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GRADUATE COLLEGE

CHARACTERIZATION OF THE INHIBITION OF DNA SYNTHESIS IN REOVIRUS-INFECTED L CELLS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

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BY

ANDREW FINLAY FROST

Norman, Oklahoma

1975

CHARACTERIZATION OF THE INHIBITION OF DNA SYNTHESIS

IN REOVIRUS-INFECTED L CELLS

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TO DIANA

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CHARACTERIZATION OF THE INHIBITION OF DNA SYNTHESIS IN REOVIRUS-INFECTED L CELLS

CHAPTER I

INTRODUCTION

A variety of experimental approaches have been employed by cell and molecular biologists in examining the regulation of transcriptive and replicative functions of cellular genetic material. Many of these approaches require manipulation with substances such as a protein synthesis inhibitor, and then the effects of this treatment on deoxyribonucleic acid (DNA) functions are examined. Regardless of how complex or indirect the modes of action may be, any treatment of a eukariotic cell which results in a measurable deviation from normal cellular metabolism must have an absolute molecular basis. However, the specificities of action of inhibitors of cellular RNA and DNA synthesis are not restricted to these functions (19) and, though each may have a single predominant effect, other influences on the cell make it difficult to establish clear cause and effect relationships. Such problems are indications of the extreme complexity of cellular structure and function. Mechanisms regulating cellular transcription and DNA replication, which appear to be strictly controlled, are poorly understood.

May viruses are known to cause alterations of normal biosynthetic

activity in infected cells (2, 71). These alterations occasionally result in permanent cell alteration and transformation (39, 73), but more often result in virus production and cell degeneration and death. Elucidation of the specific virus-directed changes which allow both the maintenance of functional integrity of the cell and the synthesis of viral components could lead to a better understanding of the particular virus replicative cycle and of normal cellular regulatory processes.

Reovirus is an RNA virus which affects cellular nucleic acid synthesis. The first measurable effect of reovirus infection on gross cellular biosynthesis is the inhibition of cellular DNA synthesis (17, 18, 41, 122). This is not a common effect among those RNA viruses which have been intensively studied. Most other RNA viruses which inhibit cellular DNA synthesis probably do so as a secondary effect since inhibition occurs either concomitantly with or after such other alterations as reduction of protein and RNA synthesis. Blue tongue virus (BTV), an animal virus which is structurally very similar to reovirus (54, 136), inhibits translation of cellular messenger RNA (mRNA) and causes disaggregation of polysomes (57, 84) in infected cells. This effect is detectible slightly before inhibition of cellular DNA synthesis is seen (56). Evidence indicates these effects may be due to cytotoxicity of the double stranded RNA (dsRNA) genome of BTV (84). Inhibition of DNA synthesis is seen in cells infected with Newcastle's Disease Virus (NDV), a paramyxovirus, but not before inhibition of cellular protein synthesis (20, 30, 47, 48). Mengovirus, one of the picornaviruses, rapidly inhibits ribosomal RNA (rRNA) and messenger RNA (mRNA) synthesis in infected L-cells (2, 24, 85). Cellular DNA and protein syntheses are

inhibited and a progressive disaggregation of polysomes is seen (79, 94, 134). The inhibition of DNA synthesis probably is secondary to the many other effects of picornavirus infection (29, 47, 48). Vescicular stomatitis virus (VSV), a rhabdovirus, inhibits RNA and protein synthesis in two stages in several infected cell lines (12, 55). The first stage corresponds to the onset of observable cytoplathic changes in the cells (83); the second occurs later in the infection cycle (141).

Since the replication of most RNA animal viruses is believed to occur entirely in the cytoplasm (2, 14, 20), it is not surprising that they do not inhibit the synthesis of cellular DNA in the initial stages of their replicative cycles. However, data from nuclear transplantation (26, 44, 46) and cell fusion experiments (25, 50, 51) in many different cell lines indicate that events in the cytoplasm do play important roles in nuclear regulation (101).

Structurally, the reovirion is bounded by two capsid shells, both of which display an icosahedral symmetry and are composed entirely of viral protein capsomers from which only seven structural polypeptides can be identified (43, 62, 113, 114, 143, 144). The outer and inner capsid diameters are approximately 75 nm and 52 nm, respectively (82). The inner capsid bounds the viral core structure which contains the 10 unique dsRNA genome segments (42, 135), having a total molecular weight of approximately 1.5×10^7 daltons. The genome segments, which can be separated on sucrose gradients, fall into three size classes, L, M, and S, with sedimentation coefficients of approximately 14, 12, and 10.5S, respectively (8, 111, 140). Reovirions also contain an assortment of small single-stranded RNA (ssRNA) molecules (5, 72, 118) which are

enclosed in the viral core structure (63). About 50% of these ssRNA molecules are composed primarily of adenylic acid (90). It is not known what, if any function these oligonucleotides (A-rich RNA) play in viral replication. It has been proposed that they are products of abortive transcription within progeny virions in infected cells (7, 90). Treatment of L-cells with purified A-rich RNA has, however, resulted in transient inhibition of DNA synthesis (49, 88).

The intracellular replication of reovirus begins when the virion is adsorbed to the cell surface and is engulfed and contained in a phagocytic vacuole which migrates to the interior of the cell and fuses with lysosomes (23, 124). After about 50% of the outer capsid protein is digested away by lysosomal digestion, (13, 126) the resultant subvirus particle (SVP) is apparently transported to the perinuclear regions of the cell, where accumulation of viral products, beginning about 8 h post-infection at 37 C, has been followed by a variety of techniques (23, 35, 43, 126, 132). These viral inclusions are often seen in association with spindle fibers of the mitotic apparatus of infected cells (22, 124, 131). However, reovirus replication is not dependent on these tubules, and virus production is not arrested by colchicine treatment (22, 130, 132). Parental reovirions do not release free genome dsRNA into the cytoplasm (13, 124) at any time in the replicative cycle. The dsRNA is conserved within the SVP.

Shortly after partial uncoating of the parental reovirion, (41, 74), early viral mRNA is transcribed by the virion-associated RNA dependent RNA polymerase (transcriptase or DS——SS RNA polymerase) (6, 10, 119). This early mRNA synthesis continues for at least 4 h at

37 C and 10 h at 31 C (10). At this time, the formation of progeny dsRNA is detectable (74, 116), and like parental dsRNA, occurs only in association with immature virion precursors (40). These dsRNA-containing particles undergo a series of uncharacterized sequential additions of polypeptide subunits resulting in SVP's which resemble core particles (1, 89, 104, 142).

Schonberg, et al. (108), have shown that the reovirus "plus" strand or viral mRNA also serves as the template for the synthesis of the complementary "minus" strand RNA. The enzyme responsible is now referred to as the SS——DS RNA polymerase (142) and is responsible for the generation of new genome dsRNA in immature particles. Evidence gathered by Zweerink, et al. (142), indicates that "plus" strands are transcribed into "minus" strands only once and that this newly formed dsRNA remains as such and is conserved in the progeny particle. This would account for the fact that neither the "minus" strand nor the progeny dsRNA is ever found free in the cytoplasm.

The immature progeny particles containing dsRNA genomes possess transcriptase (DS——SSRNA polymerase) (103, 142) activity and are active in the production of viral mRNA. During this second stage of the virus replicative cycle, these progeny structures are responsible for the production of the bulk of viral mRNA in reovirus-infected cells. The rates of synthesis of both ds and ssRNA peak and begin to decline from about 8 h post-infection at 37 C and at about 20 h post-infection at 31 C(64). Mature progeny virus can be isolated from infected cells from about 2 h after the onset of dsRNA synthesis (64).

It has been demonstrated by Gomatos and Tamm (42) that the first detectible change in total macromolecular synthesis in reovirus

infected cells is the inhibition of DNA synthesis. Shaw and Cox (18, 122) have also found that the DNA inhibitory process is somewhat dose-dependent with respect to the input multiplicity of reovirus and is detectable as early as 3 h post-infection at 250 pfu/cell. This indicates that a component of the reovirion could at least initiate the inhibitory process. It has been found that synchronous cells, if infected prior to the onset of DNA synthesis, do not enter the DNA synthetic phase of the cell cycle (18, 30). This implies that the observed inhibition of DNA synthesis is not simply due to a decrease in the rate of synthesis, but rather an inhibition of initiation. DNA chain elongation proceeds at the same rate in both control and reovirus infected cells, but fewer points of initiation of DNA synthesis are seen by autoradiography of DNA from reovirus-infected cells (27, 47).

The inhibition of DNA synthesis is not a result of general cytotoxicity of the input virus (17, 49). Ghosted reovirions ("top component" or complete virions minus the dsRNA genome) do not alter macromolecular synthesis in treated cells, even at extremely high particle/cell ratios (49, 75). Thus, cytotoxicity cannot be ascribed to the input viral protein, even though it does indeed show kinetics of cell association (15) similar to those of whole reovirions.

Ultraviolet light-irradiated reovirions which have no infectivity but retain limited transcriptive ability inhibit cellular DNA synthesis in L-cells as effectively as an equivalent number of infectious virions (49, 122). These results and the data from top-component-treated cells indicate that some event in the reovirus replicative cycle is responsible for the inhibition of DNA synthesis.

Reovirus core particles, the limit-digestion products by -chymotrypsin (63), possess low infectivity (17) and become cell associated with a lower efficiency than whole virions (15). Cores do not affect macromolecular synthesis in treated cells (17, 49). However, these particles may have a different intracellular fate than reovirions which are partially uncoated after adsorption.

Joklik (63) and others (3, 119, 127) have reported that reovirions whose outer capsid has been partially degraded by chymotrypsin digestion produce reovirus mRNA ("plus" strands) in vitro in molar ratios inversely proportional to their molecular weights (127). This situation would be expected if each of the dsRNA genome segments were independently transcribed at the same rate of polynucleotide chain elongation. If, however, the Mg^{+2} or any of the ribonucleoside triphosphate concentrations are sub-optimal for transcriptions, the overall rates of synthesis of newly transcribed mRNA declines, and the relative rates at which each segment is transcribed are drastically altered (127). The altered pattern of in vitro mRNA synthesis approaches more closely the actual pattern of reovirus mRNA synthesis in infected cells (64). The molar ratios of reovirus mRNA species synthesized in vivo could be an indication of suboptimal concentrations of ribonucleoside triphosphates (NTP's) available for nuclear functions. It is possible that the perinuclear localization of reovirus replication and product accumulation affect the concentration of NTP's available to the nucleus. Ensminger and Tamm (27) have proposed that a nuclear membrane alteration could mediate the inhibition of DNA synthesis.

Since Bartkoski found (4) that neither the concentrations nor

activities of nuclear and cytoplasmic DNA polymerases are altered significantly at times after inhibition of DNA synthesis in reovirus infected cells, it is possible that the level of NTP's could limit nuclear function. It was also found that infected and uninfected hypotonically swollen cells, when substituted for nuclei in the in vitro DNA synthesis reaction mixture, did not differ in their abilities to incorporate labeled deoxyribonucleoside triphosphates into nuclear DNA (4). The inhibition of DNA synthesis is not the result of degradation of host DNA (122) and host DNA does not lose its ability to act as template for in vitro DNA synthesis (4).

Since the immediate purpose of this work was to expand our knowledge of reoviral inhibition of cellular DNA synthesis, evidence which favors a less direct effect by reovirus upon DNA synthesis should be examined. Much of the data already presented could support indirect inhibition of DNA synthesis through concomitant or prior inhibition of cellular protein and RNA synthesis. When synthesis of RNA, DNA and protein is followed in infected cells by radioisotope-labeled precursor incorporation, the rate of DNA synthesis is the first function to be affected by 8 h post infection. It is obvious that viral mRNA is transcribed as early as 2 h post infection, and by 8 h post infection, progeny SVP's are beginning to transcribe mRNA. Total RNA and protein synthesis are constant in infected cells until at least 14 h post-infection. Viral RNA and protein are synthesized in infected cells, but the rates of total RNA and protein labeling are constant until after the inhibition of DNA synthesis is obvious. Thus, cellular RNA and protein synthesis are apparently depressed to the extent that viral RNA

and protein are synthesized in infected cells. The question to be answered, then, is whether the inhibition of cellular RNA and protein synthesis seen in infected cells is responsible for the inhibition of DNA synthesis.

By immune precipitation of viral protein from infected cell cytoplasm, Zweerink and Joklik (143) found that inhibition of cellular protein may begin as early as 5 h post infection and is correlated with the time that the rapid second stage of viral mRNA synthesis begins. This suggests that cellular protein synthesis is inhibited by competition of viral mRNA with cellular mRNA for some component of the protein synthesizing system of infected cells (64), which ultimately results in the inhibition of DNA synthesis. Inhibition of DNA synthesis by reovirus is characteristic of that observed in cells treated with a protein synthesis inhibitor such as cycloheximide and puromycin (29). However, nuclei from cycloheximide-treated cells are not competent to synthesize DNA in vitro (53) while nuclei from reovirus-infected cells, synthesize DNA at the control levels for nuclei from uninfected cells (4).

We have examined inhibition of DNA synthesis in reovirus-infected cells using several different approaches, since there are many different possible causes which could be singly or multiply responsible for this effect. Resolving the many questions surrounding an inhibitory or regulatory phenomenon in a system as complex as a virus-infected cell may require much indirect evidence to establish the cause and effect relationships involved. We set out first, in response to criticism concerning the DNA labeling methods employed, to establish the validity of pulsing cells with labeled thymidine to measure the rate of DNA synthesis in the L-cell system.

The first facet of the investigation involved the study of alteration of macromolecular synthesis in L cells infected with different temperature-sensitive, conditional-lethal mutants of reovirus. Mutant-infected cells were investigated primarily under conditions in which the mutants did not complete the normal virus replicative cycle.

Another approach employed in this investigation was based on the observation that the site of virus replication and accumulation is the perinuclear region of the infected cells. Since reovirus infection results in the inhibition of DNA synthesis, the intracellular localization of reovirus replication was suggestive of a requirement for nuclear DNA synthesis in the completion of initial stages of viral replication. To investigate this possibility, cells which had been treated with a DNA synthesis-inhibitor prior to reovirus infection were examined after infection to ascertain the effect of the treatment on the viral replicative cycle.

A number of possible causes for the inhibition of DNA synthesis (such as precursor pool depletion or reoviral interference in the translation of cellular protein) could be studied by examining the effects of reovirus infection on the replication of viral DNA in dually-infected cells. Specifically, the ability of vaccinia and herpesvirus which replicate in the cytoplasm and nucleus, respectively, to synthesize viral DNA was examined. In addition, nuclear RNA and DNA synthesis and certain characteristics of polysome populations in reovirus-infected cells were examined at intervals after infection.

CHAPTER II

MATERIALS AND METHODS

Cells. Mouse fibroblasts, strain L-929 (106) (Flow Laboratories), adapted for growth in spinner culture, were used in all experiments. Stock cultures of L cells were maintained in minimal essential medium modified for spinner culture (MEM) (Grand Island Biological Supply Company) supplemented with 6% (v/v) heat inactivated fetal calf serum (FCS, Grand Island Biological Company). Cells were maintained in logarithmic growth at cell densities from 5.0×10^4 cells/ml to 2.0×10^5 cells/ml by dilution every 48 h with fresh, prewarmed growth medium.

HeLa cells (Flow Laboratories), adapted for growth in spinner culture, were used for the propagation of vaccinia and herpesvirus type 1. HeLa cells were maintained in the same growth medium and at the same cell densities as were L cells.

Asynchronous growth conditions. Cells to be used in experiments at higher cell densities than the stock culture were maintained as asynchronous cultures by centrifuging the cells from growth medium at 250 x g for 5 min, and resuspending them in fresh, pre-warmed growth medium at 48 h intervals. Cells used at 31 C for experiments with reovirus temperature-sensitive mutants were continually cultured at that temperature.

Synchronous growth conditions. Synchronous growth of L cells was achieved by a modification of the procedure of Littlefield (80). Cells were centrifuged at 50 h intervals and resuspended in fresh, prewarmed MEM supplemented with 10% (v/v) FCS. These cultures were seeded at an initial cell density of 5×10^5 cells/ml. Wide variation in pH of the growth medium was prevented by keeping these cultures unsealed and reducing the sodium bicarbonate concentration to 1.6 g/l.

Viable cell assay. The percentage of total cells remaining viable at times after infection was determined by trypan blue exclusion (93, 98, 100). At intervals post-infection 0.5 ml aliquots were removed from spinner cultures and the cells pelleted at 100 x g for 5 min. The cell pellet was resuspended in MEM containing no serum, and 0.05 ml 9.4% (w/v) trypan blue was added to the tube. The suspension was incubated at 37 C for 5 min and the cell suspension was then examined in a Newbauer hemacytometer. Cells which had excluded trypan blue and thus were unstained were considered viable.

Virus. Wild type (WT) reovirus type 3 used in this study was kindly provided by Dr. P. J. Gomatos. Plaque-purified virus was prepared by taking a well-isolated plaque from an infected L cell monolayer. The agar plug, picked with a small pipette, was transferred to 1 ml of sterile 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.5 (SSC) and maintained at 4 C for 24 h. Virus eluant in SSC was then pipetted onto one confluent L cell monolayer (25 cm^2 surface) from which the medium had been removed. One hour was allowed for virus adsorption and the SSC was replaced with fresh, prewarmed growth medium.

After incubation at 37 C for 36 h, the infected cells were sonicated and the resulting first-passage lysate was used to infect 10 large monolayers (75 cm^2 surface). After a 24 h incubation these monolayers were sonicated, pooled, titered, and frozen at -20C.

Plaque-purified stocks of temperature-sensitive (ts) reovirus mutants were prepared by a modification of the procedure of Fields and Joklik (33). Purification from plaques was as described for WT reovirus. The eluant ts mutant reovirus in SSC was inoculated onto monolayers which were 48 h from confluency at 31 C. The monolayers, after 2 h adsorption at room temperature, were fed with MEM supplemented with 7% (v/v) FCS, sealed in large plastic bottles and kept immersed in a constant-temperature 31 C water bath for 7 days. The monolayers were then sonicated, and second-passage ts mutant stocks were prepared in monolayer cultures as described for WT reovirus. The flasks containing monolayers were put in plastic bags and kept immersed in the 31 C water bath for 4 days.

Frozen, titered second-passage lysates of both WT and ts-mutant reovirus were used as inocula to prepare high-titer virus stocks for purification. L cells in spinner culture were pelleted at 250 x g for 5 min and resuspended to a concentration of 1.0×10^7 cells/ml in fresh prewarmed MEM without serum prior to virus adsorption. Cells were infected with 10-20 plaque-forming units (pfu)/cell and 1 h was allowed for virus adsorption. During the adsorption period, WT reovirus-infected cultures were incubated at 34 C and ts-mutant-infected cultures were incubated at room temperature or at 31 C. After adsorption the cells were diluted 10-fold with fresh growth medium at the appropriate temperature. WT reovirus-infected cultures were incubated at 34 C for 22 h. Mutant-infected cultures were incubated at 31 C for 36 h. Cultures

were then chilled by swirling in ice water, centrifuged at 3000 x g for 15 min at 4 C, and the pellets frozen at -20 C.

High-passage reovirus stocks were prepared by repeated passage of reovirus at input multiplicities between 20 and 50 pfu/cell. The cycle from inoculation to lysate preparation was repeated a minimum of 5 times before isopycnic CsCl gradient profiles were examined to establish defective virus content. Quantities of high-passage reovirus were prepared as described for plaque-purified stocks, except that the input multiplicity was 50 pfu/cell and the infected cultures were harvested at 20 h.

Herpesvirus type 1 and vaccinia were routinely propagated in HeLa cell monolayers at input multiplicities less than 1 pfu/cell. Monolayers were sonicated briefly when cytopathic effects (CPE) were obvious in 50% of the cells. This virus lysate was used as inoculum for propagation of the viruses in HeLa cells.

HeLa cell lysates containing herpesvirus or vaccinia were inoculated into half-confluent L-cell monolayers. After incubation at 37 C for a 2 h adsorption period, the inoculum fluid was replaced with MEM supplemented with 4% (v/v) FCS. Monolayers were observed daily for CPE. When 50% of the cells showed obvious CPE, the monolayers were briefly sonicated and used immediately as inoculum for other L-cell monolayers, or were used in an experiment. Vaccinia and herpesvirus were always passaged a minimum of three times in L cells prior to an experiment.

Virus purification. Reovirus was purified as described by Smith, et al. (128) with modification. The final freon-extracted aqueous

phase was layered over a 25 ml 20%-40% (w/w) glycerol gradient in 0.01 M phosphate buffer, pH 8.0, in a 38 ml centrifuge tube. Centrifugation was performed at 4 C for 45 min at 82,500 x g in the SW 27 rotor in a Beckman L2-50 ultracentrifuge. The opalescent virus band was collected from the center of the tube. Virus in approximately 30% (w/w) glycerol was then layered over a 10 ml CsCl density gradient (1.3 g/ml-1.4 g/ml) in a 17.5 ml tube and centrifuged at 4 C for 2 h at 80,000 x g in the SW 27.1 rotor. The virus band was collected cleanly with a syringe and was exhaustively dialysed against SSC at 4 C. This purified virus stock suspension was assayed and stored at 4 C.

Virus assay. The assay of WT reovirus was performed using the procedure of Shaw and Cox (122). Cells from spinner culture were adjusted to a density of 4 x 10^5 cells/ml and pipetted into small plastic petriplates (60 x 15 mm, Falcon Plastics) at 2.0 x 10^6 - 2.4 x 10^6 cells per plate. The cells were allowed to grow to near-confluency.

Dilutions of purified reovirus or lysate were then prepared in MEM. Growth medium was removed from the plates and 0.1 ml of each virus dilution was pipetted onto the center of a monolayer. Each dilution was normally assayed in triplicate. The monolayers were then incubated 1 h in a humid atmosphere of 5% (v/v) CO_2 in air for virus adsorption. Five ml of autoclavable MEM (Auto POW, Flow Laboratories) containing 1% (w/v) agar (Difco Laboratories), 3% (v/v) FCS and 100 μg/ml kanamycin was then added to the plates. After the agar had solidified, the plates were incubated at 39 C in an atmosphere of 5% (v/v) CO_2 in air for 72 h. A second overlay supplemented with 0.005% (w/v) neutral red was then added. Plaques were counted within 24 h.

The assay of reovirus ts mutants was similar to that described for WT reovirus, and was a modification of the procedure used by Fields and Joklik (33). All mutant assays from adsorption to quantitation were performed at 31 C. The second overlay was applied at 72 h and contained 18% (v/v) FCS. A third overlay containing 3% (v/v) FCS and 0.01% neutral red was applied on day 10 and titers were read on day 12 and recounted on day 14.

When new plaque-purified stocks of the mutant viruses were obtained, dual assays, one as just described and another as described for WT reovirus were performed. The latter was incubated at 39°C from adsorption to quantitation. This assay was to detect reversion or mutation to wild-type phenotype and to estimate the plating efficiency of that particular mutant strain at the two temperatures.

Vaccinia and herpesvirus were titered by inoculating a half-confluent large monolayer flask (75 cm^2 surface) of L cells with 0.5 ml of a 1:10 dilution of freshly prepared lysate. The diluate MEM contained no serum. After 2 h the inoculum was shaken out and replaced with fresh MEM containing 4% (v/v) FCS. Plaques could be counted in 72 to 96 h.

Assay for cellular DNA synthesis. An asynchronous spinner culture of L cells was harvested by centrifugation at 250 x g for 5 min and resuspended at a concentration of 3.0×10^5 cells/ml in fresh, pre-warmed MEM containing 6% (v/v) FCS and 100-150 μg/ml kanamycin. The cells were incubated for 6-10 h prior to splitting the culture into small 60 ml cultures in 125 ml tissue cultures flasks at time 0 for each experiment. All cultures were sampled identically after initial treatment and infection. Samples were withdrawn from each culture,

pipetted into air-tight screw-capped tubes, and pulse-labeled with 0.5 μCi/ml ^{3}H-thymidine. During the pulse period cells were maintained at the original temperature and were kept in suspension by occasional swirling. The pulse was arrested by the addition of 1 volume 10% (w/v) TCA. At least 2 h at 4 C was allowed for precipitation of TCA-insoluble material.

The precipitate was then collected on a 1.2 μm millipore filter or Whatman GF-C glass fiber filter (Reeve-Angel) and was washed 5 times with 1 volume 5% (w/v) TCA and pulled to dryness. Each filter was placed in a scintillation vial with 10 ml Beckman cocktail D (5 g PPO, 100 g naphthalene, 10 ml water and brought to 1 l with 1, 4-dioxane).

Alternatively, the precipitate was collected by centrifugation for 15 min at 1000 x g, washed three times with 4 ml 5% (w/v) TCA, washed twice with ethanol-ether (3:1), washed once with diethyl ether, and air dried (112). The precipitate was then resuspended in 2 ml 5% (w/v) TCA or 0.5 N $HClO_4$ and hydrolysed for 1 h at 95 C or 15 min at 70 C respectively. Radioactivity of an aliquot of the hydrolysed sample (usually 0.5 ml) was determined by liquid scintillation counting in a Beckman LS-100.

Cell fractionation. Cells in growth medium were diluted 5-fold into cold Earle's balanced salts solution (EBSS) and centrifuged at 1000 x g for 5 min at 4 C. Cell lysis was achieved by a modification of the techniques of Gielkens, et al. (38) and Perry and Kelly (95, 97). All glassware had been acid washed, autoclaved and dried. All solutions had been autoclaved and treated with 50 μl diethyloxydiformate per 100 ml as described by Palmiter (92) unless otherwise noted. All manipulations were carried out in ice water or at 4 C.

Cells were resuspended at densities greater than 10^8 cells/ml in high salt buffer (HSB, 0.05M tris-HCl, pH 9.0, 0.3 M KCl, 0.01 M $MgCl_2$, 0.001 M dithiothreitol and 1% (v/v) diethyloxydiformate). This suspension was diluted with 1 volume of 1% (v/v) NP-40 (Shell Petroleum), mixed briefly on a vortex mixer and allowed to stand in the cold for 5 min.

The suspension was then homogenized with a Potter apparatus with a tight-fitting Teflon pestle. Post-mitochondrial supernatant was prepared by centrifugation of the homogenate at 10,000 x g for 10 min. The pellet produced in this centrifugation was used in the preparation of nuclei. A polysomal pellet was obtained by layering 3 ml of the post-mitochondrial supernatant over a discontinuous gradient of 1.2 ml 1.35 M sucrose and 1.2 ml 2.0 M sucrose in 0.05 M tris-HCl, pH 7.5, 0.025M KCl, 0.005 M $MgCl_2$ (TKM buffer). The gradient was centrifuged at 114,000 x g for 24 h at 4 C in the SW 50.1 rotor. The supernatant was then removed by aspiration and the polysomal pellet was rinsed 3 times with 1 ml of sterile water at 4 C, drained and frozen at -20 C for later use. The pellet was thawed and resuspended in 0.1 ml sterile water by gentle stroking with a small glass rod. The polysome profile was examined by layering an aliquot of the suspension onto a 16.2 ml 15%-30% (w/w) glycerol gradient in TKM buffer over a 1.0 ml pad of 30% (w/w) glycerol in TKM buffer. The gradient was then centrifuged in the SW 27.1 rotor at 81,000 x g at 4 C for 120 min. Fractionation of the gradient was performed with an Isco density gradient fractionator and absorbance was monitored at 260 nm with an Isco ultraviolet absorbance monitor.

Material in the 10,000 x g pellets was resuspended by gentle pipetting in SSC with 0.5% NP-40. Nuclei were pelleted by centrifugation for 5 min at 1000 x g. Nuclear pellets were treated with 5 ml of 5% (w/v) trichloroacetic acid (TCA) and were allowed to stand at 4 C for 24 h. The pellets were then washed twice in 5% TCA and the TCA-insoluble material was pelleted, treated twice with ethanol-ether (3:1), ether extracted once and dried (112). The remaining residue was treated with 0.5 ml 0.3 N NaOH for 24 h at 37°C to hydrolyse the nuclear RNA to ribonucleotides (107, 112). The solution was then treated with 3 ml 0.5 N perchloric acid (PCA) and unhydrolysed material was allowed to precipitate for 2 h. After centrifugation at 5000 x g for 15 min, the supernatant was decanted and saved. The insoluble material was washed 3 times in 0.5 M PCA at 4 C, and the washes were discarded. The pellet was then treated with 4.5 ml 0.5 N PCA and DNA was hydrolysed by incubation for 15 min at 70 C.

Detection of reovirus-specific mRNA. Cultures at densities of 6×10^5 cells/ml were infected with 20 pfu/cell of reovirus and pulse-labeled from 8-12 h with ^{3}H-uridine at 2 μCi/ml. Cultures were treated with 0.5 μg/ml actinomycin-D (gift from Merck, Sharp and Dohme) and 100 μg/ml kanamycin sulfate at the time of infection. Reovirus mRNA was extracted from these infected cells using a modification of the procedure of Hay and Joklik (52). At 12 h post-infection the cultures were chilled and cells were pelleted by centrifugation at 2000 x g for 5 min. The pellets were washed in EBSS and resuspended in 0.01 M sodium acetate, pH 5.1, at a density of 10^7 cells/ml. The suspension was adjusted to 0.5% (w/v) sodium dodecyl sulfate (SDS) and shaken for 10

min with 2 volumes of water-saturated, redistilled phenol. Organic and aqueous phases were separated by centrifugation at 500 x g for 5 min at 4 C. The aqueous phase was re-extracted twice with water-saturated phenol and was then adjusted to 0.2 M NaCl by addition of 5.0 M NaCl. Two volumes of cold 95% ethanol were added and precipitation was allowed to proceed at -20 C overnight. The RNA was removed by centrifugation at 8000 x g for 20 min and the pellets were washed twice with ethanol at -20 C. The RNA was dissolved in SSC and the solution was made 2 M LiCl by the addition of 8 M LiCl. The solution was kept overnight at 4 C and single stranded RNA (ssRNA) was removed by centrifugation as described, washed with 70% (v/v) ethanol, and dissolved in 0.01 M tris-HCl, pH 7.5, 0.05 M NaCl (tris-saline).

The solution of ssRNA was layered in 0.2 ml aliquots onto 17.25 ml 15%-30% (w/w) glycerol gradients in tris-saline and 0.001 M ethylenedinitrilotetracetic acid (EDTA). The gradients were centrifuged in the SW 27.1 rotor at 82,500 x g at 4 C for 16 h. The gradients were then fractionated and the absorbance monitored at 260 nm with an Isco density gradient fractionator and monitor. Fractions were collected into tubes containing 100 μg bovine serum albumin and were precipitated with 0.5 ml 10% (w/v) TCA and kept at 4 C. Precipitates were collected 12 h later on 1.2 μm Millipore filters and were washed 5 times with 4 ml 5% (w/v) TCA. Radioactivity collected on the filter was determined by liquid scintillation counting.

Infectious center assay. At 3 h post-infection, a 1.0 ml aliquot of cells in growth medium was removed and diluted into 30.0 ml fresh growth medium at 4 C. After pelleting the cells by centrifugation

at 250 x g for 10 min, the cells were washed twice in 30 ml fresh, cold medium as described. The last wash was assayed for reovirus as described. The cells were then gently resuspended in 10.0 ml fresh MEM supplemented with 2% (v/v) FCS, and a 1:10 dilution of this cell suspension was prepared. Aliquots of these cell suspensions were pipetted into the centers of petri plates (60 x 15 mm). Six ml of a freshly resuspended cell culture at 2.4 x 10^5 cells/ml were then pipetted into each plate. Four h later, when all cells had attached, the medium was removed and MEM supplemented with 3% (v/v) FCS and 1% (w/v) agar was layered onto each plate as described in the virus assay procedure. One day after the second overlay, plaques were counted.

Identification of intracellular parental virus particles. Cell cultures of 100 ml at a density of 1.6 x 10^5 cells/ml were infected at a multiplicity of 50 pfu/cell. At times after infection, the cultures were chilled and the cells were pelleted and freon-extracted once, as described in the virus purification procedure. The aqueous homogenate was then applied to 2.0 ml of 20% (w/w) glycerol in SSC which had been layered on top of a 6.0 ml linear CsCl gradient (1.3 g/ml-1.5 g/ml). The gradient was centrifuged at 82,500 x g for 1 h in the SW 27.1 rotor, and was collected and monitored at 260 nm as described.

Radioisotopes. The radioisotope-labeled compounds used in this study are as indicated. The suppliers and the specific activity of each labeled compound are given as follows:

New England Nuclear

uridine (5-^{3}H) 26.4 Ci/m mole

thymidine (methyl-^{3}H) 19.9 Ci/m mole

L-amino acid mixture (^{3}H-general label)

monosodium phosphate ($NaH_2\ ^{32}PO_4$) 1.0 Ci/m mole

Amersham-Searle

uridine (2-^{14}C) 58 mCi/m mole

Assay of soluble and incorporated intracellular ^{3}H-thymidine. A modification of the procedure described by Shaw (121) was used to determine whether L cells remained permeable to thymidine at times after infection with reovirus. Infected and control cultures were prepared as described in the assay for cellular DNA synthesis. The cultures were sampled identically at intervals after infection and pulse-labeled as described. At the end of the 30 min pulse period, the samples were chilled in an icebath for 5 min, centrifuged at 250 x g for 2 min, and the growth media discarded. The cells were resuspended gently and washed twice at 4 C in MEM supplemented with 80 μg/l unlabelled thymidine. The washes were discarded, the cells were resuspended in 2.0 ml 5% (w/v) TCA, and stored at 4° C. Twelve h later, the precipitated cellular material was pelleted at 2000 x g and the supernatant saved. A 0.5 ml aliquot of the supernatant was assayed for radioactivity by liquid scintillation counting as described. This radioactivity was attributed to soluble intracellular thymidine. The precipitate was washed once in 6.0 ml of 5% (w/v) TCA and the wash discarded. DNA in the precipitate was hydrolysed in 2.0 ml 5% (w/v) TCA as described and 0.5 ml aliquots were assayed by liquid scintillation counting. This radioactivity was from ^{3}H-thymidine which had been incorporated into cellular DNA.

Thymidine incorporation into DNA of hypotonically swollen cells. Infected and control cultures were prepared as described. At intervals after infection, 2.0 ml aliquots were withdrawn from each culture and centrifuged at 200 x g for 2 min. The growth medium was decanted into another screw-capped tube, previously equilibrated in an atmosphere of 5% (v/v) CO_2 in air, and stored at 37 C. Residual medium was shaken off the cell pellet, and the cells were resuspended in 0.1 ml sterile distilled water containing 2 μCi/ml ^{3}H-thymidine and incubated 5 min at 37 C. The growth medium was then poured back into the cell suspension and incubation continued for 30 min. The aliquots were treated as described in the assay for cellular DNA synthesis. This procedure for hypotonically swollen cells was employed in tandem with the complete procedure described in the assay for cellular DNA synthesis so that the kinetics of DNA synthesis and inhibition in normal cells and swollen cells could be meaningfully compared.

Indirect DNA inhibition assay. A synchronous culture of L cells was prepared and maintained as described. Fourteen h after resuspending the cells in fresh growth medium, the culture was pulse-labeled for 2 h with 0.1 μCi/ml ^{3}H-thymidine. At the end of the pulse period, cells were pelleted by centrifugation at 250 x g for 5 min and were maintained at 37 C during centrifugation. The cells were washed once and were resuspended in growth medium at 37 C which had been taken at the same time from an identical, but unlabeled culture. The culture was allowed to finish the remaining 36 h of the 50 h synchrony-maintenance cycle. At that time, the cell culture was centrifuged as before, and resuspended in fresh growth medium. Eight h after resuspension,

the culture was split into 2 identical cultures in flasks with half the volume of the original flask. One of the cultures was inoculated with reovirus at a multiplicity of 20 pfu/cell. Aliquots of 5.0 ml each were taken from each of the cultures at 2 h intervals after resuspension and infection. The aliquots were chilled in ice and brought to 5% TCA by the addition of 5 ml 10% (w/v) TCA, and were allowed to stand overnight at 4 C. The precipitate washed with TCA, ethanol-ether (3:1) and ether and dried as described. After hydrolysis of RNA to ribonucleotides, re-precipitation with PCA and further washing, the remaining precipitate was hydrolysed in 2.0 ml PCA as described. Aliquots of this hydrolysate were used in the determination of deoxyribose by the method of Burton (11) and in radioactivity determination by liquid scintillation counting. Specific activities of the cellular DNA were calculated for both infected and control cultures at the time of each sampling.

^{32}P-labeling of nucleic acids. RNA and DNA in infected and control cells were labeled with ^{32}P by pulsing aliquots of cells with 100 μCi/ml NaH_2 $^{32}PO_4$ contained in phosphate-free growth medium supplemented with 7% (v/v) phosphate-free FCS. Aliquots of cells to be pulsed with ^{32}P were pelleted by centrifugation at 250 x g for 5 min, and washed once at 37 C in MEM to decrease the surface concentration of easily released phosphates. The cells were then pelleted as before and resuspended to ½ original volume in ^{32}P-containing medium in spinner flasks. After the 1 h pulse, the culture was chilled on ice and cells were again pelleted. The ^{32}P-containing medium was decanted into another flask which had been equilibrated with 5% (v/v) CO_2 in air, and was re-utilized in 4 more pulsing periods for a total 5 h of use. Pelleted cells were

disrupted and polysome pellets prepared as described in the cell fractionation procedure. Nuclear pellets were frozen and saved for later use.

Pulse-labeling of protein. Cell cultures were inoculated with reovirus as described in the DNA assay procedure. Aliquots of 2.0 ml were placed in screw-capped tubes containing an amount of ^{3}H-amino acid mixture for a final activity of 0.5 μCi/ml and were incubated 30 min at either 31 C or 39 C. The tubes were chilled and 2.0 ml of 10% (w/v) TCA was added. Precipitation was allowed to take place overnight at 4°C. Precipitates were collected and washed on 1.2 μm millipore filters and radioactivity determined by liquid scintillation counting.

Pulse-labeling of RNA. The procedure for pulse-labeling RNA in infected cells was the same as that employed for pulse-labeling protein, except that aliquots were pulsed with 1.0 μCi/ml ^{3}H-uridine. Precipitates were pelleted at 1000 x g for 15 min and washed three times in cold TCA. RNA was then hydrolysed by 0.3 N NaOH at 37 C for 20 h, diluted with 2 vol 10% (w/v) TCA and kept at 4 C for 4 h or more. Precipitated material was pelleted, and 0.5 ml of the supernatant assayed by liquid scintillation counting.

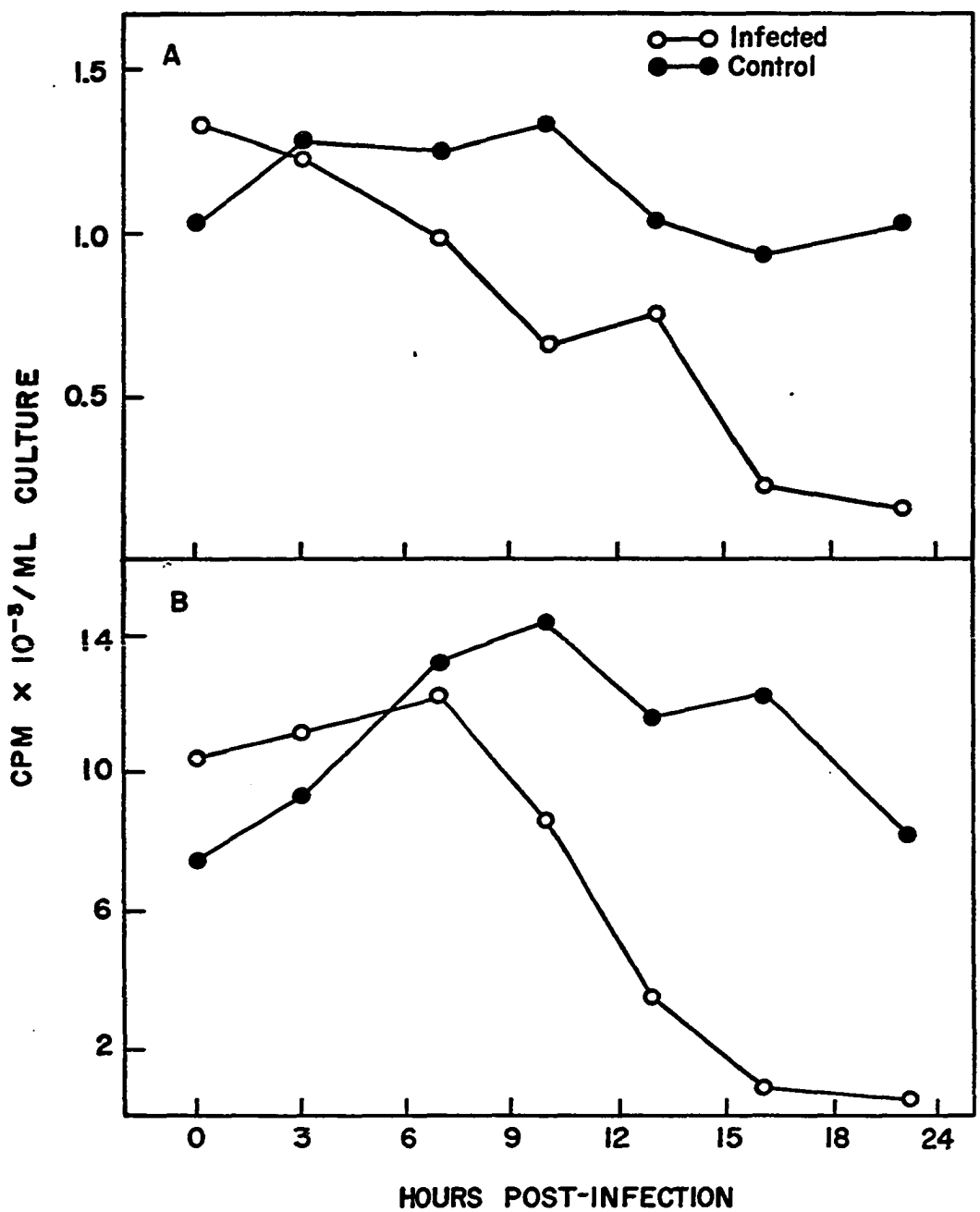
CHAPTER III

RESULTS

Comparison of ^{3}H-thymidine-uptake of control and reovirus infected L cells. The validity of ^{3}H-thymidine-labeling of cellular DNA in reovirus-infected cells was investigated. That ^{3}H-thymidine becomes cell-associated after reovirus infection has been reported previously (121), but the possibility that the association did not imply actual entrance into intracellular thymidine pools was questioned here. A decreased efficiency of thymidine transport into reovirus-infected cells could result in less ^{3}H-thymidine being incorporated into cellular DNA. To insure that cell-associated soluble ^{3}H-thymidine was actually intracellular, pulse-labeled cells were washed as described with an excess of unlabeled thymidine. TCA-insoluble counts from control and reovirus-infected cells were also compared. The results, presented in Figure 1, indicate that reovirus-infected cells are able to transport thymidine as efficiently as control cells for several hours after the inhibition of DNA synthesis is seen.

The effects of reovirus infection on ^{3}H-labeled cellular DNA. Our examination of thymidine transport in infected cells was sufficient to determine that cells remove exogenously supplied thymidine from the medium, but not whether nucleotides are transported to the nucleus

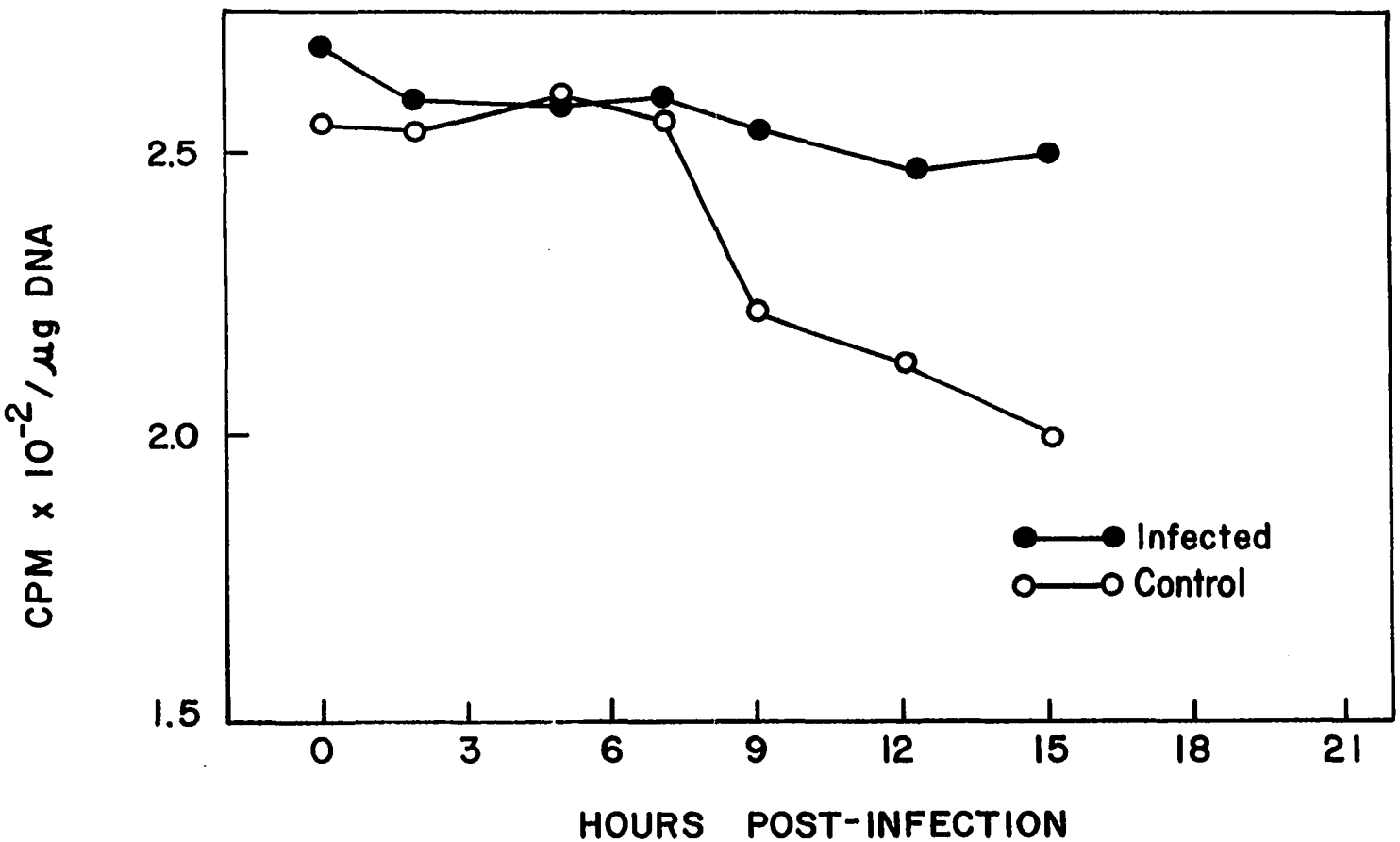
Figure 1. A comparison of radioactivity in TCA-soluble and insoluble material from control and infected L cell cultures. At times indicated, approximately 5.0×10^5 cells from each spinner culture were pulse-labeled for 30 min with ^{3}H-thymidine and then washed as described. Counts per minute of TCA soluble (Panel A) and TCA insoluble (Panel B) counts per ml of culture are shown.


A
B
Infected
Control
1.5
1.0
0.5
14
10
6
2
0
3
6
9
12
15
18
24
CPM x 10⁻³/ML CULTURE
HOURS POST-INFECTION

after phosphorylation. A more direct approach was used by examining the specific activity of previously-labeled cellular DNA in control and infected cells at intervals post-infection. Synchronous cells were used so that changes in specific activity could be focused into a span of several hours, since the theoretical maximum change in specific activity of actively growing cells through one complete cell cycle would be a 50% decrease. This decrease in specific activity could be seen only in the absence of effects such as cell damage and less efficient DNA synthesis due to heavy ^{3}H-labeling, and degradation of ^{3}H-thymidine with resultant incorporation of labeled products into acid-precipitable molecules other than DNA. The cells were infected at a time which was shown previously (15) to inhibit the entry of synchronous cultures into the DNA synthetic phase, which peaks at 15 h after resuspension. Results, shown in Figure 2, indicate that ^{3}H-thymidine incorporation accurately reflects DNA synthesis after reovirus infection.

Effects of ts-mutants on cellular macromolecular synthesis. If the inhibition of DNA synthesis were a direct result of an event or sequence of events in the viral replicative cycle, it should theoretically be possible to identify the event(s) responsible by studying cells infected with conditional-lethal mutants of reovirus. To identify such events, mutants would be used which show altered kinetics of the inhibition of cellular DNA synthesis under conditions where the normal reovirus replicative cycle is not completed. Ideally a range of mutants, some of which cause no inhibition and others which produce the same degree of inhibition as WT reovirus, would simplify this kind of investigation considerably. A definitive characterization of the mutant

Figure 2. Effect of reovirus infection on DNA synthesis measured by alterations in specific activity of ^{3}H-labeled cellular DNA in synchronized infected and control cell cultures. At intervals indicated, an aliquot from each culture was removed, and DNA content and TCA-insoluble radioactivity were determined. Results are shown as specific activity of cellular DNA at times after infection.


CPM x 10^{-2}/µg DNA
2.5
2.0
1.5
0
3
6
9
12
15
18
21
HOURS POST-INFECTION
Infected
Control

reovirus replicative cycles (including characteristics of intracellular uncoated parental particles, of progeny particles, capsid proteins, characterization of mRNA and dsRNA synthetic rates, A-rich RNA content) would be obtained with respect to one specific characteristic of WT reovirus — the ability to inhibit synthesis of cellular DNA. A more complex but still valuable approach would be the study of a series of mutants, all of which show some degree of WT-reovirus mediated inhibition.

The ts-mutants of Fields and Joklik (33) used in this study consisted of representatives from five of their recombination groups. These mutants were grouped on the basis of their ability, on co-infection with other mutants, to produce a proportion of progeny particles having WT-reovirus characteristics of infectivity and ability to produce infectious progeny at both the permissive temperature of 31 C and the nonpermissive temperature of 39 C. Mutants of a given recombination group possess the ts-lesion(s) on the same genome segment (32). Subsequently, the recombination groups were found to be coincident complementation groups with respect to the ability of a mutant, on coinfection with mutants of other groups, to produce elevated levels of ssRNA at the nonpermissive temperature (58). Several of the mutants have been found to be at least double mutants (59), but only one specific genetic lesion need be temperature-sensitive to allow the mutant to behave as a strict ts-mutant. Some of the known characteristics of these ts-mutants groups are listed in Table 1 with the plating efficiencies of the stock suspensions of mutants used in this investigation.

All of the ts-mutants transcribe all 10 species of mRNA at both permissive and nonpermissive temperatures (21). All viral proteins

Table 1.-- Characteristics of reovirus ts-mutant groups used in this investigation.

Mutant Group	Mutant	ssRNA synthesis at 39°C (%WT) (Ref. 21,64)	dsRNA synthesis at 39°C (%WT) Ref. 21,64)	Appearance of viral inclusions in infected cells at 39°C (Ref. 35)	Particles accumulating at 39°C in cytoplasm (Ref. 35)	Plating Efficiency 39°C/31°C
A	201 234	100	100	indistinguishable from WT reovirus-infected cells. Large coalesced areas of cytoplasmic fluorescence	Particles appear to have normal reovirion morphology	8.0×10^{-3} 5.0×10^{-6}
B	352	25-50	25-50	Small, round discrete areas of fluorescence of uniform size which develop more slowly than those of group A and WT reovirus	Particles similar in appearance to viral cores	5.6×10^{-6}
C	447	5	0	Immunofluorescence similar to group B Electron microscopic examination revealed material less dense and more heterogeneous than seen with group B	Particles similar in appearance to empty outer viral capsids	1.1×10^{-4}
D	357	5	<1	Factories larger than with groups B and C and were located within afibrillar matrix	Particles of two types probably empty capsids having either one or two viral protein coats	2.0×10^{-3}

detectable in WT-reovirus-infected cells are known to be translated from ts-mutant mRNA in infected cells (34). In addition, each of the mutant groups produces its own characteristic type of aberrant, non-infectious particle at the non-permissive temperature (35, 86), and immunofluorescence microscopy has shown that each group of ts-mutants produces its own type of localized viral inclusions, differing in appearance from the inclusions seen in WT-reovirus-infected cells (35).

Our investigations of total protein, RNA and DNA synthesis at times after infection in ts-mutant-infected cells have shown that, at both permissive and nonpermissive temperatures, none of the mutants tested caused altered total RNA or protein synthesis prior to the onset of the inhibition of DNA synthesis. Representative graphs showing effects on RNA and protein synthesis in WT and ts-mutant-infected cells is presented in Figure 3.

Preliminary investigations with mutant-infected cells indicated that DNA synthesis was inhibited earlier and more dramatically by group A and B mutants at the nonpermissive temperature than by WT-reovirus. Subsequently it was found that plaque formation in the mutant virus assays was not complete in ten days. Plaque production with the mutants can be extremely slow, and even after large plaques were evident, extremely small, well isolated plaques still appeared for days on the assay monolayers. Therefore assay monolayers were examined a second time one to two days after the initial plaque-counting.

The effects of the ts-mutants on DNA synthesis at both permissive and nonpermissive temperatures was examined in asynchronous L cell cultures. The results of these experiments, presented in Figures

Figure 3. Effects of WT reovirus and group C mutant 447 on total RNA and protein synthesis. Separate L-cell spinner cultures were inoculated with 10 pfu/cell of group C mutant 447 and WT reovirus at both 31 C and 39 C as described. Aliquots were pulse labeled with ^{3}H-amino acids or ^{3}H-uridine. Results are presented as counts per min in TCA insoluble material per ml culture. Radioactivity in RNA at 31 C is shown in panel A; at 39 C, panel B. Radioactivity in protein at 31 C is shown in panel C; at 39 C, panel D.

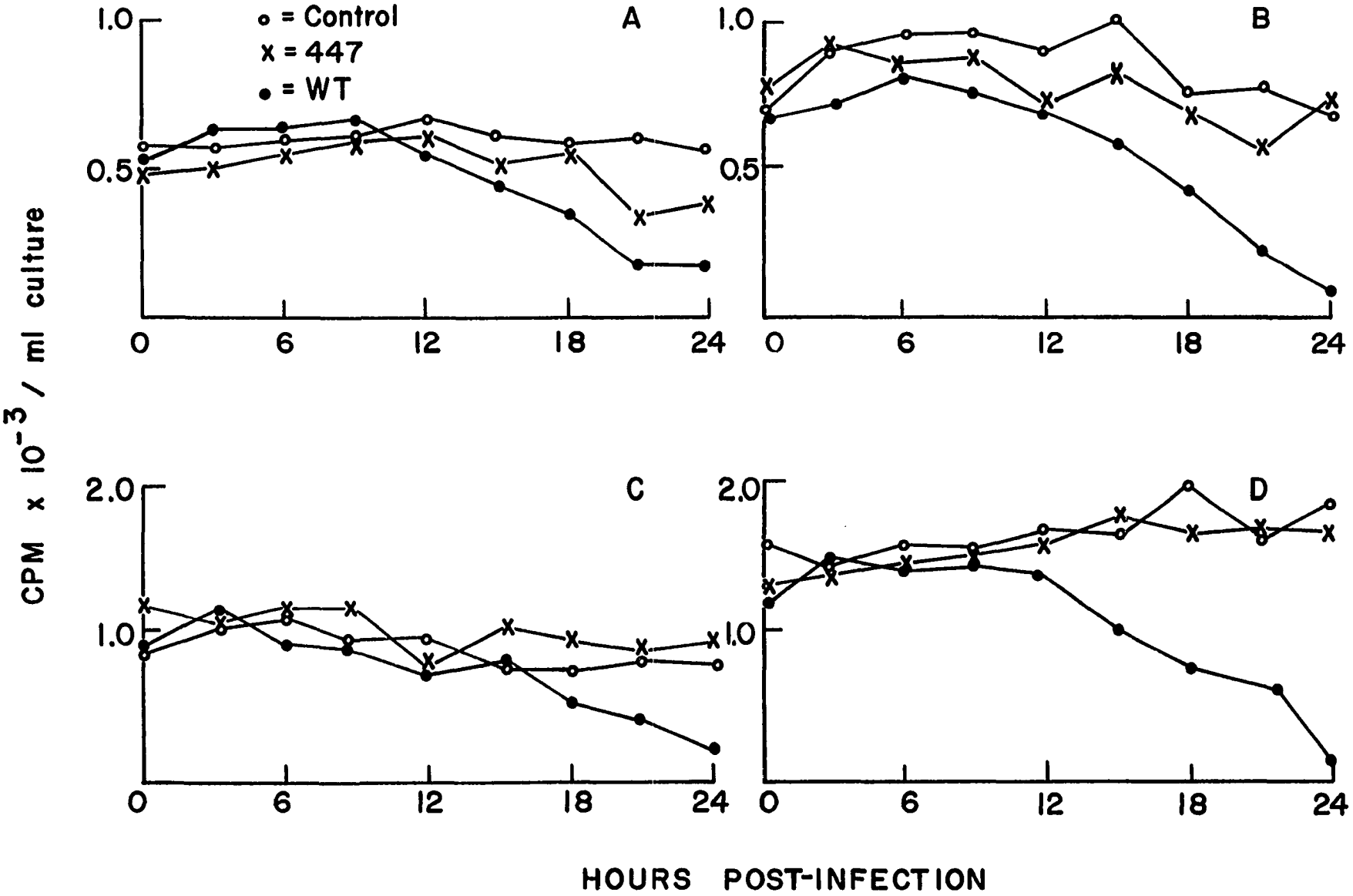

o = Control
x = 447
● = WT
A
B
C
D
1.0
0.5
2.0
1.0
0
6
12
18
24
CPM x 10^{-3} / ml culture
HOURS POST-INFECTION

Figure 4. The effects of ts-mutants on cellular DNA synthesis at the permissive temperature of 31 C. Cultures were inoculated with virus at 10 pfu/cell and aliquots of 2.0 ml from all cultures were pulse-labeled with ^{3}H-thymidine at intervals indicated. Results are presented as counts per minute TCA insoluble radioactivity per ml culture.

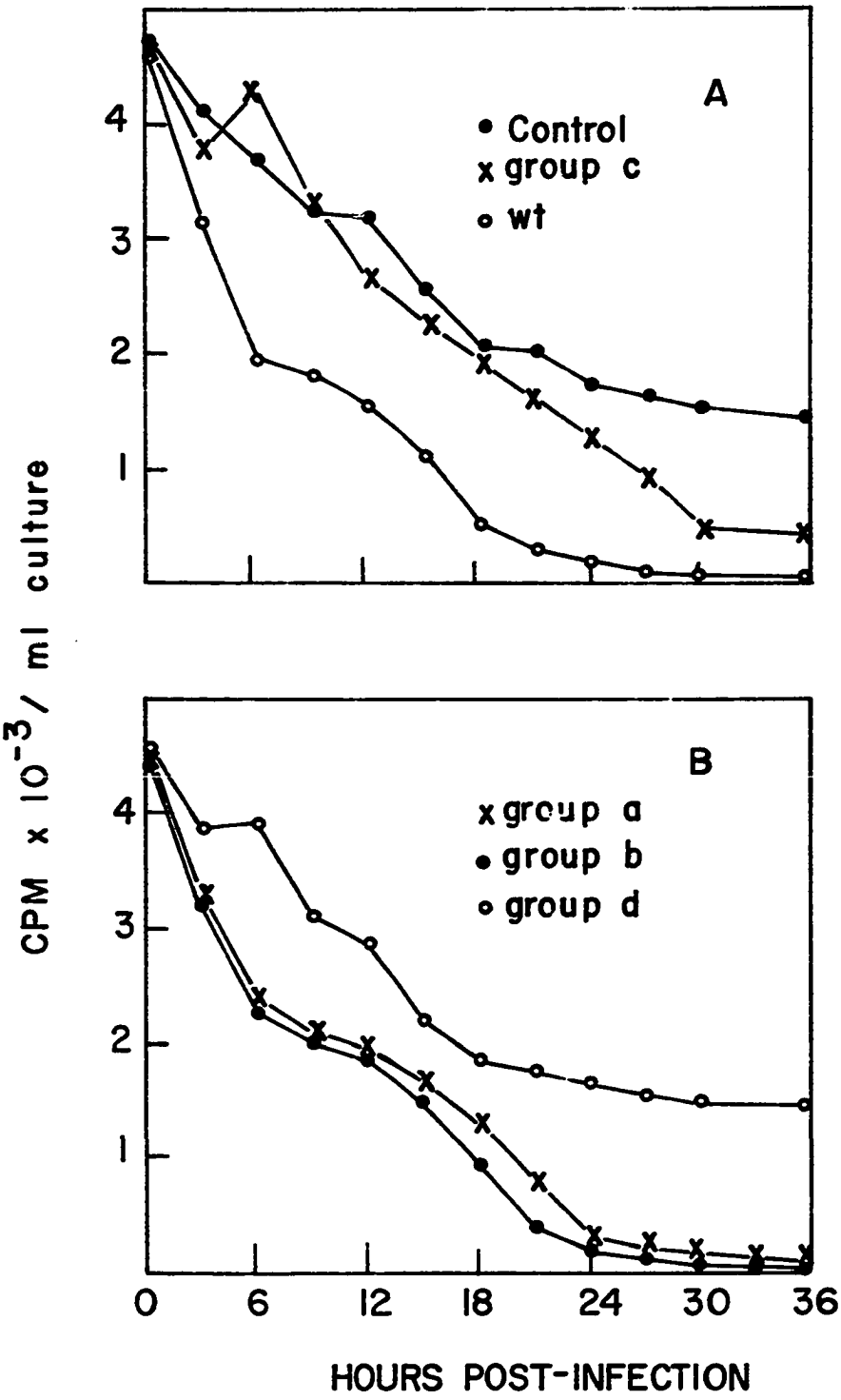
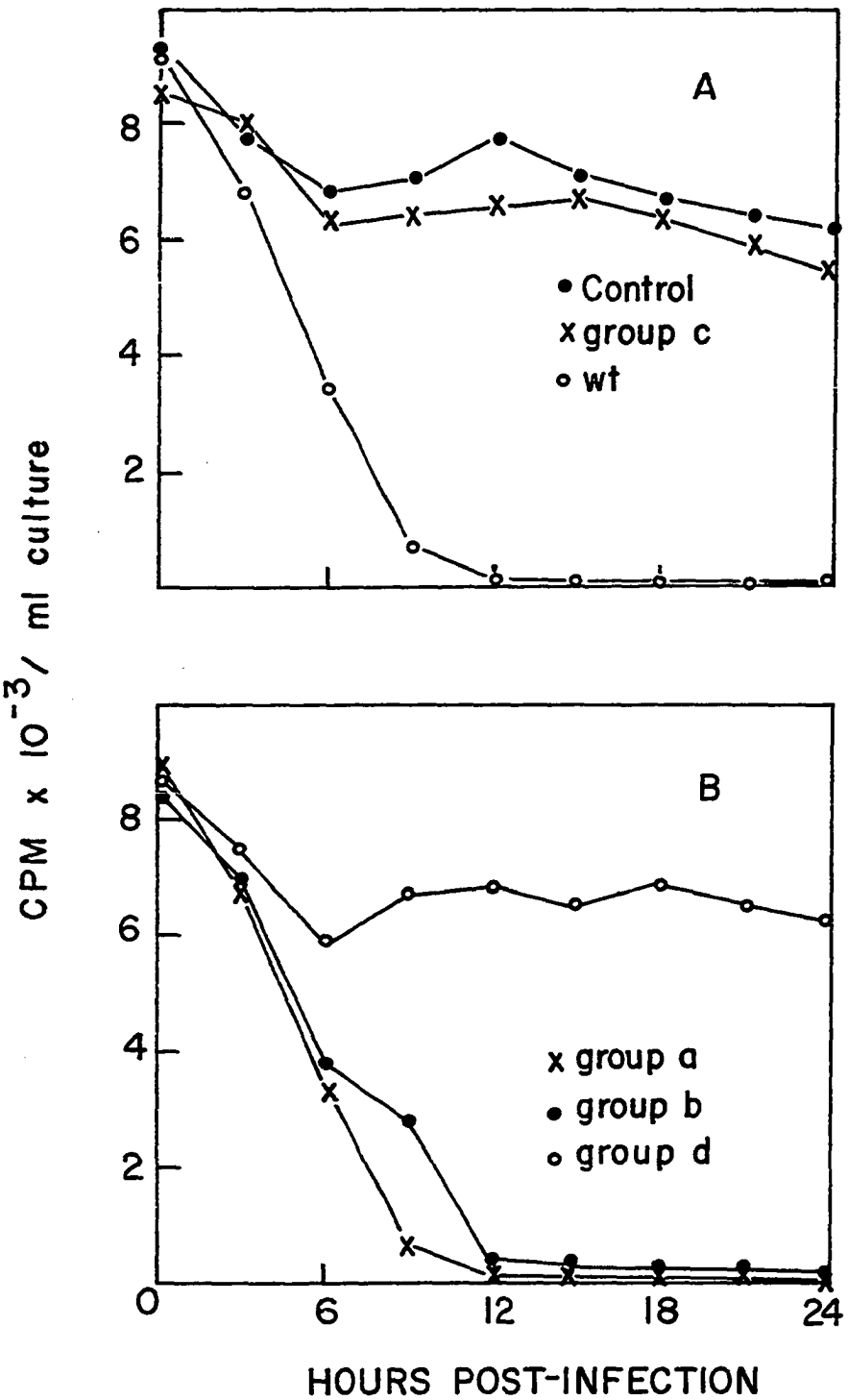

A
• Control
x group c
o wt
B
x group a
• group b
o group d
CPM x 10⁻³/ ml culture
HOURS POST-INFECTION
0
6
12
18
24
30
36
1
2
3
4

Figure 5. The effects of ts-mutants on cellular DNA synthesis at the non-permissive temperature of 39 C. Cultures were inoculated with virus at 10 pfu/ml, and aliquots of 2.0 ml from all cell cultures were pulse-labeled with ^{3}H-thymidine at intervals indicated. Results are presented as counts per minute per ml culture.


A
B
• Control
x group c
o wt
x group a
• group b
o group d
CPM x 10^{-3}/ ml culture
HOURS POST-INFECTION
0
2
4
6
8
12
18
24

4 and 5, show kinetics of the inhibition of DNA synthesis resembling those in WT-reovirus infected cells. The exceptions were mutant 447 (group C)-and mutant 357 (group D)-infected cells at the non-permissive temperature where both exhibited the inhibition, although less pronounced than WT-reovirus-infected cells. It was necessary to ascertain whether or not the cells were all being infected by the group C and D mutants at the low multiplicity of 10 pfu/cell when incubated at 39 C. The experiment was repeated, and, after allowing for virus adsorption, aliquots were removed and cells were assayed for infectious centers. It was found that only 10% of the mutant D-treated cells were actually infected, but that 100% of the cells treated with mutant C were infected. The results of these infectious-center assays were supported by immunofluorescence microscopic examinations of cells from the same cultures. These cells were washed free of unadsorbed virus, allowed to monolayer in Leighton tubes on slides, and incubated for 36 h at 31 C prior to examination by immunofluorescence microscopy.

Effects of high multiplicities of group C and D mutants on DNA synthesis. In mutant D-infected cells at 31 C, DNA synthesis was effectively inhibited. These results are presented in Figure 6. The group C mutant did not show WT-reovirus inhibition kinetics even at 10 times the normal input multiplicity (100 pfu/cell). Results are shown in Figure 7. In order to find whether the group C mutant was uncoated in the cells after adsorption as is WT-reovirus (13, 123, 125), parental virus was extracted from infected cell cultures at 5 h post-infection and centrifuged. Results of the extraction are shown on Figure 8. Both adsorbed WT-virus and group C mutant were converted to SVP's as evidenced by change in density of the input virions.

Figure 6. Effects of the Group D mutant 357 at different multiplicities of infection on cellular DNA synthesis at the non-permissive temperature of 39 C. At intervals indicated, 2.0 ml aliquots of each cell culture were pulsed with ^{3}H-thymidine for 30 min. Results are presented as counts per min in TCA insoluble material per ml of culture.

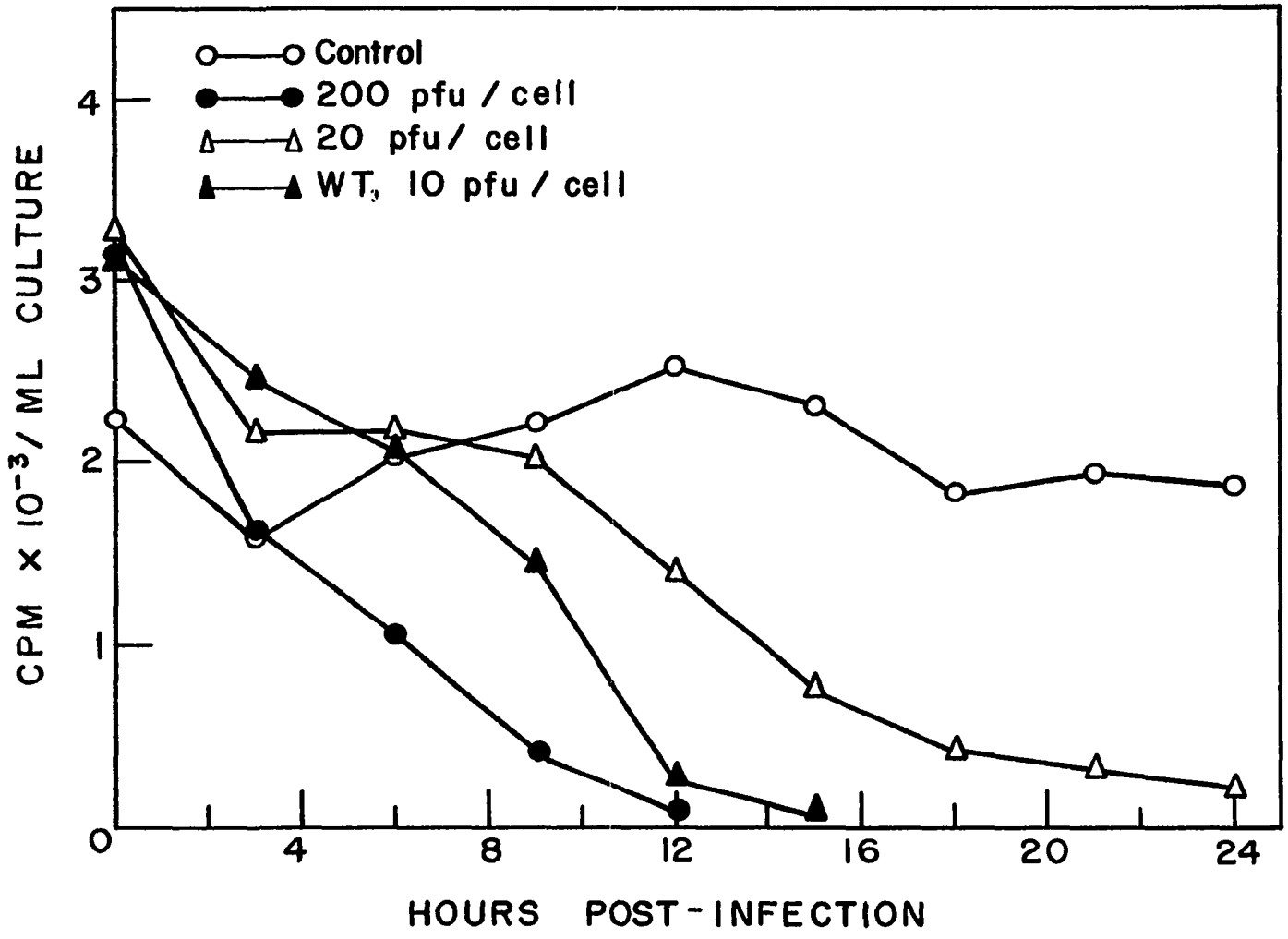
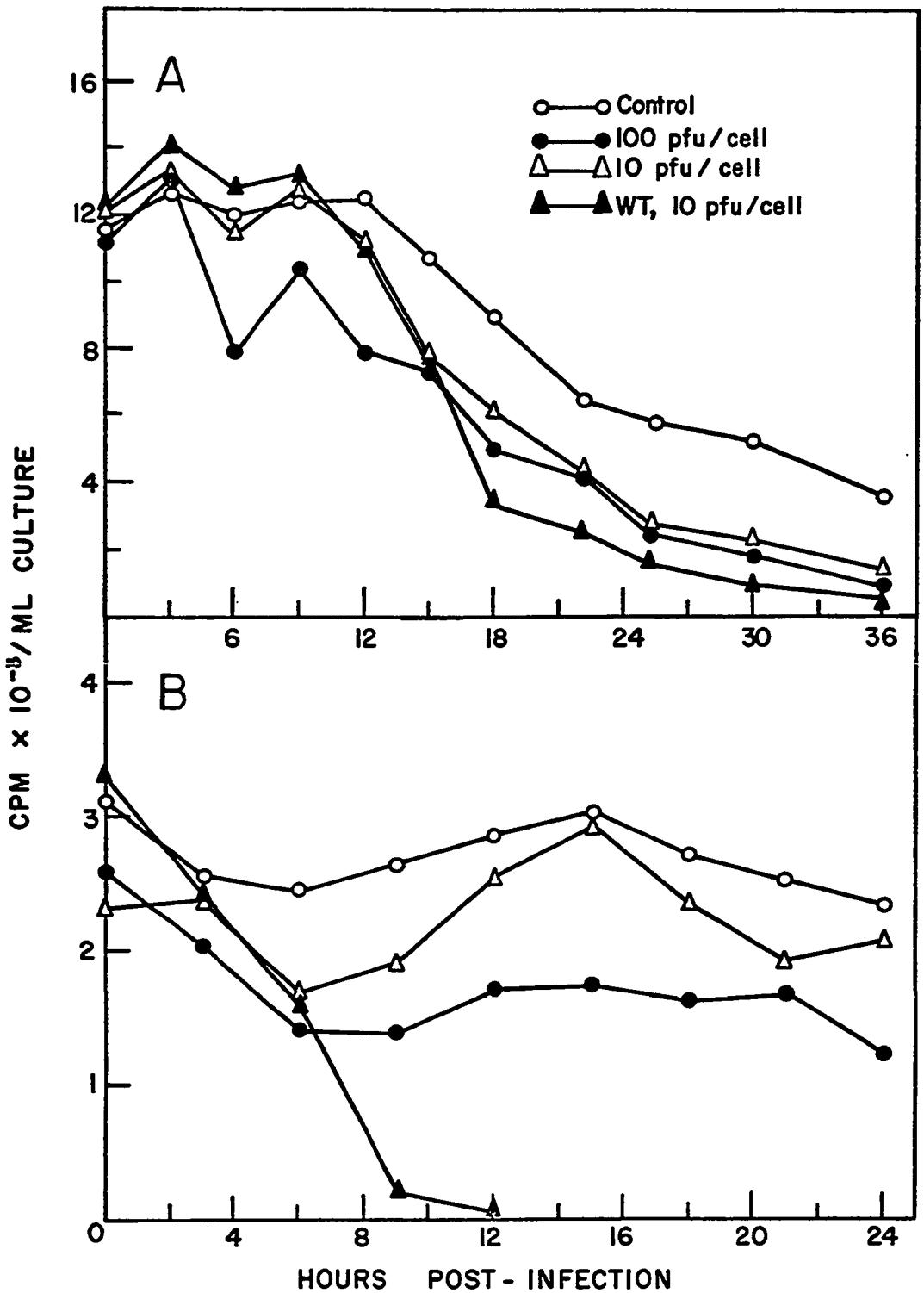

Control
200 pfu / cell
20 pfu / cell
WT, 10 pfu / cell
CPM x 10⁻³ / ML CULTURE
0
1
2
3
4
4
8
12
16
20
24
HOURS POST-INFECTION

Figure 7. Effects of increased multiplicities of infection with group C mutant 447 on cellular DNA synthesis. At intervals indicated, 2.0 ml aliquots of each cell culture were pulsed with ^{3}H-thymidine for 30 min. Results are presented as counts per min in TCA-insoluble material per ml of culture. Results in panel A were collected at the permissive temperature of 31 C; and panel B at the non-permissive temperature of 39 C.


A
B
Control
100 pfu/cell
10 pfu/cell
WT, 10 pfu/cell
CPM x 10⁻³/ML CULTURE
HOURS POST-INFECTION
0
1
2
3
4
8
12
16
20
24
30
36

Comparison of inhibition kinetics of plaque-purified virus and high passage virus. It was not possible to obtain reovirion-free defective stocks by mutant complementation of defective cores on co-infection as others have reported (129); this may have been due to differences in virus strains or cell lines. Therefore, the approach employed for investigating the inhibitory characteristics of defective reovirions was the comparison of cells infected with the same pfu/cell ratio of plaque-purified reovirus (PPV) and high-passage reovirus (HPV). HPV was purified on CsCl gradients as described, collected and monitored at 260 nm for the characteristic shouldered-peak indicative of stock virus containing defective particles. Typical gradient profiles for HPV and PPV are shown in Figure 8.

On the basis of CsCl gradient profiles, all HPV used in DNA synthesis-inhibition experiments had defective particle to normal virion ratios of at least 4:1, and sometimes as high as 10:1. Thus, at equal pfu/cell ratios, cells infected with HPV had adsorbed typically five times more particles adsorbed by cells infected with PPV. Any contribution of defective virions to the inhibition of DNA synthesis should be similar to the dose-response seen with increasing multiplicities of infection. The results, presented in Figure 9, show that this was not the case; defective virions do not significantly contribute to the inhibition of DNA synthesis in cells infected with HPV.

Effects of mitomycin-C-mediated inhibition of DNA synthesis on virus replication. The question of whether reovirus could replicate in cells which were not capable of DNA synthesis was followed by studying reovirus mRNA from cells treated with mitomycin-C (Sigma Chemical Company)

Figure 8. Comparison of gradient profiles of plaque purified virus, high-passage virus, group C mutant 447 and selected SVP's derived from each. Aliquots of high passage virus and plaque purified virus in SSC were treated with -chymotrypsin, chilled to 4°C and mixed with a 5-10 fold excess of undigested virus of the same type and centrifuged on CsCl gradients as described. Results shown in panels A (plaque-purified virus) and B (high passage virus) are presented as absorbance at 260 nm gradient profiles showing virus and core peaks. It can be seen that normal reovirions and cores band at densities of approximately 1.37 and 1.44, respectively. Defective cores band at a density of approximately 1.414, while defective virions band at a slightly greater density than normal virions. Cultures infected with mutant 447 and WT reovirus were treated as described in the procedure for identification of intracellular parental SVP's. Results are presented as dashed lines in panel C (WT reovirus) and D (mutant 447). The solid lines in panels C and D are OD_{260} profiles from identical gradients on which amounts of each virus equal to the original inocula were centrifuged.

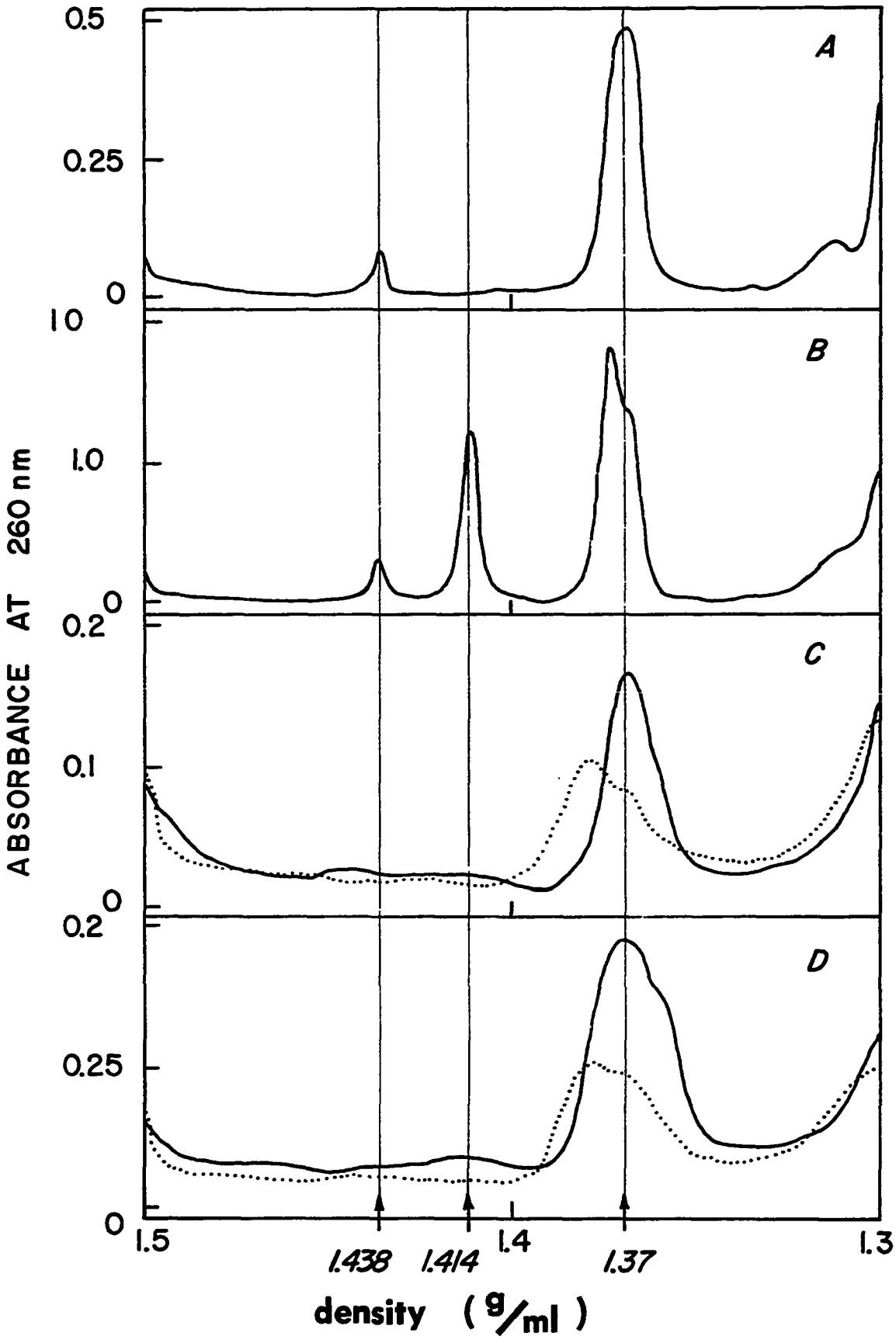

A
B
C
D
0.5
0.25
0
1.0
1.0
0
0.2
0
0.1
0
0.2
0
0.25
0
ABSORBANCE AT 260 nm
1.5
1.438
1.414
1.4
1.37
1.3
density (g/ml)

Figure 9. A comparison of inhibition of DNA synthesis in L-cells infected with high passage reovirus and plaque-purified reovirus. Cultures infected with 10 pfu/cell of either high passage virus or plaque purified virus were prepared and sampled as described in the assay of DNA synthesis. Results are presented as counts per minute in TCA insoluble material per ml culture.

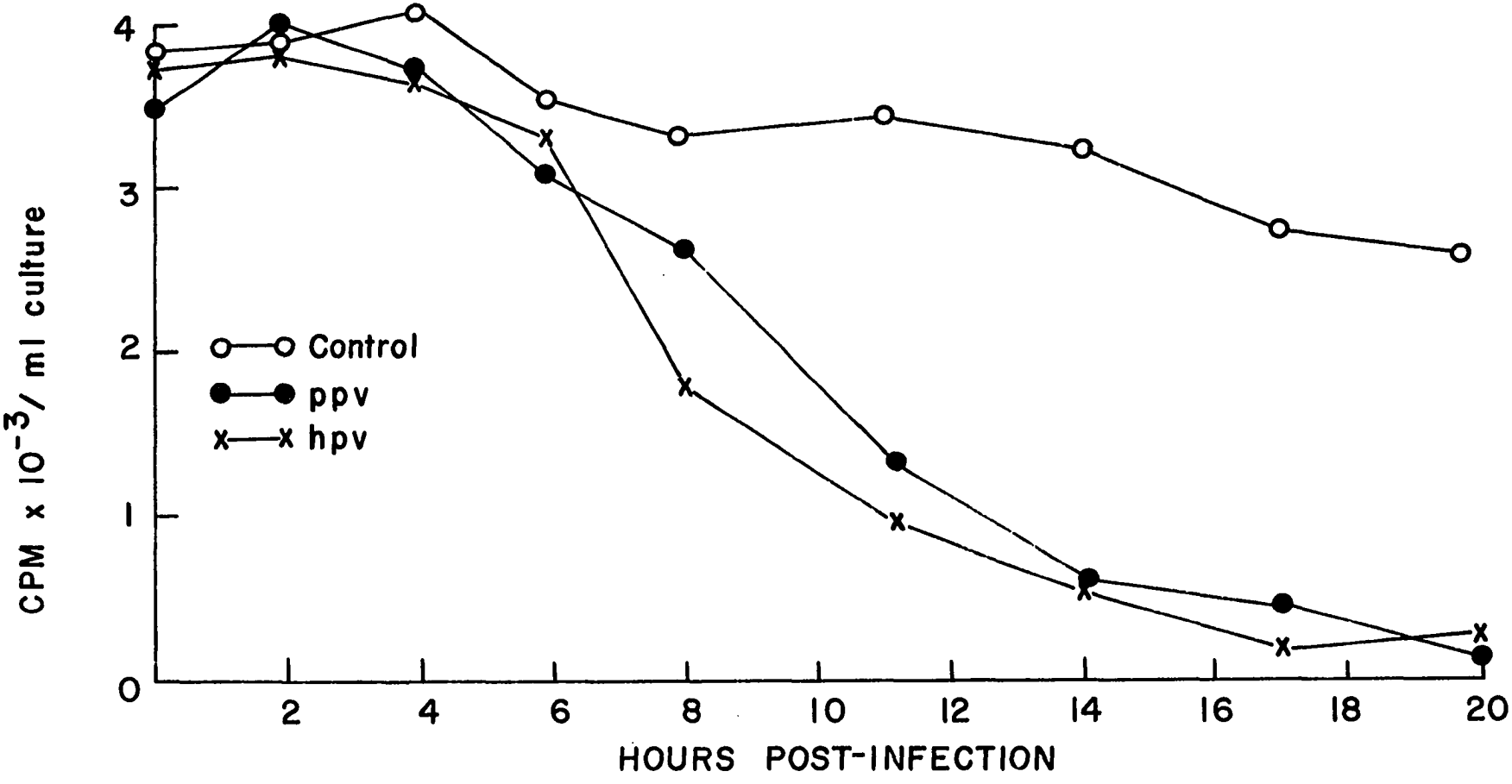
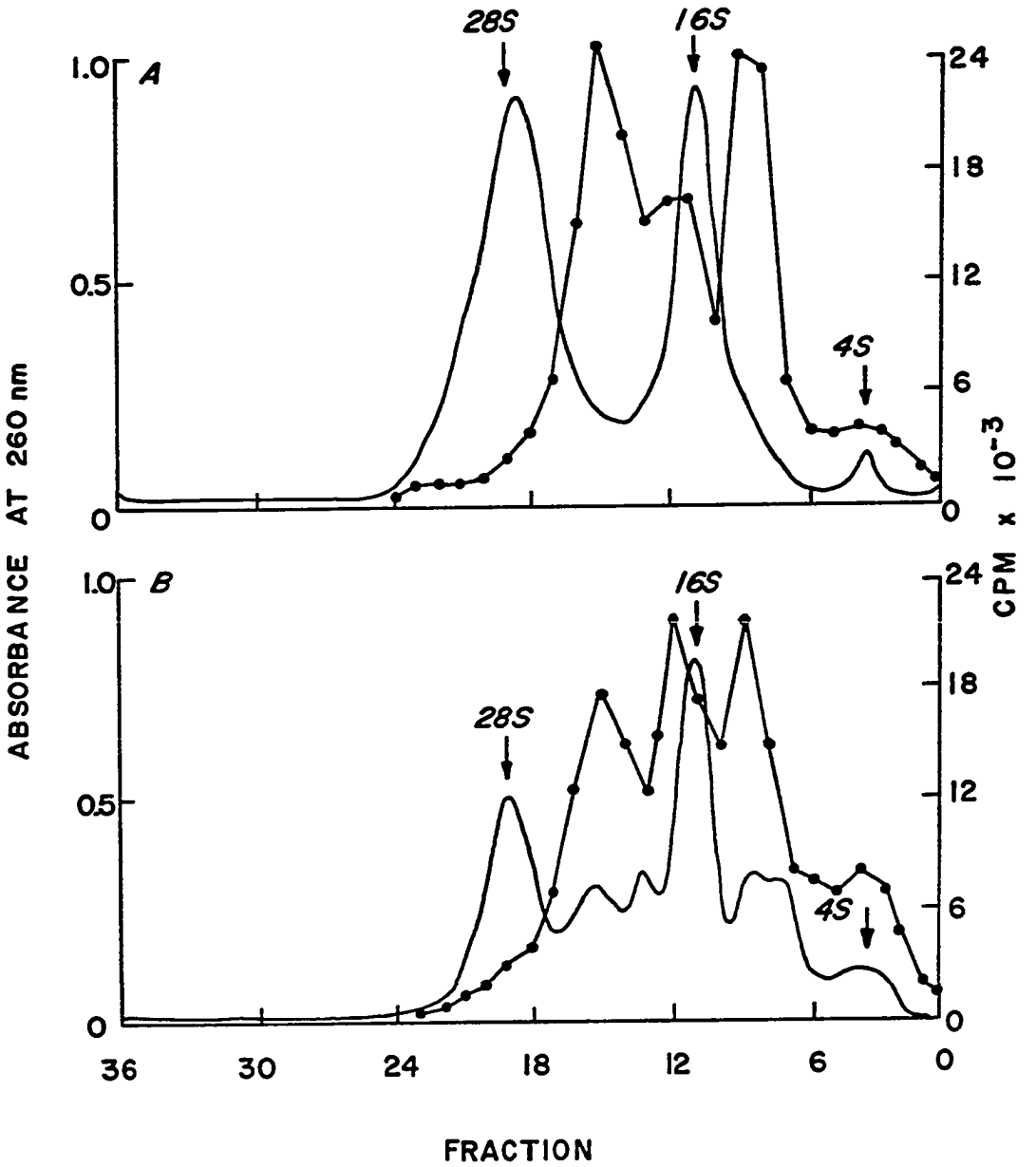

CPM x 10⁻³/ ml culture
4
3
2
1
0
Control
ppv
hpv
2
4
6
8
10
12
14
16
18
20
HOURS POST-INFECTION

Figure 10. A comparison of reovirus mRNA produced in control and mitomycin-C-treated cells. A 400 ml spinner culture was divided into two identical cultures, one of which was treated with 6 μg/ml mitomycin-C for 30 min. The cells from both cultures were washed once in MEM and resuspended in fresh medium and inoculated with 25 pfu/cell of reovirus. The cultures were pulsed from 8 h to 12 h with 2.0 μCi/ml ^{3}H-uridine. Single-stranded RNA was isolated at 12 h. The OD_{260} profiles and TCA precipitable radioactivity in fractions from 15-30% glycerol gradients are presented in panel A (control) and panel B (mitomycin-C treated). Direction of sedimentation is from right to left.


28S
16S
4S
A
B
1.0
0.5
0
24
18
12
6
0
ABSORBANCE AT 260 nm
CPM x 10⁻³
36
30
24
18
12
6
0
FRACTION

to arrest cellular DNA synthesis prior to infection. The gradient profile of ssRNA extracted from virus-infected cells was compared with that of ssRNA from infected cells which had been treated with mitomycin-C. Both profiles show the three size-classes of viral mRNA, but there are slight alterations of proportions and size distribution of the viral RNA from cells treated with mitomycin-C. It would be difficult to ascertain whether this altered ssRNA was due to an effect of residual mitomycin-C or to cell-mediated effects resulting from the inhibition of DNA synthesis. It is speculated that the effect was primarily due to mitomycin-C because of other noticeable alterations in the virus replicative cycle. The progeny virus band, purified as described on a CsCl gradient, was very flocculent, wide and diffuse. When assayed, the progeny had infectivity, but the titer was less than one-tenth that normally seen from infected cultures not treated with mitomycin-C.

Electron microscopic examination of material from the progeny virus band revealed what appeared to be large aggregates of virus particles and debris (not shown). Also, -chymotryptic digests of this material did not produce normal cores, but formed a flocculent band having a lower density than normal cores on a CsCl gradient. Progeny produced in mitomycin-C treated cells were not characterized further.

Much mRNA transcription did occur, however, and some infectious progeny was produced in mitomycin-C-treated cells. It is probable, then, that virus replication can continue in cells which cannot synthesize DNA.

Effects of reovirus replication on viral DNA synthesis. The effects of reovirus on ^{3}H-thymidine incorporation into total cellular and viral DNA was examined in vaccinia and herpesvirus infected cells.

The experimental approach was three-fold in that separate experiments were performed on cells infected with reovirus before, with, and after infection by the DNA viruses. Results are presented in Figure 11. There is obvious interference from reovirus on the DNA virus-mediated patterns of DNA synthesis seen in cells infected with either vaccinia or herpesvirus alone.

Results of examination of polysomes and nuclear RNA and DNA from control and reovirus-infected cells. A cell culture was labeled over a 24 h period with 2.0 μCi/ml ^{3}H-uridine and was approaching constant ^{3}H-label in the polysome fraction. The culture was divided into two identical cultures. One was inoculated with reovirus and the other used as a control. At intervals post-infection, 200 ml samples were incubated for 30 min with 0.05 μg/ml actinomycin-D to inhibit ribosomal RNA synthesis (96). Cells were then concentrated, washed, pulse-labeled with ^{32}P in the presence of 0.05 μg/ml actinomycin-D for 1 h, and the cells were fractionated as described. Nuclear pellets were detergent-washed a second time, and treated as described in the cell fractionation procedure. RNA determinations were made by orcinol quantitation of pentoses (87). Determinations of DNA were achieved by the method of Burton (11). Data on the distribution of radioactivity is presented in Table 2. Noticeable inhibition of both nuclear RNA and DNA synthesis is seen in infected cells between 9 and 13 h post-infection. It is possible that detergent treatment did not free the nuclear preparation of adhering reovirus and thus, some reovirus RNA may have contributed to radioactivity determinations of nuclear RNA. Its presence in the nuclear fraction would partially mask an already obvious inhibition

Figure 11. Effects of reovirus infection on DNA synthesis in cells infected with herpes or vaccinia virus. Cultures were inoculated at 0 h with 20 pfu/cell reovirus. The arrows in each panel indicate time of inoculation with herpes and vaccinia. At the times indicated, 2.0 ml aliquots of each culture were pulsed with ^{3}H-thymidine for 30 min. Results are presented as counts per min in TCA insoluble material per ml of culture.

A. Cells inoculated with herpesvirus or vaccinia 4 h after inoculation with 20 pfu/cell reovirus.

B. Cells inoculated with herpesvirus or vaccinia concurrently with 20 pfu/cell reovirus.

C. Cells inoculated with herpes and vaccinia 2 h prior to inoculation with 20 pfu/cell reovirus.

Symbols are V-vaccinia; H-herpesvirus; R-reovirus; and C-control.

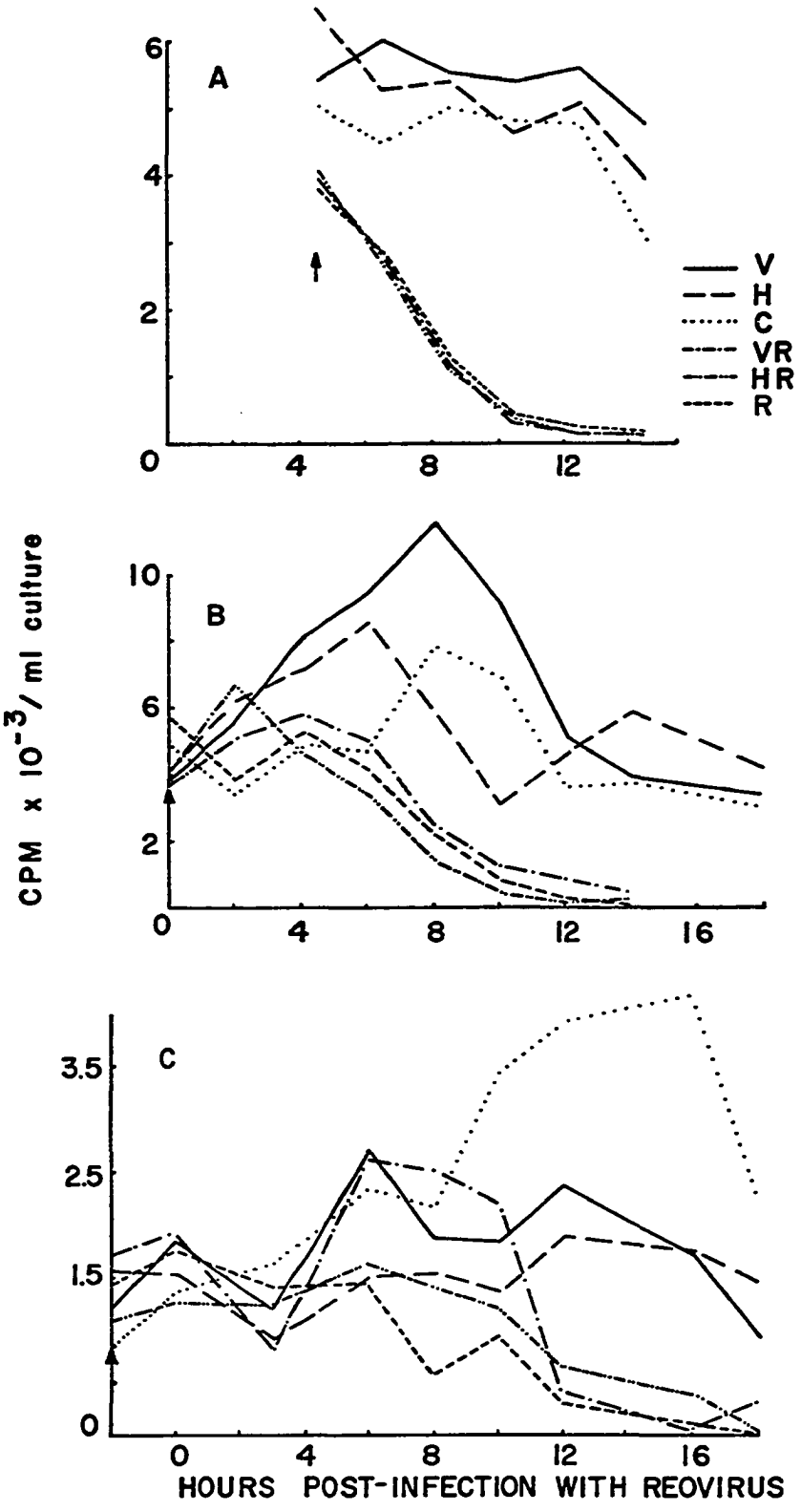

A
B
C
V
H
C
VR
HR
R
CPM x 10⁻³/ml culture
HOURS POST-INFECTION WITH REOVIRUS

Table 2.--Distribution of radioactivity in RNA and DNA in cellular fractions from control and infected cells. Infected and control cells were pre-labeled with ^{3}H-uridine and pulse labeled with $NaH_2{}^{32}PO_4$ as described, and the cells were fractionated. Radioactivity is shown as specific activity of appropriate fractions from 200 ml of cell culture

HOURS POST-INFECTION	CPM/μg NUCLEAR DNA			CPM/μg NUCLEAR RNA				CPM-POLYSOMAL RNA			
	^{32}P			^{32}P ^{3}H	^{32}P ^{3}H	^{32}P	^{3}H	^{32}P ^{3}H	^{32}P ^{3}H	^{3}H / ^{32}P	
	Control	Infected	$\frac{I}{C}$ x100	Control	Infected	I/C x 100		Control	Infected	Control	Infected
2	438	351	80.1	26,772 50,538	20,697 42,395	77.3	83.8	1200 35,750	1150 29,500	29.8	25.7
5	721	641	88.9	21,634 60,728	18,395 61,107	85.0	100.4	870 52,900	730 50,190	60.8	68.7
9	656	995	150.6	18,985 57,539	23,725 55,044	124.9	95.6	890 56,130	540 31,410	63.1	58.2
13	387	147	37.9	17,186 44,858	9,268 50,038	53.9	111.5	380 20,040	310 14,480	52.7	46.7
15	222	73	32.8	12,801 43,209	6,305 49,769	49.2	115.2	—	—	—	—

of nuclear RNA synthesis. Thus, if reovirus RNA did contribute to the RNA content of the nuclear preparations, the actual inhibition of nuclear RNA synthesis would be even more dramatic than reported here.

Polysomes were pelleted by centrifugation from the post-mitochondrial supernatant, washed, and stored at -20 C. The polysome isolation procedure described was chosen because of maximum polysome yield and minimal nuclear contamination. Nuclear contamination was measured using cells which had been pulsed with ^{3}H-thymidine for 1 h. Comparison of total TCA-precipitable radioactivity in the nuclear pellet to that in the post-mitochondrial supernatant, showed nuclear leakage in the fractionation procedure used to be less than 1%. No differences in distribution and quantity of polysomes were detectable at 5-6 h post-infection. Representative profiles are shown in Figure 12. Data showing radioactivity and differences in $^{3}H/^{32}P$ ratios from polysome preparations at times after infection is presented in Table 2.

Effect of hypotonic swelling on DNA synthesis in control and reovirus infected L-cells. DNA synthesis was monitored in both normal and hypotonically swollen control and reovirus-infected cells as described. As shown in Figure 13, the inhibition of DNA synthesis is observable during the same interval post-infection in both normal and hypotonically swollen cells.

Figure 12. A comparison of absorbance profiles of polysomes from control (A) and infected cells (B) 5 h post-infection. Direction of sedimentation is from right to left.

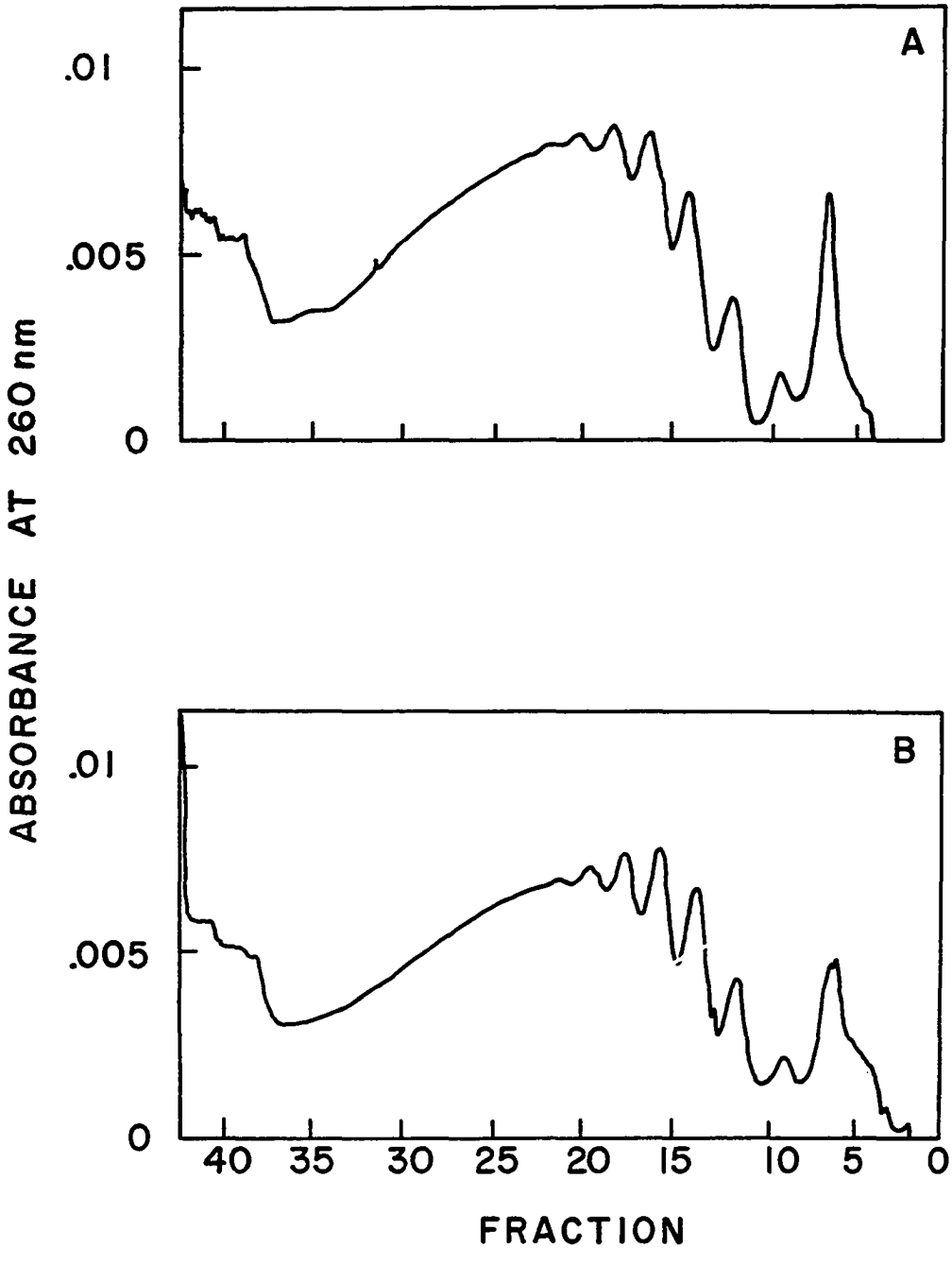
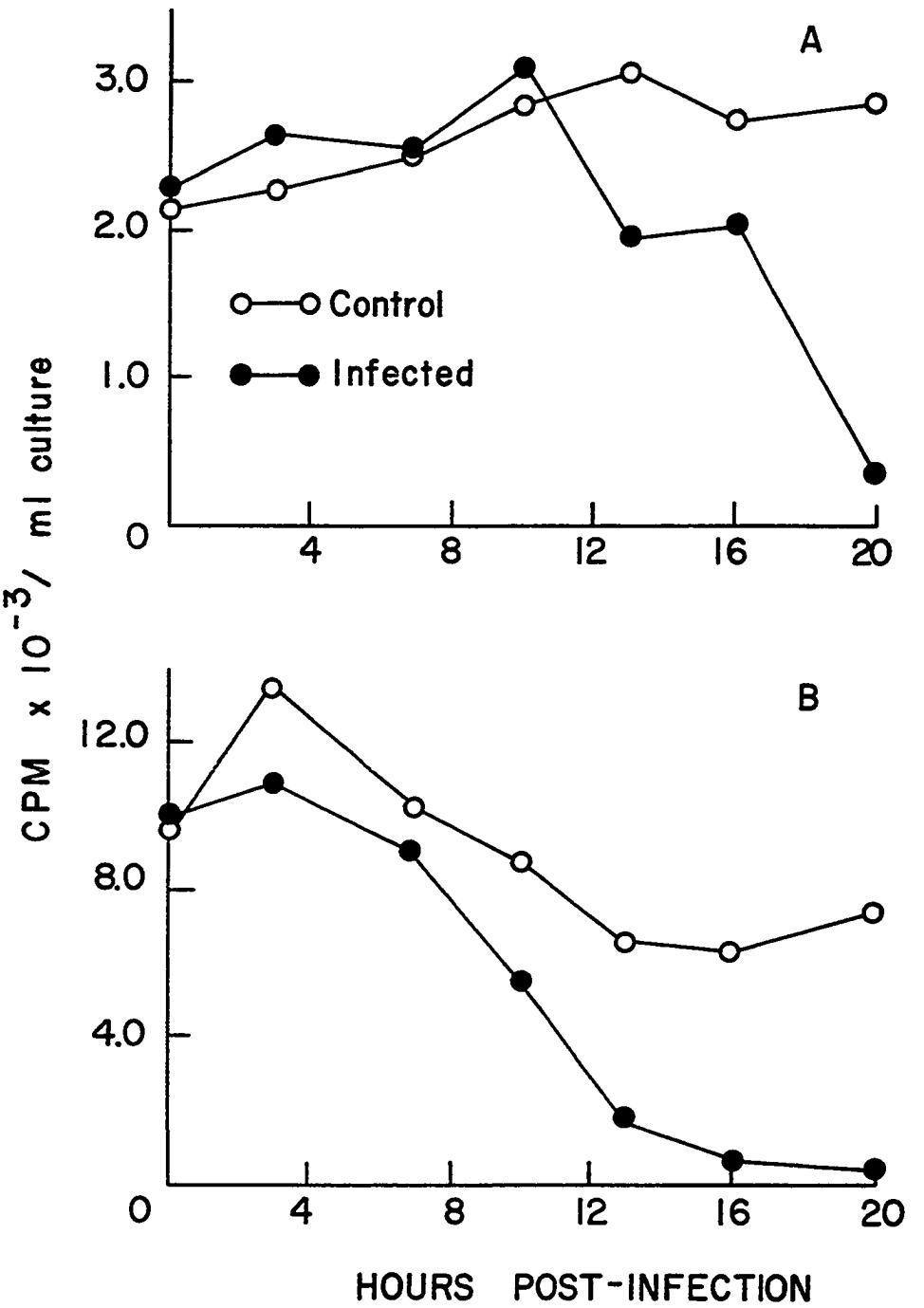

A
B
.01
.005
0
ABSORBANCE AT 260 nm
40 35 30 25 20 15 10 5 0
FRACTION

Figure 13. A comparison of reovirus-mediated inhibition of DNA synthesis in hypotonically swollen and normal L-cells. Two spinner cultures of L-cells were prepared and treated as described in materials and methods. Results are presented as counts per min in TCA-insoluble material per ml culture from hypotonically swollen cells (Panel A) and from normal cells (Panel B).


A
3.0
2.0
1.0
0
Control
Infected
4
8
12
16
20
B
12.0
8.0
4.0
0
4
8
12
16
20
CPM x 10⁻³/ ml culture
HOURS POST-INFECTION

CHAPTER IV

DISCUSSION

The results of this investigation have shown that ^{3}H-thymidine incorporation is a valid measure of DNA synthesis in normal and reovirus-infected L cells. Thymidine was found to be absorbed by infected cells at approximately the same rate as by control cells for several hours after inhibition of DNA synthesis is noted in infected cells. The specific activity of prelabeled cellular DNA in synchronous cells stabilizes after reovirus infection, indicating that cellular DNA synthesis is actually arrested. Thus. the lack of ^{3}H-thymidine incorporation into the DNA of pulse-labeled, infected cells is not simply an artifactual result of restricted nuclear membrane transport of thymidine triphosphate (TTP). The possibility of altered transport of nucleotides into the nucleus as a contributing factor to inhibition of cellular DNA synthesis cannot, however be excluded on the basis of the results of any of these experiments.

Previous investigations have shown that the top component, which contains the full complement of viral protein, does not affect cellular RNA, DNA or protein synthesis (49, 75). Similar results were reported in reovirus core-treated cells. On the other hand, infectious SVP's (15) and UV-inactivated (noninfectious) reovirions (49, 122)

inhibit cellular DNA synthesis as effectively as normal reovirions. These findings indicate that at least limited transcription and translation of viral specific RNA are necessary for inhibition to occur.

Examination of cells infected with ts-mutants supported the possibility that the levels of reovirus ssRNA and/or protein produced in infected cells influence the degree of inhibition of DNA synthesis occurring in those cells. All viral mRNA segments are produced in ts mutant-infected cells at the non-permissive temperature and are translated into identificable viral peptides (34). But, because the mutants accumulate aberrant non-infectious progeny particles, the specific ts lesion of each mutant must result in peptides altered sufficiently to result in faulty viral assembly (21, 34, 35, 60) at some stage of the infection cycle.

Group C mutant 447 is the only mutant examined in this investigation which shows a markedly lower potential than WT-reovirus for inhibition of cellular DNA synthesis. Cross, et al. (21) have shown that this mutant assembles no minus strand-synthesizing particles (ds-RNA containing progeny SVP's) and thus the second stage of viral mRNA synthesis occurring in WT-reovirus-infected cells does not occur in cells infected with mutant 447. Fields, et al. (33) reported, at the permissive temperature, mutant 447 is less efficient than WT-reovirus with respect to the production of both RNA and infectious progeny. This work supports these findings. We have found that mutant 447 has an infectious cycle 5-10 h longer than the group A and B mutants at the permissive temperature. This finding could account for the less marked inhibition of DNA synthesis that group C mutant-infected cells exhibit

at both permissive and non-permissive temperatures and support the hypothesis that viral mRNA and protein synthesis affect the degree of inhibition of DNA synthesis in infected cells. The fact that the group C mutant inhibits DNA synthesis at the non-permissive temperature indicated, however, that neither production of ds-RNA nor the presence in the cell of a large number of ds-RNA containing progeny SVP's was the primary cause of the inhibition. Indeed, the fact that all of the mutants inhibit DNA synthesis at 39°C shows that completion of the viral replicative cycle, terminating in progeny production and cell lysis, is not necessary for the inhibition. This conclusion was indicated previously in work with UV-inactivated virus particles, but the possibility that toxic products were produced during irradiation could not be ignored. All other previously-tested inhibitory particles not only transcribed ssRNA but produced infectious progeny.

It was found that the contribution of defective reovirions to the inhibition of cellular DNA synthesis was less significant than the contribution of an equal number of normal virions. On the basis of differences in physical characteristics it was plausible, however, that defective and normal virions could have dissimilar transcriptive potentials, and that defective particles synthesized RNA at a reduced rate. At least the data obtained from infection of cells with preparations containing defective virus could be resolved by the indicated relationship between the synthesis of viral protein and RNA and cellular DNA synthesis. The possibility that a specific virion protein could be the mediator of DNA inhibition in infected cells was placed in doubt by the data obtained from top component adsorption, though the fate of the

parental viral or top component protein may be entirely different in cells than that of newly synthesized (and perhaps excess) viral protein. Most of the parental virion and presumably top component is conserved and recoated upon release of progeny from productively infected cells. Top component conceivably could be degraded after a time in the cell, since these particles cannot enter into viral mRNA synthesis.

DNA synthesis in cells infected with vaccinia or herpesvirus was examined because continued viral DNA synthesis in cells infected with reovirus would have provided supportive evidence for reovirus-specific inhibition of cellular DNA synthesis. Vaccinia replicates entirely in the cytoplasm of infected cells (102), while herpesvirus DNA is replicated in the nucleus of infected cells (31). Cells which were infected with reovirus both prior to and simultaneously with either vaccinia or herpes showed no evidence of viral DNA synthesis. Total DNA synthesis in these cells was inhibited with the same kinetics observed in cells infected with reovirus alone. DNA synthesis in cells infected with reovirus 2 h after infection with either of the DNA viruses was not cleanly inhibited, but was inhibited more than in cells infected with either DNA virus alone. Vaccinia DNA is replicated from 1.5 to 2.5 h after infection and peaks at about 3 h (65). Herpes DNA replication begins approximately 4 h after infection and almost completely replaces host DNA synthesis by 7 h post-infection (66, 91). However, initiation of herpesvirus DNA synthesis in some cell types may be dependent on the cell cycle (77, 78). Thus, there is a possibility that in L cells, herpesvirus DNA synthesis may not begin synchronously after infection of asynchronous cell cultures. It is also known, that the replication

of both herpesvirus (67, 99) and vaccinia (70) is dependent on prior RNA and protein synthesis. Thus the cause of reovirus inhibition of herpes and vaccinia DNA replication is difficult to determine. A reasonable hypothesis is that reovirus-mediated events which prevent replication of other viruses could also be responsible for inhibition of cellular DNA synthesis. Interference with both viral and cellular DNA synthesis would likely involve either direct physical alteration of DNA or a much more distal event affecting the alteration of replication of all three kinds of DNA. Data reported by Bartkoski and Cox (4), Shaw and Cox (17) and others (27, 28) indicated that reovirus-mediated physical alterations of DNA are extremely unlikely. Reovirus-mediated alteration of cellular protein and RNA synthesis could conceivably result in inhibition of both viral and cellular DNA synthesis. It is probable that at the beginning of the second stage of reovirus mRNA synthesis from progeny SVP's, an increasing portion of the total RNA and protein labeled by radioactive precursor incorporation is viral. Thus, cellular RNA and protein synthesis must be inhibited to some extent in reovirus infected cells, and may be the cause of inhibition of DNA synthesis. Several findings are interpreted as indicating that the inhibition of protein synthesis is not the cause of inhibition of cellular DNA synthesis. In vitro DNA synthesizing systems prepared with nuclei and cytoplasmic fractions from reovirus infected cells, and nuclear and cytoplasmic polymerases and chromatin derived from reovirus infected cells exhibiting depressed DNA synthesis, show labeled deoxynucleotide incorporation at control levels. Small amounts of reovirus ts-mutant 447 peptides could be immunoprecipitated from infected cell

cytoplasm (34), but this mutant inhibited DNA synthesis to a lesser extent than did WT reovirus.

On the other hand, several other facts indicate that inhibition of cellular DNA synthesis is mediated by reovirus-induced inhibition of protein synthesis. The inhibition of DNA synthesis following reovirus infection has been reported to occur at the level of initiation of DNA synthesis (18, 28, 47). Similarly, the termination of DNA synthesis following puromycin or cycloheximide treatment of cells (28, 47) is thought to occur at the stage of initiation. An interesting counterpoint, however, is that unlike nuclei from reovirus infected cells, nuclei from cycloheximide-treated cells were reported unable to synthesize DNA in vitro (53). The levels and activities of several enzymes involved in DNA synthesis are known to be unaltered after DNA synthesis is depressed in cells infected with reovirus (4, 28, 121). Thus, if reoviral inhibition of cellular protein synthesis were the cause of the inhibition of DNA synthesis, the specific missing protein(s) required for DNA synthesis in reovirus-infected cells must have extremely concentration-dependent activities. Such properties might be expected of an initiator protein involved in DNA synthesis.

Inhibition of cellular protein synthesis has been examined in other virus-host cell systems. Lawrence and Thach (76) have suggested that picornaviral mRNA successfully competes with cellular mRNA for initiation factors, and thus is preferentially bound by ribosomes. Reovirus ssRNA has several interesting physical properties which could provide a basis for altered efficiency as messenger with respect to cellular mRNA. Studies (59, 64, 68, 110, 139) have indicated that

reovirus mRNA has a significant degree of secondary structure. Ward and Shatkin (138) and Ward, et al. (137) have shown that all reovirus mRNAs, the largest of which sediments at 25S, are distributed throughout even the largest polysomes from infected cells, and are linked together through protein subunits. Either of these properties could perhaps result in altered efficiency in competition with cellular mRNA for some part of the protein-synthesizing system within infected cells. That reovirus mRNA is physically distinguishable from cellular mRNA has been demonstrated in interferon treated, reovirus-infected cells (37, 45) and in in vitro protein synthesizing systems employing components produced in interferon-treated cells (105). Shatkin (115) has recently reported that reovirus mRNA is specifically methylated at the 5' terminus, and it has been known for some time that the 3' terminus of reovirus mRNA is not polyadenylated as is most cellular mRNA (133).

The possibility that reovirus mRNA could compete favorably with cellular mRNA in polysome formation was to be examined by polydeoxythymidylate (poly dT)-cellulose separation of puromycin dissociated polysome-bound viral and cellular mRNA. Although sufficiently highly labeled mRNA in large enough quantities was not obtained, data gathered from the fractionation of cells and subsequent quantitation of radioactivity in nuclear RNA and DNA, and in polysomal RNA, indicated that syntheses of nuclear RNA and DNA were inhibited at approximately the same time. Almost complete supression of both 32S and 45S ribosomal precursor RNA occurs by 10 h after reovirus infection (16). It now appears that total nuclear RNA synthesis is about 50% inhibited at 13 h post-infection. $^{3}H/^{32}P$ ratios of polysomal pellets in both reovirus-

infected and control cells were very nearly the same at times after infection. Since the cells were treated with low doses of actinomycin D to suppress cellular ribosomal RNA synthesis, ^{32}P-label incorporated during the pulse period should be contained primarily in viral and cellular mRNA and tRNA, neither of which would sediment with polysomes unless they were ribosome-bound. Radioactivity due to ^{3}H-uridine-incorporation, on the other hand, would be contained in pre-existent mRNA and primarily in ribosomal RNA. It might therefore be possible to comment on the state of ribosome loading of mRNA on the basis of $^{3}H/^{32}P$ ratios of RNA found in polysome pellets. These ratios are supportive evidence that total polysome-bound mRNA in infected cells is no less efficiently loaded with ribosomes than in uninfected cells. The total ^{3}H and ^{32}P radioactivity in polysomes indicates that polysome levels begin to decrease earlier in infected cells than in control cells, but that the rates of decrease in infected and uninfected cells are approximately the same. The apparently gradual transition from cellular to viral protein synthesis reflected by total protein curves would support the possibility that depressed mRNA synthesis in the nucleus of infected cells, rather than viral interference with polysome formation, is responsible for the early decrease in polysome levels in infected cells.

While the rate of nuclear RNA synthesis was decreasing in infected cells after 9 h post-infection, the loss or transport of ^{3}H-labeled RNA from the nucleus was slowed compared to control during the same period.

It appeared that the gross effects of reovirus on nuclei of infected cells included inhibition of DNA synthesis, inhibition of RNA

synthesis and possibly the altered transport of RNA into the cytoplasm. It appeared that, possibly due to the perinuclear localization of reovirus replication, such effects on the nucleus could be caused directly by events in the reovirus replicative cycle. The possibility that the relationship between reovirus replication and the nucleus might be more intimate than previously thought prompted the examination of reovirus replication in cells with impaired DNA function. Results indicated that reovirus was replicated in mitomycin-C-treated cells. Thus reovirus replication apparently is not dependent on replicative or transcriptive functions of nuclear DNA.

The inhibition of nucleic acid synthesis in the nucleus could be the result of suboptimal nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) concentrations, however. Lowered concentrations of these triphosphates could also result decreased efficiency of energy-requiring transport mechanisms, including nucleic acid transport from the nucleus. Evidence obtained from hypotonically swollen reovirus-infected cells indicated that when dNTP's were supplied, DNA synthesis was resumed (4). To investigate the possibility that this resumption was an artifactual result of the treatment and hypotonic swelling, DNA synthesis and thymidine permeability were measured in hypotonically swollen cells by ^{3}H-thymidine uptake and incorporation. Results indicated that inhibition of DNA synthesis in infected cells was not reversed by hypotonic swelling. The presence of actively synthesizing viral "factories" in the perinuclear region of the cell at approximately the time of inhibition of synthesis of nuclear nucleic acid indicates that a mechanism of inhibition involving NTP depletion is

indeed plausible. Similar effects of suboptimal intracellular precursor concentrations have been noted in other virus-cell systems. Shatkin and Salzman (117) reported that levels of thymidine which were sub-optimal for cellular DNA synthesis could support syntheses of certain viral DNA. The observation that the pattern of reovirus gene expression in vivo is similar to the pattern exhibited in vitro at suboptimal NTP concentrations could indicate that a similar situation exists in the cell. Viral RNA synthesis in the perinuclear region could conceivably place too great a drain on the NTP and dNTP pools to support normal biosynthetic activity in the nucleus. Proof would depend on determination not only of actual NTP and dNTP concentrations in the nucleus and perinuclear region, but of minimal concentrations of the triphosphates required for initiation and maintenance of nuclear RNA and DNA synthesis.

Another fact supporting the hypothesis of limiting nuclear function via NTP depletion is that reovirus cores also possess a nucleoside triphosphate phosphohydrolase (9, 69) that can hydrolyse all NTP's and dNTP's. The function of this enzyme is unknown, but it may be responsible for converting the terminal 5'-triphosphate groups of reovirus RNA species to diphosphates.

Fields et al. (34) have shown that although all the ts mutants tested in this investigation induce normal amounts of viral protein at 31 C (estimated at 10-30% of the total cytoplasmic proteins labeled between 4 and 15 h post-infection), the group A mutant induces only 10% viral specific protein and groups B, C, and D induce 3% or less at the nonpermissive temperature. Because of the similar characteristics of the inhibition of DNA synthesis noted in cells infected with mutants

A and B and WT-reovirus at 39 C, the degree of inhibition does not seem to be related to viral protein synthesis.

The group C mutant is the only mutant which produces no detectible dsRNA or dsRNA-containing progeny particles in infected cells. The slower and less marked inhibition of DNA synthesis in group C mutant-infected cells could be related to the facts that viral-mRNA production is much depressed and that no transcribing progeny SVP's with associated phosphohydrolase activities are produced. This supports the possibility that parental and progeny SVP's could contribute to the inhibition of nuclear RNA and DNA synthesis by decreasing the concentrations of NTP's below some threshold level required for normal nuclear function.

Due to the small quantities of viral protein produced in mutant-infected cells at 39° and the sustained levels of total protein synthesis in these cells, the possibility that inhibition of cellular protein synthesis as the primary cause of the inhibition of DNA synthesis seems remote. The possibility that altered protein synthesis does affect nuclear function in infected cells cannot be ignored, however. A portion of the radioactivity from solubilized ^{32}P liberated from TCA-treated nuclei on NaOH hydrolysis could be released from nuclear phosphoproteins, but under the conditions of the experiments described, it is probable that most of the ^{32}P label solubilized had been incorporated into RNA.

The reovirus A-rich RNA species has been shown to mediate transient inhibition of DNA synthesis in treated L cells. Speculation based on the similarity of the termini of all reovirus RNA species,

has been that A-rich RNA is a product of abortive transcription within the progeny SVP. That the group C mutant produces no progeny SVP's capable of transcription supports the possibility that the inhibition of DNA synthesis in reovirus-infected cells is caused by NTP depletion of reovirus SVP's and/or by effects of A-rich RNA transcribed by SVP's.

CHAPTER V

SUMMARY

Investigations on the kinetics of the inhibition of DNA synthesis in cells infected with reovirus ts-mutants has shown that the complete reovirus replicative cycle, ending in progeny production and cell lysis, is not necessary for the inhibition phenomenon. Further, the only ts mutant inhibiting cellular DNA synthesis less efficiently than WT virus was the mutant which produces no dsRNA-containing progeny SVP's. Reovirus SVP's are known to possess several enzyme activities, one of which is a nucleoside triphosphate phospholydrolase. There is evidence also that SVP's produce A-rich oligonucleotides as products of abortive transcription. These A-rich RNA's have been shown previously to transiently inhibit cellular DNA synthesis. Both the phosphohydrolase activity and A-rich RNA production by SVP's in infected cells could contribute to the inhibition of DNA synthesis. Since neither of these functions could be fulfilled by the group C mutant, the less severe inhibition of DNA synthesis in these mutant-infected cells could be mediated by the few parental SVP's produced from input virions.

Cells infected with WT reovirus were found to exhibit decreased nuclear DNA and RNA synthesis after 9 h post infection, a time at which progeny SVP's are rapidly being assembled in the perinuclear region. The inhibition of nuclear RNA synthesis in infected cells was not seen

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