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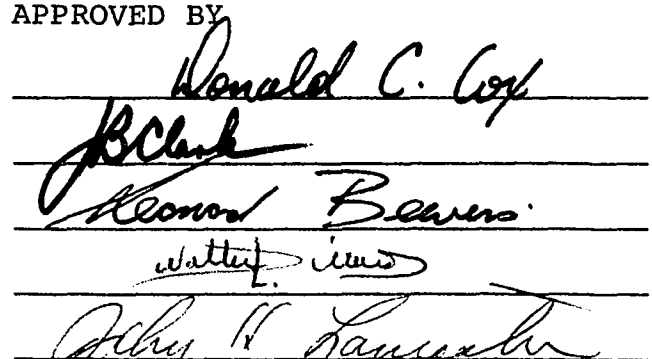
CHARACTERIZATION OF THE INHIBITION OF CELLULAR DNA SYNTHESIS BY REOVIRUS ADENINE-RICH RNA

APPROVED BY



DISSERTATION COMMITTEE

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In the name of Allah.

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CHAPTER I

INTRODUCTION

The sequence of events affecting a eukaryotic cell emerging from a division up to the end of the following division is called the cell cycle. The cell cycle consists of four phases: G_1, first gap; S, synthesis; G_2, second gap; and M, mitosis phase. During the S phase, the deoxyribonucleic acid (DNA) is replicated by a semiconservative mechanism. The chromosome is duplicated in a specific order (67) and the "Satellite" DNA's situated near the centromere of the chromosome are replicated last (25,68). Replication is initiated at the center of a replication unit and chain growth proceeds bidirectionally toward outlying termini where the newly replicated chains fuse with those synthesized in adjacent regions of DNA. At the completion of the S-phase of the cell cycle, long progeny DNA molecules, characteristic of the eukaryotic chromosome, have been synthesized (35,36). It has also been demonstrated both in bacterial and mammalian cells that initiation of DNA starts with the synthesis of an RNA

priming fragment by DNA-dependent RNA polymerase and the fragment is then extended by the DNA polymerase. The RNA primer fragment is later recognized and excised, possibly by the exonuclease activity of the DNA polymerase I (42). Studies of the initiation and termination of RNA primer and DNA chain elongation have not provided sufficient information to characterize the control of these processes and the enzymes involved.

In mouse L-cells, functioning initiation sites for DNA synthesis are distributed in clusters. The modal interval between individual sites is 40 to 50 μm (33). Infection with reovirus results in inhibition of cellular DNA synthesis in 6-8 h after infection and before the onset of cytopathologic effects (23,28,30,31). In infected cells the distance between initiation sites is increased (33), and in synchronized L-cells the initiation of the synthetic phase is completely inhibited in cells infected 8 h before the beginning of DNA synthesis (19). These observations make this cell-virus system a promising model to elucidate cellular DNA replicative control mechanisms.

The physical characteristics and the replication of the reovirion has been studied in detail. The reovirion has icosahedral symmetry and possesses two capsid shells composed of proteins. The outer shell proteins can be completely hydrolyzed by chymotrypsin leaving the inner reovirus shell intact, which is known as the core (57,64). The genome of reovirions consists of double stranded (ds) ribonucleic acid (RNA) having a molecular weight of 15 x 10^6 daltons per virion (29). It has been

established that the genome RNA consists of a heterogeneous population of 10 distinct ds-RNA fragments; three large, three medium and four small, with approximate molecular weights of 2.5×10^6, 1.4×10^6 and 0.8×10^6 daltons respectively (11,55, 59,70,72,74). Sequences of the 5´ and 3´ termini of genome RNA segments have been reported to be (p)ppGpPyp- and $-pPypApApC_{OH}$ (p = phosphate, Py = Pyrimidine) respectively (1,2). This strongly indicates that the ds-RNA segments are perfect duplexes which arise as the result of independent synthetic processes (1,2,3). Recently it has been reported that both m-RNA and the corresponding plus strand in the genome RNA contain a blocked and methylated 5´ terminus of the structure $m^7G(5´)ppp(5´)G^mpCp$---(16,27).

Reovirions also contain, in addition to the genome ds-RNA, single-stranded (ss) RNA of low molecular weight, which comprises up to 25-50% of the total amount of RNA in reovirions. The ss-RNA is rich in adenine nucleotides, and is referred to as adenine-rich (A-rich) RNA (8,41,58,65). The ss-oligoribonucleotides are 2-20 nucleotides long and are present in the central virion cavity. These oligonucleotides are lost when the outer capsid shell is degraded (6,7,38). The ss-oligonucleotides have been fractionated by DEAE-cellulose column chromotography and two-dimensional electrophoresis (10,48,65). Chromotography separated the oligonucleotides into two classes of molecules: (1) a series of molecules in which the only nucleotide is adenylic acid. These molecules

range in length from 2-20 nucleotides and have either ppp, pp or p at their 5′ termini, and can be represented by the formula $(p)(p)p(A)_{1-19}A_{OH}$. There as about 850 such molecules present in each virion and they account for some 55% of the total oligonucleotides. This class is referred as the reovirus oligoadenylates (10,48,65); (2) the second series of oligonucleotides with sequences $(p)ppGC_{OH}$, $(p)ppGCU_{OH}$, $(p)ppGCUA_{OH}$, $(p)ppGCUA(A)_{1-3}A_{OH}$, and $(p)ppGCUA(U)_{1-4}U_{OH}$, are called the reovirus 5′ -G-terminated oligonucleotides. This fraction of ss-oligonucleotides will be referred in this dissertation as heterologous oligonucleotides. There are approximately 2000 of these molecules in each virion. In addition there are some 350 other molecules from 2-8 nucleotides long (approximately 10% of the total) for which the sequences have not been determined. In total there are 3200 oligonucleotide molecules having a total molecular weight of $4-5 \times 10^6$ daltons in each reovirion. It has been proposed that these oligonucleotides are the products of abortive transcription by the enzyme RNA-transcriptase during the final stages of morphogensis of progeny virions (10,48). Recently, oligo(A) polymerase activity has been identified in reovirions. The enzyme catalyzes <u>in vitro</u> synthesis of oligo-A similar in size to that in virions by a primer-independent mechanism (14,61). Oligonucleotides are not found free in the cells, but are always associated with viral and subviral particles and are synthesized in all reovirus-infected cells (8,58).

In purified reovirions five enzymatic activities have been described:

1. The ds-RNA dependent ss-RNA polymerase, the transcriptase, which is a part of the capsid. It is inactive in the intact virion, and can be activated by partial removal of the outer capsid shell polypeptides (12,51,57). The ss-RNA dependent ds-RNA polymerase, the replicase, which is associated with subviral particles have been isolated and characterized (47,51). However, it has been suggested that the two enzyme activities may not be completely autonomous. The replicase could conceivably be converted into the transcriptase when the enzyme template complex associates with additional protein to form the subviral particle.

2. Reovirus cores can catalyze an exchange reaction between the four ribonucleoside triphosphates and inorganic pyrophosphate (71).

3. Cores also possess a nucleoside triphosphate phosphophydrolase activity which liberates inorganic phosphate from all common ribo- and deoxyribonucleoside triphosphates (13,40).

4. As described previously, reovirions contain oligo-A polymerase activity which is absent in cores. It has been suggested that oligo-A polymerase may be an alternative activity of the virion bound transcriptase and may be regulated by outer capsomere proteins (61).

5. Cores possess m-RNA modifying activities, and these are the capping activity and two methylases that result

in the formation of transcripts with blocked, methylated 5′-ends: m^7GpppG^mpC---(27).

Replication of reovirions begins when they are taken into the cell by a process of pinocytosis. The phagocytic vacuole then fuses with lysosomes. The outer capsid is partially degraded by lysosomal proteases, converting the virions into subviral particles (SVP), which are then transferred to perinuclear regions of the cell. In this area they appear as cytoplasmic inclusions and are referred to as "factories" which are the sites of accumulation of progeny viral RNA and proteins (20,63). Reovirus genome RNA is not uncoated, but remains within the SVP throughout the multiplication cycle (15,62). Partial degradation of the outer capsid shell results in full transcriptase activity of the SVP (15,45). Transcription leads to the production of two classes of m-RNA, defined according to the time of synthesis and the nature of the particle which synthesizes them during the infectious cycle. These are the early and late m-RNA's produced by the parental SVP and the progeny immature virus particles (9,50). Both early and late m-RNA's are transcribed by the virion associated transcriptase (37,73,75). The same enzyme has been proposed to be responsible for the synthesis of reovirus A-rich RNA, a component of the virion. The A-rich RNA is produced between 9 and 13 h after infection of L-cells (8). It is of interest to note that the first appearance of A-rich RNA coincides with the start of the inhibition of cellular DNA synthesis in the infected cells. The rate of

total protein synthesis does not decrease for several hours following infection (22,28,43). Reovirus specified proteins are synthesized very slowly during the early period of infection, but when the rate of synthesis of late m-RNA increases, virus specified protein synthesis predominates over host protein synthesis (78).

Since the reovirus genome is not uncoated, the information for the synthesis of progeny genome RNA and for viral protein synthesis is furnished by plus RNA strand transcripts. These plus RNA strands act as m-RNA's early in infection and as template for the synthesis of minus strand during later stages of infection. The newly synthesized minus strand does not separate from the plus stranded template (53). The genome ds-RNA is formed sequentially in particles with the S segments formed first and the L segments formed last. After the progeny genome ds-RNA is formed it serves as a template for late m-RNA synthesis (77). The syntheses of ds-RNA and ss-RNA reaches maximum levels 6-8 h after infection and then declines. Progeny virus is detectable 8-10 h after infection and in spite of severe cytopathic effects, most of the progeny virions remain cell associated (39).

As mentioned previously, infection of L-cells with reovirus brings about inhibition of cellular DNA synthesis 6-8 h after infection and at this time no cytopathology is observed (18,28). Experimental results indicated that initiation of synthesis of DNA upon new regions of chromosomal DNA replication is blocked (23,28,31,33), but reovirus infection

does not alter DNA chain growth (31). It may be postulated that the DNA inhibition observed could be the result of reduced activity of DNA synthesizing enzymes. However, no decrease in DNA synthesizing enzymes was found at 12 h post infection, a time when cellular DNA synthesis is 90% inhibited (23,60). Bartkoski has reported that neither the concentration nor activities of nuclear and cytoplasmic DNA polymerases were altered significantly at times after inhibition of DNA synthesis in reovirus infected cells. Also when nuclei, chromatin or hypotonically swollen cells were assayed for in vitro DNA synthesis, no difference was seen between infected and uninfected cells (4). The DNA inhibition is not due to degradation of host DNA or due to inactivation of DNA (at least in vitro), or loss of efficiency as a template or in vitro DNA synthesis, because these functions were unaltered when tested in vitro (4,23,30). It has also been shown that decreased thymidine incorporation in the infected cells represents a real decrease in DNA synthesis (24,60), and that DNA inhibition is not the result of depletion of precursor molecules (23,30,60).

Since infection causes an early decrease in DNA synthesis without concomittant decrease in total protein synthesis (22,28,60,78), this information suggests the action of reovirus on DNA replication may be direct and specific for one or more functions associated with initiation events for DNA replication.

The inhibition of initiation of DNA synthesis is a phenomenon observed in L-cells, which is a transformed cell

line. Duncan (21) has recently reported similar inhibition of DNA synthesis in transformed human diploid WI-38 cells. But no such inhibition was seen in the infected normal WI-38 cells. No cytopathic effects were observed up to 14 days post-infection while the cells appeared to be persistently infected by the virus (21). These observations indicate a selective recognition and inhibition of DNA synthetic mechanisms of only the transformed cells, by reovirus components and/or replication products.

The inhibition of DNA synthesis is a dose-dependent phenomenon (18). Using a multiplicity of infection of 1000 plaque-forming-units per cell, the inhibition of DNA synthesis began 2-4 h after infection and the initial rate of inhibition increased. Reovirus irradiated with ultraviolet light sufficient to abolish infectivity but not transcriptase activity, inhibits DNA replication in L-cells as efficiently as infectious virus (18,60). This suggests that either a component of the virion is capable of causing the inhibition or that the RNA genome may remain partially functional after UV-irradiation and give rise to an inhibitory component. A limited amount of viral RNA is transcribed in cells infected with UV-irradiated virus and it has been suggested that it may be the A-rich RNA (32).

However, we can not exclude the possibility that viral capsid proteins may also be inhibitory for DNA synthesis. Reovirus empty capsids (top-component) devoid of ds-RNA and

A-rich RNA can be easily separated from the virion by cesium chloride density gradient centrifugation. Reovirus top-component had no inhibitory effect on cell RNA, protein or DNA synthesis even at doses as high as 5000 particles per cell (17,32,44). This again suggested that A-rich RNA or genome ds-RNA might be responsible for inhibition of DNA synthesis. Another incomplete particle, the infectious subviral particle (SVP_i) can be obtained by limited chymotrypsin treatment of reovirions (38). This enzyme treatment results in the partial loss of the outer capsid polypeptides and 60% of the A-rich RNA, but has the full complement of genome ds-RNA. The SVP_i produced a delayed inhibition of DNA synthesis (17). Since A-rich RNA is the only virion nucleic acid released early in infection, these observations suggest that A-rich RNA may be the viral component capable of mediating DNA inhibition in L-cells. This supposition is also substantiated by an earlier report by Clinkscales (17) that reovirus cores, which have lost all of the A-rich RNA complement, do not inhibit DNA synthesis in L-cells.

It was found that reovirus A-rich RNA was capable of mediating a selective inhibition of cellular DNA synthesis (46). Inhibition began 3-4 h after treatment with A-rich RNA (1 µg/ml). However, this inhibition was transitory and cellular DNA synthesis returned to control levels in 9-10 h after infection

Reovirus A-rich RNA is the only single virion component tested which inhibits cellular DNA synthesis (32,46). Two

hypotheses can be suggested which are consistent with the inhibitory role of A-rich RNA during reovirus infection: first, the increased efficiency of inhibition resulting from high input multiplicities may be the result of the release of increased amounts of an input virion component, i.e. the A-rich RNA; and second, that the increased input multiplicity results in higher yields of progeny virions and A-rich RNA which in turn mediates increased efficiency of inhibition. To investigate these possibilities two types of experiments were conducted.

1. Cells were treated with a range of high doses of purified reovirus A-rich RNA. The objective of such experiments was to determine if higher doses of A-rich RNA could mediate an increased rate and magnitude of inhibition of DNA synthesis.

2. Cells were treated with a selected range of nucleotide doses of A-rich RNA at various time intervals. Hopefully this would simulate the conditions of natural virus infection whereby a constant amount of A-rich RNA is synthesized by the progeny SVP causing a sustained inhibition of DNA synthesis.

Earlier work indicated that synthetic polyadenylic acid having the same molecular weight as reovirus A-rich RNA had no effect on the inhibition of cellular macromolecular synthesis (46). This suggests that the heterologous oligonucleotide fraction (5′-G-terminated oligonucleotide) of the

A-rich RNA may be the inhibitory component and that the oligoadenylate fraction has no effect on cellular macromolecular synthesis. To test this hypothesis experiments were conducted with endo- and exoribonuclease-treated reovirus A-rich RNA in order to selectively abolish the inhibitory activity of specific A-rich RNA fractions. Thus, this dissertation attempts to further characterize the selective modification of cell DNA function by specific reovirus A-rich RNA oligonucleotides.

CHAPTER II

MATERIALS AND METHODS

Cells. Mouse fibroblasts, strain L-929 (52) (Flow Laboratories), adapted for growth in spinner culture, were used in all experiments. Stock cultures of L cells were maintained in minimal essential medium for spinner culture (MEM) (Grand Island Biological Supply Company) supplemented with 5% (v/v) heat inactivited fetal calf serum (FCS, Grand Island Biological Company). Cells were maintained in logarithmic growth at cell densities from 5.0×10^4 cells/ml to 2.0×10^5 cells/ml by dilution every 48 h with fresh, pre-warmed growth medium.

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

Determination of Cell Viability. Cell viability at times after treatment was determined by trypan blue exclusion (49,54). Non viable cells were those that stained blue. At timed intervals growth medium was removed from the treated

monolayers, and the cell sheet washed twice with 5 ml MEM (no serum) to remove all serum. Then 2 ml of MEM (no serum) and 0.2 ml of a 0.4% (w/v) trypan blue solution was added to the flask. Incubation was for 5 min at 37 C. The solution was removed and the cell sheet examined by light microscopy. Cells which excluded trypan blue and thus were unstained were considered viable. Total cells and cells stained blue were counted in ten random fields to determine percent viability.

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 from an infected L cell monolayer. The agar plug, picked with a small pipette, was transferred to 1 ml of sterile 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.5 (SSC) and maintained at 4 C for 24 h. Virus eluant in SSC was then pipetted onto one confluent L-cell monolayer (25 cm^2 surface) from which the medium had been removed. One hour was allowed for virus adsorption and the SSC was replaced with fresh, prewarmed growth medium. After incubation at 37 C for 36 h, the infected cells were sonicated and the resulting first-passage lysate was used to infect 10 large monolayers (75 cm^2 surface). After a 24 hr incubation these monolayers were sonicated and pooled, and the lysate was then titered, and frozen at -20 C

Virus stocks for purification were prepared by concentrating L-cells to 1 x 10^7 cells/ml in MEM without serum. The cells were infected with 10-20 plaque forming units (PFU)/

cell and allowed to adsorb at 37 C for 1 h. The infected cells were then diluted to 1 x 10^6 cells/ml with growth medium. After 24 h incubation at 34 C, the cultures were chilled and the infected cells collected by centrifugation at 5000 x g for 15 minutes. The pellets were frozen at -20C.

Virus purification. Reovirus was purified as described by Smith, et al. (64) with modification. All centrifugation and extraction was done at 4 C. The organic phases from the first three extractions were pooled and extracted 2-3 times with an equal volume of homogenizing medium. All the aqueous phases were mixed and re-extracted twice with freon. The final separated aqueous phase was layered onto a 10 ml CsCl density gradient (1.2 g/ml-1.4g/ml) in a 38 ml centrifuge tube. This was centrifuged at 4 C for 1 h at 76,600x g in the SW 27 rotor using a Beckman L2-50 preparative ultracentrifuge. The virus banded at a density of 1.37 g/ml and the band was collected cleanly with a syringe. The CsCl was removed by exhaustive dialysis against cold SSC or by pelleting the virus by centrifugation for 1 h at 82,400x g in SW 27 rotor. The virus was layered onto a 15 ml 20-40% (w/w) glycerol gradient in 0.02 M Tris pH 8.0, which had been equilibrated at 4 C for 15-20 h in a 17 ml tube. Centrifugation was performed for 45 min at 82,500x g in the SW 27.1 rotor. The virus band was collected and dialyzed exhaustively against cold SSC. This was a purified stock virus suspension and was

stored at 4 C. On the average 20-30 mg of purified reovirus was obtained from 1 liter of infected cells.

Virus assay. Reovirus was assayed on confluent monolayers of L-cells as described by Shaw (60). Cells from spinner culture were adjusted to a density of 4×10^5 cells/ml and pipetted into small plastic tissue culture plates (60 x 15 mm, Falcon Plastics) at 2.0×10^6 - 2.4×10^6 cells/plate. The cells were allowed to grow to near-confluency.

Dilutions of reovirus were prepared in MEM (no serum). Medium was removed from the monolayers and 0.1 ml of each virus dilution was pipetted onto the center of the monolayer. The plates were incubated for 1 h at 39 C in a humid atmosphere of 5% (v/v) CO_2 in air to allow for virus adsorption. Five ml of autoclavable MEM (Auto Pow, Flow Laboratories), containing 3% (v/v) FCS, 1% (w/v) agar (Difco Laboratories), and 100 ug/ml kanamycin was added to the plates and allowed to solidify. The plates were incubated at 39 C in an atmosphere of 5% (v/v) CO_2 in air for 72 h. At this time a second overlay containing 0.005% (w/v) neutral red was added. Plaques were counted within 24h after addition of the second overlay.

Purification of reovirus adenine-rich RNA. Extraction of total RNA from purified reovirus suspended in SSC containing 0.5% sodium dodecyl sulfate (SDS) was carried out by phenol extraction (48). The procedure is outlined on the flow sheet Fig. 1. Phenol-extracted RNA solution was extracted with ether to remove residual phenol. The dissolved ether was

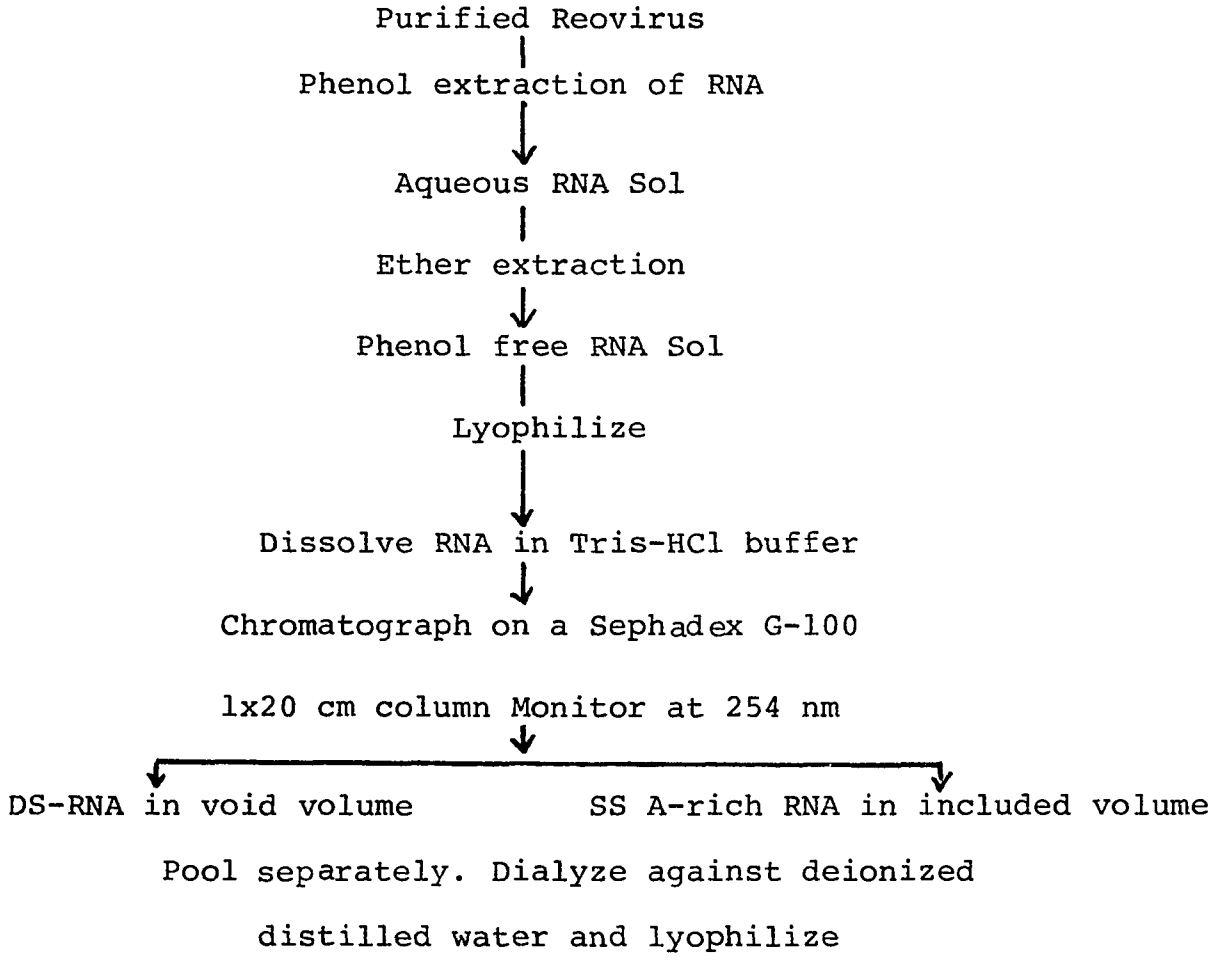


Fig. 1. Purification of reovirus adenine-rich RNA.

evaporated by passing filtered air through the solution and the final RNA solution was lyophilized. The lyophilized RNA was dissolved in 0.02 M Tris-HCl pH 8.0, and passed through a 1 cm x 20 cm column of Sephadex G-100 (Pharmacia) previously equilibrated with the same buffer (2,65). The fractions were collected with an Isco gradient collector and absorbance was monitored at 254 nm with an Isco ultraviolet absorbance monitor. The double-stranded RNA in the void volume and A-rich in the included volume were eluted with 0.02 M Tris-HCl buffer, pH 8.0, (see Fig. 3 for elution profiles). Both fractions were pooled separately and desalted by dialyzing against deionized distilled water. Desalted A-rich RNA solution was lyophilized and stored at -20C until used.

Preparation of polyadenylic acid. Synthetic polyadenylic acid (Miles Lab.) with a minimum molecular weight of greater than 100,000 daltons was subjected to alkaline hydrolysis with 0.1 N NaOH for 12 h at room temperature to reduce its molecular weight to that approximating the molecular weight of reovirus A-rich RNA (46). The pH of the hydrolyzed polyadenylic acid solution was adjusted to 6.8 and chromatographed on a 1 cm x 100 cm column of Sephadex G-50 (Pharmacia), the procedure is outlined on the flow sheet Fig. 2. Fractions were eluted with 0.1 M phosphate buffer, pH 6.8 containing 0.1% SDS and 0.2 M NaCl, and collected with an Isco gradient collector. Purified reovirus A-rich RNA was used as a molecular weight marker and only the included fractions of polyadenylic acid were collected which were eluted in the same

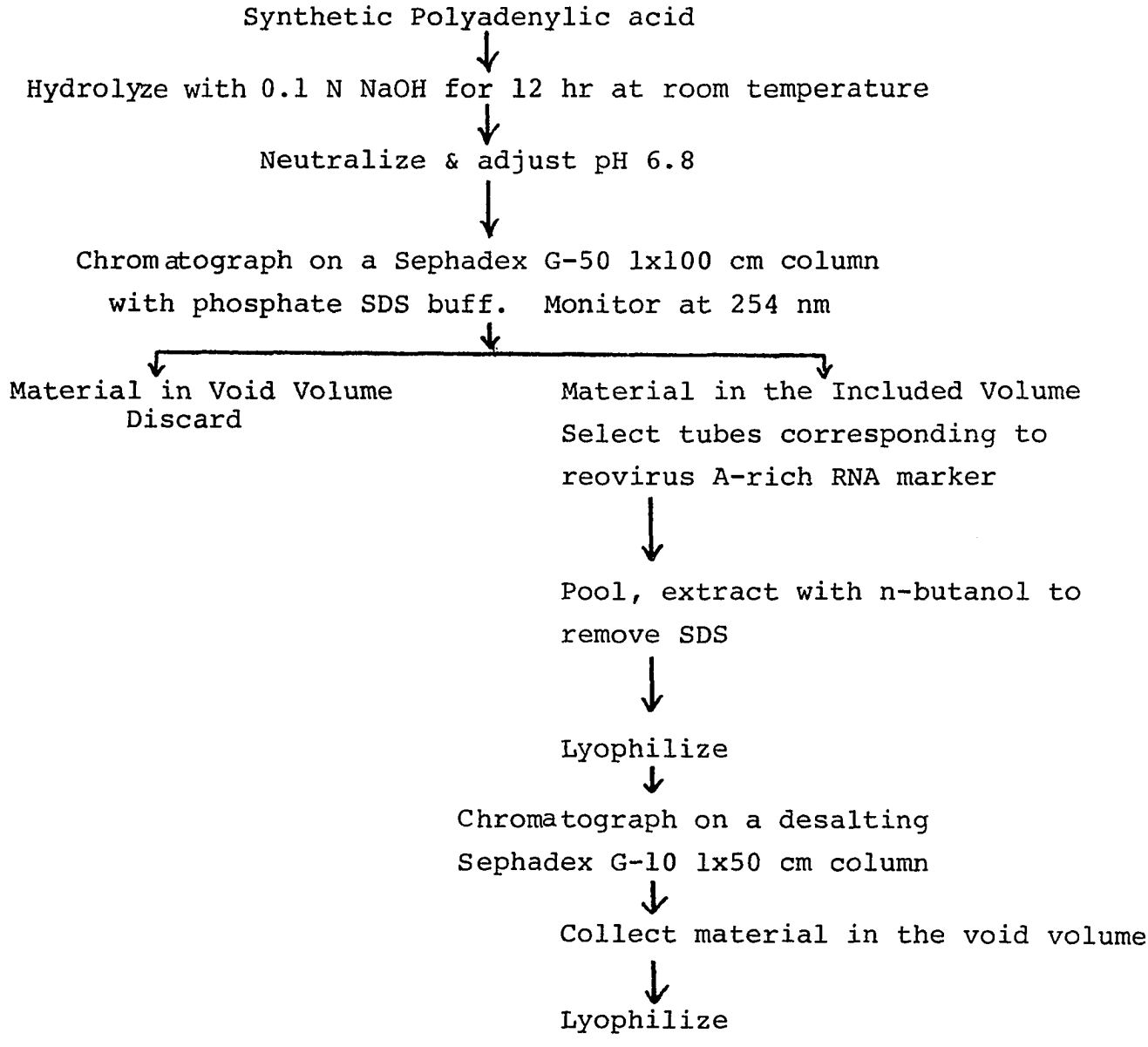


Fig. 2. Preparation of synthetic polyadenylic acid.

fractions as reovirus A-rich RNA (Fig. 4). The fractions were pooled and SDS was removed by extracting 5 X with equal volumes of n-butanol and the solution was lyophilized (48). Dried material was desalted by passing it through a 1 cm x 40 cm Sephadex G-10 column. The final salt free fractions in distilled water were pooled and lyophilized (Fig. 2). Autoclaved deionized distilled was used in the desalting column chromatography.

Quantitation of RNA. RNA in the reovirus A-rich RNA and polyadenylic acid preparations was determined by the orcinol test (56) using yeast RNA as a standard.

Determination of macromolecular synthesis. Cells in spinner cultures were adapted to 10% FCS for 1-2 weeks before making monolayers. Plastic tissue culture flasks (30 ml) were inoculated with 7 x 10^5 cells/flask in 6 ml MEM supplemented with 10% (v/v) FCS. The flasks were incubated in a CO_2 incubator for 20 h. During this time they formed semi confluent monolayers. The old medium was removed and fresh pre warmed MEM (no Serum) was then added (2 ml/flask). Monolayer cultures were treated with 1.0, 2.0 and 5.0 µg/ml of reovirus A-rich RNA or polyadenylic acid in 0.01 M Tris-HCl buffer pH 7.2 containing 4% (w/v) Sucrose. The RNA was allowed to adsorb for 2 h in CO_2 incubator and then the medium was reconstituted with 10% (v/v) FCS. Control flasks were treated with the same volume of buffer. At timed intervals duplicate treated and control flask were pulse

labelled for 30 min with 0.5 μCi/ml of ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-L-amino acids mixture for DNA, RNA and protein synthesis respectively. At the end of the labelling period, the reaction was stopped by removing the medium and the cell sheet was washed 2 x with 2.0 ml of ice cold MEM (no serum). Then 2 ml of cold 5% (w/v) trichloroacetic acid (TCA) was added and the monolayers were sonicated and stored at 4 C for at least 1 h for precipitation. The acid-insoluble precipitate was collected on millipore filters (pore size 1.2 μ). The filters were washed 3 x with 2 ml of cold 5% TCA to remove unincorporated radioactivity and dried by washing with 2 ml of 95% ethanol. The radioactivity was counted in a Beckman DPM-100 liquid scintillation spectrophotometer after dissolving the filters in 10 ml of Beckman Cocktail D (5 g diphenyloxazole, 100 g naphthalene, 10 ml water, dissolved in p-dioxane to make 1 liter).

Enzyme assay. The activity of the ribonucleases was assayed by using selected concentrations of each enzyme and yeast RNA as a substrate. The following procedures were used for each assay:

1 - Pancreatic ribonuclease: Worhington enzymes (76).

2 - Ribonuclease T_1 and T_2: Uchida & Egami (69).

3 - Ribonuclease N_1: Takai et al. (66).

4 - Snake venom phosphodiesterase was assayed by adding 2.5 μg, 5 μg, and 10 μg quantities of the enzyme to 0.01 M Tris-HCl buffer pH 8.4 containing 0.05 M $MgCl_2$. The

reaction mixture was preincubated at 37 C for 4-5 min and 0.25 ml of a 12 mg/ml yeast RNA solution was added. The reaction was allowed to proceed in a water bath at 37 C, and stopped after 15 min by adding 0.25 ml of uranyl reagent (0.75% uranyl acetate in 25% perchloric acid) making a final volume of 1.0 ml. The precipitate was immediately removed by centrifugation at 5000 rpm for 10 min at 4 C and 0.2 ml of the supernatant solution was diluted with 5.0 ml of autoclaved distilled water, and the optical density was read at 260 nm against a blank incubated without enzyme. If the dilution was not performed immediately the blank absorbance would increase with time.

Enzymatic hydrolyses of reovirus A-rich RNA. Conditions for enzymatic hydrolysis of reovirus adenine-rich RNA were:

Pancreatic ribonuclease-A: 5 µg/ml in 0.05 M Tris-HCl buffer, pH 8, containing 0.15 M NaCl, for 30 min (65).

Ribonuclease T_1: 40 units/ml in 0.2 M Tris-HCl buffer, pH 7.5, containing 0.005 M ethylenediaminetetra-acetate (EDTA) tetrasodium salt for 2 h (65).

Ribonuclease T_2: 5 units/ml in 0.1 M sodium acetate buffer, pH 4.5 for 3 h (8).

Venom phosphodiesterase: 4 units/ml (0.2 mg/ml) in 0.01 M Tris-HCl buffer, pH 8.4, containing 0.05 M $MgCl_2$ for 1 h (65).

Ribonuclease N_1: 100 units/ml (88 µg/ml) in 0.2 M Tris-HCl buffer, pH 7.0 for 3 h (66).

In all cases reaction mixtures were incubated at 37 C. The hydrolyzed reovirus adenine-rich RNA solutions were adjusted to pH 7.2 using sterile 0.1 N HCl or NaOH solutions and then diluted to the proper concentration with 0.01 M Tris-HCl buffer, pH 7.2, containing 4% (w/v) Sucrose and stored at -20 C until assayed for DNA inhibition activity as described previously.

Radioisotopes and enzymes. The source and specific activity of the radioactively labelled precursors and enzymes used in this study are as follows:

New England Nuclear

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

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

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

Sigma Chemical Company

Ribonuclease-A from bovine pancrease

90 Kunitz units/mg protein

Ribonuclease T_1 240580 units/mg protein

Ribonuclease T_2 1350 units/mg protein

Ribonuclease N_1 1140 units/mg protein

Worthington Biochemical Corporation

Venom phosphodiesterase 20 units/mg dry powder

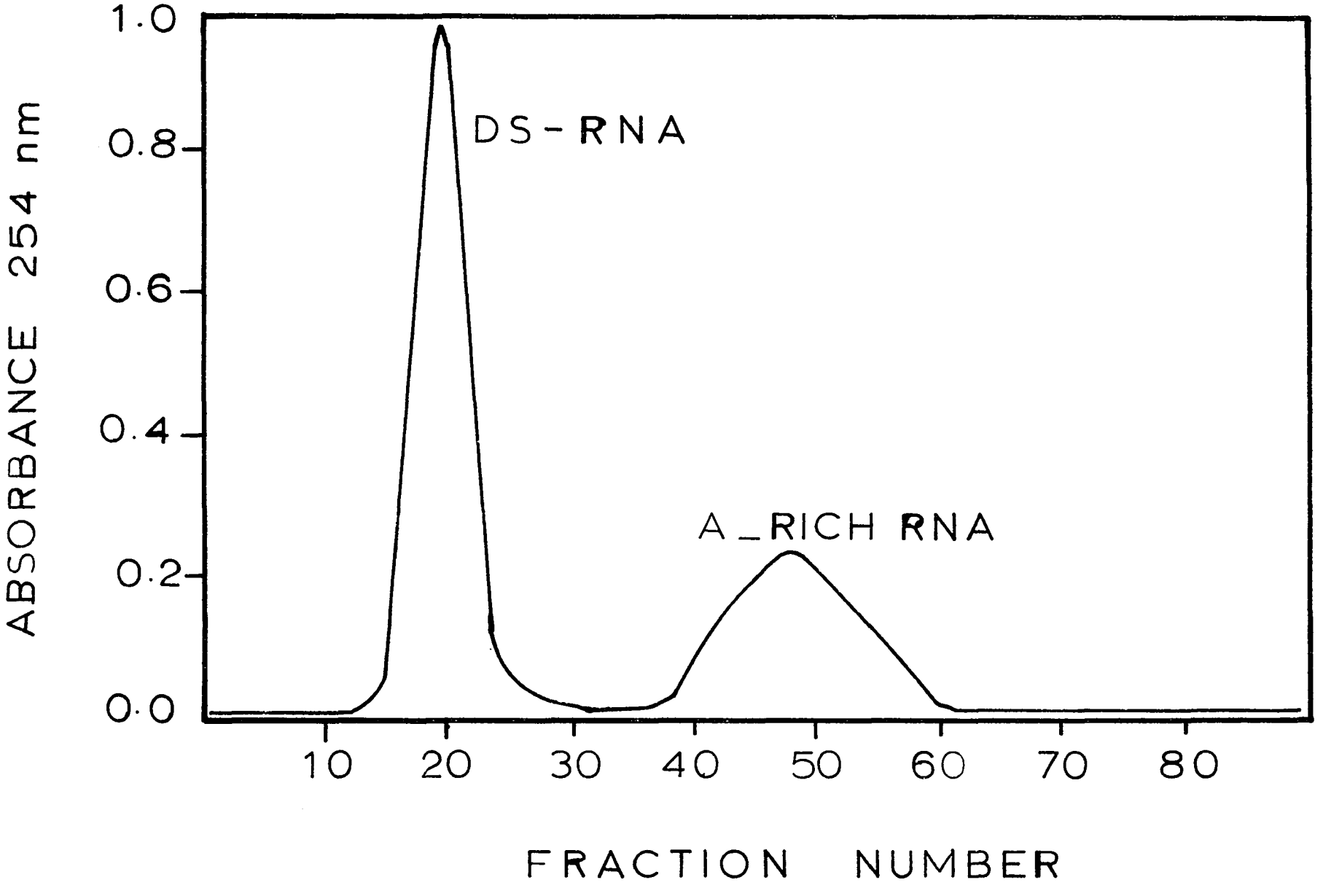
CHAPTER III

RESULTS

Reovirus adenine-rich RNA preparation. To study the effects of reovirus A-rich RNA on L-cells, it was necessary that the preparation be pure and free of any contamination with genome ds-RNA fragments and to minimize degradation by ribonucleases. This was achieved by following an established procedure of chromatographic separation of A-rich RNA from the genome ds-RNA described by Stoltzfus and Banerjee (65). To avoid degradation by contaminating ribonucleases, all buffers and solutions were autoclaved to destroy the enzymes. Total reovirus RNA was fractionated using Sephadex G-100. The elution profile is shown in Fig. 3. The ds-RNA eluted in the column void volume fractions 14-24. The single-stranded A-rich RNA was well separated and eluted in fractions 38-60. This elution profile is similar to the profile reported by Stoltzfus and Banerjee (65). The fractions containing A-rich RNA were pooled, desalted, lyophilized and stored at -20C until use.

Synthetic polyadenylic acid preparation. We have proposed that it is only the heterologous oligonucleotide fraction of the reovirus A-rich which is responsible for the

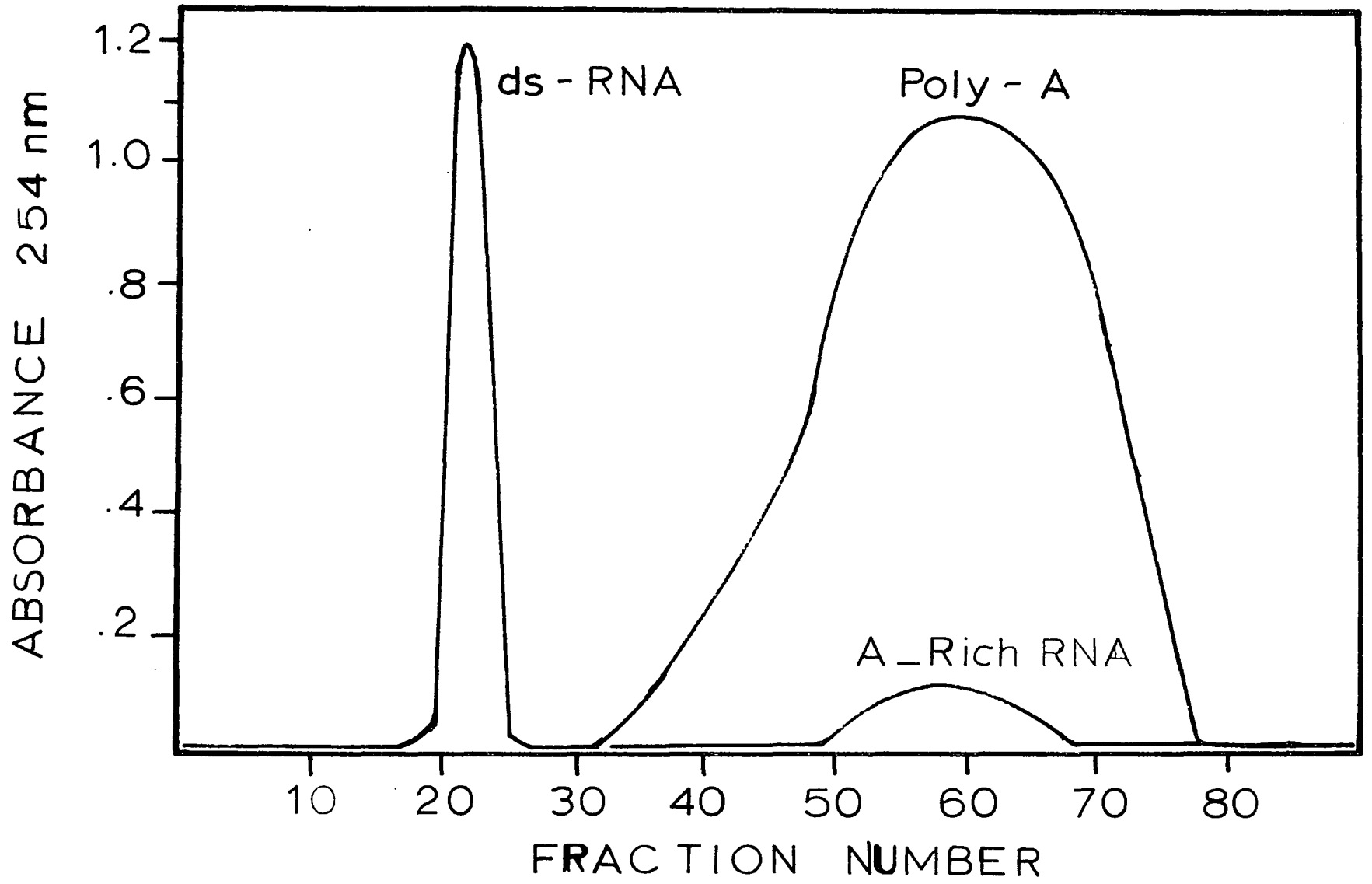
Figure 3. Sephadex G-100 profile of the fractionation of reovirus RNA. RNA was extracted with phenol and chromatographed according to the procedure described in Methods and Materials. The flow rate was approximately 30 ml/hr. 1 ml fractions were collected.


1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE 254 nm
DS-RNA
A_RICH RNA
10
20
30
40
50
60
70
80
FRACTION NUMBER

inhibition of DNA synthesis and that the oligoadenylic acid fraction has no effect on cellular DNA synthesis. An indirect approach was used to test this hypothesis. A preparation of synthetic polyadenylic with a molecular weight similar to A-rich RNA was prepared in order to study its effects on the synthesis of cellular macromolecules. Synthetic polyadenylic acid (poly-A) with a minimum molecular weight of greater than 100,000 daltons was hydrolyzed with alkali to reduce it to a molecular weight similar to A-rich RNA. The hydrolyzed sample was chromatographed on a Sephadex G-50 column to separate the fractions having molecular weight similar to A-rich RNA. Reovirus ds-RNA and A-rich RNA were used as markers in separate calibration runs. Reovirus ds-RNA eluted in the column void volume fraction 20-25 and A-rich RNA was eluted in fractions 50-70 (Fig. 4). The base-hydrolyzed poly-A eluted in the included volume as a large peak and only fractions 50-70 were selected which approximated the molecular weight of reovirus A-rich RNA. The poly-A fractions were pooled and desalted on a Sephadex G-10 column. The poly-A was lyophilized and stored until used.

Effect of low doses of reovirus A-rich RNA on cellular DNA synthesis. It has been reported that in cells infected with reovirus, inhibition of cellular DNA synthesis began 6-8 h after infection (28,60). Subsequent reports showed that an inhibition of DNA synthesis could be induced by reovirus A-rich RNA, but this inhibition was transitory and the treated cells returned to normal levels of synthesis by 9-10 h after

Figure 4. Sephadex G-50 profile of reovirus RNA and hydrolyzed polyadenylic acid. Reovirus adenine-rich RNA was used as a marker as described in Materials and Methods. The flow rate of the column was 30 ml/hr. Three ml fractions were collected. Fractions 50-70 of synthetic polyadenylic acid were collected, pooled and processed as described.


ABSORBANCE 254 nm
1.2
1.0
.8
.6
.4
.2
ds - RNA
Poly - A
A_Rich RNA
10
20
30
40
50
60
70
80
FRACTION NUMBER

treatment (32, 46). In both of the above studies reovirus A-rich RNA was separated from genome-ds-RNA by centrifugation using 5-20% sucrose density gradients. It was necessary to determine if similar inhibitory activities were mediated by reovirus A-rich RNA separated by Sephadex G-100 column chromatography.

Monolayer cultures of L-929 cells were treated with reovirus A-rich RNA at a concentration of 1 µg/ml. At timed intervals cultures were pulse-labelled with ^{3}H-thymidine and processed as described. Figure 5 illustrates the results of this experiment. Inhibition of DNA synthesis began 3-4 h after treatment. DNA synthesis remained at minimal levels until 8 h and returned to control levels by 9-10 h after treatment. Figure 5 shows 42% inhibition (average of values from 4-8 h) of DNA synthesis. Under the same experimental conditions poly-A induced no inhibition of DNA synthesis. Treated cells had control levels of DNA synthesis. These results indicate that the A-rich RNA preparation was inhibitory for DNA synthesis and that poly-A had no effect on DNA synthesis.

Effect of high doses of reovirus A-rich RNA on cellular DNA synthesis. Since a low dose of 1 µg/ml of A-rich RNA caused a transient inhibition of DNA synthesis, it was possible that increasing the dosage of A-rich RNA could produce a more pronounced and sustained inhibition of DNA synthesis. DNA synthesis was measured in cells treated with 2 µg/ml or 5 µg/ml of A-rich RNA. The results are shown in Figures 6 and

Figure 5. The effect of reovirus adenine-rich RNA and synthetic polyadenylic acid on DNA synthesis in L-cells. Monolayer cultures were treated with 1.0 µg/ml of reovirus A-rich RNA or oligoadenylic acid in 0.01 M Tris-HCl pH 7.2 containing 4% (w/v) sucrose. Control cultures were treated with the same amount of buffer. At the times indicated selected cultures in duplicate were pulse labelled with 0.5 µCi/ml ^{3}H-thymidine and processed as described in Materials and Methods. The radioactivity in acid-insoluble material is presented as counts per minute at designated intervals after treatment.

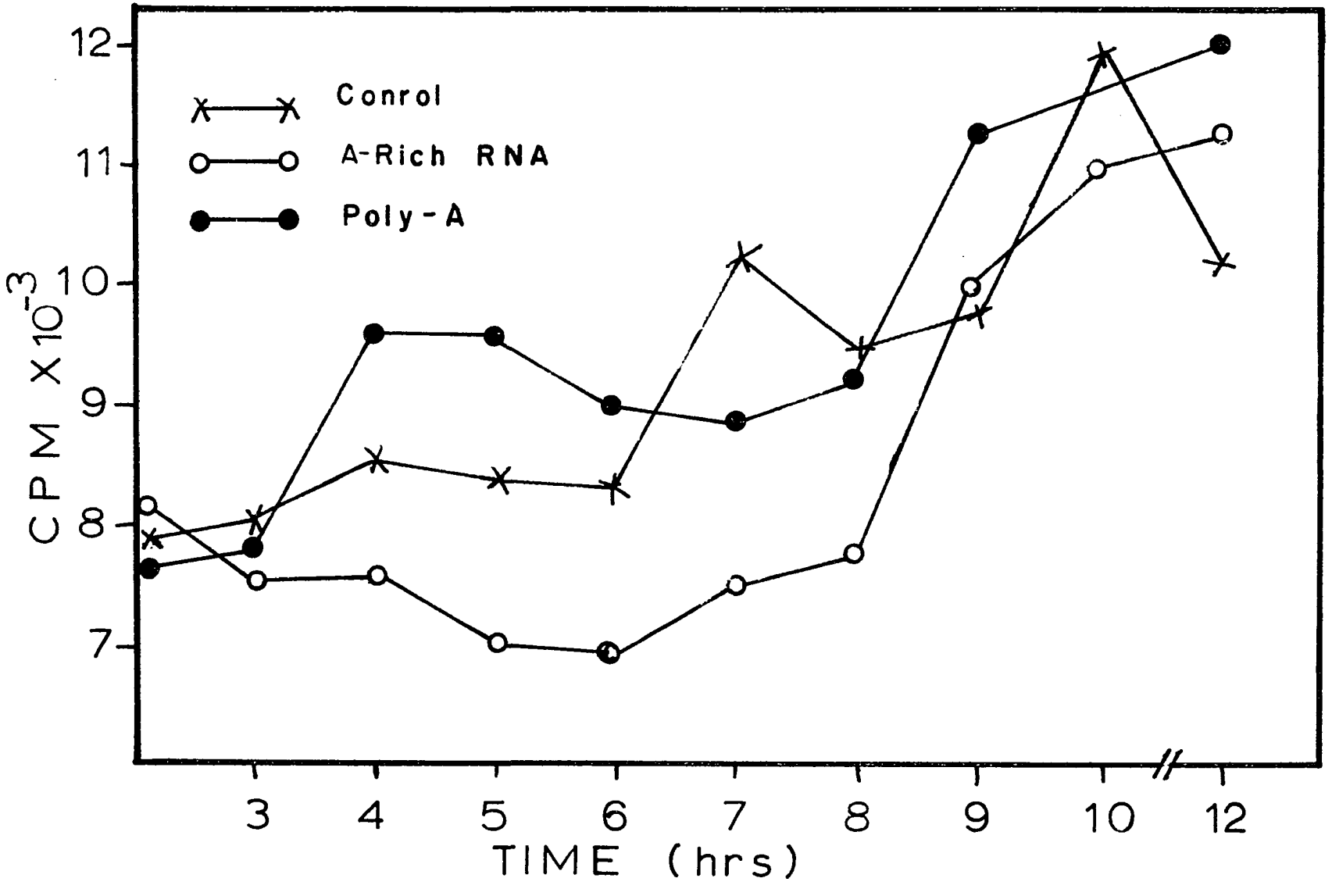

Conrol
A-Rich RNA
Poly-A
CPM X10-3
12
11
10
9
8
7
3
4
5
6
7
8
9
10
12
TIME (hrs)

Figure 6. The effect of reovirus adenine-rich RNA on DNA synthesis in L-cells. Monolayer cultures were treated with 2 μg/ml reovirus A-rich RNA and the control cultures were treated with equal volumes of buffer. At the times indicated cultures were pulse-labelled with 0.5 μCi/ml ^{3}H-thymidine for 30 min. The radioactivity in acid-insoluble material is presented as counts per minute at specified time intervals for duplicate cultures.

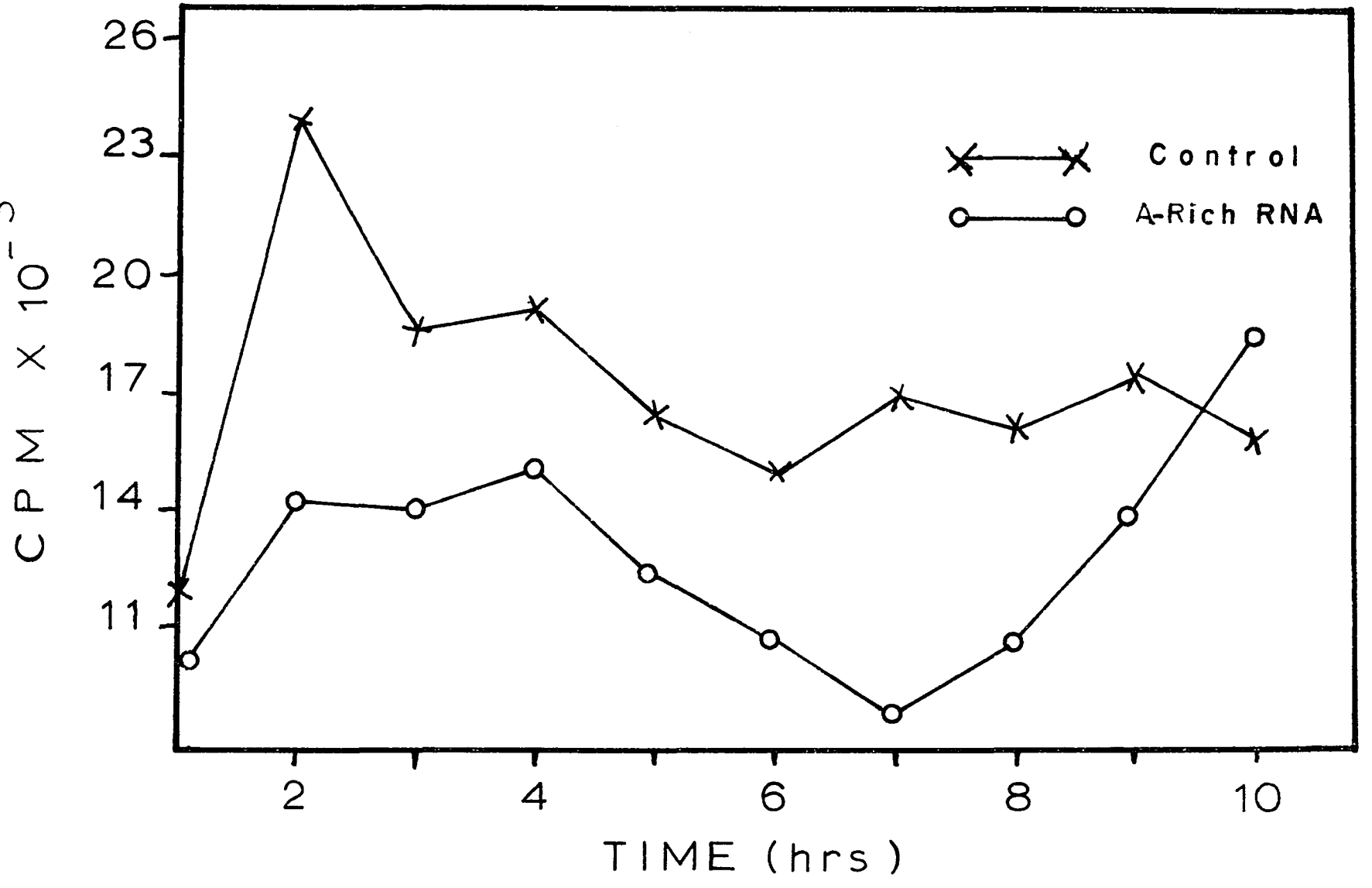
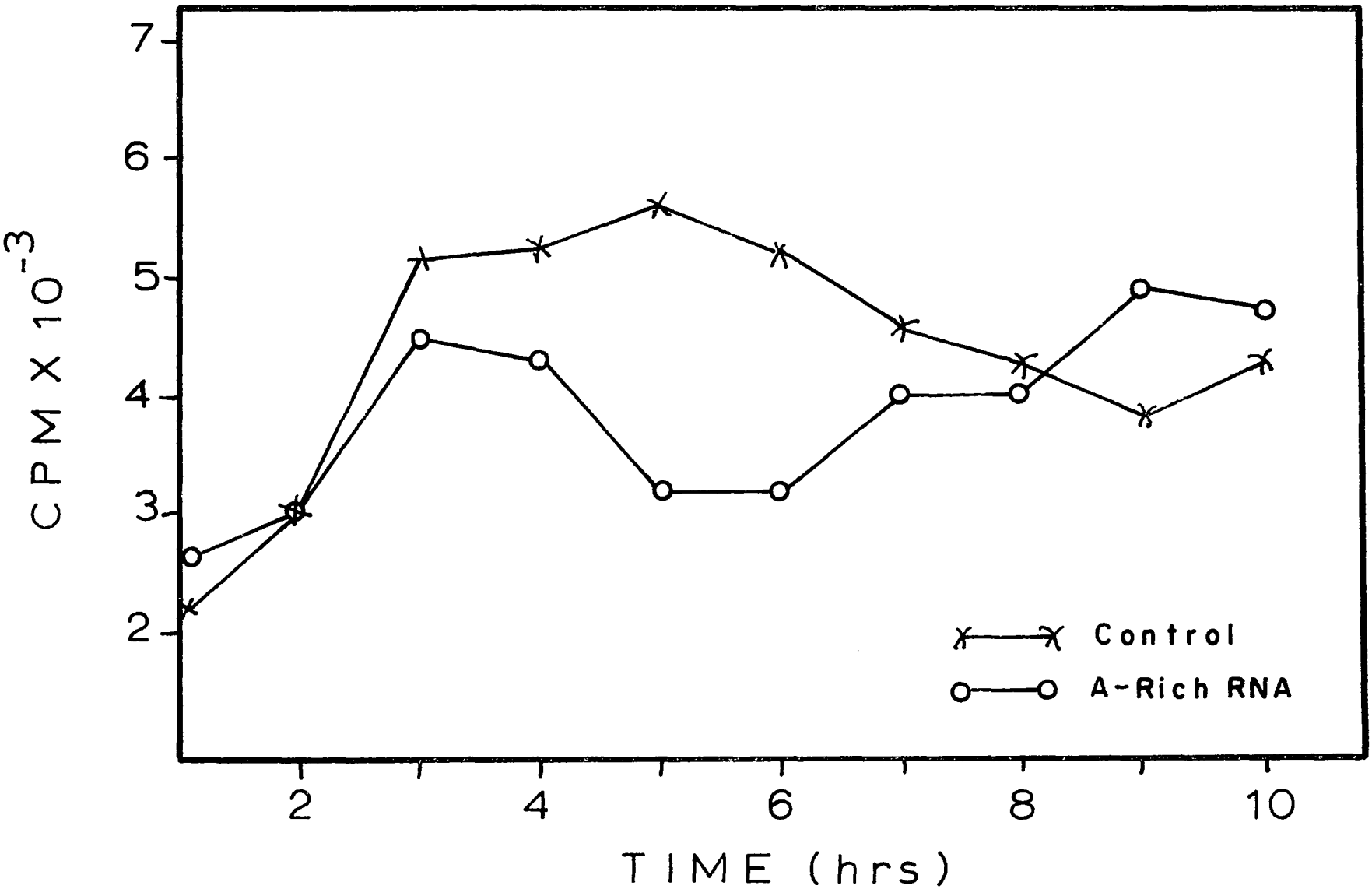

26
23
20
17
14
11
CPM X 10^-3
2
4
6
8
10
TIME (hrs)
Control
A-Rich RNA

Figure 7. The effect of reovirus adenine-rich RNA on DNA synthesis in L-cells. Monolayer cultures were treated with 5 µg/ml reovirus A-rich RNA and the control cultures were treated with equal volumes of buffer. At the times indicated cultures were pulse labelled with 0.5 µCi/ml of ^{3}H-thymidine for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute at specified times for duplicate cultures.


CPM X 10^{-3}
7
6
5
4
3
2
2
4
6
8
10
TIME (hrs)
Control
A-Rich RNA

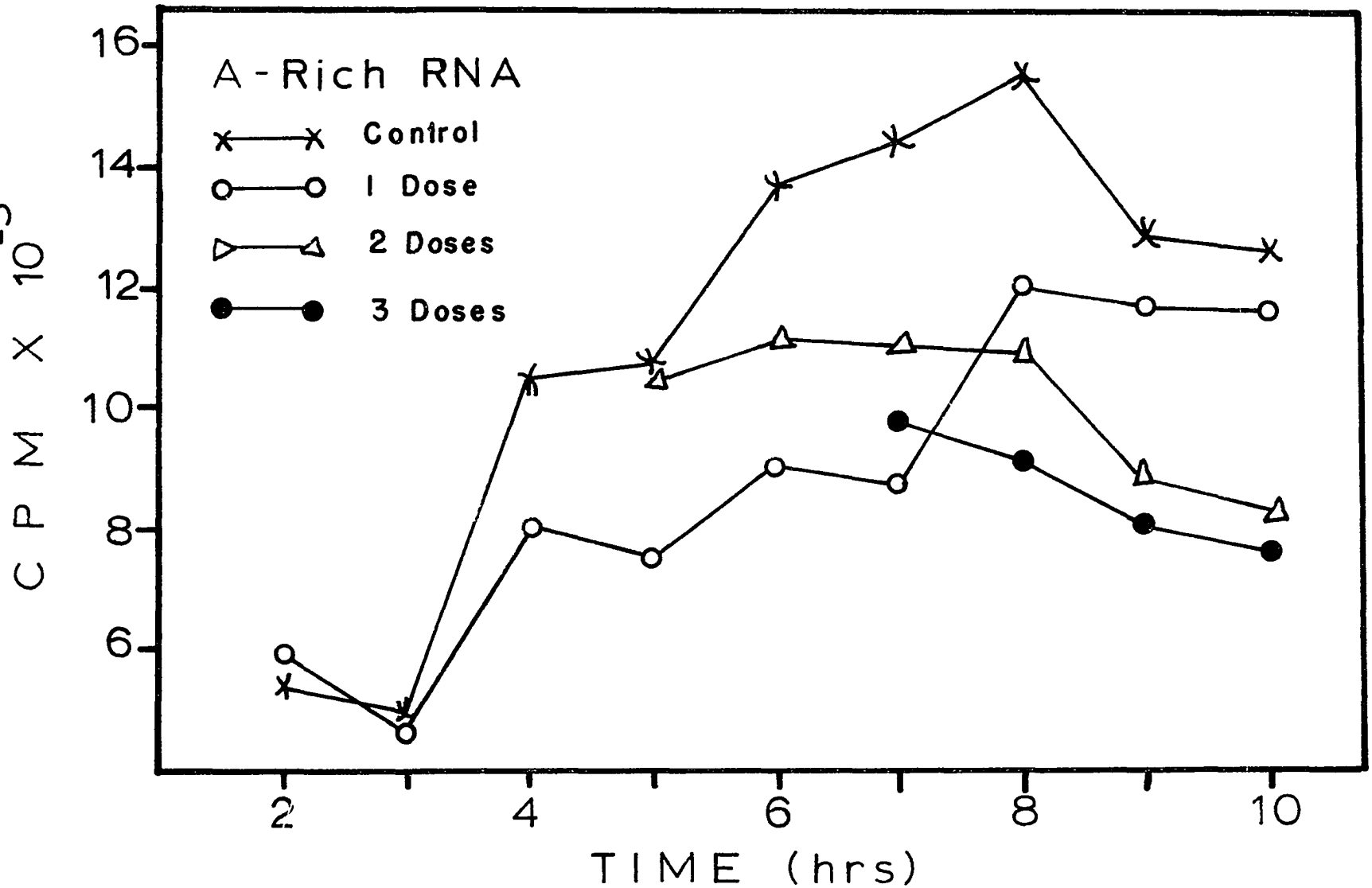
7. At both concentrations inhibition began 3-4 hr after treatment and control levels of DNA synthesis were reached by 8-9 h after treatment. The maximum levels of DNA inhibition were 47% and 46% mean of values from 4-8 h for the 2 and 5 µg/ml dosages of A-rich RNA respectively. The average DNA inhibition with a low dose of 1 µg/ml was 42% (mean of values from 4-8 h) below the control level (Fig. 5). The results showed that there is no significant difference in the degree and duration of DNA inhibition with a low (1 µg/ml) and higher (2 µg/ml and 5 µg/ml) doses of A-rich RNA.

Effect of multiple low doses of reovirus A-rich RNA on cellular DNA synthesis. Since low (1 µg/ml) and high (2 and 5 µg/ml) doses of A-rich RNA had an identical inhibitory effect on cellular DNA synthesis, sustained inhibition of DNA synthesis as observed in infected cells might require constant levels of A-rich RNA. For this reason the effect of multiple dosage of A-rich RNA on cellular DNA synthesis was examined. Monolayer cultures were treated with an initial dose of A-rich RNA followed by doses at 5 and 7 hours after the initial treatment. The times specified were selected to maintain and hopefully amplify inhibition levels achieved with the initial dose of A-rich RNA. The second dose was added at a time when inhibition was at its optimum i.e. 5 h after the initial treatment and a third dose was added when cells would have been recovering from the inhibition of DNA synthesis mediated by the initial dose of A-rich RNA.

Monolayer cultures were treated at zero time with A-rich RNA (1 μg/ml) and the RNA was allowed to adsorb for 2 h. The 2nd and 3rd doses of A-rich RNA (1 μg/ml) were given at 5 and 7 h after the initial treatment. At timed intervals, cultures were pulse-labelled with ^{3}H-thymidine and samples were processed as described earlier. The cultures treated with a single dose of A-rich RNA showed a transient inhibition of DNA synthesis as described previously, but in cultures treated with 2 and 3 doses, DNA inhibition was maintained up to 10 h at maximum levels (Fig. 8). The negative slope of the lines representing 2 and 3 dose treatment suggests a continued progressive inhibition of DNA synthesis. There was no significant difference in the degree of inhibition of DNA synthesis at 10 h in cultures treated with 2 and 3 doses. It was not clear why the cultures treated with 2 doses showed lower levels of inhibition from 5-8 h after the initial treatment but then decreased to a maximum (36% below the control) level of inhibition. During the same time period from 5-8 hr, cultures treated with one dose had an average maximum inhibition of 36% (mean of the values from 4-8 h) below control levels. The manipulation of cultures at 5 and 7 h for treatment might have some effect on this inconsistency as the cultures were exposed to suboptimal growth conditions during these times.

Effect of multiple low doses of reovirus A-rich RNA on cellular RNA and protein synthesis. Since, Monahan (46) has

Figure 8. The effect of multiple doses of reovirus adenine-rich RNA on DNA synthesis in L-cells. Monolayer cultures were sequentially treated with reovirus A-rich RNA (1 μg/ml) at 0, 5 and 7 h. The control cultures were treated with equal volumes of buffer at 0, 5 and 7 hr. At the times indicated selected cultures were pulse-labelled with 0.5 μCi/ml of ^{3}H-thymidine for 30 min. The radioactivity in acid-insoluble material is presented as counts per minute for duplicate cultures at the specified time intervals.


A-Rich RNA
Control
1 Dose
2 Doses
3 Doses
CPM X 10^{-3}
16
14
12
10
8
6
2
4
6
8
10
TIME (hrs)

reported that cells treated with a single dose of A-rich RNA 1 μg/ml had no effect on cellular RNA and protein synthesis. It was possible that the progressive inhibition of DNA synthesis by multiple doses of A-rich RNA might reflect a nonspecific general modification of macromolecular synthesis. It was of interest to determine whether repeated doses of A-rich RNA had any effect on the synthesis of these macromolecules.

The experimental data presented in Fig. 9 and 10 illustrates the effect of repeated dosage of reovirus A-rich RNA 1 μg/ml on cellular RNA and protein synthesis respectively. No inhibition of RNA and protein synthesis was observed in the cultures treated with one, two and three doses of A-rich RNA. These data indicate that the reovirus A-rich RNA is capable of selectively inhibiting only the replicative function of cellular DNA and that cellular transcription and translation processes are not affected.

Effect of multiple high doses of reovirus A-rich RNA on cellular DNA synthesis. Since multiple low (1 μg/ml) doses of A-rich RNA mediated a sustained and progressive inhibition of DNA synthesis and a maximum inhibition of 50%. It was possible that higher multiple doses of A-rich RNA might amplify this inhibition.

The data presented in Figures 11 and 12 represents the effect of multiple high doses of 2 μg/ml of reovirus A-rich RNA on DNA synthesis for 10 h and 24 h respectively. Treatment with 2 doses resulted in a 24% inhibition of DNA synthesis

Figure 9. The effect of multiple doses of reovirus adenine-rich RNA on RNA synthesis in L-cells. Monolayer cultures were sequentially treated with reovirus A-rich RNA 1 μg/ml at 0, 5 and 7 h. The control cultures were treated with equal volume of buffer at 0, 5 and 7 h. At the times indicated selected cultures were pulse labelled with 0.5 μCi/ml ^{3}H-uridine for 30 minutes. The radioactivity in acid-insoluble material is presented as counts per minute for duplicate cultures at the specified time intervals.

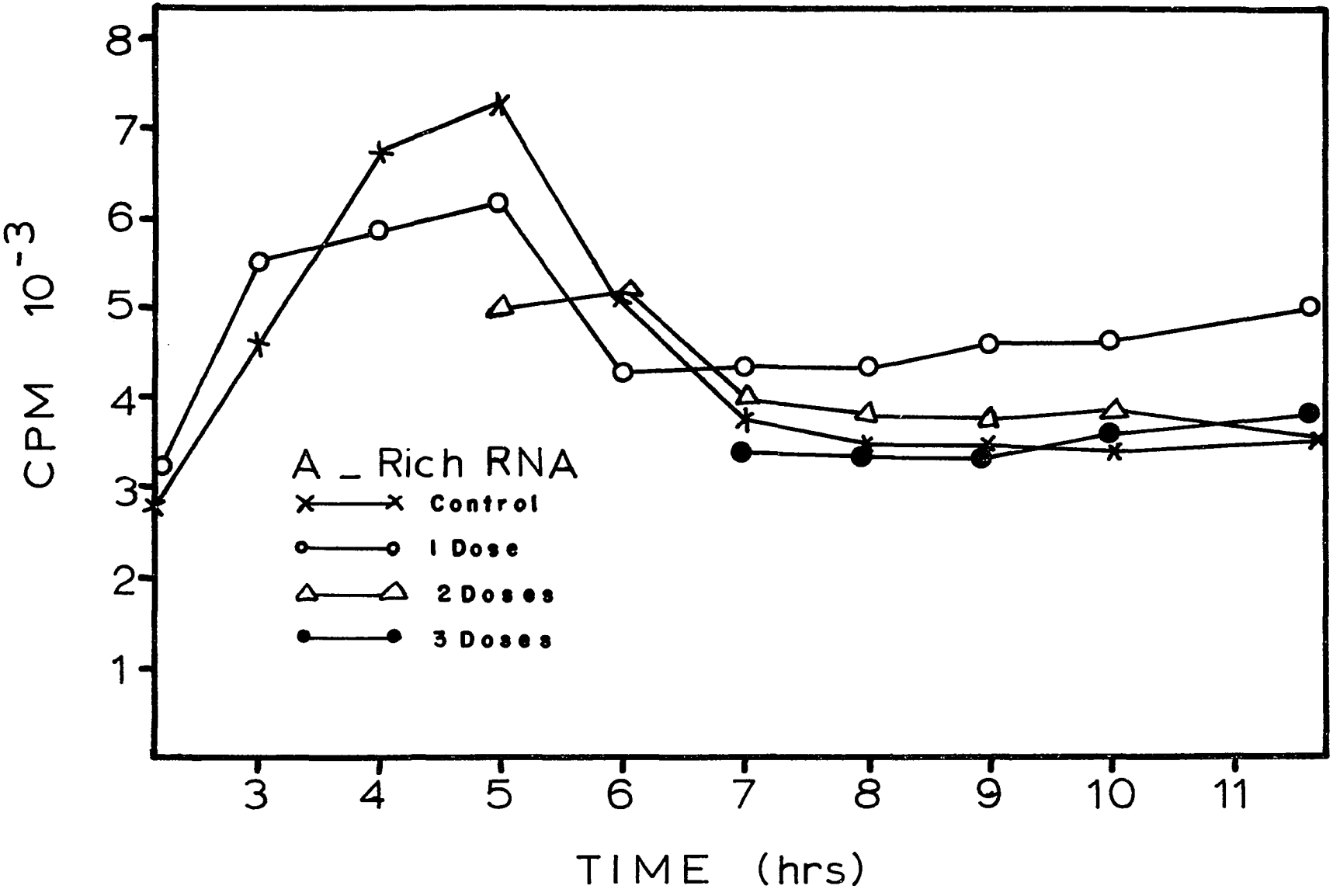

CPM 10^{-3}
8
7
6
5
4
3
2
1
3
4
5
6
7
8
9
10
11
TIME (hrs)
A _ Rich RNA
Control
1 Dose
2 Doses
3 Doses

Figure 10. The effect of multiple doses of reovirus adenine-rich RNA on protein synthesis in L-cells. Monolayer cultures were sequentially treated with reovirus A-rich RNA 1 µg/ml at 0, 5 and 7 h. The control cultures were treated with equal volume of buffer at 0, 5 and 7 h. At the times indicated selected cultures were pulse labelled with 0.5 µCi/ml of ^{3}H-L-amino acid mixture for 30 min. The radioactivity in acid-insoluble material is presented as counts per minute for duplicate cultures at the specified time intervals.

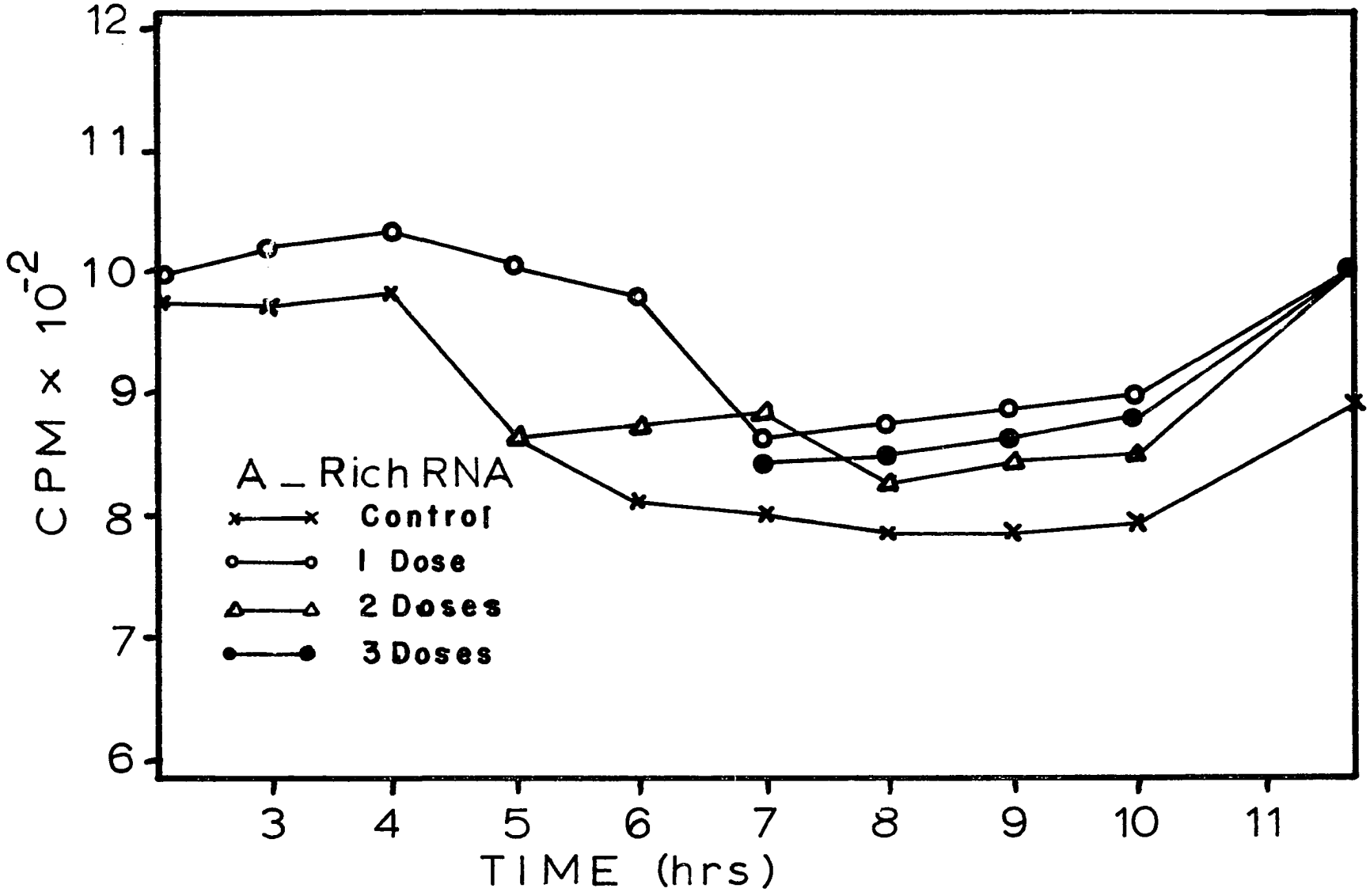

12
11
10
9
8
7
6
CPM x 10^{-2}
A_Rich RNA
Control
1 Dose
2 Doses
3 Doses
3
4
5
6
7
8
9
10
11
TIME (hrs)

Figure 11. The effect of reovirus adenine-rich RNA on DNA synthesis in L-cells. Monolayer cultures were sequentially treated with reovirus A-rich RNA (2 μg/ml) at 0, 5, and 7 h. The control cultures were similarly treated with Tris-HCl buffer. At the times indicated selected cultures were pulse-labelled with ^{3}H-thymidine 0.5 μCi/ml for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute for duplicate cultures. All points represent average values from three experiments.

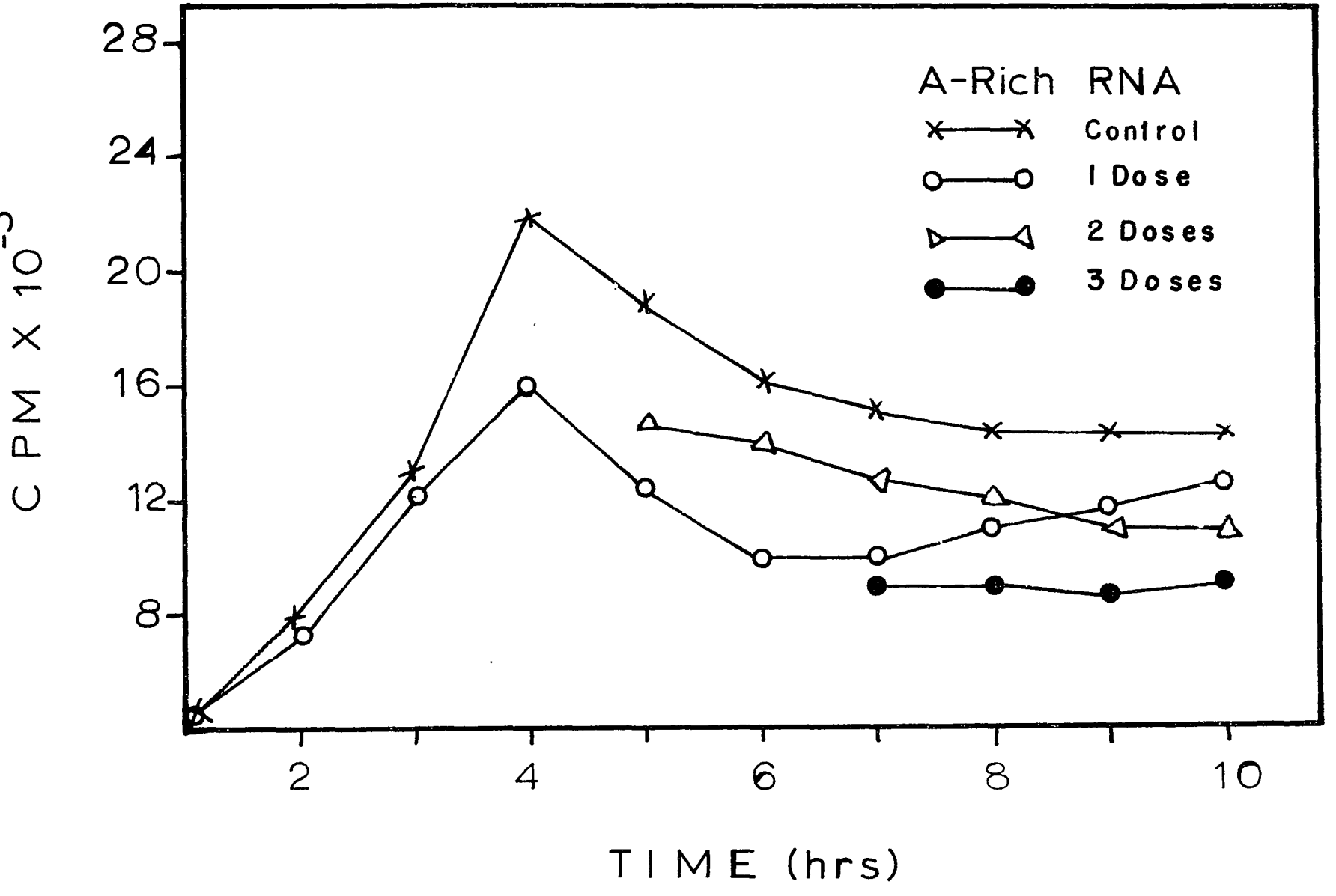
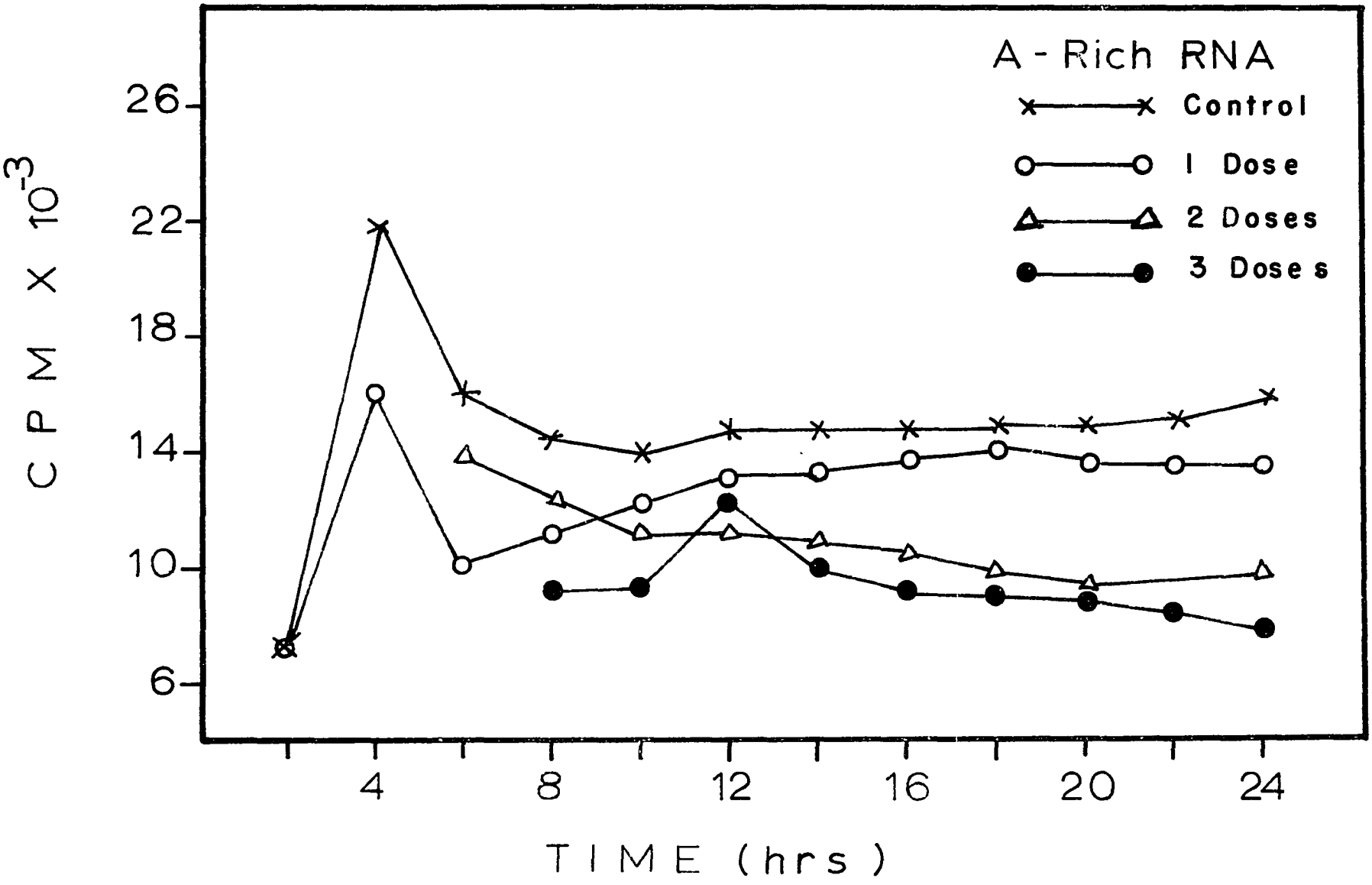

A-Rich RNA
Control
1 Dose
2 Doses
3 Doses
CPM X 10^-3
28
24
20
16
12
8
2
4
6
8
10
TIME (hrs)

Figure 12. The effect of reovirus adenine-rich RNA on DNA synthesis in L-cells. Monolayer cultures were sequentially treated with reovirus A-rich RNA 2 µg/ml at 0, 5 and 7 h. The control cultures were similarly treated with Tris-HCl buffer. At the times indicated selected cultures were pulse labelled with ^{3}H-thymidine 0.5 µCi/ml for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute for duplicate cultures. All points represent average value for three experiments.


A - Rich RNA
Control
1 Dose
2 Doses
3 Doses
CPM X 10^-3
26
22
18
14
10
6
4
8
12
16
20
24
TIME (hrs)

while 3 doses caused an inhibition of 39% of control levels of synthesis up to 10 h. The degree of inhibition increased to 38% and 50% respectively by 24 h after treatment.

Sequential treatment with three low doses (1 μg/ml) of A-rich RNA produced 39% (average of 9-10 h) inhibition by 10 h (Fig. 8), and the same level of inhibition was observed with a higher dose (2 μg/ml) of A-rich RNA under similar conditions. This indicated that no significant increase in the degree of inhibition of DNA synthesis could be achieved in cells treated with multiple high (2 μg/ml) doses of A-rich RNA.

These data indicated that the cells recover from inhibition of DNA synthesis when the inhibitory capacity of A-rich RNA is somehow overcome. Repeated dosage of A-rich RNA may mimic accumulation of viral components particularly the A-rich RNA which, when added exogenously, maintained inhibition up to 24 h after the treatment. This suggests that the persistant presence of A-rich RNA was essential for maintaining selective inhibition of cellular DNA synthesis.

Effect of synthetic polyadenylic acid on cellular DNA synthesis. Based on earlier work (46) it appeared that the heterologous oligonucleotides (5′-G- terminated oligonucleotides) might be responsible for the inhibition of cellular DNA synthesis, and that the oligoadenylate fraction had no effect on cellular macromolecular synthesis. To test this hypothesis, a sample of synthetic polyadenylic acid was prepared as described earlier, and the effect of this oligonucleotide

on cellular macromolecular synthesis was examined.

Figures 5 and 13 represent the effects of a single low dose (1 µg/ml) and high dose (5 µg/ml) respectively. No alteration in the level of DNA synthesis was observed in treated cultures which paralleled control levels of synthesis up to 10 h. Treated cultures were examined microscopically and no cytophathology was observed.

Effect of multiple doses of synthetic polyadenylic acid on cellular macromolecular synthesis. A single dose of 1 µg/ml or 5 µg/ml of poly-A had no effect on cellular DNA synthesis. It was also observed that repeated doses of A-rich RNA induced a sustained inhibition of DNA synthesis. To substantiate the hypothesis that the oligoadenylate fraction is not the inhibitory component of A-rich RNA, we tested the effect of multiple doses of poly-A on cellular macromolecular synthesis.

The data presented in Figures 14, 15 and 16 indicate that multiple doses of 1 µg/ml of poly-A caused no modification of cellular DNA, RNA or protein synthesis respectively. Therefore, it can be assumed that the heterologous oligonucleotide fragments with unique sequences might be responsible for selectively mediating the inhibition of cellular DNA synthesis.

Effect of Ribonucleases on the inhibitory activity of reovirus A-rich RNA. Based on the studies of the effect of synthetic oligoadenylate, it was suggested that the heterologous oligonucleotide fraction of the A-rich RNA may be responsible

Figure 13. The effect of synthetic polyadenylic acid on DNA synthesis in L-cells. Monolayer cultures were treated with a single dose of synthetic poly-A 5 µg/ml at 0 time. The control cultures were treated with Tris-HCl buffer, pH 7.2. At times indicated selected cultures were pulse labelled with ^{3}H-thymidine 0.5 µCi/ml for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute for duplicate cultures. All points represent mean value for 4 experiments.

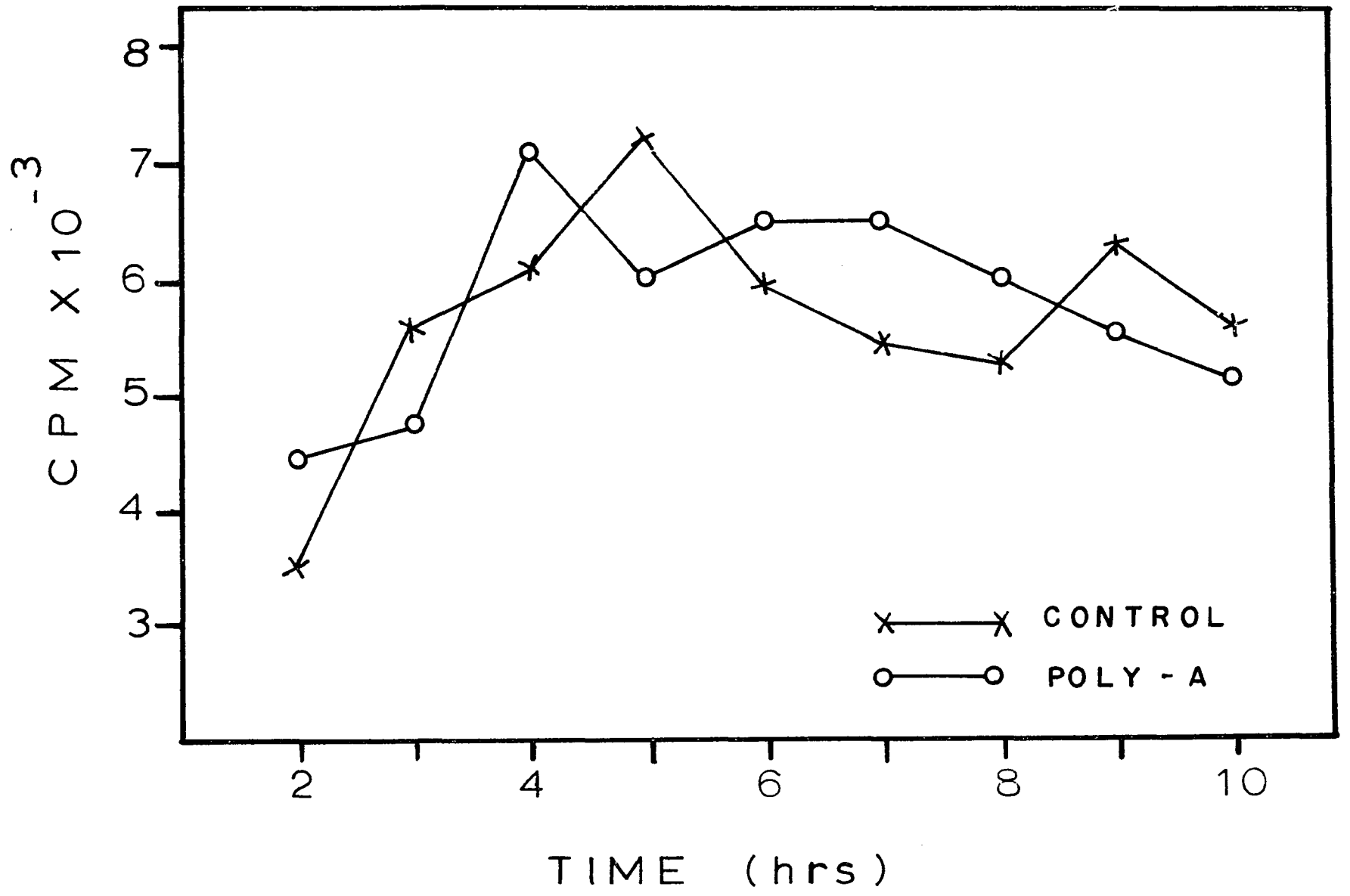

8
7
6
5
4
3
CPM X 10^-3
2
4
6
8
10
TIME (hrs)
CONTROL
POLY - A

Figure 14. The effect of synthetic polyadenylic acid on DNA synthesis in L-cells. Monolayer cultures were sequentially treated with synthetic poly-A 1 µg/ml at 0, 5 and 7 h. The control cultures were similarly treated with Tris-HCl buffer, pH 7.2. At the times indicated selected cultures were pulse labelled with ^{3}H-thymidine 0.5 µCi/ml for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute for duplicate cultures.

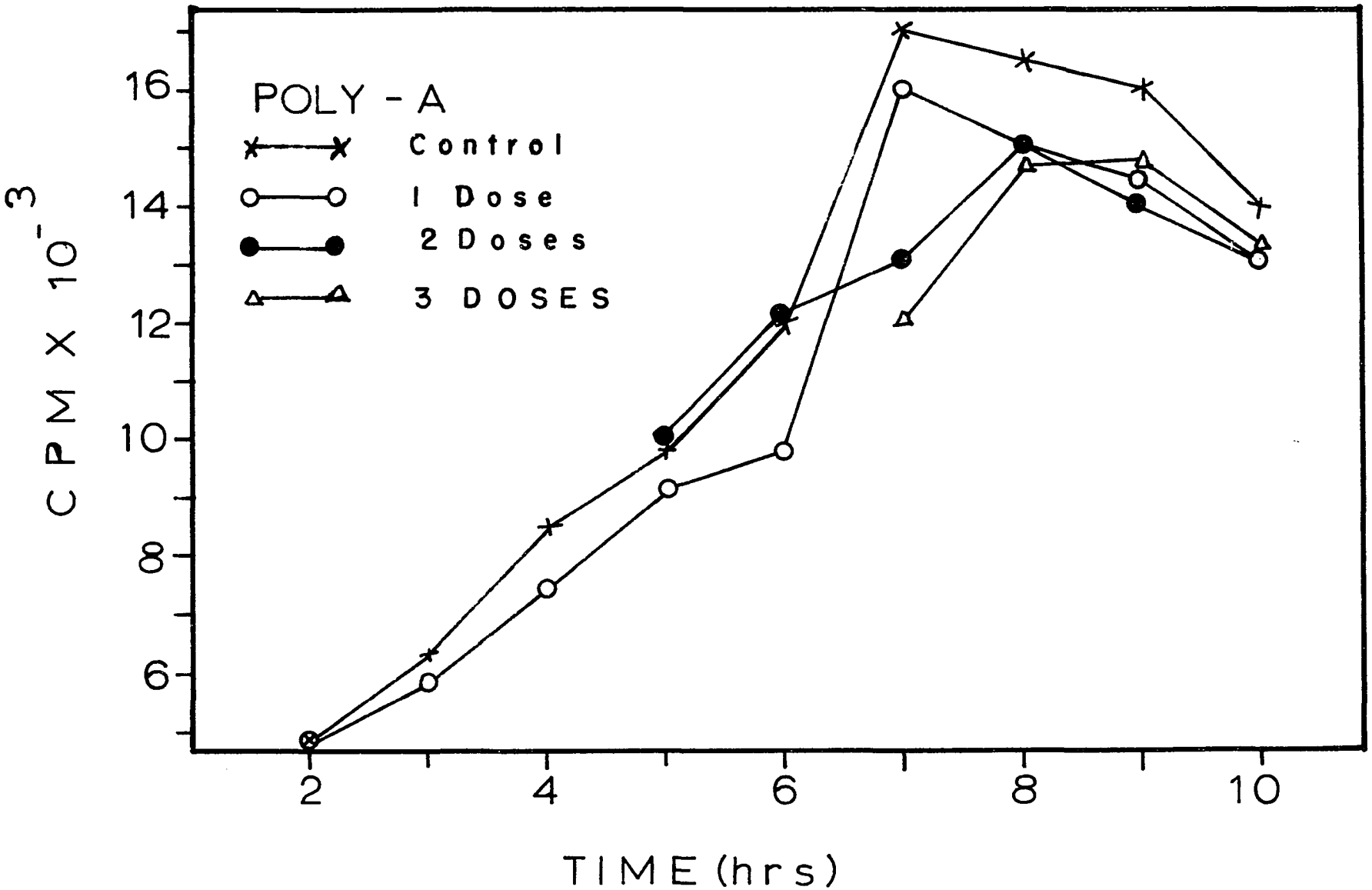

POLY - A
Control
1 Dose
2 Doses
3 DOSES
CPM X 10^-3
16
14
12
10
8
6
2
4
6
8
10
TIME (hrs)

Figure 15. The effect of synthetic polyadenylic acid on RNA synthesis in L-cells. Monolayer cultures were sequentially treated with synthetic poly-A 1 µg/ml at 0, 5 and 7 h. The control cultures were similarly treated with Tris-HCl buffer, pH 7.2. At the times indicated selected cultures were pulse labelled with ^{3}H-Uridine 0.5 µCi/ml for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute for duplicate cultures.

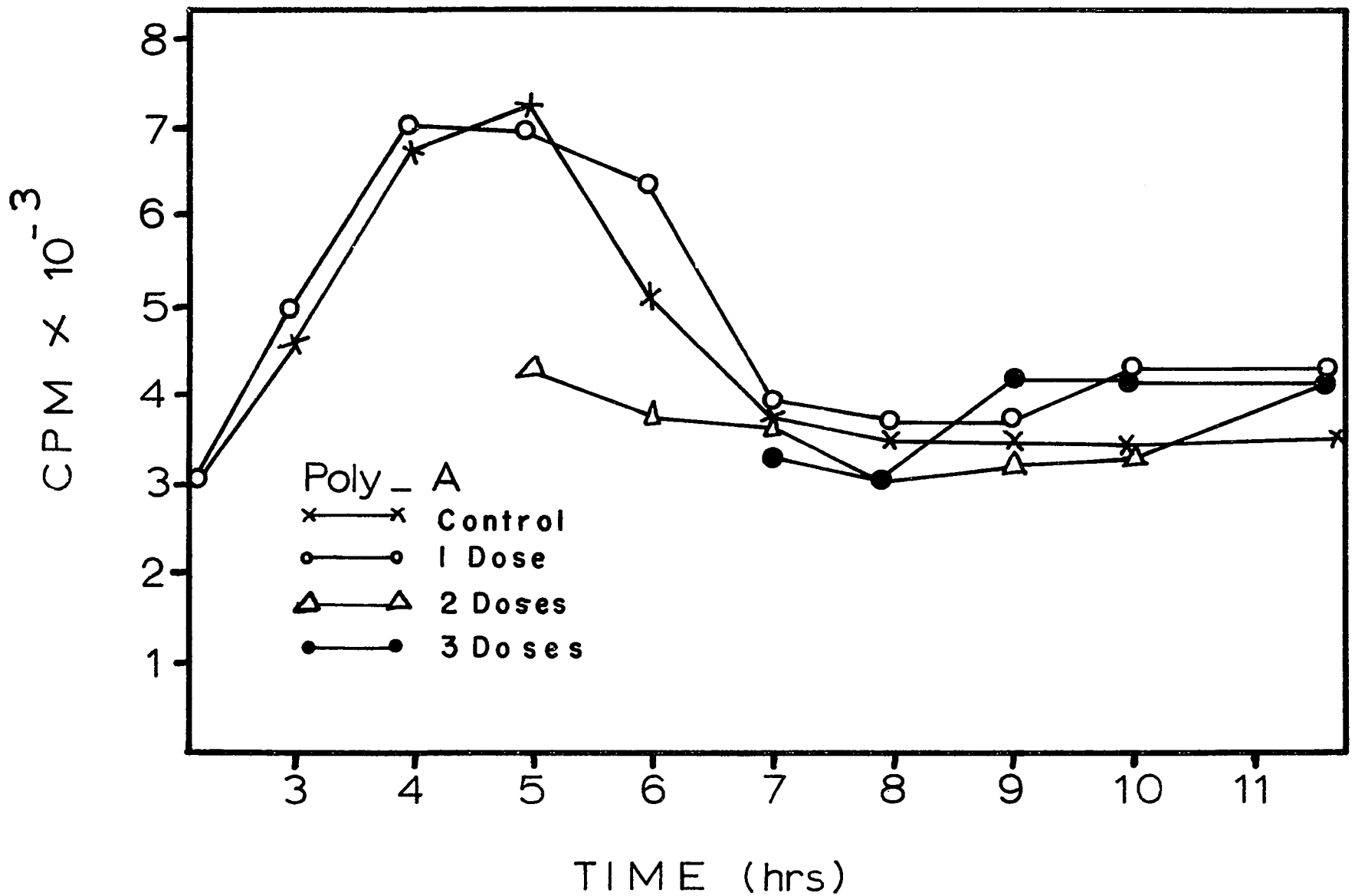
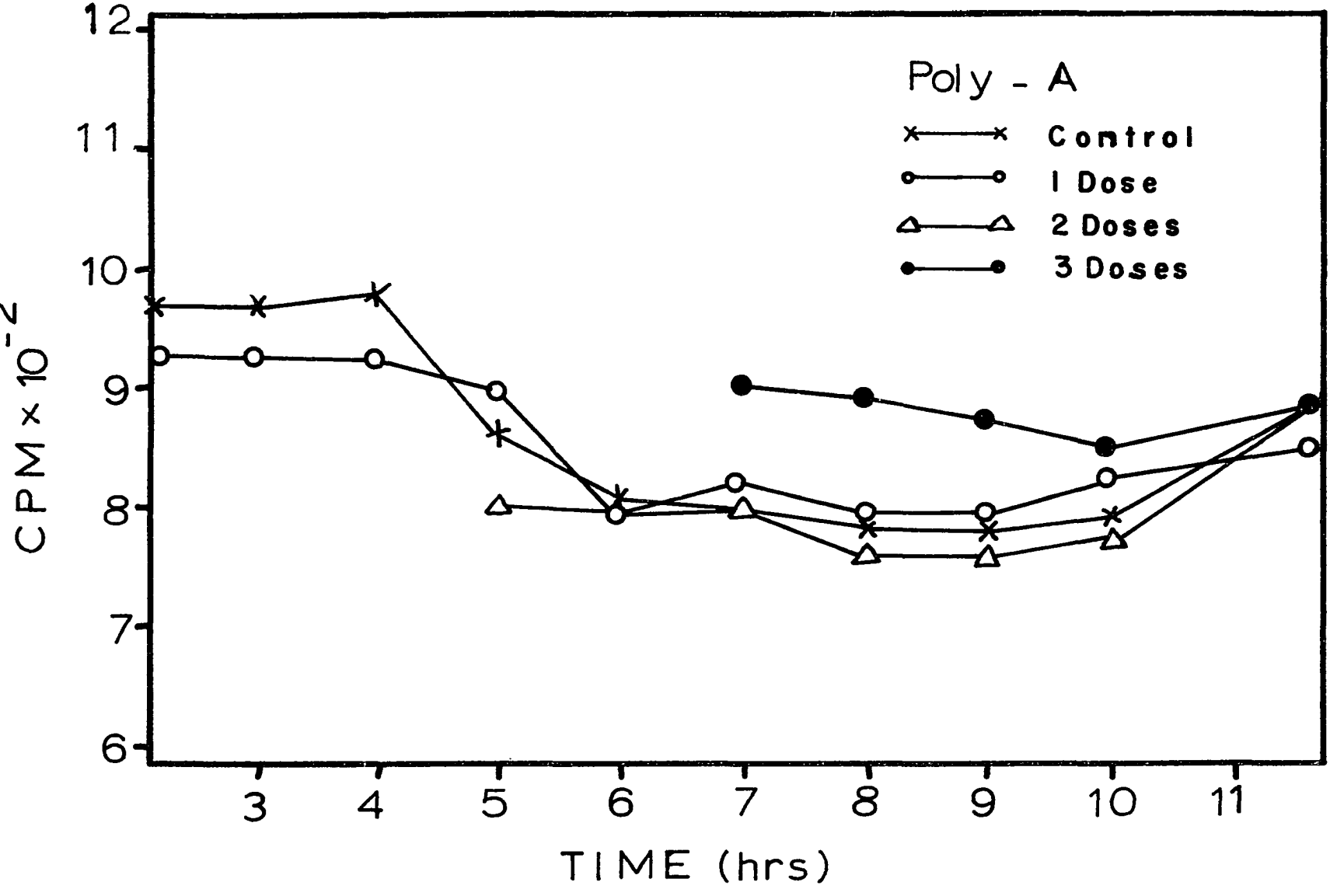

CPM X 10^-3
8
7
6
5
4
3
2
1
3
4
5
6
7
8
9
10
11
TIME (hrs)
Poly_A
Control
1 Dose
2 Doses
3 Doses

Figure 16. The effect of synthetic polyadenylic acid on protein synthesis in L-cells. Monolayer cultures were sequentially treated with synthetic poly-A 1 μg/ml at 0, 5 and 7 h. The control cultures were similarly treated with Tris-HCl buffer. At the times indicated selected cultures were pulse labelled with ^{3}H-L-amino acids 0.5 μCi/ml for 30 min. The radioactivity in the acid-insoluble material is presented as counts per minute for duplicate cultures.


Poly - A
Control
1 Dose
2 Doses
3 Doses
CPM x 10^-2
12
11
10
9
8
7
6
3
4
5
6
7
8
9
10
11
TIME (hrs)

for inhibiting DNA synthesis. To more accurately assess this possibility, the effect of various specific endoribonucleases and exoribonucleases on the inhibitory activity of A-rich RNA was examined. Specific endoribonucleases were chosen to cleave the heterologous oligonucleotides at specific nucleotide locations and exoribonucleases were used to degrade the olignucleotides to mononucleotides in an attempt to render the A-rich RNA biologically inactive.

The purified active reovirus A-rich RNA preparation was treated with ribonucleases. Ribonuclease-treated-A-rich RNA 2 µg/ml was used to sequentially treat monolayer cultures at 0, 5 and 7 h. Control cultures were similarly treated with Tris-HCl buffer and with the specific buffer system plus the enzyme used in each enzymatic hydrolysis. The results of the experiments are shown in Table 1. The ribonuclease, venom phosphodiesterase, N_1 and T_1-treated reovirus A-rich RNA did not mediate an inhibition of DNA synthesis whereas A-rich RNA treated with T_2 and Pancreatic ribonuclease could inhibit DNA synthesis. These results indicated that the oligonucleotide fragment(s) capable of mediating an inhibition of DNA synthesis were hydrolyzed by the venom phosphodiestrase, N_1 and T_1, but were not degraded by T_2 and pancreatic ribonuclease enzymes.

The venom phosphodiesterase is a non-specific exoribonuclease and hydrolyses RNA to 5′-monophosphates starting from the 3′-OH end. All the ss-oligonucleotides terminate with a 3′-OH end, this OH group is specifically required for the

Table 1. Inhibition of DNA synthesis in L-cells treated with reovirus A-rich RNA hydrolyzed with ribonucleases. Reovirus A-rich RNA was treated with each enzyme and the reaction conditions are described in Materials and Methods. Monolayer cultures were treated sequentially with ribonuclease-treated A-rich RNA 2 μg/ml at 0, 5 and 7 h. The control cultures were similarly treated with equal volumes of Tris-HCl buffer, pH 7.2 containing 4% (w/v) sucrose, and specific hydrolysis buffer system plus the enzyme. DNA synthesis was determined as described in Materials and Methods.

Nuclease	Specificity	DNA Inhibition
A-Exonuclease		
Venom phosphodiesterase[1]	require a free 3´-OH group	-
B-Endonuclease		
N_1	3´-GMP	-
T_1 [2]	3´-GMP	-
T_2 [2]	3´-AMP	+
Pancreatic ribionuclease	C-3´Py	+

(+): DNA synthesis inhibited.

(-): DNA synthesis not inhibited.

1 : Requires a free 3´-OH group and liberated nucleoside-5´-phosphate in a stepwise manner.

2 : Preferetially attacks Ap residues and releases 3´-monophosphates, but eventually all phosphodiester bonds are broken.

action of venom phosphodiesterase. The results indicated that A-rich RNA may have been completely degraded to mononucleotides resulting in the loss of its inhibitory properties.

The ribonucleases N_1 and T_1, both specifically hydrolyze between 3´-GMP and the 5´-OH group of the adjacent nucleotide of an RNA chain. Approximately 2000 of the heterologous oligonucleotides in A-rich RNA have 5´-guanosine (10,48,65). This nucleotide would be cleaved by both the ribonucleases N_1 and T_1. The results indicated that the presence of guanosine at the 5´end of these nucleotides was critical for the inhibitory action for DNA synthesis.

Pancreatic ribonuclease is a specific endonuclease which splits the bond between the phosphate residue at C-3´ in a pyrimidine nucleotide to C-5´ of the next nucleotide in the sequence. Since the enzyme had no effect on the inhibitory activity of A-rich RNA, it may be assumed that pancreatic ribonuclease did not degrade the specific fractions of A-rich RNA which were responsible for mediating the inhibition of DNA synthesis. It has been reported that pancreatic ribonuclease does not readily attack an ApA linkage (34), particularly when they are present in polymers of short chain length (5), and it has also been reported that reovirus A-rich RNA was not degraded by the enzyme (58). The purine 3´→ 5´-pyrimidine phosphodiester bond and the adjacent purine - purine bonds represented by Pu-p-Pu-p-Py-p are strongly resistant to the action of pancreatic ribonuclease (5). Based on our experimental data and the

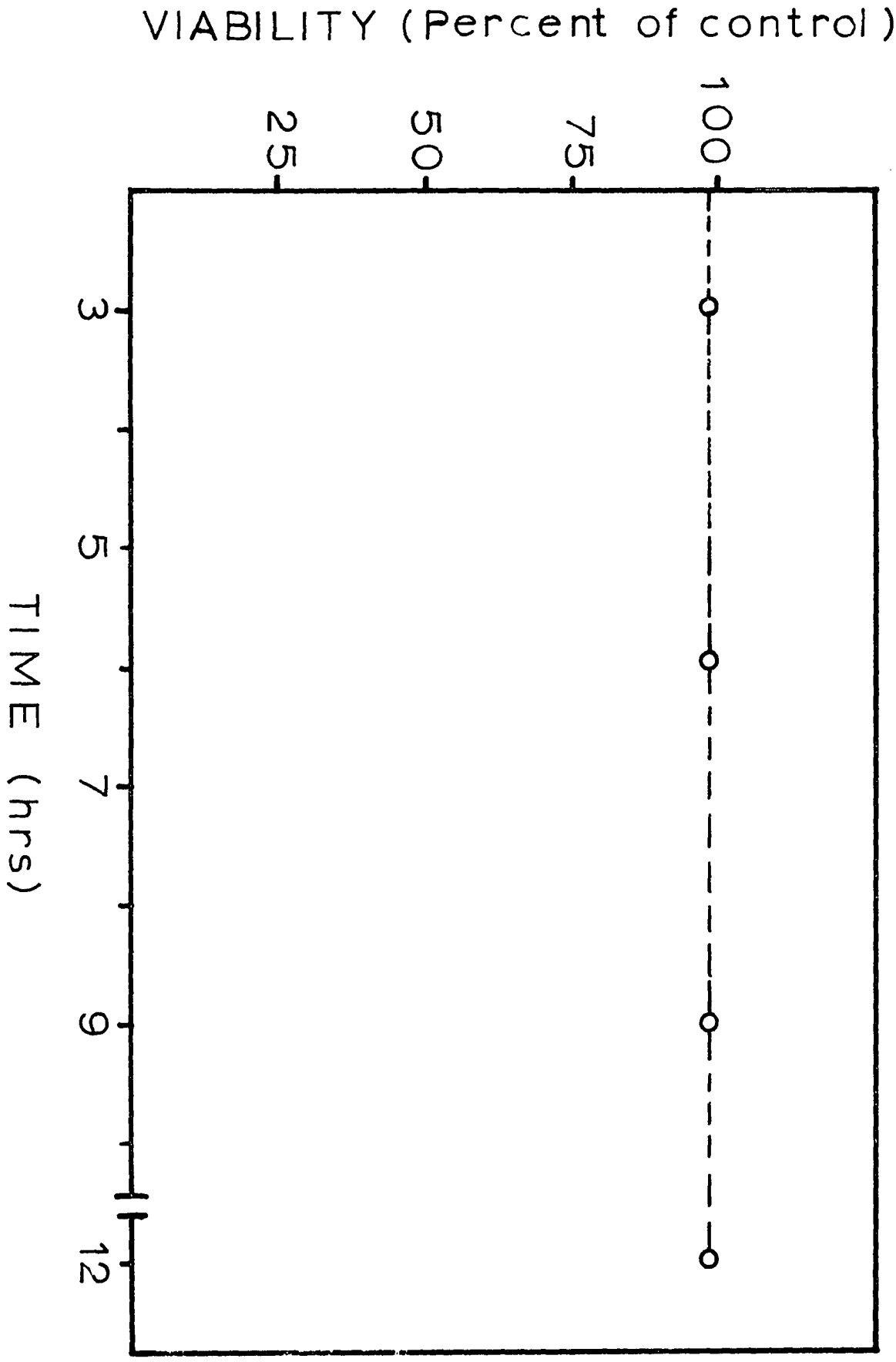
reports on the properties of this enzyme, it can be assumed that the heterologous oligonucleotide fraction of A-rich RNA was not degraded and retained its inhibitory activity.

Ribonuclease T_2, preferentially attacks Ap residues and eventually cleaves the RNA chain non-specifically at all phosphodiester bonds. There are 260 molecules with a chain length of 5-9 nucleotides in the heterologous oligonucleotide fraction of A-rich RNA which have Ap residues in their structure (48). According to the documented activity of the enzyme, all fractions of the A-rich RNA should have been degraded and the inhibitory activity should have been lost. Since A-rich RNA was active even after treatment with T_2, it is possible that the short chain heterologous oligonucleotides with a maximum chain length of up to 9 residues were not degraded. Thus, a restriction of hydrolytic activity similar to that described earlier for pancreatic ribonuclease may be taking place.

Viability of Cells. It was necessary to exclude the possibility that the inhibition of DNA synthesis by A-rich RNA was a specific event and not the indirect result of cytopathic effect of cell death. During the studies of reovirus A-rich RNA inhibition, cultures were routinely monitored microscopically for cytopathologic changes. No visible change in the appearance of cells was noted. To more accurately assess cytopathological changes cultures were also examined for any loss in cell viability by trypan blue exclusion. Monolayer cultures were treated with three doses of 2 μg/ml of A-rich RNA. At

timed intervals cultures were stained with 0.4% (w/v) trypan blue as described in Materials and Methods. The stained cultures were examined microscopically and the results are presented in Fig. 17. No significant decrease in cell viability was noted up to 12 h after treatment with A-rich RNA.

Figure 17. Viability of L-cells treated with reovirus adenine-rich RNA. Monolayer cultures were sequentially treated with reovirus A-rich RNA 2 μg/ml at 0, 5 and 7 h. At the times indicated selected cultures were stained with trypan blue, and total and nonviable cells were counted. Viability is expressed as percent of control.


VIABILITY (Percent of control)
25
50
75
100
TIME (hrs)
3
5
7
9
12

CHAPTER IV

DISCUSSION

The efficiency of inhibition of cellular DNA synthesis has been shown to be directly related to the multiplicity of infection of infectious and UV-inactivated virus particles (60). Inhibition by non-infectious particles (UV-inactivated) could not be accounted for by multiplicity reactivation and subsequent replication of virus (32). In addition, it has been shown in studies with UV-inactivated particles (60) and temperature-sensitive mutants (26) of reovirus that completion of the viral replicative cycle, terminating in progeny virus production and cell lysis, is not necessary for the inhibition of DNA synthesis. These observations suggested that an input component(s) of the reovirion and/or a transcription product(s) might be responsible for the inhibition. Attempts have been made using infectious and non-infectious subviral particles (SVP_i) to identify the viral component which could mediate this inhibition (17). This study indicated that reovirus cores (inner capsid shell and genome ds-RNA) and top component (empty capsids) had no inhibitory effect on DNA synthesis. However, a delayed inhibition of DNA synthesis was observed in SVP_i-infected cells. Top component did not inhibit the synthesis

of DNA even though it contains all virion polypeptides. However, it is missing 60% of the virion A-rich RNA. This suggested that the delay in the inhibition of DNA synthesis might have been the result of lack of release of single-stranded oligonucleotides from the input SVP_i particles. The time of first appearance of reovirus adenine-rich oligonucleotides during virus replication coincides with the beginning of the inhibition of DNA synthesis (8). This provided additional indirect evidence that A-rich RNA might be the inhibitory principle produced during reovirus infection.

Monahan has shown that purified reovirus A-rich RNA was efficiently taken up by cells in monolayer culture, and that it was the only viral component tested which was capable of mediating a selective but transient inhibition of DNA synthesis (46). In our experiments a single does of reovirus A-rich RNA 1μg/ml produced an inhibition pattern similar to that reported by others (32,46).

Since the inhibition of DNA synthesis has been shown to be a dose-dependent phenomonen, it suggested that the increased efficiency of inhibition due to high input multiplicities may be due to release of greater quantities of A-rich RNA during the early stages of infection. Maintenance of the increased rate and degree of inhibition later in infection could be the result of the production of higher yields of progeny virus which in turn would synthesize increased quantities of A-rich RNA.

The higher particle to cell ratio of UV-inactivated virus has been shown to be as effective in inhibiting DNA synthesis as infectious reovirions. UV-inactivated reovirions have been shown to be capable of limited transcription even though the genome is damaged. Initial characterization of the transcription product indicated that it was A-rich RNA (32). This information strongly indicates an apparent necessity for the contribution of a virion component, possibly A-rich RNA, from the input particle for mediating the initial inhibition of DNA synthesis. Sustained inhibition of DNA synthesis could be the result of the production of a new transcription component, possibly A-rich RNA, but not the replication of complete infectious progeny virions.

Data reported in this dissertation support the concept that initial inhibition is mediated by input A-rich RNA. Continued production of A-rich RNA is necessary for maintenance of inhibition. No significant increase in the level of inhibition was observed with an increased single dose of 5 μg/ml of reovirus A-rich RNA. It appeared that sites for the inhibitory action of A-rich RNA were saturated with a specific amount of the A-rich RNA and the remaining amount of A-rich RNA had no added effect on the inhibition of DNA synthesis. Quantities smaller than 1 μg/ml of A-rich RNA might give a minimum dose level for the inhibition of DNA synthesis, but this was not tested.

In an asynchronous culture, cells will be passing through different phases of the cell cycle, and only a fraction

of the population be in S phase at any given time. It has been reported that initiation of the synthesis of DNA upon new regions of chromosomal DNA is blocked after reovirus infection (23,24), but DNA chain growth is not altered (31). In a subsequent in vitro study with purified A-rich RNA and isolated nuclei it was demonstrated that A-rich RNA causes an inhibition of cellular DNA synthesis possibly by interfering with the cytoplasmic factor(s) necessary for initiation of DNA synthesis (46). This implies that, although the A-rich RNA is efficiently taken up by the cells (46), inhibition of DNA synthesis will take place only in those regions of the chromosome where new sites are being initiated for DNA synthesis. The sites which were initiated before contact with A-rich RNA should proceed and complete DNA synthesis. This may explain the transitory nature of inhibition with one dose of A-rich RNA since the A-rich RNA may be modified before new initiation events occur. This inhibition seems to be highly specific for specific initiation events and/or factors since activity of nuclear and cytoplasmic DNA polymerases and the DNA template are not altered even after inhibition of DNA has started (4,60).

It was mentioned earlier that sustained inhibition of DNA synthesis in reovirus-infected cells may be the result of A-rich RNA synthesized by progeny viral particles or input particle transcription during later parts of viral replication. In either case, a continuing supply of A-rich RNA is assured. To mimic such natural infection conditions, cultures were inoculated

sequentially with three doses of reovirus A-rich RNA. One dose produced a transient inhibition, but a persistent and progressive inhibition was mediated by 2 and 3 doses (Fig. 8). In addition multiple higher doses of A-rich RNA produced identical levels of inhibition. This indicated that the persistent presence of A-rich RNA was essential for maintaining the inhibition of cellular DNA synthesis. The optimum inhibitory concentration of A-rich RNA or the proper sequence of doses was not completely defined since total inhibition of DNA synthesis was not achieved by these procedures. The effect of multiple doses of A-rich RNA was selective only for the replicative function of DNA since these doses had no effect on cellular RNA and protein synthesis.

The half life of A-rich RNA in treated cells seems to be very short since a single high dose of A-rich RNA (5 μg/ml) mediated only a transitory inhibition even though the dosage was higher than the combined amount of 3 doses (3 μg) that mediated a sustained and progressive inhibition up to 10 h. It appeared that after an optimum quantity of A-rich RNA was utilized for mediating the inhibition of DNA synthesis the remaining RNA was degraded or modified within the cell before it could affect new DNA initiation events on the chromosome.

It was proposed earlier that the heterologous oligonucleotide fraction of A-rich RNA may be responsible for mediating the inhibition of DNA synthesis and that the oligoadenylate fraction has no inhibitory effect. To test this hypothesis the

effect of single low and high and multiple high doses of synthetic poly-A with molecular weight similar to A-rich RNA was examined. No alteration in the levels of cellular DNA, RNA or protein synthesis was observed. These results agree with those of Monahan (46), and indicated that DNA synthesis inhibition was not the result of non-specific inhibition by oligonucleotides of a certain molecular weight or size, or oligoadenylate nucleotide content, but the result of the specific action of one or more heterologous oligonucleotide with unique chemical or physical characteristics. However, it is not certain that reovirus A-rich oligoadenylate will have the same biological activity as synthetic oligoadenylate since the synthetic oligonucleotides did not have the 5′-terminus pppA--, which might have some function in the inhibition process.

The effect of different exo- and endo-ribonucleases on the inhibitory activity of A-rich RNA was examined in an attempt to provide additional support to the hypothesis that a specific class of A-rich RNA oligonucleotides might be specifically inhibitory. The results indicated that A-rich RNA treated with ribonuclease N_1, T_1 and venom phosphodiesterase lost its inhibitory potential. T_2 and pancreatic ribonuclease had no effect on this activity. The N_1 and T_1 nucleases are known to specifically cleave only the guanosine residue in an RNA chain and the venom phosphodiesterase hydrolyzes RNA to mononucleotides. It has been reported that nearly all of the heterologous oligonucleotides possess guanosine at the 5′ -terminus (48). This information leads us to suggest that inhibition of DNA synthesis

was the result of intact unique heterologous oligonucleotide fragments. In addition it indicates that the presence of guanosine at the 5´-termini of the oligonucleotides may be critical for the inhibitory action of A-rich RNA.

Thus, we know that in reovirus-infected cells the first detectable change in cellular metabolism is the specific inhibition of DNA synthesis. Detectable changes in RNA and protein synthesis occur only during the later stages of virus replication and are followed shortly by severe cytopathology and cell death. The initial phases of the inhibition of DNA synthesis appear to be specific events related to reovirus infection and experimental data in this and other studies indicate that this inhibition of DNA synthesis may be caused by reovirus A-rich RNA. However, in infected cells it is extremely difficult to distinguish between the specific inhibition of DNA synthesis mediated by reovirus replication and non-specific inhibition of macromolecular function late in infection resulting from cell necrosis. The experimental data presented here suggest that multiple doses of reovirus A-rich RNA are capable of selectively mediating a sustained and progressive inhibition of DNA synthesis up to 24 h but have no effect on RNA and protein synthesis or cell viability. In this case selective inhibition of DNA function can be caused by a specific virion component and replication product and its effects can be studied without obscuration by non-specific events related to the development of cytopathology.

In this study an indirect approach was used to characterize the inhibitory fraction of A-rich RNA. A-rich RNA can be fractionated by DEAE-cellulose chromatography into specific molecular fractions, and the inhibitory activity of these fractions could be determined. The effect of the active fractions on the events in the initiation of DNA synthesis should provide information which could elucidate the mechanism of action of reovirus A-rich RNA. Since A-rich RNA is taken up very efficiently by the cell, a study of its localization in cell and the condition of the active fraction after localization, could provide information regarding the specific cellular sites of inhibitory activity.

It is known that initiation of DNA starts with the synthesis of an RNA priming fragment. Since reovirus A-rich RNA may inhibit initiation of DNA synthesis by interfering with cytoplasmic initiation factor(s) in vitro, it is possible that it may have a similar activity in vivo. The chemical and structural characteristics of A-rich RNA suggest its potential to interfere with RNA-mediated initiation of DNA synthesis.

It has been reported that normal diploid and transformed human cells have different sensitivities to reovirus infection and that DNA synthesis was inhibited in transformed cells and not in normal cells (21). It will be of interest to study the effects of purified A-rich RNA on normal diploid and transformed cells to determine if differences exist in the sensitivity of the regulation of DNA replication in the two types of

cells. If only transformed cells are sensitive to the action of A-rich RNA, it could provide an effective means of characterizing fundamental differences in the regulatory properties of normal and cancer cells.

CHAPTER V

SUMMARY

This study of the effect of reovirus adenine-rich (A-rich) RNA on DNA synthesis in L-cells suggests that a single dose of A-rich RNA mediates a transitory but selective inhibition of DNA synthesis. Multiple doses of A-rich RNA which produce a sustained and progressive inhibition of DNA synthesis, have no effect on cellular RNA or protein synthesis, and the treated cells show no cytopathology or cell death. High single and multiple doses of A-rich RNA do not increase the rate or degree of inhibition of DNA synthesis.

Single and multiple doses of synthetic oligoadenylic acid (poly-A) at low and high concentrations have no effect on cellular DNA, RNA or protein synthesis. This suggests that the oligoadenylate fraction of A-rich RNA is not inhibitory for DNA synthesis.

The effects of specific endo- and exo-ribonucleases on the inhibitory activity of A-rich RNA were examined. While ribonucleases N_1, T_1 and venom phosphodiesterase destroyed the inhibitory activity of A-rich RNA, pancreatic and T_2 ribonuclease did not. This indicates that A-rich RNA is rendered

biologically inactive when heterologous oligonucleotides are either deprived of 5′-G termini or completely degraded to mononucleotides. These data support the hypothesis that the inhibitory effect is not mediated by the oligoadenylate fractions of A-rich RNA, but by the specific activity of one or more heterologous oligonucleotides.

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