IN VITRO REPLICATION OF TOBACCO MOSAIC VIRUS (TMV) RNA IN TOBACCO CALLUS CULTURES: SOLUBILIZATION AND PARTIAL PURIFICATION OF MEMBRANE-BOUND REPLICASE AND ASSOCIATION OF TMV-RNA WITH POLYRIBOSOMES IN TOBACCO CALLUS

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JAMES L. WHITE

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This is to certify that the

thesis entitled

IN VITRO REPLICATION OF TOBACCO MOSAIC VIRUS (TMV) RNA IN TOBACCO CALLUS CULTURES: SOLUBILIZATION AND PARTIAL PURIFICATION OF MEMBRANE-BOUND REPLICASE AND ASSOCIATION OF TMV-RNA WITH POLYRIBOSOMES IN TOBACCO CALLUS

presented by

James L. White

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Harry H. Murakishi
Major professor

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ABSTRACT

IN VITRO REPLICATION OF TOBACCO MOSAIC VIRUS (TMV) RNA IN TOBACCO CALLUS CULTURES: SOLUBILIZATION AND PARTIAL PURIFICATION OF MEMBRANE-BOUND REPLICASE AND ASSOCIATION OF TMV-RNA WITH POLYRIBOSOMES IN TOBACCO CALLUS

By

James L. White

A fraction which contained membrane-bound tobacco mosaic virus RNA replicase was isolated from tobacco mosaic virus-infected tobacco callus cultures. The replicase activity reached a maximum 60 hr after inoculation and then declined. The enzyme activity was insensitive to actinomycin D and DNase. The corresponding fraction from healthy callus contained essentially no activity. The viral RNA synthesis in vitro proceeded linearly for 30 min and required the four nucleotide triphosphates and Mg^{2+} ions. Mn^{2+} was a poor substitue for Mg^{2+}. During RNA synthesis the product was at least 70% resistant to RNase in 2 x SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), but completely digested by RNase in 0.1 x SSC. Analysis of the product by polyacrylamide gel electrophoresis revealed a double-stranded RNA (4.0×10^6 daltons) which appears to be replicative form and a partially RNase resistant structure similiar to replicative intermediate. Washing the membrane-bound replicase with Mg^{2+}-deficient buffer solubilized the enzyme. The solubilized enzyme was further purified by DEAE-Sephadex column chromatography. The DEAE-purified enzyme was nearly completely

depdendent upon TMV-RNA for activity. Analysis of the product on sucrose gradients revealed a double-stranded RNA with sedimentation of 16 S and smaller heterogenous RNase-sensitive products.

A procedure was developed to isolate polyribosomes from plant cell cultures. Tobacco callus extracted in 10 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose, and 140 mM tris-HCl, pH 8.2 yielded larger amounts of polysomes than cells extracted in higher or lower ionic strength or pH buffers. Optimal conditions for extraction of polysomes from soybean callus were identical except the most suitable pH for recovery was 8.5. Addition of the divalent cation chelator, ethyleneglycol-bis (2-aminoethyl ether) tetraacetic acid (EGTA) to the extraction medium improved polysomal yield from tobacco and soybean cultures. Polysomes were successfully extracted from potato, tomato, corn, and barley cell cultures in extraction medium supplemented with EGTA.

Extraction of polyribosomes from tobacco tissue cultures previously inoculated with ^{3}H-uridine labeled TMV-RNA, radioactivity was detected in large polyribosomes (>250 S). Extraction of RNA from the large polyribosomes and analysis on polyacrylamide gel electrophoresis revealed a single homogeneous peak that comigrated with authentic TMV-RNA. Analysis of the nascent polypeptides associated with TMV-RNA polyribosome revealed three polypeptides (molecular weights: 130,000; 37,000 and 17,500 daltons). The 130,000 dalton polypeptide was synthesized in the largest amount with lesser amounts of the 37,000 and 17,500 dalton polypeptides. The latter polypeptide appears to be TMV coat protein. It appears that intact TMV-RNA is associated with polyribosomes and that three polypeptides are synthesized in differing amounts. The importance in replication of TMV of the small amounts of the 2 smaller polypeptides synthesized remains to be resolved.

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By

James L. White

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This disseration is dedicated to my parents

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I will always remember the conversation and celebrations of my friends Jack Bailey, Duncan Bell, Tom Graan, Sandy Perry, Gordon Scott, Pat Erdelyi Scott, and Steve Wang.

TABLE OF CONTENTS

LIST OF FIGURES

PART 1

PART 2

PART 3

Page

LIST OF TABLES

PART 1

PART 2

PART 3

GENERAL INTRODUCTION

The development of a suitable plant tissue culture-virus system for the study of biochemical events of virus replication has been a goal for plant virologists. Early attempts to infect plant tissue cultures with virus has been reported (Kassanis et al., 1958; Wu et al., 1960; Murakishi, 1968), however only a few cells were infected and the final titer achieved was generally lower than in whole leaf tissue (Kassanis, 1957).

With the development of a plant-virus-plant tissue culture system with high titer and physiological response comparable to that obtained in leaf tissue has allowed the study of virus replication and host cell-virus interaction in a tissue culture system (Murakishi et al., 1970; 1971; Beachy and Murakishi, 1971; Pelcher et al., 1972). In inoculated tissue cultures derived from hypersensitive tobacco, a necrotic reaction occurs which is similar in appearance to that observed following inoculation of a hypersensitive leaf (Beachy and Murakishi, 1971). Pelcher et al. (1972) reported the first detailed study of viral RNA synthesis in inoculated tissue culture. The kinetics of viral RNA synthesis was correlated with the incorporation of viral RNA into complete virus and with accumulation of complete virus.

This thesis reports a study on the cell-free replication of TMV-RNA in tobacco cells and the solubilization of membrane-bound TMV replicase and its partial purification. A study was made on the conditions

required to isolate polyribosomes from tobacco and other callus cultures. Investigated was the association of TMV-RNA and polyribosomes to determine the number and nature of the polypeptides synthesized in vivo by TMV-RNA.

LITERATURE REVIEW

Virus related RNAs found in infected cells

Virus infected cells contain unique virus-induced TMV species in addition to the RNA-species encapsulated in virions. Two of these species are double-stranded replicative form (RF) and partially double-stranded replicative intermediate (RI) similar to those found in bacterial and animal cells infected with some single stranded RNA viruses. The third is a low molecular weight component (LMC-RNA) identified in TMV-infected tissues and as a component of the cowpea strain of TMV.

Replicative form

Soon after the discovery of a double-stranded form of RNA in encephalomyocarditis-infected mouse ascite tumor cells (Montagier and Sanders, 1963) such forms were identified in a number of tissues or cells infected with other RNA viruses (Ralph, 1969). Among these were extracts of plant tissues infected with TMV, turnip yellow mosaic virus, cucumber mosaic virus and a number of other viruses (Ralph and Wojcik, 1966, 1969). The best characterized plant virus RF to date is that extracted from TMV-infected tissues. It resembles that of poliovirus RF (Bishop and Koch, 1967) in being composed of an unbroken strand of viral RNA (plus strand) annealed to the complementary RNA (minus strand) (Nilsson-Tillgren, 1970; Jackson et al., 1971). Like the RF's of several bacterial virus RNAs (Franke and Hofschneider, 1966) and in

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contrast to poliovirus RF (Pons, 1964), TMV-RF is not infectious but becomes so when the strands are separated (Jackson et al., 1971). Minus strand has not been detected to exist free in extracts, but only as a component of the double-stranded forms (Siegel et al., 1973).

Replicative intermediate (RI)

A form of RNA partially double-stranded and partially single stranded is present in cells infected with bacterial and animal RNA viruses (Franklin, 1966; Baltimore, 1968). This form called replicative intermediate is believed to be composed of a double-stranded core, the same length as RF, and to have single-stranded tails which presumably are mostly plus strands in the process of being synthesized. RI has also been isolated from TMV-infected leaves and has been partially characterized. It is larger than RF as evidenced by its sedimentation in sucrose gradient and its electrophoretic mobility in polyacrylamide gels. It is intermediate between viral RNA and RF in buoyant density in cesium chloride, and is converted to a form closely resembling RF upon mild ribonuclease treatment (Nilsson-Tillgren, 1970; Jackson et al., 1971).

Low molecular weight component (LMC-RNA)

A low molecular weight component (ca. 250,000 daltons), single stranded RNA species has been detected in total extracts of TMV-infected cells (Jackson et al., 1972) and has been identified as a fragment of viral RNA. It does not contain the 5'-terminus of TMV-RNA and is present in low concentrations. It is detectable only after labeling of cellular RNA with radioactive isotopes. Siegel et al. (1973) postulated the LMC-RNA might represent the messenger RNA for viral coat protein. Subsequently, Beachy and Zaitlin (1975) demonstrated that LMC-RNA was

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associated with polyribosomes and Hunter et al. (1976) demonstrated that LMC-RNA is the messenger RNA for TMV coat protein.

Morris (1974) detected a discrete class of short nucleoprotein rods (33 nm) in addition to the expected 300 nm rods in the cowpea strain of TMV isolated from infected cowpea tissues. Bruening et al. (1976) found a third class of intermediate length rods. RNA from the short rods (similiar to LMC-RNA) directed the cell-free synthesis of a polypeptide with some properties of capsid protein. RNA from 300 nm rods (complete virus) directed the synthesis of a spectrum of polypeptides. Among these was one of approximately 130,000 daltons predominated. RNA from rods of intermediate length directed the synthesis of one or two polypeptides with molecular weights of about 30,000 daltons.

Mechanisms of RNA replication

In vivo

In the preceeding sections the evidence for the presence of double-stranded RNA forms, RF and RI, in plant tissues was presented. In this section, the role of these intermediates in the synthesis of viral RNA will be discussed. Although there is uncertainity concerning the functional conformation and the relative amounts of these forms inside the cell (Weissman et al., 1968; Bove et al., 1968), it is assumed by analogy with evidence from bacterial and animal systems that the double-stranded forms are indeed involved in replication of plant viral RNA. The few experiments performed to date tend to support this assumption.

Ralph et al. (1965) observed that when double-stranded RNA is extracted from turnip yellow mosaic virus-infected leaves labeled with

^{32}P for short time (0.5 to 4 hr), the plus strand (viral RNA) was probably labeled to a greater extent than its complement. In a similiar study, Nilsson-Tillgren (1971) observed that most of the labeled on TMV-RI was in the plus strand 60 min after addition of radioactive label.

Nilsson-Tillgren et al. (1969) and Nilsson-Tillgren (1973) concluded that the minus strands are formed more rapidly early in the replication cycle based on the rate of the appearance of the plus strand and minus strand in the relatively synchronous leaf system. After an initial rapid rise, the concentration of the minus strand then remains constant throughout the rapid synthesis of viral RNA. The final ratio between the plus and minus strand is approximately 100:1. It was determined that the concentration of RI was greater than RF at early stages, while the converse was true at latter stages. This study, although not definitive is suggestive of an asynchronous replication of the plus strand and is consistent with the concept that RI is the precursor of viral RNA (Bishop and Levintwo, 1971).

An investigation (Jackson et al., 1972) with separated cells that had been previously infected with TMV, allowed the use of pulse-chase experiments. When ^{3}H-uridine was added for 30 min, more label was detected in RF and RI than in viral RNA. After incubation of these cells for 3.5 hr in the presence of unlabeled uridine, all the label had disappeared from RI but RF still contained some radioactivity. Even longer chase periods failed to remove all the label present in RF. A great majority of the label present in the double-stranded RNAs was incorporated into viral RNA during chase periods. These experiments suggest that RI is the predominant precursor of viral RNA, although some

reservations are due to the inherent difficulty of performing pulse-chase experiments with eukaryotic systems.

In summary, RF and RI appear to be involved in viral RNA synthesis. At early times after infection RI is the predominant structure while at later times RF is predominant. RI seems to be the best candidate as the source of viral RNA, but the role of RF and RI still remains to be clearly resolved.

Cell-free RNA synthesis

The cell-free RNA synthesis studies of viral RNA polymerase from Qβ-infected bacterial cells, have been used as a model for the studies of plant viral RNA polymerases. Viral RNA (plus strands) acts as a template for viral-induced polymerase. Qβ-polymerase will recognize Qβ-RNA and its complement almost exclusively except for the homopolymer cytidylic acid. The polymerase directs the synthesis of the minus strand in the 5' to 3' direction using the plus strand as the template to form first RF and then RI. Upon completion of synthesis the minus strand acts as a template for the synthesis of new plus strands via RI. RI appears to be the immediate precursor for viral RNA (Eoyang and August, 1974). The role of RF is not clearly understood. Bishop and Levintow (1971) described it as "an end-product or expended template".

Virus-specific actinomycin D-insensitive RNA polymerase has been detected in viral infected tissues. In most cases, enzyme activity has been found in a 31,000 x g particulate fraction. In a few cases, the enzyme has been solubilized and partially characterized, however, after treatment the enzyme becomes quite unstable (Zaitlin et al., 1973; Sela and Hauschner, 1975).

Particulate cell-free extracts prepared from infected tissues of

TMV (Ralph and Wojcik, 1969), turnip yellow mosaic virus (Bove et al., 1969), brome mosaic virus (Semal and Hamilton, 1968), and cucumber mosaic virus (May et al., 1970) contain a viral-specific replicase. Enzyme activity was detected by incorporation of ^{3}H-uridine monophosphate into trichloroacetic acid-insoluble material and is dependent on the presence of all four nucleotide triphosphates and Mg^{2+} ions for maximal activity. Except for one case, cucumber mosaic virus-specified replicase, the reaction preceeds without the addition of exogenous template, indicating the template is bound to the enzyme (May et al., 1970; Siegel and Hariharasubramanian, 1974). The product is largely ribonuclease resistant and closely resembles RF isolated from infected tissues. There is evidence that replicase isolated early in the infection cycle synthesizes both plus and minus strand, while later in the cycle only the plus strand is synthesized (Bradley and Zaitlin, 1971). A component resembling RI has been isolated upon occasion, however free plus strands are usually never found as a product (Bradley and Zaitlin, 1971). Failure to detect single-stranded RNA may be due to the presence of ribonuclease in the extracts, less than optimal assay conditions, or that the enzyme system lacks a component(s) necessary for the synthesis of single-stranded RNA.

The particulate systems from brome mosaic virus- and broad bean mosaic virus-infected tissues are capable of synthesizing both double-stranded and single-stranded RNA with the predominante amount of radioactive precursor incorporated into single-stranded form (Jacquemin, 1972; Semal and Kummert, 1971). In the first two to three minutes of the reaction, labeled precursor is incorporated into a double-stranded product (RF), subsequently label begins to appear in single-stranded

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RNA. The amount of labeled double-stranded precursor remains constant after the initial rise, while single-stranded RNA concentration increases linearly for approximately 30 min. The single-stranded products are of a heterogenous size and reflect the split-genome nature of the virus. Pulse-chase experiments indicate that the labeled precursor first appears in the plus strands of RF and after further synthesis appears as free viral RNA (Semal and Kummert, 1971).

Attempts to solubilize the enzymes from particulate tissue fractions of TMV (Zaitlin et al., 1973), brome mosaic virus (Hadidi and Frankel-Conrat, 1973), turnip yellow mosaic virus (Lalfeche et al., 1972), and cucumber mosaic virus (May et al., 1969) have met with partial success. All the solubilized enzymes with the exception of turnip yellow mosaic virus-specified replicase have little activity in the absence of exogenous RNA. The enzymes from tissues infected with turnip yellow mosaic virus and cucumber mosaic virus have been shown to have little template specificity. RNAs from diverse sources being almost equally capable of stimulating incorporation of precursor into acid insoluble product. In the cases of brome mosaic virus-infected tissues, the enzyme showed some partial specificity by being stimulated best by brome mosaic virus RNA and the closely related cowpea chlorotic mottle virus RNA, and to a lesser extent by Qβ-RNA, and broadbean mottle virus RNA (Siegal and Hariharasubramanian, 1974). Sela and Haushner (1975) purified TMV-replicase to an extent where TMV-RNA was the only template that stimulated incorporation of precursor. The reason why most plant viral polymerases exhibit such a low specificity is unclear, perhaps it may be due to insufficient purification or loss of a necessary factor in purification. The product of the solubilized polymerase is

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predominantly double-stranded RNA, only the complement of the template is synthesized. The size of the viral replicases have been estimated to be approximately 130,000 to 150,000 daltons (Zaitlin et al., 1973; Hadidi and Frankel-Conrat, 1973). Whether they possess a subunit structure similiar to that of Qβ-replicase (Kamen, 1970; Weissman et al., 1973) remains to be answered, although Hariharasubramanian et al., (1973) may have detected a subunit of brome mosaic virus-specified polymerase.

While searching for viral replicase, healthy chinese cabbage (host for turnip yellow mosaic virus) and tobacco (host for TMV) were found to possess a soluble RNA polymerase (Astier-Monetcier and Cornuet, 1971; Duda et al., 1973). Enzyme activity was found to be greater in the infected plants than healthy plants. The enzyme from tobacco plants is template independent and synthesizes a small double-stranded RNA. It is stimulated by exogenous RNA in a manner that is not understood. The exogenous RNA is not a primer or template. The function of these enzymes in healthy plants remains to be resolved, but possible could be involved with gene amplification.

Translation of viral nucleic acid

Virus related proteins found in infected tissues

The genome of some small RNA phages such as R17 and f2 are approximately 1.2×10^6 daltons and contains three cistrons (Gussin et al., 1966). Most plant virus genomes are larger than these phage and one could expect that they could code for 5 to 10 proteins. One would expect to detect in infected cells other proteins besides the viral coat protein. In the past few years a large effort has been put into a search for viral specific proteins in infected cells.

The method generally used has been to detect new proteins in

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infected tissues has been co-electrophoresis of sodium dodecyl sulfate and urea-treated, differentially labeled extracts from healthy or virus-infected tissues. Despite difficulties presented by high background of host proteins synthesis, several proteins have been detected in infected macerates.

The synthesis of several proteins is stimulated in TMV-infected tissues, while others are depressed. Among the ones showing stimulation are those in the range of 155-245 x 10^3 daltons (Zaitlin and Hariharasubramanian, 1970, 1972; Sakai and Takebe, 1972). Experimental techniques do not allow the determination of whether these proteins are virus specific, however the protein of approximately 155,000 daltons appears to be viral replicase. Why the failure to detect significant amounts of new viral proteins remains to be resolved. The difficulty in interpreting data from double-labeling experiments is exemplified by the work with tomato spotted wilt virus. This complex virus contains three major structural glycoproteins. However, only one of these three proteins can be recovered and detected in extracts from infected cells (Mohamed et al., 1973).

In vitro translation of TMV-RNA

It has been demonstrated that larger plant viral RNAs stimulate the incorporation of radioactively labeled amino acids into polypeptides in cell-free protein synthesis systems form eukaryotes and prokaryotes (Efron and Marcus, 1973; Roberts and Paterson, 1973; Aach et al., 1964), but fail to produce large quantities of coat protein. This may not be unexpected since LMC-RNA has been shown to be the coat protein cistron in certain TMV strains, however, this does not rule out the possibility that viral RNA may be translated polycistronically at low levels at

certain times in the infection cycle. There is presumptive evidence that TMV-RNA acts as a mRNA. First, intact viral RNA has been found associated with ribosomes (Van Kammen, 1963; Beachy and Zaitlin, 1975). Second, upon infection TMV stimulates the synthesis of large proteins (155-245 x 10^3 daltons) (Zaitlin and Hariharasubramanian, 1973). This latter result suggests that, as with animal picornaviruses (Jacobson and Baltimore, 1968), the genome may be translated in a polycistronic manner. However, appropriate pulse-chase experiments failed to demonstrate cleavage of the large proteins into smaller products. Third, Efron and Marcus (1973) and Roberts and Paterson (1973) using TMV-RNA in a wheat embryo protein synthesizing system detected the synthesis of a peptide fraction that comigrated with authentic viral coat protein on gel electrophoresis. Comparison of a tryptic digest of this fraction with a similiar digest of *in vivo* synthesized coat protein indicated that the coat protein cistron was translated in the *in vitro* system. In contrast, Bruening *et al.* (1976) and Davies and Kaesberg (1974), using similiar systems under different conditions, failed to detect significant amounts of TMV-coat protein in the *in vitro* cell-free extracts. It appears that some RNAs of split-genome viruses are translated mono-cistronically, but how larger viral RNAs are translated remain to be resolved.

Part 1

In vitro Replication of Tobacco Mosaic Virus RNA in Tobacco Callus Cultures: Solubilization of Membrane-bound Replicase and Partial Purification

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INTRODUCTION

The study of phage-specific RNA-dependent RNA polymerase (replicase) has been aided by the success in purifying stable, template-free enzyme. Qβ-replicase has been purified to a high degree of homogeneity (Eoyang and August, 1968; Eoyang and August, 1971; Kondo et al., 1970; Kamen, 1972). The active enzyme complex consists of four polypeptides, one of which is the gene product of the replicase cistron of the phage genome (Kamen, 1972; Kondo et al., 1970). A unique property of the enzyme is its template specificity. The only nucleic acids known to serve as templates are Qβ-RNA, Qβ-complementary strand, and variants of Qβ-RNA including 6S RNA present in Qβ-infected cells.

The investigation of eukarotic viral RNA replication has been hampered by the inability to isolate stable, template-free replicase in a soluble form. Most RNA replicases isolated from infected plant or animal cells are associated with membranes (Arlinghaus and Polatnick, 1969; Horton et al., 1964; Martin and Sonnebend, 1967; Ralph et al., 1971; Zabel et al., 1974) and possess endogenous templates. Attempts to solubilize RNA replicases from membranes by using various detergents resulted in enzyme preparations that were quite unstable (Ehrenfeld et al., 1970; Hadidi and Fraenkel-Conrat, 1973; Kamen, 1972; Sela and Hauschner, 1975; Traub et al., 1975) or still contained template (Arlinghaus and Polatnick, 1969; Rosenberg et al., 1972).

An RNA polymerase from TMV-infected plants has been purified to an extent where its activity was completely dependent upon TMV-RNA for its

activity (Sela and Hauschner, 1975), however, the enzyme was quite unstable. Replicase from cowpea mosaic virus-infected tissue has been purified from membranes by using Mg^{2+}-deficient buffer. This procedure gentler than detergent disruption of membranes, resulted in soluble, template-free enzyme (Zabel et al., 1976).

During studies of viral replicases, an RNA-dependent RNA polymerase has been detected in the soluble fraction of healthy tissue homogenates in chinese cabbage (host for turnip yellow mosaic virus) (Astier-Manifacier and Cornuet, 1971) and tobacco (host for TMV) (Duda et al., 1973). The enzyme activity in tobacco leaves is stimulated upon TMV infection. Its function in healthy plants is unknown.

I investigated some properties of TMV replicase and soluble RNA-dependent RNA polymerase in TMV-infected callus; also described is a procedure to release TMV replicase from membranes, resulting in a stable, template-free enzyme. After DEAE-Sephadex chromatography the enzyme is nearly completely dependent upon TMV-RNA as a template.

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MATERIALS AND METHODS

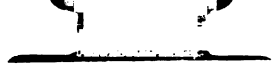
Buffers. Buffer A: sucrose 0.4 M: KCl 10 mM; $MgCl_2$ 5 mM; dithiothreitol (DTT) or dithioerythritol (DTE) 2.5 mM; Tris 150 mM, pH 8.2 at 4 C; glycerol 10% (v/v). Buffer B: KCl 10 mM; DTT or DTE 2.5 mM; NH_4Cl 25 mM; Tris 10 mM, pH 8.0 at 4 C; glycerol 10% (v/v). Buffer C: NH_4Cl 10 mM; ethyleneglycol-bis-(-amino-ethyl ether)N,N'-tetraacetic acid (EGTA) 10 mM; DTT or DTE 5 mM; Tris 100 mM, pH 8.2 at 4 C; glycerol 10% (v/v). Buffer D: NaCl 100 mM; sodium EDTA 10 mM; Tris 100 mM; 0.5% sodium dodecyl sulfate (SDS) pH 7.2. Buffer E: sodium EDTA 1 mM; sodium acetate 20 mM; Tris 40 mM, brought to pH 7.8 with glacial acetic acid containing 5% sucrose. SSC: NaCl 150 mM; sodium citrate 15 mM, pH 7.0.

Preparations of tissue cultures and inoculation. A pigmented cell culture derived from pith of tobacco (Nicotiana tabacum L. var. Havana 38) was used throughout this study. The cells were maintained and inoculated as previously described (Murakishi et al., 1970; Pelcher et al., 1972). Cell suspensions (approximately 500 mg fresh weight) in log phase of growth were aseptically inoculated by dispersion in a TMV solution (150 µg/ml) using a vortex mixer. Inoculated cells were pooled, stirred gently and washed with fresh medium (Murakishi et al., 1971). One gram samples were spread over the surface of agar and incubated at 22 to 25 C under fluorescent lights (3.7 Kerg/ cm^2.sec).

Preparation of the replicase and RNA-dependent RNA polymerase. All operations were carried out at 4 C. The glassware used in preparation

of the extracts and enzyme assays were autoclaved. Five grams of healthy or TMV-infected callus were soaked for 2 min in buffer A. The cells were then drained on a filter and gently disrupted in 8 ml of buffer A with the aid of a mortar and pestle. The homogenate was filtered through "Miracloth" and the filtrate was centrifuged at 31,000 x g for 30 min. The resultant supernatant was brought to pH 4.7 with 0.05 N HCl, allowed to sit at 4 C for 15 min, and then centrifuged at 20,000 x g for 20 min. The pellet was resuspended in buffer B (0.05 ml per gram of tissue). This solution was used as the source for the RNA-dependent RNA polymerase (Duda et al., 1973). The 31,000 x g pellet was resuspended in buffer B with the aid of a conical tissue grinder and centrifuged at 31,000 x g for 30 min. This procedure was repeated twice. The final pellet was resuspended in 0.5 ml of buffer B for each 5 grams of tissue used. This suspension was used as the source of the crude bound replicase.

Replicase and RNA-dependent RNA polymerase assay. The standard assay mixture contained 100 mM Tris (pH 8.0 at 33 C), 10 mM $MgCl_2$, 7.5 mM DTT or DTE, 25 mM $(NH_4)_2SO_4$, 10 g/ml Actinomycin D (AMD), 0.5 mmoles/ml of each ATP, CTP, GTP and 1 nmole of ^{3}H-UTP (spec. activity 43 Ci/mmole). Assays with template-free enzyme, RNA was added to the reaction mixture at a concentration of 50 µg/ml. The standard reaction mixture was 0.1 ml; the reaction was initiated by adding the enzyme. Incubation was carried out at 33 C for designated periods of time in a reciprocal shaking water bath. The reaction was terminated by transferring two 50 µl samples onto a 2.3 cm disc of Whatman 3 MM filter paper which was then placed into cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and 0.02% uracil. The discs were washed as described by Zaitlin et al. (1973). The radioactivity on the disc was determined as described



by Pelcher et al. (1972). Zero time values for assays with bound replicase were significant and were substracted from the values obtained for incubated samples. The results are expressed as picomoles of ^{3}H-UMP incorporated per mg of protein per 30 min of incubation time for bound replicase and soluble polymerase. With template-free enzyme reaction mixtures the incubation time was increased to 120 min. Protein was determined by the technique of Lowry et al. (1951).

Preparation of solubilized replicase. Solubilized replicase was prepared by adding bound replicase to buffer C and stirring the mixture for 30 to 60 min at 4 C. The solution was centrifuged at 31,000 x g for 30 min. The resulting supernatant was removed, brought to 60% saturation with $(NH_4)_2SO_4$, allowed to mix slowly for 30 min and then centrifuged at 20,000 x g for 20 min. The resulting pellet, which contained the majority of the replicase activity, was resuspended in buffer B (without glycerol), layered on a linear 7.5 to 20% glycerol gradient and finally centrifuged in a Spinco SW 50L rotor at 175,000 x g for 15 hr. Sedimentation markers were ovalbumin (45,000 daltons) and aldolase (158,000 daltons). After sedimentation, 0.5 ml fractions were collected and enzyme activity determined on 75 µl samples. The fractions comprising the enzyme activity peak were pooled and immediately applied to a small DEAE-Sephadex column. The protein was eluted in buffer C with a linear 0.05 to 0.3 M KCl gradient.

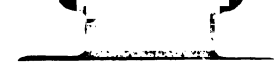
Extraction of RNA. RNA was extracted from scaled-up reaction mixtures (0.5-2.0 ml) with phenol or perchloric acid. Assay mixtures were diluted with a small buffer volume and phenol extracted as described by Bradley and Zaitlin (1971). RNA was extracted from reaction mixtures using a modification of Wilcockson's (1973) perchloric acid method.

Sodium dodecyl sulfate 5% (w/v) and $NaClO_4$ 70% (w/v) to the reaction mixture. This was vortexed for 2 min and then centrifuged at 1000 x g for 10 min. The protein-SDS complex formed a pellicle and the liquid phase containing RNA was removed and placed into cold ethanol. After standing overnight at -20 C, the precipitated RNA was removed by centrifugation and washed with 75% ethanol. The final precipitate was resuspended in buffer E.

Polyacrylamide gel electrophoresis. Acrylamide-bisacrylamide (10-0.25%, respectively) gel prepared in Plexiglass tubes (12 x 1 cm). Three cm gel (10%) were overlayed with 6 cm each of 2.5% acrylamide. The gels were allowed to polymerize for 20 min, transferred to an electrophoresis buffer and allowed to stand at 4 C for 72 hr prior to use. The gels were then placed in Plexiglass tubes, one end of which was covered with dialysis membrane and pre-run for 30 min. Approximately 20 μg of nucleic acid (including markers) was applied and electrophoresis carried out for 180 or 210 min 10 V/cm, 5 mM/gel. Gels were scanned at 260 nm before sectioning. Two 1 mm slices were added to each vial containing NCS-water (9;1, v/v), sealed, and then heated at 50 C overnight. Radio-activity was determined by a Beckman (LS-133) liquid scintillation counter.

SDS-sucrose gradient centrifugation. Perchloric acid extracted product of the reaction mixture using DEAE-Sephadex purified replicase as the enzyme source, was layered on a linear 15 to 30 % (wt/vol) sucrose density gradient in buffer D and centrifuged at 22,000 rpm in a rotor SW 25.1 for 18 hr. Two equal samples were taken from each fraction. One was treated with RNase (10 g/ml) in 2.0 x SSC for 60 min at 25 C and the other was incubated without RNase. Both samples were then made

5% in trichloracetic acid after the addition of 0.5 mg bovine serum albumin. The precipitates were collected on a Whatman GF/C filter and washed with 10 ml of 5% trichloroacetic acid, 10 ml of cold 95% ethanol, and 5 ml of ethanol:ether (1:1, vol/vol). After drying, the precipitates were solubilized with a solution of 20% NCS solubilizer, 3.75% water, and 76.25% toluene for 4 hr at 37 C. Radioactivity was determine as previously described (Pelcher et al., 1972).



RESULTS

When homogenates of TMV-infected tobacco callus were centrifuged at 31,000 x g the sedimenting material contained an RNA polymerase activity not present in the corresponding fraction from healthy material. This fraction was capable of incorporating ^{3}H-UMP into trichloroacetic acid insoluble product linearly for 30 min (Fig. 1).

Characteristics of replicase reaction in vitro. The omission of each of the unlabeled nucleotriphosphates (ATP,CTP,GTP) considerably reduced enzyme activity (Table 1). If all three nucleotide triphosphates were removed almost no activity was detected. If the 31,000 x g pellet was not resuspended and recentrifuged several times, the incorporation of ^{3}H-UMP was high even in the absence of unlabeled nucleotide triphosphates, indicating the presence of large amounts of unlabeled nucleotides in the preparation. Replicase activity is strongly dependent on the presence of Mg^{2+}. Optimal concentration of Mg^{2+} occurred in a broad range from 8 to 20 mM (not shown). The optimal Mn^{2+} concentration of 1.5 mM could replace Mg^{2+} but only gave 18% of the reaction rate of the assay containing Mg^{2+}. Replicase assay mixtures routinely contained AMD. RNA synthesis was only slightly higher in the presence of AMD. Most AMD-sensitive RNA polymerase activity was detected in the nuclear pellet (1,000 x g). Slight contamination of the 31,000 x g pellet with DNA-dependent RNA polymerase was indicated by higher incorporation of ^{3}H-UMP in assay mixtures in the absence of AMD. Treatment of the product with

Figure 1. Time course of RNA synthesis by bound TMV replicase. Enzyme reaction mixture was incubated at standard conditions. At times indicated, two 50 µl samples were removed, immediately placed on Whatmann 3 MM filter disc, washed with 5% trichloroacetic acid containing 1% $Na_4P_2O_7$, and 0.02% uracil and further processed as described in the Materials and Methods

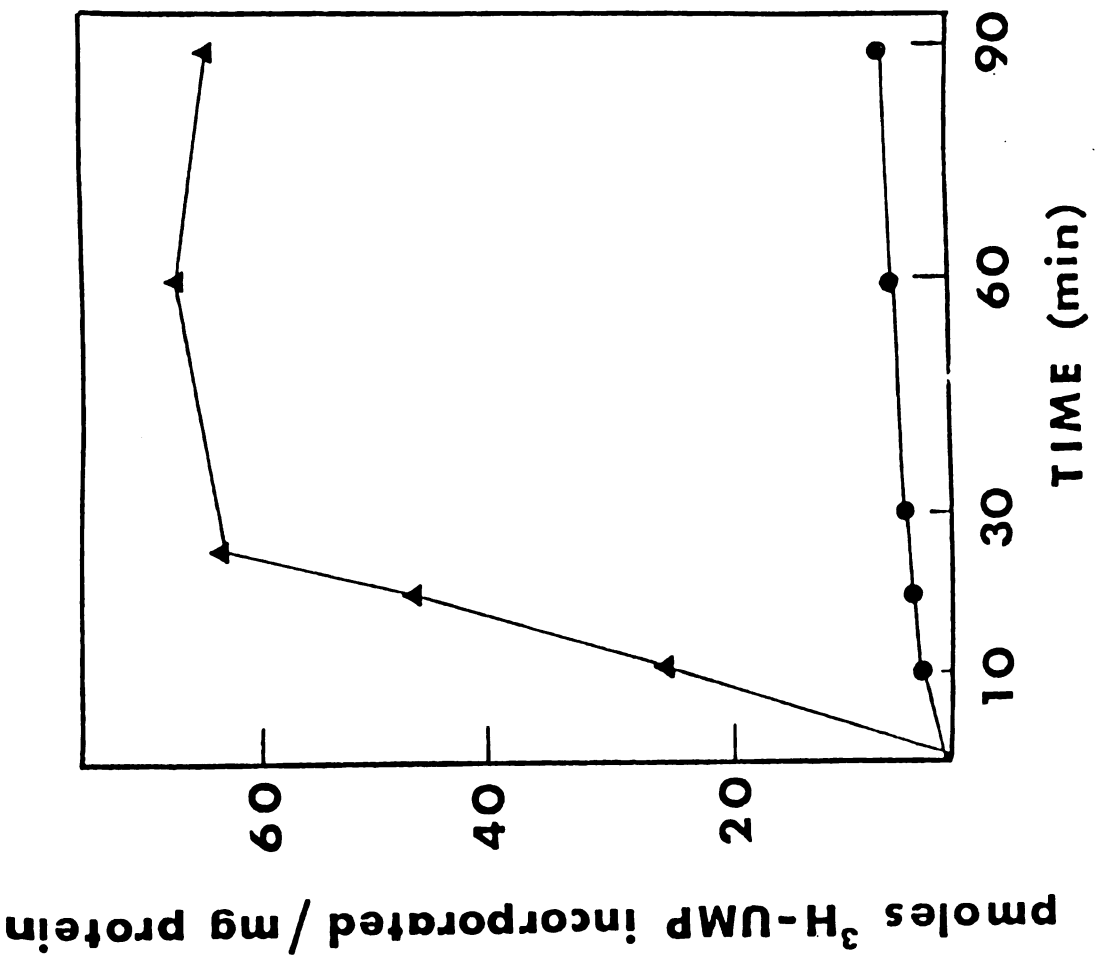
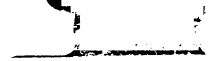

pmoles ^{3}H-UMP incorporated / mg protein
20
40
60
10
30
60
90
TIME (min)

Table 1. Requirements of bound replicase

Reaction conditions	Percent of control
Complete[a]	100
- ATP	21
- CTP	19
- GTP	23
- (ATP, CTP, GTP)	6
- Mg^{++}	8
- Mg^{++} + Mn^{++}	18
- Actinomycin D	112
- Actinomycin D + DNase (30 μg/ml)	96
+ RNase (20 μg/ml)	78
+ TMV-RNA	105
+ BMV-RNA	103

[a]The complete reaction mixture was as described in Materials and Methods.



RNase, reduced the amount of radioactivity approximately 22%. Addition of TMV-RNA or BMV-RNA did not stimulate the amount of ^{3}H-UMP incorporated into product.

Products of the bound replicase. To determine the nature of the products synthesized by replicase, samples were treated for 30 min in either high salt (2 x SSC) or low salt (0.1 x SSC) with RNase. Approximately 70% of the RNA synthesized was resistant to RNase in high salt at all time periods tested. For the first 10 min the amount of RNase-resistant product was greater than at latter times. In low salt, nearly all the product was RNase sensitive. There was no loss of trichloroacetic acid precipitable radioactivity of product incubated in the absence of RNase. It appears that most of the in vitro product is a partially-double-stranded structure (Fig. 2).

The products were further analyzed by polyacrylamide gel electrophoresis. When the products were phenol extracted two major regions of radioactivity were detected (Fig. 3). Using callus rRNAs of known molecular weights 18S (0.75 x 10^6 daltons), 25S (1.2 x 10^6 daltons) and TMV-RNA (2.05 x 10^6 daltons) the extimated molecular weight of the products were determined. Fraction number 10 had an estimated molecular weight of 4.0 x 10^6 daltons and was resistant to RNase degradation. This peak appears to be replicative form, similiar to that described by Bradley and Zaitlin (1971). Another higher molecular weight peak (approximately 5.0 x 10^6 daltons) (fraction no. 4), partially susceptible to RNase degradation, appears to be replicative intermediate. The amount of radioactive label incorporated into replicative intermediate was very low when compared to the replicative form. If the products of the replicase assay were perchloric acid extracted, a

Figure 2. Time course of total RNA synthesis and of the fraction resistant to RNase. A 2-ml reaction mixture was incubated under standard assay conditions. At the indicated times two 50 μl samples were removed. One sample was immediately assayed for total radioactivity (●); the second was incubated in 2 ml of 2 x SSC containing RNase (▲); the third was heated with RNase in (0.1 x SSC) (■); and the last sample was incubated in 2 ml of 2 x SSC without RNase (o). The reactions were terminated by the addition of trichloroacetic acid. Carrier protein was added, and the acid-insoluble precipitates were processed.

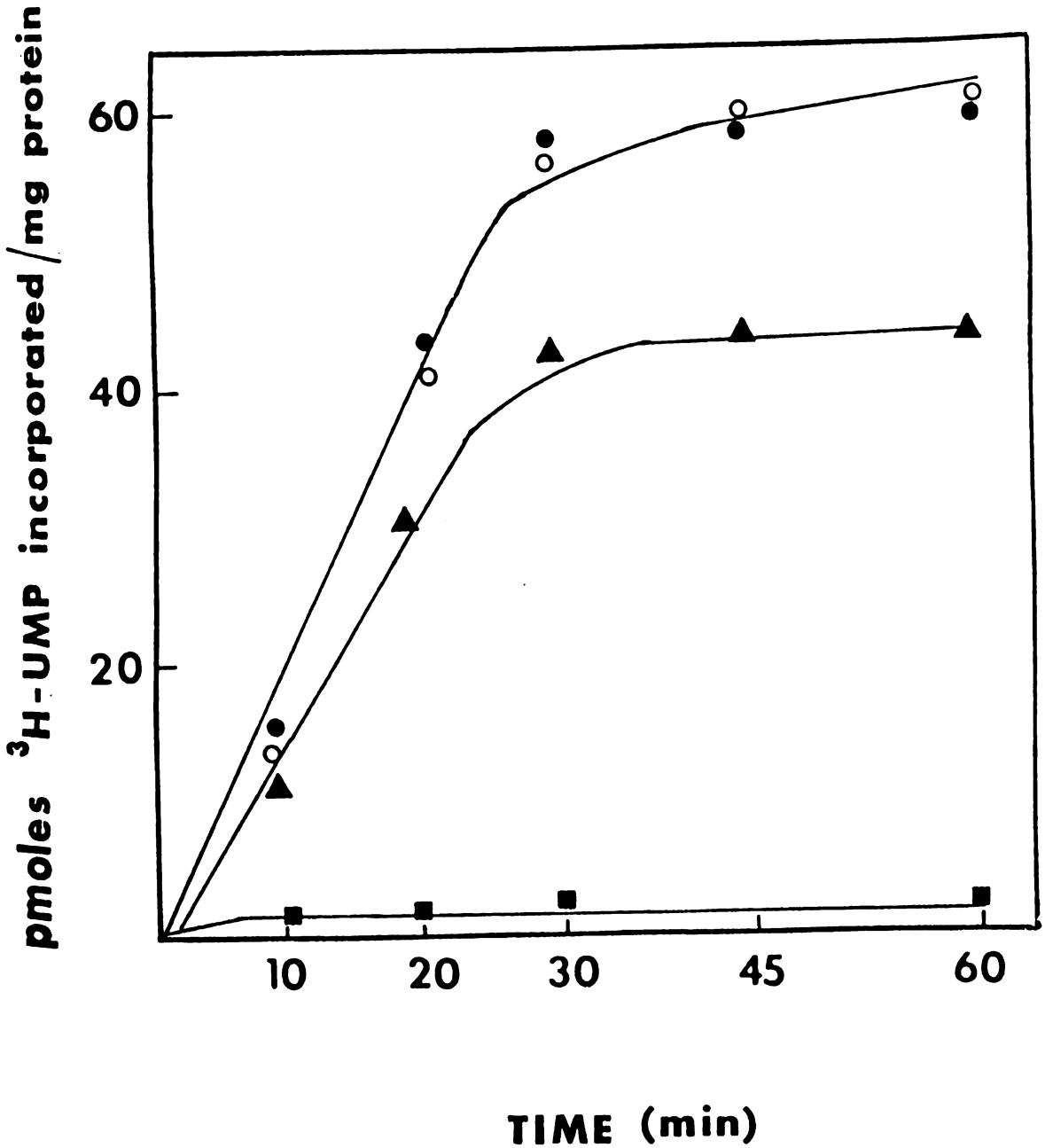
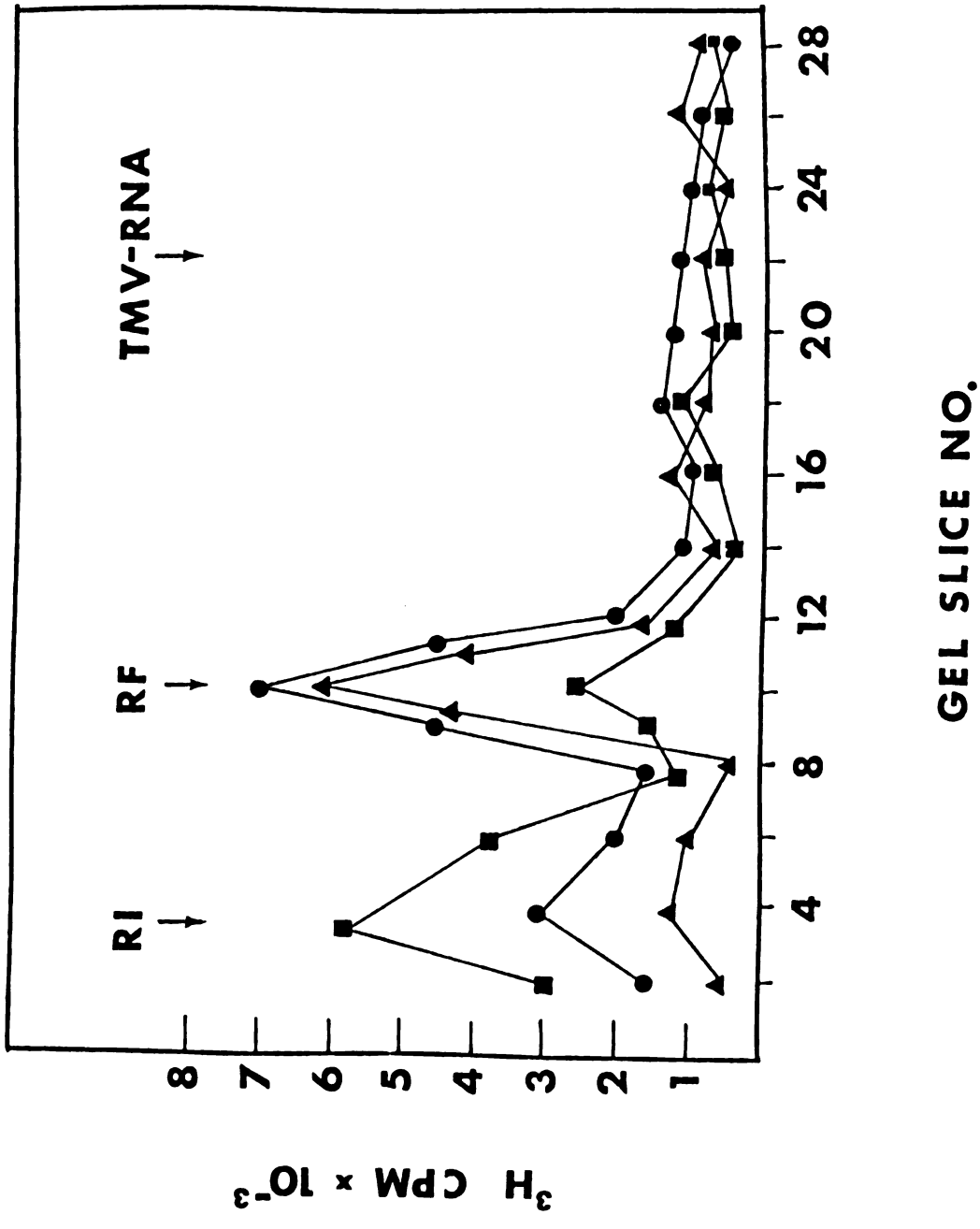

pmoles ³H-UMP incorporated /mg protein
60
40
20
10
20
30
45
60
TIME (min)

Figure 3. Polyacrylamide gel electrophoresis of labeled bound TMV replicase product. A 2-ml reaction mixture was incubated at 33 C for 30 min and subsequently deproteinized by phenol (●) or perchloric acid (■) and analyzed by polyacrylamide gel electrophoresis (210 min at 5 mA/gel). A portion of the reaction mixture extracted with perchloric acid was treated with RNase mixture before gel analysis (▲).


TMV-RNA
RF
RI
8
7
6
5
4
3
2
1
4
8
12
16
20
24
28
GEL SLICE NO.
^3H CPM x 10^{-3}

larger proportion of the radioactivity was recovered in RI than RF. No significant amount of TMV-RNA was detected. To determine if single stranded RNA was destroyed by endogenous ribonuclease activity ^{3}H-TMV-RNA was added to the reaction mixture and after 30 min incubation the amount of trichloroacetic acid precipitable activity remaining was determined. In 30 min approximately 25% of the radioactivity was rendered trichloroacetic acid soluble. It appears the reaction mixture contains an endogenous RNase activity, however, some single stranded RNA could have been detected if synthesized.

Partial purification of replicase. The use of divalent cation deficient buffers to solubilize ATPase from membranes is well established (Abrams, 1965; Steck and Fox, 1972). Cowpea mosaic virus relicase has been solubilized by the use of Mg^{2+} deficient buffers (Zabel et al., 1976). TMV replicase was released from the membranes by the addition of divalent cation-deficient buffer and stirring the mixture. The resulting supernatant contained most of the replicase activity now template-free. The enzyme was nearly completely dependent on the addition of exogenous RNA for activity. After further purification on a glycerol gradient, all tested RNAs (TMV-RNA, BMV-RNA or callus rRNA) stimulated incorporation of ^{3}H-UMP into product (Table 2). Fractions from the glycerol gradient containing enzyme activity were pooled and applied to a DEAE-Sephadex column and eluted by a KCl gradient. Using TMV-RNA as the template in the assay mixtures a single peak of activity was detected (Fig. 4).

Requirements of the solubilized replicase. Using the DEAE-Sephadex purified replicase, other RNAs besides TMV-RNA were tested for their ability to stimulate enzymatic activity (Table 2). BMV-RNA, TYMV-RNA

ble 2.

ection

mplete[a]

- TMV-

- TMV-

- TMV-

- TMV-

- (ATP

- Mg^{++}

- Mg^{++}

+ RNas

+ DNas

[a]Th

ethods.

[b]No

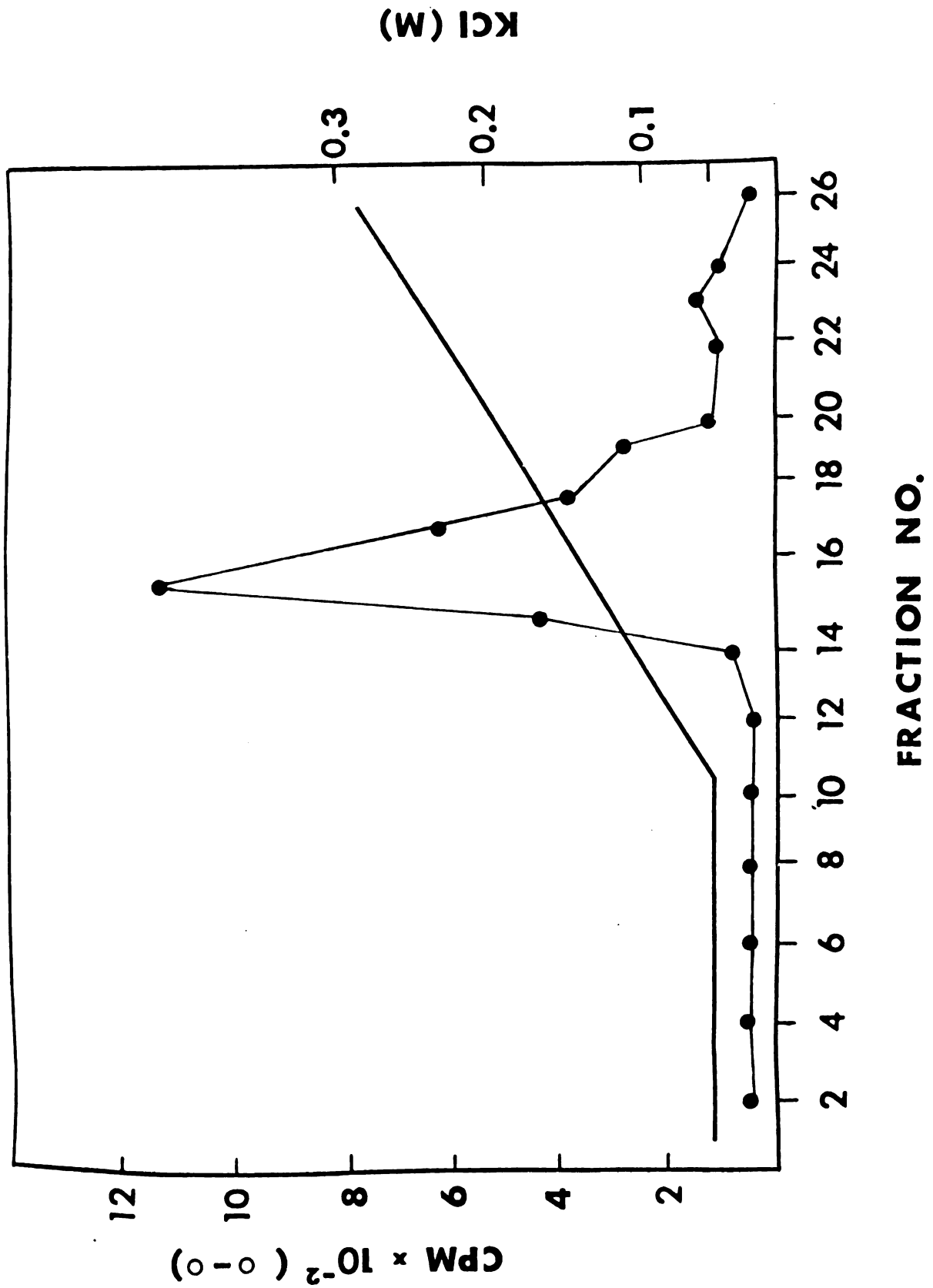
Table 2. Requirements of partially purified replicase

Reaction conditions	Gradient-purified replicase	DEAE-Sephadex purified replicase
Complete[a]	100	100
- TMV-RNA	4	2
- TMV-RNA + BMV-RNA	113	21
- TMV-RNA + TYMV-RNA	87	9
- TMV-RNA + callus rRNA	65	12
- (ATP, CTP, GTP)	3	2
- Mg^{++}	NT[b]	8
- Mg^{++} + Mn^{++}	NT	14
+ RNase	94	83
+ DNase	99	96

[a]The complete reaction mixture was as described in Materials and Methods.

[b]Not tested.

Figure 4. DEAE-Sephadex column chromatography of solubilized TMV replicase. Solubilized replicase was prepared by releasing mixing buffer C with bound TMV replicase. DEAE-Sephadex column was equilibrated in buffer C. After the flow through material had emerged, the column was treated with a linear 0.05 to 0.3 M KCl gradient (—) in buffer C. Fractions (0.5 ml) were colleged at flow rate of 10 ml per hr; 200 µl portions of each fraction were assayed for replicase activity for 90 min (●).


KCl (M)
0.3
0.2
0.1
CPM × 10⁻² (o–o)
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20 22 24 26
FRACTION NO.

or callus rRNA failed to stimulate significant amounts of ^{3}H-UMP incorporation. Omission of the nucleotide triphosphates or Mg^{2+} reduced enzyme activity considerably. The product was partially sensitive to RNase action but relatively insensitive to DNase action. Isolation of the product and analysis in sucrose gradients demonstrated a polydisperse product (Fig. 5). Most radioactivity was found in a RNase-resistant structure, which had a sedimentation coefficient of 16S and appears to be replicative form. A significant portion of the radioactive counts were in structures smaller than replicative form and RNase sensitive. Replicative intermediate or TMV-RNA was not detected.

Soluble RNA polymerase. The soluble fraction of healthy and TMV-infected callus contains and AMD-insensitive RNA polymerase. Table 3 shows the requirements of the enzyme isolated from TMV-infected cells. Although not shown the requirements of the enzyme from healthy callus were essentially identical. Enzyme activity was greatly reduced if the nucleotide triphosphates were omitted. Mn^{2+} could only replace Mg^{2+} to the extent of 22% of the Mg^{2+} stimulated reaction. AMD has little effect upon ^{3}H-UMP polymerization and its increased rate in the absence of AMD is probably due to contaminant DNA-dependent RNA polymerase. The product is resistant to DNase action but only partially resistant to RNase. Addition of TMV-RNA or BMV-RNA failed to stimulate ^{3}H-UMP incorporation. Analysis of the enzyme on glycerol gradients revealed an estimated molecular weight of 150,000 daltons. Any of the RNAs tested (TMV-RNA, BMV-RNA or callus rRNA) could be used to stimulate activity.

Kinetics of the synthesis of replicase, RNA polymerase and viral RNA. Experiments were initiated to simultaneously determine the rate

Figure 5. Sucrose-SDS density gradient centrifugation of DEAE-Sephadex-purified replicase reaction products. A 1-ml reaction mixture was incubated at 33 C and subsequently deproteinized by perchloric acid. A portion of extract was layered onto a 15 to 30% linear sucrose gradient in buffer D and centrifuged at 22,000 rpm for 18 hr in a SW 25.1 rotor. Fractions were collected from the top and two equal portions were assayed for total RNA (●) and RNase resistant RNA (o).

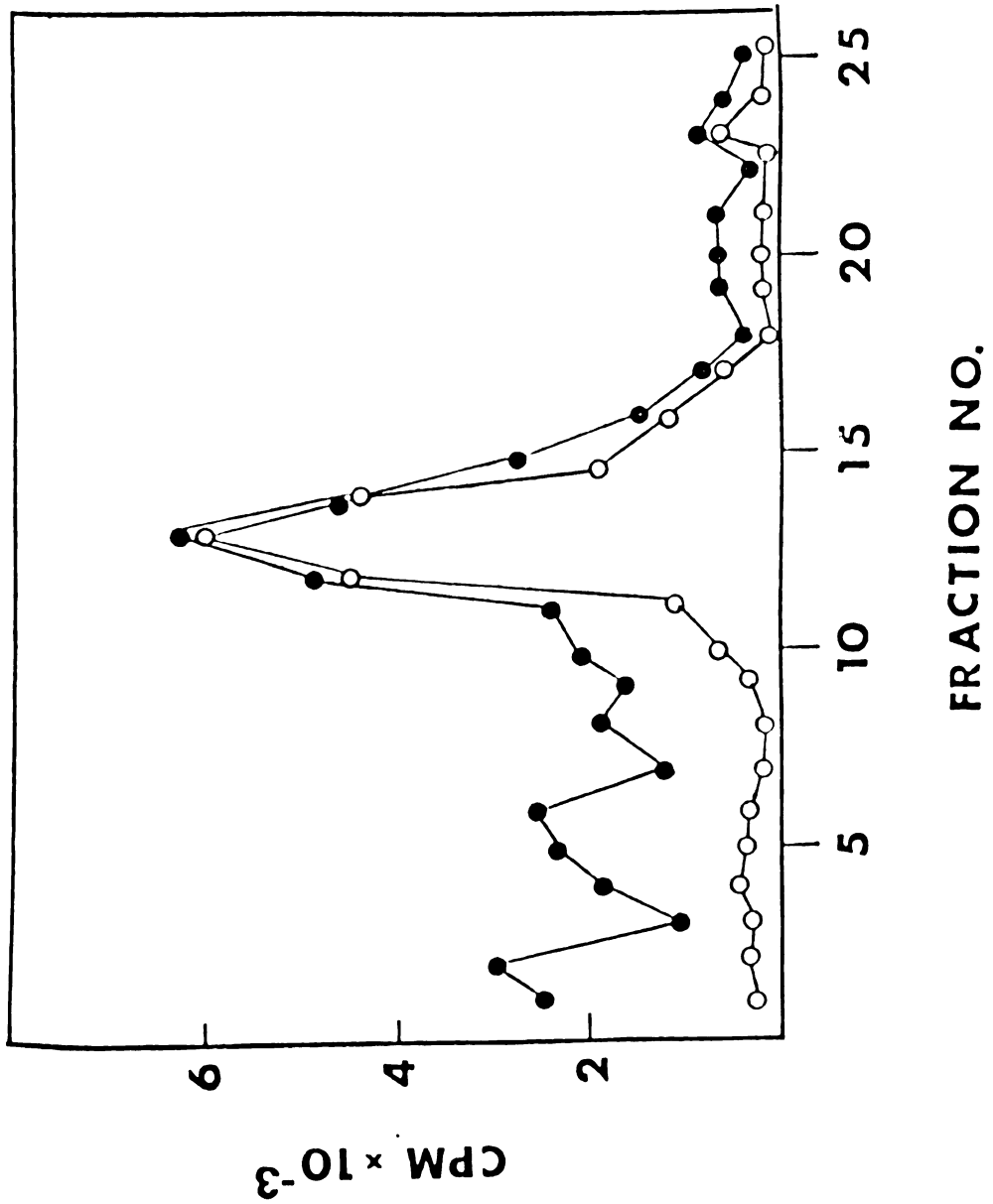

CPM × 10⁻³
2
4
6
5
10
15
20
25
FRACTION NO.

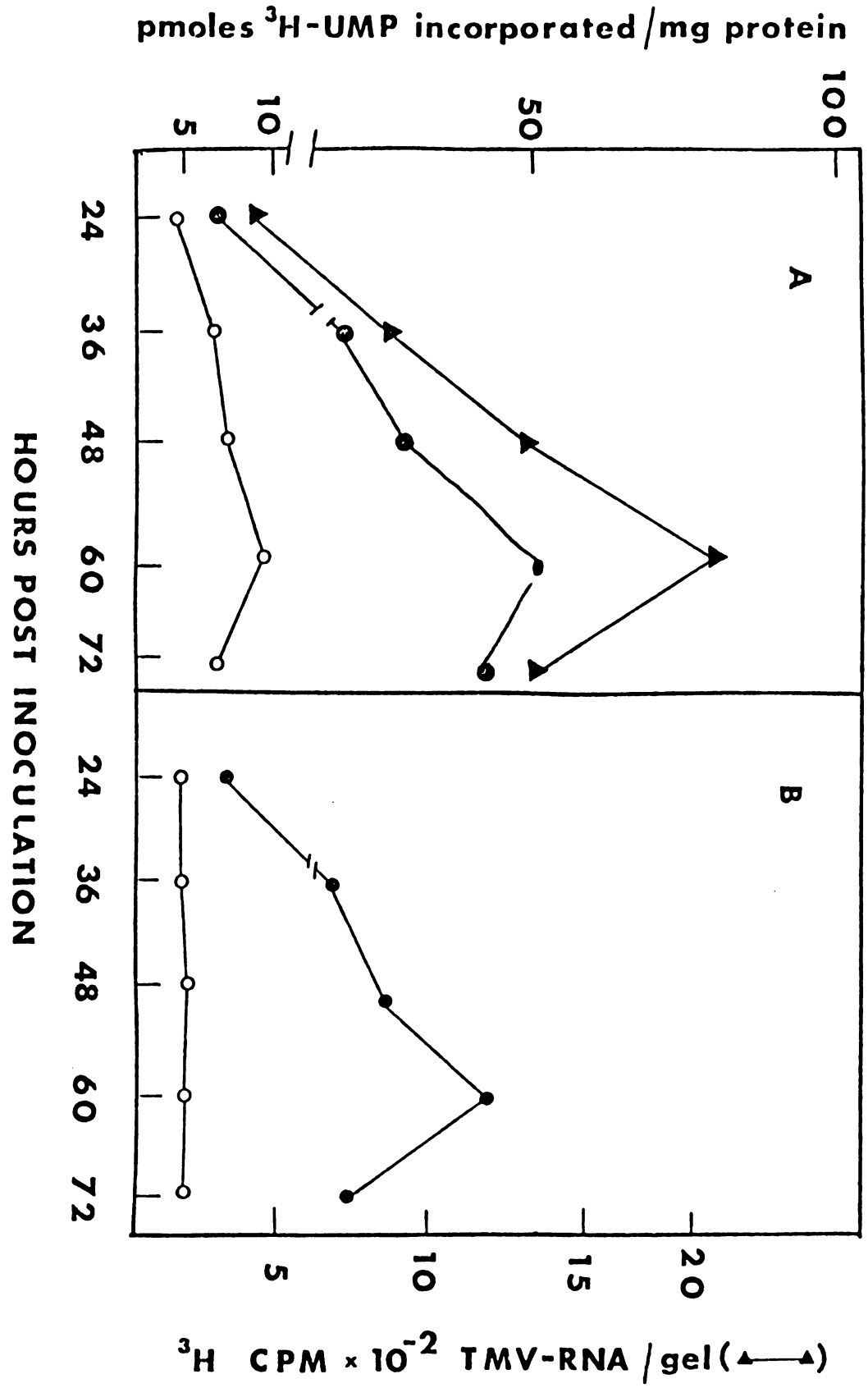
Table 3. Requirements of the soluble RNA-dependent RNA polymerase from TMV-infected tobacco tissue culture cells

Reaction conditions	Percent of control
Complete[a]	100
- ATP	15
- CTP	12
- GTP	14
- (ATP, CTP, GTP)	3
- Mg^{++}	6
- Mg^{++} + Mn^{++}	22
- Actinomycin D	117
- Actinomycin D + DNase (30 μg/ml)	92
+ RNase	84
+ TMV-RNA	92
+ BMV-RNA	89

[a]The complete reaction mixture was as described in Materials and Methods.

of appearance of replicase, TMV-RNA, and soluble RNA polyerase. At 12 hr intervals after inoculation, a sample of the inoculated cells were transferred to liquid medium containing ^{3}H-uridine (100 μCi/ml) and incubated for 4 hr. At the end of the labeling period, total RNA was extracted and subjected to electrophoresis. The rate of incorporation was made by determining the cpm/gel slice corresponding in position to the TMV-RNA optical density peak. To the remaining cell sample, viral replicase and soluble RNA polymerase were isolated and quantified. The amount of viral RNA increased in a linear manner from 24 to 60 hr after inoculation. The rate of ^{3}H-uridine incorporation into TMV-RNA increased from 60 to 72 hr after inoculation. The amount of ^{3}H-uridine incorporated into TMV-RNA at 60 hr was approximately 6 times that incorporated at 24 hr after inoculation. Replicase activity increased 10-fold from 24 to 60 hr post inoculation (p.i.) and then declined. The soluble RNA polymerase activity increased about 3 fold from 24 to 72 hr p.i. To determine whether the increase in soluble RNA polymerase was due to change in the cells present at inoculation or to new growth, mannitol was substituted for sucrose/glucose in the medium. Mannitol is an efficient osmoticum but is not metabolized by tobacco cells (Trip et al., 1967). Inoculated cells were plated on medium containing either mannitol or glucose/sucrose. Replicase which was isolated from cells incubated on medium supplemented with mannitol, showed a linear from 24 to 60 hr p.i. in activity (Fig. 6B) similiar to that of cells grown on sucrose/glucose medium (Fig. 6A). However, the RNA polymerase activity remained relatively constant throughout the duration of the experiment (Fig. 6B). The 3-fold increase in RNA polymerase in infected callus grown on medium containing glucose/sucrose may be a result of new cell

Figure 6. Rate of incorporation of ^{3}H-uridine into viral RNA, accumulation of viral RNA and the time course appearance of TMV replicase and soluble RNA polymerase. Callus was exposed to 100 μCi/ml of ^{3}H-uridine for 4 hr prior to nucleic acid extraction. Electrophoresis was carried out on 2.4% gels for 105 min at 5 mM/gel. Radioactivity determinations were confined to that portion of the gel known to contain TMV-RNA. Rate calculations were made by determining cpm/min/gel corresponding in position to the TMV-RNA optical density peak. All calculations were corrected for background radioactivity observed with nucleic acid from control callus. Total viral RNA determinations were made by converting the area under the optical density peak to micrograms RNA. Time course appearance of bound TMV replicase (●) and soluble RNA polymerase (o) and TMV-RNA (▲—▲) in callus incubated on medium containing glucose-sucrose as carbon sources (A). (B) Time course appearance of bound replicase (●) and soluble RNA polymerase (o) in callus incubated on the nonmetabolizable sugar, mannitol.


pmoles ^{3}H-UMP incorporated / mg protein
5
10
50
100
A
B
24
36
48
60
72
HOURS POST INOCULATION
5
10
15
20
^{3}H CPM × 10^{-2} TMV-RNA / gel (▲—▲)

growth and not stimulation of the enzyme in cells present at time of inoculation.

DISCUSSION

A particulate cell-free preparation from TMV-infected callus, enriched in cytoplasmic membranes, contained a RNA-dependent RNA polymerase (replicase) which appears to be viral specific. TMV replicase, like other viral replicases (Calguiri and Tamm, 1970; Eoyang and August, 1968, 1971; Ralph et al., 1971; Semal and Hamilton, 1968; Zabel et al., 1974), activity is markedly stimulated by the presence of Mg^{2+}, while Mn^{2+} is a poor substitute (Table 1). Products of the bound replicase are partially sensitive to RNase in high salt (2 x SSC) but completely sensitive in low salt (0.1 x SSC) (Fig. 2).

Analysis of the phenol-extracted products revealed the synthesis of the replicative form and only small amounts of replicative intermediate (Fig. 3). These results are consistent with those reported by other workers (Bradley and Zaitlin, 1971). However, if the product was extracted with perchloric acid, larger amounts of radioactive label were detected in replicative intermediate than in replicative form. Perchloric acid has been reported to be a simple and fast method to isolate RNA from viruses, plants and bacteria. The reason why it is superior to phenol in certain instances is not clearly understood, but since it deproteinizes solutions very rapdily it may be of great importance in deactivating ribonucleases (Wilcockson, 1973; Wilcockson and Hull, 1974).

Traditional attempts at solubilizing proteins including TMV replicase (Sela and Hauschner, 1975; Zaitlin et al., 1973), involved the

use of detergents to disrupt membranes and have met with limited success. However, TMV replicase released from membranes by the use of Mg^{2+} deficient buffer was relatively stable. Based on its sedimentation in glycerol gradients, the solublized replicase has a molecular weight of approximately 150,000 daltons. This molecular weight is in the same range as that reported for TMV-replicase from tobacco leaves (Zaitlin et al., 1973). A protein of this size has been detected as a virus stimulated protein in TMV-infected tissues (Sakai and Takebe, 1972; Zaitlin and Hariharasubramanian, 1972).

Analysis of the DEAE-Sephadex purified enzyme revealed that TMV-RNA was greatly superior as a template to the other tested RNAs. The product was partially sensitive to RNase and resistant to DNase. Of the plant viral replicases which have been purified to the template-free stage, most have shown little template specificity. Recently, however, Sela and Hauschner (1975) purified TMV replicase to an extent where it was completely dependent upon TMV-RNA as a template. Hadidi and Frankel-Conrat (1973) have purified brome mosaic virus replicase to a similiar degree. In our system, product of DEAE-Sephadex purified replicase was partially sensitive to RNase. Further analysis of the product on SDS-sucrose gradients revealed a heterogenous product. The majority of the radioactive label was found in a ribonuclease resistant structure with a sedimentation value of approximately 16S which appears to be replicative form. The remaining counts were found to have sedimentation values less than 16 S and were RNase sensitive. In no instance was complete TMV-RNA detected. DEAE-purified replicase appears to synthesize the complement of the template provided (replicative form) but fails to reinitiate RNA synthesis on the minus strand to give rise to replicative intermediate.

Duda et al (1973) reported a soluble RNA-dependent RNA polymerase (mol wt 160,000 daltons) in healthy tobacco leaves which synthesizes a small double-stranded RNA. Its activity was markedly increased upon TMV infection. The soluble fraction of healthy and TMV-infected tobacco callus contained RNA-dependent RNA polymerase which had many similiar properties (Table 3) to the enzyme from tobacco leaves. In contrast, its activity was not stimulated in the in vitro assay upon the addition of exogenous RNA as well as was the enzyme from tobacco leaves. In our experiments, RNA polymerase enzymatic activity increased approximately three-fold in healthy and TMV-infected tobacco callus 72 hr after transfer to fresh agar medium. Duda et al. (1973) reported a slightly greater than three-fold increase in this activity 5 days after inoculation with TMV when compared to healthy tissue. When healthy or TMV-infected callus was incubated on mannitol containing medium, a medium which substantially reduces cell division, the rate of enzymatic synthesis was different from comparable callus incubated on glucose/sucrose medium. RNA polymerase activity from healthy or TMV-infected callus increased approximately three-fold from 24 to 72 hr after transfer to fresh medium. If the callus was incubated on the mannitol medium no significant change in levels of the enzyme occurred. The activity of replicase was not affected by incubation of infected callus on either glucose/sucrose or mannitol containing medium. The increase in RNA polymerase enzyme in infected callus grown on medium containing glucose/sucrose may be a result of new cell growth and not stimulation of the enzyme in cells present at the time of inoculation.

During the first 60 hr after inoculation of callus cells, the rate of viral RNA synthesis increased and revealed a maximum during the 48-60

hr period. The conclusion is based on the observation that the rate of ^{3}H-uridine incorporated into viral RNA increased during each successive 4 hr labeling period 60 hr p.i. The rate declines sharply after the first 60 hr. These results are similiar to those reported by Pelcher et al. (1972). TMV replicase activity increased 10-fold and RNA polymerase activity 3-fold from 24 to 60 hr p.i. Although the function of the soluble RNA polymerase is still uncertain, its stimulation is not a prerequisite for TMV replicase activity. The ability to isolate relatively stable, template-free TMV replicase may aid in the understanding of the mechanism of viral RNA synthesis.

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LITERATURE CITED

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Part 2

Requirements for Isolation of Polyribosomes from Plant Cell Cultures

INTRODUCTION

Potential use of tissue culture for the production of natural products, genetic inprovements in crops, the recovery of disease-free clones, and as a tool to explore morphogenesis is now being realized. The role of nucleic acids in organogenesis has been investigated. Changes in patterns of RNA synthesis and certain nucleotides have been associated with shoot initiation in Cichorium intybus leaf sections. An initial period of callus formation in explants was associated with high rate of RNA synthesis, and subsequent shoot initiation was related with a rise in UMP/CMP ratio (Vassuer, 1972). Kovac (1971) noted that shoot formation in Nicotiana tissue followed an increase in RNA/DNA and histone/DNA ratios. Further studies on the changes in RNA metabolism during organogenesis will require isolation of polysomes to facilitate purification of mRNAs for further analysis in cell-free protein synthesis systems.

Procedures to isolate polysomes from plant tissues require conditions that minimize the effects of RNase. To obtain satisfactory polysome recovery extraction media have contained RNase absorbents in the presence or absence of detergents (Cocucci and Sturani, 1966; Leaver and Lovett, 1974; Ramagopal and Hsaio, 1973; Watts and Mathias, 1967; Weeks and Marcus, 1969), high ionic strength buffers (Breen et al., 1972; Davies et al., 1972; Lin et al., 1966; Sargent, 1973) and high pH (Breen et al., 1972; Davies et al., 1972; Leaver and Lovett, 1974).

The combination of high ionic strength and high pH buffers has been most effective with a variety of plant tissues (Breen et al., 1972; Davies and Larkins, 1972; Davies et al., 1972; Leaver and Lovett, 1974; Ramagopal and Hsaio, 1973). Recently, Jackson and Larkins (1976) reported that the addition of the divalent cation chelator ethylene glycol bis(2-aminoethylether)-tetracetic acid acid (EGTA) improved the yields of polysomes from unexpanded tobacco leaves and extraction of polysomes from expanded leaves required the chelator. This additive presumably reduced polysome aggregation caused by Ca^{2+} and other divalent cations. Reported is a method for isolating polysomes from six plant callus cultures in high pH and ionic strength buffer containing EGTA. This is the first report describing the conditions to isolate polysomes from plant callus cultures.

MATERIALS AND METHODS

Tissue cultures. Cell cultures used were tobacco (Nicotiana tabacum L. var. Havana 38), soybean (Glycine max L. var. Harsoy 63), wheat (Triticum aestivum L.) potato (Solanum tuberosum L.), tomato (Lycopersicon esulentum L.), corn (Zea mays), and barley (Hordeum vulgare L.). Tobacco callus were grown on the "B" medium of Murakishi et al. (1971); soybean and tomato cultures were maintained on R3 medium. R3 medium is composed of Linsmaier and Skoog (1965) minerals and carbon source supplemented with 0.5 mg pyridoxine, 0.5 mg nicotinic acid, 1.0 mg thiamine-HCl, 5.0 mg IAA, 0.3 mg kinetin, and 0.5 mg 2,4-D per liter of medium. Corn and barley cultures were maintained on B5 medium of Gamborg (1970) supplemented with 2.0 mg of 2.4-D per liter of medium. Wheat and potato cultures were maintained on Linsmaier and Skoog (1965) minerals and carbon source supplemented with 0.5 mg thiamine-HCl, 0.5 mg pyridoxine-HCl, 0.5 mg nicotinic acid, 2.0 mg 2,4-D, 2.0 mg IAA, and 0.3 mg kinetin per liter of medium. Tobacco and soybean cells were incubated under 3.7 Kerg/cm^2-sec of fluorescent light; the remaining cultures were incubated in the dark.

Preparation of polyribosomes. Two grams of cells were soaked for 2 min in 10 ml of grinding buffer (10 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose, 2.5 mM DTT, 140 mM tris-HCl, pH 8.2). The cells were then drained on a filter, and gently disrupted in 4 ml of grinding buffer with the aid of a conical tissue grinder. The resulting brei was passed through

"Miracloth" (Calbiochem.), clarified by centrifugation at 1,000 x g for 5 min and the supernatant centrifuged at 35,000 x g for 15 min. The supernatant was incubated at 4 C for 10 min after the addition of 0.2 ml of 20% Triton X-100. The mixture was subsequently layered over a cushion of 1.0 ml of extraction buffer made 0.87 M with respect to sucrose and centrifuged at 134,000 x g for 90 min. The supernatant and cushion were removed and the pellet was resuspended in the appropriate buffer with the aid of a rubber policeman. The extract was then layered over a linear sucrose gradient (4,7,7,7 ml of 100, 200, 300, and 400 mg/ml sucrose, respectively in gradient buffer) and centrifuged in a Spinco SW 25.1 rotor at 74,000 x g for 90 min. Gradients were monitored at 254 nm using an ISCO U.V. monitor and fractionator. Areas of different polysomal constituents were measured with a Lamda Instruments Corp. Portable Area Meter model L11-3000 for quantitative comparison of their absorbancy in density gradients. Most experiments were repeated three times.

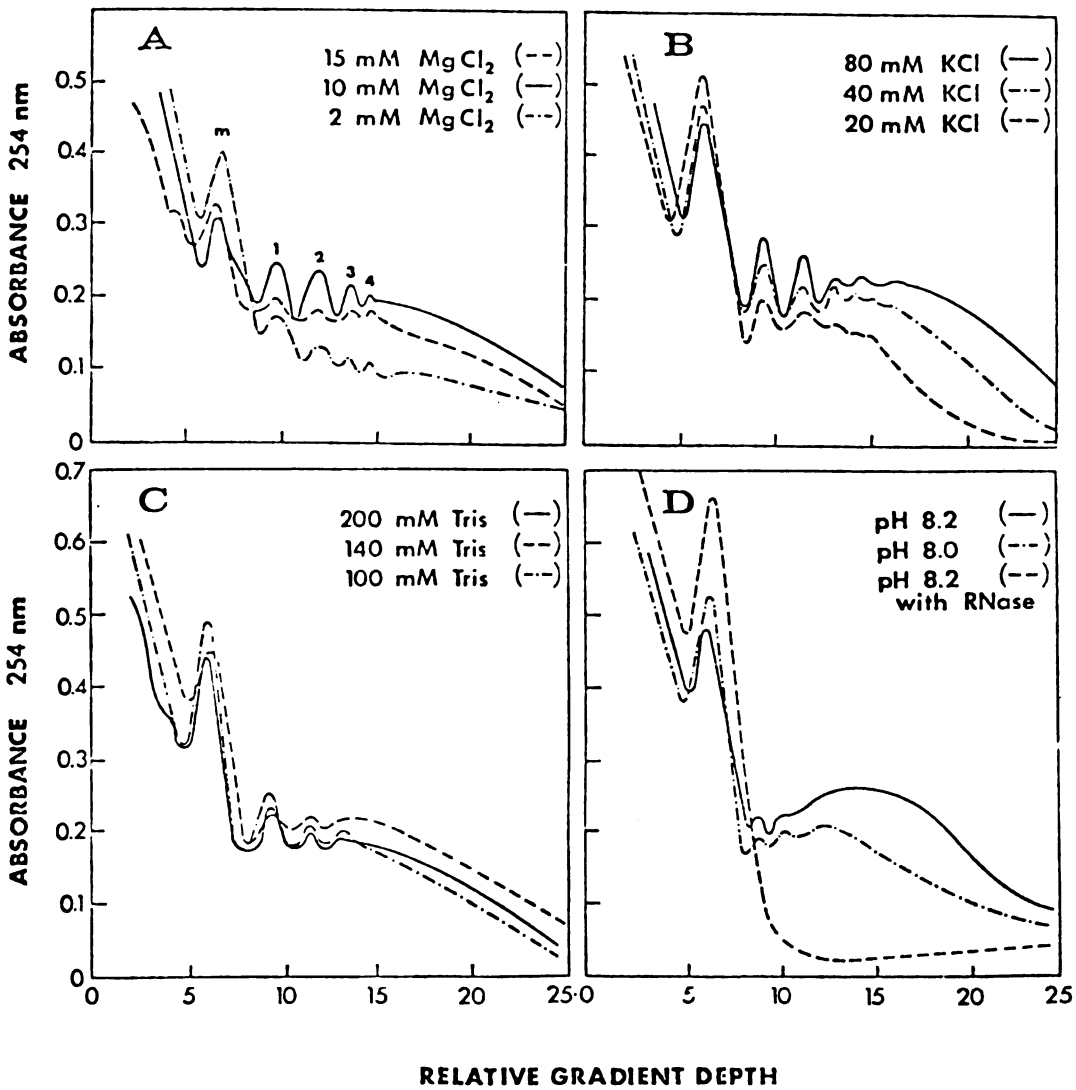
RESULTS

Previous work on conditions to isolate polysomes from expanded or unexpanded leaves (Cocucci and Sturani, 1966; Jackson and Larkins, 1976), stem segments (Davies et al., 1972), and embryos (Weeks and Margus, 1969) revealed that salt concentration, buffer concentration and pH affect polysome recovery. Therefore, concentrations of KCl, $MgCl_2$, tris or pH were varied to determine optimal conditions for the recovery of polyribosomes from callus cultures.

Polysomes were isolated from tobacco callus 60 to 72 hr after transfer to fresh medium. Several components have been identified by sedimentation rates and RNase susceptibility (Fig. 1). These are ribosomal subunits (s), monsomes (m), and classes of polymerized ribosomes which have been designated "mers" (3). The mers are presumed to be various numbers of ribosomes bound to mRNA and sediment as dimers (2-mers), trimers (3-mers), continuing through 6-mers, the last resolved by this gradient.

Recovery of polysomes from tobacco callus, as measured by increase absorbance in the gradients, was improved when the Mg^{2+} concentrations was increased from 2 mM to 10 mM (Fig. 1A). Polysomal yields were not further increased when the concentration of Mg^{2+} was raised above 10 mM. Polysome profiles were altered when concentration of KCl was increased from 20 to 80 mM (Fig. 1B). The chloride salts of Li^+, Na^+, and NH_4^+ added to the extraction medium were compared to K^+ all at 80 mM concentration. The stability was essentially the same for all these cations.

Figure 1. Effects of ionic strength and pH on surcrose density gradient profiles of polysomes extracted from tobacco callus. Polysomes were isolated as described under "Materials and Methods"; pH and ionic strength of the extraction medium varied and EGTA was omitted from the buffer. Polysomes were centrifuged through density gradients in the SW 25.1 rotor for 90 min. Extraction buffer contained: A: 20 mM KCl, 250 mM sucrose; 140 mM Tris-HCl, pH 7.8; B: 10 mM $MgCl_2$, 250 mM sucrose, 140 mM tris-HCl, pH 7.8; C: 10 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose, pH 7.8; D: 10 mM $MgCl_2$, 80 mM KCl, 140 mM tris-HCl, 250 mM sucrose. Subunits are designated s, monosomes m and mers as 2, 3, and 4.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B
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140 mM Tris (--)
100 mM Tris (-·-)
D
pH 8.2 (—)
pH 8.0 (-·-)
pH 8.2 (--)
with RNase
ABSORBANCE 254 nm
ABSORBANCE 254 nm
RELATIVE GRADIENT DEPTH

The next extraction variable examined was pH. At the buffer concentration (100 mM) and pH range used, a drop from the starting extraction pH value of 0.4 to 0.6 units was normally observed. This pH drop could be reduced to 0.1 to 0.2 units if the cells were soaked briefly in buffer before disruption. This pH change was attributed to the acidic nature of the cell and culture medium.

Extraction of polysomes in 140 mM tris resulted in best yields of high sedimenting material (Fig. 1C). Larger polyribosomes (>5-mers) were not resolved by the 100-400 mg/ml sucrose gradients as the yields of polyribosomal material increased. Better resolution of large mers was obtained by using a different gradient (4,6,6,8 ml of 150, 300, 450, 550 mg/ml sucrose, respectively in buffer). For consistency, the results reported here are for the 100-400 mg/ml sucrose gradients. Concentrations of 100 or 200 mM tris decreased polysomal yields. Extraction medium in the pH range from 7.8 to 8.4 revealed that pH 8.2 was superior (Fig. 1C;1D), polysomal profile for extraction medium pH 8.4 is not shown but was similar to 8.0). Treatment of the polysomal extract with RNase before gradient analysis converted nearly all polysomes into monosomes, indicating that there was little non-specific aggregation of monsomes into fast sedimenting material.

Addition, individually, to the extraction medium of sodium deoxycholate (1%), yeast RNA (10 mg/ml), bentonite (10 mg/ml) or diethylpyrocarbonate (0.1%) did not improve the yield of polysomes nor did freezing the cells in dry ice before disruption.

Conditions for isolation of ribosomes from soybean callus. The optimal buffer-salt combination (10 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose 140 mM Tris, pH 8.2) for obtaining polysomes from tobacco callus was

used as the starting buffer to extract polysomes from soybean callus. Increasing or decreasing the concentration of $MgCl_2$, CKl or Tris in the extraction buffer did not increase yields of polysomes (Table 1). The increase in stability of polysomes is illustrated by the increase in the ratio of >5-mers to <5-mers (Jackson et al., 1976). Jackson and Larkins (1976) reported that this method of determining the polysomal yield was superior to that of comparing ratio of monosomes to polysomes. Increasing the pH of extraction medium from 8.2 to 8.5 increased the amount of >5-mers by approximately 30%. Extraction of polysomes from soybean cells in buffer at pH 9.0 decreased large polysomal material when compared to polysomes extracted at pH 8.2 or 8.5. The optimal conditions for extraction of polysomes from soybean callus appear to be similar to those for tobacco.

Effect of EGTA on polysomal recovery from tobacco and soybean cultures. Recently, the use of EGTA, a chelator with high affinity for Ca^{2+}, Cu^{2+}, and Zn^{2+}, improved the recovery of polysomes from unexpanded tobacco leaves, while successful isolation of polysomes from expanded leaves requires the presence of the chelator (Jackson and Larkins, 1976). When polysomes were isolated from either tobacco callus or soybean callus in 10 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose, 140 mM Tris pH 8.2 (for tobacco) or 8.5 (for soybean) supplemented with 20 mM EGTA, the polysomes were destroyed. If the concentration of $MgCl_2$ was increased, in the above buffer to 35 mM, an increased yield of >5-mers were obtained (Fig. 2) from tobacco or soybean cultures. Jackson and Larkins (1976) noted that the concentration of Mg^{2+} must exceed the concentration of EGTA to obtain substantial yields of >5-mers.

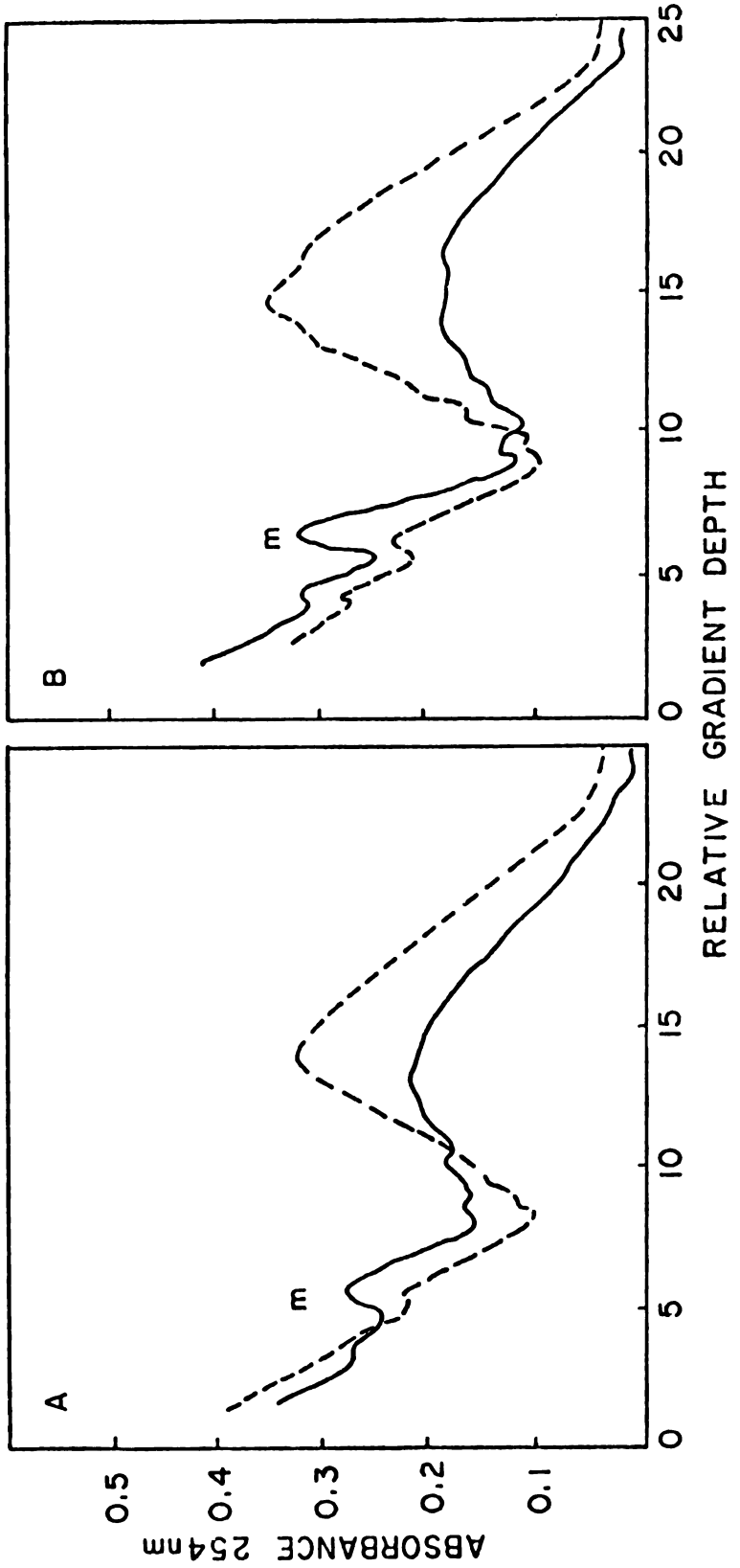
Table 1. Measurements of Sucrose Density Gradient Profiles of Polysomes from Soybean callus extracted in media of various ionic strength and pH[1].

	Relative areas of polysome profiles[2]		
	<5-mers	>5-mers	>5-mers/<5-mers
2 mM $MgCl_2$, 80 mM KCl, 140 mM Tris, pH 8.2	3.84	4.73	1.23
10 mM $MgCl_2$, 80 mM KCl, 140 mM Tris, pH 8.2	7.55	11.90	1.57
20 mM $MgCl_2$, 80 mM KCl, 140 mM Tris, pH 8.2	4.19	6.35	1.51
10 mM $MgCl_2$, 40 mM KCl, 140 mM Tris, pH 8.2	5.27	4.94	0.94
10 mM $MgCl_2$, 120 mM KCl, 140 mM Tris, pH 8.2	4.90	6.10	1.25
10 mM $MgCl_2$, 80 mM KCl, 100 mM Tris, pH 8.2	6.27	8.87	1.41
10 mM $MgCl_2$, 80 mM KCl, 200 mM Tris, pH 8.2	7.41	9.14	1.23
10 mM $MgCl_2$, 80 mM KCl, 140 mM Tris, pH 8.5	6.77	13.94	2.06
10 mM $MgCl_2$, 80 mM KCl, 140 mM Tris, pH 9.0	8.43	11.21	1.33

[1]Extraction procedure was as described in "Materials and Methods".

[2]The relative areas were measured by Lamda Instruments Portable Area Meter.

Figure 2. Effect of EGTA on extraction of polysomes from tobacco (A) and soybean (B) callus cultures. Polysomes were extracted in 10 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose, 140 mM tris-HCl, pH 8.2 for tobacco callus or pH 8.5 for soybean callus (solid line) or in the presence of 20 mM EGTA ($MgCl_2$ increased to 35 mM) (broken line). Polysomes were centrifuged through density gradient in the SW 25.1 rotor for 90 min as described in "Materials and Methods".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ABSORBANCE 254nm
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RELATIVE GRADIENT DEPTH

Isolation of polyribosomes from callus. Using the extraction medium (35 mM $MgCl_2$, 80 mM KCl, 20 mM EGTA, 140 mM Tris, pH 8.2) polysomes were extracted from tobacco, soybean, potato, tomato, corn and barley cultures. High ratios of >5-mers to <5-mers were obtained in all cases, however, the ratio for polysomes from tobacco, soybean and tomato were better than that for the other cultures. The considerably smaller amount of >5-mers isolated from corn and barley cultures may have been due to less than optimal conditions for isolation. However, the lower ratio may be due to the slow growth rate of these cultures when compared to tobacco callus (Table 2).

Table 2. Measurements of Sucrose Density Gradient Profiles of Polyribosomes Extracted in 35 mM $MgCl_2$, 80 mM KCl, 250 mM sucrose, 2.5 mM DTT, 20 mM EGTA, 140 mM Tris, pH 8.2 from callus cultures[1].

	Relative areas of polysome profiles[2]		
Callus	>5-mers	<5-mers	>5-mers/<5-mers
Tobacco	13.47	4.19	3.69
Soybean	13.86	4.29	3.23
Potato	12.16	4.74	2.58
Tomato	12.76	3.94	3.23
Corn	8.61	3.04	2.83
Barley	7.12	3.36	2.12

[1]Extraction procedure as described in "Materials and Methods".

[2]The relative areas were measured by Lamda Instruments Portable Area Meter.

DISCUSSION

We have described conditions to isolate polysomes from plant cell cultures. The pH and ionic strength of the medium greatly influence the yield of polysomes. Polysome degradation was inhibited at high pH (8.2 to 8.5) and high tris concentrations. The optimal conditions for isolation polysomes from tobacco callus and tobacco leaves was obtained by tissue extraction in 200 mM tris-HCl, pH 9.0 400 mM KCl, 200 mM sucrose, and 35 mM $MgCl_2$. It appears that optimal conditions for isolation of polysomes from tobacco callus require lower ionic strength and pH buffers. A possible reason for the great difference in KCl concentration may be due to the ability of high salt concentration to release polysomes from membranes, thus increasing yields. In our case, Triton X-100 was added to release polysomes from membranes, which may reduce the necessity for high KCl concentrations in the extraction medium.

The extraction medium (5 mM $MgCl_2$, 15 mM KCl, 250 mM sucrose, 50 mM tris-HCl, pH 7.5 plus diethylpyrocarbonate) to isolate polysomes from soybean hypocotyls is quite different from that described here for soybean callus (Lin et al., 1966). If the RNase inhibitior diethylpyrocarbonate was omitted from the extraction medium, optimal conditions required higher tris concentrations (200 mM) and high pH (8.50) conditions corresponding more closely to those for soybean callus.

One reason that a single isolation medium to isolate polysomes from callus cultures was successful, while quite different buffers are

require to isolate polysomes from various plant sources, may be due to the similarities in culturing callus. All cultures are maintained in an acidic medium (ph 5.5 to 6.0) and obtain their energy from simple sugars.

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PART 3

Association of TMV-RNA with Polyribosomes in Tobacco Callus

INTRODUCTION

Although the polycistronic translation of viral RNA in picornaviruses of animal is well documented (Levintow, 1974), the translation mode of plant viral RNAs is still uncertain. Viral RNA that codes for the coat protein from multicomponent viruses such as alfalfa mosaic and cowpea mosaic viruses appear to be moncistronic (Siegel and Hariharasubramanian, 1974). However, the mode of translation of viral RNA from other palnt viruses still must be resolved. Understanding the manner in which single large RNA molecule are translated to form two or more polypeptides is fundamental to understanding TMV replication. Three classes of mechanisms have been proposed:

1) the genome RNA has multiple initiation and termination sites (polycistronic translation)

2) the genome RNA has fewer initiation sites than there are polypeptides for which it must code; the large protein are then cleaved by specific proteases to give rise to functional proteins (post-translation cleavage)

3) some copies of genome RNA are specifically cleaved by a nuclease to give rise to moncistronic mRNAs or monocistronic RNAs are synthesized by a process involving partial transcription of genome RNA.

Experimental evidence supporting all three of these modes of TMV-RNA translation have been presented. Kiho (1968,1972) investigated the

replication of TMV in plants by studying the appearance of viral RNA in polyribosomes. He demonstrated a large TMV-specific polyribosome which contained TMV-antigenic protein in infected cells and concluded that TMV-RNA was translated by mechanism 2. However, these results have been questioned due to inadequate control and reconstruction experiments. Beachy and Zaitlin (1975) could not detect a TMV-specific polyribosome but found heterogeneous sizes of viral specific RNAs as well as some full-length TMV-RNA associated with polyribosomes. Recently, Knowland et al., (1975) and Bruening et al. (1976) reported that a small virus-specific RNA (LMC-RNA) is the mRNA for TMV coat protein. The mechanism by which this RNA arises is unknown. The results from amino acid incorporating cell-free system translation of TMV-RNA are conflicting. Efron and Marcus (1973 and Roberts et al. (1974), using a cell-free system from wheat embryo, found that TMV-RNA directed the synthesis of TMV coat protein. TMV particles assembled in the presence of treated cell-free protein extracts were shown to be radioactive and co-banded with authentic TMV in equilibrium centrifugation in cesium chloride. Bruening et al., (1976) could not detect synthesis of coat protein in wheat germ cell-free system using cowpea strain of TMV-RNA as the messenger. The predominant polypeptide synthesized had an approximate molecular weight of 130,000 daltons with small amounts of a 150,000 dalton polypeptide also detected.

The purpose of this work is to study the association of TMV-RNA and ribosomes shortly after inoculation, and to determine number and size of polypeptides associated with the viral RNA-ribosome complex.

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MATERIALS AND METHODS

Chemicals. ^{3}H-uridine (40-50 Ci/mmol), L-^{3}H-leucine (30-50 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Tissue culture, virus and inoculation. A pigmented cell culture derived from pith of tobacco (Nicotiana tabacum L. var. Havana 38) was used throughout this study. The cells were maintained and inoculated as previously described (Pelcher et al., 1973). Cells in 0.5 g batches were suspended in 3 ml of liquid medium and inoculated with a common strain of TMV at a concentration of 150 µg/ml of cell suspension. Inoculated cells were pooled, stirred gently to ensure randomization, and washed with 25 ml of fresh medium. One gram samples of cells were transferred to 7 cm discs of Whatman no. 4 filter paper in petri dishes containing 20 ml of medium solidified with 1% agar. The cells were incubated at 24 C under Gro-lux lamps. At intervals after inoculation, the cells were transferred on the filter paper to petri dishes containing 4 ml of fresh medium supplied with either 100 µCi of ^{3}H-uridine or 2.5 µCi ^{14}C-leucine and incubated under continous light for 4 to 6 hr with constant agitation. Control cells sham-inoculated with phosphate buffer (0.1 M, pH 7.6) were treated in a like manner.

Preparation of polyribosomes. Two g of cells were soaked for 2 min in 10 ml of grinding buffer (140 mM Tris, 80 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose, 2.5 mM dithiothreitol, pH 8.2). The cells were then drained on a filter, and gently disrupted in 4 ml of grinding buffer with the

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aid of a conical tissue grinder. The resulting brei was passed through "Miracloth" (Calbiochem.) clarified by centrifugation at 1000 x g for 5 min and the supernatant was centrifuged at 35,000 x g for 15 min. To the supernatant, 0.2 ml of 20% Triton X-100 was added and the mixture was incubated at 4 C for 10 min. Before U.V. analysis of polyribosomes could be accomplished, it was necessary to remove interfering phenolics and chlorophyll. The detergent-treated 35,000 x g extract was layered on a linear 10-40% sucrose gradient and centrifuged in a Spinco SW 25.1 rotor at 74,000 x g for 20 min. In preliminary experiments, it was determined that ribosomal material would penetrate the gradient within this centrifugation time while other more slowly sedimenting U.V.-absorbing material remained at the top of the gradient (Knowland et al., 1975). Following the 20 min preliminary centrifugation, the tubes were removed and a volume equal to that originally applied to the gradient was carefully pipetted off and the gradient centrifuged again for an additional 90 min at 74,000 x g. Gradients were monitored at 254 nm and fractions were collected from the top of the tube using an ISCO U.V. monitor and fractionator.

Immune precipitation of nascent TMV protein. At 48 hr post inoculation (p.i.) polyribosomes were isolated from TMV-infected or healthy cells previously pulsed for 6 hr with 2.5 μCi of ^{14}C-leucine. Two ml fractions were collected and a 0.1 ml sample was removed for each for determination of total ^{14}C-leucine incorporation into TCA insoluble proteins. To the remaining samples, five mmoles of EDTA was added to each fraction to release nascent polypeptide chains. The 50 μg of carrier TMV and IgG purified TMV (Campbell et al., 1970) were added to each fraction and the mixture was incubated overnight at 4 C.

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The resulting precipitates were collected, washed three times with 0.85% cold saline and counted after precipitation with TCA (Pelcher et al., 1973). Radioactivity specifically precipitated was calculated by correcting for non-specific precipitation of radioactivity from uninfected cell polyribosomes with TMV antisera. Non-specific precipitable counts ranged from 50 to 100 cpm. The percent antigen recovered, based on recovery of TMV coat protein, was greater than 80%.

Preparation of radioactively labeled TMV. Tissue culture cells were inoculated, transferred to filter paper and incubated on solidified medium. At 36 hr p.i., the cells were transferred to a petri dish containing 4 ml of fresh medium supplemented with either 100 μCi ^{3}H uridine or 5 μCi ^{14}C leucine and allowed to incubate under continuous light with agitation. At 72 hr p.i., the cells were harvested, washed with cold 0.25 M sucrose and frozen. The virus was isolated as previously described (Pelcher et al., 1973) and further purified by centrifugation on 10-40% sucrose gradient. Material banding in the TMV region was collected as described above. Virus purified in this manner had a specific activity of 3.0×10^{5} cpm of ^{3}H-uridine/mg of virus or 5.0×10^{4} leucine/mg of virus.

Inoculation of cells with labeled virus or TMV-RNA. Suspension cell cultures 72-96 hr after transfer to fresh medium were inoculated with labeled TMV or TMV-RNA (Murakishi, 1968) and the cells and virus mixtures were immediately transferred to 50 ml culture flasks and placed on a rotory shaker for 30 min. This additional time of virus-cell contact appeared to increase the amount of virus or RNA associated with the cells. After incubation, cells were allowed to drain on a filter, washed with 50 ml of fresh medium and transferred to a flask

containing fresh medium. At intervals, the cells were collected and polyribosomes isolated.

Isolation of RNA from polyribosomes. Polyribosomes from cells previously inoculated with ^{3}H-uridine labeled TMV-RNA were extracted and purified by sucrose density gradient centrifugation. Polyribosomes with sedimentation values greater than 250 S were collected, pooled and centrifuged at 200,000 x g in a Spinco SW 50L rotor for 2.5 hr. The pellets were resuspended in 0.4 ml of 100 mM NaCl, 1 mM Na_2EDTA, 100 mM Tris, pH 8.0 (at room temperature) and treated at 4 C with 0.1 ml of neutralized Na_2EDTA for 20 min. RNA was precipitated by the addition of 95% ethanol. Further purification and polyacrylamide gel electrophoresis is described in section 1 page .

Molecular weight distribution of nascent polypeptides. Cells 36 hr p.i., were transferred from the filter paper disc to a 50 ml flask containing 4 ml of fresh medium containing 50 µg/ml of Actinomycin D and incubated under continuous light for 2 hr with constant agitation. Subsequently, 300 µCi of ^{3}H-leucine was added and the cells incubated for 20 min. Polyribosomes were isolated and purified by centrifugation. The nascent polypeptide chains were released by SDS treatment (Jacobson et al., 1970) and polypeptides were analyzed on 5% acrylamide gels (Fairbanks et al., 1971). The gels were frozen, sliced and radioactivity determined (Pelcher et al., 1972).

RESULTS

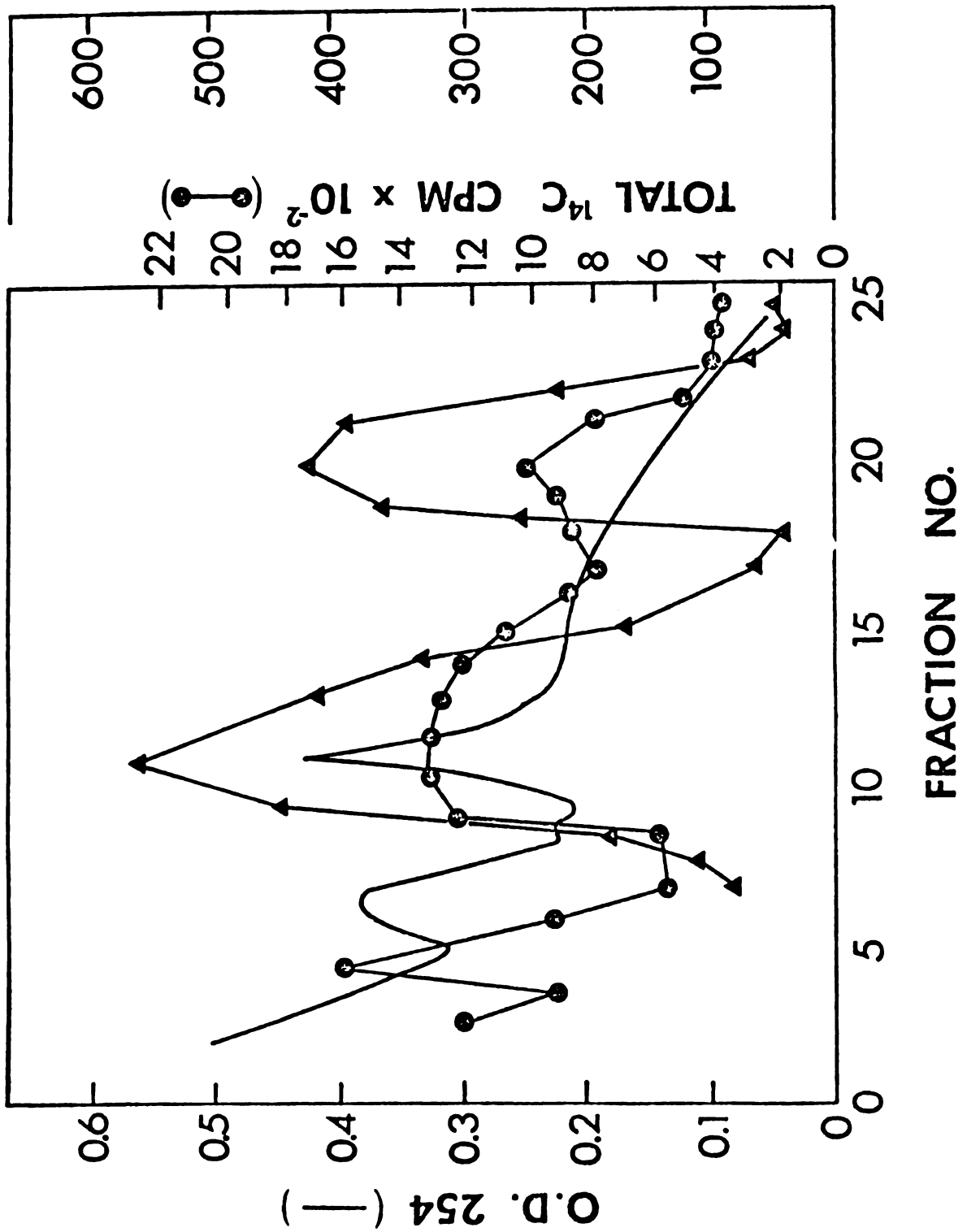
Polyribosomes from healthy and TMV-infected cells. Polyribosome prepared from ^{3}H-uridine labeled healthy and TMV-infected cells were analyzed and fractionated on sucrose gradients. The optical density and radioactivity patterns obtained for cells 60 hr after transfer to fresh medium are shown in Fig. 1A. Polyribosomal patterns obtained from infected cells 60 hr p.i. (Fig. 1B) are quite different from healthy cells due to the substantial amounts of TMV present in fraction 10-12.

Detection of TMV-coat protein. If TMV-RNA is translated by mechansim 1 or 2 (page 70) one would expect a very large polyribosome which would be synthesizing proteins code for by mRNA, including coat protein. Therefore, polyribosomes that are synthesizing TMV coat protein would be precipitated by TMV antisera if the nascent polypeptide was immunologically reactive. The polyribosomal profile from TMV-infected cells pulsed with ^{14}C-leucine and analyzed for TMV protein (Fig. 2) showed two major peaks of TMV-antigen. Based on its size, migration in the gradient, resistance to RNase degradation, fraction 9-14 appears to contain complete TMV. In fractions 18-22, another area of antigenic protein was observed. This polyribosome has a sedimentation coefficient of approximately 360 S. Treatment of polyribosomal extract before analysis on sucrose gradient with 5.0 μg of RNase eliminated greater than 90% of the radioactivity in fractions 19-22; while only a slight decrease was detected in fraction 9-14. Similiar results were

Figure 1. Polyribosomes were isolated from 2 mg of health tobacco tissue culture cells (A) or TMV-infected cells, 60 hr p.i., (B) that had been labeled for 4 hr with 100 μCi ^{3}H uridine and analyzed by centrifugation on 10-40% sucrose gradients. Solid line, optical density at 254 nm; TCA precipitable radioactivity (▲-▲)

O.D. 254 (——)
FRACTION NO.
A
O.D. 254 (——)
^{3}H CPM x 10^4 (▲—▲)
FRACTION NO.
B

Figure 2. Antigenic analysis of nascent viral proteins synthesized on polyribosomes. Polyribosomes were isolated from TMV-infected cells (48 hr p.i.) that had been labeled for 6 hr with 2.5 μCi ^{14}C leucine and analyzed by centrifugation on a 10-40% sucrose gradient. Fractions were collected and a sample was removed for determination of TCA precipitable radioactivity (●-●). To the remaining fractions, 5 mmoles of EDTA was added to release nascent polypeptides, which were then precipitated with TMV antisera and radioactivity determined (▲-▲). Optical density is shown in the solid line.


O.D. 254 (——)
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
FRACTION NO.
TOTAL ^{14}C CPM x 10^{-2} (●—●)
22
20
18
16
14
12
10
8
6
4
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ANTIGENIC PPT CPM (▲—▲)
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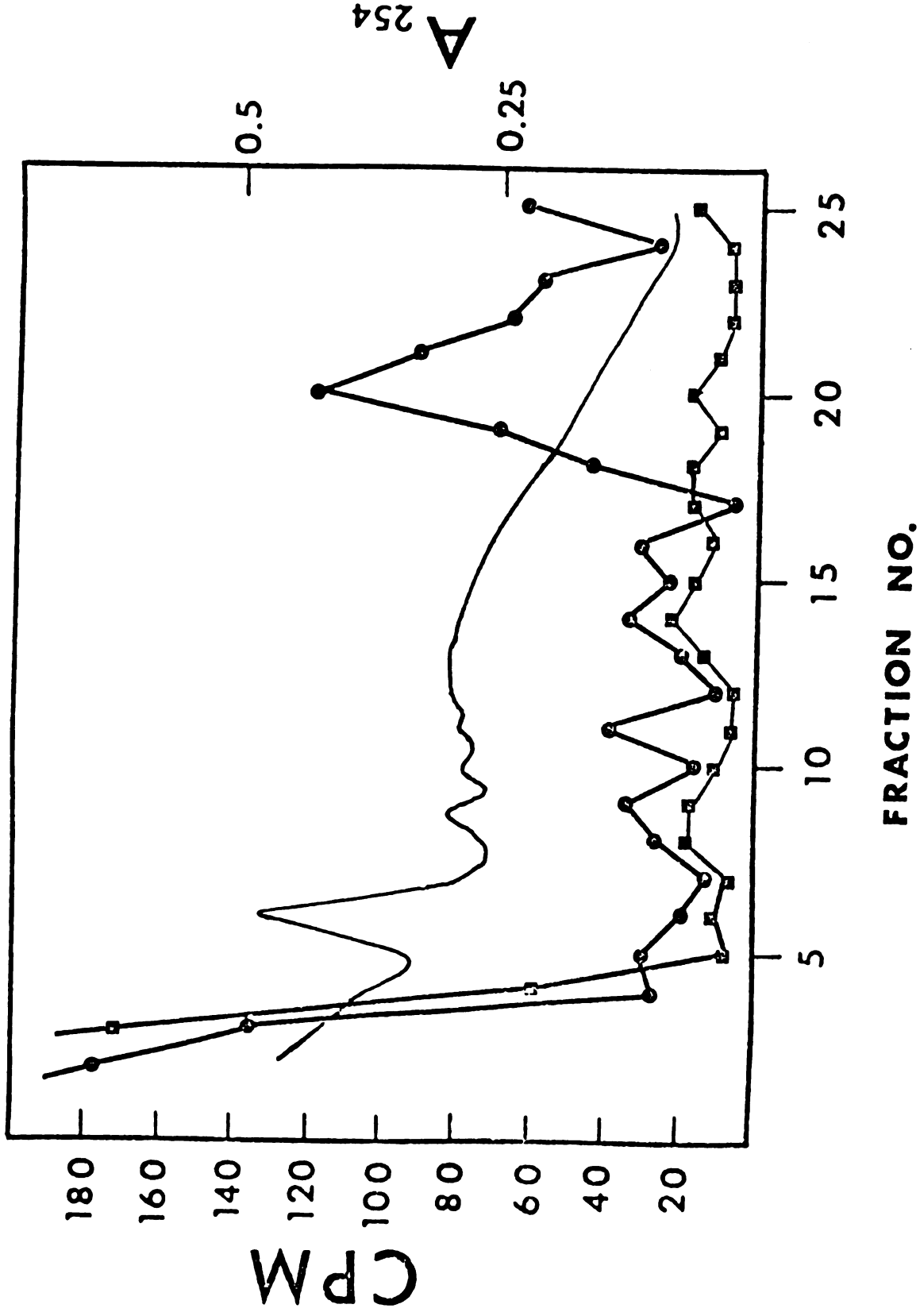
obtained when polyribosomal extracts were treated with 10 mM EDTA in place of RNase. To determine if the antigenic material found in fraction 18-22 was due to the non-specific adsorption of TMV to heavy material, polyribosomes isolated from healthy tissues previously pulsed with ^{3}H-leucine were isolated. Before layering on the gradient, ^{14}C-leucine labeled TMV (approximately 5000 or 1000 cpm) was added to half the extract and polyribosomes isolated and fractionated as described above. Although some TMV-antigenic protein was detected in fraction 18-22, only 8-12% of the TMV counts applied appeared in this region. Thus it appears that TMV coat protein is present on a large polyribosome, although non-specific adsorption of TMV coat protein or TMV to polyribosomal material cannot be completely ruled out.

Association of TMV-RNA with polyribosomes and size of nascent polypeptides. Although it appears that TMV coat protein can be detected in association with large polyribosomes, further analysis to detect TMV-RNA and nascent polypeptides associated with large polyribosomes was initiated. Cells previously treated for 1 hr with actinomycin D (50 µg/ml) were inoculated with ^{3}H-uridine labeled TMV-RNA (approximately 200,000 cpm) and incubated for 4 hr and subsequently polyribosomes isolated Fig. 3. Polyribosomes that had sedimentation values greater than 250 S were pooled, RNA isolated and analyzed on acrylamide gels (Fig. 4). Only a single homogenous peak of radioactivity was detected that co-migrated on gels with authentic TMV-RNA prepared from virions. Only a small amount of label, apparently from degraded TMV-RNA, was detected in rRNAs (18S and 25S). It appears that TMV-RNA is associated with polyribosomes, but is the genome RNA translated to give rise to TMV coat protein? Cells previously treated with actinomycin D (50 µg/ml)

Figure 3. Tobacco tissue culture cells were inoculated with ^{3}H uridine labeled TMV-RNA (approximately 200,000 cpm) and incubated for 6 hr. Polyribosomes were isolated by zonal centrifugation, fraction were collected and TCA precipitable radioactivity determined (●-●). Polyribosomal extract prepared in a similar manner and treated wtih 5 μg of RNase A just prior to zonal centrifugation (■-■). Solid line OD at 254 nm.


A 254
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CPM
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160
140
120
100
80
60
40
20
5
10
15
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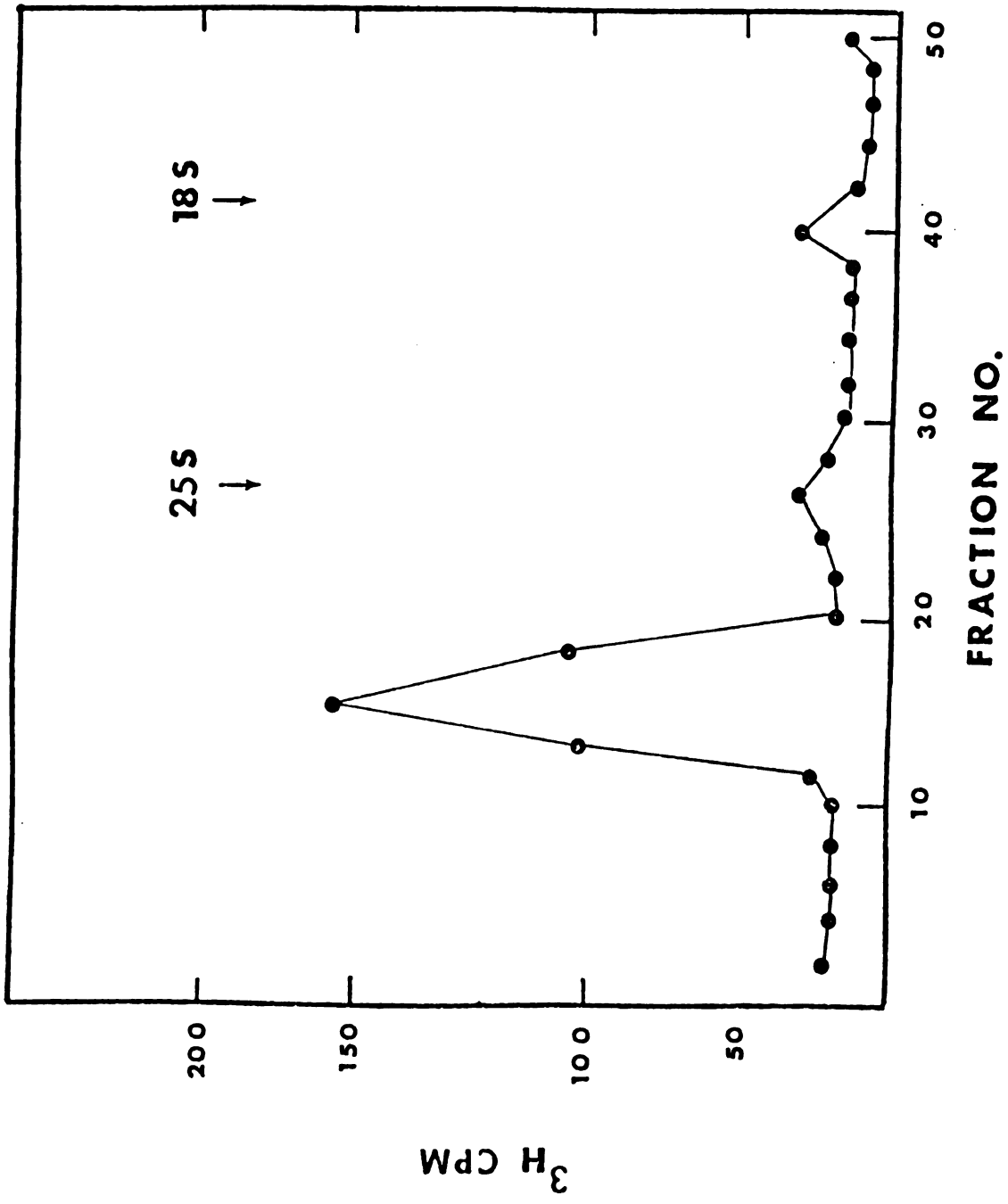
25
FRACTION NO.

Figure 4. Polyacrylamide gel electrophoresis of RNA isolated from polysomes (sedimentation >250 S, fractions 17-23 of Fig. 3). Polysomes were pelleted at 200,000 x g for 2.5 hr, the pellets resuspended in 100 mM Tris-EDTA, pH 8.0 plus 100 mM NaCl. The RNA was precipitated by the addition of ethanol and was analyzed by acrylamide gel electrophoresis (210 min at 5 mA/gel). Molecular weight standards ribosomal RNA from callus 18 S and 25 S.


18S
25S
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10
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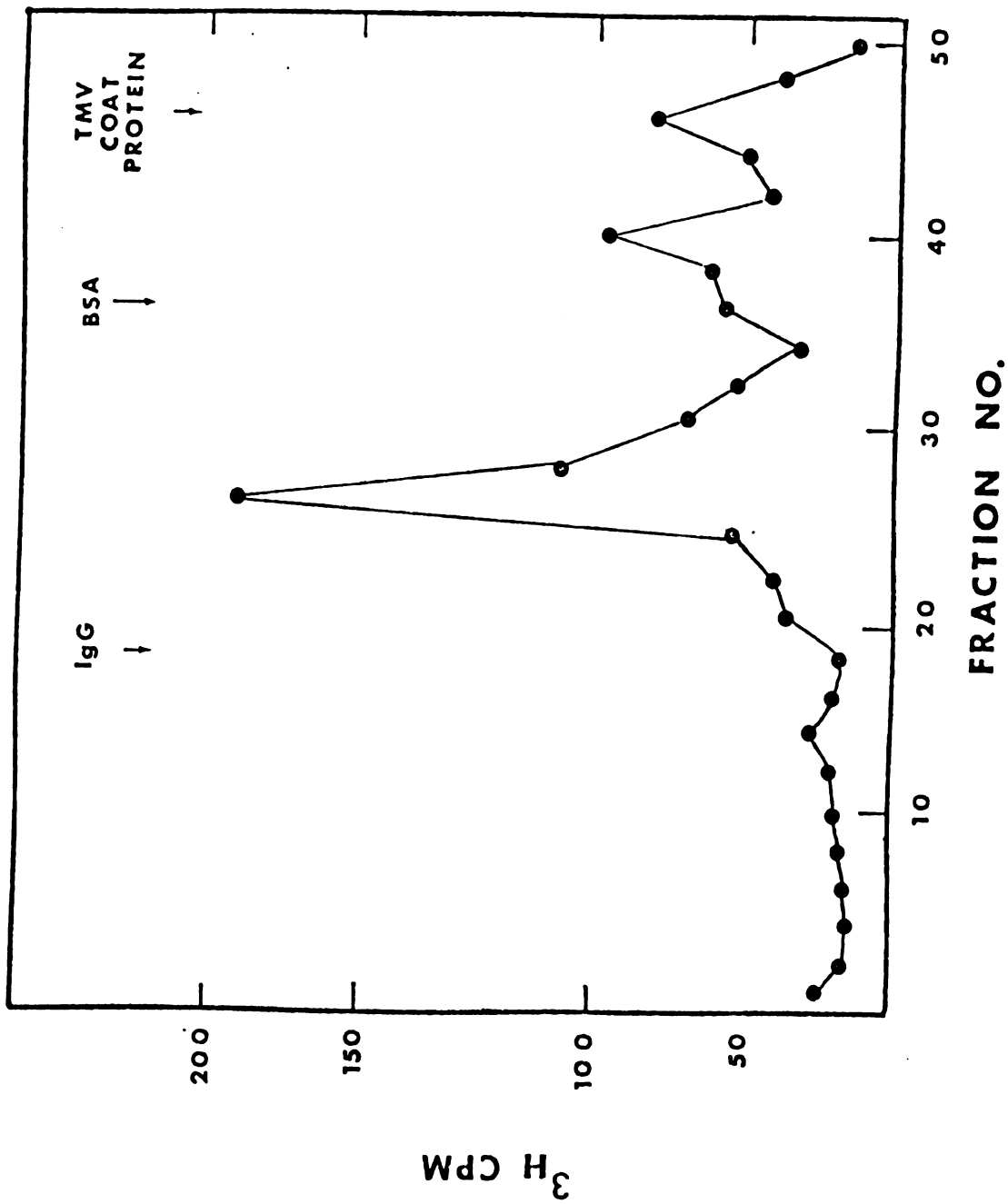
40
50
FRACTION NO.
^{3}H CPM

for 1 hr were inoculated with TMV-RNA (50 μg/ml) and incubated for 3.5 hr and pulsed for 30 min with ^{3}H-leucine. Polyribosomes which had a sedimentation value greater than 250 S were collected and pooled; nascent polypeptides chains were released by SDS and then examined in polyacrylamide gels (Fig. 5). Normal nascent chains contained little if any proteins larger than 150,000 daltons. The most prominent polypeptides were detected in fraction 28, 41, and 46 which have estimated molecular weights of 130,000, 35,000 and 17,500 daltons, respectively. The peaks in fraction 46 appears to be TMV coat protein based on its size and immunological reactivity. The majority of radioactive label was incorporated into 130,000 dalton polypeptide.

Figure 5. Size distribution of nascent polypeptides associated with TMV-RNA polyribosome. Tobacco tissue culture cells inoculated with TMV-RNA were labeled for 20 min with 300 μCi of ^{3}H-leucine. Polyribosomes with nascent chains were purified by zonal centrifugation. Nascent chains were released by SDS and analyzed on 5% acrylamide gels. Molecular weight standards were IgG (160,000 daltons) and bovine serum albumin (BSA; 45,000 daltons).


TMV COAT PROTEIN
BSA
IgG
200
150
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10
20
30
40
50
FRACTION NO.
^{3}H CPM

DISCUSSION

Three classes of mechanisms have been proposed to understand the manner in which a single large RNAs are translated to form two or more polypeptides. These mechanisms are described on page . I will summarize the evidence for the translational mode of TMV-RNA for each mechanism.

First, if TMV-RNA is translated polycistronically (mechanism 1) there are multiple initiation and ribosome binding sites present on viral RNA. Full-length TMV-RNA can be isolated from polyribosomes (Fig. 3 and 4). Beachy and Zaitlin (1975) have also detected TMV-RNA associated with polyribosomes by the use of competetion hybridization.

In actinomycin D-treated TMV-infected tobacco callus inoculated with TMV-RNA, the nascent polypeptides associated with the large polyribosomes containing TMV-RNA had a molecular weight of approximately 130,000 daltons. Smaller amounts of polypeptides of 35,000 and 17,500 daltons were detected. The latter polypeptide appears to be TMV coat protein based on its size and antigenic reactivity. The total molecular weight is consistent with the concept that they may represent nearly the total translational information of TMV-RNA.

Results of cell-free translation of TMV-RNA in wheat embryo amino acid-incorporating system revealed that small amounts of TMV coat protein is synthesized (Efron and Marcus, 1973; Roberts et al., 1974). However, Bruening et al. (1976) and Knowland et al. (1975) failed to

detect coat protein synthesis in cell-free systems using TMV-RNA as a messenger.

If TMV-RNA is translated by the second mechanism (page 69), a large polyprotein (>200,000 daltons) would be synthesized and later cleaved into functional proteins by specific proteases. Large polypeptides have been detected (155-245 x 10^3 daltons) in TMV-infected tobacco leaves (Zaitlin and Hariharasubramanian, 1972) by the use of double-labeling technique. However, attempts to chase radioactive counts from the large polypeptides into smaller products were unsuccessful. The largest polypeptides synthesized in cell-free protein synthesizing systems programmed by TMV-RNA were approximately 130,000 daltons (Bruening et al., 1976; Efron and Marcus, 1973; Roberts et al., 1974).

The third mechanism involves the use of specific nucleases to cleave viral RNA into monocistronic mRNAs. Although LMC-RNA is the mRNA for coat protein, how it arises is unknown. Analysis of in vitro and in vivo products of replicase have yet to detect synthesis of LMC-RNA or any other monocistronic messenger RNA. The search for specific nuclease to cleave TMV-RNA into monocistronic messenger RNAs, although underway for 2 years, has been unsuccessful. An additional consideration, with specific nucleases is why are some TMV-RNA molecules cleaved and others not? There is no substantial evidence on viral-specific RNAs in infected callus associated with polyribosomes the size necessary to code for 130,000 or 35,000 dalton polypeptides.

An additional consideration in analyzing the data on TMV replication studies is the difference in the nucleotide homology in the different strains of TMV. VanDeWalle and Siegel (1976) examined the nucelotide

similiarities between genomes of 12 strains of TMV by competition-hybridization. It was found that the strains fall into three groups with nucleotides being almost indistinguishable within a group but without similiarities between groups (Table 1).

Table 1. Relationships of TMV strains by competition-hybridization.*

Group 1 (U1)	Group 2 (U2)	Group 3 (Dahlemense)
J14D1	GTAMV	YTAMV
06-67		
U6		
YA		

*GTAMV (YTAMV) - green (yellow) atypical mosaic; 06-67 and J14-D1 are orchid viruses; YA-yellow acuba mosaic; U1, U2, and dahlemense are tobacco strains of TMV; cowpea mosaic and Holmes rib grass failed to hybridize to U1, U2 or dahlemense strains.

The two groups represented by U1 and dahlemense have capsid proteins which are most alike of any of the intergroup comparisons having differences in 29 of the 15 sequential amino acid positions (18%) and yet their nucleic acids show no homology. Efron and Marcus (1973) and Roberts et al. (1974) used strains of TMV that belong to group 1. Bruening et al. (1976) used cowpea strain that fails to hybridize to group 1 strains. Is the differences in the amount of coat protein synthesized reflect the heterogeneity of the TMV strains used in the cell-free protein synthesizing systems?

In conclusion, it appears that TMV-RNA may contain multiple initiation and ribosome binding sites and that at least three

polypeptides are synthesized (130,000; 30,000 and 17,500 daltons) (Fig. 5). When TMV infects a cell and viral RNA is initially translated, is the small amount of the 30,000 dalton polypeptide synthesized of biological importance or does it require a specific monocistronic messenger RNA for the cistron? If the function of this polypeptides can be elucidated, this may aid in the understanding the nature of the translation of TMV-RNA. Further investigation to determine whether other monocistroninc mRNAs are present in TMV-infected cells and the mechanism by which LMC-RNA arises would resolve many problems in the replication cycle of TMV.

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SUMMARY

A fraction which contained membrane-bound tobacco mosaic virus RNA replicase was isolated from tobacco mosaic virus-infected tobacco callus cultures. The replicase activity reached a maximum 60 hr after inoculation and then declined. The enzyme activity was insensitive to actinomycin D and DNase. The corresponding fraction from healthy callus contained essentially no activity. The viral RNA synthesis *in vitro* proceeded linearly for 30 min and required the four nucleotide triphosphates and Mg^{2+} ions. Mn^{2+} was a poor substitute for Mg^{2+}. During RNA synthesis the product was at least 70% resistant to RNase in 2 x SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), but completely digested by RNase in 0.1 x SSC. Analysis of the product by polyacrylamide gel electrophoresis revealed a double-stranded RNA (4.0×10^6 daltons) which appears to be replicative form and a partially RNase resistant structure similiar to replicative intermediate. Washing the membrane-bound replicase with Mg^{2+}-deficient buffer solubilized the enzyme. The solubilized enzyme was further purified by DEAE-Sephadex column chromatography. The DEAE-purified enzyme was nearly completely dependent upon TMV-RNA for activity. Analysis of the product on sucrose gradients revealed a double-stranded RNA with sedimentation of 16 S and smaller heterogenous RNase-sensitive products.

A procedure was developed to isolate polyribosomes from plant cell cultures. Tobacco callus extracted in 10 mM $MgCl_2$, 80 mM KCl, 250 mM

sucrose, and 140 mM tris-HCl, pH 8.2 yielded larger amounts of polysomes than cells extracted in higher or lower ionic strength or pH buffers. Optimal conditions for extraction of polysomes from soybean callus were identical except the most suitable pH for recovery was 8.5. Addition of the divalent cation chelator, ethyleneglycol-bis (2-aminoethyl ether) tetraacetic acid (EGTA) to the extraction medium improved polysomal yield from tobacco and soybean cultures. Polysomes were successfully extracted from potato, tomato, corn, and barley cell cultures in extraction medium supplemented with EGTA.

Extraction of polyribosomes from tobacco tissue cultures previously inoculated with ^{3}H-uridine labeled TMV-RNA, radioactivity was detected in large polyribosomes (> 250 S). Extraction of RNA from the large polyribosomes and analysis on polyacrylamide gel electrophoresis revealed a single homogeneous peak that comigrated with authentic TMV-RNA. Analysis of the nascent polypeptides associated with TMV-RNA polyribosome revealed three polypeptides (molecular weights: 130,000; 37,000 and 17,500 daltons). The 130,000 dalton polypeptide was synthesized in the largest amount with lesser amounts of the 37,000 and 17,500 dalton polypeptides. The latter polypeptide appears to be TMV coat protein. It appears that intact TMV-RNA is associated with polyribosomes and that three polypeptides are synthesized in differing amounts. The importance in replication of TMV of the small amounts of the 2 smaller polypeptides synthesized remains to be resolved.