THE POLYADENYLATION OF HUMAN RHINOVIRUS TYPE 14 RNA AND THE MECHANISM OF CORDYCEPIN INHIBITION OF RHINOVIRUS AND POLIOVIRUS REPLICATION

by

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This dissertation submitted by Dennis Louis Panicali has been examined by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the dissertation has received final approval with reference to content, form and accuracy of presentation.

This dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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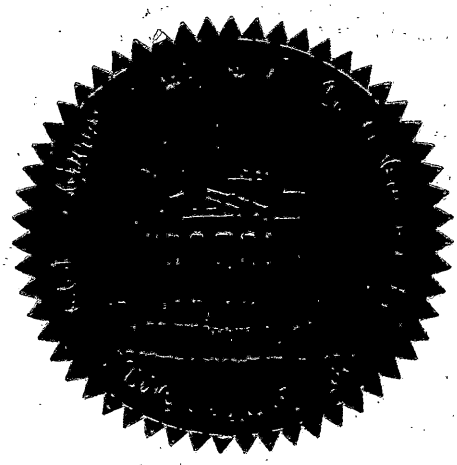

DEDICATION

This dissertation is dedicated to my wife Sandy, whose love and faith in my ability makes it all possible

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LIST OF ABBREVIATIONS

act-D	actinomycin D
3'dATP	3'deoxyadenosine triphosphate
3'dAMP	3'deoxyadenosine monophosphate
DEAE	diethylaminoethyl-
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfuric acid
EMC	encephalomyocarditis virus
ETS buffer	.01 M Tris-HCl[pH 7.2], 0.01 M EDTA, and 0.2% SDS
hnRNA	heterogeneous nuclear RNA
p. i.	post infection
HRV-14	human rhinovirus type 14
HRV-2	human rhinovirus type 2
[^{3}H]AR	^{3}H-adenosine
[^{3}H]UR	^{3}H-uridine
MEM	Eagle's minimal essential medium
MES	sodium 2-(N-morpholino) ethane sulphonate
MOI	multiplicity of infection
NETS buffer	0.01 M Tris-HCl[pH 7.2], 0.1 M NaCl, 0.01 M EDTA, and 0.2% SDS
.2 M NETS buffer	NETS buffer containing 0.2 M NaCl
PBS	phosphate buffered saline

poly(A)	polyadenylic acid
poly(dT)	polydeoxythymidylic acid
poly(U)	polyuridylic acid
RF	replicative form RNA
RI	replicative intermediate RNA
RSB	recticulocyte standard buffer
SDS	sodium dodecyl sulfate
SS	single standard RNA
TCA	trichloroacetic acid
VSV	vesicular stomatitis virus

INTRODUCTION

A. STATEMENT OF PROBLEM

It is now well established that many mammalian cellular and viral mRNAs terminate in polyadenylic acid [poly(A)] 50-250 nucleotides long. Cellular mRNAs have been shown to acquire poly(A) sequences by post-transcriptional addition. Similarly, the mRNAs of viruses which replicate in the nucleus and of at least one virus which replicates in the cytoplasm also acquire poly(A) sequences by post-transcriptional mechanisms. However, the mechanism by which the mRNA of many other cytoplasmic viruses becomes polyadenylated is not well understood.

It has been shown that the adenosine analog cordycepin inhibits the replication of some viruses but not others. In view of evidence that cordycepin preferentially inhibits nontranscriptve poly(A) synthesis in animal cells it has been suggested that resistance of viral replication to cordycepin may indicate a transcriptive mode of polyadenylation of viral mRNAs.

The genome RNAs of picornaviruses, which also serve as mRNA in the host cell, have been shown to contain poly(A). Of the picornaviruses, human rhinovirus type 14 (HRV-14) RNA was shown to have a poly(A) content apparently higher than other picornaviruses. HRV-14 replication had also been shown to be inhibited by cordycepin while poliovirus replication had been reported to be resistant or only moderately sensitive to cordycepin. Additional evidence indicated that nearly 50% of HRV-14 poly(A)

sequences might be synthesized earlier than the remaining viral RNA sequences, suggesting that preformed poly(A) sequences might be ligated to the viral RNA. In contrast, however, there was evidence that poly(A) sequences on poliovirus RNA were transcribed from a complimentary poly(U) sequence. Therefore, there was a significant possibility that HRV-14 and poliovirus mRNAs were polyadenylated by different mechanisms.

This study was undertaken to first more fully characterize the Poly(A) sequences of HRV-14 RNA, second to confirm the differential effect of cordycepin on the replication of HRV-14 and poliovirus, third to determine the mechansim by which cordycepin inhibited HRV-14 replication, and finally, to determine if the genomes of both rhinovirus and poliovirus are polyadenylated by similar mechanisms.

B. REVIEW OF THE RELATED LITERATURE:

In 1960 Edmonds and Abrams first discribed an enzyme from calf thymus nuclei that catalyzed the addition of AMP residues to the 3' end of a variety of RNAs and oligonucleotides (23). Little significance was attached to that finding since polyadenylic acid [poly(A)] sequences in RNA were unknown. In 1966 Hadjisvassiliou and Brawerman reported an adenylate rich polynucleotide associated with RNA released from rat liver polysomes (38). However, no connection was established between the adenylate rich polynucleotide and mRNA. Edmonds and Carmela in 1969 (24) showed by selection on poly(dT) cellulose that heterogeneous nuclear RNA (hnRNA) from Erlich ascites cells contained a significant portion of poly(A). They concluded however that the poly(A) was confined mainly to the nucleus (24). About the same time Lim and Canellakis demonstrated an AMP rich sequence on 9S globin messenger RNA (mRNA) released from

rabbit reticulocyte polysomes (54).

The biological importance of poly(A) was unknown until Kates and Beeson found that vaccinia virus cores which can transcribe viral DNA into RNA were capable of promoting synthesis of viral mRNA with poly(A) sequences attached (46). They also found that vaccinia RNA in infected cells contained poly(A) and subsequently found poly(A) attached to uninfected cell mRNA (45). These studies finally led to experiments which eventually established that poly(A) is covalently attached to the 3' end of both hnRNA and mRNA (19,25,51).

Since 1971, it has been well established that many mammalian RNAs contain poly(A) sequences 50-250 nucleotides long (53,109). Poly(A) has also been found in mRNAs from plants (37), yeast (39) and bacteria (102). Messenger RNAs of practically all mammalian viruses also contain poly(A) (3,4,33,44,46,67) with the exception of reovirus (108). Considering the large variety of systems in which to study poly(A) and the great amount of research that has been carried out on the various aspects of poly(A) synthesis and function, it is surprising that the mechanisms by which poly(A) is synthesized is still poorly understood and its biological function remains unknown.

Once the study of poly(A) began in depth, it was quickly shown that the mRNA of mammalian cells and viruses maturing in the nucleus appear to acquire poly(A) in the nucleus via a non-transcriptive mechanism (4,19,107). Darnell et al showed that actinomycin D (act-D) inhibited DNA-dependant RNA synthesis within a few minutes after addition to a cell culture but had no effect on poly(A) synthesis during the following two minutes (25). It was also shown that adenovirus mRNA synthesized in

the nucleus of infected cells contained poly(A); however the adenovirus genome DNA was shown to contain no poly(dA:dT) which might code for poly(A) (77,107). Additional studies using the adenosine analog cordycepin (3'deoxyadenosine) have given further proof that poly(A) is added onto hnRNA and mRNA by a non-transcriptive mechanism. Cordycepin inhibited nucleolar synthesis of rRNA by acting as a chain terminator but had no effect on the nucleoplasmic synthesis of hnRNA(74). Although cordycepin failed to inhibit hnRNA synthesis, it has been shown to inhibit the synthesis of poly(A) (18,63), and prevent newly made mRNA from appearing in the cytoplasm (2). These observations all indicate that poly(A) is synthesized post-transcriptionally by a mechanism separate from the transcription of hnRNA and mRNA. The original observation that poly(A) polymerase (terminal adenylate transferases) exist in mammalina cells (23) also supports the theory of post-transcriptional addition of poly(A); however, no evidence has yet been presented which directly links any of the known poly(A) polymerases with poly(A) synthesis in vivo.

Polyadenylation of mRNA has also been shown to occur in the cytoplasm (21,95). Pre-existing mRNAs of sea urchin embryos have been shown to be polyadenylated after fertilization (95). In addition, there appear to be enzyme(s) insensitive to cordycepin which are capable of elongating previously synthesized poly(A) in the nucleus and cytoplasm (21).

Mammalian viruses which replicate in the cytoplasm also synthesize mRNAs which contain poly(A) sequences. Vaccinia virus mRNA, as already mentioned, contains poly(A) (46). The poly(A) is apparently synthesized post-transcriptionally by a virion enzyme distinct from the viral

transcriptase (66,90). The mRNAs of other cytoplasmic viruses also are synthesized by virally specified enzymes (88). However, there is no solid evidence that polyadenylation of these viral RNAs occur solely by post-transcriptional addition. Post-transcriptional addition of poly(A) to the mRNA of cytoplasmic RNA viruses was first postulated by Marshall and Gillespie since they were unable to detect any poly(U) tracts in the genome of several of these viruses (61). This may not hold true for all cytoplasmic viruses. Poly(A) synthesis by vesicular stomatitis virus (VSV) infected L-cell extracts was shown to be coupled to viral RNA transcription (31). VSV is a single-stranded RNA virus which contains a virion associated transcriptase (11). This transcriptase can be activated in vitro by detergent treatment of isolated virions and in the presence of all four nucleoside triphosphates synthesizes mRNA which contains covalently bound poly(A). However, poly(A) is not synthesized unless all four nucleoside triphosphates are present suggesting that VSV poly(A) synthesis is coupled to transcription(29). Even though this evidence suggests a transcriptive mode of VSV polyadenylation, lack of detectable poly(U) sequences in the genome RNA (27,61) might indicate that the need for concomitant RNA synthesis only reflects a need for an appropriate primer.

Evidence for a transcriptive mode of poly(A) synthesis has been presented for poliovirus and Semliki Forest virus (83,116). Poliovirus genome RNA also serves as mRNA (plus strand RNA) in host cells and contains a poly(A) sequence covalently attached to its 3' end consisting of approximately 90 adenylic acid residues (3,114). Yogo and Wimmer first presented evidence that poliovirus poly(A) might be transcribed

when they showed that poliovirus double-stranded replicative form (RF) RNA contains a poly(U) sequence (115,117). They first reported that it was located at the 3' end of the complimentary RNA strand (minus-strand) (115) but later showed it was at the 5' end and therefore available to serve as a template for poly(A) transcription (117). Subsequently it was reported that poly(U) sequences were also present on minus-strand of the replicative intermidate (RI) RNAs (99,116). Spector and Baltimore have shown that viral RNA synthesized *in vitro* by crude viral polymerase complexes also contains poly(A) (98) and Dorsh-Häsler *et al* showed that poliovirus polymerase complex purified away from cellular terminal adenylate transferase activity still synthesizes poliovirus RNA containing poly(A) (22). Therefore they proposed that biosynthesis of poliovirus poly(A) occurs by transcription. Recently poly(U) sequences were also isolated from the minus-strand component of semliki forest virus RI RNA species (83). Semliki forest virus genome RNA also functions as mRNA in host cells and contains 3' terminal poly(A) segment which may be transcribed from the poly(U) strand on the various RI RNAs (17).

As mentioned before cordycepin preferentially inhibits *de novo* non-transcriptive poly(A) synthesis in mammalian cells (18,63). Resistance or sensitivity of virus replication and poly(A) synthesis to cordycepin was suggested as evidence for a transcriptional or non-transcriptional mechanism of polyadenylation. There was some limited evidence to support this proposal. VSV replication and poly(A) synthesis had been shown to be resistant to cordycepin (27) which corresponds to the evidence suggesting transcription of VSV poly(A)

(29,31). Conversely cordycepin inhibits the replication of Newcastle disease virus, Sendai virus, RNA tumor viruses and vaccinia virus which appear to acquire poly(A) sequences non-transcriptively (44,60,81). Recent evidence however, indicates that resistance or sensitivity to cordycepin may not be a valid indication of transcriptive or non-transcriptive polyadenylation. Maale et al have shown that cordycepin triphosphate (3' dATP) inhibits cellular RNA polymerase II as well as poly(A) polymerase (56). Cordycepin also inhibits vaccinia RNA polymerase and vaccinia poly(A) polymerase (72).

Therefore, it appears that while most cellular RNA becomes polyadenylated by post-transcriptional mechanisms, the mechanisms by which messengers of cytoplasmic viruses are polyadenylated are varied and not completely understood. They do, however provide excellent systems for continued investigation into the relationship between RNA synthesis and poly(A) synthesis in vivo and in vitro.

Although some progress has been made in elucidating the mechanism of polyadenylation of RNAs, no definitive function for the polyadenylation of RNA has been discovered. A translational function of poly(A) can be ruled out since polylysine is not found in proteins whose mRNAs contain poly(A). Poly(A) was originally thought to play an important role in the transcription, processing and transport of mRNA since inhibition of poly(A) synthesis also inhibited those functions (2,18,63,77). However, it was soon found that histone mRNA does not contain poly(A) yet is synthesized and transported as effectively as mRNAs with poly(A) (1,75). More recent studies have shown that almost 30% of Hela cell mRNAs lack poly(A) (65). A similar observation has been made in sea urchin embryos

(71). These observations along with the existence of the large number of viral mRNAs which are synthesized in the cytoplasm and contain poly(A) (88) argue against processing and transport as the sole functions of poly(A).

The existence of a large fraction of mRNA lacking poly(A) also argues against poly(A) being required for translation. In vitro translation studies of deadenylated mRNA indicates that in vitro, at least, mRNAs without poly(A) are translated almost equally as well as mRNAs with poly(A) (12,68,111). However, one translation study of deadenylated mRNAs showed that in Xenopus oocytes the efficiency of translation of rabbit globin mRNA which had the poly(A) removed decline much more rapidly than mRNA with poly(A) (42). However, another study showed that in developing sea urchin embryos inhibition of polyadenylation of maternal mRNAs in vivo does not prevent their translation (64).

Another obvious role for the presence of poly(A) is one of stabilizing mRNA. Lim and Canellakis noted a correlation between the size of poly(A) and of the messengers which carried it and suggested that poly(A) size might reflect the age of mRNA(54). Sheiness and Darnell demonstrated that the poly(A) of Hela cell cytoplasmic mRNA becomes shorter with age (89). Studies with mouse L cell mRNAs suggest however the half-life of messenger RNA is not dependant on the length of its poly(A) chain, i.e. mRNAs with short and long poly(A) sequences have the same probability of being degraded (75). Still other evidence indicates that in some systems poly(A) may serve a protective purpose. Herpes virus mRNA containing poly(A) appears to be more stable than mRNA without poly(A) (94). There have also been reports showing that poly(A) stabilizes RNA

against 3' terminal RNase activity (40).

A few other interesting observations concerning poly(A) have been made. Kwan and Brawerman have reported a particle, probably protein, which specifically binds to poly(A) in mRNA isolated from polysomes (50). Jeffery and Brawerman have shown poly(A) sequences to be associated with other nucleotide sequences of its adjacent mRNA when isolated from polysomes (43). Both findings suggest participation of poly(A) in determining secondary structure of mRNA.

All of the above findings indicate that poly(A) may be involved in the maturation, transport, stabilization and translation of some messenger RNAs. Poly(A) does not, however, appear to be essential for any of these functions and no one essential function for poly(A) in all polyadenylated RNAs has yet been found.

As mentioned previously cytoplasmic viruses provide a wide variety of systems in which to study the relationship of RNA synthesis to poly(A) synthesis in vivo and in vitro. The simplest of these viral systems to study is that of the picornaviruses. Among picornaviruses poly(A) has been found in the genomes of poliovirus (3,114), rhinovirus (58,70), mengovirus (100), encephalomyocarditis virus (EMC) (35), and Columbia S K virus (44). The picornaviruses provide an excellent system for studying the polyadenylation of cytoplasmic mRNA for several reasons; (a) the replication cycle of these viruses has been thoroughly studied, (see appendix) (b) only one mRNA is synthesized, (c) the viral genome also serves as the viral mRNA and is therefore infectious and, (d) the variety of subgroups within the picornaviruses may provide a source of variation in poly(A) content, mechanisms of

acquiring poly(A) sequences, and functions of poly(A) on cytoplasmic mRNAs.

Most thoroughly studied of the picornaviruses is poliovirus. The poliovirus genome contains at its 3' end a poly(A) sequence approximately 90 nucleotides long (114). As discussed above poliovirus double-stranded RF RNA and RI RNA both contain a poly(U) sequence at the 5' end of the complimentary (minus) strand (99,116,117), which suggests a possible transcriptive mode of poliovirus poly(A) synthesis. Further support of this assumption has come from the studies showing that viral-specific RNAs synthesized by crude polymerase complexes also contain poly(A) sequences, indicating that the replication complex is the site of viral poly(A) synthesis (22,98).

Spector and Baltimore have shown a critical biological function for poliovirus poly(A) (96). Removal of the poly(A) by digestion with ribonuclease H results in a large decrease in the infectivity of the viral RNA. They have also shown that there is no detectable viral RNA synthesis in cells infected with deadenylated viral RNA. However, there appears to be no loss of translation capability in vitro (101), indicating that poliovirus poly(A) may play a role in penetration of the viral RNA into the cell or an important regulatory role at an early step in the replication of the RNA.

Similar observations concerning the necessity of a poly(A) sequence for infectivity of picornavirus RNAs have been made with EMC virus. EMC viral RNAs lacking or containing only short segments of poly(A) were isolated by their inability to bind to oligo(dT)-cellulose. These poly(A) deficient RNAs were found to have a specific infectivity 200 times less

than EMC RNA with full length poly(A) sequences (35,41).

Nair and Owens first demonstrated poly(A) on human rhinovirus type 14 (HRV-14) RNA (70). Subsequently, others studied the poly(A) content of human rhinovirus type 2 (HRV-2) RNA (58,59). Several characteristics of the poly(A) of rhinovirus RNA appear to be different from what has been observed for the poly(A) of poliovirus RNA. HRV-14 RNA appears to have a poly(A) content significantly higher than that of poliovirus RNA and HRV-14 may synthesize up to 50% of its poly(A) sequences before the rest of the viral RNA (1). Cordycepin has also been shown to inhibit HRV-14 replication (1) while poliovirus has been reported to be resistant or only moderately sensitive to cordycepin (52,115). The final and probably the most significant difference between rhinovirus and poliovirus is an observation made by Macnaughton and Dimmock that HRV-2 RF and RI RNAs do not contain poly(U) (58).

MATERIALS AND METHODS

A. METHODS

1. Cells and Virus:

Calf serum-adapted HeLa cell line (Flow Laboratories, Inc., catalog no. 0-26340) was used throughout the course of this study except for certain experiments so noted where S-3 HeLa cells (Grand Island Biological Co., catalog no. H-3001) were used. Cells were grown in monolayer cultures at 37°C in Eagle's minimal essential medium (MEM) containing 5% calf serum, 100 units penicillin, 100 µg neomycin, and 2.5 µg fungizone per ml. Human rhinovirus type 14, poliovirus type 2 and antiserum specific for these viruses were obtained from the National Institute of Allergy and Infectionus Diseases, Bethesda, MD. Virus stocks and purified virus used in different experiments were checked for viral contamination by neutralization with specific antisera.

2. Virus growth and Purification:

Confluent monolayers of HeLa cells were infected with either purified virus or harvest fluid from infected cells (see below) by allowing virus to attach to cells at room temperature for 0.5 h. Unadsorbed virus was then removed and the monolayers washed once with phosphate buffered saline (PBS) without Ca^{++} or Mg^{++}. HRV-14 infected cells were incubated at 34°C and poliovirus infected cells at 37°C, in MEM. When infection was complete, virus was harvested and purified according to the procedure described by Nair and Owens (70). Briefly,

virus was released from the cells by three rounds of freeze-thawing in MEM, followed by forcing the lysate through a 26 guage needle. The lysate was then clarified by low speed centrifugation. The supernatant, called harvest fluid (HF) was either frozen at -70°C for use as stock virus for infections or further processed to prepare sucrose purified virus. Purification was accomplished by pelleting the virus from the HF by centrifugation at 90,000 x g at 4°C for 4 h. The pellet was suspended in 0.02 M Tris-HCl buffer pH 7.5 and sedimented through a 5% to 25% (w/w) sucrose gradient in the above buffer at 70,000 x g at 4°C for 2 h. The gradients were then fractionated by pumping from the bottom and 1.0 ml fractions collected. Using radiolabeled virus, peak virus fractions were determined by counting 10 µl aliquots. Viral fractions were then pooled and dialyzed against the appropriate buffer depending on how the virus was to be used. In later experiments gradient fractions corresponding to the relative location of virus, the peaks were routinely pooled without counting each gradient fraction. When high titer virus was necessary for infection or for RNA extraction, virus was concentrated, after dialysis, to the appropriate volume by placing the dialysis bag in polyethylene glycol 20,000. Virus purified by sucrose gradient centrifugation was stored at 70°C.

3. Preparation of Labeled Virus:

HeLa cell monolayers were infected with either HRV-14 or poliovirus at a multiplicity of infection (MOI) of ≃ 10 unless otherwise noted. Cultures were then incubated in MEM containing 20 µCi/ml of either [^{3}H] adenosine ([^{3}H]AR), [^{3}H] Uridine ([^{3}H]UR), or carrier-free [^{32}P]-labeled phosphoric acid unless otherwise stated in the figure legends.

In some experiments where viral RNA was labeled with [^{32}P]-phosphoric acid, cultures were incubated in the presence of phosphate free MEM. Poliovirus infected cultures were harvested at 9 h post infection (p.i.) and HRV-14-infected cultures at 16 h p.i. Virus was then purified as described above.

4. Extraction of Viral RNA:

Virus purified by sucrose gradient centrifugation in TNE buffer (.01 M Tris-HCl pH 7.2, 0.1 M NaCl and .01 M EDTA) was extracted at room temperature with an equal volume of a 1:1 (v/v) mixture of phenol and chloroform in the presence of 0.5% sodium deodecyl sulfate (SDS) (76) and carrier yeast RNA (unfractionated yeast RNA). The aqueous phase was then reextracted two additional times with phenol-chloroform. The final aqueous phase was made 0.2 M with respect to NaCl and the RNA precipitated with two volumes of ethanol at -20°C for at least 4 h. RNA preparations were routinely stored in alcohol at -20°C until used.

5. Determination of poly(A) Content by Digestion with Pancreatic plus T1 Ribonucleases:

Radiolabeled viral RNA samples to be analysed were precipitated from alcohol and dissolved in RNase buffer (.01 M Tris-HCl pH 6, 0.2 M KCl). RNase resistance was determined by incubating the RNA at 37°C in the presence of 50 units/ml T1 RNase and 10 µg/ml pancreatic RNase (1). After 0.5 h incubation, acid-insoluble radioactivity was precipitated with 10% trichloroacetic acid (TCA) at 4°C for 15 min. Precipitates were then collected on nitrocellulose membrane filters (Millipore Corp. type HA, 0.45 µ) washed with five 2.0 ml aliquots of 5% TCA and dried at 70°C for 0.5 h. Radioactivity was measured by scintillation counting

(see below). Total acid precipitable radioactivity was measured in the same way, however, without RNase digestion of RNA samples.

6. 10% Polyacrylamide Gel Electrophoresis of Poly(A) Sequences Isolated from Viral RNA:

[^{3}H]AR labeled poliovirus RNA and ^{32}P-labeled HRV-14 RNA were mixed and digested with pancreatic and T1 RNases as described above. After incubation, the digest was made 0.01 M in EDTA and 0.5% in SDS and reextracted with phenol-chloroform. The RNase resistant RNA was then precipitated with two volumes of alcohol in the presence of 1 A_{260} units of yeast tRNA.

Preparation of acrylamide gels and electrophoresis of poly(A) were by the method of Pinder and Gratzer (78). The precipitate containing poliovirus and HRV-14 poly(A)s was dissolved in 25 µl of electrophoresis buffer (0.05 M Tris-HCl, pH 7.4) and mixed with an equal volume of a solution containing 0.05% Brom-Phenol Blue and 50% sucrose in electrophoresis buffer and layered on a 0.5 x 5.5 cm cylindrical polyacrylamide gel containing 10% acrylamide, 0.1% N'-methylenebisacrylamide (BIS), 0.1% ammonium persulfate, and 0.1% N,N,N',N' tetramethylethylenediamine (TEMED) in electrophoresis buffer.

Electrophoresis was for 110 min at 5mA/gel. Immediately after electrophoresis, gels were scanned at 254 nm using an ISCO gel scanner to determine the position of the 4S tRNA. The gels were then fractionated into 1-mm slices using a home made gel slicer consisting of stacked razor blades. The gel slices were placed in scintillation vials and solubilized with 30% H_2O_2 (0.25 ml/slice) at 80°C for 3 h. Ten mls of counting fluid were added to each vial and radioactivity measured in

a Beckman LS230 Liquid Scintillation Spectrometer using the half Tritum isoset for measuring [^{3}H]-poliovirus poly(A) and the variable isoset set at 300-1000 for measuring [^{32}P]-HRV-14 poly(A) radioactivity.

7. Determination of the Fraction of Poly(A)-Containing RNA and Poly(A)-Lacking RNA by Poly(U)-Sepharose Chromatography:

Poly(U)-Sepharose 4B columns were prepared according to the manufacturer's directions. 200 mgs of Poly(U)-Sepharose 4B, equivalent to ∿ 1.0 ml of swollen gel, were swollen in 5 ml of 1.0 M NaCl pH 7.5 for 5 min. The slurry was poured into a pasteur pipet plugged with glass wool and washed with 20 ml of 0.1 M NaCl pH 7.5. It was then rinsed with 20 ml of distilled water and finally equilibrated with 20 ml of NETS buffer (0.01 M Tris-HCl pH 7.2, 0.1 M NaCl, 0.01 M EDTA and 0.2% SDS). Binding and elution of viral RNAs were done according to the method of Eiden and Nichols (30). Viral RNA in 1.0 ml NETS buffer was applied to the column. The column was then washed with 5.0 ml of NETS buffer to elute "Unbound" RNA. The column was next washed with 6.0 ml of glass-distilled water to elute the poly(A)-containing "Bound" RNA. Fractions (1.0 ml) were collected and the radioactivity profile determined. The remaining "Unbound" fractions and "Bound" fractions were pooled and the RNA precipitated with two volumns of alcohol.

8. Digestion of RNA with Polynucleotide Phosphorylase:

HRV-14 viral RNA was digested with polynucleotide phosphorylase in a reaction mixture containing [^{3}H]AR viral RNA, 100 mg purified yeast RNA and 500 mg polynucleotide phosphorylase per ml of buffer (0.05 M Tris-HCl pH 8.0, 0.015 M $MgCl_2$ and 0.01 M KH_2PO_4) (91,111). Incubation was for 2 min or 10 min at 37°C. The control mixture from

which the enzyme was omitted was also incubated for 10 min. The reaction was stopped with 0.5% SDS and NaCl was added to a final concentration of 0.1 M. The RNA was then extracted with phenol-chloroform and precipitated with alcohol as described above.

9. Determination of Cordycepin Sensitivity of Virus Replication:

Replicate monolayer cultures containing approximately 10^6 cells/culture in 35 mm culture dishes were infected with HRV-14 or poliovirus at a MOI of ≈ 10. After virus adsorption for 0.5 h at room temperature, unadsorbed virus was removed and each culture washed with 4.0 ml of PBS (1ml/wash). To pairs of cultures 1.0 ml of MEM containing various concentrations of cordycepin was added as indicated in the figures, legends, or a single concentration of cordycepin was added at various times post infections. HRV-14 infected cultures were incubated at 34°C for 9 h and poliovirus infected cultures at 37°C for 6 h. After incubation the cultures were frozed at -20°C. Virus was released from cultures by three cycles of freezing and thawing and by forcing the resulting lysate through a 26 gauge syringe needle. Virus yield was determined by plaque assay (see below).

10. Plaque Assay:

A modification of the procedure used by Nair and Lonberg-Holm (69) was used for determining virus titers. Serial 10-fold dilutions of virus were made in MEM. Duplicate 0.4 ml aliquots of the appropriate dilutions were added to HeLa cell monolayers in 60 mm culture dishes which were then incubated for 0.5 h at room terperature. Unadsorbed virus was removed and the monolayers were then washed with 2 ml of PBS and overlayed with MEM containing 0.35% Ionagar and 0.2% DEAE dextran.

HRV-14 infected cultures were incubated at 34°C for three to four days, and poliovirus infected cultures incubated wt 37°C for two days for plaque development. Plaques were visualized by staining the monolayers with 1% crystal violet in 20% ethanol.

11. Determination of Cordycepin Effect on Virus-Specific RNA Synthesis:

Replicate cultures in 75-cm^2 plastic flasks (Corning Glass Works) containing approximately 2 x 10^7 cells/flask were infected with purified HRV-14 or poliovirus at an MOI of ≃ 10. After infection, cultures were incubated with MEM and cordycepin was added to a final concentration of 75 µg/ml at various times after infection. Actinomycin D (act-D) was added to a final concentration of 5 µg/ml to all cultures 1 h prior to labeling with [^{3}H]AR in order to inhibit cellular RNA synthesis. Poliovirus infected cultures were labeled from 3.5 to 4.0 h p.i. and HRV-14 infected cultures from 8 to 10 h p.i. with [^{3}H]AR (20 µCi/ml). After labeling, the radioactive medium was removed and the cultures were washed twice with 5 ml of PBS. The cells were lysed with an acetate-SDS buffer (0.01 M sodium acetate pH 6.0, 0.1 M NaCl, 0.01 M EDTA and 0.5% SDS) and the lysate was extracted with an equal volume of phenol-chloroform three times as described above. The RNA was then precipitated with alcohol twice, dissolved in DNase buffer (0.01 M Tris-HCl pH 7.5, 0.1 M NaCl, and 0.01 M $MgCl_2$), incubated with RNase-free DNase-1 (10 µg/ml) for 0.5 h at 37°C and reprecipitated with alcohol. Viral RNA species were then analyzed by polyacrylamide-agarose gel electrophoresis (see below).

12. 2% Polyacrylamide, 0.5% Agarose Gel Electrophoresis:

Gels were prepared essentially according to the procedure of Kolias and Dimmock (48). Gels contained 2% acrylamide, 0.1% bisacrylamide,

10% glycerol, .5% agarose, 0.1% ammonium persulfate, and 0.1% TEMED in Loening electrophoresis "E" buffer (0.036 M Tris-HCl pH 7.8, 0.03 M NaH_2PO_4 and 0.01 M EDTA). 0.6 x 8 cm cylindrical gels were prepared by mixing all components of the gel except the agarose in 4/5 the total volume, warming the mixture to 50°C, and adding 1/5 volume of 2.5% agarose which had been melted and cooled to 50°C. Gels were then poured while still warm into tubes which had been sealed by stretching plastic wrap over one end. Gels were overlayed with water and allowed to polymerize overnight. Prior to use the gels were inverted to give a flat surface on which to layer samples. The bottom of the tube was covered with dialysis tubing or a woven paper cloth (Handywipe towel) to prevent the gel from slipping out. Gels were pre-electrophoresed in "E" buffer containing 0.2% SDS for 1 h at 5 mA/gel. 25 µl samples of viral RNAs in "E" buffer containing 0.2% SDS were mixed with 25 µl of 0.05% Brom-Phenol Blue, 0.2% SDS and 50% RNase-free sucrose in "E" buffer and layered on the gels. Gels were run at 5 mA/gel for 2.75 h at room temperature, then sliced into 1-mm sections, and two 1-mm slices were put into each scintillation vial. Slices were then solubilized by incubation with 30% H_2O_2 (0.4 ml/vial) for 3 h at 80°C, two drops of 15% ascorbic acid and 5 ml of counting fluid were added and the radioactivity in the vials counted.

13. Isolation of Viral Intermediate RNA Species from Infected Cell Cytoplasm:

HeLa cell cultures growing in roller bottles ($\sim 1 \times 10^8$ cells/bottle) or flasks ($\sim 2 \times 10^7$ cell/flask) were infected with virus at a MOI of $\simeq$ 10. 1 h prior to labeling, act-D was added to a final

concentration of 5 µg/ml. Poliovirus infected cultures were labeled from 2.5 to 4 h p.i. and HRV-14 infected cultures 4 to 8 h p.i. with either [^{3}H]AR (25 µCi/ml), [^{3}H]UR (30-50 µCi/ml) or [^{32}P]phosphoric acid (100-200 µCi/ml). After labeling infected cells were scraped from the containers and pelleted at 750 x g for 5 minutes then washed once with 5.0 ml of ice cold PBS and suspended in reticulocyte standard buffer (RBS) (0.01 M Tris-HCl pH 7.3, 0.01 M NaCl, and 0.0015 M $MgCl_2$) at approximately 2 x 10^7 cells/ml. After 10 min at 0°C the cells were ruptured with 15 strokes in a Dounce homogenizer. Nuclei and unbroken cells were pelleted at 1500 x g for 5 min. The cytoplasmic supernatant was made up, to 0.1 M NaCl, 0.01 M EDTA, 0.5% SDS and RNA extracted from it by three rounds of phenol-chloroform extraction. The RNA was precipitated with alcohol and treated with DNase as described before.

14. Separation of Viral Intermediate RNA Species:

Baltimore and Girard (69) have shown that it is possible to separate the double-stranded RF RNA from single-stranded viral RNA and the partially single-stranded RI RNA by differential precipitation with 2 M LiCl. RF remains soluble while SS and RI are insoluble. RI and/or RF RNA can also be separated from SS RNA by agarose gel filtration. RI and RF are excluded because of their size and double-stranded nature while SS RNA is partially retained by the agarose gel (7). These procedures were, therefore, used to separate rhinovirus RNAs as well as poliovirus RNAs. Total radiolabeled viral RNA isolated from infected cells was dissolved in low salt buffer (0.01 M Tris-HCl pH 7.2, 0.01 M NaCl, 0.01 M EDTA), made 2 M in LiCl and left at -4°C for 18-24 h. The

flocculent precipitate was removed by centrifugation at 15,000 x g for 20 min. The supernatant containing RF RNA was diluted 3 fold with water and the RNA precipitated with alcohol in the presence of carrier yeast tRNA. The precipitate of SS RNA and RI RNA was redissolved in low salt buffer and reprecipitated with 2 M LiCl. The RF RNA in the supernatant was precipitated with alcohol and pooled with the RF from the first round. The SS + RI RNA precipitate was dissolved in 2.0 ml NETS buffer containing 0.5% SDS and applied to 1.5 x 90 cm column of Sepharose 2B (Pharmacia) equilibrated in the same buffer. RNA was also eluted with the same buffer at a flow rate of 6-8 ml/h. Approximately 2 ml fractions were collected and radioactivity of 20 µl aliquots counted to determine peak fractions. The excluded peak containing the RI RNA and the included SS RNA peak were pooled and precipitated with alcohol in the presence of yeast tRNA.

15. Denaturation and RNAse Digestion of RI and RF RNAs:

To measure poly(A) content of RI and RF viral RNA by RNase digestion it was necessary to denature the RNA prior to RNase treatment to avoid measuring double-stranded RNA. This was done by dissolving the RNA in 0.9 ml 0.001 M EDTA pH 7.4 in the presence of 10-20 µg yeast tRNA and heating the RNA solution in a 100°C water bath for 2 min; the solution was rapidly cooled to 0°C and mixed with 0.1 ml of 0.1 M Tris-HCl pH 7.4 and 2 M NaCl (115). 10 µg pancreatic RNase and 50 units T1 RNase were added and the RNA incubated for 0.5 h at 37°C. Acid precipitable radioactivity was measured as described before.

16. Preparation of Poly(A)-Sepharose:

Poly(A)-Sepharose was prepared essentially according to the

procedure of Yogo and Wimmer (115). 5 g of CNBr-activated Sepharose 4B (Pharmacia) was washed with 1 L of 0.001 M HCl and then with 1 L of ice cold water in a scintered glass funnel. The gel was then mixed in a test tube with 9 mg poly(A) in 10 ml of 0.3 M sodium 2-(N-morpholino) ethane sulphonate (MES) pH 6, and rotated end over end for 16 h at 4°C. The gel was then washed with 200 ml of ice cold 0.05 M MES, pH 6 to remove unbound poly(A). From the A_{260} of this wash it was estimated that approcimately 110 A_{260} units of poly(A) were bound to the sepharose. The remaining active groups on the gel were reacted with 40 ml of 1 M ethanolamine in 0.2 M, N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES) pH 7.6 and rotated in a test tube end over end for 3 h at room temperature. The gel was then washed with 2 L of ice cold 0.3 M NaCl, 0.1 M $NaHCO_3$ pH 7.5 and finally with 20 ml of 0.01 M Tris-HCl (pH 7.2) containing 0.1 M NaCl, 0.01 M EDTA and 50% glycerol and stored in the same buffer at 4°C.

17. Poly(A) Sepharose Chromatography of RF and RI RNA:

Poly(A) Sepharose columns were prepared and RNA eluted as described by Sawicki and Gomatos (83). Columns were poured to approximately 4 cm in glass wool plugged pasteur pipets and washed with 10 ml of .2 M NETS (0.01 M Tris-HCl [pH 7.2], 0.2 M NaCl, 0.01 M EDTA and 0.5% SDS). 100 µg of yeast RNA in 1 ml of .2 M NETS was passed through the column followed by 5.0 ml ETS buffer (NETS minus NaCl), 5 ml of 90% formamide in ETS and finally equilibrated again with .2 M NETS. Viral RNA intermediates were prepared for chromatography by first denaturing 0.9 ml of RNA by heating to 100°C for 2 minutes as described above in the presence of 2 µg poly(U) and rapidly cooled to 0°C. RNA was then warmed

to room temperature and 0.1 ml of 10X .2 M NETS added. RNA was applied to the columns and washed with 10 ml of .2 M NETS to elute "unbound" poly(U)-lacking RNA and then with 3 ml of ETS followed by 7 ml of 90% formamide in ETS to elute "bound" RNA containing poly(U). 1 ml fractions were collected and radioactivity of the entire fraction counted.

18. Preparation and Assay of Crude Viral Polymerase Complexes:

The procedure used for the preparation and assay of crude viral polymerase complex was a combination of those reported by Yin and Knight (112) and Spector and Baltimore (98). Approximately 4×10^8 HeLa cells growing in roller cultures were infected at a MOI of 15-25 with either HRV-14 or poliovirus and incubated in MEM containing 5 µg/ml of act-D. After incubation for 3.5 h (poliovirus) or 7.5 h (HRV-14), infected cells were scraped from the monolayers, pelleted at 750 x g for 5 min, washed once with ice cold PBS and suspended in RSB at approximately 2.0×10^7 cells/ml. After 10 min at 0°C the cells were ruptured with 15 strokes in a Dounce homogenizer. Glycerol was added to 5% and aliquots frozen at -70°C.

The crude viral polymerase complex was prepared from thawed cell homogenates by pelleting nuclei and cell debris at 1500 x g for 5 min, and centrifuging the supernatant at 130,000 x g for 45 min at 4°C in an IEC A211 rotor. The pellet derived from 10^8 cells was then resuspended in 1 ml of .05 M Tris-HCl pH 8.0, containing .01 M NaCl. This crude polymerase compled was either used immediately or frozen at -70°C and used within one month.

The complete standard in vitro polymerase reaction mixture

contained in a final volume of 1.0 ml, 0.5 ml crude viral polymerase, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 µg act-D, 1.25 mM phosphoenolpyruvate, 25 µg pyruvate kinase, 6.5 mM dithiothreitol, 0.2 mM ATP, 0.05 mM GTP and 25 µCi of [8-^{3}H] GTP (16 Ci/mM) or 0.2 mM GTP, 0.05 mM ATP and 25 µCi[8-^{3}H] ATP (18 Ci/mM). Incubation of the reaction mixtures was at 34°C for HRV-14 polymerase and 37°C for poliovirus polymerase. Reactions were stopped by mixing duplicate or triplicate 100 µl samples with 0.5 ml of cold 0.15 M sodium pyrophosphate and 0.5 ml of cold 20% TCA. After 30 min at 4°C the precipitates were collected on glass fibre filters (Whatman GF/A) and washed with five 3 ml aliquots of cold 5% TCA and 2 ml of cold 95% isopropyl alcohol. Filters were dried at 70°C and counted.

19. Sucrose Gradient Analysis of Viral RNA Synthesized in vitro:

Complete 2 ml reaction mixture with [^{3}H] GTP or [^{3}H] ATP as the labeled precursor was incubated for 45 min and diluted with 4.0 ml of acetate buffer. The product RNA in the reaction mixtures was purified by two extractions with phenol-chloroform and precipitated with alcohol. The RNA was dissolved in 2 ml of NETS buffer and sedimented through a 15-30% (w/w) sucrose gradient in the same buffer for 17 h at 20,000 rpm in a Beckman SW27 rotor. Fractions (1.2 ml) were collected and absorbance monitored at 254 nm. The positions of 18S and28S rRNA (present in the crude viral polymerase complex) were thus obtained. Radioactivity in each fraction was determined by precipitation of RNA with TCA and collecting the precipitates on glass fibre filters as described above.

20. Determination of Protein Concentrations:

Protein concentration was determined by the method of Lowry

et. al. (55). Samples were diluted to 0.4 ml and 4 ml of reagent C (50 ml of 2% Na_2CO_3 in 0.1 N NaOH plus 1 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium potasium tartrate) was added and mixed. After 10 min at room temperature 0.4 ml of Folin phenol reagent (diluted 1:1 with water) was added, immediately mixed and absorbance at 600 nm read after 15 min at room temperature. A standard curve was constructed using bovine serum albumin as standard.

21. Liquid Scintillation Counting:

All radioactivity either on filters or in aqueous samples was counted using a liquid scintillation counting fluid containing 16.5 g 2,5-diphenyloxazole (PPO) and 0.5 g 2,2'-p-phenylenebis (4-methyl-5-phenyl) -oxazole (POPOP) in 2 L of toluene and 1 L of triton X-100. Glass scintillation vials were used and counted in a Beckman LS230 Scintillation Spectrometer. Where necessary, quenching was determined using the external standard techinque.

B. MATERIALS

Eagle's minimal essential medium, calf serum, and antibiotics were obtained from Grand Island Biological Co. Phosphate-free minimal essential medium was obtained from Pacific Biologicals. T1 RNase, pancreatic RNase A, RNase free DNase, pyruvate kinase, purified yeast tRNA (type 1), cordycepin, phosphoenol pyruvate, dithiothreitol, MES, DEAE dextran, agarose, SDS, Tris, and Triton X-100 were purchased from Sigma Chemical Co. Adenosine-[8-^{3}H] (18-20 Ci/mM), Uridine-[5-^{3}H] (21 Ci/mM), carrier-free ^{32}P-phosphoric acid, [^{3}H]poly(U) (11.5 mCi/mM), [^{3}H] poly(A) (18.7-20 mCi/mM), ATP-[8-^{3}H] (16-18 Ci/mM), and GTP-[8-^{3}H] (12-16 Ci/mM) were purchased from Schwartz/Mann. The disodium salts of

ATP, GTP, CTP, and UTP along with poly(U) and RNase-free sucrose were also purchased from Schwartz/Mann. Acrylamide, BIS, and TEMED were obtained from Bio-Rad Laboratories. Poly(U)-Sepharose 4B, and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals. RNase-free Micrococcus lysodiecticus polynucleotide phosphorylase was purchased from Worthington Biochemical Corp. PPO and POPOP were purchased from Mallinckrodt Chemicals. Cordycepin triphosphate was purchased from Miles Laboratories, polyethylene glycol 20,000 from the J. T. Baker Chemical Co., HEPES from Calbiochem., Ionagar No. 2 from Colab, and poly(A) from P. L. Biochemicals. Actinomycin D was a gift from Merck and Co. All other chemicals were obtained from the Fisher Chemical Company.

RESULTS

A. Poly(A) content of HRV-14 virion RNA:

A standard method for assaying the poly(A) content of various RNAs is to determine the fraction of RNA resistant to pancreatic plus T1 RNases. Nair and Owens (70) first reported the presence of poly(A) in HRV-14 using this method. Their results indicated that the average poly(A) content of HRV-14 RNA was high and apparently greater than that of poliovirus RNA.

Table 1 shows the percent of [^{3}H]AR labeled HRV-14 and poliovirus RNA resistant to pancreatic plus T1 RNases. HRV-14 RNA has approximately 80% more RNase resistant sequences than poliovirus RNA (7.2% vs 4.2%). [^{3}H]UR labeled HRV-14 RNA was only 0.68% RNase resistant while [^{3}H] poly(A) was 100% resistant. From the molecular weights and base composition of the viral RNAs it is possible to estimate the average number of nucleotides in poly(A) per molecule of RNA. Poliovirus RNA has a molecular weight of 2.6×10^6 daltons (36), consists of about 7500 nucleotides and contains 29% AMP (84). The RNase resistant fraction of [^{3}H]AR labeled poliovirus RNA corresponds to approximately 91 nucleotides in poly(A) per molecule of RNA which is similar to published values for poliovirus poly(A) determined in the same manner (97). HRV-14 RNA has a molecular weight of 2.4×10^6 daltons (69) which is equivalent to about 6900 nucleotides and contains about 35% AMP (82). 7.2% RNase resistant [^{3}H]AR labeled RNA corresponds to an average poly(A) chain length of 173 nucleotides per molecule of RNA. The data, therefore, indicate that the size of HRV-14

TABLE 1

Pancreatic plus T1 RNase Resistance of HRV-14 and Poliovirus Virion RNA

RNA Preparation	Label	Percent RNase Resistance
HRV-14	^{3}H Adenosine	7.2 (6.2-8.8)[a]
HRV-14	^{3}H Uridine	0.68 (.38-.89)
Poly(A)		100
Poliovirus	^{3}H Adenosine	4.2 (3.8-4.5)

[a]Numbers in parentheses indicate the range of values from at least two different RNA preparations. Each value represents the average of at least triplicate determinations.

poly(A) is about twice the size of poliovirus poly(A) assuming that the fraction of polyadenylated molecules is the same for both RNAs (see below).

The difference between HRV-14 RNA and poliovirus RNA in poly(A) content was further demonstrated by determining the RNase resistant fraction of ^{32}P-labeled viral RNAs. The poly(A) content of poliovirus RNA has been estimated to be 1.14% of the total genome RNA (114). This is in good agreement with the estimate of 1.16% obtained in this study (Table 2). This estimate is equivalent to a poly(A) chain length of 87 nucleotides. In comparison, the poly(A) content of purified HRV-14 RNA is 2.12% (Table 2), corresponding to a poly(A) chain length of 146 nucleotides again nearly twice that of poliovirus poly(A), corroborating the results obtained with [^{3}H]AR labeled viral RNAs (91 vs 173 nucleotides).

Implied in the above results is that HRV-14 poly(A) is larger in size than the poly(A) of poliovirus. In keeping with this, when poly(A) sequences were isolated from HRV-14 RNA and poliovirus RNA and co-electrophoresed through a 10% polyacrylamide gel, a significant fraction of the HRV-14 poly(A) migrated more slowly through the gel than did poliovirus poly(A) (Figure 1). It will be noted, however, that over one-half of HRV-14 poly(A) consist of sequences similar in size to poliovirus poly(A). Therefore, it appears that the poly(A) sequences present in the virion RNA of HRV-14 are more heterogeneous than such sequences contained in the virion RNA of poliovirus.

B. Fraction of HRV-14 RNA molecules which contain poly(A):

In determining the average poly(A) content of HRV-14 RNA it was assumed that the fraction of molecules containing poly(A) was similar to

TABLE 2

^{32}P-Poly(A) Content of HRV-14 and Poliovirus Virion RNA

Virus	^{32}P-Labeled Viral RNA (cpm/ml)[a]		% RNase Resistant
	Acid Insoluble	RNase Resistant	
HRV-14	211,130	4,480	2.12
Poliovirus	155,160	1,800	1.16

[a]The counts-per-minute data represent averages of triplicate determinations.

FIGURE 1. Polyacrylamide gel electrophoresis of poly(A) sequences isolated from HRV-14 and poliovirus RNA.

^{32}P-labeled HRV-14 RNA and [^{3}H]AR poliovirus RNA prepared as described in Materials and Methods were mixed, digested with RNases, extracted with phenol-chloroform-SDS and precipitated with alcohol in the presence of carrier yeast tRNA. The precipitate was dissolved in electrophoresis buffer and 25 µl analyzed on a cylindrical 10% polyacrylamide gel. The position of yeast tRNA marker was obtained by scanning the gel at 254 nm. The gel was sliced into 1 mm sections, slices were solubilized and counted. Symbols: (●) ^{32}P-labeled HRV-14 poly(A); (○), [^{3}H]-labeled poliovirus poly(A). Arrow indicates the position of the absorbance peak of yeast tRNA.

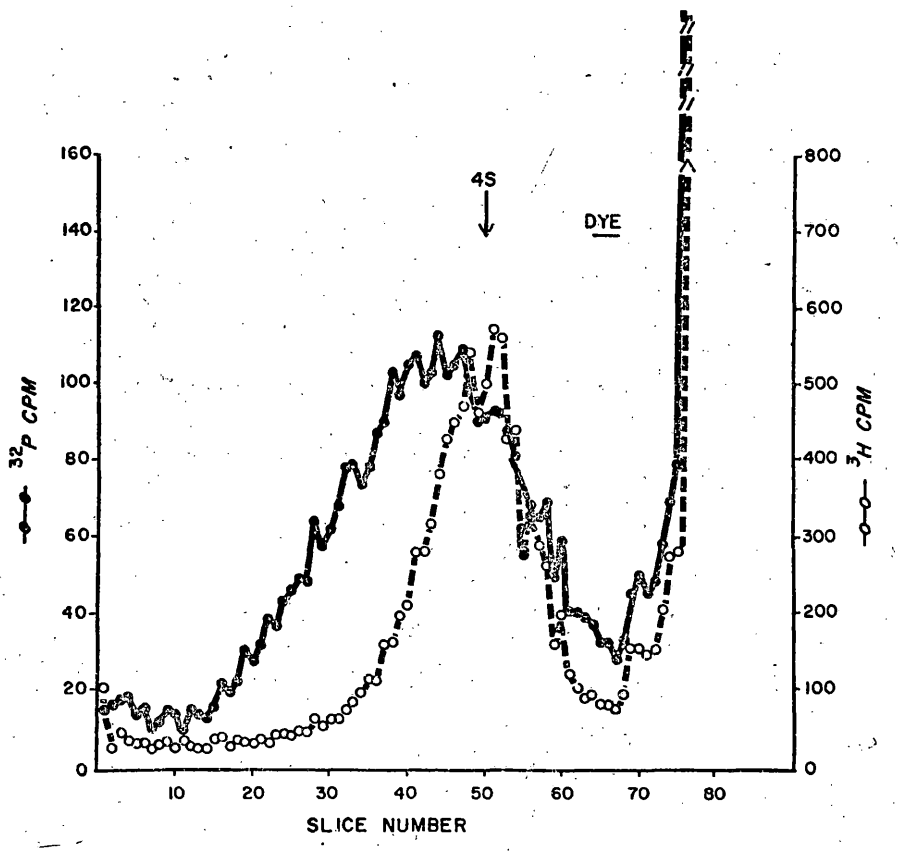
4S
DYE
^{32}P CPM
^{3}H CPM
SLICE NUMBER
0
20
40
60
80
100
120
140
160
100
200
300
400
500
600
700
800
10
20
30
40
50
60
70
80

FIGURE 1

that of poliovirus and that there was not a large fraction of HRV-14 RNA molecules which were devoid of poly(A). Spector and Baltimore (97) have shown that almost all poliovirus RNA molecules contain poly(A), by (a) determining the binding of poliovirus poly(A) to poly(U)-glass fiber filters, and (b) comparing the poly(A) size determined by polyacrylamide gel electrophoresis with the poly(A) content determined by RNase digestion.

The fraction of HRV-14 RNA molecules which contain poly(A) was measured by determining the amount of RNA binding to poly(U)-Sepharose columns. Figure 2 shows a typical elution profile of HRV-14 virion RNA. All "unbound" RNA is eluted in the first 3.0 ml of NETS buffer. Continued washing with NETS releases no additional RNA. The remaining "bound" RNA comes off immediately in the first few fractions when water is used to elute the column. Recovery of RNA from the column was usually 95-100%. Table 3 shows that on the average 74% of HRV-14 RNA was bound to the column, approximately the same percent as of poliovirus RNA. The procedure was very specific for poly(A) containing RNA since virtually no 18S + 28S rRNA was bound while poly(A) was 100% bound (Table 3).

The "bound" and "unbound" fractions of HRV-14 were rechromatographed on poly(U)-Sepharose to determine whether or not all of the "bound" RNA would rebind and if the "unbound" contained any poly(A). Unfractionated virion RNA was included as a control. The data shown in Table 4 indicates that on rechromatography about 28% of the "bound" RNA does not rebind, probably reflecting degradation of the HRV-14 RNA during the various manipulations. None of the "unbound" RNA rebinds to the poly(U)-Sepharose. However, 1.1% of the "unbound" RNA is resistant to RNase digestion indicating that those HRV-14 RNA molecules which are not bound do

FIGURE 2. Poly(U)-Sepharose chromatography of HRV-14 virion RNA.

Poly(U)-Sepharose columns were prepared as described in Materials and Methods. HRV-14 virion RNA was dissolved in 1.0 ml NETS buffer, applied to the column and eluted with 5.0 ml of NETS buffer followed by 6.0 ml of glass distilled water. 1.0 ml fractions were collected and counted.

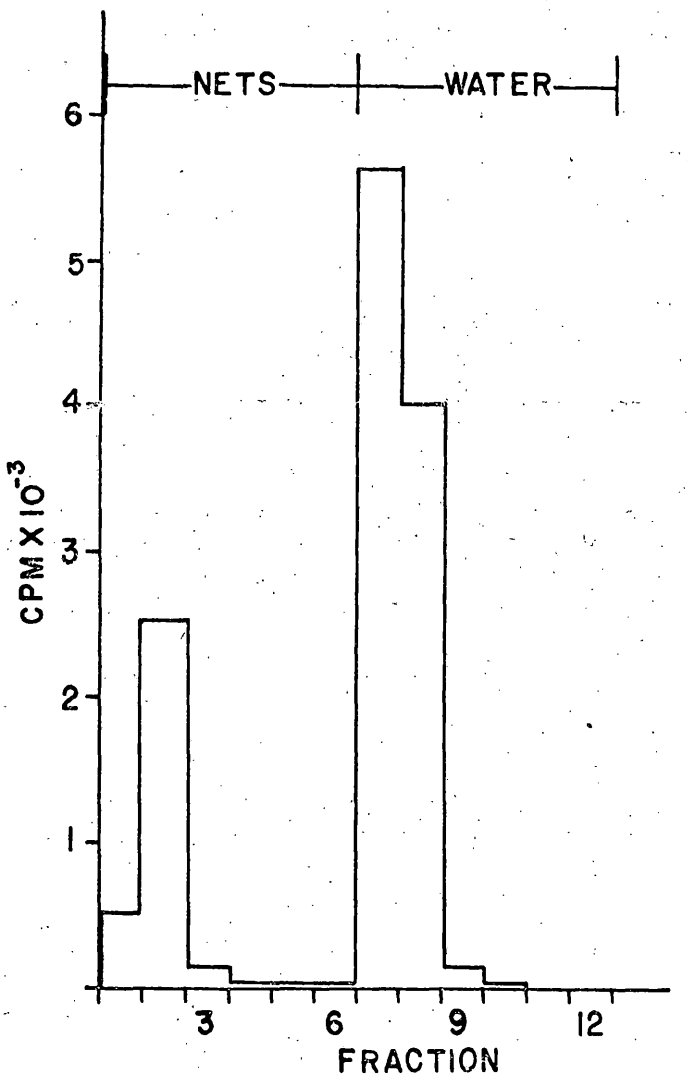

FIGURE 2

TABLE 3

Poly(U)-Sepharose Chromatography of HRV-14 Virion RNA

Preparation	Percent	
	Bound	Unbound
HRV-14 Virion RNA	74 (61-83)	26 (17-39)
Poliovirus Virion RNA	80	20
Poly(A)	100	0
18S + 28S rRNA	< 1	> 99

Numbers in parentheses represent range of three separate RNA preparation

TABLE 4

Poly(U)-Sepharose Chromatography of "Bound" and "Unbound" HRV-14 Virion RNA and RNase Resistance

RNA	Percent		Percent[a]
	Bound	Unbound	RNase Resistant
Total HRV-14 Virion RNA	61	39	6.5
Bound HRV-14 RNA	72	28	5.9
Unbound HRV-14 RNA	0	100	1.1

[a]Average of triplicate determinations.

contain a small fraction of poly(A), probably less than 30 nucleotides long and too small to bind to the poly(U)-Sepharose under the conditions used. Therefore, it appears that all HRV-14 RNA molecules probably contain poly(A) ranging from a few molecules with very short or no poly(A) at all to RNA containing full length poly(A).

C. Evidence for 3'-terminal location of poly(A) in HRV-14 RNA:

Controlled digestion with the enzyme polynucleotide phosphorylase is known to remove the 3'-terminal poly(A) from mRNA (91,111). This procedure was used to determine whether the poly(A) sequences are located at the 3'-termini of HRV-14 RNA. Viral RNA extracted from purified virions was digested with Micrococcal polynucleotide phosphorylase as described under Materials and Methods. Loss of poly(A) was monitored by the inability to bind to poly(U)-Sepharose. The results shown in Table 5 indicate that digestion with the enzyme for 10 min significantly reduced the fraction of RNA molecules binding to poly(U)-Sepharose. Incubation of viral RNA in 2 X standard saline citrate buffer for 10 min with or without polynucleotide phosphorylase did not appreciably alter the extent of binding to poly(U)-Sepharose (not shown). In view of these observations, it is unlikely that the observed loss of binding of viral RNA to poly(U)-Sepharose upon digestion with polynucleotide phosphorylase was unrelated to the poly(A) content of viral RNA or that it was due to degradation by an endonuclease which may have been present in the enzyme preparation. Therefore, the results are interpreted to mean that HRV-14 RNA terminates in poly(A) sequences.

TABLE 5

Removal of Poly(A) from HRV-14 RNA with polynucleotide phosphorylase

Length of incubation with PP[a]	Counts per minute			
	Unbound[b]	Bound[c]	Bound/Unbound	Total bound %
Control[d]	11,241	14,973	1.35	57
2 min	15,867	12,092	0.76	43
10 min	18,678	7,431	0.40	29

[a]Micrococcal polynucleotide phosphyrylase (PP) at 500 µg/100 µg of RNA per ml.

[b]Counts per minute not retained by Poly(U)-Sepharose column.

[c]Counts per minute retained by Poly(U)-Sepharose column.

[d]Samples of RNA incubated for 10 min without enzyme.

D. Cordycepin (3'deoxyadenosine) sensivity of virus replication:

Replication of HRV-14 was previously shown to be inhibited by the adenosine analogue cordycepin (70). Poliovirus replication, on the other hand, has been reported to be resistant or moderately sensitive to cordycepin inhibition (52,115). Cordycepin has been shown to inhibit mRNA synthesis in eukaryotic cells (74,77) by specifically inhibiting non-transcriptive synthesis of poly(A) (18,63). The inhibition of HRV-14 replication by cordycepin was considered as possible evidence that the poly(A) of HRV-14 RNA might be added on non-transcriptively (70) while poliovirus poly(A) might be transcribed and, therefore, be insensitive to cordycepin.

A direct comparison of the effect of cordycepin on the replication of HRV-14 and poliovirus was made to establish the relative sensitivities of the viruses to the drug in order to test the theory of a non-transcriptive vs transcriptive mechanism of polyadenylation of the two viruses. In direct contrast to what has been reported by others (52,115) it was found that poliovirus replication was nearly as sensitive to cordycepin inhibition as was HRV-14 replication. Figure 3 shows the effect of various concentrations of cordycepin on the replication of HRV-14 and poliovirus. Whereas growth of HRV-14 was almost completely inhibited by cordycepin at 25 µg/ml, slightly higher concentrations were required to comparably inhibit poliovirus replication. Both viruses were completely inhibited at 50 µg/ml.

To determine whether cordycepin was inhibiting early or late viral specific functions, the effect of cordycepin added at different times during the viral replication cycle was determined. Cordycepin at 75 µg/ml

FIGURE 3. Inhibition of HRV-14 and poliovirus replication by cordycepin: effect of cordycepin concentration.

Replicate monolayer cultures were infected with HRV-14 or poliovirus at an MOI ≈ 10. After four washes with PBS pairs of cultures were incubated with MEM containing the indicated concentrations of cordycepin for 6 h (poliovirus) or 10 h (HRV-14). Infected control cultures were similarly incubated but without cordycepin. Virus yield was determined by plaque assay. The average virus yield from each cordycepin-treated pair of cultures is plotted as a percentage of that from control cultures. Symbols: (O), HRV-14 infected cultures; (●), poliovirus infected cultures.

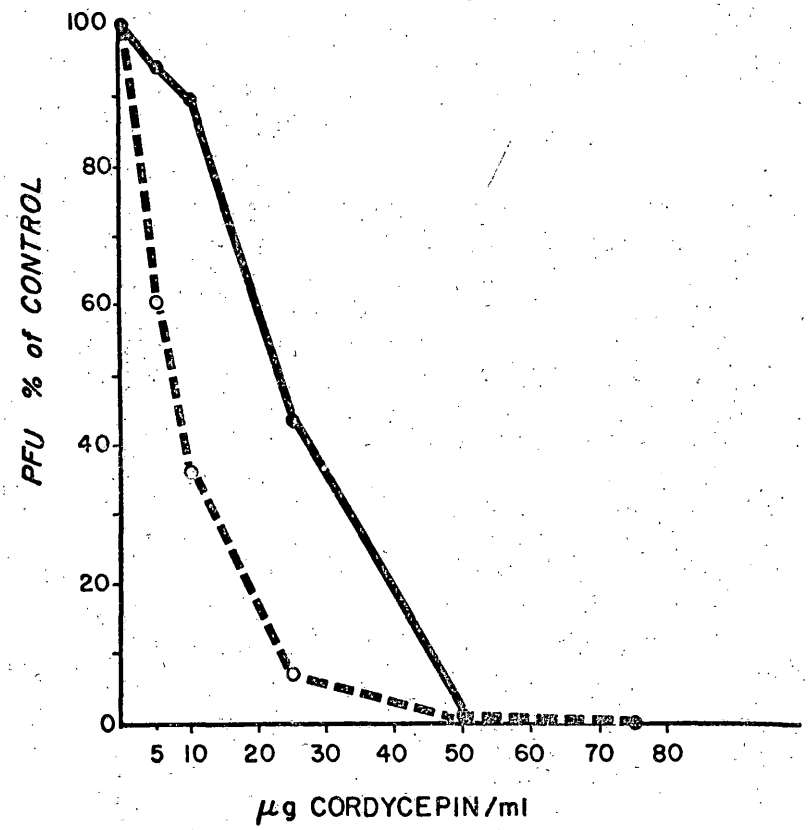
100
80
60
40
20
0
PFU % of CONTROL
5 10 20 30 40 50 60 70 80
μg CORDYCEPIN/ml

FIGURE 3

was added to HRV-14 or poliovirus infected cultures at various times after infection and virus titers determined. The results of these experiments, shown on Table 6, indicate that maximum inhibition of replication of either virus required addition of cordycepin before the onset of viral RNA synthesis. Poliovirus RNA accumulates exponentially between 1.5 and 3.0 h p.i. and precedes the appearance of infectious virus by about 0.5 h (10). Replication of HRV-14 proceeds slower than that of poliovirus and the majority of RNA synthesis occurs after 5.0 h p.i. (32,87). Increasing the MOI also had no effect on the inhibition of virus replication when cordycepin was added at the start of infection (data not shown).

In view of the preceding results the effect of cordycepin on the synthesis of viral-specific RNA was determined. It is possible to measure picornavirus-specific RNA synthesis in infected cells by inhibiting cellular DNA-dependant RNA synthesis with actinomycin D which has no effect on viral RNA-dependant RNA synthesis (85). Figures 4A and B show the effect of cordycepin (75 µg/ml) on HRV-14-specific RNA synthesis. Addition of cordycepin prior to or during viral RNA synthesis resulted in complete inhibition of subsequent synthesis of HRV-14-specific RNAs. Addition of the drug to poliovirus-infected cells at 0 or 2 h p.i. similarly inhibited all except a small fraction of poliovirus-specific RNA synthesis (Figure 5). Furthermore, both single-stranded and double-stranded RNAs were more or less equally inhibited (Figure 5).

The observation that a small fraction (less than 10%) of poliovirus-specific RNA was synthesized in the presence of cordycepin

TABLE 6

Inhibition of Virus Production by Cordycepin at 75 µg/ml at Various Times after Infection

Virus[a]	Time of Cordycepin Addition	Virus Yield[d] % of Control
HRV-14[b]	Control	100
	0 h p.i.	0.9
	3 h p.i.	1.7
	5 h p.i.	10.6
	7 h p.i.	38.8
Poliovirus[c]	Control	100
	0 h p.i.	.05
	2 h p.i.	26.8
	4 h p.i.	96.3

[a]MOI ≈ 10

[b]HRV-14 titers determined at 9 h p.i.

[c]Poliovirus titers determined at 6 h p.i.

[d]Values represent average of duplicate plaque assays on at least two separate cultures.

FIGURE 4. Effect of cordycepin on HRV-14 RNA synthesis.

Replicate monolayer cultures were infected with HRV-14 at a MOI of ≈ 10. Cordycepin (75 µg/ml) was then added at 0, 2,5 and 7 h p.i. Infected and mock infected control cultures did not receive any cordycepin. Cultures were labeled between 8 and 10 h p.i. with [^{3}H]AR (20 µCi/ml) in the presence of act-D. At the end of the labeling period cells were lysed with an acetate-SDS buffer, total RNA was extracted from each culture and treated with DNase as described in Materials and Methods. The RNAs were then dissolved in Loening electrophoresis buffer and a constant fraction of each RNA preparation was subjected to electrophoresis on 2% polyacrylamide, .5% agarose gels. The gels were fractionated, solubilized and assayed for radioactivity as described in Materials and Methods. (A) RNA from HRV-14 infected control culture (0), and mock infected control culture (●). (B) RNA from HRV-14 infected, cordycepin-treated cultures. Symbols: Cordycepin added at 0 h p.i. (●), 2 h p.i. (0), 5 h p.i. (Δ), and 7 h p.i. (Δ).

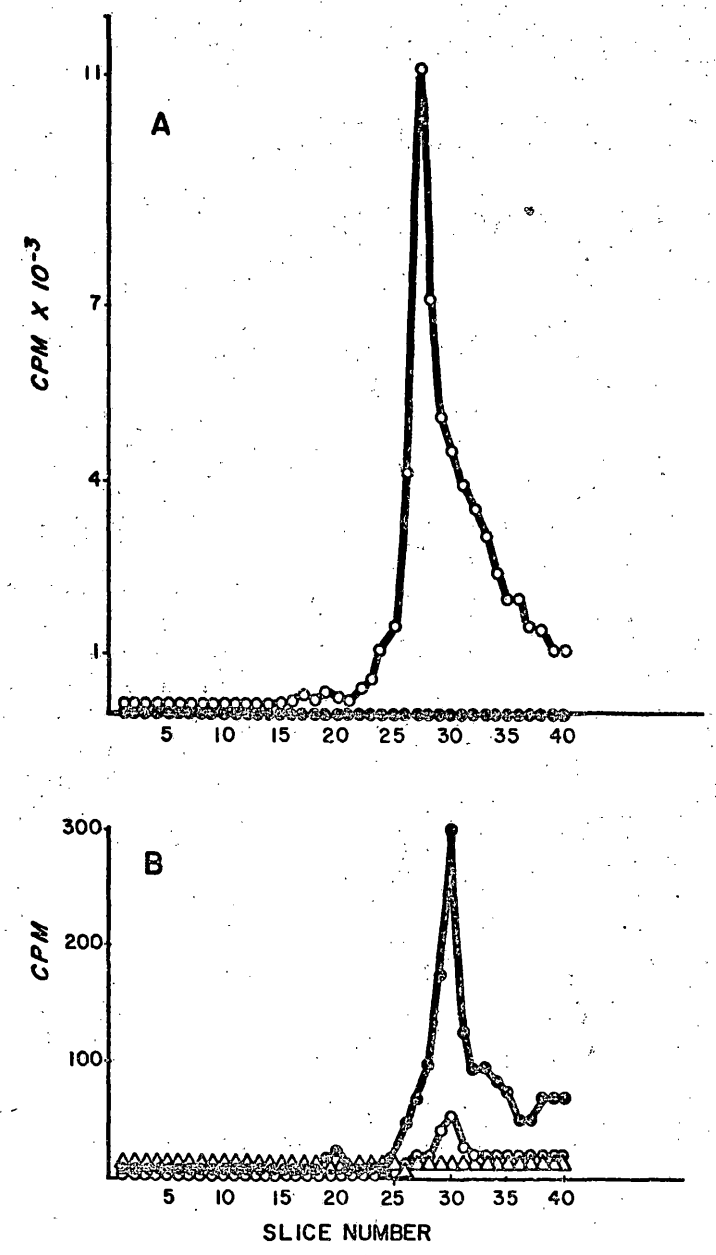
A
CPM X 10⁻³
11
7
4
1
5 10 15 20 25 30 35 40
B
CPM
300
200
100
5 10 15 20 25 30 35 40
SLICE NUMBER

FIGURE 4

FIGURE 5. Effect of cordycepin on poliovirus RNA synthesis.

Replicate monolayers were infected with poliovirus at a MOI of ≈ 10. Cordycepin (75 μg/ml) was then added at 0 or 2 h p.i. Infected cultures were labeled between 3.5 and 4.0 h p.i. with [^{3}H]AR (20 μCi/ml) in the presence of act-D. At the end of the labeling period cells were lysed with an acetate-SDS buffer, total RNA was extracted from each culture and treated with DNase. RNAs were then dissolved in Loening electrophoresis buffer and a constant fraction of each RNA preparation was subjected to electophoresis on 2% polyacrylamide, 0.5% agarose gels. The gels were fractionated, solublized and assayed for radioactivity as described in Materials and Methods. Symbols: RNA from infected control culture (●); Cordycepin added at 0 h p.i. (○), and 2 h p.i. (▲).

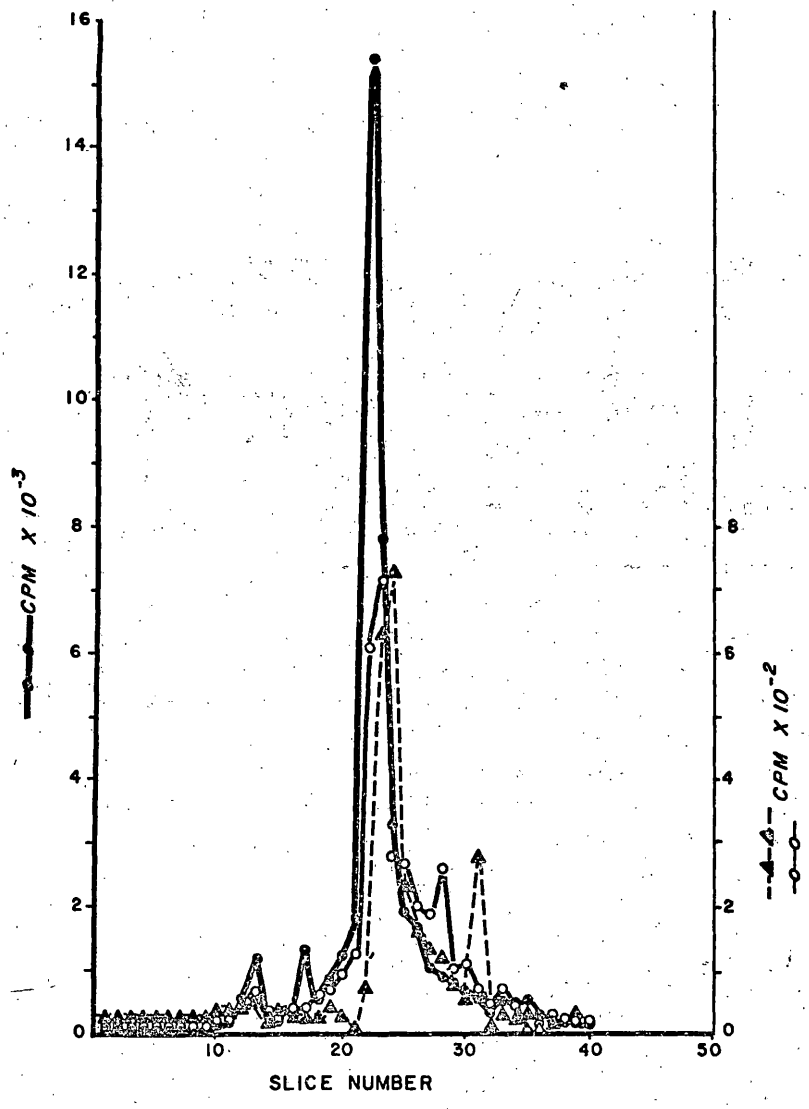
16
14
12
10
8
6
4
2
0
CPM X 10⁻³
8
6
4
2
0
CPM X 10⁻²
10
20
30
40
50
SLICE NUMBER

FIGURE 5

made it possible to determine if poly(A) was still synthesized in the presence of cordycepin. [^{3}H]AR labeled poliovirus-specific RNA synthesized in the presence or absence of cordycepin was sedimented through 15-30% sucrose gradients and the 35S SS viral RNA peak fractions pooled for analysis of poly(A) content (Figure 6).

The poly(A) content of the SS RNAs was then determined by digestion with pancreatic and T1 RNases. The results are presented in Table 7. The poly(A) content of poliovirus RNA synthesized in the presence or absence of cordycepin remains the same. Therefore, viral poly(A) synthesis appears to be no more sensitive to cordycepin than viral RNA synthesis.

The results presented above indicate that cordycepin inhibits viral replication apparently by causing an inhibition of viral RNA synthesis. The observations of Nair and Owens (70) that adenosine can reverse cordycepin inhibition of viral replication also lends support to this assumption. However, the exact mechanism by which cordycepin inhibited viral RNA synthesis remained unknown.

Several possible mechanisms of cordycepin inhibition were considered. Even though there was no detectable difference in the cordycepin sensivity of poliovirus poly(A) synthesis to viral RNA synthesis, it was possible that inhibition of HRV-14 RNA synthesis was due to an inhibition of poly(A) synthesis which somehow might be necessary for viral RNA transcription. Similarly, since cordycepin is an analogue of adenosine inhibition might be due to specific competition between cordycepin triphosphate (3'dATP) and ATP for incorporation into viral RNA. It has been demonstrated that 3'dATP inhibits <u>in vitro</u>

FIGURE 6. Sucrose gradient sedimentation profile of poliovirus specific RNA synthesized in the presence or absence of cordycepin.

Replicate cultures were infected with poliovirus at an MOI of ≈ 10. Cordycepin was then added at 0 or 2 h p.i. Cultures were then labeled between 3.5 and 4.0 h p.i. with [^{3}H]AR (20 μCi/ml) in the presence of act-D. At the end of the labeling period RNA was extracted, treated with DNase and dissolved in NETS buffer. The RNA was then layered on a 15-30% sucrose gradient also prepared in NETS buffer and centrifuged at 20,000 RPM for 17 h in a Beckman SW27 rotor. The gradients were fractionated (1.2 ml/fraction) and absorbance monitored at 254 nm. Radioactivity in each fraction was measured by counting 10 μl aliquots. Arrows indicate absorbance peaks of 18S and 28S RNAs. Brackets indicate fractions that were pooled and precipitated with alcohol in the presence of carrier yeast RNA. Symbols: RNA from infected control culture (●); RNA from infected-cordycepin treated cultures, cordycepin from 0 h p.i. (Δ), cordycepin from 2 h p.i. (○).

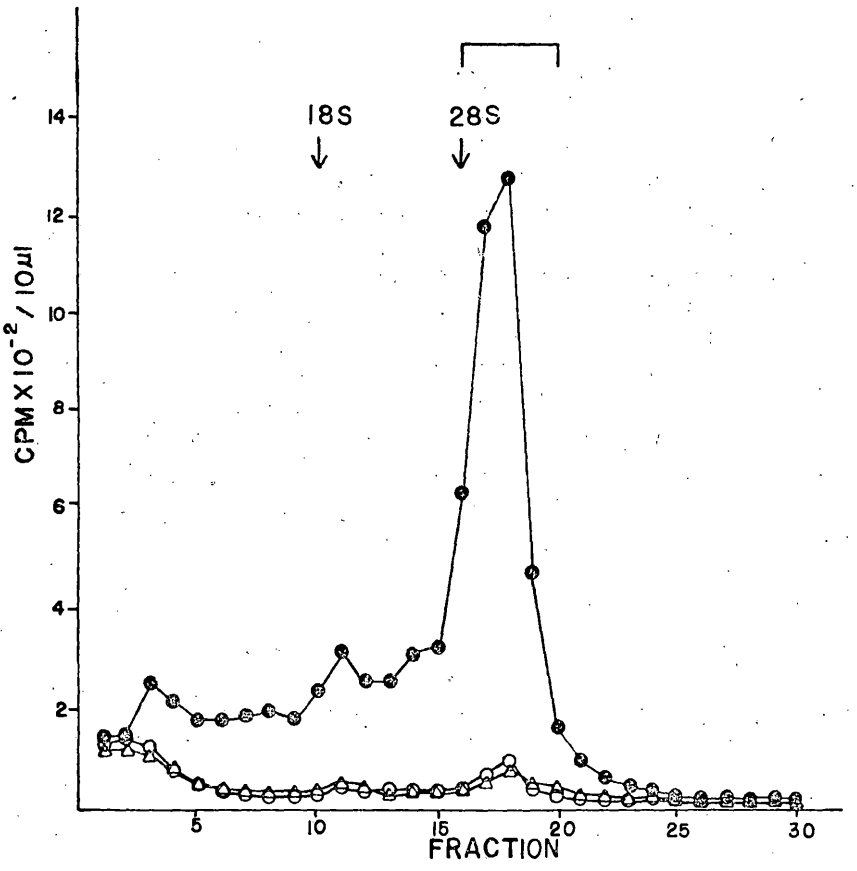

FIGURE 6

TABLE 7

Poly(A) Content of Poliovirus 35S RNA Synthesized in the Presence or Absence of Cordycepin

RNA Preparation	% RNase Resistant[a]
Control	4.3 (3.7 - 5.0)
Cordycepin 0 h p.i.	4.6 (3.8 - 5.5)
Cordycepin 2 h p.i.	5.2 (4.1 - 6.3)

[a] Each value represents average of triplicate determinations on the same RNA preparation. Numbers in parenthesis represent the range from two separate RNA preparations.

RNA synthesis by a bacterial DNA-dependant RNA polymerase (93). It has also been shown that 3'dATP inhibits eukarotic DNA-dependant RNA polymerase II *in vitro* (56). A third possibility was that inhibition of viral RNA synthesis was secondary to inhibition by cordycepin of viral protein synthesis. Because picornavirus-specific RNA polymerase is very labile (28) inhibition of viral protein synthesis would result in a rapid decrease in RNA synthesis. This last mechanism may be involved in the inhibition of Newcastle disease virus genome RNA synthesis by cordycepin (110).

E. *Cordycepin triphosphate inhibition of viral RNA synthesis in vitro*:

In order to determine which of the above mechanisms might be responsible for the inhibition of viral RNA synthesis the following experiments were performed. Cordycepin has been shown to be phosphorylated to 3'dATP in HeLa cells (56). Therefore the effect of 3'dATP on *in vitro* RNA synthesis by crude viral polymerase complexes isolated from infected cells was studied. Polymerase complexes were prepared from cells infected with HRV-14 or poliovirus or from mock infected cells. The effect of 3'dATP on [^{3}H]AMP incorporation was determined in either a complete standard assay mixture containing all four nucleoside triphosphates to measure total RNA polymerase activity or in a mixture where ATP was the only nucleotide present to measure poly(A) polymerase activity. [^{3}H] AMP incorporation with or without 0.5 mM 3'dATP is shown in Table 8. While the activity of HRV-14 and poliovirus polymerases were slightly inhibited, [^{3}H]AMP incorporation by the mock infected cell enzyme preparation was actually stimulated by 3'dATP when all four nucleoside triphosphates were present. When ATP was the sole precursor, 3'dATP caused a marked

TABLE 8

Effect of 3'dATP on [^{3}H]AMP Incorporation by Crude Viral Polymerase Complexes

Reaction Mixture[a]	Enzyme Preparation	^{3}H AMP Incorporation[b] (CPM)/100 µg Protein		% Change	
		-3'dATP	+3'dATP	Inhibition	Stimulation
Complete	Poliovirus	2056	1331	35	———
	Rhinovirus	2049	1760	15	———
	Mock Infected Cells	586	1323		125
ATP Only	Poliovirus	231	977	—	323
	Rhinovirus	426	2617	—	514
	Mock Infected Cells	338	1354	—	301

[a]The reaction mixtures were incubated for 30 min as described under methods.

[b]Averages of triplicate samples.

stimulation of [^{3}H]AMP incorporation not only by the mock infected cell preparation but also by the viral polymerase complexes. However, in the absence of 3'dATP [^{3}H]AMP incorporation obtained with the viral polymerase complexes, was comparable to that obtained with the mock infected cell preparation. Thus, apparently there was no virus-specific poly(A) polymerase activity.

The above results suggested that the cellular [^{3}H]AMP incorporating activity could have masked inhibition of viral polymerase activity by 3'dATP. To test this possibility, the effect of 3'dATP on [^{3}H]GMP incorporation by viral polymerase complexes was studied. The results are shown in Figure 7. Complexes prepared from mock infected cells showed no [^{3}H]GMP incorporation (Figure 7B). Presence of 0.5 mM 3'dATP in the reaction mixtures clearly inhibited [^{3}H]GMP incorporation by HRV-14 polymerase complex (Figure 7A) and by poliovirus polymerase complex (Figure 7B). These results therefore, confirm that the inhibition of HRV-14 and poliovirus RNA synthesis by cordycepin (shown previously) is due to direct interference by 3'dATP with viral RNA synthesis and is not due to a preferential inhibition of poly(A) synthesis nor due to inhibition of viral protein synthesis.

Though not well established by kinetic experiments, competition between 3'dATP and ATP is strongly suggested by published data (70,93). From the structure of 3'dATP one would assume RNA chain termination would result from incorporation of 3'dAMP in place of AMP and subsequent failure to provide a 3'-hydroxyl for further elongation. Whether or not this is the case is not certain, since AMP rather than 3'dATP has been found to be the residue in RNA chains terminated with

FIGURE 7. 3'dATP inhibition of [^{3}H]GMP incorporation by crude viral polymerase complexes.

(A) [^{3}H]GMP incorporation by HRV-14 polymerase complex in the absence (▲) or presence (Δ) of 0.5 mM 3'dATP. (B) [^{3}H]GMP incorporation by poliovirus polymerase complex in the absence (●) or presence (○) of 0.5 mM 3'dATP and [^{3}H]GMP incorporation by a preparation from mock infected cells (■). Each point represents the average of triplicate samples removed from standard reaction mixtures at the indicated times.

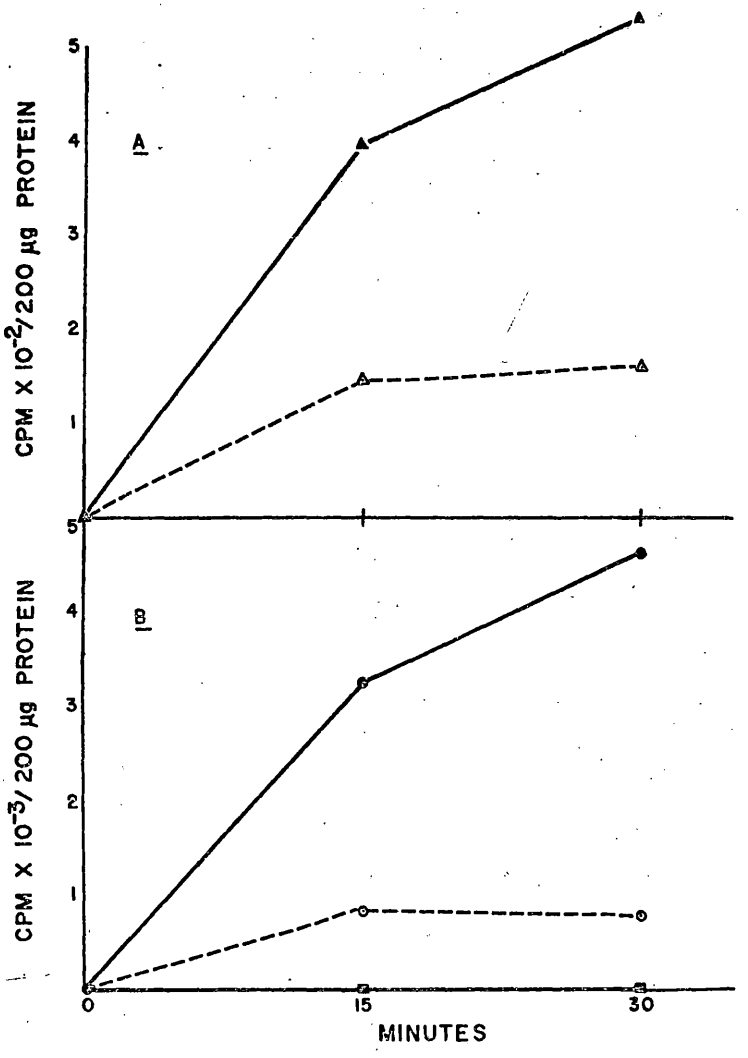
A
B
CPM X 10-2/200 µg PROTEIN
CPM X 10-3/200 µg PROTEIN
0
15
30
MINUTES

FIGURE 7

cordycepin (26). The following experiments were therefore undertaken to determine whether the mechanism by which 3'dATP inhibited viral RNA synthesis was indeed termination of RNA chain elongation.

The effect of increasing ATP or GTP concentrations in the reaction mixture on 3'dATP inhibition of [^{3}H]GMP incorporation by poliovirus polymerase complex was investigated. Figure 8 shows the effect of increasing amounts of ATP on [^{3}H]GMP incorporation in the presence of two concentrations of 3'dATP (0.125 mM and 0.25 mM). The inhibition kinetics indicate that inhibition was reversible when ATP was added along with 3'dATP. The inset to Figure 8 shows a double reciprocal plot of the data indicating competitive inhibition. The apparent Km for ATP is 1.25 X 10^{-4}M in the absence of 3'dATP and 3.33 X 10^{-4} and 4 X 10^{-4}M in the presence of 0.125 mM and 0.25 mM 3'dATP, respectively. The inhibition of RNA synthesis was not reversible, however when excess GTP was added along with 3'dATP (Figure 9) indicating that 3'dATP was competing specifically with ATP and not nucleoside triphosphates in general.

To further test the assumption that 3'dATP specifically terminates chain elongation, the effect of a 20-fold excess of ATP over 3'dATP added 10 min after the inhibitor on [^{3}H]GMP incorporation by poliovirus polymerase complex was determined. The results shown in Figure 10 demonstrate that once inhibition of RNA synthesis has occured, it can no longer be reversed with ATP. This observation is consistent with the proposal that termination of RNA chain elongation is the mechanism of cordycepin action.

Another way to test whether 3'dATP terminated RNA chain elongation is to look at the viral RNA products labeled *in vitro*. Crude poliovirus

FIGURE 8. Effect of increasing ATP concentrations on 3'dATP inhibition of *in vitro* [^{3}H]GMP incorporation by poliovirus polymerase complexes.

Reaction mixtures containing poliovirus polymerase complex, a constant amount of [^{3}H]GTP (25 μCi/ml) and increasing amounts of ATP in the presence or absence of 3'dATP were incubated at 37°C for 15 min. Acid insoluble radioactivity was determined as described in Materials and Methods. Each point represents the average of duplicate samples. Symbols: No 3'dATP (●), 0.125 mM 3'dATP (Δ), and 0.25 mM 3'dATP (■). The inset shows a double reciprocal plot of the data.

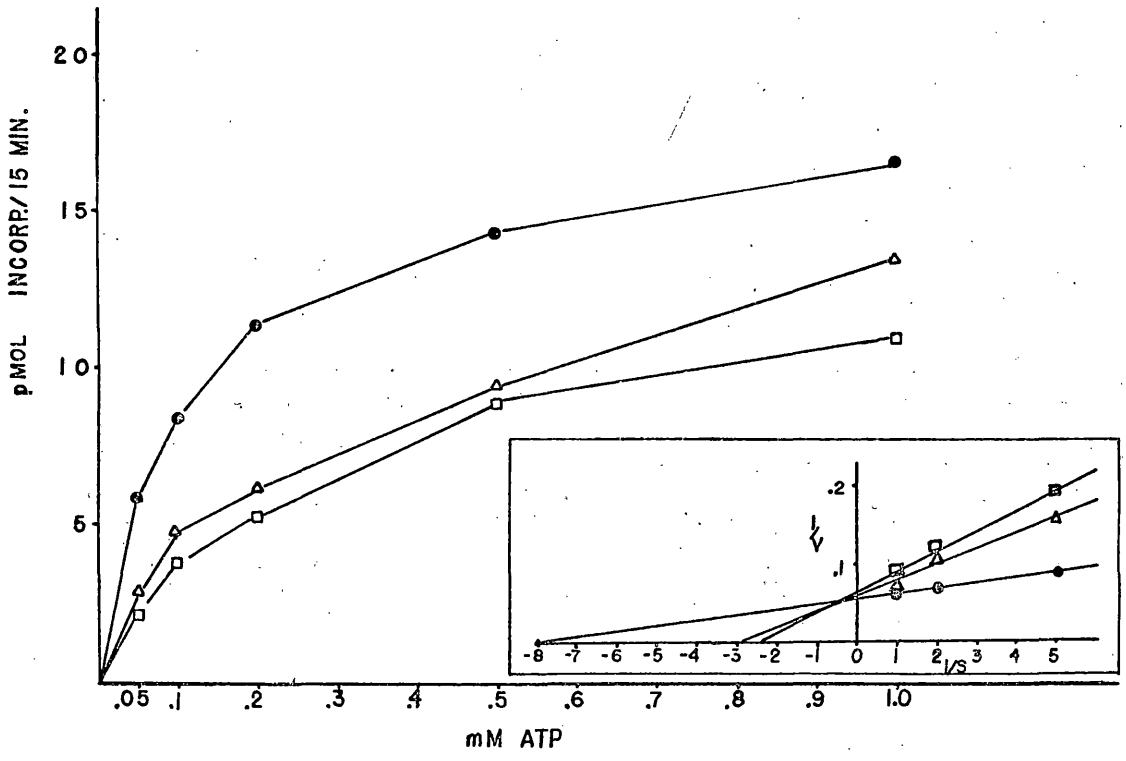
pMOL INCORP./15 MIN.
20
15
10
5
.05
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
mM ATP
1/V
.2
.1
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
1/S

FIGURE 8

FIGURE 9. Effect of increasing concentrations of GTP on 3'dATP inhibition of *in vitro* [^{3}H]GMP incorporation by poliovirus polymerase complexes.

Reaction mixtures containing poliovirus polymerase complex, a constant specific activity of [^{3}H]GTP (50 μCi/mM) and increasing amounts of GTP in the presence or absence of 3'dATP were incubated at 37°C for 30 min. Acid precipitable radioactivity was determined as described in Materials and Methods. Each point represents the average of triplicate determinations. Symbols: No 3'dATP (●), 0.25 mM 3'dATP (○).

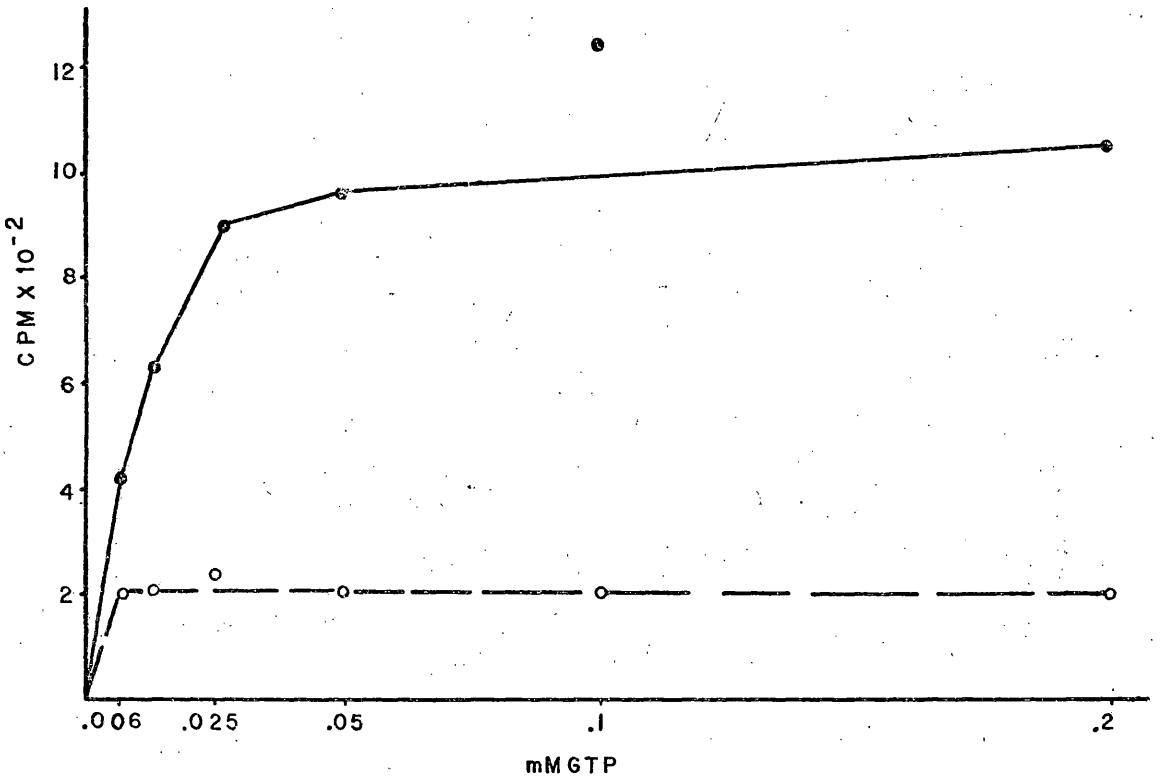

FIGURE 9

FIGURE 10. Effect of addition of excess ATP 10 min after 3'dATP, on inhibition of *in vitro* [^{3}H]GMP incorporation.

Complete reaction mixtures containing [^{3}H]GTP (25 μCi/ml) were first incubated at 37°C for 10 min with (○) or without (●,▲) 0.25 mM 3'dATP. Excess ATP (5 mM) was then added to the 3'dATP-inhibited reaction mixture (○), and to one of the control reaction mixtures (▲), and incubation continued. Acid insoluble radioactivity of 100 μl samples was determined as described previously. Each point represents the average of duplicate samples.

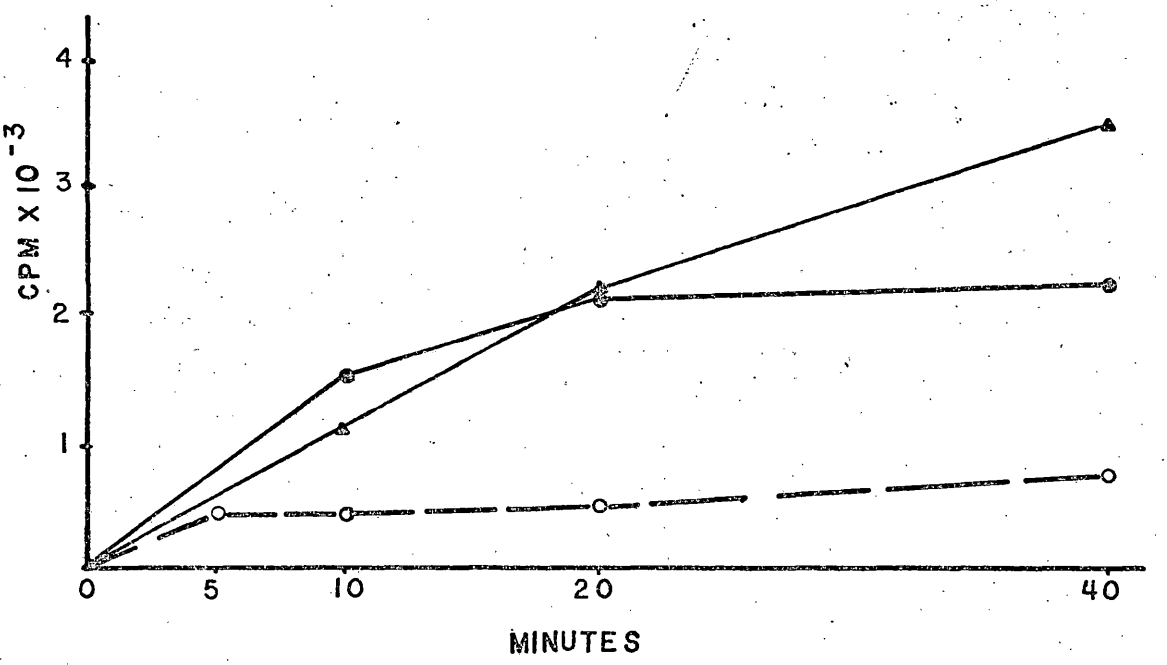

FIGURE 10

polymerase complexes synthesize in vitro SS 35S genome RNA, double-stranded 18S RF RNA and the broadly sedimenting partially double-stranded 18-28S RI RNA (5). Figure 11 shows the sucrose gradient sedimentation profiles of products synthesized by poliovirus polymerase complex in complete assay mixtures using [^{3}H]GTP or [^{3}H]ATP as the labeled precursor in the presence or absence of 3'dATP. The profiles of the products labeled with either precursor in the absence of the inhibitor were basically similar and consisted of RI, RF and SS viral RNA species. The identity of the poliovirus polymerase product sedimenting at 35S as SS viral RNA was established by infectivity assay (data not shown). The label appeared also in a low molecular weight RNA component which sedimented near the top of the gradient. In the presence of 3'dATP, the polymerase complex synthesized little if any full length 35S SS RNA (Figure 11). The [^{3}H]GMP label appeared predominantly in the low molecular weight region and minimally in the 18-28S region consisting of the RF and RI RNA. The observation that synthesis of SS viral RNA is most severely inhibited lends further support to the proposal that 3'dATP acts to terminate RNA chain elongation.

F. Synthesis of HRV-14 intermediate RNA species in the presence or absence of actinomycin D:

Macnaughton et al (57) recently reported that synthesis of human rhinovirus type 2 double-stranded RF RNA was an artifact due to the presence of act-D used to inhibit cellular RNA synthesis. Therefore, prior to analyzing the poly(A) content of the various HRV-14 intermediate RNA species it was necessary to determine whether HRV-14 was an artifact

FIGURE 11. Sucrose gradient sedimentation profiles of poliovirus RNA species synthesized *in vitro*.

2.0 ml complete reaction mixtures containing either [^{3}H]ATP or [^{3}H]GTP in the presence or absence of 3'dATP (0.5 mM) were incubated at 37°C for 45 min, RNA extracted and sedimented through a 15-30% sucrose gradient as described in Materials and Methods. 1.2 ml fractions were collected and absorbance monitored at 254 nm. RNA from each fraction was precipitated with TCA, collected on glass fibre filters and counted. Symbols: RNA labeled with [^{3}H]ATP (0- - -0). RNA labeled with [^{3}H]GTP in the absence (●-●) or presence (0-0) of 3'dATP.

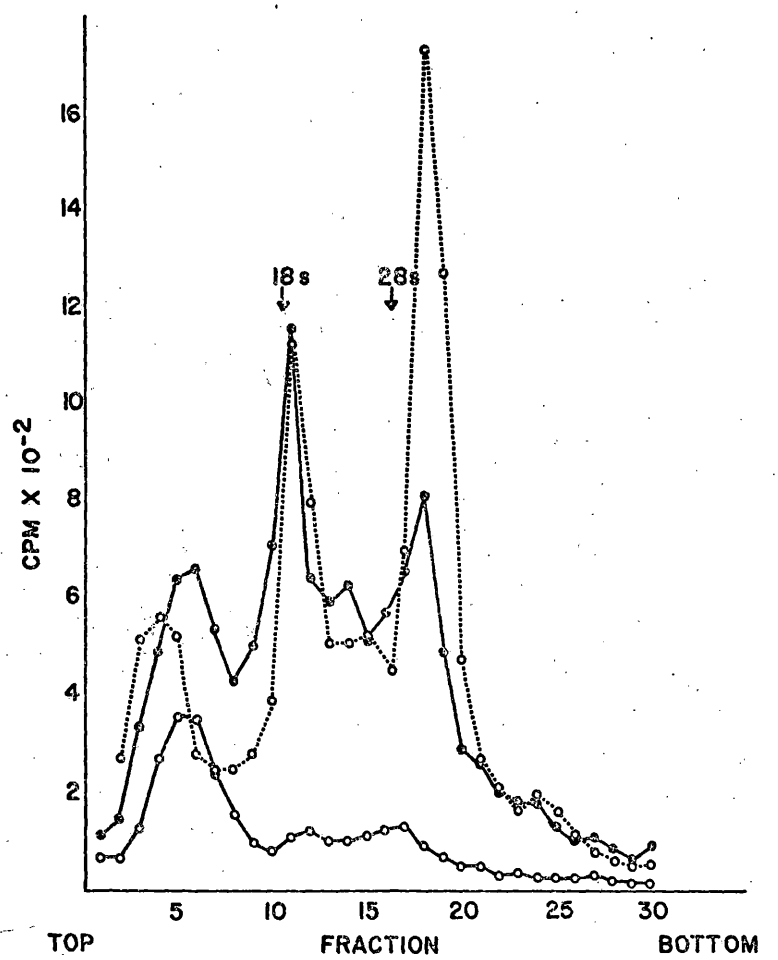
CPM X 10⁻²
16
14
12
10
8
6
4
2
18s
28s
5
10
15
20
25
30
TOP
FRACTION
BOTTOM

FIGURE 11

of act-D. [^{3}H]AR labeled RNA was extracted from HRV-14 infected cells incubated either in the presence or absence of act-D (5 µg/ml) from the start of infection. Similarly RNA was extracted from mock infected cells incubated in the absence of act-D. Each viral RNA preparation was passed through a Sepharose-2B column and the RNA excluded from the column was analyzed by electrophoresis on 2% polyacrylamide, .5% agarose gels. A composite RNA profile of all three RNA preparations is shown in Figure 12. HRV-14 RF RNA is synthesized in the absence or presence of act-D and is, therefore, not an artifact of act-D. Some high molecular weight RNA from mock infected cells is also excluded from Sepharose-2B; however, it does not interfere with determining the positions of the viral-specific RNAs.

G. Mechanism of polyadenylation of HRV-14 RNA:

Even though cordycepin appears to inhibit HRV-14 RNA synthesis and poliovirus RNA synthesis in the same manner by terminating RNA chain elongation, the possibility still remained that HRV-14 RNA was polyadenylated by non-transcriptional mechanism. In order to determine what the mechanism was a variety of aspects of HRV-14 RNA synthesis were studied.

First it was necessary to determine if the various viral intermediate RNAs contained poly(A) or if it was only present on free single-stranded RNA. To determine the poly(A) content of HRV-14 RF, RI and SS RNAs, it was first necessary to isolate each species of RNA relatively free from each other. Procedures originally developed for isolation of poliovirus RNA intermediates were applied for the separation of the various HRV-14 RNAs (6,7,9). HRV-14 SS RNA and RI RNA were first separated from RF

FIGURE 12. 2% Polyacrylamide - .5% Agarose gel analysis of HRV-14 intermediate RNA species synthesized in the presence or absence of Actinomycin D.

Replicate roller cultures were either infected with HRV-14 at a MOI ≈ 10 or mock infected. Act-D was added to one infected culture at a concentration of 5 µg/ml, the other infected culture and mock infected culture were incubated in the absence of act-D. Each culture was labeled with [^{3}H]AR (15 µCi/ml) from 4 to 8 h p.i. After labeling the cells were scraped from the bottles, swollen in RSB, broken with a Dounce homogenizer and RNA extracted from the cytoplasm as described in Materials and Methods. The RNA preparations were dissolved in NETS buffer containing 0.5% SDS and fractionated by chromatography through Sepharose-2B columns. The RNA that was excluded from the columns, containing viral intermediate RNA species was precipitated with alcohol, dissolved in electrophoresis buffer and a constant volume of each RNA was analyzed by electrophoresis on separate polyacrylamide-agarose gels. Gels were fractionated and counted as described in Materials and Methods. Purified RF and SS RNA markers were run on separate parallel gels. The figure is a composite of all three gels. Symbols: HRV-14 infected cell RNA synthesized in the presence of act-D (●); or in the absence of act-D (○); mock infected cell RNA synthesized in the absence of act-D (□).

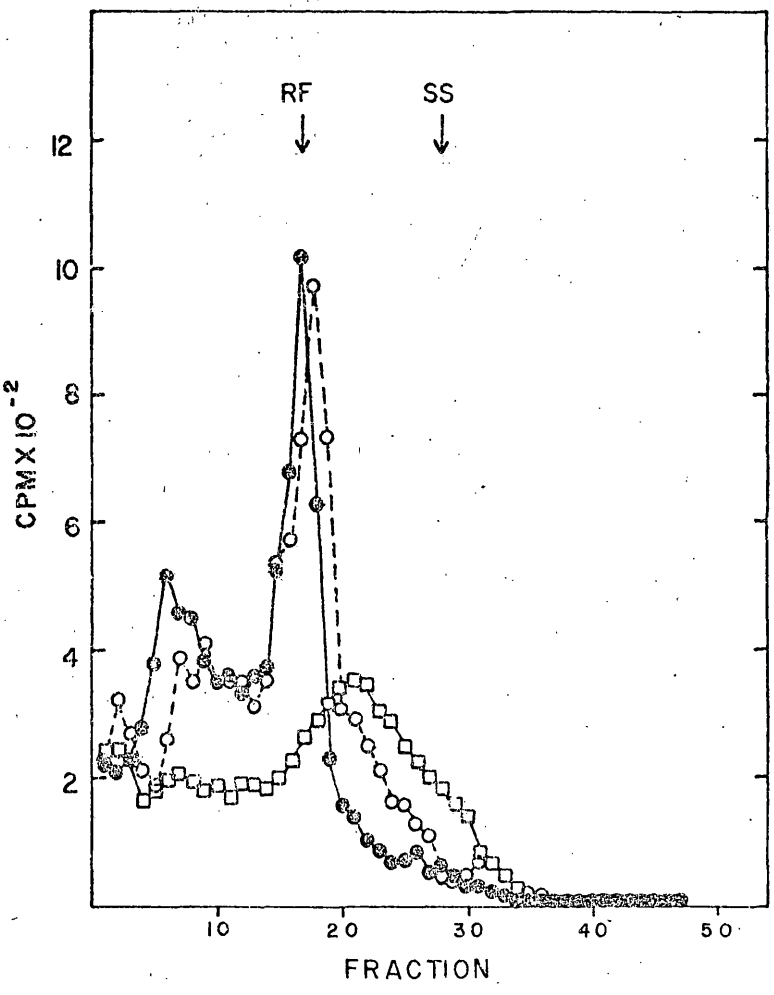

FIGURE 12

RNA by differential precipitation with 2 M LiCl, SS RNA was then separated from RI RNA by Sepharose-2B chromatography. Figure 13A shows the separation of SS RNA from RI RNA on Sepharose-2B. Re-chromatography of SS RNA is shown in Figure 13B. Analysis of the isolated RNA species on 2% polyacrylamide, .5% agarose gels shows that each purified RNA species was relatively free from cross contamination (Figure 14).

Table 9 shows the poly(A) content of the various HRV-14 intermediate RNA species as measured by resistance to pancreatic plus T1 RNases. Each RNA preparation was denatured by heating to 100°C prior to RNase digestion to avoid measuring double-stranded RNA. [^{3}H]AR labeled HRV-14 SS RNA has about the same poly(A) content as virion RNA (6.6% vs 7.2%). RI RNA has a low poly(A) content (1.5%) while RF RNA has an unexpectedly high poly(A) content (9.9%). This might be explained, however, by incomplete denaturation of the double-stranded RNA as reflected in the large RNase resistant fraction of [^{3}H]UR labeled RF RNA.

The presence of poly(A) in each of the HRV-14 intermediate RNA species was confirmed by poly(U)-Sepharose chromatography. Figure 15 shows the elution profile of each RNA. About the same fraction of SS RNA and RI RNA are bound and the elution profile closely resembles that of virion RNA (Figure 2). Only about 50% of RF RNA is bound. However, on rechromatography of RF RNA approximately 50% of the "bound" RF RNA rebinds and the same holds true for the "unbound" RF RNA, 50% rebinds (not shown).

The next experiment was undertaken to determine whether a crude HRV-14 polymerase complex was capable of synthesizing virus specific

FIGURE 13. Sepharose-2B chromatography of 2M LiCl insoluble HRV-14 SS and RI RNA.

HRV-14 specific RNA was extracted from the cytoplasm of approximately 4 X 10^8 infected cells labeled from 4 to 8 h p.i. with [^{3}H]AR (10 µCi/ml) in the presence of act-D. The RNA was then fractionated into the 2 M LiCl soluble RF RNA and the 2 M LiCl insoluble SS + RI RNA as described in Materials and Methods. (A) SS + RI RNA were then separated by chromatography on a Sepharose-2B column by eluting with NETS buffer containing 0.5% SDS at a flow rate of ~7 ml/h. 2.0 ml fractions were collected and 20 µl aliquots of each counted. Brackets indicate fractions pooled and precipitated with alcohol. (B) Rechromatography of excluded RI RNA using the same conditions as in (A).

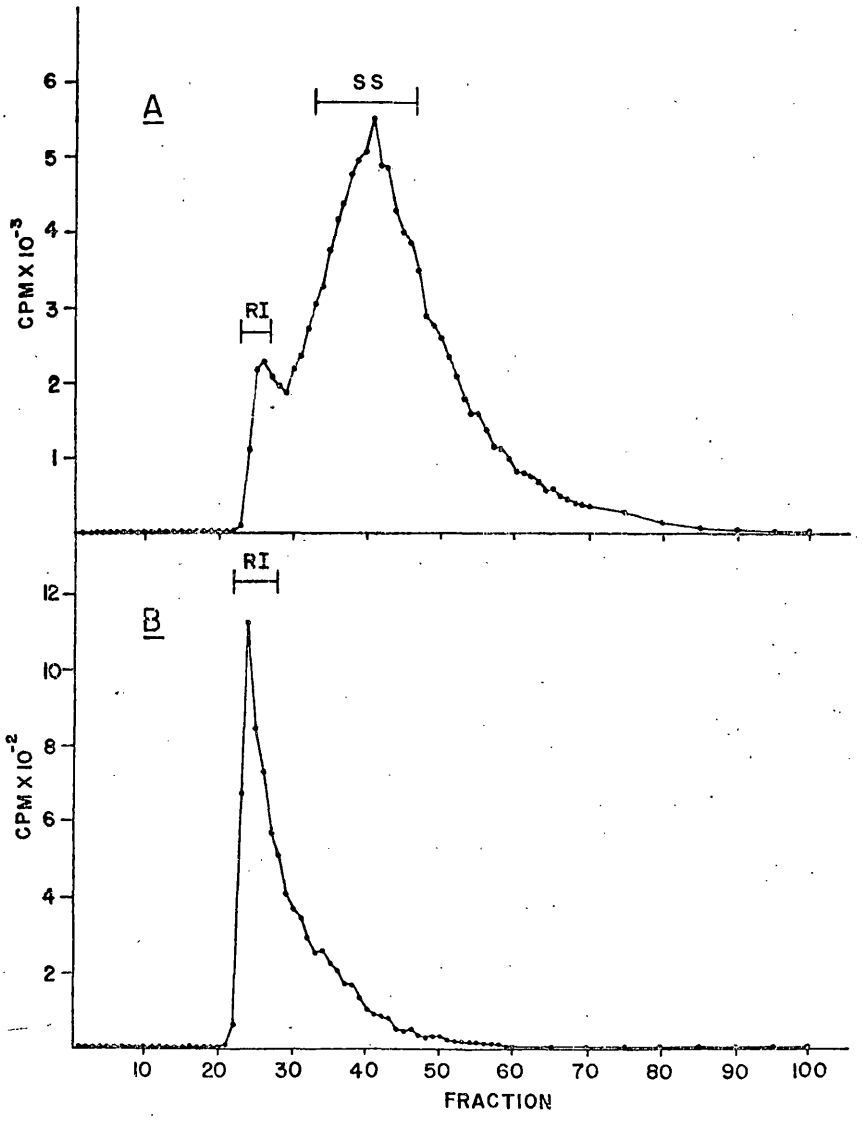
A
SS
RI
CPM X 10⁻³
6
5
4
3
2
1
B
RI
CPM X 10⁻²
12
10
8
6
4
2
10
20
30
40
50
60
70
80
90
100
FRACTION

FIGURE 13

FIGURE 14. 2% Polyacrylamide, .5% agarose gel electrophoresis of HRV-14 SS, RF, and RI RNAs.

HRV-14 SS, RF, and RI RNAs separated by LiCl precipitation and Sepharose-2B chromatography in Figure 13 were dissolved in Loening electrophoresis buffer and an aliquot analyzed by electrophoresis on 2% polyacrylamide, .5% agarose gels at 5 mA/gel for 2.75 h. Gels were sliced, solubilized and counted as described. (A) SS RNA, (B) RF RNA, (C) RI RNA.

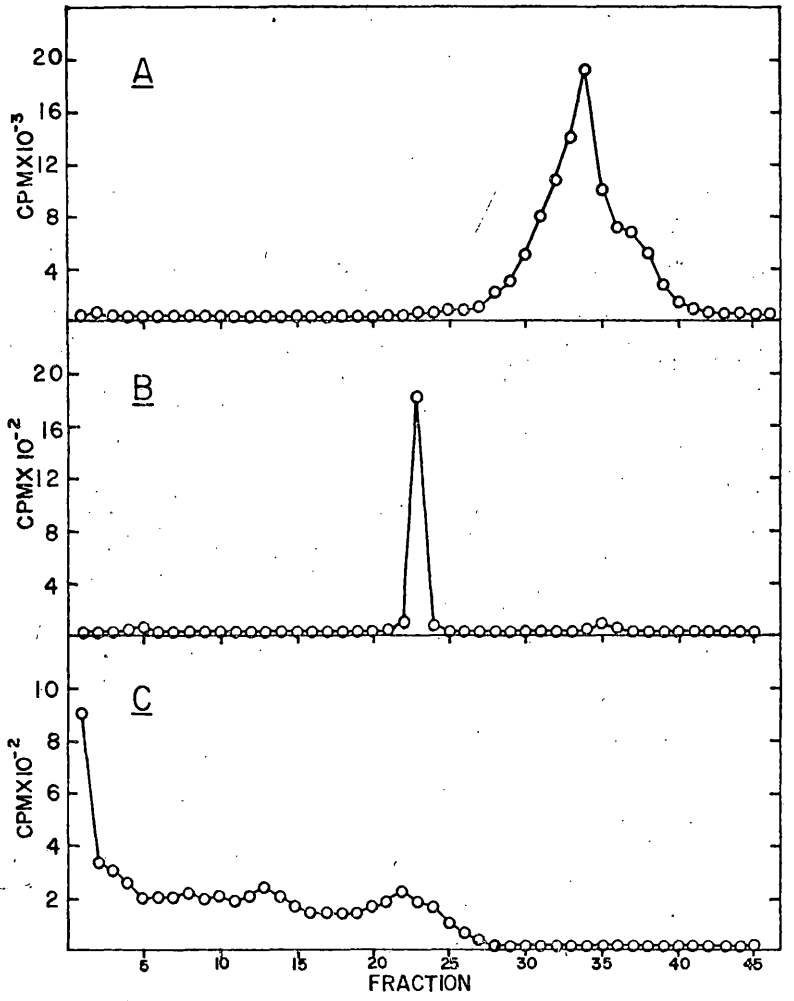
A
B
C
CPMX10⁻³
CPMX10⁻²
CPMX10⁻²
20
16
12
8
4
10
6
2
5
10
15
20
25
30
35
40
45
FRACTION

FIGURE 14

TABLE 9

Poly(A) Content of HRV-14 Intermediate Viral RNA Species as Measured by Resistance to Pancreatic and T1 RNAses

RNA[a]	Label	% RNase Resistance
SS	^{3}H Adenosine	6.6 (6.2-7.7)[b]
SS	^{3}H Uridine	0.35
RF	^{3}H Adenosine	9.9 (7.6-12.3)
RF	^{3}H Uridine	2.4
RI	^{3}H Adenosine	1.5 (1.4-1.6)

[a]Each sample was denatured by heating to 100°C for 2 min in 1 mM EDTA then rapidly cooled to 0°C prior to digestion with RNase.

[b]Numbers in parenthesis indicate the range of values from at least two different RNA preparations. Each value represents the average of at least triplicate determinations.

FIGURE 15. Poly(U)-Sepharose chromatography of HRV-14 intermediate RNA species.

Poly(U)-Sepharose columns were prepared as described under Materials and Methods. HRV-14 intermediate RNA species isolated from infected cells labeled from 4 to 8 h p.i. were dissolved in NETS buffer passed through the columns and eluted as shown. 1.0 ml fractions were collected and radioactivity counted. (A) SS RNA, (B) RI RNA, (C) RF RNA.

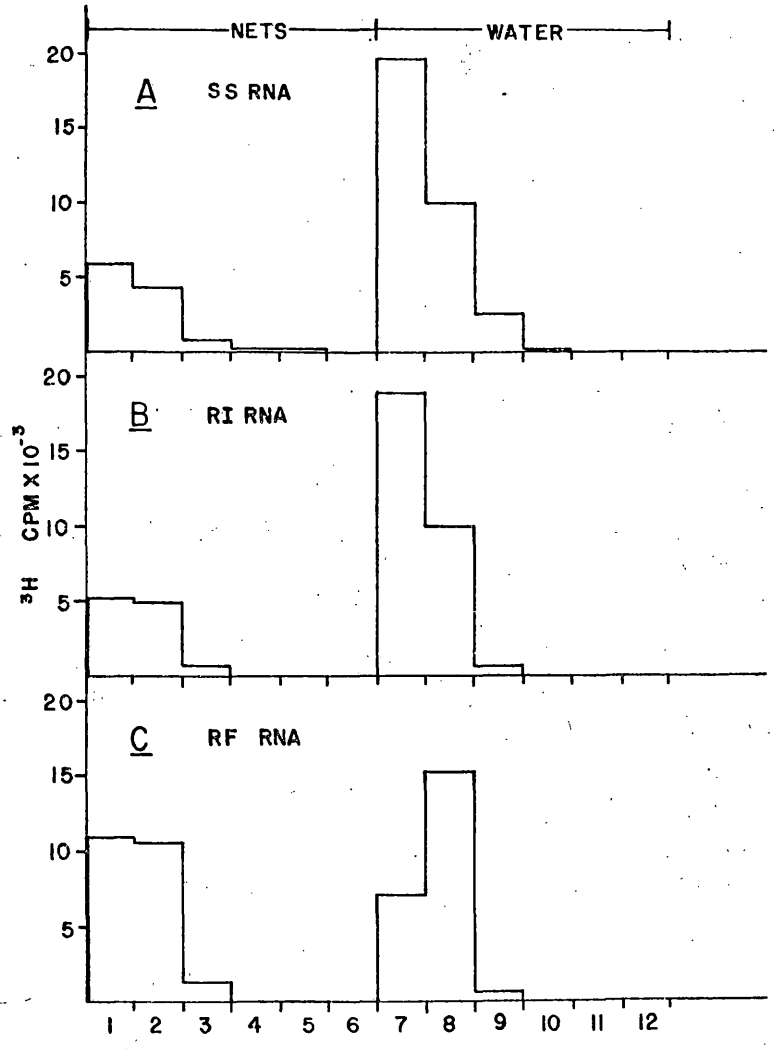
NETS
WATER
A
SS RNA
B
RI RNA
C
RF RNA
^{3}H CPM X 10^{-3}
20
15
10
5
1
2
3
4
5
6
7
8
9
10
11
12

FIGURE 15

RNA containing poly(A) *in vitro*. Figure 16 shows the sucrose sedimentation profile of products synthesized by HRV-14 polymerase complex with either [^{3}H]ATP or [^{3}H]GTP as the labeled precursor. As can be seen from the sedimentation profiles very little HRV-14 specific SS RNA is synthesized *in vitro* with either labeled triphosphate. Most of the labeled RNA appears in the range of the RI RNA, as previously reported by others (49,112). Label also appears in an undefined low molecular weight component. This may represent degraded RNA or incomplete nascent RNA chains released from the RI RNA.

To analyze *in vitro* RNA products for poly(A) content, crude HRV-14 polymerase complexes were incubated in a complete reaction mixture with [^{3}H]ATP as the labeled precursor. Viral products labeled *in vitro* were then separated into RF RNA and RI RNA by LiCl precipitation and Sepharose-2B chromatography. Poly(A) content was then determined by RNase digestion and confirmed by poly(U)-Sepharose chromatography. The results are presented in Table 10. The poly(A) label of the *in vitro* RNAs was considerably higher than RI or RF RNA synthesized *in vivo* as would be expected. It has been shown that the *in vitro* polymerase complexes only elongate previously initiated nascent RNA chains (34,112). Therefore, the proportion of label in poly(A) would be higher because of its presence at the 3'end of the RNA molecule. The *in vitro* labeled RF and RI RNAs were also bound to poly(U)-Sepharose to the same extent as the *in vivo* RF and RI RNA, confirming the synthesis of poly(A) *in vitro* (Table 10).

The final series of experiments on the mechanism of polyadenylation of HRV-14 RNA was an attempt to determine if HRV-14 intermediate RNAs

FIGURE 16. Sucrose gradient sedimentation profile of HRV-14 RNA species synthesized *in vitro*.

Complete reaction mixtures containing either [^{3}H]ATP or [^{3}H]GTP were incubated at 34°C for 45 min, RNA was extracted and sedimented through a 15-30% sucrose gradient as described in Materials and Methods. 1.2 ml fractions were collected and absorbance monitored at 254 nm. RNA from each fraction was precipitated with TCA, collected on glass fibre filters and counted. Symbols: RNA labeled using [^{3}H]ATP as the labeled precursor (●-●), or using [^{3}H]GTP as the labeled precursor (●- - -●).

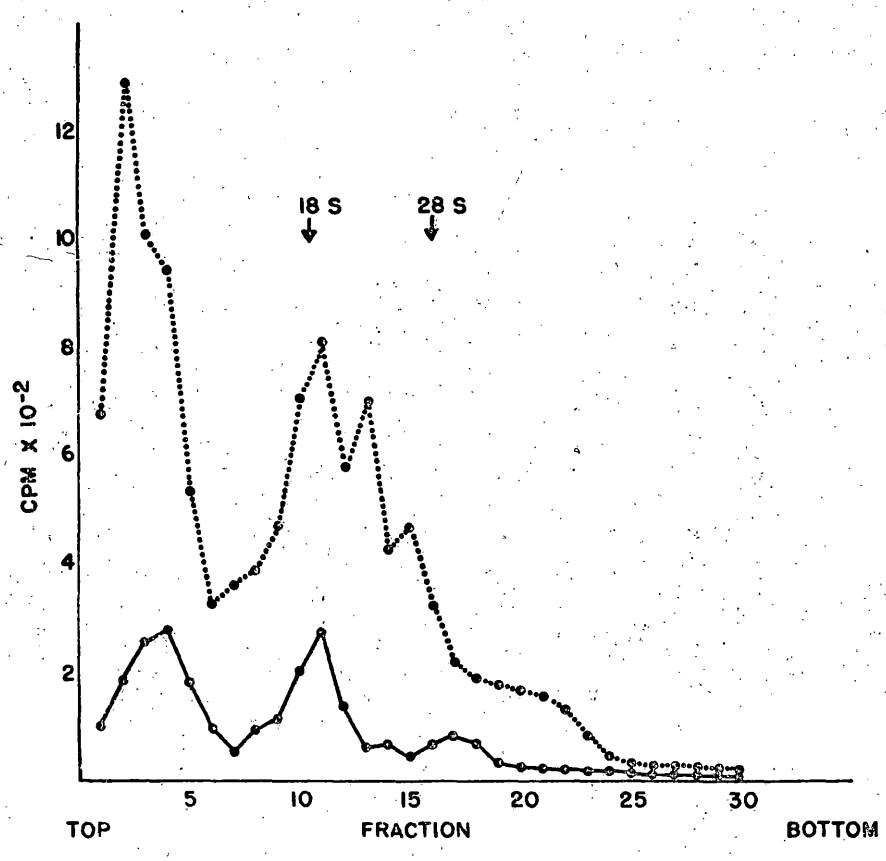

FIGURE 16

TABLE 10

RNase Resistance of [^{3}H]ATP labeled HRV-14 Products Synthesized *in vitro*.

RNA[a]	Percent RNase Resistant[b]	Percent Bound	Percent Unbound
LiCl soluble (RF)	25.5	45	55
LiCl insoluble (RI)	11.1	73	27

[a]Each sample was denatured by heating to 100°C for 2 min in 1 mM EDTA then rapidly cooled to 0°C prior to digestion with RNase.

[b]Values represent the average of triplicate determinations.

contained a poly(U) sequence from which poly(A) might be transcribed. Poly(U) has been found at the 5' end of the minus strand of poliovirus RF and RI RNA (116,117). A search for poly(U) sequences in the RF + RI RNAs of human rhinovirus type 2 failed to detect any poly(U) by chromatography on poly(A)-Sepharose columns (58). Poly(A)-Sepharose columns were also chosen as a means of detecting poly(U) sequences on HRV-14 RNAs; however, all RNA preparations were denatured in the presence of excess unlabeled poly(U). The results are presented in Figure 17. [^{3}H]-poly(U) control RNA is completely bound by the column while [^{3}H]AR SS HRV-14 RNA which contains poly(A) is not bound. A mixture of poliovirus RF + RI RNAs which have been shown to contain poly(U) sequences (116,117) were also run as a control (Figure 17C) and as expected about 25% of the RNA was bound, corresponding roughly to the amount of minus strand RNA molecules in the preparation. A mixture of ^{32}P-labeled HRV-14 RF + RI RNA was chromatographed (Figure 17D) and it also contained a fraction of RNA about 25%, that was bound to the poly(A)-Sepharose. From these results it appeared that HRV-14 RF + RI RNAs contained poly(U) sequences.

The possibility that the denatured RF + RI RNA preparations may in some way bind non-specifically to poly(A)-Sepharose was ruled out by the following experiment. [^{3}H]AR labeled HRV-14 RF + RI RNAs were heat denatured in the presence of either a large excess of poly(U) or a large excess of poly(A), then cooled at room temperature for one hour, and chromatographed on poly(A)-Sepharose. The results are shown in Figure 18. When HRV-14 RF + RI RNA was denatured in the presence of poly(U) (Figure 18A), once again about 25% of the RNA was bound to the

FIGURE 17. Poly(A)-Sepharose chromatography of viral intermediate RNA species.

Poly(A)-Sepharose columns were prepared and washed as described in Materials and Methods. (A) [^{3}H]poly(U), (B) [^{3}H]AR HRV-14 SS RNA, (C) [^{3}H]AR poliovirus RF + RI RNAs, (D) ^{32}P-HRV-14 RF + RI RNAs. All RNAs were heat denatured in the presence of 2 µg poly(U), rapidly cooled and the necessary components added to achieve their respective concentrations in .2 M NETS. The RNAs were then applied to poly(A)-Sepharose columns and eluted as indicated. 1.0 ml fractions were collected and radioactivity counted.

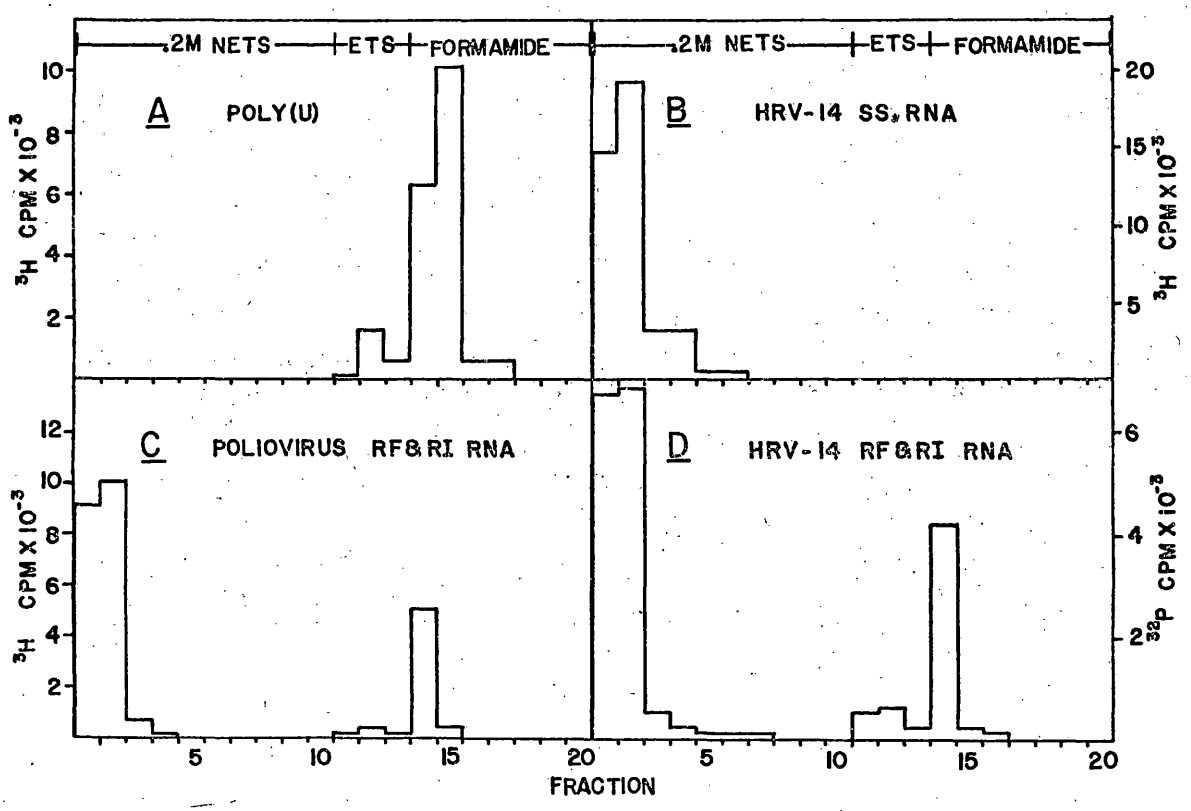
.2M NETS
ETS
FORMAMIDE
A
POLY(U)
B
HRV-14 SS RNA
C
POLIOVIRUS RF&RI RNA
D
HRV-14 RF&RI RNA
^{3}H CPM X 10^{-3}
^{32}P CPM X 10^{-3}
FRACTION

FIGURE 17

FIGURE 18. Poly(A)-Sepharose chromatography of HRV-14 RF + RI denatured in the presence of excess poly(U) or poly(A).

[^{3}H]AR HRV-14 RF + RI RNA was heat denatured in the presence of either (A) poly(U) (10 µg/ml) or (B) poly(A) (20 µg/ml), rapidly cooled and allowed to gradually warm to room temperature. The RNA then remained at room temperature for 1 h prior to chromatography on poly(A)-Sepharose columns as in Figure 17.

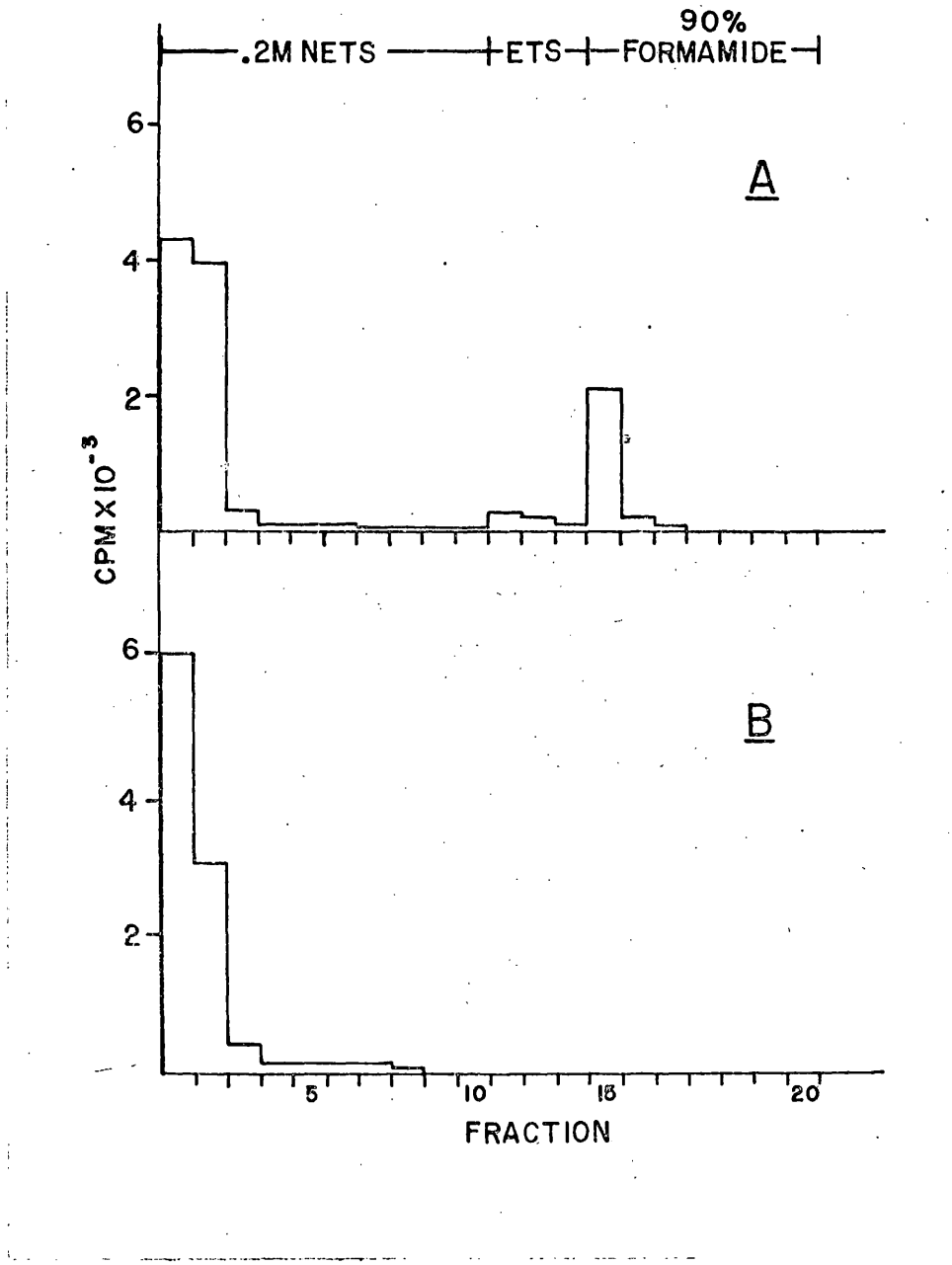
.2M NETS
ETS
90%
FORMAMIDE
A
B
CPM X 10⁻³
6
4
2
6
4
2
5
10
15
20
FRACTION

FIGURE 18

poly(A)-Sepharose. However, when HRV-14 RF + RI RNA was denatured in the presence of poly(A) none of the RNA was bound by the column as would be expected if binding to the column required the presence of a free poly(U) sequence. Therefore, HRV-14 intermediate RNAs contain poly(U) sequences of sufficient length to bind to poly(A)-Sepharose.

DISCUSSION

A. Characterization of the Poly(A) Sequences Associated with HRV-14 RNA:

Previous studies of HRV-14 and HRV-2 virion RNA have indicated that they contain poly(A) sequences (58,70). While only a rough estimate of the size of the HRV-14 poly(A) sequences was made (1), no attempt was made to determine the size of HRV-2 poly(A) sequences (58). The results obtained in this investigation show that poly(A) sequences of HRV-14 virion RNA molecules are quite heterogeneous and on the average approximately 160 nucleotides in length. The length of the poly(A) was estimated from: (1) the percentage of [^{3}H]AR labeled viral RNA resistant to pancreatic plus T1 RNases; (2) the percentage of ^{32}P-labeled viral RNA resistant to the RNases; and (3) the electrophoretic mobility of poly(A) sequences isolated from viral RNA.

All of the picornaviruses studied to date contain a poly(A) sequence in their RNA; however, the size of the poly(A) sequences varies considerably from subgroup to subgroup. Poliovirus RNA has a poly(A) sequence approximately 90 nucleotides long (114). Poly(A) sequences of Mengovirus (100) and Columbia SK virus (44) appear to be approximately 20-50 nucleotides long. EMC virus RNA was originally thought to lack an appreciably long poly(A) sequence (79), but recently it has been shown to have a poly(A) sequence about 20 nucleotides long (35). The presence of poly(A) sequences, and the variation in the size of these sequences between different subgroups of picornaviruses is also reflected in the base composition of their RNAs. The adenosine content for the various viral RNAs, on a molar percent basis, is 34-35% for

rhinovirus (14,82), 29% for poliovirus (97), 26% for mengovirus (86) and 26% for EMC virus (15). Correspondingly, each of the other three bases is less than 25% of each viral RNA (14,15,82,86,97). Therefore, the poly(A) sequence of HRV-14 RNA is by far the largest of any of the picornaviruses studied to date. This large difference in poly(A) content of HRV-14 RNA and the RNAs of other picornaviruses might bear on possible differences in the mechanism of polyadenylation of these RNAs, as will be discussed in detail later.

The ratio of infectious to total particles is much lower for rhinoviruses than for other picornaviruses. For instance, the ratio for HRV-14 is on the order of one to several thousand while the ratio for poliovirus is one to several hundred (104). Spector and Baltimore have shown that poly(A) is required for poliovirus RNA to be infectious (96). Therefore, it was considered a possibility that the reason why infectious to total particle ratio for HRV-14 was low was that a considerable fraction of HRV-14 molecules were lacking poly(A). The results of the poly(U)-Sepharose binding studies of HRV-14 virion RNA shows that this is not the case since the majority of HRV-14 RNA molecules are polyadenylated (Table 3).

This study has also demonstrated that the poly(A) of HRV-14 RNA is located at the 3'-terminus of the molecules. This was as expected considering that poly(A) is located at the 3'-termini of all cellular and viral mRNAs that have been examined (13,53,109). However, it was still necessary to determine the location before investigations on the mechanism(s) of HRV-14 polyadenylation could be made.

B. Mechanism of Cordycepin Inhibition of Picornavirus Replication:

The mechanism of cordycepin sensitivity of virus replication was studied initially because it had been suggested that sensitivity to cordycepin may indicate non-transcriptive poly(A) synthesis (70,115). This suggestion was based on an observation by Darnell et al (18) that cordycepin preferentially inhibits non-transcriptive poly(A) synthesis in mammalian cells. The observation that VSV poly(A) synthesis, which occurs only under conditions required for viral RNA transcription, was not inhibited by cordycepin (27,29,31), while other viruses which apparently acquire poly(A) non-transcriptively are inhibited by cordycepin (44,60,72,81) further supported the suggestion that the sensitivity to cordycepin might indicate a non-transcriptive mode of poly(A) synthesis.

There have been several reports in the literature that poliovirus replication was insensitive to cordycepin (52,74,115). This corresponded very nicely to the evidence suggesting that poliovirus poly(A) sequences might be transcribed from a complimentary poly(U) sequence (116,117). Nair and Owens (70) observed that HRV-14 replication was inhibited by cordycepin, suggesting that HRV-14 poly(A) might be added on non-transcriptively. It was for this reason that it was decided to determine the mechanism by which cordycepin inhibited HRV-14 replication, to see if HRV-14 poly(A) synthesis was indeed inhibited while poliovirus poly(A) synthesis was not.

Unexpectedly, the present study shows that replication of poliovirus type 2 is nearly as sensitive to cordycepin as that of HRV-14, contrary to what others have reported (52,115). These workers studied cordycepin

action on poliovirus type 1 growing in HeLa cell suspension cultures whereas in this study the action of cordycepin was measured on poliovirus type 2 in HeLa cell monolayer cultures. It is not known whether such differences would influence cordycepin action on poliovirus replication, but it seems unlikely. In a recent report by Dorsch-Häsler et al (22) the authors allude to the fact that poliovirus type 1 replication and RNA synthesis are also sensitive to cordycepin; however, they did not present any data.

Measurements of HRV-14 specific RNA synthesis in vivo showed that specific RNA synthesis was completely inhibited when cordycepin was added at 2, 5, or 7 h p.i. (6, 3, or 1 h, respectively, before the addition of label). When cordycepin was added at 0 h p.i. (8 h before label) there was some residual RNA synthesis. This residual RNA synthesis probably reflected partial metabolic inactivation of the drug during the long interval between the time the drug was added and the time of labeling. It has been shown that cellular enzymes are capable of deaminating 3'dATP to 3'dITP (105).

Similarly all except a small fraction of poliovirus-specific RNA synthesis is inhibited by addition of cordycepin at 0 or 2 h p.i. (3.5 or 1.5 h, respectively before the addition or label (Table 6). Analysis of poliovirus single-stranded RNA synthesized in the presence of cordycepin showed that poly(A) was still synthesized, indicating that poly(A) synthesis was no more sensitive to cordycepin than viral RNA synthesis (Table 7).

The fact that cordycepin can inhibit viral RNA synthesis is not without precedent. Nevins and Joklik (72) have shown that cordycepin inhibits

vaccinia mRNA synthesis apparently by inhibiting mRNA transcription, and does not preferentially inhibit poly(A) synthesis. They find that mRNA synthesized in the presence of cordycepin still contains poly(A) (72). However, vaccinia virus RNA is transcribed from a DNA template and poly(A) is synthesized by an enzyme distinct from the viral transcriptase (66), while picornavirus RNA transcription is from a RNA template (52). Therefore, it may be concluded that cordycepin is able to inhibit transcription of RNA from either DNA or RNA templates.

Since cordycepin has been shown to be phosphorylated in a variety of mammalian cells to cordycepin triphosphate (3'dATP) (47,56) it was decided to use 3'dATP in an *in vitro* viral RNA polymerase system to determine the mechanism by which cordycepin inhibits viral RNA synthesis *in vivo*.

When [^{3}H]ATP was used as the labeled precursor in the presence of the other three nucleoside triphosphates 3'dATP appeared to cause a slight inhibition of *in vitro* specific [^{3}H]AMP incorporation. However, the mock infected cell enzyme preparation also showed an activity that was capable of incorporating [^{3}H]ATP into acid-precipitable material (Table 8). This activity was stimulated by 3'dATP and apparently masks 3'dATP inhibition of viral specific [^{3}H]AMP incorporation in the presence of all four nucleoside triphosphates. If one substracts the [^{3}H]AMP incorporation in the presence of 3'dATP by the mock infected cell preparation from [^{3}H]AMP incorporation by the viral complexes in the presence of 3'dATP then it appears that inhibition of viral-specific RNA transcription is virtually complete (Table 8).

[^{3}H]AMP incorporation in the absence of the other three nucleoside triphosphates by both the viral polymerase complexes and the mock

infected cell preparation was essentially equivalent (Table 8). Under these conditions there was no detectable viral specific poly(A) polymerase activity capable of incorporating ATP in the absence of the other three nucleoside triphosphates. [^{3}H[ATP incorporation in all three preparations was stimulated to the same extent by the addition of 3'dATP in the absence or presence of the other three nucleoside triphosphates.

Cellular ATP incorporating activity associated with the crude membrane complex of HeLa cells has also been observed by others (22,98). Dorsh-Häsler et al (22) reported that the activity is stimulated by Mn^{++} and an oligo (dT) primer. The activity reported here is also stimulated by Mn^{++} (data not shown). Dorsh-Häsler et al have also shown that the activity can be separated from poliovirus polymerase by solubilization of the cellular membranes with detergents (22). This activity is similar to the terminal adenylate transferase activity reported by Diez and Brawerman (21) in Chinese hamster and mouse sarcoma 180 cells. In addition Spector and Baltimore (98) have shown that this cellular terminal adenylate transferase activity is capable of adding short poly(A) sequences, 15-25 nucleotides long, to pre-existing viral and cytoplasmic RNA.

This is the first indication, however, that this cellular ATP incorporating activity is actually stimulated by 3'dATP in the presence of Mg^{++}. The mechanism of this stimulation remains to be understood. It is conceivable though that 3'dATP might inhibit a nuclease activity which normally removes adenosine residues from poly(A) (13,26). If so, the observed stimulation may reflect a stabilization of the in vitro product by 3'dATP.

The results presented in this study show that 3'dATP inhibits [^{3}H]GTP incorporation as well as [^{3}H]ATP incorporation by the viral polymerases as would be expected if 3'dATP inhibits picornavirus RNA transcription. These results also rule out the possibility that the mechanism of cordycepin inhibition of picornavirus RNA synthesis was a secondary effect due to the inhibition of protein synthesis.

The mechanism by which cordycepin inhibits poly(A) synthesis or RNA synthesis is unclear. Though not well established by kinetic studies, competition between 3'dATP and ATP is strongly suggested from published data (70,80,93). From the structure of 3'dATP one would assume that RNA chain termination would result from incorporation of 3'dAMP in place of AMP and subsequent failure to provide a 3'-hydroxyl group for the next incoming nucleotide thereby preventing further chain elongation. There is some evidence to support this theory. Shigeura and Gordon (93) have studied the effect of 3'dATP on the DNA-dependent RNA polymerase from Micrococcus lysodeikticus. They found that 3'dATP inhibited RNA transcription from a DNA template and poly(A) transcription from a poly(U) template but did not inhibit the transcription of poly(U) from a poly(A) template indicating that 3'dATP specifically interferes with AMP incorporation (93). Shigeura and Boxer (92) have shown that ^{14}C-3'dATP was incorporated into RNA by M. lysodeikticus RNA polymerase, subsequent alkaline hydrolysis of the RNA showed that all radioactivity was in the nucleoside fraction and none in the nucleotide fraction, strongly suggesting that 3'dAMP was incorporated into the terminal position of the RNA.

This is just the opposite of the situation observed by Mendecki et al (63). They have shown that in mouse sarcoma 180 cells, RNA chains that have been terminated by cordycepin contain as the 3' end residue AMP rather than 3'dAMP (63). They have postulated that 3'dATP inhibits poly(A) synthesis by inhibiting initiation rather than inhibiting elongation. In addition, Maale et al (56) have shown that 3'dATP is a relatively poor competitive inhibitor of in vitro ATP polymerization by poly(A) polymerases of HeLa cells and maize, and is no more effective than 2'dATP, the normal precursor for DNA synthesis, in inhibiting RNA transcription.

Experiments utilizing poliovirus crude polymerase complexes indicate that 3'dATP inhibition of picornaviral RNA synthesis in vitro is reversible when ATP is added along with the inhibitor; however, GTP has no effect on 3'dATP inhibition (Figures 8 and 9). Therefore, 3'dATP appears to be a specific competitive inhibitor of ATP incorporation. Since 3'dATP inhibits [^{3}H]GMP incorporation as well as [^{3}H]AMP incorporation into RNA, it appears that the drug acts to terminate chain elongation. Consistent with this proposal is the observation that poliovirus 35S SS RNA is the most severely inhibited of the various viral RNA species labeled in vitro (Figure 11). Inhibition of RNA chain initiation by 3'dATP as an alternative mechanism for its action (63) can be ruled out since it has been shown that picornavirus polymerases do not initiate RNA synthesis in vitro but merely elongate RNA chains previously initiated in vivo (34).

It is not possible from the results presented in this study to determine whether or not 3'dAMP is incorporated into the RNA chains. The observation that the inhibition could not be reversed with excess ATP

10 min after addition of 3'dATP is consistent with incorporation of 3'dAMP into RNA as well as with irreversible binding of 3'dATP to the viral polymerase. Attempts to distinguish between these two mechanisms by pre-incubating the viral polymerase complex with 3'dATP in the absence of RNA synthesis and then allowing RNA synthesis to proceed in a large excess of ATP were unsuccessful. So it remains to be determined if 3'dAMP is in fact incorporated into the growing viral RNA chain.

C. Mechanism of Polyadenylation of HRV-14 RNA:

The results presented in this study indicate that HRV-14 RF and RI RNAs contain poly(U) sequences associated with the complimentary minus-strand RNA. The presence of a poly(U) sequence in HRV-14 intermediate RNA species along with the observation that all three HRV-14 intermediate RNA species synthesized in vivo contain poly(A) sequences is consistent with a transcriptional mechanism of HRV-14 polyadenylation but do not rule out alternative mechanisms. Poly(A) may therefore be transcribed from a complimentary poly(U) sequence present on the minus-strand of the RI molecule by the viral RNA polymerase.

This mechanism of polyadenylation is further supported by the observation that HRV-14 RF and RI RNAs synthesized in vitro contain poly(A) sequences, indicating that the crude viral polymerase complex is capable of synthesizing poly(A) linked to viral RNA. The observation that there was no detectable viral-specific poly(A) polymerase activity associated with the crude viral polymerase complex is also consistent with HRV-14 poly(A) being transcribed in the replication complex by the viral polymerase.

These results are in accord with what has been reported for poliovirus. Evidence from both *in vivo* and *in vitro* experiments strongly suggests that the poly(A) of poliovirus plus-strand RNA is directly transcribed from poly(U) sequences located at the 5' end of minus-strand RNA present in the poliovirus RI RNA (22,97,98,115,116). Sawicki and Gomatos (83) also have reported the presence of a poly(U) sequence attached to the minus-strand RNA of Semliki Forest virus, an alphavirus whose genome RNA also serves as mRNA in the host cell.

The presence of a poly(U) sequence in HRV-14 minus-strand RNA is in direct opposition to what Macnaughton and Dimmock (58) have reported for HRV-2 RF and RI RNAs. They also used poly(A)-Sepharose chromatography to detect poly(U) sequences. However, they did not denature the RF and RI RNAs prior to chromatography and for that reason probably failed to detect poly(U) because of base pairing to the poly(A) of the plus-strand RNA. The reason why undenatured rhinovirus RF or RI RNAs hybridizes to poly(U)-Sepharose columns is not known but may be due to one or both of the following possibilities. First, it is known that poly(A) is capable of forming a triple helical structure with two strands of poly(U); the reverse, however, does not occur, *i.e.* a triple helix is not formed with two poly(A) strands and one poly(U) strand (106). Secondly, the poly(A) sequences of both poliovirus and Semliki Forest virus are longer than the complimentary poly(U) sequence (83,97,99,115). The same may hold true for rhinoviruses; therefore, the free poly(A) region might be available to bind to poly(U)-Sepharose, whereas there would be no free poly(U) sequences to bind to poly(A)-Sepharose. This may indeed be the case since in this study denaturation of viral RNA in the presence of excess

cold poly(A) completely prevents any HRV-14 RF + RI RNA from binding to poly(A)-Sepharose.

The results of this study also contradict two other observations concerning HRV-2 RNAs. Macnaughton et al (57) have reported that synthesis of HRV-2 double-stranded RF RNA in HeLa cells requires the presence of act-D. The results presented here (Figure 12) however, show that HRV-14 RF RNA is synthesized either in the presence or absence of act-D. The second difference between HRV-2 and HRV-14 concerns the extent of polyadenylation in the intermediate RNA species of the two rhinoviruses. Similar to HRV-14 virion RNA, about 80% of HRV-14 SS and RI RNAs (Figure 15), and about 50% of HRV-14 RF RNA bind to poly(U)-Sepharose. This is in contrast to what Macnaughton and Dimmock (58) have reported for HRV-2 intermediate RNAs isolated from infected HeLa cells. THey find that only about 10% of SS RNA and about 30% RF and RI RNAs were capable of binding to oligo (dT) cellulose (59). The apparent differences in the proportion of intermediate RNAs of the two viruses that are polyadenylated are not due to a difference in the capability of oligo (dT) cellulose to bind viral specific RNAs, since approximately 70% of HRV-2 virion RNA was bound to the oligo (dT) cellulose and about 70% of HRV-2 RF and RI RNAs synthesized in human embryonic lung cells was bound to oligo (dT) cellulose (59).

There is no simple explanation for these differences. They may reflect a difference in the strains of HeLa cells used or a fundamental difference between the two rhinovirus serotypes. Unlike the three different serotypes of poliovirus which have considerable homology in their genomes (30-50%) (118), the rhinoviruses have little or no RNA

homology (113); in addition, various rhinoviruses also differ considerably in their buoyant densities in cesium chloride (82). These observations raise the possibility of divergent origins of the rhinoviruses, despite many common physiochemical and biological properties (52). Therefore, it is possible that HRV-14 and HRV-2 may differ slightly in their mode of RNA replication which is reflected in the difference in the mode and extent of polyadenylation of the various intermediate RNAs of the two viruses.

Even though poly(U) is present in HRV-14 RI RNA two related observations concerning the polyadenylation of HRV-14 are difficult to reconcile with a transcriptive mode of polyadenylation. The original observation of Nair and Owens (70) showing that the proportion of [^{3}H]AMP residues in the poly(A) portion of rhinovirus RNA was 7.4% when the infected culture was labeled with [^{3}H]AR from 0 h p.i. but only 4.6% when labeled from 6 h p.i. (when this experiment was repeated during the course of this investigation, values of 8.6 and 4.1% were obtained for the RNase-resistant fraction of [^{3}H]AMP residues in rhinovirus RNA, corresponding to the two intervals of labeling) was interpreted to suggest possible ligation of preformed poly(A) to rhinovirus RNA (70). These results might be explained by the alternate assumption that HRV-14 RNA synthesized early have much larger poly(A) sequences than viral RNA synthesized later in infection. Since only 10-15% of viral RNA is synthesized within the first 6 h of infection (32,87) the poly(A) sequences synthesized during this time would have to be greater than 1000 nucleotides long to account for the observed doubling of label in the poly(A) portion of viral RNA. From the polyacrylamide gel

electrophoresis profiles of HRV-14 poly(A) sequences determined during the course of this investigation it is apparent that a substantial fraction of the poly(A) sequences are larger than 90 nucleotides in length but it is not possible to determine the exact size of the largest sequences (Figure 1). It is also clear that the poly(A) sequences are very heterogeneous in length. These observations are not compatible with strict transcriptional mode of polyadenylation.

The large heterogeneity of HRV-14 poly(A) sizes might be explained however, by a combination of transcriptional and post-transcriptional poly(A) sythesis. Poly(A) of a length equal to that of the poly(U) sequence might be transcribed by the viral polymerase. Additional adenylic acid residues might then be added on by terminal addition by a cellular or viral terminal riboadenylate transferase. Evidence presented in this study and by others (22,98) has shown that such an activity does exist in HeLa cells and that it is capable of adding 15-25 nucleotides to the end of pre-existing poly(A) sequences (98). Another mechanism to explain the heterogeneity of poly(A) sizes which has been postulated for poliovirus (22,98) involves slippage of the poly(A) over poly(U) sequences on the minus-strand by a mechanism similar to that reported for the Escherichia coli DNA-dependent RNA polymerase system (16).

Evidence presented in this study and the results of studies on poliovirus (22,98) and Semliki Forest virus (83) seem to indicate that the presence of poly(U) sequences and therefore the possibility of a transcriptive mechanism of polyadenylation may be a factor common to all plus-strand RNA viruses, that is, picornaviruses and togaviruses. It is not possible, however, to rule out alternative mechanisms of polyadenylation

and indeed there may be a significant amount of post-transcriptional polyadenylation of picornaviral RNAs by cellular terminal riboadenylate transferases.

SUMMARY

The results obtained during the course of this study have confirmed and expanded upon the original observation of Nair and Owens that rhinovirus type 14 RNA contains a poly(A) sequence. The poly(A) sequence is located at the 3' end of the RNA molecule as determined by enzymatic removal with polynucleotide phosphorylase. Polyacrylamide gel electrophoresis of poly(A) sequences isolated from viral RNA indicate they are heterogeneous in length and consist on the average of approximately 160 nucleotides. The majority of rhinovirus RNA molecules are polyadenylated as estimated by binding to poly(U)-Sepharose.

Cordycepin, known to inhibit rhinovirus replication, was found to inhibit the replication of poliovirus type 2, contrary to reports by other investigators. Inhibition of viral replication is due to a specific inhibition of viral RNA synthesis as shown by experiments *in vivo* and *in vitro*. The inhibition of RNA synthesis is caused by specific competition between 3'dATP and ATP resulting in termination of RNA chain elongation. This may be due to either incorporation of 3'dAMP into the growing RNA chain and the resulting lack of a free 3' hydroxyl group needed for RNA chain growth or due to an irreversible binding of 3'dATP to the viral polymerase.

Rhinovirus specific intermediate RNA species isolated from infected cells or synthesized *in vitro* contain poly(A) sequences. No evidence could be obtained for a viral specific poly(A) polymerase associated with the crude viral polymerase complex, indicating that viral poly(A) synthesis might be a function of the viral polymerase itself.

Finally, this study has presented evidence that like poliovirus and Semliki Forest virus, rhinovirus intermediate RNA species apparently contain a poly(U) sequence associated with the complimentary minus RNA strand. This suggests that rhinoviruses may transcribe poly(A) from a complimentary poly(U) sequence.

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APPENDIX

Replication of Picornavirus RNA:

Picornaviruses are small single-stranded RNA viruses whose genome RNA also serves as mRNA and according to convention is designated as the plus strand (8). The first step in the replication of picornavirus RNA occurs after the RNA has been uncoated in the cytoplasm of the host cell. The released viral mRNA is then translated by the host cell protein synthesizing machinery into a single large protein which is subsequently cleaved into the various smaller viral proteins of which one or more serve as viral polymerase(s). The polymerase(s) then utilize the parental RNA molecule as a template for the synthesis of complimentary or minus strand RNA. The mechanism by which the parental RNA serves as both mRNA and template RNA is unknown at this time. The negative strand RNA then serves as a template for the synthesis of additional plus strand RNA and the process continues (52).

It is believed that synthesis of viral RNA occurs in "Replication Complexes", which are complexes of viral RNA and viral polymerase(s) associated with various proteins and smooth cytoplasmic membranes (34). In these complexes exists the viral replicative intermediate (RI) which consist of a complete plus or minus strand RNA on which viral polymerase molecules transcribe several complimentary RNA strands simultaneously. The majority of these RIs consist of a complete minus strand from which plus strand genome RNA is synthesized (positive RI). However, it has been estimated that between 5 and 10% of the RI molecules contain a complete plus strand RNA as template from which minus strand RNA is

transcribed (negative RI) (9). It is currently believed that picornavirus RNA replication occurs by a semiconservative mechanism, i.e. as new plus strands are transcribed against a conserved minus strand they displace existing plus strands which are only hydrogen bonded to the template by a few bases.

From picornavirus infected cells it is possible to extract three viral-specific RNA species: single-strand (SS) RNA, double-strand RNA known as replicative form (RF), and partially single-strand partially double-stranded replicative intermediate (RI) RNA which appears to be a reflection of the native intracellular component of the replication complex (52).

If one follows the fate of all three of these RNA species through the course of a typical picornavirus infection cycle, RF accumulates at a slow but constant rate through the entire cycle. RI is the most rapidly labeled RNA; however, after reaching a plateau quickly its synthesis levels off. SS RNA appears after RI and RF but accumulates most rapidly during most of the infection cycle and only declines towards the end (32,73,87). The kinetics of synthesis indicate that RI is the precursor to production of SS RNA, and that RF might be a by-product and not necessarily a relevant replicative RNA (34, 73).

It is possible to prepare crude picornavirus replication complexes from infected cell cytoplasm. These complexes appear to carry out reactions which are similar to what occurs in vivo. The three viral RNA species are labeled in vitro and the sequence of labeling appears to be the same as in vivo (34,112);however, the polymerase complexes can only elongate previously initiated nascent RNA chains (20,34,49). It

was through the use of these crude polymerase complexes that the precursor-product relationship of RI and SS RNAs was confirmed (34, 62).