STUDIES OF VELVET TOBACCO MOTTLE VIRUS RNA REPLICATION BY ENZYME-TEMPLATE COMPLEXES IN EXTRACTS FROM INFECTED LEAVES

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STATEMENT

This thesis has not previously been submitted for an academic award at this or any other University, and is the original work of the author, except where due references made in the text.

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SUMMARY

1. A RNA-dependent RNA polymerase-template complex was shown to be located in the cytoplasmic fraction of VTMoV-infected leaves of N. clevelandii. No similar activity could be detected in healthy tissue.

2. Optimal enzyme activity in the 1000 g supernatant fraction from cell-free leaf extracts was at approximately 20°C and pH above 8.8 in the presence of between 14 to 20 mM Mg^{++}. Enzyme activity did not respond to the addition of K^{+} or NH_4^{+}. However, the enzyme's Mg^{++} requirement could be replaced by 2 mM Mn^{++}. The chelating agents EGTA and EDTA stimulated enzyme activity with EGTA being the more effective of the two. No reduction in enzyme activity was observed on addition of actinomycin D or α-amanitin.

3. Partial purification of the RNA-dependent RNA polymerase-template complex was achieved by polyethylene glycol precipitation but attempts to purify the enzyme further were unsuccessful. Precipitation with ammonium sulphate or sodium sulphate resulted in complete loss of enzyme activity. Products of RNA synthesis by the partially purified polymerase-template complex were shown to be similar to those synthesized by the 1000 g supernantant fraction.

4. Optimal enzyme activity in the partially purified preparations occurred under similar conditions to those observed for the 1000 g supernatant fraction. However, EDTA and EGTA were less effective in stimulating enzyme activity and only a slight response was observed to Mn^{++}. The enzyme was found to be unstable at temperatures above 20°C and pH above 7.6.

5. Products of the RNA-dependent RNA polymerase-template complex in the 1000 g supernatant fraction from VTMoV-infected leaves were identified as two species of double stranded RNA (dsRNA) with apparent M_r's of approximately 3.5×10^6 and 0.72×10^6. Analysis of the products after denaturation indicated that both RNA 1 and viroid-like RNAs were synthesised. Dot blot hybridization together with molecular hybridization analysis in solution, showed that only positive-sense RNA was synthesised. The larger dsRNA was RNA 1-specific and the smaller was specific to the viroid-like RNA. The products of *in vitro* RNA synthesis were unlike the dsRNA isolated from VTMoV-infected plants.

6. Kinetic studies using the partially purified enzyme-template indicate that a ping-pong mechanistic model best fits the observed data. An experimental strategy with a tetra reactant system for obtaining steady state kinetic data suitable for mechanistic interpretation was developed. No evidence of terminal nucleotide transferase activity being present in the enzyme preparations was obtained during the kinetic studies.

7. It is concluded that synthesis of VTMoV RNA occurs in the cytoplasm of virus-infected plant cells. Enzyme template complexes consisting of polymerase molecules which are bound to endogenous template strands and capable of completing RNA synthesis *in vitro* can be obtained from virus infected leaf tissue. These complexes can be used to study the mechanism by which viral RNA is synthesised and purification of these complexes could lead to determine whether the enzyme is of plant or viral origin.

CHAPTER 1

INTRODUCTION

1.1 VELVET TOBACCO MOTTLE VIRUS - ITS STRUCTURE, RNA COMPOSITION AND TAXONOMIC AFFINITIES

Velvet tobacco mottle virus (VTMoV) was discovered in northern South Australia (Randles et al., 1981). The virus has polyhedral particles approximately 30 nm in diameter containing about 20% RNA. It has been suggested that the virus belongs to the Sobemovirus group (Francki et al., 1984).

The coat protein of the virus particle was reported to consist of one major polypeptide of M_r about 30,000 as determined by SDS - polyacrylamide gel electrophoresis (Randles et al., 1981). Two other minor proteins of M_r about 36,000 and 33,000 were also present in the virus preparations. Subsequent work by Chu and Francki (1983) indicated that a protein of M_r about 37,000 makes up the protein shell of intact virus particles. However, this protein is degraded to lower molecular weight proteins by plant proteases during virus purification resulting in the recovery of stable virus particles with a coat protein of M_r about 30,000.

The nucleic acid composition of VTMoV consists of single stranded (ss)RNA of M_r about 1.5×10^6 (RNA 1), and two highly base-paired ss RNAs one circular (RNA 2) and the other linear (RNA 3), both of M_r about 1.2×10^5 (Randles et al., 1981). The base sequences of RNAs 2 and 3 were shown to be indistinguishable by complementary DNA (cDNA) hybridization analysis (Gould, 1981). However, no base sequence homology

exists between RNA 1, and the RNAs 2 and 3 (Gould, 1981). Two minor RNA components are also encapsidated in VTMoV particles, RNA 1a and RNA 1b, with M_rs of about 0.63 and 0.25 x 10^6 respectively. These RNAs have been shown to have base sequence homology with RNA 1.

The similarity in structure of RNAs 2 and 3 to viroids is striking and they have been referred to as viroid-like RNAs (Randles et al., 1981) or virusoids (Haseloff et al., 1982). Viroids are low molecular weight RNAs (1.1 to 1.3 x 10^5 daltons) which exist in their native conformations as extended rod-like structures characterised by a series of base-paired double-helical sections with internal loops (Diener, 1983 ; Sanger, 1984). Electron microscopy of fully denatured viroid molecules reveals covalently closed circular as well as linear molecules (McClements and Kaesberg, 1977 ; Randles and Hatta, 1979). The linear molecule has the same base sequence as the covalently closed circular RNA and both RNA species are infectious (Owens et al., 1977 ; Palukaitis and Symons, 1980 ; Sanger, 1984).

Diener (1983) lists the following five properties of viroids by which they are distinguished from viruses.

1. The pathogen exists in vivo as an unencapsidated RNA.

2. Virion like particles are not detected in infected tissue.

3. The infectious RNA is of low molecular weight.

4. The infectious RNA is replicated autonomously in susceptible cells.

5. The infectious RNA consists of one molecular species only.

The viroid-like RNAs found encapsidated in virus particles are not capable of autonomous replication in host plants and are dependent on RNA 1 (Gould et al., 1981 ; Jones et al., 1983).

In addition to VTMoV, viroid-like RNAs have been detected in three other viruses : Solanum nodiflorum mottle virus (SNMV ; Gould and Hatta, 1981). Lucerne transient streak virus (LTSV ; Tien-Po et al., 1983) and subterranean clover mottle virus (SCMoV ; Francki et al., 1983).

The viroid-like RNAs from different sources have been reported to differ in their biological properties. It was claimed that both RNA 1 and RNA 2 are required for virus replication of VTMoV and SNMV (Gould et al., 1981) and that recombination of the RNA 1 with the heterologous viroid-like RNA produced no infection of the host plants. However, in the case of LTSV, RNA 1 can infect and produce virus particles in host plants in the absence of its viroid-like RNA. In this case RNA 2 behaves as a satellite RNA (Jones et al., 1983). Furthermore, LTSV RNA 1 can support the replication of RNA 2 from SNMV (Jones and Mayo, 1983) and SCMoV (Keese et al., 1983). A culture of SNMV devoid of RNA 2 has been established by Jones and Mayo (1984) but this SNMV isolate would not support replication and encapsidation of LTSV RNA 2 suggesting that RNA 1 from SNMV is specific for SNMV RNA 2. Recently, an isolate of VTMoV devoid of RNA 2 and 3 has also been obtained (R.I.B. Francki, C.J. Grivell and K. Gibb, personal communication).

The viroid-like RNAs 2 and 3 from LTSV replicate and are encapsidated when co-infected with sowbane mosaic virus (Francki et al., 1983) southern bean mosaic virus (Paliwal, 1984) and also possibly turnip rosette virus (Jones and Mayo, 1984) all of which belong to the

Sobemovirus group but are serologically unrelated to LTSV (Matthews, 1982). None of these viruses have ever been isolated with an encapsidated viroid-like RNA in the field, affirming the apparent satellite nature of the encapsidated viroid like RNAs.

The complete nucleotide sequence of all the known viroid-like RNAs have been determined (Symons and Haseloff, 1982 ; Keese et al., 1983 ; J. Haseloff and R.H. Symons, personal communication). SNMV RNA 2 consists of 377 base residues while that of VTMoV consists of two approximately equimolar species of 366 and 365 residues. There is 95% sequence homology of VTMoV RNA 2 with that of SNMV and 92% of SNMV RNA 2 is homologous with VTMoV RNA 2 (Haseloff and Symons, 1982).

The RNA 2 from two isolates of LTSV, one from Australia (LTSV-A) and the other from New Zealand (LTSV-N), have been sequenced by Keese et al. (1983). Each isolate of RNA 2 contains 324 residues and they differ by only eight residues. However, very little sequence homology was detected between the RNAs 2 and either LTSV or SCMoV, VTMoV and SNMV (Keese et al., 1983 ; J.W. Randles and R.I.B. Francki, personal communication).

Two species of viroid-like RNA have been detected from SCMoV, one containing 327 residues and the other 388 and either one or both may occur in field isolates of the virus (Francki et al., 1983). Extensive sequence homology exists between the first 108 and last 100 nucleotide residues of the two viroid-like RNA species but there is only relatively little sequence homology between the SCMoV RNA 2 species and the other viroid-like RNAs (J. Haseloff and R.H. Symons, personal communication).

1.2 PREVIOUS STUDIES OF VTMoV REPLICATION

Of the four viruses with viroid-like RNAs only VTMoV has been studied with respect to its replication. *In vivo* studies in *N. clevelandii* have been reported by Chu *et al*. (1983). They found that the virus was detected serologically six days after inoculation in both the inoculated and systemically infected leaves. However, in the inoculated leaves, the viroid-like RNAs 2 and 3 were detected two days after inoculation and increased in concentration thereafter. The ratio of RNA 2 concentration to that of RNA 3 increased significantly during the course of the experiment suggesting the possibility that RNA 3 could be a precursor of RNA 2.

A virus specific ssRNA of M_r about 0.25×10^6, was detected between four and six days after inoculation and is thought to be RNA 1b, a minor component usually detected in VTMoV particles (Randles *et al*., 1981). It has been suggested that this RNA may be a sub-genomic m-RNA (Chu *et al*. 1983). *In vitro* translation studies of the RNA from SNMV (Kiberstis and Zimmern, 1984) and LTSV (Morris-Krsinich and Forster, 1983) have shown that efficient coat protein synthesis was mediated by a minor RNA component in each case. These minor components are partial transcripts of RNA 1 which are encapsidated in the virus particles.

Chu *et al*. (1983) have reported the detection of a single band of dsRNA of M_r about 3.6×10^6 as determined by polyacrylamide gel electrophoresis, two to four days after inoculation. A second smaller dsRNA species of M_r about 2.8×10^6 was detected eight to ten days after inoculation. Chu *et al*. (1983) analysed the dsRNAs found *in vivo* by

Southern blot analysis and showed that the larger dsRNA ($M_r \simeq 3.6 \times 10^6$) was RNA 2 and 3 - specific and the smaller ($M_r \simeq 2.8 \times 10^6$) to be RNA 1 - specific. The dsRNA which was found to be RNA 1 - specific has a molecular weight which is close to that expected for a duplex of RNA 1 (estimated to be $M_r \simeq 1.5 \times 10^6$ by Randles et al., 1981). When this dsRNA was melted, the resultant ssRNA migrated at about the same rate as RNA 1. Chu et al. (1983) suggested that the dsRNA is a replicative form of the VTMoV RNA 1. However, the size of the RNA 2 specific dsRNA is 13 to 15 times that expected for a duplex of RNAs 2 or 3 and its significance remains obscure.

There are many questions which need to be answered before a complete understanding of VTMoV replication can be achieved. One way of probing some of these questions is by studying the replication of viral RNA in an in vitro system. This approach has been taken by a number of other workers for a variety of different plant viruses some of which are discussed below.

1.3 RNA DEPENDENT RNA POLYMERASE INVOLVED IN MULTIPLICATION OF SMALL RNA VIRUSES

A number of authors have attempted to isolate RNA-dependent RNA polymerase from virus infected plants and studied viral RNA replication by adding the appropriate template RNA. However, this approach raises questions of whether the polymerases used in such studies were actually involved in viral RNA synthesis in the intact plant tissues from which they were extracted. Of particular significance in overcoming this problem is to work with preparations of RNA-dependent RNA polymerases bound to their native virus-specific RNA templates. Such enzyme-template complexes which are capable of synthesising dsRNA, have been reported

for all RNA containing virus-host systems which have been studied (Hamilton, 1974).

Until recently enzyme template complexes isolated from virus-infected plants have received little attention. The only two systems which have been characterised to any extent are those of turnip yellow mosaic virus (TYMV) in Chinese cabbage (Mouches et al., 1974 ; Mouches et al., 1984) and cowpea mosaic virus (CPMV) in cowpea leaves (Dorssers et al., 1983).

In the case of TYMV infected Chinese cabbage leaves, replication complexes have been isolated from virus-specific double membrane vesicles associated with the chloroplasts. Bove et al. (1972) have reported the partial purification of the replication complex from the virus specific vesicles with the use of the non-ionic detergent Lubol W but no further work has been done on the complex as such. However, Mouches et al. (1974 and 1984) have studied the RNA-dependent RNA polymerase freed from its native template in some detail.

The replication complex associated with CPMV infection of cowpea leaves has been partially purified and its in vitro products extensively characterised by Dorssers et al. (1983). Virus-specific vesicles are present in the cytoplasm of CPMV infected cowpea cells and are shown to contain virus specific dsRNA (Assink et al., 1973 ; De Zoeten et al., 1974) as well as RNA-dependent RNA polymerase molecules bound to an endogenous template. After washing the membrane fractions from CPMV-infected leaves to remove template-free RNA polymerases, the enzyme-template complex was solubilized with Triton X100 and purified by filtration through Sepharose 2B (Dorssers et al., 1983). In vitro dsRNA

products of the partially purified replication complex have been characterised and shown to contain labelled positive-sense viral RNA. Further characterisation of such complexes, especially after purification, will undoubtedly increase our understanding of viral RNA synthesis in plant cells.

In this thesis, work on the characterization of the enzyme-template complex detected in VTMoV-infected leaves of N. clevelandii is reported. It has been studied in crude, cell-free leaf extracts and as a partially purified preparation. The in vitro products synthesised by the enzyme-template complex have been characterised and an enzyme kinetic model for the mechanism by which it synthesises RNA has been suggested.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Virus Isolate

The velvet tobacco mottle virus (VTMoV) isolate used was that originally isolated from Nicotiana velutina (Wheeler) in South Australia (Randles et al., 1981). The isolate was maintained in the glasshouse in mechanically inoculated Nicotiana clevelandii. A list of other virus isolates used is presented in Table 2.1.

2.1.2 Chemicals and Enzymes

The chemicals and enzymes used and their sources are listed in Table 2.2. All chemicals used were of analytical or laboratory reagent grade. Labelled ribonucleoside triphosphate [α - ^{32}P] GTP and [γ -^{32}P] was kindly provided by Dr. R.H. Symons. Ribonuclease A was heated at 100°C for 10 minutes before use to render it free of other enzymes.

2.1.3 Instruments

Ultracentrifugations were carried out in either a Beckman L2-65 or L8-70 ultra centrifuge. Sorval RC2-B and RC3 instruments were used for intermediate and low speed centrifugations respectively.

Table 2.1 : Virus Isolates Used In Studies Presented In This Thesis.

Virus	Source	Reference
Lucerne transient streak virus (LTSV)	Lucerne	Tien-Po *et al*. (1981)
Red clover necrotic mosaic virus (RCNMV)	Lucerne and White clover	Gould *et al*. (1981)
Tobacco mosaic virus (U_1 strain) (U_1-TMV)	Tobacco	Seigel and Wildman, (1954)
Tobacco ringspot virus (TRSV)	*Gladiolus* sp.	Randles and Francki, (1965)
Tomato ringspot virus (Tom RSV)	*Pentas lanceolata*	Chu *et al*. (1983a)
Velvet tobacco mottle virus (VTMoV)	*N. velutina*	Randles *et al*. (1981)

Table 2.2 : Chemicals and Biochemicals Used In This Thesis.

Chemical	Source
Acrylamide	Bio-Rad Laboratories, U.S.A.
Actinomycin D.	Merk-Sharp and Co. Inc., U.S.A.
Adenosine 5'-triphosphate	Sigma Chemical Co., U.S.A.
α-Amanitin	Sigma Chemical Co., U.S.A.
Bovine serum albumin (BSA)	Sigma Chemical Co., U.S.A.
Cytidine 5'-triphosphate	Sigma Chemical Co., U.S.A.
Deoxyribonuclease 1 (DNase 1 ; bovine pancrease ; DN-EP grade)	Sigma Chemical Co., U.S.A.
Deoxyribonucleic acid (DNA ; Herring sperm ; type VII)	Sigma Chemical Co., U.S.A.
Formaldehyde	May and Baker, England
Formamide	BDH Chemicals, England
Guanosine 5'-triphosphate (type I)	Sigma Chemical Co., U.S.A.
2-Mercaptoethanol	BDH Chemicals, England
N,N' Methylene bis-acrylamide	Bio-Rad Laboratories, U.S.A.
Monothio glycerol	Sigma Chemical Co., U.S.A.
Nuclease S_1	Sigma Chemical Co., U.S.A.
β-Nicotinamide adenine dinucleotide ; reduced ($NADH-Na_2$)	Sigma Chemical Co., U.S.A.
Ovalbumin	Sigma Chemical Co., U.S.A.
3-Phosphoglycerate	Boehringer Mannheim, W. Germany
Phosphoglycerate kinase (P.G.K.)/ Glyceraldehyde phosphate dehydrogenase (GAPDH) ; (Suspension)	Boehringer Mannheim, W. Germany
Polyethylene glycol (PEG 6000)	Union Carbide, U.S.A.

Table 2.2 continued

Pronase (type IV, fungal protease)	Sigma Chemical Co., U.S.A.
Protamine sulphate (Salmon ; grade 1)	Sigma Chemical Co., U.S.A.
Ribonuclease A (RNase A ; pancreatic)	Sigma Chemical Co., U.S.A.
Ribonuclease T_1	Sigma Chemical Co., U.S.A.
Ribonucleic acid (RNA ; yeast type III)	Sigma Chemical Co., U.S.A.
Sodium dodecyl sulphate (SDS)	BDH Chemicals, Victoria
Streptomycin sulphate	Glaxco, England
N, N, N', N'' - tetramethyl-ethylenediamine (TEMED)	Sigma Chemical Co., U.S.A.
Tris (hydroxymethyl) aminomethane (TRIS)	Sigma Chemical Co., U.S.A.
Triton X100	BDH Chemicals, England

Ultraviolet absorption spectra were measured with a Unicam SP1800 spectrophotometer. Density gradients were collected and analysed with an Isco Model 183 density gradient fractionator coupled to a UA-2 recorder.

Electrophoresis procedures were done with a Bio-Rad Model 220 vertical slab electrophoresis cell and gels were dried on a Bio-Rad Model 224 gel slab drier.

2.2 METHODS

2.2.1 Virus Propogation

Plants used for virus propogation were raised in an insect-proof glasshouse under natural illumination, shaded during the summer months. Naphthelene was occasionally used to control larvae of a small Dipterous fly which originated in the German peat used in the potting soil. If left unchecked, significant leaf damage could occur. Aphid infestation was controlled by spraying plants with pyrethrum or fumigating the glasshouse with nicotine sulphate as required. Nicotiana clevelandii plants without flowers or flower buds were found to be the best hosts for the virus. The size of plants varied according to seasonal influences.

Two procedures were followed for virus stock propogation or infection of plants for polymerase studies. These were :

1. Virus stock propogation

Plants used for propogating VTMoV were dusted with 500 mesh a carborundum powder and mechanically inoculated with sap from diseased

plants and maintained in the glasshouse until severe systemic infection was apparent. Leaves showing symptoms were harvested and stored at -20°C until required for virus preparation.

2. Virus propogation for polymerase studies

N. clevelandii plants used for VTMoV-induced RNA dependent RNA polymerase studies were inoculated with either a suspension of purified virus (0.1 mg/ml in 20 mM phosphate buffer, pH 8.5) or sap from infected leaves. Purified virus was used as inoculum for the work presented in Chapter 3. Subsequently infected leaf sap was used because it was found that better levels of infection and subsequently higher levels of enzyme activity could be obtained with this inoculum. Inoculated plants were kept in a constant temperature room maintained at 25°C and grown under continuous fluorescent light of approximately 12,000 lux.

2.2.2 Purification of VTMoV

Purification of VTMoV was carried out by a method modified from Randles et al. (1981).

Infected tissue was extracted in a kitchen blender with 0.1 M phosphate buffer, pH 7.4, containing 0.1% thioglycollic acid, in the proportion of 2 ml buffer to each gram of tissue. The extract was emulsified with an equal volume of a 1:1 mixture by volume of chloroform and n-butanol and centrifuged at 27,000 g for 10 minutes. The aqueous phase was collected and centrifuged at 78,000 g for 90 minutes. The virus containing pellet was resuspended in 20 mM phosphate buffer, pH 7.4, and clarified by centrifugation at 27,000 g for 10 minutes. The

supernatant was layered over 10% sucrose in 20 mM phosphate buffer, pH 7.4, and centrifuged at 160,000 g for 60 minutes. Pellets were resuspended in buffer, clarified by centrifugation at 27,000 g for 10 minutes and stored at 4°C until required.

2.2.3 Standard *In vitro* Assay of RNA-Dependent RNA Polymerase

The method used for analysis of RNA-dependent RNA polymerase activity associated with viral infection was a modification of that used by May and Symons (1971).

Infected or healthy leaves were collected from plants as required, washed with distilled water and the main vein was removed. In the case of infected leaves, the regions showing severe symptoms were excised for enzyme preparation. The tissue was ground with a mortar and pestle in 0.1 M Tris-HCl, pH 8.8, 0.1 M NH_4Cl, 90 mM 2-mercaptoethanol and 0.4 M sucrose. A ratio of 1 gram of tissue to 2 ml of buffer was used. The leaf extract was strained through Miracloth (Calbiochem, California, U.S.A.), centrifuged at 1000 g for 10 minutes and unless otherwise stated, the supernatant was used as the source of enzyme.

The composition of the assay mixture is shown in Table 2.3. Variations are indicated in the relevant chapters. Unless otherwise stated, incubation times for the reaction were 15 min at 20°C and unincubated samples were used as controls in all experiments. Reactions were stopped by the addition of 0.4 ml of 80 mM $Na_4O_7P_2$ and 0.4 ml of 25% TCA. The samples were placed on ice for 30 minutes, centrifuged at 18,000 g for 5 minutes and the supernatant discarded. The pellets were

Table 2.3 : **<u>In vitro</u> Assay Mixture Used to Measure RNA-Dependent RNA Polymerase Activity.**

Reagent	Concentration	
	Stock solution	After addition of Enzyme
ATP	125 µM	100 µM
CTP	125 µM	100 µM
UTP	125 µM	100 µM
GTP	1.25 µM	1 µM
$MgSO_4$	10 mM	8 mM
EGTA	12.5 mM	10 mM
NH_4Cl	25 mM	20 mM
KCl	25 mM	20 mM
Tris-HCl (pH 8.8)	62.5 mM	50 mM
B.S.A.	1.25 mg/ml	1 mg/ml
2 Mercapto ethanol	31.25 mM	25 mM
Actinomycin D	12.5 µg/ml	10 µg/ml

In each assay 200 µl of stock assay mixture was used. To this enzyme preparations were added in a volume of 50 µl giving a total volume of 250 µl per assay. α-^{32}P - GTP was added so that there was approximately 0.108 µ Ci/assay. The specific activity of the α-^{32}P - GTP was approximately 1700 Ci/m mole.

resuspended in 0.01 M NaH_2PO_4 containing 5% TCA, collected under vacuum on glass fibre filter discs (Whatman, England), and washed with 15 ml 0.01 M NaH_2PO_4 containing 5% TCA followed by 10 ml ethanol and then 10 ml acetone. The dried filter discs were placed in 2 ml plastic scintillation vials, covered with 1.5 ml of toluidine based scintillation fluid containing 0.36 grams/litre POPOP and 3.6 grams/litre PPO. The amount of radioactivity was measured in a Packard model 3320 scintillation spectrometer.

2.2.4 Nucleic Acid Preparation

Phenol extraction was used to isolate RNA from virus preparations and the in vitro RNA-dependent RNA polymerase assay mixtures when reaction product was required for analysis.

Virus preparations diluted to 4 mg/ml, or undiluted enzyme assay mixtures, were added to equal volumes of buffer containing 0.2 M sodium acetate, 0.1% thiogylcerol and 1% SDS. To this an equal volume of water saturated phenol was added and the mixtures were shaken at 25°C for at least 1 hour. The phenol and aqueous phases were separated by centrifugation at 12,000 g for 10 minutes and the aqueous phase was re-extracted with phenol as outlined above. The final aqueous extract was collected and nucleic acids precipitated with 2.5 volumes of ethanol. This precipitate was collected by centrifugation at 12,000 g for 10 minutes, resuspended in sterile distilled water and reprecipitated with ethanol to remove traces of phenol.

Nucleic acid samples were stored at -75° under ethanol until used. The ethanol precipitated nucleic acid was collected by centrifugation at

Table 2.4 : Preparation of Polyacrylamide Gels for Analysis of Nucleic Acids.

Reagent	Quantity
Urea[a]	14 g
bis-acrylamide	0.05 g
acrylamide	1 g
TEMED	34 µl
10% ammonium persulphate	340 µl
10 x TBE[b] buffer	3.4 ml
H_2O	to total volume of 34 ml

a Urea was omitted when non-denaturing gels were required.

b TBE buffer contained 10.8 grams of Tris, 5.5 grams of Boric acid and 0.93 grams of EDTA per litre. The final pH was 8.5.

12,000 g for 10 minutes and the pellets were dried under vacuum. Dried nucleic acid was resuspended in sterilized water or TBE buffer (see Table 2.4) as required.

2.2.5 Electrophoresis

Acrylamide gel slabs 1.5 mm in thickness were used for analysis of nucleic acids (Chu et al., 1983). The recipe for the gels, with and without urea, and electrophoresis buffer (TBE) is given in Table 2.4. TBE buffer was used in both electrolyte tanks and a current of 35 mA per gel was applied. Samples to be electrophoresed were mixed with an equal volume of 50% glycerol containing 0.02% bromophenol blue. Gels were stained with a solution of 0.05% toluidine blue in 5% acetic acid diluted 1:5 with water and destained with water.

Electrophoresis of protein samples was carried out using a polyacrylamide gel system modified from Davis (1964). A 1.5 mm slab gel consisting of a 15 mm long stacking gel and 50 mm separating gel was used. The recipe for the gel is given in Table 2.5. The electrophoresis buffer contained 25 mM TRIS, 0.2 M glycine, 0.1% SDS, pH 8.3. Protein samples in 1% SDS and 1% 2 mercapto ethanol were heated at 100°C for 5 min. before the addition of an equal volume of 50% glycerol containing 0.02% bromophenol blue. Gels were stained with a Kodavue Electrophoresis Visualization Kit (Eastman Kodak Company, U.S.A.).

2.2.6 Autoradiography of Gel Slabs

Gels were placed on to sheets of Whatman No. 1 chromatography paper and dried under vacuum at 80°C in a Bio-Rad gel slab drier. Dried gels

Table 2.5 : Preparation of Polyacrylamide Gels for Analysis of Proteins.

Reagent	Stacking Gel ml of Reagent	Separating Gel ml of Reagent
H_2O	3.32	4.31
10% SDS	0.5	1
Buffer	A 0.625[a]	B 1.25[b]
10% Ammonium Persulphate	0.1	0.1
TEMED	0.015	0.015
Acrylamide Solution[c]	0.5	3.33
Total Volume (ml)	5	10

a Buffer A : 25.6 ml 1M H_3PO_4 + 5.7 g TRIS in 100 ml (pH 5.6)

b Buffer B : 0.3 M TRIS-HCl pH 7.9

c Acrylamide solution consisted of 30 grams acrylamide, 0.8 grams Bis-acrylamide in 100 ml.

were placed over a piece of Fuji x-ray film (Fuji Photofilm Co. LTD., Japan) between two sheets of glass, wrapped in aluminium foil and light-proof plastic bags, and kept at -75°C for the required time. The x-ray film was developed with Kodak liquid x-ray developer, type 2 and fixed with Kodak liquid x-ray fixer (Kodak (Australasia) Pty. Ltd. Victoria).

2.2.7 **Protein Analysis**

The Coomassie brilliant blue G assay for protein analysis devised by Bradford (1976) was used to estimate the protein content of enzyme preparations during purification. This assay system has been found to be suitable for measurement of protein concentration in crude animal and plant homogenates and unlike other protein assay systems, is not affected by laboratory buffers. However, the assay cannot be used when TRITON X100 is present in concentrations greater than 1% (Read and Northcote, 1981).

The assay dye reagent consisted of 10 mg Coomassie brilliant blue G, 5 ml 95% ethanol, 10 ml 85% phosphoric acid made up to 100 ml with water and filtered to remove undissolved dye. The assay was carried out in 1 ml disposable cuvettes containing 900 µl of dye reagent to which 90 µl of water and 10 µl of protein sample was added. After 10 minutes the absorbance at 595 nm was measured against a blank of 100 µl H_2O in 900 µl of dye reagent. All samples were assayed in duplicate. A curve obtained by using bovine serum albumin (BSA) as the standard protein was used to estimate protein levels in the unknown samples.

2.2.8 Analysis of Nucleoside Tri-phosphates

For successful enzyme kinetic work, it was essential that exact concentrations of the four nucleoside tri-phosphates used were known. Solutions of ATP, CTP, UTP and GTP were assayed by the method of Grassl (1974). The method does not distinguish between the nucleoside tri-phosphates so that cross contamination cannot be measured, and was assumed to be negligible.

The reaction buffer consisted of 93 mM triethanolamine, 6.5 mM 3-phosphoglycerate, 1.3 mM EDTA and 0.2 mM NADH, 2.85 ml of which was placed in 3 ml disposable plastic cuvettes.

A 100 µl sample of nucleoside triphosphate was added and the initial absorbance at 340 nm measured. The reaction was started with the addition of 40 µl of a mixture of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, and the absorbance at 340 nm measured after 10 and 12 minutes. A blank containing 100 µl of water was used to determine the increase in absorbance due to the addition of enzyme. Concentration of the nucleoside triphosphate solutions was calculated using a molar extinction coefficient $\varepsilon_{340nm} = 6.22 \times 10^6$ cm^2/mole for NADH (Netheler, 1974). The change in NADH concentration is directly proportional to the amount of nucleoside triphosphate present.

2.2.9 Spectral Measurements

Both RNA and virus concentration were estimated at spectrophotometrically using $E^{0.1\%}_{260nm}$ of 25 for RNA and 5 for VTMoV.

2.2.10 Precautions Against Ribonuclease and Bacterial Contamination

All heat stable solutions were autoclaved at 120°C for 15 minutes, glassware and disposable plastic ware was heat sterilized at 130°C for 18 hours. Electrophoresis equipment was cleaned with dilute chromic acid before use. Reagents which were heat labile were made up with autoclaved distilled water. Pipettes were flamed before use and disposable rubber gloves were used when handling RNA samples.

2.2.11 Bacterial Cultures

Escherichia coli (strain JM 101) was obtained from Dr. R.H. Symons (Dept. Biochemistry, University of Adelaide) as a frozen stock in 50% glycerol. From this a starter culture was grown up in 20 ml of minimal A medium (Table 2.6). The culture was then plated on minimal A medium agar (Table 2.6) incubated at 37°C for 24 hours and stored at 4°C. Single colonies were recultured in 20 ml of liquid medium and replated every 10 days to maintain an active culture.

2.2.12 Bacteriophage DNA Preparation

Bacteriophage M 13 containing DNA inserts of positive and negative copies of regions near the 5' and 3' ends of VTMoV RNA and a region of RNA 2 from SNMV were obtained from Drs. J. Haseloff and D. Zimmern (MRC Laboratory of Animal Biology, Cambridge, England). Details of the inserts are given in Chapter 6, Figure 6.11. It should be noted that the viroid like RNAs from SNMV and VTMoV are almost identical in base sequence (Haseloff and Symons, 1982). A M13 phage without any inserts was obtained from Dr. R.H. Symons (Dept. Biochemistry, University of Adelaide).

Table 2.6 : Minimal A Medium for Culturing E. coli.

10.5 g K_2HPO_4

4.5 g KH_2PO_4

1 g $(NH_4)_2SO_4$

0.5 g Sodium citrate.$2H_2O$

The above salts were dissolved in 400 ml of H_2O and autoclaved before the addition of the following ingredients.

10 ml 20% Glucose solution

0.5 ml 1% Thiamine HCl solution

1 ml 1M $MgSO_4 - 7H_2O$

Sterile H_2O to a final volume of 500 ml.

The above solutions were autoclaved separately except for the Thiamine HCl solution which was passed through a 22 micron filter. For agar plates Difco minimal agar was added to the medium at a rate of 15 g/500 ml.

E. coli from a single plated colony was grown in 10 ml of minimal A medium overnight at 37°C with shaking. A 1.5 ml aliquot of this culture was diluted in 500 ml of fresh minimal A medium. Of this 5 ml was removed and placed in a sterile 25 ml flask and 50 µl of M13 bacteriophage (5 mg/ml) added to provide a seeding culture. The cultures were incubated at 37°C with shaking until the absorbance reached between 0.4 and 0.6 at 600 nm. At this point the phage seeding culture was added to the bulk bacterial culture and incubated for 6 hours at 37°C with shaking.

To harvest the phage, the culture was centrifuged twice at 10,000 g for 10 minutes and the bacterial pellets discarded. Remaining bacteria were removed from the supernatant by vacuum filtration using a filter apparatus with 0.22 micron filters (Falcon Model 7103 filter). One volume of 40% polyethyleneglycol 6000 saturated with NaCl was added to every ten volumes of filtrate and the mixture was allowed to stand at room temperature for 20 minutes to precipitate the phage. The precipitate was collected by centrifugation at 10,000 g for 10 minutes and the supernatant discarded. The tubes were drained and as much as possible of the polyethylene glycol solution was removed by wiping the inside of the tube walls with sterile tissue paper and the pellets containing the phage were resuspended in 5 ml of 20 mM Tris HCl, 20 mM NaCl, 1 mM EDTA, pH 7.5. An equal volume of Tris saturated phenol was added to the phage solution, vortexed for 1 minute, stood at room temperature for 5 minutes, vortexed again for 1 minute and centrifuged at 5,000 g for 3 minutes (Tris saturated phenol was prepared by mixing equal volumes of 1 M Tris, pH 8 and phenol and removing the aqueous phase. The phenol phase was then remixed twice with 10 mM Tris 0.1 M EDTA pH 8, and stored at -20°C). The aqueous phase was collected and 1 M

sodium acetate was added to give a final concentration of 0.2 M. To this 2 volume of cold ethanol was added and the DNA precipitated at -20°C overnight.

The DNA was collected by centrifugation at 10,000 g for 15 minutes at 4°C and resuspended in 0.1 mM EDTA. A 1/2 volume of 7.5 M ammonium acetate was added to the preparation and the DNA was precipitated with 2 volumes of cold ethanol. This mixture was left at -80°C for 1 hour before centrifugation at 10,000 g for 15 minutes at 4°C. The DNA precipitate was washed with cold 70% ethanol, collected by centrifugation and resuspended in 0.1 mM EDTA. The concentration of DNA was estimated by spectral analysis using an $E^{0.1\%}_{260nm} = 25$.

2.2.13 Dot Blot Hybridization

A modified method of Southern (1975) was used to identify the relationship between <u>in vitro</u> synthesised labelled RNA and VTMoV RNA. DNA sample (10 µg DNA in 10 µl of 10 x SSC ; 1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) were spotted onto nitrocellulose filters (Millipore HAW P04770) which had been washed with 10 x SSC with the aid of a Millipore vacuum filtration apparatus (cat no XXII 047 00). The blots were baked for 2 hours at 80°C in a vacuum oven to fix the DNA to the filters. Hybridization buffer consisted of 2 x SSC, 40% deionized formamide, 10% dextransulphate and 0.1% SDS. Double stranded RNA used for hybridizing was melted in 50% formamide, 6% formaldehyde at 100°C for 5 minutes, rapidly cooled in an ice-ethanol mixture and precipitated with 2 volumes of ethanol. The precipitate was resuspended in sterile water and added to the hybridization buffer. The nitrocellulose dot blots were placed in plastic bags, into which the hybridization mixture

containing the labelled RNA was added and the bags were subsequently heat sealed. Hybridization of the labelled RNA was carried out at 45°C for 16 hours. Following the hybridization the filters were incubated for 30 minutes at 37°C in 2 x SSC containing 20 µg/ml RNase A and washed for 2 hours in 0.1 x SSC, 0.1% SDS at 55°C. After two such washes the filters were dried and exposed to x-ray film at -75°C for various times.

End labelled VTMoV RNA was also hybridized to the nitrocellulose dot blots to check that the hybridization technique was working. The VTMoV RNA was end labelled with ^{32}P by the method of Maniatis et al. (1982). 4 µl of VTMoV RNA in sterile distilled water (0.5 mg/ml) was added to 20 µCi of [γ - ^{32}P] ATP (specific activity > 3,000 Ci/m mole) and 1 µl of linker-kinase buffer (0.7 M Tris-HCl pH 7.6, 0.1M $MgCl_2$ and 50 mM dithiothreitol). The volume of this mixture was made up to 9 µl with sterile distilled water before 1 µl (10 units) of polynucleotide kinase (Boehringer Mannheim, West Germany) was added and the reactants incubated at 37°C for 15 minutes. The end labelled RNA was recovered from the reaction mixture by phenol extraction with 0.5 ml of RNA extraction buffer and 0.5 ml of phenol using the method outlined in Section 2.2.4 of this chapter.

2.2.14 **Liquid Hybridization**

Liquid hybridization of melted labelled double stranded RNA in the presence of VTMoV RNA was also carried out. Full details are given in the relevant Section of Chapter 6.

CHAPTER 3

PROPERTIES OF RNA-DEPENDENT RNA POLYMERASE IN EXTRACTS FROM VTMoV-INFECTED PLANTS

The presence of RNA-dependent RNA polymerase activity in plant tissues infected with a number of RNA containing viruses is well established (Hamilton, 1974 ; Hall et al., 1983). Although RNA-dependent RNA polymerase has been reported in healthy plant tissue the level of the enzymatic activity is always greater in virus-infected plants (Takanami and Frankel-Conrat, 1982).

Presented in this chapter are experiments on the detection, localization and biochemical properties of the RNA-dependent RNA polymerase activity in VTMoV-infected N. clevelandii plants.

3.1 DETECTION OF RNA-DEPENDENT RNA POLYMERASE ACTIVITY IN INFECTED LEAVES

The level of RNA-dependent RNA polymerase activity in N. clevelandii leaves inoculated with VTMoV was followed for eight days. Groups of plants were inoculated with a preparation of VTMoV at 24 hour intervals over a period of four days. Half-leaf samples from the inoculated leaves were collected 24 hours after the final inoculation and unfractionated cell-free extracts were assayed for polymerase activity. The plants were then left for a further four days after which the remaining half-leaves were harvested and the extracts assayed for enzyme activity. This procedure ensured that daily changes in enzyme activity were followed.

No significant levels of polymerase activity were detected during the first three days after inoculation. However, significant enzyme activity was detected four days after infection (Figure 3.1A) at a time when chlorotic lesions appeared on the leaves; the enzyme activity increased thereafter. Nine days after inoculation, the lesions became necrotic and a day later the leaves lost turgor.

Under similar conditions, Chu et al. (1983) found that VTMoV was first detected between four and six days after inoculation and increased rapidly thereafter. Thus the detection of virus-induced RNA-dependent RNA polymerase appears to preceded the rapid multiplication of VTMoV.

3.2 FRACTIONATION OF LEAF EXTRACTS CONTAINING RNA-DEPENDENT RNA POLYMERASE ACTIVITY

To investigate the distribution of RNA-dependent RNA polymerase activity, cell-free extracts from healthy and infected leaves were fractionated by differential centrifugation. When extracts from infected leaves were centrifuged at 1,000 g for 10 minutes, 83% of the polymerase activity was detected in the supernatant (S_1) fraction (Figure 3.2). However, this proportion varied from 70 to 95% in different experiments which indicates that little if any of the enzyme was associated with nuclei or chloroplasts which were pelleted by centrifugation at 1,000 g (Francki and Peters, 1978).

Subsequent centrifugation of the S_1 fraction at 17,000 g for 10 minutes resulted in 88% of the polymerase activity being recovered in the supernatant (S_{17}) fraction (Figure 3.2). The 17,000 g pellet (P_{17}) fraction contained mitochondria, chloroplast fragments and endoplasmic reticulum (Francki and Peters, 1978).

Figure 3.1

A. Increase in RNA-dependent RNA polymerase in N. clevelandii leaves following inoculation with VTMoV.

Half-leaf samples from inoculated leaves were ground with extraction buffer and filtered through miracloth. The extracts were then assayed for enzyme activity using the standard assay conditions (Chapter 2.2.3).

B. Time-Course of the Polymerase Reaction.

The 1000 g supernatant (S_1) fraction from healthy (O-O) and infected leaves (●-●) of N. clevelandii were assayed for enzyme activity for various times by the standard method (Chapter 2.2.3).

C. Temperature Optima of the Polymerase Reaction.

The polymerase reaction was carried out at various temperatures for 15 minutes using the S_1 fraction as a source of enzyme. The level of enzyme activity at 35°C was taken as 100%. Under these conditions the maximum amount of ^{32}P incorporated into the TCA precipitate was 1589 cpm in Experiment 1 and 1027 cpm in Experiment 2.

D. Effect of pH on the Polymerase Reaction.

The polymerase reaction was carried out at various pH levels for 15 minutes using the S_1 fraction as a source of enzyme. pH levels were maintained by the appropriate 50 mM tris buffer.

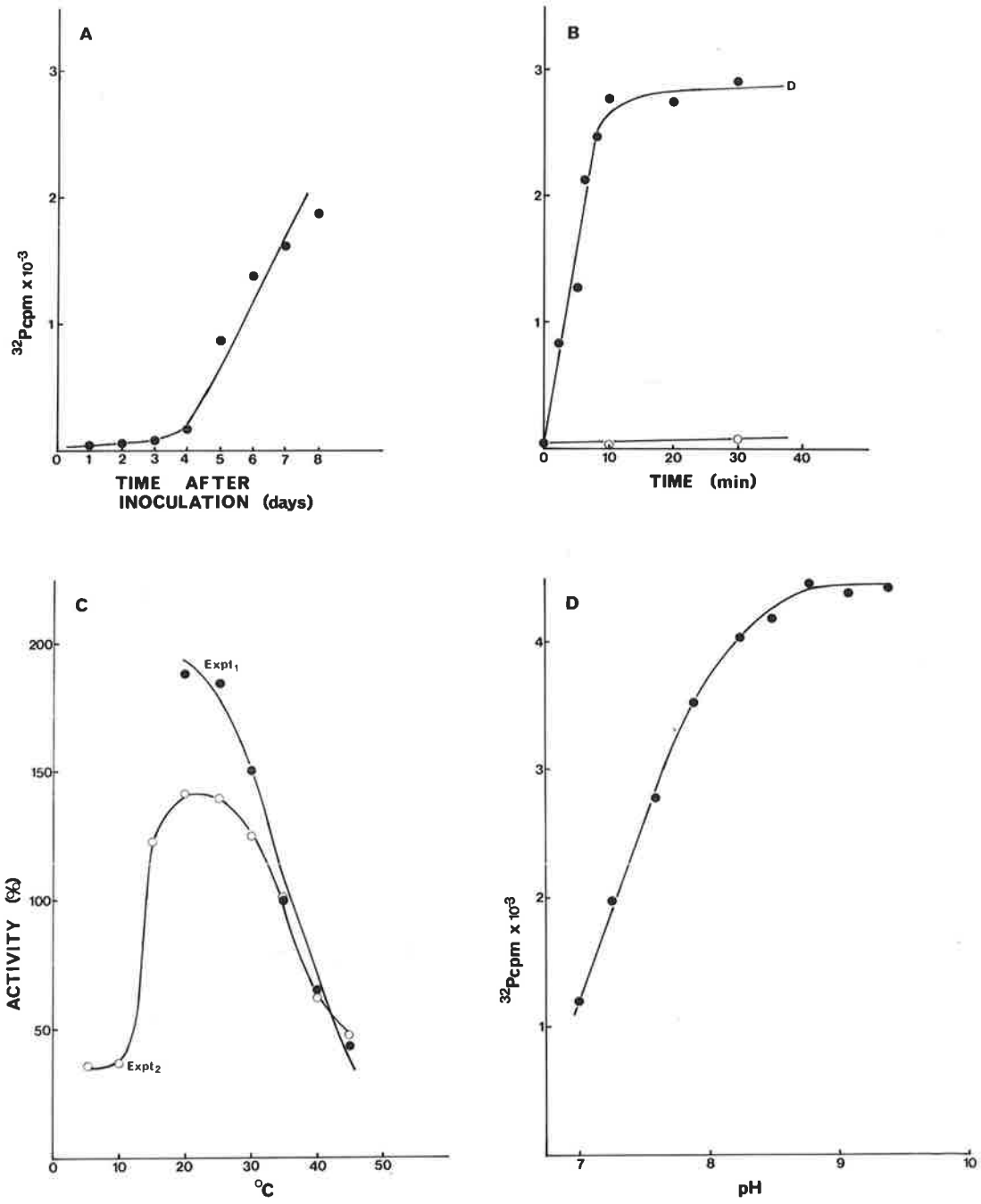

A
^{32}Pcpm x 10^{-3}
TIME AFTER INOCULATION (days)
B
D
TIME (min)
C
Expt$_1$
Expt$_2$
ACTIVITY (%)
°C
D
^{32}Pcpm x 10^{-3}
pH

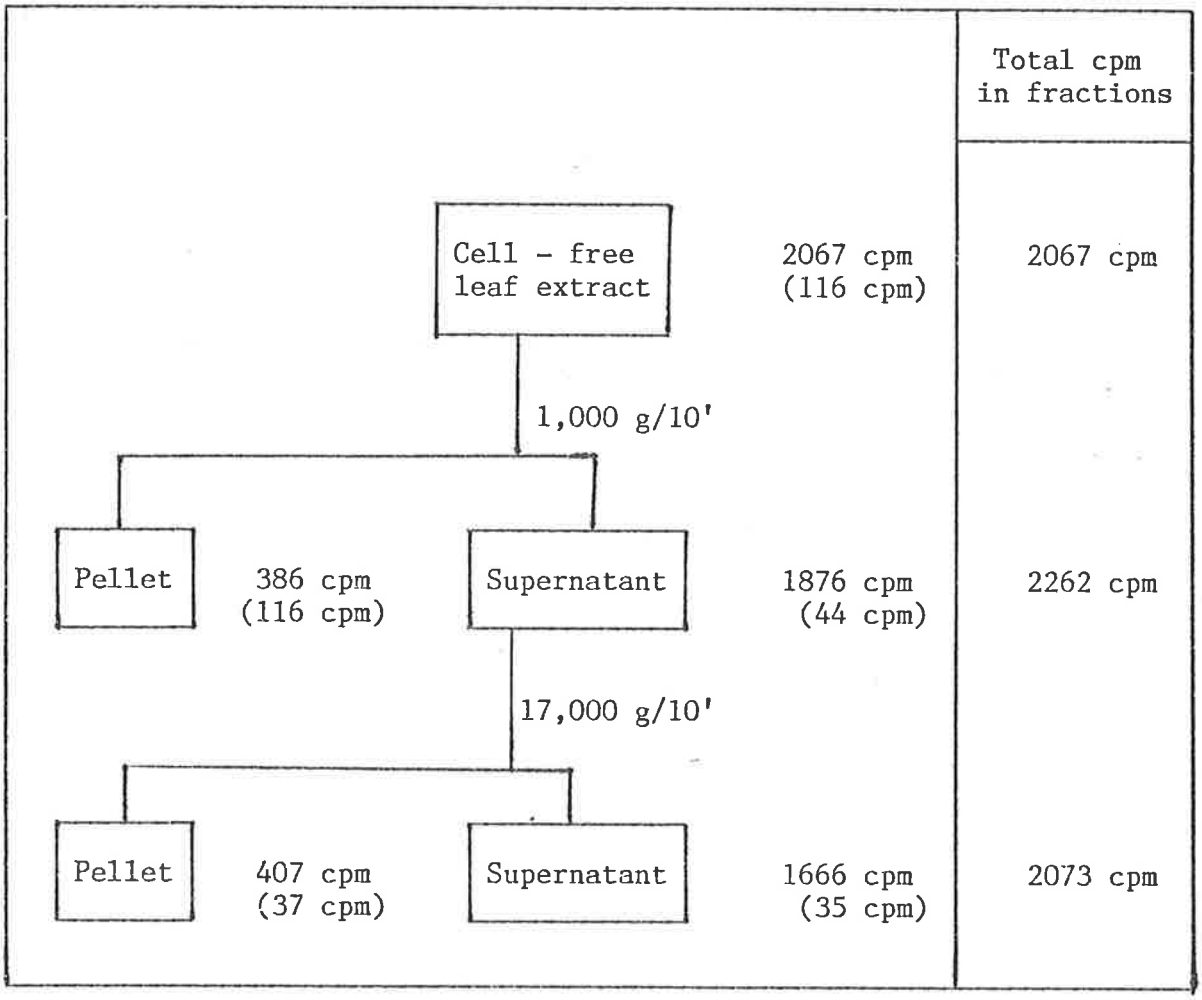
FIG.1

Figure 3.2 : RNA-dependent RNA Polymerase in Centrifugal fractions of Healthy and VTMoV-Infected N. clevelandii Leaf Extracts.

Leaves of N. Clevelandii plants which were either healthy or infected with VTMoV for seven days, were extracted into buffer, filtered through miracloth to obtain cell-free extracts and subjected to differential centrifugation. The centrifugation steps are shown in the diagram opposite with the amount of enzyme activity in each fraction from diseased leaves shown on cpm assayed by the standard method (Chapter 2.2.3). The enzyme activities detected in similar fractions from healthy plants are shown in brackets. The total enzyme activity recovered at each centrifugation step is shown in the column to the right of the flow diagram.

Zero-time treatments were done by adding leaf extract to the assay mixture and stopping any reaction immediately by adding pyrophosphate and TCA solutions. Zero-time values obtained were 38 cpm for healthy tissue treatments and 37 cpm for the diseased tissue treatments. These values have not been subtracted from the enzyme activity values given in the figure.

Figure 3.2


Total cpm in fractions
Cell - free leaf extract
2067 cpm (116 cpm)
2067 cpm
1,000 g/10'
Pellet
386 cpm (116 cpm)
Supernatant
1876 cpm (44 cpm)
2262 cpm
17,000 g/10'
Pellet
407 cpm (37 cpm)
Supernatant
1666 cpm (35 cpm)
2073 cpm

No significant RNA-dependent RNA polymerase activity was detected in healthy extracts under the assay conditions used (Figure 3.2). Most of the trace of polymerase activity present in healthy leaf extracts was pelleted at 1,000 g.

Samples of the S_1 fraction from diseased plants were centrifuged at various forces between 5,000 and 25,000 g for 20 minutes in an attempts to pellet the polymerase activity. Results presented in Table 3.1 indicate that the enzyme-template complex was not sedimented by centrifugation at 25,000 g. The variation in the small amount of enzyme detected in the pellet fractions may be due to imperfect drainage of the supernatant fraction from the centrifuge tubes used in the experiment.

The cytoplasmic location of the enzyme-template complex differs from that of other virus-host combinations reported by other authors (see Chapter 8 for details). To check that the observed location of the VTMoV-induced polymerase-template complex was not an artifact of the extraction procedure used, it was decided to check the location of the RNA-dependent RNA polymerase from leaves of N. clevelandii infected with the U_1 (common strain) tobacco mosaic virus (TMV). Ralph et al. (1971) have reported that TMV induces RNA-dependent RNA polymerase activity which is membrane-associated in tobacco leaves.

Leaves from N. clevelandii plants inoculated three days previously with TMV were fractionated by centrifugation. Extraction buffers used were the same as those for extraction of VTMoV-induced polymerase with and without 10 mM $MgSO_4$. Magnesium was added to one sample of the enzyme extraction buffer because some authors have used magnesium free-buffers to release polymerases from membranes (Zabel et al., 1976).

Table 3.1 : Effects of Centrifugation on the Distribution of the Enzyme-Template Complex in the Supernatant and Pellet.[a]

Centrifugal force (g)	Enzyme Activity (cpm)[b]		Proportion of Activity in pellet (%)
	Supernatant	Pellet	
5,000	685	0	0
10,000	779	97	11
15,000	872	95	10
20,000	712	118	13
25,000	835	0	0

a A crude cell-free extract was obtained from leaves of N. clevelandii infected with VTMoV for seven days and centrifuged at 1000 g for 10 minutes to obtain an S_1 fraction (714 cpm of enzyme activity) and a P_1 fraction (220 cpm of enzyme activity). The S_1 fraction was then centrifuged at the forces indicated for 20 minutes.

b A zero reaction time value of 30 cpm has been subtracted.

When the S_1 fraction from tissue extracted in the presence of magnesium was centrifuged at 17,000 g for 10 minutes, the S_{17} and P_{17} fractions incorporated 202 and 708 cpm of ^{32}P GMP into a TCA precipitable product which represents 22 and 78% of the activity, respectively. Similar S_{17} and P_{17} fractions from tissue extracted in magnesium-free buffer incorporated 311 and 793 cpm which represents 22 and 78% of the activity, respectively. This indicates that the TMV induced polymerase is associated principally with cell membranes as previously reported by Ralph et al. (1971) and that addition of $MgSO_4$ to the extraction buffer has no effect on the distribution of the enzyme activity between the fractions. Thus the recovery of the VTMoV-induced enzyme on the S_{17} fraction does not appear to be an artifact of the methods used but reflects its location in the cytoplasm, unassociated with any cell organelle or membrane system.

3.3 CHARACTERISTICS OF THE VTMoV-INDUCED POLYMERASE ACTIVITY IN THE S_1 FRACTION

3.3.1 Time course of the reaction

Under optimal conditions (see Sections 3.3.2 to 3.3.4) the reaction proceded linearly for approximately 10 minutes and subsequently ceased (Figure 3.1B). The following experiments were done to determine if the amount of ribonucleoside triphosphate added to the reaction mixtures were insufficient to sustain the reaction for more than 10 minutes.

The first experiment involved supplying additional amounts of all four ribonucleoside triphosphates, including labelled GTP, to the reaction mixtures after 10 minutes of incubation when enzyme activity had nearly ceased. This, however, did not result in an increase of

enzyme activity (Table 3.2) indicating that the availability of ribonucleoside triphosphate in the later stages of the reaction was not the limiting factor for RNA synthesis.

The second experiment is summarised in Table 3.3. It was carried out to determine the rate of label incorporation into RNA at various times during the reaction to check if the apparent cessation of enzyme activity was due to a balance between RNA synthesis and degradation.

Treatments 1 to 3 inclusive (Table 3.3), were standard reactions stopped at different times to confirm the normal time course of enzyme activity. In treatments 4 and 5 (Table 3.3), the reactions were started by the addition of enzyme to the reaction mixture in the absence of labelled GTP which was added 5 and 15 minutes later, respectively. The final treatment (Table 3.3) involved the addition of 0.326 µmoles of unlabelled GTP (about 1,200 times that added at commencement) to the reaction when it was essentialy complete. This was to dilute the labelled GTP to such an extent that if the cessation of enzyme activity was due to a balance between RNA synthesis and digestion, no labelled RNA would be synthesized during the final 15 minutes of incubation and there would be an apparent loss in the ^{32}P incorporated into RNA.

Data presented in Table 3.3 (treatments 1 to 5) indicate that the addition of labelled GTP at various times during the reaction did not affect the pattern of ^{32}P incorporation into a TCA precipitable product. In treatment 6 (Table 3.3) no change in the amount of label incorporated into RNA was observed after the addition of unlabelled GTP indicating that no significant degradation of the polymerase product occurred. The cessation of enzyme activity after 10 minutes of reaction time can not,

Table 3.2 : Effect of Adding Ribonucleoside Triphosphates to the Polymerase Reaction 10 minutes after Initiation of Incubation.

Time NTP Added (minutes)	Time Reaction was Stopped (minutes)	^{32}P Incorporation (cpm)[a]
0	10	1277
0	30	1567
0 + 10[b]	30	1519

a A zero reaction time value of 27 cpm has been subtracted.

b In the final treatment the reaction was supplemented with all four nucleotide triphosphate (NTP) including labelled GT_1. The amount added was sufficient to double the concentration of the nucleoside triphosphates present in the initial reaction mixture (100 µM each of ATP, CTP, UTP and 1 µm of GTP).

Table 3.3 : Response of the RNA-dependent RNA Polymerase Reaction to the addition of labelled and unlabelled GTP during incubation.

Treatment	Time ^{32}P-GTP Added (minutes)	Time GTP Added (minutes)	Reaction Time (minutes)	^{32}P Incorporation cpm[a]
1	0	-	5	5484
2	0	-	15	9238
3	0	-	30	10053
4	5	-	30	4538
5	15	-	30	824
6	0	15	30	9346

a A zero reaction time value of 74 cpm has been subtracted.

Treatment 1 to 3 inclusive, established the time-course of the reaction. In treatments 4 and 5 the reaction was started in the normal manner in the absence of ^{32}P GTP and labelled GTP was added at the time indicated. In treatment 6 unlabelled GTP (20 µl of a solution containing 10 mg/ml) was added at the time indicated, so that the labelled GTP would be diluted by unlabelled GTP. The final concentration of GTP in the treatment was 1.208 mM.

therefore, be attributed to a balance between RNA synthesis and degradation. This suggests that enzyme inactivation and/or shortage of template may have been responsible for the termination of RNA synthesis. It is also possible that the reaction was arrested after completion of nascent RNA molecules.

Addition of exogenous template, in the form of purified VTMoV RNA, to the reaction mixtures did not result in the synthesis of more labelled RNA than was observed in reaction mixtures without added RNA. This suggests that the polymerase enzyme was restricted to the native template to which it was bound.

Experiments described in this section indicate that the *in vitro* RNA synthesis by the S_1 fraction from VTMoV-infected *N. clevelandii* leaves, is confined to 10 minutes of incubation, not because of the depletion of substrate or the degradation of the synthesized RNA, but because of the inability of the enzyme to re-initiate RNA synthesis.

3.3.2 Temperature Optimum

Figure 3.1C illustrates the enzymes' activity response to temperature. The incubation time was 15 min. and maximum enzyme activity was observed at approximately 20°C. Such a low temperature optimum is suprising because in other virus-host systems the temperature optima reported for RNA-dependent RNA polymerase activities are much higher.

To explore the temperature responses of RNA-dependent RNA polymerase from other host-virus combinations, several different viruses were inoculated to *N. clevelandii* plants and, tobacco ring spot virus

(TRSV) was inoculated to several different host plant species, so that the temperature response of the polymerases from various sources could be studied.

Tomato ring spot virus (Tom RSV), lucerne transient streak virus (LTSV) and red clover necrotic mosaic virus (RCNMV) were inoculated to leaves of N. clevelandii and the temperature responses of the RNA-dependent RNA polymerase in the cell free extracts were determined (Figure 3.3, upper). The optimum temperature observed for the polymerases from all the above virus-host combinations was about 25°C when the assay time was 10 minutes. However, when TRSV-infected leaves of N. clevelandii were used as a source of polymerase activity, the optimum temperature was 30°C (Figure 3.3, lower). When TRSV-infected cucumber cotyledons were the source of polymerase activity the optimum temperature was also 30°C but the polymerase in TRSV-infected leaves of P. vulgaris showed optimal RNA synthesis at 20°C. A full discussion of these observations and comparisons with data published by other authors are presented in Chapter 8.

3.3.3 Effect of pH

Enzyme activity increased with pH up to pH 8.8 and did not change significantly between pH 8.8 and 9.4, the highest pH tested (Figure 3.1D). 50 mM Tris HCl buffer was used in the treatments and care was taken to ensure that the correct pH was maintained after the addition of the S_1 fraction to the reaction mixture.

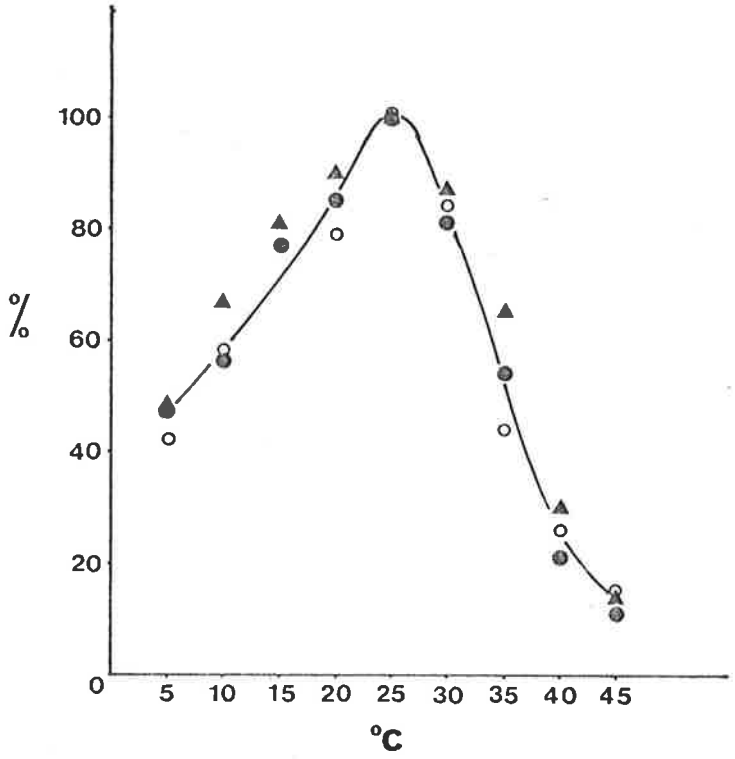
Figure 3.3 : The Temperature Response of RNA-Dependent RNA Polymerases from Various Host-Virus Combinations.

Upper : The S_1 fraction was prepared from N. clevelandii leaves infected with various viruses and the polymerase activity in that fraction was assayed at various temperatures for 10 minutes. The maximum level of enzyme activity observed was taken as 100%. Viruses used for in these experiments and the maximum levels of enzyme activity were as follows:

Lucerne transient streak virus (LTSV)	1531 cpm	●-●
Tomato ring spot virus (Tom RSV)	596 cpm	▲-▲
Red clover necrotic mosaic virus (RSNMV)	1110 cpm	O-O

Lower : Tobacco ring spot virus (TRSV) was inoculated to three different host plants and the temperature response of the polymerase activity in the S_1 fraction was determined. The assay time was 10 minutes and the maximum level of enzyme activity observed in each treatment was taken as 100%. The host plants used for these experiments and the levels of maximum enzyme activity were as follows.

N. clevelandii	2642 cpm	●-●
P. vulgaris	944 cpm	▲-▲
Cucumber cotyledons	669 cpm	O-O


100
80
60
40
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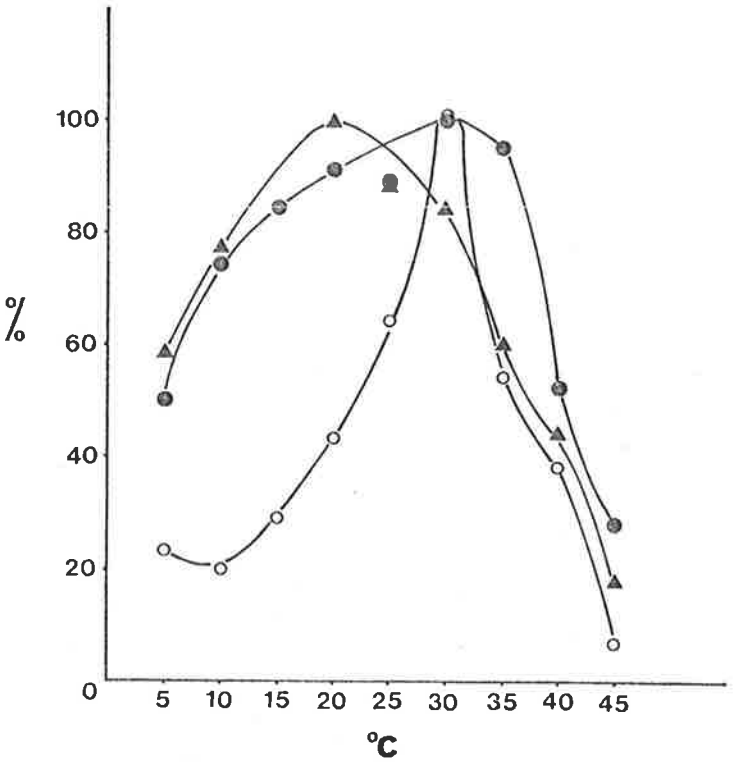
0
%
5 10 15 20 25 30 35 40 45
°C


100
80
60
40
20
0
%
5 10 15 20 25 30 35 40 45
°C

3.3.4 Effect of Cations and Chelating Agents

The monovalent cations K^+ and NH_4^+ added as chlorides had only a slight, if any, stimulatory effect on the polymerase activity at a concentration of about 5 mM (Figure 3.4A). The presence of EGTA had no effect on the response of the enzyme to K^+ (Figure 3.4B).

The enzyme had an absolute requirement for divalent cations (Figure 3.4C). In the absence of chelating agents optimal activity was observed at between 14 and 20 mM Mg^{++} and this requirement could be replaced by Mn^{++} at a level of 2 mM. Concentrations of Mn^{++} greater than 2 mM were inhibitory (Figure 3.4C).

Chelating agents had a marked effect on enzyme activity (Figure 3.4D). Both EGTA and EDTA stimulated the polymerase activity having an optimum effect between 8 and 13 mM in the presence of 14 mM $MgSO_4$, but EGTA was more effective than EDTA (Figure 3.4D). Moreover, at higher concentrations, EDTA inhibited enzyme activity whereas EGTA did not. The inhibitory effect of EDTA was probably due to the chelation of Mg^{++}.

In the presence of EGTA, the enzymes' response to different levels of Mg^{++} and Mn^{++} was altered. Both the Mg^{++} and Mn^{++} response curves show sharp optima at concentrations of 4 mM (Figure 3.4c).

Enzyme activity observed without any added Mg^{++} in the presence of EGTA and EDTA, was probably due to the presence of Mg^{++} in the S_1 fraction of the leaf extracts because no such activity was observed when partially purified enzyme-template complex was assayed (see Chapter 5).

Figure 3.4

A. Effect of Monovalent Cations on the Synthesis of RNA by the RNA-Dependent RNA Polymerase.

Reaction mixtures consisting of 50 µl of the S_1 enzyme fraction and 200 µl of assay mixture containing various levels of monovalent cations were incubated at 20°C for 10 minutes before the reactions were stopped and incorporation of ^{32}P onto TCA precipitable product measured. Both cations were used as the chloride form.

B. Effect of EGTA on the Polymerase Response to K^+.

A similar experiment to that outlined above was carried out, however, the assay mixture contained 8 mM EGTA.

C. Effect of Divalent Cations on the Synthesis of RNA by the RNA-Dependent RNA Polymerase.

The response of the polymerase enzyme to various levels of Mg^{++} and Mn^{++} in the presence and absence of 8 mM EGTA was observed. The S_1 fraction from VTMoV-infected leaves of N. clevelandii was used as a source of enzyme and assay conditions were as described in Chapter 2.

D. Effects of Chelating Agents on the Synthesis of RNA by the RNA-Dependent RNA Polymerase.

Reaction mixtures contained various levels of EGTA or EDTA and 14 mM $MgSO_4$. Reactions were stopped after 10 minutes and incorporation of ^{32}P into TCA precipitable product measured.

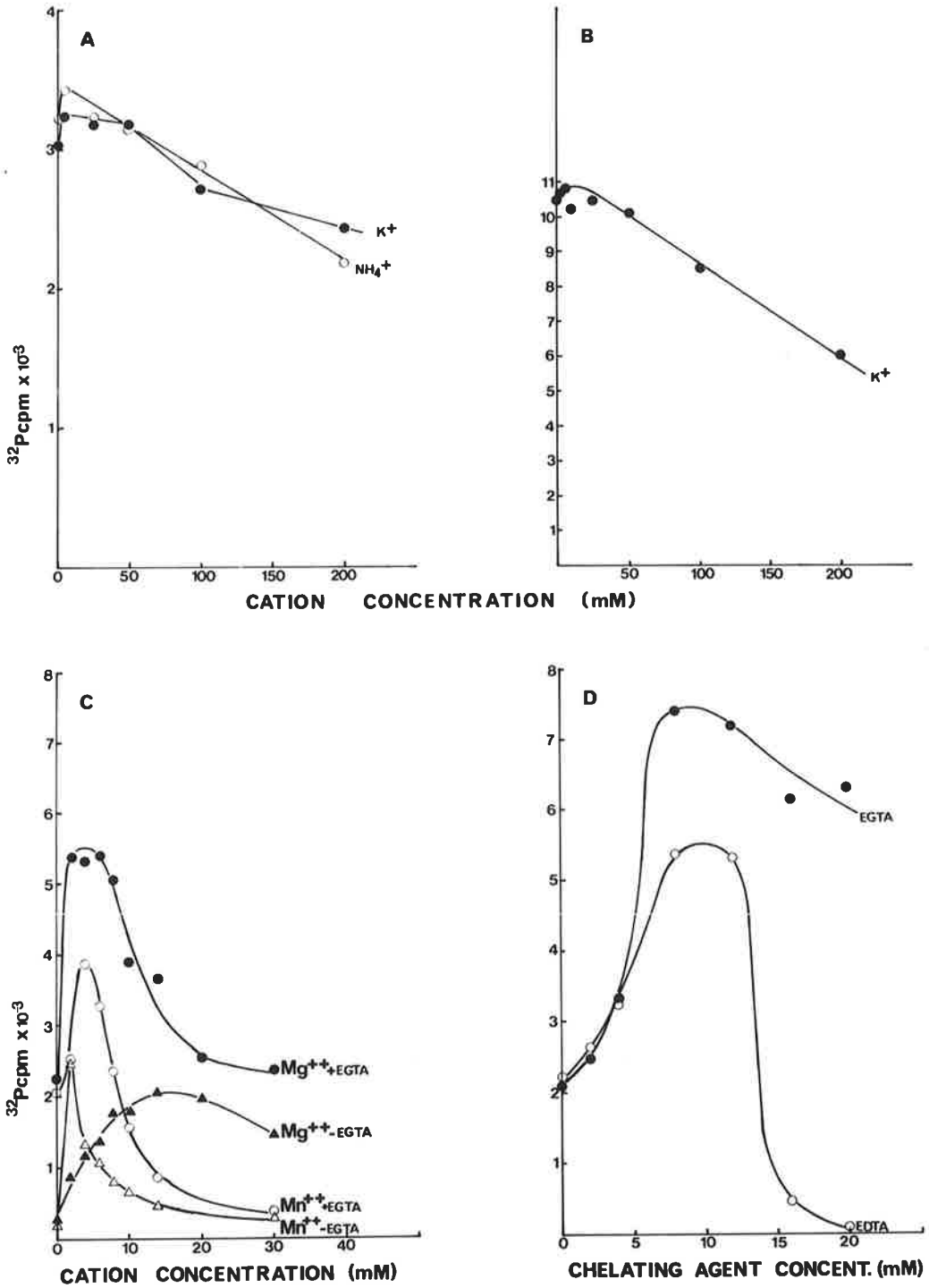

A
B
C
D
^{32}Pcpm x 10^{3}
K+
NH4+
CATION CONCENTRATION (mM)
Mg++ +EGTA
Mg++ -EGTA
Mn++ +EGTA
Mn++ -EGTA
EGTA
CHELATING AGENT CONCENT. (mM)

FIG. 3

3.3.5 Dependencies

The omission of all three unlabelled ribonucleoside triphosphates reduced enzyme activity by about 90% (Table 3.4) indicating an absence of significant amounts of terminal guanosyl transferase activity in the enzyme extract. The minimal effect of orthophosphate precludes the presence of significant amounts of polynucleotide phosphorylase activity in the extracts (Gilliland and Symons, 1968).

Absence of actinomycin D in the assay mixture or addition of DNase or Rifampicin to it, had no significant effect on the incorporation of labelled GMP into TCA insoluble product indicating that the enzyme was RNA-dependent. The partial resistance of the RNA synthesis to pancreatic RNase A is thought to be due to the double-stranded nature of both the template and product. Pyrophosphate strongly inhibited enzyme activity and can be classed as a product inhibitor as shown in Chapter 7.

The effect of various levels of actinomycin D on the polymerase activity in the unfractionated cell-free extracts of healthy and diseased leaves was also studied. Actinomycin D is an inhibitor of DNA-directed RNA synthesis (Hayman and Davidson, 1970). In the case of extracts from healthy tissues, actinomycin D significantly reduced the amount of labelled GMP incorporation into a TCA insoluble product (Table 3.5). The slight resistance of the polymerase activity observed in the extract from healthy leaves is probably due to the nuclear membrane preventing the actinomycin D from reaching the DNA dependent RNA polymerase present in the nucleus. With extracts from diseased leaves, the presence of actinomycin D reduced the observed enzyme activity by only approximately 17% at all levels of the antibiotic used. At least some of

Table 3.4 : Properties of the VRMoV-Specific RNA Polymerase From *Nicotiana Clevelandii* Leaves.

Reaction mixture	Enzyme activity (% of complete reaction mixture)		
	Experiment 1	Experiment 2	Experiment 3
Complete	100(3723 cpm)[a]	100(2610 cpm)	100(2840 cpm)
-ATP	78	62	-
-UTP	80	47	-
-CTP	74	61	-
-(ATP, UTP, CTP)	12	9	-
-ACTINOMYCIN D	96	109	-
+DNase 10 µg/ml	96	105	-
+RNase 10 µg/ml	62	53	-
+Rifampicin 50 µg/ml	92	-	-
+Yeast RNA 1 mg/ml	119	112	-
-EGTA	-[b]	34	-
-Extract	-	0	-
+Orthophosphate (10 mM)	-	-	84
+Pyrophosphate (10 mM)	-	-	10

a Experiment 1 was conducted without EGTA in the assay mixture. Experiments 2 and 3 were conducted with 10 mM EGTA in the assay mixture.

b Not tested.

Table 3.5 : Effect of Actinomycin D on the Enzyme Activity in Extracts from Healthy and Diseased Leaves of N. celvelandii.

Actinomycin D per assay (250 µl) µg	Enzyme Activity (cpm)[a]			
	Healthy	% of untreated	Diseased	% of untreated
0	265	100	2552	100
2.5	140	52	2115	83
5	110	41	2285	90
7.5	86	32	2102	82

a A zero reaction time value of 53 cpm for the healthy and 40 cpm for the diseased treatments has been subtracted.

Cell free extracts from healthy and VTMoV-infected leaves were used in this experiment. The volume of extract used was 50 µl which was added to 200 µl of enzyme assay mixture. Assay conditions were those outlined in Chapter 2, however, the amount of Actinomycin D per assay was varied as indicated.

the actinomycin-sensitive activity may be due to DNA-dependent RNA polymerase in the nuclei and chloroplasts being inhibited.

3.3 RESPONSE TO α-AMANITIN

Rackwitz et al. (1981) have demonstrated that DNA dependent RNA polymerase II from tomato and wheat germ can transcribe potato spindle tuber viroid (PSTV) RNA in vitro. Because VTMoV encapsidates a viroid-like RNA the possibility that its synthesis could be sensitive to α-amanitin was tested over a range of concentrations between 10^{-10} and 10^{-3} M. DNA-dependent RNA polymerase II is inhibited by α-amanitin at a concentration of 10^{-8} M and polymerase III at 10^{-5} M (Rackwitz et al., 1981). No significant reduction in labelled GMP incorporation into TCA insoluble products was detected below a concentration of 10^{-4} M (Table 3.6) indicating that no significant polymerase activity in the S_1 fraction could be ascribed to either RNA polymerase II or III.

3.4 CONCLUSIONS

RNA-dependent RNA polymerase activity was present in VTMoV-infected leaves of N.clevelandii four days after inoculation and this activity increased over the subsequent four days until the experiment was terminated due to the infected leaves collapsing. No polymerase activity could be detected in healthy leaves. This RNA-dependent RNA polymerase activity appears to be located in the cytoplasm of VTMoV-infected leaves.

The RNA-dependent RNA polymerase activity proceeded in vitro for 10 minutes after which little if any incorporation of ^{32}P into RNA could be

Table 3.6 : The Effect of α-Amanitin on the Activity of the RNA-Dependent RNA Polymerase.

Concentration of α-Amanitin (M)	Product formed (cpm)[a]	% of Control
0	2840	100
10^{-3}	2514	88.5
10^{-4}	2547	89.7
10^{-5}	2727	96.0
10^{-6}	2607	91.8
10^{-7}	2638	92.9
10^{-8}	2760	97.2
10^{-9}	2701	95.1
10^{-10}	2800	98.6

a A zero reaction time value of 75 cpm has been subtracted.

The S_1 fraction from leaves of N. clevelandii infected with VTMoV for seven days was used as a source of polymerase. The assay mixture and conditions are outlined in Chapter 2, however, no actinomycin D was added.

detected. This cessation of enzyme activity was found not to be due to depletion of substrate or the degradation of synthesised RNA.

Magnesium was found to be essential for RNA synthesis by the enzyme and both the chelating agents EDTA and EGTA stimulated enzyme activity, suggesting that inhibitory cations may be present in the crude enzyme extracts. The magnesium requirement of the enzyme could be replaced by manganese. All four ribonucleoside triphosphates were required for in vitro RNA synthesis indicating that RNA containing all four nucleotides was synthesised. Inhibitors of DNA-dependent RNA polymerases or DNA-dependent DNA polymerases had little effect on the polymerase activity found in VTMoV-infected leaf tissue. This suggests that the in vitro RNA synthesis was entirely RNA dependent.

CHAPTER 4

PARTIAL PURIFICATION OF RNA-DEPENDENT RNA POLYMERASE-TEMPLATE COMPLEX FROM VTMoV-INFECTED PLANTS

The purification of the virus induced RNA-dependent RNA polymerase bound to native RNA template from infected plants was undertaken so that the characteristics of the purified complex could be studied. This work was an essential prelude to enzyme kinetic work and investigation into the enzyme's mechanism of action.

4.1 PURIFICATION PROCEDURE

The purification scheme is outlined in Figure 4.1. Initial purification was obtained by differential centrifugation as outlined in Chapter 3. Centrifugation at 1,000 g removed the nuclei and chloroplasts whereas the bulk of the enzyme activity (about 86%) remained in the supernatant (S_1) fraction (Table 4.1). Subsequent centrifugation of the S_1 fraction at 18,000 g was used to remove mitochondria and fragments of large organelles (Francki and Peters, 1978), after which about 92% of the enzyme activity still remained in the supernatant (S_{18}) (Table 4.1). When the initial cell-free extracts were centrifuged at 18,000 g without previous sedimentation at 1,000 g, the S_{18} fraction was greener, indicating that chloroplasts had been damaged and had released some of their contents into solution.

To precipitate the enzyme-template complex, the S_{18} fraction was made up to 10% w/v polyethylene glycol (6,000) (PEG) and 0.6 M NH_4Cl. This mixture was stirred at 0°C for 10 minutes with a magnetic stirrer

Figure 4.1 : Summary of the Purification Procedure for the VTMoV-Induced RNA-Dependent RNA Polymerase-Template Complex from VTMoV-Infected Leaves of N. Clevelandii.

Details of enzyme activities and protein content of the various fractions are given in Table 4.1. Cell-free leaf extract was obtained by grinding VTMoV-infected leaves in buffer containing 0.4 M sucrose, 0.1 M NH_4Cl and 0.1 M Tris-HCl, pH 8.8, and filtration through Miracloth.

[a]Dry polyethylene glycol (PEG) 6,000, NH_4Cl and a 10% Triton X100 solution were added to the S_{18} fraction to give final concentrations indicated in the figure.

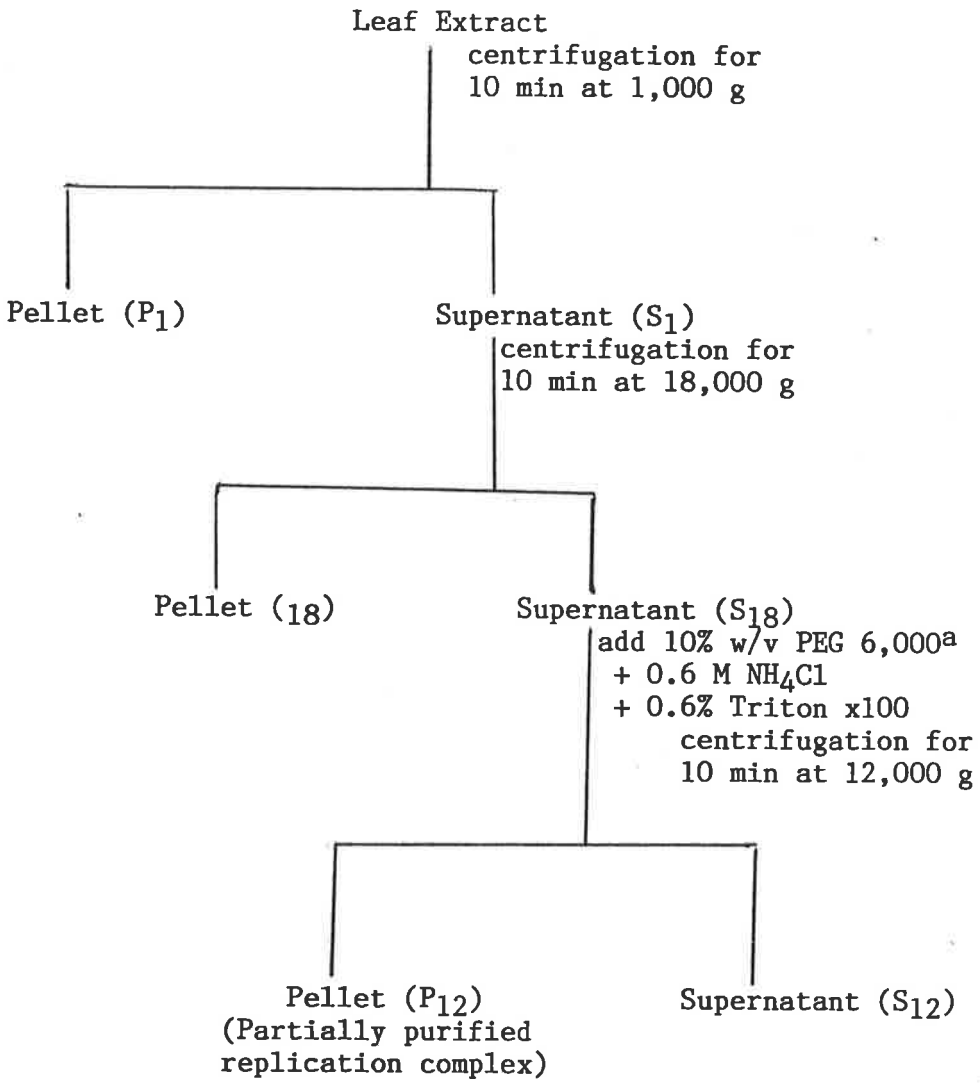

Leaf Extract
centrifugation for
10 min at 1,000 g
Pellet (P_1)
Supernatant (S_1)
centrifugation for
10 min at 18,000 g
Pellet ($_{18}$)
Supernatant (S_{18})
add 10% w/v PEG 6,000[a]
+ 0.6 M NH_4Cl
+ 0.6% Triton x100
centrifugation for
10 min at 12,000 g
Pellet (P_{12})
(Partially purified
replication complex)
Supernatant (S_{12})

Table 4.1 : RNA-Dependent RNA Polymerase Activity of the Various Fractions From VTMoV-Infected *N. clevelandii* Leaf Extracts.[a]

Step No.	Fraction	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Total Activity[b] (units)	Specific Activity (units/mg Protein)
1	Diseased leaf extract	67 (62)[c]	3.9	261 (242)	10200 (9440)	39.0
2	S_1	60 (55)	2.6	156 (143)	6600 (6050)	42.3
3	P_1	30	2.3	69	1070	15.5
4	S_{18}	55 (50)	2.3	127 (115)	5050 (4590)	39.7
5	P_{18}	20	0.7	14	420	30.0
6	S_{12}	58	0.7	41	1010	24.6
7	P_{12}	10	2.0	20	5720	286.0
1a	Healthy leaf extract	14 (10)	4.9	68 (49)	37 (26.5)	
2a	S_1	10	4.2	42	18	
3a	P_1	5	2.0	10	0	

[a] Refer to Figure 4.1 for treatments given and fraction designation.

[b] Units of enzyme activity are pico-moles GMP incorporated into TCA insoluble product/10 minutes at 20°C.

[c] The figure in brackets shows the proportion of the sample used for further treatment.

and 10% Triton X100 was added to a final concentration of 0.6%. After centrifugation at 12,000 g, 80% of the enzyme activity was recovered in the pellet (P_{12}) fraction (Table 4.1). Triton X100 was required to solubilize membrane fragments which in the absence of the detergent co-precipitated with the enzyme-template complex. Addition of Triton X100 to the S_{18} fraction before PEG precipitation resulted in poor precipitate formation and the subsequent P_{12} fraction was difficult to resuspend.

The partially purified enzyme-template complex in the P_{12} fraction was resuspended in 20 mM Tris-HCl buffer, pH 7.0, and stored at -70°C. A gradual loss of enzyme activity was observed during storage over several weeks. If glycerol was added to a final concentration of either 10 or 50% before freezing, the enzyme preparations lost activity at an increased rate. A similar result was obtained with the use of 10% dimethyl sulphoxide as a cryogenic agent. Both glycerol and dimethyl sulphoxide have been used to preserve frozen enzymes (Charm and Matteo, 1971).

The purification procedure outlined above, resulted in nearly 72% recovery of the enzyme activity present in the initial leaf extract and an eight-fold purification was achieved (Table 4.1). When required, it was stored at -70°C although prolonged storage was avoided due to the slow loss of activity.

4.2 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE PARTIALLY PURIFIED ENZYME PREPARATION

To follow the progress of the purification procedure in addition to analysis of total protein (Table 4.1), the proteins in the preparation

at various stages of purification were analysed by polyacrylamide gel electrophoresis (Figures 4.2 and 4.3). There was no obvious difference between extracts from healthy and diseased plants (Figure 4.2, tracks 1 and 2, Figure 4.3, tracks 3,4,6 and 7) which could have been attributed to the polymerase protein. The purification procedure used resulted in the partitioning of protein from the enzyme in a non-specific manner but with little loss of enzyme activity. This is illustrated in Figure 4.3 where extracts from healthy and diseased plants and the P_{12} fractions from them, are compared directly. It is interesting to note that even on the seventh day after inoculation, when the tissues were extracted, coat protein was not detected in preparations from the diseased leaves although the two viroid-like RNAs were abundant (Figures 4.4 and 4.5). The protein shells of VTMoV consist of subunits with M_r about 37,000 which are degraded to proteins of M_r about 33,000 and 32,000 by plant proteases during purification of the virus (Chu and Francki, 1983).

4.3 ELECTROPHORETIC ANALYSIS OF THE PRODUCTS SYNTHESISED BY THE PARTIALLY PURIFIED ENZYME-TEMPLATE COMPLEX

Polyacrylamide gel electrophoresis was used to analyse the *in vitro* products synthesised by the enzyme-template complex at various stages of its purification. There were two labelled RNAs, A and B synthesised *in vitro* (Figure 4.4 where the upper portion of the figure shows the gel stained to show the nucleic acids present in the preparation and the lower portion, is the autoradiograph of the dried gel). Both the products A and B were found to be synthesised by the enzyme-template complex after every step of the purification procedure (Figure 4.4). This indicates that the RNA synthesising characteristics of the enzyme-template complex in the P_{12} fraction were similar to those of the complex in cell-free extracts from VTMoV-infected leaves of

Figure 4.2 : Analysis by Polyacrylamide Gel Electrophoresis of the Protein Fractions Obtained during Purification of the Polymerase-Template Complex.

Track:

1. Extract from healthy leaves.
2. Extract from diseased leaves.
3. 1000 g Supernatant (S_1) fraction from healthy leaves.
4. 1000 g Supernatant (S_1) fraction from diseased leaves.
5. 1000 g Pellet (P_1) fraction from healthy leaves.
6. 1000 g Pellet (P_1) fraction from diseased leaves.
7. 18000 g Supernatant (S_{18}) fraction from diseased leaves.
8. 18000 g Pellet (P_{18}) fraction from diseased leaves.
9. 12000 g Supernatant (S_{12}) fraction from diseased leaves.
10. 12000 g Pellet (P_{12}) fraction from diseased leaves.
11. Protein molecular weight markers.

Details of the purification procedure are given in the text, Figures 4.1 and Table 4.1. Electrophoretic conditions are outlined in Chapter 2 and the protein samples were from the experiment summarised in Table 4.1. A volume of 10 µl of each fraction was loaded on to the gel. Molecular weight markers were :

a. Bovine serum albumin (68,000)
b. Glutamic dehydrogenase (53,000)
c. Ovalbumin (43,000)
d. Carbonic dehydrogenase (29,000) and
e. Myoglobin (17,200)

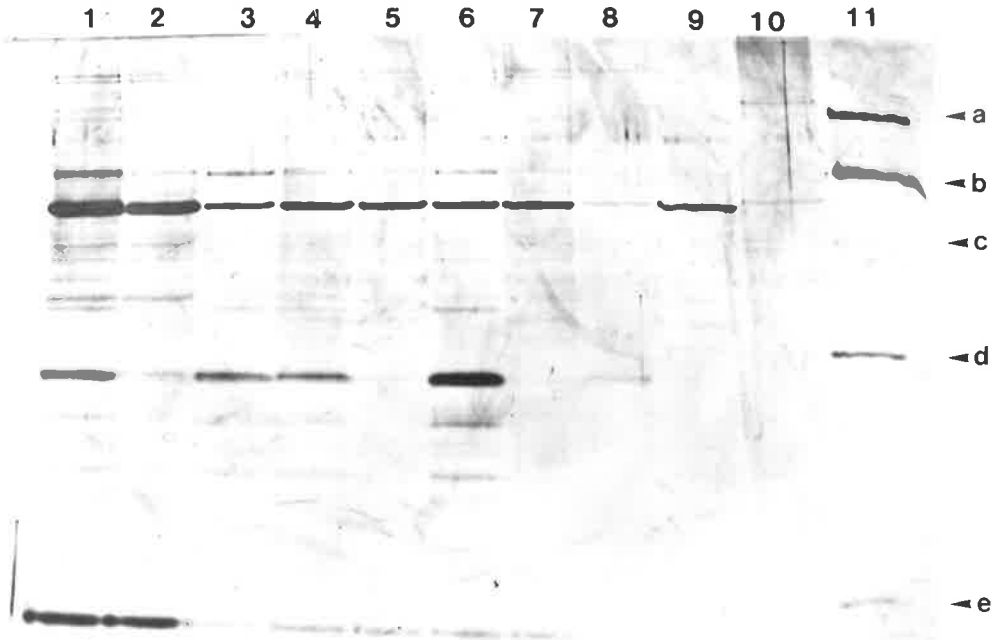

1
2
3
4
5
6
7
8
9
10
11
a
b
c
d
e

Figure 4.3 : Analysis by Polyacrylamide Gel Electrophoresis of Protein Fractions from Various Steps of Enzyme-Template Complex Purification from leaves of N. clevelandii which were either Healthy or Infected with VTMoV for Seven Days.

Track:

1 and 9	Protein molecular weight markers
2, 5 and 8	VTMoV coat protein
3	1000 g Supernatant (S_1) from healthy leaves
4	1000 g Supernatant (S_1) from diseased leaves
6	1200 g pellet (P_{12}) from healthy leaves
7	12000 g pellet (P_{12}) from diseased leaves

Details of the fractionation of the extracts are outlined in Figure 4.1 and Table 4.1. Details of the electrophoretic conditions and staining techniques are outlined in Chapter 2.

The molecular weight markers were :

a. Phosphorylase b (94,000)
b. Bovine serum albumin (68,000)
c. Glutamic dehydrogenase (53,000)
d. Ovalbumin (43,000)
e. BBMV coat protein (20,000)
f. Pancreatic ribonuclease A (13,700) and
g. Insulin (5,700)

The positions expected for the coat proteins isolated by Chu et al. (1983) are indicated by the unlabelled bars. The undegraded coat protein has a M_r of 37,000 and the degraded, 33,000 and 32,000.

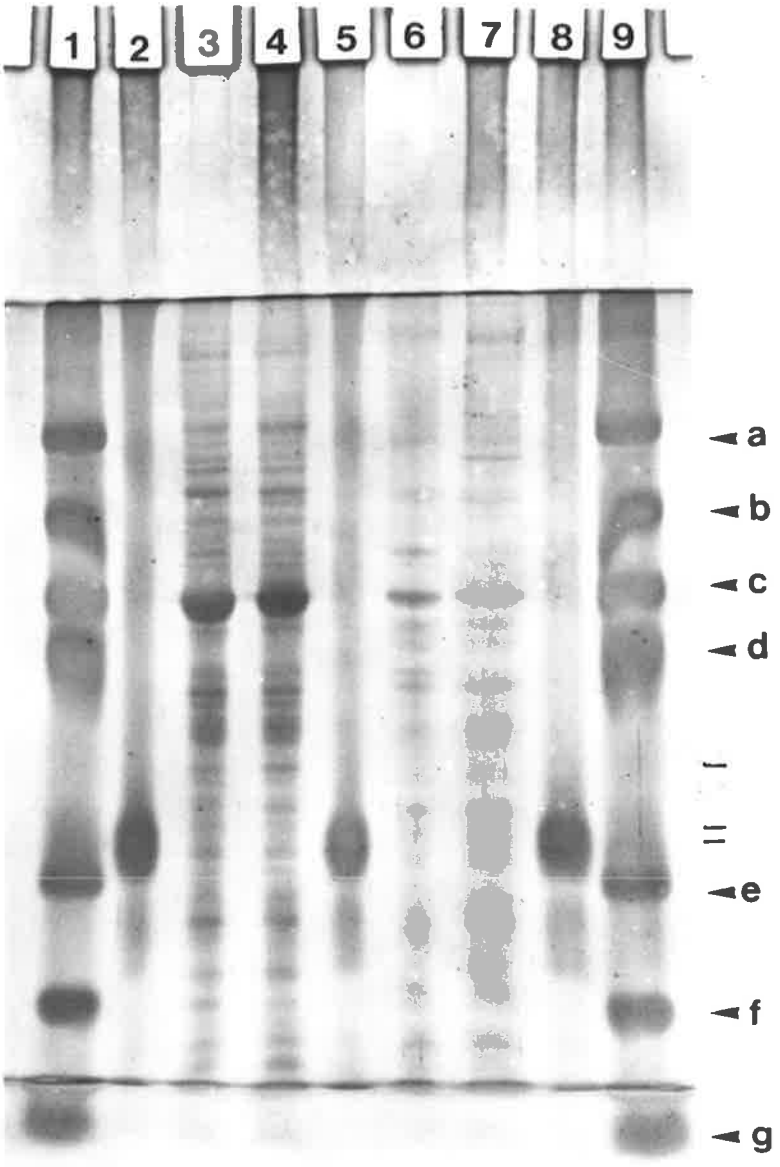

1
2
3
4
5
6
7
8
9
a
b
c
d
e
f
g

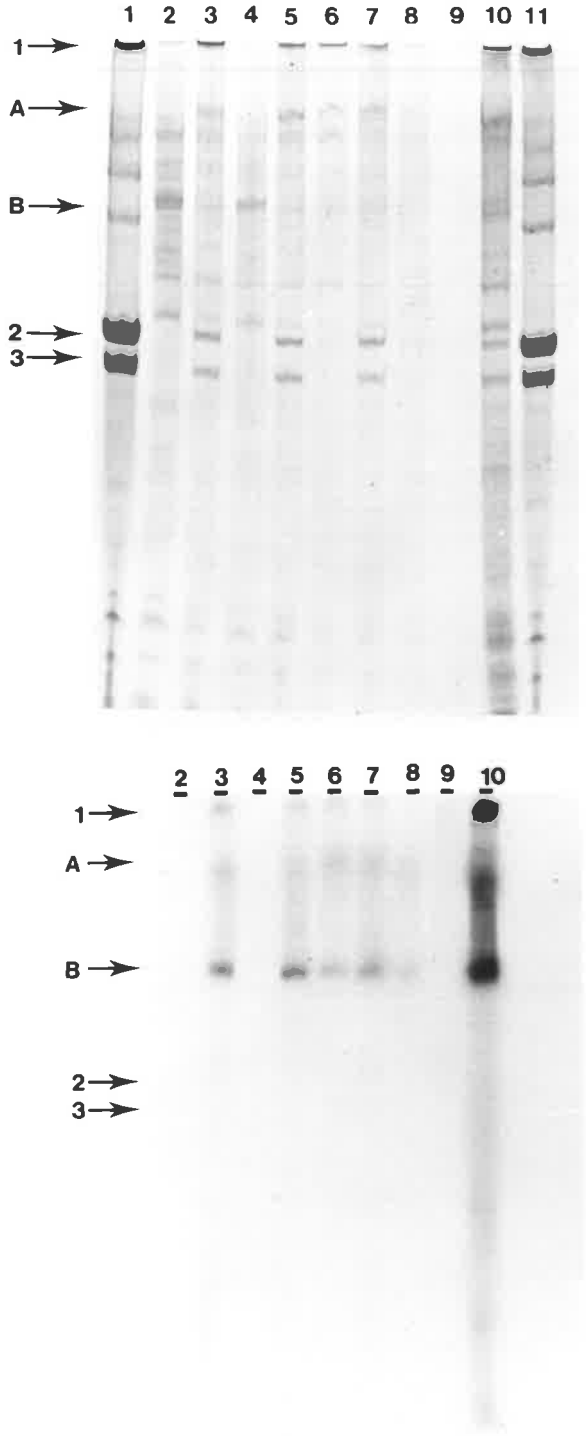
Figure 4.4 : Polyacrylamide Gel Electrophoresis of the RNA Products of the Polymerase-Template Complex at Various Stages of Purification.

The gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1 and 11	VTMoV RNA marker
2	Extract from healthy tissue
3	Extract from diseased tissue
4	S_1 fraction from healthy tissue
5	S_1 fraction from diseased tissue
6	P_1 fraction from diseased tissue
7	S_{18} fraction from diseased tissue
8	P_{18} fraction from diseased tissue
9	S_{12} fraction from diseased tissue
10	P_{12} fraction from diseased tissue

Details of the fractionation of the RNA-dependent RNA polymerase-


1 2 3 4 5 6 7 8 9 10 11
1
A
B
2
3
2 3 4 5 6 7 8 9 10
1
A
B
2
3

N. clevelandii. No ^{32}P-labelled nucleic acids were detected in samples from healthy plants (Figure 4.4, tracks 2 and 4).

Figure 4.5 illustrates a direct comparison between the nucleic acids and in vitro products synthesised by the S_1 and P_{12} fractions from healthy and VTMoV-infected leaves. In the stained gel, VTMoV RNAs 1, 2 and 3 were present in the samples from diseased tissue (Figure 4.5 upper panel, tracks 3 and 4) and the autoradiograph of the gels shows that the in vitro products A and B were present in both the S_1 and P_{12} samples from infected leaves (Figure 4.5 lower panel, tracks 3 and 4). Preparations from healthy plants did not synthesise any nucleic acids which was consistent with the lack of observed polymerase activity in such fractions.

A sample of dsRNA isolated from VTMoV-infected plants of N. clevelandii by Chu et al. (1983) was also loaded onto the gel (track 4, Figure 4.5). This dsRNA consisted of two RNA species of M_r about 3.5 and 2.8 x 10^6 which migrated as a single band with similar mobility to that of the in vitro synthesised RNA A under the electrophoretic conditions used (Figure 4.5). Further comparisons between the dsRNA isolated by Chu et al. (1983) from VTMoV-infected N. clevelandii leaves and the in vitro synthesised RNA products are described in Chapter 6.

Figure 4.5 : Polyacrylamide Gel Electrophoresis Analyses of the Products of Polymerase Activity in Preparation From Healthy and Diseased Plant Extracts.

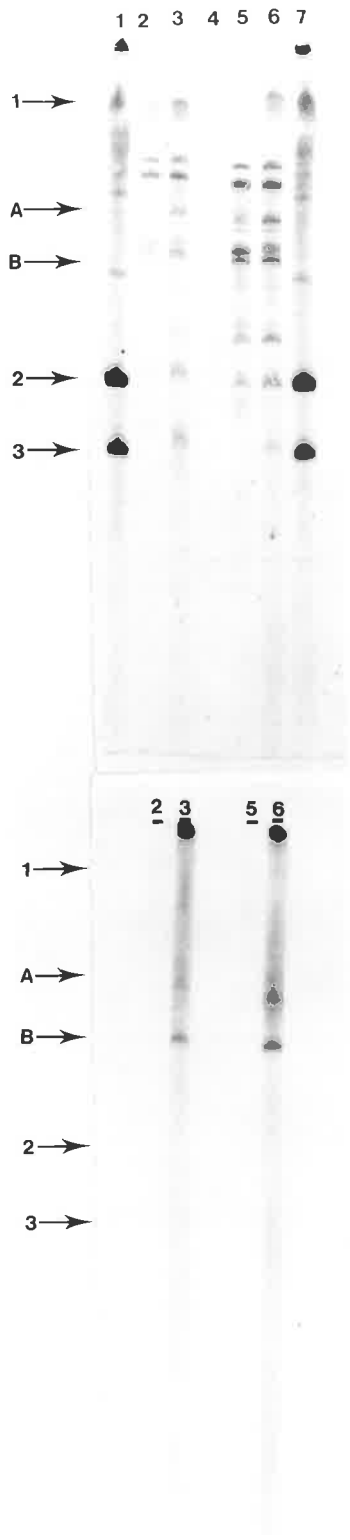
The gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1 and 7	VTMoV RNA
2	1,000 g supernatant (S_1) fraction from healthy leaves
3	1,000 g supernatant (S_1) fraction from diseased leaves
4	dsRNA isolated by Chu et al. (1983)
5	12,000 g pellet (P_{12}) fraction from healthy leaves
6	12,000 g pellet (P_{12}) fraction from diseased leaves

The fractions shown in Figure 4.3 were used as a source of enzyme to synthesise labelled RNA.

The VTMoV RNAs are labelled 1, 2 and 3 and the polymerase products which were detected by autoradiography are labelled A and B. The dsRNA in track 4 did not stain heavily and cannot be clearly seen in the photograph, however, it migrated at about the same rate as product A. Degradation of RNA 1 (Tracks 1 and 7) is probably due to storage of the RNA.


1 2 3 4 5 6 7
1
A
B
2
3
2 3 5 6
1
A
B
2
3

4.4 ATTEMPTS AT FURTHER PURIFICATION OF THE ENZYME-TEMPLATE COMPLEX

4.4.1 Use of ammonium and sodium sulphates

Attempts to use ammonium sulphate or sodium sulphate to precipitate the enzyme complex resulted in complete loss of enzyme activity. Ammonium sulphate has been successfully used by Clark et al. (1974) to purify template-free CMV-induced RNA polymerase from cucumber cotyledons. It seems possible that high salt concentrations caused disassociation of the enzyme-template complex.

4.4.2 Use of protamine sulphate and streptomycin sulphate

Protamine sulphate and streptomycin sulphate are sometimes used to remove nucleic acids from enzyme preparations by precipitation (Warburg and Christian, 1939 ; Little, 1967). Since the RNA-dependent RNA polymerase under investigation was bound to an RNA template, it was decided to investigate the possibility of purifying the enzyme by co-precipitating it with nucleic acids using protamine sulphate or streptomycin sulphate.

Tables 4.2 and 4.3 show the results obtained when the two compounds were used to precipitate the enzyme-template complex. The P_{12} fraction was used as the source of enzyme in these experiments. An apparent increase in enzyme activity after protamine sulphate precipitation was observed and was perhaps due to the removal of inhibitors of the polymerase.

Table 4.2 : Precipitation of The VTMoV-Induced Enzyme-Template Complex with Protamine Sulphate and Streptomycin Sulphate.

Precipitant mg/ml	Enzyme activity in precipitate (cpm)	
	Protamine sulphate Precipitated	Streptomycin sulphate Precipitated
1	3170	1805
2	3940	2562
4	4233	2515
8	4397	2066
12	4277	2133

In each treatment 50 µl of the partially purified replication complex (P_{12} fraction) was added to 450 µl of solution, containing the required amount of streptomycin sulphate or protamine sulphate, mixed and centrifuged at 3,600 g for 10 minutes. The supernatant was discarded and pellets resuspended in 50 µl of Tris-HCl buffer, pH 8.8, and assayed for enzyme activity. 50 µl of untreated P_{12} fraction contained the equivalent of 1760 cpm of enzyme activity.

Table 4.3 : Precipitation of the VTMoV-Induced Enzyme-Template Complex with Protamine Sulphate

Treatment		Enzyme Activity (cpm)	
			% Recovery
No treatment		2836	100
Centrifuged @ 12,000 g for 5 min	Pellet	160	5.6
	Supernatant	1832	65
8 mg/ml Protamine sulphate centrifuged @ 3,600 g for 10 min	Pellet	4047	143
	Supernatant	192	6.8

The experiment was carried out to confirm the precipitation of enzyme by protamine sulphate. Polyethylene glycol precipitated enzyme (P_{12} fraction) was used in this experiment.

Figure 4.6 : Non-Denaturing Gel Electrophoresis of the *in vitro* Products of the RNA-Dependent RNA Polymerase obtained by Various Purification Procedures.

The gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1 and 5	VTMoV RNA marker
2	*in vitro* product from the S_1 fraction
3	*in vitro* product from the P_{12} fraction
4	*in vitro* product from the protamine sulphate precipitated enzyme complex

The two *in vitro* products are indicated by A and B and the VTMoV RNAs are labelled 1, 2 and 3. The diagonal band below RNA A which can be seen in tracks 2 and 3 of the autoradiograph is due to a crack in the gel which occurred during the drying process and is not due to radioactivity.

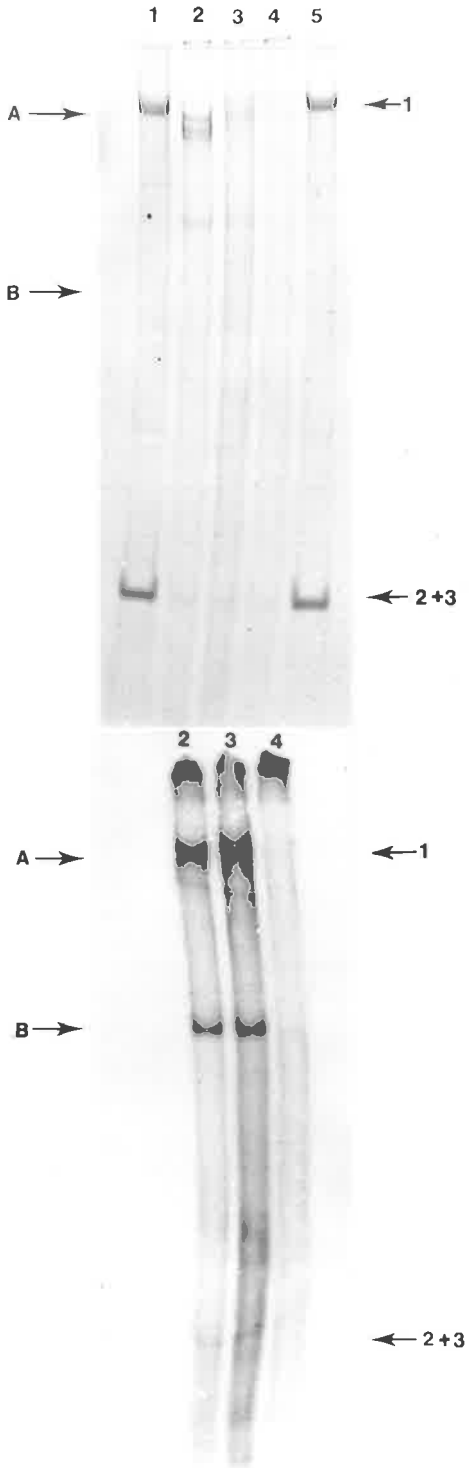

1 2 3 4 5
A
B
1
2+3
2 3 4
A
B
1
2+3

Figure 4.7 : Denaturing Gel Electrophoresis of the *in vitro* products of the RNA-Dependent RNA Polymerase Obtained by Various Purification Procedures.

The urea containing gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

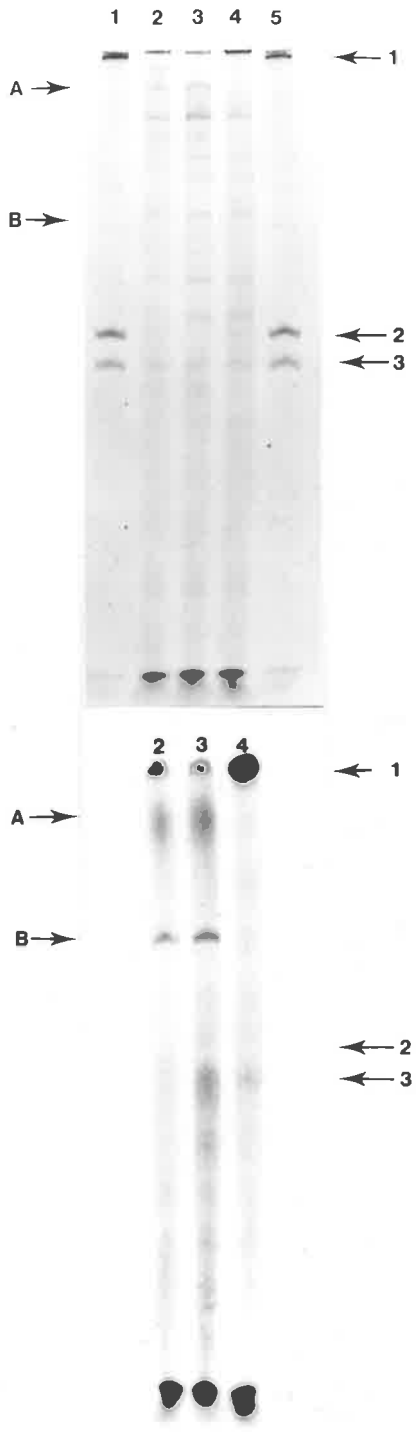
1 and 5 VTMoV RNA marker

2 *in vitro* product from the S_1 fraction

3 *in vitro* product from the P_{12} fraction

4 *in vitro* product from protamine sulphate precipitated enzyme.

The two *in vitro* products are indicated by A and B and the VTMoV RNAs are labelled 1, 2 and 3.


1 2 3 4 5
A
B
1
2
3
2 3 4
A
B
1
2
3

Products of the protamine sulphate precipitated enzyme were analysed by polyacrylamide gel electrophoresis (Figures 4.6 and 4.7). The labelled product was found to remain at the top of the gel. Extensive complexing of the nucleic acids during precipitation with protamine sulphate may be the reason for the polymerase products not entering the electrophoresis gels. Treatment of the products with SDS and pronase in an attempt to remove the protamine sulphate which may have been bound to the polymerase product, was unsuccessful in overcoming the problem. Attempts to melt the products were also unsuccessful.

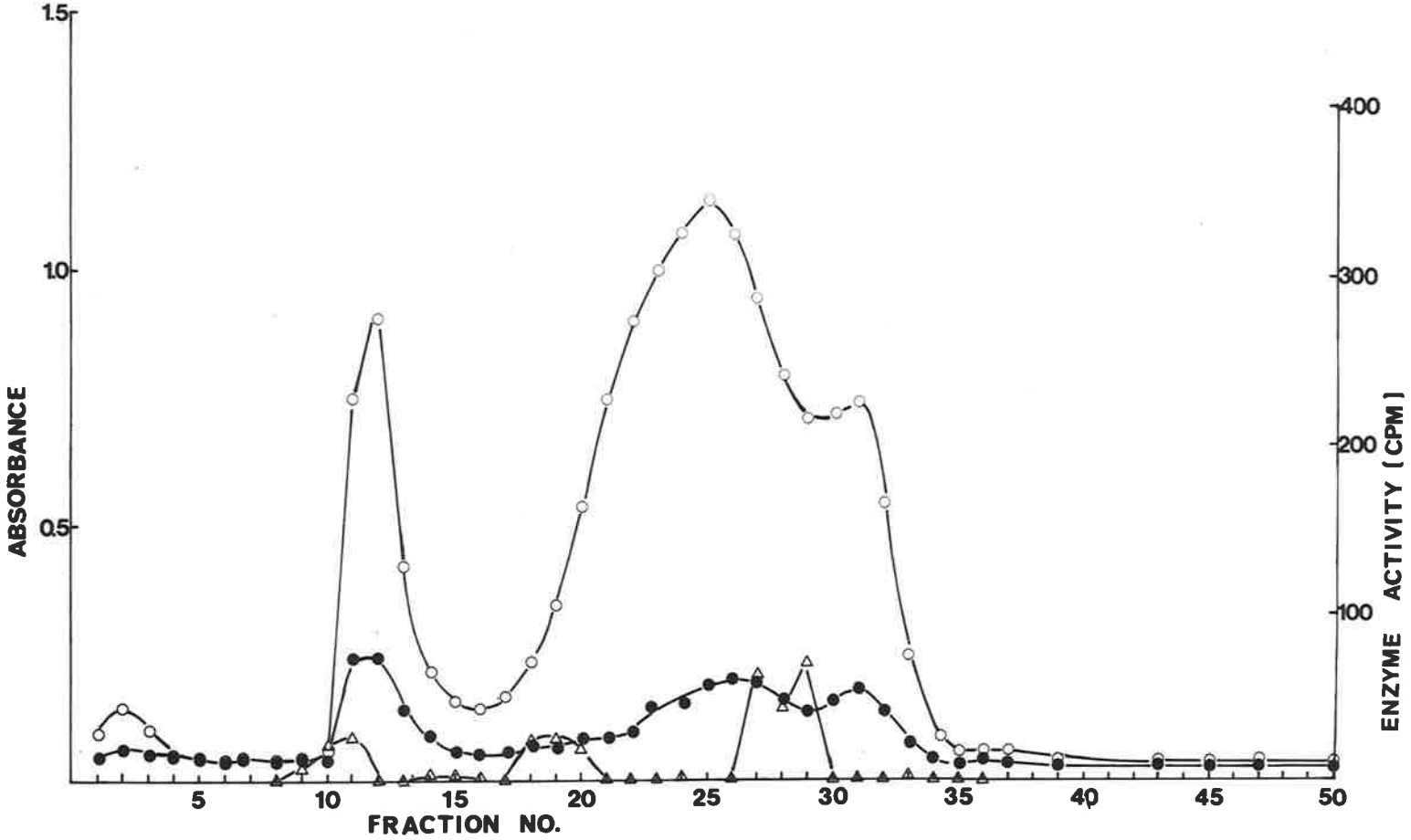
4.4.3 Use of Sepharose 2B chromatography

The successful use of Sepharose 2B chromatography to purify an enzyme-template complex from cowpea mosaic virus (CPMV)-infected cowpea leaves has been reported by Dorssers et al. (1983). Purification was based on the exclusion of dsRNA from the Sepharose 2B while ssRNA, protein and nucleoproteins such as ribosomes and virions were retained (Dorssers et al., 1983).

A column of Sepharose 2B, 1 cm in diameter and 60 cm long was loaded with partially purified enzyme (P_{12} fraction) and eluted with a buffer containing 50 mM Tris-HCl, 5 mM di-thiothreitol, 15% glycerol, 50 mM K acetate, 0.1% Triton X100, pH 8.8, at a rate of 4 ml per hour at 4°C. Fractions of 0.5 ml were collected and the absorption at 260 and 280 nm measured (Figure 4.8). Fractions 8 to 36 inclusive, were assayed for enzyme activity. Less than 1% of the total enzyme activity loaded on the column was recovered (Figure 4.8). To exclude the possibility of a co-factor becoming separated from the enzyme a 10 µl aliquot from each

Figure 4.8 : Use of Sepharose 2B Chromatography in an Attempt to Purify the RNA-Dependent RNA Polymerase-Template Complex from VTMoV-Infected Leaves of N. clevelandii.

Partially purified replication complex (P_{12} fraction) was chromatographed on a Sepharose 2B column as described in the text. The absorbance of the column fractions (0.5 ml) was determined at 260 mm (O—O) and 280 nm (●—●). Subsequently 100 µl samples from each fraction were assayed for enzyme activity. The total amount of enzyme activity loaded on the column was equivalent to 126,000 cpm and only about 2,000 cpm were recovered.


1.5
1.0
0.5
ABSORBANCE
400
300
200
100
ENZYME ACTIVITY (CPM)
5
10
15
20
25
30
35
40
45
50
FRACTION NO.

fraction was combined and a sample of this mixture was assayed for enzyme activity; however, none was detected.

4.4.4 Use of Glycerol Gradient Centrifugation

In an attempt to further purify the enzyme, 0.5 ml of the P_{12} fraction resuspended in 50 mM Tris-HCl buffer, pH 8.8, and containing 0.1% Triton X100, was layered onto a 10-30% glycerol gradient made up with Tris-Triton buffer. Centrifugation was carried out in a SW 41 rotor for two hours at 40,000 rpm, 4°C, in a Beckman L8-70 centrifuge. Fractions (0.6 ml) were collected from the gradient with the aid of an ISCO fraction collector measuring the OD at 254 nm (Figure 4.9). Fraction 21 contained resuspended pellet material from the bottom of the centrifuge tube. No significant enzyme activity was recovered from the gradient (less than 1% of that initially loaded).

4.5 CONCLUSIONS

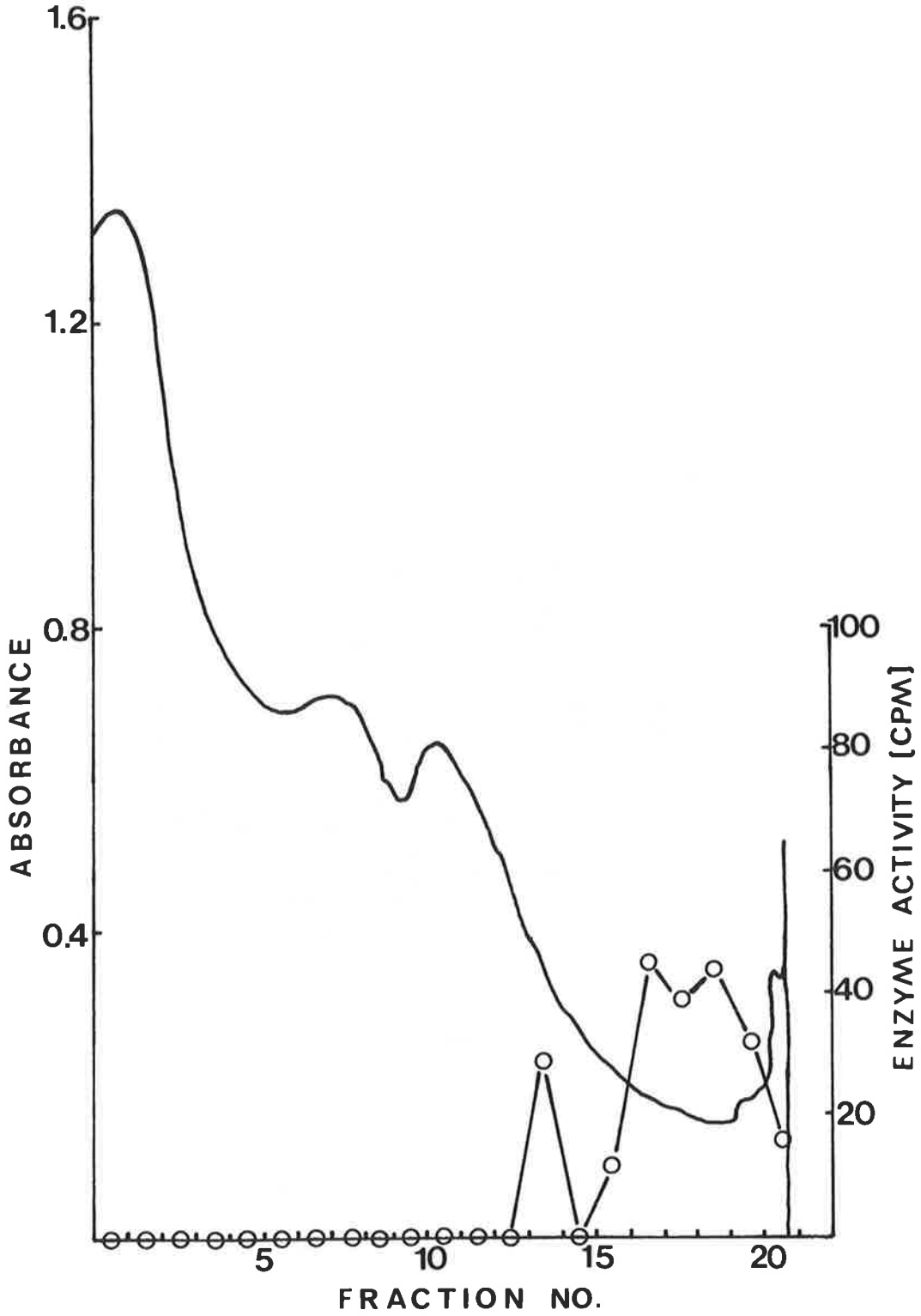
The enzyme-template complex found in VTMoV-infected leaf tissue is unstable and purification procedures such as gradient centrifugation and column chromatography resulted in almost complete loss of enzyme activity. Because the products synthesized by the enzyme-template complex purified by precipitation with protamine sulphate were different from those of the S_1 and P_{12} fractions, it was decided that this purification technique was unsuitable for preparing enzyme for use in enzyme kinetic studies. Use of streptomycin sulphate may have potential although the products of streptomycin sulphate precipitated enzyme were not analysed. In retrospect, further work on the use of these precipitation techniques is warranted. Only protamine sulphate

Figure 4.9 : Glycerol Gradient Centrifugation of the RNA-dependent RNA Polymerase-Template Complex from VTMoV-Infected Leaves of N. clevelandii.

Partially purified replication complex (P_{12} fraction) (0.5 ml) was layered on to a 10 to 30% glycerol gradient in a SW 41 centrifuge tube and centrifuged for 2 hours at 40,000 rpm, 4°C, in a SW 41 rotor using a Beckman L8-70 centrifuge. Total enzyme activity loaded was equivalent to 29,000 cpm and 21, 0.6 ml fractions were collected from the gradient with the aid of an ISCO fraction collector.

—— Absorbance at 254 nm

O——O Enzyme Activity


1.6
1.2
0.8
0.4
ABSORBANCE
100
80
60
40
20
ENZYME ACTIVITY (CPM)
5
10
15
20
FRACTION NO.

precipitation was studied in any detail because of the large increase in enzyme activity observed after precipitation.

Because the S_1 and P_{12} fractions produced similar polymerase products it was decided to use the P_{12} fraction as a source of partially purified enzyme for further characterization of the enzyme and kinetic experiments in attempts to elucidate the mode of enzyme action. These experiments are reported in subsequent chapters.

CHAPTER 5

PROPERTIES OF THE PARTIALLY PURIFIED RNA-DEPENDENT RNA POLYMERASE-TEMPLATE COMPLEX FROM VTMoV-INFECTED PLANTS

When it was decided to use the partially purified enzyme-template complex (P_{12} fraction) as described in Chapter 4, a series of experiments similar to those described in Chapter 3 were carried out. The systematic characterization of the partially purified enzyme-template preparation was an essential preliminary step before enzyme kinetic work could be attempted. The assay mixture used in these studies was the same as that used for the unpurified enzyme and is detailed in Chapter 2.

5.1 TIME COURSE OF THE POLYMERASE REACTION AND ITS RESPONSE TO TEMPERATURE.

Figure 5.1A illustrates the reaction time course of the partially purified enzyme (P_{12} fraction) at 20°C and 25°C. The P_{12} fraction displayed maximum RNA synthesis at 25°C when assayed at various temperatures between 5°C and 45°C for 10 minutes (Figure 5.1B). The enzyme reaction time course varied with temperature, at 25°C the reaction proceded for 10 minutes before no further ^{32}P was incorporated into the TCA insoluble product. However, at 20°C the initial reaction rate was slower but RNA synthesis was sustained for longer (Figure 5.1A). From these data it is concluded that the observed optimum temperature for enzyme activity is dependent on the length of time the reaction is allowed to proceed for.

5.2 THE ENZYMES' RESPONSE TO pH

Enzyme activity was assayed over a pH range between 5 and 10.6 and maximum activity was observed between pH 8.5 and 9.4 (Figure 5.1C). Three buffer systems: Tris-malate (pH 5 to 8), Tris-HCl (pH 7 to 9.1) and Glycine (pH 8.8 to 10.6) were used at a final concentration of 0.1 M in the assay mixture to obtain the pH range used in this study. The assay was done at 20°C for 10 minutes.

5.3 MONOVALENT CATIONS

Figure 5.1D shows that there is no significant response to the monovalent cations K^+ and NH_4^+ by the partially purified enzyme-template complex over the concentration range 0.5 to 100 mM. The assay was carried out at 25°C for 10 minutes.

5.4 DIVALENT CATIONS

The enzyme has an absolute requirement for divalent cations (Figure 5.2A). Optimal activity was observed between 4 and 10 mM Mg^{++} with and without EGTA. The requirements for Mg^{++} could be replaced only partially by Mn^{++} and the maximum activity was observed at a concentration of 2 and 4 mM. The response of the partially purified enzyme to divalent cations was different from that of the unpurified enzyme (Chapter 3, Figure 3.5C). This is especially evident with Mn^{++} (Figure 5.2A). The response to Mg^{++} appears to be bimodal with maxima at about 4 mM and 10 mM when EGTA was either present or absent during assay (Figure 5.2A). This effect is different to that observed when the enzyme activity was measured in the S_1 fraction (Chapter 3, Figure 3.5C).

Figure 5.1

A. TIME COURSE OF THE POLYMERASE REACTION

The P_{12} fraction from VTMoV-infected leaves of N. clevelandii was assayed for enzyme activity for various periods of time at reaction temperatures of 20°C and 25°C. Details of the assay procedure are given in Chapter 2.

B. TEMPERATURE OPTIMUM OF THE POLYMERASE REACTION

The polymerase reaction was carried out at various temperatures for 10 minutes using the P_{12} enzyme fraction. The level of enzyme activity at 35°C was taken as 100% (representing 5352 CPM) as in Figure 3.1C, Chapter 3.

C. EFFECT OF pH ON THE POLYMERASE REACTION

The polymerase reaction was carried out at various pH levels for 10 minutes at 20°C using the P_{12} fraction as the source of the enzyme-template complex. Buffers used were:

●——● 0.1 M Tris-malate

○——○ 0.1 M Tris-HCl

▲——▲ 0.1 M Glycine

pH was adjusted to the required level with KOH or HCl as required.

D. EFFECT OF MONOVALENT CATIONS ON THE POLYMERASE ACTIVITY

The P_{12} fraction was assayed in mixtures containing various amounts of monovalent cations at 25°C for 10 minutes. Both the cations K^+ and NH_4^+ used were in the chloride form.

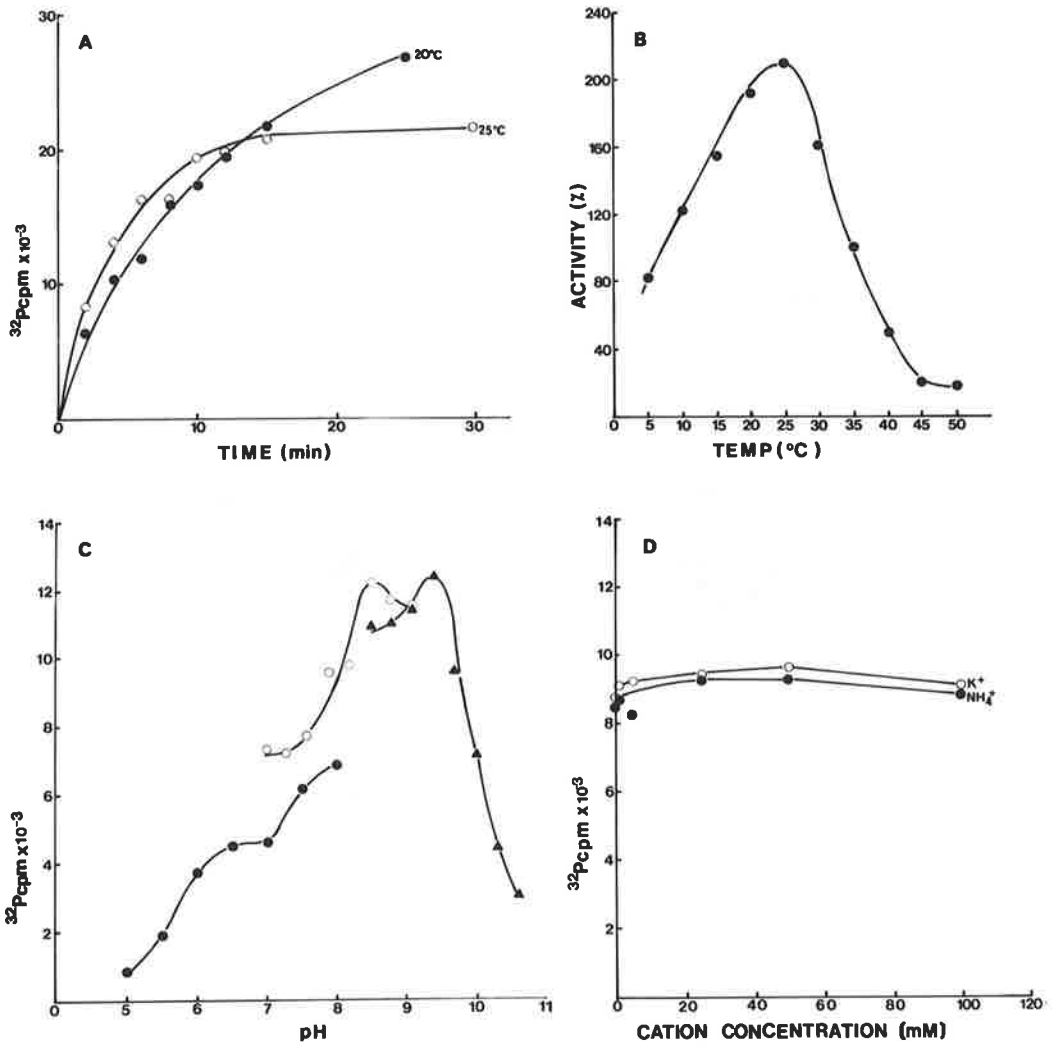

A
20°C
25°C
^{32}Pcpm x10^{-3}
TIME (min)
B
ACTIVITY (%)
TEMP (°C)
C
^{32}Pcpm x10^{-3}
pH
D
K$^+$
NH$_4^+$
^{32}Pcpm x10^{-3}
CATION CONCENTRATION (mM)

FIG.1

Figure 5.2

A. EFFECT OF DIVALENT CATIONS ON THE POLYMERASE ACTIVITY

The response of the partially purified polymerase enzyme (P_{12} fraction) to various concentrations of $MgSO_4$ and $MnCl_2$ in the presence and absence of 8 mM EGTA. Assays were done at 25°C for 10 minutes.

B. EFFECT OF CHELATING AGENTS ON THE POLYMERASE ACTIVITY IN THE P_{12} FRACTION

Reaction mixtures contained various levels of EGTA or EDTA and 8 mM $MgSO_4$. Reactions were done at 25°C and stopped after 10 minutes.

C. TEMPERATURE INACTIVATION OF THE POLYMERASE ENZYME IN THE P_{12} FRACTION

Duplicate 50 µl samples of the P_{12} fraction buffered at pH 8.8 were incubated at various temperatures for 10 minutes and brought to 25°C for 1 minute before addition of 200 µl of assay medium containing ^{32}P-GTP. The samples were then assayed for 10 minutes at 25°C as usual.

D. pH INACTIVATION OF THE POLYMERASE ENZYME IN THE P_{12} FRACTION

Duplicate 50 µl samples of enzyme in 0.05 M buffer at various pH levels were incubated at 20°C for 10 minutes before the addition of 200 µl of assay buffer containing 0.125 M Tris-HCl pH 8.8. The enzyme samples were then assayed at 20°C for 10 minutes in the usual manner. Buffers used for pH treatments were: pH 5 to 7, Tris-malate; pH 7.6 to 8.8 Tris-HCl; pH 9.4 to 10.6 Glycine.

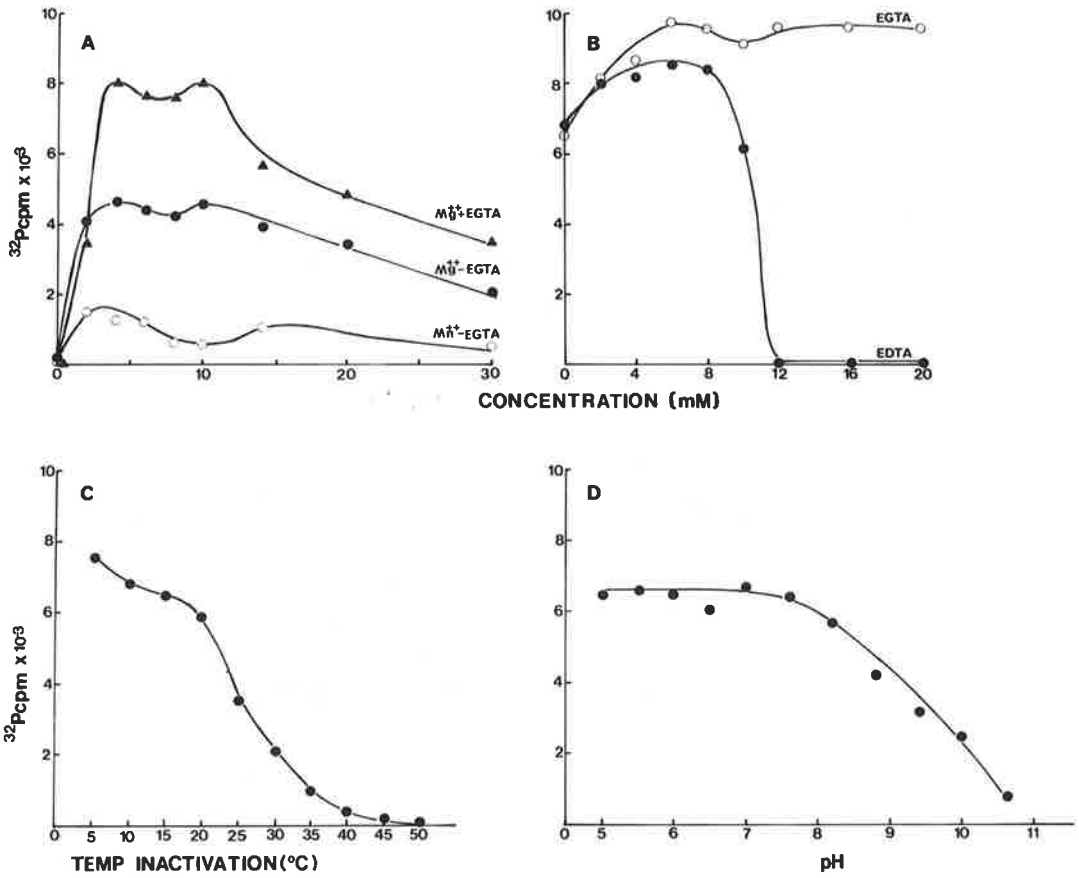

A
B
C
D
EGTA
EDTA
^{32}Pcpm x 10^3
CONCENTRATION (mM)
TEMP INACTIVATION(°C)
pH

FIG. 2

5.5 CHELATING AGENTS

Chelating agents stimulated the enzyme activity of the partially purified RNA-dependent RNA polymerase-template complex (Figure 5.2B). However, the effect was not as marked as that on the unpurified enzyme-template complex (Chapter 3, Figure 5D).

Both EGTA and EDTA stimulated the polymerase activity with EGTA being the more effective of the two. The optimum level of EDTA was between 4 and 8 mM in the presence of 8 mM Mg^{++}, but concentrations greater than 8 mM EDTA were inhibitory and at 12 mM and above, no enzyme activity was detected. This inactivation is probably due to removal of Mg^{++} from solution by EDTA. EGTA did not inhibit enzyme activity, even at a concentration as high as 20 mM.

5.6 DEPENDENCIES

Omission of UTP, CTP and ATP individually, resulted in a 89, 44 and 63% decrease in ^{32}P-GMP incorporation into TCA insoluble product, respectively (Table 5.1). The relatively low reduction of the enzyme activity when the individual ribonucleoside triphosphates were omitted from the assay, could be due to contamination of the remaining tri-phosphates with the omitted compound. Omission of all three unlabelled ribonucleoside tri-phosphates reduced enzyme activity to an insignificant level (96%). Orthophosphate at a concentration of 10 mM, reduced enzyme activity by 25%. A similar level of pyrophosphate resulted in almost complete inactivation of the enzyme.

Table 5.1 : Properties of the Partially Purified (P_{12} fraction) VTMoV-Specific RNA-Dependent RNA Polymerase from *N. clevelandii*.

Reaction Mixture	Enzyme Activity (% of complete reaction mixture)
Complete	100 (14527 CPM)
-ATP	37
-UTP	11
-CTP	56
-ATP, UTP, CTP	4
-Actinomycin D	95
+ DNase I (50 µg/ml)	99
+ RNase A (50 µg/ml)	36
+ Rifampicin (50 µg/ml)	75
-enzyme	0
+Orthophosphate (10 mM)	75
+Pyrophosphate (10 mM)	2
+Yeast RNA	108

Absence of actinomycin D or the addition of DNase to the assay mixture had no significant effect on the incorporation of ^{32}P-GMP into the product indicating that enzyme activity was entirely RNA-dependent although the presence of rifampicin in the assay mixture resulted in a 25% reduction of enzyme activity.

Addition of pancreatic RNase to the assay mixture resulted in a 64% reduction in the observed enzyme activity. This partial resistance of RNA synthesis to RNase A is thought to be due to the double stranded nature of the template and product and has been discussed in Chapter 3. Addition of yeast RNA did not significantly stimulate the enzyme activity suggesting that RNase activity present in the enzyme preparation did not significantly influence the assay.

5.7 TEMPERATURE INACTIVATION OF THE POLYMERASE

The effect of temperature on enzyme inactivation was studied by incubating samples of enzyme at various temperatures between 5 and 50°C for 10 minutes and subsequently assaying them for enzyme activity at 25°C for 10 minutes. Enzyme samples were buffered at pH 8.8, that at which the enzyme activity was assayed.

The partially purified enzyme was found to be rapidly inactivated above 20°C (Figure 5.2C). These results together with the temperature optima data (Figure 5.1B) and reaction time course studies at 20°C and 25°C (Figure 5.1A) indicate that temperature inactivation is a major problem in assaying for enzyme activity. To minimize the effect of temperature inactivation it was decided that 20°C was the best reaction temperature for enzyme kinetic studies.

5.8 pH INACTIVATION OF THE POLYMERASE

Samples of partially purified enzyme were incubated at various pH levels between 5 and 10.6 for 10 minutes at 20°C and subsequently assayed for enzyme activity at 20°C and pH 8.8 for 10 minutes.

The enzyme was found to be inactivated at pH levels above 7.6 with almost complete inactivation at pH 10.6 (Figure 5.2D). These data indicate that for successful storage and further purification of the enzyme, slightly acid pH and low temperature should be maintained.

5.9 EFFECT OF ENZYME CONCENTRATION ON RNA SYNTHESIS

The effect of enzyme concentration on the incorporation of ^{32}P-GMP into TCA insoluble product was studied. Various amounts of enzyme preparation were incubated at 20°C for 5 minutes in the standard assay mixture with a final volume of 300 µl. At low enzyme concentrations, product formation was non-linear (Figure 5.3) indicating that some inhibiting factor may be present at low enzyme levels in the assay mixture. The enzyme activity was proportional to its concentration when between 3.6 and 14.6 units were added to the assay mixture; however, the slope of the curve is greater than one. When 21.8 units of enzyme activty were used, the curve was again non-linear indicating a substrate to enzyme concentration effect, which was probably due to an insufficient concentration of GTP, which was 1 µM, in the assay mixture.

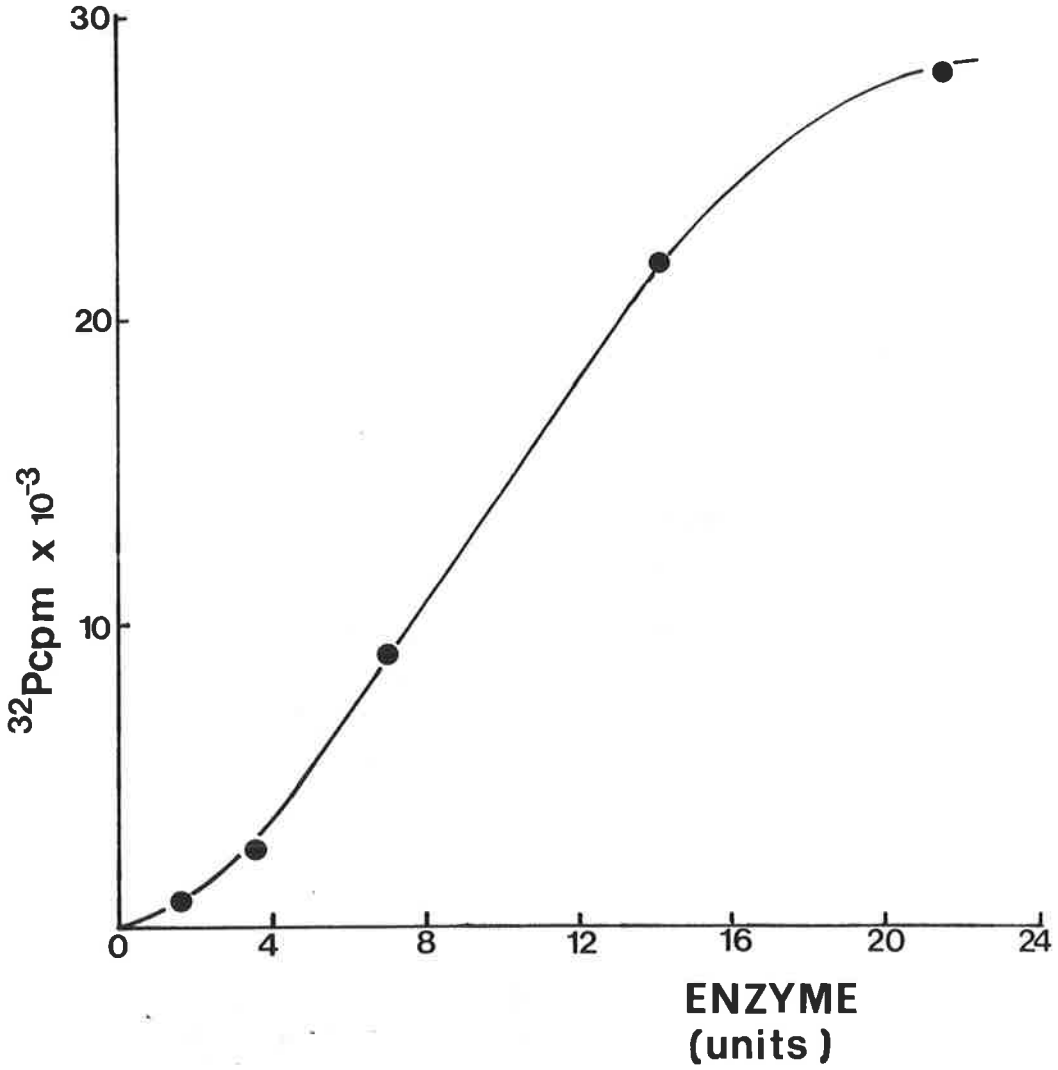
5.10 CONCLUSION

Temperature has a major influence on the stability and observed activity of the enzyme-template complex. The temperature inactivation

data suggests that inactivation of the enzyme may be partially responsible for the ceasation of enzyme activity observed when incubated at 25°C. About half of the enzyme activity was lost when the enzyme was incubated at 25°C for 10 minutes. The polymerase complex is also susceptible to damage at pH levels above 7.6. However, the high levels of enzyme activity at pH 8.5 to 9.4 compensate for any inactivation of the enzyme at these pH levels. A temperature of 20°C and pH of 8.8 was therefore selected as the best compromise between enzyme activity and enzyme inactivation for enzyme kinetic studies.

Figure 5.3 : THE EFFECT OF ENZYME CONCENTRATION ON RNA SYNTHESIS IN THE IN VITRO ASSAY SYSTEM

Increasing amounts of enzyme in the P_{12} fraction were added to the assay buffer and assayed as usual. A unit of enzyme activity is defined as the incorporation of 1 pico-mole of GTP into TCA insoluble product/ 10 minute assay at 20°C under the standard assay conditions described in Chapter 2.


30
20
10
0
4
8
12
16
20
24
^{32}Pcpm x 10^{-3}
ENZYME
(units)

CHAPTER 6

CHARACTERIZATION OF THE IN VITRO PRODUCTS SYNTHESISED BY THE RNA-DEPENDENT RNA POLYMERASE FROM VTMoV-INFECTED PLANTS

In previous chapters the presence of a RNA-dependent RNA polymerase bound to a native template in VTMoV-infected leaves of N. clevelandii was established; and some of the characteristics of in vitro RNA synthesis and partial purification of this enzyme-template complex were described. Preliminary analysis of the products revealed that two bands of radioactive RNA were synthesised in vitro by both crude cell-free extract and a partially purified (P_{12}) enzyme fraction (Chapter 4, Figures 4.4 and 4.5). Because the products of in vitro RNA synthesis were similar whether the S_2 or P_{12} enzyme fraction were used (Chapter 4, Section 4.3) it was decided to use the S_1 fraction as a source of enzyme-template complex to synthesise RNA in vitro for more extensive product analysis. Experiments were carried out to determine the nature of the RNA synthesised in vitro and to establish its relationship to VTMoV RNA.

The products were analysed by urea and aqueous polyacrylamide gel electrophoresis. Gels were stained for nucleic acids, photographed and subsequently dried before being subjected to autoradiography. Throughout this chapter electrophoresis data are presented in figures where the stained gel is shown in the upper part of the figure and the autoradiograph in the lower portion. The only exception being Figure 6.3 where the stained gel and autoradiograph are shown side by side. Products of in vitro RNA synthesis detected by autoradiography are

indicated by arrows at the edges of the figures as are the positions of VTMoV RNAs 1, 2 and 3 used as markers.

6.1 DETERMINATION OF THE MOLECULAR WEIGHTS OF THE *IN VITRO* PRODUCTS

The molecular weights of the two products, synthesised by the polymerase in the S_1 fraction (see Figure 6.1), were estimated by comparing their electrophoretic mobilities to those of the ten segments of Fiji disease virus dsRNA using molecular weight values determined by Reddy *et al*. (1975). Values of 3.5×10^6 and 0.72×10^6 were calculated as the molecular weights of the dsRNAs synthesised *in vitro* (Figures 6.1 and 6.2). The RNA of M_r 3.5×10^6 is indicated by an arrow labelled A and that of M_r 0.72×10^6 by an arrow labelled B in all figures presented in this chapter.

In urea containing gels, the product A comigrated with the dsRNA isolated from VTMoV-infected leaves by Chu *et al*. (1983) (Figure 6.2, tracks 4 and 7). Chu *et al*. (1983) isolated two species of dsRNA from VTMoV-infected plants. These dsRNAs migrated as two very close bands in urea containing gels (they are not resolved in Figure 6.2, track 6), and as two well separated bands in aqueous gels (Figure 6.3, track 2). Product A migrated slightly behind the slower moving *in vitro* labelled dsRNA isolated by Chu *et al*. (1983) (Figure 6.3, tracks 2 and 3) whose molecular weight they estimated to be about 3.6×10^6. The product B had an electrophoretic mobility unlike any of the VTMoV-specific ds RNAs detected in infected plants by Chu *et al*. (1983) (Figure 6.3).

The time course of *in vitro* synthesis of the two products A and B was studied by stopping the polymerase reaction at various times (Figure

Figure 6.1 : Molecular Weight Determination of the dsRNA Synthesised *In vitro* by the RNA-Dependent Polymerase Template Complex from VTMoV-Infected Leaves of *N. clevelandii*.

Semi-logarithmic plot relating electrophoretic mobilities of the *in vitro* products (open circles) with the dsRNA components of FDV. The M_r values used for the nucleic acid components of FDV are those of Reedy *et al*. (1975) and are tabulated below.

Component	$M_r \times 10^{-6}$
1	2.92
2	2.52
3 + 4	2.48
5	2.18
6	1.88
7	1.48
8	1.27
9 + 10	1.18

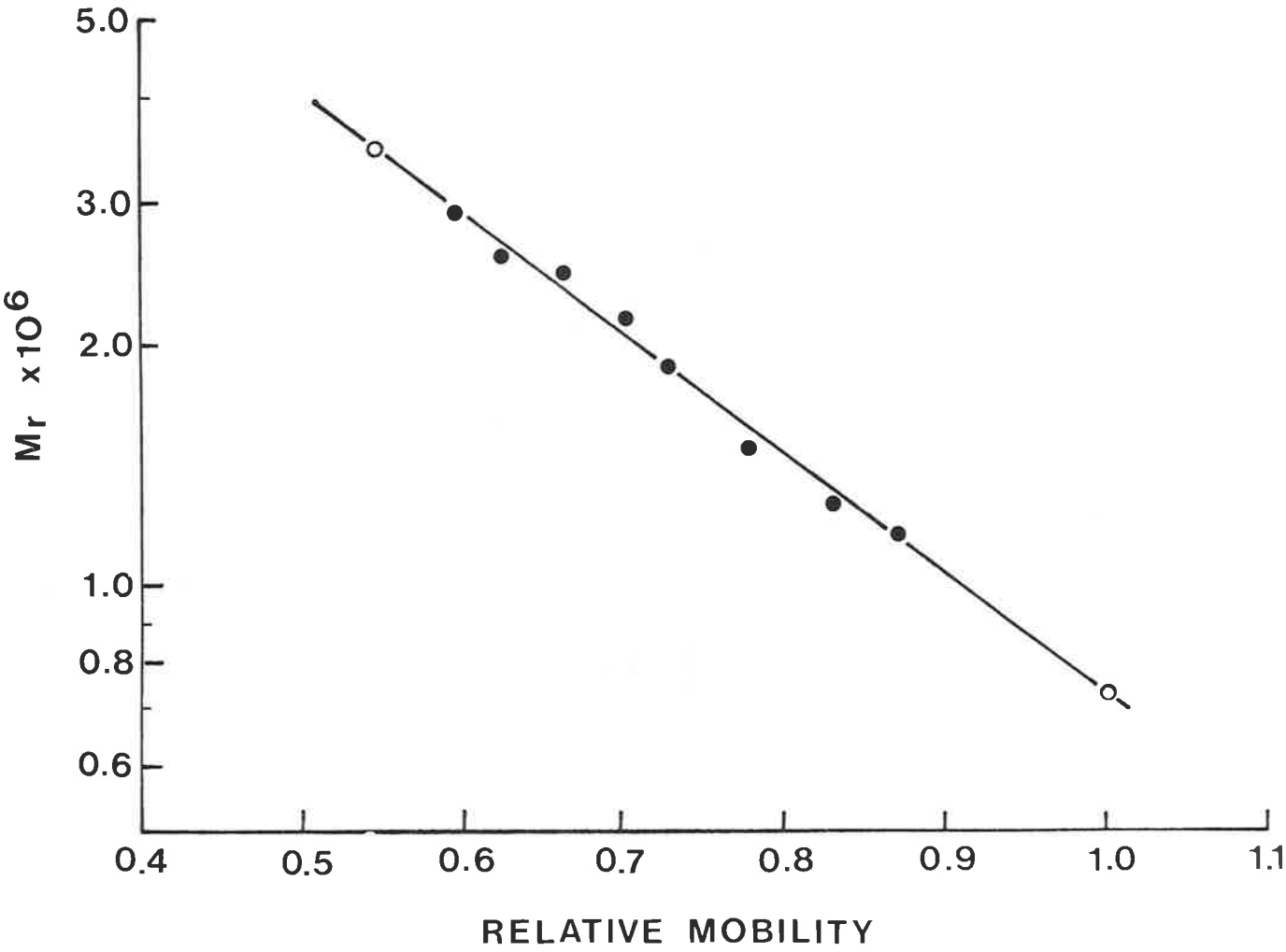

5.0
3.0
2.0
1.0
0.8
0.6
M_r x10^6
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
RELATIVE MOBILITY

Figure 6.2 : Molecular Weight Estimation of the In vitro Products by Polyacrylamide Gel Electrophoresis.

The urea-containing gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1, 5 & 10 FDV RNA markers.

2 & 7 in vitro product.

3 & 9 VTMoV RNA markers.

4 & 8 in vitro product heated at 100°C in H_2O for 1.5 min.

6 in vitro dsRNA supplied by Chu et al. (1983).

Details of the methods used to obtain nucleic acid samples, electrophoresis and autoradiography are given in Chapter 2. Note the detection of labelled viroid-like RNAs 2 and 3 in lanes 4 and 8 (lower panel) where the products heated at 100°C for 1.5 minutes were loaded in the gel. The positions of the VTMoV RNA are indicated by arrows labelled 1, 2 and 3 and the two in vitro products are indicated by A and B.

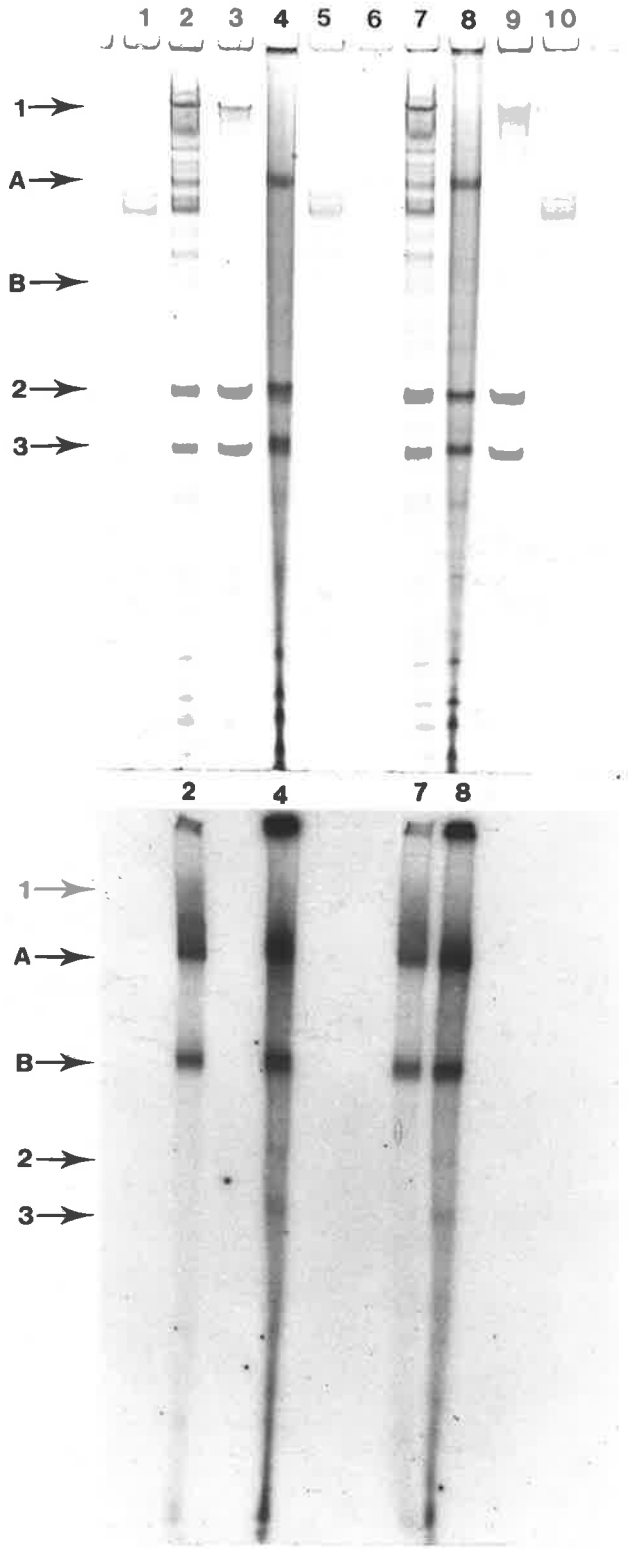

1 2 3 4 5 6 7 8 9 10
1
A
B
2
3
2 4 7 8
1
A
B
2
3

Figure 6.3 : Comparison of the *In vitro* Products of the RNA-Dependent RNA Polymerase-Template Complex in the S_1 Fraction from VTMoV-Infected leaves of *N. clevelandii* and the dsRNA Obtained From Infected Leaves by Chu *et al*. (1983).

In vitro labelled products were compared with the *in vivo* ds RNA, obtained by Chu *et al*. (1983) from VTMoV-infected leaves of *N. clevelandii* by non-denaturing polyacrylamide gel electrophoresis (see Chapter 2 for details of the methods used). The non-denaturing gel slab was stained with toluidine blue and photographed (left half of panel) before being dried and autoradiographed (right half of panel).

Track:

1. ^{32}P labelled VTMoV RNA marker
2. ^{32}P labelled dsRNA from VTMoV-infected plants supplied by Chu *et al*. (1983)
3. *In vitro* products

Note the high molecular weight product (A) migrated at about the same rate as the larger *in vivo* dsRNA. No dsRNA which migrated at the same rate as the *in vitro* product B was detected in the samples of *in vivo* ds RNA. The positions of the VTMoV RNAs are indicated by arrows labelled 1, 2 and 3 and the *in vitro* products are indicated by A and B.

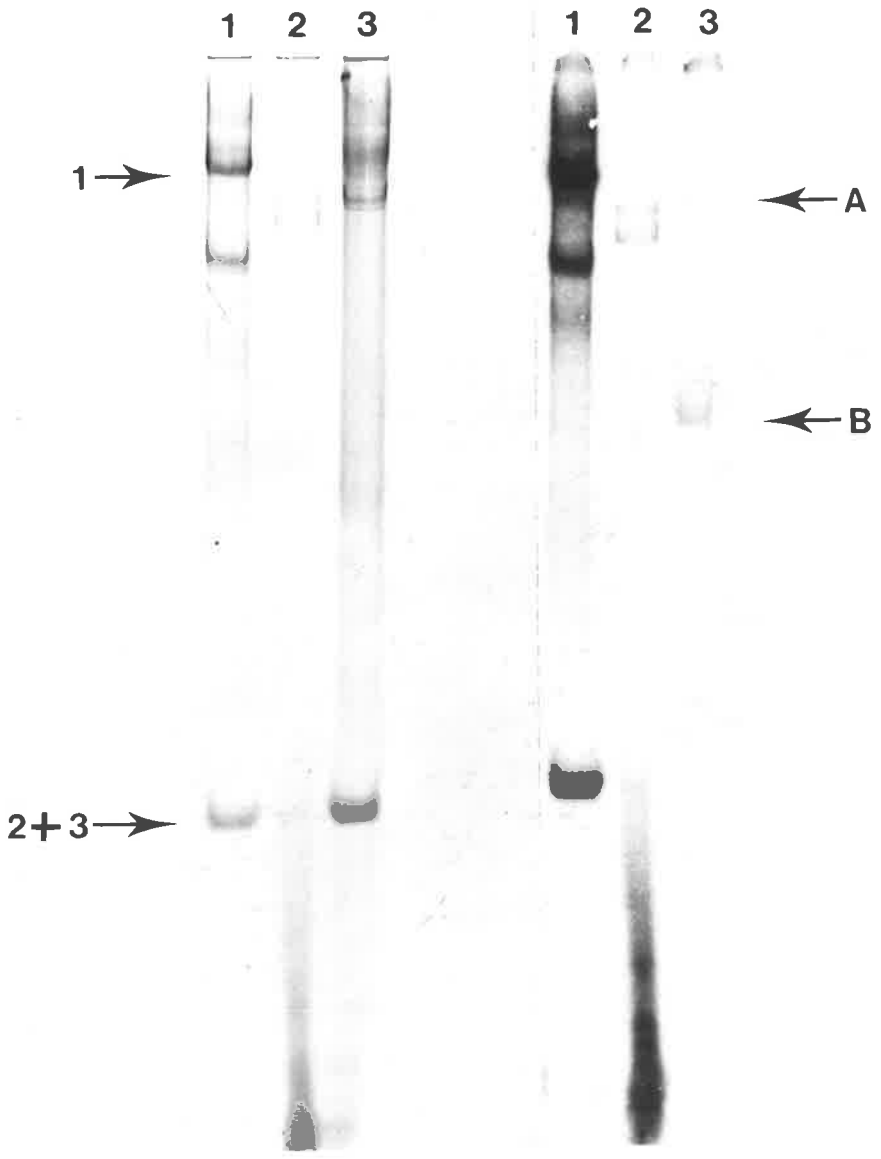

1
2
3
1
2
3
1
2+3
A
B

Figure 6.4 : Relationship Between the *In vitro* Polymerase Reaction Time Course and the Products of *In vitro* RNA Synthesis.

Samples of the reaction mixture were taken at various times after the initiation of RNA synthesis by the addition of the S_1 fraction from healthy and VTMoV-infected leaves from *N. clevelandii*. Details of nucleic acid extraction from the reaction mixtures and conditions of electrophoresis are described in Chapter 2. The non-denaturing gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

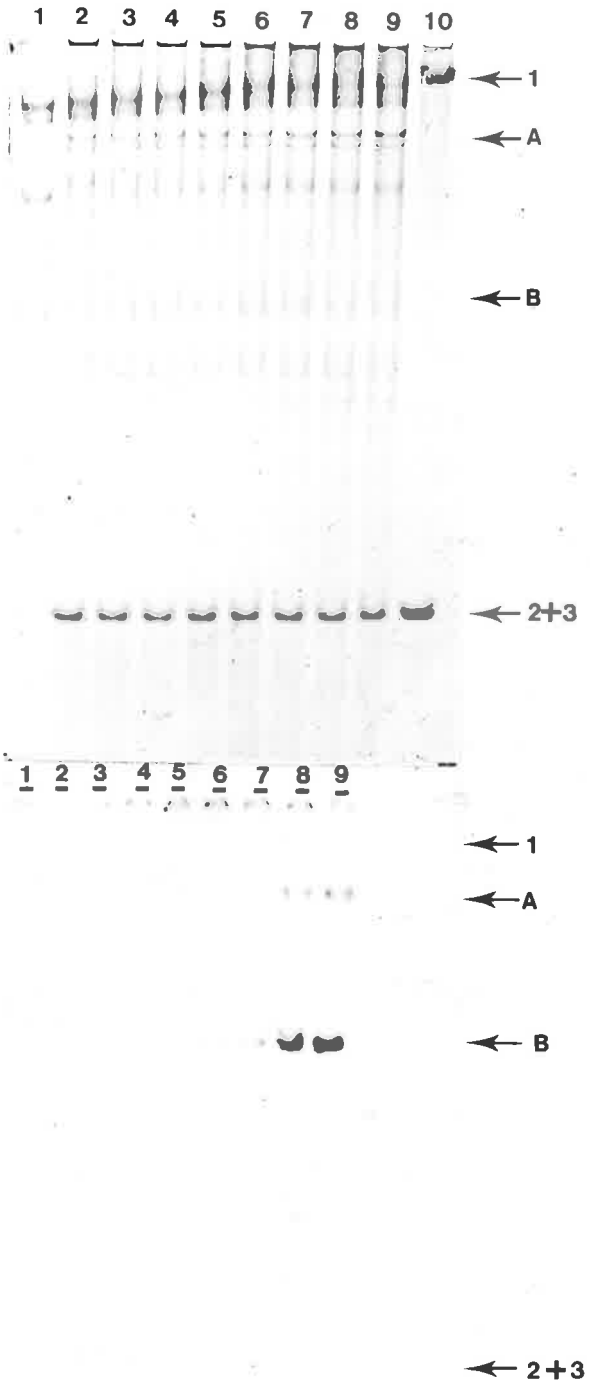
1 S_1 fraction from healthy leaves incubated in *in vitro* assay mixture for 10 minutes.

2-9 S_1 fraction from diseased leaves incubated in *in vitro* assay mixture for various times.

Track	Incubation Time (min)
2	0
3	2
4	4
5	6
6	8
7	10
8	20
9	30

Track 10 VTMoV RNA marker.

Note the absence of ^{32}P labelled RNA in the healthy leaf extract (track 1). The positions of the *in vitro* products are indicated by arrows labelled A and B and VTMoV RNA by arrows labelled 1, 2 and 3.


1 2 3 4 5 6 7 8 9 10
1
A
B
2+3
1 2 3 4 5 6 7 8 9
1
A
B
2+3

6.4). Both products A and B were present in approximately the same proportions when the reactions were terminated between 2 and 30 minutes of incubation (Figure 6.4, tracks 2 to 9). An equivalent extract from healthy leaves incubated in the in vitro assay mixture for 10 minutes showed no detectable incorporation of labelled GMP into nucleic acids (Figure 6.4, track 1). The apparent lack of any detectable degradation of plant and viral ssRNAs during incubation of the S_1 fraction indicates the absence of any significant level of RNase activity in the leaf extracts (Figure 6.4 upper panel). However, the plant and viral RNAs were sensitive to added RNase and this is shown in Figure 6.6 (Upper panel, tracks 4 and 6).

6.2 IN VIVO SYNTHESIS OF LABELLED RNA IN VTMoV-INFECTED LEAVES OF N. CLEVELANDII

The in vitro product A of the VTMoV-specific RNA polymerase had an electrophoretic mobility in urea gels, similar but not identical to those of the two dsRNAs isolated from VTMoV-infected N.Clevelandii leaves by Chu et al. (1983) (Figure 6.2, track 6). However, Chu et al. (1983) did not detect a dsRNA with an electrophoretic mobility similar to that of the in vitro product B in their nucleic acid preparations from virus-infected leaves. To determine if a RNA with the properties of the in vitro product B was present in VTMoV-infected leaves or if B was a breakdown product of a larger dsRNA, the following experiment was done.

A group of leaves from healthy plants and another from plants infected with VTMoV for seven days, were collected and sliced from the mid-rib to the outer edge with cuts about 2 mm apart (Rezaian et al., 1976). They were then divided into four equal 0.2 gram samples, two of

healthy and two of diseased leaves, and each sample was placed in a plastic petri dish and irrigated with 0.5 ml of distilled water containing 50 µc of ^{3}H-uridine (Uridine-5-H3 ; Radiochemical Centre, Amersham). Infiltration of the leaves was achieved by subjecting the samples to a gentle vacuum for 5 minutes.

The leaves were kept under grow-lux lamps for 2 hours at 25°C before nucleic acid extraction. One sample of tissue from healthy and one from diseased leaves was ground with 1 ml of RNA extraction buffer (0.2 M Na acetate, 0.2% thioglycerol, 1% SDS) and 1 ml of phenol, placed in plastic centrifuge tubes and shaken for two hours at 5°C. The remaining two leaf samples were ground in 1 ml of enzyme extraction buffer (Chapter 2) and allowed to stand at room temperature (approximately 28°C) for 15 minutes. After this 1 ml of RNA extraction buffer and 1 ml of phenol was added to the extracts and they were shaken for 1.5 hours at 5°C. The aqueous phase was recovered by centrifugation at 5,000 g for 5 minutes and the nucleic acids were precipitated twice with ethanol and resuspended in 100 µl of TBE buffer. A 5 µl sample from each nucleic acid preparation was assayed for ^{3}H incorporation by the TCA precipitation procedure used for assaying enzyme activity. No significant radioactivity was detected in samples from healthy leaves and about 200 cpm were detected in samples from the infected leaves.

A 10 µl sample of each nucleic acid preparation was analysed by gel electrophoresis in the presence of urea. Similar samples of the nucleic acid preparations from diseased leaves were melted at 85°C in formamide and formaldehyde, recovered by ethanol precipitation and then subjected to electrophoretic analysis. The resultant gel was stained for nucleic acids, dried and autoradiographed for two months.

Figure 6.5 : *In vivo* Synthesis of RNA in VTMoV-Infected Leaves of *N. clevelandii*.

Tritium-labelled RNA was obtained from leaf tissue as described in the text (Section 6.2). The urea-containing gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Tracks:

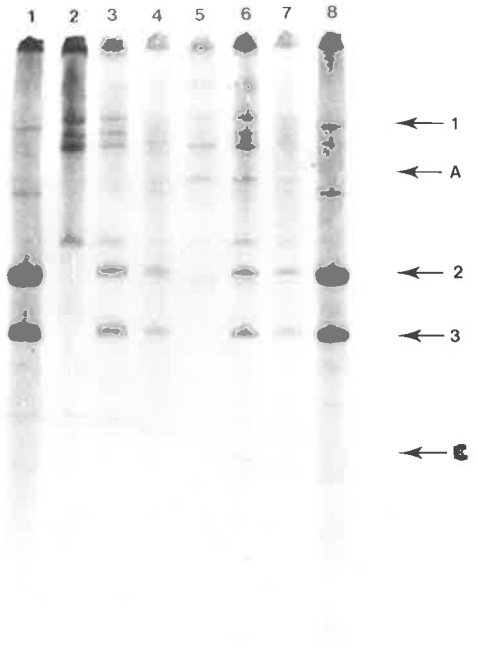
1 & 8 VTMoV RNA marker

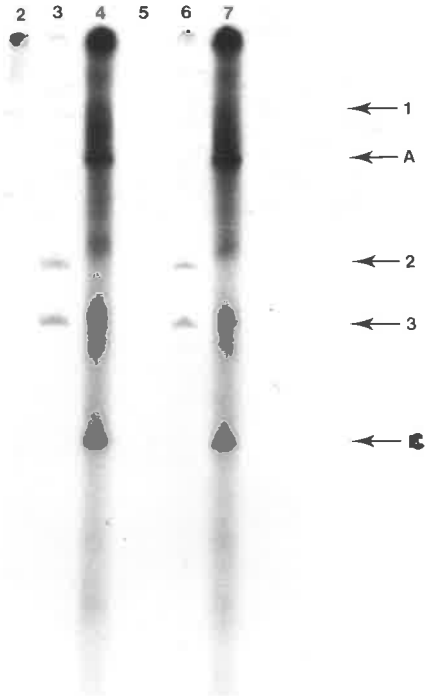
2 & 5 Nucleic acids extracted from healthy tissue after incubation with tritiated uridine.

3 & 6 Nucleic acids extracted from diseased tissue after incubation with tritiated uridine. The nucleic acids were melted in 50% formamide 6% formaldehyde at 85°C for 5 minutes.

4 & 7 Nucleic acids extracted from diseased tissue after incubation with tritiated uridine.

Samples loaded on tracks 2, 3 and 4 were from tissue which was first ground in extraction buffer (0.1 M NH_4Cl, 0.1 M TRIS-HCl, 0.4 M sucrose pH 8.8) and allowed to stand at room temperature before phenol extraction. Samples loaded on tracks 5, 6 and 7 were from tissue extracted directly into phenol. Note the presence of labelled RNAs 1, 2 and 3 in track 6 but only 2 and 3 in track 3.


1 2 3 4 5 6 7 8
1
A
2
3


2 3 4 5 6 7
1
A
2
3

As can be seen in Figure 6.5 no bands of labelled RNA were detected in the healthy leaf treatments (Tracks 2 and 5). However two major and two minor bands of labelled RNA were detected in the preparation from diseased leaf tissue (tracks 4 and 7). One of the major labelled RNAs had an electrophoretic mobility similar to that of the _in vitro_ product A (Figure 6.5) and the other was unlike any _in vitro_ product and is labelled C in Figure 6.5. The electrophoretic mobility of this second RNA species (C) appears to be similar to that of the RNase digestion product of the _in vitro_ dsRNA which is described in the following section and can be seen in Figure 6.7 (tracks 4 to 8). The two minor bands of radioactivity appear to have electrophoretic mobilities similar to those of VTMoV RNAs 2 and 3. No radioactivity was detected in the gel with the mobility expected for the _in vitro_ product B, suggesting that product B may be unique to the _in vitro_ assay system or that it occurs _in vivo_ only in trace amounts.

The two procedures used for nucleic acid extraction of the leaf tissues did not result in different RNA species being detected which precludes the possibility that B was a breakdown product of some larger labelled dsRNA in the _in vitro_ system.

6.3 EVIDENCE THAT THE _IN VITRO_ PRODUCTS ARE DOUBLE-STRANDED RNA

The products synthesised _in vitro_ by the RNA-dependent RNA polymerase in the S_1 fraction from cell-free extracts of VTMoV-infected plants of _N. clevelandii_, were subjected to various nuclease and hydroxide treatments to establish their nature.

Figure 6.6 : Characteristics of the *In vitro* Products.

The non-denaturing gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1. VTMoV RNA marker.
2. *In vitro* product incubated in DNase buffer with DNase 1 (2µg/ml).
3. *In vitro* product incubated in DNase buffer alone.
4. *In vitro* product incubated in 2 x SSC with RNase A (1µg/ml).
5. *In vitro* product incubated in 2 x SSC alone
6. *In vitro* product incubated in 0.01 x SSC with RNase A (1µg/ml).
7. *In vitro* product incubated in 0.01 x SSC alone.
8. *In vitro* product incubated in S_1 nuclease buffer with S_1 nuclease (10 units/assay).
9. *In vitro* product incubated in S_1 nuclease buffer alone.
10. *In vitro* product treated with 0.4 M KOH.
11. Untreated *in vitro* product.
12. Nothing loaded.
13. ^{32}P labelled VTMoV RNA.

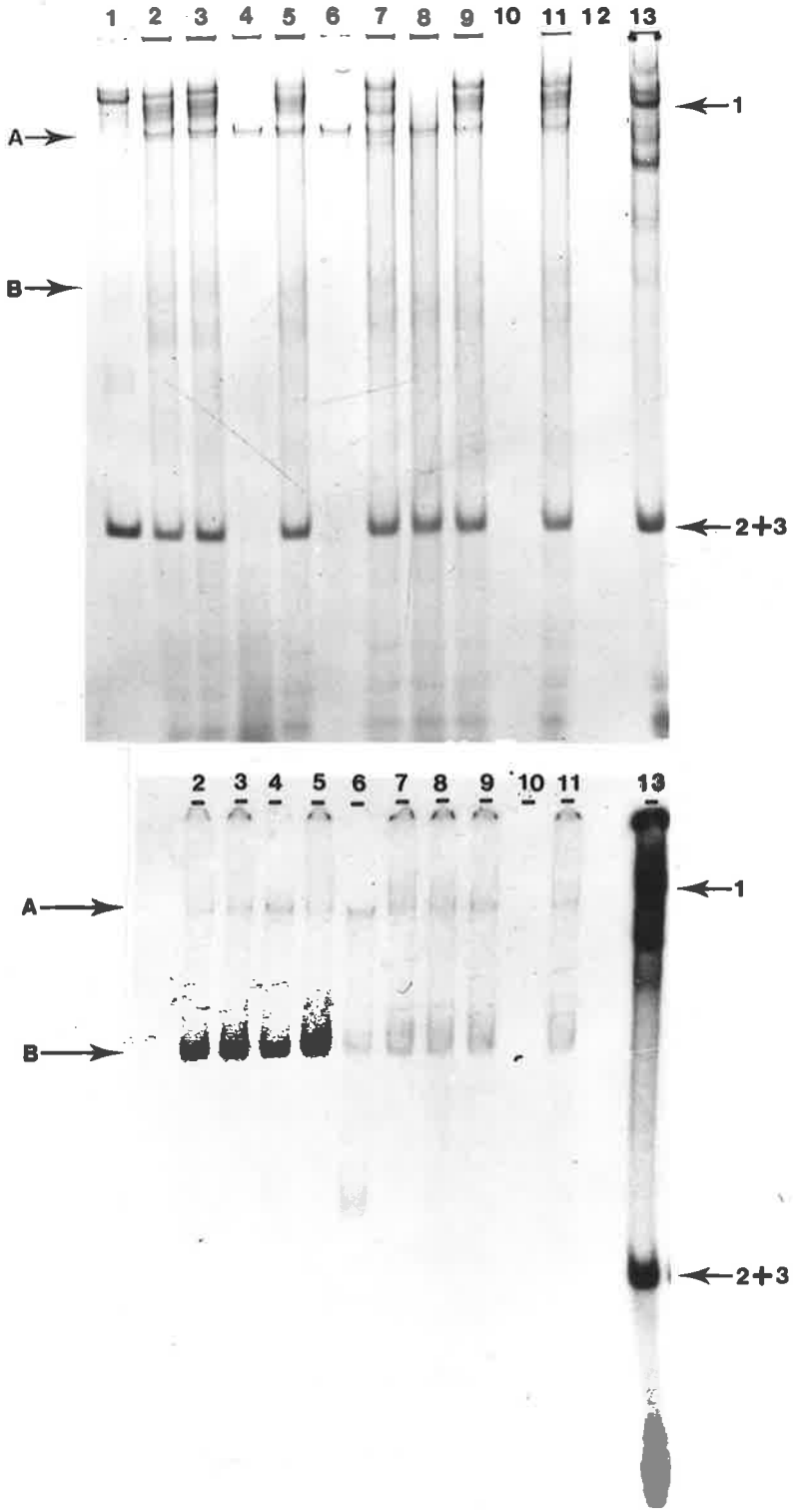
Note: a. The presence of both *in vitro* product species (A and B).

b. Complete hydrolysis of *in vitro* products by KOH.

The DNase buffer contained 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM $CaCl_2$ at pH 6. The DNase 1 used was Sigma product No. D5010.

The S_1 nuclease buffer contained 0.03 M sodium acetate, 0.3 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, pH 4.6.

The *in vitro* labelled RNAs are indicated by arrows A and B and the VTMoV RNAs are labelled 1, 2 and 3. Full details are given in Section 6.3 of the text.


1 2 3 4 5 6 7 8 9 10 11 12 13
A
B
1
2+3
2 3 4 5 6 7 8 9 10 11 13
A
B
1
2+3

Both products A and B were resistant to DNase (Figure 6.6, tracks 2 and 3) but were completely hydrolysed by incubation in 0.4 M KOH for 15 minutes at room temperature (Figure 6.6, track 10). This indicates that both products are RNA. Treatment with S_1 nuclease and RNase A in 2 x SSC had no effect on the products, indicating that the RNAs are double stranded (Figure 6.6, tracks 4, 5, 8 and 9). Incubation of the products with RNase A for 10 minutes at 37°C in 0.01 x SSC only partially digested the products (Figure 6.6, tracks 6 and 7). This apparent partial resistance of the products to RNase A was further explored by incubating them with RNase A (10 µg/ml) in 0.01 x and 2 x SSC for various times. Reactions were stopped by phenol extraction and the nucleic acids were recovered from the aqueous phase by ethanol precipitation.

The <u>in vitro</u> products were susceptible to RNase A in 0.01 x SSC but only slightly susceptable in 2 x SSC (Figure 6.7, tracks 4 to 8 and 11 to 15). A stable breakdown product which was resistant to RNase A was observed in all RNase treatments (Band C in Figure 6.7). Addition of RNase to the products but without incubation (Figure 6.7, upper panel, tracks 4 and 11), resulted in the digestion of ssRNA indicating that addition of phenol to the reaction mixtures did not completely remove the RNase from the nucleic acids in the samples. The digestion of the untreated product (Figure 6.7, and track 10) and radioactive VTMoV marker RNA (Figure 6.7, tracks 9 and 16), are thought to be due to the added RNase A which survived phenol extraction and ethanol precipitation.

Figure 6.7 : The Effect of RNase A on the dsRNA Synthesised *In vitro* by the VTMoV-Induced RNA-Dependent RNA Polymerase-Template-Complex in the S_1 Fraction from VTMoV-Infected leaves of *N. clevelandii*.

The urea-containing gel slab stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1. VTMoV RNA marker.

2,9 & 16 ^{32}P-labelled VTMoV RNA

3 & 10 Untreated *in vitro* products

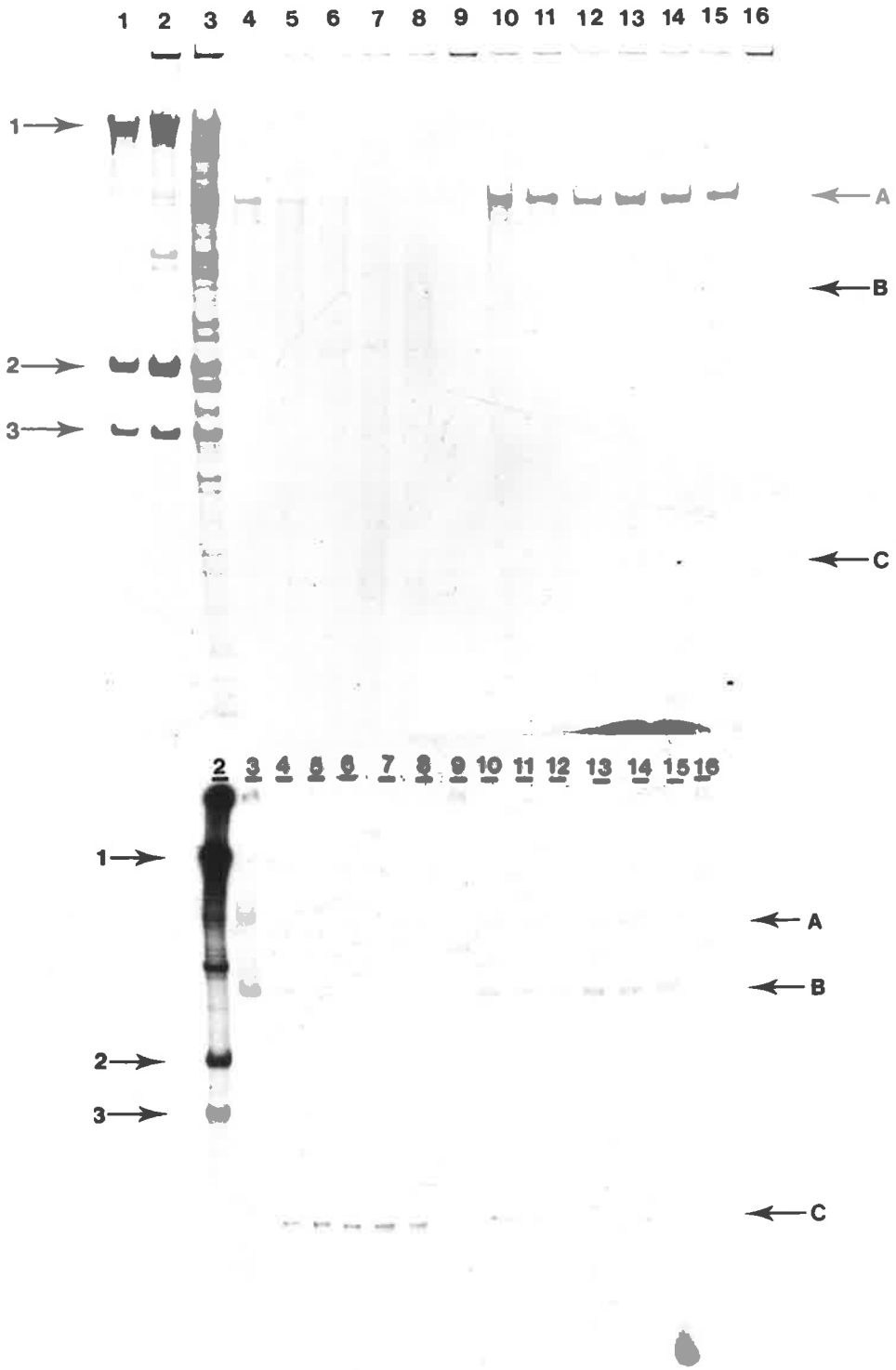
4 - 8 *In vitro* products treated with 10 µg/ml RNase A in 0.01 x SSC for various times.

Track	Reaction time (min)
4	0
5	15
6	30
7	60
8	120

11 - 15 *In vitro* products treated with 10 µg/ml RNase A in 2 x SSC for various times.

Track	Reaction time (min)
11	0
12	15
13	30
14	60
15	120

Experimental details are given in Chapter 2 and Section 6.3 of this chapter. The position of the VTMoV RNAs are indicated by arrows labelled 1, 2 and 3 and the *in vitro* products by A and B. The RNase A-resistant degradation product is indicated by the arrow labelled C.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
1
2
3
A
B
C
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
1
2
3
A
B
C

6.4 ANALYSIS OF THE MELTED IN VITRO PRODUCTS

The in vitro products were subjected to polyacrylamide gel electrophoresis after melting in the presence of formamide and formaldehyde or in distilled water (Chu et al., 1983).

Figure 6.8 (track W) shows that heating at 100°C for 5 minutes in water did not melt the labelled dsRNA. However, when heated at 85°C for two minutes in the presence of formamide and formaldehyde, the products melted into RNAs which migrated at the same rate as VTMoV RNA 1 and RNA 3 (Figure 6.8, track F). When VTMoV RNA was heated in the presence of formamide and formaldehyde, RNAs 2 and 3 displayed a slight change in electrophoretic mobility (Figure 6.8, compare tracks V and VF). The reason for this change in electrophoretic mobility is not known, however, the importance of subjecting the VTMoV RNAs used as markers to exactly the same treatments as the in vitro product samples can not be over emphasised.

6.5 SEPARATION OF THE dsRNA PRODUCTS BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

Products synthesised in vitro were loaded onto a linear-log sucrose gradient (Brakke and Van Pelt, 1970) as outlined in Chapter 2. The gradient contained sucrose solution made up with TBE buffer and was centrifuged at 38,000 rpm for 16 hours at 4°C in a Beckman Spinco SW41 rotor before being fractionated into twenty 0.6 ml fractions. Each fraction was precipitated with ethanol and any precipitate recovered was resuspended in 100 µl of TBE buffer and subjected to polyacrylamide gel electrophoresis (Figure 6.9).

Figure 6.8 : Gel Electrophoretic Analysis of the Melted dsRNA Synthesised *In vitro* by the RNA-Dependent RNA Polymerase from VTMoV-Infected Leaves of *N. clevelandii*.

Details of the treatments are given in Section 6.4 of this chapter. The urea containing slab gel was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

VF VTMoV RNA heated in formamide and formaldehyde.

P Untreated *in vitro* products.

V Untreated VTMoV RNA.

F *In vitro* product heated in formamide and formaldehyde.

W *In vitro* product heated in water.

Note the change in electrophoretic mobility of the viroid-like RNAs 2 and 3 after heating in formamide and formaldehyde. The positions of the treated VTMoV RNAs are indicated by arrows in the left of the figure and the *in vitro* products A and B on the right.

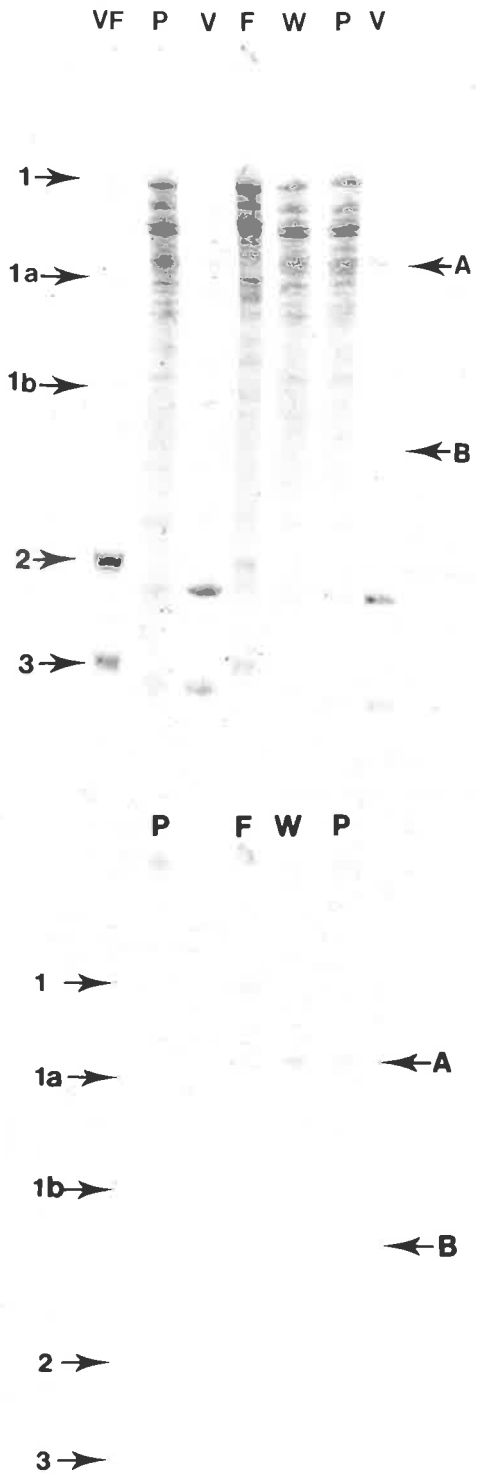
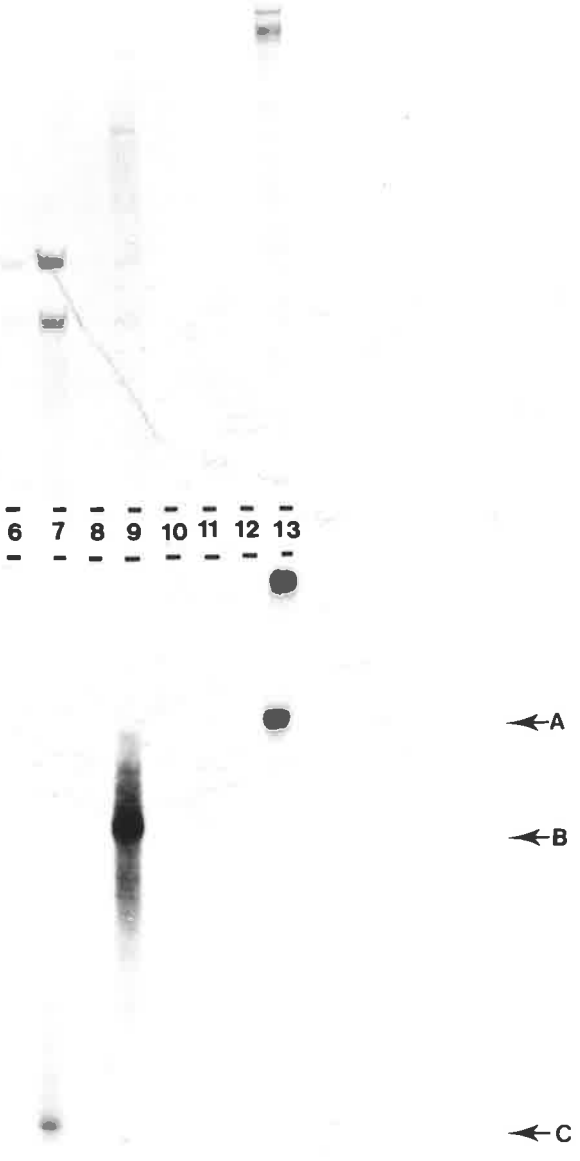

VF P V F W P V
1
1a
1b
2
3
A
B
P F W P
1
1a
1b
2
3
A
B

Figure 6.9 : Electrophoretic Analysis of the Sucrose Gradient Fractions Containing *In vitro* Products.

Samples of the first 20 fractions from a sucrose gradient were loaded on the gel (see text for details). The positions of fractions 6-13 are indicated, the other fractions did not contain any detectable radioactivity or RNA. Note the separation of the two *in vitro* labelled dsRNA species A and B and the additional product C, which is thought to be a breakdown product of B. Radioactivity located at the top of track 13 is thought to be aggregated dsRNA A. The urea containing gel was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).


6
7
8
9
10
11
12
13
A
B
C

Radioactive products were recovered almost exclusively in fraction 7, 9 and 13 from the gradient (Figure 6.9). Fraction 13 contained product A and some material which failed to enter the gel. Product B was located in fraction 9 but was contaminated with a small amount of product A. A low molecular weight product (C) was also detected in fraction 7 and this is thought to be a breakdown product of the in vitro synthesised dsRNA B. The detection of C is thought to be due to RNase contamination of the sucrose gradient, because the breakdown product was normally only detected when subjected to RNase treatment (see Figures 6.6 and 6.7).

Figure 6.10 shows the electrophoretic analysis of the three product-containing fractions after treatment with RNase A in 2 x SSC, to test for their RNase susceptibility. Also shown are the results of melting the products in fractions 7, 9 and 13 in the presence of formamide and formaldehyde. Both products B and C melted into material which migrated at the same rate as VTMoV RNA 3 (Figure 6.10, tracks 7F and 9F). When product B (fraction 9) was treated with RNase A, it was degraded to a small molecular weight RNA which migrated to the same position as the labelled RNA C found in fraction 7 (Figure 6.10, track 9R). Similar degradation products resulting from RNase A treatments can be seen in Figures 6.6 and 6.7 of this chapter. The narrowing of the RNA bands towards the bottom of the gel where RNase-treated samples were electrophoresed is due to salt which was present in these samples.

The product A in fraction 13, did not melt into a single sharp band with the electrophoretic mobility of RNA 1 as was observed in Figure 6.8. This is thought to be due to the dsRNA being nicked by exposure to RNase during centrifugation of the gradient.

Figure 6.10 : Analysis of the Fractionated *In vitro* Products by Polyacrylamide Gel Electrophoresis.

The *in vitro* labelled dsRNAs A, B and C separated on a sucrose gradient (see Figure 6.9) were given various treatments in an attempt to elucidate the relationship between them and VTMoV RNA (see Section 6.5 of this chapter for further details). The urea-containing gel slab was stained with toluidine blue and photographed (upper panel) before being dried and autoradiographed (lower panel).

Track:

1 VTMoV RNA.

2 VTMoV RNA heated in formamide and formaldehyde.

Other Tracks, dsRNAs from fractions 7, 9 and 13 from a sucrose gradient (see Figure 6.9) were subjected to various treatments before electrophoresis.

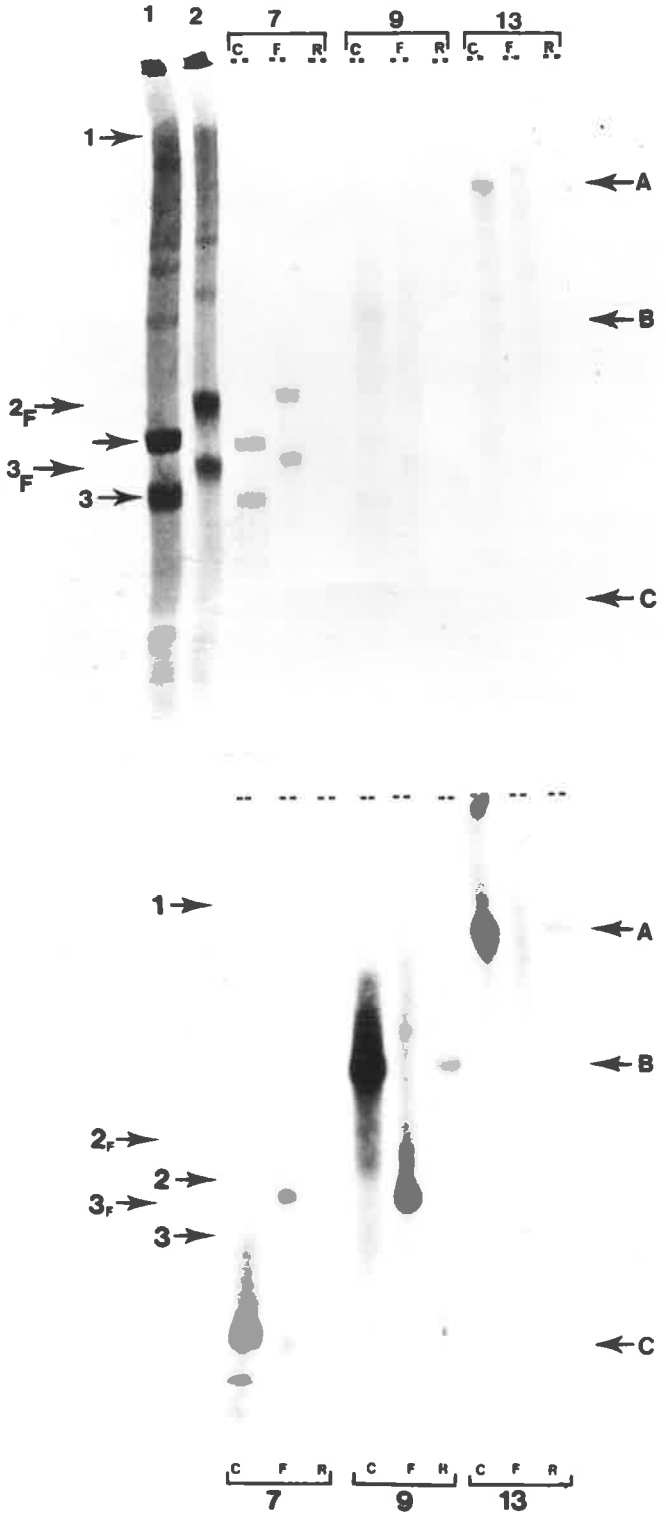
Treatments were :

C Control, no treatment.

F RNA melted in formamide and formaldehyde.

R Unmelted RNA treated with RNase in 2 x SSC.

The positions of the various RNA species are indicated by arrows. The VTMoV RNAs are labelled as 1, 2 and 3; 2_F and 3_F indicate the positions to which RNAs 2 and 3 migrated after heating with formamide and formaldehyde, respectively. The *in vitro* labelled products recovered from the gradient are indicated by A, B and C.


1
2
7
9
13
C F R
C F R
C F R
1
2F
3F
3
A
B
C
1
2F
2
3F
3
A
B
C
C F R
C F R
C F R
7
9
13

6.6 DOT-BLOT HYBRIDIZATION ANALYSIS OF THE IN VITRO PRODUCTS

The in vitro products synthesised by the polymerase in the S_1 fraction of VTMoV-infected leaves were analysed by dot-blot hybridization as outlined in Chapter 2. These experiments were based on the hybridization of ^{32}P labelled RNA synthesized in vitro to M13 DNA preparations containing plus or minus sense inserts of sequences from VTMoV RNA. Three blots were hybridized with the ^{32}P labelled in vitro products after various prehybridization treatments and a fourth blot was hybridized with ^{32}P end-labelled VTMoV RNA as a control (Figure 6.11).

Hybridization between the melted RNA products was observed only with DNA containing negative (-) sense inserts from both the 3' and 5' end regions of RNA 1 (see the legend of Figure 6.11 for details of inserts) and a small section of RNA 2 from Solanum nodiflorum mottle virus (SNMV) RNA 2 which has extensive sequences homologous to VTMoV RNA 2 (Haseloff and Symons, 1982). No hybridization was observed with DNAs having positive (+) sense VTMoV inserts or with the control DNA (M_{13} DNA without inserts) (Figure 6.11). These data indicate that only (+) sense VTMoV RNA is synthesized in vitro by the RNA-depedent RNA polymerase from infected N. clevelandii.

A high level of background radioactivity was observed when the melted in vitro synthesised dsRNA product was hybridized to the blots (Figure 6.11A). This background could be reduced by prehybridization of the nitrocellulose blots with nucleic acids extracted from healthy N. clevelandii leaves (Figure 6.11B). However, predigestion of the in vitro products with RNase A in 2 x SSC before melting and subsequent hybridization almost completely removed background radiation (Figure

Figure 6.11 : Dot-Blot Hybridization of the *In vitro* Products.

Dot-blot hybridization analysis of melted *in vitro* products was carried out as described in Chapter 2.

Treatments were:

A: Hybridization of *in vitro* products to DNA on nitro-cellulose paper which was not pre-hybrydized with the nucleic acid extract from healthy plants.

B: Hybridization of *in vitro* products to DNA on nitro-cellulose paper which was pre-hybridized with the nucleic acid extract from healthy plants.

C: Hybridization of *in vitro* products, pretreated with RNase A in 2 x SSC, to DNA on nitro-cellulose paper which was not pre-hybridized with the nucleic acid extract from healthy plants.

D: Hybridization of ^{32}P end-labelled VTMoV RNA to DNA on nitro-cellulose. No post-hybridization RNase A treatment was applied.

DNA spots bound to the nitrocellulose are indicated by arrows. The DNA treatments were.

1 Negative-sense DNA copy of RNA 1 containing base residue numbers 31 to 260 from the 5' end of the VTMoV RNA 1 which contains a total of approximately 1302 base residues.

2 Negative-sense DNA copy of RNA 1 containing residue numbers 1030 to 1182 located at the 3' end of the VTMoV RNA 1 which contains the coat protein genes.

3 Negative-sense DNA copy of SNMV viroid-like RNA containing base residue numbers 131 to 220.

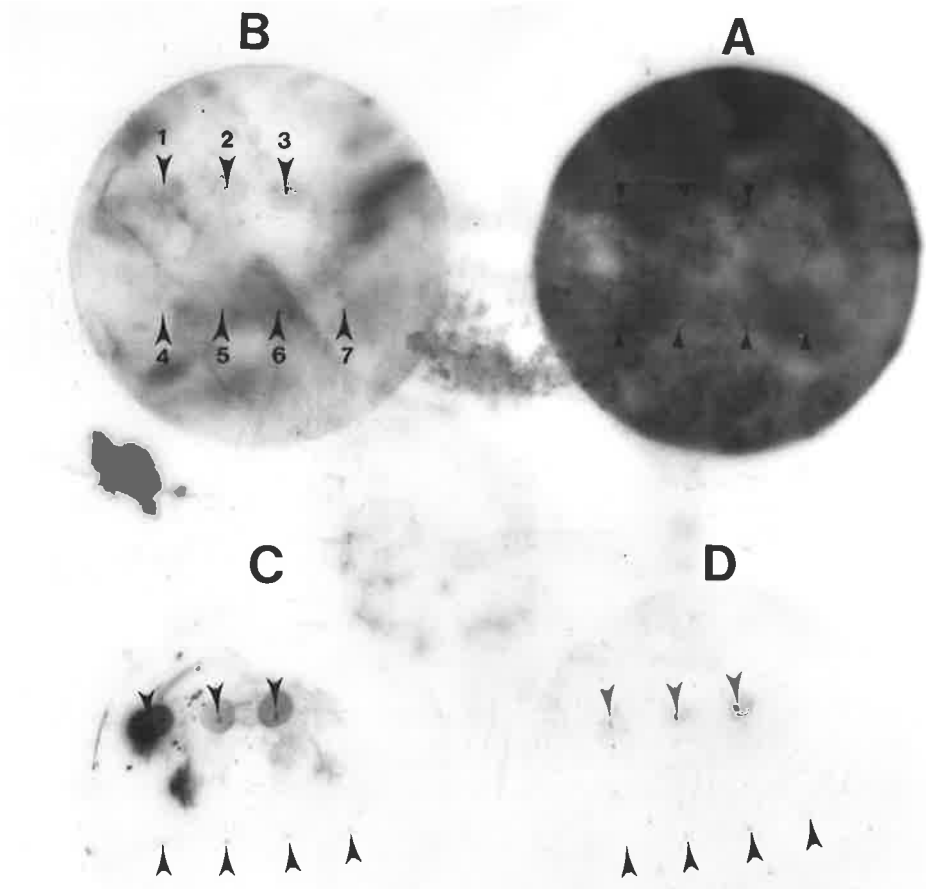
4 Positive-sense DNA copy of RNA 1 as in treatment 1.

5 Positive-sense DNA copy of RNA 1 as in treatment 2.

6 Positive-sense DNA copy of SNMV viroid-like RNA as in treatment 3.

7 M13 bacteriophage DNA without any inserts.

Note: The high background radioactivity present in treatments A and B which could not be removed by a post hybridization treatment


B
A
1
2
3
4
5
6
7
C
D

consisting of digestion with RNase A in 2 x ssc (20 µg/ml) for 30 minutes at 37°C. Pre-hybridization with yeast RNA or herring sperm DNA did not reduce the level of background radiation. Pre-treatment of the in vitro products with RNase A (treatment C) was successful in reducing background radiation. The lines on the autoradiograph are due to the plastic used to separate the nitrocellulose paper from the x-ray film.

6.11C). Only small amounts of background radiation were observed when end labelled VTMoV RNA was hybridized to the nitrocellulose blots (Figure 6.11D). This treatment was used as a control to ensure that VTMoV RNA was in fact hybridizing.

6.7 LIQUID HYBRIDIZATION OF THE IN VITRO LABELLED dsRNA PRODUCTS IN THE PRESENCE OF VTMoV RNA

To characterise the in vitro labelled dsRNA synthesized by the RNA-dependent RNA polymerase-template complex in the S_1 fraction from VTMoV-infected N. clevelandii leaves, liquid hybridization experiments were carried out. With these experiments a further check of the polarity of the in vitro labelled RNA and estimates of the amounts of the various species of VTMoV RNA synthesised in vitro, could be made.

In vitro synthesised RNA was subjected to RNase A treatment in 2 x SSC and the dsRNA was recovered by phenol extraction and ethanol precipitation. It was melted and then hybridised in the presence of VTMoV RNA. The basis of these experiments was that if RNA synthesised in vitro was of the same polarity as the VTMoV RNA, then by re-anealing the melted in vitro products in the presence of unlabelled VTMoV RNA, the labelled RNA would be displaced and could be digested by RNase A in 2 x SSC. By using a preparation of RNA 2 and 3 which had been separated from RNA 1 by sucrose density gradient centrifugation (Gould, 1981) as well as RNA 1 from the K1 isolate of VTMoV, the relative proportions of the RNA species synthesised in vitro could be estimated. The K1 isolate of VTMoV does not contain the viroid like RNAs 2 and 3 (R.I.B. Francki, C. Grivell, and K. Gibb personal communication).

Labelled dsRNA was synthesised using the S_1 fraction from leaves infected with VTMoV for six days using the standard *in vitro* assay described in Chapter 2; however, 50 µ Ci of ^{32}P-labelled GTP per 10 ml of assay mixture was used. The *in vitro* products were digested with 20 µg/ml of RNase A in 2 x SSC at 37°C for 30 minutes before addition of an equal volume of 0.1% SDS containing 10 µg/ml pronase, and further incubation at 37°C for 30 minutes. Subsequently, RNA extraction buffer and phenol were added and the sampled were shaken for 2 hours at 5°C. The aqueous phase containing the *in vitro* labelled ds RNA was precipitated and washed with ethanol. This treatment was used to ensure that the unlabelled VTMoV RNA synthesised *in vivo* prior to extraction of the leaves did not participate in the hybridization reaction.

A sample of the *in vitro* labelled ds RNA was melted in 50% formamide, 6% formaldehyde at 100°C for 5 minutes, chilled rapidly with liquid nitrogen and precipitated with ethanol. The precipitated RNA was resuspended in sterile distilled water.

Hybridizations were carried out in triplicate in 1.5 ml Eppendorff tubes. Hybridization mixtures contained 0.1% SDS, 2 x SSC, melted dsRNA (5 µg per tube) and VTMoV RNA as required. The final volume was 30 µl and this was overlaid with two drops of sterile Ondina oil (Shell Co., Australia). The samples were floated in a 16 litre water bath at 100°C and allowed to cool slowly to 40°C overnight (Rezaian and Francki, 1974).

After hybridization, 200 µl of 2 x SSC containing 20 µg/ml RNase A was added to the required tubes, and incubated at 37°C for 30 minutes. Where low salt treatment was required, the RNase A was added in water.

After the RNase treatment, 400 µl of 80 mM pyrophosphate containing 1 mg/ml yeast RNA was added as carrier, and 400 µl of 25% TCA was used to precipitate the RNA. The precipitates were collected and their ^{32}P content estimated by the method outlined in Chapter 2.

Results of this experiment are shown in Figure 6.12. Between 81 and 90% of the labelled RNA self reannealed in treatments without any added VTMoV RNA. The data in Figure 6.12 indicate that VTMoV RNA 1 and the mixture of RNAs 2 and 3 competed to a similar degree with the hybridization of the in vitro labelled RNA. This indicates that RNA 1 and the viroid-like RNAs (RNA 2 and RNA 3) were synthesised in about equal proportions. These experiments also confirm the conclusion reached from the dot-blot experiments that positive-sense viral RNA was synthesised in vitro by the RNA-dependent RNA polymerase-template complex.

When unfractionated VTMoV RNA was used in the hybridization experiment, about 4000 cpm of labelled RNA remained undisplaced even at the highest level of competitor RNA used (Figure 6.12). When unhybridized melted product was treated with RNase A in 2 x SSC, 4690 cpm was recovered by TCA precipitation which suggests that not all the dsRNA used in the hybridization experiment had melted. This unmelted RNA may have survived the hybridization procedure and this could account for the incomplete removal of radioactive RNA in the hybridization experiment. Digestion of hybridized and unhybridized melted dsRNA with RNase in 0.01 x SSC, further reduced the recovery of labelled RNA by TCA precipitation (see legend to Figure 6.12) indicating that most of the ^{32}P label was that incorporated into RNA.

Figure 6.12 : Liquid Hybridization of the In vitro Products in the Presence of VTMoV RNA.

Melted in vitro labelled dsRNA was hybridized with unlabelled VTMoV RNA as described in the text (Section 6.7). Self re-anealing was between 81 and 90% in the absence of added VTMoV RNA.

Treatments were:

▲—▲ Addition of RNA from K_1 isolate of VTMoV (RNA 1).

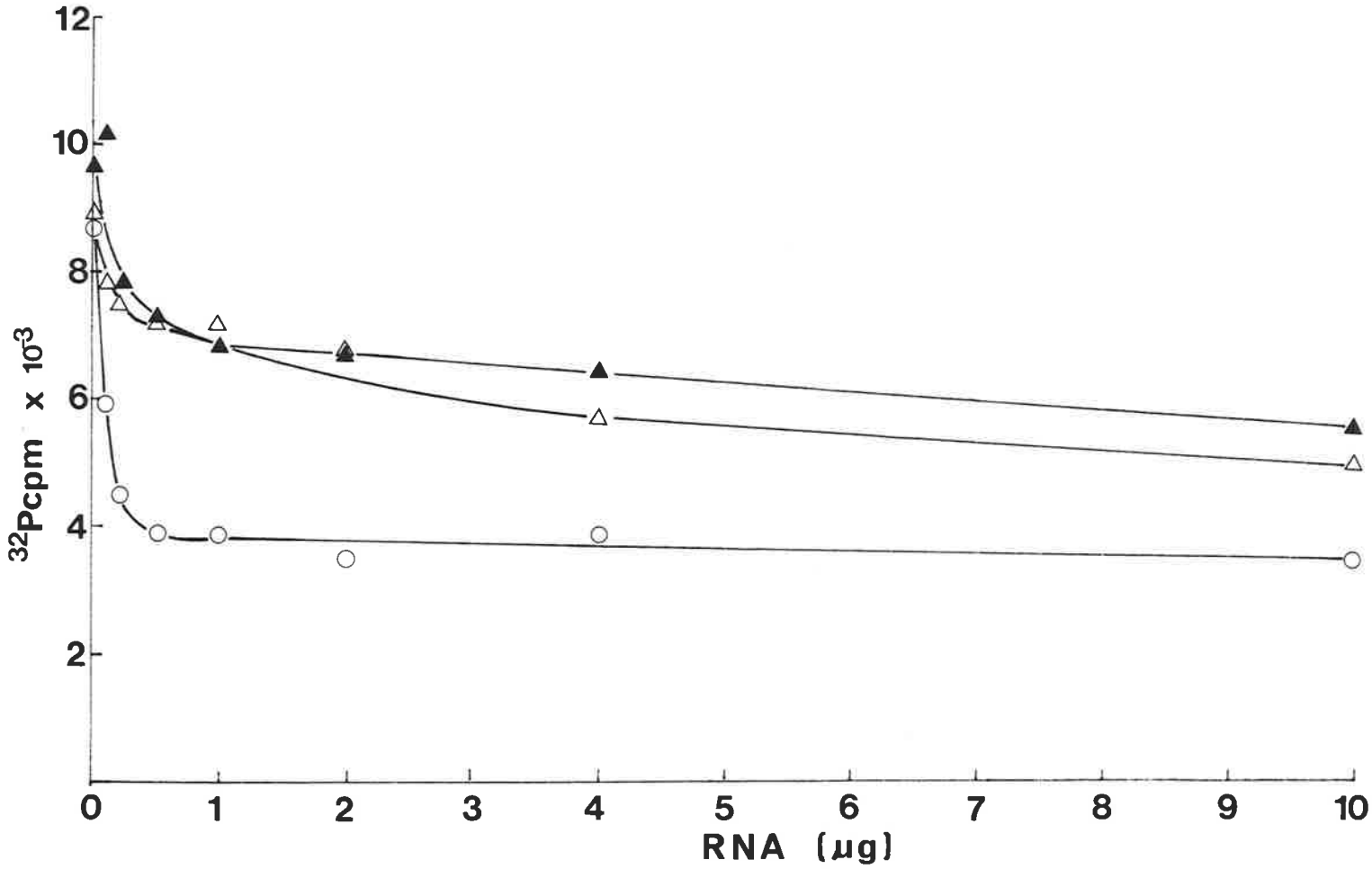
△—△ Addition of RNA 2 and 3 purified by gradient centrifugation.

O—O Addition of RNAs 1, 2 and 3 from VTMoV.

The results of additional treatments were as follows:

Hybridization	RNase A treatment	^{32}P-labelled RNA recovered (cpm)
-	none	13620
-	in 2 x SSC	4690
+[a]	in 0.01 x SSC	2530
-	in 0.01 x SSC	1680

[a] Hybridization in the absence of added VTMoV RNA.


12
10
8
6
4
2
^{32}Pcpm x 10^{-3}
0
1
2
3
4
5
6
7
8
9
10
RNA (µg)

6.8 CONCLUSION

The RNA-dependent RNA polymerase-template complex extracted from VTMoV-infected leaves of N. clevelandii, synthesized two species of RNA (A and B) in vitro. Both RNA species were found to be dsRNA with A having a M_r of about 3.6×10^6 and B, about 0.72×10^6.

In vivo experiments in which VTMoV-infected leaf tissues were infiltrated with tritiated uridine resulted in detection of an RNA species of similar but not identical electrophoretic mobility to the in vitro product RNA A. Although no RNA similar to B was detected by in vivo labelling, a low molecular weight RNA was detected. The electrophoretic mobility of RNA C was similar to that of a breakdown product detected when the in vitro product B was exposed to RNase A. This suggests that RNA B may be a precursor of RNA C; however, the absence of RNA B in the in vivo labelled RNA samples can only be explained if RNA B is only present in minute quantities and is rapidly converted to RNA C in intact leaf tissue.

Analysis of the melted RNAs A and B by polyacrylamide gel electrophoresis and molecular hybridization experiments, indicate that only positive sense VTMoV RNA was synthesised in vitro with A containing nucleotide sequences of RNA 1 and B, those of the viroid-like RNAs 2 and 3. Hybridization displacement studies, using unlabelled VTMoV RNA as a competitor for the in vitro labelled RNA, indicate that RNA 1 and the viroid like RNA were labelled in approximately equal proportions.

CHAPTER 7

KINETICS OF THE RNA-DEPENDENT RNA POLYMERASE REACTION

Synthesis of RNA or DNA by either RNA or DNA template-dependent polymerases proceeds in a series of steps that usually involve ; polymerase to template binding, followed by nucleotide chain initiation, elongation, termination and finally enzyme release (Chamberlin, 1970). Data presented in the previous chapters indicates that the *in vitro* synthesis of VTMoV-specific RNA by the enzyme-template complex isolated from infected *N. clevelandii* leaves involved only RNA chain elongation. There is no evidence that the products of *in vitro* synthesis were released from the enzyme-template complex or that new enzyme-template complexes were formed.

Studies of RNA chain elongation are possible with the enzyme-template system isolated from VTMoV-infected leaves of *N. clevelandii* because The dsRNA appears to be synthesised with no evidence that the enzyme becomes free to accept other RNA templates during the reaction. The complex isolated from VTMoV-infected tissue consists of polymerase molecules which are bound to endogenous template. Such molecules can resume *in vitro* the elongation of RNA strands initiated *in vivo*.

Under the assay conditions described in Chapter 2, the enzyme-template complex incorporates ^{32}P-GTP into RNA at a constant rate for four minutes followed by a reduced rate over the remainder of the experiment at 20^{o}C (Figure 5.1A). This reduction in reaction rate can be attributed to nucleotide chain termination and enzyme inactivation (see Chapter 5).

All rate measurements given in this chapter were derived from the first two minutes of the reaction. It is assumed being made that nucleotide chain termination and enzyme inactivation over this period is insignificant and that the rate measurement obtained is a reasonable estimate of the initial velocity of the reaction. The amount of enzyme used for kinetic work was within the range where the rate of RNA synthesis is directly proportional to the amount of enzyme-template complex added as shown in Chapter 5 (Figure 5.3). These conditions are similar to those used by Rhodes and Chamberlin (1974) in the study of RNA synthesis by DNA dependent RNA polymerase from Eschirichia coli.

7.1 STEADY STATE KINETIC MODEL FOR CHAIN ELONGATION

In developing a model for RNA chain elongation by RNA-dependent RNA polymerase, use was made of a model proposed by Rhodes and Chamberlin (1974) for RNA chain elongation by DNA-dependent RNA polymerase. A general steady state mechanism for polymerase reactions has been discussed by McClune and Chow (1979).

With the polymerase-RNA template complex four states are assumed. These are designated as ET_A, ET_C, ET_G and ET_U and defined as ; the enzyme-template complex containing an incomplete RNA chain with a 3' terminal ribonucleotide residue occurring on the template at an AMP, CMP, GMP or UMP respectively, and ready to accept the complementing base residue.

Taking the complimentary pair ET_A and UTP as an example the simplest sequence of steps for chain elongation involves the following series of reactions

$$ET_A + MgUTP \quad \overset{K_u}{} \quad ET_A(MgUTP) \qquad (1)$$

$$ET_A(MgUTP) \quad \overset{k}{} \quad ET_x + MgPPi \qquad (2)$$

Where x represents the next nucleotide residue along the template, ET_A is the enzyme-template complex free of substrate and $ET_A(MgUTP)$ the binary enzyme-template and substrate complex. K_u is the dissociation equilibrium constant for reaction (1) and k is the catalytic rate constant for the appearance of product (MgPPi) and elongation of the RNA chain being synthesised.

Step (1) involves the reversible binding of substrate to the enzyme-template complex and step (2) the formation of the phosphodiester bond and translocation of the enzyme to the next nucleotide residue on the template. Similar reaction pathways can be written for each of the four ribonucleoside triphosphates involved in RNA synthesis.

In the above example the value of the dissociation constant is given by

$$K_u = \frac{[ET_A][MgUTP]}{[ET_A(MgUTP)]} \qquad (3)$$

From the above equation we can express the concentration of the enzyme-template complex in terms of the single active enzyme from $ET_A(MgUTP)$.

Thus :

$$[ET_A] = \frac{K_u}{[MgUTP]} \quad [ET_A(MgUTP)] \qquad (4)$$

The mass conversion equation expressing the molecular distribution of the total enzyme template complex ($[ET_A]_T$) among the two species is :

$$[ET_A]_T = [ET_A] + [ET_A(MgUTP)] \tag{5}$$

Under saturating substrate conditions (i.e. when the substrate concentration is very much larger than enzyme-template concentration) we have :

$$[ET_A]_T = [ET_A(MgUTP)] \tag{6}$$

and the observed reaction velocity (ν_u) should be the maximum velocity (V_u) thus

$$V_u = k[ET_A]_T \tag{7}$$

In cases where the substrate is limiting, the observed velocity (ν_u) will be porportional to $[ET_A(MgUTP)]$ and hence :

$$\nu_u = k[ET_A(MgUTP)] \tag{8}$$

Dividing (8) by (7) we have :

$$\frac{\nu_u}{V_u} = \frac{k[ET_A(MgUTP)]}{k[ET_A]_T} = \frac{[ET_A(MgUTP)]}{[ET_A]_T} \tag{9}$$

Dividing the numerator and denominator by $[ET_A(MgUTP)]$ we have :

$$\frac{\nu_u}{V_u} = \frac{[ET_A(MgUTP)]/\ [ET_A(MgUTP)]}{[ET_A]_T\ /\ [ET_A(MgUTP)]} = \frac{1}{[ET_A]_T\ /\ [ET_A(MgUTP)]} \qquad (10)$$

Substituting (5) we have :

$$\frac{\nu_u}{V_u} = \frac{1}{\frac{[ET_A] + [ET_A(MgUTP)]}{[ET_A(MgUTP)]}}$$

$$= \frac{1}{1 + [ET_A]/[ET_A\ (MgUTP)]} \qquad (11)$$

Finally, substituting the value of $[ET_A]$ from (4) we have :

$$\frac{\nu_u}{V_u} = \frac{1}{1 + \frac{K_u}{[MgUTP]}} \qquad (12)$$

or

$$\frac{V_u}{\nu_u} = 1 + \frac{K_u}{[MgUTP]} \qquad (13)$$

A similar steady state rate equation can be written for each substrate using the above model.

The overall steady state rate equation for a system requiring all four nucleoside triphosphates is

$$\frac{V}{\nu} = 1 + \frac{K_a}{A} + \frac{K_c}{C} + \frac{K_g}{G} + \frac{K_u}{U} \qquad (14)$$

Where ν is the initial velocity (observed velocity) of the reaction at concentrations of MgATP, MgCTP, MgGTP, and MgUTP of A, C, G and U respectively ; V is the maximum velocity obtained at saturating substrate concentrations ; and K_a, K_c, K_a and K_u are the dissociation constants for each substrate. The dissociation constant (K_x) will be equal to the Michaelis constant if k is negligible. This is the case if reaction (2) is the rate limiting step.

Equation (14) falls into the general class of kinetic mechanisms which Cleland (1963) has described as ping-pong reactions. The steps outlined in (1) and (2) are probably complex ; for example step (2) is likely to involve the formation of a phosphodiester bond, release of pyrophosphate, isomerization and translocation of the enzyme to align the active site with the next template base. However, for a ping-pong reaction mechanism the presence of these substeps in the reaction sequence does not alter the kinetic equation (Cleland, 1963).

In the system we are studying there are two possible modes of RNA synthesis :

1. The polymerase is moving along a single stranded RNA template resulting in the formation of a dsRNA strand.
2. The enzyme is copying from a dsRNA template in a similar fashion shown for DNA-dependent RNA polymerase transcription of Coliphage T7 DNA (Hyman and Davidson, 1970).

The latter system could be visualized as a polymerase molecule moving along a dsRNA template releasing as RNA ahead of it and forming a new dsRNA strand after it.

Until now, the effect of base ratio of the template, on the steady state rate equation has been ignored. Transcription proceeds by a stepwise addition of ribonucleotide to the growing RNA chain as directed by the nucleotide sequence in the transcribed RNA strand. Since a finite amount of time is required for each addition of a nucleotide and it is reasonable to expect each of the four nucleotides to have different incorporation rates. The base sequence composition of the template will thus have an influence on the overall reaction rate.

If f_x is the mole fraction of ribonucloetide x in the product and t_x the mean time for addition of ribonucleotide x to the chain, after the previous ribonucleotide has been incorporated (where x = AMP, CMP, GMP or UMP) ; the average velocity of incorporation, ν, (in units of nucleotides per unit time per chain) and the average time of incorporation per nucleotide, t, are given by :

$$\frac{1}{\nu} = t = \Sigma f_x t_x \qquad (15)$$

From equation (14) we have

$$\frac{1}{\nu} = \frac{1}{V}\left(1 + \frac{K_a}{A} + \frac{K_c}{C} + \frac{K_g}{G} + \frac{K_u}{U}\right) \qquad (16)$$

By incorporating equation (15) into equation (16) we have

$$\frac{1}{\nu} = \frac{1}{V}\left(1 + \frac{f_a K_a}{A} + \frac{f_c K_c}{C} + \frac{f_g K_g}{G} + \frac{f_u K_u}{U}\right) \qquad (17)$$

This can be generalized into the equation

$$\frac{1}{\nu} = \frac{1}{V} + \frac{1}{V}\frac{\Sigma f_x K_x}{[x]} \qquad (18)$$

where x corresponds to ATP, CTP, GTP or UTP.

The new steady state rate equation taking base composition of the product (and therefore template) into account is

$$\frac{V}{\nu} = 1 + \frac{f_a K_a}{A} + \frac{f_c K_c}{C} + \frac{f_g K_g}{G} + \frac{f_u K_u}{U} \qquad (19)$$

In a typical experiment one ribonucleoside triphosphate Sy (substrate y) is adjusted to a lower concentration than the other three so that it is rate limiting and this concentration is varied. Equation (17) predicts a Linewever-Burk plot (Lineweaver & Burk, 1934) of $1/\nu$ versus $1/[Sy]$ should be a straight line with a slope of

$$\frac{d\frac{1}{\nu}}{d[Sy]} = f_y K_y \qquad (20)$$

and the intercept on the $1/\nu$ axis (at $1/[Sy] = 0$) is

$$\frac{1}{\nu}_{(1/[Sy] = 0)} = \frac{1}{V} + \frac{1}{V}\sum_{x \neq y}\frac{f_x K_x}{[x]} \qquad (21)$$

From equation (19) it is predicted that values of $f_x K_x$ and V for a given ribonucleoside triphosphate can be obtained directly by varying one substrate while the other three are present in excess. However, this approach is impractical as an experimental procedure because competitive substrate inhibition is likely to occur at high substrate concentration. This inhibition is due to nucleoside triphosphates which are not complimentary to the template base at the active enzyme site randomly entering the active site in the manner of reaction (1) and thus inhibiting chain elongation. This form of inhibition has been shown to occur with DNA-dependent RNA polymerase by Rhodes and Chamberlin (1974).

To overcome this problem the approach proposed by Fromm (1967) for three substrate enzyme systems was used. Fromm's approach required one substrate to be varied whilst holding the other two reactants at a fixed level in the general concentration range of their Michaelis constants. This experiment is then repeated but at a different concentration of fixed substrates, the ratio of fixed substrates in the two studies being maintained (Fromm, 1967).

In the study of the RNA-dependent RNA polymerase-template complex one ribonucleoside triphosphate was varied at a lower concentration than the other three which were used at a ratio of 1:1:1. The procedure of Fromm (1967) was then used to obtain the kinetic data.

From equation (19) it can be deducted that by using the experimental procedure outlined above Lineweaver-Burk plots give a series of parallel lines. By replotting the $1/\nu$ axis intercepts against the reciprocal concentration of the fixed substrates, V for the varied substrate may be estimated. The slope of the lines gives an estimate of

$f_x K_x$. Unfortunately, the dissociation constant (K_x) cannot be calculated because the value of f_x is not known for this system.

7.2 RESULTS OF THE ENZYME KINETIC EXPERIMENTS

To test the degree to which RNA synthesis fits the previously outlined kinetic model the rate of RNA chain elongation was measured as the rate of GTP incorporation at a variety of nucleoside triphosphate concentrations. Rate measurements were made at various concentrations of GTP while holding the concentration of the other three substrates constant in the manner described in the preceeding section of this chapter.

Data obtained are presented graphically in Lineweaver-Burk reciprocal plots (Figure 1A) and appear to have a form which is characteristic of a ping-pong kinetic mechanism. The lines of Figure 1A were fitted using the method of least squares (Pollard, 1977) which also gives estimates of the y axis intercepts and slope of the lines.

From equation (19) it is predicted that a ping-pong mechanism would give a series of parallel lines. The calculated slopes of the lines in Figure 7.1A were plotted as a function of the reciprocal of the concentration of the nucleoside triphosphates which had previously been held constant (Figure 7.2). The method of least squares was again used to fit a line through the points and a slope of 0.008 was calculated for that line. Although a slope of 0 was expected for a ping-pong mechanism a slope of 0.008 can be attributed to experimental error alone. The overall slope of the lines in Figure 7.1A was obtained as the y intercept of the line in Figure 7.2 and has a value of 0.78. This

Figure 7.1

A. (On left). Plot of the reciprocal of the initial reaction velocity (minutes/picomoles) versus the reciprocal of GTP concentration (micromolar^{-1}) at varying levels of each of ATP, CTP and UTP. Assay was by the protocol given in the text and the concentrations of ATP, CTP and UTP were varied as indicated below.

○—○ 9 μM

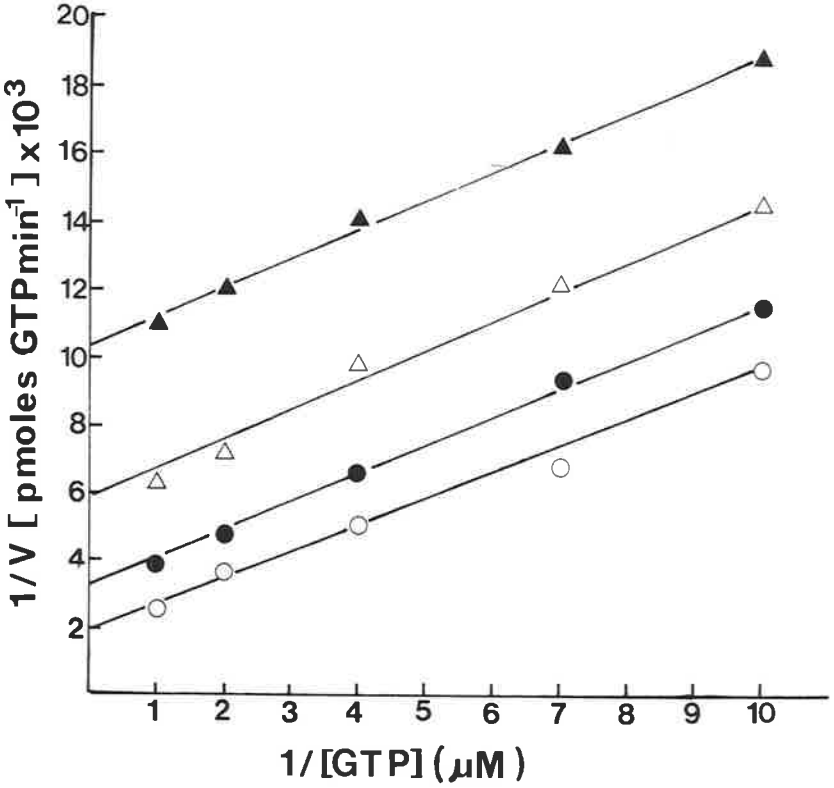
●—● 4.5 μM

△—△ 2.25 μM

▲—▲ 1.125 μM

The amount of enzyme used was 4.437 units per assay.

B. (On right). A replot of the intercepts of the lines in Figure 7.1A with the y axis (units as in Figure 7.1A) versus the reciprocal of the concentrations of each of ATP, CTP and UTP being designated as [XTP] (micromolar^{-1}).


1/V [pmoles GTP min^{-1}] x 10^3
20
18
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
1/[GTP] (μM)

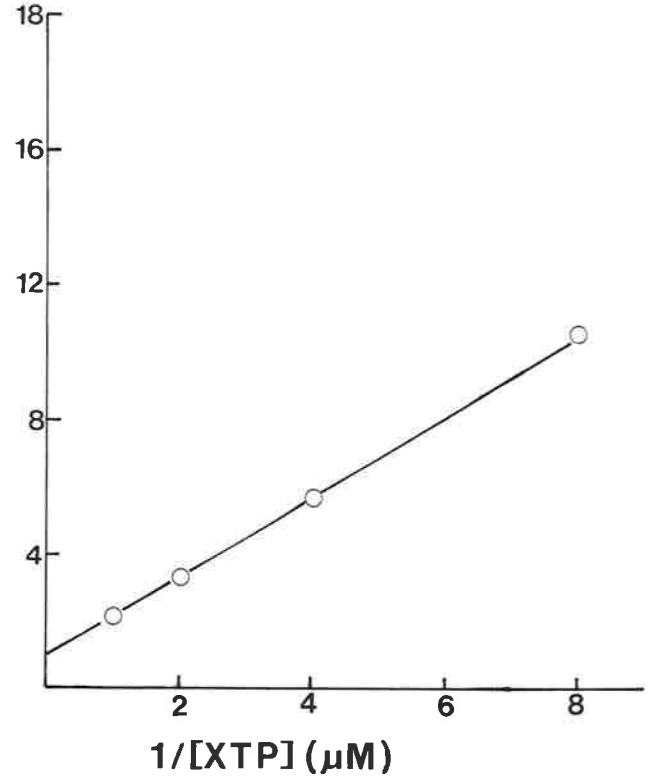
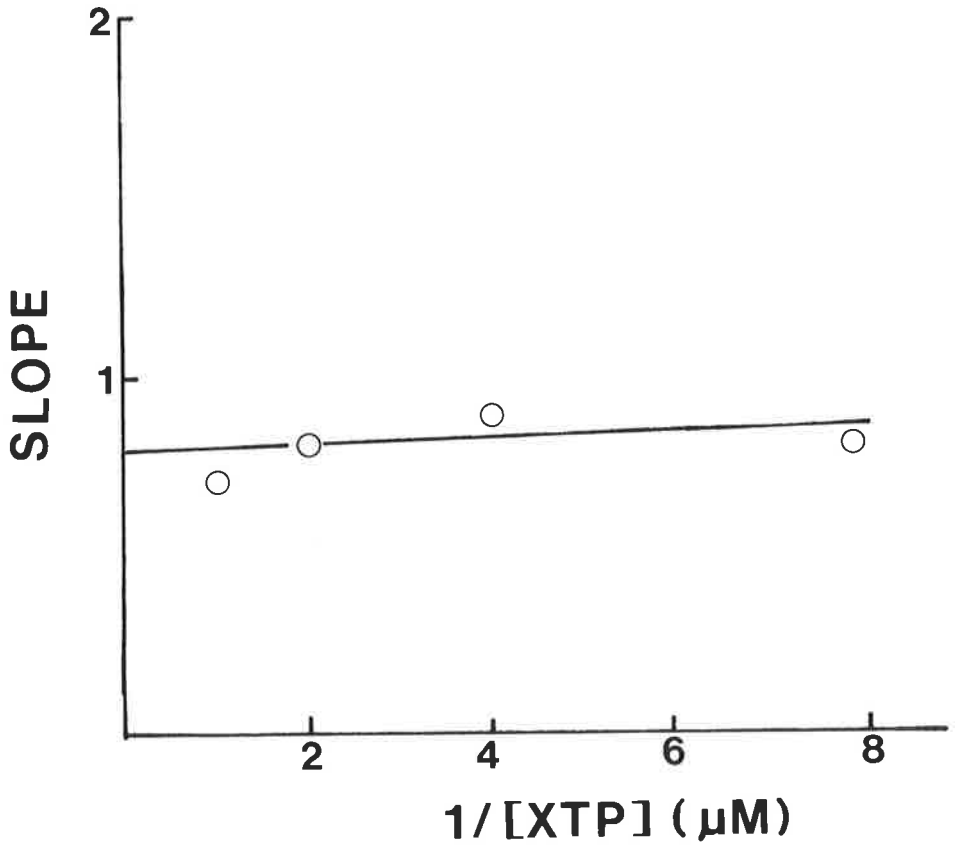

18
16
12
8
4
2
4
6
8
1/[XTP] (μM)

Figure 7.2

Plot of the slopes of the lines in Figure 7.1A against the reciprocal of the concentrations of the three ribonucleoside triphosphates ATP, CTP and CTP being designated as [XTP] (micromolar $^{-1}$). Further details are given in the text.


SLOPE
2
1
2
4
6
8
1/[XTP] (μM)

value can be substituted in equation (20). However, the Ks value for GTP cannot be calculated because the value of the mole fraction of GMP (f_y in the equation) for the product is not known.

The value of 1/V was obtained by plotting the y axis intercepts of each of the lines in Figure 7.1A as a reciprocal of the concentrations of the nucleoside triphosphates which had been held constant. As expected from equation (21) this treatment of the data gives a linear relationship from which the value 1/V can be obtained as the intercept of the y axis (Figure 7.1B). A value of 0.95 was calculated for 1/V using the method of least squares to fit a line through the points in Figure 7.1B.

7.3 CONCLUSIONS

A ping-pong mechanistic model incorporating the tetra-reactant nature of RNA synthesis could be used to describe the reaction mechanism by which RNA-dependent RNA polymerase from VTMoV-infected plants synthesises RNA *in vitro*.

The enzyme kinetic data indicates that all four ribonucleoside triphosphates are required for the RNA polymerase reaction to proceed. The series of parallel lines observed in Figure 7.1A preclude the possibility of end-labelling reactions being responsible for the incorporation of ^{32}P-GMP into RNA. If significant end-labelling occurred, reduced reaction rates would have been observed with increasing concentrations of the unlabelled nucloeside triphosphates ATP, CTP and UTP. This would have been due to the dilution of the labelled GTP by the other unlabelled nucloeside triphosphates so that

any non-template directed RNA chain elongation would result in reduced rates of ^{32}P-GMP incorporation.

The kinetic data presented, suggest that the enzyme has only one substrate binding site and no allosteric modification of enzyme activity is effected by the nucloeside triphosphates.

CHAPTER 8

GENERAL DISCUSSION

8.1 DETECTION OF RNA-DEPENDENT RNA POLYMERASE ACTIVITY IN VTMoV-INFECTED PLANTS

Extracts from healthy leaves of N. clevelandii did not contain any detectable RNA-dependent RNA polymerase by the in vitro assay system described in Section 2.2.3. However, when leaves of N. clevelandii were inoculated with VTMoV, RNA-dependent RNA polymerase was detected four days after inoculation and increased in amount thereafter, until the leaves collapsed about 5 days later. The polymerase activity detected in VTMoV infected leaves did not require the addition of RNA template to the assay mixture and was only slightly effected by the addition of RNase A. This indicates that the template associated with the polymerase enzyme was relatively stable and hence probably dsRNA. The enzyme and template appear to exist as a complex similar to that detected in CPMV-infected cowpea leaves described by Dorssers et al. (1983).

Experiments designed to determine the cellular location of the VTMoV-induced polymerase-template complex indicate that the enzyme is restricted to the cytoplasm. The complex was not sedimented by centrifugation at 25,000 g for 20 minutes which sedimented cell organelles and cellular membrane material indicating that the complex was not membrane-bound. The cellular locations of virus-induced RNA polymerases from other reported host plant-virus combinations, which were detected by in vitro assay techniques without the use of added template, are listed in Table 8.1. Most of these reports indicate that

Table 8.1 : Reported Cellular Location of Virus-induced RNA-dependent RNA Polymerases

Location	Virus	Reference
Cytoplasm	Tobacco ringspot virus (TRSV)	Rezaian et al. (1976)
	Velvet tobacco mottle virus (VTMoV)	This thesis
Cytoplasm and Cytoplasmic membrane	Cucumber mosaic virus (CMV)	May et al. (1970)
	Tobacco necrosis virus (TNV)	Fraenkel-Conrat (1976)
Cytoplasmic membranes	Alfalfa mosaic virus (AMV)	Weening and Bol (1975)
	Broad bean mottle virus (BBMV)	Semal (1969)
	Brome mosaic virus (BMV)	Hardy et al. (1979) Bujarski et al. (1982)
	Cowpea chlorotic mottle virus (CCMV)	White and Dawson (1978)
	Cowpea mosaic virus (CPMV)	De Zoeten et al. (1974)
	Tobacco mosaic virus (TMV)	Ralph et al. (1971)
Chloroplast envelope	Turnip yellow mosaic virus (TYMV)	Lafleche et al. (1972)
Nuclear Membrane	Pea enation mosaic virus (PEMV)	Powell et al. (1977)

the polymerases are either located in the cytoplasm or associated with cytoplasmic membranes.

The replication of VTMoV RNA is of particular interest because it ecapsidates two forms of ssRNA 1 of M_r about 1.5 x 10^6 and is similar to that found in many other viruses with polyhedral particles (Mathews, 1982), and the viroid-like RNA consisting of circular and linear molecules, RNA 2 and RNA 3, respectively (see Chapter 1 for details of their properties). The structural similarity between viroids and VTMoV RNAs 2 and 3 is not, however, reflected in their biological properties. Viroids are naked RNAs capable of infecting and replicating autonomously in host plants. On the other hand the viroid-like RNAs from VTMoV are dependent for their replication on RNA 1 (Gould et al., 1981).

Data presented in this thesis indicate that the viroid-like RNAs 2 and 3 together with RNA 1, replicate in the cytoplasm of VTMoV-infected leaf cells utilizing an RNA-dependent RNA polymerase. In contrast, viroid replication appears to be limited to the nucleus of infected cells (Diener, 1971 ; Schumacher et al., 1983 ; Semancik and Harper, 1984 ; Sanger, 1984). The enzyme involved in viroid synthesis has yet to be conclusively identified but it has been shown to be sensitive to α-amanitin in both protoplasts (Muhlback and Sanger, 1979) and cell free extracts (Flores and Semancik, 1982). No such inhibition of VTMoV induced RNA-dependent RNA polymerase by α-amanitin was observed in this work (Chapter 3, Table 3.6).

Only two enzymes are known to be inhibited by α-amanitin, DNA-dependent RNA polymerase II and DNA-dependent RNA polymerase III (Rackwitz et al., 1981). Sanger (1984) has pursued the possibility of

DNA-dependent RNA polymerase II as the one responsible for viroid replication. However, Semancik and Harper (1984) have produced evidence that DNA-dependent RNA polymerase I and III may also be involved in viroid replication. The possibility of a different and as yet unidentified polymerase, which is also sensitive to α-amanitin being involved in viroid replication should not be overlooked.

The origin of the polymerases involved in virus replication has not been clearly established (Hall et al., 1983) however, due to the small size of viroid RNAs (240 - 380 nucleotides) a host enzyme is most likely responsible. In the case of viroid-like RNA systems, it seems that either a host or viral RNA 1-coded enzyme could be involved.

Only one biochemical similarity between the enzyme involved in VTMoV and viroid replication system has been observed. The Mg^{++} and Mn^{++} response curves of the cytoplasmic VTMoV-induced RNA-dependent RNA polymerase are similar to those reported for the synthesis of citrus exocortis viroid in nuclei-rich cell-free preparations from Gynura aurantiaca (Semancik and Harper, 1984). Although the significance of this is not understood it should be noted that only partial replacement of the Mg^{++} requirement of the virus-induced RNA-dependent RNA polymerase by Mn^{++} has previously been reported in plants infected by other viruses (Zabel, 1978 ; White and Dawson, 1980 ; Clerx and Bol, 1978).

It can be concluded that although the viroid-like RNAs of VTMoV and viroids display many similar physical properties, there are important differences in the modes of their replication. Whereas viroids appear to replicate in plant cell nuclei utilizing enzymes usually involved in the

transcription of nuclear DNA into RNA, viroid-like RNA replicates in the cytoplasm utilizing an RNA-dependent RNA polymerase. The source of the RNA-dependent RNA polymerase is unknown but it is almost certainly not that of the viroid-like RNA itself which *in vitro*, at least, lacks messenger activity (Francki *et al.*, 1984).

8.2 SHORTNESS OF THE *IN VITRO* REACTION

Time course studies of the *in vitro* polymerase reaction revealed that RNA synthesis ceases soon after 10 minutes when the enzyme-template complex in the S_1 and P_{12} fractions from VTMoV-infected leaves, was incubated at 25°C and pH 8.8. This time course of *in vitro* RNA synthesis is similar, but shorter, to the reported observation on CPMV-(Zabel *et al.*, 1972) and BMV-(Hardy *et al.*, 1979) induced replicase where *in vitro*, RNA synthesis ceased after a reaction time of about 30 minutes. In the case of AMV, the induced replicase continued RNA synthesis for even longer, about 60 minutes (Clerx and Bol, 1978).

The reason for the cessation of RNA synthesis was explored in a variety of experiments using both the S_1 and P_{12} fractions from VTMoV-infected leaves as sources of the enzyme-template complex. It was found that adding nucleoside triphosphates to reaction mixtures after, or shortly before, cessation of RNA synthesis by the complex in the S_1 fraction did not result in the resumption of RNA synthesis (Chapter 3). This indicates that exhaustion of substrate was not responsible for the cessation of enzyme activity. Other experiments designed to test the stability of the *in vitro* labelled RNA synthesized by the replicase complex indicated that cessation of enzyme activity could not be attributed to an equilibrium between RNA synthesis and degradation.

The effect of pH and temperature on the stability of the partially purified RNA-dependent polymerase-template complex (P_{12} fraction) was studied and both these factors were found to influence enzyme inactivation. It was found that the enzyme lost activity significantly by exposure to buffers at pH levels above 7.6 at 20°C, and to temperatures above 20°C at pH 8.8 (the pH used in the *in vitro* assay). Also, RNA synthesis at 20°C showed a slower reaction rate than at 25°C, but at 20°C, the reaction proceeded for longer and resulted in the synthesis of more labelled RNA. These observations indicate that inactivation of the enzyme during the *in vitro* assay was at least partly responsible for the early cessation of RNA synthesis.

Although high temperatures and pH levels were found to inactivate the RNA-dependent RNA polymerase, a pH of 8.8 was used in the assay because this was the pH at which maximum enzyme activity was observed. The optimum temperature for enzyme activity was found to be between 20 and 25°C. This is lower than the temperature optimum observed by most authors (Table 8.2) for other virus-induced RNA-dependent RNA polymerases. The reason for this is probably due to the use of a pyruvate kinase system for nucleoside triphosphate regeneration during assay. This system was originally used by Semal *et al*. (1964) in their work on RNA synthesis in extracts from TMV-infected tobacco leaves. Pyruvate kinase has a temperature optimum of 37°C and with the low concentrations of nucleoside triphosphates used in most of the reports, the nucleoside triphosphate regenerating system may have masked the true optimal temperature of the enzyme systems studied. This suggestion could be tested by comparing the temperature optimum for *in vitro* polymerase assay systems with and without a pyruvate kinase nucleoside triphosphate regeneration system.

Table 8.2 : Reported Temperature Optima and Assay Temperatures for Various Virus-induced Polymerases

Virus	Host Plant	Assay Temperature (°C)	Optimal Temperature (°C)	Presence of Pyruvate Kinase in the assay (+ or -)	Reference
Alfalfa mosaic virus (AMV)	broad bean	35	NP[a]	+	Weening & Bol (1974)
Broad bean mottle virus (BBMV)	broad bean	30	NP	+	Semal (1969)
Brome mosaic virus (BMV)	barley	30	NP	-	Hardy *et al*. (1979)
Cowpea mosaic virus (CPMV)	cowpea	30	34	+	Zabel *et al*. (1974)
Cucumber mosaic virus (CMV)	cucumber	37	NP	+	Gilliland & Symons (1968)
		37	NP	-	Gill *et al*. (1981)
Pea enation mosaic virus (PEMV)	pea	30	NP	+	Powell *et al*. (1977)
Tobacco mosaic virus (TMV)	*N. glutinosa* & *N. tobaccum*	25	NP	+	Semal *et al*. (1964)
	N. tobaccum	35	NP	+	Bradley & Zaitlin (1971)
Tobacco ringspot virus (TRSV)	cucumber	37	>35	+	Rezaian *et al*. (1976)
Turnip yellow mosaic virus (TYMV)	Chinese cabbage	30	NP	+	Mouches *et al*. (1974)

[a]NP = Not Published.

Cessation of RNA synthesis may be due to the polymerase completing the nascent RNA strand and subsequently being unable to reinitiate RNA synthesis on a new template; in a similar fashion to that described by Dorssers *et al*. (1983) for the enzyme template complex isolated from CPMV-infected cowpea leaves. Experimental evidence presented in this thesis indicates that the native template associated with the polymerase is probably dsRNA.

It would appear that once *in vitro* synthesis of VTMoV RNA is completed on a single template molecule, the enzyme is unable to accept another because addition of VTMoV RNA to the polymerase reaction mixture did not stimulate *in vitro* RNA synthesis. However, the addition of ds RNA was not tried so that the possibility of the enzyme being able to only accept dsRNA as template must not be overlooked as the reason for the lack of enzyme activity observed with exogenous templates.

8.3 PRODUCTS OF *IN VITRO* RNA SYNTHESIS

Electrophoretic analysis of the RNA synthesised *in vitro* by the VTMoV-induced RNA-dependent RNA polymerase-template complex from *N. clevelandii* revealed the presence of two species of ^{32}P labelled dsRNA. The molecular weights of these were estimated as about 3.5×10^6 (product A) and 0.72×10^6 (product B). When the labelled dsRNAs were melted, ssRNAs, which migrated at rates similar to VTMoV RNA 1 and RNA 3 were detected. Only a trace of labelled RNA which migrated at a similar rate to RNA 2 was detected in some experiments. Dot blot and liquid hybridization experiments indicate that only positive-sense VTMoV-RNA was synthesised *in vitro* and approximately the same amount of ^{32}P was incorporated into RNA 1 as into viroid-like RNA.

When VTMoV-infected leaf tissue from N. clevelandii was infiltrated with tritiated uridine, two in vivo products (A' and C') were obtained. Product A' was similar in its electrophoretic mobility to that synthesised in vitro, however, product C' was unlike any of the observed products of in vitro RNA synthesis. The in vivo product C' had similar electrophoretic mobility to that of the stable breakdown product observed when in vitro labelled dsRNA B was subjected to RNase A digestion in 0.01 x SSC. This suggests that the in vitro product B may be synthesized in vivo but is then immediately processed to yield C' which is thought to be a duplex of the VTMoV viroid-like RNA.

The dsRNA species synthesised in vitro by the polymerase template complex were also different from the two dsRNAs isolated from VTMoV-infected N. clevelandii plants by Chu et al. (1983). One of the dsRNA species isolated by these authors had base sequences specific to RNA 1 and a M_r of about 2.8×10^6. The other dsRNA containing base sequences specific to the viroid like RNA, had a M_r of about 3.6×10^6 and is probably a double-stranded polymer containing 13-15 copies of the viroid-like RNA. These dsRNAs reported by Chu et al. (1983) may be by-products of VTMoV-RNA synthesis because they were shown to accumulate at later stages of infection when virus synthesis was already declining.

The origin of VTMoV RNA 2, which is a covalently closed circular molecule, is not clear. However, ligation of the linear RNA 3 is a possible mechanism by which RNA 2 may be derived.

8.4 ENZYME KINETICS OF VTMoV RNA SYNTHESIS BY THE RNA-DEPENDENT RNA POLYMERASE-TEMPLATE COMPLEX

To my knowledge, no previous work on the enzyme kinetics of viral RNA synthesis or on RNA-dependent RNA polymerase from plants has been reported. The majority of kinetic data relating to RNA synthesis is based on DNA-dependent RNA polymerase and has been reviewed by Kumar (1981). Workers studying DNA-dependent RNA polymerase use synthetic DNA templates, consisting of homo-polymers and co-polymers of deoxyribonucleotides, allowing the use of only one or two nucleoside triphosphates as substrates for kinetic studies. The only work I am aware of where naturally occurring DNA (containing all four bases) was used in polymerase studies, is that of Cooke et al. (1983). These authors studied initiation and RNA synthesis by DNA dependent-RNA polymerase II, using cauliflower mosaic virus DNA as template. However, no enzyme kinetic work was included in these studies.

Reported in this thesis is the development of an experimental approach to studying the enzyme kinetics which takes into account the tetra-reactant nature of the polymerase reaction, and its use to obtain enzyme kinetic data. All four nucleoside triphosphates were shown to be required for in vitro VTMoV RNA synthesis by the RNA-dependent RNA polymerase-template complex isolated from VTMoV infected leaves of N. clevelandii.

It was found that an enzyme kinetic model based on a ping-pong mechanism fitted the observed data. RNA synthesis was template-directed and no evidence of end-labelling or non-template directed RNA chain elongation was observed. This is further evidence that, in the in vitro

assay system, the replication complex completes synthesis of VTMoV RNA which was initiated *in vivo*.

Rhodes and Chamberlin (1974) have reported a ping-pong mechanism for the reaction of DNA-dependent RNA polymerase from *E. coli*. Their work is based on the use of synthetic templates allowing the use of only two substrates to obtain enzyme kinetic data. The kinetics of RNA synthesis by DNA-dependent RNA polymerase II from wheat germ, using synthetic DNA as template and either one or two substrates, has also been reported (Job *et al.*, 1984). In this system a simple ping-pong mechanism was not observed and this was attributed to the possibility of several nucleoside triphosphate binding sites on the enzyme.

The observation of linear double reciprocal plots of $1/v$ versus 1/(concentration of nucleoside triphosphate), with the use the VTMoV induced RNA-dependent RNA polymerase-template complex from leaves of *N. clevelandii* (Chapter 7), suggests that the enzyme has a single ligand binding site. It is unlikely that any allosteric regulation of this enzyme occurs.

8.5 SCOPE FOR FUTURE WORK

Reported in this thesis, is the isolation of a replication complex from VTMoV infected leaves of *N. clevelandii* which is capable of completing, *in vitro*, the synthesis of VTMoV RNA which was initiated *in vivo*. Use of these replication complexes to study RNA synthesis *in vitro* has great potential to increase an understanding of viral RNA synthesis, even though, the products of *in vitro* RNA synthesis are not identical to dsRNAs synthesized *in vivo*. The usefulness of the system is, also,

limited because the products of *in vitro*, synthesis do not appear to be released from the template, and the enzyme appears to be unable to reinitiate RNA synthesis on new template molecules. However, the development of an experimental approach for obtaining enzyme kinetic data, and establishment that VTMoV RNA synthesis proceeds via a ping-pong mechanism, opens the way for future enzyme inhibitor studies and possible development of specific inhibitors of viral RNA synthesis.

Purifying the polymerase enzyme together with its native template further, opens the possibility of obtaining highly purified preparations of the polymerase protein. Precipitation of the enzyme-template complex with protamine sulphate and streptomycin sulphate could perhaps be further explored as a means of achieving purification of the polymerase protein. Once preparations of highly purified polymerase protein are obtained they can be analysed to determine if they are host plant or virus encoded.

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