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PLANT VIRUS-INDUCED RNA POLYMERASE

Thesis submitted for the degree

of

Doctor of Philosophy

by

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from

The Department of Biochemistry
UNIVERSITY OF ADELAIDE.

JULY, 1971.

STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in any other University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except when due reference is made to such material in the text.

JOHN T. MAY

PAPERS PUBLISHED OR IN THE PRESS

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SUMMARY

An investigation has been carried out on the properties of an RNA-dependent RNA polymerase detected in cucumber cotyledons infected with cucumber mosaic virus (CMV). Healthy cucumber cotyledons were devoid of such an enzyme. The significant findings of this investigation were:

1. CMV-induced RNA polymerase activity was detected both in soluble (soluble at 16,000 g for 10 min.) and particulate (sedimented at 16,000 g for 10 min.) fractions of disrupted CMV-infected cucumber cotyledons.

2. Soluble RNA polymerase. A method has been described for the partial purification of the soluble CMV-induced RNA polymerase which was free of detectable plant ribonuclease. The soluble RNA polymerase purified in this manner had a molecular weight of 123,000 as determined by sucrose density gradient centrifugation, and was not associated with a viral RNA template. The soluble RNA polymerase copied added RNA by complementary base pairing; however, there was no unique specificity for CMV-RNA as turnip yellow mosaic virus (TYMV)-RNA, tobacco ringspot virus (TRSV)-RNA, tobacco mosaic virus (TMV)-RNA and yeast ribosomal RNA were also copied. Also the RNA present in the enzyme preparation (50 - 80 μg/ml) was copied by the soluble RNA polymerase in the absence of added RNA but this copying was only optimal with a concentration of 5 - 13 mM $MgSO_4$ in the assay medium. Further, with the

presence of 5 - 13 mM $MgSO_4$ in the assay medium the soluble RNA polymerase copied added polyC template by complementary base pairing while polyA, polyG and polyU were copied at a rate no greater than 2 - 3% of that of polyC.

The product of the soluble RNA polymerase reaction using either added yeast RNA or CMV-RNA as template, was a polyribonucleotide of which 80 - 90% was resistant to digestion by pancreatic ribonuclease, had a sedimentation value of about 5S and a melting temperature near 92° in standard saline-citrate.

3. Particulate RNA polymerase. In particulate fractions, two virus-induced RNA polymerase activities were found, one requiring the addition of RNA before activity could be detected, the other not requiring added RNA. The particulate RNA polymerase activity dependent on added RNA was solubilised during the polymerase assay by the $MgSO_4$ (29 mM) present in the assay medium. All the properties investigated of this enzyme, including its molecular weight of about 120,000 as determined by sucrose density gradient centrifugation after solubilisation in the presence of $MgSO_4$, indicated that it was very similar or identical to the RNA polymerase found in the soluble phase. By contrast, when the particulate RNA polymerase was solubilised by freezing and thawing, it had a molecular weight of about 150,000.

The particulate RNA polymerase not dependent on added RNA gave a product which was mostly double-stranded (more than 80% resistant to digestion with pancreatic

ribonuclease), had a peak sedimentation value on sucrose gradient centrifugation of about 8S and a melting temperature of 93° in standard saline-citrate. However, nearest-neighbour analysis of the product and hybridisation studies did not provide a definite answer on the nature of the RNA in a presumed RNA polymerase-template RNA complex.

4. It is considered that the various types of virus-induced RNA polymerases found both in soluble and particulate fractions of CMV-infected cucumber cotyledons represent various incomplete forms of the actual enzyme responsible for the *in vivo* replication of viral RNA.

INTRODUCTION

INTRODUCTION

The programme of work described in this thesis was carried out because crude extracts of cucumbers infected with either cucumber mosaic virus (Q-CMV, Q-representing Queensland isolate), tobacco ringspot virus (TRSV) or rose virus (RV), were found to contain a plant virus-induced RNA-dependent RNA polymerase (Gilliland and Symons, 1968). The aim of this work was to characterise both the plant virus-induced RNA polymerase activity and the product it made; and hence determine its relationship to the *in vivo* replication of plant virus RNA. The majority of the work described in this thesis was concerned with the viral-induced RNA polymerase from CMV-infected cucumber cotyledons; CMV contained a single-stranded RNA genome of 1×10^6 molecular weight (Francki *et al.*, 1966).

The CMV and TRSV-infected cucumber systems had previously been used for a study of virus-induced changes in the level of activity of the ribonucleotide kinases (Gilliland *et al.*, 1966; Gilliland and Symons, 1967). Such systems are presently being used by our laboratory to investigate the nature of the RNA and protein species induced into the host plant by plant viruses.

Viruses are widespread in nature afflicting animal, bacterial and plant cells alike. They are nucleoproteins, the nucleic acid component of which is either DNA (single or double-stranded) or RNA (single or double-stranded). However, the genome of most plant viruses isolated is single-stranded

RNA of 1 - 2.5 x 10^6 molecular weight. The exceptions are cauliflower mosaic virus (double-stranded DNA), rice dwarf virus (double-stranded RNA), wound tumour virus (double-stranded RNA) and potato spindle tuber virus (possibly double-stranded RNA; Diener and Raymer, 1969).

This Introduction will consider the evidence and theories that explain the replication process of small RNA viruses (single-stranded RNA of 1 - 2.5 x 10^6 mol. wt.) which appeared within the literature up to 1971. The majority of the evidence originated from studies using bacterial viruses.

A. GROWTH PROPERTIES OF SMALL RNA VIRUSES.

The following discussion of the growth properties of small RNA viruses will be given in general outline only.

(1) Effect of RNA virus-infection on host cells. Changes in host cell metabolism accompanied RNA phage infection, but the extent of these changes depended on the particular phage (Watanabe and Watanabe, 1970). Synthesis of bacterial RNA was only slightly affected by infection with f2 or Qβ and less than half the protein and RNA synthesised after infection was recovered in phage particles (Ellis and Paranchych, 1963; Watanabe *et al*., 1968). In contrast, despite the physical and chemical similarities of the RNA phages, infection with R17, R23 or ZIK/1 resulted in extensive inhibition of bacterial protein and ribosomal RNA synthesis (Ellis and Paranchych, 1963; Bishop, 1965; Hudson and Paranchych, 1967; Watanabe and Watanabe, 1970). In the case of R23, 60% of RNA synthesised during infection was encapsulated into

phage particles and almost all the protein synthesised late in infection was coat protein (Watanabe *et al.*, 1968). However, the precise mechanism whereby host cell RNA and protein synthesis is inhibited by RNA phage infection is still not fully understood. Lysis of infected bacteria occurred between 22 and 60 minutes, and each cell yielded up to 40,000 virus particles, of which, however, only a fraction (5 - 50%) were viable depending on the phage strain and the growth conditions.

In most types of cell, small animal viruses such as picornaviruses (*e.g.*, poliovirus, foot and mouth disease virus) caused a rapid suppression of host cell protein synthesis (Hausen and Verwoerd, 1963; Holland, 1963; McCormick and Penman, 1967) and suppressed both messenger RNA and ribosomal RNA synthesis (Fenwick, 1963; Summers *et al.*, 1967). Mature viruses appeared within the cell several hours after infection.

(2) Virus-induced proteins. In the host, the viral RNA genome itself was found to serve as a messenger that directed the synthesis of viral-specific proteins. A small RNA genome of 1×10^6 molecular weight was predicted to code for 3 or 4 proteins with an average molecular weight of 30,000 (Lodish, 1968). Three such virus-specific proteins were detected in phage MS2 (RNA of mol. wt. 1×10^6) - infected *E. coli* protoplasts, in which host RNA synthesis was halted with actinomycin D (an antibiotic that stops host DNA-dependent RNA polymerase activity) (Vinuela *et al.*, 1967; 1968). Such phage RNA was recognised as providing

information for the synthesis of coat protein (Nathans *et al*., 1962; Lodish, 1968), a minor component of the virus coat (one molecule per virus particle) designated maturation protein or A protein (Steitz, 1968; Lodish and Robertson, 1969) and the replicating enzyme, RNA-dependent RNA polymerase (Garwes *et al*., 1969; Jeppesen *et al*., 1970; Kamen, 1970).

The animal virus, poliovirus (RNA of mol. wt. 2×10^6) was found to induce four capsid (coat) proteins and 10 non-capsid proteins in Hela cells treated with actinomycin D (Summers *et al*., 1965; Summers and Maizel, 1968). This exceptionally large number of virus-induced proteins (some having mol. wts. greater than 30,000) did not agree with the expected 7 - 8 proteins of 30,000 molecular weight. However, it was later found that the 14 different virus-specific polypeptides detected did not represent 14 different species independently translated from the genome. Rather, the species arose by cleavage of larger precursor proteins, possibly from one primary gene product (Jacobson and Baltimore, 1968; Summers and Maizel, 1968). It was suggested that this phenomenon could be general for all small RNA animal viruses (Holland and Kiehn, 1968).

The presence of large precursor protein molecules reflected a difference in the growth behaviour between poliovirus and the RNA bacteriophages so far investigated.

Tobacco mosaic virus (TMV; a plant virus with an RNA of mol. wt. 2.1×10^6) was recently reported to induce 5 virus-specific proteins in actinomycin D-treated tobacco

plants (Zaitlin and Hariharasubramanian, 1970), one of which was identified as the viral coat protein.

(3) Control of viral-directed protein synthesis. For some time it had been evident that a specific mechanism was needed to explain the large excess of viral coat protein synthesised late in infection as compared to the synthesis of the other two known virus-induced proteins (maturation and polymerase proteins) used in RNA phage replication.

Lodish and Zinder (1966a) suggested that the viral coat protein of the RNA phage acted directly or indirectly as a 'repressor' of the synthesis of non-coat proteins *in vivo*, particularly the RNA polymerase whose production ceases midway through infection cycle. Several workers confirmed that small amounts of coat protein bound to bacteriophage RNA and specifically stopped the translation of the non-coat genes (Richelson and Nathans, 1967; Robertson *et al.*, 1967; Sugiyama and Nakada, 1968; Ward, 1968; Ward *et al.*, 1968). The possibility therefore existed that small RNA viruses used coat protein *in vivo* to regulate the production of non-coat proteins.

(4) Site of synthesis of viral components. The plant and animal host cell are considerably more complex than the bacterial cell which does not contain nucleus, nucleolus, mitochondria and other organelles. The RNA of small RNA animal viruses was transcribed, translated, replicated and the progeny virus assembled, within the cytoplasm of the cell (Crocker *et al.*, 1964) and in association with membranes (Penman *et al.*, 1963; Penman *et al.*, 1964; Dales *et al.*, 1965; Amako and Dales, 1967a,b; Skinner *et al.*, 1968).

It was recently demonstrated that the replication of poliovirus RNA was associated with the smooth cytoplasmic membranes while poliovirus protein synthesis was associated with the rough membranes (Caliguri and Tamm, 1969; Caliguri and Tamm, 1970a,b). However, views on the site of synthesis of plant viral components are varied and to some extent contradictory. Viral-specific proteins have been reported to be made in the cytoplasm (Schlegel and Smith, 1966; Ceck, 1967), nucleus (Reddi, 1966a) and the chloroplasts (Zaitlin and Boardman, 1958). Protein synthesis and virus assembly, however, probably occur in the cytoplasm (Zaitlin *et al.*, 1968) and in contact with membranes. It was considered that the site of replication of the plant viral RNA was the chloroplasts (Bové, 1967c; Ralph and Clarke, 1966), the nucleolus (Bové, 1967a; Bové *et al.*, 1969) or peripheral vesicles of the chloroplasts (Ushiyama and Matthews, 1970). The RNA replication work described above has all been carried out using turnip yellow mosaic virus (TYMV), but even so, there was no consistent conclusion as to the site of synthesis of the TYMV-RNA. Thus, the actual site or sites of synthesis of plant virus components had not yet been determined (see Esau, 1968).

B. PROBLEMS OF VIRAL-RNA REPLICATION

The replication of the single-stranded RNA genome presented an intriguing problem. Viral RNA synthesis could not be attributed to enzyme systems responsible for RNA synthesis in the normal host as replication of the RNA viruses proceed in the presence of actinomycin D (Summers *et al*., 1965; Vinuela *et al*., 1967). Doi and Spiegelman (1962) eliminated the involvement of DNA in phage RNA replication by the homology test in which they failed to demonstrate a hybrid between denatured normal or infected host DNA and RNA from the phage MS2. Similarly, Cooper and Zinder (1962) showed that DNA was not an intermediate by obtaining undiminished yields of phage from f2-infected *E. coli* under conditions of thymine deprival and added fluorodeoxyuridine which reduced DNA synthesis to 3% of the normal level. However, such a pathway via DNA intermediates, may exist for large animal tumour viruses (*e.g.*, Rous sarcoma virus, single-stranded RNA of 10×10^6 mol. wt.), where an RNA-dependent DNA polymerase has been found associated with the purified particle (Baltimore, 1970; Spiegelman, *et al*., 1970; Temin and Mizutani, 1970). These large viruses apparently have a different life cycle (replicating process) to the RNA phage and other small RNA viruses.

One model of small RNA virus replication stemmed from studies on the single-stranded DNA phage ØX174. Upon infection of *E. coli*, the single-stranded DNA was converted to a double-stranded DNA species (Sinsheimer *et al*., 1962).

This double-stranded DNA in turn served as a template for the formation of single-stranded copies via an asymmetric synthesis. As a single-stranded RNA virus would have the same general problems as a single-stranded DNA virus, it was quite possible that the RNA had a similar pathway in its life cycle. However, because such a model implied an RNA-RNA transmission of information the existence of a viral-induced RNA-dependent RNA polymerase was predicted. The remainder of this Introduction will be concerned with studies performed both *in vivo* and *in vitro* which indicate the mechanism by which the genome of small RNA viruses is replicated.

C. REPLICATION OF VIRAL RNA: *IN VIVO* AND *IN VITRO* STUDIES

A considerable source of our knowledge concerning viral RNA stemmed from studies of the intact, virus-host cell systems, and these will be presented in general outline only. These studies followed two different approaches; the host cell was infected with an RNA virus whose RNA had been labelled with radioactive precursors and the fate of the parental RNA was then followed in the infected cell. The other approach was to briefly incubate infected cells, in the presence of suitable radioactive precursors (*e.g.*, uridine-^{14}C), under conditions in which host cell RNA synthesis was blocked (*e.g.*, with ultra-violet irradiation or actinomycin D). The newly synthesised labelled RNA was extracted by deproteinisation procedures employing phenol

and/or sodium dodecyl sulphate (SDS), and examined by physico-chemical methods. Such studies suggested the involvement of a double-stranded RNA structure in the replication of RNA viruses. However, the conclusion drawn from *in vivo* work has been questioned by Weissmann and coworkers (Borst and Weissmann, 1965; Weissmann *et al.*, 1968). These workers used an *in vitro* system to follow the replication of the phage Q^{β}-RNA, (using the isolated phage Q^{β}-induced RNA replicase) and suggested that in this particular case at least, no truly double-stranded RNA structure was involved in viral RNA replication. These workers postulated that the deproteinisation techniques used during the *in vivo* work resulted in the formation of RNA artifacts. The *in vitro* work of Weissmann and coworkers will be discussed in this section also.

(1) *Fate of parental RNA*. The interpretation of experiments designed to follow the fate of labelled parental RNA was complicated by the fact that the viability of the RNA viruses was often low (phages 5 - 50%; poliovirus 0.1%) and there was some uncertainty as to whether the given labelled fraction was actually participating in the replication process. Studies on the animal RNA viruses were also complicated by the fact that 80% of the absorbed virus was not uncoated. Further, parental strands were conserved within the host and were not found in progeny viruses (Doi and Spiegelman, 1963) thus limiting this technique for determining the replication cycle.

Sedimentation analysis of the content of host cells infected with ^{32}P-labelled RNA phage indicated that

within minutes after infection the parental RNA was associated with the 70S ribosomal fraction (Doi and Spiegelman, 1963; Erikson *et al*., 1964; Erikson and Franklin, 1966; Godson, 1968). Similar analysis of the deproteinised RNA from ^{32}P-labelled phage R17-infected *E. coli* showed that part of the parental RNA (27S; mol. wt. 1×10^6) sedimented in a sucrose density gradient at 16S and had become resistant to pancreatic ribonuclease digestion (double-stranded RNA of mol. wt. 2×10^6; Erikson *et al*., 1964; Kelly and Sinsheimer, 1964). After heat denaturation, the 16S fraction yielded a radioactive RNA molecule which sedimented at 27S (Erikson *et al*., 1965), the characteristic value of the single-stranded R17 RNA (mol. wt. 1×10^6).

Studies on the animal RNA virus, mengovirus (RNA mol. wt. 2×10^6) showed that 50% of the labelled parental RNA was located in large polysomal aggregates which were actively synthesising protein (Toby, 1964a,b). However, in this particular case the deproteinised parental RNA was not found in a ribonuclease-resistant form, in contrast to the phage situation.

(2) Virus-specific RNA species formed in the host during labelling experiments. Basically similar results have been reported for bacterial, animal and plant RNA viruses by the use of *in vivo* labelling techniques. Host cell RNA synthesis was blocked by the addition of actinomycin D (bacteria in protoplast form) or, in the case of bacterial host, by ultra-violet light but the latter method gave varying degrees of damage to the host-virus system. A suitable

radioactive precursor (e.g., uridine-^{14}C) was supplied to the host-virus system and the RNA formed during the virus growth cycle was extracted (deproteinised with phenol or SDS) and examined by sucrose density gradient centrifugation, polyacrylamide gel electrophoresis or methylated albumin keiselghur (MAK) column chromatography.

In the small RNA animal virus systems the major deproteinised RNA component isolated was 37S (mol. wt. 2×10^6) viral progeny RNA and a pancreatic ribonuclease-resistant 20S form (double-stranded RNA of mol. wt. 4×10^6; designated 'replicative form') when analysed on sucrose gradients. Some radioactivity sedimented between the 20S and 37S RNA species and has been identified as double-stranded RNA with nascent single RNA strands (designated 'replicative intermediate', Erikson and Erikson, 1967; Oberg and Philipson, 1970).

Deproteinised RNA from E. coli infected with RNA phage, which had been pulse labelled during infection also contained three types of viral RNA; a broad band at 16S containing ribonuclease-resistant RNA predominated over the viral RNA peak (27S) with brief labelling periods, but longer pulses showed the 27S peak predominating (Fenwick et al., 1964). RNA phage-infected E. coli also contained the 'replicative intermediate' RNA (16 - 27S; 2 - 5 nascent single RNA chains on double-stranded RNA; Vandenberghe et al., 1969). However, MS2 and Qβ phage-infected E. coli contained, in addition to the other RNA species, a ribonuclease-resistant 6S RNA species ('abnormal RNA') (Kelly et al., 1965;

Banerjee _et al._, 1969b).

Deproteinised RNA from TMV-infected tobacco plants treated with actinomycin D and $^{32}P_i$ also contained a labelled ribonuclease-resistant viral RNA structure (Itoh and Hirai, 1966; Ralph and Wojcik, 1969).

(3) _The role of the double-stranded RNA_. Double-stranded viral-induced RNA has been isolated from several virus-infected systems in which it has been sought (animal, bacterial and plant systems). However, all such double-stranded RNA species have been isolated by techniques which involve deproteinisation (using phenol or SDS) of the host extracts. The double-stranded RNA isolated in this manner was resistant to pancreatic ribonuclease digestion but destroyed by ribonuclease 111 (Robertson _et al._, 1968), an enzyme which has now been shown to be specific for double-stranded RNA. The two strands of the double-stranded RNA consisted of viral RNA (referred to as plus or positive strand) and a strand complementary in base composition to viral RNA (referred to as minus or negative strand; Langridge _et al._, 1964; Weissmann _et al._, 1964). As the double-stranded DNA structure formed in ØX174 replication was involved in the replication cycle (discussed in 'Problems of viral RNA replication') it was tempting to ascribe a similar role to the double-stranded RNA.

Evidence that the double-stranded RNA played an active role in the replication of RNA viruses was shown when animal-virus induced double-stranded RNA was found to infect tissue cultures (Montagnier and Sanders, 1963; Baltimore _et al._,

1964). However, in contrast to the animal system, double-stranded RNAs isolated from phage-infected bacteria had to be heat denatured before becoming infectious for E. coli protoplasts (Amman et al., 1964; Franke and Hofschneider, 1966). Infectivity of normal or heat-denatured virus-induced double-stranded RNA of small plant viruses has not been reported.

Further evidence which suggested that the double-stranded RNA was an intermediate in RNA replication came from pulse chase experiments using R17-infected E. coli. Fenwick et al. (1964) supplied uridine-^{3}H to R17 phage-infected cells in which host RNA had been inhibited by irradiation with ultra-violet light. If the RNA was labelled for 10 sec., the extracted labelled RNA was found to be in the 16S, pancreatic ribonuclease-resistant region of a sucrose gradient. When the cells were further incubated in unlabelled medium after the initial 10 sec. labelling period, there was a marked increase in label in the 27S viral-RNA region, with a corresponding decrease in the amount of labelled ribonuclease-resistant RNA. This suggested a precursor-product relationship between 16S RNA (double-stranded RNA) and 27S progeny viral RNA (single-stranded RNA). Billiter et al. (1966) confirmed this loss of radioactivity from the double-stranded RNA structure using phage MS2-infected E. coli.

Thus it is believed from in vivo studies that the double-stranded RNA acted as a template for the production of virus-like RNA and is an important intermediate in the

replication of small RNA viruses.

(4) Mechanism of RNA replication from the double-stranded RNA-conservative versus semiconservative replication. Only two different theories concerning the mechanism of replication of viral-RNA from double-stranded RNA have received any support in the literature. These were the asymmetric conservative and asymmetric semiconservative hypothesis. The proposition common to both was that the replication was an asymmetric process, only one strand (plus strand or viral-like RNA) being produced in any quantity (Erikson et al., 1965). However, once progeny plus strands were produced by either mechanism they could then cycle to produce more ribonuclease-resistant structures, associate with the ribosomes to produce more viral proteins or be incorporated into progeny virus particles.

The semiconservative mechanism was first proposed by Weissmann et al. (1964) and Fenwick et al. (1964). According to this hypothesis, the replicating enzyme RNA-dependent RNA polymerase catalysed the synthesis of a minus strand, making use of the parental strand as template. This reaction was common to both theories and resulted in the synthesis of a completely double-stranded RNA structure (replicative form). Either the same enzyme or a second polymerase then used the duplex as a template to release new single strands by displacement of the positive strand of the duplex. This mechanism was similar to the one used to explain DNA-RNA hybrids when E. coli RNA polymerase was provided with single-stranded DNA templates (Chamberlin and Berg, 1964; Sinsheimer and Lawrence, 1964).

On the other hand, the conservative mechanism proposed that the double-stranded RNA template remained intact, and the progeny RNA (plus strands) are produced on the template without displacement of either strand.

This conservative mechanism was well characterised in the synthesis of RNA from double-stranded DNA by E. coli DNA-dependent RNA polymerase (Bremer and Konrad, 1964; Jehele, 1965) and ØX174 progeny molecules from the double-stranded ØX174 intermediate (Sinsheimer et al., 1962; Denhardt and Sinsheimer, 1965). In most reports authors have concluded that their results were in accord with the semiconservative model, but few have attempted experiments to distinguish this hypothesis from the alternative one.

Differentiation between the two different models was achieved by following the fate of the parental RNA after it had been incorporated into the duplex structure. The conservative model predicted that the parental RNA should remain in the double-stranded structure, when the new positive strands were synthesised, while the semi-conservative model postulated their displacement (see Lodish and Zinder, 1966b; Billiter et al., 1966; Kelly and Sinsheimer, 1967 and Erikson and Erikson, 1967). Results obtained with wild type RNA f2 phage showed a slow and incomplete loss of parental label from the double-stranded form (Lodish and Zinder, 1966b); however, the difficulties with this type of experiment were that the displaced parental RNA may be reincorporated into a duplex structure thus indicating a conservative mechanism. However, if the f2 mutant ts-6 (a temperature sensitive

mutant) was used then synthesis of plus strands occurred in the absence of minus strand synthesis at 43° (Lodish and Zinder, 1966b) thus overcoming the reincorporation problems encountered in wild type phage experiments. In an experiment described by Lodish and Zinder (1966b), cells were infected with ^{32}P-labelled f2 ts-6 and incubated at 34°, allowing maximum formation of ^{32}P-labelled double-stranded RNA. The infected cells were then transferred to 43° (whereby no minus strands were made) and within one min. 50% of the labelled RNA was displaced, 90% being released within 9 min. of the temperature shift. Hence, under conditions where minus but not plus strand synthesis was blocked, parental RNA molecules were released, suggesting a semiconservative type of replication. However, given a semiconservative mechanism for replication it was apparent that the failure to find parental RNA in progeny animal and bacterial viruses (Davis and Sinsheimer, 1963; Doi and Spiegelman, 1963; Homma and Graham, 1965) still needed explanation. It would not be unlikely that in fact both mechanisms operate during the replication of small RNA viruses.

It also must be pointed out that the involvement of double-stranded RNA in the replication process of viral RNA has been suggested mainly from _in vivo_ studies which involved the use of deproteinised RNA products (phenol or SDS extracted RNA). Weissmann _et al._ (1968) from _in vitro_ work, using the isolated Qβ phage replicating enzyme and avoiding deproteinisation of RNA products, proposed another mechanism which disregarded truly double-stranded RNA as an intermediate in

$Q\beta$ phage RNA replication. This mechanism and its implications on the other mechanisms of viral RNA replication are discussed below.

(5) Comparison of viral RNA produced *in vivo* and viral RNA produced *in vitro*, and implications on the mechanism of viral RNA replication. The *in vitro* synthesis of phage RNA has been used as a model system for studies on the replication procedure of RNA phage (Weissmann *et al.*, 1968; Spiegelman *et al.*, 1967; August *et al.*, 1970). One of the chief attributes of this system was that the *in vitro* reaction closely resembled the *in vivo* process even to the extent of yielding infectious RNA (infectious for *E. coli* protoplasts).

Thus the $Q\beta$ phage-induced RNA replicase isolated by the method of Pace and Spiegelman (1966) utilised $Q\beta$-RNA, *in vitro*, as template for the ultimate synthesis of more infectious, self-replicating $Q\beta$-RNA. The question arose whether this replication involved preliminary formation of viral minus strands and hence a double-stranded RNA intermediate as shown by *in vivo* studies. Spiegelman's group reported the presence of a pancreatic ribonuclease-resistant structure (15S) after short (2 min.) incubations of the replicase with $Q\beta$-RNA template (27S) and after treatment of the product with SDS (Pace *et al.*, 1967a,b,c). Further investigations of the deproteinised products of the replicase reaction revealed the presence of both double-stranded RNA (replicative form), double-stranded RNA with nascent single strands (replicative intermediate) as well as infectious RNA

(Pace *et al.*, 1967a,b,c). Thus, all three RNA species obtained from this *in vitro* work corresponded well to those already found to exist during *in vivo* studies. However, both systems employed deproteinisation techniques in the isolation of the viral RNA species.

Weissmann *et al.* (1968) studied the rate of production and types of Qβ-RNA produced by the RNA replicase (also isolated by the method of Pace and Spiegelman, 1966) during *in vitro* replication, prior to deproteinisation with phenol or SDS. Feix *et al.* (1967) observed that the formation of intact virus-like Qβ-RNA was preceded and accompanied by synthesis of full-length viral minus strands which were pancreatic ribonuclease-sensitive and therefore not part of any double-stranded helix. One main product in the early stages of production of minus strands was a 40S sedimenting complex. The pancreatic-ribonuclease resistance of this 40S protein-RNA complex was only 10% which was extremely low when compared to replicative form RNA obtained by phenol or SDS extraction of *in vivo* or *in vitro* (RNA replicase) systems. However, once the 40S complex was treated with phenol the ribonuclease-resistance of the extracted RNA increased to 73%. It was believed that the 40S complex consisted of plus and minus strands held by the protein in a particular conformational state which was favourable to annealing during deproteinisation. Weissmann *et al.* (1968) also found that double-stranded Qβ phage-RNA (isolated by phenol extraction of Qβ phage-infected *E. coli*) was not used as template in the Qβ-RNA replicase reaction, suggesting that such a duplex

structure was not involved in the replication cycle and was merely an artifact probably as a result of enzyme or protein removal. However, it was also noted that purified non-infectious (non-infectious for E. coli protoplasts) Qβ minus strands promoted vigorous synthesis of infectious RNA in the Qβ-RNA replicase reaction, the initial rate of RNA production being 5 times that found for even Qβ-RNA itself. This finding indicated that the template for the production of viral RNA was the minus strands. It should be noted that minus strands themselves were not infectious in vivo because they could not produce the RNA-dependent RNA polymerase, the first step in RNA virus-infection in vivo. In light of the results obtained by Weissmann and co-workers, a mechanism was proposed which ignored the involvement of truly double-stranded structures in the replication process (see Weissmann et al., 1968). This mechanism accounted for the sensitivity of intermediates to pancreatic ribonuclease and the ease with which they are converted to partially ribonuclease-resistant structures by deproteinisation. The most significant features of this mechanism of viral RNA replication were the 'open structure' suggested for the intermediates of both stages, the formation of minus strands and the production of new viral RNA. The 'open structure' existed only in the presence of protein which arranged the two strands in such a conformational state that on removal of protein they annealed, giving rise to artifactual replicative form and replicative intermediate RNA species. The mechanism in addition to explaining Weissmann's in vitro

replication studies had the agreeable feature of eliminating the problem of deciding whether the replication occurs by a conservative or semiconservative mechanism (as all RNA intermediates detected _in vivo_ are artifacts of the deproteinisation procedure). Most _in vivo_ double-stranded structures isolated have been extracted by techniques which involved the use of phenol and hence were probably the deproteinised forms of the intermediates. However, partially pancreatic ribonuclease-resistant material could be recovered from the Qβ-RNA replicase reaction without either phenol or SDS extraction (Weissmann _et al._, 1968; less than 10% of the total RNA). Therefore, it is not possible even at this stage to state what role, if any, double-stranded RNA plays _in vivo_ or if in fact the mechanism suggested above applies. Engelhardt _et al._ (1968) suggested that double-stranded RNA structures were not involved in RNA synthesis at all, but in coat protein synthesis.

Thus at present there are two basic models being considered for the structure of the replicating complex involving the complementary strand as template for synthesis of progeny RNA. They are: 1. viral RNA is synthesised from a double stranded RNA intermediate either by a conservative or semiconservative mechanism; 2. viral RNA is synthesised from a free complementary strand. However, this second mechanism is the result of work on only _one_ isolated RNA-dependent RNA polymerase from a phage-infected bacteria and has not yet been shown to be the case for small RNA animal and plant viruses.

D. REPLICATION OF VIRAL RNA - STUDIES WITH VIRAL-INDUCED RNA POLYMERASE

Viral replication *in vivo* is known to be carried out by a novel enzyme system arising in host cells upon infection with small RNA viruses, i.e., the RNA-dependent RNA polymerase. The existence of such an enzyme was first postulated to explain the RNA- RNA exchange of information during the replication cycle. Several laboratories have reported the isolation of RNA virus-induced RNA polymerase activities from infected animal, bacterial and plant systems. The finding of both single-stranded and double-stranded RNA after deproteinisation of the products of several isolated viral-induced RNA polymerases agreed with the mode of replication proposed by the *in vivo* work. The important properties of the virus-induced RNA polymerase preparations and their products are described for bacterial, animal and plant virus systems with extensive discussion being confined to the Qβ phage-induced RNA replicating enzyme.

(1) Bacteria - RNA virus systems

(a) MS2 phage-induced RNA synthetase. Weissmann *et al.* (1963; 1964) isolated an RNA synthesising enzyme from *E. coli* infected with MS2 phage. After partial purification (twenty-fold) the preparation was free of DNA-dependent RNA polymerase activity but contained endogenous viral minus strands. Radioactive RNA was synthesised by the partly purified enzyme, using radioactive nucleoside triphosphates as substrates and no stimulation of activity was found upon addition of MS2 (plus) RNA. The base composition of the

product was similar to MS2 RNA (Ochoa _et al._, 1964). Only a small part of the product of the RNA synthetase was resistant to pancreatic ribonuclease when treated prior to deproteinisation. However, after phenol or SDS treatment, 50% of the product was found to be resistant to pancreatic ribonuclease digestion (Borst and Weissmann, 1965). This result suggested that plus and minus strands within the MS2 replicating complex _in vivo_ might not be tightly bonded and the ribonuclease-resistant structure observed after standard extraction methods was merely an artifact (see also product of Qβ phage-induced RNA replicase discussed previously in 'Comparison of viral RNA produced _in vivo_ and viral RNA produced _in vitro_ and implications on the mechanism of viral RNA replication'). The ribonuclease-resistant product of the MS2-induced RNA synthetase isolated after phenol extraction was indistinguishable from the MS2 specific double-stranded RNA produced _in vivo_ with respect to all physico-chemical properties. Analysis of the product of the RNA synthetase by hybridisation techniques (Weissmann, 1965) showed that the radioactivity in the duplex was almost entirely due to plus strands. It was therefore concluded that the non-labelled minus strands found in the duplex were present in the enzyme preparation prior to incubation and probably bound to the enzyme as an _enzyme-viral RNA complex_ (replicating complex).

(b) <u>f2 phage-induced RNA polymerase</u>. August *et al.* (1965) utilised the non-permissive host *E. coli* S26 infected with sus-11, an amber mutant of the f2 phage, as a source of enzyme. This host-phage system did not yield phage particles but produced 5 - 8 fold increase in the amount of viral RNA polymerase and virus-specific double-stranded RNA (Lodish *et al.*, 1964). The hundred-fold purified preparation of RNA polymerase required RNA of any variety (viral, soluble or ribosomal) as a primer although semi-crude preparations lacked the requirement for added primer RNA. The nearest-neighbour frequency of the product was different for TYMV-RNA or f2 RNA primer suggesting that the added RNA determined the nucleotide sequence of the product. Its base composition, and the finding that, after deproteinisation, more than half of the product was resistant to pancreatic ribonuclease digestion indicated that a double-stranded RNA was formed.

(c) <u>Qβ phage-induced RNA replicase - isolated by Spiegelman and co-workers</u>. Haruna *et al.* (1963) isolated from *E. coli* infected with phage MS2 or Qβ, an enzyme, that under the appropriate ionic conditions specifically required the addition of MS2 or Qβ-RNA for activity. In contrast to the two enzyme preparations described already, Qβ-RNA replicase isolated by Haruna and Spiegelman (1965) catalysed the synthesis of Qβ-RNA which was infectious for *E. coli* protoplasts, producing several times the amount of input Qβ-RNA (1 μg of Qβ-RNA/40 μg of protein). Also the RNA replicase was found to replicate 'variants' of Qβ-RNA

(Mills *et al.*, 1967; which were only 17% of the original Qβ-RNA but were self-duplicating in the reaction) and Qβ-RNA minus strands (Spiegelman *et al.*, 1968; Weissmann *et al.*, 1968). The mechanism by which infectious Qβ-RNA was produced has been previously discussed in 'Comparison of viral RNA produced *in vivo* and viral RNA produced *in vitro* and implications on the mechanism of viral RNA replication'. As the Qβ phage-induced RNA replicating enzyme has been purified by two groups (Spiegelman's and August's) by different methods which yielded enzymes of different physical properties, the preparation and properties of these Qβ phage-induced replicating enzymes will be described in this and the following section.

(i) Purification of Qβ-RNA replicase (Pace *et al.*, 1968). The primary concern of this purification was to isolate an enzyme which produced a biologically active product (RNA that was infectious) and not to purify the enzyme as many fold as possible. Qβ phage-infected *E. coli* Q13 cells were lysed by freezing and thawing followed by grinding and the supernatant obtained after centrifugation was treated with protamine sulphate and the enzyme precipitated with $(NH_4)_2SO_4$. The RNA replicase was dissolved and chromatographed on a DEAE-cellulose column. These procedures were designed to remove ribonuclease activities, DNA-dependent RNA polymerase activity, polynucleotide phosphorylase activity and poly-adenylate synthetase activity. Mature virus particles retained to this stage were eliminated by subjecting the enzyme to buovant density centrifugation in CsCl, followed

by a sedimentation purification of the enzyme through a 5 - 20% sucrose density gradient. The sucrose gradient led to the partial separation of the Qβ-RNA replicase into a 'heavy' and a 'light' component. The 'heavy' component was found to be a polyC-directed polyG polymerase, and neither component alone could initiate the formation of infectious Qβ-RNA in the replicase reaction. The properties of the Qβ-RNA replicase are discussed below.

(ii) Properties of the Qβ-RNA replicase. As mentioned above, two components of Qβ-RNA replicase were partially separated by sucrose density gradient centrifugation of the enzyme. Their relative positions in the gradient indicated that one (designated 'heavy' and detected by its ability to copy polyC only) was approximately 130,000 and the other (designated 'light') about 80,000 in molecular weight (Eikhom and Speigelman, 1967). However, after sucrose density gradient centrifugation of the replicase enzyme, intact Qβ-RNA was still copied to produce infectious RNA by a protein which had a molecular weight of 110,000 (apparent mol. wt.). Thus it was concluded and subsequently proved that the Qβ-RNA replicase activity which copied intact Qβ-RNA was a composition of both 'heavy' and 'light' components. Neither of these components, adequately purified from one another, could initiate extensive polynucleotide synthesis when challenged with Qβ-RNA. It should be noted that the 'light' component was detected by the addition of the 'heavy' component thus making it competent to copy intact Qβ-RNA.

(iii) Host components. The RNA of phage Qβ has been shown to have a molecular weight of 1 x 10^6 (Overby et al., 1966; Bishop et al., 1967) and could, therefore, code for about 110,000 molecular weight equivalents of protein. Of this 50,000 was accounted for by the viral coat protein (mol. wt. approximately 14,000) and the A protein (mol. wt. approximately 37,000; Roberts and Steitz, 1967; Vinuela et al., 1968; Nathans et al., 1969). This left only about 60,000 molecular weight equivalents (see Garwes et al., 1969; Kamen, 1970; Kondo et al., 1970) to be shared between the two replicase units found by Spiegelman, which sum to almost 3 - 4 times (210,000) the molecular weight. This discrepancy suggested that either the replicase components consisted of small identical subunits or that one of the replicase components was a host-specified protein. Eikhom et al. (1968) showed this latter hypothesis to be true by demonstrating the existence in uninfected E. coli cells of a protein having all the properties of the 'light' component of the Qβ-RNA replicase. Thus, when the host 'light' component was combined with purified 'heavy' component, an enzyme was reconstituted which would copy Qβ-RNA to produce biologically functional Qβ-RNA. Thus, it would seem that the 'heavy' component (130,000 mol. wt.) was unique to E. coli infected with Qβ phage; however, even this component was larger than would be expected for a primary gene product of the Qβ-RNA genome.

(d) Qβ phage-induced RNA polymerase - isolated by August and co-workers. The Qβ replicating enzyme isolated by this method had different physical properties to that prepared by the method of Pace et al. (1968). The enzyme procedure described below yielded a 2,000 - 5,000 fold purified Qβ-RNA polymerase, which in the presence of a 'factor fraction' and Qβ-RNA produced a biologically competent Qβ-RNA product (infectious for E. coli protoplasts).

(i) Purification of the Qβ-RNA polymerase (Eoyang and August, 1968). A crude extract was obtained by retaining the supernatant after centrifugation of frozen and ground infected E. coli Q13 cells. The supernatant was subjected to a liquid-polymer extraction of nucleic acids, and the Qβ-RNA polymerase was precipitated with $(NH_4)_2SO_4$. The precipitate was dissolved and the enzyme fractionated on a DEAE-cellulose column. The fractions which contained Qβ-RNA polymerase activity were pooled, the protein concentrated with $(NH_4)_2SO_4$ and passed down a hydroxyapatite column. These chromatography steps were interesting as they resulted in two important fractions, the core enzyme (150,000 mol. wt.; see below) and a 'factor fraction' which had to be added to the core enzyme before Qβ-RNA was replicated in the Qβ-RNA polymerase reaction mixture. This 'factor fraction' was found in the $(NH_4)_2SO_4$ concentrated fraction of the 0.20 - 0.25 M tris-HCl eluants (or 0.15 M NaCl eluant, Shapiro et al., 1968) of DEAE-cellulose chromatography pooled with the 0.15-0.25 M $(NH_4)_2SO_4$ eluants of hydroxyapatite chromatography (Franze de Fernandez

et al., 1968). The Qβ-RNA polymerase was chromatographed through a Sephadex G-100 column and finally sedimented in a 5 - 20% glycerol density gradient. Thus the definition of the Qβ-RNA polymerase described here is that enzyme which required the addition of the '*factor fraction*' before Qβ-RNA can be replicated.

(ii) *Properties of the Qβ-RNA polymerase*. The molecular weight of the Qβ-RNA polymerase has been stated as 150,000 (August *et al.*, 1968; August *et al.*, 1970) while a molecular weight of 130,000 has been shown by Kondo *et al.*, (1970). This enzyme has now been shown to consist of one phage genome coded protein (69,000) and three host proteins (Kamen, 1970; Kondo *et al.*, 1970; discussed below). The enzyme has been shown to copy polyC or any synthetic heteropolynucleotide containing cytidylate (Hori *et al.*, 1967) synthesising a strand complementary to the template polymer. The enzyme replicated minus (complementary) strands of Qβ-RNA (August *et al.*, 1968; August, 1969; Banerjee *et al.*, 1969a) producing infectious Qβ-RNA. Also the enzyme replicated an unusual 6S-RNA which was isolated from Qβ-infected *E. coli* cells and was found associated with the partially purified RNA polymerase (the DEAE-cellulose fraction), producing a non-infectious 6S-RNA product (Banerjee *et al.*, 1969b). Whether this 6S-RNA, which was pancreatic ribonuclease-resistant and composed of 110 - 130 nucleotides, was derived in some manner from Qβ-RNA or present in the host cell was not known. However, the Qβ-RNA polymerase would only replicate Qβ-RNA to produce an

infectious Qβ-RNA product in the presence of the 'factor fraction' (Banerjee et al., 1967; Hori et al., 1967; Franze de Fernandez, 1968; August et al., 1969).

(iii) Host components. Since the original discovery of the 'factor fraction' (Banerjee et al., 1967; Hori et al., 1967) which was required for the Qβ-RNA polymerase to replicate Qβ-RNA, this fraction has been found to be present in healthy E. coli cells (prepared as described for infected cells) and has been resolved into two components, both of which are required in the Qβ-RNA replication reaction (Franze de Fernandez et al., 1968; Shapiro et al., 1968). One factor (Factor 1) was separated from the enzyme during the DEAE-cellulose chromatography procedure of enzyme purification, was heat stable and partly resistant to proteases, and sedimented at 3S (approximately 70,000 mol. wt.). The second factor was isolated in the hydroxyapatite procedure, was heat and pepsin sensitive and sedimented at 1.5S (approximately 40,000 mol. wt.). Both were present in extracts of uninfected cells (Franze de Fernandez et al., 1968; Shapiro et al., 1968). The role of these in the Qβ-RNA polymerase reaction appeared to be at some step between the association of Qβ-RNA and enzyme and the initiation of synthesis (August et al., 1968; August, 1969). This conclusion was based on the observations that the factors do not affect either the kinetics or formation of an enzyme-viral RNA complex.
In their absence, however, no synthesis was detected with Qβ-RNA as template. It is also noteworthy that in contrast

to the requirement for Qβ-RNA directed synthesis, the factors had no effect in the reactions directed by complementary minus stands (August et al., 1968) polyC or copolymers containing cytidylate (August et al., 1968) or 6S-RNA (Banerjee et al., 1969b). The fact that two factors were required only with Qβ-RNA as template suggested that these agents do not react directly with the enzyme but are in some way related to the role of Qβ-RNA. This postulate was supported by the fact that saturation of the reaction with 'factor fraction' was unrelated to the amount of enzyme; however, as the Qβ-RNA was increased a greater concentration of 'factor fraction' was required for maximum incorporation of nucleotide (Franze de Fernandez et al., 1968). August et al. (1970) suggested that Factor 1 could be an initiation protein while Factor 11 could modify the primary structure of Qβ-RNA or even remove the terminal adenylate residue of Qβ-RNA.

Another interesting development with the Qβ-RNA polymerase was the discovery of host components in the enzyme itself. Kondo et al. (1970) have shown that the Qβ-RNA polymerase prepared by a slightly modified version of the method of Eoyang and August,(1968; and presented above) was composed of the phage polymerase gene product of molecular weight 69,000 (as expected, see 'host proteins' of Q -RNA replicase), and three host proteins of molecular weights 74,000, 47,000 and 33,000 respectively. All molecular weights were estimated after disruption of the β -RNA polymerase with SDS and mercaptoethanol and separation of the resultant proteins by SDS-polyacrylamide gel

electrophoresis (Kondo *et al*., 1970). However, as all four polypeptides were part of the RNA polymerase it was necessary to account for the fact that their total molecular weights (223,000) greatly exceed the value of 130,000 estimated from the sedimentation properties of the enzyme (Kondo *et al*., 1970). Such an apparent discrepancy may be due to the enzyme being highly assymetric (mol. wt. of 130,000 was estimated assuming the enzyme was a globular protein) or if it was in association-dissociation equilibrium with its subunits. However, the actual reason for this discrepancy is not known. A similar conclusion on the number and the molecular weights of the polypeptides of Qβ-RNA polymerase has been made by Kamen (1970). Using the Qβ-RNA polymerase isolated from Qβ coat amber mutant (amber-12)-infected *E. coli* the RNA polymerase was deduced to be composed of one single phage-specific polypeptide (65,000 mol. wt.) and three host specified polypeptides (mol. wts. 70,000, 45,000 and 35,000). None of the host proteins described had the same mobilities as subunits from *E. coli* DNA-dependent RNA polymerase (Kondo *et al*., 1970). However, Travis *et al*. (1970) reported that a combination of the 45,000 and 35,000 host units from disrupted Qβ-RNA polymerase preferentially stimulated the synthesis of ribosomal RNA by *E. coli* DNA-dependent RNA polymerase *in vitro* a function also performed by a factor (psi) found in uninfected *E. coli*.

Thus the replication process of phage RNA has proved to be considerably more complex than first envisaged, not only with the types of replicative intermediates but also the composition of the enzyme itself. A comparison between the properties discussed of the Qβ-RNA replicase (isolated by Spiegelman) and the Qβ-RNA polymerase (isolated by August) are presented in Table 1.

(2) <u>Animal-RNA virus systems</u>. Baltimore and Franklin (1963) first demonstrated the induction of an RNA polymerase by RNA animal viruses using L-cells infected with mengovirus (RNA mol. wt., 2×10^6). The enzyme activity was associated with a particulate cytoplasmic fraction (microsomes) which on incubation with nucleoside triphosphates yielded a double-stranded RNA product (after deproteinisation). The RNA polymerase activity which was independent of added viral RNA template has since been demonstrated in microsomal fractions of numerous animal virus-infected cells (Plagemann and Swim, 1966; Scholtissek and Rott, 1969a,b; Mahy *et al.*, 1970). Deproteinised products of animal virus induced RNA polymerase reactions contained labelled double-stranded and sometimes virus-like single-stranded RNA (Martin, 1967).

The only report of a small animal virus-induced RNA polymerase which was activated (three-fold) by the addition of viral RNA has been using partly purified extracts of polio virus-infected Hela cells (Gargiglio *et al.*, 1969).

(3) Plant-RNA virus systems. Studies using plant virus-induced RNA polymerase have not been as successful as the Q β phage-induced RNA polymerase studies, due to the difficulty in isolating such a replicating enzyme. Even the site of the viral-induced RNA polymerase within the plant cell was not known (see 'Site of synthesis of viral components' discussed previously); all animal virus-induced RNA polymerases were apparently attached to membrane preparations (see Martin, 1967).

Astier-Manifacier and Cornuet (1964, 1965a,b) first reported procedures for the isolation of TYMV-induced RNA polymerase activity from infected Chinese cabbage leaves. The synthesis of RNA by the enzyme was not stimulated by the addition of viral RNA suggesting that the enzyme was firmly bound to its template, a situation found in most animal virus-systems and one phage system (see 'MS2 phage-induced RNA synthetase'). However, the activity of this enzyme preparation was extremely low and it remained for Bové (1967a,b) and Ralph and Wojcik (1966a) to report significant plant virus-induced RNA polymerase activity using TYMV-infected Chinese cabbage leaves. In contrast to the soluble enzyme preparation used by Astier-Manifacier and Cornuet (1964) these other workers found the RNA polymerase activity associated with particulate preparations (sedimented at 16,000 g for 10 min.) of disrupted plant tissue. However, in both preparations formation of an RNA product was only dependent on the four ribonucleoside triphosphates and not on added viral RNA primer. Hybridisa-

tion experiments revealed that the radioactive RNA synthesised in cell-free particulate fractions of TYMV-infected Chinese cabbage leaves, was mostly viral-like RNA (Bové, 1967b). Bove et al. (1968) presented evidence that the double-stranded TYMV-RNA produced was not an artifact of RNA isolation (see 'MS2 phage-induced RNA synthetase'), and that a structure which was 90% resistant to pancreatic ribonuclease digestion does exist during the in vivo replication of the plant virus. However, the presence of plant ribonuclease in such preparations may have selected double-stranded RNA during the incubation used in the assay of TYMV-RNA polymerase.

Soon after the detection of the TYMV-induced RNA polymerase there were reports of similar activities in particulate fractions of several virus-infected plant systems (Ralph and Wojcik, 1969; Semal and Hamilton, 1968; Semal, 1969a,b). However, these particulate preparations also contained host DNA-dependent RNA polymerase activity (Bové, 1967a,b,c; Semal and Hamilton, 1968).

Reports of plant virus-induced RNA polymerase activities recently appeared in the literature which were both soluble and free of host DNA-dependent RNA polymerase activity. These soluble RNA polymerase activities required the addition of RNA for activity and were detected in extracts of virus-infected cucumbers (Gilliland and Symons, 1968; May et al., 1969; 1970; May and Symons, 1971) and TMV-infected tobacco plants (Britshammer, 1970).

Of worthy mention was a preliminary report by

Sela (1970) of the formation of an active TMV-RNA polymerase, in vitro, by the addition of TMV-RNA to a cell-free protein synthesising system. This approach bypassed production of the enzyme in the infected plant host and the problems associated with the detection and isolation of such activity.

(4) Summary. The inconsistency in the template requirements found for the viral RNA polymerase systems in vitro (animal, bacterial and plant) pose a yet unsolved problem. The enzyme has been isolated either as an enzyme-viral RNA complex (not requiring any added RNA primer) or free of bound viral RNA. In the latter case the enzyme either copied added viral RNA (phage Qβ-induced RNA polymerase or replicase) or any RNA species (f2 phage-induced RNA polymerase, CMV-induced RNA polymerase). The priming effect of RNA isolated from sources other than the virus particle itself with RNA polymerase preparations was unusual. However, recent findings with the phage Qβ-induced RNA polymerase introduced the problem of 'specificity factors' which govern the specificity of the viral replicating enzyme for RNA. Thus, it was possible that incomplete portions of the viral-induced RNA replicating enzymes were isolated but only the complete enzyme associated with 'specificity factors' copied viral RNA in preference to other RNA species.

E. CONCLUSIONS

The relevant data and theories concerning the replication of small RNA viruses has been discussed. The most prominent feature of the replication process was the synthesis of a specific polymerase which catalysed the synthesis of viral RNA via a replicative complex within the infected cell. Whether this complex was a double-stranded RNA intermediate (composed of one plus and one minus strand of viral RNA) or a free minus strand was not clear. However, it was clear that the viral-induced RNA polymerase played an important role in the replication of small RNA viruses. If the phage Qβ-induced RNA polymerase is typical of all small RNA virus replicating enzymes, then the RNA polymerase may be far more complex than first imagined with host proteins being used in the replication process.

The study reported in this thesis described an attempt to characterise both a soluble and particulate cucumber mosaic virus (CMV)-induced RNA polymerase detected in infected cucumber cotyledons. The CMV-induced RNA polymerase studied theoretically constituted one of the three or four proteins coded for by the CMV-RNA genome (RNA mol. wt. 1 x 10^6). The investigation into the nature and properties of plant virus-induced RNA polymerases was of considerable importance to our understanding of the mechanism of replication of plant viruses, of which little is known. One long term aim of this project was to achieve plant viral RNA replication with an isolated viral-induced RNA polymerase.

TABLE 1: COMPARISON OF TWO VIRAL RNA REPLICATING ENZYME PREPARATIONS FROM Q^{β} PHAGE-INFECTED *E. COLI*

	Q^{β} phage-induced replicating enzyme	
	RNA replicase (Pace *et al.* 1968)	RNA polymerase (Eoyang and August 1968)
Molecular weight of enzyme	110,000 (apparent) 210,000 (actual)	130,000 - 150,000
Templates copied (or replicated)	polyC Q^{β}-RNA 'variants' [b]Q^{β}-RNA minus strands [b]Q^{β}-RNA	polyC 6S-RNA [b]Q^{β}-RNA minus strands [b]Q^{β}-RNA plus Factor 1 plus Factor 11[a] cytidine containing synthetic polynucleotides
Molecular weights of protein components		
(a) Host protein	80,000	74,000 47,000 33,000
(b) Viral protein	130,000	69,000

All aspects of this table have been discussed in the text, and only the known protein components and template requirements are presented.

[a] Factor 1 (mol. wt. 70,000) and Factor 11 (mol. wt. 40,000) constitute the 'factor fraction' described in the text which was essential for Q^{β}-RNA to be replicated by the Q^{β}-RNA polymerase.

[b] Use of these templates led to the production of Q^{β}-RNA which was infectious for protoplasts of *E. coli*.

CHAPTER ONE

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

In order to avoid unnecessary repetition, those materials and methods that are used more than once in the following chapters of this thesis are described below.

1. Materials. Unlabelled nucleotides, pyruvate kinase (suspension of 10 mg/ml in 2 M $(NH_4)_2SO_4$), pancreatic ribonuclease-A, pancreatic deoxyribonuclease (ribonuclease-free), haemoglobin (Type 1), polyadenylic acid (polyA), polycytidylic acid (polyC), polyguanylic acid (polyG), polyuridylic acid (polyU) and salmon DNA (Type 111) were purchased from Sigma Chemical Co., St. Louis, Missouri. PolyC has also been obtained from Miles Laboratories, Elkhart, Indiana. α-^{32}P-Ribonucleoside 5'-monophosphates with a specific activity of 2 milliCuries/µmoles were prepared as described by Symons (1966, 1968) and were converted to the triphosphate as described by Symons (1969). ATP-^{3}H was obtained from Schwarz Bioresearch Inc., Orangeburg, New York. CMV and TRSV from infected cucumber plants were purified as described by Scott (1963) and TMV from infected tobacco plants was prepared by differential centrifugation. TYMV-RNA (Honesty strain) was the same preparation as that used by Symons et al. (1963). Baker's yeast ribosomal RNA was prepared by the method of Crestfield et al. (1955). Yeast transfer RNA was prepared as described by Holley et al. (1961) and E. coli RNA as described by Bolton (1966). Phosphoenolpyruvate (PEP; cyclohexylammonium salt) was a gift of Dr. R.H. Symons, University of Adelaide, South Australia;

this was ion-exchanged to the potassium salt by the method of Clarke and Kirby (1966). Actinomycin D was kindly donated by Merck, Sharp and Dohme, Rahway, New Jersey. Sucrose (analytical reagent) was obtained from the Colonial Sugar Refineries, Sydney, New South Wales, or Mann Research Laboratories, New York, New York (special enzyme grade) and used without further purification. All other chemicals were either analytical or laboratory reagent grade.

2. Viruses and plants. All virus strains and plants were kindly donated by Dr. R.I.B. Francki, University of Adelaide, South Australia. Viruses used throughout this thesis were CMV (Q-strain), TRSV and TMV (U1 strain). Cucumber seedlings (Cucumis sativus L. var 'Polaris') were germinated in a glasshouse. Seven to ten days after planting the seed, the cotyledons were lightly dusted with Carborundum powder and infected by an inoculum (serial inoculation) prepared by grinding infected plants with a small amount of water in a mortar and pestle. At intervals of three or four months purified virus (Q-CMV) was used as inoculum. Control plants were rubbed with an homogenate prepared from healthy plants, however results obtained from these controls were identical to untreated healthy plants. Plants were grown either under glasshouse conditions or in a constant temperature room (24 - 28°) using fluorescent lighting at an intensity of 100 - 150 ft-c for a 12 hour day. For assay of virus-induced RNA polymerase, infected plants were harvested 7 - 13 days after infection with CMV. In all, over 60,000 cucumber plants were inoculated with plant viruses during the course

of this project. Under our conditions, distinct symptoms of CMV appeared 7 - 9 days after infection with CMV (see Figure 1). The fact that there was a relationship between CMV-induced RNA polymerase activity and appearance of CMV symptoms on the primary leaves of the cucumber plants, allowed the selection of the best tissue for enzyme extraction. TMV was similarly grown on tobacco plants.

3. Assay of plant ribonuclease activity. The method was similar to that of Reddi (1966b) and involved the measurement of the rate of hydrolysis of yeast RNA to acid-insoluble material at 37° and pH 7.2.

The plant extract (0.15 ml) was incubated at 37° for 25 min. with 1.35 ml of 0.3% (w/v) yeast RNA, 0.1 *M* Tris-HCl buffer pH 7.2. The reaction was stopped by chilling in ice and then the addition of 0.5 ml of cold 3 *N* perchloric acid. After thorough mixing, the suspension was allowed to stand for 5 min. and the RNA precipitate removed by centrifugation at 5,000 *g* for 10 min. To 0.25 ml of the supernatant 4.0 ml of water was added and the optical density at 260 mμ was measured. The enzyme extract was added after the acid in the control experiment. Plant ribonuclease activity was expressed relative to that of pancreatic ribonuclease.

4. Assay of RNA polymerase activity. Enzyme assays were based on the incorporation of α-^{32}P-labelled ribonucleoside triphosphate into acid-insoluble material by a modification of the procedure of Gilliland and Symons (1968). The

standard enzyme assay medium contained in a final volume of 0.375 ml:

45 mμmoles α-^{32}P NTP (usually UTP or GTP unless stated; 5 - 8 x 10^5 cpm/assay).

240 mμmoles of each of the three unlabelled nucleoside triphosphates (neutralised to pH 8.0 with NaOH before addition to the assay medium).

2.5 μg actinomycin D.

0.5 μmole PEP (K^+).

25 μg pyruvate kinase.

500 μg bovine serum albumin.

5.0 μmole KCl.

10.8 μmoles $MgSO_4$ (unless stated otherwise; 29 mM).

9.0 μmoles 2-mercaptoethanol.

10.0 μmoles NH_4Cl.

12 μmoles Tris-HCl buffer pH 8.5.

1.0 mg yeast RNA (unless stated otherwise).

Plant protein (in 0.1 ml), the amount of protein depending on the polymerase preparation used; actual amounts are quoted in the text.

Variations within the stated conditions are given in the text. Assays were incubated at 37° for 30 min. (unless stated otherwise) and the reaction was stopped by chilling in ice. All assays were carried out in duplicate. To each assay was added 5.0 ml of an ice-cold solution containing 5% trichloroacetic acid, 2% NaH_2PO_4, and 2% $Na_4P_2O_7$, and the acid-insoluble material was filtered onto a glass fibre disc (Whatman GF/A or GF/C, 2.5 cm in diameter) in a Millipore apparatus, where it was slowly washed (5 - 10 min.)

with 120 ml of the ice-cold acid solution. Residual trichloroacetic acid was removed by washing the glass-fibre disc with 5.0 ml of cold ether. In assay mixtures that contained added pancreatic ribonuclease or low levels of added RNA (less than 1.0 mg/assay), 1.0 mg of yeast RNA was added immediately before acid precipitation to ensure complete recovery of the RNA product.

The ether washed discs were dried in an oven (100° for 5 - 10 min.) and then counted in a Packard liquid scintillation spectrometer. Zero time incubation mixtures were treated in the same manner for the estimation of blank values which were normally 30 - 60 cmp per assay.

5. Nearest neighbour analysis of the RNA polymerase product.

Usually for these analyses, assays were carried out with the specific activity of the labelled substrate five times that of the standard assay (i.e., about 2.5×10^{6} cpm/assay). The collection of the product on the glass fibre disc involved a slower wash (30 min.) with acid solution (200 ml). The glass-fibre discs with labelled product were covered with 1.5 ml of 0.3 N KOH and incubated in stoppered tubes at 37° overnight to hydrolyse the RNA to 2'(3')-mononucleotides. Potassium ions were exchanged for ammonium ions by passing the digest slowly through a column containing 2 - 3 grams of Zeokarb 225 (NH_4^+), which was then washed with 6.0 ml of water. The solution was taken to dryness on a rotary evaporator, and the residue was dissolved in a small volume (0.25 ml) of 50% ethanol (v/v) - 1% NH_4OH (w/v). Duplicate samples were dried as bands 5 cm wide on Whatman 3 MM

chromatography paper, and the four mononucleotides were separated by one-dimensional chromatography for 16 hours in isopropanol - water - 25% (w/v) ammonia (7 : 2 : 1) followed in the same direction, after drying the chromatogram, by 24 hours in tertiary butanol-isobutyric acid-water (30 : 46 : 24) (made to pH 3.7 with 25% (w/v) ammonia and containing 0.4 mM EDTA). Final R_f values were: 2'(3')-GMP, 0.11; 2'(3')-UMP, 0.17; 2'(3')-CMP, 0.24; 2'(3')-AMP, 0.35. The labelled nucleotides were located under ultra-violet light, cut out, and counted in a Packard liquid scintillation spectrometer. Yield of radioactivity in the mononucleotides was 75% of the radioactivity present in the original RNA product, allowing for decay of the ^{32}P-label while employing this technique.

6. Isolation of the product of the RNA polymerase reaction. After incubation, the reaction mixture containing the radioactive product (5 - 20 times the normal assay size and about 2.5 x 10^6 cpm of labelled substrate/assay) was shaken with SDS (final concentration 0.5 - 1.0%) and Chelex resin (Na^+, 50 mg/ml) (Feix et al., 1967). An equal volume of 78% aqueous phenol was added, and the mixture was shaken vigorously for 10 min. After centrifugation at 5,000 g for 10 min. the aqueous layer was collected and most of the phenol was removed by shaking with an equal volume of ether. The ether-water mixture was centrifuged at 5,000 g for 10 min. and the aqueous layer was removed, and the RNA was precipitated from the aqueous layer by the addition of two volumes of ethanol. The precipitate was dissolved in

0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 (SSC) and used for the studies on the nature of the polymerase product. Yield of labelled product was 50 - 70%.

7. Extraction of plant viral RNA. RNA was extracted from CMV, TMV and TRSV by the following procedure, all steps being performed at 4°. Virus preparations (4.0 mg virus/ml) in 50 mM Tris-HCl buffer, pH 8.5, 0.5% SDS, were added to an equal volume of 78% aqueous phenol, and the mixture shaken vigorously for 15min. After centrifugation at 5,000 g for 10 min., the aqueous layer was re-extracted twice with an equal volume of 78% aqueous phenol. The aqueous layer, after a total of three phenol extractions and centrifugations, was shaken with an equal volume of ether. The water-ether mixture was centrifuged at 5,000 g for 10 min., the ether layer was removed and the aqueous layer again shaken with an equal volume of ether. Finally the aqueous layer was adjusted to 0.05 M NaCl by the addition of concentrated NaCl solution and the RNA precipitated by the addition of two volumes of ice-cold ethanol. The precipitate was dissolved in 50 mM Tris-HCl, pH 8.5, and stored at -15°. The yield of viral RNA was 45 - 50%.

8. Preparation of denatured DNA. A solution of native DNA which had been dissolved in water (200 μg/ml, unless indicated otherwise) was heated in a sealed Pyrex glass tube at 100° for 15 min. then frozen in an ethanol-dry ice mixture and allowed to thaw.

9. <u>RNA estimations</u>. RNA was determined spectrophotometrically on the assumption that native RNA had an optical density at 260 mμ of 24, at a concentration of 1.0 mg/ml. However, RNA estimations were carried out on <u>extracts</u> of RNA polymerase preparations (both healthy and infected plant extractions contained material present which affected RNA estimations by optical density at 260 mμ or oricinol reactions) by alkaline hydrolysis (solution made 0.3 <u>N</u> KOH and left at 37° overnight) followed by separation and estimation of the resultant 2'(3')-mononucleotides by paper chromatography. The technique used was similar to that described for the 'Nearest-neighbour analysis of the RNA polymerase product'. Thus, the overnight digested plant material (1.0 ml of plant extract plus 0.5 ml of 1.0 <u>N</u> KOH) was passed through a Zeocarb 225 (NH_4^+) column (2 - 3 grams) and washed with 6 ml of water. The solution was taken to dryness on a rotary evaporator, and the residue was dissolved in 0.25 ml of 50% ethanol (v/v) - 1% ammonia (w/v). The sample was dried as a band 5 cm long on Whatman 3 MM chromatography paper which was run overnight in isopropanol-water - 25% (w/v) ammonia (7 : 2 : 1) after which the chromatogram was dried. The 2'(3')-mononucleotides were located under ultra-violet light as two faint spots (incomplete separation of the four mononucleotides at this step; see 'Nearest-neighbour analysis of the RNA polymerase product' for complete separation). The mononucleotides were cut out and eluted overnight in 5.0 ml of 0.1 <u>N</u> HCl along with the appropriate blanks. Estimation of the amount of mononucleotide material was made on the assumption that

1 mg/ml of 2'(3')-mononucleotides has an optical density at 260 mμ of 30.

10. <u>Protein estimations</u>. Protein was estimated by the method of Lowry <u>et al</u>. (1951) using bovine serum albumin as standard.

11. <u>Scintillation fluid</u>. This was a solution of 0.3% (w/v) 2,5, diphenyloxazole (PPO) and 0.03% (w/v) 1,4-bis-2- (4-methyl-5-phenyloxazolyl benzene) (dimethyl POPOP) in toluene. Both PPO and POPOP were scintillation grade and were obtained from Packard Instrument Co., LaGrange, Illinois.

12. <u>Enzyme units.</u> A unit of RNA polymerase is defined as that amount which incorporated 1 μμmole of α-^{32}P-labelled ribonucleoside triphosphate into acid-insoluble material per min. under the specified assay conditions. <u>Specific enzymatic activity</u> is defined as units of enzyme activity per milligram of plant protein.

In order to express total RNA polymerase activity, the number of units per gram fresh weight of plants has been used.

FIGURE 1. CUCUMBER MOSAIC VIRUS-INFECTION OF CUCUMBER COTYLEDONS.

CMV infection produces a mosaic of light green to yellowish on the primary leaves, 7 - 9 days after infection.

The photograph shows a cucumber cotyledon (approximately twice the actual size) nine days after infection.

CMV-INFECTED

HEALTHY

CHAPTER TWO

EXTRACTION OF A SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE FROM INFECTED PLANTS

EXTRACTION OF A SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE FROM INFECTED PLANTS

INTRODUCTION

Evidence presented by Gilliland and Symons (1968) indicated that crude soluble extracts of cucumber cotyledons infected with CMV contained a virus-induced RNA polymerase. The work described in this chapter was primarily concerned with improving the extraction and assay of the CMV-induced RNA polymerase activity from infected cucumbers. Before the question of whether or not the CMV-induced RNA polymerase was bound to a viral RNA template could be answered (as proposed by Gilliland and Symons, 1968), removal of contaminating plant ribonucleases was considered necessary. As a consequence of this work an improved extraction method was developed which allowed the RNA polymerase to be purified five-fold as well as being free of detectable plant ribonuclease activity.

METHODS

1. EXTRACTION METHOD A. Preparation of crude enzyme extracts (as used by Gilliland and Symons (1968)). All operations were performed at 4°. Homogenates were prepared by grinding healthy or infected plants (7 - 13 days after infection) in a mortar and pestle; for each gram fresh weight of tissue, 2.0 ml of the following extraction buffer was added before grinding; 1.6% yeast RNA, 5 mM $MgSO_4$, 50 mM Tris-HCl buffer

pH 8.5. The homogenates were strained through cheesecloth and centrifuged at 10,000 g for 10 min.; the supernatant was used for the RNA polymerase assay. This crude extract containined 5.5 - 8.0 mg of protein per ml and 0.2 - 0.35 μg of plant ribonuclease activity per ml.

2. EXTRACTION METHOD B. Preparation of modified crude enzyme extracts. The modified crude enzyme extracts were prepared essentially as described for EXTRACTION METHOD A except that 90 mM 2-mercaptoethanol and 100 mM NH_4Cl replaced the 1.6% yeast RNA in the extraction medium.

3. EXTRACTION METHOD C. Preparation of partly purified enzyme extracts. All operations were performed at 4°. Homogenates were prepared by grinding healthy or infected plants (7 - 13 days after infection) in a mortar and pestle; for each gram fresh weight of tissue, 2.0 ml of the following extraction buffer was added before grinding; 50% saturated $(NH_4)_2SO_4$, 100 mM NH_4Cl, 90 mM 2-mercaptoethanol, 50 mM Tris-HCl buffer pH 8.5. The homogenate was squeezed through a fine nylon cloth and centrifuged at 25,000 g for 10 min. The pellet was then resuspended in twice the original volume of extraction buffer and centrifuged at 25,000 g for 10 min. The pellet was then resuspended in the original volume of extraction buffer without $(NH_4)_2SO_4$, and the suspension was centrifuged at 25,000 g for 10 min. The supernatant was used for assay of RNA polymerase activity in extracts of infected cucumbers and contained 1.0 - 2.0 mg of protein per ml and negligible plant ribonuclease activity. Both healthy and infected plant extracts contained 50 - 80 μg of RNA

per ml (100 - 160 µg RNA/gram fresh weight of plants).

4. Assay of RNA polymerase activity. As previously described in Chapter 1, using 10.8 µmoles $MgSO_4$/assay (29 mM), 1.0 or 1.6 mg of added yeast RNA (as stated) and UTP-^{32}P as the labelled substrate.

RESULTS

1. Effect of yeast RNA, 2-mercaptoethanol and NH_4Cl in the extraction medium on the activity of soluble RNA polymerase in crude extracts of healthy and infected plants. Gilliland and Symons (1968) have shown that, for detection of RNA polymerase activity in crude extracts of CMV-infected cucumber cotyledons, it was essential to add yeast RNA (1.6%) during the homogenisation of the tissue (see also EXTRACTION METHOD A); the role of the yeast RNA was presumed to counteract the effect of plant ribonucleases (see also Cocucci and Sturani, 1966). Thus, before purification of the virus-induced RNA polymerase was attempted, the conditions of extraction of the RNA polymerase as described by Gilliland and Symons (1968; see EXTRACTION METHOD A) were investigated with the aim of increasing the activity of the enzyme in crude extracts, and of the elimination of the requirement for yeast RNA in the extraction medium.

The effects on RNA polymerase activity in crude extracts of the addition of 1.6% yeast RNA, 90 mM 2-mercapto-ethanol and 100 mM NH_4Cl to a basic extraction medium of 5 mM $MgSO_4$, 50 mM Tris-HCl buffer pH 8.5, are shown in Table 2. (Whenever yeast RNA was not present in the

extraction medium, it was essential to add it to the enzyme assay otherwise no activity was obtained under the stated assay conditions.) The results show that mercaptoethanol and NH_4Cl increased the activity in crude extracts of infected plants prepared in the presence of yeast RNA, and there was little effect on the low activity found in extracts of healthy plants. When no yeast RNA was added to the basic Tris-HCl-$MgSO_4$ buffer, no virus-induced RNA polymerase activity was found, in agreement with the previous results of Gilliland and Symons (1968). However, activity was found when either mercaptoethanol or NH_4Cl was added and there was further stimulation when both were added together (Table 2). Maximum activity was obtained in the presence of all three compounds, i.e., yeast RNA, 2-mercaptoethanol and NH_4Cl.

The nature of the protective effect of yeast RNA on RNA polymerase activity is not known. Since mercaptoethanol and NH_4Cl could replace yeast RNA in the crude extracts (see EXTRACTION METHOD B), and it was also found that these two compounds had negligible effect on plant ribonuclease activity, the protective action of yeast RNA cannot be due to some effect on ribonuclease activity as Gilliland and Symons (1968) proposed.

2. Partial purification of soluble CMV-induced RNA polymerase and removal of plant ribonucleases. Before the question of whether or not the CMV-induced RNA polymerase was bound to a viral RNA template (as proposed by Gilliland and Symons, 1968), removal of contaminating plant ribonucleases was

considered necessary, in addition to removal of the need for added yeast RNA in the extraction medium. Fortunately, cucumber ribonucleases were found to be soluble in 50% saturated $(NH_4)_2SO_4$ (see also Reddi, 1966b) while the CMV-induced RNA polymerase was insoluble. Eventually, the method developed (see EXTRACTION METHOD C) was to extract the plant tissue twice with extraction medium made 50% saturated with $(NH_4)_2SO_4$ in order to remove plant ribonucleases before the solubilisation of the RNA polymerase. This method allowed the RNA polymerase to be purified five-fold relative to the specific activity of a crude homogenate with a recovery of 50% and with greater than 99% removal of plant ribonuclease activity. Such a preparation will be referred to throughout this thesis as the partly purified RNA polymerase extract. The alternative approach of extraction of the RNA polymerase into crude extracts (by EXTRACTION METHOD B) followed by its precipitation with 50% saturated $(NH_4)_2SO_4$ removed only 75 - 85% of the plant ribonuclease activity, with only a two-fold purification of the RNA polymerase activity.

However, an important feature to note was the fact that yeast RNA (1.0 mg) must still be added to the assay medium, in order to detect the CMV-induced RNA polymerase activity, even though it can be omitted during the extraction procedure.

3. Detection of soluble CMV-induced RNA polymerase activity in various host plants. Having developed a procedure for the extraction of viral-induced RNA polymerase activity free of contaminating ribonuclease activity, it was considered important to determine whether other host plants infected with CMV contained this activity. Table 3 shows the RNA polymerase activity detected in partly purified extracts of CMV-infected plants. Extracts from CMV-infected cucumbers or rockmelons contained the greatest amount of detectable RNA polymerase activity. Other infected plant (all plants developed symptoms of CMV) extracts investigated contained less than 10% of the CMV-induced RNA polymerase activity found in infected cucumber extracts. The source of viral-induced RNA polymerase for the work described in several chapters of this thesis was partly purified extracts of cucumbers infected with cucumber mosaic virus.

4. Effect of CMV-infection on the activity of soluble RNA polymerase in partly purified extracts of cucumber cotyledons. The results presented in Figure 2 show the partly purified RNA polymerase activity in the same batch of healthy and CMV-infected cucumber cotyledons as a function of time after infection. There was a significant increase in activity above the control level on the third day after infection. The activity of the extracts from healthy plants rose to a plateau level about nine days after infection, while negligible activity was obtained with the healthy plant extracts over the same period of time. Infected plants were harvested 7 - 13 days after CMV infection for

all subsequent work.

DISCUSSION

A central feature throughout the work described in this thesis was the problem as to whether the CMV-induced RNA polymerase existed bound to viral RNA template (an enzyme-viral RNA complex) or free of bound viral RNA. Previously the CMV-induced RNA polymerase activity was only detectable when yeast RNA (1.6%) was included in the extraction medium (Gilliland and Symons, 1968); thus interpretation of results as to whether the polymerase was indeed bound or not to an RNA template was particularly difficult. However, the need for the presence of yeast RNA in the extraction medium was abolished by the addition of 2-mercaptoethanol and NH_4Cl to the extraction medium. As these two compounds had little effect on plant ribonuclease activity it was unlikely that added yeast RNA was protecting an enzyme-RNA complex from plant ribonuclease degradation, a role proposed by Gilliland and Symons (1968). The extraction of CMV-induced RNA polymerase was further improved by the addition of 50% saturated $(NH_4)_2SO_4$ to the extraction medium. This enabled the CMV-induced RNA polymerase activity to be separated from the plant ribonuclease activity. Thus a preparation of CMV-induced RNA polymerase free of added yeast RNA and plant ribonuclease was developed. However, in order to detect CMV-induced RNA polymerase activity under the stated conditions it was necessary to include yeast RNA in the assay medium (1.0 or 1.6 mg/assay have both been used

in this chapter). This phenomenon will be discussed further in the following chapter.

Applying the new extraction method (EXTRACTION METHOD C; see Methods) to several CMV-infected plant tissues it was found that the best source of detectable CMV-induced RNA polymerase was extracts of infected cucumbers (day 7 - 13 after infection). However, 60% of the maximum activity present in CMV-infected cucumber extracts was found in extracts of infected rockmelons, a plant closely related to cucumbers. One puzzling feature was the fact that almost negligible levels of RNA polymerase activity were found in CMV-infected plants other than cucumbers and rockmelons, a feature which still remains unresolved. CMV-infected Nicotiana tobacum and glutinosa plants have been checked several times for CMV-induced RNA polymerase activity over a period of two - three years with the same negative result.

TABLE 2: EFFECT OF 2-MERCAPTOETHANOL AND NH_4Cl IN THE EXTRACTION MEDIUM ON SOLUBLE RNA POLYMERASE ACTIVITY IN CRUDE EXTRACTS OF CUCUMBERS INFECTED WITH CUCUMBER MOSAIC VIRUS

Extraction medium[a]	Specific enzymatic activity[b]	
	Healthy plants	Infected plants
Yeast RNA	0.4	5.3
+ 2-mercaptoethanol	1.2	10.6
+ NH_4Cl	0.3	6.7
+ (2-mercaptoethanol and (NH$_4$Cl	0.8	12.6
No yeast RNA	0.4	0.6
+ 2-mercaptoethanol	0.6	2.5
+ NH_4Cl	0.6	2.0
+ (2-mercaptoethanol and (NH$_4$Cl	0.7	7.0

[a]The crude enzyme extracts were prepared using an extraction medium containing 50 mM Tris-HCl buffer pH 8.5, 5 mM $MgSO_4$, with or without the following additions: 1.6% yeast RNA, 90 mM 2-mercaptoethanol, 100 mM NH_4Cl.

[b]The extract was assayed as described in Methods with UTP-^{32}P as the labelled substrate. Yeast RNA (1.6 mg) was added to the assay medium when not present in the extraction medium.

Specific enzymatic activity: μμ moles of labelled nucleoside triphosphate incorporated per min. per milligram of plant protein under the defined assay conditions.

TABLE 3: DETECTION OF SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE ACTIVITY IN VARIOUS HOST PLANT EXTRACTS

Host plant	Specific enzymatic activity	
	Healthy plants	Infected plants
CUCURBITACEAE[a]		
Cucumber	0.8	22.0
Rockmelon	0.7	13.4
Pumpkin	1.0	2.3
Marrow	0.9	2.2
Momordica charantia	0.9	2.1
Watermelon[b]	1.0	1.9
SOLANACEAE[a]		
Nicotiana clevlandii	1.3	2.2
Nicotiana glutinosa	1.1	2.0
Nicotiana tabacum	0.9	1.8
Datura stramonium[b]	1.7	2.3
Capsicum frutescens[b]	1.0	0.9
SCROPHULARIACEAE[a]		
Antirrhinum majus	0.4	0.4

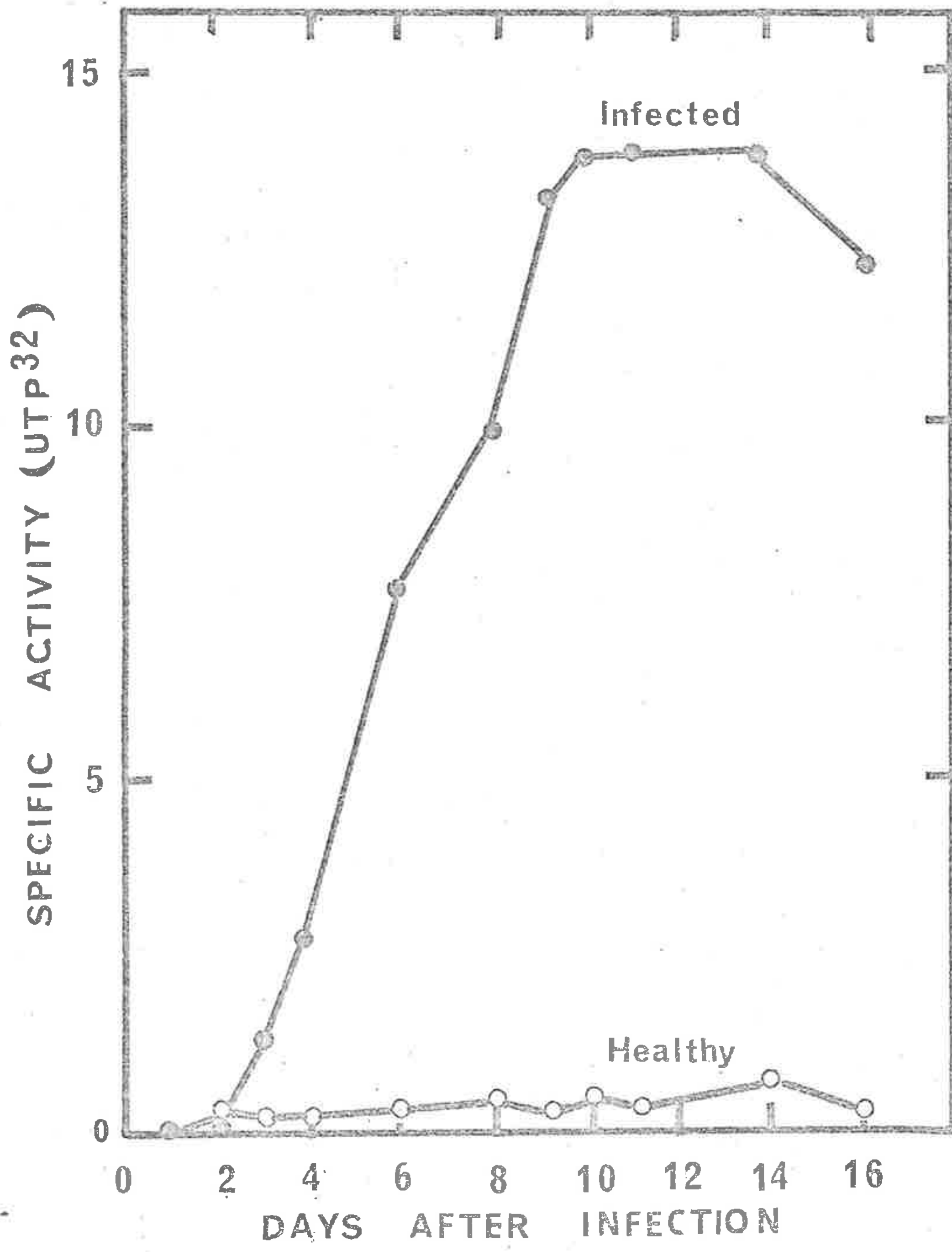
[a]The Family to which the plants belong.

[b]Extraction method removed less than 25% of the total plant ribonuclease activity.

Partly purified RNA polymerase extracts of all the CMV-infected host plants indicated (harvested 7-9 days after infection) were prepared by EXTRACTION METHOD C and contain 0.8 - 1.5 mg of protein/ml., and negligible plant ribonuclease activity (unless indicated). Assays were performed as described in Methods with 1.0 mg of yeast RNA added to the assay medium and UTP-^{32}P as the labelled substrate.

FIGURE 2: EFFECT OF CUCUMBER MOSAIC VIRUS INFECTION ON THE ACTIVITY OF SOLUBLE RNA POLYMERASE IN PARTLY PURIFIED EXTRACTS OF CUCUMBER COTYLEDONS

Partly purified RNA polymerase extracts were prepared by EXTRACTION METHOD C from CMV-infected cucumbers. Assays were performed as described in Methods with 1.0 mg of yeast RNA added to the assay medium.


Infected
Healthy
SPECIFIC ACTIVITY (UTP32)
DAYS AFTER INFECTION
15
10
5
0
0
2
4
6
8
10
12
14
16

CHAPTER THREE

PROPERTIES OF A SOLUBLE PARTLY PURIFIED PLANT VIRUS-INDUCED RNA POLYMERASE FROM CUCUMBER COTYLEDONS INFECTED WITH CUCUMBER MOSAIC VIRUS

PROPERTIES OF A SOLUBLE PARTLY PURIFIED PLANT VIRUS-INDUCED RNA POLYMERASE FROM CUCUMBER COTYLEDONS INFECTED WITH CUCUMBER MOSAIC VIRUS

INTRODUCTION

The properties of the partly purified CMV-induced RNA polymerase (prepared from infected cucumber cotyledons by EXTRACTION METHOD C described in the previous chapter) are presented in this chapter. The results show that the CMV-induced RNA polymerase copied any RNA supplied to it in the assay medium and that it was not bound to viral RNA. This enzyme system contrasted to that found in cell-free particulate fractions isolated from Chinese cabbage leaves infected with turnip yellow mosaic virus (TYMV)(Bové, 1967a,b; Ralph and Wojcik, 1966a), bromegrass mosaic virus (BMV)-infected barley leaves (Semal and Hamilton, 1968; Semal and Kummert, 1970), tobacco mosaic virus (TMV)-infected tobacco leaves (Ralph and Wojcik, 1969), broad bean mosaic virus (BBMV)-infected broad bean leaves (Semal, 1969a), alfalfa mosaic virus (AMV)-infected tobacco leaves (Semal, 1969b) and cowpea chlorotic mottle virus (CCMV)-infected cowpeas (Semal, 1969b), where the RNA polymerase was presumably present as a particulate, enzyme-viral RNA complex.

Throughout this chapter, unless otherwise stated, CMV-induced RNA polymerase activity present in the partly purified cucumber extracts was detected by the addition of 1.0 mg of yeast RNA to the assay medium. When yeast RNA

(which is shown to act as a template) was omitted from the defined assay medium (containing 29 mM $MgSO_4$; see also Chapter 8), no RNA polymerase activity was detected.

MATERIALS AND METHODS

1. Materials. General materials were previously described in Chapter 1. Lipoamide dehydrogenase, prepared from pig heart by the method of Massey (1960), was kindly donated by the late Professor M.R. Atkinson, Flinders University, South Australia. Rifampicin was obtained from Mann Research Laboratories, New York.

2. Preparation of partly purified RNA polymerase extracts. Extracts were prepared from CMV-infected cucumbers 7 - 13 days after infection by EXTRACTION METHOD C described in the previous chapter.

3. Assay of RNA polymerase activity. As previously described in Chapter 1, using 10.8 μmoles $MgSO_4$/assay (29 mM), 1.0 mg of yeast RNA (unless stated otherwise) and UTP-^{32}P (or GTP-^{32}P where stated only) as the labelled nucleoside triphosphate. Partly purified RNA polymerase preparations usually incorporated 750 - 2,000 cpm of labelled nucleotide under the standard conditions.

4. Nearest-neighbour analysis of the RNA polymerase product. As previously described in Chapter 1.

RESULTS

1. General properties of the soluble CMV-induced RNA polymerase

(a) Stability of the RNA polymerase preparation. The partly purified CMV-induced RNA polymerase preparation was found to retain 80% of its activity at 4° (in ice) after five hours but all detectable activity was lost overnight at 4° or -15°. The stability of the enzyme was increased by the addition of 5 - 10% sucrose to the preparation, as 50% of the original activity was detected after storing one week at -2°. For longer storage, however, the enzyme was stored at -15° in 50% glycerol, where 60% of the original activity was detectable after one month. In all work described, partly purified RNA polymerase extracts were prepared each day, and were not stored.

(b) The effect of temperature and time of incubation. The results presented in Figure 3 show that the activity of the RNA polymerase was optimal at 37° and that the incorporation of labelled substrate into product was linear at this temperature for at least 60 min. An unusual feature of these results was the very low level of activity of RNA polymerase at 20° as compared to that of 30°. The reason for this is not known.

(c) The effect of concentration of substrate and added RNA. Apparent Km values of 0.041 mM and 0.062 mM for UTP and GTP as the labelled substrate, respectively, were determined from Lineweaver-Burk plots of the results of Figure 4A. The RNA polymerase assay medium used in all subsequent work

described in this thesis contained 0.12 mM labelled nucleoside triphosphate (45 mμmoles/assay) and 0.64 mM unlabelled nucleoside triphosphates (240 mμmoles/assay).

RNA polymerase activity was also followed using various concentrations of RNA from three sources. Figure 4B shows that the assay system was saturated at a concentration of 1.0 mg per assay for yeast-RNA and 0.25 mg per assay for yeast transfer RNA and TYMV-RNA, under the stated assay conditions. At all concentrations, yeast RNA gave the highest activity.

(d) The effect of pH of the assay medium. The RNA polymerase activity detected in the presence of yeast RNA in the assay medium was optimal over a broad range of pH (7.5 - 8.8) but the activity greatly decreased below pH 7.0. In all work described the RNA polymerase activity was extracted and assayed at pH 8.5.

(e) The effect of various ions in the assay medium. Detection of CMV-induced RNA polymerase activity was completely dependent on magnesium ions in the assay medium, omission of these resulted in complete loss of RNA polymerase activity (see also Figure 17, Chapter 8). The magnesium ions (29 mM; 10.8 μmoles $MgSO_4$/assay) could not be replaced by the other divalent ions (Mn^{2+}, Ca^{2+}; 0 - 13 mM; Ni^{2+}, Cu^{2+}, Zn^{2+}; 0 - 2 mM) in the assay medium, as no RNA polymerase activity could be detected. Further, the addition of Mn^{2+}, Ca^{2+} or Cu^{2+} (1 - 5 mM) to an assay medium containing 29 mM $MgSO_4$ resulted in complete loss of CMV-induced RNA polymerase activity. In contrast, NH_4^+ or K^+ ions over the concentration

range of 0 - 54 mM in the assay medium had negligible effect on the RNA polymerase activity.

(f) The effect of rifampicin in the assay medium. Rifampicin is an antibiotic which has been found to halt bacterial DNA-dependent RNA polymerase activity *in vitro* by binding to the enzyme and was effective at a concentration of 0.1 - 0.2 μg/ml (Hartmann *et al.*, 1967; Bandle and Weissmann, 1970). This antibiotic had no effect on mammalian DNA-dependent RNA polymerase and its effect on plant DNA-dependent RNA polymerase has not been reported, although it is believed to stop the chloroplast DNA-dependent RNA polymerase of *Chlamydomonas reinhardi* (Surzycki, 1969). However, this antibiotic was found to have no effect on the CMV-induced RNA polymerase activity even at concentrations as high at 20 μg/ml.

(g) Nucleoside triphosphate requirements of the assay system. The dependence of the measurement of RNA polymerase activity on the presence of all four triphosphates using either labelled UTP or GTP was investigated by omitting either one or three of the unlabelled triphosphates from the assay medium. For assays with labelled GTP, there was considerable dependence of the RNA polymerase activity on the presence of all four triphosphates for extracts of infected plants. For assays using UTP as the labelled substrate there was less dependence on the presence of all four triphosphates than in the case for labelled GTP assays (Table 4). This decreased dependence was most likely related to results obtained for healthy plants that showed appreciable increase in apparent RNA polymerase activity in the absence of CTP

(either alone or with the omission of the other two unlabelled triphosphates; see also Gilliland and Symons, 1968). This effect has not been investigated further; but it can be explained by a terminal incorporation of UTP into transfer RNA (see also Chapter 8) which is inhibited by CTP; such an effect has been reported for a rat liver enzyme by Daniel and Littauer (1963).

(h) The effect of various added DNA and RNA samples. Since the presence of yeast RNA was required in the assay medium (but not in the extraction medium), other polynucleotides were tested to see if they could be substituted for yeast RNA. When salmon DNA was added to the assay medium, the RNA polymerase activity was sensitive to actinomycin D (DNA-dependent RNA polymerase activity), but this was found to be present in partly purified extracts of both healthy and infected plants (Table 5). In contrast, when yeast RNA was added to the assay medium, RNA-dependent RNA polymerase activity was detected only in virus-infected plant extracts and was insensitive to actinomycin D. Plant viral RNA was found to give the same (TMV-RNA) or less (CMV-RNA and TYMV-RNA) RNA-dependent RNA polymerase activity than yeast RNA when added to the assay system, at the same concentration (Table 5) and under the assay conditions defined.

Thus, the RNA-dependent RNA polymerase activity was present only in virus-infected plant extracts and this activity was only detected when RNA was present in the assay medium.

2. Evidence that the soluble RNA polymerase does not contain viral RNA but does require added RNA as template. As the RNA polymerase activity was only obtained when RNA was present in the assay medium, it was necessary to determine whether the RNA added was acting as a template for the RNA polymerase or was protecting an enzyme-viral RNA complex, a role originally proposed for the added RNA by Gilliland and Symons (1968).

(a) The effect of synthetic polynucleotides. If the added RNA was protecting an enzyme-viral RNA complex, then polyU, polyC and yeast RNA should all cause a similar incorporation of UTP-^{32}P, GTP-^{32}P and ATP-^{3}H into the product of the RNA polymerase reaction. However, if the added RNA acted as a template, then RNA polymerase would only be detected when complementary base pairing between the added RNA and the labelled substrate was possible. Thus, GTP-^{32}P incorporation into acid-insoluble material would only occur with polyC and yeast RNA as templates, ATP-^{3}H with polyU and yeast RNA and UTP-^{32}P with only yeast RNA. Table 6 shows that RNA polymerase activity was detected only when complementary base pairing between the added RNA and the labelled substrate was possible (see also Chapter 8); hence the results indicate that the added polynucleotides were acting as templates and were not protecting an enzyme-viral RNA complex. PolyA was not used in this work because of its insolubility under the stated assay conditions. Low and variable specific activities with no added RNA template to the assay were again detected.

(b) Nearest-neighbour analysis of the RNA polymerase product. Two templates, yeast RNA and TYMV-RNA, which vary markedly in their base ratios, were used in the assay medium with either UTP-^{32}P or GTP-^{32}P as the labelled substrate. Nearest-neighbour analyses (Table 7) showed that the products were quite different when these templates were used. After alkaline degradation of the products obtained using TYMV-RNA and yeast RNA, 43% and 20%, respectively, of the total radioactivity incorporated was found as 2'(3')-GMP. These values are indicative of the added RNA template where TYMV-RNA has a molar ratio of 38.6% CMP (Symons et al., 1963) and yeast RNA a molar ratio of 20% CMP (Crestfield et al., 1955). Thus, nearest-neighbour analysis also indicated that the CMV-induced RNA polymerase copied the added template.

(c) Resistance of the RNA polymerase to preincubation with pancreatic ribonuclease. Preincubation of the partly purified plant extract with pancreatic ribonuclease (1 or 5 μg/ml) at 37° for 10 minutes had no effect on subsequent RNA polymerase activity assayed after the removal of added ribonuclease. This experiment was possible because it was found that 98% of the added ribonuclease, which interfered with the assay (see next chapter), was soluble when the RNA polymerase was precipitated in 50% saturated $(NH_4)_2SO_4$. This experiment showed that the enzyme was not dependent on ribonuclease-accessible RNA in the enzyme preparation, but it does not exclude the possibility of non-accessible or ribonuclease-resistant RNA bound to the enzyme.

(d) Sedimentation analysis of the RNA polymerase. The RNA polymerase was centrifuged through a 5 - 20% sucrose gradient (Figure 5), using pig heart lipoamide dehydrogenase as marker, to obtain an estimate of the molecular weight. Using the equation of Martin and Ames (1961) and taking the molecular weight of pig heart lipoamide dehydrogenase as 100,000 (Massey *et al.*, 1962) the molecular weight of the RNA polymerase was calculated to be 123,000. From similar sucrose gradient results using catalase (mol. wt. 250,000) and haemoglobin (mol. wt. 68,000) as markers, the molecular weight was estimated to lie between 120,000 and 130,000. These results indicated that the RNA polymerase was not associated with a viral RNA template (mol. wt. 1×10^6) and that it was unlikely to contain RNA of any significant size.

The molecular weight obtained for this plant virus-induced enzyme is close to that found under similar conditions for the RNA-free RNA replicase of Qβ phage-infected *E. coli* (Eikhom and Spiegelman, 1967). However, the latter enzyme (isolated by the method of Pace *et al.*, 1968) is composed of two separable subunits of molecular weights 80,000 and 130,000. The possibility that the RNA polymerase studied here consists of more than one subunit is considered throughout this work.

DISCUSSION

The results presented above have indicated that the soluble CMV-induced RNA polymerase activity found in partly purified extracts of infected cucumbers, was not associated with a viral RNA template. The RNA polymerase was found to copy several RNA templates by complementary base pairing. These results were in contrast to several other cell-free plant systems where the virus-induced RNA polymerase was particulate and presumably present as an enzyme-viral RNA complex (Bove, 1967a,b,c; Semal and Hamilton, 1968). The RNA polymerase studied here had several properties similar to the RNA polymerase found in a non-permissive strain of E. coli infected with an amber mutant of the bacteriophage f2 (August et al., 1965; Shapiro and August, 1965; see also the Introduction of this thesis). The activity of the RNA polymerase extracted from these two sources was sensitive to pancreatic ribonuclease, zinc or manganese ions in the assay medium but insensitive to actinomycin D. Both enzymes copied a variety of RNA templates supplied, however, synthetic polynucleotides could only serve as a template for the CMV-induced RNA polymerase (discussed further in Chapter 8) as they were not copied by the f2-induced RNA polymerase.

TABLE 4: NUCLEOSIDE TRIPHOSPHATE REQUIREMENTS OF THE ASSAY SYSTEM USED FOR THE MEASUREMENT OF SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF HEALTHY AND CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBERS

System	Specific enzymatic activity			
	Healthy plants		Infected plants	
	UTP-^{32}P	GTP-^{32}P	UTP-^{32}P	GTP-^{32}P
Complete	0.4	0.1	15.4	19.4
- ATP	0.0	0.0	1.8	0.4
- GTP	0.6	-	2.0	-
- CTP	6.0	1.0	12.1	0.3
- UTP	-	0.0	-	1.4
- ATP, - UTP, - CTP	-	0.2	-	0.2
- ATP, - GTP, - CTP	5.2	-	5.5	-

RNA polymerase assays were carried out, with either UTP-^{32}P or GTP-^{32}P as the labelled nucleoside triphosphate substrate, as described in Materials and Methods and in the presence of 1.0 mg of added yeast RNA.

Specific enzymatic activity: μμmoles of labelled nucleoside triphosphate converted to acid-insoluble material per min. per milligram of plant protein under the defined assay conditions.

TABLE 5: EFFECT OF VARIOUS DNA AND RNA SAMPLES ON SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF HEALTHY AND CUCUMBER MOSAIC VIRUS INFECTED CUCUMBERS

Expt. No.	DNA or RNA added (250 μg/assay)	Specific enzymatic activity	
		Healthy plants	Infected plants
1.	None	0.8	2.6
	Yeast RNA	0.3	16.8
	Yeast transfer RNA	0.8	6.0
	Native salmon DNA	0.0	2.4
	Native salmon DNA (minus actinomycin D)	3.3	4.6
	Denatured salmon DNA	0.8	1.7
2.	None	-	1.0
	Yeast RNA	-	18.1
	TYMV-RNA	-	9.0
	CMV-RNA	-	13.4
	TMV-RNA	-	20.0

Partly purified enzyme extracts were prepared (by EXTRACTION METHOD C) and RNA polymerase activity was measured as described in Materials and Methods, using UTP-^{32}P as the labelled substrate; but the 1.0 mg of yeast RNA added as template to each assay was replaced by various DNA and RNA samples (250 μg/assay).

TABLE 6. EFFECT OF YEAST RNA AND SYNTHETIC POLYNUCLEOTIDES ON SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBERS

RNA or polynucleotide (250 μg/assay)	Specific enzymatic activity		
	GTP-^{32}P	UTP-^{32}P	ATP-^{3}H
None	0.3	1.7	1.8
Yeast RNA	13.4	19.6	17.8
PolyU	0.0	0.1	8.0
PolyC	13.4	0.0	0.0

The RNA polymerase was assayed as described in Materials and Methods, except that yeast RNA and polynucleotide were present at 250 μg/assay. In all cases the three unlabelled nucleoside triphosphates were present in the assay medium with the labelled nucleoside triphosphate.

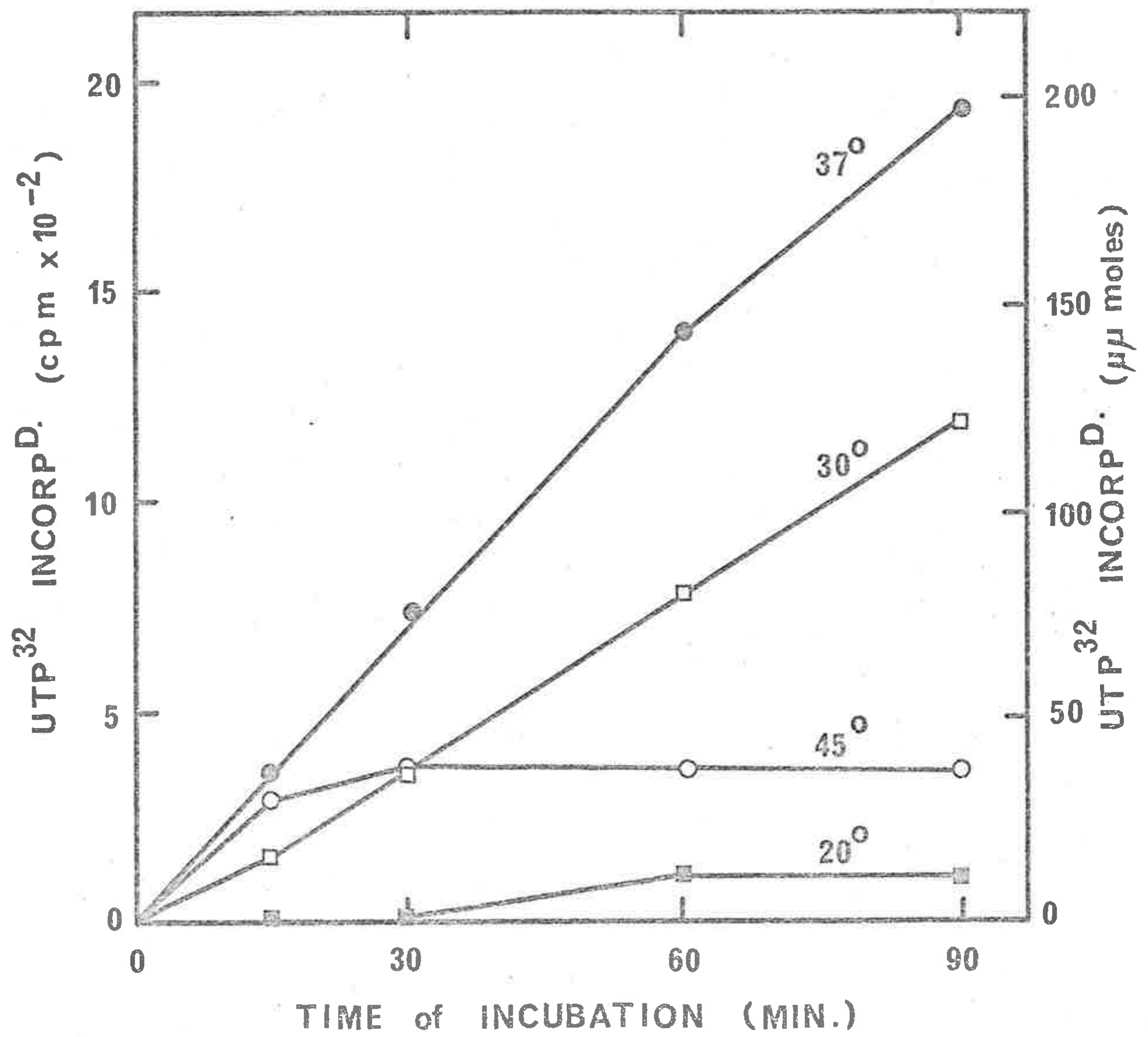
TABLE 7. NEAREST-NEIGHBOUR ANALYSIS OF THE SOLUBLE RNA POLYMERASE PRODUCT FORMED IN THE PRESENCE OF TWO RNA TEMPLATES

Template	Labelled Substrate	Isolated 2'(3')-mononucleotides (% of total radioactivity)			
		AMP	CMP	GMP	UMP
Yeast RNA	UTP-^{32}P	24.3	24.9	17.8	33.0
	GTP-^{32}P	28.5	23.0	24.0	24.5
TYMV-RNA	UTP-^{32}P	19.9	15.8	43.2	21.1
	GTP-^{32}P	21.5	13.5	42.6	22.5

Each estimation was carried out twice on three partly purified enzyme preparations. The products were formed in the assay medium described in Materials and Methods with 1.0 mg of either yeast RNA or TYMV-RNA. Values quoted are the mean percent of the total radioactivity, the standard error of the mean never being greater than $\pm$ 0.5.

FIGURE 3. THE EFFECT OF TEMPERATURE AND TIME OF INCUBATION ON SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBERS

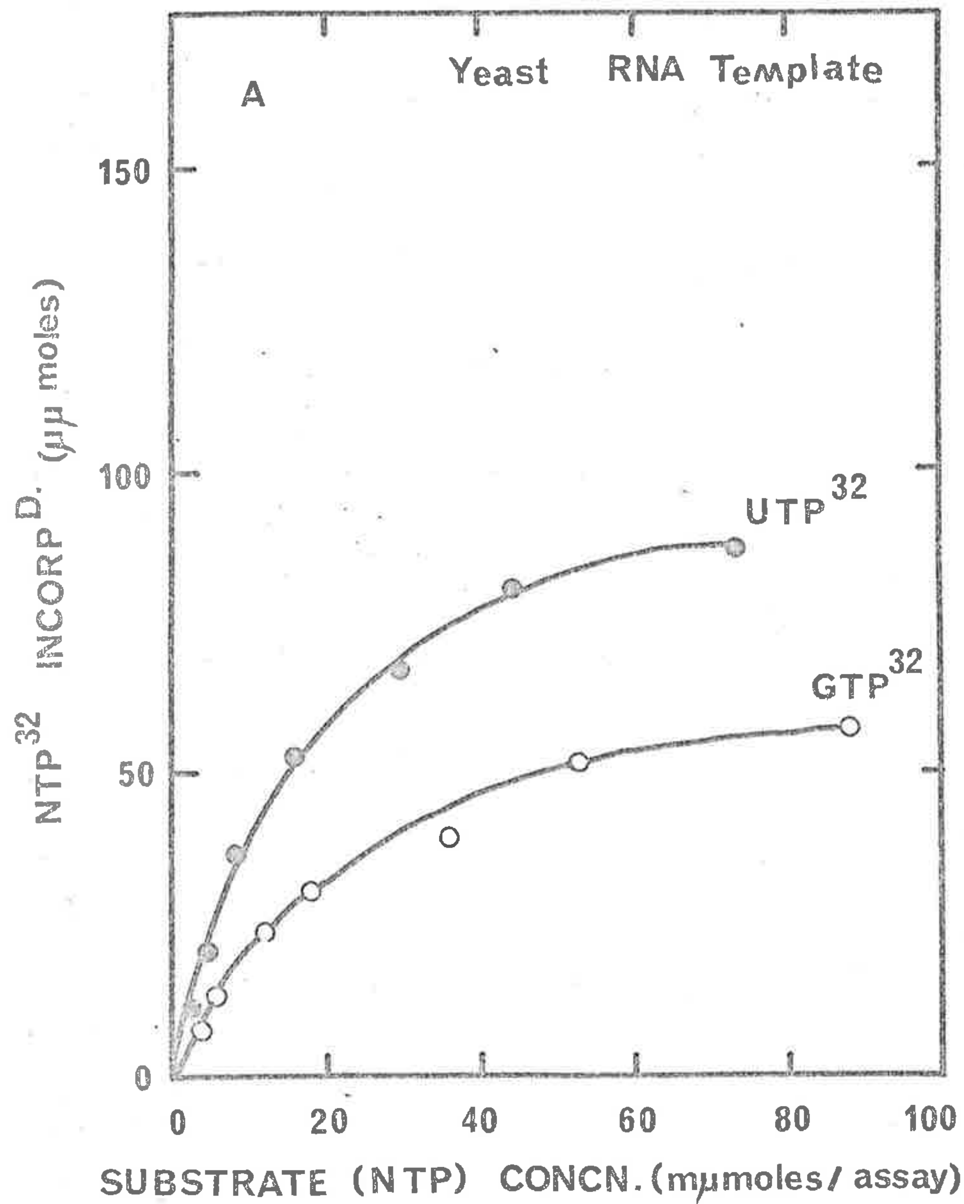
The partly purified RNA polymerase preparation was assayed as described in Materials and Methods, using yeast RNA (1.0 mg) as the added RNA, but the time of incubation was varied from 0 - 90 min. and the temperature from 20 - 45°, using 0.15 mg of plant protein per assay.



UTP^{32} INCORP$^{D.}$ (c p m x 10^{-2})
20
15
10
5
0
37°
30°
45°
20°
0
30
60
90
TIME of INCUBATION (MIN.)
200
150
100
50
0
UTP^{32} INCORP$^{D.}$ ($\mu\mu$ moles)

FIGURE 4: EFFECT OF CONCENTRATION OF LABELLED SUBSTRATE AND OF RNA ON THE SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBERS

The RNA polymerase assays were carried out as described in Materials and Methods, using 0.13 mg of plant protein and 240 μmoles of each of the unlabelled nucleoside triphosphates per assay with the modifications as shown, and with 1.0 mg of yeast RNA as template for Part A. Incubation at 37° for 30 min.


A
Yeast RNA Template
NTP^{32} INCORP$^{D.}$ (μμ moles)
150
100
50
0
UTP^{32}
GTP^{32}
0
20
40
60
80
100
SUBSTRATE (NTP) CONCN. (mμmoles / assay)

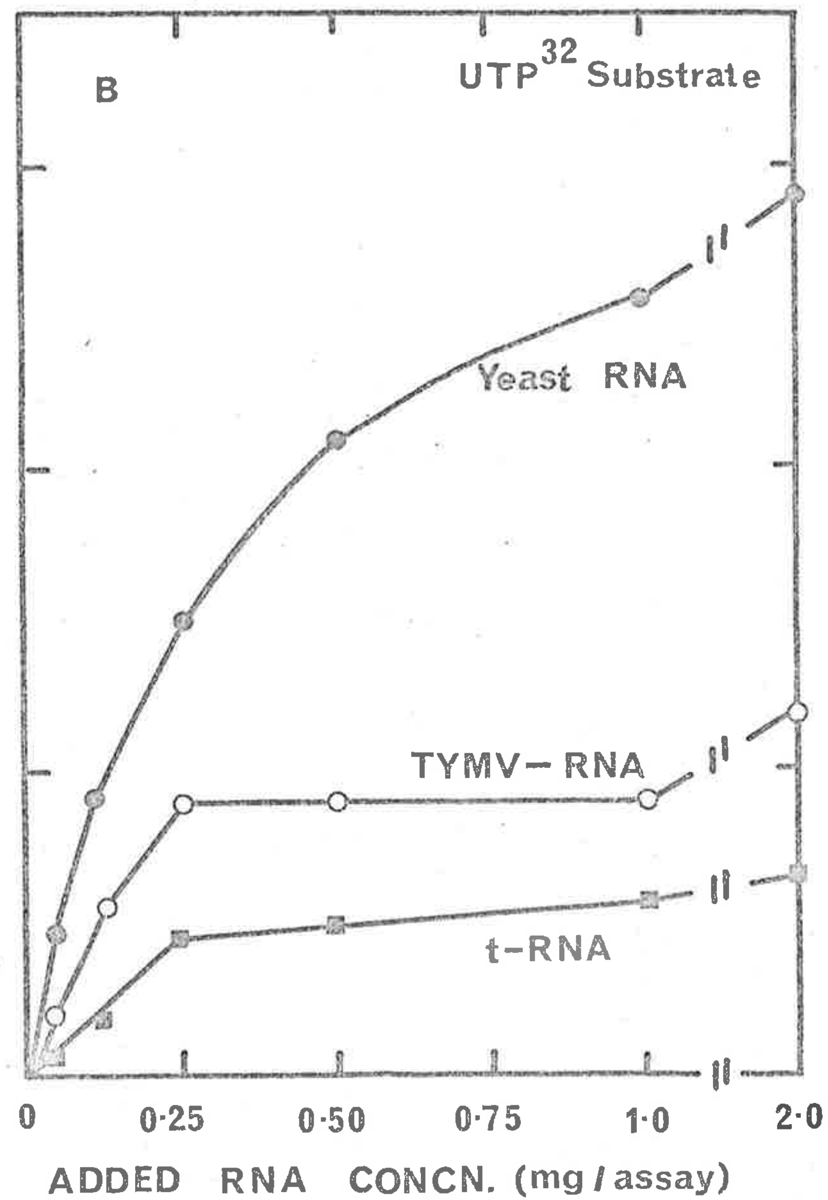
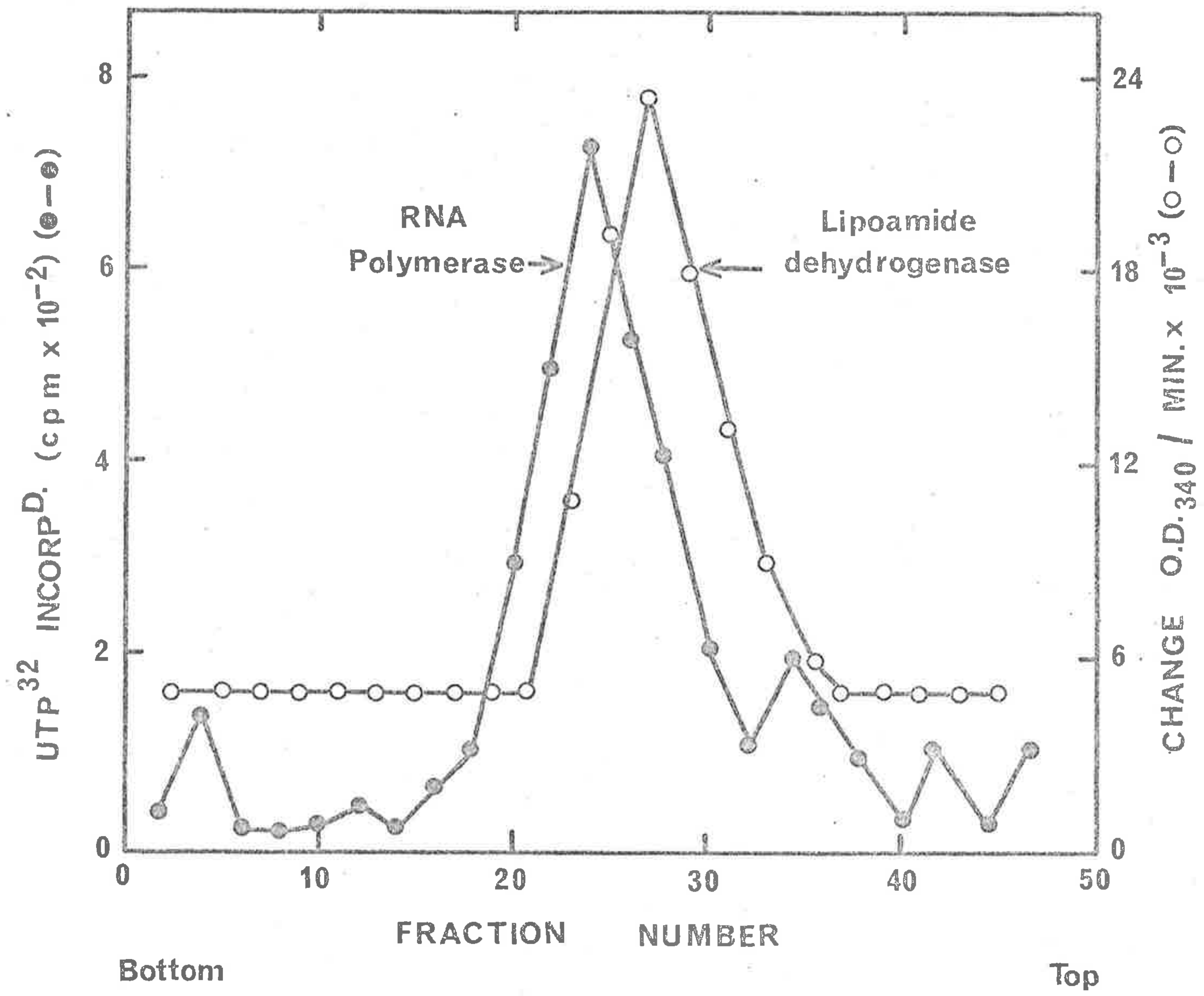

B
UTP^{32} Substrate
Yeast RNA
TYMV-RNA
t-RNA
0
0·25
0·50
0·75
1·0
2·0
ADDED RNA CONCN. (mg / assay)

FIGURE 5: SEDIMENTATION ANALYSIS OF THE SOLUBLE PARTLY PURIFIED RNA POLYMERASE

Partly purified enzyme (1.0 mg of plant protein in 0.2 ml; four-five fold more concentrated than normally used in RNA polymerase assays) was centrifuged through a 4.4 ml gradient of 5 - 20% sucrose in 0.1 M NH_4Cl, 90 mM 2-mercaptoethanol, 5 mM $MgSO_4$, 50 mM Tris-HCl buffer pH 8.5, in a Beckman SW39 rotor at 33,000 rpm for 15 hours at 3°. Two drop fractions (0.085 ml) were collected from the bottom of the cellulose nitrate tube and alternate fractions assayed for RNA polymerase activity (see Materials and Methods) or for lipoamide dehydrogenase activity by following the oxidation of NADH by dl-lipoic acid spectrophotometrically at 340 mμ as described by Massey et al. (1962). Recovery of the RNA polymerase activity (RNA polymerase assayed in the presence of 1.0 mg of yeast RNA) was 96%.


RNA
Polymerase
Lipoamide
dehydrogenase
UTP32 INCORPD. (c p m x 10−2) (●—●)
CHANGE O.D.340 / MIN. x 10−3 (○—○)
FRACTION NUMBER
Bottom
Top
0
10
20
30
40
50
2
4
6
8
6
12
18
24

CHAPTER FOUR

THE NATURE OF THE PRODUCT OF THE SOLUBLE PARTLY PURIFIED CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE

THE NATURE OF THE PRODUCT OF THE SOLUBLE PARTLY PURIFIED CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE

INTRODUCTION

The characterisation of the product of the soluble RNA polymerase reaction comprised part of an attempt to further understand the role of the CMV-induced RNA polymerase activity detected in partly purified extracts of infected cucumbers, in the replication of viral RNA *in vivo*. As yet only the phage Qβ-induced RNA replicase has been found to produce a single-stranded RNA species when provided with viral RNA template and the four triphosphates (discussed in the Introduction). Other viral polymerase systems almost invariably produced double-stranded, pancreatic ribonuclease-resistant products. Such ribonuclease-resistant structures were the products of other plant viral-induced RNA polymerases and had a sedimentation coefficient near 15S(Bové *et al.*, 1968; Semal and Hamilton, 1968). Thus the nature of the product of the soluble CMV-induced RNA polymerase, when supplied with either yeast or CMV-RNA template, was investigated with respect to size and resistance to pancreatic ribonuclease.

METHODS

1. Assay of RNA polymerase activity. Assays were carried out as described in Chapter 1 with either yeast RNA (1.0 mg/assay) or CMV-RNA (120 µg/assay) as the added template and UTP-^{32}P as the labelled nucleoside triphosphate substrate. Partly purified soluble RNA polymerase (prepared by EXTRACTION METHOD C) was used in all assays.

2. Isolation of the product of the soluble RNA polymerase reaction. This was carried out as previously described in Chapter 1.

RESULTS

1. The effect of pancreatic ribonuclease on the formation of the product. Total product formed declined with increase of pancreatic ribonuclease concentration (Figure 6). This effect was probably due to the degradation of the added template RNA (yeast RNA) since most of the product was resistant to further digestion with pancreatic ribonuclease (Figure 6; see also below).

2. Resistance of the product to pancreatic ribonuclease. When either viral RNA (CMV-RNA) or yeast RNA (50 µg or more) was used as a template for the RNA polymerase, more than 80% of the product was found to be resistant to digestion by pancreatic ribonuclease (5 - 100 µg/ml at 37° for 30 min.) either before or after deproteinisation of the medium (Figure 7). This resistance to ribonuclease indicated the presence of

a double-stranded RNA structure. Similar results have been reported for the product of the TYMV-induced RNA polymerase reaction (Bové et al., 1968), which was resistant (90%) to pancreatic ribonuclease both before and after phenol extraction.

3. Thermal transition temperature (Tm) of the phenol-extracted RNA polymerase product. Estimation of the thermal transition temperature (Tm) of the phenol-estracted product (formed in the presence of yeast RNA template) could be achieved only if the product was first digested with pancreatic ribonuclease (12 µg/ml) to give a 'ribonuclease resistant structure' (about 80% of the original product) and this gave a Tm of 92° in SSC (Figure 8). Removal of the pancreatic ribonuclease used in this treatment by a further deproteinisation with phenol, failed to alter the Tm of the product. An estimation of the Tm of the product not pretreated with ribonuclease was complicated by the fact that temperatures greater than 120° degraded the product; at 120° very little breakdown of presumed double-stranded RNA to single-strands had occurred. Hence, the treatment with pancreatic ribonuclease prior to heat denaturation had presumably modified the product in some manner making it more susceptible to heat denaturation or had destroyed single-stranded yeast RNA template which was not copied by the RNA polymerase (only 0.01% of the added yeast RNA was copied, see also below), and may interfere with the denaturation process. Possibly the inability to denature the product without prior pancreatic ribonuclease digestion is due to product being in a 'looped' form which simply flips

back into a double-stranded form when cooled. The pancreatic ribonuclease may preferentially attack the loop where it is single-stranded and the product becomes denaturable.

Also the phenol extracted product formed in the presence of added CMV-RNA template (120 µg/assay) had a Tm of 93° in SSC. However, in contrast to the above case, pretreatment with pancreatic ribonuclease (12 µg/ml) was not required for the estimation of the Tm of this product.

4. Sedimentation analysis of the phenol-extracted product.

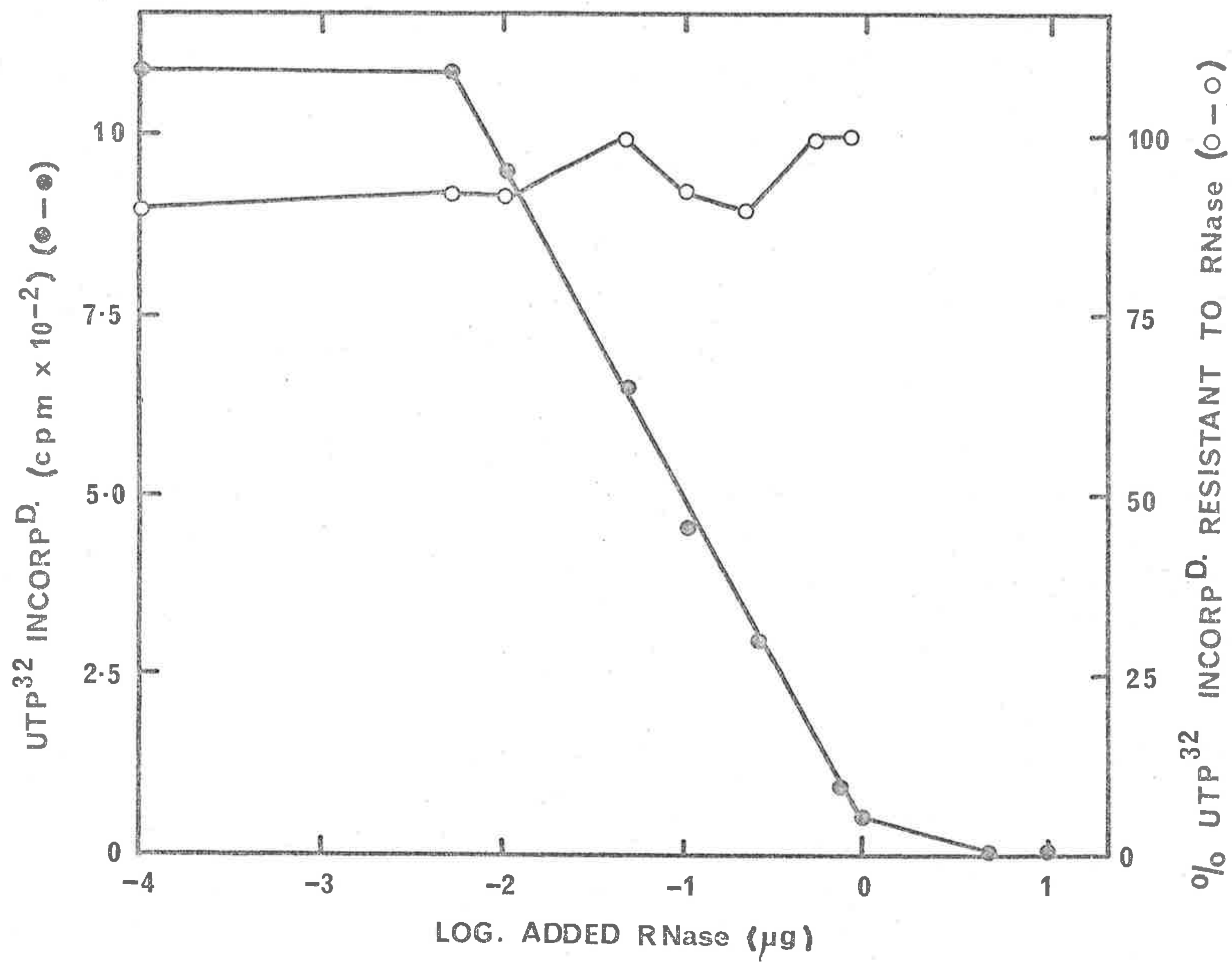
The phenol-extracted product of the RNA polymerase was centrifuged through a 5 - 20% sucrose gradient for 5 hours at 33,000 rpm using *E. coli* RNA 23S, 16S and 4S as marker. The yeast RNA added to the RNA polymerase assay consisted mostly of portions of RNA ranging in size from 4 - 16S (Figure 9), the phenol-extracted product having a sedimentation value of 5S. CMV-RNA contained two peaks of RNA 23S and 14S (see Kaper *et al.*, 1965) which yielded a product with a sedimentation value of about 6S. However, it is difficult to draw any significant conclusions from these results on the size relationship between template and product since in each assay tube the RNA made by the CMV-induced RNA polymerase is only 0.01% of the input template RNA.

DISCUSSION

The results presented in this chapter show that the soluble RNA polymerase produced a pancreatic ribonuclease-resistant and presumably double-stranded structure with either added yeast RNA or CMV-RNA template. Of interest was the fact that with the CMV-induced RNA polymerase system the behaviour of the RNA product toward the action of pancreatic ribonuclease was in agreement with the properties of the products obtained in the other plant virus RNA polymerase systems (Bové et al., 1968; Semal, 1970), in that the RNA product in its native state (before deproteinisation) was almost completely resistant to pancreatic ribonuclease digestion. This situation differed from that described for an RNA phage by Borst and Weissmann (1965) and by Feix et al. (1968) where the product of the nucleotide incorporation was sensitive to pancreatic ribonuclease in the native state, but become ribonuclease resistant upon deproteinisation. However, in general, comparisons between the products of plant virus RNA polymerases and ribonuclease-free phage-induced RNA polymerases are made difficult because of the differences between endogenous ribonucleases (although low in partly purified CMV-infected cucumber extracts) and in the efficiency of nucleotide incorporation.

FIGURE 6. EFFECT OF PANCREATIC RIBONUCLEASE ACTIVITY IN THE ASSAY MEDIUM ON THE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBERS

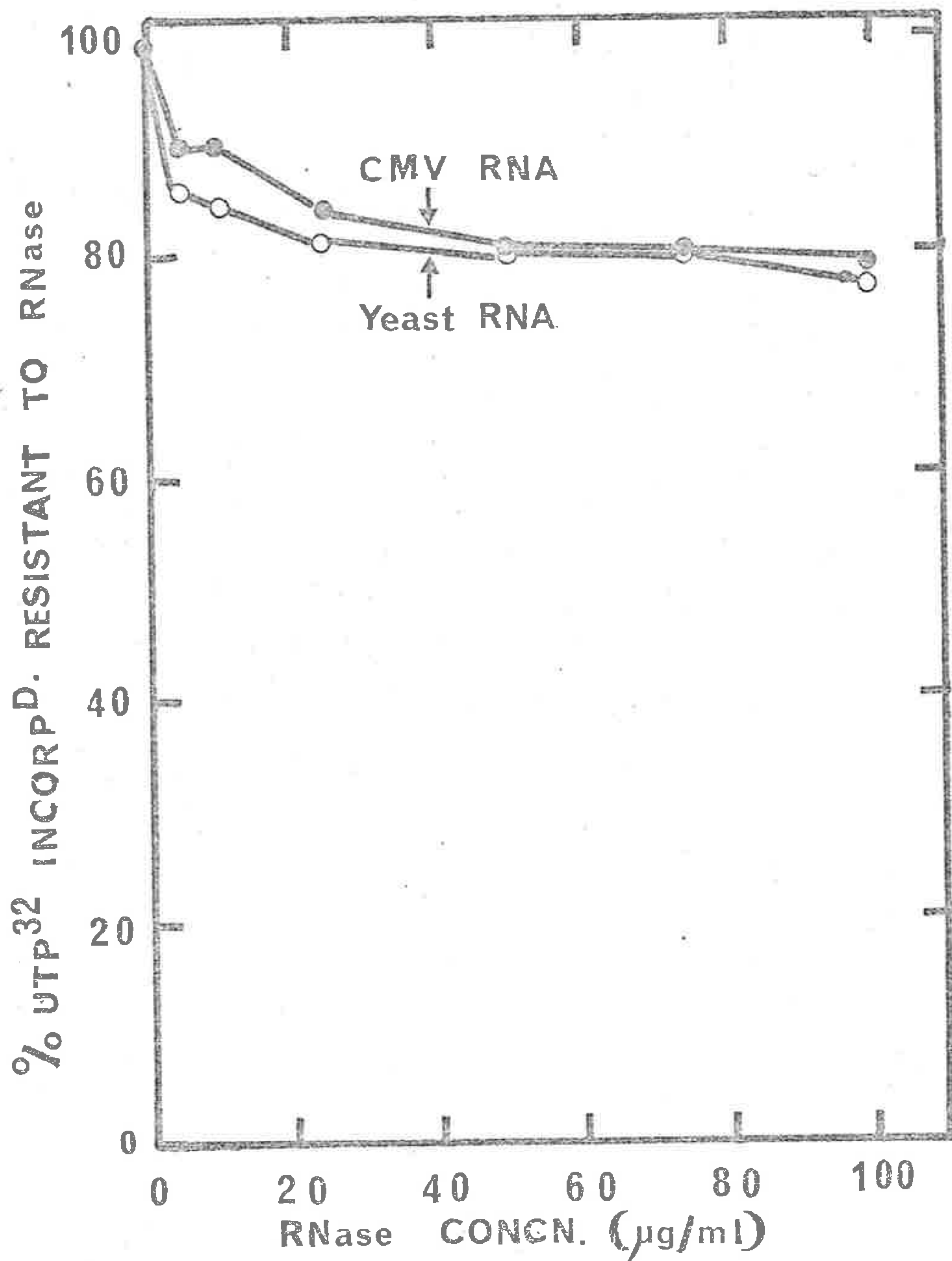
Assay of partly purified RNA polymerase (prepared by EXTRACTION METHOD C) were carried out as described in Methods, with 1.0 mg of yeast RNA, and 0.13 mg of plant protein per assay. However, pancreatic ribonuclease (0 - 10 μg) was added to the assay tubes (● — ●). At the end of the incubation in a parallel experiment, an extra 5 μg of pancreatic ribonuclease was added to each assay tube which was incubated for a further 30 min. at 37° to determine resistance of the polymerase product to ribonuclease digestion before acid precipitation (o —— o).



UTP^{32} INCORP$^{D.}$ (c p m × 10^{-2}) (●—●)
10
7·5
5·0
2·5
0
100
75
50
25
0
% UTP^{32} INCORP$^{D.}$ RESISTANT TO RNase (○—○)
−4
−3
−2
−1
0
1
LOG. ADDED RNase (μg)

FIGURE 7. EFFECT OF PANCREATIC RIBONUCLEASE ON THE PHENOL EXTRACTED PRODUCT OF THE RNA POLYMERASE REACTION

The product of the RNA polymerase reaction, using 1.0 mg of yeast RNA or 120 μg of CMV-RNA and labelled UTP-^{32}P added to the assay medium, was isolated as described in Methods. The product was dissolved in SSC andsamples (0.2 ml) were incubated with pancreatic ribonuclease (0 - 100 μg/ml) at37^{o} for 30 min. Acid-insoluble material remaining after this treatment was collected as described in Chapter 1.



100
80
60
40
20
0
CMV RNA
Yeast RNA
% UTP32 INCORPD. RESISTANT TO RNase
0
20
40
60
80
100
RNase CONCN. (µg/ml)

FIGURE 8. THERMAL DENATURATION CURVE OF THE PHENOL-EXTRACTED PRODUCT OF THE RNA POLYMERASE REACTION

The product obtained using 1.0 mg of yeast RNA per assay (using UTP-^{32}P as the labelled substrate in the assay) was isolated as described in Methods, dissolved in SSC and half the sample was incubated with pancreatic ribonuclease (12 μg/ml) at 37° for 30 min. Samples of the untreated and ribonuclease-treated product were then incubated in sealed tubes for 10 min. at the temperatures indicated. The solutions were frozen in an ethanol-dry ice mixture, thawed and digested with pancreatic ribonuclease (12 μg/ml) at 37° for 30 min. Acid-insoluble material was collected and counted as described in Chapter 1.

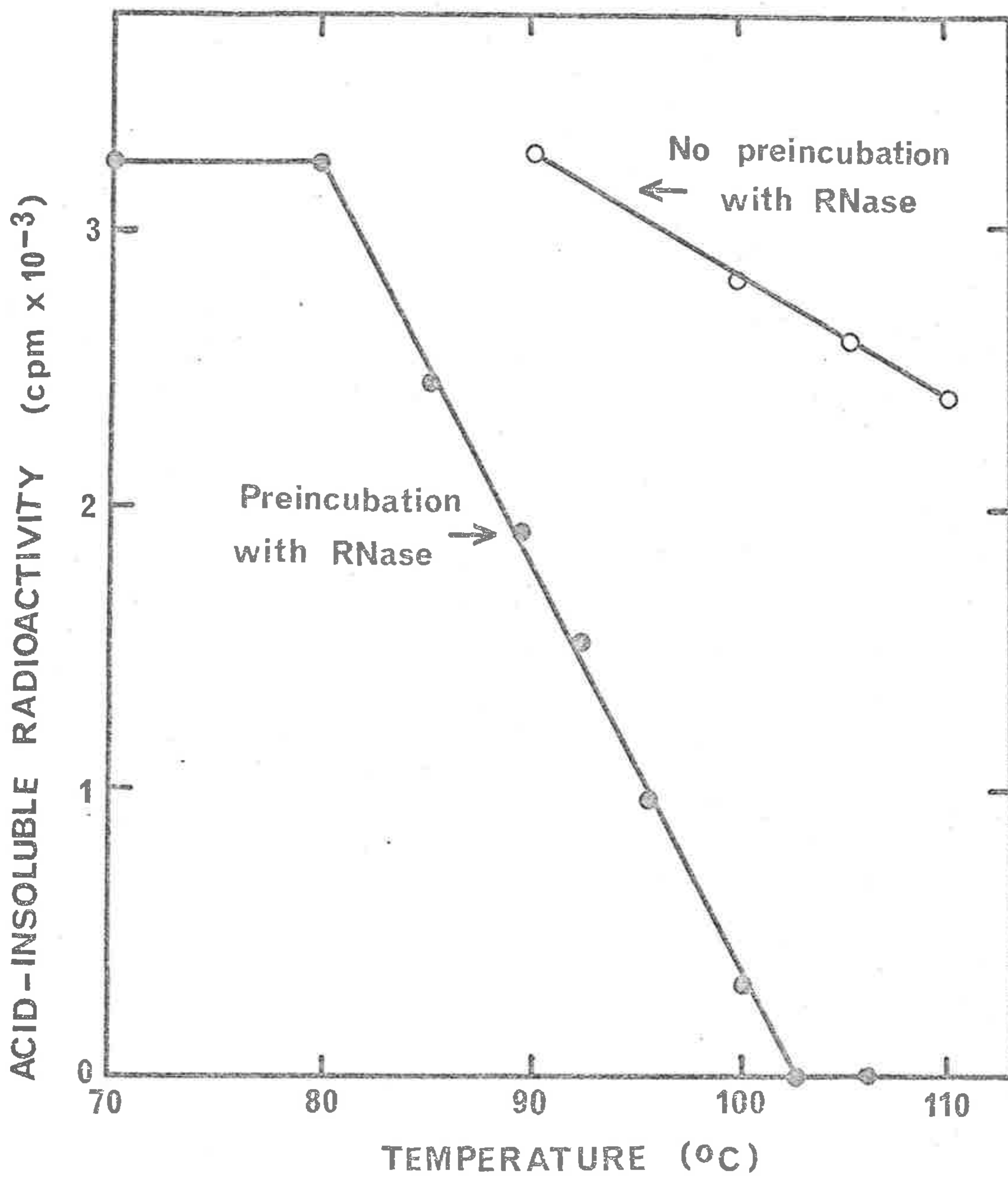
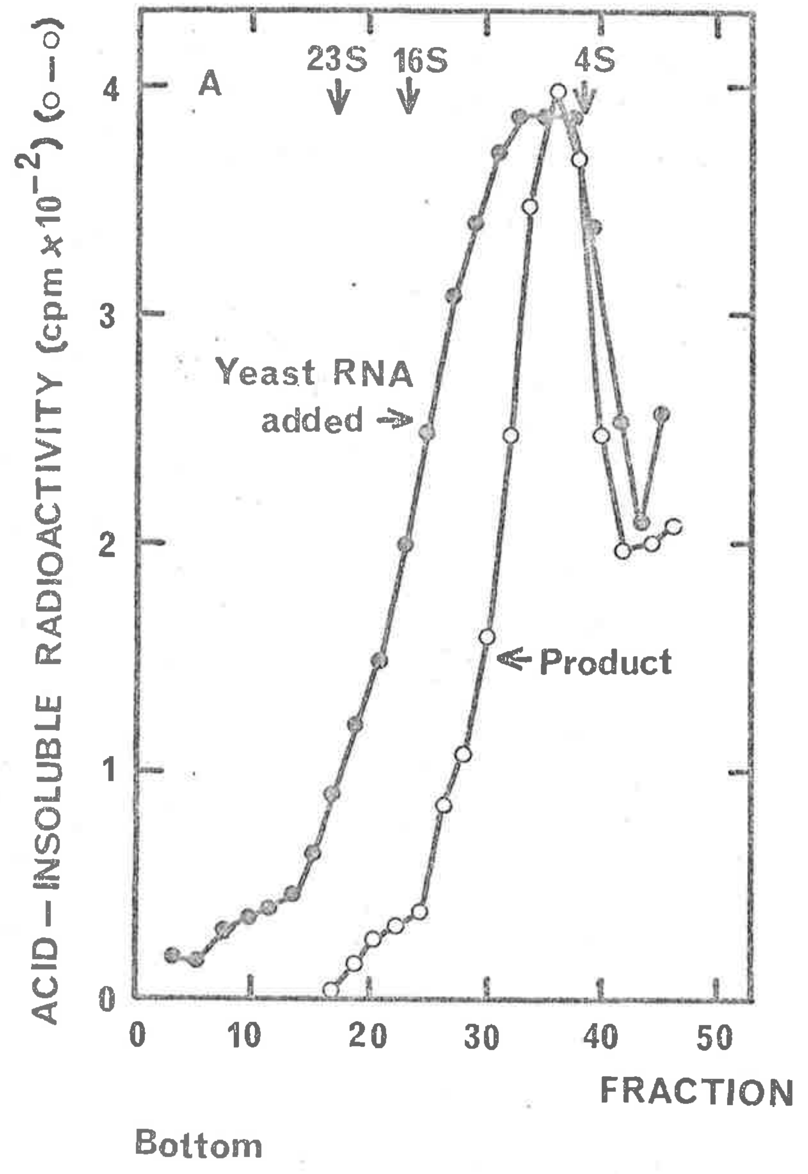
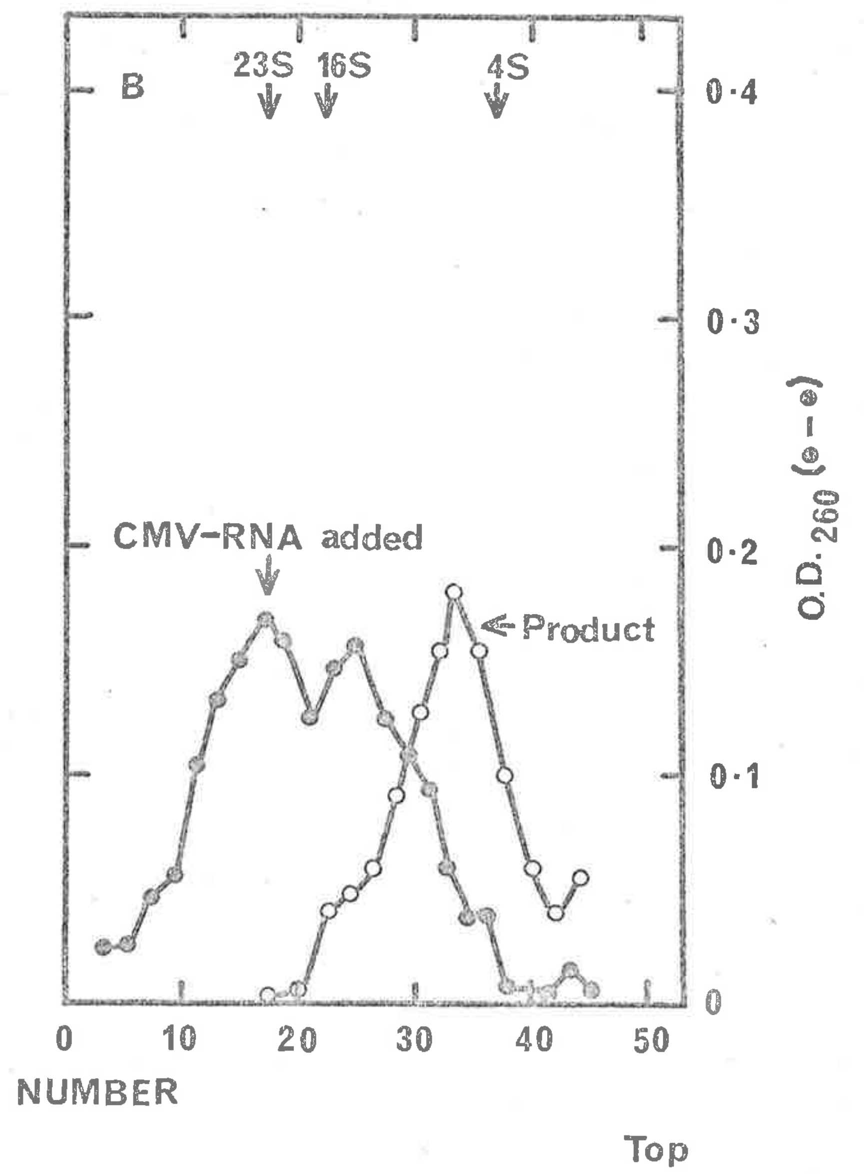

No preincubation
with RNase
Preincubation
with RNase
ACID-INSOLUBLE RADIOACTIVITY (cpm x 10⁻³)
0
1
2
3
70
80
90
100
110
TEMPERATURE (°C)

FIGURE 9. SEDIMENTATION ANALYSIS OF THE PHENOL-EXTRACTED PRODUCT OF THE RNA POLYMERASE REACTION

Experiment A. The product was isolated as described in Methods using 1.0 mg of yeast RNA per assay as template and UTP-^{32}P as the labelled substrate. The product was dissolved in SSC and samples (0.2 ml) containing added *E. coli* RNA (23S, 16S and 4S) as marker centrifuged through a 4.4 ml gradient of 5 - 20% sucrose in SSC, in a Beckman SW39 rotor at 33,000 rpm for 5 hours at 3°. Two drop fractions (0.085 ml) were collected from the bottom of the tube and alternate fractions used for the estimation of acid-insoluble radioactivity of the product as described in Chapter 1 or for the determination of optical density at 260 mμ after the addition of 2.5 ml of water; the latter results gave the optical density profile of the marker *E. coli* RNA but only the positions of 4S, 16S and 23S peaks are shown. The optical density profile of the yeast RNA used as template was determined in a separate tube at the same time under the same conditions.

Experiment B. As for experiment A except that 120 μg CMV-RNA per assay was used as template instead of yeast RNA. Likewise, the optical density of the CMV-RNA used as template was determined in a separate tube under the same conditions.


A
23S
16S
4S
Yeast RNA added
Product
ACID – INSOLUBLE RADIOACTIVITY (cpm x 10^{-2}) (o – o)
0
1
2
3
4
0
10
20
30
40
50
FRACTION
Bottom


B
23S
16S
4S
CMV-RNA added
Product
O.D.$_{260}$ (● – ●)
0
0·1
0·2
0·3
0·4
0
10
20
30
40
50
NUMBER
Top

CHAPTER FIVE

DETECTION AND PROPERTIES OF A CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE IN PARTICULATE FRACTIONS OF INFECTED CUCUMBER COTYLEDONS

DETECTION AND PROPERTIES OF A CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE IN PARTICULATE FRACTIONS OF INFECTED CUCUMBER COTYLEDONS

INTRODUCTION

As mentioned in previous chapters, plant virus-induced RNA polymerase activity has been reported in particulate (chloroplast and mitochondrial) fractions of virus-infected plant tissue (see also Introduction). Results obtained with such particulate fractions of other infected plants suggested that the RNA polymerase was present as an enzyme-viral RNA complex (a replicating complex; Bové, 1967a,b,c; Semal and Hamilton, 1968). Thus particulate fractions of CMV-infected cucumber cotyledons were investigated with the aim of determining whether such a complex existed in these fractions also.

Upon investigation of particulate fractions of CMV-infected cucumber cotyledons, a viral-induced RNA polymerase was found associated with these fractions. This and the following two chapters will describe several aspects of the RNA polymerase activity associated with particulate fractions. Thus, in addition to the soluble RNA polymerase described in the previous chapters, a viral-induced RNA polymerase was present in particulate fractions of CMV-infected cucumber cotyledons.

METHODS

1. EXTRACTION METHOD D. Preparation of particulate enzyme extracts. All preparations were performed at 4°. Homogenates were prepared by grinding healthy or infected plants in a mortar and pestle; for each gram fresh weight of tissue, 2.0 ml of the following extraction buffer was added before grinding; 0.3 M sucrose (10.2% w/v; added to maintain integrity of plant organelles), 0.1 M NH_4Cl, 90 mM 2-mercaptoethanol, 50 mM Tris-HCl buffer pH 8.5. The homogenate was squeezed through a fine nylon cloth and centrifuged at 150 g for 5 min. and the pellet discarded. The supernatant was centrifuged at 16,000 g for 10 min. to yield a pellet (16,000-P, the particulate fraction) and a 16,000 g supernatant (16,000-S, the soluble fraction). The pellet was washed twice by suspension in and sedimentation from twice the original volume of extraction buffer and was finally suspended in half the original volume of extraction buffer without sucrose (i.e., 1.0 ml per gram fresh weight of plants). The 16,000-P fraction contained 3.5 - 4.0 mg of protein per ml and the 16,000-S fraction contained 6.8 - 7.5 mg of protein per ml. The 16,000-P fraction contained negligible plant ribonuclease activity in contrast to the 16,000-S fraction which contained detectable plant ribonuclease activity (0.2 - 0.35 µg per ml).

2. Assay of RNA polymerase activity. Assays were performed as previously described in Chapter 1 using 10.8 μmoles $MgSO_4$/assay (29 mM) and GTP-^{32}P as the labelled substrate (unless otherwise stated). Also 0.35 - 0.4 mg of 16,000-P protein (0.1 ml of particulate preparation) or 0.68 - 0.75 mg of 16,000-S protein (0.1 ml of soluble preparation) were added with or without 1.0 mg of yeast RNA. All assays gave linear incorporation of the labelled nucleotide into product for 30 min. Particulate and soluble fractions normally incorporated 2,000 - 3,000 cpm per assay of GTP-^{32}P under the standard assay conditions with 1.0 mg of yeast RNA present.

RESULTS

1. Distribution of CMV-induced RNA polymerase activity in extracts of infected cucumbers. The results presented in Table 8 show that in the presence of added yeast RNA in the assay medium (containing 29 mM $MgSO_4$), 66% of the CMV-induced RNA polymerase activity was located in the soluble fraction (16,000-S; see Methods) of extracts of CMV-infected cucumbers, 5% in the 150 g pellet, 13% in the 1,000 g pellet and 16% in the 16,000 g pellet. Thus, differential centrifugation showed that the particulate fraction contained a total of one-third of the total RNA polymerase activity. However, when yeast RNA was omitted from the assay medium, low but significant RNA polymerase activity could be detected in the soluble (16,000-S) and particulate fractions of infected cucumber extracts (see Table 8). 70% of the total RNA polymerase activity independent of added RNA was located in

the particulate fraction. Thus, particulate fractions contained two CMV-induced RNA polymerase activities, one dependent on and one independent of the addition of yeast RNA. Healthy plant extracts contained neither of these activities (see Table 8). The particulate fraction sedimenting between 150 g and 16,000 g (16,000-P fraction) was used in all the subsequent experiments on the particulate RNA polymerase. Washing of this fraction with either extraction buffer with or without sucrose (see Methods) failed to release any particulate RNA polymerase activity (see also Chapter 7), indicating the absence of cytoplasmic (soluble) RNA polymerase contamination.

2. Effect of CMV-infection on RNA polymerase activity in particulate and soluble fractions. Figure 10 shows the appearance of the CMV-induced RNA polymerase in the particulate (16,000-P, assayed in the presence and absence of yeast RNA) and soluble (16,000-S, assayed in the presence of yeast RNA) fractions as a function of time after infection. There was a significant increase in activity in both fractions above the negligible healthy plant level (healthy plant soluble and particulate fractions were assayed in the presence and absence of added yeast RNA) on the third day after infection. The level of the RNA-dependent activity in the soluble fraction (16,000-S) reached a plateau on about day 9, whereas the level of the particulate RNA polymerases (RNA dependent plus RNA independent) decreased after day 10 of infection. Plants used in all subsequent experiments involving particulate CMV-induced RNA polymerase have been infected for 7 - 13 days.

The soluble (16,000-S) preparation when assayed under the stated assay conditions without yeast RNA, gave negligible RNA polymerase activity.

3. General properties of the particulate CMV-induced RNA polymerase activity

(a) Effect of $MgSO_4$ concentration of the assay medium. The effect of $MgSO_4$ concentration on the RNA polymerase activity is shown in Figure 11. The RNA polymerase activity dependent on added yeast RNA was found to be optimal at 20 - 29 mM $MgSO_4$ (7.5 - 10.8 μmoles/assay). However, the activity independent of added RNA had a broad $MgSO_4$ optimum of 2.7 - 29 mM (1 - 10.8 μmoles/assay).

(b) Nucleoside triphosphate requirement. The dependence of RNA polymerase activity on the presence of all four ribonucleoside triphosphates (using GTP-^{32}P as the labelled substrate) was investigated by omitting either one or three of the unlabelled triphosphates from the assay medium (Table 9). There was almost complete dependence on the presence of all four triphosphates for both the RNA polymerase activity dependent on, and the RNA polymerase activity independent of, added yeast RNA.

(c) The effect of synthetic polynucleotides. Table 10 shows the effect of the addition of yeast RNA, polyU or polyC to assays of healthy and CMV-infected particulate extracts using ATP-^{32}P, GTP-^{32}P, UTP-^{32}P, or CTP-^{32}P as the labelled substrate. The 16,000-P fraction from healthy plants incorporated UTP-^{32}P and especially CTP-^{32}P into acid

insoluble material in the presence of the other three unlabelled ribonucleoside triphosphates, both in the presence and absence of yeast RNA. This activity over and above that found with ATP-^{32}P and GTP-^{32}P as labelled substrate was also shown by the 16,000-P fraction from infected plants and can be explained by the terminal incorporation of UTP and CTP into transfer RNA present in the 16,000-P fraction (see Daniel and Littauer, 1963; Gilliland and Symons, 1968; see also Chapters 3 and 8). Because of this effect GTP-^{32}P was always used for the routine assay of the RNA polymerase in all subsequent work. Results using GTP-^{32}P and ATP-^{32}P as the labelled substrate indicated that maximum RNA polymerase activity was detected when complementary base pairing between the added RNA or synthetic polynucleotide was possible.

The detection of some activity that was independent of added RNA and of base pairing between labelled substrate and added template suggested that RNA (host and/or viral) present in infected plant extracts was also being copied. The total RNA content of the 16,000-P fraction was found to be 100 - 120 µg/ml (100-120 µg/gram fresh weight of plants; see Chapter 1 for method). This means that 10 - 12 µg of RNA was added with the 16,000-P fraction during each enzyme assay. This RNA has been isolated (plant particulate RNA, see following chapter) and examined by polyacrylamide gel electrophoresis (method of Loening, 1967). However, the RNA consisted of two major species of 1.1 x 10^6 (23S) and 0.56 x 10^6 (16S) molecular weight. However, no apparent viral-

induced RNA species were detected when this RNA was compared with RNA extracted from particulate preparations (16,000-P) of healthy plants (see also Vedel and D'aoust, 1970).

(d) The effect of pancreatic ribonuclease. In the presence of 15 μg/ml (5 μg/assay) of pancreatic ribonuclease in the assay medium, neither the added RNA dependent nor the added RNA independent RNA polymerase were detected.

(e) The effect of added DNA. The addition to the assay medium of 250 μg of native or heat-denatured salmon DNA (replacing yeast RNA), both in the presence and absence of actinomycin D (2.5 μg), had no effect on the CMV-induced RNA polymerase activity. Hence, no detectable DNA-dependent RNA polymerase activity was present in the 16,000-P fraction.

DISCUSSION

The results presented above have shown the presence of a virus-induced RNA polymerase in both soluble (16,000-S) and particulate fractions (16,000-P) of cucumbers infected with CMV. In the particulate fractions, two virus-induced RNA polymerase activities were found, one requiring the addition of RNA and one not requiring the addition of RNA to the assay medium. This latter activity was not found in either crude (16,000-S), or partly purified (see Chapter 2, EXTRACTION METHOD C), extracts of soluble RNA polymerase under the defined assay conditions (but see Chapter 8).

The amount of labelled nucleoside triphosphate incorporated into acid insoluble product (2.5 - 3.5 μμmoles/min/mg of protein) by the particulate CMV-induced RNA polymerase independent of added RNA was similar to that reported by Bové (1967a,b,c) for the TYMV-induced RNA polymerase and appreciably greater than that reported for other virus-infected plant systems (see the Introduction of Chapter 3). If the basic assumption of the work described is that the virus-induced RNA polymerase under investigation is responsible for the *in vivo* replication of viral RNA, then the particulate fraction described here may contain the CMV RNA-RNA polymerase complex, which was detected by the RNA polymerase activity independent of yeast RNA. Thus the RNA polymerase activity which incorporated labelled nucleoside triphosphates into an RNA product (see also following chapter) in the absence of added RNA template was similar to the activities described in other plant virus-infected particulate systems. It was hoped that an investigation into the nature of the product of the particulate CMV-induced RNA polymerase independent of added RNA would confirm the prediction of an enzyme-viral RNA complex.

TABLE 8. DISTRIBUTION OF CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE IN CRUDE EXTRACTS OF INFECTED CUCUMBERS

Fraction	Total units of enzyme activity/gram fresh weight			
	Plus yeast RNA		Minus yeast RNA	
	Healthy	Infected	Healthy	Infected
Pellet, 150 g	0.0	13.7	0.0	1.0
Pellet, 1,000 g	0.0	37.4	0.0	3.4
Pellet, 16,000 g	1.0	45.0	0.0	5.0
Cytoplasmic (soluble) extract (16,000-S)	0.0	190	0.0	4.0

The enzyme was prepared as described in Methods (EXTRACTION METHOD D) with the modification that the homogenate was squeezed through a double layer of very fine nylon cloth, and centrifuged at 150 g for 5 min., the supernatant collected and centrifuged at 1,000 g for 10 min. and finally at 16,000 g for 10 min. The pellets and the final supernatant (16,000-S, cytoplasmic extract) were collected. Assays were performed with and without added yeast RNA using GTP-^{32}P as the labelled substrate. Results are expressed as total enzyme units per gram fresh weight of leaves. Plants were harvested 10 days after infection.

A unit of RNA polymerase is defined as that amount which incorporated 1 µµmole of labelled nucleoside triphosphate into acid-insoluble material per min. under the specified assay conditions.

TABLE 9. NUCLEOSIDE TRIPHOSPHATE REQUIREMENTS OF THE ASSAY SYSTEM USED FOR MEASUREMENT OF PARTICULATE RNA POLYMERASE

Reaction mixture	Specific enzymatic activity			
	Plus yeast RNA		Minus yeast RNA	
	Healthy	Infected	Healthy	Infected
Complete	0.1	14.3	0.0	3.5
-ATP	0.2	0.4	0.0	0.1
-CTP	0.0	0.2	0.0	0.0
-UTP	0.0	0.4	0.0	0.0
-ATP, -CTP, -UTP	0.0	0.1	0.0	0.4

The particulate fraction (16,000-P) was prepared as described in Methods (EXTRACTION METHOD D). RNA polymerase activity was assayed as described in Methods using GTP-^{32}P as the labelled substrate, with or without added yeast RNA. Unlabelled nucleoside triphosphates were omitted as indicated.

Specific enzymatic activity: µµmoles of labelled nucleoside triphosphate incorporated into acid-insoluble material per min. per milligram of protein.

TABLE 10. EFFECT OF POLYRIBONUCLEOTIDES ON PARTICULATE RNA POLYMERASE

RNA added (250 µg/assay)	Specific activity with labelled substrate indicated: Healthy plants				Infected plants			
	GTP-^{32}P	ATP-^{32}P	UTP-^{32}P	CTP-^{32}P	GTP-^{32}P	ATP-^{32}P	UTP-^{32}P	CTP-^{32}P
None	0.0	0.3	5.0	49.8	2.1	2.7	9.4	60.0
Yeast	0.0	1.4	5.5	54.6	13.8	15.3	18.0	76.9
polyU	0.0	0.4	4.9	46.1	4.5	19.8	8.2	59.4
polyC	0.0	0.2	5.5	50.1	21.7	4.3	8.4	60.5

The RNA polymerase in the 16,000-P fraction was assayed as described in Methods except that yeast RNA and polynucleotides were present at 250 µg/assay and with α-^{32}P-ribonucleoside triphosphates as indicated in the presence of the other three unlabelled triphosphates.

FIGURE 10. EFFECT OF CUCUMBER MOSAIC VIRUS INFECTION ON THE ACTIVITY OF RNA IN PARTICULATE AND SOLUBLE FRACTIONS OF INFECTED CUCUMBERS

Total enzyme units per gram fresh weight of plants is plotted as a function of days after infection for infected and healthy plants. Soluble (16,000-S) and particulate (16,000-P) fractions were prepared and assayed as described in Methods using 10.8 µmoles $MgSO_4$ per assay (29 mM) and GTP-^{32}P as labelled substrate (plus or minus 1.0 mg of yeast RNA).

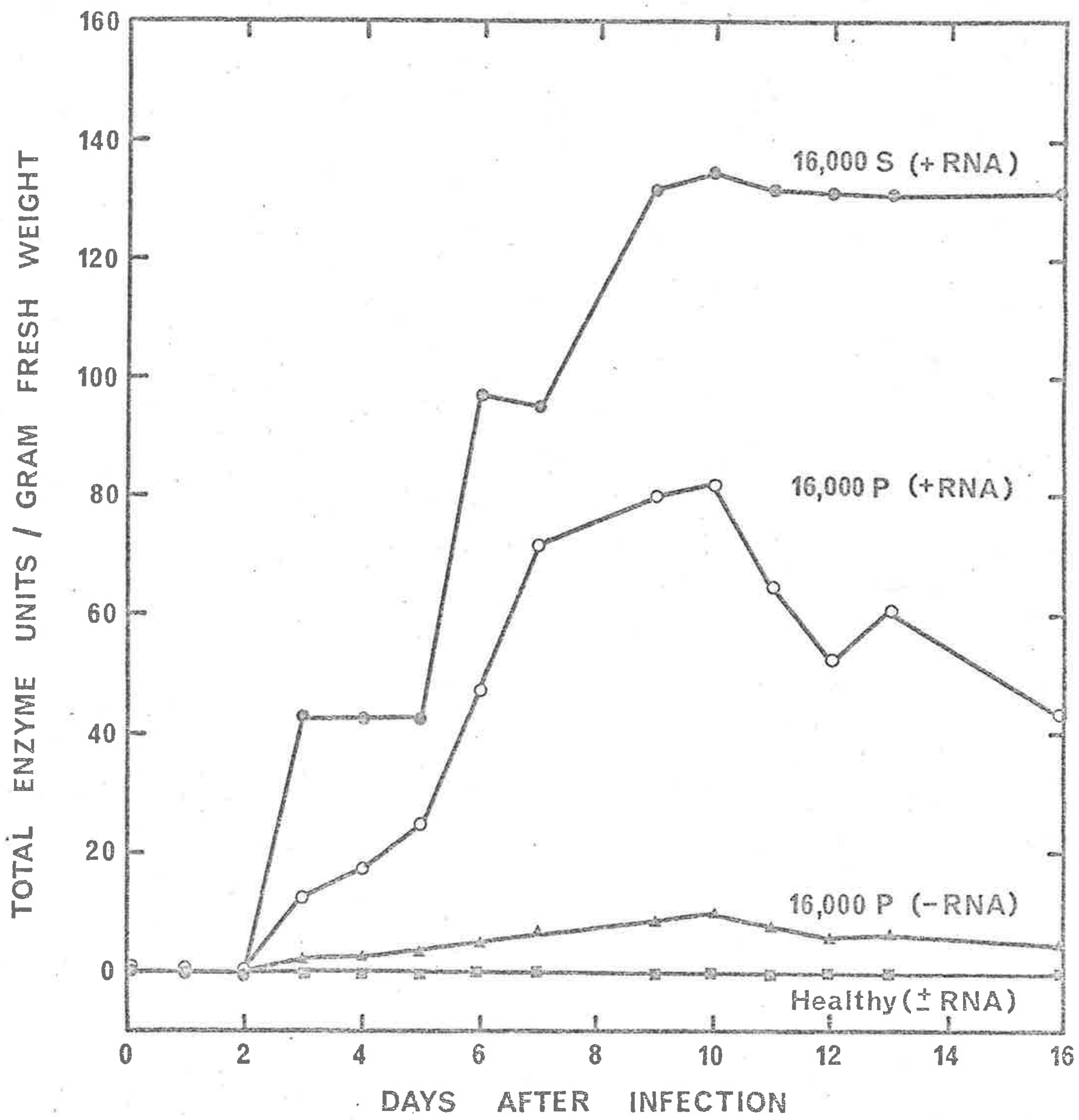
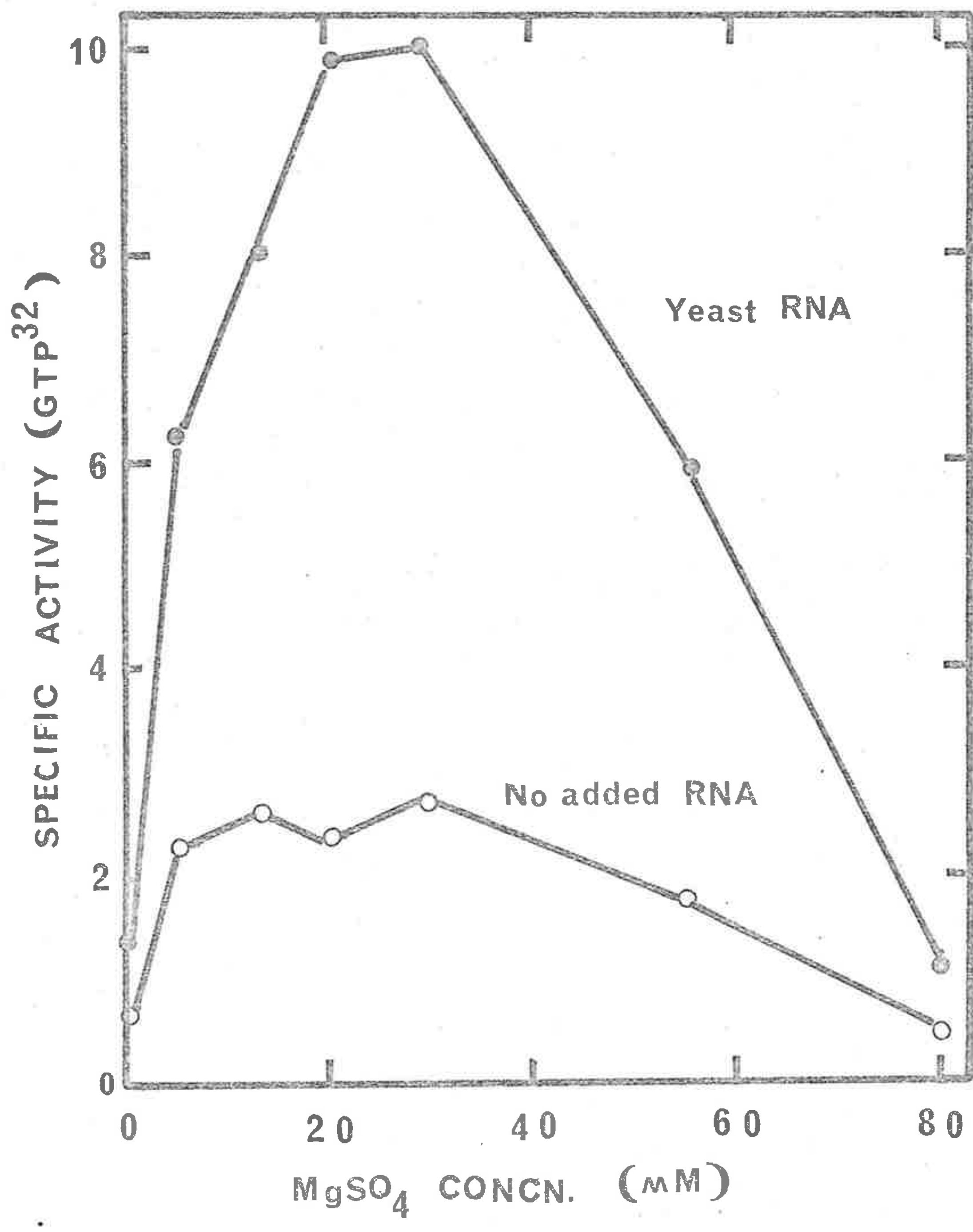

TOTAL ENZYME UNITS / GRAM FRESH WEIGHT
DAYS AFTER INFECTION
16,000 S (+RNA)
16,000 P (+RNA)
16,000 P (-RNA)
Healthy (± RNA)
0
20
40
60
80
100
120
140
160
2
4
6
8
10
12
14
16

FIGURE 11. THE EFFECT OF $MgSO_4$ CONCENTRATION ON PARTICULATE RNA POLYMERASE ACTIVITY

The RNA polymerase in the particulate fraction (16,000-P) was assayed as described in the Methods at varying $MgSO_4$ concentrations (0 - 80 mM; 0 - 30 μmoles/assay), both in the presence and absence of 1.0 mg of yeast RNA.



SPECIFIC ACTIVITY (GTP^{32})
Yeast RNA
No added RNA
0
2
4
6
8
10
0
20
40
60
80
$MgSO_4$ CONCN. (mM)

CHAPTER SIX

THE NATURE OF THE PRODUCT OF THE CUCUMBER MOSAIC VIRUS-INDUCED PARTICULATE RNA POLYMERASE

THE NATURE OF THE PRODUCT OF THE CUCUMBER MOSAIC VIRUS-INDUCED PARTICULATE RNA POLYMERASE

INTRODUCTION

It was hoped that an investigation into the nature of the product of the particulate CMV-induced RNA polymerase reaction would confirm the presence of an enzyme-viral RNA complex. The template present in such complexes is usually the complementary minus strand of viral RNA and thus such complexes produce viral-like plus strands (Bové, 1967a,b; Astier-Manifacier and Cornuet, 1965; Weissmann, 1965) whether found in animal, bacterial or plant virus-infected systems (see also Introduction). We have thus investigated the nature of the product of the particulate RNA polymerase reaction with respect to size and resistance to pancreatic ribonuclease. Hybridisation experiments were also attempted to determine if viral RNA was produced.

METHODS

1. Assay of particulate RNA polymerase activity. Assays were performed as described in Chapter 1 using GTP-^{32}P as the labelled substrate, with or without added yeast RNA (1.0 mg) and in the presence of 29 mM $MgSO_4$ (10.8 µmoles/assay). Particulate RNA polymerase (16,000-P) were prepared by EXTRACTION METHOD D (see Chapter 5).

2. Isolation and nearest neighbour-analysis of the product of the particulate RNA polymerase reaction. These were carried out as previously described in Chapter 1 except that the phenol extraction was performed at 60°.

3. Hybridisation studies with the labelled RNA product. After phenol extraction of the product, the RNA was dissolved in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 (2 x SSC). For each treatment, 10 µg of the labelled RNA in 0.15 ml was mixed with 50 - 200 µg of the stated RNA in 0.15 ml in a Pyrex tube which was sealed. The samples were heated at 120° for 10 min. in a glycerol bath, then at 85° for 2 hours, and finally at 65° for 1 hour. After cooling to room temperature, the samples were incubated with 30 µg/ml of pancreatic ribonuclease for 30 min. at 37°. Acid-insoluble radioactivity was collected and counted as described in Chapter 1.

4. Isolation of RNA from particulate plant extracts (16,000-P). The 16,000-P fraction prepared from infected cucumbers (EXTRACTION METHOD D, Chapter 5) was shaken vigorously with an equal volume of 78% aqueous phenol at 4° for 10 min. and SDS (final concentration 1.0%). After centrifugation at 5,000 g for 10 min., the aqueous layer was re-extracted twice with an equal volume of 78% aqueous phenol. The aqueous layer, after a total of three phenol extractions and centrifugations, was shaken with an equal volume of ether. The ether-water mixture was centrifuged at 5,000 g for 10 min., the ether layer was removed, and the RNA was precipitated by the addition of two volumes of

ethanol. The yield of RNA was 100 μg/gram fresh weight of plants.

RESULTS

1. The nature of the product of particulate RNA polymerase dependent on added RNA. The product of this enzyme was not associated with fractions which sedimented at 16,000 g after the assay (most probably because this RNA polymerase was solubilised during the assay; see next chapter). The phenol-extracted product (formed in the presence of added yeast RNA or CMV-RNA template) of this enzyme showed the same resistance to digestion (80%) by pancreatic ribonuclease as the product of the soluble RNA polymerase previously described (see Chapter 4). In addition similar melting properties were obtained; for example, it was necessary to predigest the product (formed in the presence of added yeast RNA template) with pancreatic ribonuclease before a melting profile could be obtained and this gave a Tm of 90° in SSC. Because all these results were essentially the same as those already presented in Chapter 4, they are not reproduced again.

2. The nature of the product of the particulate RNA polymerase independent of added RNA

(a) Phenol extraction of the product. More than 80% of the product of this enzyme was associated with the fraction that sedimented at 16,000 g after the assay. Hot phenol extraction (60°) of the assay medium which contained no added RNA recovered 60% of the labelled product while

extraction at 4° yielded only 10 - 20% of the product.

(b) Resistance to digestion by pancreatic ribonuclease. More than 80% of the product was resistant to pancreatic ribonuclease (5 - 100 μg/ml at 37° for 30 min) either before or after hot phenol deproteinisation of the assay medium. This indicates that most of the product was in a double-stranded form.

(c) Thermal transition temperature (Tm). The melting curve in SSC of the hot phenol-extracted product showed that the Tm was 93° (Figure 12). No predigestion with pancreatic ribonuclease was needed to achieve a melting profile as was found for the product of the partly purified soluble CMV-induced RNA polymerase (see Chapter 4) and of the particulate enzyme described here which was dependent on added RNA.

(d) Sedimentation analysis. The hot phenol-extracted product was centrifuged through a 5 - 20% sucrose gradient for 5 hours at 33,000 rpm using *E. coli* RNA (23S, 16S and 4S) as marker (Figure 13). The product had a sedimentation peak of 8S with a pronounced shoulder at 14S. Digestion of the product with 50 μg/ml of pancreatic ribonuclease at 37° for 30 minutes in SSC (80% still acid-precipitable) did not alter the sedimentation profile of the remaining material. If this product was intact double-stranded CMV-RNA (mol. wt. 2.0×10^{6}; Kaper *et al.*, 1965) then it would sediment at approximately 14S (S value calculated from the formula of Studier, 1965, for native double-stranded DNA). However, after heat denaturation (120° for 10 min. in SSC or 100° for 10 min.

in 0.1 x SSC) and rapid cooling, the labelled product, 90% of which was now susceptible to digestion by pancreatic ribonuclease, had a sedimentation value of 5S (see Figure 13) which corresponds to a molecular weight of approximately 40,000.

These results therefore indicated that the labelled product made under the assay conditions described, was mostly part of a low molecular weight fragment associated non-covalently and in a ribonuclease resistant form with a larger double-stranded structure (as indicated diagramatically in Figure 14), with a peak (8S) molecular weight of about 0.5×10^6. Also the size of the labelled product made was independent of the time of incubation of the RNA polymerase assay.

(e) Nearest-neighbour analysis. The nearest-neighbour analyses of the product formed by the 16,000-P fraction in the presence and absence of four RNA samples and using either GTP-^{32}P or ATP-^{32}P as the labelled substrate are shown in Table 11. CMV-RNA and RNA isolated from the particulate (16,000-P) fraction of infected cucumbers was supplied to the RNA polymerase dependent on added RNA for comparison with the product of the RNA polymerase independent of added RNA. The aim here was to determine whether or not this latter RNA polymerase was using either of these RNA molecules or the minus strand of CMV-RNA as template in particulate preparations. Unfortunately, no definite conclusions can be made although the results obtained (Table 11) in the absence of added RNA indicate that the

RNA polymerase was more likely to have copied plus than minus strands if these were present in the particulate extract.

The results obtained using TYMV-RNA (38.6% CMP) and yeast RNA as the added RNA (Table 11) with the particulate RNA polymerase were similar to those already given in Chapter 3 for the partly purified soluble RNA polymerase.

(f) Hybridisation studies. The product of the CMV-induced RNA polymerase reaction independent of added RNA was heat denatured (120° for 10 min. in SSC) in the presence and absence of RNA, and then reannealed as described in Methods. In the absence of added RNA, reannealing to produce a ribonuclease-resistant structure was low and variable (13 - 40%). Further, the addition of 50 - 200 μg of RNA (CMV, TYMV, yeast and plant particulate RNA) had no effect on the extent of reannealing. The failure of the CMV or plant particulate RNA to increase or decrease the amount of product reannealed and the low and variable reannealing observed therefore provided little information on the nature of the presumed RNA template associated with the particulate RNA polymerase. It appears that further hybridisation studies will depend on the further purification of the particulate enzyme.

DISCUSSION

The investigation into the nature of the product of the particulate RNA polymerase activity independent of added RNA yielded results which have not indicated whether the RNA polymerase was copying minus strands of CMV-RNA (similar to the TYMV-induced RNA polymerase; Bové, 1967a,b), plus strands of CMV-RNA (similar to the Newcastle disease virus-induced RNA polymerase; Scholtissek and Rott, 1969b), or perhaps some non-viral RNA template. Thus it cannot be conclusively proven whether or not particulate fractions contain an enzyme-viral RNA complex. In all the other particulate plant virus-infected systems studied, only Bove (1969a,b) has conclusively shown that the virus-induced RNA polymerase synthesised viral-specific RNA (plus-strands).

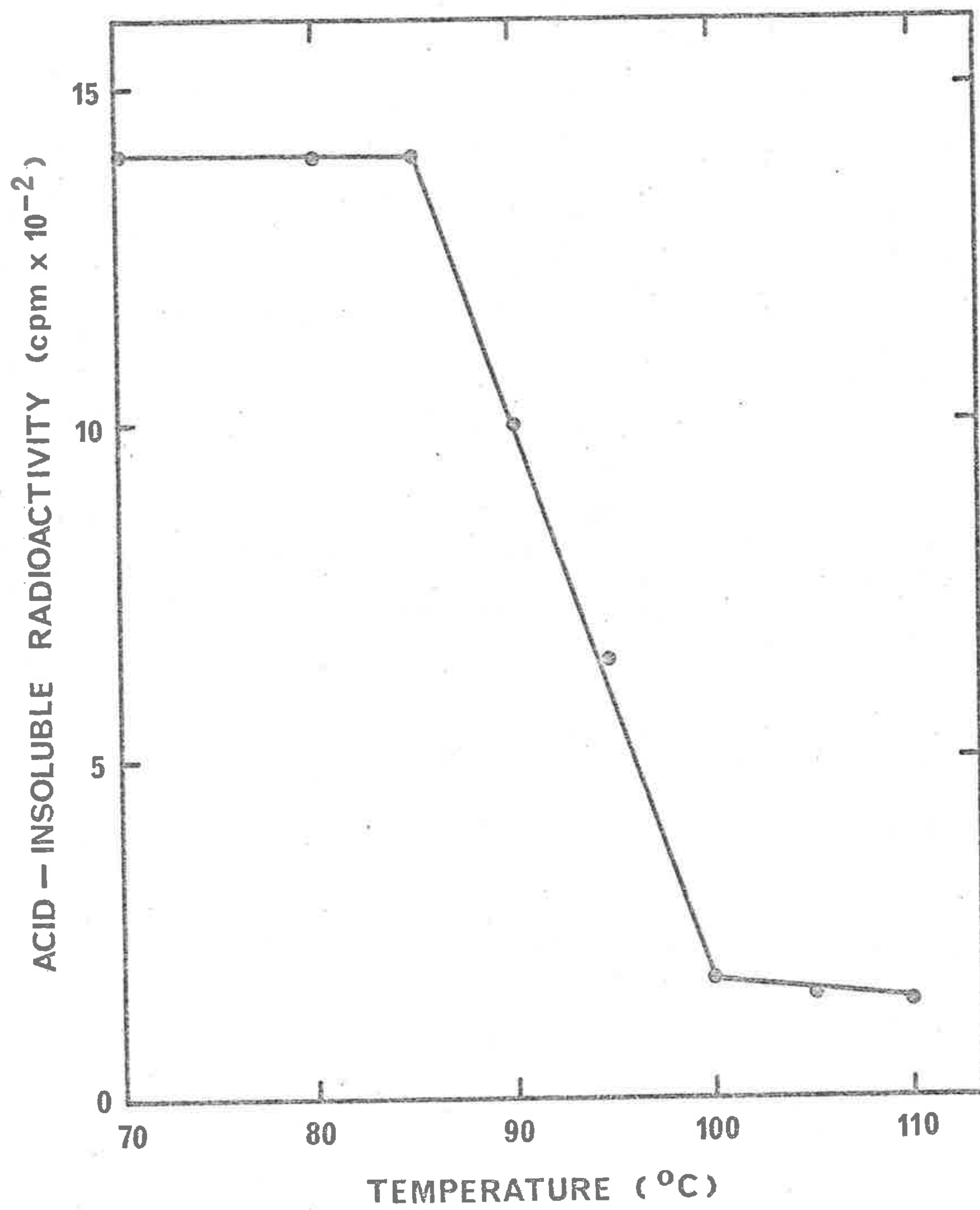
TABLE 11. NEAREST-NEIGHBOUR ANALYSIS OF THE PRODUCT FORMED IN THE PRESENCE OF VARIOUS RNA TEMPLATES BY THE PARTICULATE RNA POLYMERASE

RNA added (1.0 mg/assay)	Isolated 2'(3')-mononucleotides (% of total radioactivity)							
	ATP-^{32}P as labelled substrate				GTP-^{32}P as labelled substrate			
	AMP	CMP	GMP	UMP	AMP	CMP	GMP	UMP
None	27.0	26.5	24.7	22.0	25.7	21.1	23.6	24.7
CMV-RNA	27.7	28.3	24.6	19.1	29.2	21.7	26.7	22.3
Particulate plant RNA	26.5	29.2	25.1	19.0	26.6	25.1	26.1	22.0
TYMV-RNA					21.9	13.3	41.5	23.3
Yeast RNA					27.1	23.5	24.3	25.1

Each estimation was carried out twice on each of three separate particulate preparations (16,000-P). Values quoted are the mean percent of the total radioactivity, the standard error of the mean never being greater than ± 0.5. The particulate plant RNA was isolated from the 16,000-P fraction of infected cucumbers as described in Methods. The α-^{32}P-nucleoside triphosphates used in the assay medium are indicated.

FIGURE 12. THERMAL DENATURATION CURVE OF THE PHENOL-EXTRACTED PRODUCT OF THE PARTICULATE RNA POLYMERASE ACTIVITY INDEPENDENT OF ADDED RNA

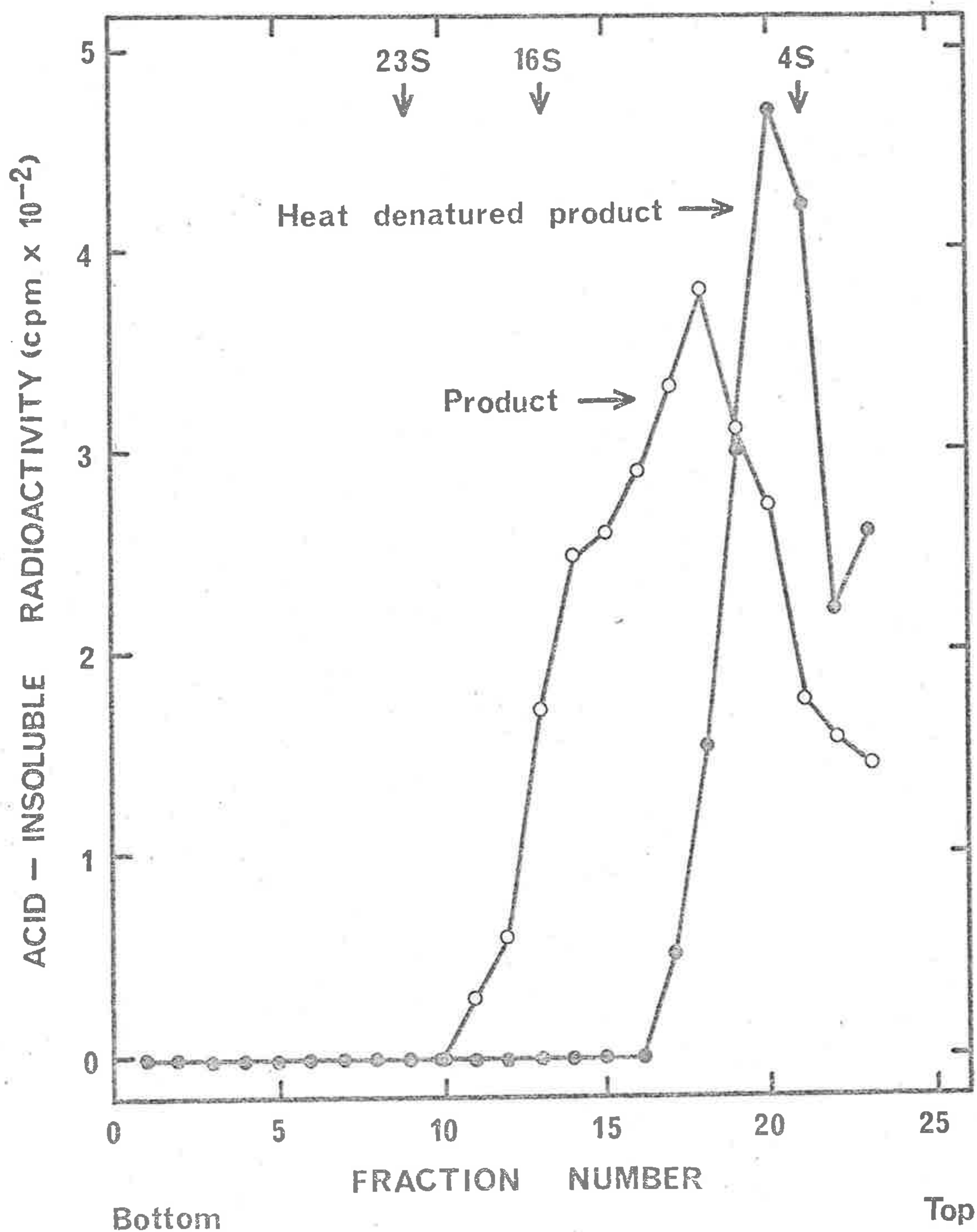
The product was isolated as described in Methods from an assay scaled up 15 times and dissolved in SSC. Samples (equivalent to one normal assay) were incubated in sealed tubes for 10 min. at the temperatures indicated. The solutions were then frozen in an ethanol-dry ice mixture, thawed and digested with pancreatic ribonuclease (18 µg/ml) at 37° for 30 min. Acid-insoluble material was collected and counted as described in Chapter 1.



ACID – INSOLUBLE RADIOACTIVITY (cpm x 10⁻²)
15
10
5
0
70
80
90
100
110
TEMPERATURE (°C)

FIGURE 13. SEDIMENTATION PROFILE IN A SUCROSE GRADIENT OF THE PHENOL-EXTRACTED PRODUCT OF THE PARTICULATE RNA POLYMERASE ACTIVITY INDEPENDENT OF ADDED RNA

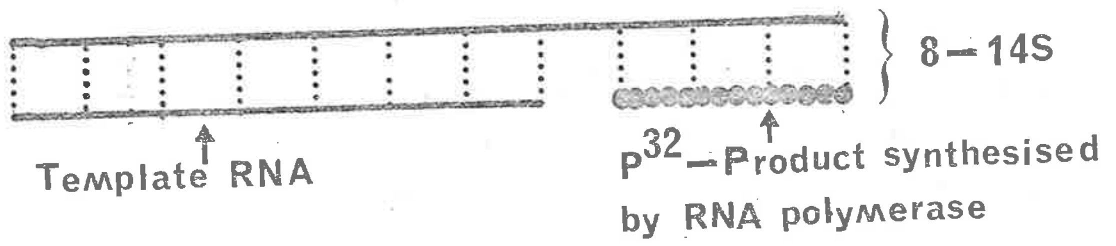
The product was isolated as described in Methods, dissolved in SSC, and half of the sample heated at 120° for 10 min. then frozen in an ethanol-dry ice mixture and thawed. Samples (0.2 ml; equivalent of three normal assays) of the product before and after heat denaturation and marker *E. coli* RNA (23S, 16S and 4S) were each layered onto a separate 4.4 ml gradient of 5 - 20% sucrose in SSC. The samples were centrifuged in a Beckman SW39 rotor at 33,000 rpm for 5 hours at 3°. Four drop (0.2ml) fractions were collected from the bottom of the tube and used for the estimation of acid-insoluble radioactivity. The optical density profile at 260 mμ of the marker *E. coli* RNA was determined but only the positions of the 23S, 16S and 4S peaks are given.



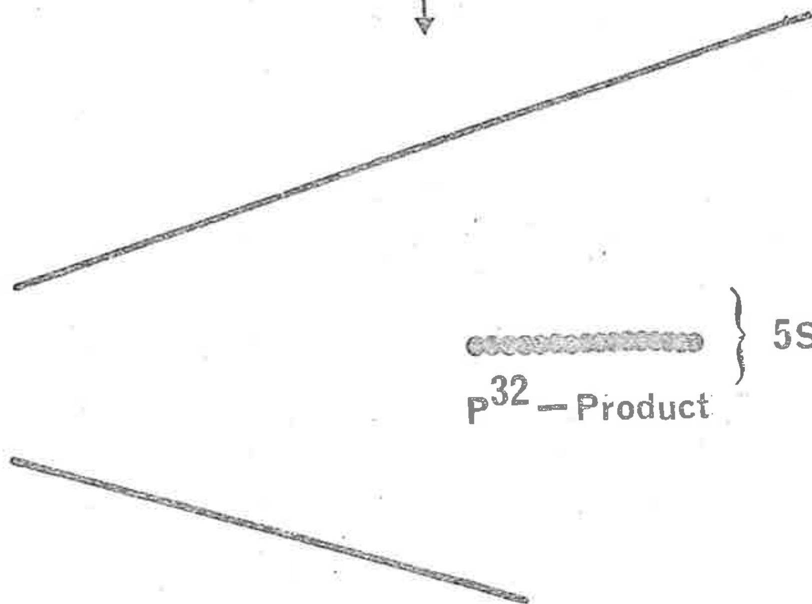
23S
16S
4S
Heat denatured product
Product
ACID – INSOLUBLE RADIOACTIVITY (cpm x 10^{-2})
FRACTION NUMBER
Bottom
Top
0
1
2
3
4
5
0
5
10
15
20
25

FIGURE 14. DIAGRAMATIC REPRESENTATION OF THE STRUCTURE OF THE PHENOL-EXTRACTED PRODUCT OF THE PARTICULATE RNA POLYMERASE ACTIVITY INDEPENDENT OF ADDED RNA BEFORE AND AFTER HEAT DENATURATION

A diagramatic representation of the structure of the phenol-extracted product of the particulate RNA polymerase activity independent of added RNA is presented. This is based solely on sedimentation data and resistance of the product to pancreatic ribonuclease digestion. Thus, the labelled product made is a low molecular weight fragment (5S) which is associated non-covalently in a pancreatic ribonuclease resistant form (in SSC) with template RNA present in the particulate fractions of CMV-infected cucumbers. However, once this double-standard structure (pancreatic ribonuclease resistant in SSC) was disrupted by heating (10 min. at 120° in SSC) the low molecular weight product was released (5S; still acid-insoluble) and became sensitive to pancreatic ribonuclease digestion.



120°/ 10 min. in SSC



CHAPTER SEVEN

SOLUBILISATION OF THE CUCUMBER MOSAIC VIRUS-INDUCED PARTICULATE RNA POLYMERASE ACTIVITY AND ITS RELATIONSHIP TO THE RNA POLYMERASE FOUND IN THE SOLUBLE PHASE

SOLUBILISATION OF THE CUCUMBER MOSAIC VIRUS-INDUCED PARTICULATE RNA POLYMERASE ACTIVITY AND ITS RELATIONSHIP TO THE RNA POLYMERASE FOUND IN THE SOLUBLE PHASE

INTRODUCTION

The results presented in the preceding two chapters have indicated that particulate fractions contain an RNA polymerase activity that can be detected either in the presence or absence of added yeast RNA template. The results presented indicated that the properties of the RNA polymerase activity dependent of added yeast RNA and of its product were similar, if not identical, to those found for the soluble partly purified RNA polymerase described in Chapters 3 and 4. In addition, in the previous chapter it was mentioned that the product of the particulate RNA polymerase activity dependent on added yeast RNA was not associated with fractions which sedimented at 16,000 g after the assay, suggesting that this particulate RNA polymerase activity was solubilised during the assay. This chapter describes the solubilisation of the particulate RNA polymerase.

MATERIALS AND METHODS

1. Materials. General materials were previously described in Chapter 1. Crystalline yeast cytochrome b_2 (EC 1.1.2.3), prepared from baker's yeast by the method of Symons and Burgoyne (1966), was kindly donated by Dr. R.H. Symons, University of Adelaide, South Australia.

2. Preparation of partly purified soluble RNA polymerase. Prepared by EXTRACTION METHOD C (See Chapter 2).

3. Preparation of particulate RNA polymerase (16,000-P). Prepared by EXTRACTION METHOD D (Chapter 5).

4. EXTRACTION METHOD E - $MgSO_4$ solubilisation of the particulate enzyme. Particulate RNA polymerase extracts (16,000-P) were prepared as described above and made 70 mM with respect to $MgSO_4$ and incubated at 37° for 15 minutes (30 min. if volume was more than 2.0 ml), cooled in ice and centrifuged at 25,000 g for 10 min. The supernatant was collected and used as the '$MgSO_4$ solubilised RNA polymerase'.

5. EXTRACTION METHOD F - Solubilisation of the particulate enzyme by freezing and thawing. Particulate RNA polymerase extracts (16,000-P), prepared as described above, were twice frozen in an ethanol-dry ice mixture followed by thawing in a water bath at 37° (liquid temperature did not rise above 4°). The preparation was centrifuged at 25,000 g for 10 min. and used as the 'frozen and thawed solubilised RNA polymerase'.

6. Assay of RNA polymerase activity. Assays were carried out as described in Chapter 1 in the presence of 29 mM $MgSO_4$ (10.8 μmoles/assay; unless stated otherwise), with GTP-^{32}P as the labelled substrate. Various RNA polymerase preparations (as described above) were used in the assay medium (the preparation used is stated in the text), with or without added yeast RNA (1.0 mg).

RESULTS

1. Solubilisation of the particulate RNA polymerase activity dependent on added RNA. The aim here was to find procedures for the solubilisation of the particulate RNA polymerase dependent on added RNA so that its properties could be compared with the enzyme normally found in the soluble phase of extracts of infected plants.

Treatment of the particulate fraction (16,000-P) with $(NH_4)_2SO_4$ (preparation made 50 - 70% saturated) or twice freezing the preparation in an ethanol-dry ice mixture followed by thawing (see EXTRACTION METHOD F above), released approximately 50% of the particulate RNA polymerase dependent of added RNA into the soluble phase (not sedimented at 16,000 g for 10 min.). Sonication at 0° (Dawe Type 1130A Soniprobe at full power, cumulative time 25 seconds) gave variable release (10 - 50%). Triton X-100 (5% final concentration) solubilised all chloroplast material (Francki et al., 1965) in the 16,000-P fraction but failed to solubilise any RNA polymerase activity dependent on added RNA. Likewise, three washings of the particulate fraction by suspension in and sedimentation (16,000 g for 10 min.) from extraction buffer minus sucrose (see EXTRACTION METHOD D, Chapter 5) failed to release any RNA polymerase activity dependent on added RNA (see also Chapter 5). The total recovery of RNA polymerase activity dependent on added RNA after all these treatments ranged from 85 - 100%. However, if $MgSO_4$ was added to a final concentration of 70 mM to the particulate (16,000-P) fraction and the mixture incubated at 37° for 15 minutes (see EXTRACTION METHOD E in Methods), 90% of

the RNA polymerase activity dependent on added RNA was solubilised (see Figure 16; there is a 1.5 fold activation of total RNA polymerase activity dependent on added RNA by this method). Under the same conditions, but in the absence of $MgSO_4$, about 75% of the total activity was lost and there was negligible solubilisation of the enzyme.

It was found that 80% release of RNA polymerase activity dependent on added RNA also occurred during the usual enzyme assay (particulate fractions, 16,000-P were the source of RNA polymerase) which contained only 29 mM $MgSO_4$ and only 20% of the enzyme could be sedimented (16,000 g for 10 min.) at the end of the incubation. As the product of this enzyme was also not sedimented (see previous chapter), it is quite possible that enzyme release occurred prior to the synthesis of any product. This would explain why the properties of the enzyme were similar to the enzyme normally isolated from the soluble phase of plant extracts (see Chapters 3 and 4). The other components of the assay medium (K^+, NH_4^+, PEP, BSA etc.), failed to release the RNA polymerase activity dependent on added RNA into a soluble phase as found with $MgSO_4$. Other divalent ions (Zn^{2+}, Mn^{2+}, Ca^{2+}, 70 mM) did not solubilise the RNA polymerase activity dependent on added RNA.

2. Sedimentation analysis of the solubilised RNA polymerase activity dependent on added RNA. The RNA polymerase dependent on added RNA and released from the 16,000-P fraction by incubation in the presence of 70 mM $MgSO_4$ (EXTRACTION METHOD E) was centrifuged through a 5 - 20% sucrose gradient with haemoglobin (mol. wt. 68,000) as marker. Soluble RNA

polymerase (prepared by EXTRACTION METHOD C, Chapter 2) was centrifuged at the same time under the same conditions. Since similar RNA polymerase activity profiles were obtained, it appeared as though the RNA polymerase normally found in the soluble phase of infected cucumber extracts could have been the same as that released from the particulate fraction by the incubation with $MgSO_4$. This conclusion is consistent with all the results presented in the two previous chapters on the properties of the particulate RNA polymerase activity dependent on added RNA.

By contrast, the sedimentation analysis of the RNA polymerase dependent on added RNA and solubilised from the particulate fraction (16,000-P) by freezing and thawing (Figure 15) showed that this enzyme had a molecular weight of 150,000, or about 30,000 higher than that of the soluble RNA polymerase (mol. wt. 123,000, see Chapter 3). (The molecular wt. value of the soluble RNA polymerase did not alter upon freezing and thawing of the partly purified soluble enzyme preparation.) The same result was obtained in five separate experiments, in some of which crystalline yeast cytochrome $\underline{b}_2$ (mol. wt. 170,000; Appleby and Morton, 1959; Symons and Burgoyne, 1968) together with haemoglobin were used as markers. Further investigation of the properties of the enzyme solubilised by freezing and thawing failed to show any differences from the enzyme normally found in the soluble phase. The possible significance of these results is considered in the Discussion.

3. <u>Solubilisation of the RNA polymerase not dependent on added RNA for activity</u>. Figure 16 shows that by incubating particulate fractions (16,000-P) with 70 <u>mM</u> $MgSO_4$ for various times, an RNA polymerase activity independent of added RNA was released into the soluble phase (activity increased 5 - 8 fold above the activity found in the particulate fraction). This solubilised activity was very dependent on the $MgSO_4$ concentration of the assay medium (optimal activity 5 - 13 <u>mM</u>; see also following chapter) in contrast to the corresponding activity in the starting material (see Figure 11, Chapter 5). Figure 16 also shows that, after treatment with 70 <u>mM</u> $MgSO_4$ at 37° for 15 min., the particulate fraction (pellet obtained after 16,000 <u>g</u> centrifugation of the treated fraction) contained RNA polymerase activity independent of added RNA at about the same activity to that found prior to $MgSO_4$ treatment, indicating that all the activity was not released by the $MgSO_4$. One possible explanation of these results is that in addition to the solubilisation of the 123,000 molecular weight RNA polymerase which is free of bound RNA, the $MgSO_4$ treatment released some 16,000-P RNA (50 - 70 μg/ml of 16,000-P preparation; released approximately half the RNA present in the 16,000-P preparation, see also Chapter 5) which acts as template in the absence of added yeast RNA (see also following chapter). However, the RNA polymerase activity independent of added RNA not released from the particulate fraction by the $MgSO_4$ treatment could be the intact RNA polymerase-viral RNA complex.

Also of interest was the fact that the $MgSO_4$ solubilised RNA polymerase activity independent of added RNA was not released by 29 mM $MgSO_4$ at 37°, nor did concentrations greater than 70 mM $MgSO_4$ release any more activity. Other divalent ions (Zn^{2+}, Mn^{2+}, Ca^{2+}, 70 mM) or monovalent ions (70 mM K^+, NH_4^+, at 37° for 15 min.) failed to release the RNA polymerase activity independent of added RNA. Why indeed only $MgSO_4$ released such activities at 37° is not understood.

Freezing and thawing of particulate fraction (see EXTRACTION METHOD F in Materials and Methods) was the only other method of those listed before (see 'Solubilisation of the particulate RNA polymerase activity dependent on added RNA') which was found to release the RNA polymerase activity independent of added RNA from particulate fractions (50% of the activity obtained by release with $MgSO_4$ at 37° when assayed in the presence of 5 - 13 mM $MgSO_4$).

DISCUSSION

The basic assumption of the work described is that the CMV-induced RNA polymerase system under investigation is responsible for the *in vivo* replication of viral RNA. If this is so then the particulate fraction (16,000-P) referred to in the last three chapters, may contain the CMV-RNA-RNA polymerase complex which was detected by RNA polymerase activity that was independent of added RNA, and presumed to be present in particulate fractions of other plant virus-infected

systems. Incubation of the particulate fraction with $MgSO_4$ at 37° released RNA polymerase activity into a soluble form and all evidence indicated that the RNA polymerase dependent on added RNA solubilised in this way was very similar to the RNA polymerase activity dependent on added RNA and normally found in the soluble phase of infected plant extracts (see Chapters 3 and 4). Whether this solubilised enzyme was originally associated with a viral RNA template or was bound in some other way to the particulate fraction is not known.

An interesting development has been the reproducible observation that the RNA polymerase solubilised by freezing and thawing and dependent on added RNA had a molecular weight of 150,000 which is about 30,000 higher than the enzyme solubilised by incubation with $MgSO_4$ and the enzyme normally isolated in the soluble phase. This indicates that we have retained, during solubilisation by freezing and thawing, an extra component(s) which was lacking from the other soluble forms of the enzyme. It is considered possible, therefore, that we may have a multicomponent RNA polymerase system similar to that described for the $Q\beta$-RNA polymerase (Eikhom et al., 1968; Franze de Fernandez et al., 1968) and that the non-specific RNA-dependent RNA polymerase found in soluble extracts of infected plants is the basic virus coded polymerase which, in association with CMV-RNA and one or more host proteins, forms a particulate complex that replicates CMV-RNA.

A further interesting development in the study of the CMV-induced RNA polymerase was the release of an RNA polymerase activity independent of added RNA from particulate fractions, the specific activity of which was similar to the RNA polymerase activity measured in the presence of added yeast RNA. However, evidence presented in the following chapter indicates that this activity is probably due to the solubilised RNA polymerase activity copying RNA released from the 16,000-P fraction, and not due to the presence of an RNA polymerase-viral RNA complex. RNA polymerase activity independent of added RNA was still found associated with particulate fractions at about the same activity either before or after solubilisation techniques were employed. Also it is known that the product of this reaction remains in the 16,000 g pellet after the enzyme assay (see Chapter 6). Thus, the RNA polymerase-viral RNA complex may not be solubilised by the techniques described. Further investigations into the particulate RNA polymerase activity independent of added RNA will be carried out on more extensively purified particulate preparations.

FIGURE 15. SEDIMENTATION PROFILE OF THE RNA POLYMERASE DEPENDENT ON ADDED RNA AND RELEASED FROM THE PARTICULATE FRACTION (16,000-P) BY FREEZING AND THAWING

The enzyme released by freezing and thawing (0.9 mg of protein in 0.6 ml) and haemoglobin marker (2.0 mg) were centrifuged through a 10 ml gradient of 5 - 20% sucrose in 0.1 M NH_4Cl, 90 mM 2-mercaptoethanol, 5 mM $MgSO_4$, 50 mM Tris-HCl buffer pH 8.5, in a Beckman SW41 rotor at 35,000 for 16 hours at 3°. Four drop (0.19 ml) fractions were collected from the bottom of the tube and alternate fractions were assayed for RNA polymerase activity (see Materials and Methods with 1.0 mg of added yeast RNA per assay) or optical density at 413 mμ. Partly purified soluble RNA polymerase (1.2 mg of protein; see EXTRACTION METHOD C, CHAPTER 2) plus haemoglobin (2 mg) were centrifuged together at the same time under the same conditions in a separate tube. Peak molecular weights were calculated as described in Chapter 3. Recovery of RNA polymerase activity was 95% for both peaks.

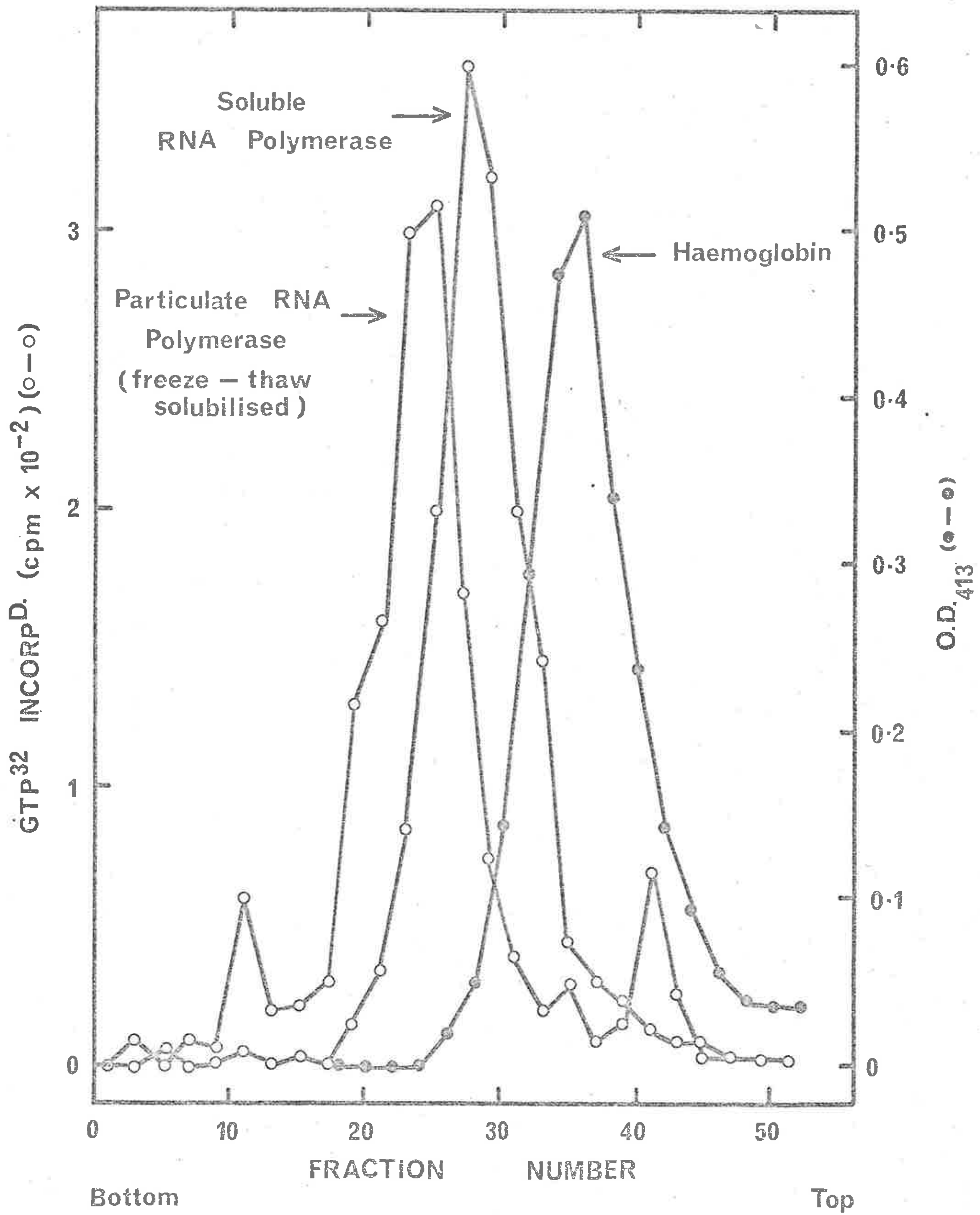
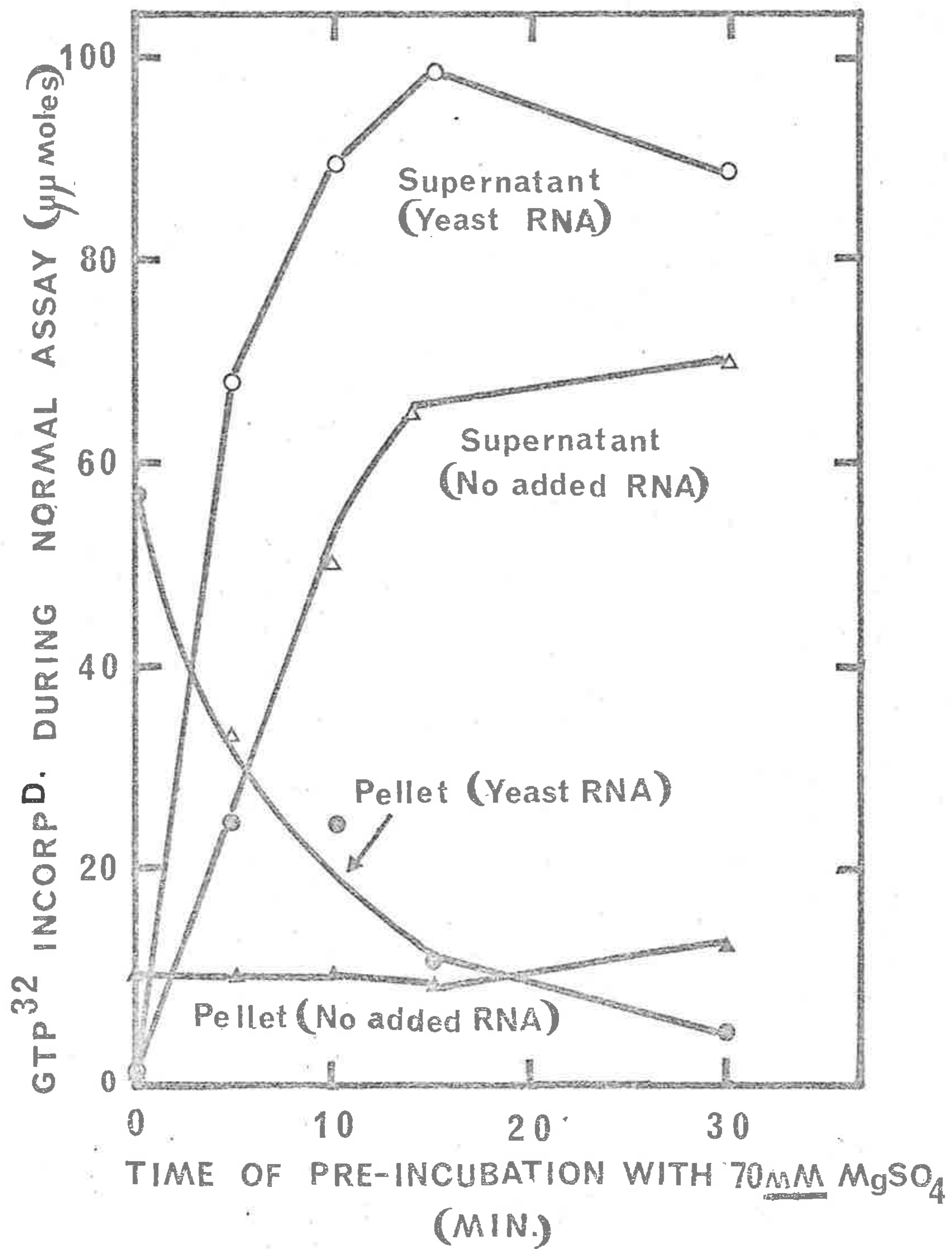

Soluble
RNA Polymerase
Particulate RNA
Polymerase
(freeze – thaw
solubilised)
Haemoglobin
GTP32 INCORPD. (cpm x 10−2) (○—○)
O.D.413 (●—●)
0
1
2
3
0·1
0·2
0·3
0·4
0·5
0·6
10
20
30
40
50
FRACTION NUMBER
Bottom
Top

FIGURE 16. SOLUBILISATION WITH $MgSO_4$ OF THE PARTICULATE RNA POLYMERASE ACTIVITIES DEPENDENT AND INDEPENDENT OF ADDED RNA FROM PARTICULATE FRACTIONS

The particulate fraction (2.0 ml; 16,000-P) was incubated in the presence of 70 mM $MgSO_4$ for the times indicated (0 - 30 min.). The preparation was then centrifuged at 25,000 g for 10 min. and the pellet and supernatant were collected. The pellet was resuspended in 2.0 ml of 50 mM Tris-HCl buffer pH 8.5, 0.1 M NH_4Cl and 90 mM 2-mercaptoethanol (extraction buffer minus sucrose, see EXTRACTION METHOD D, Chapter 5). The supernatant (0.1 ml) was assayed for 30 min. as described in Materials and Methods with GTP-^{32}P as the labelled substrate, and 7.0 µmoles $MgSO_4$/assay (that present in 0.1 ml of supernatant), both in the presence and absence of 1.0 mg of yeast RNA.

The resuspended pellet (0.1 ml) was assayed in a similar manner (7.0 µmoles $MgSO_4$ being present in the assay medium).


GTP32 INCORPD. DURING NORMAL ASSAY (μμ moles)
100
80
60
40
20
0
0
10
20
30
TIME OF PRE-INCUBATION WITH 70mM MgSO4
(MIN.)
Supernatant
(Yeast RNA)
Supernatant
(No added RNA)
Pellet (Yeast RNA)
Pellet (No added RNA)

CHAPTER EIGHT

SPECIFICITY OF THE SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE FOR RNA AND SYNTHETIC POLYNUCLEOTIDE TEMPLATES

SPECIFICITY OF THE SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE FOR RNA AND SYNTHETIC POLYNUCLEOTIDE TEMPLATES

INTRODUCTION

An important property of the RNA polymerase induced in *E. coli* after infection with the RNA bacteriophage Qβ is its template specificity. The purified enzyme replicated Qβ-RNA (Haruna and Spiegelman, 1965), its complementary minus strand (Banerjee *et al.*, 1969a), RNA molecules described as 'variants' of Qβ-RNA (Mills *et al.*, 1967), and the unusual 6S-RNA species induced in *E. coli* by Qβ phage (Banerjee *et al.*, 1969b). In addition to these RNA species, synthetic polynucleotides acted as templates for the RNA polymerase provided they contain cytidylate, with the synthesis of a strand complementary to the template polymers (Hori *et al.*, 1967; Eikhom and Spiegelman, 1967; Eikhom *et al.*, 1968).

This chapter is primarily concerned with the specificity of the soluble CMV-induced RNA polymerase for both synthetic polynucleotides and RNA templates. In previous chapters we have shown that the soluble CMV-induced RNA polymerase was mostly dependent on, but not specific for, a variety of RNA templates which it copied by complementary base pairing.

Also of importance is the fact that soluble CMV-induced RNA polymerase activity (mol. wt. 123,000) can be prepared free of detectable plant ribonucleases from either a 'soluble' cytoplasmic fraction (see EXTRACTION METHOD C - partly purified enzyme extracts, Chapter 2) or a 'particulate' fraction (16,000-P; EXTRACTION METHODS E AND F, Chapter 7) of disrupted CMV-infected cucumber cotyledons. Because of the ease of preparation, partly purified soluble extracts of CMV-induced RNA polymerase from the 'soluble' fraction (EXTRACTION METHOD C, Chapter 2) have been used throughout this chapter. However, soluble enzyme preparations from these two fractions gave similar results when comparisons were made during the present work.

METHODS

1. Preparation of partly purified soluble RNA polymerase. As prepared by EXTRACTION METHOD C, described previously in Chapter 2. Both healthy and infected plant extracts contained 50 - 80 µg of RNA per ml.

2. Assay of the soluble RNA polymerase activity. Carried out as described in Chapter 1 with GTP-^{32}P as the labelled substrate. RNA or polynucleotide templates were added as stated. Unless stated otherwise all assays were performed in the presence of 13 mM $MgSO_4$ (5 µmoles $MgSO_4$/assay). Linear incorporation of labelled nucleoside triphosphate was obtained for 60 min. with both the presence or absence of added RNA or polynucleotide templates in the assay medium.

3. Isolation and nearest-neighbour analysis of the product of the RNA polymerase reaction. These were carried out as previously described in Chapter 1.

4. Extraction of plant RNA. The source of plant RNA used in this chapter was the partly purified enzyme extract prepared as described above from both healthy and infected plants. Extracts were phenol-SDS extracted essentially as described in Chapter 1 for viral RNA but the addition of NaCl to the aqueous layer was omitted. The yield of RNA was 60 - 85% of the total RNA present as estimated by alkaline digestion of the enzyme extracts (described in Chapter 1).

RESULTS

1. The effect of magnesium concentration on the activity of the partly purified soluble CMV-induced RNA polymerase. As previously stated, soluble RNA polymerase preparations originating from 'soluble' or 'particulate' fractions of CMV-infected cucumbers gave similar results whenever comparisons were made during the present work. Only the results obtained with partly purified soluble RNA polymerase extracts (prepared as described in Methods) are given here.

In previous investigations of the soluble CMV-induced RNA polymerase, an assay medium which contained 29 mM $MgSO_4$ (10.8 μmoles/assay) was used and the results showed that the RNA polymerase activity was dependent on the presence of added RNA in the assay medium (Chapters 2 and 3). However, it has since been found that, by lowering the magnesium ion

concentration of the assay medium to 5 - 13 mM (2 - 5 μmoles/ assay), added RNA was no longer required for soluble RNA polymerase activity and the utilisation of polyC as a template was increased five-fold (Figure 17). On the other hand, there was little effect on the RNA polymerase activity measured in the presence of yeast RNA when the $MgSO_4$ concentration was varied from 5 - 30 mM. At a $MgSO_4$ concentration of 13 mM, the variation in concentration of K^+ or NH_4^+ from 0 - 54 mM or of pH from 8.0 - 8.8 had negligible effect on the soluble RNA polymerase in the presence or absence of added RNA. Replacement of Mg^{++} in the assay medium with either Ca^{2+} or Mn^{2+} (0 - 13 mM) gave negligible RNA polymerase activity dependent on added RNA or polynucleotide. Dialysis of the partly purified RNA polymerase preparation (see Methods) against 50 mM Tris-HCl buffer pH 8.5, 90 mM 2-mercaptoethanol, 100 mM NH_4Cl, for 2 hours at 4° (5 ml of enzyme preparation/litre of dialysis medium) did not alter the magnesium optimum profile of the soluble RNA polymerase in the presence of polyC or yeast RNA. However, 50% of the activity independent of added RNA was lost when assayed in the presence of 5 mM $MgSO_4$ but the activity at the other magnesium concentrations was unaffected.

However, although polyC was utilised as a template by the soluble RNA polymerase to a greater extent at 5 - 13 mM $MgSO_4$, there was little effect on the utilisation of polyU and polyG (250 μg/assay) templates by the RNA polymerase (Figure 18). PolyA was not used here due to its insolubility in the assay medium which contained greater than 5 mM $MgSO_4$

(see below also). Similar results to those shown in Figure 18 were obtained whether the assays were performed in the presence or absence of the three unlabelled triphosphates (see also below). Further information regarding the template activities of synthetic polynucleotides is presented in the following section.

CMV-RNA (25 - 125 μg/assay) was also utilised as a template by the soluble RNA polymerase to a greater (two-fold) extent when the $MgSO_4$ concentration of the assay medium was lowered from 29 mM (see Table 5, Chapter 3) to 13 mM (see also Figure 19).

2. Characterisation of the template activity of RNA and of synthetic polynucleotides. It was previously shown that the soluble CMV-induced RNA polymerase copied polyC, polyU, TYMV and yeast RNA by complementary base pairing (Chapter 3). Thus no specificity was demonstrated for any of these RNA or polynucleotide species by the RNA polymerase at the magnesium concentration of 29 mM $MgSO_4$, used in these assays. The utilisation of several RNA and polynucleotide species by the soluble RNA polymerase in the presence of 5 - 13 mM $MgSO_4$, under which conditions other activities associated with the enzyme were apparent, has now been investigated.

(a) The template activity of synthetic polynucleotides. Table 12 shows the effect of four synthetic polynucleotides (polyA, polyC, polyG and polyU) and yeast RNA on the soluble RNA polymerase activity independent of added RNA or polynucleotides, using four labelled nucleoside triphosphates

as substrates. Assays were performed both in the presence and absence of the other three unlabelled triphosphates and in the presence of 5 mM $MgSO_4$, since polyA was insoluble at higher concentrations of $MgSO_4$ (see also above).

Assays performed in the presence of all four nucleoside triphosphates showed that the addition of polynucleotides (250 μg/assay) suppressed in all but two cases, the soluble RNA polymerase activity measured in the absence of RNA or polynucleotides (Table 12). Addition of polyC gave a ten-fold increase in GTP-^{32}P incorporation into product, while suppressing (greater than 90%) the incorporation of the other labelled nucleotides tested. Further, there was increased incorporation (two-fold) of GTP-^{32}P on the addition of polyG but this activity was also found in healthy plant extracts and in the absence of the three unlabelled triphosphates (Table 12). The role of such an enzyme, present in soluble extracts of both healthy and infected plants, which presumably added GMP from GTP onto the terminal 3'-hydroxyl of polyG, is not known. Excluding this activity, healthy plant extracts were devoid of any detectable soluble RNA polymerase activity, dependent or independent of added RNA as measured by the incorporation of GTP-^{32}P (Table 12).

Table 12 also shows that the omission of unlabelled nucleoside triphosphates from the assay medium reduced by more than 85% the soluble RNA polymerase activity in the absence of added RNA or polynucleotides. However, such omission in the presence of added polynucleotides had a variable effect as there were both increases and decreases in the soluble RNA polymerase

activity. These variable results are probably related to the observation that the omission of unlabelled triphosphates in the presence of yeast RNA halted GTP-^{32}P incorporation while the other three labelled triphosphates were still incorporated at a reduced rate (Table 12). The appearance of increased polymerase activity in both healthy and infected plant extracts on the omission of one or more triphosphates from the assay medium has previously been reported (Gilliland and Symons, 1968; see also Table 4, Chapter 3) and is probably due to incorporation of nucleotides into transfer RNA (see Daniel and Littauer, 1963; see also Chapters 3 and 5). The results obtained here further emphasise the need to use GTP-^{32}P as the labelled nucleoside triphosphate in general RNA polymerase assays (see also Chapter 5). Also of worthy mention was the fact that soluble RNA polymerase preparations did not incorporate high levels of CTP-^{32}P into acid insoluble material (see Table 12) as previously found with particulate extracts of RNA polymerase (see Table 10, Chapter 5). Such CTP-^{32}P incorporation was found only in particulate (16,000-P) extracts of both healthy and infected cucumbers and was not affected by a range of 5 - 29 mM $MgSO_4$ in the assay medium.

As will be considered below, all evidence indicates that, in the absence of added RNA or polynucleotide, the soluble virus-induced RNA polymerase was copying RNA present in the partly purified plant extracts. The results of Table 12 show that the addition of polyA suppressed by at least 75% and of polyC, polyG and polyU by at least 98%

the copying of this RNA since its maximum template activity in the presence of any one polynucleotide must be given by the <u>lowest</u> specific enzymatic activity obtained with any of the labelled triphosphates in the presence of the other three unlabelled triphosphates. The most obvious explanation is that the added polynucleotide successfuly competed with the natural RNA for the active site of the polymerase. However, only in the case of polyC was there any clear evidence that the added polynucleotide was actually being copied by complementary base pairing. The variable activities obtained with added polyA, polyG and polyU may have been due to some unusual enzyme activities present in our partly purified plant extracts (see above) or to incomplete suppression by polyA of the RNA polymerase activity independent of added RNA or polynucleotide. However, the very marked preference of the soluble RNA polymerase for polyC as template is further emphasised from the data of Table 12 which show that, even if polyA, polyG and polyU were copied, their maximum template activity was no more than 2 - 3% of that of polyC.

(b) <u>The template activity of various RNA samples</u>. Various RNA species, polyU and polyC were added in amounts from 0 - 125 µg to the assay medium (13 <u>mM</u> $MgSO_4$) and their effect on the soluble RNA polymerase activity independent of added RNA and with GTP-^{32}P as the labelled substrate is shown in Figure 19. An increase in RNA polymerase activity was found with polyC (see also above), CMV-RNA, TMV-RNA, TRSV-RNA, <u>E. coli</u> RNA and infected plant RNA but there was no specificity by the enzyme for CMV-RNA alone. On the other hand, the addition

of healthy plant RNA, yeast transfer RNA, TYMV-RNA and especially polyU lowered the RNA polymerase activity. Of prime importance was the observation that RNA extracted from CMV-infected plant extracts increased the RNA polymerase activity while RNA from healthy plants tended to lower the activity.

(c) Nearest-neighbour analysis of the products formed both in the presence and absence of added polyC, yeast RNA and TYMV-RNA. To ascertain whether or not added RNA species were copied by the soluble CMV-induced RNA polymerase, nearest-neighbour analyses were performed on the products formed both in the presence and absence of varying amounts of yeast RNA, TYMV-RNA and of polyC in the assay medium with GTP-^{32}P as the labelled substrate (Table 13).

After alkaline hydrolysis of the products found in the presence and absence of TYMV-RNA (75 - 1000 μg), 36.5 - 41.0% and 26.9%, respectively, of the total radioactivity incorporated was found in 2'(3')-GMP. Thus, the values obtained in the presence of TYMV-RNA were indicative of the added RNA which has a molar composition of 38.6% CMP (Symons et al., 1963). A marked change was also found in the amount of radioactivity in 2'(3')-CMP. The results obtained in the presence and absence of yeast RNA (75 - 1000 μg) showed that 27.1 - 28.5% and 23.9%, respectively, of the total radioactivity was found in 2'(3')-AMP. The values reported here for yeast RNA were very similar to those previously presented (Chapter 3) for the product of the soluble CMV-induced RNA polymerase

primed by yeast RNA but at the higher $MgSO_4$ concentration of 29 mM. Hence, the addition of 75 µg or more of TYMV or yeast RNA to the assay medium resulted in a change in the nature of the product. Thus, the original soluble RNA polymerase activity measured in the absence of added RNA or polynucleotide was suppressed by the addition of RNA species which were then copied by the RNA polymerase. Likewise, the addition of polyC suppressed the soluble RNA polymerase activity independent of added RNA or polynucleotide (see also above) and it was copied by complementary base pairing (Table 13).

3. Characterisation of the soluble CMV-induced RNA polymerase activity measured in the absence of added RNA or polynucleotides.

A CMV-induced RNA polymerase activity independent of added RNA and which incorporated GTP-^{32}P into a double-stranded RNA product (8 - 14S) was previously described for particulate fractions of CMV-infected cucumbers (see Chapter 6) and it was considered that this particulate fraction may contain an RNA polymerase-viral RNA complex (Chapters 5 and 7). The major aim of the following experiments was to investigate the nature of the soluble RNA polymerase activity independent of added RNA.

(a) General properties. As considered above and in Table 12, 85% of the soluble RNA polymerase activity independent of added RNA was lost if three of the nucleoside triphosphates were omitted from the assay medium. Similar results were

obtained when only one nucleoside triphosphate was omitted, which indicates that the soluble RNA polymerase activity independent of added RNA was not due to random polymerisation of nucleotides. This is consistent with the nearest-neighbour analyses of the product of the reaction (Table 13).

The results presented in Table 14 provide further information on the nature of this soluble RNA polymerase activity in partly purified extracts. The lack of effect of inorganic phosphate added at seven times the concentration of nucleoside triphosphates indicated the absence of polynucleotide phosphorylase activity while the inhibitory effect of inorganic pyrophosphate was consistent with the utilisation of nucleoside triphosphates with the release of pyrophosphate. The reaction was sensitive to pancreatic ribonuclease (1 µg/assay) although once completed, the reaction product was 93% resistant to pancreatic ribonuclease digestion (10 µg/assay). Consistent with this result was the finding of low levels (20% of those described here) of soluble RNA polymerase activity independent of added RNA in crude extracts of infected cucumbers assayed in the presence of 5 - 13 mM $MgSO_4$. These crude soluble extracts contained ribonuclease activity (see EXTRACTION METHODS A, C and D (16,000S)) in contrast to the ribonuclease-free, partly purified extracts used in the present work.

The 20% decrease in soluble RNA polymerase activity independent of added RNA by the addition of deoxyribonuclease (25 µg) to the assay medium and the negligible effect of the

omission of actinomycin D, provide evidence against significant host plant DNA-dependent RNA polymerase, a conclusion supported by the observation that native DNA did not significantly prime healthy plant extracts to incorporate labelled GTP. However, although the addition of native DNA (200 μg) to the assay medium reduced the soluble RNA polymerase activity by only 10%, denatured DNA (200 μg) reduced the activity by 80%. These results indicated that single-stranded, but not double-stranded, DNA could suppress the RNA polymerase activity independent of added RNA.

(b) The product of the reaction. As described above, the product of the soluble RNA polymerase reaction independent of added RNA or polynucleotides was 93% resistant to digestion by pancreatic ribonuclease. This result was confirmed and extended by using a phenol-extracted product (see Methods) which was more than 90% resistant to pancreatic ribonuclease digestion (5 - 100 μg/ml) for 30 min. at 37° in SSC; the product gave a thermal transition temperature (Tm) of 97° in SSC and a sedimentation coefficient of 6 - 8S in sucrose density gradients.

(c) Sedimentation profile of soluble RNA polymerase activity. Figure 20 shows the sedimentation profile of the soluble CMV-induced RNA polymerase activity, assayed in the absence of added RNA and in the presence of polyC or yeast RNA, after centrifugation through a 5 - 20% sucrose density

gradient for 5 or 17 hours at 36,000 rpm. No separation of polyC or yeast RNA template copying activity (recovery 100 - 110% in six experiments; 5 - 6S; mol. wt. 123,000; see Chapter 3) was found under the conditions described. However, the soluble RNA polymerase activity independent of added RNA was detected after 5 hours centrifugation (40 - 45% recovery relative to loaded activity), but the activity was considerably lower after 17 hours centrifugation (4 - 20% recovery, 5 experiments). By contrast, this same activity was completely recovered when the enzyme preparation was stored in 5 - 10% sucrose at 4° for 17 hours. Thus, decay of this activity occurred during the centrifugation and one possible explanation could be the removal of a template which sedimented at a faster or slower rate than the RNA polymerase activity (5 - 6S).

DISCUSSION

The results presented above have indicated that, in the presence of 5 - 13 mM $MgSO_4$ in the assay medium, the soluble CMV-induced RNA polymerase (prepared from either 'soluble' or 'particulate' extracts; see Introduction) bound polyC and several RNA species which it then copied by complementary base pairing. However, in the case of polyA, polyG and polyU the situation was not so clear. Because these polynucleotides effectively suppressed the template activity of the RNA present in the partly purified enzyme preparation, it seems likely that they were bound to the enzyme but there is no definite evidence

that they also acted as templates. Even if they did, the data of Table 12 shows that their maximum template activity was no more than 2 - 3% of that of polyC. This very marked preference by the soluble RNA polymerase for polyC over polyU was a function of the Mg^{++} concentration since the template activity of polyC was markedly reduced at high $MgSO_4$ concentrations (40 mM) and approached that of polyU (Figure 18). However, polyG failed to act as a template for the soluble RNA polymerase activity at any concentration of $MgSO_4$ tested (0 - 40 mM), using CTP-^{32}P as the labelled nucleoside triphosphate (Figure 18). Of considerable interest, however, is the marked preference shown for polyC and for heteropolynucleotides containing cytidylate by the purified phage Qβ-RNA polymerase (Hori et al., 1967; Eikhom and Spiegelman, 1967).

Whether soluble RNA polymerase activity measured in the absence of added RNA or polynucleotide was due to the presence of an enzyme-viral RNA complex in the partly purified soluble extracts of cucumbers or to an RNA polymerase activity which utilised free, deoxyribonuclease-insensitive template present in the extracts is not entirely clear although all the evidence would favour the latter hypothesis. The dependence of this activity on the presence of all four nucleoside triphosphates in the assay medium and the subsequent double-stranded nature of the product indicate a reaction primed by part at least of the RNA present in our extracts (50 - 80 μg/ml). The decay of this activity during sucrose density gradient

centrifugation may be interpreted as the removal of a template species of higher or lower sedimentation value than the RNA polymerase activity (5 - 6S), implying only a weak association between the enzyme and RNA template. Thus the question arises as to whether the template present in our extracts is host RNA, a viral RNA species involved in the replication process or an 'unusual' viral RNA species analogous to the 6S-RNA found in Q^{β} phage-infected E. coli (Banerjee et al., 1969b) or perhaps a mixture of two or more species. The precise nature of this template RNA has not been determined although the results of Figure 19 indicate that part at least is a virus-induced RNA species.

The lack of specificity of the partly purified soluble CMV-induced RNA polymerase for CMV-RNA could be due to the presence of enzyme activities or other components inhibitory to the replication process in vitro or to the lack of the appropriate factors which govern the specificity of the RNA polymerase for the viral RNA in vivo (Eikhom and Spiegleman, 1967; Stavis and August, 1970). Support for the latter hypothesis was provided by the utilisation of several RNA templates by the CMV-induced RNA polymerase while the size of the RNA polymerase extracted from the particulate fractions can vary (see previous chapter), suggesting a multi-component enzyme system. However, of considerable importance is the fact that the $Q\beta$ phage-induced RNA replicase lacked dependence on added $Q\beta$-RNA when only partly purified (see Pace et al.,

1968; Banerjee et al., 1969b) and showed specificity for its viral RNA template only after considerable purification. Thus, further investigations into the specificity and nature of the CMV-induced RNA polymerase require the use of more highly purified RNA polymerase preparations.

TABLE 12. EFFECT OF YEAST RNA AND POLYRIBONUCLEOTIDES ON SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBER COTYLEDONS

RNA or polynucleotide added (250 μg/assay)	Specific enzymatic activity									
	Infected plants								Healthy plants	
	ATP-^{32}P		CTP-^{32}P		UTP-^{32}P		GTP-^{32}P		GTP-^{32}P	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
None	9.6	0.1	12.4	1.1	12.2	1.5	12.1	0.0	0.1	0.0
Yeast RNA	9.0	4.1	8.3	4.2	9.6	5.3	9.1	0.1	0.0	0.1
PolyA	3.2	4.1	3.2	3.4	8.1	4.4	3.1	0.9	0.0	0.0
PolyU	9.3	3.4	1.0	0.4	0.2	0.1	1.4	0.1	0.0	0.0
PolyG	1.2	0.0	0.1	0.0	0.1	3.1	25.5	32.3	26.2	29.3
PolyC	0.4	0.3	1.3	0.5	0.2	1.0	124.5	145.1	0.0	0.0

Table 12 (Cont'd.)

(a) Assays were carried out as described in Methods using the labelled substrate as indicated and in the presence of the other three unlabelled nucleoside triphosphates but the $MgSO_4$ concentration was modified to 5 mM (2.0 µmoles $MgSO_4$/assay) to avoid precipitation of polyA in the assay medium.

(b) As for (a) but the unlabelled nucleoside triphosphates were omitted.

Specific enzymatic activity. µµmoles of labelled nucleoside triphosphate incorporated into acid-insoluble material per min. per milligram of plant protein.

TABLE 13. NEAREST-NEIGHBOUR ANALYSIS OF THE PRODUCTS FORMED BOTH IN THE PRESENCE OR ABSENCE OF ADDED POLYC, YEAST RNA OR TYMV-RNA

RNA or polynucleotide added (µg/assay)		Isolated 2'(3')-mononucleotides (% of total radioactivity)			
		AMP	CMP	GMP	UMP
None		23.9	30.4	26.9	18.8
Yeast RNA	75 µg	27.1	26.6	24.5	21.8
	250 µg	28.5	24.4	23.2	23.9
	1000 µg	28.4	23.1	23.4	25.2
TYMV-RNA	75 µg	21.2	18.1	36.5	23.2
	250 µg	20.8	16.4	39.7	23.1
	1000 µg	20.8	17.5	41.0	20.7
PolyC	75 µg	3.3	3.3	89.6	3.8
	250 µg	3.0	2.3	92.3	2.4

Products were formed in the assay medium described in Methods using GTP-^{32}P as the labelled substrate in the presence of the three unlabelled triphosphates. RNA or polynucleotides were added to this medium as indicated. Each estimation was carried out twice on each of two partly purified soluble RNA polymerase preparations (see also Methods). Values quoted are the mean percent of the total radioactivity, the standard error of the mean never being greater than ± 0.8.

TABLE 14. PROPERTIES OF THE ASSAY SYSTEM USED FOR THE MEASUREMENT OF SOLUBLE RNA POLYMERASE ACTIVITY INDEPENDENT OF ADDED RNA

System[a]	Specific enzymatic activity	
	Healthy plants	Infected plants
Complete	0.1	15.4
+ yeast RNA (250 μg)	0.2	14.4
+ polyC (250 μg)	0.0	140.2
+ RNase (1 μg) during assay	0.0	0.0
+ RNase (10 μg) after assay[b]	0.1	14.3
+ Pi (5 μmoles)	0.0	13.3
+ PP_i (5 μmoles)	0.0	0.0
- actinomycin D	0.2	15.3
+ DNase (25 μg)	0.2	12.5
+ native salmon DNA (200 μg)[c]	0.6	13.7
+ denatured salmon DNA[c]	0.2	3.1

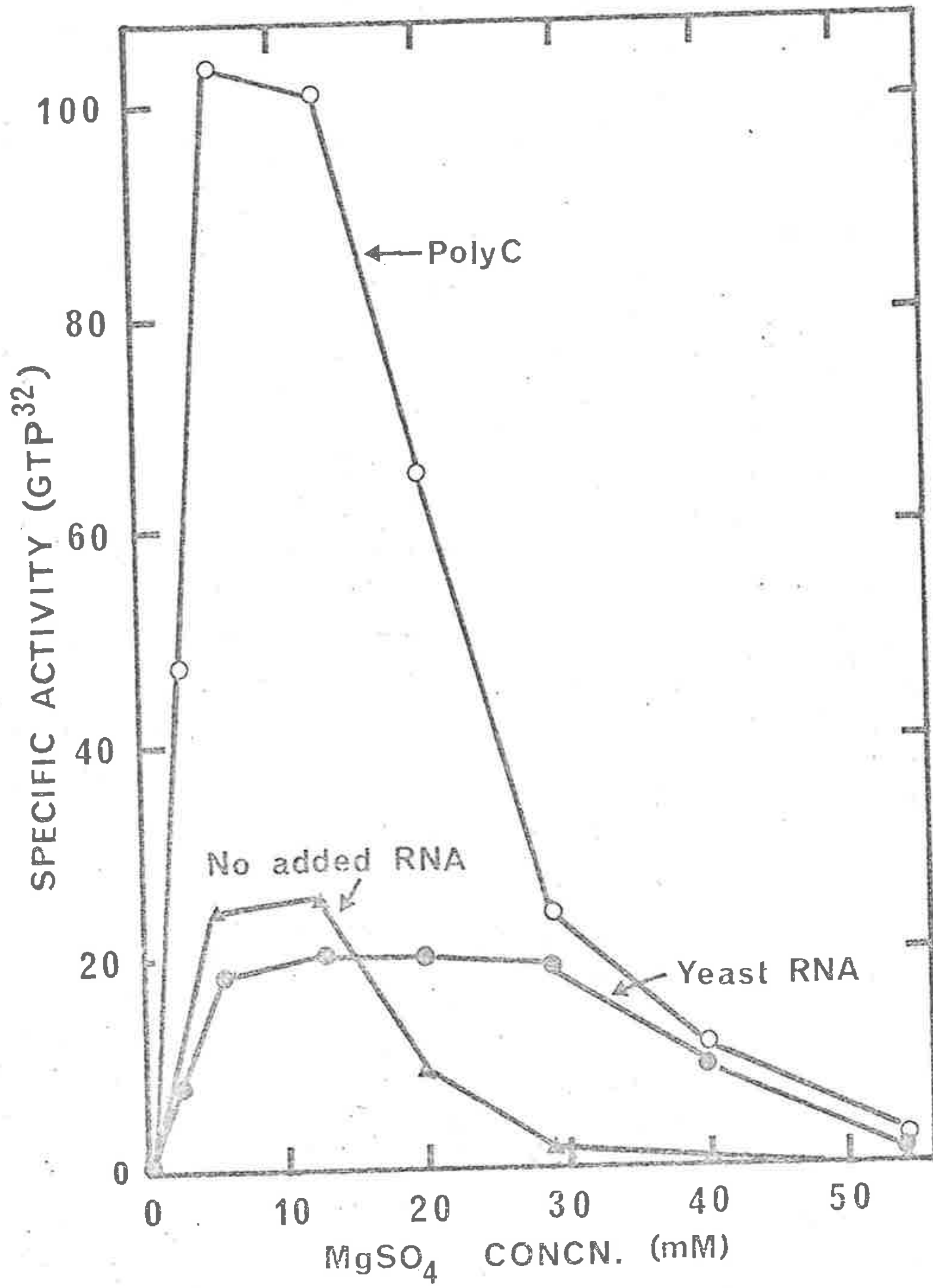
[a] Assays were carried out as described in Methods, with GTP-^{32}P as the labelled substrate and with the alterations listed.

[b] After the reaction was completed, pancreatic ribonuclease (10 μg) and 1.0 mg of unlabelled GTP were added and the assay incubated for a further 30 min. at 37°.

[c] Comparable activities were obtained when actinomycin D (2.5 μg) was omitted from these assays.

FIGURE 17. THE EFFECT OF $MgSO_4$ CONCENTRATION ON SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE ACTIVITY

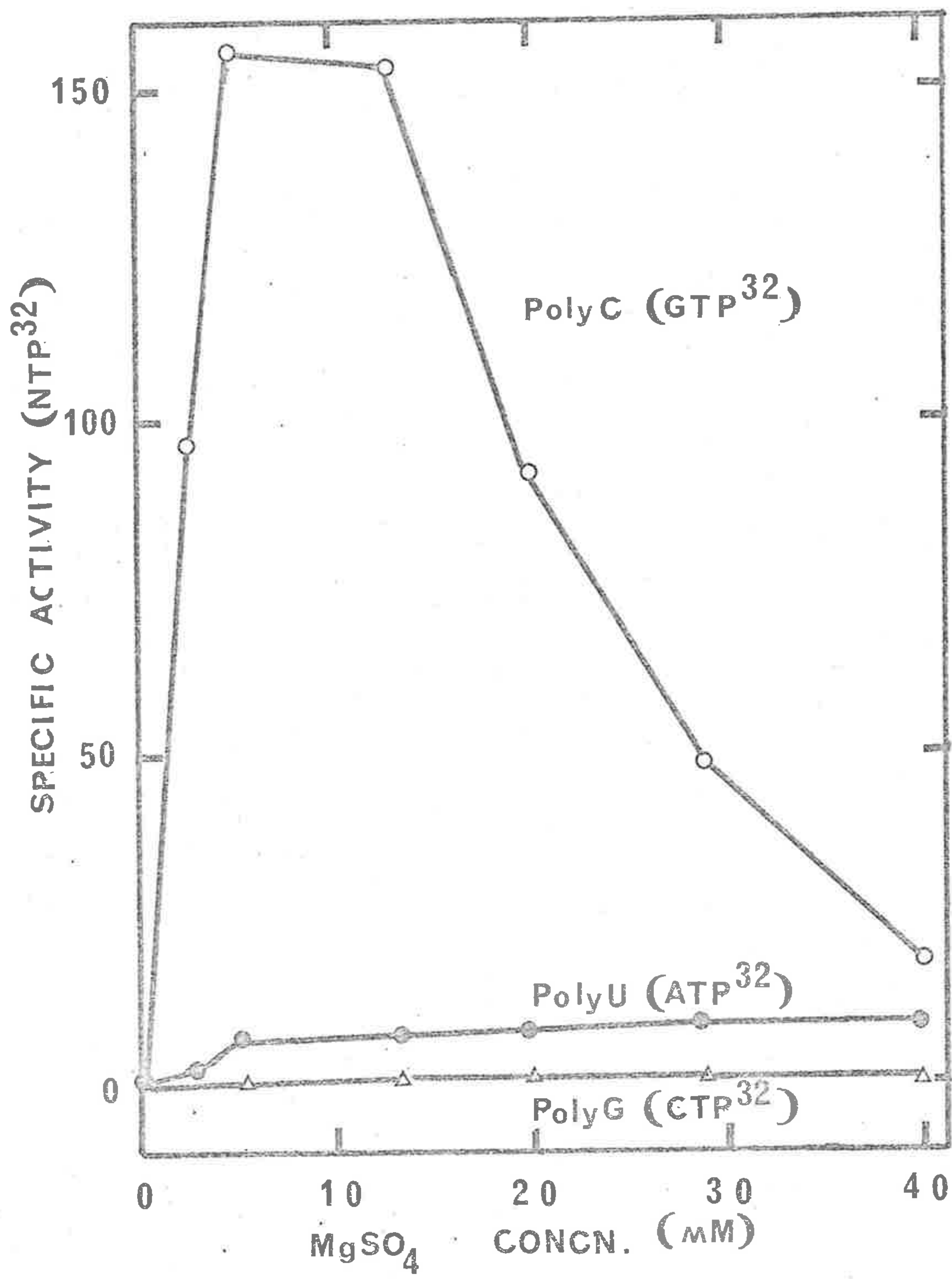
The partly purified soluble RNA polymerase preparation was prepared and assayed as described in Methods at varying concentrations of $MgSO_4$ (0 - 54 mM $MgSO_4$; 0 - 20 μmoles/assay) and in the absence of added RNA, in the presence of 250 μg/assay of yeast RNA and in the presence of 250 μg/assay of polyC.



PolyC
No added RNA
Yeast RNA
SPECIFIC ACTIVITY (GTP^{32})
100
80
60
40
20
0
0
10
20
30
40
50
$MgSO_4$ CONCN. (mM)

FIGURE 18. THE EFFECT OF $MgSO_4$ CONCENTRATION ON UTILISATION OF POLY-C, POLY-U AND POLY-G TEMPLATES BY THE SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE

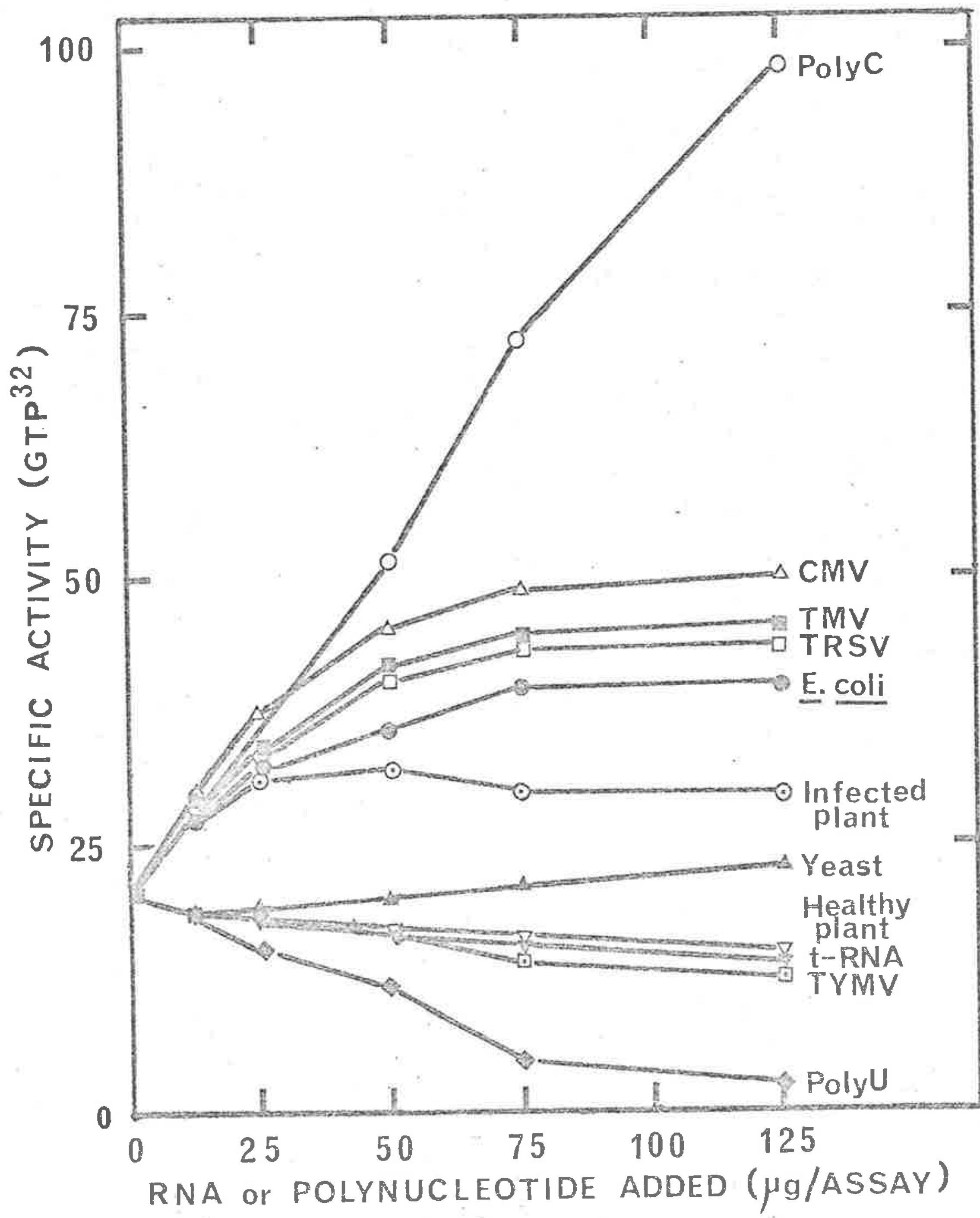
The partly purified soluble RNA polymerase was assayed as described in Methods at varying concentrations of $MgSO_4$ (0 - 40 mM $MgSO_4$; 0 - 15 µmoles/assay), in the presence of 250 µg of polyC, polyG or polyU and all four nucleoside triphosphates. The labelled nucleoside triphosphate used in each assay is indicated. (Specific activity of the RNA polymerase activity measured in the presence of 1.0 mg of yeast RNA was 19.1, 21.0 and 22.1 for GTP-^{32}P, ATP-^{32}P and CTP-^{32}P substrates respectively, at 5 - 20 mM $MgSO_4$).



150
100
50
0
SPECIFIC ACTIVITY (NTP32)
PolyC (GTP32)
PolyU (ATP32)
PolyG (CTP32)
0
10
20
30
40
MgSO4 CONCN. (mM)

FIGURE 19. SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE ACTIVITY IN THE PRESENCE OF VARIOUS RNA AND POLYNUCLEOTIDE SAMPLES

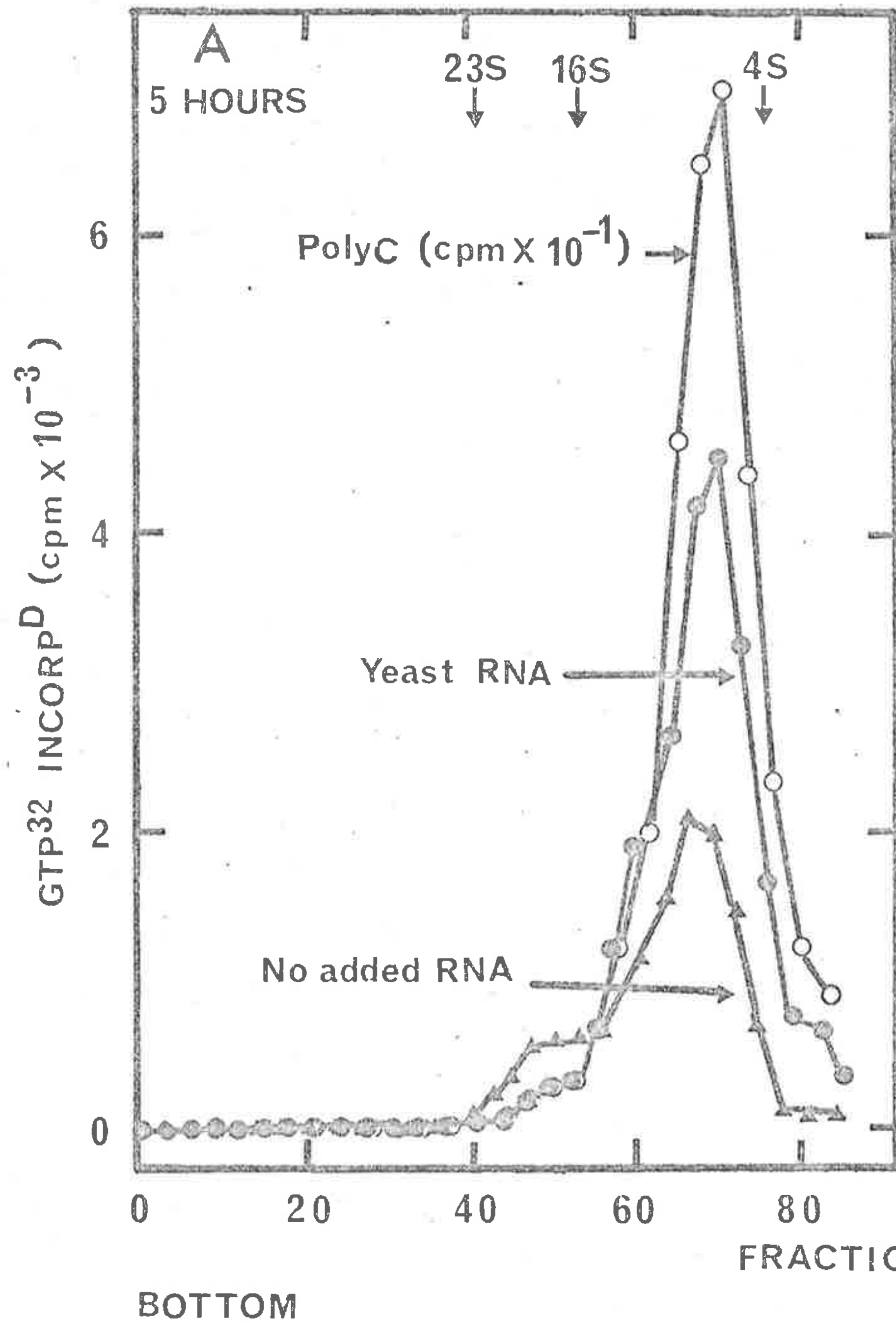
The soluble partly purified RNA polymerase was assayed as described in Methods in the presence of varying amounts of different RNA and polynucleotide species as indicated.



SPECIFIC ACTIVITY (GTP^{32})
100
75
50
25
0
PolyC
CMV
TMV
TRSV
E. coli
Infected plant
Yeast
Healthy plant
t-RNA
TYMV
PolyU
0
25
50
75
100
125
RNA or POLYNUCLEOTIDE ADDED (μg/ASSAY)

FIGURE 20. SEDIMENTATION ANALYSIS OF THE SOLUBLE CUCUMBER MOSAIC VIRUS-INDUCED RNA POLYMERASE

Partly purified soluble enzyme (3.0 mg of protein and 0.15 mg of RNA in 0.6 ml; see Methods) was centrifuged through a 11.6 ml gradient of 5 - 20% sucrose in 0.1 M NH_4Cl, 90 mM 2-mercaptoethanol, 5 mM $MgSO_4$, 50 mM Tris-HCl buffer pH 8.5, in a Beckman SW41 rotor at 36,000 rpm for 5 hours (Experiment A) or 17 hours (Experiment B). Three drop fractions (0.13 ml) were collected from the bottom of each tube and every third fraction assayed for RNA polymerase activity (see Methods) in the absence of added RNA, in the presence of 1.0 mg of yeast RNA and in the presence of 100 μg of polyC. Results for the RNA polymerase activity in the presence of polyC are expressed as one-tenth of their actual value (i.e. cpm x 10^{-1}). Recovery of RNA polymerase activity using yeast RNA or polyC as template was 110% after both 5 and 17 hours centrifugation. Recovery of the RNA polymerase activity assayed in the absence of added RNA or polynucleotide was 45% after 5 hours centrifugation and 4% after 17 hours. During experiment A, marker E. coli RNA (23S, 16S and 4S; 5 mg in 0.6 ml) was centrifuged at the same time through


A
5 HOURS
23S
16S
4S
PolyC (cpm X 10^{-1})
Yeast RNA
No added RNA
GTP^{32} INCORP^{D} (cpm x 10^{-3})
0
2
4
6
0
20
40
60
80
BOTTOM

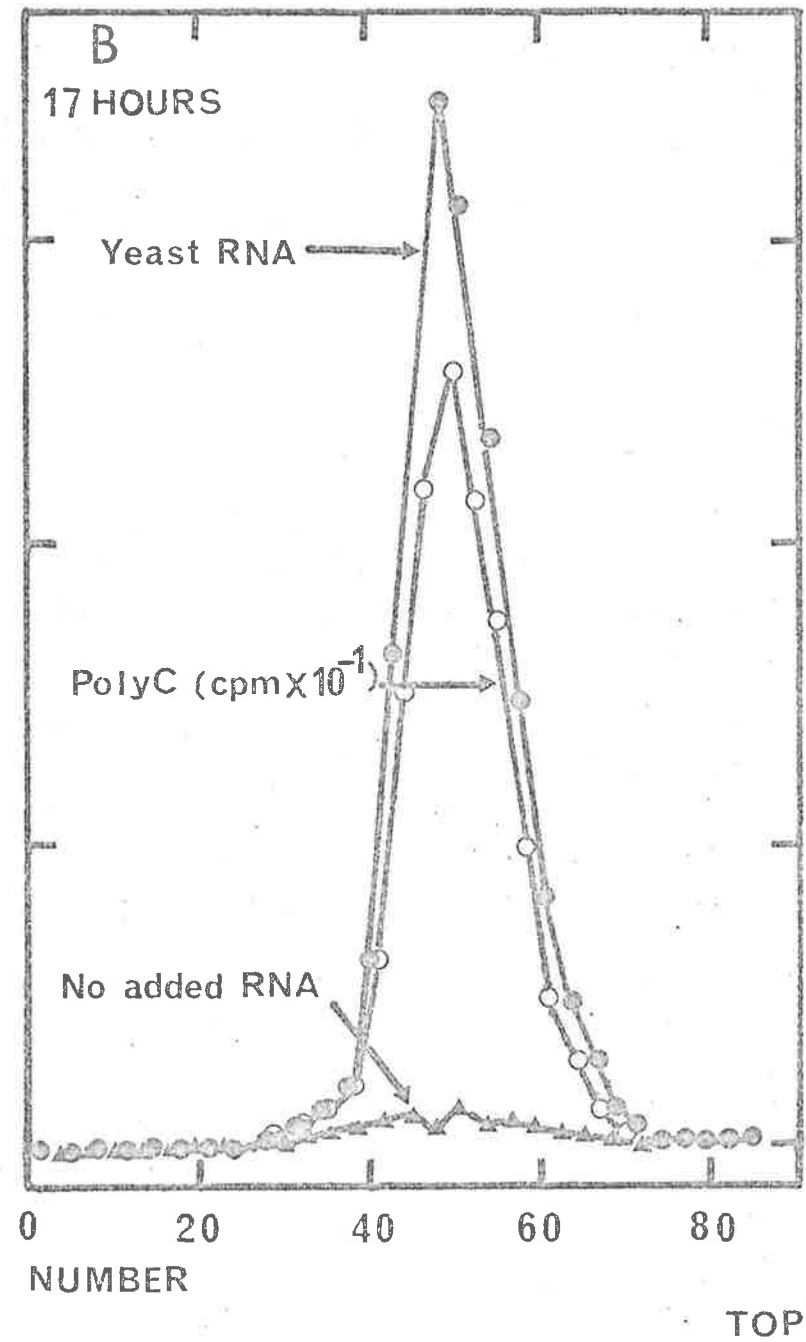

B
17 HOURS
Yeast RNA
PolyC (cpm X 10^{-1})
No added RNA
0
20
40
60
80
FRACTION NUMBER
TOP

FIGURE 20 (Cont'd.)

an identical gradient to that described above. Fractions were collected and the optical density profile at 260 mμ of the RNA was determined but only the positions of the 23S, 16S and 4S peaks are shown.

GENERAL DISCUSSION

GENERAL DISCUSSION

The results presented in this thesis have allowed a partial characterisation of the virus-induced RNA polymerase found both in soluble and particulate extracts (see Figure 21 for summary of extracts used) of cucumber cotyledons infected with cucumber mosaic virus. The basic assumption of the work described is that the CMV-induced RNA polymerase under investigation is responsible for the *in vivo* replication of CMV-RNA, but no evidence presented here directly confirms this assumption. Also no evidence has been found which indicates that the enzyme studied is *viral-coded* and is not an induced host plant enzyme.

1. *Soluble RNA polymerase*. The results presented have indicated that the soluble virus-induced RNA polymerase (mol. wt. 123,000) found in partly purified extracts infected with CMV was not associated with viral-RNA template. The RNA polymerase was found to copy several natural RNA templates and polyC by complementary base pairing and to produce a pancreatic ribonuclease-resistant and presumably double-stranded RNA with yeast RNA and CMV-RNA. A marked preference by the soluble RNA polymerase for polyC over polyA, polyU and polyG, is of considerable interest as the purified Qβ phage-induced RNA polymerase also

shows this marked preference for polyC and heteropolynucleotides containing cytidylate (Hori *et al*., 1967; Eikhom and Spiegleman, 1967). Indeed two puzzling features of the CMV-induced RNA polymerase are its lack of specificity for CMV-RNA template and the observation that negligible enzyme activity is detected in extracts of CMV-infected plants other than cucumbers or rockmelons. The lack of specificity of the partly purified CMV-induced RNA polymerase for CMV-RNA could be due to the presence of enzyme activities or other components inhibitory to the replication process *in vitro* or to the lack of the appropriate factors which govern the specificity of the RNA polymerase for the viral RNA template *in vivo* (Eikhom and Spiegleman, 1967; Stavis and August, 1970). Support for this latter hypothesis was provided by the utilisation of several RNA species by the CMV-induced RNA polymerase while the size of the RNA polymerase from particulate extracts can vary (see below, Chapter 7) suggesting a multicomponent enzyme system. However, of considerable importance is the fact that Q_{β} phage-induced RNA replicase lacked dependence on Q_{β}-RNA when only partly purified (see Pace *et al*., 1968; Banerjee *et al*., 1969b) and showed specificity for viral RNA template only after considerable purification. Thus further investigation into the specificity and nature of soluble CMV-induced RNA polymerase needs to be carried out using more highly purified RNA polymerase preparations.

2. Particulate RNA polymerase. Several plant virus-infected particulate plant extracts (see Introduction and Chapter 5) contain a presumed enzyme-viral RNA complex which is probably involved in the *in vivo* replication process of the RNA viruses. It was postulated from the work described in this thesis, that particulate fractions isolated from CMV-infected cucumbers also contain such a complex, although no conclusive proof that these fractions produce viral RNA *in vitro* has been demonstrated. An interesting development has been the solubilisation of an RNA polymerase activity from particulate fractions of CMV-infected cucumbers by incubation with $MgSO_4$ at 37° or by freezing and thawing. The general properties of these solubilised enzymes are similar if not identical to the RNA polymerase normally found in the soluble phase, except for the reproducible observation that the enzyme released by freezing and thawing had a molecular weight of 150,000 (about 30,000 higher than the other soluble RNA polymerase(s)). This indicates that we have retained, during solubilisation by freezing and thawing, an extra component(s) which was lacking from the other soluble forms of the enzyme. It is considered possibly, therefore, that the various types of RNA polymerase found in soluble and particulate fractions of infected cucumbers, infected with CMV, represent various incomplete forms of the presumed RNA polymerase responsible for replication of the plant viral RNA *in vivo*. Thus,

although the actual cellular location of the _complete_, presumed multicomponent CMV-induced RNA polymerase cannot be defined as yet, it is most likely (from the results presented) present in the particulate fraction. Further investigation of this fraction is being carried out to isolate the _complete_ CMV-induced RNA polymerase.

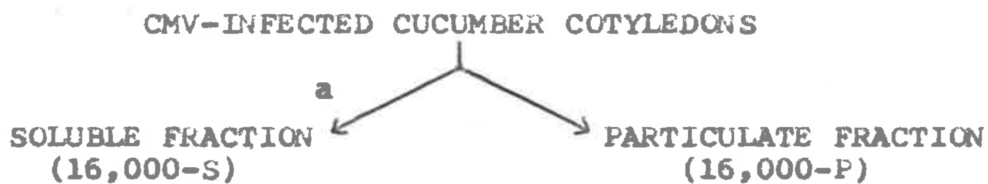
The long term goals of this work were to achieve both viral RNA synthesis _in vitro_ and a detailed understanding of the enzymology of plant viral RNA replication.

FIGURE 21. SUMMARY OF RNA POLYMERASE ACTIVITIES DETECTED IN SOLUBLE AND PARTICULATE EXTRACTS OF CUCUMBER MOSAIC VIRUS-INFECTED CUCUMBER COTYLEDONS

[a] This step usually involved the use of 50% saturated $(NH_4)_2SO_4$ to remove plant ribonucleases (see EXTRACTION METHOD C, Chapter 2).

[b] Activities expressed in the presence of 5 - 13 mM $MgSO_4$ in the assay medium (see Chapter 8).

[c] The properties mentioned are found with the RNA polymerase normally found in the soluble phase and are similar if not identical to the RNA polymerase solubilised from particulate fractions by 70 mM $MgSO_4$ at 37°.



Viral-induced RNA polymerase.

Viral-induced RNA polymerase.

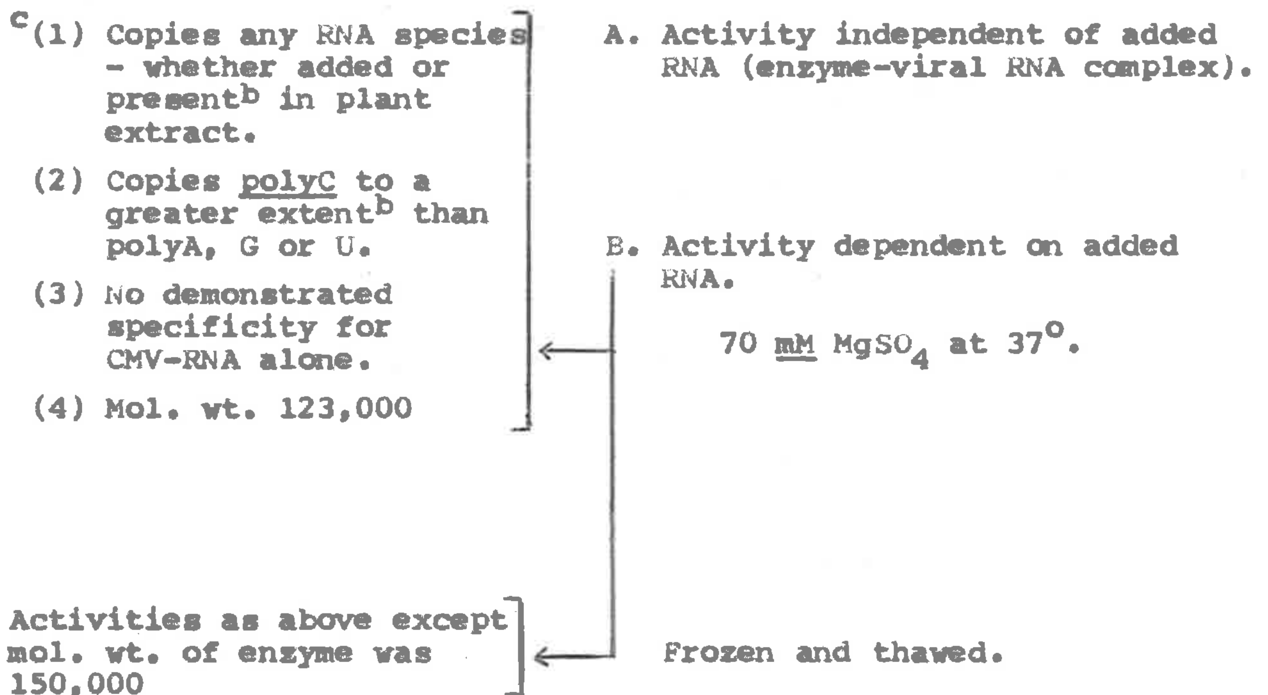


FIGURE 21

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