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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE EFFECT OF SELECTED REOVIRUS SUBVIRAL PARTICLES ON DNA FUNCTIONS IN L CELLS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

By

C. WORTH CLINKSCALES

Norman, Oklahoma

1974

THE EFFECT OF SELECTED REOVIRUS SUBVIRAL PARTICLES ON

DNA FUNCTIONS IN L CELLS

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To Robbi and Damon

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THE EFFECT OF SELECTED REOVIRUS SUBVIRAL PARTICLES ON DNA FUNCTIONS IN L CELLS

CHAPTER I

INTRODUCTION

The specific mechanisms for regulation of cellular deoxyribonucleic acid (DNA) functions are not clear. Both the transcriptive and the replicative functions appear to be strictly regulated. A frequent result of infection of cells with many viruses is the alteration of these regulatory processes (1, 36). Study of virus-specific alteration of cellular DNA transcriptive and/or replicative functions could provide a model for characterization of these regulatory phenomena. Analysis of a model cytocidal virus system is complicated by the replication cycle (55) and resulting degeneration and death of the cell (1). The oncogenic virus-cell interaction is equally complex. Thus, demonstration of viral-specific mechanisms for the alteration of cellular DNA functions has been difficult.

The reovirus infected cell exhibits certain characteristics suggesting a significant potential for investigation of these mechanisms (32, 57, 58). First, viral specific inhibition of cellular DNA replicative function is the first detectable macromolecular alteration observed in infected L-cells (13, 24, 62). Second, cellular

protein and ribonucleic acid (RNA) synthesis seem not to be affected until late in reovirus replication (19, 24, 38). These facts indicate that viral modification of cellular DNA function is a specific selective event and not a distal result of general cytopathologic changes resulting from virus replication.

Third, reovirus inhibition of cellular DNA synthesis occurs approximately 8 hours after infection with low multiplicities (24, 38). This allows sufficient time to study the mechanisms involved in the viral-specific inhibition of cellular DNA function. Fourth, the time required to show the inhibition can be reduced if high multiplicities of reovirus are used to infect L-cells (13, 62). When ultra-violet (UV) light is used to reduce the infectivity of high multiplicities of reovirus, the inhibitory effect is unchanged (13, 62). These data suggest that the input virus particles may contain a quantity of inhibitory substance and/or a more rapid synthesis of such a substance after infection with high multiplicities of reovirus.

Fifth, reovirus replicates in the cytoplasm of the host cell and peri-nuclear inclusion bodies are visible during replication (12, 23, 53, 65, 66). Studies with ferritin-antibody conjugation techniques and autoradiography (16) have not detected reovirus proteins or nucleic acid in the host cell nucleus (15). These results indicate a separation of the site of virus replication and the site of inhibition of cellular DNA synthesis which suggests production or release of the inhibitory factor at cytoplasmic virus replication sites and a subsequent modification of DNA function in the host cell nucleus. Ensminger and Tamm have suggested that an alteration of the nuclear membrane by the perinuclear virus replication may alter cellular DNA functions (20).

Sixth, the reovirus genome is composed of double-stranded (ds) RNA (25, 26, 40, 41, 48, 64, 73) which is segmented and consists of 10 fragments which separate into 3 distinct size classes (2, 22, 31, 37, 49, 61, 69, 70, 71, 76). This characteristic provides a mechanism for genetic analysis of the relationship between specific genome segments and the production of functional virus polypeptides, one or more of which may possess the ability to modify DNA function. In addition to the ds RNA, there are adenine-rich (A-rich) oligonucleotides of single-stranded (ss) RNA contained in the mature virions (3, 4, 6, 50, 59, 60, 67). This A-rich RNA has been shown in our laboratory (49) to be inhibitory for cellular DNA synthesis. Complete virions also contain 7 structural capsid polypeptides which compose two distinct protein coats each having icosahedral geometry (23, 32, 56, 57, 74, 75). Any one or a combination of the nucleic acid and protein components of the reovirion could mediate the inhibition of cellular DNA synthesis. The inhibition of DNA synthesis could be maintained by the synthesis of the component(s) during the virus replication cycle.

Seventh, several studies have demonstrated the presence of functional enzymes in mature reovirus particles. One enzyme transcribes viral RNA (9) and another converts nucleoside triphosphates to nucleoside diphosphates (10, 34). This evidence strengthens the idea that mature reovirus particles contain not only structural proteins but also biological and metabolically active molecules.

The objective of the studies described in this dissertation was to utilize specific subviral particles to study virus-directed inhibition of cellular DNA synthesis. Using these specific subviral

particles which lack various components one could establish the molecular composition required for viral mediated inhibition of cellular DNA replication (28). Double stranded RNA has been shown to inhibit protein synthesis by reticulocyte lysates (18). A subviral particle devoid of RNA (Top component) but containing all the structural proteins was used to test the requirement of ds RNA in the inhibition process. Equivalent particles, termed Ghosts, from the T-even series of bacteriophage have been shown to inhibit host macromolecular synthesis (17). These ghost particles (Top component) would also allow us to investigate the structural capsid protein's participation, if any, in mediating the inhibition process.

The fiber antigen, an external capsid component of adenovirus type 5, has been shown to inhibit host cell macromolecular synthesis (7, 42). A reovirus subviral particle which lacks the outer capsid proteins (core) was used for treating cells and investigating cellular DNA synthesis after this exposure. Since top component (TC) and cores do not contain significant infectivity, it was desirable to obtain a subviral particle in which specific viral components were absent but which still retained infectivity. To date one subviral particle (SVP_1) produced by limited chymotrypsin digestion is available. This digestion procedure removes certain outer capsid proteins (33) and approximately half of the A-rich RNA (62). SVP_1 is a perfect tool to study virus-directed inhibition of cellular DNA replicative function. Recent studies (20, 63) indicate the inhibition of cellular DNA synthesis was not a result of reovirus-directed inhibition of the function or synthesis of certain enzymes involved in the synthesis or polymerization of DNA precursors (20, 63). Degradation of the cellular DNA

genome is not detected after infection (63) and thymidine pools remain unchanged (63). It has been shown that the inhibition is related to a reduced number of initiation sites for DNA synthesis (27) and that initiation of DNA synthesis during the cell cycle is prevented after reovirus infection (14, 21).

The complex sequence of events required for initiation of cellular DNA synthesis may have been blocked at some point by reovirus. A subviral particle should offer a unique experimental approach to altering the reovirus-mediated inhibition of host cell DNA synthesis and indicate the specific components of reovirus required to inhibit host cell DNA synthesis.

CHAPTER II

MATERIALS AND METHODS

Cells

Mouse fibroblasts, strain L929 (54) (Flow Laboratories), adapted for growth in spinner culture, were used in all experiments. L-cells were cultivated 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 picking 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 virus eluted 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. (64). 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 cleanly. 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 SW 27.1 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 by a procedure of Dr. J. W. Shaw (personal communication). 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 media was removed, and 0.1ml 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 (43). 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. In order,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.

Preparation of reovirus top component

After the first CsCl density gradient during the purification

procedure, a top component (T.C.) band was collected separate from the virus band. The top component was further purified by additional sucrose and CsCl density gradient as described previously and centrifuged in the SW 50.1 rotor for 2 hours at 4C at 189,000 x g. The final top component particles were collected as a homogeneous band with a density of 1.30 g/ml, dialysed exhaustively against cold SSC and stored at 4C until used.

Preparation of reovirus cores

Purified reovirus stock was layered on a final CsCl (1.2g/ml-1.4 g/ml) density gradient and centrifuged as described for top component. The homogeneous, opalescent virus band with a density of 1.37 g/ml was collected and dialysed exhaustively against cold SSC. The virus was incubated for 1 hour at 37C in the presence of 100 μg/ml chymotrypsin (Sigma Chemical Company). The particles produced were stored in ice bath for analysis or experimentation.

Preparation of reovirus infectious subviral particles (SVP_1)

The virus was isolated and collected as described for cores, and then dialysed exhaustively against cold 0.01 M tris, pH 8.0, and 0.15 M sodium chloride (tris-saline) (61). The virus was diluted until the viral protein concentration was 200 μg/ml and then 100 μg/ml chymotrypsin was added. After incubation for 1 hour at 37C the digestion was stopped in an ice bath. The particles were stored in ice until analysed or used for an experiment.

Cellular DNA synthesis assay

Asynchronous cells were centrifuged at 250 x g for 5 minutes

and resuspended in fresh prewarmed MEM supplemented with 5% FCS and 100 μg/ml kanamycin. After resuspension to a final concentration of 5×10^5 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 to the culture treated with the specific particle being tested. Duplicate samples (approximately 10^6 cells) were pulse labeled with ^{3}H-thymidine (19.9 Ci/mM, New England Nuclear) at 0.5 μCi/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 5% (w/v) TCA by centrifuging 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 spectrometer using Beckman cocktail D (5 g PPO, 100 g naphthalene, 10 ml water, to 1 liter with 1-4 dioxane) as the fluor.

Macromolecular quantitation

DNA was determined by the method of Burton (11) using salmon sperm DNA as a standard.

RNA was determined by the method of Mejbaum (47) using yeast RNA as a standard.

Protein was determined by the method of Lowry, et al. (46) using bovine serum albumin as a standard.

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

Labeling of viral components

Cells were prepared and infected as described previously. At 5 hours post infection the appropriate isotope was added. In order to label the RNA, ^{3}H-uridine (26.4 Ci/mM, New England Nuclear) 1 μCi/ml of media was used. Viral protein was labelled with ^{3}H-amino acids (New England Nuclear) 1 μCi/ml of media. At 18 hours post infection cells were harvested by centrifugation and the virus purified.

Adsorbtion studies

The cells were exposed to each of the radioactively labeled particles to be studied. At various times after treatment an aliquot of cells was removed and washed twice with MEM. The cell-associated acid-precipitable radioactivity was determined.

Gradient analysis of various subviral particles

The particles (T.C., cores, SVP_1) to be analysed were layered on a CsCl density gradient (1.3 g/ml to 1.5 g/ml) and centrifuged in the SW 50.1 rotor at 189,000 x g for 24 hours. At this time the particles reached equilibrium density and the gradients were fractionated on an Isco density gradient fraction collector. Random fractions were read on a Bausch and Lomb refractometer and the density calculated.

Viral growth curve

Cells were chilled in ice and the virus or subviral particles were added. Following an adsorbtion period of two hours the cells were removed by centrifugation at 4C at 250 x g for 5 minutes, washed one time with cold MEM and resuspended to 5 x 10^5 cells/ml in 37C MEM supplemented with 5% (v/v) FCS. At various times samples were removed, sonicated, and assayed for infectivity.

Preparation and purification of reovirus single stranded RNA

The method of Hay and Joklik (29) was modified. After infection of the cells with 10-20 pfu/cell and the addition of Actinomycin D (gift of Merck, Sharp, and Dohme) to a final concentration of 0.5 μg/ml to inhibit cellular RNA synthesis (35), the cells were incubated at 37C and at selected times post infection the cells were pulsed for 2 hours with either ^{3}H-uridine or ^{3}H-adenine (6.1 Ci/mM, New England Nuclear) at a concentration of 2 μCi/ml.

At the end of the pulse period the cells were chilled and centrifuged at 2000 x g for 5 minutes. They were washed with cold MEM and centrifuged again. The cells were resuspended in 0.01 M sodium acetate, pH 5.1 (10^{7} cells/ml), and made 0.5% (w/v) with respect to sodium dodecyl sulfate (SDS). This mixture was extracted with two volumes of water-saturated phenol by shaking 10 minutes and separating the phases by centrifugation at 1000 x g for 10 minutes. The aqueous layer and the interphase were re-extracted with phenol 2 times. Finally the aqueous phase was carefully removed and made 0.2 M with respect to NaCl and 2 volumes of cold 95% (v/v) ethanol were added. After storage overnight at -20C, the precipitated RNA was removed by centrifugation at 8000 x g for 15 minutes. The RNA was washed 2 times with cold 70% (v/v) ethanol and dissolved in SSC. The solution was made 2 M with respect to LiCl and stored overnight at 4C. The precipitated RNA was collected by centrifugation as above, washed with 70% (v/v) ethanol and dried under a stream of air. The dried RNA was dissolved in 0.01 M tris, pH 7.5, in 0.05 M NaCl and frozen at -60C.

Analysis of reovirus ssRNA

Purified RNA samples were layered on a 15-30% (w/w) sucrose gradient containing 0.01 M tris, pH 7.5, 0.05 M NaCl, 0.001 M EDTA. Centrifugation was carried out at 94,000 x g in the SW 27.1 rotor at 4C for 18 hours. The gradients were fractionated on an Isco density gradient fraction collector and the absorbance at 260 nm was monitored. For assay of radioactivity, the fractions were precipitated with 1 ml of 10% (w/v) TCA after addition of 100 μg of bovine serum albumin as a carrier. The precipitates were collected on 1.21 μm millipore filters and washed with 5% (w/v) TCA. The filters were placed in vials with fluor and the radioactivity was measured by liquid scintillation counting.

SDS-Polyacrylamide gel electrophoresis

The protein samples were dissociated and reduced by the method of Weber and Osborn (72). The methods of Zweerink, et al. were essentially followed. Ten per cent polyacrylamide gels (0.5 cm x 11 cm) were made with 10% (w/v) acrylamide, 0.1 M phosphate buffer, pH 7.2, 0.267% (w/v) bis acrylamide, 6 M urea, 0.02 M EDTA, 0.1% (w/v) SDS, 0.1% (w/v) TEMED and 0.08% (w/v) ammonium persulfate (64, 74, 75). The running buffer was 0.1 M Na phosphate, pH 7.2, 0.1% (w/v) SDS, and current was 5 ma/gel. After 22 hours the gels were removed from the tubes and fixed with 10% TCA for 5 hours to remove the urea. Staining was with 0.025% (w/v) Coomassie Blue (Sigma Chemical Company) in 10% TCA for 18 hours (61). The gels were destained in 7% (v/v) acetic acid until background was removed.

CHAPTER III

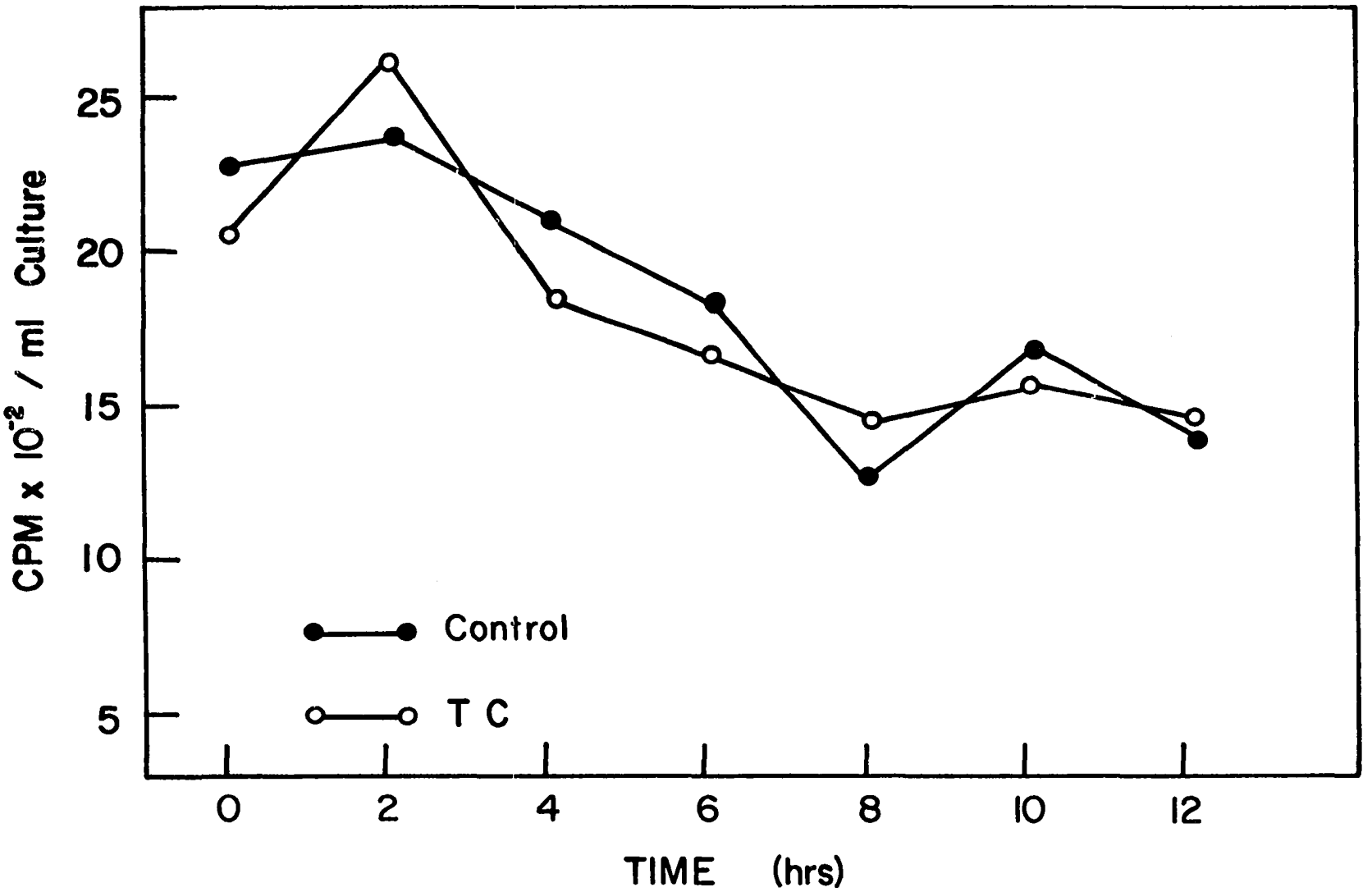
RESULTS

Effect of Reovirus Top Component

In an effort to investigate the role, if any, of reovirus coat polypeptides in mediating the inhibition of cellular DNA replication, top component (T.C.) was used to treat asynchronous cell cultures. Since T.C. contains all 7 structural polypeptides present in complete reovirions, but lacks RNA, this subviral particle could define the role of structural polypeptides in the inhibition of DNA synthesis. The T.C. was isolated by cesium chloride density gradient centrifugation and identified according to its sedimentation characteristics. The results of T.C. centrifugation to equilibrium are represented in Figure 5, panel A. The density at which T.C. banded was 1.30 gm/ml. Our results on the physico-chemical characteristics and electron microscope observations of T.C. were consistent with published literature values (64).

The culture was divided into two equal parts. Buffer was added to one, and top component added to the other. Cellular DNA synthesis in each sub-culture was monitored at selected times after treatment. Each point represents the average of duplicate samples. The results from a typical experiment are shown in Figure 1. There was no detectable difference in DNA synthesis shown between T.C.-treated and

Figure 1. DNA synthesis of spinner L cells treated with 200 μg of top component per culture. At the times indicated approximately 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 as an average of duplicate samples.


CPM x 10^{-2} / ml Culture
25
20
15
10
5
Control
T C
0
2
4
6
8
10
12
TIME (hrs)

untreated cultures (39). In each experiment, the protein concentration of the T.C. added to the test culture was similar to the quantity of viral protein present in preparations of mature reovirus particles at a multiplicity of 10 PFU/cell, although, the T.C. preparation contained only 0.001 PFU/cell.

Effect of Reovirus Core Particles

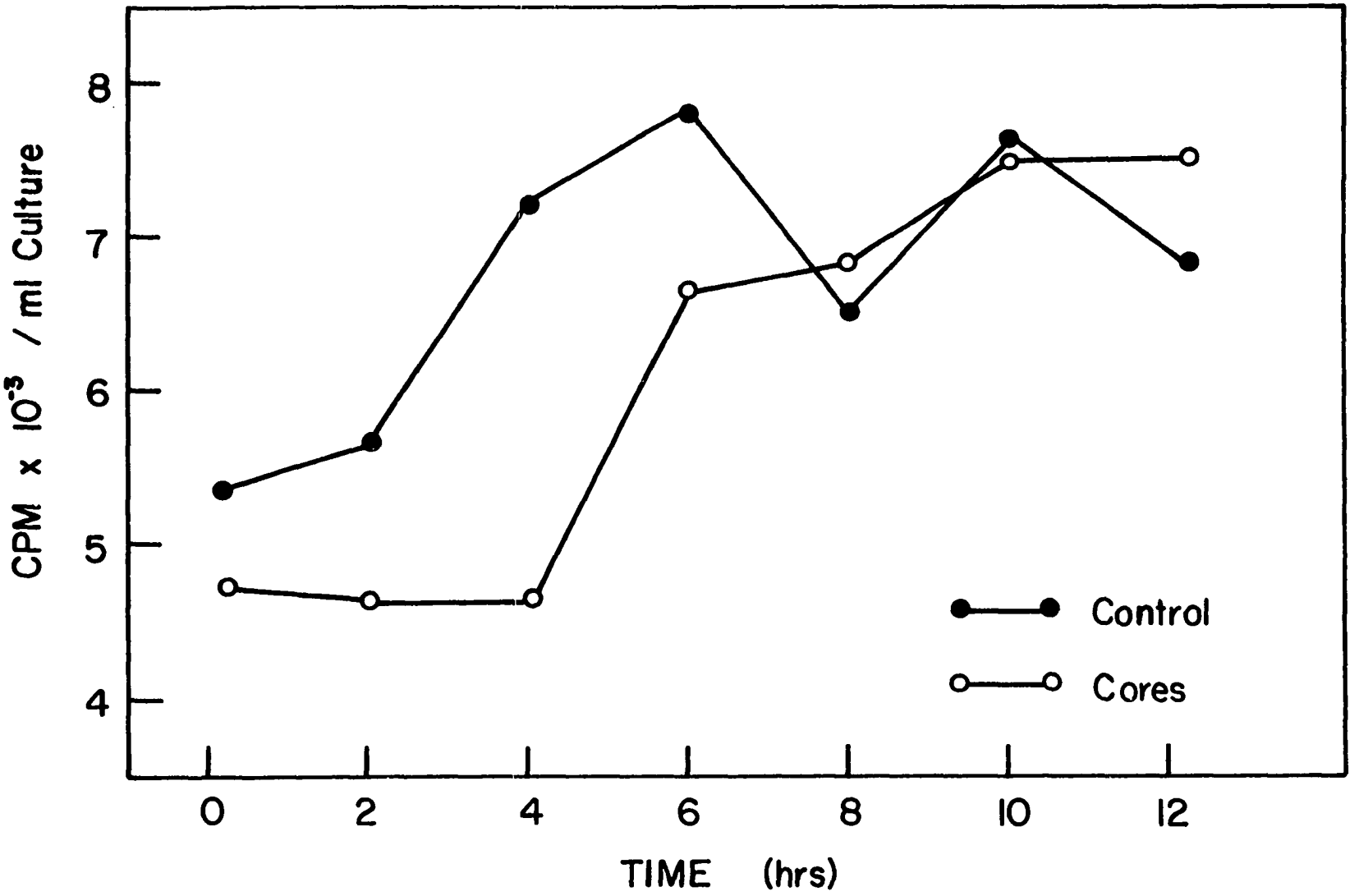
Since the 7 structural polypeptides alone seemed not to mediate the inhibition process, core particles missing the outer coat polypeptides and oligoadenylic acid molecules were tested. The core particles were composed of 3 inner capsid polypeptides and the ds RNA genome. After the outer capsid polypeptides were removed by chymotrypsin, infectivity was reduced 10,000 fold. Analysis of core particle polypeptides by electrophoresis in 10% acrylamide gels confirmed polypeptide content of these particles reported earlier (64). The core particles were more dense than mature reovirions and banded after density gradient centrifugation at 1.43 gm/ml.

These particles were used to treat spinner cells and DNA synthesis was monitored. The results are shown in Figure 2. The slight delay in DNA synthesis in the core-treated culture was not seen consistantly in repeated experiments. Core treatment did not cause a progressive decrease in DNA synthesis similar to that observed in reovirus-infected cultures.

Effect of Reovirus SVP_1

Since T.C. and cores were missing specific viral components, these subviral particles were useful in these studies, although T.C. and cores were not significantly infective. In addition, the physical

Figure 2. DNA synthesis of spinner cells treated with 150 μg of protein from core particles per culture. At the times indicated approximately 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 as an average of duplicate samples.


CPM x 10^{-3} / ml Culture
8
7
6
5
4
0
2
4
6
8
10
12
TIME (hrs)
Control
Cores

state(s) of these subviral particles after cell association were not known. Because of the inhibition of DNA synthesis by UV inactivated reovirus and the dose response of that inhibition, the physical particles were implicated. In contrast, if infectivity was absent in a subviral particle so was the inhibition. Therefore, an infectious subviral particle such as SVP_1 was desirable.

The SVP_1 was produced by limited digestion of reovirus with chymotrypsin and isolated by density gradient centrifugation at 1.39 gm/ml. as shown in Figure 5, panel D. The polypeptides present in SVP_1 were the 3 inner capsid polypeptides and an outer capsid polypeptide which appeared to be degraded to 80% its original size. Approximately 40% of the oligo A (A-rich) RNA remained with the SVP_1, no more than one-half of the infectivity was lost during the digestion. In addition, the entire genome was present in the infectious SVP_1.

It was of interest to study the characteristics of any inhibition of DNA synthesis after exposure of spinner cells to SVP_1. Treatment of spinner cells with SVP_1 resulted in a progressive decrease in cellular DNA synthesis with time. At 12 hours post-infection DNA synthesis in the SVP_1-treated culture was approximately 50% of that in uninfected control cells, while reovirus-infected cells were synthesizing DNA at 10% the rate shown by control cells. These results are presented in Figure 3.

It appeared during the course of these experiments that more cells were intact at 12 hours post-infection in the SVP_1-treated cultures than in the reovirus-treated cultures even when the multiplicities of infection were similar. This observation was tested by

Figure 3. A comparison of DNA synthesis of spinner L cells treated with SVP_1 or infected with reovirus at 10 PFU/cell. At the times indicated approximately 10^6 cells from each culture were pulse-labeled with ^3H-thymidine. Counts per minute per ml of culture in the TCA-insoluble material are presented as an average of duplicate samples.

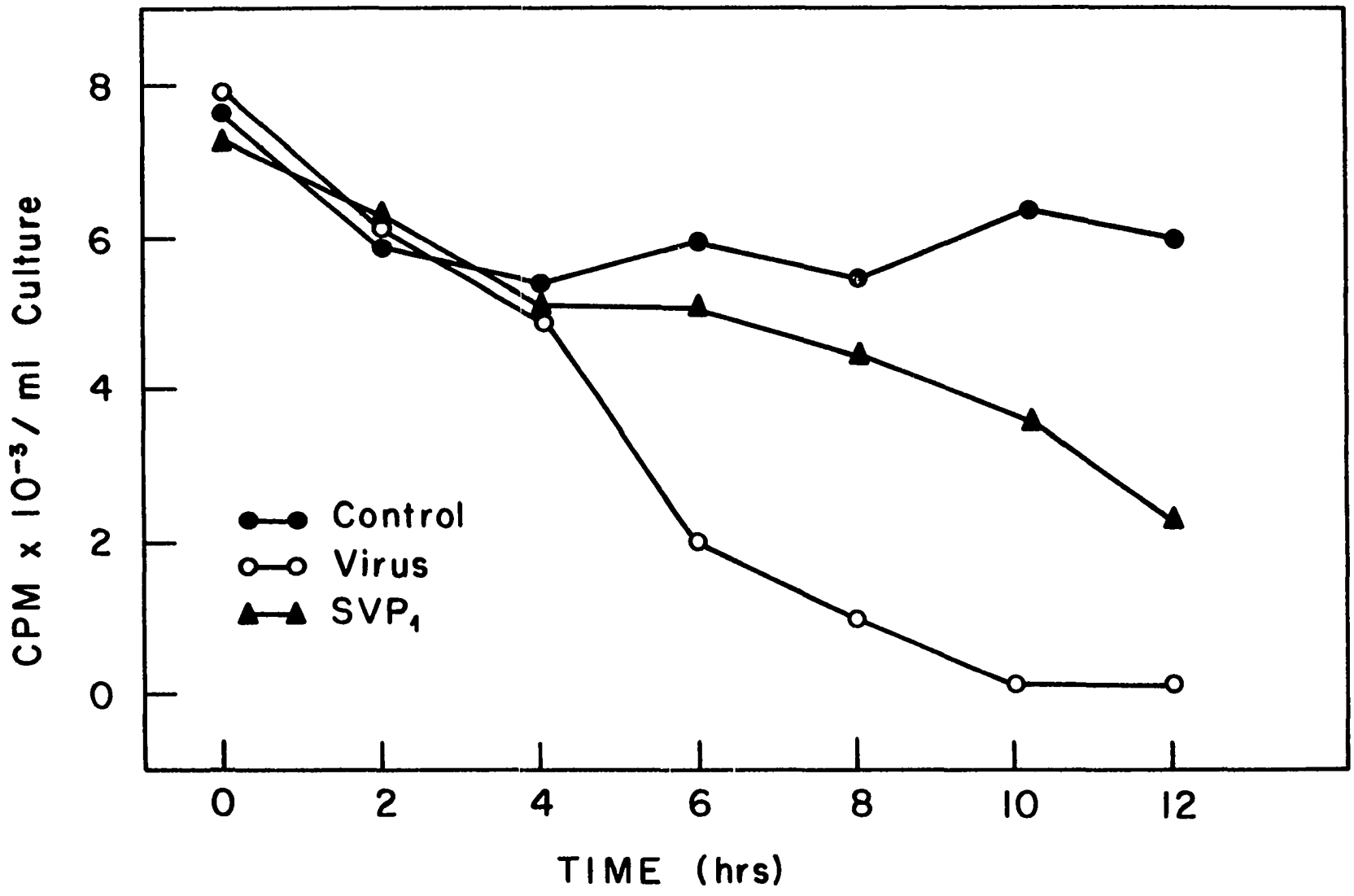

CPM x 10^{-3} / ml Culture
8
6
4
2
0
0
2
4
6
8
10
12
TIME (hrs)
Control
Virus
SVP_1

Figure 4. A comparison of DNA synthesis of spinner L cells treated with SVP_1 or infected with reovirus at 50 PFU/cell. At the times indicated approximately 10^6 cells from each culture were pulse-labeled with ^3H-thymidine. Counts per minute per 10^2 viable cells in the TCA-insoluble material are presented as an average of duplicate samples.

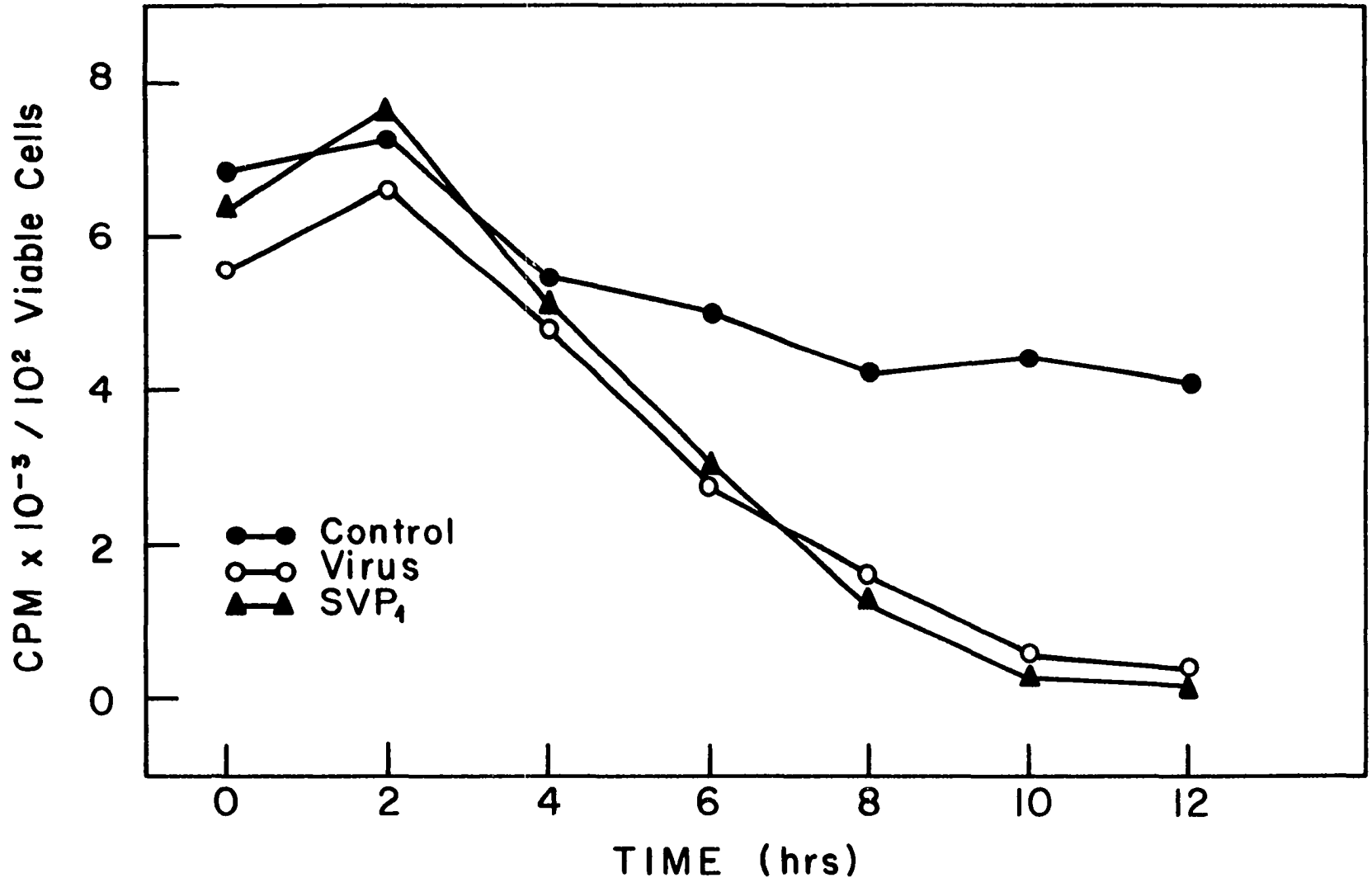
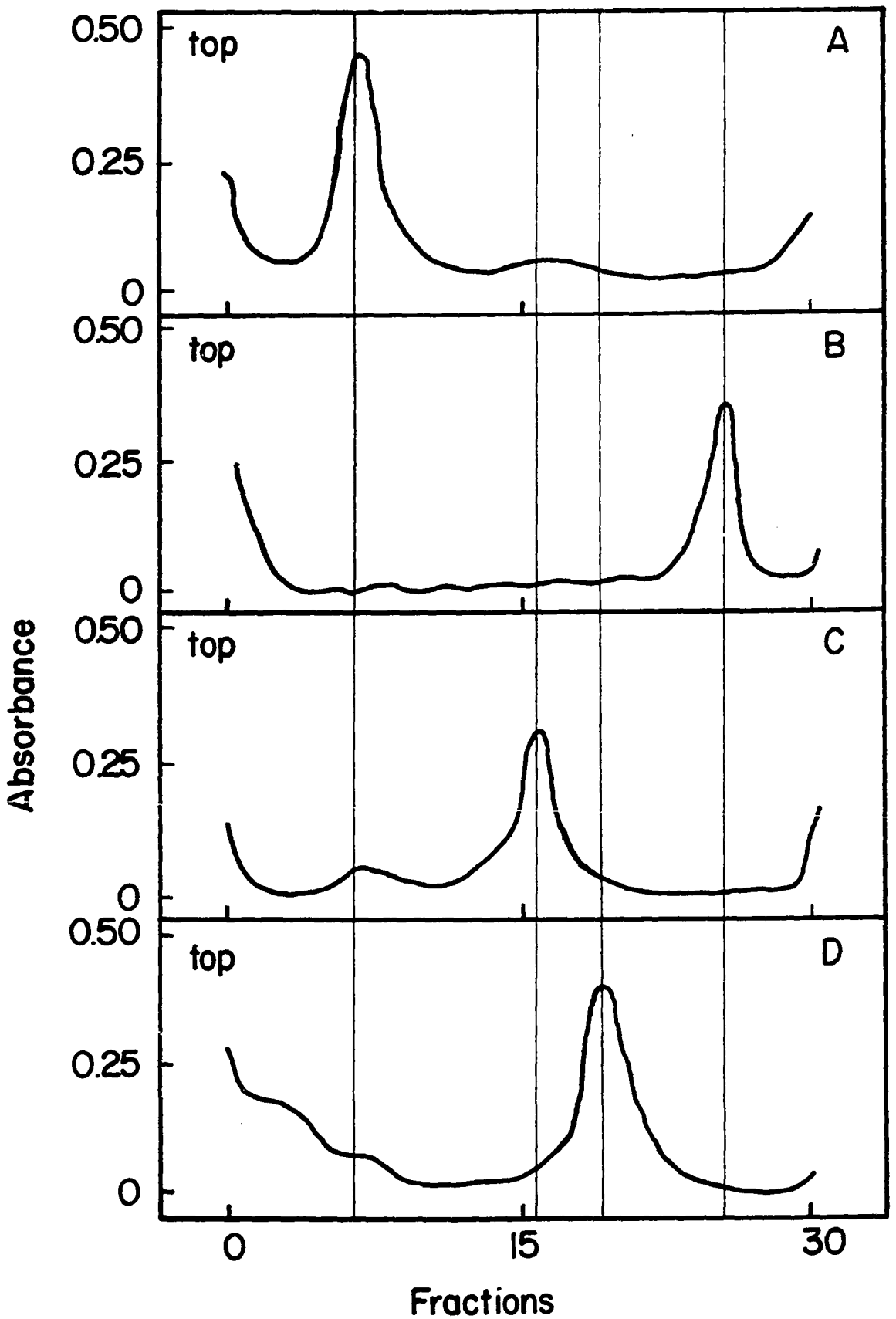

CPM x 10⁻³ / 10² Viable Cells
8
6
4
2
0
0
2
4
6
8
10
12
TIME (hrs)
Control
Virus
SVP1

Figure 5. A comparison of the equilibrium buoyant density of reovirus and selected subviral particles. Absorbance per gradient fraction is presented (A, Top component; B, Cores; C, reovirus; D, SVP_1).


0.50
0.25
0
top
A
B
C
D
Absorbance
0
15
30
Fractions

repeating the experiment described above, but at the time of each pulse-labeling an aliquot of cells was removed and stained with a vital stain (0.05% w/v neutral red) to compare the viability of cells in control, virus-infected, and SVP_1-treated cultures at each test period. These results are plotted in Figure 4.

There appeared to be no difference in the time course of the inhibition process in reovirus and SVP_1-treated cultures when relative cell viability was taken into consideration. At 12 hours post infection, both reovirus-infected and SVP_1-treated cultures showed approximately 10% of the DNA synthesis of control cultures.

Association of Subviral Particles with Cells

It was possible that the difference in the inhibition of DNA synthesis following treatment with complete reovirus or subviral particles was due to dissimilar uptake of different particles by cells.

To examine the possibility of a functional rather than structural difference between each of these subviral particles, radioactively labeled subviral particles were prepared and isolated as described previously. The percentage of cell associated radioactivity was determined over a period of 3 hours.

As shown in Figure 6, panel A, approximately 80% of the top component radioactivity became cell-associated in 3 hours while only 40% of the core particle radioactivity was associated with cells after the same time (panel B).

Panels C and D show essentially identical results, approximately 85% of particle radioactivity was cell-associated in 3 hours. Panel C represents radioactive reovirus while panel D represents radioactive SVP_1. This data did not indicate the physical state of these

Figure 6. A comparison of the cell association of various radioactive subviral particles. At the indicated times 5×10^6 cells were removed, washed three times with cold MEM, and precipitated with TCA. Percentage of the total TCA-insoluble radioactivity with time are presented. (A, Top component; B, Cores; C, Reovirus; D, SVP_1).

% Radioactivily Associated
0
25
50
75
100
A
B
C
D
TIME (hrs)
0
1
2
3

particles after cell association, but it is possible the lack of inhibition of DNA synthesis, at least following core treatment, may have been due to inefficient particle uptake.

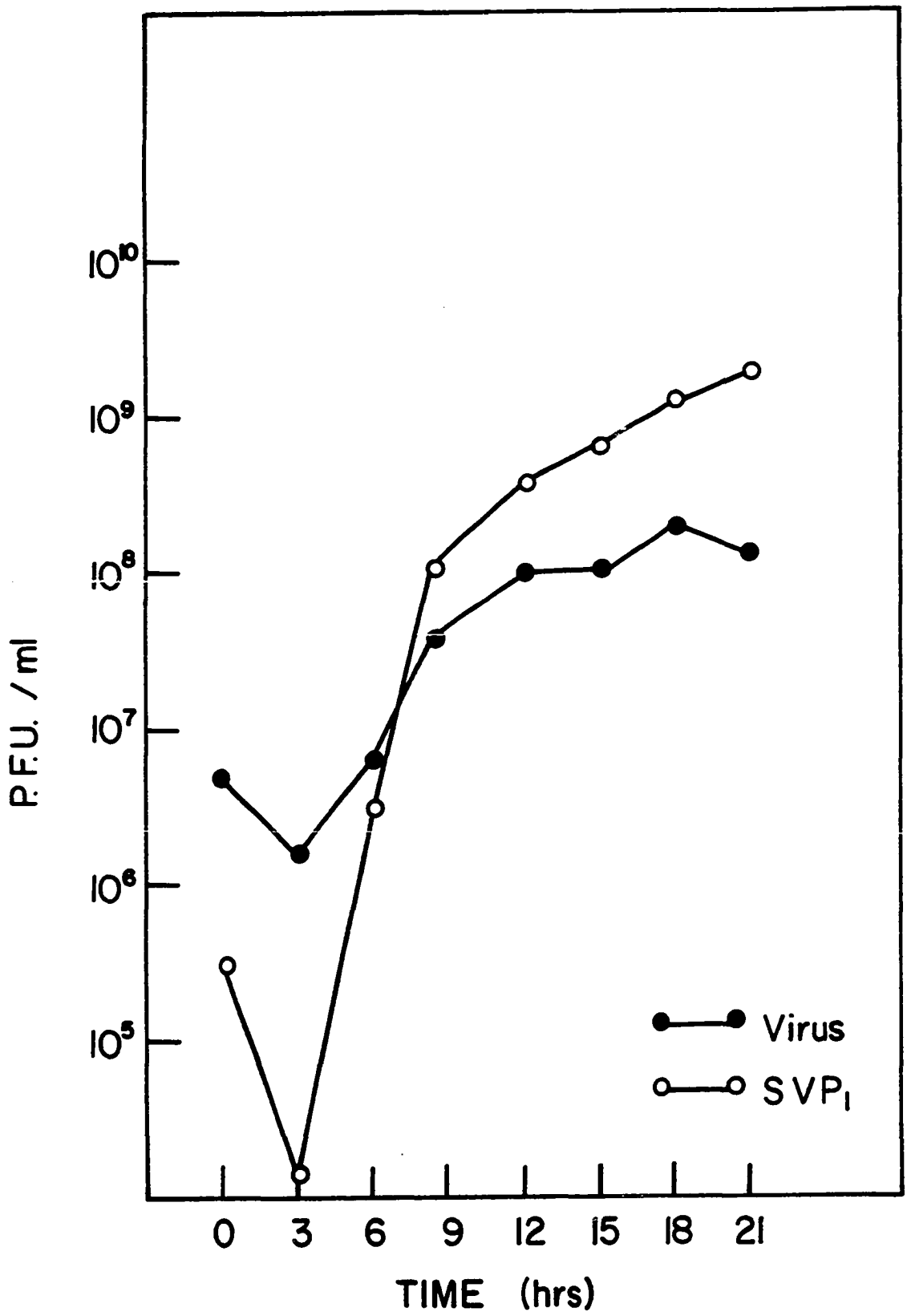
SVP_1 Growth Curve

Since reovirus and SVP_1 exhibited similar kinetics of inhibition of DNA synthesis at low multiplicities of infection, but differed in cytopathic effects, it was of interest to compare their relative rates of virus replication. Cells were infected with complete reovirus and SVP_1 and assayed every 3 hours for the presence of intracellular infective virus. The results of these experiments are shown in Figure 7. Only very minimal differences in their replication cycles were observed. While reovirus and SVP_1 produce infective particles at approximately the same time the final titers obtained in SVP_1-treated cultures were higher.

ss RNA Synthesis for SVP_1-Treated Cells

A similarity in reovirus and SVP_1 replication was observed. In addition, reovirus and SVP_1 showed almost identical kinetics of inhibition of DNA synthesis. In contrast, the cytopathic effect of SVP_1 on spinner cells was considerably reduced, therefore, it was of interest to investigate specific events during the replication cycle of reovirus and SVP_1. Previous reports (5,29) have suggested that viral-directed single stranded RNA serves as messenger-RNA which directs viral protein synthesis. Therefore this class of virus-induced ss RNA could be pulse-labeled with ^{3}H-uridine and analysed using sucrose gradients. The relative times of appearance and the rates of synthesis were investigated in spinner cells infected with reovirus and SVP_1. The

Figure 7. A comparison of the infectivity yields of spinner cells in cultures treated with SVP_1 and infected with reovirus at 50 PFU/cell. After adsorbtion the cells were washed twice with media to remove unadsorbed particles. Titers are expressed as PFU/ml of culture at selected times.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
P.F.U. / ml
0
3
6
9
12
15
18
21
TIME (hrs)
Virus
SVP$_1$

results of these experiments are shown in Figure 8.

In the interest of clarity the SVP_1-directed single stranded RNA synthesis was graphed with untreated control cell ss RNA synthesis. The virus-directed ss RNA synthesis was essentially identical and the period of time during which peak ss RNA synthesis occurred was 8-10 hours post infection with both SVP_1 and reovirus-infected cells. The time course of synthesis of ss RNA in SVP_1 and reovirus infected cells was comparable qualitatively and quantitatively with this assay system.

Effect of SVP_1 on Synchronous DNA Synthesis

SVP_1 was missing specific viral structural proteins and only 40% of the oligo A content remained. SVP_1 exhibited functional characteristics similar to complete reovirus such as 1) cell association, 2) rates of production of infective particles, 3) the time course of intracellular ss RNA synthesis, and 4) the kinetics of inhibition of DNA synthesis. In contrast the cell death rate was significantly reduced in cultures treated with SVP_1.

It has been suggested that reovirus prevents spinner cells from initiating the DNA synthetic phase (14). Therefore, it was important to investigate whether SVP_1 could also prevent entry into S phase of the cell cycle.

SVP_1 and reovirus were added to aliquots of synchronous cultures 8 hours before the onset of cellular DNA synthesis. Both SVP_1 and reovirus prevented initiation of S phase as shown in Figure 9.

The next question was how much time was required to prevent initiation of the S phase. This was preliminarily investigated by making assumptions which were consistant with previous data. First, the

Figure 8. The time coarse of synthesis of single stranded RNA of spinner cells treated with SVP_1 at 50 PFU/cell. (SVP_1; ····Control;). The cells were treated with SVP_1 and at selected times pulse-labeled with ^3H-uridine (A, 4-6 hours; B, 6-8 hours; C, 8-10 hours; D, 10-12 hours). Each sample was chilled, extracted, purified, and centrifuged in a sucrose gradient. Counts per minute of TCA-insoluble material in each fraction are presented.

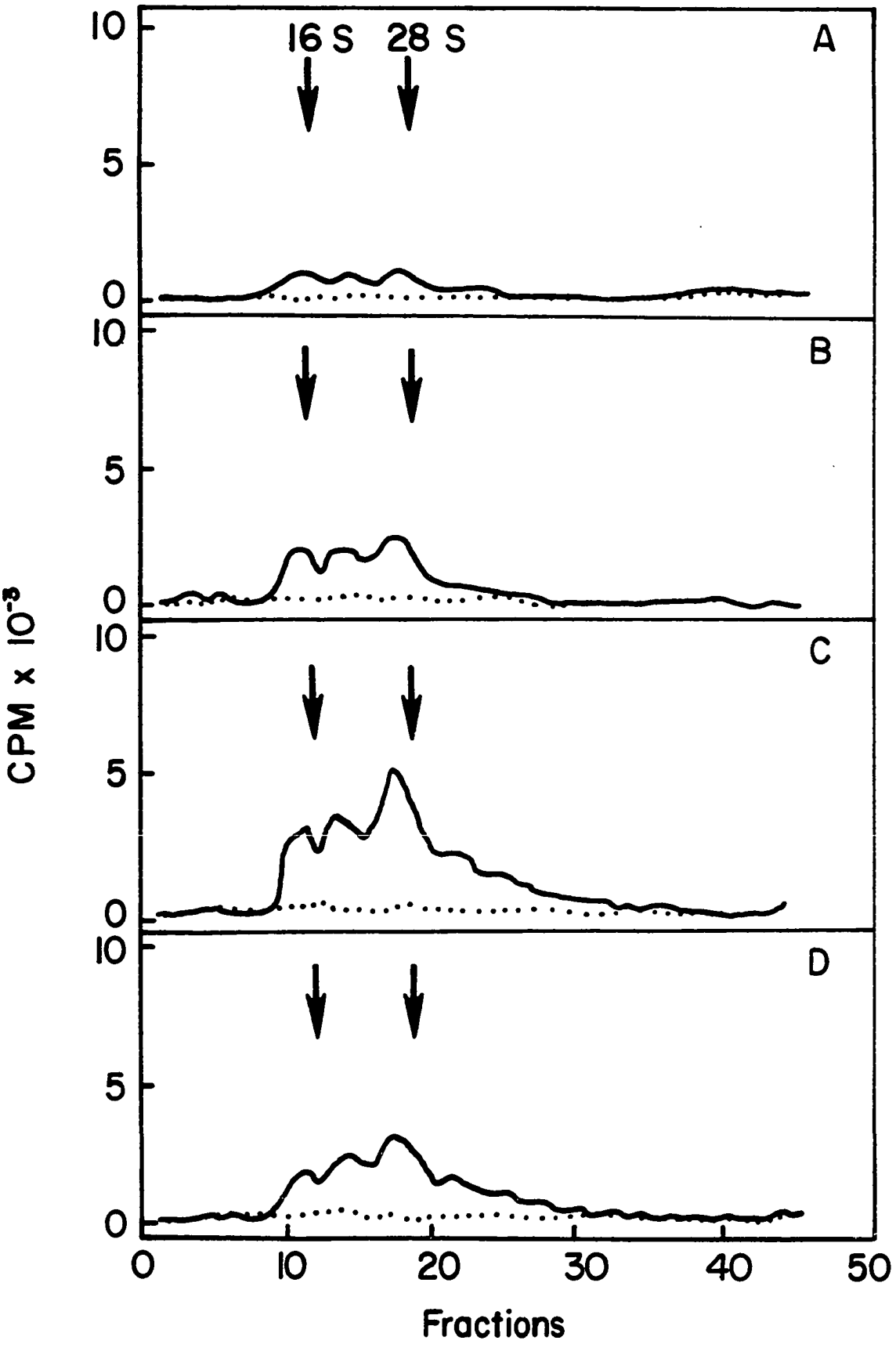
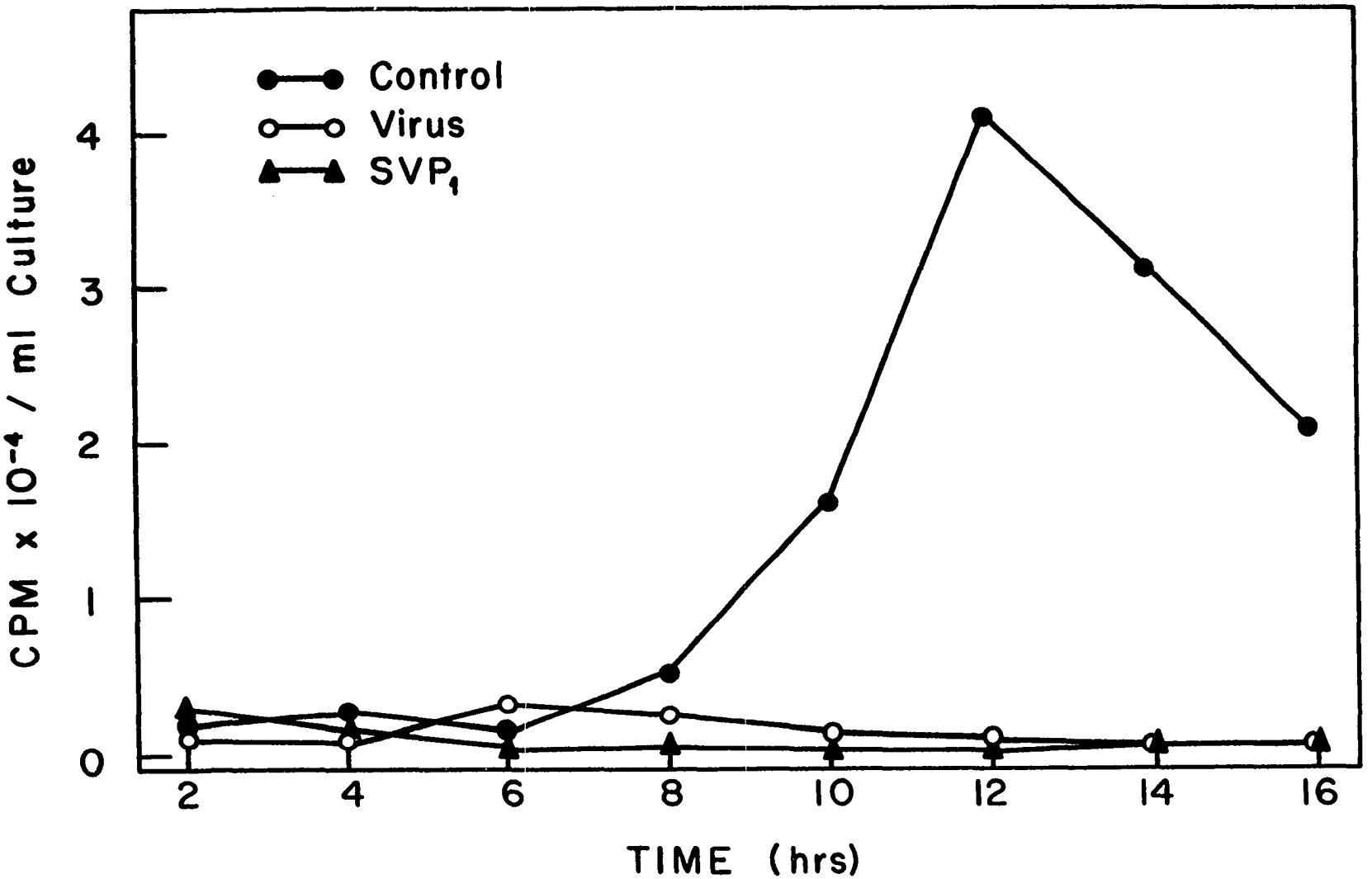

16 S
28 S
A
B
C
D
10
5
0
CPM x 10⁻³
0
10
20
30
40
50
Fractions

Figure 9. DNA synthesis of synchronized spinner cells treated with SVP_1 or infected with reovirus at 10 PFU/cell. Cells were pulse-labeled with ^3H-thymidine at the times selected. Counts per minute per ml of culture in the TCA-insoluble material are presented as an average of duplicate samples.


Control
Virus
SVP1
CPM x 10-4 / ml Culture
0
1
2
3
4
2
4
6
8
10
12
14
16
TIME (hrs)

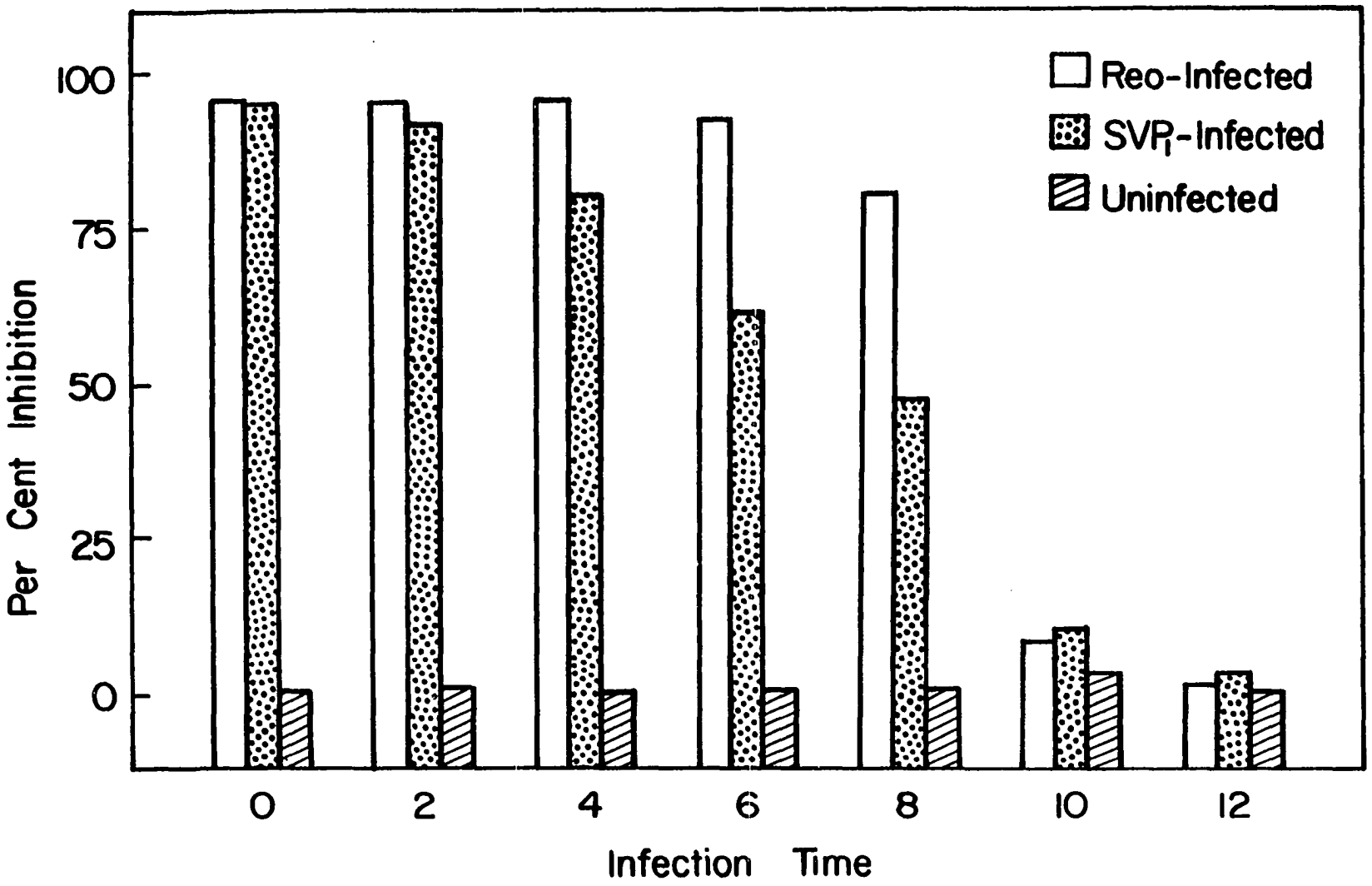
peak synthesis of DNA in synchronous cells occurred 12-14 hours after resuspension in fresh media and this would be an adequate index of whether initiation of S phase had taken place. Second, if DNA synthesis was not observed at 12 hours after resuspension some alteration would have occurred.

Synchronous spinner cells were resuspended and at selected times reovirus or SVP_1 were added and DNA synthesis was monitored at 12 hours after resuspension which was the normal peak of the DNA synthetic phase. The results are presented in Figure 10.

Control cultures always showed 100% DNA synthesis because these cells had entered S phase and at 12 hours were synthesizing DNA at maximal rate. As shown in Table 1, reovirus-infected cells are 80% inhibited when infected 8 hours after resuspension and only 9% inhibited if infected 10 hours after resuspension. Any time of infection with reovirus up to 8 hours after resuspension prevents significant DNA synthesis while after 9 hours DNA synthesis almost reaches control levels.

Control cultures were represented by 0% inhibition of DNA synthesis because these cells had entered S phase and at 12 hours were synthesizing DNA maximally. As shown in Figure 10, the DNA synthesis detected in reovirus-infected cells was at least 80% inhibited when infection occurred any time prior to the normal onset of S phase. In addition, infection with reovirus after this hour resulted in less than 8% inhibition when monitored during the time of peak DNA synthesis. A time of infection with reovirus up to 8 hours after resuspension prevented significant DNA synthesis, while after 10 hours DNA synthesis

Figure 10. A comparison of the per cent inhibition of synchronized spinner L cells treated with SVP_1 or reovirus. At various times after resuspension aliquots of cells were treated with SVP_1 or reovirus and the graph presents the per cent inhibition compared to control levels. 12 hours after resuspension approximately 10^6 cells from each culture were pulse-labeled with ^3H-thymidine.


Reo-Infected
SVP1-Infected
Uninfected
Per Cent Inhibition
100
75
50
25
0
0
2
4
6
8
10
12
Infection Time

almost reaches control levels.

The inhibition of cellular DNA synthesis in SVP_1-treated cells showed a progressive decrease the later SVP_1 was added to the cells. There was not a sharp decrease in the inhibition as shown after reovirus infection. It appeared complete reovirus inhibits the initiation of the cellular DNA synthetic phase to a greater degree and required less time to accomplish the inhibition than SVP_1.

CHAPTER IV

DISCUSSION

Alteration of host cell macromolecular function by structural component(s) of animal viruses and bacteriophage have been reported. The most definitive study of this type has been performed using adenovirus. Both the adenovirus fiber and penton components were found to disrupt cellular macromolecular synthesis (7, 42).

Reovirus has been shown to specifically alter cellular DNA replicative function. After infection with reovirus, cellular DNA synthesis progressively decreases until it reaches a minimum 12 hours post infection. Hereafter this progressive decrease in cellular DNA synthesis will be referred to as 'the inhibition'.

The time of the beginning of 'the inhibition' after reovirus infection appears to be dose-dependent and related to the number of physical particles associated with infected cells (13, 62). The results presented here seemed to indicate a requirement for infectivity of viral particles to mediate 'the inhibition' process. This was supported by several experimental observations, 1) the polypeptides composing top component were not capable of mediating 'the inhibition', 2) core particles containing the inner capsid polypeptides and the complete ds RNA genome were similarly unable to mediate 'the inhibition', 3) in every preliminary experiment with SVP_1, when infectivity was lost concomittant

loss of inhibitory capacity was observed, and 4) infectious SVP_1 mediated a progressive decrease in cellular DNA synthesis and this SVP_1-directed inhibition appears to be identical with complete reovirion-directed inhibition. Therefore, the components of the SVP_1 (Table 1) seemed to be required for efficient infection and mediation of 'the inhibition'. SVP_1 contains the complete ds RNA genome and approximately 40% of the (A-rich) oligo A composition found in reovirions and 5 specific polypeptides (61). It is of interest to note that the one subviral particle (SVP_1) which exhibits 'the inhibition' is the only subviral particle which retains a portion of the oligo A content. This and other work in our laboratory continues to indicate a role of reovirus A-rich RNA in inhibition of cellular DNA synthesis. In addition, a σ_1 polypeptide may be retained in SVP_1, but because of the small quantity it is difficult to detect. μ_2 polypeptide is cleaved with chymotrypsin to a polypeptide (G) which is 80% its original molecular weight (61) and seems essential for infectivity.

Elucidation of the biochemical mechanisms through which rec-virions mediate 'the inhibition' of cellular DNA replicative function becomes increasingly complex. These results indicate a requirement for the particles to be infectious, and therefore possible explanations for the inhibition by UV-irradiated reovirus could be 1) the limited function of virion components after UV-irradiation without production of mature virions or 2) the inhibition of DNA synthesis due to a general cytotoxic effect and not the viral-mediated specific inhibition or 3) a combination of both of these.

The possibility of cytotoxic effects was supported by work with other virus-cell systems (8, 51, 52, 73) and with reovirus type

Table 1. A Comparison of the Structural Components of Specific Subviral Particles

Components	Reovirions (64)	Top Component (64)	Cores (64)	SVP_1 (61)
I. Nucleic Acid				
1. ds RNA	1.00	——	1.00	1.00
2. oligo A	1.00	——	——	0.40
II. Polypeptides				
1. λ_1	1.00	1.00	1.00	1.00
2. λ_2	1.00	1.00	1.00	1.00
3. μ_1	1.00	1.00	1.00	1.00
4. μ_2	1.00	1.00	——	G [1]
5. σ_1	1.00	1.00	——	? [2]
6. σ_2	1.00	1.00	1.00	1.00
7. σ_3	1.00	1.00	——	——

1. Reduction in the size of μ_2 to approximately 80% of original radioactivity (61).
2. Appears to be retained, but quantitation is difficult (61).

2 (44, 45). In most cases a large virus inoculum was required to produce this effect (30) and only a large dose of reovirus was used (13, 62). In contrast to this explanation, it was recently reported that after 60 minutes of UV-irradiation the cytotoxic effect of reovirus type 2 was lost (68). However, reovirus irradiated for 60 minutes did not lose its capacity to inhibit DNA synthesis. Thus it seemed that reovirus or any altered particles of reovirus must have specific components with at least limited functionality in order to modify host DNA functions.

It was observed early in the experiments with SVP_1 that the death rate of cells treated with SVP_1 was not as rapid as the death rate of cells infected with complete reovirions. It was of interest to compare various characteristics of the SVP_1 and reovirus replication cycles. The characteristics studied to date are 1) the time-course of production of infectious particles, 2) the time-course of synthesis of ss RNA isolated from actinomycin D treated, SVP_1 treated or reovirus infected cells, and 3) the ability of SVP_1 to prevent initiation of cellular DNA synthesis in synthronized cells treated 8 hours before initiation of S phase. In all cases, these characteristics were very similar for SVP_1 and reovirus.

A significant difference was noted in the time required for the SVP_1 to prevent initiation of DNA synthesis in synchronized cells. It appeared that reovirus could prevent initiation of DNA synthesis when added 2 hours prior to the onset of DNA synthesis. In contrast, SVP_1 showed significantly less efficient inhibition when added 2 hours prior to the onset of DNA synthesis. In contrast, SVP_1 showed significantly less efficient inhibition when added 2 hours prior to S phase.

Previous reports had suggested that no new initiation points of DNA synthesis were observed after infection although previously initiated sites of synthesis continued (27). This was consistant with the data presented here and seemed to indicate a multiphasic nature of 'the inhibition' process. It was possible that the input particles begin 'the inhibition' by the solubilization or release of viral component(s) into the cytoplasm of the host cells.

Therefore, the dose response can be explained by assuming an increase in the quantity of viral components released with increase in number of particles entering the cell. Next, 'the inhibition' process could be maintained by the synthesis in the cytoplasm of new viral components which mediate the inhibition. At low multiplicities of reovirus or SVP_1, inhibition was detected at 6–8 hours post-infection which precedes maximum viral component synthesis but nevertheless, synthesis was detectable. Conversely, at high multiplicities of reovirus or SVP_1, the release of viral components (i.e. oligo A and/or outer capsid polypeptides) was increased and then 'the inhibition' process was observed earlier. It then would be possible that, in later stages of the replication cycle, the newly synthesized viral products were involved in 'the inhibition' process. As was shown in Figure 10, initiation and continuation of S phase was prevented more effectively by complete reovirus than incomplete SVP_1. Because of the absence of a portion of the oligo A (A-rich) RNA from the SVP_1, and the presence of a modified outer capsid polypeptide (G), the specific molecule(s) which mediated the inhibition process cannot as yet be defined. It could be either one or a combination of oligo A RNA or the modified capsid polypeptide (G).

In conclusion, it was not clear which specific molecule(s) present in complete reovirions and SVP_1 were responsible for 'the inhibition' although there was evidence which suggests the oligo A RNA components played a role in this alteration of cellular replicative function. First, oligo A component was present in complete reovirions and approximately 40% of this oligo A RNA remains SVP_1 associated. Second, oligo A component should be present in ultraviolet irradiated reovirus and since RNA of polyadenylic acid composition should be resistant to damage from UV-irradiation. Third, L cells in monolayers have been shown to exhibit an inhibition of DNA synthesis when exogenous purified oligo A was added to the cultures (49). Fourth, synchronous cultures of L cells are prevented from initiating S phase after infection with complete reovirus as late as 2 hours before the onset of DNA synthesis, while the SVP_1 containing reduced amounts of oligo A RNA cannot prevent significant DNA synthesis when added 2 or 4 hours prior to initiation of the S phase.

These data did not explain the details of the mechanism for the inhibition of cellular DNA synthesis after reovirus infection, but supported the role of the oligo A component in the direct or indirect inhibition of cellular DNA synthesis.

CHAPTER V

SUMMARY

This study demonstrated the effect of reovirus subviral particles on cellular DNA synthesis. Reovirus ghost particles or top component devoid of nucleic acid did not affect DNA synthesis. Similarly, reovirus core particles with the outer capsid polypeptides digested did not alter cellular DNA replicative function. In addition, these subviral particles were not infective.

Approximately 85% of the radioactivity of top component was cell associated and 38% of radioactive core preparations were cell associated in 3 hours. Therefore, this could not explain the lack of inhibition of cellular DNA synthesis.

Treatment of purified reovirus with chymotrypsin in the presence of specific cation requirements results in an infectious subviral particle (SVP_1). The SVP_1 was missing specific outer capsid polypeptides and 60% of the virion associated A-rich RNA content. The SVP_1 did inhibit DNA synthesis as efficiently as the intact reovirions. A comparison of the intact reovirions and SVP_1 showed similar growth patterns and the time-course of single-stranded RNA synthesis was the same.

When L-cells were synchronized and infected with either SVP_1 or complete reovirions 8 hours before S phase of the cell cycle, the

treated cells failed to enter the DNA synthetic phase. A significant reduction was observed in the cytocidal properties of the SVP_1 when compared to intact reovirus.

The changes observed in the cytocidal properties of SVP_1 suggested the inhibition of DNA synthesis was independent of the cytopathology. Specific capsid polypeptides were not essential for a productive infection or inhibition. These results suggested, at least, limited transcription and/or translation are required for inhibition of cellular DNA synthesis to occur in mouse L-cells.

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