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Cell cycle regulation of redoxyendonuclease activity in human neuroblastoma cells.

Deepika Walpita

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CELL CYCLE REGULATION OF REDOXYENDONUCLEASE ACTIVITY IN HUMAN NEUROBLASTOMA CELLS

DISSERTATION

Submitted to the College of Arts and Sciences
of
West Virginia University

In partial fulfillment of the requirement for the degree of
Doctor of Philosophy

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Deepika Walpita

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West Virginia

1997

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INTRODUCTION

All cells are exposed to a constant barrage of endogenous and exogenous agents that cause alterations in the structure of DNA. For many years, it was believed that the maintenance of genetic continuity was based solely on the inherent stability of the DNA molecule. However, a number of physical and chemical agents, such as ionizing radiation, ultraviolet light, thermal radiation, or reactive chemicals, induce a wide variety of damage in DNA. Damage is defined as any modification of DNA that alters its coding properties or its normal function in replication or transcription.

Oxygen Free Radicals

Oxidation, one type of damage, results in numerous DNA lesions of potential biological significance (Imlay and Linn, 1988; Wallace, 1988; Halliwell and Aruoma, 1991; Sies and Menck, 1992; Dizdaroglu, 1992). Although oxygen is essential for aerobic life forms, the metabolism of oxygen can be toxic to the organism. While mammals obtain the majority of their cellular ATP by the controlled, four electron reduction of oxygen to form water by the mitochondrial electron transport system, oxygen can accept less than four electrons to form reactive oxygen metabolites capable of reacting with cellular macromolecules (Floyd, 1990; Halliwell and Aruoma, 1991; Harris, 1992). Oxygen-free radicals are produced in cells by environmental agents, such as ionizing radiation, ultraviolet light, toxic chemicals and drugs (Floyd, 1990; Ames and Gold, 1991; Halliwell and Aruoma, 1991; Sies, 1991 Harris, 1992). In addition, reactive oxygen species are generated in normal cells as a result of the catalytic activity of a variety of enzymes.

leakage from electron transfer, or the product of chemical redox or transition metal reactions (Sies, 1991). Free radicals are not solely the by-products of aerobic metabolism or the agents of environmental toxins. Oxygen free radicals, produced by such immune cells, as activated macrophages, serve as cytotoxic agents in the host immune surveillance system.

Each oxygen free radical has its own distinct chemistry and cellular distribution. The most common forms of reactive oxygen intermediates produced are the superoxide radical anion ($O_2^{\cdot}$) and the highly reactive hydroxyl radical ($OH^{\cdot}$) (Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Sies and Menck, 1992; Dizdaroglu, 1992). However, reactive oxygen species is often used as a collective term that includes not only the oxygen-centered radicals, but also some potentially dangerous nonradical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), and ozone (O_3).

The superoxide anion is the primary product generated by the univalent reduction of molecular oxygen (Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Sies and Menck, 1992; Dizdaroglu, 1992). In aqueous environments, the superoxide anion is in equilibrium with its protonated form (Table 1). Although superoxide anion is relatively unreactive, several of its derivative compounds, including its protonated form, are capable of oxidizing organic molecules. When the superoxide anion and its protonated form approach equal molar concentrations, spontaneous dismutation occurs, and hydrogen peroxide is generated as a direct product (Table 1). Hydrogen peroxide is also produced as a direct result of a double reduction of molecular oxygen without the intermediate formation of superoxide anions.

Table 1. Mechanisms for the formation of oxygen-derived radicals and their metabolites.

Electron reduction of oxygen

$$O_2 + e^- \rightarrow O_2^-$$

Spontaneous dismutation of O_2^-

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (or } {}^1O_2)$$

Haber-Weiss reaction

$$O_2^- + H_2O_2 \rightarrow O_2 \text{ (or } {}^1O_2) + HO^- + HO^\cdot$$

Fenton reaction

$$O_2^- + M^{n+1} \rightarrow M^n + O_2$$
$$M^n + H_2O_2 \rightarrow M^{n+1} + HO^- + HO^\cdot$$

$$O_2^- + H_2O_2 \rightarrow O_2 + HO^- + HO^\cdot$$

Iron-hydrogen peroxide-chloride system

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^\cdot$$
$$HO^\cdot + Cl^- \rightarrow Cl^\cdot + HO^-$$

$$Cl^\cdot + H_2O_2 \rightarrow HOCl + HO^\cdot$$

Myeloperoxidase-hydrogen peroxide-chloride system

$$H_2O_2 + Cl^- \xrightarrow{MPO} OCl^- + H_2O$$
$$OCl^- + H_2O_2 \rightarrow H_2O + Cl^- + {}^1O_2$$
$$OCl^- + RNH_2 \rightarrow RNHCl + HO^-$$
$$OCl^- + RNHCl \rightarrow RNCl_2 + HO^-$$

Singlet oxygen is formed as a product of several reactions (Sies and Menck, 1992). For example, singlet oxygen is generated by direct absorption of radiation, by the direct photolysis of ozone in polluted air, or by irradiation of superoxide anions. Intracellular and phagocytic generation of singlet oxygen occurs during dismutation of superoxide, as a product of Haber-Weiss reactions, or as the result of the interaction of hypochlorite with hydrogen peroxide (Table 1). Singlet oxygen is highly electrophilic and will readily react with electron-rich compounds.

Additional oxygen metabolites are generated by the reactions of various peroxidases with hydrogen peroxide in the presence of halides (Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Dizdaroglu, 1992). For example, myeloperoxidase, eosinophil peroxidase, and lactoperoxidase each catalyze the reaction of hydrogen peroxide and halides to form hypohalous acids (Table 1). The iodide derivatives are the most reactive on a molar basis; however, in most biological systems, hypochlorous acid is the predominant species formed. Although relatively stable, as stated above, hypochlorous acid is reduced, by hydrogen peroxide to singlet oxygen, which will readily react with biological substrates, particularly those rich in unsaturated bonds.

The hydroxyl radical is a potent reactive oxygen species that can attack all biological molecules, usually setting off free radical chain reactions (Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Dizdaroglu, 1992). This radical is the product of the classic Haeber-Weiss reaction when hydrogen peroxide is directly reduced by superoxide anions (Table 1). Alternatively, a hydroxyl radical is generated by metal-catalyzed transport of an electron from superoxide anion to hydrogen peroxide. In this reaction, called the Fenton reaction, superoxide reduces trace metals and generates oxygen (Table 1). The reduced

form of the metal then reacts with hydrogen peroxide to regenerate the initial oxidized form of the metal, the hydroxide ion, and the hydroxyl radical. In addition, the hydroxyl radical is produced by the radiation-dependent splitting of water. It should be noted that most organisms are exposed to some level of ionizing radiation.

Reactive oxygen species also include peroxyl radicals ($RO_2^{\cdot}$), polyunsaturated lipid oxide-free radicals ($RO^{\cdot}$), lipid hydroperoxides (RO_2H), and other peroxides (ROOR) (Imlay and Linn, 1988; Halliwell and Aruoma, 1991; Dizdaroglu, 1992). Polyunsaturated fatty acids are very susceptible to oxidative damage by free radicals (Ursini et al., 1991). Hydroperoxides are considered the major product of membrane lipid peroxidation which involves peroxyl radical-dependent chain reactions among the polyunsaturated fatty acyl moieties, leading to membrane damage (Ursini et al., 1991). Not only will these peroxides alter the structural integrity and biochemical function of membranes, but such lipid peroxyl compounds also function as intermediates in the transfer of electrons to other target molecules.

Cellular Antioxidant Defense Mechanisms

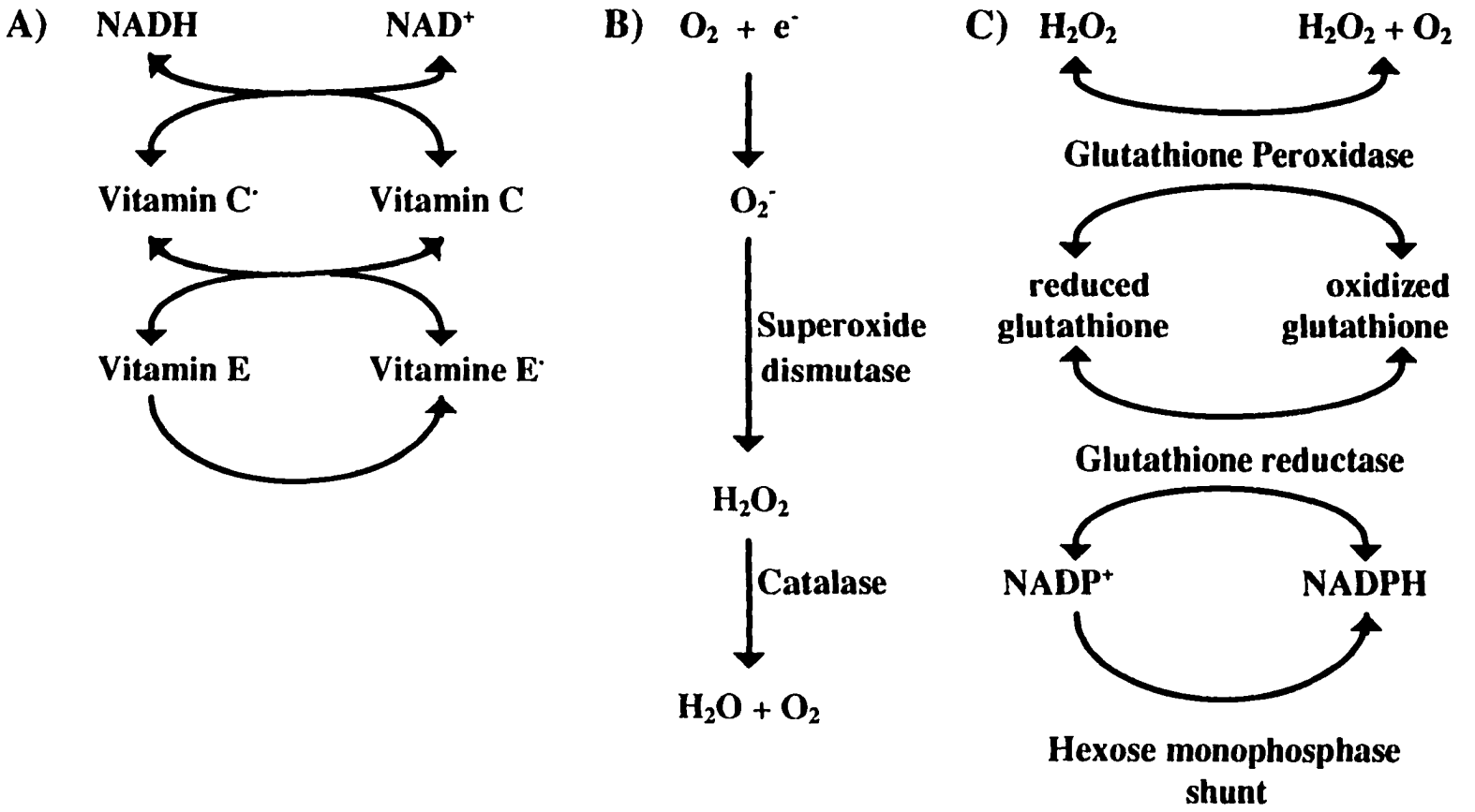
A delicate balance exists between the cellular systems that generate various oxidants and those that maintain antioxidant defense mechanisms. The longest known biological antioxidant is α-tocopherol or vitamin E (Burton and Traber, 1990; Mascio et al., 1991;Rose and Bode, 1993). Vitamin E was identified in relatively high concentrations in both cellular and mitochondrial membranes. Studies showed that vitamin E functions as a free-radical scavenger to protect cell membranes against lipid peroxidation by free radicals. Vitamin E does not alter the production of free radicals

within the biological system, but serves to scavenge and terminate free-radical reactions by forming tocopherol dimers or quinones. Vitamin E also functions synergistically with ascorbic acid, vitamin C, to terminate free-radical reactions (Figure 1). Vitamin C can function as a secondary antioxidant that can react with vitamin E radicals to regenerate vitamin E. The vitamin C radicals are then reduced by NADH reductase with NADH serving as an electron acceptor.

The three major antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and catalase, are found in all aerobic organisms with a few exceptions (Floyd, 1990; Sies, 1991; Harris, 1992; Fridovich, 1995). The primary mechanism for clearance of superoxide anions from biological systems is the superoxide dismutase enzyme. This enzyme was first isolated in 1969 by McCord and Fridovich. However, at least three separate forms of superoxide dismutase were characterized and each enzyme has at least one transition metal in its active site. In eukaryotes, Mn-superoxide dismutase is a mitochondrial enzyme located in the inner membrane but synthesized by nuclear-encoded genes. Evidence suggests that the Cu,Zn-superoxide dismutase is primarily a peroxisomal enzyme, although some studies suggest that the enzyme is also found in the nucleus. In addition, an extracellular form of the Cu,Zn-superoxide dismutase was found in a number of biological fluids, including plasma, lymph, and synovial fluid. Superoxide dismutase catalyzes the dismutation of superoxide anions to hydrogen peroxide and oxygen (Figure 1). The hydrogen peroxide, itself an oxidant, then must be converted to a less toxic compound.

A two enzyme system appears to be necessary to metabolize hydrogen peroxide (Figure 1). Catalase, a cytoplasmic heme-containing enzyme present in mitochondria and

Figure 1. Examples of antioxidant defense mechanisms.



other organelles, catalyzes the divalent reduction of hydrogen peroxide to water (Floyd, 1990, Sies, 1991, Harris, 1992). Catalase, a heme protein, is ubiquitously distributed in tissues of all species studied. A second enzyme, glutathione peroxidase, is also present in significant concentrations in the cytoplasm of cells. This enzyme requires selenium for activity and converts hydrogen peroxide to water, using glutathione as an electron donor. In addition, glutathione peroxidase metabolizes lipid hydroperoxides to relatively unreactive hydroxyl fatty acids. The antioxidant activity of glutathione peroxidase is tightly coupled to the intracellular concentrations of glutathione, glutathione reductase, and NADPH. Once oxidized, glutathione is reduced by glutathione reductase with NADPH serving as the reducing agent. Since the primary source of NADPH is the hexose monophosphate shunt, an intact shunt system is necessary to maintain the activity of this antioxidant defense system. A number of glutathione peroxidases were identified, including a plasma enzyme which is antigenically distinct from its cytosolic counterparts.

There is a dynamic equilibrium between the antioxidant defense capacity and the oxidative potential. When responding to an increase in superoxide radicals, cells must not only increase the level of superoxide dismutase, but also catalase and glutathione peroxidase (Harris, 1992). While superoxide dismutase accelerates the conversion of superoxide to hydrogen peroxide, too much of this enzyme in relation to the activities of hydrogen peroxide-removing enzymes is deleterious. Oxidative stress can occur if either antioxidant levels are depleted or if the formation of reactive oxygen species increases, or both (Sies, 1991). Antioxidant levels are compromised by malnutrition, toxic chemicals or therapeutic drugs, rendering cells more sensitive to oxidative damage.

Types of Oxidative DNA Damage

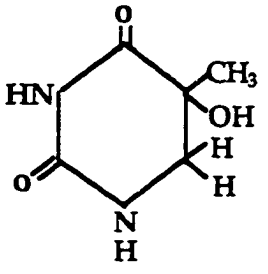
Oxidative DNA damage caused by free radicals is implicated in a number of biological processes, including aging (Holmes et al., 1992; Stadtman, 1992; Ames et al., 1993; Knight, 1995; Yu and Yang, 1996), apoptosis (Richter, 1993; Wook and Youle, 1994; Slater et al., 1995; Ratan and Baraban, 1995; Simonian and Coyle, 1996), mutagenesis and carcinogenesis (Simic, 1994; Feig, 1994; Darley-Usmar et al., 1995; Anderson, 1996; Wiseman and Halliwell, 1996). A significant amount of oxidative damage occurs as a part of normal cellular metabolism (Ames et al., 1993) with by-products of metabolic reactions causing extensive damage to DNA. This damage accumulates with age, and is thought to be a major contributor to aging and the induction of cancer (Ames and Gold 1991; Ames et al., 1993). It is estimated that in adult rats there are approximately 10^6 oxidative adducts in cellular DNA with 10^5 new adducts formed daily per cell (Ames and Gold, 1991). In comparison, it is estimated that human cells encounter approximately 10^3 oxidative hits per day from the products of normal metabolic processes (Ames et al., 1993). Cancer rates are high in rats and an important factor appears to be the basal metabolic rate which is approximately 7 times higher in rats than in humans. This could significantly increase the level of endogenous oxidants produced as by-products of metabolism (Ames et al., 1993). These results suggest that the metabolic rate of the organism is proportional to the incidence of cancer and inversely proportional to its life span.

Oxygen free radicals damage nucleic acids producing many types of lesions, including single- and double strand breaks in the sugar-phosphate backbone and a diverse array of base modifications (Halliwell and Aruoma, 1991). For example, the highly

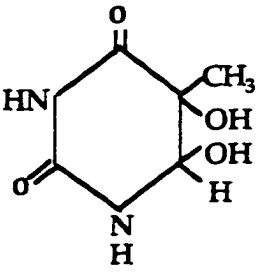
reactive hydroxyl radical can attack all components of DNA, including the sugar residue, yielding reactive sugar radicals (Halliwell and Aruoma, 1991; Breen and Murphy, 1994). Reactions of sugar radicals can lead to base release and DNA strand breaks (Randerath et al., 1992). Interactions of hydroxyl radicals with the deoxyribose moiety results in cleavage of the N-glycosylic bond with release of the free base, generating an abasic site.

While hydroxyl radicals can attack sugar molecules, the DNA bases are the primary target for reactions with a variety of oxygen free radicals (Breen and Murphy, 1994). Purine bases are modified by interaction with free radicals, forming 8-oxoguanine by oxidation at the C-8 position as the predominant purine lesion (Floyd, 1990). Oxidant damage to pyrimidines include saturation of the 5,6 double bond of both thymine and cytosine, producing a class of base alterations referred to as ring-saturated pyrimidines (Wallace, 1988; Dizdaroglu, 1992). The structures of many of these are shown in Figure 2. Hydroxyl radical addition to the double bond of thymine occurs at both the C-5 and the C-6 position. Abstraction of hydrogen from the methyl group occurs 9% of the time, generating multiple products (Simic and Jovanovic, 1986). The major C-5 hydroxyl adduct is oxidized at the C-6 position; the resulting cation is quenched with water to give *cis* and *trans* isomers of 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) (Dizdaroglu, 1992; Breen and Murphy, 1995). Less frequently the C-6 hydroxyl radical is reduced and protonated, producing the 6-hydroxy-5,6-dihydrothymine adduct (Breen and Murphy, 1995). Saturation of the 5-6 double bond of cytosine also occurs, although many of the cytosine derivatives are less stable than the oxidized thymines. Therefore, there is less information available about the ring-saturated cytosines. However, several oxidized

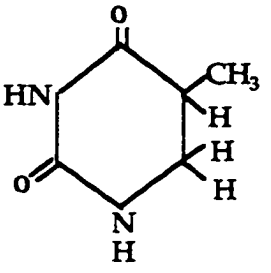
Figure 2. Structures of representative ring-saturated pyrimidines.



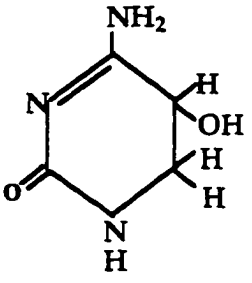
5-hydroxy-6-hydro-thymine



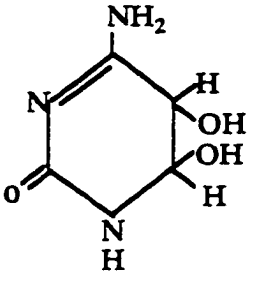
thymine glycol



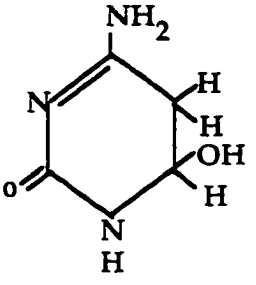
5,6-dihydrothymine



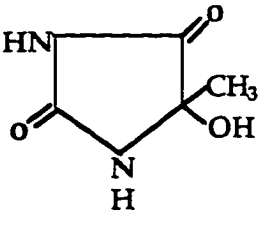
5-hydroxy-6-hydro-cytosine



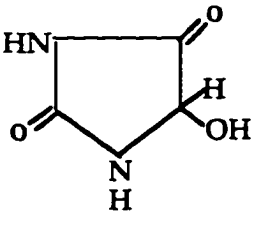
cytosine glycol



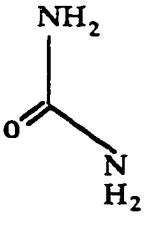
5,6-dihydro-6-hydroxycytosine



5-hydroxy-5-methyl-hydantoin



5-hydroxyhydantoin



urea

cytosines were reported, including cytosine glycol and 5,6-dihydrocytosine (Dizdaroglu, 1992; Breen and Murphy, 1995). One major stable oxidation product of cytosine, 5-hydroxycytosine, is present in the DNA of normal tissues at steady-state levels similar to those of 8-oxoguanine (Wagner et al, 1992).

A number of studies showed the formation of ring-saturated pyrimidines following exposure of DNA or cells to physical agents, such as UV and ionizing radiation, to chemical compounds, including hydrogen peroxide and metal ions, doxorubicin, osmium tetroxide, potassium permanganate, or to chemical carcinogens, such as N-hydroxy-2-naphthylamine and ferric nitrilotriacetate (Dizdaroglu, 1992; Breen and Murphy, 1995). In co-culturing experiments, stimulated macrophages induced the formation of ring-saturated pyrimidines in the DNA of NIH3T3 cells (Lewis and Adams, 1985). In the presence of catalase, formation of ring-saturated pyrimidines was blocked, suggesting that reactive oxygen species released by the activated macrophages produced oxidative damage in the fibroblast DNA.

Biological Effects of Ring-Saturated Pyrimidines

While much information is available about the structure of oxidized pyrimidines, little is known about their effects on cellular processes and survival. Studies on the damaging potential of oxygen-free radicals revealed alterations at both thymines and cytosines. Using a variety of bacterial mutagenesis systems, it was showed that exposure of DNA to singlet oxygen (Decuyper-Debergh et al., 1987), iron (Feig and Loeb, 1993), or copper ions (Tkeshelashvili et al., 1991) resulted in the formation of mutations at sites

of pyrimidines. In all cases, more base substitutions occurred at sites of cytosines than thymines. Addition of free radical scavengers abolished the mutagenic effects. Passage of a hydrogen peroxide-damaged shuttle vector into mammalian cells also showed mutations at cytosines (Moraes et al., 1989). Similarly, the treatment of mammalian cells with hydrogen peroxide resulted in the production of alterations at pyrimidines and again altered cytosines predominated (Moraes et al., 1990). Reid and Loeb (1993) demonstrated that reactive oxygen species produce tandem double mutations at sites of cytosines. The precise identity of the mutagenic lesions in these studies remains speculative.

The biological effect of one ring-saturated pyrimidine, thymine glycol, was examined. Basu et al. (1989) introduced a single thymine glycol site-specifically into M13 phage DNA and transfected this into *E. coli* cells and allowed the cells to replicate. The results showed the formation of T to C mutations, indicating that thymine glycol in DNA is mutagenic (Basu et al., 1989). Thymine glycol also was shown to be a replicative block to *E. coli* DNA polymerase I in a variety of *in vitro* systems. Thymine glycol, produced in M13 phage DNA by osmium tetroxide, inhibited DNA synthesis by *E. coli* DNA polymerase I fragment, and sequencing analysis showed that DNA synthesis terminated opposite the putative thymine glycol lesion (Ide et al., 1985). Thymine glycol, produced in M13 phage DNA by potassium permanganate, was highly inhibitory to DNA elongation and DNA synthesis was stopped opposite thymine glycol residues (Rouet and Essigmann, 1985). Another *in vitro* study using single-stranded phage DNA containing thymine glycol was used as a template for DNA synthesis by *E.coli* DNA polymerase I with similar inhibitory results, indicating that DNA synthesis stops opposite the thymine glycol lesion

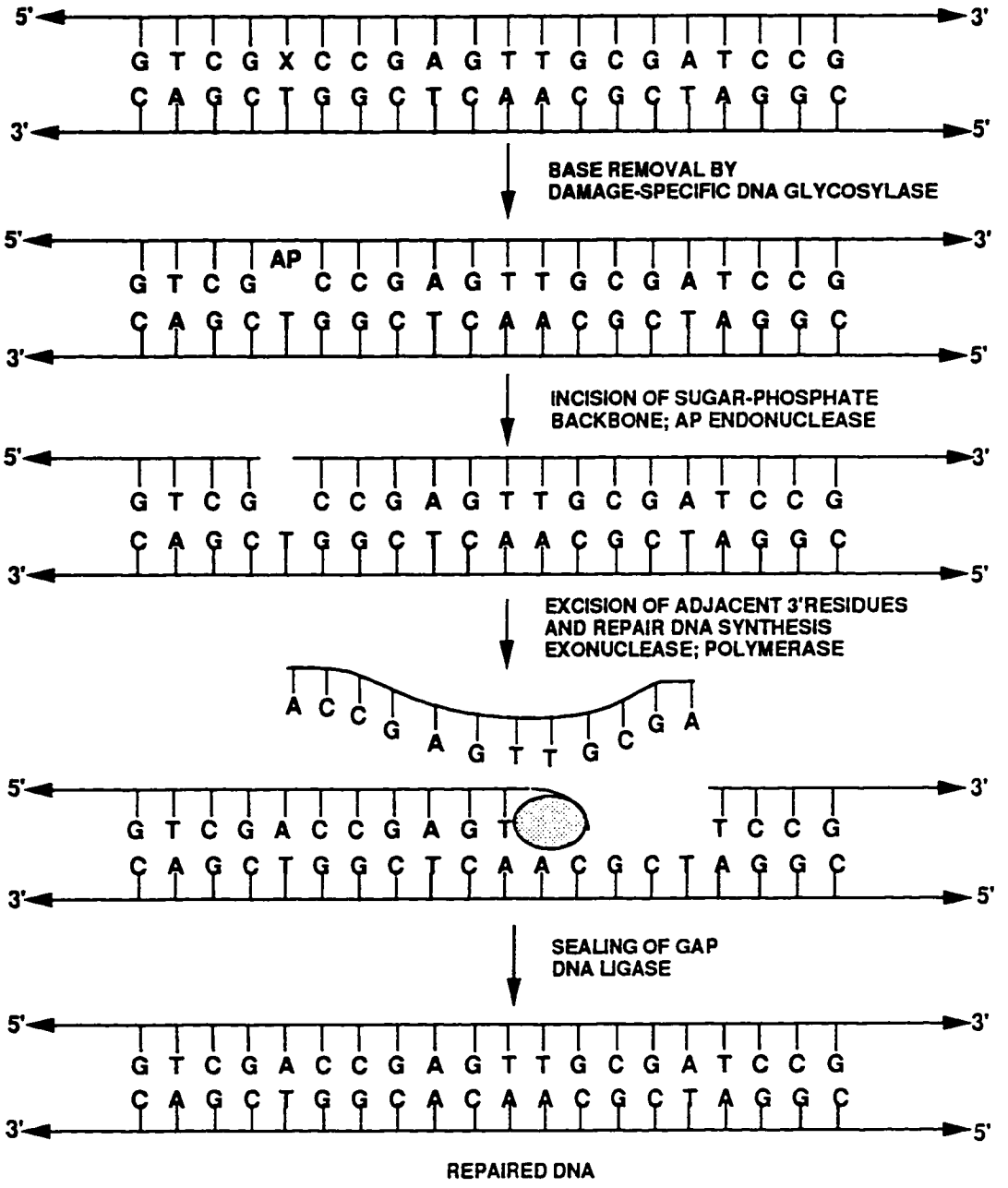
(Hays and LeClerc, 1986). Results from these studies suggest that DNA base adducts, such as thymine glycols, are potentially lethal to the cell if they are not repaired prior to DNA replication and transcription. Further, the presence of thymine glycol in the coding sequences of transcribed genes may block the action of RNA polymerases and therefore, inhibit protein synthesis. Thus, it appears likely that ring-saturated pyrimidines may play an important role in the cytotoxicity and cell lethality associated with exposure to reactive oxygen species.

Repair of Oxidative DNA Damage

Experiments with bacterial and mammalian cells showed that ring-saturated pyrimidines were actively removed from damaged DNA via the base excision repair pathway (Friedberg, 1995; Seeberg et al., 1995). Cells must maintain the integrity of their genetic information to insure proper functioning. Since a single change in DNA structure may be mutagenic, carcinogenic, or even lethal, it is not surprising that a diverse array of mechanisms for repairing DNA evolved at the cellular level (Friedberg, 1995; Seeberg et al., 1995). Such repair mechanisms involve a series of biochemical reactions which lead to the elimination of the damage and restoration of the intact DNA structure, thereby reducing the possibility of mutation, transformation or cell death.

Base excision repair, involves the enzymatic removal of damaged DNA adducts and their replacement with new monomeric units, using the opposite strand as a template (Friedberg, 1995; Seeberg et al., 1995). In this process, a base defect or abnormal base in DNA is recognized by a damage-specific DNA glycosylase which cleaves the N-glycosylic bond between the damaged base and the sugar-phosphate backbone (Figure 3). The

Figure 3. Schematic representation of base excision repair.



hydrolysis of the glycosylic bond results in the release of a free base and produces an apurinic or apyrimidinic (AP) site. AP endonucleases subsequently incise the DNA at the base-loss sites in the duplex DNA, hydrolyzing the phosphodiester bonds immediate to the 5' or 3' of each AP site to produce nicks in the DNA backbone. Another class of enzymes, exonucleases which are not repair specific enzymes, excise the nicked site along with adjacent nucleotides, resulting in an oligonucleotide gap. This gap is filled by the action of a DNA polymerase using the intact opposite strand as a template. Finally, DNA ligase joins the 3' end of the newly synthesized segment with the 5' end of the original strand. The DNA glycosylases are damage-specific, while the other enzymes in the base excision repair pathway are common to all types of damage. The known DNA glycosylases are divided into two groups (Table 2). Type I consists of those enzymes that were purified free from contaminating nuclease activity. The type II enzymes have an associated AP endonuclease activity that is an integral part of the enzyme.

Base excision repair maintains the genetic integrity of the cellular DNA by eliminating the damage and restoring the intact DNA structure. These processes are possible only in duplex regions of the DNA, since the complementary strand provides a template for retrieval of information lost during excision of the lesion (Friedberg, 1995; Seeberg et al., 1995). This process obviously cannot operate on single-stranded regions of the DNA, such as those found at the replication fork, where the parental strands have separated.

Table 2. DNA Glycosylases

DNA DAMAGE	ENZYME
	Type I DNA Glycosylases
uracil	uracil-DNA glycosylase
hypoxanthine	hypoxanthine-DNA glycosylase
3-methyladenine 7-methylguanine 3-methylguanine	3-methyladenine-DNA glycosylase
hydroxymethylthymine	hydroxymethylthymine-DNA glycosylase
hydroxymethylcytosine	hydroxymethylcytosine-DNA glycosylase
hydroxymethyluracil	hydroxymethyluracil-DNA glycosylase
	Type II DNA Glycosylases
ring-saturated pyrimidines	endonuclease III redoxyendonuclease
formamidopyrimidine 8-oxoguanine	8-oxoguanine-DNA glycosylase
pyrimdine dimers	pyrimdine dimer-DNA glycosylase

Endonuclease III and Redoxyendonucleases

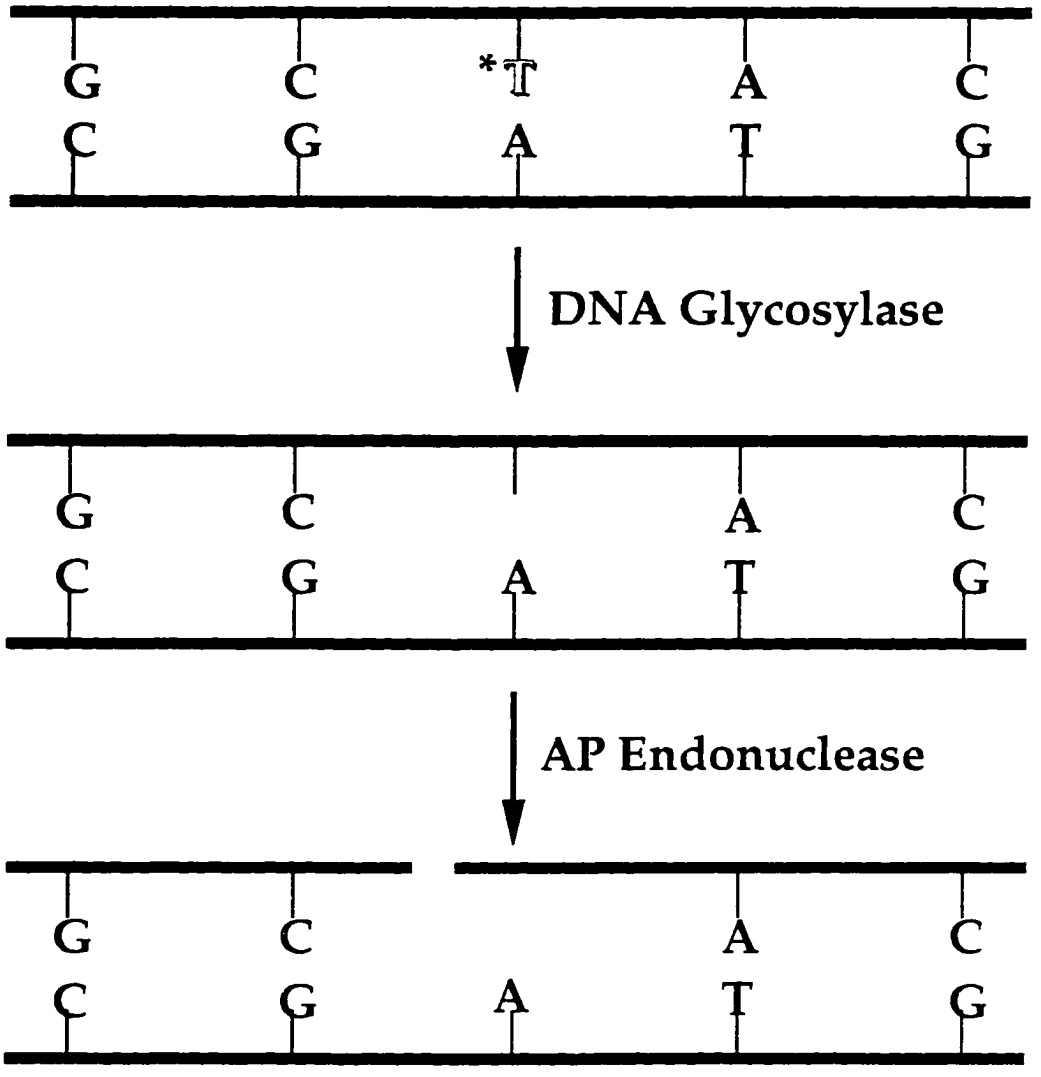
In bacteria, endonuclease III (nth) initiates the excision of ring-saturated pyrimidines from damaged DNA (Sancar and Sancar, 1988; Friedberg, 1995; Seeberg et al., 1995). Endonuclease III was first reported as an endonucleolytic activity present in crude extracts of *E. coli* which was directed against UV-irradiated DNA (Braun and Grossman, 1974). This protein was purified in 1976 by Radman, who demonstrated enzyme-induced single-strand breaks in the UV-irradiated substrate; however, the enzyme did not recognize pyrimidine dimers. While it was originally thought that thymine glycol was the lesion recognized in UV-irradiated DNA by endonuclease III, enzymatic release of this base from a UV-irradiated substrate was not demonstrated (Gates and Linn, 1977). Both Gates and Linn (1977), and Katcher and Wallace (1983), demonstrated the incision of X-irradiated or OsO_4-treated DNA following incubation with endonuclease III. The latter group also showed that the enzyme incised the DNA substrate at more sites than were accounted for by the release of thymine glycol, the major lesion in oxidized DNA (Katcher and Wallace, 1983). This suggested that endonuclease III was recognizing DNA base damages in addition to thymine glycol. The presence of a DNA glycosylase in *E. coli* which releases free urea from a potassium permanganate-oxidized DNA substrate was also demonstrated (Breimer and Lindahl, 1980). This activity was later determined to be endonuclease III (Breimer and Lindahl, 1984). In addition, several studies revealed that endonuclease III recognized and released damaged cytosines from UV-irradiated substrates (Weiss and Duker, 1986; 1987; Weiss et al., 1989). Breimer and Lindahl (1984) were the first to suggest that endonuclease III recognizes a common structural feature of damaged pyrimidines, such as the absence of the 5,6-double bond or the

nonplanar structure of the base derivatives which have lost this bond. Examination of the mechanism of action revealed that this protein has two associated enzymatic activities, a DNA glycosylase that releases ring-saturated pyrimidines from damaged DNA and an AP endonuclease activity (Figure 4), and therefore is classified as a type II DNA glycosylase (Kow and Wallace, 1987).

The gene encoding *E.coli* endonuclease III was cloned and the protein purified to apparent homogeneity from a bacterial clone which over-expresses the protein. Characterization of the enzyme by X-ray crystallography, elemental analysis, and electron paramagnetic resonance studies show that endonuclease III is an iron-sulfur protein containing four Fe-4S clusters with a helix-hairpin-helix structure (Cunningham et al., 1989; Kuo et al., 1992). These authors suggested that endonuclease III is the prototype of a new class of iron-sulfur proteins. The primary role of the iron-sulfur cluster is to position basic residues for interaction with the DNA phosphate backbone. The helix-hairpin-helix motif is also thought to be involved in DNA binding, since thymine glycols were found to interact specifically in this region (Kuo et al., 1992).

Enzymes, termed redoxyendonucleases, with similar catalytic properties as bacterial endonuclease III were found in a variety of other species including yeast (Gossett et al., 1988), Drosophila (Breimer, 1986), and cow (Doetsch et al., 1987). The human counterpart to bacterial endonuclease III was isolated from a number of human cell types, including lymphoblasts (Brent, 1979; Lee et al., 1987; Weiss et al., 1989) and HeLa cells (Higgins et al., 1987). While these proteins have not been characterized as extensively as the bacterial enzyme, analysis of enzyme-released reaction products showed that the mammalian redoxyendonucleases also act through glycosylic release of altered bases

Figure 4. Mechanism of action of the bacterial endonuclease III and mammalian redoxyendonuclease.



followed by incision of the DNA at the resultant AP sites (Doesch et al., 1987; Gallagher et al., 1989; Weiss et al., 1989). This indicates that the mammalian redoxyendonucleases have an identical substrate specificity and mechanism of action as bacterial endonuclease III.

DNA Repair and the Cell Cycle

Studies aimed at characterizing the activity of DNA glycosylases show that some of these enzymes are regulated during the cell cycle. Cells transverse an ordered series of steps to pass from one phase of the cell cycle to another. The cell cycle is subdivided into 4 phases: G1, when the cell prepares to synthesize DNA; S, during which DNA synthesis takes place; G2, in which preparations for cell division are made; and M, when mitosis occurs. Cells that are not actively dividing may be either terminally differentiated in G0, and therefore unable to enter the cell cycle, or in a state of temporary arrest (Hartwell et al., 1994; Evan et al., 1995; Kamb, 1995; Leake, 1996). In addition to G0 arrest, cells may be arrested at G1 or G2 checkpoints.

Checkpoints ensure the orderly progression of cell cycle events through the co-ordination of cellular signals. Disruption of these processes leads to the formation of mutations and chromosomal aberrations (Hartwell et al., 1994; Kaufmann, 1995; Liu et al., 1995; Murname, 1995; Tlsty, 1995; Kaufmann and Paules, 1996). Alterations in cell cycle control can occur when cells are treated with DNA damaging agents. DNA damage induced by a variety of damaging agents triggers delays in cell cycle progression at three points in the cell cycle. The delays involve progression from G1 into S phase, within S phase, and G2 into M phase, thereby increasing time for DNA repair pathways to remove

the lesions before cells traverse to the next phase. If these delay responses fail, damaged DNA is replicated before repair occurs, giving rise to additional genetic alterations.

Gombar et al. (1981) first reported that both the uracil- and 3-methyladenine-DNA glycosylase activities were modulated during cell proliferation. The specific activities of both enzymes were increased in rat liver tissue extracts following partial hepatectomy, reaching maximal levels of 2-3 fold above basal amounts 18-24 hours later. These results were extended for the uracil-DNA glycosylase in a series of cell culture experiments. In several different fibroblasts and lymphoblast cell lines, analysis showed that the uracil DNA glycosylase activity increased just prior to the peak of DNA strand replication with a subsequent decrease in activity when DNA synthesis was maximal (Sirover, 1979; Gupta and Sirover, 1980; 1981; Duker and Grant, 1980; Gombar et al., 1981; Slupphaug et al., 1991). Basal levels of enzyme activity was observed in quiescent cells. A similar increase in uracil-DNA glycosylase mRNA was observed in extracts prepared from both fibroblasts and lymphoblasts prior to entering S phase, thus, confirming the results obtained by enzyme analysis. Transcription of this gene was reduced to basal levels during the peak of S phase. In addition, uracil DNA glycosylase mRNA levels increased 2-3 fold during the G1 phase of human lung fibroblast cell and HeLa cell cycle (Muller and Cardonna, 1993). Taken together, these data suggest that the cell cycle regulation of uracil-DNA glycosylase occurs in a biphasic pattern with increases in both G1 and early S phases followed by a reduction in mid to late S. Cells which are no longer actively dividing have a reduced capacity to repair damaged DNA, as compared to cycling cells in G1 and early S phases.

SPECIFIC AIMS OF THIS RESEARCH

Although previous studies demonstrated the cell cycle regulation of uracil-DNA glycosylase, there is no information available about the regulation of enzymes that initiate the repair of oxidative DNA damage. Moreover, there are no data comparing the rate of repair of ring-saturated pyrimidines in the DNA of actively dividing and quiescent cells following oxidant challenge. Thus, the aims of this study are 3-fold:

1. to examine the levels of redoxyendonuclease in extracts of actively dividing and quiescent human neuroblastoma cells;
2. to determine whether redoxyendonuclease activity is regulated with the cell cycle; and
3. to assess the capacity of neuroblastoma cells to repair ring-saturated pyrimidines induced during different stages of the cell cycle.

The results of these studies provide information about the ability of cycling and non-cycling cells to repair oxidative DNA damage.

Neuroblastoma cells were used in this project to investigate the DNA repair capability of dividing and quiescent cells of neuronal origin. The development of the nervous system involves a balance between neuronal cell proliferation, differentiation into non-mitotic mature neurons, and the death of a substantial number of neuronal cells (Budhram-Mahadeo, et al, 1992). There is a high risk period in early development since a large proportion of cells are not differentiated to the point that proliferative capacity is lost (Mott, 1995). During the proliferative period, mutations in any cell cycle control gene can lead to subsequent mutations, initiating tumor formation. Thus, the balance between cell

proliferation and neuronal differentiation is important to the etiology of neuronal tumors, such as neuroblastomas (LoPresti et al., 1992).

Neuroblastoma is one of the most common tumors of infants and is derived from sympathetic neuroblasts (Humpl, 1995). The location and growth of tumors varies. The spontaneous regression of benign tumors is observed, as wells as malignant development with resistance to any therapy (Humpl, 1995). Although neurons are typically non-dividing, malignant transformation in the initiation of neuroblastomas confers unlimited growth potential on these cells. Since chemotherapeutic agents act by damaging DNA and interfering with cell division, it is important to understand the regulation of these processes in neuroblastoma cells.

Neurons are metabolically active cells that are critical for the functioning of the organism (Bondy, 1995). Mature neurons are terminally differentiated to a replicatively quiescent state and thus, the organism cannot replace dead or damaged neuronal cells. Oxidative DNA damage to neuronal cells is thought to play an important role in a variety of neurodegenerative diseases, as well as in aging (Bondy, 1995; Verity, 1994; Ratan and Baraban, 1995). The use of neuroblastoma cells facilitates a study of the DNA repair capacity of neuronal cells that are metabolically active but typically quiescent. Since the DNA of these cells is constantly exposed to oxygen free radicals generated as by-products of cellular metabolism, it is important to understand the ability of these vital cells to withstand the oxidant challenge.

Neuronal cells also can be extensively damaged during stroke, as a result of oxygen depletion. Stroke is characterized by a number of neurological events that are attributed to ischemia, an interruption in blood flow to the brain (Choi, 1990; Chan, 1996;

Small and Buchan, 1996). Because brain tissue has a high rate of oxidative metabolism with no nutrient reserves in its cells, a continuous supply of oxygen must be available for the neuronal cells to function (Hawkins, 1985; Choi, 1990; Chan, 1996; Small and Buchan, 1996). If reoxygenation of ischemic tissue does not occur quickly, neuronal damage results, leading to brain damage or death of the patient (Krause et al., 1988).

When oxygen is reintroduced to the ischemic tissue, there is an increased production of oxygen free radicals by the xanthine oxidase and cyclo-oxygenase pathways which metabolize hypoxanthine and arachidonate, respectively, resulting in the burst of superoxide as a by product (Bakhle, 1983; McCord, 1985). Brain tissue is particularly sensitive to oxygen free radical damage because of the high levels of polyunsaturated lipids in neuronal cell membranes (Floyd, 1990). Thus, in addition to superoxides, reoxygenation also causes the generation of lipid hydroperoxides, another source of intracellular free radicals, through lipid peroxidation (Krause et al., 1988). While it was once believed that the injury to the brain due to ischemia is caused by both the lack of oxygenation and the subsequent reoxygenation of the brain tissue, evidence now indicates that the injury occurs almost exclusively during the reoxygenation phase due to oxygen free radical-mediated oxidative events (Floyd, 1990; Chan, 1996; Small and Buchan, 1996). Once these free radicals are formed they can target any molecule in a cell and exert their biological effects (Werns and Lucchesi, 1990). Thus, the generation of free radicals within neuronal cells and subsequent oxidative DNA damage may be important in the pathology associated with stroke.

MATERIALS AND METHODS

Preparation of Radiolabeled Polynucleotide

Radioactive polynucleotide, poly(dA)-poly(dT), was prepared by the nick translation procedure (Maniatis et al., 1982). Twenty μCi of [^{3}H]-TTP (ICN Radiochemicals, Specific activity = 56 Ci/mM) was dried in a microfuge tube under vacuum for 15 min and the following components were added: 2.5 mg poly(dA)-poly(dT) (Sigma), 10 μM dATP (Promega), 20 units of DNA polymerase I (Promega), 5.0×10^{-3} μg of DNase I (Sigma), nick translation buffer {0.5M Tris-HCl (pH 7.5), 0.1M $MgSO_4$, 1.0 mM dithiothrietol (DTT), 500 μg/mL BSA} in a total volume of 100 μL.

The reaction mixture was incubated for 4 hours at 16°C after which the radiolabeled polydeoxyribonucleotide was separated from unincorporated radionucleotides by gel filtration chromatography. The mixture was loaded onto a Sephadex G-50 gel filtration column (10 cm x 1 cm) and the polynucleotide was eluted with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Ten fractions (5 drops/fraction) were collected and 2 μL aliquots of each fraction were counted by liquid scintillation spectroscopy in Scintiverse E (Fisher Scientific). The fractions containing the first peak of radioactivity were pooled as radiolabeled poly(dA)-poly(dT).

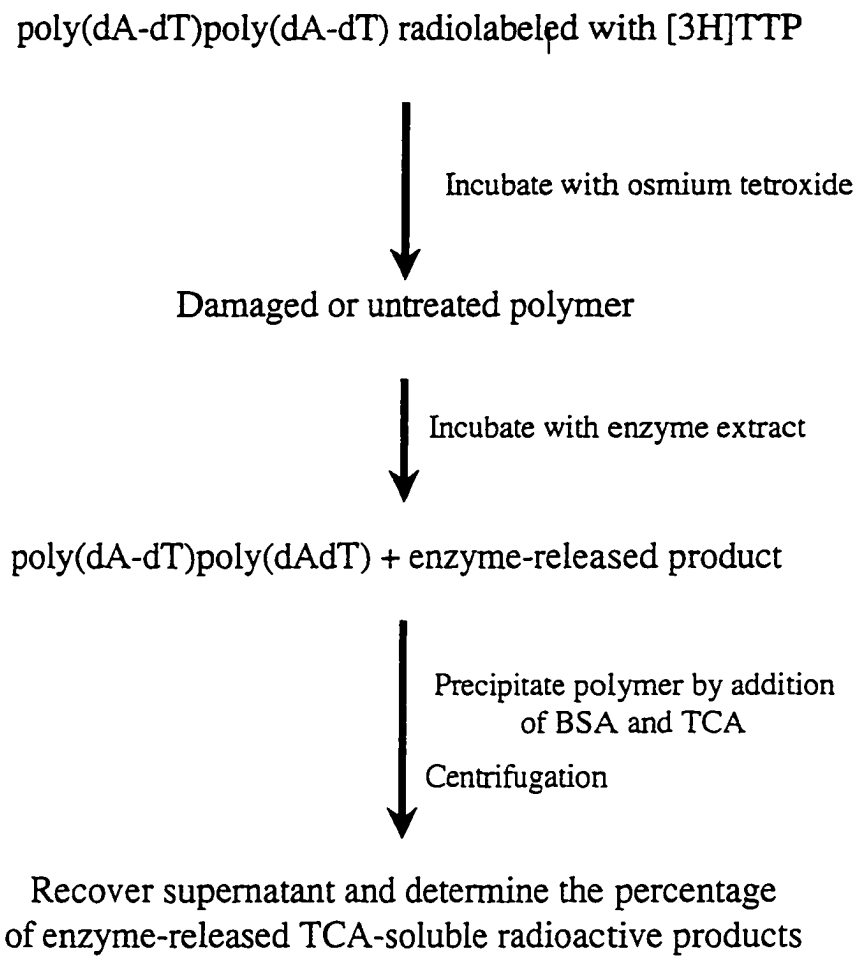
Preparation of Oxidized Substrate

Oxidized, synthetic polynucleotide, poly(dA)-poly(dT), radiolabeled with [^{3}H]-dTTP was used as a substrate to detect the neuroblastoma redoxyendonuclease activity. The polymer was heated for 2 min. at 82°C to separate the strands; osmium tetroxide (Sigma Chemical Co.) was added to a final concentration of 0.25% and the polynucleotide was incubated at 82°C for 15 min. After the oxidation reaction, osmium tetroxide was removed by ether extraction. Two volumes of anhydrous ethyl ether was added to the oxidized polymer, the sample was mixed, and subjected to centrifugation at 13,500 rpm for 5 min. The upper ether layer was removed and the ether extraction step was repeated twice. Following ether extraction, one-tenth volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of ice-cold reagent alcohol was added and the sample was incubated at -80°C for 15 min in an isopropanol bath. The precipitated polymer was isolated by centrifugation at 13,500 rpm for 15 min. The resultant pellet was rinsed twice with 70% cold ethanol and dried under vacuum. The oxidized polymer was resuspended in endonuclease III/redoxyendonuclease reaction buffer (40 mM KH_2PO_4, pH 7.5, 1 mM EDTA).

Assay for Redoxyendonuclease Activity

Endonuclease III or redoxyendonuclease activities were determined using a base release assay (Figure 5). Oxidized and untreated polymer were incubated with protein extracts in reaction buffer at 37°C for 2 hours. The polynucleotide was precipitated by

Figure 5. Outline of the base release assay.



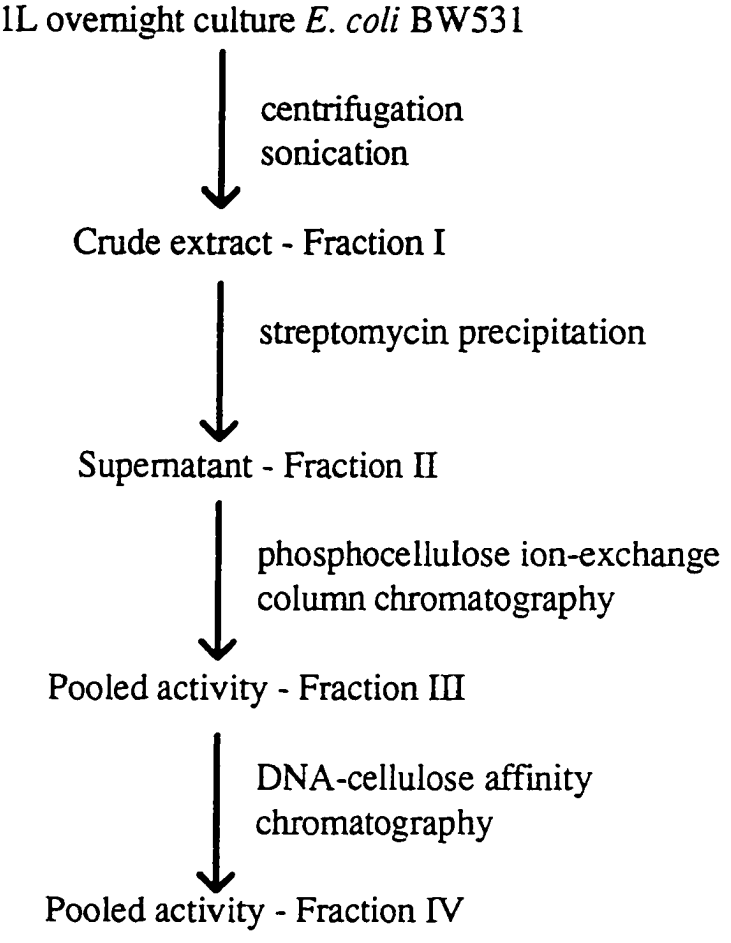
addition of BSA and TCA to final concentrations of 0.5% and 10%, respectively. The precipitates were incubated at 0°C for 15 min. and subjected to centrifugation at 13,500 rpm for 5 min. The radiolabeled, enzyme-released bases were determined by liquid scintillation spectroscopy in Scintiverse E.

The bases cleaved by endonuclease III or redoxyendonuclease were identified by high pressure liquid chromatography (HPLC) analysis. Following the enzyme reaction, the polymer was precipitated by the addition of 5 µL of 2.0 M sodium acetate, 100 µL of denatured calf thymus DNA (2.0 mg/mL) and 350 µL of absolute ethanol at -20°C. The supernatant was recovered by centrifugation and evaporated to dryness. The residue was dissolved in 100 µL of 2.5% methanol/2.5 mM potassium phosphate, pH 3.0 and a 90 µL aliquot was applied to a 25 cm reverse phase C18 silica column (Whatman Partisil 5-ODS). The column was eluted with 2.5% methanol/2.5 mM potassium phosphate, pH 3.0 at a flow rate of 2.0 mL/min. The radioactive content of fractions was determined by liquid scintillation spectrometry. Optical density of authentic marker compounds was monitored at 254 nm. This method was used to separate thymine glycol from thymine and thymine hydrate (Cunningham and Weiss, 1985).

Purification of *Escherichia coli* Endonuclease III

Endonuclease III was partially purified from *E. coli* strain BW 531 by the procedure of Weiss and Duker (1986) (Figure 6). This overproducing strain contains a plasmid encoding the bacterial gene for endonuclease III. Briefly, 1.0 L of bacterial

Figure 6. Overview of the purification scheme for the isolation of the bacterial endonuclease III.



culture was grown overnight at 37°C in an orbital shaking incubator. The bacteria were collected by centrifugation at 4°C and the resultant pellet was resuspended in 50 mM KH_2PO_4, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, and 10% glycerol (Buffer B). All subsequent steps were performed at 4°C. The mixture was sonicated to disrupt the bacterial membranes and the cellular debris was removed from the sonicate by centrifugation. The nucleic acids were precipitated from the supernatant by the addition of streptomycin sulfate to a final concentration of 2.5%. Following centrifugation, the supernatant was applied to a phosphocellulose ion-exchange column (2.5 x 30 cm) and the bound enzyme was eluted with a linear phosphate gradient (50 mM to 1.0 M) in Buffer B. Enzyme activity was detected using the base release assay, as described above. Endonuclease III-containing fractions were pooled, dialyzed against Buffer B supplemented with 50 mM NaCl and applied to a double-stranded DNA-Sepharose column (1.5 x 15 cm). Endonuclease III was eluted from the column resin with a linear NaCl gradient (50 mM to 1.0 M) in Buffer B. Enzyme fractions, as detected by the base release assay, were pooled, dialyzed against Buffer B containing 50% glycerol, and frozen at -80°C. Enzyme activity was retained for more than 1 year with less than 10% activity loss.

Protein Quantification

The concentration of protein in the cell extracts was determined by the method of Lowry (1951). Bovine serum albumin (BSA) at a concentration of 4 mg/mL in distilled water was used as the standard protein. BSA (100 µL), at concentrations from 0 to 500

μg, and cell extracts were treated with 100 μL of 2 N NaOH and the protein was hydrolyzed at 100°C for 10 min in a boiling water bath. The hydrozylates were cooled to room temperature and 1 mL of freshly mixed complex-forming reagent was added to each tube. Complex-forming reagent was prepared immediately before use by mixing the following 3 solutions: solution A (2% sodium carbonate in distilled water), solution B (1% copper sulfate in distilled water), and solution C (2% sodium potassium tartrate in distilled water) in the proportion 100:1:1, respectively. After incubation for 10 min at room temperature, the reactions were treated with 100 μL of 1 N Folin reagent, mixed, and incubated for 30 min at room temperature. The absorbance of each sample was determined at 750 nm and the protein content of the cell extracts was quantified based on a standard curve generated from the absorbance of the known BSA concentrations.

Growth of Human Neuroblastoma Cells

The human neuroblastoma cell line, SK-N-SH, was obtained from the American Type Culture Collection and maintained in a sterile, humidified atmosphere containing 5% CO_2 at 37°C. The cells were grown as monolayers in Eagle's Minimal Essential Medium (MEM; Sigma), supplemented with 10% bovine calf serum (HyClone labs. Inc.), 2 mM L-glutamine (Cellgro), 0.012 M sodium bicarbonate, and 10 μg/mL penicillin/streptomycin (Cellgro). Cells were grown in 75 cm^2 flasks (CoStar).

Preparation of Cycling and Non-cycling Cells

Cells grown to 50% confluence were washed 3 times with phosphate-buffered saline (PBS) and incubated in a 1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium (Sigma) with no serum (Figure 7). The cultures were maintained in 0% serum for 36 hrs to induce cell cycle arrest. Cells maintained in low serum containing media enter the G_0 phase of the cell cycle within a short time period. Half the cultures were incubated with 10% serum in MEM to initiate synchronous cell cycles, while the non-cycling cells were maintained in 0% serum media.

Assay for Cellular DNA Synthesis

DNA synthesis was determined according to Whitson and Itakura (1992). Cells were grown to 50% confluence in 100 mm tissue culture dishes and serum-arrested, as described above. Cycling and non-cycling cells were incubated with 1 µL [3H]-thymidine (Dupont-New England Nuclear, specific activity = 0.15uCi/mL) /culture dish starting at time 0 upon addition of media supplemented with 10% serum to half the cultures (Figure 8). After 1 hour, the medium was removed, the cells were washed 3 times with PBS, incubated with 0.5 mL of solubilizing buffer (20 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100) and the culture plates were shaken for 20 min. at room temperature. The cells were recovered with a rubber scraper and the cell extracts were collected into glass test tubes. Ten percent trichloroacetic acid (TCA; 1.5 mL) was added to the extracts to precipitate the nucleic acids and the tubes were placed on ice for 20 min. The cell extract

Figure 7. Overview of the method to prepare cycing and noncycling human neuroblastoma cells.

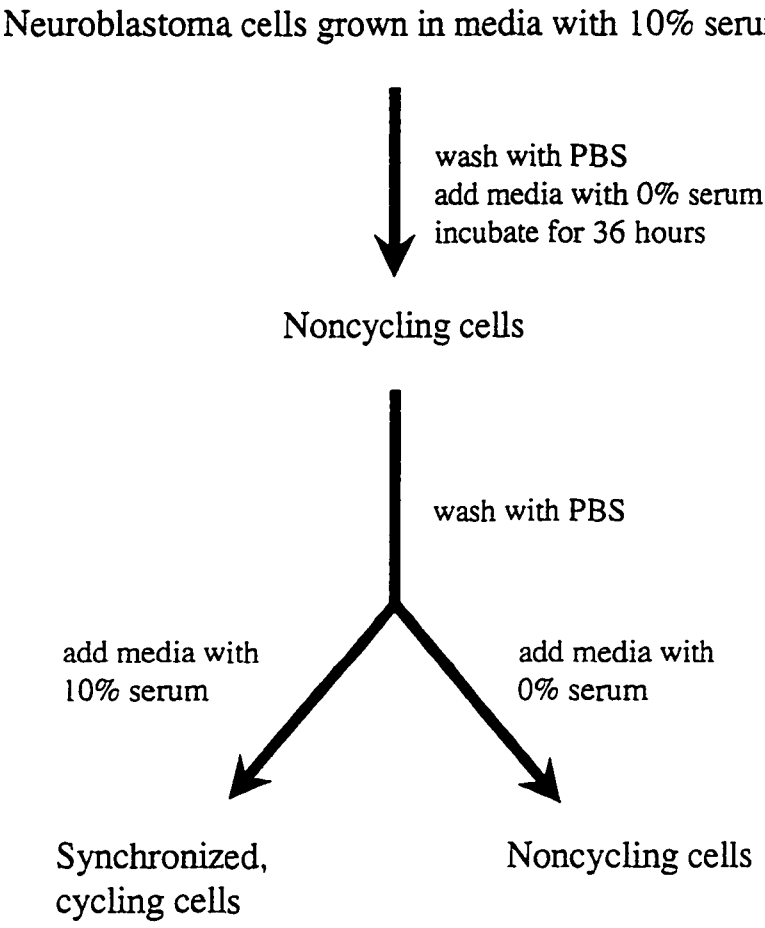
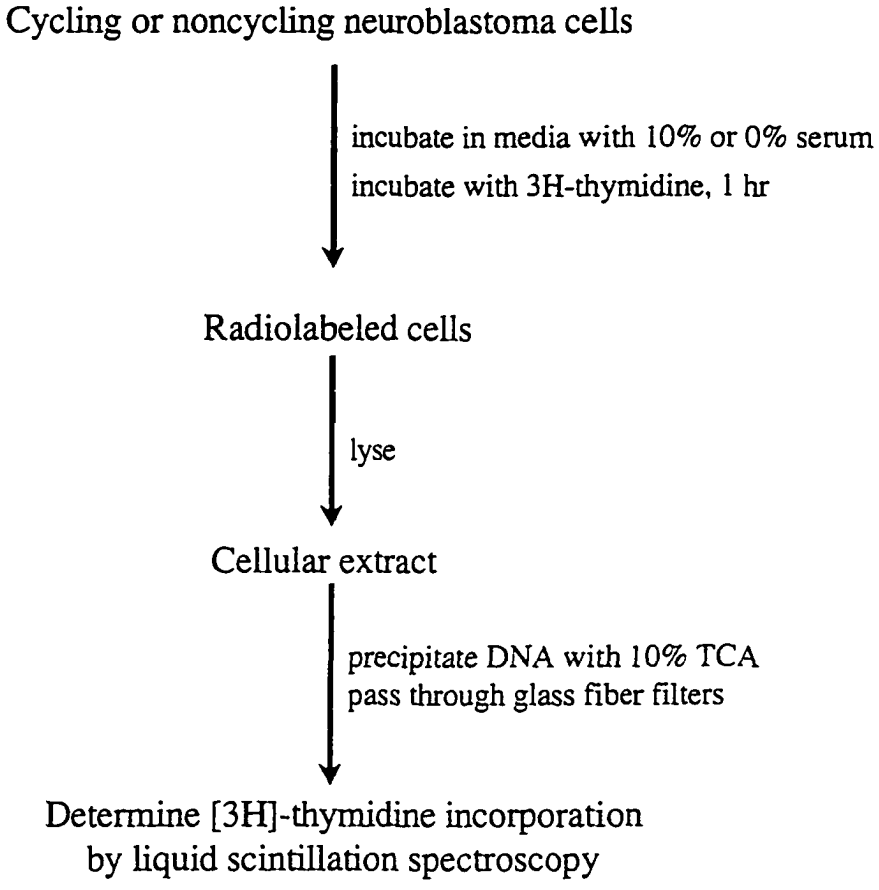


Figure 8. Outline of the DNA synthesis assay.



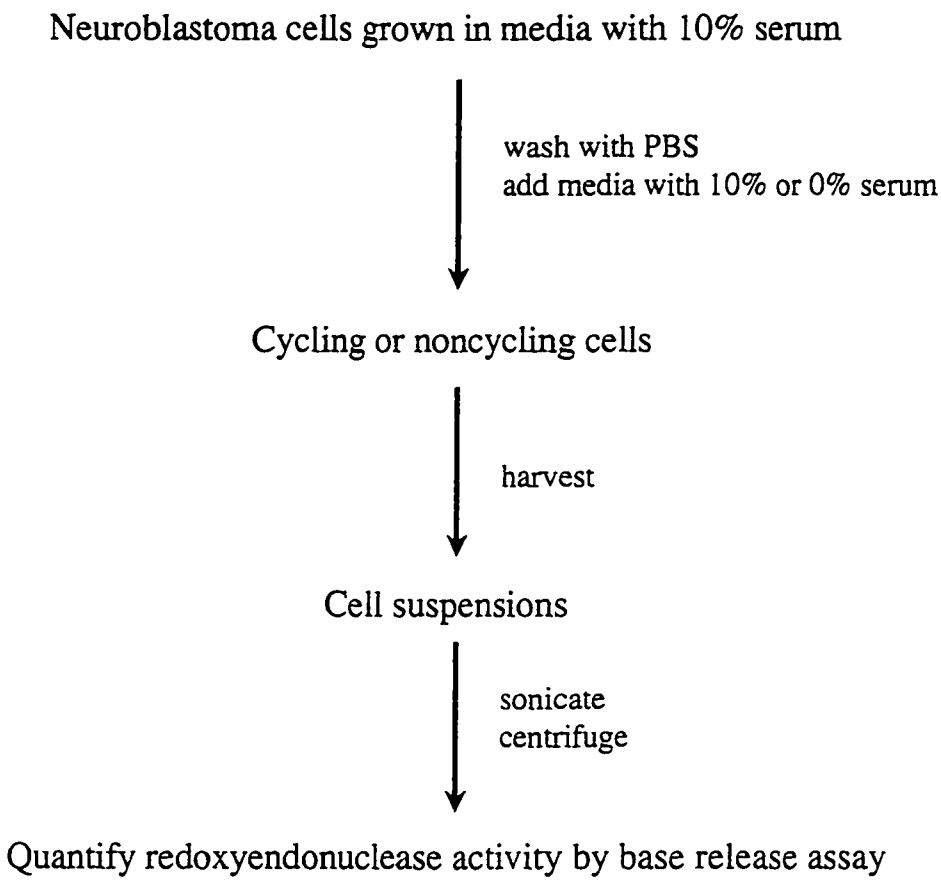
mixture was filtered through Whatman GF/C glass fiber filters to recover the precipitated material. The filters were washed twice with cold 10% TCA, dried in an oven for 20 min. and radioactive content was determined by liquid scintillation spectroscopy in POPOP.

Preparation of Cell Extracts

Monolayer cultures of cycling and non-cycling cells were washed once with PBS at the indicated time points and the cells were detached from the culture dishes by incubation with 1 x Trypsin (Sigma) for 1 min at room temperature (Figure 9). The Trypsin solution was removed and incubation of the cultures was continued at 37°C for 2 min. The detached cells were resuspended in cold PBS and cell pellets were collected by centrifugation at 1500 rpm for 10 min at 4°C. The supernatant was discarded and the cells were washed in cold PBS and cell pellets (approx. $3x10^6$ cells/pellet) collected by centrifugation. The supernatant was discarded and the cell pellets were quick-frozen in liquid N_2 and stored at -80°C until use.

Crude cell extracts were prepared by resuspending each cell pellet in 300 µL sonication buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM ß-mercaptoethanol, 10% glycerol) and disrupted by sonication twice for 5 seconds on ice using a microtip needle probe in an W-380 Sonicator. The cell lysate was subjected to centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 20 min at 4°C. The supernatant containing the enzymatic activity was quick-frozen in liquid N_2 and stored at -80°C until use in enzyme assays.

Figure 9. Overview of the procedures for the detection of the redoxyendonuclease activity in human neuroblastoma cells.



Treatment of Cells for *In Vivo* DNA Damage

Cells were grown to 50% confluence and radiolabeled with 2 μL of [3H]-thymidine per culture, to detect the formation of endonuclease III-sensitive sites to oxidative DNA damage. Cells were washed once with PBS and incubated with different concentrations of osmium tetroxide in PBS for 0.5 hr at 37°C. Cells were assayed for DNA synthesis as described to indicate cell survival.

After cells were damaged for 1 hr, osmium tetroxide was removed and cells were washed 3 times with PBS buffer. The cells were harvested using the Trypsin treatment described above and subjected to centrifugation. The resultant cell pellets were frozen at -80°C until use.

Isolation of Cellular DNA

Cell pellets were resuspended in 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 mM NaCl and 1% SDS) and the cell lysate was incubated with 1 mg/mL Proteinase K (Fisher Scientific) for 2 hr at 37°C. The DNA was extracted with an equal volume of buffer-saturated phenol:chloroform (1:1) and extracted twice more with chloroform. The aqueous and organic layers were separated by centrifugation for 10 min at 2,500 rpm. The clear, top aqueous layer was recovered and dialyzed against endonuclease III reaction buffer.

Alkaline Sucrose Density Gradient Centrifugation

Radiolabeled DNA samples (50,000 cpm per reaction) were incubated with or without endonuclease III purified from *E. coli* (Weiss and Duker, 1986) in a total volume of 100 µL for 2 hr at 37°C. The enzymatic reaction was terminated by incubating the samples an additional 15 min with 25 µl of Proteinase K (1 mg/mL) at 37°C. Reactions were made alkaline by adding 50 µL of 1 N NaOH to denature the DNA double strands and incubated for an additional 15 min at 37°C.

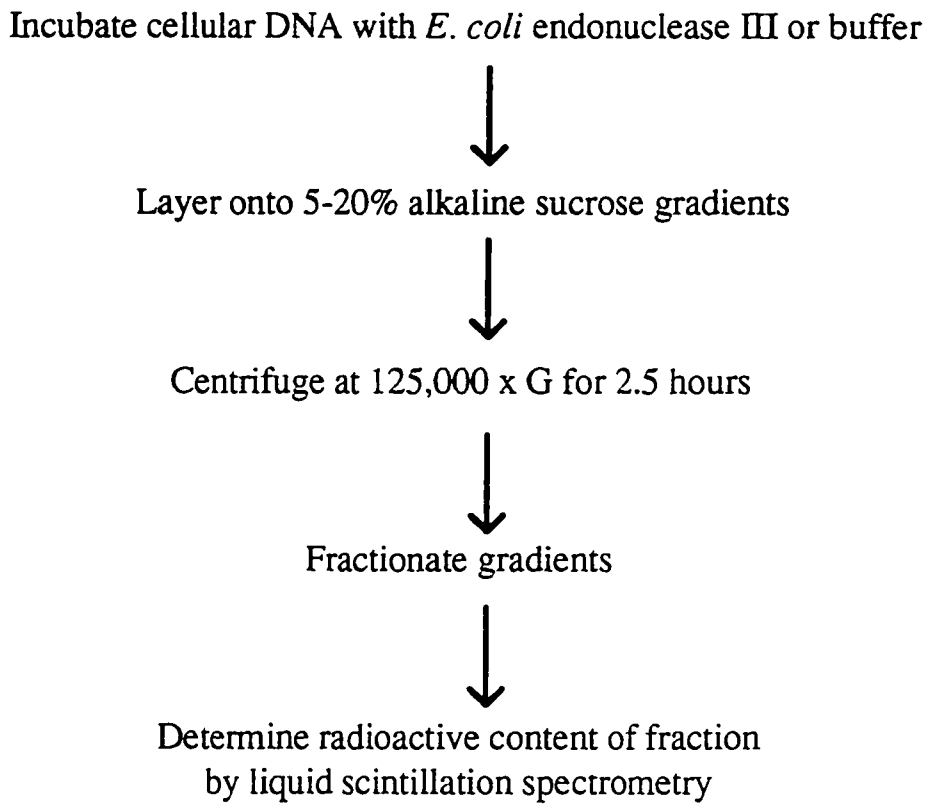
After incubation, each reaction mixture was layered onto a 15 mL 5-20% alkaline sucrose density gradient and subjected to centrifugation at 35,000 rpm in a Sorvall SW 50.1 rotor for 2.5 hrs at 20°C. The gradients were fractionated into 250 µL aliquots and 100 µL of 3 N HCl was added to each aliquot to neutralize the samples. The radioactivity in each aliquot was determined by liquid scintillation spectroscopy.

The weight-average molecular weight (MW) of the DNA in each gradient fraction was calculated as follows: $MW=\Sigma(Ci \times Mi)/\Sigma Ci$, where Ci and Mi are the cpm and molecular weight of the i th fraction, respectively. The number of strand breaks in 10^8 Daltons of DNA is equal to $(1/Mn \times 10^8)-1$, where Mn is one-half Mw (Brent, 1979). Only those fractions contributing to the major peak for each profile were included in the calculations.

In Vivo Repair of Ring-Saturated Pyrimidines

To determine the rate of repair of endonuclease III-sensitive sites, neuroblastoma cells grown to 50% confluence were damaged with $5x10^{-6}$ % osmium tetroxide in PBS for 0.5 hr at 37°C (Figure 10). After 0.5 hr, the damaging agent was removed and cells were washed 3 times with PBS and incubated in MEM with 10% serum at 37°C. Cells were harvested at indicated time points and the DNA was extracted, incubated with endonuclease III and the enzyme reaction products were separated by alkaline sucrose density gradients, as described above.

Figure 10. Outline of the procedure for the detection of cellular repair of ring-saturated pyrimidines in human neuroblastoma DNA.



RESULTS

Enzyme Purification

Endonuclease III, the bacterial enzyme that releases ring-saturated pyrimidines from damaged DNA, was purified from an *E.coli* strain BW531 to use as a molecular probe for the detection of oxidative base damages. The results of a typical endonuclease III purification are shown in Table 3. Bacterial cell walls were disrupted by sonication and following removal of the cellular debris by centrifugation, nucleic acids were removed from the extract by precipitation with streptomycin sulfate. The resulting material was applied to a phosphocellulose ion-exchange column. While endonuclease III bound to the ion-exchange resin, the majority of the cellular proteins passed through the column. Endonuclease III was eluted from the resin with a linear phosphate gradient (50 mM - 1M KH_2PO_4), with the majority of enzymatic activity eluting between approximately 200-400 mM phosphate (Figure 11). This step resulted in a 16.5-fold purification with an enzyme activity yield of approximately 68%. Fraction III extract was further purified by affinity chromatography using a DNA-cellulose resin. Endonuclease III bound to the DNA-cellulose and was eluted with a linear 0-1.0 M NaCl gradient (Figure 12). Fractions were collected and assayed against an OsO_4-treated poly(dA)-poly(dT) substrate. The fractions containing peak enzymatic activity, greater than 30% release of TCA-soluble radioactive material, were pooled. This step resulted in a 345-fold purification with about a 40% yield. Fraction IV extract was used for all subsequent studies.

Table 3. Purification of endonuclease III from Escherichia coli BW531.

Fraction	Volume (mL)	Units mL	Protein (mg/mL)	Units mg protein	Yield (%)	Purification (fold)
Crude extract (FI)	50	117	72.0	1.63	100.0	-
Streptomycin sulfate precipitation (FII)	82	56	27.0	2.07	78.4	1.27
Phosphocellulose column chromatography (FIII)	106	38	1.4	27.10	68.8	16.65
DNA cellulose column chromatography (FIV)	42	54	0.096	562.50	38.8	345.10

1 unit = 1.0% release of TCA-soluble radioactive material per minute incubation at 37°C.

Figure 11. Phosphocellulose column chromatography of Fraction II. OsO_4-treated (closed symbols) or untreated (open symbols) poly dA-dT, radiolabeled with [^{3}H]-TTP, was incubated with 20 μL enzyme extract from the column fractions for 30 minutes at 37°C. The molarity of KH_2PO_4, as determined by optical refractometry, is indicated by the broken lines.

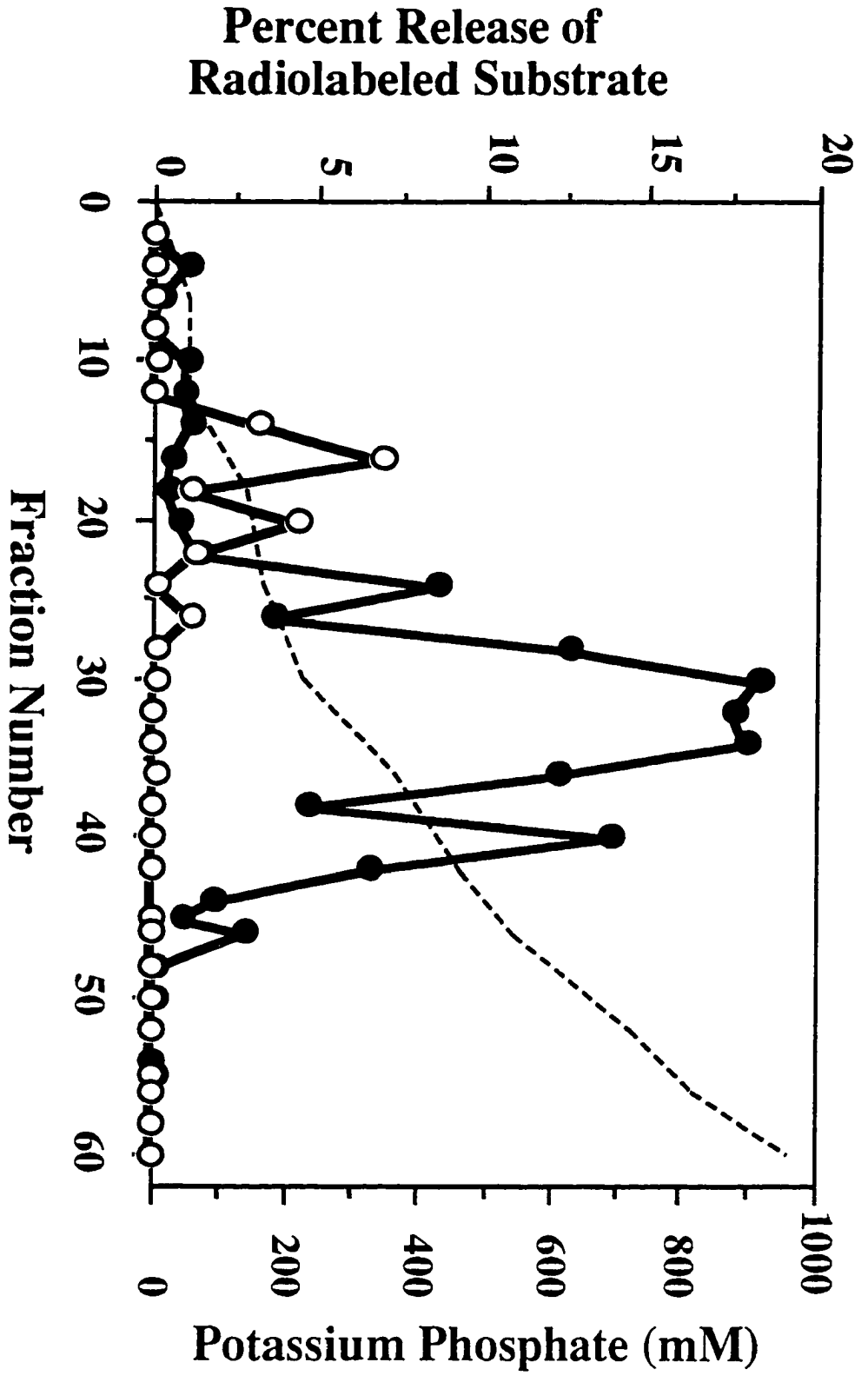


Figure 12. DNA-cellulose column chromatography of Fraction III. OsO_4-treated (closed symbols) or untreated (open symbols) poly dA-dT, radiolabeled with [^{3}H]-TTP, was incubated with 5 μL enzyme extract from the column fractions for 30 minutes at 37°C. The molarity of NaCl, as determined by optical refractometry, is indicated by the broken lines.

Characterization of Oxidized Base Release Assay

A base release assay using poly(dA)-poly(dT) radiolabeled with [^{3}H]-TTP was developed to detect redoxyendonuclease activity in extracts of human neuroblastoma cells. The polymer was damaged with OsO_4 which converts the radiolabeled thymines to thymine glycol (Burton and Reilly, 1966). The glycosylase activity of both the bacterial endonuclease III and mammalian redoxyendonucleases recognize and release thymine glycols from damaged DNA. Thus, endonuclease III and redoxyendonuclease activities were quantified by detection of TCA-soluble radioactive material following precipitation of the enzyme-treated polymer and calculated as percent base release.

Initially, the purified endonuclease III was used to establish the optimum conditions for oxidation of the substrate needed for redoxyendonuclease quantification. As shown in Figure 13, release of radiolabeled material was dose-dependent with increased oxidation of the substrate. At OsO_4 concentrations greater than 0.5%, complex formation between polymer molecules occurred, inhibiting endonuclease III activity. Studies showed that endonuclease III recognizes structural features common among the class of ring-saturated pyrimidines, such as loss of the 5,6 double bond or the non-planar structure of these lesions (Breimer and Lindahl, 1984). It is possible that the decrease in enzyme activity is due to alterations in the structure of the substrate, such that endonuclease III either fails to recognize the damaged bases or the enzyme no longer processes along the polymer.

Linear enzyme kinetics were observed with protein concentration to 25 μg (Figure 14) and time of incubation to 3 hours (Figure 15). With increased protein or incubation time, saturation kinetics were observed. There was minimal release of radioactive material

Figure 13. Oxidation dose for *in vitro* formation of thymine glycol in DNA. Poly (dA)-poly(dT) radiolabeled with [^{3}H]-TTP was treated with increasing concentrations of OsO_4 followed by incubation with 10 µL of endonuclease III for 1 hour at 37°C. The OsO_4-treated and untreated polymers are represented as closed and open symbols, respectively. The data points are the average of duplicates and are representative of three experiments.

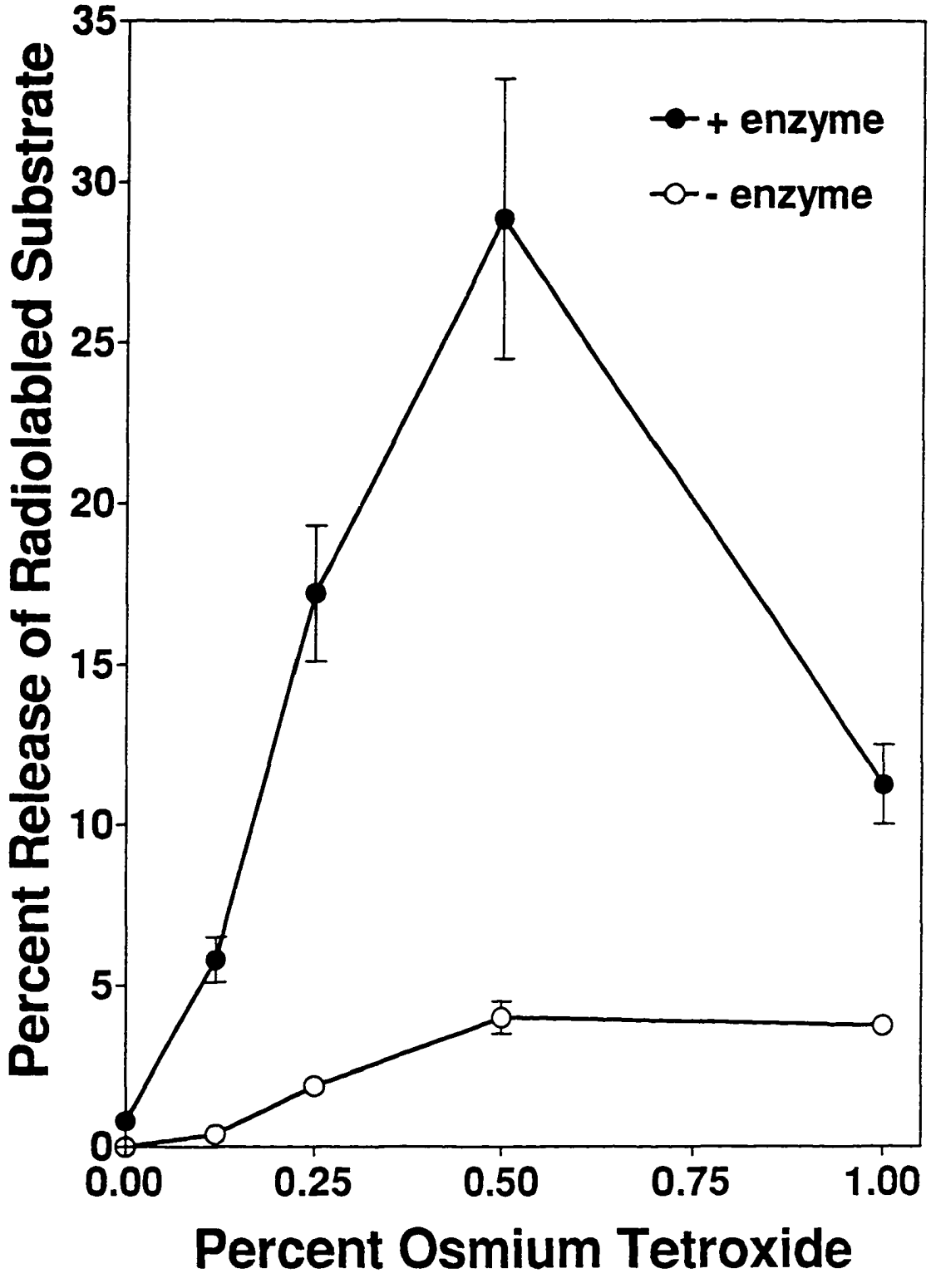


Figure 14. Enzyme concentration curve of endonuclease III. Radiolabeled OsO_4-treated (closed symbols) or untreated (open symbols) poly (dA)-poly (dT) was incubated with increasing concentrations of endonuclease III for 1 hour at 37°C. The data points are the average of duplicates and are representative of three experiments.

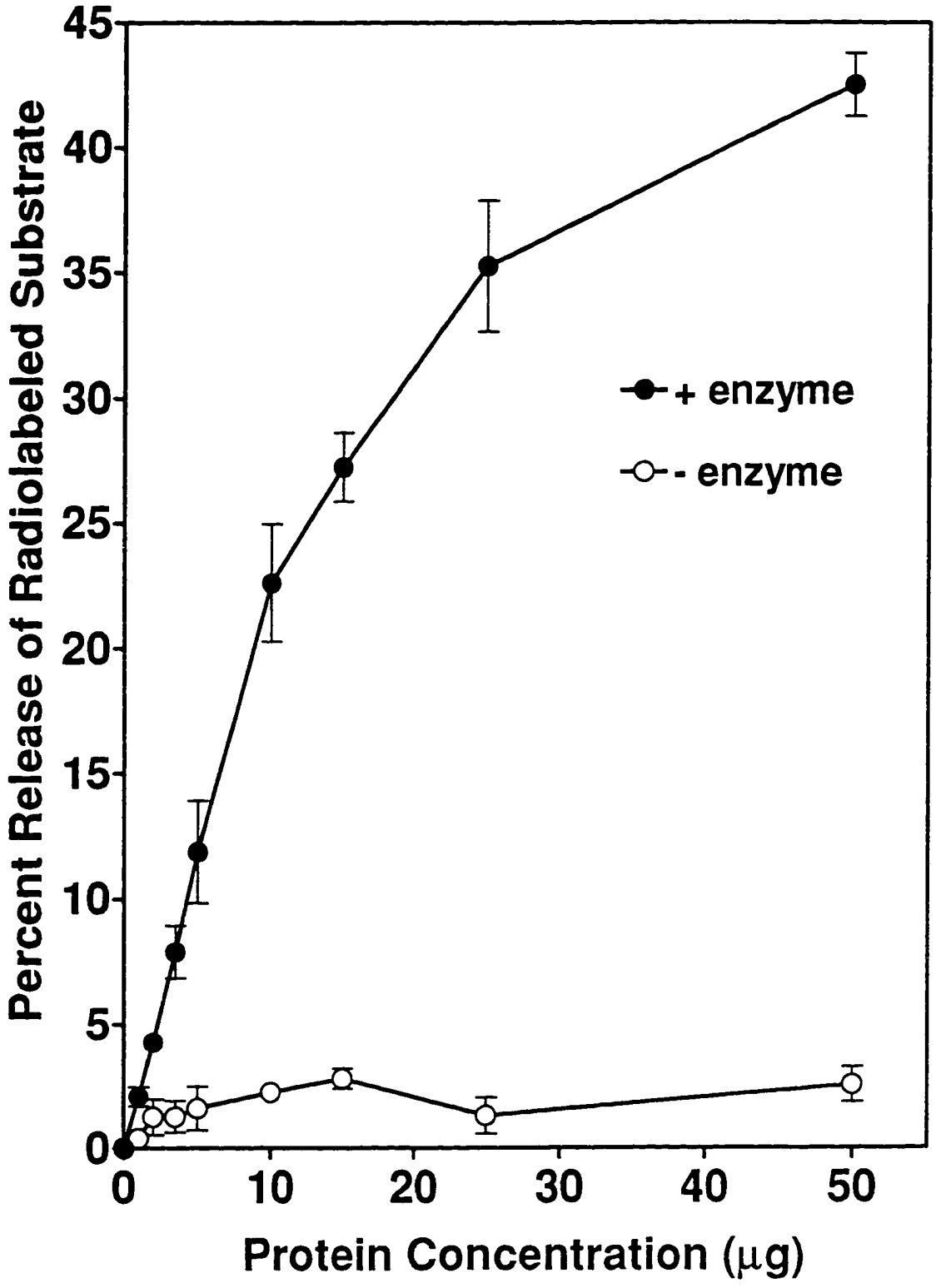
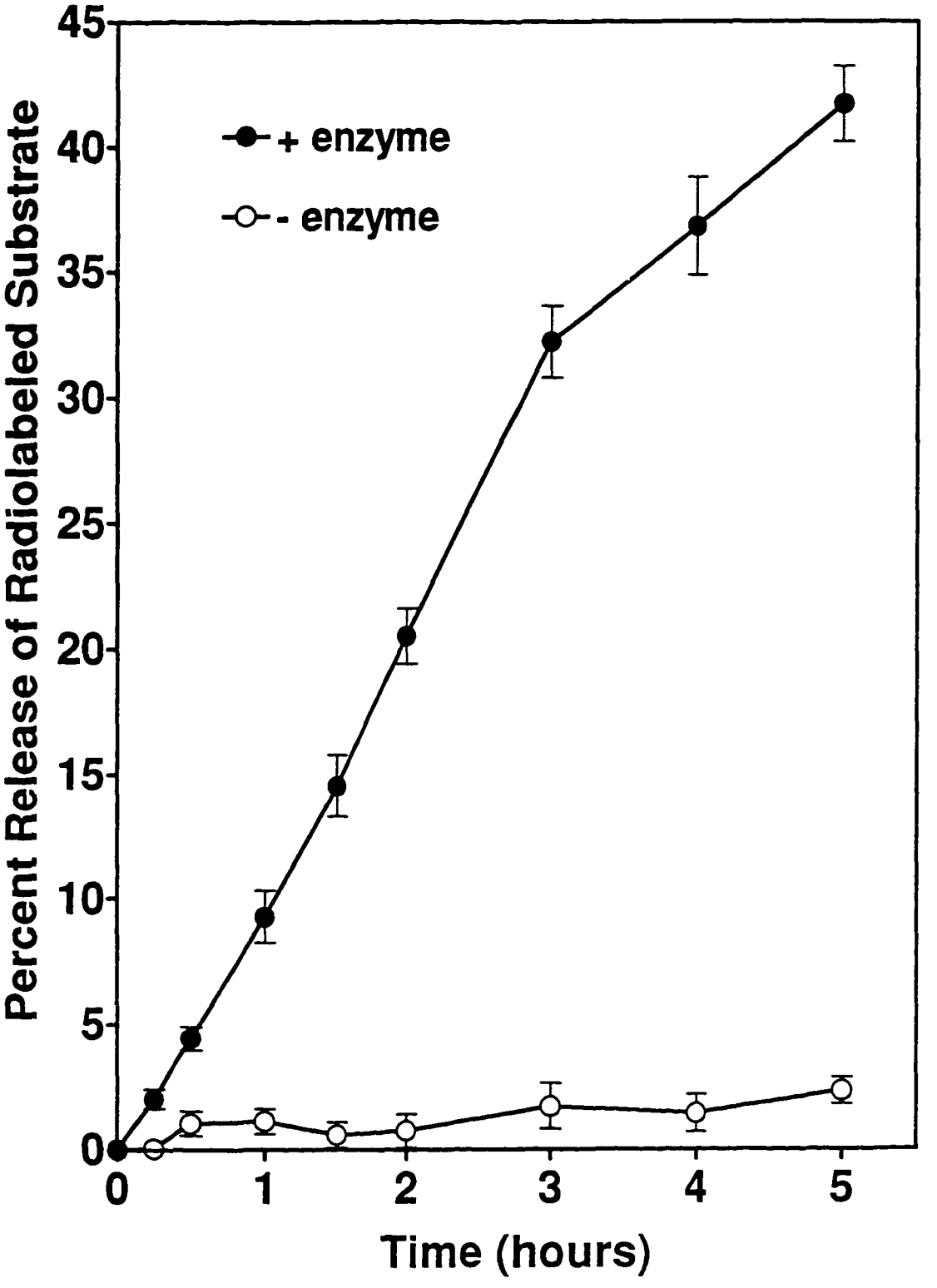


Figure 15. Incubation time course. OsO_4-treated (closed symbols) or untreated (open symbols) poly (dA)-poly (dT) radiolabeled with [^{3}H]-TTP was incubated with 10 µL of endonuclease III at 37°C for increasing time periods. The data points are the average of duplicates and are representative of three experiments.



using untreated polymer as a substrate, indicating that the enzyme specifically recognizes oxidized thymines.

Redoxyendonuclease Activity in Neuroblastoma Cells

The polynucleotide release assay was used to determine the level of redoxyendonuclease activity in dividing neuroblastoma cells. With increasing concentrations of neuroblastoma cell extracts, there was an increase in redoxyendonuclease activity, with 50 μg protein releasing approximately 3.5% of the total radioactive substrate (Figure 16). Cell extracts incubated with the untreated poly(dA)-poly(dT) showed minimal release of radioactive material, indicating that the enzymatic activity in the cell extract is specific for the oxidized substrate. As a positive control, purified endonuclease III was incubated with substrate, resulting in a release of 15% of the total radioactive material (data not shown). Thus, the substrate was not limiting in the redoxyendonuclease assays.

Redoxyendonuclease-released material was recovered and subjected to analysis by HPLC to confirm that the enzymatic activity quantified in the neuroblastoma extracts excised thymine glycol from the oxidized substrate. The retention time of the TCA-soluble material eluted from a reverse phase C-18 silica column was compared to that of authentic marker compounds. The predominant base released from the oxidized polymer by the neuroblastoma extract exhibited the same retention time as authentic thymine glycol (Figure 17). No appreciable release of undamaged thymine residues was observed. In addition, incubation of the oxidized polymer without the enzyme did not result in the excision of thymine glycol. These results indicate that enzymatic activity quantified by the

Figure 16. Enzyme concentration curve of human neuroblastoma cell extract. OsO_4-treated (closed symbols) or untreated (open symbols) poly(dA)-poly(dT) radiolabeled with [^{3}H]-TTP was incubated at 37°C for 2 hours with increasing concentrations of a protein extract prepared from cycling neuroblastoma cells. The data points are the average of duplicates and are representative of three experiments.

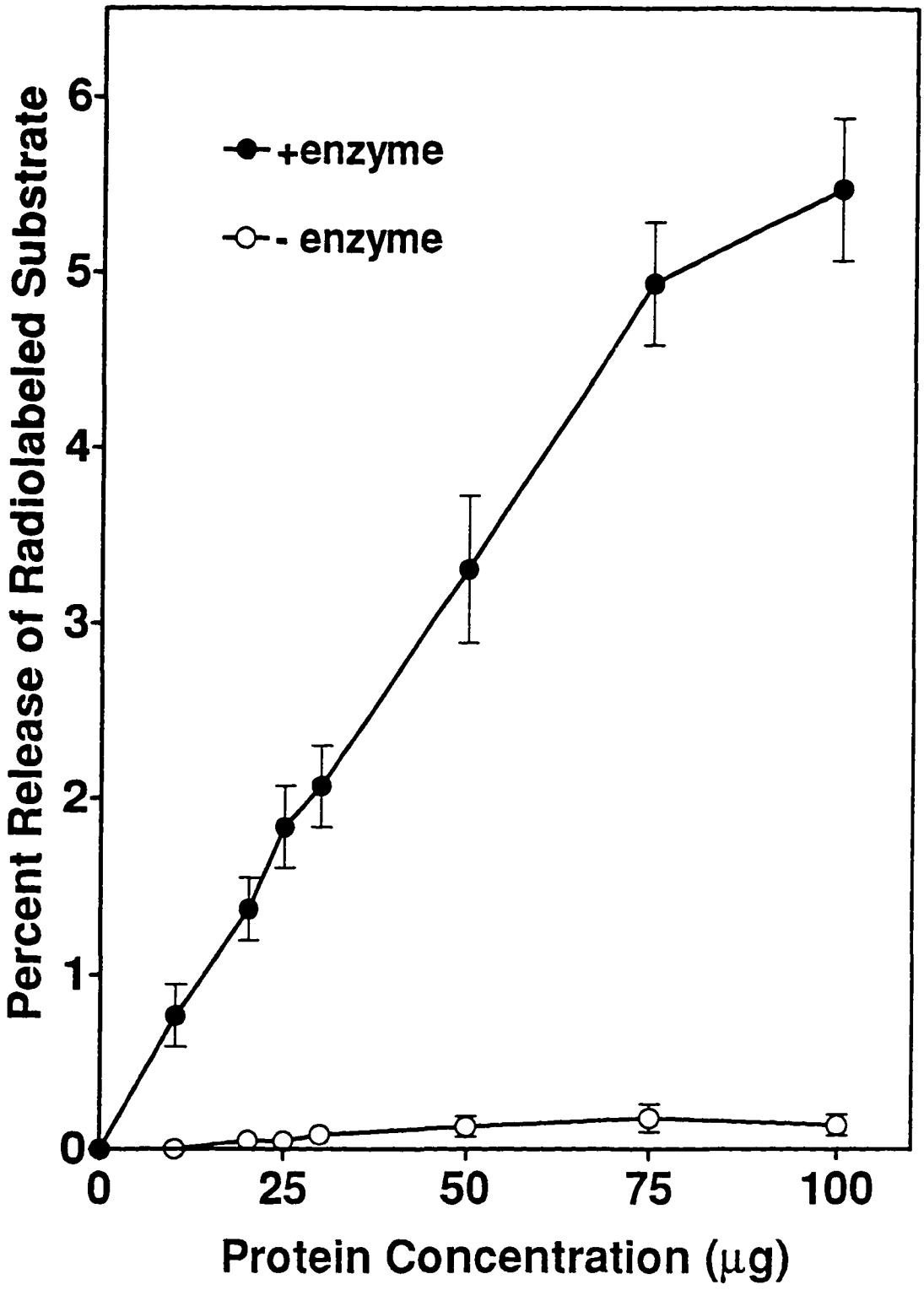
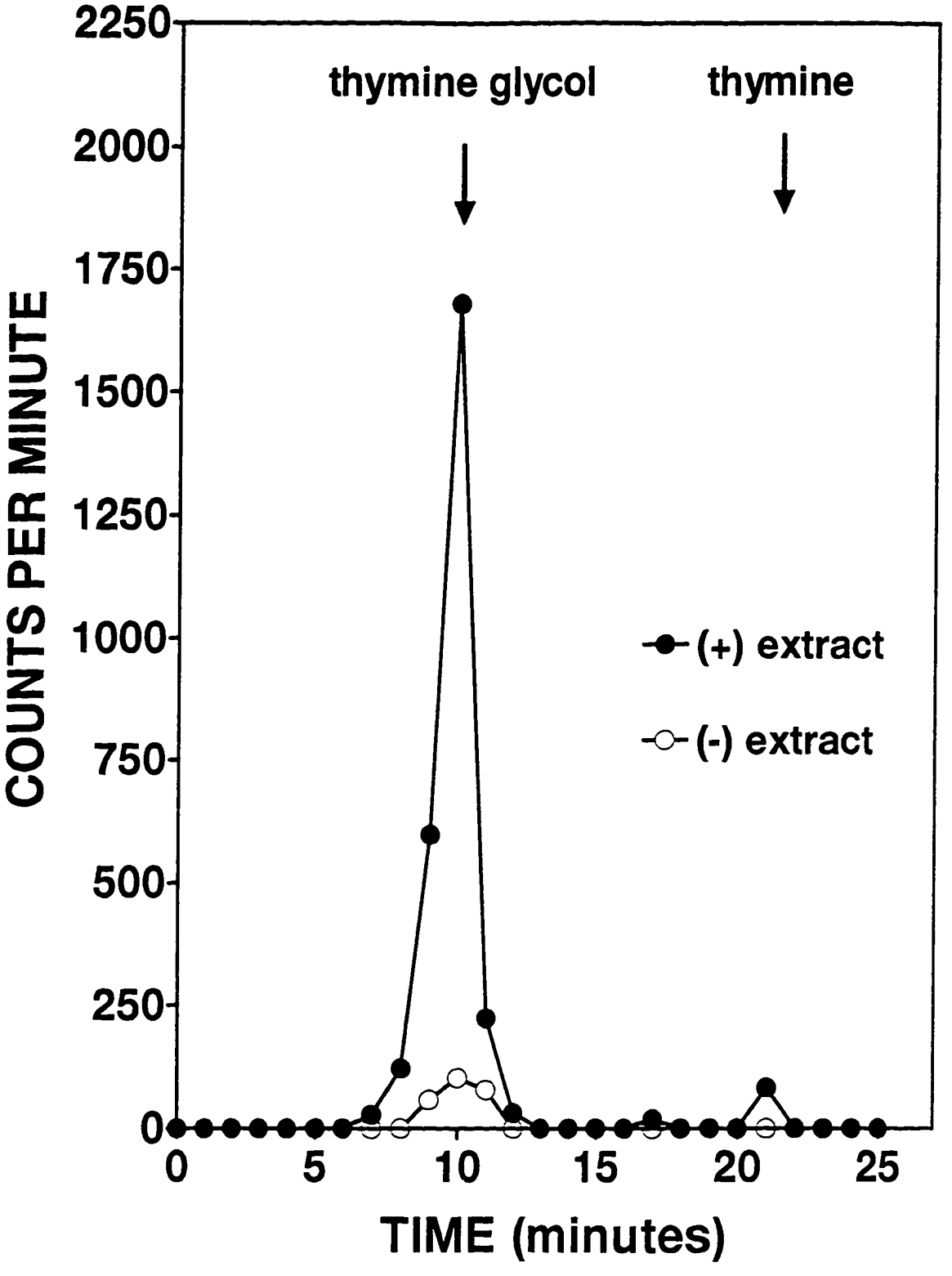


Figure 17. HPLC profile of ethanol-soluble products released from oxidized polynucleotide by a human neuroblastoma cell extract. Radioactivity profile of ethanol-soluble supernatants of oxidized [^{3}H]-thymidine-labeled poly(dA)-poly(dT) incubated with a protein homogenate of human neuroblastoma cells (closed circles) or reaction buffer (open circles), as determined by HPLC analysis on a reverse phase C18 silica column eluted with distilled water. The elution time of marker compounds, thymine and thymine glycol, are as indicated.



base release assay is specific for ring-saturated pyrimidines, as expected for the mammalian redoxyendonuclease.

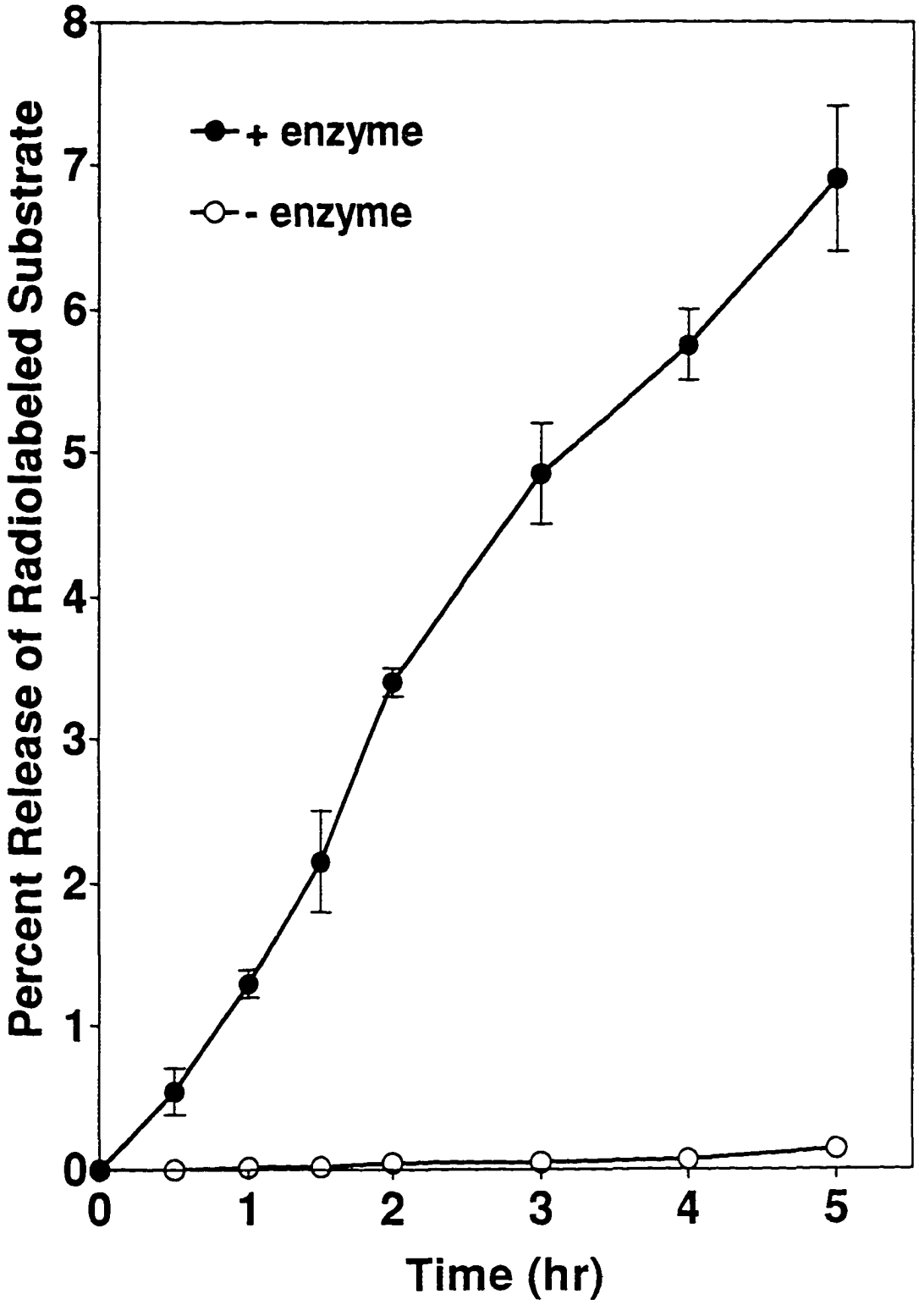
Increased time of incubation also resulted in higher redoxyendonuclease activity in extracts from human neuroblastoma cells. Linear kinetics were observed following the incubation of 50 μg of cell extract with oxidized poly(dA)-poly(dT) to 5 hours (Figure 18). A two hour incubation period, which gave rise to 3.5% base release, was chosen as the assay time for subsequent experiments. Cell extracts incubated with untreated polymer for the indicated incubation times showed minimal release of TCA-soluble radioactive material, indicating that with longer incubation times the enzyme only recognized specific damaged bases.

Preparation of Quiescent Cells

The levels of redoxyendonuclease activity in dividing and quiescent cells were compared to determine whether non-dividing cells have the same repair capacity as actively dividing cells. Cultures of quiescent cells were prepared by incubation with serum-free media for the indicated times followed by quantification of DNA synthesis (Table 4). Failure to incorporate [^{3}H]-thymidine into cellular DNA indicated that the cells were no longer cycling.

Initially, the neuroblastoma cells were maintained in media containing 0.5% or 0.25% serum for three days. Following this, the cells were either maintained in the same media or switched to media containing 10% serum, as a positive control. After 20 hours, [^{3}H]-thymidine was added to all cultures and the cells were incubated for an additional one hour before the incorporation of radioactivity into the cellular DNA was determined.

Figure 18. Incubation time course. OsO_4-treated (closed symbols) or untreated (open symbols) poly(dA)-poly(dT) radiolabeled with [^{3}H]-TTP was incubated with 50 µg of a human neuroblastoma cell extract at 37°C for increasing time periods. The data points are the average of duplicates and are representative of three experiments.



Cells fed media supplemented with 0.5% serum incorporated approximately 5% of the amount of radiolabel incorporated by cycling cells (controls), while the cells incubated with 0.25% serum-containing media incorporated approximately 4% of the amount incorporated by a population of synchronously dividing cells (Table 4). Reducing the time of treatment in media with 0.5% or 0.25% to two days still resulted in the incorporation of 6% and 5% of the [^{3}H]-thymidine of control cells, respectively. These results indicate that a small population of cells continue to cycle when maintained under these conditions.

Subsequently, human neuroblastoma cells were fed media totally depleted of serum for 3 days, to obtain a quiescent population of cells. However, after three days more than 50% of the cells detached from the culture dish. Incubation of human neuroblastoma cells in serum-free media for two days resulted in approximately 0.5% incorporation of [^{3}H]-thymidine as compared to control, indicating that almost all of the cells were not cycling (Table 4). However, under these conditions, approximately 30% of the cells detached from the culture dish. By reducing the incubation time in serum-free media to 36 hours, approximately 0.7% of the label was incorporated into DNA (Table 4), while only 15% of the cells detached from the culture dish. Therefore, cells were maintained in serum-free media for 36 hours to prepare quiescent cell cultures for subsequent studies.

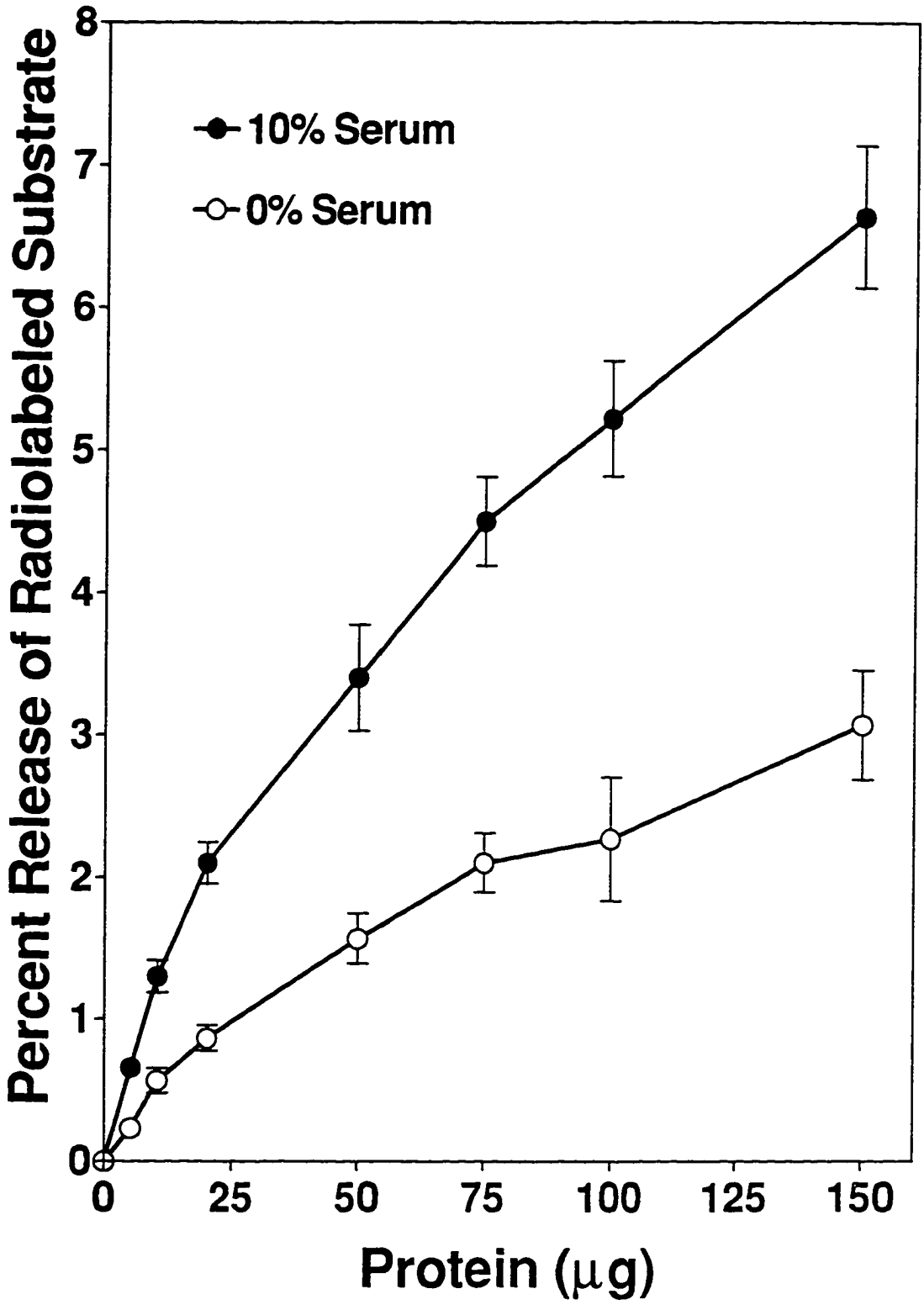
Redoxyendonuclease Activity in Dividing and Quiescent Cells

The levels of redoxyendonuclease activity were compared in neuroblastoma extracts prepared from actively dividing and quiescent cell populations. As shown in Figure 19, the redoxyendonuclease activity was greater than 2-fold higher in actively dividing cells than cells maintained in serum-free media. These results suggest that

Table 4. Incorporation of [^{3}H]-thymidine in the DNA of neuroblastoma cells grown in media containing various amounts of serum.

	Percent Serum Concentration	Percent Total [^{3}H]-thymidine incorporation
3 DAYS		
	10.00%	100.0%
	0.50%	5.2%
	0.25%	4.2%
	0.00%	0.0%
2 DAYS		
	10.00%	100.0%
	0.50%	6.0%
	0.25%	4.8%
	0.00%	0.5%
1.5 DAYS		
	10.00%	100.0%
	0.50%	8.0%
	0.25%	5.2%
	0.00%	0.7%

Figure 19. Effect of serum on redoxyendonuclease activity. OsO_4-treated poly(dA)-poly(dT) radiolabeled with [^{3}H]-TTP was incubated for 2 hours at 37°C with increasing concentrations of protein extracts from neuroblastoma cells maintained in media with 10% serum (closed symbols) or 0% serum (open symbols). The data points are the average of five separate experiments.



quiescent cells have a reduced capacity to remove ring-saturated pyrimidines from their DNA as compared to actively dividing cells and thus, are likely more susceptible to the accumulation of DNA damage.

Cell Survival

Cell survival studies were performed to determine a dose range for treatment of actively dividing neuroblastoma cells with OsO_4 which produces both detectable levels of oxidative DNA damage and a high degree of cell viability. Cell survival was determined by both trypan blue exclusion and the ability of the cells to incorporate [^{3}H]-thymidine following OsO_4 treatment. Logrithmically growing cells treated with $1x10^{-6}$% OsO_4 (v/v) for 30 minutes had the highest cell viability of the tested conditions with approximately 80% cell survival as assessed by trypan blue exclusion (Table 5). These cells incorporated [^{3}H]-thymidine 36 hours after induction of the damage, indicating that the cell population recovered from the damage-induced cell cycle arrest. Treatment of neuroblastoma cells with $1x10^{-5}$% OsO_4 resulted in approximately 70% cell viability with observable incorporation of [^{3}H]-thymidine at approximately 48 hours after the chemical treatment. At the highest concentration of the damaging agent, $5x10^{-5}$% OsO_4, cell viability was reduced to approximately 50%. Incorporation of [^{3}H]-thymidine into these cells was delayed until approximately 72 hours after treatment, indicating that this prolonged time period was required to allow the cells to recover sufficiently from the extensive cellular damage to re-enter the cell cycle. Populations of neuroblastoma cells that were not treated with the oxidizing agent had 100% cell viability and incorporated [^{3}H]-thymidine was detected in the cell cultures at every time point throughout the experiment.

Table 5. Neuroblastoma cell viability after treatment with osmium tetroxide.

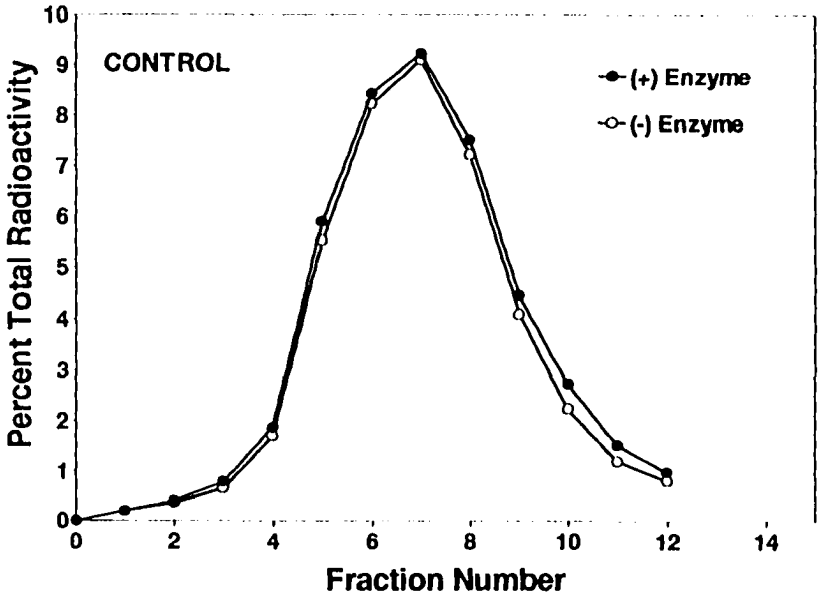
Percent OsO_4	Cell viability (Percent)	[^{3}H]-thymidine incorporation							
		0 hr	2 hr	6 hr	12 hr	24 hr	36 hr	48 hr	72 hr
0%	100%	X	X	X	X	X	X	X	X
1×10^{-6}%	80%	-	-	-	-	-	X	X	X
1×10^{-5}%	70%	-	-	-	-	-	-	X	X
5×10^{-5}%	50%	-	-	-	-	-	-	-	X

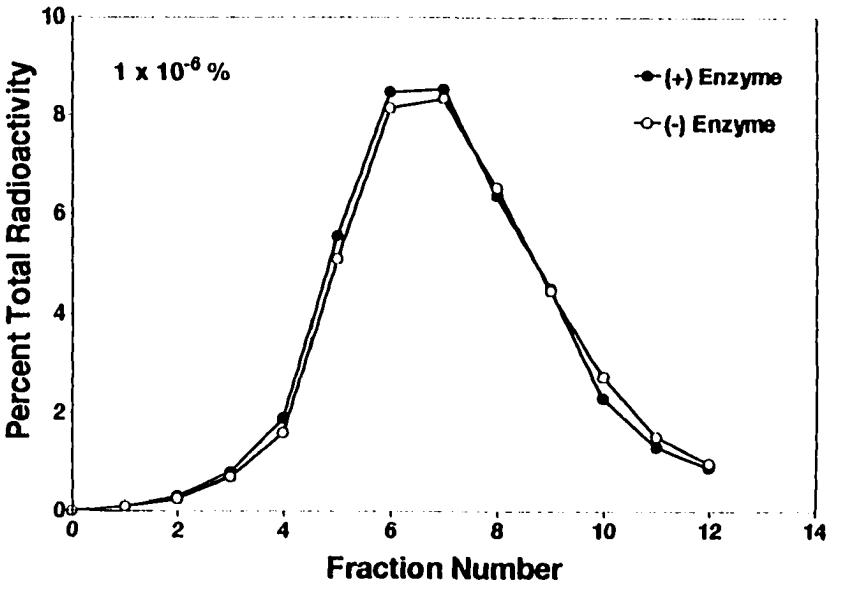
In vivo Formation and Repair of Oxidative DNA Damage

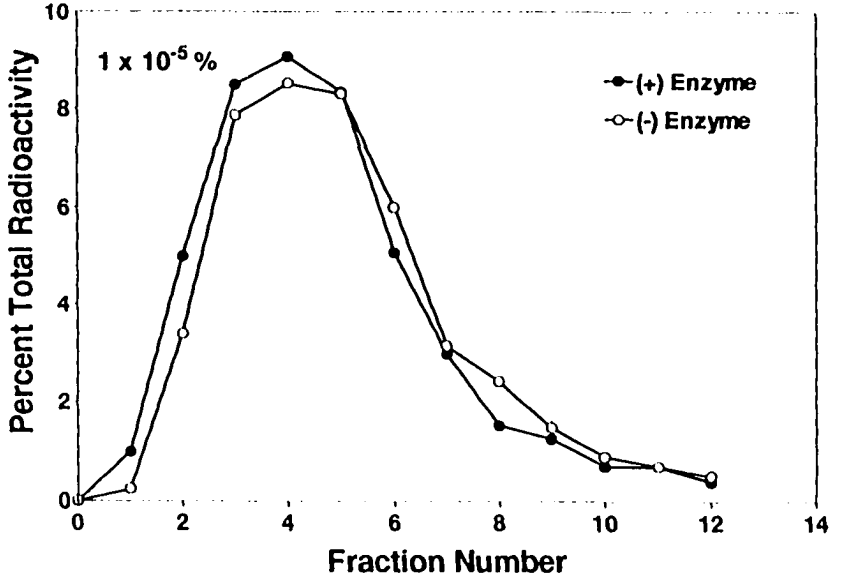
Human neuroblastoma cells were examined for their ability to excise ring-saturated pyrimidines from their cellular DNA following treatment with OsO_4. Based on the cell survival data, initial experiments were performed to determine a dose of the oxidizing agent that produced detectable levels of ring-saturated pyrimidines. Cells were treated with $1x10^{-6}\%$, $1x10^{-5}\%$, and $5x10^{-5}\%$ OsO_4 and the formation of endonuclease III-sensitive sites was determined by alkaline sucrose density gradient centrifugation.

Figure 20 shows the alkaline sucrose gradient profiles of cellular DNA following treatment of the cells for 30 minutes with the indicated doses of OsO_4. Observed variations in molecular weight between DNA preparations from each dose were eliminated by calculating the number of endonuclease III-induced DNA strand breaks per 10^8 Daltons of DNA for each gradient. This is seen as a shift in the gradient profiles from superimposable curves to noticeable differences with increasing dose of the oxidizing agent. Treatment of cells with $1x10^{-6}\%$ OsO_4 resulted in a modest shift to the left in the distribution of [^{3}H]-thymidine (Figure 20b) which reflects approximately 0.24 enzyme-induced breaks per 10^8 Daltons of cellular DNA (Figure 21). As the concentration of OsO_4 was increased, the shift in the distribution of the [^{3}H]-thymidine in DNA samples treated with endonuclease III became more pronounced and an increase in enzyme-induced DNA strand breaks was observed. Based on these results, a concentration of $1x10^{-5}\%$ OsO_4 was used to examine the in vivo repair rates of oxidant-induced thymine glycol lesions. Cellular DNA extracted from untreated cells was used as a control. The control DNA had approximately 0.08 strand breaks per 10^8 Daltons DNA, indicating that there is a small amount of endogenous oxidative damage to cellular DNA.

Figure 20. Alkaline sucrose density gradients of DNA isolated from cells oxidized with increasing concentration of OsO_4. Neuroblastoma cells were oxidized with A) 0% B) 1x10-6% C) 1x10-5% D) 5x10-5% OsO_4 for 0.5 hour at 37°C. Closed symbols represent DNA which was incubated with endonuclease III and open symbols represent DNA incubated with buffer. Sedimentation was from left to right. Weight-average molecular weight (Mw) is equal to the $\Sigma(Ci \times Mi)/(Ci)$, where Ci and Mi are the cpm and molecular weight, respectively, of the i^{th} fraction. Only those fractions contributing to the major peak for each profile were included in the calculations. Gradients were calibrated using PM2 form II DNA.







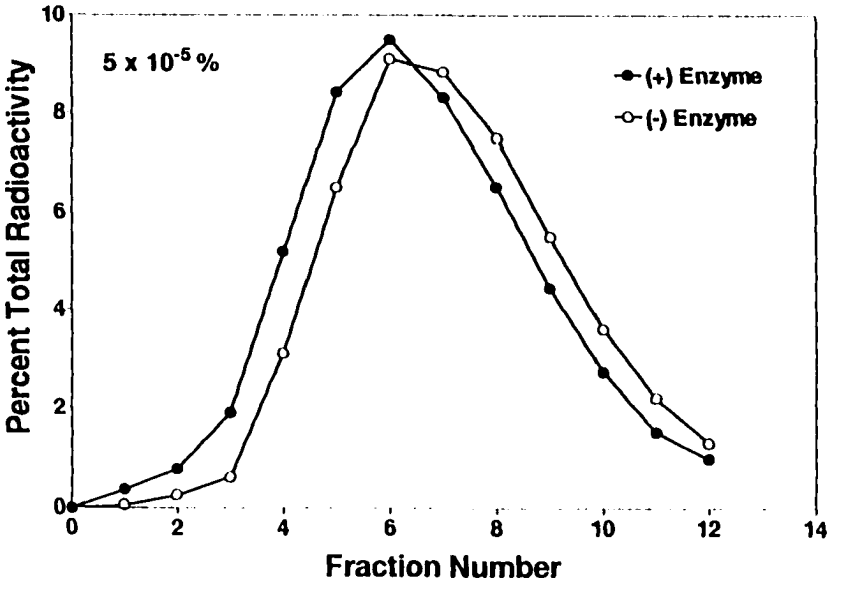
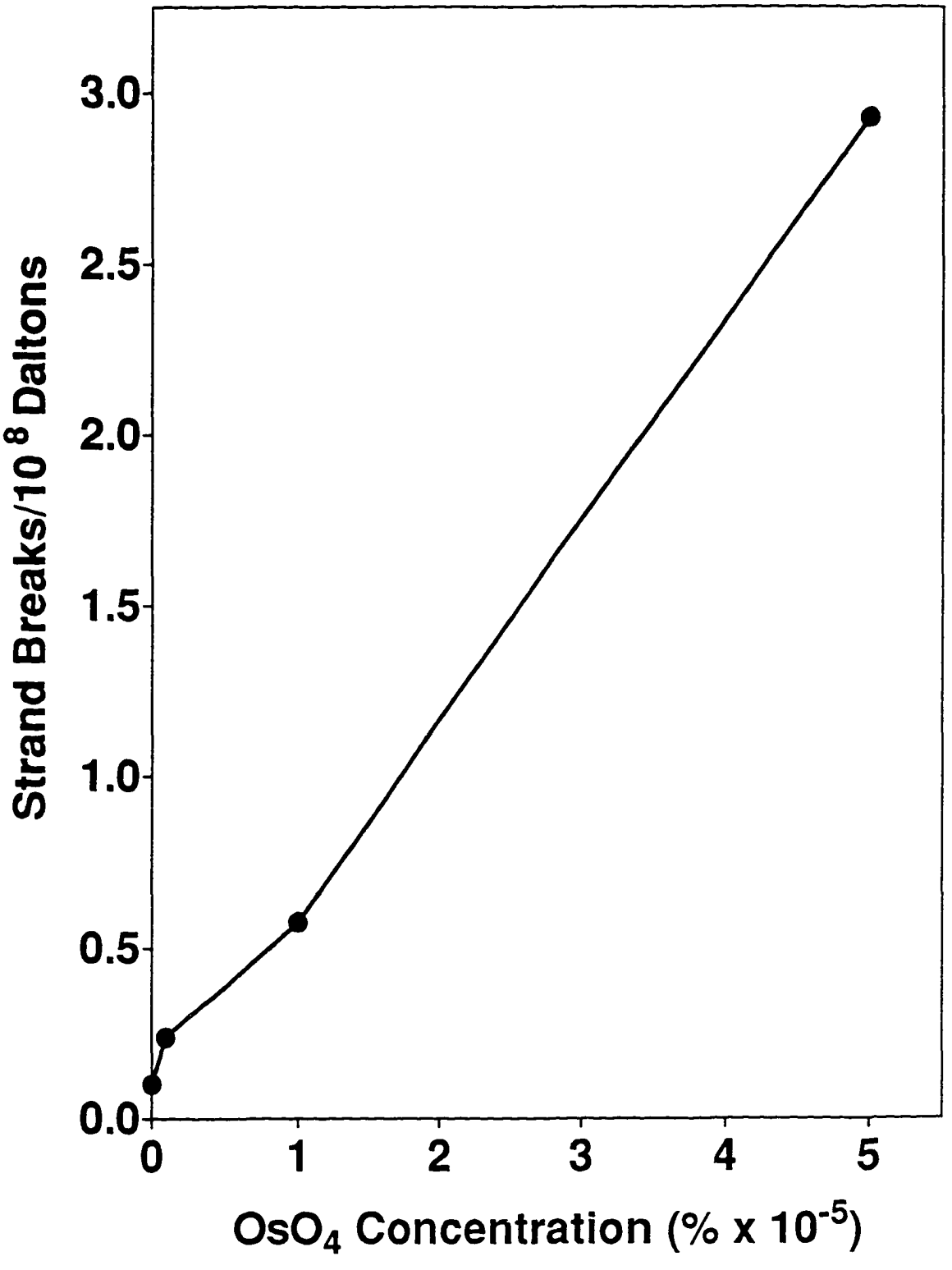


Figure 21. Oxidation dose for the *in vivo* formation of thymine glycols in DNA with increasing concentrations of OsO_4. The number of strand breaks per 10^8 Daltons DNA was calculated from the curves in Figure 20 as $(1/Mnx10^8)-1$ where Mn is the number average molecular weight which is taken as 1/2Mw. and anaylzed by alkaline sucrose density gradient centrifugation. The data points are the average of duplicates and are representative of three experiments.



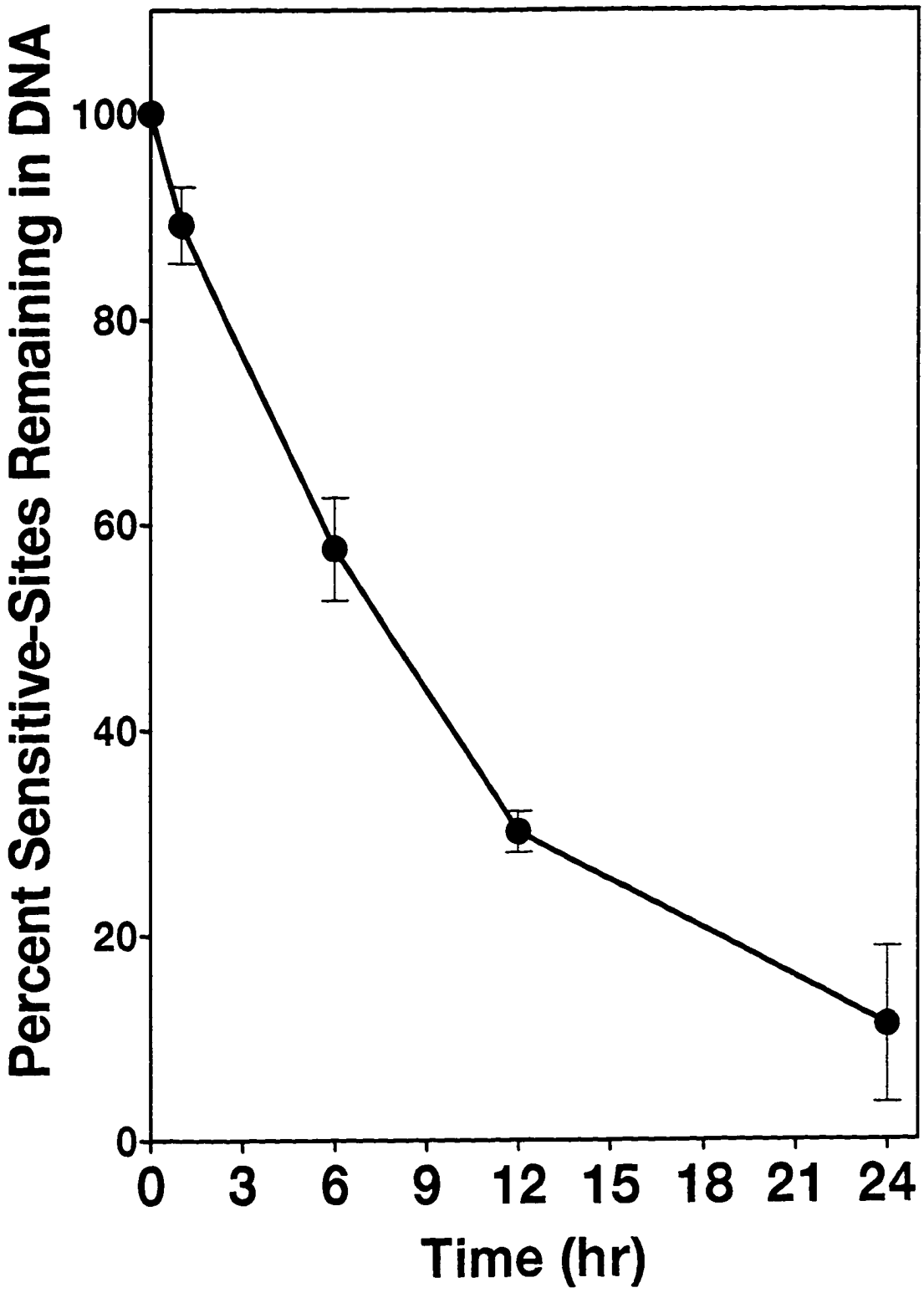
Repair of Ring-Saturated Pyrimidines in Dividing and Quiescent Neuroblastoma Cells

The alkaline sucrose density gradient centrifugation method was used to examine whether quiescent neuroblastoma cells repair oxidant DNA damage as effectively as actively growing cells. Treated cells were harvested at various times following removal of OsO_4 and the extracted, cellular DNA was analyzed for the presence of endonuclease III-sensitive sites. Asynchronously dividing cells were maintained in media supplemented with 10% serum during the repair period, while quiescent cells were grown in serum-free media.

As shown in Figure 22, approximately 50% of the enzyme-sensitive sites were repaired within 6 hours of removal of the oxidizing chemical when actively dividing cells were treated with $1x10^{-5}$% OsO_4. Within 12 hours approximately 30% of the endonuclease III-sensitive sites remained in the cellular DNA and by 24 hours essentially all of the remaining lesions were excised. The results of these experiments demonstrate that ring-saturated pyrimidines are readily excised from the DNA of actively dividing neuroblastoma cells.

Quiescent cells treated with the same concentration of OsO_4 ($1x10^{-5}$%) were also examined for their ability to repair ring-saturated pyrimidines from their cellular DNA. Within two hours after removal of the damaging agent, the majority of the cells began to detach from the cell culture dish and by six hours all the cells detached, indicating that at this concentration of the oxidizing agent, non-dividing neuroblastoma cells are not capable of withstanding the oxidant challenge. Non-dividing cells treated with a buffer control

Figure 22. Time course for *in vivo* repair of thymine glycols in dividing neuroblastoma cells. Dividing cells were oxidized with $1x10^{-5}\%$ OsO_4 for 0.5 hour and allowed to repair the DNA. Cellular DNA was extracted from cells at the indicated times and anaylzed by alkaline sucrose density gradient centrifugation. The number of strand breaks per 10^8 Daltons was calculated as described for Figure 21. The data points are the average of duplicates and are representative of three experiments.

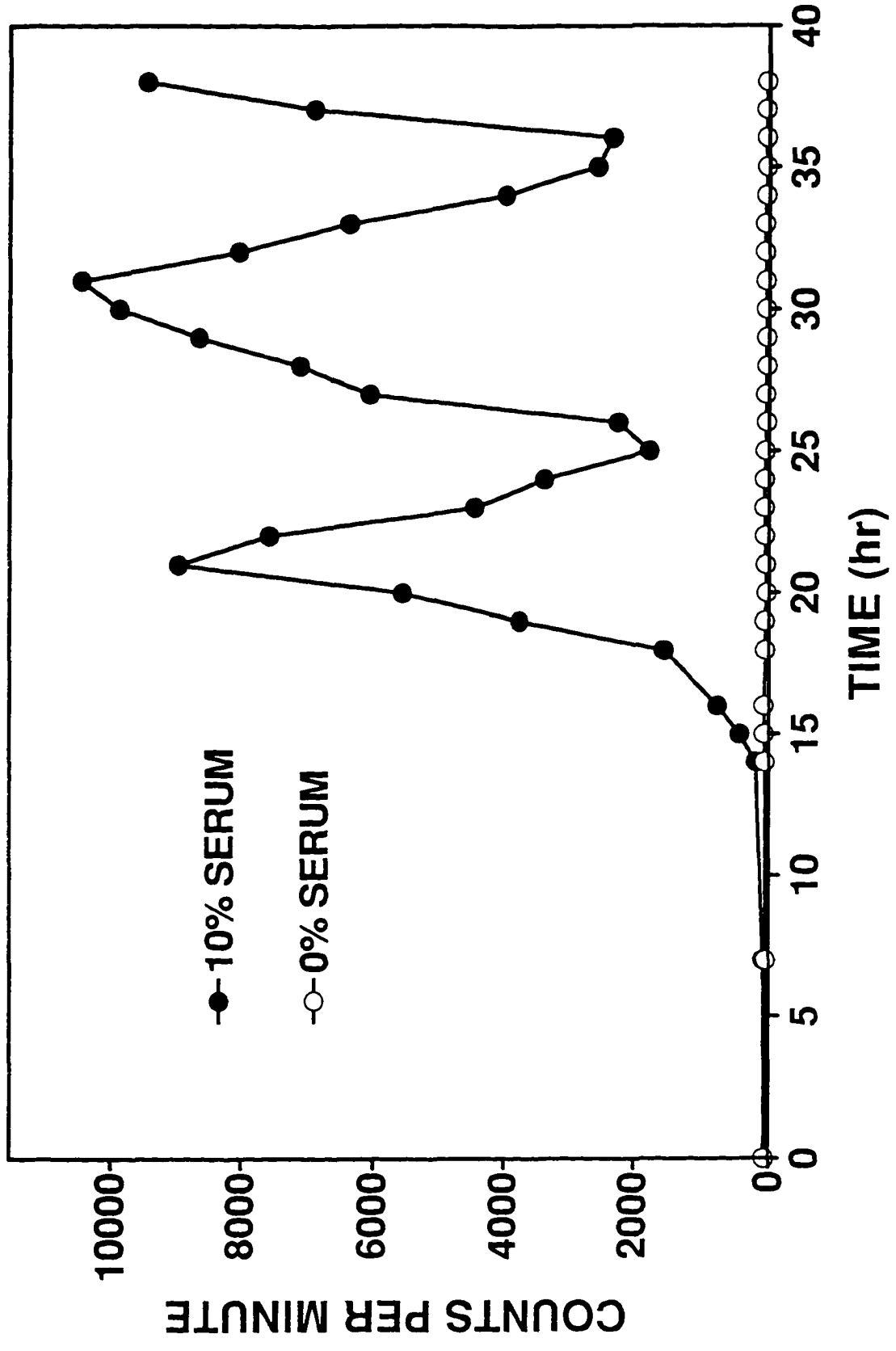


showed no evidence of cell mortality, suggesting that the observed effects were due to exposure to the chemical agent.

Redoxyendonuclease Activity during the Cell Cycle

The level of redoxyendonuclease activity at different stages of the cell cycle was examined to determine whether the enzyme activity is cell cycle regulated. Neuroblastoma cells were arrested by serum deprivation and then stimulated to enter the cell cycle by the addition of serum to the culture media, to obtain homogeneous populations of cells at various stages of the cell cycle. The duration of the neuroblastoma cell cycle was determined by monitoring the incorporation of [^{3}H]-thymidine into cellular DNA during S phase of the cell cycle. As shown in Figure 23, DNA synthesis begins at approximately 15 hours after serum stimulation and peaks between 19 and 23 hours. By 25 hours, the neuroblastoma cells completed S phase and within two hours S phase of the second cell cycle is initiated. The second S phase peaks at 31 hours and is completed by 35 hours after the addition of serum. Each successive S phase peak is reached in approximately 10 hours, indicating that the cell cycle of human neuroblastoma cells under the cell culture conditions used is approximately 10 hours. The initial lag period observed in the first cycle is due to the time required for the cells to recover from arrest induced by serum-deprivation. Over time, the population of neuroblastoma cells becomes progressively asynchronous, as evidenced by the broadening of the S phase peak. Cells maintained in serum-free media did not incorporate [^{3}H]-thymidine throughout the experiment, indicating that these cells are not cycling. However, these quiescent cells were still viable,

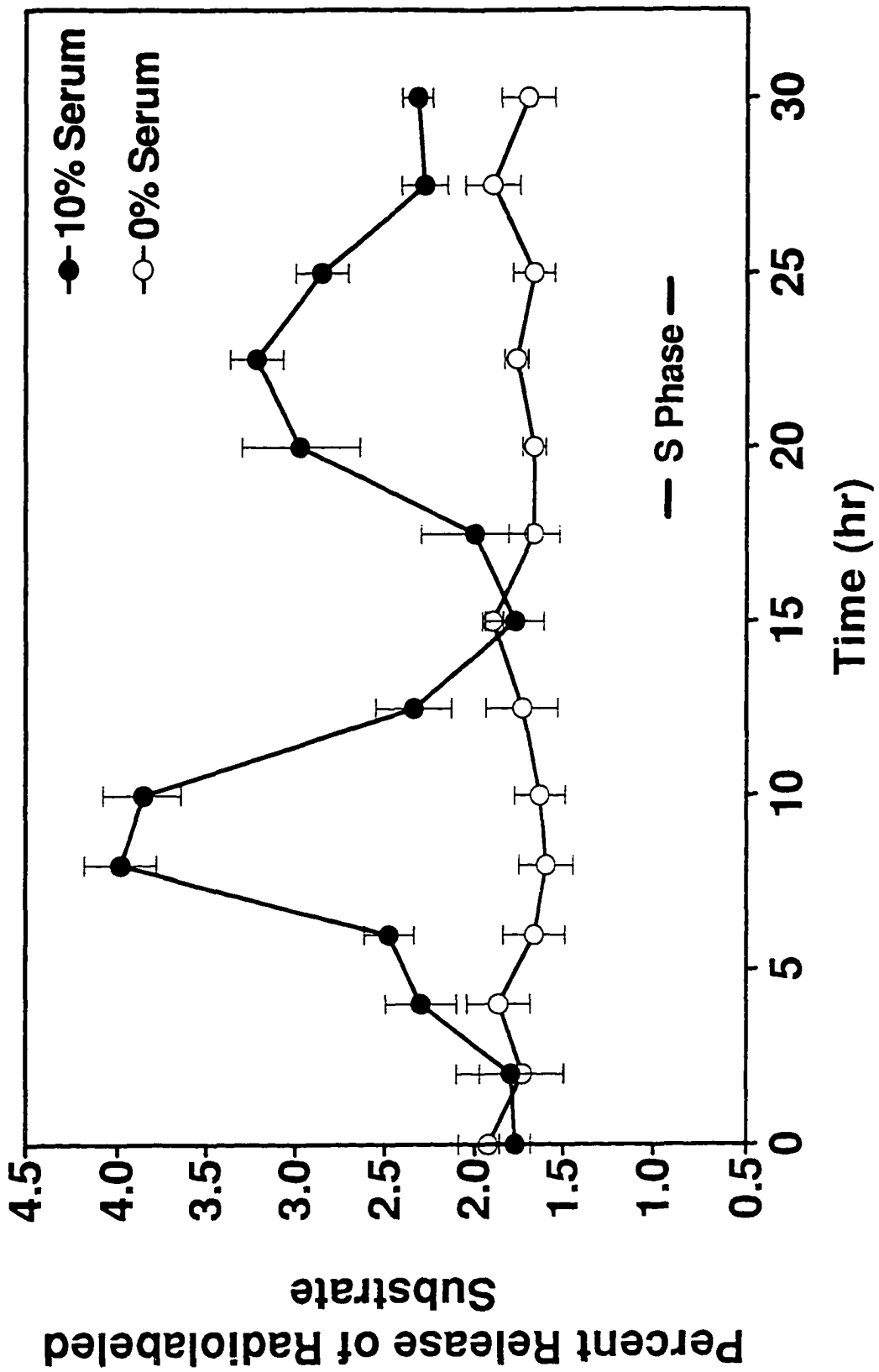
Figure 23. Incorporation of [^{3}H]-thymidine into the DNA of human neuroblastoma cells. Neuroblastoma cells were arrested by serum deprivation for 36 hours and stimulated to enter the cell cycle by the addition of 10% serum to the media at time 0. DNA synthesis was determined at the indicated times by the incorporation of [^{3}H]-thymidine into the cellular DNA. The closed symbols represent cells stimulated to enter the cell cycle by the addition of 10% serum to the media, while the open symbols represent the data points of cells maintained in serum-free media.



as evidenced by their ability to enter the cycle when supplemented with media containing 10% serum at the 36 hour time point (data not shown).

Cells were harvested at various times following serum stimulation and redoxyendonuclease activity was quantified, to determine the level of enzymatic activity during different stages of the cell cycle. Serum was added at time 0, when the cells were arrested in G0. Analysis of redoxyendonuclease activity in cell extracts prepared at this time showed a basal level of enzyme activity, comparable to that observed in quiescent cells (Figure 24). Five hours after the addition of serum, as cells entered the G1 phase of the cell cycle, enzyme activity increased. Redoxyendonuclease activity peaked within 10 hours, with a 2-fold increase observed as compared to quiescent cells, suggesting that oxidative DNA repair capacity is elevated during the early G1 phase of the cell cycle, prior to the initiation of DNA synthesis. At subsequent time points, enzyme activity decreased and by 15 hours post-serum stimulation, the level of redoxyendonuclease activity returned to that of quiescent cells. Enzyme activity remained low until cells reached S phase, at approximately 22-23 hours. In parallel experiments, the redoxyendonuclease activity was quantified in extracts prepared from control, non-dividing neuroblastoma cells harvested at the times indicated. The enzyme activity remained constant, between 1.5 and 2.0% release of the radiolabeled substrate, suggesting that quiescent cells maintain a basal level of redoxyendonuclease activity. These results demonstrate that the redoxyendonuclease activity is regulated throughout the cell cycle, with the greatest enzyme activity observed during the early G1 and late S phases.

Figure 24. Redoxyendonuclease activity at different stages of the cell cycle. Radiolabeled, oxidized poly (dA)-poly(dT) was incubated with 50 μg of neuroblastoma protein extracts from synchronized cells (closed symbols) or from quiescent cells (open symbols) at 37°C for 2 hours. The data points are the average of duplicates and are representative of five experiments.



In Vivo Repair at Different Stages of the Cell Cycle

The rate of repair of ring-saturated pyrimidines was examined following oxidant challenge at various times to determine whether neuroblastoma cells are more susceptible to oxidative injury at particular stages of the cell cycle. Neuroblastoma cells were synchronized by serum deprivation for 36 hours and then serum-stimulated to re-enter the cell cycle at time zero (0). At the indicated times after addition of serum-supplemented media, cells were treated with OsO_4 and examined for the incorporation of [^{3}H]-thymidine and for the presence of ring-saturated pyrimidines in cellular DNA. Cells exposed to the oxidizing agent during G0 did not re-enter the cell cycle, as evidenced by their failure to incorporate [^{3}H]-thymidine for at least 4 days after OsO_4 removal. Alkaline sucrose density gradient centrifugation analysis of the DNA showed that greater than 80% of the endonuclease III-sensitive sites remained after 190 hours (Figure 25). These results indicate that arrested cells do not have the capacity to efficiently repair oxidative DNA base alterations and overcome the damage-induced arrest.

When synchronous cultures of neuroblastoma cells were incubated with OsO_4 during G1, 10 hours after addition of serum-containing media, ring-saturated pyrimidines were excised from the cellular DNA with a half-life of approximately 12 hours (Figure 26). Repair of these lesions was complete within 48 hours of removal of the oxidizing chemical. Analysis of S phase cells treated at the 20 hour time point revealed that endonuclease III-sensitive sites were removed from the DNA of these cells within 24 hours, with a half-life of 5 hours (Figure 27). These data demonstrate that cells in G1 and S phase are capable of efficient repair of oxidative DNA damage.

Figure 25. Time course for *in vivo* repair of thymine glycols in neuroblastoma cells in G0 phase of the cell cycle. Cells maintained in serum-free media for 36 hours were fed media containing 10% serum and $1x10^{-5}$% OsO_4 for 0.5 hour. The oxidizing agent was removed and the cells were maintained in media containing 10% serum until harvest. Cellular DNA was extracted from cells at indicated times and anaylzed by alkaline sucrose density gradient centrifugation. The number of strand breaks per 10^8 Daltons was calculated as described for Figure 21. The data points are the average of duplicates and are representative of three experiments.

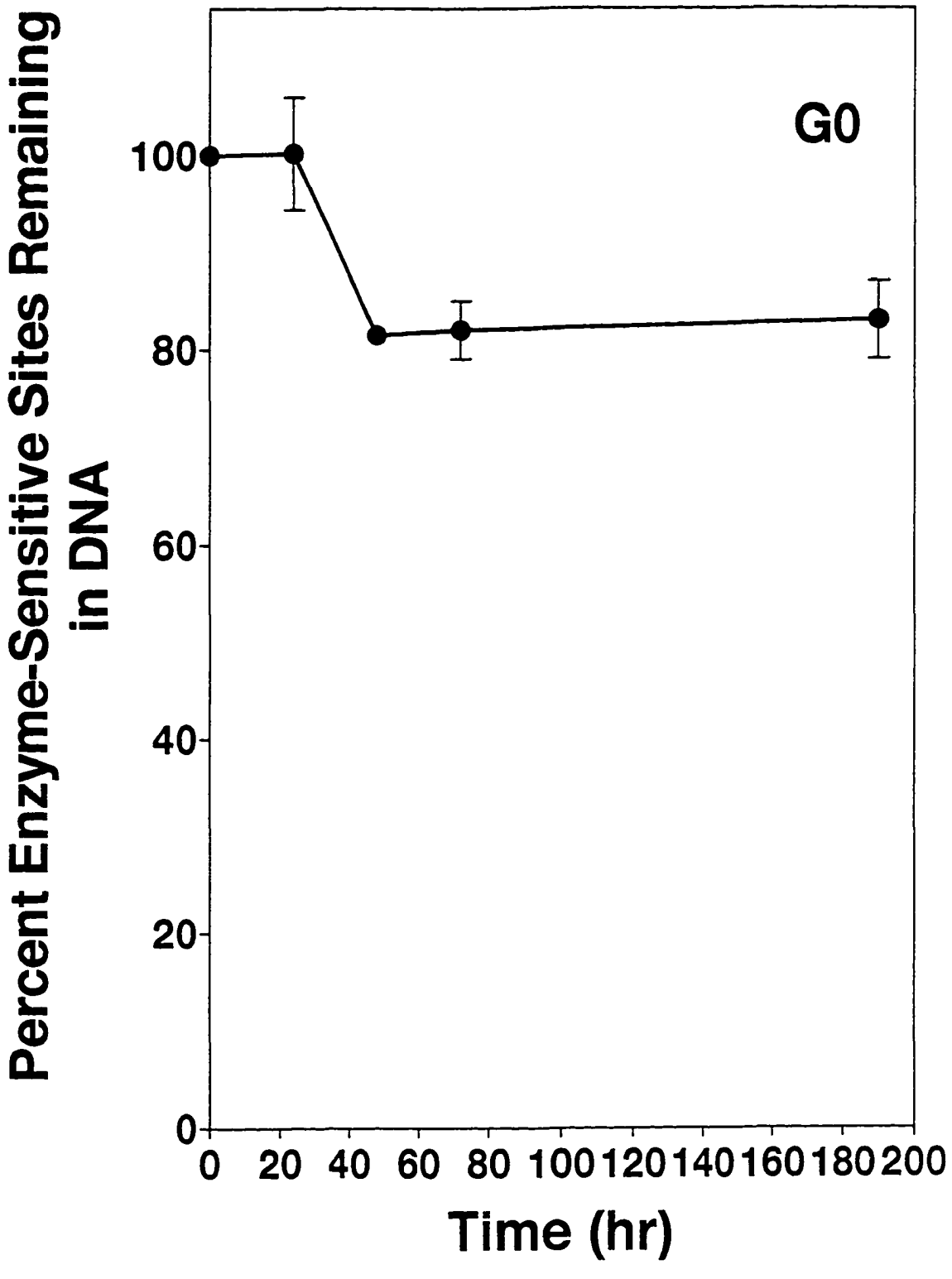


Figure 26. Time course for *in vivo* repair of thymine glycols in neuroblastoma cells in G1 phase of the cell cycle. Cells maintained in serum-free media for 36 hours were fed media containing 10% serum and then treated 10 hours later with $1x10^{-5}$% OsO_4 for 0.5 hour. The oxidizing agent was removed and the cells were maintained in media containing 10% serum until harvest. Cellular DNA was extracted from cells at indicated times and anaylzed by alkaline sucrose density gradient centrifugation. The number of strand breaks per 10^8 Daltons was calculated as described for Figure 21. The data points are the average of duplicates and are representative of three experiments.

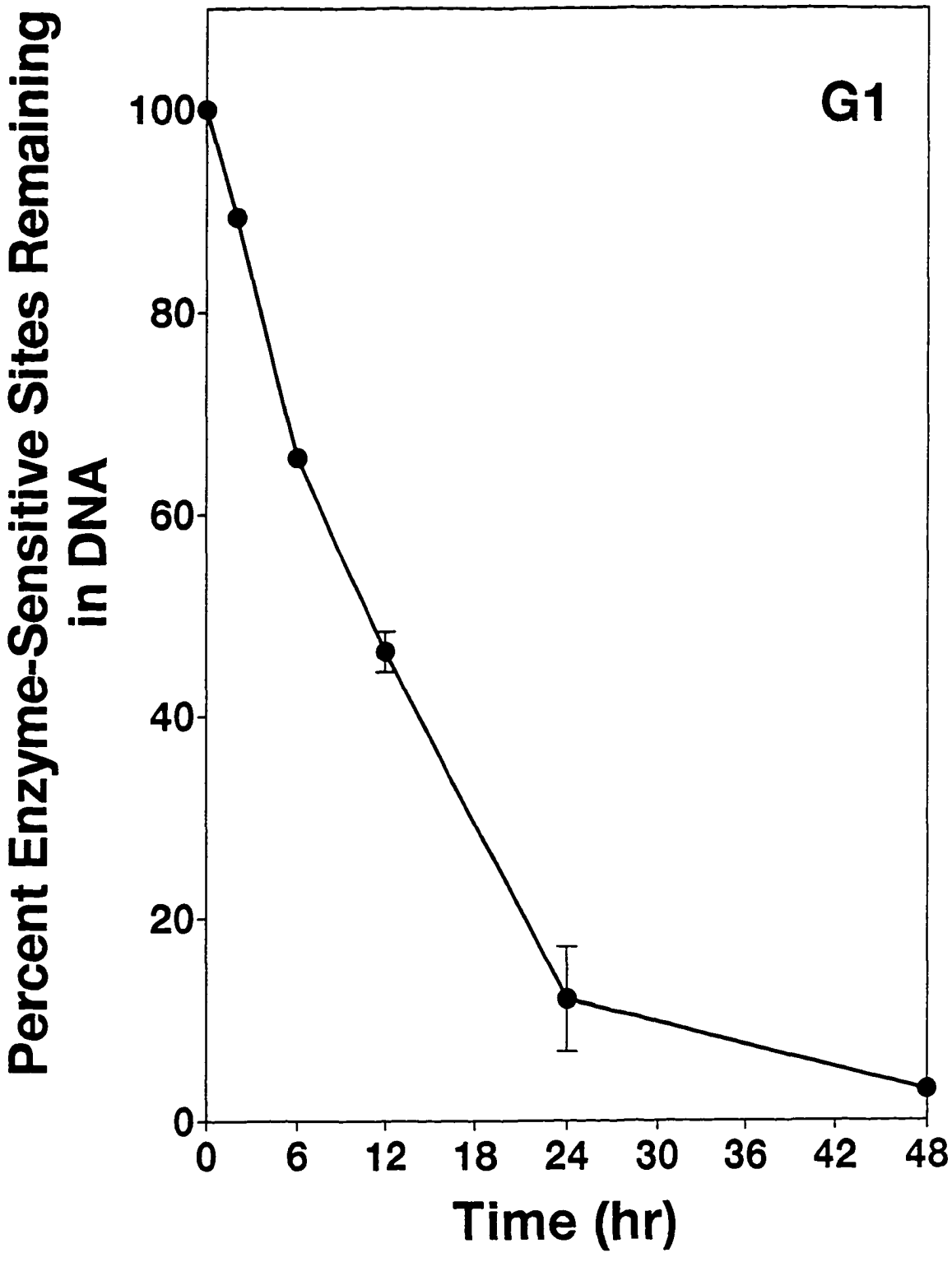
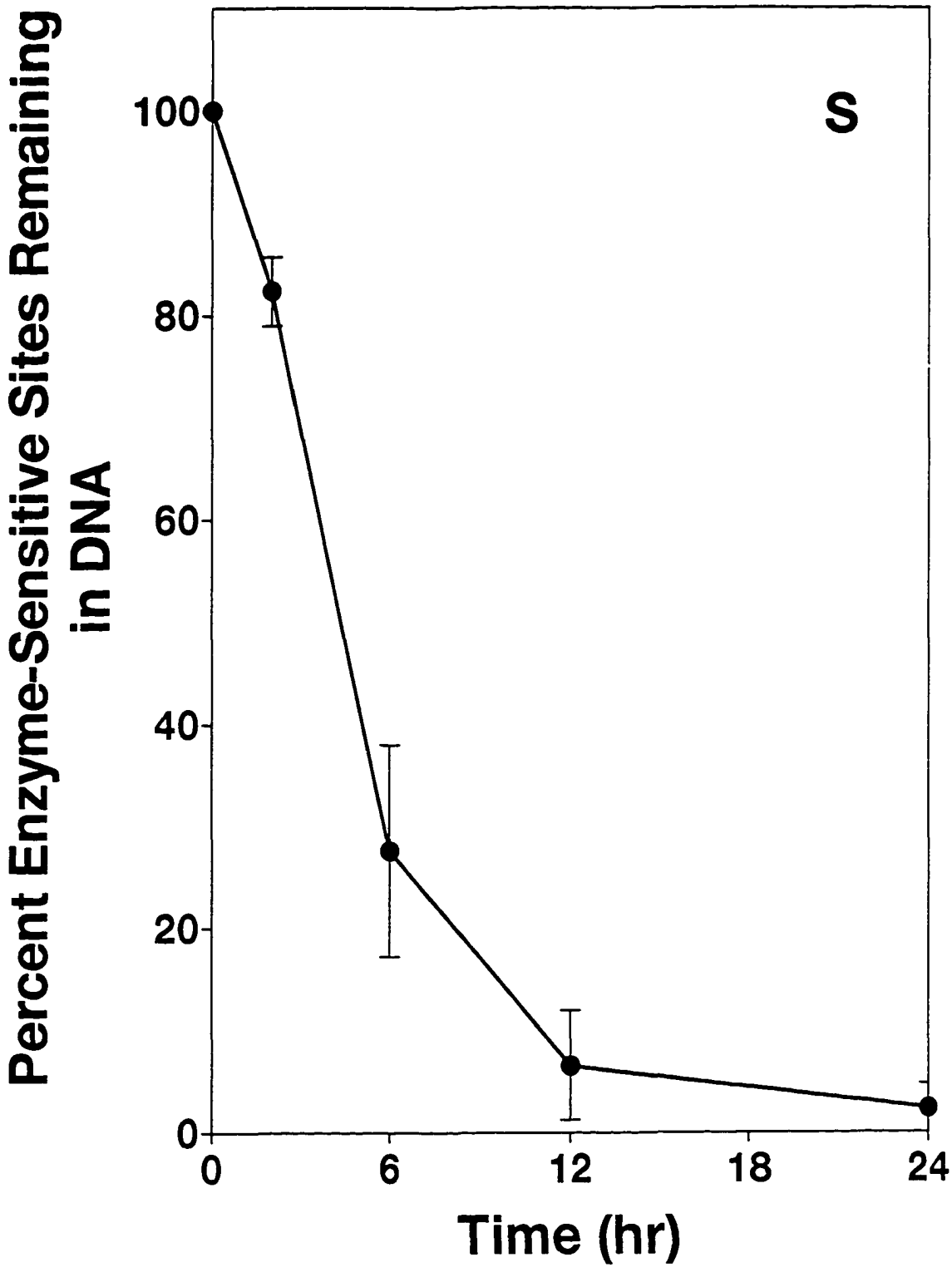


Figure 27. Time course for *in vivo* repair of thymine glycols in neuroblastoma cells in S phase of the cell cycle. Cells maintained in serum-free media for 36 hours were fed media containing 10% serum and then treated 20 hours later $1x10^{-5}$% OsO_4 for 0.5 hour. The oxidizing agent was removed and the cells were maintained in media containing 10% serum until harvest. Cellular DNA was extracted from cells at indicated times and anaylzed by alkaline sucrose density gradient centrifugation. The number of strand breaks per 10^8 Daltons was calculated as described for Figure 21. The data points are the average of duplicates and are representative of three experiments.



The differences in the rate of excision of these lesions during G1 and S phase can be explained by variations in the levels of redoxyendonuclease activity at the time of exposure to the oxidizing agent. The redoxyendonuclease activity peaks at about 10 hours after addition of serum-supplemented media, the time of addition of the OsO_4 to the cells in G1 (Figure 24). At this point, the levels of this enzyme decrease until the later stages of DNA synthesis. When S phase cells were treated 20 hours after serum-stimulation, the redoxyendonuclease activity is increasing as cells are leaving this stage of the cell cycle. Since the enzyme levels are rising, repair of ring-saturated pyrimidines is more rapid compared to G1 phase cells, in which enzymatic activity decreases as the cells encounter the DNA damage. Thus, the results of the DNA repair studies are consistent with analyses of the levels of redoxyendonuclease activity throughout the cell cycle.

DISCUSSION

The redoxyendonuclease activity was greater than 2-fold higher in extracts from actively dividing neuroblastoma cells as compared to that of a quiescent cell population. These results are similar to reports that another DNA repair enzyme, uracil-DNA glycosylase, exhibited increased enzymatic activity in proliferating lymphoblasts and fibroblasts as compared to quiescent cells (Sirover, 1979; Gupta and Sirover, 1981). Uracil-DNA glycosylase gene expression was elevated during fibroblast cell proliferation with an associated increase in enzyme activity (Meyer-Siegler et al., 1992). Similarly, a 2-3 fold increase in uracil-DNA glycosylase and 3-methyladenine-DNA glycosylase activities was found in regenerating rat liver extracts as compared to sham-operated controls (Gombar et al., 1981). These studies demonstrate that dividing cells have greater DNA repair activity compared to non-dividing cells and therefore, potentially have an increased capacity to excise DNA base damage.

The repair capacity of proliferating and quiescent neuroblastoma cells was examined by challenging cultures with non-toxic doses of OsO_4. Logarithmically-growing cells actively excised ring-saturated pyrimidines from their DNA. These lesions were removed essentially in 24 hours, with a half-life of 8 hours. Similar studies examining the repair of ultraviolet light-induced ring-saturated pyrimidines in cultured human lymphoblastoid cells demonstrated a 3 hour half-life for excision with all detectable DNA base damages removed in approximately 5 hours (Weiss and Gallagher, 1993). The differences observed in the repair rates could be due to several factors. The predominant

base damage produced by exposure to osmium tetroxide is thymine glycol (Burton and Reilly, 1966), while the major ring-saturated pyrimidine induced in DNA by exposure to ultraviolet light is cytosine hydrate (Dizdaroglu, 1992; Breen and Murphy, 1995). It is possible that specific ring-saturated pyrimidines are excised from damaged DNA at different rates. Alternatively, the disparity in the repair rate may simply be reflective of the inherent differences in the two cell types or in the cultures conditions. Lymphoblastoid cells are grown in suspension, while, neuroblastoma cells are adherent. As these are the only two studies examining the repair rate of ring-saturated pyrimidines in mammalian cells, further investigation of additional cell types is needed to address these questions.

While the cycling neuroblastoma cells actively removed the ring-saturated pyrimidines from their DNA, similarly damaged quiescent cells did not tolerate the oxidant insult. Reported differences in base excision repair also were observed in quiescent and mitogen-stimulated lymphocytes damaged with dimethyl sulfate (Shrader, 1992). Quiescent lymphocytes were less proficient than dividing cells in repairing DNA following exposure to this damaging agent. Dividing lymphocytes treated with dimethyl sulfate showed a 7-fold increase in repair activity compared to quiescent cells. The predominant damage induced in DNA by dimethyl sulfate is methylated purines (Grunberger and Singer, 1983) which are excised by the 3-methyladenine-DNA glycosylase (Gallagher and Brent, 1982)).

Reports indicate that quiescent cells also have a reduced capacity to excise DNA damages repaired by the nucleotide excision repair pathway. In this process, the damage site along with adjacent nucleotides are excised by a multi-protein complex and an

oligonucleotide segment is released (Friedberg et al., 1995). Nucleotide excision repair is one of the predominant mechanisms for the removal of large adducts from damaged DNA. Studies by Jensen and Linn (1988) using differentiated neuroblastoma cells showed that bulky DNA damages, produced by benzo[a]pyrene treatment, had a half-life of 12 days compared to a 2 day half-life in mitogen-stimulated cells. In addition, a 2-3 fold increase in repair activity was observed in dividing cells irradiated with ultraviolet light as compared to damaged quiescent cells (Shrader, 1992). The major DNA damages produced by ultraviolet light are pyrimidine dimers (Wang, 1976). Taken together, these studies indicate that quiescent cells have a significantly slower rate of both base and nucleotide excision repair than cells which are actively dividing and therefore, are more susceptible to DNA damage accumulation following exposure to chemical or physical agents.

The basal levels of base and nucleotide repair activity in quiescent cells may be sufficient to excise the DNA damage arising from daily metabolic processes. However, if the cells are subjected to a sudden influx of a damaging agent, this limited repair capacity may not be sufficient to handle the resultant elevated DNA insults. Under conditions of oxidant injury, as occurs to neuronal cells during a stroke, the limited capacity of these non-dividing cells to repair the DNA base lesions produced poses a major problem to the cell and ultimately to the organ. Quiescent, but metabolically active, cells with unrepaired DNA damage could give rise to altered or non-functional proteins. If these proteins are involved in cell cycle regulation, unchecked cell proliferation can cause cellular

transformation and carcinogenesis, while growth arrest can result in cell death and eventual organ failure.

Quantification of the redoxyendonuclease activity at different stages of the cell cycle showed that this enzyme activity is cell cycle-regulated in a bi-phasic pattern. Greater enzyme activity was observed in G1 than at the G1/S border, when DNA synthesis occurs. These results indicate that increased DNA repair is initiated prior to DNA replication. It is essential for the survival of the cell and the maintenance of genetic integrity that DNA damages are effectively removed before DNA synthesis. Tolerance of DNA damage can lead to errors during DNA replication which are passed to daughter cells. Such mutations can give rise to nonfunctional proteins, faulty gene regulation, and aberrant daughter cell function. Alternatively, some types of DNA damages block the action of DNA polymerases, thereby preventing replication, and ultimately causing cell death.

The decrease in redoxyendonuclease activity observed during the initiation of S phase may be a protective mechanism to minimize excision of oxidative DNA damage without complete repair of the damaged site. In the base excision repair pathway, the opposite undamaged DNA strand is used as a template by DNA polymerase to incorporate new nucleotides into the gap produced by excision of the lesion and adjacent nucleotides. Thus, proper base excision repair requires a double-stranded DNA substrate and cannot occur in single-stranded regions of the DNA, such as the replication fork. Excision of an oxidative base damage from DNA by the redoxyendonuclease results in the formation of a base loss site. If the excision repair is not completed, studies show that many DNA

polymerases will incorporate an adenine opposite a base loss site during DNA synthesis (Strauss et al., 1982; Strauss, 1991). Thus, a mutation will occur, unless the original damaged base was a thymine.

A second increase in redoxyendonuclease activity was observed in mid to late S phase of the cell cycle. This provides the cell with an additional round of DNA repair prior to cell division. DNA damaging agents, such as oxygen free radicals, are produced continually as by-products of cellular metabolism. The second peak of DNA repair activity will excise any damages produced during S phase and G2 before the daughter cells are finally formed.

This biphasic pattern of cell cycle regulation was observed for another DNA repair enzyme, the uracil-DNA glycosylase (Mansur et al., 1993; Muller and Caradonna, 1993; Nagelhus et al., 1995). As shown with the redoxyendonuclease, two peaks of uracil-DNA glycosylase activity were observed with the first detected in early to mid G1 and the second during S phase. The enzyme activity was correlated with a concomitant increase in both uracil-DNA glycosylase gene expression and protein synthesis. A major difference was observed between the findings with this enzyme and the redoxyendonuclease. The studies showed that the peak of uracil-DNA glycosylase activity is significantly higher in the S phase as compared to G1 (Mansur et al., 1993; Muller and Caradonna, 1993; Nagelhus et al., 1995). This difference in the regulation of the redoxyendonuclease and uracil-DNA glycosylase could be due to the type of damaged bases that the two enzymes recognize. The base damages recognized by the redoxyendonuclease are formed throughout the cell cycle, resulting from exposure to a variety of damaging agents. On the

other hand, uracil is produced in the DNA by either the deamination of cytosine or by incorporation during DNA replication (Friedberg, 1995). It is advantageous for the cell to have higher uracil-DNA glycosylase activity during S phase to remove the uracils inserted during DNA replication

The observed differences in the DNA repair rate of cycling neuroblastoma cells challenged with an oxidizing agent is reflective of the cell cycle regulation of the redoxyendonuclease activity. Cells damaged during either the S or G1 phase of the cell cycle completely repaired the DNA alterations, although the repair rate was significantly slower during G1. At the time the damaging agent was introduced during S phase, the level of the redoxyendonuclease activity was increasing, facilitating the excision of these lesions. However, at the damaging time point in G1, the enzyme activity was declining, necessitating a somewhat longer period for removal of the DNA lesions. When cells in G0 were challenged with the oxidizing agent, no excision of the ring-saturated pyrimidines from the cellular DNA was observed. For these studies, cells were serum-arrested prior to introduction of the DNA damage and then supplemented with serum-containing media to induce re-entry into the cell cycle. However, rather than entering the cell cycle like undamaged neuroblastoma cells, the majority of cells rounded up and detached from the culture dish, indicating that the dose of osmium tetroxide used was toxic. Interestingly, a small percentage of the cells underwent permanent arrest and differentiation as evidenced by extending neurite outgrowth. There was no evidence of the repair of ring-saturated pyrimidines from the surviving fraction of neuroblastoma cells. Thus, it appears that cells that are actively growing at the time DNA damage occurs have a higher DNA repair

capacity than non-cycling cells. Further, the presence of DNA damage in these cells results in the terminal differentiation or extensive cell killing of the cell population. This series of studies is the first analysis of the ability of cells to repair any DNA alterations introduced at different stages of the cell cycle. The results suggest that DNA repair of ring-saturated pyrimidines is cell cycle-dependent and that the phase of the cell cycle during which the DNA damage occurs is a crucial factor in determining cell survival.

Exposure of the neuroblastoma cells to OsO_4 resulted in cell cycle arrest. Increasing concentrations of the oxidizing agent resulted in both an increase in the number of endonuclease III-sensitive sites and in the length of time required for cell cycle initiation. Studies show that dividing cells can delay progression through the cell cycle to facilitate the DNA repair process (Enoch and Norbury, 1995; Liu et al., 1995; Kaufmann, 1995; Murname, 1995; Lohrer, 1996; Kaufmann and Paules, 1996). For example, Murname (1995) showed that cells in G1 delay entry into S phase, S phase cells delay DNA replication, and G2 cells delay entry into mitosis following treatment with ionizing radiation. This arrest provides additional time for DNA repair to occur before the cell enters critical periods of the cell cycle.

As an extension of the studies described in this dissertation, an examination of the proteins involved in the cell cycle arrest observed with the neuroblastoma cells is currently under investigation. One critical cell cycle control protein is the tumor suppressor gene product, p53, whose function is to halt abnormal growth in normal cells and thereby prevent tumorigenesis (Kastan et al., 1992; Kastan et al., 1995; Smith and Fornance, 1996). A small change in the p53 gene, such as a mutation in one of its 393 amino acids,

can eliminate the function of the protein. More than half of human cancers studied carry a p53 gene mutation with p53 mutations linked to 70% of the colorectal cancers, 50% of the lung cancers, and 40% of the breast cancers (Friedberg, 1995).

In response to DNA damage or unfavorable conditions for DNA synthesis, p53 up-regulates the expression of target genes such as the gene for p21 $^{waf-1}$ whose product in turn blocks cell proliferation in G1 phase of the cell cycle (Cox and Lane, 1995; Kastan et al., 1995). p21 $^{CIP1/WAF1}$ is an inhibitor of Cdk2 and Cdk4, cyclin dependent kinases, whose activities are required for the cell to progress from G1 to S phase (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). p21 also inhibits proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase δ *in vitro*, thereby slowing replication in the presence of damage to facilitate DNA repair (Florez-Rozas et al., 1994). GADD45, another gene product activated by p53 in response to DNA damage, is also associated with PCNA (Smith et al., 1994). Overexpression of GADD45 inhibits DNA synthesis and enhances the excision repair process (Prelich et al., 1987; Shivji et al., 1992; Kastan et al., 1992; Hollander et al., 1993; Sanchez and Elledge, 1995). By inhibiting cell cycle progression, p53 provides the cell the time to repair DNA damage or to wait for more favorable conditions for DNA replication (Friedberg, 1995).

Thus, regulation of the cell cycle is a very crucial process for maintaining genetic integrity and cell cycle regulation is present in all eukaryotes studied. Recent reports indicate that cell cycle regulation may exist even in bacteria and regulatory proteins are being isolated (Bridges, 1995; Vinella and D'Ari, 1995; Roush, 1996). This suggests that cell cycle regulation is a fundamental biological process that exists in all organisms.

The same non-lethal concentration of OsO_4 used to treat cycling neuroblastoma cells were toxic to non-cycling neuroblastoma cells. While the cycling cells were able to repair all of the ring-saturated pyrimidines, similarly damaged quiescent cells did not tolerate the oxidant insult. The majority of the noncycling cells rounded up and detached from the culture dish while the remainder underwent permanent arrest and differentiation, as indicated by the formation of long neurite extensions. At very high concentrations of OsO_4, the cell viability of the cycling cells also decreased and the majority of the cells died within 6 hours of exposure to the chemical agent. This indicates that the DNA repair machinery is not capable of repairing extensive damage to cellular DNA, although at higher concentrations membrane damage could be responsible for some of the toxic effects observed. In both cases, it is possible that accumulation of oxidative DNA damage, either by overwhelming the DNA repair systems with excessive exposure to the damaging agent in the case of the cycling cells or due to limited DNA repair capacity in the case of the noncycling cells, caused the induction of apoptosis, programmed cell death.

In addition to cell cycle arrest, accumulation of DNA damage may also induce apoptosis in multicellular organisms, and thereby eliminate cells whose genetic alterations are beyond repair (Enoch and Norbury, 1995). Based on the results of this dissertation work, studies are currently underway to determine whether treatment of both quiescent and cycling neuroblastoma cells with oxidizing agents will result in the induction of apoptosis. When DNA damage is beyond repair, it is advantageous for the cell to "commit suicide" to protect the organism at the expense of individual cells. Studies of the p53 gene showed that its expression is not only associated with cell cycle arrest in G1

following DNA damage but also with some of the apoptotic pathways (Kastan et al., 1995). One study examined the role of p53 in neuronal death in adult rat brains. Adult rats administered an excitotoxin glutamate analogue, kainic acid, showed increased levels of p53 mRNA in neurons with morphological features of apoptotic damage in the targeted brain region (Sakhi et al., 1994). Therefore, p53 induction can also be a marker of irreversible injury in postmitotic cells such as neurons (Sakhi et al., 1994). The p53 protein is thought to activate apoptosis by regulating 2 homologous proteins that have opposite effects on the process. The product of the proto-oncogene Bcl2, protects the cell from apoptosis while the induction of Bax protein increases the concentration of Bax:Bax homodimers and Bax:Bcl2 heterodimers which then act as apoptotic signals (Oitvai et al., 1993). In some cell types, the up-regulation of p53 is concomitant with the down-regulation of Bcl2 and the up-regulation of Bax and apoptosis (Miyashita and Reed 1995). Loss of apoptosis through genetic changes in this pathway can be a critical component of tumorigenesis (Kastan et al., 1995).

Thus, the tumor suppressor protein p53 has multiple functions as a transcription factor. Cells that have normal p53 functions can coordinate multiple responses to DNA damage. When cells encounter DNA damage, the level of p53 protein increases (Cox and Lane, 1995) allowing the cell to induce apoptosis through the transcriptional activation of the Bax gene (Miyashita and Reed, 1995), if the damage is not repairable. If the DNA damage can be removed, the cell can induce DNA repair through p53-stimulated expression of the GADD45 gene.

An examination of the function of p53 in our neuroblastoma system will provide further evidence for the importance of DNA repair mechanisms in the protection of neuronal cells. Cellular DNA damage is a critical problem in brain cells such as neurons which are actively metabolizing, non-dividing cells. When brain cells undergo oxidative damage, as may occur during stroke, extensive cell death is likely to occur. Since neurons are not capable of cell division, the organism cannot replace the dying cells, potentially leading to loss of organ function. Alternatively, it is possible that after oxidative damage, some of the neurons remain viable and try to maintain their normal metabolic activities. However, using the damaged DNA could give rise to altered proteins that are either non-functional or lethal to the cell. This can have such deleterious effects as cellular transformation and eventual tumorigenesis.

This dissertation study provides the first data on redoxyendonuclease activity in cycling and quiescent cells, the regulation of this enzyme during the cell cycle and the effect of the level of enzyme activity on the cellular DNA repair. This work contributes to an understanding of the complex topic of cell cycle regulation which is interlinked with DNA damage and repair. Information regarding DNA repair capabilities at critical stages of the cell cycle and repair capacity in non-mitogenic cells such as neurons may help in developing strategies to treat diseases that develop as a result of accumulation of DNA damage.

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ABSTRACT

Redoxyendonuclease activity was detected in extracts of human neuroblastoma cells using a base-release assay specific for thymine glycol in DNA. The level of redoxyendonuclease activity was more than 2-fold higher in dividing cells compared to quiescent cells, suggesting that quiescent cells may have a reduced capacity to repair oxidative DNA base damages. Cells were synchronized by serum deprivation and then stimulated to enter the cell cycle by the addition of serum to determine enzyme activity at different stages of the cell cycle. The redoxyendonuclease activity was regulated in a biphasic manner with a peak in early G1 and a peak in S phase. This suggests that at specific times during the cell cycle actively growing cells may be more resistant to oxidative DNA damage due to increased repair capacity.

The repair capacity of neuroblastoma cells was quantified as the decrease in enzyme-sensitive sites determined by alkaline sucrose density gradient centrifugation following treatment with the oxidant osmium tetroxide. Actively dividing cells repaired the oxidative damage in approximately 24 hours, while the quiescent cells failed to excise the damaged sites and subsequently died. These results indicate that non-dividing cells do not effectively repair oxidative DNA damage, as compared to the dividing cells. Similarly, quiescent cells, treated with osmium tetroxide and fed a serum-enriched media, failed to re-enter the cell cycle and did not repair the oxidative damage. The data indicate that non-dividing cells, such as neurons, do not have the capacity to repair excess oxidative damage and may suffer the biological consequences of DNA damage accumulation, including cellular death, mutagenesis or carcinogenesis. When synchronized cells were damaged

with osmium tetroxide, there were differential DNA repair rates, depending on the stage of the cell cycle. These DNA repair rates coordinated with the redoxyendonuclease activity profile.

The results of the studies described contribute to a further understanding of the DNA repair pathways which are interlinked with complex processes of cell cycle regulation.

SIGNATURE PAGE

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