INFLUENCE OF NIACIN METABOLISM ON MALIGNANT TRANSFORMATION IN VITRO

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INTRODUCTION

The requirement for niacin in humans was first demonstrated in 1937. This finding was the result of many years of nutritional and biochemical studies. These studies ultimately led to the cure of the disease, pellagra (Fouts et al., 1937; Harris, 1937; Smith et al., 1937). The administration of niacin alleviated within 24-72 hr the diagnostic symptoms of pellagra. These symptoms include the redness and swelling of the mucous membranes, nausea, vomiting, diarrhea, mental confusion and active, reddish skin lesions, which are considered to be the most diagnostic symptom of the disease. The biochemical mechanism by which niacin alleviates these symptoms is not fully understood.

Much was known about the chemistry and biochemistry of niacin and its metabolism before its role in the prevention of pellagra was confirmed. Niacin, also known as nicotinic acid, was first chemically synthesized by Huber in 1867 and its identity was confirmed in 1870 (Huber, 1867; 1870). Engler derivatized the amide form, nicotinamide (Nam), in 1894 (Engler, 1894).

The conversion of nicotinic acid (Na) and tryptophan (Trp) from food to Nam occurs in the liver and kidney (Ikeda et al., 1965). In cells of other tissues, Nam is converted to nicotinamide mononucleotide (NMN) in the cytoplasm. NMN is converted to nicotinamide adenine dinucleotide (NAD) in the nucleus (Dietrich et al., 1966).

The regulation of cellular levels of NAD is not well understood. NAD levels are known to vary depending on the metabolic state of the cell. NAD levels are lower in tumors than normal cells (Jedeikin and Weinhouse, 1955; Glock and McLean, 1957; and Wintzerith et al., 1961) and in regenerating tissues than non-dividing cells (de Burgh, 1957; Ferris and Clark, 1971). During exponential growth of normal cells in culture, NAD levels fluctuate three-fold (Jacobson et al., 1974). Cells from patients with Fanconi's anemia, a disease characterized by defects in the ability to repair damaged cellular deoxyribonucleic acid (DNA), have lower than normal NAD^+ levels (Berger et al., 1982).

The coenzyme role of NAD in oxidative metabolism, specifically in the citric acid cycle, fatty acid oxidation, and glycolysis are well understood. The transfer of electrons from NADH formed in these processes

to O_2 is the major source of adenosine triphosphate (ATP) in aerobic organisms. NAD also serves as the substrate for nicotinamide adenine dinucleotide phosphate (NADP(H)) biosynthesis. This coenzyme serves an analogous role for transfer of electrons in biosynthetic reactions.

Besides its coenzyme role, NAD serves as a substrate in several classes of reactions in which the nicotinamide portion of the molecule is cleaved and adenosine diphosphate ribose (ADP-ribose) moieties are transferred to an acceptor. These ADP-ribosylation reactions are classified according to the nature of the acceptors and the number of ADP-ribose moieties attached.

The first of these reactions to be identified was that catalyzed by NAD glycohydrolase with water as an acceptor (Handler and Klein, 1942). The enzyme is also referred to as NAD nucleosidase. The enzyme is associated with the outer plasma membrane of cells (Muller and Schuber, 1980). Its function in mammalian cells is not well understood, although it is thought to serve a scavenger role in the mammalian gut (Kakehi et al., 1972).

The second class of ADP-ribosylation reactions involves the transfer of a single ADP-ribose unit to an acceptor protein. This reaction is catalyzed by

mono(ADP-ribosyl) transferases and is referred to as mono(ADP-ribosyl)ation. Various proteins have been shown to serve as acceptors. Numerous bacterial toxins ADP-ribosylate host proteins *in vitro*. The most well understood example of this was the *in vitro* mono(ADP-ribosyl)ation and resultant inactivation of elongation factor 2 (EF2), an enzyme of eucaryotic protein synthesis, by diphtheria toxin (Honjo et al., 1968). Other examples include the *Pseudomonas aeruginosa* toxin, that also modifies EF2 (Iglewski and Kabat, 1975), and cholera toxin (Cassal and Pfeuffer, 1978 and Gill and Meren, 1978), *E. coli* enterotoxin LT (Moss and Richardson, 1978) and islet-activating protein (Katada and Ui, 1982) that activate adenylate cyclase. Recently, the first demonstration of an *in vivo* self-modification in a bacteria by mono(ADP-ribosyl) transferase has been shown. Pope et al. have shown that the iron protein of nitrogenase in *Rhodospirillum rubrum* is inactivated by covalent modification by mono(ADP-ribosyl)ation of an arginyl residue (Pope et al., 1985).

In addition to their existence in bacterial cells, mono(ADP-ribosyl) transferases are also known to occur in the cytoplasm of eucaryotic cells. For example, the avian

erythrocyte ADP-ribosyltransferase has been shown to modify several erythrocyte proteins in vitro (Moss and Vaughan, 1978) and the hepatocyte ADP-ribosyl transferase modifies hepatocyte membrane proteins in vitro (Beckner and Blecher, 1981).

The third class of ADP-ribosylation reactions involves the transfer of successive ADP-ribose residues to protein and is referred to as poly(ADP-ribosylation) (Chambon et al., 1966; Hayaishi and Ueda, 1977; Sugimura, 1973). Poly(ADP-ribose) is synthesized in the nucleus of eucaryotic cells by the enzyme poly(ADP-ribose) polymerase, also known as poly(ADP-ribose) transferase or poly(ADP-ribose) synthetase (Hilz and Stone, 1976; Hayaishi and Ueda, 1977). The enzyme is stimulated by DNA strand breaks. The highest level of activation has been achieved by double stranded nicked DNA without terminal 5' phosphate groups at the flush-ends (Benjamin and Gill, 1979; Benjamin and Gill, 1980a).

The structure of poly(ADP-ribose) is shown in Figure 1. The linear ADP-ribose units are linked together in a 2',1" α ribose-ribose linkage (Miwa et al., 1977). The length of the linear chain has been estimated to contain up to 65 residues when measured by polyacrylamide

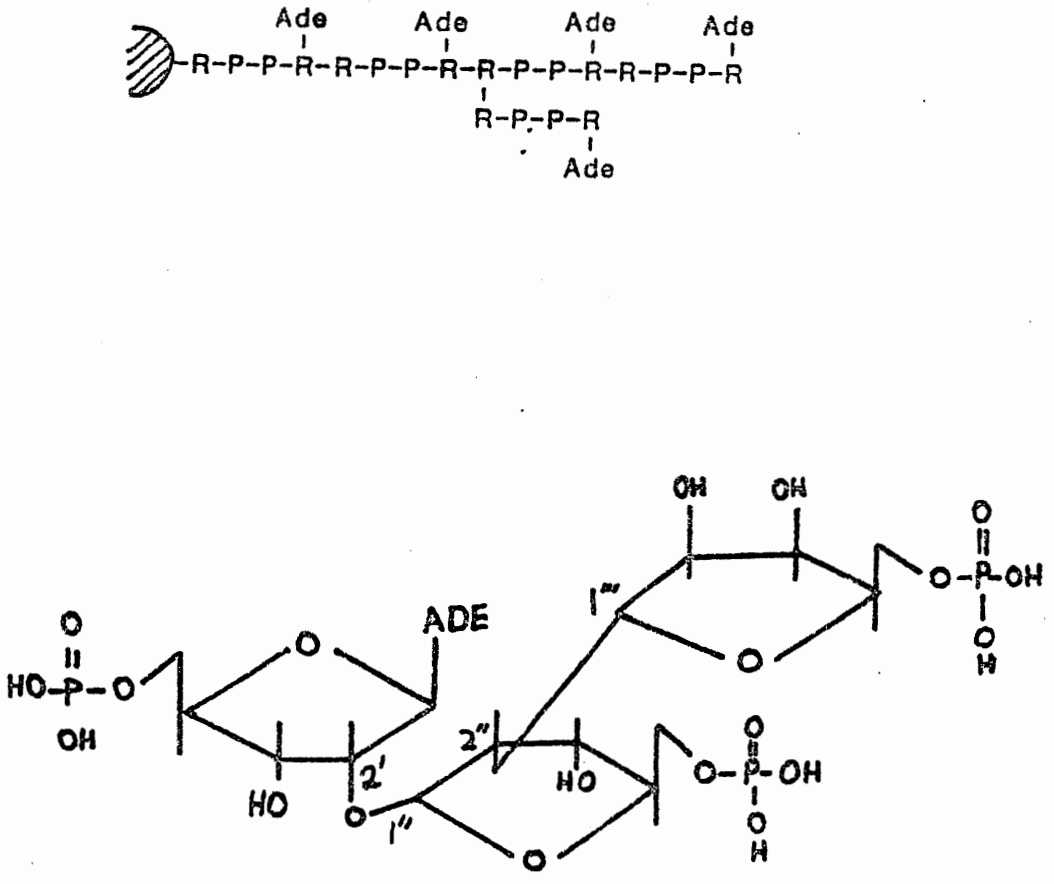


Figure 1. Structure of poly(ADP-ribose). Details of the branch point structure are shown in the lower drawing.

electrophoresis (Tanaka, 1978) and up to 300 residues when measured by electron microscopy (Hayaishi et al., 1983). Branching has also been found in the polymer (Miwa et al., 1979) and may occur as often as every 15 residues (Alvarez-Gonzalez and Jacobson, 1985). The resulting linkage is a 2",1'" ribose-ribose linkage (Miwa et al., 1979).

The proteins that serve as in vitro acceptors of the polymer include poly(ADP-ribose) polymerase (Yoshihara et al., 1977), histones (Nishizuka et al., 1968; Smith and Stocken, 1973), and nonhistone proteins (Jump et al., 1979). Histone H1 has been shown to be modified in vivo (Smith and Stocken, 1975; Ueda et al., 1975). However, very little is known regarding the nature of acceptors in vivo.

Poly(ADP-ribose) is linked to lysine and glutamic acid residues of proteins as shown by in vitro studies (Ueda and Hayaishi, 1984). Studies have also shown that these linkages are found in vivo (Alvarez-Gonzalez and Jacobson, 1985).

Proteins have been shown to be extensively modified by poly(ADP-ribose) in vitro. Poly(ADP-ribose) polymerase may have attached to it as many as 15 chains of poly(ADP-

ribose), each containing >80 ADP-ribose units (Kawaichi et al., 1981). Proteins may be even more highly modified *in vivo*. Recently, it was shown that cells treated with heat shock and mutagens produced polymers containing an average of 244 ADP-ribose units and 6.6 branch points (Alvarez-Gonzalez and Jacobson, 1985).

Polymer levels have been measured to better understand the function of poly(ADP-ribose). Several chemical methods have been developed that are briefly described. An early method employed labeling permeabilized cells with [ade-^{14}C]NAD and measuring radioactivity incorporated into the acid-insoluble pools (Levi et al., 1978). A problem with this method is that in the process of permeabilization, additional DNA strand breaks are made. A second method involved tritium adenosine labeling and purification of a tritium-labeled Ado-Rib derived from poly(ADP-ribose) by high performance liquid chromatography (Kanai et al., 1982). This method lacks specificity since many adenine containing compounds are labeled and may be measured in addition to poly(ADP-ribose). A more recent method which has proven to be specific and applicable to intact cells has been developed. This method involves use of an immobilized boronate resin that binds molecules containing

1,2 cis-diol groups. In a later step, fluorescent derivatives of adenine-containing compounds are made which are detected by HPLC (Sims et al., 1980). Alternatively, cells labeled with [^{3}H-adenine] of high specific activity are extracted and applied to the boronate resin. Radioactive label incorporated into poly(ADP-ribose) is measured by liquid scintillation counting (Jacobson, personal communication).

Quantitation of polymer levels has been difficult due to the rapid turnover of poly(ADP-ribose) in vivo. Studies using intact 3T3 mouse embryo fibroblasts treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Juarez-Salinas et al., 1979) and CF-3 cells treated with ultraviolet light (UV light) (Jacobson et al., 1983) have led to estimated turnover rates of less than one minute. Rapid turnover rates have been observed in vitro following X-rays (Benjamin and Gill 1980a, b), but the polymer is stable in many preparations of isolated nuclei and permeabilized cells (Berger et al., 1979; Levi et al., 1979). Rapid turnover rates measured in vivo indicate that the polymer is perhaps needed only transiently in any given location in damaged cells.

To elucidate the role(s) of poly(ADP-ribose)

metabolism in cellular processes, two major approaches have been taken. These approaches are necessary due to the fact that there are no known mutants of poly(ADP-ribose) polymerase, nor has the gene coding for the polymerase been cloned.

The approach taken by many laboratories to elucidate the role of poly(ADP-ribose) has been to use inhibitors of poly(ADP-ribose) polymerase to find differences that occur when poly(ADP-ribosyl)ation is blocked. Effective inhibitors of the polymerase include the nicotinamides (Preiss et al., 1971), methylxanthines (Levi et al., 1978), thymidine (Preiss et al., 1971), and aromatic amides (Purnell and Whish, 1980), such as 3-aminobenzamide (3-AB) and 3-methoxybenzamide (MBA). The acid analogues of these last two compounds and nicotinic acid do not serve as inhibitors (Hilz and Stone, 1976).

A second approach used in determining the roles of poly(ADP-ribose) involves limiting the amount of cellular NAD, the substrate for poly(ADP-ribose) polymerase. Dividing cells nutritionally deprived of nicotinamide have drastically lower NAD levels (Jacobson et al., 1979). This approach was developed using the 3T3 mouse embryo fibroblast line. Growth of these cells in nicotinamide-free

Dulbecco's modified Eagle's medium and 10% dialyzed fetal calf serum was identical to cells grown in the presence of nicotinamide over 5 generations.

Using these approaches, numerous roles have been postulated for poly(ADP-ribose). Poly(ADP-ribose) may have a direct role in DNA repair (Durkacz et al., 1980; Berger and Sikorski, 1981; Creissen and Shall, 1982). Polymer levels rise in response to DNA damage. When permeabilized cells are treated with X-rays, micrococcal nuclease, or DNase I, poly(ADP-ribose) synthesis is stimulated (Benjamin and Gill 1980a, b). SV40 transformed 3T3 cells (SVT2 cells) treated with MNNG have levels 150-fold higher than untreated cells and the NAD levels are lowered (Juarez-Salinas et al., 1979). L1210 cells treated with high levels of MNNG (136 μM) accumulate poly(ADP-ribose) with a concurrent lowering of both NAD and ATP (Berger et al., 1982). Recently, Jacobson et al. (1984) reported the first study to demonstrate the effects of poly(ADP-ribose) polymerase inhibitors in vivo. MNNG-treated C3H10T1/2 cells have a seven-fold increase in polymer levels over control cells. It was shown that MBA inhibited the increase in intracellular levels of poly(ADP-ribose) by approximately 82%.

Poly(ADP-ribose) may play a role in regulating the cell cycle. Many workers have seen a decrease in poly(ADP-ribose) polymerase activity in cells in S phase and an increase in G_2 or G_1 (Haines et al., 1969; Smulson et al., 1971; Kidwell and Watts, 1974).

Cytotoxicity of DNA damaging agents is increased in the presence of poly(ADP-ribose) polymerase inhibitors. These inhibitors include 5-methylnicotinamide (Nduka et al., 1980; Durrant et al., 1981), MBA, methylxanthines, theobromine, theophylline, and caffeine (Nduka et al., 1980; Durrant and Boyle, 1982). Inhibitors have also been shown to delay rejoining of DNA strand breaks caused by DNA damaging agents (Durkacz et al., 1980).

Also, the mutation frequency is increased with the application of inhibitors following DNA damage. V79 Chinese hamster cells treated with high doses of UV light (30 Joules/m^2) and caffeine have greater numbers of UV-induced 8-azaguanine-resistant mutants compared to cultures treated with UV alone (Bhattacharjee et al., 1982). Conversely, the frequency of 8-azaguanine resistant mutants was decreased in the presence of benzamide after MNNG treatment (Bhattacharyva and Bhattacharjee, 1983). In CHO cells, 3-AB increased the frequency of ethyl

methanesulfonate (EMS) and MNNG-induced 6-thioguanine-resistant cells but did not effect the frequency of EMS-induced ouabain-resistant cells (Schwartz et al., 1985). Since ouabain-resistant cells arise through point mutations, these authors suggested that 3-AB increases only the frequency of deletion mutations and does so by increasing the frequency and duration of DNA strand breaks.

It has been hypothesized that poly(ADP-ribosyl)ation is required for coordination of DNA repair with cell division (Jacobson et al., 1984). This is evidenced by the fact that cells treated with both inhibitors of poly(ADP-ribose) polymerase and MNNG lost the ability to divide and showed a decrease in the rate of subsequent rounds of DNA synthesis, but the rates of ribonucleic acid (RNA) and protein syntheses were similar to cells treated with MNNG alone. In contrast to exponentially dividing C3H10T1/2 cells, confluent cells that have been treated with MBA and MNNG survive as well as cells treated with MNNG alone (Jacobson et al., 1985a). Also, both dividing and quiescent cells have additional numbers of DNA strand breaks due to the presence of MBA. Additional evidence for the coordination of DNA repair with cell division was presented by Jacobson et al. (1985b). Cells synchronized

by isoleucine-deprivation and treated in G_1 with MNNG and MBA are able to complete only one full round of cell division. The cells then accumulate in G_2, in contrast to cells treated with MNNG alone.

The C3H10T1/2 mouse embryo fibroblast cell line that was used in the studies of this dissertation was developed as an in vitro model of malignant transformation (Reznikoff et al., 1973a, b). In a typical transformation assay, cells are seeded at a low density (1000 cells per 60 mm dish) and are treated with a chemical carcinogen. Control cultures are treated with the vehicle for dispensing the carcinogen. Cultures are refed when the carcinogen is removed and weekly thereafter. After six weeks the cells are fixed with methanol and stained with 10% Giemsa. Foci are usually observable macroscopically as darkly stained patches of cells. Foci are categorized into three types according to the following criteria. Type I foci are areas of tightly packed cells. Type II foci are darkly stained multilayers and the cells may exhibit apolarity. Type III foci are deeply stained and multilayered and the cells are arranged in a criss-cross fashion. Both type II and III foci may exhibit a corded morphology. Type II and III foci are considered to be malignantly transformed. When living

cells from foci are removed from the dish and injected into irradiated syngeneic mice, type II foci produce tumors in about 50% of the mice, while type III foci are tumor producing about 85% of the time (Reznikoff et al., 1973a). Type I foci do not produce tumors when injected into mice.

Recent improvements on the transformation assay system have been made by Bertram and coworkers and these modifications have been utilized in the present study. The use of penicillin-streptomycin as an antibiotic in the culture medium has been replaced by gentamicin sulfate since the use of penicillin-streptomycin in the assay inhibits the appearance of transformed foci (Bertram, 1979). A short term screening for selection of sera which support transformation in vitro has been developed (Bertram, personal communication). When C3H10T1/2 cells are grown in a serum that does not give exponential growth and does not support a stable stationary phase, a variable number of spontaneous type I foci develop. An acceptable serum lot can thus be identified by screening growth characteristics over a two week period. Another improvement that has been made on the assay system is the limiting of serum growth factors by reducing the serum concentration to about 5% just prior to confluence (Bertram, 1977). This allows transformed foci

to develop that would have been inhibited by a high density monolayer and by antitumor factors in the serum.

One of the most important developments in the C3H10T1/2 transformation assay is the observation that transformation frequency is related to the number of population doublings that occur during the exponential growth phase of the assay. Thus, when conducting comparative transformation assays, the number of survivors in each variable must be matched for the various treatments (Mordan et al., 1983).

This study was designed to investigate the role of poly(ADP-ribose) in malignant transformation. C3H10T1/2 cultures were treated with chemical carcinogens: 3-methylcholanthrene (MCA), ethylmethane sulfonate (EMS), and MNNG, and their transformation rates were compared to those that were treated in the presence of an inhibitor of poly(ADP-ribose) polymerase. In additional experiments, chemically induced transformation rates were compared in control cultures and cultures whose NAD pools had been depleted by growth in nicotinamide-free medium. The two approaches were used in order to confirm the results since each approach contains limitations. NAD and ATP levels were determined in the NAD-depletion experiments to

determine the extent of their involvement in affecting transformation frequency.

Transformation was studied in confluent cultures that were NAD-depleted or treated with inhibitors of poly(ADP-ribose) polymerase in order to determine whether the involvement of poly(ADP-ribose) was independent of events during DNA replication.

In order to determine whether the ascorbate-induced inhibition of transformation reported by Benedict et al. (1980) is due to a change in the redox state of the cells, NAD(H) and NADP(H) levels were measured in cells that were concurrently starved for nicotinamide and fed ascorbic acid. Auxillary experiments are described that aided in adapting the C3H10T1/2 cell line as a model system for studying the role of ADP-ribosylation in response to DNA damage, including studies on serum testing and attempts at finding alternatives to serum as a medium supplement.

MATERIALS AND METHODS

Cells and Cell Culture

C3H/10T1/2(CL8) mouse embryo fibroblasts (C3H/10T1/2 cells) were kindly provided by the late Dr. Charles Heidelberger, Comprehensive Cancer Center, University of Southern California, Los Angeles, CA. The cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. They were routinely subcultured at 5 x 10^4 cells per 25 cm^2 flask in Eagle's basal medium (BME) (GIBCO, Grand Island, NY) containing 10% heat-inactivated (30 min, 56°C) fetal bovine serum (FCS) (GIBCO, Grand Island, NY or Reheis Chemical Co., Phoenix, AZ or Hyclone Laboraties, Logan, UT) and sodium bicarbonate (2.2 g/L). Cells of passage 13 or less were used. When C3H10T1/2 cells were to be depleted of NAD, they 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 gentamycin (25 mg/L) (Schering Corp., Kenilworth, NJ or Sigma Chemical Co., St. Louis, MO) for 3-5 generations.

Cell counts were performed by washing the cell

monolayer twice with phosphate buffered saline (PBS) (0.01 M NaH_2PO_4-Na_2HPO_4, 0.15 M NaCl, pH 7.2) and removing the cells from the flask with 0.05% trypsin-EDTA in PBS with gentle agitation. The trypsin activity was quenched by adding medium containing serum and a portion of the cells was diluted with isotonic saline. Cells were enumerated with a Coulter Counter model ZBI.

The heat inactivated FCS was dialyzed in cellulose dialysis tubing (8,000 mw cutoff) (Thomas Scientific Co., Philadelphia, PA) against five changes of dialysis buffer (0.9 mM Na_2HPO_4-NaH_2PO_4, 0.11 M NaCl, pH 7.2) over a period of 3 days. The dialysis tubing was prepared by simmering for 1 hr under a sterile hood in 2 L of each of the following solutions per 40 feet of tubing: 2 washes with 50% ethanol, 2 washes with 10 mM $NaHCO_3$, 1 wash with 1 mM EDTA, 2 washes with distilled H_2O. The tubing was stored at $4^{o}C$ in 50% ethanol. Before filling with serum, the bags were rinsed with sterile, distilled H_2O and dialysis buffer.

Transformation Assays

Transformation assays were a modification of the procedure of Bertram (Bertram, 1977). In experiments using exponentially dividing cells and inhibitors of

poly(ADP-ribose) polymerase, control cultures were seeded at 1 x 10^3 to 1 x 10^4 cells per 60 mm dish. Cultures to be treated with cytotoxic agents were seeded at higher densities to correct for lower survival. Twenty-four hr after seeding, the cells were treated with carcinogens and/or inhibitors. After treatment, the medium was removed, cells were washed with PBS, and BME containing 10% FCS was added. When cells reached confluence the medium was replaced with BME containing 5% FCS. Cultures were refed weekly and were fixed at about five weeks post-confluence with absolute methanol and stained with 10% Giemsa in dilute PBS (1:10). Type II and III foci (Reznikoff, et al., 1973a), those which were massively piled and densely stained, were scored as malignant transformations. Transformation frequency was expressed as the number of type II and III foci per 1 x 10^4 survivors which were determined as described below.

In experiments which measured transformation frequency in confluent cultures, cells that had been in the stationary phase of growth for a minimum of 48 hr were treated with carcinogens and inhibitors, then reseeded 48 hr after treatment into the transformation assay.

In some experiments cells were starved for

nicotinamide for 3-4 population doublings before assay of carcinogen induced transformation.

Survival Studies

Cell survival rates were determined by colony forming assays (CFA) in which C3H10T1/2 cells were seeded at 300-400 cells per 60 mm dish. Treatment of these cells was parallel to treatment of cells in the transformation assay. Twenty-four hr after seeding, duplicate dishes were fixed with methanol-acetic acid (3:1 v/v) and stained with 1% methylene blue (w/v). The cells remaining attached to the dishes were microscopically counted on a Bausch and Lomb Stereozoom microscope to determine plating efficiency. At approximately seven to nine days following treatment when the control dishes had $\geq$50 cells per colony, the cells were fixed and stained as for determination of plating efficiency. Colonies of $\geq$25 cells and $\geq$50 cells were counted. Survival was expressed as the number of colonies divided by the number of cells that plated (Reznikoff et al., 1973a).

Extraction and Measurement of Pyridine Nucleotides

NAD^+ and $NADP^+$ and their reduced forms 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 and immediately neutralized with ice cold 0.37 M H_3PO_4. The reduced forms were oxidized by the addition of 0.125 ml 2 mM phenazine ethosulfate (PES) per ml of base. These samples containing the total NAD pool were frozen at -20°C until assay. NAD was measured by an enzyme cycling assay (Jacobson and Jacobson, 1976).

Extractions of pyridine nucleotides were modified in the studies of effects of ascorbate on redox states in control and nicotinamide-depleted cells in that PES was omitted. The reduced forms of pyridine nucleotides were quantitated by heating a portion of the unneutralized sample from above at 60°C for 10 min to destroy the oxidized coenzymes. The samples were then neutralized and assayed immediately. The sensitivity of the NAD assay was modified by adjusting PES and enzyme concentration and increasing cycling time in order to detect 3-30 pmoles NAD per sample.

Extraction and Measurement of ATP

ATP levels were determined by extracting as described for total pyridine nucleotides. The cell extract was precipitated with 40% trichloroacetic acid (w/v) and the supernatant was extracted four times with an equal volume of ether. The supernatant was diluted in 75 mM

glycylglycine, 15 mM $MgCl_2$, pH 7.4, and assayed for ATP content by a luciferin-luciferase bioluminescence assay in a Lumitran L3000 ATP photometer (Baker and Jacobson, 1984).

Chemical Treatment

The carcinogens N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and 3-methylcholanthrene (MCA) and the poly(ADP-ribose) polymerase inhibitor, 3-methoxybenzamide (MBA) were obtained from Sigma Chemical Co., St. Louis, MO. Each was dissolved in acetone immediately before use and was applied to the medium on the dish using a P20 pipetman (Rainin Instrument Co, Inc., Woburn, MA). The final concentration of acetone was $\leq$0.5% in the medium. The control dishes were treated with medium containing acetone only. All dishes in an experiment were treated with the same concentration of acetone. The carcinogen ethyl methanesulfonate (EMS) (Sigma Chemical Co., St. Louis, MO) was delivered directly into the medium of the dish with a P20 pipetman. Chemical treatment began 24 hr after cells were seeded. When inhibitors were used, they were applied to the dishes first, then the carcinogen was applied for the specified treatment time. The media were removed, the cells were washed with PBS, and control medium was applied. The inhibitor or acetone was reapplied so that the total

inhibitor treatment time was 24 or 48 hr.

Ascorbate studies

Cells were seeded in 100 mm dishes in BME containing 10% FCS. Twenty-four hours after seeding, the cells were washed twice with PBS and the medium was replaced with SDME containing 10% DFCS with or without nicotinamide. A portion of the control and nicotinamide deprived dishes were treated with 5.68 μM ascorbate dissolved in PBS. These media were replenished daily. After 3-5 population doublings, the cells were extracted for nicotinamide adenine dinucleotide phosphate (NAD(P)). Percent reduced NAD or NADP was calculated.

Isoleucine Synchrony

Cells were synchronized in G_1 by isoleucine deprivation (McCormick and Bertram,1982). Confluent C3H10T1/2 cells were refed and at two days postconfluence they were seeded into 35 mm dishes in isoleucine-free BME containing 10% DFCS. Twenty-four to 36 hr after seeding, the cells were released by replacing the medium with BME containing 15% FCS and 0.76 mM isoleucine (release medium). Various times after release, cells were treated with MNNG and MBA in acetone. Twenty-four hr later, the medium was removed, the cells were washed with PBS, and BME + 10% FCS was

added. Survival rates were determined as described above.

Pulse Labeling

Cells that were synchronized by isoleucine deprivation were pulse labeled at various times after release. The medium was removed from the 35 mm dishes and serum-free release medium containing excess isoleucine and 1 μCi [^{3}H]-Thd/ml was added. The cells were incubated for 60 min at 37^{o}C, then washed two times with cold PBS. The cells were extracted three times with 0.075 ml 2% sodium dodecyl sulfate (SDS) and scraped with a rubber policeman. The extract was transferred with a pasteur pipet to filter paper. The papers were washed twice in ice cold 10% TCA for 15 min, then four times in 95% ethanol for 10 min. The filters were dried overnight at room temperature and counted in glass vials containing 5 ml organic scintillation fluid 1,4-bis[5-phenyl-2-oxazolyl]-benzene (POPOP) in an LS8000 series liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Growth in MCDB 402

Cells were seeded in MCDB 402 onto poly-D-lysine coated dishes. MCDB 402 was prepared as described by Shipley and Ham (Shipley and Ham, 1981). Poly-D-lysine hydrobromide was dissolved in ultrapure water at 100 μg/ml

and sterilized via a 0.2 um acrodisc filter assembly (Gelman, Ann Arbor, MI) attached to a 6 cc plastic syringe. Two ml were poured onto each 35 mm dish, and the excess was aspirated after 10 min at room temperature. The coated surface was washed twice with sterile ultrapure water.

T3 Treatment

3,3',5-Triiodo-L-thyronine (T3) was added during isoleucine deprivation. A stock solution of 1 mM T3 in n-propyl alcohol was prepared and acidified by the additon of 1 drop of 5 N NaOH. The stock was diluted in medium to give a final concentration of 2 nM T3.

RESULTS

The C3H10T1/2 Cell Line as a Model for in Vitro Transformation

The C3H10T1/2 cell line was developed as a model for *in vitro* assay of malignant transformation (Reznikoff et al., 1973b). In this assay, C3H10T1/2 cells were grown in medium supplemented with fetal calf serum. The characteristics of the fetal calf serum vary from lot to lot, with some lots containing unknown factors that are inhibitory to growth. In other lots, the factors produce a large number of type I foci.

The occurrence of spontaneous type I foci can be eliminated by routinely screening sera lots for their suitability in transformation assays (Reznikoff et al., 1973a). Two screening methods were employed in this study. In the first method, various lots of sera were tested in a "mock" transformation assay in which untreated cells were grown in medium containing test sera for six weeks (Reznikoff, et al., 1973a). At the end of this period, the dishes were fixed and stained, and the cell lawns were examined for foci. The serum that did not produce type I

foci was chosen for use in the transformation assay. Plate 1 shows a photograph of a control dish grown using a suitable serum.

In the second method, a simplified procedure for sera testing was employed (Bertram, personal communication). Cells were seeded at a density of about eight population doublings below confluence, fed every three days, and allowed to grow for three weeks. Cell growth was monitored daily by cell enumeration. Sera that supported a doubling time of approximately 16.5 hr and a stable cell density at the stationary phase of growth were selected for use in the transformation assay. The growth curves of Figure 2 illustrate cell growth rates using two acceptable lots of sera, lots A and C, and one serum lot that was unacceptable, lot B. The doubling time of cells grown in sera A and C was 16-20 hr and the growth was exponential, while the growth of cells in serum B was not exponential and the doubling time approached 40 hr.

Once a suitable serum has been chosen, carcinogen-induced transformation can be studied. In order to measure transformation rates accurately, survival rates must be known and carefully controlled (Mordan et al., 1983). The survival rates were determined by either growth curves in

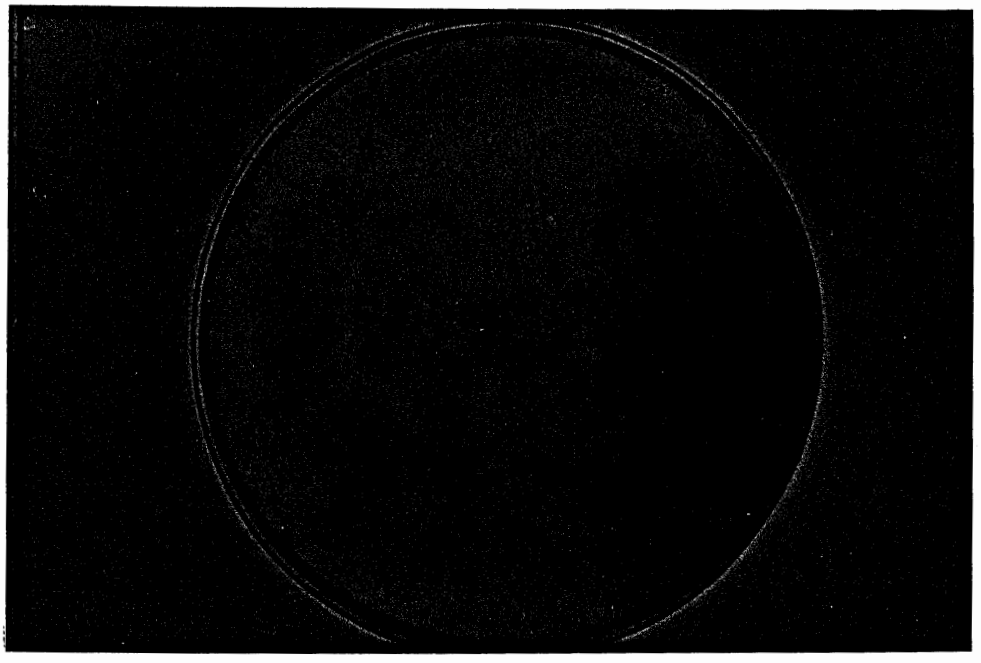


Plate 1. A typical control dish from a transformation assay in which quality fetal calf serum was used. Note that the cell lawn is fairly uniform. No spontaneous foci are present.

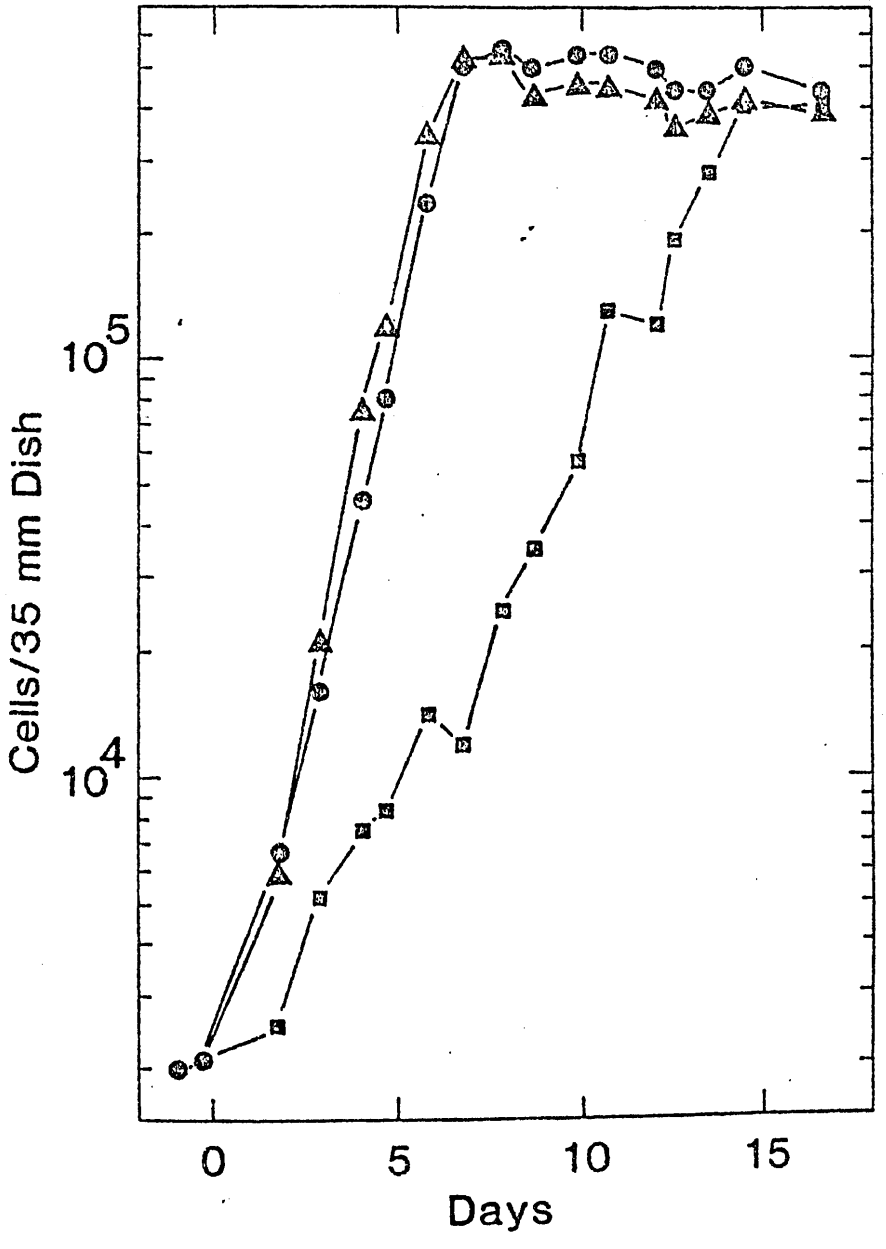


Figure 2. Screening of fetal calf serum for suitability in the C3H10T1/2 transformation assay. Cells were seeded in BME + 10% FCS (Reheis lot A) and after 24 hr, the test media were applied. Cells were refed every three days. The data are presented as the average of duplicate analyses. Lot A (V55203) (●), B (X62904) (■), C (X62603) (▲).

the case of MCA induced transformation, or by colony forming assays for the other carcinogens tested. Survival rates were dependent on both dose and length of carcinogen treatment.

In preliminary experiments, carcinogen-induced transformation was inhibited by the use of penicillin-streptomycin as the antibiotic in the culture medium (Bertram, 1979). Due to this phenomenom, its use was discontinued and gentamicin was used in subsequent transformation assays.

Alternatives to fetal calf serum supplemented medium

As mentioned earlier, the use of FCS as a medium supplement has several disadvantages due to its undefined nature. In addition, it is expensive and the screening process is time consuming. Thus, alternative sources of nutrients were tested for their ability to support and control growth of C3H10T1/2 cells. The ultimate goal in testing was to obtain a chemically defined serum substitute.

The first medium supplement tested was a commercially available mixture of 5 mg/ml each of transferin and insulin plus 5 μg/ml selenium (ITS). This mixture is diluted at 1 ml ITS per liter of medium. Cells grown in the control

medium (BME + 10% FCS) had a doubling time of approximately 17 hr, while cells cultured in BME + 2% FCS supplemented with ITS had a doubling time of about 24 hr, as shown in Figure 3. Although cell growth in this combination of ITS and low serum was exponential, the lengthening of the doubling time was undesirable. Cells that were cultured in BME and ITS did not thrive. Thus, if ITS was to be used as a medium supplement for this cell line, it would have been necessary to supplement the medium additionally. Thus, ITS did not appear to be a suitable alternative to serum using this assay.

Ham and Shipley made a key contribution to the scientific literature by formulating serum-free media for the culture of mammalian cells. A recent formulation, MCDB 402, has been developed (Shipley and Ham, 1981), which contains, along with the essential amino acids and salts, essential fatty acids and trace minerals that are supplied by serum under the usual culture conditions. When Swiss 3T3 cells are seeded into the medium supplemented with insulin, dexamethasone, and bovine plasma fibronectin, the cells attach to polylysine-coated culture surfaces and remain viable for several days. However, the cells do not divide unless stimulated by the addition of fibroblast

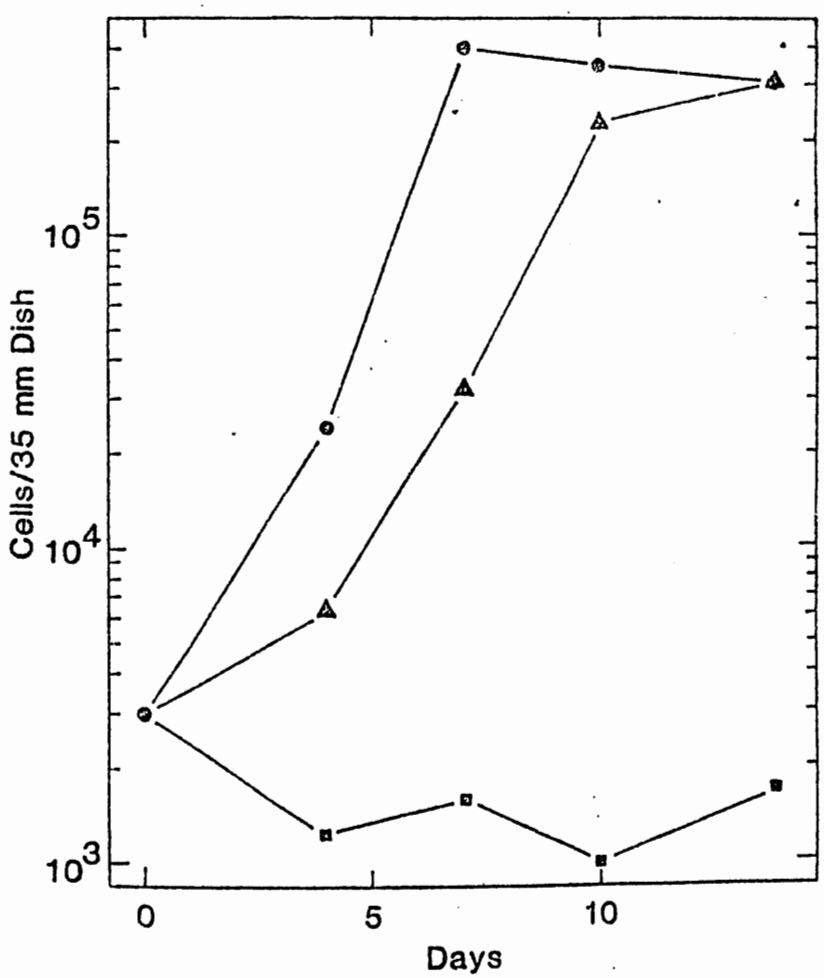


Figure 3. Effect of ITS on growth of C3H10T1/2 cells. Cells were seeded in BME + 10% FCS and 6 hr later the medium was replaced with BME containing the indicated supplement. The data are the average of duplicate analyses. Cells per dish in BME containing 10% FCS (●), 2% FCS and ITS (▲), ITS alone (■).

growth factor (Shipley and Ham, 1983).

An attempt was made in the present study to use MCDB 402 to culture C3H10T1/2 cells. Figure 4 shows that MCDB 402 supplemented with 10% FCS yielded a cell doubling time of approximately 24 hr. When the serum concentration was reduced to 2% and ITS was added, the doubling time increased to 48 hr. Elimination of FCS and supplementaion of MCDB 402 with ITS alone did not support growth of the cells. MCDB 402 did not support growth as well as BME either in the presence of 10% FCS or a combination of FCS and ITS. Thus, MCDB 402 was not an alternative medium for these studies.

A third approach to serum supplementation was to partially replace serum with another commercial preparation, SerXtend. SerXtend contains in addition to the nutrients in ITS, other growth factors (mitogen stimulation factor, fibroblast growth factor, and epidermal growth factor) and hormones. As shown in Figure 5, BME containing 5% FCS and SerXtend supported the growth of C3H10T1/2 cells as well as medium containing 10% FCS. The cost to supplement a liter of medium with 5% FCS and SerXtend saves 17-26% of the cost of using serum alone. Although this combination saves money, additional testing would be needed

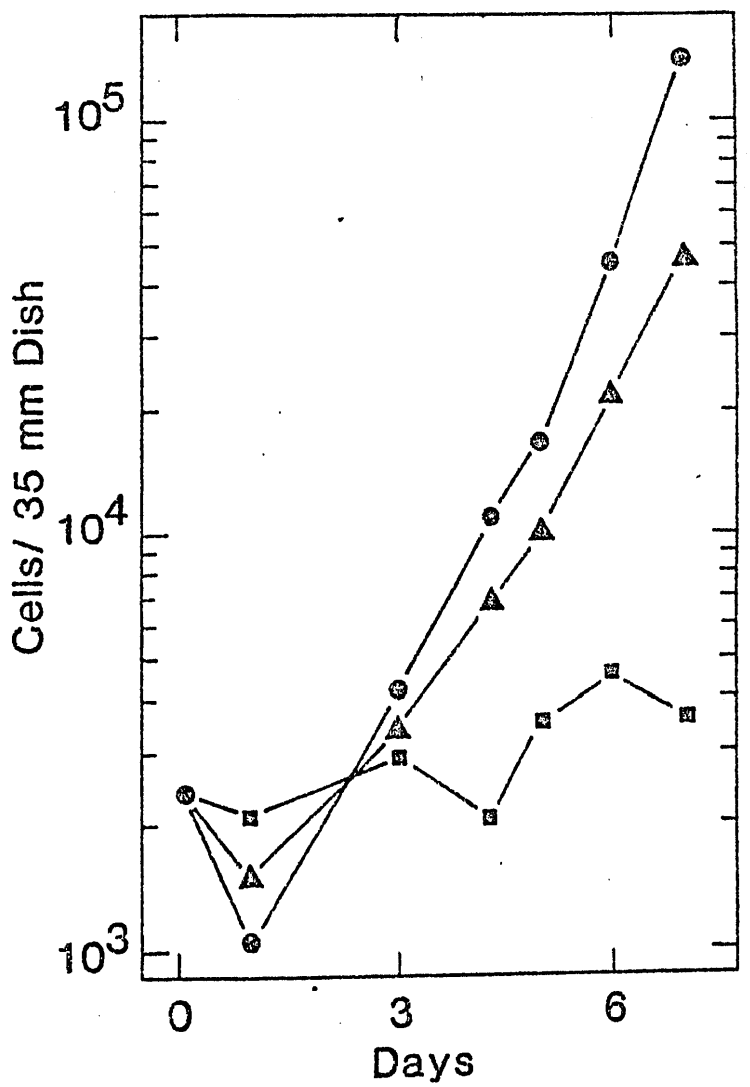


Figure 4. Effect of ITS and FCS in MCDB 402 on supporting growth of C3H10T1/2 cells. Cells were seeded in MCDB 402 containing 10% FCS into poly-D-lysine coated dishes. After 2 hr, the medium was removed and MCDB 402 containing the indicated supplements was added. The medium was replenished every 3 days. The data represent duplicate analyses. Growth in the presence of MCDB 402 containing 10% FCS (●), ITS + 2% FCS (▲), ITS alone (■).

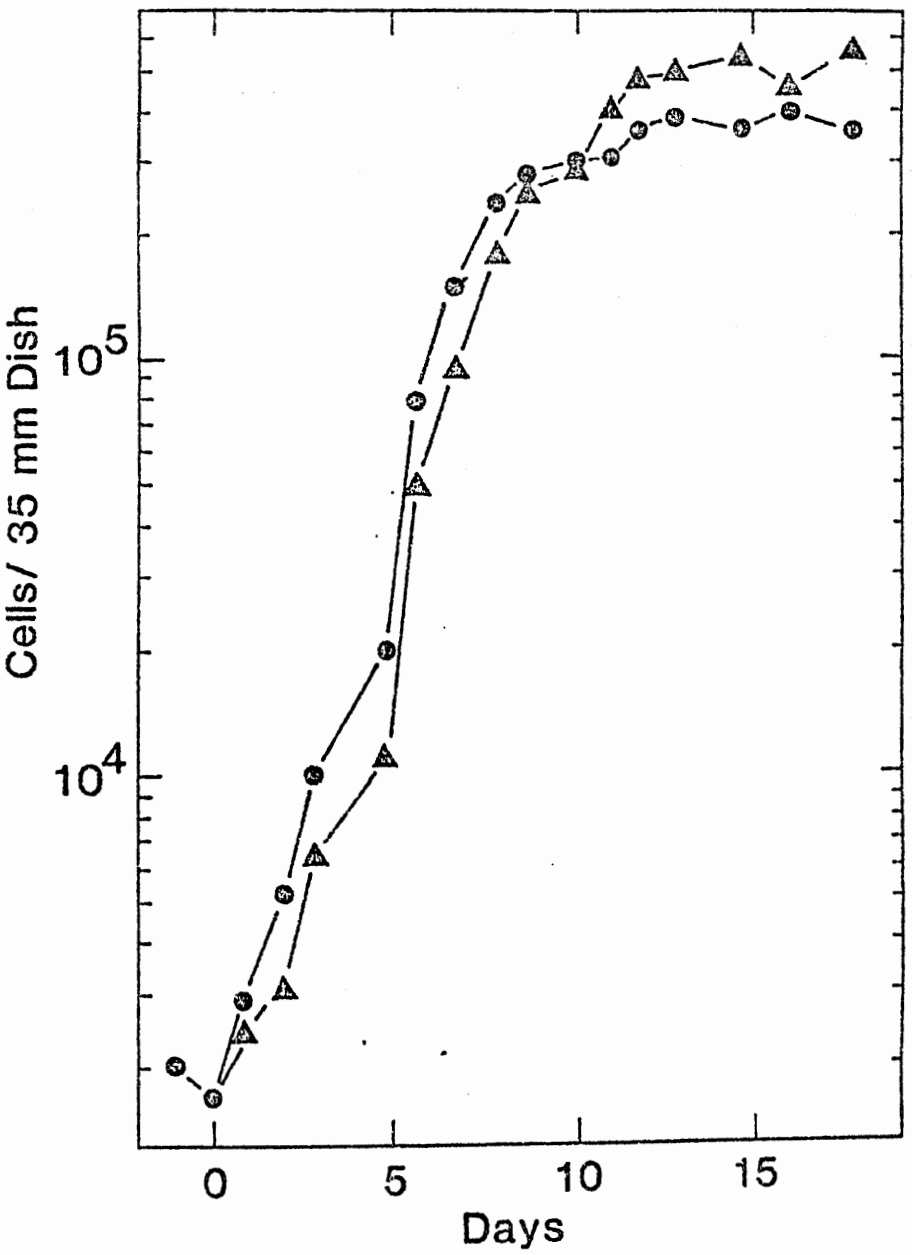
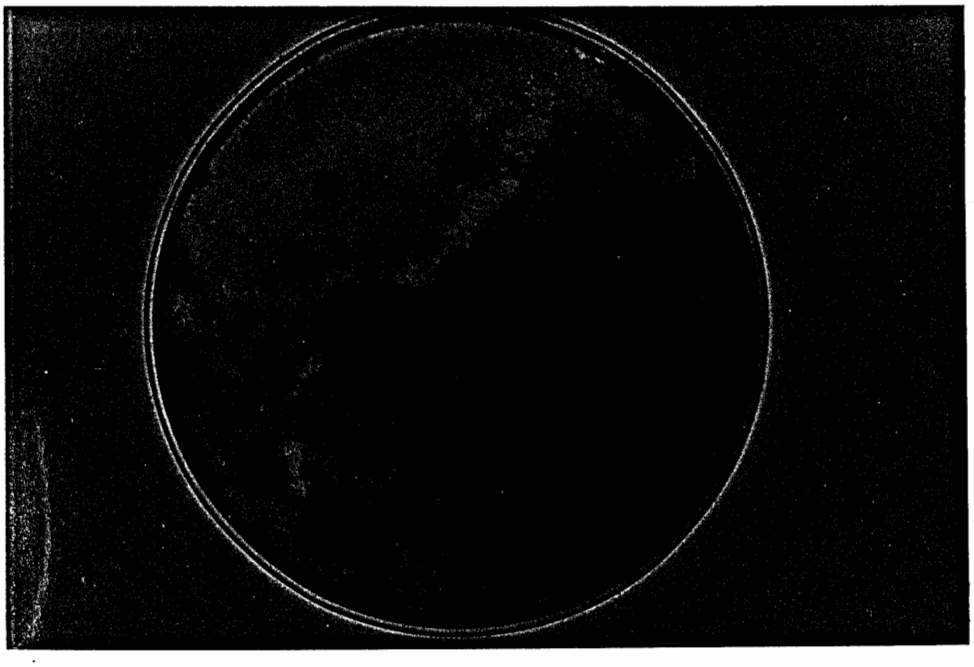


Figure 5. Effect of SerXtend on supporting growth of C3H10T1/2 cells. Cells were seeded in BME + 10% FCS. Twenty-four hr later, the medium was replaced with BME containing 10% FCS (●), or 5% FCS and SerXtend (▲). The data represent duplicate analyses.

to eliminate the need for serum.

Effect of Inhibitors of Poly(ADP-ribose) Polymerase on Carcinogen Induced Transformation in Exponentially Dividing C3H10T1/2 Cells

MCA is a potent carcinogen that has been used extensively in the development and improvement of the C3H10T1/2 cell transformation system (Reznikoff et al., 1973). Other investigators have shown transformation rates on the order of 10^{-4} using MCA as the inducing carcinogen (IARC/NCI/EPA Working Group, 1985). For this reason, MCA was selected as the initial carcinogen of choice for studying the effects of poly(ADP-ribose) polymerase inhibitors on carcinogen induced transformation. Plate 2 shows a photograph of an MCA treated (37 μM) dish containing all 3 types of foci: type I, type II, and type III. In order to distinguish the three types, the edges were examined microscopically. Plate 3 shows the edge of a type I focus. Notice that the cells of the focus are more tightly packed than the surrounding cell lawn. The edge of a type II focus is shown in Plate 4. The cells of the focus are deeply piled and darkly stained. The cells at the edge of the focus are polar. A type III focus is shown in Plate 5. The cells are darkly stained as they are in a



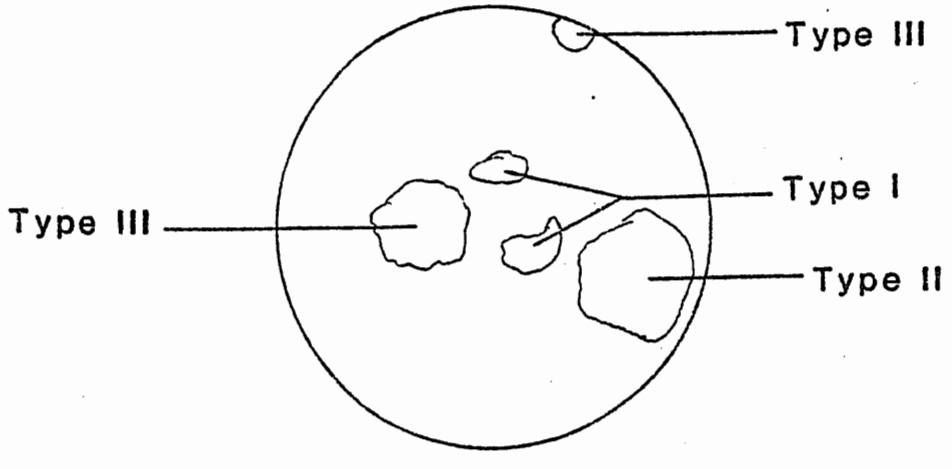


Plate 2. A typical dish treated with 37 μM MCA and stained after 6 weeks of growth. Several foci are present. The bottom drawing identifies the foci types.

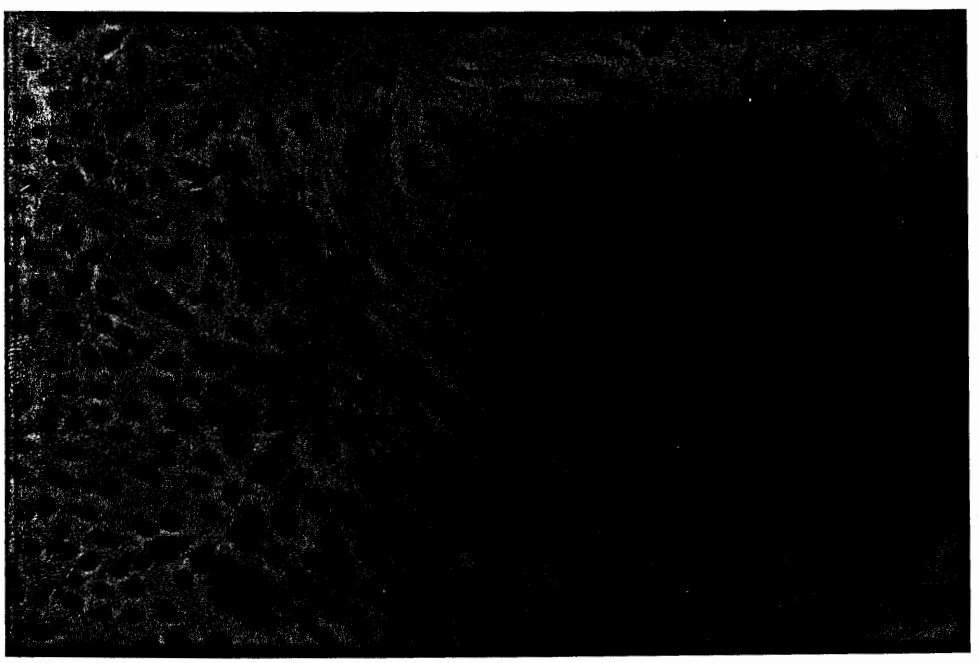


Plate 3. The edge of a type I focus. The cells of the focus are tightly packed compared to the cell lawn.

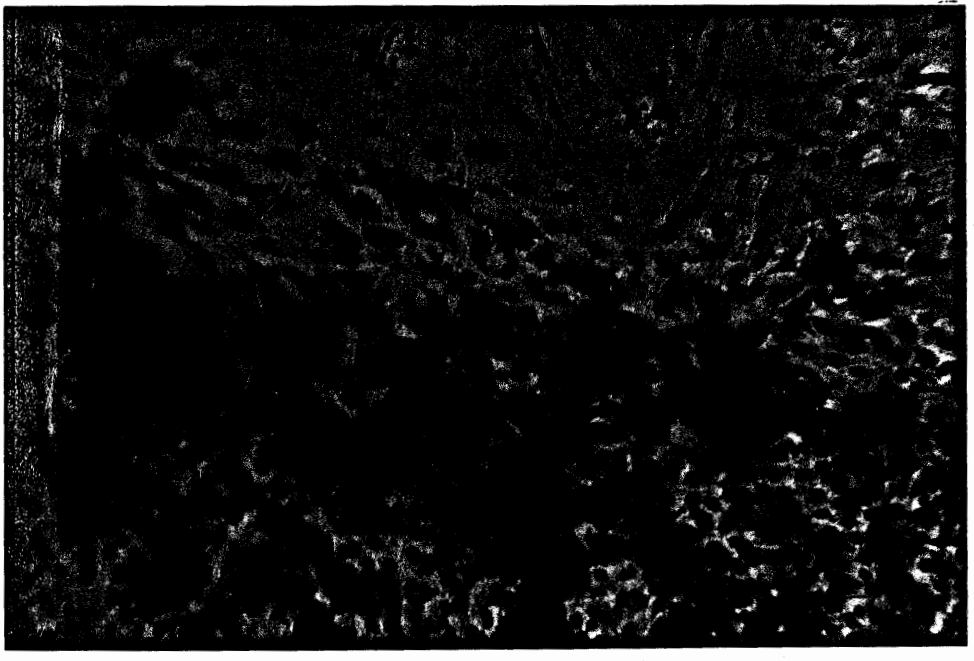


Plate 4. The edge of a type II focus. The cells are multilayered and deeply stained.

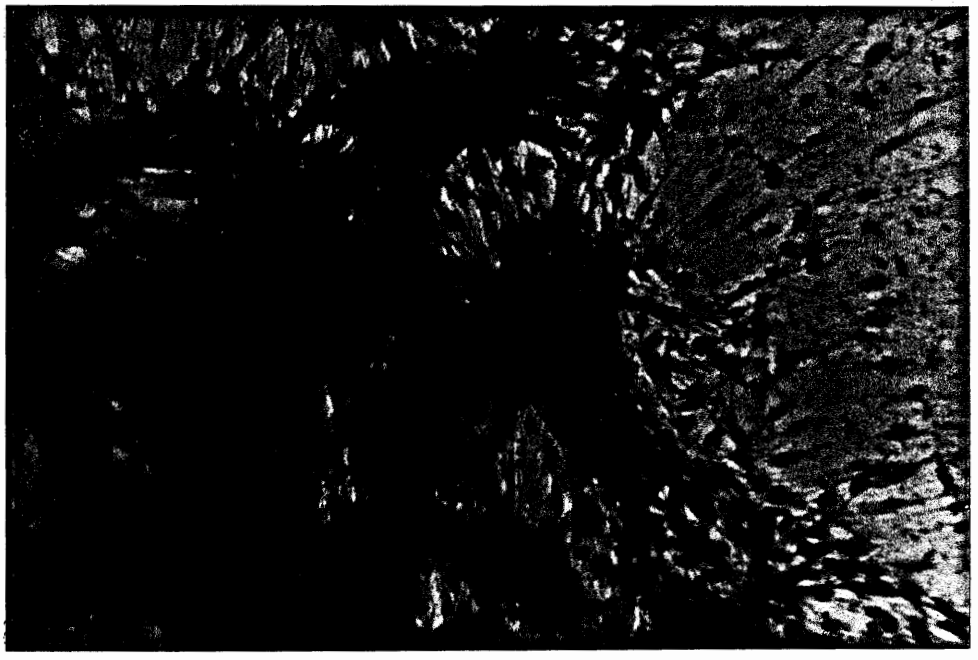


Plate 5. The edge of a type III focus. The cells are massively piled and darkly stained. Cells at the edge of the focus are crisscrossed.

type II focus. However, the cells are more deeply piled and the cells at the edge of the focus exhibit a criss-crossed appearance.

When exponentially dividing C3H10T1/2 cells were treated with 37 μM MCA as described above, a transformation frequency of 3.49 foci per 10^4 survivors was obtained. The data are shown in Table 1. The addition of the poly(ADP-ribose) polymerase inhibitor, MBA (0.5 mM) during the period of carcinogen exposure was then tested. As can be seen in Table 1, the presence of the inhibitor increased the transformation frequency by 11%. As shown in Figure 6, control and MBA treated cultures grew at the same rate. Likewise, cells treated with both MCA and MBA grew at the same rate as those treated with MCA alone. Thus, the presence of the inhibitor had no effect on survival in this experiment.

It has since been shown that inhibitors of poly(ADP-ribose) polymerase greatly enhance the cytotoxicity of alkylating agents (Nduka et al., 1980). However, the inhibitors have little cocytotoxic effect with DNA damaging agents such as MCA which form bulky adducts (Walker, 1984). When this information became available, experiments were begun using alkylating agents to induce transformation.

Table 1

EFFECT OF MBA ON 3-METHYLCHOLANTHRENE INDUCED TRANSFORMATION

TREATMENT	SURVIVORS/DISH	TOTAL SURVIVORS	#FOCI/#DISHES	TRANSFORMATION FREQUENCY X 10^{-4}	NUMBER OF POPULATION DOUBLINGS
Control	1900	153900	0/50	0	6.9
MBA	2100	172200	0/82	0	6.9
MCA	1700	146000	51/80	3.5	6.4
MCA + MBA	1400	113700	44/77	3.9	6.3

C3H10T1/2 cells were seeded at 2500 cells/60 mm dish. After 24 hr, cells were treated with 37 μM MCA and 0.5 mM MBA for 24 hr. Data are pooled from 5 experiments. Survivors were determined by growth curves.

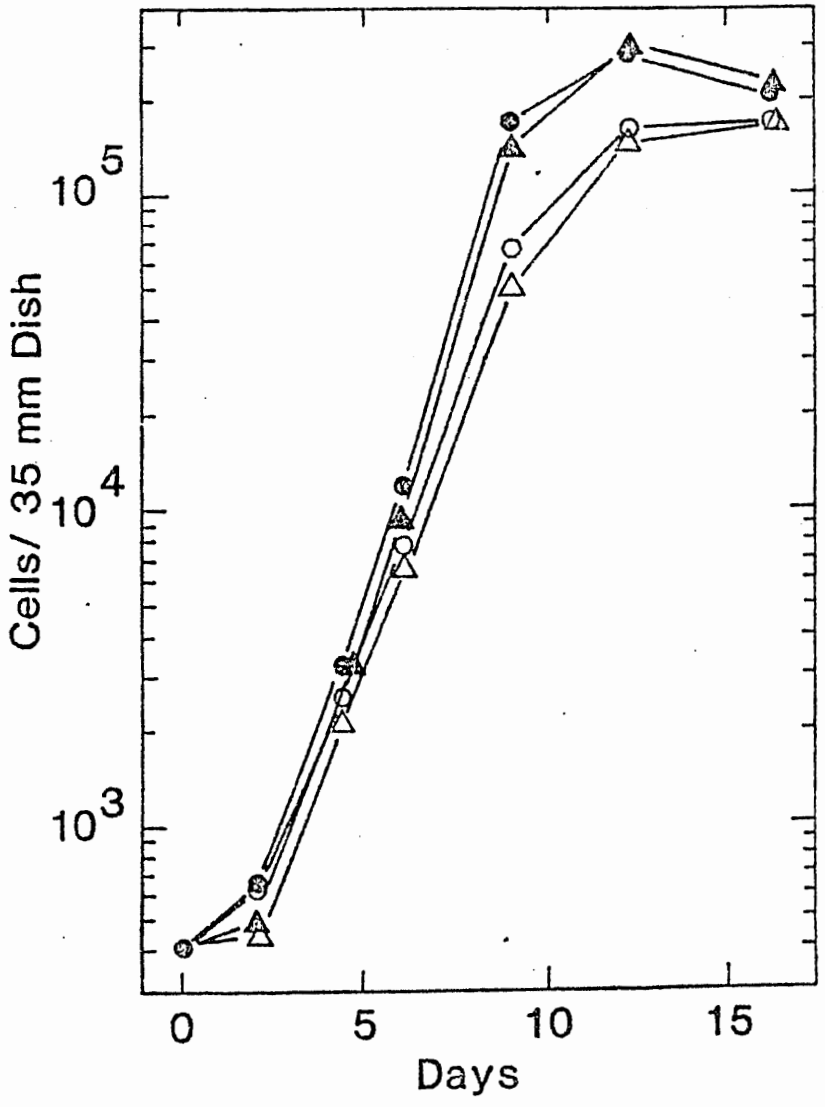


Figure 6. Cell division during transformation assay following MCA and MBA. Twenty-four hr after seeding, cells were treated with 37 μM MCA and 0.5 mM MBA in acetone for 24 hr. Cells treated with acetone alone (●), MBA (▲), MCA (○), MCA and MBA (△).

Ethyl methanesulfonate (EMS) is a carcinogen whose toxicity is increased by inhibitors of poly(ADP-ribose) polymerase. The growth effects with the combined treatment of 0.4 mM EMS and 1 mM MBA for 24 hr is illustrated in Figure 7. Initially a slight cell killing occurred. The overall growth rate of EMS and MBA treated cells was the same as that of cells treated with EMS alone. Also, the same saturation density was attained.

Figure 8 shows the preliminary study of the cocytotoxic effects of 1 mM MBA on cells treated with 0-4 mM EMS. The additon of inhibitor decreased survival by as much as 95% at the highest dose of carcinogen. In order to design a transformation assay in which the number of survivors in EMS treated cells was similar to that in EMS plus MBA treatment, a dose of 0.4 mM EMS was selected. The experiment was designed to carefully match the number of survivors in the control and test cultures by selecting a low dose of EMS and adjusting the number of cells seeded. However, the design with this dose of alkylating agent did not give a detectable transformation frequency in this cell line. In order to detect transformation using this carcinogen, extensive preliminary studies would have had to be done in order to determine the appropriate dose.

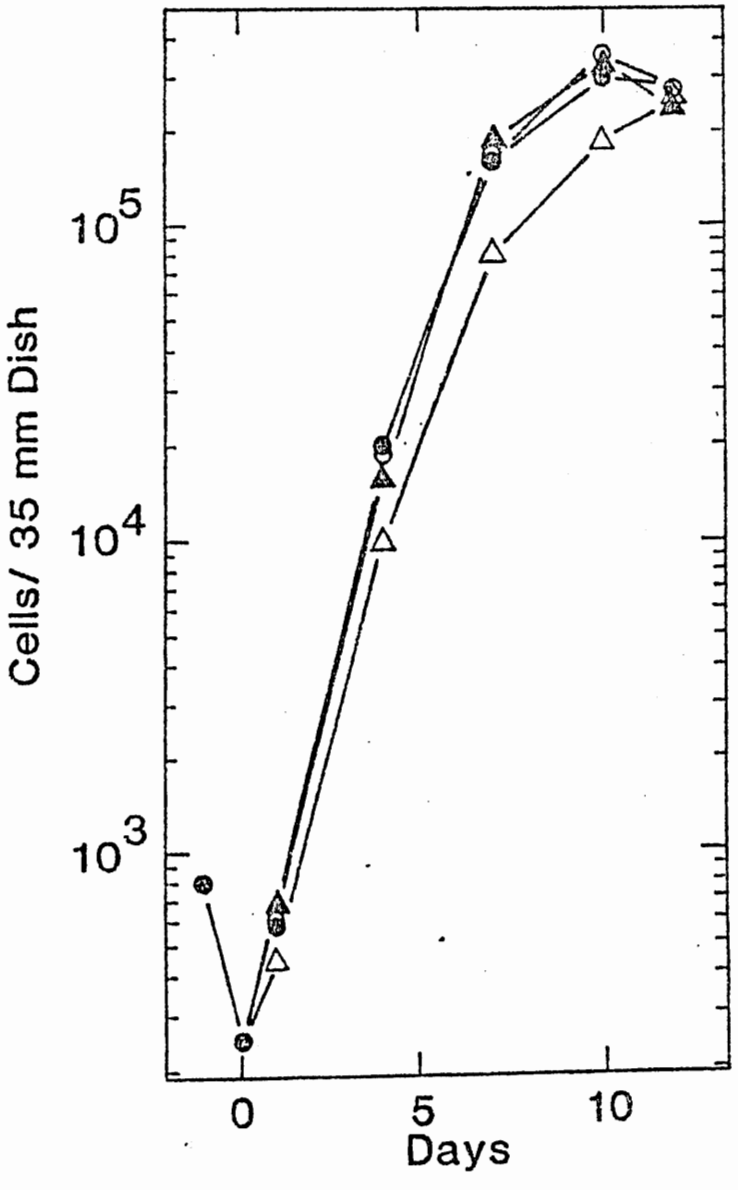


Figure 7. Cell division during transformation assay following EMS and MBA treatment. Twenty-four hr after seeding, cells were treated with EMS and 1 mM MBA for 24 hr. Cells treated with acetone (●), MBA (▲), EMS (○), EMS and MBA (△).

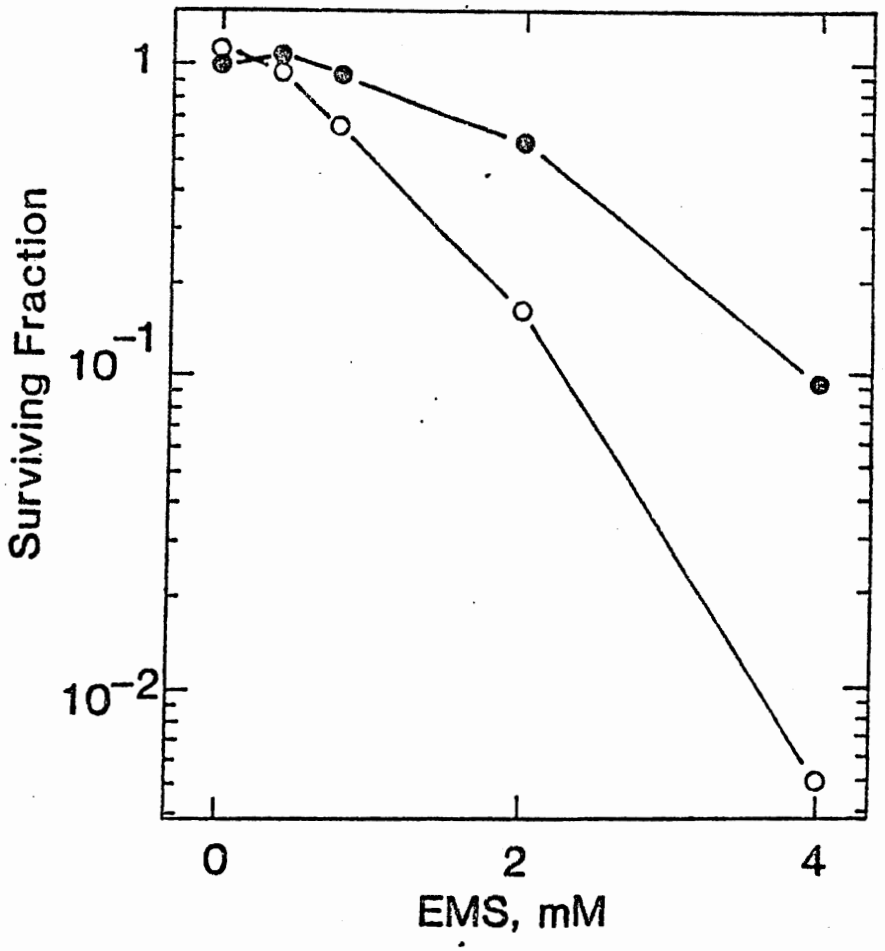


Figure 8. Cell survival following EMS and MBA treatment. Twenty-four hr after seeding, cells were treated with 0-4 mM EMS and 1 mM MBA for 24 hr. Colonies containing 50 or more cells were counted. Surviving fraction is calculated as the number of colonies divided by the number of cells that plated expressed relative to the acetone control. Cells treated with EMS (●) or EMS and MBA (O).

Previous studies in the Jacobson laboratories have used MNNG as the DNA damaging agent to study poly(ADP-ribose) metabolism following DNA damage. After MNNG damage, cells accumulate poly(ADP-ribose) levels that are up to 150-fold higher than in control cells (Jacobson et al., 1980). In addition to quantitation of polymer levels, survival rates (Jacobson et al., 1980), repair replication (Jacobson et al., 1985a), and the cell cycle (Jacobson et al., 1985b) have been studied following MNNG-induced damage. The selection of MNNG to induce transformation allows for a comparison to be made between the previously studied events and the studies of this dissertation.

In order to control for survival in the transformation assays, preliminary colony forming assays were performed. Figure 9 shows the data in which exponentially dividing C3H10T1/2 cells were treated with 0-6.8 μM MNNG. In cultures treated with MNNG alone, cytotoxicity increased with dose. The curve has a shoulder for doses below 3.4 μM and is linear up to 6.8 μM. Survival was decreased to 20% of control at 6.8 μM MNNG when MBA was included in the carcinogen treated dishes. In addition, the shoulder portion on the curve was no longer detectable.

Transformation experiments were designed so that the

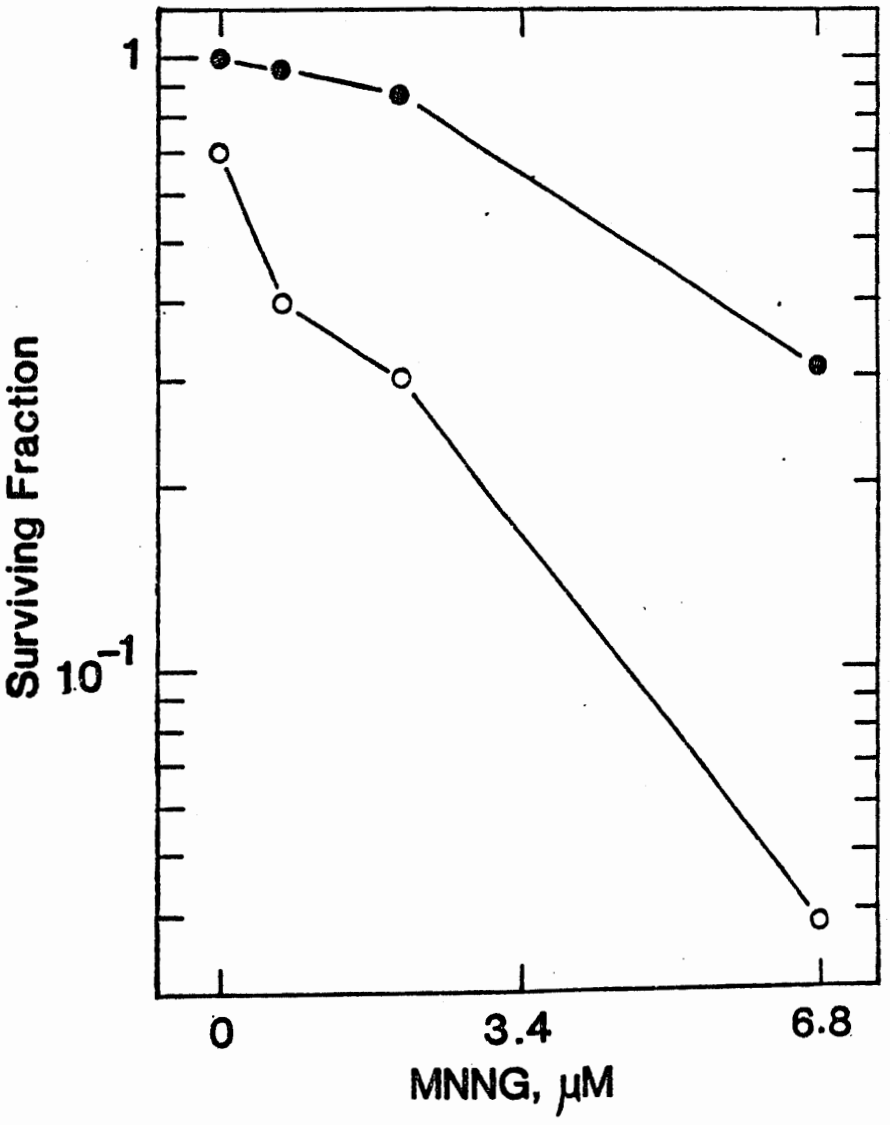


Figure 9. Survival of C3H10T1/2 cells following MNNG and MBA treatment. Twenty-four hr after seeding, cells were treated with MNNG (●) or MNNG and MBA (O).

cells on each dish underwent a minimum of 10 population doublings before the cell monolayer became confluent. The expression of transformation frequency is maximal and independent of the number of cells plated if greater than 10 populations occur (Mordan et al., 1983). Exponentially dividing cells were seeded into dishes and treated with 2.0 μM MNNG and 1 mM MBA for 24 hr. The data of Table 2 shows that there were no spontaneously transformed foci in either the acetone treated controls or MBA treated dishes. The cells treated with MNNG had a low, although detectable transformation frequency of 0.44×10^{-4}. A dramatic 12 fold increase in transformation frequency was observed in cells treated with the combination of MNNG and the poly(ADP-ribose) polymerase inhibitor, MBA over cultures treated with MNNG alone.

Effect of Nicotinamide Deprivation on Carcinogen-Induced Transformation in Exponentially Dividing Cells

When 3T3 cells in culture were grown in the absence of nicotinamide (SDME + 10% DFCS) for several generations (-Nam cells), the NAD content dropped to as low as 5-10% of the control levels (+ Nam cells). The growth rate of these cells is identical to controls (Jacobson and Jacobson, 1976). This method of nutritionally depleting the cellular

Table 2

MALIGNANT TRANSFORMATION IN EXPONENTIALLY DIVIDING C3H10T1/2 CELLS

TREATMENT	CELLS SEEDED/DISH	SURVIVORS/DISH	TOTAL SURVIVORS	#FOCI/# DISHES	TRANSFORMATION FREQUENCY X 10^{-4}	POPULATION DOUBLING
Control	1.0 X 10^3	730	35040	0/48	0	10.8
MBA	1.0 X 10^3	904	43392	0/48	0	10.5
MNNG	1.0 X 10^3	813	68312	3/84	0.44	10.7
MNNG + MBA	1.2 X 10^4	287	28374	15/99	5.3	12.1

C3H10T1/2 cells were seeded at 4000 cells per 60 mm dish for all treatments except for cells to be treated with MNNG plus MBA which were seeded at 1.2 x 10^4 cells/dish. Twenty-four hr later, cells were treated with 2 μM MNNG and 1 mM MBA for 24 hr. Data are pooled from 2 experiments. Survivors were determined by colony forming assays.

NAD levels resulted in limiting the availability of substrate for poly(ADP-ribose) polymerase. C3H10T1/2 cells were selected for optimal growth in Basal medium Eagle's (BME) and 10% FCS. Attempts were made to culture these cells in nicotinamide-free BME containing 10% DFCS. The cells did not divide when supplied with this medium. However, as shown in Figure 10, they could be grown in SDME + 10% DFCS in which 3T3 cells are grown. The growth of C3H10T1/2 cells in the presence or absence of nicotinamide was identical except that the -Nam cultures reached confluence at a slightly lower density than the +Nam cultures (4.5×10^5 vs 5.5×10^5 cells per dish). The final saturation density for these cells varied from experiment to experiment depending upon the density at which they were first given nicotinamide-free medium. The population of cells doubled four to five times before reaching confluence.

Initially, MCA was used as the carcinogen in these studies. Figure 11 shows the growth of C3H10T1/2 cells that were cultured in the absence of nicotinamide for three generations, then reseeded into the same medium and treated with 18.5 μM MCA for 24 hr. The cells were allowed to grow an additional 24 hr at which time the medium was replaced

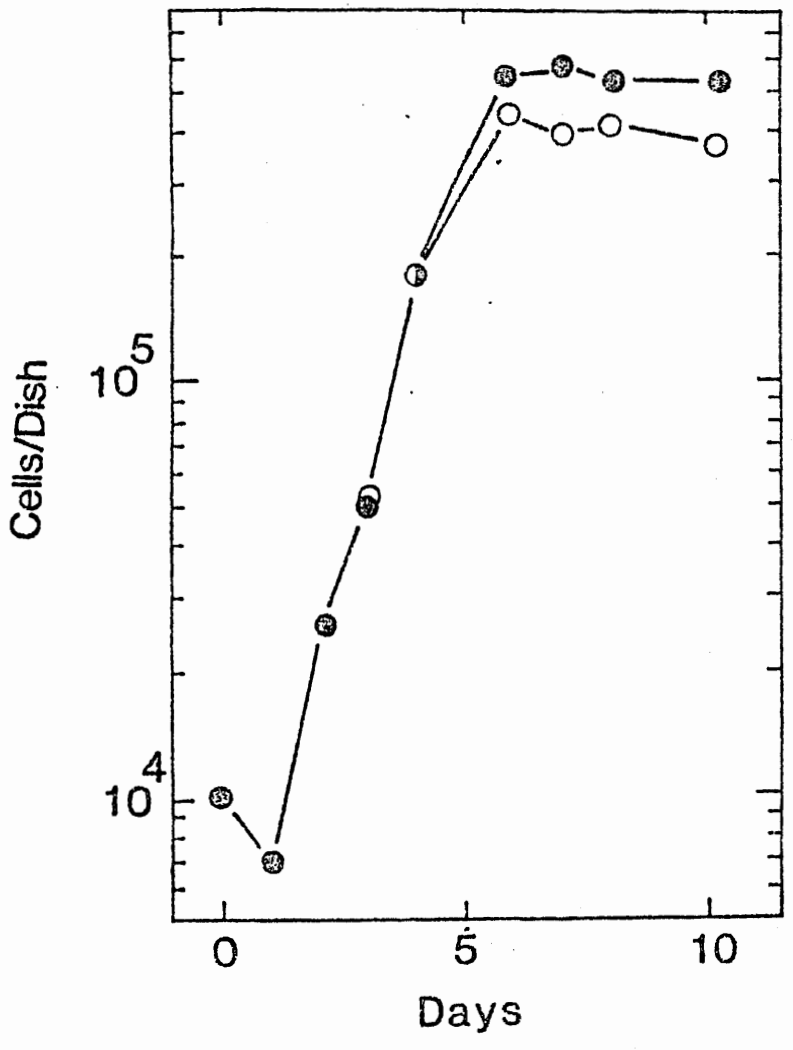


Figure 10. Effect of nicotinamide-free medium on cell division in C3H10T1/2 cells. When cells reached 2.6 X 10^4 cells/dish, SDME + 10% DFCS with nicotinamide (●) or without nicotinamide (○) was added.

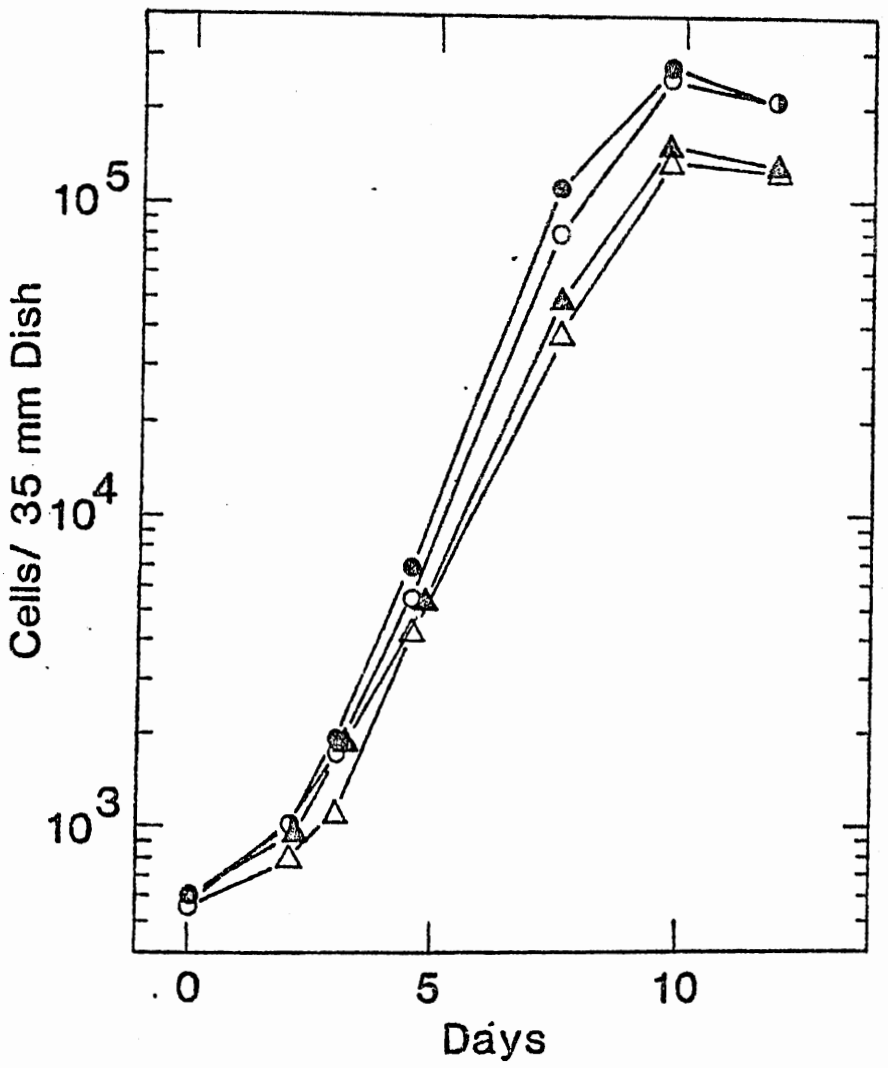


Figure 11. Cell division during transformation assay following MCA and MBA. Cells that had been grown for 3 generations in SDME + 10% DFCS in the presence or absence of nicotinamide were reseeded in the same media at 400 cells/dish and treated 24 hr later with 37 μM MCA in acetone for 24 hr. Control or nicotinamide-free medium was replenished for an additional 24 hr. Control (+Nam) cells (●), NAD-depleted (-Nam) cells (○), +Nam cells treated with MCA (▲), -Nam cells treated with MCA (△).

with BME + 10% FCS containing nicotinamide. Growth was similar for control and nicotinamide-deprived cultures. The growth rate for the carcinogen treated cultures was slower than for controls (21-22.5 hr doubling time vs. 18-19.5 hr doubling time) and reached a lower saturation density (1.2×10^5 vs 2.1×10^5 cells/dish), but did not differ due to the nicotinamide-deprivation.

The frequency of spontaneously transformed foci in exponentially dividing cultures of C3H10T1/2 cells is very low (IARC/NCI/EPA Working Group, 1985). As is shown in Table 3 no detectable foci were observed in either the controls or in the cultures that had been depleted of 90% of their NAD. The +Nam, MCA treated cultures expressed a transformation frequency of 0.63 foci/10^4 survivors. However, the -Nam, MCA treated cultures showed a transformation frequency that was twice as high as the treated +Nam cells of 1.28 foci/10^4 survivors.

As shown earlier, the transformation frequency of exponentially dividing cultures treated with the combination of MNNG and MBA was 12 times greater than MNNG alone, while the transformation frequency of cells treated with MCA and MBA was only 1.1 times higher. Thus the presence of the inhibitor enhanced the transforming

Table 3

EFFECT OF NIACIN DEPRIVATION ON 3-METHYLCHOLANTHRENE INDUCED TRANSFORMATION

TREATMENT	SURVIVORS/DISH	TOTAL SURVIVORS	#FOCI/#DISHES	TRANSFORMATION FREQUENCY $\times 10^{-4}$	NUMBER OF POPULATION DOUBLINGS
CONTROL CULTURES					
Control	2110	42200	0/20	0	6.0
37 μM MCA	2375	47500	3/20	0.63	5.9
NAD-DEPLETED CULTURES					
Control	1950	39000	0/20	0	6.0
37 μM MCA	1959	39000	5/20	1.3	6.1

C3H10T1/2 cells were grown for 3 generations in the presence or absence of nicotinamide. Cells were reseeded at 2500 cells/60 mm dish. Twenty-four hr later, cells were treated with 37 μM MCA for 24 hr. The medium was removed and control or nicotinamide-free medium was returned for an additional 24 hr. After this period, all cultures received control medium. Survivors were determined by growth curves.

capability of MNNG 12 fold more than it did with MCA. MBA also enhanced the cytotoxicity of MNNG more than MCA. It was of interest to study transformation and cytotoxicity with both types of carcinogens in cells that had been depleted of their NAD levels, particularly since MNNG was the carcinogen of choice for inhibitor studies.

Growth of cells was followed in a preliminary transformation assay. Figure 12 shows that control (+Nam cells) and NAD depleted cells (-Nam cells) had doubling times of 14 and 13.2 hr, respectively. However, cells treated with 34 μM MNNG grew more slowly (26 and 23 hr doubling times) than the acetone treated controls. Interestingly, a 2.5-fold increase in cytotoxicity was observed in the -Nam, MNNG treated cultures over that of +Nam, MNNG treated cultures when measured 24 hr after treatment.

Again, these series of experiments to test the effect of NAD-depletion on transformation were begun by first examining the effect of nicotinamide deprivation on cytotoxicity following MNNG. Survival was determined in -Nam cells by means of colony forming assays. The data are presented in Figure 13. The survival rate in -Nam cultures (no MNNG treatment) was not significantly different from +Nam control cultures. However, survival was significantly

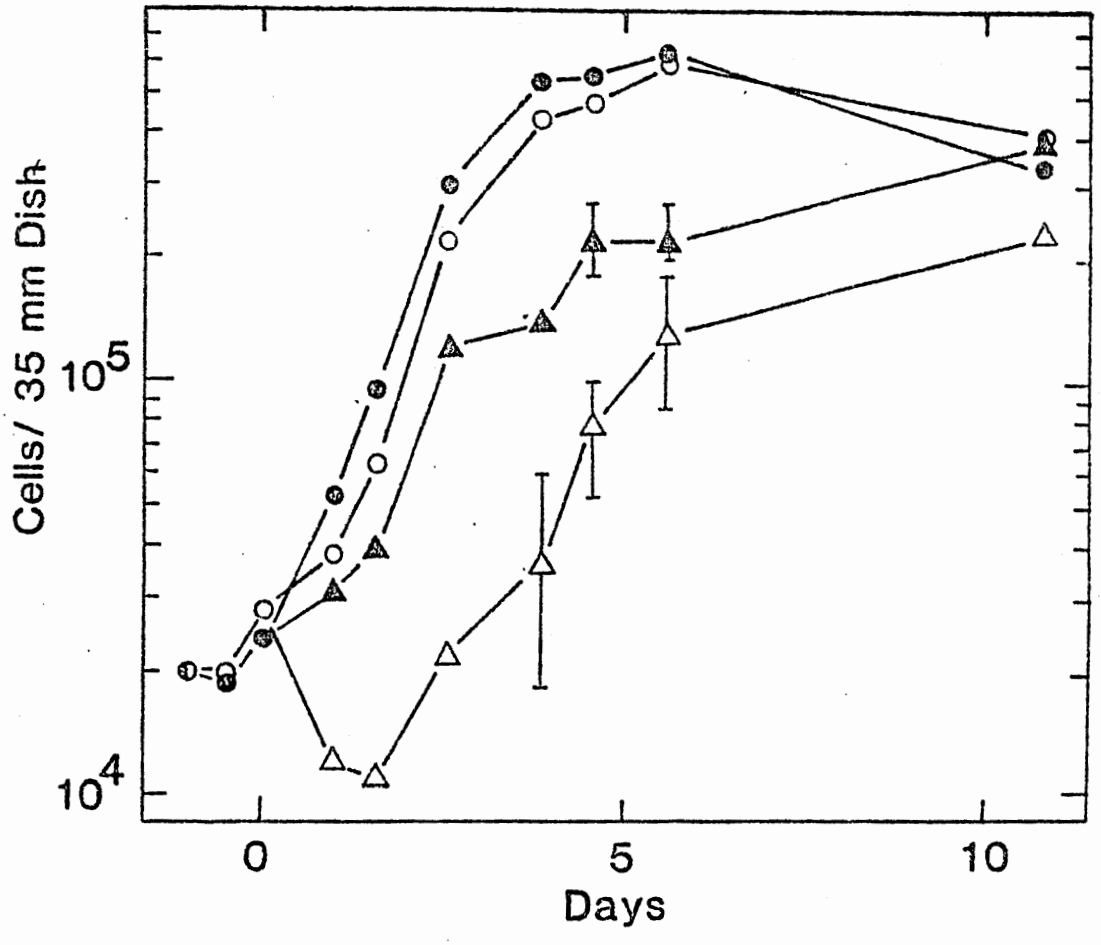


Figure 12. Growth of NAD-depleted C3H10T1/2 cells following MNNG treatment. Cells were grown in SDME + 10% DFCS in the presence (+Nam) or absence (-Nam) of nicotinamide for 3 generations before reseeding in the same media. After 24 hr they were treated with 34 μM MNNG for 20 min. The treatment media were removed and the original media were replenished for 24 hr. Control +Nam cells (●), -Nam cells (O), +Nam + MNNG (▲), -Nam + MNNG (Δ).

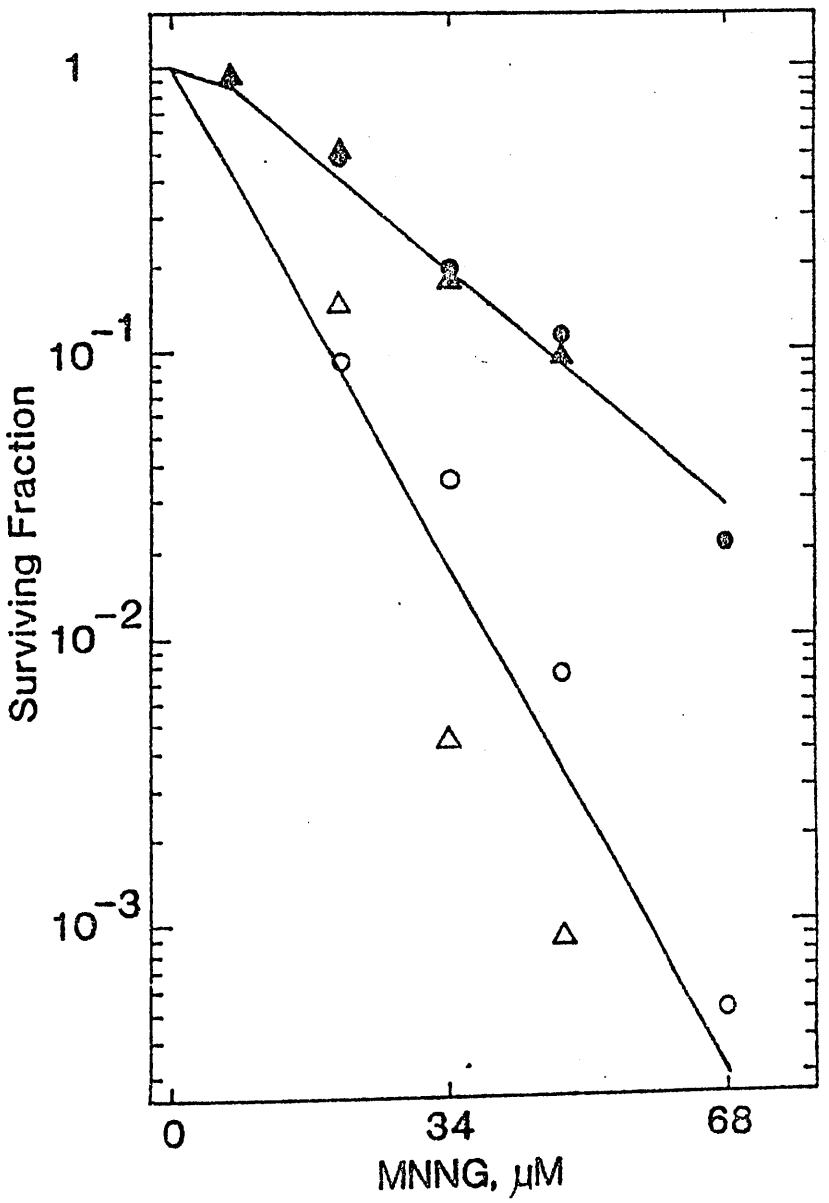


Figure 13. Survival of NAD-depleted C3H10T1/2 cells following MNNG. Control and NAD-depleted cells were reseeded at 300-1000 cells/60 mm dish in the presence (+Nam) or absence (-Nam) of nicotinamide. After 24 hr, cultures were treated with MNNG dissolved in acetone for 20 min. +Nam and -Nam media were replenished for 24 hr. Data are from two separate experiments. +Nam (●,▲) or -Nam (○,△).

decreased in NAD-depleted cells over that in control cells. At 10% survival for the +Nam cells, -Nam cultures showed a 26 fold decrease in survival.

It is highly preferred not to use DFCS in the transformation assay because important growth control factors could have been removed by the dialysis procedure. However, the use of DFCS could not be avoided since that was the only way to insure that the cells would not be supplied with pyridine nucleotides. Also, C3H10T1/2 cells were selected for growth in BME, but since the cells did not divide when nicotinamide-free SBME and DFCS was used, SDME was used. Using these altered growth conditions, the cell monolayer had a different appearance than it did with standard growth conditions. A photograph of typical dishes is shown in Plate 6. In some cases patches of cells with altered morphology appeared. These were type I foci. The cells in these foci were more tightly packed and did not have the normal fibroblastic appearance. These foci were very small; they contained less than fifty cells. These type I foci were more plentiful in the NAD-depleted cultures than in the controls. They were larger and more densely stained in the MNNG-treated plates than in the controls and appeared to be dose dependent since there were

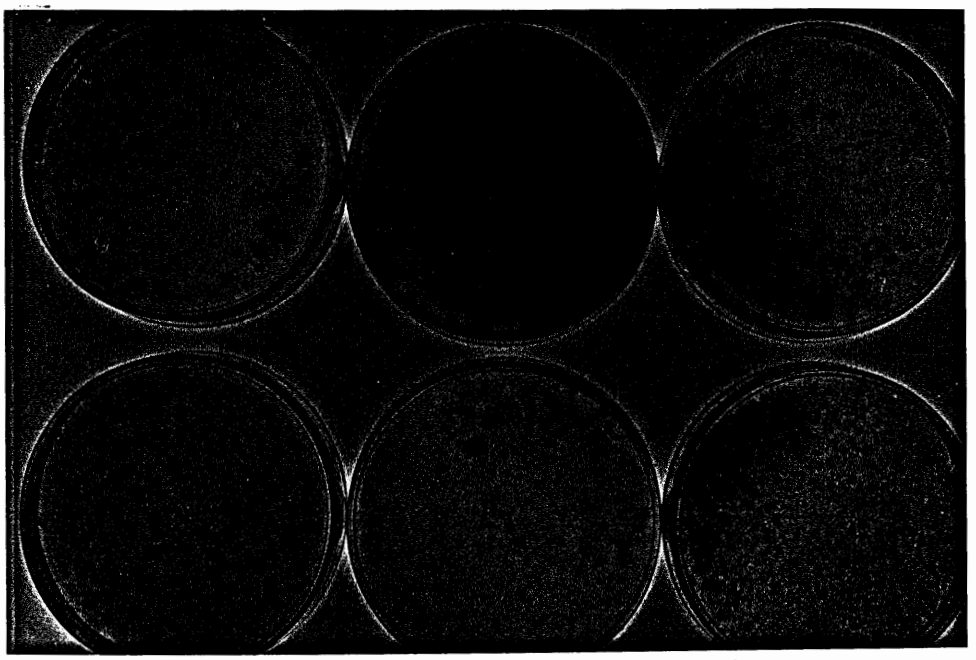


Plate 6. Dishes of cells grown in SDME plus dialyzed FCS and treated with MNNG. Dishes on the top row contain cells grown in the presence of nicotinamide. Those on the bottom row were grown in the absence of nicotinamide. Controls are on the left.

more in the 34 μM MNNG than in the 20.4 μM MNNG treated dishes.

Malignant transformed foci (type II or III) were not detected in either the nicotinamide-containing or nicotinamide-free cultures that did not receive MNNG. These data are shown in Table 4. At each dose of MNNG there were more absolute numbers of foci in the -Nam cultures than in the +Nam cultures, where the same number of survivors were plated for each dose. Thus, the increased number of foci per 10^4 survivors in the -Nam cultures can be considered to be valid, according to established criteria for the transformation assay (Mordan, 1983). The ratio of the transformation frequency of MNNG treated, -Nam cultures to that of MNNG treated, +Nam cultures remained constant with dose, at about 1.4. This value was on the same order as that obtained with MCA and nicotinamide-deprivation, where the ratio was two.

The increase in transformation frequency in NAD-depleted cells could be the result of several events. The results could be interpreted to mean that when poly(ADP-ribosyl)ation events are inhibited, an increase in transformation frequency results. Alternatively, or additionally, a secondary effect could be responsible for

Table 4

EFFECT OF NIACIN DEPRIVATION ON MALIGNANT TRANSFORMATION

(EXPONENTIALLY DIVIDING CELLS)

TREATMENT	CELLS SEEDED/DISH	SURVIVORS/DISH	TOTAL SURVIVORS	#FOCI/# DISHES	TRANSFORMATION FREQUENCY X 10^{-4}	POPULATION DOUBLING
CONTROL CULTURES						
Control	3.1 X 10^3	689	34457	0/50	0	10.9
20 μM MNNG	5.0 X 10^3	234	23400	13/100	5.6	12.5
34 μM MNNG	1.0 X 10^4	133	13068	37/100	28	13.3
NAD-DEPLETED CULTURES						
Control	3.1 X 10^3	843	42142	0/50	0	11.6
20 μM MNNG	1.0 X 10^4	240	23520	18/100	7.6	12.4
34 μM MNNG	2.0 X 10^4	115	11040	43/100	39	13.5

C3H10T1/2 cells were grown for 3 generations in the presence or absence of nicotinamide. Cells were reseeded into 60 mm dishes and 24 hr later treated with MNNG for 20 min. The medium was removed and control or nicotinamide-free media was returned for 24 hr. At the end of this period, all cultures received control medium for the remainder of the assay. Seeding densities for the various treatments were as follows. Control cultures: Control 3.1 X 10^3 cells/dish, 20 μM MNNG 5 X 10^3 cells/dish, 34 μM MNNG 1 X 10^4 cells/dish. NAD-depleted cultures: Control 3.1 X 10^3 cells/dish, 20 μM MNNG 1 X 10^4 cells/dish, 34 μM MNNG 2 X 10^4 cells/ dish. Survivors were determined by colony forming assays.

the increase in transformation frequency. When cells are treated with chemical carcinogens, the NAD level drops (Jacobson et al., 1980). Since the NAD level of cells is intimately related to the energy charge, an experiment was carried out to examine both NAD and ATP levels in nicotinamide-depleted and control cultures following MNNG treatment. The data of Figure 14 shows that the NAD level dropped drastically in +Nam cultures with increasing dose of MNNG, to as low as 14% of untreated controls in cells treated with 68 μM MNNG. The NAD content of the -Nam cells prior to MNNG treatment was approximately 6% of the untreated control cells and dropped to a level below detection in this experiment following treatment with 34 μM MNNG. Figure 15 shows that in contrast to the NAD content, ATP levels were not altered by MNNG treatment up to 68 μM in control cells. However, the ATP levels of the -Nam cultures dropped to as low as 27% of untreated controls following 68 μM MNNG. Thus, the increases in transformation may be due to the secondary effect of depletion of cellular ATP.

The Effect of Inhibitors of Poly(ADP-ribose) Polymerase on Survival in Synchronized Cultures

Rates of survival, mutation and transformation vary

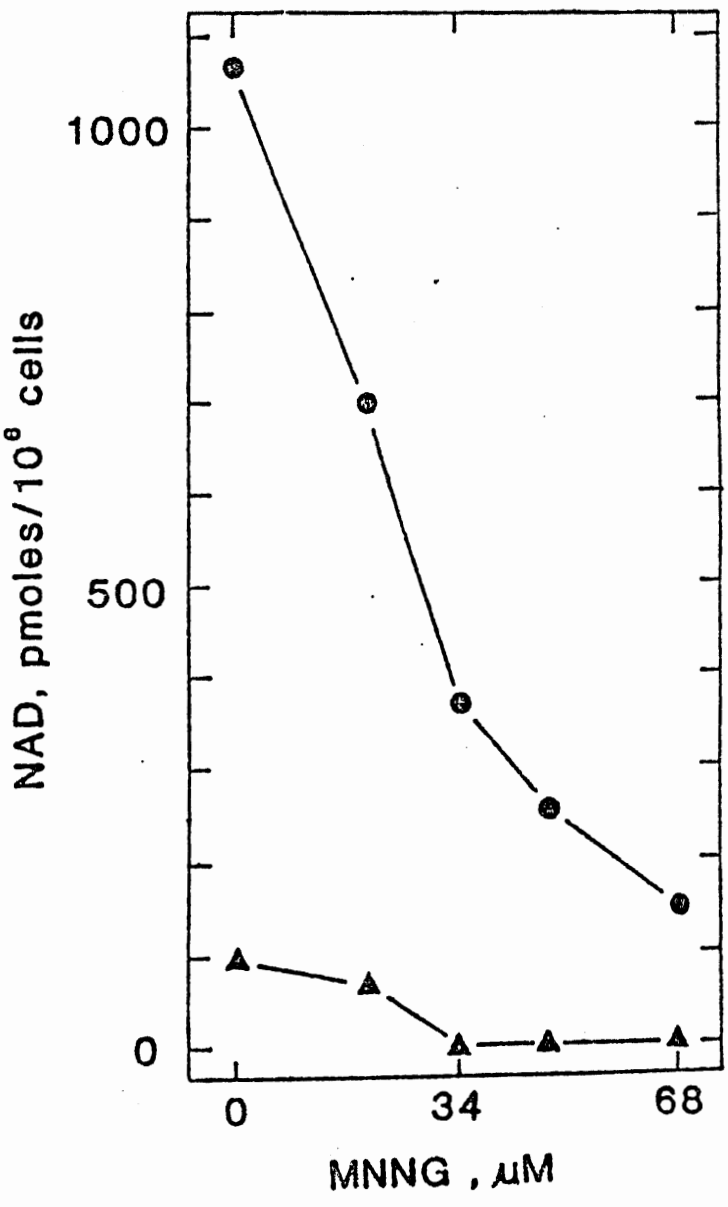


Figure 14. Cellular NAD content in nicotinamide deprived C3H10T1/2 cells following MNNG treatment. Cells were seeded in BME + 10% FCS and 24 hr later, the medium was removed and SDME + 10% DFCS with (+Nam) and without (-Nam) nicotinamide was added. Cells were refed once. After 96 hr, cultures were treated with MNNG for 20 min. Three hr later, NAD was extracted. NAD content in +Nam cell (●) or -Nam cells (▲).

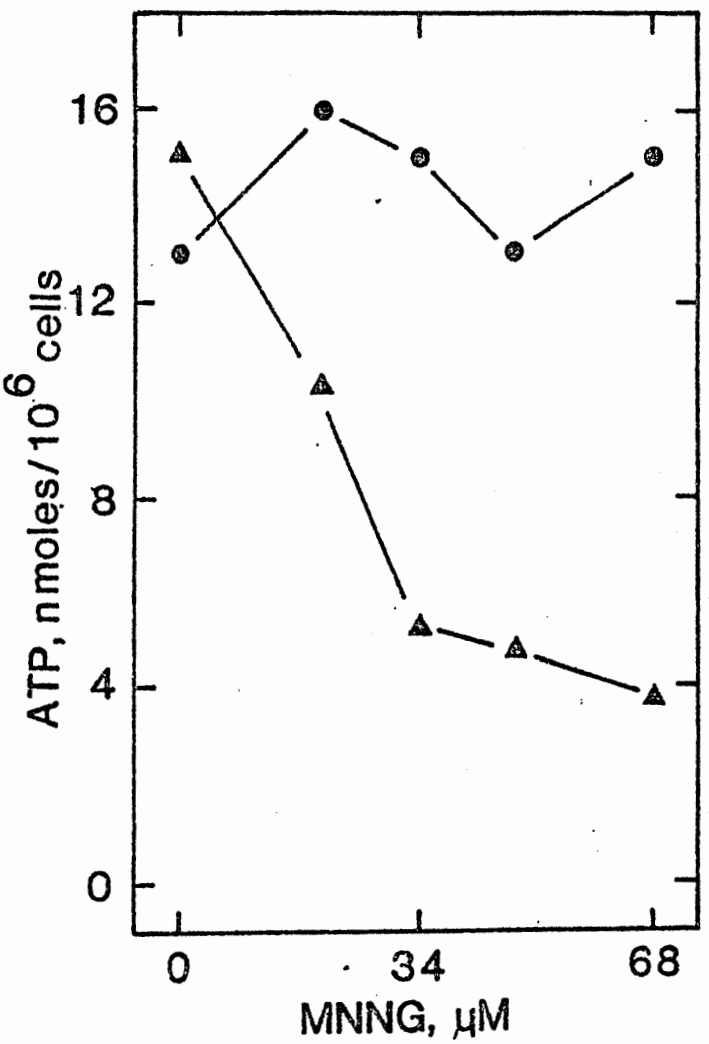


Figure 15. Cellular ATP levels in niconinamide-deprived C3H10T1/2 cells following MNNG treatment. Cells were treated as described in Figure 16 and ATP was extracted in +Nam cells (●) and -Nam cells (▲).

depending on the phase of the cell cycle during which cells are treated with MNNG (McCormick and Bertram, 1982). Transformation frequency is low in asynchronously dividing cells treated with MNNG, as shown above. It has been postulated that poly(ADP-ribose) may be involved in regulating the cell cycle (Haines et al., 1969) or in coordinating cell cycle events with DNA repair (Jacobson et al., 1984). Studies were begun to determine the role of poly(ADP-ribose) in survival in cells treated during G_1 and at the G_1/S border of the cell cycle. Cells were synchronized in G_1 by isoleucine deprivation (McCormick and Bertram, 1982). They were treated with MNNG and MBA for 24 hr beginning at 1 or 8 hr after release from isoleucine deprivation. In order to follow the progression of the cell cycle after treatment, cells were pulse labeled with [methyl-^{3}H] thymidine for 60 min at 0, 8, and 14 hr after release. Figure 16 shows that cells pulsed at 8 hr began to incorporate radioactivity and at 14 hr reached maximal incorporation. Therefore, the cells treated with carcinogen at 1 hr after isoleucine release were in G_1 and those treated at 8 hr were at the G_1/S border. The results of survival studies in synchronized cultures are shown in Figure 17. The data show that survival decreased with

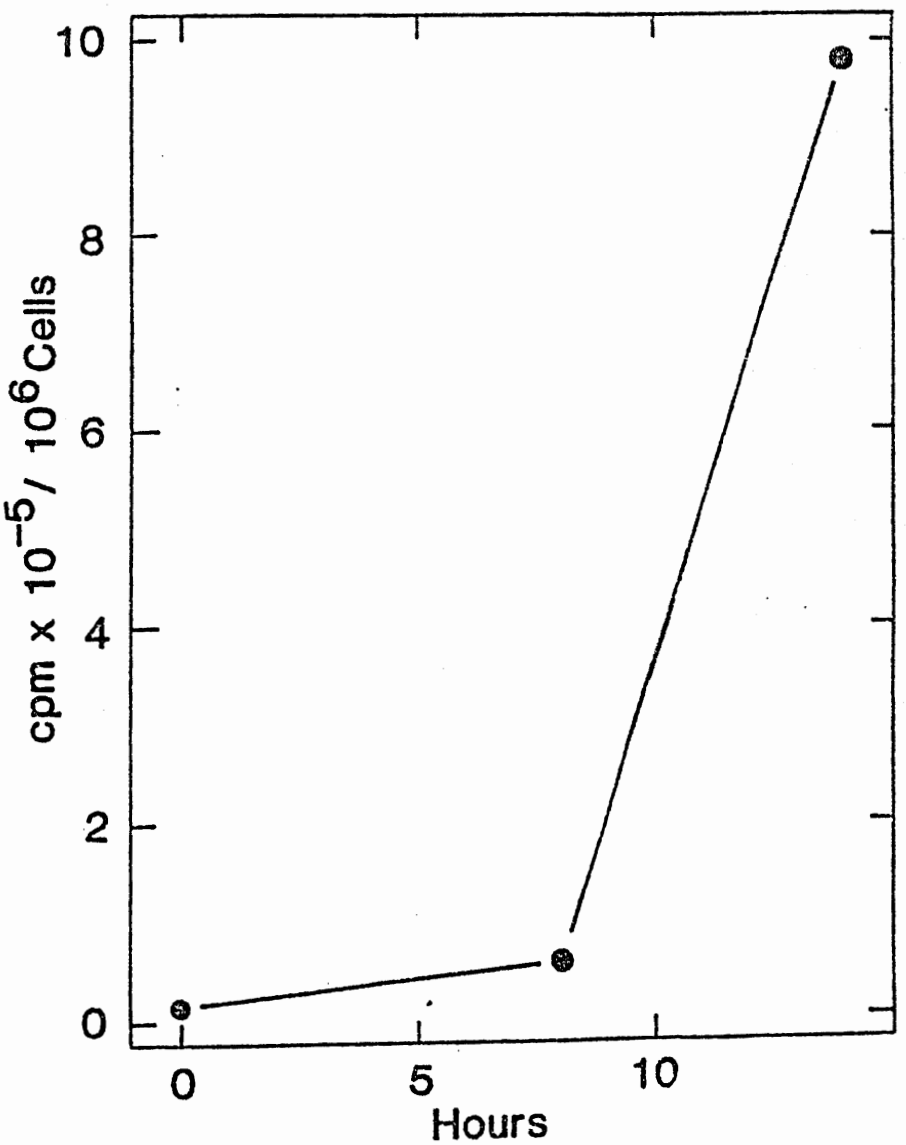


Figure 16. Time course of DNA synthesis in C3H10T1/2 cells released from isoleucine deprivation. Cells were seeded at 800 cells/dish and treated as described in figure 9. Cells were pulse labeled at the indicated times after release with 1 μCi/ml [methyl-^{3}H] Thd and the acid insoluble pools were extracted (●).

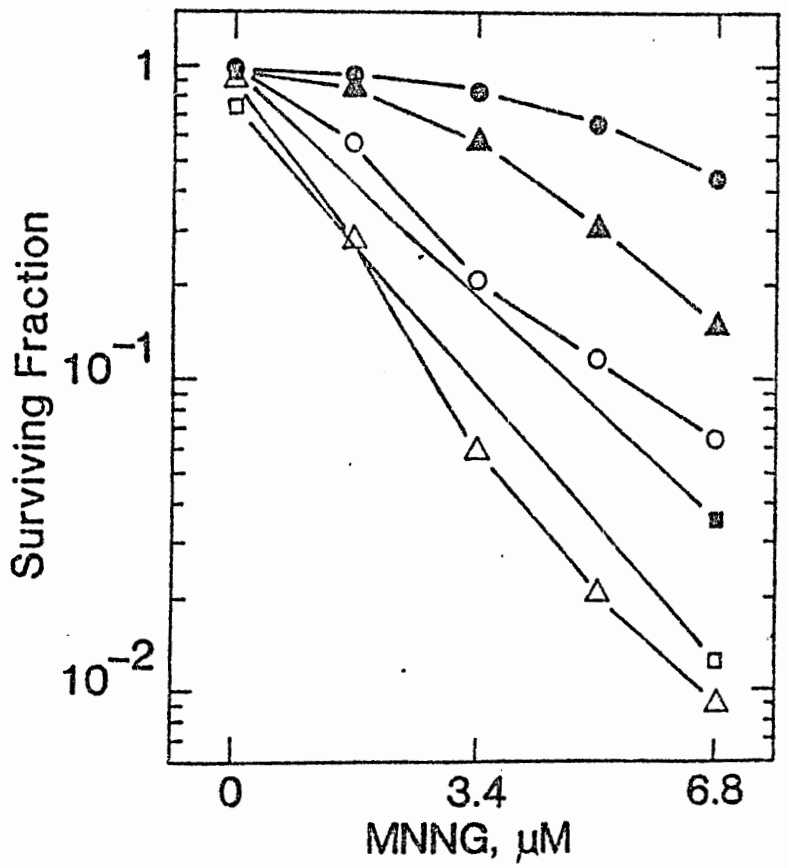


Figure 17. Survival of C3H10T1/2 cells synchronized by isoleucine deprivation and treated with MNNG and/ or MBA. Forty-eight hr post-confluent cultures were reseeded in isoleucine-free BME containing 10% DFCS. Thirty-six hr later, the cells were released by the addition of BME containing 15% FCS and 100 μg/ml isoleucine. At the indicated times following release, cells were treated with MNNG and 0.5 mM MBA. Survival is expressed relative to the acetone control for each time point. Closed symbols represent cells treated with MNNG alone 1 hr (●), 4.5 hr (▲), and 8 hr (■) after release. Open symbols represent cells treated with MNNG and MBA 1 hr (○), 4.5 hr (△), and 8 hr (□) after release.

increasing doses of MNNG. At each dose survival was lower in cultures treated with both MNNG and MBA than in cells treated with MNNG alone. In addition, survival decreased when cells were treated at later times after release from isoleucine deprivation. Presumably, this was due to the greater sensitivity of cells treated in S phase than those treated in G_1. The low survival rates obtained in synchronized cells treated with both MNNG and MBA would result in transformation assays that would be prohibitively large and costly using this cell line.

Thyroid hormone (T3) may be a necessary component in the induction of transformation (Borek et al., 1983). T3 is normally present in FCS at a concentration of 2nM, and may be removed from sera by dialysis. An additional isoleucine synchrony experiment was performed. In an attempt to increase transformation is cases where DFCS was used, the medium was supplemented with T3. T3 (2 nM) was added during the period of isoleucine deprivation in order to compensate for its absence in DFCS. Figure 18 shows that survival in cells synchronized in the presence of T3 and treated 7.5 hr after release was drastically reduced. Thus, although T3 may be needed for induction of transformation, it has a negative effect on cell survival.

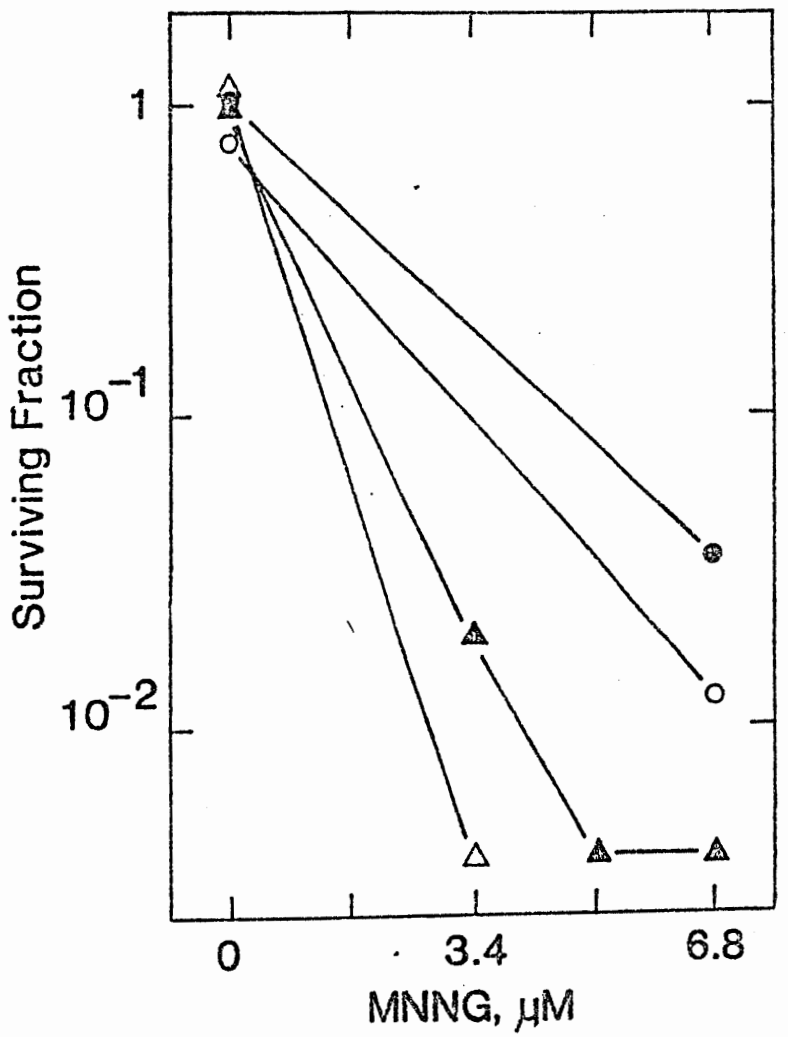


Figure 18. Survival in C3H10T1/2 cells synchronized by isoleucine deprivation and supplemented with T_3 and treated with MNNG and MBA. Cells were treated as described in Figure 17 except T_3 was added during the period when DFCS was used. Cells treated 8 hr after release with MNNG (●) and MNNG + MBA (O). Cells synchronized in the presence of T_3 and treated 7.5 hr after release with MNNG (▲) and MNNG + MBA (Δ).

Experiments in this study that utilized nicotinamide deprivation required DFCS. Transformation was detectable without resorting to the addition of T3. Thus, T3 could have been present in the DFCS, or else it was not required for expression of transformation under these culture conditions. Therefore T3 and isoleucine deprivation was not pursued further.

Effect of Inhibitors of Poly(ADP-ribose) Polymerase on Transformation in Confluent Cells

The role of poly(ADP-ribose) in transformation could be due to its involvement in DNA repair. Alternatively, it could be due to activities involved in the coordination of DNA repair with cell division. In order to distinguish between these two possibilities, experiments were begun using cultures treated with MNNG and MBA in the confluent state. In order to correct for survival in the transformation experiments, survival was first studied. The data from colony forming assays is shown in Figure 19. The cells were treated with both 3.4 or 6.8 μM MNNG and 1 mM MBA for 20 min, and with MBA for an additional 48 hr. At the end of this time period, the cells were reseeded into a colony forming assay. It can be seen that there was no difference in survival when cells were treated with the

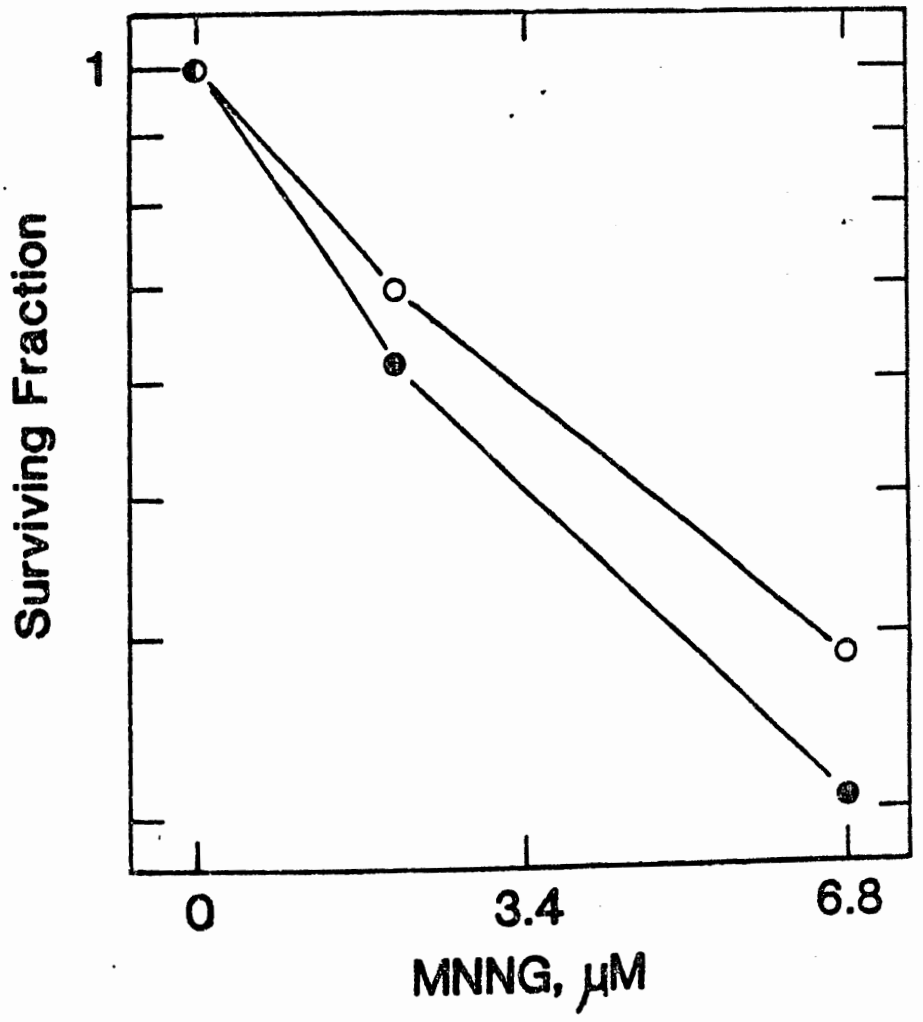


Figure 19. Survival of confluent C3H10T1/2 cells following MNNG and MBA treatment. Cells were held at confluence 48 hr and treated for 20 min with MNNG and MBA. MBA or acetone containing media were replaced and cells were held at confluence 48 hr prior to seeding into colony forming assay. Treatment with MNNG (●) or MNNG and MBA (O).

combination of MNNG and MBA as compared to MNNG alone. This is in contrast to the enhancement of MNNG-induced cytotoxicity by MBA in exponentially dividing cells shown previosly.

Transformation rates were studied using postconfluent cells treated with 20 μM MNNG and 1 mM MBA for 20 min. MBA was returned for an additional 48 hr. As shown in Table 5, transformation frequency was virtually identical in cells treated with MNNG alone and cells treated with both MNNG and inhibitors of poly(ADP-ribose). Again, this is in contrast to the situation seen in exponentially dividing cells.

Effect of Nicotinamide Deprivation on Transformation in Confluent Cells

In exponentially dividing cells, NAD-depleted cells that were damaged with MNNG were ATP deficient. They also had lower rates of survival and higher rates of transformation than damaged, +Nam cells. Experiments were designed in order to test whether nondividing cells that were depleted of their NAD levels showed similar responses to DNA damage. C3H10T1/2 cells were grown in either the presence or absence of nicotinamide until they reached confluence. The cells were fed and allowed to remain in

Table 5

MALIGNANT TRANSFORMATION IN CONFLUENT C3H10T1/2 CELLS

TREATMENT	CELLS SEEDED/DISH	SURVIVORS/DISH	TOTAL SURVIVORS	#FOCI/# DISHES	TRANSFORMATION FREQUENCY X 10^{-4}	POPULATION DOUBLING
Control	3.1 X 10^3	771	39341	0/51	0	10.7
MBA	3.1 X 10^3	818	40896	2/50	0.49	10.7
MNNG	8.4 X 10^3	1415	72167	3/51	0.42	9.8
MNNG + MBA	8.4 X 10^3	1608	80409	3/50	0.37	9.7

C3H10T1/2 cells were treated 48 hr postconfluence with 20 μM MNNG and 1 mM MBA for 20 min. MBA was returned for an additional 48 hr. Cells were reseeded at approximately 3.1 X 10^3 cells/dish for control and MBA treated dishes and 8.4 X 10^3 cells/ dish for MNNG and MNNG + MBA treated dishes. Survivors were determined by colony forming assays.

the confluent state an additional 48 hr. They were then treated with 0, 20.4, or 34 μM MNNG in acetone for 20 min. The carcinogen containing medium was removed. Control or nicotinamide-free medium was returned to the appropriate dishes. After an additional 48 hr, the cells were reseeded into nicotinamide containing medium for the transformation assay and colony forming assay. As shown in Figure 20 the +Nam control cultures had only a slightly higher rate of survival than the -Nam control cultures. The MNNG treated cells that had been depleted of their NAD before MNNG treatment had very similar survival rates when compared to those grown in nicotinamide. This is in contrast to studies in exponentially dividing cells that showed lower survival in MNNG treated, nicotinamide-depleted cells.

The transformation frequencies of the MNNG treated cultures showed only small increases in transformation. The data are shown in Table 6. The nicotinamide-depleted cultures treated with 20.4 μM MNNG had nearly twice as many foci per 10^4 survivors than the +Nam, 20.4 μM MNNG treated cultures. The number of survivors for this treatment were evenly matched. However, the +Nam, 34 μM MNNG treated cultures had 1.5 times as many foci per 10^4 survivors as the -Nam, 34 μM treated cells. Survival was difficult to

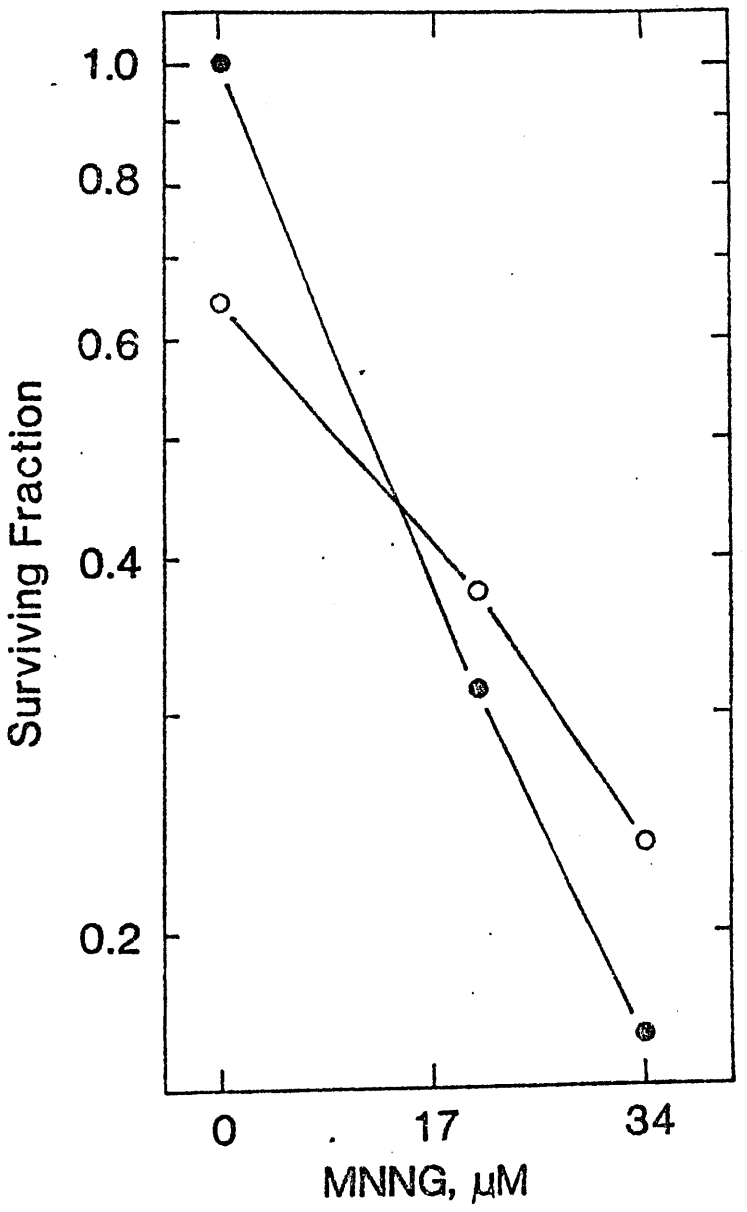


Figure 20. Survival of confluent, NAD-depleted C3H10T1/2 cells following MNNG treatment. Cells were seeded in BME + 10% FCS and 24 hr later the medium was replaced with SDME + 10% DFCS with or without nicotinamide. Cells were refed after 2 days and at confluence. Forty-eight hr postconfluence, cells were treated with MNNG in acetone for 20 min. The control and nicotinamide-free media were replaced and the cells were held for 48 hr at confluence prior to reseeding into BME + 10% FCS for colony forming assay. +Nam cultures (●) and -Nam cultures (O).

Table 6

EFFECT OF NIACIN DEPRIVATION ON MALIGNANT TRANSFORMATION

(QUIESCENT CELLS)

TREATMENT	CELLS SEEDED/DISH	SURVIVORS/DISH	TOTAL SURVIVORS	#FOCI/# DISHES	TRANSFORMATION FREQUENCY X 10^{-4}	POPULATION DOUBLING
CONTROL CULTURES						
Control	9.6 X 10^3	887	41689	0/50	0	10.5
20 μM MNNG	1.3 X 10^4	486	22842	2/47	0.88	11.4
34 μM MNNG	2.7 X 10^4	524	24628	17/47	6.9	11.3
NAD-DEPLETED CULTURES						
Control	5.2 X 10^3	387	19350	4/50	2.1	11.7
20 μM MNNG	1.2 X 10^4	507	24843	4/49	1.6	11.3
34 μM MNNG	4.8 X 10^4	1306	65300	29/50	4.4	9.93

C3H10T1/2 cells were grown for 4.5 generations in the presence or absence of nicotinamide. Cells were treated 48 hr postconfluence with MNNG for 20 min. Control or nicotinamide-free medium was returned for 48 hr. Cells were reseeded at the following densities. Control cultures: Control 9.6 X 10^3 cells/dish, 20 μM MNNG 1.3 X 10^4 cells/dish, 34 μM MNNG 2.7 X 10^4 cells/dish. NAD-depleted cultures: Control 5.2 X 10^3 cells/dish, 20 μM MNNG 1.2 X 10^4 cells/dish, 34 μM MNNG 4.8 X 10^4 cells/dish. Survivors were determined by colony forming assays.

control in this experiment. Because high numbers of survivors were plated in the -Nam, 34 μM MNNG dishes, the apparent transformation frequency was decreased.

Spontaneous Transformants in Confluent Cultures

Ordinarily, control cultures do not develop transformed foci. Transformed foci were not detected in the acetone control dishes and this agrees with the results seen in exponential cultures. However, a high rate of transformation was detected in the confluent cells treated with MBA alone (Table 5). These "spontaneous" transformants that appeared in the absence of DNA damage were not detected in exponentially dividing cells.

Likewise, the similar results were observed in the nicotinamide-depletion experiments (Table 6). The +Nam controls did not contain foci. However, there was a high rate of spontaneously transformed foci in the -Nam control dishes. It is interesting to note that there were spontaneously occurring foci in both the -Nam confluent cultures and the inhibitor treated, confluent cultures mentioned earlier.

NAD Redox Levels in the Presence of Ascorbate in Nicotinamide Deprived and Control C3H10T1/2 cells

Transformation is prevented when cells are treated

with ascorbate as late as 21 days after the exposure of cells to carcinogens (Benedict et al., 1980). Transformation can also be reversed by ascorbate (Benedict et al., 1982). The effects of ascorbate on the oxidation state of the pyridine nucleotides, NAD and NADP, were examined in control and nicotinamide-deprived C3H10T1/2 cells in order to determine whether changes in the redox state could be related to the effect of ascorbate on transformation. This was also of interest since differences were observed in the transformation frequency of control cells and cells that were depleted of their NAD.

As shown in Figure 21 when ascorbate was fed to cells at a daily dose of 5.7 μM there was no effect on the growth rate of either nicotinamide-depleted or control cultures. In order to measure NAD levels in the cells, the standard NAD assay was modified to detect the low levels of NAD in nicotinamide-deprived cells. To increase the sensitivity of the assay, enzyme concentration and cycling time were first increased, as shown in Figure 22. Optimal cycling time was 90 min with a concentration of 0.24 mg/ml alcohol dehydrogenase (ADH). These conditions were used in an assay to measure NAD standards of 0-40 pmoles per assay. The assay was not linear. It was hypothesized that the

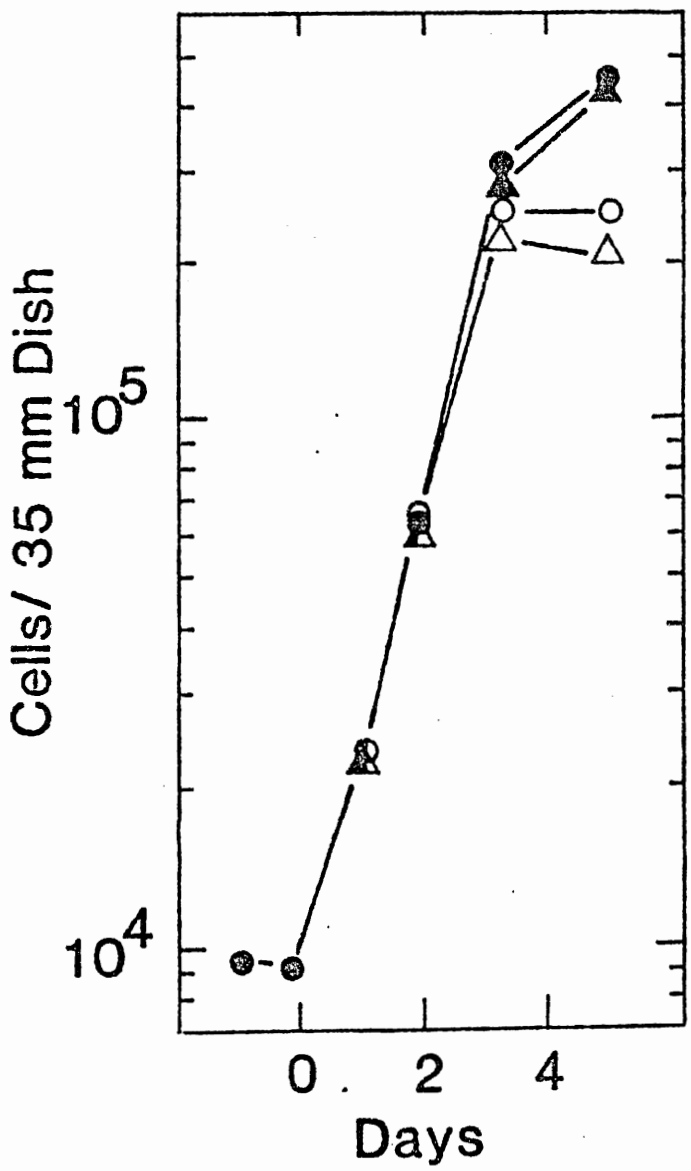


Figure 21. Growth of nicotinamide-depleted C3H10T1/2 cells in the presence of ascorbate. Twenty-four hr after seeding, cells were fed SDME + 10% DFCS with or without nicotinamide and 5.7 μM ascorbate. Cells were refed daily. +Nam cells (●), -Nam cells (○), +Nam + ascorbate (▲), -Nam + ascorbate (△).

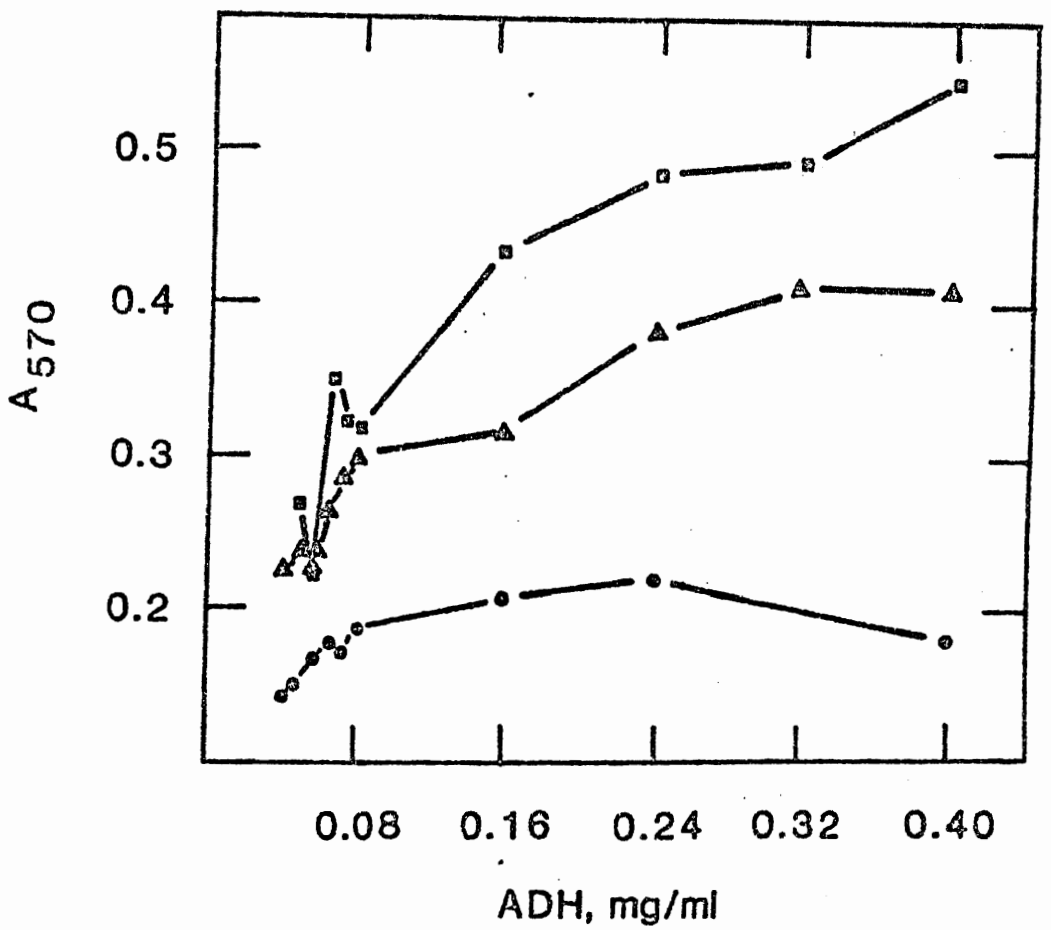


Figure 22. Effects of enzyme concentration and cycling time on NAD assay. Thirty-one pmoles/assay NAD was measured using 30 (●), 60 (▲), and 90 (■) min cycling time and 0.04 to 0.4 mg/ml ADH.

nonlinearity was due to a limiting amount of phenazine ethosulfate (PES) in the assay mix. Thus, various concentrations of PES were used in an assay utilizing optimal ADH concentration (0.24 mg/ml) and cycling time (90 min). As shown in Figure 23, it was found that 4 mM PES gave a linear response with the greatest slope. The data in Figure 24 show standard curves using optimal PES, two cycling times and two enzyme concentrations. To save enzyme cost, a 90 min cycling time using 0.08 mg/ml ADH was chosen as the standard assay condition for measuring NAD in the ascorbate studies. Figure 25 shows the percent of NAD that was in the reduced form. The presence of ascorbate in the culture medium did not significantly alter the percent of NAD in the reduced form in either control or nicotinamide-deprived cells. However, the nicotinamide-deprived controls contained 2-fold higher percent reduced NAD levels than the control cultures after 4.8 generations in nicotinamide-free medium. Figure 26 shows the percent of NADP that was in the reduced form. No significant differences were observed due to ascorbate or nicotinamide-deprivation. Although ascorbate did not affect the redox levels of NAD and NADP in control cultures and cultures deprived of nicotinamide for 3 to 5

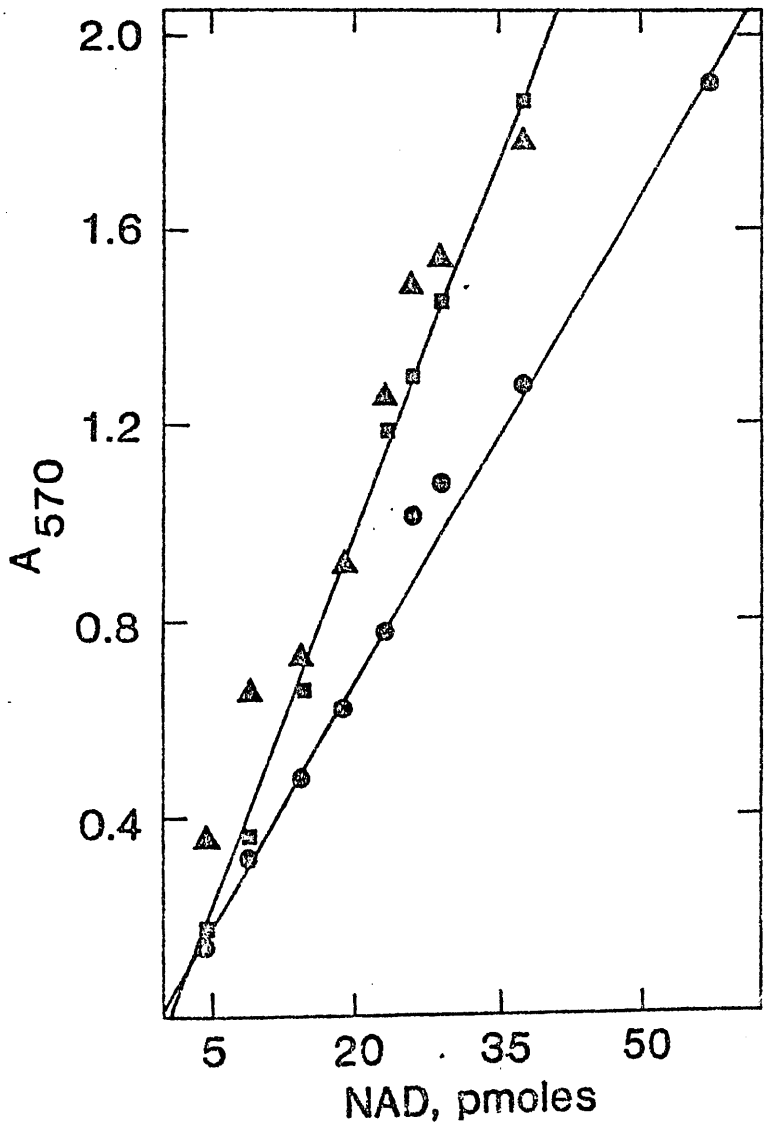


Figure 23. Effect of PES concentration on NAD assay. NAD standards were assayed using 0.24 mg/ml ADH for 90 min cycling time. PES concentrations were 2 mM (●), 4 mM (▲), and 6 mM (■).

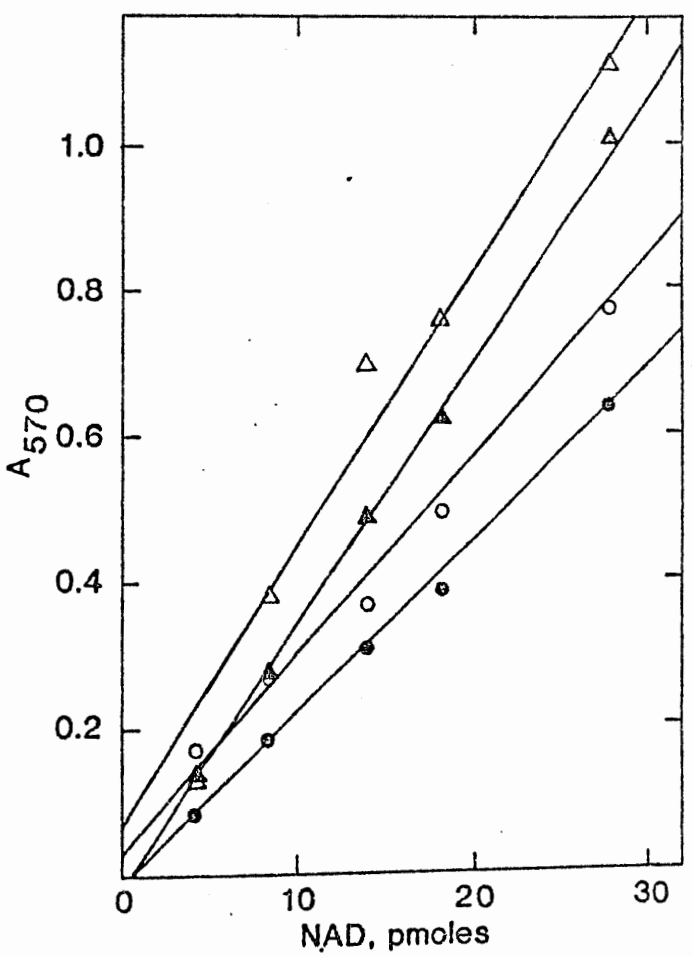


Figure 24. Effect of enzyme concentrations and cycling time on NAD assay with optimal PES. NAD standards of 0-30 pmoles/assay tube were measured using 6 mM PES. Closed symbols: 60 min cycling time with 0.08 mg/ml ADH (●) or 0.16 mg/ml ADH (▲). Open symbols: 90 min cycling time using 0.08 mg/ml ADH (○) or 0.16 mg/ml ADH (△).

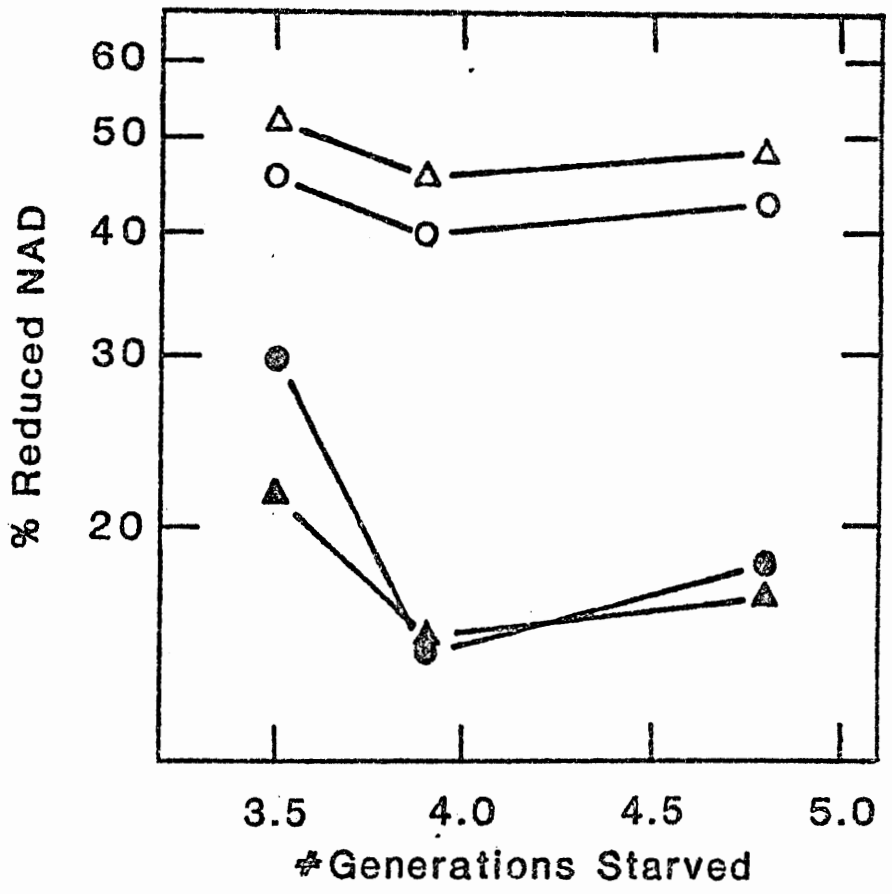


Figure 25. Effect of ascorbate on the oxidation-reduction levels of NAD in nicotinamide-deprived cells. NAD(H) was extracted from C3H10T1/2 cells that had been fed ascorbate (5.7 μM) during the period of nicotinamide-deprivation. Percent reduced NAD in control cells grown in the presence (▲) or absence (●) of ascorbate, and nicotinamide-deprived cells grown in the presence (△) or absence (○) of ascorbate.

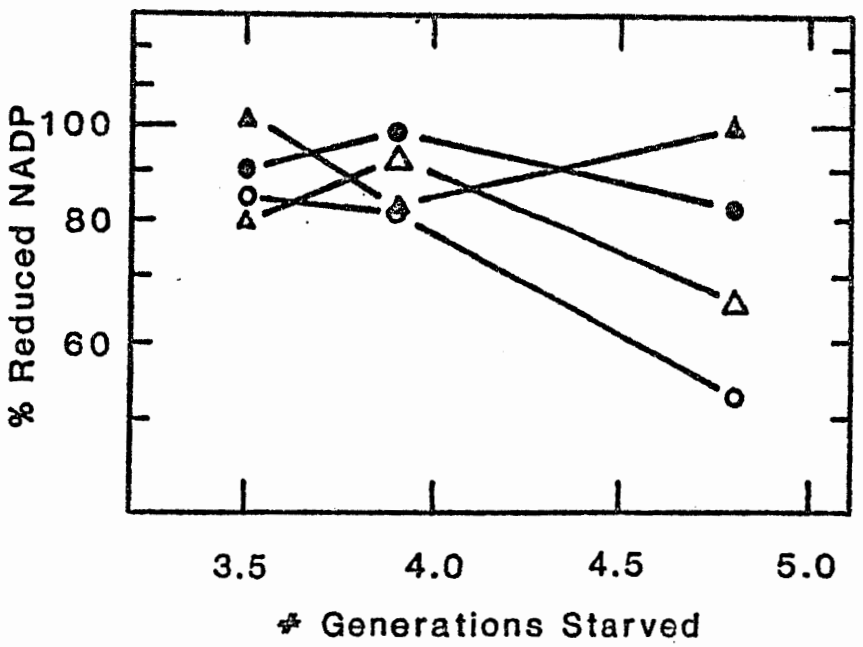


Figure 26. Effect of ascorbate on the oxidation-reduction levels of NADP in nicotinamide-deprived cells. Cells were grown as in Figure 26. NADP(H) was extracted. Percent reduced NADP in cells grown in the presence (▲) or absence (●) of ascorbate, and nicotinamide-deprived cells grown in the presence (△) or absence (○) of ascorbate.

generations, it may affect the transformation rates by some other mechanism. Moreover, ascorbate may alter the levels in DNA damaged cells. These questions require further study.

DISCUSSION

The major objective of this study was to investigate the role of poly(ADP-ribose) metabolism in malignant transformation. Evidence has accumulated that poly(ADP-ribose) is involved in the cellular recovery from DNA damage (Jacobson et al., 1985c). If poly(ADP-ribose) is involved in cellular recovery following DNA damage, then alteration of the poly(ADP-ribose) metabolism may lead to increases in the transformation frequency. In order to probe this question, studies were conducted using exponentially dividing C3H10T1/2 cells, a model cell line for the study of malignant transformation. The first approach to studying altered poly(ADP-ribose) metabolism utilized an inhibitor of poly(ADP-ribose) polymerase.

In the present study, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a potent alkylating agent, was applied to C3H10T1/2 cells in the presence of the polymerase inhibitor, MBA. A marked (12-fold) increase in transformation frequency was seen over cultures treated with MNNG alone (Table 2).

During the course of this study, a similar result was

reported by Lubet et al. (1984) using a different system. 3T3 cells were utilized with ethyl methane sulfonate (EMS) as the alkylating agent and 3-aminobenzamide (3-ABA) as the inhibitor of poly(ADP-ribose) polymerase. An enhancement of transformation frequency was observed. These workers also saw an enhancement of cytotoxicity with EMS and MBA. However, the number of survivors plated for each treatment were not carefully matched. It is especially important when using C3H10T1/2 cells to match the number of survivors for the various treatments. This is necessary since transformation rates have been shown to be affected by the number of population doublings, which is determined by the number of survivors plated (Mordan et al., 1983). Lubet et al. did not fully address this issue in their studies using 3T3 cells. Thus, the calculated transformation frequencies may be overestimated.

The increases in transformation frequency using inhibitors found in these two studies are in contrast to the data reported by other investigators. Kun et al. (1983) observed that when human fibroblasts treated with methylazoxymethanol acetate (MMA) were grown in benzamide, transformation was inhibited. Borek et al. (1984a) reported decreases in transformation frequency in C3H10T1/2

cells and hamster embryo cells following X-rays or UV when benzamide or 3-ABA was present. In another report, Borek et al. (1984b) observed an enhancement of transformation frequency due to inhibitors following treatment by ethylating agents, such as EMS but no increases in cells treated with methylating agents. However, these investigators did not present data showing increases in cytotoxicity with the inhibitors. The cytotoxic damage caused by alkylating agents was shown to be enhanced by inhibitors of poly(ADP-ribose) polymerase by Jacobson et al. (1984) and was confirmed by the studies of this dissertation (Figure 9 and Figure 8). Since no cytotoxicity data was presented by Kun et al. or Borek et al., and transformation rates depend on cell survival, these published data are difficult to evaluate.

It is interesting to note that in this study, when exponentially dividing C3H10T1/2 cells were treated with the carcinogen 3-methylcholanthrene (MCA) and the poly(ADP-ribose) polymerase inhibitor, MBA, only a slight increase (11%) in transformation was observed over cultures treated with MCA alone (Table 1). The damage caused by MCA treatment is in the form of bulky adducts. It was shown in this study (Figure 6) and by others (Walker, 1984) that

inhibitors of poly(ADP-ribose) have little cocytotoxic effect with DNA damaging agents such as MCA, which form bulky adducts.

The increase in transformation frequency observed when using a poly(ADP-ribose) polymerase inhibitor could be due to a direct involvement of poly(ADP-ribose) metabolism in DNA repair. Alternatively, it could be due to its involvement in the coodination of DNA repair with cell division, as postulated by Jacobson et al. (1984). In order to investigate this possibility, experiments were designed to study transformation in nondividing cells.

The poly(ADP-ribose) polymerase inhibitor, MBA, was applied to confluent cells following MNNG treatment. Confluent cells treated with MNNG and MBA had the same transformation frequency as those treated with MNNG alone (Table 5). Thus, poly(ADP-ribose) did not demonstrate a rate limiting role in the prevention of transformation in confluent cells. This is in contrast to the data obtained in exponentially dividing cells.

To further investigate the possible involvement of poly(ADP-ribose) in transformation events at confluence, a second method of metabolic alteration was used. Cells were depleted of their NAD levels prior to reaching confluence.

Confluent, NAD-depleted cells that were treated with MNNG showed a small increase in transformation frequency over MNNG treated controls (Table 6). Thus, by using two methods of altering poly(ADP-ribose) metabolism, inhibitors and NAD-depletion, little or no effect on the carcinogen-induced transformation frequency of nondividing cells was detected. Therefore, the involvement of poly(ADP-ribose) in DNA repair may be indirect and may indeed involve the coordination of DNA repair with cell division. This is supported by the data of Jacobson et al. (1985a). They report that survival in confluent cultures after inhibitors is higher than in dividing cells, although both dividing and confluent cultures contained DNA strand breaks due to the presence of MBA.

An interesting observation was obtained from the experiments using confluent cultures. Confluent cultures treated with MBA alone and NAD-depleted, confluent cultures showed high rates of spontaneous transformation (Table 5 and Table 6). These foci appeared in the absence of DNA damage. The occurrence of the foci must have been a consequence of the treatment conditions since foci did not appear in dishes treated with acetone alone in the inhibitor studies. In addition, the appearance of the foci

was not due to altering the culture conditions in the nicotinamide-deprivation experiments since no foci were observed in the nicotinamide-containing control cultures.

In addition to the use of inhibitors in exponentially dividing cells, NAD-depletion was used. NAD-depletion limits the substrate for poly(ADP-ribose) polymerase. An increase in transformation frequency of 1.4-fold was observed in MNNG-treated, NAD-depleted cells over MNNG treated controls (Table 4). As expected, MNNG treatment caused a lowering of the NAD pools of control and NAD-depleted cells (Figure 14). However, further study revealed that concurrent with a lowering of NAD levels in the nicotinamide-deprived, treated cultures was a lowering of the ATP content (Figure 15). However, ATP levels remained constant in the nicotinamide-fed cells. Thus, the increase in transformation frequency seen in the nicotinamide-deprived MNNG-treated cells may not be due to a direct involvement of poly(ADP-ribose) metabolism, but instead may be due to a decrease in the energy pool, which would affect DNA repair rates.

However, another explanation for the increase in transformation in NAD-depleted cultures was provided by the ascorbate studies. The redox levels in control and

NAD-depleted cells showed an important difference. The percent reduced NAD (NADH) was 2-fold higher in the cells grown in the absence of nicotinamide than in the control cells (Figure 25). Since a large portion of the NAD pool was in the reduced form, very little of it was available for synthesis of poly(ADP-ribose). Thus, poly(ADP-ribose) was not available for the prevention of transformation. It would be interesting to measure these levels in DNA damaged cells.

Niacin deprivation suggested a role for poly(ADP-ribose) in transformation. However, the exact role is not conclusive. Nonetheless, the data obtained is informative from a nutritional standpoint. The RDA for niacin was established in order to prevent the clinical appearance of the disease pellagra. However, marginally low levels of nicotinamide can be ingested without producing the clinical symptoms of pellagra. In many cases of niacin-deprivation in humans, skin lesions, which are the diagnostically important symptom of the disease, do not appear (Spivak and Jackson, 1977). However, other organs of the body may be affected, such as the intestines, the oral cavity, and the nervous system.

Interestingly, lesions in pellagrins occur in areas of

the body exposed to environmental stresses. It is also interesting to note that these cells are the primary cells undergoing constant cell division in the human. In particular the skin, mouth, and intestinal tract are affected by UV light, digestive enzymes, and bile acids, respectively. Such stresses, when applied to cells in culture, cause DNA damage that can be detected as increases in cytotoxicity and mutation frequency (Bhattacharjee et al., 1982), and transformation frequency (Chan and Little, 1976; Mondal and Heidelberger, 1976; Kaibara et al., 1984). The study presented in this dissertation provides evidence that NAD-depleted cells in culture are unable to overcome the stress inflicted by DNA damaging agents as efficiently as control cells. Thus, some of the symptoms of pellagra may be in part caused by the inability of the NAD-depleted cells to overcome the DNA damage inflicted by environmental stresses, certainly in dividing cells. The decreased synthesis of poly(ADP-ribose) may be a contributing factor in the inability of these cells to repair DNA damage normally.

As a whole, the human population does not ingest adequate levels of nicotinamide as determined by the RDA (Doll and Peto, 1981). This study showed that decreases in

nicotinamide metabolism *in vitro* resulted in increases in transformation. Transformed foci cause tumors when injected into the appropriate hosts (Reznikoff et al., 1973a). Thus, events that result in transformation may be the same events as those that result in formation of tumors *in vivo*. Therefore, alterations of nicotinamide metabolism that result in increases in transformation *in vitro* could conceivably result in carcinogenesis *in vivo*.

Support for this hypothesis is provided by epidemiological evidence. Dietary niacin deficiency has been linked to the high incidence of esophageal cancer in humans residing in a region of South Africa (Warwick and Harrington, 1973). Animal studies have shown a similar correlation. Rats fed a diet deficient in niacin died earlier due to N-nitrosodimethylamine-induced renal tumors than those fed nicotinamide (Miller and Burns, 1984). Interestingly, it was found that nicotinamide supplementation of a basic grain diet suppressed bracken fern-induced carcinogenicity in rats (Pamukeu et al., 1981). This is of interest since it has been shown that Japanese who eat this plant daily have three times as great a risk of developing esophageal cancer as nonconsumers (Hirayama, 1979).

The involvement of niacin metabolism in transformation is very complex. Clearly, poly(ADP-ribose) metabolism was involved in the prevention of malignant transformation in exponentially dividing C3H10T1/2 cells. This was demonstrated by increases in transformation when this metabolism was altered by inhibitors or by niacin deprivation. Lowering of the ATP pool was a secondary effect found in exponentially dividing cells that were grown in the absence of nicotinamide.

Poly(ADP-ribose) was shown to be involved in preventing transformation in dividing cells but not in non-dividing cells. These data are consistant with a possible role for poly(ADP-ribose) in the coordination of DNA repair with cell division. Further investigation will be required to determine the extent of involvement of poly(ADP-ribose) metabolism in the prevention of diseases such as pellagra and cancer that result from DNA damage in dividing cells.

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