CUCUMBER MOSAIC VIRUS-INDUCED PARTICULATE RNA REPLICASE

A thesis submitted to the University of Adelaide, South Australia, for the degree of Doctor of Philosophy,

by

DALIP SINGH GILL, M.Sc.(Hons.) Biochemistry
(Punjab Agricultural University, India)

Adelaide University Centre for Gene Technology,
Department of Biochemistry,
University of Adelaide, Adelaide, South Australia.

March, 1983.

CONTENTS

CHAPTER TWO SOLUBILIZATION OF CMV-INDUCED RNA REPLICASE FROM THE PARTICULATE FRACTION

SUMMARY

The particulate RNA-dependent RNA polymerase (RNA replicase) induced in cucumber plants by infection with cucumber mosaic virus (CMV) has been solubilized, purified and characterized. The purified CMV-induced RNA replicase was investigated to determine whether the enzyme contains any full length translation product of the CMV genomal RNAs. The products synthesized by both the crude and purified RNA replicase were also characterized.

The RNA replicase was solubilized by incubation of the washed particulate fraction of CMV-infected plants in buffered 150 mM $MgSO_4$ at 37°C for five min. The effect of $MgSO_4$ in solubilization of the CMV-induced RNA replicase was compared with twelve other salts and three different detergents. Both magnesium and sulphate were required for the maximum release of RNA replicase. Solubilization of RNA replicase with $MgSO_4$ was stimulated with increase in temperature up to 40°C. At 37°C in the presence of 150 mM $MgSO_4$, solubilization of RNA replicase was spontaneous. No such enzyme activity was solubilized from healthy cucumber plants when treated under identical conditions.

The solubilized RNA replicase was purified by polyethyleneglycol precipitation and phosphocellulose column chromatography. Enzyme was further purified by column chromatography either on poly(C)-cellulose or alternatively on heparin-Sepharose or on Cibacron blue F3GA agarose. The specific activity of the poly(C)-cellulose step enzyme of 24 and 907 units/mg protein (1 unit of enzyme activity equals 1 nmol of GMP incorporated into RNA per min at 37°C) with CMV RNA and poly(C) as templates, respectively, was similar

to that of the 10,000-fold purified soluble enzyme. Polypeptide composition of the enzyme fractions at various steps of purification was analysed by SDS-polyacrylamide gel electrophoresis. Seven major polypeptides (of M_r 28,000 to 110,000) along with trace amounts of several other polypeptides were present in the poly(C)-cellulose step enzyme. When heparin-Sepharose step enzyme was further purified by stepwise chromatography on poly(C)-cellulose, followed by Cibacron blue F3GA agarose, a major polypeptide of M_r 100,000 co-purified with RNA replicase activity. Results indicated that this polypeptide is the catalytic subunit of the RNA replicase whereas other six polypeptides may or may not be the components of the enzyme. The polypeptide composition and various kinetic parameters of purified CMV-induced particulate RNA replicase studied were similar to that of the purified soluble CMV RNA replicase reported by Kumarasamy and Symons (*Virology* 96, 622-632, 1979).

Out of the seven major polypeptides present in the poly(C)-cellulose step enzyme, three (with M_r 110,000; 100,000 and 35,000) were found unique to CMV infection as they were not present in similarly purified control extracts from healthy plants. The possibility that these three polypeptides (M_r 110,000; 100,000 and 35,000), which had approximately the same electrophoretic mobilities on SDS-polyacrylamide gels as the *in vitro* translation products of CMV RNAs 2, 1 and 3, respectively, were the gene products of three genomal RNAs of CMV (RNAs 1, 2 and 3) was investigated. The full length translation products of the three largest RNAs of the strain P, Q and T of CMV were

detectably different in mobility from each other, whereas the polypeptide patterns of RNA replicase induced by the corresponding strains of CMV were indistinguishable from each other on SDS-polyacrylamide slab gel electrophoresis. It was concluded that none of these proteins (M_r 110,000; 100,000 and 35,000) of RNA replicase was a translation product of any of the CMV RNAs. The comparison of peptide maps of the translation products of CMV RNAs 1, 2 and 3 with the enzyme polypeptides (of M_r 110,000; 100,000; and 35,000), using CNBr cleavage and partial digestion with *Staphylococcus aureus* V-8 protease, confirmed the above conclusion.

The purified CMV-induced particulate RNA replicase showed little template specificity. It copied brome mosaic virus RNA, alalfa mosaic virus RNA, cowpea mosaic virus RNA and even yeast rRNA, in addition to CMV RNA when supplied as templates. Purified enzyme also accepted avocado sun-blotch viroid and the virusoid of subterranean clover mottle virus as templates to synthesize a small fraction of full length transcripts. Because of this lack of template specificity and absence of viral gene products in the purified CMV-induced RNA replicase and of the long held view that the *in vivo* replication of CMV RNAs occurs in the particulate fraction of infected plants (May *et al.*, *Virology* 41, 653-664, 1970), the properties of RNA replicase in the washed particulate fraction, which was the starting material for the solubilization of the particulate enzyme, were investigated. When this particulate fraction was incubated in the presence of α-^{32}P-NTPs without any added template, the label was incorporated into full length CMV RNAs. The particulate fraction did not respond to exogenous

RNAs. As there was no further increase in the incorporation of α-^{32}P-NTPs after 10 min of incubation and also incorporation was only slightly inhibited by 10 µg/ml heparin, it was concluded that in the particulate fraction RNA replicase was bound to CMV RNAs with pre-initiated nascent chains of transcripts, which were completed on further incubation in the *in vitro* RNA replicase assay rather than re-initiating new ones.

In conclusion, our current model of the membrane bound RNA replicase in CMV-infected cucumber seedlings envisages a virus-induced host protein of M_r 100,000 as the catalytic subunit together with the translation products of CMV RNAs 1, 2 and 3 presumed to be present in the membrane bound RNA replication complex to ensure the specificity and regulate the whole process.

STATEMENT

This thesis contains no material which has been accepted for the award of any degree or diploma in any University. However, the work described in Chapter Six was done in collaboration with Mr K.H.J. Gordon, who carried out *in vitro* translations of CMV RNAs, electroelution and *S.aureus* protease digestions of radiolabelled proteins. To the best of my knowledge and belief, this thesis contains no material published or written by any other person, except where due reference is made in the text.

Signed

DALIP SINGH GILL

ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for the opportunity to undertake my Ph.D. studies at the Department of Biochemistry, University of Adelaide.

I express my sincere thanks to Dr. R.H. Symons for his ever available advice, encouragement and criticism during the course of investigations and preparation of the manuscript.

I wish to thank the following people for their helpful discussions and advice on several occasions: Dr. G.E. Bruening, Dr. R. Kumarasamy, Dr. P.L. Molloy and Dr. P. Palukaitis. I am grateful to Mr. K.H.J. Gordon for his willing co-operation and to the other members of our laboratory for their encouragement and useful suggestions.

I also wish to thank Dr. R.I.B. Francki, Department of Plant Pathology, University of Adelaide, for use of glass house facilities and providing all virus strains and plants used throughout this work.

I thank to Miss J.L. Rosey and L. Waters for technical assistance and preparing diagrams and photographs for this thesis. Thanks are due to Mrs. J. Brooker for typing thesis and Dr. G. Srivastava for his help in proof reading it.

Financial assistance in the form of the University Research Grant Scholarship from the University of Adelaide and in the form of the grants from the Rural Credits Development Fund of the Reserve Bank of Australia are thankfully acknowledged.

ABBREVIATIONS

AMV	alfalfa mosaic virus
ASBV	avocado sunblotch viroid
BMV	brome mosaic virus
CMV	cucumber mosaic virus
CPMV	cowpea mosaic virus
PSTV	potato spindle tuber viroid
PSV	peanut stunt virus
SCMoV	subterranean clover mottle virus
TAV	tomato aspermy virus
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
TYMV	turnip yellow mosaic virus
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Ci	curie
cpm	counts per minute
CTP	cytidine-5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleside-5'-triphosphate
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetate
g	gram
GTP	guanosine-5'-triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonate

mA	millamperes
MOPS	3-(N-morpholino)ethanesulphonic acid
M_r	molecular weight
mRNA	messenger ribonucleic acid
NTP	nucleoside-5'-triphosphate
PEG	polyethyleneglycol
Poly(A)	polyriboadenylic acid
Poly(C)	polyribocytidylic acid
Poly(G)	polyriboguanylic acid
Poly(U)	polyribouridylic acid
POPOP	1,4-bis[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
RFs	replicative forms
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
tRNA	transfer ribonucleic acid
Tris	tris(hydroxymethyl)aminomethane
UTP	uridine-5'-triphosphate
Oligo(U)	oligoribouridylic acid
PVP	polyvinylpyrrolidone
V_f	final volume
v/v	volume by volume
w/v	weight by volume

CHAPTER ONE

GENERAL INTRODUCTION AND SCOPE OF THE PROJECT

Section 1.1 RNA REPLICATION

The fundamental mechanism of RNA replication is similar to that of DNA replication. Synthesis of new complementary RNA strands proceeds in the 5' → 3' direction by sequential addition of nucleoside monophosphates in an order dictated by the Watson-Crick base pairing rule on the parental RNA templates. No cellular RNA molecules have been reported so far to serve as templates for the *in vivo* synthesis of new RNA strands. Replication of genomes of RNA viruses (viroids?) demands the involvement of a new enzyme as an intermediate in the cycle between parental and progeny genomes. The nature of this enzyme depends on the type of the virion RNA.

Baltimore (1971) recognized six classes of viruses (two of DNA viruses and four of RNA viruses) depending upon the type of virion nucleic acid and the route of information transfer from virion nucleic acid to the viral mRNA found in the infected cells. The virion single stranded RNA (ssRNA) or single stranded DNA (ssDNA) of the same polarity as that of mRNA is considered (+)RNA or (+)DNA, respectively. A nucleic acid complementary to the mRNA is (-)DNA or (-)RNA. Double stranded DNA (dsDNA) or double stranded RNA (dsRNA) is the (±)DNA or (±)RNA, respectively. The four classes of RNA viruses are:

I. (±)RNA in virions

II. (+)RNA in virions, new (+)RNA transcribed from (-)RNA *in vivo*

III. (-)RNA in virions

IV. (+)RNA in virions, new (-)RNA transcribed from (±)DNA *in vivo*.

The viruses of class I, III and IV carry in their

virions molecules of a nucleic acid polymerase which, on infection, allows the growth cycle to start. In viruses of class I and III, i.e., with (±)RNA and (-)RNA genomes, these polymerase molecules are RNA-dependent RNA polymerase and described as virion RNA transcriptase and virion mRNA polymerase, respectively. Several animal and plant viruses with (±)RNA and (-)RNA genomes are known. The polymerase carried by viruses of class IV is an RNA-dependent DNA polymerase, also called reverse transcriptase, which copies viral RNA to double stranded DNA, which in turn is integrated into host DNA, from where viral RNA or mRNA is transcribed by host enzymes. No class IV plant virus has been described.

The situation with class II viruses i.e., with (+)RNA genomes is different as their virions do not carry any polymerase activity. Consistent with the finding of dsRNA in the cells or tissues infected with these viruses, the RNA synthesizing enzymes which generate RNA complementary to viral RNA and then progeny of viral RNA molecules are RNA-dependent RNA polymerases, also called RNA replicases. The most important feature common to all the viruses of class II described here is the induction of RNA replicase activity in the virus-infected cells or tissues. Although a similar RNA-dependent RNA polymerase activity is detected in some healthy plants at very low levels, there is a marked increase in the RNA replicase activity upon viral infection (Table 1.1) (Astier-Manifacier and Cornuet, 1971, 1978, 1981; Duda *et al.*, 1973; Bol *et al.*, 1976; Fraenkel-Conrat, 1976, 1979; Stussi-Garaud *et al.*, 1977; Romaine and Zaitlin, 1978; Clerx and Bol, 1978; Ikegami and Fraenkel-Conrat, 1978a; Chifflot *et al.*,

TABLE 1.1

PROPERTIES OF PLANT VIRUS RNA REPLICASES

Virus	Soluble or Particulate Form	Stimulation Upon Virus Infection	Method of Enzyme Solubilization	Enzyme Subunits		Nature of the Products Synthesized on		Template Specificity Towards the RNA of Infecting Virus
				Host Specified	Virus Specified	Endogenous Template	Exogenous Template	
CPMV[a]	Particulate	6 - 20 times	80% with Mg^{2+} deficient buffers	Purified enzyme contained a single polypeptide M_r 130,000	None in the purified enzyme fraction	Small and heterogenous in size	-	Controversial
			20% with Triton X-100	-	-	Full length dsRFs	-	-
BMV[b]	Particulate	50 times	Dodecyl-β-D-maltoside, Nonidet P-40, Triton X-100	Many host proteins were present	A viral polypeptide (M_r 110,000) was present in partially purified enzyme fraction	Full length dsRFs	Full length dsRFs	More specific for BMV RNAs
AMV[c]	Soluble & Particulate	2 - 3 times	Lubrol, Mg^{2+}-deficient buffer	Several	None	-	Small and heterogenous in size but of (+) and (-) type	Non-specific
TYMV[d]	Particulate	Several times	Lubrol	Three polypeptides (M_r 30,000; 45,000 and 50,000) in most purified enzyme fraction	A virus specific polypeptide (M_r 120,000) was present in the purified enzyme fraction	-	-	TYMV RNA was preferred template
TMV[e]	Soluble & Particulate	6 - 7 times	Clemsol, Mg^{2+}-deficient buffer, Nonidet P-40	Several	None	-	-	Non-specific but particulate enzyme from tobacco callus was more specific for TMV RNA

TABLE 1.1 (Cont!d..)

a. Zabel *et al.*, 1976; 1979; Dorssers *et al.*, 1981; 1982; 1983.

b. Hardy *et al.*, 1979; Bujarski *et al.*, 1982; Hall *et al.*, 1982.

c. Weening and Bol, 1975; Bol *et al.*, 1976; Chifflot *et al.*, 1980; Linthorst *et al.*, 1980; Linthorst, 1982.

d. Mouches *et al.*, 1974; 1976; 1981.

e. Zaitlin *et al.*, 1973; Sela and Hauschner, 1975; White and Murakishi, 1977; Romaine and Zaitlin, 1978.

1980; Duda, 1979).

The RNA viruses of class II include RNA bacteriophages, picornaviruses, togaviruses and most of the RNA plant viruses. The RNA plant viruses of class II readily fall into two groups.

Group I: Viruses with undivided genomes, also called monopartite RNA viruses; e.g., TMV and TYMV.

Group II: Viruses with divided genomes or multipartite RNA viruses (Jaspars, 1974).

The viruses of the Group II are unique to plant systems. Depending upon the number of elements of the genome, they are described either as a bipartite (Bruening, 1977) or a tripartite genome (Van Vloten-Doting and Jaspars, 1977). The best studied viruses with bipartite genomes are CPMV and TRV. The viruses containing a tripartite genome are further classified into two subgroups (Van Vloten-Doting, 1976); the coat protein independent [e.g., CMV, TAV, PSV (cucumoviruses) and BMV, CCMV (bromoviruses)] and coat protein dependent (e.g., alfalfa mosaic virus group). A brief review of RNA replicases of a few viruses of class II, which are the best studied among their groups and RNA-dependent RNA polymerases of uninfected plants is given below. The RNA replicase induced by cucumber mosaic virus (CMV) is discussed in subsequent chapters.

A. THE RNA REPLICASES OF (+)RNA VIRUSES

i. Qβ RNA Replicase

The Qβ RNA replicase was first found by Haruna and Spiegelman (1965) in *E. coli* cells infected with bacteriophage

Qβ. Since then it has been studied extensively in a large number of laboratories and has become the best system for the study of *in vitro* RNA replication. The earlier work on Qβ RNA replicase was reviewed in detail by Kamen (1975) and more recent findings have been extensively reviewed by Blumenthal and Carmichael (1979). A brief account of its structure and properties is given below.

I. Structure of Qβ RNA replicase:

The Qβ RNA replicase, purified to homogeneity, consists of four non-identical subunits, only one of which (M_r 65,000) is the gene product of Qβ RNA. The other three, present in the uninfected *E. coli* cells, are 30S ribosomal protein S_1 (M_r 70,000) and the protein elongation factors EF-Tu and EF-Ts (M_r 45,000 and 35,000, respectively). Their functional roles in Qβ RNA replicase and in uninfected *E. coli* cells have been reviewed by Blumenthal and Carmichael (1979). The enzyme complex has an aggregate molecular weight of 215,000 and can be dissociated reversibly into two tight subcomplexes consisting of S_1·phage replicase protein and of EF-Tu·Ts.

Qβ RNA replicase normally replicates Qβ RNA [(+) and (-) strands], "6S" RNAs found *in vivo* in Qβ infected *E. coli* cells, Qβ variant RNAs synthesized *in vitro* and cytidylate rich synthetic polymers. The *in vitro* replication of (+) Qβ RNA requires, in addition to complete Qβ RNA replicase, another host protein called host factor (Franze *et al.*, 1968; Kamen, 1975). Qβ RNA replicase lacking S_1 has been isolated and can transcribe some templates (Kamen *et al.*, 1972).

II. Template recognition by Qβ RNA replicase:

Qβ RNA replicase is remarkable in its ability to replicate selectively Qβ RNA and to ignore most of the other natural RNAs. GTP is the only initiating ribonucleoside triphosphate. In the case of Qβ RNA template, the replicative intermediate is single stranded.

The discrimination between natural RNAs involves the recognition of secondary structure and/or the tertiary structure of the template. The property of template selection resides in the phage-coded subunit of the enzyme. Qβ RNA replicase has the ability of binding tightly to the internal sites of Qβ RNA and "6S" RNAs in the presence of GTP and host factor. One of these binding sites, called S-site, which overlaps with the initiation site of coat protein synthesis on Qβ RNA, is independent of Mg^{2+} and does not appear to be important for the RNA replication process. The binding at another site, called M-site, which is at about 2550 to 2870 nucleotides from the 3'-end of Qβ RNA, requires the presence of Mg^{2+} and is considered to be essential for template activity of the enzyme (Meyer *et al.*, 1981). A model has been proposed by Meyer *et al.* (1981), in which the template specificity is attributed to the binding of internal RNA regions to the RNA replicase, resulting in specific spatial orientation of the RNA by which the inherently weak but essential interaction at the 3'-end is allowed to occur and to lead to the initiation of RNA synthesis.

"6S" RNAs and variant RNAs are recognised, because of their secondary structure, by binding of the Qβ RNA replicase to the internal sites of these RNAs (Mills *et al.*, 1977)

whereas the transcription of synthetic polymers depends on the recognition of their 3'-ends. All variant RNAs are synthesized *de novo* and can exist as single stranded or double stranded RNA (Biebricher *et al.*, 1982).

The template specificity of Qβ replicase has been overcome by replacing Mg^{2+} with Mn^{2+}, by using a primer in the assay medium or by adding a short stretch of C-residues to the 3'-end of the RNA to be copied (Blumenthal and Carmichael, 1979). Blumenthal (1980) has suggested that high template specificity of Qβ replicase is the result of the requirement for high levels of GTP for transcription of restricted templates as different templates require different GTP concentrations for initiation. Because of the novel structure of Qβ RNA replicase and its high degree of template specificity for Qβ RNA, the molecular mechanism of which is still unknown, Qβ RNA replicase is perhaps unique among the well studied replication systems.

ii. Poliovirus RNA Replicase

Poliovirus is the prototype of the group of animal viruses, the picornaviruses, which is understood in most detail. Poliovirus RNA replicase is responsible for the replication of the genomal RNA of poliovirus. The complete sequence of the 7,433 bases long virion RNA has been determined, (Kitamura *et al.*, 1981). The RNA molecule is polyadenylated at 3'-end and is covalently linked to a protein of 22 amino acids, called VPg (genome linked viral protein), at the 5'-end. Poliovirus RNA replicase shares several properties with other animal virus RNA replicases, in the sense that, like bacteriophage RNA replicases, they all show

an interplay of both host- and viral-encoded proteins, with RNA polymerase activity associated with the viral gene product (Brown, 1981; Newman *et al.*, 1979; Clegg *et al.*, 1976; Lund and Scraba, 1979; Traub *et al.*, 1976; Flanegan and Baltimore, 1979).

Two forms of RNA polymerase activity has been detected in the cells infected with poliovirus; polyuridylic acid polymerase and RNA replicase (Flanegan and Baltimore, 1977; 1979; Dasgupta *et al.*, 1979; 1980). Poly(U)-polymerase activity, which sediments at 4 - 5S, copied poly(A) complexed with oligo(U) primer. This enzyme activity was solubilized from the membrane fraction of poliovirus-infected cells and contained a single viral protein p63 (M_r 63,000), also called NCVP4 (Flanegan and Baltimore, 1979). The RNA replicase activity that copied natural RNAs sedimented at 7S and was isolated from the soluble phase of poliovirus-infected cells which also contained poly(U)-polymerase activity. Both the activities purified together from the soluble phase and the purified fraction contained two major viral proteins p63 and NCVP2 (M_r 77,000). Purified enzyme copied a variety of RNAs with some preference for poliovirus RNA (Dasgupta *et al.*, 1979). When soluble RNA replicase was purified in a similar method as described in Dasgupta *et al.* (1979), except that the enzyme was eluted in the final step from poly(U)-Sepharose with a linear salt gradient instead of stepwise elution, only poly(U)-polymerase but no RNA replicase activity was recovered (Dasgupta *et al.*, 1980).

The RNA replicase activity was restored from highly purified poly(U)-polymerase by the addition of the host factor

purified from a high salt ribosomal wash prepared from uninfected HeLa cells (Dasgupta *et al.*, 1980). The function of host factor in uninfected cells is unknown.

Poliovirus RNA replicase purified to apparent homogeneity by an alternative procedure contained a single virus specific protein p63 (NCVP4) and required oligo(U) as primer (Van Dyke and Flanegan, 1980). When the partially purified RNA replicase or highly purified RNA replicase plus host factor were used in the assay containing poliovirus RNA or non-polyadenylated RNAs in the absence of oligo(U), the product synthesized was twice the size of the template. It was suggested that this product was covalently linked to the template which acts as primer as well (Van Dyke *et al.*, 1982; Flanegan *et al.*, 1982).

The virion RNA, (-) strands, and nascent strands of replicative intermediates are covalently linked to VPg at their 5'-end (Flanegan *et al.*, 1977; Lee *et al.*, 1977; Golini *et al.*, 1978; Pettersson *et al.*, 1978). Based on the presence of VPg and the absence of a triphosphate (pppNp) at the 5'-end, it was suggested that VPg acts as a primer for RNA synthesis (Nomoto *et al.*, 1977; Wimmer, 1982). Data indicated (Baron and Baltimore, 1982a; Semler *et al.*, 1982; Wimmer, 1982) that VPg enters in the RNA replication complex in the form of a percursor (possibly P3-9, M_r 12,000) which is cleaved off after initiation of RNA synthesis. VPg maps within the region of the genome coding for replication proteins, and its sequence is included within the coding region for NCVP-1b, the large precursor to NCVP-2 from which poliovirus RNA replicase protein p63 (NCVP-4b), also called

P3-4b, is derived (Kitamura *et al.*, 1981; Baron and Baltimore, 1982a). If VPg plays any role in the initiation of RNA synthesis, it probably does so in one or more of its precursor forms, which is consistent with the role played by the terminal protein of adenovirus DNA (Challberg and Kelly, 1981; Lichy *et al.*, 1981).

iii. Tobacco Mosaic Virus RNA Replicase

Tobacco mosaic virus (TMV) is a monopartite plant virus with its genomic information contained in a single strand RNA molecule. The complete sequence of 6397 nucleotides of TMV RNA has recently been reported (Goelet *et al.*, 1982). TMV RNA encodes for the two major translation products (M_r 125,941 and a readthrough polypeptide of M_r 183,253) (Goelet *et al.*, 1982). Infection involving TMV generates RNA molecules which are subsets of genomic RNA and act as efficient mRNAs for specific viral translation products. Two such TMV infection associated RNAs are I_2 RNA or sRNA and LMC RNA encoding for the proteins of M_r 30,000/29,000 and coat protein, respectively, in various translation systems (Jackson *et al.*, 1972; Seigel *et al.*, 1973; Hunter *et al.*, 1976; Bruening *et al.*, 1976; Beachy and Zaitlin, 1977). The *in vivo* functions of all these polypeptides except the coat protein are unknown. The translation and replication of TMV has been reviewed extensively (Zaitlin *et al.*, 1976; Zaitlin, 1979; Hirth, 1980; Davies, 1979; Bruening, 1981; Hall *et al.*, 1982; Bruening *et al.*, 1979).

An RNA replicase activity in TMV-infected plants has been reported in several laboratories. RNA replicase activity was found both as soluble enzyme in cytoplasmic extract and as

insoluble enzyme bound to the membranes of TMV-infected plants. Soluble enzyme was partially purified by Brishammer and Juntti (1974). Membrane bound TMV RNA replicase was solubilized with detergents and partially purified (Zaitlin *et al.*, 1973; Sela and Hauschner, 1975). White and Murakishi (1977) solubilized template dependent and virus specific RNA replicase from the membrane fraction of TMV-infected tobacco callus. A non-specific RNA replicase was reported in the soluble phase of healthy and infected callus. No membrane bound activity was detected in healthy callus.

Significant comparative work was reported by Romaine and Zaitlin (1978) on the soluble RNA replicase from uninfected and TMV-infected tobacco leaves. The RNA replicase fractions purified from healthy and TMV-infected plants had similar polypeptide patterns after SDS polyacrylamide gel electrophoresis. None of the polypeptides was unique to TMV infection. Both enzymes were indistinguishable from each other with respect to the number of kinetic parameters tested. It was concluded that the enhanced soluble RNA replicase activity following TMV infection is due to stimulation of a host RNA-dependent RNA polymerase, rather than the genesis of viral coded RNA replicase. Further, the possibility that a viral coded protein (M_r 130,000) is a constituent of TMV RNA replicase was not ruled out.

Interestingly, Fraenkel-Conrat and colleagues believe that the presence of RNA-dependent RNA polymerase is the normal feature of the healthy plants and TMV exclusively uses this non-specific host RNA-dependent RNA polymerase for

replication of its RNA (Ikegami and Fraenkel-Conrat, 1978a, 1978b, 1979a, 1979c; Takanami and Fraenkel-Conrat, 1982). Their hypothesis is based on the similarity in the enzymatic and physico-chemical properties of the RNA replicase isolated from healthy tobacco plants and plants infected with TMV and other viruses, irrespective of the quantitative level or specific activity of the RNA replicase. As pointed out by Hall *et al.* (1982) the activities of their enzyme preparations (Ikegami and Fraenkel-Conrat, 1979c) were generally too low to show any clear cut difference in enzymatic properties.

iv. Cowpea Mosaic Virus RNA Replicase

Cowpea mosaic virus (CPMV) contains a bipartite genome, consisting of two RNA (B and M) molecules which are encapsidated separately in two (bottom and middle) nucleoprotein particles (Van-Kammen, 1972; Jaspars, 1974; Bruening, 1977, 1981). Like poliovirus RNA (Frisby *et al.*, 1976; Nomoto *et al.*, 1977; Kitamura *et al.*, 1981) both RNAs of CPMV bear a VPg at the 5'-end and a poly(A) tail at the 3'-end (El Manna and Bruening, 1973; Stanley *et al.*, 1978; Daubert *et al.*, 1978; Daubert and Bruening, 1979) and are translated into a polyprotein from which structural or functional proteins are derived via proteolytic processing (Pelham, 1979; Rezelman *et al.*, 1980). On inoculation of protoplasts with separated components, the B component RNA was replicated and expressed independently, whereas M component did not, showing that B RNA plays a crucial role in the replication of both CPMV RNAs (Goldbach *et al.*, 1980).

An RNA replicase activity associated with a membrane

fraction of CPMV-infected cowpea plants was solubilized by the use of Mg^{2+}-deficient buffer (Zabel *et al.*, 1974) and was partially purified (Zabel *et al.*, 1975, 1976). Partially purified enzyme was shown specific for CPMV RNA as a template by the use of a nitrocellulose membrane filter binding assay (Zabel *et al.*, 1979), but later on this property of template specificity when tested under similar conditions was discounted (Ikegami and Fraenkel-Conrat, 1979c; Dorssers *et al.*, 1981, 1982). On further purification, the RNA replicase activity solubilized by the use of a Mg^{2+}-deficient buffer was shown to be associated with the single host encoded polypeptide of apparent M_r 120,000. The virus specific polypeptides which were present in initial extracts could be separated upon chromatography of the partially purified enzyme on poly(U)-Sepharose column, without affecting the sedimentation rate and other properties of RNA replicase. It was suggested that none of the viral encoded components present in partially purified enzyme is part of the RNA replicase. Using the same purification procedure, an RNA replicase activity was purified from mock inoculated cowpeas and had identical chromatographic properties to the RNA replicase purified from CPMV-infected plants, while both enzymes co-sedimented upon glycerol density gradient centrifugation (Dorssers *et al.*, 1981, 1982). On the basis of these results, Dorssers *et al.* (1982) have proposed that the viral genome does not encode for the polymerase subunit of RNA replicase, but only contributes the protein factors that make it template specific. This model is similar to one proposed by Romaine and Zaitlin (1978) for TMV RNA replicase.

More recently a CPMV RNA replication complex has been isolated from the membrane fraction of CPMV-infected cowpeas, after the solubilization of host encoded RNA-dependent RNA polymerase with Mg^{2+}-deficient buffer (Dorssers *et al.*, 1983). This CPMV RNA replication complex was solubilized with Triton X-100 and then purified by Sepharose 2B chromatography. After purification, the RNA replication complex resumed *in vitro* to complete the nascent chains initiated *in vivo*. The products synthesized by the RNA replication complex were shown to be the size of CPMV RNAs and complementary to virion RNAs. The polypeptide composition and viral coded nature of this RNA replication complex is still to be investigated.

v. Brome Mosaic Virus RNA Replicase

Brome mosaic virus (BMV), the representative of the bromoviruses, encapsidates four RNAs in three types of virions (designated H, M, and L) (Lane, 1974; Kaesberg, 1976a; Haenni *et al.*, 1982). Three largest RNAs constitute the BMV genome and are completely dependent on each other for their replication and formation of progeny virions (Lane and Kaesberg, 1971; Bancroft and Lane, 1973). All the four RNAs are functionally monocistronic in *in vitro* conditions and also code for the polypeptides of comparable mobility in BMV-infected protoplasts (Kaesberg, 1976a; Sakai *et al.*, 1979; Okuno and Furusawa, 1979). Bromoviruses along with other plant viruses have been reviewed by several authors (Jaspars, 1974; Lane, 1974; Kaesberg, 1976a, 1976b, 1977; Van Vloten-Doting and Jaspars, 1977; Lane, 1979; Davies, 1979; Atebekov and Morozov, 1979; Bruening, 1981; Van Vloten-Doting, 1976;

Bruening *et al.*, 1979).

An RNA replicase activity in BMV-infected barley plants was reported by Semal and Kummert (1970, 1971a, 1971b) and Kummert and Semal (1972). Cell free synthesis of full length RFs of BMV RNAs by crude replicase preparations was also reported by Kummert (1974). RNA replicase from BMV-infected plants was solubilized from the particulate fraction with Triton X-100 and was partially purified by $(NH_4)_2SO_4$ precipitation and sucrose density gradient centrifugation (Hadidi and Fraenkel-Conrat, 1973). Triton solubilized enzyme was unable to synthesize the full length RFs of BMV RNAs 1 and 2 as compared to the bound enzyme (Hadidi, 1974). However, the presence of a polypeptide (M_r 34,500) of mobility comparable to the translation product of RNA 3 was reported in the Triton X-100 solubilized RNA replicase, which was unique to BMV-infected plants (Hariharasubramanian *et al.*, 1973). No specific role was assigned to this polypeptide.

A template-dependent and template specific RNA replicase was solubilized with Nonidet P-40 from the membrane fraction of BMV-infected barley leaves and was partially purified (Hardy *et al.*, 1979). The RNA replicase activity of the enzyme preparation was stimulated 15 fold upon addition of BMV RNAs and synthesized full length RFs of all four BMV RNAs. The enzyme had a high degree of template specificity for BMV RNAs.

Recently, Bujarski *et al.* (1982) and Hall *et al.* (1982) have reported the enhancement of the RNA replicase activity and the template specificity of enzyme for BMV RNAs on treatment of the membrane fraction of BMV-infected barley leaves,

with the non ionic detergent dodecyl β-maltoside (12-M). The detergent treated RNA replicase fraction could accept added BMV RNAs as template and synthesize full length RFs. SDS-gel electrophoresis of the 12-M solubilized enzyme fraction after partial purification revealed several polypeptides. Most of these except one, which co-migrated with the *in vitro* translation product of BMV RNA 1, were common to the extract purified in an identical way from mock inoculated barley leaves. Tryptic digests of the BMV specific polypeptide were similar to those obtained from the *in vitro* translation product of BMV RNA 1. No particular function was assigned to this polypeptide (Bujarski *et al.*, 1982). The translation products of BMV RNAs 1 and 2 could be detected in protoplasts infected with purified RNAs 1 and 2 (Kiberstis *et al.*, 1981). The authors have suggested their possible participation in BMV RNA replicase.

More recently by micrococal nuclease treatment of the detergent treated membrane fraction from BMV-infected barley leaves, the increase in the template specificity of BMV RNA replicase for BMV RNAs was observed (Mr. Miller, 1982 personal communication at I.U.B., Perth). Nuclease treated enzyme could initiate RNA synthesis on added BMV RNAs as template to synthesize full length RFs, showing that their purified preparations probably contained intact RNA replicase. Unfortunately due to the low degree of purification achieved, the host/viral encoded nature of the polymerising activity remained unresolved.

vi. Alfalfa Mosaic Virus RNA Replicase

Alfalfa mosaic virus (AMV) is an example of a "protein-dependent" tripartite genome plant virus with heterocapsidic bacilliform virions. The minimum requirement for infectivity is the three largest RNAs plus either a few molecules of coat protein per RNA molecule or RNA 4, which in turn generates coat protein molecules *in vivo* (Bol *et al.*, 1971; Smit and Jaspars, 1980; Smit *et al.*, 1981). Each of the four AMV RNAs behaves as a monocistronic messenger in *in vitro* translation systems (Mohier *et al.*, 1975; Thang *et al.*, 1975; Gerlinger *et al.*, 1977). So far no virus specific protein, other than coat protein, in virus-infected plants or protoplasts has been reported (Van-Tol, 1981). However, when cowpea protoplasts are infected with virions containing RNAs 1 and 2 the synthesis of virion specific RNA could be detected suggesting the role of gene products of RNAs 1 and 2 in RNA replication (Nassuth *et al.*, 1981a; 1981b). AMV has been reviewed in detail along with other plant viruses (Van Vloten-Doting and Jaspars, 1977; Lane, 1979; Davies, 1979; Atabekov and Morozov, 1979; Bruening, 1981; Hall *et al.*, 1982; Bruening *et al.*, 1979).

Weening and Bol (1975) found that extracts of AMV-infected broad bean leaves contained a membrane bound replicative structure consisting of an RNA polymerase associated with its template which incorporated radioactive NTPs into virion type RNA. A membrane bound and soluble RNA replicase was isolated from the extracts of uninfected and AMV-infected tobacco leaves (Le Roy *et al.*, 1977; Clerx and Bol, 1978). No difference other than the increase in level of RNA replicase

activity after infection was found in the enzyme from healthy and infected tobaccos. Later on from the same laboratory, partial purification of the soluble RNA replicase from uninfected and AMV-infected tobacco plants was reported (Chifflot *et al.*, 1980; Sommer *et al.*, 1981a, 1981b). The membrane bound and soluble enzymes were identical and also no difference was found in the enzyme isolated from healthy or AMV-infected plants. The authors believe that viral infection of a plant causes an increase in the level of cellular RNA replicase rather than the synthesis of a new viral-coded RNA replicase.

Recently Linthorst (1982) compared the RNA replicase activities from healthy and AMV-infected plants using several experimental approaches. SDS-gel electrophoresis of glycerol gradient purified enzyme from both healthy and AMV-infected plants showed almost identical polypeptide pattern except that the preparation from infected plants contained coat protein and a polypeptide of M_r 20,000. The polypeptides in the fractions containing enzyme activity had molecular weights of 100,000, 68,000, 59,000 and 40,000. No significant differences were found in the products synthesized by the enzyme purified from healthy and infected plants. Linthorst (1982) has concluded that the healthy enzyme is capable of transcription of viral RNA but viral encoded protein(s) are necessary for efficient transcription and specificity.

B. RNA-DEPENDENT RNA POLYMERASES FROM UNINFECTED PLANTS

Unlike bacteria and animal tissues RNA replicase

like RNA-dependent RNA polymerase activity has been isolated from some plants. These plants include tobacco (Ralph and Wojcik, 1969; Duda, 1979; Duda *et al.*, 1973; Bol *et al.*, 1976; Stussi-Garaud *et al.*, 1977; Romaine and Zaitlin, 1978; Ikegami and Fraenkel-Conrat, 1978a, 1978b; Frankel-Conrat, 1976, 1979), chinese cabbage (Astier-Manifacier and Cornuet, 1971, 1981), tomato leaves (Boege and Sanger, 1980; Boege *et al.*, 1982a, 1982b) and cowpeas (White and Dawson, 1978; Dorssers *et al.*, 1982). This host RNA-dependent RNA polymerase activity is generally in the soluble phase of uninfected plants whereas virus specific RNA replicases are mostly membrane-bound, but upon infection an increase in the pre-existing soluble enzyme has commonly been observed. However purification of host encoded RNA-dependent RNA polymerases (which were presumed to be virus coded) from the membrane fraction of virus-infected plants has recently been reported (Dorssers *et al.*, 1982; Linthorst, 1982). No remarkable differences have been found in the soluble and solubilized enzymes from virus-infected and uninfected tissues (Fraenkel-Conrat, 1976; Clerx and Bol, 1978; Romaine and Zaitlin, 1978; White and Dawson, 1978; Dorssers *et al.*, 1982).

Enzymes isolated from healthy plants can transcribe non-specifically a variety of RNAs when offered as templates. The product synthesized is mostly double stranded, RNase resistant, heterodisperse and small in size. These enzymes like virus specific RNA replicase are not inhibited by the inhibitors of DNA-dependent RNA polymerases, but are inhibited by pyrophosphate and RNase. The reported molecular weight varied in the range of 140,000 to 200,000. However, no such

plant polymerase has been proven to fulfil the definition of an RNA replicase as defined by Hall *et al.* (1982), i.e., to use (+) stranded RNA as a template to make many more faithful copies of full length (+) stranded RNA.

There is no known role of an RNA-dependent RNA polymerase in uninfected plants. These enzymes have been suggested to be responsible for the replication of viroid RNAs, which have insufficient genetic information to code for any protein for their own replication (Boege and Sanger, 1980). Sanger's opinion is based upon the *in vitro* synthesis of full length transcripts when viroid RNA was used as template for the RNA-dependent RNA polymerase purified from healthy tomato leaves (Boege *et al.*, 1982a; 1982b). Sanger's group, on the basis of their *in vitro* studies (Rackwitz *et al.*, 1981), have also suggested the involvement of DNA-dependent RNA polymerase II in viroid RNA replication. From *in vitro* properties alone it is hard to speculate what happens *in vivo*. Existence of similar enzymes has occassionally been reported in animals tissues (Downey *et al.*, 1973), but their proposed role in amplification of mRNA have been discounted (Boyd and Fitschen, 1977).

Section 1.2 CUCUMBER MOSAIC VIRUS

Cucumber mosaic virus (CMV) is a tripartite plant RNA virus, with three functional species of single stranded RNA, packaged in three types of icosahedral particles (Lot and Kaper, 1976a; 1976b). CMV has a wide host range. Many different strains have been reported e.g., the Y-strain (Kaper *et al.*, 1965), the Q-strain (Francki *et al.*, 1966)

and the S-strain (Van Regenmortel, 1967). Tomato aspermy virus (TAV) and peanut stunt virus (PSV) are two other members of the cucumovirus group closely related to CMV (Holings and Stone, 1971; Mink, 1972).

A. PROPERTIES AND COMPOSITION OF VIRIONS

CMV particles are isometric and about 28 - 30 nm in diameter. The capsid consists of 180 identical protein subunits (M_r 26,200). Virion molecular weight is about 5.5×10^6 (reviewed by Francki *et al.*, 1979). The RNA content of CMV is about 18% (w/w) (Francki *et al.*, 1966). G:A:C:U = 24:23:23:30 (Habili and Francki, 1974a). CMV contains four major RNA species (RNAs 1 - 4), their molecular weights are $1.23 - 1.35 \times 10^6$, $1.06 - 1.16 \times 10^6$, $0.77 - 0.85 \times 10^6$, and $0.33 - 0.35 \times 10^6$ respectively (Peden and Symons, 1973; Reijnders *et al.*, 1974; Mossop *et al.*, 1976; Takanami *et al.*, 1977). RNA 1 and 2 are encapsidated separately and RNA 3 and 4 probably in the same particle. Thus virus preparations contain three different types of particles having almost similar sedimentation properties (Francki *et al.*, 1979).

All the four RNAs of CMV are capped by $m^7G^{5'}ppp^{5'}NP$ at their 5'-ends (Symons, 1975) and the 3'-ends can be amino-acylated with tyrosine (Kohl and Hall, 1974). Sometimes CMV virions contain small unrelated RNAs which are completely dependent for their replication and encapsidation on CMV genomal RNAs; these are called satellite RNAs. Two of these, one called CARNA 5 (Kaper and Waterworth, 1977; Richards *et al.*, 1978) and another one CMV sat RNA (Gould *et al.*, 1978) are well characterized. The base sequence of CMV sat RNA

shows that it has a truncated version of the tRNA like structure at its 3'-end (Gordon and Symons, 1983). Coat protein is the only other component of CMV virions (Francki *et al.*, 1979).

B. STRUCTURE AND GENE CONTENT OF CMV GENOME

The three largest RNAs of cucumber mosaic virus are unique (Gould and Symons, 1977) and are sufficient for infection (Peden and Symons, 1973; Lot *et al.*, 1974). Hybridization studies have shown that the complete sequence of RNA 4 is at the 3'-end of RNA 3 of CMV (Gould and Symons, 1977). Base sequence analysis of the 3'-terminal residues of four RNAs of Q-CMV showed that the sequences of RNAs 3 and 4 are identical and there is an extensive homology with RNAs 1 and 2 (Symons, 1979). Further the 3'-terminus of each of the four CMV RNAs has a tRNA like secondary structure (Symons, 1979; Gunn and Symons, 1980). This property of tRNA like secondary structure at the 3'-end is in common with another type member tomato aspermy virus and also with brome mosaic virus (BMV) (Wilson and Symons, 1981; Alhquist *et al.*, 1981). The similarity in the property of aminoacylation and in the secondary structure at the 3'-ends of the RNAs of these tripartite plant viruses may be significant for the replication or encapsidation of their RNAs (Haenni *et al.*, 1982).

The *in vitro* translation studies of purified RNAs isolated from CMV show that the CMV genome codes for four proteins of apparent M_r 95,000; 110,000; 35,000 and 24,500 (coat protein) which are directly translated from RNAs 1, 2, 3 and 4 respectively (Schwinghamer and Symons, 1975; 1977). Under *in vitro* translation conditions all the four RNAs of CMV act as monocistronic messengers. RNA 4, the entire sequence of

which is contained in RNA 3 is an efficient messenger for *in vitro* synthesis of coat protein and is considered to be the subgenomic mRNA for *in vivo* synthesis of coat protein (Habili and Francki, 1974b; Schwinghamer and Symons, 1977).

The complete base sequence of the 2193 residues of CMV RNA 3 (Q-strain) has been determined by using cloned DNA fragments (Gould and Symons, 1982). It also gave the base sequence of 1027 residues of CMV RNA 4 (M_r ~~34,900~~ 349,000). The nucleotide sequence of RNA 4 predicted the sequence of 236 amino acids of coat protein (M_r 26,200) and also confirmed its monocistronic nature. Determination of the base sequence of RNA 3 confirmed the dicistronic nature of RNA 3 and also gave the location of the 5'-terminal cistron coding for the 333 amino acid residues of the *in vitro* translation product, the 3A protein (M_r 36,700). The coat protein gene of RNA 3 is ~~therefore~~ silent in *in vitro* translation and presumably *in vivo* also (Gould and Symons, 1982).

There is a remarkable difference in the proportion of RNA 1 and 2 utilized to code for their *in vitro* translation products (M_r 95,000 and 110,000 respectively). Most of the sequence (about 88%) of RNA 2 appears to function directly as messenger, whereas it is only 65% for RNA 1. Recent progress in base sequence determination of RNA 1 has shown its dicistronic nature, with the potential to code for two polypeptides, proteins 1A and 1B (M_r 95,000 and 35,000 respectively) with their cistrons located near 5'- and 3'-ends, respectively (Gould and Symons, personal communication). The cistron for the smaller polypeptide (M_r 35,000) seems to be silent in *in vitro* translations of RNA 1. However, a small

RNA translatable into a protein of the same molecular weight has recently been isolated from CMV-infected cucumber plants. The base sequence of this small RNA is in common with CMV RNA 1 as indicated by the nitrocellulose gel blots probed with ^{32}P-labelled probes prepared from RNA 1 fragments cloned in the bacteriophage vector M13 (Gordon *et al.*, personal communication). The small RNA is presumed to be the *in vivo* subgenomic CMV RNA derived from RNA 1, but the validity of its messenger nature in *in vivo* conditions is still to be investigated.

Unfortunately, despite of numerous efforts using various techniques none of the full length translation products of CMV RNAs 1, 2 and 3 have been detected in CMV-infected cucumber seedlings or cowpea protoplasts (Gonda and Symons, 1979). *In vivo* validity of the translation products of three largest RNAs of CMV is still a dilemma whereas the coat protein, the gene product of RNA 4, is readily detectable in CMV-infected cowpea protoplasts after labelling with radioactive amino acids and gel electrophoresis (Gonda and Symons, 1979). However, a few proteins (M_r 78,000; 62,000; 48,000, coat protein and two smaller polypeptides) were induced upon infection of cucumber plants with CMV (Ziemiecki and Wood, 1976). The functional meaning of these proteins except coat protein is still obscure.

Section 1.3 SCOPE OF THE PROJECT

Our research group, at the University of Adelaide, is studying the structure of CMV genome and molecular mechanism of its replication in cucumber plants. A brief account of

CMV is given in the previous section. Upon infection of cucumber seedlings with CMV an RNA-dependent RNA polymerase (RNA replicase) activity appears in both soluble and particulate fractions (Gilliland and Symons, 1968; May *et al.*, 1969; 1970; May and Symons, 1971; Kumarasamy and Symons, 1979a). No such activity has been found in healthy cucumber plants. The soluble form of this enzyme was purified about 10,000 fold and characterized in detail (Kumarasamy and Symons, 1979a).

Very little work has been done on the particulate form of CMV-induced RNA replicase (May *et al.*, 1970). In virus-infected plants, virus specific RNA replicase activity is usually restricted to the particulate fraction (Bol *et al.*, 1976; Zabel *et al.*, 1976; White and Murakishi, 1977; Bujarski *et al.*, 1982). Therefore it was considered important to carry out the detailed study of the particulate form of CMV-induced RNA replicase, with the main emphasis on the investigation of the possibility of the participation of viral gene products in RNA replicase and the role of RNA replicase in virus-infected plants.

The work described in this thesis is mainly on the particulate form of CMV-induced RNA replicase, isolated from CMV-infected cucumber seedlings. A standard procedure, routinely used for the solubilization and purification of particulate form of the enzyme was developed. The purified CMV-induced particulate RNA replicase was studied to investigate the possibility of its viral coded nature. In order to study the role of CMV-induced RNA replicase in CMV-infected plants, the products synthesized by the particulate fraction

and RNA replicase purified from CMV-infected plants has been analysed.

CHAPTER TWO

SOLUBILIZATION OF CMV-INDUCTED RNA REPLICASE FROM THE PARTICULATE FRACTION

Section 2.1 INTRODUCTION

Upon infection of cucumber seedlings with CMV an RNA-dependent RNA polymerase (RNA replicase) activity appears in the particulate as well as soluble phase. No such activity has been detected in healthy cucumber plants (Gilliland and Symons, 1968, May *et al.*, 1969; 1970; May and Symons, 1971; Clark *et al.*, 1974; Kumarasamy and Symons, 1979a). The partial purification of the soluble form of this enzyme was attempted by May and Symons (1971). The enzyme was later purified about 100 fold by Clark *et al.* (1974) and more extensive purification was carried out by Kumarasamy and Symons (1979a). Various catalytic properties and requirements of the extensively purified enzyme were studied in detail. After 10,000 fold purification, fractions containing RNA replicase activity were shown to contain about nine polypeptides; most of these were common to similar preparations prepared from healthy cucumber plants. A polypeptide of M_r 100,000 along with trace amounts of two other polypeptides (M_r 110,000 and ~33,000) were specific for RNA replicase preparations. The enzyme copied a variety of natural RNAs without any specificity, but had a remarkable preference for poly(C), with a much lower activity for poly(U) and very little for poly(A) and poly(G) (Kumarasamy and Symons, 1979a).

However, at the start of my experimental work very little work had been done on the particulate form of the CMV-induced RNA replicase (May *et al.*, 1970). Two forms of RNA replicase in the crude preparation of particulate fraction were reported. One required the addition of RNA before the enzyme activity could be detected and other did not require

the addition of RNA (May *et al.*, 1970). Like other virus specific replicases (Table 1.1), the particulate form of CMV-induced RNA replicase was presumed to be the *in vivo* form of the enzyme. Therefore, it was considered important to carry out a detailed investigation of the particulate form of the enzyme in order to study the mechanism of viral RNA replication. The immediate requirement of this investigation was the solubilization of the enzyme from the particulate fraction where it is presumed to be membrane bound. In this chapter the establishment of a standard and reproducible procedure used routinely for the solubilization of the membrane bound RNA replicase from the particulate fraction is described.

Section 2.2 MATERIALS AND METHODS

A. GENERAL MATERIALS

α-^{32}P-GTP was prepared by the method of Symons (1977) and was kindly provided by Dr. R.H. Symons. Unlabelled ribonucleoside triphosphates (ATP, UTP, CTP and GTP), polycytidylic acid [poly(C)], pyruvate kinase [suspension of 10 mg/ml in 2 M $(NH_4)_2SO_4$] and bovine serum albumin were purchased from Sigma Chemical Co. Phosphoenolpyruvate (Cychohexyl ammonium salt) was prepared and ion exchanged to the potassium salt by the method of Clarke and Kirby (1966) and was kindly provided by Dr. R.H. Symons as a 0.1 M stock solution. Actinomycin D was obtained from Merck, Sharp and Dohme, Rahway, New Jersey.

B. VIRUSES AND PLANTS

Viruses and plants used were kindly provided by Dr. R.I.B. Francki, Waite Agricultural Research Institute, Glen Osmond. CMV (Q-strain) was grown and purified and viral RNA was isolated as described by Peden and Symons (1973). Cucumber plants (*Cucumis sativus* L. var polaris) were germinated either in a growth room or in a green house. Eight to twelve days after planting, the seedlings were dusted with carborundum powder and the cotyledons were inoculated by rubbing with a solution of purified virus (0.4 mg/ml) in 5 mM sodium borate and 0.5 mM EDTA. The control plants were untreated healthy or mock inoculated in the above solution. Plants were sprayed with tap water immediately after inoculation. Plants were grown in the constant temperature growth room (24°C - 26°C) under fluorescent light at an intensity of 700 lux for a 14 h day or under green house conditions. About 7 to 10 days after inoculation, the symptom bearing primary leaves together with cotyledons were harvested for enzyme extraction.

C. BUFFER SOLUTIONS USED IN THE ENZYME EXTRACTION AND SOLUBILIZATION PROCEDURES

Buffer A: 50 mM Tris-HCl, pH 8.5, 100 mM NH_4Cl, 10% sucrose (w/v), 90 mM 2-mercaptoethanol.

Buffer B: 50 mM Tris-HCl, pH 8.5, 100 mM NH_4Cl, 90 mM 2-mercaptoethanol.

D. ASSAY OF RNA REPLICASE ACTIVITY

Enzyme assays were based on the incorporation of α-^{32}P-GTP into acid-insoluble material essentially as described by May and Symons (1971). The composition of the standard

enzyme assay medium in a volume of 0.1 ml was as follows:

20 mM Tris-HCl, pH 8.5

0.12 mM of α-^{32}P-GTP, ~0.5 µCi/assay

0.6 mM of each of unlabelled ATP, UTP and CTP (neutralized with triethylamine and stored in 0.1 mM EDTA, at -15°C)

2 µg pyruvate kinase

2 mM phosphoenol pyruvate (K^+ salt)

2 - 3 µg actinomycin-D

25 µg bovine serum albumin

5 mM KCl

13 mM $Mg(CH_3COO)_2$

30 mM 2-mercaptoethanol

50 - 60 mM NH_4Cl

CMV RNA and poly(C) were used as templates at 0.2 mg/ml. Assays with poly(C) as template contained 6 mM Mg-acetate and 1.0 mM each of the four ribonucleoside triphosphates. The reaction was started by adding 5 to 30 µl of the enzyme extract. Incubation was at 37°C for 60 min in small glass-tubes covered with parafilm. The reaction was stopped by spotting 50 µl of reaction mixture on 3 MM Whatman paper squares (1.5 x 2.0 cm), which were then washed batchwise in ice-cold 5% TCA (w/v) containing 2% Na_2HPO_4 (w/v) and 2% $Na_4P_2O_7$ (w/v) for seven minutes while stirring slowly. Washing was repeated for five times each time with fresh solution. The paper squares were then soaked in chilled ether: acetone (3:1, v/v) for five min and dried for 30 - 60 min in an oven at 110°C. Blank assays were run in an identical way but without enzyme.

E. SCINTILLATION COUNTING

The radioactive samples dried on the 3 MM Whatman paper were counted in toluene based PPO-POPOP scintillation fluid prepared by dissolving 35 g of PPO (2,5-diphenyloxazole) and 3.5 g of POPOP [1,4-bis-(2-(5-phenyloxazolyl)benzene)] in 1 litre of toluene. The counting for ^{32}P was done in a Packard Tri-carb liquid scintillation spectrometer using discriminator settings of 50 - 1000 and a gain setting at 1%.

F. PROTEIN ESTIMATIONS

Protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard. Protein was precipitated from the solubilized samples with 15% ice cold TCA (w/v) for an h or longer. The protein precipitate, collected after centrifugation at 10,000 g for 15 min, were washed with cold ether:acetone (3:1 by volume), dried under vacuum, finally dissolved in 1 N NaOH and an aliquot used for the estimation of protein concentration. For the estimation of total protein in the particulate fraction, the particulate pellet was washed repeatedly in 80% ice cold acetone to extract the pigments. The dried acetone washed pellet was heated in 1 N NaOH for 4 min at 90°C and then left overnight at room temperature. The solubilized total protein was recovered in the supernatant after centrifugation and an aliquot used for protein estimation.

G. ENZYME UNITS

A unit of RNA replicase activity is defined as that amount of enzyme protein, which incorporated 1 nmol of nucleotide (GMP) per min into acid insoluble material.

H. SPECIFIC ENZYME ACTIVITY

Specific enzyme activity is defined as the number of units of enzyme activity per milligram of protein.

I. EXTRACTION AND SOLUBILIZATION OF CMV-INDUCED RNA REPLICASE FROM PARTICULATE FRACTION

All operations were carried out at 4°C. The homogenates were prepared by grinding X gram of healthy or CMV-infected cucumber seedlings with 2 X ml of Buffer A in a Waring blender at low speed for 90 seconds. The homogenate was then squeezed through a layer of nylon cloth and centrifuged at 22,000 g for 25 min in the Beckman B-21 centrifuge. The supernatant containing the soluble enzyme was discarded and the pellet was resuspended in 4 X ml of Buffer A using a Potter-Elvijhem homogeniser and centrifuged at 22,000 g for 25 min. This washing step of resuspending the pellet in 4 X ml of Buffer A and centrifugation was repeated two times to ensure the complete removal of soluble enzyme physically entraped in it. The well washed pellet obtained in this way was called particulate fraction and was used for solubilization.

For solubilization, the particulate fraction from X g of leaf material was resuspended in 0.056 X ml of 2 M $MgSO_4$ plus Buffer B to a final volume of 0.75 X ml. The suspension was incubated at 37°C for five minutes after the temperature of the suspension reached 37°C. The light green supernatant containing the solubilized RNA replicase activity was recovered after centrifugation of the suspension at 22,000 g for 30 min at 4°C. The supernatant was dialysed against ten times volume of Buffer A with one change over 16 hours. This

dialysis step was necessary to remove the $MgSO_4$ which otherwise interferred in the assay and also in subsequent purification. The dialysed supernatant was called crude particulate enzyme and was used for the assay of enzyme activity or for further purification. Various steps of extraction and solubilization of RNA replicase activity from 200 g of CMV-infected cucumber leaves are summarized in a flow sheet (Fig. 2.1).

Section 2.3 RESULTS

A. SOLUBILIZATION OF CMV-INDUCED RNA REPLICASE FROM PARTICULATE FRACTION WITH VARIOUS SALTS

The solubilization of RNA replicase from the particulate fraction with $MgSO_4$ during the assay originally observed by May *et al.* (1970) has been further investigated by testing the effect of various salts. The particulate fraction was prepared and washed by resuspending in and centrifuging from Buffer A, as described under Materials and Methods. Three washings were found enough for complete removal of soluble RNA replicase activity and most of other non-specific soluble proteins. The RNA replicase activity and protein released on incubation of the washed pellet in the presence of six different salts (at final concentration of 150 mM) separately relative to the control without any added salt are given in Table 2.1. The release of RNA replicase activity assayed with CMV RNA and poly(C) as templates was the highest when the pellet was incubated in the presence of $MgSO_4$, whereas five other salts ($MgCl_2$, $Mg(CH_3COO)_2$, K_2SO_4, Na_2SO_4 and $(HN_4)_2SO_4$ tested released 17 to 42% of the activity released with $MgSO_4$. The RNA

FIGURE 2.1

SOLUBILIZATION OF CMV RNA REPLICASE FROM PARTICULATE FRACTION

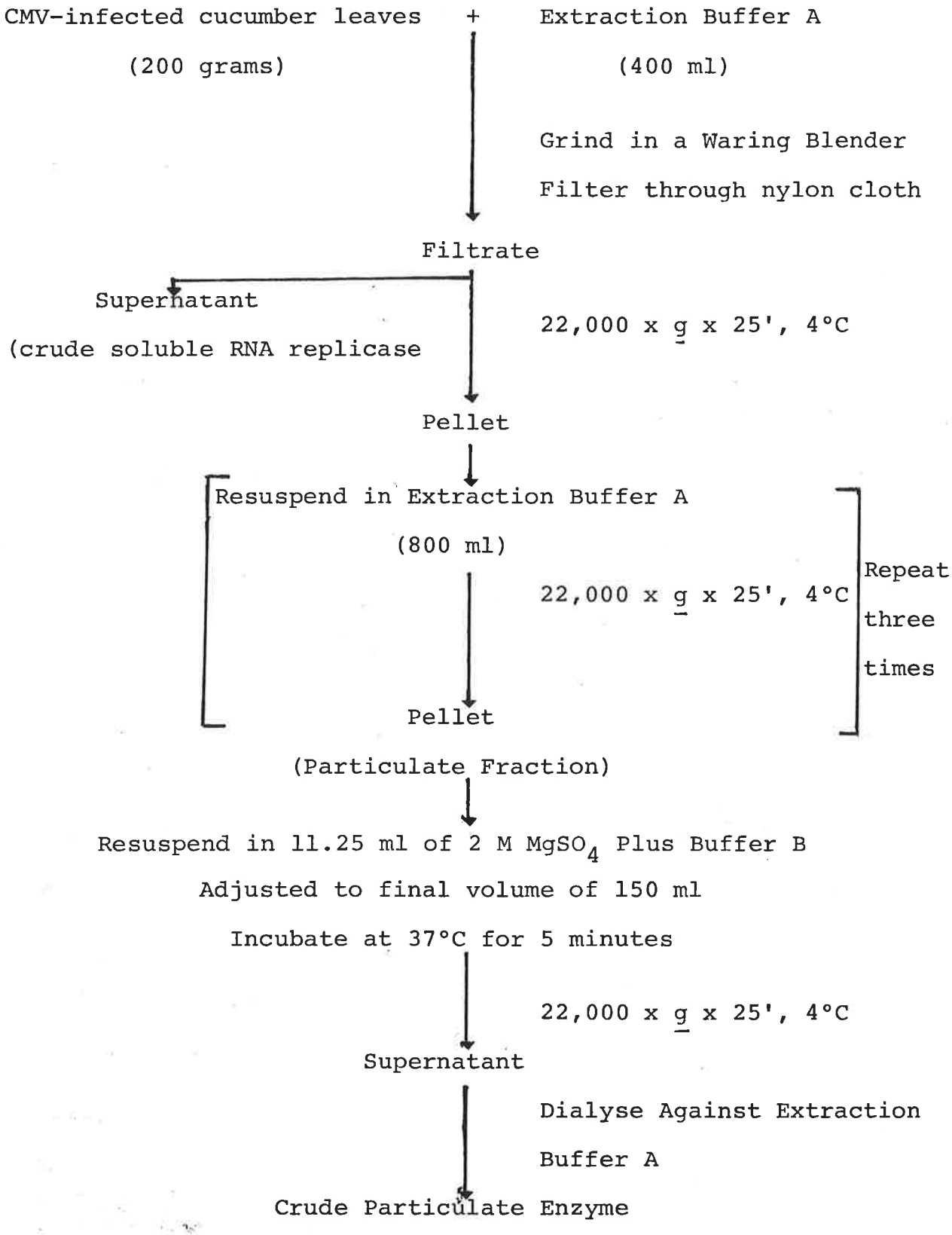


TABLE 2.1

SOLUBILIZATION OF RNA REPLICASE ACTIVITY FROM THE PARTICULATE FRACTION OF CMV-INFECTED CUCUMBER SEEDLINGS WITH DIFFERENT SALTS

Added Salt [150 mM]	Total protein released relative to control	RNA replicase activity released relative to control	
		CMV RNA	Poly(C)
None - control	1.00[a]	1.00[b]	1.00[b]
$MgSO_4$	1.23	17.50	29.10
$MgCl_2$	1.23	6.30	11.10
$Mg(CH_3COO)_2$	0.93	3.40	6.40
$ZnSO_4$	0.27	0.9	0.4
K_2SO_4	1.18	3.9	4.8
Na_2SO_4	1.23	6.1	7.3
$(NH_4)_2SO_4$	1.05	7.4	8.8

TABLE 2.1 (cont'd...)

The well washed particulate fraction was prepared from CMV-infected cucumber leaves as described in Materials and Methods (Section 2.2I). The washed pellet was resuspended in Buffer B to give a uniform suspension. Samples (5 ml, corresponding to 6.7 g of leaves) of this suspension adjusted to a final concentration of 150 mM of indicated salt were incubated at 37°C for 5 min after the temperature of the suspension reached 37°C and then centrifuged at 22,000 g for 30 min at 4°C. All the supernatants obtained after centrifugation were dialysed exhaustively in the same flask against Buffer A. The RNA replicase activity was assayed using 20 µl of the supernatant per 100 µl of standard assay with CMV RNA and poly(C) as templates. All values are expressed relative to the control with no added salt and are the average of two separate experiments.

a Control value of 0.23 mg/ml of protein released (5.6% of the total protein in the particulate fraction).

b Control values of RNA replicase activity released were 0.0065 units/ml and 0.114 units/ml with CMV RNA and poly(C) as templates respectively.

replicase activity released with $ZnSO_4$ was exceptionally low (lower than control). In contrast to this wide variation in the release of RNA replicase activity with all these salts, there were only small variations in the release of total protein. Which varied from 5 to 7% of the total protein present in the particulate fraction, except for $ZnSO_4$. None of the seven other salts, $MnCl_2$, $CaCl_2$, $ZnCl_2$, NaCl, KCl, LiCl and NH_4Cl, released RNA replicase activity more than two fold from the no salt control, while $MgSO_4$ gave a 17.2 fold and 16.1 fold increase with CMV RNA and poly(C) as templates, respectively (Table 2.2). These results indicated that both magnesium and sulphate ions were necessary for the maximum release of CMV-induced RNA replicase activity from the particulate fraction prepared from CMV-infected plants. No such activity was detected in healthy plants when solubilized and tested in an identical way.

B. SOLUBILIZATION OF CMV-INDUCED RNA REPLICASE FROM PARTICULATE FRACTION WITH DETERGENTS

Table 2.3 shows the results of CMV-induced RNA replicase activity solubilized from the particulate fraction with Lubrol, Nonidet P-40 and Triton X-100 (1% v/v) in comparison to the RNA replicase activity solubilized with $MgSO_4$ (150 mM) and in a control experiment with no added salt. These three detergents under the conditions tested, solubilized from 20 to 60% of the enzyme activity solubilized with $MgSO_4$, when assayed with CMV RNA and poly(C) as added templates. However, the $MgSO_4$ gave relatively template dependent enzyme whereas, the release of template independent

TABLE 2.2

SOLUBILIZATION OF RNA REPLICASE ACTIVITY FROM THE PARTICULATE FRACTION OF CMV-INFECTED CUCUMBER SEEDLINGS BY DIFFERENT SALTS

Added Salt (150 mM)	RNA replicase activity released relative to control	
	CMV RNA	Poly(C)
Control[a]	1.00[b]	1.00[b]
$MgSO_4$	17.30	16.10
$MnCl_2$	0.98	1.00
$CaCl_2$	0.82	1.30
$ZnCl_2$	0.25	0.22
NaCl	1.37	1.20
KCl	1.63	1.25
LiCl	1.28	1.20

TABLE 2.2 (cont'd..)

a Particulate fraction was prepared from CMV-infected plants and resuspended in Buffer B as described in Materials and Methods. Samples (5 ml) of this suspension after adding the indicated salt to the final concentration of 150 mM were incubated at 37°C for 5 min after temperature reached 37°C. Supernatants obtained after centrifugation were dialysed overnight in the same flask against Buffer A. Controls without any added salt were treated in identical way. Samples (20 µl) of dialysed supernatants were used in standard assay to estimate RNA replicase activity released..

b The RNA replicase activity in the control experiment was 22.8 and 291.8 pmols of GMP incorporated per h per assay when CMV RNA and poly(C) were used as added templates, respectively.

TABLE 2.3

RELEASE OF RNA REPLICASE ACTIVITY FROM PARTICULATE FRACTION OF CMV-INFECTED CUCUMBER SEEDLINGS BY DIFFERENT DETERGENTS

Experiment	RNA replicase activity released relative to control[a]		
	None	CMV RNA	Poly(C)
Control	1.00[b]	1.00[b]	1.00[b]
+ Lubrol	4.22	3.00	5.06
+ Nonidet P-40	5.85	1.75	3.89
+ Triton X-100	6.37	2.08	1.93
+ $MgSO_4$	2.57	5.06	10.80

TABLE 2.3 (cont'd...)

a The well washed particulate fraction was prepared from CMV-infected plants as described in Materials and Methods (Section 2.2I). The washed pellet was resuspended in Buffer B. Samples (5 ml) of this suspension containing the indicated detergent (1% v/v) or 150 mM $MgSO_4$ were incubated at 37°C for 5 min after the temperature reached 37°C and then centrifuged at 22,000 g for 30 min at 4°C. The supernatants recovered were dialysed collectively in the same flask against Buffer A. The RNA replicase activity was assayed with or without added template using 20 µl of the supernatant in 100 µl assay medium and was expressed relative to the control treated in a similar way but without any added detergent or salt.

b Control values of RNA replicase activity were 0.34, 1.0 and 0.87 nmoles of GMP incorporated per h per g of leaf material without any added template or with CMV RNA or poly(C) as added templates.

(i.e., assayed in the absence of added template) RNA replicase with detergents was 1.6 to 2.5 times higher than with $MgSO_4$. The use of detergents released a large amount of green material from the particulate fraction, along with enzyme activity, which was difficult to separate for the further purification, whereas the supernatant containing enzyme solubilized with $MgSO_4$ was clear and light pale green in colour.

C. STANDARDIZATION OF THE CONDITIONS FOR THE SOLUBILIZATION OF CMV-INDUCED RNA REPLICASE WITH $MgSO_4$

To obtain reproducibly maximum release of RNA replicase from the particulate fraction with $MgSO_4$, various conditions of solubilization were optimised and the results obtained are given below.

i. On incubation of the particulate fraction with increasing concentrations of $MgSO_4$, a rapid increase in the release of enzyme activity was observed upto 100 mM $MgSO_4$ after which there was a plateau at 100 - 300 mM $MgSO_4$ (Fig. 2.2). A concentration of 150 mM was chosen as standard for routine work.

ii. On incubation of a suspension of the particulate fraction in Buffer B plus 150 mM $MgSO_4$ at various temperatures, there was a marked increase in the RNA replicase activity released into the supernatant upto 40°C (Fig. 2.3) and then a sharp fall in CMV RNA as well as poly(C) copying enzyme activities at 45°C. When the particulate fraction was incubated under identical conditions in the absence of $MgSO_4$ as a control there was a small release of RNA replicase upto 25°C and thereafter a fall in enzyme activity released

FIGURE 2.2

EFFECT OF $MgSO_4$ CONCENTRATION ON SOLUBILIZATION OF RNA REPLICASE ACTIVITY FROM PARTICULATE FRACTION

The washed particulate fraction was prepared from CMV-infected cucumber seedlings and suspended in Buffer B as described in Materials and Methods (Section 2.2I). The five ml fractions of this suspension containing $MgSO_4$ at the final concentration indicated in the Figure were incubated at 37°C for five min after the temperature of suspension reached 37°C and then centrifuged at 22,000 g for 30 min at 4°C. Supernatants recovered were dialysed exhaustively in the same flask against Buffer A. The RNA replicase activity was measured by incubating 20 µl of the supernatants in standard 0.1 ml assay medium for 1 h at 37°C using CMV RNA (o—o) and poly(C) (×—×) as templates. Results are expressed as specific activity (units/mg of protein).

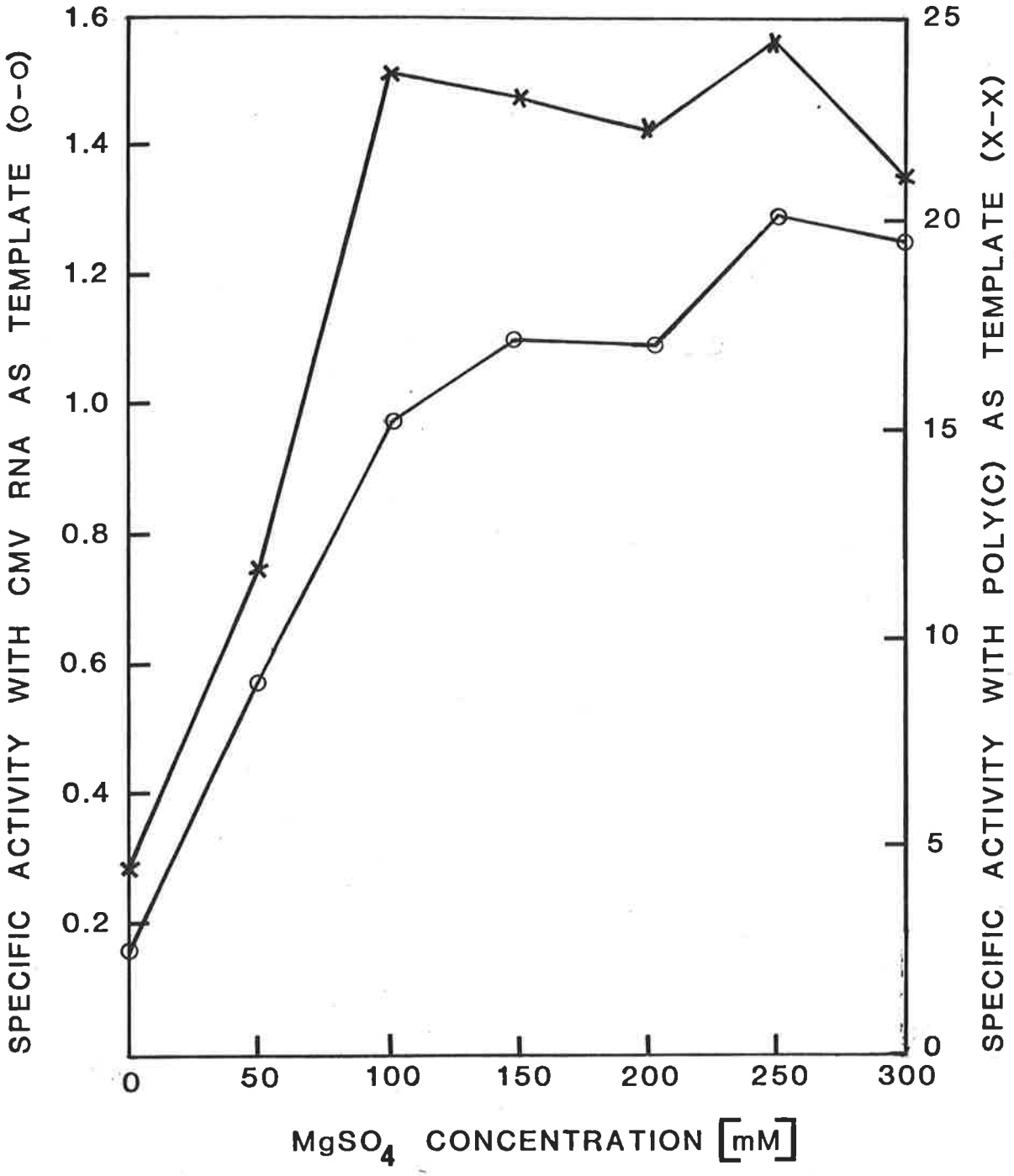
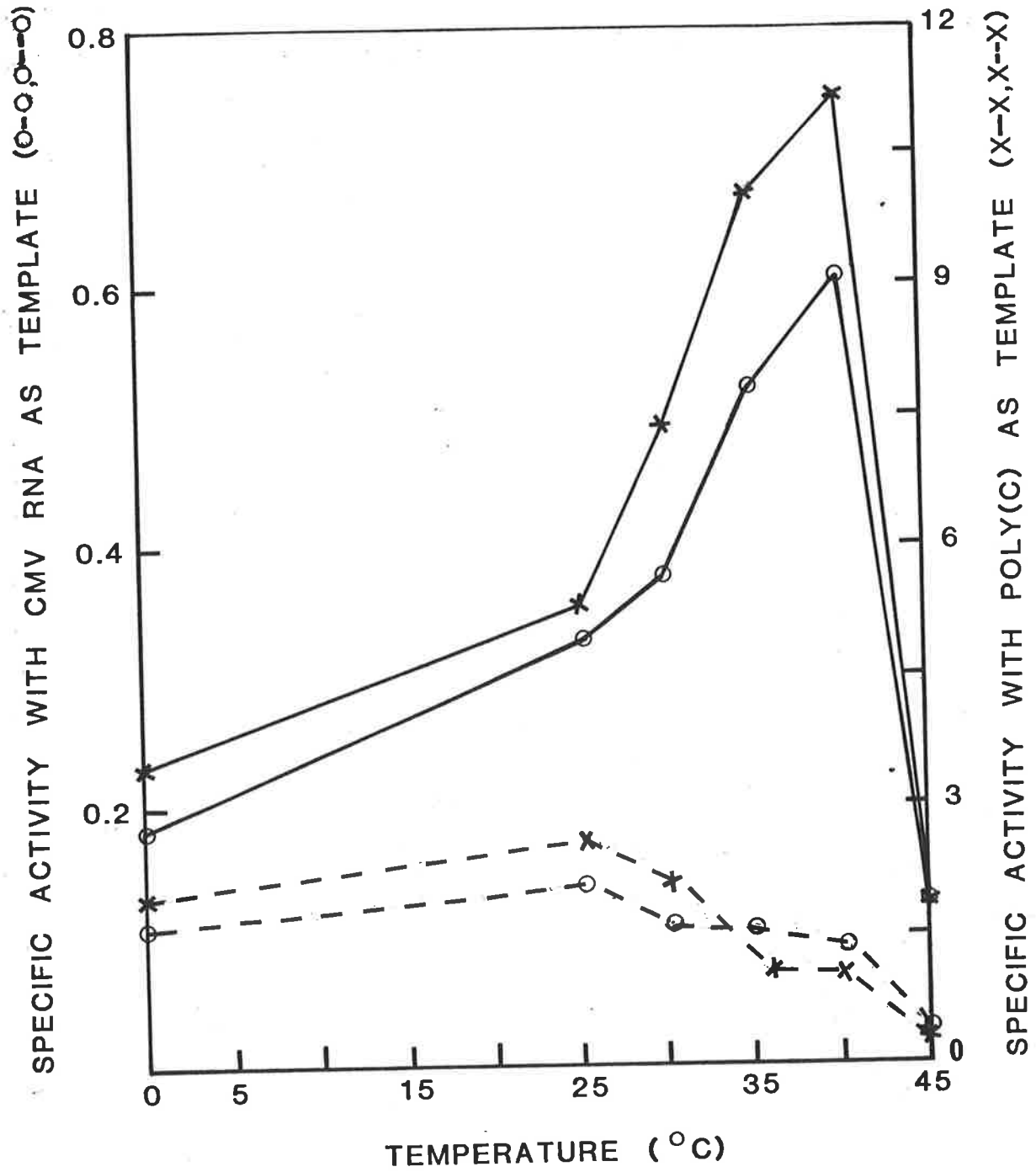

SPECIFIC ACTIVITY WITH CMV RNA AS TEMPLATE (O-O)
SPECIFIC ACTIVITY WITH POLY(C) AS TEMPLATE (X-X)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
25
20
15
10
5
0
0
50
100
150
200
250
300
MgSO4 CONCENTRATION [mM]

FIGURE 2.3

EFFECT OF TEMPERATURE ON SOLUBILIZATION OF CMV RNA REPLICASE ACTIVITY FROM PARTICULATE FRACTION IN PRESENCE AND ABSENCE OF $MgSO_4$

The washed particulate fraction was prepared from CMV-infected cucumber seedlings and suspended in Buffer B as described in Materials and Methods (Section 2.2I). The 5 ml fractions of this suspension were incubated in the presence (o—o , ×—×) or absence (o--o, ×-×) of 150 mM $MgSO_4$ for 5 min after the suspension reached indicated temperature and then centrifuged at 22,000 g for 30 min at 4°C. The supernatants containing solubilized RNA replicase were dialysed against Buffer A. RNA replicase activity was measured by incubating 20 µl aliquots of dialysed supernatants in standard assay system for 1 h at 37°C using CMV RNA (o—o , o--o) and poly(C) (×—× , ×-×) as templates. Results are expressed as specific activity (units/mg protein).


SPECIFIC ACTIVITY WITH CMV RNA AS TEMPLATE (O—O, O--O)
SPECIFIC ACTIVITY WITH POLY(C) AS TEMPLATE (X—X, X--X)
0.8
0.6
0.4
0.2
12
9
6
3
0
0
5
15
25
35
45
TEMPERATURE (°C)

was observed (Fig. 2.3). These results showed that temperature up to 40°C enhanced the solubilization of RNA replicase by $MgSO_4$. The protein released over the range of temperatures tested in Figure 2.3 varied from 0.22 to 0.31 mg/ml and no correlation between protein released and temperature of incubation was observed. On the basis of the above results, a temperature of 37°C was chosen as routine for the solubilization of RNA replicase.

iii. The effect of time of incubation on the solubilization of RNA replicase with 150 mM $MgSO_4$ at 37°C is shown in Figure 2.4. The maximum release of RNA replicase assayed with CMV RNA and poly(C) as templates was obtained as soon as the temperature reached 37°C (zero time) and there was little change for the next 10 min after which both the activities declined slowly. A standard time of 5 min was chosen for the routine solubilization of CMV-induced RNA replicase from the particulate fraction.

Solubilization of RNA replicase with $MgSO_4$ in the presence of 10% sucrose (w/v) in Buffer B was 80% of that was obtained in the absence of sucrose (results not given). Therefore Buffer B was chosen instead of Buffer A to resuspend the particulate fraction before solubilization.

D. DISTRIBUTION OF CMV-INDUCED RNA REPLICASE IN PARTICULATE FRACTIONS OBTAINED FROM CMV-INFECTED CUCUMBER PLANTS

Results presented in the Table 2.4 show the RNA replicase activity present in various particulate fractions by differential centrifugation of the homogenate. Each of these fractions was washed twice before the solubilization of RNA replicase activity with $MgSO_4$ under standard conditions.

FIGURE 2.4

EFFECT OF TIME OF INCUBATION ON SOLUBILIZATION OF CMV RNA REPLICASE ACTIVITY FROM PARTICULATE FRACTION

The washed particulate fraction was prepared from CMV-infected cucumber seedlings and suspended in Buffer B as described in Materials and Methods (Section 2.2I). This suspension (30 ml) containing 150 mM $MgSO_4$ was incubated in a flask at 37°C. When the temperature reached 37°C, 5 ml samples were removed at the times indicated and put on ice. At the end all samples were centrifuged at 22,000 g for 30 min at 4°C and supernatants containing RNA replicase activity were dialysed extensively. RNA replicase activity was measured by incubating 20 µl aliquots of dialysed samples in standard assay for 1 h at 37°C using CMV RNA (o—o) and poly(C) as (×—×) templates. The results are expressed as specific activity (units/mg protein).

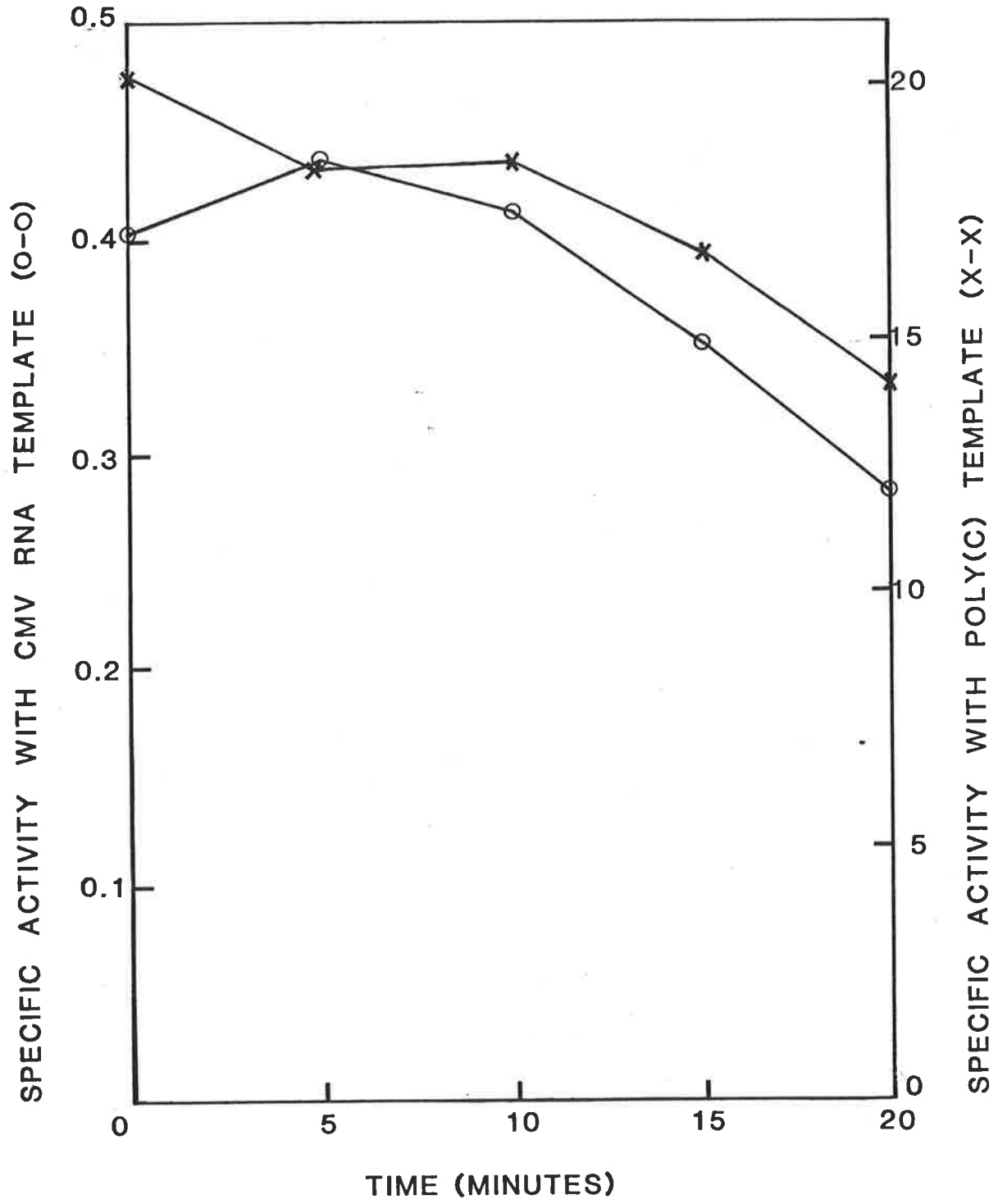

0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
SPECIFIC ACTIVITY WITH CMV RNA TEMPLATE (O-O)
SPECIFIC ACTIVITY WITH POLY(C) TEMPLATE (X-X)
TIME (MINUTES)

TABLE 2.4

DISTRIBUTION OF RNA REPLICASE ACTIVITY IN PARTICULATE FRACTION

Particulate Fraction	Percent of RNA replicase activity released with $MgSO_4$		
	None	CMV RNA	Poly(C)
160 g	11.1	12.8	10.5
1,000 g	38.0	37.3	36.7
22,000 g	51.9	50.2	52.8

The crude homogenate prepared from CMV-infected cucumber seedlings as described in Materials and Methods was first centrifuged at 160 g for 5 min. The pellet obtained was called the 160 g fraction. The supernatant was then centrifuged at 1000 g for 7 min and the pellet obtained was called the 1000 g fraction. The supernatant recovered was then centrifuged at 22,000 g for 25 min and the pellet obtained was called the 22,000 g fraction. All three fractions were washed twice in Buffer A and then used for enzyme solubilization in a standard procedure as described in Materials and Methods.

a The total activity (100%) in all the three assays, each with 20 µl of different enzyme fraction per 100 µl assay medium, was 0.216, 0.42 and 10.17 nmoles of GMP incorporated per h with no added RNA, CMV RNA or poly(C) as templates.

The supernatants after dialysis were assayed for RNA replicase activity using CMV RNA, poly(C) and without any added template. Half of the total enzyme was solubilized from the 22,000 g fraction whereas about 37% and 10 - 13% of the total RNA replicase was recovered from the 1000 g and 160 g particulate fractions, respectively. These results show that all the fractions tested contained significant amounts of enzyme activity. For obtaining the maximum yield of enzyme activity in the standard routine procedure, all the three fractions were pelleted together at 22,000 g and were used subsequently for the solubilization of RNA replicase after washing as described in section 2.2I. Results presented in the Table 2.4 also show that RNA replicase is mainly associated with the cell organelles pelleted at 1000 g and 22,000 g which included chloroplasts and mitochondria, respectively.

E. CHARACTERIZATION OF SOLUBILIZED CRUDE PARTICULATE RNA REPLICASE

The CMV-induced RNA replicase activity solubilized from the particulate fraction with $MgSO_4$ was measured as a function of time of incubation and of enzyme concentration used in the assay in order to find linear conditions of assay.

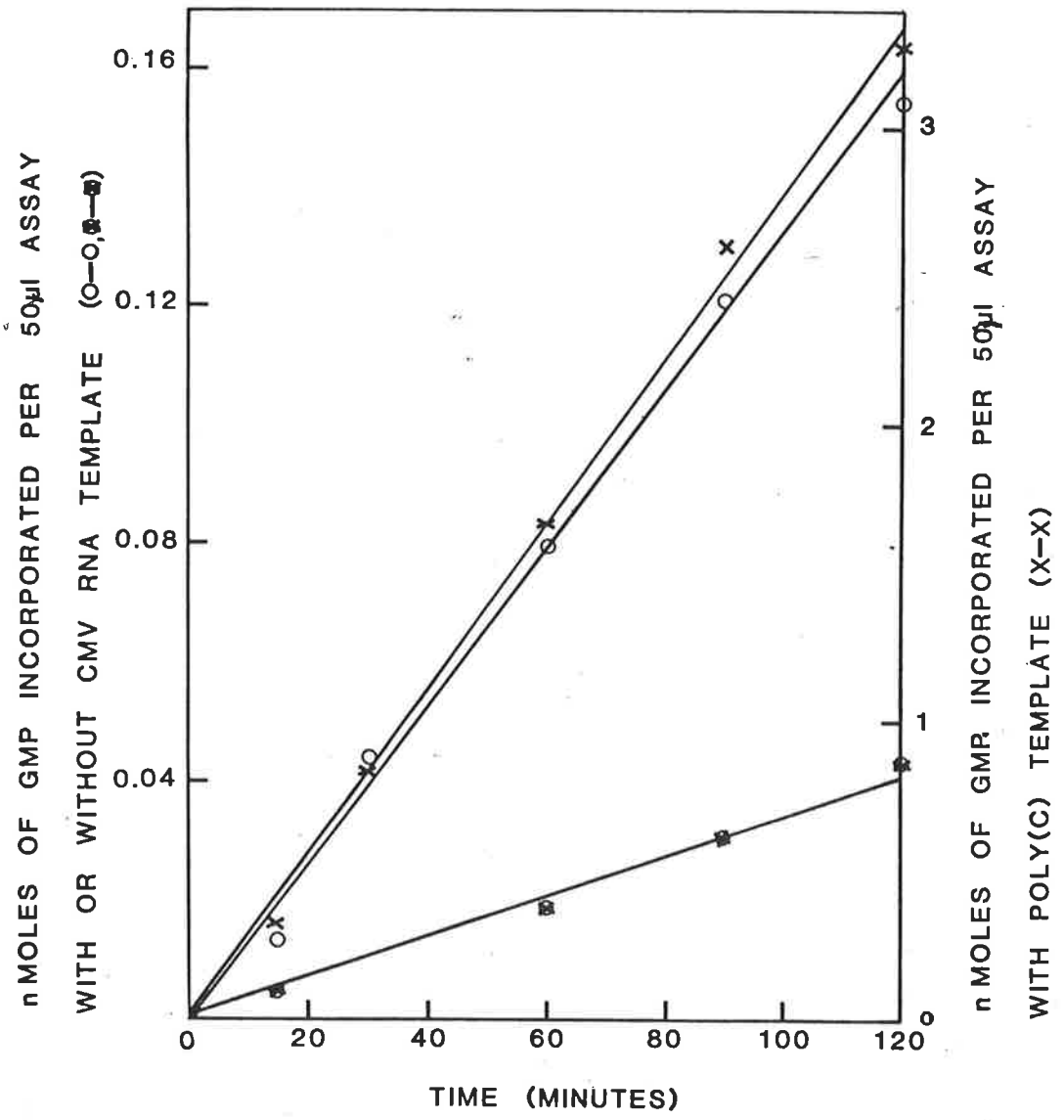
i. The RNA Replicase Activity as a Function of Time of Incubation

Incorporation of labelled GTP into acid-insoluble material increased linearly with time upto 2 h in the absence or presence of added templates (Fig. 2.5). A standard time of 60 min for enzyme assays was chosen for

FIGURE 2.5

RNA SYNTHESIS AS A FUNCTION OF TIME OF INCUBATION

The crude particulate RNA replicase was prepared from CMV-infected cucumber seedlings as described in Materials and Methods (Section 2.2I). RNA synthesis was measured by incubating 70 µl of enzyme extract with 280 µl of assay medium (V_f 350 µl) in absence (⊗—⊗) or presence of CMV RNA (o—o) or poly(C) (×—×) template. Aliquots (50 µl) were removed at the indicated times and spotted on Whatman 3 MM paper strips presoaked in 5% TCA (w/v) and total RNA replicase activity was measured as described in Materials and Methods.


n MOLES OF GMP INCORPORATED PER 50µl ASSAY
WITH OR WITHOUT CMV RNA TEMPLATE (O—O, ■—■)
n MOLES OF GMP INCORPORATED PER 50µl ASSAY
WITH POLY(C) TEMPLATE (X—X)
0.16
0.12
0.08
0.04
3
2
1
0
0
20
40
60
80
100
120
TIME (MINUTES)

most of the work described later.

ii. The RNA Replicase Activity as a Function of Enzyme Concentration in the Assay Medium

When different amounts of enzyme extract (5 to 30 µl/100 µl) were used in the assay medium with or without added templates, a linear increase in the rate of incorporation of labelled GTP into acid insoluble material was observed up to 20 µl of enzyme (Fig. 2.6). For further work, 20 µl of the dialysed enzyme extract per 100 µl of the assay medium were used. However, in some cases when the level of enzyme activity in plants was very high, lower amounts of enzyme were used in the assays. Thus the amount of enzyme varied depending upon the specific activity of the enzyme in the crude extract which varied directly with the severity of symptoms on infected plants.

F. EFFECT OF STORAGE OF THE SOLUBILIZED CRUDE PARTICULATE ENZYME EXTRACT ON THE CMV-INDUCED RNA REPLICASE. ACTIVITY

When the solubilized crude enzyme extract after dialysis was stored at 4°C, a rapid fall (up to 50%) in both the CMV RNA and poly(C) copying activities was observed in the first 72 hours (Fig. 2.7) with a slow rate of loss of enzyme activity thereafter. Hence, crude particulate enzyme after overnight dialysis was further processed as soon as possible on the following day.

FIGURE 2.6

RNA SYNTHESIS AS A FUNCTION OF ENZYME CONCENTRATION IN RNA REPLICASE ASSAY

The crude particulate RNA replicase was prepared from CMV-infected cucumber seedlings as described in Materials and Methods (Section 2.2I). RNA synthesis was measured by measuring the total RNA replicase activity per assay when 5 - 30 µl of enzyme extract was incubated in standard 0.1 ml assay medium. RNA replicase activity was measured without any added template (⊗—⊗), or with CMV RNA (o—o) or poly(C) (×—×) as added templates in the assay medium.

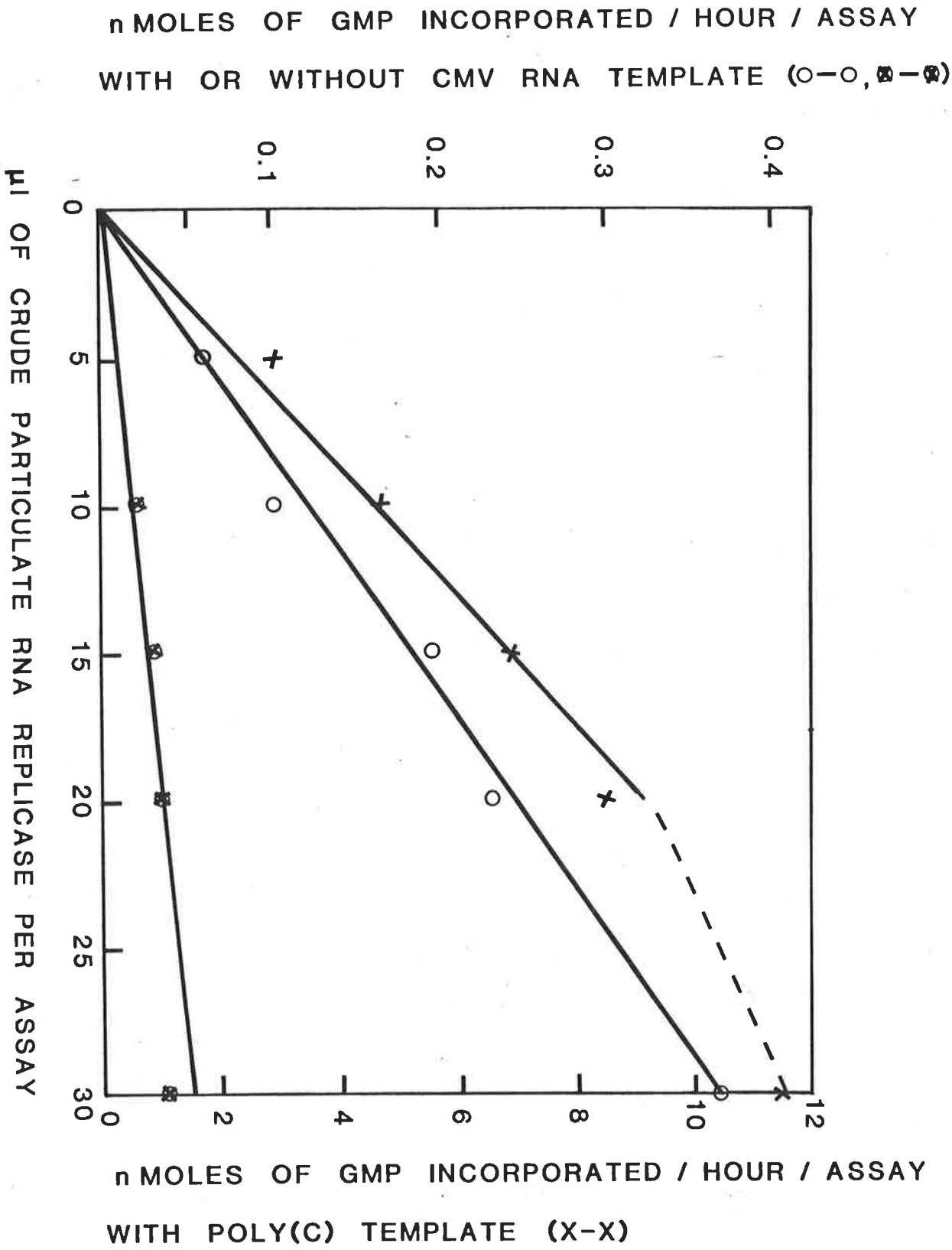
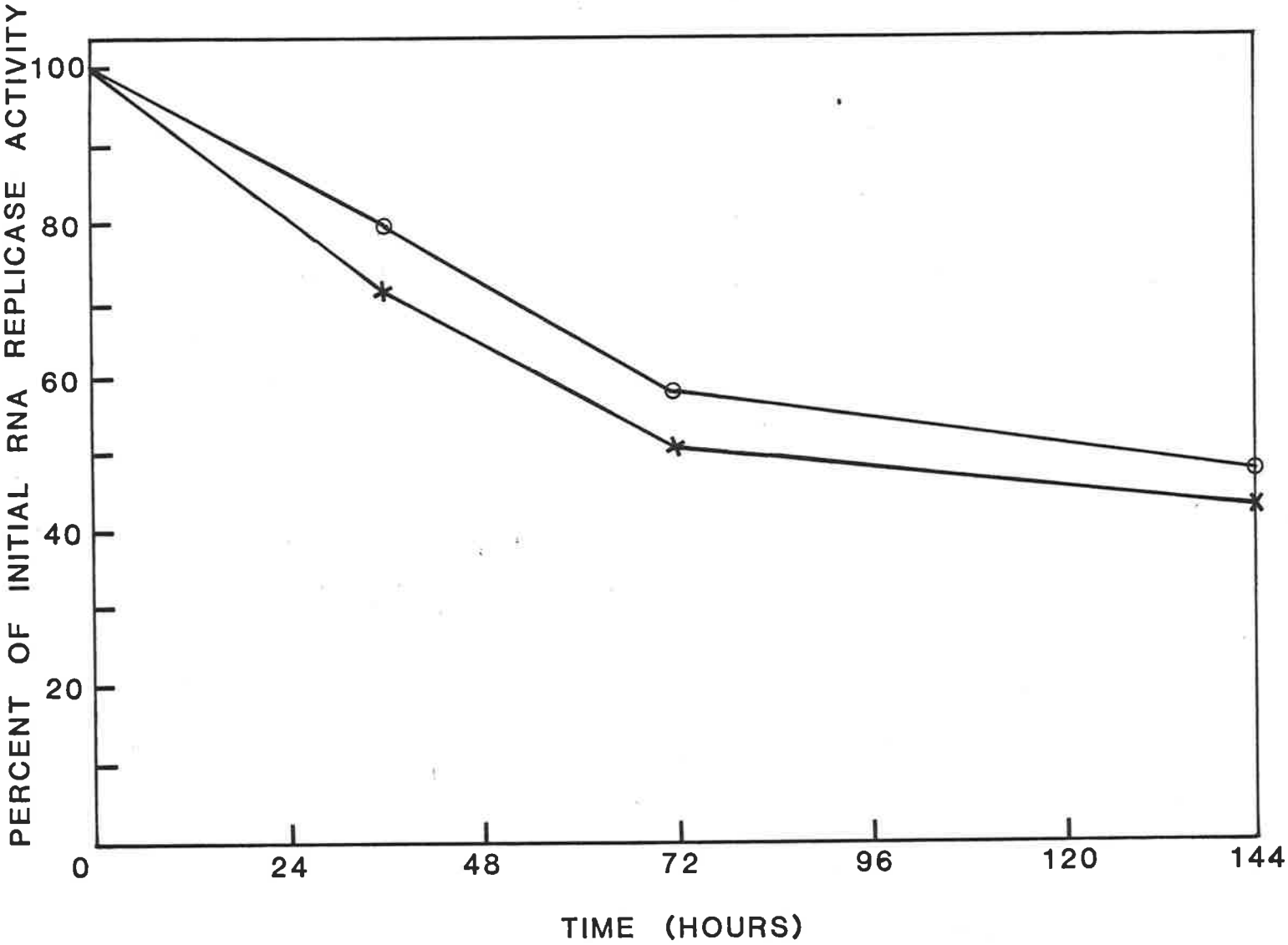

n MOLES OF GMP INCORPORATED / HOUR / ASSAY
WITH OR WITHOUT CMV RNA TEMPLATE (○—○, ⊠—⊠)
0.1
0.2
0.3
0.4
μl OF CRUDE PARTICULATE RNA REPLICASE PER ASSAY
0
5
10
15
20
25
30
0
2
4
6
8
10
12
n MOLES OF GMP INCORPORATED / HOUR / ASSAY
WITH POLY(C) TEMPLATE (X-X)

FIGURE 2.7

LOSS OF CMV-INDUCED PARTICULATE RNA REPLICASE ACTIVITY IN CRUDE PARTICULATE ENZYME EXTRACT ON STORAGE AT 4°C

The crude particulate RNA replicase extract was prepared from CMV-infected cucumber seedlings as described in Materials and Methods (Section 2.2I) and was stored at 4°C. RNA replicase activity was measured by incubating 20 µl of enzyme extract after indicated times using CMV RNA (o—o) or poly(C) (x—x) as templates. The initial RNA replicase activities after dialysis was 0.6 and 12.4 units/assay, respectively, and were taken as 100%.


PERCENT OF INITIAL RNA REPLICASE ACTIVITY
100
80
60
40
20
0
24
48
72
96
120
144
TIME (HOURS)

Section 2.4 DISCUSSION

The establishment of a standard method which has been used reproducibly many times for the solubilization of the RNA replicase from the particulate fraction prepared from the CMV-infected cucumber plants is described in this chapter. Various non-ionic detergents and buffers deficient in divalent metal ions have been used for the solubilization of RNA replicases from the membrane fraction prepared from various virus-infected plants (Zaitlin *et al.*, 1973; Mouches *et al.*, 1974; Zabel *et al.*, 1976; Fraenkel-Conrat, 1976; Le Roy *et al.*, 1977; White and Murakishi, 1977; Clerx and Bol, 1978; Hardy *et al.*, 1979; Bujarski *et al.*, 1982). Our procedure of solubilization is unique to all these procedures as it requires both magnesium and sulphate ions. The exact nature of this synergistic effect of these two ions in solubilization of RNA replicase is unknown. It is presumed that in the particulate fraction the enzyme is bound to its RNA template and that when magnesium is added at high concentration it binds to the RNA and displaces the RNA replicase into the soluble phase. This is consistent with the observation that the solubilized RNA replicase is mainly free of template and its activity is greatly stimulated on addition of exogenous templates. However, the specific requirement of sulphate ion is not accounted for by this hypothesis. Three detergents tried were only 20 to 60 percent successful as $MgSO_4$ in solubilizing the template dependent RNA replicase. The detergents presumably act by binding hydrophobically to poorly soluble nonpolar moieties, creating water-soluble complexes. In doing so, detergents

disperse the membrane systems into a form not sedimentable at 22,000 g when centrifuged for 30 min. The specificity of $MgSO_4$ in solubilizing the RNA replicase was shown by minor variations in the extent of protein released from the membrane fraction (5 to 7% of the total protein) in the absence or presence of a wide range of salts used. Many other proteins were solubilized non-specifically from the particulate fraction irrespective of the presence or absence of $MgSO_4$ or any other salt tested except ZnSO4 or $ZnCl_2$. Of practical importance was the 15 - 20 fold purification achieved by this simple step to produce a clear light green coloured extract which was very convenient for further purification.

Results obtained during the optimization of various conditions of solubilization showed that an increase in temperature in the absence of $MgSO_4$ did not solubilize the RNA replicase activity, but an increase in temperature enhanced the process of solubilization with $MgSO_4$. Moreover, solubilization was spontaneous at 40°C, after which a rapid fall in enzyme activity released was observed, probably due to thermal denaturation of enzyme protein. Therefore, for the efficient and rapid solubilization of CMV-induced RNA replicase from the particulate fraction a combined effect of temperature and 150 mM $MgSO_4$ was required.

From the results presented here and in Kumarasamy and Symons (1979a), it is apparent that the CMV-induced RNA replicase in cucumber seedlings is distributed between soluble and tightly bound particulate forms. In the particulate fraction most of the enzyme activity is in the 22,000 g

and 1000 g fractions. It has been observed from a large number of preparations over three years of study (data not shown) and also in May *et al*. (1970) that the proportion of RNA replicase between soluble and particulate fractions varies depending upon the stage of infection. If the plants were harvested in early stages of infection (7 - 10 days after inoculation) more enzyme was obtained from the particulate fraction and less from the soluble phase, whereas from the plants harvested at later stages of infection (10 - 16 days after inoculation) more enzyme was obtained from the soluble phase as compared to the particulate fraction. From this observation it is suggested that the enzyme first appears in the particulate fraction from where it leaches out into the soluble phase.

CHAPTER THREE

PARTIAL PURIFICATION OF CMV-INDUCED PARTICULATE RNA REPLICASE

Section 3.1 INTRODUCTION

After having solubilized the CMV-induced RNA replicase from the particulate fraction of cucumber seedlings, it was essential to purify the enzyme extensively in order to elucidate its molecular structure and properties. An efficient and rapid procedure is described in this chapter for the partial purification of the particulate RNA replicase. The procedure used in the purification of the soluble CMV-induced RNA replicase, initially developed by Clark *et al.* (1974), was further improved and extended by Kumarasamy and Symons (1979a) to give 10,000 fold purification. Most of the steps of the procedure described in this chapter for the partial purification of CMV-induced particulate RNA replicase were adopted after some modifications from the procedure described by Kumarasamy and Symons (1979a) for the extensive purification of the soluble CMV-induced RNA replicase.

Section 3.2 MATERIALS AND METHODS

A. MATERIALS

Polyethyleneglycol-6000, poly(C), acrylamide, N,N'-methylene-bis-acrylamide, amberlite MB-1 and PPO were from Sigma Chemical Co. Phosphocellulose P-11 and cellulose powder CF-11 were from Whatman, England. ^{3}H-KBH_4 (3.9 - 6.4 Ci/mmol) was obtained from The Radiochemical Centre, Amersham.

B. BUFFER SOLUTIONS USED IN THE ENZYME PURIFICATION

Buffer A: 20 mM Tris-HCl, pH 8.5, 5% polyethyleneglycol-6000 (w/v), 1 mM EDTA, 30 mM 2-mercaptoethanol and 2.0 M NH_4Cl.

Buffer B: 20 mM Tris-HCl, pH 8.5, 10% glycerol (v/v), 30 mM 2-mercaptoethanol and 1 mM EDTA.

Buffer C: 20 mM Tris-HCl, pH 8.5, 30% glycerol (v/v), 30 mM 2-mercaptoethanol and 1 mM EDTA.

Buffer D: 20 mM Tris-HCl, pH 8.5, 30% glycerol (v/v), 30 mM 2-mercaptoethanol, 1 mM EDTA and 10 mM Mg-acetate.

Buffer solutions were autoclaved without 2-mercaptoethanol which was added just before use.

C. RNA REPLICASE ASSAY

The RNA replicase assays using CMV RNA or poly(C) template were essentially the same as described in Section 2.2D except that actinomycin D, pyruvate kinase and phosphoenolpyruvate were omitted in the assays with enzyme fractions other than the crude particulate enzyme.

D. PRETREATMENT OF PHOSPHOCELLULOSE

Phosphocellulose was washed first by decantation with 0.1 N HCl in 50% ethanol and then with water, followed by decantation with 0.1 N NaOH in 50% ethanol and finally washed extensively with water as described by Wickner (1973). Washed phosphocellulose was stored as a slurry in 20 mM Tris-HCl, pH 8.5 at 4°C.

E. PREPARATION OF POLY(C)-CELLULOSE

Poly(C)-cellulose was prepared from a mixture of dry poly(C) and ethanol washed Whatman cellulose (CF-11) powder by ultraviolet irradiation according to the method described by Carmichael (1975, 1979).

F. PARTIAL PURIFICATION OF THE CMV-INDUCED PARTICULATE RNA REPLICASE BY FOUR STEP PROCEDURE

All operations of extraction and purification were carried out in the cold room at 4°C.

Step 1. Preparation of crude particulate RNA replicase Extract:

Crude particulate enzyme extract was prepared from leaves (200 g) of CMV-infected (or healthy in control experiments) cucumber seedlings as described in Section 2.2I. The light green supernatant containing the $MgSO_4$-solubilized crude particulate RNA replicase after dialysis against extraction buffer was called Step 1 enzyme.

Step 2. Polyethyleneglycol precipitation and high salt solubilization of CMV-induced particulate RNA replicase:

To one volume (which is usually 100 ml from 200 g of leaves) of Step 1 enzyme extract was added an equal volume of stock solution containing 44% polyethyleneglycol (PEG)-6000 (w/v), 50 mM 2-mercaptoethanol and 1.0 M NH_4Cl, while stirring. The solution was then stirred thoroughly for 30 min. The greenish precipitate containing RNA replicase was recovered by centrifugation at 22,000 g for 30 min. The precipitate was resuspended in Buffer A (8 - 10 ml) using a Potter-Elvijhem homogenizer and the suspension was centrifuged at 22,000 g for 15 min to give a clear supernatant called the Step 2 enzyme. The pigmented material, if any, was separated as insoluble material in this step. Step 2 enzyme could be stored frozen at -80°C for about eight weeks without appreciable loss of activity. Step 2 enzyme was dialysed overnight with one change against one litre of Buffer B just before the chromatography on phosphocellulose in the next step of the purification.

Step 3. Stepwise column chromatography on phosphocellulose:

A column of phosphocellulose (8 ml of Whatman P-11 pretreated as described in Section 3.2D) was equilibrated with Buffer C containing 0.1 M NH_4Cl. Step 2 enzyme after dialysis was adjusted to a final concentration of 0.1 M NH_4Cl by adding solid NH_4Cl and loaded onto the pre-equilibrated column at a flow rate of 5 ml per h using a peristaltic pump. The column was then washed with two column volumes (16 ml) of equilibration buffer. The RNA replicase was eluted stepwise with Buffer C containing 0.6 M NH_4Cl and 2 ml fractions were collected and assayed for enzyme activity. The fractions with the RNA replicase activity were pooled and called Step 3 enzyme or phosphocellulose step enzyme. Step 3 enzyme was kept at -80°C until the next step of purification.

Step 4. Stepwise column chromatography on poly(C)-cellulose:

A one ml column of poly(C) cellulose (prepared as described in Section 3.2E) was constructed in a pasteur pipette and was equilibrated with Buffer D containing 0.1 M NH_4Cl, until the A_{260} nm of the flow through buffer was less than 0.05. A part (1 to 2 ml) of the Step 3 enzyme was diluted with five volumes of Buffer D and loaded onto the pre-equilibrated column of poly(C)-cellulose at a slow flow rate (2.5 ml per h). The column was washed with nine column volumes (9 ml) of the equilibration buffer and then with nine column volumes of Buffer D containing 0.2 M NH_4Cl. RNA replicase was eluted stepwise with Buffer D containing 1.0 M NH_4Cl and 2 M urea. Fractions (0.5 ml) were collected and assayed for enzyme activity. Peak fractions of enzyme activity were pooled and called either Step 4 enzyme or poly-

(C)-cellulose step enzyme which was kept at -80°C before use.

G. *IN VITRO* LABELLING OF PROTEINS BY REDUCTIVE METHYLATION FOR ANALYSIS BY SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS

Protein samples were labelled with ^{3}H-KBH_4 by reductive methylation as described by Kumarasamy and Symons (1979b) with minor modifications. The samples containing small amounts of protein(2 - 20 µg) were dialysed extensively against double distilled water at 4°C and then freeze-dried. Freeze-dried material was dissolved in 50 µl of 50 mM sodium borate (in some of the experiments described in later chapters deionised formamide, 20 µl/50 µl of reaction volume, was added and samples were heated for 90 sec at 100°C to denature the protein). The labelling reaction was carried out on ice in a fumehood. The reaction was started by adding 3 µl of 60 mM formaldehyde and after 30 sec, 0.5 to 1.0 mCi of ^{3}H-KBH_4 in 1 to 2 µl of 10 mM KOH was added and the reaction was allowed to proceed for another 30 min or more on ice. The reaction was stopped by adding 5 µl of 0.5 N HCl and protein samples were precipitated with 10% cold TCA (w/v) after addition to 20 - 50 µg of BSA as carrier. The precipitate was washed twice with chilled ether-acetone (3:1, v/v) mixture and resuspended in sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol (v/v) 5% 2-mercaptoethanol and 0.002% bromophenol blue) described by Laemmli (1970). In some experiments, the reaction was stopped by adding two volumes of water and three volumes of 2 X Laemmli sample loading buffer and then heating for 2 min in a boiling water bath in the fumehood.

H. SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF PROTEINS

Discontinuous polyacrylamide gel electrophoresis was carried out in the Tris-glycine-SDS buffer system of Laemmli (1970) as described by Schwinghamer and Symons (1977). The slab gels (15 x 12 x 0.2 cm) were cast in a Hoefer vertical slab gel apparatus. The separating gel was made from a stock solution of 40% acrylamide (w/v) and 0.33% bis-acrylamide (w/v) (deionised with mixed bed resin, Amberlite MB-1) in glass distilled water. The separating gel was poured to a height of 9.0 cm. The stacking gel, which contained deionised 3% acrylamide (w/v) and 0.08% bisacrylamide, was poured above the separating gel to a height of 3.0 cm. A comb was then inserted between the two glass plates with the teeth immersed to a depth of 5 mm. Protein samples, which has been heated for 2 min at 100°C in 40 to 70 µl of sample buffer (Laemmli, 1970), were loaded in the wells between the teeth of the comb. Electrophoresis was performed at 25 mA constant current at room temperature until the bromophenol blue dye reached near the bottom.

I. FLUOROGRAPHY AND AUTORADIOGRAPHY

After electrophoresis, the gels were fixed in isopropanol:acetic acid:water (25:10:65, by volume), for 2 h or overnight. The gels were then dehydrated by soaking in DMSO (300 ml per gel) for one hour. After this, gels were impregnated in 20% naphthalene (w/v) and 0.5% PPO (w/v) in DMSO (150 ml per gel) for 3 h on a slow gyratory shaker followed by soaking in distilled water for 90 min. Gels were then placed on a sheet of Whatman 3 MM paper and covered with

a thin film of polyethylene sheet (Glad wrap) and dried under vacuum and heat using a BIO-RAD gel slab dryer.

Fluorography was carried out at -80°C for variable lengths of time using Fuji Rx medical X-ray film to record ^{35}S- or ^{3}H-labelled bands on the gel (Bonner and Laskey, 1974; Laskey and Mills, 1975). Autoradiography of ^{125}I- or ^{35}S-labelled proteins separated on an SDS-gel was carried out at 4°C by exposing Fuji Rx medical X-ray film on the dried or wet polyacrylamide gel. The films were developed with Kodak X-ray developer for two to five minutes followed by rinsing in water and then fixed in Ilford hypam rapid fixer for 2 min. Fixed films were rinsed in water and dried.

Section 3.3 RESULTS

A summary of the results obtained when 200 g of CMV-infected leaves were processed through the four step procedure of extraction and purification described above is given in Table 3.1. RNA replicase activity was monitored at each step by using CMV RNA and poly(C) as templates in the standard RNA replicase assay. Salient features of the four step procedure are given below.

A. STEP 1. SOLUBILIZATION OF CMV-INDUCED PARTICULATE RNA REPLICASE

The solubilization of RNA replicase activity from the particulate fraction of CMV-infected cucumber seedlings has been described in Chapter Two.

TABLE 3.1

SUMMARY OF THE PURIFICATION OF THE CMV-INDUCED PARTICULATE RNA REPLICASE[a]

Step No	Fractions	Total Protein (mg)	Total Activity (units)		Specific activity (units/mg protein)		Yield (%)		Purification	
			CMV RNA	Poly (C)	CMV RNA	Poly (C)	CMV RNA	Poly (C)	CMV RNA	Poly (C)
1.	Solubilized supernatant	57.3	26.2	826	0.46	14.4	100	100	1	1
2.	PEG high salt supernatant	-[b]	30.2	962	-	-	116	117	-	-
3.	Phosphocellulose	2.1	14.0	480	6.7	228	53	58	14	16
4.	Poly(C)-cellulose	0.14	3.4	127	24.3	907	13	15	53	63

a Data were the average of three separate experiements, each starting with 200 g of CMV-infected cucumber seedlings. RNA replicase assays were done with CMV RNA and poly(C) as templates.

b The polyethyleneglycol in this fraction prevented the estimation of protein concentration.

B. STEP 2. POLYETHYLENEGLYCOL PRECIPITATION AND HIGH SALT ELUTION OF CMV-INDUCED PARTICULATE RNA REPLICASE

PEG-6000 at a final concentration of 11% (w/v) in the presence of 0.3 M NH_4Cl has been used successfully in the precipitation of the soluble RNA replicase (Kumarasamy and Symons, 1979a). However, under these conditions only 40 and 57% of the particulate RNA replicase activity was precipitated when assayed with yeast RNA and poly(C) as templates, respectively (Table 3.2). On increasing the concentration of PEG-6000 to 22% (w/v) in the presence of 0.55 M NH_4Cl, precipitation of particulate RNA replicase activity also increased to 61 and 108% when assayed with yeast RNA and poly(C) as templates, respectively (Table 3.2). This requirement for the higher concentration of PEG-6000 for the complete precipitation of RNA replicase was presumably due to the low protein concentration in the Step 1 enzyme extract.

The effect of variations of NH_4Cl concentration on the efficiency of PEG precipitation was also investigated and results obtained are given in Fig. 3.1. The optimum final concentration of NH_4Cl for maximum precipitation of RNA replicase activity was 0.4 to 0.6 M. The greater than 100% recovery of RNA replicase activity is presumably due to the removal of inhibitory materials. The enzyme was eluted from the precipitate in a buffer containing 2.0 M NH_4Cl as described by Kumarasamy and Symons (1979a). Step 2 enzyme was mainly free of endogenous template and pigmented plant material.

TABLE 3.2

PRECIPITATION OF CMV-INDUCED PARTICULATE RNA REPLICASE WITH POLYETHYLENEGLYCOL (PEG)

Experiment	RNA replicase activity relative to the initial crude enzyme extract[a]		
	None	Yeast RNA	Poly(C)
Crude particulate enzyme extract	100[b]	100[b]	100[b]
PEG (11%, w/v) precipitate	0	40	57
PEG (22%, w/v) precipitate	0	61	108

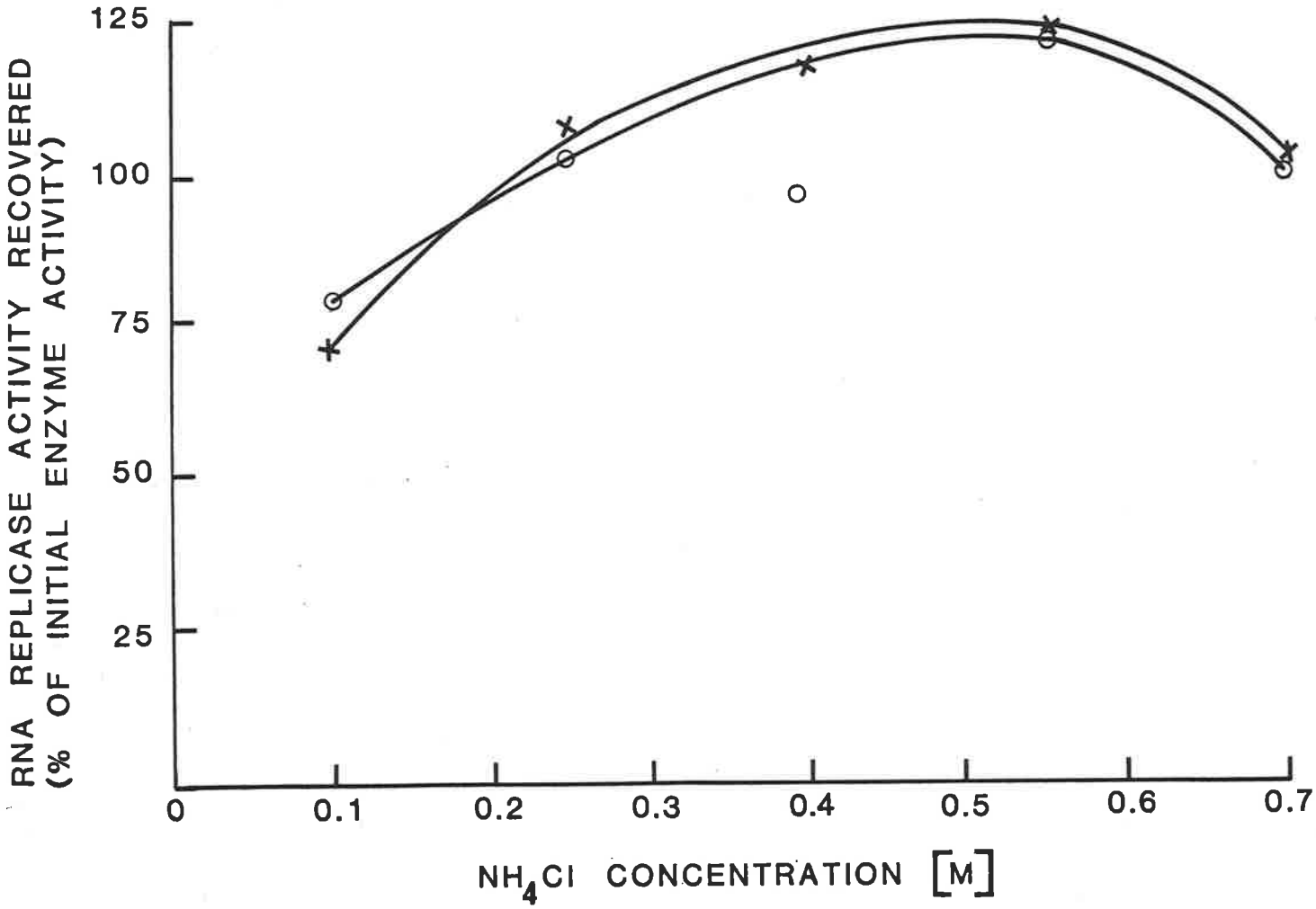
[a] The crude particulate enzyme extract of RNA replicase was prepared as described under Materials and Methods. Ten ml of the dialysed RNA replicase was precipitated with PEG at the indicated final concentration and enzyme was eluted in Buffer A as described under Materials and Methods. Enzyme (4 µl/assay) eluted in Buffer A was assayed for RNA replicase activity without any added template and with yeast RNA or poly(C) as templates.

[b] RNA replicase activity in crude particulate enzyme extract was 0.21, 0.82 and 43 nmoles of GMP incorporated per h per g of leaf material without any added template and with CMV RNA or poly(C) as templates, respectively.

FIGURE 3.1

EFFECT OF NH_4Cl CONCENTRATION ON PEG PRECIPITATION OF PARTICULATE RNA REPLICASE

The crude particulate RNA replicase extract was prepared from CMV-infected cucumber plants as described in Section 2.2I. The 4.0 ml of stock solutions of 44% PEG 6000 (w/v) and 50 mM 2-mercaptoethanol containing 0.1, 0.4, 0.7, 1.0 and 1.3 M NH_4Cl, respectively were mixed with each of the 4.0 ml samples of crude enzyme extract and stirred for 30 min. Precipitates were obtained by centrifugation at 22,000 g for 30 min at 4°C. The RNA replicase was eluted by resuspending the precipitate in 400 µl of Buffer A followed by centrifugation. The RNA replicase activity was measured using 3 µl of PEG step enzyme per 100 µl assay with CMV RNA (o——o) and poly(C) (×——×) as templates as described in Materials and Methods. Total enzyme activity recovered was expressed as percent of the total activity (1.0 and 19.5 units with CMV RNA and poly(C), respectively) in 4.0 ml of the crude enzyme extract.


RNA REPLICASE ACTIVITY RECOVERED
(% OF INITIAL ENZYME ACTIVITY)
125
100
75
50
25
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NH4Cl CONCENTRATION [M]

C. STEP 3. STEPWISE COLUMN CHROMATOGRAPHY ON PHOSPHO-CELLULOSE

The stepwise chromatography of the particulate RNA replicase on a phosphocellulose column was essentially as described by Kumarasamy and Symons (1979a). During this step PEG-6000 was replaced with glycerol. The recovery of enzyme activity in the purification of the particulate RNA replicase on the phosphocellulose column was 53 - 58% (Table 3.1).

D. STEP 4. STEPWISE COLUMN CHROMATOGRAPHY ON POLY(C)-CELLULOSE

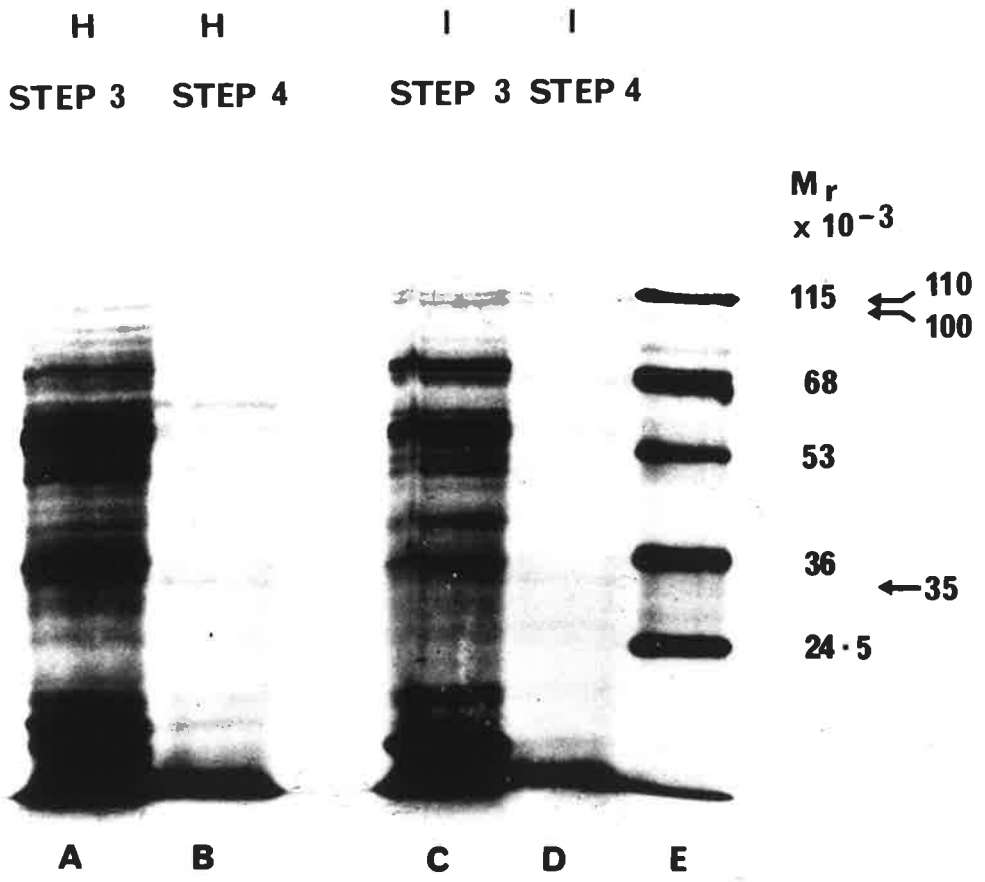
Phosphocellulose step enzyme was purified on a poly(C)-cellulose column after lowering the NH_4Cl concentration by dilution with buffer without NH_4Cl rather than by dialysis as described for the soluble enzyme (Kumarasamy and Symons, 1979a). The column was then washed thoroughly to remove non-specific proteins, a small amount of RNA replicase activity eluted during washing with buffer containing 0.2 M NH_4Cl. The recovery of enzyme activity in the purification step on poly(C)-cellulose column was only 25% (Table 3.1), but there was a high degree of protein purification obtained in this step (Fig. 3.2D).

The practical importance of the four step procedure of solubilization and purification is that it is rapid and can be completed within four days. The specific activity of the enzyme obtained was 24 and 907 units/mg of protein with CMV RNA and poly(C) as templates, respectively (Table 3.1). The apparent degree of purification on the basis of protein measurements was only 53 to 65 fold. This was

FIGURE 3.2

SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF STEP 3 AND STEP 4 PURIFIED FRACTIONS PREPARED FROM HEALTHY AND CMV-INFECTED CUCUMBER PLANTS

Small amounts of protein samples purified from the extract prepared from healthy (H) and CMV-infected (I) cucumber seedlings were labelled with ^{3}H and analysed on a 10% polyacrylamide slab gel as described in Materials and Methods. Molecular weight marker proteins (28,000 cpm) were run in a parallel track E. Protein bands were detected by fluorography at -70°C as described in Materials and Methods.


H
H
I
I
STEP 3
STEP 4
STEP 3
STEP 4
Mr
x 10-3
115
110
100
68
53
36
35
24·5
A
B
C
D
E

presumably due to the removal of most of the protein during the extensive washing of the particulate fraction prior to solubilization of the RNA replicase with $MgSO_4$.

No RNA replicase activity was observed at any of the steps when an extract from healthy plants was prepared and purified by the identical procedure. This is consistent with the lack of RNA replicase in the soluble phase of healthy cucumbers (Kumarasamy and Symons, 1979a).

E. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS OF STEP 3 AND STEP 4 ENZYME FRACTIONS

In order to assess the extent of purification and identify the virus-induced components of the particulate RNA replicase, small amounts of protein samples after phosphocellulose and poly(C)-cellulose chromatography were labelled with ^3H-KBH_4 by reductive methylation and analysed by SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. Figure 3.2 shows the comparison of the polypeptide patterns of Step 3 and Step 4 enzyme fractions obtained from CMV-infected plants and of corresponding column fractions when the extract from healthy plants was taken through the identical procedure of extraction and purification. The phosphocellulose step enzyme (Fig. 3.2C) contained a large number of polypeptides, as compared to the poly(C)-cellulose enzyme fraction (Fig. 3.2D), which contained two major polypeptides (M_r 110,000 and 100,000). This shows the great degree of purification achieved between Step 3 and Step 4. The main feature of Fig. 3.2 is the presence of two polypeptides (M_r 110,000 and 100,000) in the enzyme fractions from CMV-infected plants (Figs. 3.2C and D), which are

clearly absent from the corresponding fractions of the extract from healthy plants (Figs. 3.2A and B). There is an another polypeptide (M_r 35,000) unique to RNA replicase fractions which is of low intensity in Fig. 3.2, but more clearly visible in the results presented in subsequent chapters. These three polypeptides, unique to the particulate RNA replicase from CMV-infected cucumber plants, have also been reported to be specifically present in the highly purified soluble CMV-induced RNA replicase (Kumarasamy and Symons, 1979a).

Section 3.4 DISCUSSION

The four step procedure described here for the solubilization and partial purification is very convenient and fast. Although there was only 53 - 65 fold purification over the particulate crude RNA replicase extract, the specific activity of the purified enzyme obtained was very high (24 and 900 units/mg with CMV RNA and poly(C) as templates, Table 3.1) and was comparable with the extensively purified soluble CMV-induced RNA replicase (68 and 800 units/mg with CMV RNA and poly(C) as templates, Kumarasamy and Symons, 1979a) and also with the 95% purified Qβ replicase (100 and 800 units/mg with Qβ RNA and poly(C) as templates, respectively; Carmichael *et al.*, 1976; Blumenthal, 1979). The recovery of enzyme activity after Step 3 was low as compared to that obtained in a similar step in the purification of the soluble enzyme (Kumarasamy and Symons, 1979a). The four fold increase in the specific activity obtained in the Step 4 is less than one would expect from the difference in the protein composition

of the enzyme fractions of Step 3 and Step 4 shown in Fig. 3.2. This inconsistency in the increase in specific activity with the protein removed between the Step 3 and Step 4 may arise due to the loss of some functional sub-unit(s) of the enzyme in the final step of purification. This possibility has been considered more seriously in the extensive purification of the particulate RNA replicase described in Chapter Four.

The dominant feature of the particulate RNA replicase purified from CMV-infected cucumber seedlings is the presence of two high molecular weight polypeptides (M_r 110,000 and 100,000) and one low molecular weight poly-peptide (M_r 35,000). These three polypeptides along with others have been also reported to be present specifically in the purified soluble CMV RNA replicase (Kumarasamy and Symons, 1979a). The electrophoretic mobility of these three enzyme polypeptides is comparable to the *in vitro* translation products of the three largest RNAs of CMV, synthesized in plant and animal cell free translation systems (Schwinghamer and Symons, 1977). The possibility that these three poly-peptides are CMV gene products or host proteins induced as a result of viral infection has been investigated in detail and is described in Chapter Six.

CHAPTER FOUR

EXTENSIVE PURIFICATION OF CMV-INDUCED PARTICULATE RNA REPLICASE

Section 4.1 INTRODUCTION

The attempted extensive purification of the particulate RNA replicase described in this chapter was considered important from several points of view. The low recovery of enzyme activity in the Step 3 and the inconsistency in the four fold increase in the specific activity with the much greater degree of purification achieved as indicated from the comparison of the electrophoretic patterns of the Step 3 and Step 4 enzyme fractions of the four step procedure of purification described in the previous chapter demanded the improvement of this procedure or development of an equally efficient alternative method of purification. The important need of extensive purification was to find out which of the several polypeptides of the poly(C)-cellulose purified enzyme was/were required for RNA replicase activity. Considering all these factors, the particulate RNA replicase was subjected to two different schemes of purification. The extent of purification after each step was judged from the polypeptide composition of enzyme fractions analysed by SDS-gel electrophoresis. The soluble CMV RNA replicase was also purified for comparison with purified CMV-induced particulate RNA replicase.

Section 4.2 MATERIALS AND METHODS

A. GENERAL MATERIALS

Sepharose 4B and heparin were purchased from Pharmacia Fine Chemicals and Sigma Chemical Co., respectively. Cyanogen bromide was obtained from Ajax Chemicals (Australia). Cibacron blue F3GA agarose was from Amicon Corp. Other materials were as described in previous Chapters.

B. BUFFER SOLUTIONS USED IN THE PURIFICATION OF PARTICULATE RNA REPLICASE

Buffers A, B, C and D were as described in Chapter Three, Section 3.2B.

Buffer E: 20 mM Tris-HCl, pH 7.3, 30% glycerol (v/v), 30 mM 2-mercaptoethanol, 1 mM EDTA, and 10 mM Mg-acetate

Buffer F: 20 mM Tris-HCl, pH 8.5, 30% glycerol (v/v), 30 mM 2-mercaptoethanol, 1 mM EDTA, and 1.0 M NH_4Cl.

C. PREPARATION OF HEPARIN-SEPHAROSE

Sepharose 4B was activated with CNBr as described by March *et al.* (1974) and heparin was coupled to activated Sepharose by the method of Teissere *et al.* (1977). Heparin-Sepharose was stored at 4°C in 20 mM Tris-HCl, pH 8.5.

D. RNA REPLICASE ASSAY

RNA replicase activity was assayed as described in Section 2.2D except that assays with poly(C) as template contained 1.0 mM GTP as the only ribonucleotide.

E. THE COLUMN CHROMATOGRAPHY PROCEDURES USED IN THE ATTEMPTED EXTENSIVE PURIFICATION OF THE PARTICULATE RNA REPLICASE

All the operations of enzyme purification were carried out at 4°C. All equipment used was autoclaved beforehand. The PEG step enzyme was prepared from crude CMV-induced particulate RNA replicase as described in Section 3.2F. The procedures described here were used in most of the experiments; however, the size of columns and volume of enzyme samples used were varied occasionally.

F. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON PHOSPHOCELLULOSE

The chromatography of particulate RNA replicase on a phosphocellulose column was basically the same as described in Chapter Three. But from here onwards the PEG step enzyme was diluted by adding nine volumes of Buffer C instead of dialysis to lower the salt concentration. The 6 ml phosphocellulose column was equilibrated with Buffer C containing 0.2 M NH_4Cl. The diluted PEG step enzyme (60 to 80 ml) was loaded onto the pre-equilibrated column at a flow rate of 5 - 8 ml/h controlled with a peristaltic pump. The column was then washed with 12 ml of equilibration buffer and finally the enzyme was eluted with Buffer C containing 0.6 M NH_4Cl. Two ml fractions were collected using a Gilson fraction collector and were assayed for RNA replicase activity. The peak fractions containing RNA replicase activity were pooled and stored at -80°C until used.

G. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON HEPARIN-SEPHAROSE

An 8 ml column of heparin-Sepharose was washed with Buffer D containing 1.0 M NH_4Cl and 6 M urea, then with sterile glass distilled water, and finally equilibrated with 0.2 M NH_4Cl in Buffer D. 6 - 10 ml of phosphoecllulose step enzyme was diluted with two volumes of Buffer D and then loaded on the column at a flow rate of ~8 ml/h. The column was washed with two column volumes of equilibration buffer and then with four column volumes of Buffer D containing 0.3 M NH_4Cl. RNA replicase activity was eluted with 0.6 M NH_4Cl in Buffer D. Two ml fractions were collected and assayed for RNA replicase

activity. The peak column fractions with enzyme activity were pooled and stored at -80°C until further use.

H. STEPWISE COLUMN CHROMATOGRAPHY OF THE PARTICULATE RNA REPLICASE ON POLY(C)-CELLULOSE

The heparin-Sepharose or phosphocellulose step purified particulate RNA replicase was further purified on a poly(C)-cellulose (1 - 2 ml) column essentially as described in Chapter Three except that the enzyme was dialysed for two hours against 100 volumes of Buffer D prior to loading on the poly(C)-cellulose column pre-equilibrated with Buffer D containing 0.1 M NH_4Cl. The column was washed with two column volumes of the buffer used for equilibration and then with nine column volumes of Buffer D containing 0.2 M NH_4Cl. The RNA replicase was eluted with 1.0 M NH_4Cl in Buffer D. Fractions (0.5 - 1.0 ml) were collected and assayed for RNA replicase activity. Active fractions were pooled and stored at -80°C until used.

I. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON CIBACRON BLUE F3GA AGAROSE

A 3.0 ml column of Cibacron blue F3GA agarose was washed with 1.0 M NH_4Cl and 6 M urea in Buffer D, then with sterile glass distilled water and finally equilibrated with Buffer E containing 0.1 M NH_4Cl. Poly(C)-cellulose or phosphocellulose step purified enzyme (1.5 to 3 ml) was diluted by 10 fold or 6 fold, respectively, with Buffer E and loaded onto the column at the rate of 4.5 ml/h. The column was washed with four column volumes of the buffer used for equilibration. The RNA replicase activity was eluted with Buffer F, collecting 1 ml fractions. Fractions were

assayed for RNA replicase activity and active fractions were stored frozen at -80°C.

J. PURIFICATION OF SOLUBLE FORM OF CMV-INDUCED RNA REPLICASE

The first supernatant (soluble phase) from the extract prepared from CMV-infected cucumber plants (9th day after inoculation) by the procedure described in Section 2.2I was recovered for the purification of soluble RNA replicase. The RNA replicase was precipitated with PEG 6000 by adding one volume of stock solution containing 44% PEG 6000 (w/v), 1.0 M NH_4Cl and 50 mM 2-mercaptoethanol to three volumes of soluble phase and then stirring for 15 min (Kumarasamy and Symons, 1979a). The precipitate was recovered by centrifugation at 22,000 g for 15 min and the RNA replicase was eluted by resuspending the precipitate in Buffer A followed by centrifugation. The supernatant containing RNA replicase, called soluble PEG step enzyme, was further purified sequentially on a phosphocellulose, heparin-Sepharose and finally on a poly(C)-cellulose column in a similar way as described above for the particulate RNA replicase. The polypeptide composition at each step of purification was analysed by SDS-gel electrophoresis after labelling the protein samples with ^3H-KBH_4.

K. *IN VITRO* LABELLING AND SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF PROTEINS

The *in vitro* labelling of enzyme protein samples with ^3H-KBH_4 by reductive methylation, SDS-polyacrylamide slab gel electrophoresis and fluorography of labelled proteins were essentially as described in Section 3.2G, H and I, resp-

ectively.

Section 4.3 RESULTS

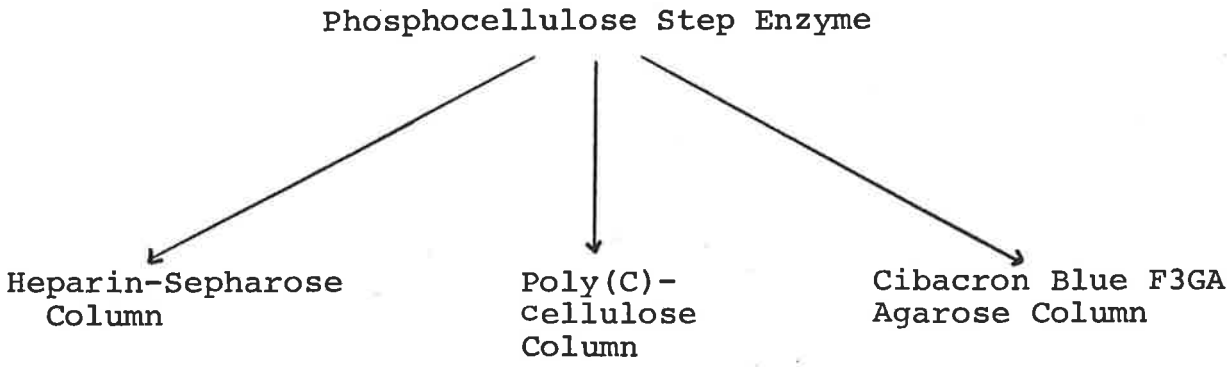
The purification of the particulate RNA replicase up to the phosphocellulose column was taken as the basic procedure, after which two further purification schemes were tried. In scheme A, phosphocellulose step enzyme was subjected to purification on a variety of chromatography columns to find an efficient purification step as an alternative to a poly(C)-cellulose column (Fig. 4.1A). In scheme B, phosphocellulose step enzyme was purified by three consecutive chromatography steps to find out which of the polypeptides co-purified with RNA replicase activity up to the final step (Fig. 4.1B). Chromatographic characteristics of the enzyme on each of the columns tried are given below.

A. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON PHOSPHOCELLULOSE

The low recovery of the enzyme activity after the phosphocellulose column reported in the previous chapter was found to be due to loss of RNA replicase activity during dialysis of the PEG step enzyme. However, 70 - 80% of the enzyme activity was recovered with high reproducibility when the salt concentration of the PEG step enzyme was lowered to 0.2 M by 10 X dilution rather than by dialysis, before loading on the phosphocellulose column. The results of a typical phosphocellulose column are given in Fig. 4.2A. The phosphocellulose step particulate RNA replicase contained several polypeptides (Figs. 4.3A and 4.4A). The two polypeptides (M_r 110,000 and ~15,000) were variably either of low intensity

FIGURE 4.1

A.


Phosphocellulose Step Enzyme
Heparin-Sepharose Column
Poly(C)-cellulose Column
Cibacron Blue F3GA Agarose Column

B.

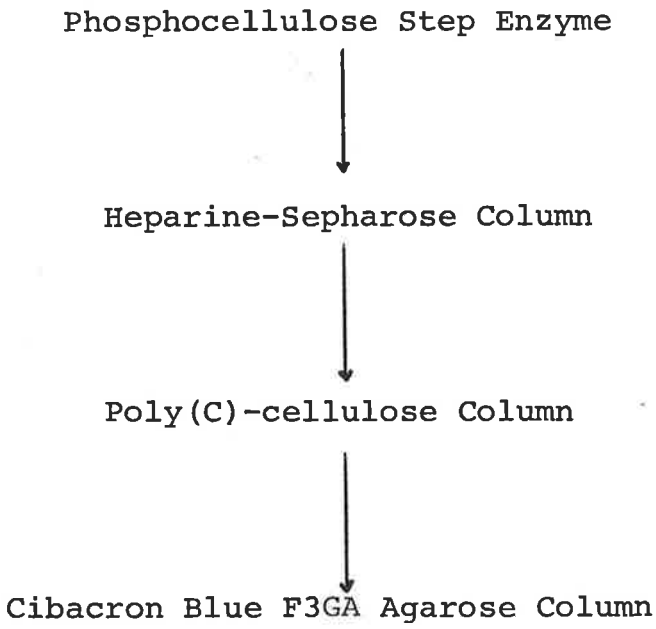

Phosphocellulose Step Enzyme
Heparine-Sepharose Column
Poly(C)-cellulose Column
Cibacron Blue F3GA Agarose Column

FIGURE 4.2

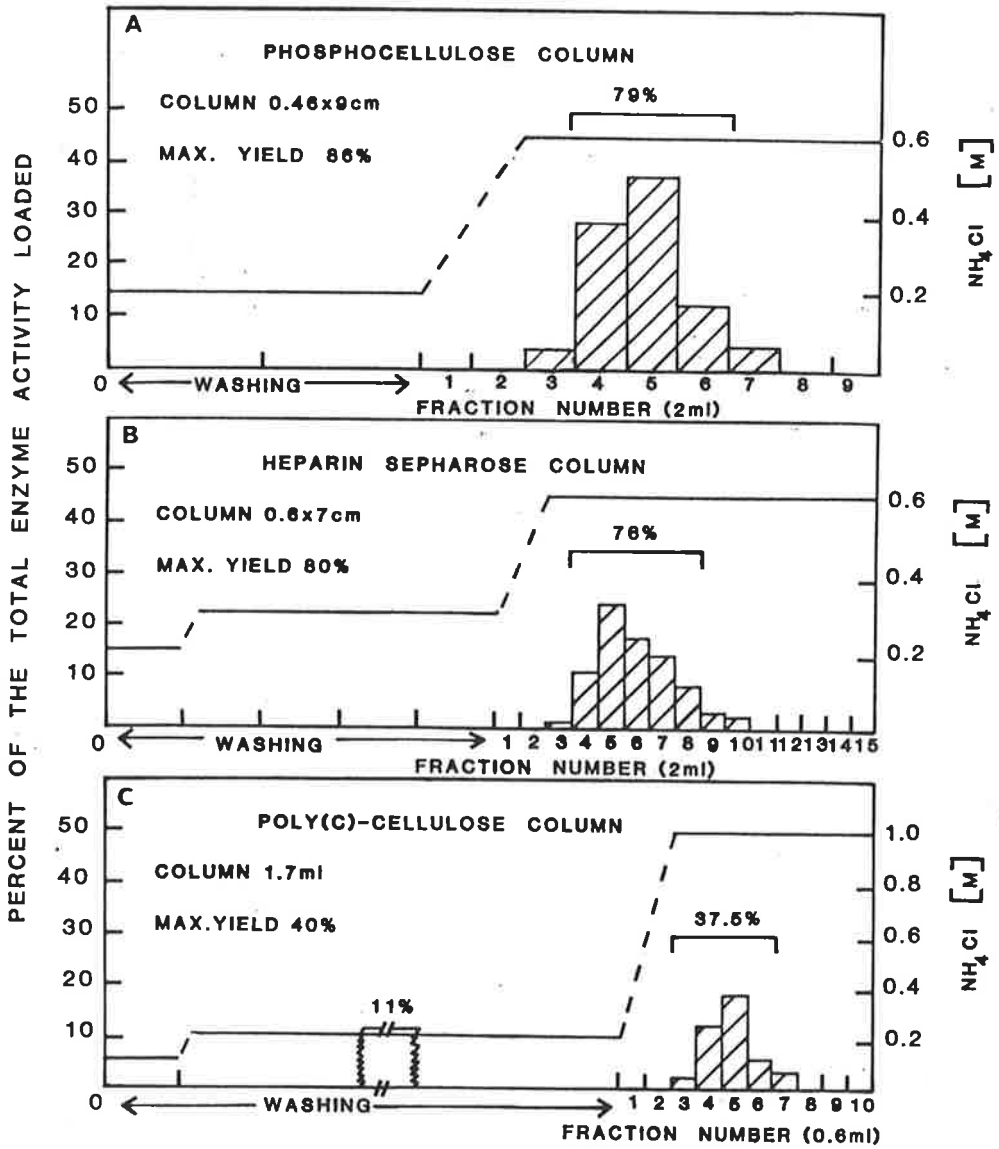

A
PHOSPHOCELLULOSE COLUMN
COLUMN 0.46x9cm
MAX. YIELD 86%
79%
WASHING
FRACTION NUMBER (2ml)
B
HEPARIN SEPHAROSE COLUMN
COLUMN 0.6x7cm
MAX. YIELD 80%
76%
WASHING
FRACTION NUMBER (2ml)
C
POLY(C)-CELLULOSE COLUMN
COLUMN 1.7ml
MAX.YIELD 40%
37.5%
11%
WASHING
FRACTION NUMBER (0.6ml)
PERCENT OF THE TOTAL ENZYME ACTIVITY LOADED
NH4Cl [M]

FIGURE 4.3

SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF PHOSPHOCELLULOSE STEP CMV PARTICULATE RNA REPLICASE FRACTIONS PURIFIED BY VARIOUS AFFINITY COLUMNS

The phosphocellulose step enzyme fractions (A) were purified by column chromatography, on heparin-Sepharose (B), on poly(C)-cellulose (E) or on Cibacron blue agarose (F), as described in Materials and Methods. Unbound protein passing through the heparin-Sepharose column and poly(C)-cellulose column during the washing stage are shown in (C) and (D), respectively. All protein samples were labelled with ^{3}H-KBH_4 by reductive methylation and run on the SDS-12% polyacrylamide slab gel as described in Materials and Methods. Molecular weights are derived from ^{3}H-labelled markers.

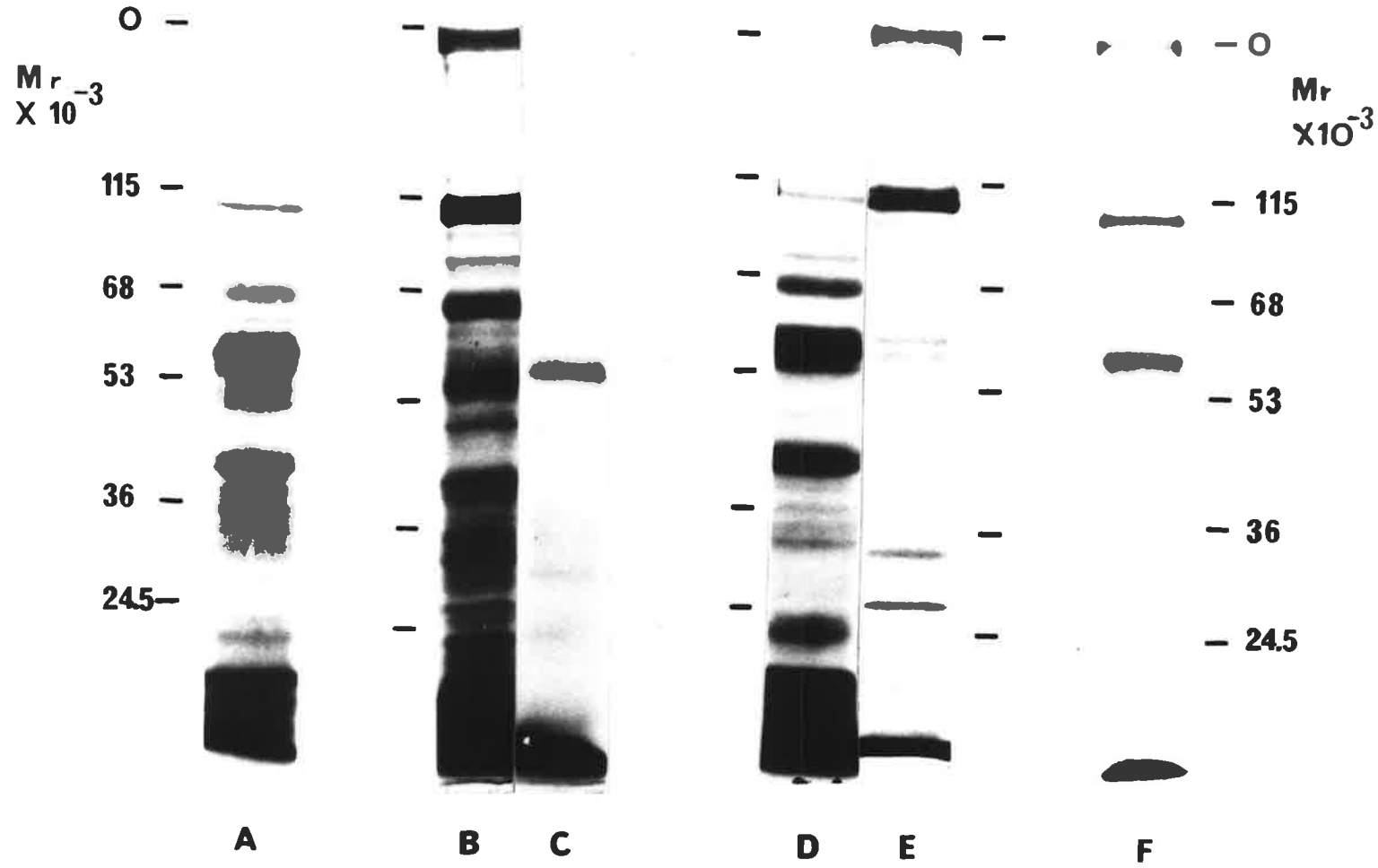
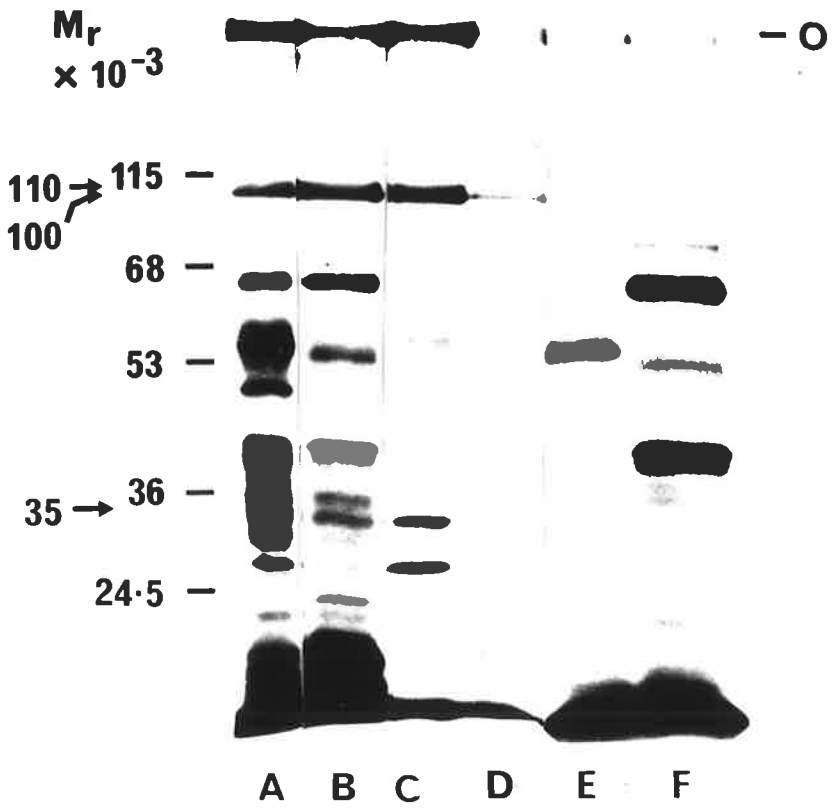

O
Mr
X 10-3
115
68
53
36
24.5
A
B
C
D
E
F
O
Mr
X10-3
115
68
53
36
24.5

FIGURE 4.4

SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF CMV PARTICULATE RNA REPLICASE AT VARIOUS STAGES OF SEQUENTIAL PURIFICATION

A preparation of phosphocellulose step enzyme (A), was sequentially purified by column chromatography in the following order: heparin-Sepharose (B), poly(C)-cellulose (C), and Cibacron blue agarose (D), as described in Materials and Methods. Unbound protein passing through the heparin-Sepharose column and through the poly(C)-cellulose column during the washing stage are shown in (E) and (F), respectively. All protein samples were labelled with ^{3}H-KBH_4 by reductive methylation and run on the same 12% polyacrylamide slab gel as described in Materials and Methods. Molecular weights are derived from ^{3}H-labelled markers (Kumarasamy and Symons, 1979b). O indicates the top of the gel.


Mr
× 10-3
O
110
100
115
68
53
35
36
24·5
A
B
C
D
E
F

or were completely absent from some enzyme preparations depending upon the time period for which enzyme fractions were kept stored before labelling with ^{3}H-KBH_4 (see Chapter Five, Section 5.3 J).

B. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON HEPARIN-SEPHAROSE

Heparin coupled to Sepharose has been successfully used in the purification of DNA-dependent RNA polymerase I, II and III (Spindler *et al.*, 1978; Sasaki *et al.*, 1979; Teissere *et al.*, 1977). As heparin is a potent inhibitor of CMV-induced RNA replicase (Section 5.3G), it was considered worthwhile to use it as an affinity ligand in the enzyme purification. Results of a typical experiment of chromatography of RNA replicase on heparin-Sepharose 4B column are given in Fig. 4.2B. The RNA replicase was bound to heparin-Sepharose in Buffer D containing 0.2 M NH_4Cl and was then washed with Buffer D containing 0.3 M NH_4Cl without any elution of enzyme activity. Recovery of total enzyme activity varied between 70 - 80%. When the column was developed with 0.6 M NH_4Cl in Buffer D, enzyme was eluted as a broad peak in about two column volumes, but a sharper peak could be obtained using 1.0 M NH_4Cl in elution buffer. The polypeptide pattern of a typical heparin-Sepharose step enzyme from the particulate fraction is shown in Figs. 4.3B, and 4.4B. A doublet of polypeptides (M_r ~59,000) along with trace amounts of several other polypeptides was mainly obtained on washing the heparin-Sepharose column with buffer containing 0.3 M NH_4Cl (Figs. 4.3C and 4.4E). Unfortunately, neither GTP (6 mM) nor poly(C) (200 µg/ml) in Buffer D containing 0.3 M NH_4Cl

could elute any RNA replicase activity from heparin-Sepharose.

C. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON POLY(C)-CELLULOSE

The results of a typical experiment on the purification of particulate CMV-induced RNA replicase on a poly(C)-cellulose column are given in Fig. 4.2C. Almost all the proteins in the enzyme samples either purified by a phosphocellulose column or by a heparin-Sepharose column bound to poly(C)-cellulose in the presence of 0.1 M NH_4Cl in Buffer D. However, on washing of the column with 0.2 M NH_4Cl in Buffer D most of the proteins were eluted (Figs. 4.3D and 4.4F, when the phosphoecllulose or heparin-Sepharose step enzyme fractions were purified, respectively). The strange feature of this step was that 10% of the CMV RNA and 15% of poly(C) copying activity of the total activity loaded also eluted from the column and was found spread throughout the washing step (Fig. 4.2C, wavy box) rather than as a separate distinct peak in the initial few fractions.

The main fraction of enzyme activity was eluted as a sharp peak with 1.0 M NH_4Cl in Buffer D. The recovery of CMV RNA-copying activity and poly(C)-copying activity in the main peak varied between 15 - 30% and 20 - 50% of the total activity loaded, respectively. The two enzyme fractions, obtained with 0.2 M NH_4Cl and 1.0 M NH_4Cl in Buffer D did not differ significantly in their ratio of CMV RNA copying activity to poly(C) copying activity (results not given). This observation and the uniform spreading of enzyme activity in the washing step with 0.2 M salt in Buffer D shows that the enzyme fractions

eluted with 0.2 M NH_4Cl and 1.0 M NH_4Cl are not two separate functional forms of enzyme but that the 0.2 M NH_4Cl fraction is due to slow elution (leaching) of RNA replicase activity.

The polypeptide patterns of phosphocellulose and heparin-Sepharose step enzyme fractions purified on a poly(C)-cellulose column is shown in Figs. 4.3E and 4.4C, respectively. In the purification step of RNA replicase on a poly(C)-cellulose column there was a high degree of protein purification obtained but there was unavoidable loss of some enzyme activity during the washing of the poly(C)-cellulose column with buffer containing 0.2 M NH_4Cl.

D. STEPWISE COLUMN CHROMATOGRAPHY OF PARTICULATE RNA REPLICASE ON CIBACRON BLUE F3GA AGAROSE

The Cibacron blue F3GA dye has been used as an affinity ligand in the purification of various enzymes of nucleic acid metabolism (Thompson *et al.*, 1975; Sugiura, 1980; Kumarasamy and Symons, 1979a). In our purification procedure for the particulate RNA replicase, the enzyme was bound to a Cibacron blue F3GA agarose column in the presence of 10 mM Mg-acetate and 0.1 M NH_4Cl in Buffer E, pH 7.3 and enzyme was eluted in the absence of Mg^{2+} with Buffer F which contained 1.0 M NH_4Cl at pH 8.5. The polypeptide pattern of the phosphocellulose step enzyme purified by Cibacron blue F3GA agarose (Fig. 4.3F) shows that several low molecular weight (M_r <53,000) polypeptides and a doublet at M_r ~58,000 were removed during this step. The recovery of CMV RNA and poly(C) copying activities was 68 - 85% of the total enzyme activity loaded onto the column.

When a Cibacron blue F3GA agarose column was used as the

final step in sequential purification scheme of Fig. 4.1B, only one major polypeptide (M_r 100,000) co-purified with the RNA replicase activity (Fig. 4.4D). The enzyme fraction could copy both CMV RNA and poly(C) and the recovery of total enzyme activity was 50 - 60% of the enzyme activity loaded onto the column. The results presented in Fig. 4.4 show that a major polypeptide (M_r 100,000), which co-purified with RNA replicase activity up to the final step of the sequential purification procedure, is associated with the catalytic activity of RNA replicase. Both poly(C) and CMV RNA copying activities were associated with this polypeptide. Recently, Baron and Baltimore (1982b) have shown that, in poliovirus RNA replicase, both poly(U)-polymerase and RNA replicase activities are associated with the single viral polypeptide p63.

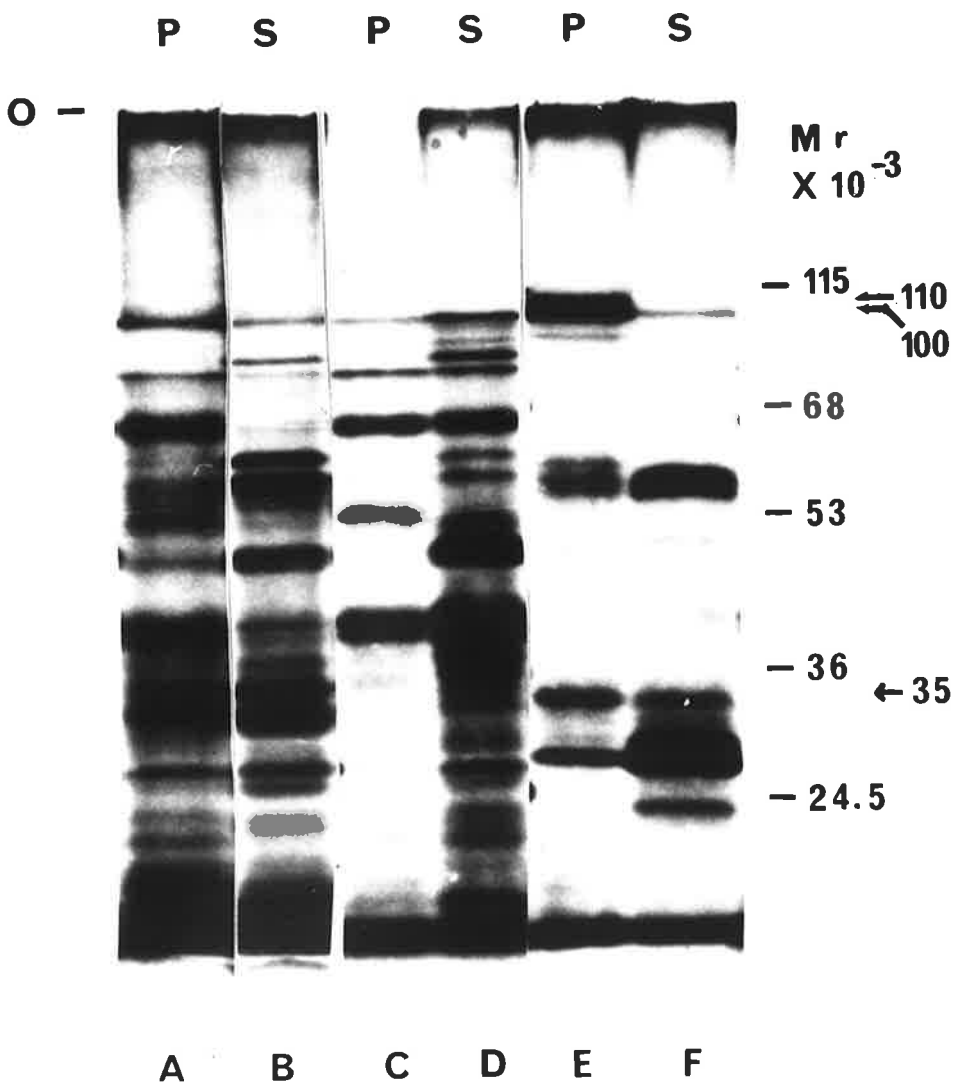
E. COMPARISON OF PROTEIN PATTERNS IN THE COLUMN FRACTIONS OF CMV-INDUCED PARTICULATE AND SOLUBLE RNA REPLICASE

The comparative polypeptide pattern of particulate and soluble CMV-induced RNA replicase fractions at the final two chromatography steps of purification is shown in Fig. 4.5. There was a great difference in the number of polypeptides in the enzyme fractions purified from the particulate fraction and the soluble phase up to the heparin-Sepharose column chromatography step (Figs. 4.5A and B). Even the 0.2 M NH_4Cl in Buffer D washing fraction from the poly(C)-cellulose column of the soluble enzyme (Fig. 4.5D) contained several polypeptides in addition to those present in the corresponding 0.2 M NH_4Cl washing fraction of the particulate enzyme (Fig. 4.5C). However, the particulate and soluble enzyme fractions

FIGURE 4.5

A COMPARISON OF PROTEINS IN PURIFIED FRACTIONS OBTAINED FROM CMV-INDUCED SOLUBLE AND PARTICULATE RNA REPLICASE

The preparations of soluble (S) and particulate (P) RNA replicase purified on a phosphocellulose column were sequentially purified by column chromatography on heparin-Sepharose and then on poly(C)-cellulose columns as described in Materials and Methods. Proteins in the column fractions were labelled with ^{3}H-KBH_4, electrophoresed on a SDS-12% polyacrylamide slab gel and fluorographed as described under Materials and Methods. Proteins in the soluble enzyme obtained after chromatography on heparin-Sepharose and poly(C)-cellulose columns were run in (B) and (F), respectively and those in the particulate enzyme obtained after chromatography on heparin-Sepharose and poly(C)-cellulose columns were run in (A) and (E), respectively. The enzyme proteins eluted with 0.2 M NH_4Cl in Buffer D from poly(C)-cellulose columns of soluble and particulate enzyme fractions were run in (D), and (C), respectively. Molecular weights are derived from ^{3}H-labelled marker proteins. O indicates the top of the gel.


P S P S P S
O –
Mr X 10-3
– 115
110
100
– 68
– 53
– 36
← 35
– 24.5
A B C D E F

eluted with 1 M NH_4Cl in Buffer D from the respective poly(C)-cellulose column had most of the polypeptides (M_r 110,000; 100,000 a doublet at ~58,000; 35,000 and 28,000) in common (Figs. 4.5E and F, respectively.) These polypeptides have been found in variable amounts in most RNA replicase fractions prepared in different ways (Figs. 4.3, 4.4, 4.5 and Kumarasamy and Symons, 1979a). However, two polypeptides of M_r ~22,000 and 29,500 were unique to the soluble enzyme whereas polypeptides of M_r 62,000 and 90,000 were unique to the particulate enzyme but their significance is unknown. There was a great difference in the stoichiometric ratio of the polypeptides (especially M_r 110,000; 100,000 and 28,000) of the CMV-induced RNA replicase purified from soluble and particulate fractions. This might be related to the stage of viral infection of plants at which plants were harvested since it is feasible that enzyme first appears in the membrane fraction and then leaches out into the soluble phase.

Section 4.4 DISCUSSION

All evidence indicates that the major polypeptide of M_r 100,000 which co-purified with both CMV RNA-copying and poly(C)-copying activities of RNA replicase up to the final step of our sequential chromatographic purification procedure described above is the catalytic subunit of CMV-induced RNA replicase. The poly(C)-cellulose step enzyme purified by the scheme of Fig. 4.1A, which is analogous to the partial purification scheme described in Chapter Three, contained seven major polypeptides and has been used in most of the work described in subsequent chapters. The seven polypeptides

(Fig. 4.3E) included two main virus-induced (Chapter Three) polypeptides of M_r 110,000 and 100,000, a triplet in the range of M_r 58,000 to 65,000; another virus induced polypeptide of M_r ~35,000 and a low M_r polypeptide (M_r 28,000). In addition to these seven major polypeptides two more low M_r polypeptides (M_r <15,000) and trace amounts of other polypeptides were occasionally present depending upon the extent of washing of the poly(C)-cellulose column before the elution of RNA replicase enzyme activity. The triplet of polypeptides between M_r 58,000 and 65,000 and two smaller polypeptides (M_r 35,000 and 28,000) were selectively removed from the enzyme fraction by chromatography on heparin-Sepharose and Cibacron blue agarose columns, respectively (Figs. 4.3B, C and F, respectively) without significant effect on the yield and catalytic properties of the RNA replicase. Therefore, these five polypeptides are not necessary for the catalytic activity of CMV-induced RNA replicase. Similarly, the virus-induced M_r 110,000 polypeptide was clearly absent from the enzyme fraction eluted with 0.2 M NH_4Cl in Buffer D from the poly(C)-cellulose column. In addition, it disappeared from enzyme fractions stored at -80°C, so it is also not required for catalytic activity of RNA replicase. The functional significance of these six polypeptides which co-purify with the catalytic subunit and RNA replicase activity up to the poly(C)-cellulose step from the soluble phase and the particulate fraction of CMV-infected cucumber plants is unknown. They may or may not be the components of the enzyme; if they are they could have some regulatory role in the replication reaction.

CHAPTER FIVE

CHARACTERIZATION OF CMV-INDUCED PARTICULATE RNA REPLICASE

Section 5.1 INTRODUCTION

The characterization of some enzymatic properties of the CMV-induced particulate RNA replicase described in this chapter was considered necessary from several points of view. The standard RNA replicase assay set by May and Symons (1971) was based on the characteristics of the soluble RNA replicase. It was quite feasible that the particulate RNA replicase might have different enzymatic characteristics than its soluble form. Therefor, optimum conditions for various components of the assay system were standardised for the particulate RNA replicase. For some of the enzymatic properties, optimization was carried out simultaneously for the phosphocellulose and poly(C)-cellulose step enzymes to see if these were affected during the course of enzyme purification.

Section 5.2 MATERIALS AND METHODS

A. GENERAL MATERIALS

RNase A, RNase T_1, heparin and sodium salts of salmon sperm DNA and calf thymus DNA were obtained from Sigma Chemical Co. Rifampicin was from Mann Research Laboratories, New York. Sephadex G-50 was obtained from Pharmacia Fine Chemicals, GF/A filters were from Whatman, England. Other materials were as described in previous chapters.

B. RNA REPLICASE ASSAY

The enzyme activity of CMV-induced RNA replicase was measured in the standard RNA replicase assay as described in Section 2.2D except that assays with poly(C) as template contained 1.0 mM GTP as the only ribonucleoside triphosphate and the other modifications as described in legends of respective

figures and tables. The phosphocellulose step purified CMV-induced particulate RNA replicase after purification on poly(C)-cellulose or on heparin-Sepharose as described in Chapter Four was used in the RNA replicase assay as indicated.

C. ALKALINE HYDROLYSIS OF THE ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED BY CMV-INDUCED RNA REPLICASE

The ^{32}P-labelled RNA synthesized in a standard RNA replicase assay containing α-^{32}P-GTP unlabelled ATP, UTP and CTP and CMV RNA as template was extracted twice with phenol and chromatographed over a 15 ml column of Sephadex G-50 in STE buffer (10 mM Tris-HCl, pH 7.3, 100 mM NaCl, 1 mM EDTA). The peak fractions of radioactively labelled RNA in the exclusion volume of the column were pooled, RNA precipitated with 2.5 volume of ethanol and collected by centrifugation. The RNA precipitate (20,000 cpm, Cerenkov counting) was dissolved in 50 µl of 10% piperidine (v/v) and incubated at 95^{o}C for 90 min in a tightly closed Eppendorf tube (Bock, 1967). After incubation, the contents of the tube were dried down under vacuum and then dissolved in 2 µl of water.

D. SEPARATION OF ALKALI HYDROLYSED RNA PRODUCTS OF CMV RNA REPLICASE BY TLC

The mixture of 2'(3')-mononucleotides, formed upon alkaline digestion of RNA products of CMV RNA replicase, was separated by two dimensional ascending thin layer chromatography on a Polygram plastic sheet, precoated with cellulose (Polygram: CEL 300 PEI/UV, (12 x 15 cm)). An aliquot of piperidine hydrolysed ^{32}P-labelled products was spotted with a glass capillary on the plastic sheet at 2 cm from either of the two edges. Separation in the first dimension was in H_2O:NH_4OH:

isobutyric acid (181:6.2:312 by volume) and in the second dimension was in H_2O:HCl:isopropanol (15:15:70 by volume) until the solvent front reached near the top (Dr. G. Bruening, personal communication). The developed chromatogram was air dried, covered with thin plastic film (Glad wrap) and autoradiographed at -80°C. The four radioactive spots were cut out with a razor and counted for ^{32}P in toluene based scintillation fluid to find their relative radioactivity.

E. RIBONUCLEASE TREATMENT OF THE ^{32}P-LABELLED RNA PRODUCTS

The ^{32}P-labelled RNA products synthesized by poly(C)-cellulose step enzyme (25 µl) in RNA replicase assay (V_f 500 µl) containing α-^{32}P-GTP (45 µCi) and CMV RNA (0.1 mg) were isolated by standard phenol-$CHCl_3$ (1:1) extraction and ethanol precipitation (see Section 7.2E) and digested with RNase A-T_1 mixture: (A), without melting as described by Duda *et al.* (1973), or; (B), after melting and annealing as described by Gould and Symons, (1977).

A. ^{32}P-labelled RNA samples (V_f 60 µl) were incubated at 37°C for 20 min with or without RNase A-T_1 mixture (20 µg/ml-0.4 µg/ml, respectively) under indicated conditions of salt used. 1.0 X SSC was 0.15 M NaCl and 0.015 M Na_3-citrate, pH 7.0. Digestion was terminated by the addition of 1.0 ml 10% TCA and then BSA (100 µg) was added as a carrier. After 30 min on ice TCA precipitates were collected onto GF/A filters, washed 4 x 5 ml with cold 5% TCA containing 2% Na_2HPO_4 and 2% $Na_4P_2O_7$ and twice with cold ether:ethanol (1:1). The radioactivity retained on filters was then counted by liquid scintilation spectrometery.

B. The 40 µl of ^{32}P-labelled RNA product in hybridization buffer (0.18 M NaCl, 0.01 M Tris-HCl, pH 7.0, 1 mM EDTA, 0.05%

SDS and 0.1 mg/ml CMV RNA) was transferred to a siliconised capillary tube (100 µl) which was then sealed at both ends. The capillary tubes were immersed in boiling water for 2 min and then incubated at 60°C for the indicated length of time. Annealing (hybridization) was terminated by chilling the capillaries on ice. The percent hybrid formation was assayed using ribonuclease T_1 and A. For the RNase assays contents of each capillary were added to 500 µl of 2 X SSC, 5% glycerol, 20 µg/ml *E. coli* rRNA. Two samples, each of 200 µl, were taken and to one was added RNase A-T_1 mixture (1.72 µg - 10 units, respectively), the other serving as a control. Both samples were then incubated at 37°C for 20 min. Undigested RNA·RNA hybrids were TCA precipitated, filtered, washed and the acid-insoluble radioactivity was measured as described above.

Section 5.3 RESULTS

A. PROPERTIES OF CMV-INDUCED PARTICULATE RNA REPLICASE

Table 5.1 shows the general properties of the particulate RNA replicase. The omission of Mg-acetate in the assay medium decreased the enzyme activity significantly but not completely because the enzyme sample itself contained 10 mM Mg-acetate. However, the effect of Mg^{2+} was studied separately (Section 5.3C). BSA was found necessary in the assay as a carrier. Absence of 2-mercaptoethanol from the assay medium decreased the CMV RNA copying activity but poly(C)-copying activity was increased. Actinomycin D (25 µg/ml) inhibited the replicase activity by 15 - 18%, its effect was further studied in detail (Section 5.3G). The presence of pyruvate kinase and phosphoenol pyruvate did not affect the CMV RNA copying activity

TABLE 5.1

PROPERTIES OF CMV-INDUCED PARTICULATE RNA REPLICASE

Reaction conditions	RNA replicase activity as percent of control with templates of	
	CMV RNA	Poly(C)
Complete[a]	100[b]	100[b]
- KCl	105	136
- $Mg(CH_3COO)_2$	11.2	59.3
- BSA	38.2	57.4
- 2-mercaptoethanol	80.3	109.3
+ Actinomycin D (2.5 µg/100 µl)	85.5	82.5
+ Pyruvate kinase (1 µg/100 µl) and phosphoenolpyruvate (2.5 mM)	100	36.8
+ Pi (10 mM)	65.8	65.3
+ PPi (5 mM)	18.4	10.7

a. The complete assay mixture (V_f 0.1 ml), contained 20 mM Tris-HCl, pH 8.5, 5 mM KCl, 13 mM Mg-acetate, 25 µg BSA, 25 mM 2-mercaptoethanol, NTP and CMV RNA or Poly(C) as described in Materials and Methods. Reaction was started by adding 7 µl of poly(C)-cellulose step enzyme. Incubations were at 37°C for 1 h. All the assays contained ^{32}P-GTP (0.5 µCi).

b. The RNA replicase activity of complete assay mixture was taken as 100% and treated as control. The control values with CMV RNA and poly(C) as templates were 1.52 and 23.5 nmoles of GMP incorporated per h per assay, respectively.

but due to some unknown reason poly(C)-copying activity was inhibited by 63% in the presence of pyruvate kinase and phosphoenol pyruvate. PPi inhibited the CMV-induced RNA replicase activity showing the presence of RNA-dependent RNA polymerase activity. The significant inhibition (35%) by Pi was not clear. Omission of CTP or UTP from the enzyme assay mixture strongly reduced the incorporation of α-^{32}P-GTP (Table 5.2), the significant incorporation (16%) observed in the absence of added ATP was due to the presence of unlabelled ATP in α-^{32}P-GTP preparation (Symons, 1977). The omission of three of the four ribonucleosidetriphosphates from the enzyme assay resulted in the total absence of the incorporation (Table 5.2).

B. EFFECT OF NH_4Cl CONCENTRATION IN THE ASSAY MEDIUM ON ENZYME ACTIVITY OF PARTICULATE RNA REPLICASE

NH_4Cl, being a constituent of most of the buffer solutions of enzyme samples is added in variable amounts in the assay medium along with enzyme protein. Figure 5.1 shows the RNA replicase activity of poly(C)-cellulose step enzyme and phosphocellulose step enzyme with CMV RNA and poly(C) as templates, at various concentrations of NH_4Cl in the assay medium. The enzyme activity with both the templates was maximum between 40 - 80 mM NH_4Cl. CMV RNA copying activity was more sensitive to higher concentrations of NH_4Cl as it started declining after 80 mM NH_4Cl whereas poly(C)-copying activity stayed at the plateau level up to 150 mM NH_4Cl after which it declined rapidly. The effect of NH_4Cl at different concentrations on poly(C)-copying activity with poly(C)-cellulose step enzyme and phosphocellulose step enzyme was essentially the same.

TABLE 5.2

REQUIREMENT FOR ALL THE FOUR NUCLEOTIDE TRIPHOSPHATES

Reaction mixture	Radioactivity incorporated/h/assay (cpm)	
Complete[a]	46900[b]	(100%)
- ATP	7460	(16%)
- CTP	1084	(2.3%)
- UTP	2728	(5.8%)
- ATP - CTP - UTP	334	(<1%)

a. Complete assay (0.1 ml) contained 0.12 mM GTP and 0.6 mM each ATP, CTP and UTP. All the assays contained poly(C)-cellulose step enzyme (5 µl), CMV RNA (10 µg), α-^{32}P-GTP (0.48 µCi) and other components of RNA replicase assay as described in Materials and Methods.

b. Results are expressed as acid-insoluble radioactivity incorporated after 1 h incubation at 37°C. A background of 448 cpm incorporated in control assays without enzyme was subtracted from all the values given.

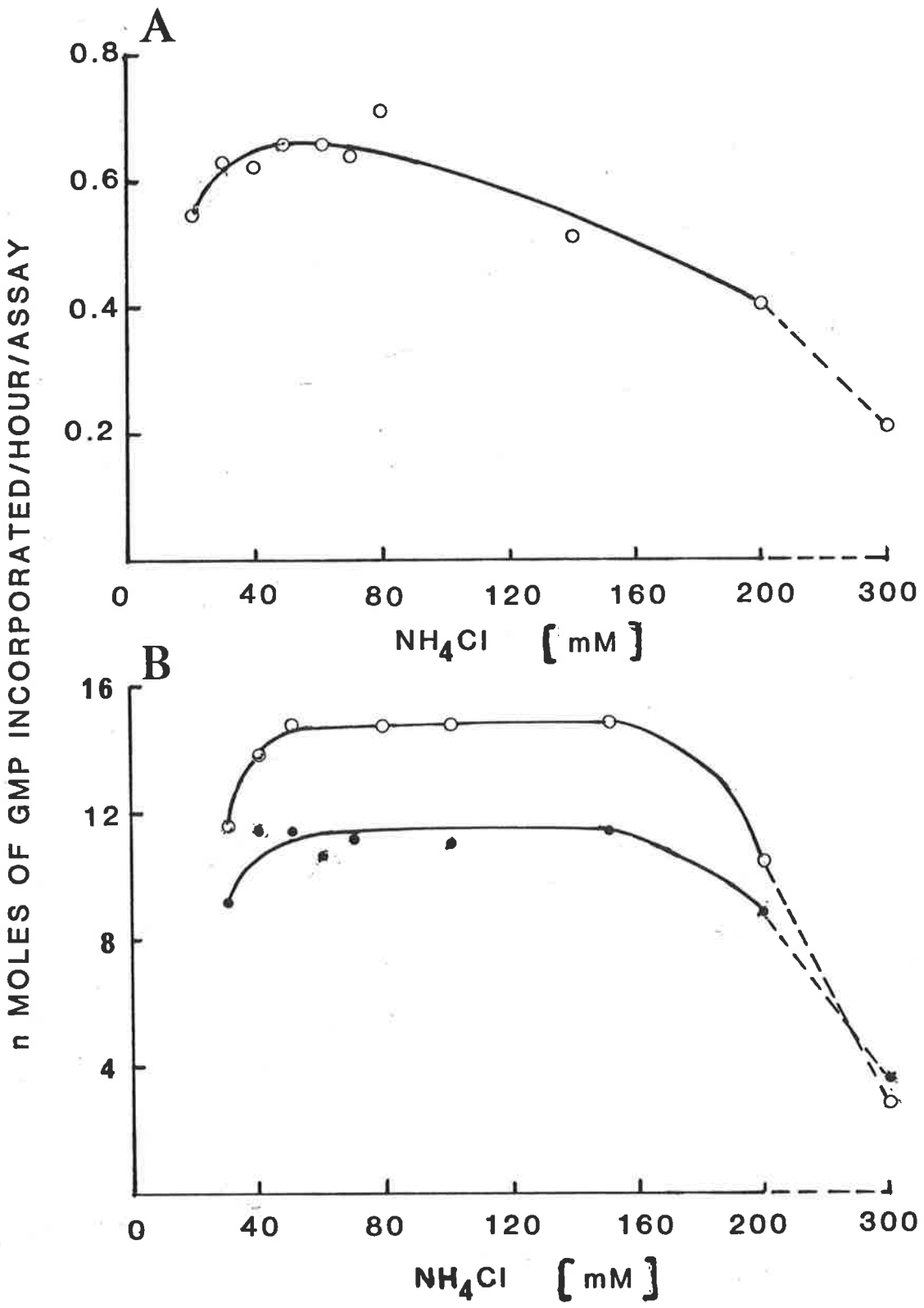
FIGURE 5.1

EFFECT OF NH_4Cl CONCENTRATION IN ASSAY MEDIUM ON ENZYME ACTIVITY OF PARTICULATE RNA REPLICASE

The RNA replicase enzyme activity was assayed with CMV RNA (10 µg/100µl) and poly(C) (20 µg/100 µl) using poly(C)-cellulose step enzyme (2 µl in A, 3 µl in B per assay) or phosphocellulose step enzyme (5 µl per assay) as described under Materials and Methods except that different indicated amounts of NH_4Cl were used in the assays. The incubations were at 37°C for 1 h after which acid precipitable radioactivity was measured as described in Materials and Methods and expressed as nmols of GMP incorporated per h per assay.

A. Poly(C)-cellulose step enzyme with CMV RNA as template

B. Poly(C)-cellulose step enzyme (o—o) and phosphocellulose step enzyme (•——•) with poly(C) as template.


A
B
n MOLES OF GMP INCORPORATED/HOUR/ASSAY
0.8
0.6
0.4
0.2
16
12
8
4
0
40
80
120
160
200
300
NH4Cl [mM]

C. EFFECT OF Mg-ACETATE CONCENTRATION IN THE ASSAY ON THE ENZYME ACTIVITY OF PARTICULATE RNA REPLICASE

The effect of Mg-acetate in the assay on the enzyme activity of poly(C)-cellulose and phosphocellulose step particulate RNA replicase with CMV RNA and poly(C) templates is shown in the Fig. 5.2. As with NH_4Cl, CMV RNA copying activity of the poly(C)-cellulose step enzyme was seen to be more sensitive than the poly(C) copying activity at higher concentrations of Mg-acetate. The former declined slowly after 16 mM Mg-acetate, while the latter stayed at a plateau level up to 40 mM Mg-acetate (Fig. 5.2). The Mg-acetate concentration between 8 - 16 mM was a common optimum for both CMV RNA and poly(C) templates. In most of the RNA replicase assays 13 mM Mg-acetate was used. The RNA replicase activity of the phosphocellulose step enzyme in the absence of Mg-acetate was less than 4% of the maximum at 13 mM (Fig. 5.2B) showing the absolute requirement of Mg^{2+} ion for enzyme activity of the RNA replicase. The enzyme activity of poly(C)-cellulose step in the absence of Mg^{2+} was not determined because the enzyme sample itself contained 10 mM Mg-acetate. The effect of Mg-acetate concentration in the RNA replicase assay on the enzyme activity of poly(C)-cellulose and phosphocellulose step enzyme with poly(C) as template was essentially the same (Fig. 5.2B).

D. ENZYME ACTIVITY OF PARTICULATE RNA REPLICASE AS A FUNCTION OF GTP CONCENTRATION IN THE ASSAY MEDIUM

The enzyme activity of the particulate RNA replicase with CMV RNA and poly(C) templates as a function of GTP substrate is shown in Figs. 5.3A and B, respectively, along with their

FIGURE 5.2

EFFECT OF Mg-ACETATE CONCENTRATION IN ASSAY ON ENZYME ACTIVITY OF PARTICULATE RNA REPLICASE

The enzyme activity of CMV-induced particulate RNA replicase was assayed with either CMV RNA (10 µg per assay) or poly(C) (20 µg per assay) using poly(C)-cellulose step enzyme (5 µl per assay) or phosphocellulose step enzyme (3 µl per assay) as described in Materials and Methods except different indicated concentrations of Mg-acetate were used. The incubations were at 37°C for 1 h after which acid precipitatable radioactivity was measured as described in Section (2.2D) and expressed as nmoles of GMP incorporated per h per assay.

A. Poly(C)-cellulose step enzyme with CMV RNA template.

B. Poly(C)-cellulose step enzyme (o—o) and phospho-cellulose step enzyme (•—•) with poly(C) template.

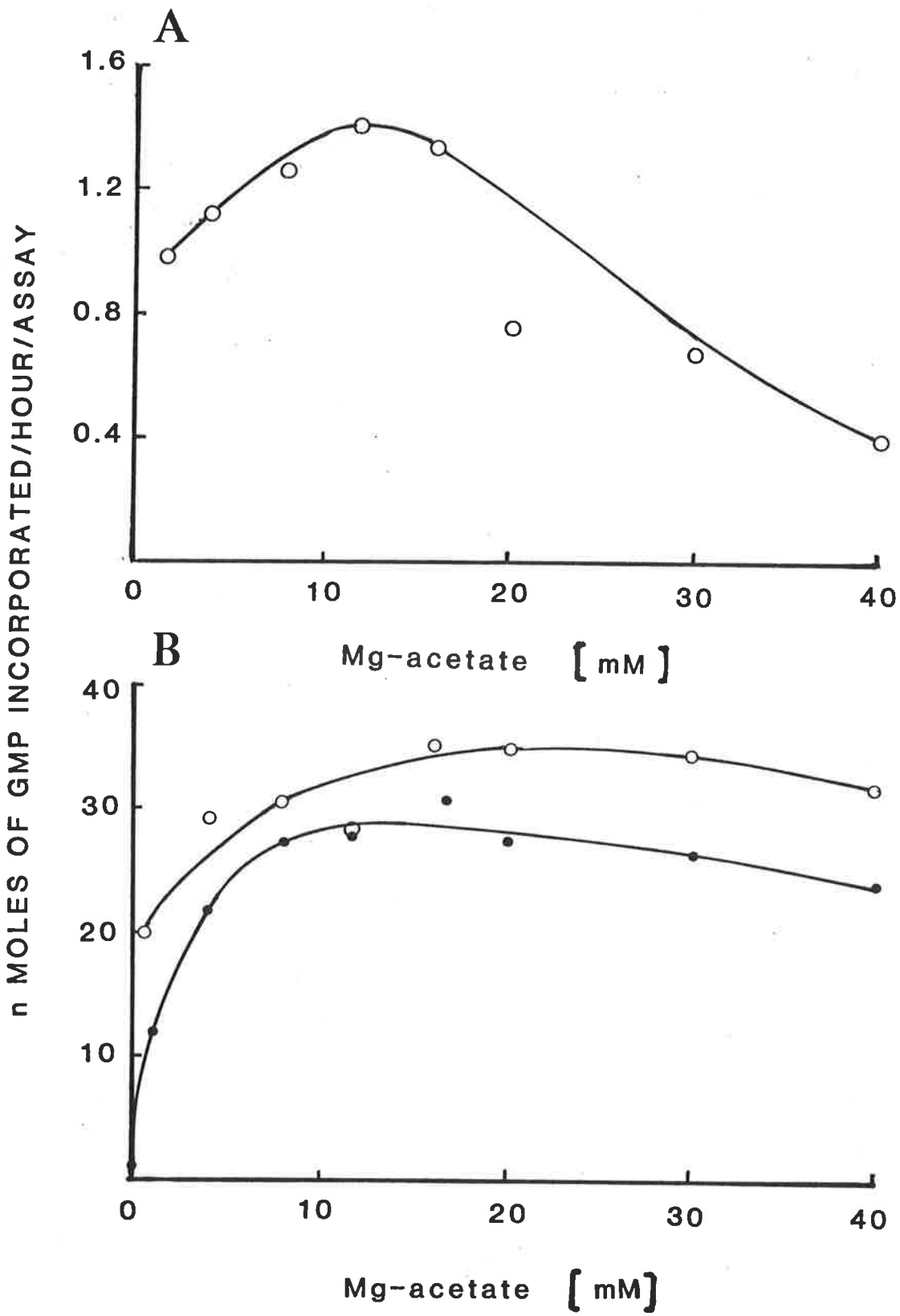

A
1.6
1.2
0.8
0.4
0
10
20
30
40
Mg-acetate [mM]
B
40
30
20
10
0
10
20
30
40
Mg-acetate [mM]
n MOLES OF GMP INCORPORATED/HOUR/ASSAY

FIGURE 5.3

RNA REPLICASE ACTIVITY AS A FUNCTION OF GTP CONCENTRATION IN ASSAY MEDIUM CONTAINING CMV RNA OR POLY(C) AS TEMPLATE

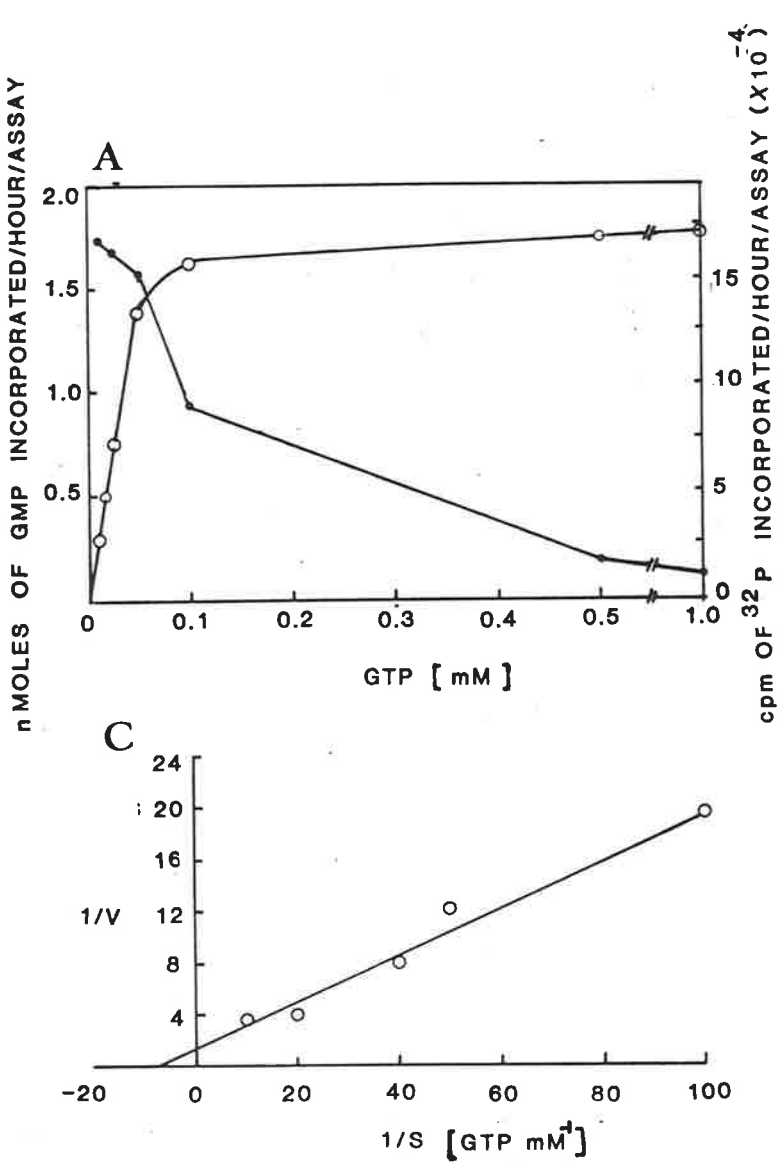
The RNA replicase activity of poly(C)-cellulose step enzyme with CMV RNA and poly(C) as templates at indicated concentrations of GTP was measured as described in Materials and Methods. (A), The 100 µl assays contained 5 µl of enzyme sample, 10 µg CMV RNA, 0.6 mM each of unlabelled UTP, CTP, ATP and 0.01 to 1.0 mM unlabelled GTP. (B), The 100 µl assays contained 3 µl of enzyme sample, 20 µg poly(C), 0.1 to 1.5 mM unlabelled GTP as the only ribonucleotide. α-^{32}P-GTP (0.25 and 0.35 µCi) was included in each of the assays of A and B, respectively. After 1 h incubations at 37°C acid-insoluble radioactivity was measured and expressed as ^{32}P-cpm (•—•) and nmols of GMP (o—o) incorporated per h per assay. Results given are average of duplicate assays.

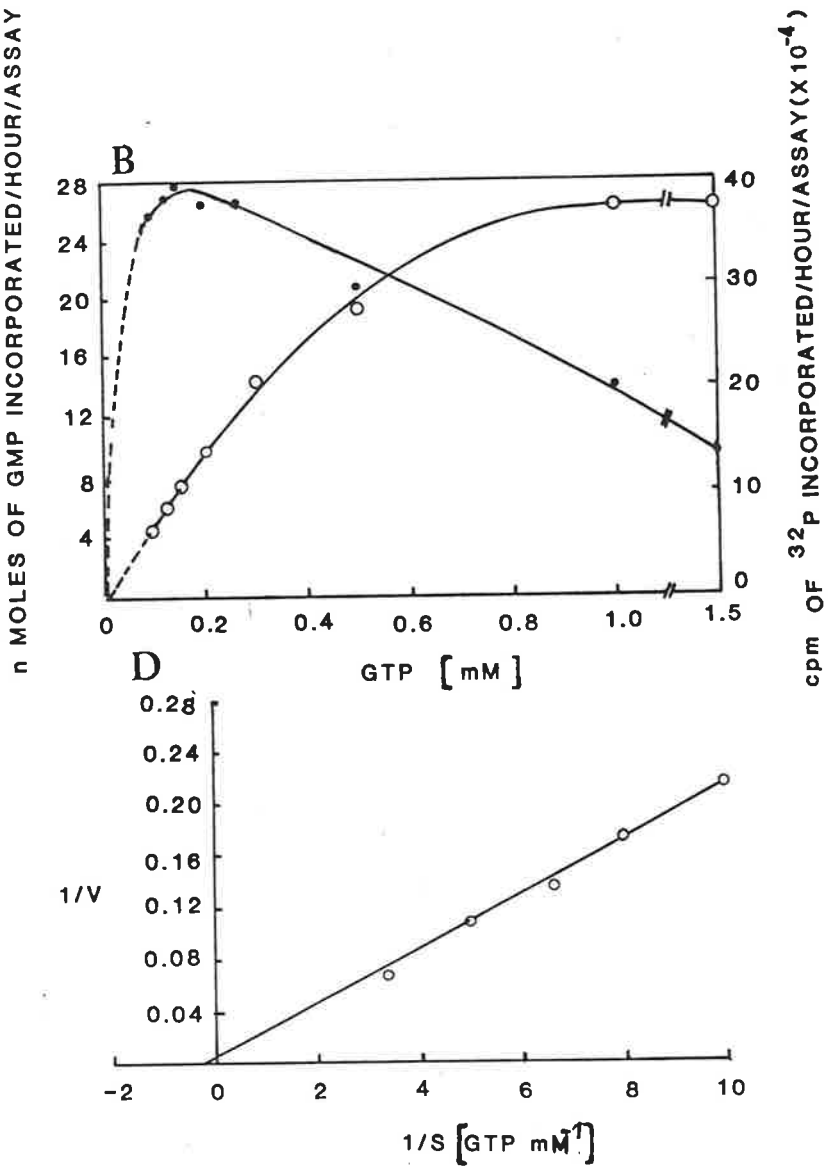
A. CMV RNA copying activity as a function of GTP concentration.

B. Poly(C)-copying activity as a function of GTP concentration.

C. Double reciprocal plot of (A).

D. Double reciprocal plot of (B).


A
n MOLES OF GMP INCORPORATED/HOUR/ASSAY
cpm OF ^{32}P INCORPORATED/HOUR/ASSAY (X10^{-4})
GTP [mM]
C
1/V
1/S [GTP mM^{-1}]


B
n MOLES OF GMP INCORPORATED/HOUR/ASSAY
cpm OF ^{32}P INCORPORATED/HOUR/ASSAY(X10^{-4})
GTP [mM]
D
1/V
1/S [GTP mM^{-1}]

respective double reciprocal plots (Figs. 5.3C & D). The CMV RNA copying activity of poly(C)-cellulose step enzyme increased linearly up to 0.05 mM GTP and reached at substrate saturation level at 0.1 mM GTP (Fig. 5.3A). The 0.12 mM GTP was used in the standard RNA replicase assays with CMV RNA as template. This is the same concentration which has been used for soluble CMV RNA replicase (Kumarasamy and Symons, 1979a). There was a significant incorporation of label at 0.12 mM GTP whereas at higher GTP concentrations (as demanded by the high Km values of 0.14 mM (Fig. 5.3C) to saturate the assay system) the incorporation of the label was very low (Fig. 5.3A).

The poly(C)-copying activity of the poly(C)-cellulose step enzyme increased uniformly up to 1.0 mM GTP and thereafter seems to reach a saturation level (Fig. 5.3B). The maximum incorporation of the label was observed at 0.2 mM GTP thereafter it decreased slowly. 1.0 mM GTP concentration, which apparently saturated the assay system, was used in standard assays with poly(C) as template irrespective of the higher GTP concentration demanded by the high Km value (3.3 mM, Fig. 5.3D). Unfortunately, the assays of Figs. 5.3A and B do not meet the ideal conditions of the Henri-Michaelis-Menten equation because about 25% of the GTP substrate had been used over the assay period due to the high specific activity of the enzyme sample used in these assays. Therefore the differences in the observed high Km values (0.14 and 3.3 mM GTP with CMV RNA and poly(C) as templates, respectively, Figs. 5.3C and 5.3D) for the particulate RNA replicase and those of its soluble form (0.03 and 0.75 mM GTP with CMV RNA and poly(C) as templates, Kumarasamy and Symons, 1979a) were not considered significant. The apparent Km values for GTP with poly(C) as

template for the poly(C)-cellulose step enzyme (3.3 mM, Fig. 5.3D) and for the phosphocellulose step enzyme (3.6 mM, data not given) were not significantly different.

E. ENZYME ACTIVITY OF CMV-INDUCED PARTICULATE RNA REPLICASE AS A FUNCTION OF TEMPLATE CONCENTRATION

CMV RNA and poly(C)-copying activities of poly(C)-cellulose step enzyme increased linearly with template concentration up to about 2.0 µg of CMV RNA and 10 µg of poly(C) per 100 µl of assay medium, respectively (Fig. 5.4). The amount of template required to saturate 5 µl of poly(C)-cellulose step enzyme in 100 µl of assay medium was found to be approximately 10 and 15 µg in the case of CMV RNA and poly(C), respectively, under the given assay conditions.

F. THE RNA REPLICASE ACTIVITY AS A FUNCTION OF TIME OF INCUBATION OF ASSAY REACTION

The Fig. 5.5 shows the RNA replicase activity of poly-(C)-cellulose and phosphocellulose step enzyme with CMV RNA and poly(C) templates as a function of time. The RNA replicase activity was essentially linear up to 90 and 60 min with CMV RNA (Fig. 5.5A) and poly(C) (Fig.5.5B) templates, respectively, and thereafter a slower increase in GTP incorporation with time up to 240 min was observed. GMP incorporation with both the enzyme fractions increased in a similar fashion with CMV RNA and poly(C) templates, respectively (Fig. 5.5A and B). A time of 60 min was used in standard RNA replicase assays.

G. EFFECT OF ACTINOMYCIN D, RIFAMPICIN AND HEPARIN ON ENZYME ACTIVITY OF PARTICULATE RNA REPLICASE

Actinomycin D is known to inhibit DNA-dependent RNA polymerases of bacteria and of eukaryotic organelles as a result

FIGURE 5.4

ENZYME ACTIVITY OF CMV-INDUCED PARTICULATE RNA REPLICASE AS A FUNCTION OF TEMPLATE CONCENTRATION

The enzyme activity of poly(C)-cellulose step purified RNA replicase (5 µl/100 µl assay) was determined as described under Materials and Methods except different indicated amounts of CMV RNA or poly(C) as template were used. Incubations were at 37°C for 1 h. The enzyme activity was expressed as nmoles of ^{32}P-GMP incorporated per h per assay.

A. CMV RNA as template (o—o)

B. Poly(C) as template (×—×)

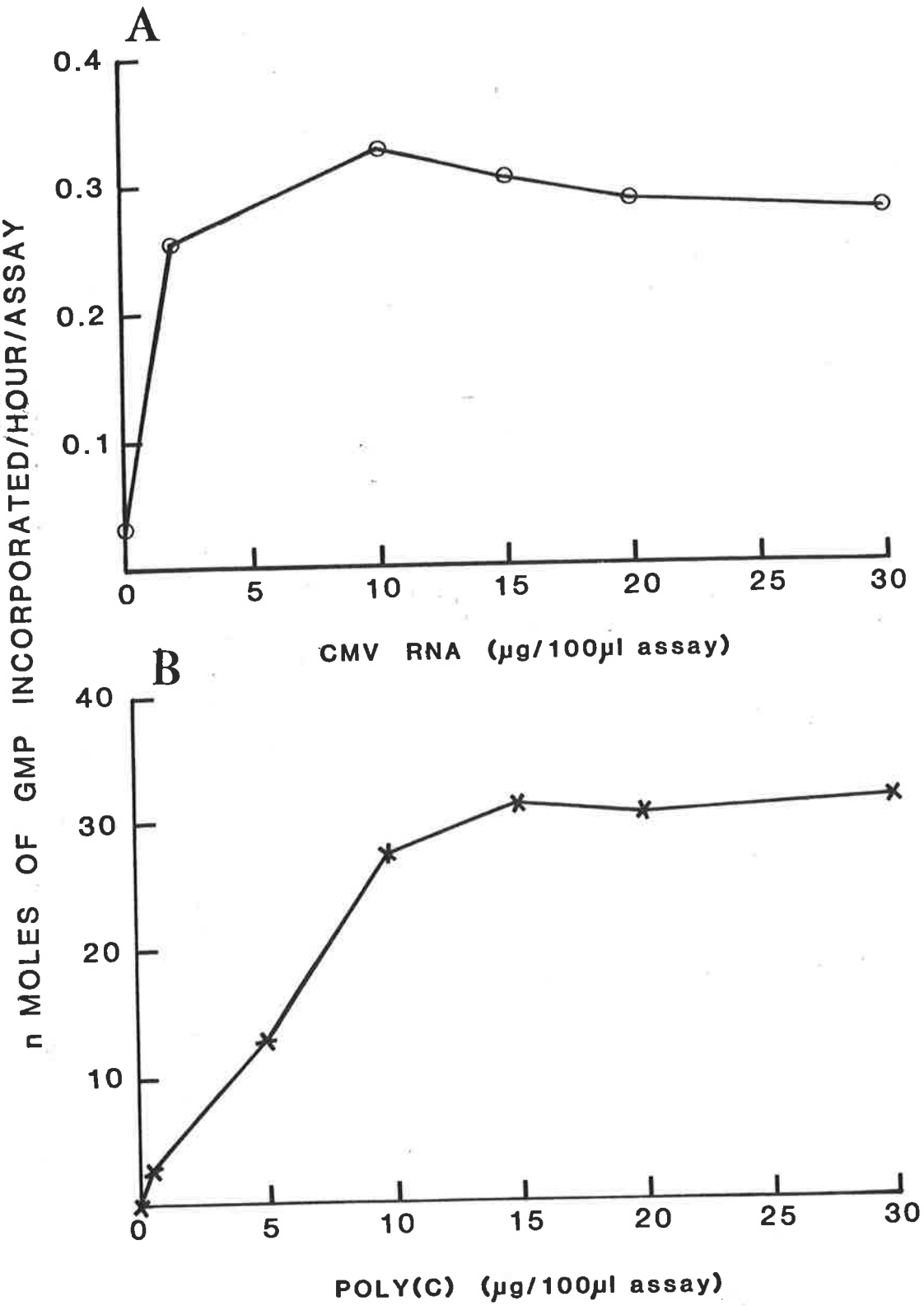

A
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
CMV RNA (µg/100µl assay)
B
40
30
20
10
0
5
10
15
20
25
30
POLY(C) (µg/100µl assay)
n MOLES OF GMP INCORPORATED/HOUR/ASSAY

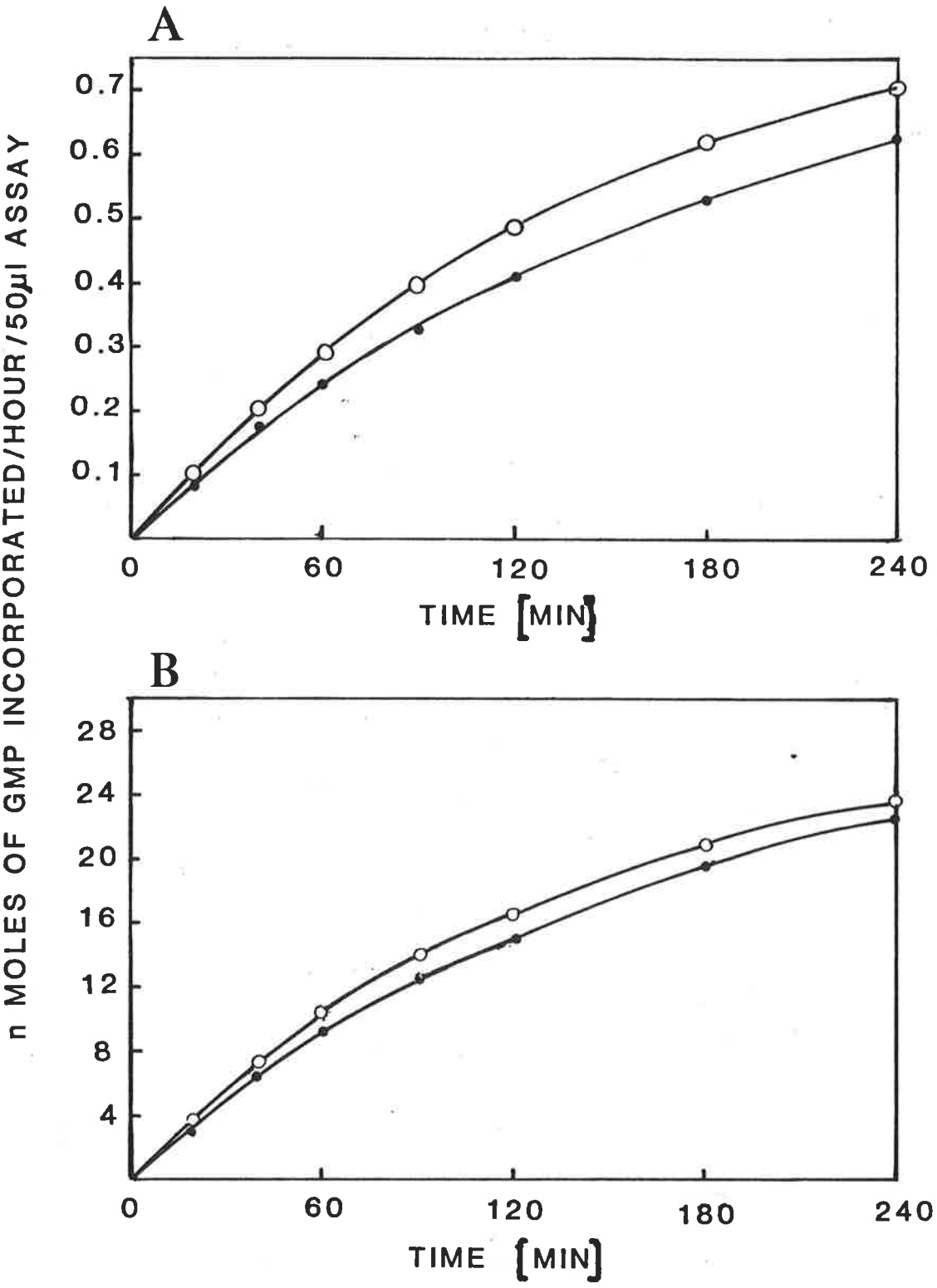
FIGURE 5.5

RNA REPLICASE ACTIVITY AS A FUNCTION OF TIME OF INCUBATION OF ASSAY REACTION

The RNA replicase reaction mixtures (1 ml) containing phosphocellulose step enzyme (15 µl/ml) or poly-(C)-cellulose step enzyme (12 µl/ml) with CMV RNA or poly-(C) as templates were assayed at 37°C as described in Materials and Methods. At the indicated times, duplicate aliquots (50 µl) were removed and acid-insoluble radio-activity was measured. Results were expressed as nmoles of GMP incorporated per 50 µl after indicated times.

A. CMV RNA template with
   i. poly(C)-cellulose step enzyme (o—⊖)
   ii. phosphocellulose step enzyme (•—•)

B. Poly(C) template with
   i. poly(C)-cellulose step enzyme (o—o)
   ii. phosphocellulose step enzyme (•—•)


A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
60
120
180
240
TIME [MIN]
B
28
24
20
16
12
8
4
0
60
120
180
240
TIME [MIN]
n MOLES OF GMP INCORPORATED/HOUR/50µl ASSAY

of its interaction with the DNA template. 50 - 100% inhibition of T3 and *E. coli* RNA polymerases was observed at actinomycin D concentrations of 0.3 - 20 µg/ml (Kupper *et al.*, 1973; Chamberlin and Ring, 1973). When actinomycin D was used in assays of CMV RNA replicase, a 50% inhibition of CMV RNA replicase was observed with 0.84 to 1.1 mg/ml of actinomycin D (Fig. 5.6B). The requirement of a high concentration of actinomycin D indicated that inhibition of CMV RNA replicase with the antibiotic was non-specific.

Rifampicin inhibits bacterial DNA-dependent RNA polymerase activity *in vitro* at the chain initiation step, and was effective at the concentration of 0.1 to 20 µg/ml for complete inhibition (Bandle and Weissmann, 1970; Chamberlin and Ring, 1973). Like eukaryotic RNA polymerases, rifampicin did not affect CMV RNA replicase activity up to 0.3 mg/ml (Fig. 5.6A).

Heparin inhibits DNA-dependent RNA polymerases by binding to the site on the enzyme normally occupied by the template DNA, due to its polyanionic nature (Chamberlin, 1974). Heparin was also found to inhibit CMV-induced RNA replicase activity acutely. 50% inhibition of CMV RNA and poly(C)-copying activities was observed at heparin concentrations of 23 and 84 ng/ml, respectively (Fig. 5.6C).

The different concentrations of actinomycin D and heparin required for 50% inhibition of CMV RNA and poly(C)-copying activities suggests that the two enzyme activites were different at least in some aspects.

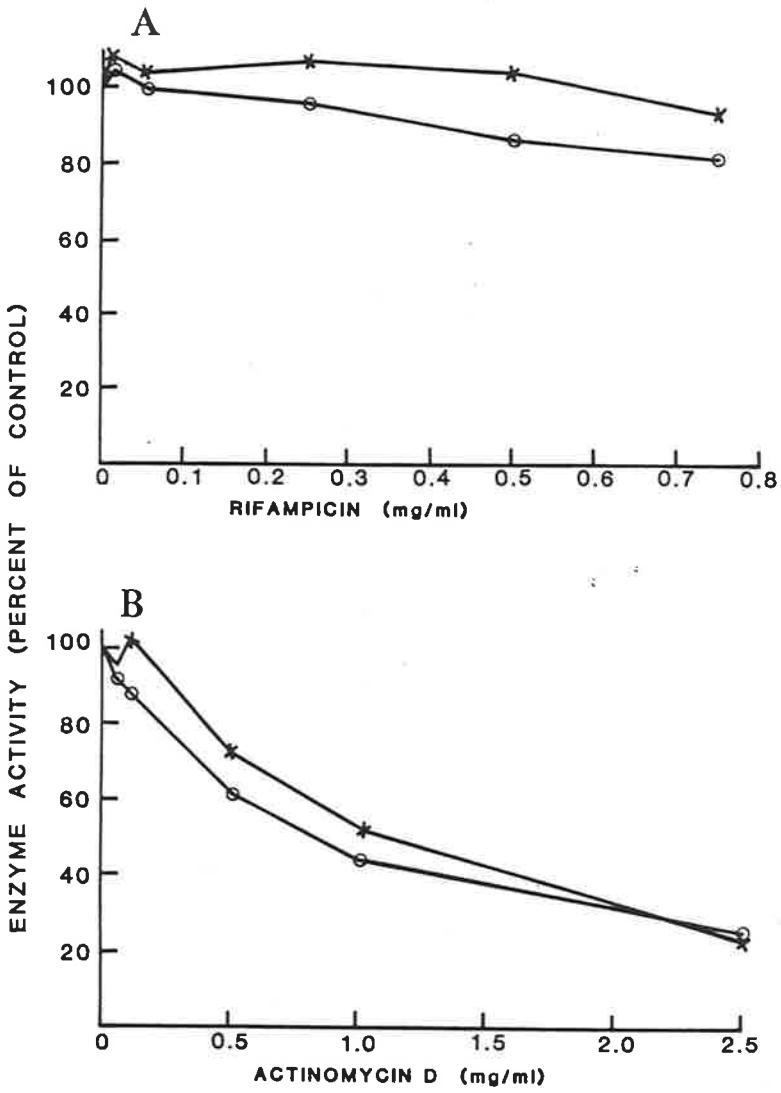
H. ANALYSIS OF THE DISTRIBUTION OF THE LABEL IN THE RNA PRODUCTS OF PARTICULATE RNA REPLICASE

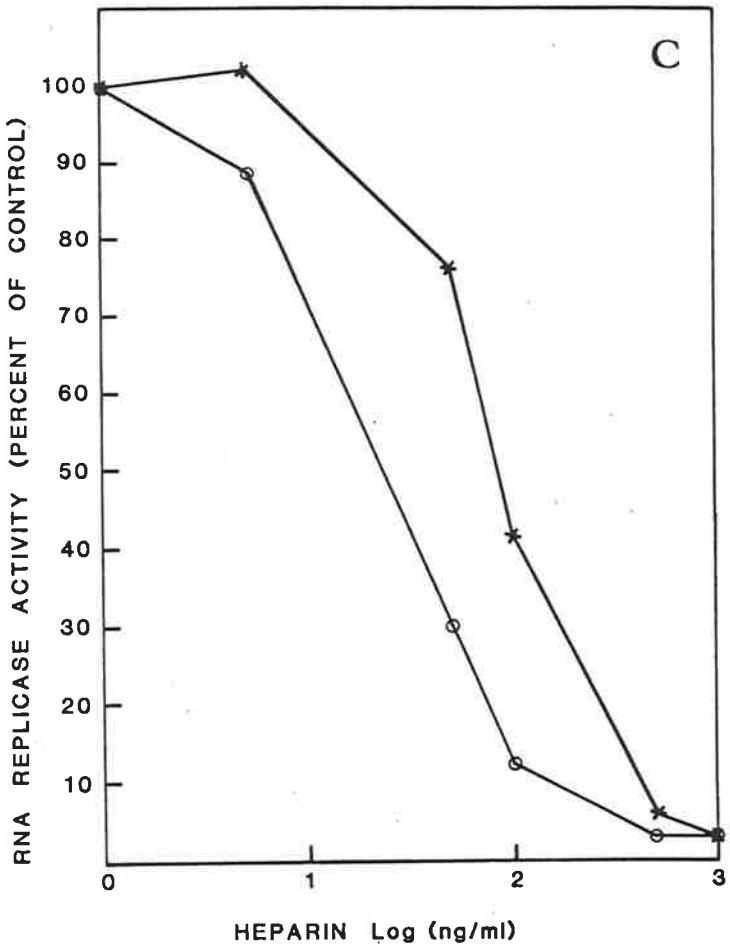
The results obtained, when ^{32}P-labelled RNA products

FIGURE 5.6

EFFECT OF RIFAMPICIN, ACTINOMYCIN D AND HEPARIN ON ENZYME ACTIVITY IN RNA REPLICASE ASSAY

The RNA replicase activity of heparin-Sepharose step enzyme (A & B, 10 µl/100 µl) and phosphocellulose step enzyme (C, 10 µl/100 µl) with CMV RNA (o——o) and poly(C) (×——×) as templates was measured as described in Materials and Methods, except, rifampicin (A), actinomycin D (B) or, heparin (C) at indicated concentration was included in the assay medium. After 1 h incubation at 37°C acid-insoluble radioactivity was measured and expressed as percent of the control assays. The RNA replicase activity in control assays measured in the absence of any added inhibitor was (A) 1.1 and 18.9, (B) 1.1 and 20, (C) 0.37 and 11.4, nmoles of GMP incorporated per h per assay with CMV RNA and poly(C) as templates, respectively.


A
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
RIFAMPICIN (mg/ml)
B
ENZYME ACTIVITY (PERCENT OF CONTROL)
100
80
60
40
20
0
0.5
1.0
1.5
2.0
2.5
ACTINOMYCIN D (mg/ml)


C
RNA REPLICASE ACTIVITY (PERCENT OF CONTROL)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
HEPARIN Log (ng/ml)

of CMV-induced particulate RNA replicase synthesized by using α-^{32}P-GTP and unlabelled ATP, CTP, UTP with CMV RNA as template in the assay medium, were hydrolysed with alkali and separated by TLC as described in Materials and Methods, are shown in Fig. 5.7. The ^{32}P-label as detected by autoradiography was recovered in all four 2'(3')-ribonucleoside monophosphate (A:G:C:U::29:29:20:22), indicating the variation in nearest neighbours of the α-^{32}P-GMP incorporated into the product.

I. SUSCEPTIBILITY OF THE PHENOL EXTRACTED PRODUCT TO RNase DIGESTION

Table 5.3 shows the effect of RNase treatment of the phenol extracted product of CMV-induced RNA replicase. The product was completely susceptible to nuclease action in 0.1 X SSC or lower salt concentrations. However, the product was 67% resistant to RNase in 2 X SSC. When the heat denatured product was allowed to anneal with its template in excess, its RNase resistance was recovered by 65% in 18 h which is equal to RNase resistance before melting. These results of Table 5.3 show that the product of CMV-induced RNA replicase was largely double stranded.

J. THE EFFECT OF STORAGE ON POLYPEPTIDE COMPOSITION OF RNA REPLICASE ENZYME SAMPLES

Fig. 5.8 shows the comparison of the polypeptide composition of a sample of the same phosphocellulose step enzyme labelled with ^{3}H at two and nine weeks after purification. Results show that on storage of the enzyme sample frozen at -80^{o}C before labelling with ^{3}H for gel analysis there was detectable breakdown of at least two polypeptides (M_r 110,000 and ~15,000) indicated by arrows in Fig. 5.8A which were absent in Fig. 5.8B.

FIGURE 5.7

NEAREST NEIGHBOUR BASE ANALYSIS OF THE RNA PRODUCTS SYNTHESIZED BY CMV RNA REPLICASE USING α-^{32}P-GTP AS LABELLED PRECURSOR

The ^{32}P-labelled RNA products were synthesized by particulate CMV RNA replicase (phosphocellulose step enzyme), using α-^{32}P-GTP as the labelled precursor and unlabelled ATP, UTP and CTP, with CMV RNA as template in the standard assay system described in Materials and Methods. The RNA products were isolated, hydrolysed with alkali and the hydrolysate was fractionated by two dimensional TLC as described under Materials and Methods. The positions of various labelled mononucleotides were detected after autoradiography and comparison with standard unlabelled markers visualized under UV light. The radioactivity in the various spots was measured and when expressed as the proportion of the total ^{32}P in the dinucleotides read in 5'→3' direction was A-G:G-G:C-G:U-G::0.29:0.29:0.20:0.22.

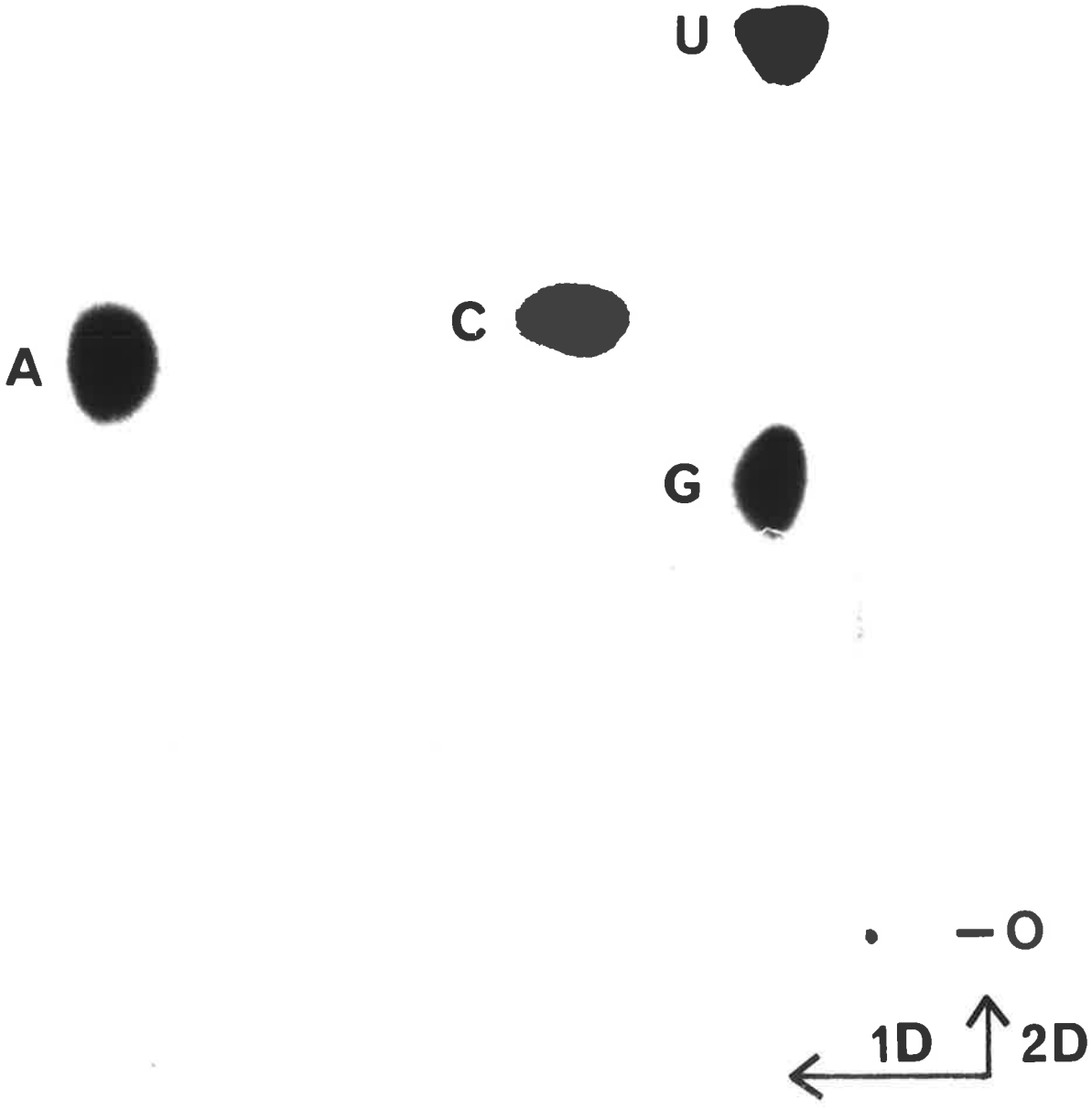

U
C
A
G
O
1D
2D

TABLE 5.3

RESISTANCE OF RNA PRODUCT TO NUCLEASE DIGESTION

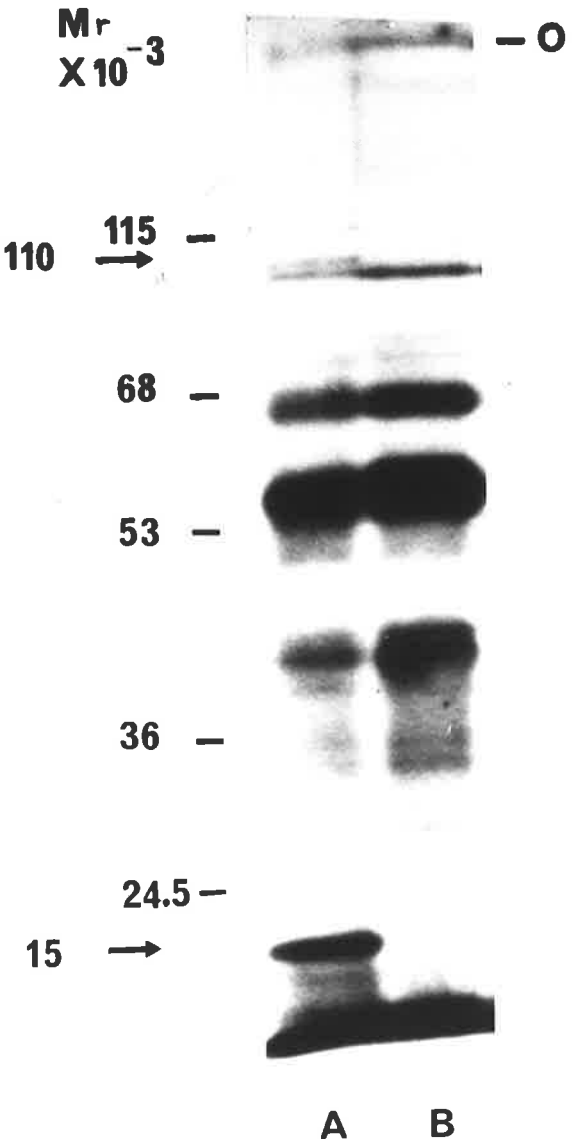
Conditions	Incubation without RNase (cpm)	Incubation with RNase (cpm)	Percent RNase resistance $\frac{\text{cpm (+) RNase}}{\text{cpm (-) RNase}} \times 100$
A. Before Melting			
1.0 mM EDTA	168,200	1,430	0.8
0.1 X SSC	159,400	2,480	1.6
2.0 X SSC	182,300	122,900	67.4
B. After Melting and Annealing			
0 h	35,200	2,780	8.0
12 h	36,240	10.250	28.0
18 h	37,000	23,900	65.0

The ^{32}P-labelled RNA products were synthesized, isolated and incubated: (A), before melting or; (B), after melting and annealing with or without RNase A-T_1 mixture as described under Materials and Methods under indicated conditions. The acid-insoluble radioactivity was measured as described under Materials and Methods and the percent RNase resistance was calculated from the ratio of the duplicates incubated either in the presence or absence of RNase A-T_1 mixture.

FIGURE 5.8

SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF A SAMPLE OF PHOSPHOCELLULOSE STEP ENZYME LABELLED AT DIFFERENT TIME INTERVALS

Small amounts of the same phosphocellulose step purified particulate CMV RNA replicase were labelled with ^{3}H by reductive methylation as described in Section 3.2G at two times after purification. The enzyme sample before labelling was kept stored in a buffer (20 mM Tris-HCl, pH 8.5; 30% glycerol (v/v), 1 mM EDTA, 30 mM 2-mercapto-ethanol and 0.6 M NH_4Cl) at $-80^{o}C$. The protein samples labelled at 2 and 9 weeks after purification on phospho-cellulose column were electrophoresed in Lanes A and B, respectively, on a SDS-12% polyacrylamide slab gel and detected by fluorography as described in Sections 3.2H and I. Arrows indicate the positions of the bands which were affected during storage of the enzyme sample.


Mr
X10-3
O
110
115
68
53
36
24.5
15
A
B

This indicated that enzyme samples contained some proleolytic activity which was asctive under the storage and handling conditions used. As a consequence of this effect, the large virus-induced polypeptide (M_r 110,000) was present in variable amounts in the enzyme samples used for SDS-gel analysis in the results shown in this study.

Section 5.4 DISCUSSION

The results of this chapter showed the presence of an RNA polymerizing enzyme in the samples purified from the particulate fraction of CMV-infected cucumbers as it appeared to meet all characteristics of an RNA-dependent RNA polymerase. This CMV-induced RNA replicase was not inhibited by rifampicin and was only slightly inhibited by the low concentration of actinomycin D effective for the complete inhibition of DNA-dependent RNA polymerase. The acid-insoluble product synthesized by the partially purified CMV-induced RNA replicase upon the addition of CMV RNA template appeared to be the product of polymerisation of all four ribonucleotide precursors, since its synthesis was dependent upon all the four NTPs and the ^{32}P-label, as indicated by nearest neighbour analysis, was distributed over all the four nucleotides and moreover the product, most probably, was complementary to the added template, since at least a part of the product was double-stranded as shown by resistance towards RNase digestion. Inhibition of CMV-induced RNA replicase with 5 mM pyrophosphate is another indication of RNA polymerising activity. Like other RNA-dependent RNA polymerases isolated by other workers from virus-infected or uninfected plant tissues (Fraenkel-Conrat, 1976; Ikegami and Fraenkel-Conrat, 1979b; Zabel *et al.*, 1979;

Hardy *et al.*, 1979; Linthorst, 1982), CMV-induced particulate RNA replicase was also substantially inhibited by low concentrations of phosphate.

The enzymatic characteristics of the CMV-induced particulate RNA replicase studied in this chapter and of the soluble CMV RNA replicase reported earlier (Kumarasamy and Symons, 1979a; Kumarasamy, 1980), were very similar. This finding of similarity in the catalytic properties of the particulate and the soluble forms of the CMV RNA replicase is similar to that reported for AMV RNA replicase (Linthorst, 1982). Enzyme activity of the CMV-induced particulate RNA replicase was dependent on Mg^{2+} and was stimulated with NH_4Cl; 8 - 16 mM Mg-acetate and 40 - 80 mM NH_4Cl were required for maximum enzyme activity. The RNA replicase assay medium became saturated with CMV RNA and poly(C) as template, when used at concentrations of 10 - 20 µg/100 µl assay. The various conditions used in the assay system of the particulate RNA replicase were kept the same as those used by Kumarasamy and Symons (1979a), in the assays of the soluble RNA replicase, except that in the assays with poly(C) as template 1.0 mM GTP was the only ribonucleotide used. The presence of ATP, CTP and UTP in the assays with poly(C) template was not required (results not given), but a high concentration of GTP was necessary. RNA replicase activity was linear up to 60 min, when CMV RNA or poly(C) were used as templates in the standard assay medium; after this time a slow decline from linearity was observed.

The various catalytic characteristics of the phosphocellulose step RNA replicase studied before and after purification on a poly(C)-cellulose column were also similar. These results ruled out the possibility of the loss of some catalytic subunit of the

enzyme during the purification on poly(C)-cellulose column, as was suspected on the basis of results discussed in Chapter Three and Four. This was further confirmed by the co-sedimentation of RNA replicase activity upon sucrose density gradient centrifugation of phosphocellulose and poly(C)-cellulose step enzyme fractions (results not given).

CHAPTER SIX

INVESTIGATION OF POSSIBLE PARTICIPATION OF VIRAL GENE PRODUCTS IN PURIFIED CMV-INDUCED PARTICULATE RNA REPLICASE

Section 6.1 INTRODUCTION

Results given in Chapter Three and Four indicated that the catalytic subunit (M_r 100,000) and two other polypeptides (M_r 110,000 and 35,000) of CMV-induced particulate RNA replicase were unique to CMV-infected plants. These three enzyme polypeptides, with electrophoretic mobilities comparable to the *in vitro* translation products of three largest RNAs of CMV (Schwinghamer and Symons, 1977), appeared to be the gene products of CMV RNAs. This possibility was investigated further in this Chapter by the direct comparison of three enzyme polypeptides (M_r 110,000; 100,000 and 35,000) with the *in vitro* full length translation products of CMV RNAs 1, 2 and 3, by CMV strain specific differences and by peptide mapping studies.

Section 6.2 MATERIALS AND METHODS

A. GENERAL MATERIALS

Creatine phosphate, creatine kinase and HEPES were obtained from Sigma Chemical Co. *S. aureus* V8 protease was from Miles. The Q, P and T strains of CMV (Habili and Francki, 1974, Rao *et al.*, 1982) were kindly provided by Dr. R.I.B. Francki. Viral RNA, extracted from purified virus essentially as in Peden and Symons, (1973), was fractionated on preparative 2.8% polyacrylamide slab gels (Symons, 1978) and RNAs were further purified by preparative sucrose gradient centrifugation as in Schwinghamer and Symons (1975).

B. PURIFICATION OF CMV-INDUCED PARTICULATE RNA REPLICASE

The particulate RNA replicase was isolated from cucumber seedlings infected with the indicated strain of CMV and was purified by column chromatography on phosphocellulose

and then on poly(C)-cellulose essentially as described in Chapter Four.

C. *IN VITRO* LABELLING OF ENZYME PROTEINS

The proteins in the purified enzyme samples were labelled with ^{3}H-KBH_4 in the presence of 40% deionised formamide by reductive methylation as described under Section 3.2G or with ^{125}I as described in Radioiodination Techniques (Amersham Review 18).

D. DANSYLATION OF MARKER PROTEINS

A mixture of marker proteins containing sheep liver pyruvate carboxylase (M_r 115,000), BSA (M_r 68,000), L-glutamate dehydrogenase (rabbit muscle, M_r 53,000), Lactate dehydrogenase (M_r 35,000) and CMV coat protein (M_r 24,500, Kumarasamy and Symons, 1979b) was labelled with dansyl chloride as described in Schetters and McLeod (1979).

E. *IN VITRO* TRANSLATIONS OF CMV RNAS

A cell-free lysate of rabbit reticulocytes was prepared as described by Ranu and London (1979), then treated with micrococcal nuclease (Pelham and Jackson, 1976) and used for translation of purified CMV RNAs. Reaction mixtures (20 - 50 µl) contained 65% lystate (v/v) (20 µM in hemin), 10 µg/ml creatine kinase, 10 mM creatine phosphate, 1 mM Mg-acetate, 70 mM K-acetate, 20 mM HEPES-KOH, pH 7.5, 100 µM spermidine-HCl, 1 mM dithiothreitol, 100 µg/ml rat liver tRNAs, 75 µM of each of the unlabelled amino acids, 20 - 40 µg/ml purified viral RNA, and 200 - 500 µCi/ml of ^{3}H-leucine (120 Ci/mmol) or ^{35}S-methionine (1300 Ci/mmol) (both from New England Nuclear). For the synthesis of more highly labelled translation products, the concentration of labelled amino acid was increased to 1.0

Ci/ml.

F. SDS-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS OF PROTEINS

Radiolabelled enzyme fractions and ^{3}H- or ^{35}S-labelled translation products were electrophoresed on SDS-10% or 12% polyacrylamide slab gels (Laemmli, 1970) and fluorographed or autoradiographed as described under Sections 3.2H and I.

G. ELECTROPHORETIC ELUTION OF PROTEINS FROM GELS FOR PEPTIDE MAPPING

Radiolabelled enzyme proteins and translation products of CMV RNAs were fractionated by SDS-10% polyacrylamide gel electrophoresis. The positions of ^{3}H-labelled proteins were located by using either dansylated marker proteins with mobilities comparable to translation products of CMV RNAs or ^{35}S-labelled translation products (detected by autoradiography of wet gels for 16 - 20 h at 4°C). The bands of interest were excised from the wet gels and proteins were recovered by electroelution in the buffer system of Laemmli (1970).

Gel slices each containing protein sample were equilibrated for 30 - 40 min in Eppendorf tubes containing 1.0 ml of equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS) and placed in a 1 ml disposable plastic syringe (internal diameter 5 mm), immediately above a 10 mm plug of 1.5% agarose in anode buffer (0.125 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS). The gel slices were then sealed in place with about 0.5 ml of 1% agarose in equilibration buffer containing 0.05% bromophenol blue. The bottom of a 1.5 ml Eppendorf tube was cut off carefully to ensure a smooth surface cut. A small piece of dialysis membrane was glued on to cover the opening at the bottom of the tube using cyanoacrylate ester glue (Symons, 1978)

and about 150 - 200 µl of anode buffer was added. A hole with a diameter equal to the ourter diameter of 1 ml syringe (8 mm) was cut in the lid of the Eppendorf tube, allowing the syringe to be just pushed through the lid, until the 1.5% agarose plug just made contact with the buffer over the dialysis membrane. The syringe was then glued in place to provide an airtight seal.

The upper tank (cathode) contained 25 mM Tris-glycine, pH 8.3, 0.1% SDS and the lower tank (anode) contained 125 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS. Electroelution was carried out at 2 - 3 mA/tube (150 - 200 V) for about 1 h, after all the blue dye had entered the Eppendorf tube (about 3 h total).

Twenty-five micrograms of bovine serum albumin (BSA) was added as carrier to each Eppendorf tube and the sample was dialysed for 2 - 3 h against anode buffer, to remove glycine which would otherwise be precipitated at the next step. After addition of 4 volumes of acetone, the protein samples were precipitated overnight at -15°C. In control experiments overall recovery was found to be essentially complete by comparison of the amount of radioactivity eluted from the gel slices with that present in other gel slices containing identical protein samples.

H. PEPTIDE MAPPING OF PROTEINS WITH *S. AUREUS* V8 PROTEASE

Radiolabelled protein samples (20,000 - 40,000 cpm of ^{3}H- or 5000 - 10,000 cpm of ^{35}S-labelled protein) were digested with *S. aureus* V8 protease (Miles) under conditions based on those of Cleveland *et al.* (1977). The electroeluted proteins in buffer containing 0.125 M Tris-HCl, pH 6.8, 1 mM

EDTA, 10% glycerol (v/v), 2% SDS, 25 µg of unlabelled BSA, and V8 protease (500 µg/ml) in the final volume of 40 µl were digested at 37°C for 3 h. After addition of 0.05% bromophenol blue, the digests were separated on SDS-18% polyacrylamide slab gels and fluorographed as described above.

I. PEPTIDE MAPPING OF PROTEINS WITH CNBr

^{3}H-Leucine-labelled translation products of CMV RNAs and the ^{3}H-labelled polypeptides of the CMV-induced RNA replicase were electroeluted and precipitated as above. After drying the protein samples (50,000 cpm of each) plus 50 µg of unlabelled BSA were dissolved in 20 µl of 75% formic acid. Digestions were started by adding 5 µl of CNBr (0.2 g/ml in 75% formic acid) and carried out under nitrogen in Eppendorf tubes for 60 h at 25°C in the dark. The tube contents were then diluted with 1 ml of water and lyophilized; this process was repeated four times. The digestion products were separated on a SDS-18% polyacrylamide slab gel and fluorographed as described above.

Section 6.3 RESULTS

A. COMPARISON OF POLYPEPTIDES OF CMV-INDUCED PARTICULATE RNA REPLICASE WITH *IN VITRO* TRANSLATION PRODUCTS OF CMV RNAs 1, 2 AND 3

A direct comparison of electrophoretic mobilities of polypeptides of RNA replicase purified from Q-CMV-infected cucumber plants and of the *in vitro* translation products of the three largest RNAs of Q-CMV was made on SDS-polyacrylamide slab gels (Figs. 6.1A and B). A comparison of the gel pattern of poly(C)-cellulose step purified Q-CMV RNA replicase

FIGURE 6.1

COMPARISON OF CMV RNA REPLICASE POLYPEPTIDES WITH *IN VITRO* TRANSLATION PRODUCTS OF CMV RNAs 1, 2 AND 3 BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

The *in vitro* translation products of three largest RNAs of Q-CMV and the ^{3}H-labelled protein samples of phosphocellulose and poly(C)-cellulose step purified particulate Q-CMV RNA replicase were prepared and analysed by SDS-polyacrylamide slab gel electrophoresis as described under Materials and Methods. The positions of marker proteins are given on the left-hand side.

A. 10% polyacrylamide-SDS slab gel:

(1) Poly(C)-cellulose step Q-CMV RNA replicase;

(2) Translation products of Q-CMV RNA 1;

(3) Translation products of Q-CMV RNA 2;

(4) Mixture of 1 & 2;

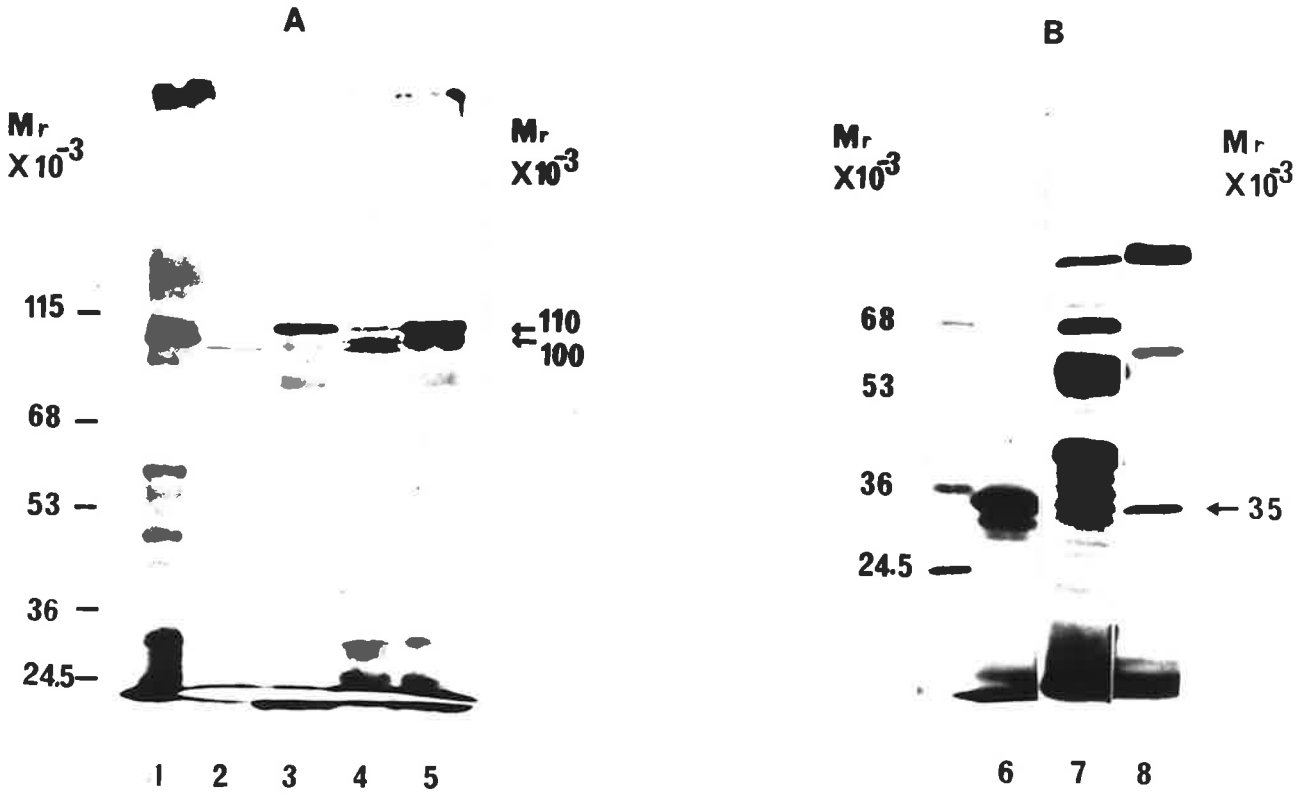
(5) Mixture of 1 & 3

B. 12% polyacrylamide-SDS slab gel:

(6) Translation products of Q-CMV RNA 3;

(7) Phosphocellulose step Q-CMV RNA replicase;

(8) Poly(C)-cellulose step Q-CMV RNA replicase.


A
Mr
X10-3
115
68
53
36
24.5
Mr
X10-3
110
100
1 2 3 4 5
B
Mr
X10-3
68
53
36
24.5
Mr
X10-3
35
6 7 8

and the Q-CMV RNAs 1 and 2 translation products (Fig. 6.1; Lanes 1, 2 and 3, respectively) with a mixture of RNA replicase with the translation products of Q-CMV RNA 1 (Fig. 6.1; Lane 4) and of Q-CMV RNA 2 (Fig. 6.1; Lane 5) shows clearly that the M_r 110,000 polypeptide of CMV RNA replicase was identical in electrophoretic mobility to full length translation product of Q-CMV RNA 2. However, the M_r 100,000 polypeptide of RNA replicase was significantly larger (M_r about 5,000) than the full length translation product of Q-CMV RNA 1.

Similarly a comparison of the polypeptide pattern of phosphocellulose and poly(C)-cellulose step purified enzyme samples of Q-CMV RNA replicase (Fig. 6.1; Lanes 7 and 8, respectively) with the *in vitro* translation products of Q-CMV RNA 3 (Fig. 6.1; Lane 6) shows that the electrophoretic mobility of the M_r 35,000 polypeptide component of RNA replicase, unique to CMV-infected plants, was similar (but not identical) to the *in vitro* full length translation product of Q-CMV RNA 3.

B. DIFFERENT STRAINS OF CMV INDUCE RNA REPLICASE WITH IDENTICAL POLYPEPTIDE COMPONENTS

As shown by the results given in the preceding section, three polypeptides (M_r 110,000; 100,000 and 35,000) of the particulate RNA replicase, unique to CMV-infected cucumber plants, had approximately the same electrophoretic mobilities on SDS-polyacrylamide slab gels as the full length translation products of CMV RNAs 2, 1 and 3, respectively. The possibility whether any of these RNA replicase components are actually viral translation products was tested by comparing the mobilities of these polypeptides in phosphocellulose and poly(C)-cellulose step purified RNA replicase preparations

from cucumber plants infected with three different strains (Q, P and T) of CMV. Should any RNA replicase polypeptides be viral gene products, they would be expected to show variation in their mobilities on SDS-gels similar to that found among the translation products of the RNAs from these different strains of CMV.

The *in vitro* full length translation products of P-CMV RNA 1 and T-CMV RNA 2 had electrophoretic mobilities on SDS-12% polyacrylamide slab gel identical to the *in vitro* full length translation products of the corresponding RNAs of Q-CMV (Fig. 6.2; Lanes 9, 12, 7 and 8, respectively). However, the full length translation product of P-CMV RNA 2 (M_r 105,000) was slightly smaller than that from Q-CMV RNA 2 (Fig. 6.2; Lanes 10 and 8, respectively) and similarly the full length translation product of T-CMV RNA 1 (M_r 100,000) was detectably larger than that of Q-CMV RNA 1 (Fig. 6.1; Lanes 11 and 7, respectively).

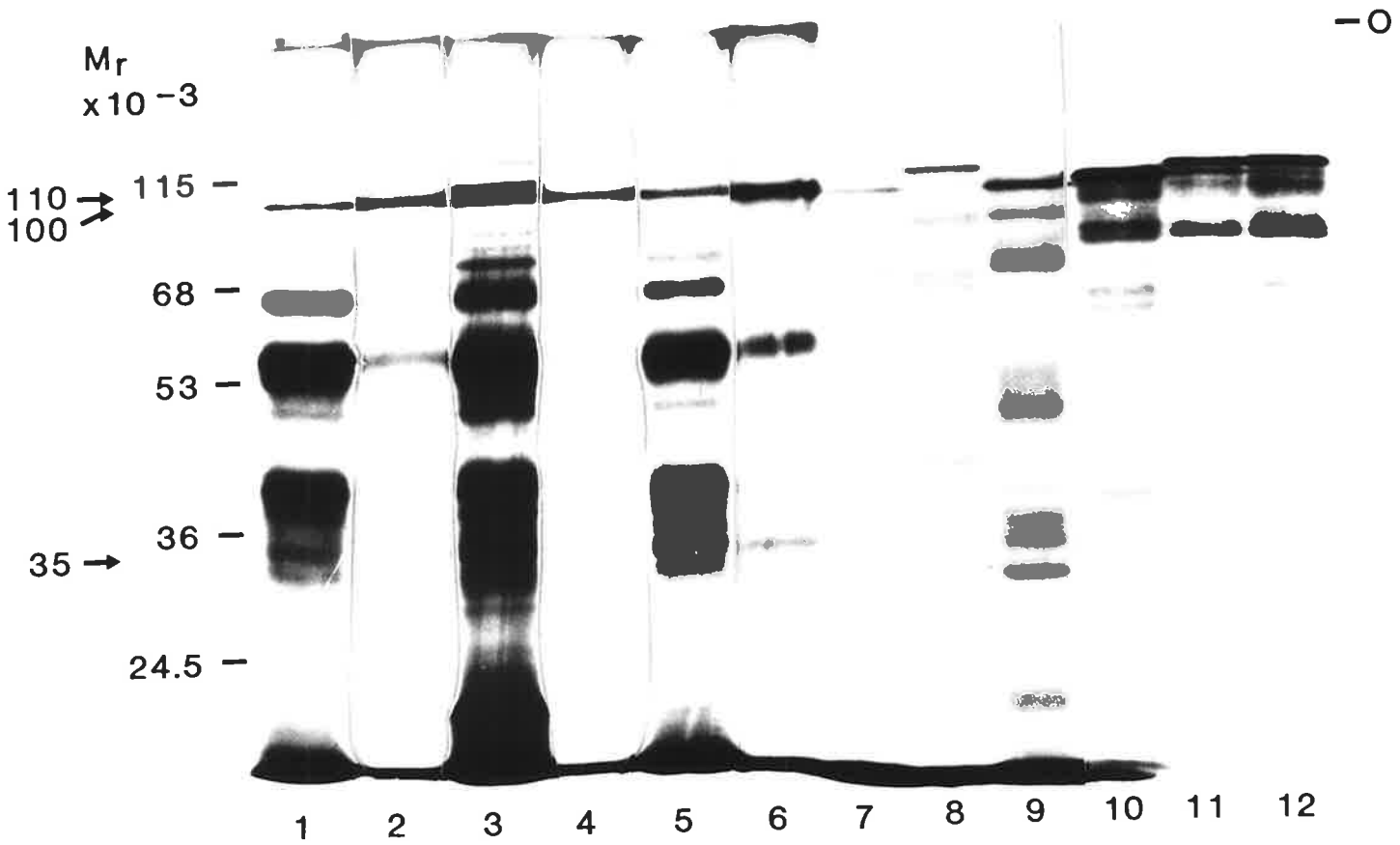
In contrast, the polypeptides of M_r 110,000 and M_r 100,000 in the phosphocellulose and poly(C)-cellulose step purified preparations of RNA replicase from the cucumber plants infected with Q, P and T strains of CMV had identical electrophoretic mobilities on a SDS-12% polyacrylamide slab gel (Fig. 6.1; Lanes 1, 2, 3, 4, 5 and 6). However, there were slight variations in the intensity of various polypeptides (especially the M_r 110,000 in poly(C)-cellulose step enzyme fractions) of RNA replicase preparations which was considered unimportant in this study and has been discussed in Section 5.3J.

The *in vitro* full length translation product of T-CMV RNA 3, with the electrophoretic mobility of M_r 38,000, was clearly larger than the full length translation products of

FIGURE 6.2

COMPARISON OF *IN VITRO* TRANSLATION PRODUCTS OF RNAs 1 AND 2 AND OF THE RNA REPLICASE INDUCED BY Q, P AND T STRAINS OF CMV ON SDS-POLYACRYLAMIDE SLAB GEL

The protein in the particulate RNA replicase samples purified by phosphocellulose column (Lanes 1, 3 and 5) and then by poly(C)-cellulose column (Lanes 2, 4 and 6) from cucumber plants infected with Q (Lanes 1 and 2), P (Lanes 3 and 4) and T (Lanes 5 and 6) strains of CMV, was labelled with ^{3}H-KBH_4 and analysed by SDS-12% polyacrylamide slab gel electrophoresis as under Materials and Methods. Purified RNAs were translated at 20 µg/ml in rabbit reticulocyte cell-free systems in the presence of ^{3}H-leucine and electrophoresed on SDS-12% polyacrylamide gel as under Materials and Methods. Translation products of Q-CMV RNAs 1 and 2 were run in Lanes 7 and 8, those from P-CMV RNAs 1 and 2 in Lanes 9 and 10, and from T-CMV RNAs 1 and 2 in Lanes 11 and 12, respectively. Molecular weights were derived from the positions of ^{3}H-markers. Note that Lane 11 shows that the T-RNA 1 used for *in vitro* translations was contaminated with T-CMV RNA 2 (see Lane 12).


Mr
x10 -3
115
110
100
68
53
36
35
24.5
-O
1
2
3
4
5
6
7
8
9
10
11
12

RNA 3 (M_r 35,000) of the P and Q strains of CMV (Fig. 6.3; Lanes 1, 2 and 3 respectively) when analysed on a SDS-13% polyacrylamide slab gel. However, the electrophoretic mobilities of the M_r 35,000 polypeptides in the poly(C)-cellulose step purified RNA replicase preparations from cucumber plants infected with the T, P and Q strains of CMV were indistinguishable (Fig. 6.3; Lanes 4, 5 and 6, respectively), when analysed on the same 13% polyacrylamide slab gel.

The results given here show that none of the full length translation products of the three largest RNAs of CMV was a component of purified CMV RNA replicase.

C. ABSENCE OF FULL LENGTH TRANSLATION PRODUCTS OF CMV RNAs IN PURIFIED RNA REPLICASE WAS CONFIRMED BY PROTEIN FINGERPRINTING

The purified polypeptides (M_r 110,000; 100,000 and 35,000) of Q-CMV-induced particulate RNA replicase and the *in vitro* full length translation products of the three-largest RNAs of Q-CMV were compared by peptide mapping with V8 protease digestion and chemical cleavage with CNBr. The comparison of V8 protease digests of electroeluted M_r 110,000 and 100,000 polypeptides of RNA replicase (Fig. 6.4; Lanes 1 and 2, respectively) shows that there was very little homology in the electrophoretic mobilities of the peptides derived from the two enzyme polypeptides with those from any of the full length translation products of the two largest CMV RNAs, (Fig. 6.4; Lanes 3 and 4). Some bands generated from the enzyme polypeptides co-migrated with bands in the peptide maps of the RNA 1 translation product; others, especially among the large ones, did not.

The peptide patterns given in Fig. 6.4 were sufficiently different to confirm the conclusion that neither the M_r 110,000

FIGURE 6.3

COMPARISON OF THE *IN VITRO* TRANSLATION PRODUCTS OF RNA 3 AND PROTEINS OF RNA REPLICASE INDUCED BY T, P AND Q STRAINS OF CMV ON SDS-POLYACRYLAMIDE SLAB GEL

The proteins in the particulate RNA replicase samples prepared from cucumber plants infected with T (Lane 4,), P (Lane 5) and Q (Lane 6) strains of CMV after purification on poly(C)-cellulose column, were labelled with ^{3}H-KBH_4 and analysed by SDS-13% polyacrylamide slab gel electrophoresis as described under Materials and Methods. Purified RNA samples were translated at 40 µg/ml in the presence of ^{3}H-leucine in the reticulocyte cell free system as described under Materials and Methods. Translation products of RNA 3 of T, P and Q strains of CMV were run in Lanes 1, 2 and 3, respectively. Molecular weights were derived from the positions of ^{3}H-markers. Note that Lane 1 indicates that the T-CMV RNA 3 was contaminated with degraded larger RNAs.

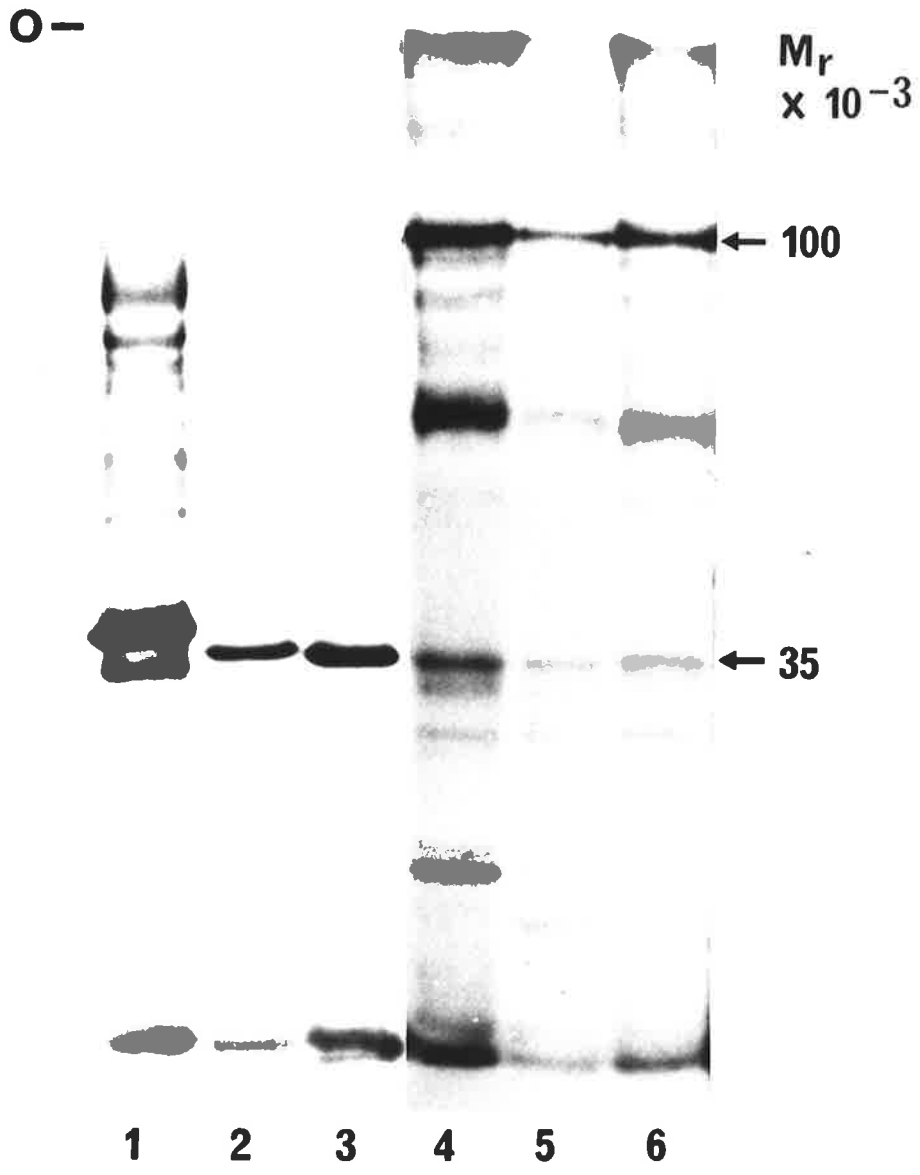
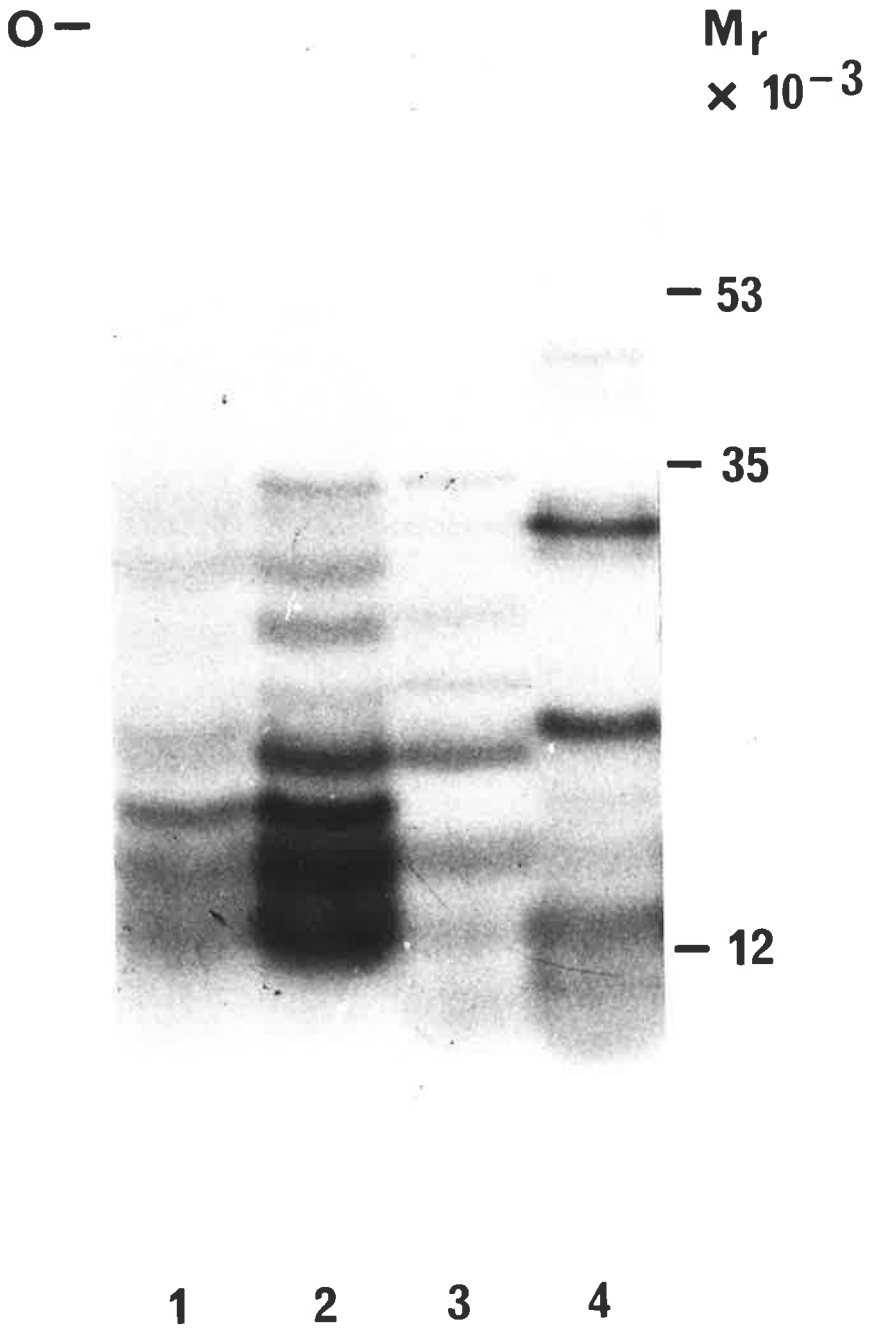

O–
M_r
x 10^{-3}
100
35
1
2
3
4
5
6

FIGURE 6.4

PEPTIDE MAPPING OF 110K AND 100K BANDS OF CMV-INDUCED RNA REPLICASE AND OF THE TRANSLATION PRODUCTS OF CMV RNAs 1 AND 2 WITH V8 PROTEASE

The ^{3}H-leucine labelled translation products and polypeptides of poly(C)-cellulose step RNA replicase labelled with ^{3}H-KBH_4 were separated by electrophoresis on a SDS-10% polyacrylamide slab gel. After recovery from the SDS-polyacrylamide gel and digestion with V8 protease as under Materials and Methods, all the samples were electrophoresed on a SDS-18% polyacrylamide gel and detected by fluorography. The V8 protease digests of Q-CMV RNA replicase polypeptides M_r 110,000 (or 110K) and 100,000 (or 100K) were in Lanes 1 and 2, respectively, and of full length translation products of Q-CMV RNAs 1 and 2 were run in Lanes 3 and 4, respectively. Molecular weights were derived from co-electrophoresis of marker proteins.


O –
Mr
× 10−3
– 53
– 35
– 12
1
2
3
4

nor the M_r 100,000 polypeptide of RNA replicase was the gene product of the two largest RNAs of CMV. These results were further confirmed by the difference shown in the comparison of the peptide patterns obtained after fragmentation with CNBr from the electroeluted enzyme proteins (M_r 110,000 and 100,000) and the translation products of Q-CMV RNAs 1 and 2 (Fig. 6.5). Unfortunately, due to some unknown reason, complete digestion of electroeluted proteins with CNBr was found difficult to achieve under different experimental conditions tried. Interestingly, the comparison of peptide patterns of V8 protease digests (Fig. 6.4; Lanes 1 and 2) as well as of CNBr cleavage products (Fig. 6.5; Lanes 3 and 2) of M_r 110,000 and 100,000 polypeptides of RNA replicase show some similarity in the structure of these two polypeptides.

The proteins used in the studies described in Fig. 6.4 and 6.5 were labelled at different amino acids; the translation products with ^{3}H-leucine and the enzyme polypeptides by reductive methylation with ^{3}H-KBH_4 at lysine amino acids (Rice and Means, 1971). However, comparison of the peptide maps of the translation products labelled with either ^{3}H-leucine or ^{35}S-methionine showed that there were no significant differences between them (data not shown). Hence, the different location of the radioactive label detected by fluorography, leucine or methionine for the translation products and lysine for the enzyme polypeptides, was not critical when comparing their peptide maps.

The comparison of the electrophoretic mobilities of the peptides generated with *S. aureus* V8 protease (Fig. 6.6A) from the electroeluted M_r 35,000 component of Q-CMV-induced RNA

FIGURE 6.5

SDS-POLYACRYLAMIDE GEL PEPTIDE MAPS AFTER CNBr FRAGMENTATION OF TRANSLATION PRODUCTS OF CMV RNAs 1 AND 2 AND OF 110K AND 100K ENZYME POLYPEPTIDES

^{3}H-leucine labelled *in vitro* full length translation products of Q-CMV RNAs 1 and 2 and ^{3}H-labelled M_r 110,000 (110K) and M_r 100,000 (100K) polypeptides of poly-(C)-cellulose purified Q-CMV RNA replicase were electro-eluted and then treated with CNBr in presence of equal amounts (50 µg) of unlabelled carrier BSA as described under Materials and Methods. The digested samples of 100K and 110K enzyme proteins (Lanes 2 and 3, respectively) and of translation products of Q-CMV RNAs 1 and 2 (Lanes 1 and 4, respectively) were fractionated on SDS-18% polyacrylamide slab gel and fluorographed as described under Materials and Methods.

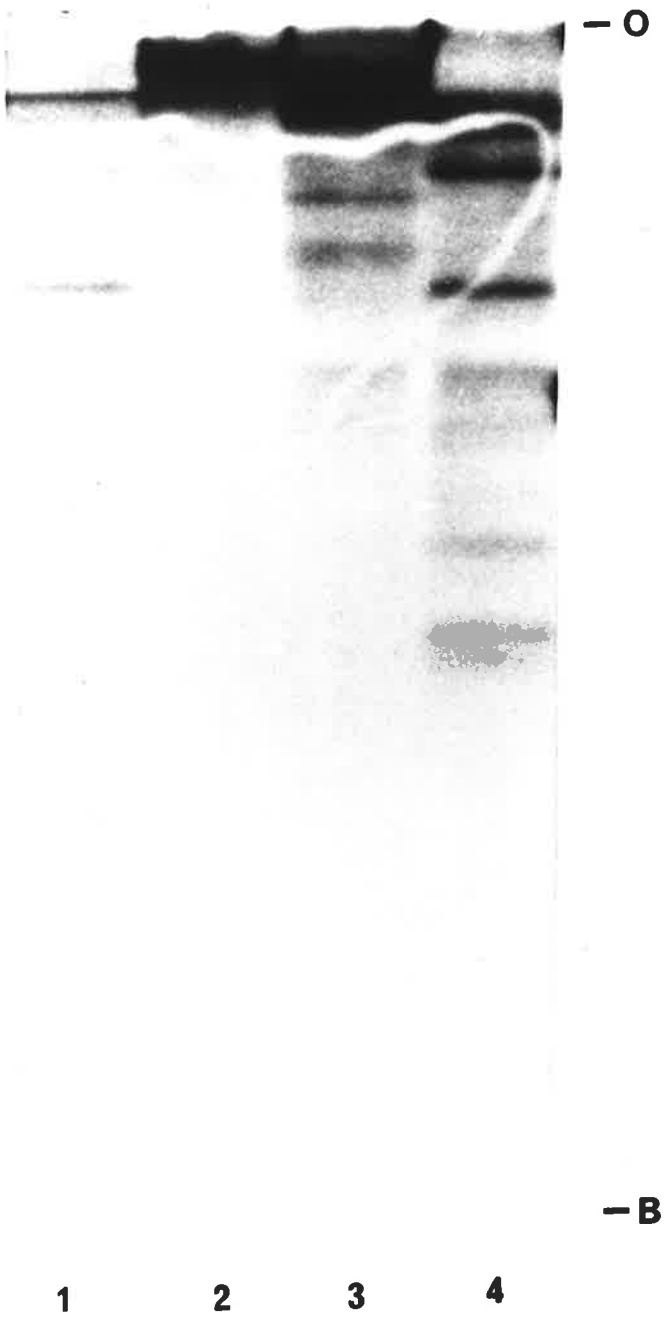

– O
– B
1
2
3
4

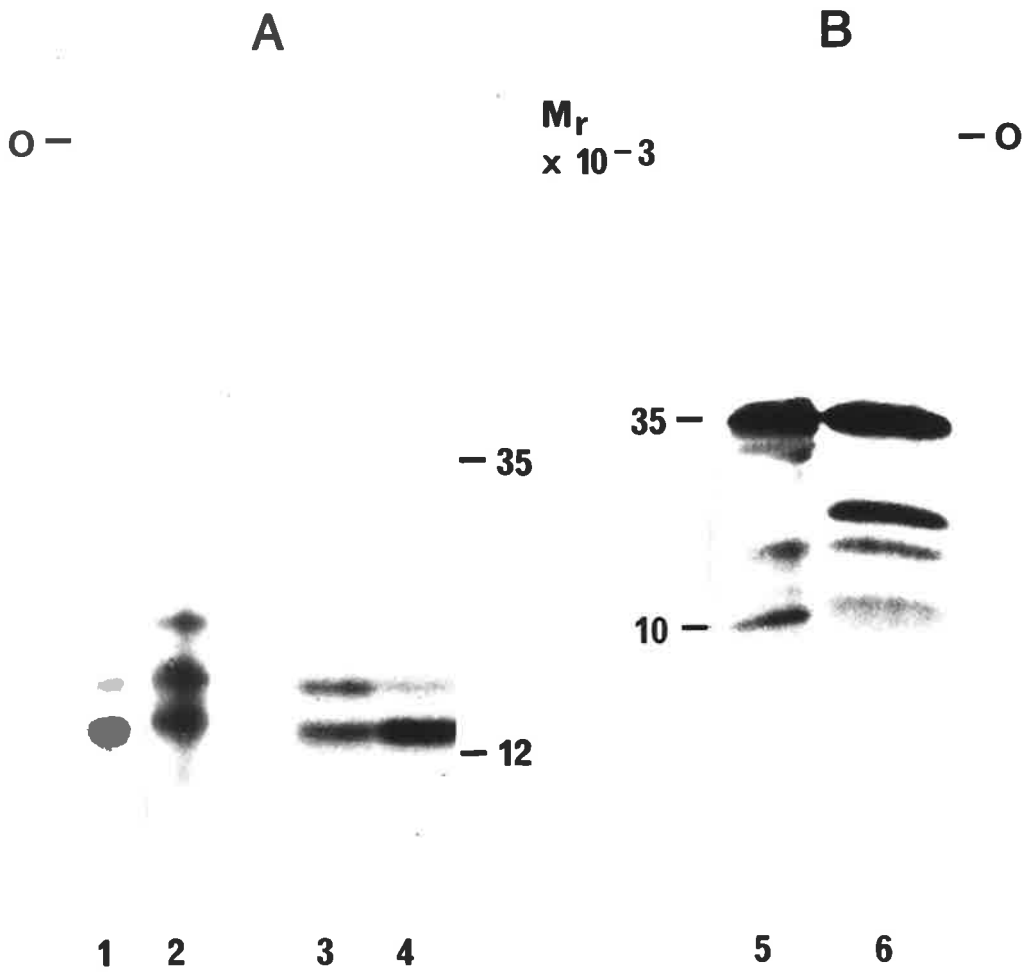
FIGURE 6.6

PEPTIDE MAPPING OF THE TRANSLATION PRODUCTS OF Q-CMV RNA 3 AND THE M_r 35,000 POLYPEPTIDE OF THE Q-CMV RNA REPLICASE

Q-CMV RNA 3 was translated in the reticulocyte cell-free system in the presence of either ^{3}H-leucine or ^{35}S-methionine. The RNA replicase preparations were purified by chromatography on phosphocellulose and poly(C)-cellulose, before labelling with ^{3}H-KBH_4 or ^{125}I as described under Materials and Methods.

A. Gel slices containing ^{35}S-labelled translation products (Lane 1) and ^{125}I-labelled M_r 35,000 enzyme polypeptide (Lane 2) were placed in the wells of a SDS-18% polyacrylamide gel and digested with 5 µg/well of *S. aureus* V8 protease during electrophoresis, as described by Cleveland *et al.* (1977). In another experiment to show that digestion of the translation products gives the same peptide map irrespective of the labelled amino acid, ^{3}H- and ^{35}S-labelled products of RNA 3 (Lanes 3 and 4, respectively) were electroeluted, digested with V8 protease, and run on a SDS-18% polyacrylamide gel.

B. The ^{3}H-labelled full length translation products of Q-CMV RNA 3 and M_r 35,000 polypeptide of poly(C)-cellulose step purified Q-CMV RNA replicase were electroeluted and cleaved with CNBr in presence of equal amounts of carrier BSA as described in Materials and Methods. The digested protein samples of translation products of Q-CMV RNA 3 (Lane 5) and of M_r 35,000 enzyme polypeptide (Lane 6) were separated on a SDS-18% polyacrylamide slab gel and then fluorographed as described under Materials and Methods.


A
B
O –
– O
M_r
x 10^{-3}
– 35
– 12
35 –
10 –
1
2
3
4
5
6

replicase (Fig. 6.6; Lane 2) and the full length translation product of Q-CMV RNA 3 (Fig. 6.6; Lane 1) shows that these two proteins were different. Although different amino acids were radiolabelled in the two protein samples (^{35}S-methionine in the translation products and ^{125}I-tyrosine in the enzyme polypeptides), the peptide maps of ^{3}H-leucine and ^{35}S-methionine labelled translation products of Q-CMV RNA 3 were identical (Fig. 6.6; Lanes 3 and 4). The non-identity of the M_r 35,000 protein of CMV RNA replicase and the CMV RNA 3 translation product was further confirmed by the difference in the peptide maps obtained by CNBr digestion of these two proteins (Fig. 6.6; Lanes 5 and 6).

Section 6.4 DISCUSSION

The earlier work on CMV RNA replicase showing the absence of RNA replicase activity in healthy cucumber plants and its strong induction upon infection with CMV (May *et al.*, 1969; 1970), the difference in the properties of membrane bound RNA replicases induced in cucumber plants upon infection with CMV and TRSV (Peden *et al.*, 1972), and the uniqueness of three polypeptides (M_r 110,000; 100,000 and 35,000) to the soluble RNA replicase (Kumarasamy and Symons, 1979a) provided circumstantial evidence that RNA replicase induced upon viral infection of cucumber plants was virus specific. Results given in Sections 3.3E and 6.3A showed that three polypeptides (M_r 110,000; 100,000 and 35,000), unique to the particulate CMV RNA replicase, had electrophoretic mobilities similar to the *in vitro* translation products of genomal RNAs of CMV supported our previous findings. On the basis of this evidence, it appeared that these

three polypeptides (M_r 110,000; 100,000 and 35,000) were gene products of CMV RNAs 2, 1 and 3, respectively. The minor differences in the size of the M_r 110,000 and 35,000 proteins of RNA replicase and the *in vitro* translation products of CMV RNAs 1 and 3, respectively were thought to be due to *in vivo* post-translation modifications of these proteins before they become enzyme components.

The detailed analysis reported in this chapter of the above assumption has shown that the purified CMV RNA replicase does not contain any of the full length translation products of the three largest RNAs of CMV. The *in vitro* full length translation products of each of the CMV genomal RNAs of the Q, P and T strains of CMV varied in their electrophoretic mobilities, whereas the polypeptide patterns of the RNA replicase preparations purified from cucumber plants infected with the corresponding strains of CMV were indistinguishable from one another on SDS-polyacrylamide slab gels, showing that full length translation products of CMV genomal RNAs were not the components of the enzyme. The non-identity of the three polypeptides (M_r 110,000; 100,000 and 35,000) in CMV-induced RNA replicase with the *in vitro* full length translation products of CMV RNAs 2, 1 and 3, respectively, was confirmed by the comparison of their peptide maps obtained after *S. aureus* V8 protease digestion as well as by chemical cleavage with CNBr.

Hence, the data presented here indicated that the three polypeptides (M_r 110,000; 100,000 and 35,000) in the purified RNA replicase were host encoded, induced upon viral infection, despite their similar electrophoretic mobilities on SDS-polyacrylamide gels to the *in vitro* translation products of the

genomal RNAs of CMV. Therefore, RNA replicase activity solubilized with $MgSO_4$ from the membrane fraction of CMV-infected plants, which was considered to be associated with the M_r 100,000 polypeptide (Chapter Four), is host encoded. Similar results have been reported for the CPMV RNA replicase (Dorssers *et al.*, 1982).

The replication of CMV depends upon CMV RNAs 1, 2 and 3 (Peden and Symons, 1973; Lot *et al.*, 1974; Rao and Francki, 1981). Assuming that the translation products of CMV RNAs 1, 2 and 3 are actually responsible for RNA replication in some way, our findings imply three possible roles for the translation products, as being either;

1) extensively processed before incorporation into a replication complex, or,

2) not directly involved in the replication complex, but responsible for induction or regulation of viral replication through the synthesis of host proteins, or,

3) directly involved in the assembly of the viral replication complex but in such a fashion (e.g., as tightly bound membrane components) that they were not isolated under the conditions of solubilization and purification used in this study.

CHAPTER SEVEN

ANALYSIS OF THE RNA PRODUCTS SYNTHESIZED BY THE PARTICULATE FRACTION AND PURIFIED RNA REPLICASE FROM CMV-INFECTED PLANTS

Section 7.1 INTRODUCTION

The results presented in Chapter Six indicated that RNA replicase activity purified from the particulate fraction of CMV-infected plants was due to the host proteins induced upon viral infection. In order to find the biochemical role, if any, played in viral RNA replication by this host RNA-dependent RNA polymerase (or RNA replicase) enzyme activity, the appearance of which is associated with CMV infection of cucumber plants, it was decided to look for the template RNA associated with this enzyme in the particulate fraction prior to solubilization as well as in the crude enzyme extract solubilized from the particulate fraction of CMV-infected plants. The RNA products transcribed from this enzyme bound endogenous RNA template in the *in vitro* RNA replicase assay were characterized. This also allowed an investigation of the ability of unsolubilized RNA replicase (which was presumed to represent the *in vivo* form of the enzyme) to synthesize virus specific RNA products. The characterization of the RNA products synthesized by the purified particulate RNA replicase is also reported in this chapter.

Section 7.2 MATERIALS AND METHODS

A. GENERAL MATERIALS

MOPS, HEPES, PVP-T40, salmon sperm DNA, dCTP, dATP, dTTP, dGTP were purchased from Sigma Chemical Co. Ficoll (M_r 4000,000) was obtained from Pharmacia Fine Chemicals. Avian myeloblastosis virus reverse transcriptase was obtained from the Office of Program Resources and Logistics (National Cancer Institute, Bethesda). AMV, TMV, CMV and ASBV RNAs were

prepared and kindly provided by Dr. R.H. Symons, CPMV RNA was prepared and kindly provided by Dr. George E. Bruening. SCMoV RNAs were purified and kindly provided by Mr. Paul Keese. BMV RNA was prepared and kindly provided by Dr. A.O. Jackson. α-^{32}P-dCTP was prepared by the method of Symons (1977) and kindly provided by Dr. R.H. Symons. Other materials were as described in previous Chapters.

B. STOCK SOLUTIONS

i. Extraction Buffer A

50 mM Tris-HCl, pH 8.5

100 mM NH_4Cl

10% sucrose (w/v)

90 mM 2-mercaptoethanol.

ii. Solutions used in RNA extraction:

Phenol-Chloroform mixture

1 volume redistilled water-saturated phenol

1 volume chloroform (AR grade)

0.1% 8-hydroxyquinoline (w/v)

Buffer STE

10 mM Tris-HCl, pH 7.3

100 mM NaCl

1 mM EDTA

iii. Buffer Solutions used in gel electrophoresis:

10 X TBE

0.89 M Tris-base (Trizma), pH 8.3

0.89 M Boric acid

0.025 M Na_2-EDTA

40 X MOPS-NaOH

1.0 M MOPS

0.5 M NaOH

50 X HEPES-NaOH

1.0 M HEPES

0.6 M NaOH

15 X TAE

0.6 M Tris-acetate, pH 7.4

0.3 M Na-acetate

0.03 M EDTA

Formamide-dye mixture

95% Formamide

10 mM EDTA

0.05% Xylene cyanol F.F.

0.05% Bromophenol blue

MOPS sample loading buffer (1.5 X)

5 µl 40 X MOPS-NaOH

45 µl 12 M HCHO

150 µl Formamide-dye mixture

10 µl RNA sample in H_2O was mixed with

20 µl of MOPS-sample loading buffer.

HEPES sample loading buffer (1.5 X)

10 µl 50 X HEPES-NaOH

90 µl 12 M HCHO

300 µl Formamide-dye mixture

10 µl of RNA sample in H_2O was mixed with

20 µl of HEPES sample loading buffer

iv. Solutions used in nitrocellulose hybridization

20 X SSC

3.0 M NaCl

0.3 M Na_3-citrate

Prehybridization buffer

0.75 M NaCl

75 mM Na_3-citrate

0.02% (w/v) each BSA, Ficoll, PVP-T40

0.25 mg/ml Salmon sperm DNA (heat denatured and sonicated

50 mM sodium phosphate, pH 6.5

50% (v/v) deionised formamide

5 mM EDTA

0.2% (w/v) SDS

Hybridization concentrate (1.1 X)

11% (w/v) Dextran sulphate

0.66 M NaCl

66 mM Na_3-citrate

0.016% (w/v) each BSA, Ficoll, PVP-T40

0.22 mg/ml Salmon sperm DNA

(heat denatured and sonicated)

44 mM sodium phosphate, pH 6.5

44% (v/v) deionised formamide

5 mM EDTA

0.2% (w/v) SDS

C. PREPARATION OF THE PARTICULATE FRACTION FROM CMV-INFECTED CUCUMBER PLANTS

The particulate fraction from uninfected or CMV-infected cucumber plants was prepared essentially as described in Section 2.2I with minor modifications. All equipment and

buffers used for extraction were autoclaved one day before use. Leaves (10 - 20 g), of CMV-infected plants were ground in a pestle and mortar with extraction buffer A (2 ml/g of leaves) and then filtered through one layer of nylon cloth. The filtrate was centrifuged at 1000 g for 7 min, the pellet containing the cell debris and nuclei discarded and the supernatant was further centrifuged at 22,000 g for 25 min. The pellet obtained was washed twice by resuspending in and centrifuging from extraction buffer A (4 ml/g of leaves). The washed pellet was resuspended in extraction Buffer A (0.5 ml/g of leaves); this suspension was called the particulate fraction and was used in RNA replicase assays usually at 30 µl per 100 µl of assay medium.

D. RNA REPLICASE ASSAY

Assays for the measurement of acid-insoluble activity were essentially as described in Section 2.2D but assays for the analysis of labelled products by hybridization or by gel electrophoresis were modified as described below. The assay medium, 0.1 to 2.5 ml, contained the following constituents:

20 mM Tris-HCl, pH 8.5

0.6 mM of each of the unlabelled ATP, UTP and CTP

0.5-2 µM α-^{32}P-GTP (25 - 150 µCi)

13 mM Mg-acetate

5 mM KCl

25 mM 2-mercaptoethanol

25 µg/ml actinomycin D

50 - 60 mM NH_4Cl (from the enzyme sample).

The assays with particulate fraction did not contain any added RNA as template. The reactions were started by adding

either particulate fraction (30 µl/100 µl), or $MgSO_4$-solubilized crude RNA replicase (20 µl/100 µl , prepared as in Section 2.2I) or poly(C)-cellulose step RNA replicase (5 µl/-100 µl). Incubations were at 37°C for 15 and 30 min for particulate fraction and $MgSO_4$-solubilized crude or purified particulate RNA replicase, respectively.

E. ISOLATION OF ^{32}P-LABELLED RNA PRODUCTS OF RNA REPLICASE ASSAY

The RNA replicase reactions were stopped by vortexing the reaction mixture with an equal volume of phenol-chloroform mixture. The aqueous phase recovered by centrifugation at 10,000 g for 1 - 5 min, was re-extracted with an equal volume of phenol-chloroform and washed by mixing with and centrifuging from two volumes of ether. The RNA products were precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol to the ether washed aqueous phase for 1 h in dry ice or overnight at -20°C. The RNA was collected by centrifugation at 10,000 g for 15 min, at 4°C. Alternatively, the aqueous phase was chromatographed over a Sephadex G-50 column (20 X 1.1 cm, or 2 ml constructed in a graduated 2 ml pipette) in STE Buffer. Either one ml or one drop fractions were collected, respectively and monitored by Cerenkov counting of radioactivity in a Tri-carb liquid scintillation spectrometer using discriminator settings of 50 - 1000 and a gain setting of 50%. The peak fractions of radioactively labelled RNA in the exclusion volume of the column were pooled, the RNA precipitated with 2.5 volumes of ethanol either by keeping in dry ice for one hour or at -20°C overnight. Precipitates were collected by centrifugation at 10,000 g for 15 min at 4°C, rinsed with chilled 70% ethanol,

dried under vacuum and redissolved in water or sample buffer for gel electrophoresis.

F. SYNTHESIS OF DNA COMPLEMENTARY TO CMV RNA

cDNA to CMV RNA was synthesized with reverse transcriptase by the procedure described for the synthesis of the first strand of DNA on RNA by Gould and Symons (1982). The reaction mixture (20 µl) contained 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 40 µg of salmon sperm DNA as primer, 3 µg CMV RNA, 2 µl reverse transcriptase, 10 mM DTT, 1 mM each dATP, dGTP, dTTP and either 1 mM dCTP for the synthesis of unlabelled cDNA to be spotted on nitrocellulose paper or 0.1 mM dCTP containing α-^{32}P-dCTP (60 µCi) for the synthesis of ^{32}P-labelled cDNA to be used as radioactive probe. The incubations were at 42^{o}C for 30 min after which reactions were stopped by heating at 100^{o}C for one min. Reaction contents were incubated at 60^{o}C for 15 min after the addition of 2 µl of 5 M NaOH to hydrolyse the CMV RNA. After neuteralization with 2 µl of 5 M HCl the solution was extracted twice with the phenol-$CHCl_3$. Radioactive cDNA was separated from free α-^{32}P-dCTP by passing the aqueous phase over a Sephadex G-50 column (2 ml) in STE buffer. The peak fractions of radioactively labelled cDNA in the exclusion volume of the column were pooled. Unlabelled cDNA after phenol-$CHCl_3$ extraction or ^{32}P-labelled cDNA after separation on the Sephadex G-50 column was precipitated with ethanol, redissolved in water and used.

G. THE EXTRACTION OF RNA FROM THE PARTICULATE FRACTION OF HEALTHY OR CMV-INFECTED CUCUMBER PLANTS

For RNA extraction, the particulate fraction prepared from uninfected or CMV-infected cucumber plants was mixed with

0.1 volume of 10% SDS (w/v) and 0.1 volume of 3 M Na-acetate and was then shaken with an equal volume of the phenol-$CHCl_3$ for five min. The aqueous phase was recovered after centrifugation at 10,000 rpm for 10 min in HB-4 rotor of Sorvall centrifuge and the RNA precipitated with 2.5 volumes of ethanol at -20°C for 1 h or longer. The RNA was collected by centrifugation at 10,000 rpm for 15 min. Ethanol precipitation was repeated once more, the RNA pellet obtained was rinsed with chilled 70% ethanol, dried under vacuum and was finally redissolved in water.

H. HYBRIDIZATION OF DENATURED RNA OR DNA SAMPLES ON NITROCELLULOSE

The hybridization of denatured nucleic acids on nitrocellulose paper was done essentially as described by Thomas (1980). A sheet of nitrocellulose paper was soaked in H_2O for five min and then in 20 X SSC, for 30 min. In dot blot assays nucleic acid samples (1 to 2 µg) in 2 µl of H_2O were directly spotted on the presoaked sheet of nitrocellulose. The nitrocellulose paper was air dried and then baked at 80°C for 2 h in vacuo. The pre-hybridization was carried out for 20 h in pre-hybridization buffer in sealed plastic bags in an oven maintained at 42°C. For hybridization, the radioactive probe (^{32}P-cRNA or -cDNA) in 0.1 volume of 2 mM EDTA was denatured by heating at 80°C for 90 sec and then snap cooled on ice, mixed with 0.9 volumes of 1.1 X hybridization concentrate, sealed with pre-hybridized nitrocellulose in a plastic bag and incubated at 55°C for 24 h in a water bath. The nitrocellulose sheet was then washed in 2 x SSC containing 0.1% SDS four times, each for 5 min, at room temperature, and then twice in 0.1 X SSC containing 0.1% SDS and once in 0.02 X SSC containing 0.1% SDS, each for

15 min, at 50°C. The washed nitrocellulose sheet was covered with a layer of thin polyethylene sheet and autoradiographed.

Section 7.3 RESULTS

A. COMPARATIVE ANALYSIS OF THE RNA PRODUCTS SYNTHESIZED BY PARTICULATE FRACTION AND RNA REPLICASE BEFORE AND AFTER PURIFICATION

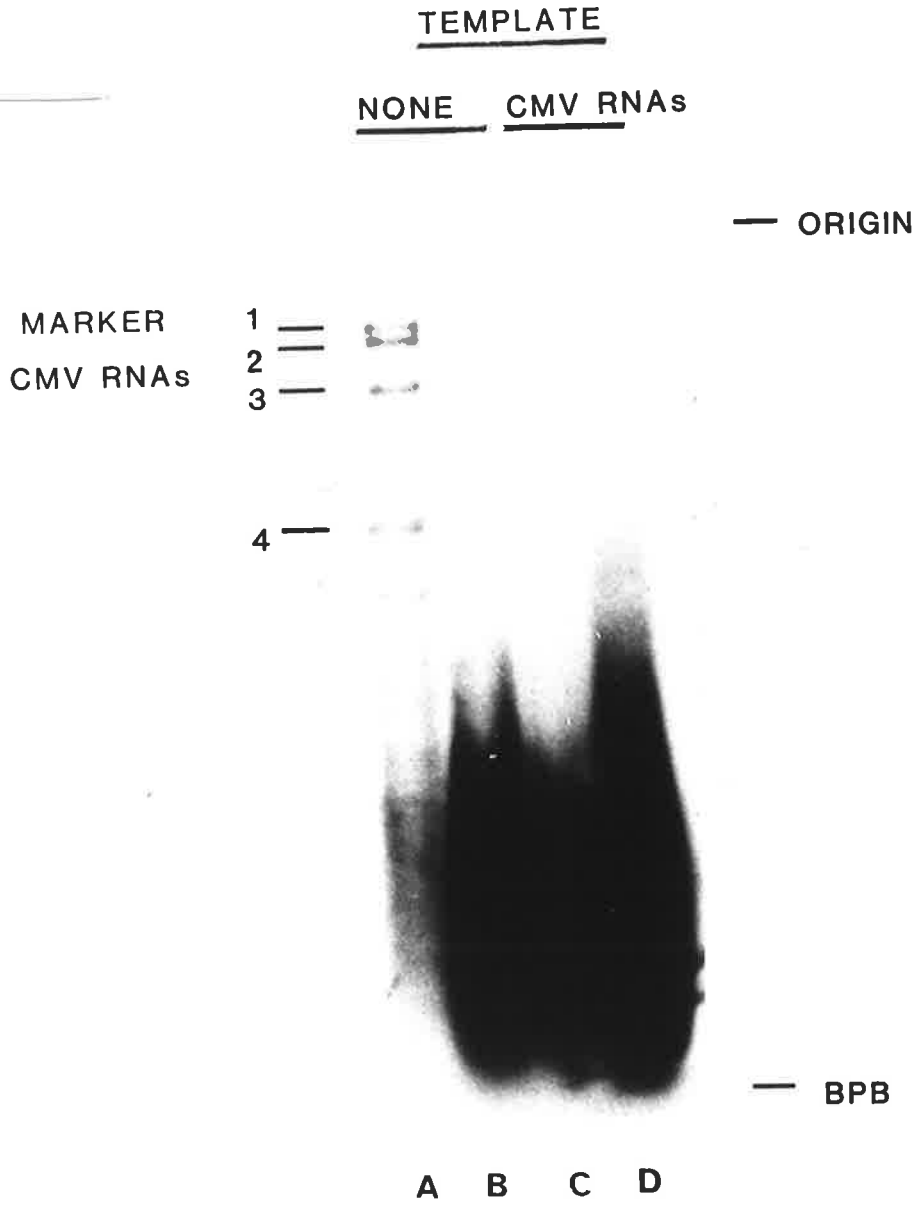
The Fig. 7.1 shows the analysis under denaturing conditions on a HCHO-agarose gel (Lehrach *et al.*, 1977) of the ^{32}P-labelled RNA products synthesized by incubating (A), the particulate fraction; (B), the $MgSO_4$-solubilized crude RNA replicase without any added template; (C), the $MgSO_4$-solubilized crude RNA replicase, and (D), the poly(C)-cellulose step RNA replicase with CMV RNA as a template. The particulate fraction incorporated α-^{32}P-GTP into five major products, four of which co-migrated with markers of CMV RNAs (Fig. 7.1A). These products of the particulate fraction were characterized further to check the possibility that they were the transcripts of CMV RNAs and the results are given below.

The ^{32}P-labelled RNA products synthesized by the $MgSO_4$-solubilized crude RNA replicase due to endogenous RNA templates present in the enzyme sample (i.e., in the absence of any added template) were small and heterogenous in size (Fig. 7.1B). In order to find out the nature of the endogenous template in the $MgSO_4$-solubilized crude RNA replicase, the ^{32}P-labelled RNA products (cRNA) were used as probes and hybridized with the RNAs isolated from the particulate fraction prepared from uninfected or CMV-infected cucumber plants. Purified CMV RNAs 2 and 3, total virion RNA of CMV, RNAs of unrelated viruses and *E. coli*

FIGURE 7.1

FORMALDEHYDE-MOPS -AGAROSE GEL ELECTROPHORESIS OF ^{32}P-LABELLED RNA PRODUCTS

The ^{32}P-labelled RNA products synthesized by the particulate fraction, (A); $MgSO_4$-solubilized crude RNA replicase, (B) and (C); and poly(C)-cellulose step RNA replicase, (D); with, (C) and (D) or; without, (A) and (B) added CMV RNA as template in the RNA replicase assay were isolated by phenol-$CHCl_3$ extraction and gel filtration as described under Materials and Methods. The 10 µl of RNA samples (1500; 50,000; 40,000 and 350,000 cpm of A, B, C and D, respectively) were mixed with 20 µl of MOPS sample loading buffer, heated at 80^{o}C for 75 sec, snap cooled and fractionated by electrophoresis on a 1.6% agarose-formaldehyde (2 M) (14 x 14 x 0.3 cm) gel in MOPS-NaOH buffer system (Lehrach *et al.*, 1977). After electrophoresis gel was stained with toluidine blue to detect the CMV RNA markers, electrophoresed on the same gel in a separate lane, dried under vacuum and low heat and autoradiographed.


TEMPLATE
NONE
CMV RNAs
ORIGIN
MARKER
CMV RNAs
1
2
3
4
BPB
A
B
C
D

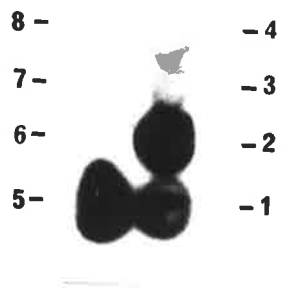
tRNAs were used as controls. The radiolabelled RNA products hybridized with the purified CMV RNAs 2 and 3 (which were presumably free from any plant RNA), total CMV RNA, RNA isolated from CMV-infected plants as well as from uninfected cucumber plants and with none of the unrelated RNAs (Fig. 7.2A). These results of Fig. 7.2A show that ^{32}P-labelled products synthesized by $MgSO_4$-solubilized RNA replicase in the absence of added template were complementary to the CMV RNA and host plant RNA which indicated that either these RNAs were bound as templates to $MgSO_4$-solubilized RNA replicase or were present as free RNA in the enzyme sample.

The RNA products synthesized by the $MgSO_4$-solubilized crude RNA replicase and by the poly(C)-cellulose step RNA replicase with CMV RNAs as template were small and heterodisperse in size when analysed on a denaturing gel (Figs. 7.1C and D, respectively). These results were similar to those obtained with purified soluble CMV RNA replicase (Kumarasamy and Symons, 1979a). The ^{32}P-labelled RNA products synthesized by the purified particulate CMV RNA replicase with CMV RNA as template were complementary to CMV RNAs 2 and 3 and virion RNA as shown by the dot-blot assay (Fig. 7.2B). ^{32}P-labelled RNA products also hybridized to RNA isolated from the particulate fraction prepared from CMV-infected plants and also to the RNA from uninfected plants (Fig. 7.2B). This hybridization with RNA of healthy plants was presumably due to the presence of host RNA contaminating the viral RNA used as samples.

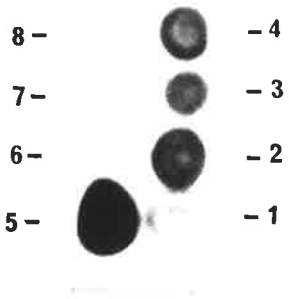
FIGURE 7.2

DOT-BLOT HYBRIDIZATION ANALYSIS OF THE ^{32}P-LABELLED RNA PRODUCTS

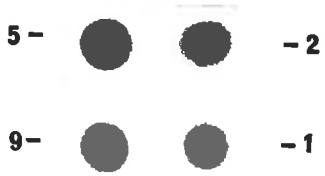
The ^{32}P-labelled RNA products synthesized by incubating the (A), $MgSO_4$-solubilized crude RNA replicase (50 µl) in RNA replicase assay (Vf 200 µl) containing 0.12 mM α-^{32}P-GTP (55 µCi) or; (B), poly(C)-cellulose step RNA replicase (8 µl) in RNA replicase assay (Vf 100 µl) containing 0.12 mM α-^{32}P-GTP (50 µCi) and (C), particulate fraction (800 µl) prepared from CMV-infected plants in RNA replicase assay (Vf 2 ml) containing 2.0 nmoles of α-^{32}P-GTP (400 µCi) in the absence, (A) and (C) and in the presence, (B) of CMV RNA as template at 37°C for 15 min were isolated by phenol-$CHCl_3$ extraction and gel filtration as described in Materials and Methods. The bulk of virion RNA in (C) was separated from the ^{32}P-labelled (ds) products by 2 M LiCl precipitation. The ^{32}P-labelled products (90,000, 70,000 and 80,000 cpm in A, B, and C, respectively), were ethanol precipitated, redissolved in H_2O and used as radioactive probes in hybridisation with indicated nucleic acid samples as described in Materials and Methods. The unlabelled cDNA to CMV RNA (9) spotted on nitrocellulose sheets and ^{32}P-cDNA to CMV RNA used as probe in (D) was prepared as described in Section 7.2F. The radioactive spots were detected by autoradiography.



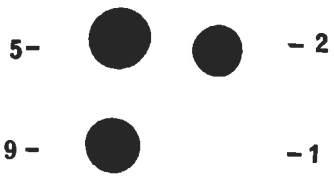
A



B



C



D

NUCLEIC ACID SAMPLES

1. RNA uninfected plants
2. RNA CMV-infected plants
3. CMV RNA 3
4. CMV RNA 2
5. Total CMV RNA
6. AMV RNA
7. BMV RNA
8. E. coli tRNA
9. cDNA to CMV RNA

B. CHARACTERIZATION OF THE ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED BY PARTICULATE FRACTION

i. The *in vitro* ^{32}P-labelled RNA products synthesized by the particulate fraction were the RNA products of CMV-induced RNA replicase

As described above, the particulate fraction from CMV-infected plants under *in vitro* conditions synthesized five major products co-migrating with CMV RNAs on gel electrophoresis (Fig. 7.1A and 7.3B). No such bands were observed when the products synthesized by the particulate fraction prepared from uninfected control cucumber plants were prepared and analysed under identical conditions (Fig. 7.3A). Therefore these RNA products were due to the enzyme activity unique to CMV-infected plants. Further, the incorporation of α-^{32}P-GTP into the RNA products required the presence of all the four NTPs in the assay medium. No such RNA products were synthesized when the particulate fraction from CMV-infected plants was incubated in the assay mediums lacking either UTP or CTP (Figs. 7.3D and E, respectively). The incorporation of α-^{32}P-GTP into the RNA products observed in Fig. 7.3C was due to the presence of non-radioactive ATP in the preparation of α-^{32}P-GTP (Symons, 1977) hence the assays of Fig. 7.3C contained all the four ribo-nucleotides. The results presented here show that the *in vitro* synthesis of RNA products by the particulate fraction was due to CMV-induced RNA replicase activity.

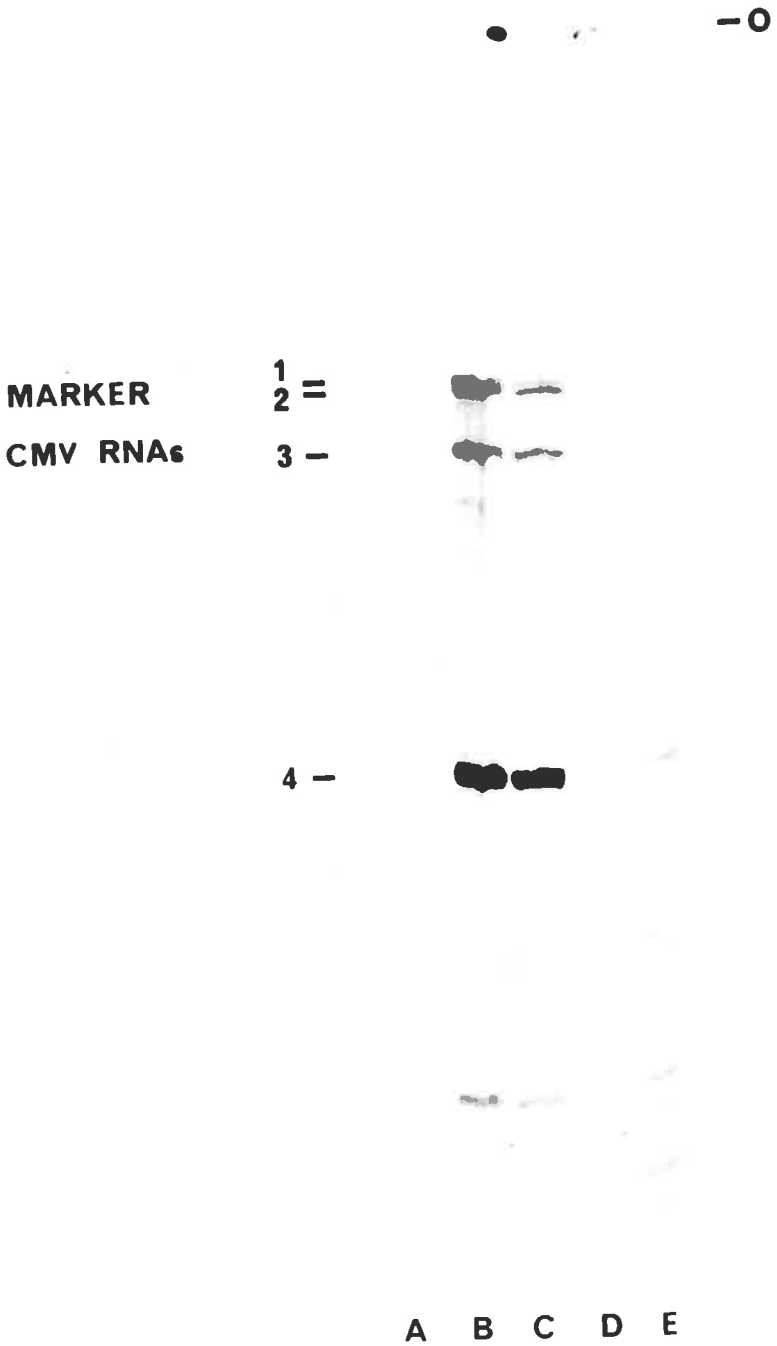
ii. The largest four major RNA products synthesized by the particulate fraction were the transcripts of CMV RNAs

Since the RNA products synthesized by the particulate fraction of CMV-infected cucumber plants were of the size of

FIGURE 7.3

TBE-UREA-POLYACRYLAMIDE GEL ELECTROPHORESIS OF ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED, (a) BY THE PARTICULATE FRACTION FROM HEALTHY AND CMV INFECTED PLANTS (b) AND IN PRESENCE OF ALL BUT ONE NTPs BY THE PARTICULATE FRACTION FROM CMV-INFECTED PLANTS

The particulate fractions (200 µl/500 µl assay) prepared from uninfected (A), and CMV-infected (B,C,D and E) cucumber plants were incubated in their respective assay media at 37°C for 15 min. Each of the five assays (Vf 500 µl) contained 0.3 nmoles of α-^{32}P-GTP (25 µCi, containing carrier ATP) and 0.6 mM each of unlabelled (A and B) ATP, UTP, CTP; (C) CTP, UTP; (D) ATP, CTP; (E) ATP, UTP; and other components of the RNA replicase assay as described under Materials and Methods. The RNA products were isolated by phenol-$CHCl_3$ extraction and ethanol precipitation as described under Materials and Methods and were heated in 20 µl of formamide-dye loading mixture at 80°C for 90 sec and snap cooled just before electrophoresis. Each sample (10 µl) was fractionated by electrophoresis on a TBE-7 M urea-3 % polyacrylamide gel (40 x 20 x 0.05 cm) as described in Sanger and Coulson, (1978) at 20 mA until xylene cyanol dye reached near bottom. The CMV RNAs run as markers in an adjacent track were detected by staining with toluidine blue. Autoradiography of ^{32}P-labelled bands was at -80°C.


-O
MARKER
CMV RNAs
1
2 =
3 –
4 –
A B C D E

the CMV RNAs it was quite reasonable that they were the transcripts of CMV RNAs. In order to check this possibility, we used two experimental approaches. There is a detectable difference in the electrophoretic mobilities in the RNA 4 of the Q and P strains of CMV, whereas each of the RNAs 1, 2 and 3 of two strains were indistinguishable when fractionated on a TBE-Urea-3% polyacrylamide gel. A corresponding difference was seen in the mobilities of the products of the size comparable to RNA 4 synthesized by the particulate fractions prepared from cucumber plants infected with Q and P strains of CMV (Figs. 7.4A and B). These results showing the strain specific differences provided further evidence that these four major RNA products were transcripts of CMV RNAs rather than of any host RNA of a size comparable to CMV RNAs.

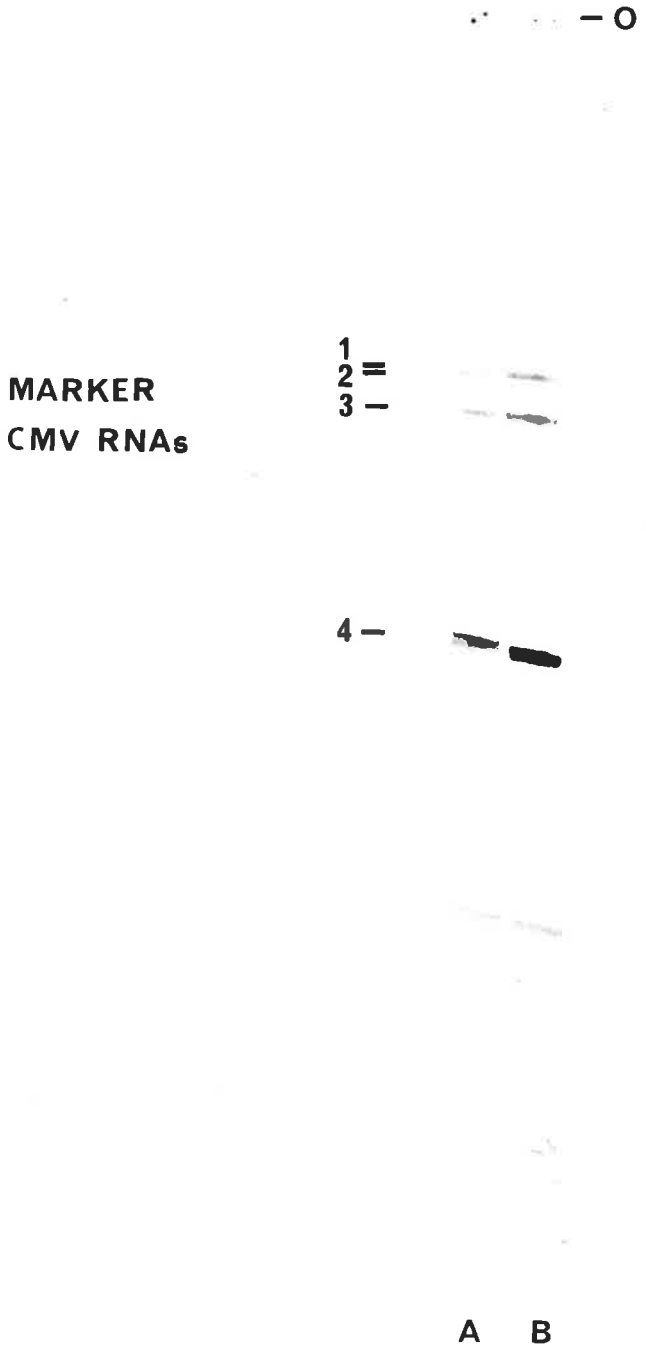
In order to confirm the above results and to find the polarity of the products, the *in vitro* ^{32}P-labelled RNA products synthesized by the particulate fraction were used as radioactive probes in the dot-blot hybridization with CMV RNA, cDNA to CMV RNA, and the RNAs extracted from uninfected and CMV-infected cucumber plants. The ^{32}P-labelled RNA products hybridized to variable extents with all the four samples of RNA (Fig. 7.2C). The hybridization of the radioactive RNA products with virion RNA, RNA from uninfected and CMV-infected plants gave similar results as were observed with the cRNA transcribed from endogenous template with $MgSO_4$-solubilized crude CMV RNA replicase (Fig. 7.2A). These results show that the some of the sequences of the radiolabelled RNA products were complementary to the CMV virion RNA and some to the host RNA.

The hybridization of the ^{32}P-RNA products with CMV virion

FIGURE 7.4

COMPARISON OF THE ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED BY THE PARTICULATE FRACTION FROM CUCUMBER PLANTS INFECTED WITH Q AND P STRAINS OF CMV

The particulate fractions (240 µl/600 µl assay) prepared from cucumber plants infected with Q and P stains of CMV were incubated in RNA replicase assay (Vf 600 µl) (A and B, respectively) containing 0.44 nmols of α-^{32}P-GTP (68 µCi) at 37°C for 15 min and the RNA products were isolated by phenol-$CHCl_3$ extraction and ethanol precipitation as in Materials and Methods. The RNA pellets were resuspended in formamide-dye loading mixture, a part of each sample was heated at 80°C for 90 sec, snap cooled and fractionated by electrophoresis on a TBE-7 M urea-3 % polyacrylamide gel as described in Fig. 7.3. The marker RNAs of Q and P strains of CMV were electrophoresed in adjacent wells on the same gel and were stained with toluidine blue. The radioactive bands were detected by autoradiography of the wet gel.


O
MARKER
CMV RNAs
1
2
3
4
A
B

RNA (i.e., + type) shows that the polarity of the RNA products was of (-) type. The attempts made to confirm the absence or presence of (+) type RNA products using unlabelled single-stranded cDNA to CMV RNA were unsuccessful, because we were unable to make single-stranded cDNA under the conditions used. The unlabelled cDNA to CMV RNA prepared by the method described for the synthesis of first strand of DNA on RNA by Gould and Symons (1982) and used in these studies was found to be double-stranded because it hybridized to the radiolabelled cDNA to CMV RNA prepared under identical conditions but in presence of α-^{32}P-GTP (Fig. 7.2D). Therefore the results presented here neither confirmed nor ruled out the possible presence of (+) type RNA products as well.

iii. Under *in vitro* assay conditions the particulate fraction completed pre-initiated nascent RNA transcripts

Little difference in the incorporation of the ^{32}P-label was observed when the particulate fraction was incubated in the RNA replicase assay for 10 min and 60 min (Table 7.1), showing that the polymerization reaction was over in the first 10 min after which there was no reinitiation of transcription. These results were further supported by the observation that no difference was seen in the intensities of the major bands when the RNA products synthesized by the particulate fraction in the presence or absence of added CMV RNA template were electrophoresed on TBE-Urea-polyacrylamide gel (results not given). The above results were further confirmed by incubating the particulate fraction in the presence or absence of heparin (10 µg/ml) in the RNA replicase assay. Heparin is an inhibitor of DNA-dependent RNA polymerase at the initiation step (Schafer *et al.*, 1973) and also a potent inhibitor of purified RNA replicase (Section 5.3G). In the assay containing heparin the incorporation of ^{32}P-label

TABLE 7.1

INCORPORATION OF α-^{32}P-GMP INTO THE RNA PRODUCTS BY PARTICULATE FRACTION WITH TIME OF INCUBATION

Time of incubation (min)	^{32}P-label incorporated (cpm)
10	9189
60	8236

The particulate fraction (100 µl) prepared from CMV-infected cucumber plants was incubated in 250 µl of assay medium, containing 25 µCi of α-^{32}P-GTP as described under Materials and Methods. The incubations were at 37^{o}C for the indicated times, after which RNA products were isolated by phenol-$CHCl_3$ extraction and gel filtration, and were counted by Cerenkov counting as described under Materials and Methods. The electrophoretic pattern of the RNA products synthesized after 10 min and 60 min were similar when analysed on TBE-7 M Urea-polyacrylamide gel (results not given).

into products was 66% of the control assay without heparin (Table 7.2). These results indicate that *in vitro* the particulate fraction only resumed to complete the elongation of nascent chains initiated *in vivo* and there was presumably no reinitiation at least to synthesize the full length transcripts of viral RNAs. There are some indications of the possibility of reinitiation by some RNA replicase molecules (presumably solubilized ones), which gave rise to the synthesis of host specific small sized heterodisperse RNA products, which could not be seen on gel electrophoresis due to continuous smearing but were apparent in the hybridization assay (Fig. 7.2C).

iv. Agarose gel analysis of ^{32}P-RNA products of particulate fraction

The electrophoresis of untreated and heat denatured ^{32}P-products of particulate fraction on an agarose gel is shown in Fig. 7.5. The untreated RNA products migrated slowly as double stranded material, but no sharp resolution was obtained (Fig. 7.5A). However, the RNA products denatured by heating in formamide co-migrated with marker CMV RNAs treated in a similar way (Fig. 7.5B). The results indicated the double stranded nature of the RNA products. However, the chromatography of RNA products on cellulose in 15% ethanol in Buffer STE (v/v) showed that only about 65% of the total RNA products were double stranded (results not given).

v. RNA products synthesized by the particulate fraction obtained before and after the $MgSO_4$-solubilization of RNA replicase

The Fig. 7.6 shows the comparison of the RNA products synthesized by the particulate fraction of CMV-infected plants which has been treated with $MgSO_4$ (150 mM) to remove the particulate RNA replicase with those synthesized by the particulate

TABLE 7.2

INCORPORATION OF α-^{32}P-GMP INTO THE RNA PRODUCTS BY PARTICULATE FRACTION IN PRESENCE OR ABSENCE OF HEPARIN

Experiment	^{32}P-incorporated (cpm)
Control	1976
Control + heparin (10 µg/ml)	1330

The particulate fraction (120 µl) prepared from CMV-infected plants, was incubated in assay medium (v_f 300 µl) in absence (control) or presence of 3 µg heparin at 37°C for 1 h, the RNA products were recovered by phenol-$CHCl_3$ extraction and gel filtration and radioactivity was measured by Cerenkov counting as described under Materials and Methods. The electrophoretic pattern of the RNA products synthesized in the absence and in the presence of heparin was similar (results not given).

FIGURE 7.5

AGAROSE GEL ELECTROPHORESIS OF THE ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED BY PARTICULATE FRACTION

The particulate fraction (1 ml) prepared from CMV-infected cucumber plants was incubated in RNA replicase assay (Vf 2.5 ml) containing 1.74 nmoles of α-^{32}P-GTP (136 µCi) at 37°C for 15 min and products (116,000 cpm) were isolated by phenol-$CHCl_3$ extraction and gel filtration as described under Materials and Methods. ^{32}P-labelled RNA samples (A and B, each about 10,000 cpm were dried down; (A) was taken up in 20 µl of 20% sucrose solution containing 2 mM EDTA; unlabelled marker CMV RNAs and (B) was taken up in 20 µl of 80% formamide containing 2 mM EDTA and 4% sucrose, heated at 80°C for 90 sec and snap cooled just before electrophoresis. RNA samples were fractionated by electrophoresis on a 1.5% agarose gel (14 x 14 x 0.3 cm) in the TAE buffer system. After electrophoresis, gel was stained with toluidine blue to detect the positions of marker RNAs, then put on Whatman 3 MM paper, dried under vacuum, and low heat and autoradiographed to detect radioactive bands.

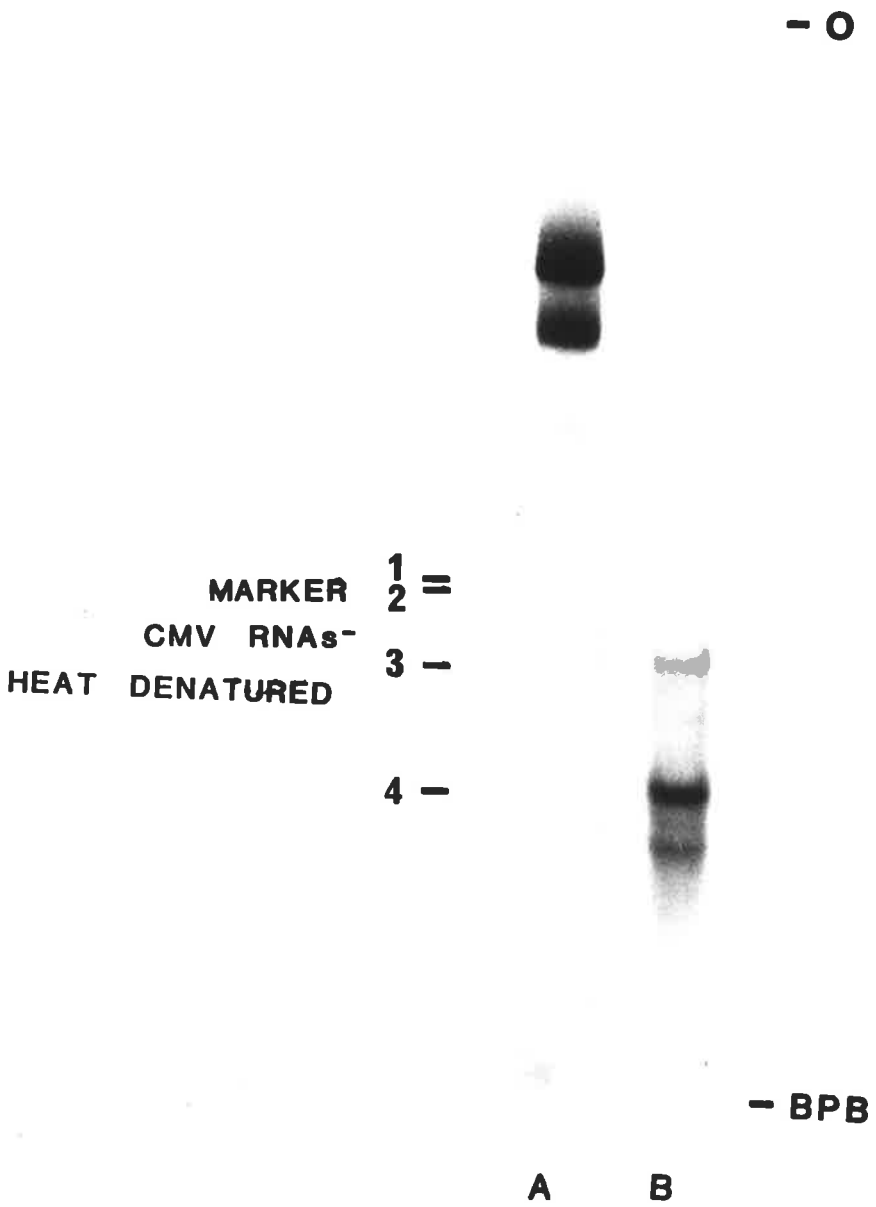
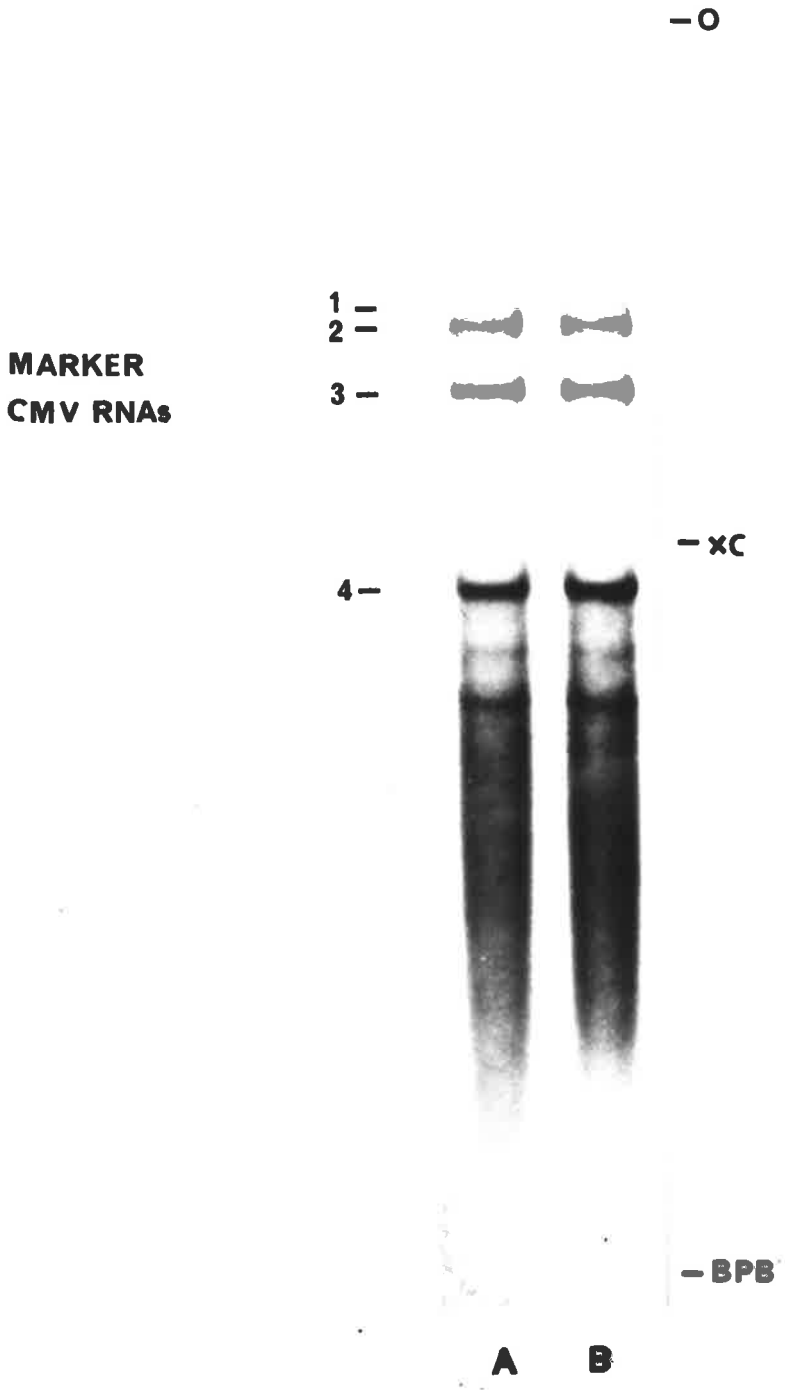

- O
MARKER
CMV RNAs-
HEAT DENATURED
1
2
3
4
- BPB
A
B

FIGURE 7.6

FORMALDEHYDE-HEPES GEL ELECTROPHORESIS OF ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED BY THE PARTICULATE FRACTION BEFORE AND AFTER SOLUBILIZATION

The particulate fraction was prepared from CMV-infected plants as described in Materials and Methods. (A), The particulate fraction (240 µl) was incubated in RNA replicase assay (600 µl) containing 1.6 nmoles of α-^{32}P-GTP (44 µCi) at 37^{o}C for 15 min and products were isolated by phenol-$CHCl_3$ extraction and gel filtration as in Materials and Methods. (B), The particulate fraction (1 ml) was incubated with $MgSO_4$ (150 mM) at 37^{o}C for 5 min (Section 2.2D). The pellet obtained by centrifugation after the removal of $MgSO_4$-solubilized RNA replicase, was washed with extraction Buffer A (1 ml), resuspended in 1 ml of Buffer A, incubated in RNA replicase assay (240 µl/600 µl) containing α-^{32}P-GTP (44 µCi) at 37^{o}C for 15 min and products were isolated as above in (A). The RNA products (4347 cpm of (A) and 3846 cpm of (B)) were fractionated on a 2 M HCHO - 1.6% agarose gel by electrophoresis in HEPES-NaOH buffer system as in Fig. 7.1. After electrophoresis gel was stained with ethidium bromide, illuminated with UV light, photographed to detect marker CMV RNAs run in a parallel track. RNA samples were transferred from agarose gel to nitrocellulose sheet in 20 X SSC and autoradiographed as in Thomas (1980).


-O
1
2
MARKER
CMV RNAs
3
-xC
4
-BPB
A
B

fraction (control) without any treatment. The RNA products synthesized by the untreated control and $MgSO_4$-treated particulate fraction were similar (Figs. 7.6A and B) but the quantitative incorporation of α-^{32}P-GTP into RNA by $MgSO_4$-treated particulate fraction was only 45% of that obtained with the untreated (control) particulate fraction and was about 4% of that with the $MgSO_4$-solubilized crude RNA replicase when assayed in the absence of any added template.

C. CHARACTERIZATION OF THE RNA PRODUCTS SYNTHESIZED BY THE PURIFIED PARTICULATE RNA REPLICASE

i. The enzyme activity of CMV-induced particulate RNA replicase with different RNA templates

The enzyme activity of the poly(C)-cellulose step purified particulate RNA replicase with different plant viral and non-viral RNAs as templates at three different concentrations was studied by measuring the incorporation of α-^{32}P-GTP into acid-insoluble material and results are presented in Fig. 7.7. All the different RNAs tested were accepted as efficient templates by the purified CMV RNA replicase. The RNA replicase activity was the highest with CMV RNA especially at the concentration below than that required to saturate the assay whereas at an RNA concentration of 0.2 mg/ml the RNA replicase activity with BMV RNA was 92% of that with CMV RNA as template. AMV RNA and rRNA were better templates than CPMV RNA.

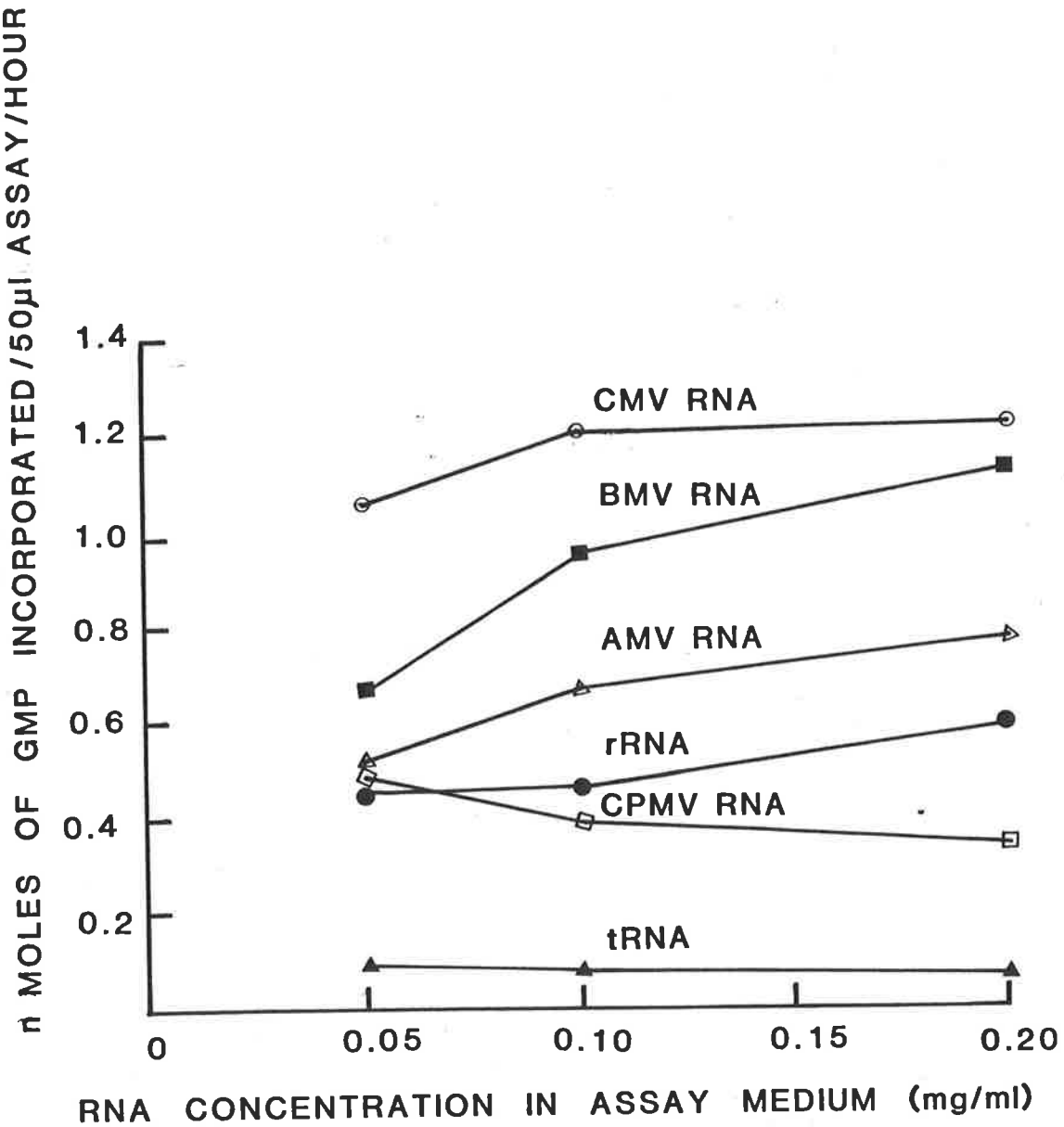
ii. CMV-induced particulate RNA replicase transcribes viroid and virusoid RNA into full length copies

Since the results presented above in this section indicate that CMV-induced RNA replicase after purification had little template specificity for CMV RNA and the RNA products

FIGURE 7.7

RNA REPLICASE ACTIVITY OF POLY(C)-CELLULOSE STEP ENZYME WITH DIFFERENT RNA TEMPLATES.

Different viral and non-viral RNAs were extracted or obtained from different sources as described under Materials and Methods. Before use, all RNAs were precipitated twice with ethanol, RNA pellets obtained were rinsed with cold 70% ethanol, dried under vacuum and redissolved in water. Each RNA sample (2.5, 5, 10 µg/50 µl) was used as templates in RNA replicase assay (Vf 50 µl) for poly(C)-cellulose step purified RNA replicase as described in Materials and Methods. After one hour incubation at 37°C the RNA replicase activity was calculated by measuring the acid-insoluble radioactivity and expressed as nmoles of GMP incorporated per h per assay.


n MOLES OF GMP INCORPORATED/50µl ASSAY/HOUR
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.05
0.10
0.15
0.20
CMV RNA
BMV RNA
AMV RNA
rRNA
CPMV RNA
tRNA
RNA CONCENTRATION IN ASSAY MEDIUM (mg/ml)

synthesized were of small heterogenous size like those of RNA-dependent RNA polymerases of healthy plants discussed briefly in the introduction. Recently (Boege *et al.*, 1982a, 1982b) the RNA-dependent RNA polymerase purified from healthy tissue of tomato plants, which synthesized heterogenously sized small RNA products with TMV RNA as a template has been shown to copy potato spindle tuber viroid (PSTV) to synthesize a small fraction of full length transcripts. Viroids are infectious, single-stranded, covalently closed, circular RNA molecules 250 - 600 residues in size (Diener, 1981). On the basis of the above results the possible role of RNA-dependent RNA polymerases of healthy plants in viroid RNA replication was suggested (Boege *et al.*, 1982a; 1982b). Purified CMV-induced RNA replicase was found to accept purified avocado sunblotch viroid (ASBV) and subterranean clover mottle virusoid (SCMoV RNA 2) (viroid like encapsidated RNA (Haseloff *et al.*, 1982)) as efficient templates. On the TBE-urea gel electrophoresis of the ^{32}P-labelled RNA products under the conditions used by Boege *et al.* (1982a) about 7 - 15% of the total products was found to co-migrate with the respective circular and linear marker viroids or virusoids used as templates (Figs. 7.8A, B, C). In contrast to the results of Boege *et al.* (1982a) who found only linear products, CMV-induced RNA replicase products co-migrated with both circular as well as linear RNA markers when the assay medium contained a mixture of linear and circular RNA templates (Figs. 7.8A and C), but products were only linear when purified linear RNA template was used in the assay medium (Fig. 7.8B).

In order to ensure the complete denaturation of the ^{32}P-labelled RNA products co-migrating with the marker RNAs, they

FIGURE 7.8

GEL ELECTROPHORESIS OF ^{32}P-LABELLED RNA PRODUCTS SYNTHESIZED BY THE PURIFIED RNA REPLICASE

(a) TBE-UREA-POLYACRYLAMIDE GEL ELECTROPHORESIS

The ^{32}P-labelled RNA products were synthesized by incubating the poly(C)-cellulose step RNA replicase (3 µl) in RNA replicase assay (Vf 50 µl) containing 0.32 nmoles of α-^{32}P-GTP (64 µCi) and, (A), 3 µg of SCMoV RNA 2 (circular and linear, or; (B), 3 µg of SCMoV (linear) or; (C), 2.5 µg of ASBV (circular and linear) as templates at 37^{o}C for 30 min and isolated by phenol-$CHCl_3$ extraction and gel filtration. The RNA samples (535 x 10^3, 514 x 10^3 and 609 x 10^3 cpm of A, B and C, respectively were ethanol precipitated and redissolved in 10 µl of 80% formamide-dye loading mixture, heated at 80^{o}C for 90 sec and snap cooled just before electrophoresis. Electrophoresis was performed on a TBE-7 M urea-3% polyacrylamide gel as described in Fig.7.3 until the bromophenol dye reached near the bottom. After electrophoresis gel was stained with toluidine blue to detect marker RNAs run in adjacent tracks. The radioactive bands were detected by autoradiography at 4^{o}C for 6 h and those which co-migrated with the marker RNAs were excised from the wet gel for further analysis.

a

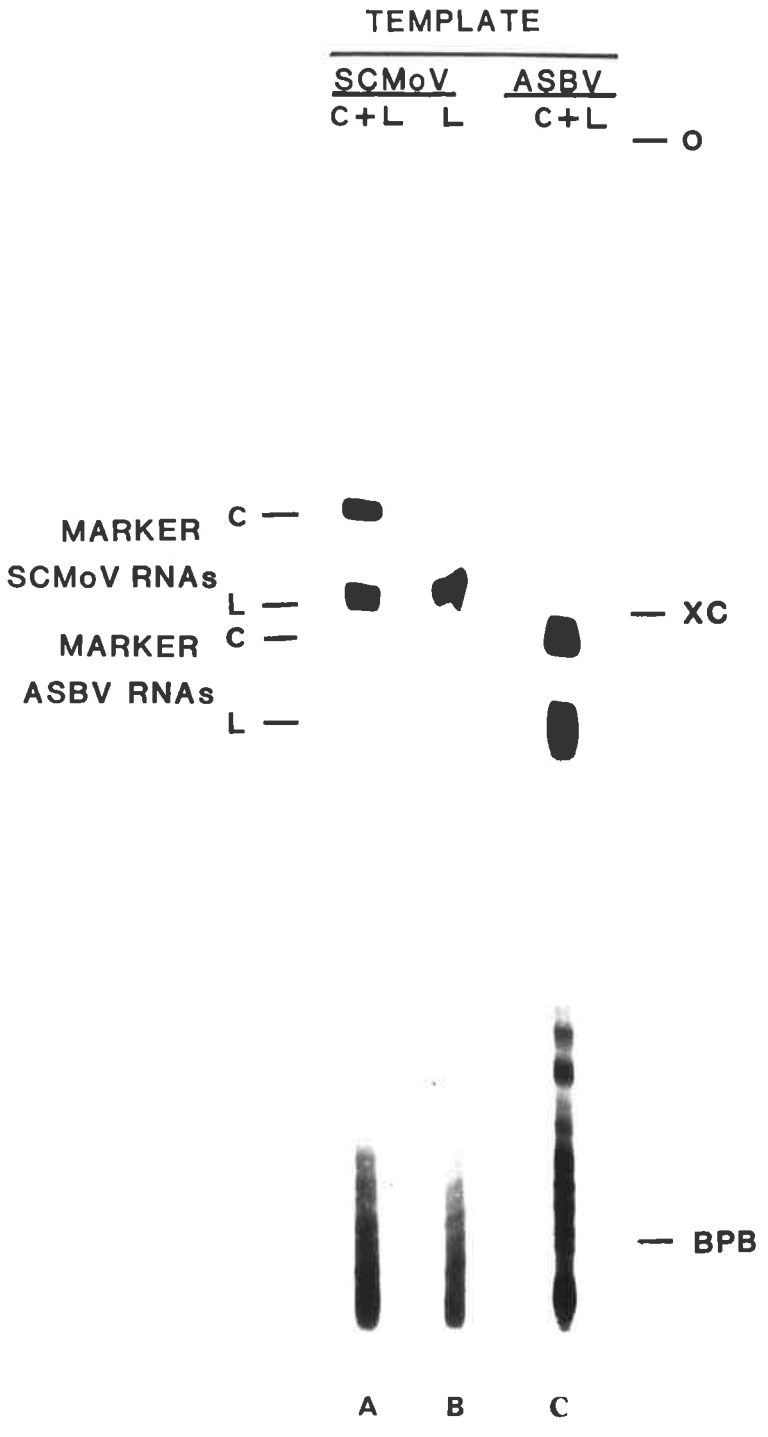
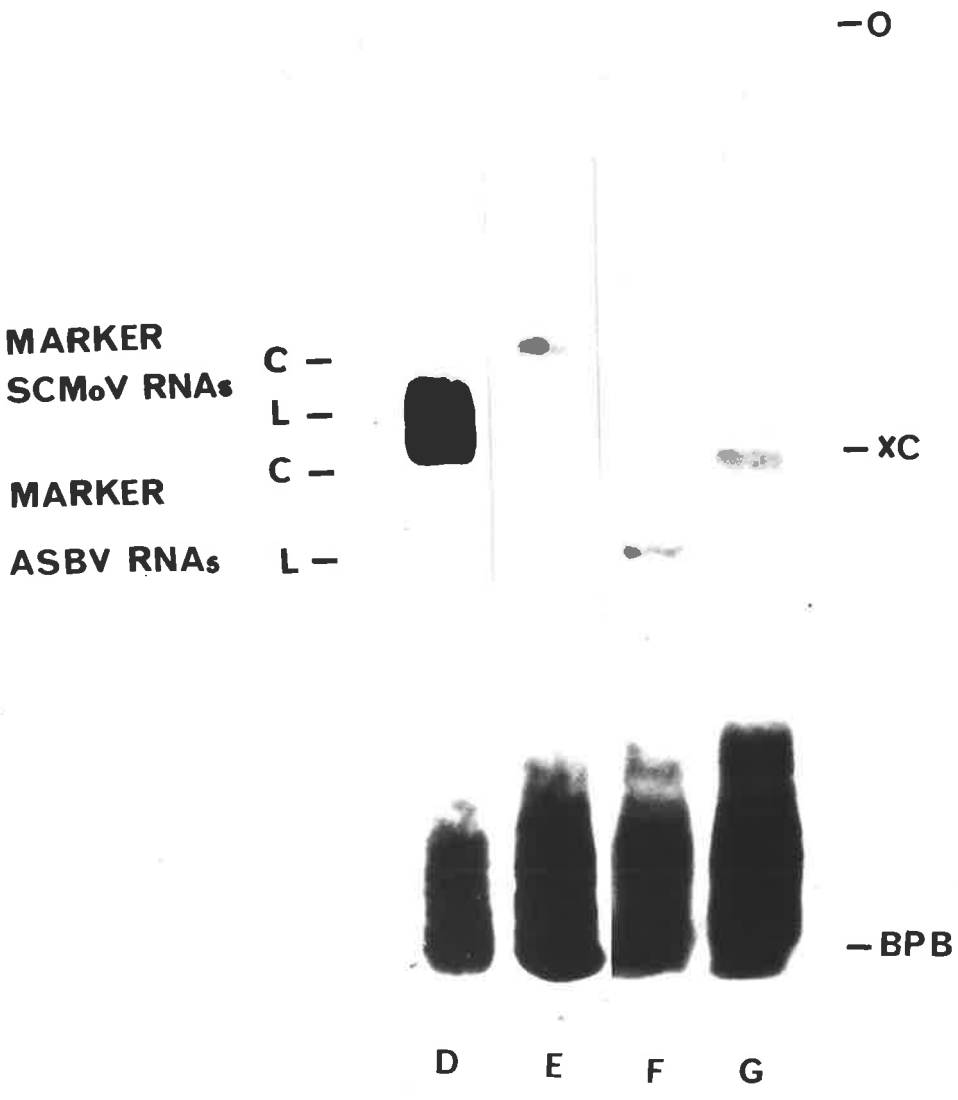

TEMPLATE
SCMoV
C+L
L
ASBV
C+L
O
MARKER
C
SCMoV RNAs
L
MARKER
C
ASBV RNAs
L
XC
BPB
A
B
C

FIGURE 7.8 cont...

(b) FORMALDEHYDE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE PRODUCTS ELUTED FROM TBE-UREA GEL

The ^{32}P-labelled RNA samples were eluted by overnight soaking of each of the TBE-Urea gel slices in 0.5 ml elution buffer (0.5 M ammonium acetate, 0.1% (w/v) SDS and 0.1 mM EDTA, Maxam and Gilbert, 1977). The RNA products were recovered by ethanol precipitation, the pellets aobtained after centrifugation were redissolved in 5 µl of H_2O plus 10 µl of HEPES-sample loading buffer (1.5 X), heated at 80°C for 75 sec and snap cooled just before electrophoresis. Electrophoresis was on a 2 M formaldehyde-3% polyacrylamide (14 x 14 x 0.1 cm) gel in HEPES-NaOH buffer system (Lehrach *et al.*, 1977) until the bromophenol dye reached near the bottom. After electrophoresis gel was stained with toluidine blue to detect marker RNAs run in adjacent tracks on the same gel. Finally, the gel was dried under heat and vacuum and radioactive bands were detected by autoradiography at -80°C.

b



were eluted from the wet TBE-urea-polyacrylamide gel by soaking gel slices in gel elution buffer (Maxim and Gilbert, 1977), denatured by heating at 80°C for 75 sec in formamide (50%, v/v) and formaldehyde (2M), snap cooled and then fractionated on a HCHO-polyacrylamide gel (Lehrach *et al.*, 1977). Polyacrylamide was used as gel matrix due to its property to resolve the circular and linear forms of RNA products (and of marker RNAs as well), whereas these had the same electrophoretic mobilities when agarose (2%) was used as gel matrix (results not given). On complete denaturation only a small fraction (Fig. 7.8b) of the ^{32}P-labelled RNA products eluted from the TBE-urea-polyacrylamide gel were full sized transcripts. The low molecular weight material observed in the HCHO-polyacrylamide gel (Fig. 7.8b) was presumably either due to breakdown of full length transcripts or due to small sized radioactive transcripts which remained associated with the template transcript complex in TBE-urea-polyacrylamide gel (Fig. 7.8a). Because of the small size of the transcript only a small portion of the template was double-stranded so it migrated in the position of the single-stranded marker RNA on TBE-urea gel (Fig. 7.8a).

The results of Figs. 7.8a and b show that the complete denaturation of dsRNA products of viroids could not be achieved on heating the RNA products in formamide and then snap cooling for their analysis on a TBE-urea gel. It was assumed that two strands of the dsRNA products (linear) separated on heating in formamide and stayed as single stranded when they were of full length, but some of the small sized transcripts hybridized back to the template RNA and migrated along with the template RNA in the TBE-urea gel (Fig. 7.8a). The RNA products co-migrating

with circular viroid and virusoidal markers, detected on the HCHO-polyacrylamide gel (Figs. 7.8E and G), were considered to be single-stranded.

Section 7.4 DISCUSSION

The results presented in this chapter indicated that the RNA replicase in the particulate fraction of CMV-infected plants was bound to CMV RNAs (possibly host RNAs also) as templates with nascent chains of transcripts in the form of a replication complex. Synthesis resumed *in vitro* to continue elongation of the nascent chains of the RNA products initiated *in vivo* and to give full length RFs of CMV RNAs as major products. There were five major RNA products synthesized in the *in vitro* RNA replicase assay with the particulate fraction in the absence of any added template. Under denaturing conditions of gel electrophoresis four of them co-migrated with four major RNAs of CMV and fifth (M_r 0.2 X 10^{-6}) was of the size of CMV RNA 4a (M_r 0.26 X 10^{-6}) Peden and Symons, 1973). These major RNA products synthesized by the particulate fraction prepared from CMV-infected plants, on the basis of their electrophoretic mobilities, strain specific variation in their size and hybridization with CMV RNAs were considered to be transcripts of CMV RNAs. These RNA products of the particulate fraction were definitely of -ve polarity whereas the possibility of some products with +ve polarity has not been ruled out.

The RNA replicase bound to the RNA replication complex did not seem to reinitiate or accept any exogenous RNA template to synthesize full length transcripts. Some heterogenous small sized transcripts of plant RNA were synthesized by the particulate

fraction presumably by the RNA replicase enzyme molecules either solubilized in the assay medium or released from the replication complex after completion of nascent transcripts of viral RNAs These host RNA transcripts were not apparent on gel electrophoresis due to their heterogenous size but were easily detectable in blot hybridization studies. Probably these host specific transcripts were synthesized non-specifically in the *in vitro* RNA replicase assay by solubilized RNA replicase on the free host RNA templates rather than by the replicative complex containing host RNA as a template.

Similarity in the RNA products synthesized by the untreated control and $MgSO_4$-treated particulate fraction (Fig. 7.6) indicated that the RNA replication complex was not completely solubilized on incubation of particulate fraction with 150 mM $MgSO_4$ at 37°C. The fraction of replication complex (about 55%) solubilized with $MgSO_4$ treatment lost its ability to incorporate α-^{32}P-NTP into full length RFs of CMV RNAs. Similar results have been reported for CPMV RNA replicase, for which the particulate fraction left after the solubilization of RNA replicase with Mg^{2+}-deficient buffer incorporated the α-^{32}P-NTPs into full length RFs of CPMV RNAs (Dorssers *et al.*, 1983).

The RNA products synthesized in the absence or presence of CMV RNA template by the solubilized crude RNA replicase were heterodisperse and small in size and were host and virus specific. This suggested that RNA replicase solubilized with $MgSO_4$ was either bound to degraded, variable small sized templates or was free of any template and transcribed non-specifically both host and viral RNAs present in the enzyme extract. The latter possibility is more likely and suggests that CMV-induced RNA replicase

after solubilization became non-specific and functionally similar to the RNA-dependent RNA polymerases of uninfected plants. There are two possible reasons for the loss of template specificity of RNA replicase upon solubilization; the integrity of membrane structure might be the essential requirement for CMV-induced RNA replicase to be functionally specific for CMV-RNAs as templates or solubilized RNA replicase required additional regulatory factors which remained unsolubilized (presumably viral gene products) to provide template specificity towards CMV RNAs.

The RNA products synthesized in the presence of CMV RNA as template by the purified CMV-induced particulate RNA replicase were similar to those synthesized by the crude particulate RNA replicase with or without viral RNA template. In contrast to purified soluble CMV-induced RNA replicase (Clark *et al.*, 1974; Kumarasamy and Symons, 1979a) the purified particulate CMV-induced RNA replicase was slightly more active with CMV RNA as template than the other viral and non-viral RNA templates tested.

Another functional similarity of purified CMV-induced RNA replicase with the RNA-dependent RNA polymerase of healthy plants (Boege *et al.*, 1982a) was that it copied viroid and virusoid in *in vitro* RNA replicase assay to synthesize a small fraction of full length transcripts. Both linear and circular RNA products were transcribed when a mixture of linear and circular RNA was used as templates. The exact mechanism of circularization of RNA products was not clear, possibly some RNA ligase activity, which co-purified with RNA replicase and was present in the enzyme preparations, was responsible for circularization of

viroid RNA products. Such *in vitro* circularization of viral progeny RNA by an RNA ligase from wheat germ has recently been reported (Branch *et al.*, 1982). Another possibility can be that the transcribing RNA replicase after completing one cycle around the circular template finds or recognises the 5'-ppp of the starting nucleotide and ligates the two ends covalently to form a closed circular transcript. As the products co-migrated with single stranded circular marker RNAs when analysed on denaturing gel (Figs. 7.8E and G), it is likely that template was nicked (probably by circularizing enzyme) to facilitate the release of product. Techniques such as 2-D fingerprinting (De Wachter and Fiers, 1972) and resistance to RNase (Duda *et al.* 1973) may be needed to establish unequivocally the nature of the transcripts of viroids or virusoids.

CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

The replication of viral RNA in (+)RNA viruses infecting bacterial and animal cells involves a dsRNA and a partially viral-encoded RNA replicase as an intermediate in the cycle between parental and progeny genomes. The recent research on RNA replication of plant RNA viruses, with the isolation, purification, and characterization of host-encoded RNA-dependent RNA polymerases from various uninfected and virus-infected plants, the characterization of their products and the continuous failure to show the direct involvement of viral gene products in viral RNA replication discussed briefly in Chapter One, led to speculation that perhaps host RNA-dependent RNA polymerase is involved in plant viral RNA replication (Zaitlin, 1979; Duda, 1979; Ikegami and Fraenkel-Conrat, 1979b; Fraenkel-Conrat, 1979; 1983). However, in addition to the analogies with bacterial and animal viruses, circumstantial evidence indicates the involvement of virus specified protein(s) in the plant virus RNA replication. The stimulation of RNA replicase activity in virus-infected plants (Table 1.1), dependence of the two smaller RNAs on the two largest RNAs in some tripartite viruses, e.g., AMV (Nassuth *et al.*, 198la) and BMV (Kiberstis *et al.*, 1981) and of the smaller of the two RNAs of some bipartite viruses, i.e., CPMV (Goldbach *et al.*, 1980) and TRV (Harrison and Nixon, 1959a; 1959b) upon the larger, for their expression and also the results with plant virus mutants (Dawson and White, 1978), strongly show that RNA-dependent RNA polymerases of uninfected plants are not sufficient to account for viral RNA replication.

For the detailed investigation of CMV-induced particulate

RNA replicase, the enzyme was solubilized from the particulate fraction of CMV-infected plants with $MgSO_4$ (150 mM) at 37°C for 5 min. The solubilization of CMV-induced RNA replicase with $MgSO_4$ is unique to the procedures used in the solubilization of other plant viral RNA replicases (Table 1.1). The exact mechanism of the synergistic effect of Mg^{2+} and $SO_4^{''}$ is unknown. It is probable that at high salt concentrations of $MgSO_4$, Mg^{2+} binds to template RNA molecules and displaces the RNA replicase into the soluble phase from a membrane bound enzyme-template complex. Although this assumption does not explain the specific requirement for $SO_4^{''}$, the RNA replicase solubilized by this procedure was mainly template free.

SDS-polyacrylamide gel analysis of the CMV-induced particulate RNA replicase after purification on a variety of ion exchange and affinity columns has shown that RNA replicase preparations contained consistently seven major polypeptides (M_r 110,000; 100,000, a triplet between 58,000 to 65,000, 35,000 and 28,000). All of these except one polypeptide (M_r 62,000) were also present in the purified soluble form of CMV-induced RNA replicase. The SDS-gel analysis of various enzyme fractions obtained in the sequential column chromatography purification procedure has shown the enrichment and co-purification of a major polypeptide of M_r 100,000 with RNA replicase activity. It was concluded that this M_r 100,000 polypeptide is the catalytic subunit of the CMV-induced RNA replicase. Six other polypeptides, which co-purified with RNA replicase activity up to the poly(C)-cellulose step, may have some regulatory functions.

The comparison of the RNA replicase partially purified

from CMV-infected cucumber plants with the similar preparations from uninfected cucumber plants showed the presence of three polypeptides (M_r 110,000; 100,000 and 35,000) unique to the RNA replicase preparations. These three enzyme polypeptides, which had electrophoretic mobilities comparable to the *in vitro* translation products of CMV RNAs 2, 1 and 3, respectively, (Schwinghamer and Symons, 1977), were also unique to the purified soluble CMV RNA replicase (Kumarasamy and Symons, 1979a). Furthermore, the three largest RNAs of CMV were required for infection of cucumber plants (Peden and Symons, 1973; Lot *et al.*, 1974). On the basis of these observations it was presumed that the major catalytic subunit (M_r 100,000) and two other components of RNA replicase of Mr 110,000 and 35,000, unique to CMV-infected cucumber plants, were the gene products of CMV RNAs 1, 2 and 3, respectively. Further support that the CMV-induced particulate RNA replicase is virus-induced was provided by the difference in the properties of the RNA replicase induced by two different viruses, CMV and TRSV, in the same host, cucumber (Peden *et al.*, 1972).

On the basis of the results presented in the Chapter Six on the direct comparison of the three enzyme polypeptides (M_r 110,000; 100,000 and 35,000) with the full length *in vitro* translation products of the three largest RNAs of CMV by peptide mapping, on the studies of strain specific differences observed in the size of translation products of the three largest RNAs of P, Q and T-CMV, and on the unchanged polypeptide pattern of RNA replicase induced by corresponding strains of CMV, it was concluded that the three polypeptides of RNA replicase were virus-induced host proteins rather than gene products of CMV

RNAs. This conclusion is consistent with the results that RNA replicase induced by CMV in cucumber plants was different in quantity, chromatographic properties, and polypeptide composition ~~than~~ from that induced in tobacco plants by the same virus (Linthorst, 1982; Takanami and Fraenkel-Conrat, 1982).

Like RNA-dependent RNA polymerases of uninfected plants (discussed in Section 1.3), purified CMV-induced particulate RNA replicase copied a variety of viral RNAs, yeast ribosomal RNA and even avocado sunblotch viroid and the virusoid of subterranean clover mottle virus with little template specificity. However, the analysis of the products synthesized by the particulate fraction, which was the starting material for enzyme solubilization, has shown that the RNA replicase in the particulate fraction, and probably in *in vivo* conditions also is bound to CMV RNAs with the nascent chains of transcripts which were completed to the genome sized products on incubation in the *in vitro* RNA replicase assay. This indicated the involvement of CMV- induced host RNA polymerase (RNA replicase) in the viral RNA replication.

In view of the results discussed in this thesis the conclusion we draw is that the CMV-induced particulate RNA replicase, which is similar to its soluble form, consists of a virus-induced host protein (M_r 100,000) as catalytic subunit which requires viral protein(s) to provide template specificity in the *in vivo* replication of CMV RNA. The exact mechanism by which the CMV gene products act in the induction of host RNA polymerase and provide it with template specificity under *in vivo* conditions is unknown. The investigation of the following aspects may shed some light on the replication of CMV.

i. Detection of CMV gene products in the particulate fraction or soluble phase of CMV-infected cucumber plants by using antibodies raised against synthetic viral peptides.

ii. Extension of the work described by Peden *et al.* (1972) to see if the M_r 100,000 protein induced by CMV infection is also associated with RNA replicase activity induced in TRSV-infected cucumber seedlings.

APPENDIX

PUBLICATIONS

Papers published

Gill, D.S., Kumarasamy, R., and Symons, R.H. (1981)
"Cucumber mosaic virus-induced RNA replicase: Solubilization and partial purification of the particulate enzyme". *Virology* 113, 1-8.

Gordon, K.H.J., Gill, D.S., and Symons, R.H., (1982)
"Highly purified cucumber mosaic virus-induced RNA-dependent RNA polymerase does not contain any of the full length translation products of the genomic RNAs". *Virology* 123, 284-295.

Symons, R.H., Gill, D.S., Gordon, K.H.J., and Gould, A.R. (1983)
"Gene content and expression of the four RNAs of cucumber mosaic virus" in *'Manipulation and Expression of Genes in Eukaryotes'* (P. Nagley, A.W. Linnane, W.J. Peacock, and J.A. Pateman, eds). pp. 373-380, Academic Press, Sydney.

Abstracts of the papers presented at conferences:

Gill, D.S., and Symons, R.H. (1980)
"Cucumber mosaic virus-induced RNA replicase: Solubilization and properties of the particulate enzyme" in *Proceedings of Australian Biochemical Society*, 13, 96.

Symons, R.H., Gill, D.S., and Gordon, K.H.J. (1981)
"Characterization of the soluble and particulate forms of the RNA replicase of cucumber mosaic virus" in *Abstracts*

of the 5th International Congress of Virology, Strasbourg, p. 250.

Gill, D.S., Gordon, K.H.J. and Symons, R.H. (1982) "Structure and properties of cucumber mosaic virus-induced RNA replicase" in *12th International Congress of Biochemistry Perth*, p. 159 (Abstracts).

REFERENCES

Ahlquist, P., Dasgupta, R., and Kaesberg, P. (1981) *Cell* 23, 183.

Astier-Manifacier, S., and Cornuet, P. (1971) *Biochim. Biophys. Acta* 232, 484.

Astier-Manifacier, S., and Cornuet, P. (1978) *C.R.Seances, Acad Sci. D.* 287, 657.

Astier-Manifacier, S., and Cornuet, P. (1981) in *Abstracts of the 5th International Congress of Virology, Stasbourg.* p. 249.

Atabekov, J.G. and Morozov, S.Y. (1979) *Adv. Virus Res.* 25, 1.

Baltimore, D. (1971) *Bacter. Rev.* 35, 235.

Bancroft, J.B. and Lane, L.C. (1973) *J. Gen. Virol.* 19, 381.

Bandle, E., and Weissmann, C. (1970) *Biochim. Biophys. Acta.* 199, 551.

Baron, M.H., and Baltimore, D. (1982a) *Cell* 28, 395.

Baron, M.H., and Baltimore, D. (1982b) *J. Virol.* 43, 969.

Beachy, R.N., and Zaitlin, M. (1977) *Virology* 81, 160.

Biebricher, C.K., Diekmann, S., and Luce, R. (1982) *J. Mol. Biol.* 154, 629.

Blumenthal, T. (1979) *Methods in Enzymology* 60, 628.

Blumenthal, T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 2601.

Blumenthal, T., and Carmichael, G.G. (1979) *Ann. Rev. Biochem.* 48, 525.

Bock, R.M. (1967) *Methods in Enzymology* 12, 224.

Boege, F., Rohde, W., and Sänger, H.L. (1982a) *Bioscience Reports* 2, 185.

Boege, F., Rohde, W., and Sanger, H.L. (1982b) in "*12th International Congress of Biochemistry, Perth*" p.160 (abstr.).

Boege, F., and Sanger, H.L. (1980) *F.E.B.S. Letters* 121, 91.

Bol, J.F., Clerx-Van Haaster, C.M., and Weening, C.J. (1976) *Ann. Microbiol. (Paris)* 127A, 183.

Bol, J.F., Van Vloten-Doting, L. and Jaspars, E.M.J. (1971) *Virology* 46, 73.

Bonner, W.M., and Laskey, R.A. (1974) *Eur. J. Biochem.* 46, 83.

Boyd, C.D., and Fitchen, W. (1977) *Nucleic Acids Res.* 4, 461.

Branch, A.D., Robertson, H.D., Greer, C., Gegenheimer, P., Peebles, C., and Abelson, J. (1982) *Science* 217, 1147.

Brishammer, S., and Juntti, N. (1974) *Virology* 59, 245.

Brown, F. (1981) *Trends in Biochemical Sciences* 6, 325.

Bruening, G. (1977) In "*Comprehensive Virology*" (H. Fraenkel-Conrat, and R.R. Wagner, eds). Vol. 11, pp. 55-141. Plenum Press, New York.

Bruening, G. (1981) In "*The Biochemistry of Plants*" (A. Marcus ed.) Vol. 6, pp. 571-631, Academic Press, New York.

Bruening, G., Beachy, R.N., Scalla, R., and Zaitlin, M. (1976) *Virology* 71, 498.

Bruening, G., Beachy, R., and Zaitlin, M. (1979) In "*Molecular Biology of Plants*" (I. Rubenstein, R.L. Phillips, C.E. Green and B.G. Gengenbach, eds.) pp. 241-272, Academic Press, New York.

Bujarski, J.J., Hardy, S.F., Miller, W.A., and Hall, T.C. (1982) *Virology* 119, 465.

Carmichael, G.G. (1975) *J. Biol. Chem.* 250, 6160.

Carmichael, G.G. (1979) *Methods in Enzymology* 60, 456.

Carmichael, G.G., Landers, T.A., and Weber, K. (1976) *J. Biol. Chem.* 251, 2744.

Challberg, M.D., and Kelly, T.J. (1981) *J. Virol.* 38, 272.

Chamberlin, M.J. (1974) In *"The Enzymes"* (P.D. Boyer, ed.) Vol. X, pp. 333-374. Academic Press, New York.

Chamberlin, M.J., and Ring, J. (1973) *J. Biol. Chem.* 248, 2245.

Chambon, P. (1974) In *"The Enzymes"* (P.D. Boiyer ed.) Vol. X, pp. 261-331.

Chifflot, S., Sommer, P., Hartmann, D., Stussi-Garaud, C., and Hirth, L. (1980) *Virology* 100, 91.

Clark, G.L., Peden, K.W.C., and Symons, R.H. (1974) *Virology* 62, 434.

Clarke, V.M., and Kirby, A.J. (1966) *Biochememm. Prep.* 11, 101.

Clegg, J.C.S., Brzeski, H., and Kennedy, S.I.T. (1976) *J. Gen. Virol.* 32, 413.

Clerx, C.M., and Bol, J.F. (1978) *Virology* 91, 453-463.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) *J. Biol. Chem.* 252, 1102.

Dasgupta, A., Baron, M.H., and Baltimore, D. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 2679.

Dasgupta, A., Zabel, P., and Baltimore, D. (1980) *Cell* 19, 423.

Daubert, S.D., and Bruening, G. (1979) *Virology* 98, 246.

Daubert, S.D., Bruening, G., and Najarian, R.C. (1978) *Eur. J. Biochem.* 92, 45.

Davies, J.W. (1979) In *"Nucleic Acids in Plants"* (T.C. Hall and J.W. Davies, eds.) Vol. II, pp. 111-149. CRC Press, Boca Raton, Fla.

Dawson, W.O., and White, J.L. (1979) *Virology* 93, 104.

De Wachter, R., and Fiers, W. (1972) *Anal. Biochem.* 49, 184.

Diener, T.O. (1981) *Scientific American* 244(1), 58.

Dorssers, L., v/d Meer, J., Van-Kammen, A., and Zabel, P. (1981) In *"Abstracts of 5th International Congress of Virology Stasbourg"* p. 249.

Dorssers, L., v/d Meer, J., Van-Kammen, A., and Zabel, P. (1983) *Virology* in press

Dorssers, L., Zabel, P., v/d Meer, J., and Van-Kammen, A. (1982) *Virology* 116, 236.

Downey, K.M., Byrnes, J.J., Jurmark, B.S., and So, A.G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 3400.

Duda, C.T. (1979) *Virology* 92, 180.

Duda, C.T., Zaitlin, M., and Siegel, A. (1973) *Biochim.Biophys. Acta.* 319, 62.

El Manna, M.M., and Bruening, G. (1973) *Virology* 56, 198.

Flanegan, J.B. and Baltimore, D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 3677.

Flanegan, J.B., and Baltimore, D. (1979) *J. Virol.* 29, 352.

Flanegan, J.B., Petterson, R.F., Ambros, V., Hewlett, M.J., and Baltimore, D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 961.

Flanegan, J.B., Tuschall, D.M., and Young, D.C. (1982) in *"12th International Congress of Biochemistry, Perth"* p. 168, (Abstr.).

Fraenkel-Conrat, H. (1976) *Virology* 72, 23.

Fraenkel-Conrat, H. (1979) *Trends in Biochemical Science* 4(3), 184.

Fraenkel-Conrat, H. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80, 422.

Francki, R.I.B., Mossop, D.W., and Hatta, T. (1979) In *"CMI/AAB Description of Plant Viruses"* No. 213. Commonwealth Mycological Inst. Kew, Surrey, England and Association of Applied Biologists.

Francki, R.I.B., Randles, J.W., Chambers, T.C., and Wilson, S.B. (1966) *Virology* 28, 729.

Franze de Fernandez, M.T., Eoyang, L., and August, J.T. (1968) *Nature (London)* 219, 588.

Frisby, D.P., Newton, C., Carey, N.H., Feliner, P., Newman, J.E.F., Harris, T.J.R., and Brown, F. (1976) *Virology* 71, 379.

Gerlinger, P., Mohier, E., Le Meur, M.A. and Hirth, L. (1977) *Nucleic Acids Res.* 4, 813.

Gilliland, J.M., and Symons, R.H. (1968) *Virology* 36, 232.

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J., and Karn, J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 5818.

Goldbach, R., Rezelman, G., and Van-Kammen, A. (1980) *Nature (London)* 286, 297.

Golini, F., Nomoto, A., and Wimmer, E. (1978) *Virology* 89, 112.

Gonda, T.J. and Symons, R.H. (1979) *J. Gen. Virol.* 45, 723.

Gordon, K.H.J., and Symons, R.H. (1982) In *"12th International Congress of Biochemistry, Perth* p. 169, (Abstr.).

Gordon, K.H.J., and Symons, R.H. (1983) *Nucleic Acids Res.* in press.

Gould, A.R., and Symons, R.H. (1977) *Nucleic Acids Res.* 4, 3787.

Gould, A.R., and Symons, R.H. (1978) *Eur. J. Biochem.* 91, 269.

Gould, A.R., and Symons, R.H. (1982) *Eur. J. Biochem.* 126, 217.

Gunn, M.R., and Symons, R.H. (1980) *F.E.B.S. Letters* 115, 77.

Habili, N., and Francki, R.I.B. (1974a) *Virology* 57, 392.

Habili, N., and Francki, R.I.B. (1974b) *Virology* 61, 443.

Hadidi, A. (1974) *Virology* 58, 536.

Hadidi, A., and Fraenkel-Conrat, H. (1973) *Virology* 52, 363.

Haenni, A.L., Joshi, S., and Chapeville, F. (1982) *Prog. Nucleic Acid Res. Mol. Biol.* 27, 85.

Hall, T.C., Miller, W.A., and Bujarski, J.J. (1982) In *"Advances in Plant Pathology"* (D. Ingram and P.H. Williams eds.) Vol. I, pp. 179-211. Academic Press, New York.

Hardy, S.F., German, T.L., Sue Loesch-Fries, L., and Hall, T.C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 4956.

Hariharasubramanian, V., Hadidi, A., Singer, B., and Fraenkel-Conrat, H. (1973) *Virology* 54, 190.

Harrison, B.D., and Nixon, H.L. (1959a) *J. Gen. Microbiol.* 21, 569.

Harrison, B.D., and Nixon, H.L. (1959b) *J. Gen. Microbiol.* 21, 591.

Haruna, I., and Spiegelman, S. (1965) *Proc. Natl. Acad. Sci. U.S.A.* 54, 579.

Haseloff, J., Mohamed, N.A., Symons, R.H. (1982) *Nature (London)* 299, 316.

Hirth, L. (1980) In *"Genome Organization and Expression in Plants"* (C.J. Leaver ed.) NATO Advanced Study Institute Series A., Vol. 29, pp. 497-510. Plenum, New York.

Hollings, M., and Stone, O.M. (1971) In *"CMI/AAB Descriptions of Plant Viruses"* No. 79. Commonwealth Mycological Institute, Kew, Surrey, England.

Hunter, T.R., Hunt, T., Knowland, J., and Zimmern, D. (1976) *Nature (London)* 260, 759.

Ikegami, M., and Fraenkel-Conrat, H. (1978a) *Proc. Natl. Acad. Sci. U.S.A.* 75, 2122.

Ikegami, M., and Fraenkel-Conrat, H. (1978b) *F.E.B.S. Letters* 96, 197.

Ikegami, M., and Fraenkel-Conrat, H. (1979a) *J. Biol. Chem.* 254, 149.

Ikegami, M., and Fraenkel-Conrat, H., (1979b) *Proc. Natl. Acad. Sci. U.S.A.* 76, 3637.

Ikegami, M., and Fraenkel-Conrat, H. (1979c) *Virology* 100, 185.

Jackson, A.O., Zaitlin, M., Siegel, A., and Francki, R.I.B. (1972) *Virology* 48, 655.

Jaspars, E.M.J. (1974) *Adv. Virus Res.* 19, 37.

Kaesberg, P. (1976a) In *"Animal Virology. ICN-UCLA Symposia on Molecular and Cellular Biology"* (D. Baltimore, S. Huang, and C.F. Fox, eds.)., Vol 4, pp. 555-566. Academic Press, New York.

Kaesberg, P. (1976b) *Prog. Nucleic Acid Res. Mol. Biol.* 19, 465.

Kaesberg, P. (1977) In *"Beltsville Symposia in Agricultural Research, (1) Virology in Agriculture"* (J.A. Romberger, ed). pp. 267-272. Allenheld Osmun and Co., Montclair, N.J.

Kamen, R.I. (1972) *Biochim. Biophys. Acta.* 262, 88.

Kamen, R.I. (1975) In *"RNA Phages"* (N. Zinder, ed.) pp. 203-234. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Kamen, R., Kondo, M., Romer, W., and Weissmann, C. (1972) *Eur. J. Biochem.* 31, 44.

Kaper, J.M., Diener, T.O., and Scott, H.A. (1965) *Virology* 27, 54.

Kaper, J.M., and Waterworth, H.E. (1977) *Science* 196, 429.

Kaper, J.M., and Re, G.G. (1974) *Virology* 60, 308.

Kiberstis, P., Loesch-Fries, L.S., and Hall, T.C. (1981) *Virology* 112, 804.

Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.J., Dorner, A.J., Emini, E.A., Hanecak, R., Lee, J.J., v/d Merf, S., Anderson, C.W., and Wimmer, E. (1981) *Nature (London)* 291, 547.

Kohl, R.J., and Hall, T.C. (1974) *J. Gen. Virol.* 25, 257.

Kumarasamy, R. (1980) Ph.D. Thesis, Adelaide University, Adelaide, South Australia.

Kumarasamy, R., and Symons, R.H. (1979a) *Virology* 96, 622.

Kumarasamy, R., and Symons, R.H. (1979b) *Anal. Biochem.* 95, 359.

Kummert, J. (1974) *Virology* 57, 314.

Kummert, J., and Semal, J. (1972) *J. Gen. Virol.* 16, 11.

Kupper, H.J., MacAllister, W.T., and Bautz, E.K.F. (1973) *Eur. J. Biochem.* 38, 581.

Laemmli, U.K. (1970) *Nature (London)* 227, 680.

Lane, L.C. (1974) *Adv. Virus Res.* 19, 152.

Lane, L.C., and Kaesberg, P. (1971) *Nature (London) New Biol.* 232, 40.

Laskey, R.A., and Mills, A.D. (1975) *Eur. J. Biochem.* 56, 335.

Lee, Y.F., Nomoto, A., Detjen, B.M., Wimmer, E. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 59.

Lehrach, H., Diamond, D., Wozeney, J.M., and Boedtker, H. (1977) *Biochemistry* 16, 4743.

Le Roy, C., Stussi-Garaud, C., and Hirth, L. (1977) *Virology* 82, 48.

Lichy, J.H., Horwitz, M.S., and Hurwitz, J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 2678.

Linthorst, H.J.H. (1982) Ph.D. Thesis, University of Leiden, Leiden, The Netherlands.

Linthorst, H.J.H., Bol, J.F., and Jaspars, E.M.J. (1980) *J. Gen. Virol.* 46, 511.

Lot, H., and Kaper, J.M. (1976a) *Virology* 74, 209.

Lot, H., and Kaper, J.M. (1976b) *Virology* 74, 223.

Lot, H., Marchoux, G., Marrou, J., Kaper, J.M., West, C.K., Van Vloten-Doting, L., and Hull, R. (1974) *J. Gen. Virol.* 22, 81.

Lowry, O.H., Rosebrough, N.J., Farr, A.D., and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265.

Lund, G.A., and Scraba, D.G. (1979) *J. Gen. Virol.* 44, 391.

March, S.C., Parikh, I., and Guatrecasas, P. (1974) *Anal. Biochem.* 60, 149.

May, J.T., and Symons, R.H. (1971) *Virology* 44, 517.

May, J.T., Gilliland, J.M., and Symons, R.H. (1969) *Virology* 39, 54.

May, J.T., Gilliland, J.M., and Symons, R.H. (1970) *Virology* 41, 653.

Maxam, A.M., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 560.

Meyer, F., Weber, H. and Weissmann, C. (1981) *J. Mol. Biol.* 153, 631.

Mills, D.R., Nishihara, T., Dobkin, C., Kramer, F.R., Cole, P.E. and Spiegelman, S. (1977) In *"Nucleic Acid-Protein Recognition"* (H.J. Vogel, ed.), pp. 533-547. Academic Press, New York.

Mink, G.I. (1972) *"CMI/AAB Descriptions of Plant Viruses"* No. 92. Commonwealth Mycological Institute, Kew, Surrey, England.

Mohier, E., Hirth, L., Le Meur, M.-A., and Gerlinger, P. (1975) *Virology* 68, 349.

Moore, G., and Crichton, R.R. (1973) *F.E.B.S. Letters* 37, 74.

Mossop, D.W., Francki, R.I.B., and Grivell, C.J. (1976) *Virology* 74, 544.

Mouchés, C., Bové, C., Barreau, C., and Bové, J.M. (1976) *Ann. Microbiol. (Inst. Pasteur)* 127A, 75.

Mouchés, C., Bové, C., and Bové, J.M. (1974) *Virology* 58, 409.

Mouchés, C., Renaudin, J., Chuchana, P., and Bové, J.M. (1981) In *"Abstracts of the 5th International Congress of Virology, Strasbourg"* p. 249.

Nassuth, A., Alblas, F., and Bol, J.F. (1981a) *J. Gen. Virol.* 53, 207.

Nassuth, A., Alblas, F., and Bol, J.F. (1981b) In *"Abstracts of the 5th International Congress of Virology, Strasbourg"* p. 248.

Newman, J.F.E., Cartwright, B., Doel, T.R., and Brown, F. (1979) *J. Gen. Virol.* 45, 497.

Nomoto, A., Detjen, B., Pozzatti, R., and Wimmer, E. (1977) *Nature (London)* 268, 208.

Okuno, T., and Furusawa, I. (1979) *Virology* 99, 218.

Peden, K.W.C., and Symons, R.H. (1973) *Virology* 53, 487.

Peden, K.W.C., May, J.T., and Symons, R.H. (1972) *Virology* 47, 498.

Pelham, H.R.B. (1979) *Virology* 96, 463.

Pelham, H.R.B., and Jackson, R.J. (1976) *Eur. J. Biochem* 67, 247.

Petterson, R.F., Ambros, V., and Baltimore, D. (1978) *J. Virol.* 27, 357.

Rackwitz, H.-R., Rohde, W., and Sanger, H.L. (1981) *Nature (London)* 291, 297.

Ralph, R.K., and Wojcik, S.J. (1969) *Virology* 37, 276.

Ranu, R.S., and London, I.M. (1979) *Methods in Enzymology* 60, 459.

Rao, A.L.N., and Francki, R.I.B. (1981) *Virology* 114, 573.

Rao, A.L.N., Hatta, T., and Francki, R.I.B. (1982) *Virology* 116, 318.

Reijnders, L., Aalbers, A.M.J., Van-Kammen, A., and Thuring, R.W.J. (1974) *Virology* 60, 515.

Rezelman, G., Goldbach, R., and Van-Kammen, A. (1980) *J. Virol.* 36, 366.

Rice, R.H., and Means, G.E. (1971) *J. Biol. Chem.* 246; 831.

Richards, K.E., Jonard, G., Jacquemond, M., and Lot, H. (1978) *Virology* 89, 395.

Romaine, C.P., and Zaitlin, M. (1978) *Virology* 86, 241.

Sakai, F., Dawson, J.R.O., and Watts, J.W. (1979) *J. Gen. Virol.* 42, 323.

Sanger, F., and Coulson, A.R. (1978) *F.E.B.S. Letters* 87, 107.

Sasaki, Y., Ishiye, M., Goto, H., and Kamikubo, T. (1979) *Biochim. Biophys. Acta.* 564, 437.

Schafer, R., Zilling, W., and Zechel, K. (1973) *Eur. J. Biochem.* 33, 207.

Schetters, H., and McLeod, B. (1979) *Anal. Biochem.* 98, 329.

Schwinghamer, M.W., and Symons, R.H. (1975) *Virology* 63, 252.

Schwinghamer, M.W., and Symons, R.H. (1977) *Virology* 79, 88.

Sela, I., and Hauschner, A. (1975) *Virology* 64, 284.

Semler, B.L., Anderson, C.W., Hanecak, R., Dorner, L.F., and Wimmer, E. (1982) *Cell* 28, 405.

Semal, J., and Kummert, J. (1970) *J. Gen. Virol.* 7, 173.

Semal, J., and Kummert, J. (1971a) *J. Gen. Virol.* 10, 79.

Semal, J., and Kummert, J. (1971b) *J. Gen. Virol.* 11, 189.

Siegel, A., Hari, V., and Kolacz, K. (1978) *Virology* 85, 494.

Siegel, A., Zaitlin, M., and Duda, C.T. (1973) *Virology* 53, 75.

Smit, C.H., and Jaspars, E.M.J. (1980) *Virology* 104, 454.

Smit, C.H., Roosien, J., Van Vloten-Doting, L., and Jaspars, E.M.J. (1981) *Virology* 112, 169.

Sommer, P., Andriamanantena, A.G., Chevallier, D., Stussi-Garaud, C., and Hirth, L. (1981) In *"Abstracts of the 5th International Congress of Virology, Strasboug"* p. 248.

Sommer, P., Andriamanantena, A.G., and Stussi-Garaud, C. (1981) *Journal of Virological Methods* 3, 229.

Spindler, S.R., Duester, G.L. and D'Alessio, J.M., and Paule, M.R. (1978) *J. Biol. Chem.* 253, 4669.

Stanley, J.S., Goldbach, R., and Van-Kammen, A. (1980) *Virology* 106, 180.

Stanley, J., Rottier, P., Davies, J.W., Zabel, P. and Van-Kammen, A. (1978) *Nucleic Acids Res.* 5, 4505.

Stussi-Garaud, C., Lemius , J., and Fraenkel-Conrat, H. (1977) *Virology* 81, 224.

Sugiura, M. (1980) *Anal. Biochem.* 108, 227.

Symons, R.H. (1975) *Mol. Biol. Reports* 2, 277.

Symons, R.H. (1977) *Nucleic Acids Res.* 4, 4347.

Symons, R.H. (1978) *Aust. J. Biol. Sci.* 31, 25.

Symons, R.H. (1979) *Nucleic Acids Res.* 7, 825.

Takanami, Y., and Fraenkel-Conrat, H. (1982) *Biochemistry* 21, 3161.

Takanami, Y., Kubo, S., and Imaizumi, S. (1977) *Virology* 80, 376.

Teissere, M., Penon, P., Azou, Y., and Ricard, J. (1977) *F.E.B.S. Letters* 82, 77.

Thang, M.N., Dondon, L., Thang, D.C., Mohier, E., Hirth, L., Le Meur, N.A., and Gerlinger, P. (1975) *INSERM* 47, 225.

Thomas, P.S. (1980) *Proc, Natl. Acad. Sci. U.S.A.* 77, 5201.

Thompson, S.T., Cass, K.H., and Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 669.

Traub, A., Diskin, B., Rosenberg, H., and Kalmer, E. (1976) *J. Virol.* 18, 375.

Van-Dyke, T.A., and Flanegan, J.B. (1980) *J. Virol.* 35, 732.

Van-Dyke, T.A., Rickles, R.J., and Flanegan, J.B. (1982) *J. Biol. Chem.* 257, 4610.

Van-Kammen, A. (1972) *Ann. Rev. Phytopathol.* 10, 125.

Van Regenmortel, M.H.V. (1967) *Virology* 31, 391.

Van Regenmortel, M.H.V., Hendry, D.A., and Baltz, T. (1972) *Virology* 49, 647.

Van Tol, R.G.L. (1981) Ph.D. Thesis, University of Leiden, Leiden, The Netherlands.

Van Vloten-Doting, L. (1976) *Ann. Microbiol (Paris)* 127A, 119.

Van Vloten-Doting, L., and Jaspars, E.M.J. (1977) IN *"Comprehensive Virology"* (H. Fraenkel-Conrat and R.R. Wagner, eds.). Vol. 11, pp. 1-47. Plenum Press, New York.

Weening, C.J., and Bol, J.F. (1975) *Virology* 63, 77.

White, J.L., and Dawson, W.O. (1978) *Virology* 88, 33.

White, J.L., and Murakishi, H.H. (1977) *J. Virol.* 21, 484.

Wickner, R.B. (1973) In *"Methods in Molecular Biology, Nucleic Acid Biosynthesis"* (A.I. Laskin, and J.A. Last, eds). Vol. 4, pp. 157-166. Marcel Dekker, New York.

Wilson, P.A., and Symons, R.H. (1981) *Virology* 112, 342.

Wimmer, E. (1982) *Cell* 28, 199.

Zabel, P., Jongen-Neven, I., and Van Kammen, A. (1975) *INSERM* 47, 143.

Zabel, P., Jongen-Neven, I., and Van Kammen, A. (1976) *J. Virol.* 17, 679.

Zabel, P. Jongen-Neven, I., and Van-Kammen, A. (1979) *J. Virol.* 29, 21.

Zabel, P., Weenen-Swaans, H., and Van-Kammen, A. (1974) *J. Virol.* 14, 1049.

Zaitlin, M. (1979) In *Nucleic Acids in Plants* (T.C. Hall and J.W. Davies, eds.) Vol. II, pp. 31-64. CRC Press Inc. Boca Raton, Florida.

Zaitlin, M., Beachy, R.N., Bruening, G., Romaine, C.F., and Scalla, R. (1976) In *"Animal Virology, ICN-UCLA Symposia on Molecular and Cellular Biology"* (D. Baltimore, S. Huang and C.F. Fox, eds.). Vol. 4, pp. 567-581. Academic Press, New York.

Zaitlin, M., Duda, C.T., and Petti, M.A. (1973) *Virology* 53, 300.

Ziemiecki, A., and Wood, K.R. (1976) *J. Gen. Virol.* 31, 373.