STUDIES ON ADP-RIBOSE POLYMER METABOLISM IN CULTURED
MAMMALIAN CELLS FOLLOWING DNA DAMAGE

THESIS

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By

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ADP-ribose polymer metabolism has been studied in human cells derived from a patient with Glutamyl Ribose Phosphate Storage Disease (GRPSD) and in mouse C3H10T1/2 cells following oxidative stress induced by hydrogen peroxide (H₂O₂). It has been postulated that GRPSD resulted from an abnormality in ADP-ribose polymer metabolism. This study has shown that these cells exhibit reduced poly(ADP-ribose) polymerase activity which is proposed to result from modification of the enzyme with ribose phosphate groups. The modification in the polymerase is proposed to be secondary to a defect in either ADP-ribosyl protein lyase or an overproduction of a cellular phosphodiesterase. The metabolism of ADP-ribose polymers was rapidly altered by H₂O₂ and there were independent effects on adenine nucleotide pools. The results suggest that ADP-ribose polymer metabolism is involved in cellular defenses to oxidative stress.
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CHAPTER I

INTRODUCTION

Since the original discovery of NAD\(^+\) by Von Euler this nucleotide has been found to be widely distributed in nature and to function as an electron carrier in various dehydrogenase systems. NAD\(^+\) contains two energy-rich bonds, a pyrophosphate bond which results in the activation of an AMP residue, and a glycosylic linkage to a quaternary ring nitrogen, yielding an activated ADP-ribose (1). Olivera et al. showed in 1967 that bacteria make use of one of the energy-rich bonds by transferring the AMP portion of NAD\(^+\) to DNA during the process of DNA ligation (2). Mandel's group in 1966 described a nuclear enzyme which was able to use the ADP-ribose portion in NAD\(^+\) to form a nucleic acid-like homopolymer in which the ADP-ribose groups are linked together (3). Shortly after, the structure of the homopolymer termed poly(ADP-ribose) was elucidated by the same group. In contrast to DNA or RNA, this polymer has a ribose (1\(^"\rightarrow\) 2\(^'\) ) ribose-phosphate-phosphate backbone as shown in Fig. 1. It exhibits variable chain length, ranging from two to more than two hundred ADP-ribosyl residues (4, 5). As one end of the polymer is attached to nuclear proteins, poly(ADP-ribose) can be said to be a posttranslational protein modification.
Fig. 1. Structure of poly(ADP-ribose) indicating the ribose-ribose-phosphate-phosphate backbone.
similar to phosphorylation, methylation, acetylation, and adenylylation.

The general reaction catalyzed by ADP-ribose transfer enzymes is shown in Fig. 2. Among protein ADP-ribosyl transferases, two subclasses have been defined: mono(ADP-ribosyl) transferases and poly(ADP-ribosyl) transferase (more commonly known as poly(ADP-ribosyl) polymerase). With regard to protein acceptors poly(ADP-ribose) polymerase catalyzes the transfer of ADP-ribose residues to the carboxylate group of glutamate or aspartate. Mono(ADP-ribosyl) transferases catalyze the transfer of an ADP-ribose residue to a specific amino acid of a specific target protein. Monomeric transfer reactions have been shown to occur in both prokaryotes and eukaryotes, although the most widely studied are the bacterial toxins. The ADP-ribosyl acceptors identified in the bacterial toxins are the guanidinium group of arginine, the sulfhydryl group of cysteine, the imidazoyl group of dipthamide and the amido group of asparagine. The specific in vivo acceptor proteins in animal cells are not known but endogenous transferase have the same range of acceptor specificity as the bacterial toxins (5). Since the focus of this study is mainly on poly(ADP-ribosyl)ation, the metabolism of polymers of ADP-ribose and its effects on some cellular functions is reviewed.
Fig. 2. General reaction catalyzed by ADP-ribose transfer enzymes. X represents the nucleophilic acceptor.
Poly(ADP-ribose) Metabolism

There is compelling evidence that poly(ADP-ribosyl)ation reactions are ubiquitously distributed among higher eukaryotes. There are also reports of poly(ADP-ribosyl)ation activity among plants and lower eukaryotes (7). The presence of poly(ADP-ribose) polymerase activity in yeast is controversial (8) and so far none has been found in prokaryotic organisms. Poly(ADP-ribose) polymerase has been purified from various tissues (9) and Shizuta et al. have identified three distinct functional domains of the enzyme (10). Following limited proteolysis of the purified enzyme, they characterized a Mr 54000 fragment as the substrate binding moiety, a Mr 46000 fragment as the DNA binding domain and a Mr 22000 polypeptide as the site of autmodification. With the recent availability of monoclonal antibodies against the DNA binding and other functional domains of the enzyme, more detailed structural and functional information on poly(ADP-ribose) polymerase can be expected (11).

Poly(ADP-ribosyl)ation proceeds in three steps, namely initiation, elongation and branching of the polymer. The initiation reaction is the covalent attachment of an ADP-ribosyl moiety from NAD$^+$ to the carboxylate group of glutamate or aspartate in an acceptor protein. The elongation reaction involves the sequential addition of the ADP-ribose
residue to the 2' hydroxyl group of the adenine-proximal ribose of the first residue. The branching reaction involves the introduction of an ADP-ribose residue which branches off from a linear portion of the polymer (6). All these reactions appear to be catalyzed by poly(ADP-ribose) polymerase. An NAD⁺ glycohydrolase activity which catalyzes the cleavage of NAD⁺ to nicotinamide and ADP-ribose is also carried out by the enzyme. The activity of the polymerase is absolutely dependent on DNA and is stimulated by Mg²⁺ and polycations such as histones or polyamines. Single-stranded DNA, although it binds very efficiently to the enzyme (12), is ineffective as an activator of the polymerase, and so is RNA. DNA-RNA hybrids are partially effective (13). Apart from activating the enzyme, DNA binding of the enzyme also affects its Km for NAD⁺ (14).

Inhibitors of poly(ADP-ribose) polymerase activity have become extremely important tools in the attempt to elucidate the biological function of ADP-ribosylation reactions. Purnell and Whish showed that substituted benzamides are potent inhibitors with Ki values of less than 2 μM (15). The studies of Sims et al. with benzamides and other analogs demonstrated that the ring nitrogen is not essential for effective inhibition but emphasized the importance of a carboxamide group for potent inhibitory action (16). In 1980, Shall's group reported for the first time the successful
application of substituted benzamides in determining the potential role of poly(ADP-ribosyl)ation reactions in DNA excision repair and in cellular recovery from DNA damage (17). Furthermore, the lack of poly(ADP-ribosyl)ation deficient mutants makes these inhibitors more valuable in the elucidation of the biological function of poly(ADP-ribosyl)ation reactions.

In eukaryotic cells, two different types of enzymes are known to attack polymers of ADP-ribose (Fig. 3). The first enzyme, poly(ADP-ribose) glycohydrolase cleaves ribose-ribose bonds of both linear and branched portions by an exoglycosidic hydrolysis mode. A second enzyme, a phosphodiesterase attacks the pyrophosphate bond of ADP-ribose polymers by exonucleolytic action. The proximal ADP-ribosyl moiety bound to carboxylate groups of glutamic acid residues of various acceptor proteins is removed by another kind of enzyme, ADP-ribosyl protein lyase (6).

Poly(ADP-ribose) glycohydrolase seems to be the most important activity in the catabolism of poly(ADP-ribose) in vivo. It was discovered by Miwa and Sugimura (18) and subsequently partially purified by Ueda et al. (19). Recently there have been more extensive purifications (20, 21) and the Km determined for poly(ADP-ribose) as ADP-ribosyl residues is below 2 µM which roughly corresponds to the constitutive poly(ADP-ribose) concentration in most tissues. Little is
Fig. 3. Schematic representation of the enzymes of ADP-ribose polymer metabolism.
known about the physiological regulation of poly(ADP-ribose) catabolism. The half life of polymers formed as a result of DNA damage by treatment of cells with carcinogens is less than two minutes (22) which is considerably shorter than the half life of constitutive polymers which are degraded in a biphasic manner with half-lives of five minutes and 7.75 hours respectively (23). Presumably, the concentration, chain length, and complexity of the polymers have an affect on the half-life of the polymer. Polymers formed in vivo in response to carcinogens also exhibit multiphasic decay characteristics (23). Wielkens et al. have proposed that the rate limiting step in the catabolism of carcinogen-induced polymers may be the removal of proximal ADP-ribosyl residue bound to the acceptor protein (24) which is removed by the enzyme ADP-ribosyl protein lyase. The ADP-ribosyl protein lyase activity was discovered and partially purified by Hayaishi's group (25) and later purified to homogeneity (26). This enzyme is unique among mammalian enzymes insofar that its reaction yields unsaturated sugars by an enzymatic elimination reaction. The structure of the split product is close to, but not identical with ADP-ribose (26). The substrate specificity of the ADP-ribosyl protein lyase is not very stringent with regard to the protein portion but specific for the mono(ADP-ribose) moiety and the carboxyl ester bond to the protein. For example, the enzyme does not split ADP-ribose histone
adducts formed chemically through Schiff base reduction or the ADP-ribosyl arginine bond (26).

Poly(ADP-ribosylation) in the Recovery of Mammalian Cells from DNA Damage

For over 50 years, it has been known that glycolysis is inhibited in cells exposed to alkylating agents. Since then, numerous reports have demonstrated that alkylating agents and ionizing radiation lower intracellular NAD$^+$ levels. This was identified as the cause for the inhibition of glycolysis by these same agents. In 1975 an important correlation was made between NAD$^+$ metabolism and poly(ADP-ribose) biosynthesis. Two groups showed that NAD$^+$ depletion caused by treatment of cells with DNA damaging agents was due to an enhanced utilization of NAD$^+$ as a substrate for poly(ADP-ribose) biosynthesis (27, 28). These and numerous subsequent reports have confirmed the importance of ADP-ribosylation reactions as a major pathway of NAD$^+$ catabolism in cells subjected to DNA damaging agents. The possibility that the NAD$^+$ lowering effect of these treatments could result from concomitant inhibition of NAD$^+$ biosynthesis or stimulation of microsomal NAD$^+$ glycohydrolase activity has been excluded (29, 30). Miller in 1975 examined the effect of DNAase I on poly(ADP-ribose) synthesis in isolated nuclei and found that DNA fragmentation may be the initial signal for poly(ADP-ribose)
synthesis (31). Benjamin and Gill demonstrated using an *in vitro* model system that double stranded restriction fragments with flush ends were more effective on poly(ADP-ribose) polymerase than overlapping ends (13). Neither NAD⁺ depletion nor stimulation of poly(ADP-ribose) polymerase were observed in UV treated xeroderma pigmentosum cells which lack the capability to perform the incision step in UV damage repair. However N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which is an alkylating agent, did stimulate the conversion of NAD⁺ into poly(ADP-ribose). These results confirmed that the production of DNA strand breaks either directly (by ionizing radiation) or enzymatically (in the process of DNA excision repair) are required for stimulation of poly(ADP-ribose) biosynthesis. Shall and coworkers in 1980 using 3-aminobenzamide subsequent to infliction of DNA damage by the alkylating agent dimethyl sulfate, concluded that poly(ADP-ribose) participates in DNA-excision repair by activating DNA ligase II (32). Since the studies of Shall and coworkers, a more complex picture has emerged regarding the involvement of poly(ADP-ribosyl)ation in DNA excision repair. Several investigators have pointed out that the elevation of DNA strand break frequency in cells subjected to alkylating agents as seen by Shall's group does not necessarily reflect blocked ligation activity but could result from an increased rate of incision (33).
The role of poly (ADP-ribosyl)ation in recovery from DNA damage can be further examined from a biological viewpoint: the clonal survival of cells at various stages of recovery from DNA damage. Studies show that cells which have to recover from DNA damage in the presence of ADP-ribosylation inhibitors exhibit reduced survival capacity. Thus, it is important to consider the effects of these inhibitors on cell cycling which can affect cell survival independent of their action on repair reactions. Boorstein and Pardee showed that the presence of 3-aminobenzamide following treatment with an alkylating agent caused an arrest in the G2 phase of the cell cycle with a delay in mitosis thereby preventing them from forming colonies (34). Similarly Jacobson et al. showed that inhibitors such as nicotinamide analogs blocked the recovery of the cell division potential in C3H10T1/2 cells treated with MNNG (35). These experiments show that following DNA damage poly(ADP-ribosyl)ation reactions are required for normal cell cycle progression.

The role of poly(ADP-ribosyl)ation in the reciprocal exchange of DNA between homologous regions of sister chromatids or sister chromatid exchanges (SCE) has also been implicated since SCE involves strand breakage and ligation mechanisms. Several investigators have shown a positive correlation between the SCE inducing potential and inhibitory effect of inhibitors of poly(ADP-ribose) polymerase (36).
However much more needs to be learned about the mechanisms of SCE induction and its relation to the biological role of chromatin-bound poly(ADP-ribose).

Research Prospectus

The research described in this thesis involves two different projects. Project I deals with the study of glutamyl ribose-5-phosphate storage disease, and project II deals with the study of oxidative stress induced by hydrogen peroxide. The two projects are related in that poly(ADP-ribose) metabolism was studied in both cases. This study of poly(ADP-ribose) metabolism broadly involved measurements of NAD\(^+\), poly(ADP-ribose), and protein-bound mono(ADP-ribose) derived from intact cultured cells. The methodology developed in this laboratory has allowed the determination of all three values from a single dish of cultured cells. Fig. 4 shows a flowchart which outlines the basic protocol followed. A more detailed explanation of these procedures is given in the methods section.

An introduction to poly(ADP-ribosyl)ation has been reviewed in chapter I. Chapter II details the materials and methods used in performing the experiments. A brief introduction about each project followed by results and discussion is found in chapters III and IV respectively.
Fig. 4. Analytical scheme for labeling of the NAD$^+$ pool and analysis of NAD$^+$ and polymeric and monomeric ADP-ribose from a single culture dish of cells.
Confluent Cell Cultures

$[^3H]$ ade labeling

Experimental Manipulation

TCA

Pellet

Supernatant

DHB-Sepharose

NAD

KOH, EDTA

DHB-Bio-Rex

Polymeric ADP-ribose

Selective Release

DHB-Bio-Rex

DHB-Sepharose

Monomeric ADP-ribose
CHAPTER II

MATERIALS

Biologicals

Human fibroblasts from a patient with glutamyl ribose-5-phosphate storage disease, and foreskin cells from an age-matched control were obtained from Dr. Julian C. Williams, Children's Hospital (Los Angeles, CA). C3H10T1/2 cells (clone 8) were obtained from Dr. C. Heidelberger, Comprehensive Cancer Center, University of Southern California (Los Angeles, CA). Dulbecco's modified Eagle's medium and gentamycin sulfate were purchased from Life Technologies (Grand Island, N.Y.). Bovine calf serum was purchased from Hyclone Laboratories (Logan, UT).

Radioisotopes

[2-8,3H] Adenine (35Ci mmol\(^{-1}\)) was purchased from ICN Radiochemicals (Irvine, CA). Scintillation vials were obtained from Fischer Scientific (Pittsburgh, PA).

Enzymes

Snake venom phosphodiesterase from Crotalus Adamanteus was purchased from Cooper (Freehold, NJ). Trypsin and
bacterial alkaline phosphatase (BAP) from *Escherichia Coli* were obtained from Sigma (St. Louis, MO) and catalase from bovine liver was purchased from Serva Feinbiochemica (Heidelberg, NY).

**General Supplies**

Adenosine, adenosine 5'-monophosphate (AMP), deoxyadenosine, adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), nicotinamide (Nam), adenosine 5'-diphosphoribose (ADP-ribose), N-methyl-N'-nitro-N-nitroso guanidine (MNNG), and hydrogen peroxide (30%) were purchased from Sigma (St. Louis, MO). The homopolymers of adenylic acid: (poly A) of variable chain lengths (100-120 residues); the dodecamer [(Ap)\(_{11}\)A]; the octamer [(Ap)\(_{7}\)A]; and the pentamer [(Ap)\(_{4}\)A] of adenylic acid were from Pharmacia (Piscataway, NJ). The materials used for affinity chromatography such as polypropylene econo-columns (0.8 in I.D.) and the cation exchange resin Bio-Rex 70 (200-400 mesh) were obtained from Bio Rad (Richmond, CA). Guanidine hydrochloride was from Fluka (Ronkonkoma, NY). For reversed-phase HPLC, an altex ultrasphere-ODS column (250 mm X 4.6 mm I.D.) was obtained from Beckman (Berkeley, CA). The partisil 10-sax column (250 mm X 4.6 mm I.D.) was obtained from Whatman Chemical Separation Inc. (Clifton, N.J.). The **μ** Bondapak C\(_{18}\) column (3.9 mm X 30 cm, 10 μm particle) was
purchased from Waters Association Inc. (Milford, Mass.). The silica-based columns for gel-filtration GF-250 and GF-450 (250 mm X 9.4 mm I.D.) were from Du Pont (Newton, Co.). All other chemicals were reagent grade.
METHODS

Cell culture

Human fibroblasts and C3H10T1/2 mouse fibroblasts (clone 8) were grown in a humidified 10% CO\textsubscript{2}-air incubator at 37°C, and maintained in Dulbecco's modified Eagle's medium and Eagle's basal medium, respectively. The medium contained 10% fetal calf serum (heat inactivated at 56°C for 30 minutes) and 2 μg/ml of gentamycin sulfate. Cells from exponentially dividing stock cultures were seeded at a density of 2.0 X 10\textsuperscript{4} cells per 35 mm dish in 2 ml of medium, and allowed to grow to confluence. Culture medium was replaced with fresh medium at 48 hour intervals.

Radiolabeling

When cell density reached confluence, the medium overlaying the cells was aspirated and replaced with fresh medium containing 20 μCi ml\textsuperscript{-1} [\textsuperscript{3}H-Adenine]. The dishes were left to incubate with the radiolabeled medium overnight (≈ 16 hours). The next day, the radiolabeled medium was replaced with fresh medium 1 hour prior to cell treatment. At this point the radiolabeled cell cultures could be manipulated experimentally according to the design of the experiment.
For experiments with the human cell line, radiolabeled cell cultures were treated with MNNG to induce the synthesis and accumulation of poly(ADP-ribose). MNNG dissolved in acetone was added to the intact cell cultures. At the appropriate times, the cells were harvested by rinsing with ice-cold phosphate buffered saline, pH 7.2, followed by the addition of 1 ml 20% ice-cold trichloroacetic acid. Acid insoluble material was removed from the dishes by scraping with a rubber policeman. The samples were then centrifuged and the supernatant saved for NAD⁺ determination. The pellet was dissolved in 0.2 ml ice-cold 98% formic acid. The samples were then diluted by the addition of 10 volumes of ice-cold deionized H₂O and 1 mg of bovine serum albumin was added to facilitate precipitation. Samples were then adjusted to a final concentration of 20% (w/v) in trichloroacetic acid with the acid insoluble fraction being collected by centrifugation.

C3H10T1/2 cells were treated with varying concentrations of H₂O₂ for different time intervals. Following treatment, cells were extracted in the same manner as described above for determination of NAD⁺ and poly(ADP-ribose). However, to determine the energy charge of the cells at the time of harvest by strong anion exchange HPLC and the ATP levels by the luciferin/luciferase method, the cells were harvested by
dissolving in 0.1 N NaOH/1 mM nicotinamide. The extract was quickly (within 2 minutes) neutralized by 0.37 M H₃PO₄, and frozen. Experimental analysis was performed within 2–3 days due to the lack of stability of ATP.

**Determination of NAD⁺**

The trichloroacetic acid supernatant was diluted to 10 ml with 250 mM ammonium formate, pH 8.6 and adjusted to pH 8.6 ± 0.2 with concentrated ammonium hydroxide. The sample was applied to a 0.5 ml DHB-Sepharose column which had been pre-washed with 10 ml of 250 mM ammonium formate, pH 8.6. The column was washed with 10 ml 250 mM ammonium formate, pH 8.6 and 2 ml H₂O. NAD⁺ was eluted with 2 × 2 ml 250 mM ammonium formate, pH 4.5. All data points for NAD⁺ measurements shown in the results section of both projects are averages of two measurements that agreed within 5% of each other.

**Extraction and Measurement of NAD⁺ and ATP Levels**

Cells were dissolved in 0.1 N NaOH/1 mM nicotinamide, taking care to limit exposure to alkali to less than 2 minutes and the extract was rapidly neutralized by H₃PO₄. An aliquot was taken and assayed for ATP content by a luciferin-luciferase bioluminescence assay in a Lumitran L3000 ATP photometer (37). This assay was always done the next day as ATP is not very stable even at -20°C. The remainder of the
cell extract was treated with phenazine ethosulfate and stored in the dark to be analyzed for NAD$^+$ by the cycling assay according to the method of Jacobson and Jacobson (38).

**Determination of Polymers of ADP-ribose**

The acid insoluble pellet was dissolved in 1 ml 6 M guanidium chloride, 250 mM ammonium acetate, 10 mM EDTA, pH 6.0. One ml 1 M KOH, 100 mM EDTA was added and the sample was incubated at 37°C for 2 hours. The sample was diluted to 10 ml with 1 M guanidium chloride, 250 mM ammonium acetate, 10 mM EDTA, pH 9.0 (buffer A) adjusted to pH 9.0 ± 0.2 and applied to a 0.5 ml column of DHB-Bio-Rex that had been pre-washed with 5 ml H$_2$O and 10 ml buffer A. The column was washed with 25 ml buffer A, followed by 10 ml 1 M ammonium bicarbonate, 1 mM EDTA, pH 9.0. ADP-ribose polymers were eluted with 5 ml of H$_2$O. All data points showing ADP-ribose polymer levels were averages of duplicate measurements that agreed within 5% of each other.

**Determination of Protein-bound Mono(ADP-ribose)**

The acid insoluble pellet was dissolved in 1 ml 6 M guanidium chloride, 250 mM ammonium acetate, 10 mM EDTA, pH 6.0 and 1 ml 100 mM MOPS, 10 mM EDTA pH 7.0 containing either 2 M ammonium chloride or 2 M hydroxylamine hydrochloride. The samples were adjusted to pH 7.0 and incubated at 37°C for 6
hours. Released ADP-ribose was isolated using DHB-Bio-Rex and DHB-Sepharose. Samples were diluted to 10 ml with buffer A and adjusted to pH 9.0 ± 0.2 and applied to 0.5 ml DHB-Bio-Rex column pre-washed with 5 ml H₂O and 15 ml buffer A. The column was then washed with 10 ml buffer A followed by 20 ml 1 M ammonium acetate, 10 mM EDTA, pH 9.0, and ADP-ribose was eluted with 1 ml 1 M HCl followed by 4 ml H₂O. 1 ml 2.5 M ammonium acetate, pH 9.0, was added to the eluate and samples were diluted to 10 ml with water. The pH was adjusted to 9.0 ± 0.2 and the sample was applied to a 0.5 ml DHB-Sepharose column prewashed with 5 ml 10 mM H₃PO₄, 25 mM KCl and 10 ml 250 mM ammonium acetate, pH 9.0. The column was washed with 30 ml 250 mM ammonium acetate, pH 9.0 and 0.5 ml 10 mM H₃PO₄, 25 mM KCl. ADP-ribose was eluted with 2 ml 10 mM H₃PO₄, 25 mM KCl. The data points showing protein-bound mono(ADP-ribose) levels in project I were averages of duplicate measurements that agreed within 5% of each other.

**Enzymatic digestion and HPLC analysis**

To determine if the radiolabel eluted from DHB-Bio-Rex consisted mainly of poly(ADP-ribose), the eluted material was lyophilized to dryness. The dried sample was dissolved in 1 ml of 50 mM MOPS, pH 7.4 and 20 mM MgCl₂, digested with 1 U each of snake venom phosphodiesterase and bacterial alkaline phosphatase for 3 hours at 37° C. 100 nmols of adenosine and
deoxyadenosine were added to the digested material and it was filtered through a 0.22μ type GS millipore membrane. The filtrate was then applied in a total volume of 1 ml to a Beckman-Altex ultrasphere-ODS reversed phase column using 7 mM ammonium formate, pH 5.8, containing 11% methanol as the running buffer. The column was eluted isocratically at a flow rate of 1 ml min⁻¹. Fractions were collected every 30 seconds. The radioactivity in the fractions was determined by liquid scintillation counting.

To determine if NAD⁺ was the major component in the eluate from DHB-Sepharose, the eluate was separated on a μBondapak C18 reversed phase column. The running buffer used was 0.1 M potassium phosphate buffer at pH 6.0 at a flow rate of 1 ml min⁻¹. Radioactivity in 0.5 ml fractions was determined as before.

Strong anion exchange (SAX) HPLC

The distribution of the radiolabel in the acid soluble nucleotide pool following treatment of C3H10T1/2 cells by H₂O₂ was determined by applying an aliquot of the neutralized NaOH/nicotinamide extract directly on to a Whatman partisol-10 SAX column. The elution was based on a gradient of buffer A (0.1 M KH₂PO₄ pH 4.7) and buffer B (1 M KCl and 0.25 M KH₂PO₄ pH 4.7) at a flow rate of 1.5 ml min⁻¹. The gradient was: 15 min 100% A, 40 min 0-100% B. The retention times of
the radiolabeled adenine compounds (NAD\(^+\), AMP, ADP, ATP) were
determined by adding them as carriers to the extract obtained
from the cells. Absorbance at 254 nm was monitored and 1 ml
fractions were collected.

**Molecular sieve chromatography of poly (ADP-ribose)**

To determine the average size of the polymers
synthesized in response to \( \text{H}_2\text{O}_2 \), they were first purified by
boronate chromatography. The eluted material was lyophilized
to dryness and redissolved in 50 μl of a solution containing
0.5 U poly A, 0.125 U each of \([(\text{Ap})_{11}\text{A}], [(\text{Ap})_7\text{A}],\) and
\([(\text{Ap})_4\text{A}]\) and 0.2 mM of ADP-ribose and AMP. The samples were
fractionated by size exclusion chromatography in Du Pont
spherical silica GS-450 and GS-250 columns connected in
series. Sodium phosphate buffer at a concentration of 0.1 M
and pH 6.8 was used as the running buffer at a flow rate of 1
ml min\(^{-1}\). Absorbance was at 254 nm and fractions of 0.5 ml
were collected.

**Colony formation assays**

For survival experiments with the human cell lines,
cells from the patient and the age-matched control were
treated with MNNG for 20 minutes and then reseeded at 300-400
cells/60mm gridded dish. After 24 hours, 2 dishes were fixed
and stained for determination of plating efficiency. At least 5 dishes at each condition were incubated an additional 6-7 days for colony formation. The medium was aspirated and the cells were rinsed twice with phosphate buffered saline. The cells were fixed by 2 successive treatments with methanol: glacial acetic acid (3:1, v/v). The dishes were dried and stained with 1% methylene blue for 30 minutes. Colonies with 25 cells or more were counted. Percent survival was determined as the percentage of the cells forming colonies at each condition relative to control.

A similar procedure was followed with C3H10T1/2 cells to determine the relative colony forming ability following treatment with H$_2$O$_2$. Experiments with cycling and non-cycling cells were carried out. To obtain data with cycling cells 300-400 cell/60mm gridded dish were seeded and allowed to attach. After 8 hours, they were treated with H$_2$O$_2$ for 1 hour. Medium containing H$_2$O$_2$ was then replaced with fresh medium, and the dishes put in the incubator for the next 6 days. Experiments with non-cycling cells involved treatment of confluent cell cultures with H$_2$O$_2$ for 1 hour followed by reseeding the cells 300-400 cells/60mm gridded dish. At the completion of the incubation time, the cells were fixed and stained as before.
CHAPTER III

PROJECT I

In 1984, Williams and collaborators described the case of a six-year old boy with a lysosomal storage disease, who after a period of progressive neurological deterioration died of renal failure at the age of 8 years (39). It was found that a small molecular weight compound had accumulated in the lysosomes of the kidney and brain of the patient. The chemical structure for this material was proposed to be glutamyl ribose-5-phosphate as shown in Fig. 5. It was demonstrated that the amino acid-sugar linkage in the accumulated compound consisted of an ester bond between the \( \gamma \)-carboxyl of glutamic acid and the reducing position of ribose. Among known \( \gamma \)-linkages between carbohydrate and protein in mammalian glycoproteins and proteoglycans, this linkage was found to be unique. The two basic types of covalent linkages between carbohydrate and protein in mammalian glycoproteins and proteoglycans are either N-linked, consisting of N-acetyl glucosamine-asparagine or O-linked consisting of N-acetylgalactosamine-serine/threonine, galactose-hydroxylysine and the xylose-serine linkage (40). The linkage found in glutamyl ribose-5-phosphate was found to be the same type of linkage as found in proteins post-
Fig. 5. Structure of glutamyl ribose-5-phosphate
translationally modified by poly(ADP-ribosyl)ation. This represents the only known example in mammalian biochemistry of a sugar amino acid ester linkage. This suggested that the structural identity of the accumulated compound is most likely derived from poly(ADP-ribosyl)ated proteins.

The discovery of Glutamyl ribose-5-phosphate storage disease provided a valuable opportunity to study poly(ADP-ribose) metabolism in these cells. The explanation for this disease proposed at the initiation of this project was that the tissues accumulating glutamyl ribose-5-phosphate were deficient in ADP-ribosyl protein lyase activity and therefore were unable to cleave the bond between the proximal ADP-ribose and glutamate. The combined action of intracellular proteases and phosphodiesterases resulted in accumulation of glutamyl ribose-5-phosphate which was unable to be broken down further. In this study, the metabolism of poly(ADP-ribose) in intact fibroblasts of the patient was studied and compared to an age-matched control.
Results

In the present study, the effect of DNA damage on poly(ADP-ribose) metabolism was examined in cells from a patient with glutamyl ribose-5-phosphate storage disease. Normal human age-matched cells were used as controls for comparison. MNNG, an alkylating agent was used to induce the synthesis of ADP-ribose polymers. Benzamide, which is an effective inhibitor of the polymerase, was also used to gain insight into the metabolism of these polymers.

Poly(ADP-ribose) is made from NAD⁺ in cells and previous studies with mouse cells have shown that when ADP-ribose polymers are induced, NAD⁺ levels are depleted. The basal levels of NAD⁺ were established to determine the extent of the variations in these levels. NAD⁺ levels were measured in cells from the patient as well as in cells from both parents. This was done because family history indicated an X-linked recessive inheritance in the patient. The NAD⁺ content in human foreskin and in two age-matched control cell lines was also determined. A brief description of each cell line and its NAD⁺ content is shown in Table I. The results of all the experiments reported here were obtained with the WS cell line as the age-matched control; however, similar results were obtained with HF and JE cell lines. Cells were grown to confluence and extracted as described in methods. MC refers to the patient cells throughout this text. Basal NAD⁺ levels
## TABLE I

*NAD*⁺ Content of Human Cell Lines Studied

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>NAD⁺ pmol/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>Glu-R-P Storage Disease</td>
<td>2819 ± 186 (N=4)</td>
</tr>
<tr>
<td>HF</td>
<td>Human Foreskin</td>
<td>2522 ± 40 (N=8)</td>
</tr>
<tr>
<td>AC</td>
<td>Father of MC</td>
<td>2542 ± 44 (N=4)</td>
</tr>
<tr>
<td>JC</td>
<td>Mother of MC</td>
<td>2454 ± 45 (N=4)</td>
</tr>
<tr>
<td>WS</td>
<td>Age-matched Control</td>
<td>2474 ± 47 (N=4)</td>
</tr>
<tr>
<td>JE</td>
<td>Age-matched Control</td>
<td>2295 ± 65 (N=4)</td>
</tr>
</tbody>
</table>
were found to be similar in all the cell lines studied.

The method used in the determination of labeled NAD$^+$ levels by liquid scintillation counting is described in chapter II in the methods section. To determine if the material from the acid soluble pool eluted from DHB-Sepharose contained only NAD$^+$, a sample of labeled material was subjected to reversed phase HPLC. The profile obtained is shown in Fig. 6. Greater than 90% of the counts comigrated with NAD$^+$. The radiolabel in the smaller peak was characterized further in this laboratory in several solvent systems and was found to be ADP-ribose (41). The source of this ADP-ribose is most likely from the fraction of the total NAD pool (NAD$^+$ plus NADH) present in the reduced form (NADH) as NADH is converted to ADP-ribose by acid treatment used for extraction of nucleotide pools (6).

Confluent cell cultures from the patient and age-matched control cell lines were treated with MNNG. NAD$^+$ levels were measured as a function of time and the results are shown in Fig. 7. NAD$^+$ levels in untreated dishes from both cell types were used as the control values. Following MNNG, NAD$^+$ levels decreased in both cell lines; however, the rate of NAD$^+$ depletion in cells from the patient was slower than in the age-matched control. At 80 minutes of MNNG treatment, NAD$^+$ levels in WS cells were down to about 20% of control levels whereas in MC cells they were only down to
Fig. 6. **Reversed phase chromatography of DHB-Sepharose elutate.** Material eluted from DHB-Sepharose was fractionated on a μBondapak C<sub>18</sub> column and radiolabel was determined as described in chapter II. The expected position of migration of ADP-ribose (ADPR) and NAD<sup>+</sup> are shown.
Fig. 7. \textbf{NAD}^+ \textit{depletion following MNNG.} Confluent cell cultures radiolabeled with $[^3\text{H}]-\text{adenine}$ were treated with 68 μM MNNG. At the time points indicated, cells were harvested as described in methods. (●) indicates cells from patient with glutamyl ribose-5-phosphate storage disease. (〇) indicates an age-matched control.
about 50%. After 80 minutes, NAD⁺ levels in MC cells did not alter much but recovered to about 40% of control in WS cells.

In a similar experiment, benzamide was used to determine if the NAD⁺ depletion was due to the synthesis of ADP-ribose polymers. Cell cultures were preincubated with benzamide for 30 minutes before the addition of MNNG. As shown in Fig. 8, addition of benzamide inhibited NAD⁺ depletion. It should be noted that the benzamide was less effective on cells from the patient than on the control.

ADP-ribose polymer levels were measured in response to MNNG as described in methods. To determine if the counts eluted from DHB-Bio-Rex resin were only from poly(ADP-ribose), the eluted material was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase. This digestion results in the generation of ribosyladenosine and diribosyladenosine from the linear internal and branched residues respectively and adenosine from the terminal residues. Reversed phase chromatography gave the profile shown in Fig. 9. Approximately 80% of the radiolabel eluted at the expected elution position of ribosyladenosine and about 15% comigrated with adenosine. This result demonstrates that the bulk of the radioactive material eluted from DHB-Bio-Rex was poly(ADP-ribose). To estimate recovery of polymers in the DHB-Bio-Rex eluate, standards of radiolabeled poly(ADP-ribose) were added to unradiolabeled cell extracts.
Fig. 8. The effect of benzamide on NAD$^+$ depletion following MNNG treatment. Radiolabeled, confluent cell cultures were equilibrated with 3 mM benzamide for 30 minutes before treatment with 272 μM MNNG. The time points indicated represent the patient cell line in the absence (●) and presence (■) of benzamide, and the aged matched control cell line in the absence (○) and presence (□) of benzamide.
Fig. 9. Reversed phase chromatography of DHB-Bio-Rex eluate digested with SVFD and BAP. Details for the experimental procedure are described in methods. The expected elution positions of adenosine (Ado) and ribosyladenosine (RAdo) are shown.
Recovery was routinely found to be between 85-90%. Polymer levels were measured in the same samples that were used to show NAD⁺ depletion in Fig. 8. Poly(ADP-ribose) data obtained as counts per minute were converted to picomoles/10⁶ cells since the specific activity of poly(ADP-ribose) is identical to that of NAD⁺. The specific activity of NAD⁺ was calculated by dividing the counts per minute of NAD⁺ in radiolabeled untreated cells by the picomoles of NAD⁺ in that sample which was measured by the cycling assay. Fig. 10 shows the results of polymer accumulation in response to MNNG. Polymer accumulation was greatly reduced in the cells from the patient. For example, at 20 minutes of MNNG treatment polymer accumulation in WS cells was almost 4 times higher than in the patient cells. The peak of polymer accumulation in both cell lines was 40 minutes although the peak level in MC cells was about 40% of peak level in WS cells. A likely explanation is that the polymerase is not as active in MC cells. However, from these data alone one cannot rule out the possibility that the MC cells have an increased poly(ADP-ribose) glycohydrolase activity. Fig. 11 shows an experiment that was done to measure the turnover of the polymers. This is an indirect method of measuring the glycohydrolase activity in the intact cells. Polymer synthesis was induced by a high dose of MNNG and near the peak of accumulation of the polymers, benzamide was added to inhibit further synthesis.
Fig. 10. Accumulation of ADP-ribose polymers following MNNG treatment. The data points indicate levels of polymer measured in the acid insoluble portion of patient cells (●) and age-matched control cells (O). The dose of MNNG used was 272 μM.
Fig. 11. Effect of benzamide on the synthesis and accumulation of poly(ADP-ribose). 272 μM MNNG was used to induce a large response. At 20 minutes, 3 mM benzamide was added to inhibit further synthesis and polymer levels were measured subsequent to the addition of benzamide. Patient cells in presence (■) and absence (●) of benzamide. Age-matched control in presence (□) and absence (○) of benzamide. Arrows indicate the point at which benzamide was added.
and polymer levels were measured subsequent to the addition of benzamide. The dose of MNNG used in this experiment was 4 times higher than that shown in Fig. 10. Again polymer accumulation was far less in MC cells than in WS cells. However the increase in polymer accumulation with the increased dose of MNNG was proportional in both cell lines. At 40 minutes following MNNG, polymer levels increased from 24 pmol/10⁶ cells to 45 pmol/10⁶ cells in WS cells and from 12 pmol/10⁶ cells to 22 pmol/10⁶ cells in MC cells. The data with regard to benzamide of Fig. 11 was re-plotted as a semi-log plot for easier interpretation (Fig. 12). The estimated half-lives of the polymers generated in response to MNNG were approximately 6 minutes for MC cells and 7 minutes for WS cells. Thus, no significant difference was observed in the turnover rate of the polymers formed in the two cell lines.

The results of these experiments indicate that the difference in the level of polymer accumulation in response to MNNG is at the level of the polymerase rather than the glycohydrolase. Since it appeared that the poly(ADP-ribose) polymerase activity is altered in some way, further experiments were performed toward that end. Prelabeled confluent cell cultures were preincubated with varying concentrations of benzamide for 30 minutes before the addition of MNNG. Polymer levels were measured as a function of increasing concentration of benzamide and a dose response
Fig. 12. Rate of poly(ADP-ribose) turnover plotted as a semi-log plot. The data shown in fig. 11 is re-plotted here to show the turnover rate of the polymer. (●) represents cells from the patient and (○) represents cells from age-matched control.
Fig. 13. Inhibition of poly(ADP-ribose) synthesis by varying doses of benzamide. Cells from the patient (●) and age-matched control (○) were preequilibrated with benzamide for 30 minutes before treatment with 68 μM MNNG for 30 minutes.
curve was constructed as shown in Fig. 13. Consistent with previous data shown in Fig. 8, benzamide was less effective in inhibiting polymer accumulation in cells from the patient in comparison to the control. Also significant is the dose of benzamide that is effective in inhibiting polymer accumulation in the normal cells. Previous studies in this laboratory have determined the IC$_{50}$ value of benzamide for the polymerase in vivo to be in the very low μM range (55) which is consistent with the data shown here in Fig. 13. IC$_{50}$ is the concentration required for 50% inhibition.

The hypothesis proposed in the beginning of this chapter would predict that protein-bound mono(ADP-ribose) levels should be greatly elevated following treatment with MNNG in the patient in comparison to the control. Protein-bound mono(ADP-ribose) levels were measured following MNNG treatment as described in methods and the results are shown in Fig. 14. At 40 minutes, the monomer levels in cells from the patient were twice that in cells from the control cell line, even though at 40 minutes the polymer levels were about half those of the control. However, accumulation was transient in both cell lines.

It was of interest to determine if the decreased polymerase response to a stress would affect the survival capability in these cells. Confluent cell cultures were treated with MNNG for 20 minutes and were reseeded as
Fig. 14. Protein-bound mono(ADP-ribose) levels following MNNG. Cells from the patient (●) and age-matched control (○) were treated with 272 μM MNNG, and cultures were harvested at the times indicated. Details of the measurements are given in methods.
Fig. 15. Survival of exponentially dividing cells treated with MNNG. Cells from the patient (●) and age-matched control (○) were exposed to MNNG for 20 minutes and colony formation assays were done as described in methods.
described in the methods. The relative colony forming ability was obtained by dividing the number of colonies counted at each condition by the number of colonies in untreated dishes. Fig. 15 shows that there was no significant difference in the survival rates of the two cell lines tested.
Discussion

Williams et al. have clearly demonstrated that the compound isolated from the tissues of the eight year old patient contains covalent linkage of glutamic acid to ribose phosphate (39). The structural identity of glutamyl ribose-5-phosphate to that found in poly(ADP-ribosyl)ated proteins suggested that glutamyl ribose-5-phosphate represents the linkage region in ADP-ribosylated proteins. On the basis of these findings, Williams proposed that the molecular defect in the patient was a functional deficiency of the enzyme ADP-ribose protein lyase which catalyzes the removal of the proximal ADP-ribosyl residue from acceptor proteins (39). The proposed relationship of glutamyl ribose-5-phosphate storage disease to cellular poly(ADP-ribose) metabolism is depicted in Fig. 16. The study of a defect in the catabolism of poly(ADP-ribosyl)ated proteins may provide very valuable insights into the physiological function of these polymers. Thus far, no disease model has been found which would clearly point to a vital role of poly(ADP-ribose) biosynthesis in the normal function and survival of mammals. In the present study, the focus was on comparing the poly(ADP-ribose) metabolism in patient cells to age-matched control cells in order to further understand the biological role of poly(ADP-ribosyl)ation.
Fig. 16. A possible relationship of glutamyl ribose-5-phosphate storage disease to cellular poly(ADP-ribose) metabolism.
N+ Ade I I
R-P-P-R Nam

poly(ADP-R) polymerase

nuclear protein

ADP-Ribose protein lyase

poly(ADP-R) glycohydrolase

CH₂-CH₂-C-O-(ADPR)_n

phosphodiesterases

CH₂-CH₂-C-R-P + AMP

proteases

Glutamyl-Ribose-5-Phosphate
These studies were made possible by the development of an efficient and highly sensitive radiolabeling method that allowed the simultaneous quantification of NAD\(^+\) as well as polymers and protein-bound monomers of ADP-ribose derived from a single dish of cultured cells (41). High specific activity \(^{3}\text{H}\)adenine was used as the source of radiolabel and it was efficiently taken up and converted to NAD\(^+\) by confluent monolayer cell cultures. The use of boronate resins that are selective in retaining compounds with two or more pairs of vicinal hydroxyl groups, in combination with the \(^{3}\text{H}\)adenine radiolabeling technique has allowed the isolation and quantitation of poly(ADP-ribose) polymers produced in response to DNA damage. This technique is non-destructive with regard to the ADP-ribose polymers and has also made it possible to characterize the polymers in terms of their size and complexity.

MNNG was used to induce the synthesis of the polymers in the cell lines under study and it was found that the cells from the patient exhibited greatly reduced accumulation of polymers of ADP-ribose when compared to age-matched controls. In agreement with this data, NAD\(^+\) depletion was greatly reduced. Poly(ADP-ribose) glycohydrolase activity was normal.

Based on the data reported in this study, there are three lines of evidence for an altered poly(ADP-ribose) polymerase activity in MC cells. These are the reduced rate
of NAD⁺ depletion, reduced rate of polymer accumulation and reduced sensitivity to benzamide in MC cells when compared to the controls. One can propose two models that are most likely to explain the altered polymerase activity and the failure to detect protein-bound monomers of ADP-ribose.

One model is consistent with that proposed by Williams and coworkers that indeed MC cells are deficient in the enzyme ADP-ribose protein lyase (39). As shown in Fig. 16, poly(ADP-ribose) metabolism is a cyclic event that once activated is repeated a number of times and a block at any step would result in the accumulation of the material immediately before the block. Also the relatively short half-lives of the polymers generated after DNA damage would indicate that this system acts very rapidly. Therefore, a lack of the enzyme lyase should result in the rapid accumulation of monomers of ADP-ribose bound to protein. When the level of these monomers was measured in MC cells following induction of the poly(ADP-ribose) metabolic system, some elevation of ADP-ribose monomer levels was detected relative to the control cell line. This result was more significant due to the fact that ADP-ribose polymer levels were always lower following MNNG in MC cells relative to the control cells. Nevertheless, the elevation in protein-bound monomer levels was transient and our data are entirely consistent with the possibility that they were being
processed by another enzyme such as the phosphodiesterase as rapidly as they were being formed. As shown in Fig. 16, phosphodiesterase action followed by cellular proteases would lead to the accumulation of the compound glutamyl ribose-5-phosphate. Also consistent with the data reported here is the second model according to which the MC cells have an increased production of phosphodiesterase, rather than a deficiency in the enzyme lyase. In this case when the polymerase is activated, the resulting protein-bound ADP-ribose monomers are rapidly deadenylated before the lyase has a chance to cleave the ADP-ribose off. Similar to the first model this would also lead to the accumulation of glutamyl ribose-5-phosphate. The altered polymerase activity is consistent with both models. As mentioned before, the polymerase itself is an acceptor of ADP-ribosyl residues and once activated undergoes many rounds of modification and removal of the modifying moiety. In any one of these models, the polymerase would be normal only in the first round and after that it would be modified by a ribose-5-phosphate residue. Judging from the reduced polymer accumulation in MC cells, it appears that it may still function but on a reduced scale. The reduced effectiveness of benzamide as a polymerase inhibitor in MC cells is also consistent with both of these models. It is known that the polymerase activity is absolutely dependent on DNA binding. Although the site for
automodification is different from the DNA binding site, it is likely that the negative charge surrounding the ribose phosphate bound to the enzyme decreases the affinity of the enzyme for DNA. In addition, the Km of the enzyme for NAD⁺ is also affected by DNA binding to the enzyme. Since benzamide is a competitive inhibitor of the polymerase, it is likely that the Km for benzamide is also affected. Thus, a reduced affinity for DNA by the polymerase would result in fewer molecules of NAD⁺ and benzamide being used as substrate and substrate analog for poly(ADP-ribose) polymerase.
CHAPTER IV

PROJECT II

Although the detailed biological role of the metabolism of polymers of ADP-ribose remains unclear, they are involved in nuclear processes following the occurrence of DNA strand breaks. Among agents known to cause DNA strand breaks are various forms of oxygen-derived free radicals which are formed intracellularly as a normal consequence of a variety of essential biochemical reactions. The most potent of these in terms of its reactivity towards DNA is the hydroxyl (OH-) radical. It makes a single strand nick in DNA by extracting a proton from deoxyribose in the DNA backbone, which then breaks down due to secondary reactions causing chain cleavage. As explained previously, such breaks in DNA would be expected to elicit the stimulation of poly(ADP-ribose) polymerase. The use of certain antioxidants as anticarcinogens in experimental animals and in vitro culture systems also suggests a role for active oxygen in the transformation process (42). In this study, poly(ADP-ribosyl)ation response has been characterized in mouse fibroblasts following exposure to the OH- radical produced by hydrogen peroxide (H₂O₂). Hydrogen peroxide in the presence of Iron(II) generates OH- radicals in a reaction termed the
Fenton reaction (43). This reaction involves the one electron reduction of $H_2O_2$ and the concomitant breaking of the O-H bond to yield $OH^+$ and $OH^-$:

$$Fe_2^+ + H_2O_2 \rightarrow Fe_3^+ + OH^+ + OH^-$$

In the present study $H_2O_2$ was used as the source of $OH^-$. Because of its low reactivity and being small and uncharged it is able to diffuse freely into cells at a rate comparable to water molecules (44). $H_2O_2$ is normally produced intracellularly, as a consequence of a wide variety of metabolic reactions, where its level is kept low by enzymes such as catalase and glutathione peroxidase which complete its reduction to $H_2O$ and molecular oxygen. It is also produced extracellularly as part of the inflammatory response. When the concentration of oxygen derived species ($H_2O_2$ and $OH^-)$ exceeds the cellular antioxidant defense system, oxidative stress is likely to occur.

Interest in the role of $H_2O_2$ and free radicals in human diseases is growing rapidly. Data from Cerutti's laboratory supports the notion that DNA is a major target of oxidant attack in vivo (45). Thus the potential of antioxidants as therapeutic agents and especially as anticarcinogens is substantial and much work is being pursued on this topic. The following study describes the alterations in pyridine and
adenine nucleotide metabolism in response to oxidant injury caused by \( \text{H}_2\text{O}_2 \). In addition, polymers of ADP-ribose generated in response to DNA damage have been characterized.
Results and Discussion

Alterations in Pyridine and Adenine Nucleotide Metabolism in response to H$_2$O$_2$

In this study, the response to H$_2$O$_2$ in C3H10T1/2 cells was measured with regard to their NAD$^+$, ATP and ADP-ribose polymer levels. Confluent cell cultures were radiolabeled with [$^3$H-Adenine] as described in methods. Fig. 17 shows the distribution of radiolabel in the acid soluble fraction in untreated cells. Anion exchange chromatography was used to separate the total adenine pool into AMP, ADP, and ATP. Although there was a significant peak comigrating with NAD$^+$, over 90% of the total counts were accounted for by the adenine nucleotides. From this point the total adenine pool in the supernatant will be referred to as total counts per minute (cpm).

Cell cultures were treated with H$_2$O$_2$ and analyzed according to the flowchart shown in Fig. 4. Experimental manipulation refers to treatment of cells with varying doses of the DNA damaging agent. Following H$_2$O$_2$ exposure, NAD$^+$ levels and the total adenine pool fell drastically within one hour. As shown in Fig. 18 (A & B) this depletion was concentration dependent. The fall in NAD$^+$ levels closely paralleled the fall in the total cpm.
Fig. 17. Distribution of the adenine pool in untreated C3H10T1/2 cells. The cells were prelabeled with $[^3H]$-adenine before extraction with NaOH/nicotinamide solution as described in methods. The expected elution positions of NAD$^+$, AMP, ADP and ATP are shown.
Fig. 18 A. **Effect of hydrogen peroxide on total counts per minute.** Radiolabeled, confluent C3H10T1/2 cells were treated with 0.1 mM (○), 0.5 mM (□), 1 mM (●), and 5 mM (■) hydrogen peroxide for the times indicated. Cultures were harvested as described in methods.

Fig. 18 B. **Effect of hydrogen peroxide on NAD⁺ levels.** An aliquot was taken from the same samples mentioned in Fig. 18 A to measure NAD⁺ levels. The symbols for concentration of H₂O₂ used are the same as in Fig. 18 A.
Addition of catalase along with \( \text{H}_2\text{O}_2 \) completely blocked the depletion of \( \text{NAD}^+ \) and the total cpm pool (Figs. 19 A & B). This result demonstrated that the effects seen were due to \( \text{H}_2\text{O}_2 \) since catalase catalyzes the decomposition of \( \text{H}_2\text{O}_2 \) to yield \( \text{O}_2 \) and \( \text{H}_2\text{O} \) and is specific for \( \text{H}_2\text{O}_2 \).

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \]

Since treatment with \( \text{H}_2\text{O}_2 \) resulted in depletion of the total counts in the acid soluble fraction, the composition of this fraction was examined. On exposure of these cells to \( \text{H}_2\text{O}_2 \), the distribution in the adenine pool was altered and is shown in Fig. 20 A & B. At 15 minutes there was a marked decrease in ATP and an increase in ADP and AMP. Previously it has been found that \( \text{H}_2\text{O}_2 \) causes a rapid conversion of metabolically available ATP to AMP and consequently to inosine or inosine monophosphate (IMP) (46). In agreement with this observation, a large peak of IMP appeared at 15 minutes. Fig. 20 B shows the composition of the total counts after exposure to \( \text{H}_2\text{O}_2 \) for 45 minutes. ATP levels had recovered somewhat and AMP and IMP levels had fallen. Table II shows the data shown in Fig. 17, and Fig. 20 A & B in a quantitative form. The fluctuations in the adenine nucleotide pool were a cause for concern since these nucleotides participate in many metabolic reactions and are major factors in maintaining cellular homeostasis (47). For this reason, it was important to determine the energy charge of the cells exposed to \( \text{H}_2\text{O}_2 \). The
Fig. 19 A. **Catalase blocks the action of hydrogen peroxide on total counts per minute.** 20 µg/ml catalase was added to the cell cultures at the same time as 1 mM H₂O₂ (☐). (●) represents 1 mM H₂O₂ with no catalase added.

Fig. 19 B. **Catalase blocks the depletion of NAD⁺ by hydrogen peroxide.** NAD⁺ was measured in the same samples that were used to measure total cpm in Fig. 19 A. The symbols used are the same as in Fig. 19 B.
Fig. 20 A. **Effect of hydrogen peroxide on the distribution of the adenine pool.** A portion of a sample treated with 1 mM H₂O₂ for 15 minutes was applied to a strong anion exchange column as described in methods. The expected elution positions of Ade, NAD⁺, AMP, IMP, ADP and ATP are shown.

Fig. 20 B. **Effect of a longer treatment of hydrogen peroxide on the adenine pool.** Cell cultures were treated with 1 mM H₂O₂ for 45 minutes and the effect on the adenine pool is in the direction of recovery. The expected elution positions of Ade, NAD⁺ AMP, ADP and ATP are shown.
energy charge of a cell has been related to control of multiple metabolic functions. Once the individual ATP, ADP, and AMP concentrations are determined, the energy charge can be calculated by the equation \((\frac{ATP + 1/2ADP}{ATP + ADP + AMP})\). For control cells with no treatment the energy charge was 0.86 (table III). However, following \(H_2O_2\) for 15 minutes it dropped to 0.34. Although, it has been reported that following a metabolic stress that lowers the energy charge, recovery of normal values of energy charge is achieved by degrading the AMP formed during periods of high ATP utilization (48). It has also been shown that in some cell types, AMP deaminase is activated by a decrease in energy charge (49) and there is subsequent conversion of IMP to nucleosides and bases (50). In agreement with this, following a longer exposure to \(H_2O_2\) (45 minutes) the AMP peak decreased in size and the IMP peak almost disappeared. The energy charge rose to almost 0.6 (table III).

ATP levels were also measured by the luciferin /luciferase bioluminescent assay as described in methods (Fig. 21). \(H_2O_2\) concentration as low as 0.1 mM brought ATP levels down to about 15% of control in 15 minutes. As seen earlier in the energy charge calculations, a slow recovery was made over the next 45 minutes. However at 1 hour, ATP levels were still down to 40% of control. Previous studies with P388D1 murine macrophage cells have shown that exposure
TABLE II

Distribution of adenine and pyridine nucleotide pool in control and hydrogen peroxide (H₂O₂) treated C3H10T1/2 cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>1 mM H₂O₂, 15 minutes</th>
<th></th>
<th>1 mM H₂O₂, 45 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>% of cpm</td>
<td>cpm</td>
<td>% of cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>3834</td>
<td>6.1</td>
<td>2622</td>
<td>8.0</td>
<td>1221</td>
</tr>
<tr>
<td>AMP</td>
<td>1506</td>
<td>2.4</td>
<td>4857</td>
<td>14.0</td>
<td>2295</td>
</tr>
<tr>
<td>IMP</td>
<td>_</td>
<td>_</td>
<td>3231</td>
<td>10.0</td>
<td>396</td>
</tr>
<tr>
<td>ADP</td>
<td>9213</td>
<td>14.7</td>
<td>3909</td>
<td>11.4</td>
<td>5532</td>
</tr>
<tr>
<td>ATP</td>
<td>32865</td>
<td>52.3</td>
<td>1587</td>
<td>5.0</td>
<td>4566</td>
</tr>
<tr>
<td>Treatment</td>
<td>Energy Charge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM H₂O₂, 15 minutes</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1 mM H₂O₂, 45 minutes</td>
<td>0.59</td>
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</tr>
</tbody>
</table>
Fig. 21. ATP levels in response to hydrogen peroxide. ATP levels were measured the day after the experiment by a bioluminescent assay. Cells were treated with 0.1 mM (○) and 0.25 mM (□) $H_2O_2$. Cultures were harvested at the times indicated and the samples were stored in -20°C immediately after extraction.
to doses of $H_2O_2$ of 0.25 mM or higher induces an irreversible
depletion of $NAD^+$ and ATP (51). The authors have considered
that these events may be analogous to that reported by Sims et al. who suggested that ATP depletion following massive DNA
breakage by MNNG may represent a suicide mechanism (52).
Accordingly, high levels of DNA damage as induced by $H_2O_2$
could activate poly(ADP-ribose) polymerase to a degree that
depletes cellular $NAD^+$ and secondarily cellular ATP levels.
As a result the cells are being killed before they have a
chance to repair DNA damage.

As mentioned earlier, $H_2O_2$ treatment of C3H10T1/2 cells
resulted in $NAD^+$ depletion. The decrease in $NAD^+$ levels was
prevented by benzamide which is an effective inhibitor of
poly(ADP-ribose) polymerase. 5 mM benzamide completely
blocked $NAD^+$ depletion following $H_2O_2$ and 0.1 mM was 90%
effective in preventing $NAD^+$ depletion (Fig. 22 A). In
addition, in contrast to catalase which blocked depletion of
the total cpm pool shown in Fig. 19 A, benzamide (up to 5 mM)
had absolutely no effect on blocking the depletion of total
counts (Fig. 22 B). As mentioned earlier, the total counts
represent the total adenylate pool. Because the addition of
catalase along with $H_2O_2$ blocked depletion of total cpm, it
was assumed that ATP depletion was also inhibited. Because
benzamide did not block the depletion of total counts, it was
of interest to determine the effect on ATP levels. Previous
Fig. 22 A. Benzamide blocks depletion of NAD$^+$ levels following H$_2$O$_2$. Cells were treated with 0.5 mM H$_2$O$_2$ in the absence (□) and presence of 0.1 mM (●) and 5 mM (○) benzamide.

Fig. 22 B. Benzamide does not prevent depletion of total counts per minute following H$_2$O$_2$. % Total cpm was determined on the same samples as in Fig 22 A. The symbols used are the same as in Fig. 22 A.
work with L1210 cells showed that nicotinamide, which is also a poly(ADP-ribose) polymerase inhibitor, prevented the rapid fall in NAD\(^+\) and ATP pools following high doses of MNNG (52). However, when ATP levels were measured following benzamide and H\(_2\)O\(_2\) in a dose as low as 0.1 mM it was found that even 5 mM benzamide had no effect in inhibiting ATP depletion. The results are shown in Fig. 23. It is clear that the mechanism of ATP depletion following H\(_2\)O\(_2\) is different from that following MNNG although poly(ADP-ribose) polymerase is activated in both cases. This result is corroborated by a recent study on endothelial cells exposed to glucose-glucose oxidase and hypoxanthine-xanthine oxidase (both are oxygen radical generating systems). It was found that 5 mM nicotinamide provided substantial protection from NAD\(^+\) depletion, but ATP depletion was not prevented by 5 mM aminobenzamide or 5 mM nicotinamide (53). This finding also suggested that the NAD\(^+\) depletion does not account for the alterations in ATP metabolism. In addition, Sims et al. showed that the depletion in ATP pools was secondary to the effects of NAD\(^+\) depletion following MNNG (52) which is in contrast to the results reported here. Fig. 21 when compared to Fig. 18 B clearly shows that ATP lowering preceded NAD\(^+\) depletion, indicating that in response to H\(_2\)O\(_2\) the decline in intracellular ATP levels was not only due to falling NAD\(^+\) levels. Furthermore, Cochrane and associates have shown that
Fig. 23. Benzamide does not prevent depletion of ATP levels following $\text{H}_2\text{O}_2$. ATP levels were measured by a bioluminescent assay. 0.1 mM $\text{H}_2\text{O}_2$ was used in the presence (●) and absence (○) of 5 mM benzamide. A higher dose of 0.25 mM $\text{H}_2\text{O}_2$ in the presence (■) and absence (□) of 5 mM benzamide was also used.
in cells exposed to \( \text{H}_2\text{O}_2 \), ADP phosphorylation is inhibited by both glycolysis and mitochondria in P388D1 cell (54).

**Polymer Levels in response to \( \text{H}_2\text{O}_2 \) and the Effect of Inhibitors of Polymerase**

ADP-ribose polymers were synthesized in C3H10T1/2 cells when exposed to \( \text{H}_2\text{O}_2 \) and this response was dose dependent (Fig. 24). The advantage of the technique used to measure polymer levels (as described in methods) was that they were isolated intact and further characterization was possible. The polymers were synthesized very rapidly, the peak being reached within 10 minutes. By 30 minutes, they were degraded and their level was down to basal levels, whereas as seen in Fig. 18 B NAD\(^+\) levels continued to drop. This is partly due to the very high turnover rate of the polymers and partly due to the DNA strand breaks being repaired rapidly so that no more polymers are generated. The synthesis of the polymers was completely blocked by addition of catalase (Fig. 25 A) just as catalase inhibited NAD\(^+\) depletion. Because catalase inactivates the OH\(^-\) radical, no DNA strand breaks are produced and as a result poly(ADP-ribose) polymerase was not activated. Catalase inhibited polymer synthesis in a dose dependent manner and a minimum concentration of 20 \( \mu \text{g/ml} \) of catalase was needed to completely block polymer synthesis (Fig. 25 B). This is consistent with the fact that 20 \( \mu \text{g/ml} \)
Fig. 24. Poly(ADP-ribose) levels following hydrogen peroxide in C3H10T1/2 cells are dose dependent.

Poly(ADP-ribose) levels were measured in counts per minute as described in methods. The cpm were converted to picomoles by first measuring the NAD+ levels in picomoles by the cycling assay. From this, a value for cpm per picomole was obtained for NAD+. This value could also be used for converting cpm of polymer to picomoles of polymer since the specific activity of the polymer is identical to NAD+. The dose of H2O2 used was 0.1 mM (○), 0.5 mM (□), and 1 mM (●).
Fig. 25 A. Catalase blocks polymer synthesis by hydrogen peroxide. The response to 1 mM H$_2$O$_2$ (●) was completely inhibited by 20 µg/ml catalase (□). Catalase was added at the same time as H$_2$O$_2$.

Fig. 25 B. The action of catalase is dose dependent. Poly(ADP-ribose) levels were measured after 10 minutes of incubation with 1 mM H$_2$O$_2$ and the appropriate dose of catalase.
A. Plot of Poly(ADP-Ribose) concentration in pmol/10E6 cells over time in minutes.

B. Plot of the percentage of Poly(ADP-Ribose) relative to control over varying concentrations of catalase in μg/ml.
Fig. 26. **Effect of benzamide on polymer levels following hydrogen peroxide.** Cell cultures were preincubated with 0.1 mM benzamide for 30 minutes prior to addition of H$_2$O$_2$ (■). A dose of 0.75 mM H$_2$O$_2$ was used. (□) represents no benzamide.
also completely blocked NAD\(^+\) depletion. As expected, benzamide also prevented the synthesis of the polymers and as little as 0.1mM benzamide brought about almost complete inhibition as shown in Fig. 26. It must be noted that 0.1 mM benzamide was only 90\% effective in inhibiting NAD\(^+\) depletion (Fig. 22 A). One explanation may be that one of the NAD\(^+\) consuming reactions in the cell is that catalyzed by NAD\(^+\) glycohydrolase. Rankin et al. have reported that studies with partially purified NAD\(^+\) glycohydrolase *in vitro* indicate that this enzyme is far less sensitive to inhibition by benzamide than poly(ADP-ribose) polymerase (55). Benzamide is inhibitory in the \(\mu\)M range for the polymerase, but in the mM range for the NAD\(^+\) glycohydrolase (55).

*Comparison of the rate of turnover of polymers generated in response to \(H_2O_2\) and MNNG*

Prelabeled cell cultures were treated with \(H_2O_2\) and MNNG in separate dishes. At the peak of accumulation of ADP-ribose polymers, benzamide was added to inhibit further synthesis of the polymers. Polymer levels were then measured at timed intervals following the addition of benzamide. The degradation curve shown in Fig. 27 was established using the time point of benzamide addition as the zero time point of polymer degradation. The half-lives of the polymers generated in response to \(H_2O_2\) and MNNG were estimated from these data.
The half life represents the time taken to degrade 50% of the polymers. The rate of degradation of these polymers provides an estimation of poly(ADP-ribose) glycohydrolase activity in the cells. Alvarez-Gonzales et al. have reported a biphasic mode of decay of polymers formed consequent to MNNG treatment in cultured hepatocytes. The initial half-life was observed to be less than 2 minutes while subsequent degradation occurred with a half-life of 7.7 h (23). In agreement with their findings, biphasic degradation was observed for MNNG induced polymers (Fig. 27). The half-life of the initial decay was found to be < 2 minutes and subsequent degradation was considerably longer. Polymers formed in response to H2O2 did not exhibit biphasic degradation. The half-life of these polymers was approximately 6 minutes. Interestingly, Jacobson and associates determined polymer catabolism following hyperthermic treatment in SVT2 cells and found that there was a 30-fold increase in the apparent half-life of the polymers (56). More recently, their experiments with C3H10T1/2 cells also showed an increase in polymer half-life following hyperthermia (57). A similar inference has been made by Singh et al. who observed persistently elevated polymer levels in human fibroblasts treated with the tumor promoter phorbol-12-myristate-13-acetate (58). Similar to H2O2, the action of this tumor promoter is thought to mediate by oxygen radicals (59).
Fig. 27. **Comparison of turnover of polymers synthesized in response to MNNG and H$_2$O$_2$.** Prelabeled confluent cell cultures were treated with 1 mM H$_2$O$_2$ for 10 minutes and 68 μM MNNG for 20 minutes. Immediately after the treatment time was over, the medium was replaced by medium containing 3 mM benzamide. Dishes were then harvested at the times shown in the figure. Polymer levels were measured as described previously. (□) represents MNNG treatment and (●) represents H$_2$O$_2$ treatment.
Relative Size Distribution of the Polymers Generated in Response to $H_2O_2$ and MNNG

There is a rapid accumulation of ADP-ribose polymers when DNA is damaged by $H_2O_2$ or MNNG. It has already been shown that the turnover rates and the mode of decay of polymers formed following MNNG and $H_2O_2$ differ considerably. It was of interest to determine whether the size and complexity of the polymers generated depended in any way on the damaging agent used. In a previous study conducted in this laboratory, polymers formed in response to MNNG and hyperthermia were characterized in terms of their size and complexity (5). In the present study the focus was on characterizing the polymers generated following treatment with $H_2O_2$ and to compare the results with MNNG induced polymers. The methodology used to size the polymers was developed in this laboratory and involved purification of polymers with a boronate resin followed by fractionation according to size by molecular sieve high-performance liquid chromatography (5). More details of the procedure followed is given in methods. First, polymers were generated by treatment with $H_2O_2$ and then they were isolated as described in methods and sized by molecular sieve chromatography. Fig. 28 A shows the profile of polymer size distribution obtained in response to treatment with $H_2O_2$ for 10 minutes, which represents the peak of polymer accumulation. The polymers that accumulated in 10
Fig. 28 A. **Molecular sieve chromatography of ADP-ribose polymers formed in response to H₂O₂.** A concentration of 1 mM H₂O₂ was used for 10 minutes. Polymers were measured as described in methods. Size estimation of polymers in each fraction was done by running a standard of poly A (126 residues) and shorter oligomers of (Ap)₁₁A, (Ap)₇A, (Ap)₄A and AMP at the same time as the polymer sample. Poly A eluted at fraction numbers 7-9 and (Ap)₁₁A, (Ap)₇A, (Ap)₄A and AMP eluted at fraction numbers 22, 23, 24, and 26 respectively.

Fig. 28 B. **Molecular sieve chromatography of polymers formed in response to MNNG.** A concentration of 68 μM MNNG was used for 20 minutes. The elution times of the standards used for polymer size estimation are the same as in Fig. 28 A.
minutes following \( \text{H}_2\text{O}_2 \) consisted of basically a long-chain or complex polymer fraction and a short-chain fraction. The relative size of the polymers was related to the different sized poly A standards used. Fig. 28 B depicts the profile obtained following treatment with MNNG for 20 minutes which is the peak of accumulation of MNNG induced polymers. The basic size distribution of the these polymers was similar to \( \text{H}_2\text{O}_2 \) induced polymers, however, there was a greater percentage of the complex polymer fraction. In addition, the broader plateau region indicated a range of medium to long-chain polymers. The degradation of these polymers was examined by addition of benzamide at the peak of accumulation of the polymers. Benzamide would inhibit further synthesis of the polymers and the level of the polymers remaining would be indicative of poly(ADP-ribose) glycohydrolase activity. Fig. 29 shows the results of adding benzamide at 10 minutes of \( \text{H}_2\text{O}_2 \) treatment and incubating for a further 16 minutes before extraction. The total counts in ADP-ribose polymers decreased progressively with longer incubation with benzamide. To study the relative rates of degradation of polymers of different size, the data were plotted as % of total cpm vs fraction number (Fig. 30 A & B). It was observed that there was preferential degradation of the long-chain fraction as indicated by the decrease in relative peak size of large polymers and increase in relative peak size of smaller
Fig. 29. Profile of polymer degradation following H$_2$O$_2$. Polymer synthesis was induced by 0.5 mM H$_2$O$_2$. At 10 minutes, 3 mM benzamide was added and polymer levels measured subsequently as described before. The elution times for the standards used in estimating polymer size are the same as in Fig. 28 A. The duration of benzamide treatment is represented by 0 (□), 4 (○), 12 (◇) and 16 (△) minutes.
Fig. 30 A & B. Preferential degradation of ADP-ribose polymers formed in response to H₂O₂. The data from Fig. 29 is plotted here as % of total cpm. The elution times for the standards used in estimating polymer size are the same as in Fig. 28 A. In Fig. 30 A the y-axis has been expanded to show the variation in the large size fraction. (□) represents 0.5 mM H₂O₂, followed by 4 minutes (○), and 16 minutes (△) benzamide.
polymers. This result is consistent with a recent report that the Km of poly(ADP-ribose) glycohydrolase decreases dramatically with increasing sizes of polymer substrates (60). Since the turnover rates of MNNG and H2O2 induced polymers were found to be different, the relative rate of degradation of these polymers was compared. To obtain data similar to that shown in Figs. 29 and 30 A & B, benzamide was added at 20 minutes of MNNG treatment and left to incubate for a further 20 minutes. The results are shown in Fig. 31. The overall result was similar to that shown in Fig. 29. The total counts in ADP-ribose polymers decreased in both peak fractions. Again, to look at the relative rates of degradation by the glycohydrolase, the data were plotted as shown in Fig. 32. Again, preferential degradation of the medium to long-chain fraction was observed as indicated by the decrease in the relative size of the higher molecular weight peak and increase in the relative size of the lower molecular weight peak. It has already been shown that polymers formed in response to MNNG have a higher initial rate of turnover than H2O2 induced polymers. Again, this is consistent with the fact that glycohydrolase has a higher affinity for large size polymers and the proportion of higher molecular weight polymers is greater following MNNG.
Fig. 31. Molecular sieve chromatography of polymers synthesized after treatment with MNNG. A dose of 68 μM MNNG was used and 3 mM benzamide was added at 20 minutes. The elution times for the standards used in estimating polymer size are the same as in Fig. 28 A. (□) represents 0 benzamide followed by 2.5 (○), 10 (◇) and 20 (△) minutes of benzamide.
Fig. 32. Preferential degradation of ADP-ribose polymers formed in response to MNNG. The dose of MNNG used was the same as in Fig. 31. The elution times of the standards used for polymer size estimation are the same as in Fig. 28 A. Duration of benzamide treatment is represented by 0 (□), 5 (○) and 20 (△) minutes.
Cytotoxicity of H$_2$O$_2$ and its Enhancement by Benzamide

Treatment with H$_2$O$_2$ leads to rapid NAD$^+$ and ATP depletion which participate in control of multiple metabolic functions and therefore can result in cell dysfunction and death. It was of interest to determine the effect of H$_2$O$_2$ on cell survival. Table IV shows the effect of H$_2$O$_2$ on relative colony forming ability which is a measure of the toxicity of H$_2$O$_2$ on cycling and non-cycling cells. The experimental technique used is as described in methods. It was observed that the cycling cells were more sensitive to the toxicity of H$_2$O$_2$ than non-cycling cells. A concentration of 0.1 mM H$_2$O$_2$ was about 2.5 times more toxic for cycling cells probably because of active DNA replication. However, the relatively high toxicity in the non-cycling cells as well suggested that DNA is not the only target for H$_2$O$_2$ induced cytotoxicity but the effects on NAD$^+$ and ATP depletion helped increase the toxicity factor. It has long been known that inhibitors of ADP-ribosylation reactions strongly potentiate the cytotoxicity of DNA damaging agents (61) indicating that these reactions are required for cellular recovery from the cytotoxic effects of these agents. To measure the enhancement of cytotoxicity by benzamide in non-cycling cells, the following experiment was performed. Confluent cell cultures were treated with 0.1 mM H$_2$O$_2$ for 1 hour in the presence and absence of varying doses of benzamide. Details of the procedure are described in
TABLE IV

Effect of hydrogen peroxide cytotoxicity on cycling and non-cycling cells

Non-cycling cells

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<thead>
<tr>
<th></th>
<th>Relative Colony Forming Ability</th>
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<tbody>
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<td>0.1mM H₂O₂</td>
<td>53</td>
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</tbody>
</table>

Cycling cells

<table>
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<tr>
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<th>Relative Colony Forming Ability</th>
</tr>
</thead>
<tbody>
<tr>
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<td>100</td>
</tr>
<tr>
<td>0.01mM H₂O₂</td>
<td>99</td>
</tr>
<tr>
<td>0.05mM H₂O₂</td>
<td>38</td>
</tr>
<tr>
<td>0.1mM H₂O₂</td>
<td>23</td>
</tr>
</tbody>
</table>
Fig. 33. **Enhancement of H$_2$O$_2$ induced cytotoxicity by benzamide.** Cytotoxicity enhancement ratio was obtained as described in results and discussion. The arrow indicates the approximate IC$_{50}$ value.
methods. The dishes were left to incubate for 5-6 days and colonies containing 25 or more cells were counted. A separate set of dishes were treated with benzamide only using identical doses, to correct for cytotoxicity caused by benzamide alone. The cytotoxicity enhancement ratio was obtained by dividing the RCFA of H$_2$O$_2$ + benzamide treatment by the RCFA of benzamide alone. Fig. 33 shows the enhancement of H$_2$O$_2$ cytotoxicity by benzamide. In a recent paper describing studies of inhibitors of ADP-ribosylation it was reported that poly(ADP-ribose) polymerase is inhibited both in vitro and in vivo by μM concentrations of benzamide (55). Whereas, in the same report it was shown that mono(ADP-riboseyl) transferases are inhibited in vitro by mM concentrations of benzamide. As shown in Fig. 33, mM concentrations of benzamide were required to enhance H$_2$O$_2$ cytotoxicity. It would be interesting to compare the mechanisms of action of H$_2$O$_2$ and MNNG on cell survival. Unpublished results from this laboratory indicate that the curve for the enhancement of cytotoxicity of MNNG by benzamide would lie to the far left in Fig. 33 that is, in the range of μM concentrations of benzamide. The estimated IC$_{50}$ for benzamide from that curve is approximately 50 μM. The approximate IC$_{50}$ for benzamide from the H$_2$O$_2$ curve is 3 mM as shown by the arrow in Fig. 33. Although very little information is available at this point about the mechanisms of H$_2$O$_2$ induced cytotoxicity, it is
proposed in this study that \( \text{H}_2\text{O}_2 \) induced cytotoxicity is mediated by effects on mono(ADP-ribosyl) transferases rather than on poly(ADP-ribose) polymerase. The analysis of the effects of varying concentrations of benzamide on other biological end points, may prove valuable for assigning their physiological effects to poly(ADP-ribose) polymerase or mono(ADP-ribose) transferases.

It is evident from this study that oxidant injury to cells rapidly alters NAD\(^+\) and ADP-ribose polymer metabolism. ATP pools are also rapidly depleted however, the data with regard to alterations in the adenine and pyridine nucleotide pools, is consistent with a mechanism for ATP depletion independent of ADP-ribose polymer metabolism. Nevertheless, the rapid stimulation of poly(ADP-ribose) polymerase upon oxidant exposure together with effects on cytotoxicity by ADP-ribosylation inhibitors indicate that poly(ADP-ribosyl)ation reactions play a role in recovery from oxidative stresses. Cellular prooxidant states as induced by various forms of active oxygen are suppressed by enzymes of the cellular antioxidant defense system. These enzymes are superoxide dismutase, catalase, peroxidase, and glutathione peroxidase. Many antioxidants are known to be anticarcinogens and it has been shown that inhibitors of poly(ADP-ribosyl)ation reactions strongly potentiate the cytotoxicity of DNA alkylating agents such as MNNG. Therefore, based on
the data presented here it is proposed that poly(ADP-ribose) polymerase may yet be another enzyme of the cellular antioxidant defense system.
BIBLIOGRAPHY


42. Ames, B. N. (1985) *Science* 221, 1256-1264


