

RNA POLYMERASE INDUCED IN L CELLS
INFECTED WITH VS VIRUS

STUDIES ON A RNA POLYMERASE INDUCED IN L CELLS
INFECTED WITH VESICULAR STOMATITIS VIRUS

by

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SCOPE AND CONTENTS:

An RNA dependent RNA polymerase induced in L-cells infected with VSV was studied. The in vitro properties of this enzyme and the nature of its synthesized products were examined. The products were characterized in terms of size, poly A content, and homology to both the infectious and defective particles of VSV. Analysis of this enzymatic activity during interference indicated a direct correlation between the in vitro activity and the in vivo interference.

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ABBREVIATIONS

Act D	=	actinomycin D
AMP	=	adenosine monophosphate
ATP	=	adenosine triphosphate
CMP	=	cytidine monophosphate
CTP	=	cytidine triphosphate
cpm	=	counts per minute
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
DOC	=	deoxycholate
EDTA	=	ethylenediaminetetra-acetic acid
G	=	guanosine
GMP	=	guanosine monophosphate
GTP	=	guanosine triphosphate
HR-LT	=	heat resistant strain of the Indiana serotype of VSV, derived from Ind-ST and producing LT particles
Ind-ST	=	Indiana serotype of VSV which produced ST particles
LT	=	long defective particles of Indiana VSV
MEM	=	Joklik modified minimal essential medium
MEMM	=	Eagle's minimum essential monolayer medium
moi	=	multiplicity of infection
MgAc	=	magnesium acetate
NBCS	=	new born calf serum

NJ = New Jersey serotype of VSV
NTP = nucleoside triphosphates
PBS = phosphate buffered saline
PEP = phosphoenol pyruvate
PFU = plaque forming unit
Poly A = polyriboadenylic acid
Poly U = polyribouridylic acid
RNA = ribonucleic acid
RNase = ribonuclease
RNP = ribonucleoprotein
rpm = revolutions per minute
RSB° = reticulocyte standard buffer
S = sedimentation coefficient in Svedbergs
SDS = sodium dodecyl sulphate
ST = short defective T particles of Indiana VSV
TCA = trichloroacetic acid
tris = tris (hydroxymethyl) amino-methane
U = units
UMP = uridine monophosphate
UTP = uridine triphosphate
UV = ultraviolet light
VSV = vesicular stomatitis virus

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INTRODUCTION

General Introduction

The discovery of TMV by Iwanowsky (1892), of Rous Sarcoma virus (Ellermann and Bang, 1908; Rous, 1911), of foot and mouth disease virus (Loeffler and Frosch, 1898) and bacteriophage by Twort and d'Hérelle (1917) laid down the foundation for molecular virology. From d'Hérelle's description of the bacteriophage in 1925 as "living being, an infravisible parasite of bacteria" remarkable advances had been made in molecular biology. A major contribution to our present ideas on the transmission of genetic information and on virus replication has stemmed from the work done with the bacteriophages.

All viruses have unique common properties which clearly distinguishes them from other forms of life. As defined by Lwoff (1957) viruses contain only one type of nucleic acid DNA or RNA, they multiply as viral nucleic acid and do not possess enzymes of energy metabolism making them parasites. Recently, however, it was found that T5, a DNA containing virus, contains some RNA (Rosenkranz, 1973). Also Rous Sarcoma virus, a RNA tumor virus, contains small amounts of DNA (Levinson et al., 1972). In view of these

discoveries alteration of the classical definition of viruses might be required. In addition to nucleic acids, all viruses also contain proteins. The more complex virions may also possess lipids, polysaccharides and several other minor components.

To date, about 600 animal viruses are known containing from five to several hundred genes and replicating and maturing at different sites within the cell. Among these are DNA viruses which contain either single or double-stranded DNA which replicates and functions like cellular DNA. Other viruses contain single or double-stranded RNA as their genetic material. These RNA viruses provide unique systems for examination of gene expression and protein synthesis.

The study of the replication of mammalian viruses provides important systems for elucidating the synthesis, function and regulation of macromolecules in mammalian cells. Also since many of these viruses are pathogens their study is of medical importance.

RNA Viruses

In the replication of RNA viruses the genetic material-RNA must function both to direct protein synthesis and to generate additional copies identical to virion RNA. The best studied system is that used by the RNA

bacteriophage and the picornaviruses of mammals. In the course of this thesis the nomenclature proposed by Baltimore (1971) will be used when referring to RNA species. Accordingly, the RNA molecules with messenger function are termed positive and their complementary strands are termed negative.

From in vitro studies of the replication of the RNA of the bacteriophage, Q β , by Weismann et al. (1968), August et al. (1968) and Spiegelman et al. (1968) the following mechanism of replication has been formulated. After invading its host, the RNA of Q β phage serves first as messenger for virus specific protein synthesis and subsequently as template for its own replication. One of the synthesized proteins, the RNA polymerase (replicase) attaches to the parental RNA and synthesizes a strand complementary to it. This negative strand in turn acts as template for the synthesis of positive strands or infectious virion RNA. Completely or partially double-stranded RNA intermediates consisting of viral RNA and its complement called replicative form and replicative intermediates have been implicated in this replication. Like the RNA bacteriophage the replication of poliovirus (a picornavirus) RNA involves the formation of double-stranded and replicative intermediate RNA (Baltimore, 1969). Polio viral RNA appears to have a single ribosomal initiation site and hence the entire genome is translated into a single large

polypeptide which is later cleaved into smaller functional proteins (Jacobson and Baltimore, 1968).

Since viral RNA and mRNA, in these two systems, are indistinguishable, transcription and replication are the same process for these viruses.

In the more complex enveloped viruses (myxoviruses, paramyxoviruses, RNA tumor viruses, and rhabdoviruses) the process of transcription and replication are separate events. The virion RNA is non infectious and transcription of the viral RNA into a complementary positive strand which serves as messenger RNA is initiated in the infected cell by a virion associated polymerase, a transcriptase. Enzyme molecules of this type have been identified in the ribonucleoprotein core of Influenza (myxovirus), Newcastle disease virus (NDV) and Sendai (paramyxoviruses) Vesicular Stomatitis virus (VSV) (rhabdovirus) (Chow and Simpson, 1971; Bishop et al., 1971; Huang et al., 1971; Hutchinson and Mahy, 1972; Baltimore et al., 1970) and their utilization in in vitro studies has shed some light on the process of transcription in these viruses.

Although partially and completely double-stranded RNA intermediates have been identified in cells infected with influenza (Nayak, 1969), NDV and Sendai (Bratt and Robinson, 1967), Sindbis (Sreevalsan and Allen, 1968) and VSV, as well as positive and negative strands, very little is

known about the replication step of these viruses. Part of the problem has been the complexity of these viruses and the inability to establish satisfactory in vitro systems. The complexity of these systems is attributed to some of the characteristics of each group of viruses. The genome of influenza virus is discontinuous and involves at least five separate segments (Duesberg, 1968; Pons and Hirst, 1968). NDV and Sendai virions contain both negative and positive strands (Robinson, 1970). The enveloped arbovirus, Sindbis, like poliovirus contains an RNA genome which is infectious, yet like the more complex enveloped viruses, RNA strands complementary to the viral genome are found on polyribosomes of Sindbis infected cells. The RNA tumor viruses transcribe their information into DNA, which may then be integrated into the host cell DNA and provide a template for the synthesis of mRNA and a template for synthesis of viral RNA (Temin, 1971). The RNA dependent DNA polymerase carried by the virion is responsible for the copying of the viral genome into DNA.

The replicative cycle of VSV in vitro and in vivo has been studied in our laboratory and by many other workers. This has been facilitated by the ability of this virus to grow to high titers in almost any cell line and by its short growth cycle.

Vesicular Stomatitis Virus (VSV)

Vesicular stomatitis virus, a member of the rhabdovirus group infects many species including man (Howatson, 1970; Tesh et al., 1972). Although the clinical symptoms in cattle and horses resemble those of foot and mouth disease, affected animals recover completely.

The two best studied strains of VSV are the serologically distinct Indiana and New Jersey serotypes. Other members of the group, the Cocal (Jonkers et al., 1964), Brazil (Andrade et al., 1964, cited by Cartwright and Brown, 1972) and Argentina (Garcia Pirazzi et al., 1963) viruses were also described. Complement fixation and neutralization test showed the Cocal and Argentina strains to be closely related to each other, and all three strains were weakly related to Indiana (Federer et al., 1967). On this basis it was proposed that Cocal, Argentina and Brazil viruses should be classified as subtypes of Indiana serotype (Federer et al., 1967). Two other morphologically similar viruses Piry (Murphy and Shope, 1971) and Chandipura (Bhatt and Rodrigues, 1967) were found immunologically related to each other and to Indiana strain by Murphy and Shope (1971). However neither Cartwright and Brown (1972) nor Crick and Brown (1973) could demonstrate any relationship between these two viruses and the VSV group.

Structure and Composition of VSV

Examination of VSV with electron microscope using negative staining and thin sections of infected cells (Howatson and Whitmore, 1962; Simpson and Hauser, 1966; McCombs et al., 1966; Bradish and Kirkham, 1966; Nakai and Howatson, 1968) have shown the virus to be bullet shaped 170 nm long and 60-70 nm in diameter (Fig. 1). The infectious particle, referred to as B particle, consists of an internal helix of nucleoprotein, which appears in electron micrographs as transverse striations, surrounded by an envelope with projecting spikes on its outer surface (Fig. 2). Several different models of the internal structure of the virion have been proposed (Simpson and Hauser, 1966; Klimenko et al., 1966; Bradish and Kirkham, 1966; Bergold and Munz, 1967; Nakai and Howatson, 1968). The last authors favour the model in which the nucleoprotein is in a form of a single helix of about 30 coils with fewer coils of diminishing diameter at the round end of the virus. About 1000 brick like subunits are attached to the helix which consists of a single stranded molecule of RNA.

The first evidence that VSV is a RNA containing virus was obtained by Prevec and Whitmore (1963). Huang and Wagner (1966) determined that virion RNA has sedimentation coefficient of 42S, and using the relationship of Spirin (1963) which relates the sedimentation coefficient to the

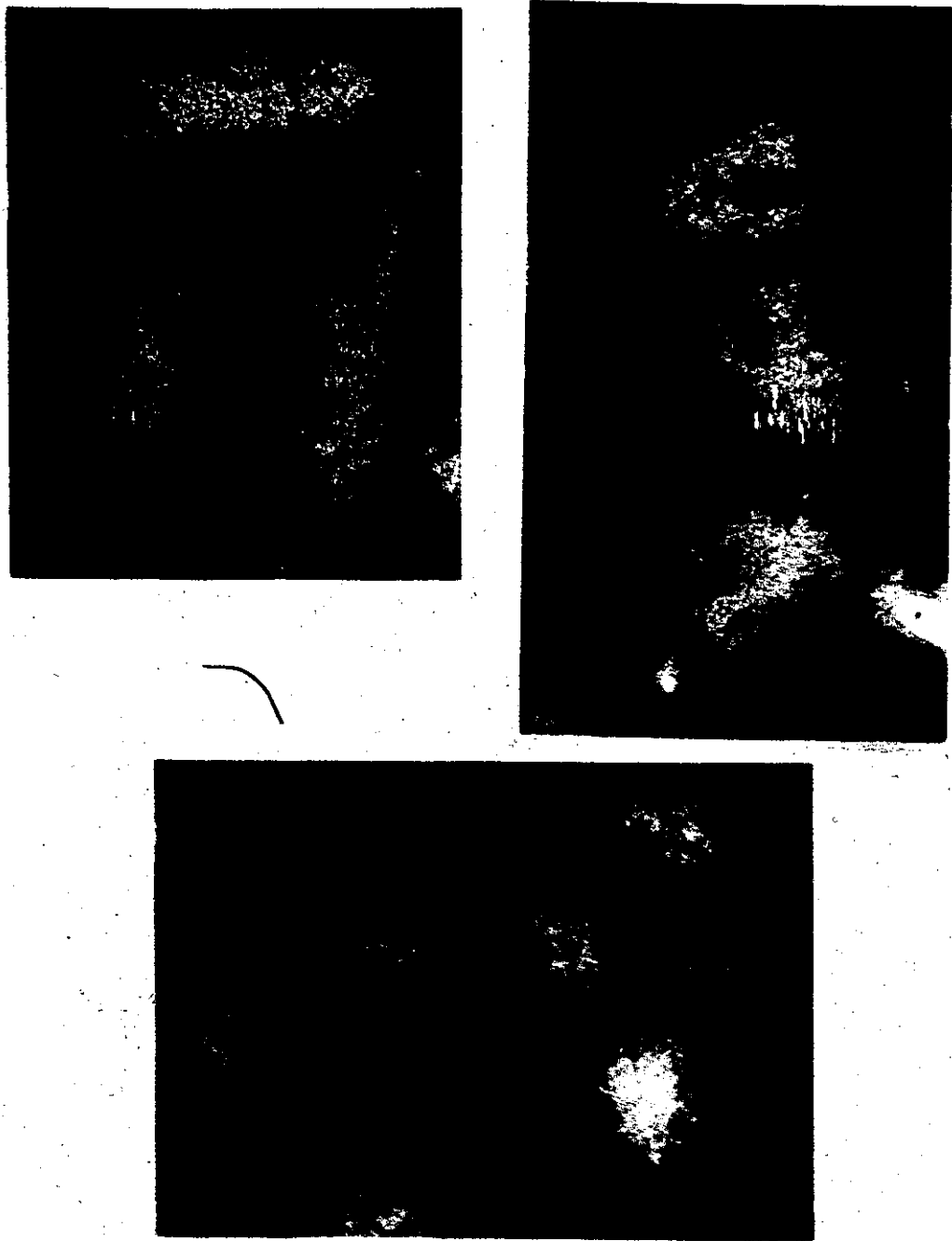


FIGURE 1. Electron Micrographs of VSV Infectious B particle (a), defective Lr particle (b), and defective ST particle (c), stained with 1% phosphotungstic acid. Magnification approximately X250,000.

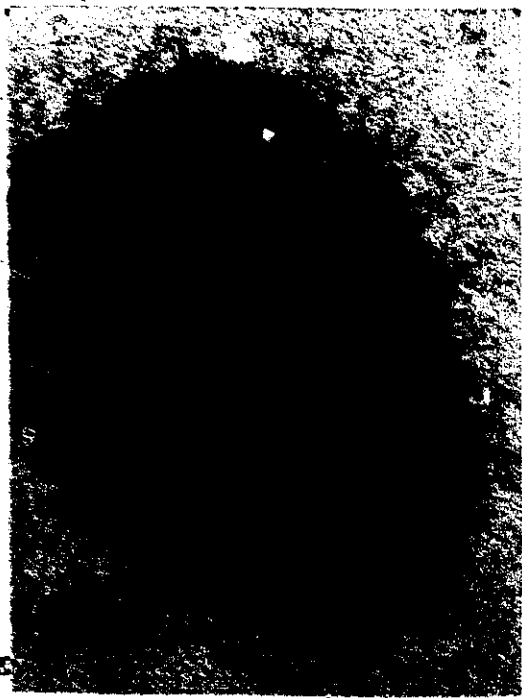


FIGURE 2. The internal structure of B particle of VSV.
Magnification approximately X340,000.

molecular weight, estimated the corresponding molecular weight to be 4×10^6 . Brown et al. (1967a) found that virus RNA which was shown to be single stranded by RNase digestion, had a sedimentation coefficient of 36-40S with corresponding molecular weight of 3×10^6 . Although different sedimentation coefficients for RNA from the infectious B particle are obtained by different workers, most of the values range between 38-42S. An exception is the RNA of sedimentation coefficient of 51S obtained from a long mutant of New Jersey serotype (Schaffer et al., 1972).

The molecular weight of Indiana VSV RNA was also determined from measurements of the lengths of nucleoprotein strands derived from disintegrated VSV particles (Nakai and Howatson, 1968). Using these figures, molecular weight of 3.5×10^6 was calculated for the RNA of B particle.

Chemical analysis have shown that VS virions contain 38% RNA, 64% protein, 13% carbohydrate, and 20% lipids (McSharry and Wagner, 1971a).

Polyacrylamide gel electrophoresis has resolved five proteins which are present both in the virion and synthesized in VSV infected cells (Kang and Prevec, 1969; Mudd and Summers, 1970; Wagner et al., 1970). These proteins have been designated as the M, NS, N, G and L proteins with estimated molecular weights of 29,000, 45,000, 50,000, 69,000 and 190,000 respectively. (Wagner et al., 1972).

The M and G proteins are components of the viral envelope. The G protein is a glycoprotein which contains carbohydrate chains (McSharry and Wagner, 1971b) and appears to comprise the viral spikes (Cartwright et al., 1970). This glycoprotein is also the major antigenic determinant of the virus, and its removal results in loss of infectivity (Cartwright et al., 1970; Kelley et al., 1972). The N protein is the structural nucleoprotein bound tightly to virion RNA (Kang and Prevec, 1969; Wagner et al., 1969). The function of NS and L proteins is unknown, but one or both of them have been implicated as components of the virion associated transcriptase (Emerson and Wagner, 1972).

The lipid bilayer which constitutes the viral envelope as well as the carbohydrate attached to viral glycoprotein and glycolipid appear to be derived from, or specified by, the host cell (McSharry and Wagner, 1971a; Burge and Huang, 1970).

Effect on Host Cell

Infection with VSV causes morphological alterations in the cell. The cytopathic effect (CPE) consists of rounding up of the cells with pseudopodal budding of the cell membranes of fibroblastic cells and cytoplasmic vacuolation in epithelial cells (David-West and Osunkoya, 1971). It was further speculated by the same authors on the basis of

microscopic examination that VSV infection blocks the cell cycle in late telophase or the early stages of G₁ phase.

At the macromolecular level, VSV was reported to inhibit cellular RNA, DNA and protein synthesis. The rapid inhibition of host RNA synthesis occurs even if replication of the virus is prevented by prior UV irradiation (Huang and Wagner, 1965; Yaoi et al., 1970a). This result suggests that either a structural component of the virus or perhaps a double-stranded RNA species produced by the virion associated transcriptase may be responsible for this inhibition. This latter hypothesis is based on the observation that double-stranded replicative intermediates of poliovirus can inhibit protein synthesis (Ehrenfeld and Hung, 1971) and the observation by Clavell and Bratt (1971) that UV-irradiation does not prevent the synthesis of double-stranded transcriptive intermediates by Newcastle Disease Virus.

Studies on the effect of UV inactivated VSV on cellular DNA synthesis have shown a selective inhibition of the flow of G₁ cells into the S phase. However infection during the S phase had no effect on DNA synthesis and on subsequent cell division (Yaoi and Amano, 1970).

Defective Particles of VSV

Many animal virus preparations contain defective particles which interfere with the replication of their

related non-defective virus. These defective particles have been proposed to play a role in viral infections (Huang and Baltimore, 1970).

VSV system is particularly suited for studying the phenomenon of interference since the defective particles are readily separable from the infectious virions.

Several undiluted passages of VSV in cell cultures give rise to infectious B particles as well as to truncated, non-infectious T particles. One type of defective particle, or ST, produced by the Indiana strain was characterized by Huang et al. (1966) who found it to be 65 nm long (Fig. 1). Another type of defective particle, or LT, associated with the heat resistant (HR) strain of Indiana VSV, is significantly larger than the ST particle. These LT particles are 100 nm in length (Fig. 1) (Petric and Prevec, 1970). Defective particles of intermediate length (82 nm) were shown to be produced by the New Jersey serotype (Hackett, 1964). Reichmann et al. (1971) has shown that different size T particles are associated with some of the temperature sensitive mutants of Indiana VSV.

Although the T particles, except for the overall length, are identical to the B particles in terms of morphology, antigenicity, and protein constituents, they contain only part of the viral genome. LT and ST contain RNA of 1/2 and 1/3 the size of the B particles respectively. The deletions

in the RNA of LT and ST are specific and not random (Schincariol and Howatson, 1972; Hallett, 1972).

The T particles can only replicate in the presence of the infectious virus, and when present in sufficient quantities they interfere with the replication of the homologous helper B particles, causing a reduction in the yield of infectious particles (Huang et al., 1966; Crick et al., 1966; Petric and Prevec, 1970). This interference is not mediated by interferon (Huang and Wagner, 1966) but requires the presence of a functional T particle nucleic acid (Huang and Wagner, 1966; Sreevalsan, 1970). The interference is also specific. ST particles interfere equally well with the production of B particles by the Indiana, HR, Argentina, Brazil, and Cocal strains, but are ineffective against the New Jersey, Piry and Chandipura viruses (Crick and Brown, 1973). In contrast the LT particles are capable of interfering with both Indiana and New Jersey serotypes (Prevec and Kang, 1970).

Multiplication of VSV

a. Adsorption and Maturation

The adsorption of virions and their mode of entry into the host cell have been studied by electron microscopy by Heine and Schnaitman (1969) and Simpson et al. (1969). According to the first authors the flat end of the virion

attaches to the cell surface, the viral envelope and cell membrane then fuse and penetration occurs by release of the nucleoprotein into the cytoplasm. According to Simpson et al. (1969) and Howatson (1970) VSV virions enter the cell by a process similar to phagocytosis. The virion attaches to the cell surface membrane and is taken into the cytoplasm by invagination to form a vesicle where uncoating occurs.

Once inside the cell, uncoating of VSV occurs effectively within 30 min of infection, with the viral coats accumulating in the Golgi apparatus (Yaoi and Ogata, 1972). Both viral RNA synthesis as measured by autoradiography (Yaoi et al., 1970b) and viral protein synthesis, detected by fluorescent antibody staining occurs in the cytoplasm (Schincariol, 1971).

Ribonucleoprotein complexes containing both virus specific proteins and virus specific RNA have been identified in the cytoplasm of infected cells. Infection with B particles alone produces complexes having sedimentation coefficients of 160S and 140S (Kang, 1971). Complexes with sedimentation coefficients of 80S and 140S are found in cells producing ST particles (Wild, 1971), while cells producing LT particles contain complexes with sedimentation coefficients of 100S and 140S (Petric and Prevec, 1970; Schincariol and Howatson, 1970).

The process of extrusion of the newly synthesized ribonucleoprotein cores from the cell to form virions occurs by budding through a portion of the host plasma membrane which

has been modified by the insertion of the M and G polypeptides (Cohen et al., 1971; David, 1973; Howatson and Whitmore, 1962). Through this process, the virions acquire an envelope which also contains some cellular constituents (Cartwright and Pearce, 1968; Hecht and Summers, 1972).

b. Replication and Transcription in Infected Cell

Studies on the multiplication of VSV in a number of different cell lines have shown that a large number of viral specific RNA species are involved.

Schaeffer et al. (1968) observed that under conditions of interference, in addition to the RNA of B and ST particles, single-stranded molecules of 15S, 6S and 31S were present in the cytoplasm of infected cells. Most of these latter species were complementary to virion RNA.

Stampfer et al. (1969) characterized the RNA species in VSV infected Chinese hamster ovary cells into two groups. Group I included the RNA made when cells were producing mainly B particles, and consisted of single-stranded 40S, 28S and 13S RNA and partially double-stranded 23-35S RNA. Under conditions of interference by ST particles a shift from group I to group II occurred. Group II consisted of single-stranded 19S, 6S and partially double-stranded 19S and 15S and completely double-stranded 13S.

Huang et al. (1970) examined the RNA species associated

with the polyribosomes in infected cells producing only B particles. RNA species of sedimentation coefficients of 28S and 13S complementary in base sequence to virion RNA were associated with the VSV specific polyribosomes. Acrylamide gel analysis of the 13S RNA indicated that at least two different RNA species were present in this group. Mudd and Summers (1970b) confirmed the heterogeneity of this RNA class and in addition showed it to contain poly A sequences.

Detailed analysis involving the separation and characterization of viral RNA species present in cells infected with B and LT particles were done by Schincariol and Howatson, (1970, 1972). Three major size classes of RNA were found by these workers in the infected cell. The most abundant class of RNA sedimented between 10S and 20S and consisted of eight species found on the polysomes. All of these were complementary to B and LT particle RNA. Of the three most abundant species among these eight only one did not anneal to ST RNA. The second class of RNA sedimenting between 20-31S consisted of species sedimenting at 21S and 31S RNA as well as 26S RNA. This latter species corresponds to LT particle RNA and was shown to contain both positive and negative strands. The 31S RNA is associated with polyribosomes and is probably equivalent to the 28S RNA described by Huang et al. (1970). The third class of RNA consist solely of RNA of sedimentation coefficient equivalent to that of B

particle RNA. This class also contains positive and negative strains.

These authors also found double-stranded (RF) and partially double-stranded RNA species (RI) whose size after denaturation corresponded to B and LT particle RNA. Wild (1971) also examined the RI complexes produced in cells infected with B particles and found that after denaturation they yielded RNA which sedimented with S values characteristic of B particle RNA together with smaller species of RNA with sedimentation coefficients of 10-19S. As in the bacteriophage and picornavirus systems, the double-stranded species present in VSV infected cells may be intermediates in the replicative and transcriptive processes. Among the single-stranded RNA species found in the infected cell the role of the 13-20S and 30S RNA appear to be that of virus specific messengers. This function is suggested by the complementarity of these RNAs to virion RNA, their association with the polysomes in the infected cell, their size which is appropriate to code for the viral proteins, and the fact that they contain poly A sequences (Soria and Huang, 1973; Ehrenfeld and Summers, 1972).

c. Transcription in vitro

In 1970 Baltimore et al. identified an RNA dependent transcriptase which is present in the viral particles of VSV.

They showed that this enzyme functions as a transcriptase in vitro, synthesizing product complementary to virion RNA.

Bishop and Roy (1971a, 1971b) showed that the in vitro product has a size range of $2-10 \times 10^5$ molecular weight.

They showed further that synthesis occurs in association with virion RNA and that completed strands are subsequently liberated as free species. Multistrand complexes are produced during this process.

The transcriptive process catalyzed by this enzyme was shown to be repetitive, that is, multiple copies of the same sequence were copied from one template. By determining the extent to which the in vitro product was able to protect labelled virion RNA from nuclease digestion after hybridization Bishop and Roy (1971b) concluded that only 30% of the template sequences were transcribed at 37°C. In contrast the product produced at 28°C was complementary to over 90% of the virion genome. At this lower temperature, the transcriptive process was sequential since free product obtained early in reaction was not complementary to RNA from ST particles, whereas in late reactions free product was complementary to RNA from ST particles (Bishop, 1971).

The sequential transcription and the small size of the products synthesized in vitro led Roy and Bishop (1973) to look for characteristic initiation sequences. These workers found that there are multiple initiation sequences.

two of which were identified as pppApCpGp... and pppGpCp... The fact that labelled tetraphosphate was found at the 5' end suggested that direction of synthesis was in the 5' to 3' direction. The presence of more than one initiation site suggests that the VSV virion transcriptase is able to start RNA synthesis at more than one site along the template. While this is compatible with the finding of multiple RNA species in vivo (Huang et al., 1970; Mudd and Summers, 1970b) and with the other results observed in vitro the biological significance of the initiation sites observed remains to be determined.

In contrast to B particles, ST particles were shown not to contain significant enzyme activity (Roy and Bishop, 1972). Huang and Manders (1972) also found that no transcription occurred in the presence of cycloheximide in cells infected with ST particles alone. This result suggests that during the replication of ST, either the defective particle utilizes the enzyme of the helper B particle or that transcription from the ST particle genome is not required. From dissociation and reconstitution experiments of the soluble transcriptase, containing proteins L and NS, and the nucleocapsid template, containing RNA and N protein, from B and ST it appears that ST particles contain transcriptase which cannot utilize their own nucleoprotein as template but can transcribe the ribonucleoprotein derived from B

particles (Emerson and Wagner, 1972).

Although no complete dissociation of the transcriptase from its RNA template has been reported to date, the proteins involved in its activity have been identified, these are L, NS and N (Emerson and Wagner, 1972). Bishop and Roy (1972) suggested the presence of two other proteins A and B in the complex. None of these proteins was found, by the latter workers, to be released from the ribonucleoprotein complex during the process of transcription. Szylágyi and Uryvayev (1973) have also shown that the RNP core obtained after disruption of the virus particles with Triton and CsCl contain the active transcriptase and all the information required to initiate infection.

As indicated above, a great deal of information about the transcription process has been obtained from in vitro studies. It is not known whether this same enzyme alone, or perhaps in combination with other viral or cellular factors, might also be responsible for the replicative process whereby viral RNA strands (negative strands) are synthesized. No information from in vitro studies is yet available on the replication system of this virus.

Polyadenylate Sequences of RNA

In view of the growing amount of literature dealing with the polyadenylate (poly A) sequences of cellular and

viral RNA, and the fact that it could serve as a handle for deciphering the processing of mRNA and its translation, it is only appropriate to review the known information in a separate section.

Poly A segments are present in the mRNA and heterogenous nuclear RNA of eukaryotes, but have not been observed in ribosomal RNA or in t-RNA (Hadjirassilious and Brawerman, 1966; Edmonds et al., 1971; Lee et al., 1971; Darnell et al., 1971a; Seeber and Busch, 1971). Poly A has also been found in the mRNAs of vaccinia virus (Kates, 1970), adenovirus (Philipson et al., 1971), VSV (Ehrenfeld and Summers, 1972; Soria and Huang, 1973), Sindbis (Eaton et al., 1972), and as part of the virion RNA of polio and Eastern equine encephalitis virus (Armstrong et al., 1972), RNA tumor viruses (Gillespie et al., 1972), Sindbis virus (Johnston and Bose, 1972) and in Mengo virus (Miller and Plageman, 1972).

The polyadenylate regions appear to be covalently linked to the remainder of the molecule (Darnell et al., 1971b; Edmonds et al., 1971; Lee et al., 1971; Darnell et al., 1971a).

In the case of vaccinia virus mRNA (Kates, 1970; Sheldon et al., 1972) and polio virus RNA (Yoshiaki and Wimmer, 1972) the poly A is located at the 3'-OH end of the RNA. Most of these poly A tracts are of the order of 150-200 nucleotides long. Regions of this length have also

been reported for heterogenous nuclear RNA (HnRNA), mRNA (Darnell et al., 1971b), vaccinia virus mRNA (Kates, 1970) and RNA tumor viruses (Lai and Duseberg, 1972). Poly A segments less than 100 nucleotides long have been reported for polio, mengo and sindbis virions (Armstrong et al., 1972; Miller and Plageman, 1972; Johnston and Bose, 1972).

The mechanism by which poly A tracts are formed is not known. Edmonds and Abrahams have isolated from the nuclei of some cells an enzyme which can sequentially add adenosine residues to the 3'-OH terminus of RNA (1960). A similar enzyme has been reported in rat liver mitochondria (Jacob and Schindler, 1972) and in the cytoplasm of mouse ascites cells (Giron and Huppert, 1972a,b). Darnell et al. (1971b) and Philipson et al. (1971) indicated that poly A is added post transcriptionally to the nuclear RNA. While the poly A of vaccinia mRNA may be copied from poly dT stretches of vaccinia DNA (Kates, 1970) no poly dT regions could be found in adenovirus genome (Philipson et al., 1971). In the cases of RNA containing viruses whose genome specifies a poly A segment containing mRNA no poly U tracts could be detected in the corresponding viral RNA (Marshall and Gillespie, 1972).

The function of the terminal adenylate rich sequences on RNA molecules is unknown. It has been suggested that these sequences may play a role in processing and transport of mRNA

from the nucleus to the cytoplasm or may have a cytoplasmic function in translation of mRNA (Armstrong et al., 1972; Darnell et al., 1971b). The latter suggestion seems appealing in view of the findings of Sheiness and Darnell (1972) that the size of poly A of mRNA molecules in the cytoplasm of HeLa cells gets shorter with age. The findings that poly A in the 3' end of both mRNA and rRNA has been suggested by Molloy et al. (1972) and Sheldon et al. (1972) to indicate that the rRNA is processed such that the 3' fragment is conserved and destined to be mRNA.

A cytoplasmic function for poly A is anticipated since the RNA viruses which replicate in the cytoplasm contain such sequences. Poly A could perhaps serve to allow differentiation between viral RNAs with a messenger function from other viral RNAs.

Purpose of Study

As reviewed above, the physiology of VSV is more complex than the model systems presented for RNA bacteriophage and picornavirus. In addition this system readily produced defective interfering particles.

In order to gain a better understanding of the molecular events and regulation involved in the replication and interference of this virus, an in vitro study of a RNA polymerase induced in infected L cells was begun. An indication that such an enzyme existed in VSV infected cells came from the preliminary report of Wilson and Bader (1965). The present studies were initiated to characterize this enzymatic activity, analyze its products in relation to virion and messenger RNA, and to determine its correlation with the process of interference.

MATERIALS AND METHODS

MATERIALS

All chemicals used were of reagent grade.

Tetralithium salts of UTP, CTP, GTP and disodium salt of ATP were obtained from Schwarz/Mann; 2'(3')AMP, 2'(3')CMP, 2'(3')UMP, 2'(3')GMP, from General Biochemicals, Inc., poly U and poly A from P-L Biochemicals; pyruvate kinase and phosphoenolpyruvate from Boehringer, Mannheim Corp.; yeast RNA and enzyme grade sucrose from Mann Research Laboratories; β mercaptoethanol, acrylamide and NN-methylene bisacrylamide from Eastman Organic Chemicals; dithiothreitol (Cleland's reagent) from Calbiochem; cycloheximide from Upjohn Company; DNase I (bovine pancreatic), ribonuclease A and T₁ ribonuclease from Worthington Biochemical Corp.; Triton X-100 from Hartman-Leddon Corp.; sodium deoxycholate (DOC) from Sigma Chemicals; Actinomycin D and the non-ionic detergent BRIJ-58 [polyoxyethylene (20) cetyl ether] were gifts from Merck, Sharp and Dohme Co. of Montreal, and Atlas Chemicals Industries Inc. respectively.

[8-³H]ATP (18-20 Ci/mmole) and [³H]-Poly U (7 mCi/mmole) were obtained from Schwarz/Mann; [8-³H]GTP (5.6 Ci/mmole),

^{14}C (1.54 mCi/mg) or ^3H (25 mCi/mg) labelled amino acid mixtures and omnifluor from New England Nuclear Corp.; [α - ^{32}P]GTP (5.3 Ci/mmole) from International Chemicals and Nuclear Corp.

METHODS

1. Growth of Cells

The cell line used in all experiments is a subline, L-60, of Earle's mouse L-cells (Earle, 1943), obtained from Dr. A. F. Howatson, University of Toronto.

The cells were grown at 37°C, in suspension culture in Joklik modified minimal essential medium (MEM), supplemented with 5% newborn calf serum (NBCS) (both obtained from Grand Island Biological Co.) (Kang, 1971).

The cells were maintained in log phase growth by regular dilution with fresh medium.

2. Growth of Virus

A. Source

The vesicular stomatitis virus used was of Indiana serotype. Both strains of this virus, Ind-ST and HR-LT, were obtained from Dr. A. F. Howatson.

Ind-ST was identical to the virus used in previously reported work (Nakai and Howatson, 1968), and HR-LT was selected by Dr. Nakai from Ind-ST stock for its resistance to heat treatment (43°C, 3 hours). The surviving virus were regrown and subjected to the heat treatment several times.

Virus particles from a single plaque (see below) were used to grow the final stock.

Both the plaque purified Ind-ST and HR-LT stocks were maintained by successive low multiplicity of infection (moi) passes of the plaque purified virus, with occasional heat treatment for HR-LT virus. Stocks yielding defective particles ("long T" from HR-LT and "short T" from Ind-ST) were produced by four undiluted passes of the above plaque pick.

B. Plaque Assay and Plaque Purification

Infectivity of the virus stock was determined in terms of Plaque Forming Units per milliliter (PFU/ml). A volume of 0.1 ml of appropriately diluted virus was placed on a full L-cell monolayer, 5×10^6 cells, in Falcon petri dish (60 x 15 mm). Following an adsorption period of 45 minutes at 37°C in an atmosphere of 5% CO₂ in air and 100% humidity, the monolayer was overlaid with 5 ml of MEMM (Eagle's minimum essential medium, Grand Island Biological Co.) containing 5% NBS and 0.9% of agar (Difco, Noble). Plaques could be observed after 20-24 hours of incubation at 37°C in a water saturated 5% CO₂ atmosphere.

In order to obtain virus stocks relatively free of defective particles, 5 successive plaque purifications were done as described by Stampfer et al. (1971). A sterile pasteur

pipette was used to lift up the agar and cell sheet over a well isolated visible plaque. The agar plug was dispersed in growth medium, retitrated and replated for a second plaque pick. The fourth plaque pick was grown at a moi of 0.01 PFU/cell in suspension cell culture.

Following 6 hours incubation (one growth cycle) the lysate was passed at moi of 0.1 PFU/cell and constituted the "plaque purified" stock.

C. Growth of Virus Stock

Cells from a growing culture were collected by centrifugation at 400 g for 10 minutes, resuspended to 10^7 cells/ml in MEM containing 2% NBCS and infected at low moi of 0.01-0.1 PFU/cell. After an adsorption period of 30-45 minutes at 37°C, MEM plus 2% NBCS was added to the infected culture to yield a final cell concentration of 10^6 cells/ml. The culture was incubated for 16-18 hours, after which the cells were pelleted, by centrifugation as above, and the supernatant containing the virus was aliquoted and stored at -60°C.

For the preparation of uridine labelled virions, the same procedure as above was followed, except that at 30 minutes post resuspension 2 µg/ml of Actinomycin D (Act. D) was added followed by the addition of ^3H -uridine (2.5 µCi/ml) 2.5 hours later.

For the preparation of labelled viral proteins, cells infected at a moi of 100 were collected by centrifugation at 3½ hours post infection and resuspended in medium containing 1/20 the concentration of amino acids present in Joklik's modified minimal essential medium. ³H-amino acids to an activity of 2.0 µCi/ml were added and virions harvested some 10-14 hours later.

3. Purification of B and T. Virus Particles

L-cells were infected with the appropriate virus stock to yield predominately B, "long T", or "short T" particles as required. The culture was harvested 12-16 hours post infection, and cells removed by centrifugation at 13,000 g for 20 minutes at 4°C in Sorvall fixed angle rotor. The virus particles in the supernatant were pelleted by centrifugation at 34,000 g for 165 minutes in a Spinco Type 19 rotor, resuspended in phosphate buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄, Dulbecco and Vogt, 1954) and frozen at -60°C. When virus pellets from the infection of 1-2 x 10¹⁰ cells had been accumulated, purification on sucrose gradients was done.

Linear gradients of 5 to 30% sucrose in PBS were prepared in 37 ml volumes in cellulose nitrate tubes of the

Spinco SW 27 rotor. A volume of 1-1.5 ml of resuspended virus was layered on each gradient. In the case of ST particles, the virus suspension was treated with 20 $\mu\text{g/ml}$ of RNase A for 30 minutes at room temperature prior to layering on the gradients. After centrifugation at 81,000 g for 35 minutes at 4°C in a Spinco SW 27 rotor, the visible light-scattering band containing the virus particles was collected from the gradients with Pasteur pipette, and concentrated by centrifugation at 81,000 g for 1 hour. The final pellet was resuspended in 1 ml of PBS and a sample taken for interference assay and observation with the electron microscope.

A. Interference Assay

To L-cells monolayers (2×10^6 cells in a 35 x 10 mm Falcon plastic petri dishes) was added 2×10^6 PFU of plaque purified particles of HR-LT strain in a volume of 0.1 ml. A similar volume of appropriate dilutions of purified "long T" or "short T" particles was also added to each plate. After adsorption at 37°C for 30 minutes, 5 ml of MEM plus 5% NCS was added and plates incubated for 16-20 hours. The yield of PFU/ml in the lysate was determined by plaque assay and the interference titre of the defective particles determined from the reduction in infectious virus yield in its presence (Bellett and Cooper, 1959). An example of

such an interference assay for LT particles is illustrated in Fig. 3.

B. Electron Microscopy

A drop of the purified viral suspension was spotted on a parlodion/carbon coated grid of 100 x 400 mesh and allowed to dry. The grid was then stained with 1% phosphotungstic acid (pH 7) and excess stain blotted off with filter paper. The grids were observed and photographed on Philips EM 300 electron microscope. Micrographs were taken at a magnification of 51,000 X.

4. Extraction of Viral RNA

The procedure used for extraction of RNA from virions was a modification of the method of Penman (1966). The purified viral suspension was diluted with STE buffer (0.01 M Tris-HCl pH 7.2, 0.1 M NaCl, 0.001 M EDTA) and sodium dodecyl sulphate (SDS) was added to a concentration of 0.5%. The viral solution was mixed with equal volumes of water-saturated phenol and chloroform containing 1% isoamyl alcohol, and heated at 60°C for 2 minutes with occasional shaking. After cooling on ice, the emulsion was centrifuged at 23,000 g for 2 minutes, at 4°C, and the upper aqueous phase was re-extracted twice more with phenol, chloroform-

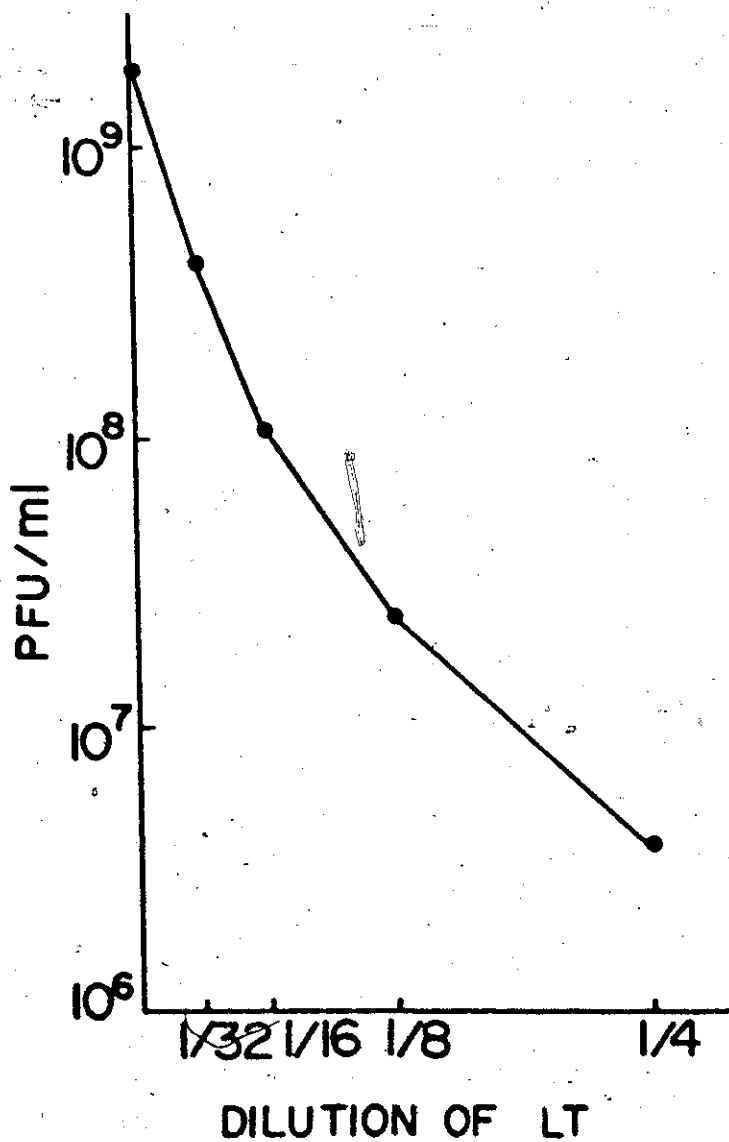


FIGURE 3. Interference Assay for LT Particles.

This graph shows the total infectious yield obtained from cells infected with a constant titre of infectious B particles and the stated dilution of interfering T particles. The details of procedure are presented in METHODS.

isoamyl solutions, followed by two extractions with ether. The ether layer was removed with a pasteur pipette and traces of ether were evaporated by blowing air over the sample.

The RNA was precipitated from the solution by storing overnight at -20°C , following the addition of two volumes of 95% ethanol and NaCl to a final concentration of 0.1 M. The precipitate was collected by centrifugation, 23,000 g, 10 minutes, 4°C , air dried and redissolved in 0.01 M Tris-HCl buffer, pH 7.5.

The concentration of RNA was determined from its absorbance at 260 nm, one optical density unit being equivalent to 40 $\mu\text{g/ml}$ of RNA (Scherrer, 1969).

With the labelled virions the radioactivity of the RNA was assayed by precipitation with trichloroacetic acid (TCA). To 0.01 ml of the labelled RNA, 0.5 ml of 10% TCA and 0.1 mg of yeast RNA, used as a carrier, were added. The resulting precipitate was collected on a 0.45 μ Millipore filter washed with 5 ml cold TCA and dried.

The filters were counted in a Beckman scintillation counter using omnifluor (4 g/liter of toluene) as the scintillation fluid.

5. Preparation of Cytoplasmic Extracts

About 2×10^9 cells from a growing culture were infected with virus at a moi of 20 PFU/cell as described

earlier. Fewer cells were mock infected to yield the uninfected controls.

After 5½ hours of incubation at 37°C, the cells were pelleted by centrifugation and washed three times by successive resuspension and centrifugation in cold PBS. The washed cell pellet was resuspended in 20 ml of 0.01 M Tris buffer and disrupted by 10 strokes of a glass Dounce homogenizer.

After removal of nuclei and cell debris by centrifugation for 10 minutes at 4°C and 6000 g, the supernatant was made 0.01 M in mercaptoethanol. Glycerol and Cleland's reagent were also added to a final concentration of 5% (V/V) and 0.002 M respectively. This constituted the crude enzyme fraction, and was aliquoted in 0.5 and 1 ml volumes and stored at -60°C. The frozen preparation was stable up to 6 months. A dialyzed extract was prepared using either frozen or freshly prepared crude enzyme fraction. Dialysis against 500-1000 volumes of buffer [0.01 M Tris-HCl pH 7.5, 5% (V/V) glycerol, 0.01 M mercaptoethanol, 0.002 M dithiothreitol] was carried out overnight at 4°C.

A. Determination of Protein

The protein concentration of the cytoplasmic extract was estimated using Warburg and Christian's (1941) formula. The absorbancy at 280 nm (A_{280}) and at 260 nm (A_{260}) was

determined and used in the following formula:

$$1.54 A_{280} - 0.76 A_{260} = \text{protein concentration in mg/ml.}$$

About 8-14 mg of protein per ml were obtained from a preparation of enzyme stock as described above. A variation of 10% was observed when protein concentration was estimated by the technique by Lowry et al. (1951).

6. Analysis of RNA Extracted from Reaction Mixtures

A. Alkaline Hydrolysis

RNA from 2.5 ml reaction mixtures was extracted by the SDS, hot phenol-chloroform, isoamyl alcohol method as described above. TCA to a final concentration of 5% was added to the final aqueous phase and the resulting precipitate was collected by centrifugation at 12,000 g for 10 minutes and washed twice with 5% TCA, once with 95% cold ethanol and once with ether. The pellet was air dried and dissolved in 0.3 ml of 0.3 M KOH and hydrolysed for 18 hours at 37°C. Hydrolysis was terminated by chilling and acidifying to pH 3.5 with HClO₄. The precipitate was removed by centrifugation at 1100 g for 10 minutes. The supernatant was analyzed by paper chromatography.

B. Paper Chromatography

Descending paper chromatography was carried out using

Whatman No. 1 filter paper as described by Lane (1963).

After developing for 17 hours at room temperature the paper was air dried and nucleosides were located under ultraviolet lamp and identified by parallel markers of 2' (3') AMP, GMP, UMP, CMP, guanosine and the alkaline hydrolysate of yeast RNA (Randerath and Randerath, 1965). The paper was cut into 1 cm pieces, placed in vials containing toluene scintillation liquid and the radioactivity determined.

C. Nearest Neighbour Frequency Analysis

An in vitro assay mixture containing [α -³²P]-GTP as the labelled substrate and 1 mg protein of dialyzed crude extract was incubated at 37°C for 1 hour. Alkaline hydrolysis and paper chromatography was carried out as described above. The relative frequency with which a given nucleotide is adjacent to any other nucleotide in the chain was determined (Josse and Swartz, 1963).

7. Centrifugation on Sucrose Density Gradients

A. Cytoplasmic Extracts

Linear gradients of 15-30% sucrose in 0.01 M Tris-HCl pH 7.5, 1 mM magnesium acetate, 1 mM mercaptoethanol, 5% (v/v) glycerol and 2 mM Cleland's reagent were prepared in 38 ml volumes in nitrocellulose tubes using a Buchler gradient maker.

About 1 ml of crude cytoplasmic extract was layered on each gradient. Centrifugation was carried out at 4°C, 81,000 g for 5 hours in a Beckman Ls-65B ultracentrifuge using a SW27 rotor. After centrifugation the gradient fractions were collected in successive 1 ml fractions with continuous monitoring of the optical density at 254 nm, using an ISCO analyzer and fraction collector. A 0.4 ml portion of each fraction was assayed for polymerase activity.

B. RNA Products

RNA extracted from reaction mixtures labelled with ^3H -GTP and dissolved in STE-SDS buffer, was analyzed by centrifugation on a linear 5-20% sucrose gradient in STE-0.1% SDS buffer. Centrifugation was carried out for 120 minutes at 15°C and 180,000 g in a Beckman SW50 rotor. Successive 15 drops fractions were collected by puncturing the bottom of the tube. The radioactivity in each tube was assayed by precipitation with yeast RNA and 10% TCA.

Sucrose gradient analysis of RNA labelled with ^3H -ATP and dissolved in STE, was carried out as above but without SDS. Centrifugation was for 180 minutes at 180,000 g and 4°C, fractions of 17 drops were collected and radioactivity determined as described before in Section 4.

8. Hybridization

A. In vitro RNA Product with Viral RNA

Annealing to form RNA-RNA hybrids was carried out as described by Huang et al. (1970). About 5000 cpm of the in vitro RNA product and increasing quantities of viral RNA in a total volume of 0.4 ml of H₂O were melted by immersing in boiling H₂O for 3 minutes. After the addition of 0.1 ml of 1.5 M NaCl and 0.1 ml of 0.15 M Na citrate, the samples were incubated at 65°C for 150 minutes. After cooling, each sample was divided into two 0.25 ml portions one of which was digested with 50 µg/ml of pancreatic RNase for 30 minutes at room temperature. All samples were acid precipitated in the presence of carrier yeast RNA (0.01 mg) and assayed for radioactivity.

B. ³H-Poly U with Poly A

Hybridization of ³H-poly U and poly A was done according to the method described by Gillespie et al. (1972). A standard 0.05 ml hybridization mixture consisting of 1 µg (7 mCi/mM) of ³H-poly U; 10 µg of poly A; 50% formamide; 0.01 M Tris-HCl pH 7.5, was melted in boiling water for 3 minutes followed by the addition of 3 X SSC (0.45 M NaCl, 0.045 M Na citrate). The mixtures were incubated for 22 hours at 37°C and cooled to 30°C for 1 hour. A 5 ml solution containing 0.01 M Tris pH 7.5,

0.01 M $MgCl_2$, 0.5 M NaCl was added to each sample. Half of each sample was digested with pancreatic RNase 20 $\mu g/ml$ and T_1 nuclease 150 U/ml at 30°C for 30 minutes. All samples were TCA precipitated in the presence of carrier yeast RNA and radioactivity determined.

The same procedure was followed for hybridization of 3H -U labelled viral RNA to poly A.

9. Analysis of Proteins Associated with Enzyme Complex

A. Preparation of Radioactively Labelled Cytoplasmic Extract

Cells pre-treated with 0.5 $\mu g/ml$ of Act. D for 20 hours, were infected with VSV at moi of 100. At 3 hours post resuspension, the cells were centrifuged and resuspended in medium containing 1/20 the concentration of amino acids present in Joklik's modified minimal essential medium. ^{14}C -amino acids at a concentration of 0.5 $\mu Ci/ml$ were added.

The cells were harvested 5½ hours post infection, washed with PBS, and disrupted in RSB° buffer (0.1 M NaCl; 0.0015 M $MgCl_2$; 0.01 M Tris, pH 7.5; Prevec, 1965) as outlined in Section 5 above. The nuclei-free supernatant was layered on 15-30% linear sucrose gradient dissolved in RSB°, and centrifuged for 3½ hours at 5°C, 81,000 g, in a SW27 rotor. The gradients were fractionated into 1 ml

portions and radioactive peaks located by spotting 0.05 ml of each fraction on Sartorius glass fiber pads and counting in toluene scintillation fluid.

B. Extractions of Proteins

Appropriate fractions of the gradients were pooled and precipitated in 2 volumes of 95% ethanol at -20°C overnight. The precipitate was collected by centrifugation at 23,000 g for 10 minutes, and air dried. Both the above precipitate and the final viral pellet labelled with ^3H -amino acids were resuspended in 0.2-0.5 ml of alkylating buffer (1% SDS; 8 M urea; 0.005 EDTA; 0.0005% phenol red; 2.5 $\mu\text{l}/\text{ml}$ of mercaptoethanol; all dissolved in 0.5 M Tris-HCl pH 8.6; Reuckert, 1965). The sample was allowed to stand at room temperature for one hour and then dialyzed overnight against 1000 volumes of dialysis buffer (0.01 M Na_2HPO_4 ; 0.0028 M KH_2PO_4 , pH 7.1, 0.1% SDS; 1% (v/v) mercaptoethanol; 0.5 M urea; Summers et al., 1965).

C. Polyacrylamide Gel Electrophoresis

The method used was that described by Kang (1971). Polyacrylamide gels were prepared in 27 cm x 0.9 cm plastic tubes sealed at one end with dialysis tubing. The gel solutions A [15% (w/v) acrylamide; 0.3% (w/v) N,N'-methylene bisacrylamide made up in running buffer], B [0.1% (v/v)

N,N,N',N'-methylethylenediamine in running buffer] and C [0.56% (w/v) ammonium persulphate in running buffer] were mixed in a ratio of 4:3:1 respectively and urea added to concentration of 0.5 M. A volume of 13-15 ml of the mixture was poured into the tubes and water was layered on top of each gel. After 45 minutes at room temperature, the water was removed and no more than 0.3 ml of the solubilized and dialyzed protein containing 20% sucrose was layered on the gel. The tubes were carefully filled with running buffer (0.1 M Na_2HPO_4 ; 0.028 M KH_2PO_4 pH 7.1-7.3, 0.1% SDS; Summers *et al.*, 1965) and mounted in a Buchler vertical electrophoresis apparatus. Running buffer was added to the chambers and electrophoresis was carried out at 1 volt/cm of gel for the initial 30 minutes, followed by 4 volts/cm of gel for the next 20-22 hours.

After electrophoresis, the gel was sliced manually with razor blades into discs of about 0.15 cm in height. For single label, each slice was placed into scintillation vial containing 10 ml of toluene scintillation fluid with 25% methanol. For dual label (^3H and ^{14}C) gel slices were depolymerized by incubating at 37°C overnight in 0.1 ml of hydrogen peroxide and then counted in 5 ml of Bray's scintillation fluid (8 g omnifluor; 300 ml of methanol; and 60 ml of ethylene glycol, in a total volume of 3.0 liters of dioxane; Bray, 1960).

RESULTS

SECTION I. Properties of the RNA Polymerase System

1. Optimal Conditions for the RNA Polymerase Activity

The enzyme assay used was a slight modification of the system described by Wilson and Bader (1965). A complete standard reaction mixture with concentrations and amounts of components is shown in Table 1. The concentrations of the reactants were derived from the following experiments.

A pH of 7.5-8.0 was chosen for the assay on the basis of the data presented in Table 2. As shown, incorporation was maximal in this pH range and fell to considerably lower values at pH 7.0 or 8.5.

The response of the assay to varying Mg^{++} concentrations is shown in Fig. 4. The optimum for activity was 10 mM. This was in agreement with Wilson and Bader's data (1965).

The incorporation of the labelled triphosphate, 3H -GTP was dependent on the presence of other 3 nucleoside triphosphates (Table 3). As indicated in this Table the incorporation of GTP dropped to 20% in the absence of all the unlabelled triphosphates. Also when each of the triphosphates was substituted one at a time by the corresponding labelled

Table 1Composition of Standard Reaction Mixture

A 5 ml solution composed of 0.6 M Tris-HCl pH 7.5-8.0; 0.1 M magnesium acetate; 0.005 M mercaptoethanol.	0.05 ml
A mixture of 0.026 M UTP, CTP and ATP in 0.01 M Tris-HCl pH 7.5 (neutralized with sodium bicarbonate)	0.05 ml
³ H-GTP, (1.16 Ci/mmole) 100 μ Ci/ml	0.01 ml
Cytoplasmic extract (infected or non infected cells)	0.1 ml
0.01 M Tris-HCl pH 7.5-8.0	0.29 ml
Total volume of solutions	0.50 ml

Amounts of Components per 0.5 ml

Tris-HCl pH 7.5-8.0	33 μ moles
Magnesium acetate	5 μ moles
Mercaptoethanol	0.5 μ moles
Mercaptoethanol present in cytoplasmic extract	1.0 μ moles
UTP, CTP, ATP	1300 μ moles
Cytoplasmic extract	0.5-1 mg protein

The reaction mixture was incubated at 37°C or 28°C for 20 minutes, chilled and 1 ml of cold 10% TCA was added. The precipitate formed was collected on nitrocellulose filters, washed 5 times with 10% TCA. The filters were dried and counted as described in METHODS.

Table 2
Effect of pH on the Assay

Reaction System	cpm/20 min/mg protein
Complete; pH 7.0	2,460
Complete; pH 7.5	13,340
Complete; pH 8.0	16,860
Complete; pH 8.5	6,780

The complete reaction mixture contained all the components described in Table 1 with the Tris-HCl buffers of the indicated pH, adjusted at room temperature.

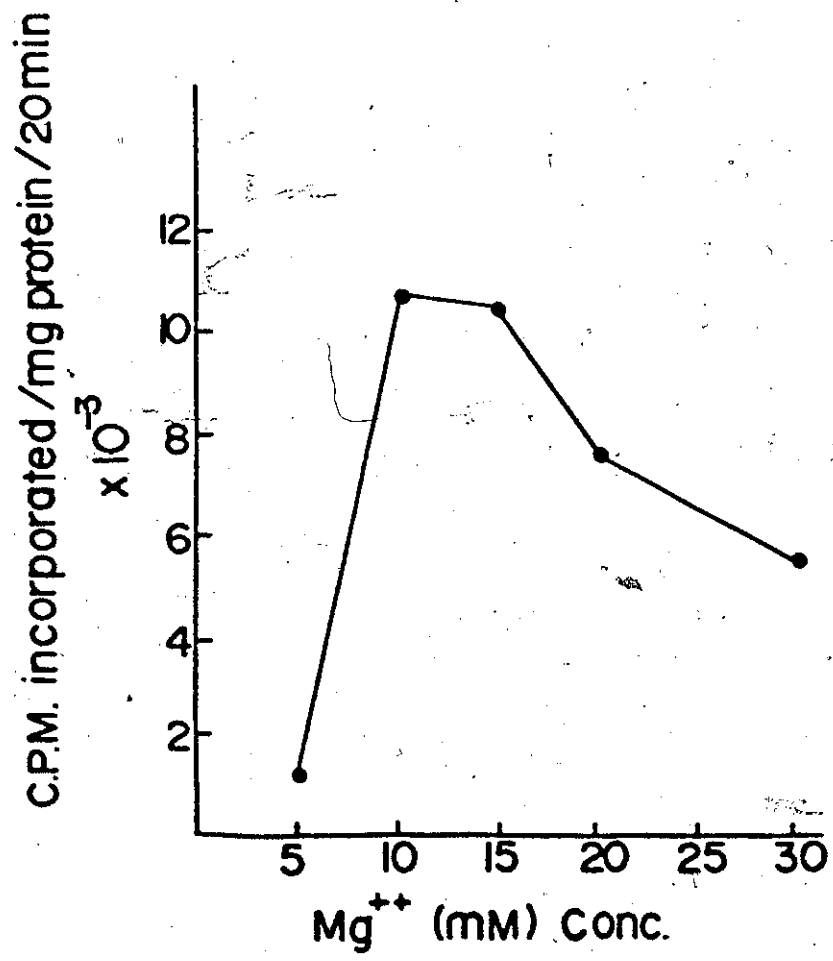


FIGURE 4. Effect of Mg⁺⁺ Ion Concentration on the Incorporation of GMP

Standard reactions mixtures were made up as described in Table 1 with the Mg⁺⁺ ion concentration changed to that recorded above and incubated at 37°C for 20 min. The products were then precipitated and radioactivity determined.

Table 3Triphosphate Requirement for GMP Incorporation

Reaction System	Expt. I		Expt. II	
	cpm*	%	cpm*	%
Complete	2443	100	9046	100
Minus ATP	579	23	2449	27
Minus CTP	464	19	2797	32
Minus UTP	1278	52	6103	65
Minus ATP, CTP, UTP	495	20	1652	18

The complete reaction mixture contained all the components described in Table 1. The other mixtures were identical except for omission of the stated phosphates. Incubation was at 37°C for 20 min.

Experiments I and II were performed with different HR-LT enzyme preparations each of which was dialyzed overnight against 500 volumes of dialysis buffer.

*Expressed as cpm/20 min/mg protein

nucleoside triphosphate the results presented in Table 4 were obtained. Each of the radioactive nucleoside triphosphates was incorporated by the polymerase into acid insoluble product.

In order to determine optimum substrate concentrations, reaction mixtures were prepared in which the concentrations of each of the nucleoside triphosphate was separately varied. The results are shown in Fig. 5. Incorporation increased to a saturating level with increasing substrate concentrations. Concentrations of 2.6 mM for UTP, CTP and ATP respectively were used in all assays, although the saturating concentrations for UTP could be well below this value. As noted in both Fig. 5 and Table 3, considerable incorporation of GTP occurred even in the absence of added UTP. Similar results were obtained using triphosphates from three different commercial sources. Paper chromatography of ATP and CTP from Schwarz/Mann under conditions which could detect 5% contamination levels failed to reveal any contaminating UTP. The possibility that 1) UTP was generated by deamination of CTP, or 2) that a non-uridine polymer was synthesized, were examined with the analysis of the nature of the synthesized product (Section II).

Fig. 6 illustrates the optimal concentration of the labelled triphosphate, ^3H -GTP, plotted both as a function of total counts (A) and pmoles incorporated (B). This

Table 4

Incorporation of Labelled Nucleoside Triphosphates by Cytoplasmic

Extract from VSV Infected L-Cells

Labelled Precursor	Concentration of NTP (mmoles)	mmoles $\times 10^{-3}$ /mg/20 min incorporated
^3H -GTP, complete (1 μCi , 1.16 Ci/mmole)	0.86	7.2
^{14}C -UTP, complete (0.54 μCi , 356 mCi/mmole)	1.5	8.2
^{14}C -CTP, complete (0.54 μCi , 92 mCi/mmole)	5	32
^{14}C -ATP, complete (0.5 μCi , 38 mCi/mmole)	12	11.2

Complete reaction mixtures having one labelled NTP at the concentration described above and the remaining 3 unlabelled triphosphates at 2.5 mM as described in Table 1 were incubated for 20 min at 37°C and the products acid precipitated and counted.

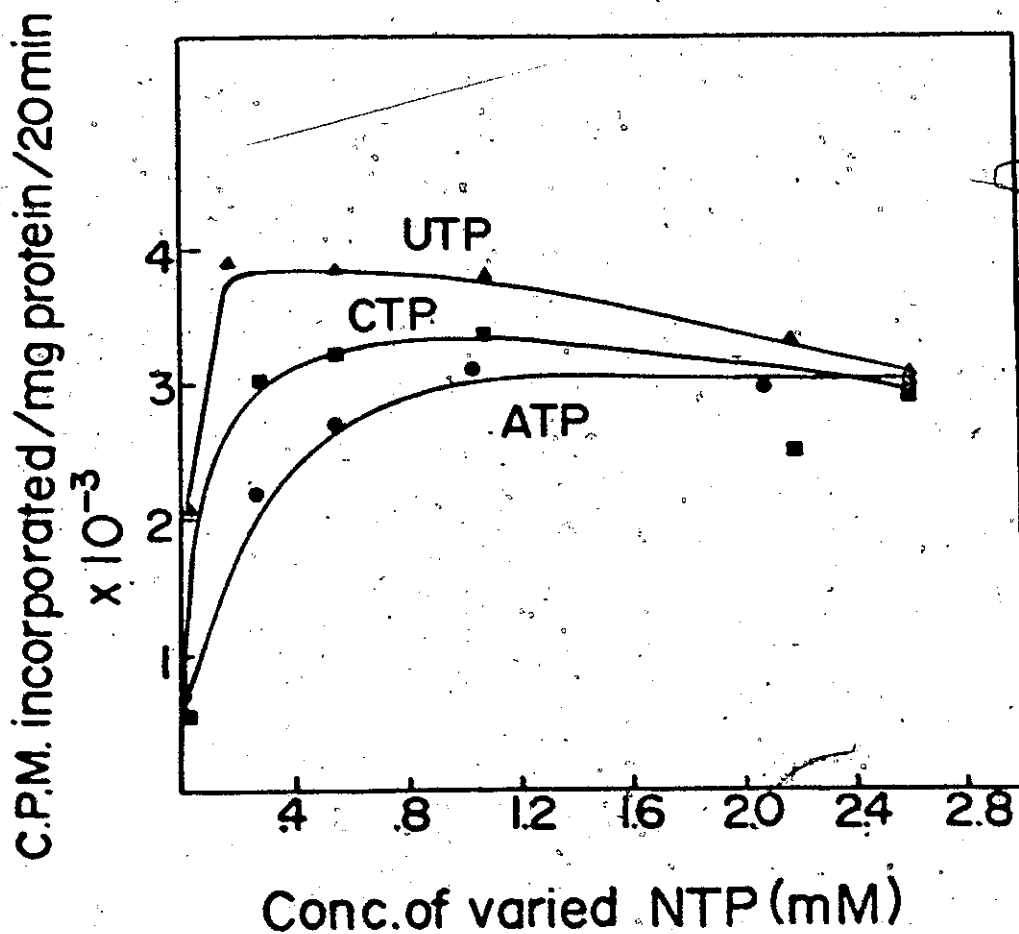


FIGURE 5. Effect of Increasing Concentrations of Unlabelled Nucleoside Triphosphates (NTP) on Incorporation of ^3H -GMP
 Reaction mixtures were set up containing ^3H -GTP and two unlabelled nucleoside triphosphates at the concentrations presented in Table 1. The concentration of the remaining triphosphate was varied as indicated in the above Figure and incubation carried out at 37°C for 20 minutes.

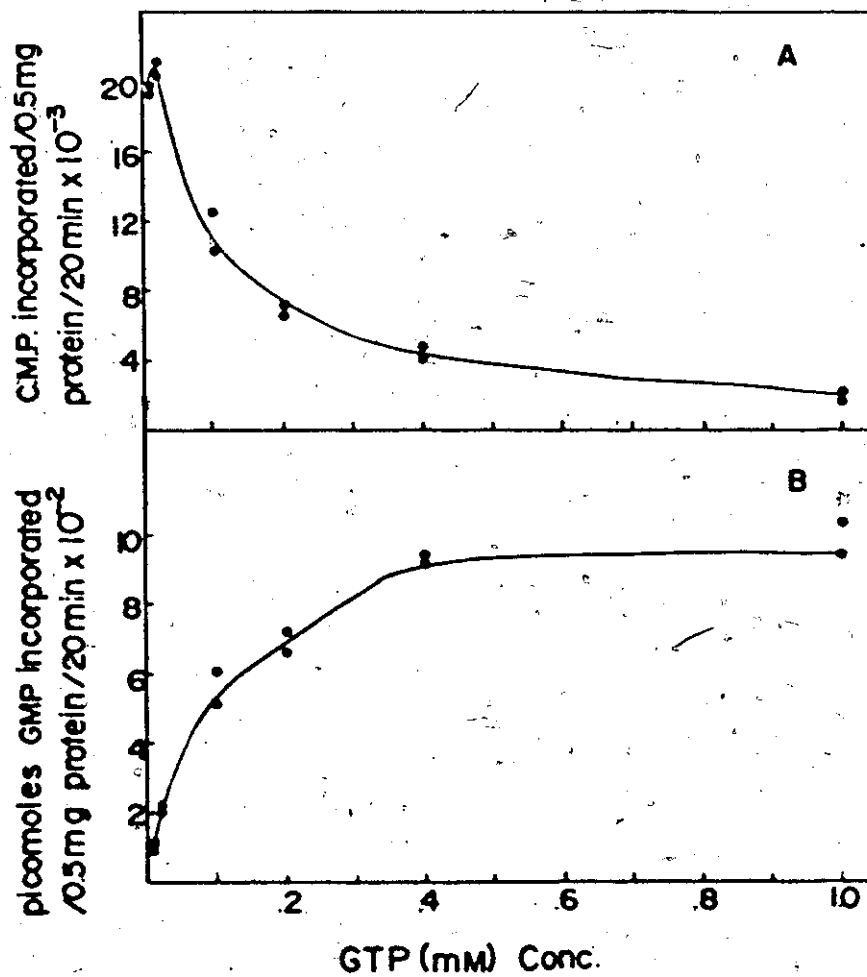


FIGURE 6. Effect of GTP Concentration on GMP Incorporation
 Reaction mixtures containing the components described in Table 1 were set up, except that different amounts of unlabelled GTP were added. Incubation was for 20 min at 37°C: A) The amount of GMP incorporated determined from the incorporated radioactivity and the specific activity B) Incorporation (CPM) of ³H-GMP.

experiment was done using standard reaction mixtures containing constant amounts of the three unlabelled triphosphates and $^3\text{H-GTP}$. Increasing amounts of unlabelled GTP were added to the respective samples. The optimal value for GTP was about 0.5 mM. The V-max value for $^3\text{H-GTP}$ utilization was 19×10^{-10} moles/mg of protein. For routine assays of the polymerase, $^3\text{H-GTP}$ of high specific activity (1.16 Ci/mmole) and a concentration of 1.7 μM was used. As shown in Fig. 6A, this concentration of $^3\text{H-GTP}$ gave maximum incorporation of radioactive counts.

When $^3\text{H-ATP}$ was used as the labelled substrate and its incorporation was plotted as a function of ATP concentration, the results presented in Fig. 7 were obtained. The optimal concentration of ATP was 2 mM and the V-max value for $^3\text{H-ATP}$ utilization was about the same as for $^3\text{H-GTP}$. Although this maximal number of labelled picomoles incorporated of both ATP and GTP is the same, a greater concentration of ATP than GTP is required to reach such levels. While the reason for less ATP incorporation than GTP at comparable substrate concentrations could perhaps be attributed to the presence of an ATPase or some other ATP scavenging system in the crude cell extract, it seems unlikely since Bishop et al. (1971) observed similar results with the virion associated transcriptases of VSV and Influenza virus.

The effect of energy generating systems on $^3\text{H-GTP}$ incorporation was examined. Energy generating systems are

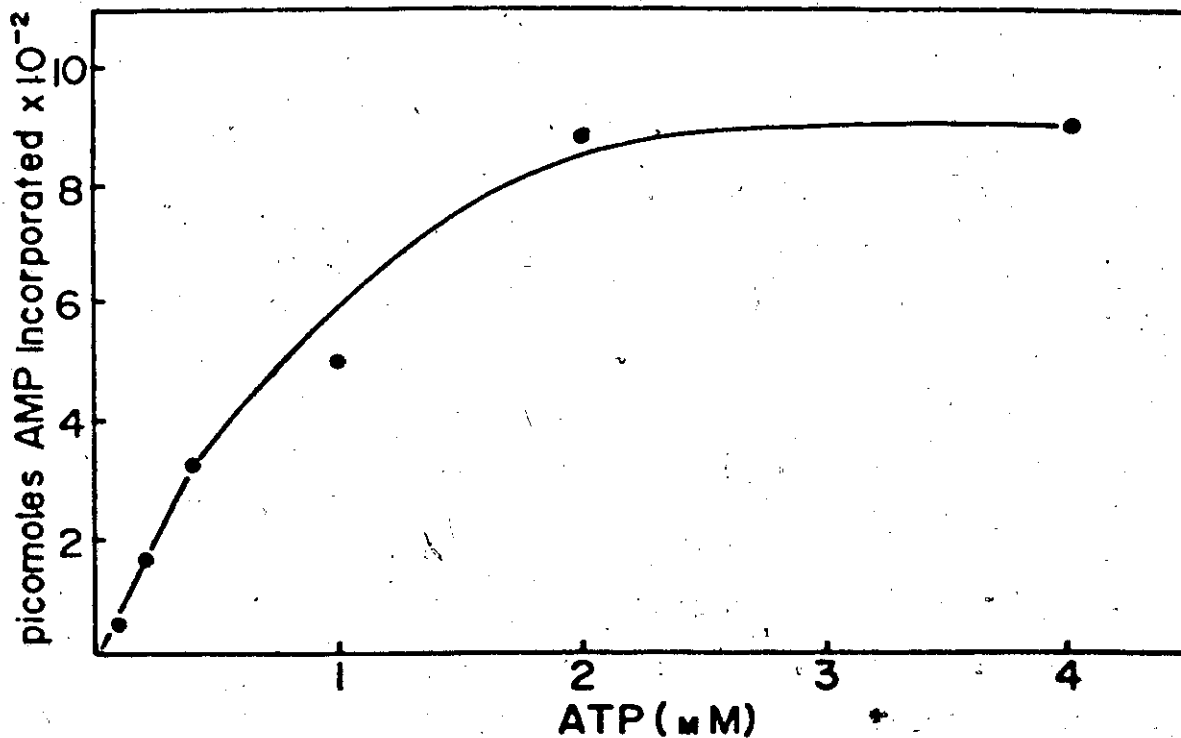


FIGURE 7. Effect of ATP Concentration on AMP Incorporation
Twelve standard reaction mixtures containing 1 μCi of ^3H -ATP (20 Ci/ μmole) as the labelled substrate were set up. Sufficient unlabelled ATP to bring the final ATP concentration to the level shown was added to pairs of mixtures. After incubation for 20 min at 37°C the duplicate samples were acidified and radioactivity determined as described in METHODS. The amount of AMP incorporated was determined from the incorporated radioactivity and the specific activity, and is expressed per 0.5 mg of protein.

usually added to ensure that any nucleoside monophosphates and diphosphates are converted to nucleoside triphosphates. The systems usually used are phosphoenol pyruvate with pyruvate kinase and creatine phosphate with creatine phosphokinase. The addition of PEP and pyruvate kinase at concentrations of 20 mM and 8 µg/ml respectively inhibited the incorporation of ^3H -GTP in the assay (Table 5). The reason for the inhibition is not known but appears to be due to PEP alone. In its presence at a concentration of 6 mM, only 11% of the activity was retained as compared to a complete reaction in the absence of PEP. When PEP, at a concentration which did not cause inhibition (0.1 mM), was added to a complete reaction mixture together with pyruvate kinase, no enhancement of incorporation was observed. Other generating systems, creatine phosphate with phosphokinase and 3-P-glyceric acid, neither inhibited nor increased incorporation. For these reasons an ATP-generating system was not usually included in the standard assay.

The incorporation of ^3H -GTP was linearly proportional to the amount of added enzyme up to at least 1.0 mg of protein (Fig. 8). A concentration of 0.5-1.0 mg of protein was used per assay.

Table 5Effect of ATP Generating System on Incorporation

Reaction System	Relative Activity
Complete	100%
plus PEP (20 mM), Pyr. Kin. (8 μ g/ml)	29
plus PEP (20 mM)	22
plus Pyr. Kinase (8 μ g/ml)	104
plus PEP (0.1 mM)	90
(0.2 mM)	83
(1.0 mM)	77
(2.0 mM)	67
(6 mM)	11
plus PEP (0.1 mM), pyr. kinase (8 μ g/ml)	83
plus 3-P-glyceric acid (20 mM)	97
plus creatine phosphate (5 mM), creatine phosphokinase (8 μ g/ml)	100

Each reaction mixture contained the components as described in Table L. Incubation was at 37°C for 20 minutes.

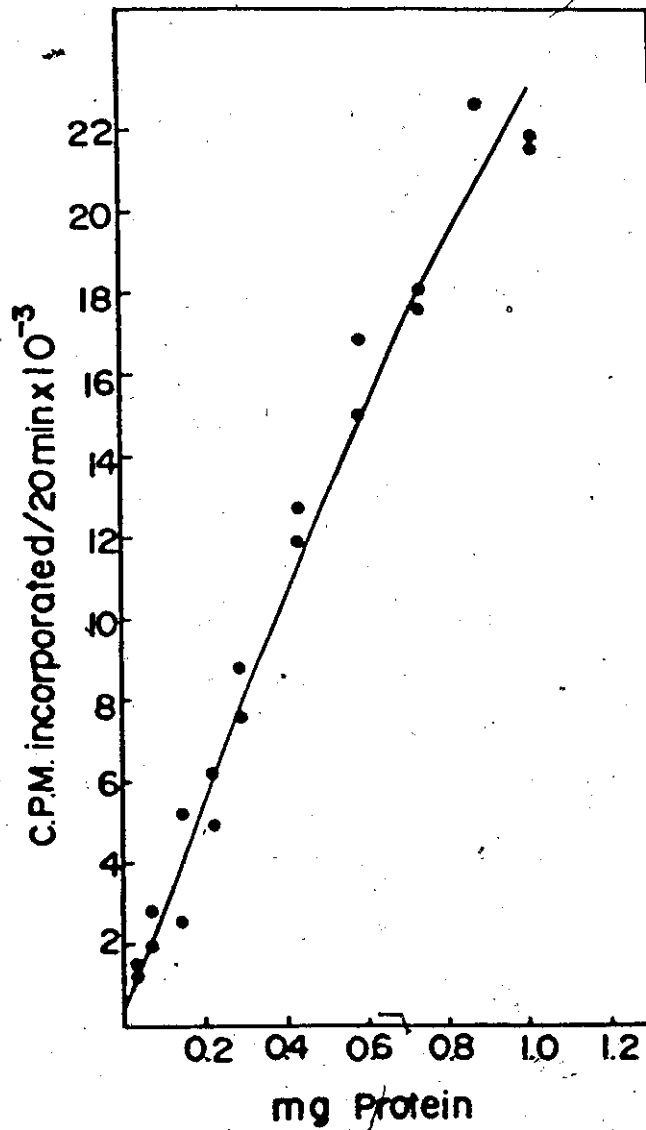


FIGURE 8. GMP Incorporation as a Function of Protein Concentration

Reaction mixtures were prepared as in Table 1, but containing different protein concentrations of the infected cytoplasmic extract. Incubation was at 37°C for 20 min.

2. Other Conditions Influencing Polymerase Activity

The effect of detergents, Mn^{++} , and salt on the assay and the thermal stability of the enzyme were examined.

In Table 6, the effect of various factors on the incorporation of 3H -GTP by cytoplasmic extracts into acid insoluble product is presented. Polymerase activity was demonstrated only in cytoplasmic extracts of infected cells. Reaction mixtures containing an extract from uninfected cells gave values which were no higher than the amount of radioactivity bound to filters in the absence of extract.

No activity was detected in the absence of Mg^{++} ions. Mn^{++} ions at the same concentrations as Mg^{++} , either in the presence or absence of Mg^{++} inhibited activity by 90%.

Incorporation was unaffected by Act. D. or DNase which demonstrated the lack of participation of DNA in the enzymatic reaction. As expected, in the presence of RNase, incorporation was reduced to control levels.

Effect of Detergents

In order to observe in vitro polymerase activity by virion-associated enzymes the virus must first be activated. Activation of enveloped viruses is most easily achieved by treating with non-ionic detergents, which presumably strip off the outer, lipid containing envelope and thus expose the nucleoprotein core (Baltimore et al., 1970; Aaslestad

Table 6

Properties of the RNA Polymerase Assay System

Experiment	Reaction Mixture	cpm/30 min/0.5 mg protein
1	Complete; uninfected cells	240
	Complete; infected cells	8000
	Complete; minus Mg Λ C ₂	190
	plus Act D (10 μ g)	7820
2	plus DNase (20 μ g)	8130
	plus RNase (10 μ g)	250
	Complete; uninfected cells	131
2	Complete; infected cells	13,980
	plus MnCl ₂ (10 mM)	164
	plus MnCl ₂ (10 mM) minus Mg ⁺⁺	167

Each 0.5 ml assay system contained the components described in Table 1, except for the designated additions. Experiments 1 and 2 were performed with different batches of HR-LT enzyme.

et al., 1971; Bishop et al., 1971). With the cellular VSV complex the addition of non ionic detergents such as Brij and Triton X-100 did not affect incorporation at the concentrations used (Table 7), which suggested that the enzyme was not enveloped. In contrast, the addition of the ionic detergent sodium deoxycholate greatly inhibited the reaction. This may have been due to the removal of one of the viral proteins (L) from the ribonucleoprotein complex by DOC (Kang, 1971), or to the precipitation of Mg^{2+} by this detergent (Kohler et al., 1968).

Effect of Salt

Since the presence of a monovalent ion is required by some enzymes for stabilization of a conformation responsible for maximal catalytic activity the effect of addition of NaCl and KCl on the reaction was examined. Concentrations of either salt up to 0.08 M stimulated enzymatic activity while greater concentrations inhibited incorporation (Fig. 9). The cytoplasmic extract used for these experiments was dialyzed first to remove intracellular ions. When the same experiment was repeated with undialyzed extract, inhibition was observed with concentrations greater than 0.01 M (Fig. 10). It appears that the crude extract does not require additional salt, and hence salt was not included routinely in the assay. The stimulation of incorporation by the dialyzed extract observed with NaCl and KCl was similar to the results reported

Table 7

Effect of Detergents on the Enzyme

Experiments	Assay System	cpm/mg protein/20 min
1	Infected; complete	9,800
	- plus 0.5% Triton X-100	8,700
	- plus 0.5% Brij	9,700
	- plus 1% Brij	10,000
2	- plus 2% Brij	9,400
	Infected; complete	5,650
	- plus 0.02% DOC	2,460
	- plus 0.2% DOC	627

Each reaction mixture contained the components as described in Table 1, except for the designated additions. Incubation was at 37°C.

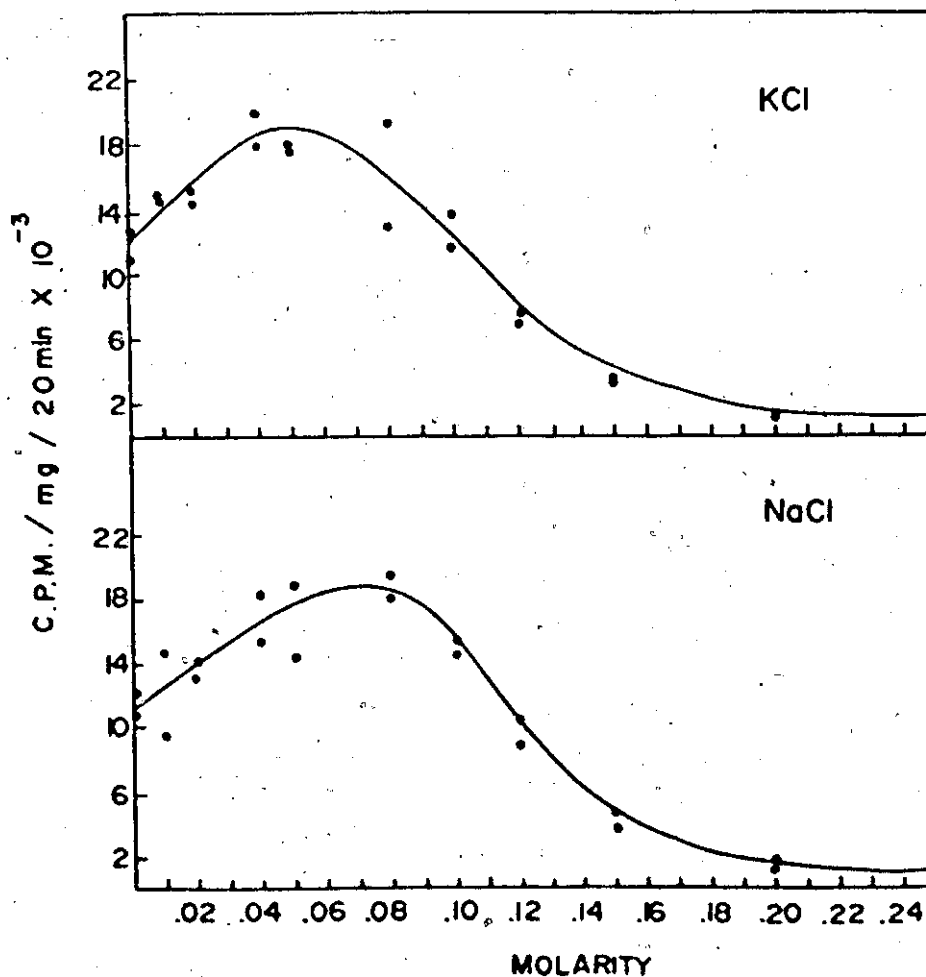


FIGURE 9. Effect of K⁺ and Na⁺ on Enzyme Activity of a Dialyzed Extract

Standard reaction mixtures as described in Table 1 were prepared with dialyzed cytoplasmic extract. KCl and NaCl were added to the mixture to the final indicated molarity. Incubation was at 37°C for 20 min.

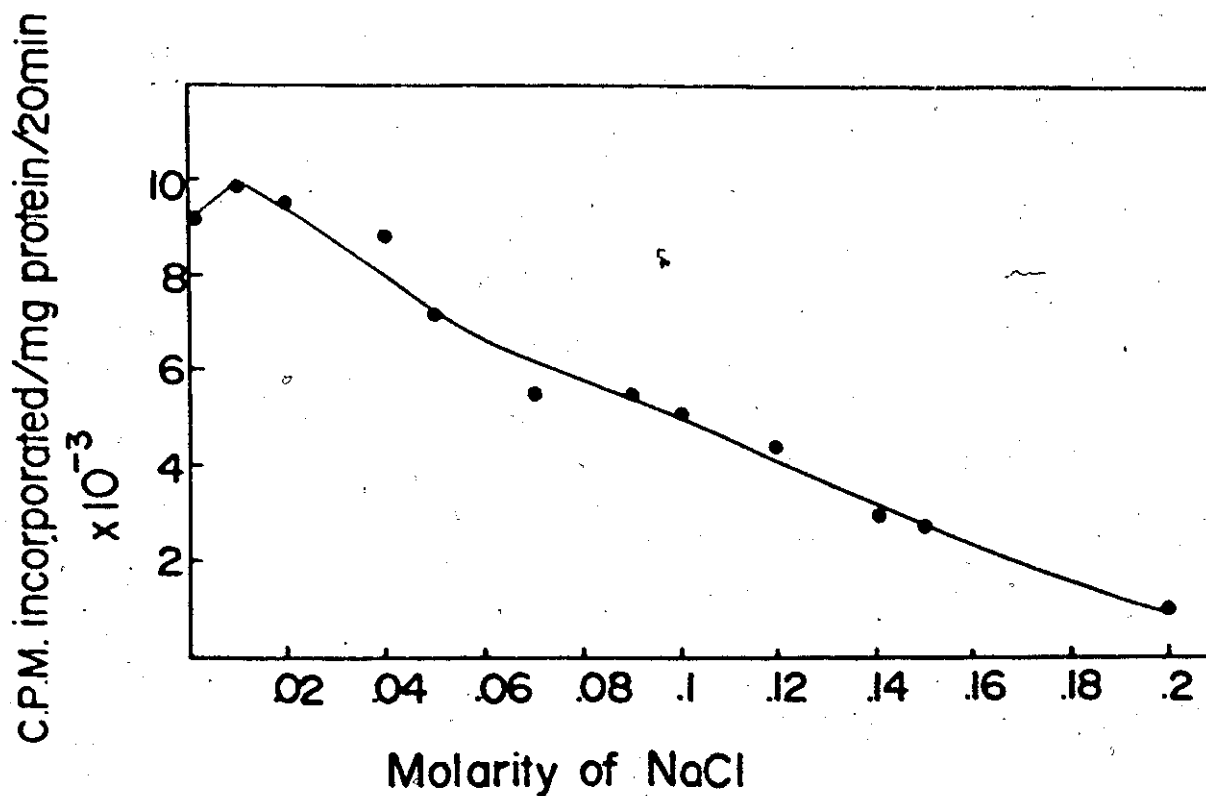


FIGURE 10. The Effect of Na⁺ on Enzyme Activity of a Crude Extract

Standard reaction mixtures containing undialyzed enzyme as in Table 1 were incubated with various amounts of NaCl at 37°C for 20 min. The acid precipitable product was collected and radioactivity determined.

by Aaslestad et al. (1971) for the virion associated polymerase.

Thermal Stability of the Enzyme

The activity of the enzyme preparation was tested after incubation in the absence of substrates, for various periods of time at 28°C and 37°C. As shown in Fig. 11, no loss of enzymatic activity occurred at these two temperatures over a period of 3 hours. The enzyme therefore appears to be reasonably thermostable in this temperature range. Table 8 shows the results of stability experiments conducted at both lower (room temperature) and higher (45°C) temperatures. As expected from the preceding result, no loss of activity was observed after three hours at room temperature, however 80% of the activity was destroyed after heating for 5 minutes at 45°C. If the three unlabelled nucleoside triphosphates were added to the enzyme prior to heating then virtually no activity was lost under these same conditions. This result suggests that the enzyme-substrate complex may be significantly more thermostable than the enzyme itself (Munyon, 1970).

3. Fractionation of RNA Polymerase Activity

The partial purification of VSV polymerase by differential centrifugation of cytoplasmic material is

C.P.M. incorporated / 0.5mg protein
/ 20 min $\times 10^{-2}$

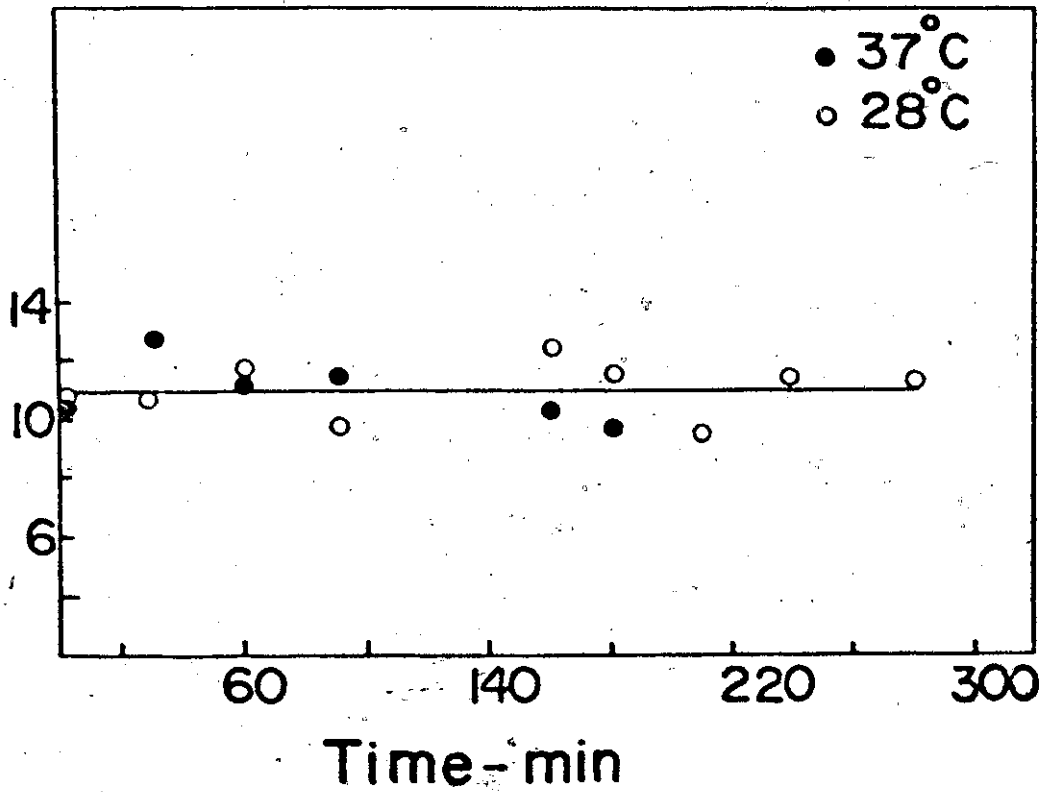


FIGURE 11. Heat Stability of the Crude Extract
Cytoplasmic extract was incubated at 37°C and at 28°C.
At the indicated times a volume equivalent to 0.5 mg
of protein was withdrawn and assayed for activity as
described in Table 1.

Table 8

Heat Sensitivity of the Enzyme

Reaction System	cpm/20 min/mg protein
Complete; infected	10,960
Complete; uninfected	110
Complete; infected, one hour at room temp.	8,070
- 2 hrs at room temp.	7,630
- 3 hrs at room temp.	7,750
- 5 minutes at 45°C	1,860
- 5 minutes at 45°C, plus NTP	8,400

To a complete reaction mixture (Table 1) was added extract that has been left at room temperature for 1, 2, 3 hours respectively. Extract that was incubated in the presence or absence of the three unlabelled NTP (2.5 mM each) at 45°C for 5 min was chilled in ice and assayed for activity.

outlined in Fig. 12. The distribution (units) of RNA polymerase activity (pmoles of $^3\text{H-GMP}$ incorporated in 20 min at 37°C) is also shown. About 80% of the polymerase activity was pelleted at 160,000 g for 75 min (P_3). This suggests that VSV polymerase, as is the case with most polymerases of mammalian RNA viruses, is associated with large intracellular structures.

To further analyze these structures, cytoplasmic extracts of cells, infected under conditions where only infectious virus was produced, were centrifuged on a 15-30% sucrose gradient for 5 hours and each 1 ml collected fraction was assayed for polymerase activity. The profile obtained is illustrated in Fig. 13. The activity was associated with two distinct regions, a large complex located in fractions 7-12 and a smaller complex present in fractions 13-20. Sedimentation coefficients based on the optical density profile of the ribosomes could not be assigned to these structures, since the polysomes were not well separated under these experimental conditions.

Since polymerase activity is associated with two distinct complexes it was of interest to follow the biogenesis of these complexes in order to determine if both were present throughout the replicative cycle or whether at some stage only one was detectable. Accordingly the appearance of two complexes at 2, 4 and 5.5 hours post infection was monitored

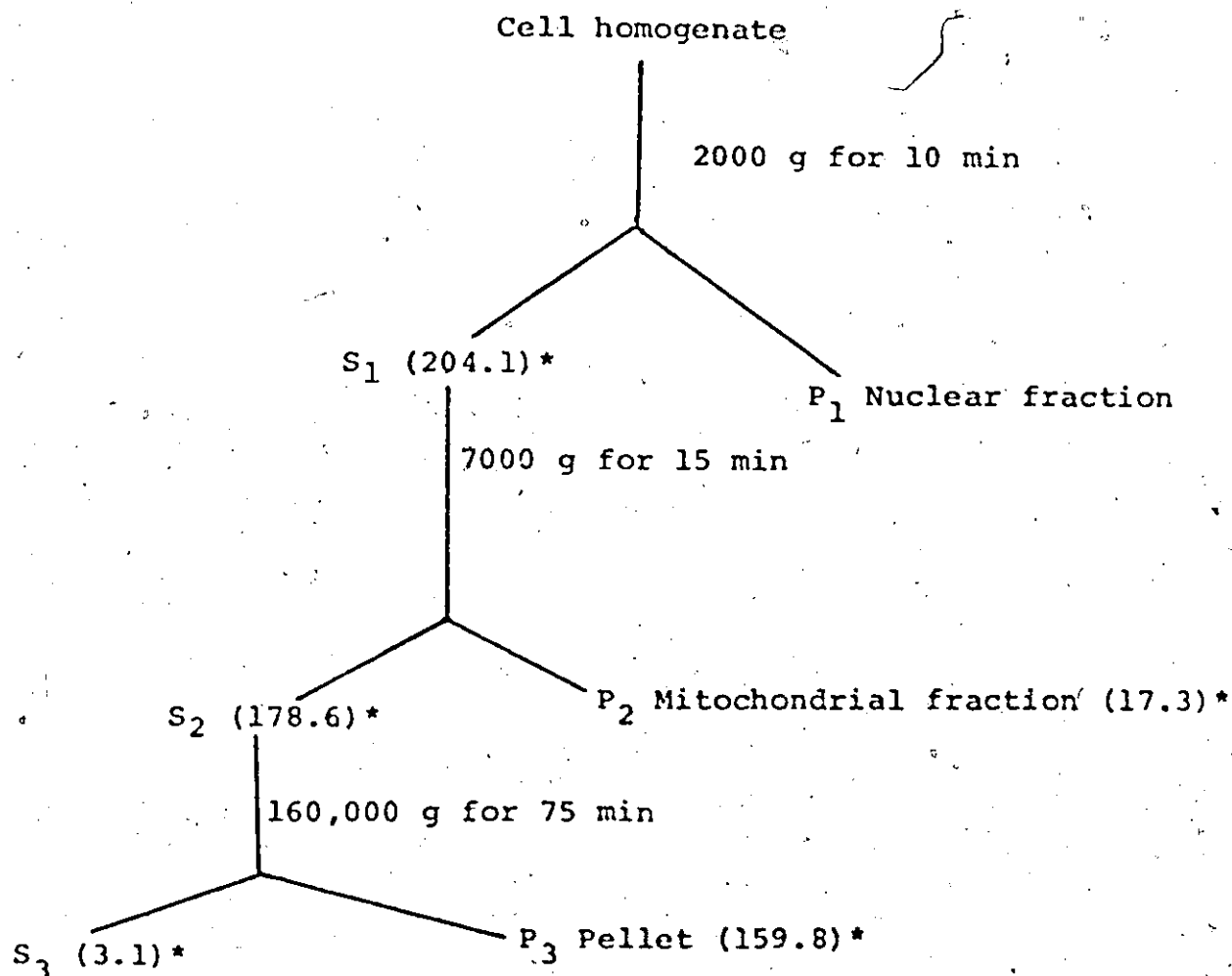


FIGURE 12. Procedure for Fractionating VSV RNA Polymerase Activity from Infected L-Cells by Sedimentation
 Infected cells were harvested, disrupted with Dounce homogenizer and nuclei spun out (P₁). The supernatant (S₁) was taken through 2 centrifugations and activity was assayed at each step as described in Table 1.

*Total units in pmoles of ³H-GMP incorporated per 20 min.

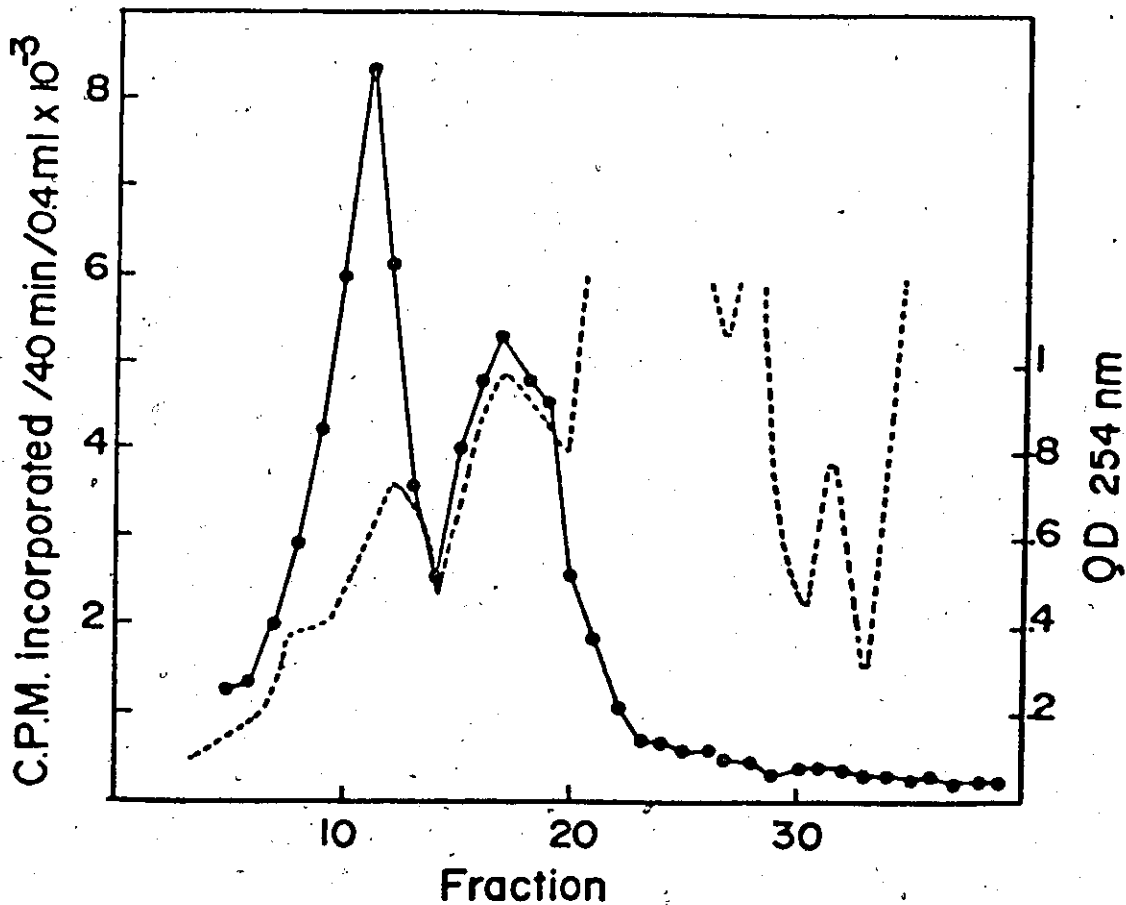


FIGURE 13. Sucrose Density Gradient Analysis of Cytoplasmic Extract from Cells Infected with HR-LT

Cytoplasmic extracts were prepared as described in METHODS, layered on a 15-30% sucrose gradient in 0.01 M Tris-HCl buffer pH 7.5, with 1 mM magnesium acetate, 1 mM mercaptoethanol, 5% (v/v) glycerol and 2 mM Cleland's reagent, and centrifuged for 5 hours at 24,000/rpm, for optical density at 254 nm ----- and for polymerase activity (●). The bottom of the gradient is to the left.

as described above. As illustrated in Fig. 14, activity was associated with large structures at the three times examined. Two distinct complexes were visible at later times, while at two hours, a low level of polymerase activity (note scale) was associated with a single region. While this material seems to have the sedimentation coefficient of the smaller complex seen at later times the significance of this observation remains to be determined.

4. Viral Proteins Associated with the Polymerase Complexes

Since the polymerase complexes coincide in sucrose gradients with the position of the ribonucleoprotein complexes previously observed in the infected cell (Kang, 1971), it was of interest to examine the protein constituents of each complex and to try to identify the proteins responsible for polymerase activity. Accordingly cells pretreated with Act D for 12 hours were infected, and split into two equal portions, one of which was labelled with ^{14}C -amino acids as described in METHODS. Both samples were harvested 5.5 hours post-infection, cytoplasmic extracts prepared and analyzed on sucrose gradients. Fractions of one gradient were assayed for polymerase activity and a profile similar to Fig. 13 was obtained. In the other gradient, the radioactive regions were identified and found to coincide with the polymerase

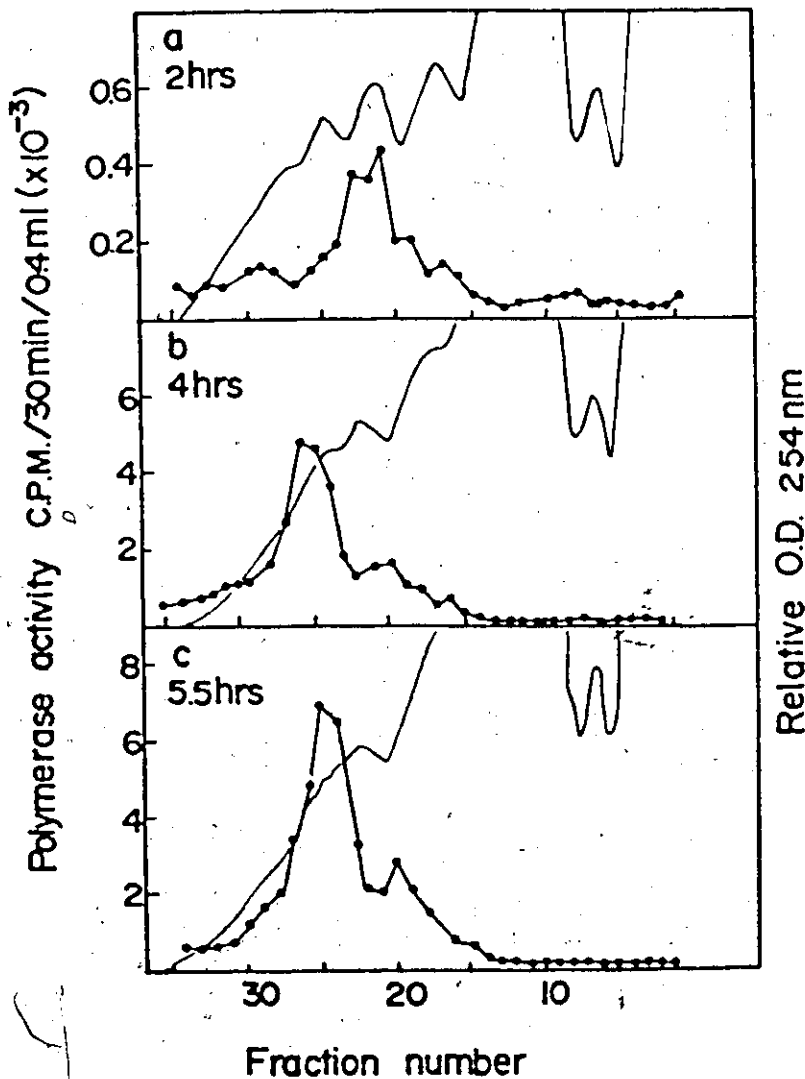


FIGURE 14. Sucrose Density Analysis of Cytoplasmic Extract from Cells Infected with HR-LT at 2, 4, and 5.5 Hours Post Infection

About 5×10^8 cells were infected with HR-LT at a moi of 50 PFU/cell. At 2, 4 and 5.5 hours post infection 1.5×10^8 cells were removed and extracts prepared as described in METHODS. One ml (7.0 mg of protein) of each was layered on the top of 15-30% sucrose gradient and centrifuged for 5 hours at 24,000 rpm, 5°C in a SW27 rotor. 1 ml fractions were collected and monitored for optical density at 254 nm— and for polymerase activity (●). The bottom of the gradient is to the left.

active complexes. The fractions corresponding to the two complexes in the labelled gradient were pooled separately, denatured and analyzed on polyacrylamide gels as outlined in METHODS. The distribution of the proteins on the gels is presented in Fig. 15.

Labelled protein present at the top of the sucrose gradient where no polymerase activity was detected consisted entirely of NS protein (Fig. 15A). This is in agreement with the observation of Wagner et al. (1970) and Kang (1971) who showed that NS is present in large amounts in the soluble fraction of the cytoplasm. Both the small (Fig. 15B) and the large (Fig. 15C) polymerase complexes contained the proteins L, N, and NS. These proteins have previously been identified as those constituting the ribonucleoprotein core of VSV virion (Kang, 1971; Petric, 1970; Wagner et al., 1969; Cartwright et al., 1970). On the basis of the above results it is concluded that viral proteins L, N, and NS are associated with polymerase activity.

To further show that ribonucleoprotein of viral origin is involved with the polymerase activity, the inhibitory effect of antisera, prepared against viral nucleoprotein core and coat protein, was tested on the polymerase assay.

The antisera were obtained from Kang (1971). The antigens used to elicit these antisera were prepared from purified virus treated with DOC and centrifuged through a

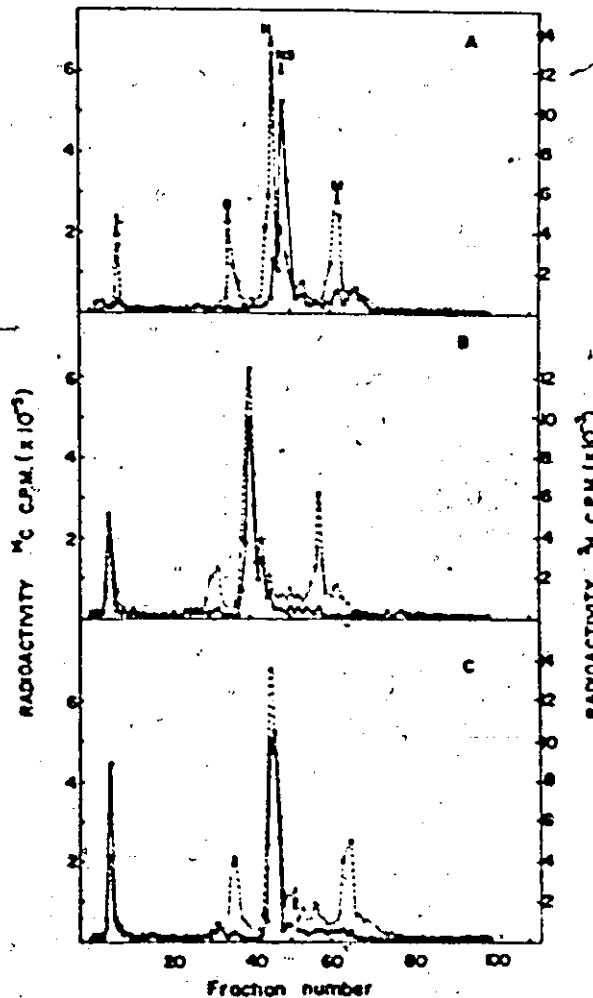


FIGURE 15. Polyacrylamide Gel Profiles of ^{14}C -Radioactive Protein Fractions Taken from Cytoplasmic Extracts of VSV Infected Cells

Radioactive proteins pooled from selected fractions of sucrose gradients were analyzed on polyacrylamide gels. Approximately 100,000 cpm of each protein was analyzed. Co-electrophoresis of ^3H -labelled purified virion proteins o--o provided the marker. A) Material obtained from the top of the gradient. B) Material taken from the low molecular weight region of the gradient comparable to fractions 13-20 in Fig. 13. C) Material taken from the high molecular weight region of the gradient comparable to fractions 7-12 in Fig. 13.

sucrose gradient. The nucleoprotein of the virus containing proteins N and NS moved down the gradient, and viral coat components containing proteins G and M remained at the top of the gradient. These two fractions were separately collected and used for preparation of the antibody (Kang, 1971).

Fig. 16 illustrates the influence of various amounts of antiserum on the RNA polymerase activity. Both RNP core and coat antisera inhibited enzyme activity as compared to control. The control used was normal rabbit serum. The inhibition observed with coat antiserum was probably due to contamination of this serum with core material. Kang (1971) has observed that the coat protein fraction prepared with DOC as described above contained some N protein in addition to G and M.

Although it was shown in the previous experiment that protein L was also associated with the active polymerase complex, no assessment of its function can be drawn from this experiment. It is uncertain whether core and/or coat antiserum contained antibodies to protein L, since neither Kang (1971) or Wagner et al. (1969) could demonstrate unequivocally the presence of L in the core fraction following DOC treatment of virions.

This experiment, therefore, demonstrates the involvement of viral proteins in the polymerase complex. Future experiments using antiserum made against individual

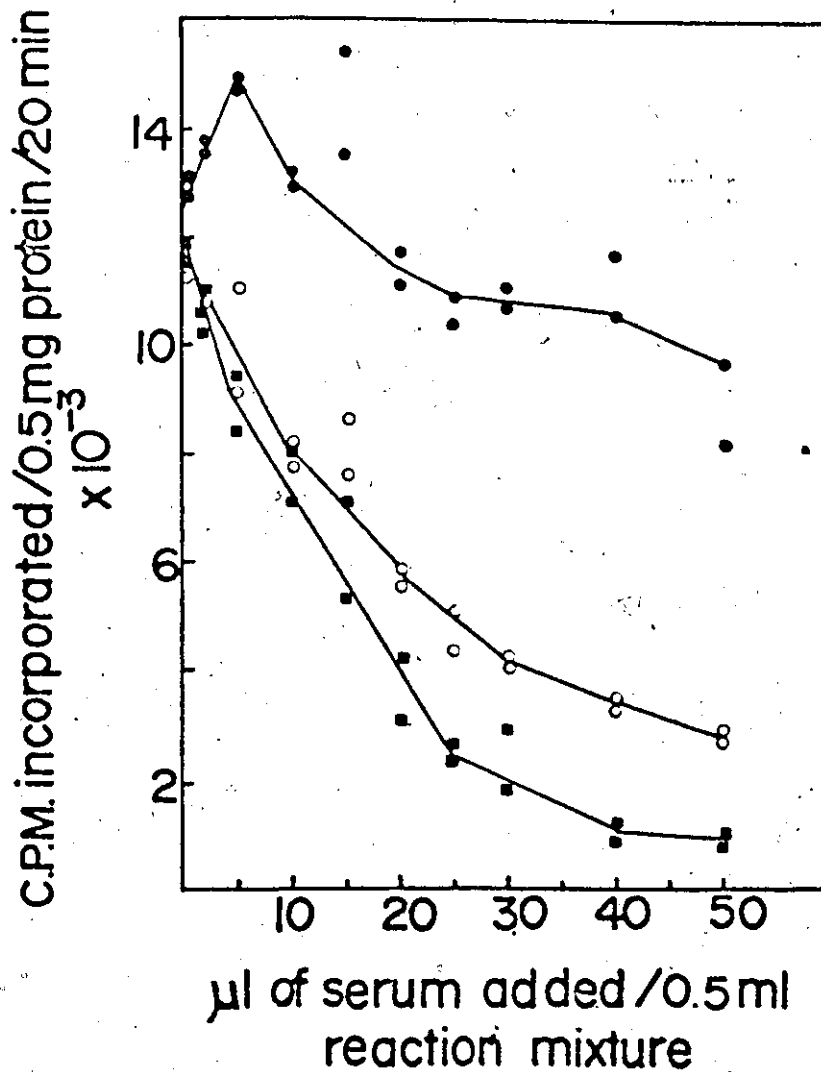
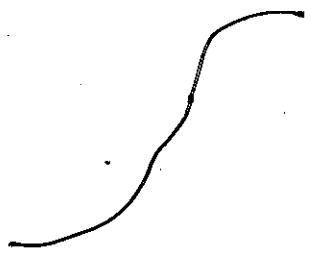


FIGURE 16. Effect of Different Immune Sera on Polymerase Activity

Polymerase activity was assayed by incubating different amounts of normal uninfected rabbit serum (●), anticore serum (■), and anticoat serum (○), with standard assay mixtures described in Table 1. Incubation was for 20 min at 37°C; samples were precipitated with TCA and collected on glass fiber filters.

viral proteins might aid in determining the specific role of each protein in the transcriptive complex.



SECTION II. Analysis of the Product of the *in vitro* Assay

1. Nature of the Product

To determine the nature of the product synthesized *in vitro* and to get an estimate of its size, nearest neighbour analysis and the nucleoside to nucleotide ratios following alkaline hydrolysis were examined.

Nearest neighbour analysis involves incubating the enzyme extract with a reaction mixture in which one of the nucleoside triphosphates is labelled with $\alpha\text{-}^{32}\text{P}$ at the 5'-position of the ribose. Following polymerization the product RNA is hydrolyzed with alkali, which cleaves the phosphodiester linkages resulting in the transfer of $\alpha\text{-}^{32}\text{P}$ phosphate residue from a 5'-nucleotide to the 3'-position of its nearest neighbour on the left. If the RNA product is a heteropolymer then radioactivity would be associated with each of the nucleoside-3'- or 2'-phosphate, and analysis of the amount of radioactivity would yield the frequency with which each of the bases occupy the position next to the base originally labelled.

When such an experiment was done using [$\alpha\text{-}^{32}\text{P}$] GTP as the labelled substrate, and the hydrolyzed bases were separated by chromatography, radioactivity was found in all four mononucleotides indicating that each of the four nucleotides was present next to GMP, and hence the synthesized

product was a heteropolymer (Table 9, Exp. I).

In order to determine if the incorporated radioactivity represents synthesis of substantial lengths of RNA rather than terminal addition of a few nucleotides to pre-existing RNA, the reaction product labelled with either ^3H -GMP or ^{14}C -CMP was hydrolyzed with KOH. The resulting nucleosides and nucleotides were separated and quantitated by paper chromatography.

If incorporation represents terminal addition of only a few nucleotides to pre-existing chains, then upon alkaline hydrolysis of product labelled with GTP or CTP a high percentage of the total radioactivity would be found in the guanosine or cytidine region respectively. The actual distribution of radioactivity of hydrolyzed product on paper chromatography is illustrated in Fig. 17, and the data is summarized in Table 10. Significant radioactivity (>98%) is found at positions corresponding to GMP, fractions 21-26 in one experiment, and fractions 13-18 in another independent experiment (Fig. 18). The radioactivity in the nucleoside fraction represents less than 2% of the total, and as indicated this amount is only slightly higher than the background radioactive level which represents about 40 counts. The G/GMP ratio indicates that product formed in the reaction was on an average 60 nucleotides long. Analysis of hydrolysate of ^{14}C -CTP labelled product (Fig. 19, Table 10,

Table 9

Nearest Neighbour Analysis of RNA Polymerase Product

Experiment	Distribution of Radioactivity			
	CpG	GpG	ApG	UpG
I Complete	11.5 ± 0.5	25.1 ± 0.6	32.3 ± 0.7	30.5 ± 0.7
II Minus UTP	11.7 ± 0.5	22.2 ± 1.1	32.0 ± 0.8	33.1 ± 2.2

A standard reaction mixture utilizing [α - 32 P]-GTP (8.6 μ Ci, 1.0 Ci/mmmole) as the labelled triphosphate and 1 mg protein of dialyzed cytoplasmic extract, with or without added UTP, was incubated at 37°C for 1 hour. Alkaline hydrolysis and paper chromatography were carried out as described in METHODS.

Results are expressed as average percentages, with standard errors determined from duplicate runs of three independent experiments.

Table 10

Distribution of Radioactivity in Alkaline Hydrolysate of Labelled Viral RNA Polymerase

Product

Experiment	Precursor	GMP	CMP	UMP	G	C	cpm in nucleoside (% of total)
I	³ H-GTP	2,392	-	-	39	-	1.6
II	¹⁴ C-CTP	-	10,079	180	-	160	1.5
III	¹⁴ C-CTP Minus UTP	-	5,600	175	-	240	4.3

Reaction mixtures containing 10 μ Cl of ³H-GTP or 1 μ Cl of ¹⁴C-CTP (92 mCi/mmole) as the labelled substrate (with or without added UTP) were incubated for 1 hour at 37°C and prepared for paper chromatography as described in METHODS.

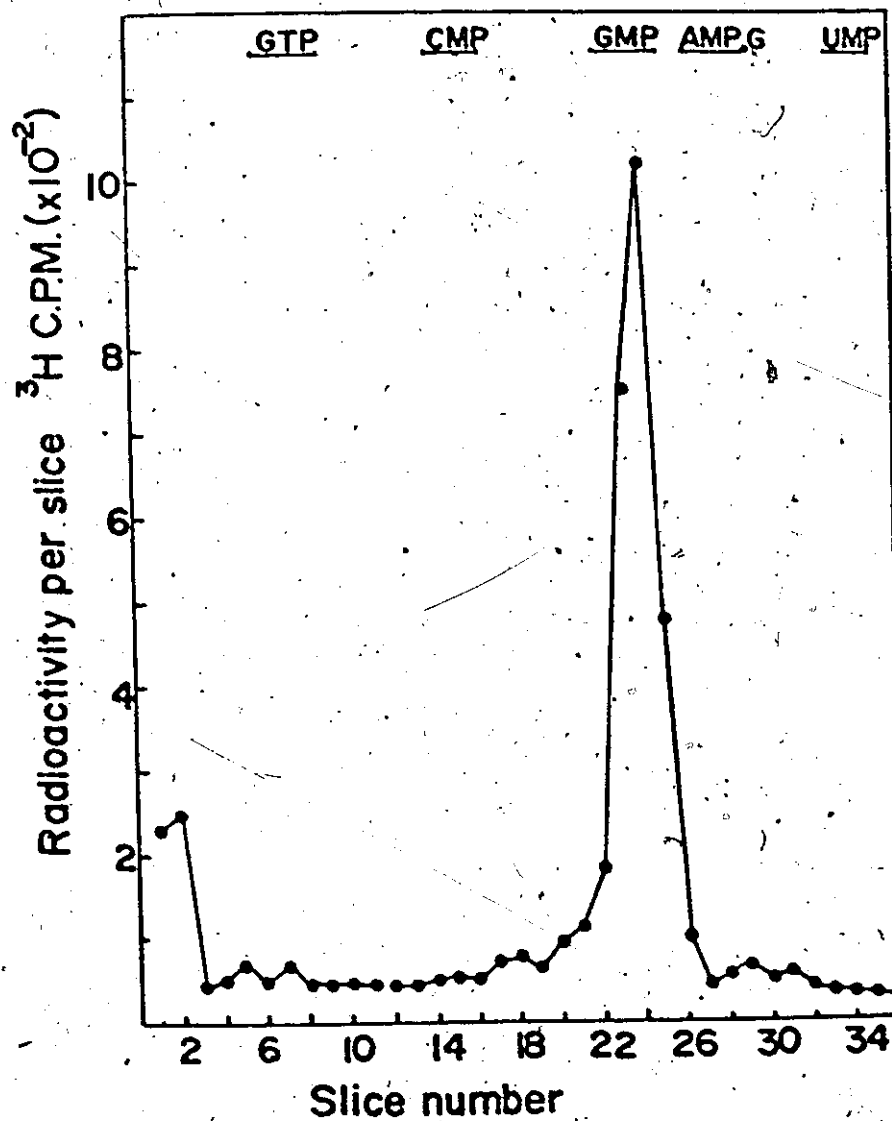


FIGURE 17. Paper Chromatographic Analysis of the Alkaline Hydrolysate of Product Labelled with ^3H -GTP
 Reaction mixtures containing 10 μCi of ^3H -GTP as the labelled substrate were incubated for 1 hr at 37°C and prepared for paper chromatography as described in METHODS. The brackets indicate the positions of each of the unlabelled markers, analyzed on the same piece of chromatography paper and determined by scanning the paper with UV light. The peak at slice numbers 1 and 2 represent material left at the origin.

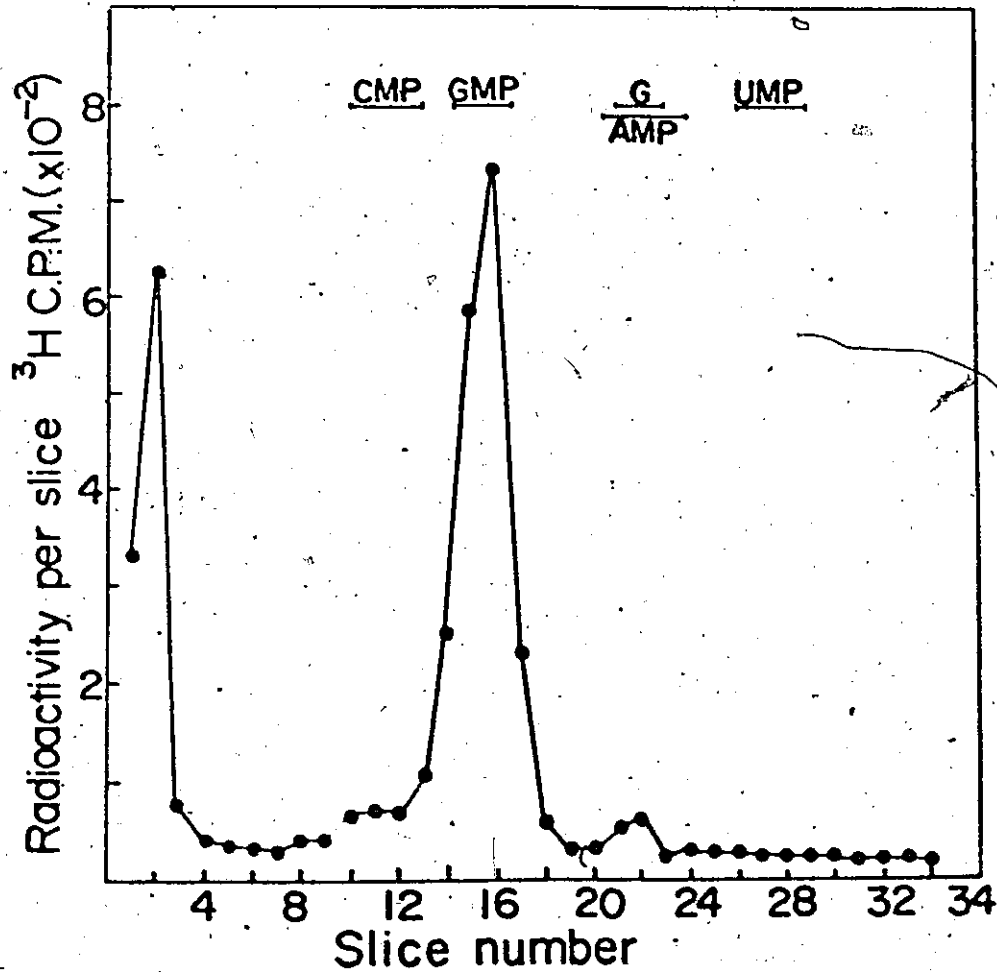


FIGURE 18. Paper Chromatographic Analysis of the Alkaline Hydrolysate of Product Labelled with $^3\text{H-GTP}$

The procedures are identical to those described in Figure 17, except that a different enzyme extract was used to synthesize the product.

Expt. II) yields similar results. Therefore the above results indicate that a heteropolymer consisting of at least 60-nucleotides was synthesized.

Since it was observed earlier that substantial incorporation of ^3H -GTP occurred in the absence of UTP, nearest neighbour analysis was repeated without this triphosphate to determine if the synthesized RNA contained UMP. The results obtained were similar to the nearest neighbour frequency obtained in the presence of UTP (Table 9, Expt. II). This suggested again that a heteropolymer was synthesized, and although exogenous UTP was not added, it was present in the reaction mixture and was incorporated next to GMP.

In order to check the possibility that UTP was generated by deamination of CTP, a product synthesized in a standard assay mixture with ^{14}C -CTP as the labelled substrate and without added UTP was digested with KOH and analyzed by paper chromatography. As seen in Fig. 19 and Table 10, Expt. III, in the absence of added UTP the radioactivity in the nucleoside fraction represented about 4% of the total indicating that this product was shorter than the RNA synthesized in the presence of UTP. About 3% of the amount of radioactivity in the product made in the absence of UTP, and 1.5% in the RNA synthesized in the presence of UTP was found in the UMP region. Although these values represent only

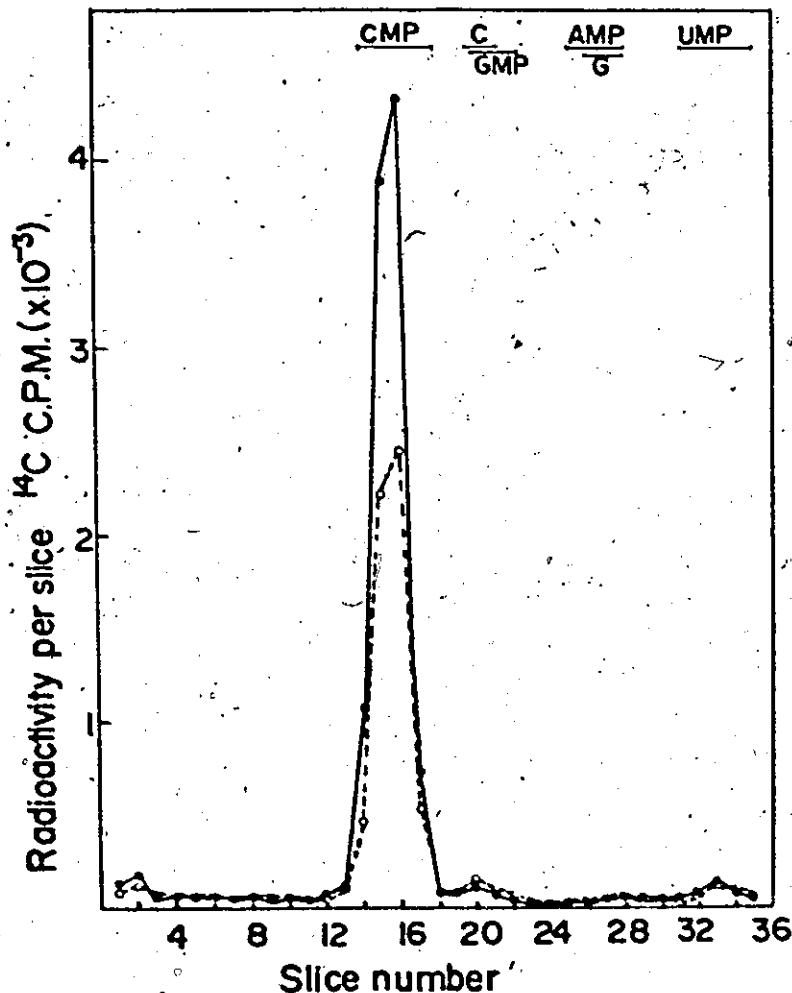


FIGURE 19. Paper Chromatographic Analysis of the Alkaline Hydrolysate of Product Labelled with ^{14}C -CTP

Reaction mixtures containing 1 μCi of ^{14}C -CTP (92 mCi/mmole) as the labelled substrate with (●) or without (o--o) UTP were incubated for 1 hr at 37°C and prepared for paper chromatography as described in METHODS. The brackets indicate the positions of each of the unlabelled markers determined by scanning the paper with UV light. The position of C was determined from the data by Lane (1963).

180 counts above background, they are associated with distinct regions as depicted in Fig. 19. If these results represent true deamination, then part of the observed incorporation of ^3H -GTP in the absence of UTP (Table 3) might be accounted for by these results.

However approximately the same amount of radioactivity was found in the region corresponding to UMP regardless of whether the product was synthesized in the presence or absence of added UTP (Table 10). It would be expected, if deamination of CTP to UTP does occur, that the labelled UTP produced in this manner would be diluted to a very high degree in the presence of unlabelled UTP and hence that much less radioactivity would be incorporated into the UMP region in the presence of exogenous UTP.

In view of this it would seem likely that the radioactivity which cochromatographs with UMP in this experiment is not actually present in UMP but in some other unidentified constituent.

2. Size of the Product

In the infected cell, it has been shown that the polymerase activity is associated with two large distinct complexes (Section I). For this reason it was of interest to analyze the size of the RNA products synthesized by each complex.

Accordingly, fractions of a cytoplasmic gradient, corresponding to the position of the two complexes were separately pooled and incubated with standard assay mixtures. The ^3H -GTP labelled RNA product was extracted with phenol and analyzed by rate zonal sedimentation on sucrose gradients. Radioactivity was detected in RNA molecules with sedimentation coefficients ranging from 5-18S, as estimated by extrapolation from the cytoplasmic RNA markers. As illustrated in Fig. 20, the size distribution of the products synthesized from the small complex is similar to that of the products of the larger polymerase complex.

3. Association of Nascent Product with RNA Template

Since the ribonucleoprotein complex serves as a template for the polymerase, it might be expected that RNA being transcribed may be initially associated with virion RNA while completed products should be released from the complex. To examine this possibility the synthetic reaction was terminated after 5, 10 and 20 min of incubation at 37°C and the products were extracted with phenol and separated in a sucrose gradient as seen in Fig. 21.

While some 95% of the RNA product synthesized after 20 min of incubation sedimented between 5 and 18S in agreement with the result presented above, the shorter incubation

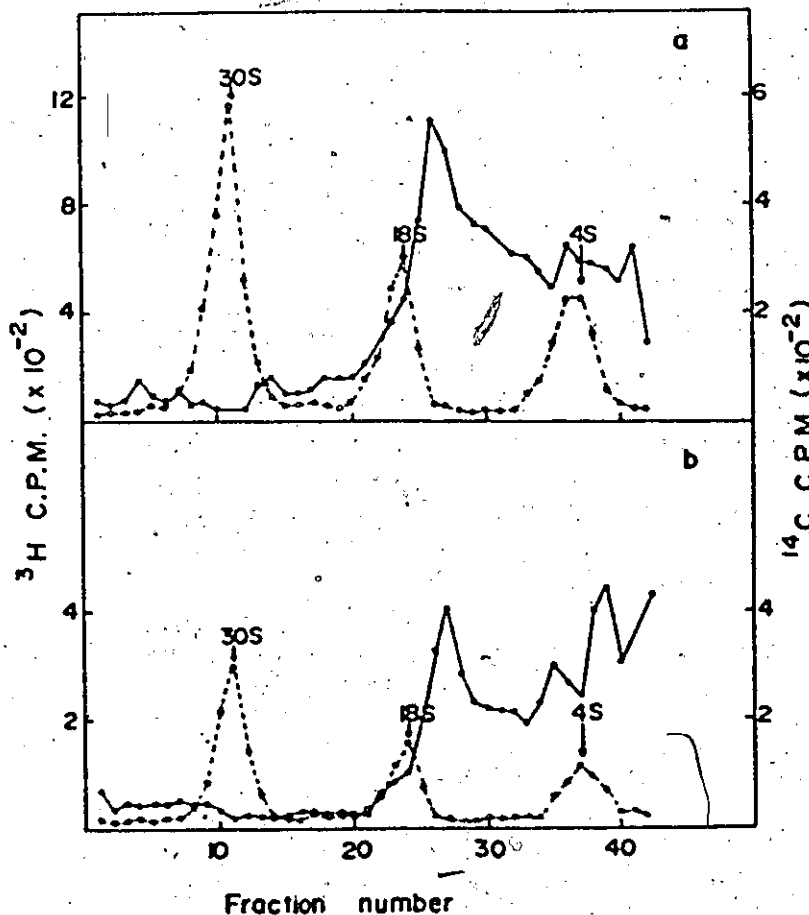


FIGURE 20. Sucrose Gradient Analysis of the RNA Product of the Ribonucleoprotein Complexes

The RNA synthesized for 1 hr, at 37°C by complexes corresponding to fractions 7-12 (a) and fractions 13-20 (b) of Figure 13 were phenol extracted and analyzed with ^{14}C -labelled ribosomal RNA (O) as marker on a 5-20% sucrose gradient in STE-0.1% SDS buffer as described in METHODS. The bottom of the gradient tube is towards the left.

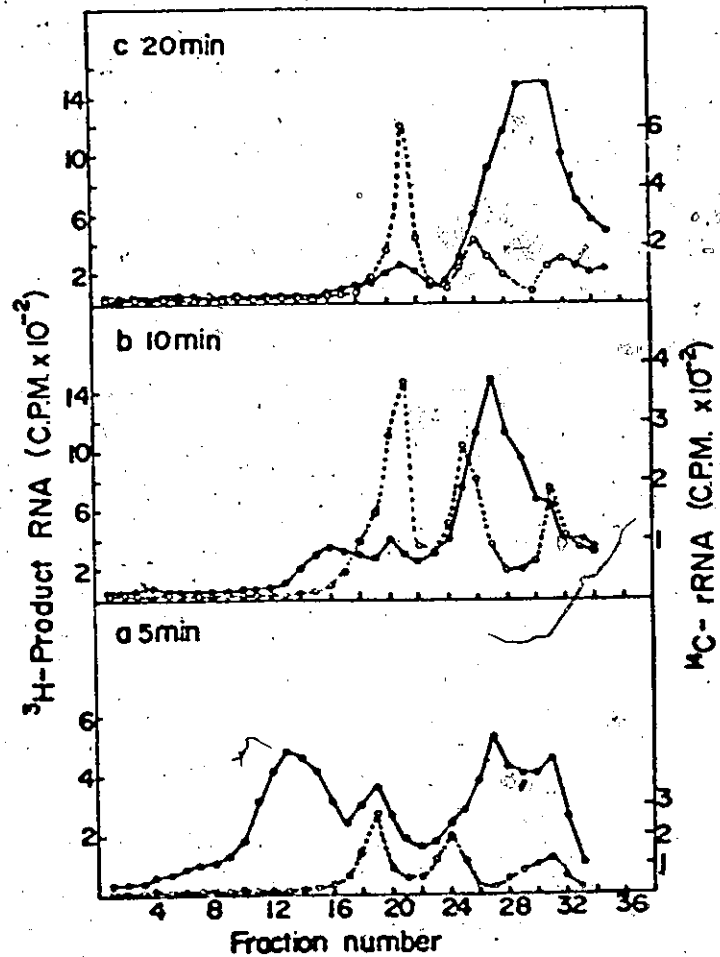


FIGURE 21. Kinetic Analysis by Sucrose Gradient Centrifugation of Product Synthesized by Polymerase

A 12-fold standard reaction mixture was incubated at 37°C, and 6x, 4x, and 2x reaction mixtures were removed at 5, 10, and 20 min respectively after start of incubation. The RNA was extracted with cold phenol-isoamyl alcohol-chloroform mixture, and centrifuged along with ^{14}C -uridine labelled L-cell ribosomal RNA as marker (o) on a 5-20% sucrose gradient in STE-0.5% SDS buffer, at 15°C and 48,000 rpm for 1.5 hours. The marker regions from the left represent 30S, 18S and 4S RNA respectively. Collected fractions were acid precipitated and radioactivity determined (●). The centrifugation profiles for 5, 10 and 20 min samples are given in (a), (b) and (c) respectively.

periods showed that a significant proportion of labelled product was associated with larger RNA species. The 5 min product, for example, showed approximately 36% of its radioactivity sedimenting at 40S, the sedimentation coefficient of B particle RNA. This result is consistent with the hypothesis that newly synthesized or nascent RNA is associated with the virion RNA template and released after synthesis from the complex. More detailed experiments of this type must be done to determine the nature of the 40S RNA complex, the nature of the material sedimenting at 30S and the rate of synthesis and release of RNA strands.

4. Kinetics of Product Synthesis

The kinetics of synthesis of product RNA were examined. At 37°C incorporation of ^3H -GTP into acid precipitable material continued in a linear fashion for the first hour and reached maximum levels after 60-90 min (Figs. 22A, 23A, 24A). Addition of more enzyme to the reaction mixture after the maximal level was reached resulted in a further stimulation of incorporation at 37°C. The latter fact suggested that the plateau of incorporation was not due to depletion of triphosphates. Similar kinetics of synthesis of product at 37°C were observed with the virion-associated polymerase (Baltimore et al., 1970).

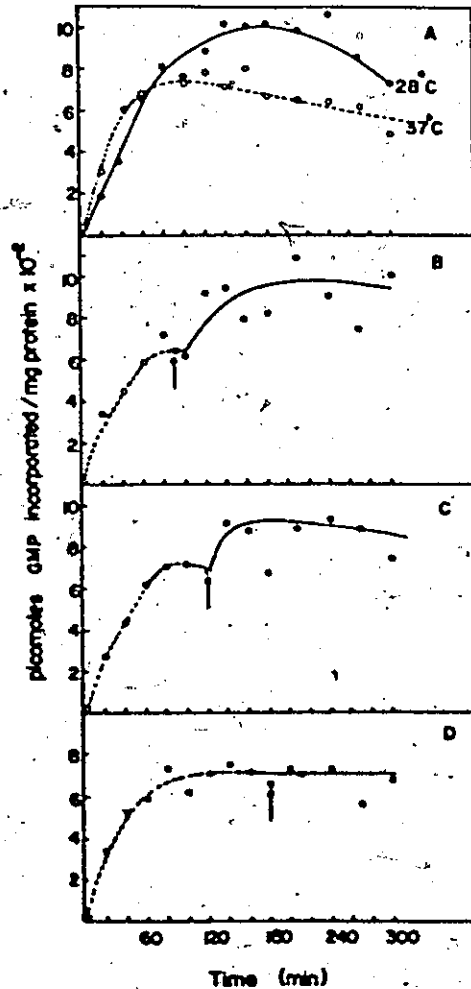


FIGURE 22. Kinetic Analysis of GMP Incorporation in Samples Shifted from 37°C to 28°C.

A 10-fold reaction mixture was prepared containing ^3H -GTP (Sp. Act. 0.04 mCi/ μmole). The mixture was divided into 5 tubes and one tube was kept at 28°C (●) (A), and one at 37°C (○) (A), these constituted the controls for continuous incorporation. Three other tubes were put at 37°C. After 90 (B), 120 (C) and 180 (D) min of incubation at 37°C tubes were transferred to 28°C and incorporation was determined at the times indicated by TCA precipitation of 50 μl of each sample.

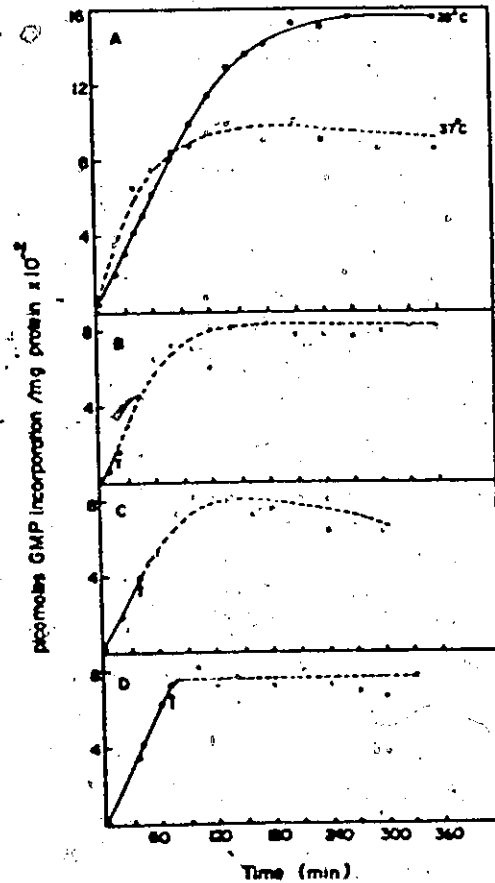


FIGURE 23. Kinetic Analysis of GMP Incorporation in Samples Shifted from 28°C to 37°C

A 10-fold reaction mixture was prepared containing ^3H -GTP (Sp. Act. 0.1 $\mu\text{Ci}/\mu\text{mole}$). The mixture was divided into 5 tubes. One portion (1 ml) was incubated at 37°C, and 4 tubes at 28°C. After each of 20, 40, and 60 min of incubation at 28°C one tube was transferred to 37°C. The incorporation of ^3H -GMP into RNA were determined in each tube by TCA precipitation of 50 μl samples at intervals shown.

- (A) Incorporation at 28°C (●), at 37°C (○).
- (B) Shift to 37°C after 20 min at 28°C.
- (C) Shift to 37°C after 40 min at 28°C.
- (D) Shift to 37°C after 60 min at 28°C.

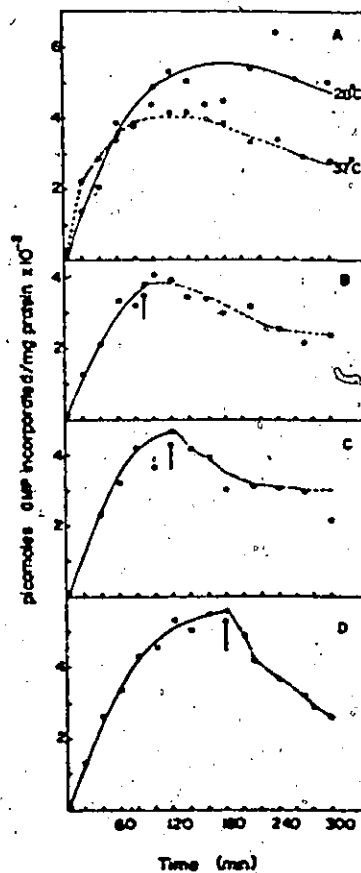


FIGURE 24. Kinetic Analysis of GMP Incorporation in Samples Shifted from 28°C to 37°C

A 10-fold reaction mixture was prepared containing ^3H -GTP (Sp. Act. 0.04 mCi/ μmole) and the experiment was done as described in Fig. 23, except that 1 ml portion was transferred from 28°C to 37°C water bath at 90, 120 and 180 min after start of incubation.

- (A) Incorporation at 28°C (●), at 37°C (○).
- (B) Shift to 37°C after 90 min at 28°C.
- (C) Shift to 37°C after 120 min at 28°C.
- (D) Shift to 37°C after 180 min at 28°C.

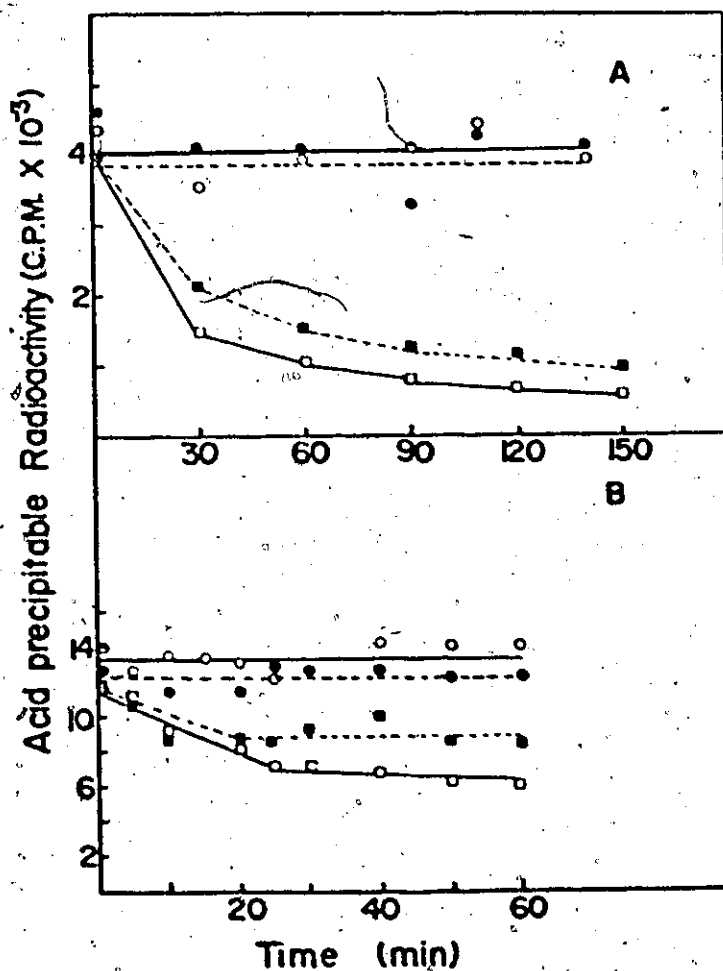


FIGURE 25. Degradation of Added RNA by a Reaction Mixture Under Assay Conditions

Four complete, separate, reaction mixtures each containing all four unlabelled nucleotide triphosphates were prepared. A constant amount of ^3H -GMP labelled RNA synthesized in a reaction mixture and purified by hot phenol extraction was added to each. To 2 of the mixtures polymerase extract was added, and one of these tubes was incubated at 28°C (\blacksquare), and the other at 37°C (\blacksquare). Similarly one of the tubes lacking the extract was incubated at 28°C (\bullet) and the other at 37°C (\circ). At the shown times, 50 μl of each tube was TCA precipitated and radioactivity determined. The two presented experiments are similar except that (A) was carried out for 150 min, and (B) for 60 min.

The kinetics of RNA synthesis were repeated at 28°C since at this lower temperature the virion associated polymerase activity proceeds linearly for several hours (Huang et al., 1971; Aaslestad et al., 1971; Bishop, 1971). The incorporation by the intracellular enzyme at 28°C was linear for 140 min, after which no further net synthesis occurred. Although the initial rate of incorporation was greater at 37°C than at 28°C, net RNA synthesis at 28°C was up to two-fold greater than at 37°C (Figs. 22A, 23A, 24A).

On prolonged incubation at either temperatures it was observed that a loss in radioactivity occurred, which suggested the presence of nucleases. In order to investigate this possibility, the fate of exogenous RNA added to a complete unlabelled reaction mixture was followed. The exogenous labelled RNA was synthesized by the enzyme and extracted with phenol as described in METHODS. Fig. 25 illustrates the degradation of this RNA as measured by loss of acid-insoluble material with time of incubation. It is evident that nucleolytic activity was present in the extract at both temperatures. Examination of the final levels of residual RNA either at 60 min (Fig. 25A) or at 2.5 hrs (Fig. 25B) indicate that slightly more degradation occurred at 37°C than at 28°C.

To further investigate the nature of the different kinetics of incorporation at 28°C and 37°C, temperature shift

experiments were done. Reaction mixtures that were initially incubated at 37°C and then transferred to 28°C after 90 and 120 min of incubation showed a rapid increase in the rate of incorporation after the shift, with gradual slowing to 28°C rate (Fig. 22B,C). However when the samples were shifted to 28°C after 180 min no further increase in incorporation was observed.

Reverse experiments were also done. The incorporation of reaction mixtures initially incubated at 28°C and then transferred to 37°C at 20, 40 and 70 min reached plateau values by 80 min in a manner identical to the sample which had been continuously incubated at 37°C (Fig. 23). On the other hand, if the reaction mixtures were shifted to higher temperatures at 90, 120 and 180 min, the acid precipitable radioactive product in each sample after the shift decreased rapidly to the level present in the sample maintained continuously at 37°C (Fig. 24B,C,D).

In the analysis of the different kinetics it is clear that we must consider both the synthesis of product RNA and its degradation at that temperature. Since in these experiments the two processes cannot be independently identified the apparent rates of reaction and the final plateau levels attained may be the net effect of these two reactions.

One of the ways to explain the difference between the incorporation observed at 28°C and 37°C is to assume that

the rate of nuclease degradation is higher at 37°C than at 28°C, but that it is constant at either temperature throughout the interval examined. Then the two-fold increase in incorporation after 60 min at 28°C as compared to 37°C can be attributed to less degradation at the lower temperature. Similarly the loss in labelled material at late times at both temperatures may be due to the action of nuclease with no concurrent RNA synthesis by the polymerase at these times. In the same manner the increase in incorporation on shift from 37°C to 28°C would be due to diminished degradation while activation of nucleases at 37°C could account for the decrease in radioactive material resulting from the 28°C to 37°C shift.

Although nucleolytic activity was present in the polymerase extracts the specific effect of this activity in producing the results observed cannot be completely assessed. For this reason we can consider an alternative hypothesis regarding the increase in amount of product synthesis at 28°C. It has been observed by Bishop (1971) using purified transcriptase from virus that the product of 28°C incubation is complementary to a larger fraction of the infectious virus genome than is the product made at 37°C. In the following experiments we utilized hybridization with RNA genomes from both infectious virus and from each of the defective particles to determine the relative extent of transcription by the intracellular

polymerase at 37°C and 28°C.

5. Hybridization of the Product to Virion RNA

The first requirement for this investigation was to determine to what extent the polymerase product synthesized at different temperatures was complementary to the infectious virion RNA. Standard reaction mixtures containing ^3H -GTP (at a Sp. Act. of 0.04 mCi/ μmole) were incubated at 37°C and 28°C respectively. RNA was extracted after 60 and 220 min of incubation at each temperature. The 60 min product was designated as "early" and the 220 min as "late" according to the kinetic experiments presented in Fig. 22A.

Annealing of both the "early" and "late" labelled RNA synthesized at 28°C and 37°C to excess unlabelled B RNA showed that the RNA product was completely complementary in base sequence to the infectious RNA. Thus only positive strands were synthesized under the above conditions. Complete hybridization of these products required some 4 μg of virion RNA under the conditions used (Fig. 26, 27). It might be noted that annealing of product RNA in the absence of added excess unlabelled viral RNA demonstrated 10-15% resistance to RNase digestion. This background level appears not to be due to double-stranded RNA, since neither RNase treatment immediately following melting or after 24 hours of hybridization

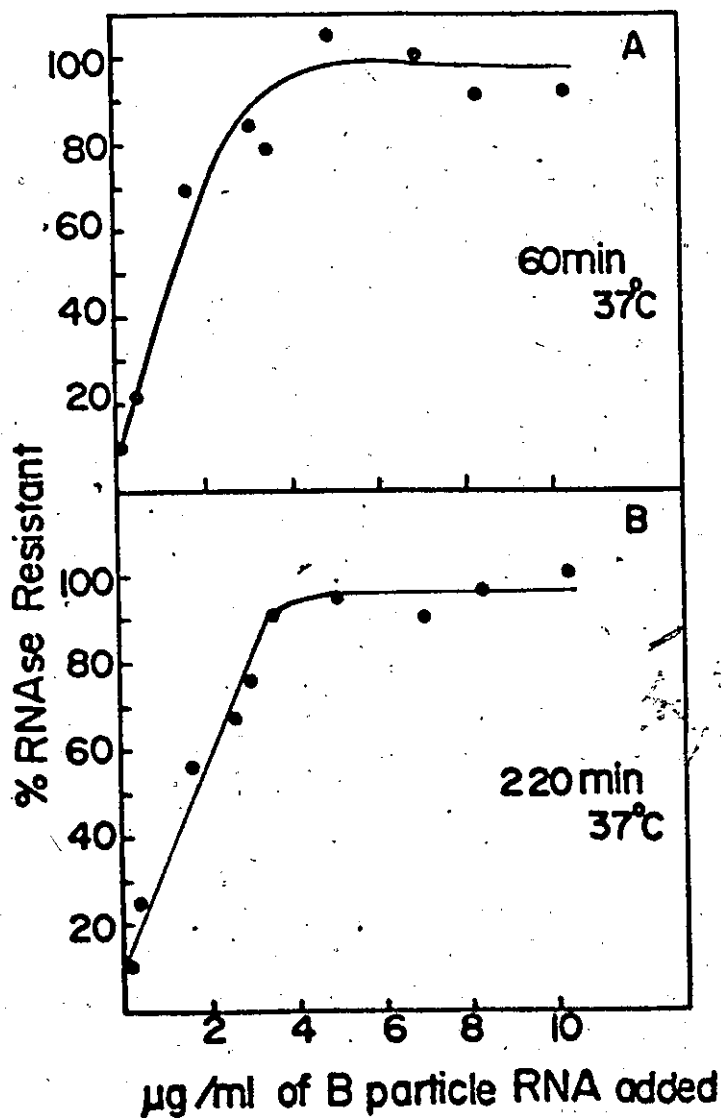


FIGURE 26. Annealing of GMP-Labelled Product RNA to Unlabelled B Particle RNA

About 3000 cpm (0.08 $\mu\text{g/ml}$) of the *in vitro* product synthesized during the first 60 min (A), and 220 min (B) at 37°C was annealed to the indicated amounts of B RNA. The total hybridization volume was 0.3 ml, and hybridization time was 5 hours at 65°C. After annealing, the complete 0.3 ml was digested by RNase A. The samples to which unlabelled virion RNA was not added, were used as control for total radioactivity.

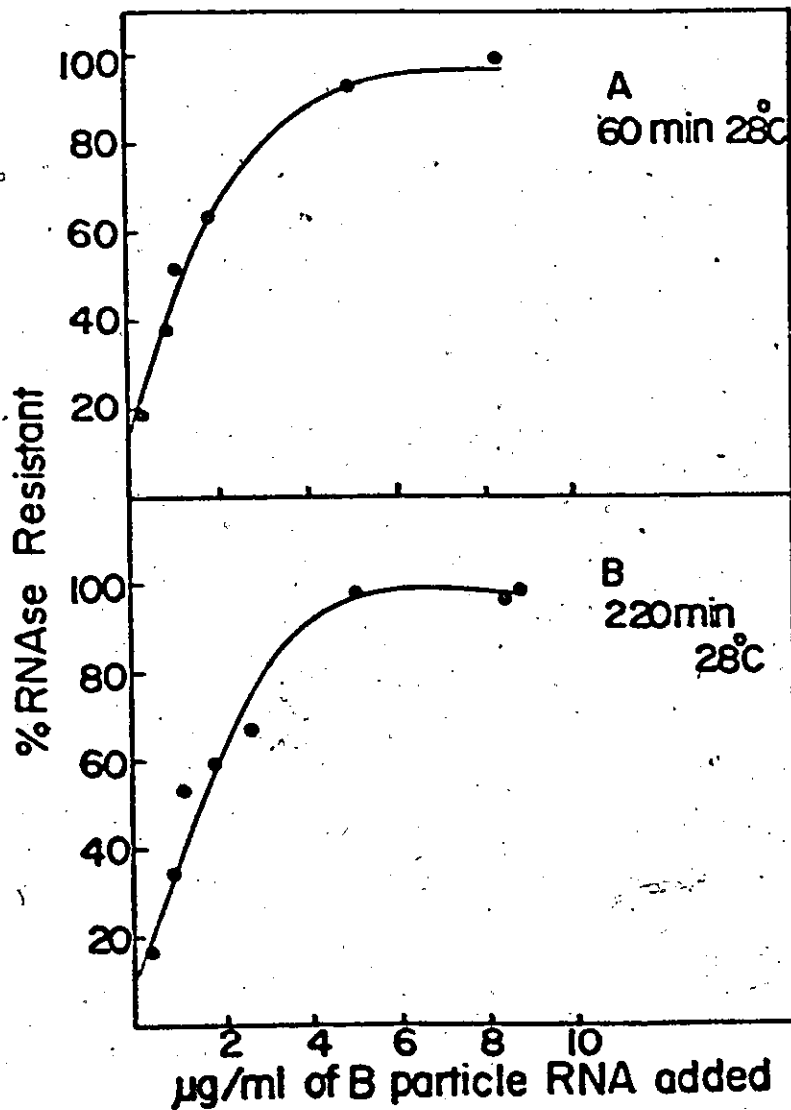


FIGURE 27. Annealing of 28°C GMP-Labelled Product to B Particle RNA

About 6000 cpm (approximately 0.08 µg/ml) of the *in vitro* product synthesized for 60 min (A) and 220 min (B) at 28°C was annealed to increasing amounts of unlabelled B RNA, in a 0.6 ml total volume for 2.5 hours. Digestion of half of each sample with ribonuclease was as described in METHODS.

appreciably changed this value.

In order to map, by use of the defective particles, the region of virion RNA which is transcribed in vitro, it was necessary to first show that the RNA of these particles is not complementary to B RNA. The addition of increasing amounts of unlabelled B particle RNA to a constant amount of labelled LT and ST particle RNA did not confer added protection to RNase digestion which suggests that both the LT and the ST RNA are not complementary to B particle RNA. Also no significant self-annealing was observed with B, LT and ST particle RNA indicating that these viral particles do not contain plus and minus strands of RNA (Table 11). The fact that the LT and ST genomes represent specific segments of the B particle genome was shown by Bishop and Roy (1971b), Schincariol (1972) and Hallett (1972).

The hybridization of both early and late RNA made at 37°C to RNA genomes from LT particles, showed almost complete annealing, indicating complementary nucleotide sequences between the two RNAs (Fig. 28). Similarly 100% of the "late" 28°C and 90% of the "early" 28°C RNA was complementary to LT (Fig. 29). Complete hybridization of these products required half as much LT RNA (2 µg) as B RNA under the conditions used. This is the expected result if LT particle RNA represents a unique half-segment of the B particle genome.

In contrast to the above results hybridization of

Table 11
Annealing of B Particle RNA and RNA of the
Defective Particles LT and ST

Labelled RNA	Unlabelled RNA μ g	Ribonuclease resistance (8)
B particles	B particles 1	12
B particles	B particles 3.5	11
LT particles	-	15
LT particles	B particles 1	15
LT particles	B particles 3.5	17
ST particles	-	18
ST particles	B particles 1	11
ST particles	B particles 4	16

Virion RNA was extracted from both labelled and unlabelled particles. The extraction and hybridization were carried out as described in METHODS. Each sample contained approximately 5000 cpm of ^3H -RNA. Each value is the average of two samples.

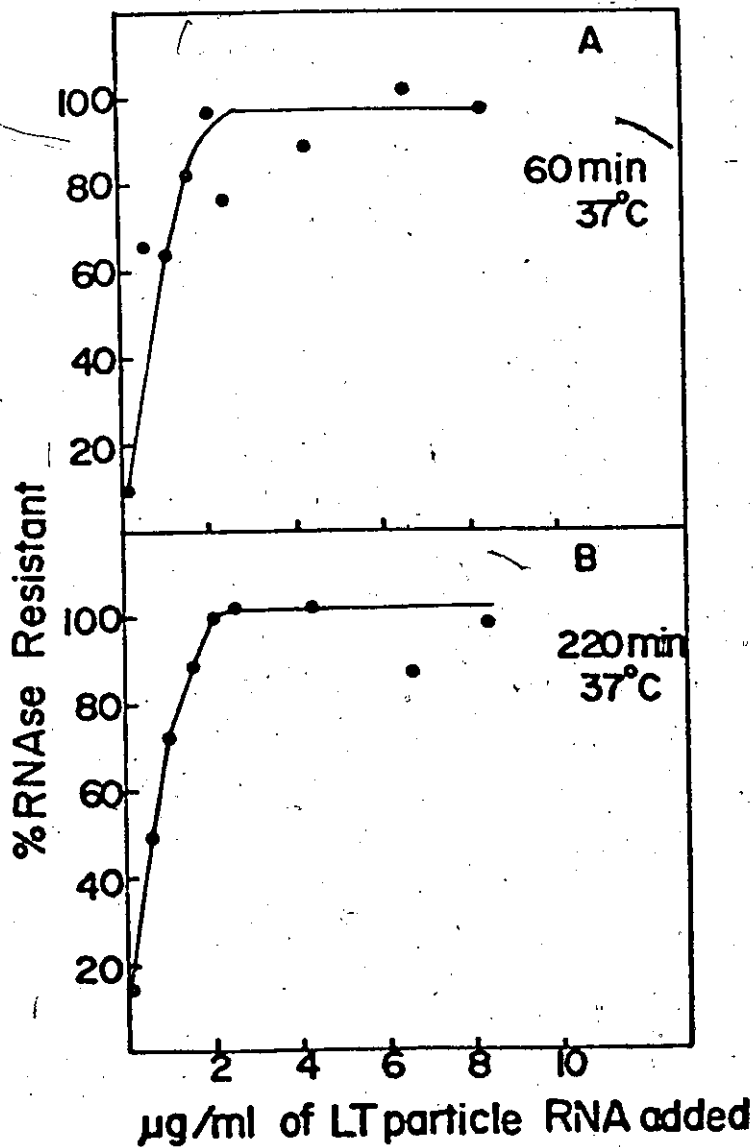


FIGURE 28. Annealing of Polymerase Product RNA to LT RNA. About 6000 cpm of the *in vitro* product synthesized at 37°C for 60 min (A) and 220 min (B) was hybridized to the indicated amounts of RNA extracted from LT particles. Hybridization conditions and determination of RNase resistance were done as described in METHODS.

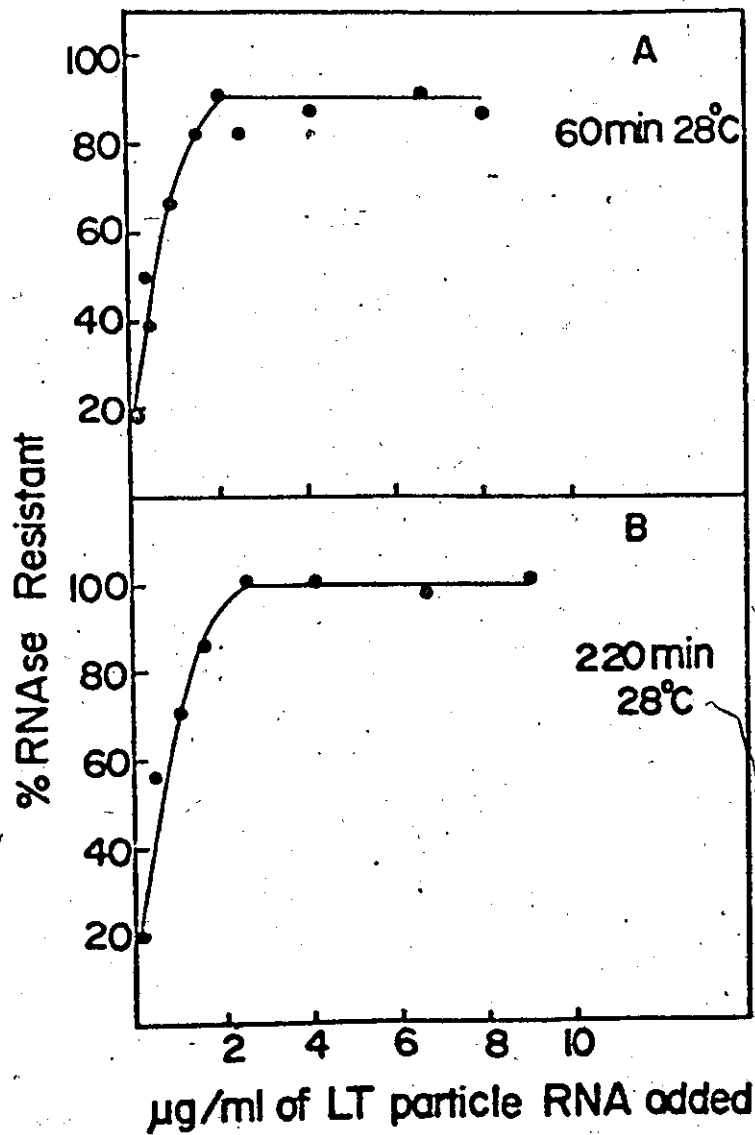


FIGURE 29. Complementary Between in vitro Synthesized RNA at 28°C and LT RNA

Annealing of approximate 6000 cpm of product RNA synthesized for 60 min (A) and 220 min (B) at 28°C with increasing amounts of LT RNA was carried out as described in METHODS.

either "early" and "late" 37°C product to increasing amounts of RNA from the ST particles showed no annealing above background levels (Fig. 30). However when either "early" or "late" 28°C product was annealed to ST RNA the result shown in Fig. 31 was obtained. While considerable scatter was observed in the individual points the general result seems to indicate some annealing of the 28°C product to ST RNA. Because of the scatter both the shape of the annealing curves and the final saturation levels are difficult to establish. It would appear however that some 10-15% of the RNA product made at 28°C by 60 or 220 min of synthesis is complementary to the ST particle genome.

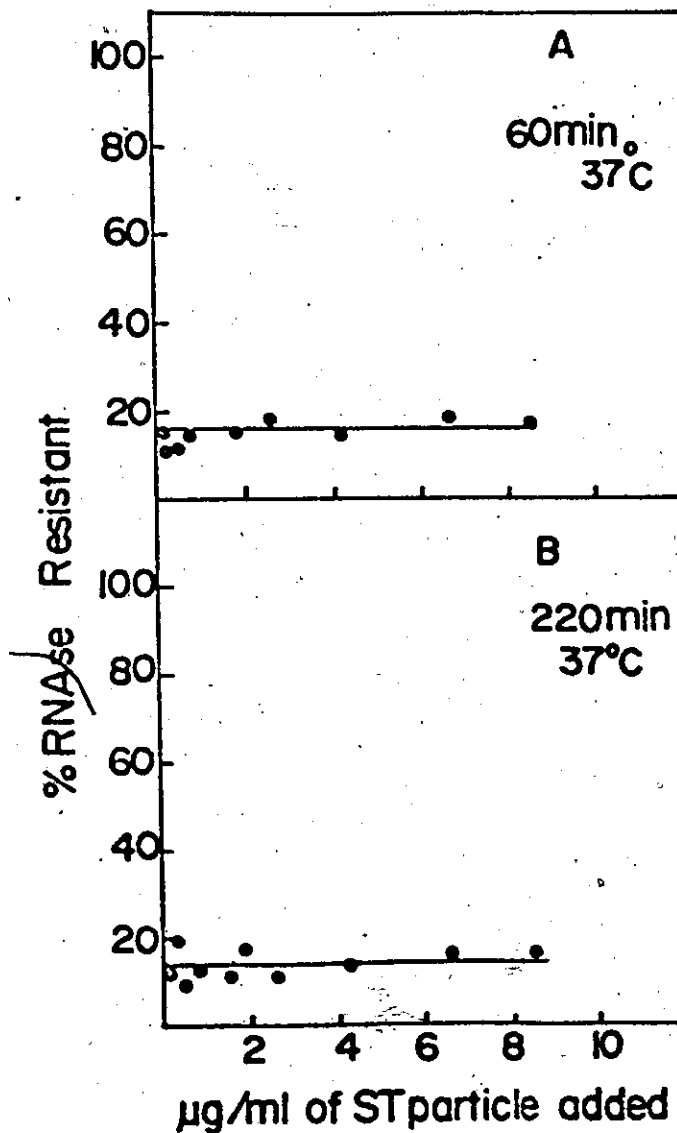


FIGURE 30. Annealing of Polymerase Product RNA (37°C) to ST RNA

Approximate 6000 cpm of ^3H -GMP RNA synthesized at 37°C for 60 min (A) and 220 min (B) was annealed to the indicated amount of RNA extracted from ST particles as described in METHODS.

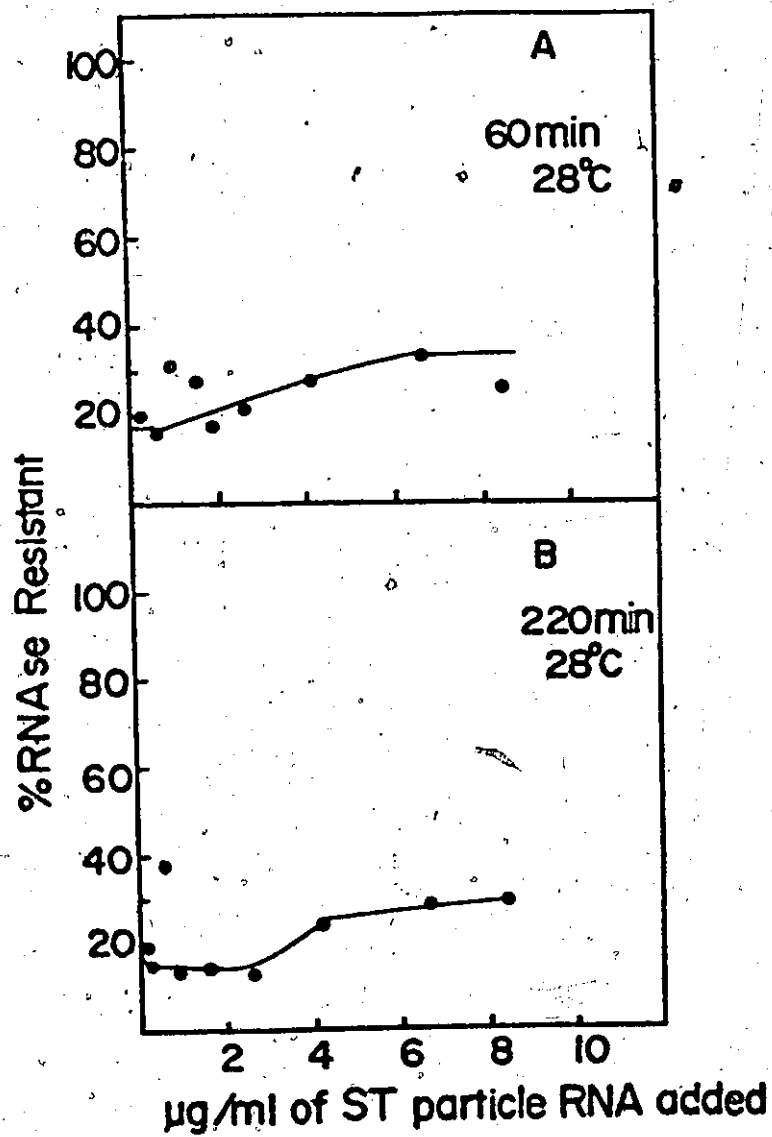


FIGURE 31. Complementarity Between the *in vitro* Synthesized RNA at 28°C and ST_b RNA
 About 6000 cpm of RNA synthesized at 28°C for 60 min (A) and 220 min (B) was annealed to increasing amounts of ST RNA as described in METHODS.

SECTION III. Poly A Synthesis by Cytoplasmic Extracts
of Infected Cells

1. Properties of the ^3H -ATP Labelled Product

While VSV specific mRNA species synthesized in vivo contain poly A segments (Soria and Huang, 1973; Ehrenfeld and Summers, 1972), the complementary RNA synthesized in vitro by the purified virion-associated transcriptase does not contain detectable A-rich sequences (D. B. Bishop - personal communication).

Substituting labelled ATP as a precursor for GTP in a standard reaction mixture, we tried to determine whether the RNA products synthesized in our system contain poly A sequences. The test for poly A sequences involved digestion of the product with pancreatic RNase and T_1 nuclease in 2 x SSC solution. Pancreatic RNase cleaves RNA next to pyrimidines, while T_1 nuclease is specific for Gp-Xp residues. The segment resistant to both these enzymes should consist almost entirely of adenylate residues. As shown in Tables 12 and 13, the ^3H -adenylate product contained some 18% ribonuclease-resistant material, while the product labelled with GMP had about 2% ribonuclease resistance. To check if this resistance was a function of the concentration of nucleases, ^3H -ATP and ^3H -GTP labelled product was subjected to digestion with increasing concentrations of the nucleases. As

Table 12

Susceptibility of the in vitro Product to Increasing
Concentrations of T₁ and Pancreatic RNase

Labelled Precursor	Total Incorporation cpm	RNase $\mu\text{g/ml}$	T ₁ U/ml	Nuclease Resistance cpm	%
³ H-ATP	3,640	5	37	530	14
³ H-ATP	2,590	10	75	625	24
³ H-ATP	4,140	20	150	730	17
³ H-ATP	3,300	30	225	760	23
³ H-ATP	2,940	40	300	540	18
³ H-GTP	38,500	5	37	490	1
³ H-GTP	36,550	10	75	590	1.6
³ H-GTP	37,080	20	150	500	1.3
³ H-GTP	30,070	30	225	310	1.0
³ H-GTP	31,320	40	300	300	1.0

One ml reaction mixtures were prepared containing either 6 μCi of ³H-ATP (22.6 Ci/mole) with 2.5 mM of the 3 unlabelled triphosphates, or 4 μCi of ³H-GTP (1.15 Ci/mole) with 2.5 mM of the 3 unlabelled triphosphates. After incubation at 37°C for 30 min each of the samples was split into two equal portions, one fraction precipitated with TCA for total radioactivity. And the other portion was made 0.3 M in NaCl and 0.03 M in Na citrate, and treated with pancreatic RNase and T₁ nuclease at concentrations indicated above for 30 min at 37°C and radioactivity determined after TCA precipitation.

Table 13

Nuclease Susceptibility of the *in vitro* Product of
a Polymerase Extract from VSV Infected Cells

Labelled Precursor	Total Incorporation cpm	Nuclease Resistant cpm	%
³ H-ATP	2,780	510	18
³ H-ATP	2,380	460	18
³ H-GTP	7,170	130	1.8
³ H-GTP	4,570	100	2
³ H-GTP	4,860	109	2
³ H-GTP	11,840	213	1.9

One ml reaction mixtures were prepared as described for Table 12, except that 2 μ Ci of ³H-GTP (1.15 Ci/mmmole) was used for the GTP labelled product. After incubation at 37°C for 30 min, the ³H-ATP samples were split into 0.4 ml portions, while the ³H-GTP samples were divided into 0.4, 0.2 and 0.1 ml. Each of the portions was TCA precipitated for total radioactivity with its corresponding fraction treated with RNase A (20 μ g/ml) and T₁ nuclease (150 U/ml) as described for Table 12.

illustrated in Table 12, no decrease in the RNase resistant material occurred with increased concentration of T_1 and RNase A. The resistance varied from 14-24% for $^3\text{H-ATP}$ product and from 1 to 1.6% for the $^3\text{H-GTP}$ labelled product. In order to ensure that the relatively high percentage of resistant RNA in the ATP labelled sample was not due simply to a high absolute background radioactivity, lesser amounts of ATP and GTP labelled product were compared as shown in Table 13. In either case, the resistance was still about 18% and 2% for $^3\text{H-ATP}$ and $^3\text{H-GTP}$ labelled products respectively.

The time course of ATP incorporation into total RNA and nuclease resistant product at 28°C and 37°C is shown in Fig. 32. Maximum synthesis of product was reached after 60 min of incubation at 37°C, while at 28°C it continued to rise. The total amount of RNA synthesized at 28°C was two-fold greater than at 37°C, a situation similar to the kinetics of $^3\text{H-GTP}$ labelled product although only about 1/5 as much RNA was made using $^3\text{H-ATP}$ as substrate as compared to GTP. The A-rich RNA appeared to be synthesized continuously during the first 60 and 120 min of incubation at 37°C and 28°C respectively.

The $^3\text{H-AMP}$ containing product synthesized after 60 min of incubation at 37°C was almost completely complementary to B particle RNA, indicating that it was transcribed off the B particle RNA. This labelled product was also totally

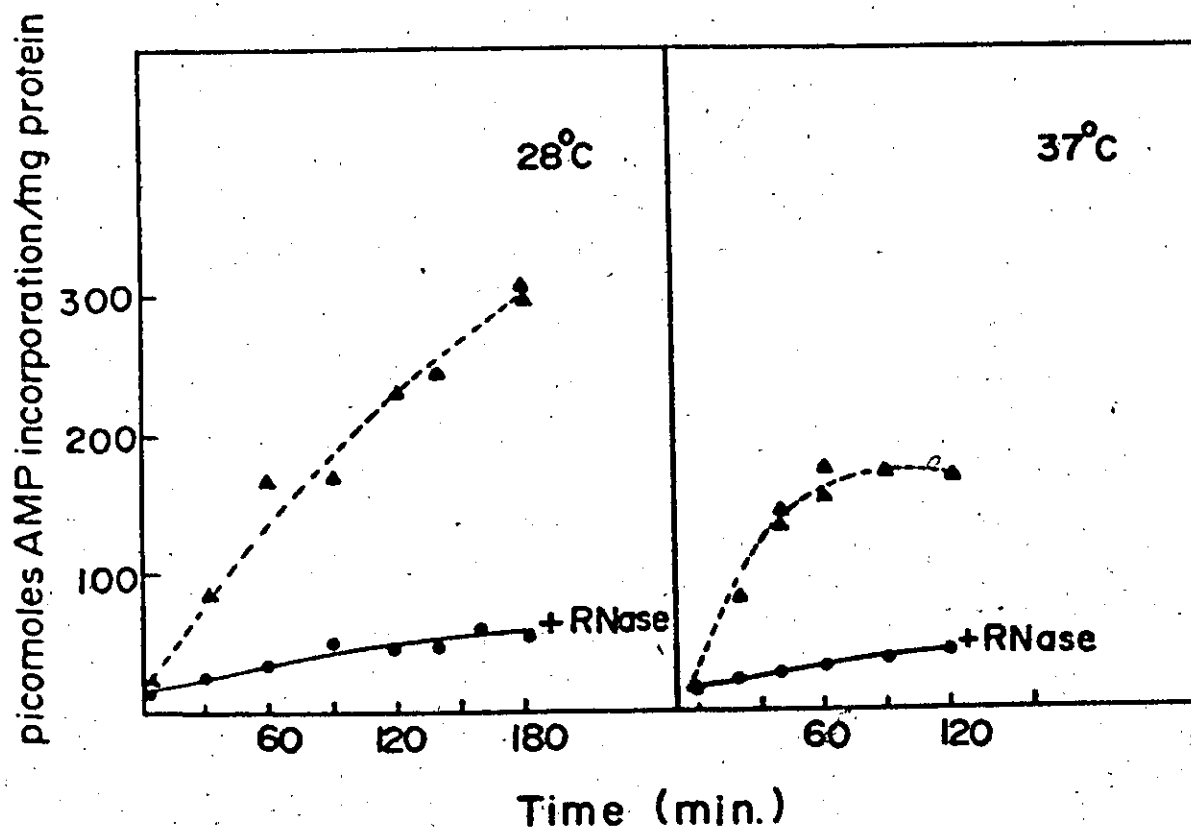


FIGURE 32. Kinetics of Incorporation of ^3H -ATP into Total Acid Insoluble and Nuclease Resistant RNA

Two one ml reaction mixtures each containing 2.5 mM CTP, UTP, GTP, 4 μCi of ^3H -ATP (60 mCi/mmol), and 2 mg of protein were prepared. One reaction mixture was incubated at 37°C and the other at 28°C. Two samples of 0.05 ml were removed from each reaction mixture at the indicated times; one sample was treated with nucleases as described in Table 13 (●) while the other was directly precipitated with cold TCA (▲).

sensitive to alkaline hydrolysis suggesting it was RNA (Table 14).

2. Size of the ^3H -ATP Product and of the A-Rich Fragments

Since the GTP-labelled transcription products of this enzyme were shown earlier to have sedimentation coefficients as large as 16S it was of interest to determine the size of the RNA containing the A-rich fragment.

The product labelled with ATP for 30 and 120 min of incubation at 37°C and for 4 hours at 28°C was phenol extracted and examined by sucrose gradient centrifugation. The labelled ATP was incorporated into 2 distinct product species of 16-18S and 4S at both temperatures (Fig. 33a,d; Fig. 34a). No significant increase in size was observed as a result of the longer labelling period or temperature difference. When a portion of each gradient illustrated in Figs. 33a,d, 34a was treated for 30 min at 37°C with pancreatic and T_1 nucleases the results presented in Figs. 33c,f and 34c were obtained. It is evident that A-rich segments (nuclease resistant) were present in regions of the gradients corresponding to the 16-18S and 4S peaks.

To determine the size of the A-rich sequences, the product of the above incubations was digested with pancreatic and T_1 nucleases for 30 min at 37°C immediately

Table 14

Properties of the *in vitro* Product Labelled with ^3H -ATP

	Total cpm	Nuclease Resistant cpm	%
^3H -ATP product self- annealed	1,970	740	37
^3H -ATP product annealed with B RNA	1,877	1,700	90
		<u>KOH Resistant</u>	
^3H -ATP product	30,000	175	0.6

For annealing with virion RNA, 5000 cpm of product RNA from a reaction mixture (containing 2.5 mM of CTP, UTP, GTP, 10 $\mu\text{Ci/ml}$ of ^3H -ATP, Sp. Act. 50 mCi/mole, and 2 mg of protein) were hybridized with 6 μg of B RNA as described in METHODS. To determine alkali stability of the ATP containing product, some 30,000 cpm of RNA obtained from the experiment presented in Fig. 33d were incubated at 37°C for 18 hours in 0.3 M KOH and the amount of TCA insoluble material (KOH resistant) determined.

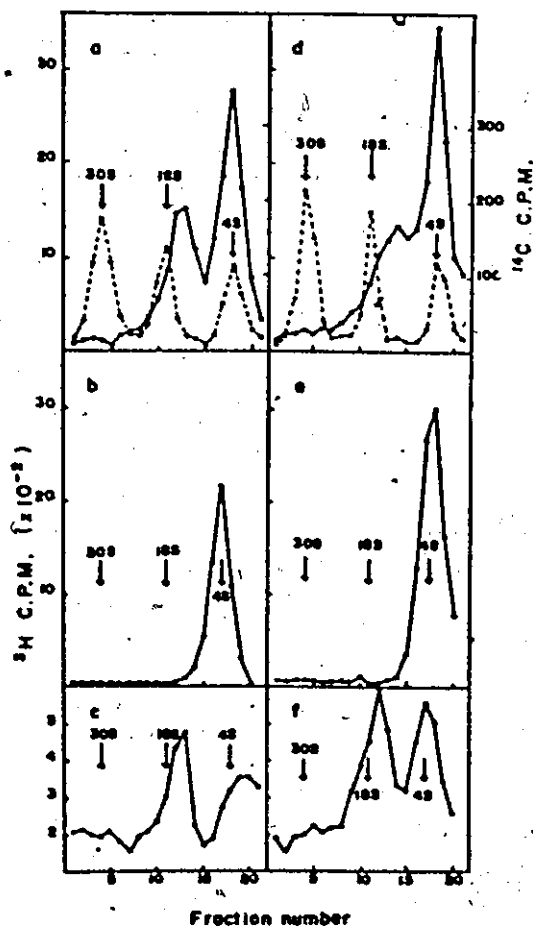


FIGURE 33. Sucrose Gradient Analysis of the A-Rich Fragments Synthesized by Cytoplasmic Extracts at 37°C

Ten ml of reaction mixture containing 2.5 mM of GTP, CTP, UTP and ^3H -ATP at 20 $\mu\text{Ci/ml}$ (20.5 Ci/mmmole) and 2 mg/ml of protein were incubated at 37°C. After 30 minutes and again at 120 minutes, 5 ml aliquots were removed and one-half of each aliquot was phenol extracted as described in METHODS while the other half was digested in RNase and T_1 in 2 x SSC at 37°C prior to phenol extraction. All four extracted RNA samples were centrifuged at 4°C and 180,000 g for 3 hours on 5-20% sucrose gradients in STE, together with ^{14}C -labelled rRNA as marker (o). The fractions collected from the nuclease pretreated samples, 30' (b) and 120' (e), were TCA precipitated directly and counted. The fractions from the other two gradients were first split into equal portions, one portion was TCA precipitated directly to give total counts [30' (a), 120' (d)] while the other was treated with pancreatic and T_1 nuclease in 2 x SSC prior to TCA precipitation to give nuclease resistant counts [30' (c), 120' (f)].

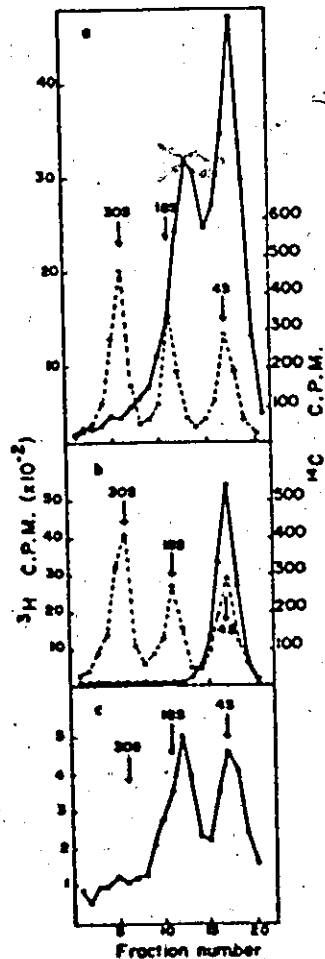


FIGURE 34. Sucrose Gradient Analysis of the A-Rich Fragments Synthesized by Cytoplasmic Extracts at 28°C

Four ml of reaction mixture as in Fig. 33, were incubated at 28°C. After 4 hours, 2 ml of the mixture was removed and RNA extracted. The remaining 2 ml was digested with nucleases for additional 30 min at 37°C followed by phenol extraction. The extracted RNA together with ^{14}C -labelled rRNA as marker was centrifuged on 5-20% sucrose gradients as in Fig. 33. The fractions from the gradient containing RNA treated with nuclease prior to centrifugation were acidified with TCA and counted (b). Each gradient fraction from the other gradient was divided into 2 equal portions. One portion was acidified and the precipitate collected to determine total ^3H -AMP incorporated (●) and ^{14}C -rRNA marker (○) (a). The other portion was digested with nuclease and radioactivity determined (c).

after incubation, then extracted with phenol and centrifuged along with marker RNA (C^{14} -ribosomal RNA) on sucrose gradients. As seen in Figs. 33b,e and 34b, the nuclease resistant A-rich fragments had sedimentation coefficients of approximately 4S. Identical results were obtained if RNA was first phenol extracted from the reaction mixture and then treated with nuclease prior to gradient analysis. Thus A-rich fragments of sedimentation coefficients of about 4S are synthesized by the polymerase extracts, and these A-rich fragments exist as both free 4S molecules and in association with large nuclease sensitive RNA molecules.

The synthesis of A-rich fragments in association with larger RNA species is dependent on the presence of all four ribonucleoside triphosphates. When a reaction mixture containing infected cell extract and only 3H -ATP without the three unlabelled triphosphates was incubated the synthesized product sedimented at 3-7S (Fig. 35C). Of this product some 27% was nuclease resistant and sedimented at less than 4S.

As it is possible that the enzyme responsible for poly A synthesis was distinct from the viral polymerase and could in fact be a cellular enzyme the incorporation of 3H -ATP by cytoplasmic extract from uninfected cells was examined. Fig. 35A indicated that a product sedimenting from 3-7S was synthesized. Some 10% of this product was nuclease resistant and had a sedimentation coefficient

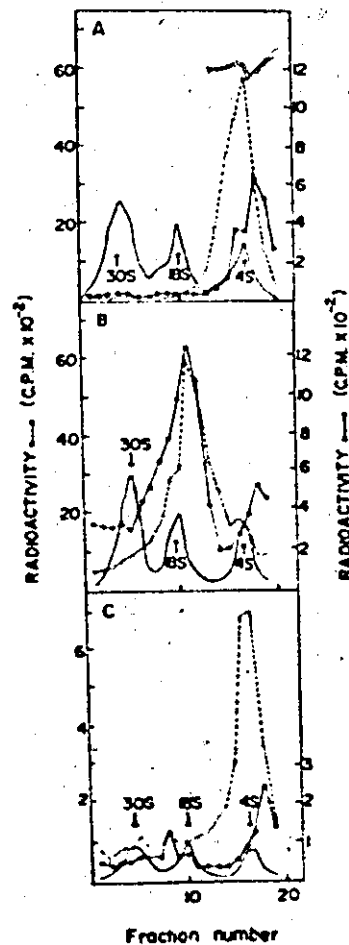


FIGURE 35. Sucrose Gradient Analysis of the Product of Extract from Uninfected Cells and from Infected Cells Minus NTP

Three 2 ml reaction mixtures were prepared as in Fig. 33 except that in A) infected extract was substituted by extract from uninfected cells, in C) reaction mixture lacked the three unlabelled triphosphates but contained infected cell extract. The reaction mixture in B was complete with infected cell extract. The product of A, B, and C was extracted with phenol and analyzed on sucrose gradients with ^{14}C -rRNA as marker and each gradient fraction assayed for total (o) as well as nuclease resistant (●) RNA.

The relative incorporation of ^3H -ATP under the three conditions was: uninfected extract = 125,000 cpm; infected extract with all triphosphates = 420,000 cpm; infected extract lacking the three unlabelled triphosphates = 40,000 cpm. The ^{14}C -rRNA marker is presented as a continuous line.

less than 4S.

In these latter two experiments the larger RNA species was absent. This suggests that 1) association of A-rich fragments with larger RNA species was catalyzed only by extracts of infected cells and 2) this association is dependent on the synthesis of heteropolymeric RNA.

3. Kinetics of Synthesis of the A-Rich RNA

Since the results in the above section demonstrated that the 4S A-rich segment existed both free and in association with the larger RNA species it was possible that the A-rich segment was first synthesized as a 4S species and then linked to the newly synthesized 16-18S RNA. If this hypothesis were correct then a brief exposure to $^3\text{H-ATP}$ (the "pulse") followed by dilution of the labelled nucleotide by adding excess unlabelled ATP (the "chase") should result in the radioactive A-rich RNA in the 4S peak moving into the larger RNA peak.

In order for such a pulse-chase experiment to be carried out it was first necessary to establish the proper dilution for the radioisotope. From the results presented in Fig. 7 it is seen that the optimum value for ATP is 2 mM, above which there is no further increase in incorporation of $^3\text{H-ATP}$. To ensure that sufficient dilution of the labelled ATP takes place during the chase experiment a concentration

of 4 mM of unlabelled ATP was chosen.

For the pulse-chase experiment, the completed reaction mixture containing ^3H -ATP and 0.4 mM ATP was incubated at 37°C. One half of the sample was removed for RNA extraction after 30 min, and a ten-fold excess of cold ATP (4 mM) was added to the remaining portion and incubation continued for a further 30 min. Under these conditions no further incorporation of radioactive ATP occurred during the last 30 min. The sucrose gradient profiles of RNA extracted after the pulse and the chase are illustrated in Fig. 36A and A'. There is no detected movement of 4S A-rich segment(A) into association with larger RNA species during the 30 min chase period (A'). The amount of label in the 4S A-rich fragment relative to the A-rich RNA in the larger species was the same during the pulse and the chase. Also after both pulse and chase, the RNA product had A-rich segments with sedimentation coefficients of 4S (Fig. 36B, B').

The above data suggests that the A-rich segments are not synthesized separately as 4S pieces and then attached to newly synthesized mRNA, since both species are present in the chase as well as in the pulse experiments. However, it is also possible that the conversion process did not occur in the chase period because of the high levels of ATP used since it was found that the incorporation of GTP by the polymerase extract in the presence of 4 mM ATP was 40% lower

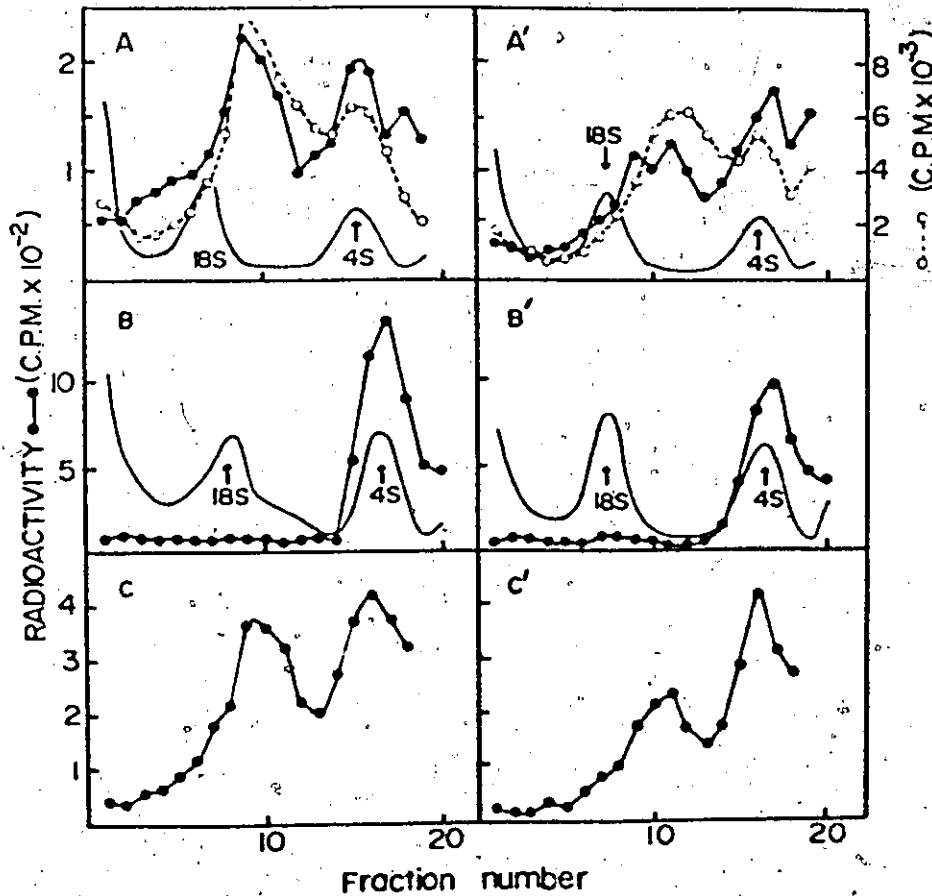


FIGURE 36. Gradient Analysis of A-Rich RNA after a 30 min Label Followed by a 30 min Chase

Eight ml of a reaction mixture containing 2.5 mM of GTP, CTP, UTP and 20 μ Ci/ml of 3 H-ATP (50 mCi/mole) was incubated at 37°C for 30 min. At this time 2 ml was removed for digestion with enzymes before RNA extraction and gradient analysis (B) and 2 ml for direct RNA extraction and gradient analysis (A). To the remaining 4 ml of sample, sufficient unlabelled ATP in a volume of 0.5 ml was added to bring the final concentration to 4 mM. Incubation was continued for a further 30 min and then 2 ml was treated with nuclease prior to gradient analysis (B') or directly extracted and analyzed on gradients (A'). Samples C and C' are identical to A and A' respectively except that the RNA was kept at 100°C in STE buffer for one min before cooling and gradient analysis. In these samples, C and C', only the amount of nuclease resistant RNA along the gradient was determined. The total RNA in A and A' (o), nuclease resistant fractions (●). The 14 C-rRNA marker is shown as continuous line. All procedures were identical to those described in Fig. 33 except that centrifugation was at 180,000 g for 240 min.

than the incorporation in the presence of optimum ATP concentration of 2 mM.

If the 4S is not a precursor to the larger RNA species, then it could perhaps arise from nucleolytic cleavage of this large RNA molecule. In either case the function of the 4S species is not known.

To determine if the A-rich fragment is hydrogen bonded to the larger RNA species, the extracted RNA was placed at 100°C for 60 sec, quick cooled and analysed on a sucrose gradient. The distribution of nuclease resistant RNA along the gradient (Fig. 36C, C') remained unchanged from that observed without heating. This suggests that the short poly A fragments are covalently linked to the 16-18S species.

4. The Mechanism of Poly A Synthesis

The poly A tract may be copied from a corresponding complementary region (poly U or poly T) in the template nucleic acid, or the poly A region may be added post-transcriptionally by an enzyme which adds AMP residues sequentially to one terminus of a heteropolymeric RNA.

In most systems examined to date evidence seems to support the latter mechanism.

It should be possible to decide between these two

mechanisms in the VSV systems since it has been shown that the heteropolymeric product is complementary to, and therefore templated by, virion RNA. If virion RNA contains polyuridylic acid (poly U) sequences as large as the synthesized poly A stretches then a direct templating mechanism would be favoured. Experiments were therefore carried out to detect poly U sequences in virion RNA.

The assay used to detect poly U regions in RNA consisted of hybridizing virion RNA labelled with uridine to non-radioactive poly A. Under the proper conditions the $^3\text{H-U}$ labelled virion RNA should form double-strands with poly A and protect it from RNase. As a control for the procedure, labelled polyuridylic acid and unlabelled polyadenylic acid were hybridized as described by Gillespie et al. (1972). Table 15 indicates that 93% of the poly U was converted to ribonuclease-resistant structures. Using these conditions, hybridization between poly A and labelled RNA isolated from virus particles failed to show any increase in RNase resistance above that level observed when no poly A was added. Hence it seems unlikely that virion RNA contains poly U tracts which serve as templates for poly A synthesis. The lack of poly U segments in the VSV RNA has also been confirmed by Marshall and Gillespie (1972).

Table 15
Hybridization of ^3H -Poly U and ^3H -U Labelled
Virion RNA with Poly A

Annealed	Total cpm	Nuclease Resistant cpm	$\%$
^3H -poly U + poly A	5,135	4,815	93
^3H -poly U alone	4,817	32	0.7
^3H -U VSV RNA + poly A	23,870	449	1.9
^3H -U VSV RNA alone	21,940	440	2.0

Duplicate sets containing approximately 1 μg (34,000 cpm/ μg) of virion RNA or 1 μg (7 mCi/mM) of ^3H -labelled polyuridylic acid (Schwarz/Mann) were hybridized with 10 μg of unlabelled poly A as described in METHODS.

SECTION IV. Enzyme Activity During Viral Replication and Interference

1. Kinetics of Appearance of RNA Polymerase in Infected Cells

The time course of appearance of polymerase activity and PFU production in L-cells infected with either the Ind-ST or the HR-LT strain is illustrated in Fig. 34. In cells infected at a moi of 20 PFU/cell, polymerase activity, as measured by the standard assay, became detectable at 2 hours post infection with both strains, and continued to increase in cells infected with HR-LT (Fig. 37b). However in Ind-ST infected cells, maximal enzymatic activity was observed at 6 hours post infection and decreased at later times (Fig. 37a). Polymerase activity was not detectable in uninfected cells (Fig. 37b). In accordance with these time courses, extract from infected cells was routinely prepared for polymerase stocks at 5.5 hours post infection.

To determine if enzyme yield could be increased by increasing the virion concentration, polymerase activity was assayed in cell extracts infected with different multiplicities of virus. As shown in Fig. 38, polymerase activity increased with increased input of virions up to multiplicity of 20. However increasing the virion concentration above 20 PFU/cell did not yield increase in activity. Similarly, the yield of infectious virus was not augmented

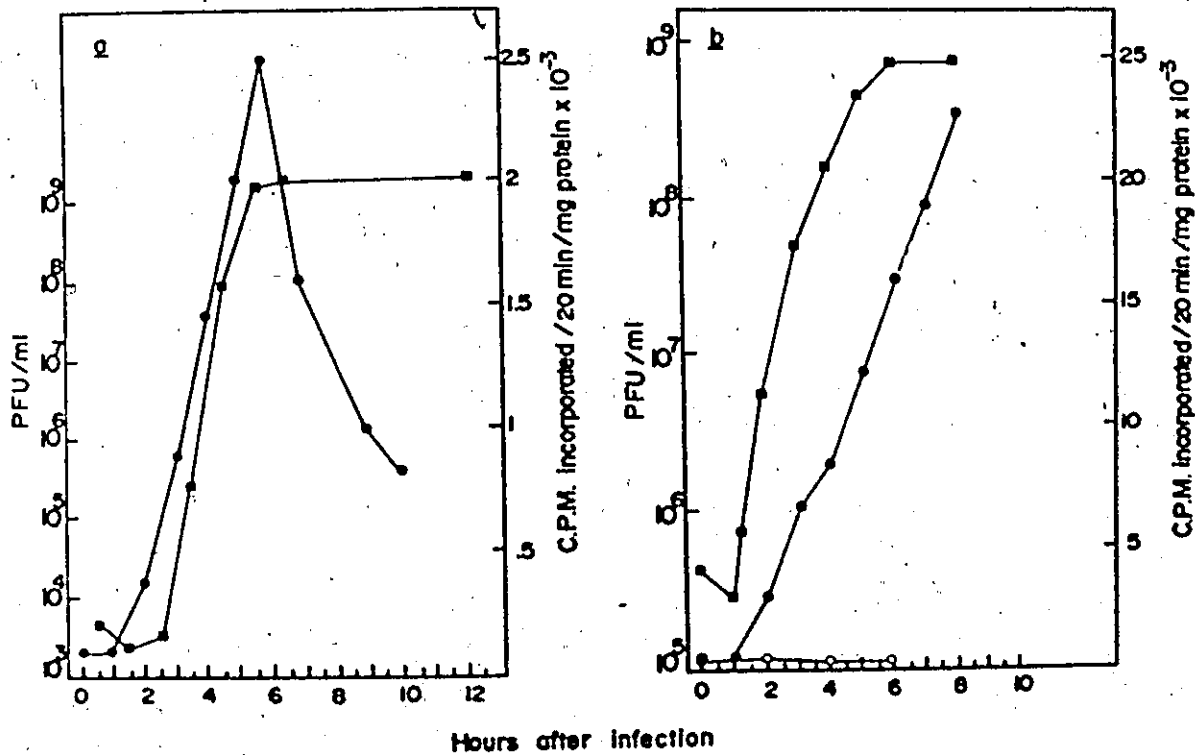


FIGURE 37. Kinetics of Appearance of VSV RNA Polymerase in Infected Cells

Cells were infected with infectious particles of either (a) Ind-ST or (b) HR-LT at moi of 20. At shown intervals 10⁸ cells were collected from the infected culture by centrifugation and aliquots of the supernatant were taken for PFU assay (■). The cells were disrupted and the cytoplasmic fractions were assayed for polymerase activity as described in Table 1 (●). Cytoplasmic extracts from uninfected cells were also assayed in the same manner (○).

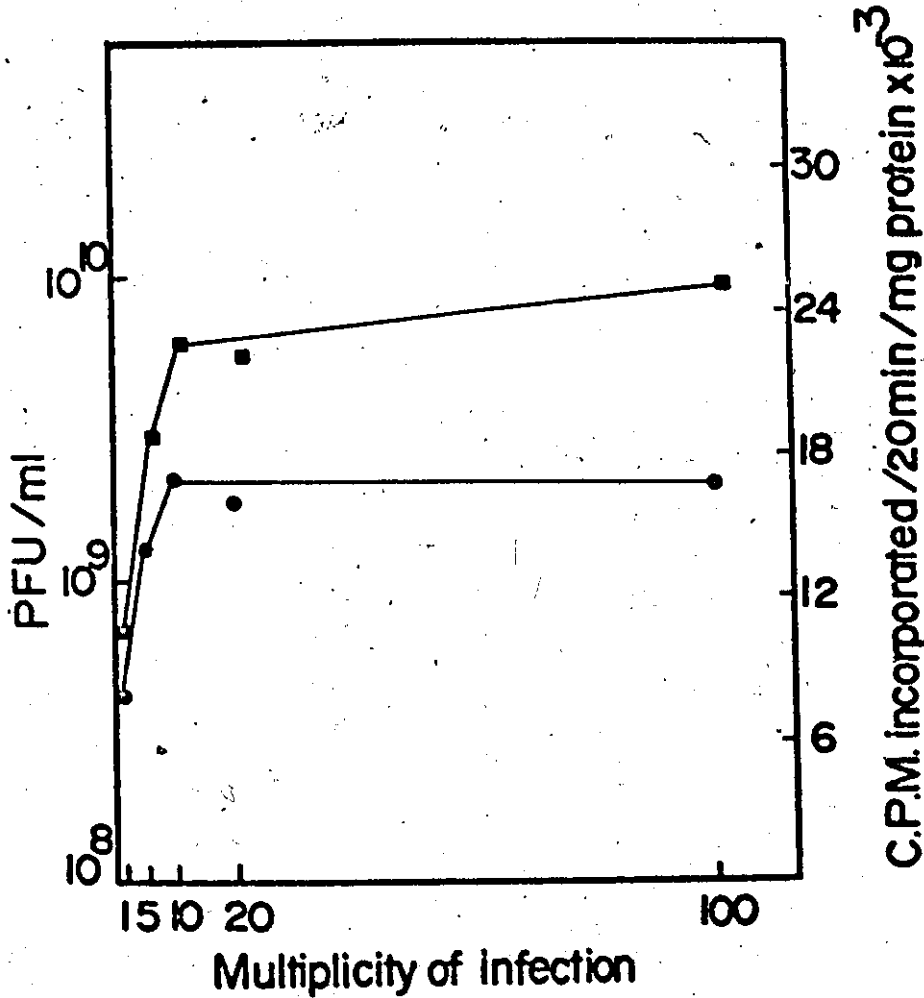


FIGURE 38. Infectious Virus Production and RNA Polymerase Activity in Cells Infected with Increasing mol of Ind-ST

About 7×10^7 cells were infected with 1, 5, 10, 20 and 100 PFU per cell. At 5.5 hr post infection samples were collected and assayed for polymerase activity (■) and virus production (●).

by increasing the multiplicities of infection above 20. The reason for this saturation effect is not known but could be related to the presence of defective virus particles in the stocks used in these experiments.

2. Polymerase Activity During Homotypic Interference

The phenomenon of interference by defective particles is well documented in VSV system. Prevec and Kang (1970) have shown that both defective particles LT and ST interfere equally well with Ind-ST and HR-LT strains of Indiana serotype. The following experiments were done to determine whether polymerase production was affected under similar conditions of homotypic interference.

The preparation of defective particles used in the following experiments and assay of their interfering activities were described in METHODS. Cells treated with Act D (0.5 $\mu\text{g/ml}$) for 12 hours before infection, were separately infected with either purified B particles of HR-LT strain, purified B plus LT particles, or with LT particles alone. At 3, 5 and 7 hours after infection, samples were taken from each culture, the cells extracted and assayed for polymerase activity.

As shown in Fig. 39, low levels of polymerase activity were present in cells infected with LT particles alone. This

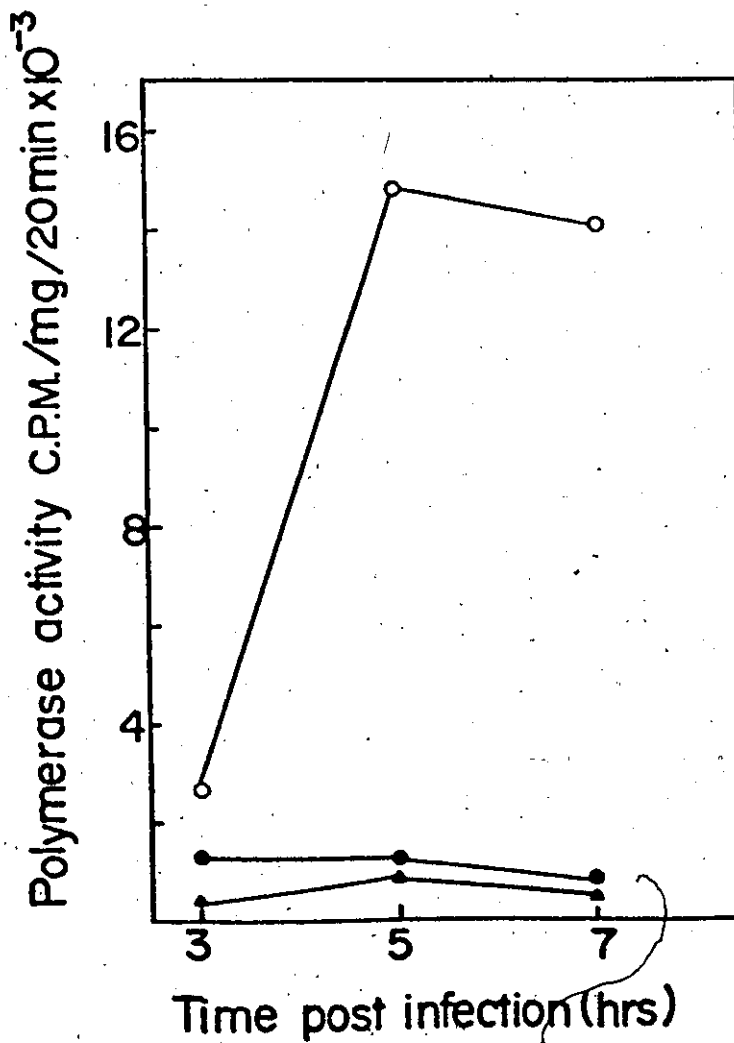


FIGURE 39. Polymerase Activity in L-Cells Coinfected with LT and HR-LT

Cells were pretreated with Act D (0.5 $\mu\text{g}/\text{ml}$) 12 hours prior to infection. The cell culture was split into 3 and infected as follows:

- culture 1. B particles of HR-LT at moi of 5 (o).
- culture 2. B particles plus sufficient LT particles to produce 100-fold decrease in infectious units (●).
- culture 3. LT alone (▲).

After 30 min of adsorption, samples were incubated at 37°C. At 3, 5 and 7 hours post infection cells were taken from each of the three cultures, washed and assayed for polymerase by the standard procedure described in METHODS.

is in keeping with the observations that defective particles by themselves are incapable of inducing any significant synthesis of viral products in infected cells (Stampfer et al., 1969). Cells infected with B particles alone showed the expected increase in polymerase activity, whereas cells infected with the same number of B particles together with LT defective particles showed no significant polymerase synthesis. Thus interference by defective particles affects the production of polymerase activity as well as the total yield of infectious virus particles.

To check the relationship between the level of polymerase activity and the degree of interference produced by different concentrations of defective particles, L-cells infected with B particles of HR-LT were co-infected with varying concentrations of ST particles in one experiment, and with varying concentrations of LT particles in the other experiment.

After 5 hours of incubation at 37°C cells were harvested and assayed for both polymerase activity and total viral infectivity. As seen in Table 16, both the number of infectious virus produced by this time and the amount of polymerase activity were reduced in parallel as the number of defective particles added was increased. When LT particles were used as the interfering defective, a similar result was obtained in that both polymerase activity and

Table 16

Effect of Various Concentrations of ST on Polymerase Activity in L-Cells

Infected with B Particles of HR-LT

Infection with	Incorporation/ mg/20 min cpm	% of control	PFU/ml	% of control
HR-LT B particles alone	11,080	100	1.3×10^9	100
ST particles alone	80	0.7	3×10^6	0.2
B + 1/16 dilution of ST	434	3.9	1.8×10^7	1.4
B + 1/32 dilution of ST	770	7	4×10^7	3
B + 1/64 dilution of ST	2,235	20	1.3×10^8	10
B + 1/128 dilution of ST	3,650	33	3×10^8	23

After Act D treatment (as described in Fig. 36) cell culture was split into 6 and infected with various concentrations of ST. The stock of ST contained sufficient particles to cause 100-fold decrease in PFU. Appropriate dilution of this stock was used in above experiments. At 5 hours post infection cells were harvested and assayed as described in METHODS.

infectious virus titre decreased with increasing additions of LT particles (Table 17). It is interesting that in this case polymerase activity seems to be somewhat less sensitive to LT particle interference than is virus yield.

3. Polymerase Activity in Heterotypic Interference

In a manner identical to homotypic interference, LT particles were also shown to be capable of heterotypic interference with B particles of New Jersey serotype. However in this case very few, if any, LT particles were produced as a result of this interference (Prevec and Kang, 1970).

When polymerase activity was examined under these conditions it was found that, as with homotypic interference, activity decreased with increasing concentrations of LT particles (Table 18).

It was previously shown by Huang and Wagner (1966) that UV irradiation of ST particles abolished their ability to interfere with virus production. To determine if interference with polymerase activity was also inhibited LT particles were irradiated with UV prior to their addition to cells infected with New Jersey particles. Table 18 illustrates that neither polymerase activity nor the yield of infectious particles decreased under these conditions. This suggests that inhibition of both polymerase activity

Table 17
Effect of Various Concentrations of LT on Polymerase Activity in L-Cells

Infected with B particles of HR-LT

Infection with	Incorporation/ mg/20 min Cpm	% of control	PFU/ml	% of control
HR-LT B particles alone	2,650	100	5.5×10^7	100
LT particles alone	116	4	-	
B + 1/16 dilution of LT	470	17	5×10^5	0.9
B + 1/32 dilution of LT	1,225	46	5.5×10^6	10
B + 1/64 dilution of LT	2,174	82	2.6×10^7	47
B + 1/128 dilution of LT	3,320	120	2.5×10^7	45

The procedure for this experiment is similar to that described in Table 16 except that LT particles are used.

Table 18

Polymerase Activity in L-Cells Coinfected with New Jersey B and LT

Infection with	Incorporation/ mg/20 min cpm	% of control	PFU/ml	% of control
New Jersey B alone	1,766	100	1.6×10^8	100
LT alone	102	6	2.5×10^3	<0.1
B + 1/32 dilution of LT	98	6	5.1×10^6	3
B + 1/64 dilution of LT	226	12	1.2×10^7	7.5
B + 1/128 dilution of LT	444	31	2.5×10^7	15
B + (1/32 dilution of LT) UV-irradiated	1,960	110	1.7×10^8	100

The procedure for this experiment was similar to that described for Table 16, except that B particles of New Jersey serotype were used. The 1/32 dilution of LT was UV-irradiated at a dose of $2400 \text{ ergs mm}^{-2}$ and then used to superinfect New Jersey B infected cells.

and virus yield require a functional LT particle genome.

4. Timing the Interference Event

As stated in the INTRODUCTION, it has been shown by Huang and Wagner (1966) and Wild (1972) that the interference effect of defective ST particles requires that they be present within the first two hours of the infectious cycle. Addition of defective particles to pre-infected cells at later times resulted in a decreased interference effect, or, if added four hours after infection, in no interference. This result suggests that interference acts at an early stage in the virus replication cycle.

The following experiments are designed to investigate the time post-infection at which polymerase synthesis became refractory to inhibition by defective particles and to determine the speed with which inhibition became maximal following defective particle addition. Accordingly, cells were infected with B particles and after a 30 min adsorption period ($t = 0$) an aliquot was removed, centrifuged and superinfected with ST. At hourly intervals thereafter this procedure was repeated. All cultures were harvested at 6 hours post-infection. Polymerase activity in each of the samples was determined.

The results from two such independent experiments are

shown in Fig. 40. Both experiments demonstrate that the greatest degree of inhibition of infectious virus occurred when T particles were added at time zero or 1.5 hours. By 3 hours post infection, the addition of T particles had little effect on virus yield.

Polymerase activity was also affected by the addition of defective particles. In experiment (A) polymerase activity was inhibited when T particles were added up to 3 hours post infection. By 3 hours, T particles had no effect on subsequent enzyme activity. In experiment (B), however, polymerase activity was still affected when T particles were added at 2.5 and 4 hours. Since these two experiments are duplicates no obvious reason for the discrepancy in result can be advanced. If the result observed in experiment (B) is not simply due to error in assay it suggests that polymerase may continue to be synthesized in excess over that required for maximal virus production. This result is in agreement with the time course of polymerase appearance and virus yield in infected cells as illustrated in Fig. 37.

To determine if the loss of interference observed at 3 hours post infection with B particles in experiment (A) can be reversed by adding increasing concentrations of ST the following experiment was done. Cells were infected with B particles, incubated for 3 hours and then superinfected with increasing concentrations of ST. Three hours after the

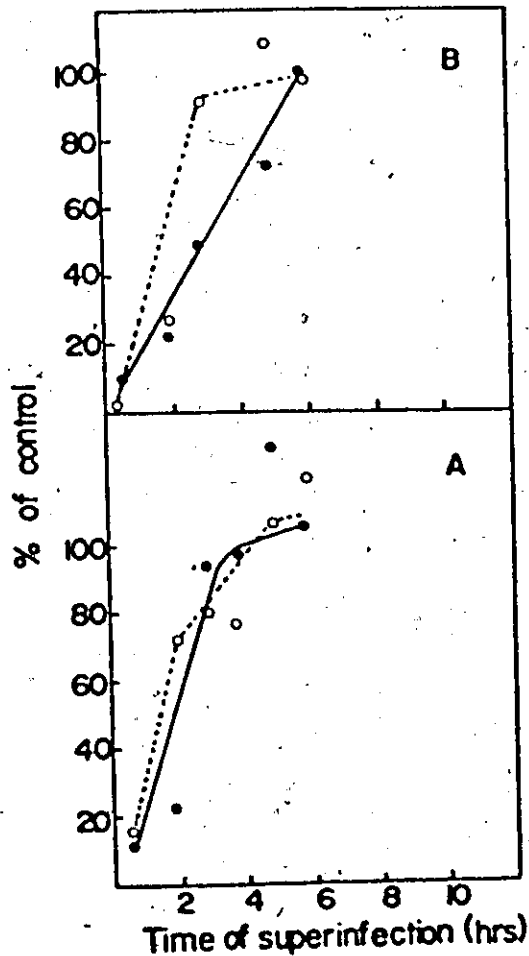


FIGURE 40. Polymerase Activity and PFU Production after Superinfection by ST at Different Times during the Replication Cycle of B

About 4.9×10^8 cells were infected with B particles of HR-LT at moi of 5. Immediately after adsorption and at hourly intervals thereafter, samples containing 7×10^7 cells were centrifuged from the culture and superinfected with ST particles. After 6 hours of incubation at 37°C cells were harvested and assayed for polymerase activity (\bullet) and PFU (\circ). (A) and (B) represent two independent experiments. The results are presented as % of control, control being the result obtained in the absence of added ST particles. The times plotted are adjusted by 30 min to allow for manipulation during superinfection with ST particles.

addition of ST, enzyme activity of the extracts, and PFU were determined. The results illustrated in Fig. 41 were obtained. Although there was a slight inverse correlation between numbers of T particles added and both polymerase activity and PFU, the overall effect was very small compared to similar concentrations of ST particles added at time zero. These results suggest that interference occurs at an early step during VSV multiplication and the addition of additional defective particles at later times has little or no effect on either polymerase activity or PFU production.

5. Kinetics of Interference

In order to gain further insight into the process of polymerase inhibition the kinetics of interference produced by T particles added at different times in the virus growth cycle was examined.

Cells were infected with B particles, and a sample of these cells was superinfected with ST (at a concentration sufficient to produce 100 fold decrease in infectious units) one hour later and incubated for 6 hours. At 3 hours post infection, the remaining cells were centrifuged and divided into 3 cultures. One portion was superinfected with the same concentration of ST particles as used in 1 hr, while cycloheximide (20 µg/ml) was added to another. The third

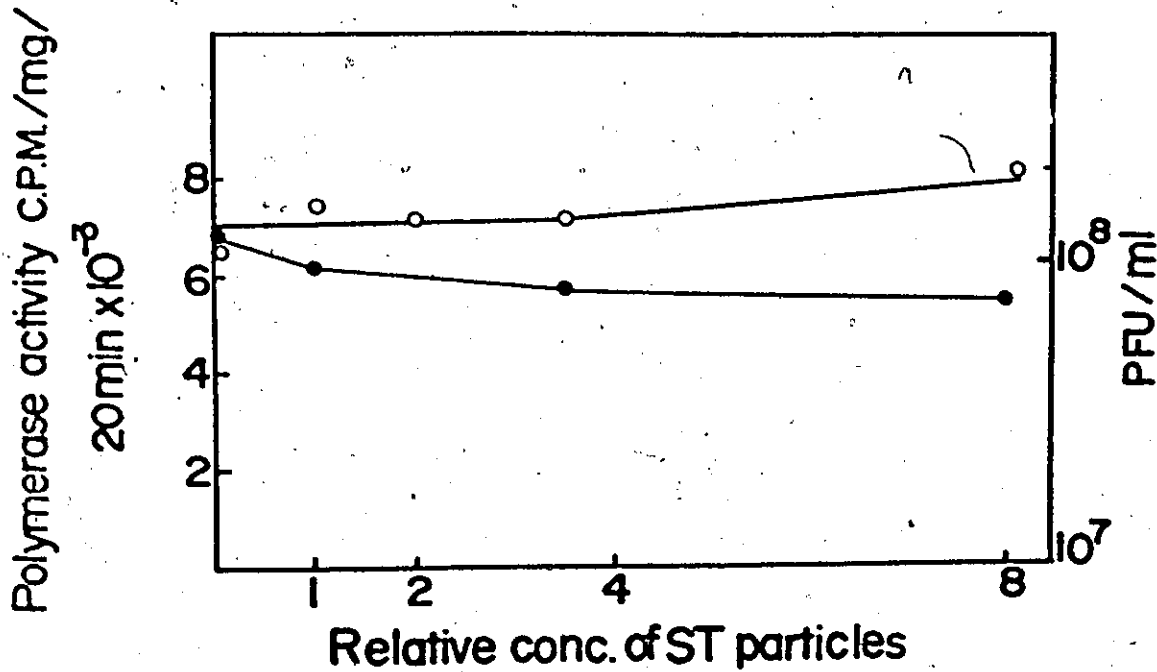


FIGURE 41. Polymerase Activity and PFU Yields After Superinfection by Various Concentrations of ST at 3 Hours Post Infection

About 3.5×10^8 cells were infected with B particles of HR-LT strain at moi of 5. After 2.5 hours of incubation the culture was split into 5 portions. One portion received no further treatment while to the other four were added interfering ST particles in relative concentrations of 1:2:4:8. The lowest concentration of ST particles used was capable of reducing the infectious yield by 100-fold if added at time zero. At 3 hours post superinfection, that is at 6 hours post infection with B alone, cells were harvested and polymerase activity (●) and PFU (○) production were determined.

culture was left as control. At each hour samples were withdrawn and polymerase activity determined.

The results are shown in Fig. 42. Polymerase activity, in samples superinfected with ST particles at 3 hrs increased identically to the control infected culture. In the culture to which ST particles were added at one hour polymerase activity continued to increase, at a somewhat diminished rate from the control, until some five hours post infection after which a decline in activity was observed.

In contrast to the gradual inhibition by ST particles, cycloheximide added at 3 hrs produced an almost immediate block on the increase in polymerase activity but the level of activity remained constant, indicating stability of the enzyme-template complex. This result shows that continued protein synthesis is essential for increased polymerase activity. Whether the essential protein is new polymerase or some other protein mediating polymerase-template association is not known.

6. Polymerase Activity in Ribonucleoprotein Complexes Produced with Continuous Undiluted Passage of Virus

From the previous work of Kang (1971) and as described earlier, cells infected with pure B particles

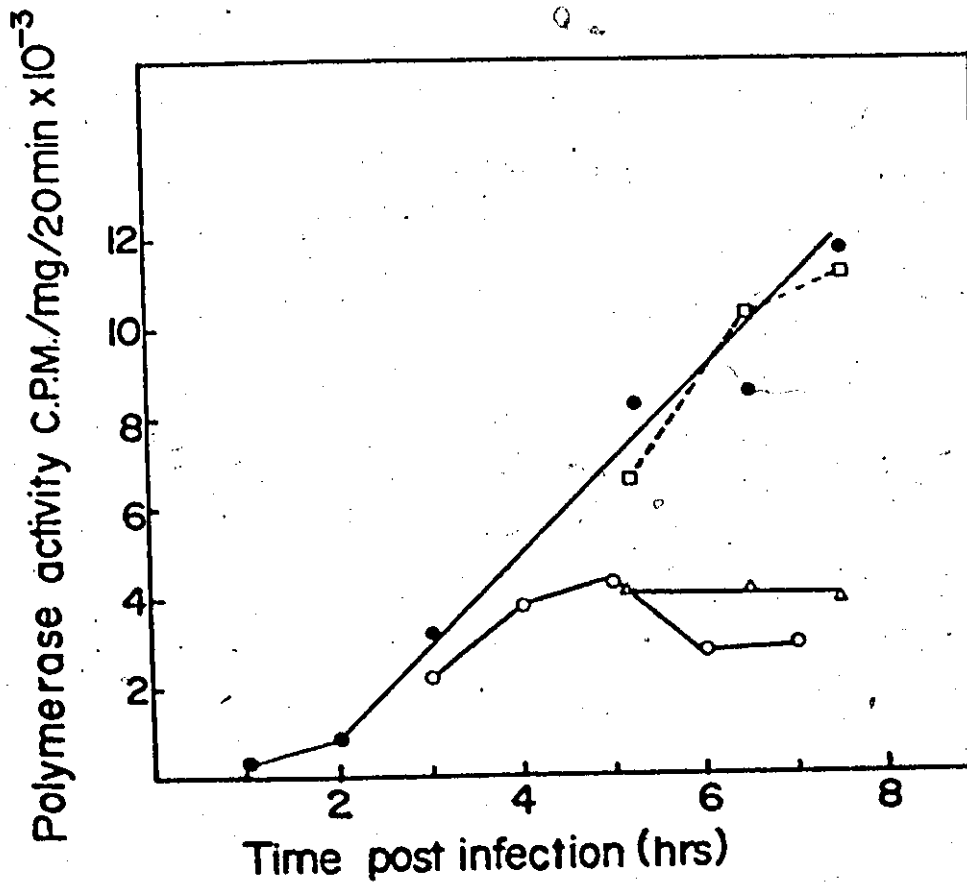


FIGURE 42. The Effect of ST and Cycloheximide, Added at Intervals After Infection with B particles on Polymerase Activity

Approximately 1.2×10^9 cells were infected with B particles of HR-LT strain at a moi of 5. This was the stock culture (●). At 1 hr after infection 3.5×10^8 cells were removed, centrifuged and superinfected with ST. At each hour (for 4 hours) 7×10^7 cells from the stock culture and culture superinfected with ST (○) were withdrawn and assayed for polymerase. After 3 hours incubation the stock culture was split into 3 portions. To one portion ST was added (○), to the other 20 μ g/ml of cycloheximide was added (Δ) and the third was left as control (●). Incubation was carried out at 37°C. Each hour, for a duration of 3 hrs, samples were withdrawn from each culture and assayed for polymerase activity. The number of ST particles used in this experiment was sufficient to cause a 100-fold reduction in the infectious yield if added at the time of infection.

accumulate two RNP complexes with sedimentation coefficients of approximately 140S and 160S. These structures are DOC sensitive and can be converted by detergent treatment to particles of reduced sedimentation coefficients with the concomitant release of L protein (Petric, 1970; Kang, 1971). Cells infected with a mixture of B and LT particles accumulate, within their cytoplasm, complexes having sedimentation coefficients of 140S and 100S (Petric, 1970). On infection with a mixture of B and ST particles the two detectable RNP complexes in the infected cells sediment at 140S and 80S (Wild, 1971).

As was shown earlier, both of the cytoplasmic RNP complexes in cells infected with pure B particles contain RNA polymerase activity. To determine if polymerase activity also coincides with the RNP complexes produced under conditions of interference, the following experiments were done. First, plaque purified stocks of HR-LT were prepared as described by Kang (1971) and Stampfer et al. (1971). These workers have shown that only after the 3rd or 4th successive high multiplicity passage of this purified virus, defective particles are produced. Therefore this plaque purified virus was passed at multiplicity of 100 PFU/cell for four consecutive times to yield stocks containing both infectious and defective particles. The yield of infectious virus at each of these passages was measured by plaque assay and plotted in Fig. 43.

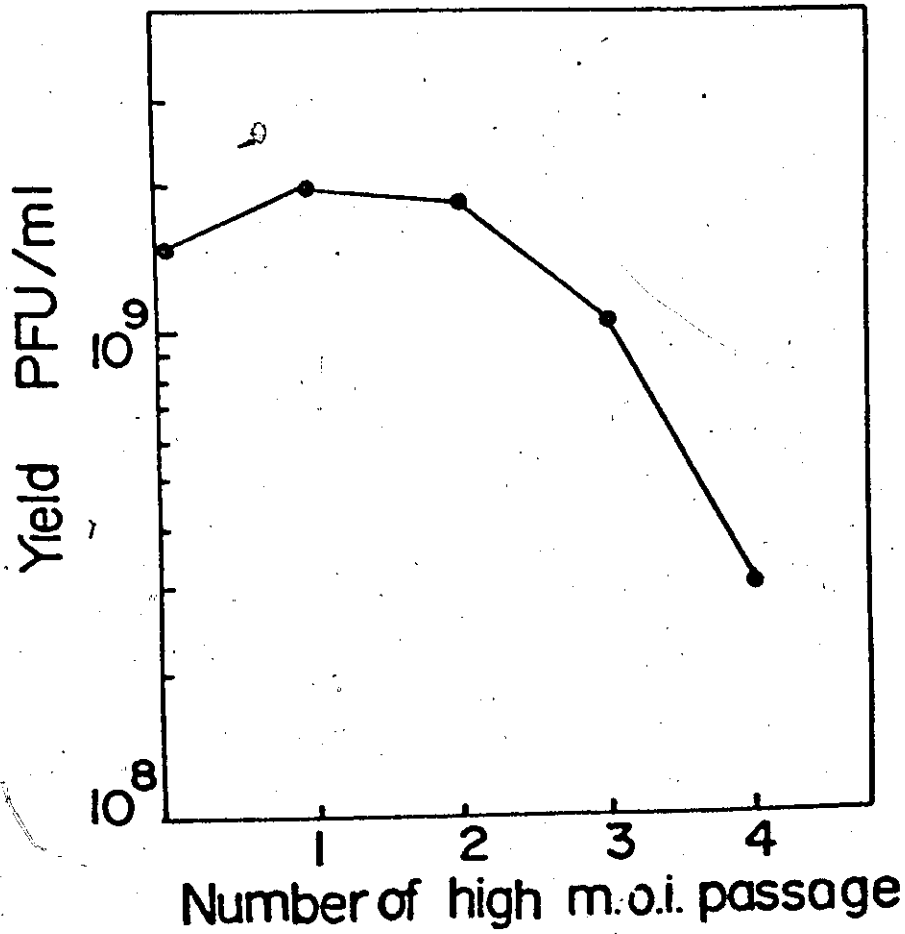


FIGURE 43. Effect of Repeated High Multiplicity Passages on Infectious Virus Yield

High moi passage stocks of HR-LT strain of VSV were prepared by successive passage, starting with a plaque purified low moi stock (zero passage) at 100 PFU/cell. Virus from each passage was titrated by plaque assay.

As seen continuous high moi passage results in loss of infectivity.

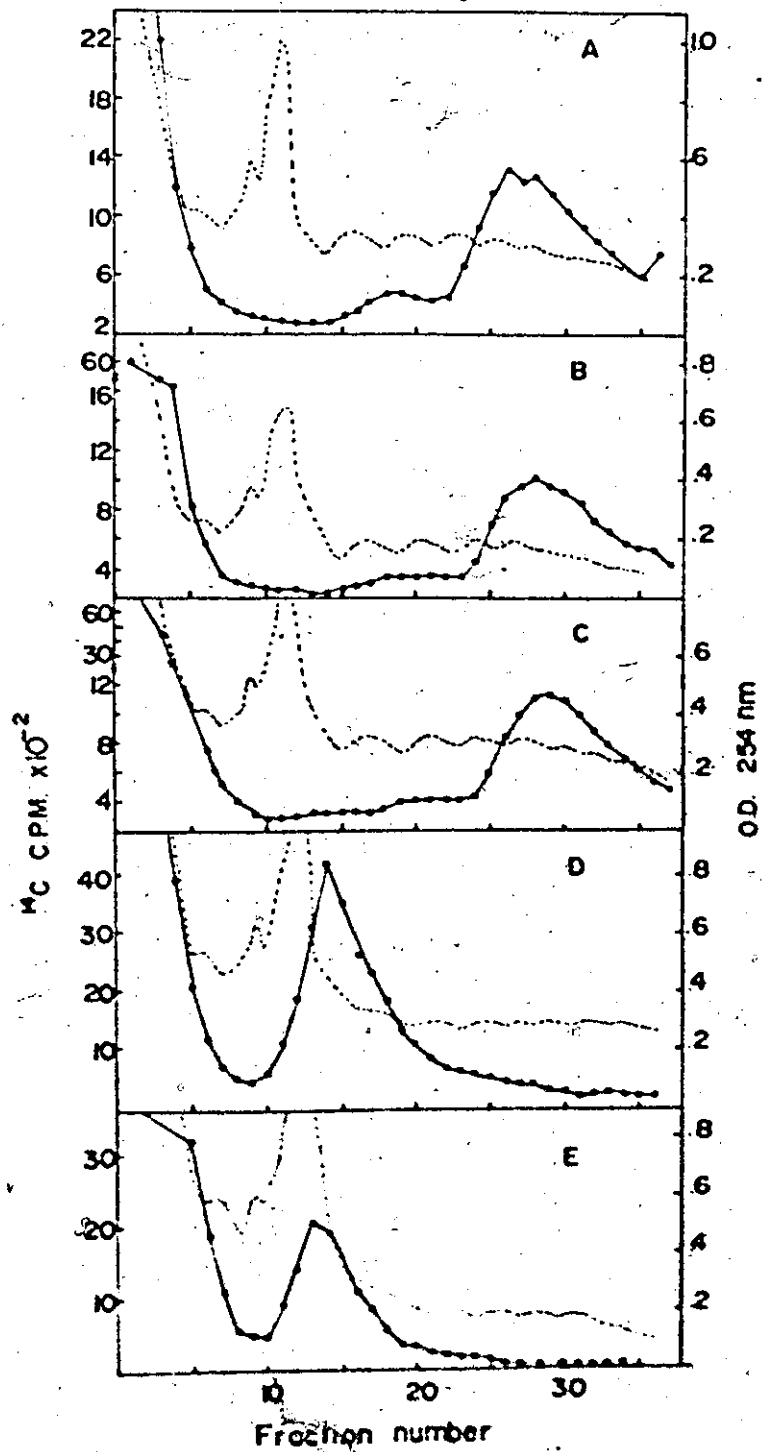
Each of these high multiplicity passages were used to infect cell cultures, and the RNP complexes produced were monitored by incorporation of labelled amino acids and polymerase activity.

The infected cell cultures at each high moi passage were labelled with ^{14}C amino acid mixture between three and five hours post infection and the cytoplasm extracts then analyzed on sucrose gradients. In extracts of the first three high moi passes (Fig. 44A,B,C) labelled viral proteins are present at the top of the gradient and in fractions sedimenting at 160S and 140S. Relatively little labelled material was present in the 140S region. A variation in the amount of material at 140S relative to the 160S region has been observed here and by Kang (1971) on various occasions. These two complexes are replaced in the 4th and 5th passages by a single complex of sedimentation coefficient of about 100S. Hence it seems that under conditions of high multiplicity passage there is a change in the nature of cytoplasmic complexes present in the infected cell. Since this change in size of RNP complex is observed at the same passage as production of substantial amounts of defective particles it would seem likely that the 100S RNP complexes may be involved in the synthesis of defective particles.

FIGURE 44. Sucrose Gradient Analysis of ^{14}C -Amino Acid
Labelled Cytoplasmic Structures Obtained from
Cells Infected with Five Successive High
MOI Passages of HR-LT

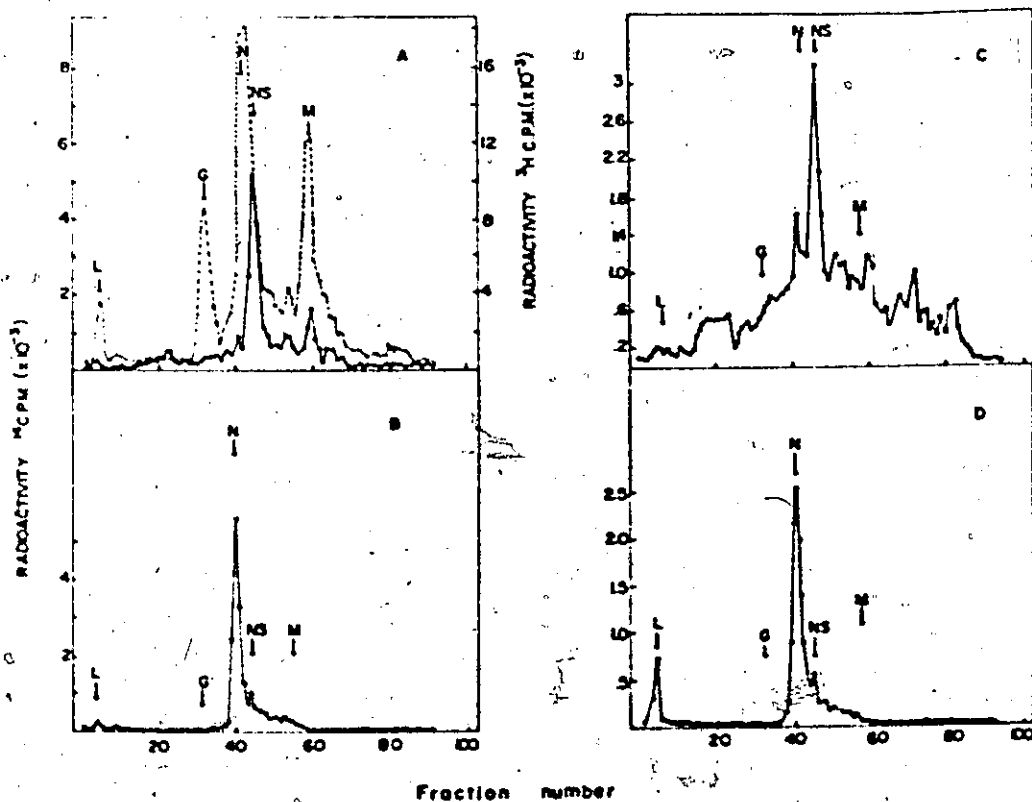
About 10^8 cells were pretreated with 0.5 $\mu\text{g}/\text{ml}$ of Act D for 12 hours and then infected with the appropriate high moi passage stock of HR-LT (prepared as described in Fig. 43) at 100 PFU/cell. The cultures were each labelled with the ^{14}C -amino acid mixture between 3 and 5 hours post-infection. Cytoplasmic extracts were prepared from the harvested cells and layered on 15-30% sucrose gradients made in RSB $^\circ$ and centrifuged for 3.5 hours at 24,000 rpm at 5 $^\circ\text{C}$, in Spinco SW27 rotor. Successive 1 ml fractions of the gradients were collected with continuous monitoring for optical density (----). A volume of 0.05 ml of each fraction was spotted on glass fiber filters and radioactivity determined.

- (A) First passage.
- (B) Second passage.
- (C) Third passage.
- (D) Fourth passage.
- (E) Fifth passage.



The labelled viral proteins present in the soluble fraction of the cytoplasm and the RNP complexes from each of the gradients of the high moi passages was examined by polyacrylamide gel electrophoresis. The labelled material at the top of the gradient and in the two complexes of the first high moi passage has already been presented in Fig. 15. As shown there the top of the gradient consisted entirely of the NS protein, while both heavier regions contained proteins L, N and some NS. To determine if the same species of proteins are present in the smaller complexes observed in higher passages the proteins of the 4th and 5th passage were examined. Fig. 45 illustrates that the same proteins are present both in the cytoplasm of the 4th high moi passage (A,B) and in the 5th high moi passage (C,D) as in the first high moi passage. The NS is found at the top of the gradients and L, N and small amount of NS are present in the 100S material. These results are in agreement with Kang's data (unpublished results).

In parallel with the above labelling experiments the same procedures were repeated in the absence of labelled amino acids and each fraction of the gradient was analysed for polymerase activity. As seen in Fig. 46, polymerase activity was found from the 1st to the 3rd passages in two structures, one at 140S and the other at 160S. At 4th or 5th passage however the activity was found associated with the



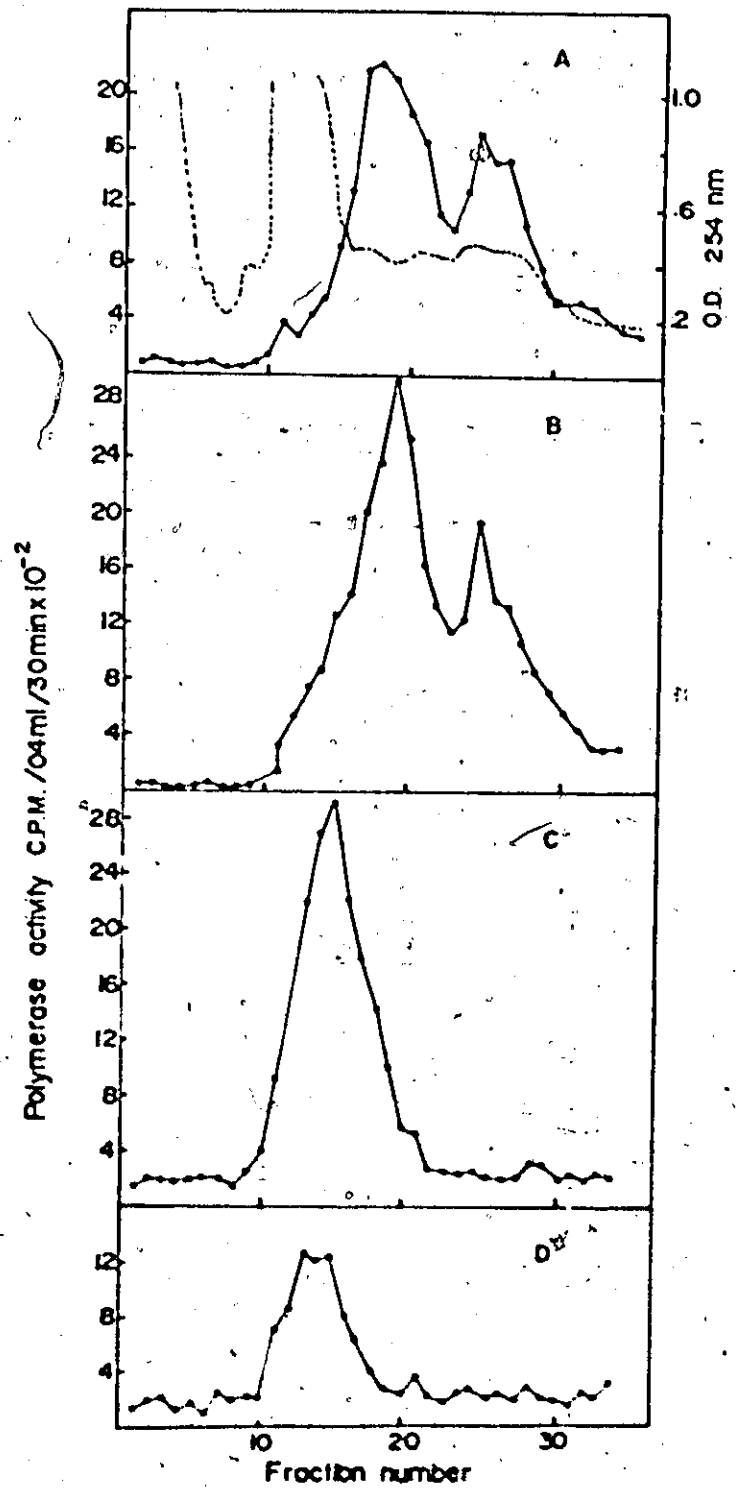
**FIGURE 45. Polyacrylamide Gel Profiles of Virus Proteins
In Cytoplasmic Extracts of Cells Infected
at Different High MOI Passage**

Radioactive proteins from selected fractions of the sucrose gradients shown in Fig. 44, were precipitated with 95% ethanol and analyzed on polyacrylamide gels. The material shown in (A) was obtained from the top of the gradient shown in Fig. 44D. The material in (B) was taken from the 100S region of the same gradient. (C) represents the material from the top of the gradient shown in Fig. 44E and (D) represents the material from the 100S region of the same gradient. All samples were co-electrophoresed with ^3H -viral protein markers.

FIGURE 46. Sucrose Gradient Analysis of Polymerase Activity in Cytoplasmic Extracts Obtained from Cells Infected with Successive High Multiplicity Passages of HR-LT

About 1.5×10^8 cells were pretreated with Act D for 12 hours and infected with the appropriate high moi passages of HR-LT at 100 PFU/cell as described in Fig. 44. At 5 hours post infection cells were harvested and cytoplasmic extracts were layered on 15-30% sucrose gradients made in RSB^o and centrifuged for 3.5 hours at 24,000 rpm at 5°C in SW27 rotor. Successive 1 ml fractions were collected and monitored for O.D. A volume of 0.4 ml of each fraction was assayed for polymerase activity at 37°C for 30 min.

- (A) First passage.
- (B) Third passage.
- (C) Fourth passage.
- (D) Fifth passage.



complex sedimenting at 100S. It appears then that the cytoplasmic 100S RNP complex characteristic of LT RNP core (Petric, 1970; Kang and Prevec, 1969) has transcriptase activity. As shown previously in Fig. 20 the products of the polymerase of the 140S and 160S regions range in size from 5S to 18S. When the synthesized product of the polymerase region in the 5th passage was phenol extracted and analyzed on sucrose gradient the result presented in Fig. 47 was obtained. As seen, the product of this complex is relatively small, sedimenting with S values no larger than 4S. Although the nature of this RNA is not known its possible significance will be discussed in the DISCUSSION.

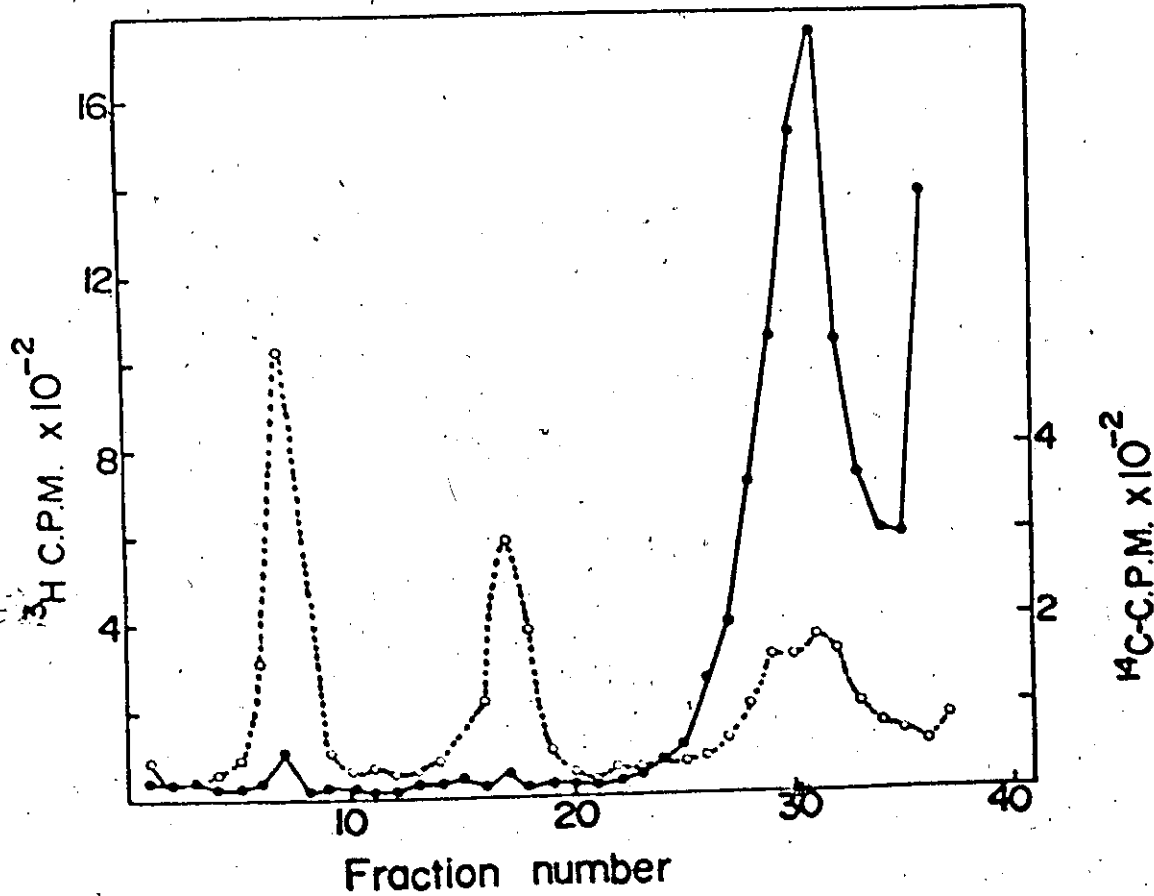


FIGURE 47. Sucrose Gradient Analysis of the RNA Product of the RNP Polymerase Complex Present in the Fifth High MOI Passage

The RNA produced by the complex corresponding to fraction 14 of Fig. 45D was phenol extracted and analyzed by sedimentation through a 5-20% sucrose gradient as described in METHODS. ^{14}C -labelled ribosomal RNA (o--o) was present as marker. The marker regions from the left represent 30S, 18S and 4S respectively.

DISCUSSION

The studies described in this thesis are concerned with the in vitro synthesis of viral specific RNA, by an enzyme induced in L-cells following vesicular stomatitis virus infection. An understanding of the mode of action of such an enzyme is necessary for elucidating the replicative process of virion RNA and its regulation in the infected cells. As indicated by the results, the enzyme is found to function as a transcriptase, synthesizing RNA strands complementary to virion RNA. During the course of this work, other investigators (Baltimore et al., 1970) found that the virion itself carries a transcriptase and have characterized the reaction products (Bishop and Roy, 1971a, 1971b; Bishop, 1971; Roy and Bishop, 1972).

The discussion will consider the properties of the cellular enzyme, its in vitro products, and the effect of interference on polymerase activity. An attempt will be made to relate these observations to what has been shown by others regarding the mechanisms of viral transcription and interference, and to postulate some models concerning the regulation of transcription.

Properties of the Enzyme Activity

Upon infection of L-cells with either the Indiana-ST or the HR-LT strain of VSV, an RNA polymerase activity is detected. This activity is measured in vitro by the ability of the cytoplasmic extract to catalyze the incorporation of ribonucleoside triphosphates into an acid insoluble product. Enzyme activity can first be detected at 2 hours post infection, and increases throughout the viral replication cycle. Using the HR-LT strain, activity continues to increase after 6 hours post infection, the time at which virion yield shows no further increase. With the Ind-ST strain however, activity decreases after 6 hours post infection. The reason for the difference in polymerase synthesis in the two strains is not known. It appears that in the HR-LT strain enzyme molecules are synthesized in excess of that required for virion production.

While the incorporation of GTP by the cytoplasmic polymerase is dependent on the presence of exogenous ATP and CTP it is only partially inhibited by the absence of exogenous UTP. Nearest neighbour analysis of the product of a reaction mixture to which UTP was not added showed that this nucleotide is present next to [α - 32 P]-GTP with the same frequency as it is in a complete reaction mixture, suggesting that a heteropolymer, rather than a non-uridine polymer is synthesized. This could be due 1) to production

of UTP in the reaction mixture by deamination of CTP,
2) to the presence of residual UTP in the dialyzed extract,
or 3) to contamination by UTP of the unlabelled triphosphates.
Alkaline hydrolysis of product RNA labelled with ^{14}C -CTP
in the absence of UTP, has shown the presence of radioactivity
in the UMP region. While this suggests that deamination of
CTP may occur a positive identification of this material
as UMP was not done so this result must be regarded with
caution.

The presence of polymerase activity in the absence
of UTP has also been observed with Sendai virion transcriptase
(Stone et al., 1971) while the omission of either ATP or CTP
had no marked effect on incorporation of GTP by reovirus
induced RNA polymerase (Watanabe et al., 1968).

The enzyme activity from cytoplasmic extracts is
independent of exogenous template and is associated with
two ribonucleoprotein complexes present in cells infected
with B particles only. Both the complex sedimenting at
160S and that at 140S contain the virus specific proteins N, L
and NS together with viral RNA. These three proteins were
shown to be required for the activity of the virion associated
transcriptase by Emerson and Wagner (1972), Bishop and Roy
(1972) and Szilágyi and Uryvayev (1973).

Treatment of the cytoplasmic complexes with DOC
results in the release of protein L with concomitant reduction

in sedimentation value (Kang, 1971) and loss of polymerase activity. This suggests that these complexes consist of viral proteins associated in DOC sensitive structures, and that protein L is required for polymerase activity. Since the two complexes found in the infected cell are similar to the cores found in the virion, it is probable that these cellular RNP represent precursors to completed virion particles. If such is the case, then it would be expected that the morphogenesis of the virions in the infected cytoplasm should involve different size complexes as the RNA combines with increasing numbers of viral specific proteins. The presence of these two complexes in excess in the cell suggests that they represent structures just prior to a rate limiting step in the assembly process.

Since the two complexes contain enzyme activity, viral specific proteins and RNA it seems likely that these structures serve as intracellular transcriptive complexes. The association of RNA polymerase with intracellular RNP complexes is not unique to VSV, but is a common feature of many other RNA viruses including influenza (Compans and Caliguire, 1973), polio (Ehrenfeld et al., 1970), EMC (Bases and Tarikas, 1969), rabies (Pinteric et al., 1963), and reovirus (Watanabe et al., 1968).

The fact that no viral proteins are released during transcription of such complexes of VSV (Bishop and Roy, 1972)

raises the question of how the virion RNA is copied while it is complexed to proteins. The viral protein NS, found to be a phosphoprotein (Sokol and Clark, 1973), was suggested by the same workers to be involved in the regulation of transcription perhaps by temporarily detaching the N protein from the nucleic acid by electrostatic interaction, thus making the virion RNA accessible to the transcriptase.

Product of the Polymerase

As stated in the INTRODUCTION, a number of species of viral RNA synthesized in the infected cell were identified. Among these, the two classes of single-stranded RNA consisting of the heterogenous class with sedimentation coefficients from 13-20S and the relatively homogenous species with sedimentation coefficients of 30S have characteristics which suggest that they serve as messengers for viral protein synthesis.

In vitro transcription by the virion associated enzyme yields RNA species comparable in size to the class of 13-20S RNA found in the infected cell. The products of the virion enzyme sediment at 10S to 21S and are complementary to virion RNA (Bishop and Roy, 1971b). Primary transcription by the virion associated enzyme following infection of cells in the presence of cycloheximide yields both the 13-20S RNA and the 30S RNA species.

As shown in this thesis the principal product produced after one hour of in vitro synthesis by the cytoplasmic enzyme has sedimentation coefficients in the range of 5-18S with the mode around 16S. Part of the heterogeneity of this material may be due to the presence of contaminating nucleases in the enzyme preparation. During shorter incubation periods (5 to 10 minutes) a larger proportion of the newly incorporated radioactivity was associated with RNA sedimenting at the position of whole virion RNA (40S). By 20 minutes, however, almost 95% of the radioactivity was found in the 5 to 18S species of RNA. Other experiments by Prevec (personal communication) have shown that the amount of labelled RNA associated with the RNP complex remained constant after three minutes of synthesis while the amount of label in the free RNA product increased linearly with time. These results suggest that the product RNA is synthesized in association with RNP and then released.

In addition to the 5-18S RNA a larger 30S species is also observed during a short synthetic period. It could be possible that this larger species of RNA is 30S mRNA which may be transcribed in vitro only during the initial stages of incubation. Alternatively however, the labelled RNA present at 30S may be new product which is complexed to virion RNA in a partially double-stranded replicative complex sedimenting

at this position. These alternatives deserve more detailed investigation.

Analysis of the in vitro product synthesized at 37°C shows it to be completely complementary to the RNA from the B and LT particles but not to the ST RNA. The lack of hybridization to ST RNA indicates that the portion of the virion RNA transcribed by the enzyme did not contain sequences in common with the ST particle. From all hybridization data it appears that a limited region of B particle RNA is transcribed.

Examination of the kinetics of product synthesis at 28°C and 37°C shows a two-fold increase in RNA synthesis at the lower temperature. Annealing of the 28°C product to ST particle RNA indicates that an additional segment of virion RNA is transcribed at 28°C as opposed to 37°C. However, since the 28°C product present after either 60 and 220 min of incubation hybridizes to the same extent with ST RNA, it appears that the same RNA species are transcribed during both early and late times at this temperature. This suggests that all the involved cistrons were transcribed continuously rather than in a sequential manner as observed with the virion transcriptase (Bishop, 1971).

With the virion associated enzyme, temperature also affected in vitro transcription. At 31°C the complete viral genome was copied but at 37°C only 30% of it was transcribed.

Further experiments (Roy and Bishop, 1973) have suggested that multiple initiation sites for transcription are present at 31°C. Whether only some of the sites are accessible to the enzyme at 37°C remains to be determined. The partial transcription at 37°C is in contrast to the situation observed in vivo during infection in the presence of cycloheximide by Huang and Manders (1972). These workers showed that under these conditions the virion-associated transcriptase copies the entire viral genome. The temperature effect on transcription may very well be an in vitro property of the system.

Optimum synthesis of in vitro product at 28°C is not unique to the VSV system. Maximum synthesis of RNA at 28°C in vitro have been reported for influenza polymerase (Bishop et al., 1971) and for the polymerase induced in cells infected with Sendai virus (Mahy et al., 1970; Stone et al., 1971). The latter investigators suggested that increased endogenous nuclease activity at the higher temperature was responsible for this effect. Our data indicates that degradation of product at 37°C is slightly higher than at 28°C. However in the light of the hybridization data presented above, it is plausible to suggest that some of the increased synthesis at 28°C is due to the availability of additional sequences for transcription at this temperature. The possibility of selective nuclease destruction at 37°C of those product

sequences complementary to the ST particle seems far less plausible.

Synthesis of Poly A

As stated in the INTRODUCTION, sequences of poly-A have been found in messenger RNA and heterogenous nuclear RNA in eukaryotes, in RNA of oncogenic and non-oncogenic RNA viruses, in mRNA of two DNA viruses, and in general are considered to be a feature of translatable, or potentially translatable RNA. Hence poly A sequences have not been found in virus RNAs that do not function directly as mRNA such as influenza, NDV and VSV (Gillespie et al., 1972).

Mudd and Summers (1970b) first reported that the RNA associated with the polysomal region in HeLa cells infected with VSV, have a high adenosine content. Soria and Huang (1973) confirmed these results and showed that all the 28S and 13-15S messenger RNA species of VSV contain sequences of poly A. The complementary RNA synthesized in vitro by the virion associated transcriptase does not contain A-rich sequences (D. Bishop, personal communication). In contrast to Bishop's results, the work in this thesis shows that the same cytoplasmic extract from infected cells which synthesizes a complementary RNA (with ^3H -GTP as labelled precursor) also synthesizes an A-rich segment associated with 16S species.

Kinetics of synthesis of ^3H -ATP labelled product show

that maximum synthesis is reached after 1 hour of incubation at 37°C with continued synthesis for longer times at 28°C as with the ³H-GTP labelled product. Poly A synthesis is similar to that of total RNA. On a sucrose gradient the newly synthesized ATP labelled RNA sediments with sedimentation coefficients from 4 to 18S with definite peaks in the radioactive distribution at sizes corresponding to 16S and 4S. Poly A segments are present in both the 16S and 4S species. With longer labelling periods neither the product nor the associated poly A segment increase in size. The ATP labelled RNA is alkali labile and is made totally nuclease resistant by hybridization to excess unlabelled B particle RNA. Thus the non-poly A segments of the product are complementary to virion RNA.

The presence of poly A both as 4S segments and in association with 16S RNA suggests that poly A may first be synthesized as a 4S segment which is then attached to a 16S heteropolymeric RNA. To check this possibility a pulse-chase experiment was carried out. While failure to observe movement of label from the smaller to the larger species suggests that this idea was incorrect, the possibility exists that the high levels of ATP employed during the chase period inhibit the normal conversion process. In this regard the incorporation of GTP by the polymerase extract is 40% lower in the presence of 4 mM ATP than at the optimum ATP

concentration of 2 mM. Alternatively it is possible that the 4S RNA is the result of nucleolytic cleavage from the larger RNA.

An estimate of the size of the poly A tracts of the in vitro product determined by nuclease treatment followed by zonal sedimentation with rRNA as markers, yields a sedimentation coefficient of 4S. From the content of poly A of the ^3H -adenylate product and the base composition of the B particle RNA (Brown et al., 1967), and based on the assumption that 16S corresponds to molecular weight of $5-6 \times 10^5$, it can be calculated that the poly A segment is about 80-100 nucleotides long.

Although direct evidence that the A-rich fragment synthesized in vitro is attached to the newly synthesized RNA molecule has not been obtained, some credence is lent to this suggestion by the fact that the synthesis of poly A attached to a 16S RNA species is dependent on the presence of the 3 unlabelled nucleotides, with very little poly A synthesized by the cytoplasmic extract in their absence. Similarly extracts from uninfected cells do not synthesize the large RNA species. The acid precipitable product from these extracts sediments in the range from 3-7S and contains about 10% nuclease resistant material.

Poly A sequences in mRNA can either be synthesized from complementary sequences in the genome as may occur in

vaccinia virus (Kates, 1970), or may be added without the use of templates as in adenovirus mRNA (Philipson et al., 1971). To determine if the poly A segments in the in vitro ATP labelled product are transcribed from poly U tracts present in the RNA of VSV virions, hybridization experiments between ³H-uridine labelled virion RNA and synthetic commercial poly A were performed. No detectable poly U segments were found in the VSV RNA. This result was later confirmed by Marshall and Gillespie (1972). The absence of a poly U template suggests that poly A is attached post transcriptionally. The enzyme catalyzing such a reaction could either be viral induced or a normal cellular enzyme. A number of such poly A synthetases have been found in the mammalian cell cytoplasm (Giron and Huppert, 1972a, 1972b; Tsiapalis et al., 1973), nucleus (Edmonds and Abrahams, 1960), and mitochondria (Jacob and Schindler, 1972) though to date no direct biological significance has been attached to these systems.

Possible Model of In Vitro Transcription

The complementarity of the synthesized product to virion RNA, its size and the suggestion that it might be associated with poly A leads us to believe that this RNA could be similar to some of the species of mRNA found in the infected cell.

From the information available to date and with some

assumptions it is possible to speculate which mRNA species have been transcribed in vitro.

The VSV genome is composed of at least five genes. This statement stems from evidence that five virus specific proteins L, G, N, NS and M can be identified either in the virion or virus-infected cell. Using the molecular weight of these proteins it can be calculated that they account for all the coding potential of the B particle genome (Kang and Prevec, 1971). A size correlation of each of these protein species to a corresponding mRNA is possible at least in part since the mRNA species can be separated into four distinct size classes. The 30S mRNA is of the required size to code for the L protein, and there is in vivo evidence using pactamycin that this protein may be a product of the 30S mRNA (Stampfer and Baltimore, 1973). The portion of the B particle genome coding for the 30S mRNA is distinct from the genome of the LT particle, since RNA from this defective particle does not anneal to the 30S RNA (Hallett, 1972). Since the in vitro product anneals completely to the LT virion RNA and since its size is considerably smaller than 30S it seems unlikely that it would contain the information for the L protein.

The 13 to 20S mRNA species have sizes which are consistent with coding function for the proteins G, N, NS and M. Schincariol and Howatson (1972) have characterized the

LT and ST particle genomes by hybridization with these mRNA species after fractionating on polyacrylamide gels into three size classes consistent with the expected sizes of messenger for proteins G, N or NS and M respectively. They observed that ST RNA hybridizes preferentially with the presumed messengers for G and M while RNA of LT particles hybridizes equally well with all three classes of messenger RNA. This then suggests that ST is probably lacking the segment of genome coding for N or NS protein. Since the in vitro product synthesized at 37°C does not anneal to ST RNA but does anneal to LT it is possible that this synthesized RNA is transcribed off the segment in the B RNA coding for the N or NS protein. If such is the case then the question is raised why is there no transcription of the other cistrons in vitro?

It has been shown in the infected cell that the RNA messenger which corresponds to the N protein is transcribed more frequently than the other messengers (Schincariol and Howatson, 1972). This is in agreement with the observation that the N protein constitutes about 40% of all viral proteins synthesized in the infected cell (Kang and Prevec, 1971). If preferential transcription of the N messenger does occur it is possible that the promoter for this gene is easily accessible to the enzyme, while more complex regulation could be required for transcription of the other cistrons.

While the above speculations seem attractive and plausible, the final solution to this speculation awaits the results of experiments to translate the synthesized product using a cell-free protein synthesizing system. In this way it is possible that the nature of the products at 37°C and 28°C can be characterized and further that the possible role of poly A sequences in protein synthesis can be determined.

Polymerase and Interference

It is demonstrated in this thesis that polymerase activity measured in vitro shows a direct correspondance to the degree of interference, as measured by infectious virus yield, in cells coinfectd with defective particles. Active enzyme is not detected in cells infected with T particles alone, and decreases during both homotypic and heterotypic interference in parallel with the yield of infectious virus.

Also correlated with interference is a change in the character of the RNP complexes in the infected cell. The two transcriptive complexes present in the B particle infected cells are replaced by a complex of lower sedimentation coefficient (100S) during LT particle interference. This new polymerase complex synthesizes in vitro a small RNA molecule with a sedimentation coefficient of 4S. The 4S RNA could represent a transcript of a segment of the LT genome. Even if this proves to be correct it is evident that the

fragments synthesized are considerably smaller than that required to code for any of the viral proteins. A fuller understanding of the nature of this product may be of fundamental importance to our understanding of the interference process. In this regard Stampfer et al. (1969) have also found that a 6S RNA species is synthesized in the infected cell during interference. More recently Reichmann (personal communication) has found that a similar small RNA species is synthesized by ST and LT particle associated transcriptase.

The mechanism or the step of replication of B particles at which T particles affect polymerase activity is not known. Two models of interference have been suggested. One of them proposes that a protein may be synthesized in an unregulated fashion from the T particle genome, this protein then having the capacity to interfere with the B particle directed synthesis of viral enzymes and RNA (Huang and Wagner, 1966). This model would imply that the genetic information in T particle can be both transcribed and translated. A large body of evidence is accumulating to suggest that defective particles are not transcribed during infection. This evidence consists of the following:

- 1) Cells infected with ST particles of Indiana and New Jersey serotypes in the presence of cycloheximide, do not synthesize any complementary messenger RNA (Huang and Manders, 1972;

Perrault and Holland, 1972a), 2) No identifiable viral antigens were found in cells infected with T particles alone (Schaffer et al., 1969; Huang and Manders, 1972), 3) Genetic studies of Reichmann et al. (1971) have shown that T particles do not complement any of the temperature sensitive mutants of VSV.

In view of the above evidence, Stampfer et al. (1969), Prevec and Kang (1970), Huang and Manders (1972) and Perrault and Holland (1972a), proposed that interference is a result of competition for some limiting viral protein, such as the replicase, synthesized as a result of translation of the mRNA copied from the helper B particle. Since the T particles are unable to synthesize this protein, they would compete with B particle RNA as a template for the replicase. The shorter segment of nucleic acid in the defective particles may confer a selective advantage on these particles, so that when present early in infection the T particle genome multiply at the expense of that of the infectious B particles. These conditions would be manifested as interference.

Our results indicate that polymerase is either not produced or is inactive in cells infected with T particles alone. In cells coinfecting with both particles, the T particles seem to interfere with polymerase early during the replicative cycle. If the competition model for replicase

or other viral protein is correct then the observed inhibition of polymerase activity is a result of inhibition of B particle replication and hence secondary transcription.

SUMMARY

A RNA-dependent RNA polymerase induced in L-cells infected with vesicular stomatitis virus was studied. Enzyme activity, measured by the incorporation of ^3H -GTP into acid-insoluble form, was observed only in the cytoplasm of infected cells. Activity first appeared at 2 hrs after infection and reached its maximum at 6 hrs in cells infected with Ind-ST, while in HR-LT infected cells, it continued to increase. Enzyme activity increased with increased input of virus particles up to 20 PFU/cell. Higher multiplicities did not augment enzyme yield or virus titers.

The in vitro properties of the enzyme assay were examined to establish optimal conditions for synthesis of its product. For activity to be manifested, Mg^{2+} , plus all four ribonucleoside triphosphates were required. The reaction was independent of exogenous template, and activity was associated with two ribonucleoprotein complexes, one sedimenting at 160S and the other at 140S. Both complexes contained virus-specific proteins N, L, and NS together with virion RNA.

The in vitro product of either the crude cytoplasmic extracts or the separated RNP complexes synthesized a single stranded RNA species with sedimentation coefficients of 5-18S. This synthesized product is a heteropolymer as demonstrated by incorporation of α -labelled nucleotide triphosphate into internal phosphodiester linkages. Preliminary results indicated that the product was initially synthesized on the template-enzyme complex and then released.

Hybridization of the RNA synthesized after one or 3.5 hrs of incubation at both 37°C and 28°C showed it to be completely complementary to RNA extracted from B or LT particles. Only the RNA synthesized at 28°C hybridized to RNA from the small defective particle. These results plus the data from temperature shift experiments suggest that transcription at 37°C is more limited than transcription at 28°C.

The poly-A content of the synthesized products was examined. Analysis of the ATP-labelled product by sucrose density gradient centrifugation showed that the RNA products of the polymerase complex include a short poly-A sequence (4S) which is covalently linked to a 16S RNA species. Since no detectable poly-U sequences were found in the virion RNA, it is concluded that the poly-A segment is added post-transcriptionally.

The correlation of enzyme activity with interference

was analyzed. Results indicate that extractable in vitro activity corresponded with the extent of homotypic and heterotypic interference.

The above results were discussed with respect to the mode of replication, regulation of transcription, and interference of VSV.

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