THE EFFECT OF MNNG ON HSV REPLICATION AND GENE EXPRESSION

THE EFFECT OF N-METHYL-N'-N-NITROSOGUANIDINE ON HERPES SIMPLEX VIRUS REPLICATION AND GENE EXPRESSION

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ABSTRACT

Experiments were carried out to determine if pretreatment of cells with N-Methyl-N'-Nitro-N-Nitrosoguanidine (a potent alkylating agent known to induce DNA repair) would affect Herpes Simplex Virus Replication. The data demonstrated a 1.5 fold increase in virus yield, a 2 fold increase in HSV specific TK activity and no change in HSV specific DNA polymerase activity in MNNG treated cultures. The effects of MNNG treatment on virus replication and enzyme expression are discussed.

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LIST OF ABBREVIATIONS

A	Adenine
AdR	Deoxyadenosine
ADM	Arginine Deficient Medium
C	Cytosine
CPE	Cytopathic Effect
CPM	Counts Per Minute
dATP	deoxy Adenosine Triphosphate
dCTP	deoxy Cytidine Triphosphate
dGTP	deoxy Guanosine Triphosphate
EDTA	Ethylene diaminetetraacetate
FUdR	5 Flouro deoxyuridine
G	Guanine
HSV	Herpes Simplex Virus
^{3}H AdR	Tritiated deoxyadenosine
^{3}H TdR	Tritiated thymidine
MEM	Minimal Essential Medium
MNNG	N-Methyl-N'-Nitro-N-Nitrosoguanidine
MOI	Multiplicity of Infection
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Units
T	Thymine
TCA	Trichloroacetic Acid
TdR	Thymidine

List of Abbreviations cont'd.

TDP	Thymidine Diphosphate
TK	Thymidine Kinase
TMP	Thymidine Monophosphate
TTP	Thymidine Triphosphate
U	Uracil
XP	Xeroderma Pigmentosum

INTRODUCTION

The study of the virus-cell interaction has primarily dealt with the control the infecting virus exercises on a host cell during the replicative cycle of the virus. An area which recently has been receiving attention is the role of the cell during the viral replicative cycle. It is known that host cell functions are required to transport viral DNA to the nucleus during HSV replication (Becker et al., 1967). As well, a uv sensitive host cell function appears to be required for early HSV protein synthesis (Fenwick, 1977).

There have been several studies on the reactivation of virus in uv-irradiated cells (Bockstahler and Lytle 1970; Day, 1970). It has been suggested that the increased reactivation of virus observed may be related to DNA repair occuring in cells due to treatment with repair inducing agents (See mayer and Defendi, 1974; Day, 1975; Lytle et al., 1976). A study on HSV replication has shown an increase in HSV production in cells undergoing DNA repair due to uv-irradiation (Coppey and Nocentini, 1976). The authors, on the basis of their results, and a consideration of the data on reactivation of inactivated virus have suggested a relationship between DNA repair and HSV replication.

The study presented here examines HSV replication and two HSV specific enzymes associated with DNA synthesis, HSV thymidine kinase and HSV DNA polymerase, in cells which have been pretreated with a potent alkylating agent known to induce repair, N-methyl-N'-nitro-N-nitrosoguanidine.

BACKGROUND

A. N-Methyl-N'-Nitro-N-Nitrosoguanidine

In the study presented, cells were treated with a chemical agent prior to viral infection. The chemical, MNNG, is a potent alkylating agent belonging to a group of compounds termed aliphatic nitrosamides. There have been extensive studies on their chemical nature, biological action and the diverse effects of these agents in both prokaryote and eukaryote systems (see reviews: Lawley, 1975; Heidelberger, 1975; Drake and Blatz, 1976; Pegg, 1977; Roberts, 1978). This portion of the introduction will be limited to a brief description of MNNG in terms of its mode of action and effects on the cell.

Early studies with alkylating agents were primarily concerned with identifying the products of alkylation, and have shown that DNA, RNA and proteins can be alkylated (Singer, 1975). Since DNA is the genetic material and the cell usually possesses many more copies of RNA compared to DNA, the products of alkylation reactions with the RNA may not produce as many mutagenic or lethal effects as those produced with DNA (Pegg, 1977). In addition to the induction of DNA repair (see: Roberts, 1978), alkylation has been shown to inhibit protein synthesis in a dose dependent manner (Cerda-Olmedo and Hanawalt, 1967;

Anderson and Burdon, 1970). Cells treated with alkylating agents have also demonstrated a dose dependent inhibition of replicative DNA synthesis, which is often accompanied with an extension of S phase (Roberts et al., 1974). In some cell lines, the inhibition of replicative DNA synthesis may not occur immediately after exposure to the alkylating agent but in the following cell cycle (Roberts et al., 1974).

The reactions alkylating agents can have with DNA have been extensively studied. Nucleotides have been observed to be alkylated at the nitrogen atoms of positions 3 and 7 of guanine, positions 1, 3, and 7 of adenine, position 3 of thymine, position 2 of cytosine and at the oxygen atom of positions 6 of guanine, 2 of cytosine and 4 of thymine (Pegg, 1977). Initial studies aimed at determining the identity of the promutagenic lesion(s) were based on the assumption that there was a correlation between the most frequently occuring alkylation product and mutagenic activity (Robert, 1978). Thus 7-methylguanine, a major product of alkylation, was assumed to be the promutagenic lesion. Loveless (1969) suggested that a minor product of alkylation, O-6 methylguanine, was the promutagenic lesion rather than 7-methylguanine. Evidence in support of O-6 methylguanine being the promutagenic lesion has come from the following studies. An in vitro

study using alkylated DNA as a template for RNA polymerase provided evidence that O-6 methylguanine frequently miscodes for uridine rather than cytosine (Gercham and Ludlum, 1973). Lawley and Martin (1975) added further evidence for the mutagenic activity of O-6 methylguanine; their work was based on a study of reversion frequencies of bacteriophage T4rII AP72 to $T4r^+$. The reversion to the wild type ($T4r^+$) phage is believed to occur through a specific transition mutation (i.e. GC→AT; Krieg, 1963). Another minor product of alkylation, O-4 methylthymine, has also been suggested as being promutagenic (Lawley and Shah, 1974) and has recently been shown to mispair with guanine (Abbot and Saffhill, 1977).

The methylation of DNA by MNNG is considered to be a 'unimolecular substition' type of reaction (i.e. SN_1, Lawley and Martin, 1975). MNNG has been observed to be activated by thiols (Shulz and McCalla, 1969), and the methylation of nucleic acids appears to be affected by the intracellular concentration of thiols (Lawley and Thatcher, 1970). The methylation of DNA by MNNG has been suggested to occur via a potent methylating species, methyl diazonium, which transfers an intact methyl group to various sites on nucleotides (Lawley and Thatcher, 1970; and Sussmuth et al., 1972).

The activity of MNNG has shown some cell cycle dependence. In bacterial systems, it has been observed that the majority of lesions caused by MNNG occur primarily at the region of DNA replication (i.e. replication fork; Cerda-Olmedo et al., 1968). Studies in mammalian systems have demonstrated that the effects of MNNG are maximal if the cells are exposed to the carcinogen just prior to, or at the onset of, DNA replication (i.e. late G_1 or early S phase). The effects of MNNG in these experiments were expressed as tumor induction (Bowden and Boutwell, 1974), cellular transformation (Marquardt, 1974), and DNA repair (Barranco and Humphrey, 1971; Peterson et al., 1974). These studies were only suggestive of the role or necessity of the replication fork for alkylation reactions in mammalian systems. Bertram and Heidelberg (1974) demonstrated that the preferred site of action of MNNG was either very close to or at the replication fork.

B. DNA Repair

In the previous section the modifications caused by MNNG to cells, in particular modifications to the DNA, were considered. To permit cell survival in a system in which there has been exposure to mutagenic and potentially lethal agents, there must be at least one mechanism to

correct damage in the DNA. Prokaryotes are known to possess three mechanisms: photoreactivation, excision repair, and postreplication repair. Eukaryotes are known to have at least the two latter types of repair (there is some controversy as to the existence of a mammalian photoreactivation system; Lehmann and Bridges, 1977). Owing to the extensive literature available on repair (reviews: Trosko and Chu, 1975; Grossman et al., 1975; Lehmann and Bridges, 1977; Roberts, 1978), only the pertinent points of repair related to the correction of modifications induced by alkylating agents will be considered.

There has been evidence in bacterial (Lawley and Orr, 1970) and mammalian (Roberts et al., 1971b) systems that cells are able to recognize and remove alkylated nucleotides. Recognition of specific lesions may be carried out by specific and different enzymes, while the actual excision and repair of lesions (i.e. O-6 Alkylguanine, dimers, etc.) is probably the same as described for the repair of dimers (Lehmann and Bridges, 1977; Roberts, 1978).

The recognition step was believed to be carried out by a specific endonuclease (II), which was thought to recognize both apurinic sites and alkylated DNA, as well

as possessing activity against both (Verly and Paquette, 1972). This has been found to be partially correct. The endonuclease probably only recognizes apurinic sites and creates single stranded breaks in the DNA (Kirtikar et al., 1972). The repair of alkylated DNA is believed to involve the generation of apurinic sites in DNA. This may occur in two ways. The first mechanism involves a β(N) glycosidase which recognizes alkylated nucleotides and removes the alkylated base generating apurinic sites (Kirtikar and Goldthwaith, 1974; Lindahl, 1976). The second mechanism is via the spontaneous hydrolysis of 7-alkylguanine (Strauss and Hill, 1970). It has been suggested that the reason some XP strains repair 7-alkylguanine with greater efficiency than O-6 Alkylguanine is due to the spontaneous appearance of apurinic sites. It appears that these cells lack the ability to recognize O-6 Alkylguanine, but can recognize and repair apurinic sites (Goth-Goldstein, 1977). Once the apurinic site is generated, the apurinic specific endonuclease (II) creates a single stranded break in the DNA (Kirtikar et al., 1974). The enzyme splits the DNA at the 3' side of the apurinic site to produce 3'OH and 5'PO_4 (Ljungquist and Lindahl, 1974, 1975). This specific endonuclease is present in both bacterial (Verly and Paquette,1972) and mammalian

cells (Brent, 1976). After the nick is created, an exonuclease digests the portion of DNA containing the apurinic site and repair replication follows (Roberts, 1978). The β polymerase (Bertuzzoni et al., 1976) then adds nucleotides in a non-semiconservative manner to the DNA (Lehmann and Bridges, 1977; Roberts, 1978). Once the nucleotides are added, a ligase seals the nicks (Cerutti, 1974).

Mammalian cells possess another system to complement excision repair; this is post-replication repair. As with excision repair, post-replication repair is basically modeled after observations in bacterial systems (Lehmann and Bridges, 1977; Roberts, 1978). There are two basic models: error prone and error free repair. Error prone repair has been modeled after 'SOS' repair in bacterial systems (Radman, 1975) and is a process involving gap filling. Here the DNA replicates normally until the replication fork reaches a lesion, at which time replication stops, leaving a gap of 1000 nucleotides (Lehmann, 1974). DNA synthesis is reinstated at what is believed to be the next Okazaki fragment (Lehmann and Bridges, 1977). The gaps produced are later filled in. This is not via a recombinational event as in bacteria, but by de novo synthesis (which suggests the process to be semi-conservative

Buhl et al., 1972; Lehmann, 1972).

The second model of post-replication repair, which is relatively error-free, was proposed independently by Higgins et al., (1976) and Fujawara et al., (1976). According to the model, normal DNA synthesis proceeds until it is blocked by a lesion in one template. DNA synthesis on the complementary strand continues past the lesion. The daughter strands of DNA then separate from the parental strands, and associate so that one daughter strand provides a template for the other; nucleotides are then added. The daughter strands then separate and reassociate with respective parental strands (one of which retains the lesion). It should be noted that in both excision and post-replication repair, a high dose of alkylating agent can inhibit both repair and replication (Roberts, 1978).

C. Herpes Simplex Virus: Replication

Herpes Simples Virus (HSV) belongs to the Herpesviridae family, of which there are over 50 members. An interesting feature of this virus is its tendency to enter a latent state within the host, followed by recurrence of its clinical manifestations. HSV type 1 is the etiological agent of 'cold sores'. HSV type 2 is the causitive agent of Herpes gentalis, which has the

epidemiology of a veneral disease. On the basis of seroepidemiological studies, HSV 2 has also been implicated as a possible pathogenic factor in cancer of the cervix (Rawls et al., 1969).

Herpes Simplex Virus is a large linear double stranded DNA containing virus, packed in an icosahedral nucleocapsid which is enveloped (O'Callaghan and Randall, 1976). The nucleic acid weighs 100 x 10^6 daltons (Becker et al., 1968) and has a high G & C content (68%, see: Roizman and Furlong, 1974). The structure of the viral DNA is unique, it is composed of a long (L) and a short (S) region possessing unique sequences which are bounded by terminal and internal repetitive sequences, giving the DNA the following appearance:

ab____unique (L)____b'a'a'c'____unique (S)____ca

(Sheldrick and Berthelot, 1974; Wadsworth et al., 1975). The orientation of the regions can alter, yielding four patterns of molecular arrangement: prototype (P), inverted long region (I_L), inverted short region (I_S) and inverted long and short regions (I_{LS}) (Wadsworth et al., 1975).

The molecular characteristics, replication, pathogencity and transforming potential of HSV have been studied extensively (see reviews: Roizman and Furlong, 1974; O'Callaghan and Randall, 1976; Clements and Hay, 1977;

Graham, 1977; Honess and Watson, 1977). Replication in the Herpesviridae family seems to be fairly similar despite the diversity found in its members with respect to their hosts and clinical manifestations (Honess and Watson, 1977). With this in mind, several of the salient points to be discussed on HSV replication have been drawn primarily from studies on HSV 1, HSV 2, and Pseudorabies.

Attachment or absorption of the virus to the host cell is rapid and not energy dependent (Hochberg and Becker, 1966). Once the capsid is located in the cytosol, uncoating begins and the viral DNA is transported to the nucleus by a process which requires some host cell functions (Ben-Porat et al., 1976). As well, the initiation of viral protein synthesis appears to require some host cell functions which are uv sensitive (Fenwick, 1977). The 'shut down' of host functions is believed to be mediated by products of one or more viral genes which appear early in infection (Haliburton and Timbury, 1976).

The viral DNA now located in the nucleus will be transcribed to produce viral RNA (Roizman and Furlong, 1974). There are three populations of RNA produced: immediate early, early, and late, all of which can be further classified into abundant and scarce subpopulations (Frenkel and Roizman, 1972). The abundant comprise 99.3 and 93.5% of total RNA produced as early and late RNA respectively,

even though this RNA arises from only 24% of the DNA (Roizman and Furlong, 1974). Prior to passage to the cytoplasm, the RNA is processed by a mechanism which may involve modification by cleavage (Wagner and Roizman, 1969) and/or adenylation (Silverstein et al., 1973). Polyadenylated RNA also appears to vary in the relative amounts of adenylation (Silverstein et al., 1976). An examination of polyadenylated and non-polyadenylated virus RNA has shown that the 5' terminal structure has a $m^7G(5')ppp5'NmpNmp$ cap (Clements and Hay, 1977).

There has been some controversy as to the identity of the viral DNA-directed RNA polymerase. Suggestions include host RNA polymerase, a modified host RNA polymerase, or a virally coded RNA polymerase (Clements and Hay, 1977). Studies have shown that the RNA polymerase is α-amanitin sensitive and has similar but not identical properties to cellular RNA polymerase II, suggesting a modified host cell RNA polymerase (Ben-Zev et al., 1976; Preston and Newton, 1976).

The regulation of viral replication appears to occur at the level of transcription of specific populations of RNA. The concept of cascade regulation was first proposed by Honess and Roizman (1974). The RNA and subsequent polypeptides formed have been divided into three classes (α, β, and γ)

according to the temporal pattern of polypeptide synthesis (Honess and Roizman, 1974).

The α class is composed of one structural and several nonstructural polypeptides whose synthesis peaks 3 to 4 hours post-infection. At least one product of the α class is believed to be required to initiate β class polypeptide synthesis and a β polypeptide(s) is believed to switch off α synthesis (Honess and Roizman, 1975). The β polypeptide synthesis peaks at 5 to 7 hours post-infection and is composed of one structural and several nonstructural polypeptides. The last polypeptide class, γ, is believed to be regulated in a similar manner to that of the β class. Thus γ polypeptide synthesis is initiated by a β class product(s) and a γ class product(s) switches off β class synthesis. The γ polypeptide synthesis peaks at 12 hours post-infection and is composed of the majority of major structural polypeptides.

The regulation is sequentially controlled as originally postulated by Honess and Roizman (1974). The production of α RNA does not require infected cell protein synthesis (Rakusnova et al., 1971; Frenkel and Roizman, 1972). The synthesis of β and γ polypeptides require protein synthesis of the previous class (i.e. α and β respectively) and new

RNA synthesis (Honess and Roizman, 1974). Studies using amino acid analogues have shown that the switch from α to β or β to γ requires at least one polypeptide product from the previous class (Honess and Roizman, 1975). Studies with hydroxyurea have shown that polypeptide synthesis is substantially reduced if DNA synthesis is inhibited (Roizman and Furlong, 1974).

Some protein synthesis occurs after the onset of transcription early in the replicative cycle of the Herpesvirus. Two β proteins with specific enzymatic functions, the viral thymidine kinase and the viral DNA polymerase, are to be briefly discussed.

Thymidine kinase (TK) functions by phosphorylating thymidine to thymidine mono-phosphate. The HSV induced thymidine kinase has been extensively studied (see reviews: Kit et al., 1975; Kit, 1976). Kit (1963) first suggested that HSV induced TK activity in infected cells. Subsequently the inducible TK activity was shown to be virally coded, first by the isolation of HSV TK^- mutants (Dubbs and Kit, 1964) and more recently by an in vitro TK synthesizing system (Preston, 1977). HSV DNA has been successfully used to biochemically transform TK^- cells to TK^+ (Bacchetti and Graham, 1977; Minson et al., 1978).

Adaptations of this technique have been utilized to identify the HSV DNA fragment carrying the TK gene (Silverstein et al, 1978). The sequence coding for the enzyme is believed to be in the long region of the viral nucleic acid (prototype arrangement); between 0.27 and 0.35 map units as determined by recombination studies (Morse et al., 1978).

HSV TK does not require DNA synthesis for its production or activity (Kit and Dubbs, 1977). The regulation of the TK gene is probably related to transcription or translation. The experiments which demonstrate this type of control require the use of TK^- cells which are either infected or transformed with HSV. Viral TK activity is then studied at various times in mock-infected or superinfected cultures. The results from these studies demonstrate that an α polypeptide is required to initiate TK synthesis and a later product, probably a γ polypeptide, is required to turn off TK gene activity (Leiden et al., 1976; Kit and Dubbs, 1977; Leung, 1978). It should also be noted that TK activity starts to decrease as DNA synthesis is initiated (Garfinkle and McAuslen, 1974). The TK enzyme can also regulate itself through allosteric feedback inhibition, that is increased amounts of TMP, TDP and TTP appear to inhibit the enzyme (Breitman, 1963).

A detailed characterization of HSV induced DNA polymerase shows it has a molecular weight of 1.8×10^5 and demonstrates optimal activity under the following conditions: high $(NH_4)_2SO_4$, low Mg^{++}, and DNA with a high G & C content (Wiessbach et al., 1973). Furthermore, the HSV DNA polymerase is inhibited by phosphonoacetic acid (Overby et al., 1974). It was not known initially if the enzyme was a modified host polymerase or a virally coded one. This was resolved when HSV temperature sensitive mutants of DNA polymerase were isolated and subsequent complementation studies showed a virus-specific enzyme with at least three genes being required for its production (Purifoy et al., 1975; Aron et al., 1975). Recently, the DNA polymerase gene was mapped near TK, between 0.43 and 0.52 map units on the long region of HSV DNA (prototype arrangement; Morse et al., 1978).

The viral nucleic acid is replicated in the nucleus by semi-conservative replication, with characteristic single stranded regions which are subsequently filled in (Ben-Porat et al., 1977a, 1977b). Initiation is believed to occur at two sites which are located at the joint regions of the DNA and on the short arm near the joint region (Friedman et al., 1977; Hirsh et al., 1977; Biswal et al.,

1978). The bulk of the DNA is synthesized 4 to 7 hours post-infection (Roizman and Furlong, 1974).

At this stage, DNA being replicated, the γ polypeptides are made and transferred from the cytoplasm to the nucleus (Ben-Porat et al., 1969). This transfer and assembly is believed to require arginine (Becker et al., 1967; Courtney et al., 1970). The absence of arginine does not appear to affect viral DNA synthesis, although a decrease of up to 95% in virus yield has been observed (Mark and Kaplan, 1971). Capsids are assembled in the nucleus and an envelope is acquired as the capsid buds out of the nucleus into the cytoplasm (Darlington, 1968; Asher et al., 1969). There is currently much controversy as to the actual path the virion takes in passing from the nucleus to the exterior of the cell (Roizman and Furlong, 1974).

D. The Influence of DNA Repair on Virus Replication and Gene Expression

Cellular DNA repair has been implicated as an important factor in the ability of virus: to transform cells (see: Casto and DiPaolo, 1973), to undergo reactivation (Day, 1970; Bockstahler and Lytle, 1970; Lytle *et al.*, 1978; Das Gupta and Summers, 1978), and to replicate (Coppey and Nocentini, 1976).

Viral transformation of cells *in vitro* and *in vivo* has been observed to be enhanced in animals and cell cultures that have been exposed to physical or chemical agents known to induce repair (Casto and DiPaolo, 1973). Casto and DiPaolo (1973) suggest that lesions produced by physical or chemical repair inducing agents, or the repair of these lesions in the DNA, could give rise to susceptible cells. The authors further speculate that the increase in viral transformation is due to an enhanced sensitivity of these cells to transformation by a potentially tumorigenic virus. An enhancement has also been observed in the biochemical transformation of TK^- cells to TK^+ by HSV provided a repair inducing agent was added after infection (Verweord and Rapp, 1978).

Early studies in bacterial systems indicate that uv-irradiated bacteriophage could be reactivated to a greater extent if the host cell DNA was undergoing repair (Wiegle

1953). This type of observation has also been seen in mammalian systems in which uv-irradiated virus (usually a DNA virus) showed enhanced reactivation if cells were exposed to uv-irradiation (Day, 1970; Bockstahler and Lytle, 1970) or chemical agents which induce DNA repair (Das Gupta and Summers, 1978; Lytle et al., 1978) prior to infection. Studies using excision repair deficient xeroderma pigmentosum cells treated with uv-irradiation (Lytle et al., 1976) or uv-irradiation and caffeine (Day, 1975), suggest that post-replication repair is involved in the enhanced reactivation of virus. It has been proposed that the DNA repair occurring after treatment with repair inducing agents in the host cell inadvertantly corrects the damaged portions of viral DNA (Radman, 1975). The mechanism involved requires: de novo protein synthesis (Das Gupta and Summers, 1978), DNA repair replication (Seemayer and Defendi, 1974; Rainbow, 1975) and an incubation period of several hours between the induction of repair and infection (Bockstahler et al., 1976). Since the enhanced reactivation of phage in uv-irradiated cells is accompanied by a high level of phage mutagenesis (Wiegle, 1953) it has been suggested that the post-replication repair in the bacterial system is of the 'SOS' or error prone type (see: Witkin, 1976). It is suspected that the

post-replication repair associated with the reactivation of virus in mammalian systems is also of the 'SOS' type (Das Gupta and Summers, 1978).

A recent study in which cells were exposed to a repair inducing agent (uv-light) prior to viral infection showed an increase in viral production (Coppey and Nocentini, 1976). It was of interest to determine if a repair inducing chemical agent would have similar effects on viral production. In the study presented here, actively growing cells were exposed to MNNG (a potent alkylating agent known to induce repair) prior to HSV 2 infection. The production of virus progeny and the activity of two HSV-specific enzymes associated with DNA synthesis, HSV thymidine kinase and HSV DNA polymerase, were examined to determine the influence of MNNG treatment of cells on HSV replication.

MATERIALS AND METHODS

A. Tissue Culture and Virological Techniques

1. Cells

Human thymidine kinase deficient cells (line 143 TK^-) were obtained from Dr. S. Bacchetti. This cell line was derived from a murine sarcoma virus-transformed osteosarcoma cell line (R970-5; Rhim et al., 1975) which had been selected for thymidine kinase deficiency by growth in medium containing BrUdR (Huebner and Croce, unpublished). Cells were routinely subcultured twice a week. Medium was decanted from confluent monolayers grown in 150 cm^2 flasks, cells were rinsed in PBS and incubated in 3 mls of Trypsin-EDTA (Gibco) for 5 to 10 minutes. The monolayer was removed; a single cell suspension was formed which was then added to new flasks containing fresh medium at a ratio of 1:5 or 1:10. Cultures were routinely checked and found negative for mycoplasma contamination by one of the following techniques: (1) the ability of 143 TK^- cells to incorporate ^{3}HTdR, or (2) laboratory diagnosis involving culturing of the contaminating organism (Crawford, 1970).

2. Media

The 143 TK^- cells were propagated in 150 cm^2 plastic

tissue culture flasks (Corning) with ∝ Minimal Essential Medium (∝ MEM, Gibco), supplemented with 10% v/v heat inactivated fetal calf serum (Gibco), 100 u/ml penicillin, 100 u/ml streptomycin, 0.03% w/v glutamine, 0.75 g/l $NaHCO_3$ and 0.01 mM Hepes buffer.

Arginine depleted medium was prepared in the same fashion except that the stock ∝MEM lacked arginine and the fetal calf serum was dialyzed against PBS for three days.

N-Methyl-N'-nitro-N-nitrosoguanidine was freshly dissolved and added to the medium for experiments which required cells to be treated with an alkylating agent. The MNNG was dissolved in double distilled water in the dark at 100 times the final concentration used (which ranged from 0 - 0.2 ug/ml).

3. Survival Curves

The 143 TK^- cells were seeded in 25 cm^2 flasks (Corning) at least 12 hours prior to use. The cells were exposed to varying concentrations of MNNG (in ∝MEM) for 1.5 hours. Cultures were then rinsed with PBS and re-fed with normal medium. To determine cell survival, the cells were trypsinized and both total and viable cell populations were determined by Trypan Blue dye exclusion.

4. Virus

The virus used in this study, Herpes Simplex Virus type 2 strain 219, an isolate from a patient with chronic cervicitis (Seth et al., 1974), was obtained from Dr. W.E. Rawls. Virus stocks were prepared by adsorbing 0.1 PFU/cell for 1 hour at 37°C in Vero cells. Medium was then added and the cultures were held at 37°C until complete CPE was observed (approximately 72 hours). The virus was harvested in the following manner: the cells were scraped with a rubber policeman, and the cell suspension was pelleted by centrifuging at 1500 rpm for 10 minutes (IEC Daimon PRJ Centrifuge). Pellets were resuspended (1 ml of αMEM for each pellet obtained from a 150 cm^2 bottle) and freeze-thawed three times. Suspensions were then centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and sonicated for 30 seconds and virus titres were determined with a standard plaque assay.

5. Plaque Assay

The HSV suspensions were diluted in a 10-fold dilution series and 0.2 ml of viral suspensions were plated on monolayers of Vero cells (60 x 15 mm plastic petri plates; Lux Scientific). After one hour of adsorption at 37°C, infected cells were overlayed with αMEM (prepared as

previously described) with the addition of 0.2% v/v Human Immune Globulin Serum (Connaught Labs). After a three to four day incubation period at 37°C, the medium was decanted, the cells were rinsed with PBS, fixed in Carnoy's Solution (Methanol 3 vol:Acetic Acid 1 vol), and stained with 0.1% w/v crystal violet. Plaques were then enumerated by the following formula:

$$\#\text{ of plaques per 0.2 ml} = \frac{\Sigma\,\#\text{ plaques}}{\Sigma(\#\text{of plates per dilution})\left(\frac{1}{\text{d.f.}}\right)}$$

where Σ equals the "sum of" and d.f. is the "dilution factor".

The following procedures were carried out to determine viral replication in MNNG treated cells: confluent 150 cm^2 tissue culture flasks of 143 TK^- cells were treated with various concentrations of MNNG for 1.5 hours. Cells were then rinsed with PBS and HSV 219 (moi = 0.1) was allowed to absorb for one hour at 37°C. Infected cultures were re-fed and harvested 24 hours later. Titres were determined by the previously described plaque assay and are expressed in PFU/ml.

B. Incorporation of Nucleotides

1. Quantitation by Scintillation Counting

Cultures were prepared by seeding 1 to 2 x 10^6 cells per 60 mm petri plate at least 12 hours prior to use.

Cultures were usually pulse-labeled for 1 hour with either 1 uCi/ml ^{3}H deoxyadenosine (generally tritiated deoxyadenosine, ^{3}H AdR, specific activity 25 Ci/mMole) or 1 uCi/ml of [methyl-^{3}H]-thymidine (tritiated at the 5 methyl position of thymidine, ^{3}H TdR, specific activity 20 Ci/mMole) as indicated in the Results. Samples were then rinsed twice in cold PBS and frozen (-20°C) until all the samples had been collected. Cells were harvested in 1 ml of PBS containing 10 mM EDTA and plates were rinsed twice with 1 ml of PBS. The cell suspensions were collected in cold test tubes, to which ice-cold (4°C) 5% (w/v) TCA (final concentration) was added. After a 10 minute incubation at 4°C, the cell suspension was filtered through glass fiber filters, which were rinsed several times with 5% TCA and once with ethanol. The filters were dried, placed in a toluene based scintillation fluid, and counted.

2. Quantitation by Autoradiography

Pulse-labeled cultures were rinsed twice in cold PBS, fixed in Carnoy's Solution (5 min) and allowed to air dry. Dried plates were put at 4°C with 5% TCA for two hours, rinsed and air dried. Nuclear track emulsion

(Kodak NTB-3) was diluted 1:2 (with filtered distilled water) and poured onto the plates, excess emulsion was drained and the plates were stored for 24 hours in light-tight boxes at -70°C, after which time they were developed in D-19 developer (Kodak) and fixed in Rapid Fix (Kodak). Plates were rinsed, dried, and stained with 0.1% w/v Giemsa. Cells were scanned for the presence, location and number of grains in the nucleus.

C. Thymidine Kinase Assay

The thymidine kinase activity was determined according to the procedure of Munyon et al., (1972). Briefly, cell cultures were harvested seven hours post-infection, washed in PBS and centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was decanted and the pellets were frozen at -70°C until use. Cell pellets were resuspended in the extracting buffer containing 10 mM Tris HCl (pH 8), 1.4 mM β-Mercaptoethanol and 1 mM ATP. Cell extracts were obtained by sonicating the cell suspension for two 30 second bursts, followed by centrifugation of the suspension at 28,000 rpm for 20 minutes at 4°C (IEC, B-60 ultra-centrifuge; rotor A-269). The supernatant was collected and diluted 1:10 or 1:20 in the reaction buffer, which contained 0.1 M Tris maleate (pH 6.5), 0.025

M KCl, 0.20 M $MgCl_2$, 1.4 mM β-Mercaptoethanol and 10 mM ATP. Aliquots ranging from 10 to 50 ul of extract were added to 10 ul of 1 uCi/ml ^{3}H TdR, brought to a final volume of 0.1 ml with the reaction buffer, and incubated for one hour at 37°C. The amount of protein used in the reaction was in the range in which the reaction was linear. The reaction was terminated by the addition of 10 ul of 1.2 mg/ml TdR. To quantitate the conversion of ^{3}HTdR to ^{3}HTMP, 50 ul of the reaction mixtures were spotted on DEAE-81 chromatography paper (which had been prespotted with cold 1.2 mg/ml TdR). Spotted papers were dried, rinsed three times in 1 mM Ammonium formate (pH 3.6), twice in distilled water, and once in ethanol. The papers were dried and counted in a toluene based scintillation fluid.

Total protein was determined by the Lowry Protein Assay (Lowry et al., 1951). Results are expressed as picomoles of ^{3}HTMP formed in 30 minutes at 37°C per µg protein.

D. Polymerase Assay

DNA polymerase assay was carried out as described by Purifoy et al., (1975). Briefly, crude extracts were prepared from washed cell pellets, which were collected and frozen (-70°C) until used. Pellets were suspended in

0.1 M Tris HCl (pH 8) and 3 mM β-Mercaptoethanol, sonicated for two 30 second bursts, centrifuged at 39,000 rpm for 20 minutes at 2°C (IEC, β-60 ultracentrifuge; Rotor A-269) and the supernatant was collected.

The cellular DNA polymerase assay was carried out in the following reaction mixture: 50 mM Tris HCl (pH 8), 8 mM $MgCl_2$, 1 mM β-Mercaptoethanol, 10 uM dGTP, 10 uM dATP, 10 uM dCTP, 10 uM dTTP, 100 ug/ml activated calf thymus DNA, and 1 u Ci/ml ^3H dTTP (specific activity 62.2 Ci/mMole). In order to obtain the optimal conditions for the HSV DNA polymerase assay, the $MgCl_2$ concentration was lowered to 3mM and 100 mM $(NH_4)_2SO_4$ was added to the cellular DNA polymerase reaction mixture. Equal volumes (100 ul) of reaction mixture and extract were added to test tubes and incubated at 37°C for 45 minutes. The amount of protein (100 ul) used was in the range in which the reaction was linear. The reaction was terminated by placing the samples on ice and adding 10 mM pyrophosphate (final concentration). To prepare for scintillation counting, 3 mls of cold 5% TCA and 0.01 mM pyrophosphate were added to the samples. After 10 minutes, the samples were filtered through glass fiber filters, rinsed several times in 5% TCA and once in ethanol. Filters were dried and placed in toluene based scintillation fluid.

Total protein was determined by the Lowry Protein Assay (Lowry et al., 1951). Results are expressed as picomoles of ^{3}H TTP incorporated per ug protein in 30 minutes at 37°C.

The exact conditions for both HSV TK and DNA polymerase reactions were established in previous experiments in our laboratory.

RESULTS

Effect of MNNG on Cell Survival and DNA Synthesis

Initial studies were aimed at examining cell survival and DNA synthesis in cells which had been exposed to MNNG. The optimal dose of MNNG to be used in subsequent experiments was determined by examining the effect of various concentrations of MNNG on cell survival and ^{3}H AdR incorporation in cells. Cells were exposed to various amounts of MNNG for 1.5 hours as described in Materials and Methods. The cells were either pulse-labeled for one hour with ^{3}H AdR and prepared for scintillation counting, or stained with trypan blue. The deoxyadenosine used in these experiments was generally labeled with tritium. Since the cells were pulse-labeled, the bulk of the radioactivity detected is most likely due to label incorporated into DNA; however, it is possible that some label may be incorporated into RNA as ^{3}H Adenosine.

The results obtained demonstrate that with increased doses of MNNG cell survival decreases (figure 1) and ^{3}H AdR incorporation per surviving cell increases (figure 2). The dose of MNNG to be used in subsequent experiments was determined from a comparison of figures 1 and 2.

Figure 1 Effect of MNNG on Cell Survival

5×10^5 143 TK^- cells were seeded in 25 cm^2 flasks. The cells were treated with the indicated amounts of MNNG for 1.5 hours. Cultures were rinsed with PBS and refed. The percentage of surviving cells was determined by Trypan Blue Dye exclusion.

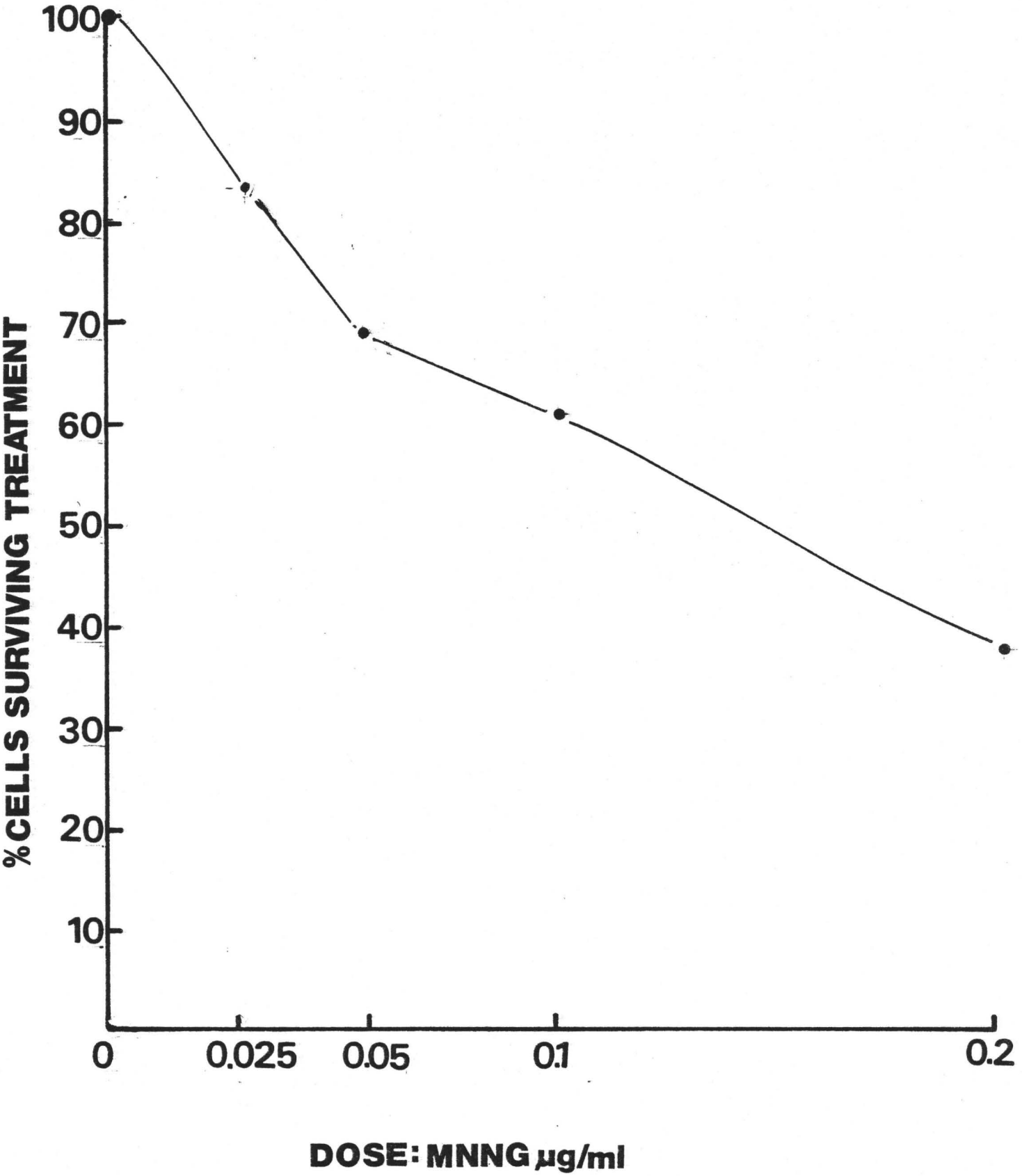
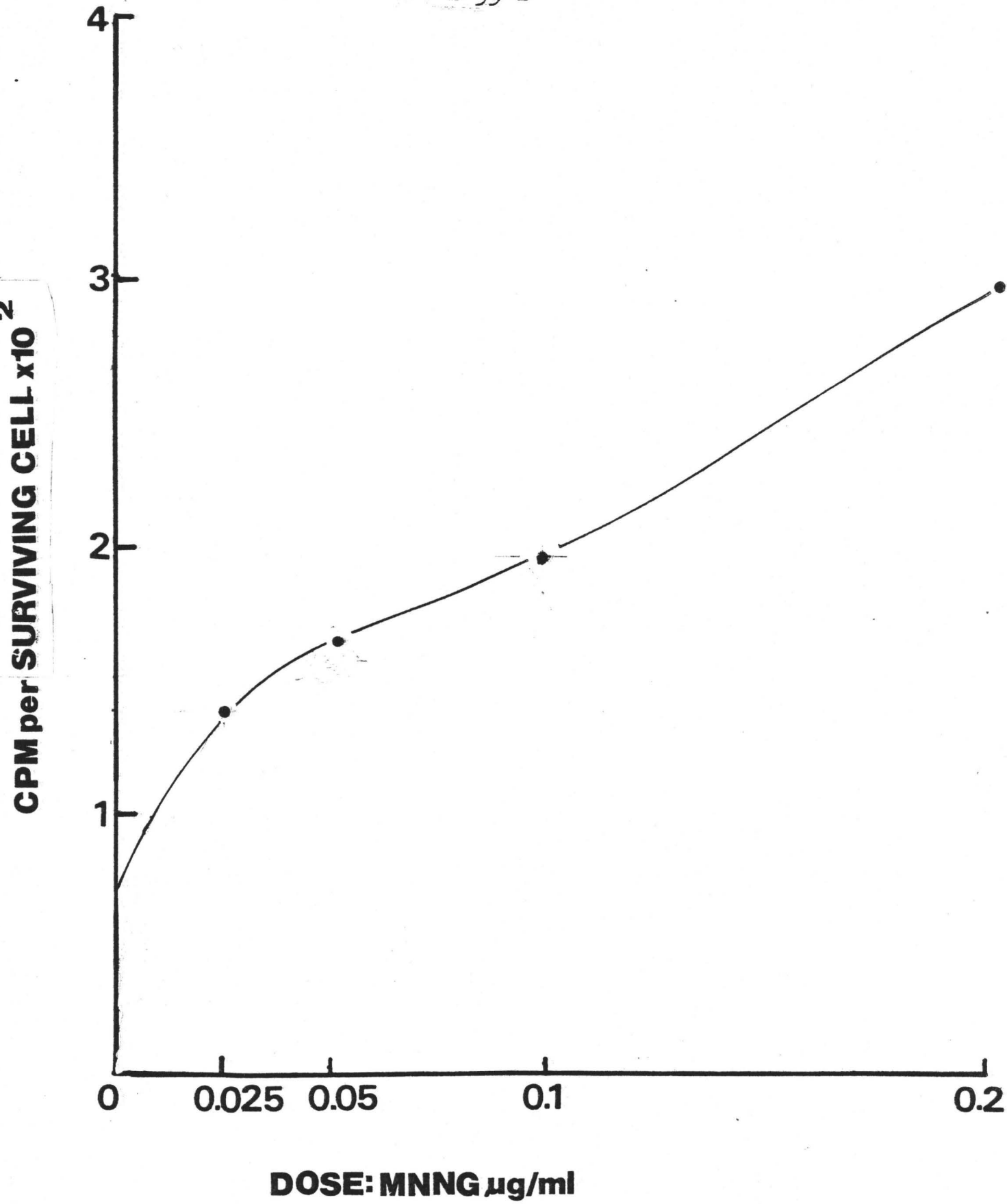

100
90
80
70
60
50
40
30
20
10
% CELLS SURVIVING TREATMENT
0
0.025
0.05
0.1
0.2
DOSE: MNNG µg/ml

Figure 2 ^{3}H AdR Incorporation After Exposure to Various Amounts of MNNG

Semiconfluent monolayers of 143 TK^{-} cells were exposed to the indicated amounts of MNNG for 1.5 hours. Cells were then rinsed in PBS and pulse-labeled with 1 uCi/ml of ^{3}H AdR for one hour. TCA precipitable counts were divided by the number of cells survivng MNNG treatment.


4
3
2
1
0
0.025
0.05
0.1
0.2
CPM per SURVIVING CELL x10 2
DOSE: MNNG µg/ml

The amount selected, 0.1 ug/ml MNNG, resulted in an acceptable percentage of cells surviving treatment (63%) and a relative high level of ^{3}H AdR incorporation (1.92 x 10^{-2} CPM per surviving cell).

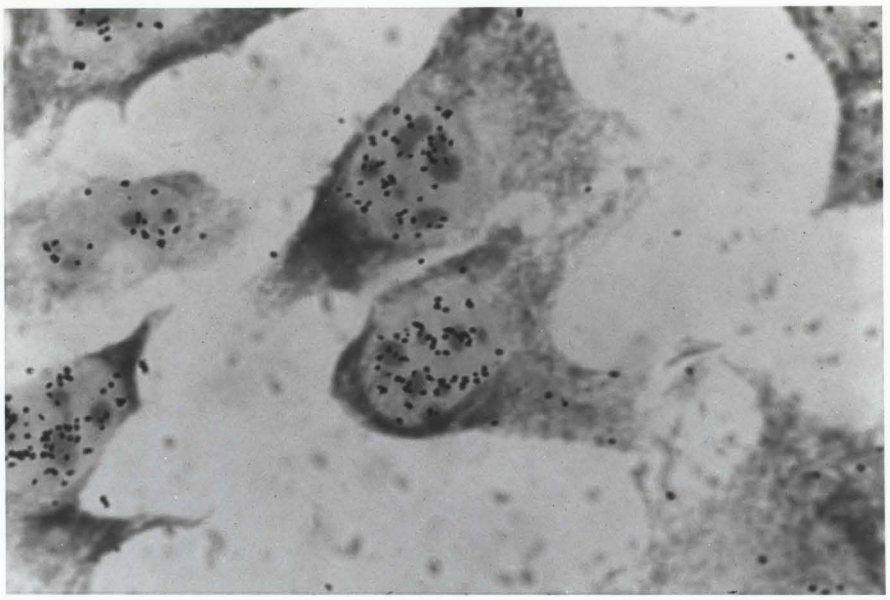
To further characterize the increase observed in ^{3}H AdR incorporation (figure 2), autoradiographs of MNNG treated cells were prepared and the percentage of labeled nuclei as well as the number of grains per nucleus were determined. Cultures were exposed to MNNG, pulse-labeled with ^{3}H AdR for one hour and prepared for autoradiography.

An examination of the distribution of grains per nuclei in autoradiographs of control (non-treated) cultures suggested the existence of three groups of cells. Negative cells were defined as having less than 6 grains per nucleus. Positive cells with more than 50 grains per nucleus were considered in S phase, those with 6 to 50 grains per nucleus were considered as positive cells which were either entering or leaving S phase or were undergoing repair. The technique cannot differentiate between these two classes of cells since they will both show an intermediate number of grains per nucleus (i.e. between 6 to 50).

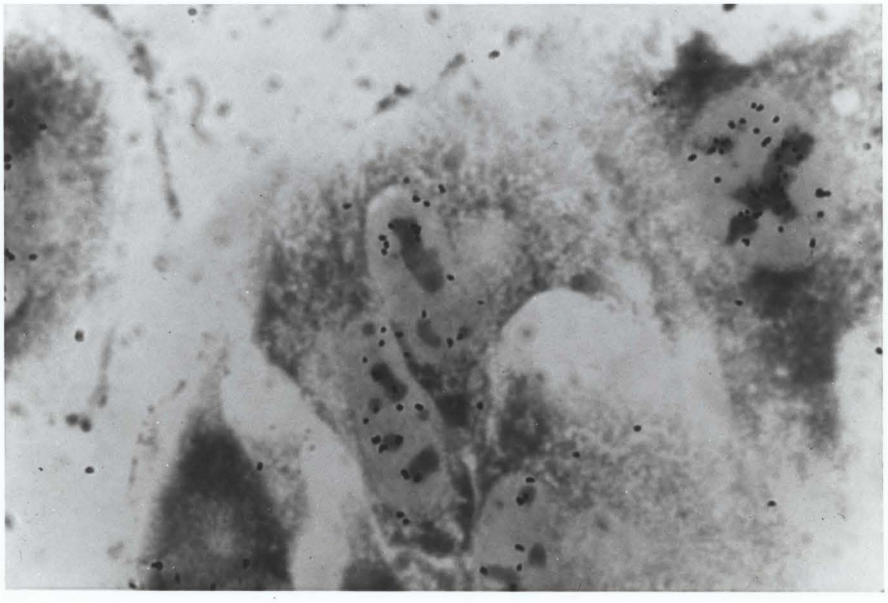
The autoradiographs depicted in figures 3a and 3b

Figure 3 ^{3}H AdR Incorporation After Exposure to MNNG (Autoradiographs)

143 TK^{-} cells were treated with 0.2 ug/ml MNNG for 1.5 hours, and pulsed-labeled for one hour with 1 uCi/ml ^{3}H AdR. Cells were fixed and developed as indicated in "Materials and Methods" and stained with Giemsa. Figure 3a is control cells and figure 3b is MNNG treated cells.



3a



3b

are representative of the differences observed in MNNG treated and non-treated cells. Figure 3a is a photograph of actively growing non-treated cells in S phase. Figure 3b is an autoradiograph representative of a culture treated with MNNG.

Table I show the number and percentage of cells grouped according to the number of grains per nucleus. The data show more positive cells present in the MNNG treated cultures. A difference in the grouping of cells according to the number of grains in the nucleus was observed in the MNNG treated cultures. A lower number of cells (12.1%) were in S phase and a substantially larger amount (81%) were positive of the intermediate class, compared to 47.5% and 20.3% for non-treated cultures respectively. The observed increase in the number of cells of the intermediate class (6-50 grains per nucleus) is compatible with both a decrease in the rate of semiconservative DNA synthesis (which may be accompanied by a lengthening of S phase) and/or the induction of DNA repair synthesis. The possibility that DNA repair is indeed occurring could be inferred from the observed reduction in the percentage of unlabeled cells in the MNNG treated cultures.

TABLE I

Number and Percentage of Cells Showing Different Amounts of Grains per Nucleus

Number of grains per nucleus		Non-treated Cells		MNNG treated Cells	
		Number	%	Number	%
Negative (less than 6)		177	32.2	40	6.9
Positive (6 or more)	Between 6 and 50	122	20.3	475	81.0
	Greater than 50	261	47.4	71	12.1
Total		550	100.0	586	100.0

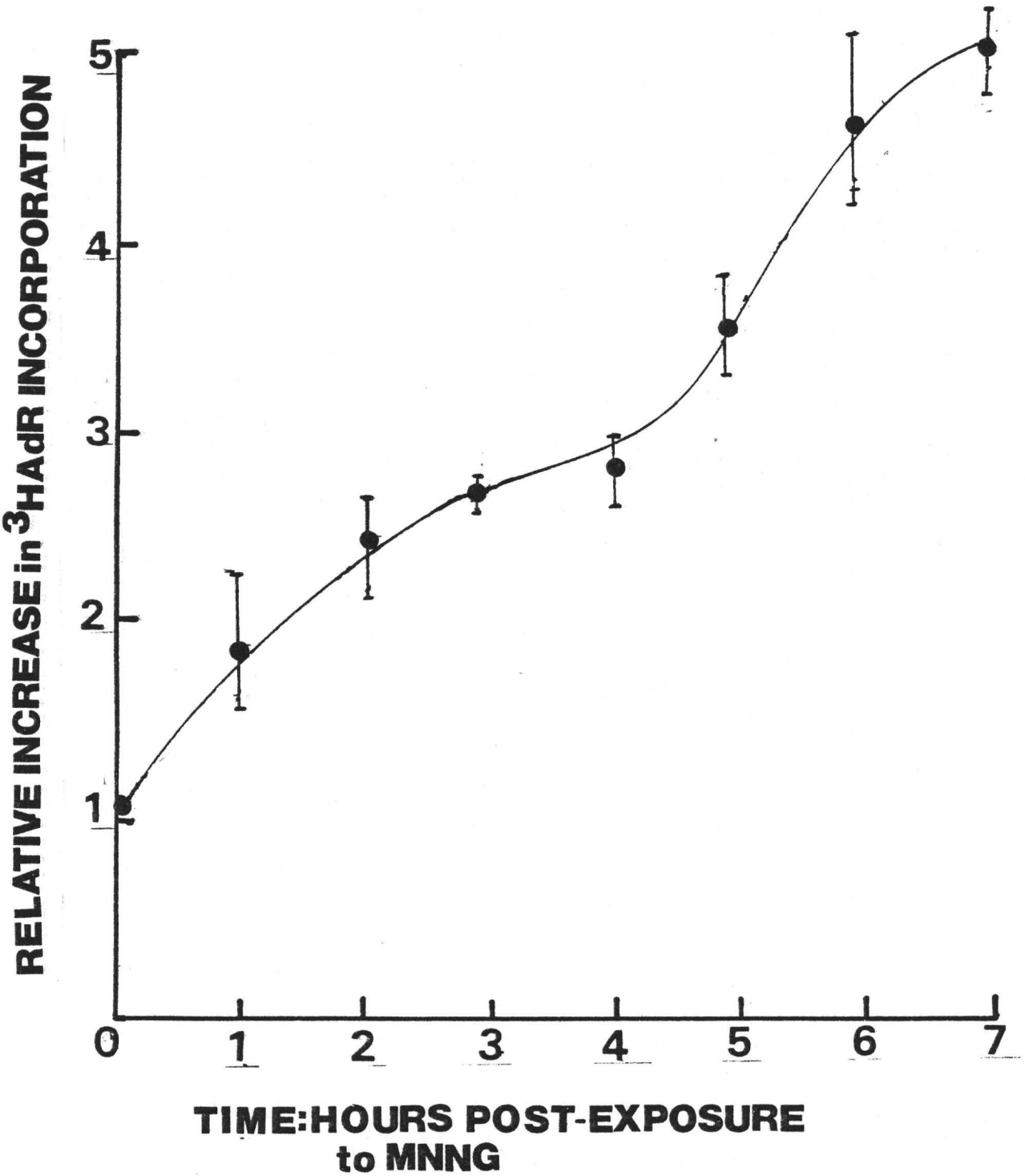
Cells (143 TK^-) were treated with 0.1 ug/ml MNNG for 1.5 hours, rinsed in PBS and pulse-labeled for one hour with 1 uCi/ml ^3H AdR. Slides were fixed and prepared for autoradiography as indicated in Materials and Methods. Results are a total of two experiments done in triplicate. All the cells in 20 randomly selected microscopic fields were recorded for each slide.

To further characterize the influence of MNNG on DNA synthesis, the relative increase in ^{3}H AdR incorporation with time was determined. MNNG treated cells in this experiment were pulse-labeled at various time post-exposure and prepared for scintillation counting. The relative increase in ^{3}H AdR incorporation was obtained from the ratio of CPM per surviving MNNG treated cell to CPM per surviving non-treated cell. The data are shown in figure 4. The values obtained differ significantly from a straight line ($p<0.001$ as determined by analysis of co-variance of parallel lines; Armitage, 1974). The results show a two component curve, which is indicative of a change in the rate of ^{3}H AdR incorporation with time.

The high increase (5 fold) in ^{3}H AdR incorporation is most likely due to alterations in the rate of semi-conservative DNA synthesis and the duration of S phase. The shape of the curve could also indicate that MNNG treatment might result in a degree of cell synchrony. DNA repair synthesis might also contribute to the observed changes in ^{3}H AdR incorporation, but to an extent which cannot be detected under the experimental conditions used.

Figure 4 The Relative Increase in ^{3}H AdR Incorporation After Exposure to MNNG

143 TK^{-} cells were exposed to 0.1 ug/ml MNNG for 1.5 hours and pulse-labeled for one hour with 1 uCi/ml ^{3}H AdR. Cultures were then prepared for scintillation counting as described in "Materials and Methods". The relative increase in ^{3}H AdR incorporation is equal to the CPM per surviving MNNG treated cell at the times indicated divided by the CPM per surviving non-treated cell.


RELATIVE INCREASE in ^{3}HAdR INCORPORATION
5
4
3
2
1
0
1
2
3
4
5
6
7
TIME:HOURS POST-EXPOSURE
to MNNG

Effect of MNNG on HSV Replication

HSV replication was studied in cells which had been pre-treated with various amounts of MNNG. Since the previous experiments demonstrated that MNNG treatment altered cell survival and DNA synthesis in 143 TK cells, it was of interest to determine if these changes would affect HSV replication. To examine this, HSV 2 was grown for 24 hours in cells which had been exposed to various amounts of MNNG for 1.5 hours, harvested, and virus titres determined. The results did not show a significant change in viral replication in cultures treated with 0.05 ug/ml or less MNNG (table II). There appears to be an increase in HSV yields in cultures treated with increasing doses of MNNG suggesting that MNNG treatment of cells prior to infection increases HSV 2 replication.

The increase in HSV yield observed in the previous experiment could be due to more cells being infected after MNNG treatment. Plaquing efficiency represents the facility with which a virus can form plaques on a given cell monolayer. The plaquing efficiency could thus provide data on the susceptability of MNNG treated cells to viral infection. Cells in this experiment were treated with different amounts of MNNG, infected with HSV

TABLE II

Titration of HSV 2

Dose MNNG (ug/ml)	Plaquing Efficiency[a] (Titre PFU/ml) Experiment		Virus Yield[b] (Titre PFU/ml) Experiment		Ratio[c]
	I	II	I	II	
0	0.89×10^7	1.49×10^7	1.98×10^7	1.68×10^7	-
0.025	1.01×10^7	2.19×10^7	1.89×10^7	1.73×10^7	0.99
0.050	1.15×10^7	1.85×10^7	1.91×10^7	1.99×10^7	1.07
0.100	1.20×10^7	1.80×10^7	2.61×10^7	2.87×10^7	1.50
0.200	1.09×10^7	1.79×10^7	2.65×10^7	2.75×10^7	1.48

[a] Triplicate monolayers of 143 TK^- cells were treated with the indicated amounts of MNNG for 1.5 hours, rinsed in PBS and infected with HSV 2. Cells were overlayed with medium as indicated for the 'Plaque Assay' in "Materials and Methods". Plaques were enumerated 48 hours later.

[b] Equal numbers of 143 TK^- cells (in duplicate) were treated with the indicated amounts of MNNG for 1.5 hours, rinsed in PBS and infected with HSV 2 (MOI=1). Virus was harvested 24 hours after infection and was titrated as indicated in "Materials and Methods".

[c] Ratio refers to PFU/ml present in MNNG treated cells to PFU/ml in non-treated cells, based on virus yield.

2, overlayed with plaque assay medium and the plaque yield was determined. The results (Table II) show a slight but not consistent increase in the plaquing efficiency of virus in MNNG treated cells. The data suggest that the increase in HSV production is not due to an increase in the number of cells being infected, but to a higher virus yield per cell.

Effect of MNNG on Enzyme Expression

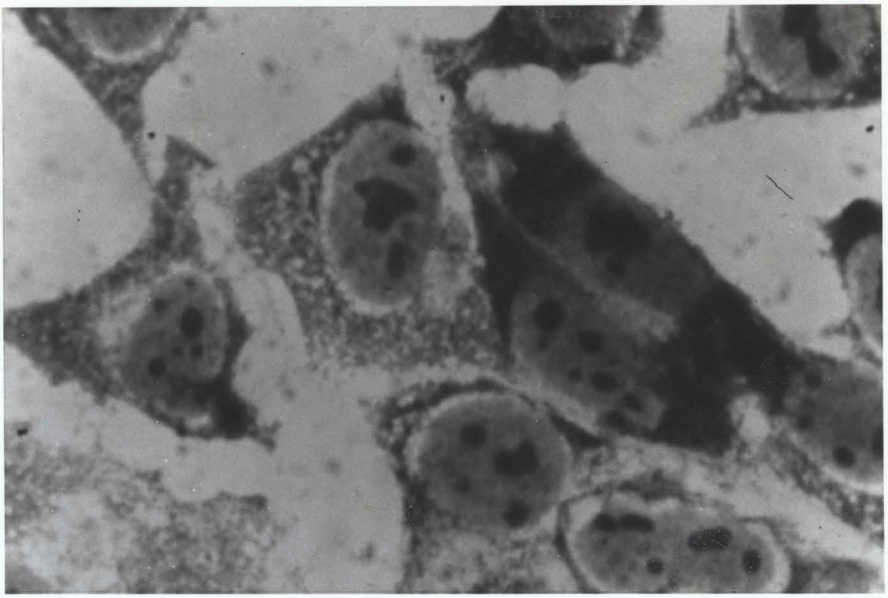
The MNNG treated cells were observed to produce greater yields of HSV than non-treated cells. In order to further examine the influence that MNNG treatment of cells had on virus replication, two viral enzymatic functions related to DNA synthesis were studied, the viral thymidine kinase and the viral DNA polymerase.

HSV induced TK can be indirectly assayed by the incorporation of ^{3}H TdR into infected cells. The cell line (143 TK^{-}) utilized in this study was deficient in the cytosol TK. The incorporation of ^{3}H TdR into DNA was then expected to be the result of HSV induced TK activity. To examine the effect of MNNG treated 143 TK^{-} cells on HSV induced ^{3}H TdR incorporation, MNNG treated cultures were infected with HSV 2, pulse-labeled with ^{3}H TdR for one hour at various times post-infection,

and prepared for autoradiography. The examination of randomly selected fields in the autoradiographs obtained suggested that each cell could be placed in any of two groups. Negative cells were defined as having less than 6 grains per nucleus. All the cells with 6 or more grains per nucleus were considered positive.

The autoradiographs shown in figures 5 to 8 are representative fields of those observed in MNNG treated and non-treated infected cells. The autoradiograph shown in figure 5 (non-treated, non-infected cells) and figure 6 (MMNG treated, HSV infected cells at 0 hours post-infection) show negative cells. The lack of grains in either the cytoplasm or nucleus in these cultures suggest the absence of significant levels of endogenous cellular ^{3}H TdR induced incorporation (e.g. mitochondrial TK or cellular TK^{+} revertants). Figure 7 is an autoradiograph of non-treated HSV infected cultures at 4 hours post-infection. The cells show an increase in both the amount of grains per nucleus and the number of positive nuclei in the infected cultures. Figure 8, prepared from MNNG treated HSV infected cultures at 6 hours post-infection, is representative of cultures with both the highest number

Figures 5-8 Growing cells were treated 0.1 ug/ml MNNG for 1.5 hours prior to infection with HSV 2 (MOI=2). Cultures were pulsed at various times post-infection for one hour with 1 uCi/ml ^{3}H TdR. Cells were fixed, developed, and stained as indicated in "Materials and Methods". Figure 5- control cells; Figure 6 - MNNG treated, HSV infected, time: 0 hour post-infection; Figure 7 - non-treated, HSV infected, time: 4 hours post-infection; Figure 8 - MNNG treated, HSV infected, time: 6 hours post-infection.



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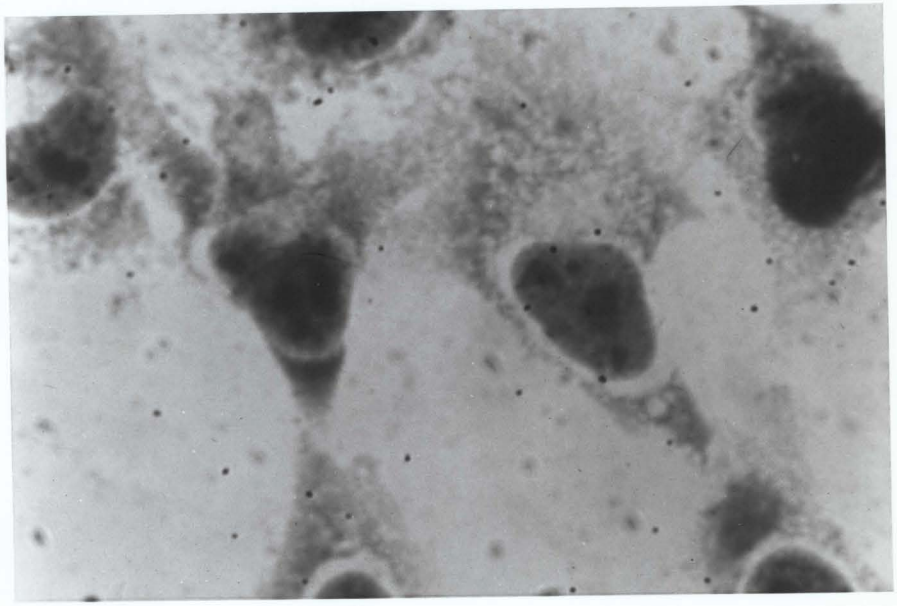

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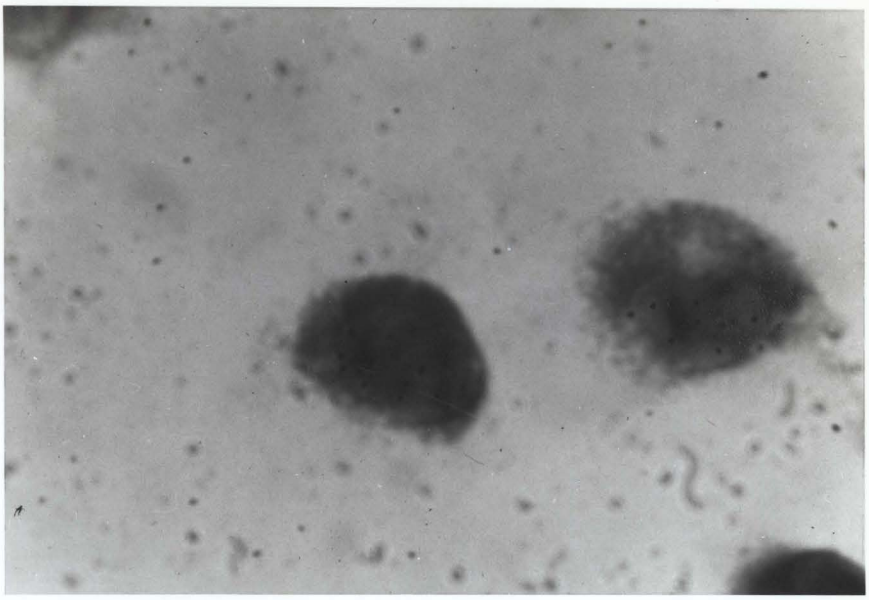

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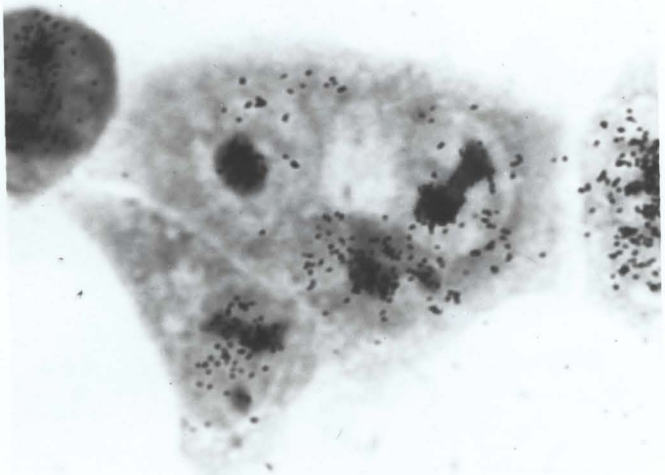


8

of positive nuclei and amount of grains per nucleus. It should be noted that throughout the course of HSV infection, the MNNG treated cells were observed to have heavier labeling than non-treated infected cultures.

Table III summarizes the results obtained from the autoradiographs in this experiment. The data show that more positive cells were present in the MNNG treated infected cultures than in the non-treated infected cultures. The highest number of positive cells were observed at 6 hours post-infection. At this time 65.1% of the MNNG treated infected cells and 52.3% of the non-treated infected cells were positive. The difference in the number of positive cells in MNNG treated and non-treated infected cultures is statistically significant ($p < 0.001$, determined by a chi square test).

To further quantitate the above results, the same basic experiment was repeated, but rather than use autoradiographic techniques, HSV induced incorporation of ^{3}H TdR was determined by scintillation counting of TCA precipitable counts. The results are shown in figure 9. A steady increase can be observed in virally induced ^{3}H TdR incorporation until a peak is reached at 6 hours post-infection. The incorporation of ^{3}H TdR in MNNG

TABLE III

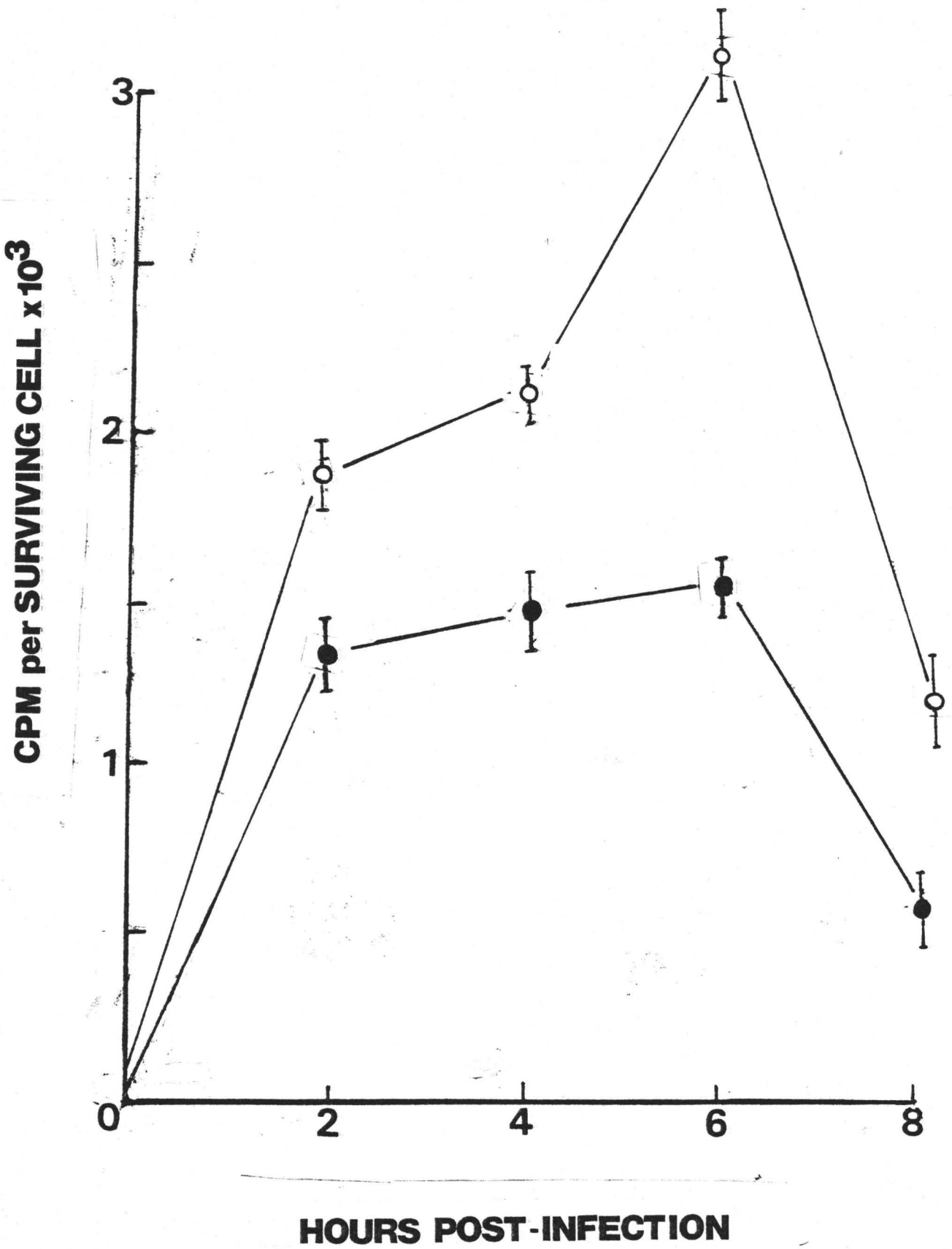
HSV 2 Induced ^{3}H TdR Incorporation in 143 TK^{-} Cells

Hours Post-Infection	% Cells with Positive Nuclei	
	-MNNG	+MNNG
0	0	0.5
4	38.7	49.8
6	52.3	65.1
8	31.25	40.8

Cells were treated with 0.1 µg/ml MNNG for 1.5 hours, rinsed with PBS and infected with HSV 2 (MOI = 2). Cells were pulse-labeled for one hour with 1 µCi/ml ^{3}H TdR at times indicated. Slides were then fixed and processed for autoradiography. Results are an average of 2 experiments done in triplicate; All the cells in 20 randomly selected microscopic fields were recorded for each slide, approximately 600 cells were scored for each time indicated.

Figure 9 HSV Induced ^{3}H TdR Incorporation in 143 TK^{-} Cells

MNNG treated (0.1 ug/ml for 1.5 hours; O——O) and non-treated (●——●) 143 TK^{-} cells were infected with HSV 2 (MOI= 2). HSV induced ^{3}H TdR incorporation is measured as TCA precipitable counts per survivng cell at the times indicated.


CPM per SURVIVING CELL x10^3
3
2
1
0
2
4
6
8
HOURS POST-INFECTION

treated and non-treated HSV infected cultures are different in magnitude and appear to be parallel. The difference in ^{3}H TdR incorporation in infected cultures between MNNG treated and non-treated cultures was found to be statistically significant at the peak of the ^{3}H TdR incorporation ($p<0.001$, determined by an unpaired t test).

The difference in magnitude between MNNG treated and non-treated HSV infected cultures expressed as the relative increase in ^{3}H TdR incorporation is shown in table IV. The relative increase was determined from the ratio of CPM per surviving cell in the MNNG treated HSV infected cultures at the times indicated, over the values obtained from non-treated HSV infected cultures. At the peak of ^{3}H TdR incorporation the difference in magnitude was maximal with a ratio of 2.30. Since the virally induced ^{3}H TdR incorporation is increased in MNNG treated cells, it can be inferred that treatment of cells with the drug prior to infection may affect viral DNA synthesis.

In order to maximize the observed increase in virally induced ^{3}H TdR incorporation, incubation periods between MNNG exposure and HSV infection were studied. To determine the optimal incubation period, cells were infected at various times post-exposure to MNNG and ^{3}H TdR

TABLE IV

Increase of ^{3}H TdR Incorporation in HSV Infected Cells Treated with MNNG

Hours Post-Infection	Increase in Incorporation of ^{3}H TdR[a]
0	1.88
2	1.25
4	1.46
6	2.30
8	2.10

[a] The increase is expressed as the ratio of CPM per cell treated with MNNG over CPM per cell not treated.

incorporation was determined at 6 hours post-infection. The data presented (figure 10) demonstrate that maximal ^{3}H TdR incorporation occurs with a one hour incubation period between MNNG exposure and HSV infection. The relative enhancement observed over non-treated infected cultures at this time is 2.35, if the incubation period between MNNG treatment and infection is extended to 4 or more hours the enhancement decreases.

The incorporation of ^{3}H TdR observed in the previous experiments is indicative of increased DNA synthesis. Studies were carried out to determine how MNNG treatment of cells prior to HSV infection affects the <u>in vitro</u> activity of viral enzymes associated with DNA synthesis, the HSV TK and the HSV DNA polymerase. <u>In vitro</u> enzyme assays were carried out with crude extracts obtained seven hours post-infection from HSV infected MNNG treated and non-treated cells.

The results for the TK assay are shown in table V. Since 143 TK^{-} cells were utilized in these experiments, the phosphorylation of ^{3}H TdR was expected to be primarily due to HSV TK. The TK activity observed in the extracts from MNNG treated cultures shows a 2.1 fold increase over non-treated infected cultures (table V, rows C and D).

Figure 10 Increase in ^{3}H TdR Incorporation at Various Times Post-exposure to MNNG

143 TK^{-} cells were treated with 0.1 ug/ml MNNG for 1.5 hours; then, at the times indicated, were infected with HSV (MOI=2). At six hours post-infection, cells were pulse-labeled with 1 uCi/ml ^{3}H TdR for one hour. Incorporation was determined from TCA precipitable counts of surviving cells. The relative increase is the ratio of CPM per surviving HSV infected MNNG treated cell over CPM per surviving HSV infected non-treated cell at the times indicated.

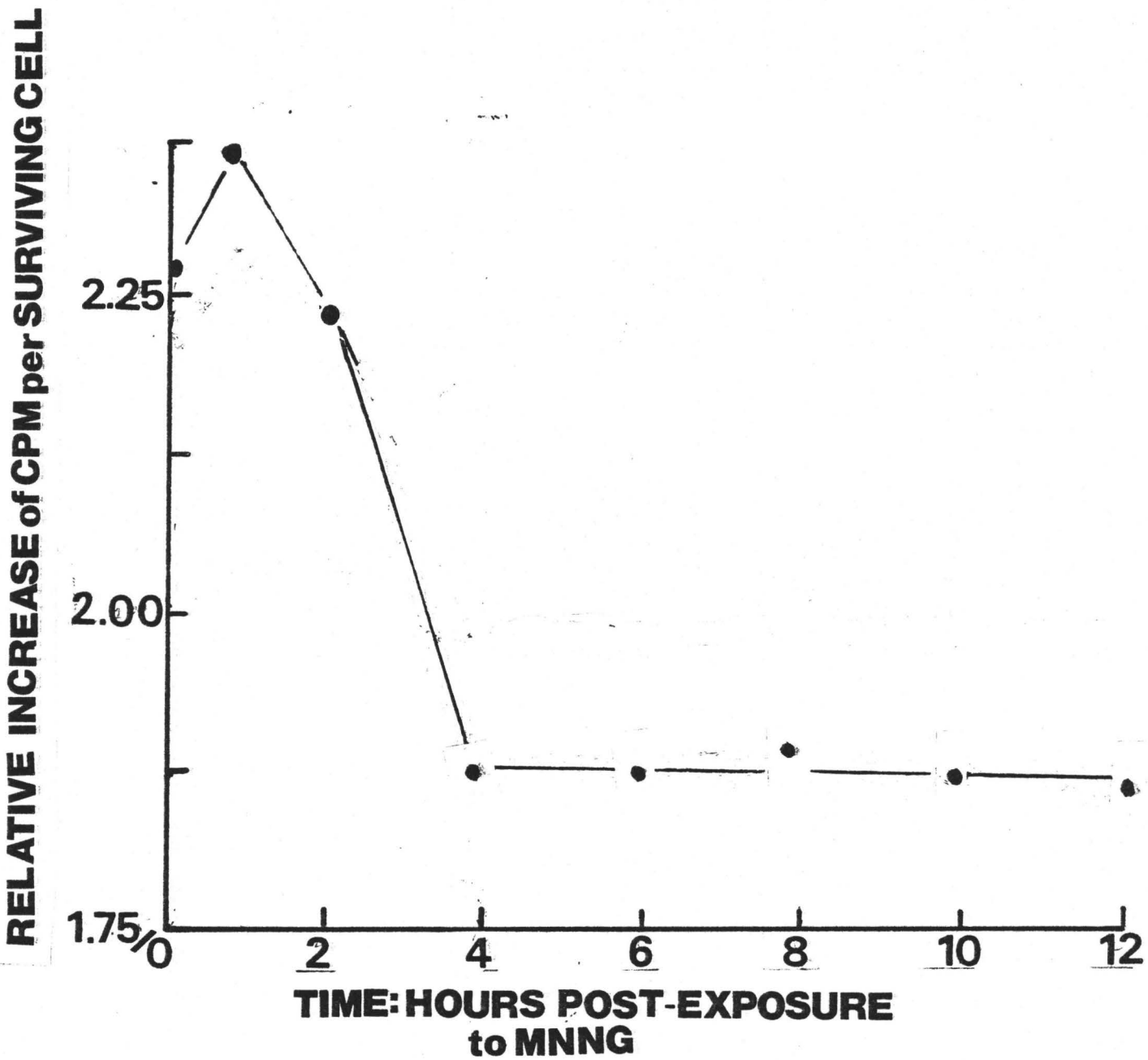

RELATIVE INCREASE of CPM per SURVIVING CELL
2.25
2.00
1.75
0
2
4
6
8
10
12
TIME: HOURS POST-EXPOSURE
to MNNG

TABLE V

Activity of Thymidine Kinase and DNA Polymerase in HSV 2 Infected Cells in the Absence and Presence of MNNG[a]

	Assay for HSV Thymidine Kinase Assay[b]		Assay for Cellular DNA Polymerase Assay[c]		Assay for Viral DNA Polymerase Assay[c]	
	Experiment I	II	I	II	I	II
(A) TK^- 143 cells	3.27×10^{-4}	4.14×10^{-4}	7.80×10^{-2}	9.52×10^{-2}	9.10×10^{-3}	8.6×10^{-3}
(B) TK^- 143 cells & MNNG	N.D.[d]	N. D.	1.00×10^{-1}	8.87×10^{-2}	9.86×10^{-3}	$6.83.10^{-3}$
(C) TK^- 143 cells & HSV 2	2.90×10^{-2}	3.32×10^{-2}	$3.63.10^{-1}$	4.63×10^{-1}	2.62×10^{-1}	2.48×10^{-1}
(D) TK^- 143 cells & HSV 2 & MNNG	5.78×10^{-2}	7.07×10^{-2}	2.75×10^{-1}	4.71×10^{-1}	2.05×10^{-1}	2.24×10^{-1}
Ratio (D)/(C)	2.01	2.10	0.76	1.02	0.78	0.90

a Duplicate cultures of 143 TK^- cells were exposed to MNNG for 1.5 hours, rinsed in PBS, infected with HSV 2 (MOI=1) and harvested seven hours post infection as indicated in Materials and Methods.

b Activity for the TK assay is expressed as picomole ^3H TdR phosphorylated per ug protein in 30 minutes at 37°C.

c Activity for the DNA Polymerase assays is expressed as picomole ^3H TTP incorporated per ug protein in 30 minutes at 37°C.

d Not Done

It is interesting to note that this increase agrees well with the enhancement in ^{3}H TdR incorporation observed in the previous experiments (table IV). The low but detectable levels of phosphorylation observed in extracts obtained from control cultures (table V, row A) are probably due to the activity of other cellular thymidine kinases known to exist in TK^{-} cells (mitochondrial TK or other minor cytosol TK; see: Kit et al, 1975).

The results for the DNA polymerase assays are shown in table V. To distinguish between cellular and viral DNA polymerase the assay conditions were altered. The addition of $(NH_4)_2SO_4$ and lowering of the Mg^{++} concentration in the assay mixture were necessary in order to obtain optimal conditions for the detection of HSV DNA polymerase (Wiessbach et al., 1973).

Extracts of non-infected cells were assayed for cellular and viral DNA polymerase (table V, row A). The results show a 10 fold decrease in the cellular DNA polymerase activity under assay conditions for HSV DNA polymerase. Extracts of infected cells assayed for HSV DNA polymerase demonstrated approximately a 30 fold increase in the incorporation of ^{3}H TTP into DNA compared to non-infected cells (table V, rows A and C). The infected cell extracts were also assayed for cellular DNA

polymerase and demonstrated approximately a 5 fold increase in DNA polymerase activity compared to non-infected cell extracts (table V, rows A and C) indicating that the assay for cellular DNA polymerase also detects a fraction of the viral DNA polymerase. The larger increase of DNA polymerase activity in infected cells observed in the assay conditions for HSV DNA polymerase suggests that the ^{3}H TTP incorporation into DNA is due to HSV DNA polymerase. However, since extracts of non-infected cells assayed for HSV DNA polymerase showed some incorporation of ^{3}H TTP into DNA (table V, row A), the possibility that the assay for viral DNA polymerase also detected cellular DNA polymerase cannot be eliminated. It should be noted that extracts obtained from non-infected MNNG treated and non-treated cells did not show any significant difference in the DNA polymerase activity under the assay conditions for both cellular and HSV DNA polymerase (table V, rows A and B). The results obtained from HSV infected MNNG treated and non-treated cells did not demonstrate any difference in DNA polymerase activity (table V, row D). This result is different from those obtained with the parameters previously examined.

DISCUSSION

This study provides evidence that treatment of 143 TK^- cells with MNNG, prior to HSV infection, affects viral production, suggesting that viral synthesis may at least be partially dependent on cellular mechanisms which are affected by MNNG treatment.

The data show a 1.5 fold increase in the production of HSV in MNNG treated cells which is reproducible and statistically significant ($p < 0.001$, table II). A previous study on HSV replication examined the increase in viral production in cells which had been exposed to another type of repair inducing gent, uv-irradiation (Coppey and Nocentini, 1976). In this study, the authors noted that the enhancement of HSV production after uv-irradiation of cells appeared to follow the kinetics of excision repair. As well, the source of nucleotides (i.e. exogenous or endogenous) used during DNA repair of uv-irradiated cells and HSV replication was examined, and the data here provided evidence suggesting that DNA repair and HSV DNA synthesis may share common metabolic pathways (Coppey, 1977). On the basis of this evidence, the author

has suggested that the ability of cells to support HSV replication may be correlated with the extent of DNA repair in cells (Coppey, 1977). Although the results do not permit any final conclusion, the data presented here do not contradict this, implying that the replication of HSV is dependent on or parallels repair mechanisms occurring in the cell.

The possibility of MNNG treated cells being more susceptible to infection was examined by comparing the plaquing efficiency of HSV in MNNG treated and non-treated cells (table II). The results obtained from this experiment did not show an increase in the production of plaques in MNNG treated cells, indicating that the enhancement in the production of the virus was not due to an increased number of cells being infected.

MNNG has been shown to react with cellular components to produce the following reactions: alkylation of DNA, RNA and protein (Pegg, 1977), a decrease in protein (Anderson and Burdon, 1970) and replicative DNA (Strauss et al., 1974) synthesis, as well as the induction of DNA repair (Roberts, 1978). The actual MNNG induced alterations to the cells responsible for the observed

results cannot be discerned from the experiments presented in this study.

Experiments which examined the time required between MNNG treatment and HSV infection of cells to produce maximal ^{3}H TdR incorporation demonstrated a different optimal incubation period from that observed by Coppey and Nocentini (1976) in uv-irradiated cells. The enhancement of virally induced ^{3}H TdR incorporation had a maximal increase (2.35 fold) with an incubation period of one hour between MNNG treatment and HSV infection (figure 10). Uv-irradiated cells required an incubation period of at least 48 hours to produce a similar enhancement (Coppey and Nocentini, 1976). If it is assumed that the induction of DNA repair may be responsible for the increase in viral production and virally induced ^{3}H TdR incorporation, then the difference in the time post-exposure to uv-irradiation and MNNG treatment could be related to differences in the type of lesions produced by these agents and/or the repair they induce.

The activity of two HSV specific enzymes associated with DNA synthesis, HSV TK and HSV DNA polymerase were studied. The results (table V) showed differences in

the relative enhancement of enzyme activity in MNNG treated HSV infected cells.

The HSV induced TK in the MNNG treated and non-treated infected cells was studied by both indirect and direct methods. Since the cell line used (143 TK^-) is deficient in TK, ^3H TdR incorporation in HSV infected cells was used as an indirect measure of HSV TK. The results of this experiment (figures 5 to 8, table III) demonstrate a 2 fold enhancement of ^3H TdR incorporation in cells treated with MNNG prior to viral infection. The phosphorylation of ^3H TdR was measured directly in extracts obtained from MNNG treated and non-treated infected cells by the <u>in vitro</u> TK assay. The results (table V, row C and D) show a 2 fold increase in the amount of phosphorylation which agrees with the increase observed in ^3H TdR incorporation.

The increase in ^3H TdR phosphorylation may be related to changes in either the specific activity of the enzyme, or in the amount of enzyme produced. In order for changes in specific activity to occur (i.e. changes in the amount of ^3H TdR phosphorylated per given amount of TK), the enzyme or substrate would have to be altered. There is no reason to suspect that there is a specific

alteration to the substrate, ^{3}H TdR, other than phosphorylation occurring under the assay conditions in extracts obtained from MNNG treated infected cells. The possibility of the enzyme being altered to increase its activity does not appear likely, since the half-life of MNNG is a few minutes (Lawley and Thatcher, 1970) and the phosphorylation of ^{3}H TdR is measured in extracts obtained 7.5 hours post-exposure to the drug. Studies on HSV replication in uv-irradiated cells also show a similar increase in ^{3}H TdR phosphorylation (Coppey and Nocentini, 1976) and it seems unlikely that MNNG treatment and uv-irradiation of cells prior to infection could alter the enzymes in a similar fashion. A final possibility to account for the increase in ^{3}H TdR phosphorylation is related to the amount of TK produced. Since virus replication is increased in MNNG treated cells, it is likely that more TK is produced in MNNG treated cells.

HSV DNA polymerase activity, unlike the parameters measured in previous experiments, did not show an increase in assays carried out on extracts obtained from MNNG treated HSV infected cells. The amount of ^{3}H TTP incorporated into DNA in MNNG treated infected cells showed a slight but not significant decrease (table V, rows C and

D).

The experiments conducted here show that HSV TK and HSV DNA polymerase have different levels of activity in MNNG treated cells but do not allow any conclusions with regard to the responsible mechanisms. HSV protein synthesis is regulated by a cascade type of mechanism. (Honess and Roizman, 1974). This has been demonstrated for TK which appears to require the prior synthesis of at least one viral regulatory α polypeptide for TK synthesis (Leung, 1978). The regulation of HSV DNA polymerase has not been well characterized, although complementation studies show at least three genes are necessary for its production (Purifoy et al., 1975). With the above information and the observation that under experimental conditions, HSV TK is enhanced and HSV DNA polymerase is not greatly affected, it can be suggested, on a speculative basis, that there could be differences in the regulation of these viral enzymes.

In conclusion, the data show that treatment of cells with MNNG prior to infection does have an influence on HSV replication: production of viral progeny is increased. The increase is not observed in all the viral products present during replication (i.e. HSV DNA polymerase).

The approach presented in this study, the treatment of cells with an alkylating agent prior to viral infection, may be of use in elucidating the control of individual proteins (by monitoring their levels and/or specific activity at various times during infection). In addition, with the appropriate modifications to the system, a similar approach could be used to study the co-operative effect of physical or chemical and viral carcinogenic agents in biochemical and oncogenic transformation.

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