POLY(ADP-RIBOSE) METABOLISM FOLLOWING DNA DAMAGE

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We hereby recommend that the dissertation prepared under our supervision by Kathleen Mary Antol entitled Poly(ADP-ribose) Metabolism Following DNA Damage.

be accepted as fulfilling this part of the requirements for the Degree of Doctor of Philosophy.

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INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is well known as an electron carrier in biological oxidation-reduction reactions. However, many other reactions of biological significance consume NAD as a substrate. The first of these reactions to be identified was the utilization of NAD as a substrate for bacterial DNA ligase. This enzyme forms a pyrophosphate bond between the 5' phosphate of the AMP portion of NAD and the phosphate on the 5' side of a nick in DNA. The energy from the hydrolysis of this bond is subsequently utilized to seal the nick (Olivera and Lehman, 1967). Another class of reactions which consumes NAD as a substrate is that catalyzed by ADP-ribosyl transferases. Two types of ADP-ribosylation reactions occur. In the first, a mono-ADP-ribosyl transferase forms a covalent bond between the ADP-ribose unit of NAD and an acceptor protein. Several bacterial toxins have mono-ADP-ribosyl transferase activity. For example, both Diphtheria and Pseudomonas toxins catalyze the modification of elongation factor 2 with a single ADP-ribose (Honjo et al., 1968; Honjo et al., 1969; Honjo et al., 1971; Iglewski and Kabat, 1975). Cholera toxin catalyzes the ADP-ribosylation of the

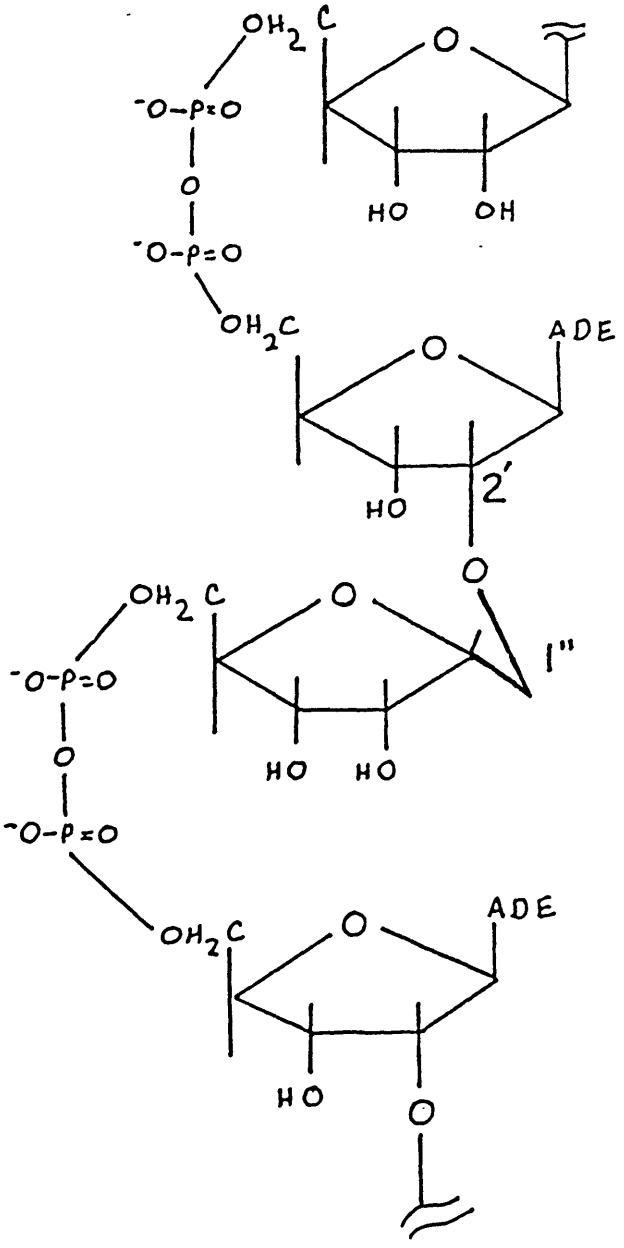
GTP-binding protein of adenylate cyclase in intestinal cells (Gill and Meren, 1978). After infecting a bacterial host, some bacteriophages have been found to synthesize proteins which have ADP-ribosyl transferase activity. These proteins from T4 phage modify host-cell RNA polymerase activity by ADP-ribosylation (Goff, 1974; Rohrer et al., 1975). Coliphage N4 ADP-ribosylates many host proteins (Pesce et al., 1976). An ADP-ribosyl transferase which activates adenylate cyclase has also been found in turkey erythrocytes (Moss and Vaughn, 1978). ADP-ribosyltransferase activity is found in most eukaryotic organisms examined to date (Purnell et al., 1980).

The studies of this dissertation are concerned with a second type of ADP-ribosylation which occurs in eukaryotic nuclei: poly(ADP-ribosylation). In this type of reaction, ADP-ribose units of NAD are polymerized into a polymer which is believed to be attached to protein, although free, unbound polymer has been found in isolated nuclei (Jump and Smulson, 1980; Benjamin and Gill, 1980a). This unusual homopolymer of ADP-ribose found in eukaryotic nuclei has been named poly(ADP-ribose). The enzyme responsible for this polymerization is called poly(ADP-ribose) polymerase (Hilz and Stone, 1976; Hayaishi and Ueda, 1977).

Evidence for the existence of poly(ADP-ribose) was first shown in 1963 by Chambon, Weill, and Mandel (Chambon et al., 1963). It was observed in isolated hen liver nuclei that nicotinamide mononucleotide (NMN) stimulated adenine-labeled ATP incorporation into an acid in insoluble product. In 1965, Fujimura, Hasegawa, and Sugimura made an equivalent observation using rat liver nuclei (Sugimura, 1973). In 1966, after exhaustive structural studies, Chambon and his workers reported that the product was poly(ADP-ribose); the detailed structure of this molecule was presented (Chambon et al., 1966). The structure of poly(ADP-ribose) was also determined by Sugimura and co-workers and Hayaishi and his workers in 1967 (Hayaishi and Ueda, 1976; Sugimura, 1973). The structure of poly(ADP-ribose) is shown in Figure 1.

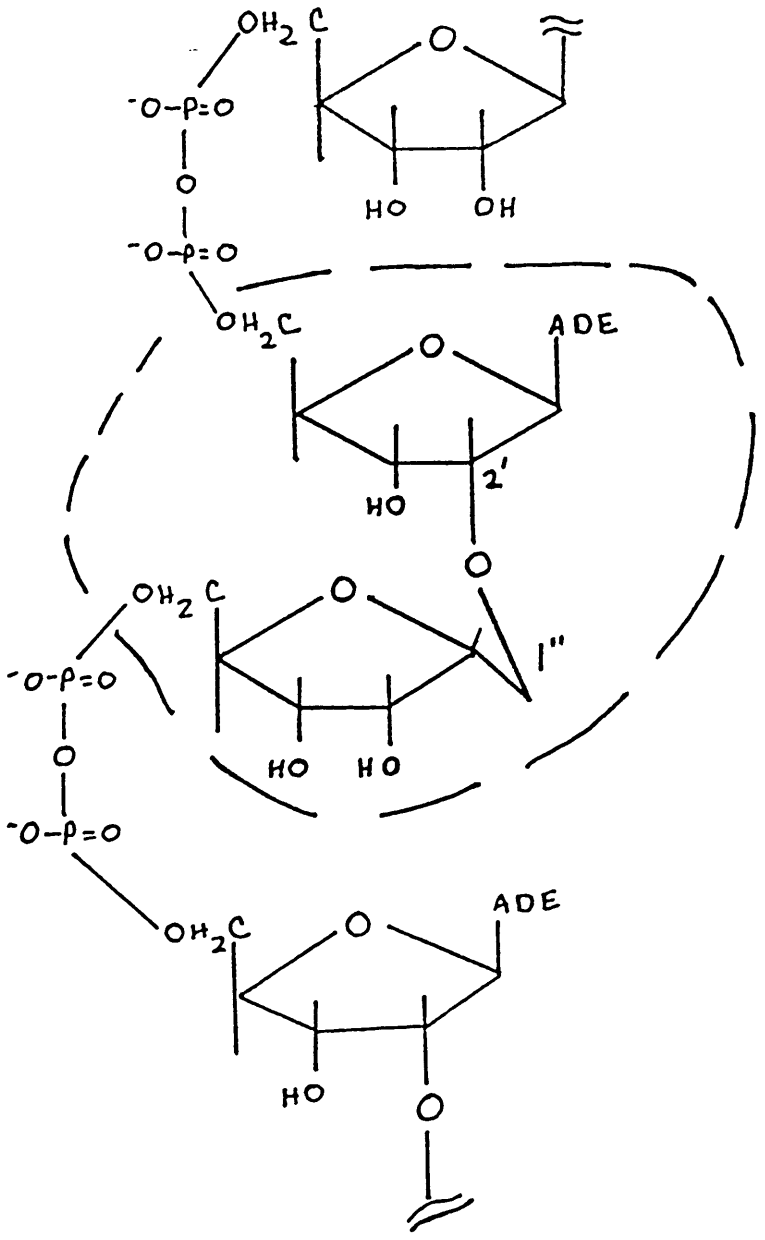
Poly(ADP-ribose) is composed of ADP-ribose moieties which are linked through a glycosidic (1" - 2') ribosyl ribose bond. The back-bone of the structure consists of pyrophosphate linkages alternating with ribose-ribose linkages and is thus chemically very different from the classic ribose phosphodiester linkages of polynucleotides (Hayaishi and Ueda, 1977). Nevertheless, poly(ADP-ribose) maintains the general characteristics of polynucleotides: It is soluble in water, precipitates with acid (Sugimura,

Figure 1. Structure of poly(ADP-ribose).


OH2C
O
-O-P=O
O
-O-P=O
OH2C
HO
OH
ADE
O
HO
2'
O
OH2C
O
1"
-O-P=O
O
-O-P=O
HO
HO
OH2C
O
ADE
HO
O

1973). However, due to the unusual linkages, poly(ADP-ribose) is neither hydrolyzed by DNase nor RNase. Snake venom phosphodiesterase (Crotalus adamanteus), however, catalyzes the hydrolysis of the polymer by cleaving the pyrophosphate bonds. The major products of this hydrolysis are ribose-5-phosphate attached to an acceptor molecule, 5'-AMP from the termini, and a number of 2'[5"-phospho-ribosyl]-5'-AMP (PR-AMP) molecules from the interior. Alkaline phosphatase (bacterial, potatoe, pancreatic) can be used to cleave the phosphates from the above products leaving ribose attached to the acceptor protein, adenosine from the termini, and ribosyladenosine from the interior residues (Sugimura, 1973). Figure 2 presents the structure of ribosyladenosine. This nucleoside is entirely unique to poly(ADP-ribose) and thus forms the basis of the technique for the quantification of in vivo levels of the polymer. Following separation of poly(ADP-ribose) from cellular DNA, RNA, and proteins by dihydroxyboryl-sepharose affinity chromatography, complete hydrolysis with snake venom phosphodiesterase and alkaline phosphatase yields ribosyladenosine. Treatment with chloracetaldehyde yields a fluorescent derivative, 1, N^6-ethenoribosyl-adenosine, which is separated from other fluorescent

Figure 2. Structure of ribosyladenosine.


OH2C
O
HO
OH
-O-P=O
O
-O-P=O
OH2C
ADE
O
HO
2'
O
OH2C
O
1"
-O-P=O
O
HO
HO
-O-P=O
OH2C
O
ADE
HO
O

compounds by reversed-phase high pressure liquid chromatography (HPLC) and quantified by fluorescence detection (Sims et al., 1980).

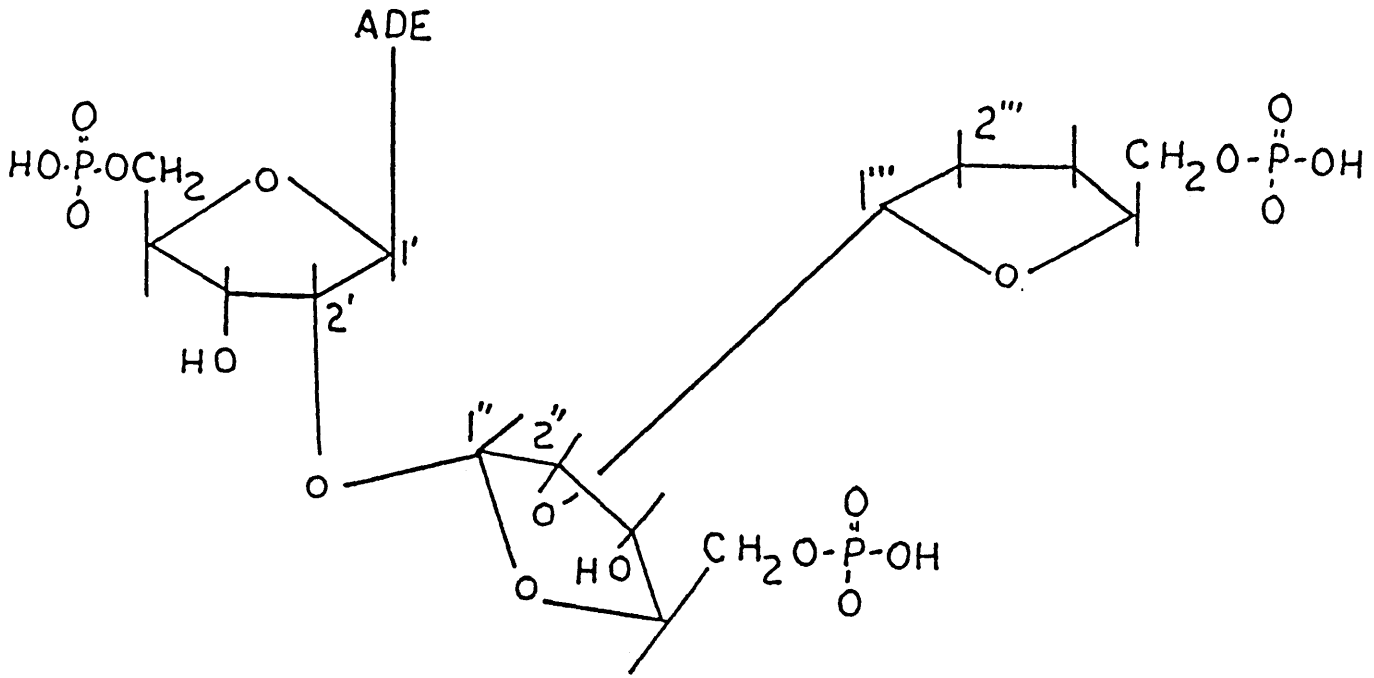
The PR-AMP portion of poly(ADP-ribose) has also been used as an analytical tool. After injecting chickens with ^{32}P-orthophosphate, ^{32}P-PR-AMP was isolated from snake venom digests of liver extracts (Doly and Mandel, 1967).

The size of the poly(ADP-ribose) molecule in biological systems is unknown. Average chain length, as determined by the ratio of total adenine residues over terminal adenine residues is less than 30 when poly(ADP-ribose) is synthesized in vitro (Tanaka et al., 1978). However, it has been observed that the molecule often behaves as a very high molecular weight during gel-filtration, gel electrophoresis, and sucrose density gradient centrifugation (Sugimura et al., 1973; Tanaka et al., 1978). Miwa et al. (1979) have explained that high molecular weight behavior could comply with short chain length if the molecule had a branched structure. A branched structure was indeed identified and the structure of the branch point has been characterized as 2'-[1''α-ribosyl-2''(1'''α-ribosyl)]adenosine 5', 5'', 5''' tris (phosphate). After digestion of poly(ADP-ribose)

synthesized *in vitro* with snake venom phosphodiesterase, the remaining branch structure is shown in Figure 3. It is estimated that branching occurs every 20-30 residues (Miwa et al., 1980). Juarez-Salinas et al. (1982) have shown that poly(ADP-ribose) is branched *in vivo*. Complete digestion of the polymer with snake venom phosphodiesterase and alkaline phosphatase converts the branch point to diribosyladenosine. When poly(ADP-ribose) from MNNG-treated SVT2 cells was completely hydrolyzed and the resulting mixture reacted with chloracetaldehyde, etheno-diribosyladenosine was detected.

Poly(ADP-ribose) polymerase is localized in chromatin (Ueda et al., 1968). It is a DNA dependent enzyme, whose activity is destroyed by DNase, but not by RNase (Chambon et al., 1966; Nishizuka et al., 1967; Ueda et al., 1968). When this activity is lost by DNase treatment, it is restored by the addition of double-stranded DNA (Chambon, 1966). Ciri et al. (1978) localized poly(ADP-ribose) polymerase in the internucleosomal linker regions of chromatin. After digesting HeLa cell chromatin with micrococcal nuclease, they found that dimer, trimer, and oligomer nucleosome fragments retained poly(ADP-ribose) polymerase activity. When mononucleosomes with remaining linker regions

Figure 3. Structure of nucleotide at poly(ADP-ribose) branch point.


ADE
HO·P·OCH2
HO
1'
2'
HO
1''
2''
O
HO
CH2O-P-OH
1'''
2'''
CH2O-P-OH

were removed, poly(ADP-ribose) polymerase activity was lost.

Poly(ADP-ribose) is also chromatin-associated; it is covalently linked to protein (Ueda et al., 1975). Nishizuka et al. (1968) showed that after incubating rat liver nuclei with [adenine-^{14}C]NAD, the acid insoluble radioactivity was associated with the histone fraction. Ueda et al. (1975) isolated poly(ADP-ribose) bound to histones from rat liver nuclei. By using a dihydroxyboryl Bio-gel P60 column, Okayama et al. (1978) showed that in rat liver nuclei histone H1 and H2B and several non-histone proteins were ADP-ribosylated. Adameitz et al. (1979) found that 40% of the ADP-ribosylated proteins from Erhlich ascites tumor cells were nucleosomal core proteins and 60% were non-histone proteins. Among the latter were 3 major species: MW 12,000, MW 31,000, and MW 125,000. In a defined in vitro system, Ferro and Olivera (1982) have discovered that free H1 is poly(ADP-ribosylated) in preference to H1 bound to DNA.

Several types of covalent linkages between poly-(ADP-ribose) and protein have been proposed including a carboxylic acid ester (Nishizuka et al., 1968; Nishizuka et al., 1979), a Schiff-base with lysine (Kun et al., 1976), or a phosphodiester linkage with phosphoserine

(Smith and Stocken, 1975). In vitro ADP-ribosylation of rat liver H1 followed by peptide analysis revealed that this histone can be ADP-ribosylated in 3 places: 2-glu, 14-glu, and 213-lys. H2B was ADP-ribosylated only at 4-glu. It was concluded that the linkage is an ester linkage with the γ-carboxyl of glu. The linkage is labile in both neutral NH_2OH and mild alkali (Ogata et al., 1980a; Ogata et al., 1980b). Yoshihara et al., (1977) found poly(ADP-ribose) is covalently linked to poly(ADP-ribose) polymerase from calf-thymus extracts. Ogata et al. (1981) showed that multiple auto-poly(ADP-ribosylation) occurs. In addition Jump and Smulson (1980) have shown the occurrence of free, unbound poly(ADP-ribose). Ferro and Olivera (1982) suggest that the amount of auto-poly(ADP-ribosylation) determines the extent of poly(ADP-ribosylation accomplished by each enzyme molecule.

Chromatin is the apparent site for both the synthesis and the degradation of poly(ADP-ribose). Two enzymes are responsible for the degradation of poly(ADP-ribose) in vivo. The first is poly(ADP-ribose) phosphodiesterase. This enzyme cleaves the pyrophosphate bonds of the back-bone of the polymer (Fatai, 1967). The second enzyme is poly(ADP-ribose) glycohydrolase. This enzyme cleaves

the ribosyl (1"-2') ribose bond (Miwa et al., 1971; Ueda et al., 1972). This enzyme is inhibited by ADP-ribose and cAMP (Ueda et al., 1972). The apparent role *in vivo* for this enzyme is the turnover of poly(ADP-ribose) (Miyakawa et al., 1972). In various rat tissues, Miwa et al. (1975) showed that it is the major degradative enzyme of poly(ADP-ribose). However, poly(ADP-ribose) glycohydrolase does not remove the final ADP-ribose residue from protein (Burzio et al., 1976). Whether or not it is removed by another enzyme is not known. Stone et al. (1977) correlated a decrease in poly(ADP-ribose) glycohydrolase activity with synthesis of H1 histone dimer in rat mammary tumor cells. It was suggested by Lorimer et al. (1977) that poly(ADP-ribose) glycohydrolase controls the formation of the H1-poly(ADP-ribose) complex.

Studies of poly(ADP-ribose) have been conducted *in vitro*, employing cell-free systems, isolated nuclei, or permeabilized cell systems. To measure the synthesis of poly(ADP-ribose) in these systems, the activity of poly-(ADP-ribose) polymerase is monitored by following the conversion of NAD into acid precipitable material. Studies have been limited to *in vitro* investigations because: 1) the NAD molecule cannot cross the plasma membrane of an intact cell. Therefore, no suitable precursor exists

for labeling ADP-ribosylated proteins (Purnell et al., 1980); 2) until recently, methodology for the isolation and direct quantification of poly(ADP-ribose) *in vivo* has not been available. Thus, the biological function of poly(ADP-ribose) has remained unknown since its discovery. One definite problem in *in vitro* studies has been the difficulty of correlating observed polymerase activity with a defined cell function (Purnell et al., 1980). Nevertheless, the participation of poly(ADP-ribose) in a variety of cellular processes has been implicated, such as DNA synthesis, cellular proliferation, cell cycle events, differentiation and development, alteration of chromatin structure, and DNA repair (Hilz and Stone, 1976; Hayaishi and Ueda, 1977; Purnell et al., 1980). Definitive evidence correlating poly(ADP-ribosylation) with any of these functions had not yet been presented, with the possible exception of DNA repair (Purnell et al., 1980).

Many studies have shown that cellular NAD levels are decreased by carcinogens and other DNA damaging agents. Roitt (1956) was the first to report that a DNA alkylating agent decreased cellular NAD levels to the extent that glycolysis was affected. Among other agents which decrease NAD levels are a glucose derivative of the alkylating agent 1-methyl-1-nitrosourea (MNU), streptozotocin

(Schein et al., 1968; Ho and Hashim, 1972; Chang, 1972; Hinz et al., 1973; Schein et al., 1973; Gunnarson et al., 1974; Smulson et al., 1975; Davies et al., 1976), neocarzinostatin (Davies et al., 1977; Goodwin et al., 1978), ionizing radiation (Campagnari et al., 1966; Hilz et al., 1963; Davies et al., 1977), nucleases (Benjamin and Gill, 1980a), MNNG (Jacobson and Jacobson, 1978a; Jacobson and Jacobson, 1978b), and many other classes of chemicals that damage DNA (Rankin et al., 1980). These in vitro studies have suggested that DNA damaging agents decrease NAD levels through the synthesis of poly(ADP-ribose). Smulson et al. (1975) found that if HeLa cell nuclei were treated with streptozotocin, NAD levels decreased while the activity of poly(ADP-ribose) polymerase activity increased; the same results were found when HeLa nuclei were treated with MNU (Smulson et al., 1977). Benjamin and Gill (1979; 1980a) found that HeLa and BSC-1 cells made permeable to nucleotides exhibited a 50% loss of NAD levels after treatment with X-rays; this decrease was concomitant with an increase of poly(ADP-ribose) polymerase activity. The same effect was observed when the cells were treated with DNase and micrococcal endonuclease (Benjamin and Gill, 1980a). In vivo studies have shown that DNA damaging agents stimulate the conversion of NAD to poly(ADP-ribose).

By comparing *in vivo* rates of NAD depletion and *in vitro* rates of poly(ADP-ribose) activity, Jacobson, M. K. et al. (1980) have shown that MNNG selectively depletes the cellular NAD pool while causing a 7-fold increase in poly(ADP-ribose) polymerase activity. Theophylline, a known inhibitor of poly(ADP-ribose) polymerase, prevented the *in vivo* lowering of NAD. Juarez-Salinas et al. (1979) isolated and quantified levels of poly(ADP-ribose) *in vivo* using the technique of Sims et al. (1980). After treating SVT2 cells with MNNG, an 200-fold increase in cellular poly(ADP-ribose) levels occurred concomitant with NAD depletion. Jacobson and Jacobson (1978a; 1978b) examined the effect of N-nitroso compounds on NAD concentrations in 3T3 cells and mitogen stimulated human lymphocytes. Large decreases in NAD were observed when both cell types were treated with direct acting carcinogens. Non-carcinogens did not affect NAD concentrations.

Of the damaging agents employed for the stimulation of poly(ADP-ribose) polymerase, all either make DNA strand breaks directly or indirectly by the activation of a DNA repair mechanism. This suggests that strand breaks in DNA signal the synthesis of poly(ADP-ribose) in *in vitro* systems. Miller (1975a), using isolated HeLa cell nuclei, was the first to show that the presence of endonucleases,

DNase I and micrococcal endonuclease stimulated the incorporation of [^{3}H]-NAD into an acid insoluble product. He concluded that poly(ADP-ribose) polymerase probably requires both DNA and an endonuclease for maximal activity. In another study, Miller showed that this stimulation was the result of the initiation of new poly(ADP-ribose) chains rather than length-addition to pre-existing chains (Miller, 1975b). In a third study, Miller (1976) reported that bleomycin, an agent which makes direct strand breaks such that DNA is fragmented, also caused the stimulation of poly(ADP-ribose) polymerase activity. If HeLa nuclei were allowed incubation time for repair of the damage, the activity of poly(ADP-ribose) polymerase returned to its control level.

Berger et al. (1980) worked with lymphocytes made permeable to nucleotides. These lymphocytes were from patients with xeroderma pigmentosum (XP), a genetic disease in which the ability to repair damage by UV irradiation is lacking. The defect in xeroderma pigmentosum is the inability to introduce endonucleolytic strand breaks into DNA for the excision of UV lesions (Hashem et al., 1980). When XP cells were treated with UV irradiation, no increase in poly(ADP-ribose) synthesis was evident. When treated with MNNG, an alkylating agent

which rapidly causes DNA strand breaks directly, a large increase in poly(ADP-ribose) synthesis was observed. Berger and Sikorski (1981) found that Micrococcus luteus UV endonuclease had no effect on poly(ADP-ribose) synthesis in non-UV treated XP lymphocytes. This endonuclease makes strand incisions in DNA at lesions caused by UV light. When XP cells were UV irradiated followed by the addition of UV endonuclease, poly(ADP-ribose) synthesis increased 2 to 4 fold. Time course studies showed that the stimulation of poly(ADP-ribose) polymerase in UV irradiated cells occurs following the formation of DNA strand breaks resulting from the endonucleolytic activity of normal excision-repair (Berger and Sikorski, 1981). Other studies have utilized the SV40 minichromosome as an in vitro model of eucaryotic chromatin. Stimulation of poly(ADP-ribose) polymerase was not observed following UV treatment unless M. luteus UV endonuclease was introduced into the system. Thus, UV damage is not sufficient to stimulate poly(ADP-ribose) polymerase activity, but the breaks made at the damaged site by the endonuclease result in the stimulation (Cohen and Berger, 1981).

Additional evidence that poly(ADP-ribose) is synthesized in response to DNA strand breaks comes from the work of Benjamin and Gill (1979; 1980a; 1980b). They

reported poly(ADP-ribose) polymerase activity in nucleotide permeable cells only when breaks in DNA were introduced. DNase I, micrococcal nuclease, or X-rays were effective in introducing these breaks and stimulating poly(ADP-ribose) polymerase activity. In a cell-free system, they also used plasmid pBR322 to program the activity of poly(ADP-ribose) polymerase. By using various specific restriction endonucleases, various classes of DNA strand breaks were introduced into the plasmid DNA. Double-stranded restriction fragments with flush-ends supported maximal poly(ADP-ribose) polymerase activity. These fragments were three times as effective as fragments with staggered ends in stimulating the enzyme. Covalently closed plasmids or synthetic homopolymers were ineffective in the stimulation of poly(ADP-ribose) polymerase (Benjamin and Gill, 1980b).

Since poly(ADP-ribose) is synthesized in response to DNA damage, particularly DNA strand breaks, it has been argued that this polymer may function in DNA repair processes. Miller (1975a; 1975b) was the first to suggest a role of poly(ADP-ribose) in DNA repair. Both poly(ADP-ribose) synthesis and DNA repair are consequences of DNA damage. Again using lymphocytes made permeable to nucleotides, Berger has demonstrated an association between

increase in poly(ADP-ribose) polymerase activity and DNA repair synthesis. A dose-dependent increase in poly(ADP-ribose) polymerase activity followed treatment of lymphocytes and HeLa cells with UV irradiation, MNNG, and N-acetoxy-acetyl-amino fluorene (AAAF) (Berger et al., 1979a). The dose-dependent increase in poly(ADP-ribose) polymerase activity was also observed after treating cells with bleomycin, gamma-radiation, and endonucleases (Berger et al., 1979b). The use of cycloheximide showed that the increase in activity did not depend on protein synthesis. In these same studies, a dose-dependent increase in unscheduled DNA synthesis (UDS) was also observed. This UDS was found to be DNA repair synthesis based on CsCl gradient analysis of density labeled DNA. Prior treatment with cycloheximide showed that the repair synthesis was not dependent on protein synthesis.

After X-ray damage to HeLa cells made permeable to nucleotides, Benjamin and Gill (1980b) found that cells could repair damaged sites if allowed to incubate at 35^{o}C. If treated with x-rays and incubated at 0^{o}C, the cells accumulated damaged sites. If cells were subsequently placed at 35^{o}C, a burst of poly(ADP-ribose) polymerase activity occurred concomitant with the elimination of the x-ray induced damage sites. In addition to these reports,

Smulson and workers have shown that when single strand breaks are induced in intact cells by MNU, the poly(ADP-ribosylation) of nuclear histones significantly enhances DNA repair *in vitro* (Smulson et al., 1977).

Unscheduled DNA synthesis (UDS) as measured by autoradiography in the presence of hydroxyurea does not occur in 3T3 cells grown in nicotinamide-free medium (Jacobson and Narasimhan, 1979). These starved cells resume unscheduled DNA synthesis upon addition of nicotinamide. 3T3 cells grown in the absence of nicotinamide are not capable of DNA repair replication, as measured by CsCl equilibrium sedimentation analysis of density labeled DNA, or poly(ADP-ribose) synthesis *in vivo*. The addition of nicotinamide to the culture medium restores both DNA repair replication and poly(ADP-ribose) synthesis. Thus, there must be a direct relationship between the ability to repair DNA and the ability to synthesize poly(ADP-ribose) (Jacobson, E. L. et al., 1980).

The use of inhibitors of poly(ADP-ribose) polymerase has become important in probing for the function of poly(ADP-ribose). *In vitro*, poly(ADP-ribose) polymerase is inhibited by 3 classes of compounds: the nicotinamides and benzamides, thymidine, methylxanthines. Clark et al. (1971) found that 5'-methylnicotinamide and nicotinamide

were both effective as inhibitors at the same concentration but 5'-methylnicotinamide was less effective, the K_i of 5'-methylnicotinamide was 2×10^{-4} M; the K_i of nicotinamide was 2×10^{-5} M. Priess et al. (1971) showed that both 5'-methylnicotinamide and nicotinamide were competitive inhibitors; thymidine was also found to fit into this category. In studying poly(ADP-ribose) activity in differentiating cardiac muscle cells, Claycomb (1976) found that the methylxanthines, including caffeine and theophylline at 1 mM were good inhibitors. Levi et al. (1978) reported potent inhibition by methylxanthines and cytokinins (N^6-substituted derivatives of adenine). Purnell and Whish (1980) tested benzamide compounds and found that those substituted in the 3-position were the most inhibitory compounds to date. 3-aminobenzamide and 3-methoxybenzamide, both competitive inhibitors, had K_i values of less than 2 μM. Sims et al. (1982) tested 33 analogues of nicotinamide and the adenine portion of NAD for poly(ADP-ribose) polymerase inhibition. The most effective inhibitors *in vitro* were the most effective in preventing NAD lowering, and the best in preventing the accumulation of poly(ADP-ribose) *in vivo* in the following order: the benzamides, the nicotinamides, and theophylline, respectively.

By the utilization of inhibitors, the results of a number of studies have suggested that the synthesis of poly(ADP-ribose) is necessary for the recovery of cells from DNA damage. The approach has been to inhibit poly(ADP-ribose) synthesis in vivo and look for cellular dysfunction. Davies et al. (1977) suggested that poly(ADP-ribose) synthesis is necessary for the survival of cells after damage with MNU. Both theophylline and 5'-methylnicotinamide decreased the survival of L1210 cells after treatment with MNU. They proposed that poly(ADP-ribose) is involved in the repair of the lesions made by alkylating agents. Nduka et al. (1980) reported enhancement of cytotoxicity in L1210 cells when treatment with MNU or gamma-radiation was followed by incubation with polymerase inhibitors methylnicotinamide, methylxanthine, and thymidine. Durkacz et al. (1980a; 1980b) has shown that the polymerase inhibitor, 3-amino-benzamide potentiates the lethality of dimethyl sulfate in permeabilized L1210 cells. This inhibitor prevents NAD depletion completely in this cell system and apparently prevents the rejoining of strand breaks as measured by alkaline sucrose density gradient centrifugation. Thus, inhibitors of poly(ADP-ribose) polymerase inhibit excision repair in these cells, but they do not inhibit incision

events or repair replication (Durkacz et al., 1981). The inhibition of DNA strand rejoining was shown not to be due to inhibition of repair synthesis. Durrant and Boyle (1982) tested the abilities of non-toxic doses of the poly(ADP-ribose) inhibitors, 5'-methylnicotinamide, 3-methoxybenamide, and caffeine, to potentiate the cytotoxicity of MNU. The use of 4 rodent cell lines revealed a differential cytotoxicity which may be dependent on cell metabolism and permeability. However, when the inhibitors were used in pairs, the data suggested that caffeine may be affecting a different process than either nicotinamide or benzamide. Durrant et al. (1981) also demonstrated that 5'-methylnicotinamide potentiated lethality after MNU treatment of L1210 cells. However, this inhibitor reduced the MNU mutation frequency, enhanced the rate of 0^6-methylguanine removal from DNA, and stimulated UDS. The data suggest that inhibition of poly(ADP-riobse) synthesis facilitates the removal of 0^6-methylguanine, a promutagenic lesion.

A number of recent studeis have examined the effect of poly(ADP-riobse) inhibitors on UDS. In each of these studies UDS was measured in the presence of hydroxyurea. Berger and Sikorski (1980) damaged human lymphocytes with UV irradiation, MNNG, or

dimethyl sulfate. The presence of nicotinamide stimulated unscheduled DNA synthesis. The amount of stimulation was dependent on the concentration of the inhibitor. Althaus et al. (1980) reported that after treatment with methyl methane sulfonate, unscheduled DNA synthesis was markedly increased in cultured hepatocytes in the presence of 25 mM nicotinamide. Althaus et al. (1982) found that the same results were obtained with benzamides, nicotinamides theophylline and thymidine. Miwa et al. (1981) reported increased (UDS) in the presence of benzamide and 3-aminobenzamide after treating lymphocytes with UV irradiation. XP lymphocytes did not show the enhancement after UV, but did after MNNG treatment. Sims et al. (1982) reported that the best stimulators of UDS were those inhibitors which were the best overall inhibitors of all aspects of poly(ADP-ribose) metabolism, benzamides, nicotinamides, and theophylline, respectively.

The ultimate aim of this study is to investigate the function of poly(ADP-ribose). Toward that end we have characterized poly(ADP-ribose) metabolism in vivo in human diploid fibroblasts following UV irradiation. The kinetics of NAD conversion to poly(ADP-ribose) have been determined and the turnover of poly(ADP-ribose) after UV irradiation has been estimated. The role of

this metabolism *in vivo* in DNA repair has been assessed by the use of the poly(ADP-ribose) polymerase inhibitor, 3-aminobenzamide. The kinetics of NAD degradation and poly(ADP-ribose) synthesis *in vivo* after treatment with the DNA alkylating agent, MNNG, have also been determined. The effect of 3-aminobenzamide on DNA repair replication following damage by an alkylating agent has been assessed and compared to the effect following UV irradiation.

Because of the implications that poly(ADP-ribose) may be involved in chromatin structure, the regulation of DNA repair, and that it is synthesized in response to DNA strand breaks, an attempt was made to assess poly(ADP-ribose) metabolism as a function of cellular senescence. The CF-3 line of human diploid fibroblasts used in this study characteristically possess a finite *in vitro* life-span. When cultured from explants of infant tissue, these cells undergo 60 $\pm$ 5 populations doublings. After this, both cell division potential and cell viability is lost. This phenomenon allows the use of human diploid fibroblasts as an *in vitro* model system for cellular senescence (Hayflick, 1977).

An hypothesis which proposes to explain the loss of doubling potential and cell viability in human diploid fibroblasts suggests that DNA repair mechanisms are

deficient or defective in cells of high population doubling levels (PDL) (Little, 1976). This hypothesis has been tested by measuring DNA repair in high PDL cells after treatment with UV light. The results have been inconclusive (Goldstein, 1971; Hart and Setlow, 1976; Smith and Hanawalt, 1976b). However, it has been discovered that the use of 0.5% fetal calf serum in culture medium places fibroblasts in an essentially non-mitotic state of arrest. The result is the extension of calendar life for the cell, but not an extension of the finite number of population doublings.

Once in the arrested state, high PDL cells not only maintain the ability to repair DNA (as measured by unscheduled DNA synthesis in the presence of hydroxyurea after DNA damage), but also exhibit an elevated level of unscheduled DNA synthesis. This level of UDS is 20-30% over and above that for either confluent or arrested low PDL cells or confluent high PDL cells. It does not occur until the first one-third of the lifespan is completed and is the greatest during the last one-third of the lifespan (Dell'Orco and Whittle, 1978).

The phenomenon of serum arrest places cells in what is presumed to be the G_o state of the cell cycle. Low serum arrest maintains cells in a state which mimics

the in vivo situation. Cellular processes are more readily defined with the removal of the metabolic demands of cellular division. Arrested cells have low endogenous levels of DNA replicative synthesis (Dell'Orco et al., 1973). Hydroxyurea, a DNA synthesis inhibitor, is routinely utilized in experiments which measure UDS to inhibit endogenous levels of DNA synthesis so that all [^{3}H]-thymidine incorporation following DNA damage may be considered DNA repair synthesis.

An explanation offered for elevated UDS in arrested cells of high PDL is that repair enzymes have greater accessibility to DNA due to less tightly bound chromosomal proteins (Dell'Orco and Whittle, 1978). Thus, aging may affect euchromatin structure. Moreover, it has been observed that nucleoids of CF-3 cells of high PDL exhibit lower superhelicity patterns and therefore have more naturally occurring DNA strand breaks than do nucleoids from low PDL cells (Lipetz, 1980).

To characterize the possible role of poly(ADP-riobse) in senescence, this study examined several parameters. Inherent NAD content and basal levels of poly(ADP-ribose) were assessed. In addition, the rates and extent of conversion of NAD following UV irradiation were determined

and correlated with the extent of repair replication in the aged cell.

MATERIALS AND METHODS

Cells and cell culture

CF-3 normal human diploid fibroblasts from newborn foreskin tissue where a gift from Dr. Robert Dell'Orco, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma. xeroderma pigmentosum cells, XP12BE, complementation group A, were purchased from the American Type Culture Collection, Rockville, Maryland. All cells were grown in Dulbecco's modified Eagles' medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (KC Biological Co., Lenexa, KS or Reheis Chemical Co., Phoenix, AZ), 0.25 µg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO), and 50 µg/ml gentamycin (Shering Corp., Kenilworth, N.J.). All experiments were performed on cells of less than 28 population doublings except for studies on aged fibroblasts. For experiments in which levels of NAD were determined, cells were seeded into 35 mm or 60 mm culture dishes. For experiments in which poly(ADP-ribose) levels were determined, cells were seeded into 100 mm dishes. After seeding, the cells were grown to confluence and mitotically arrested with 0.5% fetal calf serum for not less than 5 or more than 10 days (Dell'Orco and Whittle, 1978). Cells were fed twice weekly by replacing the medium.

Procedures for damaging DNA

To treat with UV light, the medium was removed from the dishes and the cells washed with phosphate buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2). Cells were placed under a General Electric germicidal lamp (G15T8) and irradiated with an incident flux of 0.5 J/m^2 for 10 sec. The medium was then replaced and the cells allowed to incubate. Control dishes were treated in the same way except the UV lamp was not turned on.

To treat with bleomycin (Blenoxane, lot #G3689), the medium was replaced with phosphate buffered saline, pH 7.5, containing 32 μg/ml bleomycin for 30 min. The cells were then washed once with phosphate buffered saline, pH 7.2. The medium was replaced and the cells allowed to incubate. Control plates were treated in the same way except that no bleomycin was added to the phosphate buffered saline, pH 7.5.

To treat with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma Chemical Co., St. Louis, MO), a 0.1 mM sodium acetate buffer, pH 5.0 was used to dissolve the MNNG at a concentration of 3.4 mM (0.5 mg/ml). An appropriate aliquot of this solution was diluted into medium to a final concentration of 34 μM (5 μg/ml). The concentration of acetate

in the medium was 1.0 mM or less. The cells were treated with MNNG at room temperature. Cells were then washed once with phosphate buffered saline, pH 7.2, the medium was replaced, and the cells were incubated at 37°C.

When inhibitors were tested, cells were preincubated for 1 hr in the presence of the inhibitor. Where indicated, 1-β-D-arabinofuranosylcytosine (ara C) (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 0.1 mM, 3-aminobenzamide (Pfaltz and Bauer, Inc., Stamford, CT) at a concentration of 3 mM, and novobiocin (Sigma Chemical Co., St. Louis, MO) at a concentration of 1 mM. Hydroxyurea (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 10 mM and was added following treatment with UV light and bleomycin. When added to the culture medium at 1 hr following UV treatment, 3-aminobenzamide was used at a concentration of 10 mM.

Measurement of NAD pools

Total NAD pools were extracted according to the method of Jacobson et al. (1979) which involves an ice-cole alkaline extraction which was nutralized with H_3PO_4 within 2 min. Reduced NAD was oxidized with 2 M phenazine ethosulfate (Sigma Chemical Co., St. Louis, MO). The samples were frozen until assayed for NAD content. NAD was

measured by the method of Jacobson and Jacobson (1976) which is an enzymic cycling assay initiated by alcohol dehydrogenase and amplified by MTT tetrazolium (Sigma Chemical Co., St. Louis, MO) and phenazine ethosulfate. Each time point in an individual experiment involved the extraction of duplicate dishes followed by duplicate assays of each extraction.

Measurement of poly(ADP-ribose) levels

To extract poly(ADP-ribose), the medium was removed from the dishes of cells. Immediately, 2 ml of ice-cold 20% trichloroacetic acid (w/v) were added and the dishes were set on ice. The acid precipitable material was then scraped from the dishes with a rubber policeman, followed by a 1 ml wash with TCA. The acid insoluble pellets were then collected by centrifugation, washed twice with 20% TCA for 10 min and twice with 95% ethanol for 10 min.

Poly(ADP-ribose) was quantified by the method of Sims et al. (1980) or by a modification of that method: Acid insoluble cell pellets from 1.5×10^7 to 1.0×10^8 cells were dissolved by the aid of sonification in 10 ml of 50 mM MOPS buffer, 6 M guanidine-HCL adjusted to pH 8.8-9.0. Dihydroxyboryl BioRex 70 resin prepared as described by Wielckens et al. (1981), 0.2 ml packed volume,

was added and the mixture was shaken for 2-4 hr to adsorb poly(ADP-ribose) to the resin. The resin was collected by centrifugation and washed successively with 5 ml of 50 mM MOPS, 6 M guanidine-HCL, pH 8.8, and with 2.5 ml of 0.25 M ammonium acetate buffer, pH 8.8. Poly(ADP-ribose) was then eluted from the resin with water. The polymer was converted to ribosyladenosine which was reacted with chloracetaldehyde to form 1, N^6-ethenoribosyladenosine as described previously by Sims et al. (1980). Following chloracetaldehyde treatment, the reaction mixtures (approximately 3 ml) were diluted to 10 ml and adjusted to 0.25 M ammonium acetate and the pH was adjusted to 9.0. The mixture was passed through a 0.5 ml column of dihydroxyboryl polyacrylamide resin (Affigel 601, BioRad) to absorb ethenoribosyladenosine. The column was washed with 5.0 ml of 0.25 M ammonium acetate buffer, pH 9.0 and the ethenoribosyladenosine was eluted with 2.0 ml of 200 mM sodium citrate buffer, pH 4.5. High pressure liquid chromatography was performed with a Beckman 110A liquid chromatograph equipped with a Altex Ultrasphere-ODS reversed phase column (250 mm x 4.6 mm x 0.25 inch). Fluorescence was detected with a Varian Fluorichrom filter fluorometer equipped with a deuterium light source and a 220-I Varian interference filter on the excitation side.

A varian 3-75 emission filter was used. All samples were injected in 200 mM sodium citrate buffer, pH 4.5, and elution was performed isocratically at room temperature with 7 mM ammonium formate buffer pH 5.8/100% methanol, 93:7 (v/v). The values obtained were corrected for recovery as described by Sims et al. (1980).

Measurement of DNA repair replication

DNA repair replication was measured by modifications of the method of Smith and Hanawalt (1976). This method involves the incubation of cells with 10 μM bromodeoxyuridine (Sigma Chemical Co., St. Louis, MO) as a density label and 10 μCi/ml of ^{3}H-thymidine, 65 Ci/mmole, (ICN, Irvine, CA) as a radioisotopic label. All procedures were as described except that fluorodeoxyuridine was omitted and the cells were pretreated for 1 hr with bromodeoxyuridine. In selected experiments, hydroxyurea was also omitted. Cell lysates were mixed with CsCl and adjusted to a refractive index of 1.406 - 1.407 and the samples were subjected to centrifugation at 44,000 rpm at 20^{o}C for 17 hr in a Beckman Ti65 rotor. The gradients were fractionated onto Whatman 3MM paper filters which were washed twice with ice-cold 10% TCA, twice with 5% TCA, three times with 95% ethanol. The filters were dried and counted in a toluene

base liquid scintillation cocktail. Radioactivity banding at the density of [^{14}C]-light DNA was taken as the measure of DNA repair replication. [^{14}C]-light DNA was synthesized by cells in the presence of [^{14}C]-thymidine but not in the presence of the bromodeoxyuridine density label.

Estimation of poly(ADP-ribose) turnover rate

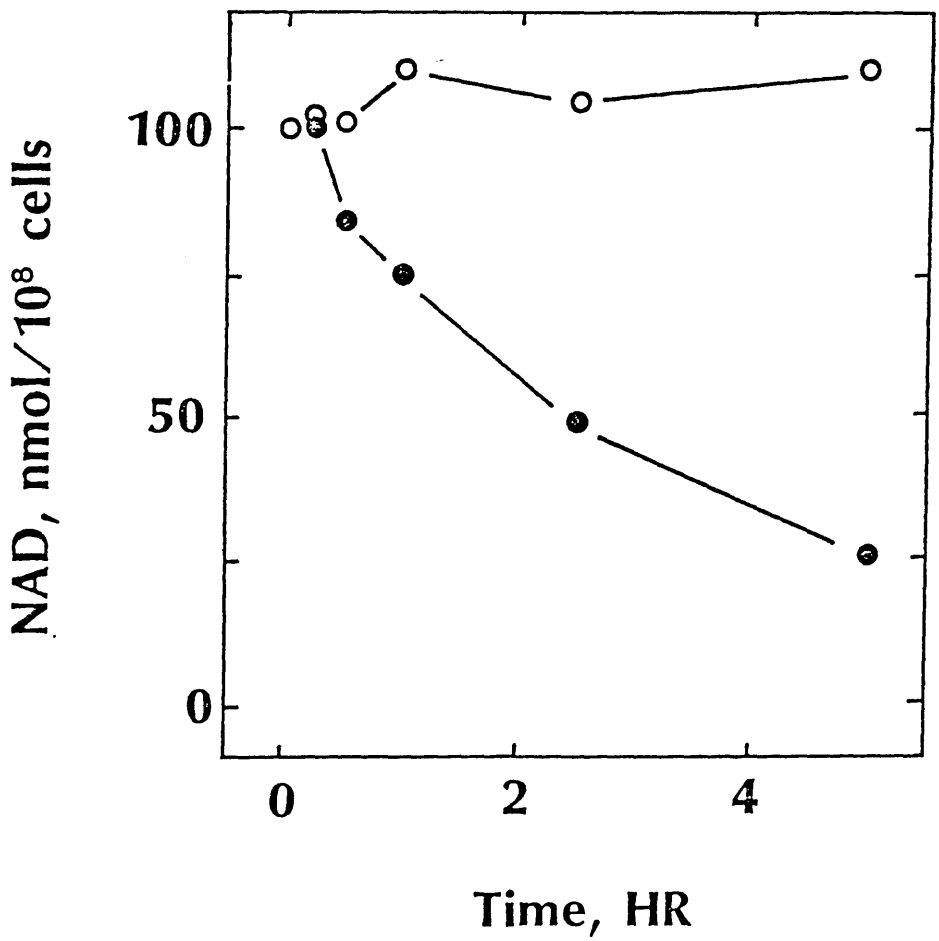
Dishes of cells were UV irradiated and at 1 hr post-irradiation time, the medium was removed and replaced with medium containing 10 mM 3-aminobenzamide. Cells were then allowed to incubate. Control plates were treated the same except 3-aminobenzamide was not added to the replacement medium. Cells were then extracted for NAD and poly(ADP-ribose) at designated times.

RESULTS

Characterization of poly(ADP-ribose) metabolism following UV irradiation

Simultaneous analyses of total cellular NAD and poly(ADP-ribose) contents in CF-3 normal human fibroblasts were perofrmed following treatment with 5 J/m^2 of UV irradiation. The kinetics of the depletion of cellular NAD following UV treatment are shown in Figure 4. The initial NAD content is approximately 100 $nmol/10^8$ cells. No detectable decrease in the NAD content is observed for the first 15 min following UV treatment. After this time, a continuous depletion of the cellular NAD pool occurs. The rate of loss is maximal at 1 hr. Between 1 and 5 hr after UV treatment, the cellular NAD pool decreases at an approximate rate of 200 $pmol/min/10^8$ cells. The accumulation of poly(ADP-ribose) in intact cells following UV treatment is shown in Figure 5. The basal level of poly(ADP-ribose) is 12 $pmol/10^8$ cells of ribosyladenosine derived from poly(ADP-ribose). Following UV treatment, the level increases rapidly to a maximum at 1 hr where accumulation is approximately 200 $pmol/10^8$ cells. The accumulation represents an increase of 100% above basal level by 15 min, 1600% by 1 hr and 1100% by 5 hr following UV treatment.

Figure 4. Total intracellular content of NAD in CF-3 cells following exposure to 5 J/m^2 UV irradiation. The data represent the average of duplicate extractions on 35 mm or 60 mm dishes of mitotically arrested cells from three experiments (n = 8-10). In general, standard deviation from the mean was less than 5%. Cell numbers for 35 mm dishes ranged from $4.8 \times 10^5 - 4.9 \times 10^5$. Cell numbers for 60 mm dishes ranged from $1.41 \times 10^6 - 1.42 \times 10^6$. The data are expressed as NAD content in $nmol/10^8$ cells as a function of time. NAD levels in unirradiated control cells, (○); UV irradiated cells, (●).


NAD, nmol/10⁸ cells
100
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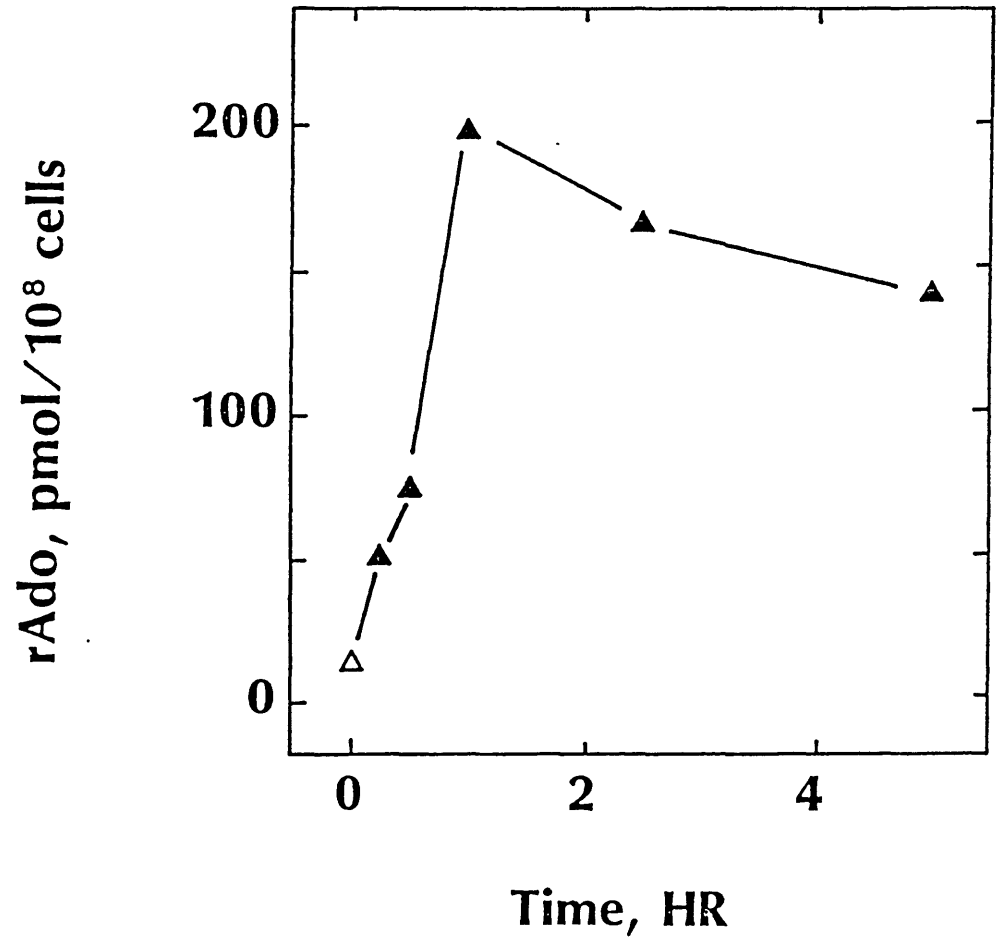
2
4
Time, HR

Figure 5. Total intracellular content of poly(ADP-ribose) in CF-3 cells following exposure to 5 J/m^2 UV irradiation. Each data point represents an analyses of extractions of from 6 - 30 100 mm dishes of mitotically arrested cells. Cell numbers ranged from 2.70×10^6 - 2.76×10^6. The data are expressed as poly(ADP-ribose) content in pmol/10^8 cells of ribosyladenosine derived from poly(ADP-ribose) as a function of time. Poly(ADP-ribose) levels in unirradiated control cells, (Δ); UV irradiated cells, (▲).


rAdo, pmol/10⁸ cells
200
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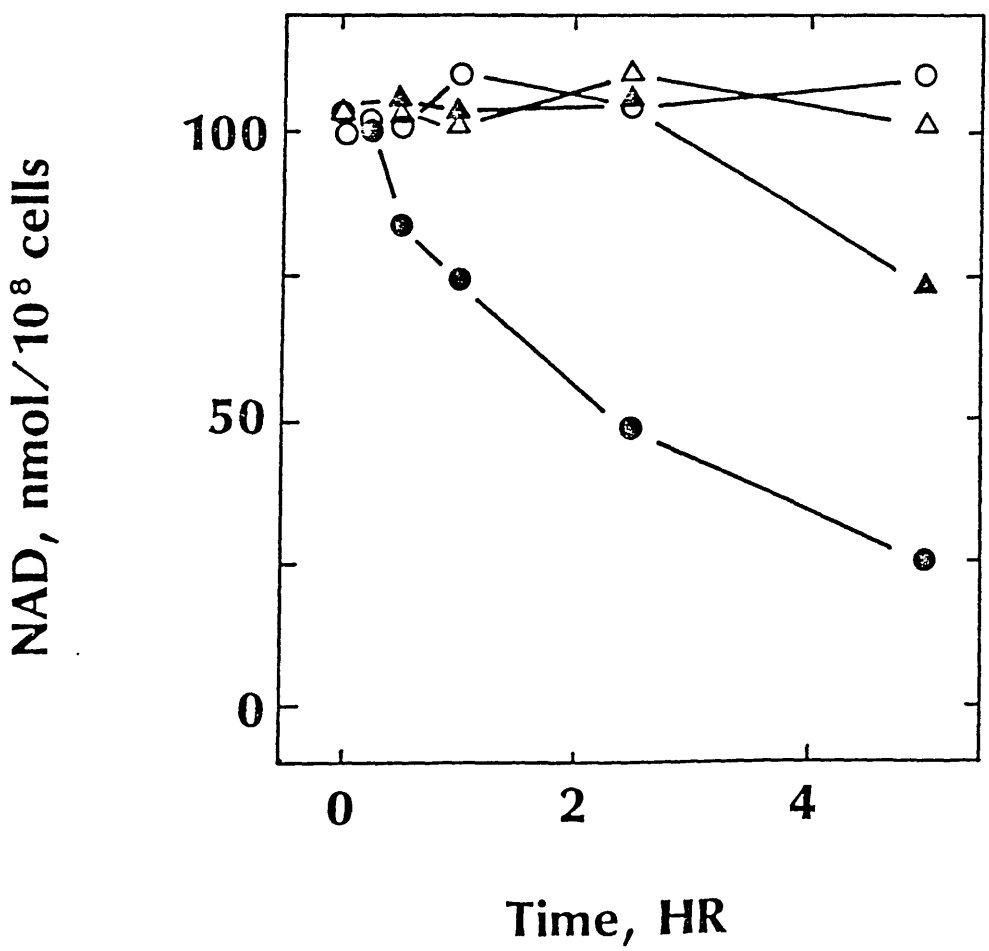
Time, HR

Effectiveness of 3-aminobenzamide in vivo

Figure 6 shows the kinetics of NAD depletion following 5 J/m^2 of UV irradiation in the presence of the poly(ADP-ribose) polymerase inhibitor, 3-aminobenzamide. When cells are incubated in 3 mM 3-aminobenzamide both prior to and following UV treatment, no detectable depletion of the cellular NAD pool occurs for 2.5 hr following UV irradiation. However, between 2.5 and 5 hr following treatment, a 30% loss of the cellular NAD pool is observed. The accumulation of poly(ADP-ribose) following 5 J/m^2 of UV irradiation in the presence of 3-aminobenzamide is shown in Figure 7. When cells are incubated in 3 mM 3-aminobenzamide both prior to and following UV treatment, no detectable accumulation of poly(ADP-ribose) occurs for 2.5 hr following UV irradiation. However, by 5 hr, a 300% increase in the poly(ADP-ribose) content is observed.

The combined data from Figures 5-7 represent the first report that poly(ADP-ribose) synthesis is a rapid response following UV irradiation of intact cells. The appearance of this polymer in vivo is blocked by an inhibitor of poly(ADP-ribose) polymerase. Moreover, poly(ADP-ribose) synthesis is tightly coupled to the time-course

Figure 6. The effect of 3-aminobenzamide on the NAD content of CF-3 cells following exposure to 5 J/m^2 UV irradiation. Cells were incubated in medium containing 3-mM 3-aminobenzamide for 1 hr prior to UV treatment and for the entire post-irradiation period. The data represent the average of duplicate analyses of extractions of 35 mm or 60 mm dishes of mitotically arrested CF-3 cells from three experiments (n = 8-10). In general, standard deviation from the mean was less than 5%. Cell numbers for 35 mm dishes ranged from $4.8 \times 10^5 = 4.9 \times 10^5$. Cell numbers for 60 mm dishes ranged from 1.41×10^6 to 1.42×10^6. The data are expressed as total intracellular NAD content in $nmol/10^8$ cells as a function of time. NAD levels in unirradiated control cells in the absence of 3-aminobenzamide, (○); UV irradiated cells in the absence of 3-aminobenzamide, (●); unirradiated control cells in the presence of 3-aminobenzamide, (△); UV irradiated cells in the presence of 3-aminobenzamide, (▲).


NAD, nmol/10⁸ cells
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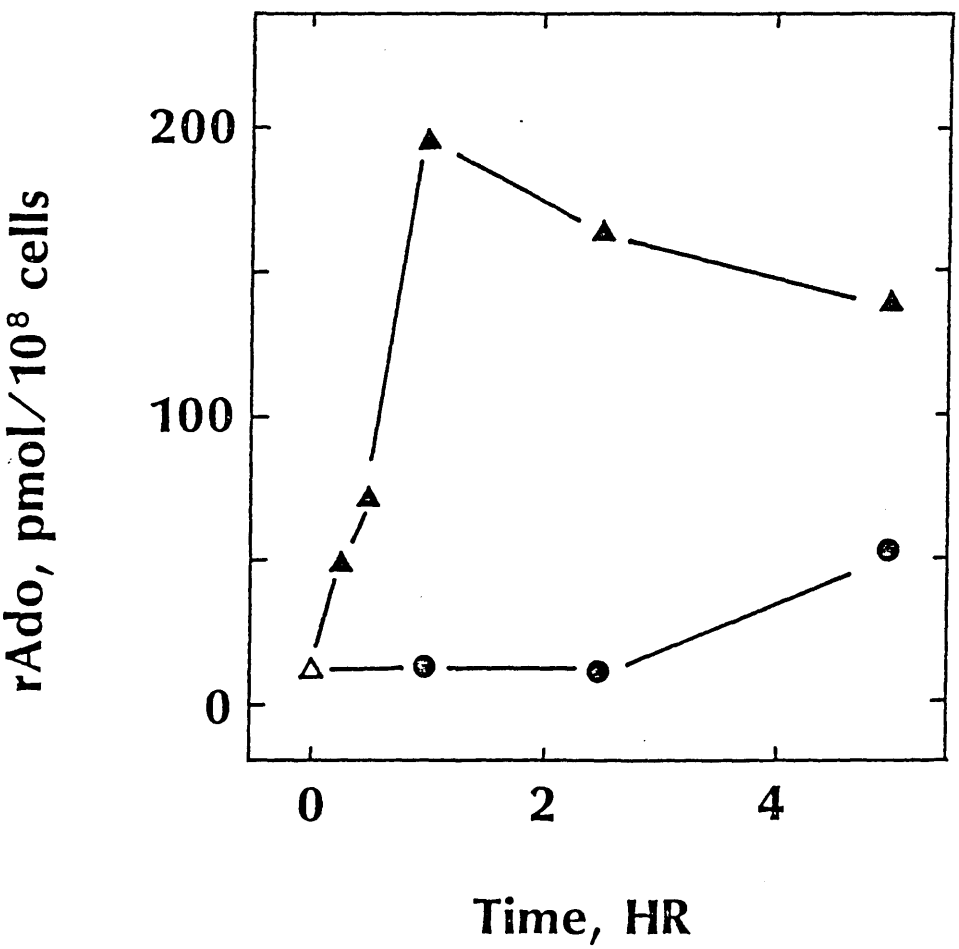
Time, HR

Figure 7. The effect of 3-aminobenzamide on the poly-(ADP-ribose) content of CF-3 cells following exposure to 5 J/m^2 UV irradiation. Each data point represents analyses of extractions of from 6 - 30 100 mm dishes of mitotically arrested cells. Cells were incubated in medium containing 3 mM 3-aminobenzamide for 1 hr prior to UV treatment and for the entire post-irradiation period. Cell numbers ranged from 2.70×10^6 - 2.7×10^6. The data are expressed as total intracellular content of poly(ADP-ribose) in pmol/10^8 cells of ribosyladenosine derived from poly(ADP-ribose) as a function of time. Poly-(ADP-ribose) levels in unirradiated control cells in the absence of 3-aminobenzamide, (Δ); UV irradiated cells in the absence of 3-aminobenzamide, (▲); UV irradiated cells in the presence of 3-aminobenzamide, (●).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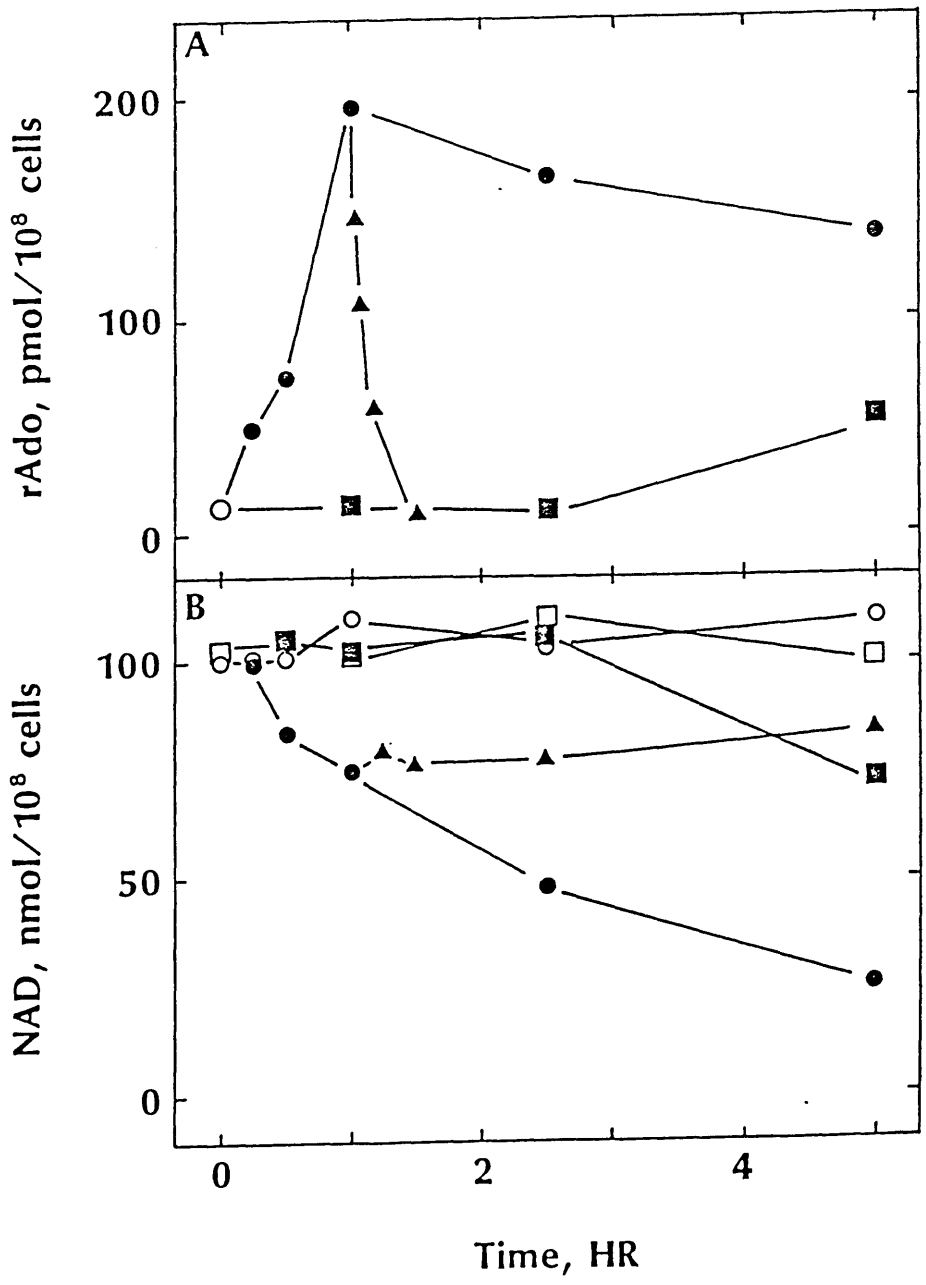
Time, HR

of NAD depletion. Thus, NAD lowering following DNA damage may be a convenient assay for the synthesis of poly(ADP-ribose). Further work is necessary, however, to rule out the possibility that other ADP-ribose transferase reactions may also be occurring. It is known that 3-aminobenzamide is an inhibitor of mono-ADP-ribosyl transferases (Moss, J., personal communication).

Assessment of Poly(ADP-ribose) Turnover Rates

Between 1 and 5 hr following UV irradiation, the poly(ADP-ribose) content remains at 150-200 pmol/10^8 cells; the rate of loss is 0.21 pmol/min/10^8 cells. During this time, the NAD pool is rapidly decreasing at a rate of 200 pmol/min/10^8. These data suggest that poly(ADP-ribose) is being turned-over very rapidly. To estimate the turnover rate of poly(ADP-ribose), 10 mM 3-aminobenzamide was added to the culture medium at the time of maximum accumulation of the polymer following UV damage (1 hr) to immediately block polymer synthesis. The turnover rate of the polymer was then estimated by simultaneous analysis of poly(ADP-ribose) levels and NAD depletion. Figure 8, panel A, shows that poly(ADP-ribose) levels decrease to 74% of maximum within 2 min and to 55% of maximum within 5 min following the addition of the inhibitor. By 30 min

Figure 8. Estimation of poly(ADP-ribose) turnover rate following UV irradiation of CF-3 cells. At 1 hr following UV treatment of dishes of mitotically arrested CF-3 cells, the medium was removed and replaced with medium containing 10 mM 3-aminobenzamide. Control plates received medium alone. At designated times, cells were extracted for NAD and poly(ADP-ribose). For NAD (panel B), the data represent duplicate analyses of duplicate extractions of 35 mm dishes of cells and are expressed as total intracellular NAD content in nmol/10^8 cells as a function of time. In general, standard deviation from the mean was less than 5%. Cell numbers ranged from 8.5 x 10^5 - 8.7 x 10^5. For poly(ADP-ribose) (panel A), the data represent analysis of from 4-8 100 mm dishes of cells and are expressed as total intracellular content of poly(ADP-ribose) in pmol/10^8 cells of ribosyladenosine derived from poly(ADP-ribose) as a function of time. Cell numbers ranged from 3.28 x 10^6 - 3.29 x 10^6. Levels of poly(ADP-ribose) obtained after the addition of the inhibitor were normalized by percentage to the maximum accumulation of poly(ADP-ribose) at 1 hr following UV irradiation. (A) Poly(ADP-ribose) levels in unirradiated cells in the absence of 3-aminobenzamide, (○); UV irradiated cells in the absence of 3-aminobenzamide, (●); UV irradiated cells in the presence of 3 mM 3-aminobenzamide, (□); UV irradiated cells to which 10 mM 3-aminobenzamide was added to the culture medium at 1 hr following treatment, (▲). (B) NAD levels in unirradiated cells in the absence of 3-aminobenzamide, (○); UV irradiated cells in the absence of 3-aminobenzamide, (●) unirradiated cells in the presence of 3 mM 3-aminobenzamide, (□); UV irradiated cells in the presence of 3 mM 3-aminobenzamide, (■); UV irradiated cells to which 10 mM 3-aminobenzamide was added to the culture medium at 1 hr following UV treatment, (▲).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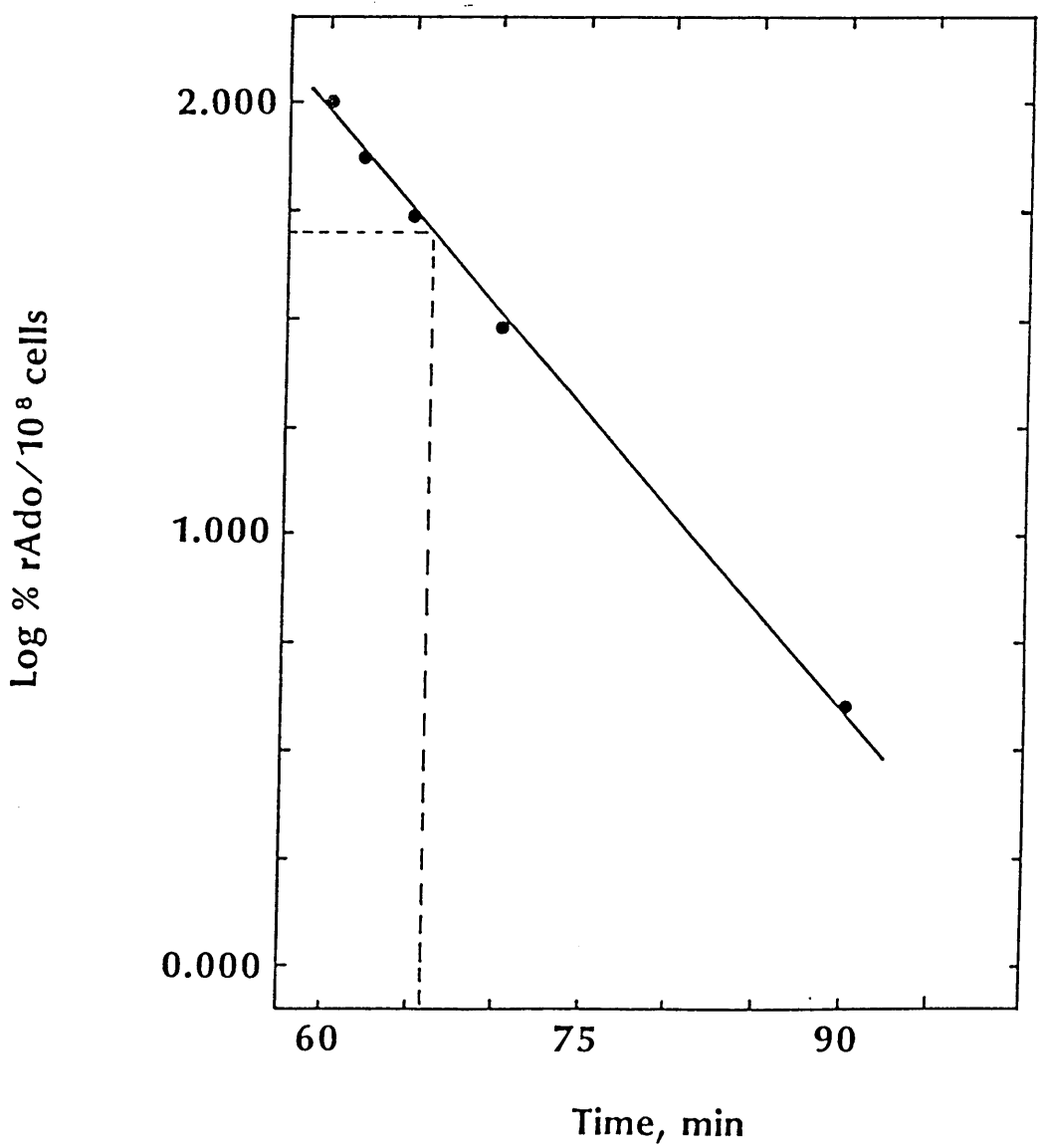
rAdo, pmol/10⁸ cells
NAD, nmol/10⁸ cells
Time, HR

following addition of the inhibitor, poly(ADP-ribose) levels are at basal level. Thus, all the poly(ADP-ribose) which was accumulated at 1 hr following UV treatment was turned-over within 30 min following inhibition of poly-(ADP-ribose) polymerase.

Figure 8, panel B, shows NAD depletion after the addition of the inhibitor at 1 hr. Since the NAD pool is decreasing at a rate (200 pmol/min/10^8 cells) which is as large as the maximum accumulated poly(ADP-ribose) pool (200 pmol/10^8 cells), quantification of the turnover rate of poly(ADP-ribose) by this approach requires that the inhibition of the polymerase be immediate. Assuming that all the NAD is converted to poly(ADP-ribose) via this reaction, continued synthesis for 1 min could account for the entire poly(ADP-ribose) pool. Determining that synthesis is blocked immediately is difficult. The size of the NAD pool is approximately 80 nmol/10^8 cells at this point in time. Since the limit of detection for a decrease in NAD pools approaches 1-5% (0.8-4.0 nmol/10^8 cells), it is not possible to determine by NAD measurement whether poly(ADP-ribose) polymerase is inhibited immediately. However, we can calculate from these data a maximum $t_{1/2}$ for poly(ADP-ribose). Figure 9 is a semi-log plot of the decay of poly(ADP-ribose) following the addition of the

Figure 9. Graphic determination of $t_{1/2}$ for poly(ADP-ribose). The data represent the log of the percent of decrease in the total intracellular poly(ADP-ribose) content of mitotically arrested CF-3 cells following the addition of 10 mM 3-aminobenzamide at 1 hr post-irradiation as a function of time. The poly(ADP-ribose) level at 1 hr post-irradiation was taken as 100%. At log of 50% of the 1 hr level, the $t_{1/2}$ for poly(ADP-ribose) was graphically determined to be 6 min.


2.000
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Log % rAdo/10⁸ cells
Time, min

inhibitor at 1 hr following UV treatment. Using the data in this figure, we calculate a $t_{1/2}$ of poly(ADP-ribose) of 6 min. We consider this $t_{1/2}$ to be a maximum estimate due to the difficulty in determining whether or not poly-(ADP-ribose) synthesis is blocked immediately upon the addition of the inhibitor.

Studies with DNA synthesis inhibitors

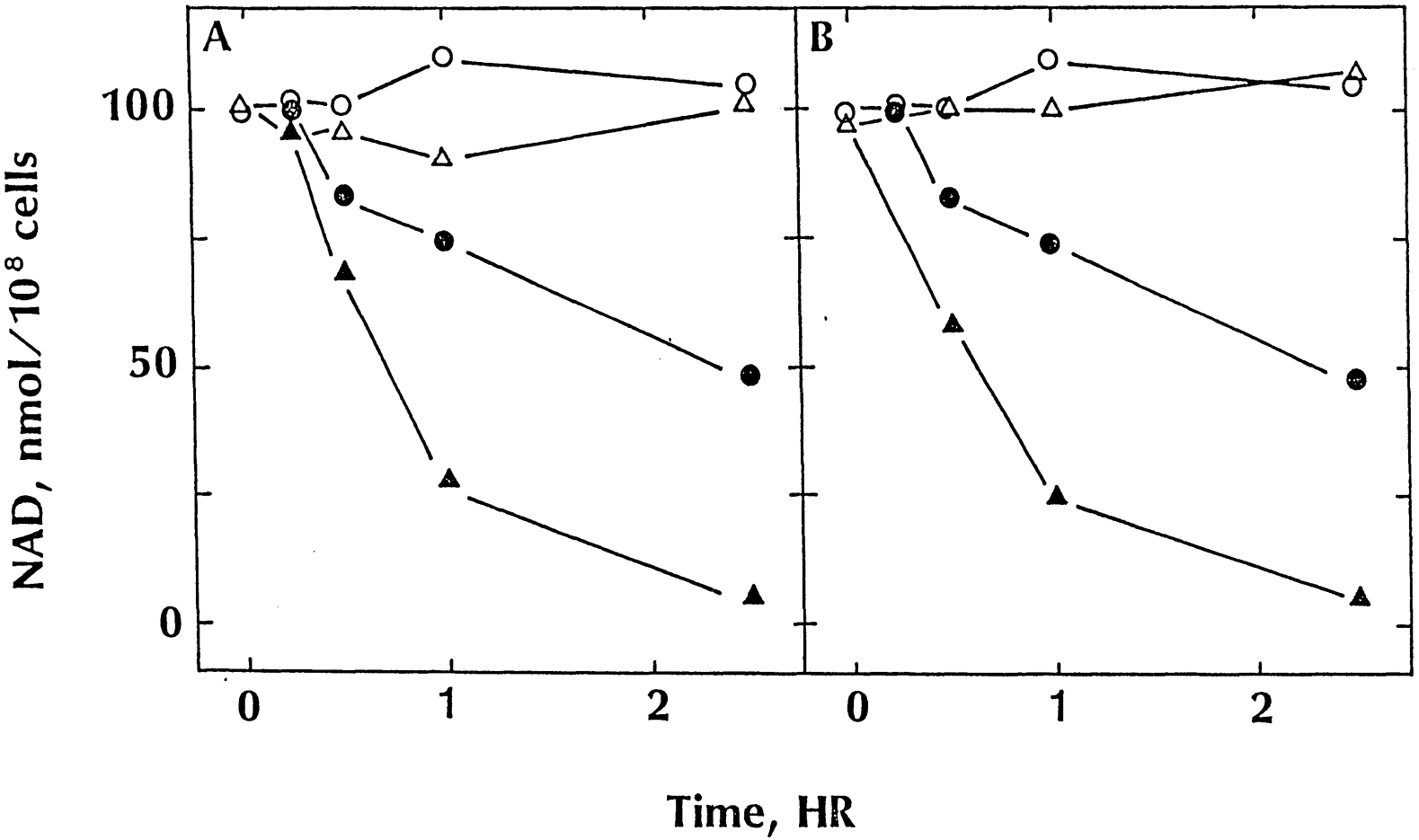
A transient increase in the number of DNA strand breaks occurs following UV treatment of human fibroblasts in culture (Collins and Johnson, 1969; Collins et al., 1977; Hiss and Preston, 1977; Johnson and Collins, 1978; Erixon and Ahnstrom, 1979; Collins et al., 1980). Following UV irradiation, the presence of the DNA synthesis inhibitors, hydroxyurea and ara C, results in an increased number of DNA strand breaks over and above those produced by UV alone (Erixon and Ahnstrom, 1979). Considerable evidence has accumulated which suggests that poly(ADP-ribose) synthesis in *in vitro* systems occurs in response to DNA strand breaks (Miller, 1975a; Cohen and Berger, 1981; Benjamin and Gill, 1980b). To determine whether this is also true in intact cells, hydroxyurea and ara C were added to the post-irradiation incubation medium to

increase the number of DNA strand breaks. If the number of strand breaks *in vivo* controls the synthesis of poly(ADP-ribose), it would be expected that both hydroxyurea and ara C would increase the rates of depletion of NAD and synthesis of poly(ADP-ribose).

Figure 10 shows the effect of 0.1 mM ara C (panel A) and 10 mM hydroxyurea (panel B) on cellular NAD levels over a 2.5 hr post-irradiation period. The presence of these 2 compounds enhances the rate of depletion of NAD by 164% and 200%, respectively, in the first hour following UV treatment. Figure 11 shows that poly(ADP-ribose) content was unchanged by the presence of ara C (panel A) or hydroxyurea (panel B) in the first 15 min following exposure to UV. However, the presence of these two compounds increases the rate of poly(ADP-ribose) synthesis by 175% and 80%, respectively, in the first hour following UV treatment. At 2.5 hr, poly(ADP-ribose) was lower in the presence of ara C or hydroxyurea. This may be explained by substrate depletion since the cells contained only 5% of their original NAD at 5 hr after treatment.

These data show that hydroxyurea and ara C, compounds which increase the number of DNA strand breaks following UV

Figure 10. The effect of ara C or hydroxyurea on the NAD content of CF-3 cells following exposure to 5 J/m^2 UV light. Mitotically arrested cells were incubated in medium containing 0.1 mM ara C for 1 hr prior to UV treatment and for the entire post-irradiation period (panel A). Other cells were incubated with 10 mM hydroxyurea following UV treatment (panel B). The data represent duplicate analyses of extractions of 35 mm dishes of cells from each of three experiments (n = 8). In general, standard deviation from the mean was less than 5%. Cell numbers ranged from 4.8×10^5 - 5.0×10^5. The data are expressed as total intracellular NAD content in $nmol/10^8$ cells as a function of time. (A) NAD levels in unirradiated control cells in the absence of ara C, (○); UV irradiated cells in the absence of ara C, (●); unirradiated cells in the presence of 0.1 mM ara C, (△); UV irradiated cells in the presence of 0.1 mM ara C, (▲). (B) NAD levels in unirradiated control cells in the absence of hydroxyurea, (○); UV irradiated cells in the absence of hydroxyurea, (●); unirradiated control cells in the presence of hydroxyurea, (△); UV irradiated cells in the presence of hydroxyurea, (▲).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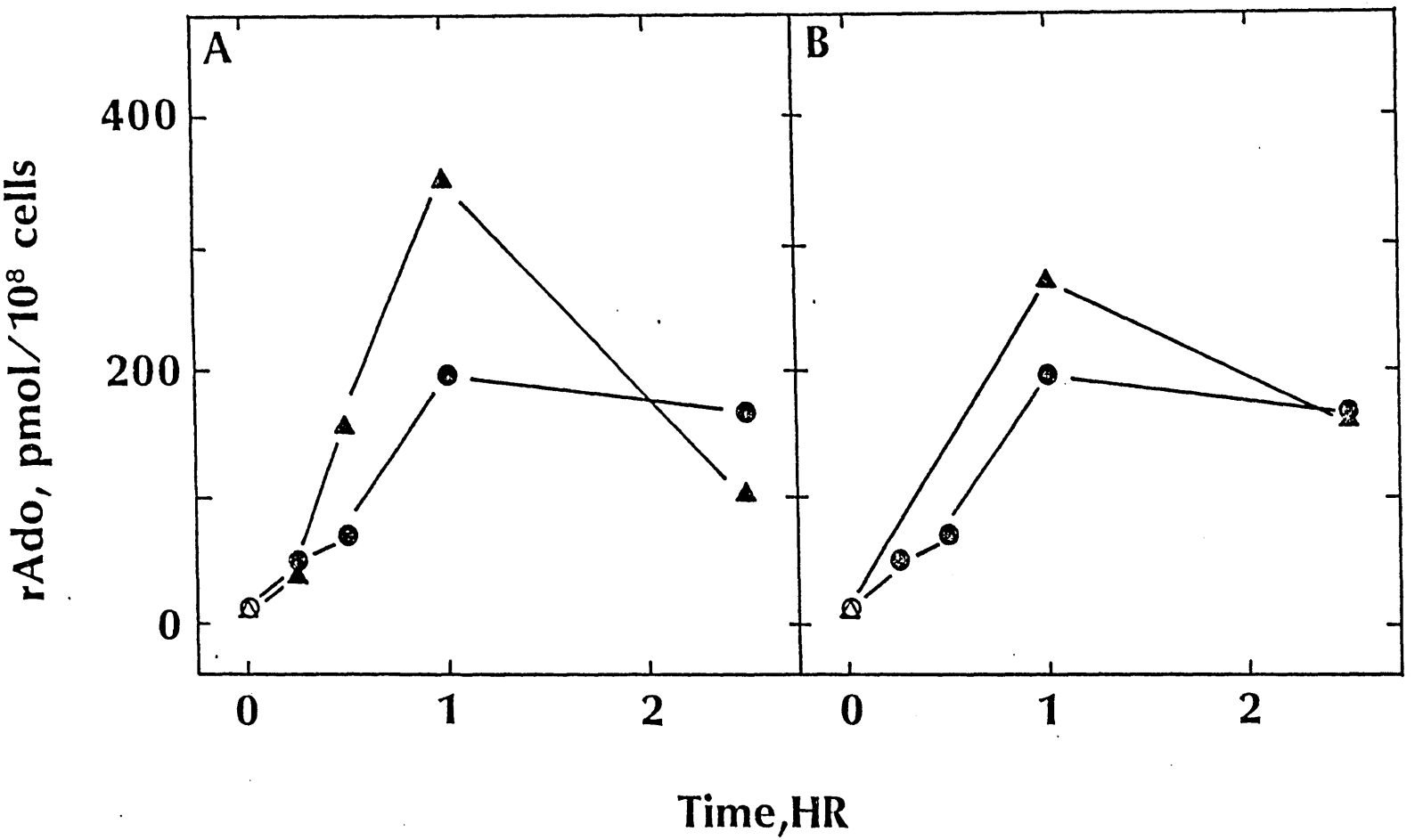
NAD, nmol/10⁸ cells
Time, HR

Figure 11. The effect of ara C or hydroxyurea on the poly(ADP-ribose) content of CF-3 cells following exposure to 5 J/m^2 UV light. Mitotically arrested cells were incubated in medium containing 0.1 mM ara C for 1 hr prior to UV treatment and for the entire post-irradiation period (panel A). Other cells were incubated in 10 mM hydroxyurea following UV treatment (panel B). Each data point represents analyses of extractions of from 6 - 30 100 mm dishes of cells. Cell numbers ranged from 2.5×10^5 - 2.7×10^6/ dish. The data are expressed as total intracellular content of poly(ADP-ribose) in pmol/10^8 cells of ribosyladenosine as a function of time. (A) Poly(ADP-ribose) levels in unirradiated control cells in the absence of ara C, (○); UV irradiated cells in the absence of ara C, (●). UV irradiated cells in the presence of ara C, (▲). (B) Poly-(ADP-ribose) levels in unirradiated control cells in the absence of hydroxyurea, (○); UV irradiated cells in the absence of hydroxyurea, (●); UV irradiated cells in the presence of hydroxyurea, (▲).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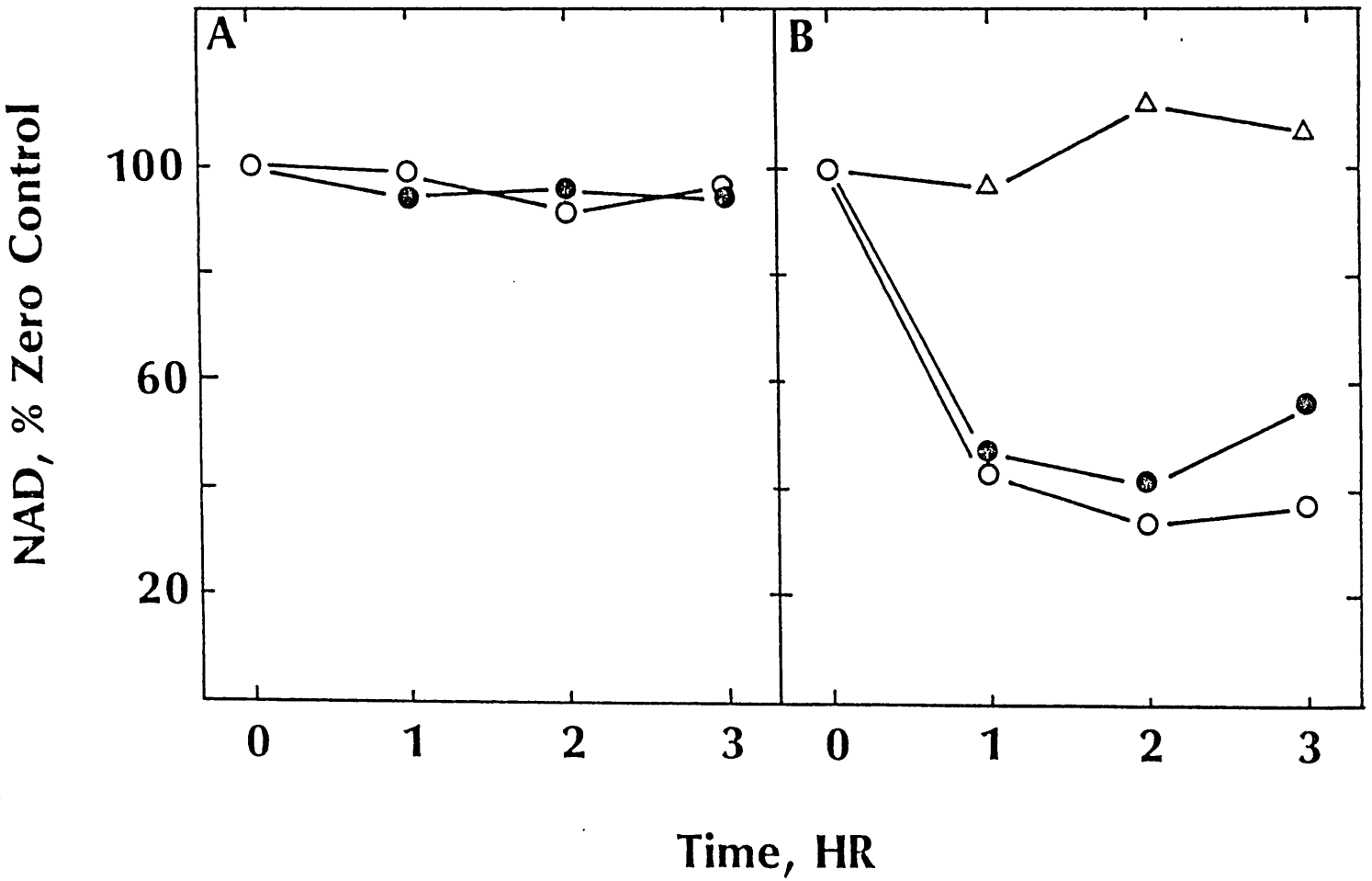
rAdo, pmol/10⁸ cells
Time,HR

treatment over and above those caused by UV irradiation alone, increase the rate of NAD lowering and poly(ADP-ribose) synthesis. However, it is possible that both hydroxyurea and ara C either simulate poly(ADP-ribose) synthesis directly after UV treatment or act synergistically with UV irradiation to stimulate poly(ADP-ribose) synthesis. To test these possibilities, the effect of UV treatment on NAD depletion was measured in the presence of hydroxyurea in xeroderma pigmentosum cells, complementation group A. These cells are known to be deficient in endonucleolytic strand breaks in DNA following UV treatment (Hashem et al., 1980). It has previously been shown that these cells can not convert NAD to poly(ADP-ribose) following treatment with UV irradiation, but can with agents which make direct strand breaks such as MNNG (McCurry and Jacobson, 1981; Berger et al., 1980). If hydroxyurea stimulates poly(ADP-ribose) synthesis directly, one would expect to see the enhancement of NAD depletion by hydroxyurea in this cell type. Figure 12 (panel A) shows that hydroxyurea does not enhance NAD depletion; in fact, NAD content is not affected at all in these cells either with or without hydroxyurea following UV treatment. Thus, the enhancement of NAD conversion to poly(ADP-ribose) by hydroxyurea in normal human fibroblasts can be attributed

Figure 12. The effect of hydroxyurea on the conversion of NAD to poly(ADP-ribose) in xeroderma pigmentosum cells. Mitotically arrested cells were incubated in the presence of 10 mM hydroxyurea following 5 J/m^2 UV light or 32 µg/ml bleomycin. The data represent duplicate analysis of duplicate extractions of 35 mm dishes of cells following exposure of XP12BE cells to 5 J/m^2 of UV light (panel A) or 32 µg/ml bleomycin (panel B). The data are expressed as percent of zero-time NAD control (NAD/10^6 cells) as a function of time. Standard deviation of each of the data points was less than 5% of the mean. Cell numbers ranged from 2.55×10^5 - 2.65×10^5. (A) NAD levels in UV irradiated cells incubated in medium alone, (○); UV irradiated cells incubated in medium containing 10 mM hydroxyurea, (●). (B) Bleomycin treated cells incubated in medium alone, (○); bleomycin treated cells incubated in medium containing 10 mM hydroxyurea, (●). Bleomycin treated cells incubated in medium containing 3 mM 3-aminobenzamide, (Δ).


A
B
100
60
20
NAD, % Zero Control
0
1
2
3
0
1
2
3
Time, HR

directly to an increased number of DNA strand breaks rather than to a synergistic effect of hydroxyurea and UV irradiation on poly(ADP-ribose) polymerase.

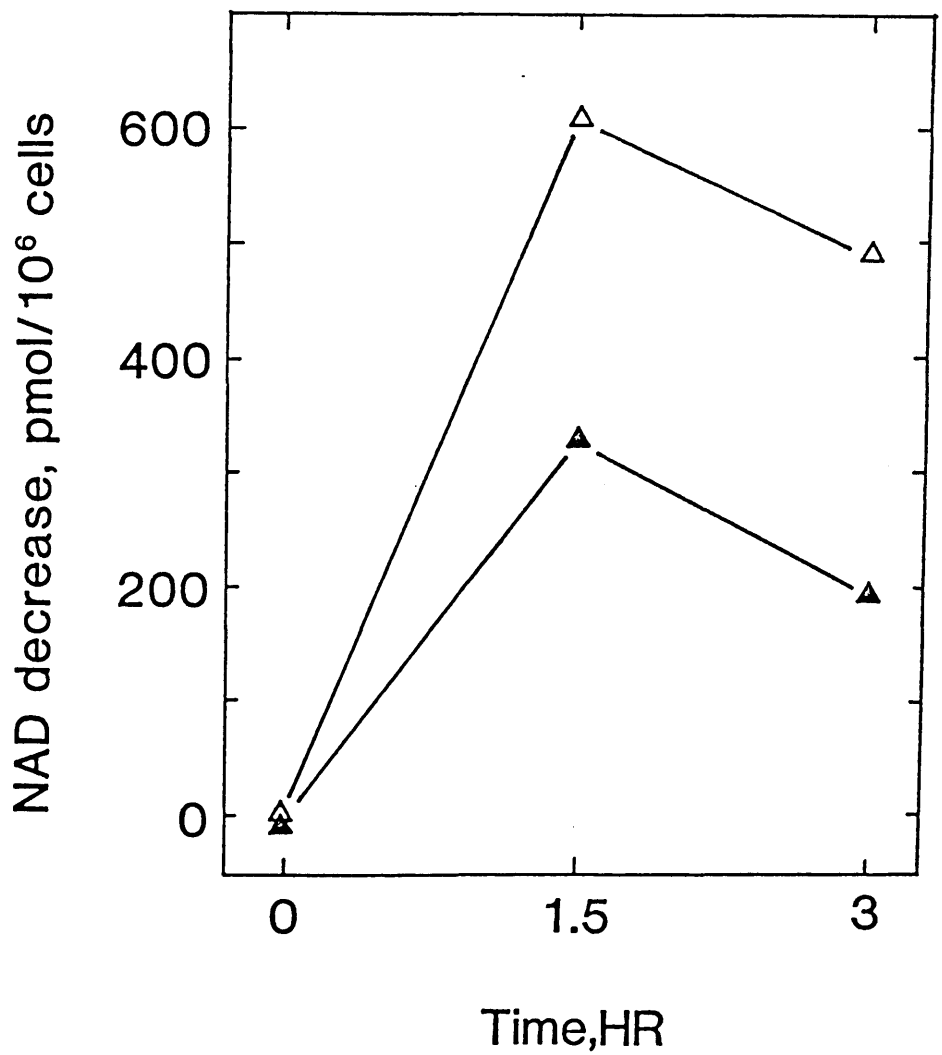
Hydroxyurea and ara C increase the number of strand breaks following UV treatment by increasing the length of time for polymerization at a DNA site which is cleaved by UV endonuclease (Erixon and Ahnstrom, 1979). Hydroxyurea would not be expected to be as effective in enhancing poly(ADP-ribose) synthesis following treatment with DNA damaging agents which make direct DNA strand breaks very rapidly or in which repair occurs by a short patch excision repair pathway. Figure 12 (panel B), shows the effect by hydroxyurea on NAD lowering following treatment of XP cells with bleomycin, an agent which makes direct strand breaks which are repaired by a short-patch pathway (Regan and Setlow, 1974; Hittlemon et al., 1974). The data show that XP cells do lower NAD levels after treatment with bleomycin. As predicted, hydroxyurea does not enhance NAD depletion. Thus, an increased rate of poly(ADP-ribose) synthesis does not occur.

For recognition and binding of DNA polymerases, the DNA double-helix may have to be slightly unwound by the action of DNA gyrase (Collins and Johnson, 1979). Novobiocin prevents the initiation of DNA synthesis

mammalian cells by inhibiting DNA gyrase (Mattern and Painter, 1978). Following UV treatment, novobiocin prevents endonucleolytic incisions (Collins and Johnson, 1979). Thus, the number of DNA strand breaks are decreased after UV treatment in the presence of novobiocin. If the synthesis of poly(ADP-ribose) *in vivo* is controlled by the number of strand breaks, it would be expected that novobiocin would inhibit the conversion of NAD to poly(ADP-ribose). Figure 13 shows the amount of NAD lost following treatment of CF-3 cells with 5 J/m^2 of UV light in the presence and absence of novobiocin. The data show that novobiocin decreases the number of pmol of NAD/10^6 cells converted to poly(ADP-ribose) by 40%. As predicted, in decreasing the number of strand breaks, novobiocin decreases poly(ADP-ribose) synthesis following UV treatment.

UV irradiation of cells has been shown to cause a transient increase in the number of DNA strand breaks (Collins and Johnson, 1969; Collins et al., 1977; Hiss and Preston, 1977; Johnson and Collins, 1978; Erixon and Ahnstrom, 1979) and the presence of either hydroxyurea or ara C has been shown to increase the number of strand breaks over and above those produced by UV alone (Erixon and Ahnstrom, 1979). Figures 10-11 show that the presence of either hydroxyurea or ara C enhances both the rate of

Figure 13. The effect of novobiocin on the conversion of NAD to poly(ADP-ribose) in CF-3 cells following exposure to 5 J/m^2 of UV light. Mitotically arrested cells were incubated in medium containing 1 mM novobiocin following UV treatment. The data represent duplicate analyses of 35 mm dishes of cells and is expressed as the NAD decrease in $pmol/10^6$ cells as a function of time. Cell numbers ranged from 6.59×10^5 - 6.65×10^5. Standard deviation from the mean was less than 5%. NAD decreased in UV irradiated in medium alone, (Δ); NAD lost in UV irradiated cells incubated in medium containing 1 mM novobiocin, (▲).


NAD decrease, pmol/10⁶ cells
600
400
200
0
0
1.5
3
Time,HR

NAD depletion and the accumulation of poly(ADP-ribose) over and above UV alone. Moreover, since Figure 12 shows that hydroxyurea does not stimulate the depletion of NAD in xeroderma pigmentosum cells which are defective in the introduction of endonucleolytic DNA strand breaks following UV treatment, the enhancement of poly(ADP-ribose) synthesis by hydroxyurea can be attributed to an increased number of strand breaks rather than to a direct effect on poly(ADP-ribose) polymerase. Furthermore, following treatment with bleomycin which makes direct DNA strand breaks, i.e. when a transient increase in DNA strand breaks does not occur, the enhancement of poly(ADP-ribose) synthesis by hydroxyurea does not occur (Figure 12). Finally, novobiocin has been shown to decrease the number of DNA strand breaks following UV treatment (Collins and Johnson, 1979). Figure 13 shows that novobiocin decreases the number of pmol of NAD/10^6 cells converted to poly(ADP-ribose).

The data of Figures 10-13 taken together support a mechanism by which the number of DNA strand breaks in intact cells is a determinant for the rate of conversion of NAD to poly(ADP-ribose).

Effectiveness of 3-aminobenzamide in the presence of hydroxyurea and ara C

Since the presence of hydroxyurea or ara C affect NAD and poly(ADP-ribose) metabolism after UV treatment, it is of interest to determine whether the poly(ADP-ribose) polymerase inhibitor, 3-aminobenzamide, functions to inhibit poly(ADP-ribose) synthesis in the presence of these compounds. Figure 14 shows the effect of 3-aminobenzamide on NAD depletion after UV treatment in the presence and absence of ara C (panel A) and hydroxyurea (panel B). Again, 3 mM 3-aminobenzamide alone blocks NAD lowering for 2.5 hr, but between 2.5 and 5 hr 30% of the NAD is consumed. When 0.1 mM ara C or 10 mM hydroxyurea is present in combination with 3-aminobenzamide NAD depletion is observed as early as 1 hr with ara C (panel A) and 2.5 hr with hydroxyurea (panel B). Figure 15 shows the effect of 3-aminobenzamide on poly(ADP-ribose) synthesis after UV treatment in the presence and absence of ara C and hydroxyurea. With 3-aminobenzamide alone, poly(ADP-ribose) synthesis is blocked for 2.5 hr. However, by 5 hr, a 300% (53 pmol/10^8 cells) increase over basal level occurs. When either 0.1 mM ara C or 10 mM hydroxyurea is present in combination with 3-aminobenzamide, poly(ADP-ribose) synthesis is observed by 1 hr.

Figure 14. The effect of ara C or hydroxyurea on NAD content in CF-3 cells in the presence of 3-aminobenzamide. Mitotically arrested CF-3 cells were incubated in the presence of 3 mM 3-aminobenzamide and 0.1 mM ara C prior to treatment with 5 J/m^2 UV light and for the entire post-irradiation period (panel A). Some cells were incubated in the presence of 3-aminobenzamide prior to UV treatment and incubated in the presence of 10 mM hydroxyurea and 3 mM 3-aminobenzamide for the entire post-irradiation period (panel B). The data represent duplicate analyses of extractions of 35 mm or 60 mm dishes of cells from three experiments (n = 8-10). In general, standard deviation from the mean was less than 5%. Cell numbers for 35 mm dishes ranged from $4.8 \times 10^5 - 4.9 \times 10^5$. Cell numbers for 60 mm dishes ranged from $1.41 \times 10^6 - 1.42 \times 10^6$. The data are expressed as total intracellular NAD contant in $nmol/10^8$ cells as a function of time. NAD content of unirradiated control cells in medium alone, (○); UV irradiated cells in medium containing 3 mM 3-aminobenzamide, (●); unirradiated control cells in medium containing 3 mM 3-aminobenzamide and 0.1 mM ara C (panel A) or 10 mM hydroxyurea (panel B), (△); UV irradiated cells in medium containing 3 mM 3-aminobenzamide and 0.1 mM ara C (panel A) or 10 mM hydroxyurea (panel B), (▲).

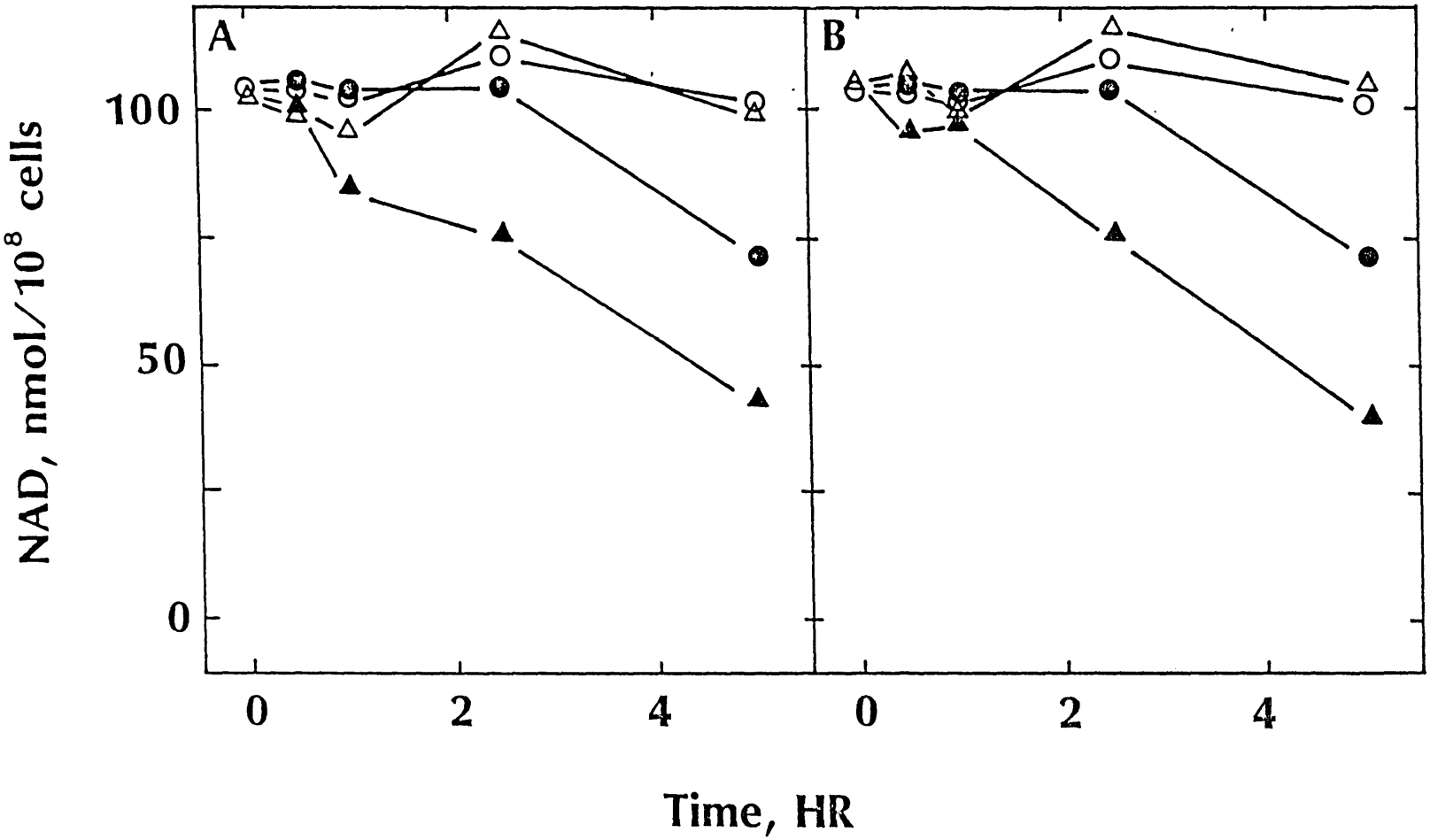
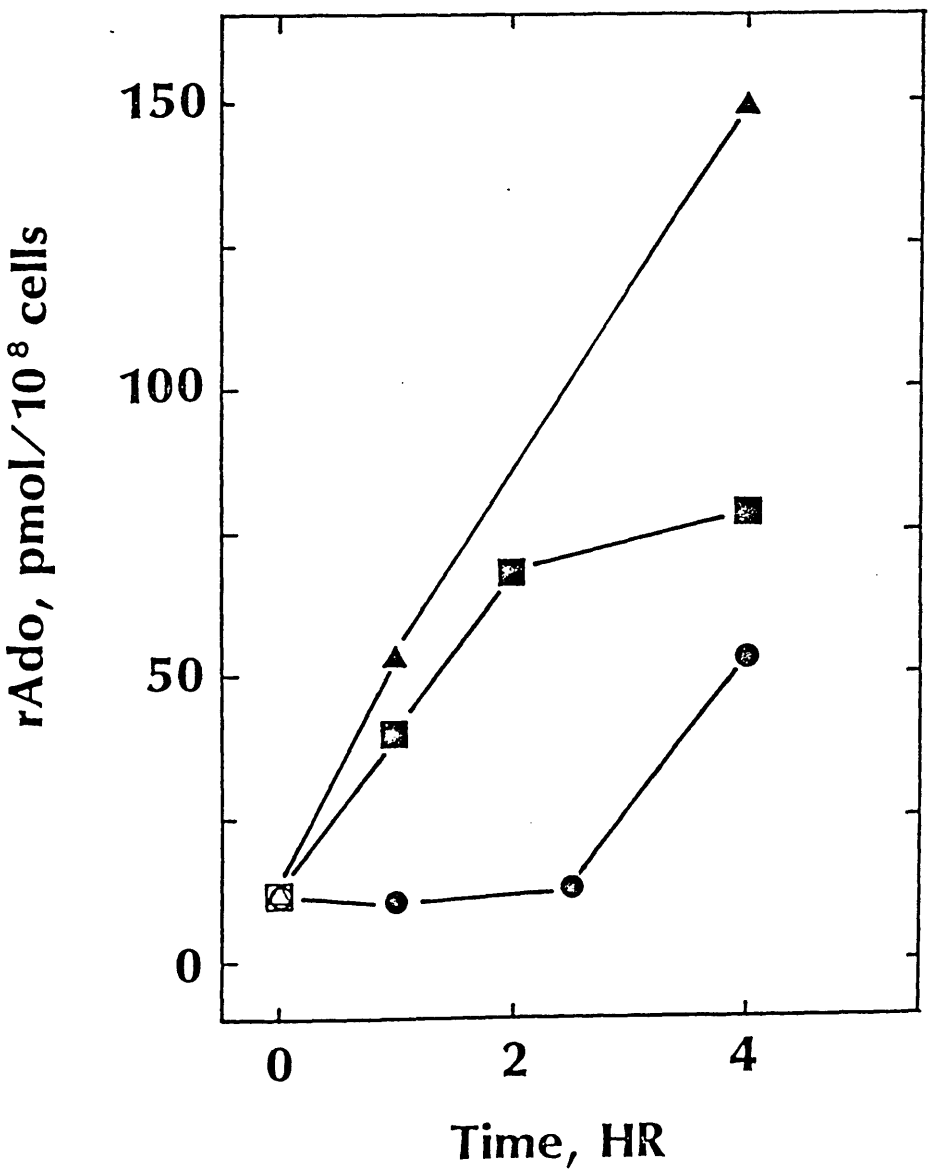

A
B
100
50
0
0
2
4
0
2
4
NAD, nmol/10^8 cells
Time, HR

Figure 15. The effect of ara C or hydroxyurea on poly-(ADP-ribose) content in CF-3 cells in the presence of 3-aminobenzamide following 5 J/m^2 UV irradiation. Mitotically arrested CF-3 cells were incubated in the presence of 3 mM 3-aminobenzamide and 0.1 mM ara C prior to UV treatment and for the entire post-irradiation period. Other cells were incubated in the presence of 3 mM 3-aminobenzamide prior to UV treatment and in the presence of 3 mM 3-aminobenzamide and 10 mM hydroxyurea for the entire post-irradiation period. Each data point represent analyses of extractions of from 6 - 30 100 mm dishes of cells. Cell numbers ranged from 2.9×10^6 - 3.2×10^6. The data are expressed as total intracellular poly-(ADP-ribose) content in pmol/10^8 cells of ribosyladenosine derived from poly(ADP-ribose) as a function of time. Poly(ADP-ribose) levels in unirradiated control cells in medium alone, (△); UV irradiated cells in medium containing 3 mM 3-aminobenzamide, (●); UV irradiated cells in medium containing 0.1 mM ara C and 3 mM 3-aminobenzamide, (■); UV irradiated cells in medium containing 10 mM hydroxyurea and 3 mM 3-aminobenzamide, (▲).


150
100
50
0
rAdo, pmol/10⁸ cells
0
2
4
Time, HR

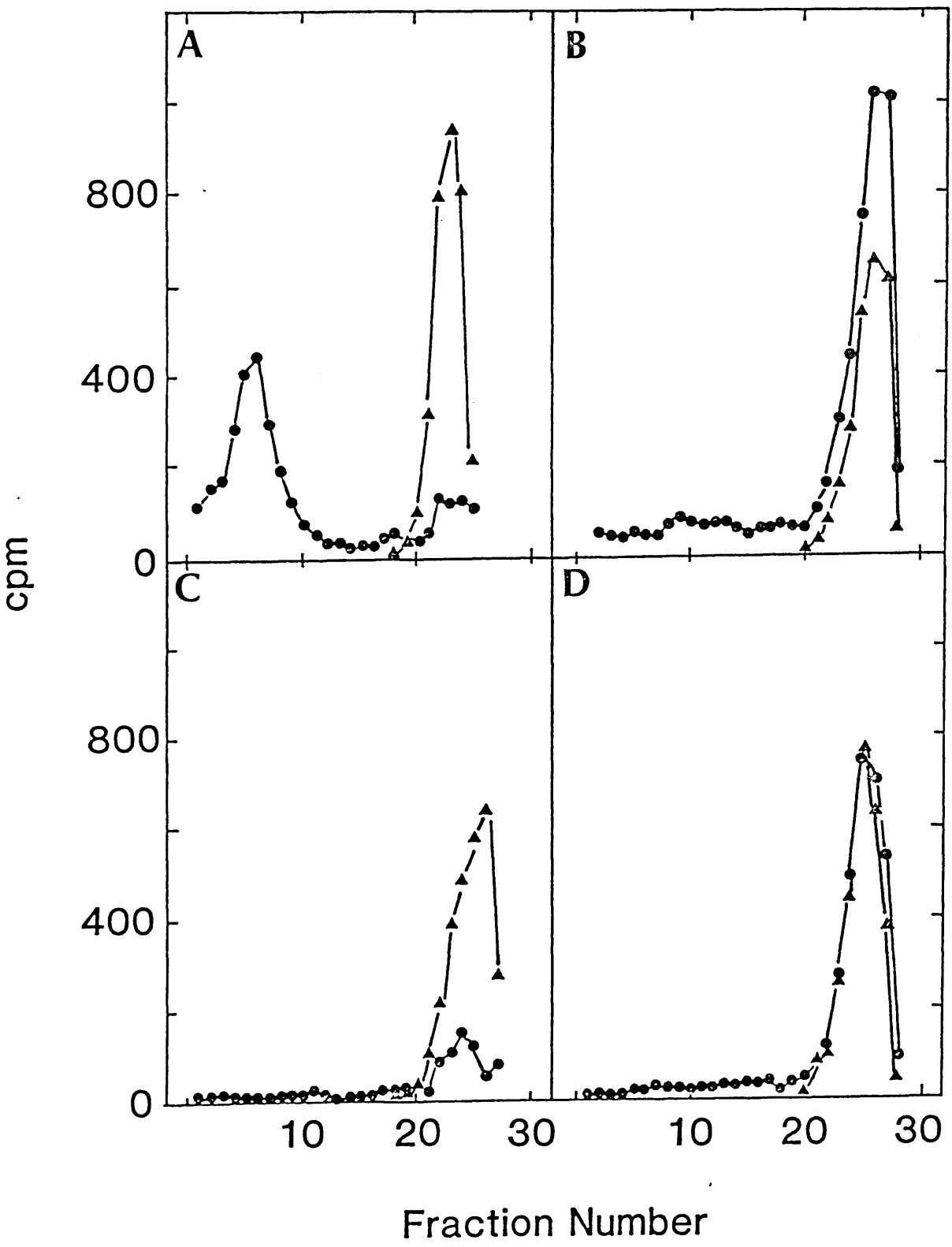
In the presence of ara C, synthesis of 40 pmol/10^8 cells occurs; in the presence of hydroxyurea, synthesis of 53 pmol/10^8 cells occurs. By comparison, this study has observed poly(ADP-ribose) synthesis following 5 J/m^2 UV irradiation in the absence of inhibitors to be 200 pmol/10^8 cells by 1 hr, the time of maximum accumulation (Figure 5). Thus, the poly(ADP-ribose) synthesized by 1 hr in the presence of ara C and 3-aminobenzamide is 20% of control; that synthesized by 1 hr in the presence of hydroxyurea and the inhibitor is approximately 30% of control. It is interesting to note that by 5 hr, in the presence of ara C (panel A), the poly(ADP-ribose) level is 60% of control; in the presence of hydroxyurea, the polymer level is greater than 100% of control.

Effect of 3-aminobenzamide on DNA repair replication

The effect of inhibitors of poly(ADP-ribose) polymerase on unscheduled DNA synthesis following DNA damage has been examined in several previous studies (Althaus et al., 1980; Berger and Sikorski, 1981; Miwa et al., 1981; Durrant et al., 1981; Sims et al., 1982; Althaus et al., 1982). These studies reported that the presence of inhibitors caused a stimulation of unscheduled DNA

synthesis. Hydroxyurea had been utilized in each of these studies to suppress DNA replicative synthesis. In the present study, it had been observed that 3-aminobenzamide does not block poly(ADP-ribose) synthesis effectively in the presence of hydroxyurea following UV treatment. Therefore, the effect of 3-aminobenzamide on DNA repair replication in the absence of hydroxyurea was examined. Repair replication was measured by a modification of the method of Smith and Hanawalt (1976a). Cells were incubated for 2.5 hr in the presence of [^{3}H]-thymidine radiolabel and bromodeoxyuridine density label. Alkaline cesium chloride density equilibrium sedimentation analyses were performed. Figure 16 shows the distribution of label on gradients of control cells and cells treated with UV in the absence or presence of hydroxyurea. In the profile of untreated control cells (panel A), a small peak of high density can be seen. This represents endogenous replicative synthesis which is completely suppressed by hydroxyurea (panel C). Following UV treatment, a peak of repair synthesis can be seen at a lower density in both the absence (panel B) and presence (panel D) of hydroxyurea. The data in Figure 16 show that the peak of high density in panel A (DNA replicative synthesis) is well separated from the peak of low density in panel B

Figure 16. DNA repair replication in mitotically arrested CF-3 cells in the presence and absence of hydroxyurea. Unirradiated CF-3 control cells (panels A and C) and UV irradiated cells (panels B and D) were incubated for 2.5 hr in the presence of [^{3}H]-thymidine (10 µCi/ml, 65 Ci/mmole), 10 µM bromodeoxyuridine, and 10 mM hydroxyurea (panels C and D only). Cells were then lysed with SDS buffer, pH 7.9 and alkaline cesium chloride equilibrium sedimentation analyses were performed according to MATERIALS AND METHODS. Each gradient contained DNA from 1.7 x 10^{6} cells. Radioactivity from [^{3}H]-thymidine, (●); radioactivity of added DNA from cells grown in the presence of [^{14}C]-thymidine, but in the absence of bromodeoxyuridine as a density marker, (▲). Total cpm from [^{3}H]-thymidine under [14]-C-light DNA peak corrected for control, (B) 4695; (D) 2987.


A
B
C
D
800
400
0
cpm
10
20
30
Fraction Number

(DNA repair replication). Thus, even in the absence of hydroxyurea, repair replication can be quantified. These data show that the amount of repair replication is supressed by 36% by hydroxyurea in this experiment. Other experiments routinely showed 30-40% inhibition by hydroxyurea.

Since repair replication can be quantified in the absence of hydroxyurea, the effect of 3-aminobenzamide on repair replication was examined under these conditions in order to assess the role of poly(ADP-ribose) in DNA repair of UV lesions. Figure 17 shows the distribution of label on gradients of UV treated control cells (panel A) and UV treated cells in the presence of 3-aminobenzamide with and without hydroxyurea. Controls which were not treated with UV show negligible repair replication (data not shown). By comparing panel A with panel C, it can be seen that little difference in the amount of repair replication occurs in UV treated cells with (panel C) or without (panel A) 3-aminobenzamide. However, a comparison of the amount of repair replication with (panel D) and without (panel B) 3-aminobenzamide in the presence of hydroxyurea shows an apparent stimulation by the presence of 3-aminobenzamide.

Figure 18 shows the time course of DNA repair replication following 5 J of UV irradiation in the absence or

Figure 17. The effect of hydroxyurea on DNA repair replication in mitotically arrested CF-3 cells in the presence and absence of 3-aminobenzamide. UV irradiated cells were incubated for 2.5 hr in the presence of [^{3}H]-thymidine (10 µCi/ml, 65 Ci/mmole), 10 µM bromodeoxyuridine (panel A). Some cells were incubated in the presence of 10 mM hydroxyurea (panel B), 3 mM 3-aminobenzamide (panel C), and the combination of 3 mM 3-aminobenzamide with 10 mM hydroxyurea (panel D). Cells were then lysed with SDS buffer, pH 7.9 and alkaline cesium chloride gradient analysis was performed according to MATERIALS AND METHODS. Each gradient contained DNA from 1.7 x 10^{6} cells. Radioactivity from [^{3}H]-thymidine (●); radioactivity of added DNA from cells grown in the presence of [^{14}C]-thymidine, but in the absence of bromodeoxyuridine as a density marker, (▲). Total cpm from [^{3}H]-thymidine under [^{14}C]-light peak corrected for control, (A) 4695; (B) 2987; (C) 4976; (D) 4876.

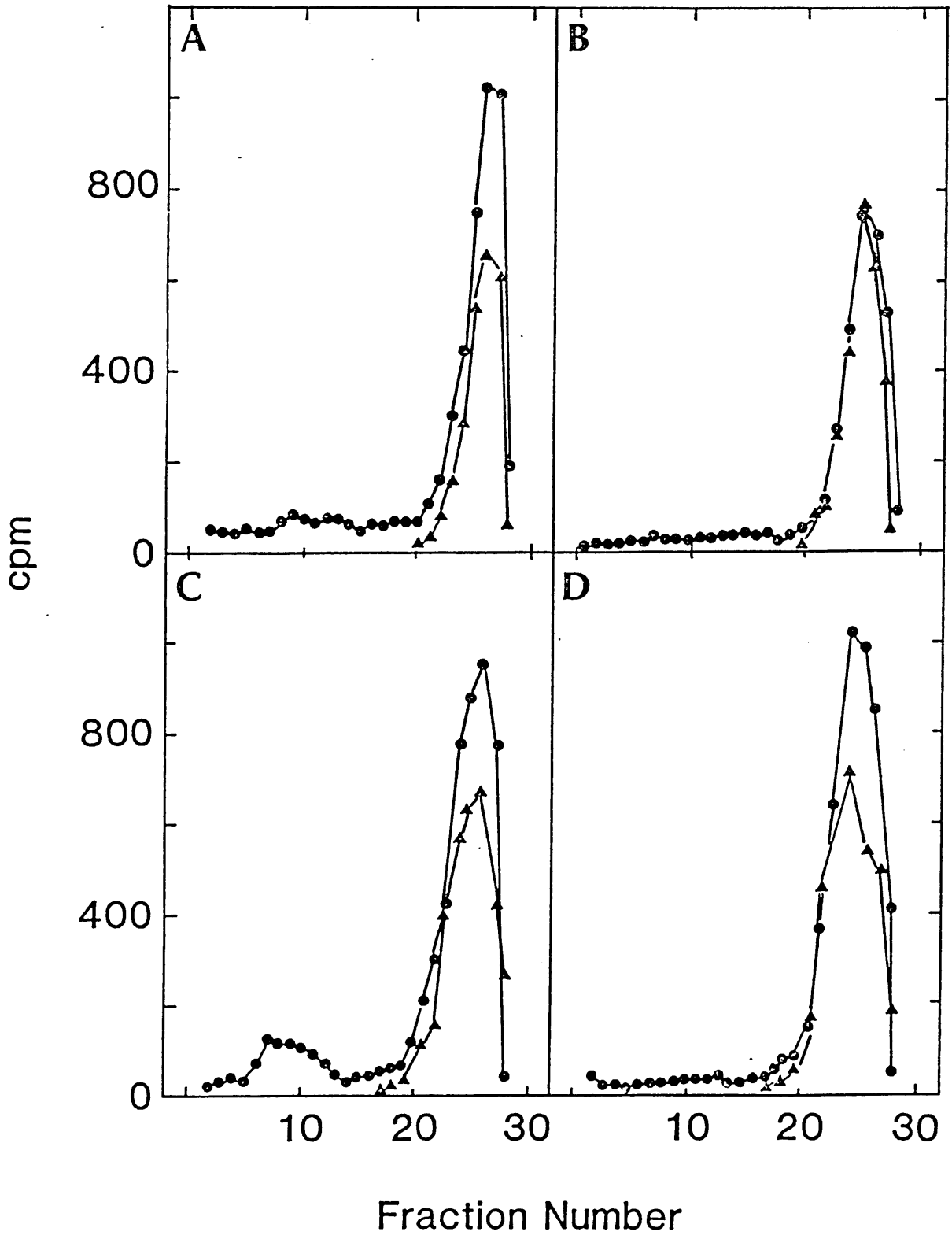
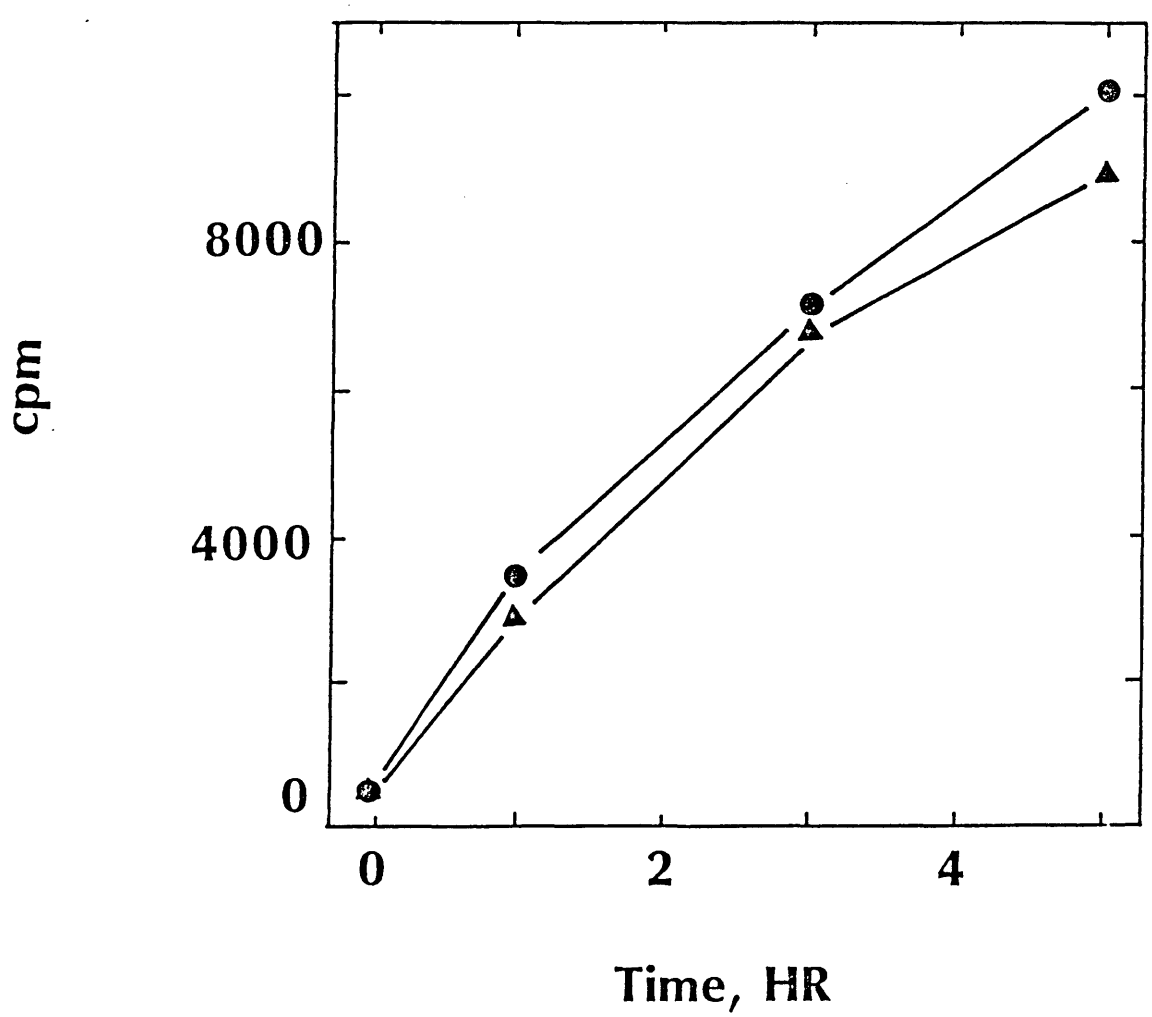

A
B
C
D
800
400
0
cpm
10
20
30
Fraction Number

Figure 18. The effect of 3-aminobenzamide on DNA repair replication in the absence of hydroxyurea following 5 J/m^2 UV irradiation of CF-3 cells. Mitotically arrested cells were incubated in medium containing 3 mM 3-aminobenzamide for 1 hr prior to UV treatment and for the entire post-irradiation period. The data represent the distribution of radiolabel in cesium chloride gradients and are expressed as cpm from [^{3}H]-thymidine incorporation banding at the density of [^{14}C]-light DNA (as described in MATERIALS AND METHODS) as a function of time. Each gradient contained DNA from 3.2 x 10^5 cells. DNA repair replication in the absence (●) or presence (▲) of 3 mM 3-aminobenzamide.


cpm
8000
4000
0
0
2
4
Time, HR

presence of 3-aminobenzamide. Hydroxyurea was not present. Under these conditions, poly(ADP-ribose) synthesis was completely inhibited for 2.5 hr (Figure 4). Thus, the data of Figure 18 show that there is little difference in the time course or in amount of repair replication in the absence of poly(ADP-ribose) synthesis.

Poly(ADP-ribose) metabolism following treatment with MNNG

UV irradiation produces many types of DNA lesions which are removed by an excision-repair system. In this study, it was of interest to examine poly(ADP-ribose) metabolism and the effect of 3-aminobenzamide on DNA repair following treatment of cells with an alkylating agent which produces another unique set of DNA lesions removed by other repair mechanisms.

MNNG, a potent carcinogen, is a DNA alkylating agent which produces a variety of lesions. Among these are the adducts, O^6-methylguanine, N^3-methyladenine, and N^7-methylguanine (Sklar and Strauss, 1981). The removal of these adducts produces single-strand breaks in DNA due to enzymatic cleavage (Lindahl, 1979). MNNG can cause direct single-strand breaks also (Lijinsky, 1976). The *in vivo* rate of poly(ADP-ribose) synthesis after large doses (0.3 mM) of MNNG has been previously studies in SVT2

cells (Juarez-Salinas et al., 1979). The rate of conversion of NAD to poly(ADP-ribose) was rapid; maximum poly(ADP-ribose) accumulation occurred at 20 min and decreased to control levels by 80 min.

The present study has examined the kinetics of poly-(ADP-ribose) synthesis in human fibroblasts following treatment with a non-toxic dose of MNNG of 5 µg/ml (34 µM). Figure 19 shows that this does causes a similar initial rate of NAD depletion as 5 J/m^2 of UV light. The potency of 3-aminobenzamide on poly(ADP-ribose) synthesis following MNNG treatment has been examined. In addition, the effect of 3-aminobenzamide on repair replication is presented in order to assess the role of poly(ADP-ribose) in DNA repair of MNNG lesions.

Analysis of NAD and poly(ADP-ribose) levels in CF-3 cells were performed following treatment with 5 µg/ml MNNG. Figure 20, panel A, shows the kinetics of NAD depletion and the accumulation of poly(ADP-ribose). The initial NAD content is approximately 120 nmol/10^8 cells. A rapid, continuous depletion of the cellular NAD pool occurs for 80 min following treatment. The rate of loss is maximal between 0-40 min where the NAD pool is decreasing at a rate of approximately 600 pmol/min/10^8 cells. NAD content is stable after 80 min and by 5 hr the net synthesis of NAD occurs.

Figure 19. NAD depletion in CF-3 cells following 5 J/m^2 UV irradiation of 5 µg/ml MNNG. The data represent duplicate analyses of duplicate extractions of 35 mm dishes of mitotically arrested cells and are expressed as percent of 0 hr control NAD/10^6 cells as a function of time. Standard deviation of the mean for each data point is less than 5%. For UV treated cells, cell numbers ranged from 6.1×10^5 - 6.5×10^5. For MNNG treated cells, cell numbers ranged from 4.8×10^5 - 4.9×10^5. NAD levels in unirradiated (○) or non-MNNG treated (△) control cells; UV irradiated cells, (●); MNNG treated cells, (▲).

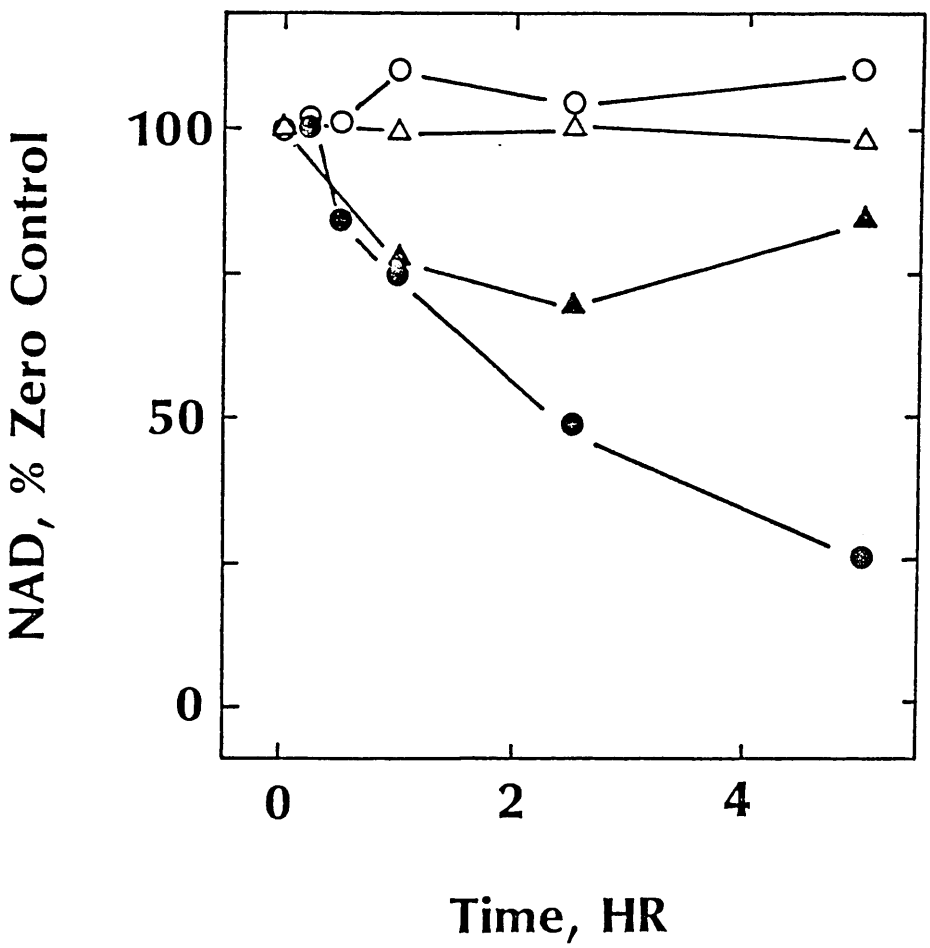
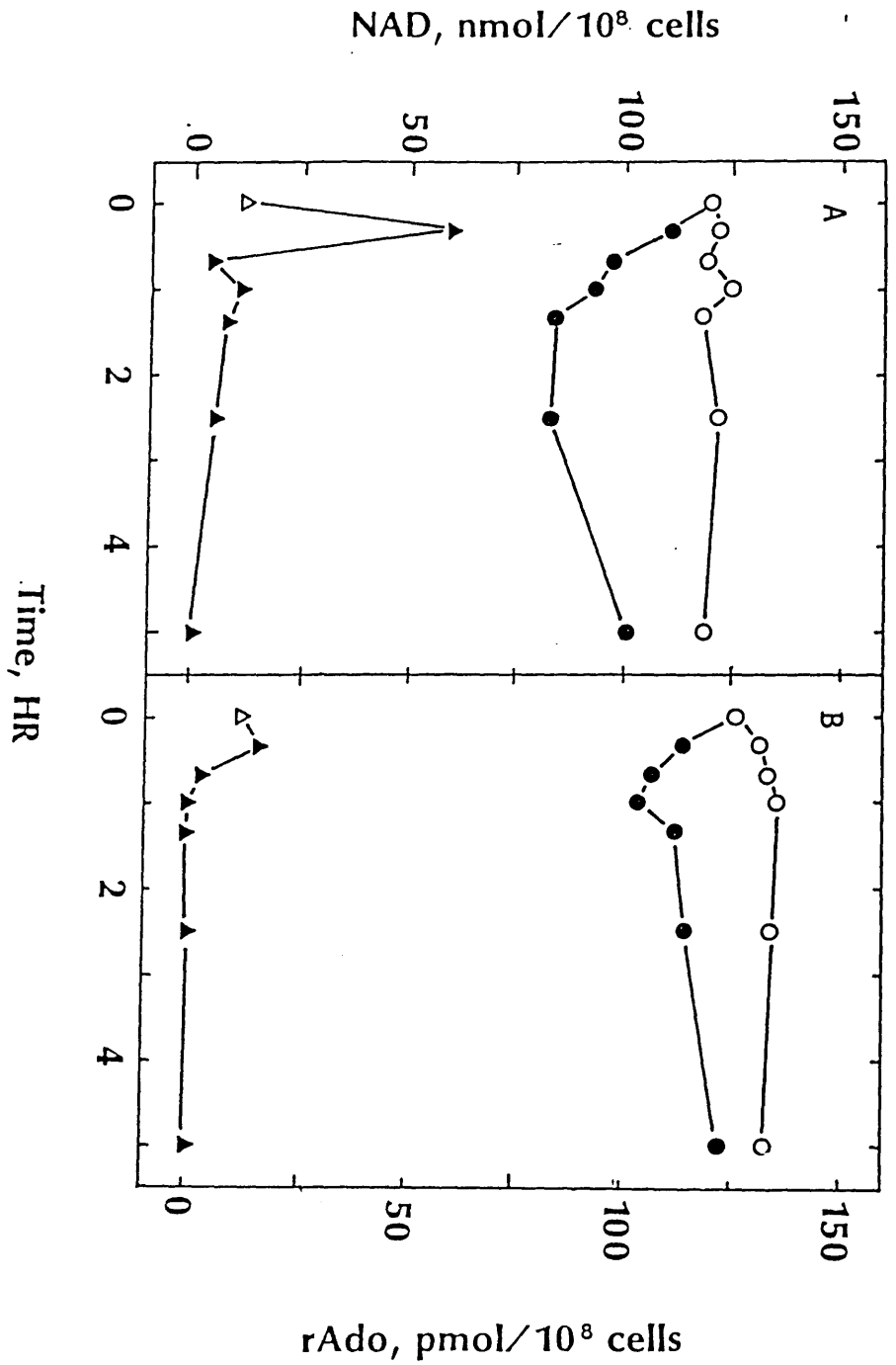

NAD, % Zero Control
100
50
0
0
2
4
Time, HR

Figure 20. Total intracellular contents of NAD and poly-(ADP-ribose) following treatment of CF-3 cells with 5 μg/ml MNNG. Mitotically arrested CF-3 cells were treated with 5 μg/ml MNNG for 20 min and incubated in the absence (panel A) or presence (panel B) of 3 mM 3-aminobenzamide. For NAD, the data represent duplicate analyses of duplicate extractions of 35 mm dishes of cells and are expressed as NAD content in nmol/10^8 cells as a function of time. Standard deviation of the mean for each data point is less than 5%. Cell numbers ranged from 6.2 x 10^5 - 6.5 x 10^5. For poly(ADP-ribose), the data represents analyses of from 5 - 30 10 mm dishes of cells and are expressed as poly(ADP-ribose) content in pmol/10^8 cells of ribosyl-adenosine derived from poly(ADP-ribose). Cell numbers ranged from 3.07 x 10^6 - 3.11 x 10^6 per each 100 mm dish. (A) NAD levels in untreated control cells (○); in MNNG treated cells, (●). Poly(ADP-ribose) levels in untreated control cells, (△); in MNNG treated cells, (▲). (B) NAD levels in untreated control cells in the presence of 3 mM 3-aminobenzamide, (○); in MNNG treated cells incubated in the presence of 3 mM 3-aminobenzamide prior to treatment and for the entire post-treatment period, (●). Poly(ADP-ribose) levels in untreated control cells, (△); in MNNG treated cells incubated in the presence of 3 mM 3-aminobenzamide prior to treatment and for the entire post-treatment period, (▲).


NAD, nmol/10⁸ cells
0
50
100
150
A
B
0
2
4
Time, HR
rAdo, pmol/10⁸ cells

Following MNNG treatment, the level of poly(ADP-ribose) increases rapidly to a maximum at 20 min. Accumulation of approximately 60 pmol/10^8 cells of ribosyladenosine derived from poly(ADP-ribose) is observed. This represents a 5-fold increase over basal level. Between 20 and 40 min, the accumulated level of poly(ADP-ribose) decreases as rapidly as it appears.

Between 20 and 40 min following treatment, the cellular NAD pool decreases at an approximate rate of 160 pmol/min/10^8 cells while the poly(ADP-ribose) maximum accumulation of 60 pmol/10^8 cells rapidly decreases to basal level. This rate is approximately 2.8 pmol/min/10^8 cells. Poly(ADP-ribose) content at maximum level can only account for 1.8% of the NAD degradation. These data are consistent with the hypothesis that poly(ADP-ribose) turnover is very rapid (Juarez-Salinas, 1979).

Figure 20, panel B, shows the kinetics of NAD depletion and poly(ADP-ribose) accumulation following 5 μg/ml MNNG treatment in the presence of 3-aminobenzamide. When 3-aminobenzamide is present in the culture medium following MNNG treatment, a 12% inhibition of NAD depletion occurs during the first 60 min. At 2.5 hr when NAD levels are a minimum in control cells without the inhibitor, 3-aminobenzamide inhibition is approximately 40%. The

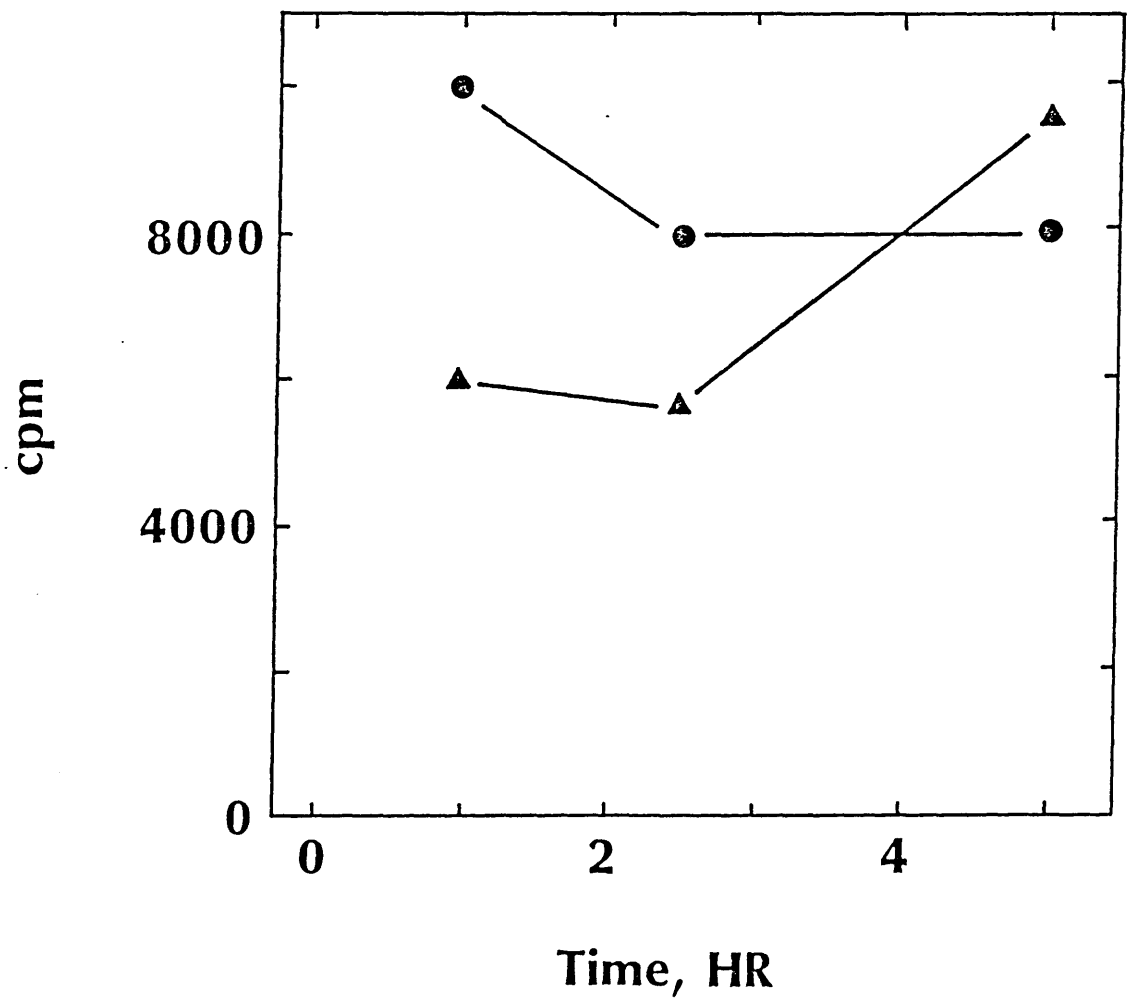
experimental protocol may account for the NAD depletion in the presence of 3-aminobenzamide following MNNG treatment. To avoid possible interaction between MNNG and 3-aminobenzamide, the inhibitor was not present during the 20 min treatment period. Although 3-aminobenzamide was added to the culture medium 1 hr prior to MNNG treatment, cells may lose this compound rapidly. Since it is known that MNNG causes DNA strand breaks very rapidly (Lijinsky, 1976), the combination of rapid DNA strand break formation and rapid loss of inhibitor may be responsible for the observed NAD depletion. Between 2.5 and 5 hr, NAD levels begin to increase steadily. Whether this increase is the result of inhibition or the net synthesis of NAD cannot be determined by this study. When 3-aminobenzamide is present in the culture medium following MNNG treatment, the level of poly(ADP-ribose) increases to a maximum at 20 min. Accumulation of 16 pmol/10^8 cells of ribosyladenosine derived from poly(ADP-ribose) level is observed. This represents 26% of the poly(ADP-ribose) level observed 20 min after MNNG treatment in the absence of 3-aminobenzamide (Figure 20, panel A). The absence of the inhibitor during treatment probably accounts for this. Between 20 and 40 min in the presence of the inhibitor, the accumulated level of poly(ADP-ribose) decreases as

rapidly as it appeared. By 60 min, levels of poly(ADP-ribose) are not detectable.

Effect of 3-aminobenzamide on DNA repair replication following MNNG treatment

The effect of 3-aminobenzamide on DNA repair replication following MNNG treatment was examined. Figure 21 shows the time-course of DNA repair replication following treatment with 5 µg/ml MNNG. Differences in both amount and time-course are observed. In the control cells, rates of DNA repair replication are the fastest at 1 hr following MNNG treatment. The control cell rate of DNA repair of 10,000 cpm/hr/10^6 cells is 35% faster than this rate in cells treated with 3-aminobenzamide. Between 1 hr and 2.5 hr, the amount of DNA repair replication decreases by 20% and remains at this level up to 5 hr. This data suggests that a significant amount of repair occurs within 1 hr following MNNG damage. In cells with 3-aminobenzamide in the culture medium prior to and following MNNG treatment, DNA repair replication is inhibited approximately 40% during the first 2.5 hr after treatment. Between 2.5 and 5 hr, however, the rate of repair replication increases 168%. After 5 hr following treatment, the level of repair replication is 95% of the 1 hr control. These data suggest that the

Figure 21. The effect of 3-aminobenzamide on DNA repair replication in the absence of hydroxyurea following treatment of CF-3 cells with 5 μg/ml MNNG. Mitotically arrested cells were incubated in medium containing 3 mM 3-aminobenzamide for 1 hr prior to MNNG treatment and for the entire post-treatment period. The data represent the distribution of radiolabel in cesium chloride gradients and are expressed as cpm for [^{3}H]-thymidine incorporation banding at the density of [^{14}C]-light DNA (as described in MATERIALS AND METHODS) as a function of time. Each gradient contained DNA from 6.7 x 10^{5} cells. DNA repair replication in the absence (●) or presence of 3 mM 3-aminobenzamide, (▲).


cpm
8000
4000
0
0
2
4
Time, HR

presence of 3-aminobenzamide after MNNG treatment does not inhibit, but may delay repair replication. In the absence of hydroxyurea, no stimulation of repair replication is observed at all by the inhibition of poly(ADP-ribose) synthesis as is observed by other investigators. The stimulation of repair replication observed by others may be attributable to the presence of hydroxyurea.

NAD conversion to poly(ADP-ribose) in cells of low and high population doubling level (PDL) following UV irradiation

Following UV treatment, mitotically arrested cells of high PDL exhibit 20-30% more UDS than low PDL cells (Dell'Orco and Whittle, 1978). Dell'Orco and Whittle have proposed that the increased UDS may be related to the observation that chromosomal proteins of aged cells are less tightly bound allowing repair enzymes greater accessibility (ibid.). Alternatively, there is evidence that high PDL cells have more naturally occurring DNA strand breaks (Lipetz, 1980). The correlation of poly-(ADP-ribose) synthesis with DNA strand breaks and the implication of its function in chromatin structure and DNA repair prompted this study to assess poly(ADP-ribose) metabolism as a function of senescence.

CF-3 cells aged in vitro to PDL 53-57 were employed

as a senescent population. Cells of PDL 26-28 were used as a control population. The NAD content/cell was measured as a function of time in young and aged cells following exposure to 5 J/m^2 UV light. Table 1, part A, shows pmol of NAD/10^6 cells from four experiments. NAD content in the aged cells is 191% higher than in the young cells. By observation, aged cells at confluency are larger in volume and size analysis on the Coulter Electronic Counter showed that cells of PDL 57 have a mean volume of approximately 2400 μm^3 while the mean volume of cells of PDL 27 is 1200 μm^3. The intracellular concentration of NAD is probably very similar in young and aged cells assuming that the intracellular compartmentation of NAD is similar. However, it is interesting to note that the basal level of NAD in the aged cells is not only larger, but it is also more variable. The coefficient of variation for NAD from experiment to experiment is 26% in the aged cells as compared to 12% in the young cells. The variation in the data is clearly due to differences in individual populations of aged cells since the standard deviation of the mean within an experiment averaged 3% (Table 1, part A).

Since the NAD content of aged cells is variable, it was of interest to determine whether the poly(ADP-ribose) content of these cells would show similar variation.

Table 1. NAD and poly(ADP-ribose) levels in high and low PDL cells following UV irradiation.

*A

	Expt.	pmol NAD/10^6 cells 0 hr	1 hr	2.5 hr	5 hr
Low PDL					
	A	986 ± 20	(710)	383 ± 1	355 ± 6
	B	986 ± 37	759 ± 32	506 ± 76	292 ± 53
	C	764 ± 3	566 ± 15	(525)	172 ± 6
	D	855 ± 68	504 ± 22	(335)	216 ± 97
	$\bar{X}$ =	898 ± 108	610 ± 133	455 ± 87	259 ± 81
High PDL					
	A	2200 ± 0	(1900)	1545 ± 13	1532 ± 78
	B	1260 ± 35	1047 ± 129	(880)	578 ± 3
	C	1415 ± 6	1274 ± 6	(970)	424 ± 1
	D	1983 ± 18	(1865)	1723 ± 18	1658 ± 32
	$\bar{X}$ =	1715 ± 449	1521 ± 427	1279 ± 417	1048 ± 637

**B

		pmol ribosyladenosine/10^8 cells
Low PDL		
	E	12
	F	7
High PDL		
	E	5
	F	331

*NAD content of mitotically arrested CF-3 cells of low and high PDL was determined following 5 J/m^2 UV irradiation and expressed in pmol of NAD/10^6 cells. The data

represent duplicate analyses of duplicate extractions on 35 mm or 60 mm dishes of cells from four experiments. In each experiment, A, B, C, D, respectively, the extractions of low and high PDL cells were performed simultaneously. Cell numbers for 60 mm dishes in experiment (A), 1.41×10^6 - 1.43×10^6, high PDL; 1.96×10^6 - 2.02×10^6, low PDL; (B) 1.24×10^6 - 1.25×10^6, high PDL; 2.26×10^6 - 2.30×10^6, low PDL. Cell numbers for 35 mm dishes in experiment (C), 1.22×10^6 - 1.26×10^6, high PDL; 1.83×10^6 - 1.85×10^6, low PDL; (D), 4.95×10^5 - 5.02×10^5, high PDL; 5.66×10^5 - 5.77×10^5.

**Basal level poly(ADP-ribose) contents of mitotically arrested CF-3 cells of low and high PDL were determined and expressed as pmol of ribosyladenosine derived from poly(ADP-ribose). The data represents extractions of 30, 100 mm dishes of cells for experiment E and 10, 100 mm dishes of cells for experiment F. In each experiment, E and F, respectively, the extractions of low and high PDL cells were performed simultaneously. Cell numbers ranged from 4.12×10^6 - 4.16×10^6 for E; 2.99×10^6 - 3.02×10^6 for F.

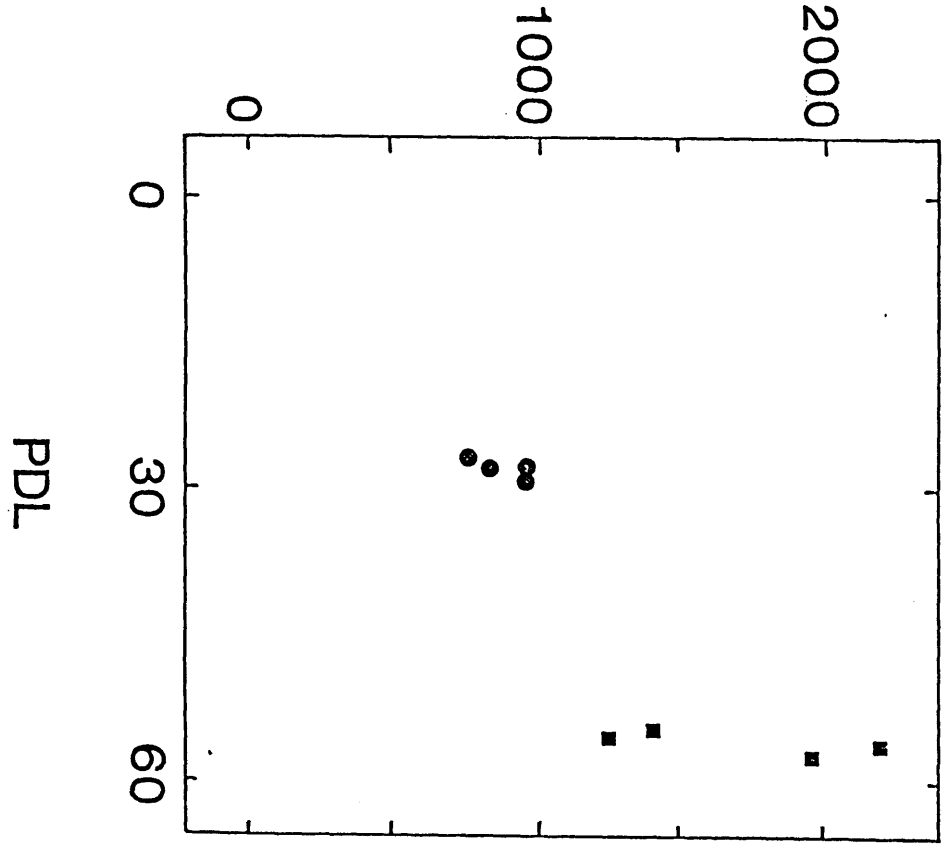
Quantification of poly(ADP-ribose) basal levels shows that aged cells have drastically varying amounts of this polymer while poly(ADP-ribose) levels in young cells is very low and constant (Table 1, part B).

The data of Figure 22 correlate the basal NAD level with the PDL of the CF-3 populations from the four experiments shown in Table 1, part A. The data again show that cells of high PDL have higher and more variable amounts of initial NAD content. The data also show that aged cells of higher PDL, 55 and 57, respectively, have greater amounts of initial NAD content than do aged cells of lower PDL, 54 and 53, respectively. The variability of the basal levels of both NAD and poly(ADP-ribose) in aged cells may be related to an accumulation of DNA strand breaks which stimulate poly(ADP-ribose synthesis in aged cells. It has been reported that CF-3 cells of high PDL have a greater number of accumulated DNA strand breaks than cells of low PDL (Lipetz, 1980). Assuming cells accumulate DNA strand breaks as senescence occurs, then it is reasonable to assume that cells nearer to the senescence point (end of life-span) would have a greater number of DNA strand breaks and therefore less NAD and more poly(ADP-ribose) than other cells. However, the PDL at which the senescence

Figure 22. NAD basal levels in young and aged CF-3 cells as a function of time. Mean initial (0 hr) NAD contents of young and aged mitotically arrested CF-3 cells from four experiments were plotted against the PDL of the respective cell population. Data are expressed as pmol of NAD/10^6 cells, basal level as a function of PDL. Young cells, (●); aged cells, (■).

pmol NAD/10^6 cells,

Basal Level



point occurs varies ± 5 PDL at approximately 60 PDL. Therefore, some cells at PDL 55, for example, are nearer to the senescence point with greater numbers of accumulated DNA strand breaks, less NAD, and more poly(ADP-ribose) than other cells at PDL 55. Thus, cells of high PDL may exhibit greater variability in initial NAD content due to the variability of the PDL at the senescence point.

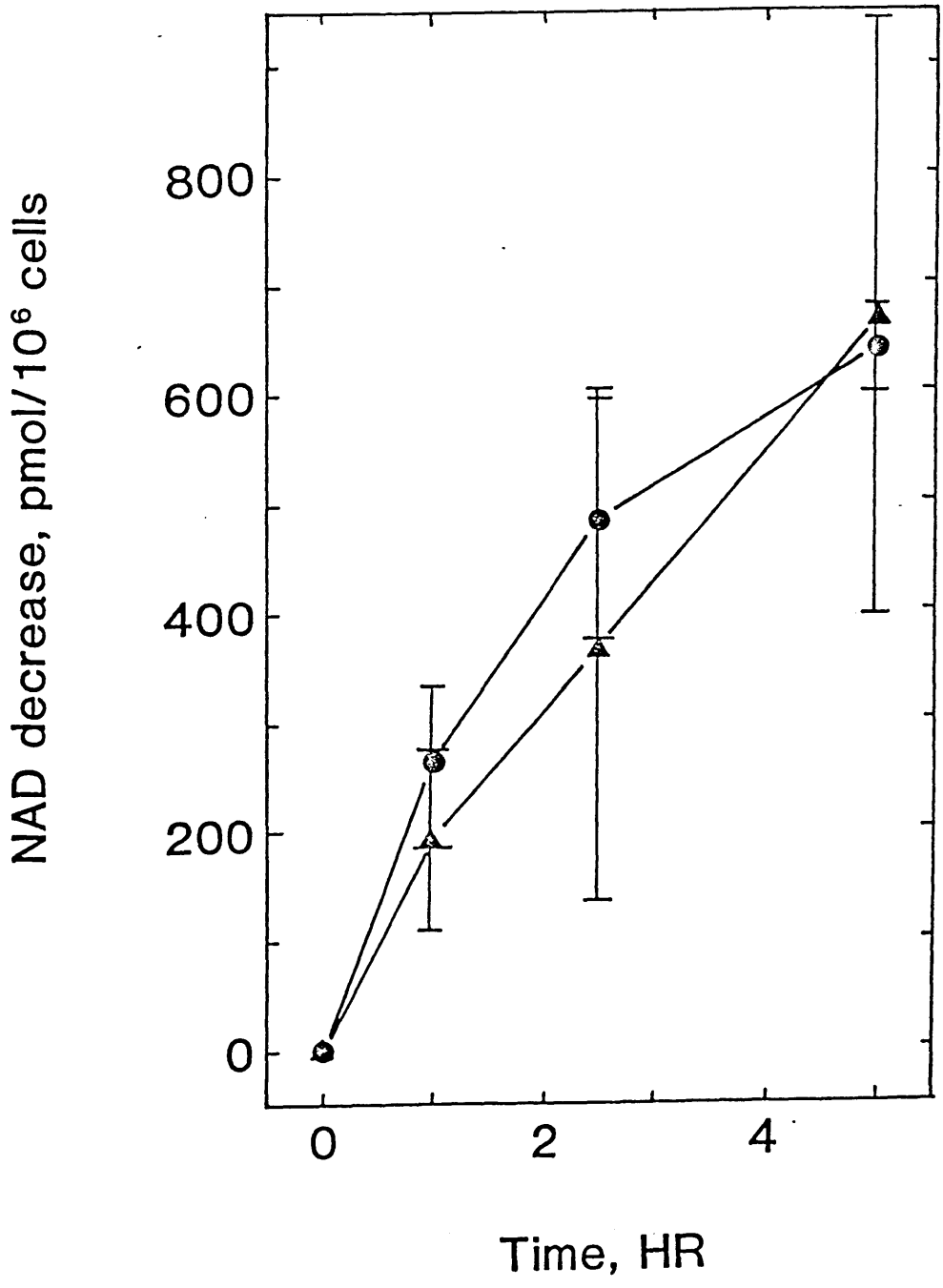
Since poly(ADP-ribose) is synthesized in response to DNA strand breaks, it was of interest to determine if the synthesis of this polymer in aged cells that demonstrate altered DNA repair responses following UV irradiation is normal. Since initial levels of NAD vary in the aged cells, the data were plotted as pmol of NAD converted to poly(ADP-ribose). Table 2 shows the mean number of pmol of NAD converted to poly(ADP-ribose) over the 5 hr post-irradiation period in four experiments. The mean value for each time point does not differ significantly between young and aged cells. The aged cells, however, show greater variability in the number of pmol of NAD converted, with a coefficient of variation of 41%, in the young cells, the coefficient of variation is 7%. This may reflect the variability in the initial NAD and poly(ADP-ribose) content as seen above. In addition, Figure 23 shows that

Table 2. Conversion of NAD to poly(ADP-ribose) in high and low PDL cells following UV irradiation*

	Expt.	Initial pmol NAD/10^6 cells	pmol NAD/10^6 cells converted to poly(ADP-ribose) 1 hr	2.5 hr	5 hr
Low PDL					
	A	986	(276)	603	631
	B	986	227	480	694
	C	764	198	(339)	592
	D	855	351	(520)	639
	$\bar{x}$ =	898 ± 108	263 ± 70	486 ± 110	639 ± 42
High PDL					
	A	2200	(300)	655	668
	B	1260	213	116	682
	C	1415	141	445	991
	D	1983	(118)	260	325
	$\bar{x}$ =	1715 ± 449	193 ± 82	369 ± 233	667 ± 272

*Dose of UV irradiation was 5 J/m^2.

Figure 23. Amount of NAD converted to poly(ADP-ribose) in high and low PDL cells following 5 J/m^2 of UV irradiation. Young and aged mitotically arrested CF-3 cells were exposed to 5 J/m^2 UV irradiation and duplicate NAD analyses of duplicate extractions of 35 mm of 60 mm dishes of cells were performed. The data represent the mean number of pmol of NAD/10^6 cells lost in both young and aged cells of four experiments. The data are expressed as NAD decrease, pmol/10^6 cells as a function of time. NAD decrease in young cells, (●); aged cells, (▲).


NAD decrease, pmol/10⁶ cells
800
600
400
200
0
0
2
4
Time, HR

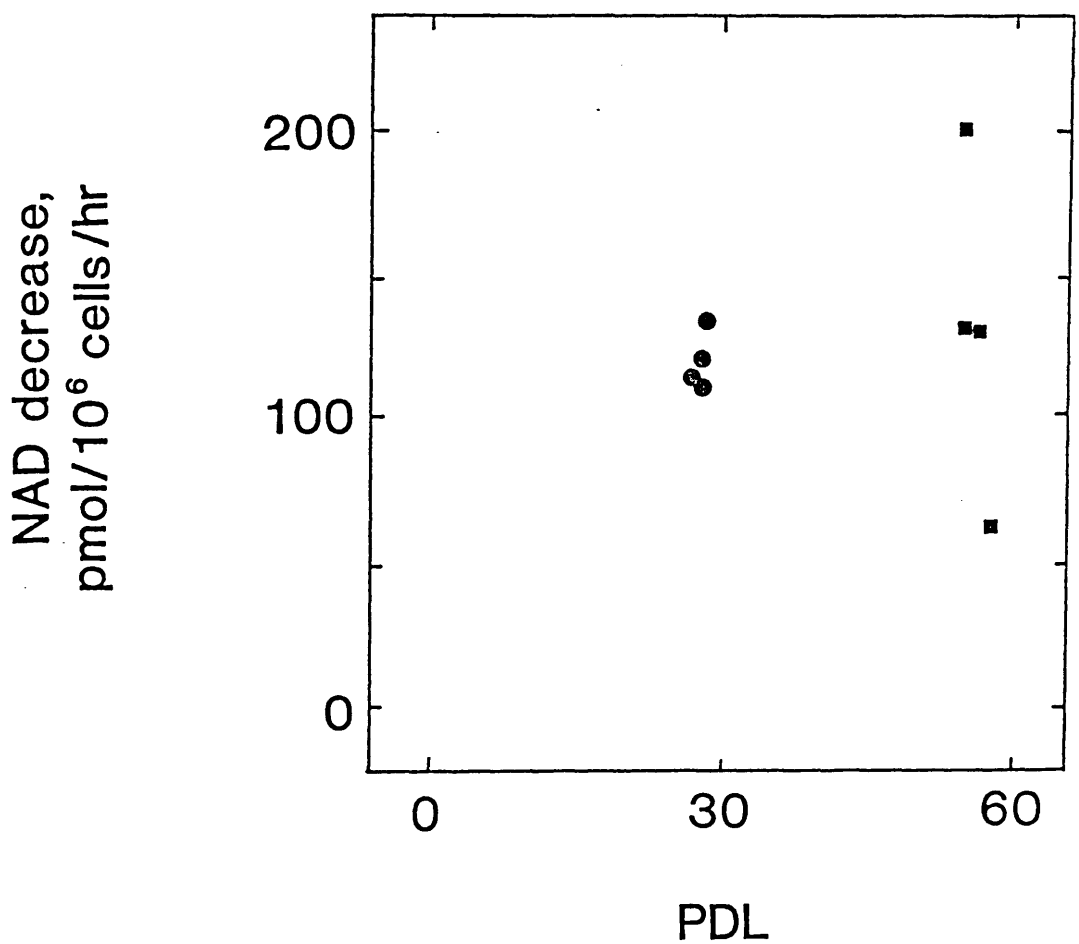
the mean number of pmol of NAD converted to poly(ADP-ribose) as a function of time is lower at earlier times for aged cell. However, due to variance between aged populations, the data show no significant differences in initial rates of NAD degradation. Table 3 shows the $t_{1/2}$ for NAD degradation for both young and aged cells. The $t_{1/2}$ was obtained from the regression lines of the data in Table 1, part A. The data show that the young cells have an average $t_{1/2}$ for NAD of 130 $\pm$ 21 min. The $t_{1/2}$ for NAD in aged cells (experiments B and C) ranges from 40% to 100% longer than that of young cells; $t_{1/2}$ of NAD is never attained in experiments A and D. This is presumably due to the high initial NAD levels in the cells of these experiments. Young cells deplete 70-80% of the cellular NAD pool in 5 hr following 5 J/m^2 of UV light. In contrast, aged cells, depending on the magnitude of the initial NAD pool, lose an average of only 40% of the cellular NAD pool even though the average amount of NAD converted to poly(ADP-ribose) is the same as in young cells at 5 hr. However, Figure 24 shows the rate of NAD depletion plotted as a function of PDL. The data show that aged cells have a wide range of rates of NAD depletion, from 63 pmol/hr/10^6 cells to 200 pmol/hr/10^6 cells as compared to young cells which have

Table 3. Half-life of NAD.

	Expt.	Initial pmol/NAD/10^6 cells	* $t_{1/2}$ NAD
Low PDL	A	986	137 min
	B	986	137 min
	C	764	146 min
	D	855	99 min
		$\bar{x}$ = 898 ± 108	$\bar{x}$ = 130 ± 21
High PDL	A	2200	never attained
	B	1260	261 min
	C	1415	182 min
	D	1983	never attained
		$\bar{x}$ = 1715 ± 449	

*Regression analyses of NAD levels at designated time points following 5 J/m^2 UV irradiation were performed for each experiment; regression lines were plotted. The time in min of 50% of the initial NAD level was taken as $t_{1/2}$ for NAD.

Figure 24. Rates of NAD depletion in young and aged CF-3 cells as a function of PDL. Rates of NAD depletion of young and aged mitotically arrested CF-3 cells from four experiments were plotted against the PDL of the respective cell populations. Data are expressed as NAD decrease, pmol/10^6 cells/hr as a function of PDL. Rates/hr of NAD depletion were obtained from regression analyses of NAD levels at designated time points over a 5 hr period following 5 J/m^2 UV irradiation. Young cells, (●); aged cells, (■).


NAD decrease,
pmol/10^6 cells/hr
200
100
0
0
30
60
PDL

rates from 111 pmol/hr/10^6 cells to 135 pmol/hr/10^6 cells.

The data of Figure 25 relate the average basal levels of NAD to the rates of NAD depletion in each experiment. Regression analysis of the data points for aged cells reveals a slope of -4.2; the same analysis of the data points for young cells reveals a slope of +7.6. These data suggest that the rate of NAD depletion in aged cells following UV treatment is not positively correlated to basal level, but that in young cells which contain lower levels of NAD, NAD depletion is positively correlated to initial level of NAD. Apparently, the higher the basal NAD content in cells of high PDL, the more slowly do they deplete NAD. However, the significance of the data is low. The coefficient of correlation for the aged cells is -0.5; that for the young cells is +0.7.

Since mitotically arrested aged cells show an altered NAD metabolism as compared to young cells, it was of interest to determine whether a correlation existed between this parameter and the increased UDS observed in mitotically arrested cells by Dell'Orco and Whittle (1978). DNA repair replication of mitotically arrested young and aged cells was quantified by cesium chloride gradient analysis. Figure 26 shows the distribution of radiolabel in the gradients following treatment with 5 J/m^2 of UV light in young cells in the presence (panel A) and absence (panel C) of 10 mM

Figure 25. Correlation of NAD basal levels in young and aged CF-3 cells as a function of rate of NAD depletion. Mean initial (0 hr) NAD contents of young and aged mitotically arrested cells from four experiments were plotted against the rates of NAD depletion of the respective cell populations. Trend lines were computed by regression analysis of the young and aged data points. Data are expressed as pmol of NAD/10^6 cells, basal level as a function of NAD decrease, pmol/10^6 cells/hr. Rates/hr of NAD depletion were obtained from regression analyses of NAD levels for 5 hr following treatment with 5 J/m^2 UV light. Young cells, (●); aged cells, (■).

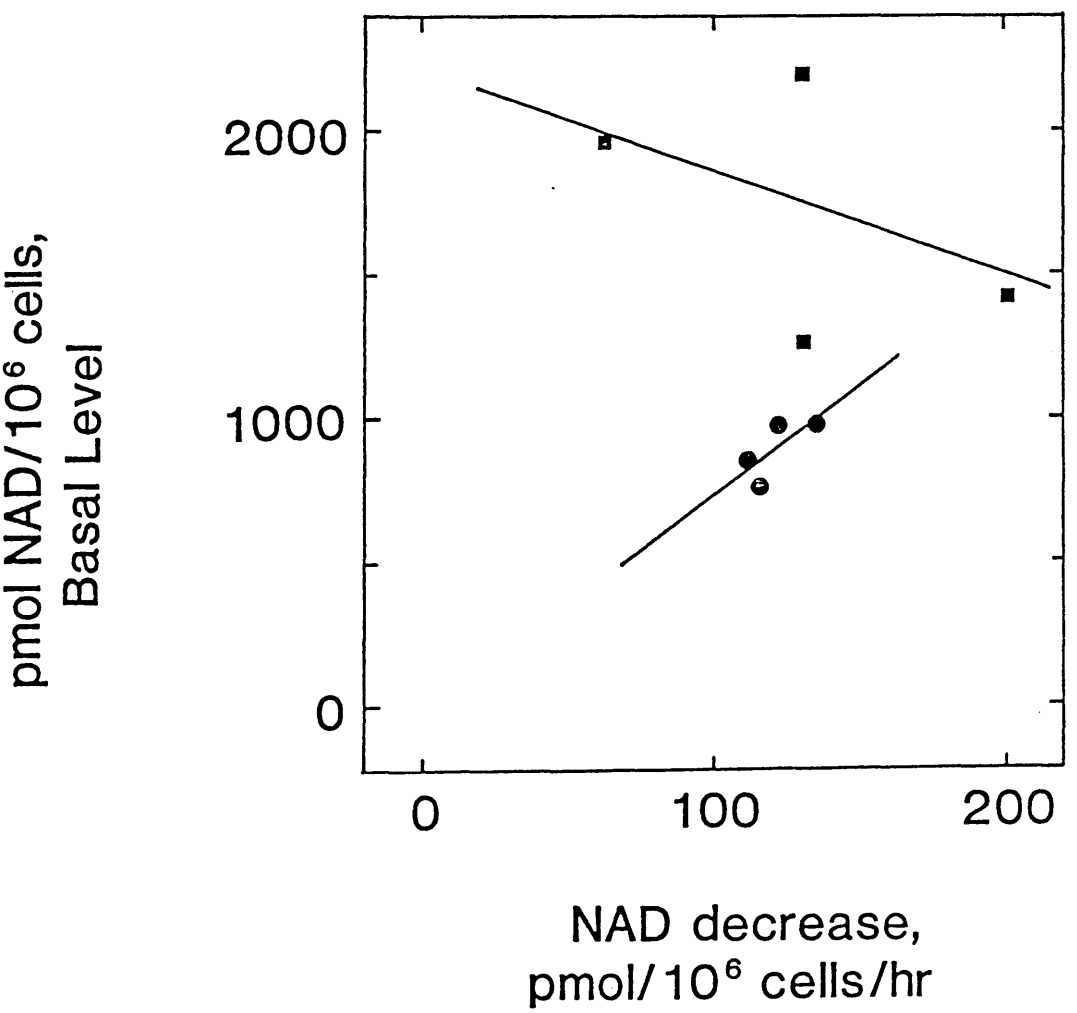
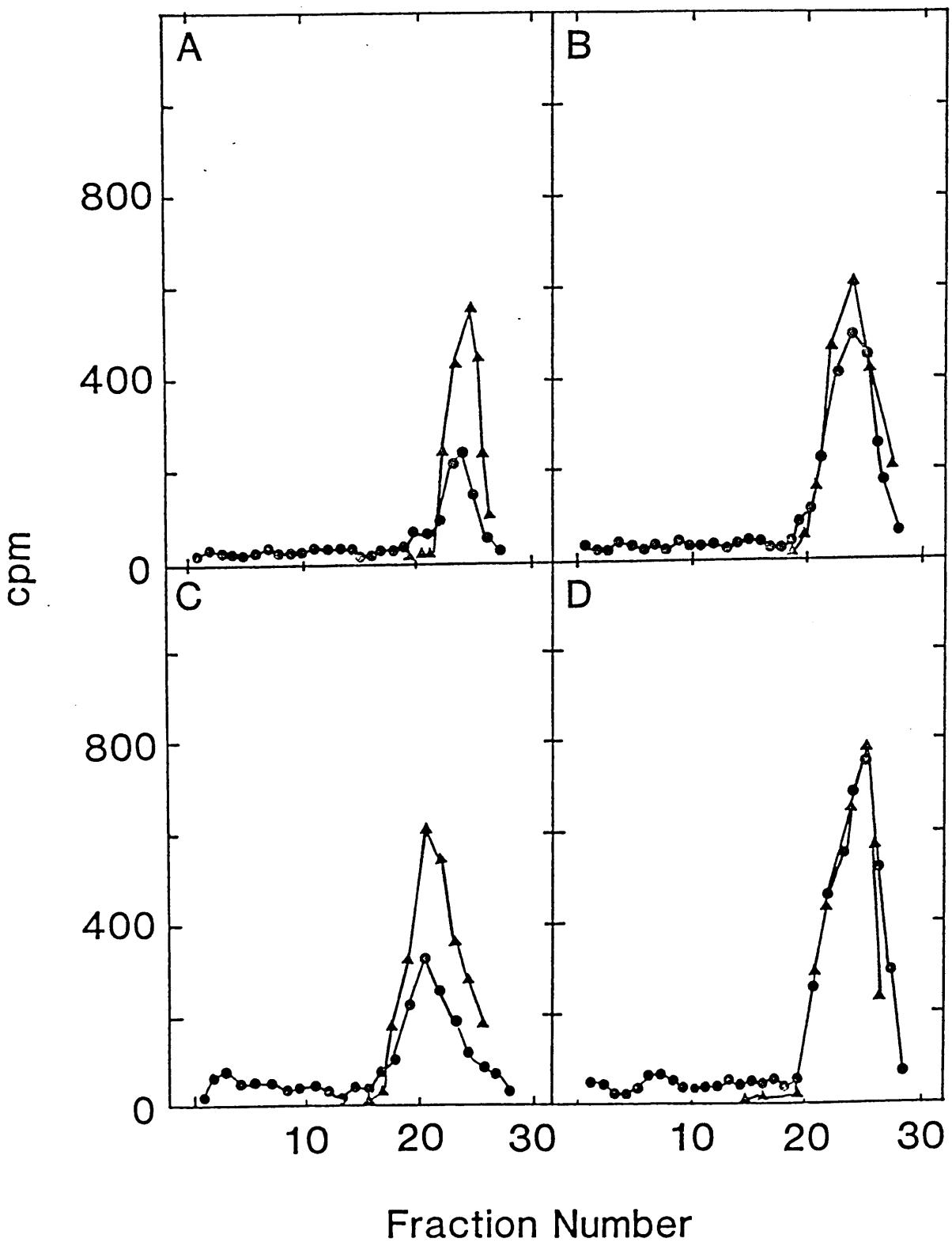

pmol NAD/10⁶ cells,
Basal Level
2000
1000
0
0
100
200
NAD decrease,
pmol/10⁶ cells/hr

Figure 26. DNA repair replication in mitotically arrested cells of low and high PDL in the presence and absence of hydroxyurea. UV irradiated cells of low PDL (panels A and C) and high PDL (panels B and D) were incubated for 5 hr in the presence of [^{3}H]-thymidine (10 μCi/ml, 65 Ci/mmole) and 10 μM bromodeoxyuridine. Some cells were also incubated with 10 mM hydroxyurea (panels A and B). Cells were then lysed with SDS buffer, pH 7.9, and alkaline cesium chloride gradient analyses were performed according to MATERIALS AND METHODS. Each gradient contained DNA from 1.7 x 10^{6} cells. Increasing density is from right to left. Radioactivity from [^{3}H]-thymidine, (●); radioactivity of added DNA from cells grown in the presence of [^{14}C]-thymidine, but in the absence of bromodeoxyuridine, as a density marker, (▲). Total cpm from [^{3}H]-thymidine under [^{14}]-C-light DNA peak corrected for control, (A) 1343; (B) 3503; (C) 512; (D) 1968.


A
B
C
D
800
400
0
cpm
10
20
30
Fraction Number

hydroxyurea and in aged cells in the presence (panel B) and absence (panel D) of 10 mM hydroxyurea. The data show that mitotically arrested aged cells have increased levels of DNA repair replication in the presence or absence of hydroxyurea. This observation is in agreement with Dell'Orco and Whittle (1978) who observed that UDS levels in mitotically arrested aged cells were 20-30% greater than in mitotically arrested young cells. UDS was measured in the presence of hydroxyurea in the experiments of Dell'Orco and Whittle (ibid.). In the present study, DNA repair replication was 280% greater in the aged cells than in the young in the presence of hydroxyurea. In the absence of hydroxyurea, the level was 160% greater than in the young. While hydroxyurea has been observed by this study to inhibit DNA repair replication in cells less than PDL 28 (Figures 16-17), it may enhance the observed stimulation of DNA repair replication in aged cells. Although mitotically arrested aged cells show an altered NAD metabolism as compared to young cells, no correlation was observed between NAD metabolism and amounts of DNA repair replication.

The above assessment of poly(ADP-ribose) metabolism as a function of senescence shows some interesting alteration in the metabolism of aged cells. Yet the quantitative study

of the involvement of poly(ADP-ribose) in the aging process does not seem feasible by this approach. Accumulation of DNA strand breaks appears to occur late in senescence (Lipetz, 1980). In a given population of cells this may occur at 60 $\pm$ 5 PDL. Thus, the synthesis of poly(ADP-ribose) may only occur at post-senescence or an extremely late stage of senescence.

DISCUSSION

The ultimate aim of this study was to investigate the function of poly(ADP-ribose). Toward that end poly(ADP-ribose) metabolism was characterized in intact human diploid fibroblasts following treatment with two DNA damaging agents: UV irradiation and the alkylating agent, MNNG. The data presented in this study are the first to demonstrate that treatment with UV irradiation results in a rapid increase of poly(ADP-ribose) content in intact cells. During the first hour following treatment with UV, the cellular NAD pool decreases maximally at a rate of approximately 380 pmol/min/10^8 cells. Between 1 and 5 hr post-irradiation, the NAD pool continuously decreases at a rate of 200 pmol/min/10^8 cells (Figure 4). By 1 hr after treatment with UV, poly(ADP-ribose) increases to a maximum level of approximately 200 pmol of ribosyl-adenosine/10^8 cells and remains elevated for up to 5 hr (Figure 5).

This study also is the first to examine the effectiveness of the poly(ADP-ribose) polymerase inhibitor, 3-aminobenzamide in intact cells. 3-aminobenzamide effectively inhibits both NAD depletion (Figure 6) and

poly(ADP-ribose) synthesis (Figure 7) for 2.5 hr following UV irradiation.

These data clearly show that decreases in NAD levels closely correlate with increases in poly(ADP-ribose) levels (Figures 4-8, 10-11, 14-15, 19). Therefore, NAD depletion represents a measure of poly(ADP-ribose) synthesis although other concomitant ADP-ribosylation reactions after DNA damage cannot be ruled out at this time.

Between 1 and 5 hr following damage by UV light, it has been observed that, in intact cells, the poly(ADP-ribose) level remains elevated at 150-200 pmol/10^8 cells of ribosyladenosine. At the same time, the cellular NAD pool is rapidly decreasing at a rate of 200 pmol/min/10^8 cells. These data are evidence that the poly(ADP-ribose) synthesized after DNA damage by UV irradiation is turned over very rapidly *in vivo*. Evidence regarding the stability of poly(ADP-ribose) after its synthesis in *in vitro* systems is conflicting. In isolated nuclei, the poly(ADP-ribose) synthesized in response to DNA damage by DNase I was found to be stable (Berger et al., 1979; Levi et al., 1981). However, in permeable cells poly(ADP-ribose) was found to be unstable following DNA damage by x-rays

(Benjamin and Gill, 1980a). Under these conditions, the polymer had a half-life of between 5 and 10 min.

In this study, the addition of the poly(ADP-ribose) inhibitor, 3-aminobenzamide, to the culture medium at the time of maximum synthesis of poly(ADP-ribose) enabled the half-life to be estimated for poly(ADP-riboes) following UV irradiation. After the addition of the inhibitor, a decrease in poly(ADP-ribose) synthesis was observed within 2 min (Figure 8, panel A). If all the NAD that is lost is converted to poly(ADP-ribose) via this reaction, then continued synthesis for 1 min could account for the entire maximum observed accumulation of poly(ADP-ribose). Thus, quantification of the turnover rate of poly(ADP-ribose) by this method requires that the inhibition of the polymerase be immediate. However, the size of the NAD pool is approximately 80 nmol/10^8 cells at this point in time. Since the limit of detection for a decrease in NAD pools approaches 1-5% (0.4-4.0 nmol/10^8 cells), it is not possible to determine by NAD measurements whether poly(ADP-ribose) synthesis is inhibited instantaneously, and, thus, whether the observed rate of poly(ADP-ribose) degradation is a true turnover rate. Therefore, these data only allow calculation of a maximum $t_{1/2}$ of poly(ADP-ribose). We have calculated the $t_{1/2}$ for poly(ADP-ribose) to be 6 min (Figure 9).

Between 1 and 5 hr following UV irradiation, the cellular NAD pool is decreasing at a rate of 200 pmol/min/10^8 cells while the cellular content of poly(ADP-ribose) remains elevated at 150-200 pmol/min/10^8cells. Again, if it is assumed that all of the NAD is converted to poly(ADP-ribose) between 1 and 5 hr, then the theoretical $t_{1/2}$ calculated by this approach for poly(ADP-ribose) is actually less than 1 min.

In vitro studies have shown that agents which cause DNA strand breaks cause stimultion of poly(ADP-ribose) polymerase activity. Miller (1975) showed that the activity of this enzyme was increased by nucleases. Benjamin and Gill (1980b) quantitatively related the number of nuclease induced DNA strand breaks with the activity of poly(ADP-ribose) polymerase. Cohen and Berger (1981) have shown that UV damage to the SV40 minichromosome is not sufficient to stimulate poly(ADP-ribose) polymerase activity, but the enzyme becomes active after the addition of miccrococcal UV endonuclease. The present study shows that the number of DNA strand breaks determines the synthesis of poly(ADP-ribose) in vivo. UV irradiation damages DNA by the formation of thymine dimers and other lesions which are removed by excision repair. The first step in this process is an endonucleolytic cleavage made in the

region of the thymine dimer. This step is rate limiting (Erixon and Ahnstrom, 1979). As a result, there is a transient increase in the number of DNA strand breaks following UV treatment of cells in culture (Collins and Johnson, 1969; Collins et al., 1977; Hiss and Preston, 1977; Johnson and Collins, 1978; Erixon and Ahnstrom, 1979; Collins et al., 1980). The DNA synthesis inhibitors, hydroxyurea and ara C, 'uncouple' the incision and re-synthesis steps of excision repair such that incised sites remain open longer than normal thereby increasing the number of strand breaks (Collins et al., 1980; Erixon and Anhstrom, 1979). This study shows that UV light causes a rapid increase in cellular poly(ADP-ribose) levels and that the addition of hydroxyurea or ara C stimulates both NAD depletion (Figure 10) and poly(ADP-ribose) accumulation (Figure 11). However, the possibility exists that both hydroxyurea and ara C directly stimulate the synthesis of poly(ADP-ribose) after UV treatment. To test this possibility, the effect of hydroxyurea on NAD depletion following UV treatment was measured in xeroderma pigmentosum cells, complementation group A. This group of cells is unable to introduce endonucleolytic strand breaks in DNA (Hashem et al., 1980). Previously, it had been shown that NAD depletion does not take place in XP cells

following UV irradiation, but does take place following MNNG treatment (McCurry and Jacobson, 1981). The data of this study show that NAD depletion is not enhanced by hydroxyurea in XP cells which do not make endonucleolytic strand breaks in DNA (Figure 12, panel A). NAD content is unaffected in these cells with or without hydroxyurea. Thus, the enhancement of NAD conversion to poly(ADP-ribose) by hydroxyurea is neither due to its direct action on poly(ADP-ribose) polymerase nor to a synergistic reaction with UV light. The enhancement is due to an increased number of DNA strand breaks.

The DNA strand breaks which occur following UV damage are dependent upon the action of an endonuclease. This endonucleolytic formation of strand breaks in DNA is rate-limiting: excision repair of thymine dimers cannot proceed until strand cleavage occurs adjacent to the lesion. DNA synthesis inhibitors such as hydroxyurea and ara C increase the transient number of strand breaks by inhibiting the rate of DNA polymerization occurring at repair sites. However, the incision step is not impaired. Thus, in the presence of hydroxyurea, endonucleolytic cleavage continues while the rate of DNA repair replication decreases. From this, a reasonable prediction would be that hydroxyurea would not enhance NAD conversion to

poly(ADP-ribose) following treatment with agents which make direct strand breaks, i.e. no endonucleolytic cleavage is necessary; no transient increase in the number of strand breaks occurs. The effect of hydroxyurea on NAD depletion was examined following treatment of XP cells with bleomycin, an agent which makes direct strand breaks. Hydroxyurea did not enhance the rate of conversion of NAD to poly(ADP-ribose) following bleomycin treatment (Figure 12, panel B). These data are consistent with a mechanism in which the number of DNA strand breaks regulates the synthesis of poly(ADP-ribose).

If poly(ADP-ribose) is synthesized in response to DNA strand breaks, then it seems reasonable to predict that a decreased number of DNA strand breaks would inhibit NAD conversion to poly(ADP-ribose). Endonucleolytic strand breaks which occur following UV damage to DNA can be decreased by the presence of novobiocin (Collins and Johnson, 1979). Novobiocin inhibits DNA gyrase, a DNA associated enzyme with two distinct activities located in separate subunits. The first activity introduces negative supercoils into DNA while the second activity unwinds or relaxes DNA (Champoux, 1978). Although novobiocin has long been known to inhibit DNA synthesis in bacteria, recently it has been found to inhibit DNA synthesis in

mammalian cells (Mattern and Painter, 1978). Novobiocin blocks the unwinding activity of DNA gyrase (Champoux, 1978). In order for recognition and/or binding of DNA-associated enzymes, the DNA double helix may have to be unwound. Collins and Johnson (1979) examined the effect of novobiocin on the number of DNA strand breaks following UV treatment of HeLa cells in the presence of hydroxyurea and ara C. Both hydroxyurea and ara C increased the number of strand breaks following UV, but the presence of novobiocin drastically reduced the number of these strand breaks. When hydroxyurea and ara C were omitted, only a small number of breaks were evident following UV in the presence of novobiocin. These breaks were assumed to be transient excision breaks which were always present. Because of the drastic reduction of strand breaks observed after UV irradiation, especially in the presence of hydroxyurea and ara C, it was concluded that novobiocin reduces breaks by preventing endonucleolytic incision. The present study quantified the pmol of NAD converted to poly(ADP-ribose) after UV irradiation of CF-3 cells in the presence and absence of novobiocin. The data show that under our conditions novobiocin decreased the amount of poly(ADP-ribose) by 40% (Figure 13). Thus, when DNA strand breaks are decreased by novobiocin following UV irradiation, the poly(ADP-ribose)

level is decreased. These data, together with data which show that the rate of NAD depletion and accumulation of poly(ADP-ribose) is increased by agents which increase the number of DNA strand breaks following UV irradiation (Figures 10 and 11). In addition, the rate of NAD depletion is not stimulated by hydroxyurea in XP cells which are defective in endonucleolytic cleavage of DNA following UV damage (Figure 12, panel A). Finally, the rate of NAD depletion is not enhanced by hydroxyurea following treatment by bleomycin which makes direct DNA strand breaks (Figure 12, panel B). These data support a mechanism by which the number of strand breaks regulates the cellular content of poly(ADP-ribose). The data show that manipulation of the number of strand breaks controls poly(ADP-ribose) levels and suggests that a relationship between DNA strand breaks and poly(ADP-ribose) synthesis occurs in vivo.

Since the synthesis of poly(ADP-ribose) occurs in response to DNA strand breaks, it may function in some aspect of DNA repair. 3-Aminobenzamide has been used to block poly(ADP-ribose) synthesis in studies that seek to determine the involvement of poly(ADP-ribose) with DNA repair mechanisms (Berger and Sikorski, 1980; Althaus et al., 1980; Miwa et al., 1981; Sims et al., 1982; Althaus et al., 1982). Studies of this type also routinely use hydroxyurea

so that unscheduled DNA synthesis can be measured. The data from the present study show that 3-aminobenzamide blocks NAD depletion and poly(ADP-ribose) synthesis up to 2.5 hr after UV treatment in the absence of hydroxyurea (Figures 6-7). However, in the presence of hydroxyurea, 3-aminobenzamide does not block the depletion of NAD (Figure 14, panel B) or the synthesis of poly(ADP-ribose) (Figure 15). Thus studies that have examined the involvement of poly(ADP-ribose) in DNA repair by utilizing 3-aminobenzamide in the presence of hydroxyurea may need re-evaluation. In studying the involvement of poly(ADP-ribose) in DNA repair, 3-aminobenzamide may be utilized in the absence of hydroxyurea. The present study has shown that it is possible to accurately quantify repair replication in the absence of hydroxyurea by utilizing CsCl gradient analysis of mitotically arrested cells (Figure 16). The data show that hydroxyurea inhibits DNA repair replication by 30 to 40%. Moreover, without hydroxyurea, when poly(ADP-ribose) formation is blocked by 3-aminobenzamide, DNA repair replication is not affected either in amount or time-course (Figure 18). Thus, the present study argues that poly(ADP-ribose) synthesis is not required for repair replication after UV damage. This does not rule out, however, that

poly(ADP-riboes) is not necessary at some later step in the excision reapir process. Several studies that have used 3-aminobanzamide to block poly(ADP-ribose) in the presence of hydroxyurea have reported the occurrence of increased levels of unscheduled DNA synthesis. The present study examined the effect of 3-aminobenzamide on repair replication in the presence and absence of hydroxyurea. The presence of hydroxyurea inhibited the amount of repair replication by 31% (Figure 17, panel B). However, hydroxyurea had no effect on the amount of repair replication in the presence of 3-aminobenzamide (Figure 17, panel D). Comparison of the amount of repair replication with and without 3-aminobenzamide in the presence of hydroxyurea shows an apparent stimulation by the presence of 3-aminobenzamide. Thus, taken together, the data show that if hydroxyurea were present in UDS studies, 3-aminobenzamide would appear to stimulate UDS.

This study also examined the kinetics of NAD depletion and poly(ADP-ribose) synthesis after DNA damage by MNNG. The kinetics of the rate of poly(ADP-ribose) synthesis and degradation following treatment with MNNG differs from the kinetics which follow UV irradiation in time-course and level of accumulation. The time of the reaction is much shorter following MNNG treatment. Maximum accumulation of

the polymer occurs at 20 min following treatment. By 80 min, the accumulation of poly(ADP-ribose) is no longer observed. (Figure 20, panel A). The MNNG data are consistent with the kinetics of poly(ADP-ribose) synthesis in SVT2 cells after MNNG treatment with doses ten times as large (Juarez-Salinas et al., 1979). The time courses observed in SVT2 cells are the human diploid fibroblasts of the present study are consistent with a mechanism of the repair of 0^6-methylguanine aklylation product which occurs by 1 hr (Shiloh, 1981; Sklar and Strauss, 1981; Medcalf and Taruly, 1981; Singer, 1976). Following MNNG treatment, poly(ADP-ribose) is turned over rapidly which also is in accordance with the studies of Juarez-Salinas et al. (1979). Thus, the time course of poly(ADP-ribose) apparently is similar and independent of the dose of MNNG. Following 5 μg/ml MNNG treatment, the cellular NAD pool decreased at a rate of 500 pmol/min/10^8 cells for 20 min. At this time, maximal accumulation of poly(ADP-ribose) occurred. Following treatment with 5 J/m^2 of UV irradiation, the cellular NAD pool decreased at a rate of 200 pmol/min/10^8 cells for 1 hr where maximal accumulation of poly(ADP-ribose) occurred. Maximal accumulation occurs over a short period of time following MNNG; after UV, the poly(ADP-ribose) level remains elevated. Between 2.5

and 5 hr following treatment, net synthesis of NAD is observed after MNNG treatment (Figure 20, panel A). During the same time-course following UV irradiation, this is not observed. Following MNNG damage to DNA, strand breaks occur very rapidly (Lijinsky, 1976). In addition, the removal of O^6-methylguanine adduct occurs with a $t_{\frac{1}{2}}$ of 1 hr. Following UV damage to DNA, however, the rate of strand break formation is dependent on endonuclease activity (Erixon and Ahnstrom, 1979). The activity of the endonuclease has been calculated to be 0.7 strand break in DNA/min following exposure to UV light of approximately 5 J/m^2. Thus, the data of the present study are consistent with the kinetics of strand break formation following damage to DNA by MNNG and UV light.

Experiments which examined the conversion of NAD to poly(ADP-ribose) in young and aged cells reveal that aged cells have greater and more variable initial NAD levels as compared to young cells (Table 1, part A; Figure 22). Although cell volume may account for this in part, it is observed that poly(ADP-ribose) levels also vary drastically in the aged cells from 5-300 pmol ribosyladenosine/10^8 cells (Table 1, part B). Thus, another possible explanation may have to do with an increased number of accumulated DNA strand breaks in high PDL cells which may stimulate poly-(ADP-ribose) synthesis. Assuming cells accumulate DNA

strand breaks as senescence occurs, then it is reasonable to assume that cells nearer to the senescence point (end of life-span) have the greatest numbers of DNA strand breaks and therefore less NAD and more poly-(ADP-ribose) than other cells. However, the PDL at which the senescence point occurs varies $\pm$ 5 PDL at approximately PDL 60. Therefore, some cells at PDL 55 are nearer to the senescence point and may have greater numbers of accumulated DNA strand breaks than other cells at PDL 55. Thus, both NAD and poly(ADP-ribose) levels may vary in the PDL at which strand breaks are accumulating.

Even though aged cells have greater cellular NAD pools, they convert approximately the same quantity of NAD to poly(ADP-ribose) at a 5 hr post-irradiation period (Table 2).

However, initial levels and rates of aged cells show greater variability in the loss of the NAD pool following UV treatment (Figure 23). This may be a function of PDL at senescence point and/or the number of accumulated DNA strand breaks. Moreover, aged cell populations exhibit a wide range of rates of NAD depletion over a 5 hr post-irradiation period, from 63 pmol/hr/10^6 cells to 201 pmol/hr/10 cells (Figure 24). In addition, some aged cell populations never attain $t_{\frac{1}{2}}$ for NAD presumably due to high initial levels of NAD (Table 3). Thus, aged cells

lose an average of 40% of the cellular NAD pool while young cells lose 70-80% following UV damage. However, the data suggest that the rate of NAD depletion in aged cells following UV treatment is not positively correlated to basal level NAD, but that in young cells which contain lower levels of NAD, NAD depletion is positively correlated to initial NAD level (Figure 25).

Mitotically arrested aged cells show 160% greater levels of DNA repair replication in the absence of hydroxyurea and 280% greater level of repair replication in the presence of hydroxyurea than young cells (Figure 26). This elevated repair replication in aged mitotically arrested cells is in agreement with Dell'Orco and Whittle (1978) who observed elevated UDS in aged arrested cells in the presence of hydroxyurea. Since this study has observed that hydroxyurea inhibits repair replication in cells less than PDL 28 (Figures 16-17), repair replication may be stimulated by hydroxyurea in cells at a certain stage of senescence.

Quantitative study of the involvement of poly-(ADP-ribose) does not seem feasible by the approach of this study. Accumulation of DNA strand breaks appears to occur near the end of life-span and this senescence point is variable + 5 PDL. Thus, the synthesis of poly(ADP-ribose)

may only occur at an extremely late stage of senecence or at post-senescence.

LITERATURE CITED

Adamietz, P., K. Klapproth, and H. Hilz. 1979. Isolation and partial characterization of the ADP-ribosylation of nuclear proteins from Erhlich ascites tumor cells. Biochem. Biophys. Res. Comm. 91:1232-1238.

Althaus, F. R., S. D. Lawrence, G. L. Sattler, and H. C. Pitot. 1982. ADP-ribosyl transferase activity in cultured hepatocytes: interactions with DNA repair. J. Biol. Chem. 257:5528-5535.

Althaus, F. R., S. D. Lawrence, G. L. Sattler, and H. C. Pitot. 1980. The effect of nicotinamide on unscheduled DNA synthesis in cultured hepatocytes. Biochem. Biophys. Res. Comm. 95:1063-1070.

Benjamin, R. C. and D. M. Gill. 1979. A connection between poly(ADP-ribose) synthesis and DNA damage. Fed. Proc. 38:619.

Benjamin, R. C. and M. D. Gill 1980a. ADP-ribosylation in mammalian cell ghosts. J. Biol. Chem. 255:10403-10501.

Benjamin, R. D. and D. M. Gill 1980b. Poly(ADP-ribose) synthesis *in vitro* programmed by damaged DNA. J. Biol. Chem. 255:10502-10508.

Berger, N. A., S. J. Petzold, and S. J. Berger. 1979. Association of poly(ADP-ribose) synthesis with cessation of DNA synthesis and fragmentation. Biochim. Biophys. Acta. 564:90-104.

Berger, N. A. and G. W. Sikorski. 1980. Nicotinamide stimulates repair of DNA damage in human lymphocytes. Biochem. Biophys. Res. Comm. 95:67-72.

Berger, N. A. and G. W. Sikorski. 1981. Poly(adenosine diphosphoribose) synthesis in ultraviolet-irradiated xeroderma pigmentosum cells reconstituted with *Micrococcus luteus* UV endonuclease. Biochem. 20:3610-3614.

Berger, N. A., G. Sikorski, S. Petzold, and K. Kurohara. 1979a. UV irradiation stimulates synthesis of poly-(adenosine diphosphoribose) in association with DNA rapair. Fed. Proc. 38:619.

Berger, N. A., G. Sikorski, S. Petzold, and K. Kurohara. 1979b. Association of poly(adenosine diphosphoribose) synthesis with DNA damage and repair in normal human lymphocytes. J. Clin. Invest. 63:1164-1171.

Berger, N. A., G. Sikorski, S. Petzold, and K. Kurohara. 1980. Defective poly(adenosine diphosphoribose) synthesis in xeroderma pigmentosum. Biochem. 19:289-293.

Burzio, L. O., P. T. Requilme, E. Ohtsuka, and S. S. Koide. 1976. Evidence for two variants of poly(adenosine diphosphate ribose) glycohydrolase in rat testis. Arch. Biochem. Biophys. 173:306-319.

Campagnari, F., J. F. Whitfield, and U. Bertazzoni. 1966. The effect of x-radiation on the nicotinamide adenine dinucleotides (NAD-NADH) content of rat thymocytes. Exp. Cell Res. 42:646-656.

Chambon, P., J. D. Weill, and J. Doly. 1966. On the formation of a novel adenylic compound by enzymatic extracts of liver nuclei. Biochem. Biophys. Res. Comm. 25:638-643.

Chambon, P., J. D. Weill, and P. Mandell. 1963. Nicotinamide mononucleotide activation of a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. Biochem. Biophys. Res. Comm. 11:39-43.

Champoux, J. J. 1978. Proteins that effect DNA conformation. Ann. Rev. Biochem. 47:449-479.

Chang, A. Y. 1972. On the mechanism for the depression of liver NAD by Streptozotocin. Biochim. Biophys. Acta. 261:77-84.

Clark, J. B., G. M. Ferris, and F. Pinder. 1971. Inhibition of nuclear and NAD nucleosidase and poly(ADP-ribose)

polymerase activity from rat liver by nicotinamide and 5'-methylnicotinamide. Biochim. Biophys. Acta. 238: 82-85.

Claycomb, W. C. 1976. Poly(adenosine diphosphate ribose) polymerase activity and nicotinamide adenine dinucleotide on differentiating cardiac muscle. Biochem. J. 154:387-393.

Cohen, J. J. and N. A. Berger. 1981. Activation of poly-(adenosine diphosphate ribose) polymerase with UV irradiated and UV endonuclease treated SV40 minichromosome. Biochem. Biophys. Res. Comm. 98:268-274.

Collins, A. R. S. and R. T. Johnson. 1969. Repair and survival after UV in quiescent and proliferating Microtis agrestis cells: Different rates of incision and different dependance on DNA precursor supply. J. Cell Physiol. 99:125-138.

Collins, A. R. S., S. L. Schor, and R. T. Johnson. 1977. The inhibition of repair in UV irradiated human cells. Mutant. Res. 42:413-432.

Collins, A. R. S. and R. T. Johnson. 1979. Novobiocin: an inhibitor of the repair of UV induced damage in mammalian cells. Nucl. Acids. Res. 7:1311-1320.

Collins, A. R. S., C. S. Downes, and R. T. Johnson. 1980. Cell cycle regulated variations in UV damage and repair capacity in Chinese Hamster (CHO-K1) cells. J. Cell. Phys. 103:179-191.

Creissen, D. and S. Shall. 1982. Regulation of DNA ligase activity by poly(ADP-ribose). Nature. 296:271-272.

Davies, M. E., H. Hallodorsson, S. Shall, and C. J. Skidmore. 1976. The action of streptozotocin on mouse leukaemia cells. Biochem. Soc. Trans. 4:635-637.

Davies, M. I., S. Shall, and C. J. Skidmore. 1977. Poly-(adenosine diphosphate ribose) polymerase and deoxyribonucleic acid damage. Biochem. Soc. Trans. 5:949-950.

Dell'Orco, R. T., J. F. Mertens, and P. F. Krause, Jr. 1973. Doubling potential, calendar time and senescence

of human diploid cells in culture. Exp. Cell Res. 77:356-360.

Dell'Orco, R. T. and W. L. Whittle. 1978. Unscheduled DNA synthesis in confluent and mitotically arrested populations of aging human diploid fibroblasts. Mech. Ag. and Develop. 8:269-279.

Doly, J. and P. Mandell. 1967. Mise en evidence de la biosynthese in vivo d'un polymere compose, le polyadenosine diphosphoribose dans les noyaex de foie de poulet. C. R. Acad. Sc. Paris. 264:2687-2690.

Durrant, L. G., G. P. Margison, and J. M. Boyle. 1981. Effects of 5'-methylnicotinamide on mouse L1210 cells exposed to N-methyl-N-nitrosourea: mutation induction, formation, and removal of methylation products in DNA, and unscheduled DNA synthesis. Carcinogenesis. 1013-1017.

Durrant, L. G. and J. M. Boyle. 1982. Potentiation of cell killing by inhibitors of poly(ADP-ribose) polymerase in 4 rodent cell lines exposed to N-methyl-N-nitrosourea or UV light. Chem. Biol. Interactions. 38:325-338.

Durkacz, B. W., N. Nduka, O. Omidiji, S. Shall, and A. Zai'ee. 1980a. ADP-ribose and DNA repair. In Novel ADP-ribosylations of regulatory enzymes and proteins. M. Smulson and T. Sugimura (eds.) Elsevier North Holland, pp. 207-216.

Durkacz, B. W., J. Irwin, and S. Shall. 1981. Inhibition of (ADP-ribose)n biosynthesis retards DNA repair, but does not inhibit DNA repair synthesis. Biochem. Biophys. Res. Comm. 101:1433-1441.

Durkacz, B. W., O. Omidiji, D. A. Gray, and S. Shall. 1980b. (ADP-ribose)n participates in DNA excision repair. Nature. 283:593-596.

Erixon, K. and G. Ahnstrom. 1979. Single-strand breaks in DNA during repair of UV-induced damage in normal human and xeroderma pigmentosum cells as determined by alkaline DNA unwinding and hydroxylapatite chromotography. Mut. Res. 59:257-271.

Fatai, M., D. Mizuno, and T. Sugimura. 1967. Hydrolysis of the polymer formed from NAD with rat liver phosphodiesterase yielding nucleoside 5'-monophosphate. Biochem. Biophys. Res. Comm. 28:395-399.

Ferro, A. M. and B. M. Olivera. 1982. Poly(ADP-ribosylation) *in vitro*. J. Biol. Chem. 257:7808-7813.

Gill, D. M. and R. Meren. 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. 75:3050-3054.

Giri, C. P., M. H. P. West, and M. Smulson. 1978. Nuclear protein modification and chromatin substructure 1. Differential poly (adenosine diphosphate) ribosylation of chromosomal proteins in nuclei versus isolated nucleosomes. Biochem. 17:3495-3500.

Goldstein, S. 1971. The role of DNA repair in aging of cultured fibroblasts from xeroderma pigmentosum and normals. Fed. Proc. 137:730-734.

Goff, C. G. 1974. Chemical structure of a modification of the *Escherichia coli* ribonucleic acid polymerase alpha polypeptides induced by bacteriophage T4 infection. J. Biol. Chem. 249:6181-6190.

Goodwin, P. M., P. J. Lewis, M. I. Davies, C. S. Skidmore, and S. Shall. 1978. The effect of gamma irradiation and neocarzinostatin in NAD and ATP levels in louse leulemia cell. Biochim. Biophys. Acta. 543:576-582.

Gunnarson, R., C. Berne, and C. Hellerstrom. 1974. Cytotoxic effects of streptozotocin and N-nitrosomethylurea on the pancreatic B cells with special regard to the role of nicotinamide adenine dinucleotide. Biochem. J. 140:487-494.

Hart, R. W. and R. B. Setlow. 1976. DNA repair in late passage human cells. Mech. Aging and Develop. 5:67-77.

Hashem, N., D. Bootsma, W. Keijzer, A. Greene, L. Conile, G. Thomas, and J. E. Cleaver. 1980. Clinical

characteristics, DNA repair, and complementation groups in xeroderma pigmentosum patients from Egypt. Cancer Res. 40:13-18.

Hayaishi, O. and K. Ueda. 1977. Poly(ADP-ribose) and ADP-ribosylation of proteins. Annu. Rev. Biochem. 46:95-116.

Hayflick, L. 1977. The cellular basis for biological aging. In Handbook of the biology of Aging. C. E. Finch and L. Hayflick (eds.) Van Rostrand Reinhold, New York, pp. 159-188.

Hinz, M., N. Katsilambros, V. Maier, H. Schatza, and E. F. Pfeiffer. 1973. Significance of streptozotocin induced nicotinamide adenine dinucleotide (NAD) degradation in mouse pancreatic islets. FEBS Lett. 30:225-228.

Hilz, H. and P. Stone. 1976. Poly(ADP-ribose) and ADP-ribosylation. Rev. Physiol. Biochem. Pharmacol. 76:1-58.

Hilz, H., P. Hlavica, and B. Bertram. 1963. Die entscheidende Bedeutung der DPNase-Aktivierung fur den DPN-Abfall in bestrahlten Aschitestumorzellen. Biochem. Z. 338:283-299.

Hiss, F. A. and R. J. Preston. 1977. The effect of cytosine arabinoside on the frequency of double-strand breaks in DNA of mammalian cells following irradiation or chemical treatment. Biochim. Biophys. Acta. 478:1-8.

Hittelmen, W. N. and P. N. Rao. 1974. Bleomycin-induced damage in prematurely condensed chromosomes and its relationship to cell cycle progression in CHO cells. Can. Res. 34:3433-3439.

Ho, C. K. and S. A. Hashim. 1972. Pyridine nucleotide depletion in pancreatic islets associated with streptozotocin-induced diabetes. Diabetes. 21:789-793.

Honjo, T., Y. Nishizuka, O. Hayaishi, and I. Kato. 1968. Diphtheria toxin dependent adenosine diphosphate

ribosylation of aminoacyl transferase II and inhibition of protein synthesis. J. Biol. Chem. 243:3553-3555.

Honjo, T., Y. Nishizuka, and O. Hayaishi. 1969. Adenosine dephosphoribosylation of amino transferase II by Diphtheria toxin. Cold Spr. Harb. Symp. Quant. Biol. 34:603-608.

Honjo, T. Y. Nishizuka, I. Kato, and O. Hayaishi. 1971. Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by Diphtheria toxin. J. Biol. Chem. 246:4251-4260.

Iglewski, B. H. and D. Kabat. 1975. NAD dependent inhibition of protein synthesis of Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. U.S.A. 72:2284-2288.

Jacobson, E. L. and M. K. Jacobson. 1976. Pyridine nucleotide levels as a function of growth in normal and transformed 3T3 cells. Arch. Biochem. Biophys. 175: 627-634.

Jacobson, M. K. and E. L. Jacobson. 1978a. Carcinogenicity of N-nitroso compounds and decreases in cellular NAD. Fed. Proc. 37:1347.

Jacobson, M. K. and E. L. Jacobson. 1978b. Alteration of NAD metabolism associated with carcinogen induced DNA damage. J. Supramol. Struct. Suppl. 2:74.

Jacobson, E. L. and G. Narasimhan. 1979. Absence of carcinogen-induced unscheduled DNA synthesis in NAD depleted 3T3 cells. Fed. Proc. 38:619.

Jacobson, E. L. R. A. Lange, and M. K. Jacobson. 1979. Pyridine nucleotide synthesis in 3T3 cells. J. Cell Phys. 99:417-426.

Jacobson, E. L., D. Juarez, and J. L. Sims. 1980. Absence of DNA repair and Poly(ADP-ribose synthesis in NAD depleted 3T3 cell. Fed. Proc. 39:1739.

Jacobson, M. K., V. Levi, H. Juarez-Salinas, R. A. Barton, and E. L. Jacobson. 1980. Effect of carcinogenic N-alkyl-N-nitroso compounds on nicotinamide adenine dinucleotide metabolism. Cancer Res. 40:1797-1802.

Johnson, R. T. and A. R. S. Collins. 1978. Reversal of the changes in DNA and chromosome structure which follow the inhibition of UV-induced repair in human cells. Biochem. Biophys. Res. Comm. 80:361-369.

Juarez-Salinas, H., J. L. Sims, and M. K. Jacobson. 1979. Poly(ADP-ribose) levels in carcinogen treated cells. Nature. 282:740-741.

Juarez-Salinas, H., V. Levi, E. L. Jacobson, and M. K. Jacobson. 1982. Poly(ADP-ribose) has a branched structure in vivo. J. Biol. Chem. 257:607-609.

Jump, D., and M. Smulson. 1980. Purification and characterization of the major non-histone protein acceptor for poly(adenosine diphosphate ribose) in HeLa cell nuclei. Biochem. 19:1024-1030.

Kun, E., A. Chang, M. Sharma, A. M. Ferro and D. Nitecki. 1976. Covalent modification of proteins by metabolites of NAD. Proc. Natl. Acad. Sci. U.S.A. 73: 3131-3135.

Levi, V., E. L. Jacobson, and M. K. Jacobson. 1978. Inhibition of poly(ADP-ribose) polymerase by methylated xanthines and cytokinins. FEBS Lett. 88:144-146.

Levi, V., H. Juarez-Salinas, and M. K. Jacobson. 1981. Increased poly(ADP-ribose) synthesis in quiescent cells not associated with DNA fragmentation. Fed. Proc. 40: 171.

Lijinsky, W. 1976. Interaction with nucleic acids of carcinogenic and mutagenic N-nitroso compounds. Prog. Nucl. Acid Res. Mol. Biol. 17:247-269.

Lindahl, T. 1979. DNA glycosylases, endonucleases for apurinic/apyrimidinic sites and base excision repair. Prog. Nucl. Acid Res. Mo. Biol. 22:135-192.

Lipetz, P. D., D. E. Brash, L. B. Joseph, H. D. Jewett, R. W. Hart, L. E. Lipetz, and R. W. Stephens. 1980. Fluorescent detection of DNA strand breakage in nucleoids derived from a subpopulation of aging human fibroblasts. Fed. Proc. 39:1739.

Little, J. B. 1976. Relationship between DNA repair capacity and cellular aging. Gerontol. 22:28-55.

Lorimer, W. S., P. R. Stone, and W. R. Kidwell. 1977. Control of histone H1 dimerpoly(ADP-ribose) complex formation by poly(ADP-ribose) glycohodrolase. Exp. Cell Res. 106:261-266.

Mattern, M. R. and R. B. Painter. 1979. Dependence of mammalian DNA replication in DNA supercoilings II: Effects of novobiocin on DNA synthesis in Chinese Hamster ovary cells. Biochim. Biophys. Acta. 563: 306-312.

Medcalf, A. S. C. and P. D. Taruly. 1981. Time-course of O^6-methyl guanine removal from DNA of N-methyl-N-nitrosourea treated human fibroblasts. Nature. 289: 796-799.

Miller, E. G. 1975a. Stimulation of nuclear poly(adenosine diphosphate-ribose) polymerase activity from HeLa cells by endonucleases. Biochim. Biophys. Acta. 395: 191-236.

Miller, E. G. 1975b. Effect of deoxyribonuclease I on the number and length of chains of poly(ADP-ribose) synthesized in vitro. Biochem. Biophys. Res. Comm. 66:280-286.

Miller, E. G. 1976. Stimulation of poly(adenosine diphosphate ribose polymerase activity by bleomycin. Fed. Proc. 36:906.

Miwa, M. and T. Sugimura. 1971. Splitting of the ribose-ribose linkage of poly(adenosine diphosphate ribose) by a calf thymus extract. J. Biol. Chem. 246:6362-6364.

Miwa, M., K. Nakatsugawa, K. Hara, T. Matsushima, and T. Sugimura. 1975. Degredation of poly(adenosine diphosphate ribose) by homogenates of various normal tissues and tumors of rats. Arch. Biochem. Biophys. 167:54-60.

Miwa, M., N. Saikawa, Z. Yamaizumi, S. Nishimura, and T. Sugimura. 1979. Structure of poly(adenosine diphosphate ribose): Identification of 2'-[1''-ribosyl-2''-(or 3''-)(1'''-ribosyl)]adenosine 5', 5'', 5'''-tris(phosphate) as a branch linkage. Proc. Natl. Acad. Sci. U.S.A. 76:595-599.

Miwa, M., M. Ishihara, S. Takishima, N. Saikawa, M. Tanaka, N. Takasuka, M. Maeda, Z. Yamaizumi, S. Nishimura, H. Saito, T. Sugimura, S. Yokoyama, and T. Miyazawa. 1980. Novel structure of poly(ADP-ribose) with a branch linkage. In Novel ADP-ribosylations of regulatory enzymes and proteins. M. Smulson and T. Sugimura (eds.) Elsevier North Holland, pp. 207-216.

Miwa, M., M. Kanae, K. Tomoko, H. Hiro-o, K. Ishihara, and T. Sugimura. 1981. Inhibitors of poly(ADP-ribose) polymerase enhance unscheduled DNA synthesis in human peripheral lymphocytes. Biochem, Biophys. Res. Comm. 100:463-470.

Miyakawa, N., K. Ueda, and O. Hayaishi. 1972. Association of poly(ADP-ribose) glycohydrolase with linear chromatin. Biochem. Biophys. Res. Comm. 49:239-245.

Moss, J. Personal communication.

Moss, J. and M. Vaughn. 1978. Hydrolysis of nicotinamide adenine dinucleotide of choleragen and its A protomer: Possible role in the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. 73:4424-4427.

McCurry, L. S. and M. K. Jacobson. 1981. Poly(ADP-ribose) synthesis following DNA damage in cells heterzygous or homozygous for the xeroderma pigmentosum genotype. J. Biol. Chem. 256:551-553.

Nuduka, Y., K. Ueda, K. Nakasawa, and O. Hayaishi. 1967. Studies on the polymer of adenosine dephosphate ribose I. Enzymatic formation from nicotinamide adenine denucleotide in mammalian nuclei. J. Biol. Chem. 242:3164-3171.

Nishizuka, Y., K. Ueda, K. Nakasawa, and O. Hayaishi. 1967. Studies on the polymer of adenosine dephosphate ribose I. Enzymatic formation from nicotinamide adenine

denucleotide in mammalian nuclei. J. Biol. Chem. 242:3164-3171.

Nishizuka, Y., K. Ueda, K. Nakazawa, and O. Hayaishi. 1968. Enzymic adenosine diphosphate ribosylation of histone and poly(adenosine diphosphate ribose) synthesis in rat liver nuclei. J. Biol. Chem. 243:3765-3767.

Nishizuka, Y., K. Ueda, H. Yoshihara, M. Yomamura, and O. Hayaishi. 1969. Enzymatic adenosine diphosphoriboseylation of nuclear proteins. Cold Spr. Harb. Symp. Quant. Biol. 34:781-789.

Ogata, N., K. Ueda, H. Kagamiyama, and O. Hayaishi. 1980a. ADP-ribosylation of histone H2B: Identification of glutamic acid residue 2 as modification site. J. Biol. Chem. 255:7610-7615.

Ogata, N., K. Ueda, H. Kagamiyama, and O. Hayaishi. 1980b. ADP-ribosylation of histone H1: Identification of glutamic acid residues 2, 14, and the COOH terminal lysine residue as modification sites. J. Biol. Chem. 255:7616-7620.

Ogata, N., K. Ueda, M. Kewauchi, and O. Hayaishi. 1981. Poly(ADP-ribose) in isolated nuclei. J. Biol. Chem. 256:4135-4137.

Okayama, H., K. Ueda, and O. Hayaishi. 1978. Purification of ADP-ribosylated nuclear proteins by covalent chromatography in dihydroxyboryl polyacrylamide beads and their characterization. Proc. Natl. Acad. Sci. U.S.A. 75:1111-1115.

Olivera, B. M. and I. R. Lehman. 1967. Diphosphopyridine nucleotide: a cofactor for the polynucleotide-joining enzyme from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 57:1700-1704.

Otake, H., M. Miwa, S. Fujimura, and T. Sugimura. 1969. ADP ribose polymer with histone. J. Biochem. 65:1450-1456.

Presce, A., C. Caadi, and G. C. Schito. 1976. Rifampicin-resistant RNA polymerase and NAD transferase activities in Coliphage N4 virions. Nature. 262:412-414.

Preiss, J., R. Schlaeger, and H. Hilz. 1971. Specific inhibition of poly(ADP-ribose) polymerase by thymidine and nicotinamide. FEBS Lett. 19:244-246.

Purnell, M. R., P. R. Stone, and W. J. D. Whish. 1980. ADP-ribosylation of nuclear proteins. Biochem. Soc. Trans. 8:215-228.

Purnell, M. R. and W. J. D. Whish. 1980. Novel inhibitors of poly(ADP-ribose) synthetase. Biochem. J. 185:775-777.

Rankin, P. W., M. K. Jacobson, V. R. Mitchell, and D. L. Busbee. 1980. Reduction of nicotinamide adenine dinucleotide levels by ultimate carcinogens in human lymphocytes. Cancer Res. 40:1803-1807.

Regan, J. D. and R. B. Stelow. 1974. Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. Cancer Res. 34:3318-3325.

Rohrer, H., W. Zilly, and R. Mailhammer. 1975. ADP-ribosylation of DNA-dependent RNA polymerase by Escherichia coli by an NAD protein ADP-ribosyltransferase from bacteriophage T4. Eur. J. Biochem. 60:227-238.

Roitt, I. M. 1956. The inhibition of carbohydrate metabolism in Ascites tumour cells by ethyleneimines. Biochem. J. 63:300-307.

Samson, L. and L. Schwartz. 1980. Evidence for an adaptive DNA repair pathway in CHO and human skin fibroblasts lines. Nature. 287:861-863.

Schein, P. S. and S. Loftus. 1968. Streptozotocin: Depression of mouse liver pyridine nucleotides. Cancer Res. 28:1501-1506.

Schein, P. S., D. A. Cooney, M. G. McMenamin, and T. Anderson. 1973. Streptozotocin diabetes: further studies on the mechanism of depression of nicotinamide adenine dinucleotide concentrations in mouse pancreatic islets and livers. Biochem. Pharmac. 22:2625-2631.

Shiloh, Y. 1981. Kinetics of O^6-methylguanine repair in houman normal and ataxia telangiectasia cell lines and corrlation of repair capacity with cellular sensitivity to methylating agents. Cancer Res. 41: 5114-5120.

Singer, B. 1976. All oxygens in nucleic acids react with carcinogenic ethylating agents. Nature. 246:333-339.

Sklar, R. and B. Strauss. 1981. Removal of O^6- methyl-guanine from DNA of normal and xeroderma pigmentosum derived lymphoblastoid lines. Nature. 289:412-420.

Sims, J. L., H. Juarez-Salinas, and M. K. Jacobson. 1980. A new highly sensitive and selective chemical assay for poly(ADP-ribose). Anal. Biochem. 106:296-306.

Sims, J. L., G. W. Sikorski, D. M. Catino, S. Berger, and N. Berger. 1982. Poly(adenosinediphosphoribose) polymerase inhibitors stimulate unscheduled dexoyribo-nucleic acid synthesis in normal human lymphocytes. Biochem. 21:1813-1821.

Smith, C. A. and P. C. Hanawalt. 1976. Repair, replication in human cells simplified determination utilizing hydroxyurea. Biochim. Biophys. Acta. 432:336-347.

Smith, C. A. and P. C. Hanawalt. 1976b. Repair replic-ation in cultured normal and transformed human fibroblasts. Biochem. Biophys. Acta. 447:121-132.

Smith, J. A. and L. A. Stocken. 1975. Chemical and meta-bolic properties of adenosine diphosphate ribose derivatives of nuclear proteins. Biochem. J. 147: 523-529.

Smulson, M. E., P. Stark, M. Gazzoli, and J. Roberst. 1975. Release of template restriction for DNA synthesis by poly(ADP-ribose) polymerase during the HeLa cell cycle. Exp. Cell Res. 90:175-183.

Smulson, M. E., P. Schein, D. W. Mullins, Jr., and S. Sudhakar. 1977. A putative role for nicotinamide adenine dinucleotide-promoted nuclear protein modification in the antitumor activity of N-methyl-N-nitrosourea. Cancer Res. 37:3006-3012.

Stone, P. R., W. S. Lorimer, and W. R. Kidwell. 1977. Properties of the complex between histone H1-poly (ADP-ribose) synthesized in HeLa cell nuclei. Eur. J. Biochem. 81:9-18.

Sugimura, T. 1973. Poly(adenosine diphosphate ribose). In progress in nucleic acid research and molecular biology. J. A. Davidson and W. E. Cohn (eds.) Academic Press, New York and London. 13:127-151.

Sugimura, T., M. Yamada, M. Miwa, T. Matsushima, T. Hidaka, M. Nazao, N. Inue, and S. Takayama. 1973. Properties of poly(adenosine diphosphate ribose) polymerase, poly(adenosine diphosphate ribose) glycohydrolase and poly(adenosine diphosphate ribose). Biochem. Soc. Trans. 1:642-644.

Ueda, K., R. H. Reeder, T. Honjo, Y. Nishizaka, and O. Hayaishi. 1968. Poly adenosine diphosphate ribose synthesis associated with chromatin. Biochem. Biophys. Res. Comm. 31:379-

Ueda, K., J. Oka, S. Narumiya, N. Miyakawa, and O. Hayaishi. 1972. Poly ADP-ribose glcohydrolase from rat liver nuclei: a novel enzyme degrading the polymer. Biochem. Biophys. Res. Comm. 46:516-523.

Ueda, K., A. Omashi, M. Kawaichi, and O. Hayaishi. 1975. Natural occurrence of poly(ADP-ribose) histones in rat liver. Proc. Natl. Acad. Sci. U.S.A. 72:205-209.

Weilckins, K., R. Bredenhorst, P. Adamietz, and H. Hilz. 1981. Protein bound polymeric and monomeric ADP-ribose residues in hepatic tissues: Comparative analysis using a new procedure for the quantification of poly(ADP-ribose). Eur. J. Biochem. 117:69-74.

Yoshihara, K., T. Hashida, H. Yoshihara, Y. Tanaka, and H. Ohgushi. 1977. Enzyme-bound produce of purified poly(ADP-ribose) polymerase. Biochem. Biophys. Res. Comm. 78:1281-1288.

Yoshihara, K., T. Hashida, Y. Tanaka, H. Ohgushi, H. Yoshihara, and T. Kamiza. 1978. Bovine thymus poly (adenosine diphosphate ribose) polymerase. J. Biol. Chem. 253:6459-6466.