INTERACTIONS BETWEEN GENOMIC RNAS AND A SATELLITE RNA OF CUCUMOVIRUSES

by

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CONTENTS

SUMMARY

1. Fourteen cucumovirus isolates were examined with respect to their RNA profiles, antigenic properties and amino acid compositions. All isolates contained four major RNA components, designated RNAs 1-4 in order of decreasing molecular weight, the three largest of which are genomic. However, some isolates had an additional RNA with a molecular weight of approximately 1.05×10^5 daltons. Antigenic properties of the isolates divided them into two serologically unrelated groups; 11 strains of CMV (two of the CMV isolates were considered to be the same) and 2 strains of TMV. Amino acid composition data confirmed this division, and in addition separated the CMV strains into 2 sub-groups.

2. Heterologous mixtures of genomic RNAs 1+2 and RNA 3 from three strains of CMV and a strain of TAV were used to investigate the genetic function of RNA 3. It was confirmed that RNA 3 specifies coat protein and it was also demonstrated that it is associated with aphid transmission. In addition it was shown that symptom expression on host plants can be determined by genetic information on different RNA components. Some host reactions appear to be associated with gene(s) located on RNAs 1 and/or 2 and others on RNA 3 alone. However, in some instances, the symptom expression appears to involve interactions between genetic information on both RNA 3 and RNAs 1 and/or 2.

3. Two types of RNA, each with a molecular weight of approximately 1.05×10^5 daltons, designated RNA 5 and Sat-RNA, have been found in purified preparations of CMV and have been characterized by molecular

hybridization analysis using ^{32}P-labelled complementary DNA (cDNA) probes. RNA 5 was shown to consist of specific cleavage products of RNAs 1-4. In contrast, Sat-RNA has a unique nucleotide sequence with no detectable homology with CMV-RNAs.

4. Sat-RNA was compared with a similar low molecular weight RNA (CARNA 5) isolated in the U.S.A. Whereas CARNA 5 is known to induce severe disease symptoms in tomato plants in the presence of its CMV helper, no such reactions could be induced by all strains of CMV examined in the presence of Sat-RNA. A comparison of the base sequences of these two satellite RNAs showed that they have approximately 70% of their nucleotide sequences in common. It would appear that this difference in their primary structures is reflected in differences in their biological properties.

5. Sat-RNA was readily transmitted from one cucumovirus strain to another, and in all strains of CMV examined, it was replicated (and encapsidated) to high levels. In contrast, Sat-RNA was produced in low amounts in the presence of TAV. As a consequence of Sat-RNA replication, the yield of its associated CMV and the proportion of CMV-RNAs 1 and 2 were both markedly reduced. Sat-RNA is unable to replicate autonomously and is hence dependent on cucumoviruses for both its replication and encapsidation. This helper function could not be fulfilled by either alfalfa mosaic virus or tobacco ringspot virus. Using cDNA transcribed to Sat-RNA as a probe, it was shown that Sat-RNA is able to survive *in vivo* for prolonged periods in the absence of its helper virus. This capacity for *in vivo* survival was also shared by the RNA of satellite tobacco

necrosis virus (STNV-RNA), but not by the genomic RNA 3 of CMV.

6. Both Sat-RNA and STNV-RNA was shown to be more resistant to nuclease digestion and inactivation in crude plant extracts than the RNAs of their respective helper viruses. It is possible that the *in vivo* survival of Sat-RNA and STNV-RNA may be related to features of their molecular structure.

STATEMENT

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

Donald W. Mossop

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ABBREVIATIONS

AMV	alfalfa mosaic virus
BMV	brome mosaic virus
CCMV	cowpea chlorotic mottle virus
CMtV	carnation mottle virus
CMV	cucumber mosaic virus
PcV	*Penicillium chrysogemum* virus
PsV	*Penicillium stoloniferum* virus
RRV	raspberry ringspot virus
TBRV	tomato black ring virus
TMV	tobacco mosaic virus
TRSV	tobacco ringspot virus
TRV	tobacco rattle virus
TYMV	turnip yellow mosaic virus
TAV	tomato aspermy virus
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
cpm	counts/min
dCTP	deoxycytosine triphosphate
Ci	Curie
DI particles	defective interfering particles
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DNase	deoxyribonuclease
ds	double-stranded
EDTA	ethylenediaminetetraacetic acid
dGTP	deoxyguanosine triphosphate
mA	milliamperes

mRNA	messenger ribonucleic acid
oligo(dT)	oligodeoxythymidylic acid
PEG	polyethylene glycol
POPOP	1,4,-bis-[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
ss	single-stranded
SSC (1x)	0.15M $NaCl_2$, 0.015M sodium citrate (pH 7.0)
TCA	trichloroacetic acid
TEMED	N,N,N',N"-tetramethylethylenediamine
T_m	melting temperature
Tris	tris(hydroxymethyl)aminoethane
dTTP	deoxythymidine triphosphate
UV	ultraviolet
TGA	thioglycollic acid

CHAPTER 1

INTRODUCTION

The Cucumovirus Group

An infectious, filterable disease agent inciting severe leaf mosaic and fruit blistering symptoms on several cucurbit species was first described, independently, by Doolittle (1916) and Jagger (1916). Since its discovery, cucumber mosaic virus (CMV) has been variously referred to as cucumber virus 1, *Cucumis* virus 1, *Marmor cucumeris*, spinach blight virus and tomato fern leaf virus (Gibbs and Harrison, 1970). The cucumovirus group, of which CMV is the type member, has the cryptogram R/1:1.1/18+1.0/18+(0.70+0.3)/18:S/S:S/C,Ve/Ap (Fenner, 1976). Other members of the cucumovirus group include tomato aspermy virus (TAV; Hollings and Stone, 1971), peanut stunt virus (PSV; Mink, 1972) and cowpea ringspot virus (CpRSV; Phatak *et al.*, 1976).

The relationships between members of the cucumovirus group, based largely on serological studies and cross-protection tests are still controversial (Habili and Francki, 1974a). These authors failed to demonstrate any serological relationship between the V strain of TAV (VTAV) and the Q strain of CMV (QCMV; Francki *et al.*, 1966). However, other work using different isolates of CMV and TAV (Hollings *et al.*, 1968; Stace-Smith and Tremaine, 1973; Devergne and Cardin, 1975) has detected distant serological relationships between TAV and CMV. Devergne and Cardin (1975) also indicated that PSV is distantly related to both CMV and TAV. However, CpRSV appears to be serologically unrelated to CMV, TAV or PSV (Phatak *et al.*, 1976). It is significant

that the apparent degree of serological relationship between TAV and CMV indicated by Hollings *et al.* (1968) and Devergne and Cardin (1975) depended on the virus isolates compared and the antiserum used. Mink *et al.* (1975) suggested that the reported serological relationships between TAV and CMV should be viewed with some caution since they established that in several instances, antisera had been prepared to a mixture of the two viruses.

On the basis of differences in the biological, chemical and serological properties of VTAV and QCMV, Habili and Francki (1974a) concluded that these two cucumovirus isolates are different viruses and not merely strains of the same virus.

Properties of the Cucumovirus Capsid

Studies on the biochemical and physical properties of CMV and related viruses were precluded until the development of suitable purification procedures (Scott, 1963; Grogan *et al.*, 1963). Subsequent studies have demonstrated that cucumoviruses have icosahedral particles approximately 28 nm in diameter which sediment as a single component with an $S_{20,w}$ value of about 98S (Gibbs and Harrison, 1970; Habili and Francki, 1974a). Capsids are composed of 180 protein subunits with a molecular weight of 24,500 daltons (Habili and Francki, 1974a) arranged in hexamers and pentamers on an icosahedral surface lattice with T=3 symmetry (Finch *et al.*, 1967).

CMV particles are readily disrupted in solutions containing high concentrations of neutral chloride salts (Kaper *et al.*, 1965; Francki *et al.*, 1966) and by the anionic detergent, sodium dodecyl sulphate (SDS, Kaper 1975a). In addition, CMV particles are degraded in the

presence of ribonuclease (RNase; Francki, 1968); properties shared by alfalfa mosaic virus (AMV) and members of the bromovirus group (Kaper, 1975a). From these observations, Kaper (1975a) concluded that these viruses are stabilized largely by RNA-protein interactions, rather than by linkages between protein subunits.

Properties of the Cucumovirus Genome

Events leading to the discovery that several groups of plant viruses contain functionally-divided (multipartite) genomes have been recently reviewed by Jaspars (1974) and Van Vloten-Doting *et al.* (1977). Although the physical heterogeneity of CMV-RNA was first observed by Diener *et al.* (1964) and Kaper *et al.* (1965), it was concluded that the lower molecular weight RNA species represented nuclease digestion products of the largest RNA component. Similarly, Bockstahler and Kaesberg (1965) commenting on the heterogeneous sedimentation behaviour of the RNA of brome mosaic virus (BMV), concluded that the smaller RNA species (0.7 and 0.3 x 10^6 daltons) were possibly generated by specific enzymic cleavage of the largest RNA component (1 x 10^6 daltons). However it was later demonstrated that the three largest RNA components of BMV, RNAs 1, 2 and 3, were all required for infectivity and that the lower molecular weight RNA 4, although encapsidated, is not obligatory for infection (Lane and Kaesberg, 1971).

Electrophoretic analysis of CMV-RNA in polyacrylamide gels (Kaper and West, 1972) revealed the presence of four major RNA components similar to those of BMV-RNA. The critical evidence that CMV contains a multipartite genome was obtained by Peden and Symons (1973) who demonstrated that, like BMV, the infectivity of CMV required the presence of the three largest RNAs (1, 2 and 3), but not RNA 4. These conclusions

were confirmed by Lot *et al.* (1974). The RNA components of QCMV, designated RNAs 1-4 in order of decreasing molecular weight, were shown by electrophoresis in 4% acrylamide - 98% formamide gels to have molecular weights of approximately 1.35, 1.16, 0.85 and 0.35 x 10^6 daltons respectively (Peden and Symons, 1973). These values are somewhat higher than those reported by Kaper and West (1972) and are the subject of continuing debate (Kaper and Diaz-Ruiz, 1977). In addition to the four major RNA components, Peden and Symons (1973) reported the occurrence of several minor components in RNA preparations of QCMV. These components with molecular weights 0.26, 0.12 and 0.05 x 10^6 were designated RNAs 4a, 5 and 6 respectively. Kaper and West (1972) also reported the presence of minor RNA components corresponding to RNAs 5 and 6.

Encapsidation of the Cucumovirus Genome

The particle weight of cucumoviruses is between 5.3 and 5.8 x 10^6 daltons (Francki *et al.*, 1966; Stace-Smith and Tremaine, 1973) with an RNA content of about 18% (Francki *et al.*, 1966; Kaper, 1975b). That is, each particle is capable of encapsidating only a little more than 1 x 10^6 daltons of RNA. CMV preparations sediment as a single homogeneous component and CMV particles stabilized with formaldehyde show only slight heterogeneity in isopycnic density gradients (Lot and Kaper, 1973; Habili and Francki, 1974a). These observations indicate that the three genomic RNA components of the cucumoviruses must be encapsidated in separate particles. However, attempts to resolve the different particle types expected in isopycnic gradients are complicated by the instability of cucumoviruses in the high salt solutions normally used in these analyses (Lot and Kaper, 1973; Habili and Francki, 1974a; Lot and Kaper, 1976a). Furthermore, the isolation of viral RNA from

formaldehyde stabilized preparations of CMV is difficult (Habili and Francki, 1974a; Lot and Kaper, 1976a). Using CMV-D, a strain which is more resistant to disruption in RbCl, Lot and Kaper (1976b) demonstrated that particles containing RNA 1 were found predominantly in the heavier gradient fractions, whereas those containing RNA 2 were predominantly in the lighter fractions. These observations indicated that the genomic RNAs of CMV are probably encapsidated in a manner similar to that proposed for the bromoviruses (Lane and Kaesberg, 1971; Bancroft and Flack, 1972; Hull, 1972). That is, the viral RNAs are encapsidated in three types of particles; one type containing one molecule of RNA 1, a second type containing one molecule of RNA 2, and a third type containing one molecule each of RNAs 3 and 4. However, it appears that the encapsidation of cucumovirus RNAs may be more complicated than that of the bromoviruses, since RNAs 3 and 4 are often not present in equimolar ratios (Lot and Kaper, 1976a; Hull, 1976). To account for the nonequimolar proportions of RNAs 3 and 4, and for the near-homogeneous buoyant density of cucumovirus preparations, Lot and Kaper (1976b) and Hull (1976) suggested that other particle types may exist containing various combinations of RNAs 2, 3, 4 and 5.

Genetic Analyses of Cucumoviruses

The demonstration that cucumoviruses contain a multipartite genome (Peden and Symons, 1973; Lot *et al.*, 1974) indicated that these viruses were amenable to the type of genetic analyses applied to other plant viruses with divided genomes (reviewed by Jaspars, 1974, and Van Vloten-Doting *et al.*, 1977). For those plant viruses in which the RNA components are encapsidated in particles with different sedimentation properties, the separation and purification of their genomic RNAs is

greatly simplified. Moreover, mixtures of the purified nucleoprotein components of these viruses would be expected to have a higher specific infectivity than those of their corresponding genomic RNAs since in an encapsidated form, their RNA would be less accessible to inactivation by RNases. In contrast, for the cucumoviruses, in which the genomic RNA components are encapsidated in particles of similar buoyant density and sedimentation properties, it is necessary to separate their genomic RNAs from isolated viral RNA by gel electrophoretic or density gradient centrifugation techniques.

Progeny viruses, whose genomic RNA complement originated from two or more parental viruses (or strains), have been referred to as 'hybrids' (Bancroft, 1972; Dingjan-Versteegh *et al.*, 1972; Marchoux *et al.*, 1974a), or pseudorecombinants (Harrison *et al.*, 1974; Habili and Francki, 1974c; Gibbs and Harrison, 1976). Since the term 'hybrid' has connotations of a diploid state, the term 'pseudorecombinant' has been used throughout this thesis.

Following inoculation of a mixture of RNAs 1+2 from VTAV (T_1+T_2) and RNA 3 from QCMV (C_3), Habili and Francki (1974c) reported the isolation of a pseudorecombinant virus (PV) which had properties of both parental viruses. PV (T_1+T_2+C_3) was serologically indistinguishable from QCMV. These results indicated that the coat protein gene of the cucumoviruses, like that of other viruses with tripartite genomes (Lane and Kaesberg, 1971; Bol and Van Vloten-Doting, 1973), is located on RNA 3.

Interestingly, symptoms induced by PV on a range of plant hosts were indistinguishable from those induced by VTAV (Habili and Francki, 1974c). From these observations, it was suggested that genetic information specifying the development of symptoms in cucumoviruses is

located on RNA 1 and/or RNA 2. Unfortunately, since the pseudorecombinant containing the genomic RNA complement $C_1+C_2+T_3$ could not be constructed their conclusions could not be substantiated in reciprocal genetic analyses. In contrast, Marchoux *et al.* (1974a, b) using several different strains of CMV, reported that in addition to specifying the coat protein and electrophoretic mobility of CMV particles, RNA 3 also contained genetic information controlling the development of symptoms in almost all the host plants examined. Marchoux *et al.* (1974a) indicated that although RNA 3 determines the size of necrotic lesions induced on *Vigna sinensis* Endl. (cowpea), genetic information located on RNA 2 determines whether the lesions are necrotic or chlorotic, and whether the virus is able to infect this host systemically. With this exception, all other host reactions were determined by RNA 3 alone, in apparent contrast to results reported by Habili and Francki (1974c). It should be noted that whereas Marchoux *et al.* (1974a) used different strains of CMV, or their mutants in these investigations, Habili and Francki (1974c) used two serologically unrelated cucumoviruses, QCMV and VTAV.

Scope of This Thesis

In the first part of this thesis are described investigations which re-examine the location of genes on cucumovirus genomic RNAs responsible for the development of host plant reactions. In this study, pseudorecombinants were constructed from heterologous mixtures of genomic RNAs from several distinct isolates of CMV, as well as VTAV, in an attempt to explain the apparently conflicting results obtained by Habili and Francki (1974c) and Marchoux *et al.* (1974a). During the course of

these investigations, a low molecular weight RNA species, termed Sat-RNA, was detected in RNA isolated from several CMV isolates, as well as their pseudorecombinants. The properties and significance of Sat-RNA were investigated and the results of these studies constitute the latter part of the thesis. Kaper *et al.* (1976) reported some unusual properties of an RNA, similar in molecular size to Sat-RNA, which they also detected in RNA preparations from CMV. The properties of this RNA, subsequently designated cucumber mosaic virus-associated RNA 5 (CARNA 5; Kaper and Tousignant, 1977) are compared to those of Sat-RNA.

CHAPTER 2

GENERAL MATERIALS AND METHODS

MATERIALS

Virus Isolates

The virus isolates used and their sources are listed in Table 2.1. QCMV, MCMV, GCMV and the V strain of TAV (VTAV) were used throughout these investigations and a comparative study of their physical, chemical and biological properties is presented in Chapters 3 and 4.

Chemicals

The chemicals and biochemicals used and their sources are presented in Tables 2.2 and 2.3. Deoxyribonucleotides, [α-^{32}P]ATP, [α-^{32}P]dATP and [α-^{32}P]dCTP were kindly provided by Dr R.H. Symons. Enzymes used in the preparation of cDNA transcripts and S_1 nuclease were provided by Mr P. Palukaitis and Dr R.H. Symons. All chemicals not listed in Tables 2.2 or 2.3 were standard analytical or laboratory reagent grade.

Buffers and Solutions

RNA preparations were suspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.3). STMg buffer, used in the enzymic digestion of host cellular DNA in total leaf nucleic acid extracts, contained 0.2M NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, pH 7.4. Buffer used in aqueous analytical and preparative gel electrophoresis was TBE (Peacock and Dingman, 1968) and contained 10.8 g Tris, 5.5 g Boric acid and 0.93 g EDTA in one litre of water.

Table 2.1. Virus isolates employed in these studies.

Virus	Source	Reference
A. Cucumovirus isolates		
QCMV	*Capsicum frutescum* L., Queensland	Francki *et al.* (1966)
VTAV	*Chrysanthemum* sp., Victoria	Habili and Francki (1974a)
MCMV	A mutant of Price's No. 6 Yellow strain, isolated by Dr K.M. Smith, England	Professor R. Markham and Professor L.L. Stubbs (personal communication)
GCMV	Unknown host species	Waite Agricultural Research Institute collection
ACMV	*Malva parviflora* L., South Australia	Dr R.I.B. Francki (personal communication)
NCMV	Unknown host species, Queensland	Dr G. Behncken (personal communication)
LCMV	*Lupinus* sp., Queensland	"
ECMV	*Gerbera* sp., Queensland	"
HCMV	*Hippeastrum* sp., Victoria	Mr R.H. Taylor (personal communication)
WAICMV	*Capsicum* sp., Western Australia	Dr G.D. McLean (personal communication)
WAIICMV	"	"
PCMV	*Spinacea oleraceae* L.	Waite Agricultural Research Institute collection
ZCMV		"
NTAV	*Nicotiana glauca* Graham, South Australia	"
B. Other isolates used		
AMV	*Trifolium* sp., Queensland	Dr G. Behncken (personal communication)
TRSV	*Gladiolus* sp., South Australia	Randles and Francki (1965)
TMV (U_1 strain)		Siegel and Wildman (1954)
MS2		Mr I. Hooper, Biochemistry Department, University of Adelaide (personal communication)
TNV) STNV)	Unknown host species	Waite Agricultural Research Institute collection

Table 2.2. Chemicals and biochemicals used in this thesis.

Chemical	Source
Acrylamide	BDH Chemicals, England
Adjuvant, Freund's complete	Calbiochem, U.S.A.
Agarose (electrophoretic grade)	BDH Chemicals, England
Amberlite MB1	Ajax Chemicals Ltd, Sydney
1,4-Bis-[2-(5-phenyloxazolyl)] benzene (POPOP)	Ajax Chemicals Ltd, Sydney
Bovine serum albumin (BSA)	Sigma Chemical Co., U.S.A.
Cytochrome c (type IV)	Sigma Chemical Co., U.S.A.
Deoxyribonuclease 1 (DNase 1; bovine pancreas; DN-EP grade)	Sigma Chemical Co., U.S.A.
2,5-Diphenyloxazole (PPO)	Ajax Chemicals Ltd, Sydney
Formaldehyde (AR grade)	May and Baker, England
Formamide (AR grade)	BDH Chemicals, England
^{125}I ($Na^{125}I$) (100 mCi/ml)	The Radiochemical Centre, Amersham
Methanesulphonic acid (4N, containing 0.2% 3-(2-aminoethyl)indole)	Piece, Illinois, U.S.A.
N,N' Methylene bis-acrylamide	BDH Chemicals, England
Ovalbumin	Sigma Chemical Co., U.S.A.
Polyethylene glycol (PEG 6000)	Union Carbide, U.S.A.
Pronase (type iv, fungal protease)	Sigma Chemical Co., U.S.A.
Ribonuclease A (RNase A; pancreatic)	Sigma Chemical Co., U.S.A.
RNase T_1	Sigma Chemical Co., U.S.A.
tRNA (Phe, from yeast)	Sigma Chemical Co., U.S.A.
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co., U.S.A.
N,N,N',N"-tetramethyl-ethylenediamine (TEMED)	Sigma Chemical Co., U.S.A.
Tris(hydroxymethyl)aminomethane (Tris)	Sigma Chemical Co., U.S.A.
Triton X-100	BDH Chemicals, England

Table 2.3. Chemicals and biochemicals used specifically in molecular hybridization studies.

	Source	Reference
Actinomycin D	Merck and Co. Inc.,U.S.A.	
dATP	Sigma Chemical Co.,U.S.A.	
[α-^{32}P]ATP	Dr R.H. Symons	Symons (1977)
[α-^{32}P]dATP	Dr R.H. Symons	Symons (1977)
dCTP	Sigma Chemical Co.,U.S.A.	
[^{3}H]dCTP (15 Ci/mM)	The Radiochemical Centre, Amersham	
[α-^{32}P]dCTP	Dr R.H. Symons	Symons (1977)
dGTP	Sigma Chemical Co.,U.S.A.	
Poly(A) polymerase (ATP : RNA adenyltransferase)	From *E. coli* B (fractionated on phosphocellulose column)	Sippel (1973)
Reverse transcriptase (Avian myeloblastosis virus)	Dr J.R.E. Wells Biochemistry Department, University of Adelaide	Originally obtained from: Office of Program Resources and Logistics, Viral Cancer Program, Viral Oncology, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Maryland 20014
S_1 nuclease (Type IV-a)	Sigma Chemical Co.,U.S.A. isolated from crude α-amylase powder	Vogt (1973)
dTTP	Sigma Chemical Co., U.S.A.	
Oligodeoxythymidylic acid (oligo(dT))	Sigma Chemical Co., U.S.A.	

Instruments

Ultracentrifugations were carried out in either Beckman Model L or Model L2-65 centrifuges and analytical centrifugations in a Beckman Model E analytical ultracentrifuge equipped with an AN-D rotor. Sorvall RC2-B and RC3 centrifuges were used for intermediate and low speed centrifugations. Ultraviolet absorption spectra were measured with a Unicam SP1800 spectrophotometer and the kinetics of RNA thermal denaturation investigated with an attached electrically heated cell holder, SP876 temperature programme controller, and a Unicam AR25 linear recorder. Sucrose density gradients were analysed with an ISCO Model 183 density gradient fractionator coupled to a UA-2 recorder. Polyacrylamide or agarose gels were scanned with a Joyce-Loebl Chromoscan.

METHODS

The methods employed in the succeeding chapters are described below. Modifications of specific methods are detailed in the appropriate chapter.

Plants, Inoculations and Virus Propagation

All cucumoviruses were routinely propagated in either *Nicotiana clevelandii* A.Gray or the *N. clevelandii* x *N. glutinosa* L. hybrid (*N.* hybrid; Christie, 1969). The former host was found more suitable for the propagation of both QCMV and GCMV. Plants were lightly dusted with Carborundum (500 mesh) and mechanically inoculated by rubbing viral nucleoprotein or RNA suspension on the leaves with a forefinger enclosed in a rubber fingerstall. Excess inoculum was washed off with tap water. Unless otherwise stated plants were maintained in an insect-proof, air-cooled glasshouse, except for several months in summer when plants

were kept in a growth cabinet at 22°C with a photoperiod of 12 hr and 12,000 lux provided by fluorescent lights.

The other viruses, AMV, TRSV and TMV, were propagated in *N. tabacum* L. cv. White Burley and the plants maintained under the growth conditions described above. TNV and STNV were both propagated in either *N. clevelandii* or *N.* hybrid.

Virus Purification

QCMV and GCMV were purified from systemically infected tissue harvested 12-14 days after inoculation essentially as described by Peden and Symons (1973). Leaf tissue was homogenized in a Waring blender in 2 volumes (w/v) of 0.5M sodium citrate buffer, pH 6.5, containing 0.5% thioglycollic acid and 5mM EDTA. The extract was strained through a double layer of muslin and shaken briefly with an equal volume of chloroform. After centrifugation at 10,000 g for 10 min, PEG was added to the clarified supernatant to 10% (w/v) and the mixture stirred for 45-60 min at 4°C. Precipitated virus was pelleted by centrifugation at 10,000 g for 10 min and suspended in extraction buffer containing 1% Triton X-100 (50 ml/100 g leaf material) by stirring for at least 30 min. The suspension was clarified by centrifugation at 15,000 g for 20 min and subjected to 2 cycles of high- and low-speed centrifugation at 78,000 g for 120 min, and 5,000 g for 10 min respectively. Virus pelleted by ultracentrifugation was suspended in 5mM borate buffer containing 1mM EDTA (pH 9.0). Virus preparations for antiserum production were further purified by centrifugation in 5-25% sucrose density gradients containing 20mM phosphate buffer, pH 7.5 (Beckman SW27 rotor, 27,000 rpm for 150 min at 4°C).

VTAV was purified by the above procedure with the following modifications; EDTA was not included in buffers at any stage of purification as it has been shown to disrupt this virus (Habili and Francki, 1974b) and virus pelleted by ultracentrifugation was suspended in 20mM phosphate buffer, pH 7.6.

Initial attempts to purify MCMV by the method of Scott (1963), or by that described above for QCMV were unsuccessful in that no virus was recovered. An investigation of the effects of organic solvents, and those of the pH, molarity and composition of extraction buffer on the infectivity of MCMV indicated that conditions which favour particle stability of this virus strain differ markedly from those of QCMV (R.I.B. Francki and C.J. Grivell, personal communication). Extraction of MCMV in the presence of chloroform completely eliminated its infectivity. Other solvents including Freon 113, carbon tetrachloride, and diethyl ether also greatly reduced infectivity of MCMV. The purification procedure (Mossop *et al.*, 1976) finally adopted for this virus strain involves extraction of leaf material in 3 volumes (w/v) of 0.1M Na_2HPO_4, containing 0.1% each of thioglycollic and diethyldithiocarbamate (pH ∿ 8.0). The extract was strained through muslin and clarified by centrifugation at 10,000 g for 10 min. Triton X-100 was added to the clarified supernatant to 1% (w/v) and stirred for 15 min. The mixture was subjected to centrifugation at 78,000 g for 120 min and pellets were resuspended in approximately one-tenth the original volume of extraction buffer. Particulate material was removed by centrifugation at 5,000 g for 10 min and the supernatant was then layered on to 5 ml of a 10% sucrose cushion prepared in 0.1M Na_2HPO_4 and centrifuged at 144,000 g for 45 min in a Beckman 65 rotor. Pellets were suspended in either 0.1M Na_2HPO_4, if the preparation was to be further purified by sucrose density gradient centrifugation, or in 10mM sodium borate buffer,

pH 7.6. For antiserum production, MCMV preparations were layered on 5-25% sucrose density gradients prepared in 0.1M Na_2HPO_4 and centrifuged at 27,000 rpm for 150 min at 4°C in a Beckman SW27 rotor. Purified virus was then suspended in 10mM sodium borate buffer, pH 7.6.

The above purification procedures produced highly purified preparations of QCMV, GCMV, MCMV and TAV in yields of approximately 300, 300, 600 and 1,000 mg viral nucleoprotein per kg of leaf material respectively.

AMV was purified by the method described by Van Vloten-Doting and Jaspars (1972). Leaf material was extracted in an equal volume (w/v) of 0.1M K_2HPO_4 containing 0.1M ascorbic acid and 20mM EDTA, pH 7.1. The slurry was rehomogenised with a 1:1 chloroform : butanol mixture (1 g/1 ml/1 ml) and clarified by centrifugation at 10,000 g for 10 min. PEG was added to the supernatant to a final concentration of 5% and the mixture stirred for 30 min. Precipitated virus was sedimented at 10,000 g for 10 min and pellets were suspended in 1/5th volume (v/w) harvested leaf material of 10mM sodium phosphate buffer, pH 7.0. The suspension was clarified by centrifugation at 15,000 g for 20 min and subjected to 2 cycles of high-speed (78,000 g for 150 min) and low-speed (5,000 g for 10 min) centrifugation. Purified virus was suspended in 10mM sodium phosphate buffer containing 1mM EDTA, pH 7.0.

TRSV was purified by a modification of the method of Rezaian and Francki (1973). Leaf tissue was blended with equal volumes (1 g/1 ml/1 ml) of 0.1M phosphate buffer, pH 7.0, and chloroform. The homogenate was strained through a double layer of muslin and clarified by centrifugation at 10,000 g for 10 min. PEG and NaCl were added to the supernatant to a final concentration of 6% and 0.3M respectively, and the mixture stirred for 30 min at 4°C. The precipitate was pelleted by centrifugation at

10,000 g for 10 min and resuspended for at least 6 hr in 0.1M phosphate buffer, pH 7.0, containing 10mM EDTA and 2% Triton X-100. Particulate material was removed by centrifugation at 15,000 g for 20 min and the suspension subjected to a cycle of high speed (160,000 g for 50 min) and low speed (5,000 g for 10 min) centrifugation. Purified virus was usually suspended in 10mM phosphate buffer, pH 7.0.

Where RNA was to be isolated from virus preparations from small amounts of leaf material for subsequent gel electrophoretic analysis, it was necessary to suspend the final pellets in 10mM borate buffer, pH 7.6, to avoid interference by high phosphate concentrations during electrophoresis.

TMV was purified by the method described by Francki and McLean (1968). Leaf tissue was blended in 1.5 volumes of 0.2M Na_2HPO_4 and strained through a double layer of muslin. Decolorising charcoal was added (0.05 g/ml) and the mixture shaken briefly and filtered through a pad of celite. DEAE-cellulose was then added to the filtrate (0.01 g/ml) and the mixture again filtered through celite. The clarified suspension was subjected to 2 cycles of high- and low-speed centrifugation and the final pellets resuspended in distilled water.

STNV was purified from leaves of *N. clevelandii* infected with TNV and STNV 4-6 days after inoculation using the procedure described by Fraenkel-Conrat (1976). Leaf material was normally frozen at -65°C before extraction. Frozen leaf tissue was pulverized in a chilled mortar and pestle, and the powder transferred to a Waring blender. An equal volume (w/v) of a buffer containing 10mM Tris, pH 8.1, 10mM KCl, 25mM NH_4Cl, 10% glycerol and 0.1% 2-mercaptoethanol was added and the mixture blended. The slurry was strained through muslin and clarified by centrifugation at

10,000 g for 10 min. STNV was pelleted by centrifugation at 78,000 g for 3.5 - 4 hr and resuspended in approximately one-tenth of the original leaf tissue weight of extraction buffer. An equal volume of chloroform was added and the mixture was shaken by hand and centrifuged at 5,000 g for 10 min. Six ml of saturated $(NH_4)_2SO_4$ were added to each 20 ml of clarified supernatant and stirred at 4°C for 2 hr. Precipitated STNV and some TNV were sedimented at 10,000 g for 10 min and suspended in about 1/50th of the original leaf tissue weight of extraction buffer. Preparations were dialysed against water for 24 hr at room temperature (20 ± 5°C) and clarified by centrifugation at 5,000 g for 10 min. STNV preparations were further purified by centrifugation on linear 5-30% sucrose gradients prepared in water, at 27,000 rpm for 8 hr in a Beckman SW27 rotor.

An isolate of TNV, free of STNV, was recovered by repeated serial transfer of large local lesion types. The isolate used in these studies had been cloned by five lesion transfers on *N.* hybrid. Attempts to purify TNV using the $(NH_4)_2SO_4$ precipitation method above were unsuccessful. It is probable that the strain of TNV used in these studies corresponds to the TNV strain B, described by Bawden and Pirie (1942), and Kassanis and Nixon (1961), which is disrupted by $(NH_4)_2SO_4$ (Babos and Kassanis, 1963). The method used for TNV purification was a modification of that described by Kassanis and Nixon (1961). Leaves of *N. clevelandii* or *N.* hybrid were harvested 3-4 days after inoculation and extracted in 2 volumes (w/v) of 20mM sodium phosphate buffer, pH 7.8, containing 0.2% TGA. The extract was frozen at -15°C for at least 8 hr and allowed to thaw at room temperature over a period of 24 hr. After centrifugation at 10,000 g for 10 min, the aqueous phase was passed through Whatman No. 4 filter paper and stirred with 1% Triton X-100 at 4°C for 30 min. TNV was sedimented by centrifugation at 78,000 g for 105 min and resuspended in about 1/20th

of the original tissue weight of 20mM phosphate buffer, pH 7.2. The suspension was centrifuged at 10,000 g for 10 min and the supernatant layered onto 4 ml of 15% sucrose in a Beckman 65 rotor tube and centrifuged at 144,000 g for 45 min. Pellets were resuspended in 20mM phosphate buffer, pH 7.2.

Bacteriophage MS2 was grown in an F^+ strain of *E. coli* and purified essentially as described by Strauss and Sinsheimer (1963). To 1 l. of medium containing 10 g bacto-tryptone (Difco), 1 g yeast extract (Difco), 5mM $CaCl_2$ and *E. coli* cells in an early log phase of growth (approximately 10^8 cells/ml) was added sufficient MS2 particles to produce a multiplicity of infection of approximately 10. The culture was shaken at 37°C for 7 hr and the lysate was centrifuged at 5,000 g for 10 min. To the supernatant was added $(NH_4)_2SO_4$ (280 g/l. of lysate) and the mixture was stirred for 60 min at 4°C. The precipitate recovered by centrifugation at 10,000 g for 10 min was suspended in a buffer containing 0.1M NaCl, 50mM Tris-HCl, pH 7.6 (10 ml/l. of lysate), and shaken briefly with an equal volume of chloroform. After centrifugation at 12,000 g for 10 min, the aqueous phase was recovered and stirred with 0.55 g of CsCl per g of aqueous phase to produce a density of approximately 1.38 g/ml. The gradient was centrifuged to equilibrium in a Beckman 50Ti rotor at 37,600 rpm for 24 hr at 4°C. Virus-containing bands were recovered with a syringe and dialysed against 0.1M Tris HCl, pH 7.0, for 24 hr at 4°C.

Isolation of Viral and *E. coli* RNA

Unless otherwise stated, RNA of all viruses used in these studies was isolated from purified virus preparations using the phenol-SDS procedure described by Peden and Symons (1973). Each virus preparation was emulsified with equal volume of 90% phenol containing 0.1%

8-hydroxyquinoline, and buffer containing 0.6M sodium acetate, 0.6% SDS and 20mM EDTA (1 ml : 1 ml : 1 ml). The mixture was shaken vigorously for 10 min at 25°C and the aqueous phase was separated by centrifugation at 10,000 g for 10 min. One-half volume of phenol was added to the aqueous phase and the extraction repeated. Traces of phenol were removed by washing the aqueous phase with 2 volumes of ether, and the RNA was precipitated by addition of 2.5 volumes of cold, re-distilled ethanol. After at least 3 hr at -15°C, the RNA precipitate was sedimented at 3,000 g for 10 min and the pellet washed once with ethanol. To facilitate their resuspension, RNA pellets were dried briefly in a Dynavac freeze-drying unit. Unless otherwise stated, RNA was suspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.3) and stored at -15°C. In later studies, the ether step was replaced by an additional ethanol precipitation.

TNV-RNA and STNV-RNA were prepared from SDS-disrupted virus preparations as described by Fraenkel-Conrat (1976). Virus preparations containing 2% SDS were incubated for 15 min at 37°C. Sodium acetate was added to 0.2M and the mixture was then extracted twice with phenol. RNA was recovered by ethanol precipitation as described above.

Cucumovirus RNAs for base ratio analyses, and their coat proteins for amino acid analyses were prepared from LiCl-dissociated virus as described by Francki *et al.* (1966). An equal volume of 4M LiCl in 20mM Tris, pH 7.2, was added to cucumovirus preparations, and kept at -15°C for at least 3 hr. The mixture was thawed slowly and the RNA precipitate recovered by centrifugation at 3,000 g for 10 min. The supernatant fraction, containing viral coat protein, was retained (see isolation of viral protein, p. 29). RNA recovered by this method was normally suspened in TE buffer containing at least 0.2M sodium acetate and further purified by ethanol precipitation.

E. coli rRNA, used as molecular weight standards in gel electrophoretic studies and as a carrier RNA in some hybridization experiments, was prepared as follows. Cells of *E. coli* K12 grown in nutrient broth (Difco) for 24 hr at 25°C were harvested by centrifugation at 10,000 g for 10 min. Pellets were suspended in STMg buffer containing 1% SDS, and transferred to a chilled mortar and pestle. A volume of 90% phenol containing 0.1% 8-hydroxyquinoline equal to that of the resuspension buffer was added and the mixture ground thoroughly. The slurry was shaken vigorously for 15 min at 25°C and the aqueous and phenol phases separated by centrifugation as above. The aqueous phase was re-extracted with a half-volume of phenol and the nucleic acids precipitated by the addition of 2.5 vols of ethanol. Strands of *E. coli* DNA was spooled out on a sterile glass rod and the remaining nucleic acids were recovered by centrifugation and were suspended in TE buffer.

MS2-RNA was isolated by phenol-SDS extraction. SDS and sodium acetate were added to purified MS2 preparations to 0.5% and 0.2M respectively and shaken vigorously with an equal volume of phenol solution. The aqueous phase was recovered and extracted a second time with a half volume of the phenol solution and again centrifuged at 10,000 g for 10 min. RNA was precipitated by the addition of 2.5 volumes of chilled ethanol and kept at -15°C.

Isolation of Total Leaf RNAs and Virus-specific ds-RNA

Nucleic acids were isolated from leaf tissue by a modification of the procedure described by Loening and Ingle (1967). Leaf tissue was pulverized in a mortar and pestle containing liquid nitrogen. Two volumes (w/v) of a 90% phenol solution containing 0.1% 8-hydroxyquinoline was then added and the mixture ground to a fine powder and transferred to a 50 ml

polypropylene centrifuge tube. To this was added 2 volumes (w/v) of a buffer containing 50mM NaCl, 10mM Tris-HCl, 10mM EDTA, 1% SDS, pH 8.5, and the mixture was shaken vigorously for 30-45 min at 25°C. The aqueous phase recovered after centrifugation at 10,000 g for 10 min was extracted twice more with phenol, and NaCl was added to a concentration of 0.5M. Total nucleic acids were precipitated with 2.5 volumes of ethanol. After at least 3 hr at -15°C the precipitate was recovered by centrifugation at 5,000 g for 10 min, lyophilised and suspended in STMg buffer. DNA was removed from such preparations by incubation with 10 µg/ml DNase I. Preparations were extracted twice with phenol as above, and total leaf RNAs were recovered by ethanol precipitation. From 3-4 g of leaf tissue, approximately 1 mg of total leaf RNA was obtained by this method.

Double-stranded RNA specific to Sat-RNA was isolated from leaf tissue as described above. It was necessary to remove ss-Sat-RNA from such preparations to avoid unwanted competition in hybridization experiments. In preliminary experiments it became evident that LiCl fractionation was not entirely effective in removing Sat-RNA. Precipitation of an RNA preparation from QCMV with 2M LiCl and subsequent analysis on agarose gels indicated that only approximately 50% of the original Sat-RNA content of the RNA preparation had been removed. Leaf RNA preparations were incubated with 10 µg/ml RNase A for 30 min at 37°C in a buffer containing 0.2M NaCl, 10mM Tris-HCl, 5mM EDTA, pH 7.4. RNase-treated preparations were then incubated with 10 µg/ml pronase for 30 min at 37°C and extracted twice with phenol. Remaining nucleic acids were precipitated with ethanol and suspended in TE buffer.

No attempt was made to remove polysaccharides contaminating leaf RNA or ds-RNA preparations, since their presence did not noticeably affect their molecular hybridization by methods described below.

RNA Base Ratio Analyses

Base ratios of viral RNAs were determined by the procedure described by Markham (1955). RNA samples were hydrolysed in 1N HCl for 1 hr at 100°C and the products were separated by paper chromatography in isopropanol-HCl. The isolated nucleic acid components were located by their UV absorbance, eluted in 0.1N HCl and their relative concentrations were determined spectrophotometrically.

Analytical Gel Electrophoresis of Viral RNAs

The RNA component composition and their molecular weights were investigated by electrophoresis under non-denaturing conditions in 2% agarose gels, and in 4% acrylamide-formamide gels respectively.

In most experiments, viral RNA was analysed in 2% agarose gels prepared in the Tris-borate-EDTA buffer system containing 10.8 g Tris, 5.5 g Boric acid, 0.93 g EDTA in 1 litre of water, pH 8.3 (TBE), described by Peacock and Dingman (1968). The autoclaved gel solution was dispensed into 15 x 0.6 cm Plexiglass tubes to a depth of approximately 13 cm, and a flat loading surface was ensured by trimming 1-2 mm off the top of the solidified gel column with a scalpel. Gels were prepared immediately prior to their use. Before loading, RNA samples (10-15 µg) were normally heated at 75°C for 5 min and cooled rapidly in an ice-water bath and sucrose was then added to each sample to a concentration of 5% (w/v). Electrophoresis was normally at 2.5 mA/gel (10 Volts/cm) for 2.5 - 3 hr at 25°C. Electrophoresis buffer, TBE, was exchanged between the reservoir tanks by a peristaltic pump. Gels were stained for 10 - 15 min with 0.05% toluidine blue 0 in 50mM sodium acetate, 5mM acetic acid, 0.1mM EDTA, pH 8.5, as described by Peden and Symons (1973) and destained in several changes of 5% acetic acid. Gels were scanned at 620 nm using a

Joyce-Loebl Chromoscan.

For molecular weight determinations viral RNA was electrophoresed in 4% acrylamide - 98% formamide gels containing 20mM diethylbarbituric acid, pH 9.0, as described by Pinder *et al.* (1974). Formamide was deionized by stirring with 4% (w/v) Amberlite MB1 resin for at least 1 hr and filtered through Whatman No. 4 filter paper. To prepare 37.5 ml of gel solution, 1.275 g acrylamide monomer, 0.225 g bis-acrylamide, 144 mg diethylbarbituric acid and 90 µl TEMED were added to approximately 30 ml of deionized formamide. The mixture was adjusted to pH 9.0 (calomel electrode, 25°C) and made up to volume with buffered formamide (pH 9.0). Finally, 0.3 ml of a fresh ammonium persulphate solution (18% w/v) was added and the mixture dispensed into 15 x 0.6 cm Plexiglass tubes to a depth of 13 cm. A flat loading surface was formed by overlaying the gels with 50% tert. butanol in buffered formamide. When the gels had polymerized, the tert. butanol was removed by gently washing the loading surface with buffered formamide. RNA samples were precipitated with ethanol, lyophilized briefly, and suspended in buffered formamide, pH 9.0, containing 10% (w/v) sucrose. Buffered formamide was added above the loading surface to a depth of approximately 1.5 cm, and 20mM NaCl was introduced into the reservoir tanks before the RNA samples were layered on the gels. RNA samples (10-15 µg) were electrophoresed at 1 mA/gel (5 Volts/cm) for 24 hr at 25°C. Electrophoresis buffer was exchanged between the reservoir tanks using a peristaltic pump. In some experiments, $CuCl_2$ was added to the RNA samples (Lehrach *et al.*, 1977) to a concentration of 1mM Cu(II) per 1mM nucleotide. Gels were stained in 0.05% toluidine blue 0 as described above and destained in several changes of distilled water.

Preparative Electrophoretic Separation of Viral RNA Components

Viral RNA components were fractionated by two-cycles of gel electrophoresis; the first in 2.6% polyacrylamide gels and the second in 2% agarose gels. To prepare 65 ml of gel solution, 8.45 ml of stock acrylamide solution (19 g acrylamide, 1 g bis-acrylamide and water to 100 ml), 6.5 ml of stock buffer (10 x TBE) and 49.5 ml distilled water were mixed. The solution was briefly degassed and 0.5 ml of ammonium persulphate (10% w/v) and 50 µl TEMED were added and the mixture dispensed into 15 x 1.0 cm Plexiglass tubes to a depth of 13 cm as described above. Electrophoresis buffer (TBE) was introduced into the reservoir tanks and the gels were pre-electrophoresed for at least 30 min at 3 mA/gel. Immediately before electrophoresis, RNA samples were heated at 75°C for 3-5 min and cooled rapidly in an ice-water bath.

Sucrose was added to 10% and the RNA samples were electrophoresed at 2 mA/gel for 0.5 hr and then at 4 mA/gel for an additional 4 hr. Normally, 100-150 µg of RNA was loaded on each gel. RNA bands were located by staining with 0.01% ethidium bromide (Schuerch *et al.*, 1975) and excised with a scalpel blade. RNA was extracted from pooled gel slices by a modification of the method described by Schwinghamer and Symons (1977). TBE buffer containing 0.1M NaCl was added (0.75 ml per gel slice) and the mixture homogenised by repeatedly forcing it through an 18 gauge needle attached to a 10 ml glass syringe. Homogenised gel slices were shaken for 16-20 hr at 4°C, extracted twice with phenol and the eluted RNA components were recovered by ethanol precipitation. Normally, RNA eluted from 7-8 gel slices was suspended in approximately 400 µl TE buffer. RNA samples were again heated at 75°C for 5 min, cooled rapidly in an ice-water bath, and layered on two, 2% agarose gels

(13 x 1 cm) prepared as described above. Conditions of electrophoresis, location and fractionation of RNA components were all essentially as described above except that the gel slices were pulverized with a glass rod. The isolated RNA components were suspended in TE buffer and stored at -15°C.

Construction, Isolation and Characterization of Pseudorecombinants

Pseudorecombinants were constructed essentially as described by Habili and Francki (1974c). Appropriate dilutions of the heterologous genomic RNAs were prepared in TE buffer and inoculated to leaves of *N. tabacum* cv. White Burley. In most experiments, inocula contained approximately 50 µg/ml each of cucumovirus RNAs 1+2 and RNA 3. Prior to inoculation, plants were kept in darkness for 12-24 hr to increase their sensitivity and to enhance the development of local lesions. Inoculated plants were maintained at 25°C under continuous illumination of 5,000 lux. Under these conditions, faint chlorotic lesions developed within 3 days. Isolated lesions were excised singly and pulverized in 20 µl of 20mM sodium phosphate buffer, pH 7.6, containing 0.01% Na_2SO_3 and inoculated to tobacco plants. Usually, between 4 and 6 local lesion isolates of each pseudorecombinant were cloned serially at least 3 times in tobacco plants, and then propagated in either *N.* hybrid or *N. clevelandii* plants. Pseudorecombinants were purified by the method used for the parental strain supplying RNA 3.

The RNA composition of each pseudorecombinant virus was examined by electrophoresis in 2% agarose gels as described above and the coat protein serologically by testing the purified virus against antisera prepared to the parental virus strains.

Aphid Transmission Studies

A colony of *Myzus persicae* (Sulz.) was reared on Chinese cabbage (*Brassica pekinensis* Rupr.) seedlings at 25°C with a photoperiod of 16 hr. Aphids were fasted for 2-4 hr, and then allowed to probe briefly (30-40 sec) on detached, systemically infected leaves of *N. clevelandii*. For comparative studies of the aphid transmissibility of pseudorecombinants and their parental viruses, leaves exhibiting the most pronounced symptoms were removed 12 days after inoculation and used for virus acquisition. After probing aphids were transferred singly to small *N. glutinosa* seedlings, and were removed the following day or sprayed with a 1% nicotine sulphate solution.

Stabilization of Viruses with Formaldehyde and Glutaraldehyde

Purified preparations of cucumoviruses were treated with either formaldehyde or glutaraldehyde as described by Francki and Habili (1972). Formaldehyde was added to virus preparation to a concentration of 0.2% and the mixture dialysed for 24 hr at 4°C against 0.2% formaldehyde in 5mM borate buffer, pH 9.0 (QCMV and GCMV), in 10mM borate buffer, pH 7.6 (MCMV), or in 20mM phosphate buffer, pH 7.6 (VTAV). Unreacted formaldehyde was removed by dialysis against the appropriate buffers without formaldehyde for an additional 24 hr.

In other experiments, virus preparations were treated with 0.25% glutaraldehyde rather than formaldehyde as it is known that the former introduces more stable cross-linking in proteins (Bowes and Cater, 1966).

Serological Techniques

Serological techniques used throughout these studies have been previously described (Francki *et al.*, 1966; Francki and Habili, 1972;

Habili and Francki, 1975). Immunodiffusion tests were done in glass Petri dishes containing 12 ml of 0.75% purified bacteriological agar (Difco) in 10mM phosphate buffer, pH 7.6, with 0.02% sodium azide added as preservative. Approximately 10 µl of viral antigen or antiserum was dispensed into wells cut 3 mm in diameter and 3.5 mm apart. Unless otherwise stated, the concentration of viral antigen used in immunodiffusion tests was adjusted to 0.75 mg/ml. Diffusion plates were incubated at 25°C and the development of precipitin bands was recorded after 2-4 days.

Antisera to each cucumovirus antigen fixed with formaldehyde or glutaraldehyde were prepared in either Swiss mice or rabbits. The route of immunization and techniques used for antiserum production in mice were as described by Francki and Habili (1972). Adult mice received three intraperitoneal injections of 20-50 µg of viral antigen emulsified with an equal volume of Freund's complete adjuvant at weekly intervals. After a further two weeks, each mouse received an intraperitoneal injection of viral antigen and 0.2-0.5 ml of a fresh Krebs ascitic tumour cell suspension (Gamble and Kinsley, 1963). Ascitic fluid was drained from anaesthetized mice 6-9 days later, and cells and particulate material removed by centrifugation at 2,000 g for 10 min. In this manner, up to 15 ml of antiserum could be obtained from each animal. Sera were stored at -15°C either frozen, or in an equal volume of glycerol.

Rabbits were immunized by a series of subcutaneous and intravenous injections. Approximately 1 mg of viral antigen emulsified with Freund's complete adjuvant was administered subcutaneously around the neck area of each animal. Three weeks later, increasing amounts of antigen (2-5 mg) were injected intravenously at weekly intervals on three occasions. Ten

days later, blood samples were collected from an ear vein and the serum recovered after clotting and removal of cellular material by centrifugation at 2,000 g for 10 min.

Antisera specific to some strains of CMV were prepared by absorption of sera to a heterologous viral antigen. To 0.5 ml of serum was added 200-300 µg of the heterologous viral antigen and the mixture incubated at 37°C for 30 min and then left at 4°C for at least 8 hr. Unreacted serum protein was recovered in the supernatant after centrifugation at 5,000 g for 10 min and was stored as described above. Usually, such antisera failed to react with the heterologous antigen in immunodiffusion tests after a single adsorption step.

Isolation of Viral Protein

Coat protein of all cucumoviruses used in these studies was prepared from LiCl-dissociated virus. The supernatant containing LiCl-soluble coat protein was dialysed against 10mM Tris-HCl containing 1mM dithioerythritol, pH 7.2, for 24 hr at 4°C (Habili and Francki, 1974a). Precipitated coat protein was recovered by centrifugation at 10,000 g for 10 min, lyophilized, and stored at -15°C for subsequent amino acid analysis.

AMV coat protein was isolated essentially as described by Kruseman *et al.* (1971). Viral preparations were made 0.5M $MgCl_2$ by the dropwise addition of 1M $MgCl_2$ and stirred gently at 4°C for 45 min. Precipitated RNA was recovered by centrifugation at 3,000 g for 10 min and the supernatant containing viral coat protein was then dialysed against 50mM sodium acetate buffer, pH 5.5, at 4°C for 24 hr. The protein solution was clarified by centrifugation at 5,000 g for 10 min and lyophilized.

Polyacrylamide Gel Electrophoresis of Viral Protein

Electrophoretic analysis of viral coat protein was carried out in 10% polyacrylamide gels in the presence of SDS as described by Weber and Osborn (1969). To prepare 30 ml of gel solution, 10 ml of stock acrylamide solution (30 g acrylamide, 0.8 g bis-acrylamide, and water to 100 ml), 3 ml of stock buffer solution (1M sodium phosphate, pH 7.2, containing 1% SDS) and 17 ml of water were mixed. The mixture was degassed, and 0.15 ml of a freshly-prepared solution of ammonium persulphate (10%) and 0.015 ml of TEMED were added. The solution was then dispensed into 12 x 0.6 cm Plexiglass tubes to a depth of 10 cm, and a flat loading surface was ensured by gently layering a small volume of water on top of the gel solution. The stock buffer solution was diluted ten-fold, 2-mercaptoethanol was added to 0.1%, and the solution used as the electrophoresis buffer.

Viral and marker proteins were prepared as described by Agrawal and Tremaine (1972). Protein (1 mg/ml) in 0.1M sodium phosphate buffer, pH 7.2, containing 4M urea, 1% SDS and 0.1% 2-mercaptoethanol were heated at 100°C for 1 min. Glycerol was added to each sample to 10%. Electrophoresis was for 9-10 hr at 8 mA/gel. Gels were stained for 4 hr in a mixture containing 0.2% Coomassie Brilliant Blue R250, 50% methanol and 7% acetic acid at 37°C, and destained in several changes of 7% acetic acid as described by Maizel (1971). The mobility of each protein relative to cytochrome c was determined from densitometer traces of gels analysed with a Joyce-Loebl Chromoscan.

Amino Acid Analyses

Amino acid compositions of cucumovirus and AMV coat proteins were determined after either HCl or methanosulphonic acid hydrolysis.

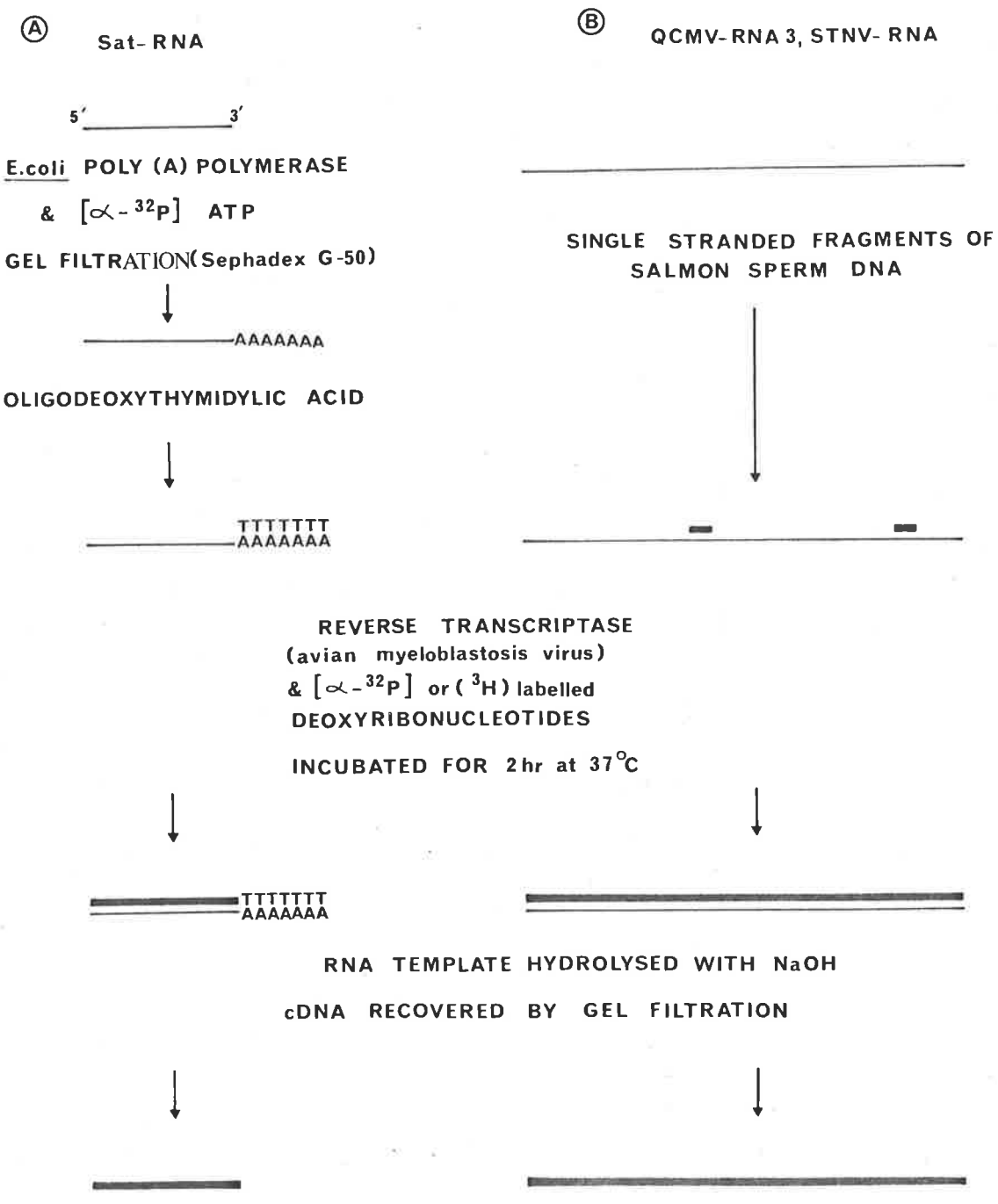
Proteins were hydrolysed with 6N HCl essentially as described by Delange *et al.* (1969). Lyophilized samples of 1-2 mg of viral coat protein were dissolved in 1 ml of 6N HCl containing a drop of 5% phenol and hydrolysed for 24, 48 and 72 hr at 110°C. Amino acids were dried, suspended in 0.1N HCl containing 12.5% sucrose and examined in either a Beckman 120C or 119 amino acid analyser.

Cysteine, methionine and tryptophan residues were determined in carboxymethylated viral protein samples hydrolysed with methanesulphonic acid essentially as described by Simpson *et al.* (1976). Viral coat proteins were carboxymethylated as described by Tachovsky and Hare (1975). Lyophilised samples of 0.5-2.0 mg of each protein were dissolved in a buffer containing 8M urea, 1M Tris-HCl, pH 8.5, and 1mM EDTA. Protein samples were reduced with the addition of 2-mercaptoethanol to a final concentration of 0.75M and kept under an atmosphere of N_2 for 3 hr at room temperature. Iodoacetate in 1M Tris-HCl, pH 8.5, was added to a final concentration of 0.34M and a pH of 8.0 maintained by addition of 10N NaOH. The reaction was allowed to proceed for 2 hr at 25°C in complete darkness. Carboxymethylated protein samples were then dialysed for 24 hr at 4°C against 10mM Tris-HCl, 0.1% 2-mercaptoethanol, pH 7.2, and precipitated protein recovered by centrifugation at 5,000 g for 10 min. Lyophilized protein samples were suspended in 1 ml of 4N methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole, and evacuated to a vacuum of 50-100 μ. Sealed vials were then incubated at 115°C for 22 hr. After hydrolysis, samples were adjusted to pH 2.0 and their amino acid compositions were analysed as above.

Preparation of Complementary DNA Transcripts

Complementary DNA (cDNA) was prepared to Sat-RNA, QCMV-RNA 3 and STNV-RNA by two distinct methods (Figure 2.1).

Figure 2.1. Preparation of cDNA to Sat-RNA, QCMV-RNA 3 and STNV-RNA. Details of the transcription of template RNAs by the oligo(dT)-primer method (Sat-RNA) or by the method employing oligodeoxyribonucleotide fragments (QCMV-RNA 3, STNV-RNA) are given in the text.


Ⓐ Sat-RNA
5′ 3′
E.coli POLY (A) POLYMERASE
& [α-32P] ATP
GEL FILTRATION (Sephadex G-50)
AAAAAAA
OLIGODEOXYTHYMIDYLIC ACID
TTTTTTT
AAAAAAA
Ⓑ QCMV-RNA 3, STNV-RNA
SINGLE STRANDED FRAGMENTS OF SALMON SPERM DNA
REVERSE TRANSCRIPTASE
(avian myeloblastosis virus)
& [α-32P] or (3H) labelled
DEOXYRIBONUCLEOTIDES
INCUBATED FOR 2hr at 37°C
TTTTTTT
AAAAAAA
RNA TEMPLATE HYDROLYSED WITH NaOH
cDNA RECOVERED BY GEL FILTRATION

Template RNAs of molecular weights in excess of approximately 0.2×10^6 may be effectively primed by the use of a random mixture of oligodeoxyribonucleotides generated by DNase treatment of salmon sperm DNA (Taylor *et al.*, 1976). The oligonucleotides anneal with randomly occurring complementary sequences on the RNA template and act as primers for cDNA synthesis by reverse transcriptase (Figure 2.1). This method is unsatisfactory for small RNA molecules the size of Sat-RNA (Gould *et al.*, 1978) presumably because such molecules would, on an average, contain fewer regions with base sequences complementary to those of the added DNA fragments.

Naturally occurring RNAs which possess poly(A) at their 3'-termini are readily transcribed by reverse transcriptase using oligodeoxythymidylic acid, oligo(dT), as a primer (Duesberg *et al.*, 1971). Recently, a method has been described for the enzymic addition of poly(A) sequences to the 3'-end of RNAs (Devos *et al.*, 1976), thereby enabling their transcription into cDNA (Figure 2.1) by the oligo(dT)-primer method.

Sat-RNA

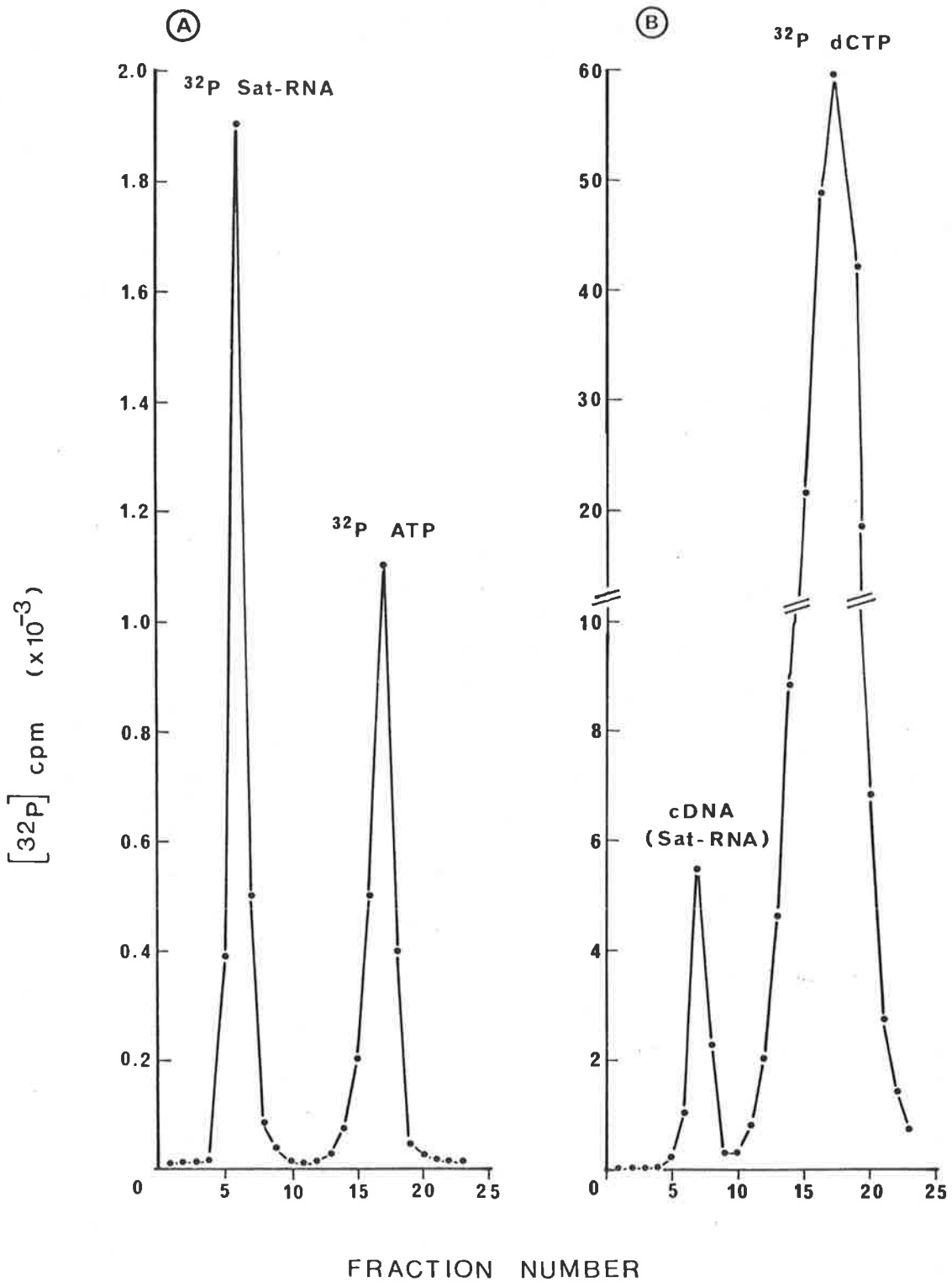
Poly(A) was added to the 3'-terminus of Sat-RNA by a modification of the procedure of Devos *et al.* (1976) as described by Gould *et al.* (1978). Sat-RNA (1-2 µg) was incubated for 30-45 min at 37°C in a reaction mixture (100 µl) containing 50mM Tris-HCl, pH 7.9, 1mM dithioerythritol, 50 µg bovine serum albumin, 10mM magnesium acetate, 2.5mM $MnCl_2$, 0.3M NaCl, 0.2mM [α-^{32}P]ATP (20 µCi/µM) and approximately 13 µg of poly(A) polymerase. Unreacted [α-^{32}P]ATP and polyadenylated Sat-RNA were separated by gel filtration on a Sephadex G-50 column (1.5 x 12 cm). RNA was eluted with 0.01mM EDTA and 30 drop fractions collected using a Gilson fraction collector. Radioactivity in each fraction was determined and peak fractions

of polyadenylated Sat-RNA were combined. A typical separation of polyadenylated Sat-RNA and [α-^{32}P]ATP is presented in Figure 2.2A. Approximately 30-60% of the total ^{32}P eluted from the column was incorporated into the 3'-terminus poly(A) tract of Sat-RNA. To the pooled fractions of polyadenylated Sat-RNA was added 3 x 10^8 cpm of either [α-^{32}P]dATP or [α-^{32}P]dCTP, and, if necessary, sufficient unlabelled dATP or dCTP to yield a final concentration of 100 μM (5 nmol). The mixture was lyophilized and dissolved in a reaction volume of 50 μl containing 50mM Tris, pH 8.3, 8mM $MgCl_2$, 2 μg of oligo(dT), 8mM dithioerythritol, 100 μg/ml actinomycin D, approximately 10 units of avian myeloblastosis virus reverse transcriptase, and 0.67mM of each of the approximate unlabelled deoxyribonucleotide triphosphates. The mixture was incubated at 37°C for 2 hr and the reaction terminated by the addition of 150 μl of 0.75% SDS and 300 μl of 0.5N NaOH. After incubation at 37°C for 2 hr to hydrolyse the RNA template, the cDNA was separated from the unreacted [α-^{32}P] deoxyribonucleotide and small ribonucleotide fragments by gel filtration on a Sephadex G-50 column as before. The column was eluted with 0.1M NH_4HCO_3 and fractions were collected and their radioactivity determined as above. A typical separation of [^{32}P]cDNA prepared to Sat-RNA, [^{32}P]cDNA(Sat-RNA), is shown in Figure 2.2B. Fractions containing cDNA were pooled and lyophilized in the presence of excess triethylamine to volatilize ammonia and triethylammonium salts. The cDNA was suspended in distilled water and stored at -15°C. Of the total ^{32}P radioactivity eluted from the column, between 0.1 and 1.0% of the [α-^{32}P] deoxyribonucleotide triphosphate had become incorporated into the cDNA. As 5 nmol of [α-^{32}P]dATP or dCTP containing 3 x 10^8 cpm was added to the reaction mix, the cDNA prepared would contain 5-50 pmol [α-^{32}P] deoxyribonucleotide. Assuming that the G:C:A:U content of Sat-RNA is 1:1:1:1, and that the average molecular weight of a DNA base is 300, it can be calculated that typical cDNA preparations contained between 6 and 60ng cDNA and between 0.3 and 3 x 10^6 cpm respectively. A newly-

Figure 2.2. Purification of polyadenylated Sat-RNA and [^{32}P]cDNA(Sat-RNA) by gel filtration on Sephadex G-50 columns. Thirty-drop fractions were collected and 10 µl aliquots of each fraction were removed for radioactivity determinations.

A. Separation of polyadenylated Sat-RNA and unreacted [α-^{32}P]ATP (column was eluted with 0.01m<u>M</u> EDTA, pH 7.0).

B. Separation of [^{32}P]cDNA(Sat-RNA) and unreacted ^{32}P-labelled deoxyribonucleotide (in this instance [α-^{32}P]dCTP). The column was eluted with 0.1<u>M</u> NH_4HCO_3, pH ∿ 7.0.


A
B
^{32}P Sat-RNA
^{32}P ATP
^{32}P dCTP
cDNA
(Sat-RNA)
[^{32}P] cpm (x10^{-3})
FRACTION NUMBER

prepared cDNA preparation would therefore have a specific activity of approximately 50,000 cpm/ng cDNA.

QCMV-RNA 3 and STNV-RNA

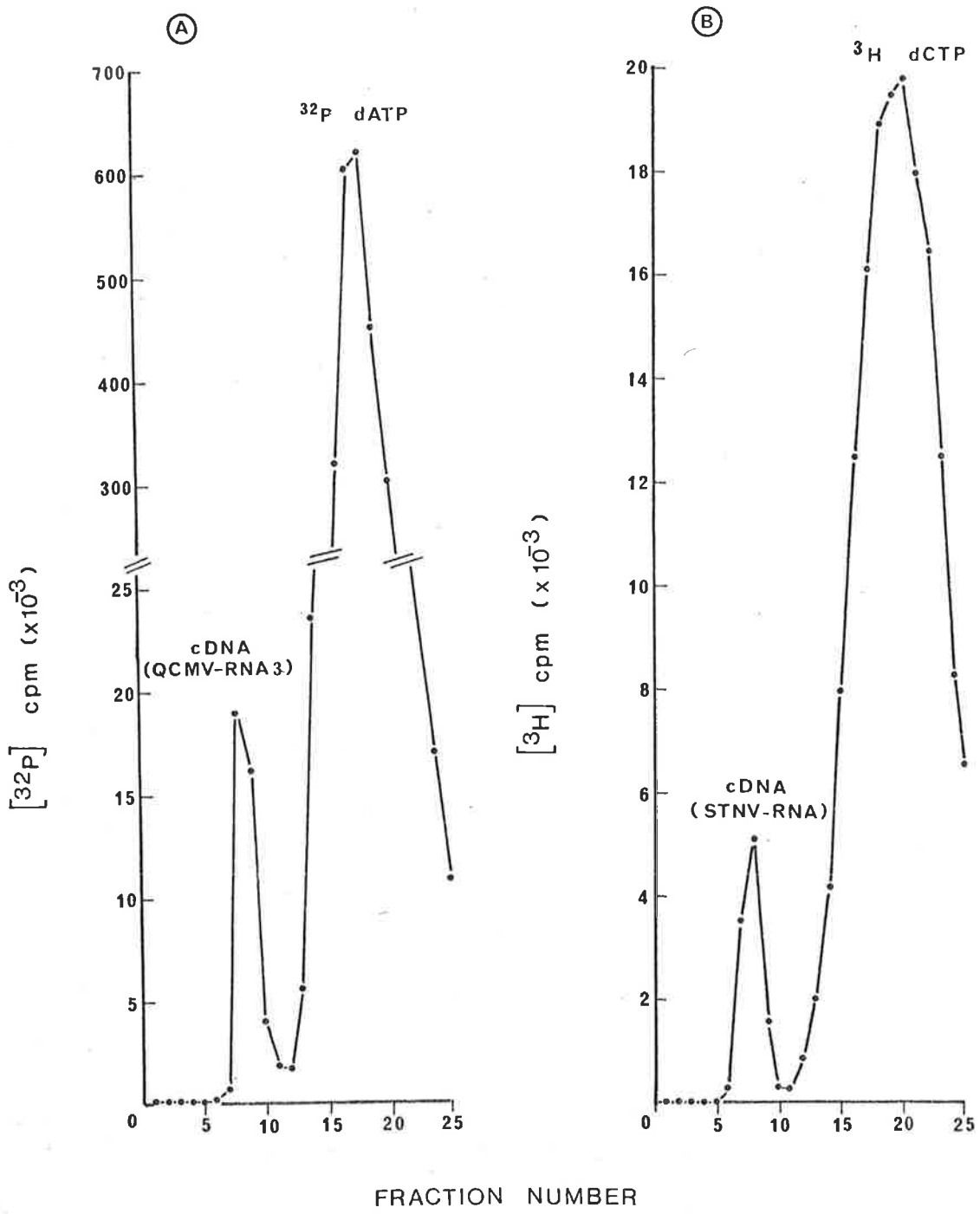
Complementary DNA was prepared to QCMV-RNA 3 and STNV-RNA essentially as described by Gould and Symons (1977). DNA fragments used to 'prime' the RNA templates were prepared as follows. A solution containing 5 mg/ml of salmon sperm DNA, 10mM magnesium acetate, 10mM Tris-acetate buffer, pH 7.4, was incubated at 37°C for 2 hr with 70 µg/ml of DNase I and then heated at 121°C for 10 min (Taylor *et al.*, 1976). A typical reaction mixture of 50 µl contained 2 µg of the RNA template, 50mM Tris-HCl, pH 8.3, 8mM $MgCl_2$, 8mM dithioerythritol, 4mM of either [α-^{32}P]dATP or [α-^{3}H]dCTP (approximately 2 x 10^8 cpm), 100 µg/ml actinomycin D, 125 µg of denatured salmon sperm DNA, approximately 10 units of avian myeloblastosis virus reverse transcriptase and 0.67mM of each of the appropriate, unlabelled deoxyribonucleotide triphosphates. The mixture was incubated at 37°C for 2 hr, the reaction terminated and the RNA template hydrolyzed with NaOH as described above. The cDNA preparations were eluted from a Sephadex G-50 column with 0.1M NH_4HCO_3 (Figure 2.3A and B) and the cDNA recovered as described before.

The incorporation of labelled deoxyribonucleotide into cDNA prepared by this method was slightly higher than that for cDNA transcribed from polyadenylated Sat-RNA. Actual incorporation of [α-^{32}P]dATP into cDNA (QCMV-RNA3) and [α-^{3}H]dCTP into cDNA(STNV-RNA) were 2.0 and 5.4% respectively. Using the assumptions given above, and taking into consideration the lowered counting efficiency of ^{3}H (approximately 30%), the effective specific activities of cDNA(QCMV-RNA 3) and cDNA(STNV-RNA) were approximately 44,000 and 12,000 cpm/ng of cDNA respectively.

Figure 2.3. Separation of cDNA(QCMV-RNA 3) and cDNA(STNV-RNA) from unreacted deoxyribonucleotides by gel filtration on Sephadex G-50 columns. Each column was eluted with 0.1M NH_4HCO_3 (pH ∿ 7.0) and the radioactivity determined as described in Figure 2.2.

A. [^{32}P]cDNA(QCMV-RNA 3).

B. [^{3}H]cDNA(STNV-RNA).

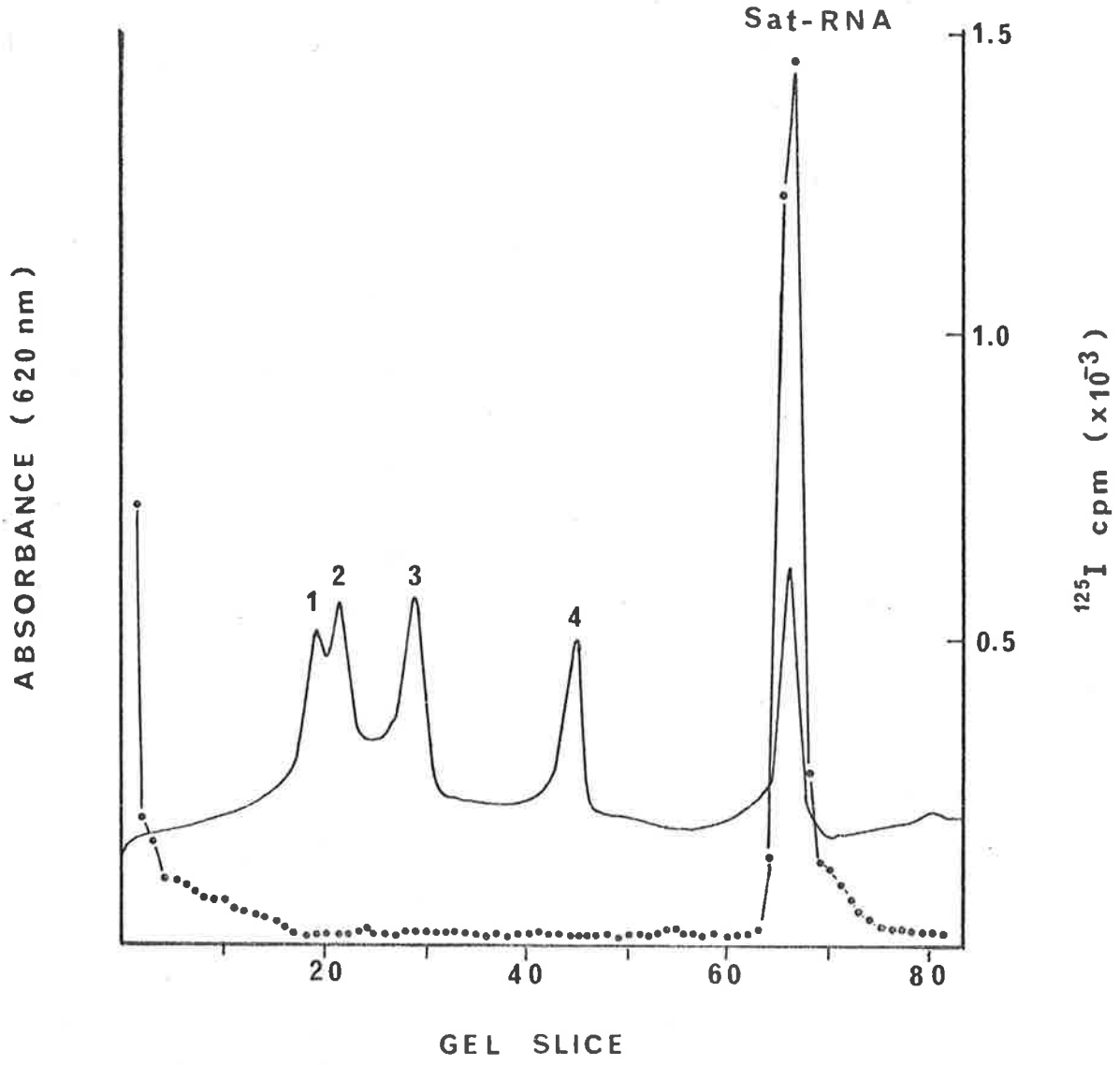

A
B
^{32}P dATP
^{3}H dCTP
cDNA
(QCMV-RNA3)
cDNA
(STNV-RNA)
[^{32}P] cpm (x10^{-3})
[^{3}H] cpm (x10^{-3})
FRACTION NUMBER

Radioiodination of Sat-RNA

[^{125}I]-labelled Sat-RNA was prepared by modifications to the procedure described by Prensky (1976). The reaction mixture of 25 µl contained 0.1M sodium acetate buffer, pH 4.7, 2.5 x 10^{-9}M KI, 2.5 x 10^{-8}M thallic trichloride, 200 µCi of carrier-free Na^{125}I, 100 µg/ml heparin, and 5 µg Sat-RNA. Heparin was included in the reaction mixture to inhibit RNases present in the commercial Na^{125}I (Heiniger *et al.*, 1973). The mixture was incubated at 60°C for 10 min, cooled on ice, and the reaction terminated by the addition of 2-mercaptoethanol to approximately 0.1M. Sodium acetate and NaI were then added to final concentrations of 0.5M and 20mM respectively and [^{125}I]-labelled Sat-RNA precipitated with 2 volumes of chilled ethanol. Instead of the additional heating and column chromatography steps suggested by Prensky (1976), [^{125}I]Sat-RNA was further purified by an additional ethanol precipitation step in the presence of excess KI. The initial ethanol precipitate was sedimented at 15,000 g for 10 min and dissolved in 0.2 ml of 0.25M sodium acetate buffer, pH 7.0, containing 50mM KI. [^{125}I]Sat-RNA was reprecipitated with 2 volumes of ethanol, recovered by centrifugation and dissolved in TE buffer.

In preliminary studies to detect ds-RNA to Sat-RNA in leaf RNA extracts, it was evident that the [^{125}I]Sat-RNA preparation contained a significant level of RNase-resistant, TCA-insoluble ^{125}I radioactivity. Paper electrophoresis (Smith, 1955) of [^{125}I]Sat-RNA hydrolysed with 0.3M KOH for 24 hr at 25°C, indicated that of the total ^{125}I radioactivity loaded only about 38% migrated as [^{125}I]CMP or its deamination product, [^{125}I]UMP (Robertson *et al.*, 1973); the remainder had not moved from the origin. Moreover, electrophoresis of [^{125}I]Sat-RNA on 2% agarose gels indicated that only about 35% of the total radioactivity loaded on the gel migrated with unlabelled Sat-RNA (Figure 2.4). RNA purified by polyacrylamide gel electrophoresis (Symons, 1978) and radioiodinated as

Figure 2.4. Electrophoresis of [^{125}I]Sat-RNA and QCMV-RNA on 2% agarose gels. Approximately 10,000 cpm of [^{125}I]Sat-RNA and 10 µg of QCMV-RNA was subjected to electrophoresis. The gel was stained in toluidine blue, destained in 5% acetic acid, and scanned at 620 nm. Slices, 1.4 mm thick, were cut with a multi-bladed apparatus and radioactivity in each slice was then determined by scintillation spectrophotometry.


Sat-RNA
1.5
1.0
0.5
1
2
3
4
20
40
60
80
ABSORBANCE (620 nm)
^{125}I cpm ($\times 10^{-3}$)
GEL SLICE

described above does not contain non-specific ^{125}I radioactivity (R.H. Symons, personal communication). It is conceivable that the non-specific ^{125}I radioactivity in the [^{125}I]Sat-RNA preparations was due to the binding of ^{125}I to some substance(s) eluted from agarose gels in the initial purification of Sat-RNA. [^{125}I]Sat-RNA was separated from the non-specific ^{125}I by two-cycles of gel electrophoresis. The position of [^{125}I]Sat-RNA was located by staining gels containing unlabelled Sat-RNA electrophoresed in parallel. [^{125}I]Sat-RNA was recovered from gel slices as described above, ethanol precipitated, and suspended in TE buffer and stored at -15^{o}C. The specific activity of [^{125}I]Sat-RNA prior to gel electrophoresis was 2 x 10^{6} cpm/µg. Assuming that the recovery of RNA from polyacrylamide gels is approximately 20% (Chapter 4), the estimated specific activity of the final [^{125}I]Sat-RNA was approximately 4 x 10^{5} cpm/µg.

RNA-cDNA Hybridization Procedures

Conditions for RNA-cDNA hybridization studies were essentially those of Kemp (1975) as modified by Gould and Symons (1977). In all hybridizations, the concentration of viral RNA relative to that of cDNA was in excess.

The extent of RNA base sequence homology between Sat-RNA isolated from various cucumoviruses, between QCMV-RNA 3 and VTAV-RNA, and between STNV-RNA and TNV-RNA were investigated as follows. RNAs were diluted in hybridization buffer (0.13M NaCl, 10mM Tris-HCl, 1mM EDTA, 0.05% SDS, pH 7.0) to either 2, or 6 µg/ml and serially diluted in two- and ten-fold steps in the same buffer. To 40 µl aliquots of each dilution was added approximately 2,000 cpm (40-200 pg) of the appropriate cDNA. The mixtures were drawn up into siliconized 100 µl capillary tubes, the ends of which were then sealed in a bunsen flame. The RNA-cDNA

mixtures were denatured by heating at 100°C for 2 min and incubated at 60°C. Unless otherwise stated, the capillaries were incubated for 2 hr. Hybridizations were terminated by chilling the capillaries in an ice-water bath.

The $R_o t$ values for the various combinations of RNA concentrations and hybridization times were calculated as:

$$R_o t = \frac{\text{RNA concentration} \times \text{hybridization time}}{\text{MW of nucleotide}} \quad (\text{mol sec litre}^{-1})$$

where RNA concentration is expressed as g/l, the hybridization time in seconds, and assuming that the average molecular weight for ribonucleotides is 340.

The extent to which the RNA-cDNA mixtures hybridized was determined using the single-strand specific nuclease, S_1 from *Aspergillus oryzae* (Ando, 1966). The sealed capillaries were opened and their contents dispensed into 420 µl of a buffer containing 30mM sodium acetate, pH 4.6, 50mM NaCl, 1mM $ZnSO_4$, 5% glycerol, and 40 µg/ml of denatured calf thymus DNA. Two, 200 µl aliquots were removed from each mixture, and to one was added approximately 2 units of S_1 nuclease, while the other served as a control. Both samples were incubated at 45°C for 30 min. The digestion was terminated by the addition to each sample of 1 ml of 10% TCA and 100 µg of BSA as a carrier. Samples were mixed thoroughly, and left on ice for at least 30 min. TCA-insoluble material was collected on Whatman GF/A filters, washed four times with 4 ml of 5% TCA, and twice with 5 ml of ether. The radioactivity on dried filters was determined by liquid scintillation spectrophotometry. The fraction of each hybrid resistant to S_1 nuclease was calculated by comparing the TCA-insoluble

radioactivity of duplicate samples incubated in the presence, and absence of S_1 nuclease. The S_1 resistance of the cDNA preparation in the absence of added RNA was deducted from each hybridization value. In most experiments, the S_1-resistance of cDNA preparations was less than 5% and varied according to the preparation of cDNA used. The $R_o t_{\frac{1}{2}}$ value, defined here as the midpoint between the levels of highest and lowest hybridization values, was determined for each RNA-cDNA combination from plots of percent hybridization *versus* Log $R_o t$ value.

In all experiments to detect Sat-RNA, QCMV-RNA 3, or STNV-RNA in total leaf RNA extracts, leaf RNA and the appropriate cDNA mixtures were hybridized to a $R_o t$ value of 10.0 mol sec litre^{-1}. This corresponds to 52.5 µg/ml of total leaf RNA diluted in hybridization buffer incubated for 18 hr at 60°C. Two, 40 µl aliquots of each leaf RNA containing approximately 2,000 cpm (40-200 pg) of the appropriate cDNA preparation were drawn up into capillary tubes, heated at 100°C for 2 min, and incubated at 60°C for 18 hr as above. The S_1-resistant fraction of the various leaf RNA-cDNA mixtures were determined as described above. In all such experiments hybridizations were done in duplicate. Hybridization values given are the mean of two duplicates which rarely differed by more than 5%.

RNA-RNA Hybridization Techniques

Conditions of hybridization of [^{125}I]Sat-RNA with leaf RNA extracts were similar to those described by Shoulder *et al.* (1974). Leaf RNA extracts from healthy *N. clevelandii*, or plants infected systemically with either CMV alone, or CMV and Sat-RNA, had been incubated with RNase to remove ss-Sat-RNA and suspended in 0.5 ml TE buffer. Each hybridization mixture contained 50 µl of RNase-treated leaf RNA and

approximately 4,000 cpm of [^{125}I]Sat-RNA in siliconized test tubes (7 x 50 mm), and was then overlayed with paraffin oil. Hybridization mixtures were denatured by heating at 100°C for 10 min, 10 x SSC added to give a final concentration of 2 x SSC, and the mixture incubated at 70°C for 18 hr. Hybridizations were terminated by chilling the tubes. From each tube, 50 µl of the hybridization mixture was removed and mixed with 400 µl of a buffer containing 10mM Tris, 0.3M NaCl, 5mM EDTA, pH 7.4, and 60 µg/ml *E. coli* rRNA. Two, 200 µl aliquots were removed from each sample. To one was added 50 µg/ml of RNase A and 10 units of RNase T_1, while the other served as a control. The duplicate samples, with and without RNases, were incubated at 37°C for 30 min. One ml of 10% TCA and 100 µg BSA were added to each sample, and the mixtures kept on ice for at least 30 min. TCA-insoluble material was collected on GF/A filters, washed five times with 4 ml of 5% TCA, and twice with 4 ml of ether. The fraction of [^{125}I]Sat-RNA hybridized was calculated by comparing TCA-insoluble radioactivity of duplicates incubated with and without RNases.

Spectrophotometry

Ultraviolet absorption spectra, and the kinetics of enzyme digestion and thermal denaturation of viral RNAs were determined using a Unicam SP1800 spectrophotometer. The concentration of viral nucleoprotein and RNA preparations were determined using the extinction coefficients listed in Table 2.4. If necessary, absorption spectra were corrected for light-scattering as described by Noordam (1973).

Scintillation Spectrophotometry

TCA-insoluble radioactivity on glass fibre filters was determined using a toluene-based fluid containing 3.5 g of PPO and 0.35 g of POPOP per litre. Radioactivity of aqueous samples was determined in a

Table 2.4. Extinction coefficients used for spectrophotometric determinations.

Virus or RNA	$E^{0.1\%}_{1\ cm,260\ nm}$	Reference
CMV, TAV	5.0	Francki *et al.* (1966) Stace-Smith and Tremaine (1973)
AMV	5.0)	Unfractionated nucleoprotein preparations
TRSV	7.5)	
TMV	2.7	Knight (1962)
Viral RNA	25.0	
Total leaf RNA	25.0	
E. coli rRNA	25.0	
$tRNA^{Phe}_{yeast}$	20.0	

scintillation fluid containing 2 parts toluene, 1 part Triton X-100, and 3.5 g of PPO and 0.35 g of POPOP per litre. The distribution of ^{125}I in agarose gels was determined after gel slices were first solubilized in 0.5 ml of a 1 NCS : 9 water mixture. ^{32}P in formamide-acrylamide gel slices was measured by Cerenkov radiation.

Precautions Against RNase

All solutions which were to come in contact with RNA were autoclaved (121°C for 10 min), where practicable. Glassware was heat sterilized (130°C for at least 3 hr). Objects unable to be sterilized by heat were washed with a solution containing 0.2M KOH in 90% ethanol and rinsed thoroughly in sterile distilled water.

CHAPTER 3

COMPARATIVE PROPERTIES OF FOUR CUCUMOVIRUS ISOLATES

Comparative studies on the physical, chemical and serological properties of QCMV and VTAV have shown that although both viruses possess characteristics of the cucumovirus group, the primary structure of their coat proteins and genomic RNAs are readily distinguishable (Habili and Francki, 1974a, 1974b, 1974c). The additional isolates of CMV, MCMV and GCMV, have distinctive biological properties and seemed suitable candidates for genetic analyses by the construction of pseudorecombinants. In this chapter are described experiments on the comparative physical and chemical properties of QCMV, GCMV, MCMV and VTAV.

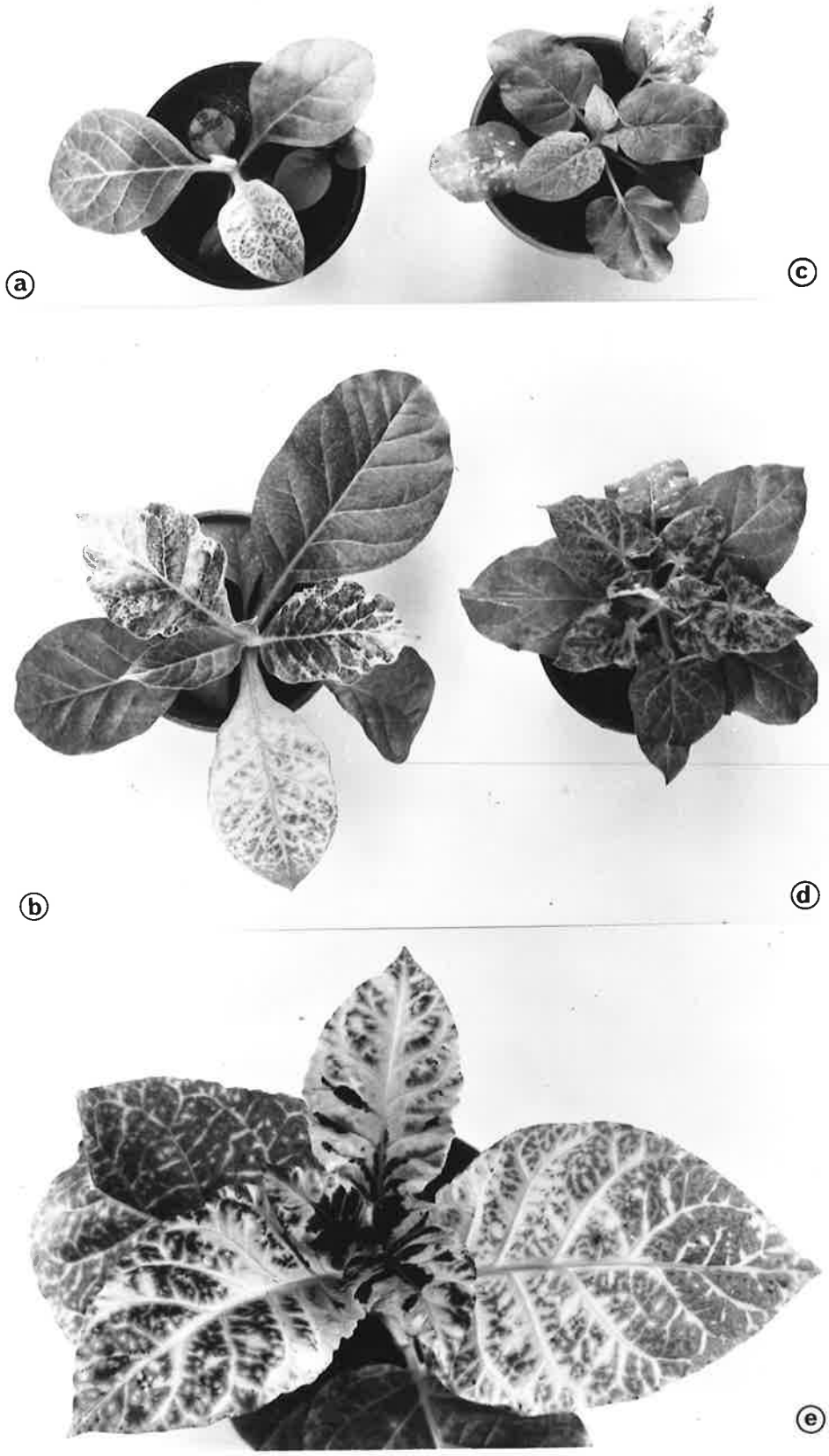
Purification and Properties of GCMV and MCMV

A previously uncharacterized cucumovirus isolate, GCMV, was recovered from a lyophilized culture maintained at the Waite Agricultural Research Institute (Table 2.1). Since GCMV is readily purified by the purification procedure used for QCMV (Chapter 2), and is serologically indistinguishable from QCMV in immunodiffusion tests (Table 3.2), the molecular weight of its coat protein and the nucleotide base composition of its RNA were not determined.

MCMV was originally isolated as a mutant of Price's No. 6 yellow strain of CMV (Price, 1934) in Cambridge, England by Dr K.M. Smith (R. Markham and L.L. Stubbs, personal communications). This isolate was initially termed the CMV 'white mutant' because of the brilliant light-yellow mosaic symptoms it produces in *Nicotiana* species (Figure 3.1).

Initial attempts to purify MCMV by the normal method used for the purification of CMV in this laboratory (Chapter 2), or that used

Figure 3.1. Symptoms of MCMV on *N. tabacum* cv. White Burley (a, b and e) and on *N. glutinosa* (c and d), 9 days (a and c) and 18 days (b, d and e) after inoculation. Plants shown in (a-d) were maintained in a growth chamber at 22°C with a photoperiod of 12 hr and 12,000 lux, while the *N. tabacum* cv. White Burley shown in (e) was maintained in an air-cooled glasshouse.

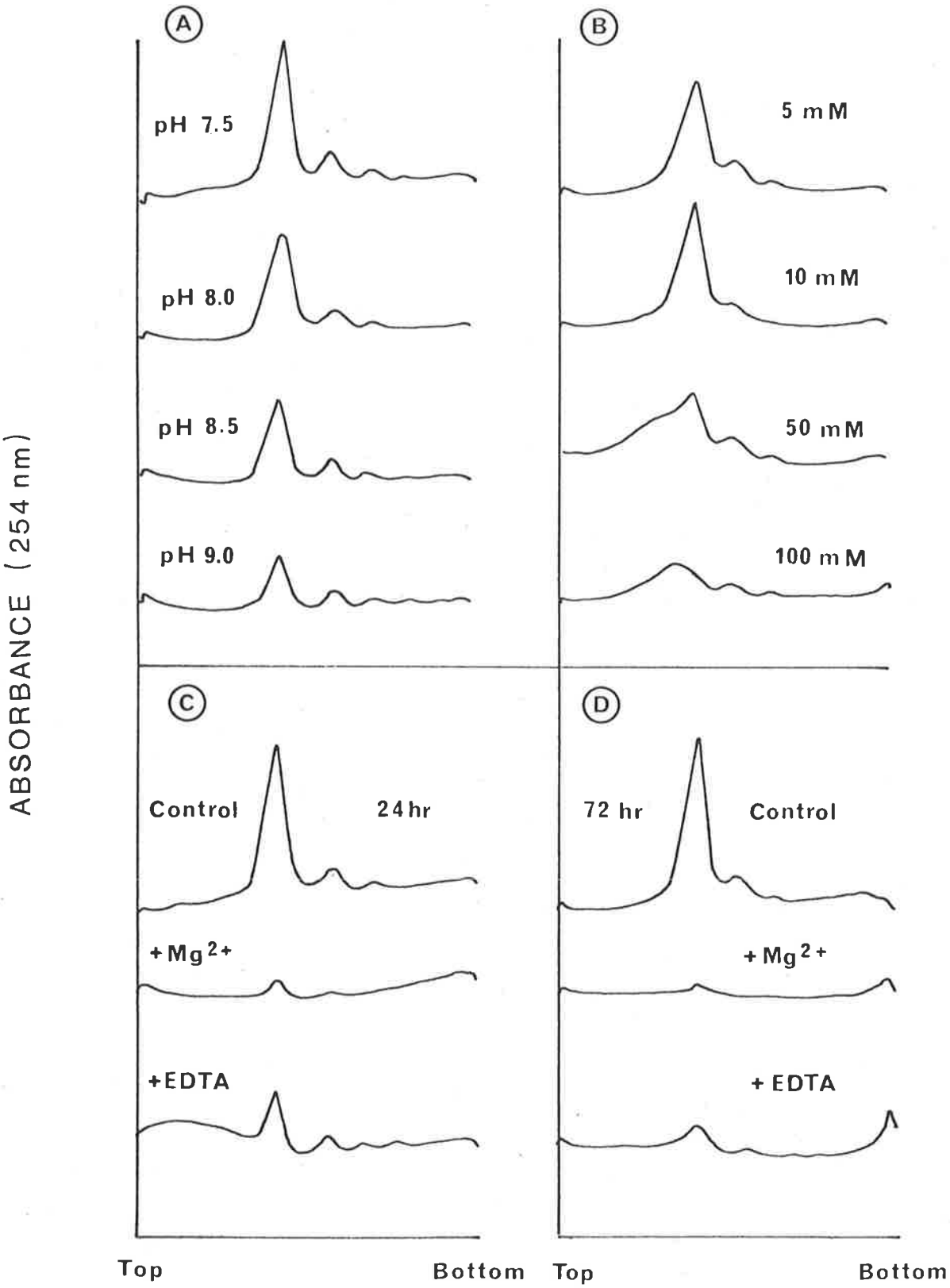

a
c
b
d
e

previously by Francki *et al.* (1966) were unsuccessful in that no virus was recovered. Subsequent experiments demonstrated the detrimental effects of organic solvents on MCMV; no virus was recovered from leaf extracts clarified with chloroform, and between 50 and 90% was lost after emulsification with solvents such as diethyl ether, carbon tetrachloride and Freon 113 (R.I.B. Francki and C.J. Grivell, personal communication). It was also observed that the ionic composition, molarity, and pH of extraction and resuspension buffers were critical if serious virus losses were to be avoided. The procedure finally adopted for the purification of MCMV (p. 15) enabled the preparation of highly purified virus in yields ranging from 300-600 mg/kg of leaf tissue. This procedure was unsuitable for the purification of QCMV; significant losses of this isolate occurred and sucrose density gradient analysis indicated a considerable proportion of virus particles in such preparations were disrupted.

The effects of molarity and pH of the resuspension buffer and the addition of Mg^{2+} and EDTA on the stability of MCMV are presented in Figure 3.2. Sodium borate buffer at a concentration of 10 mM and a pH of 7.5 is optimal for particle stability. MCMV is readily precipitated in the presence of Mg^{2+} (Figure 3.2c), a property it shares with other isolates of CMV (Takanami and Tamara, 1969; Habili and Francki, 1974b). However, unlike QCMV, MCMV was shown to be prone to degradation in the presence of EDTA (Figure 3.2c). In this respect, MCMV resembles VTAV, although the latter cucumovirus is stabilized by Mg^{2+} (Habili and Francki, 1974b).

Price's No. 6 yellow strain of CMV is readily purified by methods similar to those used for QCMV (Wood and Coutts, 1975). Conceivably MCMV, which was originally derived from Price's No. 6 strain, has undergone

Figure 3.2. Sedimentation profiles of MCMV incubated in sodium borate buffers of varying pH and molarity, and in the presence and absence of Mg^{2+} or EDTA. Virus samples of 1 mg/ml were incubated at 4°C in (a) 10mM sodium borate at various pH values for 2 days, (b) in sodium borate buffer, pH 7.5 at various molarities for 4 days, and (c and d) in 10mM sodium borate buffer, pH 7.5, in the presence and absence of 10mM $MgCl_2$ or 10mM EDTA for 24 hr (c) and 3 days (d). A sample (50 µl) of each preparation was layered on a 5-25% sucrose gradient containing 10mM sodium borate buffer, pH 7.5, and centrifuged at 50,000 rpm for 35 min at 4°C in a Spinco SW50.1 rotor. The gradients were analysed at 254 nm with an ISCO apparatus.


A
pH 7.5
pH 8.0
pH 8.5
pH 9.0
B
5 mM
10 mM
50 mM
100 mM
C
Control
24 hr
+Mg2+
+EDTA
D
72 hr
Control
+Mg2+
+EDTA
ABSORBANCE (254 nm)
Top
Bottom
Top
Bottom

mutation resulting in certain amino acid exchanges which have markedly affected properties of the capsid.

MCMV coat protein migrated together with that of QCMV in 10% polyacrylamide gels containing SDS (Figure 3.3A). The molecular weights of these proteins were confirmed to be 24,500 (Habili and Francki, 1974a) by comparing their electrophoretic mobilities with a limited number of standard proteins (Figure 3.3B).

The molar base ratios of MCMV (Table 3.1) are within the range of values reported for other cucumovirus strains (G : 23.1-24.7; A : 22.4-26.4; C : 21.2-23.2; U : 28.7-30.7 moles %). Although the molar base ratios of each RNA component have not been determined, it is most likely that they are similar since it has been recently shown that the base ratios of the RNA components of other multiparticle viruses are indistinguishable (A.J. Gibbs, personal communication).

Initial attempts to resolve RNAs 1 and 2 of MCMV under non-denaturing electrophoretic conditions were unsuccessful. However, in 4% polyacrylamide-formamide gels, MCMV-RNA was readily resolved into 4 components (Figure 3.4), characteristic of cucumoviruses (Peden and Symons, 1973; Lot *et al.*, 1974; Habili and Francki, 1974a). In subsequent studies it was shown that the resolution of MCMV-RNAs 1 and 2 in aqueous electrophoretic conditions was greatly improved by increasing the length of the polyacrylamide and agarose gel columns from 11 to 13 cm, heating the RNA sample prior to electrophoresis at 75^{o}C for 5 min followed by rapidly cooling, and by reducing the voltage gradient during electrophoresis.

Figure 3.3. Electrophoresis of MCMV and QCMV proteins in 10% polyacrylamide gels and the determination of their molecular weights.

A. Molecular weight determination of MCMV and QCMV proteins. The mobility of each protein was recorded relative to that of cytochrome c. The molecular weights of market proteins (BSA - 68,000, OA - 43,000, and cytochrome c - 11,700) are from Weber and Osborn (1969).

B. Gels were loaded with; (a) MCMV, (b) QCMV and (c) a mixture of MCMV and QCMV proteins. Electrophoresis was at 6 mA/gel for 9 hr at 25°C. Bovine serum albumin (BSA), ovalbumin (OA), and cytochrome c (Cyto c) were included as molecular weight markers.

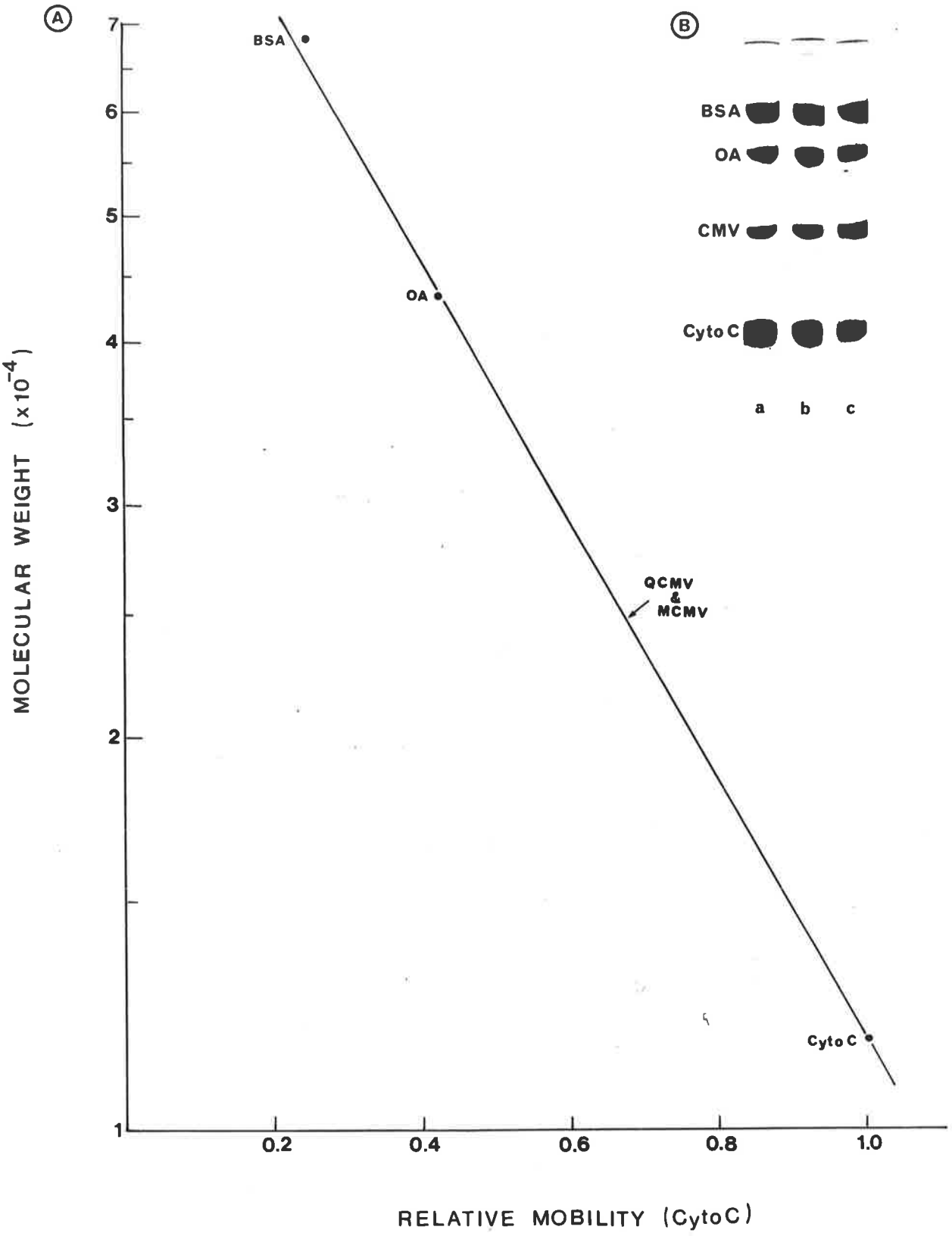
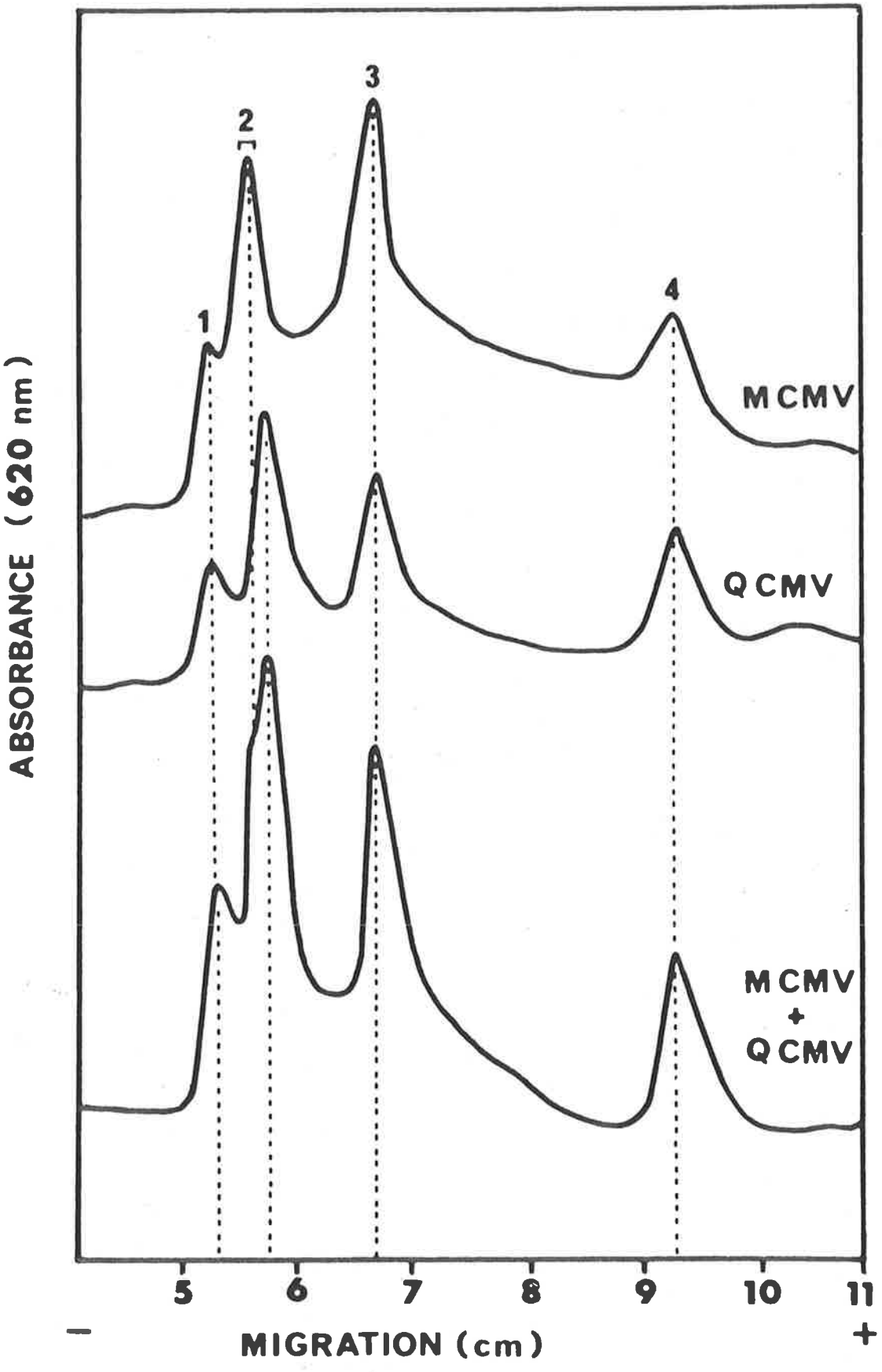

A
B
BSA
OA
QCMV & MCMV
Cyto C
MOLECULAR WEIGHT (x10^{-4})
RELATIVE MOBILITY (CytoC)
7
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
BSA
OA
CMV
Cyto C
a
b
c

Table 3.1. Molar base ratios of MCMV and other cucumovirus strains.

Virus	Reference	Mole % G	A	C	U
TAV (Canadian)	Stace-Smith and Tremaine (1972)	23.7	26.4	21.2	28.7
TAV (Canadian)	Stace-Smith and Tremaine (1973)	23.1	26.2	21.6	29.0
VTAV	Habili and Francki (1974a)	23.9±0.48	24.6±0.47	21.4±0.29	30.1±0.39
CMV (Y strain)	Kaper *et al.* (1965)	23.4±0.4	24.3±0.5	23.2±0.7	29.0±0.7
QCMV	Francki *et al.* (1966)	24.7±0.33	22.4±0.22	22.8±0.45	30.1±0.5
QCMV	Habili and Francki (1974a)	24.0±0.5	23.5±0.39	21.8±0.29	30.7±0.39
MCMV[a]	This work	24.6±0.2	23.6±0.3	22.2±0.2	29.6±0.4

a Based on six determinations of three different RNA preparations.

Figure 3.4. Analysis of MCMV-RNA and QCMV-RNA by polyacrylamide gel electrophoresis under denaturing conditions. RNA samples (10 µg) in formamide containing 20mM diethylbarbituric acid, pH 9.0, were layered on 4% acrylamide-formamide gels prepared as described in Chapter 2. Electrophoresis was at 1 mA/gel (5 volts/cm) for 24 hr at 25°C, after which the gels were stained with toluidine blue and scanned at 620 nm.


ABSORBANCE (620 nm)
1
2
3
4
MCMV
QCMV
MCMV
+
QCMV
5
6
7
8
9
10
11
−
+
MIGRATION (cm)

Properties of the Four Cucumovirus Isolates

Serological relationships between QCMV, GCMV, MCMV and VTAV are presented in Table 3.2. In immunodiffusion tests, QCMV and GCMV were indistinguishable whereas MCMV, although related to QCMV and GCMV, could be readily distinguished from these two isolates. The homologous titre of an anti-MCMV serum was reduced from 1/32 to 1/4 by absorption with QCMV, and an anti-QCMV serum from 1/32 to 1/8 by absorption with MCMV.

No serological relationship could be demonstrated between VTAV and the three isolates of CMV (Table 3.2). It is of interest to note that an anti-VTAV serum also failed to react with 9 additional isolates of CMV (Table 5.1). However, VTAV is serologically closely related to another isolate of TAV, NTAV, an antiserum to which also failed to react with any of the isolates of CMV examined (Table 5.1). These results confirm those of an earlier report (Habili and Francki, 1974a) which failed to demonstrate any serological relationships between VTAV and QCMV.

The amino acid composition of the four cucumovirus isolates is presented in Table 3.3. As expected, the amino acid composition of the isolates of QCMV and VTAV used throughout this thesis are in reasonably good agreement with those obtained by Habili and Francki (1974a). A notable exception between the amino acid analyses presented in Table 3.3, and those reported previously for QCMV and other strains of CMV (Habili and Francki, 1974a; Van Regenmortel, 1967) is the presence of cysteine residues in the coat protein of CMV. This is presumably because in the hydrolysis method used by these workers (6N HCl), cysteine is readily degraded (Knight, 1975). Hydrolysis of carboxymethylated protein samples in 4N methanesulphonic acid containing 0.2% 3-(2-aminoethyl) indole (Simpson *et al.*, 1976) enables the efficient recovery of methionine,

Table 3.2. Serological relationships between the four cucumoviruses.

Antigen[a]	Antiserum[b]			
	QCMV	GCMV	MCMV	VTAV
QCMV	32[c]	64	8	-[d]
GCMV	32	64	8	-
MCMV	8	16	32	-
VTAV	-	-	-	64

a Antigen was fixed with glutaraldehyde and used at a concentration of 0.75 mg nucleoprotein/ml.

b Antisera were diluted in two-fold steps in 20mM sodium phosphate buffer, pH 7.6. Antisera to QCMV, GCMV and VTAV were prepared in mice while that to MCMV was prepared in rabbits. All serological techniques were as described in Chapter 2.

c Reciprocal of antiserum titres in immunodiffusion tests. Homologous titres are underlined.

d No reaction observed on immunodiffusion tests with undiluted antisera.

Table 3.3. Amino acid composition of the coat protein of four cucumoviruses.

Amino acid	QCMV		QCMV[b]	GCMV		MCMV		VTAV		VTAV[b]
	Relative molar ratio[a]	Integer		Relative molar ratio[a]	Integer	Relative molar ratio[a]	Integer	Relative molar ratio[a]	Integer	
Lys	15.1	15	15	15.0	15	12.0	12	14.2	14	15
His	3.6	4	4	3.9	4	2.8	3	4.9	5	5
Arg	20.4	20	20	21.1	21	22.3	22	15.6	16	16
Asp	24.0	24	24	23.2	23	24.6	25	26.0	26	27
Thr[c]	13.5	14	14	12.7	13	14.4	14	19.0	19	19
Ser[c]	24.7	25	26	25.1	25	26.6	27	22.6	23	23
Glu	15.6	16	16	13.3	13	12.6	13	18.2	18	19
Pro	16.0	16	18	15.7	16	13.4	13	14.0	14	14
Gly	14.1	14	14	13.8	14	12.6	13	13.2	13	14
Ala	14.8	15	14	18.9	19	20.0	20	18.8	19	19
Cys[d]	0.9	1	0	0.8	1	1.9	2	1.6	2	1
Val	17.9	18	18	20.0	20	20.0	20	16.2	16	17
Met[d]	5.4	5	5	3.6	4	3.4	3	2.7	3	2
Ile	11.0	11	12	8.3	8	10.8	11	12.8	13	13
Leu	21.7	22	22	21.9	22	23.7	24	18.8	19	19
Tyr	9.0	9	9	10.4	10	8.8	9	7.7	8	8
Phe	6.1	6	6	5.5	6	5.3	5	6.5	7	7
Trp[d]	1.2	1	1	0.8	1	1.2	1	1.4	1	1
		236	238		235		237		236	239

a Means of 24, 48 and 72 hr hydrolyses of at least three separate protein preparations including one 22 hr methanesulphonic acid hydrolysis of carboxymethylated protein samples.

b Data from Habili and Francki (1974a).

c Extrapolated to zero hydrolysis time.

d Determined from methanesulphonic acid hydrolysis. Cysteine was measured as S-carboxymethyl cysteine.

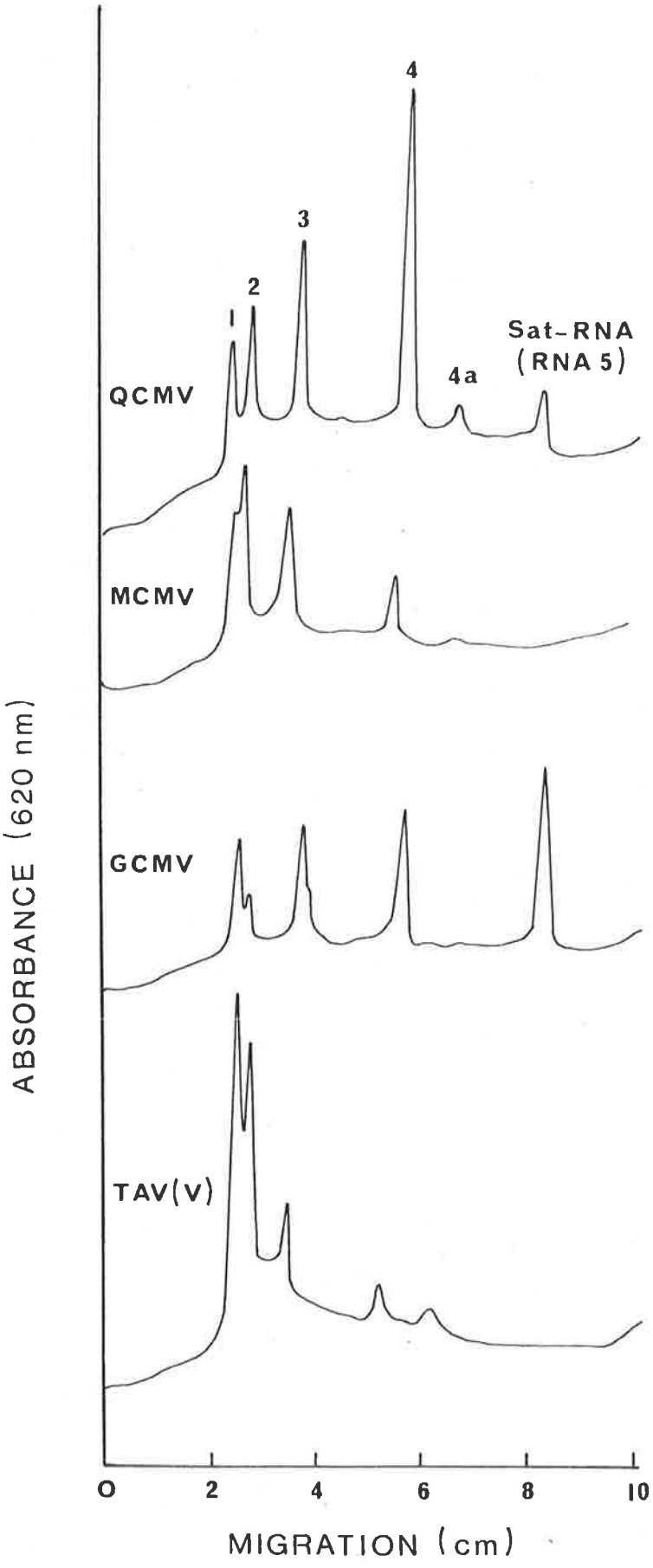
tryptophan and cysteine, and also provides a means of distinguishing cysteine (as S-carboxymethylcysteine) and half-cysteine (as S-sulphocysteine).

Although the amino acid compositions of QCMV, GCMV and MCMV are readily distinguishable, the differences between these three isolates are considerably less than those between each isolate of CMV and VTAV (Table 3.3). The amino acid compositions of an additional 9 cucumovirus isolates and 7 strains of AMV are presented in Tables 5.2 and 5.3 respectively, and a classification of these viruses based on computer analysis of their amino acid data is presented in Figure 5.2.

Isolated RNA of QCMV, GCMV, MCMV and VTAV electrophoresed in 2% agarose gels, all contained four major RNA components, termed RNAs 1 to 4 in order of decreasing molecular weight (Figure 3.5). In addition, QCMV and GCMV also contained substantial amounts of a low molecular weight RNA species, designated Sat-RNA. Experiments described in Chapters 4 and 7 demonstrate that Sat-RNA has no nucleotide sequence homology with genomic RNAs 1-4 of QCMV and depends on cucumoviruses for both its replication and encapsidation.

Although both MCMV and VTAV did not appear to contain Sat-RNA when first isolated (Figure 3.5) electrophoresis of increased amounts of their RNAs indicated the presence of low levels of an RNA component which comigrated with Sat-RNA. This RNA component, termed RNA 5, is present in varying amounts in RNA preparations of all cucumoviruses examined. Hybridization analysis using [^{32}P]cDNA transcribed from Sat-RNA, cDNA(Sat-RNA), demonstrated that RNA 5 has no nucleotide sequence homology with Sat-RNA, and is produced as a result of specific fragmentation of genomic RNAs of cucumoviruses (Gould *et al.*, 1978, and Table 4.3).

Figure 3.5. Separation of RNA components of QCMV, GCMV, MCMV and VTAV under non-denaturing conditions in 2% agarose gels. RNA samples (10-15 µg) were heated briefly (75°C for 3 min) and cooled rapidly in an ice-water bath prior to electrophoresis. Electrophoresis was at 25°C at 2.5 mA/gel for 3 hr.


4
3
2
1
Sat-RNA
(RNA 5)
4a
QCMV
MCMV
GCMV
TAV(V)
ABSORBANCE (620 nm)
0
2
4
6
8
10
MIGRATION (cm)

Electrophoresis of GCMV-RNA in 2% agarose gels occasionally resulted in the appearance of two bands for each of RNAs 3 and 4 (Figure 3.5). The relative proportions of the electrophoretically distinguishable components varied somewhat between RNA preparations. Invariably, the slower-moving RNA was the major component, and presumably the faster-moving RNA represents an altered conformational state (conformer) of the native genomic RNA since GCMV-RNAs 3 and 4 always migrated as single components in 99% formamide-acrylamide gels. Similar conformational differences have been observed in some preparations of QCMV-RNA (R.H. Symons, personal communication), ribosomal RNAs (Morris *et al.*, 1974) and with the genomic RNAs of some bromoviruses (Bancroft, 1971; Dickerson and Trim, 1978). These conformational changes have been shown to be cation-induced (Morris *et al.*, 1974; Dickerson and Trim, 1978). Conformers of GCMV-RNA 3 were also observed in some RNA preparations of a pseudorecombinant containing this RNA (Figure 4.3) indicating that the ability to assume different conformational states is a property specific to the RNA.

In almost every combination, the genomic RNA components of the four cucumovirus isolates could be distinguished by their comparative electrophoretic mobilities in 2% agarose gels (Figure 3.6). The slight differences observed in the electrophoretic mobilities of the RNA components of each isolate enabled the direct identification of the genomic RNA composition of pseudorecombinants constructed from heterologous mixtures of RNA components from these four cucumoviruses (Chapter 4).

Estimates of the molecular weights of cucumovirus genomic RNAs vary considerably (Table 3.4) depending on the choice of electrophoretic conditions and molecular weight markers used. Whenever *E. coli* RNAs are used as internal molecular weight markers, estimates for the molecular weights of CMV-RNAs 1 and 2 are invariably considerably higher than

Figure 3.6. Comparison of the electrophoretic mobilities of RNA components from QCMV, GCMV, MCMV and VTAV coelectrophoresed in 2% agarose gels in various combinations. RNA sample preparation, and the conditions of electrophoresis were as described in Figure 3.5. Arrows indicate genomic RNAs, e.g. Q_1 = QCMV-RNA 1.

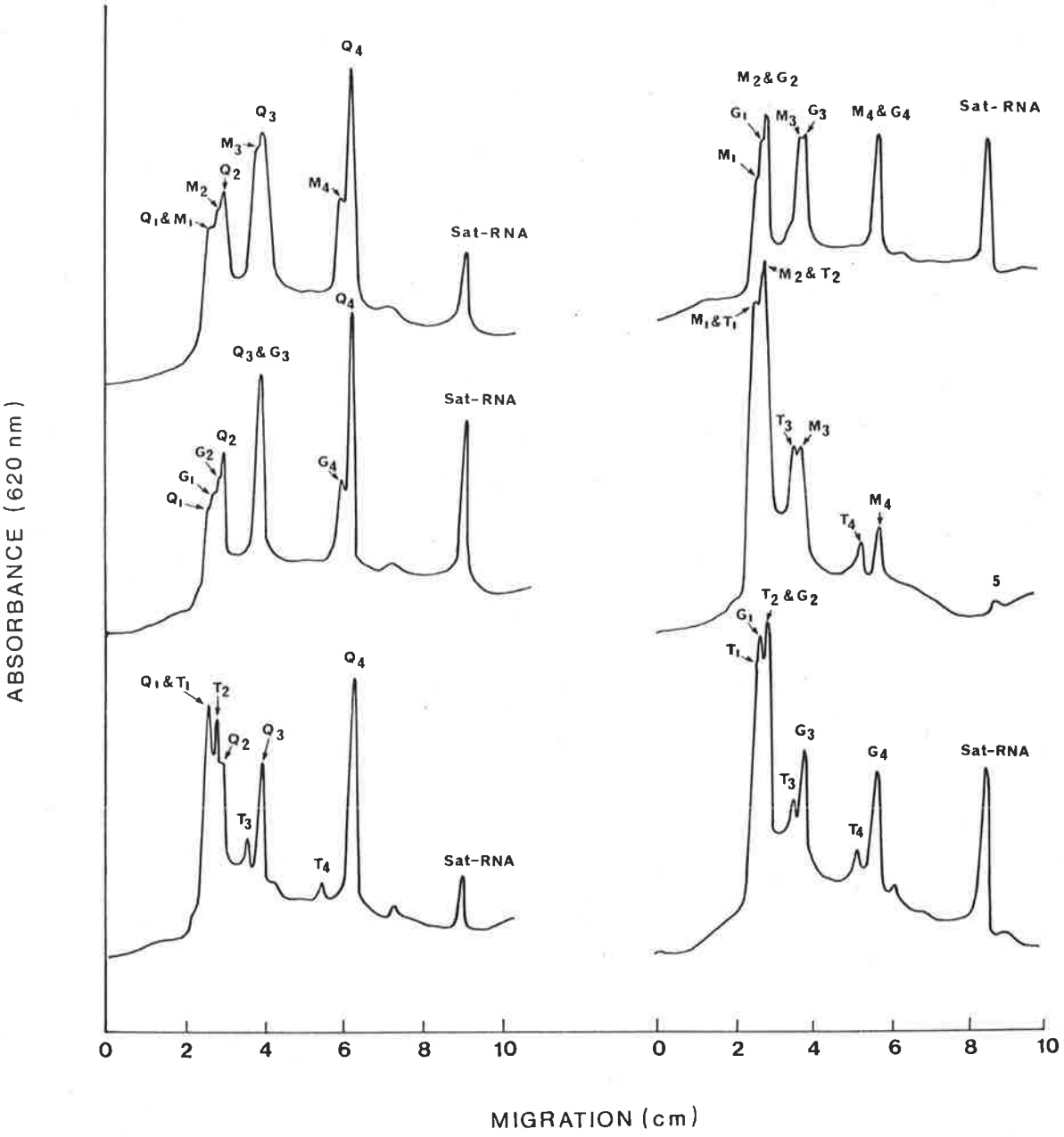

ABSORBANCE (620 nm)
Q4
Q3
M3
Q2
M2
Q1&M1
M4
Sat-RNA
Q4
Q3&G3
Sat-RNA
Q2
G2
G1
Q1
G4
Q1&T1
T2
Q2
Q3
T3
T4
Q4
Sat-RNA
M2&G2
G1
M3
G3
M4&G4
Sat-RNA
M1
M2&T2
M1&T1
T3
M3
M4
T4
5
T2&G2
G1
T1
G3
G4
Sat-RNA
T3
T4
0
2
4
6
8
10
0
2
4
6
8
10
MIGRATION (cm)

Table 3.4. Molecular weight estimates of the RNA components of several cucumoviruses.

Virus	Reference	Internal RNA standards[a] used	Electrophoresis[b] conditions	Molecular weights of RNA components ($x10^{-6}$) (sat-RNA/CARNA 5) 1	2	3	4	5	6
QCMV	Peden and Symons (1973)	*E. coli* 23S & 16S rRNA	aqueous	1.30	1.13	0.78	0.34	0.12	0.05
QCMV	"	"	formamide	1.35	1.16	0.85	0.35	-[c]	-
QCMV	Habili and Francki (1974a)	"	aqueous	1.26	1.10	0.77	0.34	-	-
QCMV	Habili (1974)	*E.coli* 23S & 16S rRNA+TMV	formamide	1.30	1.17	0.83	0.35	-	-
QCMV	This work	*E.coli* 23S & 16S rRNA+TMV +MS2	formamide	1.27	1.13	0.82	0.35	0.105	-
GCMV	"	"	"	1.27	1.18	0.82	0.35	0.105	-
MCMV	"	"	"	1.27	1.18	0.82	0.35	-	-
CMV (Strain S)	Kaper and West (1972)	TYMV, CMtV, MS2	formaldehyde-treated RNA[d]	1.01	0.89	0.68	0.33	0.11	0.01
CMV (Strain S)	Kaper and Waterworth (1973)	TYMV, MS2	"[d]	1.07	0.95	0.69	0.33	-	-
CMV (Strain S)	Kaper and Diaz-Ruiz (1977)	PsV, PcV	aqueous[e]	1.01	0.89	0.68	0.33	0.11	-
CMV (Strain S)	Reijnders *et al.* (1974)	*E. coli* 23S & 16S rRNA	8M urea, 60°C	1.21	1.06	0.79	0.33	-	-
CMV (Strain D)	Marchoux *et al.* (1973)	AMV	6M urea, 4°C	1.10	0.95	0.70	0.34	0.16	-
CMV (Strain D)	"	BMV	6M urea, 4°C	1.26	1.12	0.76	0.38	-	-
CMV (Strain D)	"	*E. coli* 23S & 16S rRNA	6M urea, room temperature	1.26	1.21	0.93	0.36	-	-
CMV (strain Y & O)	Takanami *et al.* (1977)	"	8M urea, 60°C	1.23	1.13	0.83	0.33	0.07	-
TAV (Hollings strain)	Lot *et al.* (1974)	"	aqueous	1.23	1.17	0.97	0.50	0.17	-
TAV (Hollings strain)	"	"	formaldehyde-treated RNA	1.36	1.27	1.04	0.49	-	-
VTAV	Habili and Francki (1974a)	"	aqueous	1.26	1.10	0.90	0.43	-	-
VTAV	Habili (1974)	*E.coli* 23S & 16S rRNA+TMV	formamide	1.30	1.22	0.94	0.50	-	-
VTAV	This work	*E.coli* 23S & 16S rRNA+TMV +MS2	"	1.27	1.18	0.90	0.42	-	-

a The molecular weight values used by most workers for *E. coli* 23S & 16S rRNA, and TMV-RNA were 1.07, 0.55 (Stanley and Bock, 1965) and $2.05x10^6$ respectively. The molecular weight value for MS2-RNA used in this work was $1.15x10^6$ (after Fiers *et al.*, 1976), whereas that used by Kaper and West (1972) and Kaper and Waterworth (1973) was only $1.05x10^6$.

b No distinction has been made between the pH, molarity, or ionic composition of aqueous buffer systems used by other workers, although these may have an effect on the relative mobilities of RNAs (Reijnders *et al.*, 1973).

c Not determined.

d Data based on sedimentation velocity and electrophoresis of formaldehyde-treated RNA. Molecular weights of marker RNAs used in polyacrylamide gel electrophoresis were determined from their sedimentation velocity as described by Boedtker (1968).

e ds-RNAs of CMV-S were coelectrophoresed with isolated RNA of the ds-RNA mycoviruses, PsV and PcV (Bozarth and Harley, 1976). Molecular weight estimates of the ds-RNAs of PsV and PcV were based on contour-lengths of these RNAs examined in the electron microscope.

estimates based on sedimentation analyses (e.g. Kaper and West, 1972; Kaper and Waterworth, 1973; Table 3.4).

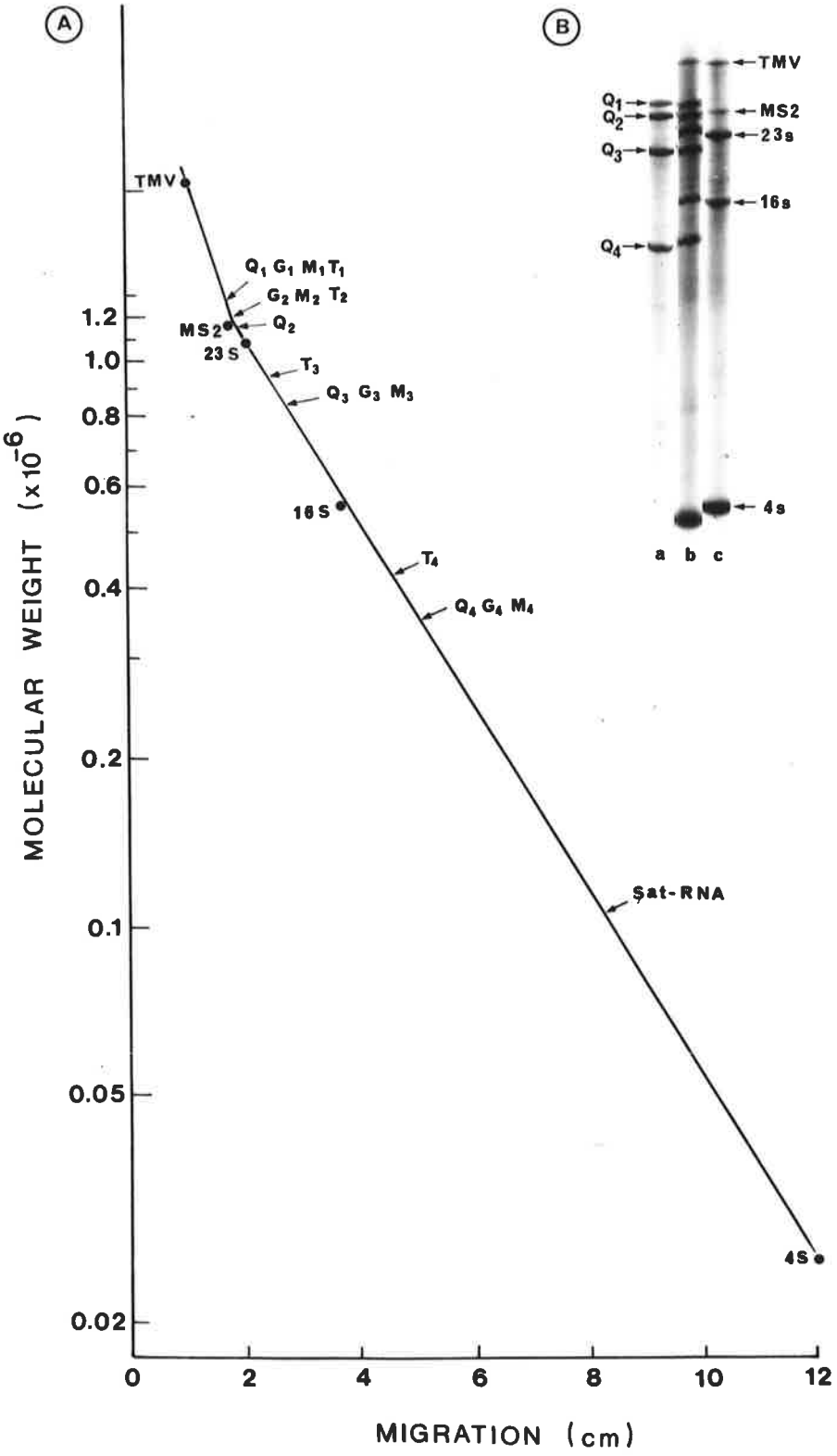
Electrophoresis of the RNA molecular weight markers, TMV-RNA, MS2-RNA, and *E. coli* 23S and 16S rRNAs and 4S tRNA in 4% polyacrylamide - formamide gels indicate that for RNAs with molecular weights in excess of approximately 1.2×10^6, the relationship between log molecular weight and electrophoretic mobility was no longer linear (Figure 3.7). Extrapolation from *E. coli* 23S and 16S rRNAs indicates a molecular weight values of 1.2×10^6 for MS2-RNA. Recently the sequence of the 3569 nucleotides in MS2-RNA has been determined (Fiers *et al.*, 1976), enabling a precise molecular weight calculation of 1.15×10^6.

Using TMV-RNA and *E. coli* rRNAs as internal markers, values for the molecular weights of RNAs 1, 2, 3 and 4 were found to be approximately 1.27, 1.13, 0.82 and 0.35×10^6 for QCMV, 1.27, 1.18, 0.82 and 0.35×10^6 for GCMV and MCMV, and 1.27, 1.18, 0.90 and 0.42×10^6 for VTAV respectively (Figure 3.7, Table 3.4). The molecular weight of Sat-RNA was found to be approximately 1.05×10^5 by interpolation from the electrophoretic mobilities of *E. coli* 23S and 16S rRNAs and 4S tRNA. When coelectrophoresed with QCMV-RNA, MS2-RNA migrated in a position intermediate between QCMV-RNAs 1 and 2. However, MS2-RNA could not be clearly resolved from RNA 2 of GCMV, MCMV or VTAV. The apparent discrepancy between molecular weight values for MS2-RNA estimated from the extrapolation of *E. coli* rRNAs (1.2×10^6, Figure 3.7), and the more accurate value calculated from its nucleotide sequence (1.15×10^6) suggests that the molecular weight values given for the RNA components of QCMV, MCMV and VTAV in the present work (Figure 3.7, Table 3.4) may slightly overestimate their real value. The presence of $CuCl_2$ (1.5 mM/mM nucleotide) in the RNA sample and

Figure 3.7. Molecular weight determination of the RNA component, of QCMV, GCMV, MCMV and VTAV by electrophoresis in 4% acrylamide-formamide gels.

A. Semi-logarithmic plot relating the electrophoretic mobilities of the cucumovirus RNA components with those of TMV-RNA, MS2-RNA, *E. coli* 23S and 16S rRNAs, and 4S tRNA. The molecular weight values used for the RNA standards were: TMV-RNA (2.05×10^6; mean of values obtained by Boedtker, 1968 and 1971), MS2-RNA (1.15×10^6; Fiers *et al.*, 1976), *E. coli* 23 S and 16S rRNAs (1.07 and 0.55×10^6 respectively; Stanley and Bock, 1965) and 4S tRNA (25,000, Buck and Ratti, 1977). The molecular weight values used for *E. coli* 23S and 16S rRNAs in this work are in reasonably close agreement with those obtained from their nucleotide sequences (Spohr *et al.*, 1976, and references therein).

B. Electrophoresis of QCMV-RNA in the presence and absence of TMV-RNA, *E. coli* 23S and 16S rRNA and 4S tRNA in 4% acrylamide-formamide gels. Conditions of electrophoresis were as described in Chapter 2. (a) QCMV-RNA alone, (b) QCMV-RNA and TMV-RNA, *E. coli* 23S and 16S rRNAs and tRNA, (c) TMV-RNA, MS2-RNA, *E. coli* 23S and 16S rRNAs and tRNA.


A
B
TMV
Q1 G1 M1 T1
G2 M2 T2
MS2
Q2
23 S
T3
Q3 G3 M3
16 S
T4
Q4 G4 M4
Sat-RNA
4S
MOLECULAR WEIGHT (x10⁻⁶)
1.2
1.0
0.8
0.6
0.4
0.2
0.1
0.05
0.02
0
2
4
6
8
10
12
MIGRATION (cm)
TMV
Q1
Q2
Q3
Q4
MS2
23s
16s
4s
a b c

electrophoresis buffers did not alter the relative mobilities of QCMV-RNAs 1 and 2, MS2-RNA, and *E. coli* 23S and 16S rRNA. It has been reported that GC-rich helical regions which may not be completely denatured in formamide at room temperature, are induced to do so in the presence of Cu(II) (Lehrach *et al.*, 1977).

Under no conditions of electrophoresis did CMV-RNA from any of the cucumoviruses examined migrate faster than *E. coli* 23S rRNA.

Conclusions

The physical and chemical properties of QCMV, GCMV, MCMV and VTAV have been compared, and although all four cucumovirus isolates share characteristics common to members of the cucumovirus group, they are readily distinguishable. Apart from QCMV and GCMV, all other isolates were readily distinguished serologically. There appears to be no serological relationship between VTAV and the three isolates of CMV. The amino acid composition of coat proteins from the four isolates are readily distinguishable. Electrophoresis of their RNAs under denaturing conditions indicate slight differences in the molecular weights of RNA components. In non-denaturing conditions, the differences in electrophoretic mobilities of their RNA components was even greater, enabling their direct identification. In addition to the four genomic RNAs, QCMV and GCMV also contained high levels of a low molecular weight RNA component, termed Sat-RNA.

In view of their distinguishable physical and chemical properties, and the distinctive symptoms they induce in a range of host species (Chapter 4), the four cucumovirus isolates examined appear to be potentially useful for genetic analyses.

CHAPTER 4

GENETIC ANALYSES OF PSEUDORECOMBINANTS CONTAINING RNA 3 FROM DIFFERENT CUCUMOVIRUS ISOLATES

Efforts to locate genes on cucumovirus RNAs 1, 2 and 3 involved in host symptom induction by the construction of pseudorecombinants have led to somewhat contradictory conclusions (see Chapter 1). In this chapter are described investigations of a method developed for the fractionation of cucumovirus genomic RNAs. Pseudorecombinants constructed from genomic RNAs 1+2 and RNA 3 from the four cucumoviruses described in the previous chapter have been used to locate genes determining the expression of certain host reactions including local lesion and systemic symptom development, and aphid transmissibility.

Development of a Procedure for the Preparative Fractionation of Cucumovirus Genomic RNAs

In preliminary experiments it was found that preparations of cucumovirus RNAs 1 and 2 recovered from polyacrylamide gels produced numerous local lesions in *Vigna sinensis* Endl. cv. Blackeye (cowpea), indicating they were contaminated with aggregates of RNA 3. To reduce the level of this contamination, and yet retain acceptable infectivity of mixtures of RNAs 1+2 and RNA 3, the effects of several denaturing agents on QCMV-RNA were investigated. Denaturing agents used to eliminate RNA secondary structure and disrupt RNA aggregates include formaldehyde (Boedtker, 1971), urea (Reijnders *et al.*, 1973; Mohier *et al.*, 1975) and 99% formamide (Pinder *et al.*, 1974). Mild heating followed by rapid cooling has also often been used to disrupt RNA aggregates prior to their

electrophoretic separation (e.g. Schwinghamer and Symons, 1975; Schuerch *et al.*, 1975). Formaldehyde was not investigated in these experiments as it causes hydroxymethylation of the nucleotide base (Palecek, 1976) which results in RNA inactivation (Franekel-Conrat, 1954).

The effects of heating in the presence and absence of urea on the fractionation and infectivity of QCMV-RNA were examined as follows. QCMV-RNA (100 µg/ml) was suspended in either TBE buffer, pH 8.3, alone or in TBE buffer containing 8M urea, pH 8.3. The RNA samples were then heated at either 75°C for 5 min or 90°C for 3 min and cooled rapidly in an ice-water bath. A sample of each RNA solution was left unheated as a control. After the addition of sodium acetate to 0.2M, RNA samples were precipitated with 2.5 volumes of ethanol and suspended in TE buffer to produce an RNA concentration of 100 µg/ml. Each sample was then inoculated to half-leaves of cowpea (Experiment 1, Table 4.1). In a parallel experiment (Experiment 2, Table 4.1), samples of QCMV-RNA in TBE buffer or TBE buffer containing 8M urea were heated as in Experiment 1. Sucrose was then added to 10% (w/v) and each sample was fractionated by a single-cycle of electrophoresis in 2.6% polyacrylamide gels. Bands corresponding to RNAs 1+2 and RNA 3 were located by staining, and the two RNA fractions eluted from homogenised gel slices as described in Chapter 2. RNAs 1+2 and RNA 3 of each treatment were resuspended in 200 µl TE buffer, and inoculated alone, or in a 1:1 (v/v) mixture on opposite half-leaves of cowpea.

The results of Experiment 1 (Table 4.1) indicate that heating in TBE buffer alone did not significantly affect the infectivity of QCMV-RNA. Moreover, preparations of RNAs 1+2 fractionated from RNA samples which had been heated prior to electrophoresis, were no longer infectious (Experiment 2), suggesting that aggregates containing RNA 3 which would ordinarily migrate in the same region as RNAs 1+2 had been dissociated by

Table 4.1. Effect of heat and 8M urea on the fractionation and infectivity of QCMV-RNA.

Treatment of RNA prior to inoculation (Experiment 1) or fractionation (Experiment 2)[a]	Mean number of lesions[b] Experiment 1 (unfractionated RNA)	Experiment 2 RNAs1+2	Experiment 2 RNAs1+2+3
TBE, unheated	152 (100%)[c]	4.0	15.5
TBE, heated 75°C for 5 min	155 (102%)	0.0	28.0
TBE, heated 90°C for 3 min	144 (95%)	0.0	19.0
Urea, unheated	33 (22%)	28.5	4.0
Urea, heated 75°C for 5 min	4 (3%)	0.0	0.0
Urea, heated 90°C for 3 min	4 (3%)	0.0	0.0

a QCMV-RNA at 100 µg/ml (Experiment 1) or samples of 100 µg of QCMV-RNA (Experiment 2) in TBE buffer alone, or TBE buffer containing 8M urea were heated as indicated, and either inoculated directly to half-leaves of cowpea (Experiment 1) or fractionated in 2.6% polyacrylamide gels as described in Chapter 2 and the purified RNA components assayed for infectivity on cowpea (Experiment 2).

b The infectivity of QCMV-RNA heated at the various conditions in TBE buffer alone, were compared on opposite half-leaves with that of the corresponding treatments of RNA suspended in TBE buffer containing 8M urea. Fractionated RNAs 1+2 and RNA 3 of each treatment in Experiment 2 were each suspended in 200 µl TE buffer. RNA components were inoculated to opposite half-leaves of cowpea, either undiluted alone, or as a 1:1 (v/v) mixture of RNAs 1+2 and RNA 3. Purified RNA 3 preparations of all treatments did not produce local lesions. In Experiment 1, lesion numbers represent the mean on 8 half-leaves, and in Experiment 2, the mean number on 4 opposite half-leaves.

c. Figures in parentheses represent the infectivity of QCMV-RNA relative to that of the unheated control in TBE buffer alone.

this treatment. Urea markedly reduced QCMV-RNA infectivity, particularly on heating. QCMV-RNA samples suspended in buffer containing 8M urea produced less distinct bands in polyacrylamide gels, and the presence of a number of additional bands near the bottom of these gels suggested significant RNA degradation had occurred. It seems unlikely that the presence of urea had exposed hidden 'nicks' in the QCMV--RNA preparation used in these experiments, since RNA heated in TBE buffer alone was highly infectious, and showed no evidence of the extensive degradation observed for RNA suspended in urea. In similar experiments, MCMV-RNA preparations incubated for 2 hr at 4°C in deionized formamide buffered with 20mM diethylbarbituric acid, pH 9.0, or dimethylsulphoxide were less than 15% as infectious as RNA preparations incubated in 20mM diethylbarbituric acid, pH 9.0, alone.

It is not known why these denaturing agents caused degradation or inactivation of cucumovirus RNAs. Since heating had no demonstrable effect on viral RNA infectivity, but did greatly reduce the residual infectivity of fractionated preparation of RNAs 1+2, this procedure was adopted for the routine fractionation of cucumovirus genomic RNAs.

Recovery and Specific Infectivity of Fractionated Genomic RNA of QCMV

To determine the yield of genomic RNAs fractionated by the two-step gel electrophoretic method and the effect of this procedure on the specific infectivity of the isolated RNA components, the following experiments were done. QCMV-RNA was fractionated by two-cycles of gel electrophoresis and the yields of RNAs 1+2, 3, 4 and Sat-RNA were determined spectrophotometrically. In addition, a sample of unfractionated QCMV-RNA was subjected to electrophoresis in 2% agarose gels and the proportion of

each RNA component was determined by integrating the areas under each RNA peak from densitometer traces as shown in Figure 5.5A. RNAs 1, 2, 3, 4 and Sat-RNA accounted for 11, 14.5, 22 and 28% respectively of the total RNA. The remainder, presumably RNA fragments and aggregates between RNA peaks, was not included in calculations of the yield or specific infectivity of the separated genomic RNAs. Of the 750 µg of QCMV-RNA fractionated by the two-step electrophoretic procedure, a total of 120 µg of RNAs 1+2, 3, 4 and Sat-RNA were recovered, representing a yield of approximately 27%. The yield of each RNA component was proportional to its contribution to the unfractionated QCMV-RNA preparation.

The infectivities of unfractionated QCMV-RNA, and mixtures of fractionated RNAs 1+2 and RNA 3 were compared on opposite half-leaves of cowpeas. The concentration of RNAs 1+2+3 in the unfractionated QCMV-RNA preparation was adjusted to 50 µg/ml in TE buffer. Fractionated preparations of RNAs 1+2 and RNA 3 were also adjusted to a total RNA concentration of 50 µg/ml such that the mixture contained approximately the same proportion of RNAs 1, 2 and 3 as the unfractionated QCMV-RNA preparation. The mean number of local lesions on 8 opposite half-leaves produced by the unfractionated QCMV-RNA preparation, and by the mixture of RNAs 1+2 and RNA 3 were 62 and 36 respectively. These results indicate that genomic RNAs of QCMV subjected to two-cycles of gel electrophoresis are approximately 60% as infectious as unfractionated RNA preparations. Electrophoretic elution of RNAs from polyacrylamide gels enables almost complete RNA recovery (Symons, 1978), however, the infectivity of QCMV-RNA was markedly reduced by this procedure (R.H. Symons, personal communication). It is possible that prolonged exposure to the photoreactive dye, toluidine blue, which was used to locate RNA components in this method,

may have resulted in RNA inactivation (Schuerch *et al.*, 1975). Moreover, electrophoretic elution involves a number of additional mechanical manipulations, thereby increasing the likelihood of the inadvertent introduction of RNases.

Although the yield of RNA recovered by the two-step gel electrophoretic procedure was only approximately 27%, the isolated RNA was largely intact and infectious. Inefficient elution of RNA from particles of acrylamide or agarose gels may account for their relatively low recovery by this method. In addition, movement of RNAs between the walls of the Plexiglass tubes and the acrylamide or agarose gel columns would also result in a loss of RNA.

Infectivity of Heterologous Mixtures of Genomic RNAs from Different Cucumoviruses

All heterologous mixtures consisting of RNAs 1+2 from one isolate and RNA 3 from another, produced local lesions on *Chenopodium amaranticolor* Coste & Reyn. (Table 4.2). Mixtures of QCMV-RNA 3 and VTAV-RNAs 1+2 produced visible local lesions in the most sensitive leaves only. It seems probable that the apparent inability of VTAV-RNA 3 to stimulate the infectivity of QCMV-RNAs 1+2 previously reported by Habili and Francki (1974c) may be attributed to difficulties in detecting local lesions induced by this particular heterologous RNA combination.

Construction and Characterization of Pseudorecombinants

Pseudorecombinants were successfully constructed from all combinations of RNAs 1+2 and RNA 3 of the four parental cucumoviruses

Table 4.2. Compatibility of cucumovirus genomic RNAs and role of RNA 3 in determining local lesion type of *C. amaranticolor*.

Source of RNAs 1+2[a]	QCMV	GCMV	MCMV	VTAV
QCMV	85[b] / 5.5 [c] ●	102 / 0.5 ◉	239 / 33 ○	160 / 18 [d] ●
GCMV	125 / 9 ●	105 / 0.5 ◉	286 / 11 ○	27 / 6 ●
MCMV	52 / 0.7 ●	287 / 21 ◉	65 / 0.8 ○	58 / 1.7 ●
VTAV	105 / 1.5 ●	126 / 2.7 ●	40 / 2.5 ●	76 / 3.5 ●

a RNAs 1+2 and RNA 3 were purified by two-cycles of gel electrophoresis as described in Chapter 2. Inocula contained either 30 µg/ml of RNAs 1+2 or 15 µg/ml each of RNAs 1+2 and RNA 3.

b Mean number of local lesions on 6 opposite half-leaves of *C. amaranticolor* of homologous or heterologous mixtures of RNAs 1+2 and RNA 3 (numerator) and RNAs 1+2 alone (denominator). Purified preparations of RNA 3 from all cucumoviruses were non-infectious.

c Lesion type induced:
- ● - very small, chlorotic (< 0.2 mm diameter where visible)
- ◉ - small, chlorotic with necrotic centre (≤ 0.3 mm diameter)
- ○ - intermediate, chlorotic with necrotic centre (c. 0.5 mm diameter)
- ◉ - large, chlorotic with necrotic centre (c. 1 mm diameter)

Lesions induced by QCMV and VTAV on opposite half-leaves were indistinguishable.

d Lesions induced by this genomic RNA combination were particularly small and visible on the most sensitive leaves only. Lesion numbers represent the mean on 3 opposite half-leaves only.

with the exception of those containing VTAV-RNA 3. Infectivity data (Table 4.2) indicate that VTAV-RNA 3 stimulates the infectivity of RNAs 1+2 from the 3 isolates of CMV. However, attempts to induce local lesions in tobacco or *C. quinoa* Willd. with mixtures of RNAs 1+2 from QCMV, GCMV and MCMV and VTAV-RNA 3 were unsuccessful. Moreover, attempts to recover these pseudorecombinants from lesions induced by genomic RNA mixtures on *C. amaranticolor* were also unsuccessful.

Local lesion isolates of the remaining pseudorecombinants were cloned by successive local lesion transfers on *N. tabacum* cv. White Burley and propagated in *N.* hybrid or *N. clevelandii*. Since the respective genomic RNAs of the parental strains are able to be identified by electrophoresis in 2% agarose gels (Figure 3.6), the genomic RNA composition of each pseudorecombinant could be verified directly. The nomenclature used throughout this thesis to identify pseudorecombinants is derived from the origin of their genomic RNAs. For example, the pseudorecombinant constructed from MCMV-RNAs 1+2 and QCMV-RNA 3 is referred to as $M_1M_2Q_3$.

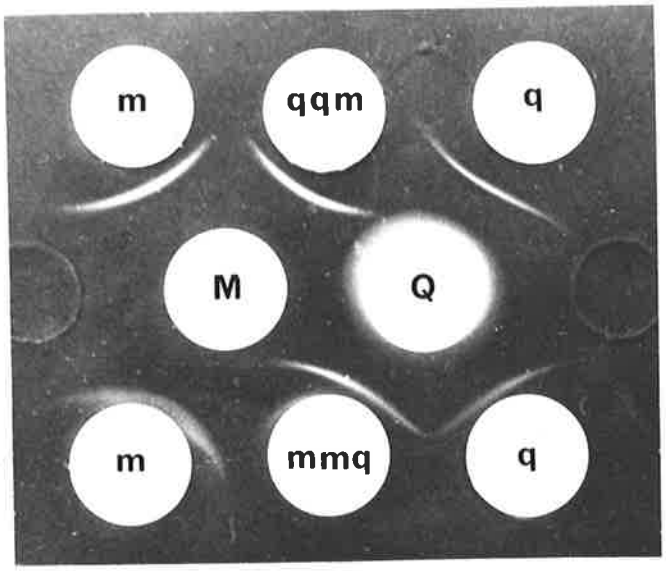
Results summarised in Figures 4.1-4.5 indicate that the pseudorecombinants constructed *in vitro* contain the expected genomic RNA composition. Of the cloned, local lesion isolates of each pseudorecombinant, a high proportion were subsequently shown to contain the expected genotype by electrophoretic analysis in 2% agarose gels. Furthermore, for those pseudorecombinants constructed from serologically distinguishable parental cucumoviruses, it was demonstrated that the pseudorecombinants are serologically identical to the parental strain providing RNA 3 (e.g. Figure 4.1A). These results confirm an earlier report by Habili and Francki (1974c) that genetic information determining cucumovirus coat protein is located on RNA 3.

Figure 4.1. Analysis of the RNA components and serological relationships of MCMV and QCMV and their pseudorecombinants.

A. Immunodiffusion test demonstrating antigenic identity of $Q_1Q_2M_3$ (qqm) with MCMV (m), and $M_1M_2Q_3$ (mmq) with QCMV (q). Antisera specific to MCMV (M) and QCMV (Q) with homologous titres of 1/4 and 1/8 respectively were prepared by absorption with heterologous antigens.

B. Electrophoretic analysis of the RNA component compositions of $M_1M_2Q_3$ and $Q_1Q_2M_3$ in 2% agarose gels.

(a)


m
qqm
q
M
Q
m
mmq
q

(b)

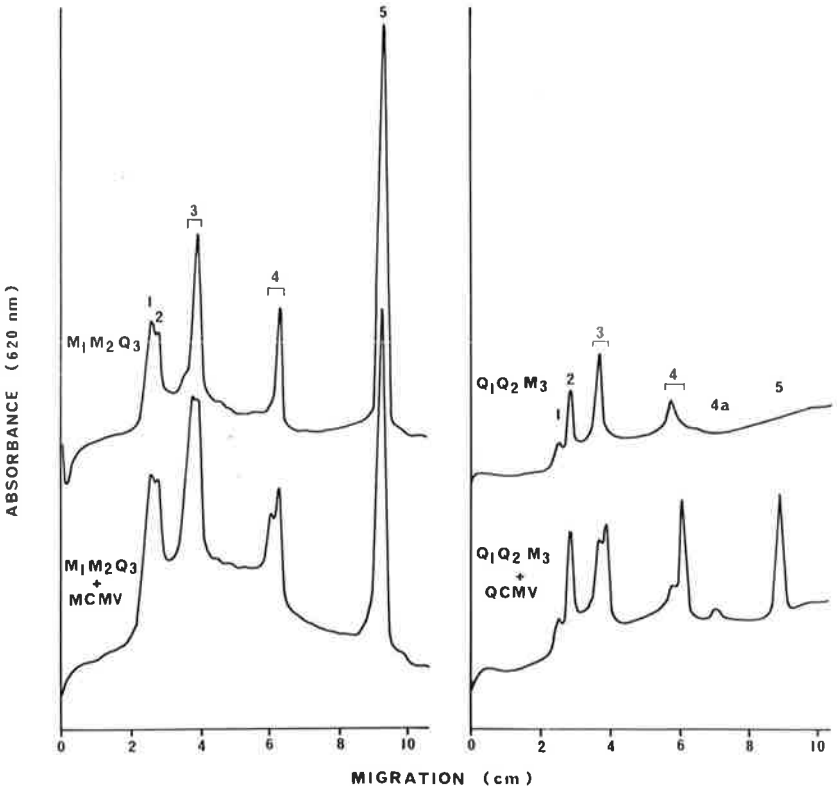

ABSORBANCE (620 nm)
M1M2Q3
M1M2Q3 + MCMV
Q1Q2M3
Q1Q2M3 + QCMV
1
2
3
4
4a
5
0
2
4
6
8
10
MIGRATION (cm)

Figure 4.2. Analysis of the RNA component of $T_1T_2M_3$ and $T_1T_2Q_3$ and their parental strains by electrophoresis in 2% agarose gels.

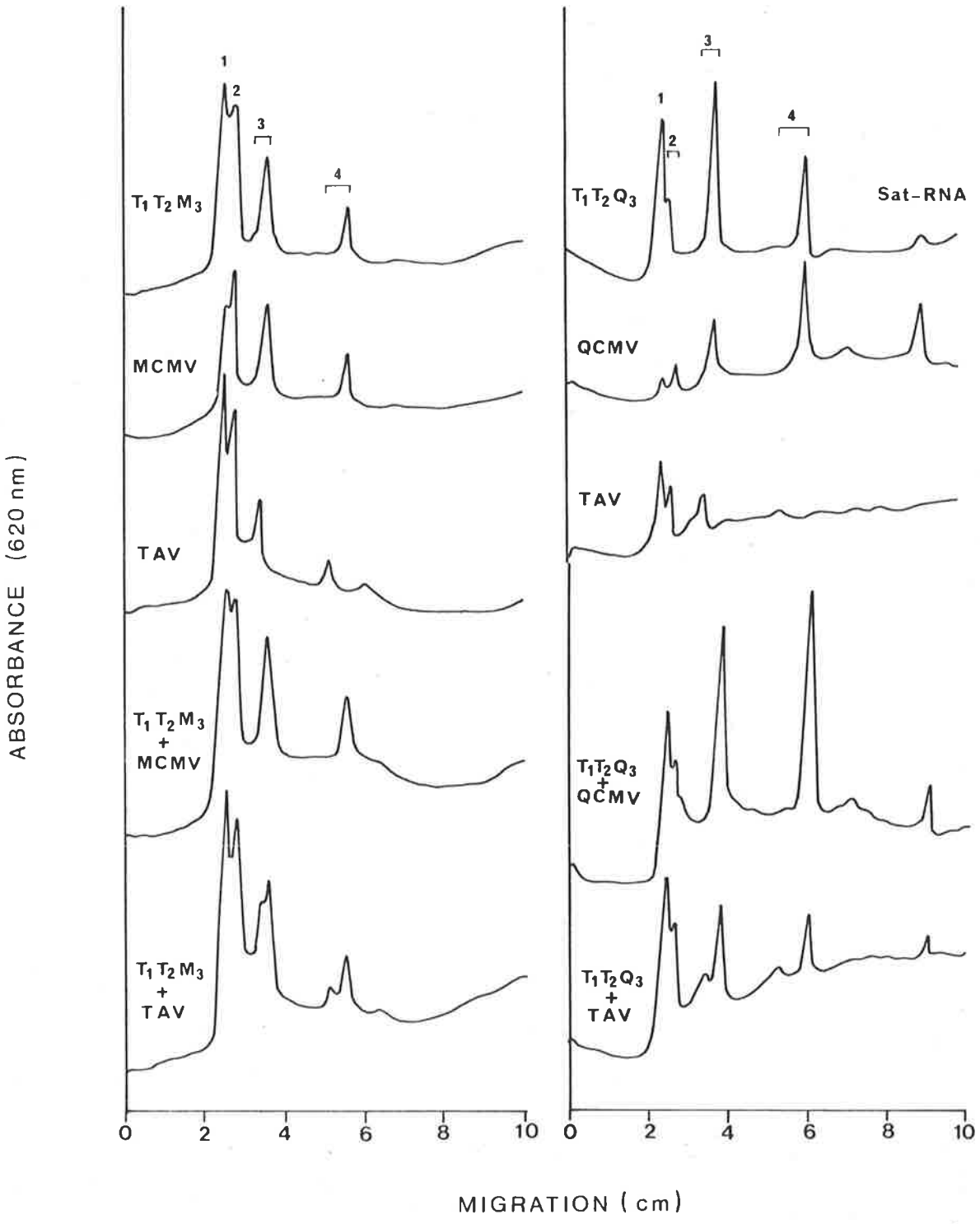

ABSORBANCE (620 nm)
1
2
3
4
T1 T2 M3
MCMV
TAV
T1 T2 M3 + MCMV
T1 T2 M3 + TAV
T1 T2 Q3
QCMV
TAV
T1T2Q3 + QCMV
T1 T2 Q3 + TAV
Sat-RNA
0
2
4
6
8
10
MIGRATION (cm)

Figure 4.3. Analysis of the RNA component composition of $M_1M_2G_3$ and $T_1T_2G_3$ and their parental strains by electrophoresis in 2% agarose gels.

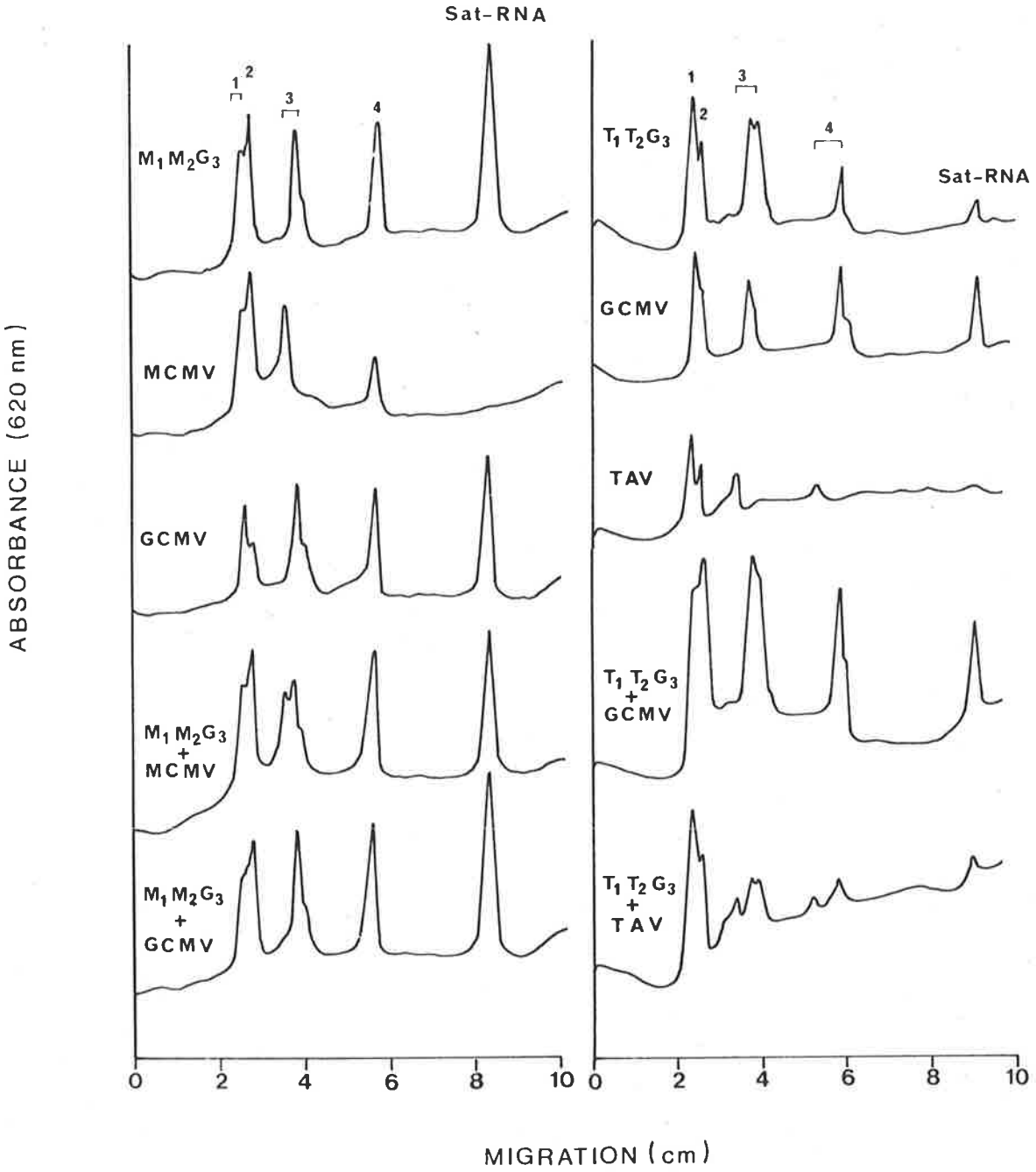

Sat-RNA
1
2
3
4
$M_1M_2G_3$
MCMV
GCMV
$M_1M_2G_3$ + MCMV
$M_1M_2G_3$ + GCMV
$T_1T_2G_3$
Sat-RNA
GCMV
TAV
$T_1T_2G_3$ + GCMV
$T_1T_2G_3$ + TAV
ABSORBANCE (620 nm)
0
2
4
6
8
10
MIGRATION (cm)

Figure 4.4. Analysis of the RNA component composition of $G_1G_2M_3$ and $G_1G_2Q_3$ and their parental strains by electrophoresis in 2% agarose gels.

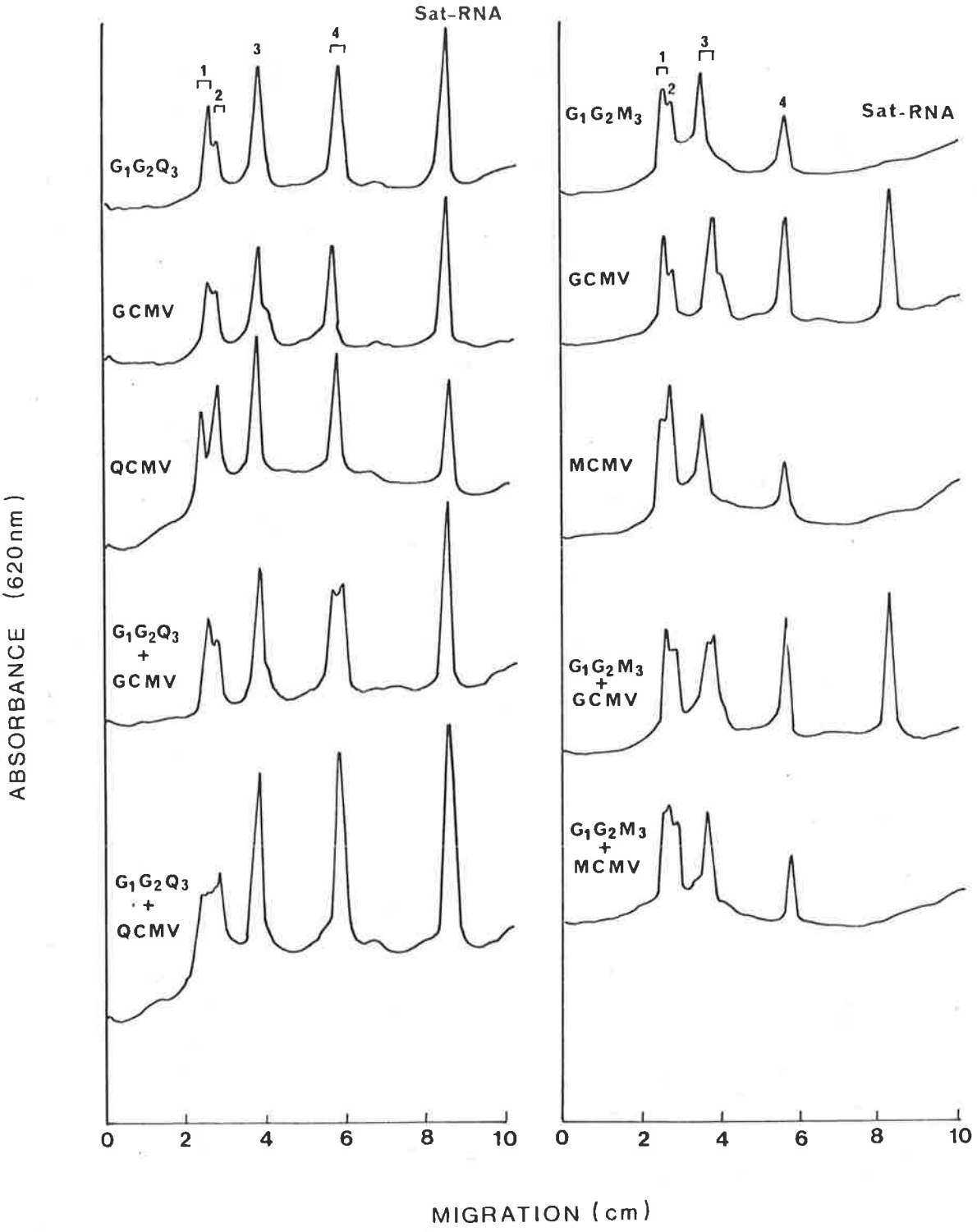
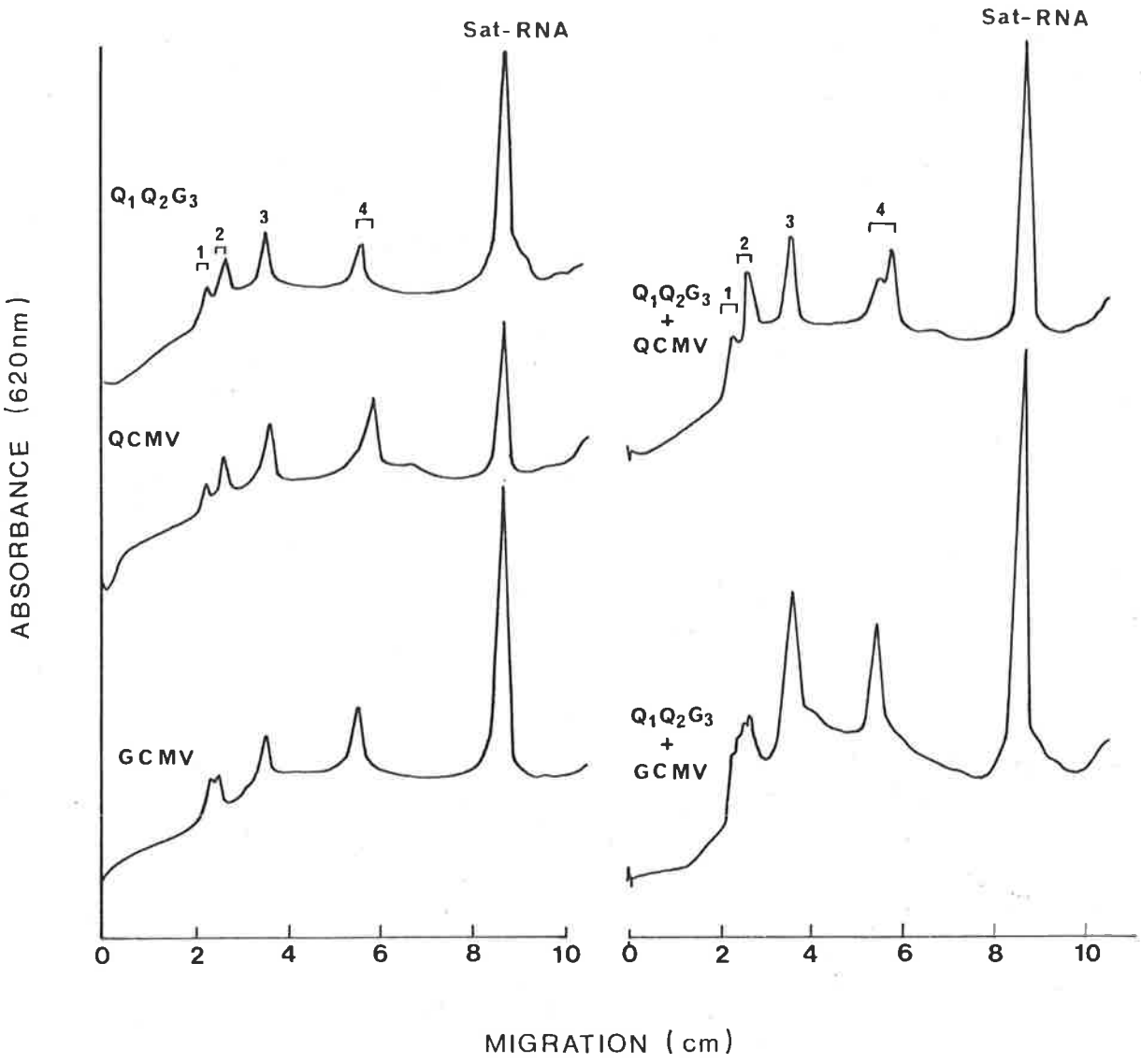

Sat-RNA
1
2
3
4
$G_1G_2Q_3$
GCMV
QCMV
$G_1G_2Q_3$ + GCMV
$G_1G_2Q_3$ + QCMV
$G_1G_2M_3$
Sat-RNA
GCMV
MCMV
$G_1G_2M_3$ + GCMV
$G_1G_2M_3$ + MCMV
0
2
4
6
8
10
ABSORBANCE (620nm)
MIGRATION (cm)

Figure 4.5. Analysis of the RNA component composition of $Q_1Q_2G_3$ and its parental strains by electrophoresis in 2% agarose gels.


Sat-RNA
Sat-RNA
$Q_1Q_2G_3$
1
2
3
4
$Q_1Q_2G_3$ + QCMV
QCMV
GCMV
$Q_1Q_2G_3$ + GCMV
ABSORBANCE (620nm)
0
2
4
6
8
10
MIGRATION (cm)

The electrophoretic mobility of RNA 4 of each pseudorecombinant was identical to that of the parental strain providing RNA 3, also confirming an earlier report that this RNA is derived from RNA 3 *in vivo* (Habili and Francki, 1974c). Subsequent work has shown that the nucleotide sequence of RNA 4 is entirely represented in RNA 3 (Gould and Symons, 1977), and that this RNA acts as a mRNA for viral coat protein in *in vitro* cell-free translation systems (Schwinghamer and Symons, 1977).

Association of Sat-RNA with Certain Pseudorecombinants

It is interesting to note that pseudorecombinants contained detectable levels of Sat-RNA when first isolated only if their RNA 3 originated from QCMV or GCMV. That is, the pseudorecombinants $M_1M_2Q_3$, $M_1M_2G_3$, $T_1T_2Q_3$, $T_1T_2G_3$, $G_1G_2Q_3$ and $Q_1Q_2G_3$ contained detectable levels of Sat-RNA, whereas $Q_1Q_2M_3$, $G_1G_2M_3$ and $T_1T_2M_3$ did not (Figures 4.1-4.5). These preliminary observations suggested that Sat-RNA may be generated *de novo* by specific fragmentation of RNA 3 from QCMV and GCMV. In this respect, it was initially considered that Sat-RNA may be a defective RNA, similar to those reported in a number of animal viruses (Huang, 1973).

An investigation of the base sequence homology between Sat-RNA and genomic RNAs of QCMV (Gould *et al.*, 1978) demonstrated that there were no detectable nucleotide sequences common to Sat-RNA and QCMV-RNAs 1-5 (Table 4.3). These results eliminate the possibility that Sat-RNA is derived from QCMV-RNA 3 *in vivo*, and conclusively demonstrate the satellite nature of Sat-RNA. Hybridization of GCMV-RNA 3 with cDNA(Sat-RNA) to a $R_o t$ value which would enable the detection of one or more copies of Sat-RNA per RNA 3 molecule, failed to indicate the presence of sequences of Sat-RNA (Table 4.3). Although these results do not eliminate the possibility that Sat-RNA is derived from sequences

Table 4.3. Determinations of the sequence homology between Sat-RNA and QCMV-RNAs 1-5 and GCMV-RNA 3[a].

RNA hybridized[b]	$R_o t_{½}$	Hybridization[c]
QCMV-RNA 1	-	1.8
QCMV-RNA 2	-	2.1
QCMV-RNA 3	-	4.6
QCMV-RNA 5	-	4.7
GCMV-RNA 3	-	4.1
Sat-RNA	1.0×10^{-3}	100.0

a Hybridization data for QCMV-RNAs 1-5 and Sat-RNA are taken from Gould *et al.* (1978), Table 2.

b RNAs purified by two-cycles of polyacrylamide slab gel electrophoresis (Gould *et al.*, 1978) were hybridized with [^{32}P]cDNA(Sat-RNA). The isolate of QCMV used in these hybridizations (Gould *et al.*, 1978; Isolate A) did not contain Sat-RNA. GCMV-RNA 3 was purified by two-cycles of gel electrophoresis as described in Chapter 2. QCMV-RNA 4 was not hybridized with cDNA (Sat-RNA) in these experiments as the nucleotide sequence of this RNA is entirely represented in QCMV-RNA 3 (Gould and Symons, 1977).

c All RNAs except GCMV-RNA 3 were hybridized to a $R_o t$ value of 0.1 mol sec litre^{-1}. GCMV-RNA 3 was hybridized to a $R_o t$ value of 0.035 mol sec litre^{-1}. Values are expressed relative to the hybridization of Sat-RNA with cDNA (Sat-RNA) which was taken as 100% and had been corrected for the S_1 nuclease-resistant hybridization value of 4% obtained for this cDNA preparation in the absence of added RNA.

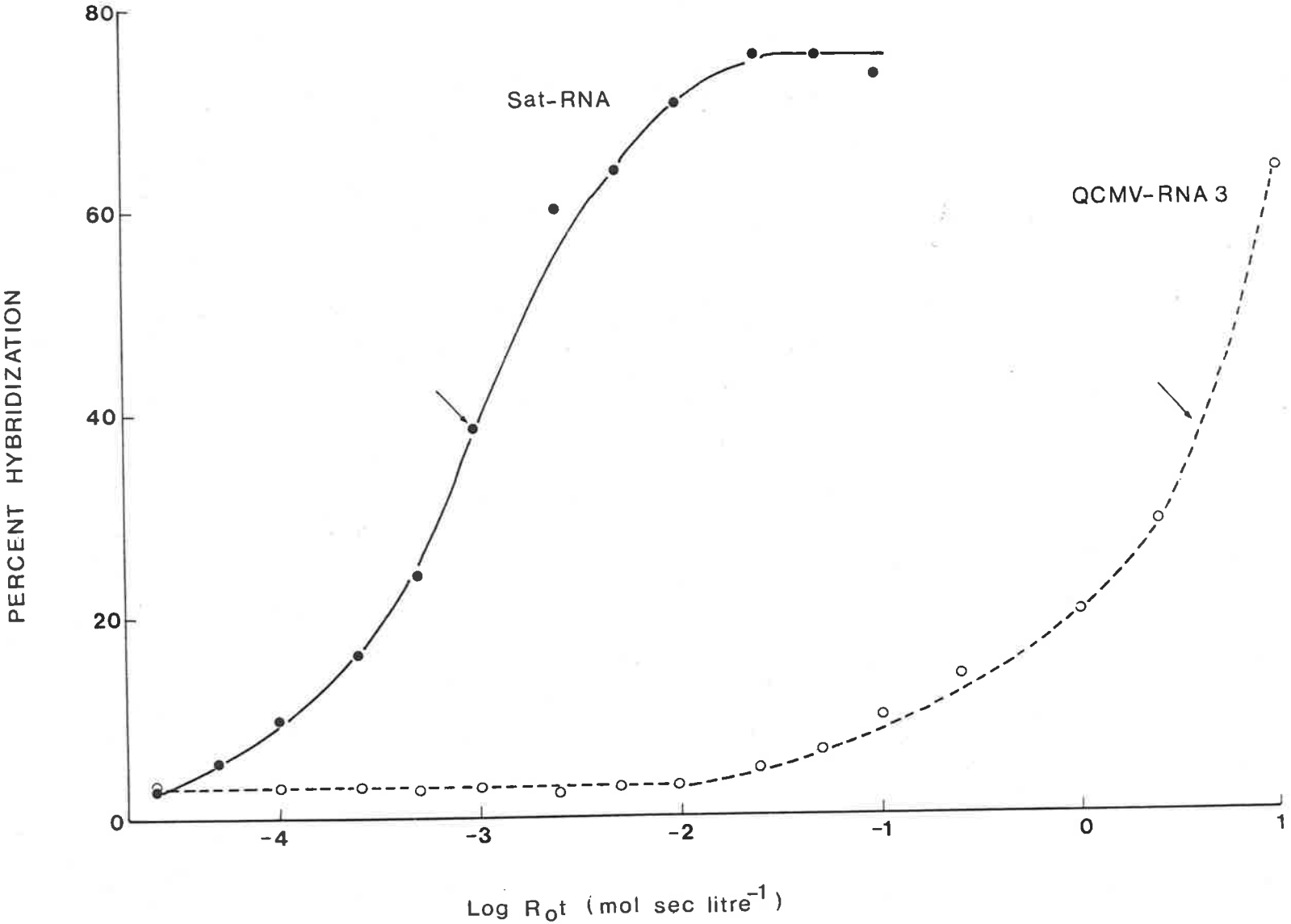
located on GCMV-RNAs 1 or 2, the pattern of inheritance of Sat-RNA by pseudorecombinants suggests that like QCMV, GCMV and Sat-RNA have few, if any, common nucleotide sequences.

Hybridization of [^{32}P]cDNA (Sat-RNA) with a preparation of QCMV-RNA 3 which had been used to construct pseudorecombinants in these studies, indicated that at high concentrations of RNA 3, sequences of Sat-RNA were detected (Figure 4.6). A comparison of the observed homologous and heterologous $R_o t_{\frac{1}{2}}$ values (1.0×10^{-3} and 5.6 mol sec litre^{-1} respectively) indicated that this preparation of QCMV-RNA 3 was contaminated with Sat-RNA to approximately 0.13% on a molar basis (Gould *et al.*, 1978). In view of the extraordinarily high specific infectivity of Sat-RNA (Table 5.5), it seems reasonable to conclude that the presence of Sat-RNA in pseudorecombinants constructed from RNA 3 isolated from both QCMV and GCMV was due to the contamination of these RNA 3 preparations with Sat-RNA.

Biological Properties of Pseudorecombinants

Pseudorecombinants constructed *in vitro* using RNAs 1+2 and RNA 3 from distinguishable parental viruses provide a means of investigating the role of the various genomic RNAs in determining biological and chemical properties of cucumoviruses, and for investigating the interaction between heterologous combinations of RNAs 1+2 and RNA 3. RNAs 1+2 of MCMV, GCMV and VTAV could not be completely resolved using the preparative electrophoretic method described in Chapter 2. Hence no attempt was made to construct pseudorecombinants in which RNAs 1 and 2 originated from different parental cucumoviruses.

Figure 4.6. Determination of the level of contamination of purified QCMV-RNA3 with Sat-RNA. QCMV-RNA3 had been purified by two cycles of gel electrophoresis. Conditions of hybridization and the assay of hybrid formation using S_1 nuclease were as described in Chapter 2. The $R_o t_{\frac{1}{2}}$ values (arrowed) for the homologous and heterologous hybridizations were 1.0×10^{-3} and 5.6 mol sec litre^{-1} respectively. Data from Gould *et al.* (1978), Figure 8. [^{32}P]cDNA(Sat-RNA) hybridized with, ● - Sat-RNA, o - QCMV-RNA 3.


80
60
40
20
0
PERCENT HYBRIDIZATION
Sat-RNA
QCMV-RNA 3
-4
-3
-2
-1
0
1
Log R0t (mol sec litre-1)

The role of the respective genomic RNAs in determining local lesion type was investigated in two ways; (1) heterologous mixtures of RNAs 1+2 and RNA 3 were inoculated on half-leaves opposite those which had been inoculated with RNAs 1 + 2 alone, as in Table 4.2, and (2) nucleoprotein preparations of cloned pseudorecombinants were inoculated on half-leaves opposite those which had been inoculated with the appropriate parental virus.

A summary of the local lesion types induced in *C. amaranticolor* by parental cucumoviruses and heterologous mixtures of their genomic RNAs is presented in Table 4.2. Mixtures of RNAs 1+2 and RNA 3 from the three isolates of CMV indicate that the predominant local lesion types induced are indistinguishable from those induced by the parental isolate of the CMV providing RNA 3 (Table 4.2, Figure 4.7). In contrast, lesions produced by heterologous mixtures of RNAs 1+2 from VTAV and RNA 3 from QCMV, GCMV or MCMV are indistinguishable from those produced by VTAV-RNA (Figures 4.8 and 4.9). Lesions induced by mixtures of RNAs 1+2 from MCMV or GCMV and RNA 3 from VTAV were also indistinguishable from those induced by VTAV (Figures 4.8 and 4.9) while those produced by mixtures of QCMV-RNA 1+2 and VTAV-RNA 3 were usually smaller and less distinct (Table 4.2, Figure 4.8).

The determinant for local lesion type on cowpea is apparently located in RNA 1 and/or RNA 2, since lesions produced on this host species by MCMV and $M_1M_2Q_3$ are indistinguishable (Figure 4.9c). Lesions induced by purified preparations of these two isolates are readily distinguishable from the smaller lesion types induced by QCMV (Figure 4.9d).

Local lesion development in *Nicotiana* species, like that in *C. amaranticolor*, appears to involve complex interactions between

Figure 4.7. Local lesions produced on *C. amaranticolor* inoculated with heterologous mixtures of genomic RNAs from QCMV, MCMV and GCMV (a) QCMV-RNAs 1+2 (left) and QCMV-RNAs 1+2 and GCMV-RNA 3 (right). Arrow indicates a local lesion induced by QCMV. (b) GCMV-RNAs 1+2 (left) and GCMV-RNAs 1+2 and QCMV-RNA 3 (right). Arrow indicates a local lesion induced by GCMV. (c) MCMV-RNAs 1+2 (left) and MCMV-RNAs 1+2 and GCMV-RNA 3 (right). Arrow indicates a lesion induced by MCMV. (d) GCMV-RNAs 1+2 (left) and GCMV-RNAs 1+2 and MCMV-RNA 3 (right). Arrows indicate lesions induced by GCMV. Inoculum concentrations were as described in Table 4.2. Plants were maintained in a growth room at 25^{o}C with continuous illumination of 5,000 lux.

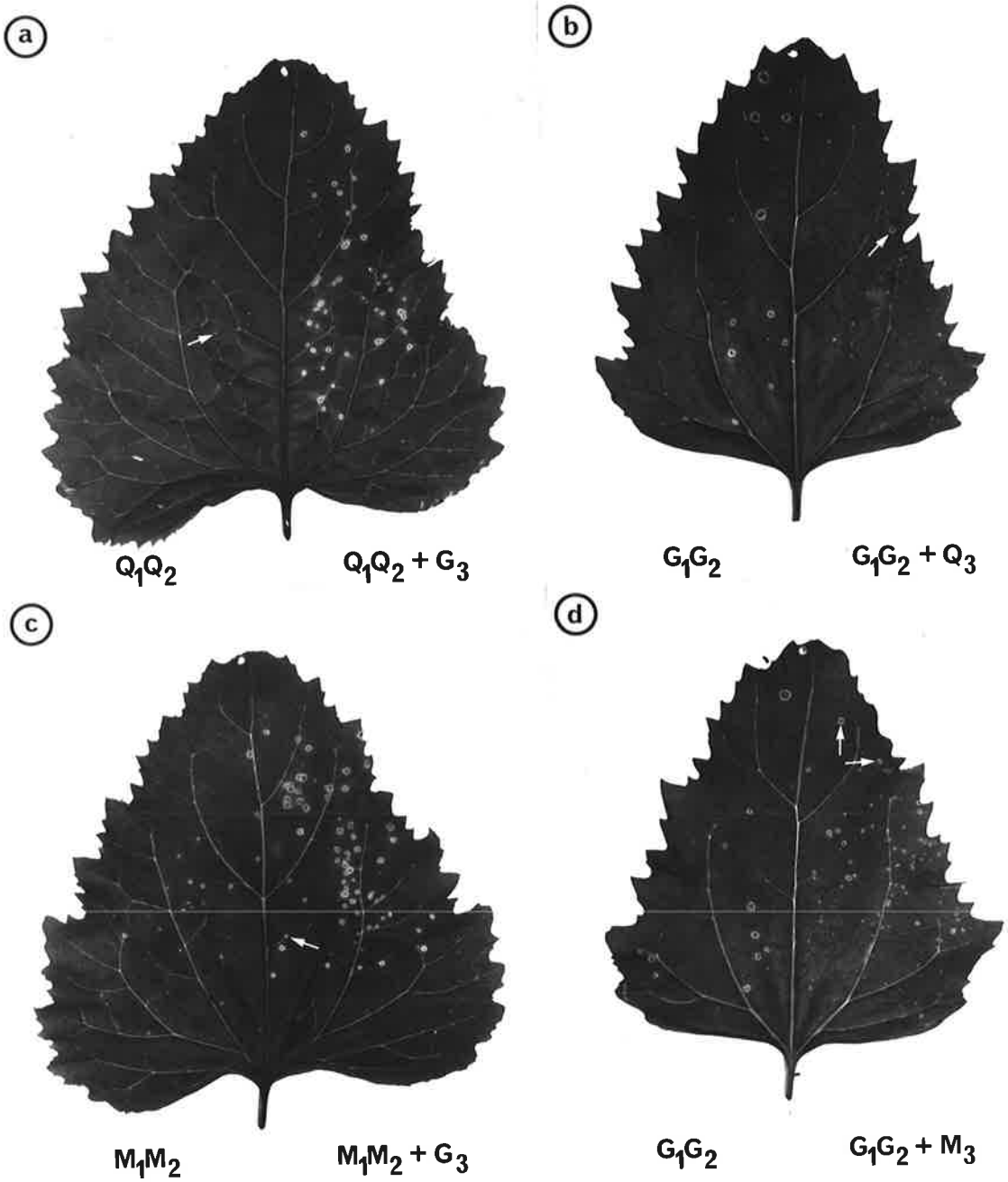

a
b
c
d
Q_1Q_2
$Q_1Q_2 + G_3$
G_1G_2
$G_1G_2 + Q_3$
M_1M_2
$M_1M_2 + G_3$
G_1G_2
$G_1G_2 + M_3$

Figure 4.8. Local lesions produced on *C. amaranticolor* inoculated with heterologous mixtures of genomic RNAs from QCMV, GCMV and VTAV. (a) VTAV-RNAs 1+2 (left), and VTAV-RNAs 1+2 and QCMV-RNA 3 (right). (b) QCMV-RNAs 1+2 (left) and QCMV-RNAs 1+2 and VTAV-RNA 3 (right). (c) GCMV-RNAs 1+2 (left) and GCMV-RNAs 1+2 and VTAV-RNA 3 (right). (d) VTAV-RNAs 1+2 and GCMV-RNA 3 (left) and VTAV-RNAs 1+2 (right). Inoculum concentrations and propagation conditions were as in Table 4.2 and Figure 4.7.

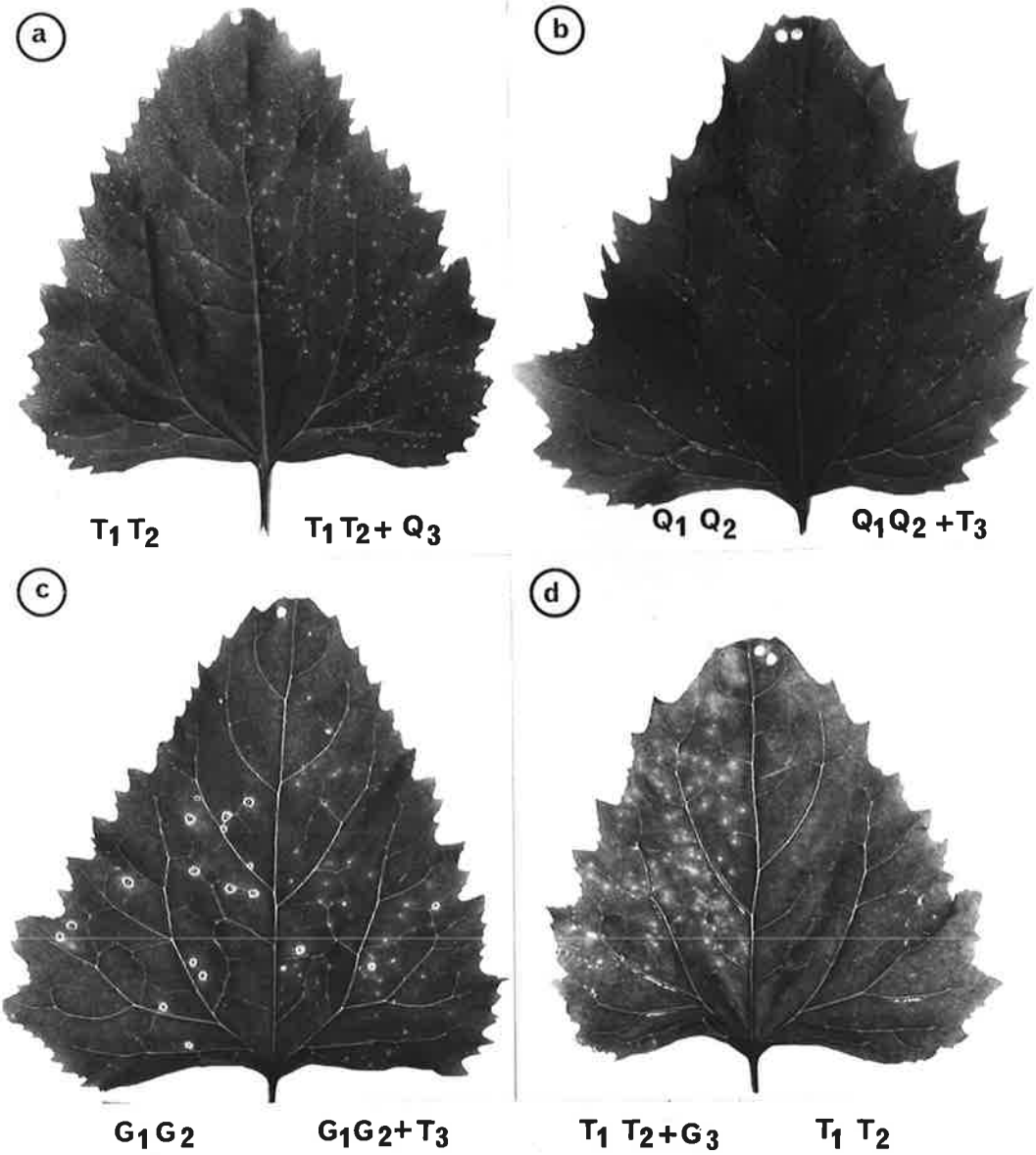
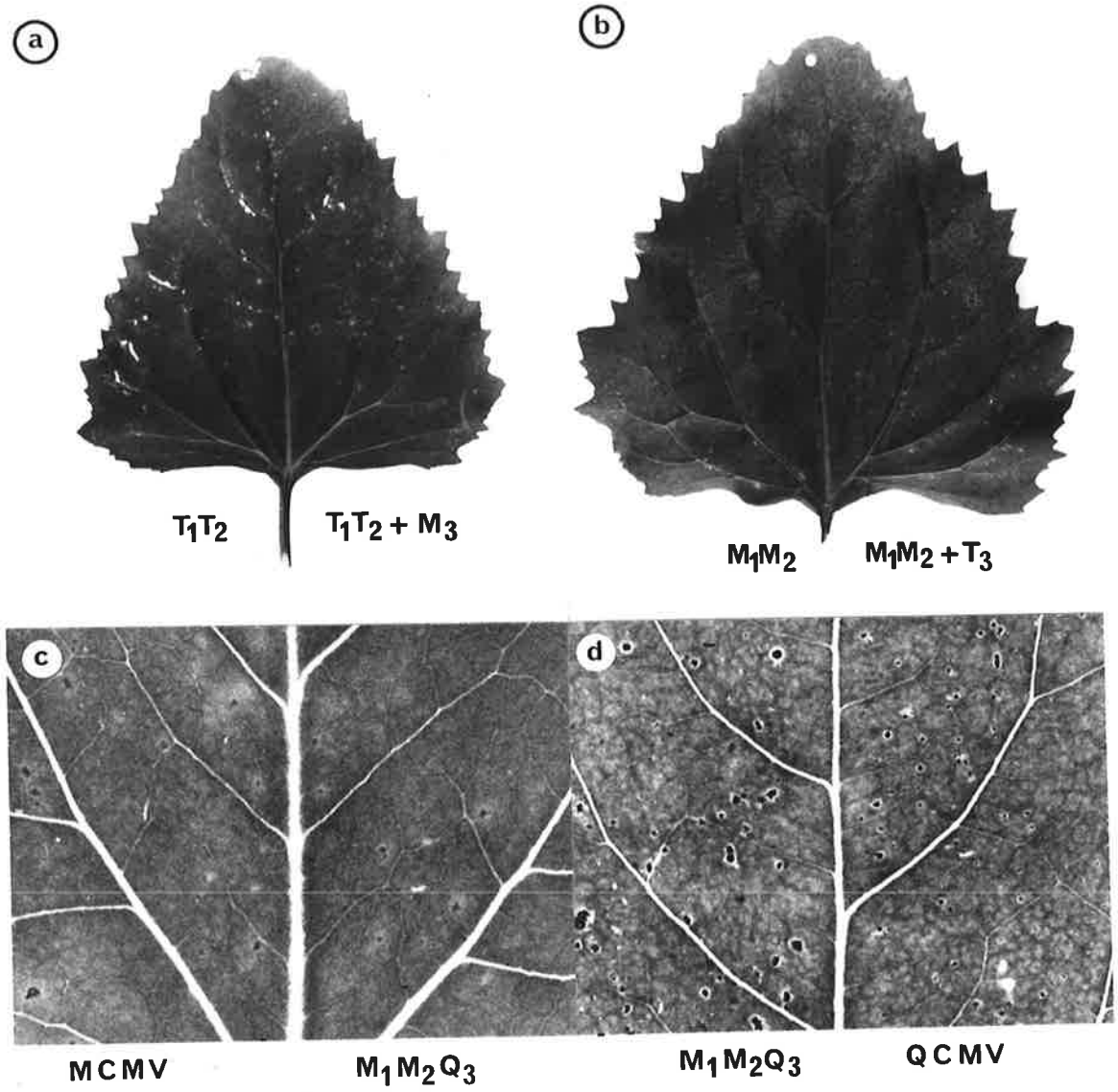

a
b
$T_1 T_2$
$T_1 T_2 + Q_3$
$Q_1 Q_2$
$Q_1 Q_2 + T_3$
c
d
$G_1 G_2$
$G_1 G_2 + T_3$
$T_1 T_2 + G_3$
$T_1 T_2$

Figure 4.9. Local lesions produced on *C. amaranticolor* by heterologous mixtures of genomic RNAs from VTAV and MCMV (a and b), and lesions produced on 'Blackeye' cowpea by MCMV, QCMV and $M_1M_2Q_3$ (c and d). (a) VTAV-RNAs 1+2 (left) and VTAV-RNAs 1+2 and MCMV-RNA 3 (right). (b) MCMV-RNAs 1+2 (left) and MCMV-RNAs 1+2 and VTAV-RNA 3 (right). Lesions induced on opposite half-leaves of cowpea inoculated with (c) MCMV (left) and $M_1M_2Q_3$ (right) and (d) $M_1M_2Q_3$ (left) and QCMV (right). Concentrations of genomic RNAs in (a) and (b) were as in Table 4.2. In (c) and (d), opposite half-leaves were inoculated with purified virus preparations at a concentration of 10 µg/ml. Propagative conditions were as in Figure 4.7.


a
b
T_1T_2
$T_1T_2 + M_3$
M_1M_2
$M_1M_2 + T_3$
c
d
MCMV
$M_1M_2Q_3$
$M_1M_2Q_3$
QCMV

cucumovirus genomic RNAs. GCMV and VTAV both produce irregular, spreading necrotic local lesions in *N. clevelandii*, *N. tabacum* cv. White Burley and *N.* hybrid (Figures 4.10 and 4.11). Pseudorecombinants containing GCMV-RNA 3, $T_1T_2G_3$, $M_1M_2G_3$ and $Q_1Q_2G_3$ (Figures 4.12, 4.13 and 4.14, respectively) also induce necrotic local lesions in these hosts, suggesting that the gene determining this characteristic is located on GCMV-RNA 3 alone. In contrast, $T_1T_2Q_3$, $T_1T_2G_3$ and $T_1T_2M_3$ induce necrotic local lesions in these *Nicotiana* species similar to those produced by VTAV (Figures 4.12 and 4.16 respectively).

Hence, it appears that the size of local lesions produced on cowpea is controlled by gene(s) on RNA 1 and/or RNA 2. In contrast, local lesion development in *C. amaranticolor* and *Nicotiana* species apparently involves a complex interaction of genetic information located on RNA 1 and/or RNA 2, as well as on RNA 3.

Systemic symptoms produced by the four parental cucumoviruses and their pseudorecombinants in several host species are presented in Figures 4.10 - 4.16 and summarized in Table 4.4. Symptoms produced by QCMV and GCMV in all hosts examined were mild and not readily distinguished. In contrast, MCMV produces a brilliant yellow mosaic symptom in all *Nicotiana* species examined (Figures 3.1 and 4.11). VTAV produces a severe mosaic symptom in *Nicotiana* species readily distinguishable from symptoms induced by the other parental isolates. In addition, VTAV unlike QCMV, GCMV or MCMV, is unable to infect *Cucumis sativus* L. (Table 4.4). Of the four parental isolates, MCMV and VTAV appeared more suitable for genetic analysis of the determinants responsible for systemic symptoms in cucumoviruses.

Figure 4.10. Symptoms produced in four *Nicotiana* species infected with QCMV (left column) and GCMV (right column) 12 days after inoculation. Host species in rows from top to bottom are *N. glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Plants were inoculated with nucleoprotein preparations of each isolate at a concentration of 100 µg/ml and maintained in a growth room at 25°C with continuous illumination of 5,000 lux.

QCMV GCMV

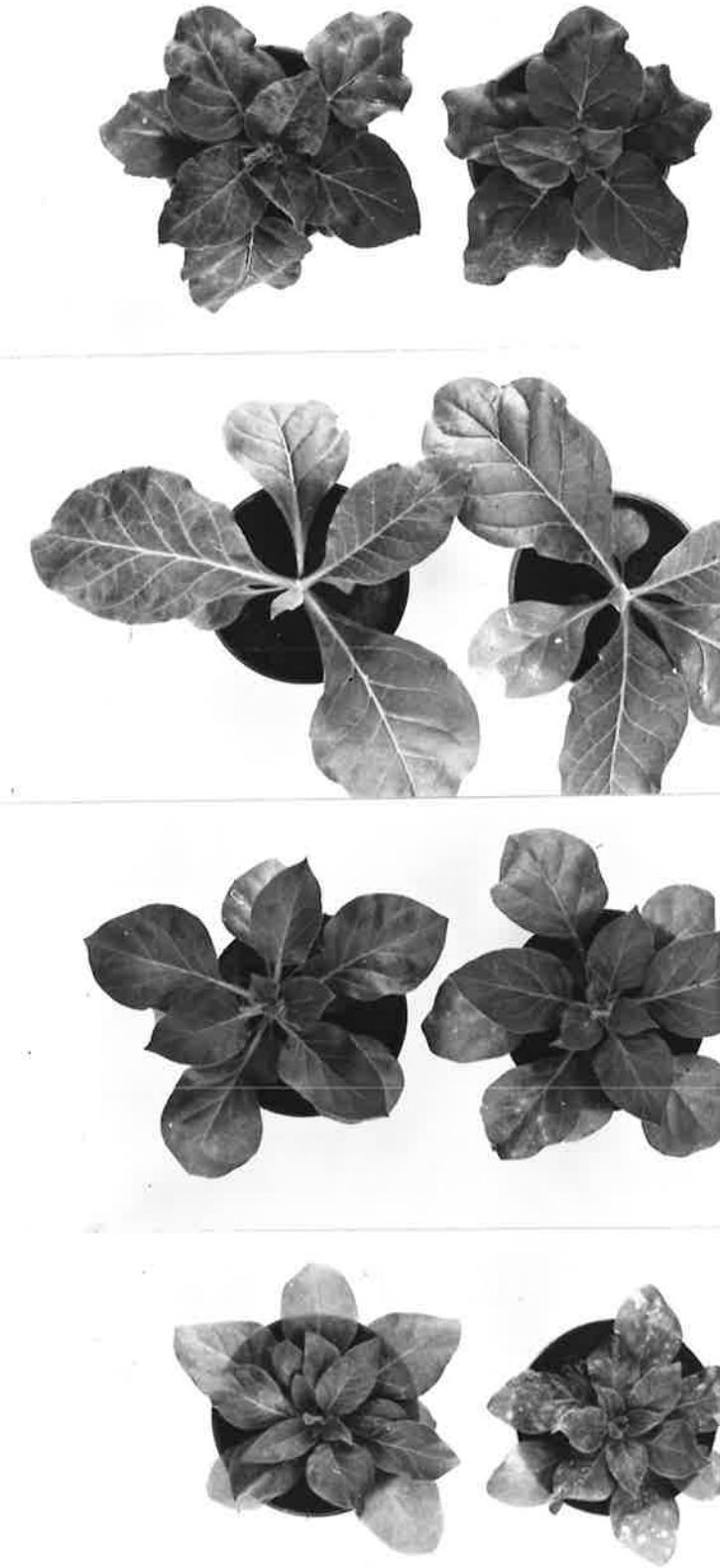


Figure 4.11. Symptoms produced in four *Nicotiana* species infected with MCMV (left column) and VTAV (right column) 12 days after inoculation. Host species in rows from top to bottom are *N.glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Inoculations and growth conditions were as described in Figure 4.10.

MCMV VTAV

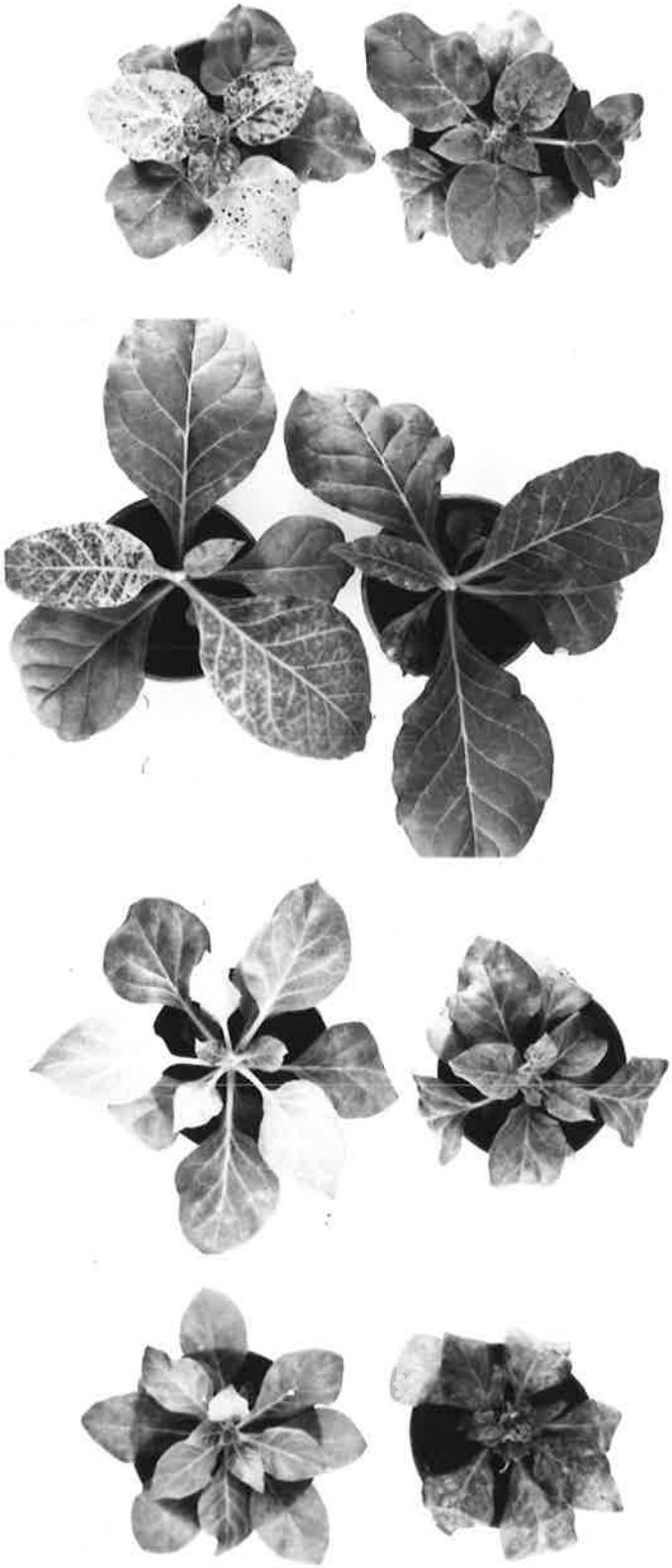


Figure 4.12. Symptoms produced in four *Nicotiana* species infected with $T_1T_2Q_3$ (left column) and $T_1T_2G_3$ (right column) 12 days after inoculation. Host species in rows from top to bottom are *N. glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Inoculations and growth conditions were as described in Figure 4.10.

$T_1T_2Q_3$ $T_1T_2G_3$

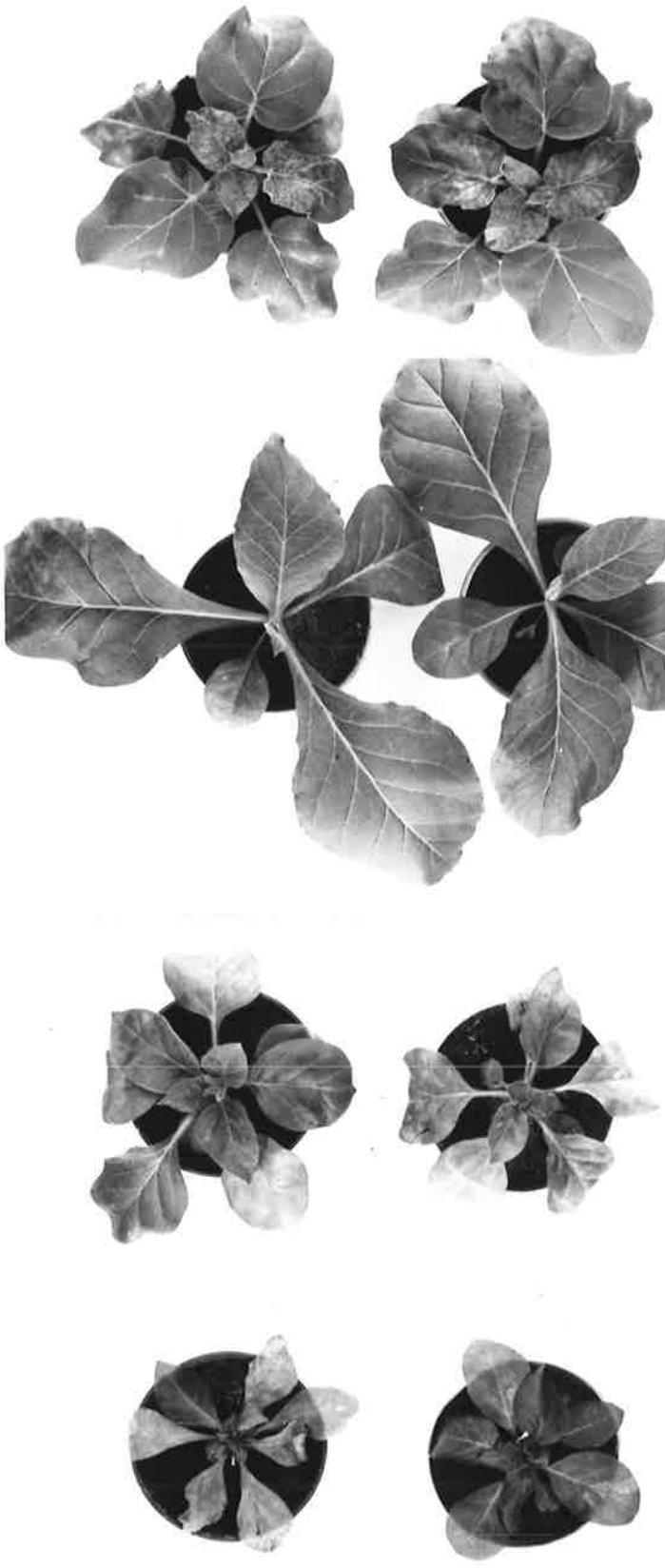


Figure 4.13. Symptoms produced in four *Nicotiana* species infected with $M_1M_2G_3$ (Left column) and $M_1M_2Q_3$ (right column) 12 days after inoculation. Host species in rows from top to bottom are *N. glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Inoculations and growth conditions were as described in Figure 4.10.

$M_1M_2G_3$

$M_1M_2Q_3$

Figure 4.14. Symptoms produced in four *Nicotiana* species infected with $Q_1Q_2M_3$ (left column) and $Q_1Q_2G_3$ (right column) 12 days after inoculation. Host species in rows from top to bottom are *N. glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Plants were inoculated with isolated RNA of each pseudorecombinant at a concentration of 200 µg/ml and maintained under the growth conditions described in Figure 4.10.

$Q_1Q_2M_3$ $Q_1Q_2G_3$

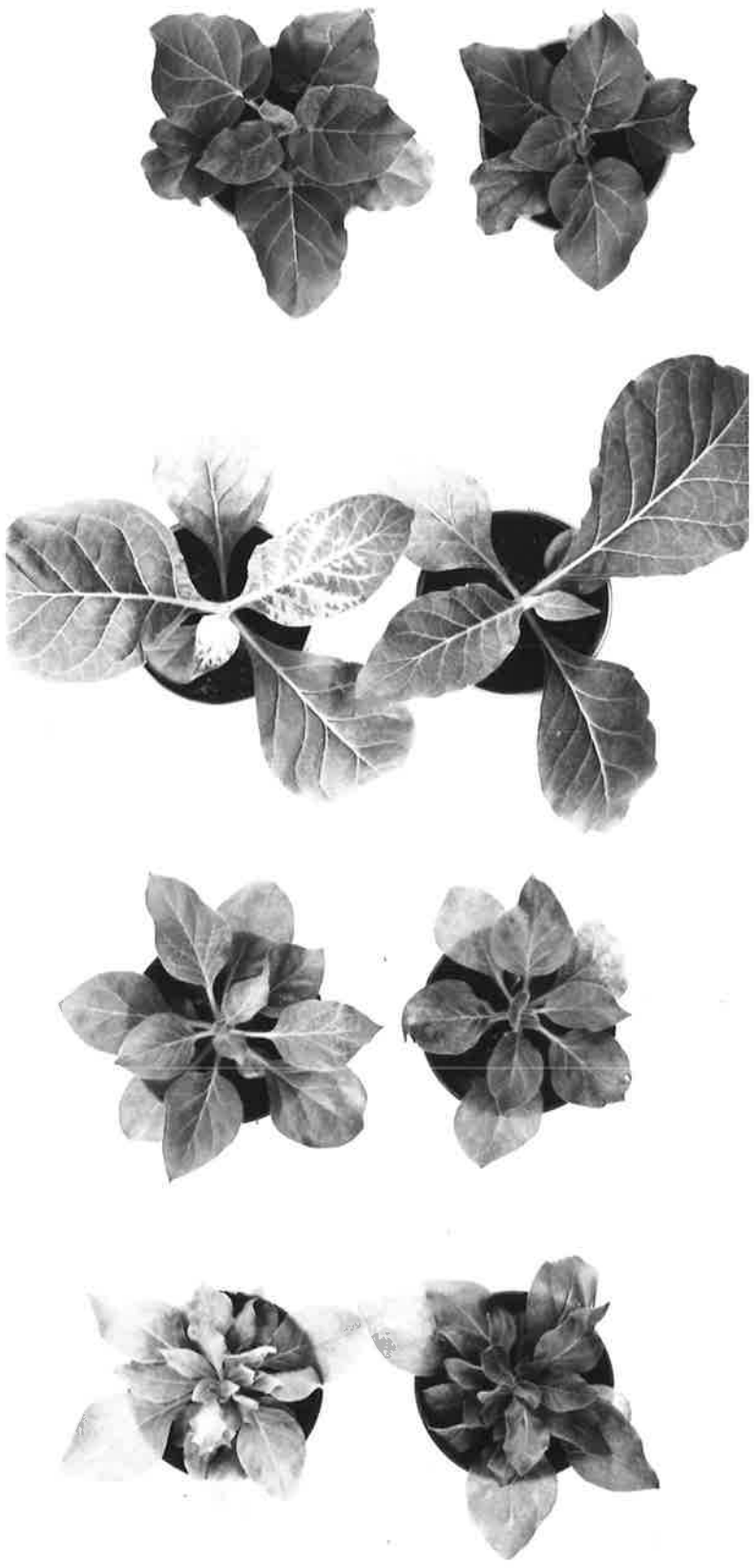


Figure 4.15. Symptoms produced in four *Nicotiana* species infected with $G_1G_2M_3$ (left column) and $G_1G_2O_3$ (right column) 12 days after inoculation. Host species in rows from top to bottom are *N. glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Inoculations and growth conditions were as described in Figure 4.10.

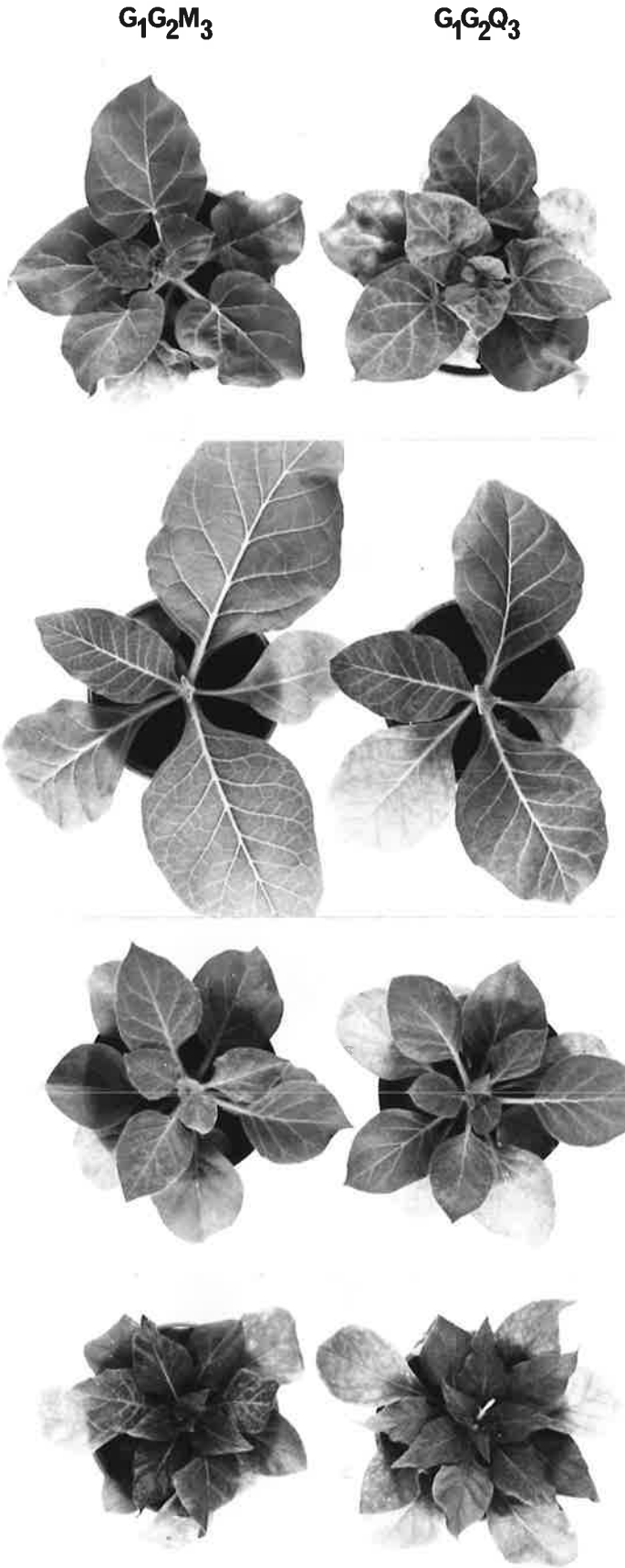

$G_1G_2M_3$
$G_1G_2Q_3$

Figure 4.16. Symptoms produced in four *Nicotiana* species infected with $T_1T_2M_3$ 12 days after inoculation. Host species from top to bottom are *N. glutinosa*, *N. tabacum* cv. White Burley, *N.* hybrid and *N. clevelandii* respectively. Inoculations and growth conditions were as described in Figure 4.10.

$T_1 T_2 M_3$

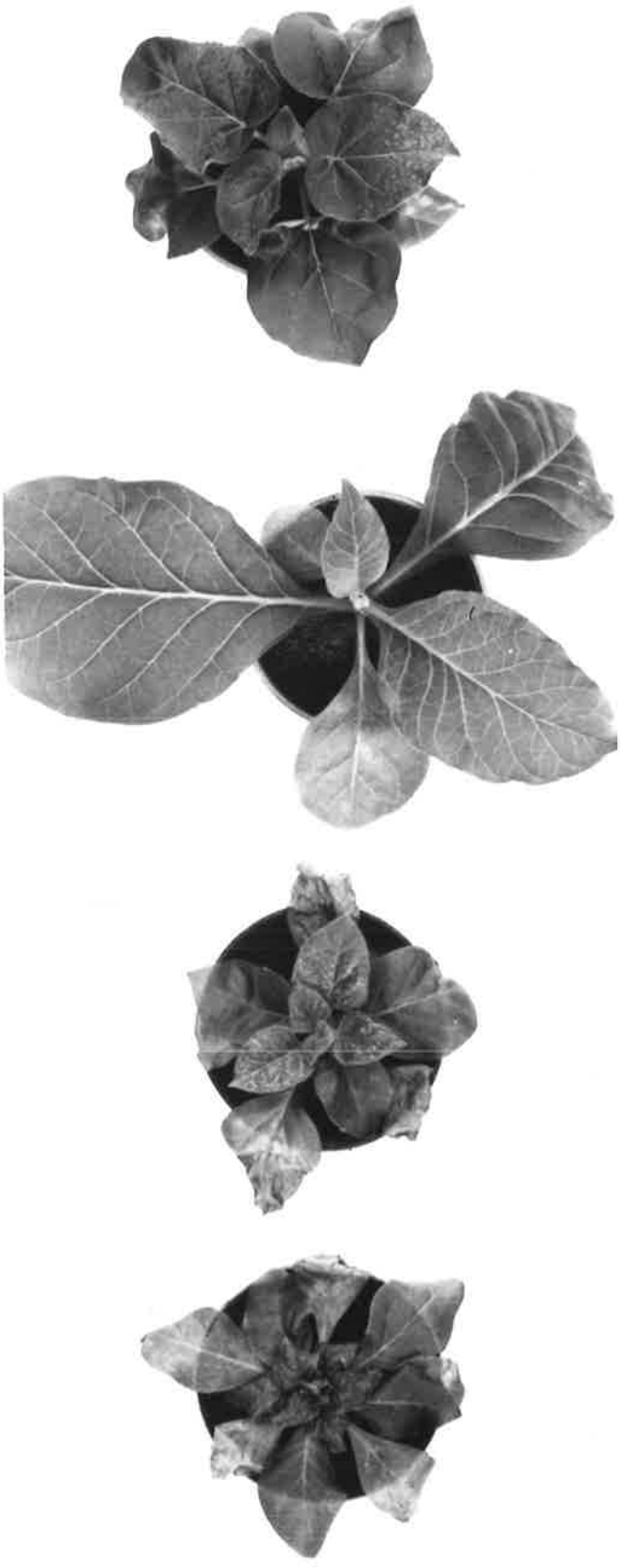


Table 4.4. Summary of symptoms produced in several host species by the four parental cucumoviruses and their pseudorecombinants.

Host	Virus[a]												
	QCMV	GCMV	MCMV[b]	VTAV[b]	$Q_1Q_2M_3$	$Q_1Q_2G_3$	$M_1M_2Q_3$	$M_1M_2G_3$	$G_1G_2Q_3$	$G_1G_2M_3$	$T_1T_2Q_3$	$T_1T_2G_3$	$T_1T_2M_3$
N.glutinosa	mos.	mild mos.	yellow mos.,dist.	severe mos.,dist.	mos.	mild mos.	mos.,sys. nec.spots	blister mottle, pale chlor.	mos	mild mos.	severe mos.,sys. nec.	severe mos.,sys nec.	mos.
N.clevelandii	mild mos.	irreg. nec. ll., mild mos.	yellow mos., dist.	irreg. nec. ll., severe mos., dist.	V.chlor. becoming general in younger leaves	irreg. nec. ll., mild mos.	mos.	irreg. nec.ll., blister-ing and iv.chlor.	mild mos.	pale V. chlor.	irreg.nec. ll.severe mos.,leaf dist.	irreg.nec. ll.severe mos.,leaf dist.	irreg.nec. ll.,pale V.chlor., leaf dist.
N. hybrid	mild mos.	irreg. nec. ll., mild mos.	yellow mos., dist	irreg. nec. ll., severe mos., dist.	pale V. chlor. becoming general in younger leaves	irreg. nec. ll., mild mos.	iv.chloro., mild mos.	irreg. nec. ll., patches of iv.chlor. and blistering	mild mos.	pale V. chlor.	irreg.nec. ll.severe mos.	irreg.nec. ll., mos.	irreg.nec. ll., V. chlor., leaf dist.
N.tabacum cv White Burley[c]	mild mos.	mild mos.	yellow mos., dist.	severe mos., dist.	V.chloro. becoming general in younger leaves	mild mos.	pale iv. chlor., mild mos.	iv.chlor. some blistering	mild mos.	pale V. chlor.	mild mos.	mild mos.	pale V. chlor.
C. sativus Polaris	d	d	d	-[e]	d	d	d	d	d	d	-[e]	-[e]	-[e]

a Plants were inoculated with purified nucleoprotein or isolated RNA preparations at concentrations of 100 or 200 µg/ml respectively, and maintained in a growth room at 25°C with continuous illumination of 5,000 lux.

b Leaf blistering frequently developed in *Nicotiana* species infected with these strains 2-3 weeks after inoculation. Under these propagative conditions plant growth is often greatly reduced and newly-formed leaves infected with MCMV, although severely distorted, may not exhibit the characteristic yellow mosaic symptoms (see Figure 3.1).

c All viruses produced faint chlorotic local lesions in tobacco (White Burley) 2-3 days after inoculation.

d Pale chlorotic local lesions produced on cucumber cotyledons 3-5 days after inoculation, and all inoculated plants develop a systemic mosaic symptom.

e Virus could not be recovered from inoculated leaves or newly-formed lesions.

Legend mos. - mosaic; dist. - leaf distortion; sys. - systemic; nec. - necrotic; chlor. - chlorosis; ll. - local lesions; V - veinal; iv. - interveinal; irreg. - irregular, spreading.

$Q_1Q_2M_3$, $G_1G_2M_3$ and $T_1T_2M_3$ all produce mild chlorosis in *Nicotiana* species (Figures 4.14, 4.15 and 4.16 respectively). However, in each case the chlorotic symptoms produced were less pronounced than those produced by MCMV, and except in *N. glutinosa*, the chlorosis was normally restricted to regions bordering leaf veins. The development of veinal chlorosis by $G_1G_2M_3$ and $T_1T_2M_3$ in *N. tabacum* cv. White Burley was not readily seen in systemically infected leaves until approximately 15 days or more after inoculation. In contrast, $M_1M_2G_3$ and $M_1M_2Q_3$ produce interveinal chlorosis, and slight blistering and distortion in systemically infected leaves (Figure 4.13). The chlorosis was consistently more pronounced in *Nicotiana* species infected with $M_1M_2G_3$ than those infected with $M_1M_2Q_3$. Moreover, *N. glutinosa* plants infected with $M_1M_2Q_3$ often developed systemic necrotic spots.

It appears that the brilliantyellow mosaic symptom produced by MCMV in *Nicotiana* species may be the result of two genes; one located on RNA 3 inducing veinal chlorosis, and one located on RNAs 1 and/or 2 inducing interveinal chlorosis, leaf blistering and distortion.

Symptoms produced by $T_1T_2Q_3$, $T_1T_2G_3$ and $T_1T_2M_3$ (Figures 4.12 and 4.16 respectively) were generally more severe than those produced by the parental CMV isolate providing RNA 3. In this respect, symptoms produced by these three pseudorecombinants more closely resemble those induced by the parental isolate, VTAV. A notable exception was the relatively mild symptoms induced by $T_1T_2Q_3$, $T_1T_2G_3$ and $T_1T_2M_3$ in *N. tabacum* cv. White Burley. RNA 3 of QCMV or GCMV did not appear to contribute any genetic information toward symptoms produced by $T_1T_2Q_3$ or $T_1T_2G_3$ respectively. The most reasonable explanation for these observations is that the relatively mild symptoms produced by QCMV and GCMV would be masked by the severe mosaic and leaf distortion

symptoms determined by genetic information located on VTAV-RNAs 1 and/or 2.

Furthermore, $T_1T_2Q_3$, $T_1T_2G_3$ and $T_1T_2M_3$, like VTAV, did not produce local lesions or systemic symptoms on *C. sativus* (Table 4.4). These results suggest that the ability of cucumovirus to infect this host is determined by genetic information located on RNA 1 and/or RNA 2. However, it is possible that genes determining the local lesion type and systemic symptoms induced by cucumoviruses on this host may be different from that which specifies the 'ability to infect *C. sativus*'.

While these investigations were in progress, Kaper and Waterworth (1977) demonstrated that a satellite RNA associated with CMV (CARNA 5) modified symptoms produced by CMV in *Lycopersicon esculentum* Mill. cv. Rutgers (tomato) and *N. tabacum* cv. Xanthi nc. Since Sat-RNA and CARNA 5 share a number of physical and chemical properties (Chapters 5 and 6), it is possible that the presence of Sat-RNA in certain pseudorecombinants introduced another element into the complex interaction of cucumovirus genomic RNAs leading to symptom expression.

Association of RNA 3 with the Aphid Transmission of CMV

Preliminary investigations in this laboratory had established that whereas QCMV is readily transmitted by a number of aphid species including *Myzus persicae* (Sulz.), MCMV is not (R.I.B. Francki, personal communication). Since the pseudorecombinants $M_1M_2Q_3$ and $Q_1Q_2M_3$ had been previously constructed from genomic RNAs of QCMV and MCMV (Figure 4.1) it was therefore possible to investigate the role of RNA 3 in the aphid transmission of CMV.

Results summarized in Table 4.5 demonstrate that $Q_1Q_2M_3$, like MCMV, was not transmitted by *M. persicae*, whereas $M_1M_2Q_3$ was transmitted with apparently even greater frequency than QCMV. The 10 *N. glutinosa* plants infected with $M_1M_2Q_3$ following single-aphid transmissions in Experiments 4 and 5 (Table 4.5) were used to inoculate a series of *N.* hybrid plants. Purified virus of each isolate was shown by immunodiffusion tests to be serologically identical to QCMV, and by agarose gel electrophoresis to have RNA composition indistinguishable from that of the $M_1M_2Q_3$ isolate infecting *N. clevelandii* plants used for virus acquisition. These results demonstrate that neither RNA 1 nor RNA 2 contribute any genetic information necessary for rendering CMV aphid transmissible.

RNA preparations from the isolates of MCMV and $Q_1Q_2M_3$ used in these experiments, unlike those of QCMV and $M_1M_2Q_3$, did not contain detectable levels of Sat-RNA (Figures 3.5 and 4.1). Since the nucleotide sequence of RNA 4 is entirely represented in RNA 3 of CMV (Gould and Symons, 1977), only RNA 3 and Sat-RNA have to be considered as being potentially involved in aphid transmission.

To investigate the role of Sat-RNA in the aphid transmission of CMV, the following experiment was done. Sat-RNA, purified from a preparation of QCMV by two-cycles of gel electrophoresis was artificially introduced into an isolate of MCMV by inoculating *N. clevelandii* plants with a mixture of MCMV-RNA and Sat-RNA at 100 and 2.5 µg/ml respectively. Apterous *M. persicae* were allowed to probe briefly on detached leaves of *N. clevelandii* systemically infected with MCMV, MCMV containing Sat-RNA or $M_1M_2Q_3$ 12 days after the plants had been inoculated, and transferred in groups of 3 aphids per plant to 10 *N. glutinosa* seedlings.

Table 4.5. Transmission of QCMV,MCMV and their pseudorecombinants by *Myzus persicae* (Sulz.).

Virus	Transmission[a] Experiments 1	2	3	4	5	Total
QCMV	1/20[b]	-	0/20	-	3/30	4/70 (5.7)
MCMV	0/20	-	0/20	-	-	0/40 (0)
$Q_1Q_2M_3$	-	0/20	0/20	0/20	0/30	0/90 (0)
$M_1M_2Q_3$	3/20	2/20	-	5/20	5/30	15/90 (16.7)

a Apterae of *M. persicae* were fasted for 2-4 hr and then allowed to probe for 30-40 sec on detached, systemically infected, young leaves of *N. clevelandii* 10-12 days after inoculation. The aphids were transferred, one to each young *N. glutinosa* seedling, which were sprayed with insecticide on the following day.

b Numerator indicates the number of plants developing virus symptoms within 3 weeks and the denominator the number of seedlings exposed to aphids.

c Figures in parentheses indicate percentage of plants infected.

MCMV in the presence or absence of Sat-RNA was not transmitted by *M. persicae*, whereas 3 of the 10 *N. glutinosa* test plants became infected with $M_1M_2Q_3$. Agarose gel electrophoresis of RNA isolated from purified virus preparations from the *N. clevelandii* plants infected with MCMV and MCMV containing Sat-RNA used in this experiment for virus acquisition, demonstrated that whereas MCMV did not contain a detectable amount of Sat-RNA, the latter contained a level of Sat-RNA comparable to that present in RNA preparations of $M_1M_2Q_3$ (Figure 4.1B). Thesè results indicate that Sat-RNA does not provide any genetic information necessary for the aphid transmission of CMV.

Conclusions

The results presented here enable the following conclusions to be drawn.

1. Heterologous mixtures of genomic RNAs 1+2 and RNA 3 from the four parental cucumoviruses all produce numerous local lesions in *C. amaranticolor* indicating that viable pseudorecombinants had been formed *in vivo* with all possible genomic RNA combinations.

2. Genetic analysis using heterologous genomic RNA mixtures and pseudorecombinants indicate that local lesion type induced by strains of CMV in *C. amaranticolor* and *Nicotiana* species is determined by RNA 3, whereas lesion type in *V. sinensis* is controlled by genetic information on RNA 1 and/or RNA 2. In contrast, local lesion development in *C. amaranticolor* and *Nicotiana* species by VTAV and pseudorecombinants containing VTAV-RNAs 1+2 is apparently determined by gene(s) located on RNA 1 and/or RNA 2.

3. The ability of cucumoviruses to infect *C. sativus* is controlled by RNA 1 and/or RNA 2.

4. Systemic symptom expression, like local lesion development, appears to involve interactions between genomic RNAs 1 and/or 2 and RNA 3.

5. Aphid transmission of cucumoviruses is determined by genetic information located on RNA 3 alone. The most reasonable explanation for the results presented in Table 4.5 is that the aphid transmissiblity of CMV is determined by properties of the viral coat protein.

6. Possible interactions between cucumovirus genomic RNAs, the host species and Sat-RNA present in certain pseudorecombinants may further complicate interpretations of the genetic analyses described in this chapter. Biological properties of Sat-RNA, and its effect on symptoms induced by cucumoviruses in a range of plant hosts are described in the following chapter.

CHAPTER 5

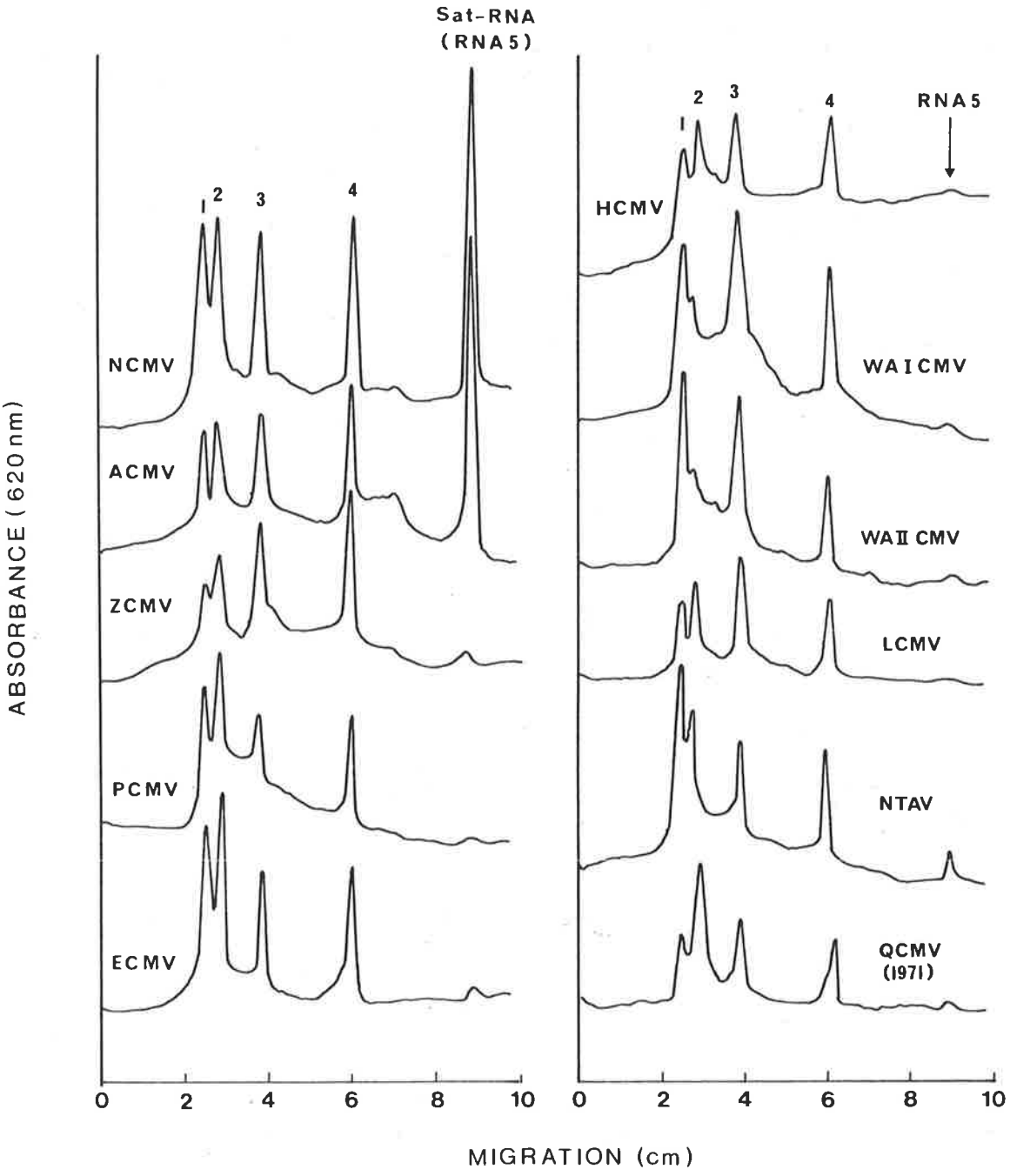
BIOLOGICAL PROPERTIES OF Sat-RNA AND ITS INCIDENCE IN CUCUMOVIRUSES ISOLATED IN AUSTRALIA

Sat-RNA was initially detected only in RNA preparations of QCMV and GCMV (Figure 3.5). Pseudorecombinants containing genomic RNA 3 from either QCMV or GCMV invariably contained Sat-RNA, even if their RNAs 1 and 2 were provided by a cucumovirus isolate which did not contain Sat-RNA (Chapter 4). These results suggested that Sat-RNA may be readily transmitted between different cucumoviruses. In this chapter are described experiments which investigate the ability of Sat-RNA to be transferred to other isolates of cucumoviruses, and its effect on their replication. A number of additional cucumovirus isolates have been examined to determine the distribution of Sat-RNA within Australia, and a comparison of the base sequence homology between Sat-RNA isolated from four different isolates is also given. Also described in this chapter are investigations to determine whether Sat-RNA, like CARNA 5 (Kaper and Waterworth, 1977) is able to modify symptoms produced by its cucumovirus helper.

Screening of Cucumovirus Isolates for the Presence of Sat-RNA

To ascertain whether Sat-RNA was widespread in other Australian cucumoviruses, RNAs isolated from purified preparations of 10 additional isolates (Table 2.1) were examined by electrophoresis in 2% agarose gels. Results presented in Figure 5.1 indicate that two other CMV isolates, ACMV and NCMV, contain significant levels of Sat-RNA. In RNA profiles of the remaining cucumovirus isolates, an RNA component whose electrophoretic

Figure 5.1. Electrophoretic analysis of RNAs isolated from various cucumovirus isolates. RNA samples (10-15 µg) in TE buffer containing 10% sucrose were heated at 75°C for 5 min, cooled rapidly in an ice-water bath, and layered onto 2% agarose gels. Conditions of electrophoresis and the location of RNA bands were as described in Chapter 2.


Sat-RNA
(RNA 5)
1
2
3
4
RNA 5
NCMV
ACMV
ZCMV
PCMV
ECMV
HCMV
WA I CMV
WA II CMV
LCMV
NTAV
QCMV
(1971)
ABSORBANCE (620 nm)
0
2
4
6
8
10
MIGRATION (cm)

mobility is indistinguishable from that of Sat-RNA, could also be detected in low amounts. This RNA component, termed RNA 5, contains a mixture of breakdown fragments derived from CMV-RNAs 1-4 and has no sequence homology with Sat-RNA (Gould *et al.*, 1978).

An isolate of QCMV recovered from leaf material lyophilized in 1971, did not contain detectable levels of Sat-RNA (Figure 5.1), suggesting that Sat-RNA was introduced into this laboratory sometime during the past seven years.

Some Properties of the Cucumovirus Isolates

The serological relationships between the 14 cucumovirus isolates examined are summarized in Table 5.1. All of the 12 CMV isolates are closely related. In contrast, VTAV and NTAV, although closely related themselves, are both serologically unrelated to any of the CMV isolates. The amino acid composition of these viruses are presented in Tables 3.3 and 5.2.

Since AMV and cucumoviruses share several biological and physical properties it seemed desirable to compare the amino acid compositions of AMV (Q) (Table 2.1) with those of the cucumovirus isolates examined in Table 5.2. Moreover, since the accurate amino acid composition of a number of AMV strains are available (Kraal, 1975), it provided an opportunity to investigate the reliability of the amino acid analyses presented in Table 5.2. A comparison of the amino acid composition of AMV (Q) with the reported values for 6 other strains of AMV is given in Table 5.3. The number of cysteine, methionine, and tryptophan residues obtained for AMV (Q) by methanesulphonic acid hydrolysis are identical to those values reported

Table 5.1. Serological relationships between 14 cucumovirus strains.

Antigen[a]	Antiserum[b] QCMV	GCMV	MCMV	WAIICMV	VTAV	NTAV
QCMV	64[c]	128	128	128	-[d]	-
GCMV	64	128	128	128	-	-
MCMV	32	64	256	128	-	-
ACMV	64	64	128	128	-	-
NCMV	32	64	128	128	-	-
ECMV	64	64	128	128	-	-
ZCMV	64	64	128	128	-	-
PCMV	64	32	128	128	-	-
LCMV	32	64	128	128	-	-
HCMV	64	32	128	128	-	-
WAICMV	64	128	128	128	-	-
WAIICMV	64	128	128	128	-	-
VTAV	-	-	-	-	128	128
NTAV	-	-	-	-	64	128

a Antigens fixed with glutaldehyde were all at a concentration of 0.75 mg nucleoprotein/ml in 20mM sodium phosphate, pH 7.6.

b Antisera were diluted in two-fold steps in 20mM sodium phosphate buffer, pH 7.6. Antisera to QCMV, GCMV and VTAV were prepared in mice while that to MCMV, WAIICMV and NTAV were prepared in rabbits. All other serological techniques were as described in Chapter 2.

c Reciprocals of antiserum titres in immunodiffusion tests. Homologous titres are underlined.

d No reaction observed in immunodiffusion tests with undiluted antisera.

Table 5.2. Amino acid composition of the coat proteins of cucumovirus isolates.[a]

Amino acid	NCMV		ACMV		ZCMV		PCMV		ECMV		WAICMV		WAIICMV		LCMV		NTAV	
	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer	Relative molar ratio	Integer
Lys	15.0	15	16.1	16	15.2	15	17.0	17	15.0	15	13.2	13	13.4	13	14.2	14	14.9	15
His	3.8	4	3.9	4	2.9	3	3.3	3	3.2	3	3.4	3	3.4	3	3.3	3	4.4	4
Arg	20.8	21	20.2	20	20.7	21	21.4	21	20.4	20	22.5	23	22.5	23	21.7	22	16.6	17
Asp	24.0	24	24.0	24	26.5	27	24.0	24	25.7	26	23.5	24	23.5	24	22.8	23	26.0	26
Thr[b]	11.8	12	12.4	12	11.5	12	12.4	12	11.9	12	11.3	11	11.3	11	13.4	13	16.1	16
Ser[b]	23.2	23	22.6	23	26.9	27	25.3	25	25.2	25	25.4	25	26.0	26	25.0	25	17.8	18
Glu	15.4	15	15.0	15	15.2	15	15.7	16	15.0	15	13.8	14	13.4	13	14.7	15	19.9	20
Pro	16.8	17	16.3	16	14.6	15	15.4	15	16.2	16	14.7	15	14.8	15	14.4	14	13.8	14
Gly	15.6	16	13.9	14	14.8	15	16.0	16	15.0	15	13.3	13	13.4	13	15.2	15	17.4	17
Ala	16.0	16	13.3	13	13.8	14	15.0	15	15.0	15	19.8	20	19.8	20	19.7	20	16.9	17
Cys[c]	1.2	1	0.7	1	1.0	1	0.7	1	0.8	1	0.9	1	0.6	1	0.8	1	4.6	5
Val	16.4	16	18.7	19	20.2	20	16.2	16	17.0	17	20.0	20	19.8	20	19.7	20	15.7	16
Met[c]	7.4	7	5.9	6	5.6	6	5.9	6	7.0	7	5.6	6	5.4	5	3.9	4	4.0	4
Ile	11.2	11	12.6	13	9.6	10	10.7	11	10.9	11	9.2	9	9.8	10	10.0	10	12.5	13
Leu	21.2	21	21.4	21	22.2	22	22.1	22	22.2	22	21.7	22	22.0	22	21.9	22	19.8	20
Tyr	9.0	9	9.9	10	6.9	7	9.0	9	7.8	8	9.5	10	9.9	10	8.9	9	6.7	7
Phe	6.2	6	5.9	6	5.5	6	5.7	6	5.7	6	5.4	5	5.8	6	6.7	7	6.8	7
Trp[c]	0.6	1	1.7	2	1.3	1	1.3	1	0.6	1	1.2	1	1.1	1	0	0	0.9	1
		235		235		237		236		235		235		236		237		237

a Means of 24, 48 and 72 hr hydrolyses of 2-4 separate protein preparations.

b Extrapolated to zero hydrolysis time.

c Determined from methanesulphonic acid hydrolysis. Cys was determined as s-carboxymethylcysteine.

Table 5.3. Comparison of the amino acid composition of AMV(Q) with other strains of AMV.

Amino acid	Q[b]		AMV strain[a]					
	Relative molar ratio	Integer	A	P	YSMV	425	S	AA-1
Lys	14.1	14	14	14	14	14	14	14
His	5.9	6	6	6	6	7	6	6
Arg	11.2	11	10	10	11	11	10	10
Asp	20.6	21	20	20	20	21	20	20
Thr[c]	13.2	13	13	13	13	13	13	13
Ser[c]	15.0	15	16	15	15	15	15	15
Glu	20.8	21	20	20	19	20	20	20
Pro	18.0	18	18	18	16	17	17	17
Gly	17.4	17	17	17	17	17	17	18
Ala	18.9	19	21	21	21	20	21	21
Cys[d]	2.7	3	3	3	3	3	3	3
Val	12.6	13	12	12	12	13	13	14
Met[d]	2.8	3	3	3	3	3	3	3
Ile	4.8	5	5	5	5	5	5	5
Leu	21.2	21	21	21	22	21	23	22
Tyr	5.2	5	5	5	5	4	5	5
Phe	15.2	15	15	16	16	18	16	16
Trp[d]	2.1	2	2	2	2	2	2	2
Total		222	221	221	220	224	223	223

a Amino acid data for AMV strains YSMV, 425, S and AA-1 are taken from Kraal (1975). Data for strains A and P were those of Tremaine and Stace-Smith (1969) recalculated by Kraal (1975).

b This work. Values are the means of 24, 48 and 72 hr acid hydrolyses of two separate protein preparations. An additional sample was also hydrolysed with methanesulphonic acid after carboxymethylation.

c Extrapolated to zero hydrolysis time.

d Determined by methanesulphonic acid hydrolysis. Cysteine was determined as s-carboxymethyl cysteine.

for the other AMV strains (Table 5.3).

A classification of 13 cucumovirus isolates (Tables 3.3 and 5.2) and 7 strains of AMV (Table 5.3) based on computer analyses (MULTCLAS program using Euclidean metric; Lance and Williams, 1967) of their amino acid compositions is presented in Figure 5.2. The amino acid composition data were analysed in two ways; principal coordinate analysis (Gower, 1966) as illustrated in Figure 5.2 A-C, and by the agglomerative hierarchical sorting method of Lance and Williams (1967) as shown in Figure 5.2 D. Using both analytical methods, the viruses are separated into four main clusters; two for the CMV isolates, one for the 2 isolates of TAV, and another for the 7 AMV strains (Figure 5.2 A-D). The resolution of the CMV isolates into the two clusters is most apparent in Figures 5.2 A, B and D, where NCMV, ECMV, QCMV, PCMV, ACMV and ZCMV are separated from LCMV, GCMV, WAICMV, WAIICMV and MCMV. It is not unexpected that MCMV should occupy a position furthermost from the other isolates of CMV (Figure 5.2 A and B) since factors affecting its particle stability and core biological properties differ markedly from those normally associated with those of CMV (Chapter 3). Moreover, MCMV is serologically distinguishable from both QCMV and GCMV (Table 3.1) and other isolates of CMV (Table 5.1).

As expected, the two isolates of TAV, and the seven strains of AMV form two homogeneous groups, well separated from each other and the two CMV clusters (Figure 5.2 A-D).

Comparison of the Base Sequences of Sat-RNA from Four Cucumovirus Isolates

Hybridization analyses using cDNA probes have recently demonstrated that the base sequences of Sat-RNA preparations from QCMV and GCMV are indistinguishable, and that Sat-RNA has no detectable sequence homology with genomic RNAs of QCMV (Gould *et al.*, 1978). Using cDNA transcribed

Figure 5.2. Classification of the cucumovirus and AMV isolates based on the amino acid compositions of their coat proteins. The amino acid composition data from Tables 3.3, 5.2 and 5.3 were analysed using the MULTCLAS program (Lance and Williams, 1967).

A, B and C. Principal coordinate analysis (Gower, 1966). The isolates of CMV are delineated by a dashed (---) line, those of TAV by a dotted line (···) and those of AMV by a solid line (——). The numbers 1, 2 and 3 on the coordinates signify the first, second and third dimensions respectively.

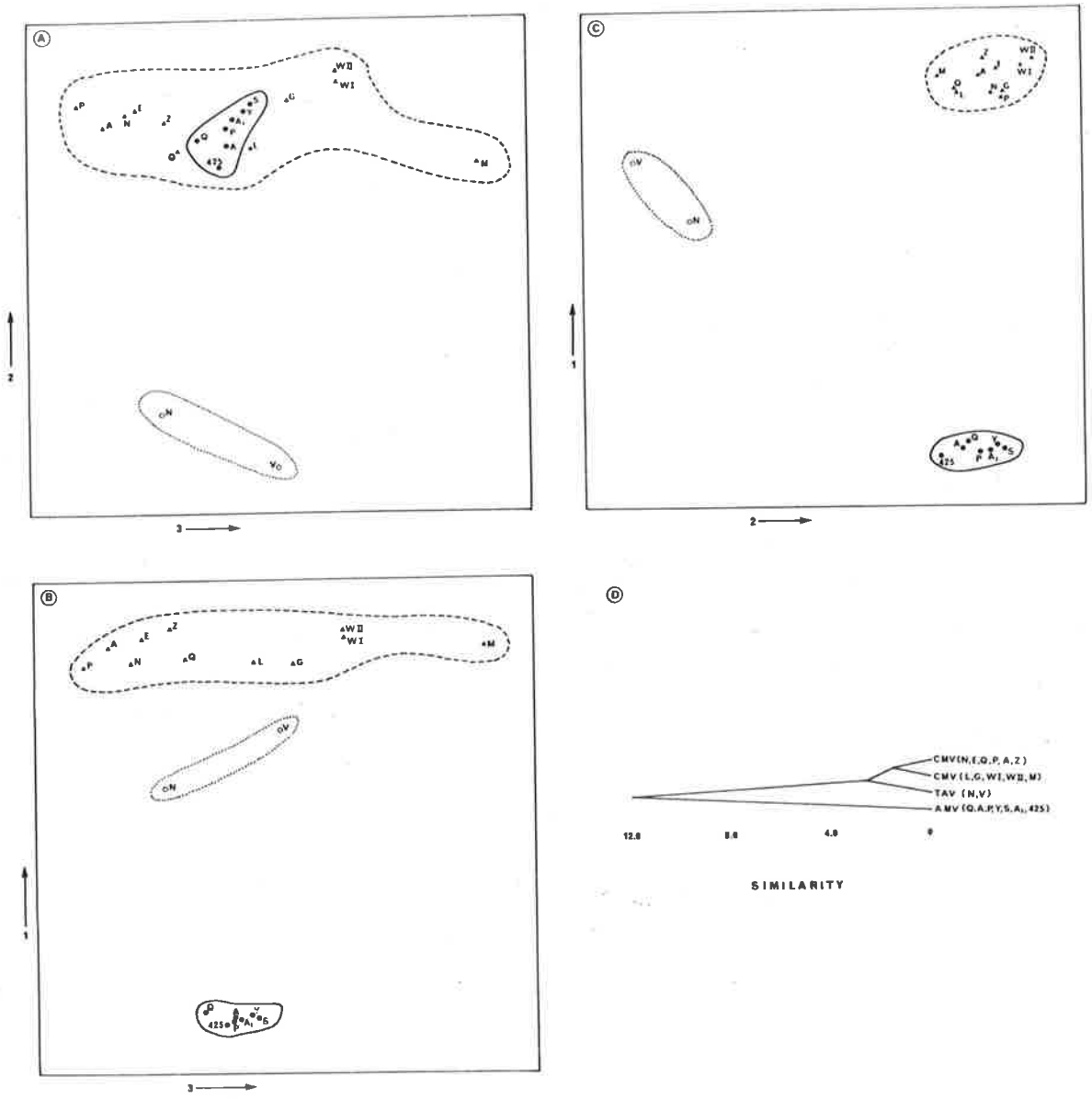
D. Dendrogram illustrating the classification of viruses using the agglomerative hierarchical sorting method (Lance and Williams, 1967). The scale indicates the level of dissimilarity at which the clusters fused.

Viruses are lettered as follows:

CMV - N = NCMV; E = ECMV; Q = QCMV; P = PCMV;
A = ACMV; Z = ZCMV; L = LCMV; G = GCMV;
WI = WAICMV; WII = WAIICMV; and M = MCMV.

TAV - N = NTAV; and V = VTAV.

AMV - Q = AMV(Q); A = AMV(A); P = AMV(P); Y = YSMV;
425 = AMV(425); S = AMV(S); and A_1 = AMV(AA-1).


A
WII
WI
P
A
N
E
Z
G
S
Y
Q
A1
V
A
I
425
M
cN
V0
2
3
C
WII
WI
M
Z
A
J
Q
A1
N
G
425
cV
cN
1
A
Q
V
S
425
P
A1
2
B
WII
WI
Z
E
A
P
N
Q
L
G
M
cV
cN
1
Q
425
P
A1
V
S
3
D
CMV(N,E,Q,P,A,Z)
CMV(L,G,WI,WII,M)
TAV (N,V)
AMV (Q,A,P,Y,S,A1,425)
12.0
8.0
4.0
0
SIMILARITY

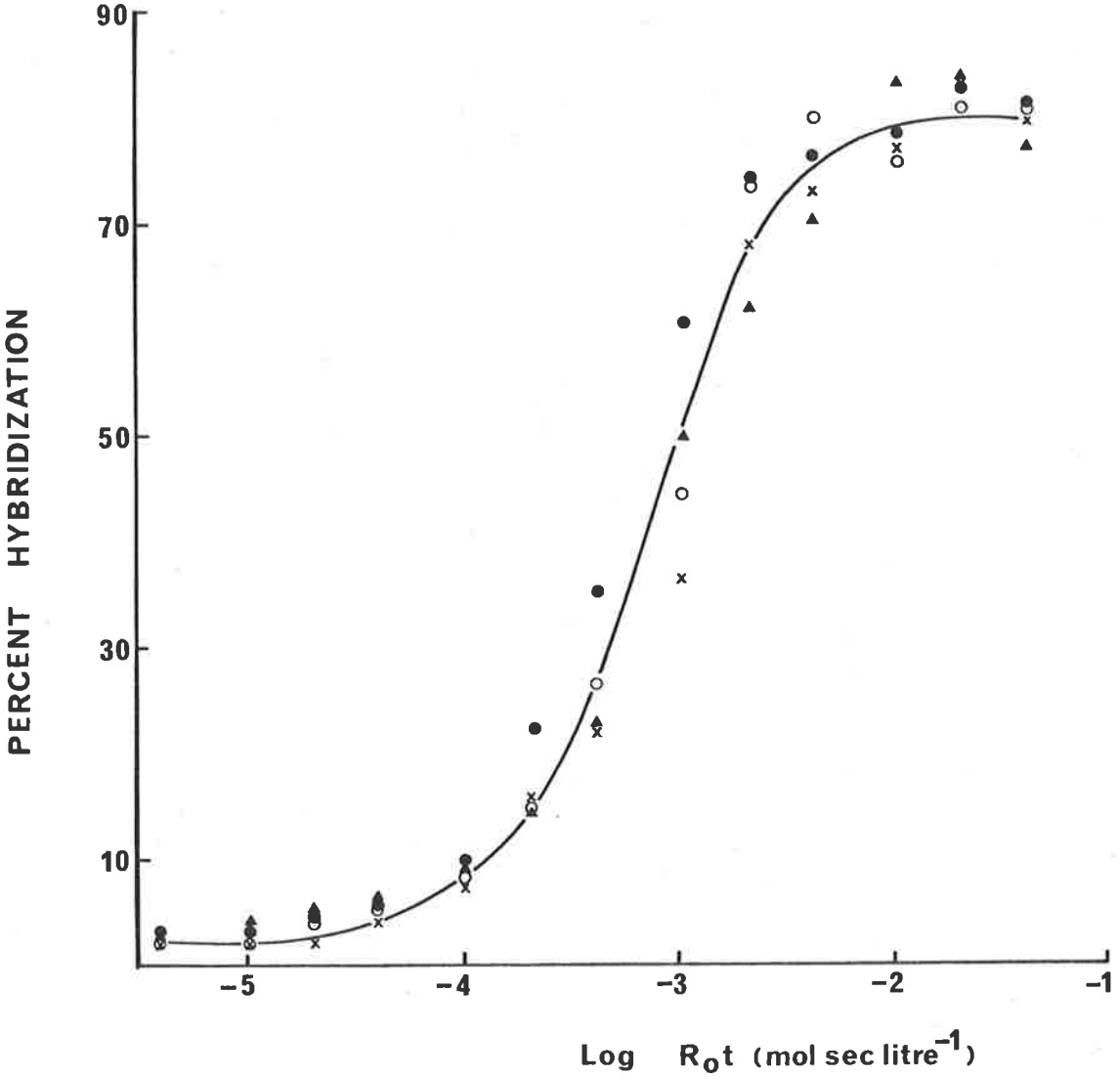
from Sat-RNA isolated from GCMV, cDNA(GCMV Sat-RNA), the extent of sequence homology between Sat-RNAs isolated from GCMV, QCMV, ACMV and NCMV was investigated. Results presented in Figure 5.3 indicate that the kinetics of hybridization of cDNA(GCMV Sat-RNA) with Sat-RNA from the various cucumovirus isolates were indistinguishable. The observed $R_o t_{½}$ values for the Sat-RNAs of GCMV and QCMV were 1.0 and 0.5 x 10^{-3} mol sec litre^{-1} respectively, and 0.8 x 10^{-3} mol sec litre^{-1} for the Sat-RNAs of both ACMV and NCMV. These values are similar to the expected $R_o t_{½}$ value of 1.0 x 10^{-3} mol sec litre^{-1} for Sat-RNA (Gould *et al.*, 1978). These data, and the fact that all Sat-RNA preparations hybridized to a similar plateau level (approximately 80%) indicate that (i) the Sat-RNA isolated from these isolates of CMV are homogenous, containing one type of RNA molecule only, and (ii) they have indistinguishable base sequences.

Transmission of Sat-RNA between Cucumoviruses

The detection of Sat-RNA in certain pseudorecombinants containing RNAs 1 and 2 from cucumovirus strains which, when first isolated did not contain Sat-RNA, suggested that this low-molecular weight RNA may be readily transmissible between cucumoviruses. The transmissibility of Sat-RNA was investigated as follows. Isolated RNA of MCMV, PCMV, ZCMV, QCMV (isolate A, Gould *et al.*, 1978), VTAV and NTAV (Figures 3.5 and 5.1) was inoculated to *N. clevelandii* plants at a concentration of 100 µg/ml, with and without added Sat-RNA (2.5 µg/ml). Inoculated plants were maintained in an air-cooled glasshouse and after 12 days viruses were purified from systemically infected leaf tissue of each treatment. RNA isolated from purified virus preparations was then analysed by electrophoresis in 2% agarose gels.

Figure 5.3. Kinetics of hybridization of [^{32}P]cDNA(GCMV Sat-RNA) with the Sat-RNAs isolated from QCMV, GCMV, ACMV and NCMV. Sat-RNAs were purified by the two-step gel electrophoretic procedure described in Chapter 2. Serial dilutions of each Sat-RNA in hybridization buffer (0.18M NaCl, 0.01M Tris-HCl, pH 7.0, 1mM EDTA, 0.05% SDS) were incubated at 60^{o}C for 2 hr with approximately 2,000 cpm of [^{32}P]cDNA. The extent of hybrid formation was determined using S_1 nuclease as described in Chapter 2. The curve describes the mean hybridization values of the various Sat-RNAs. Actual $R_o t_{\frac{1}{2}}$ values for the Sat-RNAs of GCMV and QCMV were 1.0×10^{-3} and 0.5×10^{-3} mol sec litre^{-1} respectively and 0.8×10^{-3} mol sec litre^{-1} for the Sat-RNAs of ACMV and NCMV. Sat-RNAs from:

x - GCMV, ● - QCMV, o - NCNV, ▲ - ACMV.


90
70
50
30
10
PERCENT HYBRIDIZATION
−5
−4
−3
−2
−1
Log $R_o t$ (mol sec litre^{-1})

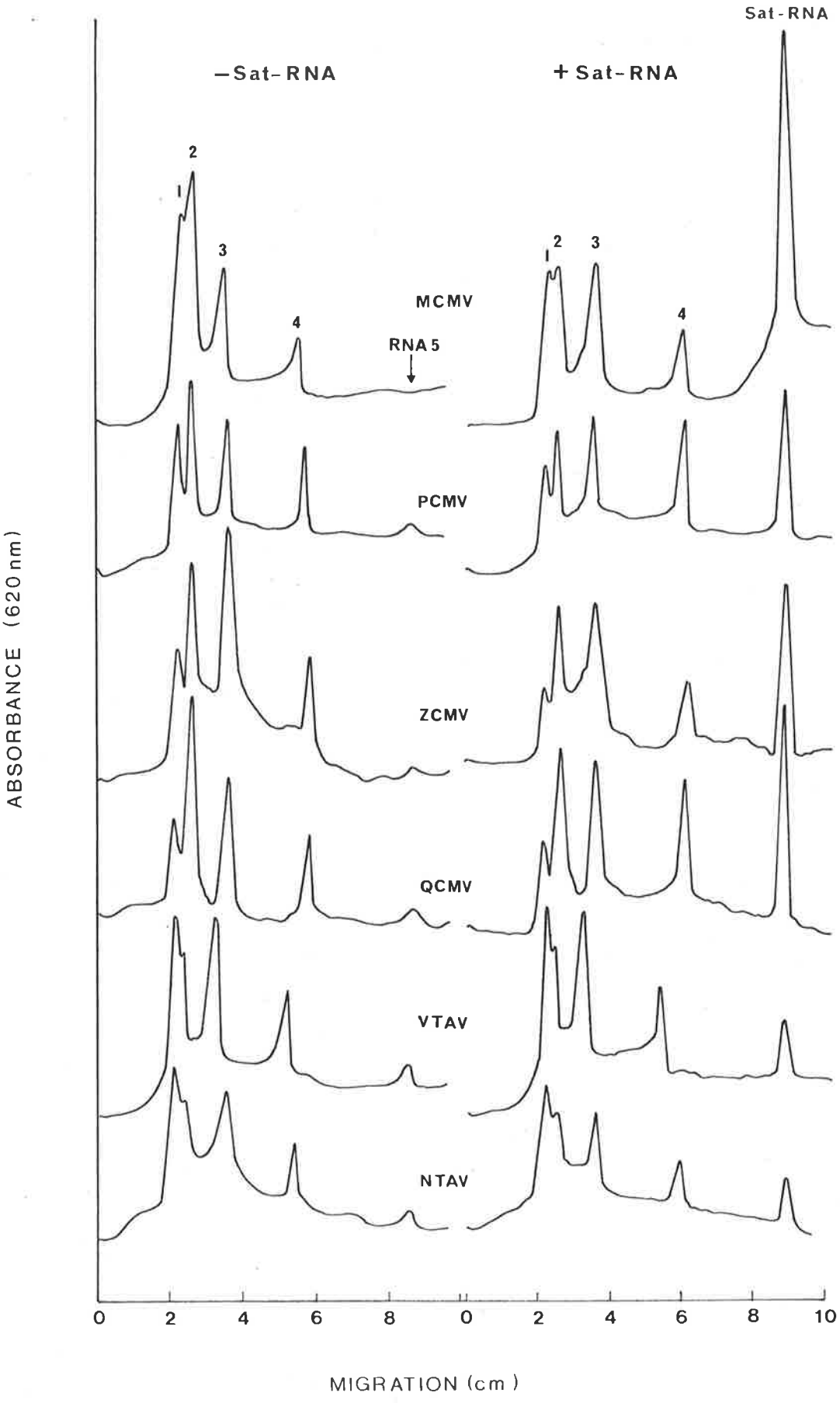
Sat-RNA was detected in RNA preparations from all cucumoviruses where the viral RNA inoculum had been supplemented with Sat-RNA (Figure 5.4). Sat-RNA was present in substantial amounts in RNA preparations from all four isolates of CMV, whereas in both VTAV and NTAV the level of Sat-RNA was considerably lower and barely distinguishable above that of RNA 5 (Figure 5.4). That Sat-RNA is able to replicate in plants infected with these two isolates of TAV was confirmed by hybridization of RNA preparations from each isolate with cDNA(Sat-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}.

The low level of Sat-RNA detected in preparations of VTAV is probably due to the reduced ability of this cucumovirus to support its replication, rather than its encapsidation, since the pseudorecombinant $T_1T_2Q_3$ and $T_1T_2G_3$ (Figures 4.2 and 4.3) also contain only low levels of Sat-RNA. Moreover, these results suggest that the replication of Sat-RNA is controlled by genetic information located on cucumovirus genomic RNAs 1 and/or 2.

Interference of Cucumovirus Replication by Sat-RNA

The recent demonstration that replication of CARNA 5 interferes with that of its helper cucumoviruses (Kaper *et al.*, 1976; Kaper and Tousignant, 1977) is similar to that reported for Defective Interfering particles of animal viruses (Huang, 1973) and satellites of other plant viruses (Schneider, 1977). Kaper *et al.* (1976) have shown that the presence of CARNA 5 in CMV preparations is associated with a reduction of both the amount of recoverable virus and its specific infectivity as well as an alteration of the proportion of genomic RNAs of the CMV helper virus. In view of the apparent similarities between Sat-RNA and CARNA 5, it was desirable to determine whether the replication of Sat-RNA also affected

Figure 5.4. Transmission of Sat-RNA between cucumoviruses. *N. clevelandii* plants were inoculated with viral RNA alone at 100 µg/ml, or a mixture of viral RNA and Sat-RNA at 100 and 2.5 µg/ml respectively. Viruses were purified from systemically infected leaf tissue 12 days after inoculation, and isolated RNA of the various preparations were then subjected to electrophoresis in 2% agarose gels as in Figure 5.1.


−Sat-RNA
+Sat-RNA
Sat-RNA
1
2
3
4
MCMV
RNA 5
PCMV
ZCMV
QCMV
VTAV
NTAV
ABSORBANCE (620 nm)
0
2
4
6
8
0
2
4
6
8
10
MIGRATION (cm)

that of its associated cucumoviruses.

In experiments described in Table 5.4, *N. clevelandii* plants were inoculated with viral RNA alone, or a mixture of viral RNA and Sat-RNA as described in Figure 5.4. Systemically infected leaf tissue was harvested 12 days after inoculation and RNA isolated from purified preparations of each virus was analysed by electrophoresis in 2% agarose gels. The proportion of each RNA component in these RNA preparations was determined by integrating areas under each RNA peak from densitometer traces (Figure 5.5 A). In these experiments it was assumed that there is a linear relationship between the amount of RNA in a peak and the amount of toluidine blue dye bound.

The results summarized in Table 5.4 demonstrate, that for the cucumovirus isolates which support the replication of Sat-RNA to high levels there is a marked reduction in both the yield of virus and the proportions of genomic RNAs 1 and 2. In contrast, the virus yields and proportions of genomic RNAs of both VTAV and NTAV were not noticeably affected by the presence of Sat-RNA. Neither VTAV nor NTAV are able to support production of Sat-RNA to high levels (Figure 5.4). Although the reduction of virus yield associated with Sat-RNA replication is convincing, its apparent effects on the relative proportions of CMV genomic RNAs are rather more difficult to interpret. When the contribution of Sat-RNA and/or RNA 5 is subtracted from the total RNA composition of each virus, the differences between the levels of genomic RNAs 1 and 2 in RNA preparations from each isolate grown in the presence and absence of Sat-RNA are greatly reduced (percentages given in parentheses; Table 5.4). However, as a result of these adjustments, the apparent levels of RNAs 3 and 4 from viruses grown in the presence of Sat-RNA are often increased

Table 5.4. Effects of Sat-RNA on the replication of several cucumovirus isolates.

Experiment	Inoculum[a]	Proportion of RNA components (%)[b] 1	2	3	4	Sat-RNA[d] (or RNA 5)	Yield[c] mg/kg	Yield[c] Virus+Sat / Virus-Sat (%)
1	MCMV	24.8	38.4	27.9	8.9	-[e]	567	18.0
	MCMV+Sat-RNA	13.5 (25.4)	14.0 (26.0)	19.2 (36.2)	6.4 (12.0)	47.0 (0)	100	
2	ZCMV	13.3	27.5	44.0	15.2	-	266	26.0
	ZCMV+Sat-RNA	10.7 (15.7)	22.1 (32.6)	26.8 (39.3)	8.4 (12.4)	32.0 (0)	69	
3	QCMV	15.6	41.0	26.6	16.8	-	87	45.0
	QCMV+Sat-RNA	11.1 (15.5)	29.8 (41.3)	18.6 (25.9)	12.4 (17.2)	28.1 (0)	39	
4	PCMV	24.2 (24.9)	30.4 (31.2)	25.1 (25.8)	17.6 (18.1)	2.6 (0)	151	35.0
	PCMV+Sat-RNA	15.2 (19.9)	18.6 (24.3)	24.0 (31.4)	18.6 (24.3)	22.8 (0)	53	
5	VTAV	26.0 (26.9)	20.9 (21.7)	31.8 (33.0)	17.7 (18.4)	3.6 (0)	540	107.0
	VTAV+Sat-RNA	21.1 (23.1)	20.4 (22.4)	32.5 (35.7)	17.1 (18.8)	8.9 (0)	590	
6	NTAV	27.5 (28.6)	22.5 (23.5)	32.0 (33.3)	14.0 (14.6)	2.7 (0)	200	75.0
	NTAV+Sat-RNA	29.3 (33.7)	21.5 (24.7)	22.5 (25.9)	13.6 (15.7)	13.1 (0)	150	

a Inocula contained cucumovirus RNA at 100 µg/ml, with and without added Sat-RNA at a concentration of 2.5 µg/ml. Viruses were purified from systemically infected leaves of *N. clevelandii* plants 12 days after inoculation, and their RNA isolated as described in Chapter 2.

b Isolated RNA preparations of each virus were analysed by electrophoresis in 2% agarose gels, and the relative proportion of their RNA components were determined as described in Figure 5.5 A. The values in parentheses represent the corrected proportions of the various RNA components after the contribution of Sat-RNA and/or RNA 5 toward the total RNA complement of each virus has been deducted.

c Yields of virus recovered as expressed as mg nucleoprotein per kg of leaf tissue and also as a percentage of virus recovered in the presence and absence of Sat-RNA.

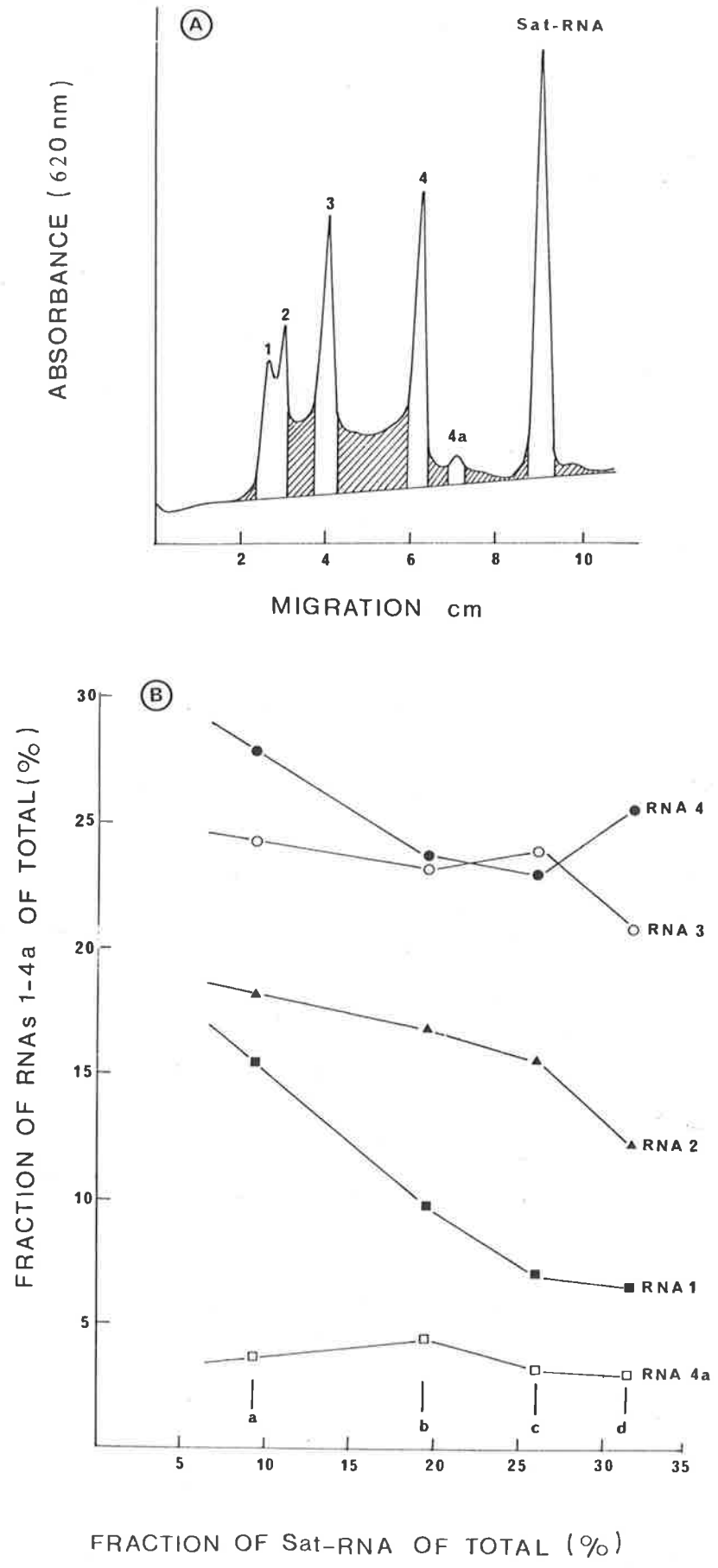
d Sat-RNA and RNA 5 could not be resolved under the electrophoretic conditions used.

e Neither Sat-RNA nor RNA 5 could be detected in 2% agarose gels.

Figure 5.5. Relationship between the level of Sat-RNA and the genomic RNA species composition of QCMV.

A. Estimation of the RNA species composition of cucumovirus RNA. Viral RNAs were electrophoresed in 2% agarose gels, stained with toluidine blue and scanned at 620 nm as described in Chapter 2. The area under each peak was integrated and expressed as a percentage of the total area under peaks corresponding to RNA 1-4a and Sat-RNA (or RNA 5). The shaded areas between RNA components were not considered since these presumably represent fragments, aggregates and mixtures of RNA species.

B. Relationship between the level of Sat-RNA and the genomic RNA species composition of four different preparations of QCMV-RNA (a-d). All four preparations were from QCMV propagated in *N. clevelandii* under the same growth conditions. The RNA species composition of each preparation was determined as above. For each preparation, the level of Sat-RNA present is indicated by arrows (abscissa).


A
Sat-RNA
ABSORBANCE (620 nm)
1
2
3
4
4a
2
4
6
8
10
MIGRATION cm
B
30
25
20
15
10
5
FRACTION OF RNAs 1-4a OF TOTAL(%)
RNA 4
RNA 3
RNA 2
RNA 1
RNA 4a
a
b
c
d
5
10
15
20
25
30
35
FRACTION OF Sat-RNA OF TOTAL (%)

(e.g. RNAs 3 and 4 of MCMV and PCMV).

In an RNA preparation from MCMV which had been successively transferred 5 times in *N. clevelandii* after the introduction of the satellite, Sat-RNA represented approximately 75% of the total RNA, whereas RNAs 1 and 2 were barely discernible. Similarly, extremely low levels of RNAs 1 and 2 have been reported by Kaper *et al.* (1976) and Kaper and Tousignant (1977) following repeated serial transfer of strains of CMV in tobacco plants in the presence of CARNA 5.

The effect of Sat-RNA replication on the proportion of genomic RNAs of its helper virus was further investigated by comparing the relative proportion of each RNA component in four RNA preparations of QCMV containing different levels of Sat-RNA. All RNA preparations were isolated from QCMV propagated in *N. clevelandii* under similar growth conditions. Results presented in Figure 5.5 B indicate that increased levels of Sat-RNA were correlated with reduced levels of genomic RNAs 1 and 2. The levels of the genomic RNAs indicated in Figure 5.5 B have not been adjusted for the various levels of Sat-RNA as in Table 5.4. Using Spearman's ranked correlation test (Colquhoun, 1971) a significant negative correlation was obtained between Sat-RNA and RNAs 1 and 2 (-1.0, $P = 0.10$). In contrast the levels of RNAs 3, 4 and 4a were not significantly correlated with increasing levels of Sat-RNAs.

Effect of Plant Host on the Replication of Sat-RNA

The replication of CARNA 5 by helper cucumoviruses has recently been shown to be influenced both by the host plant in which the virus is propagated, and by the strain of helper virus (Kaper and Tousignant, 1977). In squash (*Cucurbita pepo* L. cv. Caserta Bush) these authors reported that the level of CARNA 5 in RNA isolated from preparations of

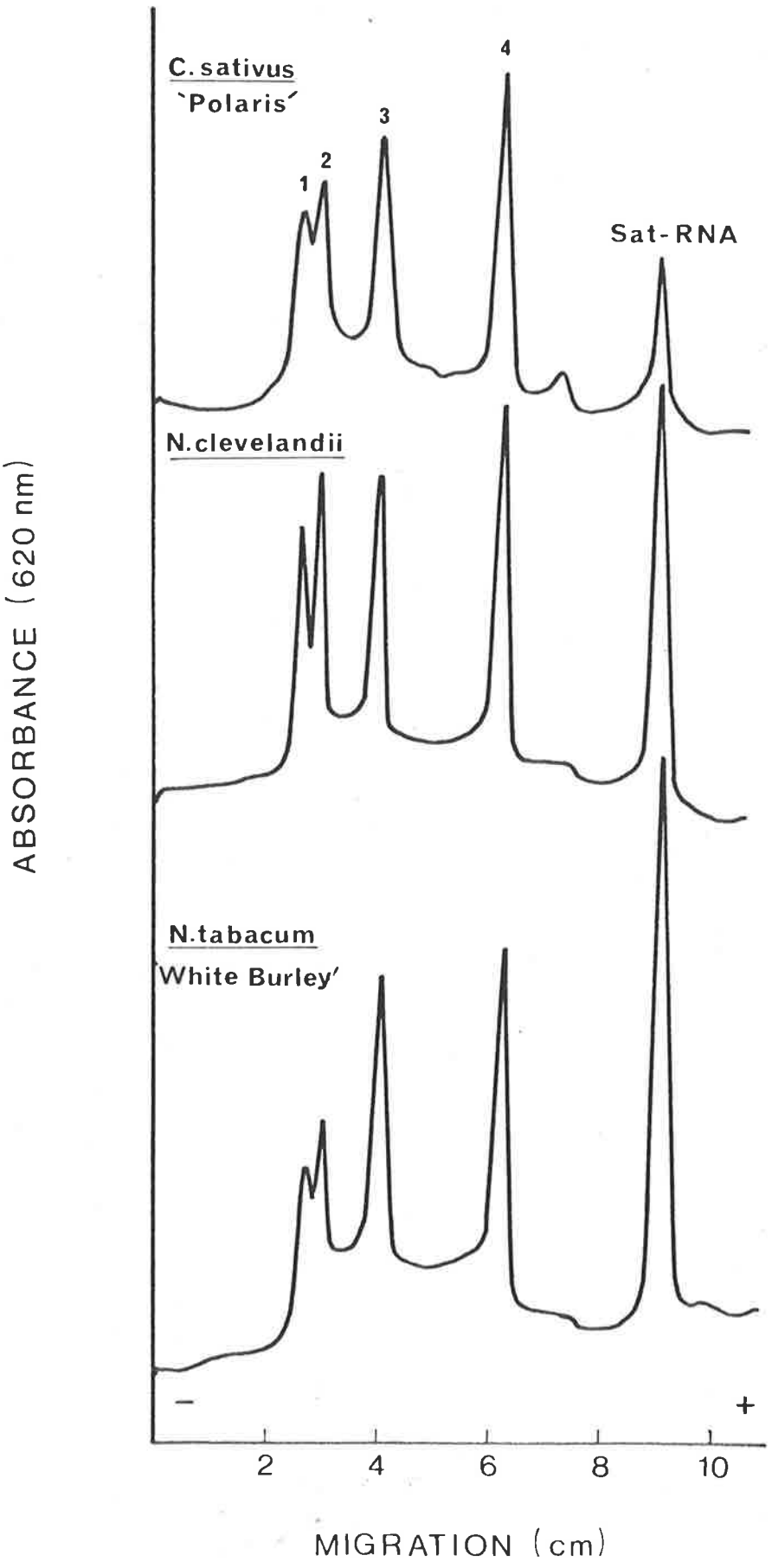
all strains of CMV examined except CMV-R, was considerably less than that in preparations propagated in tobacco (*N. tabacum* cv. Xanthi nc).

To investigate the possible effect of host plant on the level of Sat-RNA, plants of *Cucumis sativus* L. cv. Polaris (cucumber), *N. tabacum* cv. White Burley and *N. clevelandii* were inoculated with a preparation of QCMV-RNA at 100 µg/ml. The QCMV-RNA preparation contained approximately 20% by weight of Sat-RNA. Virus was purified from systemically infected leaf tissue harvested from each host species 12 days after inoculation, and isolated RNA from each preparation was then subjected to electrophoresis in 2% agarose gels. Results shown in Figure 5.6 demonstrate that the level of Sat-RNA in QCMV preparations from cucumber was considerably less than that in either tobacco or *N. clevelandii*. These results indicate that the synthesis or encapsidation of Sat-RNA, like CARNA 5, is influenced by the propagative host plant. Interestingly, the propagative host also influenced the levels of QCMV genomic RNAs 1 and 2 (Figure 5.6). Waterworth *et al.* (1978) have also reported that the RNA species composition of CMV-COM propagated in *C. quinoa* differed considerably from that of RNA isolated from virus propagated in tobacco.

Dilution End-point of Sat-RNA

It was demonstrated that purified preparations of RNA 3 from the isolate of CMV used in the construction of pseudorecombinants was contaminated with Sat-RNA to an extremely low level (Figure 4.6). Moreover, pseudorecombinants containing RNA 3 from either QCMV or GCMV invariably contained Sat-RNA when first isolated (Chapter 4). These results suggested that Sat-RNA may be highly infectious. That the base sequence homology between Sat-RNA from four CMV isolates were indistinguishable (Figure 5.3)

Figure 5.6. The effect of host plant on the level of Sat-RNA replication. Plants of each host species were inoculated with a preparation of QCMV-RNA (100 µg/ml) which contained a high level of Sat-RNA. RNA isolated from purified virus preparations from each host was electrophoresed in 2% agarose gels as described in Chapter 2.


C. sativus
'Polaris'
1
2
3
4
Sat-RNA
N. clevelandii
N. tabacum
'White Burley'
ABSORBANCE (620 nm)
−
+
2
4
6
8
10
MIGRATION (cm)

and observations that a number of the isolates of CMV which when first isolated did not contain Sat-RNA (Figure 5.1), have subsequently acquired this RNA, suggest that it may have been inadvertently spread in the course of viral propagation.

Dilutions of Sat-RNA ranging from 5×10^{-6} to 2.5 µg/ml were inoculated to *N. clevelandii* plants in TE buffer containing 200 µg/ml of ZCMV-RNA. Two plants were inoculated with a mixture of each Sat-RNA dilution and ZCMV-RNA. Another two plants were also inoculated with ZCMV-RNA alone as controls. At the concentration of ZCMV-RNA used in these experiments, confluent chlorotic local lesions developed on the inoculated leaves within 5 days. Systemically infected leaf tissue from each plant was harvested 12 days after inoculation, and their RNAs were extracted. Leaf RNA extracts were hybridized with cDNA(Sat-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}.

Results summarized in Table 5.5 demonstrate that at an inoculum concentration as low as 5×10^{-6} µg/ml, Sat-RNA was detected in leaf RNAs extracted from one of the two plants inoculated. These results indicate that preparations of Sat-RNA have an extraordinarily high specific infectivity, similar to disease agents such as potato spindle tuber viroid (Morris and Smith, 1977).

Modification of CMV-Host Interactions by Sat-RNA

Recently, Kaper and Waterworth (1977) have demonstrated that in the presence of CARNA 5, 'Rutgers' tomato plants infected with strains of CMV develop severe necrotic symptoms. However, in 'Xanthi nc' tobacco plants, the presence of CARNA 5 was reported to attenuate disease symptoms.

In order to determine if Sat-RNA is capable of modifying host

Table 5.5. Dilution endpoint of Sat-RNA.

Inoculum concentration of Sat-RNA (µg/ml)[a]	No. of plants in which Sat-RNA detected/No. of plants inoculated[b]	
	Experiment 1	Experiment 2
0 (ZCMV-RNA alone)	0/2	0/2
2.5	2/2	-[c]
2.5×10^{-1}	2/2	-
2.5×10^{-2}	2/2	2/2
2.5×10^{-3}	2/2	2/2
2.5×10^{-4}	2/2	2/2
5.0×10^{-5}	-	2/2
5.0×10^{-6}	-	1/2

a Different preparations of Sat-RNA, each purified by two-cycles of gel electrophoresis, were used in the two experiments. Dilutions of Sat-RNA were prepared in TE buffer containing 200 µg/ml ZCMV-RNA. Each dilution was inoculated to 3 leaves on each of 2 *N. clevelandii* plants, and after 12 days systemically infected leaves from each plant were harvested and their RNAs extracted.

b Leaf RNAs were hybridized with cDNA(Sat-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}. Hybridization values obtained for leaf RNAs containing Sat-RNA ranged from 65% to 85%. The absence of Sat-RNA was indicated by hybridization values similar to those obtained when cDNA(Sat-RNA) was incubated without added RNA (3.0-10.0% in the two experiments).

c Not determined.

symptoms induced by cucumoviruses, a series of experiments were done in which several host plant species were inoculated with a number of different cucumovirus isolates in the presence and absence of added Sat-RNA. In all experiments, viral RNA at 100 µg/ml was inoculated with and without, added Sat-RNA at 2.5 µg/ml, and the plants were placed in a growth cabinet at 22°C with a photoperiod of 14 hr (6,000 lux). Symptom development was observed over a period of up to 2 months.

With all cucumovirus isolates examined, the presence of Sat-RNA failed to increase the severity of symptoms produced in 'Rutgers' tomato plants (Table 5.6). Similar results were obtained when inoculated tomato plants were maintained under different propagation conditions; a growth room at 25°C with continuous illumination of 5,000 lux, or an air-cooled glasshouse. The presence of Sat-RNA actually attenuated symptoms induced by several isolates of CMV in tomato and cv White Burley tobacco plants (Table 5.6, Figures 5.7 and 5.8). 'Rutgers' tomato plants inoculated with ECMV-RNA alone developed a systemic necrotic reaction in addition to the usual leaf distortion symptom and stunting associated with other isolates of CMV. The presence of Sat-RNA in ECMV-RNA inocula greatly reduced the severity of the systemic symptoms produced (Figure 5.7 A). RNA isolated from purified preparations of ECMV propagated in 'Rutgers' tomato plants in the presence of Sat-RNA contained high levels of Sat-RNA (Figure 5.7 C). RNA isolated from virus purified from plants inoculated with ECMV-RNA alone did not contain detectable levels of Sat-RNA when electrophoresed in agarose gels (Figure 5.7 B) or when hybridized to a $R_o t$ value of 10.0 mol sec litre^{-1} with cDNA(Sat-RNA).

Attenuation of host symptoms by the addition of Sat-RNA was observed only for those CMV isolates which induced severe symptoms in tomato

Table 5.6. Symptoms produced in 'Rutgers' tomato plants inoculated with various cucumovirus isolates with and without Sat-RNA.

Strain	Inoculum[a]	
	- Sat-RNA	+ Sat-RNA
QCMV[b]	NT[c]	mild mosaic
GCMV[b]	NT	-[d]
ACMV[b]	NT	-
NCMV[b]	NT	-
PCMV	mild mosaic	mild mosaic
MCMV	yellow mosaic and leaf distortion	yellow mosaic and leaf distortion
ECMV	mosaic, leaf distortion and systemic necrosis	mild mosaic (Figure 5.6)
ZCMV	mosaic, leaf distortion	mosaic, leaf distortion
HCMV	mild mosaic	mild mosaic
WAICMV	mosaic, leaf distortion	mild mosaic (Figure 5.7a, b)
WAIICMV	mosaic, leaf distortion	mosaic, leaf distortion
VTAV	mosaic, leaf distortion	mosaic, leaf distortion
NTAV	leaf distortion, severe systemic necrosis	leaf distortion, severe systemic necrosis

a 'Rutgers' tomato plants were inoculated in groups of 3 plants with viral RNA at a concentration of 100 µg/ml, with and without Sat-RNA at 2.5 µg/ml. Plants were inoculated on the cotyledons and first true leaves. At the time of inoculation, plants were approximately 7 cm tall, and the first true leaves were approximately 1 cm in length. Plants were maintained in a growth cabinet at 22°C with photoperiod of 14 hr at 6,000 lux.

b RNA of these cucumoviruses contained Sat-RNA when first isolated.

c Not tested, since the inocula already contained Sat-RNA.

d Symptomless.

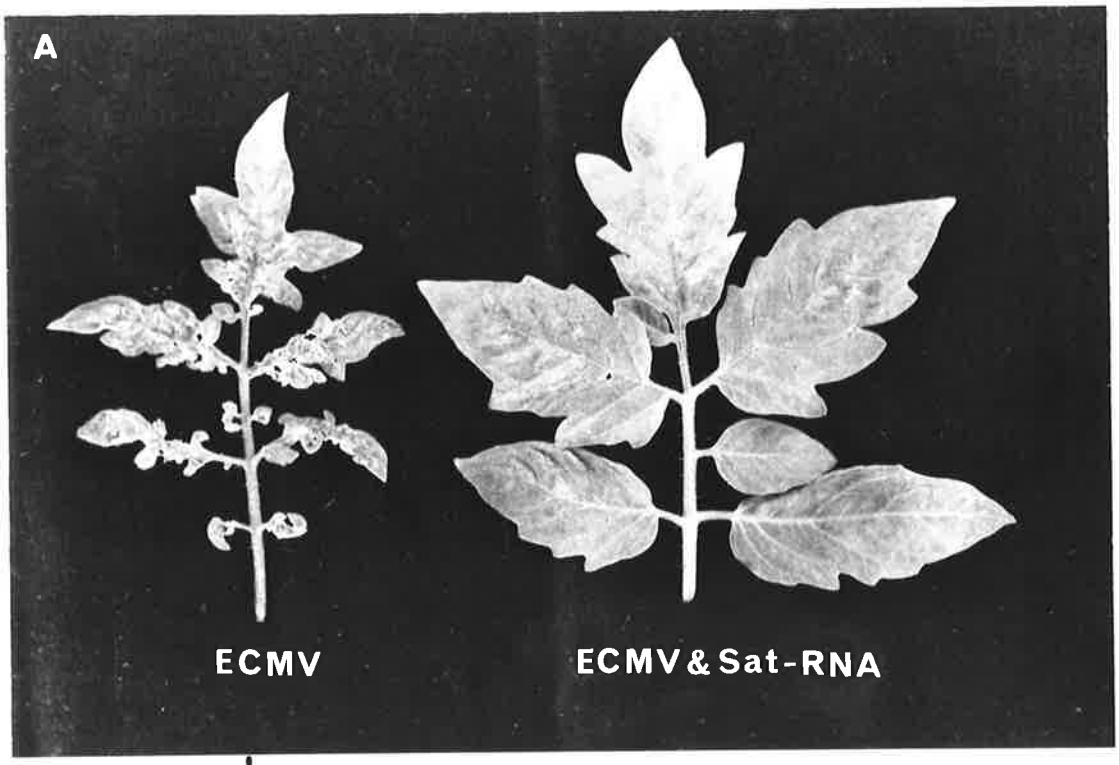
Figure 5.7. Attenuation of symptoms induced by ECMV in 'Rutgers' tomato plants in the presence of Sat-RNA. Plants were inoculated with ECMV-RNA, with and without Sat-RNA, as in Table 5.6.

A. Symptoms induced in the fifth true leaves of plants inoculated with ECMV-RNA alone (left) or ECMV-RNA and Sat-RNA (right).

Electrophoresis of RNA isolated from preparations of ECMV (above) from tomatoes inoculated with ECMV-RNA in the presence and absence of Sat-RNA. RNA samples (10-15 µg) were electrophoresed in 2% agarose gels as before.

B. RNA isolated from virus preparations from 'Rutgers' tomato plants inoculated with ECMV-RNA alone (above) and RNA isolated from virus preparations from plants inoculated with a mixture of ECMV-RNA and Sat-RNA (below).

The yields of virus recovered from 'Rutgers' tomato plants inoculated with ECMV-RNA alone, and ECMV-RNA and Sat-RNA were approximately 120 and 60 mg/kg of leaf tissue respectively.


A
ECMV
ECMV & Sat-RNA

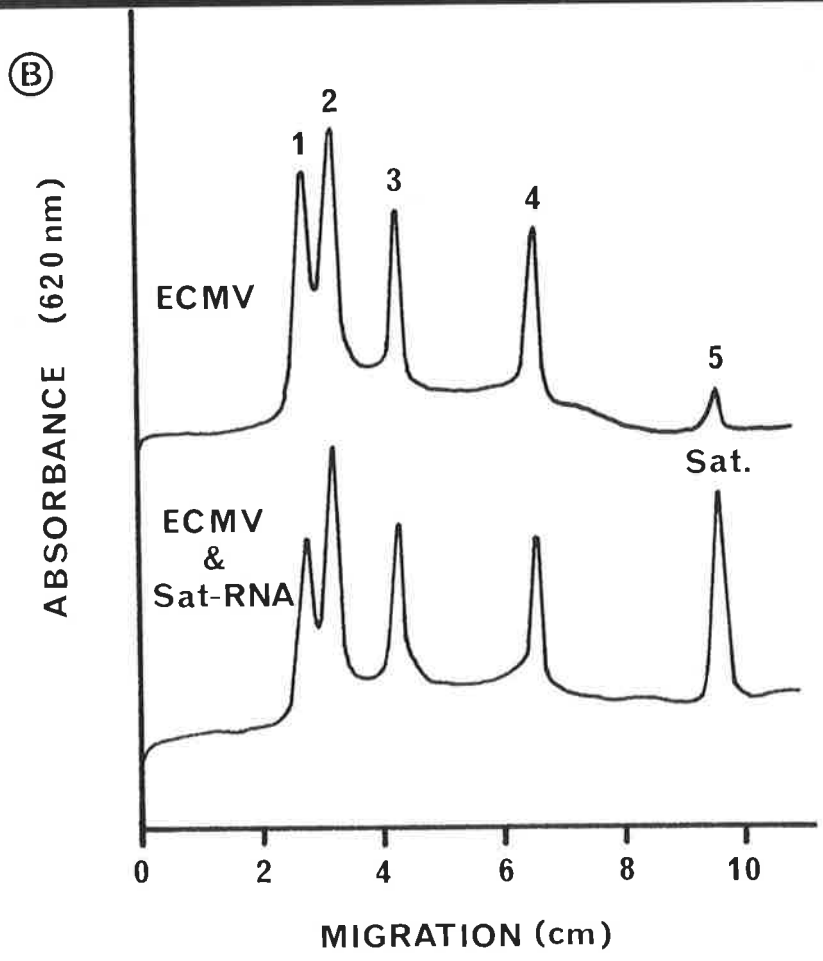
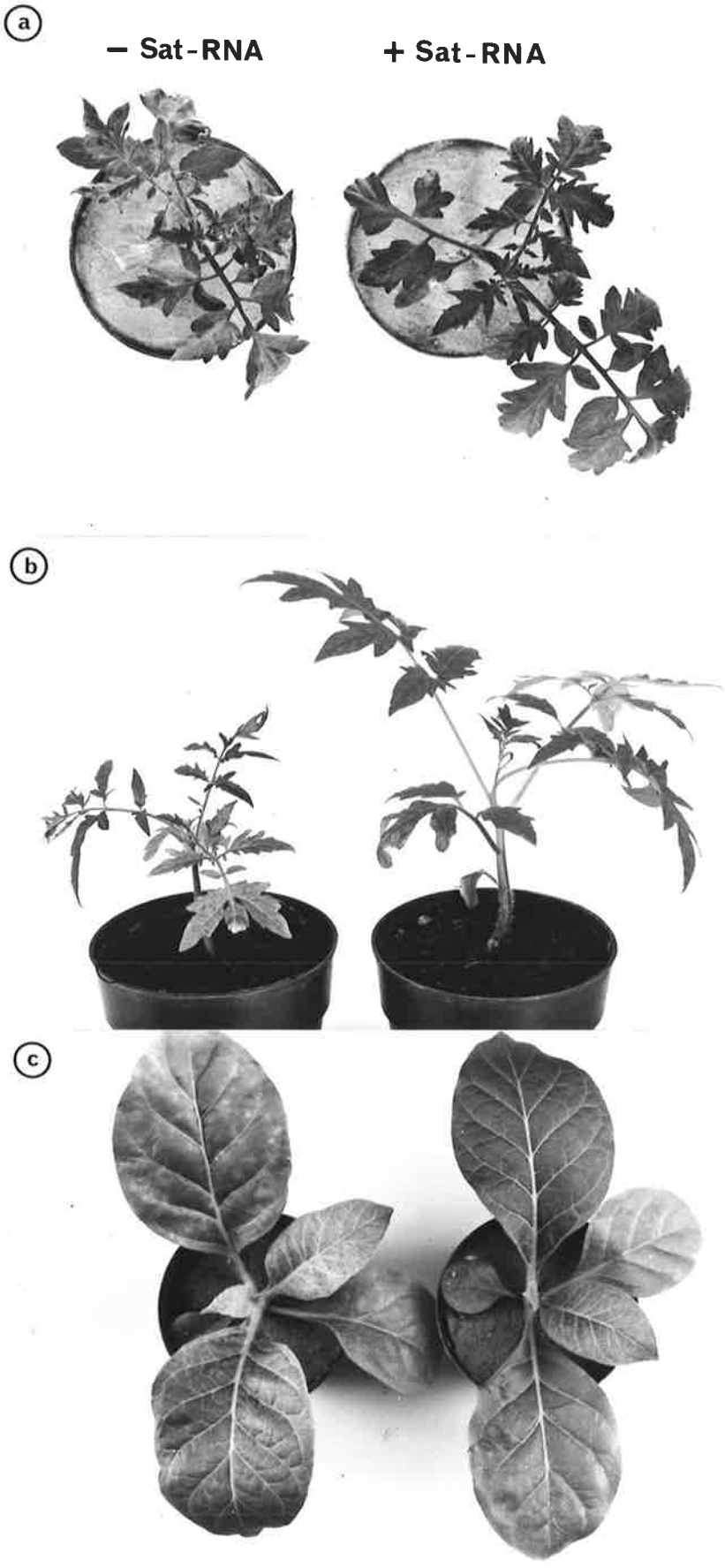

B
ABSORBANCE (620 nm)
1
2
3
4
5
ECMV
Sat.
ECMV
&
Sat-RNA
0
2
4
6
8
10
MIGRATION (cm)

Figure 5.8. Attenuation of symptoms induced by WAICMV in 'Rutgers' tomato plants and 'White Burley' tobacco plants in the presence of Sat-RNA. Plants were inoculated with WAICMV-RNA, with and without Sat-RNA as described in Table 5.6.

A. Symptoms induced in tomato plants inoculated with WAICMV-RNA alone (left) and WAICMV-RNA and Sat-RNA (right) 12 days after inoculation. Propagative conditions were as described in Table 5.6.

B. Symptoms induced in tomato plants inoculated with WAICMV-RNA alone (left) and WAICMV-RNA and Sat-RNA (right) 18 days after inoculation. Plants were maintained in an air-cooled glasshouse.

C. Symptoms induced in tobacco plants inoculated with WAICMV-RNA alone (left) and WAICMV-RNA and Sat-RNA (right) 12 days after inoculation.
Propagative conditions were as described in Table 5.6.


a
− Sat-RNA
+ Sat-RNA
b
c

and tobacco, and was invariably associated with a reduction in the yield of recoverable virus and in the production of substantial levels of Sat-RNA. Symptoms induced by both VTAV and NTAV, two isolates of TAV which support a production of Sat-RNA to low levels (Figure 5.4), were not noticeably attenuated by the presence of Sat-RNA (Table 5.6).

Attempts to Detect Sat-RNA Sequences in Nucleic Acids Isolated from Healthy Plants

It has been demonstrated that CARNA 5 was often recovered in preparations of CMV following repeated transfer of the virus in *N. tabacum* cv. Xanthi nc (Kaper and Tousignant, 1977; Waterworth *et al.*, 1978). Although these authors do not suggest how CARNA 5 was introduced into strains of CMV following their transfer in 'Xanthi nc' tobacco plants, it seems possible that this satellite may have been derived from sequences contained within the genome of this host plant species. To test the possibility that Sat-RNA also may represent a complementary copy of sequences within the genome of 'Xanthi nc' tobacco plants, DNA extracted from healthy leaf tissue of this host (a gift from Dr R.I.B. Francki) was hybridized with cDNA(Sat-RNA) to a $C_o t$ value of 2.25×10^4 mol sec litre^{-1}. At this high $C_o t$ value, it would be theoretically possible to detect one or more copies of Sat-RNA per cell DNA complement, assuming that the nucleotide complexity of tobacco DNA, like that of tomato, is approximately 2.35×10^{12} (Hadidi *et al.*, 1976).

In this experiment, cDNA(Sat-RNA) hybridized with the tobacco DNA to less than 1%, indicating that at least this selection of 'Xanthi nc' does not contain sequences common to those of Sat-RNA. Gould *et al.* (1978) have demonstrated that leaf RNAs isolated from healthy *N. clevelandii* plants also failed to hybridize significantly with cDNA(Sat-RNA). However,

these results do not exclude the possibility that Sat-RNA (or similar RNAs) are derived from sequences within the genomes of their host plants and it is difficult to dismiss the possibility that in the course of their experiments (Kaper and Tousignant, 1977; Waterworth *et al.*, 1978), CARNA 5 had not been inadvertently introduced as a contaminant into plants infected with CMV.

Conclusions

Sat-RNAs isolated from four isolates of CMV have indistinguishable base sequences as determined by molecular hybridization analysis and presumably have a common origin. This conclusion is corroborated by the demonstration that Sat-RNA is readily transmissible between different cucumoviruses, and has an extraordinarily high specific infectivity. With all isolates of CMV examined, Sat-RNA was replicated to high levels. In contrast, VTAV and NTAV, like $T_1T_2Q_3$ and $T_1T_2G_3$ (Figures 4.2 and 4.3), support the production of Sat-RNA to only low levels. These results suggest that the replication of Sat-RNA by cucumoviruses is controlled by gene(s) located on RNAs 1 and/or 2. In addition to the helper virus, replication of Sat-RNA is also influenced by the plant host species.

Sat-RNA cannot be considered a pathogen *per se*, since, unlike CARNA 5, it does not increase the severity of symptoms produced by its helper cucumoviruses in any of the host species examined. In fact in several CMV-host combinations, the presence of Sat-RNA actually attenuates the symptoms produced.

CHAPTER 6

COMPARATIVE BASE SEQUENCE HOMOLOGY BETWEEN Sat-RNA AND CARNA 5

The apparent inability of Sat-RNA to induce necrosis in 'Rutgers' tomato plants infected with a number of isolates of CMV and TAV (Figures 5.7 and 5.8, Table 5.6) suggested that Sat-RNA may differ from CARNA 5 (Kaper and Waterworth, 1977). To compare the biological properties of Sat-RNA and CARNA 5, and the extent of nucleotide sequence homology between these two satellite RNAs, a collaborative research project with Dr J.M. Kaper (Plant Virology Laboratory, Plant Protection Institute, Beltsville, U.S.A.) was undertaken. Australian quarantine authorities prohibited the propagation of CARNA 5 in our laboratory, hence experiments to compare the ability of Sat-RNA and CARNA 5 to induce necrosis in tomato plants were undertaken in Dr Kaper's laboratory. A sample of purified CARNA 5 obtained from Dr Kaper was used in molecular hybridization experiments in this laboratory to compare the nucleotide sequence homology between Sat-RNA and CARNA 5.

Experimental

The CARNA 5 preparation obtained from Dr J.M. Kaper had been isolated from CMV-S and purified by sucrose density gradient centrifugation (Kaper and Tousignant, 1978; Figure 1A). [^{32}P]cDNA was transcribed from polyadenylated Sat-RNA and CARNA 5 as described in Chapter 2. Sat-RNA and CARNA 5 were polyadenylated with equal efficiency; the incorporation of [^{32}P]ATP was approximately 59% and 51% respectively. An analysis of

the size distribution of the cDNA preparation transcribed from each satellite RNA indicates that cDNA(Sat-RNA) was approximately the same length as Sat-RNA itself (Figure 6.1 A). In contrast, the average length of cDNA(CARNA 5) was only approximately half that of cDNA(Sat-RNA) (Figure 6.1 B). Similar results were obtained in two different experiments with cDNA transcribed from this preparation of CARNA 5. It was not possible to determine whether the CARNA 5 preparation used for cDNA transcription was extensively degraded as there was insufficient material available for electrophoretic analysis.

The kinetics of hybridization of cDNA(Sat-RNA) and cDNA(CARNA 5) with their homologous and heterologous RNAs are presented in Figure 6.2. The cDNA(Sat-RNA) hybridized with Sat-RNA and CARNA 5 to approximately 85% and 64% respectively (Figure 6.2 A). The $R_o t_{\frac{1}{2}}$ values for the homologous and heterologous hybridization were 0.45×10^{-3} and 0.7×10^{-3} mol sec litre^{-1} respectively. Values obtained for the hybridization of cDNA(CARNA 5) with CARNA 5 and Sat-RNA were considerably lower (Figure 6.2 B), being 55% and 28% respectively. The $R_o t_{\frac{1}{2}}$ values for both the homologous and heterologous hybridizations were 0.9×10^{-3} mol sec litre^{-1}.

To further investigate the extent of sequence homology between Sat-RNA and CARNA 5, the thermal stability of hybrids between cDNA preparations transcribed from each satellite RNA with their homologous and heterologous RNAs was compared. The kinetics of the thermal denaturation of RNA-cDNA hybrids provide an indication of the extent of base mismatching within those regions participating in hybrid formation. The comparative thermal stabilities of the cDNA-RNA hybrids were investigated as described by Gould and Symons (1977). To ensure complete hybrid formation, the

Figure 6.1. Size distribution of [^{32}P]cDNA transcribed from Sat-RNA and CARNA 5 as determined by electrophoresis under denaturing conditions. Tube gels containing 4% acrylamide in 98% formamide were prepared as described in Chapter 2 and electrophoresed at 1 mA/gel for 16 hr at room temperature. QCMV-RNAs 3 and 4, Sat-RNA and *E. coli* tRNA were included with each cDNA sample. After staining briefly in toluidine blue and destaining in water to locate the position of marker RNAs, gels were sliced (1 mm) with a Mickle gel slicer and the radioactivity of gel slices measured by Cerenkov radiation.

A. cDNA(Sat-RNA) B. cDNA(CARNA 5)

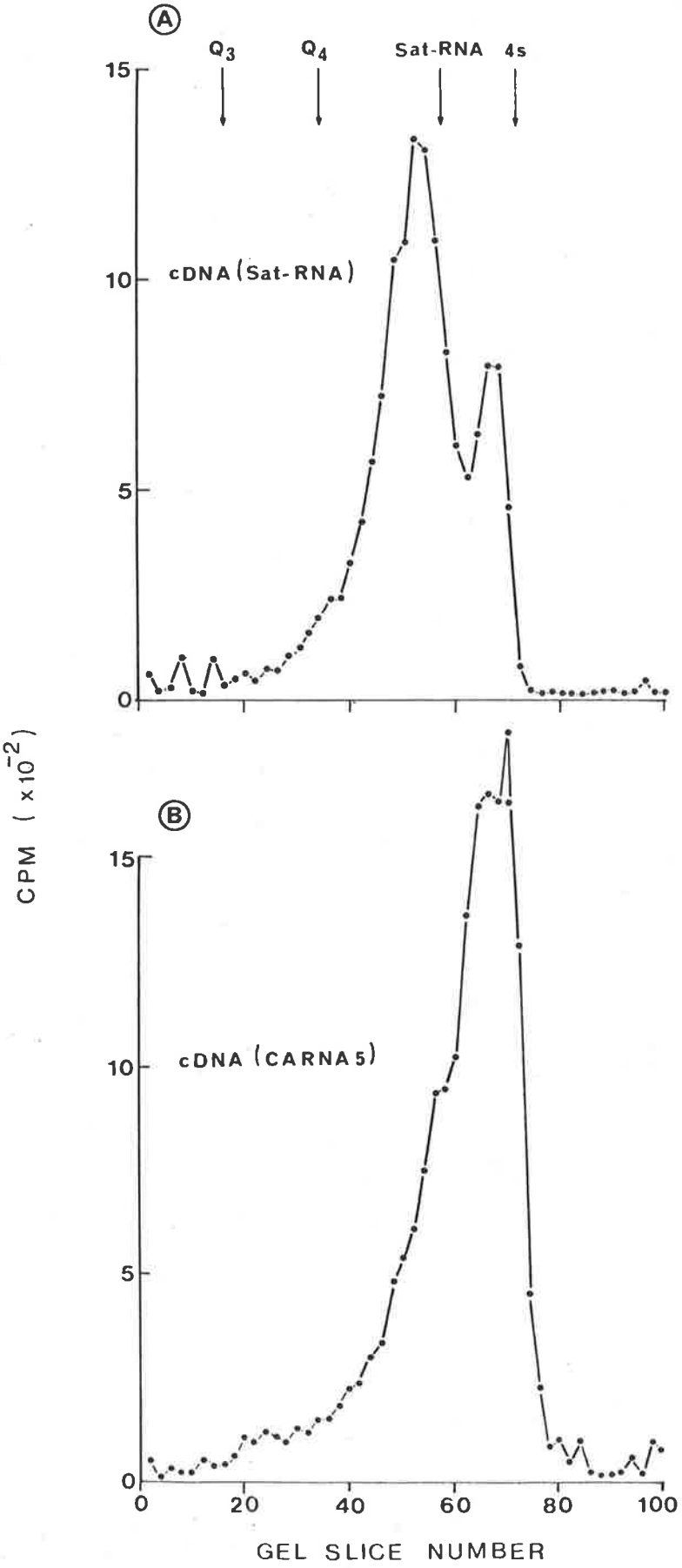
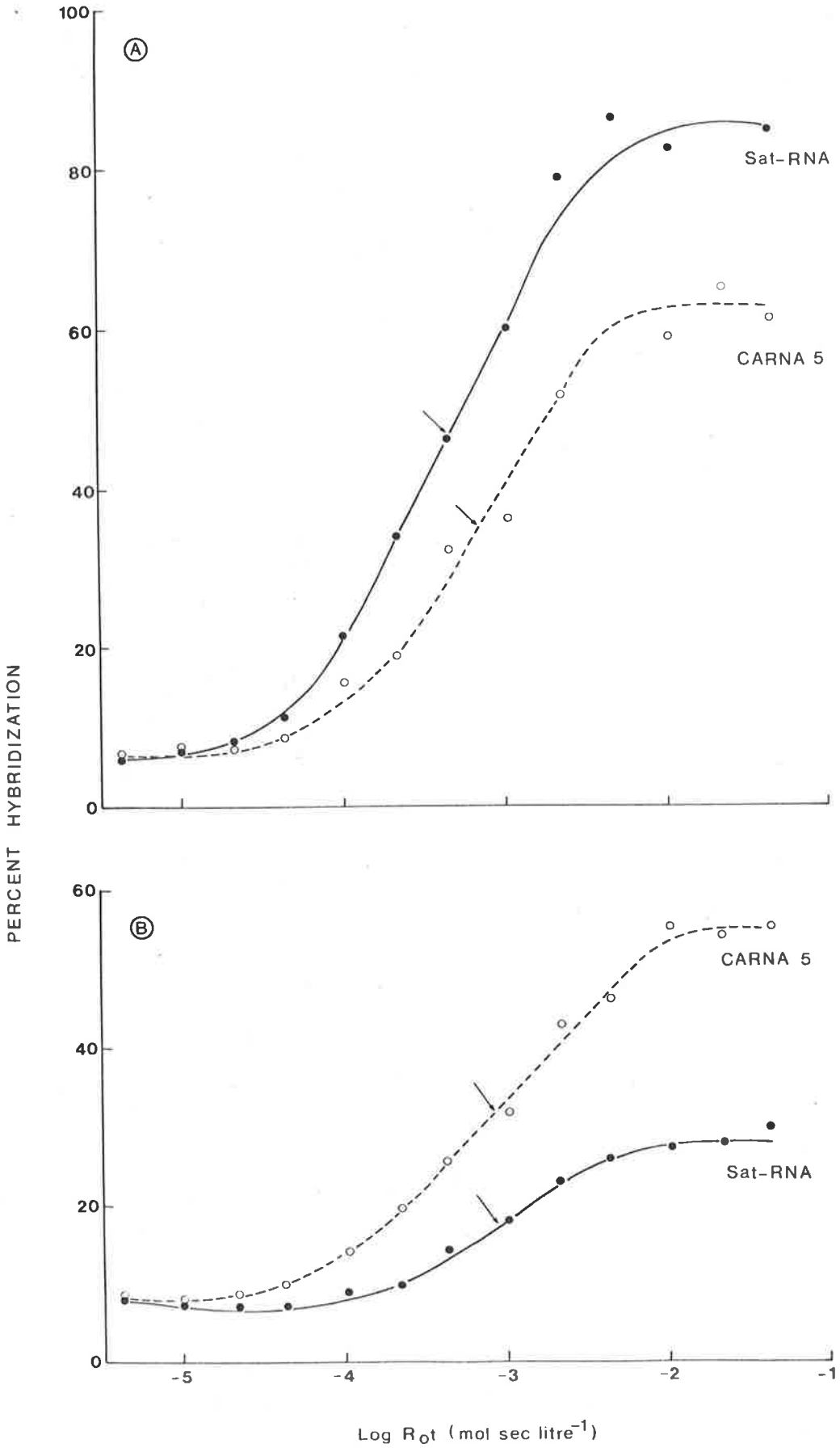

A
Q3
Q4
Sat-RNA
4s
15
10
5
0
cDNA (Sat-RNA)
B
15
10
5
0
cDNA (CARNA 5)
CPM (x10^-2)
0
20
40
60
80
100
GEL SLICE NUMBER

Figure 6.2. Kinetics of hybridization of cDNA(Sat-RNA) and cDNA(CARNA 5) with Sat-RNA and CARNA 5. The conditions of hybridization and S_1 nuclease assays were as described in Chapter 2. $R_o t_{\frac{1}{2}}$ values for each hybridization reaction are indicated by arrows.

A. cDNA(Sat-RNA) hybridized with: ● - Sat-RNA and o - CARNA 5. The $R_o t_{\frac{1}{2}}$ values for the homologous and heterologous hybridization reactions were 0.45×10^{-3} and 0.70×10^{-3} mol sec litre^{-1} respectively.

B. cDNA(CARNA 5) hybridized with: o - CARNA 5 and ● - Sat-RNA. The $R_o t_{\frac{1}{2}}$ values obtained for both hybridization reactions were 0.9×10^{-3} mol sec litre^{-1}.


100
80
60
40
20
0
Ⓐ
Sat-RNA
CARNA 5
PERCENT HYBRIDIZATION
60
40
20
0
Ⓑ
CARNA 5
Sat-RNA
-5
-4
-3
-2
-1
Log R_{ot} (mol sec litre^{-1})

homologous and heterologous RNA-cDNA mixtures were hybridized to a $R_o t$ value of 0.1 mol sec litre^{-1}. Siliconized capillary tubes containing the hybrid mixtures were then heated for 5 min at temperatures ranging from 60° to 100°C and chilled rapidly in an ice-water bath. The percentage of each hybrid mixture which was resistant to S_1 nuclease at the various incubation temperatures was determined as before.

Hybrids of cDNA(Sat-RNA) and Sat-RNA melted with a sharp thermal transition and a high T_m value (88°C) indicating that the hybrid formation was specific with no evidence of base mismatching (Figure 6.3 A). In contrast, hybrids of cDNA(Sat-RNA) and CARNA 5 melted with a much broader thermal transition and a lower T_m value (79°C), indicating a significant degree of base mismatching. Similar results were obtained for hybrids between cDNA(CARNA 5) and its homologous and heterologous RNAs (Figure 6.3 B) with the exception that the T_m values were considerably lower (78° and 68°C respectively).

The lower thermal stability of hybrids containing cDNA (CARNA 5) suggested that hybrids between this cDNA preparation and Sat-RNA (Figure 6.3 B) may not have annealed to their full extent at hybridization temperatures of 60°C. Since a temperature approximately 20°C below the T_m value is optimal for hybrid formation (Hutton, 1977) the apparent differences in the estimates of sequence homology between Sat-RNA and CARNA 5 (Figure 6.2) using cDNA(Sat-RNA) and cDNA(CARNA 5) may be ascribed to the instability of cDNA(CARNA 5) - Sat-RNA hybrids.

To investigate this possibility, the homologous and heterologous RNA-cDNA mixtures were hybridized to a $R_o t$ value of 0.1 mol sec litre^{-1} at incubation temperatures of either 60° or 45°C. Results summarized in

Figure 6.3. Thermal stability of hybrids of cDNA(Sat-RNA) and cDNA(CARNA 5), and their homologous and heterologous RNAs. Homologous and heterologous reactions were hybridized at 60°C to a $R_o t$ value of 0.1 mol sec litre^{-1}, heated for 5 min at each temperature, and cooled rapidly. The fraction of each hybrid remaining at the various temperatures was assayed as before using S_1 nuclease. T_m values are indicated by arrows.

A. cDNA(Sat-RNA) hybridized with: ● - Sat-RNA, o - CARNA 5. The T_m values obtained for the homologous and heterologous hybrids were 88° and 79°C respectively.

B. cDNA(CARNA 5) hybridized with: o - CARNA 5, ● - Sat-RNA. The T_m values for the homologous and heterologous hybrids were 78° and 68°C respectively. Note the instability of the heterologous hybrid.

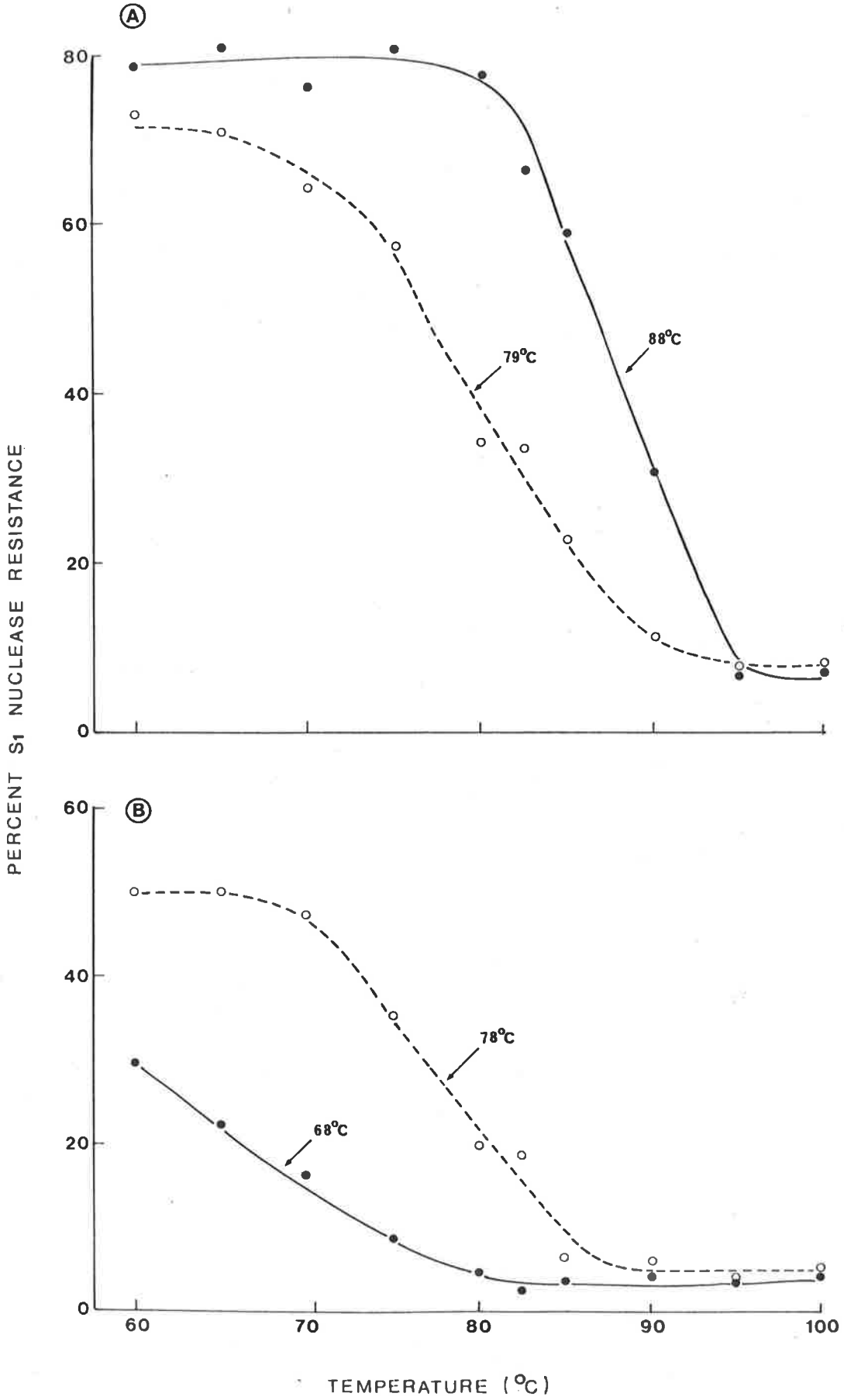

A
80
60
40
20
0
88°C
79°C
B
60
40
20
0
78°C
68°C
60
70
80
90
100
PERCENT S1 NUCLEASE RESISTANCE
TEMPERATURE (°C)

Table 6.1. Determination of the sequence homology between Sat-RNA and CARNA 5.

Hybridization mixture[a]	% hybridization[b] 60°C	45°C
cDNA(Sat-RNA) hybridized with		
Sat-RNA	100	100
CARNA 5	82.9	80.1
cDNA(CARNA 5) hybridized with		
CARNA 5	100	100
Sat-RNA	46.5	63.2

a cDNA(Sat-RNA) and cDNA(CARNA 5) were hybridized with their homologous and heterologous RNAs at either 60°C or 45°C to a R_0t value of 0.1 mol sec litre^{-1}. S_1 nuclease digestions were as described before.

b Hybridization values for the homologous reactions at each hybridization temperature were taken as 100% and the values for the heterologous hybridization reactions normalized on this basis. Values are the means of at least 3 replicates for each hybridization reaction. Actual hybridization values obtained at 60° and 45°C for the homologous hybridization reactions were 74.7% and 84.7% for cDNA(Sat-RNA) and 57.0% and 51.2% for cDNA(CARNA 5) respectively. The S_1 nuclease-resistance of cDNA(Sat-RNA) and cDNA(CARNA 5) preparations hybridized in the absence of added RNA (4.0% and 3.0% respectively) was subtracted from these values.

Table 6.1 demonstrate that decreasing the hybridization temperature from 60^{o} to 45^{o}C increased the extent to which cDNA(CARNA 5) hybridized with Sat-RNA from 46.5% to 63.2%. Reducing the incubation temperature did not significantly alter the extent of hybridization between cDNA(Sat-RNA) and CARNA 5 (Table 6.1).

Conclusions

Results presented in this chapter indicate that Sat-RNA and CARNA 5 have significant base sequence homology, although they are readily distinguishable by hybridization analysis. A comparison of the values obtained when cDNA(Sat-RNA) is hybridized with Sat-RNA and CARNA 5 indicate that the two satellite RNAs have approximately 80% of their base sequences in common (Figure 6.2, Table 6.1). However, the thermal denaturation kinetics of hybrids formed between cDNA(Sat-RNA) and CARNA 5 indicate extensive base mismatching, suggesting that the true estimate of the sequence homology between Sat-RNA and CARNA 5 is somewhat lower than that obtained by hybridization analysis. Assuming that T_m value is reduced by 1.1^{o}C per percent base mismatching (Wetmur, 1976), the corrected estimate for the extent of sequence homology between Sat-RNA and CARNA 5 is approximately 71%.

The inability of CARNA 5 to yield 'full length' transcripts in these experiments, and the anomalous behaviour of cDNA(CARNA 5) in hybridization experiments is discussed in detail in Chapter 9. However, it seems reasonable to conclude that the inability of Sat-RNA to induce systemic necrosis in tomato plants infected with CMV (Table 5.6) may well be explained on the basis that Sat-RNA and CARNA 5 have sufficient differences in their primary structure to render them biologically distinct.

CHAPTER 7

REPLICATION AND *IN VIVO* SURVIVAL OF Sat-RNA

Of the 14 cucumovirus isolates examined, Sat-RNA was detected initially in only four. It has been demonstrated that the base sequences of Sat-RNA isolated from the four strains are indistinguishable by molecular hybridization. Sat-RNA can be readily transmitted from one cucumovirus isolate to another. In this chapter are described investigations of the association between Sat-RNA, its helper cucumoviruses and their plant hosts.

Dependence of Sat-RNA Replication on Helper Viruses

It was recently suggested that the replication of Sat-RNA depends on its association with cucumoviruses (Gould *et al.*, 1978). However, in these studies, Sat-RNA was detected by its recovery in capsids of the associated cucumoviruses. Kaper *et al.* (1976) stated that inoculation with isolated CARNA 5 alone '... *gives no disease symptoms in tobacco, nor can free or encapsidated CARNA 5 be extracted from such plants*'. Unfortunately, they did not provide experimental details. Conceivably, Sat-RNA could be capable of autonomous replication but without attendant cucumovirus infection, could have escaped detection. This possibility was investigated as follows.

Groups of *N. clevelandii* plants were inoculated with Sat-RNA alone (2.5 µg/ml), QCMV-RNA alone (100 µg/ml), or with a mixture of Sat-RNA and QCMV-RNA (2.5 and 100 µg/ml respectively). Another group of plants was mock-inoculated with TE buffer alone. The isolate of QCMV used in this experiment (Gould *et al.*, 1978, Isolate A) did not contain

Sat-RNA (see Table 4.3). Total leaf RNAs were extracted from six inoculated leaves from plants of each treatment at various intervals after inoculation and hybridized with [^{32}P]cDNA(Sat-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}. Results summarized in Table 7.1 indicate that the small amount of Sat-RNA detected in leaf-RNA extracts from plants inoculated with Sat-RNA alone, decreased soon after inoculation. These results indicate that Sat-RNA is incapable of autonomous replication. The apparently significant amount of Sat-RNA detected 1 day after inoculation was possibly that still present on the leaf surface. Leaf RNAs from plants inoculated with QCMV-RNA alone failed to hybridize significantly with cDNA (Sat-RNA). In contrast, when inoculated in the presence of QCMV-RNA, Sat-RNA increased rapidly to a level which hybridized completely with the cDNA used for its detection (slightly more than 80% in this experiment, Table 7.1). The hybridization conditions used in these experiments were chosen to detect the replication of Sat-RNA rather than to determine its actual concentration in leaf RNA extracts.

Dependence of Sat-RNA Replication on Cucumoviruses

Sat-RNA has several properties in common with the satellite of TRSV (S-TRSV; Schneider, 1977). In view of these similarities, TRSV seemed a logical candidate to test for its ability to support the replication of Sat-RNA. As AMV, which has been classified in a group of its own, and cucumoviruses have a number of physical and biological properties in common (Fenner, 1976), the experimental transmission of Sat-RNA to this virus was also attempted.

AMV-RNA, TRSV-RNA and QCMV-RNA at 750, 50 and 100 µg/ml respectively were inoculated to *N. tabacum* cv. White Burley in the

Table 7.1. Detection of Sat-RNA in leaf RNA extracts from plants inoculated with Sat-RNA in the presence and absence of QCMV[a].

Time after inoculation (days)	% Hybridization[b] RNAs isolated from leaves inoculated with		
	Sat-RNA alone	QCMV-RNA alone	Sat-RNA+ QCMV-RNA
1	8.4	1.1	14.1
3	3.3	2.0	82.0
7	4.0	2.3	82.5
10	2.8	2.0	84.0
14	2.5	3.0	86.5

a Leaves of *N. clevelandii* plants were inoculated with Sat-RNA (2.5 µg/ml), QCMV-RNA (100 µg/ml) or a mixture of each (2.5 and 100 µg/ml respectively), and at the intervals indicated above, 6 leaves from plants of each treatment were harvested. Leaf RNAs were extracted as described in Chapter 2.

b Leaf RNA extracts were hybridized to a $R_o t$ of 10.0 mol sec litre^{-1} with cDNA (Sat-RNA). RNAs isolated from healthy leaves mock-inoculated with TE buffer alone hybridized with cDNA (Sat-RNA) to 1.5%. The hybridization value for this cDNA preparation hybridized in the absence of added RNA was 4.0% and has been deducted from each value. Purified Sat-RNA hybridized with cDNA(Sat-RNA) to 82.5% in this experiment.

presence and absence of Sat-RNA at a concentration of 2.5 µg/ml. The QCMV isolate used in these experiments (Gould *et al.*, 1978; Isolate A), did not contain Sat-RNA. At these inoculum concentrations, each RNA preparation produced approximately 200 lesions per half-leaf on cowpea leaves. Twelve days after inoculation, the viruses were purified from systemically infected leaf tissue from plants of each treatment, and their RNAs isolated. Viral RNAs were then examined for the presence of Sat-RNA by electrophoresis in 2% agarose gels.

Results presented in Figure 7.1 indicate that whereas Sat-RNA was present in the QCMV preparation, none could be detected in preparations of either TRSV or AMV. However, these results do not preclude the possibility that Sat-RNA replicated in the presence of AMV and/or TRSV but failed to be encapsidated by these viruses. This possibility was investigated as follows.

Groups of *N. clevelandii* plants were inoculated with AMV-RNA, TRSV-RNA or QCMV-RNA in the presence or absence of Sat-RNA using the same inoculum concentrations as described in Figure 7.1. In addition, groups of plants were also inoculated with Sat-RNA alone (2.5 µg/ml) or mock-inoculated with TE buffer alone. RNA extracted from inoculated leaves from plants of each treatment were hybridized with cDNA(Sat-RNA) to a $R_o t$ of 10.0 mol sec litre^{-1}.

Results summarized in Table 7.2 demonstrate that whereas Sat-RNA replicated in the presence of QCMV, no Sat-RNA was detected in plants infected with either AMV or TRSV.

Figure 7.1. Differential recovery of Sat-RNA in preparations of TRSV, AMV and QCMV. TRSV-RNA, AMV-RNA and QCMV-RNA at concentrations of 50, 750 and 100 µg/ml respectively were inoculated to *N. tabacum* cv. White Burley in the presence and absence of Sat-RNA (2.5 µg/ml). Viruses were purified from systemically infected leaves 12 days after inoculation, and their RNAs isolated as described in Chapter 2. RNA samples of each virus preparation were then subjected to electrophoresis in 2% agarose gels.

a. RNA isolated from virus propagated in the absence of Sat-RNA.

b. RNA isolated from virus in which the inoculum had been supplemented with Sat-RNA.

c. TRSV-RNA or AMV-RNA as in (b) co-electrophoresed with QCMV-RNA containing Sat-RNA.

d. QCMV-RNA with Sat-RNA alone.

T_s and T_l indicate the small ($\sim 1.4 \times 10^6$) and large ($\sim 2.4 \times 10^6$) RNA components of TRSV respectively. A_{1-4} and Q_{1-4} indicate the 4 major RNA components of AMV and QCMV respectively.

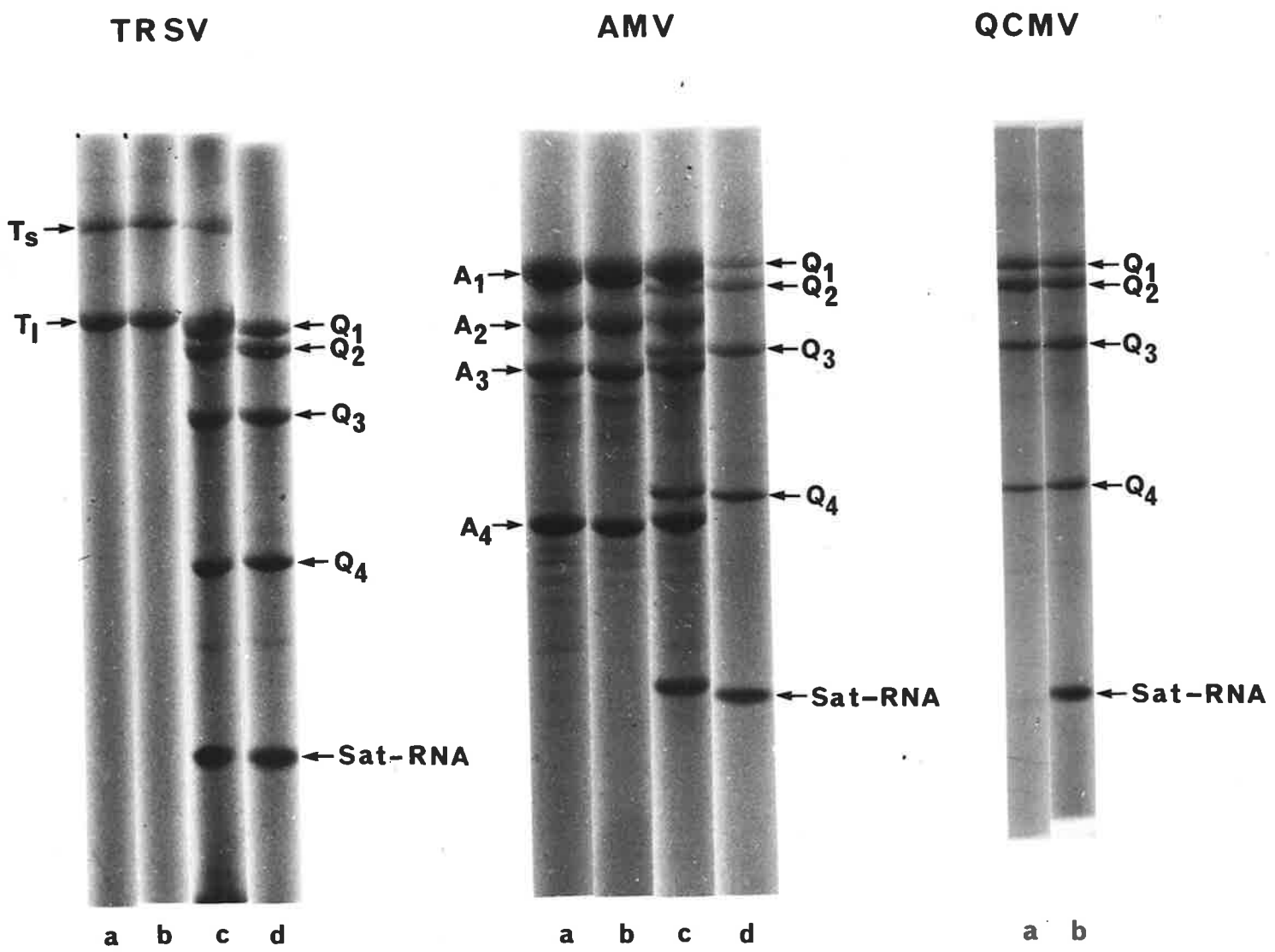

TRSV
AMV
QCMV
Ts
Tl
Q1
Q2
Q3
Q4
Sat-RNA
A1
A2
A3
A4
a
b
c
d

Table 7.2. Dependence and specificity of Sat-RNA replication on CMV.

Leaf RNAs isolated from[a] plants inoculated with	% Hybridization[b]
Buffer	2.0
Sat-RNA	2.4
QCMV-RNA	4.9
QCMV- and Sat-RNA	74.0
AMV-RNA	2.0
AMV- and Sat-RNA	2.2
TRSV-RNA	1.9
TRSV- and Sat-RNA	2.6

a QCMV-RNA, AMV-RNA and TRSV-RNA at 100, 750 and 50 µg/ml respectively (each RNA preparation produced approximately 200 lesions/half-leaf on cowpeas) were inoculated to leaves of *N. clevelandii* with and without added Sat-RNA (2.5 µg/ml). RNA dilutions were prepared in TE buffer. Leaf RNAs were extracted 5 days after inoculation.

b Leaf RNAs were hybridized with cDNA(Sat-RNA) to a $R_o t$ of 10.0 mol sec litre^{-1}. Values are the means of two replicates and have been corrected for the S_1 nuclease resistance of the cDNA(Sat-RNA) preparation (4.0%). Purified Sat-RNA hybridized to 75.0% in this experiment.

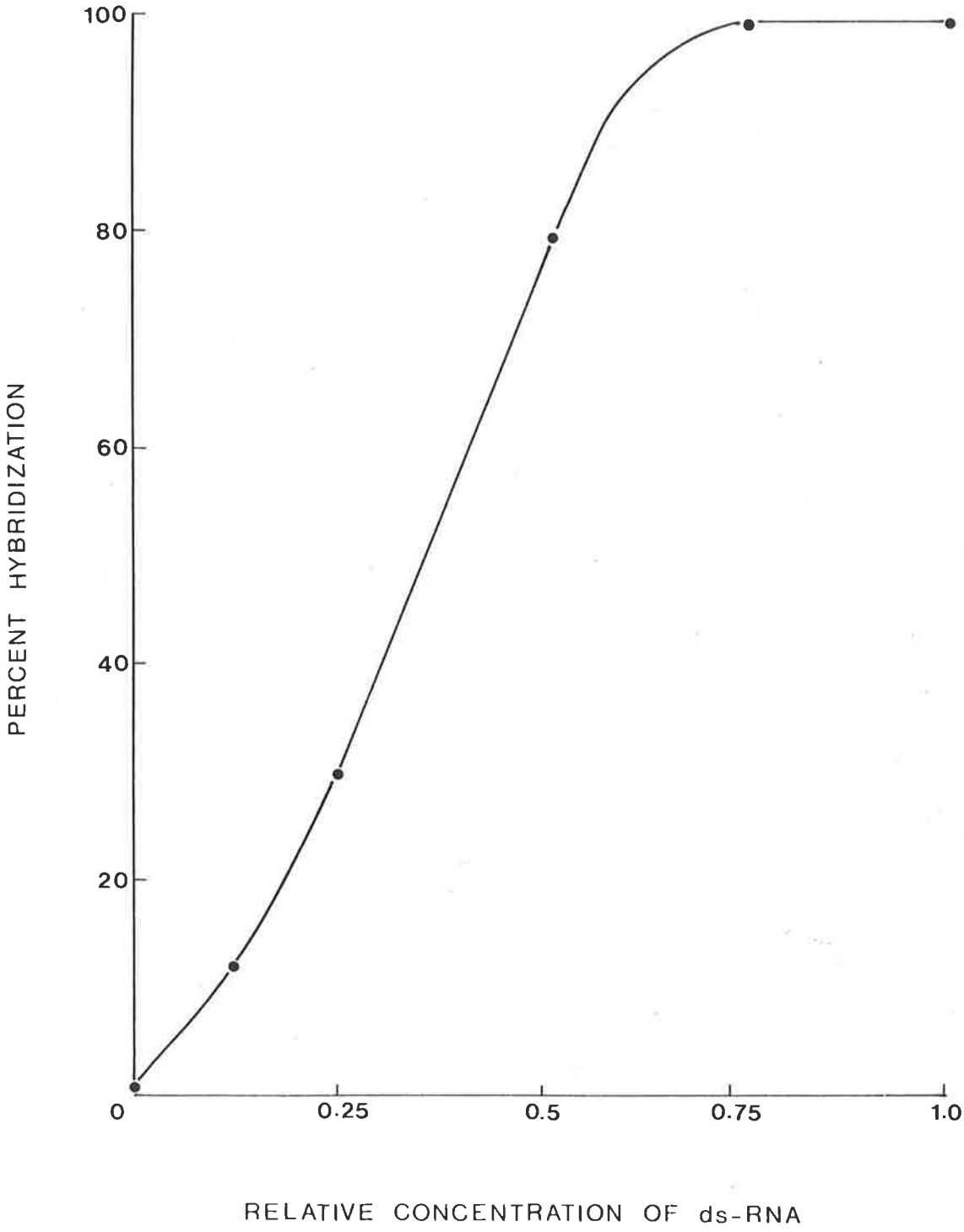
Detection of ds-RNA Associated with the Replication of Sat-RNA

The replication of single-stranded RNA viruses is invariably associated with the accumulation of double-stranded (ds) replicative forms (RF) specific to the viral infection (Zaitlin, 1977). Replicative forms consist of two complementary RNA strands; a plus strand which has the same base sequence as that in viral RNA, and its complementary minus strand. Although the role of RF in virus replication is not entirely clear (Siegel and Hariharasubramanian, 1974), its presence in infected cells is indicative of viral RNA synthesis. The presence of RF specific to Sat-RNA was investigated in RNase-treated nucleic acid preparations extracted from plants inoculated with TE buffer alone, CMV-RNA alone, Sat-RNA alone, or a mixture of CMV-RNA and Sat-RNA, using [^{125}I]Sat-RNA as a probe.

Leaves of *N. clevelandii* inoculated with ECMV-RNA at 200 µg/ml, Sat-RNA at 2.5 µg/ml, TE buffer, or with a mixture of ECMV-RNA and Sat-RNA at 200 and 2.5 µg/ml respectively were harvested 5 days after inoculation. Leaf RNAs were extracted and incubated with RNase A to remove ss-RNA. RNase-resistant nucleic acids (ds-RNA and/or DNA) from approximately 30 g of inoculated leaves from each group of plants were suspended in 500 µl TE buffer and hybridized with [^{125}I]Sat-RNA.

Results presented in Figure 7.2 demonstrate that ds-RNA specific to Sat-RNA was detected only in nucleic acid preparations from plants inoculated with both ECMV-RNA and Sat-RNA. Hence, the replication of Sat-RNA, like that of the genomic RNAs of cucumoviruses (Takanami *et al.*, 1977), is associated with the accumulation of a specific RF.

Figure 7.2. Kinetics of hybridization of ds-RNA specific to Sat-RNA with [^{125}I]Sat-RNA. Nucleic acids were extracted from inoculated leaves from each group of plants 5 days after inoculation, and treated with RNase A. Approximately 4,000 cpm of [^{125}I]Sat-RNA (∿ 20 ng) was added to each assay. Hybridization conditions and the assay of hybrid formation using RNases A and T_1 were as described in Chapter 2. [^{125}I]Sat-RNA in the absence of added nucleic acid hybridized to 0.3%. A relative concentration of 1.0 indicates the ds-RNA preparation was hybridized undiluted. The hybridization values obtained for undiluted nucleic acid preparations from leaves inoculated with TE buffer, Sat-RNA alone, and ECMV-RNA alone in this experiment were 2.6%, 3.4% and 4.0% respectively.


100
80
60
40
20
0
0.25
0.5
0.75
1.0
PERCENT HYBRIDIZATION
RELATIVE CONCENTRATION OF ds-RNA

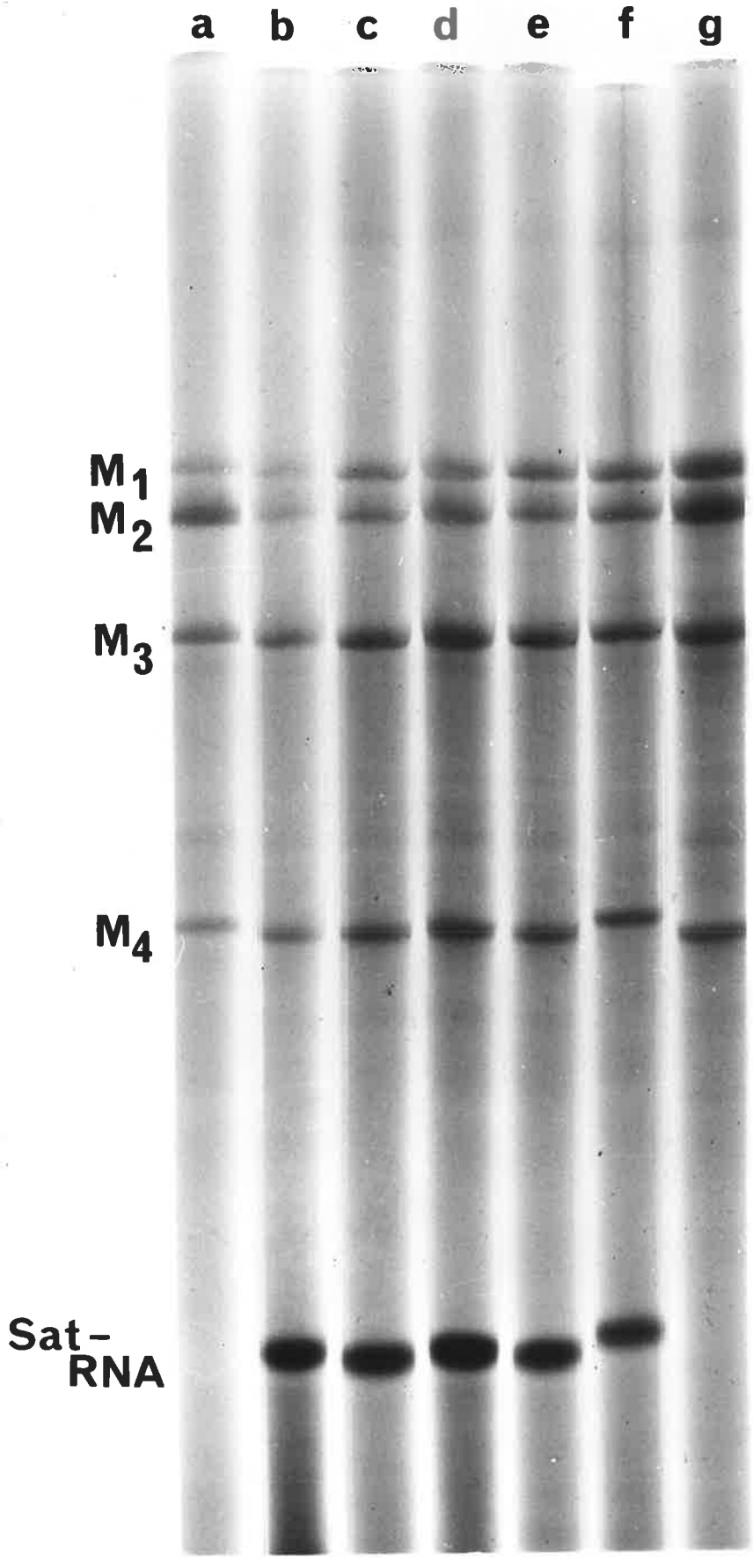
In vivo Survival of Sat-RNA in the Absence of Helper Virus

The highly infectious nature of Sat-RNA (Table 5.5) and observations that it was inadvertently spread in the glasshouse to strains of CMV which, when first isolated, did not contain this satellite prompted a study of the interaction of this unusual RNA with its helper viruses and plant hosts.

In a preliminary study of the interaction between Sat-RNA and MCMV, a strain of CMV which did not contain Sat-RNA when first isolated but which readily supports its replication to high levels (Figure 5.4), an unexpected result was obtained. MCMV preparations purified from systemically infected tissue of *N. clevelandii* which had been inoculated with Sat-RNA at 2.5 µg/ml up to 4 days before reinoculation with MCMV-RNA at 100 µg/ml contained substantial amounts of Sat-RNA (Figure 7.3). The failure to detect Sat-RNA in progeny virus when MCMV-RNA was applied to younger leaves above those which had been inoculated 4 days previously with Sat-RNA (Figure 7.3 g) indicates that Sat-RNA is not translocated in plant tissue in the absence of a helper cucumovirus. Subsequent experiments in which the interval between application of the two inocula was increased to as long as 14 days, confirmed the ability of Sat-RNA to survive *in vivo* for prolonged periods in the absence of a helper virus. However, interpretation of the results of some of these experiments is complicated by the presence of variable amounts of the genomic RNA breakdown product, RNA 5 (Gould *et al.*, 1978) in control MCMV preparation from plants inoculated with MCMV-RNA alone. Sat-RNA and RNA 5 have indistinguishable electrophoretic mobilities under the conditions of gel electrophoresis used in these studies (Figure 5.1).

Figure 7.3. Survival of Sat-RNA *in vivo*, and its subsequent recovery in preparations of MCMV.

Groups of 3 *N. clevelandii* plants were inoculated with MCMV-RNA alone at 100 µg/ml (a), or with a mixture of MCMV-RNA and Sat-RNA at 100 and 2.5 µg/ml (b), and after various intervals, with MCMV-RNA at 100 µg/ml; Sat-RNA followed by MCMV-RNA after 12 hr (c), 24 hr (d), 48 hr (e), and 96 hr (f). To another group of plants, MCMV-RNA at 100 µg/ml was applied to uninoculated leaves above those which had been inoculated 96 hr previously with Sat-RNA (g). Systemically infected leaf tissue from each group of plants was harvested 12 days after infection with MCMV. RNA isolated from purified MCMV preparations from each group of plants was then subjected to electrophoresis in 2% agarose gels.


a
b
c
d
e
f
g
M1
M2
M3
M4
Sat-
RNA

To overcome this problem, the ability of Sat-RNA to survive *in vivo* was investigated using the molecular hybridization method as described in Table 7.1. In two independent experiments groups of *N. clevelandii* plants were inoculated with Sat-RNA (2.5 µg/ml), and at either 1, 5, 7 or 10 days later, leaves were reinoculated with QCMV-RNA at a concentration of 100 µg/ml. Another group of plants was inoculated with a mixture of QCMV-RNA and Sat-RNA (100 and 2.5 µg/ml respectively). Other groups of plants were mock-inoculated with TE buffer, or inoculated with Sat-RNA alone (2.5 µg/ml) or QCMV-RNA alone (100 µg/ml). The isolate of QCMV used in these experiments was the same as that used in previous experiments (Tables 4.3 and 7.1). Six inoculated leaves from the various groups of plants were harvested, their RNAs extracted, and hybridized with cDNA(Sat-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}.

Results presented in Table 7.3 demonstrate that Sat-RNA multiplied in all plants inoculated with QCMV-RNA and Sat-RNA irrespective of the interval between application of the two inocula. In contrast no Sat-RNA was detected in leaf RNA extracts from plants inoculated with either Sat-RNA or QCMV-RNA alone. These results confirm the dependence of Sat-RNA replication on its helper cucumoviruses (Tables 7.1 and 7.2) and demonstrate that Sat-RNA can persist in inoculated leaves for at least 10 days without replicating, but can then be induced to do so by the introduction of an appropriate helper virus.

Ability of Other Plant Viral RNAs to Survive *in vivo*

The ability of Sat-RNA to survive *in vivo* in a non-replicating form presents an interesting question: what properties of this RNA or its association with plant cell components protect it from degradation by

Table 7.3. Survival of Sat-RNA *in vivo* in the absence of CMV helper.

Inoculation procedure[a]	Interval between Sat-RNA inoculation and leaf RNA extraction	% Hybridization[b] Expt 1	Expt 2
Buffer	5 days[c]	2.4	2.6
Sat-RNA alone		-	2.1
QCMV-RNA alone		5.0	3.0
Sat-RNA + QCMV-RNA (simultaneous)		-	77.7
Sat-RNA alone	6 days	2.2	-
Sat-RNA followed by QCMV-RNA after 1 day		77.0	-
Sat-RNA	10 days	-	3.2
Sat-RNA followed by QCMV-RNA after 5 days		-	78.5
Sat-RNA alone	12 days	2.5	-
Sat-RNA followed by QCMV-RNA after 7 days		80.3	-
Sat-RNA alone	15 days	-	3.3
Sat-RNA followed by QCMV-RNA after 10 days		-	82.7

a Concentrations of Sat-RNA and QCMV-RNA in the inocula were 2.5 and 100 µg/ml respectively in TE buffer.

b Different [^{32}P]cDNA preparations were used in the two experiments. In Experiment 1, purified Sat-RNA of GCMV hybridized to 79.1%. Hybridization conditions and S_1 nuclease treatments were as described in Chapter 2.

c Leaf RNAs of these treatments were all extracted 5 days after inoculation.

cellular RNases? To understand the mechanism(s) by which Sat-RNA is able to survive *in vivo* for prolonged periods it was desirable to determine if this ability is a common property of other viral RNAs.

In order to demonstrate *in vivo* survival it is necessary that the candidate RNAs are unable to replicate alone but are able to be induced to replicate and be recovered in detectable amounts by the subsequent introduction of an appropriate helper virus system. Two plant viral RNAs; genomic RNA 3 of CMV, and STNV-RNA fulfil these criteria.

QCMV-RNA 3

The pseudorecombinant, $T_1T_2Q_3$, is readily formed *in vivo* following inoculation with a mixture of VTAV-RNAs 1 and 2 and QCMV-RNA 3 (Habili and Francki, 1974c; Chapter 4). In the studies below, the ability to detect *in vivo* survival of QCMV-RNA 3 depended on its subsequent recovery as the pseudorecombinant $T_1T_2Q_3$ following introduction of VTAV-RNA. To determine whether $T_1T_2Q_3$ is able to replicate in the presence of VTAV, and to ascertain conditions which optimise its detection, the following experiment was done.

$T_1T_2Q_3$-RNA and VTAV-RNA, each at 500 µg/ml were inoculated separately or together to groups of *N. clevelandii* plants. Inoculated and systemically infected leaves of each group were harvested after 7 and 12 days respectively and partially purified preparations of each were obtained as described in Table 7.4. The concentrations of $T_1T_2Q_3$ and VTAV in each preparation were then estimated by titrating dilutions of each preparation against antisera specific to QCMV and VTAV. Results presented in Table 7.4 indicate that $T_1T_2Q_3$ replicates to high levels in the presence of VTAV. Surprisingly, $T_1T_2Q_3$ is produced in preference to VTAV, particularly in systemically infected leaf tissue.

Table 7.4. Replication of $T_1T_2Q_3$ in the presence and absence of VTAV in *N. clevelandii*.

		Inoculum[a]		
		VTAV-RNA	$T_1T_2Q_3$-RNA	VTAV-RNA & $T_1T_2Q_3$-RNA
Trial 1	Antigen detected in inoculated leaves[b]			
	VTAV	0.6[c]	-[d]	0.1
	$T_1T_2Q_3$	-	1.5	3.1
Trial 2	Antigen detected in systemically infected leaves			
	VTAV	1.4	-	0
	$T_1T_2Q_3$	-	4.1	3.7

a Three leaves on *N.clevelandii* plants were inoculated with either VTAV-RNA (500 µg/ml), $T_1T_2Q_3$-RNA (500 µg/ml) or a mixture of VTAV-RNA and $T_1T_2Q_3$-RNA (500 µg/ml of each). At these inoculum concentrations confluent local lesions developed on all inoculated leaves of *N. clevelandii* within 5 days.

b Inoculated (Trial 1) and systemically infected (Trial 2) leaves from plants of each treatment were harvested 7 and 12 days respectively after inoculation. Leaf tissue was ground in 2 volumes of 0.5M sodium citrate buffer containing 0.5% thioglycollic acid (pH ∿ 6.5) and clarified by chloroform extraction (Chapter 2). The supernatant was centrifuged at 78,000 g for 120 min and the resultant pellets resuspended in 20mM phosphate buffer, pH 7.6. EDTA was omitted from buffers to prevent the degradation of VTAV (Habili and Francki, 1974b). The virus concentrations in each of the partially purified virus preparations were determined by titrating two-fold dilutions prepared in 20mM phosphate, pH 7.6, against undiluted antisera to VTAV and QCMV (homologous titres of 1/32 and 1/64 respectively) in immunodiffusion tests. Purified VTAV and $T_1T_2Q_3$ preparations of known concentration were also titrated in adjacent wells. The lowest concentration of purified virus producing a visible precipitin band (62.5 and 31.25 µg/ml for VTAV and $T_1T_2Q_3$ respectively) was used to calculate the approximate concentration of each antigen in the various preparations.

c Various concentrations are expressed as mg/g harvested leaf tissue.

d Not detected. Antisera to VTAV and QCMV used in this experiment failed to react with purified $T_1T_2Q_3$ and VTAV respectively, even at antigen concentrations of 1 mg/ml.

The kinetics of hybridization of [^{32}P]cDNA(QCMV-RNA 3) with QCMV-RNA 3 and unfractionated VTAV-RNA are presented in Figure 7.4. The $R_o t_{\frac{1}{2}}$ value of 4.6×10^{-3} mol sec litre^{-1} for the homologous reaction is similar to that obtained by Gould and Symons (1977). Unfractionated VTAV-RNA hybridized to only approximately 2% with cDNA (QCMV-RNA 3) in this experiment, confirming the recent findings of Gonda and Symons (1978) that VTAV and QCMV have negligible base sequence homology.

The ability of QCMV-RNA 3 to survive *in vivo* was investigated by modification of the methods used for Sat-RNA. The QCMV-RNA 3 preparation used in this experiment was not infectious alone at a concentration of 20 µg/ml, but when added to QCMV-RNAs 1+2 at an equivalent concentration, stimulated the number of lesions produced on cowpeas from 5 per half-leaf for RNAs 1+2 alone, to 50 per half-leaf for the mixture of RNAs 1+2+3. Groups of *N. clevelandii* plants were inoculated with QCMV-RNA 3 alone (20 µg/ml) and at either 2, 5 or 8 days later, the leaves were reinoculated with VTAV-RNA at a concentration of 250 µg/ml. Other groups of plants were inoculated with QCMV-RNA 3 alone (20 µg/ml), VTAV-RNA alone (250 µg/ml) or a mixture of QCMV-RNA 3 and VTAV-RNA (20 and 250 µg/ml respectively). Systemically infected leaf tissue from plants of each treatment was harvested 12 days after inoculation with VTAV-RNA, or as indicated in Table 7.5, and the leaf RNAs were extracted as before.

Results presented in Table 7.5 indicate that QCMV-RNA 3 was detected only in leaf RNA extracts from plants inoculated simultaneously with QCMV-RNA 3 and VTAV-RNA. These results demonstrate that the pseudorecombinant $T_1T_2Q_3$ had been generated *in vivo* when QCMV-RNA 3 and VTAV-RNA were inoculated simultaneously. However, when inoculated alone, QCMV-RNA 3 failed to survive *in vivo* for two days.

Figure 7.4. Kinetics of hybridization of [^{32}P]cDNA (QCMV-RNA 3) with QCMV-RNA 3 and unfractionated VTAV-RNA. Hybridization conditions and assay of hybrid formation using S_1 nuclease were as described in Chapter 2. cDNA(QCMV-RNA 3) hybridized with: ● - QCMV-RNA 3
and: ▲ - VTAV-RNA.

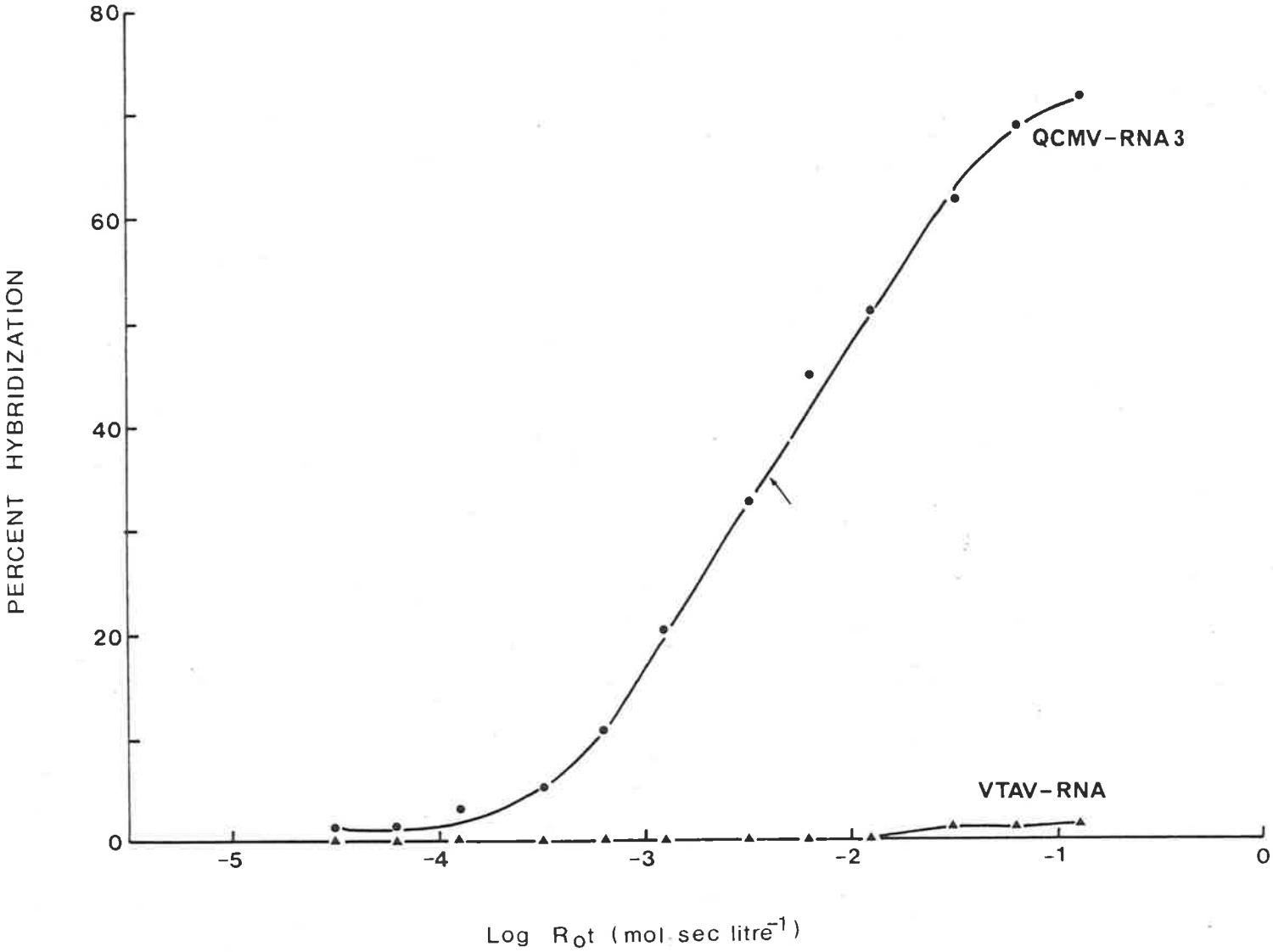

80
60
40
20
0
PERCENT HYBRIDIZATION
QCMV-RNA 3
VTAV-RNA
-5
-4
-3
-2
-1
0
Log Rot (mol sec litre-1)

Table 7.5. Inability of QCMV-RNA 3 to survive *in vivo*.

Inoculation procedure[a]	Interval between QCMV-RNA 3 inoculation and leaf-RNA extraction[b]	% Hybridization[c]
QCMV-RNA 3 alone	12 days	1.8
VTAV-RNA alone	12 days	3.1
QCMV-RNA 3 and VTAV-RNA (simultaneous)	12 days	64.0
QCMV-RNA 3 followed by VTAV-RNA after 2 days	14 days	3.3
QCMV-RNA 3 followed by VTAV-RNA after 5 days	17 days	2.1
QCMV-RNA 3 followed by VTAV-RNA after 8 days	20 days	2.7

a Concentrations of QCMV-RNA 3 and VTAV-RNA in the inocula were 20 and 250 µg/ml respectively in TE buffer.

b Leaf-RNAs were extracted from systemically infected leaves 12 days after inoculation with VTAV-RNA.

c Hybridization conditions and S_1 nuclease treatments were as described in Chapter 2. Leaf RNAs were hybridized with cDNA(QCMV-RNA 3) to a $R_o t$ value of 10.0 mol sec litre^{-1}. Purified QCMV-RNA 3 hybridized to 62% at a $R_o t$ value of 0.1 mol sec litre^{-1} in this experiment.

STNV-RNA

STNV-RNA purified by two-cycles of gel electrophoresis was not infectious when inoculated to plants alone, but when added to TNV-RNA, small necrotic lesions developed characteristic of mixed STNV-TNV infection (Kassanis, 1970; Figure 7.5 A).

Electrophoretic analysis of RNA isolated from purified STNV preparations indicated that in addition to STNV-RNA ($\sim 0.46 \times 10^6$ daltons), a smaller nucleic acid of unknown origin which will be referred to as the low-molecular weight satellite component (LMSC), was detected consistently (Figure 7.5 B). This nucleic acid, with a molecular weight approximately half that of STNV-RNA ($\sim 0.23 \times 10^6$ daltons), was not detected in RNA isolated from preparations of TNV (Figure 7.5 B).

The kinetics of hybridization of cDNA(STNV-RNA) with STNV-RNA, LMSC and TNV-RNA are presented in Figure 7.6. The $R_o t_{\frac{1}{2}}$ value obtained for the homologous hybridization of 2.5×10^{-3} mol sec litre^{-1} is similar to the value expected for an RNA template of this molecular weight (Gould and Symons, 1977). LMSC hybridized with cDNA(STNV-RNA) with a $R_o t_{\frac{1}{2}}$ value of 2.0×10^{-1} mol sec litre^{-1}, which is 80-fold higher than the $R_o t_{\frac{1}{2}}$ value for the homologous hybridization reaction. These results indicate that LMSC contains a unique sequence but was contaminated with sequences from STNV-RNA to the extent of approximately 1.25% by weight. TNV-RNA did not hybridize significantly with cDNA(STNV-RNA), confirming results obtained by Shoulder *et al.* (1974), that STNV-RNA and TNV-RNA have little base sequence homology.

Experiments to investigate the ability of STNV-RNA to survive *in vivo* were essentially similar to those described earlier for Sat-RNA and QCMV-RNA 3. Groups of *N. clevelandii* were inoculated with STNV-RNA

Figure 7.5. Infectivity assay and electrophoretic analysis of STNV-RNA and TNV-RNA preparations.

A. Lesion types induced on opposite half-leaves of *N.* hybrid 4 days after inoculation with TNV-RNA at 15 µg/ml (left) and a mixture of TNV-RNA and STNV-RNA at 15 and 1.4 µg/ml respectively (right).

B. Electrophoretic analysis of STNV-RNA and TNV-RNA in 2% agarose gels (a). QCMV-RNA was included in one gel of each RNA sample as a marker (b). QCMV alone (c). Using QCMV-RNA and *E. coli* 23S and 16S rRNAs as markers, the molecular weights of STNV-RNA, LMSC and TNV-RNA in aqueous conditions were found to be approximately 0.46, 0.23 and 1.26 x 10^6 daltons, respectively.

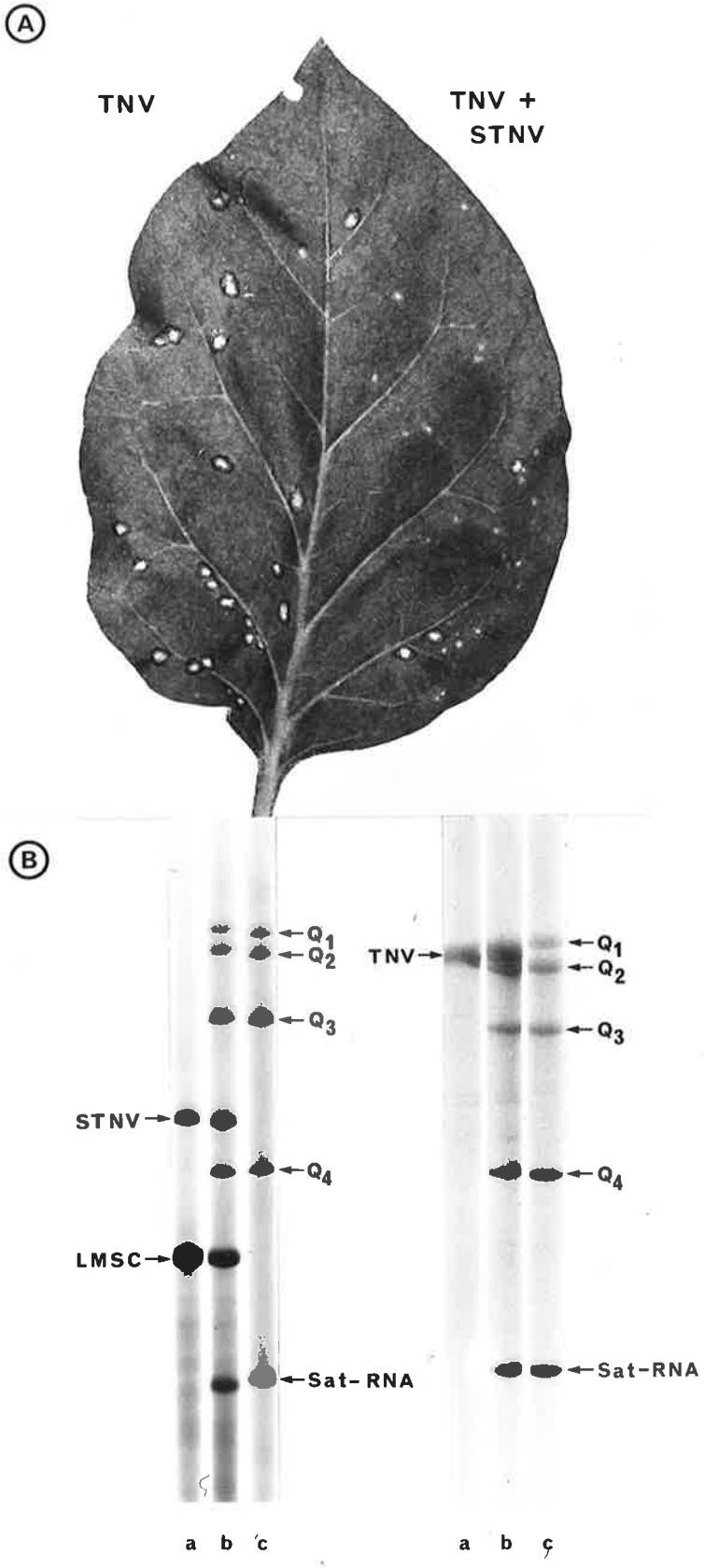
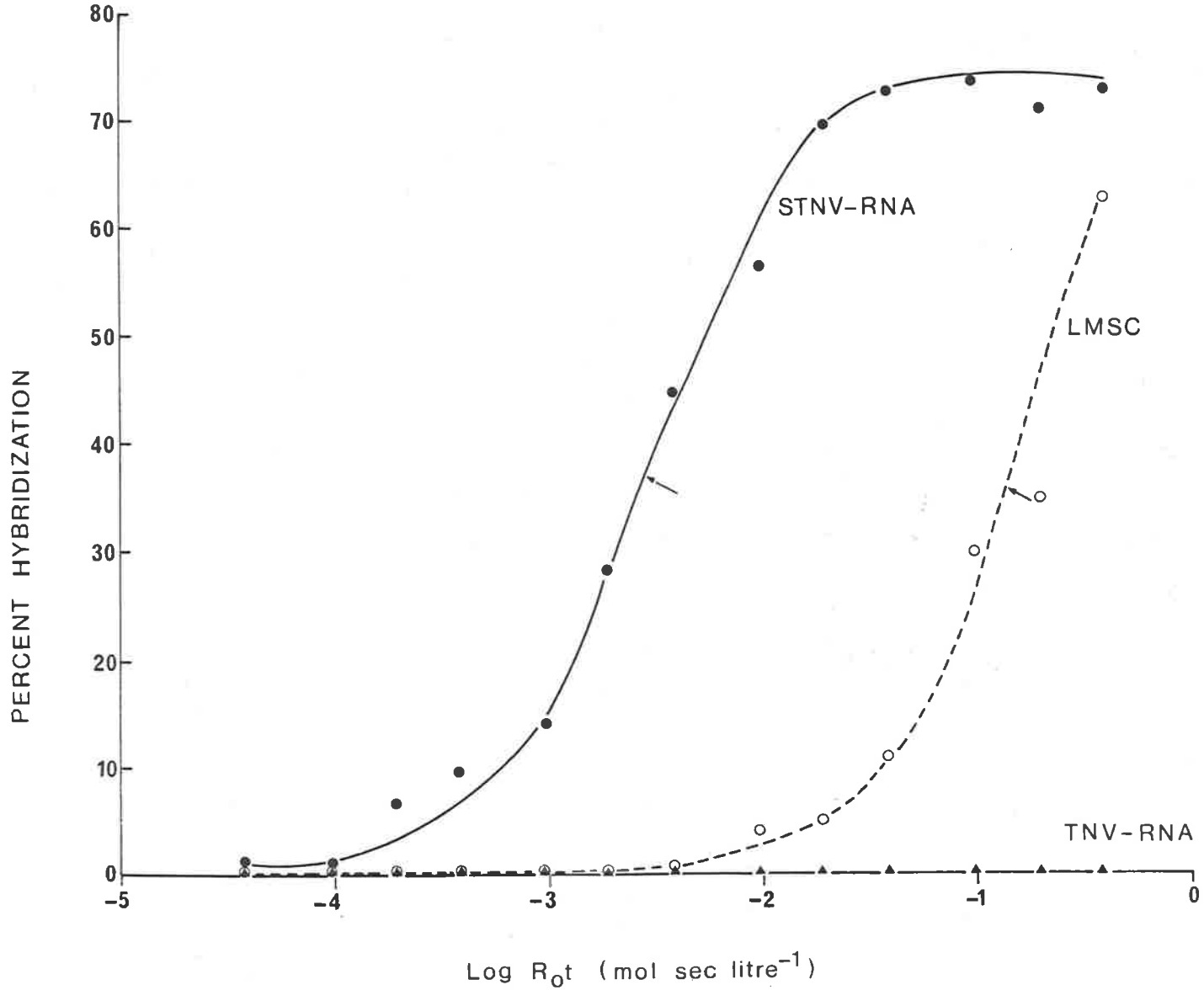

A
TNV
TNV +
STNV
B
Q1
Q2
Q3
Q4
STNV
LMSC
Sat-RNA
TNV
Q1
Q2
Q3
Q4
Sat-RNA
a b c
a b c

Figure 7.6. Kinetics of hybridization of [^{3}H]cDNA(STNV-RNA) with STNV-RNA, LMSC and TNV-RNA. STNV-RNA and LMSC were purified by two-cyles of gel electrophoresis and TNV-RNA was isolated from purified virus preparations. Conditions of hybridization and S_1 nuclease treatments were as described in Chapter 2. $R_o t_{\frac{1}{2}}$ values for hybridization mixtures containing STNV-RNA and LMSC, indicated by arrows, were 2.5×10^{-3} and 2.0×10^{-1} mol sec litre^{-1} respectively. [^{3}H]cDNA(STNV-RNA) hybridized with:

● - STNV-RNA, o - LMSC, and ▲ - TNV-RNA


80
70
60
50
40
30
20
10
0
PERCENT HYBRIDIZATION
STNV-RNA
LMSC
TNV-RNA
-5
-4
-3
-2
-1
0
Log R_0t (mol sec litre^{-1})

alone (1.4 µg/ml) and at 1, 5 or 10 days later, leaves were reinoculated with TNV-RNA at a concentration of 15 µg/ml. Other groups of plants were inoculated with STNV-RNA alone (1.4 µg/ml), TNV-RNA alone (15 µg/ml) or a mixture of STNV-RNA and TNV-RNA (1.4 and 15 µg/ml respectively). Six inoculated leaves from plants of each group were harvested either 4 days after inoculation, or 4 days after reinoculation with TNV-RNA, and their RNAs were extracted as before. In a preliminary hybridization experiment it was found that the level of STNV-RNA in some leaf RNA extracts was too low to hybridize completely with cDNA(STNV-RNA) in the reaction mixtures. Hybridization values obtained for leaf RNAs extracted from plants inoculated with TNV-RNA alone, STNV-RNA alone, STNV-RNA followed after 1 day by TNV-RNA, and a mixture of STNV-RNA and TNV-RNA were 4.0%, 5.0%, 13.7% and 92.0% respectively. These low hybridization levels were probably due to the fact that TNV induces necrotic local lesions in this host species, thereby restricting the number of plant cells contributing to virus production. To determine unequivocally whether STNV-RNA was capable of *in vivo* survival in the absence of TNV, it was considered necessary to increase the concentration of STNV-RNA in leaf RNA extracts. In a subsequent experiment (Table 7.6), the inoculated leaves from plants of each group were harvested as before and crushed in an equal volume of 20mM phosphate buffer, pH 7.8, and the extracts inoculated to another series of *N. clevelandii* plants. After 4 days, six inoculated leaves from plants of each series were harvested and their RNAs extracted. Leaf RNAs were then hybridized with cDNA(STNV-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}.

Results presented in Table 7.6 demonstrate that STNV-RNA multiplied in those plants which were inoculated with STNV-RNA and TNV-RNA, irrespective of the interval between application of the two inocula. These results indicate that STNV-RNA, like Sat-RNA, is able

Table 7.6. Survival of STNV-RNA *in vivo* in the absence of TNV.

Inoculation procedure[a]	Interval between STNV-RNA inoculation and subsequent transfer[b]	% Hybridization[c]
TNV-RNA	4 days	4.3
STNV-RNA alone		1.8
STNV-RNA + TNV-RNA (simultaneous)		85.7
STNV-RNA followed by TNV-RNA after 1 day	5 days	82.0
STNV-RNA followed by TNV-RNA after 5 days	9 days	79.5
STNV-RNA followed by TNV-RNA after 10 days	14 days	85.6

a Concentration of STNV-RNA and TNV-RNA in the inocula were 1.4 and 15 µg/ml respectively in TE buffer. A total of 6 leaves on 2 *N. clevelandii* plants were inoculated with each treatment.

b To enhance the detection of STNV-RNA in leaf RNA extracts, the concentration of STNV relative to that of TNV was increased by inoculating a sap extract of plants from each treatment to another group of *N. clevelandii* as follows. Inoculated leaves from plants of each treatment were harvested as indicated above and crushed in an equal volume (w/v) of 20mM phosphate buffer, pH 7.8. Each sap extract was then inoculated to a total of 6 leaves on 2 *N. clevelandii* plants, and after 4 days, total leaf RNAs were isolated as described before.

c Leaf RNAs were hybridized with $[^3H]$cDNA(STNV-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}.

to survive *in vivo* for at least ten days in the absence of its helper virus.

Conclusions

Results presented in this chapter indicate that (1) Sat-RNA is incapable of autonomous replication; (2) replication of Sat-RNA is dependent on a cucumovirus helper; neither TRSV nor AMV are able to fulfil this helper function; (3) associated with Sat-RNA replication is the accumulation of ds-RNA specific to Sat-RNA infections; (4) both Sat-RNA and STNV-RNA are able to survive *in vivo* for periods of at least 10 days without replicating, but can be induced to do so by the subsequent introduction of their appropriate helper viruses. In contrast, genomic RNA 3 of QCMV rapidly loses its biological activity *in vivo*.

CHAPTER 8

STUDIES ON THE *IN VITRO* STABILITY OF Sat-RNA AND STNV-RNA

Results presented in Chapter 7 indicate that whereas QCMV-RNA 3 is rapidly inactivated *in vivo*, Sat-RNA and STNV-RNA are both able to persist *in vivo* for prolonged periods without replicating. To explain the behaviour of these low molecular weight RNAs, two possibilities have been considered. The first is that these RNAs are modified *in vivo* to an RNase-resistant form, either by their conversion to a ds-RNA structure, or by their association with host cell components. The second possibility is that intrinsic properties of these RNAs render them resistant to inactivation *in vivo*. In this chapter are described experiments designed to test these possibilities. Firstly an attempt was made to detect RNase-resistant, ds-RNA in plants inoculated with Sat-RNA alone. Secondly, the kinetics of thermal denaturation and S_1 nuclease digestion of Sat-RNA and STNV-RNA are described, and their resistance to inactivation in plant sap extracts is compared to that of the RNA genomes of their helper viruses.

The Possible *in vivo* Survival of Sat-RNA as a ds-RNA

The demonstration that healthy plant tissue contains RNA-dependent RNA polymerase (e.g. Duda *et al.*, 1973; Fraenkel-Conrat, 1976; Le Roy *et al.*, 1977) suggested that the ability of small RNA molecules to survive *in vivo* for prolonged periods may be due to their conversion to a ds-RNA form which renders them more resistant to inactivation by cellular

RNases. This possibility was investigated by comparing the RNase resistance of purified Sat-RNA with that of RNA from plants which had been previously inoculated with Sat-RNA.

Sat-RNA at a concentration of 25 µg/ml in TE buffer was inoculated to 2 *N. clevelandii* plants, and after 5 days the inoculated leaves were harvested (approximately 6.0 g) and their total nucleic acids extracted (≡ INF, Table 8.1). As a control, nucleic acids were also extracted from a similar weight of leaves of *N. clevelandii* which had been mock-inoculated with TE buffer alone 5 days previously. Purified Sat-RNA was then added to this nucleic acid preparation to a concentration of 2.5 µg/ml (≡ H, Table 8.1). Each nucleic acid preparation was then divided into 3 samples and 10 x SSC was added to each to a final concentration of 2 x SSC. To one sample of each nucleic acid preparation (INF and H) was added RNase A (25 µg/ml) and the mixtures were incubated at 37°C for 30 min. Another sample of each was heated at either 95°C for 3 min (Experiment 1, Table 8.1) or at 100°C for 10 min (Experiments 2 and 3, Table 8.1) and cooled rapidly in an ice-ethanol bath prior to incubation with RNase A. SSC was omitted from the heat treated samples in Experiments 2 and 3. The remaining sample of each nucleic acid preparation was left untreated. RNase digestions were terminated by incubating each sample with pronase (20 µg/ml) for 30 min at 37°C. Samples were then extracted twice with phenol and their nucleic acids recovered by ethanol precipitation and suspended in 200 µl TE buffer. Each sample was then heated at 100°C for 3 min, cooled rapidly in an ice-water bath, and ZCMV-RNA (Figure 5.1) added to a concentration of 200 µg/ml. Mixtures were inoculated onto 3 leaves on each of 2 *N. clevelandii* plants. Other groups of *N. clevelandii* plants were

Table 8.1. Attempts to detect ds-RNA in nucleic acid preparations from *N. clevelandii* inoculated with Sat-RNA alone.

Inoculum	% Hybridization Experiment 1	Experiment 2	Experiment 3
ZCMV-RNA alone	5.2	6.0	5.3
ZCMV-RNA + Sat-RNA	87.0	71.5	80.0
INF nucleic acid preparation:[a]			
untreated	61.0	74.0	87.4
RNase, 2 x SSC	36.0	14.7	0.7
heated, RNase	66.2	1.3	1.0
H nucleic acid preparation:[b]			
untreated	77.4	70.5	82.2
RNase, 2 x SSC	8.75	37.7	1.2
heated, RNase	3.0	0.7	4.4

a INF = nucleic acids extracted from leaves of *N. clevelandii* inoculated 5 days previously with Sat-RNA at a concentration of 25 µg/ml.

b H = nucleic acids extracted from leaves of *N. clevelandii* mock-inoculated 5 days previously with TE buffer alone. Sat-RNA purified by two-cycles of gel electrophoresis was added to a concentration of 2.5 µg/ml.

Nucleic acid preparations (INF and H) were divided into 3 samples. One sample of each was left untreated; while another was incubated with 25 µg/ml RNase A in 2 x SSC. The remaining sample was heated at either 95°C for 3 min (Experiment 1) or 100°C for 10 min (Experiments 2 and 3) and incubated with 25 µg/ml RNase for 30 min at 37°C. After digestion with 20 µg/ml pronase for 30 min at 37°C, and phenol extraction, nucleic acid preparations were recovered by ethanol precipitation and suspended in TE buffer. Each sample was heated at 100°C for 3 min and cooled rapidly on ice. ZCMV-RNA was added to 200 µg/ml, and the various mixtures inoculated to 3 leaves on each of 2 *N. clevelandii* plants. Other groups of plants were inoculated with ZCMV-RNA alone (200 µg/ml) or a mixture of ZCMV-RNA and Sat-RNA (200 and 2.5 µg/ml respectively). After 7 days, inoculated leaves from plants of each treatment were harvested and their RNAs isolated. Leaf RNA extracts were hybridized with cDNA(Sat-RNA) to a $R_o t$ of 10.0 mol sec litre^{-1}.

inoculated with ZCMV-RNA alone (200 µg/ml) or a mixture of ZCMV-RNA and Sat-RNA (200 and 2.5 µg/ml respectively). After 7 days, inoculated leaves from each group of plants were harvested and their RNAs isolated as before. To detect Sat-RNA, leaf RNAs from each treatment were hybridized with cDNA(Sat-RNA) to a $R_o t$ value of 10.0 mol sec litre^{-1}. The results of Experiments 1 and 2 (Table 8.1) indicate that the infectivity of Sat-RNA in nucleic acid preparations of treatments INF and H was not completely eliminated by RNase digestion. In contrast, in Experiment 3, Sat-RNA was not detected in any of the leaf RNA extracts from plants inoculated with nucleic acid preparations which had been treated with RNase. Since Sat-RNA had been incompletely inactivated by RNase digestion in both INF and H nucleic acid preparations in Experiments 1 and 2, it would appear that this RNA is relatively resistant to enzymic degradation. This conclusion was confirmed in subsequent experiments (Figure 8.3 and Table 8.2). Experiments described in Table 8.1 are inconclusive, although the absence of any differential RNase-resistance of Sat-RNA between the INF and H nucleic acid preparations in Experiment 3 would suggest that the *in vivo* survival of Sat-RNA is not due to its conversion to a ds-RNA form. These experiments do not preclude the possibility that the survival of Sat-RNA is due to their association with host cellular components *in vivo*, since the nucleic acid preparations were treated with pronase and extracted with phenol.

Kinetics of Thermal Denaturation of Sat-RNA and STNV-RNA

The kinetics of thermal denaturation of nucleic acids provides an indication if they are single- or double-stranded (Geiduschek *et al.*, 1962, and references therein), and the degree to which they are stabilized by intramolecular base-pairing (Henco *et al.*, 1977, and references therein). In the following studies, QCMV-RNAs 1+2, 3 and 4, Sat-RNA, STNV-RNA,

Table 8.2. Comparative stability in sap extracts of Sat-RNA, STNV-RNA and the RNAs of their helper viruses.

Incubation time[a] (min)	Survival[b] Experiment 1		Experiment 2	
	NTAV-RNA	Sat-RNA	TNV-RNA	STNV-RNA
0	+	+	+	+
30	-	+	+	+
60	-	+	-	+
120	-	+	-	+
240	-	+	-	+
480	-	+	-	-

a NTAV-RNA and Sat-RNA at concentrations of 100 and 2.5 µg/ml respectively were incubated at 0°c in a 1/10 dilution of a *N. clevelandii* sap extract in TE buffer (Experiment 1). In Experiment 2, TNV-RNA and STNV-RNA at concentrations of 7.5 and 0.75 µg/ml respectively were incubated in a 1/5 dilution of a *N. clevelandii* sap extract in 5mM sodium phosphate buffer, pH 7.4. In Experiment 1, groups of 2 *N. clevelandii* plants were inoculated with aliquots of the sap extract containing NTAV and Sat-RNA at the intervals indicated. One plant in each series received no further treatment to test for the development of symptoms by NTAV. Leaves on the other plant in each series were immediately reinoculated with QCMV-RNA at a concentration of 200 µg/ml. After 12 days, RNA was extracted from systemically infected leaves of these plants and hybridized with cDNA(Sat-RNA) to a R_0t value of 10.0 mol sec litre^{-1}. In Experiment 2, groups of 3 *N.* hybrid plants were inoculated with the sap extract containing TNV-RNA and STNV-RNA, also at the intervals indicated above, and 1 plant of each group received no further treatment to test for the development of local lesions by TNV. The remaining 2 plants in each group were immediately reinoculated with TNV-RNA at 15 µg/ml. After 4 days, inoculated leaves from plants of each group were pulverized in an equal volume of water and the resultant extracts used to inoculate a second series of *N. clevelandii* plants. Inoculated leaf tissue from each series of plants was harvested after 4 days, pulverized in an equal volume of water and samples of each tested in immunodiffusion tests against an antisera prepared to STNV. This antiserum failed to react with sap extracts containing TNV alone, or purified preparations of TNV.

b The presence (+) and absence (-) of Sat-RNA in leaf RNA extracts (Experiment 1) or STNV (Experiment 2) was determined by hybridization with cDNA(Sat-RNA) or by immunodiffusion tests using an anti-STNV serum respectively. The survival (+) of NTAV-RNA and TNV-RNA in sap extracts was determined by the development of characteristic systemic symptoms in the case of NTAV, and by local lesion development in the case of TNV.

In each experiment, the helper virus RNA preparations, QCMV-RNA in Experiment 1 and TNV-RNA in Experiment 2, were both shown to be free of their respective satellites.

TNV-RNA, tRNA and the cadang-cadang associated ccRNA-1 (Randles *et al.*, 1976) in 0.1 x SSC were heated at 1°C per min in stoppered quartz cuvettes, and increases in their absorbance at 260 nm were monitored continuously.

The thermal denaturation of QCMV-RNAs 1+2, 3 and 4 (Figure 8.1) and TNV-RNA (Figure 8.2) was broad, typical of ss-RNAs which do not contain extensive base-pairing (Miura *et al.*, 1966; Randles *et al.*, 1976). In contrast, ccRNA-1, the low molecular weight replicating RNA associated with the cadang-cadang disease of coconuts (Randles *et al.*, 1976) exhibited a sharp increase in absorption with a T_m of approximately 56°C. Recent investigations indicate that ccRNA-1 has a number of properties similar to those of several viroids (Randles *et al.*, 1976; Randles and Palukaitis, 1978), and may also be a covalently closed circular RNA stabilized by regions of internal base-pairing (Sänger *et al.*, 1976). The kinetics of thermal denaturation of ccRNA-1 (Figure 8.1) is very similar to those of both citrus exocortis viroid (CEV) and cucumber pale fruit viroid (CPFV) reported by Henco *et al.* (1977).

The thermal transition of Sat-RNA appears intermediate to those of QCMV-RNAs 1-4 and ccRNA-1 (Figure 8.1). A comparison of the thermal denaturation kinetics of Sat-RNA, STNV-RNA and *E. coli* total tRNA (Figure 8.2) indicates that all three RNAs are stabilized by similar amounts of base-pairing. Henco *et al.* (1977) have demonstrated that although yeast $tRNA^{Phe}$, CEV and CPFV have a similar amount of base-pairing, the kinetics of thermal denaturation of tRNA differs significantly from that of the two viroids. As in Figure 8.2, these authors demonstrated that the thermal denaturation of tRNA is spread over a temperature range of more than 40°C, which they suggest, is a reflection of '... *the more*

Figure 8.1. Kinetics of thermal denaturation of QCMV-RNAs 1+2, 3 and 4, Sat-RNA and ccRNA-1. RNAs (5-15 µg) in 1 ml of 0.1 x SSC were heated at 1°C per min in stoppered, quartz glass cuvettes and the increase in absorption at 260 nm was monitored continuously.

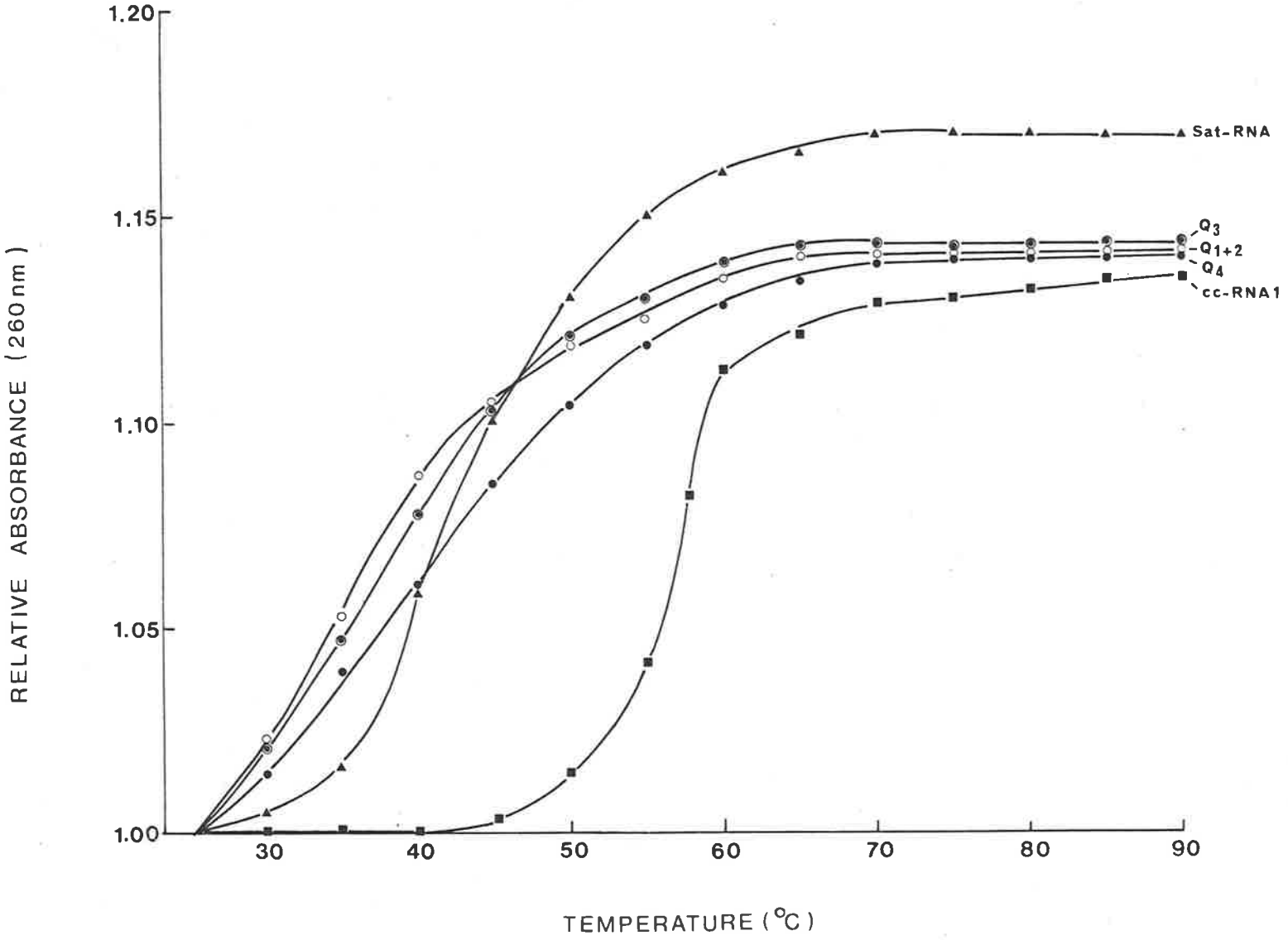
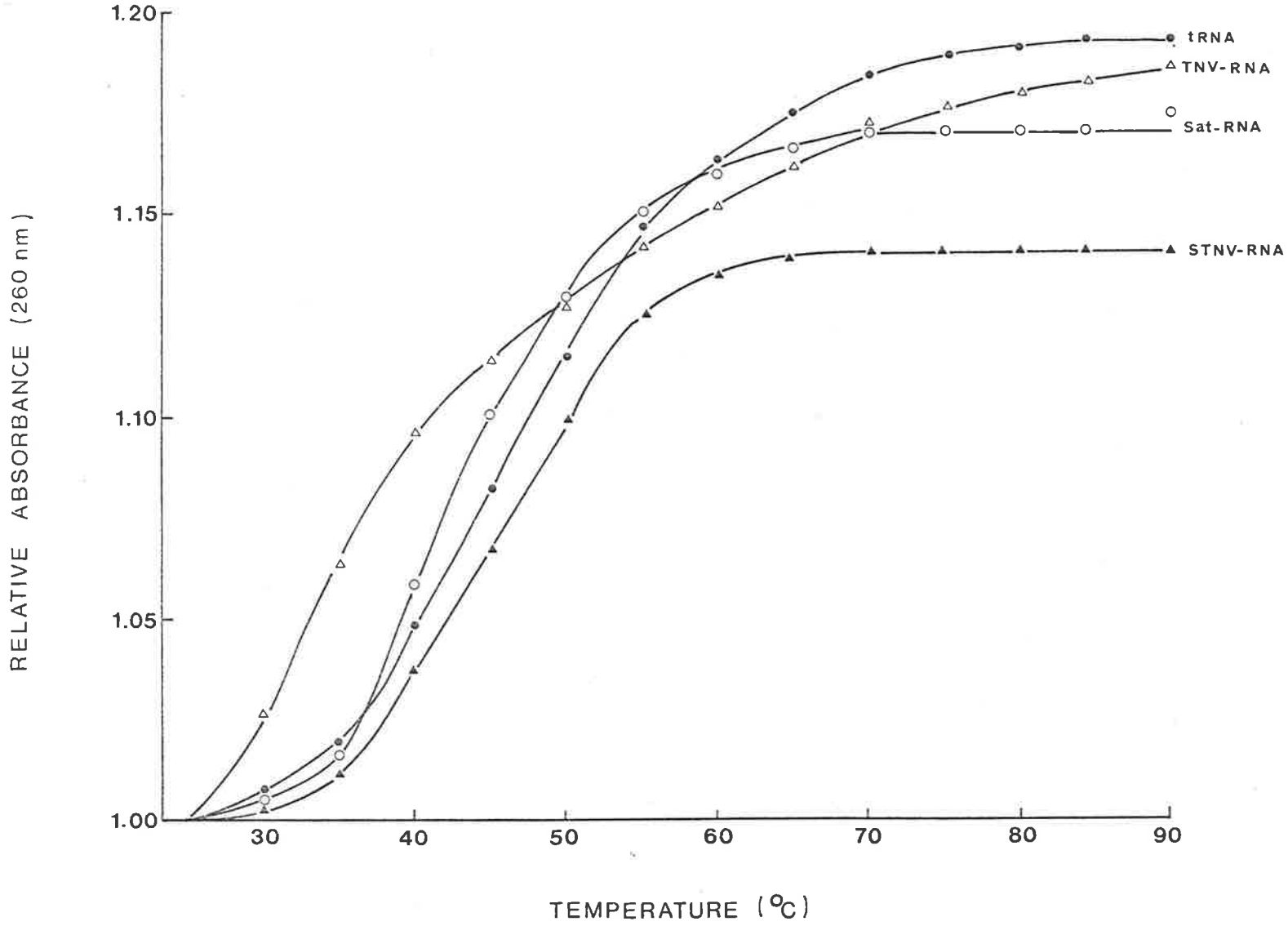

1.20
1.15
1.10
1.05
1.00
RELATIVE ABSORBANCE (260nm)
30
40
50
60
70
80
90
TEMPERATURE (°C)
Sat-RNA
Q3
Q1+2
Q4
cc-RNA1

Figure 8.2. Kinetics of thermal denaturation of TNV-RNA, Sat-RNA, STNV-RNA and *E. coli* total tRNA. RNA (5-10 µg) in 1 ml of 0.1 x SSC were heated at 1°C per min as described in Figure 8.1.


1.20
1.15
1.10
1.05
1.00
RELATIVE ABSORBANCE (260 nm)
30
40
50
60
70
80
90
TEMPERATURE (°C)
tRNA
TNV-RNA
Sat-RNA
STNV-RNA

or less sequential process of the melting of the different branches of its clover-leaf structure'. In contrast, these authors also suggest that the steep thermal transition observed for viroids reflects the cooperative melting of an extensive double-helical region rich in G:C base-pairs.

The similarity of the melting profiles of Sat-RNA and STNV-RNA with that of tRNA suggests that these RNAs contain a number of short base-paired regions rather than the extensive double-helical structures characteristic of viroids (Henco *et al.*, 1977).

Kinetics of Nuclease Digestion of Sat-RNA and STNV-RNA

The kinetics of digestion of nucleic acids with specific nucleases provides an indication as to whether they are DNA or RNA, single- or double-stranded and also the extent to which they are stabilized by base-pairing (Bishop and Koch, 1967; Paleček, 1976; Randles *et al.*, 1976). The S_1 nuclease purified from *Aspergillus oryzae* (Ando, 1966; Vogt, 1973) specifically cleaves single-stranded nucleic acids. RNase A, however, in low Na^+ concentrations, or at high enzyme : substrate ratios is also capable of digesting double-stranded RNA (Edy *et al.*, 1976).

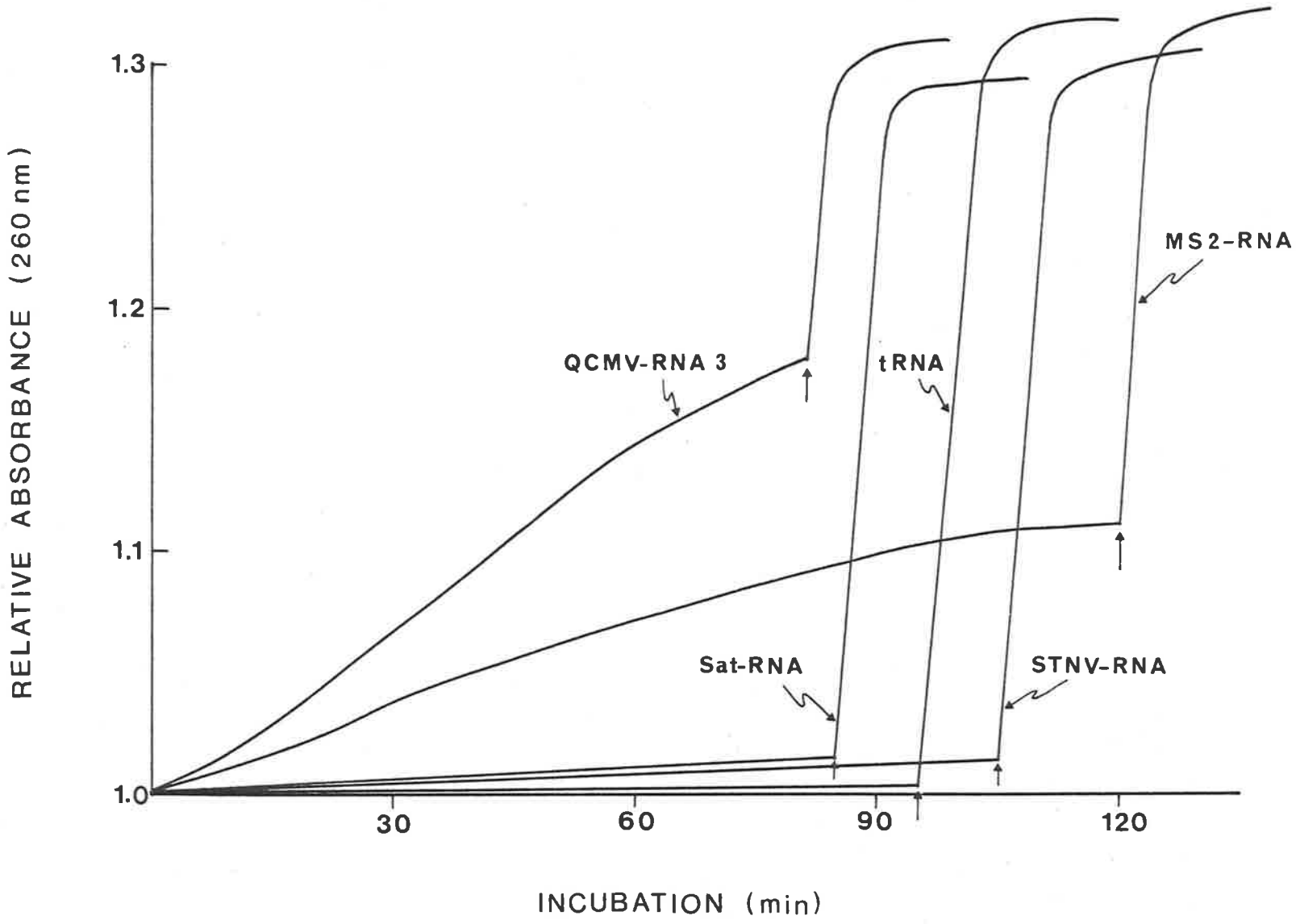
RNA preparations were diluted to contain approximately 4-10 µg/ml in one ml of a buffer containing 0.3M NaCl, 50mM sodium acetate, pH 5.7 (Rushizky and Mozejko, 1977) and 1mM $ZnSO_4$. S_1 nuclease was added to a concentration of 1 unit per µg of RNA, and the mixture incubated at 30°C in a stoppered quartz glass cuvette. The increase in absorption at 260 nm was recorded continuously, and after 80 to 120 min, RNase A was added to 10 µg/ml and the digestion allowed to proceed to completion. It

should be emphasized that the incubation conditions chosen in these experiments were sub-optimal for S_1 nuclease activity. S_1 nuclease activity is optimal at a pH of 4.5, and a temperature of 45^{o} (Ando, 1966; Vogt, 1973). The presence of Zn^{2+} was mandatory for the detection of RNA digestion by S_1 nuclease.

Results presented in Figure 8.3 demonstrate that whereas QCMV-RNA3 and MS2-RNA were readily digested by S_1 nuclease, Sat-RNA, STNV-RNA and yeast $tRNA^{Phe}$ exhibited considerable resistance to degradation by this enzyme. However, all RNAs were rapidly digested with RNase A, even at the relatively high Na^{+} concentration used in these experiments. These results suggest that like yeast $tRNA^{Phe}$, Sat-RNA and STNV-RNA contain a number of base-paired regions separated by single-stranded loops. The relatively small increases in absorption observed for these RNAs in the presence of S_1 nuclease may be due to the digestion of these single-stranded loop regions. It is interesting to note, that although tRNA and MS2-RNA have a similar proportion of their nucleotide bases in double-stranded regions (Fiers, 1973), the apparent resistance of these two RNAs to S_1 nuclease is very different (Figure 8.3). The explanation for their differential digestion by S_1 nuclease is not clear, although Crouch (1976) has suggested that the small molecular size of tRNA and its complex tertiary structure may possibly explain its stability.

It is known that tRNA exhibits considerable stability to degrading nucleases, particularly exonucleases (Cramer, 1971). The removal of the 5'-terminal phosphate of *E. coli* $tRNA^{Phe}$ by alkaline phosphatase is possible only at temperatures close to the melting temperature (Stern *et al.*, 1969). This is consistent with the highly-structured molecular configuration observed for this class of RNA (Cramer, 1971; Clark, 1977).

Figure 8.3. Kinetics of digestion of QCMV-RNA 3, MS2-RNA, Sat-RNA, STNV-RNA and yeast $tRNA^{Phe}$ by S_1 nuclease and RNase A. RNA (4-10 µg/ml) was incubated at 30°C in a buffer containing 0.3M NaCl, 50mM sodium acetate, 1mM $ZnSO_4$, pH 5.7, and 1 unit of S_1 nuclease per µg of RNA. RNase A was added to a concentration of 10 µg/ml at times indicated by arrows. Increases in the absorption at 260 nm were monitored continuously using a Varian Superscan 3 spectrophotometer.


RELATIVE ABSORBANCE (260 nm)
1.3
1.2
1.1
1.0
30
60
90
120
INCUBATION (min)
QCMV-RNA 3
tRNA
MS2-RNA
Sat-RNA
STNV-RNA

Comparison of the Stability of Sat-RNA and STNV-RNA with that of the RNA of their Helper Viruses in Sap Extracts

The stability of Sat-RNA and STNV-RNA in sap extracts of *N. clevelandii* was investigated as follows. A sap extract was prepared by crushing *N. clevelandii* leaf tissue in an equal volume (w/v) of 0.1M phosphate buffer, pH 7.4, and clarified by centrifugation at 10,000 g for 10 min. The clarified supernatant was diluted either 10-fold in TE buffer containing 2.5 µg/ml of Sat-RNA and 100 µg/ml of NTAV-RNA (Experiment 1, Table 8.2) or 5-fold in 5mM phosphate buffer, pH 7.4, containing 0.75 µg/ml of STNV-RNA and 7.5 µg/ml TNV-RNA (Experiment 2, Table 8.2). Diluted sap extracts containing either Sat-RNA or STNV-RNA and RNA of their respective helper viruses were incubated at 0°C. The first sample was removed immediately after the addition of the diluted sap extract (0 min), and thereafter samples were taken at the intervals indicated in Table 8.2.

Samples taken from the sap extract containing Sat-RNA and NTAV-RNA (Experiment 1) were inoculated to 3 leaves on each of 2 *N. clevelandii* plants. Leaves on one plant of each series were immediately reinoculated with QCMV-RNA at a concentration of 200 µg/ml, and after 12 days, RNA was extracted from systemically infected leaf tissue, and hybridized with cDNA(Sat-RNA). The remaining plant of each group served as a control, to determine the rate of inactivation of NTAV-RNA in the diluted sap extract. This isolate of TAV induces severe systemic necrotic symptoms in infected *N. clevelandii* plants. In addition, leaf RNAs were also extracted from systemically infected *N. clevelandii* plants inoculated with QCMV-RNA alone (200 µg/ml) and

hybridized with cDNA(Sat-RNA). These leaf RNAs failed to hybridize with cDNA(Sat-RNA) indicating that the isolate of QCMV used to rescue Sat-RNA in this experiment was free of Sat-RNA.

In experiment 2, samples taken from the diluted sap extract containing STNV-RNA and TNV-RNA were inoculated to 3 leaves on each of 3 *N.* hybrid plants at the intervals indicated in Table 8.2. One plant received no further treatment to determine the rate of inactivation of TNV-RNA. Also, as a control, 3 *N.* hybrid plants were inoculated with TNV-RNA alone (15 µg/ml). Leaves on the remaining 2 plants of each series were then immediately reinoculated with TNV-RNA alone, at a concentration of 15 µg/ml. After 4 days, inoculated leaves from plants of each series were combined, pulverized in an equal volume of water and the resultant extracts were then used to inoculate a series of *N. clevelandii* plants. Inoculated leaves from this second series of plants were harvested after 4 days, pulverized in an equal volume of 10mM phosphate buffer, pH 7.4, and the presence or absence of STNV was tested by immunodiffusion. The anti-STNV serum used in this experiment had a homologous titre of approximately 1/256 and was diluted 1/40 before use; this serum failed to react with leaf extracts from control plants inoculated with TNV-RNA alone, or with purified preparations of TNV. In earlier experiments, it was found that STNV was not consistently detected in leaf extracts from plants inoculated with mixtures of STNV-RNA and TNV-RNA. Hence, in this experiment, the concentration of STNV in leaf extracts was increased by a second passage in *N.clevelandii*.

Results summarized in Table 8.2 indicate that both Sat-RNA and STNV-RNA are considerably more resistant to inactivation in sap extracts than the RNA of their respective helper viruses. Sat-RNA was still infectious after 8 hr incubation in the sap extract, whereas STNV-RNA

was able to be recovered after 4 hr but not 8 hr incubation. However, a comparison of their relative stabilities in these two experiments is not strictly possible since the concentration of STNV-RNA on a molar basis was considerably less than that of Sat-RNA in Experiment 1. Moreover, the sap extract used in Experiment 2 was in a higher concentration than that in Experiment 1.

Conclusions

Attempts to detect ds-RNA structures containing Sat-RNA in leaf RNA extracts from plants which had been inoculated with this RNA alone were unsuccessful. Although modification of Sat-RNA and STNV-RNA by host-specified polymerases, or their association with normal cellular components cannot be dismissed, it seems likely that other factors may be involved in their *in vivo* survival. The kinetics of the thermal denaturation and S_1 nuclease digestion of both Sat-RNA and STNV-RNA are very similar to those of tRNA (Figures 8.1 - 8.3). These results suggest that the proportion of their nucleotide bases involved in base-pairing is similar for these three RNAs, and also that these base-paired regions are probably not very long, or rich in G : C pairs. The resistance of Sat-RNA and STNV-RNA to enzymic inactivation was also confirmed in an investigation of their stability in plant sap extracts (Table 8.2).

CHAPTER 9

GENERAL DISCUSSION

Properties of the Cucumovirus Isolates

Isolates of CMV examined in this thesis are readily distinguishable from those of TAV on the basis of their amino acid compositions (Figure 5.2) and their antigenic specificities (Table 5.1). These results confirm those of Habili and Francki (1974a) and indicate that, although CMV and TAV share a number of physical and chemical properties characteristic of the cucumovirus group (Harrison *et al.*, 1971), they are clearly different viruses. Throughout these investigations, the taxonomic status of the 11 CMV isolates has not been assumed. Although attempts have been made to define the taxonomic status of the virus 'strain' (Gibbs and Harrison, 1976), the delineation between strains of the same virus or between isolates of the same strain still involves some subjective judgements. On the basis of differences in their amino acid compositions (Table 5.2, Figure 5.2) and biological properties, it is considered that most of the isolates of CMV studied should be considered as different strains. Only WAICMV and WAIICMV are so similar (Figure 5.1 and 5.2, Tables 5.2 and 5.6) that they can be considered as isolates of the same strain (WACMV). A computer analysis of the amino acid compositions of the viral coat proteins indicates that the ten strains of CMV form two clusters (Figure 5.2). However, serological data (Table 5.1) do not correlate particularly well with the groupings indicated by the computer classification of the amino acid composition data. A more thorough investigation of their serological relationships is warranted, in which antisera to each strain is collected from immunized animals at various intervals after administration of the respective antigen. Using a similar approach, Devergne and Cardin (1975)

were able to establish two serological groups of CMV. It would be of interest to determine whether the two serological groups defined by these authors correlate with those based on amino acid composition of viral proteins (Figure 5.2). A comparison of the nucleotide homology between RNAs 1-4 of QCMV, PCMV, MCMV and VTAV (Gonda and Symons, 1978), is in good agreement with the relationship between these cucumoviruses proposed on the basis of their amino acid composition (Figure 5.2) and antigenic specificity (Table 5.1). Although it would appear necessary to include considerably more cucumovirus strains in comparative studies of their nucleotide sequence homology, these preliminary results suggest that molecular hybridization may be useful in the classification of plant viruses. A possible advantage of this approach is that it would enable differences in the sequence homology between viral RNAs to be expressed quantitatively taking account of the entire viral genome.

The molecular weights of genomic RNAs of cucumoviruses have been the subject of some controversy in recent years. Estimates of the molecular weight of CMV-RNAs vary considerably, particularly those for RNAs 1 and 2 (Table 3.4). It is significant that molecular weight values obtained for CMV-RNAs 1 and 2 based on their electrophoretic mobilities in polyacrylamide gels relative to those of *E. coli* rRNAs, are invariably higher than those obtained by Kaper and West (1972), Kaper and Waterworth (1973) or Kaper and Diaz-Ruiz (1977). These authors have used as internal molecular weight standards, RNAs whose molecular weights were determined from sedimentation analyses or contour length measurements in the electron microscope. Molecular weight estimates of CMV-RNAs 1 and 2 determined by polyacrylamide gel electrophoresis using *E. coli* rRNAs as internal standards are considered too high by Kaper and Diaz-Ruiz (1977) since they appear to offend the basic constants accepted for CMV (Kaper

1975b; Kaper and Re, 1974). It should be noted that Kaper and his colleagues have chosen a value of 5.3×10^6 as the mean particle weight of CMV, although values obtained in different laboratories range from 5.0×10^6 (Dupont *et al.*, 1968) to 5.8×10^6 (Francki *et al.*, 1966). In view of the large variation in values obtained for the particle weight of CMV, it seems possible that conclusions concerning the limiting amount of RNA able to be encapsidated in CMV particles based on the particle size of CMV (Kaper and Re, 1974) may be spurious. It would appear that a re-examination of the particle weight of CMV is desirable, preferably using several independent procedures.

Genetics of Cucumoviruses

Genomic RNAs 1 and 2 of MCMV, GCMV and VTAV could not be clearly resolved in the preparative gel electrophoretic procedure used in these investigations. It was decided that initially an attempt should be made to investigate the genetic content of RNA 3 and, hence, data in this thesis provides unequivocal information concerning genes located only on RNA 3.

In all possible heterologous combinations, genomic RNAs 1+2 and RNA 3 from four different strains of CMV or TAV were infectious on *C. amaranticolor* (Table 4.2). It seems most likely that the apparent incompatibility of mixtures of QCMV-RNAs 1+2 and VTAV-RNA 3 on this host, previously reported by Habili and Francki (1974c), was due to host plant insensitivity since lesions induced by this particular combination are extremely small and visible only on the most 'sensitive' leaves (Table 4.2). It is possible that the apparent incompatibility of heterologous genomic RNA mixtures of BMV and CCMV (Bancroft, 1972), RRV and TRSV (Harrison *et al.*, 1972) and strains of tobacco rattle virus (TRV; Ghabrial and Lister, 1973) may be explained on the basis that these pseudorecombinants produce extremely small lesions which escaped detection on the indicator

plants chosen. Alternatively, it has been suggested that the 'replicase subunit' specified by the genomic RNA of one strain does not recognised the complementary genomic RNA of another (Van Vloten-Doting *et al.*, 1977). Conceivably, observations that heterologous genomic RNA mixtures are less infectious, or produce much smaller lesions than the homologous mixtures (e.g. Bancroft, 1972; Ghabrial and Lister, 1973; Randles *et al.*, 1977) may be due to a reduced affinity of the virus specified 'replicase subunits' for the heterologous RNA. However, it is not clear how this suggestion could explain why some heterologous genomic RNA mixtures are completely compatible while the reciprocal genomic RNA combinations are not.

Although mixtures of CMV-RNAs 1+2 and VTAV-RNA 3 were infectious on *C. amaranticolor* (Table 4.2), attempts to recover these pseudorecombinants by local lesion transfer were unsuccessful. The explanation for this phenomenon remains obscure. *In vitro* reconstitution experiments had previously indicated that QCMV-RNA is efficiently encapsidated with VTAV coat protein, and *vice versa* (Habili, 1974). These results suggest that coat protein specified by VTAV-RNA 3 *in vivo* should be able to associate with CMV-RNA. It is possible that coat protein itself may affect viral RNA synthesis *in vivo*. Indirect evidence of the influence of coat protein, or perhaps genetic information other than the coat protein gene located on RNA 3, was obtained from the behaviour of VTAV and its pseudorecombinant $T_1T_2Q_3$ in mixed infections (Table 7.4). In plants inoculated with both VTAV and $T_1T_2Q_3$, the latter dominated the progeny viral population and markedly inhibited the production of VTAV coat protein. Apparently, particles containing QCMV-RNA 3, or those constructed with QCMV coat protein have a competitive advantage over the corresponding VTAV particles. It is of interest to note that similar findings have been reported for heterologous combinations of long and short nucleoprotein particles of

2 strains of TRV (Ghabrial and Lister, 1973). These authors reported that in plants inoculated with a mixture of long and short particles of TRV-Z and short particles of TRV-Y, a 'hybrid' virus composed of long particles of strain Z and short particles of strain Y predominanted. This anomalous preferential synthesis and/or encapsidation of heterologous genomic RNAs could be further investigated in protoplast systems in which the synthesis of specific RNAs and proteins could be followed. Such an approach may also be useful to investigate the basis of the apparent incompatibility of certain heterologous genomic RNA combinations.

Genetic analysis of determinants specifying symptom production by cucumoviruses (Chapter 4), indicate that the situation is more complicated than earlier reports had suggested (Habili and Francki, 1974c; Marchoux *et al.*, 1974a). The distribution of genes specifying local lesion type determined from genetic analyses using heterologous mixtures of genomic RNAs from serologically related strains of CMV is relatively straightforward with no apparent evidence of RNA interactions or epistatic effects; CMV-RNA 3 determines the lesion type on *C. amaranticolor* (Table 4.2, Figure 4.7) *Nicotiana* species (Table 4.4), in addition to the coat protein, whereas CMV-RNA 1 and/or RNA 2 determines the size of lesions on cowpea (Figure 4.9). However, Marchoux *et al.* (1974a) concluded that genetic information located on CMV-RNA 3 determines the local lesion size in cowpea. These apparently conflicting results may well be explained on the basis that genetic information located on RNA 1 and/or RNA 2, as well as on RNA 3 contributes towards the development of local lesions on this host.

Local lesions induced on *C. amaranticolor* by heterologous mixtures of genomic RNAs from VTAV and strains of CMV were usually indistinguishable from those induced by VTAV, irrespective of whether VTAV provided RNAs 1 and 2 or RNA 3 in the inocula (Table 4.2). Lesions induced by mixtures of

QCMV-RNAs 1+2 and VTAV-RNA 3 on this host are considerably smaller than those induced by VTAV (Figure 4.8). These results suggest that RNAs 1 and/or RNA 2 of VTAV, unlike that of CMV, contains genetic information which modifies the expression of the gene(s) determining local lesion type located on CMV-RNA 3. There are examples where the genomic RNA from one strain or virus affects the normal expression of a complementary genomic RNA from another virus or strain (Bancroft, 1972; Ghabrial and Lister, 1973; Randles *et al.*, 1977). However, it is debatable that this modification represents an epistatic effect since pseudorecombinants constructed from closely related strains of the same virus often do not exhibit this phenomenon.

Genes determining the systemic symptoms produced by MCMV appear to be distributed over at least two of the essential genomic RNAs, including RNA 3 (Table 4.4). In all pseudorecombinants, the presence of MCMV-RNA 3 was invariably associated with veinal chlorosis in *Nicotiana* species, although the chlorotic symptoms produced were always less pronounced than those induced by MCMV. In contrast, MCMV-RNAs 1 and/or 2 were associated with the development of interveinal chlorosis and the severity of leaf blistering and distortion. This appears to represent an additive effect; the development of characteristic symptoms by MCMV depending on genetic information located on RNA 3, as well as on RNA 1 and/or RNA 2. The severity of symptoms induced by VTAV is apparently controlled by genes located on TAV-RNAs 1 and/or 2 since symptoms induced by $T_1T_2Q_3$ and $T_1T_2G_3$ are similar to those produced by VTAV (Table 4.4). In addition to the severe leaf distortion symptoms characteristic of VTAV, $T_1T_2M_3$ also induces mild veinal chlorosis in *Nicotiana* species (Figure 4.16). It seems most likely that differences

in the conclusions reached by Habili and Francki (1974c) and Marchoux *et al.* (1974a) concerning the relative contribution of genomic RNAs 1, 2 and 3 towards symptom production in the cucumoviruses may be attributed to differences in the cucumovirus combinations used in their genetic analyses.

Future attempts to assign genes determining biological properties of cucumoviruses to particular genomic RNAs would necessitate a careful selection of parental strains. The use of spontaneous or artificially-induced mutants which can be readily distinguished from their parental viruses in such properties as their host range, lesion type and the temperature sensitivity of their replication (De Jager and Van Kammen, 1970; Bancroft and Lane, 1973; De Jager, 1976) is an approach which may provide more definitive answers. However, there is still a possibility that epistatic effects or interactions between genes located on different RNA components may be overlooked. The presence of Sat-RNA in certain pseudorecombinants and parental strains used in the investigations described in Chapter 4 may possibly further complicate the interpretations of these genetic analyses. Subsequent experiments have demonstrated that Sat-RNA may actually attenuate symptoms induced by strains of CMV in several *Nicotiana* species (Chapter 5), although it does not appear that Sat-RNA has modified host symptom expression in genetic analyses described in Chapter 4. However, in future studies it would seem desirable to exclude replicating, extra-genomic entities such as Sat-RNA from experiments involving genetic analyses.

Aphid transmission of CMV is associated with genetic information located on RNA 3 (Table 4.5). It has been previously established that the nematode transmissibility of RRV is also determined by information

located on its smallest essential RNA (Harrison *et al.*, 1974). These authors suggested that surface properties of RRV are responsible for its ability to be transmitted by nematodes. Aphid transmission of CMV does not require genetic information located on RNAs 1 or 2, or Sat-RNA. Although it seems likely that aphid transmissibility of CMV is also determined by properties of its coat protein, other possibilities must be considered. CMV-RNA 3 is known to specify a protein with a molecular weight of approximately 34,000 in *in vitro* translation systems (Schwinghamer and Symons, 1977). It seems unlikely that this polypeptide represents a 'helper' substance similar to that necessary for the aphid transmission of other plant viruses (Pirone, 1977), since CMV can be transmitted by aphids probing a purified virus preparation (Megahed and Pirone, 1966). Recently, Clarke and Bath (1977) have suggested that aphid transmissibility of pea enation mosaic virus may be associated with the appearance of a second structural protein in viral capsids. It seems most unlikely that this 34,000 dalton polypeptide constitutes a minor capsid component of CMV since there is no evidence for the presence of such a protein in the cucumovirus capsid and it is difficult to suggest how such a molecule would fit into the projected structural model of the CMV capsid (Finch *et al.*, 1967).

Both the concentration and cellular location of virus in infected plants from which virus is acquired may be of importance for its acquisition by an aphid (Pirone, 1969), but are difficult to determine experimentally. Relative concentrations of isolates with QCMV-like and MCMV-like coat proteins are difficult to estimate with precision since they require different extraction procedures for optimum virus recovery (Chapter 3). Furthermore, studies on the cellular distribution of isometric

viruses such as CMV pose problems since their particles are not readily distinguished from ribosomes (Honda and Matsui, 1974). To determine unequivocally whether properties of the coat protein are responsible for the aphid transmission of CMV, a worthwhile approach would be to compare the transmissibility of reconstituted viruses containing either QCMV or MCMV coat protein in membrane-feeding experiments. Although the mechanism of non-persistent aphid transmission of plant viruses remains obscure (Garrett, 1973; Harris, 1977) it would seem that the ability of virus particles to be absorbed to and released from the insect's feeding canals during probing (Taylor and Robertson, 1974) may be of paramount importance.

For almost all plant viruses examined with multi-partite genomes, the gene specifying viral coat protein is located on the smallest essential RNA component (Van Vloten-Doting *et al.*, 1977 and references therein). However, it is evident that for plant viruses with tripartite genomes at least, the smallest essential RNA (RNA 3), itself, probably does not represent the *in vivo* mRNA for coat protein synthesis (Schwinghamer and Symons, 1977). Recent evidence indicates that in BMV, AMV and CMV the sub-genomic RNA 4, which is derived *in vivo* from RNA 3 is the coat protein mRNA (Shih and Kaesberg, 1973; Mohier *et al.*, 1975; Schwinghamer and Symons, 1977). With the exception of the coat protein gene, there are relatively few generalizations possible concerning the location of genes determining symptom development, or controlling stages of virus synthesis. For example, for most viruses examined there are at least two genes, located on different RNA components, which determine local lesion development in host plant species (Jaspars, 1974, and references therein). Although genetic analyses provide an indication of the location of genes determining host plant reactions, and in some instances, genes affecting

aspects of viral synthesis, a clearer understanding of the molecular basis of viral replication and virus-host interactions may result from the isolation and characterization of virus-induced polyribosomes and proteins from infected plant tissue or protoplasts.

Properties of Sat-RNA, and its Interaction with Helper Viruses

Sat-RNAs isolated from RNA preparations of four different strains of CMV could not be distinguished using the molecular hybridization technique (Figure 5.3). In addition, there were no apparent changes in the sequences of Sat-RNA isolates from one cucumovirus strain after it had been propagated in another strain (Gould *et al.*, 1978). Furthermore, the absence of detectable sequence homology between Sat-RNA and QCMV-RNAs 1-4 demonstrates that this low molecular weight replicating RNA is a true satellite RNA (Gould *et al.*, 1978). A comparison of the T_1 ribonuclease digestion products of CARNA 5 and CMV-RNAs separated by two-dimensional polyacrylamide gel electrophoresis also demonstrated that CARNA 5 has no sequences in common with CMV-RNAs 1-4 (Lot *et al.*, 1977). Diaz-Ruiz and Kaper (1977) using the competitive RNA-RNA hybridization method, independently reached a similar conclusion.

Sat-RNA has a molecular weight of approximately 105,000 (Chapter 3), similar to that reported for CARNA 5 (Kaper *et al.*, 1976), the satellite of TRSV (S-TRSV; Schneider, 1977), and several viroids (Sänger *et al.*, 1976). Like CMV-RNAs 1-4, the 5' end of both Sat-RNA and CARNA 5 have a $M^7G_{ppp}G_p$ 'cap' (P. Palukaitis, personal communication; Lot *et al.*, 1977). However, unlike CMV-RNA, the 3' terminal sequences of both Sat-RNA and CARNA 5 do not end with an adenosine residue (Symons, 1975; P. Palukaitis, personal communication; Lot *et al.*, 1977). It is of

interest to note that attempts to add [^{32}P]ATP to the 3' terminus of Sat-RNA using nucleotidyl transferase purified from wheat germ extracts were unsuccessful (P. Palukaitis, personal communication) suggesting that unlike CMV-RNA (Kohl and Hall, 1974), Sat-RNA is probably unable to be aminoacylated. Both CARNA 5 and Sat-RNA are able to direct the *in vitro* synthesis of polypeptides in wheat germ translation systems (Owen and Kaper, 1977; P. Palukaitis, personal communication).

Results presented in Chapter 5 indicate that Sat-RNA was able to be transmitted to all strains of CMV and TAV tested. In all strains of CMV, Sat-RNA was replicated (and encapsidated) to relatively high levels. In contrast, VTAV and the pseudorecombinants $T_1T_2Q_3$ and $T_1T_2G_3$ do not readily support the replication of Sat-RNA (Figures 5.4, 4.2 and 4.3) suggesting that genetic information located on RNA 1 and/or RNA 2 influences the efficiency of Sat-RNA replication. As a consequence of Sat-RNA replication, the yield of its associated CMV, and the level of CMV-RNAs 1 and 2 were both markedly reduced (Table 5.4). Similar effects were not observed in preparations of VTAV or NTAV propagated in the presence of Sat-RNA.

It would then appear that Sat-RNA and CARNA 5 are similar in that (i) their replication appears to depend on a cucumovirus helper, (ii) the efficiency of replication of both is influenced by the helper virus strain (probably by genetic information located on RNA 1 and/or RNA 2) and the propagative host, (iii) in strains of CMV which support their replication, these satellite RNAs depress both the yield and the levels of genomic RNAs 1 and 2 of their associated helper viruses, and (iv) successive passage of both satellite RNAs in association with their helper viruses enriches their relative proportions in the total encapsidated RNA (Kaper *et al.*, 1976; Kaper and Tousignant, 1977; results presented in Chapter 5). In these respects, Sat-RNA and CARNA 5

resemble Defective Interfering (DI) particles of animal viruses (Huang, 1973; Huang and Baltimore, 1977), STNV (Kassanis, 1968) and S-TRSV (Schneider, 1977). RNA-3 of tomato black ring virus (TBRV) also appears to have a number of properties characteristic of defective or satellite RNAs (Murant *et al.*, 1973). Both Sat-RNA and CARNA 5 can be distinguished from DI particles in that they contain genetic information not present in the genome of their helper viruses, and unlike satellite viruses such as STNV and the adeno-associated viruses (Fenner *et al.*, 1974), they utilize coat protein of their helper virus for encapsidation (Table 9.1). At present, it is not known whether S-TRSV-RNA and TRSV-RNA contain common nucleotide sequences (Schneider, 1977). The complete absence of sequence homology between satellite RNAs or satellite viruses and their helper viruses would be rather unlikely since some common sequences would be expected for replicase recognition. Moreover, the association between helper viruses and satellite RNAs or satellite viruses is often extremely specific (reviewed by Schneider, 1977). As an explanation for the apparent absence of sequence homology between satellites and the genomes of their helper viruses, it is possible that their replication may be mediated by host RNA-dependent RNA polymerases stimulated as a result of coinfection with their helper viruses. However, at present it is difficult to see how such non-specific RNA polymerases (Ikegami and Fraenkel-Conrat, 1978) could explain the relatively specific satellite-helper virus interactions observed.

At present it is not understood how DI particles, satellite RNAs and satellite viruses interfere with the replication of their helper viruses. In an attempt to explain the interference of polio virus replication by its DI particles, Baltimore *et al.* (1974) suggested that they may compete with the standard polio virus genome for common replicase

Table 9.1. Properties distinguishing DI particles, satellite viruses and satellite RNAs.

	Criteria	DI particles	Satellite viruses (STNV)	Satellite RNAs (Sat-RNA, CARNA 5)
1.	Reproduce only in presence of helper	$+^{a}$	$+^{b}$	$+^{d}$
2.	Encapsidation in helper coat protein	$+^{a}$	$-^{b}$	$+^{e}$
3.	Interference with the replication of helper	$+^{a}$	$+^{b}$	$+^{f}$
4.	Represents a part of helper genome	$+^{a}$	$-^{c}$	$-^{g}$

References cited above

a Huang (1973); Huang and Baltimore (1977).

b Kassanis (1968).

c Shoulder *et al.* (1974).

d Mossop and Francki (1978).

e Kaper *et al.* (1976); Mossop and Francki (1977).

f Kaper *et al.* (1976); this thesis (Chapter 5).

g Gould *et al.* (1978); Lot *et al.* (1977); Diaz-Ruiz and Kaper (1977).

and host protein synthesis systems, as well as for polio virus-specified coat protein for their encapsidation. More recently, Schnitzlein and Reichmann (1977) have demonstrated that the specificity of interference of the negative-strand virus vesicular stomatitis virus (VSV) by DI particles was related to the type of viral protein encapsidating the DI particles. These authors suggested that nucleocapsid proteins may have a regulatory role in the transcription of VSV-RNA. Conceivably, the interference of Sat-RNA and CARNA 5 with the replication of their helper viruses may involve factors other than competition for limited amounts of virus-specified replicase or coat protein, since both satellite RNAs are able to direct the synthesis of low molecular weight polypeptides *in vitro*. It would be of interest to determine whether these satellite RNAs are translated *in vivo* in the presence of their helper viruses. Analysis of proteins synthesized in plant protoplasts infected with CMV in the presence and absence of Sat-RNA is a possible experimental approach to test if the genetic formation of Sat-RNA is expressed *in vivo*.

Another consideration worthy of investigation is the effect of the accumulation of relatively large amounts of ds-RNA specific to CMV satellite-RNAs (Takanami *et al.*, 1977; Diaz-Ruiz and Kaper, 1977) on the *in vivo* translation of CMV-RNAs 1-4. It has been demonstrated that ds-RNA inhibits *in vitro* translation in rabbit reticulocyte systems (Content *et al.*, 1978, and references therein), although earlier experiments had failed to demonstrate any inhibition of *in vitro* translation in a wheat germ system with ds-RNA of cowpea mosaic virus (Reijnders *et al.*, 1975). The enrichment of satellite RNAs and satellite viruses by successive passage in 'permissive' host plants may possibly be explained on the basis of the replicative advantage imparted on such RNAs by virtue of their small size as has been

demonstrated for a low molecular weight variant RNA of Qβ phage (Mills *et al.*, 1967).

The most apparent difference between Sat-RNA and CARNA 5 is the failure of Sat-RNA to induce severe systemic necrosis in tomato plants coinfected with CMV (Table 5.6). A comparison of the hybridization of cDNA(Sat-RNA) with Sat-RNA and CARNA 5 indicates that after the hybridization data had been corrected for base mismatching these two satellite-RNAs have approximately 70% of their nucleotide sequences in common (Table 6.1). However, hybridization analyses of Sat-RNA and CARNA 5 using cDNA(CARNA 5) indicated even less sequence homology between these RNAs (Table 6.1). It was evident that cDNA transcribed from CARNA 5 was considerably smaller than expected (Figure 6.1B). Since transcription of polyadenylated RNA proceeds from the 3' end (Devos *et al.*, 1976), it is possible than cDNA preparations transcribed from CARNA 5 contained complementary sequences from, or near, the 3' end of CARNA 5 only. The apparent discrepancy between estimates of the sequence homology between Sat-RNA and CARNA 5 using cDNA(Sat-RNA) and cDNA(CARNA 5) could possibly be explained if sequences near the 3' end of these two satellite RNAs differed more than those near the 5' end. It is not understood why CARNA 5, unlike Sat-RNA, was unable to yield full-length transcripts. Possibly the lyophilized sample of CARNA 5 received from Dr Kaper had been extensively degraded in transit, or in our laboratory. Unfortunately, there was insufficient CARNA 5 material available to analyse by gel electrophoresis. Recently, Frolova *et al.* (1977) suggested that the inability of mitochondrial mRNA to be transcribed completely was due to the presence of either modified nucleotides or extensive regions of base-pairing in the template RNA which 'blocked' the progress of reverse transcriptase. However, Lot *et al.* (1977) were unable to demonstrate the presence of unusual or

modified nucleotides in CARNA 5. It is also possible that CARNA 5 may have become extensively degraded during the polyadenylation or transcription reactions. It has been recently suggested that RNases present in commercial preparations of BSA included in the polyadenylation reaction mixture may be responsible for the degradation of template RNAs (P. Palukaitis, personal communication). If contaminating RNases were responsible for degradation of CARNA 5 during the polyadenylation reaction, it is surprising that Sat-RNA was not also fragmented.

The molecular hybridization technique used in these studies, like the competitive hybridization method, suffers from several disadvantages which preclude absolute statements concerning the degree of base sequence homology between RNA species. The formation of hybrids between nucleic acid molecules requires a minimum number of complementary bases. For RNA-cDNA mixtures this stable minimum length is probably 10-20 bases (Walker, 1969), although this estimate would be influenced by factors such as salt concentration, hybridization temperature and nucleotide base competition. Hence, single-base substitutions separated by regions smaller than the stable minimum length would result in an under-estimate of sequence homology.

The procedure used to assay the extent of hybrid formation using S_1 nuclease introduces another possible source of error. It has been demonstrated that S_1 nuclease cleaves single-stranded nucleic acids more readily than it does the small loop regions resulting from base mismatching (Dodgson and Wells, 1977). This would be expected to over-estimate the extent of sequence homology between RNAs with similar, but not identical, base sequences. Moreover, the S_1 nuclease resistance of homologous RNA-cDNA hybrids in these studies rarely exceeded 85% (range 60-92%). Double-stranded nuclease activity has been reported

in preparations of S_1 nuclease (Hahn and Van Ness, 1976; Gonda and Symons, 1978) and this may, in part, be responsible for the incomplete S_1 nuclease resistance of homologous RNA-cDNA hybrids. Another possible explanation is that with the hybridization conditions employed in these investigations, some regions of the RNA molecules may preferentially anneal to form hair-pin like configurations rather than hybridize with their cDNAs.

Despite these objections, molecular hybridization offers several important advantages over the competitive RNA-RNA hybridization method, especially in determining the sequence homology between the RNA components of viruses with divided genomes (Gonda and Symons, 1978). An examination of the hybridization kinetics provides a direct measure of both the sequence homology between RNAs, and the extent to which one RNA is contaminated with sequences from another (e.g. Figures 4.6 and 7.6). Furthermore, since there is a linear relationship between the molecular weight of an RNA template and its expected $R_o t_{\frac{1}{2}}$ value under given hybridization conditions (Hell *et al.*, 1976; Gould *et al.*, 1978), the nucleotide complexity of an RNA can also be determined from the hybridization kinetics of the homologous RNA-cDNA mixture. Since cDNA of high specific activity can be readily obtained, molecular hybridization can be used as a probe for the detection of extremely low levels of a specific RNA. This approach was used in this thesis to detect the presence of either Sat-RNA, QCMV-RNA 3 or STNV-RNA in total leaf RNA extracts.

Satellites: Their *in vivo* Survival and Possible Origins

Results presented in Figure 7.3 and Table 7.3 demonstrate that Sat-RNA is able to persist *in vivo* for prolonged periods. Since it was

also demonstrated that Sat-RNA is unable to replicate autonomously (Tables 7.1, 7.2 and 7.3), it would appear that its ability to survive intact *in vivo* may be associated either with its inherent resistance to nuclease digestion, or its conversion *in vivo* to some resistant form. Attempts to investigate the capabilities of different viral RNAs for *in vivo* survival are restricted by the limited number of suitable systems available. Two viral RNAs, namely genomic RNA 3 of QCMV, and STNV-RNA appeared particularly suitable candidates since neither are capable of replication in the absence of their complementary genomic RNAs or helper virus (Table 7.5; Kassanis, 1968). Furthermore, both RNAs have little or no nucleotide sequence homology with that of the complementary genomic RNAs or helper virus employed for their subsequent recovery (Figure 7.4; Figure 7.6; Shoulder *et al.*, 1974).

Results presented in Table 7.6 indicate that STNV-RNA, like Sat-RNA, is able to survive *in vivo* for prolonged periods in the absence of its helper virus, TNV. This property is not shared by QCMV-RNA 3 (Table 7.5) which is apparently rendered biologically inactive soon after inoculation. Hence it appears that the ability to survive *in vivo* may be restricted to a particular class of low molecular weight RNAs.

Experiments designed to detect ds-RNAs in leaf tissue inoculated with Sat-RNA alone were inconclusive (Table 8.1) and hence, it is not possible to conclude that the *in vivo* survival of Sat-RNA (or STNV-RNA) does not involve a host-mediated conversion of these RNAs to some RNase resistant form. However, their relative resistance to S_1 nuclease digestion (Figure 8.3) and their stability in crude plant extracts (Table 8.2) suggests that the *in vivo* survival of Sat-RNA and STNV-RNA

may be related to features of their molecular structure. The *in vivo* stability of tRNA is considered to be due to features of its secondary and tertiary structure, and its small molecular size (Cramer, 1971; Crouch, 1976). Both Sat-RNA and STNV-RNA, like tRNA, appear to be stabilized by considerably more secondary structure than the genomic RNAs of the helper viruses (Figure 8.1 and 8.2), although the kinetics of thermal denaturation alone would not be expected to satisfactorily resolve absorption increases due to base-stacking and base-pairing (Boedtker and Gesteland, 1975). These authors suggest that a more reliable estimate of secondary structure is obtained by comparing the hyperchromicity of RNA before and after reaction with formaldehyde (Boedtker, 1968).

Conceivably, the extraordinarily high specific infectivity of Sat-RNA (Table 5.5) may be a consequence of its *in vitro* stability. Recently, it has been shown that reovirus mRNAs which contained a $M^7G_{ppp}G$ 5'-terminal 'cap' were more stable than mRNAs containing an 'unblocked' 5' end in several *in vitro* translation systems (Furuichi *et al.*, 1977). These authors suggested that mRNAs containing a 5'-terminal $M^7G_{ppp}G$ cap are protected against 5'-exonucleolytic degradation. It is possible that the $M^7G_{ppp}G$ 5'-terminal 'cap' of Sat-RNA may contribute to its *in vitro* stability, however not that of STNV-RNA since the 5' end of this RNA, like that of TNV-RNA, does not contain this structure (Lesnaw and Reichmann, 1970). Furuichi *et al.* (1977) have also suggested that the apparent *in vitro* stability of certain mRNAs including STNV-RNA which lack a 5'-terminal $M^7G_{ppp}G$ 'cap' structure, may be due to their association with protein(s) as in

polio virus RNA (Lee *et al.*, 1977), or to 5'-terminal secondary structure.

It is interesting to speculate why Sat-RNA and STNV-RNA should have evolved such stable molecular structures since both RNAs are encapsidated, and are transmitted by the same vector as that of their helper viruses; aphids in the case of Sat-RNA (Chapter 4) and *Olpidium brassicae* in the case of STNV (Kassanis, 1968, and references therein). Considering the relatively large amounts of Sat-RNA detected in some RNA preparations of CMV, it seems likely that a substantial proportion of CMV particles may contain Sat-RNA alone, or a number of possible combinations of both Sat-RNA and genomic RNAs. That CMV particles can package Sat-RNA alone was recently demonstrated in reconstitution experiments by Lot and Kaper (1976b). Following their introduction into susceptible cells of their plant hosts, the satellites, whether in the presence or absence of their helper viruses, would presumably undergo immediate uncoating since this is probably a function of host enzymes over which infecting particles have no control (Matthews, 1970, and references therein). If either encapsidated Sat-RNA or STNV enter a host plant cell unattended by their helper viruses, their RNAs would be unable to replicate, and would thus be exposed to inactivation by cellular RNases. Conceivably, their *in vitro* stability may have evolved in response to such selection pressures, since their survival would depend on their ability to remain intact until the subsequent arrival of their appropriate helper viruses, either by secondary infection of the cell, or more likely, by invasion from adjoining cells. It is interesting to note that Kassanis (1968) reported that STNV was able to be rescued 5 days after inoculation by the subsequent introduction of TNV. In these experiments the author used STNV particles, and attributed its apparent survival to the stability of the virus particles.

Sat-RNAs and viroids share several properties including their molecular size and *in vitro* stability. However, there are several important differences in their mode of replication. Viroids do not appear dependent on helper viruses for their replication (Diener, 1972, and references therein), and whereas the replication of satellite RNAs of CMV is RNA-dependent (Takanami *et al.*, 1977; Diaz-Ruiz and Kaper, 1977; Figure 7.2), that of viroids appears to be DNA-dependent (Diener and Smith, 1975). Hence, similarities in their molecular stability may have been acquired during parallel evolution in response to the absence of protective structures at some stages of their life-cycle.

The origin of satellite RNAs and satellite viruses is of considerable interest and two possibilities seem worthy of consideration: (1) they are derived from genetic information carried on, or specified by their plant hosts, or (2) they originate from the genome of a virus other than their helper. Attempts to detect sequence homology between Sat-RNA, and leaf RNAs from healthy *N. clevelandii* plants (Gould *et al.*, 1978) or DNA extracted from healthy *N. tabacum* cv. Xanthi nc plants were unsuccessful. To substantiate or reject this hypothesis it may be necessary to compare the base sequences of satellites with those of cellular DNA and RNA isolated from an extremely large number of host species since it would be impossible to predict a likely host plant from which they may have been derived.

The alternative hypothesis suggests that satellites are derived from sequences contained in the genome of viruses which differ from their helper viruses. At present, there is relatively little evidence to suggest that a situation analogous to DI particles of animal viruses (Huang and Baltimore, 1977) exists in plant viruses (Schneider, 1977). A number of

plant viruses, including CMV, encapsidate virus-specied RNAs not required for their infectivity (Jaspars, 1974, and references therein). In most cases these RNAs are thought to represent *in vivo* mRNAs since they direct the *in vitro* synthesis of virus-specific proteins. For example, RNAs 4 of BMV, AMV and CMV have been shown to direct the synthesis of viral coat protein (Shih and Kaesberg, 1973; Rutgers, 1977, and references therein, and Schwinghamer and Symons, 1977, respectively). A similar situation was shown to exist in the case of TYMV (Pleij *et al.*, 1977; Higgins *et al.*, 1978). In addition to a low molecular weight RNA specifying coat protein, the cowpea strain of TMV also encapsidates larger mRNAs which appear analogous to RNA 3 of viruses with tripartite genomes (Bruening *et al.*, 1976). Whether these non-essential, encapsidated RNAs are similar to the DI particles of animal viruses is debatable, since they appear to fulfil a necessary mRNA function *in vivo*. Moreover, their discrete molecular size suggests that such RNAs may arise either as a result of specific fragmentation of the genomic RNAs, or from their partial transcription (Takanami *et al.*, 1977). Therefore, by this convention, DI particles of plant viruses would be expected to contain RNA derived from the viral genome which did not fulfil some necessary function in viral synthesis, and which interferes with the replication of the standard viral genome. Conceivably, the defective RNA of one virus may represent the satellite RNA of another if it is capable of acting as a helper. RNA 5, which has several of the characteristics of a defective RNA as defined above, would be a logical candidate to test. It is possible that the RNA 5 from one cucumovirus strain may be able to be replicated by another distinct strain, in which case, this RNA would then be considered as a satellite similar to Sat-RNA studied in this thesis.

APPENDIX

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