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A COMPARISON OF *IN VITRO* DNA SYNTHESIZING SYSTEMS FROM REOVIRUS INFECTED AND UNINFECTED CELLS

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A COMPARISON OF *IN VITRO* DNA SYNTHESIZING SYSTEMS

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DISSERTATION COMMITTEE

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To Carolyn

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A COMPARISON OF *IN VITRO* DNA SYNTHESIZING SYSTEMS FROM REOVIRUS INFECTED AND UNINFECTED CELLS

CHAPTER I

INTRODUCTION

The study of DNA synthesis in eukaryotic organisms is usually accomplished by measuring a physiological function since methods based on genetic studies, such as those dealing with mutants of bacteria or viruses, are generally not available. In many instances in the study of the mechanisms of DNA synthesis (4,8,9,18) the utilization of sub-cellular systems or isolated enzymes has proved beneficial to the elucidation of mechanisms involved in the DNA synthetic process (8,18,28 41,49,53).

Along with the use of isolated DNA synthetic systems, it was often beneficial to employ viral agents capable of selectively altering various cellular physiological processes in order to use this alteration to elucidate normal cellular control mechanisms (2,29).

Many problems may arise in the study of viruses as agents capable of selecting modifying host macromolecular synthesis. In many cases the alteration may not be specific for a particular macromolecular function (i.e., DNA synthesis) but may be secondary (16). The viral replicative cycle may be such that cell degeneration closely follows the

desired effect (45). The desired agent should be one which, while being specific for a particular function, also has a replicative cycle which allows one to observe the effect and if needed manipulate the cells as desired. An example of this would be the use of sub-cellular systems from the infected cells (1,53,56).

Reovirus, a double stranded RNA virus, appears to be an agent which has the desired specificity for a particular cellular function, the inhibition of DNA synthesis (12,14) and a growth cycle which enables one to study this process (19). As one of the first detectable events, reovirus infected cells exhibit the specific inhibition of cellular DNA synthesis (11,12,19). Gross cellular protein and RNA synthesis do not seem to be affected until later in the infective process (14,19,46). This inhibition occurs eight (8) hours post infection with low multiplicities of infection (19) allowing time to study the inhibitory process before the onset of viral induced cell cytopathology.

Previous work had shown that inhibition of DNA synthesis by reovirus was dose dependent (11,46), with higher multiplicities of infection shortening the time between infection and the onset of the inhibition of DNA synthesis. This dose dependent effect was unaffected when ultraviolet light was used to reduce infectivity (11,46). The inhibition could also be mediated by certain "A-Rich" components of the virion (22). Recent work with reovirus sub-viral particles indicates that a specific viral replication event may be needed to mediate this inhibition (13).

Data from our laboratory (12), as well as others (23), pointed to the specificity of inhibition for the initiation step of DNA synthesis.

The use of various sub-cellular DNA synthetic systems from infected cells, therefore, may further characterize the inhibitory process and possibly define the step in replication where the inhibition occurred. The systems utilized in these experiments included the assay of DNA polymerase activity. The rational in employing an in vitro DNA synthesizing system, utilizing isolated DNA polymerase, was based on the fact mentioned above that inhibition studies indicated inhibition of DNA synthesis by reovirus seemed to be specific for the initiation step (12,23). We considered that a possible mechanism for this inhibitory process could be mediated through a decrease in the activity of cellular DNA polymerases. Many workers recently have shown the presence of two (2) general classes of polymerase, cytoplasmic and nuclear (4,8,20,54) and that there seems to be a possible relationship between them (6,24,47). The polymerases also appear to show some variability with the cell cycle (7,8,51).

Several other systems also have been studied. These systems offered a more complete view of the possible inhibitory process and also offered a better opportunity to duplicate the inhibitory process in vitro. The systems included isolated nuclei, chromatin, and hypotonically swelled cells. All of these systems have been shown to be able to simulate DNA synthesis in vivo. Along with DNA synthetic systems, a second system closely associated with the cells ability to carry out DNA synthesis has been studied (10,49). The system measures the ability of isolated nuclei to carry out the adenosine diphosphoribosylation of nuclear proteins through the transfer of the adenosine diphosphoribose moeity of NAD to the nuclear proteins. This process has been shown

possibly to be related to the ability or lack of ability of the cell to carry out DNA synthesis. To our knowledge this was the first correlation of viral infection and Poly (ADP) polymerase activity.

The experiments described in this work detail the results observed concerning the inhibitory process of reovirus utilizing these systems. They also describe our results concerning the functioning of these cellular components in uninfected cells.

CHAPTER II

METHODS AND MATERIALS

Cells

Mouse fibroblasts, strain L929 (42) (Flow Laboratories), adapted for growth in spinner culture, were used in all experiments. L-cells (L929) were cultured in Minimal Essential Medium (MEM, Grand Island Biological Company) supplemented with 5-10% (v/v) heat inactivated fetal calf serum (FCS, Grand Island Biological Company). L-cells were maintained in logarithmic growth by dilution with fresh prewarmed growth medium every 48 hours to a cell concentration 0.5 to 1 X 10^5 cells/ml.

Virus

Reovirus type 3, kindly provided by Dr. P. J. Gomatos, was used in the study. Plaque-purified virus was prepared by removing a large, well isolated plaque with a pipette. The agar plug containing the virus was eluted for 24 hours at 4C in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.5 (SSC). The eluted virus was placed on one confluent L-cell monolayer culture (25 sq. cm surface area) and incubated at 37C for 36 hours. The infected cells were removed by sonication. This was considered one passage and was repeated by infecting 10 confluent L-cell monolayer cultures with the lysate from passage one.

Virus stocks for purification (passage 3-5) were prepared by concentrating cells to 10^7 cells/ml in MEM without serum. The inoculum, 10-20 plaque forming units pfu/cell, was added to the concentrated cells and allowed to adsorb for 1 hour at 34C. The cells were then diluted to 10^6 cells/ml with MEM supplemented with 5% (v/v) FCS. After 24 hours at 34C the cultures were chilled, and the cells and virus harvested by centrifugation at 5000 x g for 15 minutes. The pellets were resuspended for purification.

Virus purification

Reovirus was purified according to a modified procedure of Smith et al. (48). When the final aqueous phase was separated, it was layered over a 10 ml CsCl density gradient (1.2 g/ml-1.4 g/ml) in a 38 ml centrifuge tube. Centrifugation was for 1 hour and 76,600 x g in the SW 27 rotor using a Beckman L2-50. The virus band at a density of 1.37 g/ml was collected. The CsCl was removed by exhaustive dialysis against cold SSC or pelleting the virus for 1 hour at 82,500 x g in the SW 27 rotor. The virus was layered on a 20-40% (w/w) linear sucrose gradient and centrifuged for 1 hour at 82,500 x g in the SW 27 rotor. The virus band was collected and dialysed exhaustively against cold SSC. This was a purified virus stock suspension which was stored at 4C until used.

Virus assay

Reovirus was titered on confluent L-cell monolayers as previously described (46). Spinner cells were added to small petri plates (60 x 15 mm, Falcon Plastics) at a concentration of 3.2×10^6 cells/plate. After 1 to 4 hours at 39C the cells had attached, the medium was removed, and

0.1 ml of each virus dilution was pipetted into the center of each cell sheet. The cultures were incubated for 1 hour at 39C in an atmosphere of 5% (v/v) CO_2 in air. Five ml of autoclavable MEM (Auto Pow, Flow Laboratories) containing 3% (v/v) FCS and 1% (w/v) agar (CoLab) was added and allowed to solidify. The plates were incubated at 39C in an atmosphere of 5% (v/v) CO_2 in air for 72 hours. At this time a second overlay containing 0.005% (w/v) neutral red was added. An additional 24 hours incubation was required before the plaques were visible.

Asynchronous cell growth

To maintain asynchronous cell growth and achieve higher cell density than stock cultures, the cells were centrifuged at 200 x g for 5 minutes and resuspended in fresh prewarmed MEM supplemented with 5% (v/v) FCS every 48 hours.

Synchronous cell growth

Cells were synchronized by serum starvation using a modification of the procedure of Littlefield (35). Cells were centrifuged and resuspended in fresh prewarmed MEM supplemented with 10% FCS at a density of 5×10^5 cells/ml and recycled every 50 hours. To compensate for pH changes caused by the high cell density the flask was not sealed and the sodium bicarbonate concentration was reduced to 1.6 g/liter.

Isolation of DNA polymerase

For analysis of enzyme content the procedure utilized was essentially that of Chang et al. (8). Samples containing 2-5 x 10^8 cells were washed with 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 50 mM

Tris · HCl (pH 7.5) (Buffer A). The cells were then homogenized in buffer with a glass homogenizer fitted with a teflon pestle. As an alternative procedure cells were swelled in low ionic strength buffer, 1 mM Tris · HCl (pH 8.0), 1 mM EDTA, for 15 minutes and lysed in a Dounce homogenizer by 25 strokes from a tight fitting pestle and brought to 0.25 M with respect to sucrose. Cell breakage, checked microscopically was greater than 95%. The homogenate was centrifuged in a Sorvall RCB-2, at 2,000 RPM for 10 minutes and the supernatant fraction (cytoplasmic fraction) was saved. The nuclear fraction was resuspended in buffer A containing 0.5% Triton X100 (v/v). The suspension was allowed to stand for 15 minutes at 4C, and the nuclei were collected by centrifugation. The nuclei were washed 3 times by centrifugation and resuspended in buffer A without Triton X100. The final nuclear pellet was resuspended in buffer A, brought to 0.2 M in potassium phosphate (pH 7.5) and allowed to extract for 30 minutes at 4C. The nuclear extract and cytoplasmic fraction were clarified at 40,000 RPM in the SW 50.1 rotor for 90 minutes in the Beckman L2-50. The cytoplasmic fraction and nuclear extracts were then dialyzed against 0.1 M potassium phosphate (pH 7.5) for 2 to 3 hours. The cytoplasmic fractions and nuclear extract were then ready for sucrose gradient analysis.

DNA polymerase assay

DNA polymerase assays were carried out with activated Salmon Sperm DNA (14) as a template. A 20 ul portion from each gradient fraction was mixed with 100 ul of a mixture containing 50 ug of activated DNA. 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 mM Tris · HCl (pH 7.4) (for

cytoplasmic polymerase) and (pH 8.8) (for nuclear polymerase),).1 mM each of dATP, dCTP, and dGTP, and 2 uCi/ml ^{3}H-dTTP specific activity (50.4 Ci/mM). After 15 minutes incubation at 37C, 50 ul of the reaction mixture was transferred to a Whatman #3 filter disc. The discs were then processed according to the procedure of Bollum (3).

Isolation and assay of nuclei

Cells were pelleted, swollen in a hypotonic buffer, 1 mM Tris · HCl (pH 8.0), 1 mM EDTA and lysed with 25 strokes in a Dounce homogenizer utilizing a tight fitting pestle. The breakage was checked microscopically and was greater than 95%. The nuclei were pelleted from the homogenate. The soluble protein fraction (CF) was prepared from the supernate by ultracentrifugation (100,000 x g for 1 hour at 0C). CF was stored at -70C until used in nuclear assays. The nuclei were resuspended in a nuclei wash buffer, 60 mM Tris · HCl (pH 8.0), containing 5 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM EDTA, 11 mM glucose and 60 mM NaCl.

The nuclei suspended in the wash buffer were distributed to assay tubes. The tubes were centrifuged at 800 x g to pellet the nuclei and the supernate wash solution was decanted.

In a typical assay of DNA replicase activity 0.4 ml of either cytoplasmic fraction or reaction buffer as indicated was added to each tube followed by 0.2 ml of the standard assay mixture. The final concentrations of the components in the basic assay system were 9 mM $MgCl_2$, 1 mM EDTA, 2 mM 2-mercaptoethanol, 70 mM glucose, 5 mM potassium phosphate, 70 mM NaCl, 70 mM Tris · HCl (pH 7.9), 5 mM ATP, and 0.5 mM each dATP, dCTP, dGTP, and ^{3}H-TTP specific activity (50.4 Ci/mM). The nuclei were

resuspended with a pasteur-pipet and incubated at 37C for the indicated times.

To measure the amount of radioactivity incorporated into DNA, the reaction was stopped by the addition of an equal amount of 0.8 M perchloric acid and 0.01 M sodium pyrophosphate. The samples were allowed to precipitate overnight and the precipitate collected by centrifugation. The pellet was washed 3-4 times by suspension and centrifugation in 0.4 M perchloric acid and 0.005 M sodium pyrophosphate in a GLC-1 centrifuge at 1000 x g.

The precipitate was suspended in 0.5 ml of 10% TCA (w/v) and hydrolyzed in a water bath at 100C for 1 hour. The contents of the tube and a 0.5 ml water rinse were transferred to scintillation vials containing 10 ml of Beckman Cocktail D. The radioactivity was measured in a Beckman DPM-100 liquid scintillation counter.

Isolation and assay of chromatin

For the isolation of chromatin the cells were processed and nuclei isolated as described above. The nuclei were washed once with a mixture of 0.25 M sucrose, 5 mM Tris · HCl (pH 8.0), 3 mM $CaCl_2$, and 1 mM EDTA. Chromatin was prepared by washing the nuclei with the media in the following order: 1.) 0.075 M NaCl, 25 mM EDTA (pH 8.0); 2.) 50 mM Tris · HCl (pH 8.0) 1 mM EDTA; 3.) 10 mM Tris · HCl (pH 8.0), 1 mM EDTA twice; 4.) 1 mM Tris · HCl (pH 8.0), 1 mM EDTA.

The crude chromatin fraction obtained by washing was resuspended in medium (4) and sheared by passing twice through a 21 g hypodermic needle. The suspension was placed on top of a medium containing 1.7 M

sucrose, 10 mM Tris · HCl (pH 8.0), and 1 mM EDTA and centrifuged at 27,000 RPM in a SW 27 rotor in a Beckman L2-50 centrifuge for 1 hour.

The clear gelatinous pellet was suspended in the standard reaction mixture for the assay of DNA polymerase activity. The standard reaction mixture contained 30 mM Tris · HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM KCl, 2 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM ATP and 20 uM each dATP, dGTP, dCTP and ^{3}H-TTP specific activity (50.4 Ci/mM).

The mixture was incubated at 37C for the appropriate time and processed as described above for nuclei.

Isolation of nuclei and assay for Poly (ADP) polymerase activity

The nuclei for the assay of Poly (ADP) polymerase activity were isolated and washed as described above for preparation of nuclei for DNA synthesis.

The nuclei were resuspended in a standard reaction mixture composed of 40 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol containing ^{3}H-NAD specific activity (3.46 Ci/mM) to which non-radioactive NAD was added to give a specific activity of 2.0 u Ci/u mole. The mixture was incubated at 25C for 15 minutes and processed as described above for nuclei and chromatin.

Cellular DNA synthesis assay

Asynchronous or synchronous cells were centrifuged at 250 x g for 5 minutes and resuspended in fresh prewarmed MEM supplemented with 5% FCS and 100 ug/ml kanamycin. After resuspension to a final concentration of 5 x 10^5 cells/ml the cultures were incubated 4-10 hours before the culture was split and the experiment begun. In each experiment an

uninfected control culture and a virus infected culture were sampled identically. Duplicate samples (approximately 10^6 cells) were pulse labeled with ^{3}H-thymidine specific activity (19.9 Ci/mM) at 0.5 uCi/ml for 30 minutes at 37C. Labeling was stopped with the addition of an equal volume of cold 10% (w/v) trichloroacetic acid (TCA). At least 1 hour was allowed for precipitation at 4C and then each sample was washed with 5 ml cold (w/v) TCA by centrifugating at 1000 x g for 10 minutes. After 3 washings with 5% TCA and 1 washing with 95% ethanol, the samples were allowed to dry. Each sample was resuspended in 1 ml 10% TCA and hydrolysed for 1 hour in a 95C water bath. An aliquot of each sample was counted in a Beckman DPM-100 liquid scintillation spectrophotometer using Beckman Cocktail D (5g PPO, 100 g naphthalene, 10 ml water, made to 1 liter with 1-4 dioxane) as the fluor.

Cellular protein synthesis assay

Asynchronous or synchronous cells were processed as described above for quantitation DNA synthesis. To measure protein synthesis cells were pulsed with ^{3}H-amino acids specific activity (32 Ci/mM) at 0.5 uCi/ml for 30 minutes at 37C. Labeling was stopped by the addition of ice cold MEM without serum. The cells were collected by centrifugation and precipitated with 5% TCA (w/v). The precipitate was collected on millipore filters and radioactivity was determined by placing the filter disc in 10 ml fluor and counted in a Beckman DPM-100 scintillation spectrophotometer.

Sucrose density gradient centrifugation

For density gradient analysis of DNA polymerase activity,

cytoplasmic fraction and nuclear extracts were layered on a 5-20% (w/v) sucrose gradient containing 0.1 M potassium phosphate (pH 7.5) and 1 mM 2-mercaptoethanol and centrifuged for 16 hours at 40,000 RPM in a SW 50.1 rotor in a Beckman L2-50 ultracentrifuge. The gradients were fractionated on an Isco density gradient fraction collector and the fractions assayed for activity as described above.

Macromolecular quantitation

DNA was determined by the method of Burton (5) using salmon sperm DNA as a standard.

RNA was determined by a method of Mejbaum (37) using yeast RNA as a standard.

Protein was determined by the method of Lowry, et al. (36) or Schacterle and Pollack (36,43) using bovine serum albumin as a standard.

Absorbance was measured using a Beckman DB-G dual beam grating spectrophotometer.

Materials

^{3}H-thymidine (19.9 Ci/mM), ^{3}H-TTP (50.4 Ci/mM), ^{3}H-NAD (3.46 Ci/mM) and ^{3}H-amino acids (32 Ci/mM) were purchased from New England Nuclear. All non-labeled triphosphates and kanamycin were purchased from Sigma Chemical Company.

CHAPTER III

RESULTS

DNA polymerase activity in reovirus infected asynchronous cells

To examine possible variations in the levels of cellular cytoplasmic and nuclear DNA polymerases, the activities of these enzymes were assayed utilizing asynchronous cells. The cells were harvested and the enzymes isolated 12 hours after viral infection with 10-20 pfu/cell. At that time DNA synthesis in the infected culture was less than ten per cent of the non-infected culture, Figure 1.

This isolation procedure monitors two classes of DNA polymerase (8) based upon their cellular location, cytoplasmic or nuclear. The nuclear polymerase, the smaller molecular weight species, has been reported to be the only polymerase found in the nucleus (4,8,20,54). The cytoplasmic polymerase was the predominant form in the cytoplasm and was also found associated with small amounts of nuclear polymerase (4,8,20, 25,54). The results obtained in this study are in agreement with other workers using the L929 (8) and other cell lines (24). We find only one species of polymerase in the nucleus. Activity was found in the cytoplasm indicating the presence of both nuclear and cytoplasmic polymerase. The results of these findings are shown in Figures 2 and 3. It must be made clear that while nuclear polymerase was found in the cytoplasmic

Figure 1. DNA synthesis of asynchronous cells infected with 10-20 pfu/cell of reovirus. Control o———o, infected ●———●. At the times indicated cells from each culture were pulsed with ^{3}H-thymidine. Counts per minute in the TCA-insoluble material are presented.

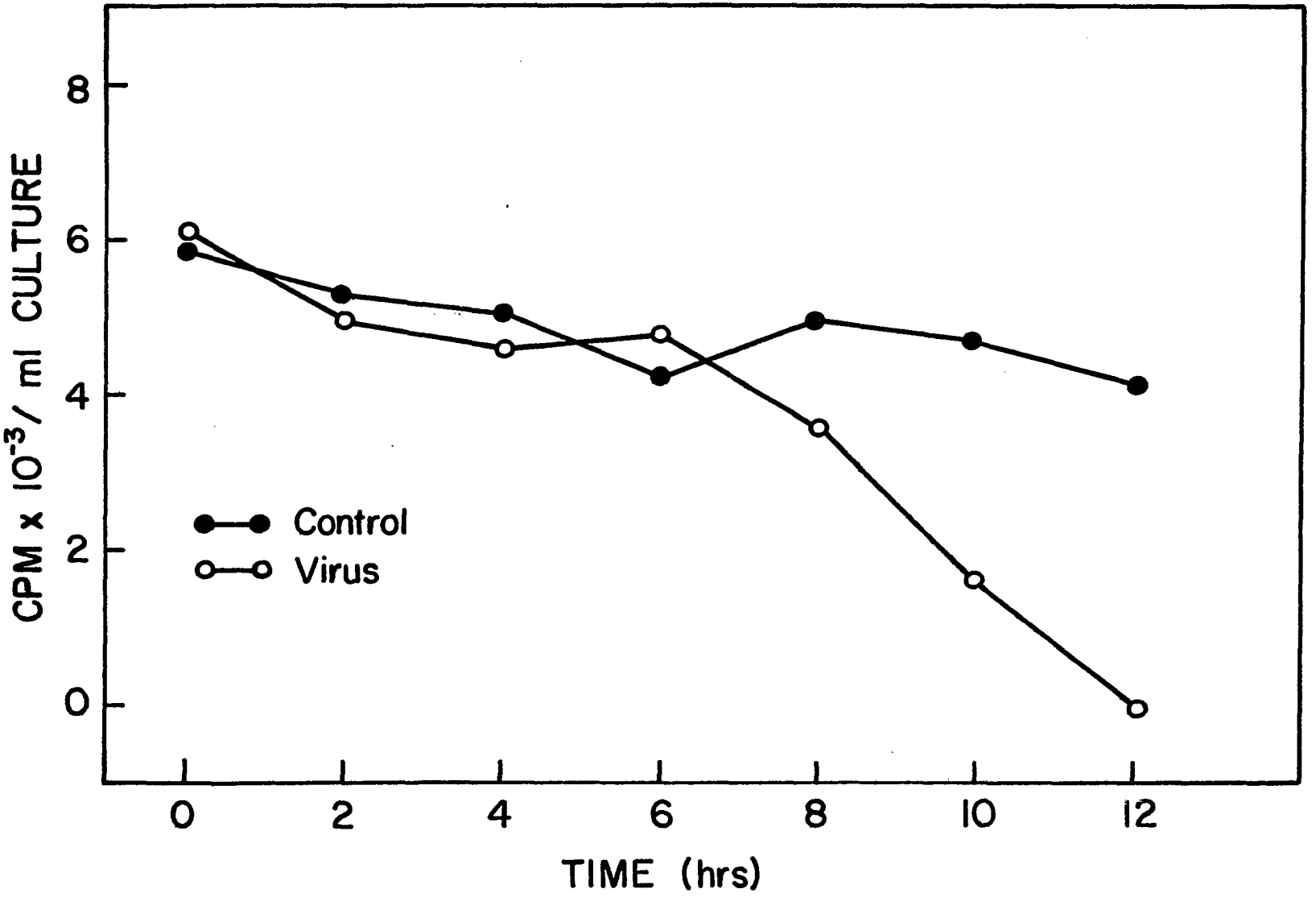

CPM x 10^{-3}/ ml CULTURE
8
6
4
2
0
Control
Virus
0
2
4
6
8
10
12
TIME (hrs)

Figure 2. Levels of nuclear DNA polymerase activity in infected and uninfected L-cells. Nuclear extracts were layered onto linear 5-20% sucrose gradients, centrifuged for 16 hours at 4C at 40,000 rpm in the SW 50.1 rotor of a Beckman L2-50 ultracentrifuge. Gradients were fractionated and assayed as described in methods and materials. Sedimentation is from left to right, control ●———●, infected o———o.

For comparison the arrows represent the position of bovine serum albumin and aldolase respectively when centrifuged under the identical conditions.

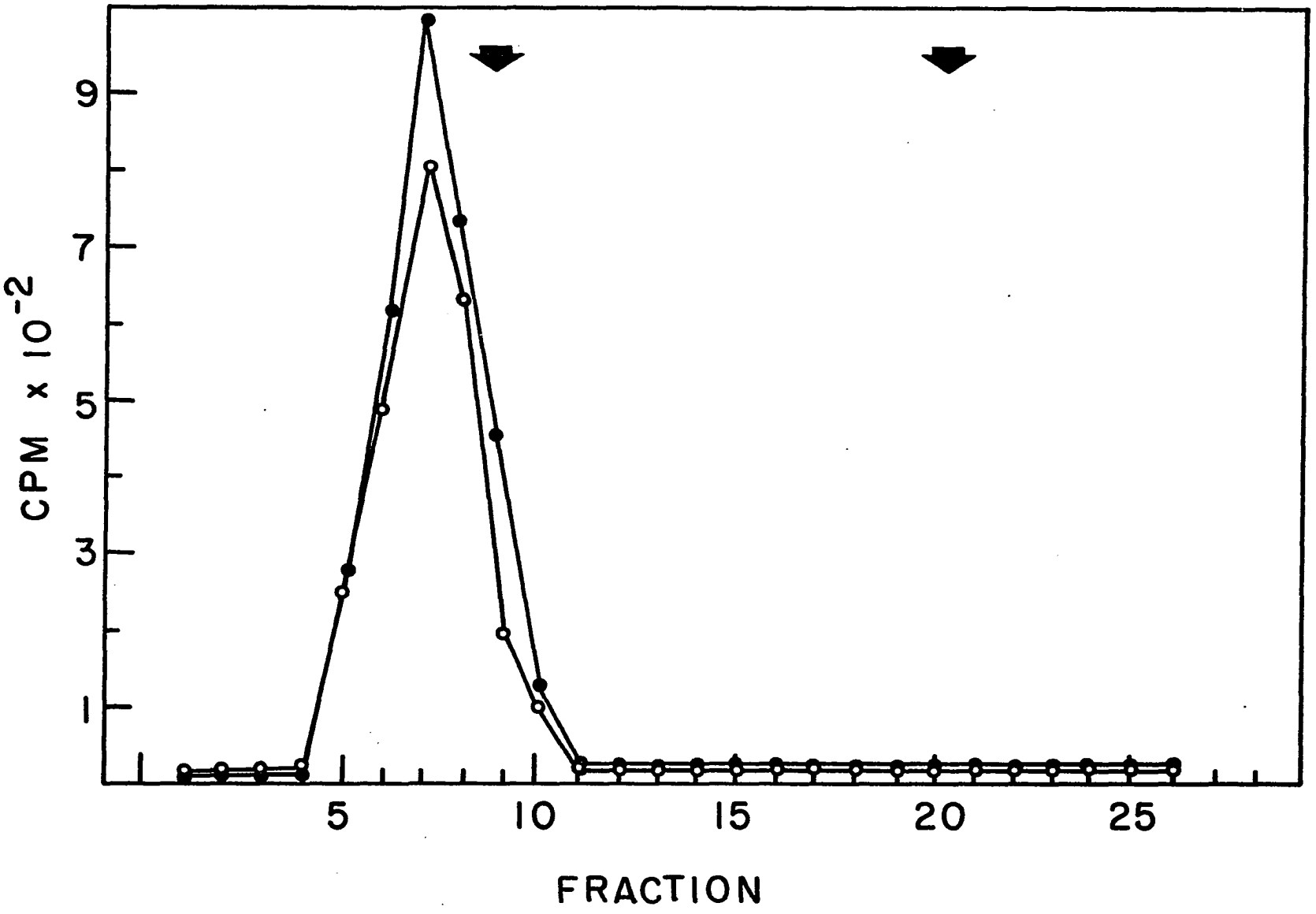
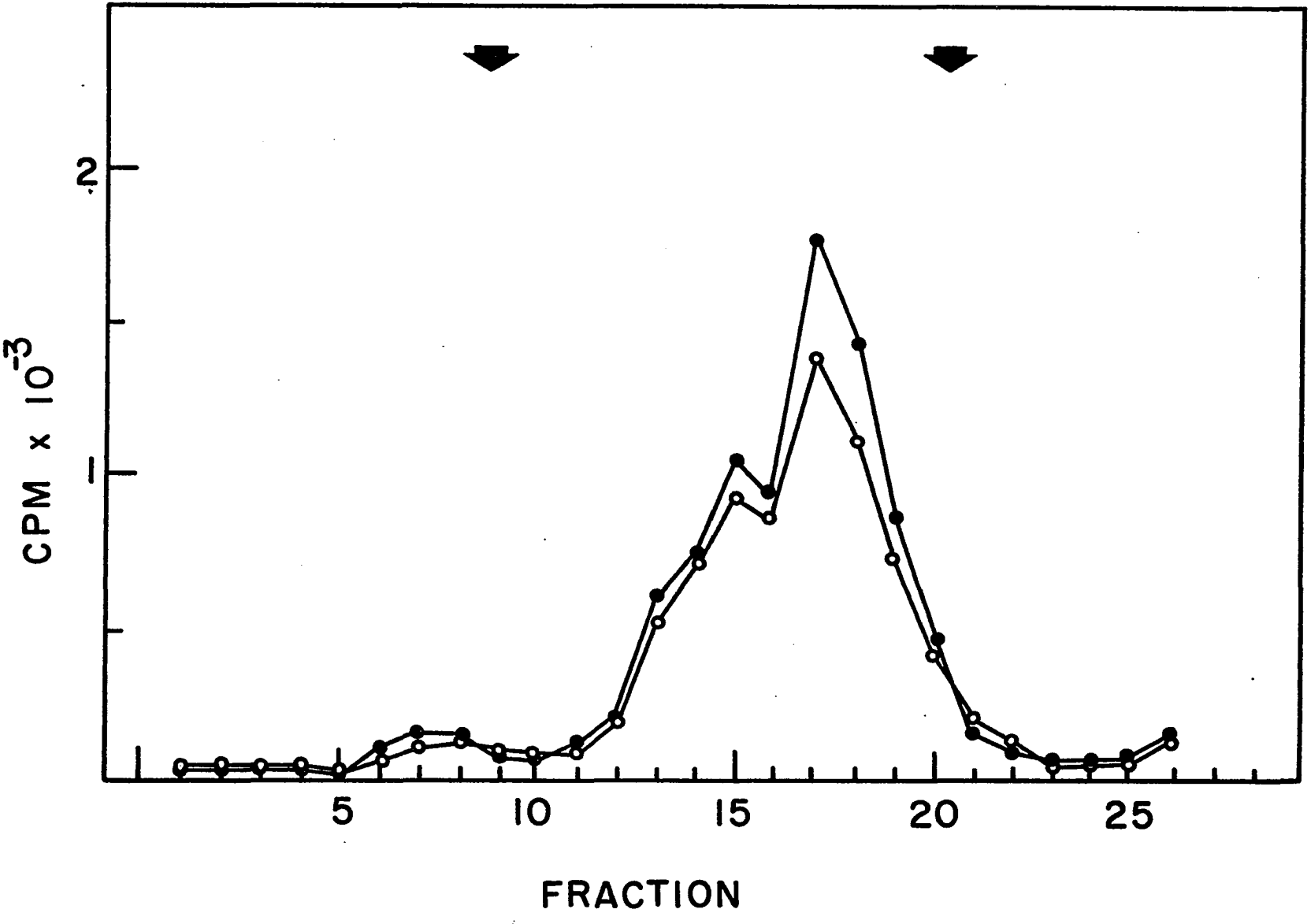

CPM x 10-2
9
7
5
3
1
5
10
15
20
25
FRACTION

Figure 3. Levels of cytoplasmic DNA polymerase activity in infected and uninfected L-cells. Cytoplasmic extracts were layered onto linear 5-20% sucrose gradients, centrifuged for 16 hours at 4C at 40,000 rpm in the SW 50.1 rotor of a Beckman L2-50 ultracentrifuge. Gradients were fractionated and assayed as described in methods and materials. Sedimentation is from left to right, control ●——●, infected o——o.

For comparison the arrows represent the position of bovine serum albumin and aldolase respectively when centrifuged under the identical conditions.

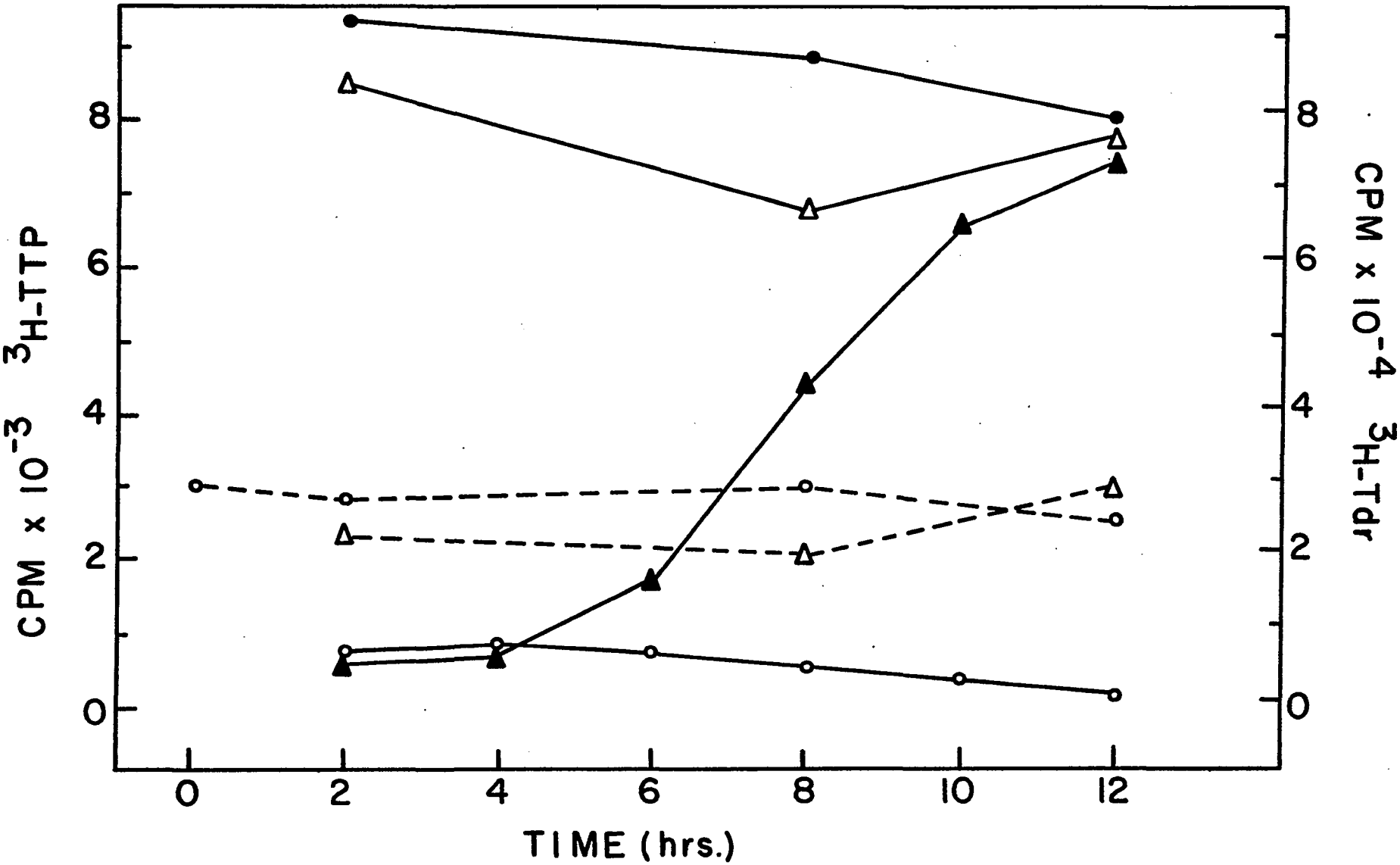

CPM x 10^{-3}
2
1
5
10
15
20
25
FRACTION

fraction, we cannot exclude the possibility that its presence may be due to leakage from the nucleus during isolation. The positions of the enzymes in the gradients yield sedimentation values in agreement with published data (8). Figures 2 and 3 illustrate the levels of activity of these enzymes in cells infected with reovirus. As was shown by the centrifugation patterns and levels of activity there appears to be no alteration in the polymerase activities or physical characteristics due to reovirus infection. As reported by Chang, et al. (8) and others (57) the sedimentation pattern of the cytoplasmic polymerase indicates the presence of multiple species or multiple forms of the same species of polymerase. It is possible that as many as three polymerases are in this peak (57).

DNA polymerase activity in synchronous cells following reovirus infection

As shown in the previous section we could discern no difference in cytoplasmic or nuclear polymerase activities following reovirus infection of asynchronous cells. It was considered that another possible mechanism for viral inhibition of DNA synthesis could have been by prevention of the maintenance of levels of nuclear polymerase, if indeed the cytoplasmic polymerase complex served as a nuclear polymerase precursor (8,24,25,47). Synchronous cells were utilized in these experiments, since this system seemed better suited to detect changes in levels of polymerase activities (8,9,51) as the cell proceeded from a non-synthetic to an active synthetic state. Cells were synchronized as described previously and infected at the time of release from synchrony. Following infection samples were taken and assayed for polymerase activity. The

Figure 4. Levels of nuclear and cytoplasmic DNA polymerase activity in reovirus infected synchronous L-cells. Uninfected cell nuclear polymerase o- - - -o, infected cell nuclear polymerase Δ- - - -Δ, uninfected cell cytoplasmic polymerase o——o, infected cell cytoplasmic polymerase Δ——Δ, uninfected cell DNA synthesis Δ——Δ, infected cell DNA synthesis o——o. Cells were infected at time 0 with 10-20 pfu/cell. Polymerase and DNA synthetic activities were measured as described in methods and materials.


CPM x 10^-3 3H-TTP
CPM x 10^-4 3H-Tdr
TIME (hrs.)
0
2
4
6
8
10
12

results are shown in Figure 4. The data indicate that at a time when cells are totally prevented from entering DNA synthesis by reovirus infection, the levels of activity of both nuclear and cytoplasmic DNA polymerase remain relatively unchanged. While the overall level of activity of the infected cell cytoplasmic polymerase is lower than in control cells, this slight decrease appears insufficient to explain the noted decrease in DNA synthetic activity. The data in the graph also show that at a time when DNA synthetic activity, in control cells, was at a maximum we fail to detect any change in nuclear polymerase activity. The infected cells show a total lack of synthetic capability but show similar levels of nuclear activity indicating that a constant level of activity may be present in the nucleus at all times regardless of the period in the cell cycle (8,51). The data fail to show any significant change in the levels of cytoplasmic polymerase as cells proceed from an inactive to active period of DNA synthesis as has been demonstrated in other systems (8,51). The latter observation could be explained by the method of synchronization and will be discussed later.

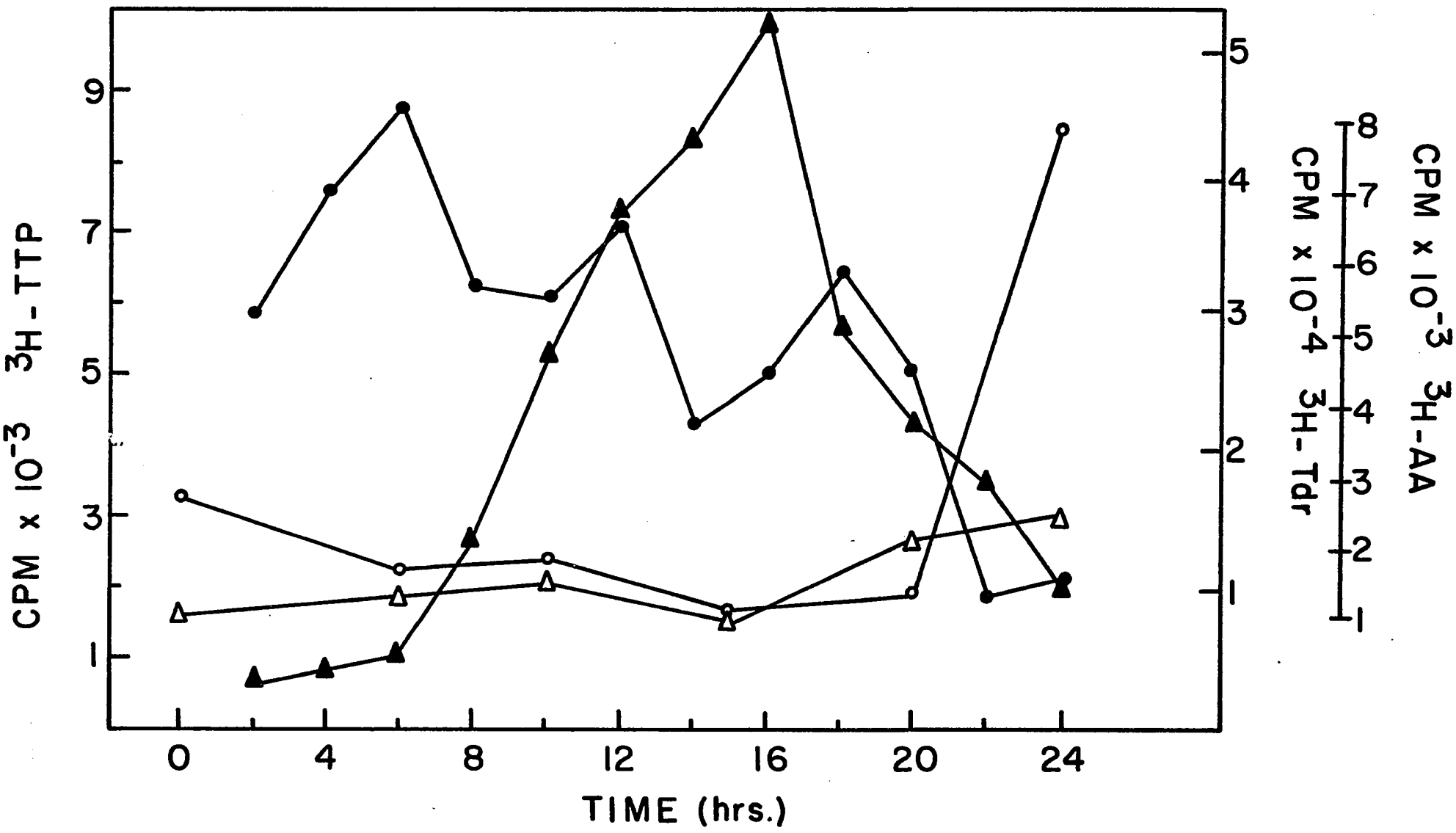
Induction of DNA polymerase

It was shown in the previous section that the levels of cytoplasmic and nuclear polymerase remained relatively constant regardless of the stage of the cell cycle. These results were in opposition to published results (8,51) which indicated variability in the polymerases, and we were interested in whether polymerase activities in our cells could show this same variability if we manipulated them properly. To achieve this, synchronized cells were maintained in culture for 100 hours rather

than the 50 hours described in methods and materials. At the end of this period cells were harvested at selected intervals and DNA polymerase, DNA synthetic, and protein synthesis levels were monitored for 24 hours following the release of the cells from the stationary phase induced by the culture conditions. The results are shown in Figure 5.

The results illustrate that under these conditions there was a decrease in levels of cytoplasmic polymerase activity, and that the cytoplasmic polymerase activity appears to respond to a growth stimulus as indicated by others (8,51). This response to growth appears in our system to be totally dissociated from DNA synthetic activity, and could rather be related to the end of a cell cycle. The data show that the time corresponding to the increase in cytoplasmic polymerase was associated with the time and when these cells would be expected to begin division. The results are in agreement with those of Chang, et al. (8) but our conclusions differ. Chang, et al. (8) interpreted the increase in cytoplasmic polymerase activity to be in response to cell proliferation and DNA synthesis. They assayed polymerase activity 48 hours after release from the stationary phase and demonstrated an increase in activity which was interpreted as a response to growth. In these experiments we followed polymerase activity in the 24 hour period immediately following release and the results seem to indicate that the levels of cytoplasmic polymerase activity in the cell under our conditions do not correlate to the DNA synthetic activity at that time, but possibly to the end of the cell cycle. This work also demonstrates the constant level of activity of the nuclear polymerase throughout the cycle, a result which is consistent with published data (8,51). The data in the graph also indicate that there was

Figure 5. Induction of nuclear and cytoplasmic polymerase in mouse L-cells. Nuclear polymerase ▲——▲, cytoplasmic polymerase o——o, DNA synthesis ▲——▲, protein synthesis ●——●. Cells were released from the stationary phase at time 0 and activities were assayed at the indicated times. DNA polymerase, DNA synthetic and protein synthetic activities were measured as described in methods and materials.


CPM x 10^-3 3H-TTP
CPM x 10^-4 3H-Tdr
CPM x 10^-3 3H-AA
TIME (hrs.)
0
4
8
12
16
20
24
1
3
5
7
9
2
4
6
8

no relationship between induction of the cytoplasmic polymerase activity and cellular protein synthesis, as no increase in protein synthetic activity corresponded to any increase in polymerase activity.

The effect of inhibition of protein synthesis on DNA polymerase activity

During the course of these studies on DNA polymerase activity we were in doubt as to the stability of the DNA polymerases since we were unaware of any studies examining this property of the enzymes. To elucidate the stability of the polymerases, cells maintained as described in the previous section were blocked for 5 hours prior to the isolation of the polymerase (see Figure 5) with cycloheximide. The results are shown in Table I.

The data in the table show a differential stability of the polymerase depending on the period of the cell cycle. The cytoplasmic and the nuclear polymerases show a turnover during periods of rapid DNA synthesis, and were relatively stable during periods of low DNA synthetic activity. These results were in contrast to changes seen in normal cells in which protein synthesis is not blocked. The results could indicate a protein synthetic step needed to regenerate both nuclear and cytoplasmic polymerase and the concomitant decrease in both polymerases may indicate a relationship between cytoplasmic and nuclear polymerases.

The effect of reovirus on the DNA synthetic capacity of isolated nuclei

Further attempts to characterize a possible mechanism of reovirus mediated inhibition of cellular DNA synthesis, utilized a system of isolated nuclei, shown by others to be capable of carrying on DNA synthesis initiated *in vivo* (18,27,28,31). The requirements for DNA synthesis in isolated nuclei are shown in Table II.

Table I. The effect of cycloheximide on cellular DNA polymerases. Cells were treated with 20 ug/ml cycloheximide 5 hours before the samples were taken and DNA, protein and polymerase activities were assayed as described in methods and materials. The time column indicates the time after 0 hour in Figure 5. All values listed are given as per cent of control values in Figure 5.

EFFECT OF CYCLOHEXIMIDE ON CELLULAR POLYMERASES

Time	DNA	Protein	Cytoplasmic Polymerase	Nuclear Polymerase
6	< 5	< 5	86	100
10	< 5	< 5	68	87
15	< 5	< 5	23	58
20	< 5	< 5	81	72

Table II. Requirements for DNA synthesis in isolated nuclei

SYSTEM	ACTIVITY (%)
Complete[1]	100
-ATP	20
-dATP	42
-dGTP	39
-dCTP	64
-dATP,dGTP,dCTP	30
-Cytoplasmic fraction	29

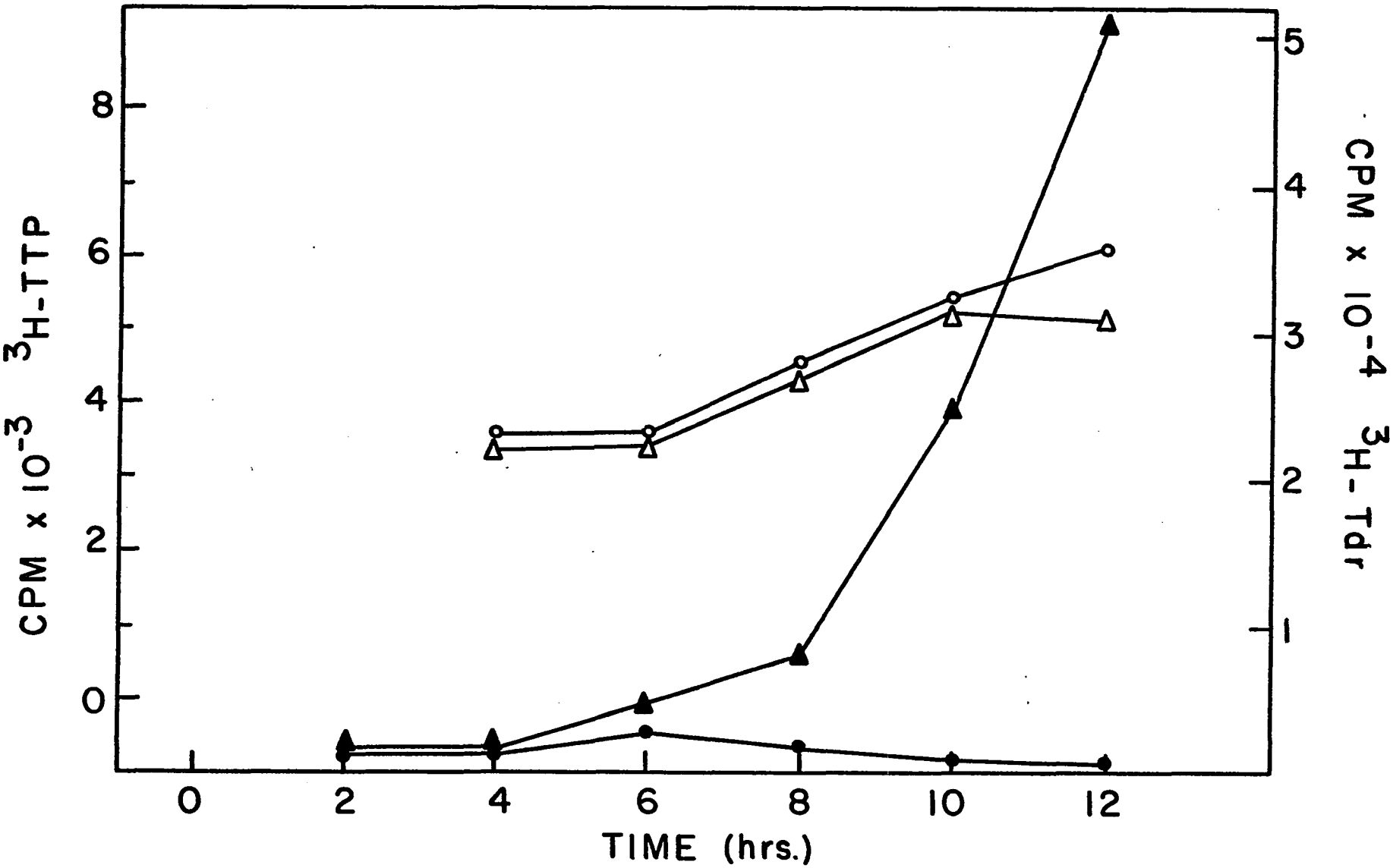
1. Nuclei were assayed as described in methods and materials.

As the data in Table II indicate, the nuclei require the four triphosphates and ATP for maximum activity similar to the requirements demonstrated by others (17,32,39). The data also show a need for a soluble cytoplasmic fraction (CF) (27,31,32). When nuclei were isolated from synchronized cells at various times after infection, we found that the nuclei from infected cells did not reflect the inhibition of DNA synthesis shown in vivo (Figure 6). In fact, the nuclei showed a pattern of synthesis nearly equivalent to uninfected cells. Whatever the mechanism of inhibition, it appeared that it can be overcome by isolating the nuclei and supplying them with the precursors for DNA synthesis. The cytoplasmic fraction (CF) from either infected or uninfected cells worked equally well in the nuclei system (data not shown).

During the work with the isolated nuclei we became aware that hypotonically treated cells become permeable to trisphosphates (27). It was hoped that perhaps the hypotonically treated reovirus-infected cells would show some degree of inhibition when placed in a nuclei reaction mixture and processed as were the nuclei. The results were similar to those obtained with nuclei; the inhibitory process was not detectable.

The ability of isolated chromatin to support DNA replication was also tested. The chromatin isolated and assayed as described in methods and materials yielded results compatable with previous work with nuclei. Infected cell chromatin was as competent as uninfected chromatin to synthesize DNA. These data seemed to indicate the presence of a bound polymerase (58) and no gross damage to the DNA template (15).

Figure 6. DNA synthesis in isolated L-cell nuclei from synchronous cells. Nuclei from uninfected cells o——o, nuclei from infected cells ▲——▲, DNA synthesis in uninfected cells ▲——▲, DNA synthesis in infected cells ●——●. Cells were isolated, assayed, and DNA synthesis was measured as described in methods and materials.


CPM x 10-3 3H-TTP
CPM x 10-4 3H-Tdr
TIME (hrs.)
0
2
4
6
8
10
12
0
2
4
6
8
1
2
3
4
5

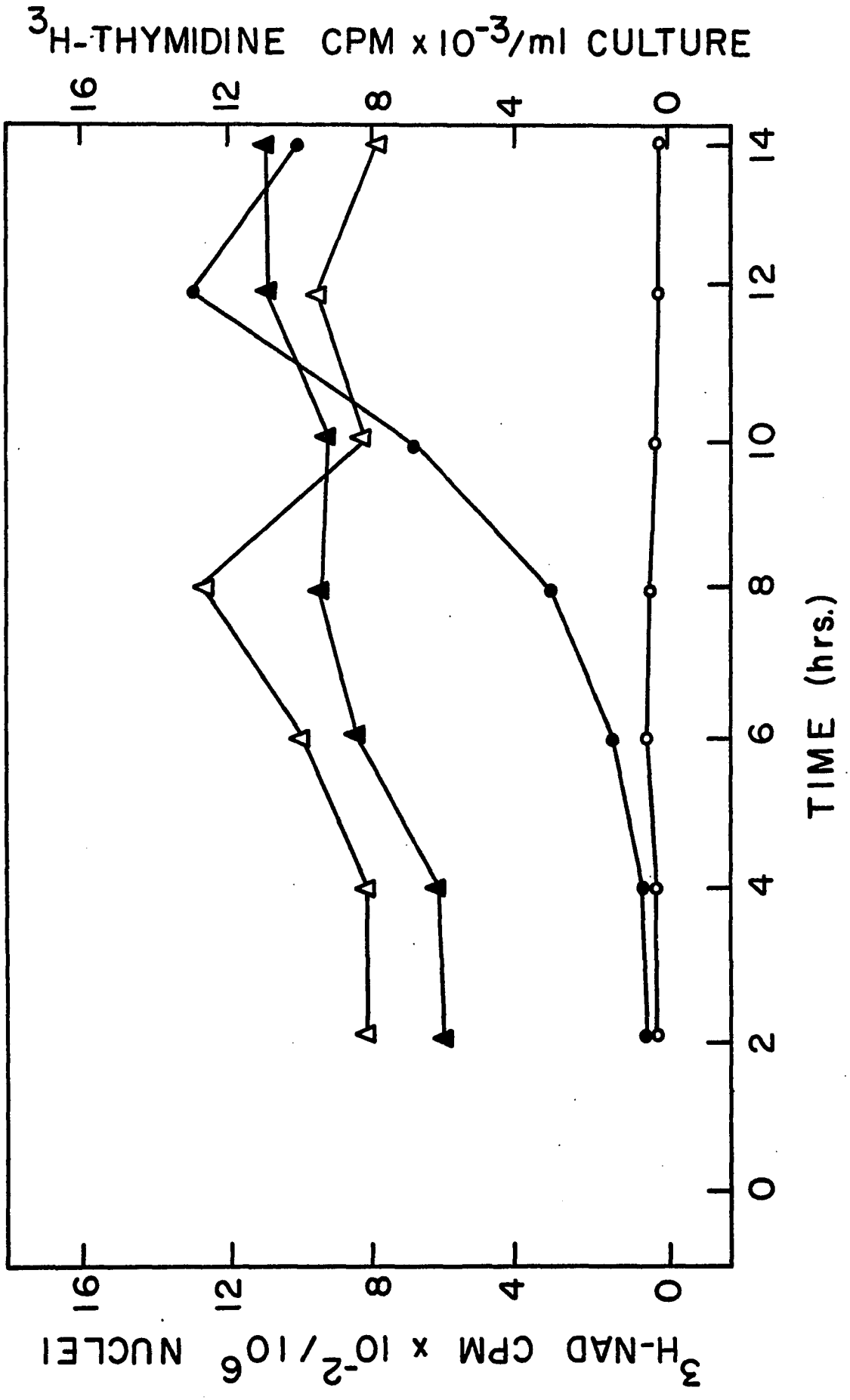
Poly (ADP-Rib) polymerase activity in reovirus infected cells

Since no inhibition of the DNA synthetic systems could be detected in vitro, we decided to examine a system which has been implicated to be intimately associated with levels of DNA synthesis, depending on the cell type (10,34,38,40,49,57). This system examines the capability of isolated nuclei to carry out the adenosine diphosphoribosylation of nuclear proteins in the presence of NAD, as described in methods and materials. As indicated, the process was considered by many to be intimately associated with DNA synthesis (10,34,38,40,49,52). It was of interest to see if we could detect any change in this activity either as a result of viral infection or as a function of the cell cycle. With poly (ADP-Rib) synthesis reported to be so closely related to DNA synthesis it was hoped that inhibition of DNA synthesis by reovirus would cause some fluctuation in its activity. The results are shown in Figure 7.

The graph shows there was only a relatively minor change in the level of poly (ADP-Rib) polymerase activity with the transition of the cell from non-active to active DNA synthesis in uninfected cells. Only after the cells had proceeded several hours into S-phase do we see a rise in activity. This was in contrast somewhat to published results with L-cells (10) using a different technique for measuring poly (ADP-Rib) polymerase activity, and other cell lines using the same procedure (34,38,40). The same pattern was seen in infected cells, however, a small but reproducible increase was seen 6-8 hours post infection.

To examine the possibility that the increase in poly (ADP-Rib) polymerase activity was significant, the pattern of reovirus inhibition of DNA synthesis in the presence of nicotinamide (NAM), an inhibitor

Figure 7. Adenosine diphosphoribose polymerase activity in synchronous L-cells. Poly (ADP-Rib) polymerase activity in nuclei from uninfected ▲——▲, infected cells ▲——▲. Cells were infected with 10-20 pfu/cell at time 0. DNA synthesis in uninfected cells ●——●, DNA synthesis in infected cells o——o. Poly (ADP-Rib) polymerase activity in isolated nuclei was assayed and DNA synthesis measured as described in methods and materials.


^{3}H-THYMIDINE CPM x 10^{-3}/ml CULTURE
16
12
8
4
0
0
2
4
6
8
10
12
14
TIME (hrs.)
^{3}H-NAD CPM x 10^{-2}/10^6 NUCLEI

of poly (ADP-Rib) polymerase (10,13), was followed. Nicotinamide (NAM) caused an overall depression of cellular DNA synthesis, which was opposite to published data obtained with HeLa cells (50) and had no effect on reovirus inhibition of DNA synthesis. The results could be interpreted to mean that adenosine diphosphoribosylation plays no role in reovirus inhibition of DNA synthesis or that possibly the effect of NAM and reovirus are complementary and any effect was masked.

CHAPTER IV

DISCUSSION

The inhibition of DNA synthesis by reovirus has been shown by several workers (12,15,23) to be specific for the inhibition of DNA synthesis. Efforts to demonstrate particular viral proteins as mediators of this inhibitory process have not been successful (13). To date the only virus associated product capable of mediating an inhibition similar to that seen with reovirus are the oligo A ("Adenine-rich") oligonucleotides associated with the virus (22).

The purpose of this research was to examine at the molecular level reovirus inhibition of cellular DNA synthesis utilizing several different sub-cellular systems. The systems used included isolated DNA polymerases, nuclei and chromatin, all shown to be capable under the proper conditions, of synthesizing DNA (17,18,27,28,31,32). The ability of isolated nuclei to carry out the adenosine diphosphoribosylation of nuclear proteins was also examined. This process has been reported to be associated with the cells' capacity to synthesize DNA (34,38,40,49).

The activity levels of two classes of cellular DNA polymerases, cytoplasmic (6-8S) and nuclear (3.4S) (8) were shown to be unaffected by reovirus infection, although DNA synthesis was inhibited by greater than 90%. These results were the same whether synchronous or asynchronous

cells were employed and were in agreement with earlier workers conclusions from a study of cytoplasmic polymerase activity (21) in reovirus infected cells.

While examining the effect of reovirus infection on cellular DNA polymerases, the regulation of the polymerases as a function of the cell cycle was also examined. Of the polymerases studied, only the cytoplasmic polymerase showed variability. The nuclear polymerase was stable, maintaining uniform levels regardless of the stage of the cell cycle. The data indicate that when cells are maintained in conditions that cause a decrease in cytoplasmic polymerase activity, regeneration of this activity was apparently associated with the onset of cell division, and not necessarily associated with DNA synthesis as reported by other workers (8,51). It appears that the levels of cytoplasmic polymerase activity normally maintained by the cell was far in excess of the levels needed for the cell to progress through its DNA synthetic period. This assumes, of course, that the cytoplasmic polymerase is needed or plays a role in DNA synthesis, a relationship not conclusively demonstrated (51).

When the stability of the cytoplasmic and nuclear polymerases were followed after inhibition of protein synthesis with cycloheximide, the polymerases were shown to be relatively stable. Only when cycloheximide was present during periods of active DNA synthesis did the polymerases show any tendency to decrease in activity and this decrease in activity showed a partial temporal relationship (see Table I). This was in contrast to the polymerase activity in synchronized, reovirus infected cells, which did not change. The failure of reovirus to cause a decrease

in activity of the polymerases may indicate that the inhibition of DNA synthesis by reovirus was not due to inhibition of host cell protein synthesis as some have suggested (30). This observation was supported by similar work utilizing isolated nuclei (26).

Isolated nuclei from reovirus infected cells failed to show the inhibition of DNA synthesis exhibited *in vivo*. The nuclei from infected cells were as capable as nuclei from uninfected cells of carrying on DNA synthesis initiated *in vivo*. It appeared that whatever the cause of the inhibitory process mediated by reovirus, it can be overcome by isolating the nuclei and providing them with precursors for DNA synthesis. Such is not the case when an inhibitor of protein synthesis was used to block DNA synthesis. The nuclei from these cells were not fully capable of synthesizing DNA (26). These observations were in agreement with the data on the activity of DNA polymerase described above and indicate that the inhibition of host cell protein synthesis by reovirus is not responsible for inhibition of DNA synthesis. When hypotonically swelled cells or isolated chromatin were assayed for their capacity to support DNA synthesis no detectable difference was seen between components from infected or uninfected cells.

The adenosine diphosphoribosylation of nuclear protein assayed in these experiments showed a consistent increase at the onset of reovirus inhibition of host cell DNA synthesis, indicating that it may play a role in the inhibitory process. It cannot, however, be ruled out that this increase was unrelated to the inhibition. In fact nicotinamide, an inhibitor of Poly (ADP-Rib) polymerase, fails to cause any change in the pattern of inhibition of DNA synthesis by reovirus. However, as

mentioned previously, nicotinamide was inhibitory and any effect on DNA synthesis inhibition by reovirus may be masked.

The above data support the following conclusions concerning reovirus inhibition of cellular DNA synthesis. First, the inhibitory process causes no damage to the cellular DNA synthetic apparatus (i.e., DNA polymerases, nuclei, chromatin), and appears not to be due to cell cytopathology resulting from infection. Second, the inhibitory process, as measured utilizing sub-cellular DNA synthesizing systems (i.e., DNA polymerases, nuclei, etc.), was unlike that detected when a similar inhibition was caused by the inhibition of protein synthesis due to cycloheximide (26). Finally, whatever the mechanism of inhibition it may be overcome by removing the nuclei and supplying them with precursors.

While the above data and conclusions do not point to the specific mode of inhibition of DNA synthesis by reovirus, they do indicate some characteristics of the inhibitory process and suggests further experiments. The inhibitor, if such a specific product exists, was diffusible and does not appear to be tightly bound to any cell component necessary for DNA synthesis. The inhibition process could result from failure of the DNA precursor (i.e., triphosphates) to reach the proper enzymes. This type of inhibition could result from lack of transport to the nucleus, changes in precursor pools, or failure of the enzymes maintaining these pools. The data support any of the above possibilities but does not allow us to distinguish between them. It will be of future interest to examine in detail the synthesis of DNA by sub-cellular systems from infected cells. Further experiments are needed to compare the products made in these DNA synthesizing systems from infected and uninfected

cells. The enzymes required for maintenance and production of DNA precursors must be examined, as well as the ability of their DNA precursors to become available for DNA synthesis.

The experiments described in this paper do not point to the mode of inhibition of DNA synthesis by reovirus. However, they do indicate that a specific viral mediated event was necessary for the inhibition and that inhibition of DNA synthesis was not due entirely to cell cytopathology or inhibition of protein synthesis caused by the viral infection.

CHAPTER V

SUMMARY

These experiments explored the capacity of sub-cellular DNA synthesizing systems (DNA polymerases, nuclei, chromatin) to synthesize DNA *in vitro* following reovirus infection. The activity of Poly (ADP-Rib) polymerase was also assayed in infected cells.

Isolation and assay of nuclear and cytoplasmic polymerase following fractionation of the polymerases on sucrose gradients indicated no difference between infected and control cells. Utilizing uninfected cells, the induction of cytoplasmic polymerase following release of cells from the stationary phase indicated that there was no relationship between the induction of cytoplasmic polymerase activity and DNA synthesis. The nuclear polymerase maintained constant levels of activity regardless of the period in the cell cycle. Both the nuclear and cytoplasmic polymerase were stable in the presence of cycloheximide, except during periods of high DNA synthetic activity.

When nuclei, chromatin or hypotonically treated cells were assayed for DNA synthetic capacity, no difference was seen between infected and uninfected cells.

The activity of Poly (ADP-Rib) polymerase was consistently higher in reovirus infected cells. The increase in this activity, while

slight, corresponded to the onset of reovirus inhibition of cellular DNA synthesis.

These results indicate that whatever the mechanism of inhibition it can be overcome by isolating the nuclei and supplying them with precursors. The data also suggest that the inhibition of DNA synthesis is not a result of inhibition of protein synthesis or a result of cell cytopathology following infection.

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