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GRADUATE COLLEGE

THE EFFECT OF REOVIRUS ADENINE-RICH RNA ON L CELL MACROMOLECULAR SYNTHESIS AND ITS POSSIBLE ROLE IN THE REPLICATIVE CYCLE OF THE VIRUS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

THOMAS M. MONAHAN

Norman, Oklahoma

1972

THE EFFECT OF REOVIRUS ADENINE RICH RNA ON L CELL MACROMOLECULAR SYNTHESIS AND ITS POSSIBLE ROLE IN THE REPLICATIVE CYCLE OF THE VIRUS

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DISSERTATION COMMITTEE

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I wish to express my gratitude to my major professor Dr. Donald C. Cox for his suggestions, criticism, and encouragement throughout the course of this work.

For Carolyn and T_3

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THE EFFECT OF REOVIRUS ADENINE RICH RNA ON L CELL MACROMOLECULAR SYNTHESIS AND ITS POSSIBLE ROLE IN THE REPLICATIVE CYCLE OF THE VIRUS

CHAPTER I

INTRODUCTION

The nature of cellular reproduction and the life cycle of cells has been one of the most intensively studied areas of modern biology. The cell cycle has been divided into four different phases (21) designated M, G1, S, and G2, and defined by the morphological and biochemical events which occur during each of these periods. The time interval from prophase to telophase has been designated M or mitosis. Gap 1 (G1), has been characterized by an increased rate of ribonucleic acid (RNA) and protein synthesis. During this phase a small molecular weight soluble protein is ultimately produced which is necessary for the initiation of S or the Deoxyribonucleic acid (DNA) synthetic phase (46, 14). Gap 2 (G2) is a resting state between S phase and mitosis and is characterized by reduced rates of cellular macromolecular synthesis. Every variety of cell possesses characteristic

time intervals in which each of these cellular events are completed.

Since many viruses are known to disrupt cellular macromolecular synthesis during viral replication, the cellular events which occur during the cell cycle have proven to be an important part of the study of virus-host cell interactions. In this study I have been particularly interested in alterations of cellular macromolecular synthesis, during the phases of the cell cycle, in relation to the replication of reovirus type 3.

One of the intriguing features of many virus infections is the inhibition of DNA synthesis and/or the alteration in the transcriptive and translation processes which occur in the cell cycle of the infected cell (32, 33, 39). Whether these changes in cellular macromolecular synthesis are necessary for virus replication remains unclear. Although the mechanisms of virus induced alterations in cellular macromolecular synthesis are not well understood characterization of these mechanisms could provide a valuable means of studying the regulation of DNA function in normal as well as virus infected cells.

There may be numerous mechanisms by which viruses alter DNA functions. Certain members of the picornavirus group such as poliomyelitis and mengovirus (33, 39) and certain myxoviruses appear to produce a virus-specific protein which may act directly or indirectly in the inhibition of

cellular DNA synthesis or transcription. Adenovirus infection results in the inhibition of cellular DNA synthesis following the accumulation of a specific virus coat protein within the host cell (32). Reovirus possesses certain unique physical, chemical and biological properties which suggest its suitability as a model system for definitive characterization of viral modification of cellular genome function.

The physical and biological characteristics of reovirus have been extensively studied in recent years. The virus contains double stranded and single stranded RNA surrounded by a protein capsid (22). The capsid consists of an inner core protein surrounding the RNA and an outer protein shell. Reovirus possesses cubic symmetry of the icosahedral type and the complete virion has a diameter of 75nm (16). The outer coat of the virion is necessary for efficient infection (22), since the removal of the outer capsid by chymotrypsin digestion decreases virion infectivity. However, the outer coat does not seem to play any other discernible role in the replicative cycle of the virus. The core is functionally more significant during the replicative cycle since it contains a transcriptase (44). The cores contain the double stranded RNA and catalyze the synthesis of new single stranded RNA while fully conserving the viral genome RNA within the core (22).

Reovirus contains double stranded RNA (dsRNA). This was first reported by Gomatos and Tamm (19) and has since

been confirmed by numerous investigators (16, 30, 24, 48). The evidence for the double stranded nature of the reovirus genome has been determined using a number of different techniques. The RNA exhibits a very sharp melting profile, is resistant to ribonuclease digestion and base composition analysis indicates equality of adenine and uracil as well as guanine and cytosine (2, 42). X-ray diffraction patterns have confirmed that reovirus dsRNA exists in the form of a double stranded helix (30).

The dsRNA occurs in 3 distinct size classes or fragments. The molecular weights of the 3 fragments are 0.8, 1.4, and 2.5×10^6 daltons for the small, medium, and large fragments respectively (4, 50). Since the reovirus genome is thought to have a molecular weight of 10^7 daltons and a total length of 8 um (18) one must assume that more than one of each of these fragments is incorporated into each virion. Investigations using polyacrylamide gel electrophoresis have shown that each virion contains 3 of the large, 3 of the medium and 4 of the small fragments (41). The chemical determination of the number of 3' - OH groups per virion yields a number of about 20 (36) while end group analysis (49) reveals that these fragments are probably not linked within the virion. Furthermore, there is no cross hybridization between the molecules making up the 3 size classes (50) and the various segments are transcribed specifically into single-stranded RNA molecules (3, 51), indicating that these ten molecular species are not

fragments or products of random breakage resulting from isolation.

In addition to the dsRNA complement, reovirus contains a single-stranded adenine-rich RNA (A-rich RNA) (2, 4, 42). Although it is not known how the A-rich RNA is formed nor why it is encapsidated a number of its unique characteristics have been elucidated.

The A-rich RNA appears to be a constituent of the virus core (35), although some investigators (28) suggest that it is found between the outer coat and virus core. According to the latter hypothesis any treatment that would remove the outer coat, would then also remove the A-rich RNA. However, removal of the outer capsomeres may merely allow the A-rich RNA to leak out of the core (22). Reovirus which does not contain A-rich RNA has also been reported (28). However, in this report the methods used to purify the virus may have resulted in the removal of the A-rich RNA from the virion. Viral infectivity is also decreased by any treatment that causes the loss of A-rich RNA (22).

Work with infected and "mock" infected cells indicates that this A-rich RNA is not synthesized in uninfected L cells (2) and that in infected cells little if any A-rich RNA is synthesized before 8 hours post infection (2). Detectable amounts of A-rich RNA appear in infected cells between 9 and 13 hours after infection (2) which coincides with the replication of the double stranded genome. Furthermore, it has

been demonstrated that this A-rich RNA will not hybridize to any of the dsRNA fragments (2). Each virion contains approximately 2000 of these A-rich oligonucleotides constituting approximately 25% of the total RNA present (1, 2, 42).

The base composition has been determined by several investigators (1, 2, 42) and is 88 to 89% adenine, 10% uracil, and 1% each of guanine and cytosine. A value of 1.85 S has been shown for A-rich RNA which corresponds to a molecular weight of approximately 5300 daltons and a chain length of 15 nucleotiles (42).

A more recent analysis of this A-rich RNA demonstrates that it represents a highly heterogeneous population of molecules with respect to both size and base composition (1). Sixty per cent of the molecules are from 6-12 nucleotides long and the overall size range is from 2 to 15 nucleotides (1). While most of the molecules have a 5' ppp and a 3'-OH some of the smallest molecules have an unusual, or unidentified, component at the 5' terminus (1). In addition the smaller molecules contain a higher proportion of pyrimidines and a trinucleotide of composition pppGpCpU has been identified (1, 22).

Recent discoveries of adenylic acid rich sequences in the heterogeneous nuclear RNA of normal (9, 10, 12) as well as transformed cells (31) have prompted renewed interest in the A-rich RNA of reovirus. In addition polyadenylic acid sequences have been shown in vaccinia virus mRNA (23) and in

the virus specific mRNA of adenovirus infected cells (37). Further work along these lines has shown that non-template single-stranded polyribonucleotides can strongly and specifically inhibit the DNA polymerase of Rausher murine leukemia virus. In these studies polyuridine is a much more effective inhibitor than polyadenine, although the latter is still inhibitory for the enzyme (47).

Reovirus is a unique ribovirus because of its relatively long latent period (7, 8, 16). After an initial 2 hour adsorption period infectious virus titers do not increase for 7 hours (16). The cell associated virus then increased exponentially from 9-13 hours and reached a maximum at 15-24 hours (16). Subsequent work has shown that reovirus RNA appears in infected cells 7-8 hours after infection which coincides with the start of virus induced inhibition of cellular DNA synthesis (15, 29). Reovirus infection apparently has no early effect on the synthesis of cellular RNA or protein synthesis (15). By increasing the multiplicity of infection it has been shown that the inhibition of cellular DNA synthesis occurs as early as 3 hours post-infection (7, 8). Ultraviolet light inactivation of the infective virus particles does not alter the inhibitory capacity of the virus (8) and the inhibition of cellular DNA synthesis was noted to occur earlier during infection as the levels of inactivated virus were increased. Thus, whether the virus was infectious or non-infectious the time at which DNA synthesis was

inhibited seemed to be solely dependent on the number of virus particles present. The dose response effects of these infective and non-infective virus particles suggests that the inhibitor of cellular DNA synthesis is an integral part of the virus and is resistant to ultraviolet radiation. One could speculate that this earlier inhibition of cellular DNA synthesis could be due to a cytotoxic effect and subsequent cellular death but this does not seem to be the case (7).

Further experiments (13) using synchronous cell cluture have suggested that L cells are prevented from entering into S phase following reovirus infection. The possibility was investigated that reovirus infection may inhibit DNA synthesis through a reduction in necessary enzyme activities (13). Those enzyme activities necessary for DNA synthesis were not significantly decreased even at 12 hours post-infection, a time when cellular DNA synthesis is inhibited by 80% or more (13). Recent work has also demonstrated that the inhibition of DNA synthesis cannot be accounted for by a reduced rate of DNA chain elongation (20). Thus it would seem that the initiation of replication of new DNA chains is the step most sensitive to inhibition by reovirus. Even though the cause of the inhibition at this step in the cell cycle is not readily apparent, hypotheses have been presented (8, 13) which would explain this phenomenon.

The inhibition of DNA synthesis by reovirus may be due to the specific inhibition of the synthesis or activity

of initiator protein. Alternatively, the inhibition of DNA synthesis might somehow affect the initiation process by affecting the integrity of the nuclear membrane since reovirus has been shown to localize in perinuclear inclusions (13). Another possibility is that the transport of initiator substances across the nuclear membrane is impeded by virus-specific products (13). Finally, the nucleic acid component of the viral genome may either code for an inhibitor, interact with the DNA synthesizing system directly, or proteinaceous components of the infectious virion may serve as the inhibitor (8).

The main thrust of my research has been to elucidate the mechanisms of reovirus inhibition of DNA synthesis in particular and its effects on other cellular macromolecular synthesizing systems in general. In particular I have investigated the effect of reovirus A-rich RNA on cellular macromolecular synthesis.

As previously described this heterogeneous population of A-rich molecules possess a number of characteristics which makes it a likely candidate for this inhibitory process. Avian reovirus A-rich RNA has been shown to bind lysyl tRNA to E. coli ribosomes (27) and thus it may act as a mRNA to form a basic protein. Since the genetic code for lysine is A-A-A (52) this is a distinct possibility. The finding of polyadenylic acid sequences in other virus infected cells (23, 37) along with the high adenylic acid concentration in

heterogeneous nuclear RNA (9, 10, 12) tends to implicate the A-rich RNA of reovirus as being potentially important, possibly having a widespread general function. The fact that homoribopolymers such as poly (U) and poly (A) can inhibit DNA polymerase activity (47) in addition to the fact that polyadenylic acid sequences are particularly U. V. resistant (43) makes the A-rich RNA of reovirus even more attractive as an inhibitor.

This investigation is restricted to a study of the DNA inhibitory function of reovirus RNA and emphasizes the role of A-rich RNA in the inhibitory process. In addition, I have examined the role of this A-rich RNA in isolated nuclei "in vitro" DNA synthesizing systems. By utilizing these techniques I was able to investigate the role of A-rich RNA on the soluble protein initiator produced late in G1 phase, and active during S phase of the cell cycle.

CHAPTER II

METHODS AND MATERIALS

Cells

Mouse fibrablasts, strain L-929 were obtained from Flow Laboratories, Rockville, Maryland. The cells were maintained in suspension culture with Eagle's spinner modified minimal essential medium (MEM) (Schwarz Bioresearch) supplemented with 7% (v/v) fetal calf serum (FCS) (Kam Laboratories Inc.). Stock cell suspensions were maintained at an average cell density of 2×10^5 cells/ml by dilution with fresh prewarmed MEM every 48 hours.

Monolayer cultures were established from stock suspension cultures using basal Eagle's medium (BME) (Grand Island Biological Company) and 10% FCS. Cells at a concentration of 7×10^5/ml were added to 30 ml plastic culture flasks (Falcon Plastics) and allowed to attach to the surface of the flask at room temperature for 2 hours. After being incubated at 37 C for 24 to 36 hours the cells formed confluent monolayers and were then utilized for experimental purposes at this time.

Virus

The Dearing strain of reovirus type 3 was kindly provided by Dr. P. J. Gomatos. The virus was propagated in L cells in suspension cultures. The cells at a concentration of $1x10^6$ cells/ml were concentrated by centrifugation (650xg for 5 min) and resuspended in fresh pre-warmed medium containing 2% (v/v) FCS at 25% of the volume of the original suspension culture. Two passage plaque purified virus was added to the concentrated cell culture at a multiplicity of 10 plaque forming units (PFU) per cell and allowed to adsorb at 37 C for 2 hours. Fresh pre-warmed medium was then added to the culture sufficient to return the cell concentration to $1x10^6$ cells/ml and the FCS concentration to 10%. The virus-infected cell culture was then incubated at 37 C for 18 hours, centrifuged briefly (6000xg) and the pellet stored at -70 C until purification. Virus with labeled nucleic acid was obtained in the same manner by propagating the virus in cells grown in a medium containing 1 μCi/ml of ^{14}C adenine (5-15 mCi/mM) (New England Nuclear).

Virus purification

Reovirus was purified from infected cells according to the procedure of Smith et al. (44), with the following modifications. The aqueous phase of the flourocarbon treated virus-cell pellets were layered onto a preformed cesium chloride density gradient (1.2-1.4 gm/ml) and centrifuged for 1 hr at 113,000xg in the SW 27 rotor of the Beckman L2-50

ultracentrifuge. The virus bands were collected cleanly by puncturing the centrifuge tube with an 18 gauge needle and drawing the virus bands into a 10 ml syringe. The virus bands were then diluted with 30 ml of 0.1M sodium chloride, 0.015M sodium citrate pH 7.5 (SSC) and pelleted at 131,000xg for 1 hr. The virus pellet was resuspended overnight in 5 ml SSC and then layered onto a 14 ml preformed 20-40% w/w sucrose gradient and centrifuged for 1 hr at 116,000xg in the SW 27 rotor. These viral bands were collected cleanly and again pelleted after being diluted with SSC. This final pellet was taken up in 10 ml of 0.3M sodium chloride, 0.01M Tris-HCL pH 7.2 containing 0.001M EDTA (0.3M STE) and stored at -70 C until the purified virus was needed.

Virus assay

Virus plaque titration was performed essentially as described by Gomatos et al. (16). Cell monolayers were prepated in 60x15 mm plastic petri dishes (Falcon Plastics) at 37 C in an atmosphere of 5% CO_2 (v/v) in air. Approximately 24 hours before the cells reached confluency 0.1 ml of each virus dilution in monolayer medium without serum was introduced onto the center of each cell sheet and gently distributed over the culture surface. The virus was allowed to adsorb for 30-45 min at 37 C. Following adsorption 5 ml of MEM monolayer medium containing 0.9% (w/v) lonagar (Difco) supplemented with 3% (v/v) FCS was added. The cultures were then incubated at 37 C in an atmosphere of 5% CO_2 (v/v) in

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Preparation of synthetic polyriboadenylic acid

Polyadenylic acid with a minimum molecular weight of greater than 100,000 daltons (Miles Laboratories) was subjected to hydrolysis in 0.1N NaOH for 12 hr at room temperature to reduce its molecular weight to that approximating the molecular weight of reovirus A-rich RNA. ^{3}H-polyadenylic acid with a minimum molecular weight of greater than 50,000 daltons was sheared by repeatedly forcing the suspension through a one inch 26 gauge needle. These labelled and unlabelled RNA preparations were then fractionated on a 2.5x40 cm column of G-50 Sephadex (Pharmacia) which was eluted with Tris-buffer. E. coli transfer RNA (Schwarz Bioresearch) and insulin were used as molecular weight markers. Only those fractions of synthetic polyadenylic acid with a molecular weight which approximated 5,000 daltons were collected and used for experimentation.

Uptake studies

A modified method of Koch and Bishop (26) was used to test the efficiency of uptake of reovirus A-rich RNA. Cells were pretreated for 5 min with either methylated bovine serum albumin (40 µgm/ml) or DEAE dextran (50 µgm/ml) prior to treatment with A-rich RNA ^{14}C (1µgm/ml) or the labelled A-rich RNA was added directly to cells in 4% (w/v) sucrose in Tris-buffer without pretreatment. The monolayers were washed twice at selected time intervals after treatment with 2 ml of

tissue culture medium without serum and then sonicated for 30 sec in 5 ml of cold 5% (v/v) trichloroacetic acid (TCA). The samples were allowed to stand for 30 min at 4 C, the precipitates collected on glass fiber filters, and acid insoluble radioactivity determined by liquid scintillation counting. Uptake studies involving the synthetic poly A and dsRNA were done in the same manner with no cellular pretreatment.

Determination of macromolecular synthesis

Monolayer cultures were grown to confluency in 30 ml plastic tissue culture flasks (Falcon Plastics) as previously described. Reovirus adenine-rich RNA and polyadenylic acid in 4% (w/v) sucrose in Tris-HCL buffer were each diluted to a nucleic acid concentration of 1.0 µgm/ml with monolayer medium supplemented with 10% FCS. The growth medium was replaced with 2 ml of the medium containing a specific nucleic acid preparation. Control cultures received identical medium containing no nucleic acid. DNA, RNA or protein synthesis was determined by pulse labelling for one-half hr in duplicate test and control cultures at selected time intervals after specific nucleic acid treatment using ^{3}H-thymidine (5-10 Ci/mM), ^{3}H uridine (20 Ci/mM) or ^{3}H-L-amino acid mixture (New England Nuclear) respectively. At the end of the labelling period the medium was removed and cold (4 C) 5% (v/v) TCA was added to the test cultures. After storage in

the cold (4 C) for 45 min the acid insoluble precipitate was collected by centrifugation at 1000xg for 5 min at 4 C. The precipitate was then washed twice with 5 ml of cold 5% (v/v) TCA. After the final wash the precipitate was resuspended in 10 ml of Aquasol (New England Nuclear), and counted in a Beckman DPM-100 Scintillation counter following a 12 hour dark adaption period. Quantitative determination of DNA was performed according to the method of Burton (5).

Synchronous growth conditions

Cells in spinner culture were synchronized by starvation. The procedure was similar to that described by Littlefield (34) and consisted of centrifuging and resuspending cells every 72 hours in MEM supplemented with FCS to give a final concentration of 10% (v/v). This procedure was used to prepare synchronized nuclei for the in vitro DNA synthesis experiments.

Preparation of L cell nuclei

Cells were propagated in suspension culture as previously described. The isolation of the L cell nuclei was essentially the procedure described by Kidwell and Mueller (25). The cells were allowed to reach a density of $1x10^6$ cells/ml and were then centrifuged at 809xg for 2 min. The cell pellet was washed twice with BME, resuspended in 5 ml of a RSB solution diluted 2:1 (0.1M sodium chloride, 0.01M Tris-HCL pH 7.4, 0.0015M magnesium chloride) and allowed to swell

for 30-45 min in an ice bath. After swelling the cells were homogenized with 20-30 strokes in a Dounce homogenizer (Wheaton Glass Co.) using a tight fitting pestle. Centrifugation at 809xg for 2 min pelleted the nuclei which were washed twice to remove traces of cytoplasm. The nuclei were resuspended in the DNA reaction mixture buffer (0.12M Tris-HCL pH 8.0, 0.022M glucose) to a final concentration of nuclei of at least $1x10^6$ nuclei/ml.

Preparation of cytoplasmic factor

The low molecular weight, soluble protein cytoplasmic factor (CF) necessary for induction of DNA synthesis was prepared according to Kidwell and Mueller (25). The cells were propagated in suspension culture as previously described using synchronous or asynchronous conditions for growth. Cells were collected by centrifugation at 809xg for 2 min and the nuclei removed as previously described. The cytoplasmic preparation was saved and centrifuged at 100,000xg for 1 hour at 2 C in the SW 27 rotor of a Beckman L2-50 preparative ultracentrifuge. The resulting supernatant containing the CF factor was then frozen at -70 C until needed.

DNA synthesis in isolated nuclei

The DNA reaction mixture which contained $1-2x10^6$ nuclei/ml and 5.0 mM ATP, 0.5 mM dATP, dGTP, dCTP (Sigma Chemicals), 5.0 µCi/ml dTTP (18.3 Ci/mM) (New England

Nuclear), 9.5 mM magnesium chloride was incubated at 37 C without shaking. At selected time intervals 0.3 ml samples were removed and the reaction stopped by the addition of an equal amount of cold (4 C) 10% (v/v) TCA. The TCA precipitated samples were allowed to stand in the cold for 30 min. The TCA insoluble material was collected on glass fiber filters (New Brunswick Scientific) and washed with 10 ml of cold 5% (v/v) TCA. The glass fiber filters were then placed in vials containing 10 ml Aquasol (New England Nuclear) for scintillation counting. All nuclear preparations received either 1.0 µgm/ml A-rich RNA, 1.0 µgm/ml synthetic poly A or were "mock" treated with an equal volume of Tris buffer.

CHAPTER III

RESULTS

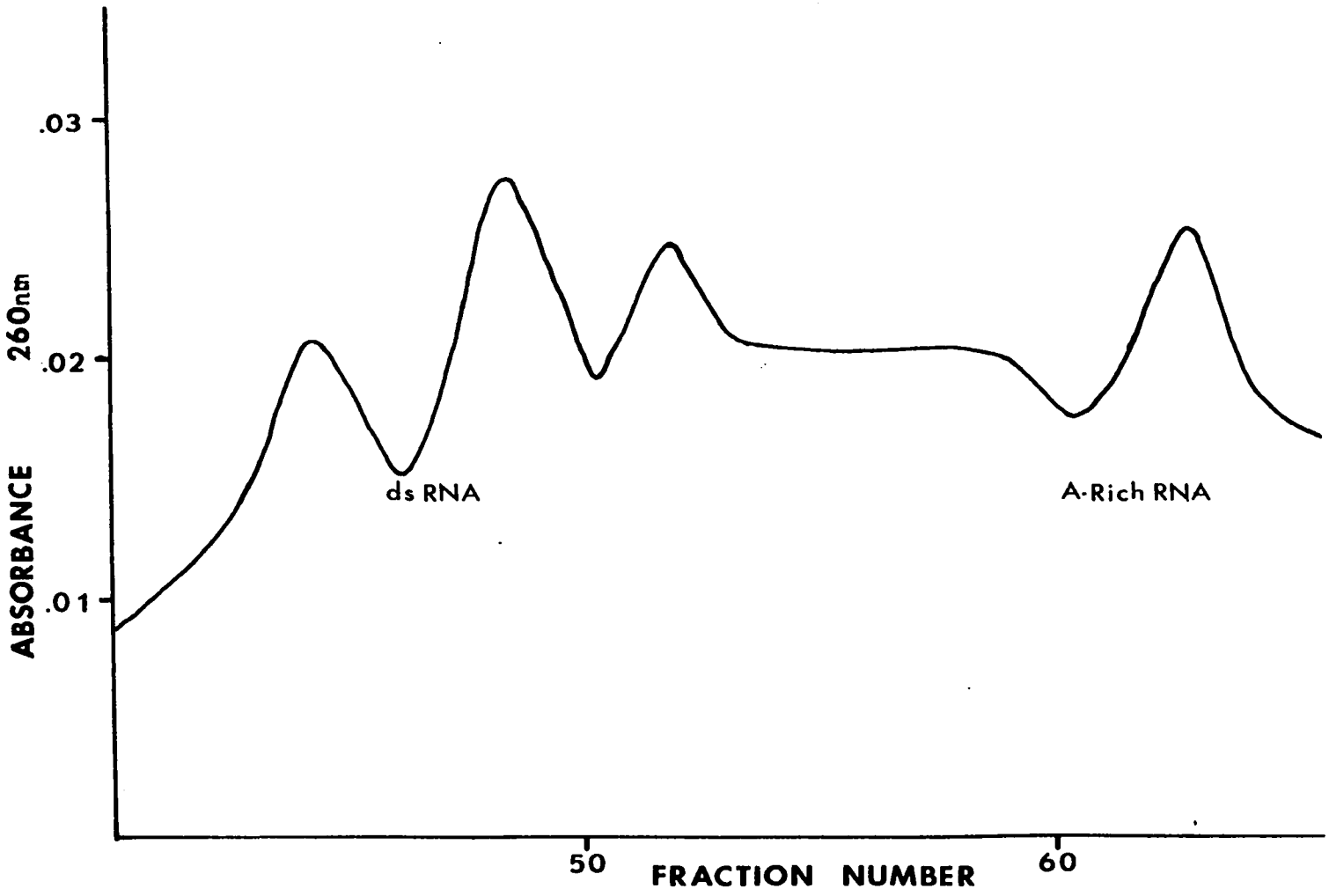
Reovirus nucleic acid preparation

Figure 1 shows the sedimentation pattern of reovirus RNA. This pattern of the different RNA species is consistent and reproducible when using RNA extracted from purified virus. The 3 species of ds genome RNA are easily identified and the A-rich RNA is always found at the top of the gradient as shown. The isolated reovirus RNA's were collected and frozen until required for experimentation.

Synthetic polyriboadenylic acid preparation

A synthetic polyriboadenylic acid (poly A) with a molecular weight of greater than 100,000 daltons was base hydrolyzed to obtain an adenine rich RNA similar in size to reovirus A-rich RNA. Only that portion of peak D which approximated a molecular weight of 5000 daltons was collected and used for experimental purposes. The elution pattern for sheared ^{3}H labelled synthetic poly A was similar to peak D.

Fig. 1.--Sedimentation pattern of reovirus RNA. Purified viral RNA in tris buffer was layered onto a preformed 36 ml 5-20% w/w sucrose gradient and centrifuged for 18 hr at 131,000xg in the SW 27.1 rotor of the Beckman L2-50 ultracentrifuge. The dsRNA and A-rich RNA as indicated were collected and frozen until used.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A-Rich RNA
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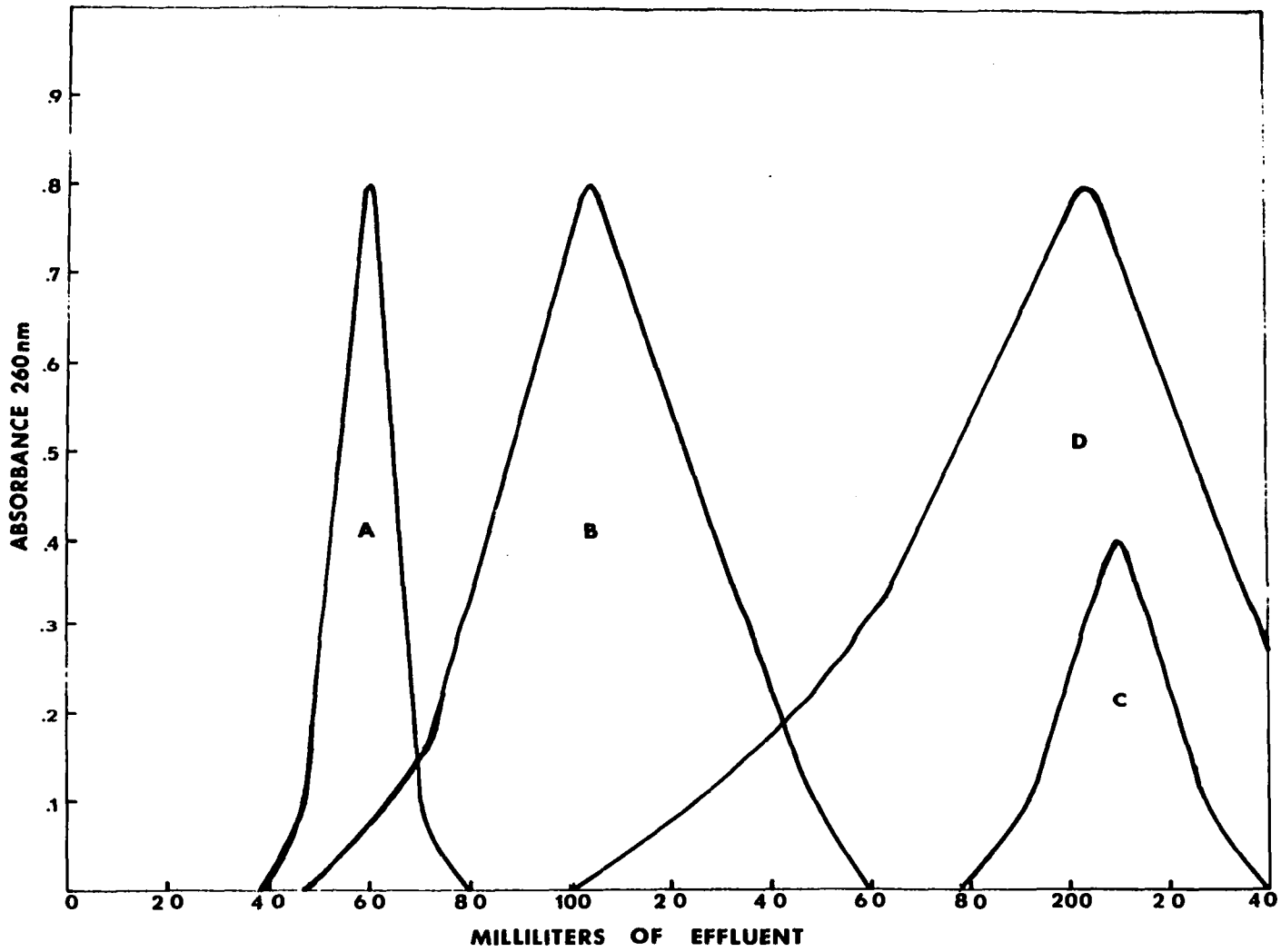
Fig. 2.--Fractionation of base-hydrolyzed polyadenylic acid on a 2.5x40 cm column of Sephadex G-50. Fractions were eluted with buffer containing 0.01 M Tris-HCL (pH 7.2) with 0.14M NaCl and 1 mM $MgCl_2$. (A) Elution pattern for polyadenylic acid (M.W. 100,000 daltons). (B) Elution pattern for E. coli t-RNA. (C) Elution pattern for insulin (M.W. 5800 daltons). (D) Elution pattern for base hydrolyzed polyadenylic acid. This figure represents a composite of individual sample absorbance tracings using an ISCO ultraviolet monitor.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Uptake studies

Radioactively labelled reovirus A-rich RNA, dsRNA and synthetic poly A were prepared and purified as previously described. The reovirus A-rich RNA was taken up rapidly and efficiently by L cells without polycationic treatment (Figure 3A). The cells pretreated with DEAE dextran were the least efficient in the uptake of A-rich RNA. At 2 hrs the cells which had received no pretreatment and those which had been pretreated with methylated bovine serum albumin appeared to take up the A-rich RNA with equal efficiency. However, after 2 hrs untreated cells were still taking up A-rich RNA while those pretreated with methylated bovine serum albumin had essentially stopped taking up RNA. Therefore, a 2 hr adsorption period was allowed in all experiments and the cell cultures were not pretreated with any polycations.

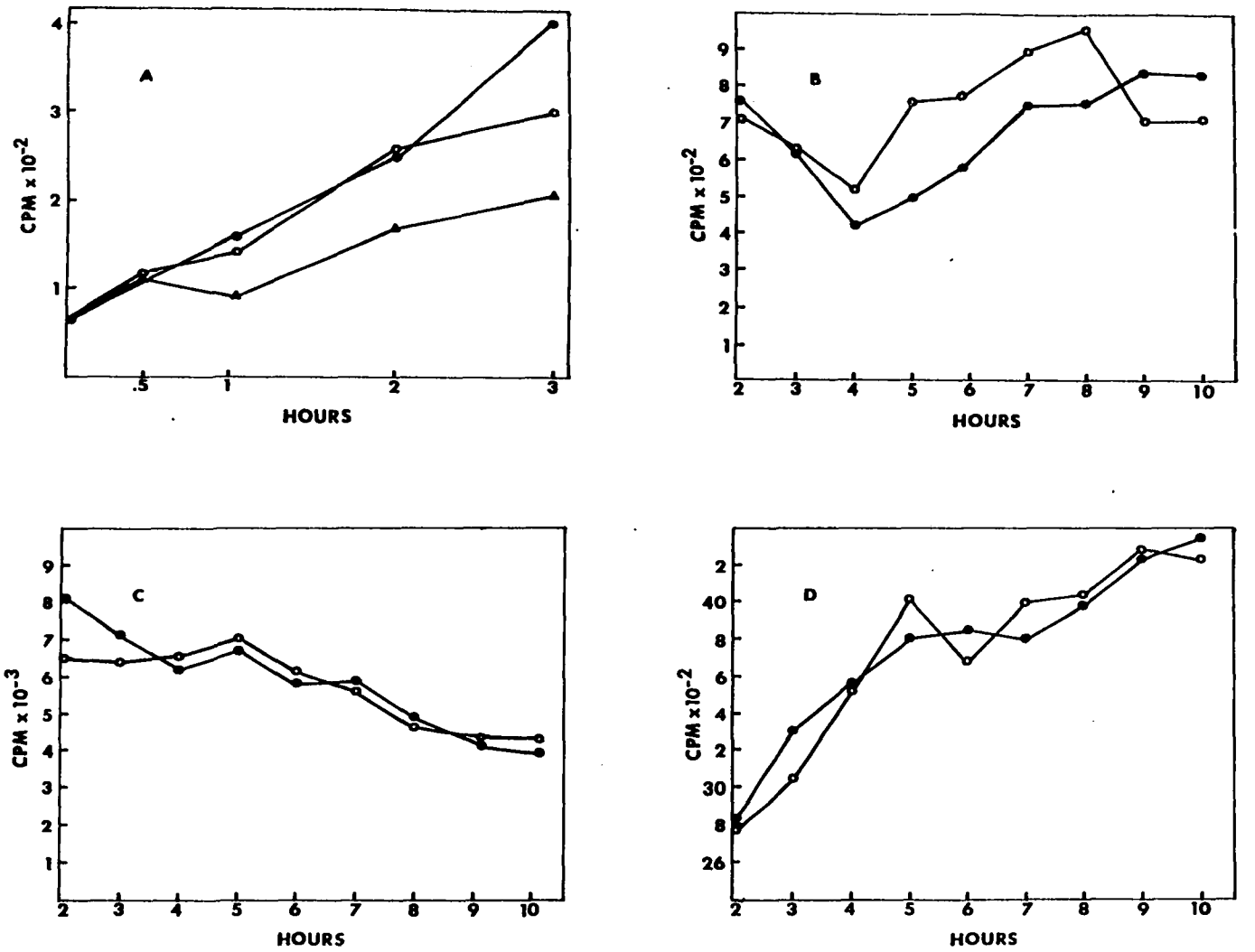
Figures 4A and 5A show the uptake of synthetic poly A and reovirus dsRNA respectively. No cellular pretreatment with polycations was used and the synthetic poly A was apparently taken up as rapidly and efficiently as was A-rich RNA. Double-stranded genome RNA, however, was not taken up efficiently and the data indicated very little of this RNA was cell-associated.

Effect of reovirus A-rich RNA on cellular DNA, RNA and protein synthesis

Evidence for the inhibition of L cell DNA synthesis is presented in Figure 3B. This inhibition appeared to be a

Fig. 3.--Uptake of reovirus adenine-rich RNA by L cells and its effect on cellular DNA, RNA, and protein synthesis.

The uptake of ^{14}C-labelled reovirus adenine-rich RNA by L cells was measured (A). Monolayer cultures were either pretreated with 50 µgm/ml DEAE dextran (▲) or 40 µgm/ml methylated bovine serum albumin (O) or received no polycationic pretreatment (●). All cultures were then exposed to 1.0 µgm/ml ^{14}C-labelled A-rich RNA. At the times indicated selected cultures were washed and cell-associated acid-precipitable radioactivity determined. All points represent an average value from 3 cultures. The effects of reovirus(●) A-rich RNA on cellular DNA (B), RNA (C) and protein synthesis (D), control cultures were "mock" infected using buffer containing 4% (v/v) sucrose (O). Following a 2 hr uptake period cultures were pulse-labelled for 30 min at selected time intervals with ^{3}H thymidine, ^{3}H uridine or ^{3}H-L amino acids (0.5 µCi/ml). Acid precipitable radioactivity was then measured.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CPM x 10-2
HOURS

Fig. 4.--Uptake of base hydrolyzed synthetic polyadenylic acid by L cells and its effect on cellular DNA, RNA, and protein synthesis.

The uptake of base hydrolyzed synthetic ^{3}H-polyadenylic acid by L cells was measured (A). The cells were treated with 1.0 µgm/ml ^{3}H poly A in buffer containing 4% (w/v) sucrose. At the times indicated selected cultures were washed and cell-associated acid precipitable radio-activity determined. All points represent an average value from 3 cultures. The effect of poly A on cellular DNA (B), RNA (C), and protein (D) synthesis was tested. Each test culture received 1.0 µgm/ml poly A (●) and control culture were "mock"-infected using a 2 hr uptake period, cultures were pulse-labelled for 30 min at selected time intervals with ^{3}H-thymidine, ^{3}H uridine, or ^{3}H-L amino acids (0.5 uCi/ml). Acid precipitable radioactivity was then measured.

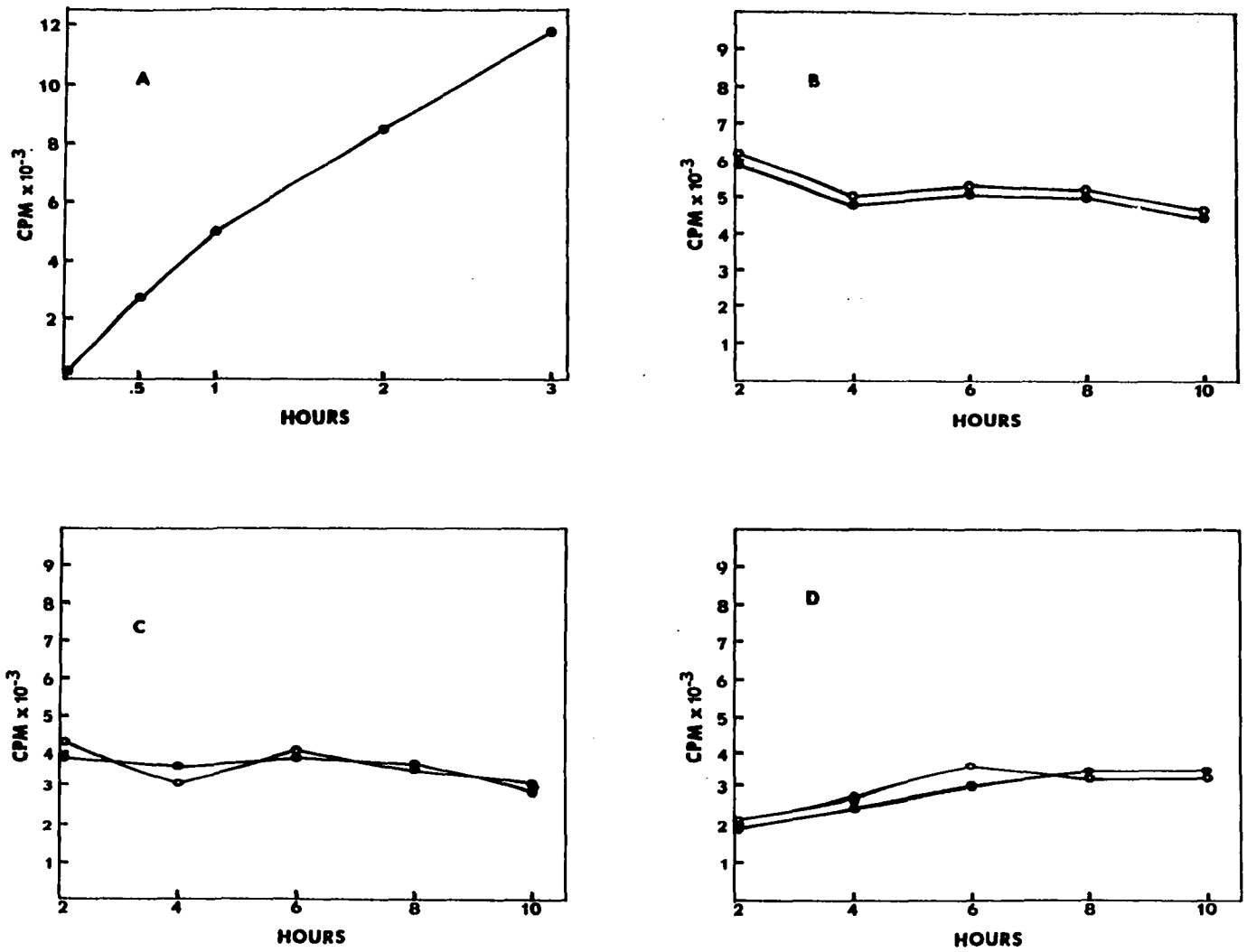
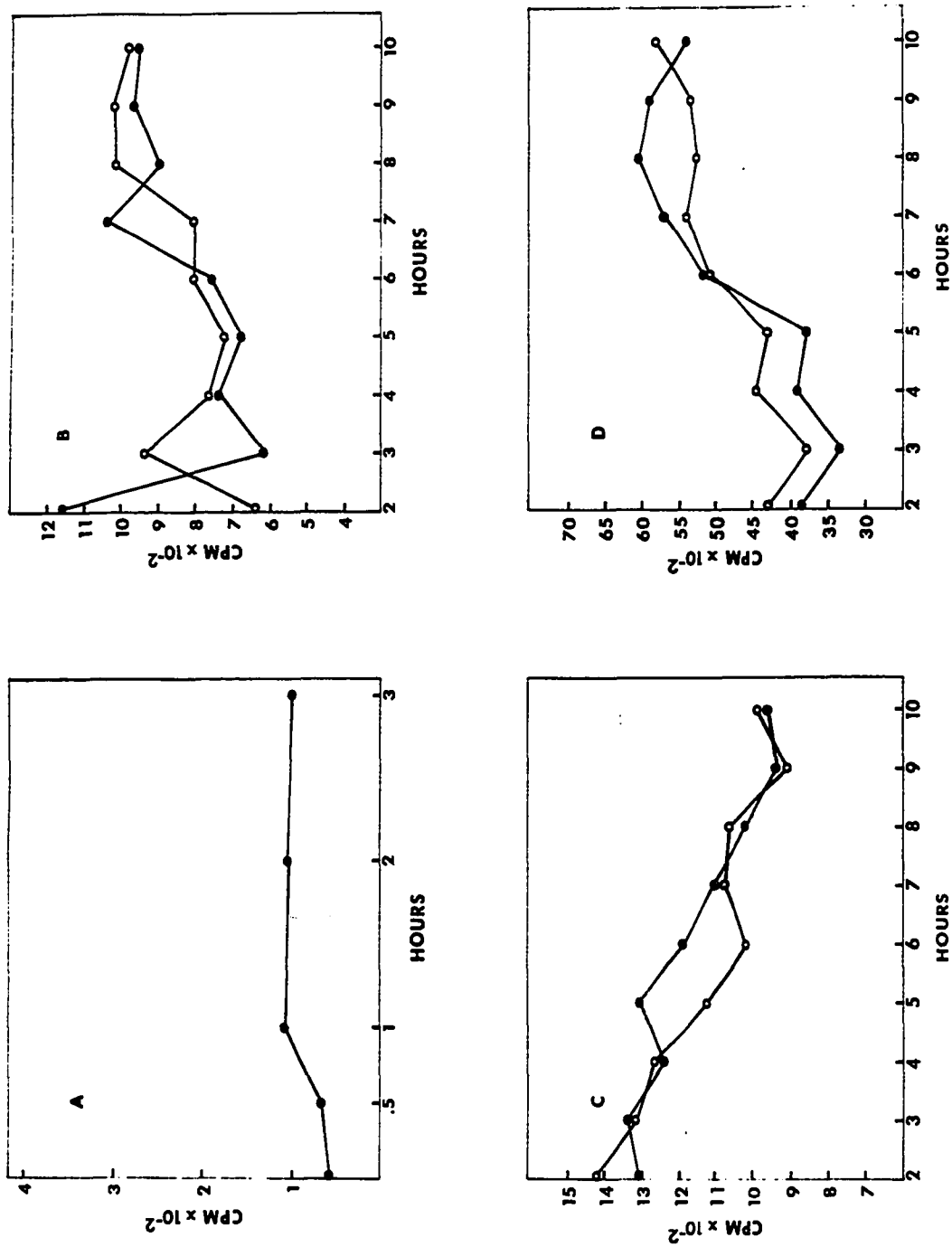

A
B
C
D
CPM x 10^-3
HOURS
.5
1
2
3
4
6
8
10

Fig. 5.--Uptake of reovirus double-stranded RNA by L cells and its effect on cellular DNA, RNA, and protein synthesis.

The uptake of reovirus double-stranded RNA by L cells was measured (A). The cells without pretreatment were treated with 1.0 µgm/ml of the ^{14}C-labelled dsRNA in buffer containing 4% (w/v) sucrose. At the times indicated selected cultures were washed and cell-associated acid precipitable radioactivity determined. All points represent an average from 3 cultures. The effect of dsRNA (●) and control cultures were "mock"-infected using buffer containing 4% (w/v) sucrose (○). Following a two hr uptake period cultures were pulse-labelled for 30 min at selected time intervals with ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-L amino acids (0.5 µCi/ml). Acid precipitable radioactivity was then measured.

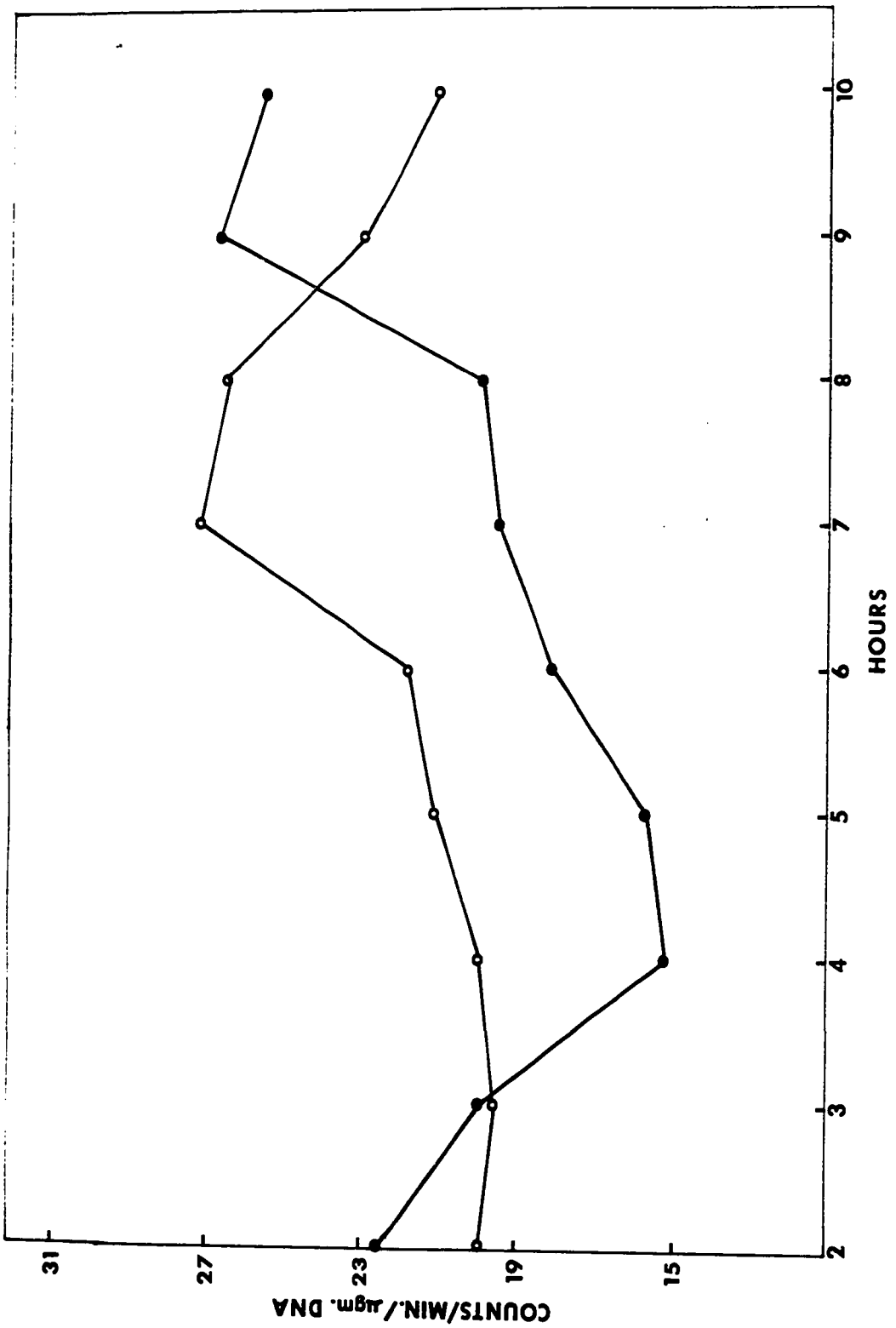

A
B
C
D
CPM x 10-2
HOURS

transitory phenomenon the pattern of inhibition appeared was identical upon repeated experimentation. The inhibition of cellular DNA synthesis occurred between 3 and 4 hours in contrast to viral inhibition which occurred 7-8 hours post infection with 10PFU/cell. However, the cells appeared to recover and reached control levels of synthesis between 8 and 9 hours after treatment with reovirus A-rich RNA.

When synthesis was measured as a function of the specific activity of DNA, the pattern of inhibition was unaltered although, the degree of inhibition appeared to be more significant (Figure 6). The level of inhibition shown in this figure ranges from 25 to 35% below control levels of synthesis. In all the experiments which showed this inhibition of DNA synthesis by A-rich RNA a level of inhibition less than 22% was never observed. Thus, although the inhibition appears to be transitory. This pattern was seen consistently in this system.

The data presented in Figure 3C and D illustrates the effect of A-rich RNA on RNA (C) and protein synthesis (D) respectively. There seems to be no apparent effect on these cellular macromolecular synthesizing systems by A-rich RNA. Therefore, it appeared that reovirus A-rich RNA was capable of selectively inhibiting DNA synthesis rather than generally inhibiting all template activity. For this reason it was of interest to determine if this selective property was unique or if other polynucleotides of similar nucleotide

Fig. 6.--The effect of A-rich RNA on cellular DNA synthesis measured by changes in the specific activity of DNA. Monolayer cultures were treated with 1.0 μgm/ml A-rich RNA (●) or "mock" infected using buffer (○) containing 4% (w/v) sucrose. Following a 2 hr uptake period, test and control cultures were pulse labelled with ^{3}H thymidine (0.5 μCi/ml) for 30 min at selected time intervals after treatment. The cultures were treated with cold 5% (v/v) TCA, the precipitate washed twice with 5% (v/v) TCA and hydrolyzed at 95 C for 30 min in 5 ml of 5% (v/v) TCA. One ml aliquots were taken and tested for radioactivity and DNA concentration by the diphenylamine reaction.


COUNTS/MIN./µgm. DNA
31
27
23
19
15
2
3
4
5
6
7
8
9
10
HOURS

composition and molecular weight could effect similar alterations in the replicative function of cellular DNA.

Effect of synthetic polyriboadenylic acid on cellular DNA, RNA, and protein synthesis

The data presented in Figure 4B indicated there was no alteration in the synthesis of DNA following treatment of cells with poly A. There also appeared to be no change in the synthesis of cellular RNA or protein (Figures 4C and D respectively) after exposure of the cells to poly A. It seemed therefore, that the ability of reovirus A-rich RNA to selectively inhibit cellular DNA synthesis was unique to this molecule and not merely a non-specific manifestation of its adenine-rich base composition, low molecular weight, or chain length.

Effect of reovirus double stranded RNA or cellular DNA, and protein synthesis

The effect of reovirus dsRNA on DNA synthesis in L cells is presented in Figure 5B. RNA and protein synthesis also appear to be unaffected by reovirus dsRNA (Figures 5C and D). The patterns of cellular macromolecular synthesis appear to be erratic. However, repeated experimentation demonstrated no reproducible effect of dsRNA on macromolecular synthesis.

Effect of reovirus A-rich RNA on synchronized L cell cultures

Cell cultures were synchronized and concentrated to $1x10^6$ cells/ml in a total volume of 50 ml. As shown in Figure 7 the A-rich RNA did not show its inhibitory effect until the cells entered the S or DNA synthetic phase of the cell cycle. In addition the synthetic poly A did not appear to be inhibitory. Therefore, the A-rich RNA, like the virus, appeared to exhibit its inhibitory effect on the initiation of DNA synthesis.

The "in vivo" fate of reovirus A-rich RNA

Radioactively labelled reovirus A-rich RNA was added to a concentrated suspension culture of asynchronously growing cells and a nuclear isolation procedure was used to separate the nuclei from the cytoplasm. The data illustrated in Figure 8 demonstrated that the A-rich RNA was efficiently taken up into the cytoplasm of the L cells until 5 hours after treatment began. A rapid rise was also seen in the isolated nuclei but leveled off and remained at much lower levels as compared to the cytoplasm. After 5 hours the amount of A-rich RNA found in the cytoplasm of the cells diminished, while amounts found in the isolated nuclei remained fairly constant.

Fig. 7.--The effect of reovirus adenine-rich RNA and synthetic polyadenylic acid on synchronized cultures of L cells.

The cell cultures were synchronized and concentrated to $1x10^6$ cells/ml in a total volume of 50 ml. The cells received either 1.0 µgm/ml A-rich RNA (●), 1.0 ugm/ml synthetic poly A (Δ), or were "mock" treated (0) with tris-buffer containing 4% (w/v) sucrose. RNA was added at time 0, serum was added at time 2, and the RNA was allowed to adsorb for 2 hr. After the 2 hr adsorption period cultures were tested for DNA synthesis using ^{3}H-thymidine (0.5 µCi/ml) for 30 min. Aliquots were precipitated with cold 5% (v/v) TCA, allowed to stand in an ice bath for 30 min, and collected on glass fiber filters for determination of radioactivity.

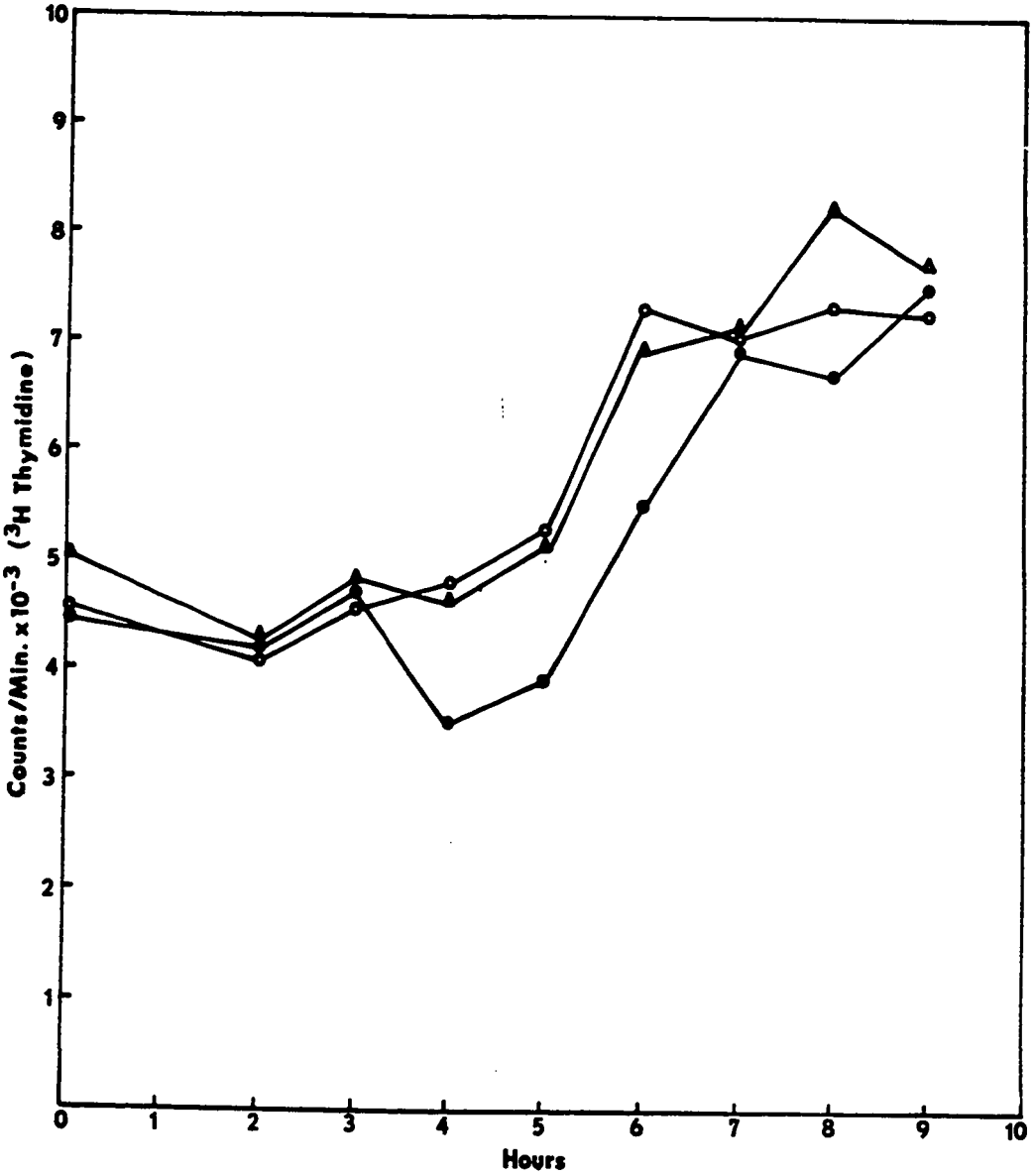
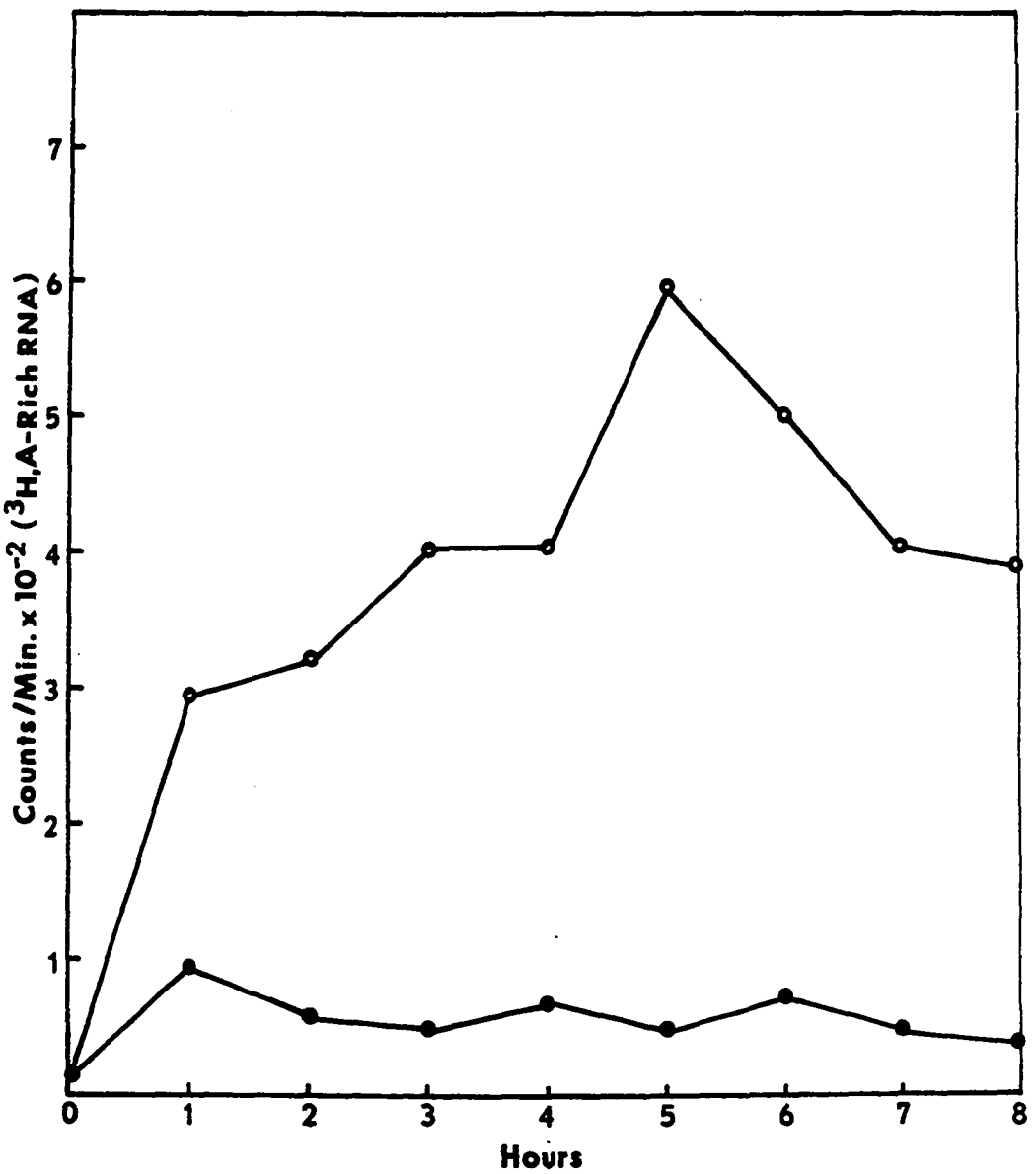

Counts/Min. x10-3 (3H Thymidine)
Hours
0
1
2
3
4
5
6
7
8
9
10

Fig. 8.--The "in vivo" fate of reovirus adenine-rich RNA.

An asynchronous culture of cells was concentrated to $2x10^6$ cells/ml in 50 ml. To this was added 2.0 µgm/ml ^3H-adenine labelled reovirus A-rich RNA. At hourly intervals thereafter aliquots were removed and the nuclei and cytoplasm were isolated. The samples were treated with cold (4 C) TCA and allowed to precipitate for 30 min in an ice bath. The precipitates were collected on glass fiber filters and the radioactivity determined by liquid scintillation counting. Cytoplasmic extract (0) and nuclei extracts (●) are shown.


Counts/Min. x 10^{-2} (^{3}H,A-Rich RNA)
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Hours

Effect of A-rich RNA and synthetic poly A on DNA synthesis in isolated nuclei

The experiments described above suggest a specific inhibition of cellular DNA synthesis by reovirus A-rich RNA. However, it is not clear if this inhibition is mediated by translation of the A-rich RNA to yield an inhibitory polypeptide or if the A-rich RNA acts directly on the DNA template or on one or more of the components necessary for the initiation of DNA synthesis. In order to further clarify the mode of inhibition DNA synthesis was measured in isolated nuclei where the potential for translation is minimized, and the effects of A-rich RNA and polyadenylic acid on DNA synthesis in these preparations was measured. The effects of these molecules on cytoplasmic factors and their ability to induce DNA synthesis in isolated nuclei was also measured.

The DNA synthetic pattern for nuclei isolated from unsynchronized cells which received no cytoplasmic factor (CF) is illustrated in graph A of Figure 9. As shown the control and synthetic poly A treated nuclei synthesized similar levels of DNA. The reovirus A-rich RNA-treated nuclei maintained control levels of synthesis shortly after treatment followed by a reduction in DNA synthesis, and ultimately returned to control levels. This transient inhibition pattern resembles that seen in previous experiments and may be due to the inhibitory process acting on only those nuclei from cells which have not yet started to synthesize DNA. It should be

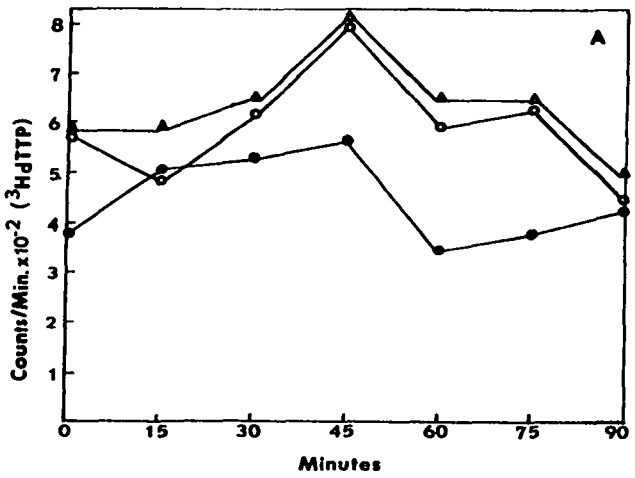
Fig. 9.--The effect of A-rich RNA on DNA synthesis in isolated L cell nuclei. All nuclei and CF were prepared as previously described. The isolated nuclei experiments all utilized 1.0 µgm/ml A-rich RNA, 1.0 µgm/ml synthetic poly, or the nuclei were "mock" treated with buffer. At the selected time intervals 0.3 ml nuclei samples were precipitated with an equal volume of cold (4 C) 10% (v/v) TCA. These samples were allowed to stand in an ice bath for 30 min and collected on glass fiber filters. At least 10 ml of cold 5% (v/v) TCA was used to wash each sample which were then placed in scintillation vials for determination of radio-activity.

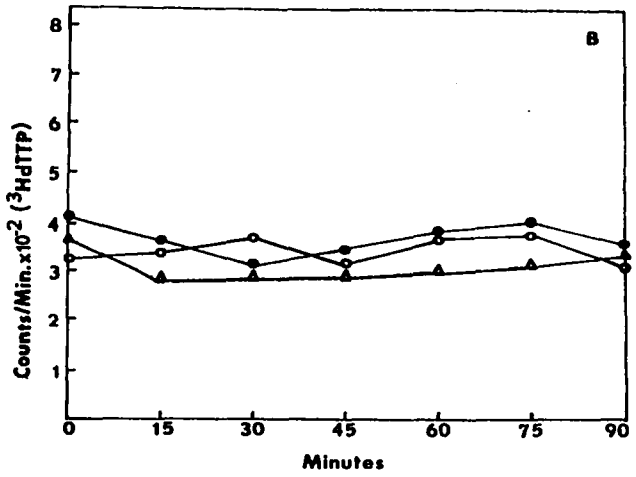
Nuclei from unsynchronized cells (graph A). RNA was added at time 0. Control nuclei (0), nuclei + A-rich RNA (●), nuclei + synthetic poly A (Δ).

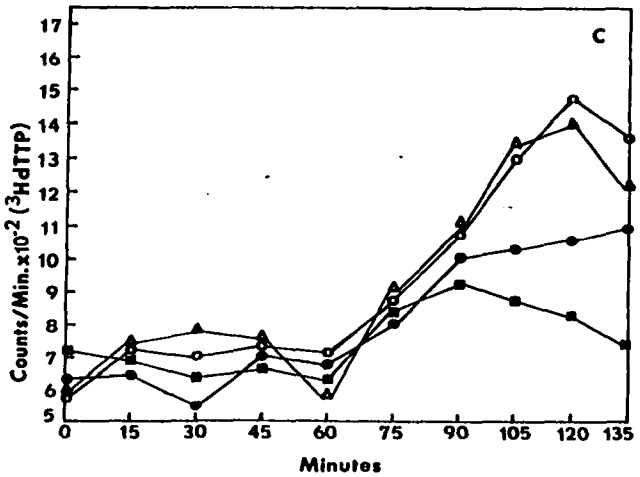
Nuclei from synchronized cells (graph B). RNA was added at time 0. Control nuclei (0), nuclei + A-rich RNA (●), nuclei + synthetic poly A (Δ).

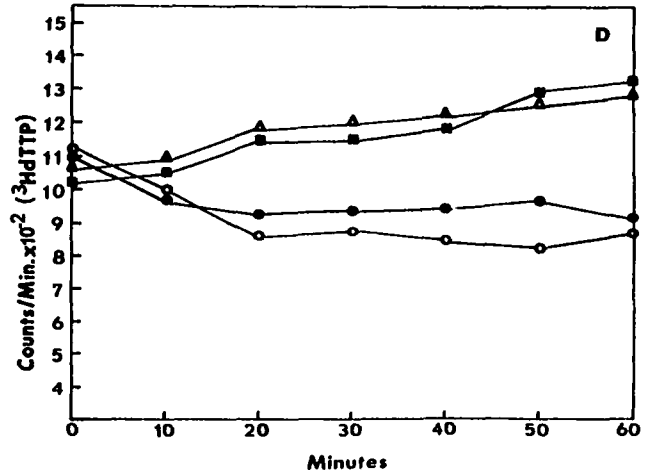
Nuclei from synchronized cells + cytoplasmic factor (CF) (graph C). RNA was added at time 0, CF was added at 60 min. Control nuclei + CF (0), control nuclei not treated with CF (■), nuclei + CF + synthetic poly A (Δ), nuclei + CF + A-rich RNA (●).

Nuclei from synchronized cells + CF pretreated for 15 min with RNA. Pretreated CF added at time 0 with appropriate RNA. Control nuclei + CF (■), nuclei not treated with CF (0), nuclei + CF + synthetic poly A (Δ), nuclei + CF + A-rich RNA (●).


A
Counts/Min.x10-2 (3HdTTP)
Minutes
0 15 30 45 60 75 90
1 2 3 4 5 6 7 8


B
Counts/Min.x10-2 (3HdTTP)
Minutes
0 15 30 45 60 75 90
1 2 3 4 5 6 7 8


C
Counts/Min.x10-2 (3HdTTP)
Minutes
0 15 30 45 60 75 90 105 120 135
5 6 7 8 9 10 11 12 13 14 15 16 17


D
Counts/Min.x10-2 (3HdTTP)
Minutes
0 10 20 30 40 50 60
4 5 6 7 8 9 10 11 12 13 14 15

noted that in this system it has been shown that L cell nuclei have a very limited capacity to synthesize DNA, and after 60-75 min their ability to synthesize DNA declines.

The results shown in Figure 9B provided evidence for the lack of any effect, by A-rich RNA or synthetic poly A, on DNA synthesis in nuclei from synchronized cell cultures. The nuclei were isolated from synchronized cell cultures after the cells had been allowed to enter S phase or DNA synthesis. The treated nuclei showed no inhibition of DNA synthesis which indicated that the action of A-rich RNA on DNA synthesis must occur before or at the time of initiation of new synthesis.

To test this hypothesis, nuclei and CF were isolated from cells actively synthesizing DNA (Figure 9C). The nuclei were incubated for 60 min with the appropriate RNA or buffer at which time the CF was added to selected nuclear reaction mixtures. The evidence indicated that the nuclei not receiving the CF did not show increased levels of DNA synthesis. Those reaction mixtures receiving the CF showed a dramatic and significant increase in levels of DNA synthesis. However, the A-rich RNA inhibited DNA synthesis while synthetic poly A caused no modification of the synthetic pattern.

Nuclei from synchronized cells not yet in S-phase and CF from cells actively synthesizing DNA were isolated (Figure 9D). The purified CF was treated with A-rich RNA, synthetic poly A, or Tris buffer, and then the mixtures were incubated at 37 C for 15 min. Data are presented in graph D which seem

to implicate A-rich RNA as an inhibitor of the cytoplasmic factor necessary for the initiation of DNA synthesis. The levels of DNA synthesis in control nuclei as well as those treated with synthetic poly A were very similar which indicated no inhibitory effect by poly A. Of considerably greater importance is that the level of DNA synthesis in nuclei receiving the CF pretreated with A-rich RNA was significantly less than that of control levels and approached that of nuclei receiving no CF.

CHAPTER IV

DISCUSSION

The results of this investigation suggest that reovirus A-rich RNA is capable of mediating a selective inhibition of cellular DNA synthesis. Earlier work has shown that reovirus infection of L cells is selective for the inhibition of DNA synthesis since there is apparently no inhibition of RNA or protein synthesis prior to or at the time of inhibition of DNA synthesis (7, 15). By using U.V. inactivated virus particles, Shaw and Cox (7, 8) have shown that replication of the virus is not necessary for the inhibition of L cell DNA synthesis. The inhibition of DNA synthesis was shown to be a direct result of increasing multiplicities of U.V. inactivated virus and could not be accounted for by multiplicity of reactivation and subsequent replication of the virus (7). Thus, it would appear that a subunit of reovirus could possibly mediate the inhibition of DNA synthesis in L cells.

The properties of reovirus A-rich RNA that make it physicochemically unique are the same characteristics that

have made it appealing as an inhibitor of L cell DNA synthesis. Reovirus A-rich RNA was chosen for the following reasons: a. A-rich RNA has a low molecular weight making it relatively easy to separate from other viral components. b. The A-rich RNA is taken up rapidly and efficiently (Figure 3A) by L cells without polycationic treatment. c. Sequences of polyadenylic acid have been shown to be relatively resistant to U.V. irradiation (43). d. Since avian reovirus A-rich RNA was shown to bind lysyl t RNA to E. coli ribosomes (27) the possibility existed that mammalian reovirus A-rich RNA could be translated into a basic, potentially inhibitory protein such as polylysine.

Evidence for the selective inhibition of L cell DNA synthesis was presented in Figures 3 B, C and D. The inhibition began 3-4 hours (Figure 3B) after the cells were exposed to A-rich RNA and seemed to recover by 9-10 hours after treatment began. While this inhibition appeared to be transitory the levels of inhibition were 20-30% below control levels of synthesis. By comparing the specific activities of cellular DNA synthesis in treated and untreated cells the levels of DNA synthesis in cells treated with A-rich RNA was 30-40% below control levels of synthesis (Figure 6). The levels of RNA and protein synthesis in cells treated with A-rich RNA were unaffected. Thus, it appears that reovirus A-rich RNA is selective for the inhibition of cellular DNA synthesis. Although the inhibition of DNA synthesis appears to be

transitory, the degree of cellular DNA inhibition was a consistant and reproducible occurrence.

The transitory nature of the inhibition of L cell DNA synthesis by A-rich RNA could be explained on the basis of its possible interaction with and inhibition of DNA polymerase in the cytoplasm or nucleus. An alternate explanation may be that since the cells were growing asynchronously when treated with A-rich RNA the recovery of the cells may have been due to cells entering the S phase before the RNA could act. A similar hypothesis would be that the inhibition which was observed was complete and the resulting return to normal levels of synthesis was caused by cells entering DNA synthesis at a time when the A-rich RNA was not effective. Thus, it would appear that the ability of A-rich RNA to inhibit DNA synthesis may be dependent upon its being in contact with some factor during a brief but critical time period in the cell replication cycle.

A number of questions arise concerning the selective, although transitory, nature of inhibition of cellular DNA synthesis by reovirus A-rich RNA. Is it unique to reovirus A-rich RNA or would any polyriboadenylic acid of similar size and base composition cause this same effect? What role would dsRNA play in this inhibition if the single stranded A-rich RNA was contaminated with fragments of dsRNA? Is A-rich RNA translated to produce a basic inhibitory protein? Finally, at what point during the cell cycle does A-rich RNA cause the inhibition of cellular DNA synthesis?

The inhibition of DNA synthesis by A-rich RNA is not a non-specific event mediated by the adenine content, molecular weight or chain length of this molecule. The data obtained using the synthetic poly A supports this since no effect can be seen by this molecule on any cellular macromolecular synthesizing system (Figures 4B, C and D). Thus, not only is A-rich RNA selective for DNA inhibition it is a specific event unique to this mixture of oligonucleotides.

Ehrenfeld and Hunt (11) using an "in vitro" protein synthesizing system have shown that the double-stranded intermediate of poliovirus can inhibit protein synthesis. Addition of large amounts of bovine enterovirus double-stranded RNA to cell cultures has shown that it leads to rapid cell death (6). The effect of reovirus dsRNA on cell cultures was then examined to eliminate the possibility that dsRNA was causing the inhibition of DNA synthesis. The uptake experiments using the same conditions used for A-rich RNA indicated that little if any dsRNA was taken up by L cells. Further work showed that reovirus dsRNA had no effect on DNA, RNA or protein synthesis under the conditions used in this study. Therefore, even if the A-rich RNA was contaminated with fragments of the dsRNA the inhibitory effect can be ascribed only to the action of A-rich RNA.

The question of whether A-rich RNA is translated is difficult to answer; it could be. The data shows that the A-rich RNA remains mainly in the cytoplasm of treated cells.

The time it remains in the cytoplasm is much greater than needed for translation of a basic inhibitory protein. The subsequent drop in the levels of A-rich RNA found in the cytoplasm may be due to cellular degradation after its mRNA function has been completed.

A small amount of A-rich RNA also has been shown located in the nucleus (Figure 8) and its activity here may be of importance. A-rich RNA may interfer with synthetic activity by binding to the template in such a way as to inhibit DNA synthesis.

The interference with initiation factors found in the cytoplasm seems to be the most plausible mechanism for A-rich RNA inhibition of DNA synthesis. Although the translation mechanism cannot be totally excluded, the way in which A-rich RNA inhibits the initiation of DNA synthesis (Figure 7) lends support to this idea. Additional evidence that A-rich RNA interfers with initiation factors comes from the method in which CF is prepared. Some of the factors which are needed for translation, such as ribosomes, are removed by the centrifugation and thus A-rich RNA could not produce a basic protein.

The "in vitro" DNA synthesizing system in isolated nuclei provided further evidence for the mechanism of A-rich RNA inhibition of DNA synthesis (Figure 9). The A-rich RNA again was shown to be unique for the inhibition of DNA synthesis and not a non-specific event mediated by poly A

sequences. The data showed that the step most sensitive to inhibition was the initiation of DNA synthesis and once DNA synthesis had begun no inhibition was noted. Finally the evidence indicated that A-rich RNA causes the inhibition of cellular DNA synthesis by interfering with the cytoplasmic factor or factors necessary for initiation of DNA synthesis.

In conclusion the data presented in this investigation suggest that reovirus A-rich RNA is selective for the inhibition of DNA synthesis. A-rich RNA, as well as synthetic poly A is taken up rapidly and efficiently by L cells and the transitory inhibition of DNA synthesis is not due to a non-specific event mediated by adenine content. Reovirus dsRNA apparently has no role in the inhibition of DNA synthesis under the conditions used in these experiments. The site of action of reovirus A-rich RNA appears to be in the cytoplasm and it apparently interfers with the steps leading to initiation of DNA synthesis. Finally, the isolated nuclei investigations suggest that A-rich RNA inhibits cellular DNA synthesis by apparently interfering with the cytoplasmic factor or factors necessary for the initiation of DNA synthesis.

Although the data presented here suggest that reovirus A-rich RNA is specifically and selectively inhibitory to L cell DNA synthesis and that this inhibition may be mediated by the interference with the cytoplasmic factors which initiate DNA synthesis the molecule still retains an aura of mystery. There is still no information as to how this

molecule reproduces itself, nor is there data explaining the incorporation of this molecule into the virion. Therefore, a number of different experiments should be devised to further elucidate the role of this A-rich RNA during the replicative cycle of reovirus.

The exact mechanism by which the initiation of cellular DNA synthesis is inhibited and the time during the cell cycle that reovirus A-rich RNA is active are areas of investigation which could further clarify the way in which A-rich RNA inhibits initiation of DNA synthesis. By using increasing amounts of A-rich RNA it may be possible to determine whether the transitory nature of this inhibition of cellular DNA synthesis is due simply to a dose response phenomenon. If this is done in conjunction with the addition of other viral components a relationship may be shown between inhibition of DNA synthesis and other viral components.

Since A-rich RNA represents a mixture of oligonucleotides one fraction of this mixture may be the active inhibitor of DNA synthesis. However, the A-rich portion may be a carrier for the more active fractions or a synergistic effect between the different molecules may be necessary for the inhibition observed. Finally, is there a relationship between reovirus A-rich RNA and cellular heterogeneous A-rich RNA and could cellular A-rich RNA also inhibit DNA synthesis?

It becomes increasingly clear that the data presented in this investigation represents a small step in the

understanding of this unusual molecule. The primary objective of this study has been satisfied and it is hoped that the information presented here will aid in our understanding of how this virus alters the regulator mechanisms of L cells.

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