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A KINETIC STUDY OF THE INHIBITION OF DNA SYNTHESIS IN L-CELLS BY REOVIRUS TYPE 3

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A KINETIC STUDY OF THE INHIBITION OF DNA SYNTHESIS IN L-CELLS BY REOVIRUS TYPE 3

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To Phyllis

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CHAPTER I

INTRODUCTION

The replicative cycle of the eukaryotic cell has been divided into four phases, each of which characterizes the cell according to its stage of nuclear development from one mitosis to the next. Each phase is defined by a time interval during which specific morphological and biochemical changes occur. The appearance of each phase is sequential and the sum of the time intervals of each phase equals one generation time. It is characteristic that cultured mammalian cells at any given time are in different phases of their replicative cycle unless they reach high population densities or they become starved for essential growth requirements. Under these conditions a significant number in a population may become synchronized or phased.

The four phases of the replicative cycle, in sequence of occurrence, are symbolically defined as: G_1, S, G_2, and M (29). The G_1 phase is the time interval between the

end of mitosis and the initiation of deoxyribonucleic acid (DNA) synthesis. The S or DNA synthetic phase is the time required for duplication of nuclear genetic material. G_2 is the time interval between the end of the S phase and the initiation of mitosis, and M the time required to complete one mitosis.

In the discussion to follow, emphasis will be placed on the S or DNA synthetic phase since reovirus infection results in the inhibition of this particular phase of the replicative cycle (17,22,37). This does not preclude the possibility (12) that other phases of the replicative cycle are also inhibited following reovirus infection or that inhibition of the S phase is a result of reovirus-directed alteration during other phases.

Initiation and continued synthesis of DNA in mammalian cultures depends upon specific metabolic events. In mouse fibroblasts continued protein synthesis is a necessary requirement not only for initiation but for the continued synthesis of DNA (45). Puromycin inhibits protein synthesis and when added to cultures of mouse fibroblasts during the S phase markedly inhibits the synthesis of DNA. Since the synthesis of histones occurs concurrently with the synthesis of DNA in these cells, histone synthesis may be necessary for DNA replication.

Ribonucleic acid (RNA) synthesis, at least during the G_1 phase, may also be necessary for DNA replication.

Either actinomycin D or chromomycin succinate, inhibitors of RNA synthesis, can inhibit the synthesis of early replicative DNA if either is present during the G_1 phase (19). However, addition of either drug during the middle of the S phase does not inhibit late replicating DNA. Thus the S phase of a eukaryotic cell is dependent on events that occur during the G_1 phase of the replicative cycle.

There is increasing evidence that DNA synthesis may also be controlled by nuclear-cytoplasmic interaction. DNA synthesis can be "switched on" in a G_1 nucleus before the G_1 phase is complete. This has been shown in hybrid cells (26,27), in transfer experiments using Xenopus egg cytoplasm (24,25), and in nuclear transfer experiments using G_1 and S nuclear phases of the protozoan Stentor (14). It has also been reported that a G_2 phase nucleus can be "switched on" before the G_2 phase is complete if it is transplanted to a S phase cytoplasm. Also an S phase nucleus can be "switched off" before completion of the S phase by transplanting it to a G_2 phase cytoplasm (52). However, more recent evidence suggests that a G_2 phase nucleus must first undergo mitosis before being "switched on" (49). It was suggestive, therefore, that the condition of the cytoplasm as well as events occurring during the G_1 phase of the cell cycle directly influence the S phase. It is pertinent to mention at this

point that reovirus, whose replication occurs entirely in the cytoplasm and not in the nucleus, can inhibit DNA synthesis in L-cells (21).

The mechanism of viral inhibition of host cell DNA synthesis is not known. However, since inhibition of RNA or protein synthesis either precedes or parallels the inhibition of DNA synthesis in many virus infected mammalian cells, it has been suggested that these early alterations account for the inhibition of DNA synthesis (11). While this may be true for other cytoplasmic RNA viruses (2,31,42,56,58,72) and for vaccina, a cytoplasmic DNA virus (33), it is not true for reovirus (17,22). There apparently is no inhibition of RNA or protein synthesis prior to or at the time of inhibition of DNA synthesis in reovirus infected L-cells (17,21). Although inhibition of protein synthesis has been reported in reovirus infected L-cells in spinner culture (37), this was shown to be a result of increased sensitivity of infected cells to agitation which resulted in cell lysis (17). If infected spinner cells were transferred to stationary cultures no inhibition of protein synthesis occurred and there was no reduction in cell number.

Since reovirus inhibition of cellular DNA synthesis was a specific event, an event not explainable by prior inhibition of RNA or protein synthesis, it warranted further investigation. The use of reovirus for this

investigation was justified not only because it was an ideal biological tool with which to study viral inhibition of cellular DNA synthesis, but because it was a unique mammalian virus, one which could be used to study other heretofore unexplained biological and biochemical phenomena. The ultimate goal of this investigation was not so much to answer all questions about viral alteration of cellular DNA function, but instead to reveal other interesting areas of research using reovirus as an investigative biological and biochemical tool. The following description of reovirus is intended to emphasize its unique characteristics as a mammalian virus and to point out its applicability to this investigation.

Reovirus has been isolated from a large number of animal species in nature (54). Since reovirus was isolated from both respiratory and enteric sources and could claim no single host species, it was an orphan, thus the name reo (respiratory, enteric, orphan). The relationship of reovirus to specific disease is not known.

Reovirus was reported as an icosohedral virus measuring 60-70 nm in diameter (54) with a molecular weight of 70×10^6 (23). It was resistant to ether treatment (23) and therefore probably contained no lipid material. Its capsid was unusual in that it contained two layers of protein (47,60,69). The outer protein layer was not necessary for infection as was shown by its removal with chymotrypsin

(60). Chymotrypsin treatment reduced the diameter of the virion from 70 to approximately 50 nm (60). The inner layer of protein contained the reovirus genome.

The reovirus genome was unlike any viral nucleic acid isolated from mammalian viruses. It was a double-stranded, helical, RNA molecule (39) which apparently segmented on isolation into 10 independent double-stranded RNA species (10). The nucleic acid of reovirus, when isolated by a variety of techniques and sedimented through linear sucrose density gradients, separated repeatedly into three class sizes, L (large), M (medium), and S (small) (4,20,30,34,70). Each virion probably contained 3L, 3M, and 4S pieces of double-stranded RNA since the L, M, and S fractions from sucrose gradients could be further resolved by polyacrylamide gel electrophoresis into 10 (3L, 3M, and 4S) double-stranded RNA molecules (60).

As would be expected for such a molecule, preexisting cellular nucleic acid transcriptases would not use the double-stranded RNA genome of reovirus as a template for the synthesis of single-stranded messages (38,61). Also, it was shown that the parental reovirus genome did not denature and serve as single-stranded messages <u>in vivo</u> (46,60,64). In spite of these findings, reovirus-specific, single-stranded RNA was found associated with cellular ribosomes (53). The ribosome-associated, single-stranded

RNA would hybridize with denatured, double-stranded reovirus RNA.

Since preexisting cellular transcriptases did not exist for the reovirus genome and since the reovirus genome was conserved in infected cells, it was difficult to explain the presence of reovirus-specific, single-stranded RNA in cells soon after infection. Recent findings (6,63,65) suggested that the reovirus virion included, in addition to its nucleic acid, transcriptase molecules, possibly one for each double-stranded RNA segment (65). This was demonstrated by the finding that ribonucleoside triphosphates were incorporated into single-stranded RNA molecules in vitro when purified reovirus virions were used for templates. The single-stranded RNA products hybridized with denatured, double-stranded RNA from purified reovirus. RNA synthesis proceeded in vitro only if virion protein coats were altered by heat shock or chymotrypsin treatment. This suggested that the intact virion either rendered the genome inaccessible to precursor molecules or that it prevented enzyme activity (65).

Thus it was possible for the first time to study the requirements for transcription of double-stranded RNA molecule in vitro and potentially possible to correlate physical sites on the reovirus genome with reovirus-specific proteins. Since transcription of each segment of the reovirus genome resulted in equivalent numbers of

each product *in vitro* (65) but not *in vivo* (71), regulation of transcription probably occurred in infected cells. Thus, the study of regulation of transcription would also be possible using reovirus.

Another unique feature of reovirus was its association with single-stranded, adenine-rich RNA (3,4,35,62). Since the single-stranded RNA was found only with cell-associated virus, but not virus released from infected cells, and since it could be removed from the virions without affecting their infectivity, it probably was not a complement of the reovirus genome (36). The single-stranded, adenine-rich RNA hybridized neither with denatured cellular DNA nor with denatured double-stranded reovirus RNA and could not be isolated from uninfected cells (3,62). The origin and function of the adenine-rich RNA is not known.

More pertinent to the present work was the fact that reovirus had a long eclipse period in L-cells which varied with the multiplicity of infection (64). The eclipse period varied from 4.5 to 7 hours at input multiplicities of 100 and 5 PFU/cell respectively (64). This was not due to earlier infection of cells at high input multiplicities since infection was synchronized by allowing virus to adsorb at 4° C. After the adsorption period a synchronized wave of pinocytosis occurred when cells were placed at 37° C.

Also important to the present work was the fact that adsorption and penetration was slow (21,37,64) and that the productive cycle of reovirus was long (21,37). Only 50% of the plaque forming units added to suspension culture adsorbed during the first hour (21) and three hours were required to achieve 90% adsorption (37). First infectious virus appeared approximately 8 hours after infection and maximum yields of infectious virus occurred at 16 hours. This productive period was extremely long when compared to poliovirus (an RNA virus) whose productive cycle is completed by 6-8 hours after infection (13).

Also pertinent to the present work was the report that reovirus became associated with the mitotic tubules of the spindle apparatus and caused the formation of perinuclear inclusion bodies in infected L-cells (12). Also, it has been suggested that cells were stimulated to enter mitosis more rapidly than uninfected cells as was shown by a higher mitotic index above that of controls after 6 hours. Formation of perinuclear inclusion bodies and association with the mitotic spindle were not necessary for the replication of reovirus. This was shown by treatment of cells before infection with colchicine. Treatment of cells with colchicine for 6-8 hours resulted in disappearance of mitotic tubules and only dense intracytoplasmic inclusion bodies were formed. Approximately equal numbers of virus particles were present in untreated and

colchicine-treated cells.

A thorough morphological and biochemical description of reovirus infection has been reported by Silverstein and Dales (64). They determined the fate of isotopically-labeled reovirus in L-cells by correlating biochemical analyses with electron microscopic observations at various times after infection. Infection was synchronized. A single wave of pinocytosis occurred at 37° C if reovirus adsorption occurred 2 hours before this time at 4° C.

Immediately following pinocytosis, reovirus appeared in lysosome-associated phagocytic vacuoles. Virus was first detected in lysosomes as early as 25 minutes after pinocytosis. Hydrolysis of protein coats proceeded at a rapid rate in lysosomes and in later stages paralleled the rate of appearance of uncoated viral RNA. Both processes were nearly completed by 5 hours after pinocytosis.

Although preexisting protolytic enzymes in lysosomes degraded viral coat proteins, preexisting nucleases failed to degrade the exposed double-stranded RNA throughout the course of infection. Uncoated RNA was detected as early as 1.5 hours after virus uptake, a time when 15-20% of the coat protein had been hydrolyzed.

Viral RNA synthesis was initiated approximately 4 hours after infection and included both double and single-stranded RNA. While the single-stranded RNA, presumably messenger, left the phagocytic vacuoles, the parental

double-stranded RNA did not. Progeny virus started forming approximately 4 hours after pinocytosis and could be isolated from the phagocytic vacuoles of disrupted cells.

These data (64) were collected from monolayer cultures of L-cells infected with 5 or 100 PFU/cell and they have demonstrated the general features which characterize the infectious cycle of reovirus. It should be mentioned that the time course of occurrence of a single event can vary significantly when cultural conditions (stationary or spinner) vary for the same experiment or when the multiplicity of virus infection is different under the same cultural conditions.

This manuscript is a kinetic description of the events that occur in reovirus infected L-cells under conditions of high and low virus multiplicities, in particular as it applies to the synthesis of cellular DNA.

CHAPTER II

MATERIALS AND METHODS

Cells

The continuous cell line of mouse fibroblasts, strain L-929 (57), adapted for suspension culture (Flow Laboratories) was cultivated in MEM spinner medium supplemented with 7% fetal calf serum. L-cells were maintained in logarithmic growth by cultivation in air-tight flasks with an air volume to medium volume ratio of 3:2. Suspension of cells was accomplished by use of a teflon-coated, magnetic stirrer bar, the revolution of which was sufficient to cause a small vortex to extend no more than one-half of an inch beneath the surface of the medium. The cell concentration was maintained between $5x10^4$ and $2x10^5$ cells/ml by dilution with fresh prewarmed MEM spinner medium every 48 hours. The average generation time was 24 hours under these conditions.

Virus

Reovirus type 3, kindly provided by Dr. P.J. Gomatos, was used in this study. Reovirus was propagated in L-cells in spinner culture by concentrating spinner cells to $5x10^6$ cells/ml in MEM spinner medium containing

no serum. Approximately 10 PFU/cell (plaque forming units per cell) were used for infection and were allowed to adsorb to the concentrated cell suspension for 2 hours at 37° C. After the adsorption period cells were diluted to $5x10^5$ cells/ml with fresh prewarmed MEM spinner medium containing 3% fetal calf serum. Sixteen hours after infection cells were chilled in an ice bath then were centrifuged briefly (6000 x g). The cell pellet was frozen at -60° C until purification.

Virus Purification

Reovirus was purified from infected cells according to the procedure of Bellamy et al. (4). Generally, reovirus was subjected to only one cesium chloride density gradient centrifugation and was then dialyzed extensively (3, 2-liter changes) against calcium-magnesium free, phosphate-buffered saline. Purified reovirus was frozen at -60° C.

Virus Assay

Reovirus was titered on L-cell monolayers. The procedure was essentially that of Gomatos et al. (21) except that cells were grown in sealed bottles and not under an atmosphere of 5% carbon dioxide and 95% air. Approximately $3.2x10^6$ spinner cells were added to each 30 ml plastic bottle (Falcon Plastics). After cell attachment for 1 hour at room temperature, the medium was replaced with MEM monolayer medium supplemented with 7% fetal calf

serum. The monolayers were used no later than 24 hours after confluent cell sheets were formed. A medium change was usually made one day prior to the addition of virus. The day the monolayers were to be used, they were washed twice with BME monolayer medium containing no serum. BME monolayer medium was also used for virus dilutions and the pH was adjusted with sodium hydroxide rather with sodium bicarbonate. To each monolayer, 0.25 ml of a virus dilution was added and virus was allowed to adsorb for 2 hours at room temperature. After the adsorption period, 5 ml of MEM monolayer medium containing 0.9% Ionagar and supplemented with 3% serum was added. On the third day after the first overlay, a second 5 ml overlay was added. Three days later, the third and final 5 ml overlay was added which included .005% neutral red. Plaques were counted 24 hours after the third overlay.

Ultraviolet (UV)-Inactivation of Virus

Purified reovirus in calcium-magnesium free, phosphate buffered saline was inactivated using a General Electric lamp emitting 95% of its wavelength at 253.7 nm. Reovirus, approximately 10^9 PFU/ml, was irradiated in wells of a spot plate, 0.4 ml/well, for periods up to 60 minutes. Exposure to approximately 11 ergs/sec/mm^2 was sufficient to reduce the infectious titer of reovirus 10^2 to 10^3 fold in 15 minutes. Inactivation of reovirus was conducted at 4° C.

Media

The tissue culture media employed in these studies were: BME monolayer medium, MEM monolayer medium, and MEM spinner medium. Antibiotic supplements to tissue culture media were penicillin (50 units/ml) and streptomycin (50 ug/ml) and were included in all media. Serum supplements to tissue culture media were made with fetal calf serum (Flow Laboratories) which had been heat-inactivated at 56° C for 30 minutes. Stock cultures were propagated and experiments were conducted at a temperature between 36-37° C unless otherwise stated. Cell enumerations were estimated by direct cell counts in a hemacytometer.

BME spinner medium consisted of basal medium Eagle (15) in Earle's salts, modified to contain 10 times the phosphate concentration and no calcium chloride. MEM spinner medium consisted of minimal essential medium (16) in Earle's salts modified to contain 10 times the phosphate concentration and no calcium chloride. This medium was purchased in dehydrated form (Schwarz Bioresearch). MEM monolayer medium consisted of minimal essential medium in Earle's salts. This medium was dehydrated and autoclavable (Flow Laboratories). BME monolayer medium was basal medium Eagle (15) in Earle's salts.

Asychronous Growth Conditions

In order to increase the cell concentration over that of stock spinner cultures, and maintain asychronous growth

conditions for experiments, cells from a stock culture were centrifuged and resuspended in fresh prewarmed MEM spinner medium to the proper cell concentration. All experiments involving asychronous spinner cultures were conducted with initial cell concentrations of $2x10^5$ to $5x10^5$ cells/ml.

In order to maintain asychronous growth of cells in stationary cultures, aliquots containing $1.5x10^5$ cells were removed from a stock spinner culture and were added directly to a 30 ml plastic bottle. After cell attachment at 37° C for 1 hour, the spinner medium was carefully removed and was replaced with 5 ml of prewarmed BME monolayer medium supplemented with 7% fetal calf serum. Cells grown under these conditions were not confluent and remained asychronous for 24-48 hours.

Synchronous Growth Conditions

Cells in spinner culture were synchronized by starvation. The procedure was similar to that described by Littlefield (43) and consisted of centrifuging and resuspending cells every 72 hours to $5x10^5$ cells/ml in BME spinner medium supplemented with 10% fetal calf serum. Under these conditions the number of cells would double only during the first 24 hours. In order to overcome a large decrease in pH due to high cell concentrations during the 72 hours of incubation, the spinner flasks were not sealed air-tight. This required reducing the

normal sodium bicarbonate concentration (0.22%) to a value that would prevent high pH values resulting from carbon dioxide loss. Cells maintained under these conditions remained synchronized from one medium change to the next. Greater than 70% of the cells were synchronized by this method as determined by mitotic index.

^{3}H-thymidine

Tritiated thymidine, labeled in the methyl position, with a specific activity of 17.9 Ci/mM (Curies per millimole) was purchased from New England Nuclear Corporation.

^{3}H-uridine

Tritiated uridine, labeled in the 5 position, with a specific activity of 26.6 Ci/mM was purchased from New England Nuclear Corporation.

^{14}C-amino acids

Carbon 14 labeled amino acids L-arginine (198 uCi/mM), L-glutamic acid (218 uCi/mM), and L-leucine (240 uCi/mM) were purchased from ICN (International Chemical and Nuclear Corporation).

Cell Labeling Procedures

DNA, RNA, and protein synthesis were measured by the rate of incorporation of specific precursor molecules which were isotopically labeled. DNA synthesis was measured by the incorporation of ^{3}H-thymidine, RNA synthesis by the incorporation of ^{3}H-uridine, and protein synthesis by ^{14}C-amino acid incorporation. The counts

per minute (cpm) in the TCA-insoluble material of labeled cells was recorded and used as a measure of synthetic rates.

Stationary cultures were pulse-labeled for 30 minutes with ^{3}H-thymidine (0.5 uCi/ml culture) at various times after infection to determine the rate of incorporation of thymidine into the TCA-insoluble material and to determine if there were a significant difference between the thymidine pools of infected and control cultures. After the labeling period the medium was removed from culture flasks and the cell surfaces were washed twice with ice cold BME monolayer medium containing no serum. After the second wash 2 ml of cold 5% TCA was added to each bottle. After 30 minutes in the cold the TCA was removed. This was the TCA-soluble cellular material and was centrifuged briefly (6000 x g) to remove any insoluble precipitate. The cells, which were fixed to the bottle surface by TCA, were washed twice with 2 ml of cold 5% TCA. The washings were discarded. After the second wash 2 ml of cold 5% TCA was added and the cells were shaken loose by touching each bottle briefly to the probe of a sonicator. This was repeated and was added to the first 2 ml. The combined 4 ml of material was hydrolyzed at 95% C in a water bath for 15 minutes. The insoluble material remaining after hydrolysis was removed by centrifugation. The supernatant was saved.

DNA, RNA, and protein synthesis were determined in spinner cultures at various times after infection by pulse-labeling cells with either ^{3}H-thymidine (0.5 uCi/ml of culture) for 30 minutes or ^{3}H-uridine (1.0 uCi/ml of culture) for 1 hour or ^{14}C-amino acids (.045 uCi/ml of culture) for 30 minutes. Five ml aliquots were removed from control and test cultures at various times after infection and were placed in screw-capped culture tubes containing the appropriate isotope, and were shaken gently for the pulse-labeling period in a wrist action shaker. After the labeling period an equal volume of cold 10% TCA was added to each tube and the tubes were placed at 0-4° C for 30 minutes. The TCA-insoluble material was collected by centrifugation (6000 x g) for 10 minutes and was washed three times with 5 ml of cold 5% TCA. The insoluble material containing the ^{14}C-amino acids was resuspended by sonication in 2.5 ml of 5% TCA. The insoluble material labeled with ^{3}H-thymidine or ^{3}H-uridine was hydrolyzed in 2.5 ml of 5% TCA for 45 minutes in a 95° C water bath. After hydrolysis the remaining insoluble material was removed by centrifugation and the supernatant was saved.

Radioactivity Determination

The radioactive counts in the various labeled samples were recorded by a Beckman liquid scintillation counter. Counting errors were 5% or less. Beckman

cocktail D scintillation fluid consisting of 5 gm of diphenyloxazole (Beckman), 100 gm of naphthalene (Beckman), and dioxane (Fisher) to make one liter was used for these determinations. Ten ml of scintillation fluor was mixed with 0.5 ml of each sample.

Deoxyribonucleic Acid (DNA) Determination

The method of Colter et al. (9) was used for purification of DNA. Quantitative estimates of the amount of DNA were determined spectrophotometrically by the method of Burton (7) using calf thymus DNA (Calbiochem) as the standard. Absorbance was recorded by an Hatachi 124 double beam spectrophotometer.

Thymidine Kinase Assay

A radioactive assay method was used for the determination of thymidine kinase activity. This method was essentially that of Ledinko (40). Thymidine phosphates were separated from thymidine by descending paper chromatography on Whatman No. 1 paper using an aqueous mixture of isopropanol and ammonia as the solvent (8). Five microliter samples of the reaction mixture (3.3 uCi of ^{3}H-thymidine per milliliter of reaction mixture) were placed on the paper. Radioactive spots corresponding to that of TMP (thymidine-5'-monophosphoric acid, Sigma) were cut from the paper and were placed directly into scintillation vials for counting. Ten ml of scintillation counting fluid covered each paper sample.

Protein Determination

Quantitative estimates of the amount of protein were determined by the method of Oyama and Eagle (50) using crystalline bovine serium albumin (Calbiochem) as the standard. Absorbance was recorded by an Hatachi 124 double beam spectrophotometer.

Sucrose-Gradient Analysis of Cellular DNA

The method of Colter (9) was used to isolate DNA from control and infected (300 PFU/cell) cells 8 hours after infection. Prior to infection, asychronous spinner cells (2.71×10^5 cells/ml) were labeled with 0.5 uc/ml ^{3}H-thymidine for 40 hours. DNA isolated from control and infected cultures was dissolved in 2 ml of 0.2M potassium phosphate buffer, pH 7.0. One-tenth ml of each sample (60,000-80,000 cpm) was sedimented through a 5 ml linear sucrose gradient (5-20% sucrose) by centrifugation at 39,000 rpm in the SW 39 rotor (Beckman, Spinco Division). Cell DNA was sedimented at 18° C for 4 hours. After centrifugation, 7 drop fractions of each gradient were collected directly in scintillation counting vials and the cpm of each fraction was determined. DNA from control and infected cultures was analyzed on pH 8 and pH 11.5 sucrose gradients.

CHAPTER III

RESULTS

Preliminary Investigations

Initially, this investigation was concerned with an attempt to isolate a DNA inhibitory substance(s) from infected cells and to add this inhibitor back to uninfected cells. Since the inhibition of cellular DNA synthesis did not occur until 8 hours after infection (21, 37), inhibitor isolation was attempted after this time in the hope of obtaining it in sufficient quantities. It was reasoned that if an inhibitor were isolated and added back to uninfected cells, inhibition of DNA synthesis would occur earlier than 8 hours. It was assumed that uninfected cells would readily take up the inhibitor.

When crude cell extracts were prepared from infected cells and added back to uninfected cells, inhibition of cellular DNA synthesis was detected as early as 3 hours after addition of the extract. However, when the extract was assayed for infectious reovirus, it contained approximately 250 plaque forming units (PFU) for each cell in the assay culture. Only 10 PFU/cell were used to infect the culture from which the inhibitor was isolated, and

8 hours elapsed before cellular DNA synthesis started to be inhibited. Two possible explanations were immediately apparent: Either the multiplicity of infection directly affected the time of inhibition of DNA synthesis or earlier inhibition of DNA synthesis was due to the presence of the isolated inhibitor, and the presence of infectious virus in the extract was coincidental.

To test these possibilities, an aliquot of the cell extract was centrifuged at 130,000 x g (gravity) for 30 minutes. At this centrifugal force reovirus would be pelleted. After centrifugation, the supernatant was assayed in uninfected cells by measuring DNA synthesis. No inhibition was detected as late as 8 hours after addition of the extract. It appeared, therefore, that reovirus and the "inhibitor" were the same. To confirm these findings it was necessary to determine the effect of increasing multiplicities of infection on the time of inhibition of DNA synthesis.

Effect of 10, 10^2, and 10^3 PFU/Cell on DNA Synthesis

One spinner culture was divided into four separate cultures. One served as an uninfected control; the other three cultures were infected with purified reovirus, one with 10 PFU/cell, one with 10^2 PFU/cell, and one with 10^3 PFU/cell. At various times after infection (time 0), aliquots were removed from each culture and were pulse-labeled with ^{3}H-thymidine. Counts per minute (cpm) in the

TCA-insoluble material per ml of culture at the specified times after infection were recorded as shown in figure 1.

It was apparent that the multiplicity of infection not only affected the time of inhibition of cellular DNA synthesis but also the rate at which the inhibition progressed. At low multiplicities (10 PFU/cell) of infection, inhibition of DNA synthesis did not occur until 6-8 hours after infection. This was in agreement with previous reports (37). At higher multiplicities (10^2 or 10^3 PFU/cell) the time of inhibition occurred as early as 2-4 hours post infection (PI), and the rate at which inhibition occurred increased.

Effect of High Multiplicities of Infection on RNA and Protein Synthesis

In order to determine if early inhibition of DNA synthesis at high multiplicities of infection was a specific event and not a result of suppression of other closely related host cell functions, it was necessary to determine the effects of high virus multiplicities on the synthesis of RNA and protein. Two cultures were used for each experiment. One culture was infected with 250 PFU/cell; the other served as the control. Aliquots were removed from control and infected cultures at selected times after infection (time 0) and were pulse-labeled with ^{14}C-amino acids to label protein in one experiment and ^{3}H-uridine to label RNA in the second experiment.

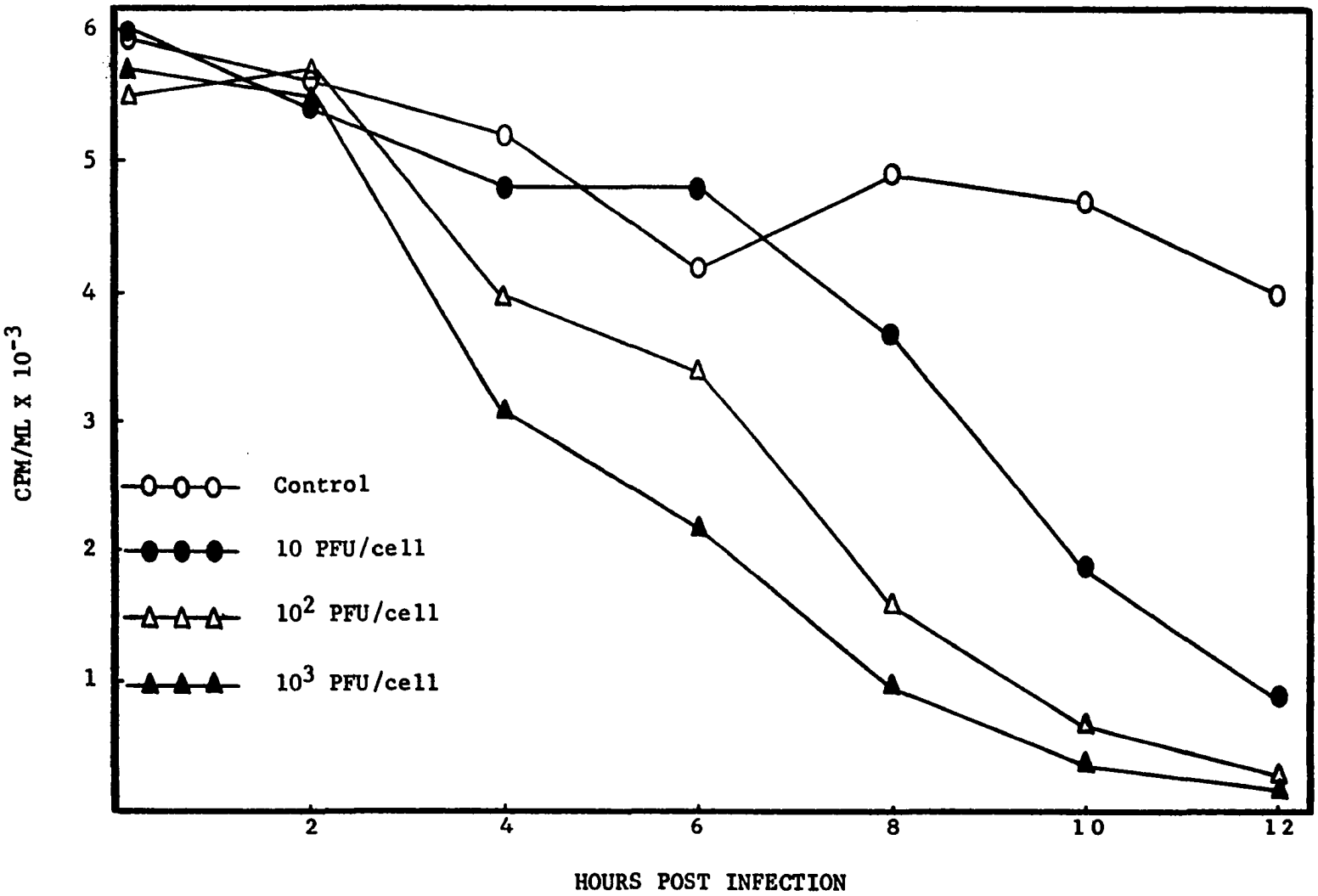

Figure 1. DNA synthesis of spinner cells infected with 10, 10^2, and 10^3 PFU/cell. At the times indicated 1.5×10^6 cells from each culture were pulse-labeled with ^{3}H-thymidine. Counts per minute per ml of culture in the TCA-insoluble material are presented.

Counts per minute per ml in the TCA-insoluble material was recorded at selected time intervals after infection. The results are represented in figures 2 and 3.

There was no decrease in RNA synthesis as late as 10 hours PI, and no significant change in protein synthesis was detected until 6-8 hours PI. These results indicated that high multiplicities of infection resulted in specific inhibition of DNA synthesis in spinner cells.

Virus Yields of Cells Infected with 10 and 250 PFU/Cell

The virus yields of cultures infected with 10 and 250 PFU/cell were compared at selected times after infection to determine if there was any difference between the virus productivity of cells infected with high and low multiplicities. Spinner cells in the logarithmic stage of growth were divided into 2 cultures. One culture was infected with 10 PFU/cell; the second culture with 250 PFU/cell. At selected times after infection, 2 ml aliquots were removed from each culture and were placed in 5 ml sonicating tubes. The cells were sonicated briefly and the cellular debris was then removed by centrifugation at 6000 x g for 1 minute. The supernatants were assayed for infectious virus. The titers (PFU/ml of culture) of each culture at the selected times after infection (time 0) are represented in figure 4.

These results indicated that cells infected with 10 or 250 PFU/cell started producing infectious virus after

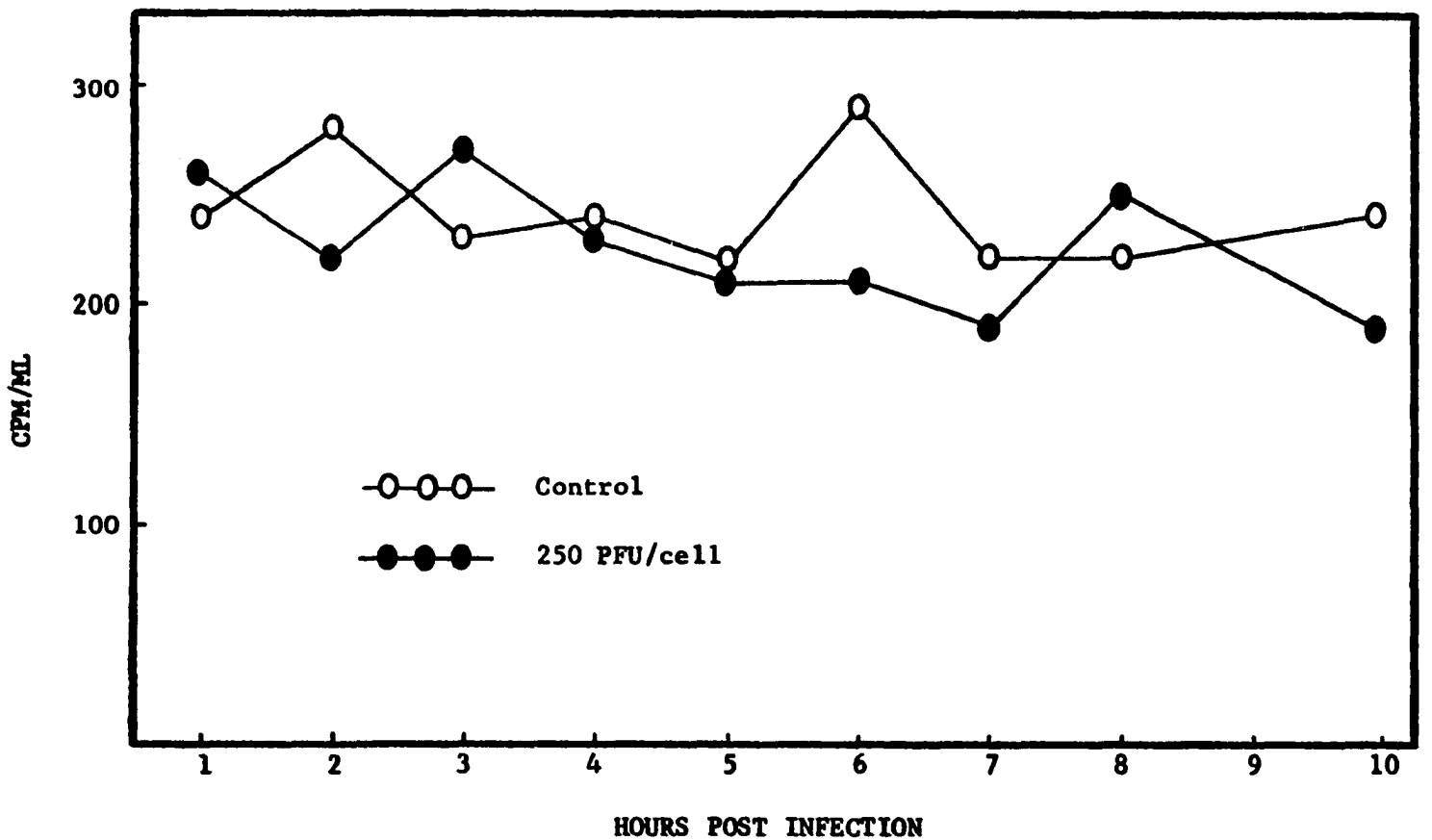

Figure 2. Total RNA synthesis of spinner cells infected with 250 PFU/cell. At the times indicated $3x10^6$ cells from each culture were pulse-labeled with ^{3}H-uridine. Counts per minute per ml of culture in the TCA-insoluble material are presented.

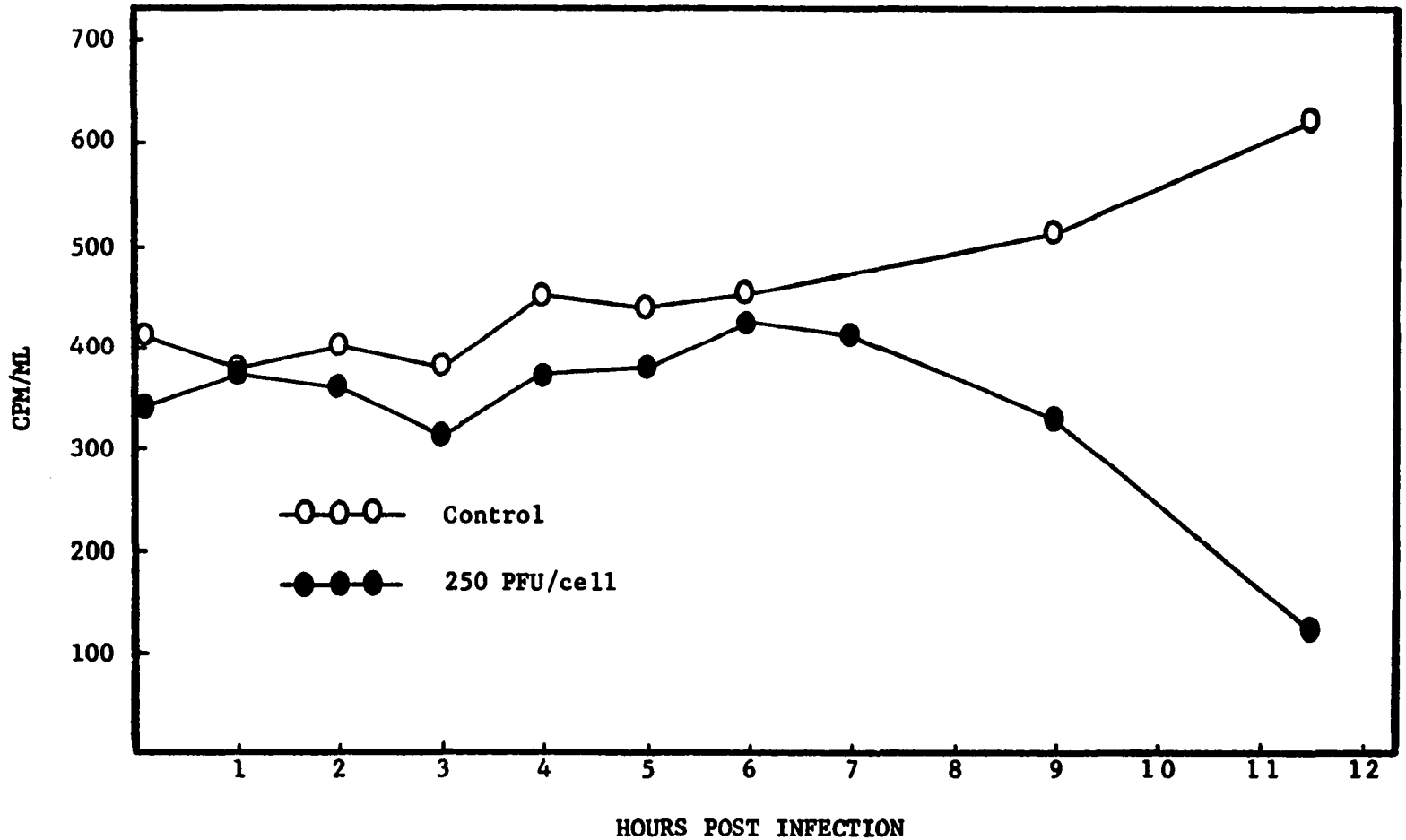

Figure 3. Total protein synthesis of spinner cells infected with 250 PFU/cell. At the times indicated 1.5×10^6 cells from control and infected cultures were pulse-labeled with ^{14}C-amino acids. Counts per minute per ml of culture in the TCA-insoluble material are presented.

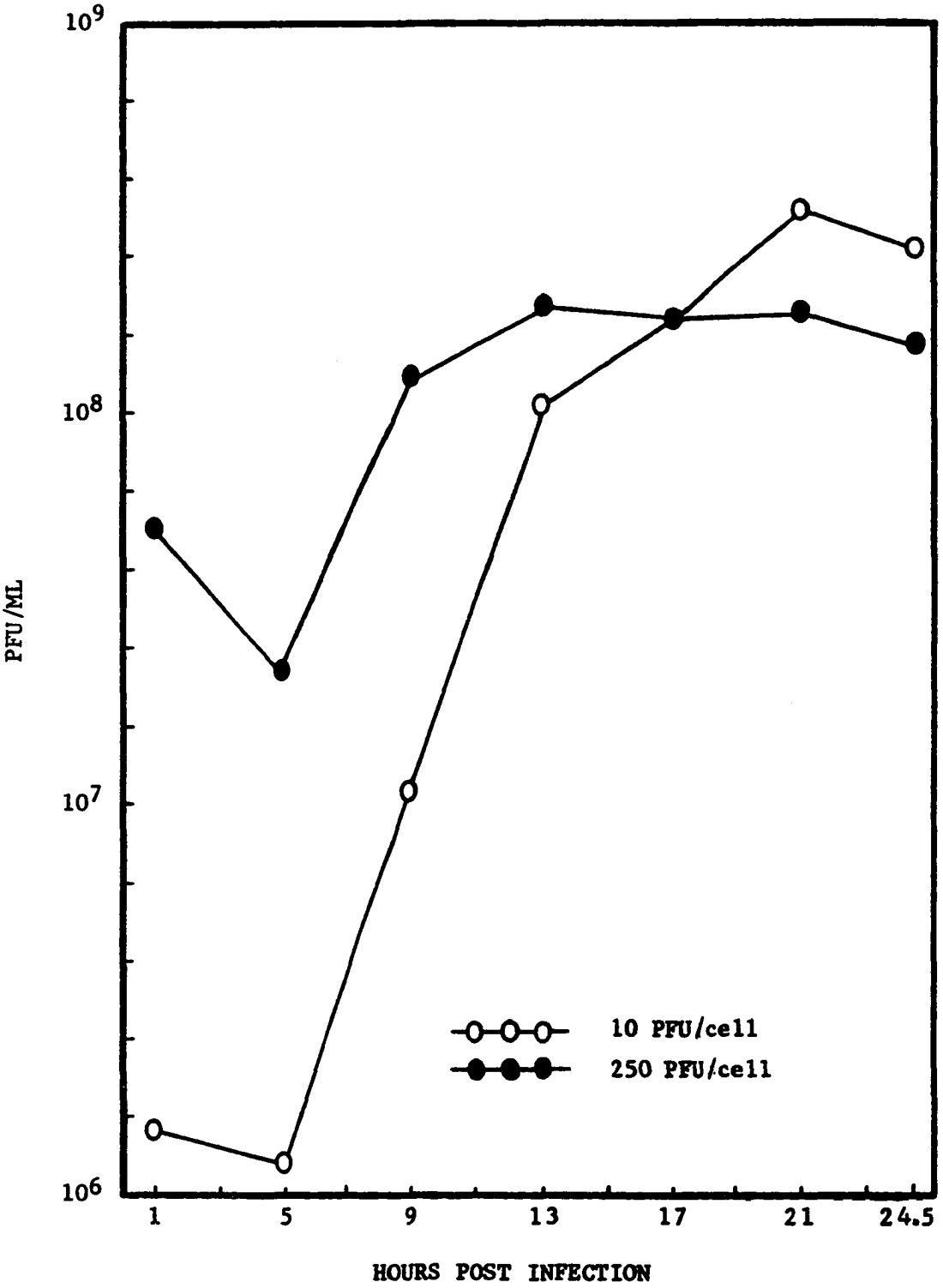

Figure 4. A comparison of the virus yields of spinner cells in cultures ($5x10^5$ cell/ml) infected with 10 and 250 PFU/cell. Titers are expressed as PFU/ml of culture at the times indicated.

5 hours PI. Maximum virus production occurred at 13 hours PI in the culture infected with 250 PFU/cell as compared to 21 hours PI in cells infected with 10 PFU/cell. Approximately equivilant amounts of virus were present in both cultures at the end of the logarithmic phases of virus production. However, 24 hours PI the amount of virus in the culture infected with 10 PFU/cell was two fold greater than the amount of virus in the culture infected with 250 PFU/cell. This experiment suggested that comparable numbers of cells in each culture were infected, and that infectious virus was not formed in either culture before 5 hours PI. At this time (5 hours PI), DNA synthesis was inhibited more than 70% in cultures infected with high multiplicities (250 PFU/cell), and was not inhibited in cultures infected with low multiplicities (10 PFU/cell) until 8 hours PI.

Ultraviolet (UV) Inactivation of Reovirus

Work by others have indicated a definite dose-response of cells to infection with poliovirus (1,28). High multiplicities of poliovirus, under conditions that prevented poliovirus replication (guanidine), resulted in suppression of protein and RNA synthesis of Hela cells (28). The intact virion, at least in the case of adenovirus, was not necessary for suppression of host cell macromolecular synthesis (41). Purified fiber antigen of adenovirus type 5 irreversibly inhibited the

synthesis of DNA, RNA, and protein synthesis in KB cells (41). More pertinent to the present work was the report by Silverstein and Dales (64) who showed that the eclipse period of reovirus-infected L-cells could be reduced from 7 to 4.5 hours by infection with 5 and 100 PFU respectively.

Since the time of inhibition of DNA synthesis was dependent upon the number of infectious reovirus particles infecting a cell, it was of interest to determine if the physical particle alone (noninfectious virus) altered the synthesis of DNA. In order to obtain high particle to PFU ratios, purified suspensions of reovirus were inactivated with UV light.

Figure 5 represents the reduction in infectious titer (PFU/ml) of an aliquot of purified reovirus after exposure to UV light for time periods up to 15 minutes. The initial titer at time 0 (2.5×10^9 PFU/ml) was reduced 10^2 to 10^3 fold after 15 minutes of exposure. The rate of inactivation for the first 6 minutes was greater than the rate of inactivation after this time. The possible significance of this type of inactivation curve has been discussed by McClain and Spendlove (48).

DNA Synthesis by Cells Infected with High Particle to PFU Ratios

In order to determine the effect of the physical particle on the synthesis of DNA, an aliquot of purified reovirus was exposed to UV light for 60 minutes at

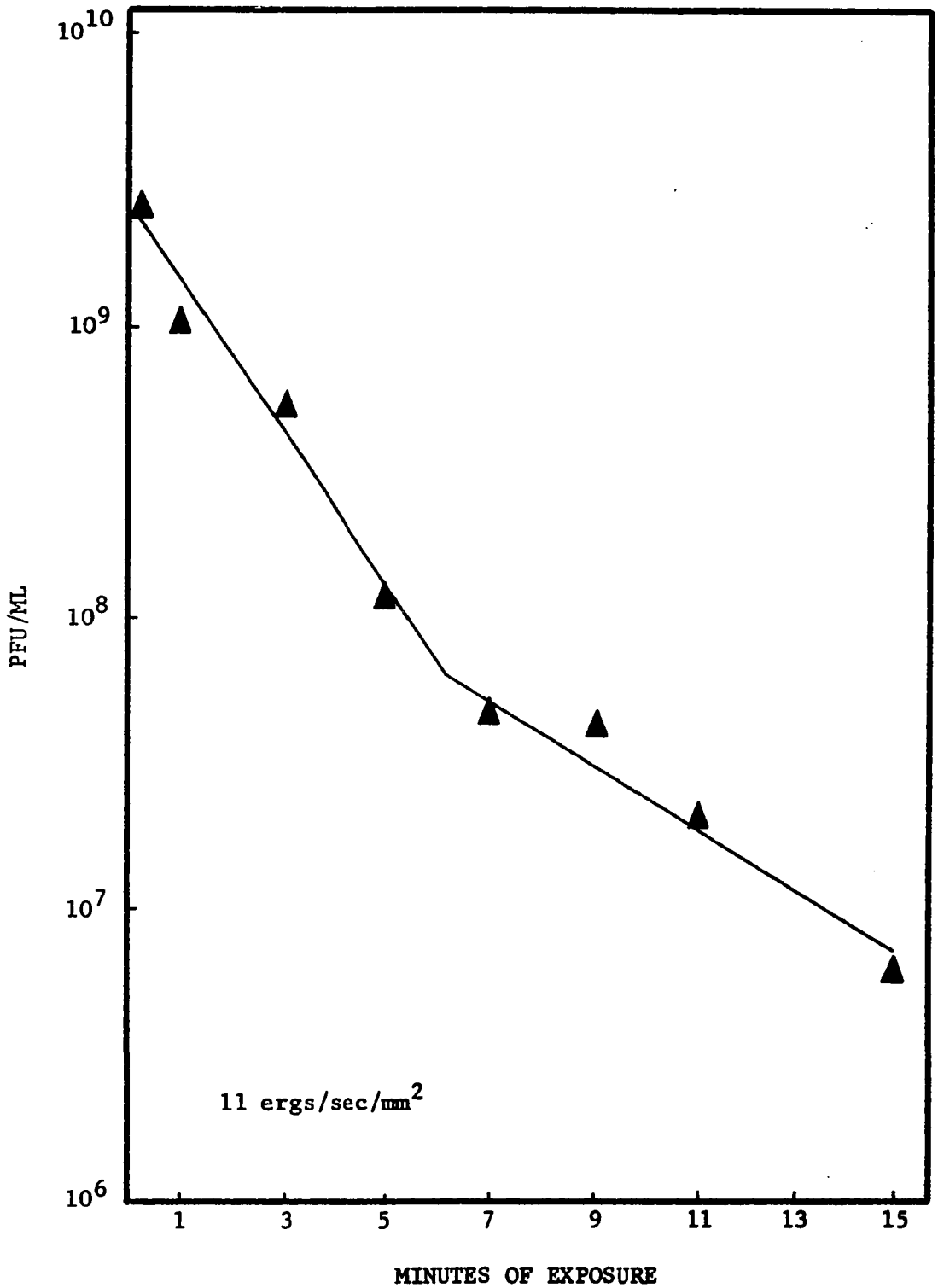

Figure 5. Ultraviolet inactivation of reovirus. Purified suspensions of reovirus in phosphate-buffered saline were exposed to UV light. At the times indicated residual infectious virus in a suspension was determined by plaque assay on L-cell monolayers. Titers are expressed as PFU/ml of suspension.

approximately 11 ergs/sec/mm^2 and was then frozen at -60° C for at least 24 hours prior to use. For the experiment a spinner culture containing cells in the logarithmic stage of growth was divided into 2 cultures. One culture served as the control (uninfected); the other was infected with 250 UV-inactivated particles/cell. At selected times after infection, 5 ml aliquots from control and infected cultures were pulse-labeled with ^{3}H-thymidine. The cpm in the TCA-insoluble material per ml of culture are represented at the selected times after infection in figure 6. The results indicated that high particle to cell ratios are as effective as high PFU to cell ratios in inhibiting DNA synthesis early during the infectious cycle.

Thymidine Pools of Infected and Uninfected Cells

The incorporation of ^{3}H-thymidine into mammalian cells as a measure of DNA synthesis may yield misleading data, particularly if thymidine pool changes occur in cells (66). The following experiment was conducted to determine if high multiplicities of reovirus infection altered the ability of L-cells to maintain a constant pool of exogenously supplied ^{3}H-thymidine.

Fourteen asychronous stationary cultures were used. Seven cultures were infected with 300 PFU/cell at time 0; seven were uninfected and served as controls. At selected times after infection, the cpm in the TCA-soluble and

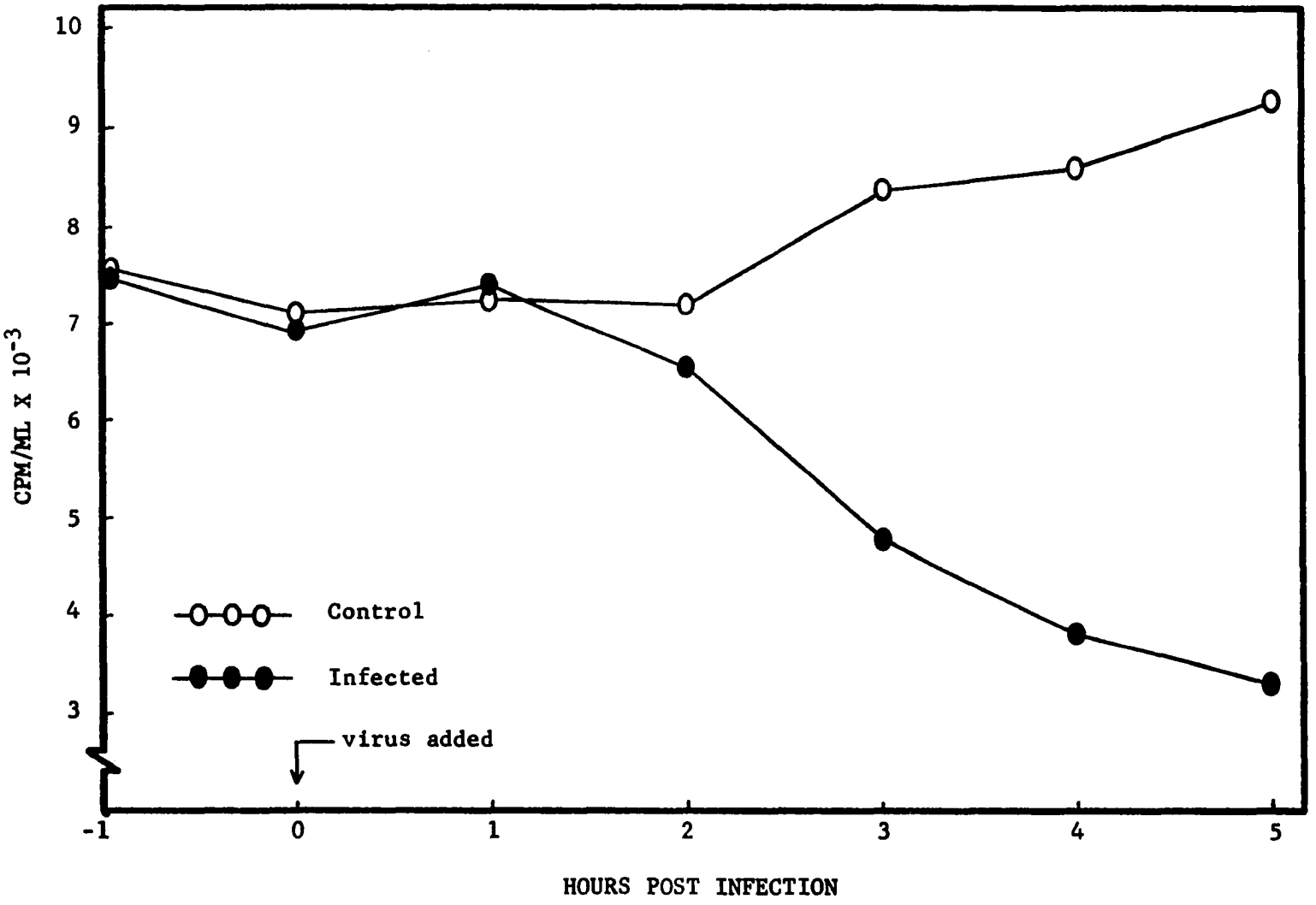

Figure 6. DNA synthesis of spinner cells infected with UV-inactivated virus. Reovirus was inactivated for 1 hour as described in figure 5 and was added to a spinner culture at 250 UV-inactivated particles per cell. At the times indicated 2.7×10^6 cells were removed from control and infected cultures and were pulse-labeled with ^{3}H-thymidine.

insoluble material of infected and control cultures was determined. The results are represented in figure 7. These results indicated that DNA synthesis (TCA-insoluble material) of infected cells started to be inhibited as early as 4-6 hours PI while the cpm in the TCA-soluble material (thymidine pool) remained constant, even as late as 8 hours PI. After 8 hours, however, there was a marked and progressive decrease in the cpm of the TCA-soluble material of infected cells. These results established that inhibition of DNA synthesis in infected cells was not due to thymidine pool changes.

Comparison of Sedimentation Profiles and Acid-Insolubility of DNA from Infected and Uninfected Cells

In order to investigate the possibility that DNA might be rendered acid-soluble following reovirus infection, cellular DNA was heavily labeled by growing spinner cells for 48 hours in a medium containing ^{3}H-thymidine. After the labeling period the cells were washed and divided into two cultures, one of which was infected with approximately 337 PFU/cell. At selected times after infection (time 0) aliquots were removed from infected and control cultures and the cpm in the TCA-soluble and insoluble material of each aliquot was determined. The TCA-soluble counts (expressed as % of the total counts of each aliquot) at selected times after infection are presented in Table 1. There was no difference between the two sets of figures to

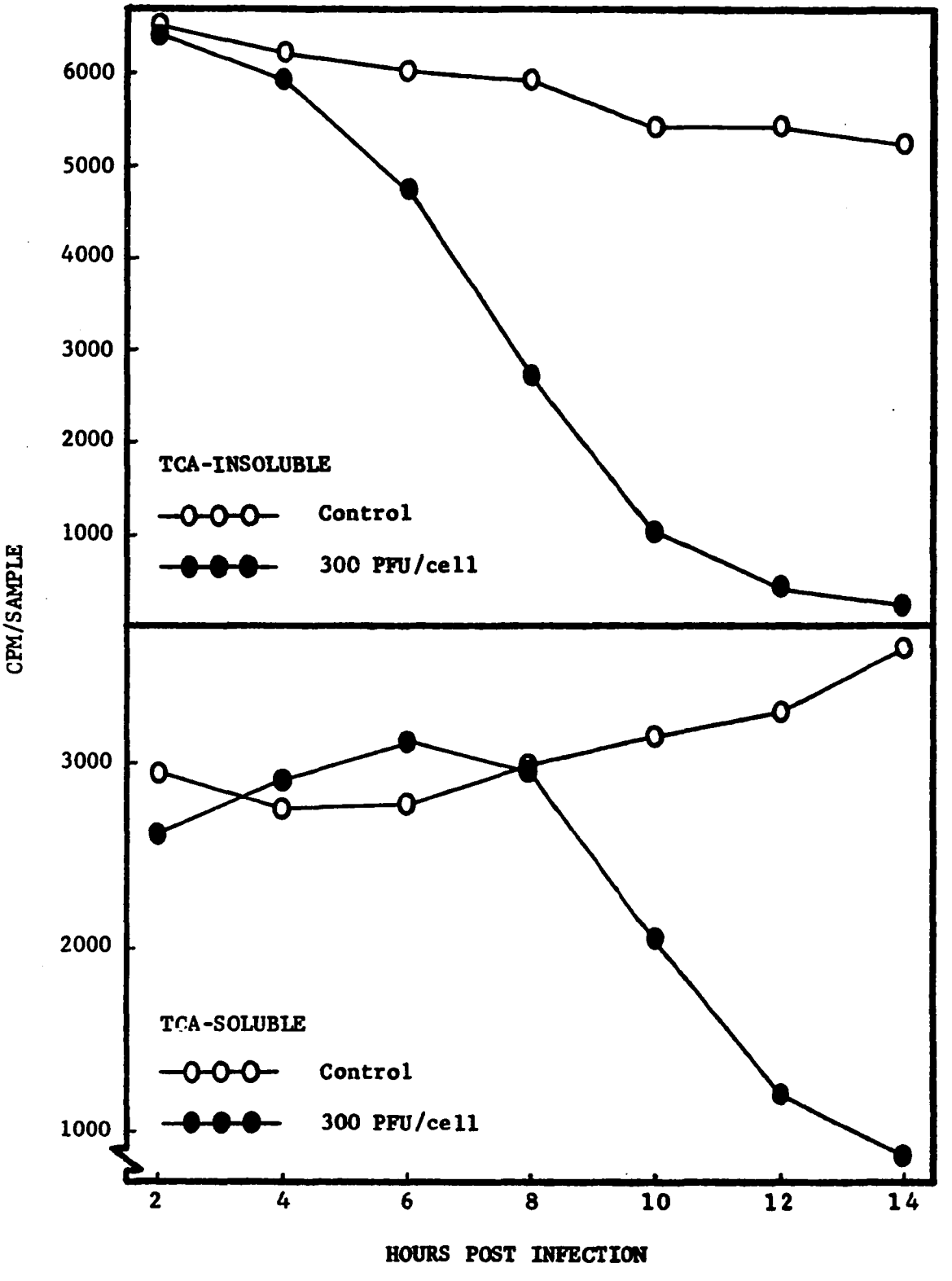

Figure 7. Comparison of the incorporation of ^{3}H-thymidine into the TCA-soluble and insoluble cellular material of control and infected stationary cultures. At the times indicated, samples (6.52×10^{5} cells) from control and infected cultures were pulse-labeled with ^{3}H-thymidine.

Table 1. Percent acid-soluble counts of infected and uninfected spinner cells labeled with ^{3}H-thymidine.*

HOURS POST INFECTION	CONTROL (UNINFECTED)	INFECTED (337 PFU/CELL)
0	0.21	0.23
2	0.17	0.18
4	0.14	0.14
6	0.13	0.13
8	0.10	0.12

*Cellular DNA was labeled with 3.3 uCi/ml of culture medium for 48 hours. Cells were washed free of exogenous isotope and were resuspended in fresh medium. Labeled cells were divided into 2 cultures, one of which was infected at time 0. At the times indicated, samples (5.4×10^{7} cells) were removed from each culture. Counts per minute in the TCA-soluble material relative to the total counts of each sample are presented above.

suggest that DNA was rendered acid soluble. In support of this, no difference was detected between the sedimentation profiles of the heavily labeled DNAs when they were isolated from control and infected cells 8 hours PI and were sedimented on neutral and alkaline linear sucrose gradients (figures 8 and 9). The two plots in each figure were not aligned with markers. Sedimentation was conducted in two tubes in the SW 39 rotor (Beckman Instruments). Drops were collected from the bottom of each tube (6 drops per fraction) and each fraction was measured for radioactivity. These results are supported by a recent publication by Ensminger and Tamm (18).

Thymidine Kinase Activity of Infected Cells

Since thymidine kinase synthesis of L-cells occurs just prior to the synthesis of DNA (44), inhibition of its synthesis or activity in reovirus-infected cells might account for inhibition of DNA synthesis. This possibility, therefore, was investigated. The results, presented in Table 2, suggested that no inhibition of thymidine kinase activity occurred in reovirus infected cells. Comparison of the enzyme activities per cell in control and infected cultures at the selected times after infection suggested that inhibition of thymidine kinase synthesis did not occur. It has been reported recently (18) that DNA polymerase and deoxycytidylate deaminase are also not inhibited in reovirus-infected cells.

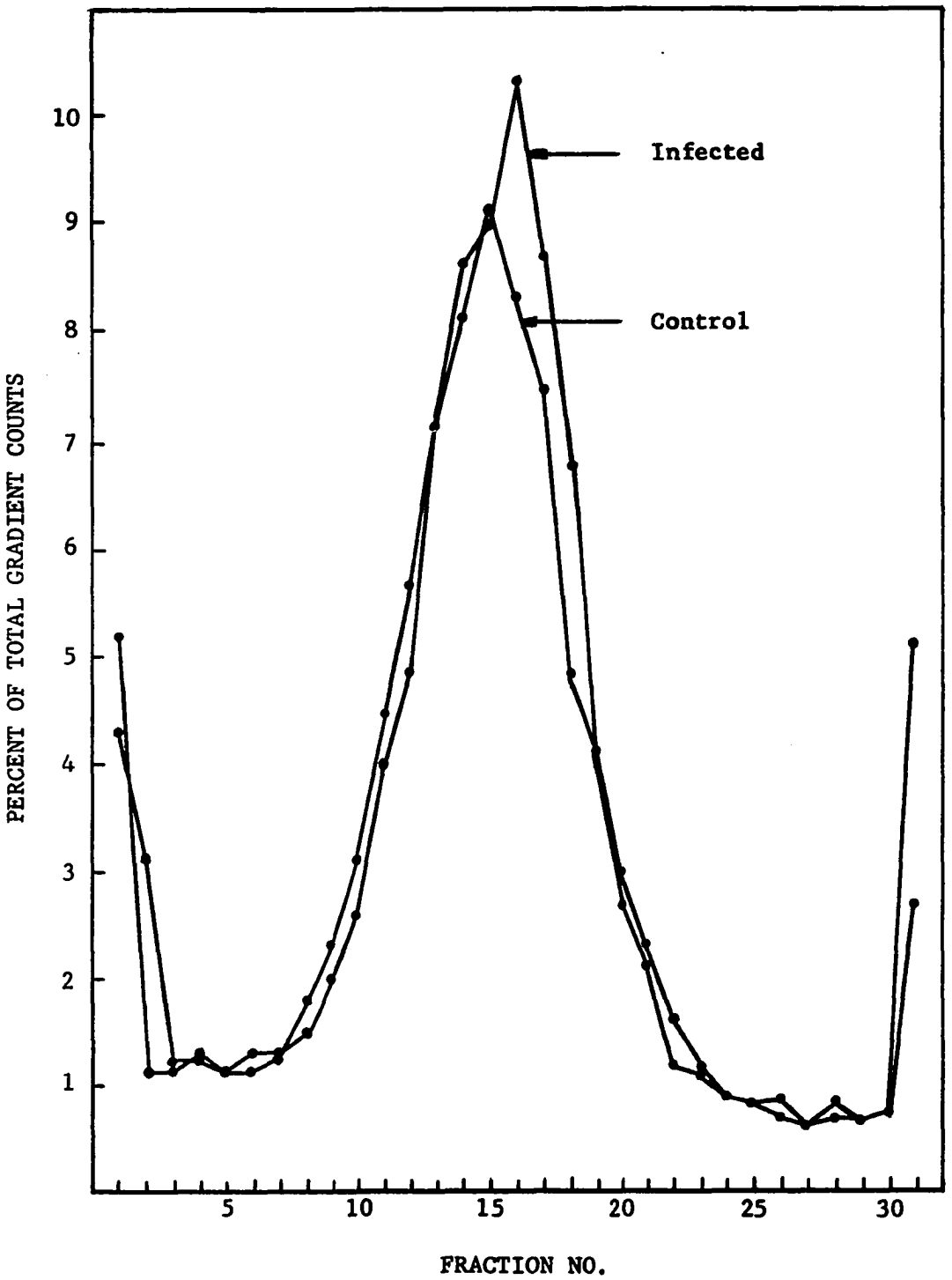

Figure 8. Sedimentation profiles of DNAs isolated from control and infected (300 PFU/cell) spinner cultures 8 hours after infection. One-tenth ml of each sample (60,000 cpm ^{3}H-thymidine) sedimented through a 5-20% sucrose gradient, pH 8.

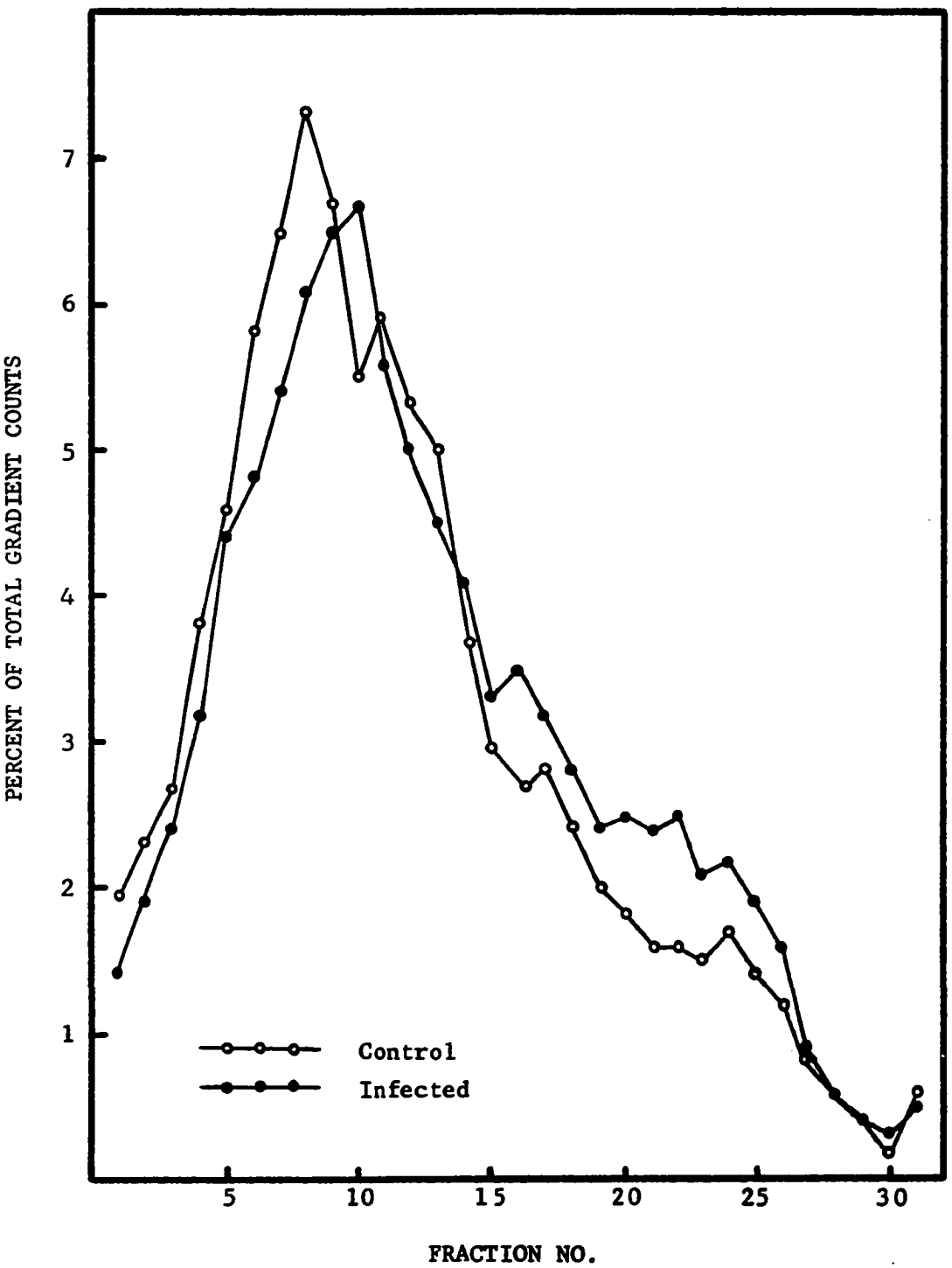

Figure 9. Sedimentation profiles of DNA isolated from control and infected cultures 8 hours after infection. Conditions are the same as figure 8 with the exception that these sucrose gradients were pH 11.5.

Table 2. Specific activity of thymidine kinase in infected (250 PFU/cell) and uninfected spinner cells.*

HOURS POST INFECTION	CONTROL	INFECTED[a]	CONTROL	INFECTED[b]
1	2.4	3.1	26.7	29.5
2	2.2	2.6	26.3	26.6
3	2.2	2.8	25.3	28.7
4	2.2	3.0	26.9	28.1
5	2.3	2.8	26.7	29.8
6	1.9	2.8	28.1	28.9

[a]TdR converted to TdR phosphates/30 minutes/ug cell protein (0.01 pmoles).

[b]Counts/minute of TdR converted to TdR phosphates/30 minutes/cell.

*Thymidine kinase activity was determined by the procedure of Ledinko (Cancer Research, 27, 1159, 1967) and involved the use of ^{3}H-thymidine. Cell protein from 2×10^{6} cells was used to determine each figure above.

Inhibition of DNA Synthesis in Synchronized L-Cells

Gomatos and Tamm (21) using autoradiographic techniques demonstrated that inhibition of cellular DNA synthesis following reovirus infection occurred in cells actively synthesizing DNA. They suggested that the point of initiation of DNA synthesis was not blocked. However, since multiple sites for the initiation of DNA synthesis exist in mammalian chromosomes (67,68), and since DNA synthesis does not occur at the same time at each site, use of asychronous cells to determine inhibition at these sites might yield misleading results. The use of synchronized cultures, however, can be used to determine if inhibition of DNA synthesis occurs early during the S phase of the cell cycle. Figure 10 shows the results of reovirus inhibition of DNA synthesis in a synchronized population of L-cells in spinner culture. Under the conditions of this synchronizing procedure (see materials and methods) uninfected cells begin to synthesize DNA approximately 8 hours after resuspension in fresh medium. DNA synthesis was not initiated at this time in infected cells. Cells in this experiment were infected with 10 PFU/cell at time 0, just after resuspension in fresh medium. Since the period of DNA synthesis in figure 10 corresponded closely to that calculated for the S phase of L-cells, 6-7 hours (43), these results suggested that reovirus infection prevented the initiation of the S phase.

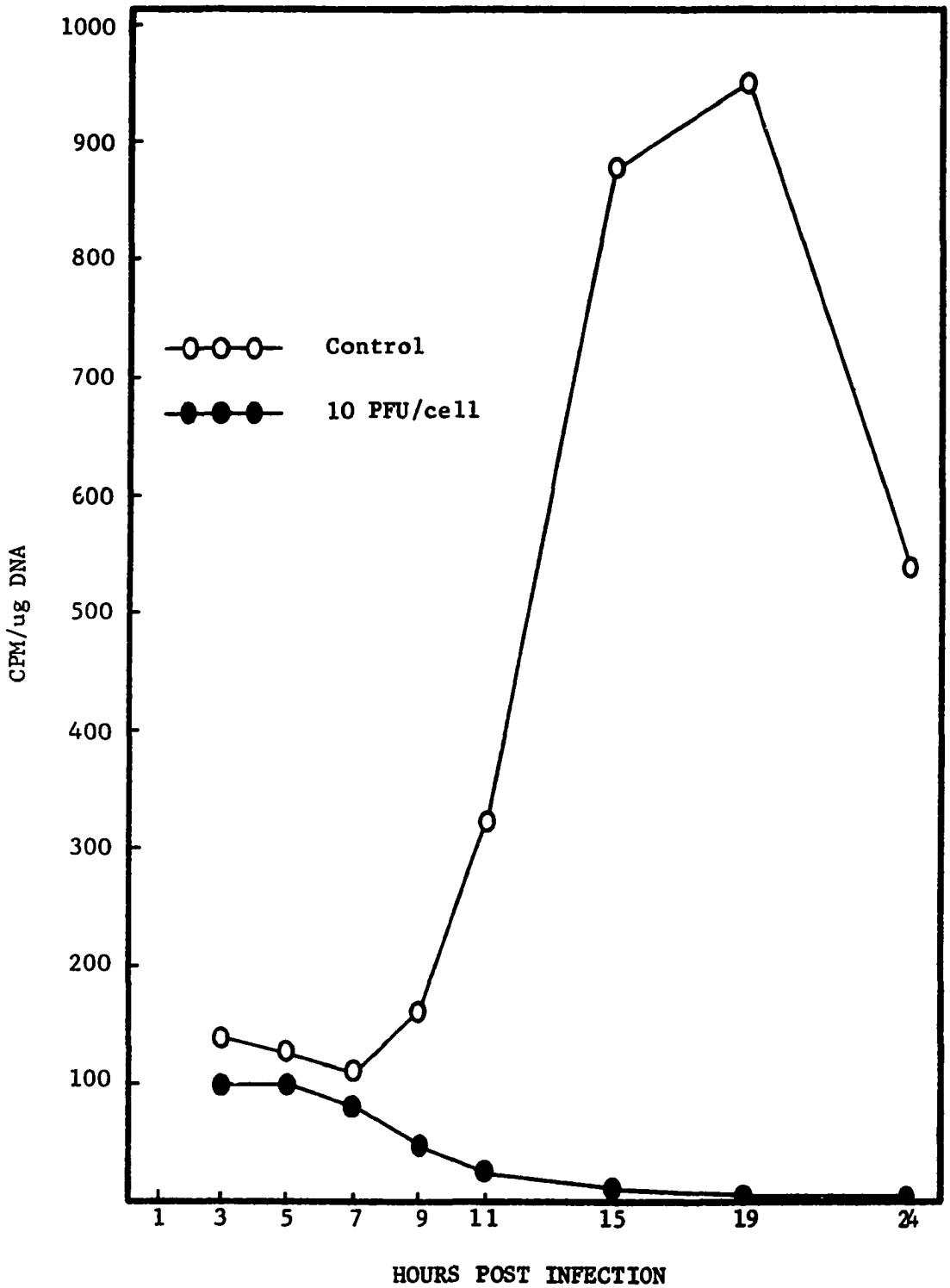

Figure 10. DNA synthesis of synchronized spinner cells. Cells were pulse-labeled with ^{3}H-thymidine at the times indicated. Each point represents cpm/ug of DNA from 2.5×10^{7} cells.

That this is a likely mechanism is supported by recent work by others (18).

CHAPTER IV

DISCUSSION

A preliminary investigation revealed that high multiplicities of reovirus inhibited the synthesis of L-cell DNA as early as 3 hours after infection. Since the time of inhibition was reported (37) to occur at 8 hours after infection with low virus multiplicities, an investigation was conducted to determine the reason for the temporal relationship between reovirus multiplicity and the time of inhibition of DNA synthesis.

The results of this investigation indicated that inhibition at high multiplicities inhibited only the replication of cellular DNA and not its capacity to function as a template for the synthesis of RNA. This suggested that cellular DNA was not degraded by high multiplicities of reovirus. This was supported by the failure to detect any differences between the DNAs from control and infected cells when they were analyzed for acid solubility and for differences in sedimentation through neutral and alkaline sucrose gradients (figures 8 and 9, Table 1).

Since no apparent inhibition of protein synthesis

could be detected in infected cells at 3 hours, the dose response of L-cells to reovirus was a specific event during the infectious cycle, an event that was reflected only by the time of inhibition of DNA synthesis. The reduction in thymidine incorporation (^{3}H-thymidine was used to measure DNA synthesis) at 3 hours was a true reflection of inhibition and not one accountable for by changes in thymidine pools. Cells were not degraded at 3 hours as was shown (figure 4) by their ability to produce infectious virus which appeared in infected cells after 5 hours. Inhibition could not be accounted for by a reduction in thymidine kinase activity or its synthesis (Table 2) nor was it due to the inhibition of deoxycytidylate deaminase or DNA polymerase activities (18). The activities of these enzymes are necessary for cellular replication of DNA.

It is only possible to speculate about the mechanism of inhibition of DNA replication as it occurs in reovirus-infected L-cells. Reovirus probably inhibits the initiation of DNA replication either by preventing infected cells from entering the S or DNA synthetic phase (figure 10) or by inhibiting the synthesis at new DNA initiation sites once cells have entered the S phase (18). The results of this investigation suggest that reovirus virions or virion components (RNA or protein) are responsible for inhibition. The fact that there is a specific dose-response

of L-cells to reovirus (figure 1) and that inhibition occurs even when the activity of reovirus has been destroyed by UV light (figure 6) supports this hypothesis.

This hypothesis accounts for the lack of early inhibition at low multiplicities by suggesting that cellular hydrolysis of low virus multiplicities does not result in the production or release of a sufficient amount of the inhibitor. Inhibition, however, would occur later (8 hours after infection) when a sufficient number of viral components are synthesized by infected cells. In this connection it is interesting that inhibition at low multiplicities occurs at the same time that progeny virus first appears in infected cells, at 8 hours (37).

Inhibition at high multiplicities, according to the hypothesis, would not necessarily require the synthesis of virus-specific components at 3 hours since a sufficient number of viral components would have been supplied to cells by high multiplicities of infection. These components would become available for inhibition following cellular hydrolysis of viral protein coats. This hypothesis would account for the inhibition of DNA synthesis by high multiplicities of UV-inactivated virus. It correlates well with the finding (figure 4) that progeny virus was not present in cells at the time of inhibition at high multiplicites, and that reovirus-specific

RNA synthesis has not been detected in infected cells before 3 hours (37,59,64).

That biologically active, subviral components are capable of inhibiting macromolecular syntheses of host cells is supported by work with other virus-host systems (1,28,41). In this connection, Holland (28) has reported that high multiplicities of poliovirus, under conditions which prevented poliovirus replication (guanidine), inhibited Hela cell macromolecular syntheses. Ackermann et al. (1) have shown that the degree of inhibition of Hela cell DNA synthesis increased with increasing multiplicities of poliovirus. And Levine and Ginsberg (41) have reported that an adenovirus virion component, the penton antigen, could inhibit the synthesis of DNA, RNA, and protein when added to KB cells.

Since reovirus replicates in the cytoplasm and not in the nucleus of cells, it might be difficult to rationalize how virion components inhibit the replication of nuclear genetic material. However, since reovirus is segregated from the rest of the cytoplasm by lysosome-associated inclusion-bodies, and since these inclusions appear in the perinuclear region of the cell, this hypothesis does not seem an unreasonable one. Virion components resulting either from enzymic hydrolysis of high virus multiplicities or by synthesis from low virus multiplicities could reach the nucleus of cells from these inclusions.

Evidence has been presented recently that the nuclear membrane of eukaryotic cells is involved in the initiation of the S phase (DNA synthetic phase) of the cell cycle (10). It is possible, therefore, that virion components act at the nuclear membrane and prevent the initiation of DNA synthesis. It has been suggested also that the perinuclear inclusions might impose adverse effects on the nuclear membrane (18). In either case it would be interesting to determine the rates of DNA synthesis *in vitro* using nuclei from control and infected cells and also from nuclei placed in the presence of reovirus virion components.

Since inhibition appears to be directed only at new initiation sites for DNA synthesis, it is possible that virion component(s) react directly with these sites. This would account not only for the inhibition of initiation but would justify the fact that once DNA synthesis has been initiated, it is not inhibited until that segment of DNA being synthesized is complete.

A virion component that might react directly with the DNA of cells and prevent initiation of its synthesis would be the adenine-rich component of reovirus (3,4, 35,62). Neither its origin nor its function are known. And it is found only in the infected cell. Should the adenine-rich component be translated in infected cells, its product, a lysine rich peptide (polyadenylic acid

codes for the synthesis of polylysine), could conceivably serve an inhibitory function. Certain histones, basic proteins possessing regulatory properties that are complexed to DNA, are rich in lysine.

Although the results of this investigation suggest a direct role of reovirus virions or virion components in inhibiting the synthesis of DNA, it is also possible that a product(s) of translation could account for the inhibition. This is possible in spite of the fact that inhibition occurs even though reovirus has been inactivated by UV light. Although UV light destroyed the plaque forming ability of reovirus, it probably did not abolish all transcription of the reovirus genome. The fact that reovirus contains 10 independent genome segments which are transcribed independently supports this possibility. Also, multiplicity reactivation of the reovirus genome has been suggested (48). It is unlikely, however, that sufficient reactivation occurs to account for the unaltered rate of inhibition occurring 3 hours after infection.

Although the synthesis of virus-specific RNA cannot be detected before 3 hours after infection, it is possible that it does occur at this time but is obscured by the synthesis of cellular RNA (59). Since the length of the latent period of reovirus-infected L-cells can be shortened from 7 to 4.5 hours by infecting cells with 100 instead of 5 PFU/cell (64), it is apparent that synthesis

of reovirus RNA occurs before 4.5 hours after infection. Whether it occurs at 3 hours or before at high multiplicities of infection has not been reported.

If the reovirus genome is transcribed and translated before 3 hours, then infection at high multiplicities would provide each cell with a sufficient number of reovirus genomes such that only a few rounds of both processes (transcription and translation) would be required to complete the synthesis of a large number of virus-specific products (inhibitors).

There would be 25 times as many genomes in cells infected with high multiplicities (250 PFU/cell) as there would be in cells infected with low multiplicities (10 PFU/cell). If the rates of transcription and translation were to be the same in cells infected at both multiplicities, one round of transcription and translation would result in a 25 to 1 ratio of the inhibitor concentrations for cells infected with high and low multiplicities respectively. Therefore, synthesis of a virus-specific product cannot be eliminated as the possible cause of inhibition at 3 hours after infection.

The data presented in this investigation suggest that a virus-specific product need not be synthesized if cells are infected with high multiplicities of reovirus, but that virus-specific syntheses are required for inhibition if cells are infected with low multiplicities of reovirus.

CHAPTER V

SUMMARY

It was reported that reovirus inhibited DNA synthesis of L-cells 8 hours after infection, a time when first progeny virus appeared in infected cells.

This investigation revealed that the time of inhibition of DNA synthesis by reovirus depended on the virus multiplicity of infection. High multiplicities (250 PFU/cell) inhibited DNA synthesis as early as 3 hours after infection, and progeny virus did not appear until after 5 hours. Inhibition occurred at 3 hours even when the plaque forming ability of reovirus had been destroyed by ultraviolet light.

At the time of inhibition at high multiplicities there was no apparent inhibition of overall RNA and protein synthesis. Inhibition of DNA synthesis could not be accounted for by changes in thymidine pools (DNA synthesis was measured by the rate of incorporation of ^{3}H-thymidine), or by a reduction in the activity or synthesis of thymidine kinase. Deoxycytidylate deaminase and DNA polymerase activities were also unaltered as was shown by others.

There was no apparent degradation of DNA of infected

cells even as late as 8 hours when high virus multiplicities were used for infection. When L-cells were synchronized and infected prior to the S-phase of the cell cycle (DNA synthetic phase), they failed to synthesize DNA at a time when control cells entered the S-phase.

It is possible that transcription and translation result in the production of a virus-specific product(s) (inhibitor) before 3 hours when cells are infected with high virus multiplicities. However, these results suggest that reovirus replication is not a necessary requirement for inhibition, and that reovirus virion or virion components prevent the initiation of DNA synthesis when they are supplied to cells in sufficient quantities by high multiplicities of infection.

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