kept moist at all times.

Dialysis

A microdialysis chamber eliminates the need to remove the reaction mixture from its vessel for dialysis. It was made by puncturing the centre of an Eppendorf tube lid with a heated Pasteur pipette (wide end) and then placing a piece of dialysis tubing across the opening of the tube. The Eppendorf lid was then closed, causing the dialysis membrane to become taut (check for leakage). The tube was inverted so that the solution to be

dialysed was resting on the membrane surface. The microdialysis chamber was attached to a beaker so that the dialysis membrane was fully immersed into the dialyzing solution. The labelled oligonucleotide was then dialysed 4 x 4h (or overnight) in each buffer (1M NaCl/50mM Tris-HCl, pH 7.6, or 50mM Tris-HCl, pH 7.6).

Preparation Of UDG Substrate For Application To The Bio-Spin Chromatography Column

The UDG substrate must be reduced to a volume of 100µl for application to the Bio-Spin Chromatography Column. To accomplish this, the UDG substrate must first be frozen in liquid nitrogen and then dried on the SpeedVac until the required volume (100µl) is attained. The sample may then be loaded directly onto the column.

APPENDIX B

Nick Translation Reagent Concentrations

dNTP mix

dNTPs were supplied as 300μM solutions. As [^{3}H]dUTP was replacing dTTP in the DNA template, then the dNTP mix was composed of dATP, dGTP and dCTP.

Nick Translation 10x Buffer

The nick translation 10x buffer consisted of: 500mM Tris-HCl, pH 7.2/100mM $MgSO_4$/1mM DTT.

Salmon Sperm DNA

Salmon sperm DNA was chromosomal and approximately 40kb in size. It was stored at a concentration of 5mg/ml and 1μg of this stock was used for nick translation reactions.

Nick Translation Enzyme

The DNA polymerase I (1U/μl) and DNase I (0.2ng/μl) enzyme mixture was stored in a 50% glycerol solution containing 50mM Tris-HCl, pH 7.2, 10mM $MgSO_4$, 0.1mM DTT and 0.5mg/ml nuclease-free BSA.

Stop Solution

The nick translation stop solution was 0.25M EDTA, pH 8.0.

APPENDIX C

RPMI 1640 Media For Cell Culture

5x RPMI 1640	52.2g
5.6% sodium bicarbonate	10g
1M HEPES	24g
Sterile Milli-Q water	5L

Adjust pH to 7.2 with 1M NaOH, filter sterilise and aliquot into sterile bottles.

1ml of 200µM L-glutamine (stored at -20°C) was added to every 100ml of RPMI 1640 media (that is, at 1%), when required for tissue culture, or after 1 month of use. L-Glutamine should be filtered before use.

Foetal Bovine Serum (FBS)

FBS was heat inactivated in 200ml aliquots at 56°C for 30min. Filter under positive pressure through 0.22µm and 0.45µm filters. Store at -20°C until required.

Hank's Balanced Salt Solution (HBSS)

Ca^{2+}, Mg^{2+} and phenol red-free 10x stock:

Solution A

NaCl	80g

KCl 4g

Sterile Milli-Q water to 500ml.

Solution B

KH_2PO_4 0.6g

Na_2HPO_4 0.6g

Glucose 10g

$NaHCO_3$ 3.5g

Sterile Milli-Q water to 500ml.

Autoclave and store solutions A and B separately.

1x Working Solution:

Solution A	5ml
Solution B	5ml
Sterile Milli-Q water	90ml

Combine under sterile conditions and store at 4°C.

Phosphate Buffered Saline (PBS)

Dissolve 1 PBS tablet in 100ml of sterile Milli-Q water and autoclave.

APPENDIX D

Dilutions Of BSA For Standard Protein Graph

Stock BSA (1.29mg/ml) was diluted in cell lysis buffer containing 0.5% Triton X-100 to a final volume of 50µl for the standard protein graph as follows:

Table D1: *Concentrations Of BSA For Standard Protein Assay.*

Final [BSA] (mg/ml)	Volume Stock BSA (µl)	Volume Buffer (µl)
0	0.0	50.0
0.4	15.5	34.5
0.8	30.0	20.0
1.2	46.5	3.5

Each standard (5µl) was then aliquoted into separate wells on the ELISA plate.

Reagent A + S For Protein Assay

The cell lysates were stored in a buffer containing Triton X-100, which is a detergent. This must therefore be compensated for in the protein assay, as detergents interfere with spectrophotometric absorbances. Reagent A (980µl) was therefore mixed with 20µl of Reagent S, and 25µl of this mixture (A + S) was aliquoted onto the 5µl of sample or standard in each well.

APPENDIX E

EXAMPLES OF A STUDENT *t* -TESTS

Exposure Of Bacterial UDG To OCl^-

Unoxidised UDG and 10^{-3}M OCl^- Sample

$$t = \frac{5887 - 4217}{\sqrt{\frac{(902)^2}{3} + \frac{(1807)^2}{3}}}$$

$$= 1.4$$

Degrees of freedom (df) $= (3 + 3) - 2$

$$= 4$$

$t(4) = 1.4, p > 0.20$

That is, the two values are not significantly different.

Exposure Of HCT 116 Cells To OCl^-

Cells Not Treated With Oxidant And 10^{-3}M OCl^- Sample

$$t = \frac{12{,}464 - 0}{\sqrt{\frac{(471)^2}{3} + \frac{(73)^2}{3}}}$$

$$= 45.3$$

df $= (3 + 3) - 2$

$$= 4$$

$t(4) = 45.3, p < 0.001$

That is, the two values are significantly different.

APPENDIX F

ABSTRACT FROM VII INTERNATIONAL CONFERENCE FOR THE SOCIETY OF FREE RADICAL RESEARCH, SYDNEY, NOVEMBER, 1994:

Oxidative Sensitivity Of Alternative Functions Of GAPDH

Scherret JH, Smith M and Baker MS. Dept of Biological Sciences, University Of Wollongong, NSW, Australia.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is composed of 4 identical 37kD subunits, which has recently been shown to also act as a DNA-base excision repair enzyme (uracil-DNA glycosylase (UDG)). We have previously shown that GAPDH is a sensitive "marker" of oxidative stress during inflammation. Here we (a) develop a biochemical assay of UDG activity and (b) determine the sensitivity of GAPDH/UDG activities to oxidants.

GAPDH exposed to H_2O_2 and OCl^- showed oxidation of susceptible thiols in the GAPDH monomer (as indicated by progressive increases in M_r as determined by electrospray mass spectrometry). Oxidants reduced (50%) GAPDH activity at ~5μM (H_2O_2) and ~10μM (OCl^-) respectively, while $NO^\bullet$ (generated by diethylamine NONOate) inhibited GAPDH with an $I_{50\%}$ = 1mM.

UDG activity (using either a [^{32}P]-5'end-labelled uracil containing-oligonucleotide or a [^{3}H]-dUTP-DNA substrate) was calibrated against authentic bacterial UDG and increased with cell (HCT 116) number between 10^4 - 2.5 x 10^5 cells (after which activity reached a plateau, reflecting the upper limit of the UDG assay).

In cells exposed to H_2O_2 and OCl^- UDG activity was retained at OCl^- concentrations of $\leq 10^{-4}M$, but were inhibited at $\geq 10^{-4}M$. This data suggests that alternate activities located on the 37kD GAPDH/UDG monomer have differential sensitivities to oxidants.