MUTAGENESIS IN CULTURED CELLS WITH ALTERED POLY(ADP-RIBOSE) METABOLISM

A DISSERTATION

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To the Provost of the Graduate School:

I am submitting herewith a dissertation by VIYADA NUNBHAKDI, entitled "Mutagenesis in Cultured Cells with Altered Poly(ADP-ribose) Metabolism". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

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To my parents— Mr. Yudth Nunbhakdi and

Mrs. Savanthong Nunbhakdi

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ABSTRACT

MUTAGENESIS IN CULTURED CELLS WITH ALTERED
POLY(ADP-RIBOSE) METABOLISM

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MAY, 1986

Poly(ADP-ribose) is synthesized in response to DNA damage caused by either chemicals or radiation. The biological function of this polymer is not known. However, most of the evidence for role(s) of poly(ADP-ribose) is based on the inhibition of the enzyme poly(ADP-ribose) polymerase by different inhibitors. When the synthesis of poly(ADP-ribose) is inhibited following DNA damage, increases are observed in cytotoxicity, chromosomal aberrations, sister chromatid exchanges and malignant transformation. This investigation was conducted to study the effect of poly(ADP-ribose) metabolism on mutagenesis in C3H10T1/2 cells. Poly(ADP-ribose) metabolism was altered by the inhibitor, 3-methoxybenzamide (MBA) or by NAD-depletion. Ouabain-resistant mutations were induced by exposing cells to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the presence and absence of poly(ADP-ribose) synthesis. Mutations at the Na^+/K^+ATPase locus were selected by growth in ouabain containing medium. In dividing cells treated with MNNG, limiting poly(ADP-ribose) synthesis by MBA or NAD-

depletion resulted in significant decreases in mutation. Since inhibiting poly(ADP-ribose) synthesis is known to enhance the cytotoxicity of MNNG and cuase cell cycle blocks, it was not clear whether the observed decrease in mutation frequency was the result of a block in the expression of mutations or whether poly(ADP-ribose) synthesis normally effects an SOS type of response to DNA damage. Therefore, analogous studies were conducted in non-dividing cells which eliminated cell cycling during the period of DNA repair and inhibition of poly(ADP-ribose) synthesis doubled the mutation rates. These data suggest that poly(ADP-ribose) may function either directly or indirectly in limiting events which lead to mutations at the Oua^r locus.

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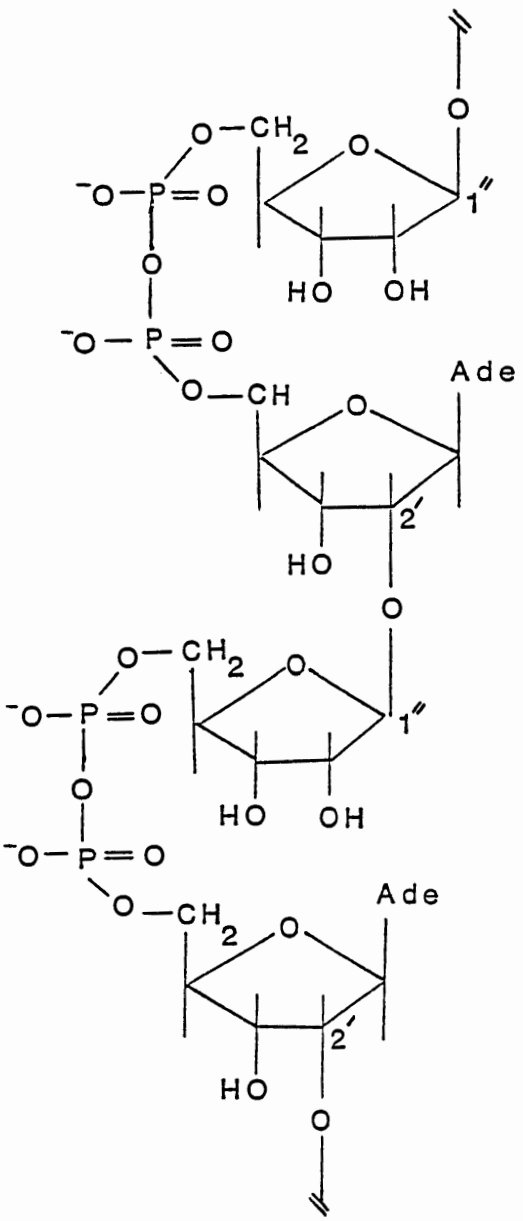
INTRODUCTION

Nicotinamide adenine dinucleotide (NAD), an oxidation-reduction coenzyme has been shown in recent years to serve as a substrate for ADP-ribosylation reactions. The NAD molecule contains two high energy bonds, the N-glycosylic bond linking the nicotinamide and ribose moieties, and the pyrophosphate bond which links the adjacent phosphate groups. NAD glycohydrolase hydrolyzes the N-glycosylic linkage of NAD, releasing adenosine diphosphate ribose (ADP-ribose), nicotinamide and a proton. ADP-ribose is transferred to a water molecule which serves as an acceptor. Alternatively, ADP-ribosyl transferases can catalyze the transfer of the ADP-ribosyl moiety of NAD to an appropriate protein acceptor. When a single unit of ADP-ribose is transferred, the reaction is referred to as mono-ADP-ribosylation and is catalyzed by a mono ADP-ribosyl transferase (mono-ADPRT). The mono-ADPRTs which have been identified to date are mostly microbial toxins, for instance, diptheria, cholera and pertussin toxins (Hayaishi and Ueda, 1977; Pekala and Moss, 1983). The diptheria toxin was the first ADP-ribosyl transferase to be discovered. The target of diptheria toxin is elongation factor (EF-2). The transferred ADP-ribose covalently attaches at a modified histidine called dipthamide. Mono-ADP-ribosylation of EF-2 results in the inhibition of protein synthesis (Hayaishi and Ueda, 1977). Besides dipthamide, the ADP-ribose moiety has also been shown to modify proteins

at other amino acids such as arginine, glutamic acid or cysteine (Moss and Vaughan, 1983; Payne et al., 1985).

The cleavage of NAD with a concurrent release of ADP-ribose and nicotinamide is catalyzed by poly(ADP-ribose) synthetase. The same enzyme, which is now called poly(ADP-ribose) transferase, transfers an ADP-ribose to a protein acceptor and subsequently transfers additional ADP-ribose groups to form a homopolymer of ADP-ribose linked via a 1''--> 2' ribose-ribose linkage (Fig. 1). Thus, this enzyme functions to polymerize a polymer of ADP-ribose and it is often called poly(ADP-ribose) polymerase. Poly(ADP-ribosyl)ation is believed to occur exclusively in eukaryotic nuclei. It has been found in the evolutionary lineage from slime molds to plants to higher mammals. However, one report indicates that this activity has been detected in the microsomal fraction of Hela cells (Ueda et al., 1983). Poly(ADP-ribosyl)ation is believed to be a posttranslational modification of chromosomal protein in eukaryotic cells (Hilz and Stone, 1976; Hayaishi and Ueda, 1977). Poly(ADP-ribose) polymerase is a chromatin-bound enzyme and has been purified from a variety of sources and snown to be a single peptide with molecular weight in the range of 110 to 130 kds (Gaal and Pearson, 1985). The enzyme contains three distinct domains of 54, 46 and 22 kds for the binding of NAD , DNA and poly(ADP-ribose), respectively (Kameshita et al., 1984). In <u>in vitro</u> systems, the maximal specific activity of this enzyme was demonstrated in chromatin structures containing eight to ten nucleosomes (Butt et al., 1979).

Figure 1. Structure of poly(ADP-ribose)


O
O—CH2
O
⁻O—P=O
1″
HO
OH
O
⁻O—P=O
Ade
O—CH
O
2′
HO
O
O—CH2
O
⁻O—P=O
1″
HO
OH
O
⁻O—P=O
Ade
O—CH2
O
2′
HO
O

Jump et al.(1979) have indicated that, the activity of poly(ADP-ribose) polymerase is high in the region of DNA replication forks. In addition, poly(ADP-ribose) polymerase absolutely requires DNA for activity (Benjamin and Gill, 1980 a,b). Mg^{2+} increases both the reaction rate and the duration of synthesis. Ca^{2+} produces an even greater increase in reaction rate and duration of synthesis (Berger et al., 1983). The activation of the polymerase occurs when the enzyme binds to the site of DNA strand breaks (Benjamin and Gill, 1980a,b). These investigators also found that the ability of DNA to support the synthesis of poly(ADP-ribose) is dependent on the number and type of DNA strand breaks. Furthermore, Benjamin and Gill (1980b) showed that single-stranded DNA is ineffective whereas double-stranded restriction fragments with flush ends are the most effective at stimulating polymerase activity.

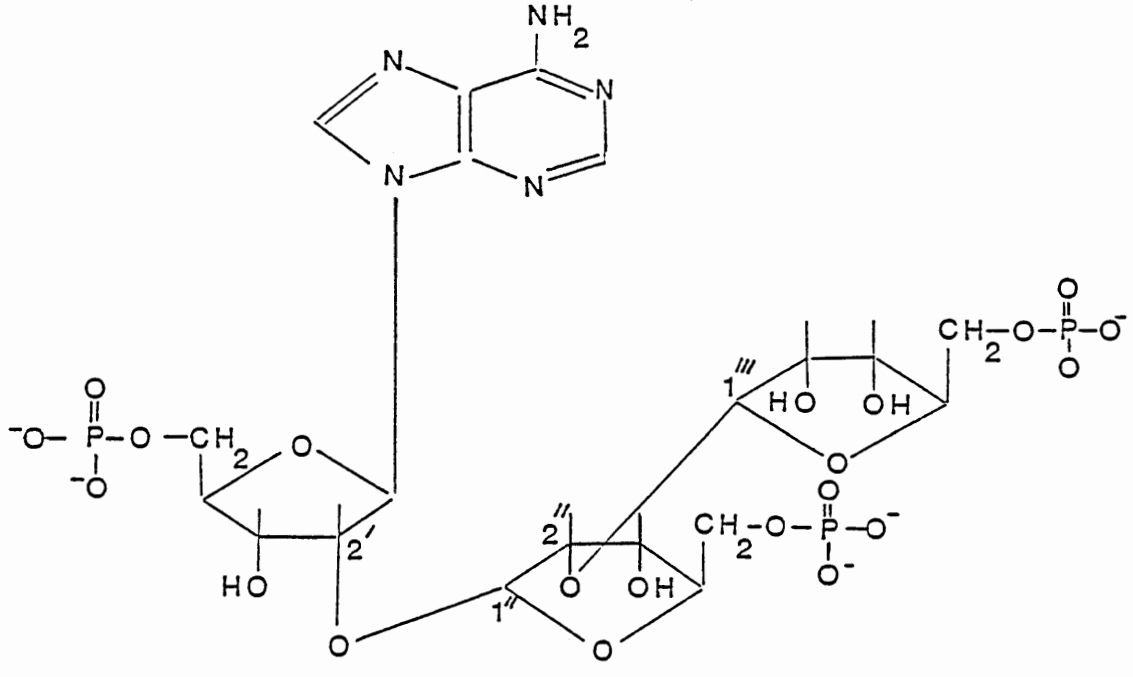
Poly(ADP-ribose) polymerase has been demonstrated to catalyze not only the initiation and elongation but also branching of poly(ADP-ribose)(Ueda et al., 1983). The linear form of this polymer has been reported to range from three to several hundred (300) units of ADP-ribose (Alvarez-Gonzales and Jacobson, 1985). The length of the polymer has been estimated by digestion with snake venom phosphodiesterase which cleaves at the pyrophosphate bonds between phosphate groups. Enzyme digestion produces 5'-AMP, iso-ADP-ribose and a ribose-5-phosphate which is attached to the protein acceptor. Ribose-5-phosphate can then be released from the acceptor by cleaving

with ADP-ribosyl histone hydrolase (Hayaishi and Ueda, 1977; Pekala and Moss, 1983). The number of 5'-AMP molecules represents the number of polymer chains. Quantification of 5'-AMP and iso-ADP-ribose makes possible the calculation of an average chain length using the following equation:

$$\text{Average chain length} = \frac{\text{5'-AMP + iso-ADP-ribose}}{\text{5'-AMP}}$$

The average chain length can be estimated from the sum of the number of molecules of 5'-AMP and iso-ADP-ribose divided by the number of molecules of 5'-AMP (Pekala and Moss, 1983). Hayaishi and Ueda (1977) have reported that the chain length of the polymer obtained from both _in vivo_ and _in vitro_ synthesis ranges from 1 to about 50 ADP-ribose units. The branched structures has been demonstrated to exist both _in vitro_ (Miwa et al., 1981) and _in vivo_ Kanai et al., 1982; Juarez-Salinas et al., 1982). The structure of the branching site is shown to be 0-α -D-ribofuranosyl-(1'''--> 2'')-0-α-D-ribofurasonyl(1''-->2')-adenosine-5'-5''-5''-Tris (phosphate)(Kanai et al., 1982). This structure is also called phosphoribosyl iso-ADP-ribose or Ado-(P)-Rib(P)-Rib(P) as shown in Figure 2. The attachment site of a branched structure can be at either the 2'' or the 3'' ribose hydroxyl group. Alvarez-Gonzales and Jacobson (1985) have shown that the large branched polymers of ADP-ribose obtained from _in vitro_ studies have an average size of 190 residues containing 5 to 6 points of branching per molecule. Polymers as large as 244 residues and containing 6 to 7 points of branching have

Figure 2. Structure of phosphoribosyl iso-ADP-ribose



been observed from in vivo studies. Taking the branched structure into account alters the validity of earlier reports of chain length the true average polymer size and the average number of branching points per molecule can be calculated using the following formulas:

$$\text{Average polymer size} = \frac{\text{AMP} + \text{PRAMP} + (\text{PR})_2\text{AMP}}{\text{AMP-(PR)2AMP}}$$

$$\text{Points of branching per molecule} = \frac{\text{(PR)2AMP}}{\text{AMP-(PR)}_2\text{AMP}}$$

where $(PR)_2AMP$ and PRAMP are produced from branched and linear internal residues, respectively (Alvarez-Gonzales and Jacobson, 1985).

Various kinds of acceptor proteins have been shown to be modified by poly(ADP-ribose). Chromatin-associated proteins, including histone proteins and nonhistone proteins (HMG), are found to serve as ADP-ribose acceptors. H1 and H2B have been shown to be ADP-ribosylated in vivo (Tanuma and Johnson, 1983; Wong et al., 1983; Adamietz and Rudolph, 1984). Additionally, histone H2B has been reported to be a major acceptor for mono-and poly(ADP-ribose) in dimethyl sulfate-treated heptoma AH 7974 cells (Adamietz and Rudolph, 1984). Under this treatment, minor amounts of other histones including H4 and non-histone proteins with molecular weights of 100-116 and 170 kd are also ADP-ribosylated. In addition to chromosomal proteins, several chromatin associated enzymes are modified by poly(ADP-ribose). The main enzyme acceptor for poly(ADP-ribose) has been shown to be the transferase itself (Ueda et al., 1983). Other chromatin enzymes which can be

poly(ADP-ribosyl)ated in vitro are DNA ligase, DNA topoisomerase I and Mg^{2+}-Ca^{2+}dependent endonuclease (Crissen and Shall, 1982; Ferro et al., 1984; Tanaka et al., 1984). Furthermore, RNA polymerase I and DNA ligase II are suggested to be poly(ADP-ribosyl)ated (Yoshihara et al., 1985; Shall, 1983). It is indicated that poly(ADP-ribosyl)ation of these enzyme proteins results in inhibition of enzyme activity.

Poly(ADP-ribose) is degraded by two types of enzymes, poly(ADP-ribose) glycohydrolase and phosphodiesterase from rat liver, snake venom and tobacco cells (Gaal and Pearson, 1985). Poly(ADP-ribose) glycohydrolase, and exoglycosylase, cleaves the ribose-ribose bonds within the linear polymer and also the ribose-ribose-ribose bonds in the branched regions of poly(ADP-ribose) (Ueda et al., 1983; Gaal and Pearson, 1985). The other type of degradative enzymes consist of phosphodiesterases which may be endonucleolytic or exonucleolytic. They split the pyrophosphate bonds to produce 5'-AMP, iso-ADP-ribose (P-Ado(P-Rib)) and ribose-5-phosphate from the linear polymer. A small amount of P-Ado(P-Rib)(P-Rib) is also produced from branched polymers (Miwa and Sugimura, 1982). Ribose-5-phosphate is linked to the acceptor protein via an ester bond which can be cleaved by ADP-ribosyl protein lyase. This enzyme recognizes the whole ADP-ribose unit remaining after poly(ADP-ribose) glycolydrolase digestion (Gaal and Pearson, 1985).

Since most known protein acceptors for poly(ADP-ribose) are involved in the metabolism or function of chromatin, many possible

biological functions have been proposed for poly(ADP-ribosyl)ation. Although there have been many attempts to elucidate the function of poly(ADP-ribose), its role is still unclear. The function is largely defined by studies involving inhibitors of the polymerase which block the synthesis of poly(ADP-ribose). This approach has been utilized since there are no known poly(ADP-ribose) polymerase mutants.

The synthesis of poly(ADP-ribose) can be inhibited by four chemical classses of inhibitors. The most widely used inhibitors are benzamide and its derivatives, such as 3-aminobenzamide (3-AB) and 3-methoxybenzamide (3-MBA). These two inhibitors are classified in the aromatic amides group (Purnell and Whish, 1980). The other three classes include nicotinamide and its derivatives, such as 5-methyl- nicotinamide; methylxanthines, such as theophylline and caffiene; and thymidine (Durkacz et al., 1980).

Many chemical inhibitors have been extensively tested for their inhibitory effect on the synthesis of poly(ADP-ribose) in several *in vitro* mammalian systems. Several of them including nicotinamide, benzamide, 3-methoxybenzamide, 3-aminobenzamide, and theophylline (Durkacz et al., 1980; Purnell and Whish, 1980) have been shown to be powerful inhibitors. However, caffeine has been shown to be a relatively poor inhibitor (Levi et al., 1978). Interestingly, the acid analogues of nicotinamide, benzamide, and 3-aminobenzamide, which are nicotinic acid, benzoic acid, and 3-aminobenzoic acid, respectively, are not inhibitory at all (Durkacz et al., 1980).

Many of the very effective inhibitors of poly(ADP-ribose) polymerase are not cytotoxic to exponentially dividing, cultured cells. For instance, 3-methoxybenzamide at a concentration of 1 mM shows no effect on survival of C3H10T1/2 mouse fibroblast cells (Jacobson et al., 1984). Bhattacharyya and Bhattacharjee (1983) have reported no inhibitory effect of benzamide on poly(ADP-ribose) synthesis in vitro at concentrations up to 6 mM for 24 hr treatment periods in V79 Chinese hamster cells. In addition, 3-aminobenzamide shows no cytotoxicity to L1210 mouse leukemic cells in concentrations up to 3 mM (Durkacz et al., 1980). However, the specificity of these inhibitors has not been established. Nicotinamide, benzamide, and benzamide derivatives are not completely specific and exhibit some side effects (Gaal and Pearson, 1985). At high concentrations e.g., 5 to 10 mM, benzamide and its derivatives inhibit mono-ADPRT (Gaal and Pearson, 1985) and purine biosynthesis (Cleaver et al., 1983). However, MBA at a concentration of 1 mM has been shown to be an effective inhibitor of poly(ADP-ribose) synthesis in intact cells. Jacobson et al. (1984) have shown that under these conditions, poly(ADP-ribose) accumulation in vivo is inhibited by 85% and no significant side effects are observed.

An alternative method for disturbing or limiting the synthesis of poly(ADP-ribose) in vivo is to cause depletion of NAD the cellular substrate of poly(ADP-ribose) polymerase. Jacobson et al. (1979) have determined the NAD content in 3T3 mouse fibroblast cells grown in control medium with nicotinamide and in nicotinamide-free medium for 5

generations. Although, the NAD content of cells grown in the absence of nicotinamide contained as little as 2% of the NAD present in the control cells after 5 generations, there was no effect on the increase in cell number.

Several lines of evidence have demonstrated that the NAD level in various mammalian cells is lowered by DNA damaging agents, including both chemical agents and irradition (Goodwin et al., 1978; Jacobson et al., 1982). The relationship of NAD lowering and DNA repair has been investigated in experiments employing Xeroderma pigmentosum (XP-12) cells, which can repair damage caused by alkylating agents but not by UV radiation and benzo(a)pyrene metabolites. NAD lowering was observed in XP cells treated with MNNG, however, no NAD lowering was observed in XP cells treated with benzo(a)pyrene metabolites (Jacobson et al., 1980). Following DNA damage and/or stress, the resulting decrease in NAD is accompanied by an increase in poly(ADP-ribose) (Juarez-Salinas et al., 1979, Sims et al., 1982, Juarez-Salinas et al., 1984). N-methyl-N'- nitro-N-niurosoguanidine (MNNG) at a concentration of 130 μM causes NAD lowering to 50% in 1 hr in 3T3 cells. Further investigations from this group have shown that MNNG effects an increase of poly(ADP-ribose) polymerase activity. This observation suggests that the lowering of NAD is not due to effects on NAD biosynthesis, but is due to an increase in poly(ADP-ribose) synthesis. Additional evidence has demonstrated that theophylline (5 mM) a potent polymerase inhibitor, prevents NAD lowering in 3T3 cells treated with MNNG, but has no effect

on cells treated with theophylline alone. This result supports the hypothesis that NAD lowering is due to increased poly(ADP-ribose) synthesis (Jacobson et al., 1979).

Synthesis of poly(ADP-ribose) results in the depletion of cellular NAD and is stimulated by various kinds of DNA strand breaks both *in vitso* (Benjamin and Gill, 1980a, b; Cohen and Berger, 1981) and *in vivo* (Jacobson et al., 1980; McCurry and Jacobson, 1981). These data suggest a potential role of poly(ADP-ribose) following DNA damage in DNA repair. In several experiments cell survival has been studied in cells following DNA damage when the synthesis of poly(ADP-ribose) is prevented. It has been suggested that the effect of inhibitors on survival in cells following DNA damage depends upon the type of cell line, the DNA damaging agent and the inhibitor used. Inhibitors of poly(ADP-ribose) polymerase can potentiate the cytotoxicity of DNA damaging agents in dividing cells (Durkacz et al., 1980; Durrant et al., 1981; Jacobson et al., 1984). Durkacz et al. (1980) first demonstrated that the inhibition of poly(ADP-ribose) synthesis in L1210 cells by 3-aminobenzamide results in an increase in cytotoxicity of dimethylsulfate (DMS). Durrant and Boyle (1980) observed similar enhancement of N-methyl-N-nitrourea (MNU) cytotoxicity by 5-methyl nicotinamide. Further studies by these investigators indicated that the degree of potentiation of cytotoxicity of a DNA damaging agent is dependent upon both agent and cell line. Recently, Jacobson et al. (1984) have described the effect of 3-methoxybenzamide on cellular recovery

following MNNG in C3H10T1/2 cells. MBA at a concentration of 1 mM which has no effect on cell survival in the absence of DNA damage, can potentiate the cytotoxicity of MNNG by decreasing survival in dividing cells. In contrast, in non-dividing cells following MNNG treatment, inhibiting poly(ADP-ribosyl)ation during the DNA repair phase has no effect on cell survival. In addition, the morphological examination of exponentially dividing, MNNG-treated cells in the presence of MBA showed abnormal nuclear structures (Jacobson et al., 1985). From this observation, it has been suggested that changes in chromatin structure may take place during poly(ADP-ribosyl)ation. Similar suggestions regarding chromatin structural changes influenced by poly(ADP-ribosyl) ation have been made by Malik and Smulson (1984). If poly(ADP-ribose) is involved in maintaining the conformation of chromatin particularly following DNA damage, one would expect to observe several biological consequences as a result of inhibiting poly(ADP-ribosyl)ation. For example, chromosomal aberrations, alteration in gene expression, sister chromatin exchanges (SCEs), malignant transformation and mutagenesis might occur.

Several additional lines of evidence suggest a relationship between poly(ADP-ribose) metabolism and chromatin structure. The structure of poly(ADP-ribose) is now known to be comprised of long, highly branched chains with a high number of electronegative charges. A rapid increase in the intracellular content of poly(ADP-ribose) with a rapid depletion of NAD has been observed when human fibroblasts (CF-3) were exposed to

UV light. The rate of degradation of poly(ADP-ribose) is shown to be very rapid with a half-life of 1-6 minutes or less (Wielckens et al., 1982; Jacobson et al., 1982). The rapid synthesis and degradation of such a structure could certainly be envisioned to participate in the condensation and/or relaxation of chromatin structures. Or perhaps these complex polymers function to stabilize higher order structures of chromatin (Alvarez-Gonzales and Jacobson, 1985). Additional evidence from in vitro studies suggests that the poly(ADP-ribosyl)ation of calf thymus nucleosomes either by purified polymerase or by the endogenous chromatin bound enzyme induces reversible relaxation of the chromatin structure through H1 modification (Aubin et al., 1983). It has been suggested that the modification of H1 during DNA repair causes a local relaxation of chromatin structure. This is due to the high rate of polymer turnover on H1 (Huletsky and Poirier, 1985). It had been suggested earlier that a poly(ADP-ribose)-H1 dimer might be a transient modification which causes localized condensation of chromatin after periods of relaxation during DNA repair or replication (Hilz and Stone, 1977). Using immunological methods in in vivo studies, a dimer complex of poly(ADP-ribosyl)ated histone-H1 was found to occur during the S/G_2 boundary of the HeLa cell cycle (Wong et al., 1983). In addition to modification to H1, Poirier and his group have showned that H2B is also poly(ADP-ribosyl)ated when a chromatin structure is relaxed by poly(ADP-ribosyl)ation of H1 (Huletsky et al., 1985). From this observation, it has been suggested that the damaged DNA might be more

accessible to repair enzymes because of histone H2B hyper(ADP-ribosyl)-ation (Huletsky et al., 1985; Adamietz and Rudolph, 1984).

In addition to poly(ADP-ribose) modification of histone proteins, nonhistone proteins (HMG) have been observed to be modified by ADP-ribose (Kameshita et al., 1983). Song and Adolph (1983) have demonstrated ADP-ribosylation of HMG proteins during the HeLa cell cycle.

It has been suggested that poly(ADP-ribosyl)ation may also be important in chromatin structural changes associated with normal cell cycle progression. Alterations of chromatin structure certainly occur during the cell cycle, for example relaxation and condensation of chromatin structures both occur, therefore many investigators have looked at the relationship of poly(ADP-ribosyl)ation to the cell cycle. By using an in vitro nuclear system of HeLa cells, it had been shown that the highest level of ADP-ribose is at metaphase (Tanura and Kanai, 1982). Studies from Holtlund et al. (1983) have shown that the level of ADP-ribosylation of metaphase cells is 4-5 times greater than interphase cells. By using ^{32}P-NAD labelling in HeLa cells and two-dimensional gels to separate the ADP-ribosylated species, Song and Adolph (1983) found a different modification pattern comparing metaphase chromosome non-histones with the nonhistone chromatin-associated proteins in interphase nuclei. However, the relationship between poly(ADP-ribosyl)ation and cell cycle progression is not well understood. Kidwell (1983) has shown that the maximal activity of

poly(ADP-ribose) polymerase is between the end of S and early G_1. Several recent studies have demonstrated that inhibitors of poly(ADP-ribose) polymerase cause an extended S phase and cell cycle arrest at the G_2 phase in different types of mammalian cells exposed to DNA-damaging agents (Boostein and Pardee, 1984; Jacobson et al., 1985; Weilckens et al., 1985). From these observations, it has been speculated that poly(ADP-ribosyl)ation may be involved in the maintenance or restoration of chromatin structure necessary for efficient DNA repair following DNA damage (Weilckens et al., 1985). However, cell cycle perturbation in alkylated, non-dividing cells apparently does not occur when the synthesis of poly(ADP-ribose) is blocked since cell survival rates are unaffected (Jacobson et al., 1985). Jacobson et al. (1985) suggest that poly(ADP-ribose) is required for chromatin alteration necessary for DNA repair during DNA replication and cell cycle progression.

Many studies have investigated the possibility that poly(ADP-ribose) is involved directly in some step of DNA repair. It was first postulated by Durkacz et al. (1980) that poly(ADP-ribose) is required for efficient DNA excision repair. By using alkaline sucrose gradients to measure the movement of small fragments of DNA to larger components during DNA repair, it was shown that inhibitors of poly(ADP-ribose) polymerase such as 3-aminobenzamide or 5-methyl-nicotinamide retarded strand-rejoining in L1210 cells exposed to DMS or MNU and other DNA damaging agents. They concluded from these data that

poly(ADP-ribosyl)ation is necessary for the DNA ligase step, the final step of DNA excision repair (Durkacz et al., 1981). Similar conclusions have been obtained from James and Lehman (1982) who observed the effects of 3-aminobenzamide on various aspects of DNA repair in human fibroblasts treated with DMS. These investigators observed a retardation of the rejoining of strand breaks and an increase in unscheduled DNA synthesis. They interpret these results to mean that the synthesis of poly(ADP-ribose) is involved in the ligation step of excision repair. However, Moran and Ebsuzaki (1985) report studies in HeLa cells treated with DMS showing that an inhibitor of poly(ADP-ribose) polymerase, 3-AB, increases the number of DNA strand breaks but does not affect the rate of strand rejoining. It had been suggested by Crissen and Shall (1982) that DNA ligase II is modified by poly(ADP-ribose) in vitro. The modification results in an increase in DNA ligase II activity observed when cells are treated with DMS. This increase is prevented by inhibiting poly(ADP-ribose) synthesis. In contrast, in vivo studies of Kreimeyer et al. (1984) failed to demonstrate the poly(ADP-ribosyl)ation of the DNA ligase following DNA damage. Althaus and Mathis (1985) have speculated that poly(ADP-ribose) primarily affects changes in chromatin structure in response to DNA damage and subsequently influences individual steps of DNA repair. By using nuclease digestion probing techniques, they determined the distribution of newly synthesized DNA repair patches in nucleosomes of hepatocytes irradiated with UV in the presence and absence of 8 mM 3-AB.

The results showed a nonuniform distribution of newly synthesized DNA repair patches in nucleosomes when poly(ADP-ribosyl)ation is inhibited. This indicates that rearrangements of nucleosomal organization during DNA excision repair is affected by ADP-ribosylation. In addition, this group examined the individual steps of DNA repair which are influenced by poly(ADP-ribose). Utilizing various kinds of carcinogens to identify ADP-ribose dependent reaction steps in the process of DNA repair, they found at least two steps (repair synthesis and ligation) which are affected by inhibition of poly(ADP-ribose) synthesis.

However, the precise contribution of poly(ADP-ribose) to DNA repair cannot be pinpointed based on the accumulated data to date. Studies comparing the influence of UV-radiation on normal human fibroblasts and on excision repair deficient Xeroderma pigmentosum fibroblasts (complementation group A) indicate that excision repair is a major mechanism for error-free repair of damaged DNA (Maher et al., 1979). When lesions are induced in the DNA by DNA-damaging agents, they are subjected to cellular repair. Unrepaired or misrepaired lesions lead to biological effects such as mutagenesis, transformation, chromosomal aberrations and sister chromatid exchanges (Zeeland et al., 1980). Inhibitors of poly(ADP-ribose) polymerase such as benzamide and nicotinamide which have been known to reduce survival, slow cell growth and enhance alkylation induced toxicity, have been found to increase sister chromatid exchange frequency in Chinese hamster ovary cells ($CHO\text{-}K_1$) (Hori, 1981; Natarajan et al., 1981; Morris and Helflich,

1984). A correlation between reduced cell survival and SCE induced by a number of simple alkylating agents has been found (Morris et al., 1982; Natarajan, 1982). Morris et al. (1982) also reported that a type of DNA damage produced by alkylating agents was responsible for inducing SCE and reducing cell survival and cell growth. Morgan and Cleaver (1982) first reported that combined treatment of an alkylating agent e.g. methylmethane sulfate (MMS) and 3-aminobenzamide synergistically increased SCE frequencies. Similar observations have been shown by Park et al. (1983) and Morgan et al. (1985). However, 3-aminobenzamide does not influence the frequencies of SCEs induced by UV or mitomycin C in Chinese hamster ovary cells (Natajaran et al., 1981, 1983).

Oikawa and his coworkers (1980) were the first to report that several inhibitors of poly(ADP-ribose) polymerase induce SCEs in the absence of DNA damaging agents. Hori (1981) examined the effect of nicotinamide-depletion on chromosome stability in CHO-K_1 resulting in an increased frequency of SCE. In addition, a combined treatment with 3-AB in nicotinamide-free medium resulted in a many fold increase in the frequency of SCEs and chromosomal aberrations. The results suggest that appropriate levels of cellular NAD and the activity of poly(ADP-ribose) polymerase are required for maintaining chromosome stability. Since SCEs have been considered as an indicator of mutagenesis (Natarajan et al., 1983), Natarajan et al. tested inhibitors of poly(ADP-ribose) polymerase for their capacity to induce mutations. The results showed that under the conditions in which 3-AB increases the frequencies of

SCEs, it does not increase the frequencies of point mutations in the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus in CHO cells.

Inhibition of poly(ADP-ribose) synthesis by agents such as 3-aminobenzamide causes increases in chromosomal aberration frequency in CHO cells following chemicals or radiation (Schwartz et al., 1985; Zwanenburg et al., 1985; Natarajan et al., 1982). Zwanenburg et al. (1985) reported that 3-AB increases the frequency of chromosomal aberrations induced by dimethyl sulfate (DMS), ethyl methanesulphonate (EMS) and 5-chlorodeoxyuridine (CldUrd) in CHO cells. However, there was no enhancement observed in cells treated with mitomycin C (MMC) (Zwanenburg et al., 1985). Previous work from this group showed that BrdUrd or CldUrd does not induce chromosomal aberrations when CHO cells are grown for 12 hr in this analogue (Zwanenburg et al., 1984). However, incubation for 24 hr in medium containing CldUrd results in numerous aberrations and this frequency is increased with 1 mM 3-AB. Utilizing the premature chromosome-condensation technique, the analysis of chromosomal aberrations in delayed G_2 cells following the incorporation of CldUrd showed numerous aberrations. The chromosomal aberration frequency is enhanced by 3-AB (Zwanenburg et al., 1985). A similar observation was shown in CHO cells induced by MNNG or EMS (Schwartz et al., 1985) where 3-AB increased the frequency of chromosomal aberrations.

Inhibition of poly(ADP-ribosyl)ation results in increased frequencies of chromosomal aberrations and sister chromatid exchanges in damaged cells. The disturbance of transformation frequency is also observed in the absence of poly(ADP-ribosyl)ation following DNA damage. Current studies in the Jacobson laboratories on the role of poly(ADP-ribose) in malignant transformation have been reported (Smith, 1985). When the synthesis of poly(ADP-ribose) is blocked by either MBA or NAD-depletion, the results of an increased transformation frequency in both conditions have been obtained in exponentially dividing C3H10T1/2 cells following MNNG. In contrast, in confluent cells following MNNG, experiments employing MBA have shown no effect on transformation frequency. Similarly, Lubet et al. (1985) have observed an increased transformation frequency obtained from BALB/3T3 clone A31-1 cells treated with MNNG when the synthesis of poly(ADP-ribose) is inhibited by 3-AB. Comparison studies on the effect of 3-AB on mutagenesis and transformation have also been conducted in CHO cells treated with DNA-damaging agents. The effect of 3-AB on transformation frequencies shows much larger and different effects than on mutagenesis. Transformation is inhibited by MMS whereas mutation from MMS is increased at the 6-thioguanine resistant locus (TG^r). However, a decrease of mutation frequency has been observed at the ouabain locus (Oua^r).

Accumulated observations on biological consequences which result from the absence of poly(ADP-ribose) synthesis in different mammalian

cell lines following DNA damage have clearly shown increases in cytotoxicity, changes of chromatin structure and increased frequencies of chromosomal aberrations, sister chromatid exchanges and malignant transformation. However, the effect of poly(ADP-ribose) on mutation rates is still in question. Both increases (Schwartz et al., 1985) and decreases (Durrant et al., 1981; Bhattacharyya and Bhattacharjee, 1983) in mutation frequencies have been reported as a result of inhibiting poly(ADP-ribosyl)ation.

The specific aim of this study was to investigate the influence of altered poly(ADP-ribose) metabolism on mutagenesis. The poly(ADP-ribose) polymerase inhibitor, MBA, is known to cause cell cycle delays in exponentially dividing cells following DNA damage (MNNG). Under the conditions where the cell cycle is delayed, if not permanently blocked, the assessment of mutagenesis may not be feasible. Therefore, an alternative study of non-dividing cells following MNNG treatment was initiated. Furthermore, this study was expanded to alter poly(ADP-ribose) metabolism with nicotinamide starvation in order to avoid the potential argument of nonspecificity of inhibitors.

MATERIALS AND METHODS

Cell line and Culture Conditions

The C3H10T1/2 (Clone 8) mouse embryo fibroblast cell line was obtained from the late Dr. C. Heidelberger, Comprehensive Cancer Center, University of Southern California, Los Angeles, California. The cells were grown in Eagle's basal medium (BME) at pH 7.2, containing 10% heat-inactivated fetal calf serum (FCS)(Reheis Chemical Co, Kankakee, IL, or Hyclone Laboratories, Logan, UT), sodium bicarbonate (2.2 g/l) and gentamicin sulfate (20 mg/l) (Sigma Chemical Co., St. Louis, MO). Cultures were maintained in Corning plastic tissue culture flasks (25 or 75 sq cm) and incubated in a humidified atmosphere of 5% CO_2 in air at 37^{o} C. Cells of passage 14 or less were used and continuously subcultured prior to confluence. For experiments employing NAD-depleted cells, C3H10T1/2 cells were grown as described for 3T3 cells, (Jacobson et al., 1979) in nicotinamide free Dulbecco's modified Eagle's medium (SDME) containing 10% dialyzed fetal calf serum (DFCS), sodium bicarbonate (2.2 g/l) and gentamicin sulfate (20 mg/l). Cells were to be depleted of NAD for 4 population doublings. Cells were removed from dishes or flasks with 0.05% Trypsin:200 mg EDTA/l (Gibco, Grand Island, NY) in phosphate buffered saline (PBS)(0.15 M NaCl, 0.01 M NaH_2PO_4-Na_2HPO_4, pH 7.2). Cell numbers were determined by counting in a Model ZBI Coulter Counter.

Fetal calf serum was heat-inactivated at 56°C for 30 min. The heat inactivated FCS was dialyzed against dialysis buffer (0.15 M NaCl, 0.5 M NaH_2PO_4-Na_2HPO_4, pH 7.2) employing an Amicon diafiltration unit equipped with a hollow fiber cartridge (Amicon Corporation Scientific Systems Divisions, Danvers, MA).

Treatment with Chemicals

MNNG was obtained from Sigma Chemical Co., St. Louis, MO. MBA was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. MNNG and MBA were dissolved in acetone. The final concentration of acetone in the medium was 0.5%. For exponentially dividing cells, stock cultures in the exponential growth phase were suspended in BME containing 10% FCS. Cells were seeded at 3×10^4 cells per 35 mm dish for studies designed to monitor population doublings and seeded at 1×10^5 cells per 100 mm dish for colony formation assays and mutagenesis assays. Approximately 24 hr after seeding, cells were treated with complete medium containing a desired concentration (1.7-3.4 μM) of MNNG with or without 1 mM MBA for 48 hr. For experiments employing exponentially dividing, NAD-depleted cells, cells were seeded in BME containing 10% FCS at 3×10^4 cells per 35 mm dish for studying cell division during starvation. Cells were seeded at 1×10^5 cells per 100 mm dish for colony formation assays and mutagenesis assays. After 24 hr, the medium was removed and SDME containing 10% DFCS with or without nicotinamide (Nam) was replenished. Control (+Nam) or depleted (-Nam) cells were grown for 4 population doublings. Before cells were reseeded for MNNG

treatment, NAD and its reduced form were extracted by the method of Jacobson and Jacobson (Jacobson and Jacobson, 1976). The cells were extracted with 0.1 M NaOH-1 mM nicotinamide (1 ml/35 mm dish) and immediately neutralized with ice cold 0.37 M H_3PO_4. The reduced form was oxidized by adding 2 mM phenazine ethosulfate (PES) (0.125 ml per 1 ml of base). NAD content was measured by an enzyme cycling assay (Jacobson and Jacobson, 1976). Control or NAD-depleted cells were then reseeded at 2 x 10^5 cells per 100 mm dish in SDME containing 10% DFCS with or without Nam. After 24 hr, cells were treated with the same medium containing MNNG concentrations in the range of 1.7-26.8 μM for 48 hr. After treatment, the media were removed, the cells were washed twice with PBS and allowed to grow in Dulbecco's modified Eagle's medium (DME) containing nicotinamide for an additional 48 hr. For experiments employing non-dividing cells, cultures were grown in BME containing 10% FCS to confluence and held for 2-3 days prior to treatment. Cells were exposed to complete medium containing 34-68 μM MNNG with or without 1 mM MBA. After 20 min treatment, the medium was removed, the cells were washed twice with PBS, and the complete medium with or without 1 mM MBA was replenished for an additional 48 hr. In the case of experiments employing non-dividing, NAD-depleted cells, cells were seeded in complete medium at 3 x 10^4 cells per 35 mm dish for determining cell division rates during starvation and at 1 x 10^5 cells per 100 mm dish for colony formation and mutagenesis assays. After 24 hr, the medium was removed and the SDME containing 10% FCS with or without Nam were

replenished. Cultures were starved for 4 population doublings and held at confluence for 2 days. Cells were treated with MNNG at 20.4-68 μM for 20 min in SDME containing 10% DFCS with or without Nam. After treatment, the cells were washed twice with PBS and fresh media (SDME containing 10% DFCS with or without Nam) were added back. After 48 hr in both dividing or non-dividing, NAD-depleted cells, the media were removed, the cells were again washed once with PBS, reseeded at 1×10^5 cells per 100 mm dish in control medium and allowed to grow for 3-10 days for mutagenic expression. Cell survival and mutation frequency were then determined by simultaneous colony formation and mutagenesis assays.

Colony Formation Assays

Cells were seeded at 250-300 cells per 60 mm gridded dish in complete medium (BME containing 10% FCS, or DME containing 10% FCS for experiments employing NAD-depleted cells. After 24 hr, two dishes were fixed and stained for determination of plating efficiency. The other 5 dishes were incubated an additional 6-7 days for colony formation. The medium was then aspirated and the cells were washed twice with PBS. The cells were fixed by two successive 1 min treatments with methanol: glacial-acetic acid(3:1, V/V). The dishes were dried and stained with 1% methylene blue in water for 10 min. The stained dishes were rinsed with water and air-dried, and colonies with 25 cells or more were counted. Percent survival was determined as the percentage of the cells forming colonies at each condition relative to controls.

The fraction forming colonies was obtained from the average number of colonies per dish divided by the number of cells which attached to the dish (Jacobson et al., 1985).

Mutagenesis Assay

Cells were reseeded in 100 mm dishes with complete medium (BME containing 10% FCS, or DME containing 10% FCS for experiments employing NAD-depleted cells) at 1 x 10^5 cells per dish, 10 dishes per condition or at 2 x 10^5 cells per dish, 5 dishes per condition. After 24 hr, the medium was removed, and medium containing 3 mM ouabain (Sigma Chemical Co., St. Louis, MO or ICN Biochemicals, Cleveland, OH) was added. Ouabain at a concentration of 3 mM was completly dissolved in complete medium by incubating at 37^{o}C for at least 2 hr (Landolph and Heidelberg, 1979). Fresh medium containing ouabain was replenished every 6 days. After 16 days, the dishes were fixed and stained by the same method described in the colony formation assay. Mutant colonies of greater than 2 mm in diameter were counted. The mutation frequency at each condition was calculated as the total number of mutant colonies observed divided by the fraction forming colonies at that condition multiplies by 10^6 to give mutants per 10^6 survivors (Landolph and Heidelberger, 1979).

RESULTS

The C3H10T1/2 cell line

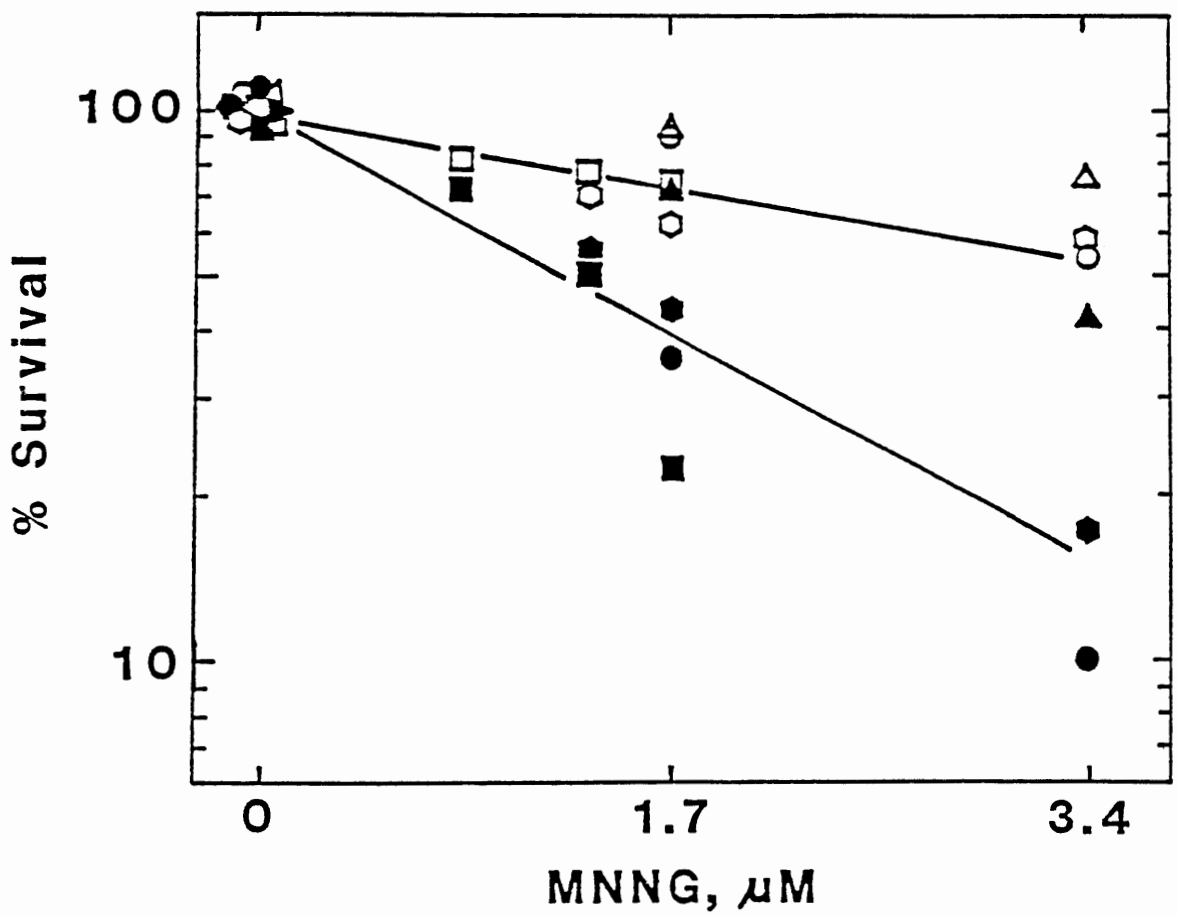
The C3H10T1/2 mouse embryo fibroblast was selected as a model to test the potential role of poly(ADP-ribose) metabolism in mutagenesis. This cell line was originally developed for and has been widely used as a model for an *in vitro* assay of malignant transformation (Reznikoff et al., 1973). Since many laboratories have become interested in the relationship between transformation and mutation, the C3H10T1/2 cell system has also been used quantitatively in the mutational induction of ouabain resistance (Oua^r) (Landolph and Heidelberger, 1980). The growth of these cells is tightly regulated by density dependent inhibition of growth. Because of this advantage, experiments employing non-dividing cells can easily be done without the application of drugs. In addition, the C3H10T1/2 cells exhibit a generation time of 12 to 15 hr. Therefore, sufficient cell numbers for experiments are rapidly attained.

Effect of poly(ADP-ribose) polymerase inhibitor on mutation frequency in exponentially dividing cells following MNNG treatment

It has been shown that inhibitors of poly(ADP-ribose)polymerase potentiate the cytotoxicity of alkylating agents (Nduka et al., 1980; Durkacz et al., 1980; Jacobson et al., 1984). Particularly, dividing cells have been shown to be more sensitive to the treatment of DNA damaging agents such as MNNG in the presence of these inhibitors

(Jacobson et al., 1984). However, MBA alone at a concentration of 1 mM has no effect on the growth of control cells (Jacobson et al., 1984). In conducting mutagenesis assays, it is desirable to find the optimal condition of treatment that allows a minimum of 10% survival (Newhold et al., 1980) in order to measure mutation frequency most accurately. Therefore, a study of the effect of MNNG dose on cell survival was initiated. Cells were seeded at 1 X 10^5 cells per 100 mm dish and 24 hr later, were treated with a range of MNNG concentrations from 0 to 3.4 μM in the absence or presence of 1 mM MBA for 48 hr. Treatment medium was removed and cells were incubated with control medium for an additional 24 or 48 hr. Cells were then reseeded at 250-300 cells per 60 mm gridded dish for colony formation assay. After 7-9 days, the number of cells capable of forming a colony of 25-50 cells or more was determined. The percent of cells surviving relative to untreated controls was plotted as a function of MNNG concentration. These data are shown in Figure 3. Survival curves obtained from MNNG-treated, dividing cells showed a linear decrease with increasing concentration. With 3.4 μM MNNG treatment alone, survival was detected at about 50%, obtained from two separate experiments. In contrast, a sharp decrease in the survival curve was obtained from MNNG-treated, dividing cells in the presence of MBA with increasing MNNG concentration. The percent survival was only 9-15% when cells were treated with 3.4 μM MNNG in the presence of MBA. Although cytotoxicity and MBA induced co-cytotoxicity were observed the survival rates at these doses were clearly above 10%. Thus, the

Figure 3. Survival of exponentially dividing C3H10T1/2 cells following MNNG and MBA treatment. Exponentially dividing cells were seeded at 1×10^5 cells/60 mm dish and treated 24 hr later with medium containing MNNG dissolved in acetone at 0, 0.67, 1.3, 1.7 and 3.4 μM in the absence (open symbols) or presence (closed symbols) of 1 mM MBA for 48 hr. Treatment medium was removed and control medium was replaced. After an additional 48 hr of growth, cells were reseeded in control medium for colony formation assays as described in METHODS. Data are expressed as the percentage of control colony forming ability. Experiment 1 (○,●), Experiment 2 (△,▲), Experiment 3 (□,■), Experiment 4 (⬡,⬢).


100
10
% Survival
0
1.7
3.4
MNNG, μM

appropriate concentration of MNNG for inducing detectable mutations in exponentially dividing cells fell in the range from 0 to 3.4 μM.

Since it is known that DNA replication and cell division are required for expression of mutagenic events, treated cells absolutely need to divide in order to assess mutation rates. One major technical difficulty in attempting to assess the effect of inhibiting poly(ADP-ribose) metabolism on mutation events is the observation that inhibitors of poly(ADP-ribose) polymerase present following DNA damage cause delays in cell cycle progression or a blockage of cells in the G_2 phase of the cell cycle (Jacobson et al., 1985). Thus the MBA treated cells require a longer time to complete the cell cycle during expression if cell cycling occurs at all. For this reason, experiments were conducted to monitor the number of population doublings during treatment and expression by measuring cell numbers daily following treatment as shown in Figure 4 and Table I. The results show that cells treated with 3.4 μM MNNG completed 4 population doublings in 72 hr whereas in the presence of MBA only 2 population doublings were obtained in the same length of expression time (72 hr) (Data shown in Table I). Other reports have demonstrated that the induction of Oua^r mutations is maximal after approximately 2 population doublings or 3 to 4 days of expression (Landolph and Heidelberger, 1979). Analyses of both the dose and time course of expression for maximal mutation frequency in the presence and absence of MBA were then conducted. The results shown in Table I reveal that in general 3-4 days of expression or 1-2 population

Figure 4. Increase in cell numbers during expression of MNNG induced mutations. Cells were seeded at 5 X 10^4 cells/60 mm dish in BME containing 10% FCS and 24 hr later, treated for 48 hr with medium containing 0.5% acetone (○), 1.7 μM MNNG in acetone (△) and 3.4 μM MNNG in acetone (□). Closed symbols indicate that 1 mM MBA was present for 48 hr. After treatment, the medium was removed and control medium was replaced for an additional 48 hr.

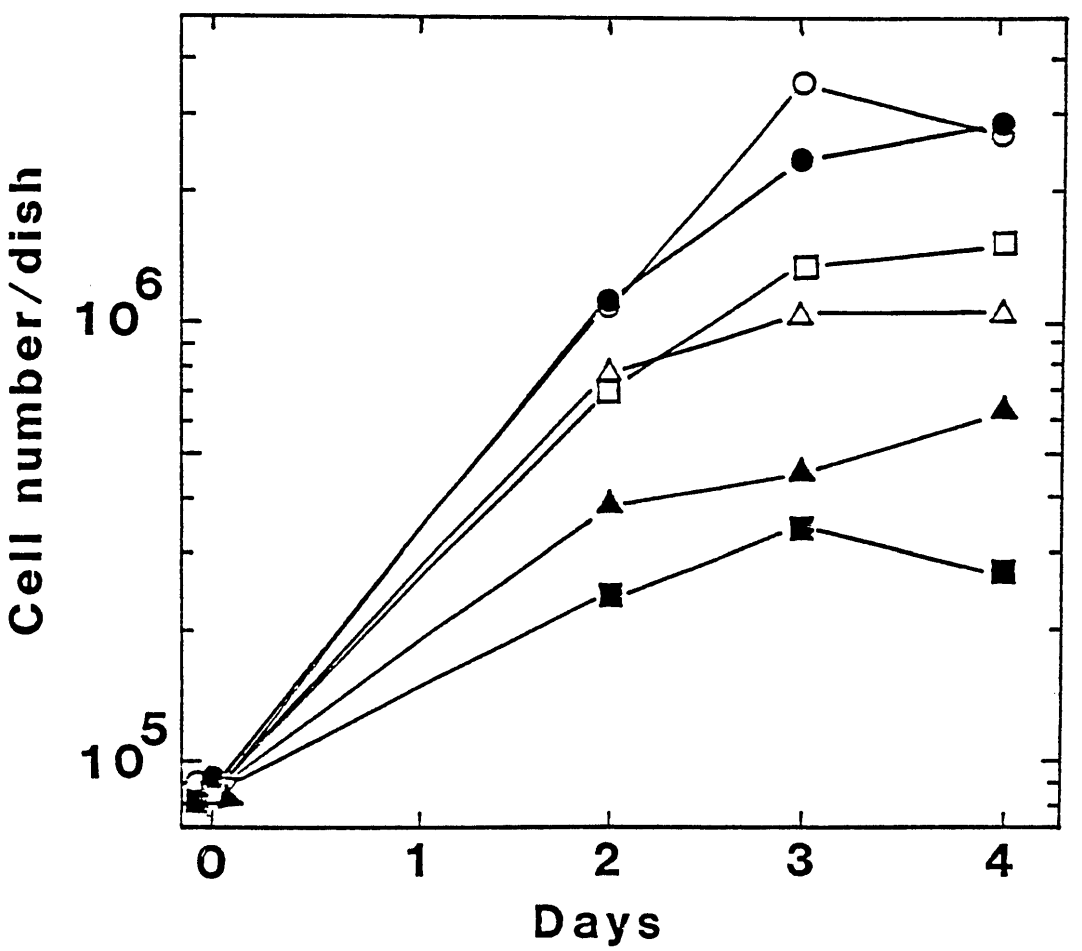

Cell number/dish
10^6
10^5
0
1
2
3
4
Days

Table I.

Effect of expression time on mutation frequency

Determination	Treatment						Days of expression
	Control	MBA	1.7 μM MNNG	1.7 μM MNNG + MBA	3.4 μM MNNG	3.4 μM MNNG + MBA	
Population	3.9	4.1	3.4	2.1	3.3	1.1	2
doublings	5.6	5.1	4.5	2.3	4.3	2.0	3
	5.3	5.3	4.7	3.2	4.5	1.6	4
Surviving	0.24	0.28	0.21	0.09	0.13	0.0	2
fraction	0.22	0.28	0.20	0.10	0.12	0.03	3
	0.36	0.37	0.24	0.17	0.18	0.03	4
Number of	0	0	12	3	12	0	2
Oua^r colonies[a]	0	0	22	0	26	1	3
	0	0	25	1	22	0	4
Mutation[b]	0	0	57	33	92	0	2
frequency	0	0	110	0	217	33	3
	0	0	104	6	122	0	4

[a] Total number of Oua^r colonies
[b] The number of Oua^r colonies per 10^6 survivors

C3H10T1/2 cells were seeded at 3 X 10^4 cells/35 mm dish and 1 X 10^5 cells/100 mm dish. After 24 hours, exponentially dividing cells were treated with 0, 1.7 or 3.4 μM MNNG in the presence and absence of 1 mM MBA for 48 hours. Population doublings were determined from cells seeded in 35 mm dishes during 2, 3 and 4 days of expression. After expression, cells were reseeded for colony formation. Surviving fraction was calculated from the observed number of colonies divided by the observed number of cells that attached (Jacobson et al., 1985). Mutation frequency was calculated as the total number of Oua^r colonies divided by the surviving fraction.

doublings exhibited the maximal rate of mutation at the Oua locus either in the presence or absence of MBA. However, evaluating growth or cell division by measuring population doublings may not be an accurate method. Some cells in the MNNG-treated cell population do not divide. Thus the observed generation time for such a population may be much longer than the actual doubling time for the portion of the population that really is dividing. For this reason, the determination of optimal expression time and population doublings were considered together in determining the maximal mutation induction in exponentially dividing cells. Figure 5 compares the mutation frequency obtained after different MNNG and/or MBA treatments as a function of expression time. The maximal mutation frequency under most conditions was observed at day 3 of expression. Using this information, experiments employing dividing cells were begun in order to assess the effect of inhibiting poly(ADP-ribose) synthesis on MNNG induced mutations. Exponentially dividing cells were exposed for 48 hr to a range of MNNG concentrations from 0 to 3.4 μM in the presence and absence of MBA. Cell growth was followed to make certain that the population divided at least one time and the expression period was 3 and 4 days. Survival and mutation frequency were assayed. Tables II and III show the data from which mutation frequencies were calculated after MNNG treatment in dividing cells after days 3 and 4 of expression, respectively. Figures 6 and 7 are graphic comparisons of mutations obtained after varying doses of MNNG in the presence and absence of MBA. The mutation frequency

Figure 5. Comparison of mutation frequency as a function of expression time. Cells were seeded at 1 X 10^5 cells per 100 mm dish. After 24 hr, cells were treated with 1.7 μM (O) or 3.4 μM (Δ) MNNG for 48 hr. Closed symbols, ● and ▲ represent 1.7 and 3.4 μM MNNG in the presence of 1 mM MBA for 48 hr, respectively. Cells were reseeded at days 2, 3 and 4 after treatment for colony formation and mutagenesis assays. Mutation frequencies were determined and plotted as a function of expression time.

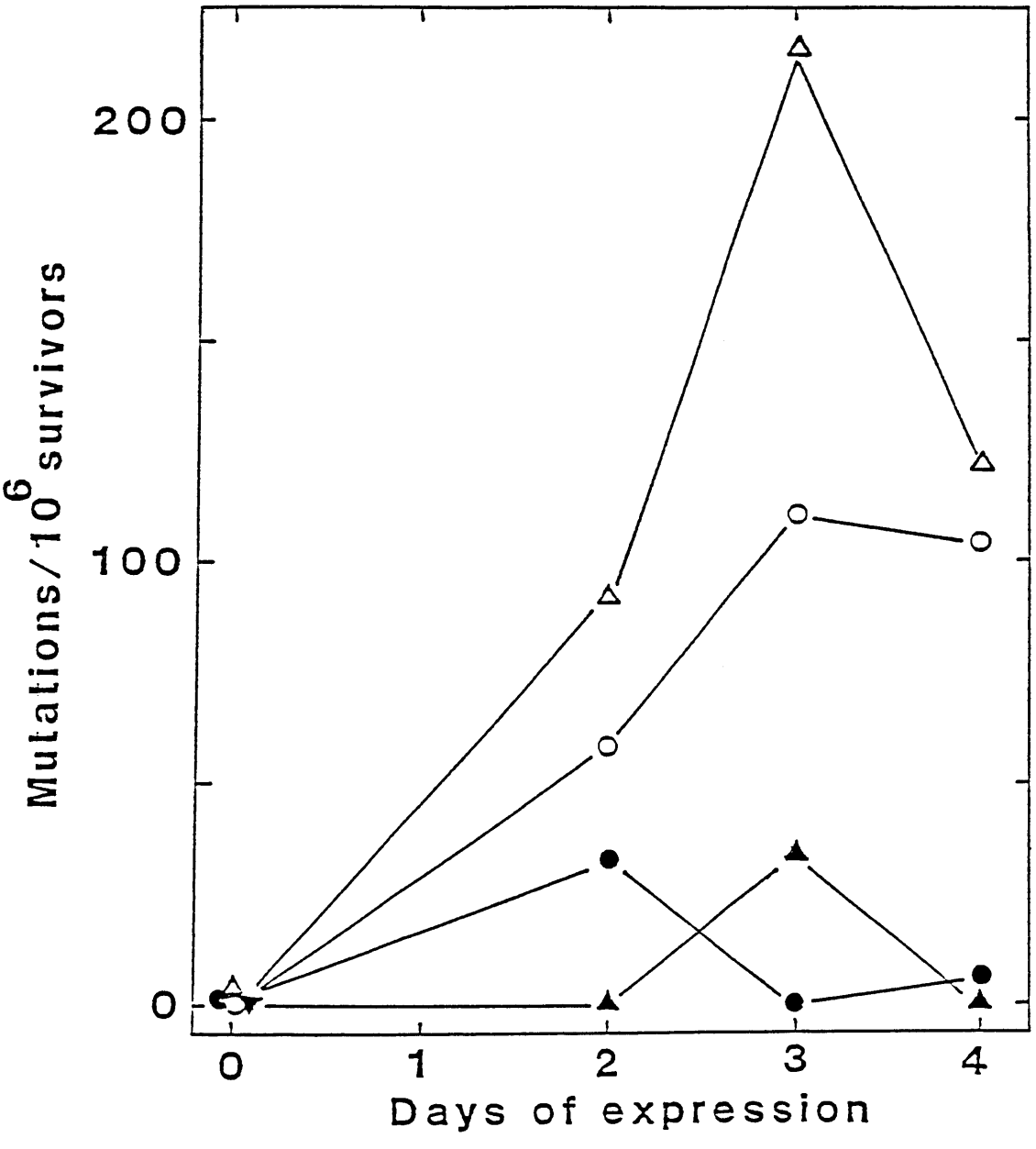

200
100
0
Mutations/10^6 survivors
0
1
2
3
4
Days of expression

Table II.

Mutation induction after MNNG treatment on day 3 of expression in exponentially dividing cells

Treatment	Population doublings	Surviving fraction	Number of Oua^r colonies	Mutation frequency
Experiment 1				
Control	5.6	0.22	0	0
MBA	5.1	0.28	0	0
1.7 μM MNNG	4.5	0.20	22	110
1.7 μM MNNG + MBA	2.3	0.10	0	0
3.4 μM MNNG	4.3	0.12	26	217
3.4 μM MNNG + MBA	2.0	0.03	1	33
Experiment 2				
Control	3.3	0.29	0	0
MBA	2.6	0.30	0	0
1.7 μM MNNG	3.0	0.27	3	11
1.7 μM MNNG + MBA	3.0	0.19	1	5
3.4 μM MNNG	2.8	0.22	21	95
3.4 μM MNNG + MBA	1.8	0.13	1	8

Mutation induction after MNNG in the presence and absence of 1 mM MBA for 48 hr. Treatment medium was removed and control medium was replaced. Population doublings were determined. After day 3 of expression, cells were reseeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined.

Table III.

Mutation induction after MNNG treatment on day 4 of expression in exponentially dividing cells

Treatment	Population doublings	Surviving fraction	Number of Oua^r colonies	Mutation frequency
Experiment 1				
Control	4.0	0.22	0	0
MBA	4.0	0.22	0	0
0.67μM MNNG	3.4	0.18	4	22
0.67μM MNNG + MBA	2.0	0.16	4	25
1.3 μM MNNG	3.4	0.17	14	82
1.3 μM MNNG + MBA	2.1	0.11	6	55
1.7 μM MNNG	3.5	0.16	24	150
1.7 μM MNNG + MBA	1.8	0.05	4	80
Experiment 2				
Control	3.6	0.24	0	0
MBA	3.4	0.23	0	0
1.3 μM MNNG	3.1	0.17	2	12
1.3 μM MNNG + MBA	2.8	0.13	2	15
1.7 μM MNNG	3.3	0.15	7	47
1.7 μM MNNG + MBA	2.2	0.10	1	10
3.4 μM MNNG	3.1	0.14	37	264
3.4 μM MNNG + MBA	1.6	0.04	7	175

Mutation induction after MNNG treatment on day 4 of expression in exponentially dividing cells. Twenty-four hr after seeding, cells were treated with 0, 0.67, 1.3 or 1.7 μM MNNG in experiment 1; and 0, 1.3, 1.7 or 3.4 μM MNNG in experiment 2 in the absence and presence of 1 mM MBA for 48 hr. Population doublings were determined. After day 4 of expression, cells were reseeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined.

Figure 6. Graphic comparison of mutation frequency in exponentially dividing cells after 3 days of expression. Twenty-four hr after seeding, cells were treated for 48 hr with medium containing 0, 1.7 or 3.4 μM MNNG in the absence (open symbols) and presence (closed symbols) of 1 mM MBA. Treatment medium was removed and control medium was added back for an additional 24 hr. Cells were reseeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined. Data are plotted as mutations/10^6 survivors. Experiment 1 (○,●), Experiment 2 (△,▲).

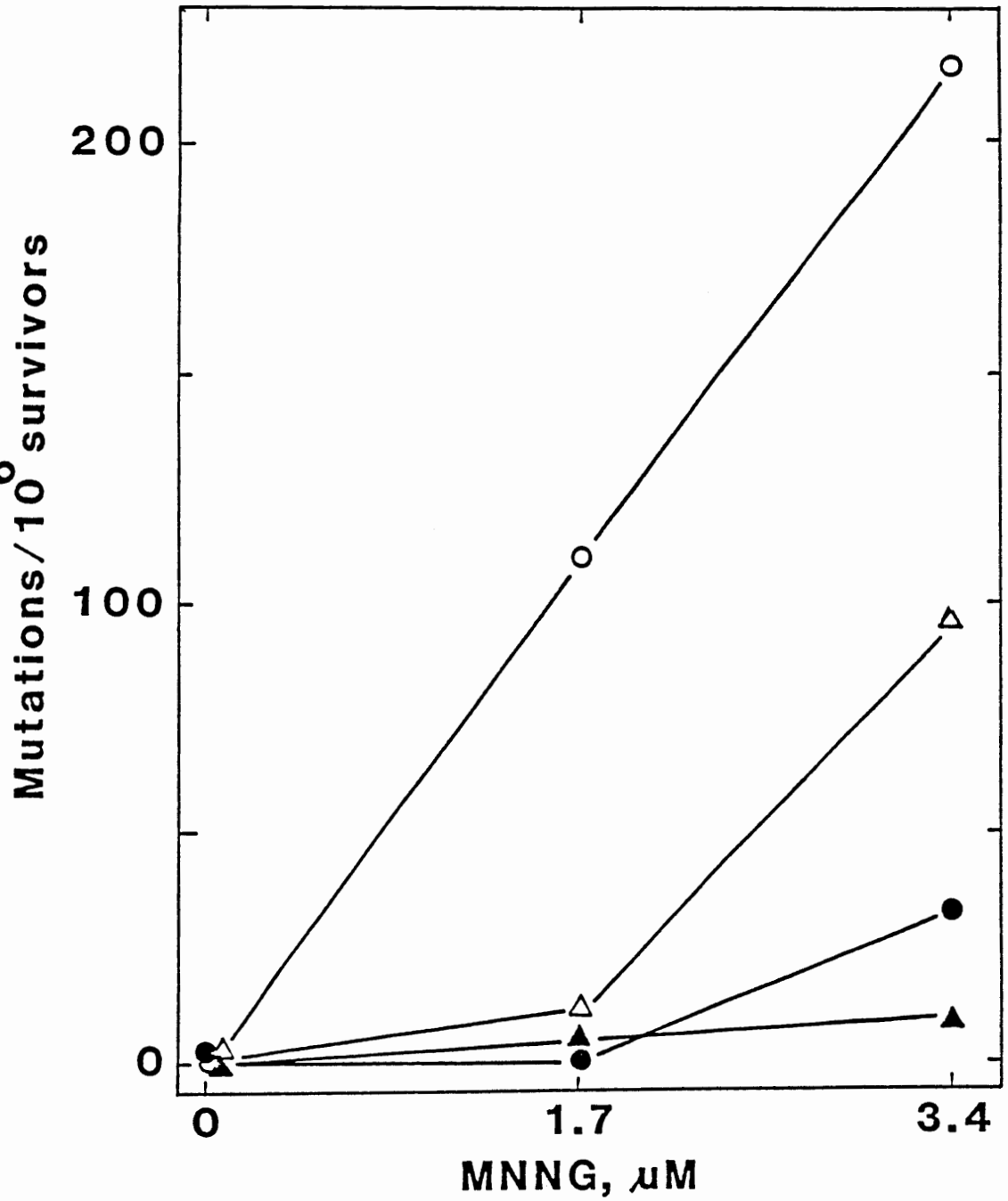
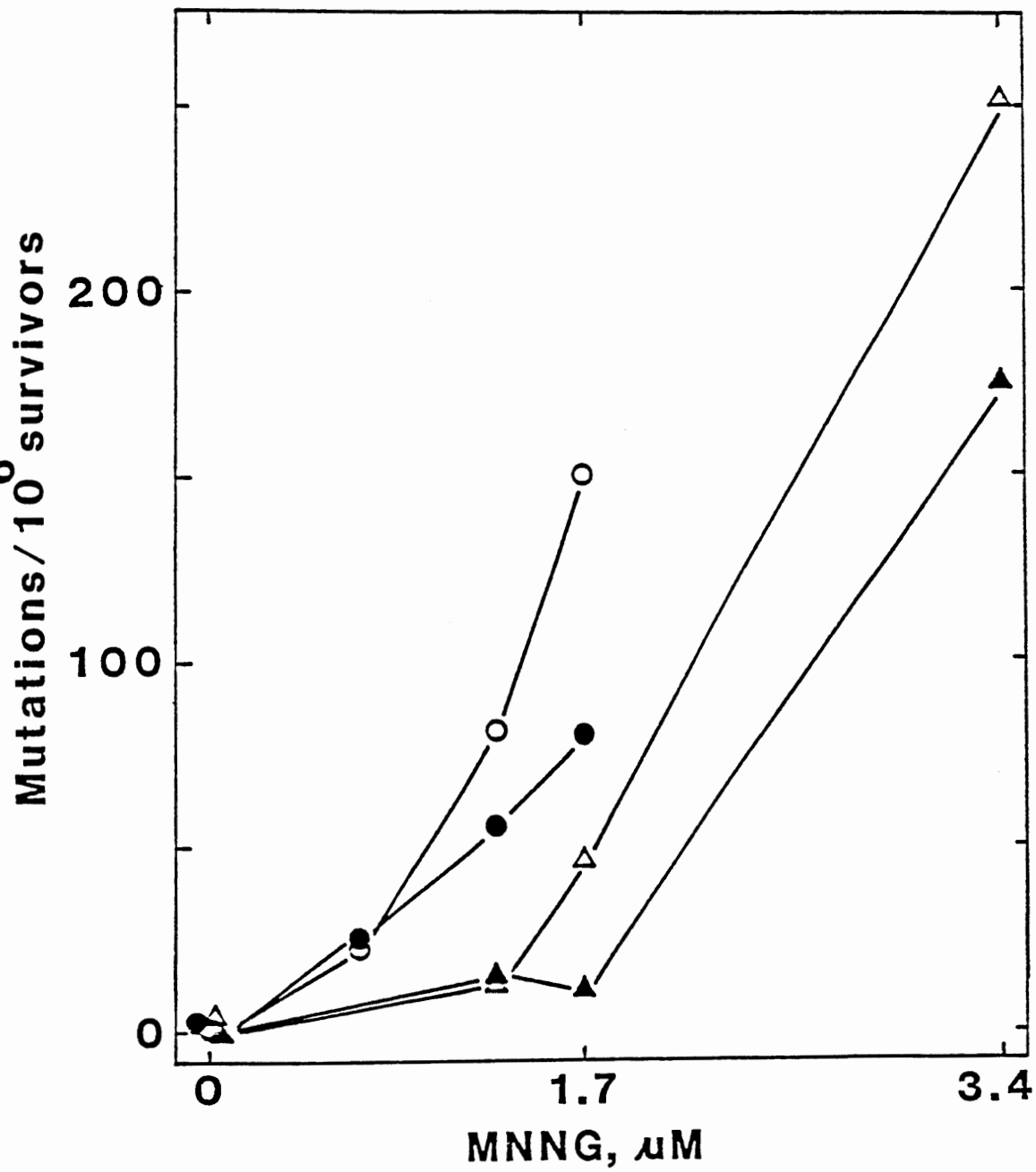

200
100
0
Mutations/10^6 survivors
0
1.7
3.4
MNNG, μM

Figure 7. Graphic comparison of mutation frequency in exponentially dividing cells after 4 days of expression. Twenty-four hr after seeding, cells were treated for 48 hr with medium containing 0, 0.67, 1.3 or 1.7 μM in experiment 1 and 0, 1.3, 1.7 or 3.4 μM MNNG in experiment 2 in the absence (open symbols) and presence (closed symbols) of 1 mM MBA. Treatment medium was removed and control medium was added back for an additional 48 hr. Cells were then reseeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined. Data are plotted as mutations/10^6 survivors. Experiment 1 (○,●), Experiment 2 (△,▲).


Mutations/10^6 survivors
200
100
0
0
1.7
3.4
MNNG, μM

increased in response to an increase in MNNG concentration. However, all 4 experiments with either 3 or 4 days of expression clearly showed a decrease in mutation frequency in the presence of MBA as shown in Tables I, II, and III, and Figures 5, 6 and 7. Table IV summarizes these data. Experiments 1 and 2 (Table II) showed 6.5-and 12-fold decreases in mutation frequency, respectively, when mutagenesis assays were conducted after 3 days of expression whereas experiments 3 and 4 showed 1.8-and 1.5-fold decreases in mutation frequency, respectively when the assays were conducted after 4 days of expression. The decrease observed from experiments 1 and 2 in Table III was about 2 to 6 times less than those observed from experiments 1 and 2 in Table II.

As shown in Table IV, the decrease in mutation frequency following MNNG treatment in the presence of MBA varied between experiments. This variation may be due to differences in detection of mutant colonies. Mutation frequency is calculated as the observed numbers of mutant colonies divided by surviving fraction, and fold decrease is calculated as a ratio of mutation frequencies. Only one mutant colony was detected in experiments 1 and 2 where dividing cells were treated with 3.4 μM MNNG in the presence of MBA. Since these observed number of mutant colonies are very low, they may not represent an accurate estimate of mutation frequency.

Effect of poly(ADP-ribose) polymerase inhibitor on mutation frequency in non-dividing cells following MNNG treatment.

It has been shown that non-dividing cells show little or no

Table IV.

Summary of the effect of MBA on mutation frequency in exponentially dividing cells

Experiment	MNNG (μM)	Days of Expression	Fold decrease in MF[a] in pressence MBA
1	3.4	3	6.5
2	3.4	3	11.8
3	1.7	4	1.8
4	3.4	4	1.5

[a] Fold decrease in mutation frequency.

Summary of the effect of MBA on mutation frequency in exponentially dividing cells following MNNG. Data from Tables II and III were used to calculate fold decreases in mutation frequency.

enhancement of cytotoxicity of MNNG in the presence of MBA (Jacobson et al., 1985). Therefore, experiments were initiated to investigate the effect of inhibiting poly(ADP-ribose) metabolism on MNNG induced mutations in non-dividing cells. This approach provides the opportunity to eliminate differential cell division rates during expression in the presence and absence of MBA. The non-dividing or confluent cells were obtained by growing C3H10T1/2 cells in the control medium to confluence. The cells were held at confluence for 2 days. Previous work from the Jacobson laboratories demonstrated that non-dividing cells treated with MNNG for 20 min in the presence and absence of MBA for 48 hr had the same survival rates (Jacobson et al., 1985).

These results suggested that confluent cells were much less sensitive to MNNG in the presence and absence of MBA than dividing cells. Therefore experiments were initiated to determine the optimum dose of MNNG to induce mutations in confluent cells. Non-dividing cells were treated with doses of MNNG ranging from 34 to 68 μM for 20 min in the presence and absence of MBA. The treatment media were removed and medium containing MBA was added back for an additional 48 hr incubation. The surviving fraction and the number of Oua^r colonies were then determined and mutation frequency was calculated. Figure 8 shows survival curves obtained after one day of expression whereas survival curves obtained after 4 and 5 days of expression are shown in Figure 9. The survival rate for MNNG-treated non-dividing cells in the presence of

Figure 8. Survival of non-dividing cells following MNNG and MBA treatment after 1 day of expression. C3H10T1/2 cells were treated at postconfluence for 20 min with medium containing 0, 51 and 68 μM MNNG in the absence (open symbols) and presence (closed symbols) of 1 mM MBA. Treatment medium was removed and medium containing 1 mM MBA was replenished for 48 hr. Cells were reseeded at 1 X 10^5 cells/100 mm dish in control medium. After 24 hr, cells were harvested and seeded for colony formation assays. Percent survival was calculated as the percentage of control colony forming ability. Experiment 1 (○,●) Experiment 2 (△,▲).

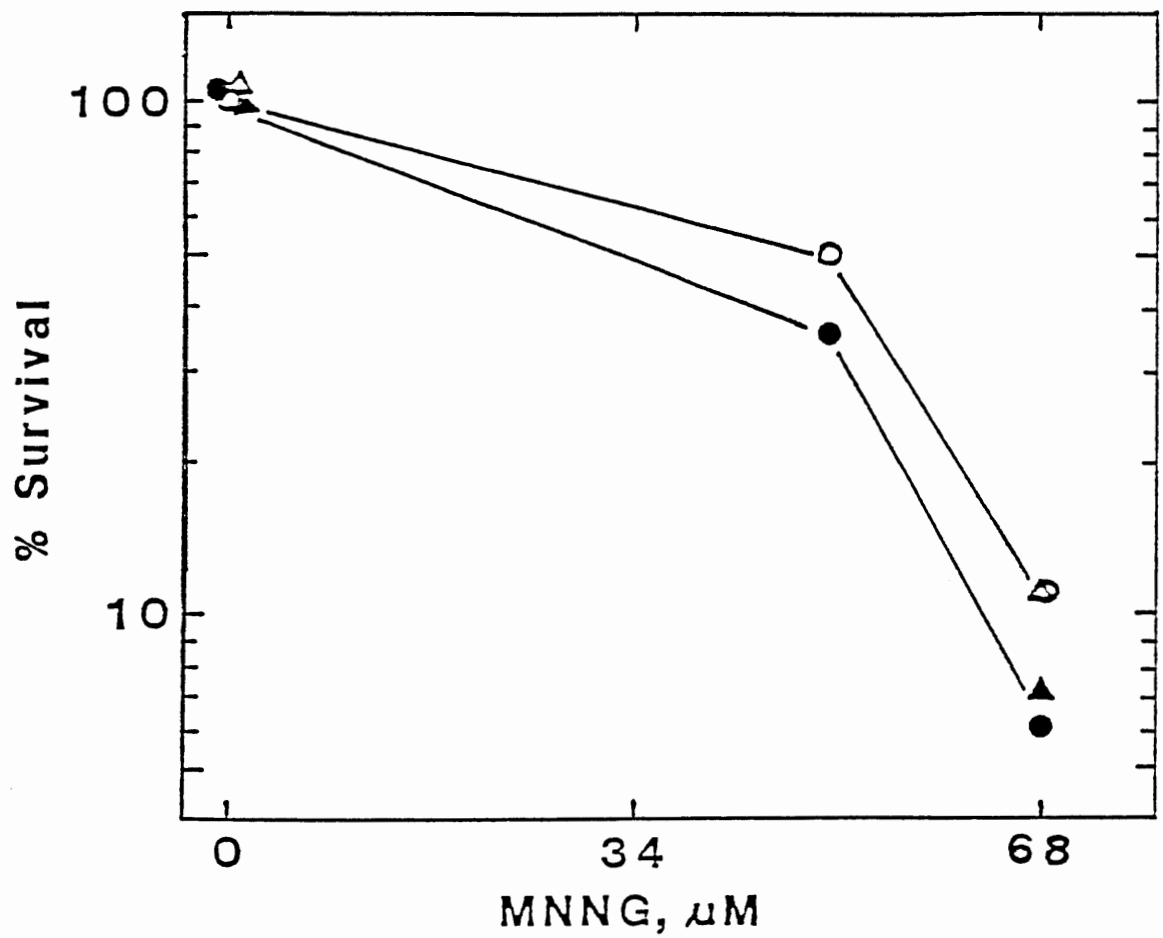
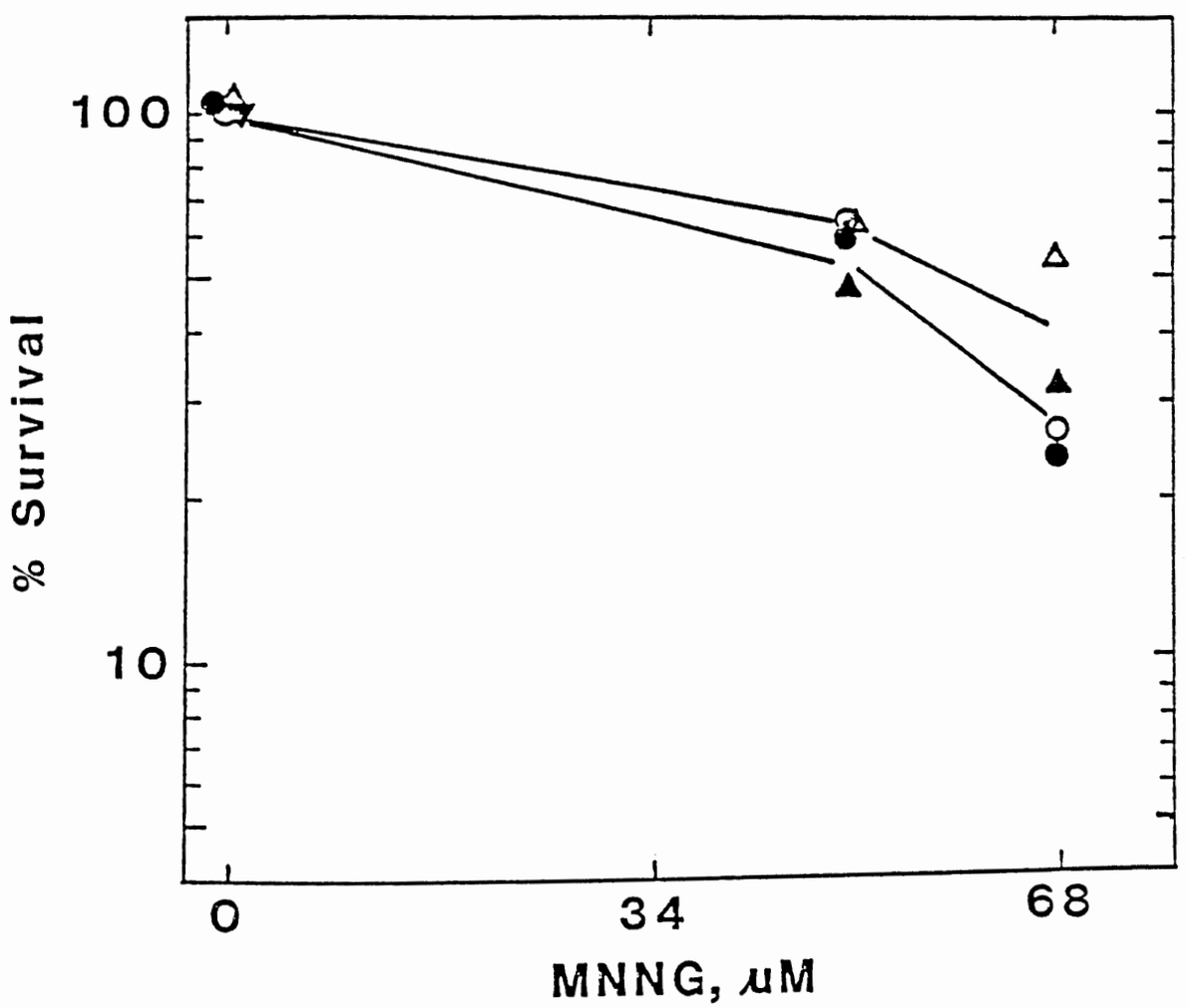

100
10
% Survival
0
34
68
MNNG, μM

Figure 9. Colony forming ability of non-dividing cells following MNNG and MBA treatment after 4 and 5 days of expression. Cells were treated at postconfluence with medium containing 0, 51 and 68 μM MNNG in the absence (open symbols) and presence (closed symbols) of 1 mM MBA for 20 min. Treatment medium was removed and medium containing 1 mM MBA was replenished for 48 hr. Cells were reseeded at 1 X 10^5 cells/100 mm dish in control medium. After 4 and 5 days of expression, cells were harvested and seeded for colony formation assays. Percent survival was calculated as the percentage of control colony forming ability. Experiment 1, 4 days of expression (○,●), Experiment 2, 5 days of expression (△,▲).


100
10
% Survival
0
34
68
MNNG, μM

MBA appears the same as that of MNNG-treated cells. This is in contrast to survival rates in experiments employing exponentially dividing cells. Cells treated with MNNG in the presence of MBA show a 3-fold decrease in survival compared to those in the absence of MBA. In Figure 8, 10% survival was observed when determined after 1 day of expression. Figure 9 shows that survival was above 20% when determined after 4 and 5 days of expression. Mutation frequency was calculated after MNNG treatment followed by 1 day of expression. Data from 2 experiments are shown in Table V. The results from both experiments showed an increased mutation frequency of MNNG-treated cells in the presence of MBA. Increases in cell number during treatment and expression were simultaneously followed. Treated cell populations from 2 experiments showed a minimum of one population doubling as shown in Tables VI and VII. Also, Tables VI and VII show the results obtained from mutation induction after MNNG treatment followed by 4 and 5 days of expression, respectively. These data again showed that the inhibitor, MBA, increased mutation frequency 1.7-to 1.2-fold. The effect of MBA on mutation frequency of MNNG-treated, confluent cells is summarized in Table VIII.

Although, the expression period varied from 1 to 5 days in these 4 separate experiments, the effect of MBA on mutation frequency in non-dividing cells following MNNG treatment was very similar. Graphic comparisons of mutation frequencies in non-dividing cells after different treatments with 1 and 4 or 5 days of expression are shown in

Table V.

Mutation induction after MNNG treatment in non-dividing cells followed by 1 day of expression

Treatment	Surviving fraction	Number of Oua^r colonies	Mutation frequency
Experiment 1			
Control	0.18	0	0
MBA	0.17	0	0
51 μM MNNG	0.09	45	500
51 μM MNNG + MBA	0.06	32	533
68 μM MNNG	0.02	16	800
68 μM MNNG + MBA	0.01	22	2200
Experiment 2			
Control	0.26	0	0
MBA	0.27	0	0
68 μM MNNG	0.03	28	933
68 μM MNNG + MBA	0.02	26	1300

Mutation induction after MNNG treatment in non-dividing cells followed by one day of expression. Cells were treated at postconfluence with 0, 51, 68 μM MNNG in experiment 1 and 0 or 68 μM MNNG in experiment 2 for 20 min in the absence and presence of 1 mM MBA. Treatment medium was removed and medium containing 1 mM MBA was replenished for 48 hr. Cells were reseeded at 1×10^5 cells/100 mm dish for expression. After 24 hr, cells were harvested and seeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined.

Table VI.

Mutation induction after MNNG treatment on day 4 of expression in non-dividing cells

Treatment	Population doublings	Surviving fraction	Number of Oua^r colonies	Mutation frequency
Control	2.2	0.19	0	0
MBA	2.2	0.17	0	0
51 μM MNNG	1.5	0.12	72	600
51 μM MNNG + MBA	1.3	0.10	ND[a]	ND[a]
68 μM MNNG	2.5	0.05	60	1200
68 μM MNNG + MBA	3.0	0.04	83	2075

[a] not determined.

Mutation induction after MNNG treatment in non-dividing cells followed by 4 days of expression. Cells at postconfluence were treated with 0, 51 or 68 μM MNNG in the absence and presence of 1 mM MBA for 20 min. Treatment medium was aspirated and medium containing 1 mM MBA was replaced. After 48 hr, cells were reseeded at 3×10^4 cells/35 mm dish and 1×10^5 cells/100 mm dish in control medium. After 4 days of expression, cells reseeded in 100 mm dish were harvested and seeded for colony formation and mutagenesis assays. Population doublings were determined from cells reseeded in 35 mm dishes. Surviving fraction and mutation frequency were determined.

Table VII.

Mutation after MNNG treatment on day 5
of expression in non-dividing cells

Treatment	Population doublings	Surviving fraction	Number of Oua^r colonies	Mutation frequency
Control	3.5	0.22	0	0
MBA	4.1	0.19	0	0
51 μM MNNG	3.2	0.14	41	293
51 μM MNNG + MBA	3.0	0.09	32	356
68 μM MNNG	1.9	0.12	63	525
68 μM MNNG + MBA	3.0	0.06	37	617

Mutation induction after MNNG treatment in non-dividing cells followed by 5 days of expression. Cells at postconfluence were treated with 0, 51 or 68 μM MNNG in the absence and presence of 1 mM MBA for 20 min. Treatment medium was aspirated and medium containing 1 mM MBA was replaced for 48 hr. Cells were reseeded in control medium for deterimnation of population doublings and expression. After 5 days, cells were harvested and seeded for colony formation and mutagenesis assays. Population doublings were determined during expression. Surviving fraction and mutation frequency were also determined.

Table VIII.

Summary of mutation frequencies determined from experiments employing MBA in non-dividing cells following MNNG

Experiment	MNNG (μM)	Days of expression	Fold increase in MBA
1	68	1	2.8
2	68	1	1.4
3	68	4	1.7
4	68	5	1.2

Summary of the effect of MBA on mutation frequency in non-dividing cells following MNNG. Data from Tables V, VI, VII were used to calculate fold increases in mutation frequency.

Figures 10 and 11, respectively.

Effect of nicotinamide-depletion on mutation frequency in exponentially dividing cells following MNNG treatment

Since the poly(ADP-ribose) polymerase inhibitor, MBA, has not yet been shown to be a specific inhibitor, an alternative approach to limiting poly(ADP-ribose) synthesis was introduced. Cellular NAD levels were depleted by growing cells in nicotinamide-free medium. The growth rates of control (+Nam) and NAD-depleted cells (-Nam) from 3 separate experiments is shown in Figure 12. No significant difference in growth was obtained between control and depleted cultures. The cells which were starved for 4 population doublings were reseeded for treatment. The NAD levels were determined by an enzymic cycling assay (Jacobson and Jacobson, 1976) at the time of reseeding. The NAD lowering during growth in depleted medium was calculated by comparing the NAD content per 10^6 cells in the depleted cells to the NAD content per 10^6 cells in the control cells. Data from 3 experiments are shown in Table IX. NAD levels were lowered to about 30% of control. Figure 13 shows the survival rates of exponentially dividing, NAD-depleted and control cells treated with 1.7 and 3.4 μM MMNG. The results show that NAD-depleted, exponentially dividing cells are less damaged relative to control cells. In exponentially dividing cells, survival of NAD-depleted cells treated with 3.4 μM MNNG showed a survival rate of 80% of control whereas only 9-15% survival was observed from experiments employing MBA to inhibit poly(ADP-ribose) synthesis. Therefore, it was essential that a dose of

Figure 10. Graphic comparison of mutation frequency in non-dividing cells after 1 day of expression. Cells at postconfluence were treated with 0, 51, 68 μM MNNG in the absence (open symbols) and presence (closed symbols) of 1 mM MBA for 20 min. Medium containing 1 mM MBA was replaced for 48 hr. Cells were reseeded in control medium. After 1 day of expression, cells were seeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined. Data are plotted as mutations/10^6 survivors. Experiment 1 (○,●), Experiment 2 (△,▲).

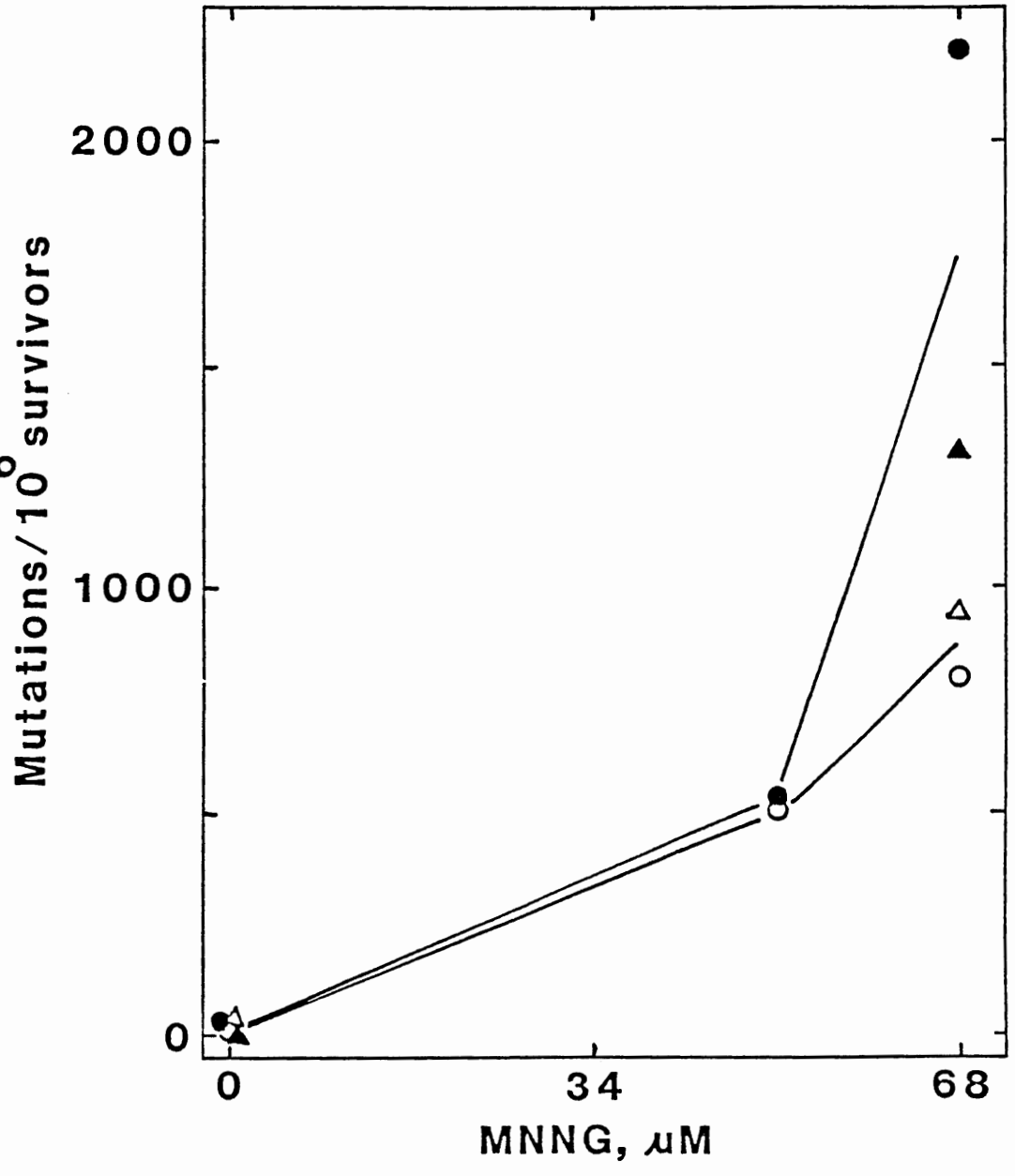

2000
1000
0
Mutations/10^6 survivors
0
34
68
MNNG, μM

Figure 11. Graphic comparison of mutation frequency in non-dividing cells after 4 or 5 days of expression. Cells at postconfluence were treated with 0, 51 or 68 μM MNNG in the absence (open symbols) and presence (closed symbols) of 1 mM MBA for 20 min. Medium containing 1 mM MBA was replaced for 48 hr. Cells were reseeded in complete medium for expression. After 4 or 5 days, cells were seeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined. Data are plotted as mutations/10^6 survivors. Experiment employing 4 days of expression (○,●), Experiment employing 5 days of expression (△,▲).

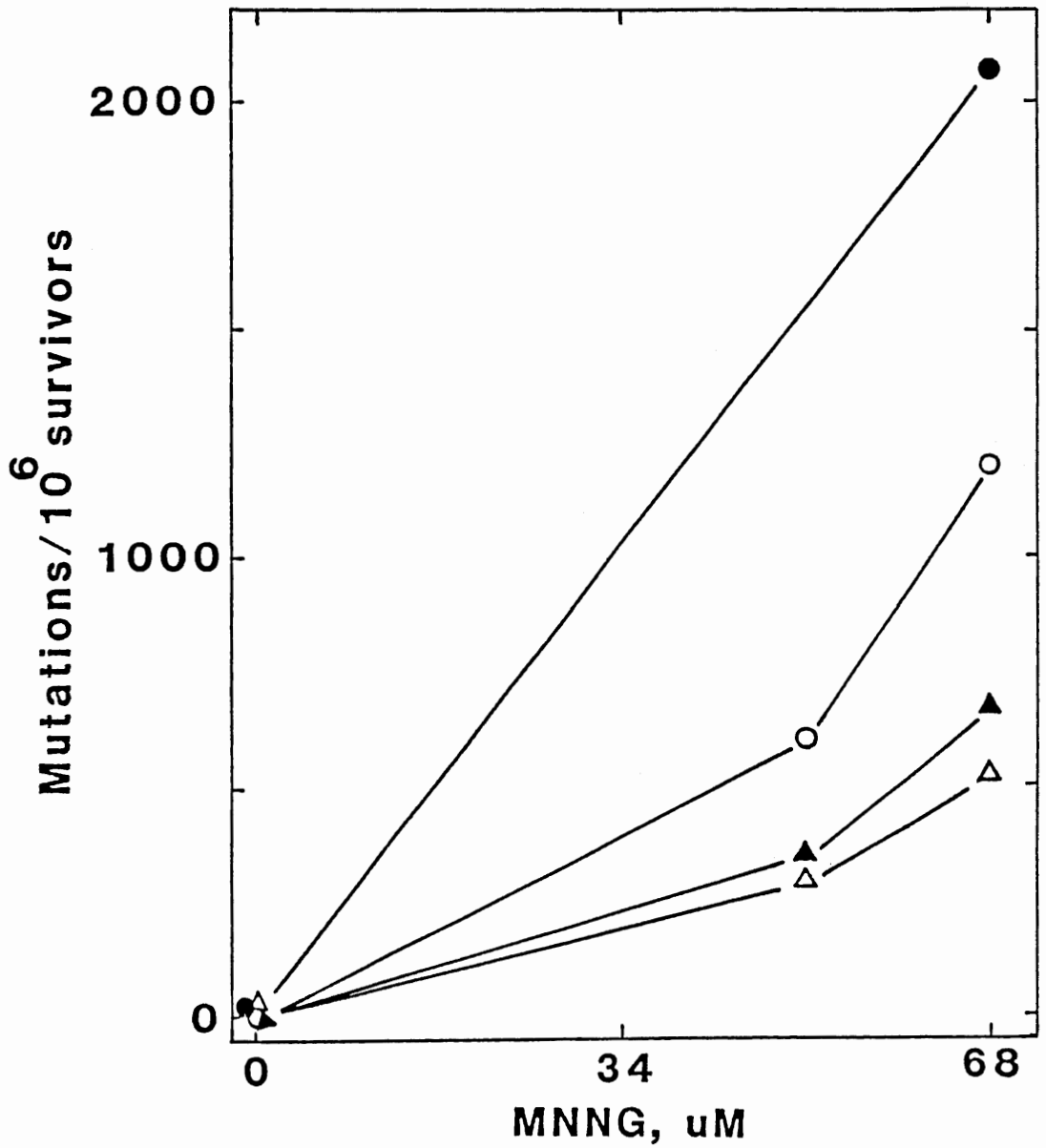

2000
1000
0
Mutations/10^6 survivors
0
34
68
MNNG, uM

Figure 12. Effect of nicotinamide-free medium on cell growth in C3H10T1/2 cells. Cells were seeded at 5 x 10^4 cells/60 mm dish. After 24 hr, complete medium was removed, and SDME + 10% DFCS with nicotinamide (open symbols) or without nicotinamide (closed symbols) was added. Experiment 1 (○,●), Experiment 2 (△,▲), Experiment 3 (□,■).

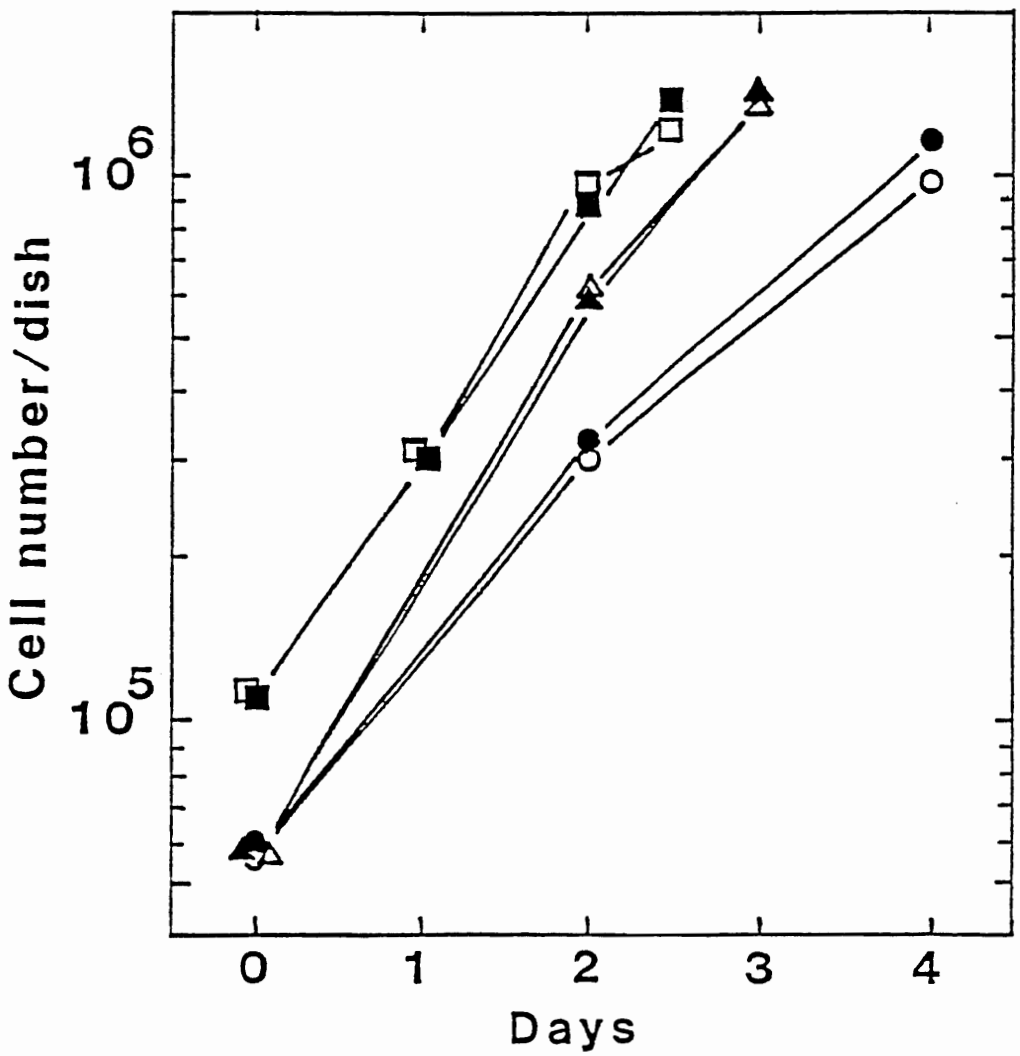

Cell number/dish
10^6
10^5
0
1
2
3
4
Days

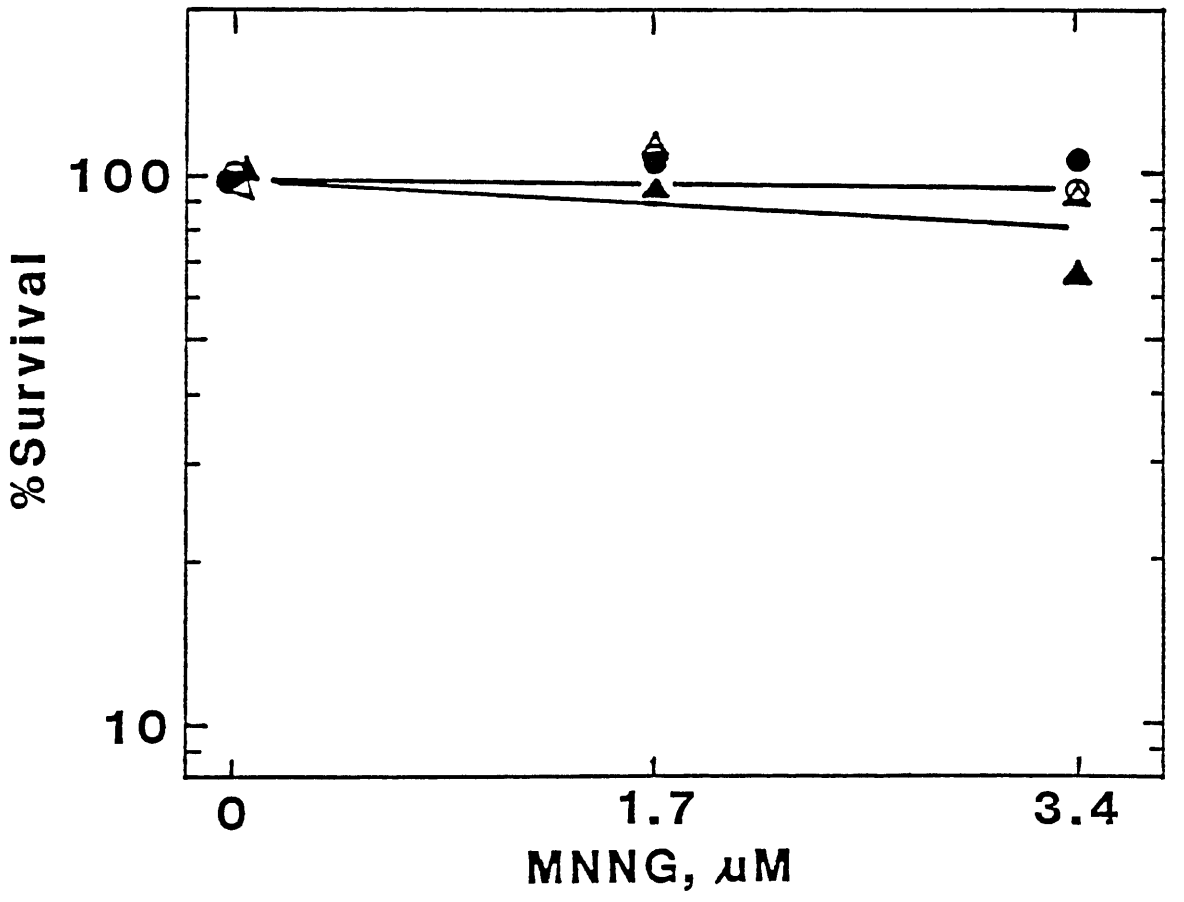
Table IX.

NAD levels in control and NAD-depleted cells

Experiment	NAD content (pmole/10^6 cells)		% of Control
	+Nam	-Nam	
1	943	249	26
2	1318	439	34
3	1206	367	31

Cells were grown in SDME containing 10% DFCS with nicotinamide (+Nam) and without nicotinamide (-Nam). After 4-5 population doublings, cell numbers/dish at each condition were determined. Simultaneously, cells were extracted and NAD contents were measured by an enzyme cycling assay (Jacobson and Jacobson, 1976). Data are expressed as picomoles of NAD and NADH per 10^6 cells and NAD content in -Nam cells was expressed as the percentage of NAD content in +Nam cells.

Figure 13. Survival of NAD-depleted, exponentially dividing cells following a low dose of MNNG. Cells were grown for 3-4 population doublings in SDME containing 10% DFCS with or without Nam. Cells were reseeded at 1 x 10^5 cells/100 mm dish and 24 hr later treated for 48 hr in the medium containing 0, 1.7 and 3.4 μM MNNG dissolved in acetone with Nam (open symbols) and without Nam (closed symbols). DME containing 10% FCS was added for an additional 24 and 48 hr. After the end of each expression time, cells were seeded for colony formation assays. Percent survival was calculated as the percentage of control colony forming ability. Experiment 1, 3 days of expression (○,●), Experiment 2, 4 days of expression (△,▲).


100
10
% Survival
0
1.7
3.4
MNNG, μM

MNNG be determined to allow optimal detection of mutants. In addition, determination of survival rates, the number of population doublings and expression time were investigated. Table X shows the effect of expression time on mutation frequency in NAD-depleted, exponentially dividing cells following MNNG. The control and NAD- depleted cells were reseeded and 24 hr later treated with MNNG at 1.7 and 3.4 μM for 48 hr. After 3 or 4 days of expression, survival and mutagenesis assays were conducted. The number of population doublings was also determined during treatment and expression. Mutation frequency was clearly detectable at either 3 or 4 days of expression. However, the data were not significant in these experiments utilizing 0 to 3.4 μM MNNG since a very low mutation frequency was detectable. In order to maintain the same relative enhancement of cytotoxicity as observed in experiments employing inhibitors of poly(ADP-ribose), an increased dose of MNNG was then used to induce mutations in NAD-depleted, dividing cells. Figure 14 shows the effect of nicotimamide starvation on survival in NAD-depleted, exponentially dividing cells following MNNG treatment. The dosage of MNNG up to 26.3 μM was selected for 48 hr treatment in control and NAD-depleted cells. An expression time of 4 days was selected to allow for a minimum of one population doubling. Survival and mutagenesis assays were conducted. Data from 3 experiments showed that the survival of NAD-depleted cells treated with 26.8 μM MNNG was decreased to 4% whereas survival observed from the control cells was above 10%. Differences in cytotoxicity between control and NAD-depleted

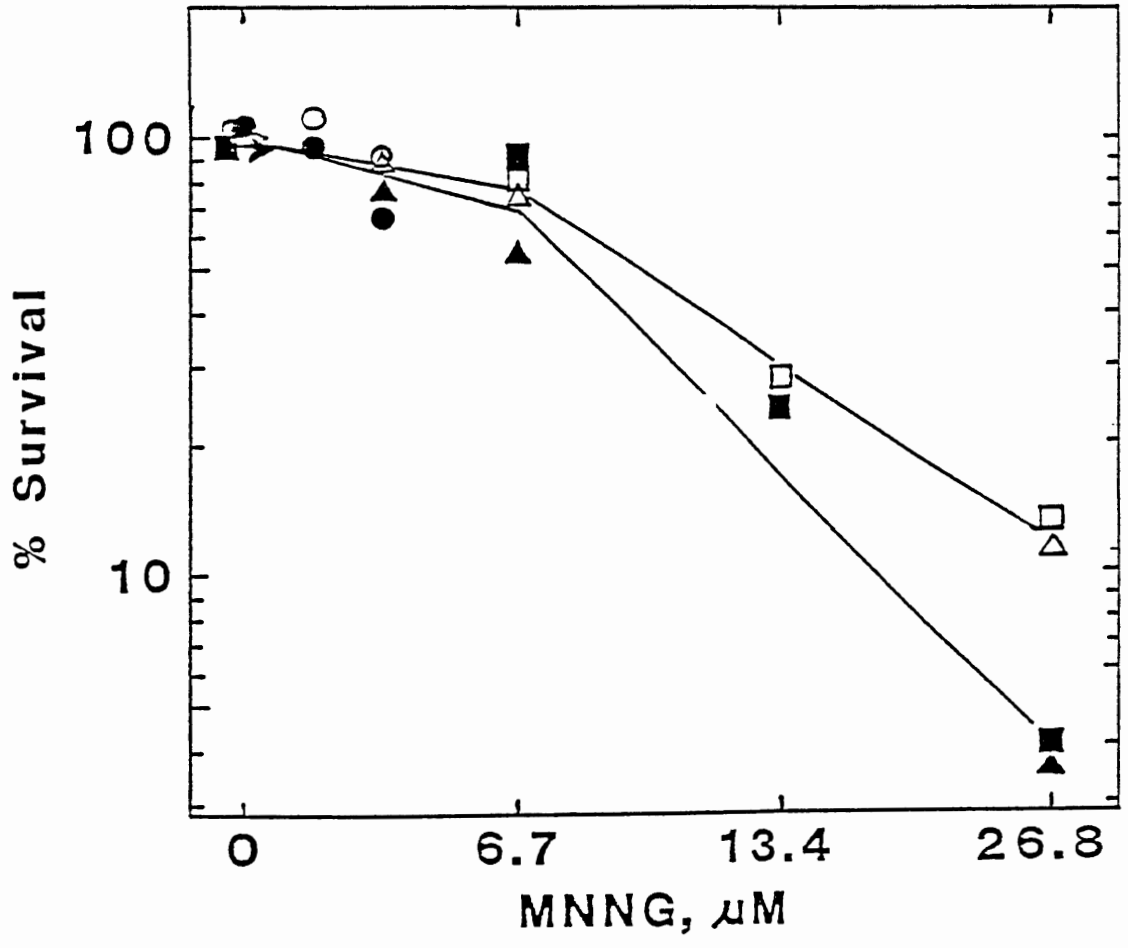
Table X.

Effect of expression time on mutation frequency in NAD-depleted, exponentially dividing cells following MNNG

Determination	Control cells			NAD-depleted Cells			Days of expression
	0 μM MNNG	1.7 μM MNNG	3.4 μM MNNG	0 μM MNNG	1.7 μM MNNG	3.4 μM MNNG	
Population	3.0	2.9	2.7	2.6	2.3	2.2	3
doublings	3.6	3.4	3.5	3.9	3.7	3.7	4
Surviving	0.31	0.33	0.29	0.33	0.34	0.39	3
Fraction	0.39	0.44	0.35	0.38	0.36	0.25	4
Number of	0	6	5	0	1	2	3
Oua^r colonies	0	1	2	0	0	5	4
Mutation	0	18	17	0	3	5	3
frequency	0	2	6	0	0	20	4

C3H10T1/2 cells were grown for 3-4 population doublings in the presence and absence of nicotinamide. Cells were reseeded at 1 x 10^5 cells/100 mm dish in the same medium and 24 hr later were treated for 48 hr with 0, 1.7 or 3.4 μM MNNG in acetone. Treatment medium was removed and DME containing 10% FCS was added. After 24 and 48 hr, cells were seeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined.

Figure 14. Survival of NAD-depleted, exponentially dividing cells following MNNG. Cells were grown for 3-4 generations in the presence and absence of Nam. Cells were seeded at 1 x 10^5 cells/100 mm dish and 24 hr later treated for 48 hr with 0, 1.7 or 3.4 μM MNNG in experiment 1; 0, 3.4, 6.7 or 26.8 μM MNNG in experiment 2; and 0, 6.7, 13.4 or 26.8 μM MNNG in experiment 3 in control (open symbols) and nicotinamide-free medium (closed symbols). Treatment medium was removed and DME containing 10% FCS was replaced. After 4 days of expression, cells was reseeded for colony formation assays. Percent survival was calculated as the percentage of control colony forming ability. Experiment 1 (○,●), Experiment 2 (△,▲), Experiment 3 (□,■).


100
10
% Survival
0
6.7
13.4
26.8
MNNG, μM

cells were observed when the dose of MNNG used was at least 6.7 μM. The number of population doublings were determined during treatment and expression for 2 experiments as shown in Table XI. Figure 15 shows the increase in cell numbers during treatment and expression. The results indicated that in experiments employing NAD-depletion, MNNG-treated cells exhibited a minimum of 1 population doubling on day 4 of expression. Analysis of the mutation frequencies in NAD-depleted cells treated with MNNG showed significant decreases in mutation as seen in Table XII. The graphic comparison of mutation frequency in response to MNNG concentration is plotted in Figure 16. The NAD-depleted cells treated with 3.4 and 26.8 μM MNNG showed 1.75-fold and 1.9-fold decreases in mutation frequency, respectively. The data are summarized in Table XIII. These results were obtained from 3 separate experiments which showed very similar mutation rates in response to MNNG. Therefore, utilizing both NAD-depletion and an inhibitor to block the synthesis of poly(ADP-ribose) results in decreases in mutation frequency.

Effect of nicotinamide-depletion on mutation frequency in non-dividing cells following MNNG treatment

Cells were grown for 4 population doublings in nicotinamide-free medium and held at confluence for 2 days. Before MNNG treatment, the levels of NAD in both control and NAD-depleted cells were determined. As shown in Table XIV, the NAD contents of depleted cells obtained from 2 experiments were 25 and 22% of control, respectively. Cells were

Table XI.
Population doublings during treatment and expression control and NAD depleted cells after day 4 of expression

MNNG (μM)	Number of population doublings +Nam	-Nam
Experiment 1		
0	4.1	4.9
1.7	3.9	4.7
3.4	4.0	4.7
Experiment 2		
0	3.7	3.7
3.4	3.4	3.5
6.7	2.8	2.6
26.8	1.1	1.0
Experiment 3		
0	4.5	4.7
6.7	4.1	4.0
13.4	3.3	2.8
26.8	1.5	0.9

Cells were grown in SDME containing 10% DFCS with or without Nam for 3-4 population doublings. Cells were seeded at 1 x 10^5 cells/100 mm dish in the same medium and 24 hr later were treated with 0, 3.4 or 6.7 μM MNNG in experiment 1; and 0, 6.7 or 26.8 μM MNNG in experiment 2; and 0, 6.7, 13.4 or 26.8 μM MNNG in experiment 3 for 48 hr. Treatment medium was removed and DME containing 10% FCS was replenished for an additional 48 hr. Population doublings at each condition were determined.

Figure 15. Increase in cell number during 48 hr treatment and 4 days of expression. Cells were grown for 3-4 generations in the presence and absence of Nam. Cells were seeded at 1 x 10^5 cells/100 mm dish and 24 hr later treated for 48 hr with 0(○), 6.7(△), 13.4(□) or 26.8(⬡) μM MNNG in the medium with Nam (open symbols) and without Nam (closed symbols). Treatment medium was removed and DME containing 10% FCS was replenished for 48 hr. Data are obtained from the mean of two experiments.

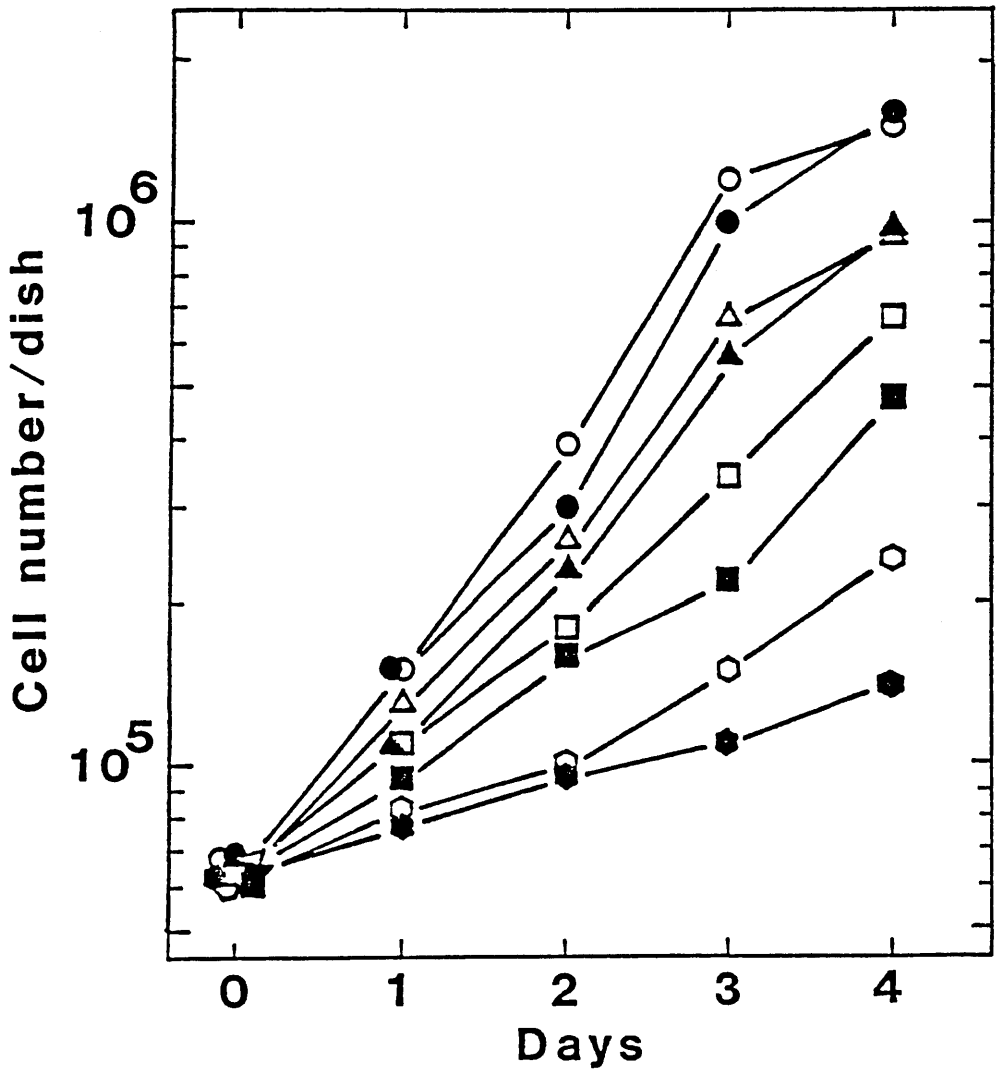

Cell number/dish
10^6
10^5
0
1
2
3
4
Days

Table XII.

Effect of NAD-depletion on mutation frequency in exponentially dividing cells following MNNG treatment

MNNG (μM)	Surviving fraction	Number of Oua^r colonies	Mutation frequency
Experiment 1			
Control cells			
0	0.39	0	0
1.7	0.44	6	14
3.4	0.35	5	14
NAD-depleted cells			
0	0.38	0	0
1.7	0.36	1	3
3.4	0.25	2	8
Experiment 2			
Control cells			
0	0.49	0	0
3.4	0.43	18	42
6.7	0.36	85	236
26.8	0.06	52	867
NAD-depleted cells			
0	0.56	0	0
3.4	0.42	13	31
6.7	0.31	37	119
26.8	0.02	9	450

Table XII. continued

Experiment 3				
Control cells				
0	0.39	0		0
6.7	0.31	42		135
13.4	0.11	54		491
26.8	0.05	44	(43.7)	880
NAD-depleted cells				
0	0.33	0		0
6.7	0.35	17		49
13.4	0.09	23		256
26.8	0.02	7		350

After growing in the presence and absence of Nam for 3-4 generations, control and NAD-depleted cells were seeded at 1 x 10^5 cells/100 mm dish. After 24 hr, cells were treated in the same medium with 0, 1.7 or 3.4 μM MNNG in experiment 1; 0, 3.4, 6.7 or 26.8 μM MNNG in experiment 2; and 0, 6.7, 13.4 or 26.8 μM MNNG in experiment 3. After 48 hr treatment, medium was removed and DME containing 10% FCS was added. After 48 hr, cells were reseeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined.

Figure 16. Graphic comparison of mutations in NAD-depleted, exponentially dividing cells following MNNG after 4 days of expression. Cells were grown for 3-4 generations in control or nicotinamide-free medium. Cells were seeded and 24 hr later treated with medium containing 0, 1.7 or 3.4 μM MNNG in experiment 1; 0, 3.4, 6.7 or 26.8 μM MNNG in experiment 2; and 0, 6.7, 13.4 or 26.8 μM MNNG in experiment 3 in the presence (open symbols) and absence (closed symbols) of Nam. After 48 hr, DME containing 10% FCS was replaced. After an additional 48 hr, cells were reseeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined. Data are plotted as mutations/ 10^6 survivors. Experiment 1 (○,●), Experiment 2 (△,▲), Experiment 3 (□,■).

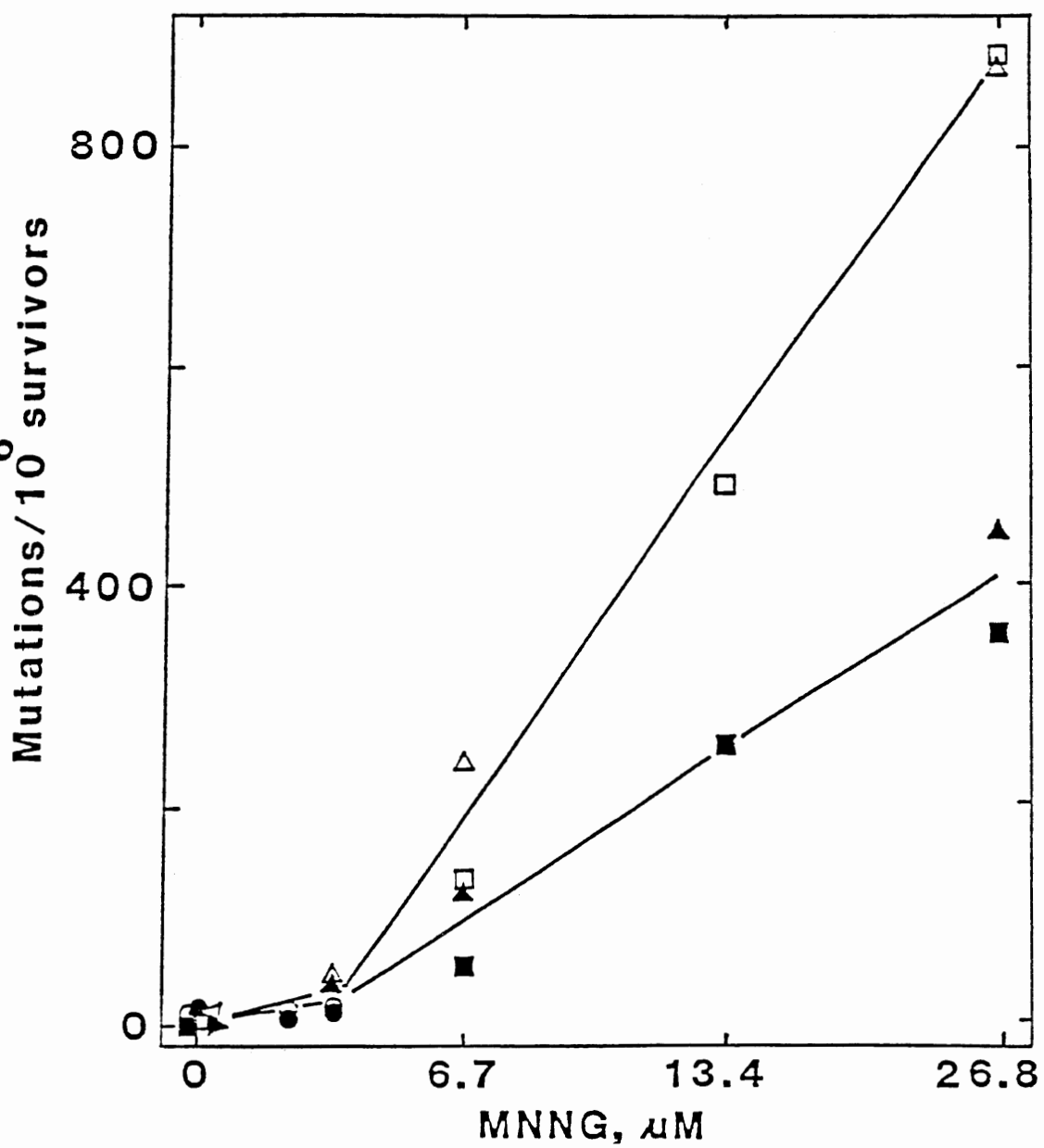

800
400
0
Mutations/10^6 survivors
0
6.7
13.4
26.8
MNNG, μM

Table XIII.

Summary of mutation frequency from experiments employing NAD-depletion in exponentially dividing cells following MNNG

Experiment	MNNG (μM)	Days of expression	Fold decrease in mutation frequency
1	3.4	3	1.75
2	26.8	4	1.92
3	26.8	4	2.45

[a] fold decrease in mutation frequency in NAD-depleted cells relative to control cells.
Data from Table XII were used to calculate the fold decreases in mutation frequency.

Table XIV.

NAD levels in control and NAD-depleted cells at confluence

Experiment	NAD content(pmole/10^6 cells) +Nam	-Nam	% of Control
1	3600	930	25
2	1032	224	22

Cells were grown in SDME containing 10% DFCS in the presence and absence of nicotinamde. At postconfluence, cell numbers/dish at each condition were determined. Simultaneously, cells were extracted and NAD contents were measured by an enzyme cycling assay (Jacobson and Jacobson, 1976). Data are expressed as picomoles per 10^6 cells and NAD content in -Nam was expressed as the percentage of NAD content in +Nam.

treated with 51 to 68 μM MNNG for 20 min. Figure 17 shows the increase in cell numbers of NAD-depleted cells treated with MNNG. The growth rates of control and MNNG-treated cells appeared the same. The number of population doublings was determined by measuring the increase in cell numbers during treatment and expression. The data are shown in Table XV. The results showed that at day 3 of expression, at least one population doubling was observed at each treatment. When the effects of MNNG concentration, an expression time and number of population doublings were available, experiments employing NAD-depletion in non-dividing cells were begun using MNNG with concentrations up to 68 μM for 20 min treatment. The medium containing MNNG was removed and media with or without nicotinamide were replenished. After 48 hr, treated cells were reseeded in control medium (+Nam). The cells were allowed to divide. Survival and mutagenesis were then assayed after 3 days of expression. The rates of survival in control and NAD-depleted, non-dividing cells after MNNG treatment appeared the same. The data are shown in Figure 18. No significant enhancement in cytotoxicity of MNNG was observed in NAD-depleted, non-dividing cells. The mutation frequencies of NAD-depleted, non-dividing cells treated with MNNG showed mutation rates similar to those of the control cells. The data are shown in Table XVI. The graphic comparisons of the effect of nicotinamide-depletion on mutation rates in non-dividing cells following MNNG are shown in Figure 19. In one experiment, the control and NAD-depleted, non-dividing cells were treated with MNNG at

Figure 17. Increase in cell number during expression following treatment of NAD-depleted, non-dividing cells. Control or NAD-depleted cells at postconfluence were treated with 0 (○), 51 (△) or 68 (□) μM MNNG for 20 min. Closed symbols indicate NAD-depleted cells which were treated with MNNG. Control or nicotinamide-free medium was added for 48 hr. After treatment, cells were reseeded in DME containing 10% FCS for an additional 48 hr.

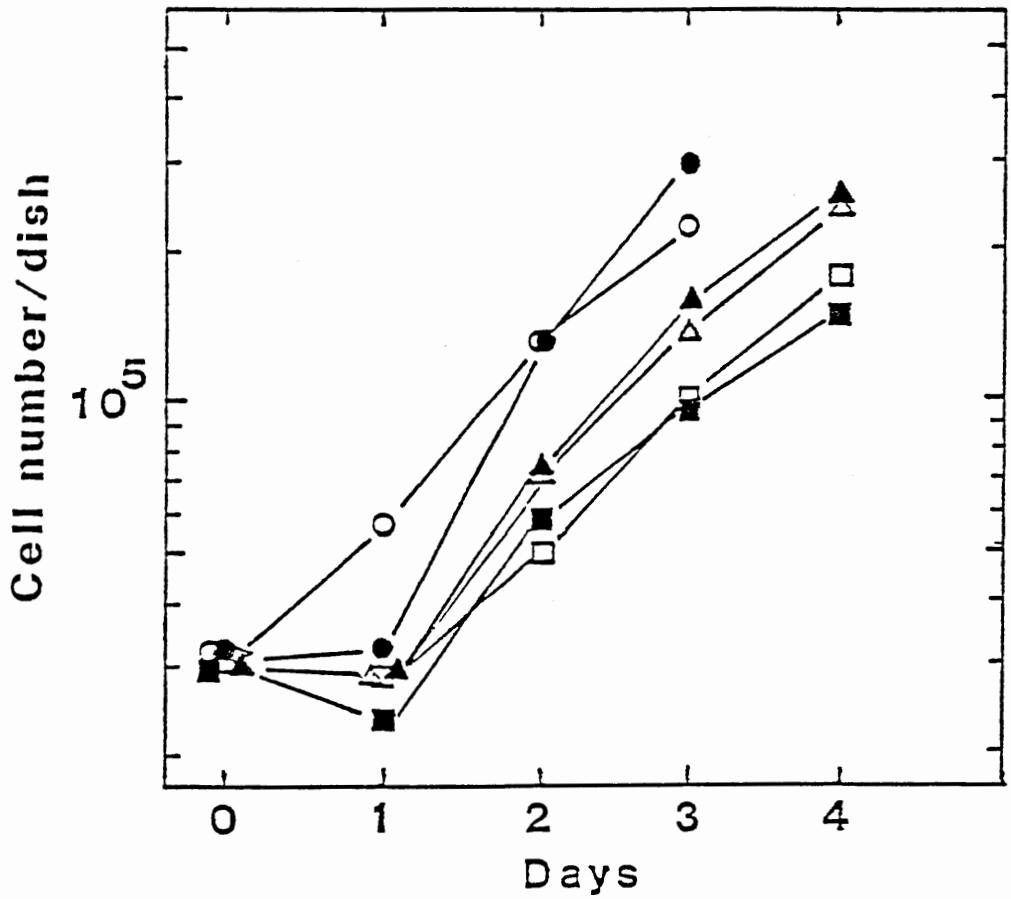

Cell number/dish
10^5
0
1
2
3
4
Days

Table XV.

Population doublings during treatment and expression in control and NAD-depleted cells

MNNG (μM)	Number of population doubling	
	+Nam	-Nam
0	2.8	3.2
51	2.1	2.2
68	1.6	1.6

Cells were grown to confluence in the presence or absence of Nam and treated with 0, 51 or 68 μM MNNG for 20 min. Control or nicotinamide-free medium was added for 48 hr. Cells were reseeded in DME containing 10% FCS for 3 days of expression. Population doublings at each condition were determined.

Figure 18. Colony forming ability of NAD-depleted, non-dividing cells following MNNG. Control or NAD-depleted cells were treated for 20 min at postconfluence with medium containing 0, 20.1 or 33.5 μM MNNG in experiment 1; and 0, 51 or 68 μM MNNG in experiment 2 in the presence (open symbols) and absence (closed symbols) of Nam. Control or nicotinamide-free medium was added for 48 hr. Cells were reseeded at 1×10^5 cells/100 mm dish in DME containing 10% FCS. After 3-7 days of expression, cells were harvested and seeded for colony formation assays. Percent survival was calculated as the percentage of control colony forming ability. Experiment 1, 5-7 days of expression (○,●), Experiment 2, 3 days of expression (△,▲).

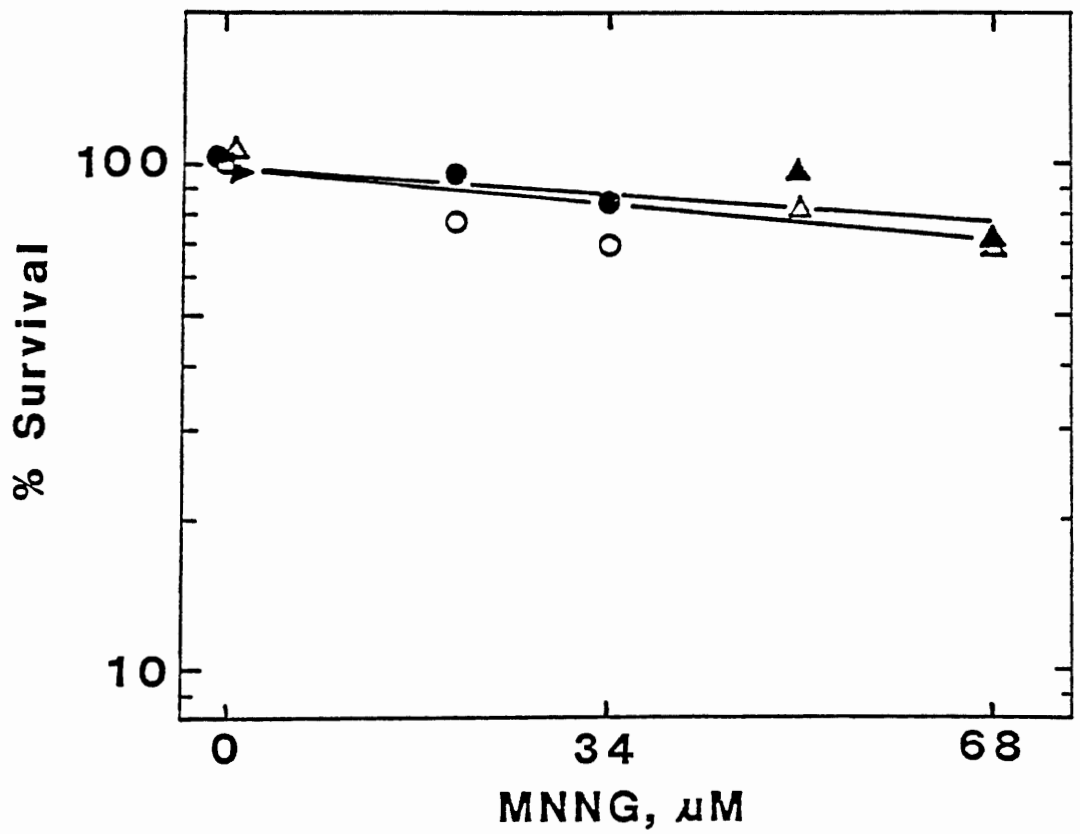

100
10
% Survival
0
34
68
MNNG, μM

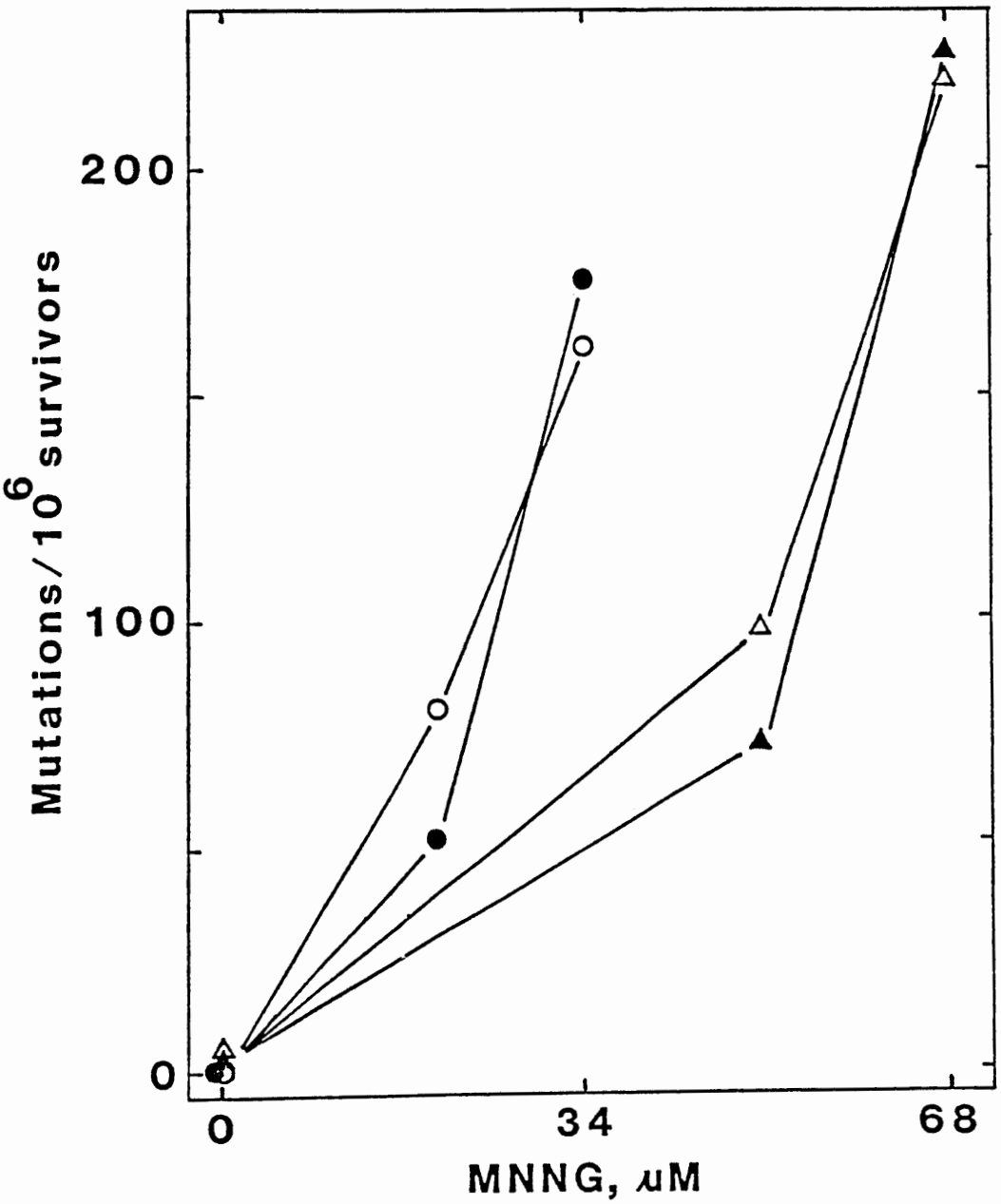
Table XVI.

Effect of NAD-depletion on mutation frequency in non-dividing cells following MNNG treatment

MNNG (μM)	Surviving fraction	Number of Oua^r colonies	Mutation frequency	Days of expression
Experiment 1				
Control cells				
0	0.26	0	0	5
20.1	0.20	16	80	5
33.5	0.18	29	161	7
NAD-depleted cells				
0	0.24	0	0	5
20.1	0.23	12	52	5
33.5	0.20	35	175	7
Experiment 2				
Control cells				
0	0.42	2	5	3
51	0.35	34	97	3
68	0.28	61	218	3
NAD-depleted cells				
0	0.35	1	3	3
51	0.36	26	72	3
68	0.25	56	224	3

Control or NAD-depleted cells were treated at postconfluence with 0, 20.1 or 33.5 μM MNNG in experiment 1; and 0, 51 or 68 μM MNNG in experiment 2 for 20 min in the presence and absence of Nam. Control and nicotinamide-free medium was returned for 48 hr. Cells were reseeded at 1×10^5 cells/100 mm dish for expression. After 3-7 days, cells were harvested and seeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined.

Figure 19. Graphic comparison of mutation frequency in NAD-depleted, non-dividing cells following MNNG. Control or NAD-depleted cells at postconfluence were treated with 0, 20.1 or 33.5 μM MNNG in experiment 1; and 0, 51 or 68 μM MNNG in experiment 2 for 20 min in the presence (open symbols) and absence (closed symbols) of Nam. Control or nicotinamide-free medium was returned for 48 hr. Cells were reseeded in DME containing 10% FCS at 1 x 10^5 cells/100 mm dish. After expression, cells were seeded for colony formation and mutagenesis assays. Surviving fraction and mutation frequency were determined. Data are plotted as mutations/10^6 survivors. EXperiment 1, 5 and 7 days of expression (○,●), Experiment 2, 3 days of expression (△,▲).


200
100
0
Mutations/10^6 survivors
0
34
68
MNNG, uM

concentrations of 20.1 and 33.5 μM. The mutation frequency was determined after these cells were allowed to divide for 5-7 days as indicated in Table XVI. In the other experiments, 51 and 68 M MNNG concentrations were used in treatments and the mutation frequency was assayed after 3 days of expression. However, very similar mutation rates were observed in both experiments. The effect of MBA on mutagenesis resulted in an increased mutation rate in non-dividing cells following MNNG whereas NAD-depletion did not seem to cause any further effect on mutagenesis.

DISCUSSION

The major purpose of this study was to investigate the influence of poly(ADP-ribose) metabolism in mutagenesis. Although the biological role for poly(ADP-ribose) has not been fully established, it has been implicated in an array of cellular events. Accumulated evidence indicates that it may involve changes in the structure of chromatin.

Several experiments have led to suggestions that changes at the chromosomal level, such as DNA repair, chromosomal aberrations, transformation, sister chromatid exchanges and mutagenesis, may involve the metabolism of poly(ADP-ribose). These suggestions have largely come from studies of the biological consequences of inhibiting poly(ADP-ribose) synthesis following DNA damage. An enhancement of cytotoxicity and a cell cycle delay have been observed in mammalian cells following DNA damage in the absence of poly(ADP-ribose) synthesis (Jacobson et al., 1985). Additionally, a number of observations have shown that the frequency of chromosomal aberrations, sister chromatid exchanges and malignant transformation increase when synthesis of poly(ADP-ribose) is blocked in cells treated with DNA damaging agents (Lubet et al., 1985; Zwanenberg and Natarajan, 1985; Smith, 1985). Therefore, it was of interest to investigate the effect of altered poly(ADP-ribose) metabolism on mutagenesis.

C3H10T1/2 cells were used as a model system during the course of this work. Mutation of the cells was induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a potent alkylating agent known to induce mutation primarily by methylation of the O^6 position of guanine (Smith and Grisham, 1983). The alteration of poly(ADP-ribose) metabolism was accomplished by inhibiting poly(ADP-ribose) polymerase with MBA, or by nutritionally depleting the substrate of poly(ADP-ribose), NAD. The mutagenic events were detected at the Na^+/K^+ ATPase locus, measured by ouabain resistance. Oua^r mutations at this locus are known to result from point mutations (Baker et al., 1974).

In the present study, MBA caused a decrease in mutation frequency, when experiments were conducted in exponentially dividing cells following MNNG treatment (Figures 6 and 7). A similar result was previously reported by Durrant et al. (1981) using 5-methylnicotinamide as a polymerase inhibitor. They found a decreased mutation frequency at the HGPRT locus in mouse L1210 cells exposed to N-methyl-N-nitrourea (MNU) in the absence of poly(ADP-ribose) synthesis. In addition, Ohnishi et al. (1982) reported that 3-aminobenzamide enhanced the cytotoxicity induced by MNNG and UV radiation and decreased the mutation frequency to methanol-resistance in amoeba. Recently, utilizing MNNG to induce mutations in V79 Chinese hamster cells, Bhattacharyya and Bhattacharjee (1983) observed that benzamide reduced mutation frequency at the HGPRT locus. More recently, Zwanenberg et al. (1985) reported that 3-AB did not increase the frequency of mutations induced by ethyl

methanesulphonate (EMS), N-ethyl-N-nitrosourea (ENU) and 5-chlorodeoxyuridine (CldUrd) in V79 cells at the HGPRT locus. Although high concentrations of benzamide (5-10 mM) and its analogues have been shown to inhibit purine biosynthesis (Cleaver, 1983; Borek et al., 1984) in addition to poly(ADP-ribose) synthesis, low concentrations (1 mM) of these compounds including MBA, do not show such non-specific side effects on metabolism. An additional approach which does not involve the use of inhibitors, the depletion of cellular NAD content, was also used in this study to limit poly(ADP-ribose) synthesis. Similar reductions in mutation frequency from either approach strongly suggested that the observed changes in mutation frequency were due to altered poly(ADP-ribose) metabolism. In contrast to these observations, Schwartz et al. (1985) reported an increased mutation frequency in CHO cells when the synthesis of poly(ADP-ribose) was inhibited by 3-AB following EMS or MNNG. They measured both deletion and point mutations. However,both 1 and 10 mM 3-AB increased deletion mutations at the HGPRT locus; point mutations at the Oua^r locus were unaffected.

The studies described above indicate that many factors, including inhibitors, inducers, cell types and selective agents for mutations may influence the results when attempting to assay the potential role of poly(ADP-ribose) in mutagenesis. It has been reported that monofunctional alkylating agents produce a number of different DNA adducts in CHO cells (Heflich et al., 1982; Beranek et al., 1983). Here, MNNG, a monofunctional alkylating agent which causes DNA lesions

of more than one type (Newhold et al., 1981) has been used in all experiments. These DNA lesions are removed by independent DNA repair mechanisms (Grisham and Smith, 1983). If MNNG causes several types of DNA damage, mutations might be expected to arise from the inadequate repair of any one type of MNNG induced lesion. Inhibitors of poly(ADP-ribose) polymerase might prevent the correct repair of such a lesion either directly or by an indirect effect. However, the selection system chosen may not be able to detect the lesion which is not properly repaired. In that event, no effect of poly(ADP-ribose) polymerase inhibitors would be detected. Therefore, the right combination of these factors is required in order to observe an effect on mutation frequency. N-hydroxy-2-aminofluorene was used to induce mutations in CHO cells (Heflich et al., 1985). This compound is known to cause only a single DNA lesion in CHO cells (Morris et al., 1984). Poly(ADP-ribose) polymerase inhibitor, benzamide (5 mM), enhanced the cytotoxicity of N-hydroxy-2-aminofluorene while showing very little effect on mutation at the HGPRT locus. Since the inducer caused only one type of DNA lesion and benzamide did not effect the removal of the lesion, a strong argument can be made for little effect of poly(ADP-ribose) metabolism (Helflich et al., 1985).

In conducting mutagenesis assays, it is necessary to study cell survival and mutagenesis simultaneously, since mutation frequency is calculated from the observed number of mutant colonies divided by the fraction forming colonies (Landolph and Heidelberger, 1979). In

addition, it is important that survival of cells after treatment be high enough (above 10%) to measure accurately mutation frequency (Newhold et al., 1981). Several lines of evidence have shown that inhibitors of polymerase increase cytotoxicity of DNA damaging agents and cause delays in cell cycle progression (Jacobson et al., 1985), therefore damaged cells in the presence of inhibitor require a longer time to complete the cell cycle.

In this study, slow cell growth was observed in MNNG-treated cells in the presence of MBA (Figure 4). The enhancement of cytotoxicity by MBA was observed (Figure 3) and shown to be dependent on MNNG dose. Since expression of mutagenic events is known to require DNA replication and cell division, it is essential that treated cells divide. The current study determined the optimal MNNG dose and time course for detection of mutation frequency. The results revealed that 3-4 days of expression (Figure 7) or 1-2 population doublings (Figure 7 and Table 1) gave the maximal rate of mutation at the Oua locus in experiments employing exponentially dividing cells. These data agreed with the observations of Landolph and Heidelberger (1979). This group reported that the induction of Oua^r mutations is maximal after approximately two population doublings or 3 to 4 days of expression. However, Schwartz et al. (1985) reported that induced mutation frequency at the Oua locus did not vary over an expression period of 2-8 days. Since their studies did not report the observed number of mutant colonies, survival and number of population doublings, it would be difficult to evaluate the

data on mutation rates. The current study also measured the number of population doublings as increases in cell number during treatment and expression by following growth curves. This method only measures the rate at which the entire population doubles which may not reflect on what portion of a given population is dividing. Taken together with the difficulties of co-cytotoxicity and cell cycle block in exponentially dividing cells, further investigation was then designed to use non-dividing cells. It has been reported that there is no co-cytotoxicity in non-dividing cells following MNNG treated in the presence of MBA for 48 hr (Jacobson et al., 1985). This approach eliminated variables due to differences in cell division rates. When conducting the mutagenesis assay in non-dividing cells, MNNG treatments of up to 60 μM, for 20 min, were used in the absence or presence of MBA for 48 hr. The effect of MBA resulted in a significant increase in mutation frequency (Figures 10 and 11). This is in contrast to the result obtained in exponentially dividing cells. The decreased mutation frequency observed in exponentially dividing cells may be due to the invalidity of the mutagenesis assay resulting from cell cycle delays.

Since many studies have shown the nonspecific effects of poly(ADP-ribose) polymerase inhibitors (Cleaver, 1983), this study also used NAD-depletion to alter poly(ADP-ribose) metabolism. The effect of NAD-depletion was a decreased mutation frequency in exponentially dividing cells following MNNG treatment (Figure 16). This observation was similar to the those obtained from experiments employing inhibitors

in exponentially dividing cells. Since there were similar results from both approaches, it could be argued that the altered mutation rates were due to limiting poly(ADP-ribose) metabolism. It might also be argued that the decreases observed here are an artifact of the approach and that it is not possible to assess mutation frequency in a cell that has sustained a type of damage that prevents cell division. Further investigation was conducted in NAD-depleted, non-dividing cells. NAD-depletion showed no effect on mutation frequency in non-dividing cells after MNNG treatment (Figure 19). This observation did not agree with that obtained using non-dividing cells employing MBA which showed a significant increase in mutation frequency. However, the NAD content of starved cells was only depleted to 22-25% of the control (Table XIV). With this incomplete inhibition of poly(ADP-ribose) polymerase, the activity of poly(ADP-ribose) metabolism is still present. It would be expected that an identical result of increased mutation frequency of non-dividing cells using both approaches of inhibition would be obtained if NAD-depletion was as effective in limiting poly(ADP-ribose) synthesis as is MBA.

Accumulated results from other studies and observation made in this investigation suggest that poly(ADP-ribose) may function in stabilizing chromatin structure following DNA damage, thus limiting events which lead to mutagenesis in cells which are non-dividing. Limiting of poly(ADP-ribose) synthesis following DNA damage in dividing cells is possibly a lethal event and precludes an accurate estimate of the effect

of inhibiting poly(ADP-ribose) synthesis on mutation frequency. Alternatively, these data could be interpreted to mean that poly(ADP-ribose) normally effects an SOS-like response and causes an increase in mutations which is not observed when poly(ADP-ribose) synthesis is limited. Due to the observation in non-dividing cells, the latter argument is less likely.

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