

STUDIES ON THE TRSV COAT PROTEIN AND ITS SYNTHESIS

IN-VIVO AND IN-VITRO

by

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SUMMARY

1. It was demonstrated that the top (T), middle (M) and bottom (B) particle components of tobacco ringspot virus (TRSV) are differentially dissociated in the presence of sodium dodecyl sulphate (SDS), in acid and alkali pHs and on mild heating. The results indicate that the M and B particles are stabilised by both protein-protein and protein-RNA interactions whereas the T particles are stabilised by protein-protein interactions only. Based on the above results a new method, not involving the use of acid, was devised to isolate intact coat protein polypeptides free from SDS and RNA.
2. The coat protein of TRSV dissociated in the presence of SDS, urea, and 2-mercaptoethanol (MCE) separated into six components on electrophoresis in polyacrylamide gels. It was established that none of the components was of host plant origin and that they were all chemically and antigenically similar. The estimated molecular weights of the components ranged from about 14,000 to more than 110,000, approximating to a polymeric series of 1, 2, 3, 4 and 8; over 85% of the protein was recovered in a band corresponding to a molecular weight of about 57,000. The relative proportions of all the components did not alter significantly when the protein was subjected to various treatments including S-carboxymethylation prior to electrophoresis. Analysis of the amino acid compositions and tryptic peptides of the protein established that the TRSV capsid is constructed from a single polypeptide species containing approximately 119 amino acid residues and has a molecular weight of about 13,000. Based on these results, it appears that the TRSV capsid is composed of 240 polypeptide subunits arranged as an icosahedron with a $T = 4$ surface lattice structure.
3. TRSV coat protein synthesis in cucumber cotyledons was inhibited by cycloheximide but not by chloramphenicol indicating that cytoplasmic 80 S and not chloroplastic 70 S ribosomes are involved in the synthesis

- of TRSV proteins *in-vivo*.
4. Electrophoresis of RNA preparations isolated from TRSV in 4% polyacrylamide gels containing 98% formamide showed that the molecular weights of the two components, RNA-S and RNA-L, are $1.44 \pm 0.02 \times 10^6$ and $2.26 \pm 0.02 \times 10^6$ respectively. These two RNA species were successfully separated by two cycles of preparative agarose gel electrophoresis. Infectivity assays of these RNAs indicate that both RNA species are required for infectivity.
 5. The TRSV RNAs were translated *in-vitro* separately and before fractionation using the wheat germ cell-free systems of Marcu and Dudock (1974) and Davies *et al.* (1976). The results obtained show that the **Marcu & Dudock** system was much more efficient. Translation of the separated RNAs shows that RNA-S is a more efficient template than RNA-L. Analyses of the translated products by polyacrylamide gel electrophoresis and by tryptic peptide mapping indicate that RNA-S and RNA-L directed the synthesis of entirely different ranges of polypeptides with molecular weights ranging from 9000 to 125,000 for RNA-S and 4000 to 230,000 for RNA-L; while the natural unfractionated RNA yielded mainly RNA-S products. Although about 90% of RNA-S and all of RNA-L appears to be translated, no TRSV coat protein was detected in the translational products of either of the RNAs. These results suggest that in TRSV the RNAs probably function as monocistronic mRNAs and that the viral coded proteins (except for the coat protein) are synthesized by post-translational cleavage of precursor proteins. However, peptide analysis of the low molecular weight products indicates that they are not post-translational cleavage products.
 6. Results are presented which confirmed the existence of a protein covalently linked to TRSV RNA (VPg) which is required for virus infectivity. It was also established that the VPg is not required for translation of the RNAs *in-vitro* and thus it may be required for

replication of viral RNA. The molecular weight of the VPg was estimated using *in-vitro* $^3\text{H-NaBH}_4$ labelled as well as *in-vivo* ^{14}C -leucine labelled RNA preparations. The results of both methods were similar, indicating that a protein with a molecular weight of approximately 64,000 - 66,000 is associated with the RNA.

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 and acknowledgements are made in the text.

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ABBREVIATIONS

AILV	artichoke Italian latent virus
AMV	alfafa mosaic virus
ArMV	arabis mosaic virus
BMV	brome mosaic virus
CarMV	carnation mottle virus
CCMV	cowpea chlorotic mottle virus
CLRV	cherry leaf-roll virus
QCMV	cucumber mosaic virus, Q strain
CpMV	cowpea mosaic virus
CRLV	cherry rasp leaf virus
EMC	encephalomyocarditis virus
GCMV	grapevine chrome mosaic virus
GFLV	grapevine fanleaf virus
MLRV	myrobalan latent ringspot virus.
RRV	raspberry ringspot virus
SLRV	strawberry latent ringspot virus
VTAV	tomato aspermy virus, V strain
TBRV	tomato black ring virus
TBSV	tomato bushy stunt virus
BTMV	tobacco mosaic virus, bean strain
U-1 TMV	tobacco mosaic virus, wild strain
TRSV	tobacco ringspot virus
TomRSV	tomato ringspot virus
TYMV	turnip yellow mosaic virus
VEV	vesicular exanthema virus

Ac	acetate ion
ATP	adenosine 5' triphosphate
bisacrylamide	methylene bisacrylamide
BPB	bromophenol blue
BSA	bovine serum albumin
CBB	coomassie brilliant blue
CNBr	cyanogen bromide
cpm	counts per min
cv	cultivar
Cyt-c	cytochrome c
DCC	diphenyl carbamyl chloride
DMSO	dimethyl sulphoxide

DNA	deoxyribonucleic acid
DTE	dithioerythreitol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetraacetic acid
GDH	glutamate dehydrogenase
GHCl	guanidinium chloride
GTP	guanosine 5' triphosphate
HEPES	N-2'-hydroxyethylpiperazine-N1-2-ethane sulphonic acid
IDI	integer deviate indices
KAc	potassium acetate
Lys	lysozyme
MCE	2-mercaptoethanol
MgAc	magnesium acetate
Mol. Wt.	molecular weight
Myo	myoglobin
NaAc	sodium acetate
NaBH ₄	sodium borohydride
OD	absorbance (optical density)
Oval	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAS	Periodic-Acid-Schiff reagent
PEG 4000	polyethylene glycol 4000
PMSF	phenyl methyl sulphonyl fluoride
PolyA	polyadenylate
POPOP	1,4,-bis-[2-(5-phenyloxazolyl)] benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
RNA-L	larger genomic RNA component of nepoviruses
RNA-S	smaller genomic RNA component of nepoviruses
RNase	ribonuclease
ds-RNA	double-stranded-RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
ss-RNA	single-stranded-RNA
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
spp	species
TBE	Tris-borate-EDTA buffer, pH 8.3
TBES	TBE containing 0.1M sodium acetate

TCA	trichloroacetic acid
TE	20mM Tris-acetate buffer, pH 7.6, 0.2 mM EDTA
Tris	tris (hydroxymethyl) aminoethane
Try	trypsin
T, M, B	top, middle and bottom particle components of nepoviruses respectively
Urea-SDS-MCE	6M urea, 1% SDS, 2% MCE
UV	ultraviolet
VPg	protein component associated with the viral genomic RNA
V/V	volume:volume
W/W	weight:weight

CHAPTER I

GENERAL INTRODUCTIONA. The Nepovirus Group

Nepoviruses constitute a rapidly expanding taxonomic group of plant viruses, made up of more than 30 distinct members and strains (Table 1). However, they can be classified into several antigenically distinct subgroups (Francki and Hatta, 1977; Harrison and Murrant, 1977). They have a group cryptogram of $(R/1 : 2.4/42 + \frac{1.4-2.2}{27-40} + \frac{2 \times 1.4}{46} : S/S : S/C, Ve/Ne)$ (Harrison and Murrant, 1977). Nepo is the acronym for *nematode-polyhedral* which refers to two of the main characteristics of the viruses distinguishing them from those of the other groups (Cadman, 1963); the ability to be transmitted by nematode vectors (Martelli, 1975), and possession of polyhedral particles 28 - 30nm in diameter (Francki and Hatta, 1977). The viruses do not multiply in their vectors but do remain infectious in nematodes for several weeks (*Longidorus* spp.) or months (*Xiphinema* spp.) but ^{the vectors} cease to transmit after moulting (Harrison and Murrant, 1977).

Other distinguishing characteristics of the viruses include:

- (a) They can be mechanically transmitted as well as being seedborne (Lister and Murrant, 1967). Survival in seeds is an important means of persistence and distribution in the field (Murrant and Taylor, 1965; Murrant and Lister, 1967).
- (b) They have wide host ranges involving many genera and families of seed plants including both herbaceous and woody plants, monocotyledons and dicotyledons.
- (c) The viruses induce characteristic ringspot and mottle symptoms in newly invaded tissues but later, leaves produce less severe symptoms or they are symptomless even though the virus is still present. Many plants infected through seeds are symptomless (Lister and Murrant, 1967; Hanada and Harrison, 1977).

Table 1

LIST OF NEPOVIRUSES

NEPOVIRUS SUBGROUP	MAIN NATURAL VECTOR	SEDIMENTATION COEFFICIENT OF PARTICLES	MOL. WT. OF COAT PROTEIN SUBUNIT†	MOL. WT. OF RNA UNDER DE-		REFERENCES
				NATURING RNA-L	CONDITIONS (x10 ⁶) RNA-S	
I. Definite Subgroups						
A. Tobacco ringspot virus (TRSV) R/1:2.3/40+1.4/28+2x1.4/44:S/S:S/Ne	<i>Xiphinema americanum</i>	53,91,126 S	57,000			Stace-Smith (1970); Mayo et al. (1971); Harrison et al. (1972a); Harrison and Murant (1977); Salazar and Harrison (1977).
Potato black ringspot virus		49,88,117 S				
B. Arabis mosaic virus (ArMV) R/1:2.4/41+1.4/29+2x1.4/44:S/S:S/Ne	<i>X. diversicaudatum</i>	53,93,126 S	54,000	2.15	1.64	Murant (1970b); Hewitt et al. (1970); Mayo et al. (1971); Francki and Hatta (1977); Martelli et al. (1978); Hewitt (1950); Dias (1950); Goheen and Hewitt (1962); Martelli and Hewitt (1963).
**Raspberry yellow dwarf virus Grapevine fanleaf virus R/1:2.4/* +1.4/* :S/S:S/Ne	<i>X. italiae</i> , <i>X. index</i>	50,86,120 S		2.13	1.63	
+Grapevine yellow mosaic virus +Grapevine vein banding virus Rhubarb mosaic virus Hop line pattern virus						
C. Raspberry ringspot virus (RRV) R/1:2.4/43+1.4/30+2x1.4/46:S/S:S/Ne	<i>Longidorus macrosoma</i> (English) <i>L. elongatus</i> (Tyre)	50,91,125 S	54,000			Murant (1970a); Mayo et al. (1971); Harrison et al. (1972a); Francki and Hatta (1977).
**Raspberry Scottish leafcurl virus **Red currant ringspot virus						
D. Tomato blackring virus (TBRV) R/1:2.5/38+1.5/28:S/S:S/Ne	<i>L. attenuatus</i>	55,97,121 S	60,000	2.08	1.63	Murant (1970c); Martelli and Quacquarelli (1972); Kenten (1972); Murant et al. (1973); Bancroft (1968); Francki and Hatta (1977); Harrison and Murant (1977); Martelli et al. (1977); Martelli et al. (1978); Kenten (1977); Dunez et al. (1976).
**Potato bouquet virus R/1:2.5/38+1.5/28:S/S:S/Ne	<i>L. attenuatus</i>	55,97,121 S				
+Beet ringspot virus R/1:2.5/33+1.5/28:S/S:S/Ne	<i>L. elongatus</i>	55,97,121 S				
Artichoke Italian latent virus R/1:2.4/41+1.5/34:S/S:S/Ne	<i>L. apulus</i>	55,96,121 S		2.1	1.68	
Grapevine chrome mosaic virus R/1:*/31+*/40:S/S:S/*	unknown	-,92,117 S		2.08	1.63	
Cacao necrosis virus */*: */(22-30)+ */(35-41):S/S:S/C	unknown	54,101,129 S				
Chicory chlorotic ringspot virus +Potato pseudoacuba virus						
**Lettuce ringspot virus +Celery yellow vein virus						
Tomato top necrosis virus Myrobalan latent ringspot virus R/1:2.6/38+1.9/28:S/S:S/*	unknown	52,102,126 S -,105,115 S	53,000	2.15	1.91	
E. Tomato ringspot virus (TomRSV) R/1:2.3/40+2.2/39:S/S:S/Ne	<i>X. americanum</i>	53,119,127 S	58,000			Stace-Smith (1970a); Schneider et al. (1974); Martelli et al. (1978); Francki and Hatta (1977); Cadman and Lister (1961); Dias (1975); Allen and Dias (1977).
+Grape vine yellow vein virus Peach rosette mosaic virus R/1:2.5/*+2.1/*:S/S:S/Ne	<i>X. americanum</i>	52,115,134 S				
**Peach yellow bud mosaic virus						

Table 1 cont'd.

F.	Cherry leafroll virus (CLRV) R/1:2.4/43+2.1/40:S/S:S/Ne +Elm mosaic virus R/1:2.4/43+2.1/40:S/S:S/Ne +Golden elderberry virus R/1:2.4/43+2.1/40:S/S:S/Ne	<i>X. diversicaudatum</i> <i>X. coxi</i> <i>X. diversicaudatum</i> <i>X. diversicaudatum</i>	- , 115, 128 S	54,000			Cropley and Tomlinson (1971); Jones and Mayo (1972); Martelli et al. (1978); Varney and Moore (1952); Hansen (1967).
G.	Strawberry latent ringspot virus (SLRV) R/1:2.6/37+2x1.6/42:S/S:S/Ne	<i>X. coxi</i> <i>X. diversicaudatum</i>	58, - , 126 S	29,000+ 44,000			Murant (1974); Mayo et al. (1974); Francki and Hatta (1977).
II. <u>Tentative members or subgroups</u>							
A.	Mulberry ringspot virus (MRSV) R/*:*/*:S/S:S/Ne	<i>L. martini</i>	50,96,126 S				Tsuchizaki (1975).
B.	Chicory yellow mottle virus (CYMV) R/1:2.2/43+2.0 /40:S/S:S/*	unknown	50, (66,78, 84-109), 117,126 S				Martelli et al. (1978); Quacquarelli et al. (1974).
C.	Cherry rasp leaf virus (CRLV) R/1:2.0/*+1.5/*:S/S:S/Ne	<i>X. americanum</i>	56,96,128 S	24,000+ 22,500			Stace-Smith and Hansen (1976); Harrison and Murant (1977).
D.	Artichoke yellow ringspot virus (AYRV)	unknown			2.17	1.8	Martelli et al. (1978).
E.	Artichoke vein banding virus (AVBV)	unknown			2.14	1.53	Martelli et al. (1978).
F.	Grapevine Bulgarian latent virus (GBLV) R/1:2.2/41+2.1/39:S/S:S/*	unknown	52,120,127 S	54,000			Martelli et al. (1977); Martelli et al. (1978).
G.	Satsuma dwarf virus (SDV)	unknown					Martelli et al. (1978).

+ Possibly strains

** Possibly synonyms

‡ Obtained from polyacrylamide gel electrophoresis of viral proteins

(d) They are relatively stable *in-vitro*. They remain infectious for several months in purified preparations at 4°C or several years in freeze dried sap, or up to several weeks in sap at 4°C. Thermal inactivation points vary from 55 - 70°C (Harrison and Murrant, 1977).

(e) The viruses occur in the cytoplasm of infected cells and are often found in membranous tubules. Crystalline aggregates are uncommon.

Individual Nepovirus members and the diseases they cause, tend to have restricted natural distributions determined mainly by the nematode vectors but as a group they are found in most parts of the world. However, many Nepoviruses have spread widely to parts very far from their origin through use of infected seeds and planting materials and an abundance of weed hosts (Harrison, 1977; Harrison and Murrant, 1977).

B. Structure of Nepoviruses

Nepoviruses are small ribonucleic acid (RNA) viruses (Brown and Hull, 1973) with limited packing capacity in their particles. With all of these small RNA viruses containing large amounts of genetic material (including those within the Nepovirus, Comovirus, alfalfa mosaic virus (AMV), Bromovirus and Cucumovirus groups), the genomes are found to be functionally divided into components which are compartmented into two or more types of particles each containing a part of the genome. Viruses with such genomic and particle heterogeneity are referred to as viruses with functionally divided genomes or multipartite viruses (Van Kammen, 1972).

(i) Composition of Nepoviruses

In most Nepoviruses, three types of isometric particles (28 nm in diameter with angular outlines) sedimenting at 50 - 55, 90 - 120 and 120 - 130 S are found, and are designated Top (T), Middle (M) and Bottom (B) components respectively. The T particles are empty capsids but both the M and B particles contain RNA. In some isolates of strawberry latent ringspot virus (SLRV), no M

components are detected and in cherry leaf roll virus (CLRV), myrobalan latent ringspot virus (MLRV) and grapevine chrome mosaic virus (GCMV), no T components are found (Table 1).

(ii) Structure and Properties of Nepovirus Capsids

The capsids of the T, M and B particles of Nepovirus appear identical in size, shape and protein composition. However, with arabis mosaic virus (ArMV) and SLRV, the T, M and B components show some variations in their electrophoretic behaviour (Clarke, 1976). This suggests that in these viruses the RNA may be involved in determining the surface charge of the particles.

Most Nepoviruses have apparent coat protein polypeptide subunit molecular weights of about 55,000 daltons when analysed by polyacrylamide gel electrophoresis (Table 1) and hence appear to be much larger than those of other small plant viruses. Using these molecular weight data of the polypeptides and the molecular weight of the protein shell (calculated from the Svedberg equation and the percentage of RNA in the particles), Mayo *et al.* (1971) concluded that the Nepovirus capsid is probably a T = 1 icosahedral structure consisting of 60 molecules of a single type of polypeptide. However, this model is incompatible with electron microscopic evidence on tobacco ringspot virus (TRSV) and ArMV which indicates that the capsids of these viruses are composed of 42 morphological subunits with T = 4 icosahedral structure (Chambers *et al.*, 1965; Agrawal, 1967). Two Nepoviruses do not have molecular weights of 60,000 for their coat protein polypeptide subunits; in SLRV they are 29,000 plus 44,000 and in ^{rasp leaf virus} cherry λ (CRLV) they are 24,000 plus 22,500. These viruses are not likely to have a T = 1 structure. Most Nepoviruses form stable T components containing no RNA, suggesting that the capsids are mainly stabilized by protein-protein interactions. This may allow the particles to encapsidate varying amounts of RNA without becoming unstable.

Nepoviruses have specific associations with their nematode vectors (see Table 1) and this specificity seems dependent on the properties of the virus capsids. However, serologically related viruses have different specific nematode vectors, suggesting that other factors besides the serological properties of the capsids are also involved in determining vector specificity (Harrison, 1964; Taylor and Murrant, 1969; Harrison *et al.*, 1974; Harrison and Murrant, 1977).

(iii) Structure and Organisation of Nepovirus Genomes

Nepoviruses contain single-stranded (ss)-RNA genomes. The genome of each member is divided into two components, one large and one small. Both species of RNAs are necessary for infectivity (Harrison *et al.*, 1972a, 1974, Quacquarelli *et al.*, 1976; Randles *et al.*, 1977).

Under non-denaturing conditions the large RNA components of Nepoviruses (RNA-L) appear to have molecular weights ranging from 2.0 to 2.6×10^6 while the small RNA components (RNA-S) from 1.4 to 2.1×10^6 (Table 1). However, the same RNAs electrophoresed under denaturing conditions in polyacrylamide gels appear to have molecular weight values with differences of up to 20% from those obtained under non-denaturing conditions. It is also interesting to note that in all cases, the RNA-S components appear relatively longer while the RNA-L components appear shorter under denaturing conditions. (Compare molecular weight data in cryptogram and those measured under denaturing conditions in Table 1).

The RNA-L components are encapsidated in the B particles (1 molecule per particle) whereas the RNA-S components are present in the M particles (1 molecule per particle) and in some Nepoviruses e.g. TRSV and raspberry ringspot virus (RRV), in the B particles as well (2 molecules per particle) (Diener and Schneider, 1966; Murrant *et al.*, 1972; Mayo *et al.*, 1973; Martelli *et al.*, 1978).

In TRSV, tomato black ring virus (TBRV) and MLRV, satellite-like RNAs are sometimes present. Each of these RNAs replicates only in cells infected with its own "helper" virus and they are encapsidated in

the helper virus capsid. In TRSV, the satellite-RNA molecules have molecular weights of 0.9×10^5 (Schneider, 1971; Schneider *et al.*, 1972), while in TBRV and MLRV, they have molecular weights of about 5×10^5 (Murant *et al.*, 1973; Delbos *et al.*, 1976).

C. Genetics of Nepoviruses

The genomes of Nepoviruses are functionally divided. In TRSV, the observation that there is very little sequence homology between the two RNA species (Rezaian and Francki, 1974) suggests that they carry different genes. Studies of pseudo-recombinants of RRV and TBRV (Harrison *et al.*, 1972b; Randles *et al.*, 1977) showed that RNA-L carries genes determining host range, seed transmissibility and types of symptoms produced, whereas RNA-S carries genes determining serological specificity, nematode transmissibility and some symptom reactions. These results suggest that the coat protein gene is located on RNA-S. Virulence was found to be dependent on both RNA species (Harrison *et al.*, 1974; Hanada and Harrison, 1977; Harrison and Murant, 1977) suggesting the existence of interaction and co-ordination in the expression of these genes on the two RNA species.

D. Occurrence and Synthesis of Virus in Infected Cells and Tissues

Nepovirus particles are detected in all parts of infected plants including seed, pollen and meristemic tissue. Many Nepoviruses (ArMV, CLRV, grapevine fanleaf virus (GFLV), SLRV and RRV) induce the formation of vesiculated membranous inclusion bodies containing ribosomes in the cytoplasm of infected cells, often close to the nucleus (Harrison and Murant, 1977). In TRSV infected cells, extensive cytoplasmic membrane vesiculation was observed throughout the cytoplasm ^{but} without forming distinct inclusions and is not always in proximity to the nucleus (Francki and Hatta, 1977). Particles of CLRV, SLRV (Walkey and Webb, 1968), TRSV (Davison, 1969; Crowley *et al.*, 1969), tomato ringspot virus (TomRSV) (de Zoeten and Gaard, 1969), GFLV (Saric and Wrischer, 1975) and RRV (Harrison and Murant, 1977) often occur

inside membranous tubules, which may pass through plasmodesmata. Particles of ArMV occur in spherical aggregates in the cytoplasm (Gerola *et al.*, 1965). In other instances small crystal-like aggregates of virus particles are found in the cytoplasm of infected cells (Gerola *et al.*, 1966; de Zoeten and Gaard, 1969; Robertset *et al.*, 1970).

There is some evidence that the Nepoviruses are replicated in the cytoplasm. The RNA-dependent RNA polymerase induced by TRSV is found in the cytoplasm of infected tissues at the beginning of the most rapid phase of synthesis of the virus (Rezaian, 1974; Rezaian *et al.*, 1976).

The vesiculated membranous inclusion bodies in the cytoplasm induced by many Nepoviruses often contain ribosomes and in RRV and TBRV are the sites of accumulation of viral antigens (Harrison *et al.*, 1974; Barker and Harrison, 1977). Thus these inclusion bodies may be the major sites of viral protein synthesis. With SLRV and TRSV, tubules containing virus particles or aggregates of virus particles are also found in these inclusion bodies, suggesting that assembly of virus components may also take place there.

E. Salient Features of TRSV

TRSV is the type member for the Nepovirus group. It has the cryptogram R/1 : 2.3/40 + 1.4/28 + 2x1.4/44 : S/S : S/Ne. It was first described by Fromme *et al.* (1927).

TRSV has a wide host range including species in more than 38 genera representing more than 17 families (Price, 1940). It causes ringspot symptoms in tobacco, cucumber, soya bean, ornamentals and other economically important crops. It is mostly transmitted by sap inoculation and by the nematode vector, *Xiphenema americanum* (McGuire, 1964; Bergeson *et al.*, 1964); it is also commonly seed transmitted (Stace-Smith, 1970). The most reliable diagnostic host species include cucumber and cowpea. Many strains of the virus are recognised but

the major ones include the tobacco green ringspot strain of Valteau (1932), tobacco yellow ringspot strain of Valteau (1932), tobacco ringspot virus number 1 of Price (1936), eucharis mottle strain of Kahn *et al.* (1962), anemone necrosis strain of Hollings (1965), satellite-containing strain of Schneider (1969) and the gladiolus strain of Randles and Francki (1965). The virus is relatively stable in sap, although the degree of stability varies with different reports. Freeze dried sap containing virus was infectious after 5 years (Hollings, 1965) but fresh sap is inactivated after heating at 65°C for 10 minutes (Lister *et al.*, 1963).

When analysed by analytical ultracentrifugation, three main particle components designated T, M, and B, in order of increasing molecular weights are found (Stace-Smith *et al.*, 1965). The three particle components are isometric with diameters of 28 - 30 nm and are serologically identical (Stace-Smith, 1970; Francki, private communication). They have sedimentation coefficients of 53, 91 and 126 S containing 0, 28 and 40% RNA respectively (Stace-Smith, 1970). The molecular weights of the particles are about 3.3 (T), 4.9 (M) and 5.7 (B) x 10⁶ daltons (Stace-Smith, 1970). The average $E_{260}^{(0.1\%)}$ is about 7. Components sedimenting faster than the B particles are often found in preparations of TRSV but these were shown to be aggregates of T, M and B particles (Rezaian, 1974).

The TRSV genome is made up of two ss-RNA components (Diener and Schneider, 1966). The reported molecular weights of these RNA components measured by polyacrylamide gel electrophoresis under non-denaturing conditions are 1.4 (RNA-S) and 2.3 to 2.4 x 10⁶ (RNA-L) (Murant *et al.*, 1972; Rezaian, 1974). Unlike most Nepoviruses, it has been difficult to demonstrate that both RNA components of TRSV are required for infectivity. Purified B component particles alone are infectious (Stace-Smith *et al.*, 1965). Sucrose density gradient fractions containing separated TRSV particle or RNA components showed

that infectivity is correlated with the B particle component or the RNA-L peaks respectively (Francki, private communication, Harrison *et al.*, 1972). Thus, it was proposed that the M component contains one molecule of RNA-S per particle while the B component consists of particles containing either one molecule of RNA-L or two molecules of RNA-S. Rezaian and Francki (1974) in their competitive RNA-RNA annealing experiments using TRSV-specific double-stranded (ds)-RNA showed that there is complete nucleotide sequence homology between the RNA-S from the M and B components, and very little sequence homology between the RNA-L and RNA-S. Studies by Harrison *et al.* (1972a) showed that highly purified preparations of RNA-L are still infectious although infectivity was increased several folds by the addition of purified RNA-S. Further, using equilibrium centrifugation in caesium chloride (CsCl), Murant *et al.* (1972) and Francki (private communication) were able to detect two types of B particles in TRSV, one of which is believed to contain two molecules of RNA-S. These observations suggest that both RNA-S and RNA-L in TRSV are required for infectivity. However, it remains to be established that the infectivity in the RNA-L preparation of Harrison *et al.* (1972a) is due to the presence of traces of RNA-S in the RNA-L preparations.

The calculated molecular weights of the protein shells of TRSV particles are similar, being about 3.4×10^6 daltons (Mayo *et al.*, 1971). Analysis of SDS-disrupted TRSV particles in polyacrylamide gels demonstrated the presence of one type of polypeptide with a molecular weight of 57,000 (Mayo *et al.*, 1971). Amino acid analyses of TRSV protein suggested that the molecular weight of the polypeptide subunit is a multiple of about 20,000 (Stace-Smith *et al.*, 1965). Thus Mayo *et al.* (1971) proposed that the capsid of TRSV (and other Nepoviruses) is composed of 60 structural subunits each consisting of a single polypeptide with a molecular weight of 57,000. On the other hand, Chambers *et al.* (1965),

using the rotation technique on electron micrographs of negative stained TRSV particles and model building, proposed that the capsid has 5:3:2 symmetry and is an icosahedron with 42 capsomeres and hence should consist of 240 polypeptide subunits (Caspar and Klug, 1962). Similarly, a $T = 4$ surface lattice structure has also been proposed for ArMv (Agrawal, 1967).

Rezaian and Francki (1973) investigated the replication of TRSV *in-vivo* in infected cucumber cotyledons. They observed that the virus increased linearly between 2 and 5 days after inoculation. The relative proportion of M and B components did not vary significantly between 3 and 12 days after inoculation. A virus induced RNA-dependent-RNA polymerase was also detected one day after inoculation. At the same time a polydispersed population of low molecular weight ds-RNA containing nucleotide sequences complementary to TRSV RNA was also detected and its concentration increased in unison with the activity of the RNA-dependent-RNA polymerase (Rezaian and Francki, 1973). The function of this ds-RNA is unclear. Both the RNA-dependent-RNA polymerase activity and the virus specific ds-RNA are confined mainly to the cytoplasm of infected cucumber cells (Rezaian, 1974). Similarly, cytological studies of tissues developing TRSV induced lesions showed that structural changes indicating increased metabolic activity were observed in the cytoplasm but not in any of the organelles (Francki and Hatta, 1977). These included development of numerous Golgi bodies, extensive vesiculation of the cytoplasmic membranes and increase in the areas of the cytoplasm containing well developed rough endoplasmic reticulum. All these observations suggest that TRSV is synthesized in the cytoplasm.

Studies by Atchison and Francki (1972) indicated that the tubules in which TRSV particles are often located (Davison, 1969; Crowley *et al.*, 1969) are not likely to be involved in virus replication but may be involved in the translocation of virus particles from cell to cell.

F. Scope of This Thesis

The original aim of this thesis was to study the translation of TRSV RNAs *in-vitro*, as a contribution towards the understanding of the mechanism of TRSV multiplication. However, many physical, biological and biochemical properties of TRSV were still obscure and had to be studied before this aspect of the thesis could begin. Based on this consideration, the following studies encompass the scope of this thesis:

(1) Due to the controversy surrounding the structure and the subunit molecular weight of the TRSV capsid it was decided first to study the viral coat protein in detail. An understanding about the molecular weight and other properties of TRSV coat protein is a prerequisite for its identification among the translational products of TRSV RNA.

(2) Accurate measurements of the molecular weights of messenger RNAs (mRNAs) are required to estimate their coding capacities. To obtain accurate molecular weight data for TRSV RNAs they have now been measured under denaturing conditions.

(3) Although it was suggested that both RNA species of TRSV are required for infectivity, it was difficult to prepare RNA-L of TRSV free from infectivity (Harrison *et al.*, 1972a; Francki, unpublished data). Rezaian and Francki (1974) also detected a 13% sequence homology between RNA-S and RNA-L of TRSV. If the above observations are due to cross contamination of RNA-S in RNA-L preparations, then it would be difficult to identify the specific translational products of RNA-S and RNA-L. Thus experiments are done to isolate pure RNA-S and RNA-L preparations.

(4) The cellular site of synthesis of TRSV protein had not been fully established and was investigated.

(5) Translation of the relatively large RNAs of Nepoviruses *in-vitro* have not been attempted until recently. Fritsch *et al.* (1978) translated TBRV RNAs *in-vitro* but the translational products were not

fully characterised. Experiments were undertaken to translate the two RNA species of TRSV separately as well as before separation. The products were analysed by polyacrylamide gel electrophoresis and tryptic peptide mapping.

(6) A recent report indicated that a protein component, covalently linked to the TRSV RNA, is required for infectivity (Harrison and Barker, 1978). Experiments were done to determine whether this protein is involved in the translation of the viral RNAs.

CHAPTER II

GENERAL MATERIALS AND METHODS

I. MATERIALS

A. Virus Isolates, Viral Proteins and RNAs

TRSV used was originally isolated from *Gladiolus* in South Australia (Randles and Francki, 1965) and was maintained by propagation in cucumber seedlings.

Tobacco mosaic virus (TMV) used was the common (U-1) strain (Siegel and Wildman, 1954) and the bean strain (BTMV; Whitfeld and Higgins, 1976). Both were propagated in tobacco (*Nicotiana tobaccum*, cultivar (cv) White Burley).

Cucumber mosaic virus (CMV) protein and RNA, isolated from the Q-strain of this virus (QCMV; Francki *et al.*, 1966), tomato aspermy virus (TAV) (V strain; Habili and Francki, 1974) RNA and *Escherichia coli* ribosomal RNA (rRNA) were provided by Dr. D. Mossop; encaphalomyocarditis virus (EMC) RNA was provided by Prof. E.M. Martin of Flinders University.

The TomRSV studied in this thesis was a virus isolated from a plant of *Pentas* growing locally (unpublished data).

B. Chemicals and Biochemicals

Chemicals and biochemicals used and their sources are listed in Tables 1 and 2 respectively and in Chapter VI. All chemicals used were of analytical (AR) or laboratory reagent grades. Ribonucleases (RNases) were pre-heated at 90°C for 10 min prior to being used to render it free from proteolytic activities.

C. Instruments and Apparatus

All instruments used are either listed in Table 3 or described in the text.

II. METHODS

A. Plant and Virus Propagation

All plants used for virus propagation were raised in an insect-proof air-cooled glasshouse at 20 - 25°C.

Table 1Basic chemicals used in this thesis

<u>Chemicals</u>	<u>Sources</u>
Acrylamide	BDH Chemicals, England
Actinomycin D	Merck and Co., Inc., U.S.A.
Adjuvant, Freund's Complete	Calbiochem, U.S.A.
Agarose (Electrophoretic grade)	BDH Chemicals Ltd., England
Amberlite MB-1 (Regenerated Form)	Ajax Chemicals Ltd., Australia
Ammonium bicarbonate	May and Baker Ltd., England
Ammonium persulphate	Ajax Chemicals Ltd., Australia
1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP)	Ajax Chemicals Ltd., Australia
Chloramphenicol, crystalline	Sigma Chemical Co., U.S.A.
Cyanogen bromide	Ajax Chemicals Ltd., Australia
Cycloheximide	Upjohn Co.
Dimethylsulphoxide (DMSO)	Ajax Chemicals Ltd., Australia
2,5-diphenyloxazole (PPO)	Koch-Light Laboratories Ltd., England
Dithiothreitol (DTT)	Sigma Chemical Co., U.S.A.
Ethidium bromide	Sigma Chemical Co., U.S.A.
Ethylenediaminetetra-acetic acid (EDTA) disodium salt	Ajax Chemicals Ltd., Australia
Ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetraacetic acid (EGTA)	Sigma Chemical Co., U.S.A.
Formamide	BDH Chemicals, England
Guanidinium chloride	Merck and Co., Inc., U.S.A.
Iodoacetamide	Sigma Chemical Co., U.S.A.
2-mercaptoethanol	BDH Chemicals, England
N,N'-methylene bis-acrylamide	BDH Chemicals, England
NCS tissue solubilizer	Amersham/Searle Corp., U.S.A.
Ninhydrin	Merck and Co., Inc., U.S.A.
Phenanthrenequinone reagent	Provided by Dr. M.E. Tate, University of Adelaide.

Table 1 cont.

<u>Chemicals</u>	<u>Sources</u>
Phenol	BDH Chemicals, England
Phenylmethyl sulphonyl fluoride (PMSF)	Sigma Chemical Co., U.S.A.
Polyethylene glycol 4000 (PEG 4000)	Union Carbide, U.S.A.
Sodium dodecyl sulphate (SDS), electrophoretic grade	Sigma Chemical Co., U.S.A. Biorad Laboratories, 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	Sigma Chemical Co., U.S.A.
Urea	BDH Chemicals, England

Table 2

Basic biochemicals used in this thesis

<u>Biochemicals</u>	<u>Sources</u>
Bovine serum albumin (Fraction V)	Sigma Chemical Co., U.S.A.
Cytochrome c, horse heart, Type VI	Sigma Chemical Co., U.S.A.
Glutamate dehydrogenase	Sigma Chemical Co., U.S.A.
Insulin (40 units/ml)	Commonwealth Serum Lab., Australia
L-[U- ¹⁴ C] leucine, 250 μ Ci/4.5 ml; 324 mCi/ mmole	The Radiochemical Centre, Amersham, England
L-[4,5- ³ H] leucine, 5 mCi/5 ml; 54 mCi/ mmole	The Radiochemical Centre, Amersham, England
Lysozyme, egg white, grade III, 23000 units/mg solid	Sigma Chemical Co., U.S.A.
Myoglobin, equine skeletal muscle, Type I	Sigma Chemical Co., U.S.A.
Ovalbumin, grade V	Sigma Chemical Co., U.S.A.
Protease, Type VI, from <i>Streptomyces</i> <i>griseus</i> containing up to 30% calcium acetate, 4 units/mg solid	Sigma Chemical Co., U.S.A.
³ H-protein hydrolysate, 1 mCi/ml	The Radiochemical Centre, Amersham, England
Ribonuclease-A, bovine pancreatic, Type III A, 100 Kunitz units/mg solid	Sigma Chemical Co., U.S.A.
Ribonucleases T1 and T2	Dr. R.H. Symons, University of Adelaide
Trypsin, bovine, DCC treated, Type XI, 7300 BAEE units/mg protein	Sigma Chemical Co., U.S.A.
L-[U- ¹⁴ C] valine, 250 μ Ci/5 ml; 270 mCi/mmole	The Radiochemical Centre, Amersham, England

Table 3

Instruments and apparatus used in this thesis

<u>Functions</u>	<u>Instruments and Apparatus</u>
Ultracentrifugation	Beckman model L) Beckman model L2-65) Spinco rotors
Low speed centrifugation	Sorval RC-2 with HL-8 rotor Sorval RC-3 with SS34 and GSA rotors
Absorption spectrophotometry	Unicam SP1800 spectrophotometer Unicam AR25 linear recorder
Density gradient analysis	ISCO model 183 density gradient fractionator ISCO model UA-2 ultraviolet analyser ISCO model 230 fraction collector
Tube gel electrophoresis	Apparatus made in our laboratory from plastic containers inserted with 12 plexiglass tubes (15 cm x 0.5 cm) or 8 tubes (15 cm x 1 cm)
Slab gel electrophoresis	Biorad model 220 dual vertical slab gel electrophoresis cell
Power pack for gel electrophoresis	Vokam SAE 2761 D.C. power supply unit
Gel scanning	Joyce-Loebl chromoscan unit
Measurement of radioactivity	Packard Tri-Carb model 3320 liquid scintillation spectrometer
Electron microscopy	Joel JEM 1000CX electron microscope
Paper chromatography	Shandon paper chromatography tank
High voltage paper electrophoresis	Paton Industries Flat Bed water cooled unit
pH measurement	Radiometer Copenhagen pH titrator, Type TTT1
Thin layer chromatography	Shandon TLC Chromatank

TRSV was propagated in cucumber seedlings. Ten days old cucumber seedlings with well expanded cotyledons, were used. Purified TRSV (100 µg/ml in water) was mechanically inoculated with a thumb and forefinger onto both surfaces of the cotyledons which had been dusted with carborandum (500 mesh). Excess inoculum and carborandum were washed off with water. The inoculated plants were transferred to a constant temperature room maintained at 25°C and grown under continuous fluorescent light of approximately 12,000 lux.

TMV was propagated in young tobacco plants by mechanical inoculation on 3 - 4 expanded leaves. The inoculated plants were grown in the constant temperature room described above.

TomRSV was propagated in cucumber seedlings as described for TRSV except that the inoculum was prepared from well infected cucumber cotyledons or leaves showing systemic lesions. The tissue was ground with sand in 0.1M phosphate buffer, pH 7.0, at 1 ml per gram tissue in an ice cooled mortar before being used. The inoculated plants were either grown in the constant temperature room or in the glasshouse.

B. Purification and Analysis of Viruses

(i) Virus Purification

TRSV was purified from cucumber cotyledons and leaves showing ringspot symptoms. A method modified from that of Rezaian and Francki (1973) was used. All steps were done at 0 - 4°C. Tissues were harvested 5 days after inoculation, cooled to 4°C, and homogenised thoroughly in a blender with 1.5 volumes of 0.1M phosphate buffer, pH 7.0, (W/V) in a cold room. The homogenate was shaken with 1 volume of chloroform (V/V) and then clarified at 10,000g for 20 min. Virus was precipitated from the supernatant by adding polyethylene glycol (PEG) 4000 and sodium chloride (NaCl) to 6% and 0.3M respectively and stirring the mixture continuously for 30 min. The precipitate was centrifuged at 10,000g for 20 min and resuspended by stirring overnight in a cold room in 0.1M phosphate buffer, pH 7.0, containing 2% triton X-100. Next day, the virus suspension was

clarified at 10,000g for 10 min and ethylenediaminetetraacetate (sodium salt) (EDTA) dissolved in 0.1M phosphate buffer, pH 7.0, was added to a final concentration of 10mM. The mixture was left in the cold room for 6 hr before being centrifuged at 160,000g for 60 min using either the Spinco 65 or the 50 Ti rotor. The virus pellets were resuspended overnight by continuous stirring at 4°C. The above purification procedure yielded highly purified preparations of TRSV when viewed in the electron microscope even before sucrose density gradient centrifugation. Yield was increased from 20 mg to 30 - 35 mg/100 gm tissue by resuspending the PEG and high speed centrifugation pellets overnight. Aggregated virus particles, detected in sucrose gradients (Rezaian and Francki, 1973), were reduced when the virus was exposed to EDTA for extended periods prior to high speed centrifugation and by storing the virus in the presence of 1mM EDTA.

When required, TRSV was further purified in sucrose or CsCl density gradients. For sucrose density gradient centrifugation, the virus was loaded at 5 - 10 mg/tube onto a 5 - 25% sucrose gradient in 0.1M phosphate buffer, pH 7.0, and centrifuged at 27,000 rpm for 125 min in a Spinco SW27 rotor. After centrifugation, the virus was collected from the gradients with the aid of the ISCO gradient analyser and then concentrated by centrifugation at 160,000g for 100 min or 78,000g for 210 min in a Spinco 30 rotor. For CsCl gradients, 0.5 ml of a 4 mg/ml virus suspension was layered on 4.5 ml of a 40% CsCl solution and then centrifuged at 35,000 rpm for 60 hr in a Spinco SW50 rotor. After centrifugation, the gradients were collected into fractions of 8 drops each and virus was detected by measuring the absorbance at 260 nm. Fractions containing the virus were pooled and dialysed against 0.1M phosphate buffer, pH 7.0, and then concentrated by centrifugation at 160,000g for 60 min. The virus pellets from either gradients were resuspended overnight in 0.1M phosphate buffer, pH 7.0, containing 1mM EDTA, clarified, and stored at 4°C.

TMV was purified as described by Francki and McLean (1968) from tobacco leaves harvested 10 days after inoculation. Tissues were homogenised in 1.5 volumes of 0.2M Na_2HPO_4 (W/V) and strained through muslin cloth. Decolorising charcoal was added (0.01 gm/ml) and the mixture filtered through celite. The filtrate was treated with DEAE-cellulose (0.01 gm/ml) and the mixture again filtered through celite. The filtrate was centrifuged at 78,000g for 120 min at 4°C and the virus pellets were resuspended in distilled water. The virus suspension was clarified and subjected to a second cycle of high and low speed centrifugation as described above. The final virus suspension was stored at 4°C.

TomRSV was purified from infected cucumber cotyledons and leaves harvested 7 days after inoculation (10 days if grown in the glasshouse). The same method used for the purification of TRSV was used for TomRSV except for the following procedures:

- (1) The homogenised tissues were first filtered through two layers of cheese cloth and the residue was extracted again with half a volume of 0.1M phosphate buffer, pH 7.0, (W/V) by grinding thoroughly in a mortar. The extract was filtered again and the filtrates pooled. The filtrate was then clarified with 1 volume of chloroform (V/V).
 - (2) The virus was precipitated with 0.3M NaCl + 8% PEG 4000.
 - (3) The virus suspension was subjected to two cycles of high speed (160,000g/60 min) and low speed (10,000g/10 min) centrifugations before being further purified on sucrose density gradients as described above.
- (ii) Preparative Fractionation of TRSV Particle Components

The TRSV particle components, T, M and B were fractionated by sucrose density gradient centrifugation as described for TRSV purification in Section II B (i). Each tube was loaded with 4 - 5mg of purified virus preparation. After centrifugation, the virus components were fractionated using the ISCO ultra-violet (uv) gradient analyser unit. The same components from each tube was pooled, concentrated by centrifugation (160,000g/60 min) and subjected to a second cycle of sucrose gradient

centrifugation, except that 1 - 2mg of each component was loaded per tube. Care was taken throughout the fractionation procedure to ensure that the bottom component collected was free from aggregated particles. The fractionated TRSV particle components were stored in 0.1M phosphate buffer, pH 7.0, containing 1mM EDTA.

(iii) Analytical Centrifugation of TRSV Particle Components

TRSV particle components were analysed by centrifugation at 49,000 rpm for 25 min in 5 - 25% sucrose gradients using the Spinco SW50 rotor at 4°C. Approximately 50 - 100 µg of virus was loaded per tube. After centrifugation the gradients were analysed by measuring their absorbance at 254 nm using the ISCO uv gradient analyser.

C. In-vivo Labelling of TRSV Protein

Various radioactive amino acids were used to prepare TRSV containing radioactive protein components. They are specified in the individual experiments involved. As far as possible, all procedures were done under aseptic conditions. Cucumber cotyledons were excised 2 days after inoculation with 0.2mg/ml of TRSV. Ten gm samples of tissues were washed in sterile distilled water, blotted dry and sliced parallel to the veins without cutting the mid-rib. The tissues were placed in large petri dishes and each sample was then infiltrated with either 20 µCi of ¹⁴C-labelled amino acid or 200 µCi of ³H-labelled amino acid diluted to 1 ml in water. After allowing for the radioactive amino acids to be completely absorbed into the tissues, 10ml of sterile 0.01M sodium phosphate buffer, pH 7.0, was added to each sample and incubated under continuous fluorescent light ^{of about 12,000 Lux} at 25°C for 48 hr. Ten ml aliquots of buffer were added to each sample at intervals to prevent the tissues from drying out. After incubation, the tissues were rinsed thoroughly with sterile phosphate buffer. TRSV was isolated from each sample to which 5mg of purified TRSV has been added as carrier. Yields and radioactivity of virus preparations were monitored at various purification steps.

D. Preparation of Viral RNA

(i) Preparation of TRSV RNA

A modified phenol-sodium dodecyl sulphate (SDS) method was used to extract the RNA from TRSV. Freshly prepared virus was resuspended in sterile 0.1 M sodium acetate (NaAc) containing 1 mM EDTA at 2 mg/ml after high speed centrifugation (160,000g/60 min). It was mixed with an equal volume of sterile 0.1 M NaAc containing 2% SDS and 10 mM EDTA, pH 7.6 (adjusted with NaOH). The virus mixture was shaken with an equal volume of water-saturated-phenol (90% phenol in water, W/W), containing 0.1% 8-hydroxyquinoline at 25°C for 3 hr. After centrifugation at 5000g for 10 min, the aqueous phase was re-extracted twice with one-third volume of phenol at 25°C for 10 min and the pooled aqueous solutions were precipitated with 2.5 volumes of re-distilled ethanol in the presence of 0.2 M NaAc. The RNA precipitates were sedimented, washed 3 times with ethanol and freeze dried. No salt appears to be precipitated with the RNA when NaAc was used as the extraction medium in contrast to phosphate buffer which produced salt crystals. The RNA was washed by dissolving it in 20 mM Tris (hydroxymethyl) amino-methane-acetate (Tris-Ac) buffer, pH 7.6, containing 0.1 M NaAc and 0.2 mM EDTA and reprecipitating it with ethanol. The final dried RNA preparation was dissolved in 20 mM Tris-Ac buffer, pH 7.6, containing 0.2 mM EDTA (TE buffer) and stored at -70°C in small aliquots.

A pronase-SDS phenol/m-cresol method modified from the method of Harrison *et al.* (1974) was also used for the extraction of TRSV RNA. However, this method was not satisfactory since lower yields and less infectious RNAs were obtained and it was not used for routine RNA preparation.

(ii) Preparation of TMV RNA

TMV RNA was extracted from the virus by the modified phenol-SDS method described above except for the following changes. Virus prepared as previously described was further clarified with 0.5 volume of chloroform and then centrifuged at 78,000g for 120 min. The virus pellets were

resuspended in the NaAc-EDTA buffer at 10 mg/ml and was extracted with phenol and SDS at 25°C for 2 hr. The RNA was resuspended in TE buffer and stored at -70°C.

E. Analysis of TRSV RNA Components

(i) Analytical Centrifugation of TRSV RNA Components

TRSV RNA components were analysed by centrifugation in sucrose density gradients containing 5 - 25% sucrose in 0.01M phosphate buffer, pH 7.0. Each tube was loaded with 15 - 20 µg of RNA and centrifuged at 49,000 rpm for 3 hr at 4°C using a Spinco SW50 rotor. After centrifugation, the gradients were analysed by measuring the absorbance at 254 nm using the ISCO uv gradient analyser.

(ii) Analytical Gel Electrophoresis of RNAs

TRSV and other RNAs were routinely analysed by electrophoresis in 2.0% agarose gels or 2.6% polyacrylamide gels using the Tris-borate-EDTA, pH 8.3, buffer system (TBE, containing 10.8 gm Tris, 5.5 gm boric acid and 0.98 gm EDTA in 1 litre of water) (Peacock and Dingman, 1968). To prepare the agarose gels, agarose was added to TBE buffer and autoclaved for 10 min to dissolve and sterilise the agarose. The hot gel solution was shaken vigorously to ensure a homogenous gel prior to dispensing into sterile 15 cm x 0.5 cmplexiglass tubes to a depth of approximately 13 cm. After the gels had set, 2 - 3 mm of agarose were trimmed off the top of each gel with a sharp scalpel to produce a flat loading surface and to maintain a constant gel length. Gels were used immediately after being made. RNA samples (25 µg TRSV RNA and 10 µg/RNA for others in 50 µl TBE buffer containing 10% sucrose and 0.0005% bromophenol blue (BPB)) were heated at 70°C for 3 min, cooled rapidly in an ice-water bath and then loaded. They were packed into the gels by electrophoresis at 1 mA/gel for 30 min and then electrophoresed at 2.5 mA/gel for 4 hr at 25°C. With such short electrophoresis times, circulation of the electrophoresis buffer was found unnecessary. After electrophoresis, the gels were stained for 15 min with toluidine blue (0.05% in 50mM NaAc, 5mM acetic acid and 0.1M EDTA, pH 8.5; Peden and

Symons, 1973) and destained in several changes of 5% acetic acid. RNAs in the gels were detected by scanning them at 620 nm using the Joyce-Loebl Chromoscan unit. During electrophoresis, the agarose gels may shrink unevenly causing variations in migration of RNAs from gel to gel. However, this was found to be minimised by using gels of equal lengths in each experiment.

For more critical analysis, the RNAs were electrophoresed in 13 cm long 2.6% aqueous polyacrylamide gels prepared from stock acrylamide solution (19% acrylamide plus 1% N,N'-methylene bis-acrylamide (bisacrylamide) in water) which had been pre-treated with 1% activated charcoal. A flat loading surface was formed on the gels by overlaying the polymerising gel solutions with water. After the gels had polymerised, the gel surfaces were washed with TBE buffer. The gels were left at 4°C for at least 2 hr and then pre-electrophoresed at 3 mA/gel for 30 min prior to loading the RNA samples. RNA samples were prepared, dissociated and electrophoresed as described above for agarose gels. After electrophoresis, the gels were stained and scanned as described above.

F. Preparation of Viral Coat Proteins

(i) Preparation of TMV Coat Protein

TMV coat protein was prepared by the cold acetic acid method (Fraenkel-Conrat, 1966).

(ii) Preparation of TRSV Coat Protein

The methods used for isolation of TRSV coat protein are described in Chapters III and IV.

G. Analysis of Proteins and Peptides by SDS-Polyacrylamide Gel Electrophoresis

The following polyacrylamide gel electrophoresis (PAGE) systems were used in various experiments to analyse proteins and peptides:

- (a) SDS-continuous system of Weber and Osborn (1969).
- (b) SDS-discontinuous system (Laemmli, 1970).
- (c) Urea-SDS-continuous system of Swank and Munkre (1971).

(i) Preparation of Protein Samples and Other Materials for Electrophoresis

The same method was used to dissociate and reduce TRSV (100 µg/gel) and other protein (10 - 15 µg per protein) samples to be electrophoresed (including molecular weight marker proteins) in all the PAGE systems. However, different sample buffers were used. Protein samples (1 - 2 mg/ml), dissolved in either 0.125 M Tris-HCl buffer, pH 6.8, (Laemmli system) or 0.1 M phosphate buffer, pH 7.6, (Weber and Osborn system) or 0.1 M orthophosphoric acid - Tris buffer, pH 6.8, (Swank and Munkre system) were dissociated by heating in 6 M urea, 1% SDS, 2% 2-mercaptoethanol (MCE) (urea-SDS-MCE) and 0.0005% BPB for 2 min in boiling water baths prior to loading.

All cylindrical gels were cast in plexiglass tubes 5 mm internal diameter and 15 cm long. A 30% acrylamide stock solution containing acrylamide : bisacrylamide ratio of 30 : 1 and pre-treated with 1% activated charcoal was used. (Too low a bisacrylamide content tends to cause the gels to shrink and swell excessively during staining and destaining.) When slab gels (Chapters VI and VII) were used, they were prepared from 30% acrylamide stock containing acrylamide : bisacrylamide ratio of 150 : 1 which had also been pretreated with activated charcoal. The gels and electrophoresis buffers used in the various systems were prepared as described by the various authors unless otherwise stated.

(ii) Electrophoresis Procedures

(a) Weber and Osborn PAGE system: Cylindrical gels, 12 cm long and containing up to 10% acrylamide, were used. Volumes up to 50 µl were loaded onto each gel. All other electrophoresis procedures were as described by Weber and Osborn (1969) and electrophoresis was done at 25°C.

(b) Swank and Munkre PAGE system: This method was used for the analysis of cyanogen bromide (CNBr) peptide fragments (see Chapter IV) and the TRSV RNA-linked protein (see Chapter VII), using cylindrical and slab gels respectively.

(c) Laemmli PAGE System: Cylindrical and slab gels were used routinely with this system to analyse TRSV coat protein (Chapter IV), translational products (Chapter VI) and the TRSV RNA-linked protein (Chapter VII). Sample volumes of up to 150 μ l were loaded onto cylindrical gels each consisting of a 10 - 12 cm long 13% separating gel and a 1 cm long 2.5% stacking gel. Electrophoresis was done at 25°C. The gels were initially electrophoresed at 1.5 mA/gel for 1 hr or until the whole sample had formed a sharp band in the stacking gels and then at 3 mA/gel for 5 hr. Slab gel electrophoresis is described in Chapter VI.

(iii) Detection of Protein and Peptide Bands

After electrophoresis, gels were routinely stained in a solution containing 0.2% coomassie brilliant blue (CBB), 50% methanol and 10% acetic acid in water at 37°C for 90 - 120 min depending on gel concentration. They were then destained for 48 hr at 37°C in several changes of 25% methanol and 7% acetic acid in water. If required, the destained gels were scanned at 575 nm.

(iv) Estimation of Molecular Weights

The migration of the protein and peptide bands, including marker proteins, and the BPB marker in the gels were measured. The molecular weights (Mol. Wts.) of unknown proteins were estimated from their relative mobilities with respect to the BPB using bovine serum albumin (BSA), glutamate dehydrogenase (GDH), ovalbumin (Oval), QCMV coat protein (CMV), trypsin (Try), myoglobin (Myo), ribonuclease-A (RNase-A), lysozyme (Lys), cytochrome-c (Cyt-c) and insulin as marker proteins. The Mol. Wts. of these proteins are listed in Table 4.

H. Serological Techniques

(i) Preparation of Antiserum to TRSV

Antiserum to TRSV was prepared in rabbits. One mg of purified virus resuspended in 1 ml 0.01 M phosphate buffer, pH 7.0, and emulsified with 1 ml of Freund's complete adjuvant, was injected subcutaneously.

Table 4

Molecular weights and extinction coefficients of
biological materials used as physical constants

A. Molecular Weights

<u>Biological Materials</u>	<u>Molecular Weights</u>	<u>Reference</u>
Bovine serum albumin	68,000	Weber and Osborn, 1969
Glutamate dehydrogenase	53,000	"
Ovalbumin	43,000	"
CMV coat protein	24,600	Habili and Francki, 1974
Trypsin	23,000	Weber and Osborn, 1969
TMV coat protein	17,500	Zaitlin and Israel, 1975
Myoglobin	17,200	Weber and Osborn, 1969
Ribonuclease-A	13,700	"
Lysozyme	13,900	Lehninger, 1975
Cytochrome c	12,400	Sigma Chemical Co., U.S.A.
Insulin	5,700	Swank and Munkre, 1971
EMC RNA	2.6×10^6	E.M. Martin, personal communication
TRSV RNA-L	2.26×10^6	This work
TRSV RNA-S	1.44×10^6	This work
TMV RNA	2.05×10^6	Boedtke, 1968, 1971
<i>E. coli</i> 23S rRNA	1.07×10^6	Stanley and Bock, 1965
<i>E. coli</i> 16S rRNA	0.55×10^6	"
QCMV RNA 1	1.27×10^6)	Mossop, 1978 Peden and Symons, 1973 Habili, 1974
QCMV RNA 2	1.13×10^6)	
QCMV RNA 3	0.82×10^6)	
QCMV RNA 4	0.35×10^6)	

B. Extinction Coefficients

<u>Biological Materials</u>	<u>E 0.1% (260) 1 cm</u>	<u>Reference</u>
TMV	2.7	Knight, 1962
TRSV	7	Rezaian, 1974
TRSV M component	7	"
TRSV B component	10	"
TomRSV	7	
RNA	25	

This was repeated 1 week later, and then intravenous booster injections of 1.5 mg of virus was given 2 weeks after the 2nd injection. The rabbits were bled at weekly intervals starting 1 week after the 2nd injection. The blood was allowed to clot at 25°C for 2 hr and then at 4°C overnight before being centrifuged at 750g for 5 min. The antisera were stored in 50% glycerol at -15°C in 5ml aliquots.

(ii) Immuno-diffusion Assays

Immuno-diffusion assays were done by the double-gel diffusion technique (Francki and Habili, 1972) in 1.5 mm thick sterile 0.75% agar gel containing 0.01M phosphate buffer, pH 7.6, 0.14M NaCl and 0.02% sodium azide. Antisera were diluted when required with 0.9% NaCl immediately before use. Virus antigens to be tested were dissolved in and diluted with 0.1M phosphate, pH 7.0. Ten µl samples were loaded into the wells and the serological plates were normally left at 25°C and observed at intervals.

I. Spectrophotometric Measurements

(i) Measurement of Protein Concentration

Concentrations of protein preparations (including TRSV protein) were measured spectrophotometrically by the use of the following formula: Protein concentration (mg/ml) = 1.55 OD₂₈₀ - 0.76 OD₂₆₀ (Layne, 1957).

(ii) Measurement of Virus and RNA Concentrations

Concentrations of purified virus or RNA were measured spectrophotometrically using the formula: Concentration (mg/ml) = $\frac{OD_{260}}{E_{1\text{ cm}}^{0.1\%}(260)}$

Where $E_{1\text{ cm}}^{0.1\%}(260)$ is the absorbance at 260nm of a 0.1% solution of the virus or RNA through a light path of 1 cm. The $E_{1\text{ cm}}^{0.1\%}(260)$ values for RNA and the viruses used in this thesis are tabulated in Table 4.

J. Measurement and Detection of Radioactivity

(i) Measurement of Radioactivity

All radioactive measurements were done using a liquid scintillation spectrometer. The specific radioactivity values of various radioactive

virus or protein preparations (expressed as counts per min (cpm)/ml or cpm/mg) were determined by adsorbing 5 - 10 μ l aliquots from each preparation onto G/FA glass fibre discs and the radioactivity was measured after addition of 5 ml of scintillation fluid containing 3.5 gm 2,5-diphenyloxazole (PPO) and 0.35 gm 1,4-bis 2-(5-phenyloxazolyl) benzene (POPOP) per litre of toluene (Atchinson and Francki, 1972). These values were used to calculate the volumes of radioactive samples required to give the amounts of radioactivity which will enable the radioactive materials to be detected in gels and tryptic peptide chromatograms. Discriminator setting of 20 - 1000 and gain settings of 50% and 15% for ^3H , and ^{14}C respectively were used. Each sample was counted for at least 10 min.

(ii) Detection of Radioactivity in Gels, Thin Layer and Paper Chromatograms

Distributions of radioactivity in cylindrical gels were measured by cutting the gels into slices 1.45 mm thick and measuring the radioactivity of each gel slice. Each gel slice was incubated in 5 ml of PPO-POPOP-NCS scintillation fluid (Schwinghamer and Symons, 1975; prepared by mixing 200 ml PPO-POPOP scintillation fluid with 30 ml NCS tissue solubilizer) at 40°C until the gels had completely dissolved and then counted. Spectrometer settings were set as above for gels containing one radioisotope. Gels containing two radioisotopes were counted by double labelling technique using discriminator settings of 20 - 200 and 200 - 1000, and gain settings of 50% and 15% for ^3H and ^{14}C respectively. For plotting radioactivity distribution, counts in the ^{14}C channel were corrected for 54% recovery and counts in the ^3H channel were corrected for a 10% ^{14}C spill-over (of total corrected ^{14}C counts) and then corrected for 82% recovery.

Distributions of radioactivity in slab gels were detected by fluorography described in Chapter VI while those in tryptic peptide chromatograms were analysed by autoradiography (Chapter IV) or fluorography (Chapter VI).

K. Precautions Against Ribonucleases and Bacterial Contamination

All materials in contact with RNA were rendered as free from RNase contamination as possible. Buffers and solutions of inorganic salts including SDS, dialysis membrane (in water), sucrose (dry), BPB, rubber tubing and other heat resistant materials were autoclaved at 120°C at 75 psi for 10 - 15 min. Glassware were kept in a 130°C oven overnight. Heat-labile chemicals (including acrylamide, dithiothreitol (DTT), amino acids etc) and enzymes were made up in sterile buffers or water. Centrifuge tubes, plexiglass tubes and electrophoresis apparatus were sterilised by rinsing in alcoholic-KOH (containing 10% KOH in 90% ethanol) and then washed several times with sterile distilled water immediately before use. Pronase was pre-incubated at 37°C for 2 hr. Sterilised materials were maintained RNase-free by keeping them sealed at room temperature, storing at -15°C or 4°C as necessary. RNA preparations were stored at -70°C in small aliquots. All inoculation of RNAs were done using new disposable plastic gloves or rubber fingerstalls.

Precautions were also taken to prevent bacterial contamination during *in-vivo* synthesis of radioactive viral components. All glassware and buffers used were sterilised by heating or autoclaving. All tissues were rinsed thoroughly with sterile distilled water and sliced with sterile scalpel blades.

L. Infectivity Assay of TRSV and TRSV RNA

All infectivity assays were done by local lesion assays using cowpeas arranged in latin squares (Clark, 1969). Ten day old cowpeas grown in an insect-proof, air-cooled glasshouse, showing only the first leaves, were kept in darkness for 24 - 36 hr to increase their susceptibility to infection. Plants were inoculated immediately after removal from darkness. For TRSV RNA, the leaves were sprayed lightly with Carborundum (500 mesh) and 10 µl samples of RNA inocula, 25 µg/ml in 0.1M phosphate buffer, pH 7.2, were applied on each half-leaf. Each half-leaf was inoculated by rubbing the inoculum thoroughly over the

whole leaf surface as soon as the inoculum was applied. No washing of excess inoculum from the leaves was necessary and the inoculated plants were kept in the constant temperature room under continuous light as described previously for propagating TRSV. Lesions were counted 3 days after inoculation. The procedures for infectivity assays of intact TRSV were essentially the same as for RNA, except no precautions against RNase were necessary and lesions were counted 4 days after inoculation.

M. Measurement of pH

In most cases, pH was measured with a pH meter. When the volumes were too small to permit the use of a pH meter, pH paper was used e.g. adenosine 5' triphosphate (ATP) and guanosine 5' triphosphate (GTP) solutions were tested by applying 10- μ l aliquots onto pH papers.

N. Physical Constants

Table 4 lists the molecular weight and extinction coefficient ($E_{1\text{cm}}^{0.1\%}$ (260)) data used in the thesis.

CHAPTER IIISTABILITY OF TRSV AND ISOLATION OF TRSV COAT PROTEINI. INTRODUCTION

In order to obtain meaningful data on the composition of viral proteins from amino acid analyses and tryptic peptide maps, the protein preparations used must contain intact polypeptides and be free from SDS and RNA which are known to interfere with these analytical methods (Viswanatha *et al.*, 1955; Fraenkel-Conrat and Ramanchandran, 1959; Smith and Markham, 1950). However, difficulties in isolating intact TRSV coat protein were anticipated since Francki (private communications) failed to obtain satisfactory preparations by the acetic acid (Fraenkel-Conrat, 1966) or the phenol (Rappaport *et al.*, 1965) methods. Thus in this chapter, experiments were done to isolate TRSV protein by other methods and to devise a new method for isolating the viral protein.

II. MATERIALS AND METHODSA. Isolation of TRSV Protein by Established Methods(i) Formic Acid Method

The formic acid method of Miki and Knight (1965) was used. TRSV (10 mg/ml) was mixed with 98% formic acid to make 67% formic acid and incubated at 37°C for various times indicated. The protein was precipitated when dialysed exhaustively at 4°C against distilled water, and was centrifuged down and freeze dried.

(ii) Phenol Method

Purified virus in 0.1M phosphate buffer, pH 7.0, (2 mg/ml) was mixed with 2 volumes of 90% phenol containing 0.1% 8-hydroxyquinoline and 1 volume of water (Rappaport *et al.*, 1965). The mixture was shaken at 25°C for 30 min and then separated into the phenol phase, the inter-phase and the aqueous phase by centrifugation at 5000g for 20 min. The phenol phase was washed with an equal volume of water and the protein was precipitated out with 5 volumes of acetone. The inter-phase and aqueous phase were each re-extracted with phenol and after removal of the aqueous phase, the

second phenol + inter-phase was mixed with 5 volumes of acetone. The two acetone mixtures were left overnight at room temperature and the protein precipitated was centrifuged, washed twice with acetone and freeze dried.

(iii) Hydrochloric Acid Method

Protein was isolated from TRSV using 1 M HCl as described by Stace-Smith *et al.* (1965).

(iv) Trichloroacetic Acid Method

TRSV was disrupted by treatment in 10% trichloroacetic acid (TCA) at 90°C for 10 min and the protein isolated by repeated washing with distilled water (Ralph and Bergquist, 1967).

B. Stability Studies on TRSV Particles

Samples of TRSV (0.2 mg/ml) were incubated at various temperatures in the presence of different combinations of SDS concentrations and pH conditions in 0.1 M phosphate buffer, pH 7.0, for various time periods as denoted in the text. After incubation, the virus preparations were analysed for the presence of intact virus particles and RNA components by centrifugation in 5 - 25% sucrose density gradients as described in Chapter II. In some experiments, the virus was also tested for infectivity after the treatments. Untreated TRSV stored at 4°C in 0.1 M phosphate buffer, pH 7.0, was used as control.

III. RESULTS

A. Isolation of TRSV Coat Protein by Established Methods

Several established methods were found to be unsuccessful for isolating TRSV coat protein free from viral RNA and SDS for the following reasons:

(a) When TRSV protein was isolated by disrupting the virus in 6 M urea and/or 2% MCE at 37°C for various time periods and the protein isolated by ammonium sulphate precipitation, the protein obtained was invariably contaminated with RNA, even after removal of intact virus particles by high speed centrifugation.

(b) When the phenol method (Rappaport *et al.*, 1965) was used, no protein was recovered.

(c) When methods involving acids were used, which include (a) the 67% formic acid method (Miki and Knight, 1965); (b) the 1 M HCl method (Stace-Smith *et al.*, 1965) and (c) the TCA method (Ralph and Bergquist, 1967), numerous extra peptide bands were observed in the protein preparations compared to the protein profile of urea-SDS-MCE disrupted virus when they were analysed in SDS-polyacrylamide gels (Fig. 1). These results indicate that the TRSV proteins contain many peptide bonds susceptible to hydrolysis by acids. Baltz and Van Regenmortel (1974) have shown that the Asp-Pro peptide bonds in the proteins of tomato bushy stunt virus (TBSV), TMV and turnip yellow mosaic virus (TYMV) are readily hydrolysed by formic acid during isolation of the proteins. Similarly, Anderer (1963) has reported that TCA hydrolysed the Asp-Pro peptide bonds of TMV protein to produce low molecular weight degradation products. Thus a new method was required to isolate the TRSV protein.

B. Stability Studies on TRSV Particles

In order to devise a new method to isolate intact TRSV coat protein from purified virus it is necessary to identify and then dissociate the bonds holding the viral protein and nucleic acids together. TRSV is very stable *in-vitro* and can remain infectious for more than two years when stored at 4°C in 0.1 M phosphate buffer, pH 7.0. In his studies, Kaper (1972) suggested that TRSV is stabilised mainly by protein-protein interactions. However, it was also suggested that some Nepoviruses are also stabilised by RNA-protein interactions (Harrison and Murant, 1977).

(i) Effects of SDS

The particle components of TRSV were found to be differentially stable in SDS at pH 7.0, under low temperature conditions. None of the viral components were affected by 1% SDS at 4°C (Table 1). The M and B components of TRSV treated with 1% SDS for up to 3 hr at 37°C in 0.1 M

Table 1

Effect of SDS on TRSV particle components under mild temperatures

Treatments ^a	Recovery of particle components(%) ^b			Total recovery of particles(%)
	T	M	B	
0% SDS, 4°C (Control)	1.5	25	73.5	100
1% SDS, 4°C	1.5	25	73.5	100
0% SDS, 37°C/3 hr	1.5	25	67.2	94
0.1% SDS, 37°C/1½ hr	0.5	24.5	69.8	95
0.1% SDS, 37°C/3 hr	0.42	24.6	67.5	92
1% SDS, 37°C/½ hr	0.20	24.8	67.9	93
1% SDS, 37°C/1½ hr	0.15	23.3	67.8	91
1% SDS, 37°C/3 hr	0.12	22.4	61.7	84

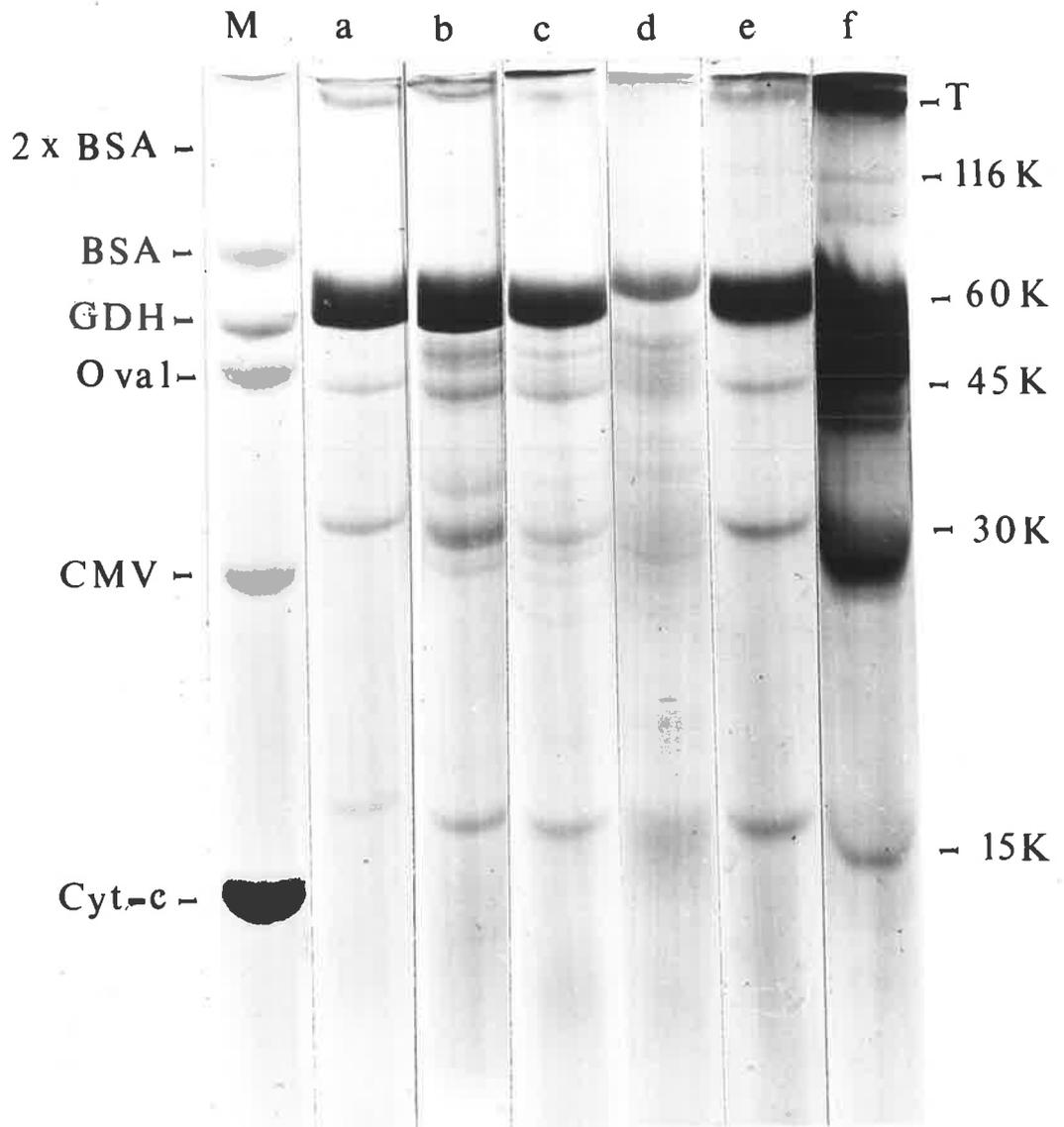
^a Samples of TRSV (1 ml, 0.2 mg/ml) were incubated in 0.1 M phosphate buffer, pH 7.0, at the temperatures and in the presence of the concentrations of SDS indicated.

^b After incubation, each virus preparation (50µg) was analysed by sucrose density gradient centrifugation as described in Chapter II. The recovery of the various particle components were estimated by integrating the areas under the peaks in the gradient absorbance profiles obtained by measuring the absorbance of the gradients at 254 nm using the ISCO uv gradient analyser. Recoveries are expressed as a percentage of the total recovery of all the components in the control virus preparation.

Figure 1. Polypeptide profiles of TRSV coat protein preparations isolated by various methods (see Materials and Methods)
Approximately 100 μ g of TRSV protein from each preparation dissociated in 6 M urea, 1% SDS and 2% MCE were loaded and electrophoresed on 13% discontinuous SDS-polyacrylamide gels as described in Chapter II.

- Gel M, marker proteins (see Chapter II);
- gel a, protein from intact TRSV (see Chapter IV);
- gel b, protein prepared by TCA method;
- gel c, protein prepared by formic acid method;
- gel d, protein prepared by HCl method;
- gel e, protein isolated by temperature-pH method,
pH 8.5;
- gel f, protein isolated by temperature-pH method,
pH 4.5.

In gel f, the protein was less soluble and difficulties in estimating the amounts of protein loaded was experienced.



phosphate buffer, pH 7.0, were still unaffected while the T component was dissociated almost completely by SDS under these conditions (Table 1). Results of the infectivity assays on the treated TRSV preparations indicate that the viral RNAs were still infectious after the SDS treatment and confirmed the conclusions that the M and B components, containing the viral RNAs, were intact (Table 2).

Although TRSV was completely disrupted in only 0.1% SDS when incubated at 60°C for 30 min at pH 7.0, the virus was also completely disrupted when incubated in the absence of SDS under the same temperature and pH conditions but the protein precipitated (Table 3).

(ii) Effects of Temperature

Fig. 2 summarises the effect of temperature on the dissociation of TRSV particle components in the presence of 0.2% SDS at pH 7.0. The results show that the rate of disruption of the T component is more rapid than the B or M components while the B component is more susceptible to temperature treatment than M. The results again show that heating at 60°C dissociates all particles (Fig. 2).

(iii) Effects of pH

When TRSV was incubated at 25°C for 30 min in the presence of 0.2% SDS in 0.1 M phosphate buffers of between pHs 4.5 and 10.0, the different types of particles were found to dissociate differentially under different pH conditions (Fig. 3). All the particles were most stable at pH 7.0. At pHs above 7.0, only the B component appears to be selectively dissociated with increasing pH while at pHs below 7.0, T, M and B components were all susceptible to dissociation. When the virus was incubated at 37°C for 30 min in the presence of 0.2% SDS at various pHs, similar effects of pH on the different particle components were also observed but increasing the temperature increased overall dissociation of all the components (Fig. 3). The results suggest that the degradation of T observed at acidic conditions was due to pH effects as well as SDS-temperature effects while its degradation observed under alkaline

Table 2

Infectivity of TRSV treated with SDS at mild temperature

TRSV concentration	Number of local lesions/half cowpea leaf ^a		
	Untreated ^b	TRSV control ^c	Incubated TRSV ^d
0.1 mg/ml	326	346	339
0.05 "	384	365	340
0.025 "	248	289	190
0.0125 "	151	193	134

^a Each value is the average of 12 replicates arranged in a Latin Square design.

^b TRSV stored at 4°C in 0.1 M phosphate buffer, pH 7.0.

^c TRSV stored at 4°C in 0.1 M phosphate buffer, pH 7.0, containing 1% SDS.

^d TRSV incubated at 37°C for 3 hr in 0.1 M phosphate buffer, pH 7.0, containing 1% SDS.

Table 3
Effect of SDS at 60°C on TRSV particles

Treatments ^a	Recovery of particle components (%) ^b			Total recovery of particles (%)
	T	M	B	
Control	2.18	16.0	81.82	100
0.1% SDS	0	0	0	0
0.25% SDS	0	0	0	0
0.5% SDS	0	0	0	0
1% SDS	0	0	0	0
0% SDS	completely disrupted and precipitated			0

^a All samples were incubated at 60°C for 30 min in 0.1 M phosphate buffer, pH 7.0, except for the control which was stored at 4°C in 0.1 M phosphate buffer, pH 7.0.

^b Recovery was estimated as described in Table 1.

Figure 2. Effects of mild heat treatment on the dissociation of TRSV particle components in the presence of SDS

Samples of TRSV (1.0 ml, 0.2 mg/ml) were incubated at the various temperatures in the presence of 0.2% SDS in 0.1 M phosphate buffer, pH 7.0, for 30 min. After incubation, the virus (50 µg) was analysed by sucrose density gradient centrifugation as described in Chapter II. The relative recoveries of the various particle components were estimated by intergrating the areas under the peaks in the gradient absorbance profiles obtained by measuring the absorbance of the gradients at 254 nm using the ISCO uv gradient analyser. Recoveries are expressed as a percentage of those recovered in the control TRSV preparation which was kept at 4°C.

● — ● — ● T

▲ — ▲ — ▲ M

○ — ○ — ○ B

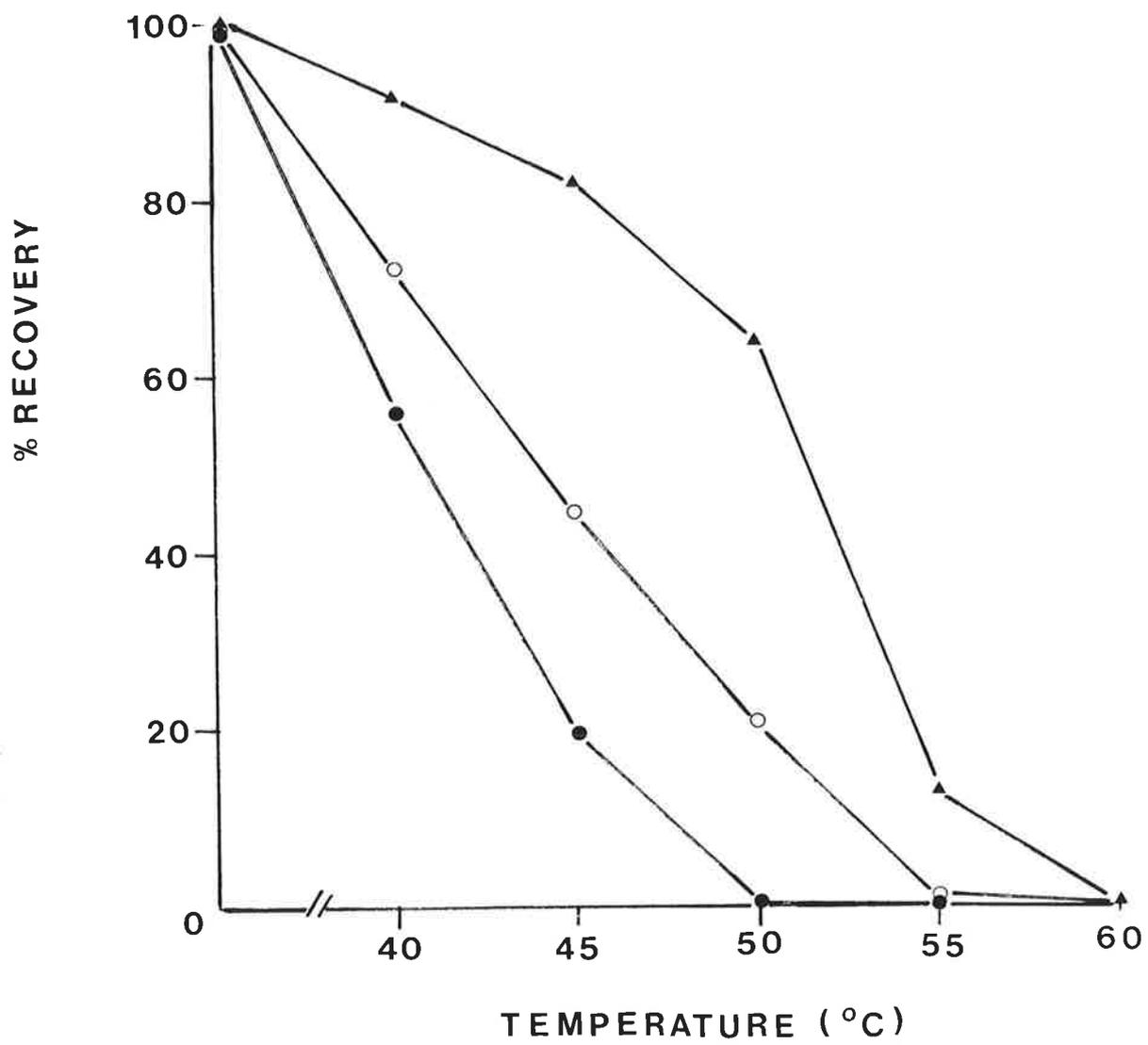


Figure 3. Effects of pH on the dissociation of TRSV particle components at 25°C and 37°C in the presence of SDS
Samples of TRSV (1 ml, 0.2 mg/ml) were incubated in 0.1 M phosphate buffer of various pHs indicated in the presence of 0.2% SDS at 25°C or 37°C for 30 min. After incubation, the virus preparations were analysed in sucrose gradients and the relative recoveries of the various particle components were estimated as described in Figure 2.

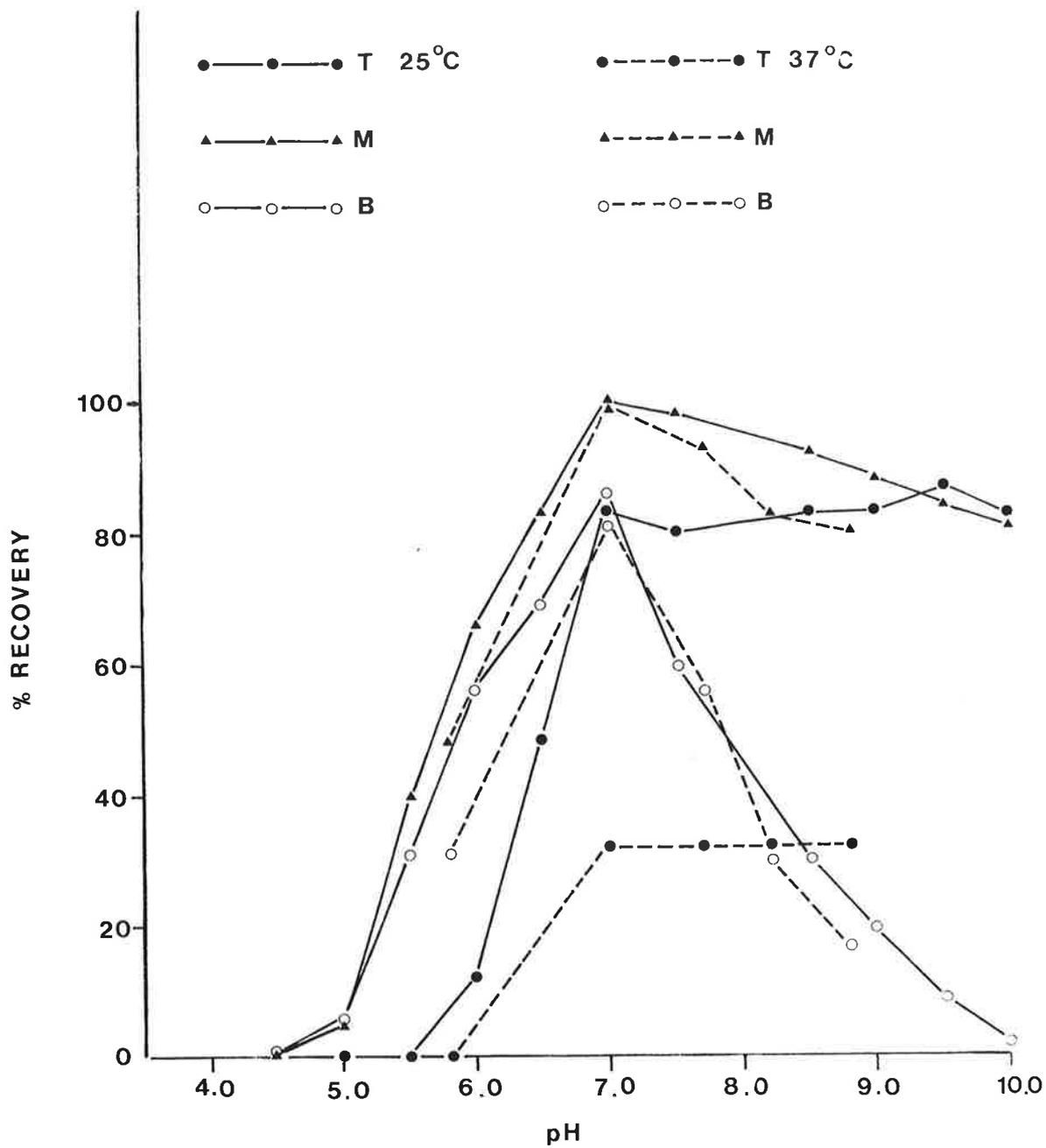
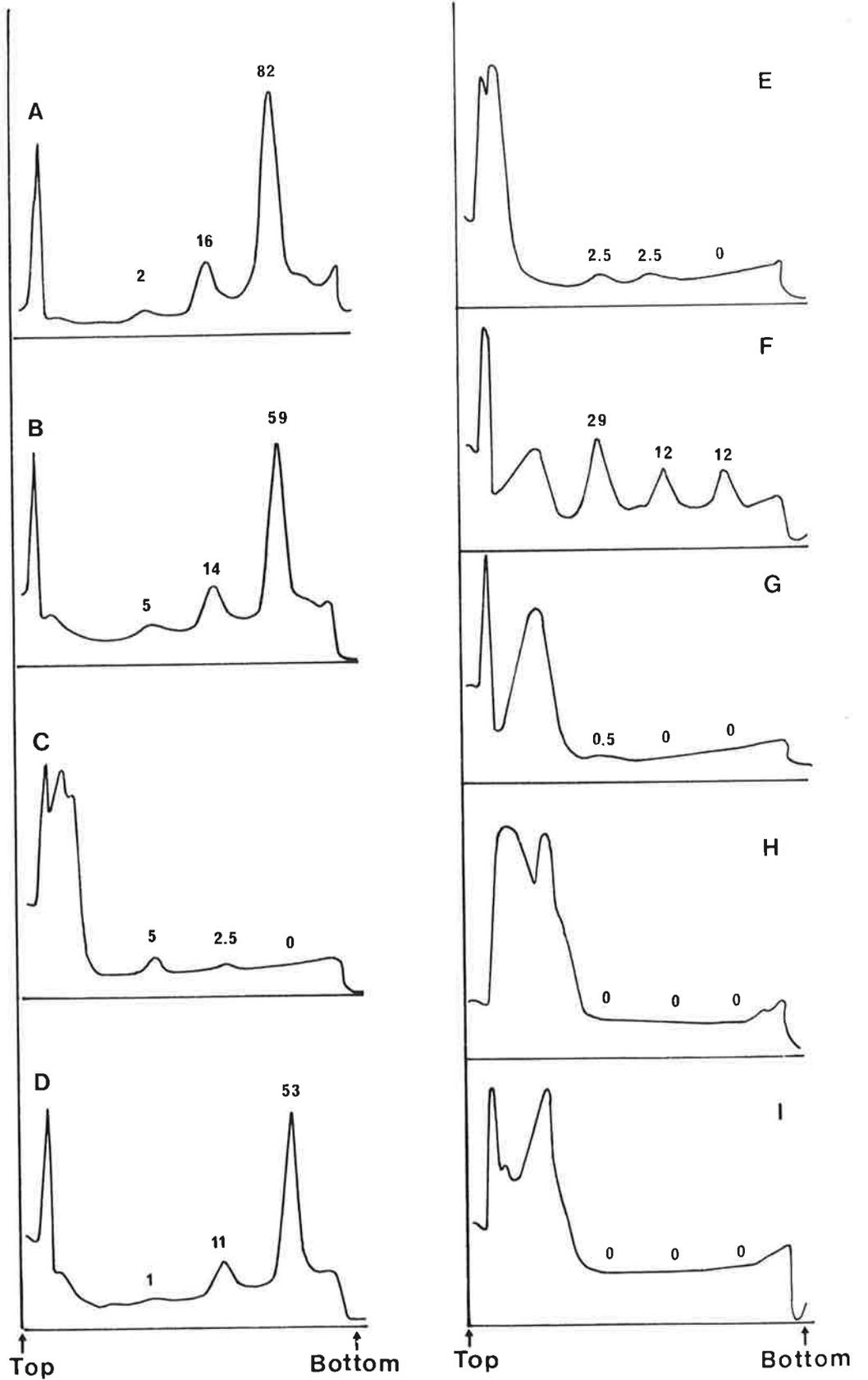


Figure 4. Effects of temperature and pH and SDS on the dissociation of TRSV particle components

Samples of TRSV (1 ml, 0.2 mg/ml) were incubated in 0.1 M phosphate buffer of various pHs and at the various temperatures indicated below. After incubation, the virus preparations were analysed in sucrose gradients and the relative recoveries of the various particle components were estimated as described in Figure 2. However, the recoveries are expressed as a percentage of the total recovery of all the components in the control virus preparation and are the values indicated on the respective peaks.

- Profiles A, control TRSV (-SDS, pH 7.0, 4°C);
B, 37°C for 30 min, pH 7.0, -SDS;
C, 60°C for 30 min, pH 7.0, -SDS;
D, 37°C for 30 min, pH 4.5, - SDS;
E, 60°C for 30 min, pH 4.5, - SDS;
F, 37°C for 30 min, pH 9.5, - SDS;
G, 60°C for 30 min, pH 9.5, - SDS;
H, 60°C for 30 min, pH 4.5, 0.1% SDS;
I, 60°C for 30 min, pH 9.5, 0.1% SDS.

RELATIVE ABSORBANCE AT 254 nm



DEPTH OF TUBES

conditions was mostly due to SDS-temperature effects, since increasing the pH above 7.0 did not increase disruption of the T component at either 25°C or 37°C (Fig. 3). Further experiments were done to investigate the effects of pH in the absence of SDS and the results (Fig. 4) suggest that complete dissociation of TRSV particles at low temperature (37°C) requires the presence of SDS (Fig. 4, D and F). However, at high temperature (60°C), SDS is not required for complete dissociation of the particles (Fig. 4, E and G).

C. Isolation of TRSV Coat Protein by Temperature-pH Method

The results presented in section B and summarised in Table 4 show that all the TRSV particle components were dissociated when the virus was heated at 60°C and that this was achieved in the absence of SDS (Fig. 4). Thus experiments were done to isolate TRSV coat protein in the absence of SDS by disrupting the virus under various combinations of temperature and pH conditions. The results showed that protein prepared at pH 4.5 contained some degradation products (Fig. 1, f). On the other hand, alkaline conditions complemented the effects of temperature by (a) reducing the temperature and time required for complete disruption of the virus and (b) enhance dissociation of TRSV at high concentration of the virus while retaining polypeptide integrity (Fig. 1, e). Based on these results, a simple and rapid method of isolating the viral protein free from RNA and SDS was developed.

TRSV protein was isolated from purified virus by heating it at 2 mg/ml in 0.1 M phosphate buffer, pH 8.5, at 55°C - 60°C. On heating, the protein was precipitated while the RNA and any partially-disrupted or undisrupted virus remained in solution. This usually takes about 90 min. At virus concentrations above 2 mg/ml, a longer time is required which tends to denature and degrade the protein rendering it less soluble. The protein precipitate was sedimented, washed several times with cold distilled water for 2 - 3 min to remove residual RNA and freeze dried. The supernatants were analysed in a uv spectrophotometer to estimate the

Table 4

Differential effects of temperature, pH and SDS on TRSV
particle components^a

TRSV components	Temperature (pH 7.0)		1% SDS (pH 7.0)	pH (37°C/30min)	
	37°C/30min	60°C/30min	(37°C/30min)	4.5 ^b	9.5
T	Resistant	Susceptible	Susceptible	Partially Susceptible	Resistant
M	Resistant	Susceptible	Resistant	Partially Susceptible	Resistant
B	Resistant	Susceptible	Resistant	Partially Susceptible	Susceptible

^a Results based on 0.2 mg/ml virus concentration and other conditions specified in the text.

^b Complete dissociation required SDS.

amounts of RNA and undisrupted virus present. Washing was stopped when no more RNA or nucleoprotein was detected in the supernatant. Protein prepared by this technique produced similar polypeptide profiles in SDS-polyacrylamide gels as those of the virus preparations disrupted in urea-SDS-MCE reagents (compare Fig. 1, a and e). Although the protein preparations were often not readily soluble in 0.1 M Tris-HCl buffer, pH 8.5, they were very soluble in buffers containing 0.1% SDS and 2% MCE. When SDS and MCE were removed from the solubilised protein by dialysing it exhaustively against buffer or double distilled water at 25°C, the protein became readily soluble in the buffer after warming at 37°C for 2 - 3 min. The yield and quality of the protein solution was measured spectrophotometrically and found to contain no significant amounts of RNA. The estimated yield of protein was about 85%.

IV. DISCUSSION AND CONCLUSIONS

The results in this chapter show that TRSV protein contains many acid-labile peptide bonds which are hydrolysed when the protein is prepared by conventional methods involving the use of acids. Thus acidification of the virus during preparation of the coat protein has to be avoided. Studies on the stability of the virus particles under various temperature and pH conditions has led to a new method of isolating the protein which has the following advantages:

- (a) The protein is relatively free from RNA.
- (b) The protein is free from SDS or other protein denaturing agents.
- (c) The method permits large amounts (e.g. 50 - 100 mg) of protein to be isolated rapidly each time.
- (d) The protein prepared is not degraded.
- (e) High yields can be obtained.

The differential stabilities of the various TRSV particle components to SDS and heat treatments and to various pH conditions (Table 4) indicate that they must be stabilised by different interactions or bonds. The T component must be stabilised by protein-protein interactions

alone while the M and B components, which are more stable than the T component to SDS and heat treatment, must depend on both protein-protein and protein-RNA interactions for their structural integrity. SDS appears to dissociate mainly the protein-protein interactions in TRSV but it is only effective at temperatures above 25°C. At higher temperatures (60°C), SDS appears to merely render the protein soluble since heat treatment dissociates both protein-protein and protein-RNA interactions. Alkaline conditions dissociate the B component selectively (Figs. 3 and 4 F) and appear to disrupt only the protein-RNA interactions in these particles since accumulation of empty capsids occurred (Fig. 4, F). On the other hand, acid conditions appear to be non-selective and dissociated all the particles partially (Fig. 4, D) thus acting on both protein-protein and protein-RNA interactions. The results also show that the B component is less stable than the M component to heat treatment (Fig. 2). Thus it appears that the protein-RNA interacts differently in these particles to render them differentially stable to alkaline pH and heat treatment.

CHAPTER IV

THE CHEMICAL SUBUNIT OF TRSV COAT PROTEIN

I. INTRODUCTION

The T, M and B components of TRSV appear to be similar in size, shape and antigenic properties (Stace-Smith, 1970; Mayo *et al.*, 1971). Thus the coat protein of these particle components appears to be identical. From electron microscopic studies of negatively stained virus particles using the rotation technique for image analysis, Chambers *et al.* (1965) concluded that the TRSV capsid has a T = 4 surface lattice structure. Similar studies on arabis mosaic virus (ArMV), another nepovirus, also suggested that it has a similar capsid structure (Agrawal, 1967). A T = 4 icosahedral structure (with 42 capsomeres) will consist of 240 polypeptide subunits (Caspar and Klug, 1962). This model was rejected by Mayo *et al.* (1971) as incompatible with their data on the sizes of the TRSV coat protein polypeptide subunit. Based on polyacrylamide gel analysis of SDS-disrupted virus, they concluded that the TRSV capsid is composed of one type of polypeptide with a molecular weight of about 57,000, and proposed that it is built of 60 structural subunits each consisting of one such polypeptide. However, both techniques are subject to possible artifacts and further investigations based on other methods of analysis are necessary to establish the true structure of the TRSV capsid. In this chapter, results of detailed studies on TRSV coat protein are presented which indicate that the TRSV capsid is built from 240 identical polypeptides of about 13,000 molecular weight.

II. MATERIALS AND METHODS

A. Isolation of Unfractionated (Total) TRSV Coat Protein

Unfractionated TRSV protein was isolated by the temperature - pH method described in Chapter III. These protein preparations were used in amino acid analyses, cyanogen bromide cleavage and tryptic peptide mapping experiments described in Section III C.

B. Analysis of TRSV Protein in Polyacrylamide Gels and Chemical Modification of TRSV Protein

TRSV protein was analysed by electrophoresis in both continuous (Weber and Osborn, 1969) and discontinuous SDS-polyacrylamide gels (Laemmli, 1970) as described in Chapter II. In some experiments they were analysed by the urea-SDS continuous system (Swank and Munkre, 1971). TRSV and TRSV coat proteins were also chemically modified as required prior to electrophoresis to ensure complete dissociation of any disulphide bonds present (Glazer, 1976).

(i) Chemical Modification

The TRSV proteins were chemically modified by the following methods:

(1) Reduction: TRSV protein was either further reduced with 5mM dithiothreitol (DTT) when dissociated in urea-SDS-MCE reagent (Wu and Breuning, 1971) or with sodium borohydride (NaBH_4) at a final concentration of 0.25% at 25°C for 30 min (Bailey *et al.*, 1970) prior to dissociation in urea-SDS-MCE for electrophoresis.

(2) S-carboxymethylation: TRSV protein was reduced with MCE and then S-carboxymethylated in the presence of nitrogen as described by Geelen *et al.* (1972) and Tachovsky and Hare (1975). After S-carboxymethylation, the protein was dialysed against double distilled water, freeze dried and dissociated in urea-SDS-MCE for electrophoresis.

(3) Oxidation: Protein preparations were oxidised with performic acid by a method modified from that of Hirs (1967). Fresh 3 - 5% performic acid was prepared by mixing 1 volume of 30% hydrogen peroxide with 19 volumes of 98% formic acid and incubated at 25°C for 2 hr. Thirty min before the performic acid was ready, protein samples were suspended in ice-cold 98% formic acid - methanol mixture (5:1) at 0.2 ml/mg protein and kept at 0°C. The performic acid was cooled to 0°C before being added to the cold protein suspensions at 1 ml/10 mg protein. Oxidation was allowed to proceed at 0°C for 2 hr, and then stopped by adding 10 volumes

of cold double distilled water. The protein was freeze dried, resuspended in water and freeze dried again before being dissociated in the urea-SDS-MCE reagent.

(ii) Detection of Glycosylated Protein

After electrophoresis the gels were tested for the presence of glycosylated proteins by staining them with Periodic-Acid-Schiff reagent (PAS) as described by Segrest and Jackson (1972), using insulin as a control glycoprotein.

C. Preparative Fractionation of TRSV Protein Components

Method 1: This method employs a preparative polyacrylamide gel fractionation and electrophoretic elution procedure modified from that of Stephens (1975). About 500 μ g aliquots of virus or viral proteins dissociated in urea-SDS-MCE reagent (2 mg/ml) were electrophoresed in each of a series of 13% polyacrylamide gels, 10 mm in diameter and 130 mm long by the discontinuous PAGE system (Laemmli, 1970) described in Chapter II. The samples were electrophoresed for 1 hr at 3 mA/gel and then for 5 hr at 6 mA/gel. After electrophoresis, one gel was stained with CBB to locate the polypeptide components relative to the BPB marker from which the positions of these protein components in the other gels were located. Each of the various protein components was excised from the gels and pooled. They were rinsed thoroughly in double distilled water and then ground up in a loose-fitting tissue homogeniser in the presence of 0.1 M Tris-HCl, pH 6.8, containing 0.1% SDS and 0.1% MCE. The homogenised gel fractions were incubated at 37°C for 1 hr before being loaded into 1 cm diameter and 15 cm long glass tubes each with a 3 cm long 4% agarose plug and a dialysis bag containing 25% glycerol in 10 ml of electrophoresis buffer at the bottom end. The agarose was in 0.375 M Tris-HCl, pH 8.8, and electrophoretic elution was carried out at 3 mA/tube for 18 - 20 hr using the 0.05 M Tris-glycine electrophoresis buffer, pH 8.3, but containing no SDS. After electrophoresis, the current was reversed for 5 min at 5 mA/tube before removing

the dialysis bags containing the concentrated protein components. The isolated protein components were dialysed exhaustively against double distilled water at 4°C, freeze dried and stored at -15°C.

Method 2: TRSV protein was dissociated in urea-SDS-MCE reagent and dialysed exhaustively against several changes of double distilled water at 4°C and were then freeze dried. The protein was redissolved in 0.05 M Tris-glycine buffer, pH 8.3, at 10 mg/ml by warming at 37°C for 5 - 10 min. One to 1.5 mg aliquots of the protein solution was loaded onto each of six 5 - 25% sucrose gradients made in the same buffer and centrifuged at 44,000 rpm in a Spinco SW50 rotor for 30 hr at 15 - 20°C. Each gradient was fractionated into 20 x 0.25 ml fractions with the aid of the ISCO gradient fractionator and uv analyser unit, and corresponding fractions from the gradients were pooled. Fifty to 100 µl samples from each pooled fraction were analysed for protein composition by discontinuous SDS-PAGE. Fractions containing the same protein component were pooled, dialysed against double distilled water and the amounts of protein recovered were estimated spectrophotometrically. The protein components were freeze dried and redissolved in the Tris-glycine buffer described above at 5 mg/ml. The proteins were refractionated by the same procedures a second time. The final TRSV protein components were freeze dried and stored at -15°C.

D. Amino Acid Analysis of TRSV Protein

Amino acid composition of TRSV protein was analysed as described by Delange *et al.* (1969). Samples of TRSV protein were dissolved in 6N HCl (1 ml/mg protein) containing aqueous phenol (1 drop of a 5% solution per ml HCl) by heating the samples in boiling water for 10 min. Three equal sub-samples were taken and hydrolysed at 110°C for 24, 48 and 72 hr respectively. Equal aliquots of the three hydrolysates were analysed in a beckman 120 amino acid analyser, and the molar ratios of the amino acids recovered were calculated. The cysteine content was determined separately by hydrolysis of the performic acid oxidised

protein and determining the cysteic acid content as described by Moore (1963), except that the protein was oxidised with 3 - 5% performic acid as described in Section B, (i), (3) above. Unoxidised protein was used as control. The cysteic acid content was estimated by reference to the molar ratio of aspartic acid recovered in the same analysis. Tryptophan content was calculated from the molar ratio of tryptophan to tyrosine of the protein, estimated by the spectrophotometric method of Beaven and Holiday (1952).

The complete amino acid composition data were subjected to FITMOL analysis as described in Gibbs and McIntyre (1970).

E. Trypsin Digestion and Tryptic Peptide Mapping

Various protein samples to be digested with trypsin were first oxidised with performic acid (Hirs, 1967) as described in Section II B, (i), (3). Trypsin digestion was as described by Knowland (1974). The oxidised protein was dissolved in fresh 2% ammonium bicarbonate buffer, pH 8.4, (adjusted with dilute ammonium hydroxide) at 1 - 2 mg/ml and then digested with fresh diphenyl carbamyl chloride (DCC) - treated trypsin (prepared in the same buffer at 1 mg/ml) at a trypsin : protein ratio of 1 : 50 (W/W) for 2 hr at 37°C in sealed round bottom flasks. An identical amount of trypsin was then added to the digests and the digestion allowed to proceed for a further 2, 8 or 18 hr at 37°C as required. To samples digested for periods in excess of 20 hr, a third aliquot of trypsin was added after the initial 20 hr of digestion. Digestion was stopped by adding 2 volumes of double distilled water followed by immediate freeze drying. The lyophilysed tryptic peptides were resuspended in double distilled water and freeze dried again before being dissolved in pyridine : acetic acid : water (25 : 1 : 225; V/V), pH 6.5, at 10 - 20 mg/ml.

The tryptic peptides were either analysed in one dimension by ascending chromatography only, or in two dimensions by high voltage electrophoresis followed by descending chromatography. Two dimensional tryptic peptide mapping was done on standard Whatman No. 3 MM Chromato-

graphy papers essentially as described by Bennett (1967). Electrophoresis was carried out with a water-cooled flat-bed high voltage electrophoresis unit, using the pyridine-acetate buffer described above. The chromatography papers were pre-electrophoresed at 2000 volts for 90 min at 25°C immediately before use. Peptide samples (1 mg in 50 - 75 µl per sample) were loaded onto the wet papers in decreasing volumes and electrophoresed at 1,500 volts for 90 min. After electrophoresis, the papers were air dried in a fume hood at 22°C overnight. They were subjected to descending chromatography in n-butanol-pyridine-acetic acid-water (90 : 60 : 18 : 72, V/V) for 12 hr at 22°C and then air dried for 24 hr as described above.

Peptides separated only in one dimension were chromatographed on thin layers of cellulose essentially as described by Burns and Turner (1967). The thin layer plates were pre-eluted first with 1% acetic acid and then with the solvent used for separating the peptides. Samples (10 - 20 µl) each containing 100 µg of peptides, were applied to the dried plates with a 2 µl microcapillary tube. Up to 9 samples could be applied on the same plate and separated simultaneously, thus permitting direct comparison of the samples. The samples were air dried before being chromatographed at 22°C for 5 hr. Several solvents were compared and n-butanol-pyridine-acetic acid-water (90 : 60 : 18 : 72) was found to be the most satisfactory. The plates were dried for 24 hr as described above prior to staining.

The total number of peptides were detected by staining with fresh solutions of 0.3% ninhydrin in acetone, buffered with 1% acetic acid and 1% pyridine (Easley, 1965). Paper chromatograms were stained by dipping them through a trough containing 100 ml of the ninhydrin solution, while thin layer chromatograms were stained by pouring 20 ml of ninhydrin solution evenly over the whole plate. The chromatograms were air dried for 20 hr and then at 40°C for 10 min.

Peptides containing arginine were detected with the phenanthrene-quinone reagent applied as described above (Yamada and Itano, 1966). Those containing histidine and tyrosine were detected by spraying the chromatograms with the Pauly reagent (Easley, 1965) and a fine light spray was required to produce sharp distinct spots. Some chromatograms were stained successively with two or more compatible reagents to detect the above amino acids (Easley, 1965; Easley *et al.*, 1969; Glazer *et al.*, 1976) but the results were not as good as those obtained from chromatograms stained once with a single reagent.

Tryptic peptides containing ^{14}C -leucine or ^{14}C -valine were detected by autoradiography of the 2-dimensional paper chromatograms containing peptides from TRSV labelled *in-vivo* with the appropriate radioactive amino acid. Autoradiography was done by placing two sheets of Kodak RP54 X-Omat X-ray films on both sides of the chromatograms. The films were exposed at room temperature for the periods required and then developed for 5 - 10 min and fixed for 5 min at 25°C using Kodak X-ray film developer and fixer.

F. Cyanogen Bromide Cleavage of Proteins

TRSV and other proteins were cleaved using fresh 2% cyanogen bromide (CNBr) in 70% formic acid at 0.5 mg/ml ^{protein concentration} as described by Swank and Munkre (1971). The proteins were normally incubated at 25°C for 16 hr unless otherwise stated. BTMV containing no methionine (Rees and Short, 1975) and horse myoglobin containing 2 methionine residues at the 55th and 131st positions (Dautrevaux *et al.*, 1969) were cleaved under identical conditions and used as controls. Control protein samples were also incubated in 70% formic acid only. Cleavage reactions were stopped by adding 10 volumes of double distilled water and the samples were freeze-dried. More water was added to the sample and then freeze-dried again. The CNBr peptide fragments were dissociated as described in Chapter II and then analysed by gel electrophoresis using the continuous urea-SDS PAGE system of Swank and Munkre (1971) designed for low molecular weight

polypeptides. Cylindrical gels with gel concentrations of 16 and 18% were used. The gels were pre-electrophoresed at 2 mA/gel for 30 min before loading the samples (50 μ l). Electrophoresis was done at 2 mA/gel for 2 hr followed by 3 mA/gel for 15 (16% gels) and 20 hr (18% gels). The mol. wts. of the CNBr fragments were estimated from their relative mobilities using various protein markers as described in Chapter II.

G. Serological Techniques

Antiserum to the TRSV 60K protein component was prepared in rabbit as described in Chapter II except that 0.7 mg of 60K protein isolated by sucrose density gradient centrifugation and resuspended in 0.5 ml of 0.05 M Tris-HCl, pH 8.0, was emulsified with 0.5 ml of Freund's complete adjuvant and injected intramuscularly into the rabbit. The injection was repeated subcutaneously 1 week later, and then a booster injection of 1.5 mg of protein was given 2 weeks after the second injection. The rabbit was bled and the blood was processed as described in Chapter II.

All double-gel immuno-diffusion assays were done as described in Chapter II. The TRSV proteins were dissolved in 0.1 M Tris-HCl buffer, pH 8.5, at 0.2 mg/ml and diluted with the same buffer when required. Ten μ l samples were loaded into the wells and serological plates were incubated at 25^o, 37^o or 50^oC.

III. RESULTS AND DISCUSSION

A. Analysis of TRSV Coat Protein by Electrophoresis in Polyacrylamide Gels

(i) Protein Profiles of TRSV Coat Protein in SDS-Polyacrylamide Gels

Six bands were consistently detected on SDS-polyacrylamide gels when 50 μ g or more of TRSV coat protein per gel were electrophoresed after dissociation in 6 M urea, 1% SDS and 2% MCE (Fig. 1). Estimation of the relative proportions of these bands showed that more than 85% of the protein migrated as a wide band corresponding to a polypeptide with Mol. Wt. of 54,000 to 60,000 (60K protein) (Fig. 1, Table 1). This band

Figure 1. Separation of TRSV protein components by electrophoresis in 13% discontinuous polyacrylamide gels (Laemmli, 1970)

A. Protein components detected in TRSV preparations

Gel a, marker proteins only (indicated on the left). They are:

Bovine serum albumin dimers (2 x BSA),

Bovine serum albumin (BSA),

Glutamate dehydrogenase (GDH),

Ovalbumin (Oval),

QCMV coat protein (CMV),

Trypsin (Try),

Lysozyme (Lys);

gel b, TRSV dissociated in urea-SDS-MCE

reagent (100 µg virus loaded). The various protein

components are designated 15K, 30K, 45K, 60K, 116K

and T and are indicated on the right;

gel c, protein markers plus dissociated TRSV.

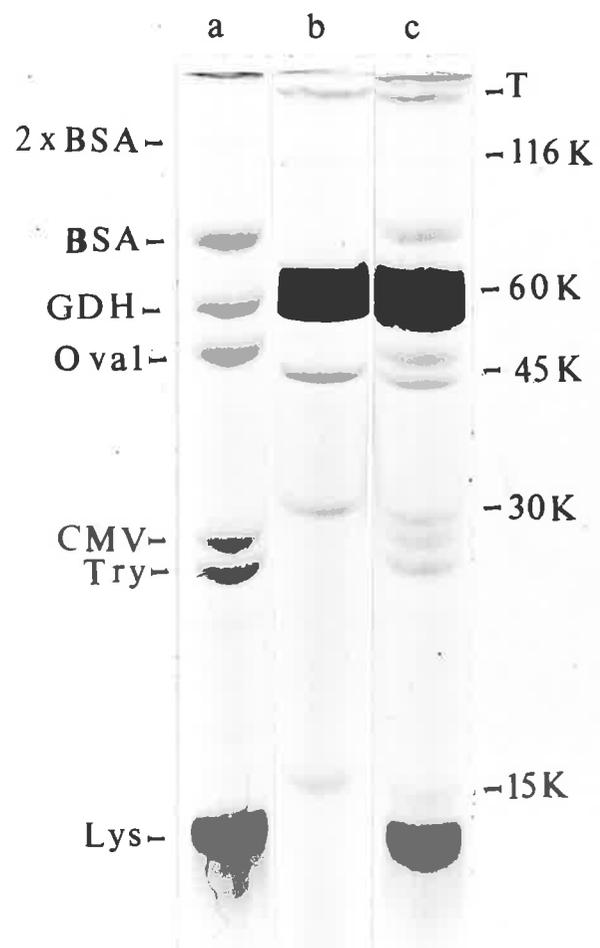
B. Protein components detected in preparations of unfractionated

(UF) and T, M and B particle components of TRSV

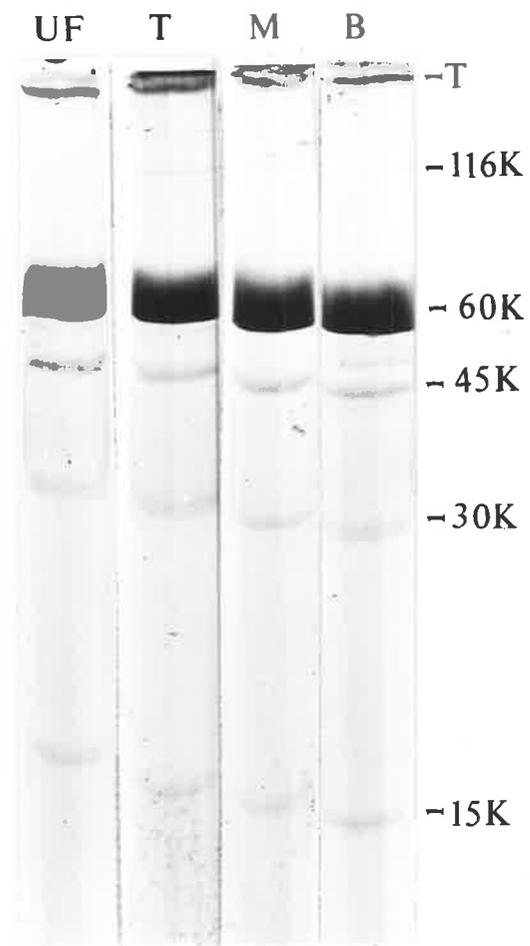
Approximately 100 µg of each virus preparation was dissociated

and loaded on each gel. The various TRSV protein components

are indicated on the right.



(A)



(B)

Table 1

Estimated molecular weights and relative proportions
of polypeptide components from TRSV protein separated
by polyacrylamide-gel electrophoresis

Polypeptide Component ^a	Estimated Molecular Weights ^b		Relative Amounts of Polypeptides (%) ^e	
	Discontinuous System ^c	Continuous System ^d	Discontinuous System	Continuous System
T	- ^f	-	2.5	2.5
116K	110,000	117,000	0.75	0.75
60K	55,500	58,000	87.5	87.5
45K	41,000	44,000	2.75	2.75
30K	26,400	29,000	4.0	4.0
15K	14,000	14,000	2.5	2.5

^a See Fig. 1 for positions of components in polyacrylamide gels.

^b Estimated by comparison with a series of standard marker proteins (see (Fig. 2)).

^c Mean estimates from three separate experiments each using a different protein preparation which was electrophoresed on 5 replicate 13% gels (Laemmli, 1970).

^d Mean estimates from two separate experiments each using a different protein preparation which was electrophoresed on 5 replicate 10% gels (Weber and Osborn, 1969).

^e Estimated by integrating areas under the protein peaks detected in scans of the gels measured with a chromoscan unit at 575 nm. Each value is a mean estimate from five different protein preparations each electrophoresed on 2 replicate gels.

^f Dash indicates that the molecular weight of the T component could not be reliably estimated by the methods used.

appears to correspond with the only polypeptide band detected by Mayo *et al.* (1971) when they electrophoresed TRSV coat protein on polyacrylamide gels. The remaining 15% of the protein migrated as 5 minor bands (15K, 30K, 45K, 116K and T) (Fig. 1 and Table 1). Similar protein profiles were obtained when TRSV protein was analysed by either the continuous (Weber and Osborn, 1969) or the discontinuous (Laemmli, 1970) system of PAGE and the Mol. Wts. of the protein components determined by these different systems were found to be similar (Fig. 2 and Table 1). When a TRSV protein preparation was centrifuged in a sucrose density gradient (Fig. 3), the same six protein components detected on gels (Fig. 1) were also separated (Fig. 3). Thus the six polypeptide bands from TRSV preparations obtained in the polyacrylamide gels were not artifacts of the polyacrylamide gel electrophoresis technique. Since the discontinuous system of PAGE produced sharper bands and takes a shorter time to complete, this system was used in all subsequent experiments. Electrophoresis of TRSV protein preparations in gels of 10%, 12.5% and 15% by the discontinuous system invariably produced very similar profiles of protein components with similar relative proportions and molecular weights (compare Tables 1 and 2).

After electrophoresis, the protein bands were detected by different staining and destaining procedures. These included staining the gels for various times from 1 hr to overnight at 25°C or 37°C, using either acetic acid/methanol as fixative (Chapter II) or 50% TCA as fixative (Laemmli, 1970) and destained with or without methanol in the destaining buffer. The results showed that relatively stronger bands were obtained in gels stained for longer times at 37°C or when they were fixed and then stained in the presence of TCA. However, destaining took at least twice as long under these conditions, especially when methanol was absent from the destaining buffer. When gels were only stained for 1 - 2 hr at 25°C without prefixing with TCA the minor bands were difficult to detect.

Figure 2. Estimation of the molecular weights of the TRSV protein components by the continuous (Weber and Osborn, 1969) and discontinuous polyacrylamide gel electrophoresis (Laemmli, 1970) systems

TRSV and marker proteins were electrophoresed in separate gels (10% gels for the continuous system and 13% for the discontinuous system). Relative mobilities of the proteins were measured with respect to bromophenol blue using the following protein markers (molecular weights in brackets):

- 1, bovine serum albumin dimers (2 x BSA) (136,000);
- 2, BSA, (68,000);
- 3, glutamate dehydrogenase (GDH) (53,000);
- 4, ovalbumin (Oval) (43,000);
- 5, QCMV coat protein (CMV) (24,600);
- 6, myoglobin (Myo) (17,200);
- 7, cytochrome c (Cyt-c) (12,400).

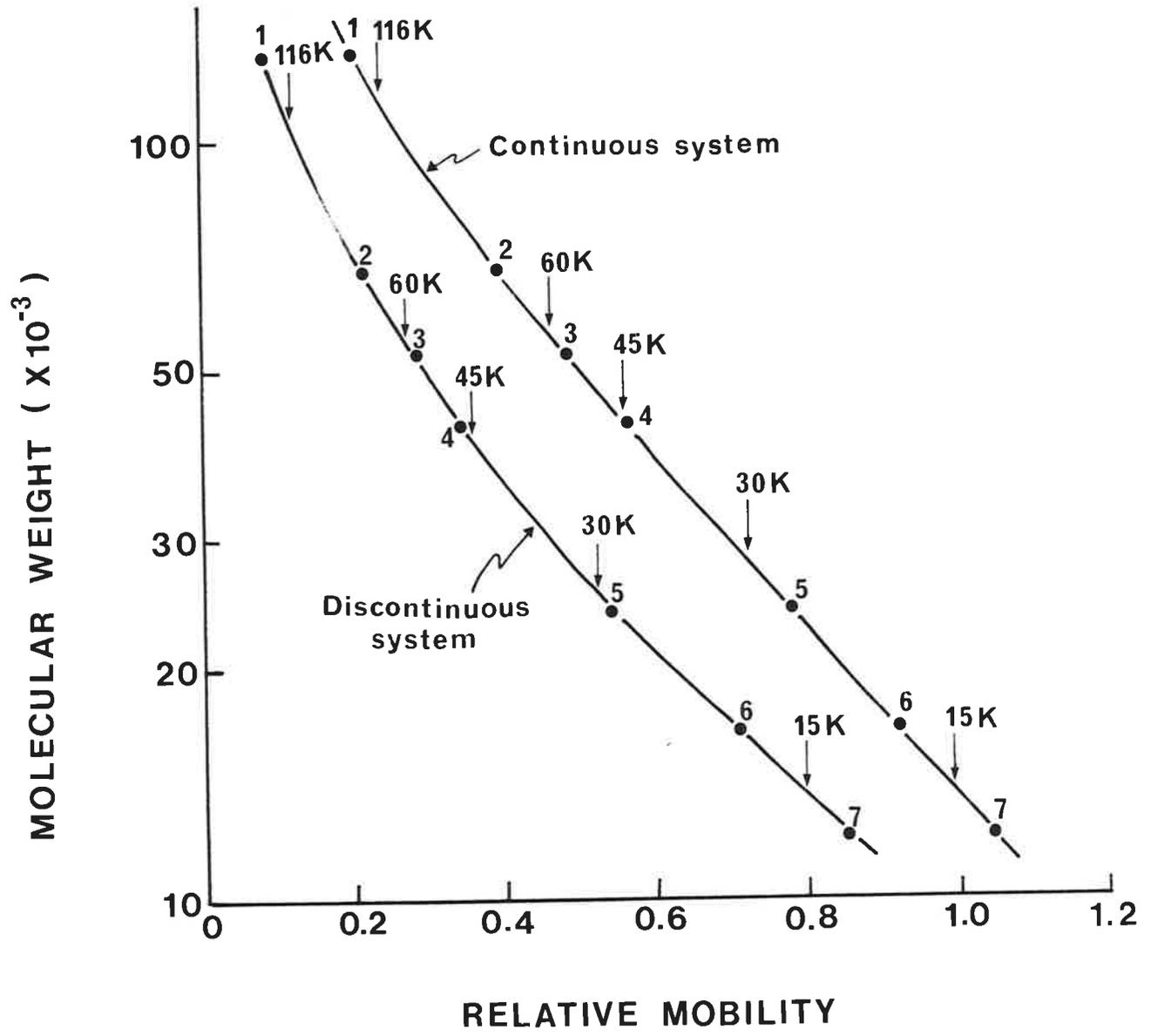


Figure 3. Separation of TRSV protein components by sucrose density gradient centrifugation

A. Ultraviolet absorbance spectrum of sucrose density gradient containing separated TRSV coat protein components

Unfractionated TRSV protein preparations were centrifuged in 5 - 25% sucrose gradients (1 mg/tube) as described in Materials and Methods. The gradients were analysed and fractionated with an ISCO uv gradient analyser which measures absorbance at 254 nm. The top and bottom of the gradients are indicated.

B. The gradient fractions collected (numbered 1 - 20) were analysed for TRSV protein components by discontinuous SDS-PAGE as described in the text. TRSV protein components similar to those detected in gels in Fig. 1 are observed in the various fractions.

RELATIVE ABSORBANCE AT 254 nm

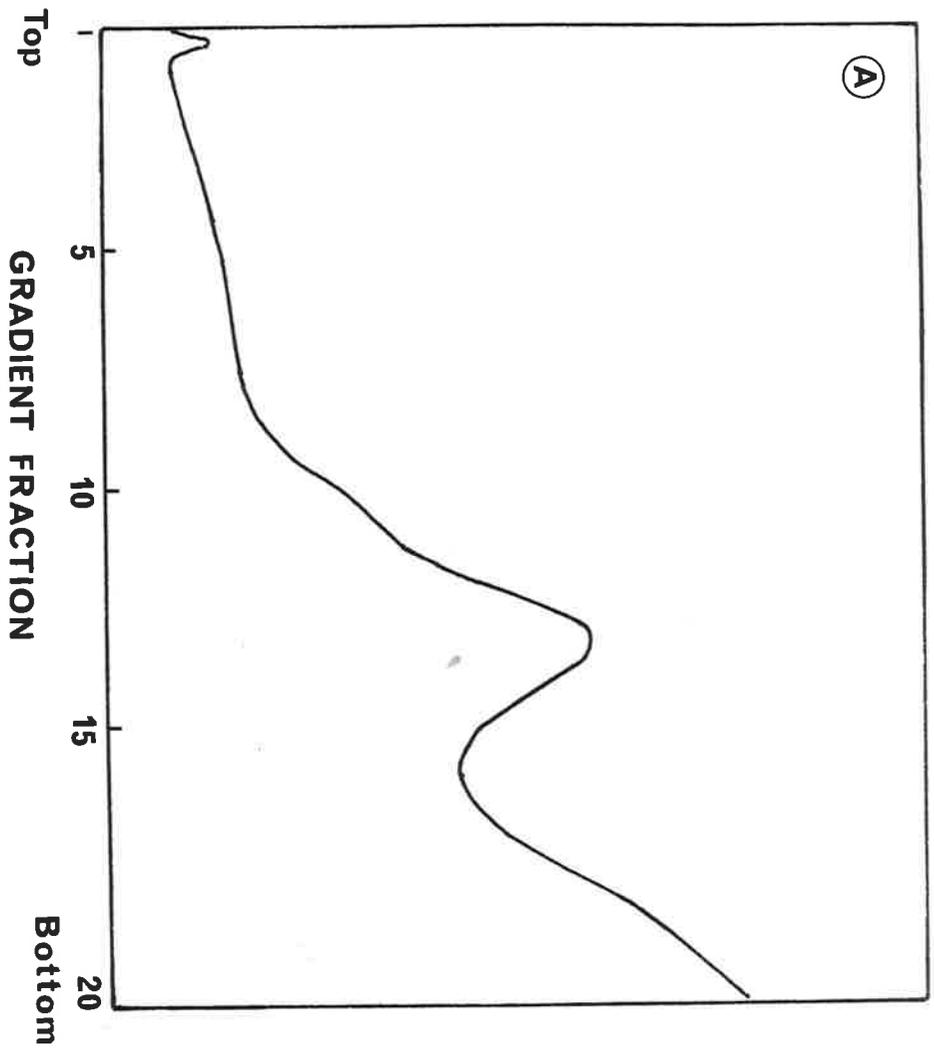


Table 2

Electrophoresis of TRSV coat protein in discontinuous
SDS-polyacrylamide gels of various concentrations

Polypeptide Component	Gel Concentrations					
	15%		12.5%		10%	
	Mol. Wt.	Rel. Propn.	Mol. Wt.	Rel. Propn.	Mol. Wt.	Rel. Propn.
T	- ^a	2.5	- ^a	2.5	- ^a	2.75
116K	110,000	0.5	110,000	0.75	108,000	0.75
60K	55,000	87.5	54,000	87.5	54,000	87.5
45K	42,000	3.0	41,000	3.0	41,000	2.75
30K	28,000	4.0	27,500	3.75	27,000	4.0
15K	14,500	2.5	14,500	2.5	- ^a	- ^a

The molecular weights (Mol. Wt.) and relative proportions (Rel. Propn.) of the protein bands were estimated as described in Table 1.

^a Dash indicates that the molecular weights and/or relative proportions of the T and 15K protein components could not be reliably estimated at the respective gel concentrations used.

The number and relative proportions of TRSV coat protein components from all the virus preparations used in the study (over 30 preparations) were similar. The isolated T, M and B particle components all yielded protein profiles similar to those of the unfractionated TRSV preparations (Fig. 1). Similar protein profiles were also obtained whether the intact virus or its isolated protein was dissociated and electrophoresed.

(ii) Are the Minor Polypeptide Components Degradation Products of 60K Protein

Although six protein bands were detected in TRSV coat protein, the bulk of the material migrated as the 60K component. Thus it was possible that the polypeptide subunit of TRSV coat protein was the 60K component as concluded by Mayo *et al.* (1971) while the T and 116K proteins were aggregates and the 15K, 30K, and 45K components degradation products. It seemed possible that degradation of the polypeptide may have occurred *in-vivo* during virus accumulation in the host tissues since under the experimental conditions used, maximum virus was attained 5 days after inoculation (Rezaian and Francki, 1973) when the cotyledons were starting to become necrotic. Also, virus may have degraded during purification and storage. These possibilities have been investigated and the results are as follows:

- (a) TRSV was purified from tissues harvested 2, 5 and 10 days after inoculation. The cucumber cotyledons were just showing mild symptoms at 2 days and at 10 days they were severely necrotic. However, analysis of the protein from all these virus preparations showed no detectable differences in the protein profiles (Fig. 4A).
- (b) No difference was observed in the polypeptide components of TRSV preparations purified in the presence or absence of a ^{plant} protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF), added to the extraction buffer at 0.5 mM (Wallace, 1974) and when the virus was purified with fresh sterile or old non-sterile buffers (Fig. 4B).

Figure 4. Effects of various procedures used in the purification of TRSV on its protein profiles in discontinuous SDS-polyacrylamide gels

In each of the experiments approximately equal amounts of TRSV protein (100 µg) were loaded on each gel.

A. Effect of time of harvest of infected tissue

Gel M, protein markers;
gel a, TRSV purified from tissues harvested 2 days after inoculation;
gel b, TRSV purified from tissues harvested 5 days after inoculation;
gel c, TRSV purified from tissues harvested 10 days after inoculation.

B. Effect of PMSF in the extraction buffer and of sterile and old buffers

Gel a, TRSV purified with fresh buffer containing PMSF;
gel b, TRSV purified with fresh buffer containing no PMSF;
gel c, TRSV purified with fresh sterile buffer;
gel d, TRSV purified with old non-sterile buffer.

C. Effect of timing of virus purification

Gel a, rapidly purified TRSV (12 hr);
gel b, slowly purified TRSV (96 hr);
gel c, homogenate incubated overnight at 25°C.

D. Effect of storage of tissues prior to purification

Gel a, TRSV purified from freshly harvested tissues;
gel b, TRSV purified from decaying tissues.

E. Effect of temperature of purification

Gel a, TRSV purified at 0°C;
gel b, TRSV purified at 25°C.

F. Effect of age of TRSV preparation

Gel a, freshly prepared TRSV;
gels b - d, TRSV stored for 4, 8 and 16 weeks at 4°C.

(c) TRSV was purified by the following procedures: (1) purified as rapidly as possible; a procedure which took only 12 hr from harvest to purification, including a final sucrose density gradient centrifugation step; (2) purified slowly in which the virus was resuspended overnight at all resuspension steps so that the total purification time was 96 hr or (3) purified by the standard method except that the tissues were homogenised and incubated at 25°C overnight prior to purification.

Analyses of the proteins of all these virus preparations showed that although there were some new minor bands detected in those isolated by the procedures (2) and (3), there were no increases in the relative proportions of the 15K, 30K or 45K minor polypeptide components (Fig. 4C).

(d) TRSV was purified at 0°C or 25°C, and from freshly harvested leaf materials and materials which were stored at 4°C for various time periods ranging from overnight to several weeks when the tissues showed signs of decay. Polyacrylamide gel analysis of the proteins of these virus preparations showed that the relative proportions of the 15K, 30K and 45K components were unaltered but new minor protein bands were observed in virus prepared from decomposing tissues (Fig. 4D) and those purified at the higher temperature (Fig. 4E).

(e) Purified TRSV was stored for various times from 0 - 16 weeks at 4°C under sterile and non-sterile conditions. Analysis of the protein of these virus preparations showed that long term storage sometimes produced new degradation products without significantly affecting the relative proportions of the 15K, 30K, and 45K components (Fig. 4F).

Thus the above results indicated that the 15K, 30K and 45K protein components are not products of *in-vivo* partial degradation of the virus which could have occurred in severely necrotic tissues. They are also not the products of enzymatic, bacterial or chemical breakdown of the 60K component since no matter what precautions were taken to ensure that there were no degradation of virus, the same low molecular weight minor polypeptide components (15K, 30K and 45K) were still present in

similar amounts as observed in the standard virus preparations. On the other hand, under conditions which encouraged enzymatic or bacterial degradation of the virus, several new weak protein bands with Mol. Wts. ranging from 20,000 - 50,000 were detected while the profiles of the 15K, 30K and 45K proteins were unchanged (Fig. 4).

(iii) Absence of Host Protein Contaminating the TRSV Preparations

The possibility that the minor polypeptide components from TRSV preparations observed on polyacrylamide gels (Fig. 1) were of host origin had to be considered. This seemed unlikely because of the following results:

(a) Viruses purified only by differential centrifugation did not yield higher proportions of the minor polypeptides than viruses further purified by sucrose density gradient centrifugation. Similarly, viruses purified by sucrose gradient centrifugation followed by isopycnic density gradient centrifugation in CsCl produced a protein profile similar to that of the viruses purified by only sucrose gradient centrifugation (Fig. 5A).

(b) TRSV purified from different hosts, tobacco, French beans, cowpeas and cucumber seedlings, using the same purification method, yielded similar protein profiles in polyacrylamide gels (Fig. 5B).

However, to obtain further evidence that all of the polypeptides detected in TRSV (Fig. 1) were of viral origin, the following experiment was done. Cucumber seedlings were divided into four groups, of which two were inoculated with 200 µg/ml TRSV (D plants), while the other two were inoculated with sterile buffer only (H plants). All plants were grown under identical conditions as described in Chapter II. Twenty-four hours later, 13 gm of cotyledons were excised from each group of plants, rinsed thoroughly in sterile distilled water, and blotted dry. Incisions were made on the cotyledons on both sides of the mid-ribs parallel to their veins. One group of cotyledons from the H plants and one from the D plants were each wetted with 100 µCi ³H-labelled protein hydrolysate and subjected to gentle vacuum to facilitate the uptake of radioactive amino acids. The remaining groups of cotyledons were treated

Figure 5A. Discontinuous polyacrylamide-gel electrophoretic analysis of the protein composition of TRSV at different stages of purification

Gel M, protein markers;

gel a, TRSV purified by differential centrifugation only;

gel b, TRSV purified through sucrose density gradient;

gel c. TRSV purified through both sucrose and caesium chloride density gradients.

Approximately 100 μg of TRSV protein were loaded on gels a-c.

B. Protein profiles of TRSV purified from different hosts in discontinuous polyacrylamide gels

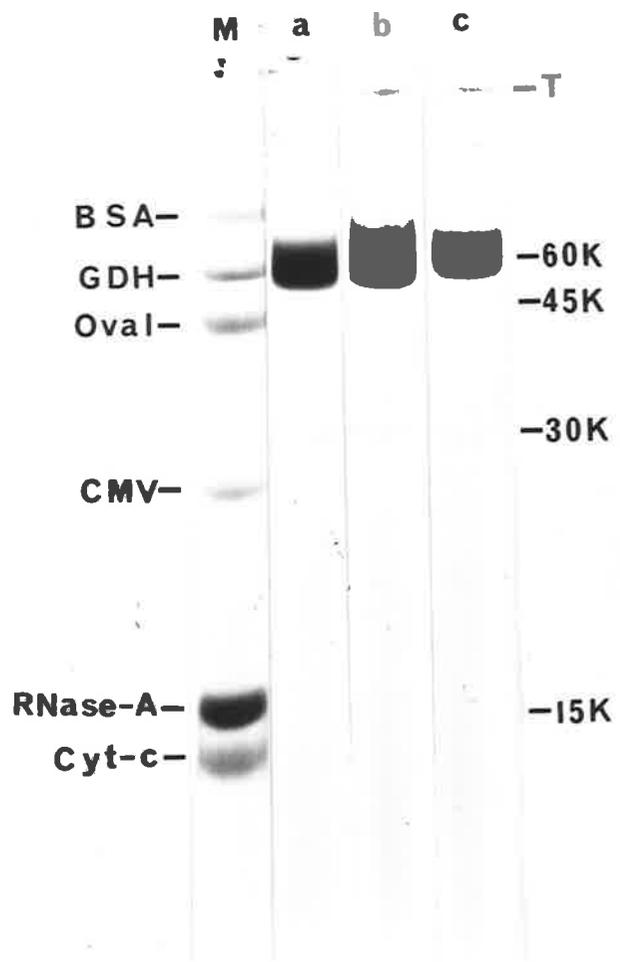
Gel M, protein markers;

gel a, TRSV from tobacco (80 μg);

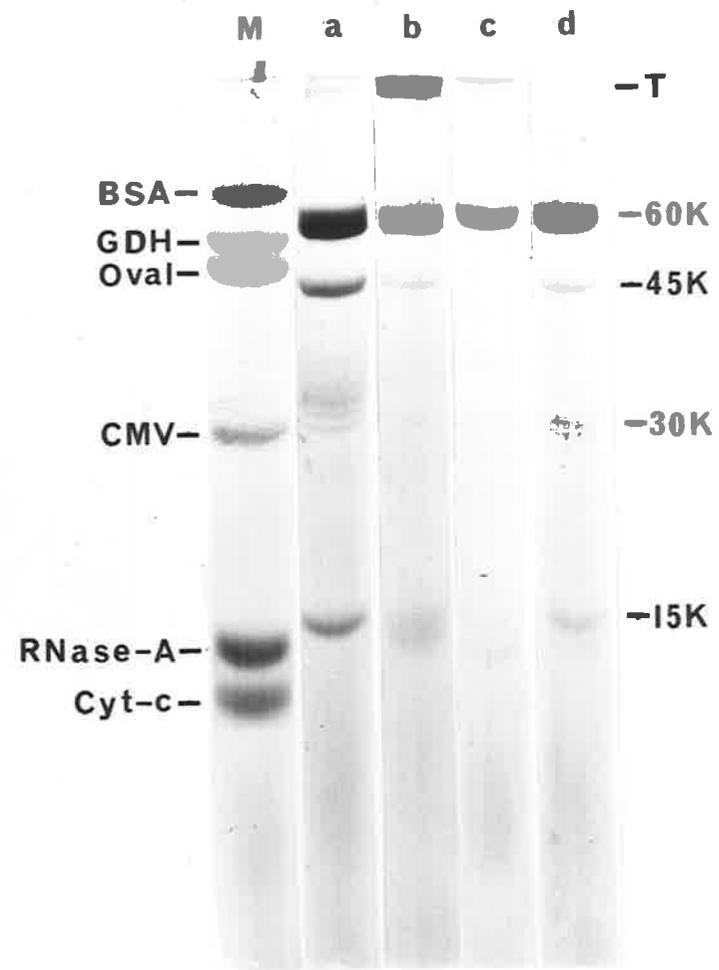
gel b, TRSV from French beans (50 μg);

gel c, TRSV from cowpeas (30 μg);

gel d, TRSV from cucumber seedlings (50 μg).



A

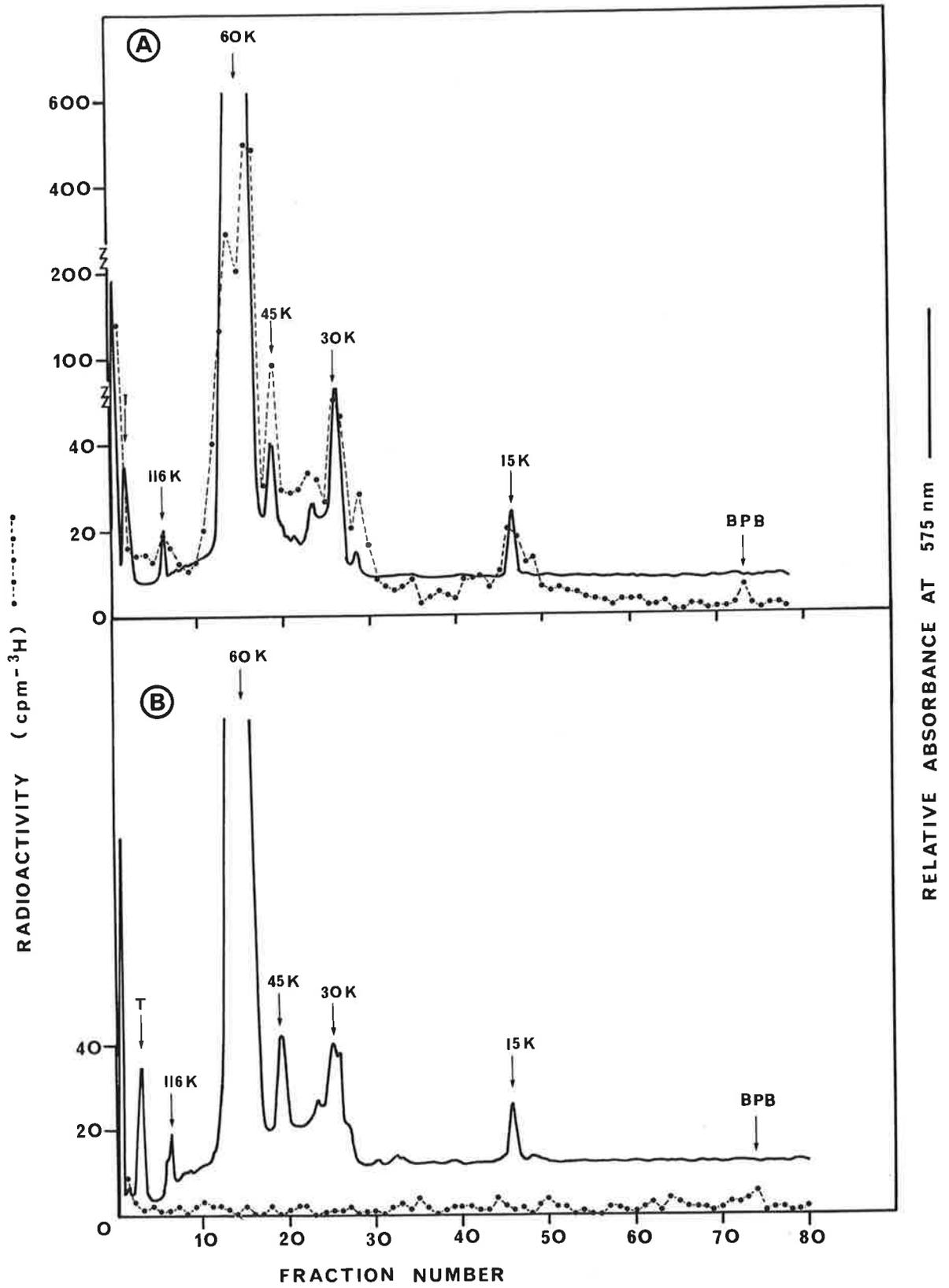


B

in a similar manner but without any labelled material added. All four groups of cotyledons were floated in petri-dishes containing 10 ml of Vickery's culture solution, pH 6.8, and were left under continuous fluorescent light of 400 lux at 25°C. More culture solution was added to the tissues as it became depleted. After 72 hr, the various groups of tissues were rinsed thoroughly with 0.1 M phosphate buffer, pH 7.0, and blotted dry. The sample of D cotyledons exposed to ³H-labelled protein hydrolysate was mixed with the sample of H cotyledons incubated in the absence of label, and the sample of H cotyledons exposed to the radioactive amino acids was mixed with the sample of D cotyledons incubated in the absence of label. Thus, the first mixture contained TRSV which had multiplied in the presence of ³H-labelled amino acids (labelled virus) while the second contained viruses which had multiplied in the absence of radioactive precursors (unlabelled virus) but contained labelled host proteins. Virus was purified from the two tissue mixtures by the standard procedure. As expected, higher specific radioactivity was obtained in the labelled virus than the unlabelled virus. Samples of the virus preparations were dissociated in urea-SDS-MCE and analysed by SDS-PAGE in duplicate gels. One gel of each duplicate was stained with CBB, the others were sliced and the radioactivity in each gel slice counted. The labelled virus preparation would produce radioactive peaks in the gel corresponding to all the stained protein bands whether they are of viral or host origin, while the unlabelled virus preparation would only produce radioactive peaks corresponding to protein bands which are of host origin and protein bands of viral origin would be unlabelled. Analysis of the gels showed that all six polypeptides were detected in the labelled virus preparation by both staining and measurement of radioactivity (Fig. 6A). However, in the unlabelled virus preparation the same six polypeptides were detected by staining but not by radioactivity (Fig. 6B). These results confirmed that none of the polypeptides detected in TRSV are of host origin.

Figure 6. Detection of protein components of TRSV separated in discontinuous polyacrylamide-gels by staining with coomassie brilliant blue (absorbance at 575 nm) and by the presence of ^3H -labelled amino acids (radioactivity)

- A. Virus prepared from a mixture of healthy, unlabelled leaves and TRSV-infected, labelled leaves and
B, a mixture of healthy, labelled leaves and TRSV-infected, unlabelled leaves. (See text for details of experimental design.)



(iv) Absence of Polysaccharide in TRSV Protein

The possibility that some of the protein components associated with TRSV may be glycoproteins was tested in the following experiment. Duplicate polyacrylamide gels were loaded with equal amounts of either urea-SDS-MCE dissociated TRSV protein, cyanogen bromide fragments of TRSV protein, or protein markers (BSA, GDH, Oval, CMV coat protein, Myo, RNase-A, Cyt-c and insulin) and were electrophoresed by the continuous urea-SDS-system. Insulin is known to be a glycoprotein. After electrophoresis, one gel from each duplicate was stained with CBB and the others with PAS reagent. The results show that both TRSV polypeptides and CNBr fragments and the marker proteins retained the CBB stain but only the marker proteins (Myo, Cyt-c and insulin) retained the PAS stain (Fig. 7). Thus none of the TRSV protein components observed in SDS-polyacrylamide gels is a glycoprotein.

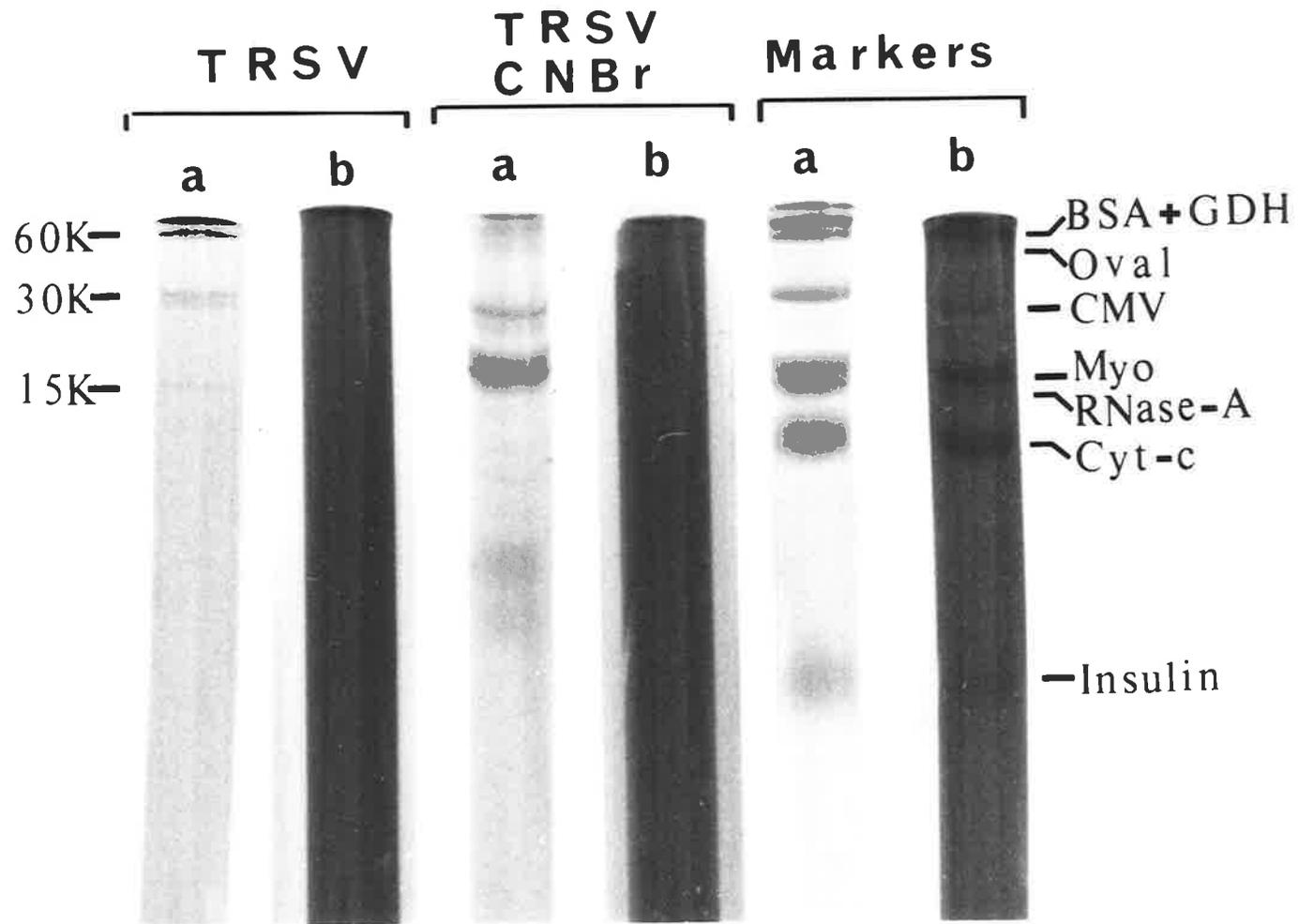
(v) Tests for the Presence of Aggregated Polypeptides in TRSV Proteins

From the data summarised in Table 1, it appears that the estimated Mol. Wts. of the polypeptide components of TRSV approximate to multiples of the smallest component (15K protein), which has a Mol. Wt. of about 14,000 (Table 1). Aggregates of viral proteins have been observed in SDS-polyacrylamide gels e.g. in TYMV, (Matthews, 1974), but the relative proportions of the aggregated materials were very small. Aggregated forms may occur in SDS-gels because of incomplete dissociation of the virus capsid or due to chemical and/or physical reassociation of the subunits by the formation of hydrogen or hydrophobic bonds, inter-molecular disulphide bridges, or other cross-linking covalent bonds (Rice, 1974). Thus it is possible that the larger proteins are aggregates of the 15K component but was not considered before since the bulk of the viral protein appeared as the 60K component (Table 1). The following experiments were done to test this possibility.

Figure 7. Detection for glycoprotein in TRSV coat protein and in cyanogen bromide fragments of the coat protein after electrophoresis of these proteins and peptides in 18% continuous urea-SDS-polyacrylamide gels (Swank and Munkre, 1971)

Gels a, stained with CBB to stain all proteins;
gels b, stained with Periodic-Acid-Schiff reagent
to detect glycoproteins.

The identity of TRSV protein components are indicated on the left and that of the marker proteins are on the right.



(a) Dissociation of TRSV in Urea, SDS and MCE and other Reagents

TRSV was dissociated under different combinations of urea, SDS, MCE and DTT and then analysed in SDS-polyacrylamide gels. All six components were detected only in virus dissociated with SDS, urea and a reducing agent (MCE or DTT) (Fig. 8A, f-h). If either the reducing agents (Fig. 8A, d) or urea (Fig. 8A, e) or both (Fig. 8A, b and c) were absent from the dissociation buffer, the minor components 15K, 45K and 116K were not as readily detectable and the other protein bands appeared weaker. In the absence of SDS, or when its concentration was only 0.1%, the virus was incompletely dissociated and no protein bands were observed except at the tops of the gels. However, increasing the concentrations of SDS, urea and reducing reagents did not increase the relative proportion of the 15K protein or alter the protein profile of TRSV (Fig. 8A, g and h). Similarly, addition of MCE and DTT to the polyacrylamide gels and electrophoresis buffer did not affect the protein profile of TRSV. Since the polypeptide profiles in gels b - e (Fig. 8A) which contain TRSV protein dissociated in the presence or absence of urea were similar, the possibility that the minor components were derived by carbamylation of the 60K protein by cyanate which could have been present in the unrecrystallised urea (Stark, 1965; Cole and Mecham, 1966) can be excluded. Other denaturants were also used instead of urea to dissociate the viral protein. When TRSV was dissociated with 6 or 12 M guanidinium chloride (GHC1) at pH 7.0, most of the protein became insoluble due to precipitation of the SDS by GHCl and they remained at the tops of the gels (Fig. 8A, i-1). There was also no difference when TRSV was dissociated in the presence of up to 90% formamide and electrophoresed in aqueous gels (Fig. 8B).

The possibility that TRSV protein subunits are aggregated by divalent cations and hydrogen bonds has been investigated as well. Although addition of the divalent cations, Ca^{++} and to a lesser extent Mg^{++} to the sample dissociation buffer induced aggregation of the TRSV protein components (especially the high molecular weight protein

Figure 8. Polypeptide profiles in discontinuous SDS-polyacrylamide gels of TRSV dissociated in 0.125 M Tris-HCl buffer, pH 6.8, in the presence of various reagents

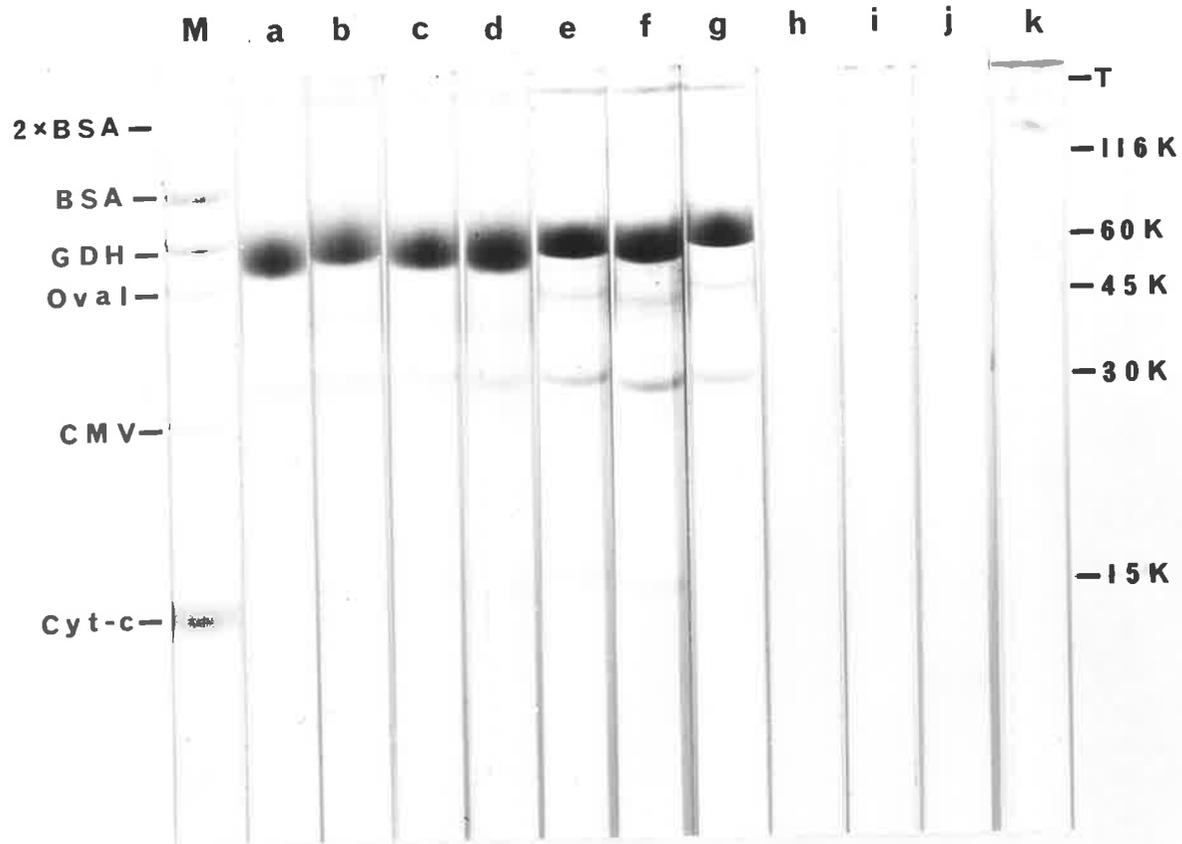
Approximately 100 μ g of TRSV were dissociated and loaded onto each gel.

A. Effects of urea, SDS, MCE, DTT and GHCl

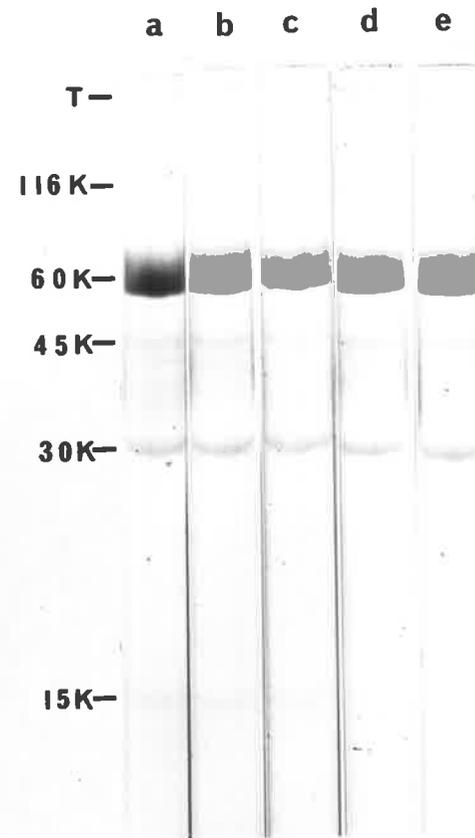
- Gel M, marker proteins dissociated by the standard method (see Materials and Methods);;
- gel a, TRSV dissociated in the presence of 1% SDS;
- gel b, 5% SDS;
- gel c, 1% SDS + 6 M urea;
- gel d, 1% SDS + 2 % MCE;
- gel e, 1% SDS + 6 M urea + 2% MCE (standard method);
- gel f, 1% SDS + 6 M urea + 2% MCE + 10 mM DTT;
- gel g, 5% SDS + 12 M urea + 10% MCE;
- gel h, 1% SDS + 6 M GHCl;
- gel i, 1% SDS + 6 M GHCl + 2% MCE;
- gel j, 1% SDS + 6 M GHCl + 2% MCE + 10 mM DTT;
- gel k, 5% SDS + 10% MCE + 12 M GHCl.

B. Effect of dissociation of TRSV in the presence of formamide

- Gel a, TRSV dissociated by the standard method;
- gel b, standard method + 10% formamide;
- gel c, standard method + 25% formamide;
- gel d, standard method + 50% formamide;
- gel e, standard method + 90% formamide.



(A)



(B)

components) in SDS-polyacrylamide gels (Fig. 9) addition of the chelating agents, EDTA and/or ethyleneglycol-bis-(β -aminoethyl ether) N, N'-tetraacetic acid (EGTA), and/or NaCl (1 M) did not cause further dissociation of the 60K component into the 15K component (Fig. 9).

In another experiment, TRSV preparations were dissociated in 6 M urea, 1% SDS and 2% MCE under different temperatures and pH conditions and for various time periods. When the virus was dissociated at pH 4.5, 7.0 or 9.6, similar protein profiles were observed whether the dissociation was done at 37°C for 3 hr, 60°C for 30 min, or 100°C for 2 min (Fig. 10A). Heating at 37°C and 100°C for prolonged periods failed to increase the amounts of the 15K, 30K or 45K but did cause the reduction of the T component and the formation of a number of new bands migrating between those of the 15K and 60K components (Fig. 10B and C). The fact that incubation at lower temperature (37°C, Fig. 10A) did not produce more of the minor low molecular weight components (15K, 30K and 45K) indicated that these proteins were not derived from the action of protease enzyme which could be present in the sample (Weber and Osborn, 1975). The results showed that although harsher dissociation conditions produced breaks in the polypeptide chains, it did not affect the relative proportions of the 15K, 30K and 45K protein components (Fig. 10B and C). Thus, it would appear that these components are not breakdown products of the 60K or other higher molecular weight proteins produced during dissociation with urea-SDS-MCE reagent. There is also no evidence to suggest that the higher molecular weight proteins are aggregates formed by over-heating as described by Wong *et al.* (1978). Thus all of the above results indicated that the 15K, 30K and 45K proteins are intact polypeptides derived from dissociation of the TRSV capsid but not necessarily from the 60K protein. Since conditions which encouraged dissociation of protein aggregates, especially those formed by hydrogen bonding, did not increase the amounts of these protein components, it may be concluded that the 60K protein cannot be further dissociated by urea-SDS-MCE reagent.

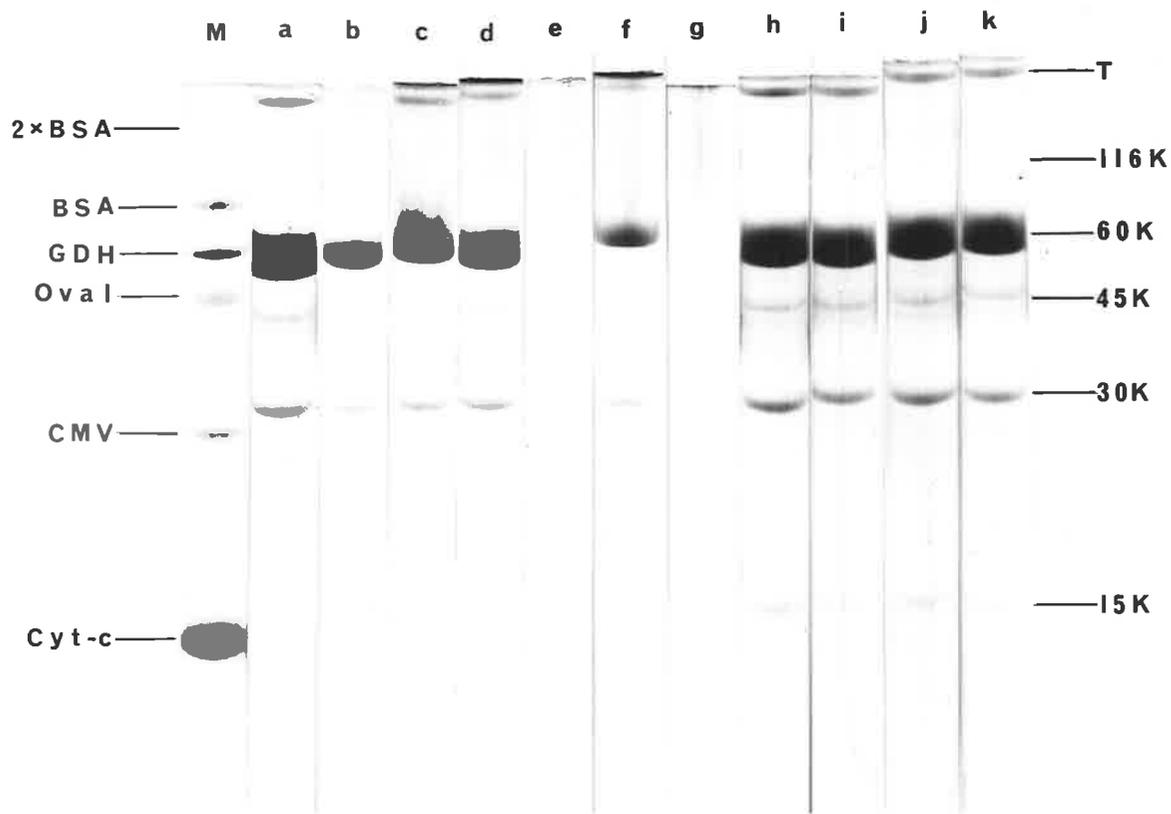
Figure 9. Effects of divalent ions, NaCl and chelating agents on the dissociation of TRSV in the presence of 1% SDS, 2% MCE and 6M urea

The results presented are from two separate experiments:

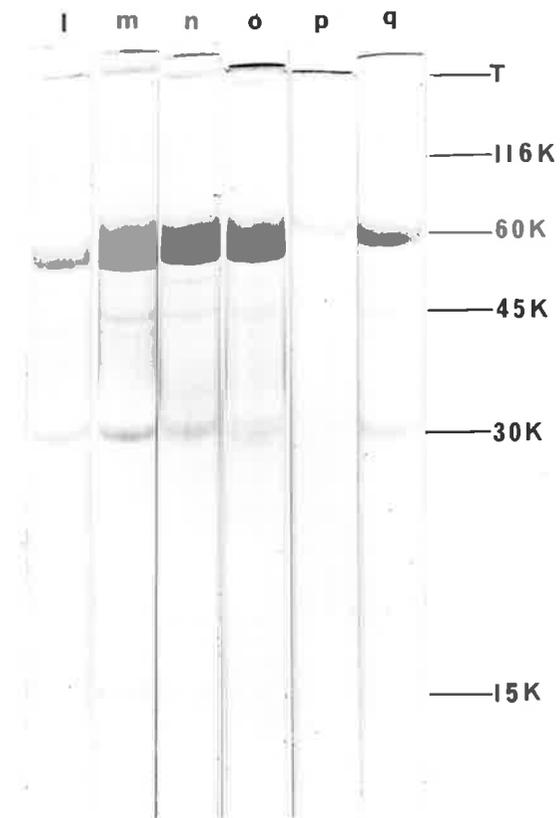
A (gels M, a - k); B (gels l - q).

In each experiment, TRSV samples (100 μ g) were mixed with the respective reagents and left for 1 hr before being dissociated by heating. On heating, precipitates were observed in samples b, c, e, g, l, p and q. The proteins were analysed by discontinuous SDS-PAGE.

Gel M, marker proteins dissociated by the standard method;
gel a, TRSV dissociated by the standard method;
gel b, + 50 mM MgCl_2 ;
gel c, + 100 mM MgCl_2 ;
gel d, + 1 M NaCl ;
gel e, + 100 mM CaCl_2 ;
gel f, + 10 mM EDTA and 1 M NaCl ;
gel g, + 100 mM MgCl_2 and 100 mM CaCl_2 ;
gel h, + 5 mM EDTA ;
gel i, + 10 mM EDTA ;
gel j, + 25 mM EDTA ;
gel k, + 50 mM EDTA ;
gel l, + 50 mM CaCl_2 ;
gel m, + 25 mM EGTA ;
gel n, + 50 mM EGTA ;
gel o, + 50 mM EDTA + 50 mM EGTA ;
gel p, + 50 mM EDTA + 50 mM EGTA + 1 M NaCl ;
gel q, + 50 mM CaCl_2 + 50 mM EGTA .



(A)



(B)

Figure 10. The effects of pH, time and temperature on the dissociation of TRSV into protein components separated by discontinuous polyacrylamide-gel electrophoresis

In each respective experiment (A - C), equal amounts of TRSV were dissociated and loaded on each gel.

Gel M, marker proteins dissociated under standard conditions (see Materials and Methods).

A. Effects of temperature and time of dissociation in 0.125 M Tris-HCl with varying pH.

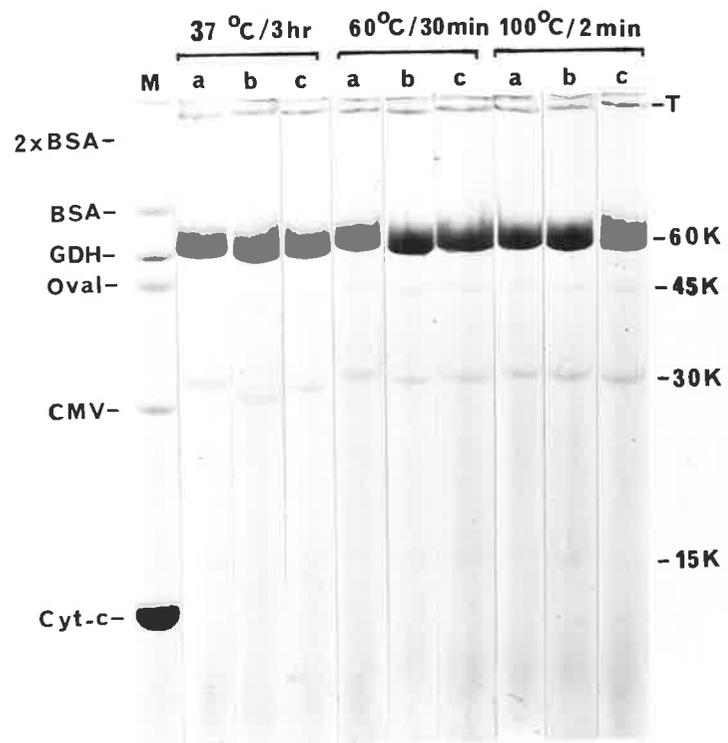
Gels a, dissociated at pH 4.5;

gels b, at pH 7.0;

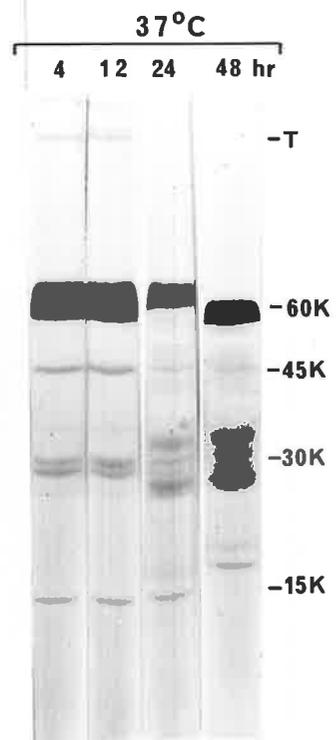
gels c, at pH 9.6.

B. Effect of increasing time of dissociation at 37°C in the standard dissociation medium (see Materials and Methods).

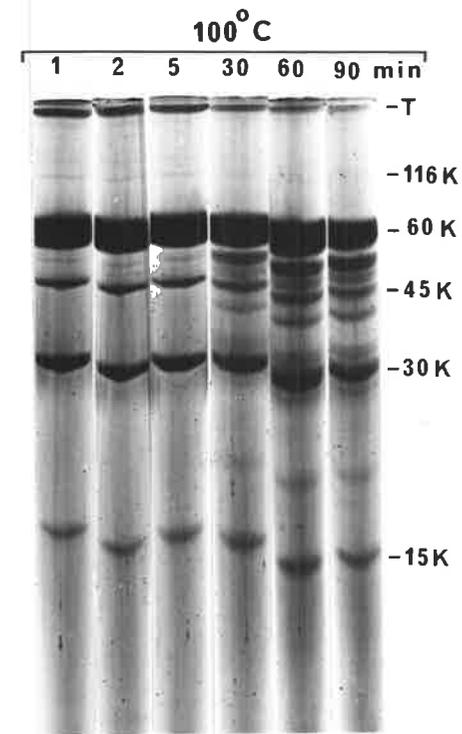
C. Effect of increasing time of dissociation at 100°C.



(A)



(B)



(C)

(b) Chemical Modification of TRSV Protein Prior to Electrophoresis in Polyacrylamide Gels

The possibility that the high molecular weight polypeptides of TRSV (30K, 45K, 60K, 116K and T) were aggregates of the 15K component formed by inter-molecular covalent bonds was investigated by analysis of the viral protein in polyacrylamide gels after they had been S-carboxymethylated, reduced with NaBH_4 or oxidised with performic acid. These treatments will normally destroy any disulphide bonds and may eliminate other cross-linking covalent bonds due to oxidative or reductive reactions present in the aggregated proteins (Hirs, 1967; Means and Feeney, 1971; Glazer, 1976). Analyses of the treated proteins showed that there was no significant difference in the protein profiles between the S-carboxymethylated and the un-carboxymethylated protein preparations (Fig. 11A), indicating the absence of inter-subunit disulphide bonds in the larger proteins. However, in the gel containing NaBH_4 reduced protein preparation all the six protein components of TRSV (Fig. 1) were absent but instead a diffused heterogenous protein band was observed around the 10,000 - 15,000 daltons region (Fig. 11B). Similarly, a series of stronger protein bands corresponding approximately to the 15K, 30K and 45K proteins together with a corresponding reduction in the relative proportions of the T, 116K and 60K protein components were observed in the gel containing performic acid oxidised protein preparation compared to the untreated protein (Fig. 11C). On the other hand, performic acid oxidised BTMV coat protein did produce several higher molecular weight components indicating aggregation of the polypeptide (Fig. 11C). Since performic acid oxidation and NaBH_4 reduction also produced other protein bands in TRSV besides the normal protein components, it seems unlikely that the 15K, 30K and 45K are merely degradation products of the other higher molecular weight protein components formed by oxidation-reduction reactions. On the other hand, these results could be interpreted to indicate that the higher molecular

Figure 11. Effects of chemical modification of TRSV protein on the protein components detected in discontinuous polyacrylamide gels (A and B) and continuous urea-SDS gels (C)

In each respective experiment comparable amounts of TRSV and TMV proteins were dissociated and loaded on each gel.

A. Effect of S-carboxymethylation

Gel M, protein markers dissociated by standard methods;

gel a, untreated TRSV dissociated under standard conditions;

gel b, TRSV dissociated after S-carboxymethylation.

B. Effect of NaBH₄ reduction

Gel M, protein markers dissociated by standard method;

gel a, untreated TRSV dissociated by standard method;

gel b, TRSV dissociated after reduction with NaBH₄.

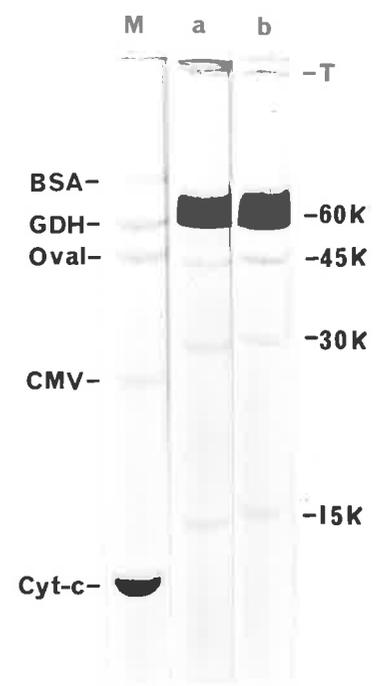
C. Effect of performic acid oxidation

Gel M, protein markers dissociated by standard method;

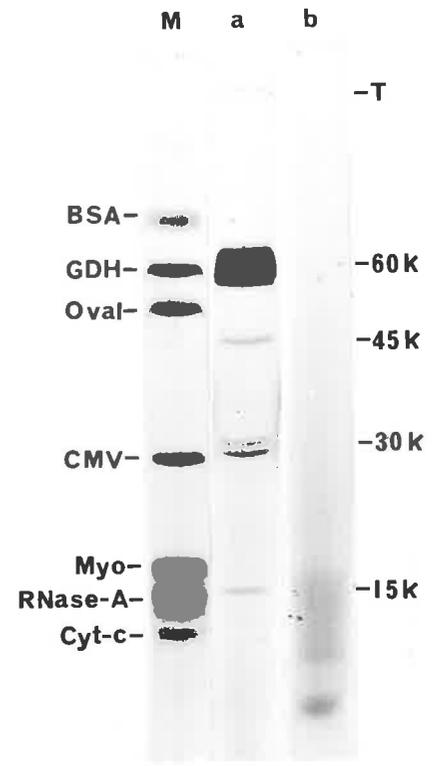
gels a, untreated TRSV and TMV coat proteins dissociated by standard method respectively;

gels b, TRSV and TMV coat proteins dissociated after performic acid oxidation respectively.

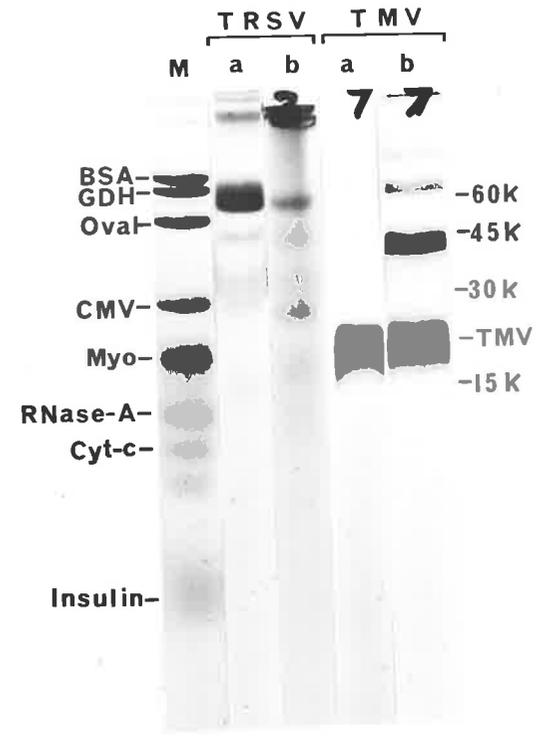
Position of TMV coat protein (TMV) is indicated.



(A)



(B)



(C)

weight protein components may be aggregates of the 15K polypeptide bonded by inter-molecular covalent bonds which may be formed from oxidation-reduction reactions.

B. Identity of the Polypeptide Components Associated with TRSV

From the data presented above, it appears that all the six polypeptides detected in preparations of TRSV (Fig. 1) are of viral origin and that none are degradation products of larger proteins. Thus two other possible explanations remained: (1) that the proteins are distinct but integral parts of TRSV capsid or (2) that they are aggregates of the 15K component. The results of performic acid and NaBH_4 treatments and the observation that the estimated molecular weights of these polypeptides approximates to multiples of that of the 15K component indicate that the TRSV capsid contains only one type of polypeptide (15K) and which formed aggregates yielding the 30K, 45K, 60K, 116K and T components. To confirm this, the following experiments were done to compare various properties of the six polypeptide components.

(i) Dissociation and Reassociation Properties of the Components

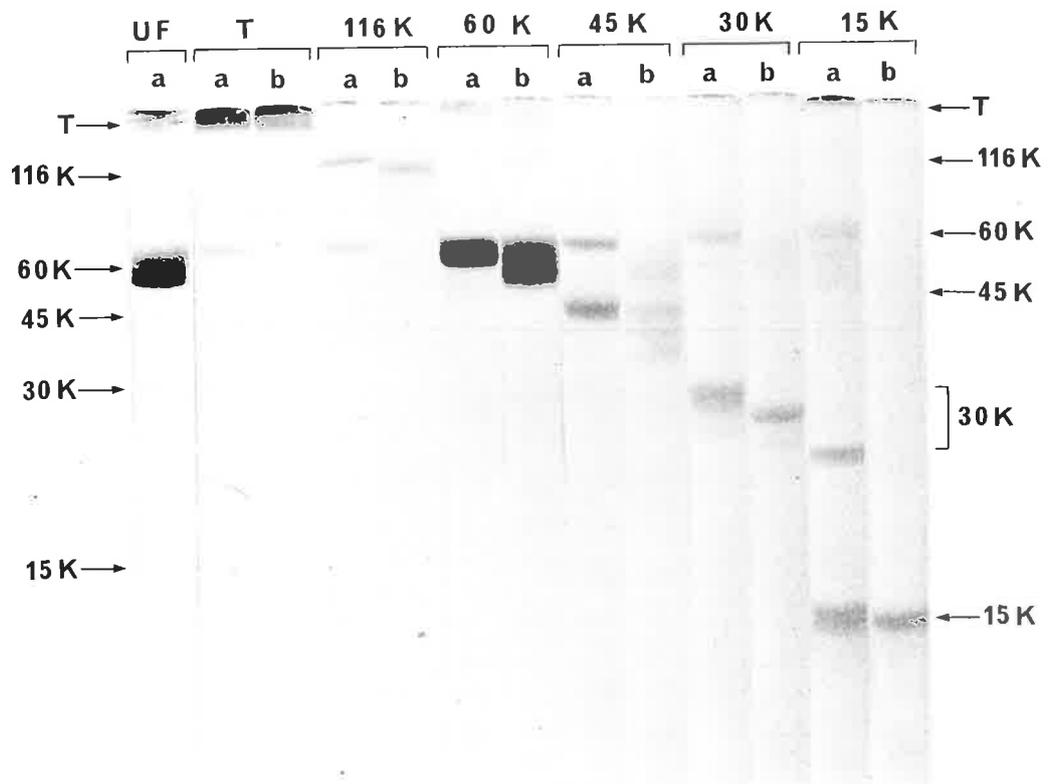
The six polypeptide components were isolated by preparative polyacrylamide gel electrophoresis and each sample was divided into two equal aliquots, a and b. To each of aliquots a, the standard dissociation reagents (urea, SDS and MCE) were added, heated and then subjected to polyacrylamide gel electrophoresis. The aliquots b were similarly electrophoresed except that the proteins were not heated or treated with the dissociation reagents. The results of the experiments, summarised in Fig. 12, indicate that the isolated T and 116K components produced significant amounts of 60K component when they were redissociated prior to re-electrophoresis but not when they were not redissociated. In preparations of isolated 60K component, no readily detectable amounts of other components were observed on either redissociated or untreated materials, but the 60K protein band was more homogenous when the sample was redissociated (Fig. 12). However,

Figure 12. Discontinuous polyacrylamide gel re-electrophoresis of TRSV protein components isolated by preparative gel electrophoresis (see Materials and Methods)

Gels a, components were re-electrophoresed after re-dissociation by the standard method;

gels b, components were re-electrophoresed after being dissolved in 0.125 M Tris-HCl buffer, pH 6.8, only;

gel UF, unfractionated TRSV protein dissociated by the standard method.



redissociation of the isolated 45K, 30K and 15K components also caused the appearance of significant amounts of 60K material which was absent in the untreated samples (Fig. 12). In the case of the isolated 15K component, some 30K component was also detected which was also absent if the protein was not redissociated. Thus in all the isolated protein components, treatment with dissociation reagents produced the 60K component. These results support the view that the five slower-migrating protein components are aggregates of the 15K component and also indicate that dissociation of TRSV protein with the urea-SDS-MCE reagent leads to, and favours the formation of the 60K component. Since equal amounts of proteins were present in the redissociated and untreated samples it appears that only part of the protein loaded was detected in the gels when it was not redissociated (Fig. 12). This suggests that in the absence of urea-SDS-MCE reagent some of the isolated proteins had reaggregated to form high molecular weight polymers which did not migrate into the gels. It seems also that the various isolated protein components are relatively stable since only a small proportion of each component is changed to the 60K component compared to the relative proportion of the 60K component in the unfractionated protein. Thus it appears that the 60K component produced by each of the isolated protein components was not formed directly from the various isolated protein components but formed by redissociation of the reaggregated high molecular weight protein mentioned above. This is supported by the previous results which showed that no change in the relative proportions of the various protein components was observed when TRSV was dissociated for extended periods in urea-SDS-MCE reagents (Fig. 10B and C).

(ii) Antigenic Properties of the Components

Anti-TRSV and anti-60K protein sera were tested against preparations of the six polypeptide components of TRSV recovered from gels and against the intact virus by immuno-diffusion assays. The experiments, illustrated in Fig. 13, showed that a strong confluent precipitin line (m) was formed between the anti-60K protein serum (p)

Figure 13. Serological reactions in agar-gel between the antisera prepared against intact TRSV (v) and isolated 60K protein component (p) and the TRSV protein components isolated by preparative gel-electrophoresis (T, 116K, 60K, 45K, 30K and 15K) and a preparation of intact TRSV(V)

Ten μ l aliquots of TRSV protein components and intact TRSV (0.2 mg/ml) were loaded onto the respective wells. The antisera were diluted 4 folds and 10 μ l samples were also loaded. The plate was incubated at 25^oC for 48 hr. Three main precipitation lines, middle (m), outer (o) and inner (i) were detected.

and all of the six polypeptides but no reaction with the intact virus (V) was detected. In addition to line m, two fainter lines were also observed (o and i, Fig. 13) between the anti-60K serum and some of the polypeptide components antigens. Line o was observed between wells containing anti-60K serum and those containing the polypeptide component antigens although it was strongest with the 60K, and 30K components (Fig. 13). Line i was observed only in wells containing the 116K, 45K, 30K and 15K components antigens but was produced in reactions with both the anti-60K and anti-TRSV (v) sera (Fig. 13). The anti-TRSV serum formed a strong precipitin line (capsid-specific precipitin line) against its homologous antigen (V) which at first appeared to be confluent with line i; however, subsequent tests demonstrated that all the precipitin lines represent distinct antigen-antibody reactions (Fig. 14). The titres of the various TRSV antigens with respect to each precipitin line produced when reacted against the anti-60K and anti-TRSV sera are shown in Table 3. It is interesting to note that with the undiluted antisera, only the 15K and 45K protein components also reacted with the virus antiserum in the same way as they reacted with the 60K protein antiserum.

These observations indicated that all of the six polypeptide components of TRSV protein are antigenically similar, although there appear to be some antigenic sites on some of the components which are absent on others. Furthermore, it appears that the intact virus and the isolated protein components contained different antigenic properties.

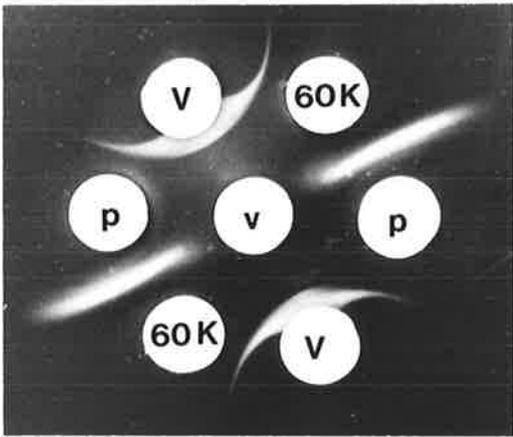
(iii) Amino Acid Composition

Although greater amounts of the various TRSV protein components could be prepared by sucrose density gradient centrifugation, this method was less satisfactory than the polyacrylamide gel electrophoresis method due to three reasons: (a) The polypeptide components prepared were not totally free from contamination by other components; (b) the T, 116K and 45K components could not be readily isolated by this method and (c) most of the protein aggregated into high polymeric form during

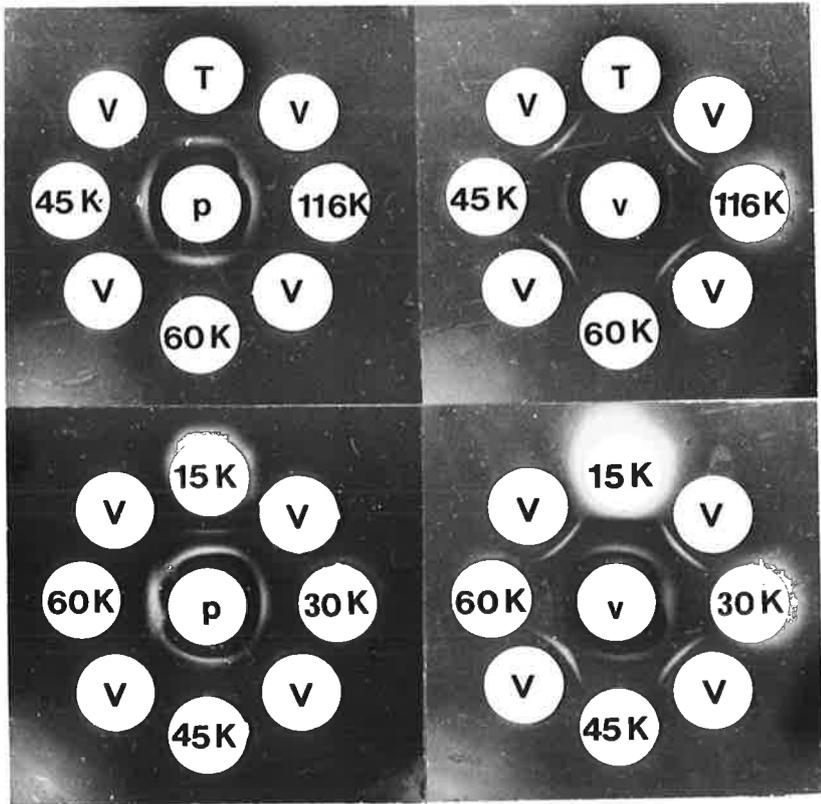
Figure 14. Serological properties of intact TRSV (V) and its protein components (T, 116K, 60K, 45K, 30K, 15K)

The antigens and antisera were loaded as described in Figure 13.

- A. Serological reactions between the antisera prepared against the intact TRSV (v) and isolated 60K protein component (p) and their respective homologous antigens. The agar plates were incubated at 25°C for 24 hr.
- B. Distinct serological reactions observed between the antisera prepared against intact TRSV (v) and isolated 60K protein component (p) and the TRSV antigens (Intact TRSV (V) and the viral protein components).
The agar plates were incubated at 25°C for 24 hr.



(A)



(B)

Table 3

Serological reactions of TRSV antigens with antisera prepared against TRSV 60K polypeptide component and intact TRSV particles

TRSV Antigen ^d	Anti-60K Serum ^b				Anti-TRSV Serum ^c			
	i	m	o	Capsid Specific	i	m	o	Capsid Specific
T	- ^e	16	4	-	-	-	-	-
116K	16	8	4	-	8	-	-	-
60K	-	16	8	-	-	-	-	-
45K	64	8	4	-	64	2	-	-
30K	64	8	8	-	32	-	-	-
15K	64	8	4	-	32	2	2	-
Intact virus	-	-	-	-	-	-	-	516

^a The titres were expressed as the reciprocal of the maximum dilution of the antiserum producing a visible precipitation line in gel-diffusion tests. Titres were recorded after incubating the immuno-diffusion plates at 25°C for 3 and 5 days and the values obtained were identical for these two incubation times.

^b Antiserum produced in response to isolated TRSV 60K polypeptide component purified by polyacrylamide gel electrophoresis.

^c Antiserum produced in response to purified intact TRSV.

^d The antigens (0.2 mg/ml) were diluted with their respective buffers and applied as described in Figure 13 and Materials and Methods.

^e Dash indicates that no reaction was detected when the antigen was tested against the undiluted antiserum.

The positions of the i, m, o and capsid specific precipitation lines produced as a result of the antigen-antiserum reactions are shown in Figure 13.

centrifugation. On the other hand, because of difficulties in obtaining sufficient quantities of the minor components from gels, only one hydrolysis time of 24 hr could be done with protein preparations isolated by this method. Thus partial amino acid compositions of one preparation of each of the protein components isolated from the same virus preparation by both the sucrose density gradient centrifugation method as well as by the preparative polyacrylamide gel electrophoresis method were determined. The results (Table 4) show that the amino acid compositions of the various TRSV protein components are very similar to that of the unfractionated protein, whether they were isolated by sucrose density gradient centrifugation or polyacrylamide gel electrophoresis. Statistical analysis using the t-test indicates that only a few of the residues appeared to be significantly different (Table 4).

(iv) Cyanogen Bromide Cleavage Products

After reaction with CNBr which converts the methionine residues into C-terminal homoserine lactone residues (Lehninger, 1975), all the TRSV polypeptide components (except the 15K component) isolated from gels produced similar sized peptide fragments when they were analysed in urea-SDS-polyacrylamide gels (Fig. 15). Five major CNBr peptide bands (CB1-5) were detected in the T, 116K, 60K, 45K and 30K protein preparations while only 2 of these bands (CB4 and CB5) were observed in the 15K component. These 2 CNBr fragments found in the 15K component were consistently observed in the CNBr cleavage products of all the other TRSV proteins and the results indicate that they may represent the actual CNBr cleavage products of the TRSV coat protein subunit (see section III C (ii) for further details). Similar results were observed whether the proteins were digested at 25°C or 40°C, the latter of which should give up to 96% conversion of methionyl residues (Kraal, 1972).

(v) Tryptic Peptide Analysis

Due to difficulties in obtaining sufficient quantities of the highly purified TRSV protein components from polyacrylamide gels, only

Table 4

Relative partial amino acid compositions of TRSV polypeptide components

Amino ^a Acid	Relative Molar Ratios of Amino Acids in Components ^b									
	15K		30K		45K	60K		116K	T	Unfrac- tionated ^c
	I	II	I	II	I	I	II	I	I	
Asp	11.7	10.2	11.1	10.9	10.5	10.1	10.3	11.5	9.9	10.7±0.46
Thr	7.4	8.8	7.7	8.9	8.3	8.5	9.4	7.6	8.8	8.7±0.40
Ser	11.1	9.5	10.9	9.4	10.5	8.9	9.1	11.7*	9.5	9.3±0.55
Glu	11.6*	8.2	12.0*	9.4	9.5	8.6	8.3	11.4*	9.1	8.9±0.54
Pro	4.6*	6.4	6.0	7.3	7.4	8.3	8.1	6.2	5.4*	7.1±0.48
Ala	9.4*	10.8	10.1	10.3	10.0	10.6	10.0	9.2*	10.5	10.7±0.28
Val	8.4	9.3	9.0	8.4	8.3	7.6	7.6	7.0	8.6	8.0±0.53
Met	1.7	1.9	1.4	2.4	1.5	2.0	2.8	1.3	2.1	2.0±0.62
Ile	7.6	7.1	6.5	6.5	7.6	7.7	7.1	6.9	8.0	7.3±0.26
Leu	8.5	8.8	8.4	8.6	8.5	8.6	8.6	8.4	8.6	9.0±0.15
Tyr	4.7	4.4	3.8	4.4	4.0	3.8	3.9	4.1	4.2	4.0±0.20
Phe	4.7	5.3	4.7	5.9	5.3	6.0	6.1	4.8	5.7	6.1±0.40
Lys	5.9	5.7	5.8	4.6	5.7	5.9	4.8	5.5	6.1*	5.2±0.23
His	2.5	2.7	3.0	2.9	3.7	3.9	3.9	3.5	3.8	3.2±0.36
Arg	6.3*	6.1*	5.3	5.3	4.7	5.0	5.2	6.1*	5.3	5.1±0.23

^a Cys and Trp were not determined because of insufficient material; Gly was not included because samples were contaminated with this amino acid from the Tris-glycine buffer used in the fractionation procedure.

^b Values calculated assuming that the polypeptides all contain 119 amino acids (see Table 5).

^c Results taken from data in Table 5 (IDI value of 0.23), and the standard errors were calculated from the 8 separate analyses described in Table 5.

I Components recovered from polyacrylamide gels after electrophoretic separation as described under Materials and Methods. Due to insufficient materials, the results were based on analysis of one preparation of each component isolated from the same virus preparation hydrolysed for 24 hr only. Hydrolysates were then analysed in duplicate. Val was corrected assuming 85% recovery and Ile assuming 80% recovery. Both Thr and Ser were corrected assuming 95% recovery.

II Components isolated by the sucrose density gradient centrifugation method as described in Materials and Methods. The 45K, 116K and T components could not be fractionated by this method. The results were based on

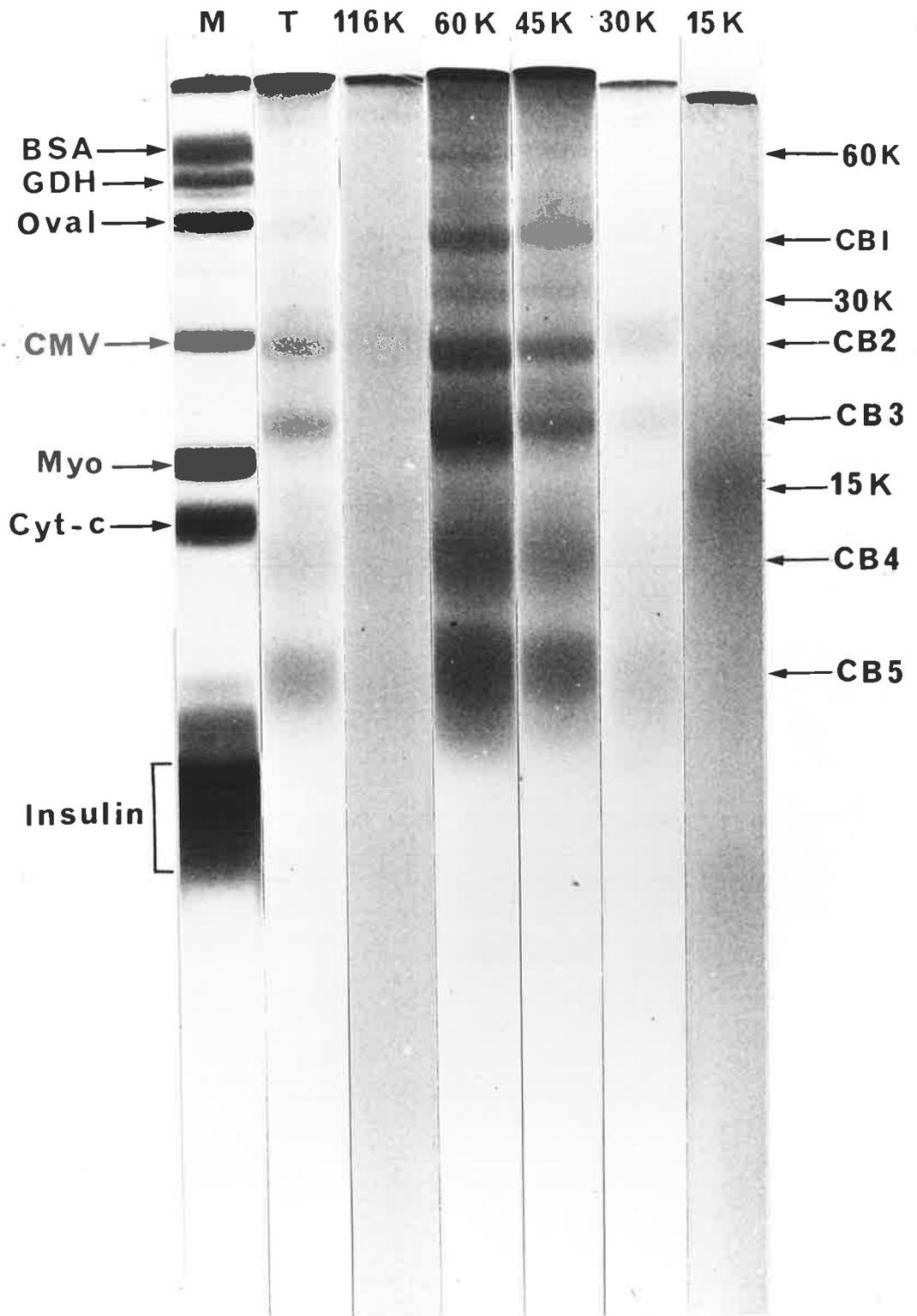
Table 4 cont'd.

analysis of one preparation of each component isolated from the same virus preparation hydrolysed for 24, 48 and 72 hr and each hydrolysate analysed in duplicate. The values presented were calculated as described in Table 5.

* Amino acid contents significantly different from the corresponding values in the unfractionated TRSV protein at 99% confidence limit (99% $t_7 = \pm 3.50$) but not at 99.9% confidence limit (99.9% $t_7 = \pm 5.41$) using the t-test for significance of difference.

The rest are not significantly different at 99% confidence limit.

Figure 15. Analysis of the CNBr fragments of the TRSV protein components (T, 116K, 60K, 45K, 30K and 15K) isolated by preparative gel electrophoresis in 16% continuous urea-SDS polyacrylamide gels. Due to insufficient materials in some of the minor components, varying amounts of CNBr fragments of each component were loaded. Gel M, protein markers. The 5 main CNBr fragments detected (CB1-5) are indicated on the right.



the 15K, 30K and 60K components separated by sucrose density gradient centrifugation were analysed. These protein components, relatively free from cross-contamination with the other proteins, were subjected to trypsin digestion and the peptides were compared with those of the unfractionated TRSV protein. Although lack of sufficient materials precluded two-dimensional separation of the peptides, the results obtained by separating the peptides of the different protein preparations simultaneously in one dimension on thin layers of cellulose (Fig. 16) indicate that the peptide maps of the three components are similar to those of the unfractionated protein, whether stained for total peptides (Fig. 16A); peptides containing histidine and tyrosine (Fig. 16B), or peptides containing arginine (Fig. 16C). On the other hand, tryptic peptides of U-1 TMV coat protein separated under the same conditions produced entirely different maps when stained with the phenanthrene-quinone reagent (Fig. 17A) or the Pauly reagent (Fig. 17B).

C. Estimation of the Molecular Weight of the Chemical Subunit of TRSV Coat Protein

All the results of the previous experiments indicate that the six polypeptide components from TRSV protein separated by polyacrylamide gel electrophoresis are all intact polypeptides forming part of the viral capsid and are chemically and antigenically similar. Thus it is concluded that the TRSV capsid is formed from one type of polypeptide subunit represented by the 15K protein and with a molecular weight of approximately 14,000 (Table 1), while the other components are a series of aggregates of this polypeptide subunit. To provide further evidence for this conclusion and to obtain a more accurate estimate of the molecular weight of the TRSV polypeptide subunit, the unfractionated TRSV protein was subjected to detailed amino acid analysis, CNBr cleavage and 2-dimensional tryptic peptide mapping.

Figure 16. Separation and detection of tryptic peptides of TRSV protein components isolated by sucrose density-gradient centrifugation (15K, 30K, 60K) and unfractionated TRSV protein (UF) by ascending chromatography on thin layers of cellulose. A. Chromatograms were stained with ninhydrin to detect all peptides. B. Stained with the Pauly reagent to detect peptides containing histidine and tyrosine. C. Stained with the phenanthrenequinone reagent to detect peptides containing arginine.

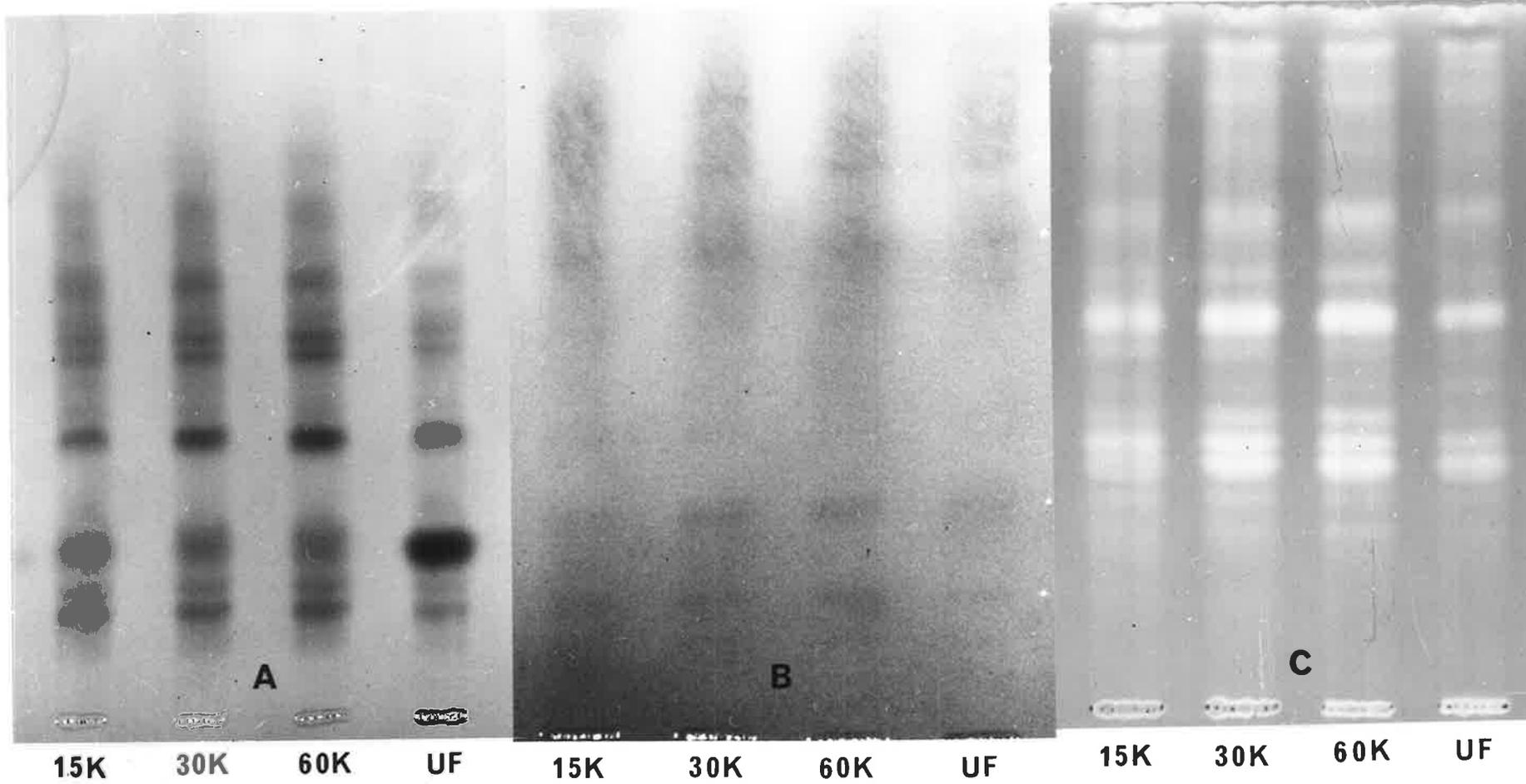


Figure 17. Analysis of the tryptic peptides of U-1 TMV and unfractionated TRSV coat proteins by ascending chromatography on thin layers of cellulose.

- A. Chromatogram was stained with phenanthrenequinone reagent to detect for peptides containing arginine using an arginine sample as control. The positive spots were indicated as circled areas.
- Track a, arginine control;
track b, U-1 TMV protein tryptic peptides;
track c, unfractionated TRSV protein tryptic peptides.
- B. Chromatogram was stained with the Pauly reagent to detect for histidine (His) and tyrosine (Tyr) containing peptides, using a histidine sample as control. Positive spots are indicated by arrows and the proteins on which they were detected are also indicated. As expected TMV protein did not give any positive histidine spots. The marker dyes used (O and G) are also indicated.
- Track a, histidine control;
tracks b and c, as above.

(i) FITMOL Analysis of TRSV Coat Protein Amino Acid Composition

The complete amino acid composition of TRSV coat protein (molar ratio) was calculated from analysis of several protein preparations and the results (Table 5) were subjected to the FITMOL analysis (Gibbs and McIntyre, 1970). After FITMOL analysis, a curve with minimum integer deviate indices (IDI) values corresponding to polypeptides with 65, 119 or 180 amino acids (Fig. 18) was obtained. Such polypeptides would have molecular weights of 7,127; 12,988 and 19,680, respectively and their expected amino acid compositions are also presented in Table 5. The FITMOL estimate of 12,988 (Table 5) is more consistent with the monomer molecular weight of 14,000 estimated by polyacrylamide gel electrophoresis (Table 1). If 12,988 is the correct molecular weight for the monomer and if the five protein components (15K, 30K, 45K, 60K and 116K) resolved by polyacrylamide gel electrophoresis (Fig. 1 and Table 1) represent monomer and aggregates of two, three, four and eight identical polypeptides respectively, we would expect these components to have molecular weights of about 13,000, 26,000, 39,000, 52,000 and 104,000 respectively. These values are not greatly different from the corresponding values estimated by polyacrylamide gel electrophoresis (Table 1).

(ii) Cyanogen Bromide Cleavage Analysis

The results of CNBr cleavage analysis of isolated TRSV coat protein showed that a total of 5 main CNBr peptide bands (CB1-CB5) were observed when the cleavage products were analysed in urea-SDS polyacrylamide gels (Fig. 19A). The estimated molecular weights of these cleavage products are about 26,500 (CB1), 20,000 (CB2), 15,500 (CB3), 8,200 (CB4), and 6,400 (CB5) (Fig. 20). Similar results were obtained when intact virus particles were digested for various times with CNBr (Fig. 19B). The total molecular weight of the 5 main CNBr peptides of TRSV protein is about 75,000 although amino acid analysis results (Table 4) indicate that a polypeptide with 4 methionine residues (= 5 CNBr cleavage peptides) should only have a molecular weight of

Table 5

Amino acid composition of TRSV coat protein

Amino Acid	Amounts of Amino Acids Recovered (μ moles) ^a				FITMOL IDI ^f					
	24 hr	48 hr	72 hr	Value Taken	0.33		0.23		0.27	
Cys ^b	0.0320	0.0282	0.0312	0.0305	1.05	1	1.92	2	2.91	3
Asp	0.1703	0.1691	0.1705	0.1700	5.85	6	10.71	11	16.21	16
Thr ^c	0.1328	0.1249	0.1210	0.1380	4.75	5	8.70	9	13.16	13
Ser ^c	0.1375	0.1235	0.1140	0.1480	5.10	5	9.33	9	14.11	14
Glu	0.1400	0.1414	0.1415	0.1410	4.85	5	8.89	9	13.44	13
Pro	0.1104	0.1126	0.1125	0.1118	3.85	4	7.05	7	10.66	11
Gly	0.1533	0.1554	0.1560	0.1549	5.33	5	9.76	10	14.77	15
Ala	0.1719	0.1678	0.1703	0.1700	5.85	6	10.71	11	16.21	16
Val ^d	0.1106	0.1251	0.1273	0.1273	4.38	4	8.02	8	12.14	12
Met	0.0328	0.0330	0.0309	0.0322	1.11	1	2.03	2	3.07	3
Ile ^d	0.1055	0.1136	0.1154	0.1154	3.97	4	7.27	7	11.00	11
Leu	0.1425	0.1408	0.1434	0.1422	4.90	5	8.96	9	13.56	14
Tyr ^c	0.0628	0.0629	0.0618	0.0630	2.17	2	3.97	4	6.01	6
Phe	0.0970	0.0970	0.0971	0.0970	3.34	3	6.11	6	9.25	9
Lys	0.0823	0.0830	0.0847	0.0833	2.87	3	5.25	5	7.94	8
His	0.0501	0.0513	0.0525	0.0513	1.77	2	3.23	3	4.89	5
Arg	0.0796	0.0810	0.0814	0.0807	2.78	3	5.09	5	7.69	8
Trp ^e	-	-	-	0.0315	1.08	1	1.99	2	3.00	3
Total number of amino acids					65		119		180	
Calculated molecular weight					7,127		12,988		19,680	

^a Values calculated from analyses of 8 separate protein preparations (except Cys and Trp) each hydrolyzed for the three different times and each hydrolysate analysed in duplicate as described in Materials and Methods.

^b Determined on three performic acid oxidised protein preparations.

^c Extrapolated to zero hydrolysis time.

^d Values obtained from the 72 hr hydrolysis time were taken as correct.

^e Determined spectrophotometrically from 4 analyses of 2 different protein preparations.

^f See Figure 18 for details of analysis.

Figure 18. FITMOL analysis of the TRSV amino acid composition data (values taken) presented in Table 5.

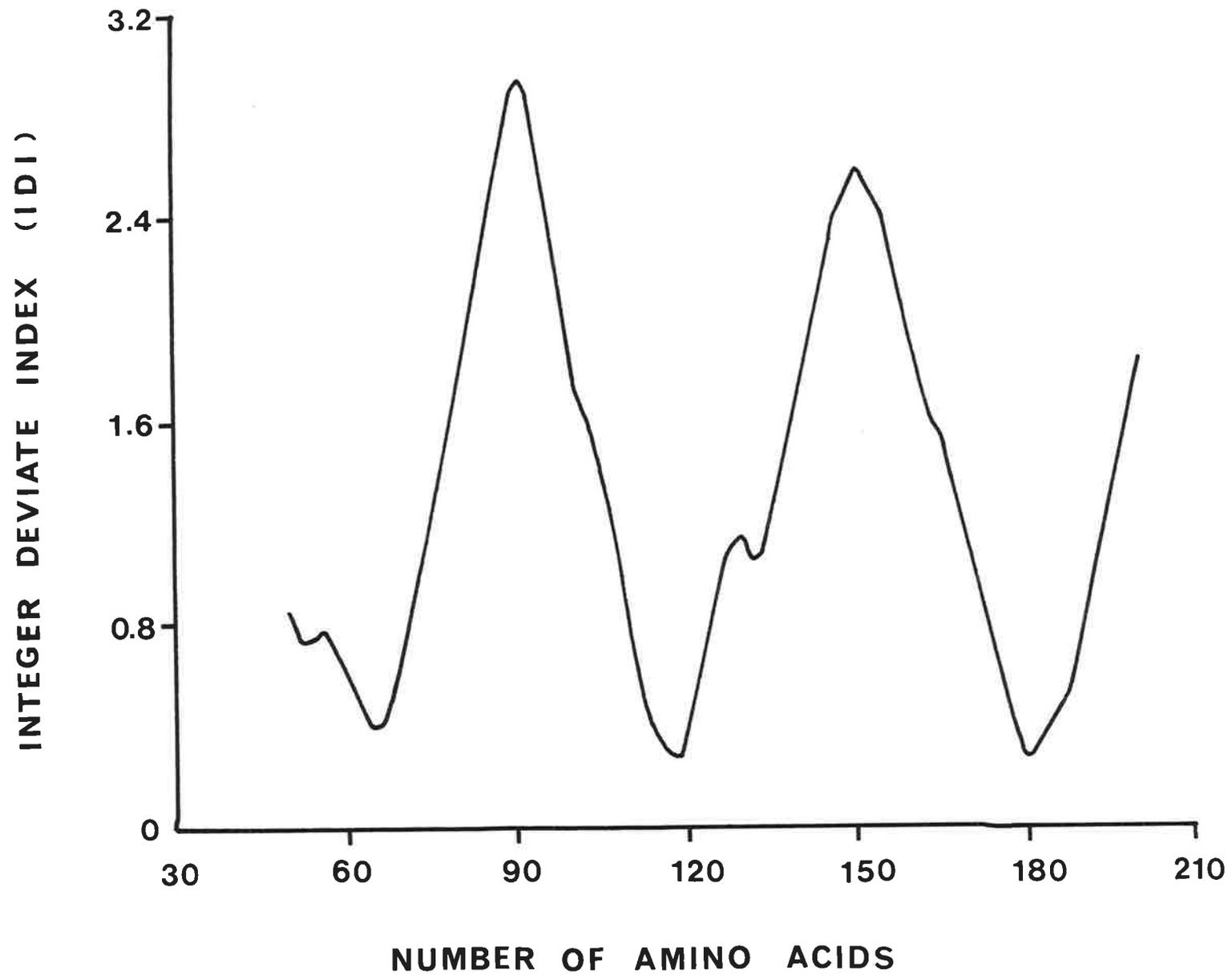


Figure 19. Analysis of the cyanogen bromide fragments of various proteins by continuous urea-SDS polyacrylamide gel electrophoresis

- A. Analysis of the CNBr fragments of isolated TRSV protein digested for various times in CNBr using 16% gels (see Materials and Methods).
- B. Analysis of the CNBr fragments of intact TRSV digested for various times in CNBr using 16% gels.
- C. Analysis of the CNBr fragments of myoglobin (Myo) digested for the various times indicated in 16% gels.
- D. Analysis of the CNBr fragments of QCMV (CMV) and BTMV protein (TMV) digested under standard conditions in 18% gels.

In all the experiments above,

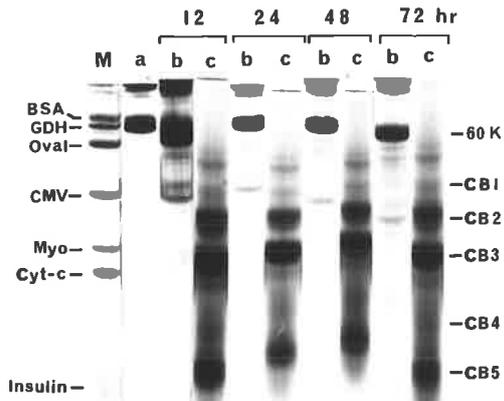
gels M, marker proteins;

gels a, untreated protein (75 $\mu\text{g}/\text{sample}$);

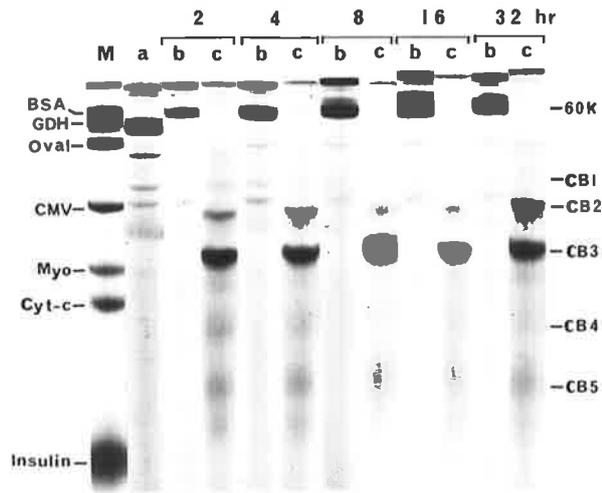
gels b, protein treated in 70% formic acid only
(75 $\mu\text{g}/\text{sample}$);

gels c, protein treated in 2% CNBr + 70% formic acid
(150 $\mu\text{g}/\text{sample}$).

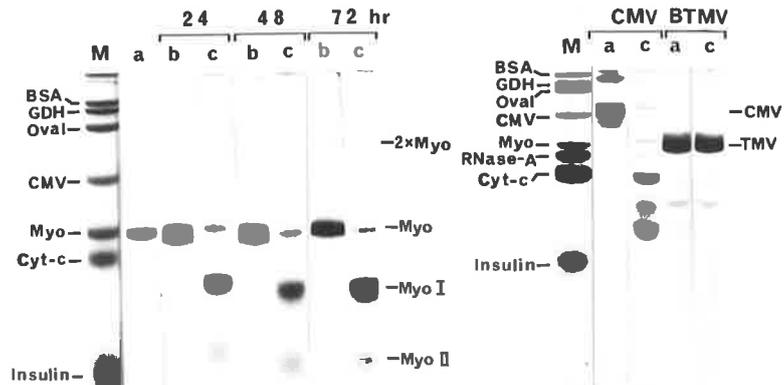
The identities of the TRSV protein and myoglobin CNBr fragments (CB1-5 and Myo I and II respectively) are indicated on the right.



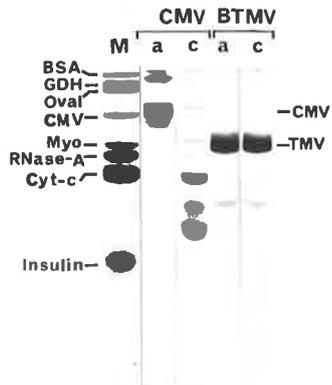
(A)



(B)



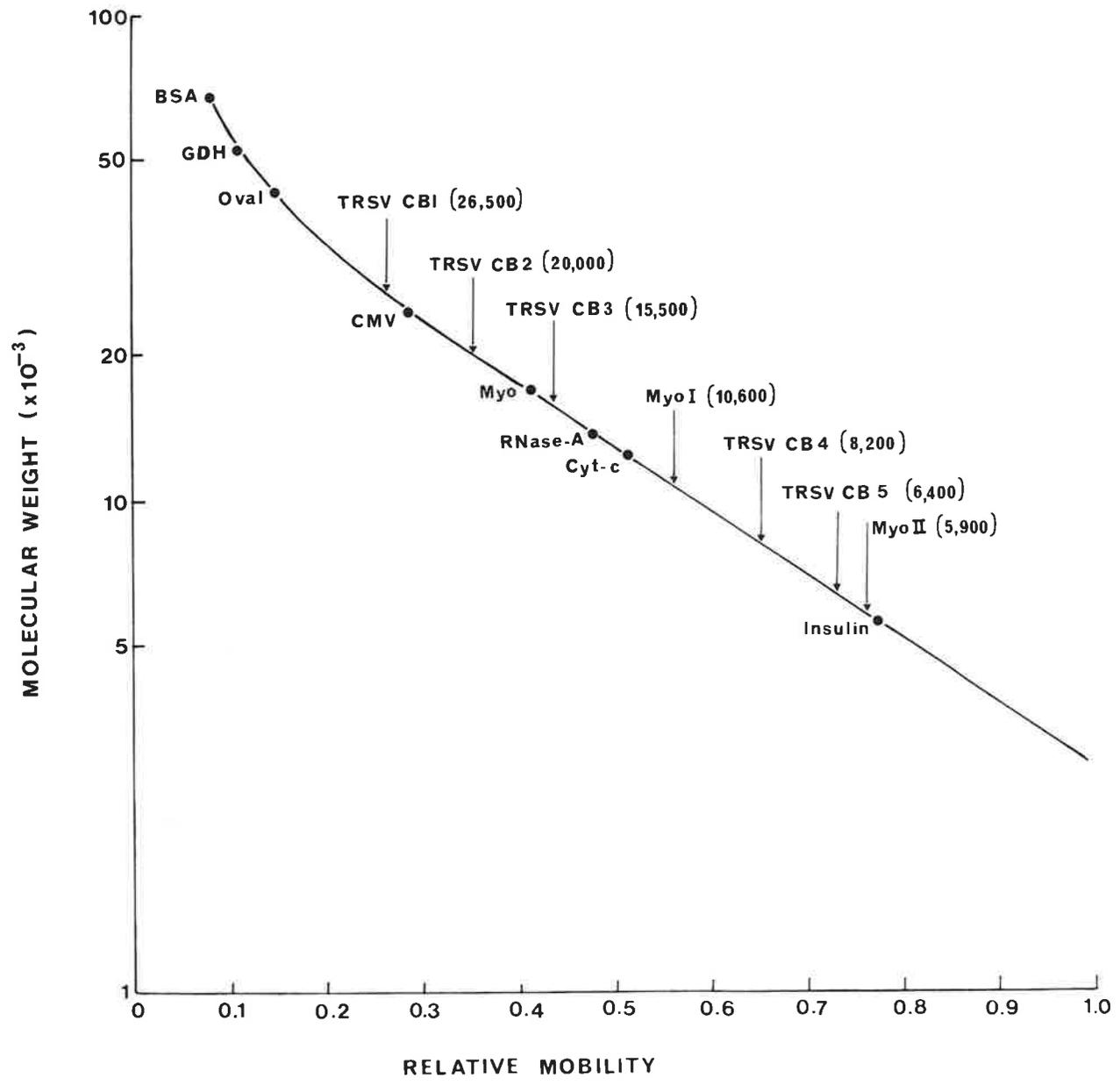
(C)



(D)

Figure 20. Estimation of the molecular weights of the CNBr fragments of TRSV protein and myoglobin

Protein markers and the CNBr fragments were electrophoresed separately in 16% urea-SDS gels. Relative mobilities were measured with respect to the bromophenol blue marker, and were calculated based on 4 replicate gels for each protein. The estimated molecular weights of the CNBr fragments of TRSV protein (CB1-5) and myoglobin (Myo I and II) are indicated.



about 26,000. On the other hand, a polypeptide of 54,000 molecular weight (60K component) would have 9 CNBr peptides.

Under the same experimental conditions, myoglobin, which contains two methionine residues per molecule (Dautrevaux *et al.*, 1969), produced 2 CNBr cleavage products from a maximum of 3 expected from its methionine contents (Fig. 19C). However, the molecular weights of the myoglobin CNBr fragments are 10,600 (Myo I) and 5,900 (Myo II) (Fig. 20) which were as expected from the positions of the respective methionine residues in the polypeptide chain (Dautrevaux *et al.*, 1969). BTMV coat protein, which contains no methionine residues (Rees and Short, 1975), remained uncleaved (Fig. 19D), while QCMV coat protein, more CNBr peptide bands than expected from its methionine contents (Habibi and Francki, 1974) were also observed and the total molecular weight of all the main CNBr peptides was also more than that of the intact protein molecule (Fig. 19D). Thus it seems that the results of CNBr cleavage experiments do not always provide conclusive evidence on the number of methionine residues.

With TRSV coat protein the results are probably complicated by the presence of several stable polypeptide aggregates in the protein. The molecular weights of the TRSV CB4 and CB5 CNBr fragments added up to 13,500 (approximately equal to the Mol. Wt. of the 15K component) and they are the only main cleavage products of the 15K component (Fig. 15). Since the 15K protein component is the basic subunit of the other protein components, it is concluded that CB4 and CB5 are the only complete CNBr cleavage products detected in TRSV coat protein. The indication that there are only two CNBr cleavage products instead of a possible three expected from the amino acid composition may be due to one of the methionine residues remaining uncleaved or to its product being so small as not to be retained by the gels.

Efficient cleavage of the polypeptide chain probably depends on the susceptibility of the methionine residue to cleavage which in turn

is dependent on its exposure to CNBr reagent and interference by neighbouring amino acid residues, e.g. Met-Ser and Met-Thr may not be cleaved (Gross, 1967). The higher molecular weight CNBr fragments (CB1-3) are probably partially cleaved products caused by either (i) CNBr not cleaving at some Met residues in the protein aggregates or (ii) CNBr may cleave at Met but does not cleave the bonds responsible for aggregating the 15K component into the 60K component. These possibilities are supported by the observation that even after digestion for 48 hr with CNBr some uncleaved and partially cleaved myoglobin still remained (Fig. 19C) and that the CNBr fragments CB1-3 are only observed in the higher molecular weight components of the TRSV protein (Fig. 15). Furthermore, since TRSV protein is susceptible to cleavage by acids, some of the minor CNBr cleavage products observed in the gels (Fig. 19) are probably due to cleavage by the formic acid used as solvent in the reaction. Results of the CNBr cleavage experiment described above are incompatible with the possibility that the polypeptide subunit of TRSV coat protein is the 60K component but are compatible with the suggestion that the viral capsid is built from only one type of polypeptide with a molecular weight of approximately 13,000 (Table 5).

(iii) Analysis of Tryptic Peptides

The tryptic peptide mapping and specific amino acid staining techniques used for analysing TRSV protein have been tested using U-1 TMV protein as a control and the results obtained with the latter (Fig. 21) agree very well with the published results (Knowland, 1974). Unfractionated TRSV protein was digested with trypsin for 4, 10, 20, 40 or 70 hr and the peptides were separated in two dimensions. There are very little differences between the peptide maps obtained with these digests after they have been stained with ninhydrin to detect all the peptides (Table 6). All subsequent protein preparations were digested for 20 hr and the maps obtained were very reproducible. A representative two dimensional tryptic peptide map of TRSV protein

Figure 21. Two dimensional tryptic peptide map of U-1 TMV coat protein digested for 10 hr under the same conditions as TRSV coat protein

The chromatogram was prepared and stained with ninhydrin as described in Materials and Methods. (Similar maps to this were obtained for 4, 20 and 40 hr digestion). Spots G and O are the marker dyes used in the experiment.

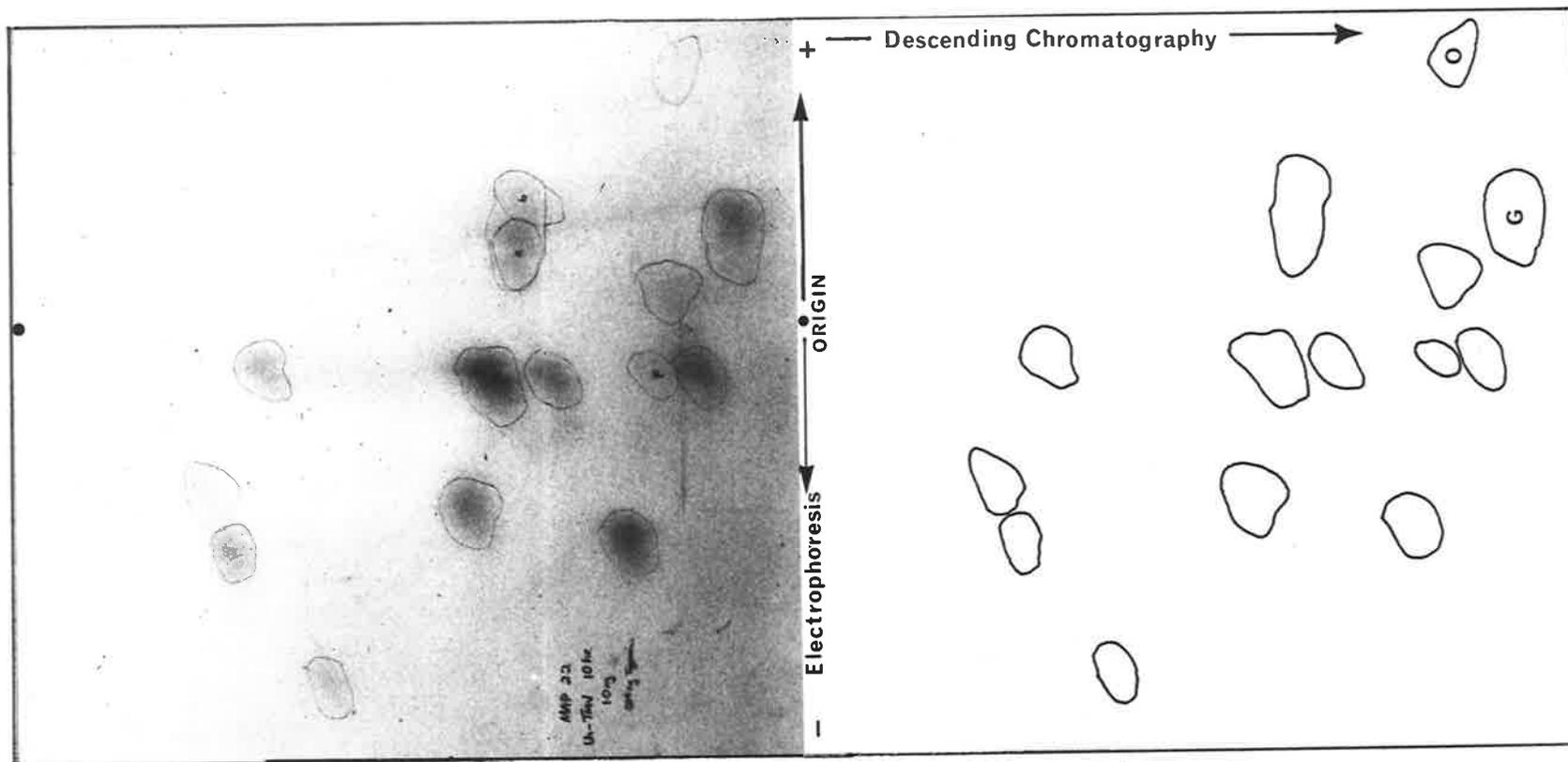


Table 6

Intensity of the peptides detected in the peptide maps of TRSV protein digested for various time periods with trypsin

Peptide ^a	Time of Trypsin Digestion (hr)					
	4	10	20 ^b	40	68	68 Insoluble ^c
1	2	2	2	2	1	1
2	2	2	2	2	2	2
3	3	3	3	3	3	1
4	3	3	3	3	3	3
5	3	3	3	3	3	2
6	3	2	2	2	2	1
7	3	3	3	4	2	2
8	3	3	3	4	3	2
9	4	4	3	3	3	3
10	4	5	4	4	5	3
11A	3	2	3	3	3	3
11B	1	3	3	2	3	1
12	3	3	3	3	3	1
13	1	3	3	3	2	2
14	2	3	3	3	3	1
15	1	2	3	3	3	2
16	1	2	2	3	2	1
a	0	1	2	2	1	0
b	1	1	1	1	1	0
c	1	2	1	1	1	1
d	1	1	0	1	0	0
e	3	2	1	1	2	2
f	1	1	2	3	2	2
g	1	1	1	0	0	0
h	1	1	0	0	0	0
i	1	1	0	0	0	1
j	2	1	1	0	0	0
k	2	1	0	0	0	0

^a See Figure 22 for positions of these peptides.

^b Standard time of trypsin digestion is 20 hr.

^c Water insoluble materials recovered from the protein samples after digestion with trypsin for 68 hr.

Values 0 - 5 indicates approximate intensity of the peptide spots.

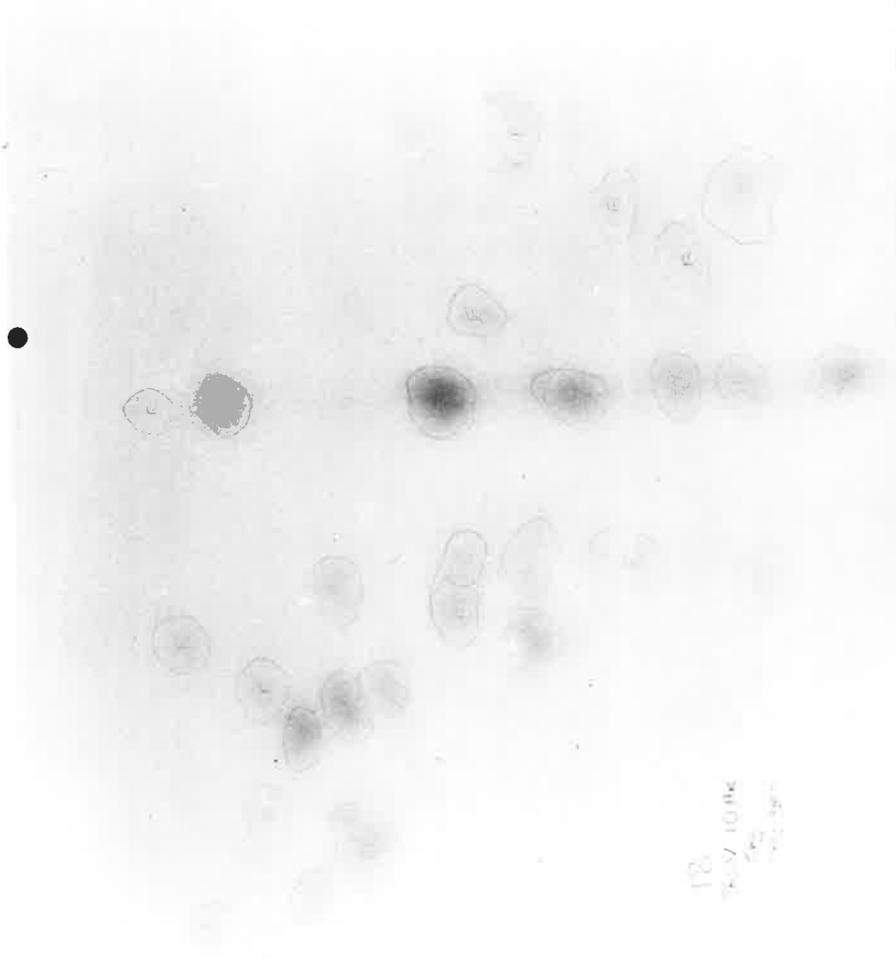
stained with ninhydrin (Fig. 22) indicates that 16 characteristic spots were consistently detected (designated by numbers corresponding to those in Table 6). Eleven additional spots were also detected (designated by letters corresponding to those in Table 6) but they were usually faint and non-persistent, varying in intensity with various times of trypsin digestion (Table 6). This suggests that they may have been either partially digested polypeptides or further degradation products of the tryptic peptides.

The presence of leucine and valine in the individual peptides were detected using TRSV coat protein separately labelled with the appropriate ^{14}C -labelled amino acids, while those peptides containing arginine, histidine and tyrosine were detected by specific staining and the results are summarised in Table 7. Most of these amino acids could not be detected in any of the faint spots a - k in Fig. 22 (Table 7). The results show that peptide spot 11 (Fig. 22) probably represents two tryptic peptides which were not completely resolved since it stained positively for both histidine (11A) and tyrosine (11B) (Fig. 22 and Table 7). The above results indicate that TRSV coat protein contains 16 or 17 tryptic peptides and also precludes the possibility that there could be more than 28.

Since trypsin cleaves a polypeptide chain specifically at the lysine and arginine residues, protein fragmenting into 17 tryptic peptide would be expected to contain 16 residues of arginine plus lysine (Bennett, 1967). This confirms the previous conclusion that the TRSV coat protein subunit is not the 60K component since with its molecular weight of approximately 54,000 and expected lysine plus arginine contents (Table 5) it would produce at least 40 tryptic peptides. Similarly it seems unlikely that the subunit molecular weight is 7,127, the smallest polypeptide predicted by the FITMOL analysis (Fig. 18) since this protein will produce only 7 tryptic peptides (Table 5). Since TRSV protein contains equal amounts of these two amino acids, this would

Figure 22. Two dimensional peptide map of unfractionated TRSV coat protein digested for 10 hr

Chromatogram was prepared and stained with ninhydrin as described in text. The peptides numbered 1 - 16 are those which were readily detectable in all mapping experiments; those labelled a - k were not always detected (see text for full explanation). Spots G and O are the marker dyes used in the mapping procedure.



12
 TRAVELINK
 1/2
 1/2

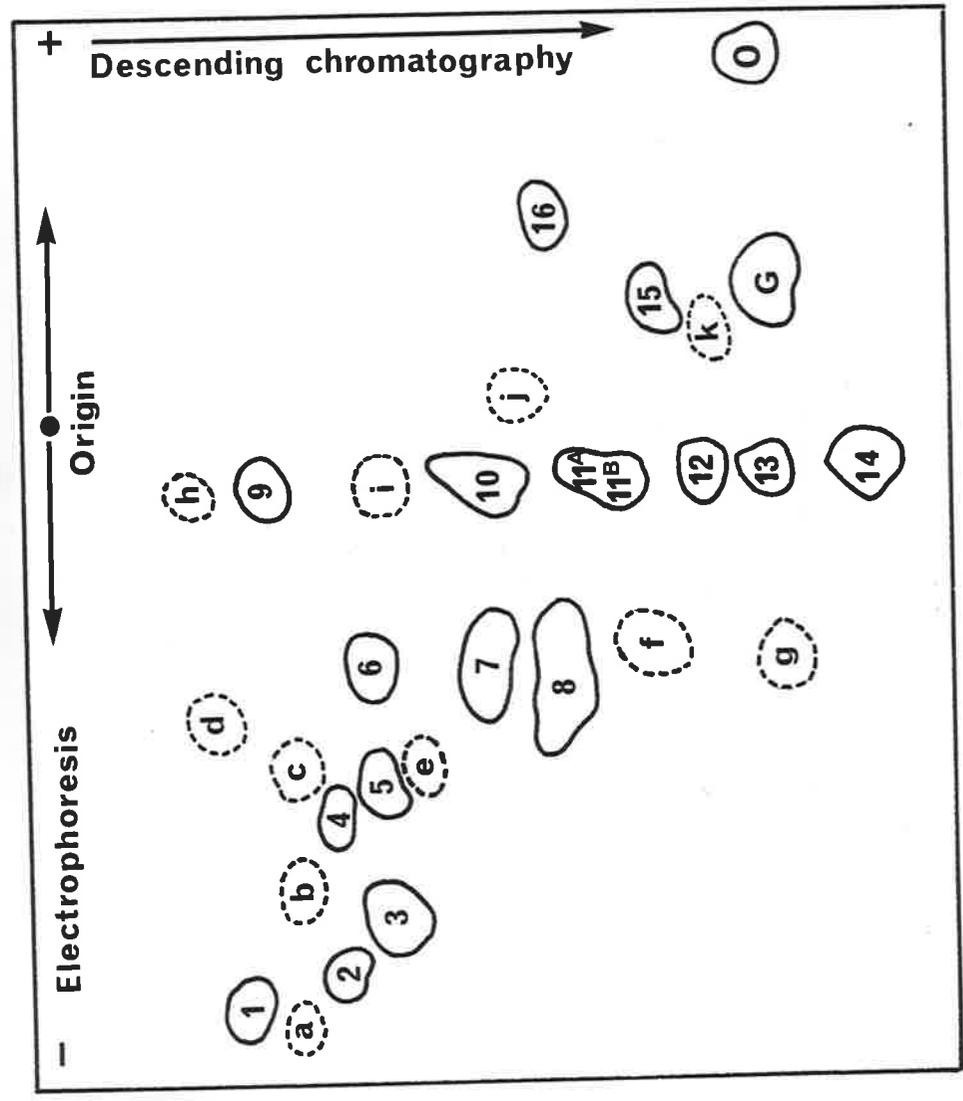


Table 7

Detection of specific amino acids in tryptic peptides
from TRSV coat protein

Peptide Number ^a	Amino Acids Detected ^b				
	Leu ^c	Val ^c	Arg ^d	His ^e	Tyr ^e
1	-	-	+	-	-
2	-	-	-	-	-
3	-	-	+	-	-
4	-	-	+	-	-
5	-	±	+	-	+
6	-	-	+	±	±
7	±	+	+	-	-
8	±	+(2?)	+	-	-
9	-	-	-	-	-
10	+	+	+	-	+
11 (A and B) ^f	+	+	-	+(A)	+(B)
12	+	+	-	+	-
13	+	±	-	-	-
14	+	+	-	-	±
15	+	+	-	±	+
16	±	-	-	-	-
Total observed	6-9	7-10	8	2-4	4-6
Predicted for poly- peptide with Mol. Wt. of 19,680 ^g	14	12	8	5	6
Predicted for poly- peptide with Mol. Wt. of 12,988 ^g	9	8	5	3	4

^a See Fig. 22 for positions of peptide spots on map. All the weak peptide spots (a-k) in Fig. 22 did not produce any positive reaction for any of the amino acids except that leucine was detected in peptides i and f.

^b - = not detected; + = detected; ± = detection uncertain.

^c Detected by autoradiography of peptide maps of protein labelled with the appropriate ¹⁴C-labelled amino acids.

^d Detected with phenanthrenequinone reagent on tryptic peptide maps of both 20 hr and 40 hr digests. The results from the two maps were similar.

^e Detected with Pauly reagent on two tryptic peptide maps of 40 hr digestion. The results of the two maps were very similar.

^f Spot probably contained two peptides A and B (see Fig. 22).

^g Calculated from FITMOL plot (see Table 5).

suggest a molecular weight of 19,680 for the polypeptide subunit (Table 5), which is one of the values predicted by FITMOL analysis (Fig. 18). However, this estimate is not supported by the molecular weight data of TRSV protein estimated by polyacrylamide gel electrophoresis (Table 1). On the other hand, there appears to be better agreement between the observed numbers of the specific amino acids detected in the tryptic peptides with the corresponding amino acid predicted for the FITMOL estimate of 12,988 molecular weight than with those predicted for the FITMOL estimate of 19,680 molecular weight (Table 7). The high number of arginine containing peptides (Table 7) could be over-estimated due to the phenanthrenequinone reagent also reacting with peptides containing tryptophan (Easley *et al.*, 1969).

A TRSV polypeptide subunit of 13,000 would contain only five arginine and five lysine residues (Table 5) and should only fragment into 11 tryptic peptides; this at first appears inconsistent with the observations presented in Fig. 22 and Table 7. However, it could be unreliable to determine the exact molecular weight of a polypeptide based on the observed number of tryptic peptides it produces, since there are several explanations which would account for fewer or greater than the expected number of peptides observed in a tryptic peptide map. In TRSV, there are several possible reasons why more than the expected number of tryptic peptides have been observed:

- (1) TRSV protein appears to have a significant number of acid-labile peptide bonds (see Chapter III) like the Asp-Pro bond observed in some viral proteins (Anderer, 1963; Baltz and Van Regenmortel, 1974). Further evidence for this was shown when TRSV protein was treated with acids such as formic or performic acid which caused a significant increase in the number of new protein bands detected in polyacrylamide gels (Fig. 23). Thus, performic acid treatment of TRSV protein in the presence of 98% formic acid prior to trypsin digestion may produce extra non-specific peptide spots.

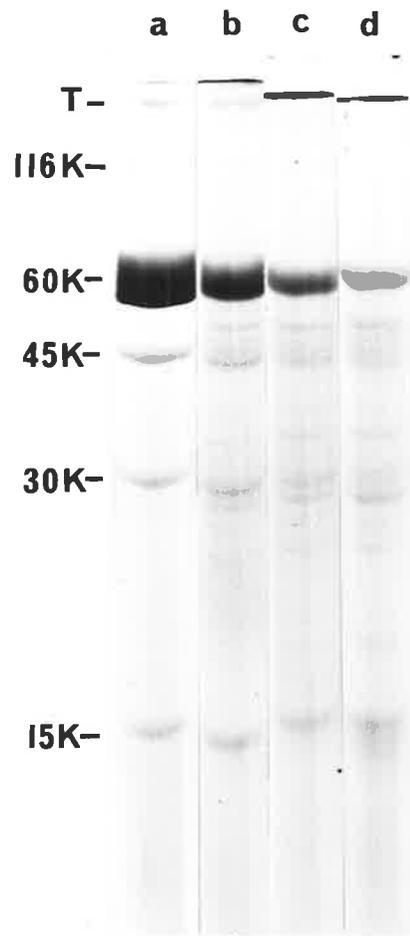
Figure 23. The effects of acids on TRSV protein

A. Effect of formic acid

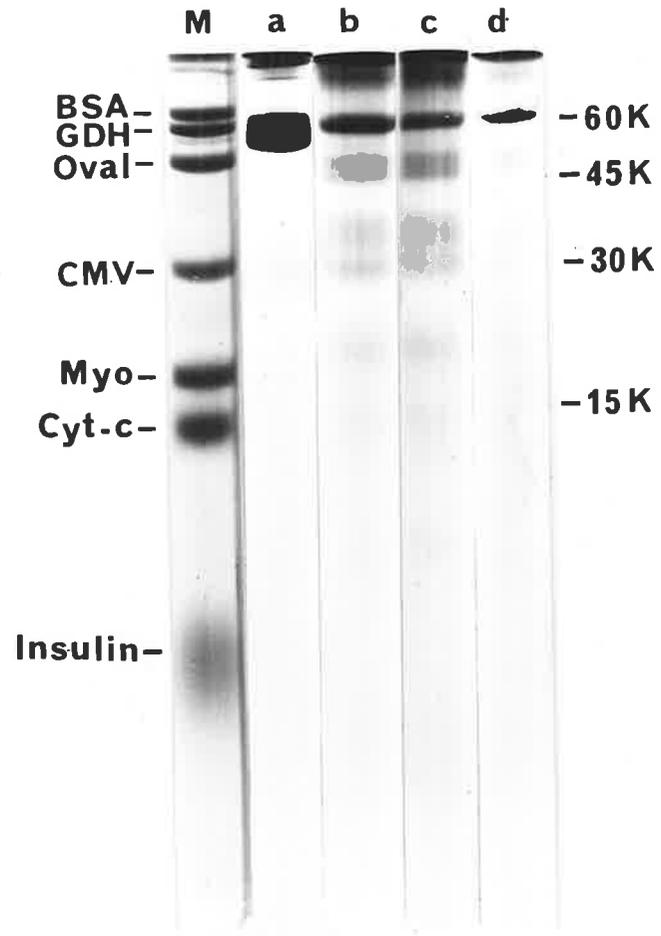
Gel a, TRSV dissociated under standard conditions
(see Materials and Methods);
gels b - d, TRSV protein preparations obtained
using the 67% formic acid method as described
by Miki and Knight (1965) after incubation
of virus in the acid for 10, 20 and 48 hr
at 37°C respectively and then dissociated
as in (a). The TRSV proteins (100 µg/gel)
were separated by the discontinuous system
of polyacrylamide gel electrophoresis.

B. Effect of performic acid oxidation

Gel M, marker proteins dissociated under standard
conditions;
gel a, TRSV protein dissociated under standard
conditions;
gels b - d, TRSV protein oxidised with performic
acid (Hirs, 1967) for 3, 7 and 24 hr
respectively at 0°C and then dissociated
as above. The proteins (100 µg TRSV
protein/gel) were separated by the continuous
urea-SDS PAGE system.



(A)



(B)

(2) It seems possible that some peptide bands involving arginine and lysine residues are more resistant to cleavage by trypsin leaving significant amounts of both cleaved and uncleaved peptides (Hill, 1965; Bennett, 1967) e.g. Lys-Glu-Glu and Arg-Glu-Glu may be resistant to tryptic cleavage (K. Gough, private communication).

(3) The presence of two adjacent basic residues e.g. X-Y-Lys(1)-Lys(2)-A-B may cause cleavage at a combination of positions (1), (2) and both (1) and (2) thereby giving rise to extra peptides (K. Gough, private communication).

(4) The strong bonds that produce the aggregates of TRSV protein may prevent complete digestion of the polypeptide by trypsin and thus produce extra peptides.

These reasons would also contribute to an over-estimation of the number of peptides containing arginine and the uncertainty of whether or not a specific amino acid was present in some of the peptides (Table 7). On the other hand, the reasons for producing fewer than the expected number of tryptic peptides may include: (1) uncleaved peptide bonds e.g. Lys-Pro, Arg-Pro (K. Gough, private communication), (2) blocked-terminus peptide which does not react with ninhydrin (Rydon and Smith, 1952; K. Gough, private communication) and (3) incomplete resolution of neutral peptides at pH 6.5. However, these reasons do not appear to play an important role in the analysis of TRSV protein.

IV. CONCLUSIONS

The bulk of dissociated TRSV coat protein migrates as a major band corresponding to a polypeptide with a molecular weight of between 54,000 and 60,000 when electrophoresed in SDS-polyacrylamide gels. However, some material remained at the top of the gel and some migrated as four other distinct minor bands (Fig. 1). In similar experiments, Mayo *et al.* (1971) detected only the major band and concluded that the TRSV coat protein contains a single type of polypeptide with a molecular weight of about 57,000. The results presented in this chapter

support the conclusions that none of the protein components detected as distinct bands in the gels is of host origin, that they are not degradation products of the 60K component, and that all are chemically indistinguishable. The estimated molecular weights of the components approximate to a ratio of 1:2:3:4:8. All these data support the view that the TRSV capsid is composed of one type of protein which is difficult to dissociate into its monomeric form but instead, tends to form stable aggregates producing the 30K, 45K, 60K, 116K and T components. Furthermore, it appears that the tetramers (60K component) are most stable since over 85% of the protein was detected as this form. The reason why the tetramers and the other aggregates are so stable remains obscure. They could not be further dissociated by additional treatment in urea, SDS and MCE or with GHCl or formamide, indicating that they are not stabilised by normal protein-protein interactions which stabilise quaternary structures of protein e.g. hydrogen bonds or hydrophobic interactions. It also appears that they are not stabilised by disulphide bonds since they were not dissociated by S-carboxymethylation. However, the effects of performic acid oxidation and sodium borohydride reduction (Fig. 11) suggest that they are probably stabilised by inter-molecular covalent bonds formed by oxidation-reduction reactions.

Data pertaining to the size of TRSV coat protein polypeptide monomer seem equivocal. Based on the FITMOL analysis of the amino acid data, three possible molecular weights can be considered: 7,127, 12,988 and 19,680 (Table 5). The number and apparent molecular weights of the protein components detected in polyacrylamide gels (Fig. 1 and Table 1) support the value of 12,988. If the monomers were 19,680 then the 60K component would have to be a trimer and not a tetramer; hence, two and not three minor components with molecular weights of approximately 20,000 and 40,000 would have been detected below the 60K component, representing monomers and dimers respectively. The results of cyanogen bromide cleavage experiments also indicate that the 12,988 molecular weight is

most likely to be correct. A molecular weight of 19,680 would produce four fragments with a total molecular weight of approximately 20,000 but the results showed that the three smallest fragments added up to over 30,000 molecular weight. Although the total number of tryptic peptides detected (Fig. 22, Table 7) appears to be consistent with the TRSV coat protein subunit being 19,680 molecular weight, there are many reasons why a protein may yield a greater number of tryptic peptides than expected from its total lysine plus arginine content (Harris and Hindley, 1965; Bennett, 1967; Burns and Turner, 1967; Hill, 1965). Furthermore, TRSV protein could be expected to produce more extra peptides than most other proteins and thus an accurate estimate of its molecular weight cannot be obtained by tryptic peptide mapping alone but requires conjunctive usage of several techniques as suggested by Laver (1969). None of the data from the above experiments support the lower molecular weight value of 7,127. Thus, based on all the evidence presented, it is concluded that the TRSV coat protein polypeptide subunit has a molecular weight of about 13,000.

CHAPTER VIN-VIVO SITE OF SYNTHESIS OF TRSV COAT PROTEINI. INTRODUCTION

Rezaian (1974) observed that most of the TRSV-specific double-stranded RNA and RNA-dependent RNA polymerase remained in the 17,000g supernatant after infected cucumber cotyledons were fractionated into (a) 17,000g supernatant (cytoplasmic fraction); (b) 500 - 17,000g pellet (mitochondrial fraction) and (c) 500g pellet (chloroplast and nuclear fraction). Thus he concluded that TRSV RNA is synthesized in the cytoplasm and not in any of the organelles. The work described in this chapter was done to determine whether the viral coat protein is synthesized by the cytoplasmic 80 S or the chloroplastic 70 S ribosomes so that a suitable cell-free protein synthetic system could be selected for the translation of the viral RNAs.

The effects of two antibiotics on the synthesis of TRSV protein *in vivo*, chloramphenicol which inhibits protein synthesis on 70 S ribosomes and cycloheximide which inhibits synthesis on 80 S ribosomes (Zaitlin *et al.*, 1968; Ellis and MacDonald, 1970; Graham *et al.*, 1970), were investigated. Evidence presented indicates that the TRSV coat protein is synthesized by the 80 S cytoplasmic ribosomes.

II. EXPERIMENTAL DETAILSA. *In-vivo* Labelling of TRSV Protein with ¹⁴C-Leucine in the Presence of Antibiotics

Chloramphenicol used was the D-threo-isomer which is more specific for 70 S ribosomes.

TRSV protein was labelled *in-vivo* with ¹⁴C-leucine under aseptic conditions. Various labelling conditions were tried but the following proved to be the most successful. Cotyledons were excised from healthy and infected cucumber seedlings 2 days after inoculation with purified TRSV (0.2 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0) or with sterile phosphate buffer (healthy plants). Seven samples of

infected cotyledons and one of healthy, each weighing 0.5 gm, were excised and cut into strips as described in Chapter II. The tissues were floated in 5 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing various concentrations of cycloheximide or chloramphenicol (Table 1). Infiltration of antibiotics into the tissues was achieved by applying gentle negative pressure. After 90 min of incubation under fluorescent light at 25°C (to allow the antibiotics to act), antibiotics and buffer were removed and 5 μ Ci of 14 C-leucine was added dropwise onto each tissue sample. After 10 min of absorption, fresh buffer containing the appropriate amounts of antibiotics was added to the tissues. Incorporation of 14 C-leucine was allowed to continue under fluorescent light at 25°C for 5 hr, after which the tissues were washed exhaustively with 0.1 M phosphate buffer, pH 7.0, blotted dry and used immediately for virus purification.

B. Recovery of TRSV and Estimation of Incorporation of 14 C-Leucine into Viral Protein

TRSV was purified from each sample as described in Chapter II. Each tissue sample was ground up in 5 ml of 0.1 M phosphate buffer, pH 7.0, containing 3.72 mg of purified unlabelled carrier virus. Radioactivity in the crude tissue homogenate was measured by counting 50 μ l aliquots of extract which were spotted onto filter paper discs. The amounts of radioactivity recovered in the tissue homogenates are estimates of the uptake of 14 C-leucine by the tissues. Virus recovered from each sample was resuspended in 1 ml of 0.1 M phosphate buffer, pH 7.0, and the yield was estimated spectrophotometrically and the radioactivity in the virus preparations was assayed by precipitation with TCA as described by Atchison and Francki (1972). Corrections were made for losses of virus during purification and differences in uptake of 14 C-leucine by tissues in each treatment. However, the virus synthesized *de novo* in the infected tissues was not taken into account because they varied with different treatments and the increase in virus content due to this was estimated to be less than 10% of the carrier virus added.

III. RESULTS AND DISCUSSION

The results of the experiments are summarised in Table 1. Based on the studies by Ellis and MacDonald (1970) and Graham *et al.* (1970) the concentrations of chloramphenicol and cycloheximide used in this study exclude any possibility that the chloramphenicol concentration is not high enough to inhibit 70 S ribosomes or that the cycloheximide is also inhibiting 70 S ribosomes as well. The results show that radioactivity in the virus preparation purified from the infected tissues incubated in the absence of antibiotics (control) is over 700% higher than that of the virus isolated from the healthy tissues. Since the amounts of virus recovered from these two tissue samples were similar, this could not be accounted for by an increase in TRSV content from virus synthesized in the infected tissues, thus indicating that there is actual *in-vivo* incorporation of ^{14}C -leucine into viral protein in the infected tissues. This conclusion is further supported by the observation that all the virus preparations isolated from the infected cucumber cotyledons contained more radioactivity than virus from healthy tissues despite one of the infected tissue samples (4 $\mu\text{g}/\text{ml}$ cycloheximide) having had lower uptake of ^{14}C -leucine than the healthy tissue. The results also show that all except one of the infected tissue samples took up more ^{14}C -leucine than the healthy tissue.

Chloramphenicol did not reduce the uptake of leucine and appears to have stimulated the incorporation of the amino acid into viral protein. This is probably caused by indirect effects of the antibiotic on cell metabolism (Graham *et al.*, 1970). However, the stimulation effect seems to be a short term effect since longer period of incubation with the antibiotic (24 hr) actually reduced incorporation of leucine into viral protein by up to 20% (results not shown).

At high concentrations of cycloheximide, both the uptake and incorporation of leucine were reduced significantly. Cycloheximide is known to delay uptake of organic compounds, especially amino acids

Table 1

Effect of chloramphenicol and cycloheximide on leucine incorporation into the coat protein of TRSV multiplying in cucumber cotyledons

Treatment ^a	¹⁴ C-leucine ^b taken up by tissues		% Recovery of carrier TRSV added ^c	TCA in-soluble ¹⁴ C-leucine incorporation into virus ^d			
	cpm x 10 ⁻⁶	% of control		Before correction ^e		After correction ^f	
				cpm x 10 ⁻³	% of control	cpm x 10 ⁻³	% of control
Healthy tissues; no antibiotics	4.93	66	56.9	2.94	9.3	0	0
Infected tissues; no antibiotics (control)	7.44	<u>100</u>	55.2	31.50	<u>100</u>	27.06	<u>100</u>
+chloramphenicol (50µg/ml)	7.21	97	48.2	46.09	146	43.13	159
+ " (150µg/ml)	6.96	94	47.2	36.26	115	34.35	127
+ " (400µg/ml)	7.38	99	47.2	38.44	122	34.32	127
+cycloheximide (0.25µg/ml)	6.70	90	61.7	31.68	100	30.72	113
+ " (1.0µg/ml)	5.35	72	54.7	10.12	32	9.62	36
+ " (4.0µg/ml)	4.03	54	54.0	5.19	16	5.15	19

^a See text for experiment details.

^b Uptake of ¹⁴C-leucine by each tissue sample (0.5gm) estimated by counting duplicate aliquots of the tissue homogenates of 50 µl each.

^c Yield of purified virus from each tissue sample expressed as a % of the total carrier TRSV added.

^d Values estimated by counting duplicate aliquots of 200µl each from each virus preparation which were then corrected for 100% recovery of added carrier virus.

^e Before correction for differential uptake of ¹⁴C-leucine by tissue samples and for contaminating radioactivity in carrier virus added to the healthy tissue.

^f After correction for above (e).

(Ellis and MacDonald, 1970). Thus, the inhibition of incorporation of leucine into TRSV protein by cycloheximide may be due to both reduction in uptake of leucine by the tissue as well as inhibition of 80 S ribosomes. However, the following results suggest that the inhibition of virus protein synthesis was achieved mainly through the inhibition of ribosomes rather than through a reduction of leucine concentration in the tissues:

1. Cycloheximide at 0.25 $\mu\text{g/ml}$ reduced uptake of leucine without affecting the incorporation of the amino acid into the viral protein.
2. Infected tissue samples showing active virus synthesis had higher uptake of leucine than the healthy tissue suggesting that the uptake of leucine is dependent on the rate of its incorporation.
3. Less than 0.5% of the ^{14}C -leucine taken up by the tissue is incorporated into virus protein, suggesting that the 50% reduction in uptake of the amino acid is not a major factor limiting its incorporation into TRSV protein.
4. Even after corrections were made in the incorporation of leucine into TRSV protein to compensate for the differences in uptake of the amino acid due to the various treatments, cycloheximide still inhibited viral protein synthesis by up to 80%.

Another major side effect of cycloheximide which would be expected to reduce the incorporation of ^{14}C -leucine into viral protein is the disruption of energy supply by uncoupling the oxidative phosphorylation reaction (Ellis and MacDonald, 1970). However, the total incubation time in the presence of cycloheximide is probably too short for the tissues to be depleted of all energy sources and it is unlikely that the lack of ATP or GTP was responsible for the observed inhibitory effect. Thus it is concluded that cycloheximide inhibits the synthesis of TRSV protein mainly by inhibiting the activity of the 80 S cytoplasmic ribosomes.

CHAPTER VIMOLECULAR WEIGHT, INFECTIVITY STUDIES AND *IN-VITRO* TRANSLATION OFTRSV RNAI. INTRODUCTION

Diener and Schneider (1966) detected two RNA species in TRSV with molecular weights of 1.2×10^6 (RNA-S) and 2.2×10^6 (RNA-L). However, these values were calculated from sedimentation velocity data without the use of appropriate RNA molecular weight markers. Other studies, done under non-denaturing conditions in polyacrylamide gels have yielded different molecular weight values, 1.4×10^6 for RNA-S and 2.3 to 2.4×10^6 for RNA-L (Rezaian and Francki, 1973; Murant *et al.*, 1972). However, it has been shown that the primary and secondary structures of RNA can affect their mobilities in gels under non-denaturing conditions (Pinder *et al.*, 1974; Spohr *et al.*, 1976; Lehrach *et al.*, 1977). Thus the molecular weights of TRSV RNAs were determined under denaturing conditions in 98% formamide gels.

Diener and Schneider (1966) and later Harrison *et al.* (1972a) found that only RNA-L was associated with infectivity. This led to the suggestion that RNA-S may be a degradation product of RNA-L but is packaged as a separate nucleoprotein component. Later, it was found that the two RNA species had very little nucleotide sequence homology (Rezaian and Francki, 1974). However, it has been difficult to demonstrate conclusively that both RNA species of TRSV are required for infectivity although it could be shown that TRSV RNA-S stimulated the infectivity of RNA-L (Harrison *et al.*, 1972a). On the other hand, studies of other Nepoviruses, RRV, TBRV and GFLV have shown that both RNA species are required for infectivity although in RRV, the RNA-L was also associated with infectivity (Harrison *et al.*, 1972a; Quacquarelli *et al.*, 1976; Randles *et al.*, 1977). An explanation for these observations was that some of the RNA-S of TRSV and RRV aggregated to form dimers which were not separated from the RNA-L (Murant *et al.*, 1972; Harrison *et al.*,

1972a). Evidence is now presented which shows that infectivity is not associated with either RNA-S or RNA-L of TRSV but that both RNA species are indeed required; and that RNA-S tends to form dimers which migrate slightly slower than RNA-L.

In the past few years, many reports have been published on the translation of the positively stranded genomic RNAs of a number of plant viruses (Table 1). The main aims of these investigations have been firstly, to examine the gene content and gene distribution and to locate and identify the functions of specific genes on the genomes of the viruses, especially those with divided genomes. Secondly, translation of viral mRNAs may provide information on the mechanisms of virus synthesis, especially that of coat protein, and the co-ordination of the various RNA components of multipartite viruses in virus replication. Thirdly, information on the mechanism of protein synthesis in eukaryotic cells may be obtained from *in-vitro* translation experiments using viral mRNAs.

Certain viral genomic RNAs are more readily translated *in-vitro* than others. This may be due to differences in the structure and organisation of their genomic RNAs which reflect their mechanisms of coat protein gene expression. As can be seen in Table 1, no complete report on the translation of any Nepovirus RNAs have been published. This may indicate that the RNAs of Nepoviruses are difficult to translate *in-vitro*. In this chapter, the messenger activities of TRSV genomic RNAs were studied and the results suggest that *in-vitro*, the viral coat protein is not synthesized by translation of the genomic RNAs.

II. MATERIALS AND METHODS

A. Determination of the Molecular Weights of TRSV RNAs by Electrophoresis in Polyacrylamide Gels Containing 98% Formamide

The molecular weights of the TRSV RNAs were estimated by electrophoresis under denaturing conditions in 4% acrylamide-98% formamide gels containing 20 mM diethylbarbituric acid, pH 9.0, as described by

TABLE 1

In-vitro translation of coat proteins of plant viruses with positive sense single-stranded-RNAs

Virus Group	Virus	Mol. Wt. of Genomic RNAs (x 10 ⁻⁶)	Possess separate mRNA for coat protein	Coat protein Mol. Wt.	Translation of coat protein from genomic RNAs in-vitro	References
Tobamovirus	TMV	2.05 (+0.28)	Yes (subgenomic)	17,500	No	Efron and Marcus (1973); Knowland (1974); Knowland et al.(1975); Zaitlin and Israel (1975); Bruening et al.(1976); Zaitlin (1977); Beachy and Zaitlin (1977).
Tymovirus	TYMV	1.9-2.3(+0.25)	Yes (subgenomic)	20,000	No	Pleij et al.(1976); Benicourt and Haenni (1976); Klein et al.(1976); Pleij et al.(1977); Szybiak et al.(1978); Benicourt et al. (1978); Mellema et al. (1979).
Tobacco necrosis virus	TNV	1.3	No	22,600	Yes	Kassanis (1977); Salvato and Fraenkel-Conrat (1977).
Carnation mottle virus	CarMV	1.4	No	38,000	Yes	Waterworth and Kaper (1972); Nelson and Tremaine (1975); Salomon et al.(1978).
Tombusvirus	TBSV	1.5	-†	41,000	-	Martelli et al.(1977a).
Potexvirus	PVX	2.1	-	18-27,000	-	Lesemann and Koenig (1977); Koenig and Lesemann (1978).
Potyvirus	PVY	3.5	-	32-34,000	-	Matthews (1979).
Closterovirus	SBYV	2.3-4.3	-	23,000	-	Matthews (1979).
Carlavirus	CLV	2.3	-	30,000	-	Matthews (1979).
Luteovirus	BYDV	2.0	-	24,000	-	Rochow and Israel (1977).
Tobravirus	TRV	2.3(+0.6-1.3)	Yes (co-virus?) ⁺	22,000	No?	Mayo et al.(1976); Bruening (1977); Fritsch et al. (1977); Pelham (1979b).
Nepovirus	TRSV	2.4, 1.4		13,000	No?	Francki and Hatta (1977); Chu and Francki (1979); Gergerich et al. (1979).
	TBRV	2.5, 1.5		52,000	No?	Randles et al.(1977); Harrison and Murrant (1977); Fritsch et al. (1978).
Comovirus	CpMV	1.37, 2.02	No	44,000+ 25,000	Yes	Geelen et al.(1972); Reijnders et al.(1974); Pelham and Jackson (1976); Davies et al. (1977); Pelham (1979a).
Pea enation mosaic virus	PEMV	1.7, 1.4(+0.3)	-	28,000+ 22,000	-	Matthews (1979).
Hordeivirus	BSMV	1.4, 1.3, 1.2(+?)	Yes?	21,000	Yes?	Lane (1974a); Dolja et al. (1976); Palomar et al.(1977).
Alfalfa mosaic virus	AMV	1.04, 0.73, 0.62 (+0.25)	Yes (subgenomic)	24,300	No	Van Ravenswaay Claasen et al.(1967); Mohier et al. (1975); Van Vloten-Doting et al.(1975);

Table 1 cont'd.

Ilavirus	TSV	1.1,0.9,0.7(+0.35)	Yes (subgenomic)	25,000	No	Kraal et al. (1976); Heijtkink et al. (1977). Clark and Lister (1971); Ghabrial and Lister (1974); Van Vloten-Doting (1976).
Bromovirus	BMV	1.09,0.99,0.75 (+0.28)	Yes (subgenomic)	20,000	No	Shih and Kaesberg (1973); Lane (1974b); Shih and Kaesberg (1976); Lane (1977).
	CCMV	1.1,1.0,0.7(+0.3)	Yes (subgenomic)	20,000	No?	Davies and Kaesberg (1974); Bancroft and Horne (1977).
	BBMV	1.1,1.0,0.7(+0.3)	Yes (subgenomic)	20,000	No?	Davies and Kaesberg (1974); Bancroft and Horne (1977).
Cucumovirus	CMV	1.35,1.16,0.85 (+0.35)	Yes (subgenomic)	24,600	No	Schwinghamer and Symons (1975, 1977); Francki et al. (1979).

TNV, tobacco necrosis virus;
PVX, potato virus X;
PVY, potato virus Y;
SBYV, sugar beet yellowsvirus;
TSV, tobacco streak virus;

BBMV, broad bean mottle virus;
CLV, carnation latent virus;
BYDV, barley yellow dwarf virus;
TRV, tobacco rattle virus;
PEMV, pea enation mosaic virus;
BSMV, barley stripe mosaic virus.

† Dash indicates information not available.

+ Question mark indicates results not fully established.

Pinder *et al.* (1974) and modified by Mossop (1978). Samples of 15 - 20 μg TRSV RNA per gel were electrophoresed at 3mA/gel for 24 hr at 25°C. The electrophoresis buffer was circulated between the reservoir tanks. Gels were stained in toluidine blue O as described in Chapter II. Various RNAs were used as internal markers. These are EMC RNA (2.6×10^6) (Matthews and Korner, 1970; Burness, 1970; Martin, E.M., private communication); U-1 TMV RNA (2.05×10^6) (Boedtker, 1968, 1971); *E. coli* 23 S rRNA (1.07×10^6) and 16 S rRNA (0.55×10^6) (Stanley and Bock, 1965); and QCMV RNAs ($Q_1 - Q_4$, 1.27, 1.13, 0.82, 0.35×10^6 ; Mossop, 1978; Peden and Symons, 1973; Habili, 1974). TRSV RNAs were co-electrophoresed with these RNA markers and the molecular weights of RNA-S and RNA-L were estimated from their relative mobilities.

B. Preparative Gel Electrophoretic Fractionation of TRSV RNAs

The TRSV RNA components, RNA-S and RNA-L, were fractionated and purified by two cycles of agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer, pH 8.3, using procedures modified from those of Mossop (1978). In the first cycle, the RNAs were separated in 1.75% agarose gels. The gels were prepared as described in Chapter II for analytical gels except that they were cast in eight 15 cm x 1.0 cm plexiglass tubes to a depth of 13 cm. The gels were loaded with RNA (1.5 mg/ml in TBE containing 10% sucrose and 0.0005% BPB, 150 $\mu\text{g/gel}$) which had been heated at 70°C for 1 min. Electrophoresis was first at 1.5 mA/gel for 1 hr, and then at 2.5 mA/gel for 6 hr at 25°C. Circulation of electrophoresis buffer was not needed. After electrophoresis, the RNA bands were located by staining for 15 - 30 min with 0.01% ethidium bromide containing 20 mM NaAc (Schuerch *et al.*, 1975) and then excised. The gel slices were stored at -70°C prior to extraction. The RNA components were extracted from gel slices accumulated from 3 - 4 such experiments with TBES buffer (TBE containing 0.1 M NaAc) at 1 ml/gel slice by homogenising them in a conical centrifuge tube with a glass rod. Freezing the gels helped the homogenising procedure. The

pulverised gels were shaken for 10 - 16 hr at 4°C, and then centrifuged. The supernatants were collected and stored at -15°C. The gel fragments were re-extracted twice with 0.5 ml/gel slice of TBES buffer as described above and once with a mixture of one volume each of phenol and TBES buffer. The supernatants containing the respective RNA components were pooled and extracted twice with one-third volume of phenol to remove the ethidium bromide and residual agarose particles. The final RNA extracts were kept at 0°C for 30 min and then centrifuged at 5000g for 20 min to remove as much of the phenol-agarose aggregates as possible. Finally, the RNAs were recovered by precipitation with 3 volumes of ethanol at -15°C. The RNA components were resuspended in 25µl TBE buffer per gel slice and their concentrations determined spectrophotometrically.

The second cycle was essentially a repeat of the first except that 2.2% agarose gels were used. The once fractionated TRSV RNA components were heated at 70°C for 3 min before being loaded at 50 µg/gel. Electrophoresis was increased to 8 hr at 2.5mA/gel. Any other bands besides those of the RNA loaded were discarded. The isolated RNA-S and RNA-L preparations were resuspended in TE buffer, pH 7.6, (10 µl/gel slice) and their concentrations determined spectrophotometrically.

C. Infectivity Assays of TRSV RNA-S and RNA-L

All infectivity assays were done on cowpeas using the Latin square design as described in Chapter II. The isolated TRSV RNA-S and RNA-L were inoculated either separately or after mixing at various concentrations and ratios of RNA-S to RNA-L. Unfractionated RNAs were used as controls in all experiments.

D. Heat Treatment of TRSV RNA

Samples of unfractionated TRSV RNA (50 µg in 50 µl TBE) when required were heated at 70°C for 3 min and then analysed by electrophoresis in 2% agarose gels as described in Chapter II.

The same RNA preparation was also heated at 25 µg/ml in 0.1 M phosphate buffer, pH 7.2, for various periods of time ranging from 0 - 15 min at 70°C and then cooled rapidly in ice. The treated RNAs were then assayed for infectivity as described in Chapter II.

In another experiment, the RNA was diluted two fold in 0.1 M phosphate buffer, pH 7.2, and then heated at 70°C for 3 min prior to inoculation on cowpeas.

E. In-vitro Translation of TRSV RNA

(i) Materials

Materials used specifically for cell-free protein synthesis assays and their sources are listed in Table 2. All materials used were rendered free from ribonucleases by methods described in Chapter II. Acetate (Ac) instead of chloride salts were used throughout (except CaCl₂) since it has been reported that Cl⁻ inhibits translation (Davies *et al.*, 1977). ¹⁴C-leucine labelled TRSV coat protein was prepared by labelling TRSV *in-vivo* with ¹⁴C-leucine as described in Chapter II. The labelled virus was dissociated in urea-SDS-MCE reagent in 0.125 M Tris-HCl, pH 6.8, and stored at -15°C.

(ii) Preparation of Cell-Free Extracts (S-30)

Translation of TRSV RNA *in-vitro* was done using cell-free extracts prepared from either commercial wheat germ (Marcu and Dudock extract) or from isolated wheat embryos (Davies extract). All steps in the preparation of the extracts were done in a cold room at 0 - 4°C.

(a) Davies Extract. The method of Davies *et al.* (1977) was used. Embryos were isolated from 100% viable wheat (*Triticum tritici*, cv. Halberd) seeds as described by Shih and Kaesberg (1973) using sieves with British standard mesh scales of 10, 16 and 22. The use of sieves with mesh scale of 22 instead of 30 removed most of the broken embryos. The freshly isolated embryos were floated on a mixture of carbon tetrachloride and cyclohexane (500:220, V/V) and then rapidly skimmed off from the organic solvents and air dried. One gram of the embryos

Table 2

Materials used specifically for *In-vitro*
protein synthesis and their sources

<u>Material</u>	<u>Source</u>
Wheat embryo	Cultivar Halberd, Department of Agronomy
Wheat germ (fresh)	Adelaide Milling Co., Port Adelaide, S. Aust.
L-[4,5- ³ H]Leucine, 54Ci/ mmole, 5mCi/5ml	The Radiochemical Centre, Amersham, England
L-[U- ¹⁴ C] Leucine, 324mCi/ mmole, 250 μ Ci/4.5ml	The Radiochemical Centre, Amersham, England
Spermidine phosphate	Sigma Chemical Co., U.S.A.
L-amino acid kit	Sigma Chemical Co., U.S.A.
N-2'-hydroxyethylpiperazine- N1-2-ethane sulphonic acid (HEPES)	Sigma Chemical Co., U.S.A.
Potassium acetate	BDH Chemicals, England
Magnesium acetate	May and Baker Ltd., England
Adenosine 5'-triphosphate (ATP), disodium salt, equine muscle	Sigma Chemical Co., U.S.A.
Guanosine 5'-triphosphate (GTP), sodium salt, equine muscle	Sigma Chemical Co., U.S.A.
Creatine phosphate, di-Tris salt	Sigma Chemical Co., U.S.A.
Creatine phosphokinase, Type I, rabbit muscle, 100-110 units/mg protein	Sigma Chemical Co., U.S.A.
Dithioerythreitol and dithiothreitol	Sigma Chemical Co., U.S.A.
Ribonuclease A, bovine pancreatic, Type IIIA, 100 Kunitz units/mg solid	Sigma Chemical Co., U.S.A.
Casamino acids	Difco Laboratories, U.S.A.
Sephadex G25 (Medium)	Pharmacia, Sweden

was ground vigorously in a pre-cooled pestle and mortar with 1 gm of crushed glass for 30 seconds. Two ml of extraction buffer containing 120 mM potassium acetate (KAc), 5 mM magnesium acetate (MgAc), 1 mM dithioerythritol (DTE) and 5 mM N-2'-hydroxyethylpiperazine-N1-2-ethane sulphonic acid (HEPES) was added to the finely ground embryos and then ground for a further 60 sec to produce a thick paste. A further 8 ml of extraction buffer was then added and the mixture was ground for another 60 sec. The resulting extract (pH = 6.4 - 6.5) was centrifuged at 30,000g for 10 min and the supernatant was drawn off with a pasteur pipette, excluding the top fatty layer and the thick layer between the pellet and the supernatant. The pH of the supernatant was adjusted with 0.02 volume of 500 mM HEPES-KOH, pH 7.6, and then centrifuged again at 30,000g for 10 min. The resulting supernatant (pH 6.8 - 7.0) was dialysed at 0°C against 2 changes of buffer of 1 litre each containing 20 mM Tris-acetate, 120 mM KAc, 5 mM MgAc and 1 mM DTE, pH 7.6, for a total of 20 - 22 hr. The supernatant (S-30) which became turbid on dialysis was snap frozen and stored in liquid nitrogen or stored at -80°C in small aliquots.

(b) Marcu and Dudock Extract. Commercial wheat germ stored at 4°C in sealed sterile bottles, was used to prepare a cell-free extract using a method modified from that described by Marcu and Dudock (1974). Two gm of dry wheat germ were ground vigorously for 60 sec in a cooled mortar with an equal weight of crushed glass. Four ml of extraction buffer containing 20 mM HEPES, 120 mM KAc, 1 mM MgAc, 2 mM CaCl₂, 1 mM DTE, and adjusted to pH 7.4 with KOH, was then added to the powdered mixture and grinding continued for another 30 sec. The resultant thick paste was centrifuged at 30,000g for 12 min and the supernatant (pH 6.3 - 6.4) was removed with a pasteur pipette, excluding the top fatty layer. The volume of the supernatant was about 2.2 - 2.5 ml and all of this was loaded onto a sephadex G-25 (medium) column (22 x 1.2 cm) which was previously equilibrated with 10 x bed volumes of elution buffer containing 20 mM HEPES, 120 mM KAc, 5 mM MgAc and 1 mM DTE, pH 7.6 (adjusted with

KOH). The extract was eluted with the same buffer at a flow rate of 2 ml/min and 0.5 ml fractions were collected 5 min after start of elution. Fractions with absorbance at 260 nm (OD_{260}) greater than 90 were pooled and centrifuged for 20 min at 30,000g. The supernatant was collected and the pH was found to be about 7.0 - 7.1. This final extract (S-30) was divided up into small aliquots (0.3 - 0.5 ml) and stored at -80°C or in liquid nitrogen. The pH of the extraction buffer was lowered from pH 7.6 to pH 7.4 to ensure that the pH of the homogenate was less than 6.5 to prevent the release of host mRNA (Marcus *et al.*, 1974). The Marcu and Dudock extract was found to be more efficient in translating high molecular weight mRNAs into large polypeptides (Marcu and Dudock, 1974; Powell, Dept. of Biochemistry, University of Adelaide, personal communication) and was used throughout the later part of this work.

(iii) *In-vitro* Protein Synthesis Assay

In-vitro translation of RNA was always assayed in duplicate in 50 μl reaction volumes using the systems of Davies *et al.* (1977) (Davies System) and Marcu and Dudock (1974) (Marcu and Dudock System). Table 3 lists the complete assay reagent mixtures used in these two systems for optimum translation of TRSV RNA. Any variation from these are described in the Results section. The standard incubation condition was 1 hr at 30°C unless otherwise stated. In all translation experiments these control were used: (1) zero time incubation (estimate of background); (2) no mRNA added (estimation of endogenous incorporation) and (3) QCMV RNA incorporation (estimates activity of the extracts). U-1 TMV and V-TAV RNAs were also translated as controls in some experiments.

After incubation, the amounts of radioactive leucine incorporated into acid insoluble material was measured and used as an estimate of *in-vitro* protein synthetic activity. In those experiments where only the translational activities were investigated, the reactions were stopped by addition of 1 ml of cold 5% TCA containing 1% casamino

Table 3

Composition of reagent mixtures optimal for *in-vitro* translation of

Reagent	<u>TRSV RNAs</u>	
	Davies	Marcu and Dudock
Tris-acetate buffer, pH 7.6	10mM	-
HEPES-KOH, pH 7.6	20mM	14mM + 6mM (pH 7.2) from extract
Magnesium acetate	3mM	2.5mM
Potassium acetate	90mM	100mM
Other K ⁺ (KOH)	10mM	10mM
ATP, pH 7.5	2.5mM	1mM
GTP, pH 7.5	0.375mM	20μM
Creatine phosphate	10mM	8mM
Dithioerythritol	1mM	2mM
Creatine phosphokinase	0.5μg/50μl	1.5μg/50μl
Spermidine	0.4mM	0.2mM
Other unlabelled amino acids	125μM each	25μM each
Labelled leucine:	(³ H-leucine (800μCi/μmole)	(³ H-leucine (54Ci/mmole)
	(1μCi/50μl (25μM)	(1μCi/50μl (0.4μM)
	(or	(or
	(¹⁴ C-leucine (324mCi/mmole)	(¹⁴ C-leucine (324mCi/mmole)
	(0.25μCi/50μl (15μM)	(0.25μCi/50μl (15μM)
Extract: Davies extract	25μl/50μl	25μl/50μl
Marcu and Dudock extract	15μl/50μl	15μl/50μl

Table 3 contd.

RNA* : TRSV RNA	2µg/50µl	4µg/50µl
TRSV RNA-S	-	2µg/50µl
TRSV RNA-L	-	2µg/50µl
QCMV RNA	1.5µg/50µl	1.5µg/50µl
VTAV RNA	1.5µg/50µl	1.5µg/50µl
U-1 TMV RNA	2µg/50µl	4µg/50µl
Water	to 50µl	to 50µl

The reagents were added in the sequence listed.

RNAs were dissolved in 20mM Tris-acetate, pH 7.6, containing 0.2mM EDTA, and creatine phosphokinase in 20mM Tris-acetate, pH 7.4.

ATP and GTP were adjusted to pH 7.5 with 1M KOH after dissolving in water. GTP was corrected for 20% loss during storage and transport (Sigma). Creatine phosphate was dissolved in water. 25µl of Davies extract per 50µl reaction volume was estimated to contain 10mM Tris-acetate, pH 7.6, 2.5mM MgAc, 60mM KAc, and 0.5mM DTE, while 15µl of Marcu and Dudock extract provided 6mM HEPES, 0.3mM DTE, 1.5mM MgAc and 36mM KAc.

³H-leucine was used when the translational products were not analysed by fluorography or tryptic peptide mapping.

¹⁴C-leucine was used when the translational products were to be analysed by fluorography and peptide mapping.

* The RNA concentrations used were optimal for the respective RNAs.

acids. The precipitated reaction mixtures were kept in ice for at least 45 min and then collected on Whatman GF/A glass fibre filters, washed 5 times (4 ml each) with cold 5% TCA containing 1% casamino acids and then 3 times (4 ml each) with ethanol-ether mixture (1:1, V/V). The filters were dried in an oven for 30 min and then counted in 2 ml of PPO-POPOP scintillation fluid in propylene tubes as described in Chapter II. Where the translational products were also analysed, the reactions were normally terminated by placing the tubes in ice; for large volumes of translation reactions, they were incubated in 50 $\mu\text{g/ml}$ RNase-A at 30°C for 30 min. Fifteen μl from each of the duplicated reactions were precipitated with 0.5 ml TCA-casamino acid mixture and measured for radioactivity incorporated into acid-insoluble materials as described above. The remaining translational products were pooled and stored immediately at -15°C until required.

(iv) Analysis of Translational Products by Discontinuous SDS-Polyacrylamide Gel Electrophoresis

Aliquots of the translational products (usually 5 - 20 μl) and authentic TRSV coat protein were made up to 50 μl containing 6 M urea, 1% SDS, 2% MCE, 0.0005% BPB in 0.125 M Tris-HCl, pH 6.8. The samples were heated in boiling water for 2 min before being electrophoresed in cylindrical or slab gels by the discontinuous SDS-PAGE system (Laemmli, 1970). Electrophoresis in cylindrical gels was carried out as described in Chapter II. After electrophoresis, the gels were stained and destained at 37°C before being sliced and counted in NCS-liquid scintillation fluid. The molecular weights of the translational products detected were estimated using protein markers as described in Chapter II. Cylindrical gels were used only in double-labelling experiments, analysis of tritium labelled translational products, and to estimate the relative proportions of the different translational products. Slab gels were used routinely in later experiments when ^{14}C -leucine was available and detection of the

translational products was done by fluorography (Bonner and Laskey, 1974).

Electrophoresis in slab gels (Laemmli, 1970) was done using the Bio-rad model 220 vertical slab gel electrophoresis apparatus. The bisacrylamide:acrylamide ratio in the separating gel was lowered to 1:150 to prevent cracking when the gel was dried for fluorography. The slab gels cast were 1.5 mm thick and 14 cm wide, with the separating gels (15%) 10 cm deep, and the stacking gels (3%, acrylamide:bisacrylamide = 30:1) 1 cm deep. Protein samples (50 μ l) were electrophoresed at 10 mA/gel slab for 1½ hr and then at 20 mA/gel slab for 4½ to 5 hr constant current. After electrophoresis, the gels were stained in 0.25% CBB in methanol:acetic acid:water (50:10:40; V/V) at room temperature overnight and destained in methanol:acetic acid:water (35:7:58; V/V) for 48 hr at 37°C. They were then impregnated with PPO using the procedures described by Bonner and Laskey (1974). The gels were soaked in 20 volumes of dimethylsulphoxide (DMSO) (400 ml per gel) for 30 min. The process was repeated with another 20 volumes of fresh DMSO. The gels were then soaked in 4 volumes of DMSO containing 22% PPO (W/V) for 3 hr. Finally, they were washed with 3 changes of distilled water (20 volumes each) for 20 min each. All the above processes were done at room temperature on a shaker. The PPO-impregnated gels were dried under heat and vacuum for about 1 hr onto Whatman 3 MM chromatography papers (Maizel, 1971), and then exposed to Kodak RP 54 X-Omat X-ray films at -70°C to -80°C for various times. After exposure, the films were developed for 7 - 10 min and fixed for 5 min. The molecular weights of the translational products were estimated from their relative mobilities using protein markers co-electrophoresed on the same gel slab.

(v) Analysis of Translational Products by Tryptic Peptide Mapping

(a) Preparation of Tryptic Peptides of Translational Products

The individual ¹⁴C-leucine labelled translational product component of RNA-S (S1-S14) and the total ¹⁴C-leucine labelled

translational products of TRSV RNA-S, RNA-L and unfractionated TRSV RNA were analysed by tryptic peptide mapping. Recovery of the radioactive products was monitored at various stages of tryptic peptide preparation by counting samples of the products spotted on filter paper discs.

The various translational products of TRSV RNA-S were fractionated by discontinuous polyacrylamide gel electrophoresis followed by electrophoretic elution as described in Chapter IV, using 0.5 cm diameter x 12 cm long cylindrical gels. A series of 10 gels each loaded with 28,000 cpm of ^{14}C -leucine labelled translational products (= 15 μl reaction mixture) made up to 50 μl and dissociated in 6 M urea, 1% SDS and 2% MCE in 0.125 M Tris-HCl, pH 6.8, described above, were electrophoresed at 1 mA/gel for 1 hr followed by 3 mA/gel for 5 hr. After electrophoresis, one of the gels was sliced and counted to locate the positions of the various radioactive translational products relative to the BPB marker. Each of these translational products was located and excised from the unstained gels, then pooled, extracted and recovered electrophoretically from the gel slices as described in Chapter IV. Half a mg of unlabelled carrier TRSV protein was added to each eluted RNA-S translational product component. The various preparations of translational products and the ^{14}C -leucine labelled authentic TRSV coat protein were dialysed exhaustively against double distilled water and then freeze dried. The freeze-dried proteins were digested with trypsin as described in Chapter IV. The tryptic peptides were dissolved in pyridine:acetic acid:water (25:1:225, V/V) at approximately 5 - 10 mg protein/ml.

(b) Tryptic Peptide Mapping

The tryptic peptides of the TRSV RNA translational products were compared with those of the *in-vivo* labelled TRSV coat protein by separating them on the same thin layer cellulose plate by ascending chromatography as described in Chapter IV. The radioactive tryptic

peptides were detected by flurography as described by Randerath (1970). The dried thin-layer plates were impregnated with PPO by pouring 15 ml of a 7% PPO solution in diethyl ether (W/V) rapidly onto each plate. The plates were exposed to X-ray films as described for gels.

F. Preparation of Total Leaf RNA

Total single-stranded (ss)-RNAs were extracted from healthy and TRSV infected cucumber cotyledons 3 days after inoculation by a method modified from that of Hunter *et al.* (1976). The tissues were washed in double distilled water and blotted dry. They were snap frozen and ground to a powder in the presence of liquid nitrogen. The powdered tissues were scraped into a centrifuge tube while frozen and one volume of 0.1 M sodium acetate, pH 7.6, containing 10 mM EDTA and 2% SDS (W/V) and one volume of water saturated phenol (W/V) were added. The mixtures were shaken at 25°C for 30 min and then centrifuged at 5000g for 10 min. The aqueous phases collected were further extracted 3 times, each with an equal volume of phenol. RNAs were then precipitated with 2½ volumes of ethanol, washed 3 times with ethanol, freeze dried and resuspended in 20 mM Tris-acetate, 0.2 mM EDTA, pH 7.6, (TE) buffer at the rate of 1 ml/gm of tissues used. The RNA preparations were heated at 70°C for 3 min and then clarified at 5000g for 20 min. The RNAs were subjected to another cycle of ethanol precipitation as described above before being fractionated with LiCl. Equal volumes of RNA and 4 M LiCl were mixed and left at 4°C overnight for the ss-RNAs to precipitate which were recovered by centrifugation at 5000g for 20 min. The ss-RNA preparations were resuspended in TE buffer containing 0.1 M sodium acetate and subjected to a cycle of ethanol precipitation before being dissolved in TE buffer. The RNAs were then divided into small aliquots and stored at -80°C.

III. RESULTS

A. Molecular Weights of TRSV RNA Components Under Denaturing Conditions

The molecular weights of TRSV RNA-S and RNA-L were estimated by co-electrophoresing the TRSV RNAs with EMC and TMV RNAs and *E. coli* rRNAs in 98% formamide gels (Pinder *et al.*, 1974) (Fig. 1A). The mean molecular weights and their standard errors were calculated from four separate experiments and the values obtained are presented in Table 4. The results show that the molecular weights obtained under denaturing conditions are very similar to those obtained by other workers under non-denaturing conditions (Table 4). In all experiments, the RNA-L migrated only slightly slower than TMV RNA (Fig. 1A).

In separate experiments using QCMV RNAs as markers, the RNA-S migrated slower than QCMV RNA-1 (Fig. 1B). The molecular weight for QCMV RNA-1 under denaturing conditions have been estimated to be 1.27, 1.30 and 1.35 x 10⁶ by Mossop (1978); Habili (1974) and Peden and Symons (1973) respectively. Thus it is unlikely that the TRSV RNA-S has a molecular weight of less than 1.3 x 10⁶. In these experiments, the apparent molecular weights of TRSV RNA-S and RNA-L (1.36 and 2.12 x 10⁶ respectively) are both lower than those obtained using *E. coli* and TMV RNAs as markers (Table 4). However, these ^{values} are not as accurate as the latter since there is still some controversy regarding the molecular weights of CMV-RNAs (Francki, private communication) and that the TRSV RNAs are obtained by extrapolation when QCMV RNAs were used as markers.

Comparison of the molecular weights of TRSV RNAs obtained in this study with those obtained by Murant and Taylor (1978) under denaturing conditions using 8 M urea at 60°C shows that both methods yielded similar results (Table 4). It appears that the variation observed in RNA-L (Table 4) is due to variation in the markers used rather than in the methods used.

Figure 1. Estimation of the molecular weights of TRSV RNA-S and RNA-L in 98% formamide-4% polyacrylamide gels using the following RNA molecular weight markers:

A. EMC RNA (2.6×10^6)

U-1 TMV RNA (2.05×10^6)

E. coli 23 S rRNA (1.07×10^6)

E. coli 16 S rRNA (0.55×10^6)

TRSV RNA (20 μ g/gel) was co-electrophoresed with the markers (10 μ g/RNA).

The molecular weights of RNA-S and RNA-L were estimated from the mean relative mobilities of the RNAs calculated for each of 4 separate experiments each involving 3 replicate gels.

B. QCMV RNAs

$Q_1 = 1.27 \times 10^6$

$Q_2 = 1.13 \times 10^6$

$Q_3 = 0.82 \times 10^6$

$Q_4 = 0.35 \times 10^6$

TRSV RNA (20 μ g/gel) was co-electrophoresed with QCMV RNA markers. The molecular weights of RNA-S and RNA-L were estimated from the mean relative mobilities calculated from 3 replicate gels.

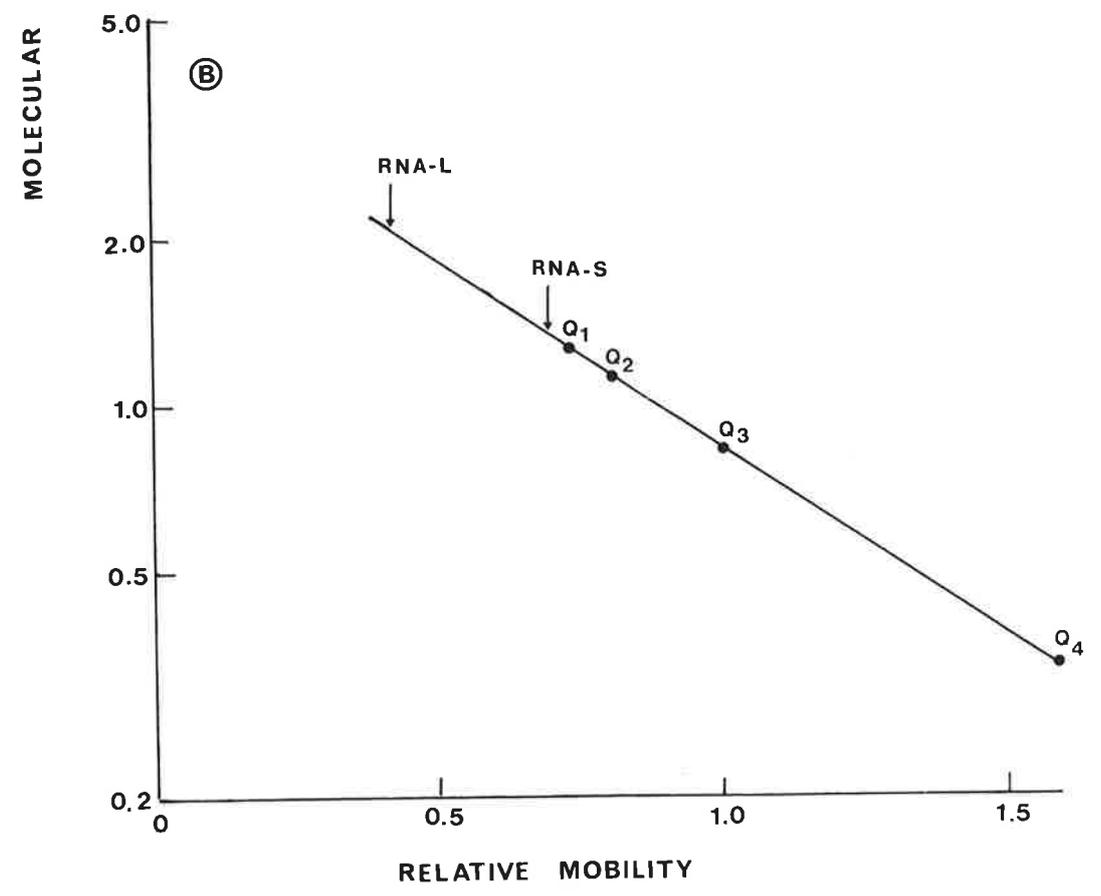
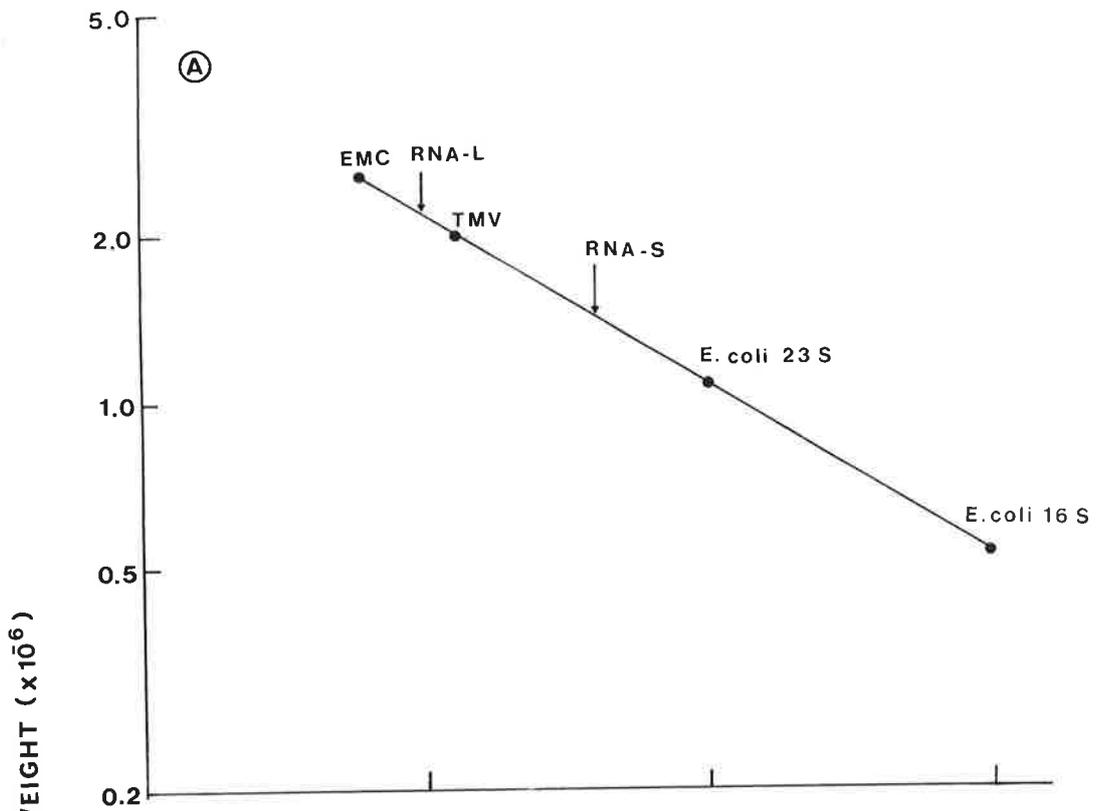


Table 4

Molecular Weights of TRSV RNA Components

Reference	Markers	RNA-S ($\times 10^6$)	RNA-L ($\times 10^6$)	Method
Diener and Schneider (1966)	None	1.2	2.2	Sedimentation (S_{20}, w)
Murant <i>et al.</i> (1972)	Pea tissue rRNAs RRV RNAs	1.4	2.4	Aqueous-SDS polyacrylamide gels
Rezaian and Francki (1973)	Tobacco leaf rRNAs	1.4	2.3	Aqueous composite polyacrylamide- agarose gels
This work ^a	EMC and TMV RNAs and <i>E.</i> <i>coli</i> rRNAs	1.44 \pm 0.02	2.26 \pm 0.02	Formamide poly- acrylamide gels
Murant and Taylor (1978)	TMV RNA <i>E. coli</i> rRNAs	1.46 \pm 0.02	2.08 \pm 0.01	60°C in poly- acrylamide gels containing 8M urea

^a The molecular weights of TRSV RNA-S and RNA-L were estimated from their relative mobilities with respect to the RNA molecular weight markers as shown in Figure 1.

The molecular weight values were first estimated from each experiment containing three replicate gels and the average molecular weight values were calculated from 4 separate experiments.

B. Infectivity of TRSV RNA Components

(i) Fractionation of TRSV RNA-S and RNA-L

Two cycles of agarose electrophoresis was found to be better than one cycle of polyacrylamide gel electrophoresis followed by one cycle of agarose gel electrophoresis in the fractionation of TRSV RNA-S and RNA-L. The RNAs were more readily extracted from agarose than from polyacrylamide resulting in higher recovery of the RNA components. In the first cycle, the RNA loaded was only heated for 1 min at 70°C to reduce contamination of RNA-S by broken RNA-L and to dissociate as much aggregated RNA as possible. In the second cycle, they were heated at 70°C for 3 min to dissociate nicked molecules still held together by secondary structures and aggregates of RNA-S. Yields of up to 25% were obtained after the first cycle and up to 20% of this was recovered after the second cycle.

(ii) Infectivity of TRSV RNA-S and RNA-L

The infectivity of the fractionated TRSV RNA-S and RNA-L were tested after the first cycle and after two cycles of gel fractionation. Table 5 shows that although some infectivity was still observed with TRSV RNA-L after one cycle of fractionation, it was stimulated by TRSV RNA-S by up to 10 fold. However, after the second cycle of fractionation no infectivity was produced by either RNA-S or RNA-L alone but a mixture of the two RNA components produced substantial infectivity (Table 5). It was observed that the infectivity obtained with the mixtures of fractionated RNA were much lower than the unfractionated RNA even when the proportion of RNA-S:RNA-L was 2:1 (approximate to the natural relative proportion of RNA-S and RNA-L). Since the unfractionated TRSV RNA was more infectious than the once fractionated RNA mixtures which in turn were more infectious than the twice fractionated RNA mixtures, it indicates that infectivity was reduced in the course of the fractionation procedures.

Table 5

Infectivity of the isolated TRSV RNA components^a

RNA component	Conc. ($\mu\text{g/ml}$)	Total number of lesions in 8 replicate half-leaves			
		Once fractionated RNA components		Twice fractionated RNA components	
		Expt. 1	Expt. 1	Expt. 2	Expt. 3
RNA-S	10	4	-	-	0
	25	12	0	0	0
	50	-	0	0	-
RNA-L	10	14	-	-	0
	25	44	0	0	0
	50	-	0	0	-
RNA-S	5 + 5	161	-	-	64
+	12.5+				
RNA-L	12.5	433	144	104	136
	25 + 25	-	256	160	-
Unfractionated TRSV RNA	10	1008			960
	25	2304	2012	2848	2072
	50	-	3816	4280	-

^a All experiments were done using the Latin square design, using 8 replicate cowpea half-leaves per treatment. The values presented are the total number of lesions in the 8 half-leaves.

(iii) Effects of Heating on Aggregation and Infectivity of TRSV RNA

Equal amounts of unfractionated TRSV RNA (50 µg) was analysed in agarose gels before and after it had been heated at 70°C for 3 min prior to electrophoresis. Results of the experiment showed that at least two extra high molecular weight RNA bands (A and B, Fig. 2, gels b and d) were observed when unheated TRSV RNA was electrophoresed in agarose gels. Heating the RNA immediately prior to electrophoresis however removed bands A and B and at the same time increased the intensity of the lower molecular weight bands (RNA-S and RNA-L) (Fig. 2, gels c and e). The molecular weights of the RNAs in bands A (RNA-A) and B (RNA-B) estimated from 3 electrophoresis experiments using *E. coli* rRNAs and TMV RNA as markers showed that their molecular weights were about 2.8×10^6 and 3.4×10^6 respectively (Fig. 3). Thus RNA-A is probably a dimer of RNA-S while RNA-B is probably an aggregate of one molecule of RNA-S and one of RNA-L.

The same preparation of TRSV RNA used for the above experiment was also heated for various time periods and at various concentrations and then analysed for infectivity. The results (Fig. 4) show that heating did not reduce the infectivity of the RNA preparations, confirming that the two lowest molecular weight TRSV RNA bands observed in the gels (Fig. 2) were not degraded RNAs.

C. In-vitro Translation of TRSV RNAs(i) Basic Factors Affecting the Efficiency of Incorporation of Radioactive Amino Acids Into Translational Products

The choice of a suitable method for termination of translation reaction is important in translation of TRSV RNAs. Several methods have been widely used in various studies of cell-free translation of viral RNAs. These included (a) precipitation with hot TCA (Marcu and Dudock, 1974; Marcus *et al.*, 1974); (b) hydrolysis with NaOH followed by TCA precipitation (Schwinghamer and Symons, 1975); (c) RNase treatment followed by cold TCA precipitation (Skotnicki *et al.*, 1976) and (d)

Figure 2. Effect of heating TRSV RNA prior to electrophoresis on its electrophoretic profile in agarose gels

Samples of unfractionated TRSV RNAs were either not subjected to heating or were heated at 70°C for 3 min prior to electrophoresis in 2% agarose gels. Electrophoresis was carried out at 2.5 mA/gel for 4 hr.

Gel a, *E. coli* rRNAs + TMV RNA (10 µg + 10 µg);

gel b, TRSV RNA unheated (50 µg);

gel c, TRSV RNA heated (50 µg);

gel d, TRSV RNA unheated + *E. coli* rRNAs and TMV RNA (50 µg + 10 µg + 10 µg);

gel e, TRSV RNA heated + *E. coli* rRNAs + TMV RNA (50 µg + 10 µg + 10 µg).

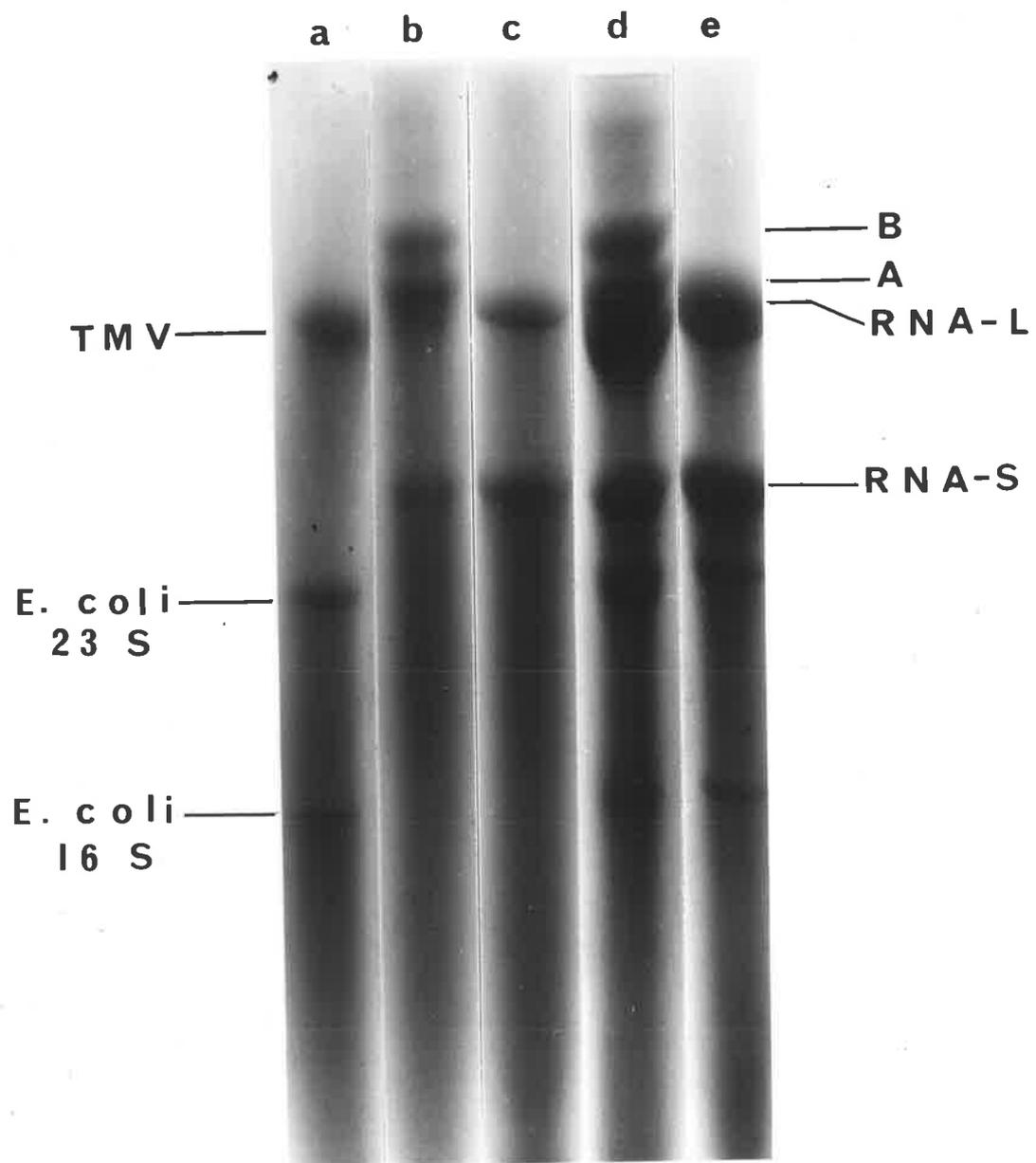


Figure 3. Estimation of the molecular weights of TRSV RNA species A and B in agarose gels

Unfractionated TRSV RNAs were co-electrophoresed with TMV and *E. coli* rRNAs in 2% agarose gels as described in Figure 2 and were not heated prior to electrophoresis in order to expose the RNA bands A and B. The molecular weights of A and B were estimated from the average relative mobilities of the RNA species with respect to TRSV RNA-S calculated for each of three separate experiments each containing 4 replicate gels. TRSV RNA molecular weights were taken as 1.44×10^6 for RNA-S and 2.26×10^6 for RNA-L.

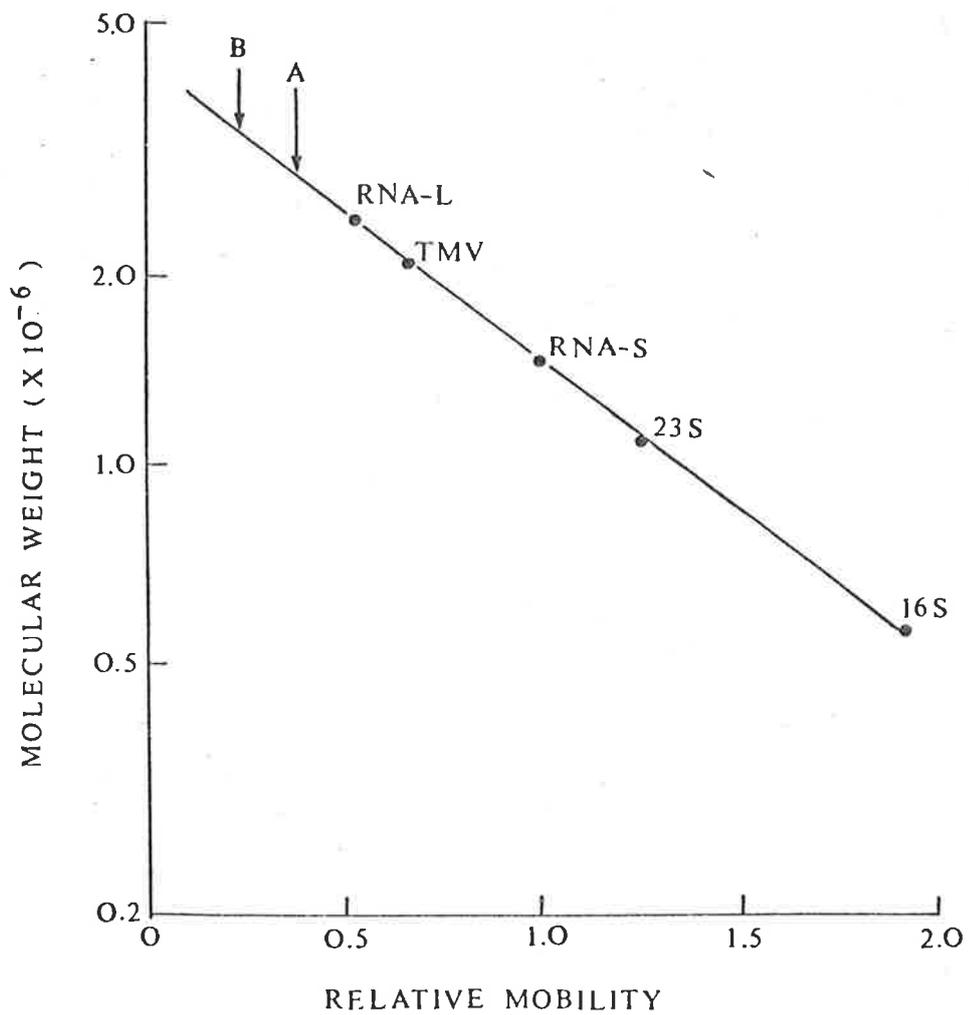
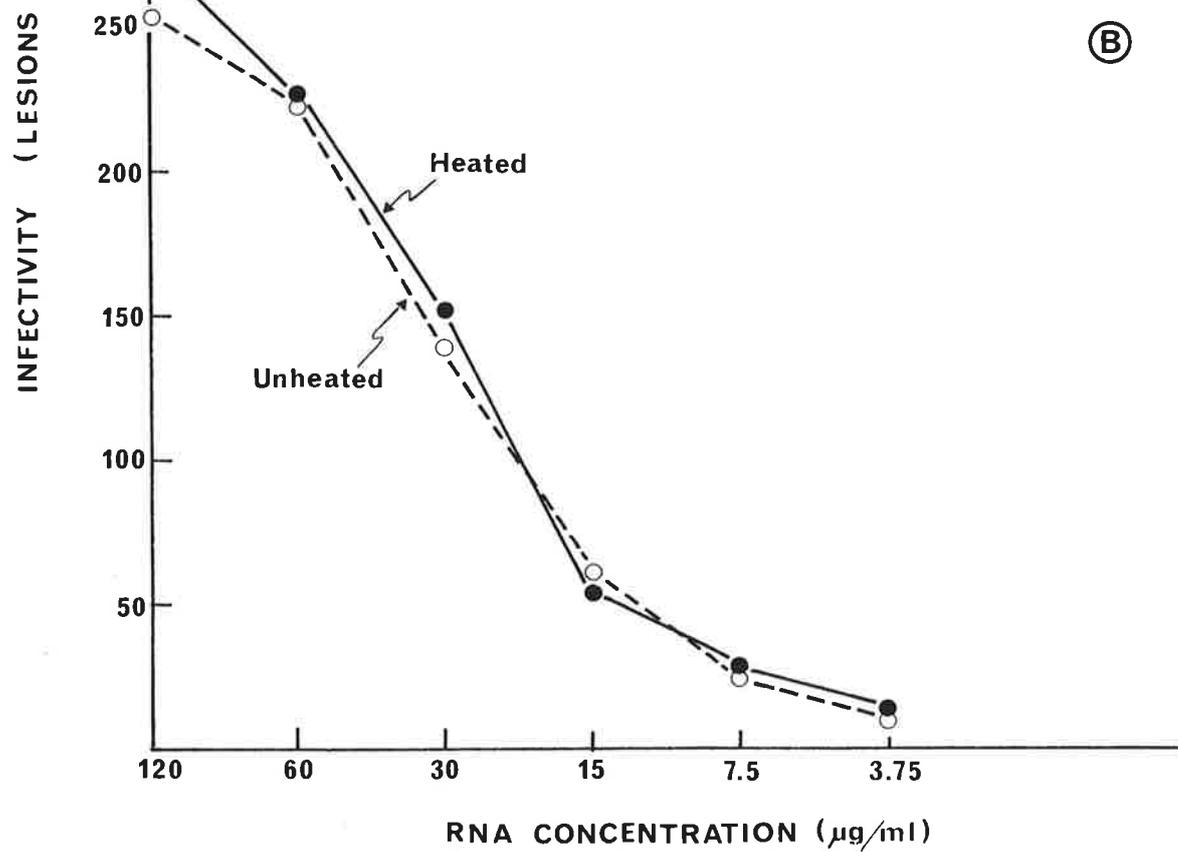
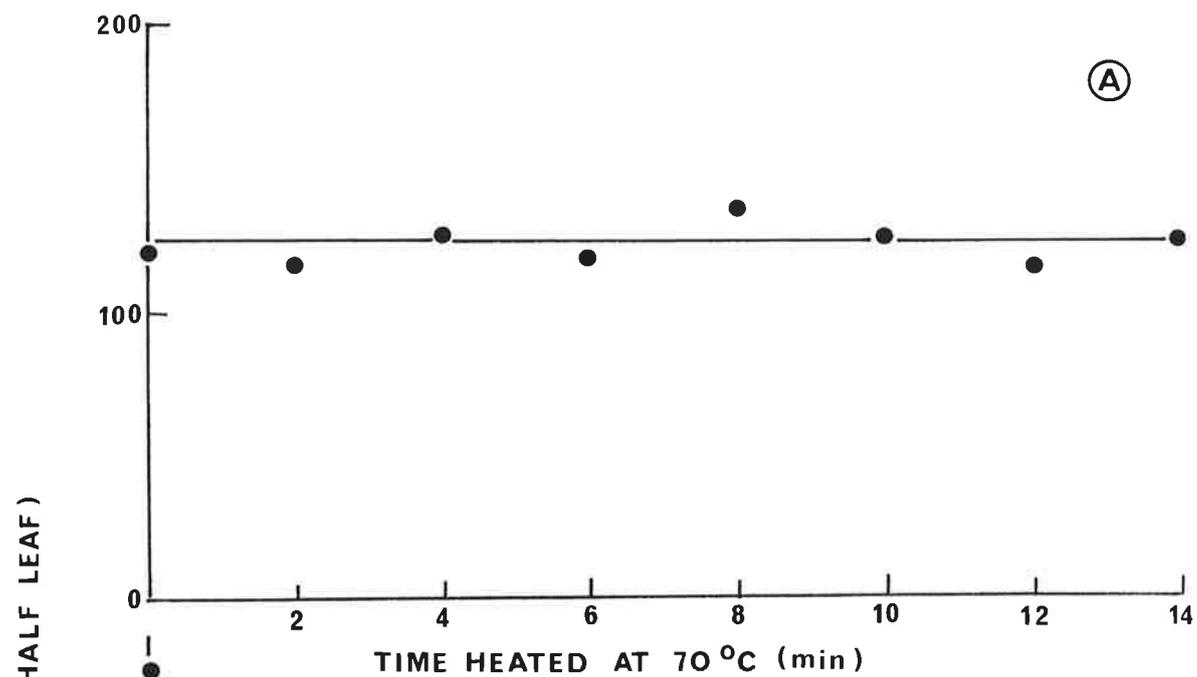


Figure 4. Effect of heating TRSV RNA on infectivity

A. Samples of unfractionated TRSV RNA (25 µg/ml) were heated for various times at 70°C as described in the text. Each RNA sample was inoculated on 16 cowpea half-leaves using the Latin Square design and the average lesion number per half-leaf was plotted.

B. Unfractionated TRSV RNA was diluted two fold using an initial RNA concentration of 120 µg/ml. Each diluted RNA preparation was either unheated or heated at 70°C for 3 min prior to inoculation on 6 cowpea half-leaves per treatment using the Latin square design.



precipitation with cold TCA (Shih and Kaesberg, 1973). The first three methods hydrolyse all aminoacyl-t-RNAs while method (d) estimates amino acids incorporation into both proteins and t-RNAs. However, the first two have a disadvantage that there may be hydrolysis of acid and alkali-labile peptide bonds in the translational products by hot TCA and NaOH. The third method is rather time consuming for routine work involving numerous samples and may be complicated by side reactions during further incubation with RNase, e.g. pronase activity. Thus in this study method (d) was used to estimate total incorporation of radioactive amino acids into proteins because of its simplicity and suitability to the analysis of large numbers of assays in a limited time. It is less likely to cleave acid-labile bonds which are found to be present in TRSV coat protein (Chapters III and IV). For analysis of the translational products, the translation reactions were stopped by rapid cooling at -15°C or RNase treatment (50 $\mu\text{g}/\text{ml}$) rather than TCA or NaOH precipitation which may cause irreversible denaturation of synthesized polypeptides and production of protein fragments.

Background counts (zero-time incubations) were rather high and variable at first. This was found to be due to (a) reaction mixture seeping into the edge of the dry GF/A filter discs and (b) rapid binding of radioactive leucine to t-RNA even at 0°C . These problems were overcome by wetting the filter discs prior to addition of reaction mixtures and precipitating the zero-time samples immediately on addition of the extracts; thus reducing background counts from over 400 cpm to 150 cpm/50 μl in the Marcu and Dudock system using ^3H -leucine.

Radioactivity due to t-RNA bound labelled leucine was confirmed by the observation that lower counts were obtained in the -mRNA treatments when translation reactions were terminated by RNase digestion (Table 6). The results indicate that about 80% of endogenous incorporation is due to t-RNA bound ^{14}C -leucine. There appears to be negligible amounts of t-RNA bound ^{14}C -leucine in the zero-time, TRSV and QCMV RNA treatments.

Table 6

Effect of RNase digestion on recovery of acid-insoluble radioactivity
from translation reaction mixtures

Treatment	cpm/50 μ l		% difference
	-RNase ^a	+RNase ^b	
Background	1100	1200	+9%
-mRNA	8850	2750	-69%
QCMV RNA	142,000	141,500	-0.5%
TRSV RNA	196,500	197,500	+0.5%

All translation reactions were done in duplicate using 0.25 μ Ci/50 μ l ¹⁴C-leucine as described in Table 3. Counts are the means of duplicate assays which differed by less than 10%.

- ^a Reactions were terminated by freezing at -15°C for 30 min and then precipitated with cold TCA.
- ^b Reactions were terminated by RNase digestion (50 μ g/ml) at 30°C for 30 min and then precipitated with cold TCA.

(ii) Translation of Unfractionated TRSV RNA Using the Davies and the Marcu and Dudock Translation Systems

In these studies, the efficiency and optimum requirements, and the translational products synthesized by the two systems were compared.

(a) Properties of the Translational Systems

Typical uv spectra of the two types of wheat extracts (Fig. 5) show that the Marcu and Dudock extract had a higher OD_{260/280} ratio (1.54) than that of Davies extract (1.37). This may be due to the presence of more t-RNA in the Marcu and Dudock extract. Both extracts had to be stored at below -70°C to maintain activity but storage in liquid nitrogen did not increase the shelf-life of the extracts when compared with those stored at below -70°C. Under optimum conditions, the Davies extracts had a half-life of only 2 weeks while Marcu and Dudock extracts retained their activity for up to 6 months. Both types of extracts varied in activity from one preparation to another (Table 7) but the Davies extract was more variable.

Both translational systems had low background radioactivity and endogenous incorporations. Results obtained with the Davies system (Fig. 6A, e) show that the background did not vary significantly with increasing specific radioactivity when the amount of radioisotope is constant but did increase with increasing amounts of radioisotope in the reaction mixture (Fig. 6A, f; 6B, f). This suggests that the background radioactivity was not due to incorporation of leucine to protein but are due to incomplete washing of the free radioisotopes. On the other hand, in both systems the endogenous incorporations were dependent on specific radioactivity indicating that they are due to active (enzyme-catalysed) incorporation of ³H-leucine.

(b) Optimum Conditions for Translation in the Two Systems

Attempts were made to do all optimisation experiments under comparable conditions e.g. using the same RNA and extract preparations. The results are summarised in Figs. 6 and 7. In both systems, endogenous

Figure 5. Ultraviolet spectrums of the wheat germ cell-free extracts prepared by the methods of Davies *et al.* (1977) and Marcu and Dudock (1974)

The final extracts (S-30) were diluted 50 times in double distilled water before being measured with the Unicam SP 1800 ultraviolet spectrophotometer.

Absorbance for the extracts were:

Davies extract, $OD_{260} = 0.995$, $OD_{280} = 0.725$;

Marcu and Dudock extract, $OD_{260} = 1.76$, $OD_{280} = 1.14$.

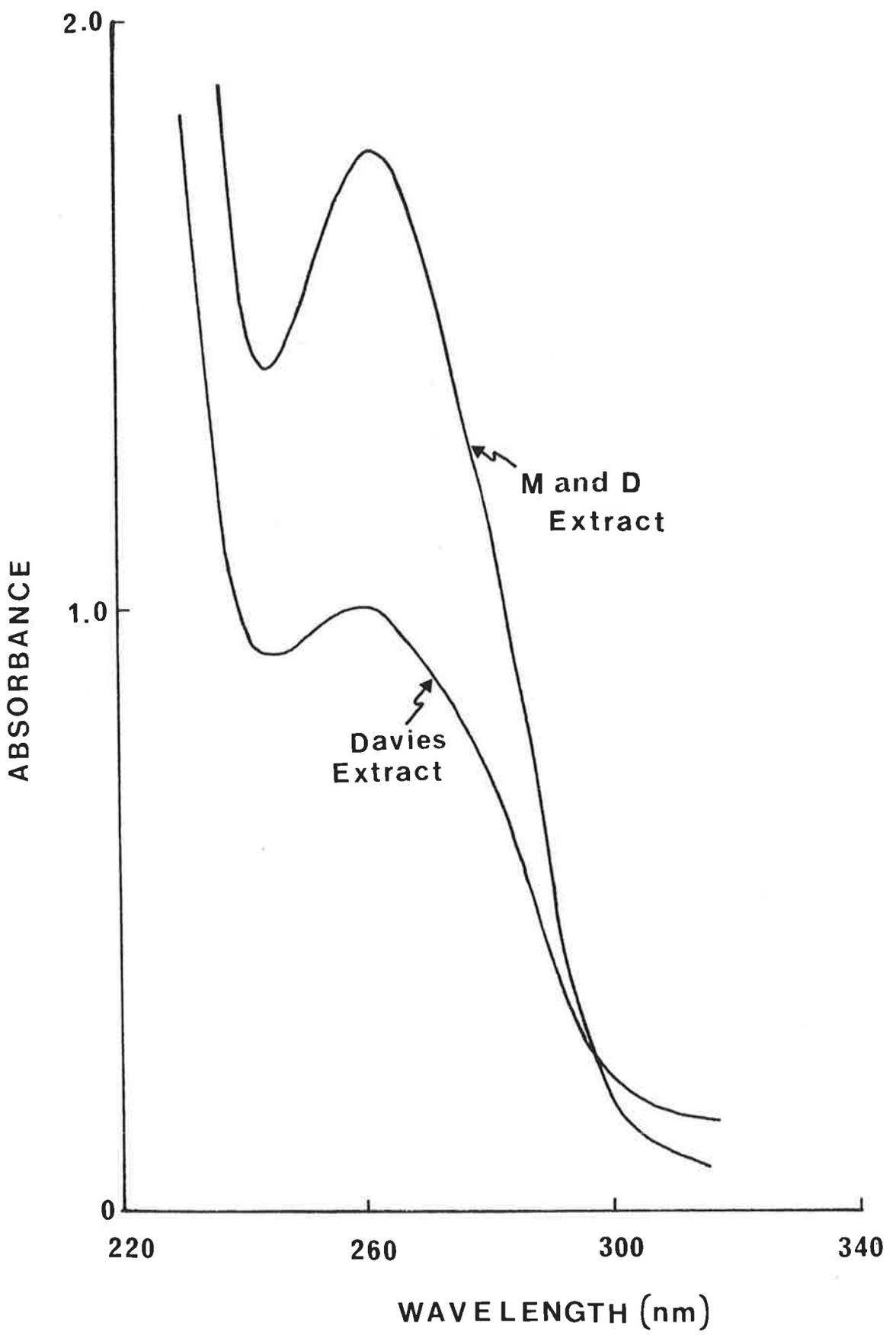


Table 7Incorporation of ^3H -leucine by various extract preparations^a

Davies Extracts				Marcu and Dudock Extracts			
Extract	-mRNA	TRSV	QCMV	Extract	-mRNA	TRSV	QCMV
I	150	850	1600	I	1200	33,500	22,600
II	180	1450	2580	II	1000	26,200	22,700
III	160	680	1150	III	1280	33,100	28,700
IV	180	1470	2750	IV	1460	33,200	30,200
V	170	870	1670	IVa	1450	26,800	24,300
VI	220	1798	2810				
VIa	200	380	705				

^a The results presented were from various experiments testing the extract activity under standard optimum conditions presented in Table 3. In all experiments the new extract was tested with the previous extract as control and using fresh reagents.

Extract VIa is extract VI which had been stored at -70°C for 3 weeks and IVa is extract IV stored at -70°C for 4 months.

Figure 6A. Optimisation of conditions for the translation of unfractionated TRSV RNA in the Davies translation system

The following reagent concentrations were used unless otherwise stated: Mg^{++} , 3.0 mM; K^+ , 90 mM;

Spermidine, 0.4 mM; RNA, 2 μ g/50 μ l;

3H -leucine, 800 μ Ci/ μ mole, 1.0 μ Ci/50 μ l.

- a. Mg^{++} optimum; \pm spermidine, 0.4 mM.
- b. K^+ optimum; no spermidine added, and 0.5 μ Ci/50 μ l 3H -leucine.
- c. Spermidine optimum.
- d. RNA optimum; two preparations of RNAs were tested, A and B.
- e. Specific radioactivity; 0.5 μ Ci/50 μ l was used.
- f. Leucine concentration; 800 μ Ci/ μ mole specific radioactivity was maintained.

○ ——— ○ ——— ○ background radioactivity.
● - - - ● - - - ● endogenous (-mRNA) incorporation.
● ——— ● ——— ● TRSV RNA directed incorporation.

^3H -LEUCINE INCORPORATED (cpm / 50 μl)

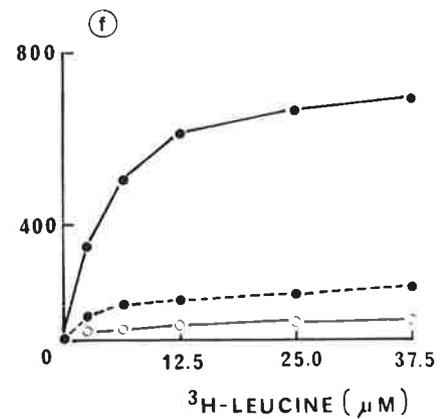
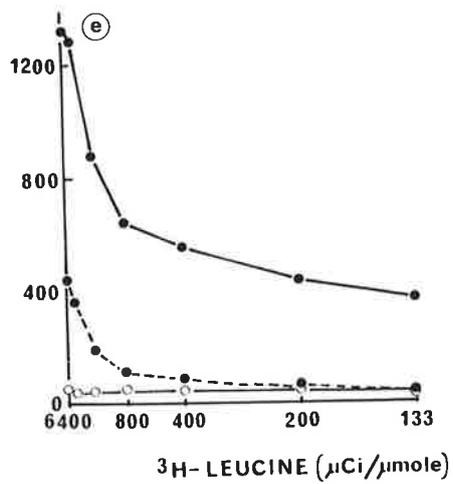
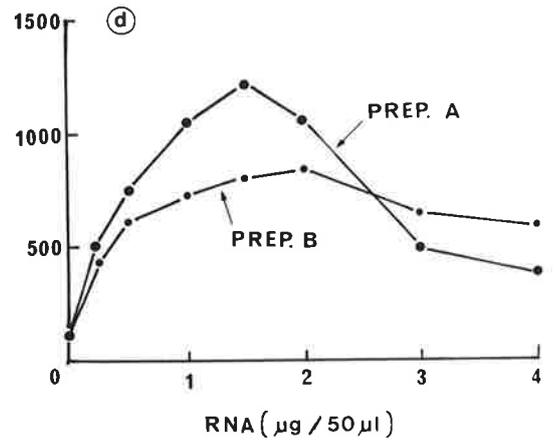
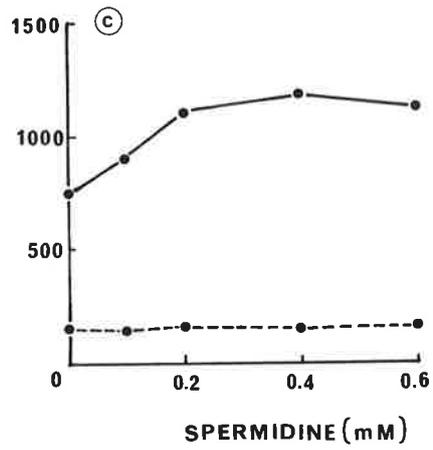
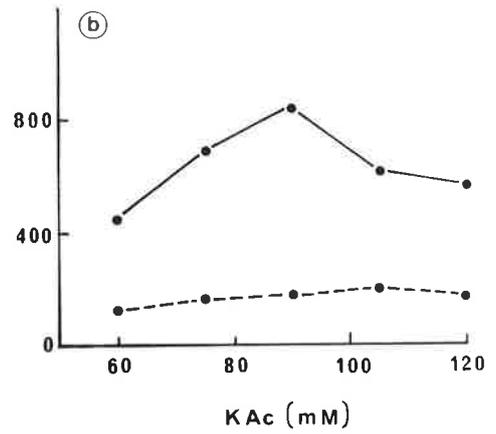
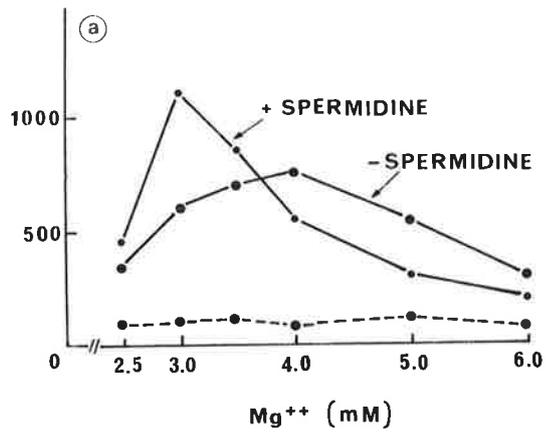
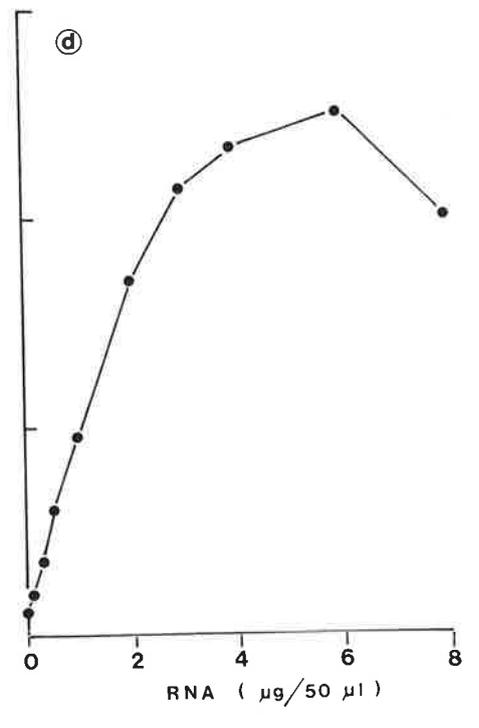
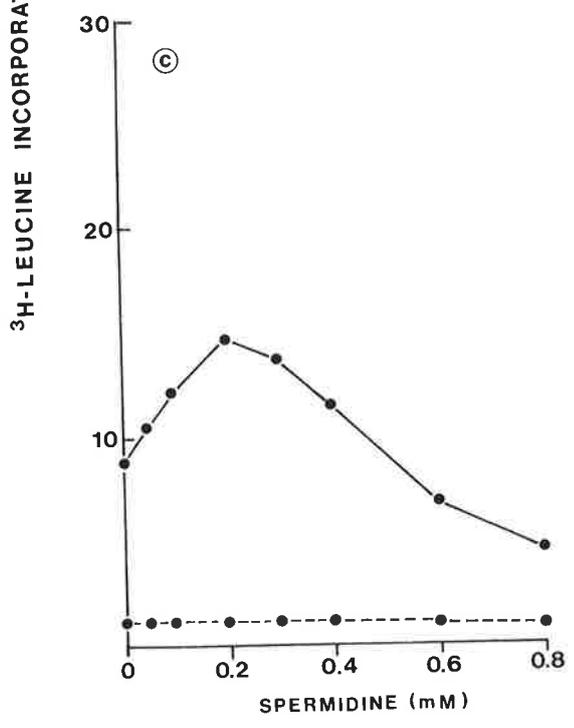
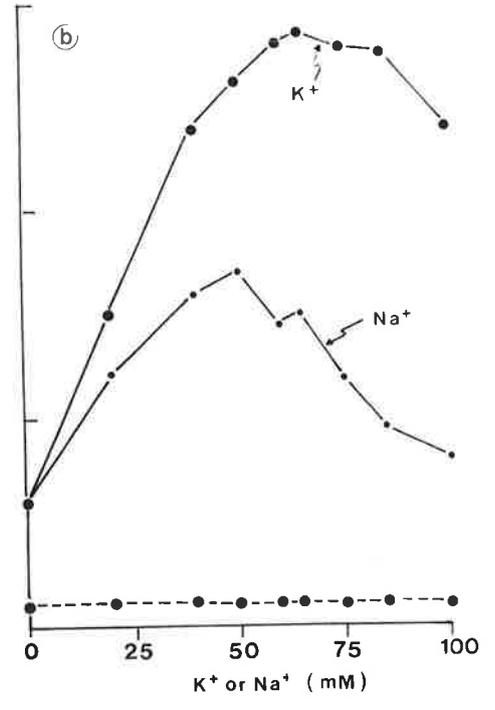
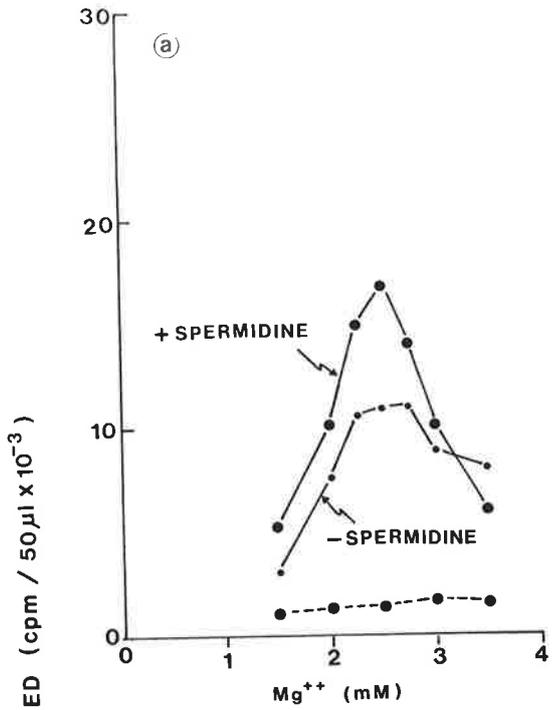
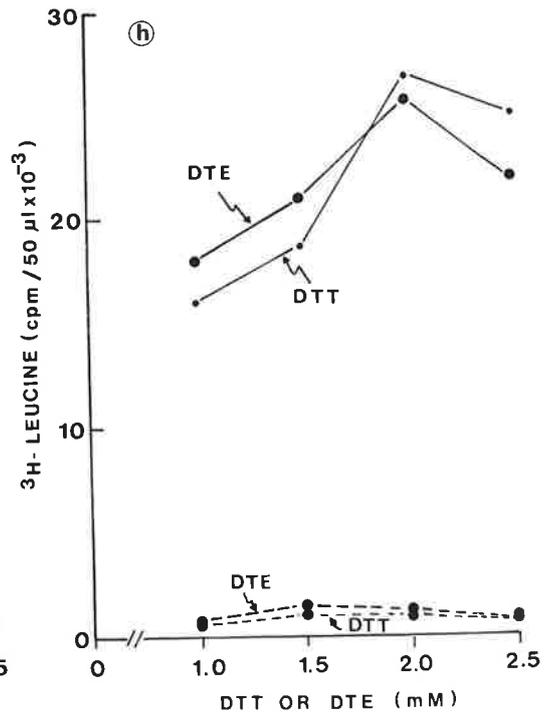
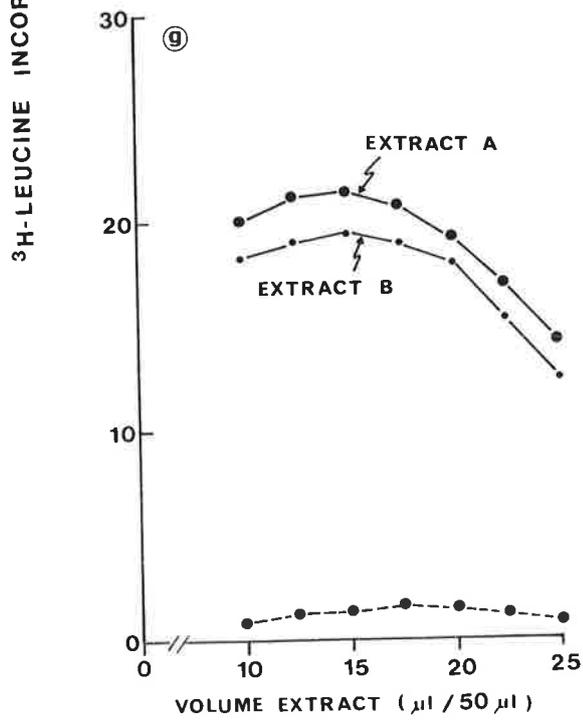
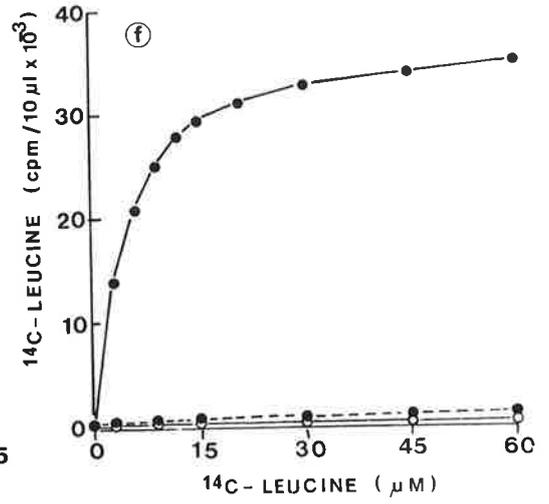
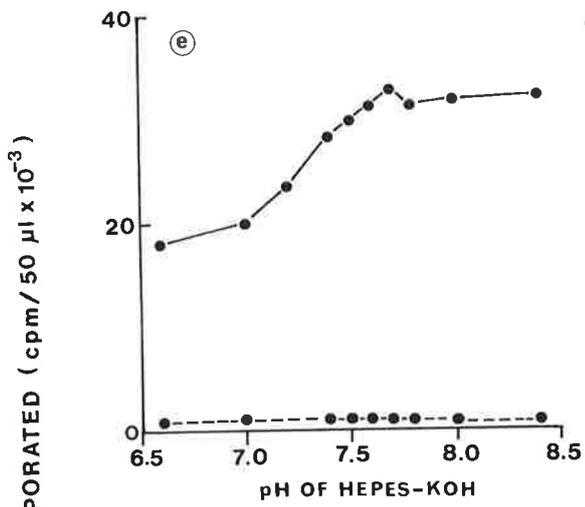


Figure 6B. Optimisation of conditions for the translation of unfractionated TRSV RNA in the Marcu and Dudock translation system

The following reagent concentrations were used in these experiments unless otherwise stated: 2.5 mM Mg^{++} ; 100 mM K^+ ; 4 μg TRSV RNA/50 μl ; 0.2 mM spermidine; 1 μCi /50 μl 3H -leucine (54 Ci/mmmole); 15 μl extract/50 μl .

- a. Optimisation of Mg^{++} in the presence and absence of 0.2 mM spermidine; 2 μg /50 μl RNA was used.
 - b. Optimisation of monovalent ions; amounts of k^+ and Na^+ shown are mM of the ions added to 36 mM K^+ supplied by the extract. .
 - c. Optimisation of spermidine; 2 μg RNA/50 μl .
 - d. Optimisation of RNA concentration.
 - e. Optimisation of pH; the pH shown is the pH of the HEPES-KOH buffer used in the reaction; pH of the extract used was 7.0.
 - f. Optimisation of leucine concentration; ^{14}C -leucine (312 $\mu Ci/\mu mole$) was used.
 - g. Extract concentration; all reagents present in the extract have been taken account of (i.e. 20 mM HEPES, 100 mM K^+ , 2.5 mM Mg^{++} and 2 mM DTE were maintained in all reactions).
 - h. Reducing agents, DTT and DTE, were tested.
- — ○ — ○ — ○ background radioactivity.
● - - - - ● - - - - ● - - - - ● endogenous (-mRNA) incorporation.
● — ● — ● — ● TRSV RNA directed incorporation.





incorporations did not vary significantly with reagent concentrations. In contrast, after optimisation of reagent concentrations, incorporation by TRSV RNA increased significantly in both systems. The optimum K^+ ion concentrations were very similar for both systems while Mg^{++} ion optimum varied only slightly (Table 3). The effect of Na^+ ion was tested using the Marcu and Dudock system only, and the results showed that although Na^+ ions increased translation activity in the absence of K^+ , its optimum concentration was only 50 mM (+ 36 mM K^+ in extract) and was not as efficient as K^+ (Fig. 6B, b). TRSV RNA was translated efficiently under a wide range of K^+ concentrations in both systems, similar to CpMV (Davies *et al.*, 1977). Spermidine was found to stimulate translation in both systems at lower concentrations of Mg^{++} (Fig. 6A, a; 6B, a) to a maximum of 80% (Fig. 6A, c; 6B, c). In the Marcu and Dudock system, the spermidine did not alter the Mg^{++} optimum while in the Davies system, absence of spermidine required a higher Mg^{++} concentration which, however, did not compensate for the spermidine effect.

A higher optimum concentration of reducing agents was obtained for the Marcu and Dudock system (Fig. 6B, h). More mRNA was required to saturate the Marcu and Dudock system than the Davies system (Fig. 6A, d; 6B, d). These results may be due to the greater translational activity associated with the former system. However, the mRNA concentration optimum was not affected by the activity of the extract preparation used but was dependent on the activity of the RNA preparations (Fig. 6A, d). These results suggest that the RNA concentration optimum is probably dependent on the relative abundance of ribosomes and intact RNA molecules binding to them.

The Marcu and Dudock system translated TRSV RNA efficiently over a wide pH range (Fig. 6B, e) but the optimum pH is about pH 7.4. Extract concentration is also an important factor affecting efficient translation of TRSV RNA and inhibition occurred with increasingly high concentrations (Fig. 6B, g).

The effects of specific radioactivity was investigated with the Davies system. Incorporation of ^3H -leucine decreased rapidly as specific radioactivity of the radioisotope was decreased (Fig. 6A, e), indicating that leucine readily saturates the reaction mixture. Addition of limiting amounts of non-radioactive leucine immediately decreased incorporation rapidly, indicating that endogenous leucine concentration in the extract was negligible. In another experiment, the results showed that the saturation concentration of leucine was about $12.5\ \mu\text{M}$ in the Davies system (Fig. 6A, f) and about $30\ \mu\text{M}$ in the Marcu and Dudock system (Fig. 6B, f).

(c) Kinetics and Efficiency of Translation of TRSV RNAs in the Two Systems

The kinetics of amino acid incorporation by the two translation systems showed that at 30°C active translation lasted for only 30 - 45 min in the Davies system but continued for a period of 60 - 75 min in the Marcu and Dudock system after optimisation (Fig. 7). Thus, the greater efficiency of incorporation of ^3H -leucine by the Marcu and Dudock system may be due to its faster rate of incorporation as well as longer period of active translation. Further studies with the Marcu and Dudock system (Fig. 7B) show that at 25°C , the initial rate of translation was lower than at 30°C but active translation was maintained for a longer period at the lower temperature. This may be due to RNAs, enzymes and co-enzymes required for translation being more stable at the lower temperature.

Translational activity of TRSV RNA in the Marcu and Dudock system was increased by more than 3 fold after optimisation (Table 8). Comparatively, a much lower increase was obtained with the Davies system after optimisation (see Fig. 6A). The results in Table 9 show that both the extract and reagent systems of Marcu and Dudock were more efficient than the extract and reagent systems of Davies in translating TRSV and CMV RNAs. TRSV RNA stimulated incorporation of leucine by up to 81 fold in the Marcu and Dudock system but only up to 13 fold in the Davies

Figure 7. Kinetics of translation of unfractionated TRSV RNA

Translation reactions were done in duplicates and separate reactions were done for each incubation time.

A. Davies system: Translation was done under optimal conditions as described in Table 3.

Legends for the curves are as in Fig. 6A.

B. Marcu and Dudock system:

• Curve a, endogenous (-mRNA) incorporation after optimisation. The translation conditions were as in Table 3. Similar curve was obtained before optimisation (see curve b below).

• Curve b, TRSV RNA directed incorporation before optimisation. The conditions were as in Table 3 except that 2 $\mu\text{g}/50 \mu\text{l}$ RNA and no spermidine were used. Translation was done at 30°C.

• Curves c and d, TRSV RNA directed incorporation after optimisation. The translation conditions were as given in Table 3. Translation reactions were done at 25°C (curve c) and 30°C (curve d).

³H-LEUCINE INCORPORATED

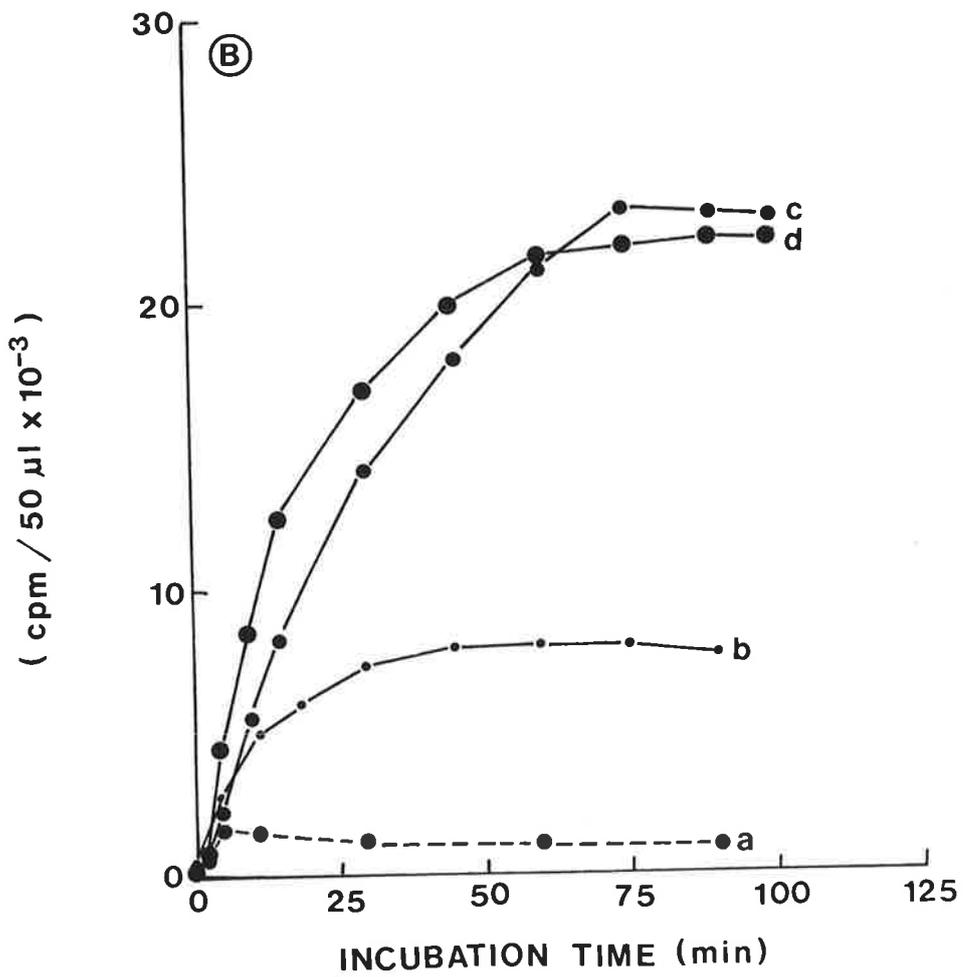
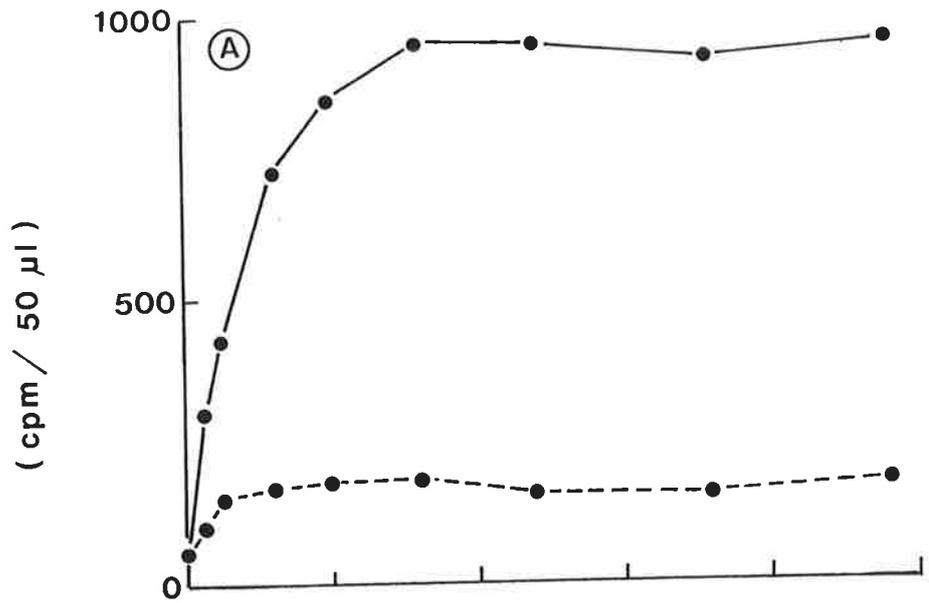


Table 8

Effect of Optimisation of Translation Conditions on Incorporation of ^3H -leucine by Unfractionated TRSV RNA in the Marcu and Dudock System

	Before Optimisation ^a			After Optimisation ^b		
	Nett cpm/ 50 μ l	Stimulation	% Incorporation ^c	Nett cpm/ 50 μ l	Stimulation	% Incorporation ^c
-mRNA	1000	1	0.8%	1200	1	0.9%
TRSV RNA	9450	9.5	7.7%	33,600	31	27%
QCMV RNA ^d	28,000	28	23 %	26,600	25	22%

The same extract preparation was used in both experiments.

^a Translation conditions before optimisation were:

RNA concentrations, TRSV 2 $\mu\text{g}/50 \mu\text{l}$; CMV 1.5 $\mu\text{g}/50 \mu\text{l}$; no spermidine added, pH of HEPES-KOH = 7.4.

^b Conditions after optimisation were:

RNA concentrations: TRSV 4 $\mu\text{g}/50 \mu\text{l}$; CMV 1.5 $\mu\text{g}/50 \mu\text{l}$; 0.2mM spermidine, pH of HEPES-KOH = 7.6.

^c Based on the estimation that 1 μCi ^3H -leucine produced 123,000 cpm.

^d QCMV RNA was translated under similar conditions as TRSV RNA except that the RNA concentrations used were optimal for the individual RNAs.

Table 9

Amino acid incorporation directed by unfractionated TRSV and QCMV RNAs
under various combinations of extract and optimised reagent systems

Systems ^a	Radioisotope	cpm/50 μ l				Stimulation ^b	
		Zero time	-mRNA	TRSV RNA ^c	QCMV RNA ^c	TRSV RNA	QCMV RNA
Davies extract	³ H-leu (800 μ Ci/ μ mole)	85	185	1280	3240	13	32
+ Davies reagents	³ H-leu (54 Ci/ mmole)	105	810	2800	5600	4	8
Davies extract	¹⁴ C-leu (324 μ Ci/ μ mole)	980	1600	4230	8260	7	13
+ Marcu and Dudock reagents	³ H-leu (54 Ci/ mmole)	110	960	3400	6200	4	7
Marcu and Dudock extract	³ H-leu (800 μ Ci/ μ mole)	120	235	3200	3840	28	33
+ Davies reagents							
Marcu and Dudock extract	³ H-leu (800 μ Ci/ μ mole)	100	290	8400	7700	44	40
+ Marcu and Dudock reagents	³ H-leu (54 Ci/ mmole)	190	1200	30,700	28,000	31	28
	¹⁴ C-leu (324 μ Ci/ μ mole)	1200	3000	146,000	128,000	81	71

³H-leucine used was 1 μ Ci/50 μ l reaction volume.

¹⁴C-leucine used was 0.25 μ Ci/50 μ l reaction volume.

^a Davies reagent system included all reagents used in the Davies translation system excluding extract, mRNA and radioisotope. The Marcu and Dudock reagent system included all the reagents used in the Marcu and Dudock translation system excluding the extract, mRNA and radioisotope.

^b Stimulation is the ratio of mRNA directed incorporation to endogenous (-mRNA) incorporation.

^c QCMV RNA was translated under conditions optimised for TRSV RNA except that the optimum CMV RNA concentration (1.5 μ g/50 μ l) was used. TRSV RNA concentrations used were those optimal for the respective reagent systems (see Table 3).

system (Table 9). The Davies system appears to be more efficient in translating CMV and TAV RNAs than TRSV RNA while the Marcu and Dudock system appears to translate all these RNAs efficiently (Tables 9 and 10). These results and results of analysis of the translational products described below suggest that the Marcu and Dudock system is more efficient in translating large mRNAs than the Davies system which will only translate small mRNAs efficiently. Thus the cell-free systems as well as the size of the mRNAs are important factors affecting the efficiency of translation of viral RNA *in-vitro*.

(d) Analysis of Translational Products

The translational products of unfractionated TRSV RNAs synthesized in both translation systems under optimised conditions were analysed in polyacrylamide gels. In both systems, many radioactive peaks, each apparently representing one or more protein products, were detected (Figs. 8 and 9, and compare Fig. 13) and their molecular weights and relative proportions were estimated and presented in Table 11. The two systems appeared to translate similar ranges of products. The major difference was that the Marcu and Dudock system synthesized a greater proportion of larger polypeptides (Table 11). The total molecular weights of the products in both systems (approximately 637,000) would be more than double the coding capacity of RNA-S + RNA-L (approximately 360,000). Thus some must be either post-translationally cleaved products or partial translation products.

Although some products migrated to similar regions as the TRSV coat proteins components (Fig. 8A and 9A), preliminary investigations using antiserum to TRSV coat protein to detect for the viral coat protein in the translational products indicated that there is very little coat protein-like polypeptides in the products synthesized by the Marcu and Dudock system. Only 6 - 8% of the acid-insoluble radioactivity were precipitated by the antiserum (Fig. 10). However, this method is not very reliable since the antigenic site may comprise only a small part

Table 10

Incorporation of ^3H -leucine by various RNAs under the optimised Davies
and Marcu and Dudock translation systems

mRNA	Davies system ^a		Marcu and Dudock system ^b	
	Nett Incorporation ^c	Stimulation	Nett Incorporation ^c	Stimulation
-mRNA	160	1	1050	1
TRSV RNA	1690	10.5	33,400	31.8
QCMV RNA	2850	17.8	26,500	25.2
TAV RNA	2450	15.1	26,600	25.3

^a RNA concentrations used (per 50 μl) were optimal for the respective viral RNAs; they were: TRSV, 2 μg ; QCMV, 1.5; TAV, 1.5. All messengers were then translated under optimal conditions for TRSV RNA as tabulated in Table 3.

^b RNA concentrations used (per 50 μl) were same as above except that in TRSV it was 4 μg . All messengers were then translated under optimal conditions for TRSV RNA.

^c Incorporations shown are cpm/50 μl .

Figure 8. Comparative analysis of the translational products of unfractionated TRSV and QCMV RNAs synthesized in the Davies system by electrophoresis in 13% cylindrical discontinuous polyacrylamide gels

- A. ^3H -leucine labelled TRSV RNA products (6000 cpm) were analysed by co-electrophoresis with ^{14}C -leucine labelled authentic TRSV coat protein (3000 cpm). After electrophoresis the gel slices were counted under double labelling conditions. The products and coat proteins were also individually electrophoresed in sister gels as controls and the molecular weights and relative proportions of the products were estimated from the singly labelled gels. The radioactive protein peaks numbered 1-14 correspond to those presented in Table 11 and the positions of TRSV coat protein peaks are also indicated.
- B. ^3H -leucine labelled QCMV RNA products (6000 cpm) and ^3H -leucine labelled endogenous (-mRNA) products (600 cpm) were analysed by electrophoresis in sister gels in the same experiment. The relative positions of the protein markers in the sliced gels are indicated in the figure.

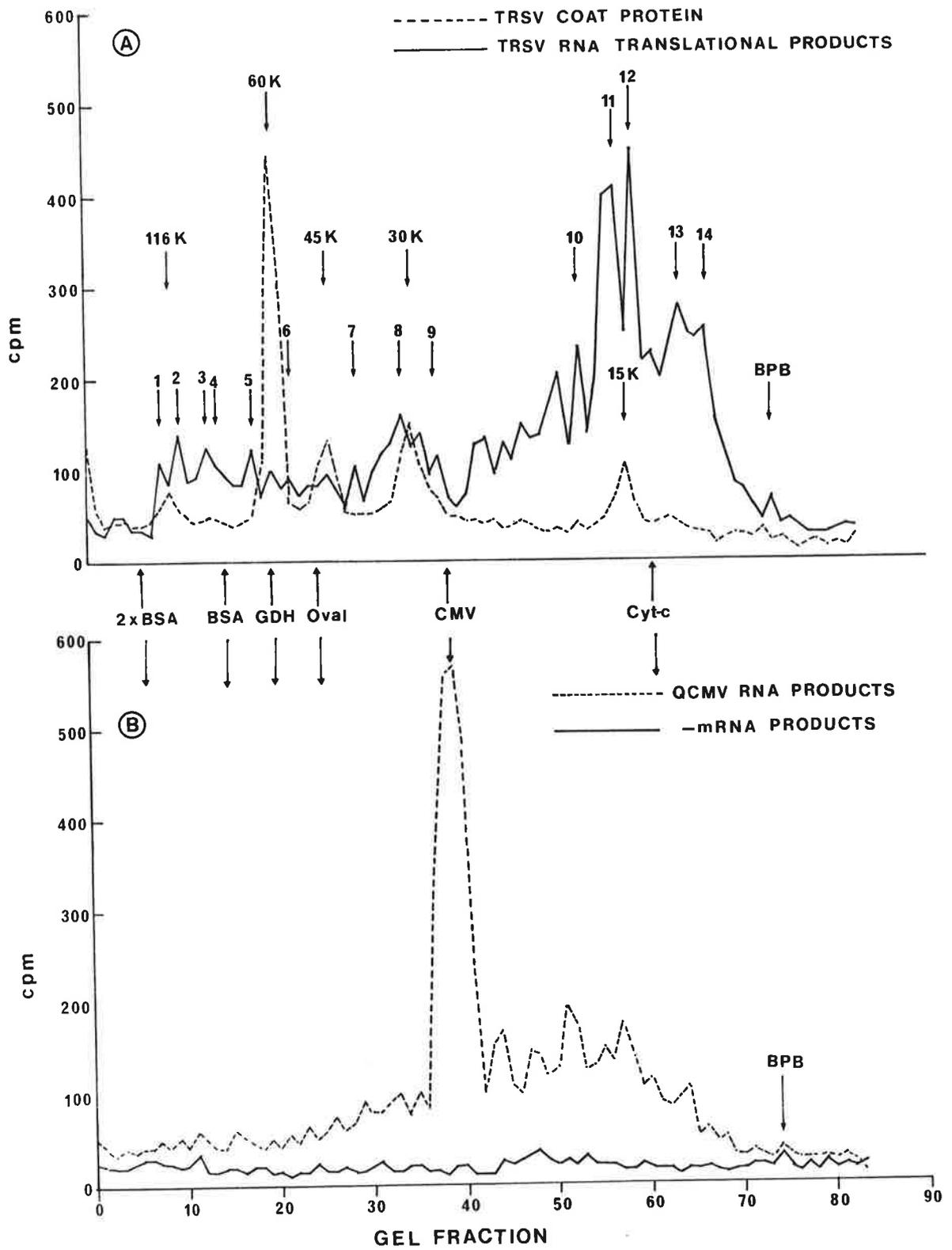


Figure 9. Comparative analysis of the translational products of unfractionated TRSV RNAs and QCMV RNAs synthesized by the Marcu and Dudock system by electrophoresis in 13% cylindrical discontinuous gels.

- A. ^3H -leucine labelled TRSV RNA products (14,000 cpm) were analysed by co-electrophoresis with ^{14}C -leucine labelled authentic TRSV coat protein (3000 cpm). After electrophoresis the gel slices were counted under double labelling conditions. The products and coat proteins were also individually electrophoresed in sister gels as controls. The molecular weights and relative proportions of the TRSV RNA translational products were estimated using the singly labelled gels. The protein peaks 1 - 14 correspond to those presented in Table 11.
- B. ^3H -leucine labelled QCMV RNA products (18,000 cpm) and ^3H -leucine labelled endogenous products (500 cpm) were electrophoresed in sister gels in the same experiment. The relative positions of the protein markers in the sliced gels are indicated in the figure.

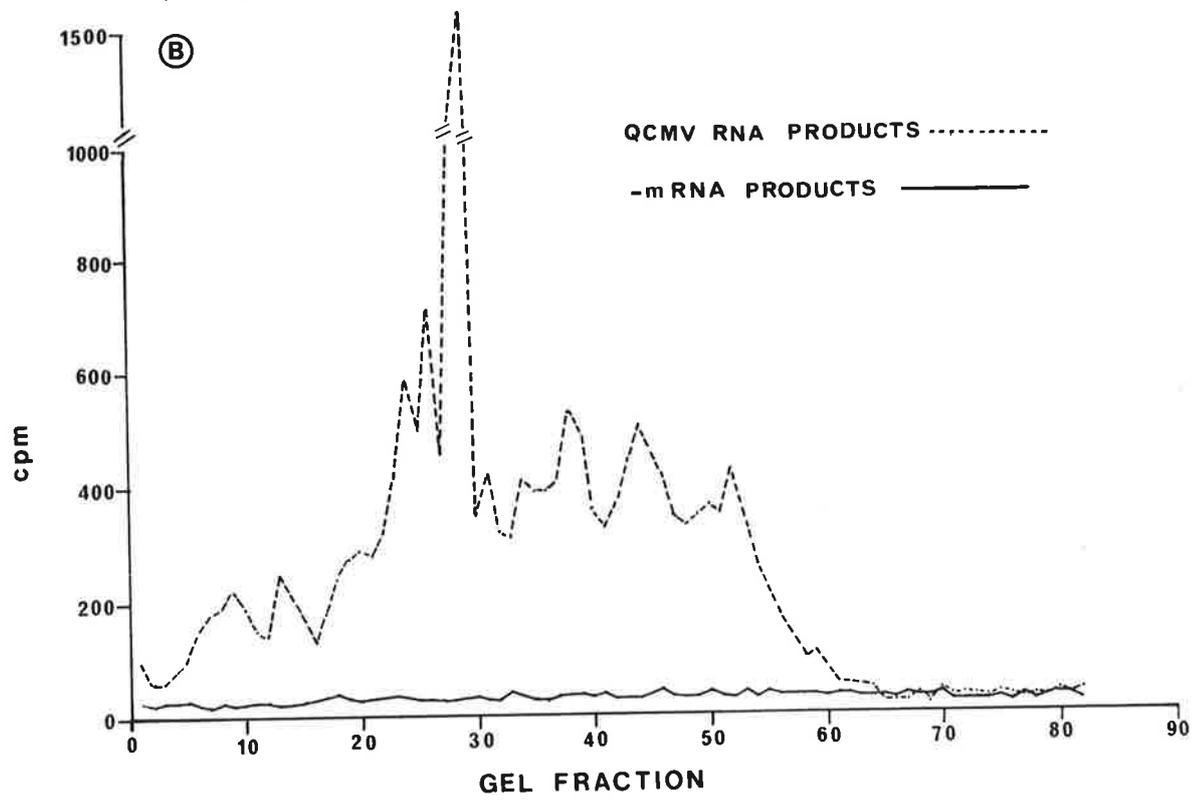
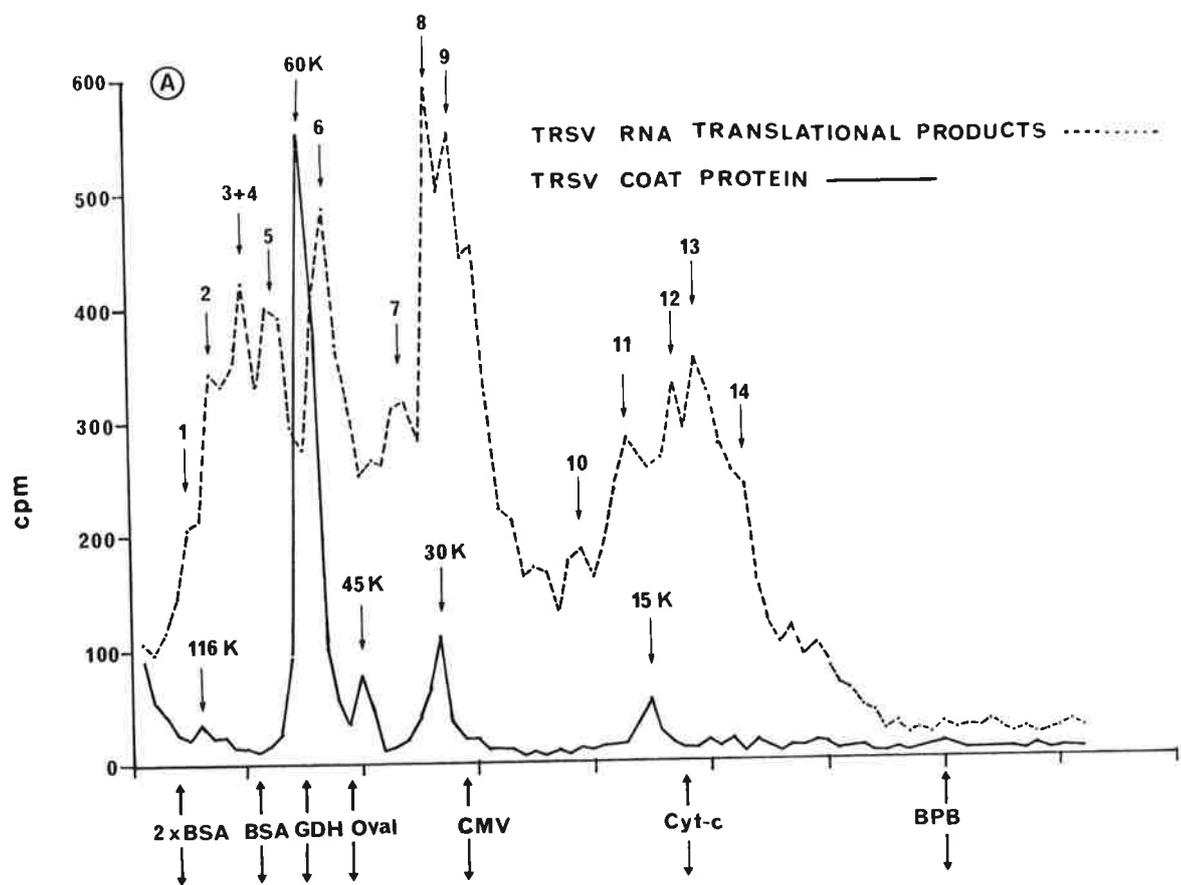


Table 11

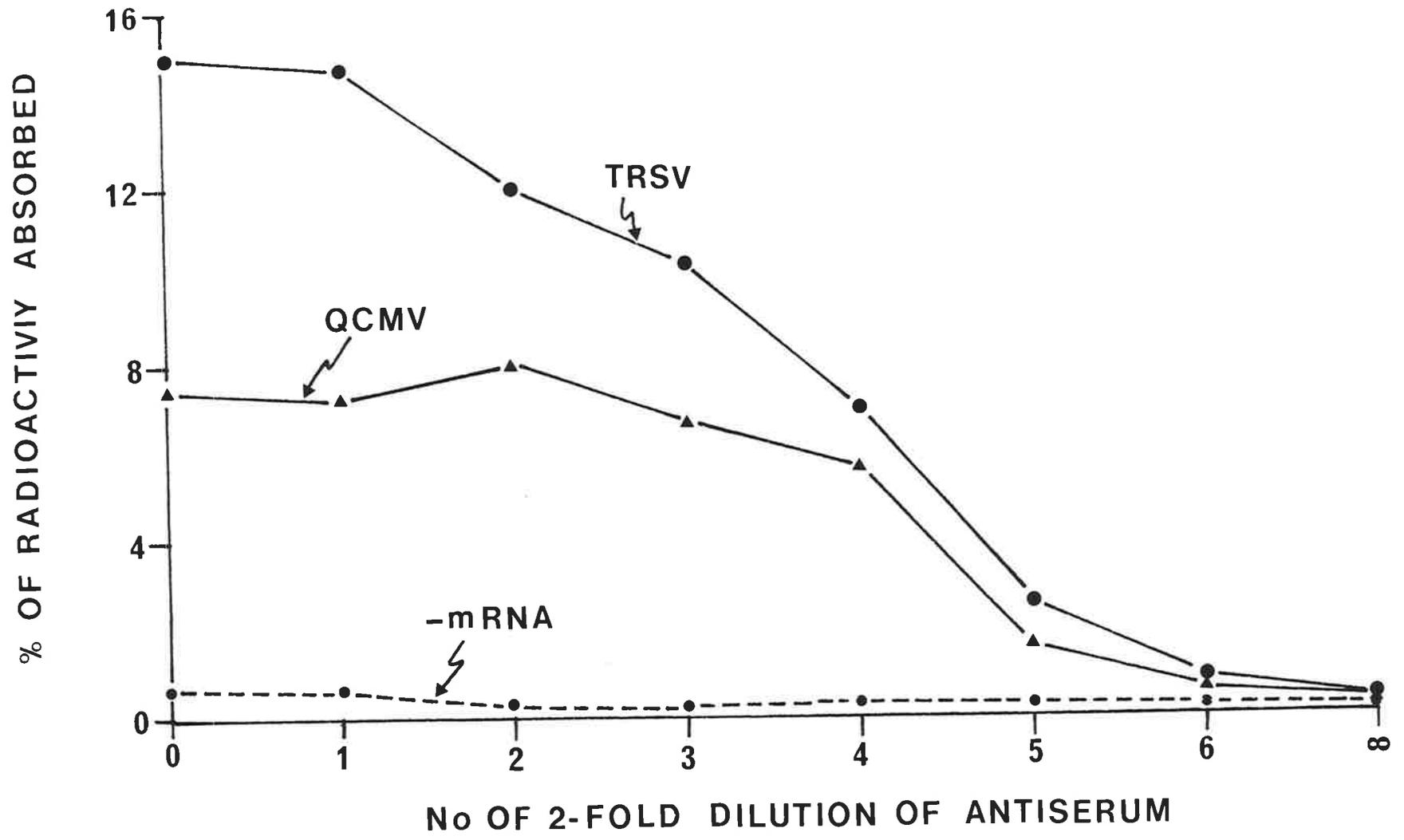
Composition of unfractionated TRSV RNA translational products synthesized
by the Davies system and the Marcu and Dudock system

Davies System			Marcu and Dudock System		
Translational Products	Mol. Wt.	Relative Proportion(%)	Products	Mol. Wt.	Relative Proportion(%)
1	120,000	2.8	1	120,000	4.8
2	95,000	3.8	2	95,000	5.3
3	78,000	2.8	3	78,000	4.6
4	74,000	2.6	4	74,000	4.9
5	65,000	3.4	5	66,000	16.2
6	50,000	2.8	6	50,000	12.2
7	35,000	2.8	7	35,000	9.9
8	29,000	8.5	8	29,000	9.5
9	26,000	7.4	9	26,000	13.6
10	17,000	14.7	10	17,500	4.9
11	15,000	16.8	11	14,700	7.6
12	13,500	11.5	12	13,500	4.2
13	11,000	12.5	13	11,000	6.8
14	9,000	7.6	14	9,000	4.1

The same RNA preparation was used for preparing the translational products. Data presented were obtained by electrophoresis of the ¹⁴C-leucine labelled translational products in duplicate 13% cylindrical gels. Gels were sliced and counted and the areas under each peak estimated. Molecular weights were determined using the standard protein molecular weight markers ran in sister gels. In the same analysis, the molecular weights of TRSV coat protein components were 116K, 108,000; 60K, 54,000; 45K, 41,000; 30K, 28,500; 15K, 13,500.

Figure 10. Serological detection for TRSV coat protein in the translational products of unfractionated TRSV RNA synthesized in the Marcu and Dudock system

A modified method of Skotnicki *et al.* (1976) was used. Aliquots of 25 μ l from each of the ^3H -leucine-labelled endogenous (-mRNA), CMV and TRSV RNA translational products synthesized by the Marcu and Dudock system was added to 0.5 ml of carrier TRSV coat protein (0.2 mg/ml). The mixtures were incubated at 37°C for 3 hr in the presence of equal volumes of antiserum prepared against TRSV 60K protein diluted in 2-fold series. The precipitates were then kept at 4°C overnight before being centrifuged down, washed with saline (3 x) and resuspended in TCA. The precipitates were then recovered on filter discs. The radioactivity precipitated by the antiserum (expressed as a percentage of the initial TRSV RNA incorporated radioactivity added for ^{the} TRSV and -mRNA products, and as a percentage of initial CMV RNA incorporated radioactivity ^{added} for CMV products) was plotted against the antiserum concentrations. No antiserum is taken as infinite dilutions.



of the coat protein polypeptide chain and non-specific absorption may occur.

Analysis of the CMV RNA directed translational products synthesized in the Marcu and Dudock, and Davies systems showed that both contained a major radioactive peak with relative mobility identical to that of the QCMV coat protein band electrophoresed in sister gel (Figs. 8B and 9B). However, the Davies system also produced less of the higher molecular weight products observed in the Marcu and Dudock system (Figs. 8B and 9B). Thus these results suggest that the Marcu and Dudock system is more efficient in the translation of larger mRNAs and may resemble translation *in-vivo* more than the Davies system.

(iii) Translation of TRSV RNA-S and RNA-L Using the Marcu and Dudock System

(a) Optimum Conditions for Translation

The optimum concentrations of reagents required for translation of TRSV RNA-S and RNA-L were investigated (Fig. 11) and the results are compared with those of unfractionated TRSV RNA (Table 12). All the RNAs had similar requirements except for RNA and leucine concentrations. RNA-L was stimulated less by Mg^{++} , K^+ and spermidine than RNA-S (Fig. 11, a - c).

The translational activity of RNA-L was much lower than that of the RNA-S (Fig. 11). Correlated with its lower translational activity, RNA-L required lower concentrations of leucine to saturate the translation reaction than RNA-S (Fig. 11, f and Table 12). The kinetics of amino acid incorporation by RNA-S and RNA-L (Fig. 11, g) show that the latter has a shorter period of active translation as well as a lower initial rate. Both RNA-S and RNA-L had lower translational activities than their parental unfractionated RNA (Table 13). However, more infectious RNA preparations were translated more efficiently and in some preparations of RNA-S, the translational activity approached that of the unfractionated RNA (Table 13). Correlated with their translational activities,

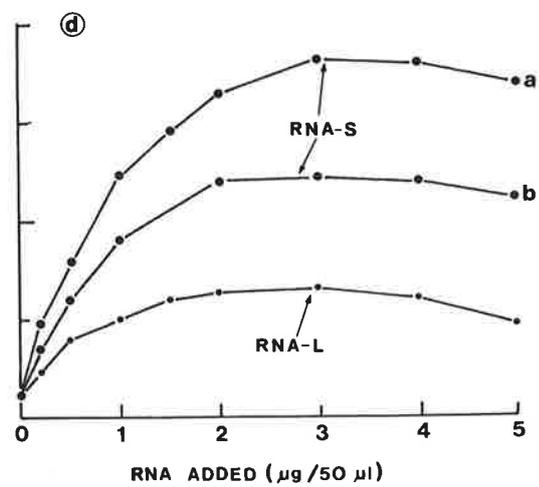
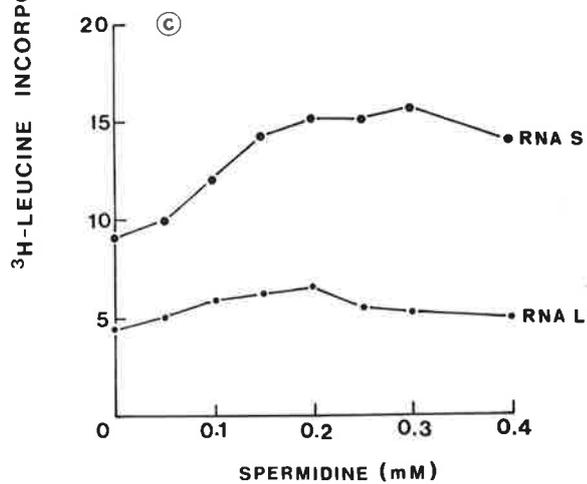
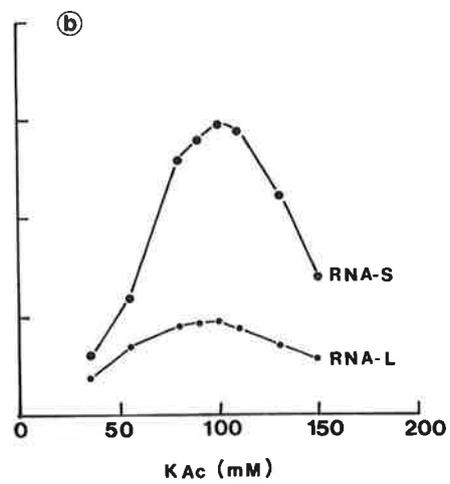
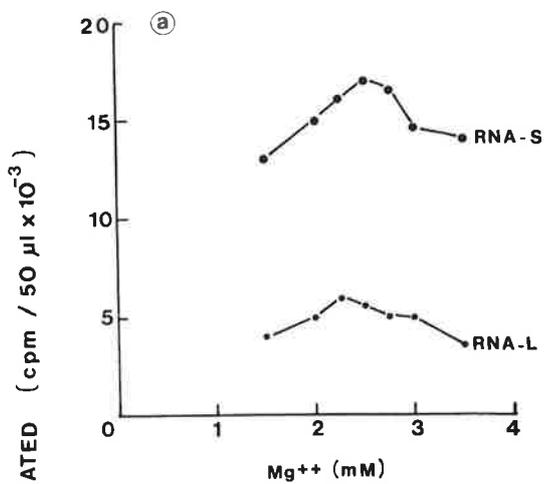
Figure 11. Optimisation of conditions for the translation of TRSV RNA-S and RNA-L in the Marcu and Dudock system

The following reagent concentrations were used in these experiments unless otherwise stated:

2.5 mM Mg^{++} ; 100 mM K^+ ; 0.2 mM spermidine;

2 μ g RNA/50 μ l; 1 μ Ci/50 μ l 3H -leucine (54 Ci/mmmole).

- a. Optimisation of Mg^{++} concentration.
- b. Optimisation of K^+ concentration.
- c. Optimisation of spermidine concentration.
- d. Optimisation of RNA concentration. Curve a, RNA concentration optimum of an RNA-S which was more infectious. Curve b, RNA optimum of a less infectious RNA-S preparation.
- e. Effect of heating RNA-S on its optimum concentration for translation.
Curve a, RNA was heated at 65°C for 3 min prior to translation.
Curve b, RNA was not heated prior to translation.
- f. Optimisation of leucine concentration; ^{14}C -leucine (324 μ Ci/ μ mole) was used in this experiment.
- g. Kinetics of translation after optimisation; translation reactions were done in duplicates and separate reactions were done for each incubation time.



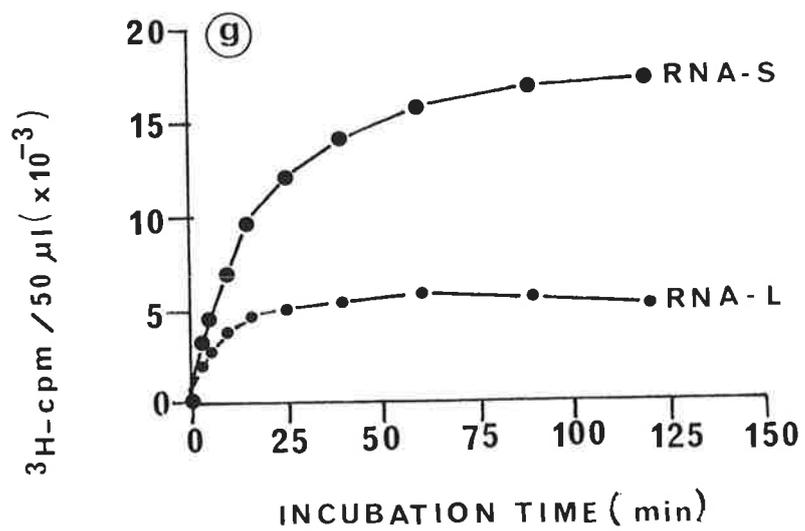
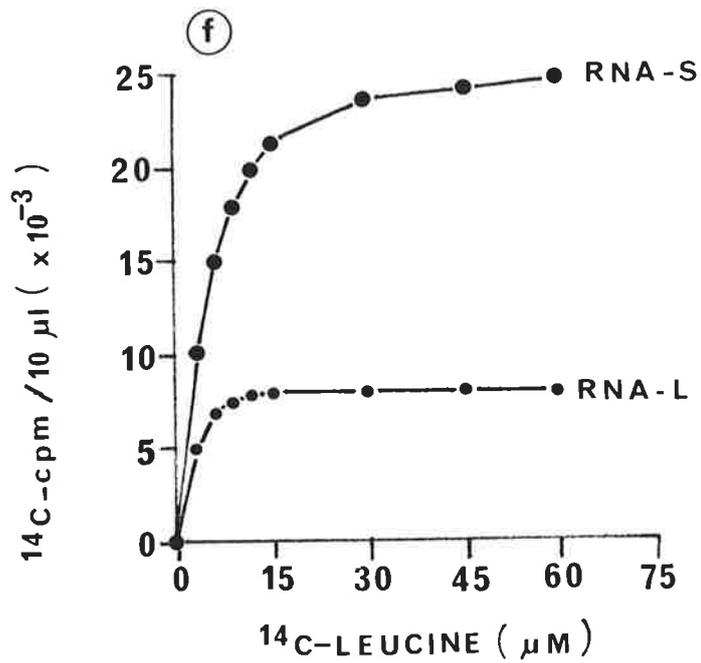
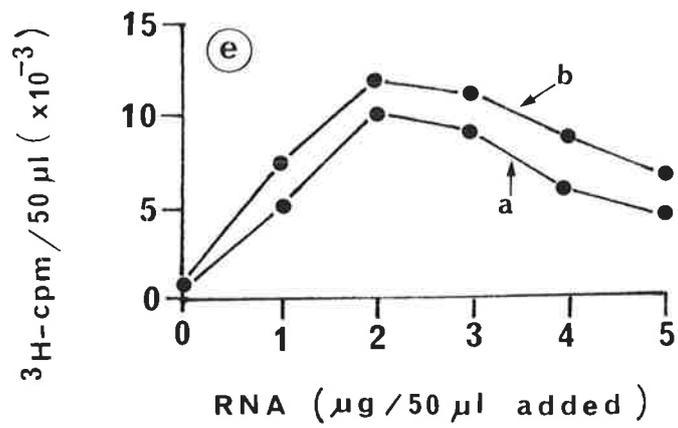


Table 12Optimum conditions for translation of RNA-S and RNA-L and unfractionated (UF)TRSV RNA

Conditions tested	RNA-S	RNA-L	UF TRSV RNA
Mg ⁺⁺ conc.	2.5 mM	2.25mM	2.5 mM
K ⁺ conc.	100 mM	100 mM	100 mM
Spermidine conc.	0.2-0.3 mM	0.2 mM	0.2 mM
RNA conc.	2-3µg/50 µl	2µg/50µl	4-6µg/50µl
Leucine conc.	30 µM	9 µM	30 µM

The Marcu and Dudock cell-free system was used.

All translational conditions were as presented in Table 3 except for the reagents tested of which a whole range of concentrations were tested.

Table 13

Variation in infectivity and translation activity of various
TRSV RNA preparations

RNA Preparation	Unfractionated TRSV RNA		TRSV RNA-S and RNA-L		
	Translational ^a activity	Infectivity ^b	Translational ^a activity		Infectivity ^b
			RNA-S	RNA-L	
I	19,880	120	4,400	1100	13
II	19,100	132	13,000	4200	24
III	33,500	241	21,000	6400	35
IV	33,200	214	20,500	5700	37
V	29,600	206	24,800	8200	42

^a Marcu and Dudock cell-free translation system was used and conditions were as described in Table 3. Each batch of TRSV RNA-S and RNA-L was tested for translational activity in the presence of its unfractionated RNA preparation as control. The values are incorporated ³H-leucine, cpm/50 μ l.

^b Infectivity was assayed on cowpea half-leaves as described in Chapter II using 25 μ g/ml inoculum for unfractionated RNA and a total of 25 μ g/ml (equal amounts of each component) inoculum for the fractionated RNAs. The values are average numbers of lesions per half-leaf from 10 replicates per RNA treatment.

unfractionated TRSV RNA had higher RNA optimum than RNA-S which had a higher RNA optimum than RNA-L (Table 12). The optimum RNA-S concentration was found to vary with the RNA preparations (Fig. 11, d). A less infectious RNA-S preparation was found to be less active in translation and had a RNA optimum similar to that of RNA-L (Fig. 11, d). In another experiment to investigate whether the aggregation of RNA-S affects RNA optimum (Fig. 11, e), heated and untreated RNA-S gave the same RNA concentration optima although the heated RNA incorporated less leucine (Fig. 11, e). Thus it appears that the translational activity and RNA optimum observed *in-vitro* may vary depending on the affinity of the mRNA for ribosomes and the quality of the RNA preparations.

(b) Translational Products of TRSV RNA-S and RNA-L

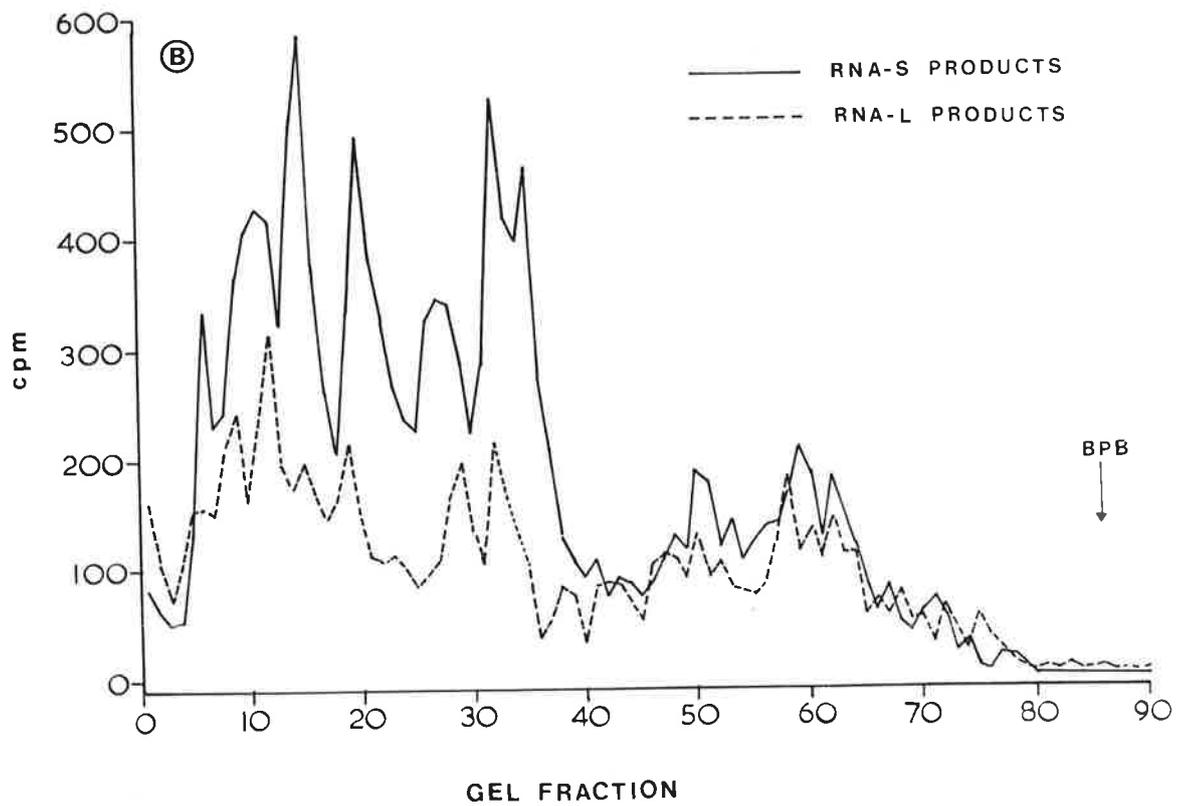
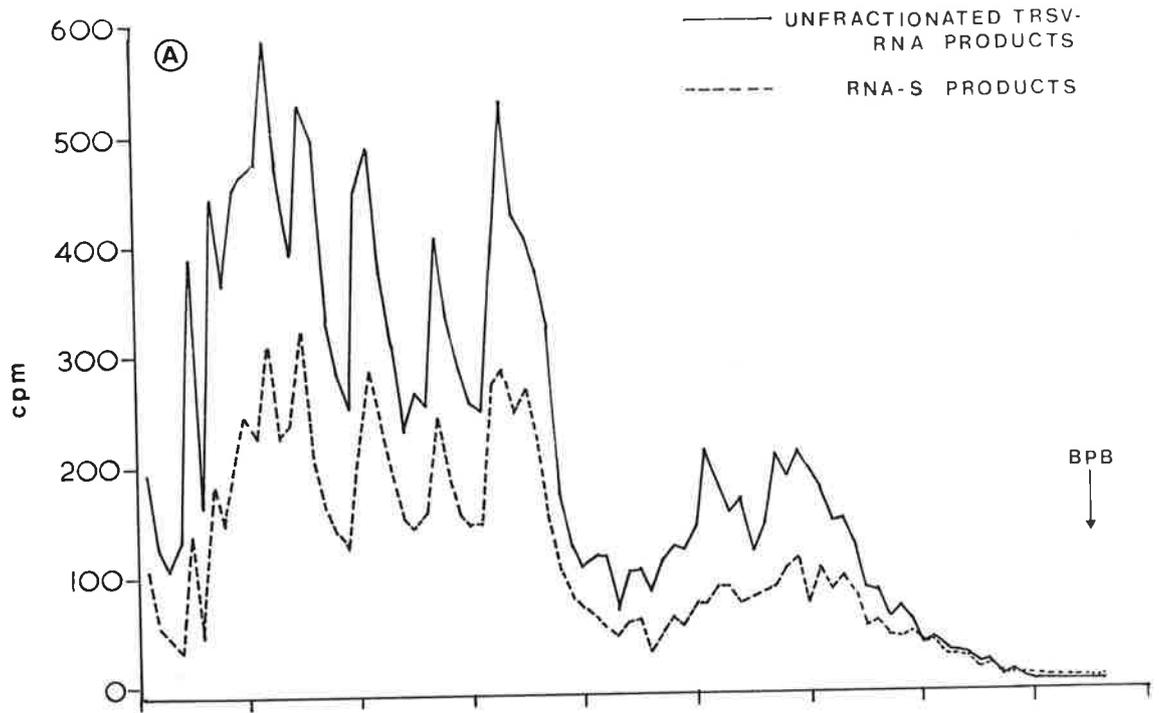
The translational products of TRSV RNA-S and RNA-L were analysed and compared with those of unfractionated TRSV RNA synthesized by the Marcu and Dudock system by electrophoresis in cylindrical and slab polyacrylamide gels (Figs. 12 and 13). The molecular weights and relative proportions of the products detected are presented in Table 14. The results revealed that unfractionated TRSV RNA and RNA-S directed the synthesis of almost identical ranges of products in terms of both sizes and relative proportions (Fig. 12A, Table 14) while RNA-L was translated into a range of products different from these RNAs (Figs. 12B, 12C and 13; Table 14). In some experiments, RNA-S and unfractionated TRSV RNA produced different product profiles in polyacrylamide gels in the sense that the latter directed the synthesis of both RNA-S and RNA-L products although the amounts of RNA-L products produced was only approximately 10% of the total. Although a greater proportion of smaller products was translated by RNA-L compared to RNA-S, it also directed the synthesis of the largest polypeptide chains translated by TRSV RNA (Table 14). RNA-S synthesized very similar products when either ^{14}C -leucine (15 μM) or ^3H -leucine (0.4 μM) was used (Fig. 12D).

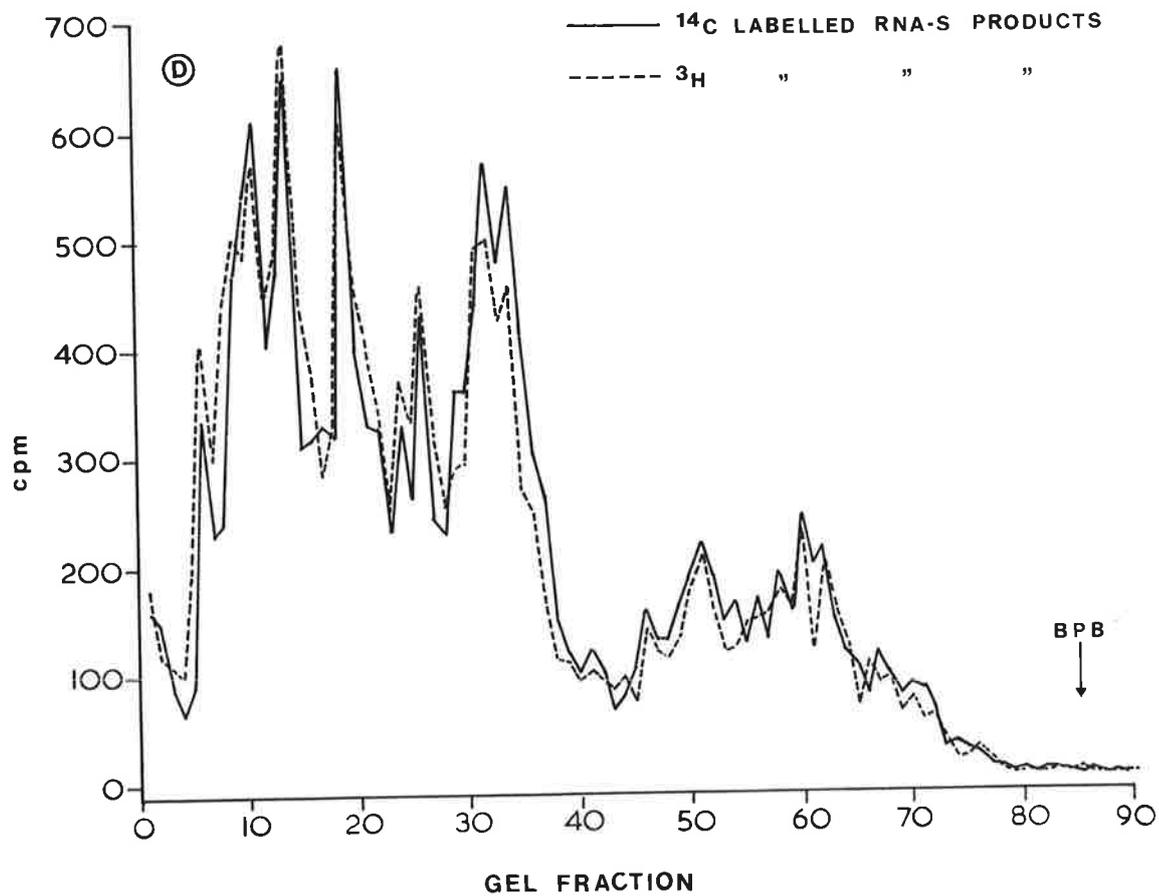
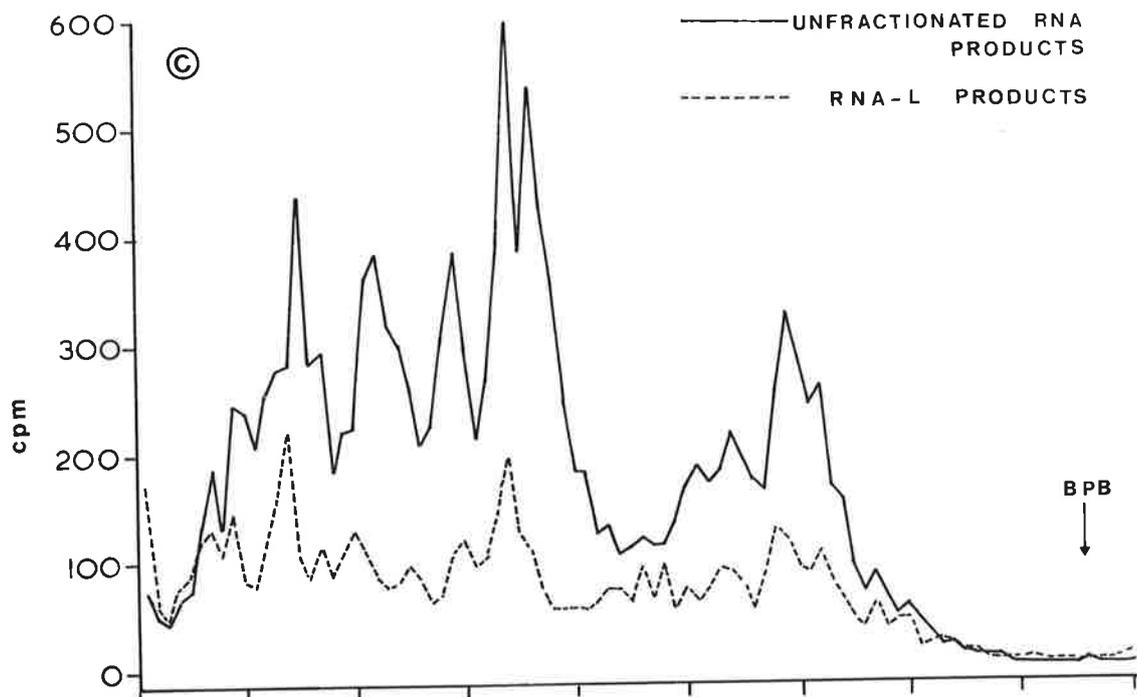
Figure 12. Comparative polyacrylamide gel electrophoretic analysis of the translational products of unfractionated TRSV RNA, RNA-S and RNA-L synthesized in the Marcu and Dudock system by double labelling technique in 13% cylindrical discontinuous gels

The following mixtures of translational products or proteins were co-electrophoresed:

- A. RNA-S + unfractionated TRSV RNA translational products (14,000 ^{14}C cpm + 18,000 ^3H cpm).
- B. RNA-S + RNA-L translational products (14,000 ^3H cpm + 10,000 ^{14}C cpm).
- C. RNA-L + unfractionated TRSV RNA translational products (10,000 ^{14}C cpm + 14,000 ^3H cpm).
- D. RNA-S + RNA-S translational products (18,000 ^3H cpm + 14,000 ^{14}C cpm).
- E. RNA-S products + authentic TRSV coat protein (18,000 ^3H cpm + 5,000 ^{14}C cpm).
- F. RNA-L products + authentic TRSV coat protein (10,000 ^3H cpm + 3,000 ^{14}C cpm).

After electrophoresis, the gel slices were counted under double labelling conditions. The profiles obtained were similar to those obtained when the respective products were electrophoresed separately in sister gels. The radioactive protein peaks numbered S1 - S14 and L1 - L18 correspond to those presented in Table 14 and the relative positions of protein molecular weight markers in the sliced gels are indicated by arrows. The molecular weights and relative proportions of the various products were estimated from the singly labelled gels.





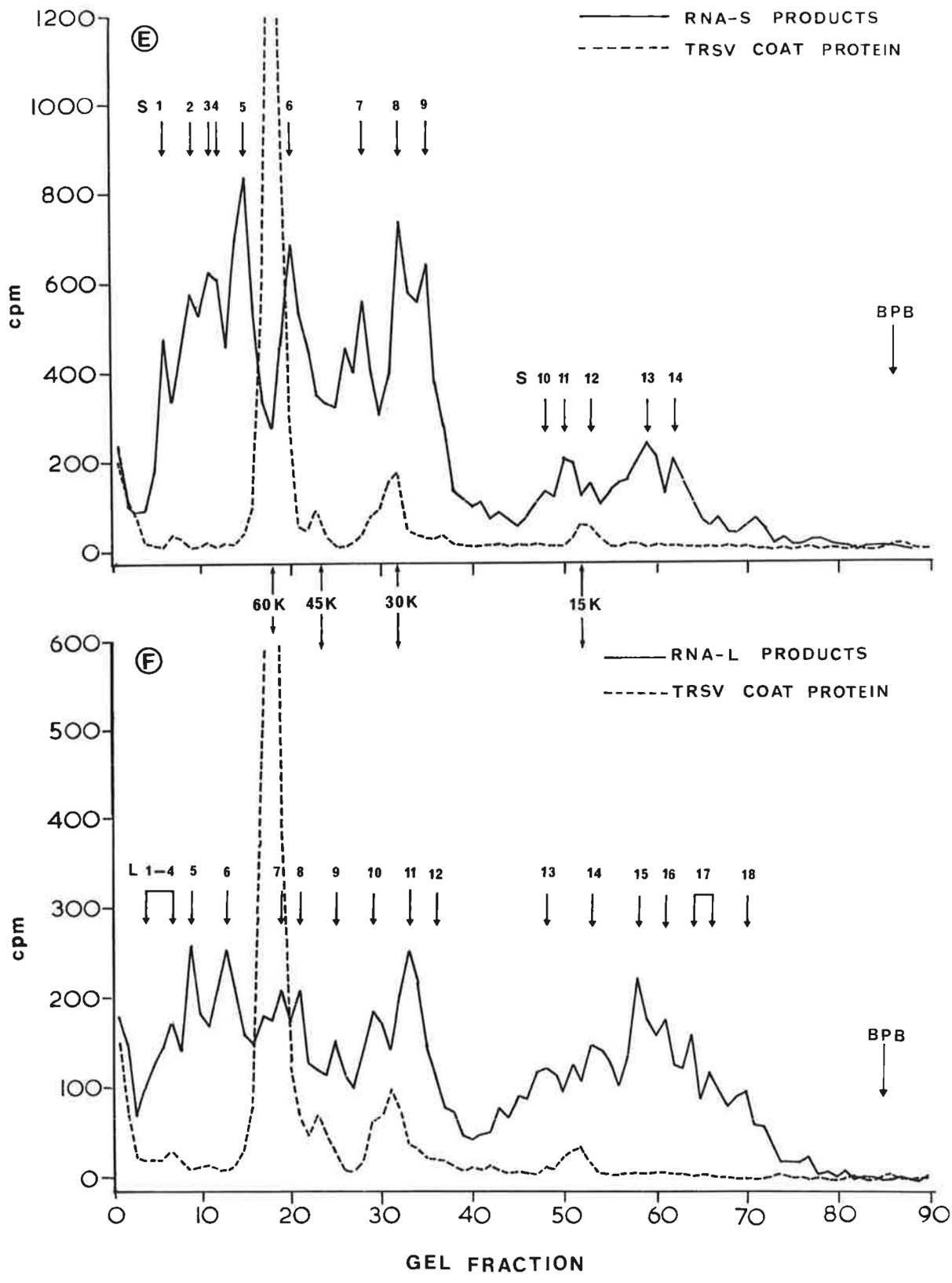


Figure 13. Analysis of the translational products of TRSV RNA-S and RNA-L by electrophoresis in 15% discontinuous slab polyacrylamide gels

Low bis-acrylamide gels were used (see methods section).

Samples loaded were:

Track a, RNA-S product (2800 cpm);
track b, RNA-S (2800) + RNA-L (4000);
track c, TRSV coat protein (2000);
track d, RNA-L (4000) + TRSV coat protein (2000);
track e, RNA-L (4000);
track f, RNA-S (3500) + coat protein (2500);
track g, RNA-S (3500);
track h, TRSV coat protein (2500);
track i, -mRNA (750);
track j, protein markers.

All the samples were ^{14}C -leucine labelled (amounts of radioactivity loaded are given in brackets). Electrophoresis was carried out at 10 mA/gel for $1\frac{1}{2}$ hr and then at 20 mA/gel slab for 5 hr. The dried gel was fluorographed for 5 weeks at -70°C and the film was developed for 10 min.

A. Dried stained gel ready for fluorography.

B. Fluorogram of the gel.

The positions of the various TRSV RNA-S (S1 - S14) and RNA-L products (L1 - L18) corresponding to those presented in Table 14 are indicated. The molecular weights of these products are estimated from the relative mobilities of the unlabelled protein markers in the original dried and stained gel and the results agree well with those estimated from cylindrical gels.

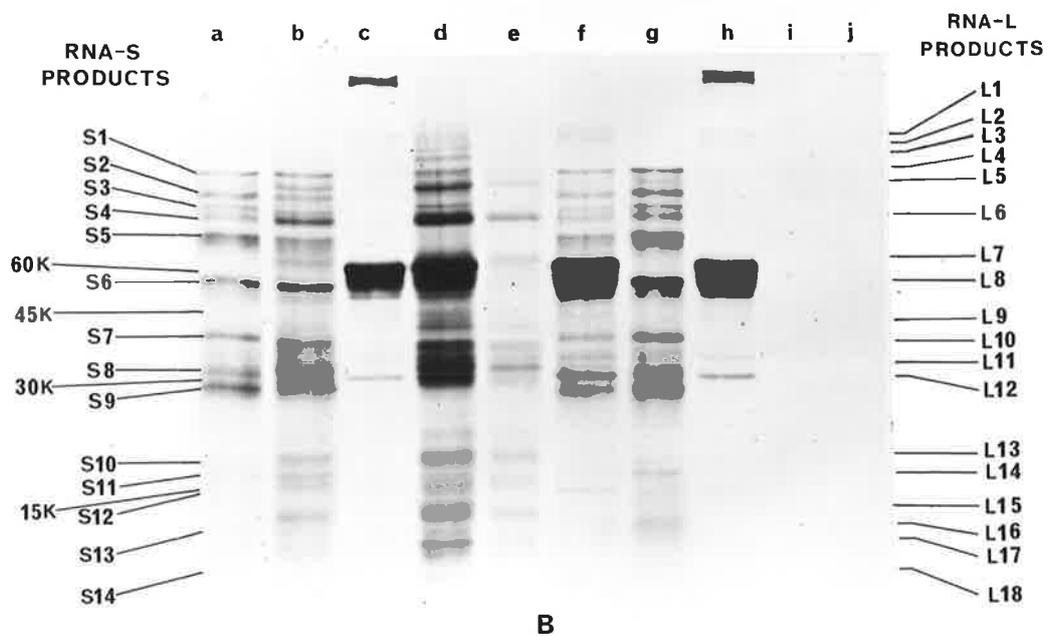
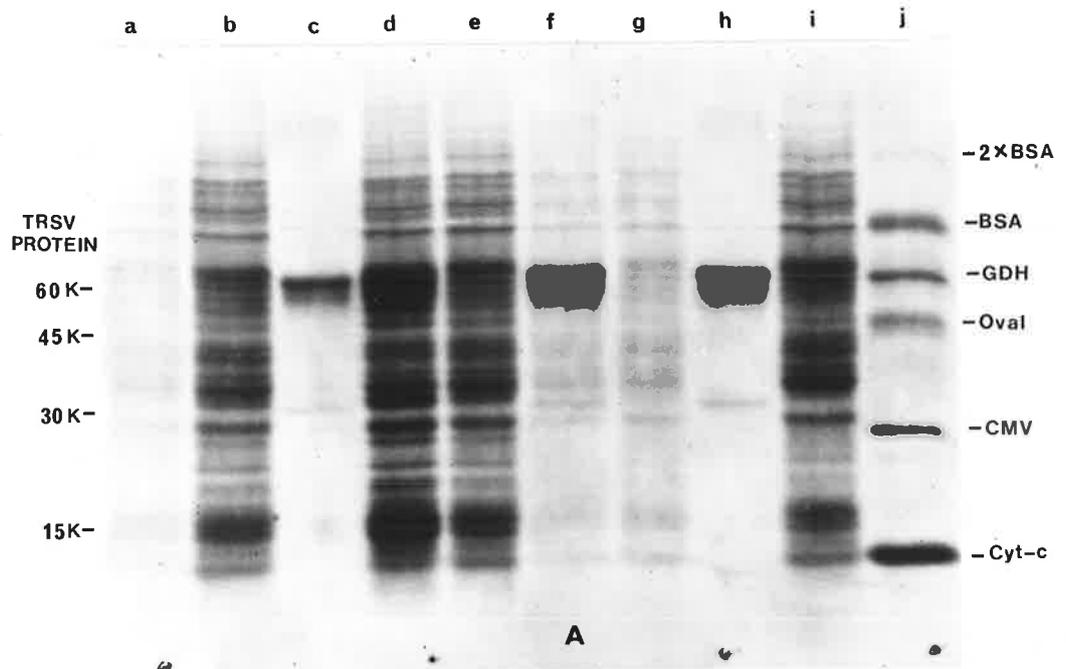


Table 14

Composition of translational products of TRSV RNA-S and RNA-L synthesized by the Marcu and Dudock system

RNA-S			RNA-L			Unfractionated TRSV RNA		
Product ^a	Mol. Wt.	%	Product ^a	Mol. Wt.	%	Product ^a	Mol. Wt.	%
S1	120,000	5.0	L1	c.230,000	1.1	1	120,000	4.8
S2	93,000	6.6	L2	c.210,000	1.5	2	95,000	5.3
S3	78,000	4.5	L3	185,000	1.9	3	78,000	4.6
S4	74,000	4.2	L4	136,000	4.4	4	74,000	4.9
S5	66,000	14.0	L5	112,000	5.9	5	66,000	16.2
S6	49,000	15.8	L6	74,000	11.7	6	50,000	12.2
S7	34,500	11.7	L7	56,000	5.6	7	35,000	9.9
S8	28,500	7.8	L8	49,000	4.3	8	29,000	9.5
S9	26,500	13.7	L9	38,000	4.9	9	26,000	13.6
S10	17,000	2.2	L10	34,000	8.7	10	17,500	4.9
S11	15,500	3.1	L11	29,500	10.6	11	14,700	7.6
S12	13,500	3.3	L12	27,500	5.1	12	13,500	4.2
S13	11,300	5.2	L13	17,200	7.3	13	11,000	6.8
S14	8,700	2.8	L14	14,800	5.9	14	9,000	4.1
			L15	12,300	9.6			
			L16	11,000	4.1			
			L17	10,000	6.5			
			L18	8,400	0.9			

RNA-S and RNA-L were translated using ¹⁴C-leucine as radioactive amino acid and the same extract preparation. The translational products were analysed in duplicated samples in both 13% cylindrical gels which were sliced and counted and in 15% slab gels which were fluorographed. Molecular weights were estimated from both the cylindrical and slab gels and the values from the two systems were very similar. The relative proportion of each product (%) was estimated by determining the areas under the radioactive peaks in the cylindrical gels. The results for the unfractionated TRSV RNA were from Table 11. Under the same analysis, TRSV coat protein components had molecular weights of: 108,000; 54,000; 40,000; 27,500 and 13,200.

^a The products numbered were the same as those designated in Figures 8 and 9, 12 and 13 and Table 11 for the respective RNAs.

The products of both RNA-S and RNA-L did not appear to correspond with the various TRSV coat protein components (Figs. 12E, 12F and 13). Although the molecular weights of some products of RNA-S and RNA-L were similar to those of the coat protein components, the relative proportions of these products were different from those of the coat protein components observed in gels.

The molecular weights of the translational products of RNA-S ranged from approximately 9,000 to 120,000 while those of RNA-L ranged from approximately 8,400 to 230,000. The molecular weights of RNA-S and RNA-L are 1.44×10^6 and 2.26×10^6 respectively. Thus, the largest products of the respective RNAs represent continual translation of approximately 80 - 85% of RNA-S and almost 100% of RNA-L. These results suggest that the TRSV RNAs probably do not contain several cistrons which are separately translated into different proteins *in-vivo*; and that the lower molecular weight products must represent either partial translation of the RNA segments, or that they are products of post-translational cleavage. The estimated size of the largest product of RNA-S is supported by the observation (Figs. 16 and 17) that it is similar to the largest product synthesized in the presence of QCMV RNA which was estimated to be 120,000 (Schwinghamer and Symons, 1975) and is smaller than the largest major product of U-1 TMV RNA which was estimated to be 140,000 (Knowland, 1974).

(iv) Detection for TRSV Coat Protein in the TRSV RNA Translational Products by Tryptic Peptide Analysis

Analysis of the translational products and determination of their molecular weights in polyacrylamide gels alone were not sufficient to indicate conclusively whether coat protein was present in the translational products of TRSV RNAs. Due to the large number of products synthesized, it is likely that some products which are not coat protein may migrate into similar positions as the coat proteins. However, it is possible that the TRSV coat protein is formed by post-

translational cleavage of precursor protein and thus products with higher molecular weights than those of the coat proteins may contain the coat protein amino acid sequence. Thus tryptic peptides of each fractionated translational product of TRSV RNA-S, where the coat protein gene is believed to be located, and the total translational products of RNA-L and unfractionated TRSV RNA were analysed and compared with those of the viral coat protein (Fig. 14 and Table 15).

After trypsin digestion, over 80% of the incorporated radioactivity in each translational product was recovered in the pyridine: acetic acid buffer-soluble fraction (Table 15) indicating that digestion has occurred. Profiles of peptide containing sample volumes of more than 50 μ l of peptides sometimes become distorted during chromatography (Fig. 14), probably due to excess carrier proteins and salting out problems (Dr. A.C. Jennings, private communication). Distortion was reduced or eliminated when samples were re-chromatographed in the same buffer. There were some radioactive materials remaining at the origins of some fractionated products but the following observations suggest that these are probably excess carrier coat proteins and translation products which did not migrate due to overloading rather than unresolved new peptides: (a) there were no differences in the patterns of peptide maps which were chromatographed only one, twice or three times in the same buffer, (b) the peptide maps of the unfractionated translational products of RNA-S (which did not contain carrier coat protein) had no radioactivity at the origin but did not produce any peptides not found in the peptide maps of the fractionated products containing radioactivity at the origin. After fluorography, the peptide maps were recorded and compared with each other and with that of TRSV coat protein (Fig. 14E).

The results confirmed the previous conclusions that unfractionated TRSV RNA directed the synthesis of mainly RNA-S translational products plus some RNA-L products, while RNA-L directed the synthesis of polypeptides entirely different from those of RNA-S. In the detailed

Table 15

Summary of translational products of TRSV RNAs analysed by tryptic peptide

Products ^a	<u>mapping</u>		
	Initial ¹⁴ C-cpm ^b	Final cpm ^c	Volume of tryptic peptides
RNA-S Products:			
S1	10,000	6,600	60 µl
S2	12,000	10,000	100 µl
S3 + S4	24,000	19,000	100 µl
S5	35,000	31,000	120 µl
S6	36,000	30,000	120 µl
S7	20,000	18,000	100 µl
S8 + S9	55,000	51,000	120 µl
S10	8,000	8,000	60 µl
S11	18,000	18,000	100 µl
S12	9,000	10,000	100 µl
S13	18,000	17,000	100 µl
S14	9,000	8,600	80 µl
Total products of RNA-S	266,000	250,000	250 µl
Total products of RNA-L	145,000	88,000	160 µl
Total products of unfractionated TRSV RNA	290,000	276,000	270 µl
Authentic TRSV coat protein	120,000	112,000	110 µl

^a All translational products analysed were labelled with ¹⁴C-leucine. They were synthesized under identical conditions by the Marcu and Dudock translational system using the same wheat germ extract and fresh RNA preparations.

TRSV RNA-S products were fractionated into the different molecular weight components (S1 - S14) detected in gels as described in the Methods section. Each component of RNA-S translational products is estimated to contain 0.2 - 0.3 mg protein from the wheat extract and 0.5 mg of unlabelled carrier viral coat protein was added to each component prior to trypsin digestion. No carrier was added to the total translational products and the ¹⁴C-TRSV coat protein.

^b Total radioactivity eluted from the gels prior to trypsin digestion after dialysis to remove free ¹⁴C-leucine.

^c Total radioactivity in the pyridine : acetic acid : water buffer soluble fraction after trypsin digestion.

Figure 14.

Analysis of the tryptic peptides of TRSV RNA translational products by ascending chromatography in thin layers of cellulose

The peptides of ^{14}C -leucine labelled TRSV RNA translational products and authentic TRSV coat protein were chromatographed as described in Chapter IV, except that in some cases, repeated elution was required when over-loading reduced resolution. The plates were fluorographed at -70°C to -80°C for various times depending on the amounts of radioactivity loaded.

- A. Analysis of the peptides of major products of RNA-S;
5000 cpm/sample was loaded and the plate was fluorographed for 5 weeks.
- Track a, S5 product peptides (Mol. Wt. similar to 60K protein component);
 - track b, S6 product peptides (Mol. Wt. similar to 60K protein component);
 - track c, total RNA-S product peptides;
 - track d, TRSV coat protein peptides;
 - track e, S7 product peptides (Mol. Wt. similar to 45K protein component);
 - track f, S8 + S9 product peptides (Mol. Wt. similar to 30K protein component);
 - track g, S11 product peptides (Mol. Wt. similar to 15K protein component).
- B. Analysis of the peptides of minor products of RNA-S;
2500 cpm/sample was loaded and fluorographed for 8 weeks.
- Track a, S2 product peptides;
 - track b, S3 + S4 peptide;
 - track c, S10 peptides;
 - track d, S12 peptides;
 - track e, S13 peptides;
 - track f, S14 peptides;
 - track g, RNA-L product peptides;
 - track h, unfractionated TRSV RNA product peptides;
 - track i, TRSV coat protein peptides.
- C. Further analysis of the peptides of products S5, S6 and S8 + S9;
2500 - 5000 cpm per sample was loaded and fluorographed for 7 weeks.
- Tracks a, c, e, g and i, TRSV coat protein peptides (5000 cpm);
 - track b, S6 (3600 cpm);
 - track d, S5 (2500 cpm);
 - track f, S8 + S9 (4000 cpm);
 - track h, S6 (5000 cpm).
- D. Analysis of the peptides of total translational products of TRSV RNA-S, RNA-L and unfractionated RNA; 20,000 cpm per sample was loaded and fluorographed for 7 days.
- Tracks a and f, TRSV coat protein;
 - track b, RNA-S;
 - track c, RNA-L;
 - track d, RNA-S + RNA-L (20,000 + 20,000 cpm);
 - track e, unfractionated TRSV RNA.

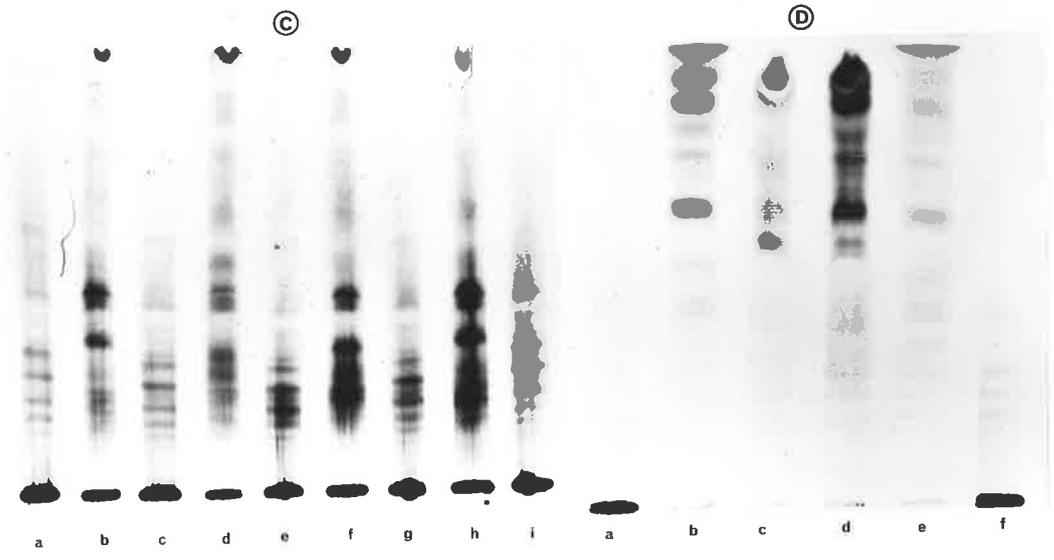
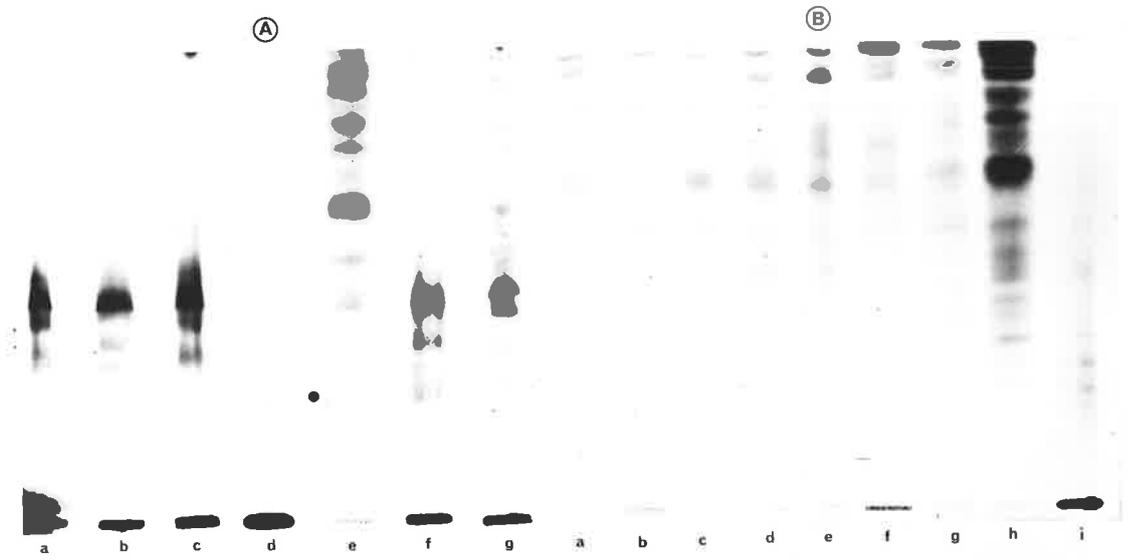
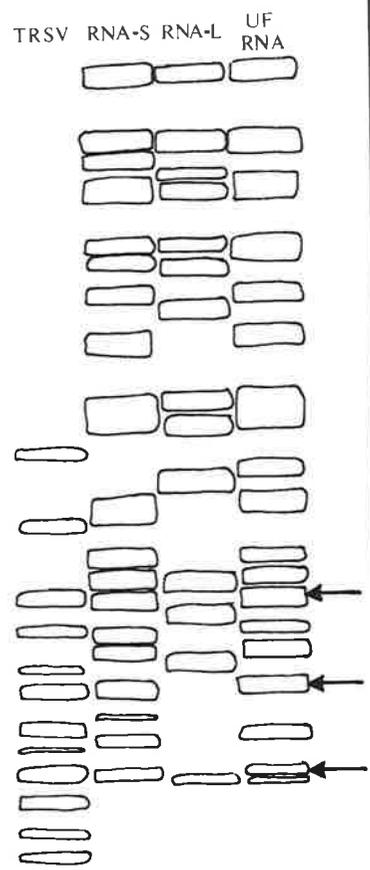
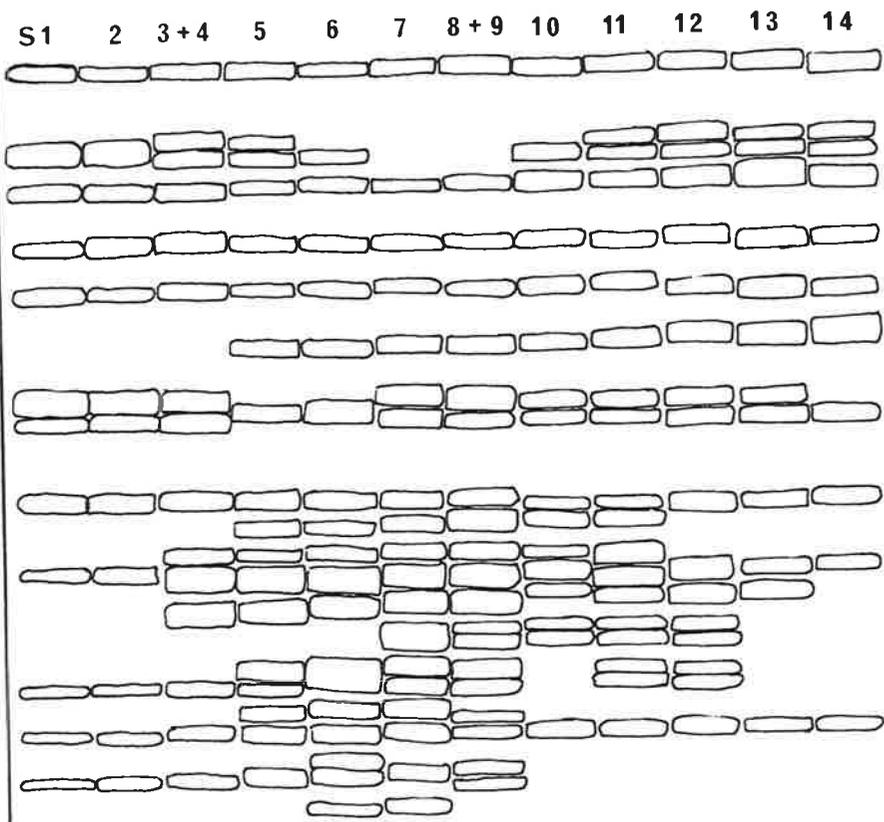


Figure 14E. Summary of the tryptic peptide maps of the translational products of TRSV RNAs.

The peptides which co-migrated with the TRSV coat protein peptides are indicated by arrows.

TRANSLATIONAL PRODUCTS

RELATIVE MIGRATION OF TRYPTIC PEPTIDES
↑
ORIGIN →



study of the individual RNA-S translational product component (Fig. 14A, C and E), almost all the translational products shared common tryptic peptides; with the peptides of the smaller products being repeated with diminishing intensity in the larger products which also contained new peptides. These results indicate that the various product components contained overlapping amino acid sequences and that they were probably synthesized by translation from common initiation sites and terminating (prematurely) at various sites rather than translation of individual cistrons or products of post-translational cleavage.

There are no tryptic peptides in RNA-L translational products which correspond to those of TRSV coat protein although the whole RNA is being translated. Thus it is unlikely that this RNA contains the coat protein gene. There are 3 peptides in TRSV RNA-S and unfractionated RNA translational products which are similar in mobility to those of TRSV coat protein (Fig. 14E shown by arrows). If they were part of the coat protein amino acid sequence they will have to be translated from the ends of the mRNA since over 80% of the RNA has been translated and found to contain no complete coat protein. However, they were not present in either the smallest or the largest translational products. Thus these "coat-protein-like peptides" are probably similar peptides from non-coat protein polypeptide chains.

(v) Effects of Translation Conditions on Translational Products of TRSV RNA

In all the experiments, there were little variation in the profiles of translational products of TRSV RNA when analysed in polyacrylamide gels if the same translation conditions were maintained. Studies by other workers have shown that variations in translational conditions would change the type of products and their relative proportions. For example, optimum K^+ ion concentration is required for translation of larger products in CpMV (Davies *et al.*, 1977) and spermidine and spermine are required for elongation and synthesis

of larger products (Rutgers, 1977). Messenger RNA concentration was also thought to be important in the regulation of translation (Zargorski, 1978; Van Tol and Van Vloten-Doting, 1979). However, various studies also show that coat proteins when synthesized *in-vitro* were translated most efficiently under optimal conditions. Thus, in the translation of TRSV RNA, there is no reason to suggest that coat protein would be translated under sub-optimal conditions if no coat protein has been detected under optimal conditions. On the other hand, there are several important factors which affect the incorporation of leucine and the translational products directed by TRSV RNA but had not been investigated in previous studies. These included extract activity, RNA activity, leucine concentration and temperature of translation.

Analysis of the translational products of different RNA preparations showed that a greater proportion of higher molecular weight products were translated in more active RNA preparations (Fig. 15). With unfractionated TRSV RNA, a significant amount of RNA-L products were also synthesized by more active RNA. Similarly, extract preparations with high translational activity also directed the synthesis of greater proportions of high molecular weight products (Fig. 16). However, in both cases, no new products or coat protein-like polypeptides were detected. There were no significant differences in the products when the leucine concentrations were sub-optimal (Fig. 17), or when translation temperature was reduced from 30°C to 25°C. Translational products remain unaltered when translation was terminated by freezing at -15°C or by RNase treatment indicating that freezing is sufficient to stop the translation reaction and also suggests that there is no post-translational proteolytic activity occurring during translation *in-vitro*.

IV. DISCUSSION AND CONCLUSIONS

The molecular weights of TRSV RNA-S and RNA-L were estimated under denaturing conditions by formamide-polyacrylamide gel electro-

Figure 15. Analysis of the translational products of different TRSV RNA preparations synthesized in the Marcu and Dudock system by electrophoresis on 15% slab gel

Different preparations of unfractionated TRSV RNA and RNA-S with different infectivities (preparations correspond to those in Table 13) were translated in the presence of ^{14}C -leucine. Reactions were stopped by freezing and 20,000 cpm of the respective products were loaded and electrophoresed. TRSV coat protein (4,000 cpm) and -mRNA products (1,500 cpm) were also electrophoresed as controls. The gel was fluorographed for 7 days. Values given in brackets below are the incorporations of the various RNA preparations.

- Track a, -mRNA (endogenous) (1,500 cpm/25 μl);
- track b, unfractionated TRSV RNA II (48,000 cpm/25 μl);
- track c, unfractionated TRSV RNA III (73,000 cpm/25 μl);
- track d, unfractionated TRSV RNA IV (73,000 cpm/25 μl);
- track e, unfractionated TRSV RNA V (73,000 cpm/25 μl);
- track f, TRSV RNA-S III (53,000 cpm/25 μl);
- track g, TRSV RNA-S IV (45,000 cpm/25 μl);
- track h, TRSV RNA-S II (37,000 cpm/25 μl);
- track i, TRSV RNA-S V (70,000 cpm/25 μl);
- track j, TRSV coat protein.

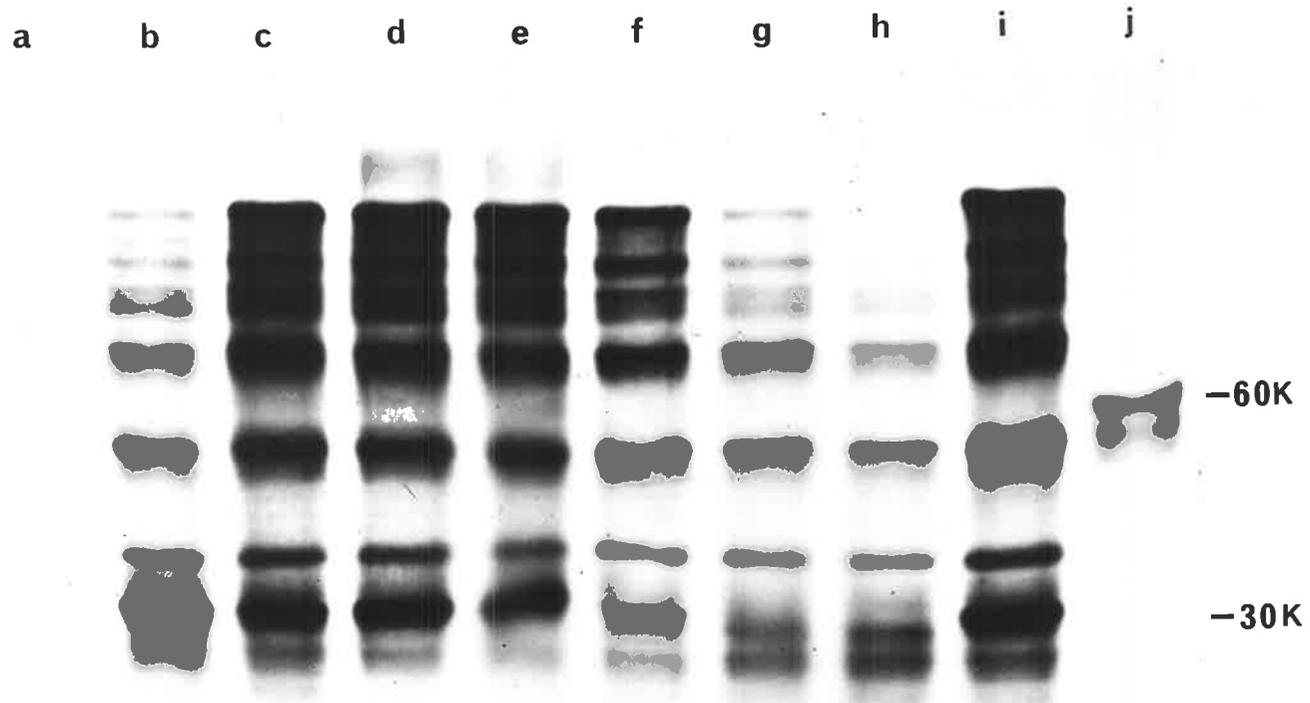


Figure 16. Analysis of the translational products of TRSV RNA synthesized in the Marcu and Dudock system using different extract preparations by electrophoresis on 15% slab gel

Unfractionated TRSV RNAs were translated in three extracts of different activities using ^{14}C -leucine. Incorporations are given in brackets. Samples of the translational products were analysed as described in Materials and Methods.

Track a, TRSV coat protein (4000 cpm);
track b, extract I (75,000 cpm/25 μl ; 20,000 cpm loaded);
track c, extract II (50,000 cpm/25 μl ; 20,000 cpm loaded);
track d, extract III (6,000 cpm/25 μl ; 6,000 cpm loaded);
track e, QCMV RNA products (extract I, 20,000 cpm loaded);
track f, TMV RNA products (extract I, 20,000 cpm loaded).
The gel was fluorographed for 7 days.

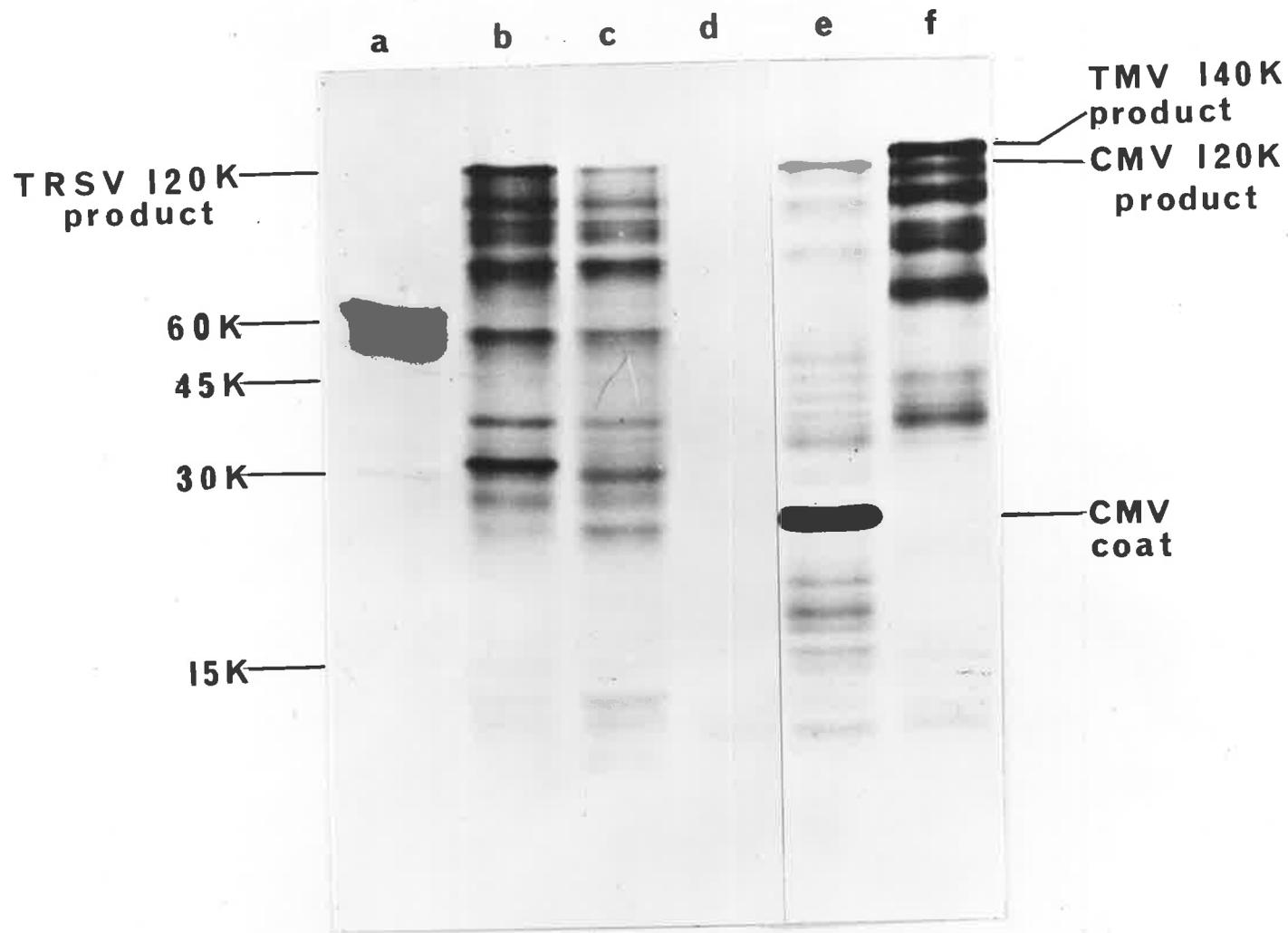
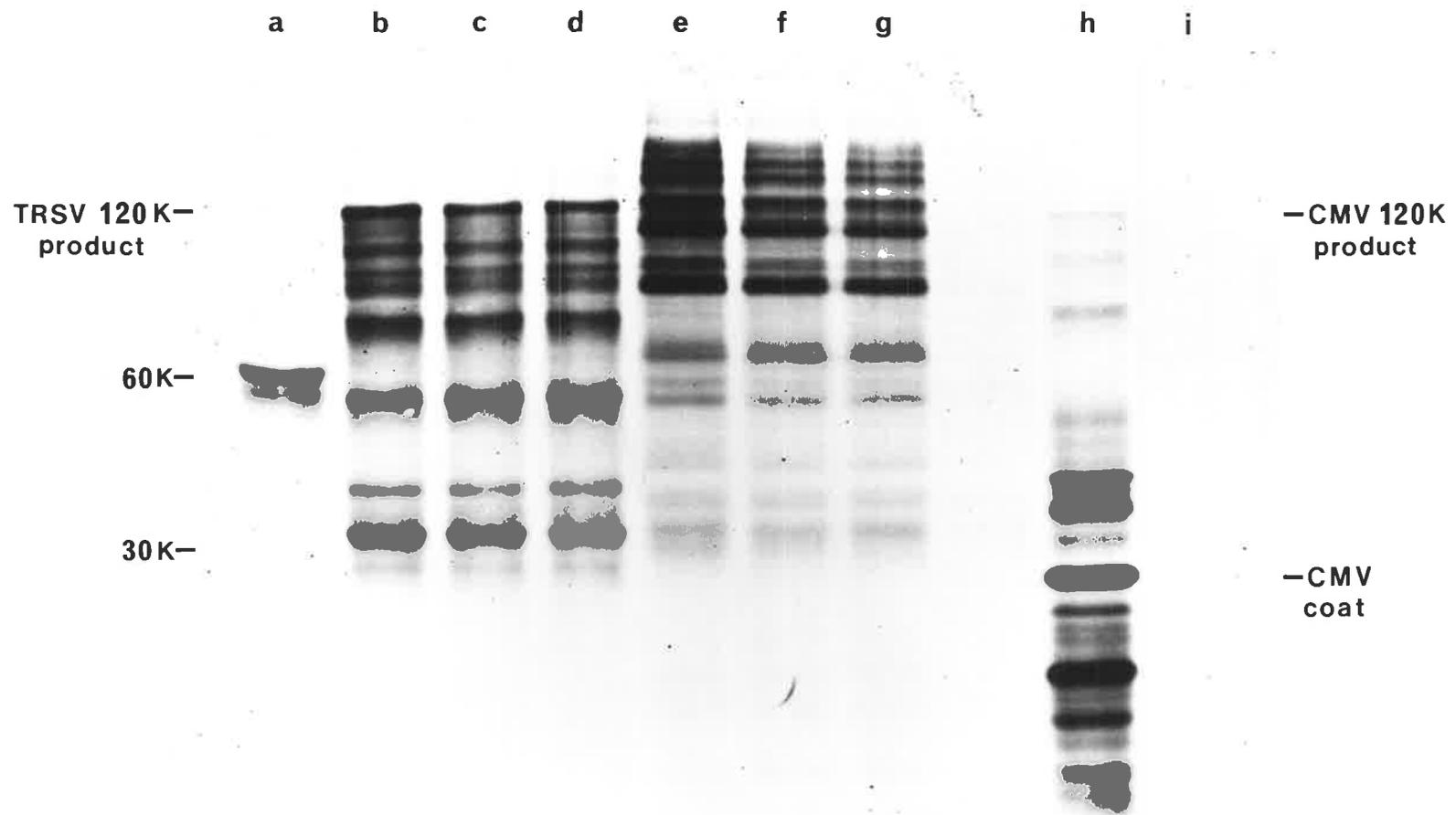


Figure 17. Analysis of the translational products of TRSV RNA synthesized in the Marcu and Dudock system under different concentrations of ^{14}C -leucine by electrophoresis in 15% slab gel

Unfractionated TRSV RNA and RNA-L were translated in the presence of varying concentrations of ^{14}C -leucine (312 $\mu\text{Ci}/\mu\text{mole}$).

Reactions were stopped by treatment with RNase and 20,000 cpm of products translated by each RNA were analysed. The gel was fluorographed for 7 days.

- Track a, TRSV coat protein (4000 cpm);
- track b, unfractionated TRSV RNA, 0.05 μCi /50 μl (3 μM);
- track c, unfractionated TRSV RNA, 0.25 μCi /50 μl (15 μM);
- track d, unfractionated TRSV RNA, 0.75 μCi /50 μl (45 μM);
- track e, TRSV RNA-L, 0.05 μCi /50 μl (3 μM);
- track f, TRSV RNA-L, 0.25 μCi /50 μl (15 μM);
- track g, TRSV RNA-L, 0.75 μCi /50 μl (45 μM);
- track h, QCMV RNA products (0.25 μCi /50 μl);
- track i, -mRNA products (0.25 μCi /50 μl).



phoresis using various RNA molecular weight markers. It is concluded that the molecular weight of TRSV RNA-L is $2.26 \pm 0.02 \times 10^6$ and that of RNA-S is $1.44 \pm 0.02 \times 10^6$. Similar results were also obtained by Murant and Taylor (1978) using different denaturing conditions. Both these results and those reported by Murant and Taylor (1978) show that the molecular weight of TRSV RNA-S under denaturing conditions is slightly higher while that of RNA-L is smaller than those measured by polyacrylamide gel electrophoresis under non-denaturing conditions (Murant *et al.*, 1972; Rezaian and Francki, 1973; Harrison and Murant, 1977). This pattern is also observed with the molecular weights of the RNA-S and RNA-L of some other Nepoviruses (e.g. ArMV, GFLV, TBRV, artichoke Italian latent virus (AILV), MLRV) when they were measured under denaturing and non-denaturing conditions (Martelli *et al.*, 1978; Murant and Taylor, 1978). This may suggest that Nepovirus RNA-S and RNA-L probably have different secondary structures while the same RNA component within the Nepovirus group may have similar secondary structure. However, it has also been suggested that the larger RNAs are probably under-estimated due to lack of reliable RNA markers above the 2.0×10^6 molecular weight region (Murant and Taylor, 1978).

Gel electrophoresis of heated and unheated TRSV RNA preparations shows that dimers of RNA-S and aggregates of RNA-S and RNA-L do exist in TRSV RNA preparations under natural conditions. This explains why infectivity of TRSV RNA components from sucrose density gradients was correlated with RNA-L fractions when analysed by Diener and Schneider (1966).

Results of infectivity assays of the fractionated TRSV RNA components (Table 5) demonstrate conclusively that both RNA-S and RNA-L can be isolated free from each other using two cycles of agarose gel electrophoresis and that both RNAs are required for infectivity. Infectivity of the mixed TRSV RNA components was low (Table 5). However, this was not due to damage sustained by the RNAs during heating (Fig. 4).

The results have shown that the Marcu and Dudock translation system translated TRSV RNA more efficiently than the Davies system. The Davies system translated a greater proportion of low molecular weight products, suggesting that its lower translational activity and shorter translation period is due to lack of elongation rather than re-initiation. The ability of the Marcu and Dudock system to translate larger RNAs efficiently may be due to its higher affinity for mRNAs, especially larger RNAs and more efficient elongation of polypeptide chains. Calculations showed that approximately 0.2 nmole of leucine was incorporated per 2.5 pmole of RNA (average Mol. Wt. = 1.5×10^6) representing a molar ratio of 80:1 respectively. Thus it is likely that re-initiation of translation occurred since most of the products represent only partial translation of the RNAs. It appears that the Marcu and Dudock system used in this work is translating TRSV RNA with similar high fidelity as other systems since U-1 TMV and QCMV RNAs used as controls were translated as efficiently, and synthesizing similar products as those reported in previous translation studies using other cell-free systems (Efron and Marcus, 1973; Roberts *et al.*, 1973; Schwinghamer and Symons, 1975).

Optimising the translation system extended the translation time as well as increased the initial rate of translation. Studies with cowpea mosaic virus (CpMV) RNA (Davies *et al.*, 1977) showed that larger and more products are translated under optimum conditions. Thus this increase in translation activity is probably due to a combination of factors including increased binding of RNA to ribosomes, increased elongation and rate of elongation, and increased re-initiation.

RNA-L generally has very flat optimisation curves and very low messenger activity compared to those of RNA-S and unfractionated RNAs. Work with other viruses e.g. alfalfa mosaic virus (AMV; Mohier *et al.*, 1975; Van Vloten-Doting *et al.*, 1975), brome mosaic virus (BMV; Zagorski, 1978; Pyne and Hall, 1979) and TYMV (Benicourt and Haenni, 1976;

Benicourt *et al.*, 1978) have suggested or shown that the larger RNAs of these viruses have lower affinities for the ribosomes than the respective smallest RNA components. Similarly the low messenger activity of RNA-L may be caused by lower affinity for the ribosomes as well as slower rate of elongation e.g. due to a less favourable secondary structure.

Fractionated RNA-S was found to have lower translational activity than unfractionated RNA. This is unlikely to be due to co-operative effect of RNA-L or RNA-L products since an increase in RNA-L products in the translational products of unfractionated RNA did not significantly increase RNA-S products. On the other hand, the correlation of translational activity with the infectivity of the respective RNA preparations (Table 13) indicates that the quality of the RNA preparation is important e.g. the presence of traces of agarose in the RNA preparations which inhibit translation (R.H. Symons, private communication) and/or due to the presence of degraded RNA in the preparations.

Thus results of the translation experiments show that efficient *in-vitro* translation of TRSV RNA depends on the efficiency of the translational system used, messenger activity of the mRNA and quality of the extract and RNA preparations. A possible explanation is that protein synthesis is dependent on the following factors: (a) availability of ribosomes, initiation and elongation factors, (b) affinity of the mRNAs for ribosomes and efficient binding of ribosomes to mRNAs, (c) proportion of mRNAs in the RNA preparation capable of binding to the ribosomes, (d) proportion of RNA molecules bound to ribosomes which are translating, and (e) rates of elongation and reinitiation. Thus to ensure efficient translation of viral mRNAs *in-vitro*, the above conditions must be met.

Analysis of the translational products of unfractionated TRSV RNA showed that many polypeptides were synthesized. The results (Table

11) are similar to those reported by Gergerich *et al.* (1979) with TRSV RNA and those obtained by Fritsch *et al.* (1978) with TBRV RNA. However, in these studies, no conclusions were made about the synthesis of the viral coat protein and the viral RNA-S and RNA-L were not translated separately to investigate their genetic contents. On the other hand, in this study, analysis of the translational products of TRSV RNA-S, RNA-L and the unfractionated RNA by tryptic peptide mapping showed that no detectable amounts of coat protein appeared to be translated *in-vitro*, although as has also been reported by Gergerich *et al.* (1979), some products with similar molecular weights to those of TRSV coat protein were detected.

Tryptic peptide analyses of the translational products of TRSV RNA-S and RNA-L showed that these RNAs translated different proteins. Although there were some translational products of RNA-S migrating to similar positions as some products of RNA-L, analysis of the tryptic peptides of RNA-L products indicated that they were different products of similar molecular weights. Translation of unfractionated RNA produced mainly RNA-S products, probably due to lower affinity of RNA-L for ribosomes. Similar patterns have been observed in translation of unfractionated BMV (Shih and Kaesberg, 1973), CMV (Schwinghamer and Symons, 1975) and AMV RNAs (Rutgers, 1977) in which mainly the respective RNA- the smallest RNA component, was translated. This may be a mechanism for translational regulation of gene expression, as has been reported for BMV (Zagorski, 1978; Pyne and Hall, 1979). RNA-L may code for proteins that are required in limited amounts (e.g. RNA polymerase) while RNA-S codes for those required in large amounts e.g. structural protein. Under unfavourable conditions, translation of RNA-L is more affected and this may indicate that the translation of this RNA is subjected to greater regulatory feedback than RNA-S.

The molecular weights of the largest translational products of RNA-S and RNA-L represent approximately 80% and 100% of their genetic

contents respectively. These results are different from those obtained by Fritsch *et al.* (1978) who indicated that complete translation of RNA-S of TBRV was achieved *in-vitro*. However, if the largest translational product of TRSV RNA obtained by Gergerich *et al.* (1979) (115,000 molecular weight) corresponds to the largest RNA-S translational product obtained in this study, then the results would also indicate that the TRSV RNA-S translated by Gergerich *et al.* (1979) was not completely translated. Since no viral coat protein was detected in these largest products of TRSV RNA, it suggests that if the same translational products were synthesized *in-vivo*, then most of the TRSV-specific proteins, except the viral coat protein, must be synthesized by post-translational cleavage of precursor proteins and that all the viral proteins, including the coat protein could not be translated from a multicistronic messenger as reported for carnation mottle virus (CarMV) (Salomon *et al.*, 1978).

Analysis of the translational products showed that a lot of smaller polypeptide chains were synthesized (Table 14). They may have been derived from one or more of the following mechanisms: (1) Partial translation of the intact RNA due to premature termination or translation from several initiation sites; (2) translation of fragmented RNA or (3) post-translational cleavage of larger products (Davies *et al.*, 1977). However, the following observations suggest that the first is the most likely or main mechanism: (i) The proportions of smaller products did not vary significantly in different RNA preparations of isolated RNA-S and RNA-L and unfractionated TRSV RNA which are expected to contain different amounts and species of fragmented RNA. Conversely, the same RNA preparation sometimes produced translational products with varying proportions of low molecular weight products. Thus translation from fragmented RNA does not seem to be important; (ii) many of the smaller products had definite molecular weights although their relative proportions may change, suggesting that they were synthesized by

initiation and/or termination at specific sites; (iii) tryptic peptide analysis indicates that the products contain overlapping amino acid sequences and (iv) incubation of the translational products at 30°C for extended periods did not increase the proportion of smaller polypeptides or a reduction of the larger ones. These observations suggest that post-translational cleavage is unlikely to be involved.

The results indicated that under favourable conditions, greater proportions of the larger polypeptides were synthesized suggesting that *in-vivo* high molecular weight products are produced. Thus many of the low molecular weight products are probably *in-vitro* artifacts due to premature termination from lack of elongation factors and/or structurally changed RNA templates. This is supported by the observation that the larger polypeptides are composed of strong clear bands on polyacrylamide gels while the lower molecular weight products (<12,000) are composed of heterogenous unclear bands (see Figs. 13 - 17). It also appears that the TRSV RNAs, like TMV (Efron and Marcus, 1973; Hunter *et al.*, 1976) and CpMV (Davies *et al.*, 1977) RNAs, may direct the synthesis of several high molecular weight products from several initiation sites instead of only one complete polypeptide chain from each mRNA.

The fact that no TRSV coat protein was detected in the translational products of TRSV RNA suggests that more research is required. Preliminary investigations were done to translate total leaf RNA from cucumber cotyledons infected with TRSV. However, the results showed that a range of products similar to that of purified unfractionated TRSV RNA was synthesized *in-vitro*.

CHAPTER VIISTUDIES ON A PROTEIN ASSOCIATED WITH THE GENOMIC RNA OF TRSVI. INTRODUCTION

Recent studies have shown that the genomic RNAs of some animal and plant viruses which lack the 5' terminal "cap" structure $m^7G(5')ppp(5')N^m_p$, have a covalently linked protein (VPg) instead (Flanegan *et al.*, 1977; Nomoto *et al.*, 1976; Frisley *et al.*, 1976; Lee *et al.*, 1977; Hewlett *et al.*, 1976; Sanger *et al.*, 1977; Hruby and Roberts, 1978; Burroughs and Brown, 1978; Klootwijk *et al.*, 1977; Daubert *et al.*, 1978; Stanley *et al.*, 1978). The VPgs appear to be relatively small, the largest reported was that found in poliovirus with a molecular weight of about 12,000 (Ambros and Baltimore, 1978).

Harrison and Barker (1978) have observed that the infectivity of RNAs isolated from two Nepoviruses, TRSV and TBRV, are also inactivated by incubation with proteinase K, indicating that they may contain a VPg. This agrees with the observations made in this study that TRSV RNA purified by the pronase-SDS method was less infectious and had lower yields than RNA prepared by the phenol-SDS method. Thus experiments were done to investigate the pronase-sensitive structure in TRSV RNA in more detail. During the course of this investigation, Mayo *et al.* (1979) showed that TRSV RNA indeed contains a VPg which has a molecular weight of about 4000.

In this chapter, the results of experiments which demonstrate that TRSV RNA contains a VPg which is essential for infectivity but not for *in-vitro* translation of the viral RNA is presented and that the VPg detected in this study has a molecular weight of about 65,000.

II. EXPERIMENTAL DETAILSA. Treatment of RNAs with Pronase

Samples of TRSV RNA (300 μ g) were incubated at 37°C for 6 hours at 150 μ g/ml in TE buffer in the presence of 0.5% SDS and 0 - 500 μ g/ml pronase (Sigma Type VI from *Streptomyces griseus*). U-1 TMV and QCMV RNAs were

similarly treated as controls. All necessary precautions were taken against any RNase activity present in the pronase or other reagents. After pronase digestion, the RNA samples were deproteinised by extracting twice with one-third volume of phenol unless otherwise stated. The RNAs were recovered by precipitation with ethanol in the presence of 0.1 M NaAc. The RNAs were washed twice with ethanol, freeze dried and each resuspended in 150 μ l TE buffer and then clarified by centrifugation at 3000g for 10 min before being stored at -70°C until needed. Untreated RNA samples (2 mg/ml) were those which received no pronase treatment but had been stored at -70°C .

B. Estimation of the Composition and Recovery of RNA after Pronase Treatment

All pronase treated RNA preparations were electrophoresed in 2.6% aqueous polyacrylamide gels (Peacock and Dingman, 1968) as described in Chapter II to check the composition and recovery of the pronase treated RNAs. Each gel was loaded with 12.5 μ l (25 μ g) of RNA from each of the RNA preparations. After electrophoresis, each gel was stained in 50 ml of 0.01% toluidine blue for 2 hr and then scanned without destaining at 620 nm. The composition and recovery of RNAs after pronase treatment was estimated by integrating the areas under the RNA peaks in scans of the stained gels. In some experiments the concentrations of the RNA samples were also estimated spectrophotometrically. Although the integration method of estimating RNA recovery was not very accurate, it has an advantage over the spectrophotometric method because the former method measured the recovery of intact RNA only while the latter may include degraded RNA. However, in most cases, the estimations obtained by both methods were similar.

C. Infectivity Assays of Viral RNAs

Relative infectivity of pronase treated TRSV and CMV RNA preparations were assayed by diluting 12.5 μ l of each RNA preparation (approximately 25 μ g) in 1 ml of 0.1 M phosphate buffer, pH 7.2, and

inoculating them on half-leaves of cowpea plants as described in Chapter II. TMV RNA was similarly assayed on *Nicotiana glutinosa*.

D. In-vitro Translation of Viral RNAs

Pronase treated RNAs were translated in the wheat germ cell-free system of Marcu and Dudock (1974) using ^{14}C -leucine (324 mCi/mmmole) as described in Chapter VI. Four $\mu\text{g}/50\ \mu\text{l}$ reaction volume were used for translation of TMV RNA while 2 $\mu\text{g}/50\ \mu\text{l}$ were used for CMV RNA. For TRSV RNA, 2 μl (approximately 4 μg) of each RNA preparation was added to each reaction.

Incorporation of leucine was estimated by the cold TCA precipitation method as described in Chapter VI. Translational products of the pronase treated RNAs (20,000 cpm/sample) were analysed by electrophoresis in 15% discontinuous slab polyacrylamide gels (Laemmli, 1970) followed by fluorography as described in Chapter VI.

E. Labelling of TRSV RNA Linked Protein Component

The protein component associated with TRSV RNA (VPg) was labelled *in-vitro* with ^3H - NaBH_4 (Kumarasamy and Symons, 1979). All reagents were cooled in ice before being used. RNAs to be labelled were resuspended in 10 mM sodium borate buffer, pH 9.1, at 10 mg/ml and kept at 0°C . One-tenth volume of 0.1 M formaldehyde solution and 0.5 $\mu\text{Ci}/\mu\text{g}$ RNA of ^3H - NaBH_4 (10 $\mu\text{Ci}/\mu\text{l}$; 13 Ci/mmol) resuspended in 10 mM KOH were added successively to the RNA. The mixture was allowed to react at 0°C for 60 min and then one-twentieth volume of 0.1 M non-radioactive NaBH_4 was added. Finally, 0.2 M sodium phosphate buffer, pH 5.8, was added to the RNA to dilute the RNA concentration to 2 mg/ml.

The VPg of TRSV RNA was also labelled *in-vivo* by incorporating ^{14}C -leucine into TRSV (Chapter IV) from which RNA preparations containing the labelled VPg were isolated. Radioactivity in the labelled RNA preparations was estimated by counting 2 μl aliquots of the RNAs dried on filter paper discs which were then washed three times in 200 ml each of cold 5% TCA and then in 200 ml of ethanol : ether mixture (1:1, V/V).

F. Analysis of the Labelled TRSV RNA

The labelled TRSV RNA preparations were analysed by electrophoresis in 2% agarose gels as described in Chapter II. When required, the RNAs were heated in TBE buffer containing various dissociation reagents prior to loading. After electrophoresis, the gels were stained briefly as described above and then scanned. Gels to be counted were sliced and counted in the presence of NCS as described in Chapter II.

G. Polyacrylamide Gel Electrophoretic Analysis of the VPg of TRSV RNA

The labelled TRSV RNAs (0.5 mg/ml) were digested with 2.5 mg/ml each of T₁ and T₂ ribonucleases (Sigma Chemical Co.) and 300 µg/ml RNase-A in 20 mM sodium acetate, 0.2 mM EDTA, pH 5.0, for 3 hr at 37°C. The digested RNA preparations were then analysed by electrophoresis in 15% continuous urea-SDS gels (Swank and Munkres, 1971) and in 10 - 13% discontinuous SDS gels (Laemmli, 1970) using slab gels with low concentrations of bisacrylamide as described in Chapter VI. After electrophoresis, the radioactive protein bands were detected by fluorography. ¹⁴C-leucine labelled TRSV coat protein components and various unlabelled protein molecular weight markers were electrophoresed in the same gel to estimate the molecular weight of the VPg detected in the gels.

III. RESULTS

A. Effect of Pronase Treatment on Infectivity and Messenger Activity of TRSV RNA

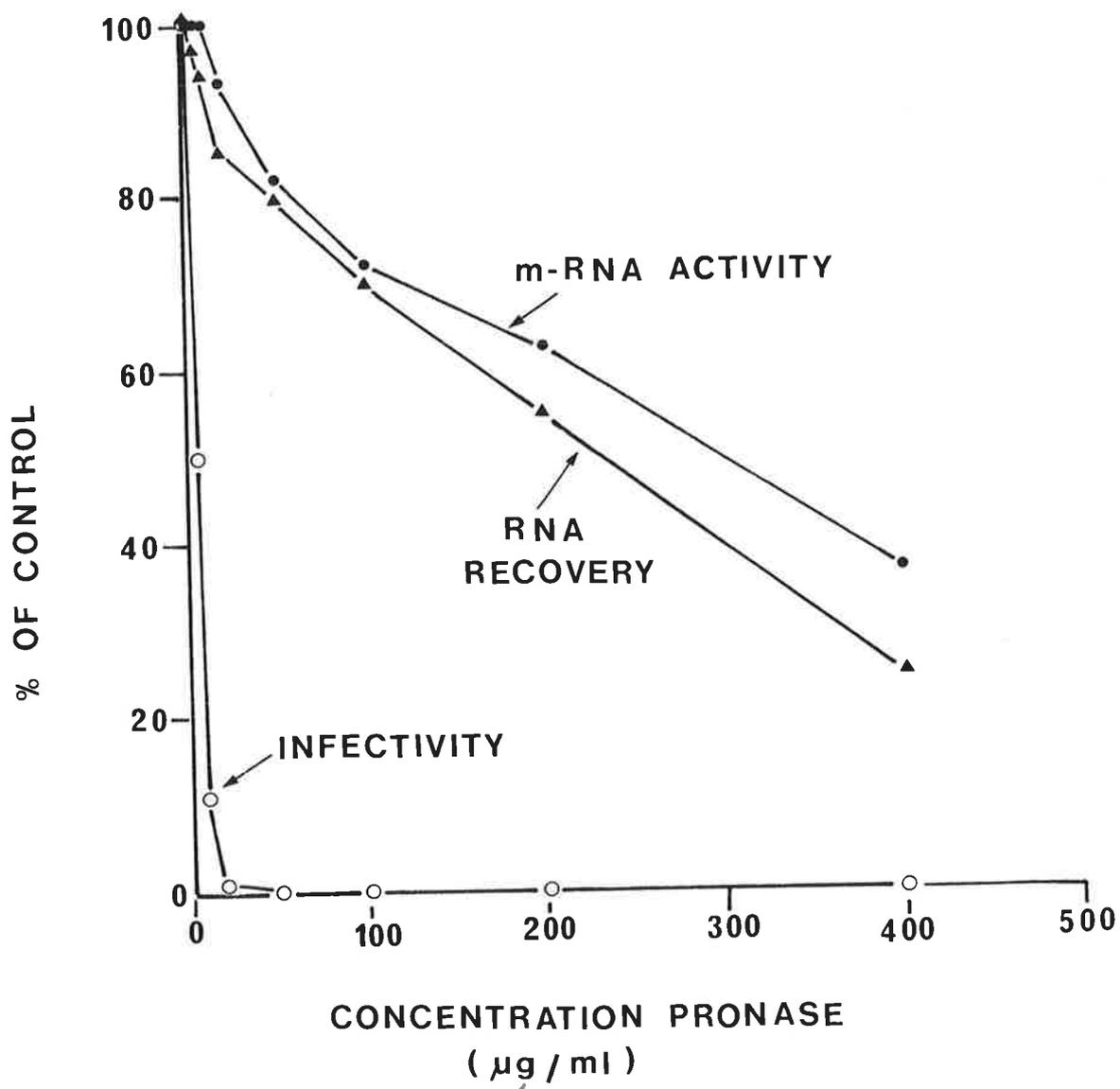
TRSV RNA isolated by the phenol-SDS method was infectious, producing 150 - 200 local lesions per half-leaf at 25 µg/ml. It retained at least 80% of its infectivity after incubation at 37°C for 6 hr in TE buffer with 0.5% SDS, but all infectivity was lost if 20 µg/ml pronase or more was added to the incubation mixture (Fig. 1). However, spectrophotometric estimation and gel analysis of the TRSV RNA after pronase treatment showed that more than 85% of the RNA was recovered intact after the treatment with 20 µg/ml of pronase. However, at higher

Figure 1. Effects of pronase treatment of TRSV RNA on its infectivity, messenger activity and RNA recovery

All RNA preparations were incubated at 37°C for 6 hr in the presence of 0.5% SDS in TE buffer at the pronase concentrations indicated. After incubation, the RNAs were extracted twice with phenol and recovered by ethanol precipitation. The RNAs recovered were analysed in 2.6% polyacrylamide gels and were found to be intact (Fig. 2). RNA recovery was estimated spectrophotometrically as well as by measuring the areas under the RNA peaks in scans of the gels after electrophoresis. Similar results were obtained. The same RNA preparations were assayed for infectivity. The average number of local lesions per half-leaf was calculated from 10 replicates arranged in a Latin square design. The RNA preparations were then translated *in-vitro*. All measurements indicated were expressed as a percentage of the RNA incubated in buffer and SDS only (control).

100% infectivity = 126 lesions per half-leaf.

100% messenger activity = 32,000 ¹⁴C-cpm/15 µl.



concentrations of pronase, less RNA was recovered (Figs. 1 and 2). This result suggests that the loss of infectivity of TRSV RNA after pronase treatment was not due to RNase breaking down the RNA.

There was insignificant reduction in messenger activity of the RNA treated with 20 $\mu\text{g/ml}$ of pronase which was completely devoid of infectivity. However, the messenger activities of the RNA preparations decreased when treated with higher concentrations of pronase (Fig. 1). The correlation of messenger activity of the RNA preparations with RNA recovery and not with infectivity suggests that the loss of translational activity was due to decreased RNA concentrations. Analysis of the translational products of these RNAs demonstrated that pronase treatment did not affect the quality of the translational products (Fig. 3). When pronase treated TRSV RNA was recovered directly by ethanol precipitation or after extracting it with phenol, the infectivity, recovery and translational activities of the corresponding RNA preparations were similar whether they were phenol-extracted or not (Table 1). These results suggest that the loss of infectivity of the pronase treated RNA was not due to residual pronase still present in the RNA preparations nor due to repeated extraction of the RNAs with phenol.

On the other hand, CMV and TMV RNAs, both of which are "capped" (Symons, 1975; Zimmern, 1975; Keith and Fraenkel-Conrat, 1975), remained infectious after treatment with 500 $\mu\text{g/ml}$ pronase (Table 2). Although the infectivity of TMV RNA was reduced to 37% after pronase treatment, the amount of RNA recovered was only 22%. Translation of these RNA preparations showed that pronase treatment did not directly affect their messenger activities and translational products (Table 2 and Fig. 4).

All the above data show that when both TRSV and TMV RNAs were treated with high concentrations of pronase ($>100 \mu\text{g/ml}$), the recoveries and messenger activities of these RNAs were greatly reduced. This poor recovery of RNA after pronase treatment was not due to enzymic breakdown of the RNA since CMV RNA was not similarly lost (Table 2).

Figure 2. Effect of pronase treatment of TRSV RNA on the RNA profile in polyacrylamide gels

Untreated TRSV RNA (gel U, 25 μg) and RNAs incubated with the amounts of pronase indicated on the top of the gels ($\mu\text{g}/\text{ml}$) as described in Fig. 1 (12.5 $\mu\text{l}/\text{sample}$) were analysed in 2.6% polyacrylamide gels without prior heating of the samples. After electrophoresis, the gels were stained with toluidine blue to locate the RNA bands. The bands of aggregated RNA detected are also indicated.

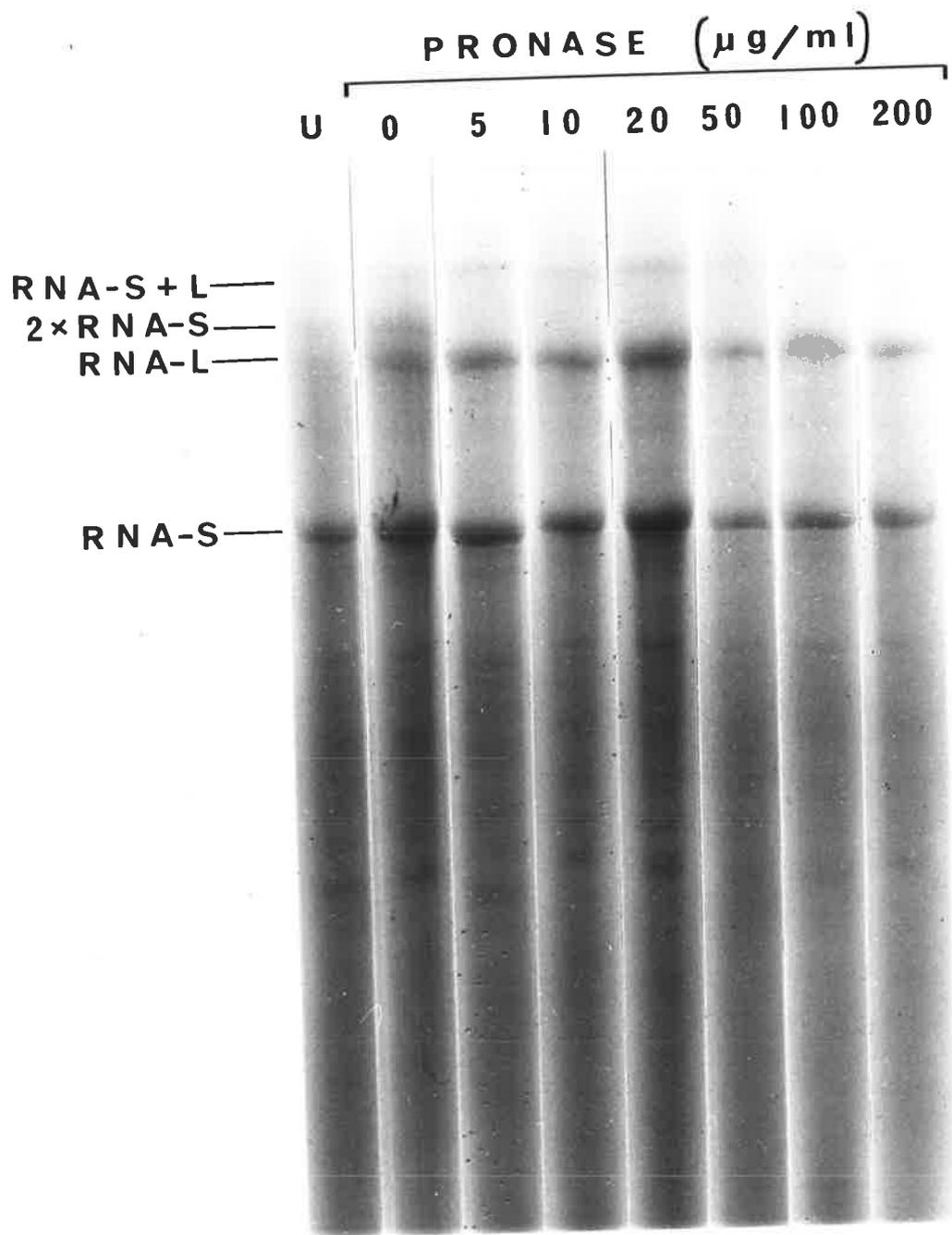


Figure 3. Polyacrylamide gel analysis of translational products of TRSV RNA before and after pronase treatment of the RNAs

TRSV RNA preparations recovered after incubation with pronase or other reagents indicated ($\mu\text{g/ml}$) in the figure were translated *in-vitro*. Aliquots of the translational products equivalent to 20,000 cpm (except for 400 $\mu\text{g/ml}$ pronase and Ca^{++} treated RNAs which had low translational activities and 5000 cpm only) were electrophoresed in 15% slab discontinuous gel. The gel was exposed by fluorography for 7 days at -70°C .

Track U, untreated RNA products;

track O, RNA incubated in buffer and SDS only;

track Ca^{++} , RNA incubated in the presence of CaCl_2 (120 $\mu\text{g/ml}$).

other tracks, RNAs incubated with the amounts of pronase indicated in $\mu\text{g/ml}$.

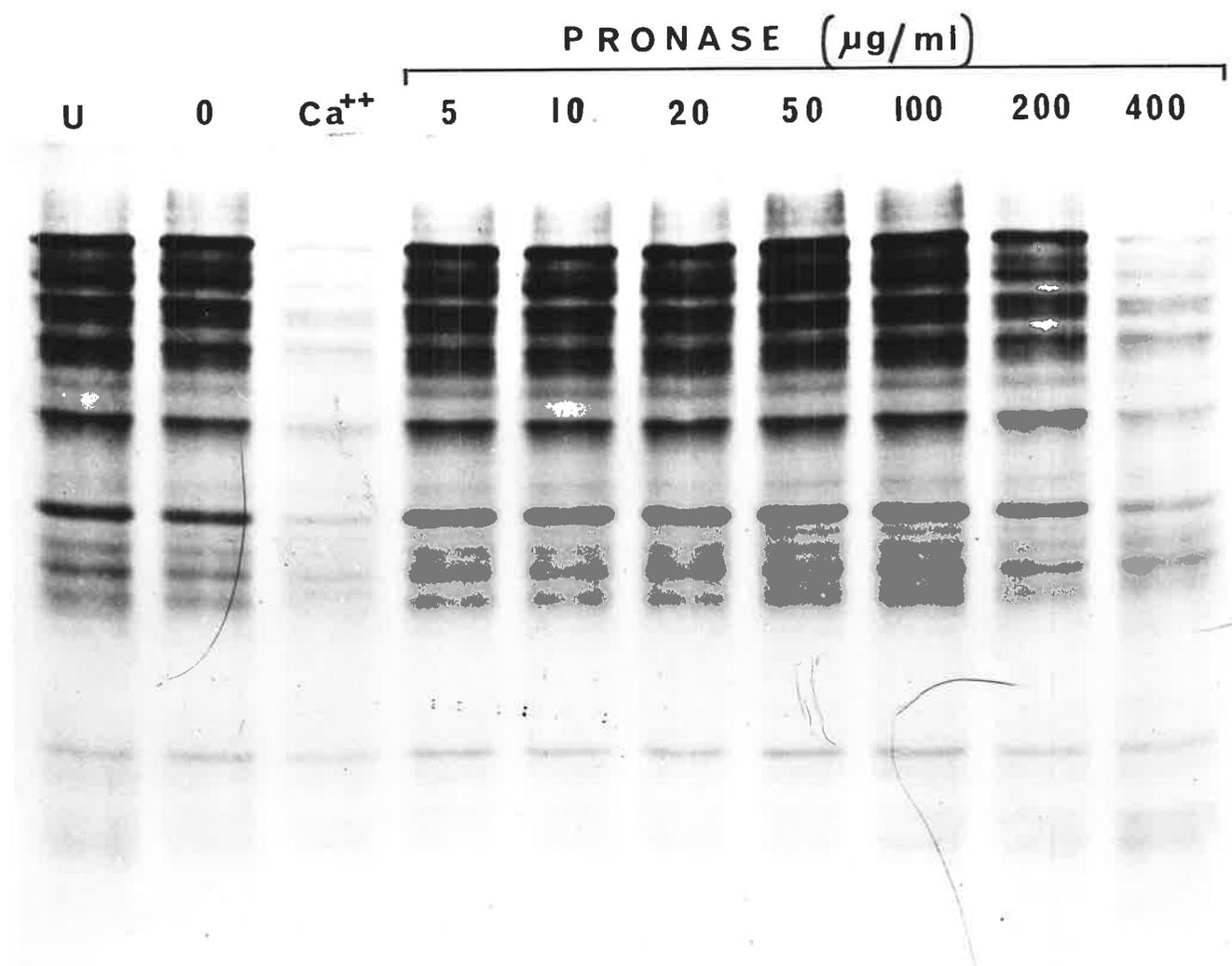


Table 1

Effects of phenol extraction on infectivity, translational activity
and RNA recovery of pronase treated TRSV RNA

RNA Treatment ^a	Not Phenol Extracted ^b			Phenol Extracted ^c		
	Infecti- vity ^d	Relative RNA Recovery ^e	Translation Activity ^f	Infecti- vity ^d	Relative RNA Recovery ^e	Translation Activity ^f
Untreated (control)	163(100)	100%	35.0(100)	163(100)	100%	35.0(100)
Pronase 0	129 (79)	88%	32.0 (91)	111 (68)	81%	31.0 (89)
Pronase 20	0.6(0.4)	82%	33.0 (94)	0.4(0.2)	80%	32.0 (91)
Pronase 100	0 (0)	74%	25.7 (73)	0 (0)	86%	27.5 (79)
Pronase 200	0 (0)	46%	7.5 (21)	0 (0)	60%	13.75(39)

^a Incubated for 6 hr at 37°C in the amounts of pronase indicated in TE buffer and 0.5% SDS.

^b The pronase treated RNAs were precipitated from the incubation mixtures directly with ethanol.

^c The incubation mixtures were extracted twice with phenol before precipitating the RNA with ethanol.

^d RNA was tested for infectivity as described in Materials and Methods. Each value is the average number of local lesions per half-leaf from 15 half-leaves. Values in brackets are percentages of control.

^e RNA recovery was estimated spectrophotometrically.

^f Translation was done as described in the text. Values are ¹⁴C-cpm/15 µl reaction volume ($\times 10^{-3}$). Values in brackets are percentages of control. Endogenous incorporation (1.0×10^3) was subtracted from each value.

Table 2

Effect of pronase and calcium chloride treatments on the recovery, infectivity and translation activity of CMV, TMV and TRSV RNAs

RNA Treatment ^a	Infectivity ^b (Lesions/half-leaf)	Translational Activity ^c (¹⁴ C-cpm/15 μ l x10 ⁻³)	Relative RNA ^d Recovery(%)
<u>TMV</u>			
untreated control	38 (100)	57.7 (100)	100
buffer only	18 (47)	54.5 (94)	40
buffer + 500 μ g/ml pronase	14 (37)	10.5 (18)	22
<u>CMV</u>			
untreated control	63 (100)	57.0 (100)	100
buffer only	36 (57)	47.0 (82)	80
buffer + 500 μ g/ml pronase	39 (62)	45.8 (80)	80
<u>TRSV</u>			
untreated control	186 (100)	29.5 (100)	100
buffer only	161 (91)	30.3 (103)	86
buffer + 500 μ g/ml pronase	0 (0)	4.8 (16)	<10
buffer + 120 μ g/ml Ca ⁺⁺	94 (50.5)	5.1 (17)	<10

^a Incubated for 6 hr at 37°C in the reagents indicated and re-extracted with phenol. Incubation buffer contained TE buffer + 0.5% SDS.

^b Mean for 18 randomised half-leaves. Figures in brackets are percentages of control RNA.

^c Assays were done as described in the text. Endogenous incorporation (1.0×10^3) was subtracted from each value. Figures in brackets are percentages of control.

^d Estimated spectrophotometrically.

Figure 4. Polyacrylamide gel analysis of the translational products of TMV and CMV RNAs before and after treatment with pronase

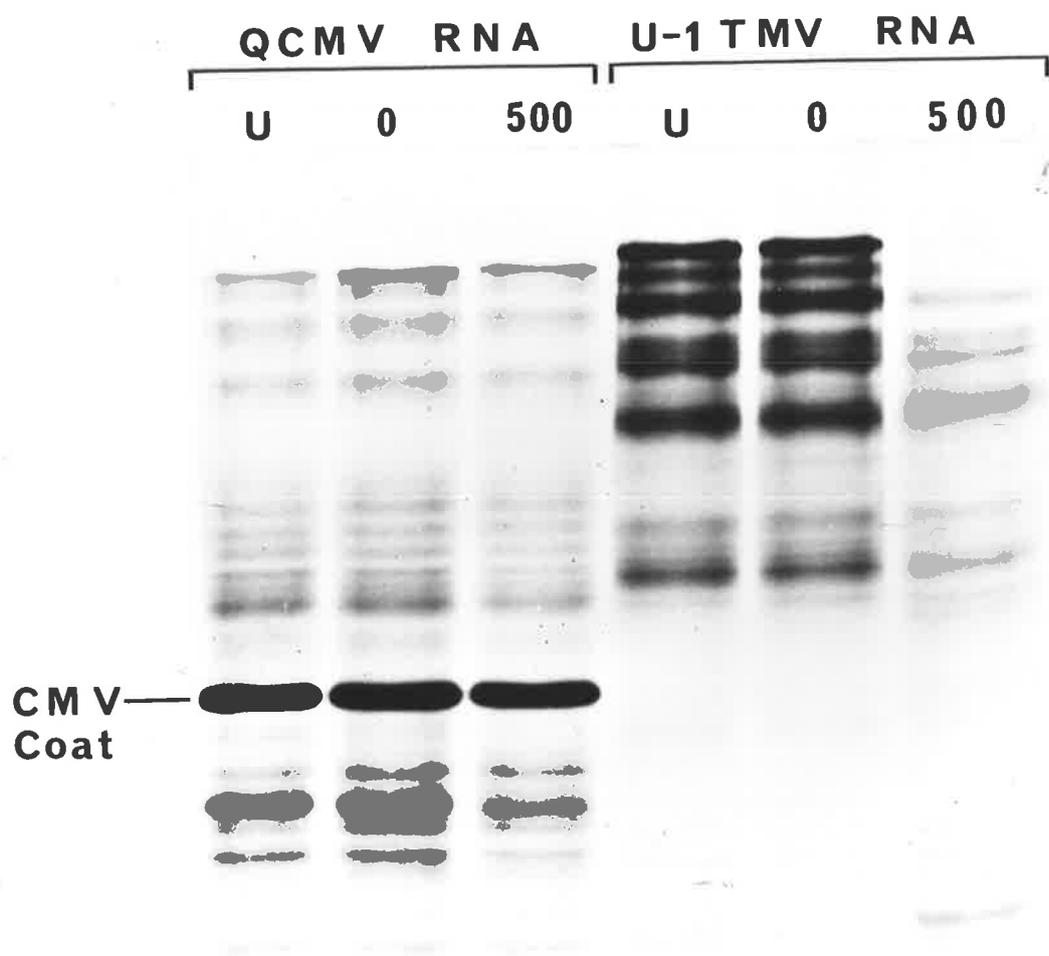
QCMV and U-1 TMV RNAs were incubated with pronase or buffer as described in Materials and Methods. The RNAs recovered were translated *in-vitro*, and the products were analysed in 15% slab discontinuous gels.

Tracks U, untreated RNA products;

tracks 0, RNAs incubated in buffer and SDS only;

tracks 500, RNAs incubated in 500 µg/ml pronase.

The low translational activity of TMV RNA incubated in the presence of 500 µg/ml pronase was due to low RNA recovery after pronase treatment and only 5000 cpm was loaded compared to 20,000 cpm for other products.



Furthermore, polyacrylamide gel electrophoresis of the pronase-treated RNA failed to reveal any heterogeneity of the RNA in the gels (Fig. 2). However, the following observations suggest that the poor RNA recoveries were due to precipitation of the RNAs by Ca^{++} ions present in the pronase preparations, which contained about 30% (W/W) of calcium acetate. In one experiment, incubation of TRSV-RNA in the presence of 120 $\mu\text{g}/\text{ml}$ calcium chloride (CaCl_2), a concentration equal to that present in 400 $\mu\text{g}/\text{ml}$ pronase, reduced the recovery and translational activity of the RNA to levels similar to those obtained after treatment with 400 - 500 $\mu\text{g}/\text{ml}$ pronase (Table 2 and Fig. 3). Ca^{++} ions appear to have less effect on CMV RNA (Table 2) but this is probably due to Ca^{++} ions precipitating the larger TRSV and TMV RNA molecules more readily than the smaller CMV RNA molecules since TRSV RNA recovered after treatment with over 400 $\mu\text{g}/\text{ml}$ pronase or with Ca^{++} ions was found to consist mostly of the smaller RNA-S component. It is unlikely that Ca^{++} ions remaining in the RNA preparations directly inhibited the translation reactions since CMV RNA treated with 500 $\mu\text{g}/\text{ml}$ of pronase did not show any reduction in messenger activity (Table 2). Thus it appears that reduced messenger activity in RNAs treated with high concentration of pronase is mainly due to low recovery of RNA caused by Ca^{++} .

TRSV RNA treated with 120 $\mu\text{g}/\text{ml}$ Ca^{++} ions retained 50% of the infectivity (Table 2) while that which was treated with only 20 $\mu\text{g}/\text{ml}$ pronase, which contained a maximum of 6 $\mu\text{g}/\text{ml}$ Ca^{++} ions, lost its infectivity completely (Fig. 1). Thus the loss of infectivity in pronase-treated TRSV RNA was not due to the effect of Ca^{++} ions in the pronase. Furthermore, it seems unlikely that other contaminating chemicals in the pronase denatured the TRSV RNA since it was inactivated with only 20 $\mu\text{g}/\text{ml}$ pronase while TMV and CMV RNAs remained infectious at 500 $\mu\text{g}/\text{ml}$. Thus the above results show that a protein (VPg) required for infectivity is associated with TRSV RNA.

B. Analysis of the VPg Associated with TRSV RNA

Samples of untreated TRSV RNA and those rendered non-infectious by incubating in 100 µg/ml pronase were labelled *in-vitro* with $^3\text{H-NaBH}_4$. The untreated RNA was more radioactive than the pronase treated RNA (Table 3). Analysis of these labelled RNAs in agarose gels indicated that most of the RNAs were still intact after labelling since there were little differences in RNA profiles of the respective labelled and unlabelled RNA preparations (Fig. 5). Analysis of the distributions of radioactivity in the gels indicated that there was no association of radioactivity with the RNA peaks of the pronase treated RNA, but some radioactivity (10 - 15% of that recovered in the gels) was associated with the RNA peaks of the untreated RNA (Fig. 6). This indicates that the RNA itself was not labelled but the protein component on the RNA was labelled. Infectivity assay of the untreated RNA showed that its infectivity was lost after labelling with $^3\text{H-NaBH}_4$. This and the results presented in Fig. 6 suggest that most of the VPg molecules are probably dissociated from the RNA during *in-vitro* labelling and tend to form aggregates which accumulate on the tops of the gels on electrophoresis of the RNAs.

Samples of the $^3\text{H-NaBH}_4$ labelled TRSV RNAs (untreated and pronase treated) were digested with RNases. After digestion, the RNAs retained most of their original radioactivities in the TCA insoluble material (Table 3). Samples of these RNA preparations were electrophoresed in both continuous (urea-SDS) and discontinuous polyacrylamide gels. The results of one such experiment illustrated in Fig. 7, show that no protein band was detected in the RNA sample which had been treated with pronase. However, a protein band (VPg in Fig. 7) was present in the RNA which received no pronase treatment. The protein had electrophoretic properties unlike those of the viral coat protein components (Fig. 7). The molecular weight values estimated for the VPg by both electrophoretic systems were similar, being between 64 - 66,000 (Fig. 8).

Table 3

In-vitro labelling of TRSV RNA with $^3\text{H-NaBH}_4$

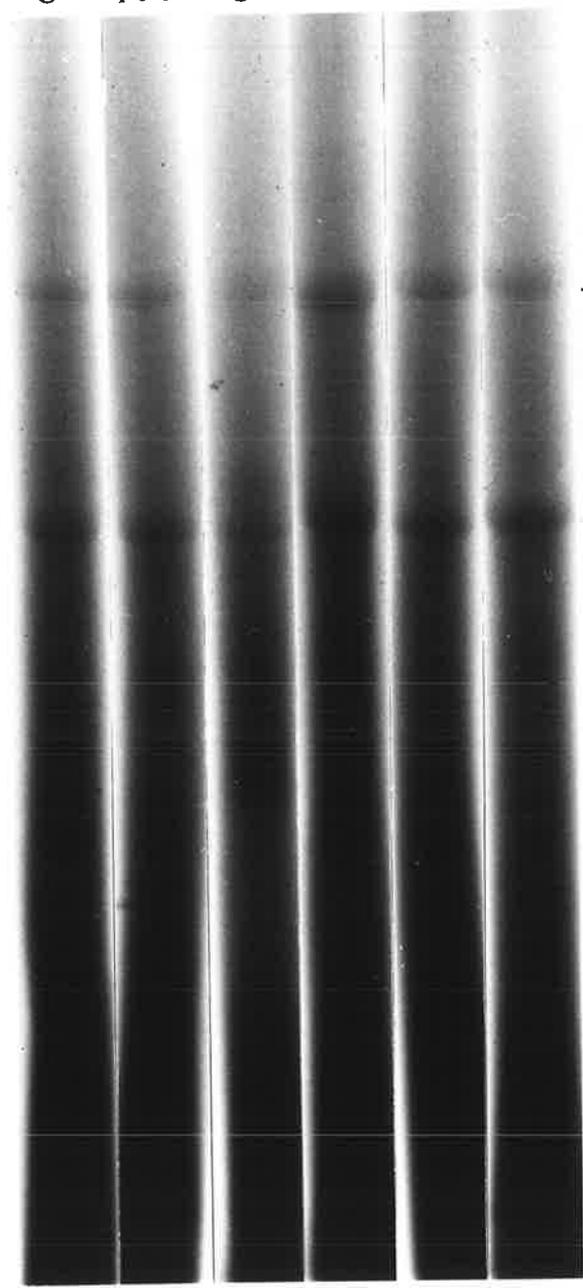
TRSV RNA	Radioactivity incorporated per μg RNA
Untreated	4,700 cpm
Untreated after RNase digestion	4,745 cpm
100 $\mu\text{g}/\text{ml}$ pronase	3,500 cpm
100 $\mu\text{g}/\text{ml}$ pronase after RNase digestion	3,290 cpm

TRSV RNA not digested with pronase (untreated) and those digested with 100 $\mu\text{g}/\text{ml}$ pronase were labelled *in-vitro* with $^3\text{H-NaBH}_4$ as described in Materials and Methods. After labelling, 2 μl samples from the RNA preparations were counted in duplicates and the average was obtained. The RNAs were then digested with RNases as described in Materials and Methods and duplicate samples (5 μl each) of the RNAs were similarly estimated for radioactivity.

Figure 5. Electrophoresis of $^3\text{H-NaBH}_4$ labelled TRSV RNA
in agarose gels

Untreated (U) and 100 $\mu\text{g/ml}$ pronase (100) treated TRSV RNAs were analysed before and after labelling with $^3\text{H-NaBH}_4$ in 2% agarose gels. The labelled and unlabelled RNA preparations are indicated on the tops of the gels. RNA samples (30 $\mu\text{g/gel}$) were heated at 70°C for 3 min in buffer (gels a) or in buffer containing 1% SDS (gels b) prior to electrophoresis. After electrophoresis, the gels were stained in 0.01% toluidine blue for 2 hr and photographed without destaining. The stain at the bottom part of the gels appears to be due to disproportionate penetration of stain into the gels.

Unlabelled		Labelled			
a	a	a	b	a	b
U	100	U	U	100	100



— RNA-L

— RNA-S

Figure 6. Distribution of radioactivity in agarose gels containing $^3\text{H-NaBH}_4$ labelled TRSV RNAs

Untreated (A) or pronase (100 $\mu\text{g/ml}$) treated (B) TRSV RNAs were labelled *in-vitro* with $^3\text{H-NaBH}_4$. Samples of the labelled RNAs (10 - 15 μg ; 48,000 cpm/sample) were either heated at 70°C for 3 min in buffer only (\bullet _____ \bullet _____ \bullet _____) or in buffer containing 1% SDS (\circ _____ \circ _____ \circ _____) or unheated (\blacktriangle _____ \blacktriangle _____ \blacktriangle _____) prior to electrophoresis in 2% agarose gels. After electrophoresis, the gels were stained with toluidine blue as described in Fig. 5 to locate the RNA bands and then scanned at 620 nm. All the gels yielded similar RNA profiles. The same gels were then sliced and counted in NCS-liquid scintillation fluid as described in Chapter II.

In A, after dissociation of the RNA in SDS, a radioactive peak (indicated by arrow) was observed at the bottom of the gel, this may represent dissociated VPg.

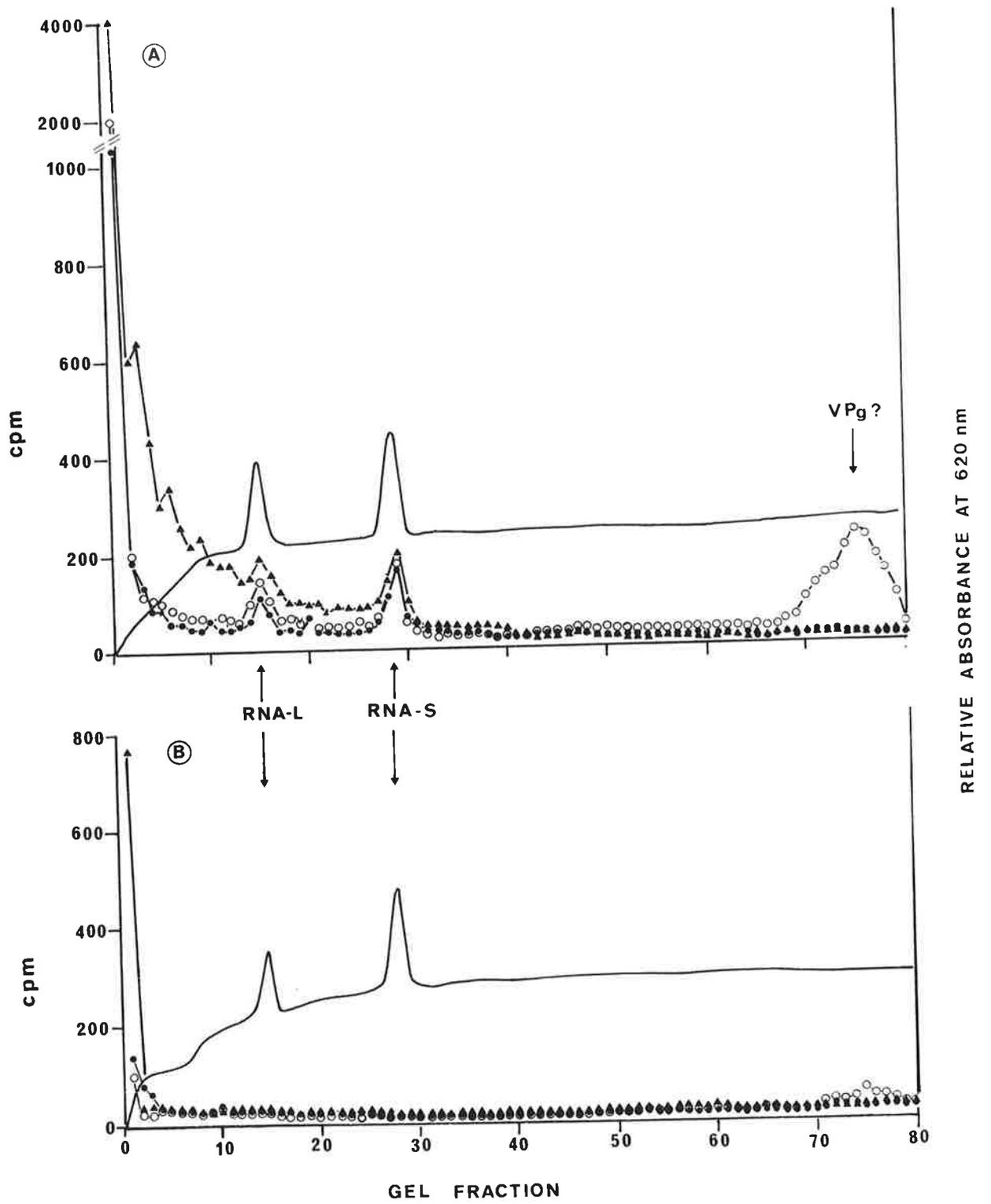


Figure 7.

A. Analysis of $^3\text{H-NaBH}_4$ labelled TRSV VPg by the continuous urea-SDS PAGE system

RNases digested $^3\text{H-NaBH}_4$ labelled untreated and pronase (100 $\mu\text{g/ml}$) treated TRSV RNA preparations were electrophoresed in 15% continuous urea-SDS slab polyacrylamide gel as described in Materials and Methods. 30,000 cpm of each RNA sample was loaded. Gel was exposed for 36 days at -70°C by fluorography. ^{14}C -leucine labelled TRSV coat protein and unlabelled marker proteins were used as markers.

Track U, TRSV RNA not digested with pronase;

track 100, TRSV RNA digested with 100 $\mu\text{g/ml}$ pronase;

track TRSV, TRSV coat protein (5000 cpm).

The VPg detected is indicated by arrow.

B. Analysis of $^3\text{H-NaBH}_4$ labelled TRSV VPg by the discontinuous SDS PAGE system

Legend is as described in A, except that the RNAs were electrophoresed in 13% discontinuous SDS slab gel and 90,000 cpm per RNA sample were loaded. Gel was exposed for 12 days.

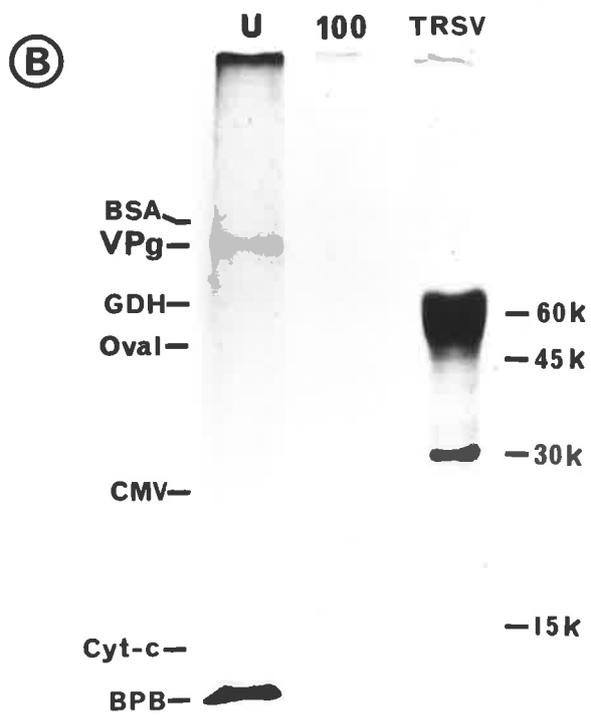
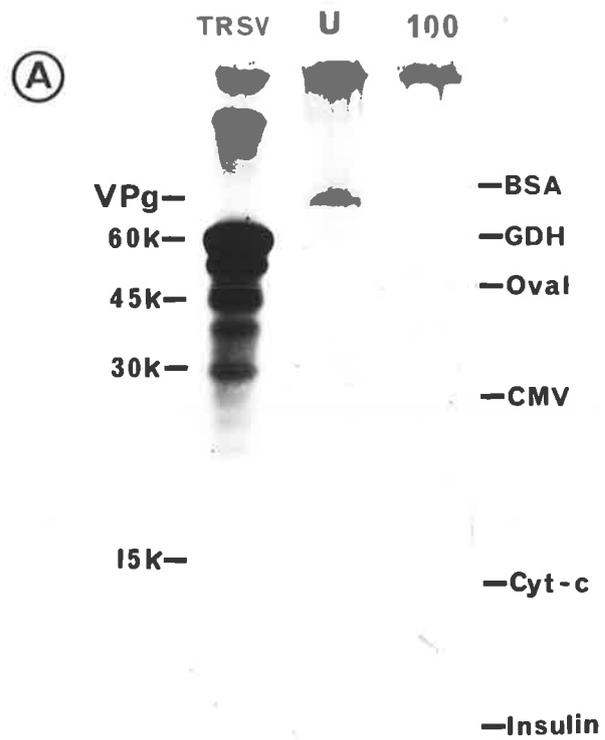


Figure 8. Estimation of the molecular weight of TRSV VPg
by electrophoresis in polyacrylamide gels

The TRSV RNA linked protein component (VPg) labelled *in-vitro* with $^3\text{H-NaBH}_4$ and *in-vivo* with ^{14}C -leucine as described in Materials and Methods was analysed by electrophoresis in continuous urea-SDS and discontinuous SDS gels (Figs.7 and 10). The relative mobilities of the VPg was estimated using various protein markers indicated in the figure.

Positions of the TRSV coat protein components (116K, 60K, 45K, 30K and 15K) and those of the VPg are indicated by arrows.

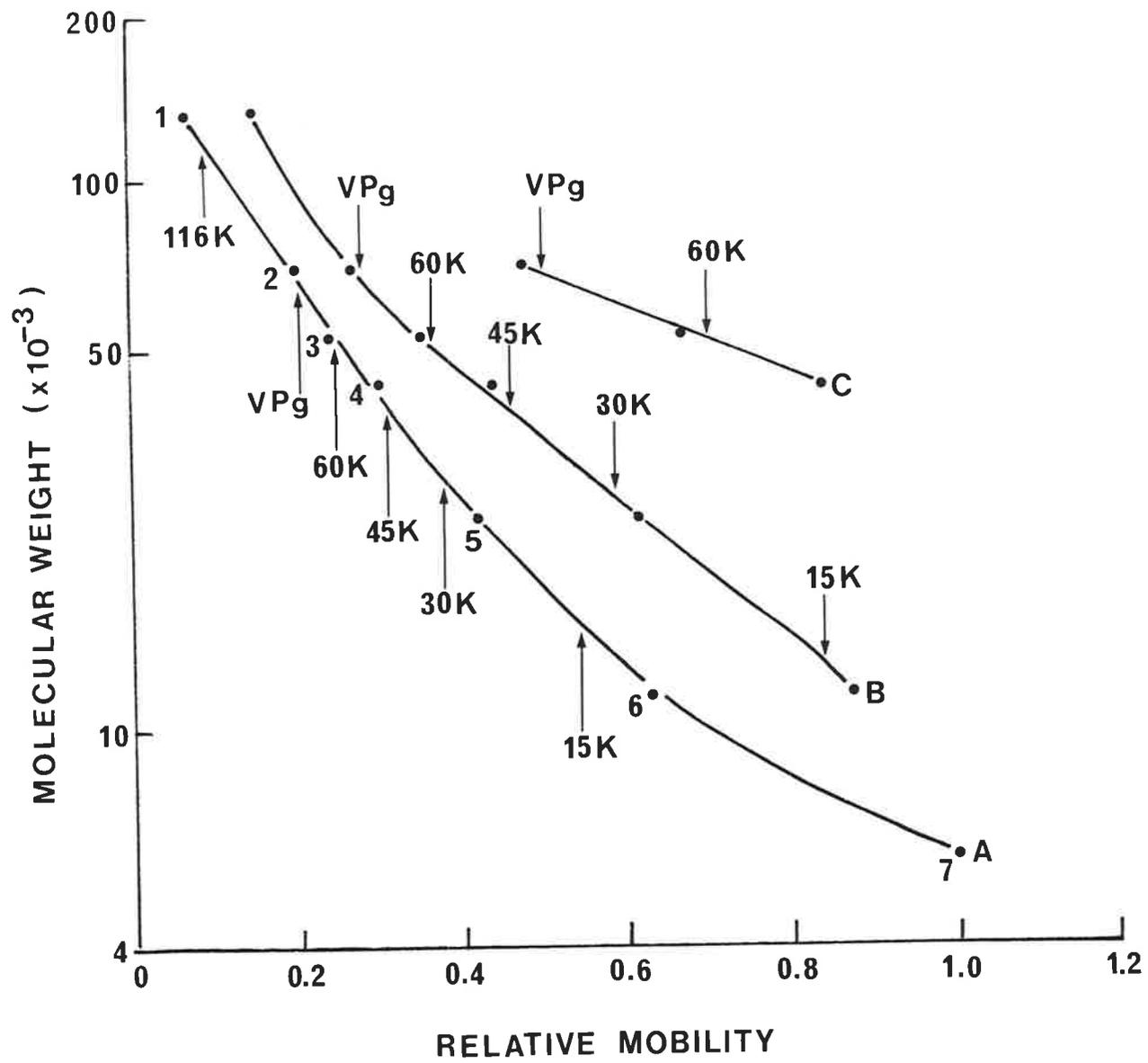
Curve A, continuous gel electrophoresis of *in-vitro* ^3H -labelled VPg (see Fig. 7A);

curve B, discontinuous gel electrophoresis of *in-vitro* ^3H -labelled VPg (see Fig. 7B);

curve C, discontinuous gel electrophoresis of *in-vivo* ^{14}C -leucine labelled VPg (see Fig. 10).

Protein Markers used were: 2 x BSA (1); BSA (2);

GDH (3); Oval (4); CMV (5); Cyt-c (6) and insulin (7).



Similar results were obtained with ^{14}C -leucine labelled TRSV RNA prepared from TRSV labelled *in-vivo* with ^{14}C -leucine. Analysis of the untreated RNA preparation and that which was digested with 100 $\mu\text{g/ml}$ pronase in agarose gels showed that radioactivity was associated with both RNA-S and RNA-L of the untreated RNA when they were electrophoresed after heating in buffer only (Fig. 9A) or after boiling in 6 M urea, 1% SDS and 2% MCE (Fig. 9B) but was not completely eliminated from the RNA treated with pronase (Fig. 9C). However, electrophoresis of these RNA preparations after RNase treatment showed that a protein band (VPg in Fig. 10) was associated only with the untreated RNA preparation. The VPg labelled *in-vivo*, similar to that labelled *in-vitro*, was found to have a molecular weight of about 66,000 (Fig. 8).

IV. DISCUSSION AND CONCLUSIONS

Evidence presented demonstrates that the removal of a protein component linked to TRSV RNA (VPg) by the proteolytic action of pronase is responsible for eliminating the infectivity of TRSV RNA. Although the effect of pronase is complicated by the presence of calcium acetate in the enzyme preparation it appears that Ca^{++} ions merely reduced the recovery of RNA without directly affecting its infectivity. The reduction of RNA recovery itself was not responsible for the loss of infectivity.

The VPg can be labelled *in-vitro* with $^3\text{H-NaBH}_4$ and *in-vivo* with ^{14}C -leucine. Its molecular weight and association with the RNA even in the presence of urea, SDS and MCE reagents indicate that it is not a component of TRSV coat protein contaminating the RNA preparations. These results confirm the report of Harrison and Barker (1978) that TRSV RNA contained a protein component linked to the genomic RNA (VPg) which is required for virus synthesis.

The VPg detected is associated with both RNA-S and RNA-L. The fact that it survived phenol-SDS extraction, and was not dissociated by boiling in 6 M urea, 1% SDS and 2% MCE indicate that it is linked to

Figure 9. Distribution of radioactivity in agarose gels
containing *in-vivo* ^{14}C -leucine labelled TRSV RNA

^{14}C -leucine labelled TRSV RNA was isolated from TRSV labelled *in-vivo* as described in Chapter II and some was incubated in 100 $\mu\text{g}/\text{ml}$ pronase as described in Fig. 1. Untreated and pronase digested RNA samples (25 $\mu\text{g}/\text{sample}$) were analysed by electrophoresis in 2.0% agarose gels. After electrophoresis, the gels were stained with toluidine blue for 2 hr and then scanned at 620 nm. The same gels were then sliced and counted in NCS-liquid scintillation fluid.

- A. Untreated RNA heated at 70°C for 3 min in sample buffer prior to electrophoresis.
- B. Untreated RNA boiled in sample buffer containing 6 M urea, 1% SDS and 2% MCE for 2 min prior to electrophoresis.
- C. Pronase digested RNA heated at 70°C for 3 min in buffer prior to electrophoresis.

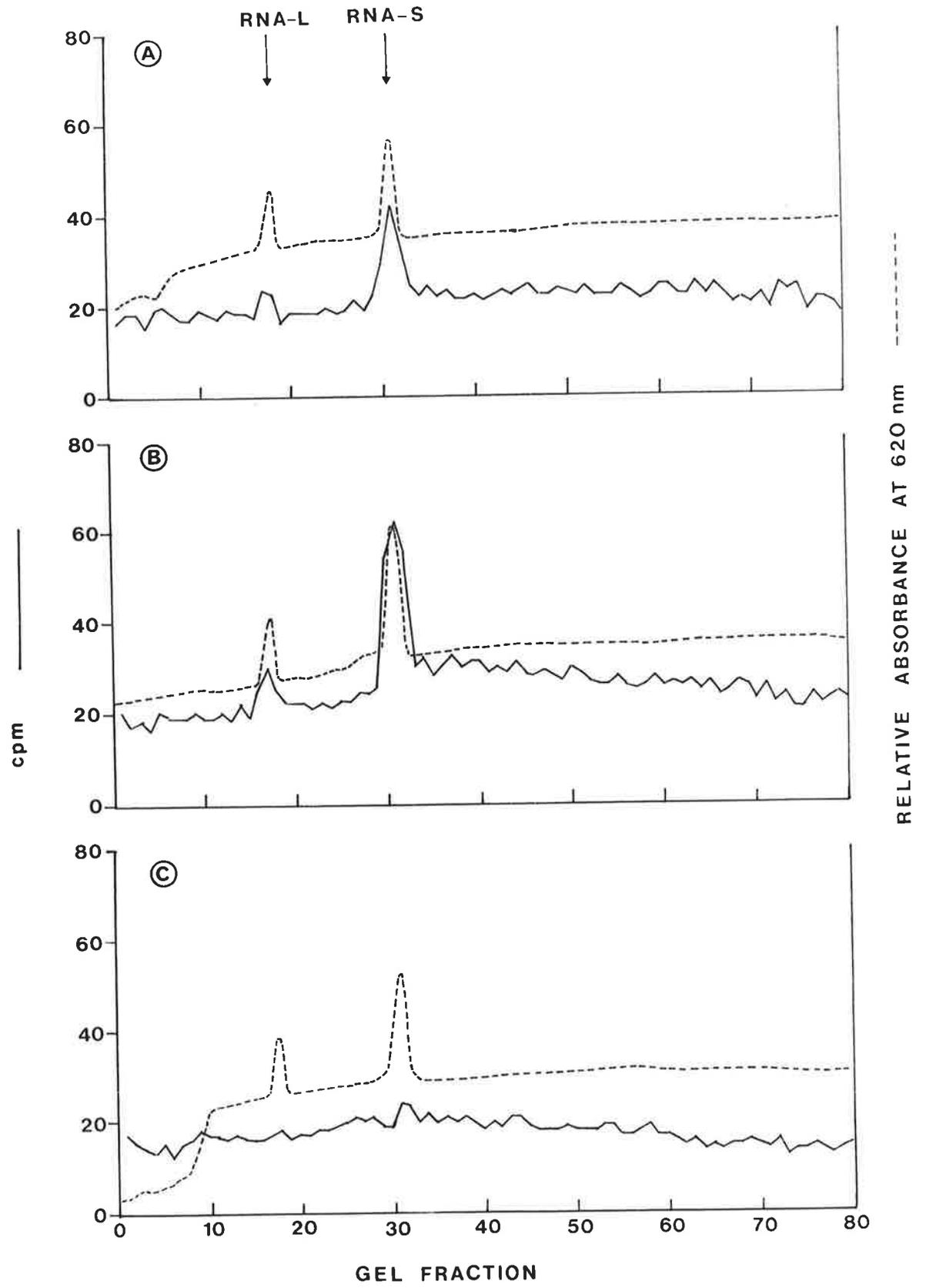


Figure 10. Analysis of *in-vivo* ^{14}C -leucine labelled TRSV VPg by electrophoresis in discontinuous SDS-slab polyacrylamide gel

In-vivo ^{14}C -leucine labelled TRSV RNA preparations were either untreated or incubated in 100 $\mu\text{g}/\text{ml}$ of pronase before being digested with RNases as described in Materials and Methods. The RNases digested RNA preparations were then electrophoresed in 10% discontinuous slab gel in the presence of protein markers as described in Fig. 7. After electrophoresis the gel was fluorographed for 14 days.

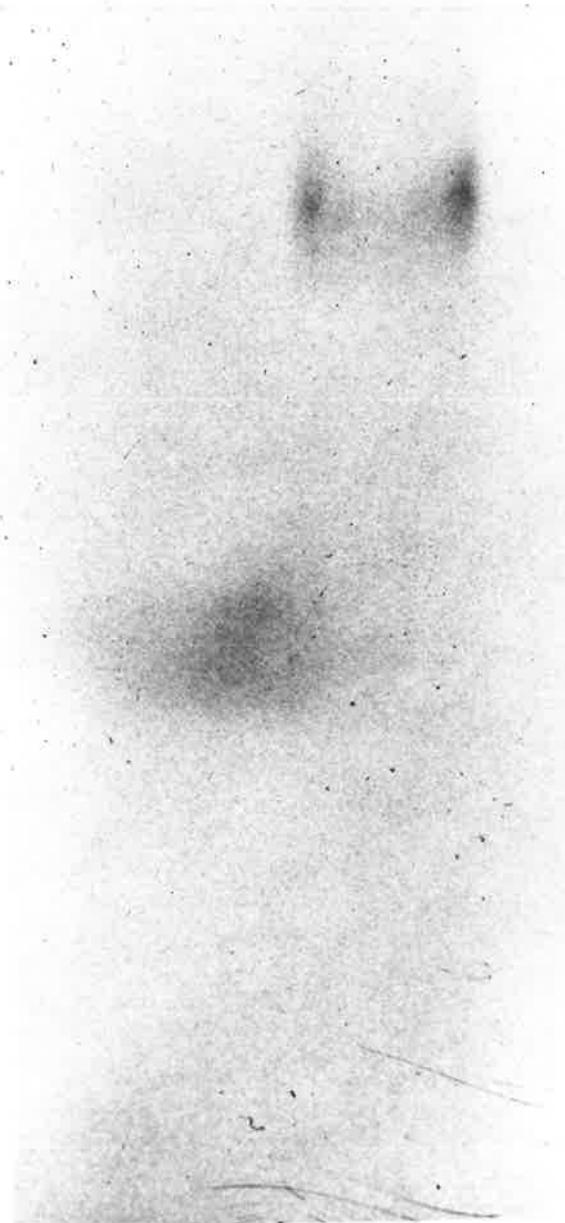
Track TRSV, 1000 cpm of ^{14}C -leucine labelled TRSV coat protein;

track U, 5000 cpm of ^{14}C -leucine labelled TRSV RNA not digested with pronase;

track 100, 5000 cpm of ^{14}C -leucine labelled TRSV RNA incubated with 100 $\mu\text{g}/\text{ml}$ pronase

The VPg detected in track U is indicated by an arrow.

TRSV U 100



— BSA

— VPg

— GDH

— 60K

— Oval

the RNA by covalent bonds. The VPg has a molecular weight of 64 - 66,000 which is different from any of the viral coat protein components (see Chapter IV). Thus the VPg detected here is much larger than the VPg reported by Mayo *et al.* (1979). Why such a discrepancy exists is unclear. The 15% continuous urea-SDS gel used (Fig. 7A) would detect proteins with molecular weights of about 4000 but no such low molecular weight materials were detected; instead only one radioactive band was detected which migrated slower than the 60K TRSV coat protein component. It is unlikely that this band represents an aggregate of a lower molecular weight protein formed during the *in-vitro* labelling process since the $^3\text{H-NaBH}_4$ labelling technique is not known to produce any aggregated proteins on SDS gels (Kumarasamy, personal communication) and since a similar protein was also detected in the *in-vivo* ^{14}C -leucine labelled RNA (Fig. 10). The observation that the VPg band (Figs. 7 and 10) was not heterogenous suggests that it is not contaminated with oligonucleotide. On the other hand, in the analysis of the VPg by discontinuous PAGE, some radioactive material was stacked behind the buffer front in the untreated RNA (Fig. 7B). It is unclear whether this is the protein observed by Mayo *et al.* (1979). Furthermore, the RNA labelled *in-vivo* still retained some radioactivity after pronase treatment (Fig. 9C) but showed no infectivity. These results may suggest the presence of two protein components in the RNA. However, it is also possible that the VPg was not completely digested by pronase in the *in-vivo* labelled RNA preparation as has been reported for poliovirus and CpMV (Nomoto *et al.*, 1977a and Stanley *et al.*, 1978). In any event, the discrepancy between the results in this study and those of Mayo *et al.* (1979) must be reconciled in future by further investigations.

The function of the VPg in TRSV is obscure. Removal of the VPg did not affect the *in-vitro* messenger activity of the RNA or alter its translational products. Thus it may be involved in the transcription of viral RNAs since it was reported that infectious viral RNA was not

replicated in its absence (Harrison and Barker, 1978). It appears that the VPg may be also involved in the aggregation of TRSV RNA-S into dimers, since it was observed that the dimer of RNA-S (see Chapter VI) was absent in the pronase treated RNA preparations but present in the untreated RNA (Fig. 2).

VPgs have been detected in several other plant and animal viruses belonging to various virus groups (Table 4). They are similar to that found in TRSV in that they all appear to be covalently linked to the genomic RNAs (Rothberg *et al.*, 1978; Flanagan *et al.*, 1977). However, they all appear to differ in molecular weights and functions (Table 4). The estimated molecular weights of the VPgs for all these viruses are less than 10,000 (Table 4) although a more recent report suggested 12,000 for poliovirus VPg (Ambros and Baltimore, 1978), and possibly even larger for the EMC VPg (Golini *et al.*, 1978). Thus the molecular weight reported here for the VPg of TRSV is very large compared to those reported for these viruses, while that reported by Mayo *et al.* (1979) is within their range (Table 4). The functions of the VPgs are unclear. Those found in viruses belonging to the family Picornaviridae have not been shown to be essential for either translation or infectivity (Sanger *et al.*, 1977; Nomoto *et al.*, 1977b; Perez Bercoff and Gander, 1978). Similarly, the VPg of CpMV which belongs to the Comovirus group was also shown to be not required for either translation or infectivity (Daubert *et al.*, 1978; Stanley *et al.*, 1978). Thus, TRSV differs from these viruses in that its VPg is required for infectivity but not for *in-vitro* translation and in this sense it is similar to vesicular exanthema virus (VEV), which belongs to the Caliciviridae (Matthews, 1979), the VPg of which is also required for infectivity (Burroughs and Brown, 1978).

Table 4

Properties of the VP_gs detected in various
plant and animal viruses

Virus Group	Virus	Molecular Weight of VP _g	Requirement for Infectivity	Requirement for Translation	Ref.
Picarno- viruses	poliovirus type 1	4000 - 6000	no	no	1,2,3
		12,000			4
	type 2	4000 - 6000	-	-	5
	foot & mouth disease virus	4000 - 6000	no	-	6
	encephalomyo- carditis virus	8000 - 10,000	no	-	4, 7
Como- virus	cowpea mosaic virus	5000	no	no	8, 9
Calici- virus	vesicular exanthema virus	10,000	yes	-	10
Nepo- viruses	TRSV	4000	yes	-	11,12
		65,000	yes	no	13
	TBRV	-	yes	-	11

References:

- | | |
|----------------------------------|---------------------------------|
| 1. Nomoto <i>et al.</i> (1977b) | 7. Hruby and Roberts (1978) |
| 2. Lee <i>et al.</i> (1977) | 8. Daubert <i>et al.</i> (1978) |
| 3. Flanagan <i>et al.</i> (1977) | 9. Stanley <i>et al.</i> (1978) |
| 4. Ambros and Baltimore (1978) | 10. Burroughs and Brown (1978) |
| 5. Golini <i>et al.</i> (1978) | 11. Harrison and Barker (1978) |
| 6. Sanger <i>et al.</i> (1977) | 12. Mayo <i>et al.</i> (1979) |
| | 13. This study |

Dash indicates data not available.

CHAPTER VIIIGENERAL DISCUSSIONA. Stability of TRSV *In-vitro*

"In all the history of virology, comparatively little attention has been paid to the question of stabilizing interactions in viruses. Yet, almost every experimental virologist is daily confronted with problems that involve these interactions (Kaper, 1972)." This statement is applicable to the situation when a new method was to be devised for isolating intact coat protein from TRSV without the use of acids.

The presence of protein-protein interactions in TRSV is supported by the presence of empty capsids. However, this appears to be not the only dominating interaction since studies of the stability of TRSV in SDS, at various pHs and to mild heating revealed that the particle components are differentially stable. Thus TRSV must be stabilised by RNA-protein interactions as well. It appears that the stability of some other Nepoviruses may be less dependent on protein-protein interactions since no T components are found (Chapter I, Table 1). Thus in contrast to Kaper (1972) who suggested that TRSV is similar to TYMV in being stabilised mainly by protein-protein interactions, it appears that Nepoviruses are also similar to CMV in being stabilised mainly by RNA-protein interactions. This is shown by the fact that TRSV has stability characteristics of both TYMV and CMV (see Chapter III and Kaper, 1972).

B. Structure of TRSV and other Nepovirus Capsids

Data pertaining to the structure of Nepovirus capsids, especially that of TRSV is highly controversial (see Chapter I and Francki and Hatta, 1977). Results of this study have shown that the majority of TRSV protein migrated as a band with a molecular weight of 57,000 when electrophoresed in polyacrylamide gels; the only protein detected by Mayo *et al.* (1971). However, in this study several other bands of protein with molecular weights of approximately 14,000, 28,000, 42,000 and 110,000 were also detected. This posed a problem in identifying the actual TRSV coat

protein subunit. Evidence has been presented indicating that all these protein components detected are chemically and antigenically similar. Their molecular weights and other results presented in Chapter IV suggest that they are various aggregated forms of the smallest component which is the actual polypeptide subunit. Amino acid analysis indicates that the TRSV coat protein subunit has a molecular weight of approximately 13,000.

None of the data obtained in this study is compatible with the conclusions reached by Mayo *et al.* (1971) that the TRSV capsid consists of 60 polypeptides each with a molecular weight of about 57,000. Such subunits with the amino acid composition reported (Chapter IV, Table 4 and Stace-Smith *et al.* 1965) would be expected to yield at least 51 peptides on digestion with trypsin and 9 cyanogen bromide fragments. None of these results have been observed. On the other hand, a subunit molecular weight of 13,000 would be compatible with the model of the TRSV capsid proposed by Chambers *et al.* (1965) in which it is composed of 240 polypeptides. Based on this model, the total molecular weight of the TRSV protein shell would be 3.12×10^6 , a value which agrees reasonably well with that of $3.3 - 3.5 \times 10^6$ calculated by Mayo *et al.* (1971) using the Svedberg equation and the percentage of RNA in M and B particles. Data presented in this thesis support the conclusion that the TRSV capsid is icosahedral with a T = 4 surface lattice structure (Caspar and Klug, 1962).

It is unclear whether other Nepoviruses may have similar capsid structures as that found in TRSV but Agrawal (1967) reported that AMV may also have a T = 4 icosahedral structure. It is possible that the capsids of most of the other Nepoviruses, if not all, may have a T = 4 structure with 240 polypeptides each with a molecular weight of around 13,000 because:

- (i) The particles of all Nepoviruses are very similar in appearance under the electron microscope, and are similar in many physico-chemical properties (Francki and Hatta, 1977; Harrison and Murant, 1977).

(ii) Most Nepoviruses including TRSV, appear to contain only one type of coat protein polypeptide with similar electrophoretic mobilities in polyacrylamide gels and molecular weights of about 55,000 (Harrison and Murant, 1977; Francki and Hatta, 1977; Chapter I, Table 1). These proteins, like that observed in TRSV, may represent tetramers of low molecular weight polypeptides (about 13,000 daltons) caused by self-aggregation. The low molecular weight polypeptide components (15K, 30K, 45K) may have not been detected or considered before, because of:

(a) protein loading was too low since yields of most Nepoviruses are very low, (b) dissociation and electrophoresis procedures and staining methods used did not enable the detection of the low molecular weight components since these procedures are important in TRSV (Chapter IV), (c) it is possible that in some Nepovirus proteins, stronger bonds are present in the tetramers which reduced the amounts of the low molecular weight components.

(iii) A tomato ringspot virus (TomRSV) isolate was purified and its protein analysed in SDS-polyacrylamide gels (data not presented in thesis). The virus preparations were relatively free from contamination when examined under the electron microscope. When this viral protein was analysed in polyacrylamide gels, a major protein band with a molecular weight of approximately 54,000, similar to that detected by Allen and Dias (1977) was observed. However, it constituted only about 70 - 80% of the protein loaded and several other protein bands, some of which may correspond to the 15K, 30K and 45K components of TRSV were also detected.

C. Aggregation and Dissociation of TRSV Coat Protein Polypeptides

Although the tetramers (60K) are the most abundant aggregates in preparations of TRSV coat protein, they are probably not integral parts of the viral capsid since all the other minor components tend to assume this structure after re-dissociation in urea, SDS and MCE (Chapter IV, Fig. 12). How the various components are formed and what

types of bonds are involved in stabilizing them, especially the 60K component remain unclear. It was observed that intact TRSV which reacted only with anti-TRSV serum ceased to react with it on dissociation in urea, SDS and MCE, and reacted with anti-60K protein serum instead. This suggests that at least two types of tertiary structures of the coat protein polypeptide are involved: (a) the original (native) structure present in the virus and (b) a new structure formed on dissociation of the capsid with urea, SDS and MCE. The fact that all the other protein components tend to become the 60K component suggests that the 60K is the most stable form and is formed preferentially on dissociation of the virus capsid; and that the other minor components are not formed from the 60K component but are less stable products formed at the same time.

The reason why the 60K component is so stable is unclear. Many proteins are known to form relatively stable aggregates, including TMV and cowpea chlorotic mottle virus (CCMV) proteins (Fraenkel-Conrat, personal communication), aldolase and crotonase enzymes (Davies and Stark, 1970). There is no conclusive evidence to indicate that any specific mechanisms are involved although it has been suggested that cross-linking peptide bonds may be formed *in-vivo* by enzymes between glutamine and lysine residues (Rice, 1974; Fraenkel-Conrat, personal communication). However, in all the other proteins, the amounts of aggregated material are relatively small compared to the monomer and it seems that a different mechanism of aggregation must be involved in TRSV protein. Indirect evidence suggests that TRSV protein possesses strong protein-protein interactions. The virus particles are relatively stable in 1% SDS and tend to aggregate *in-vitro*. The isolated protein also tends to aggregate and becomes relatively insoluble in aqueous solutions. These observations suggest that strong hydrophobic and other very strong non-polar bonds may be involved in aggregating the protein subunits. The protein appears to be able to exist in many

conformations. This suggests that several types of protein-protein interactions are involved in aggregating TRSV proteins. Thus it is likely that when the viral capsid is dissociated by urea, SDS and MCE, the conformation of the polypeptides is altered in such a way that new intermolecular covalent bonds are formed. However, more research on the protein structures by biophysicists is required to solve this problem and caution must be taken in interpreting molecular weight data of proteins from SDS-polyacrylamide gels.

D. Properties of the Particles and RNAs of TRSV and other Nepoviruses

Based on the molecular weights obtained for the TRSV coat protein subunit and RNA-S and RNA-L, it is possible to calculate a set of molecular weights and other parameters for the TRSV particle components (Table 1). These values are not very different from those obtained by Mayo *et al.* (1971) which are based on the Svedberg equation and the percentage of RNA in the particles estimated by sedimentation (Reichmann, 1965). The data in Table 1 show that there is a molecular weight difference of 0.62×10^6 and a difference of 6% in RNA content between B particles containing RNA-L and those containing two RNA-S molecules. Thus it is possible to separate these two types of B particles by CsCl analytical centrifugation as has been reported by Murant *et al.* (1972). As expected, two types of B particles were detected in TRSV when the virus was subjected to equilibrium centrifugation in CsCl (Fig. 1).

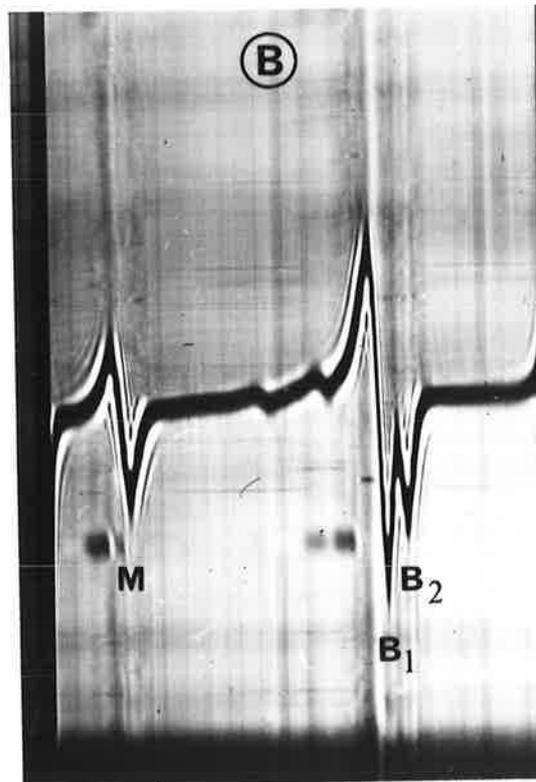
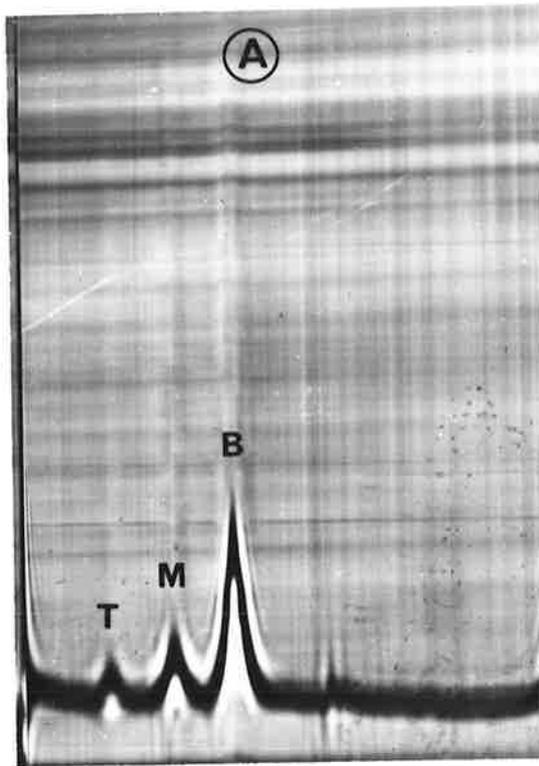
The RNA-S of several other Nepoviruses also have molecular weights of around 1.4×10^6 (ArMV, GFLV, RRV, potato black ringspot virus (PBRV), SLRV and artichoke vein banding virus (AVBV)) (Murant and Taylor, 1978; Martelli *et al.*, 1978). It is suggested that a second type of B component particle containing two molecules of RNA-S is also present in these viruses (Mayo *et al.*, 1973; Martelli *et al.*, 1978). Theoretically in equilibrium centrifugation, the B components of these viruses should each yield two buoyant density classes of particles; and this property is used as a major taxonomic characteristic for classifying the Nepoviruses (Martelli *et al.*, 1978). However, equilibrium

Table 1Data on the composition and molecular weights of TRSV component particles

	From data in this thesis	Reported in Mayo <i>et al.</i> (1971)
Mol. Wt. of coat protein	13,000	57,000
Mol. Wt. of capsid (T=4)	3.12×10^6	3.4×10^6
Mol. Wt. of RNA-S	1.44×10^6	1.4×10^6
Mol. Wt. of M particles	4.56×10^6	4.8×10^6
Percentage of RNA in M	31.6%	27%
Mol. Wt. of RNA-L	2.26×10^6	2.3×10^6
Mol. Wt. of B particles (RNA-L)	5.38×10^6	5.7×10^6
Percentage of RNA in B (RNA-L)	42%	41%
Mol. Wt. of RNA-S dimer	2.88×10^6	2.8×10^6
Mol. Wt. of B (RNA-S)	6.00×10^6	6.2×10^6
Percentage of RNA in B(RNA-S)	48%	45%

Figure 1. Separation of TRSV particle components by ultracentrifugation (A) and equilibrium centrifugation (B)

- A. TRSV (3.2 mg/ml) was centrifuged in 0.1 M phosphate buffer, pH 7.0, at 31,410 rpm in the An-D rotor for 9 min.
- B. TRSV (400 μg) was centrifuged in CsCl ($\rho = 1.45 \text{ gm cm}^{-3}$) in 1 mM phosphate buffer, pH 7.0, at 44,770 rpm in the An-D rotor for 18 hr. The two B components and the M component observed are indicated.



centrifugation studies of RRV, unlike TRSV, did not reveal any heterogeneity in the B component (Murant *et al.*, 1972). This phenomenon may be explained by the possibility that in TRSV there are more B particles containing RNA-S than in RRV. This is supported by the following observations made in this study:

- (i) RNA-S of TRSV tends to aggregate to form dimers which can be detected in agarose gels.
- (ii) Infectivity of B particles from TRSV was not increased by addition of M particles but in RRV, the M particles increased the infectivity of the B particles (Harrison *et al.*, 1972a).

Infectivity studies show conclusively that both RNA species of TRSV are necessary for infectivity as has been shown for RRV (Harrison *et al.*, 1972a), TBRV (Randles *et al.*, 1977) and GFLV (Quacquarelli *et al.*, 1976). Translation studies (Chapter VI) also show that both RNAs directed the synthesis of entirely different proteins which agrees with the finding of Rezaian and Francki (1974) that there was very little nucleotide sequence homology between the RNA species. Furthermore, pseudo-recombinant studies with RRV and TBRV by Harrison *et al.* (1974) and Hanada and Harrison (1977) have shown that the two RNA species of these viruses carry different genetic determinants. Thus all these results point to the conclusion that the two RNA species of Nepoviruses (Chapter I, Table 1) carry different genetic information required for infectivity.

E. Site of Synthesis of TRSV *In-vivo*

The results in this thesis show that TRSV protein synthesis is inhibited by cycloheximide and not by chloramphenicol, indicating that 80S cytoplasmic and not the 70S chloroplast ribosomes are involved in virus synthesis in excised cucumber cotyledons. Similar observations were reported for RRV (Harrison and Murant, 1977). In plants infected with RRV or TBRV, structures which reacted with the respective viral antiserum were also found to accumulate in the cytoplasm or cytoplasmic

inclusion bodies (Barker and Harrison, 1977). Thus in many Nepoviruses, like most other plant viruses e.g. TMV (Zaitlin *et al.*, 1968; Singer, 1972), TYMV (Bove, 1975), CpMV (Owens and Bruening, 1975), the sites of viral coat protein synthesis *in-vivo* are in the cytoplasm. These results and the finding of Rezaian (1974) that the site of RNA-dependent-RNA-polymerase activity and the localisation of TRSV specific ds-RNA are in the cytoplasmic fraction indicate that all events leading to TRSV synthesis occur in the cytoplasm of cucumber cotyledons. This conclusion is further supported by the results of electron microscopic investigations which failed to reveal any virus induced changes in any of the organelles; but did reveal an increase in the structural complexity of the cytoplasmic membrane system, suggesting an increase in cytoplasmic activities in cells of TRSV infected cotyledons during virus synthesis (Rezaian *et al.*, 1976).

F. *In-vitro* Translation of TRSV RNA and Mechanism of TRSV Coat Protein Synthesis

Since RNA-S determines serological specificity in RRV and TBRV, it suggests that the RNA-S contains the coat protein gene (Harrison *et al.*, 1974; Hanada and Harrison, 1977; Harrison and Murrant, 1977). The RNAs of TRSV were translated *in-vitro* in an attempt to locate the coat protein gene. The TRSV genomic RNAs resemble eukaryotic mRNAs in that they contain poly-adenylate (poly-A) sequences at their 3' ends (Mayo *et al.*, 1979a). This indicates that they are capable of messenger activity (Both *et al.*, 1975a; Morrison *et al.*, 1974). As expected, TRSV RNA was efficiently translated *in-vitro* in the Marcu and Dudock system. However, translation of both RNA-S and RNA-L *in-vitro* failed to detect any coat protein in any of the products (Chapter VI), although approximately 85% and 100% respectively of these RNAs were translated. This observation further supports the conclusion that the molecular weight of the coat protein of TRSV could not be 57,000 since such a large protein would require 40% of the coding capacity of RNA-S and should be detected in most of its translational products. Thus,

it is possible that the TRSV coat protein gene is located in the 15% of the RNA-S which is not translated *in-vitro*.

At present it is unclear whether the numerous products translated by TRSV RNA *in-vitro* are due to premature termination or to multiple initiation sites or both. However, it is likely that like TMV (Knowland *et al.*, 1975) and CpMV (Davies *et al.*, 1977), there is more than one initiation site on TRSV RNA; but it also seems likely that many of the products are formed from premature termination.

In translational studies of CMV, TMV and AMV RNAs where both animal cell-free system (*in-vitro*) and frog-oocytes (*in-vivo*) were used in addition to the *in-vitro* wheat germ cell-free system, it was found that all these systems synthesized similar products except that less products due to premature termination were usually obtained in the *in-vivo* system (Knowland *et al.*, 1975; Schwinghamer and Symons, 1977; Rutgers, 1977). Similarly, translation of TMV RNA in systems not derived from host plants (heterogenous systems) and in host protoplast (homogenous system) suggests that the translational products of the genomic RNA synthesized in the latter were similar to those obtained in the heterogenous systems (Paterson and Knight, 1975; Knowland *et al.*, 1975). Thus, *in-vitro* translational systems may be limited due to their variation in activity and products, lack of regulation and post-translational functions. However, the *in-vitro* products detected, including those of TRSV RNA, probably represent products of the genomic RNA synthesized *in-vivo*. Thus, it is likely that the TRSV coat protein is not being synthesized from the genomic RNAs *in-vivo*.

In-vitro translation of various viral RNAs suggests that there are at least three established mechanisms by which virus coat protein can be synthesized:

(i) Coat protein is synthesized by translation of a multi-cistronic genomic RNA containing the coat protein cistron* followed by cleavage

* To avoid confusion, a cistron is defined here as a part of the mRNA coding for a complete functional polypeptide chain.

of the resulting precursor polypeptide. This mechanism has been shown for CpMV (Pelham, 1979a).

(ii) The coat protein cistron is translated separately from other cistrons in a multicistronic genomic RNA. This mechanism was recently reported for CarMV (Salomon *et al.*, 1978) and is known in many phages e.g. R17 and MS-2. In this system the cistrons on the genomic RNA are separated by untranslatable intercistronic regions (Jeppesen *et al.*, 1970; Nichols, 1970).

(iii) In this system, the coat protein appears not to be translated from the genomic RNA which contains the coat protein cistron, but from a subgenomic mRNA transcribed from the genome and coding specifically for the coat protein. The inability of the genomic RNA to be translated into coat protein may be due to the failure of ribosomes to recognise the initiation site for the coat protein cistron e.g. due to the cistron being located in regions of high secondary structure (Knowland, *et al.*, 1975). This system has been observed in both viruses with a divided genome e.g. CMV, BMV, and AMV as well as virus with a single genomic RNA e.g. TMV (Knowland *et al.*, 1975).

There have been conflicting reports on whether TMV coat protein was synthesized from the genomic RNA (Tsugita *et al.*, 1962; Schwartz, 1967; Efron and Marcus, 1973; Roberts *et al.*, 1973; Knowland, 1974). These studies showed that only 80% of the viral RNA was translated *in-vitro* and *in-vivo*. It was at first thought that the coat protein was synthesized by post-translational cleavage of the largest translational product (Roberts *et al.*, 1974). However, it is now accepted that no coat protein is translated from the genomic RNA *in-vitro* or *in-vivo* and that the lack of coat protein synthesis *in-vitro* and in heterogenous systems is not due to lack of initiation factors but due to failure to transcribe the subgenomic coat protein mRNA (Knowland *et al.*, 1975). In CMV, BMV and AMV, the RNA-4 which codes for the coat proteins is not necessary for infection (except in AMV

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where the coat protein or its messenger is necessary for infectivity) (Peden and Symons, 1973; Schwinghamer and Symons, 1975; Shih and Kaesberg, 1973; Shih *et al.*, 1972; Van Vloten-Doting and Jaspars, 1977). In these viruses, the genomic RNAs containing the coat protein cistron (RNA-3) are required for infectivity but do not translate coat protein *in-vitro* or *in-vivo* (Van Vloten-Doting *et al.*, 1975, 1977; Mohier *et al.*, 1975; Shih and Kaesberg, 1973; Schwinghamer and Symons, 1977). The coat protein mRNAs are synthesized from the genomic RNA-3 and they do not multiply autonomously (Mohier *et al.*, 1974). Thus they are in essence subgenomic mRNAs similar to that of TMV except that they are encapsidated into particles. In fact there are some strains of TMV which encapsidate the subgenomic mRNA for coat protein in separate particles (Higgins *et al.*, 1976) indicating that TMV and these viruses are basically similar in their mechanism of coat protein synthesis. Recent studies indicate that TYMV also synthesizes its coat protein through a subgenomic mRNA (Klein *et al.*, 1976; Pleij *et al.*, 1976; Pleij *et al.*, 1977; Szybiak *et al.*, 1978; Mellema *et al.*, 1979).

It is likely that the post-translational cleavage mechanism for synthesis of viral coat proteins may be regulated by other regulatory mechanisms to enable the synthesis of large amounts of coat proteins required without producing a lot of wastage. In CpMV, it is suggested that the provision of internal initiation sites enables regulation of protein synthesis (Pelham, 1979a). On the other hand, the other two mechanisms enable regulation of translation of various proteins by controlling the translational activity of the individual mRNAs or cistrons as observed with BMV (Zagorski, 1978; Pyne and Hall, 1979). The involvement of subgenomic mRNA also has the advantage of allowing transcriptional control of gene expression as in eukaryotes.

In TRSV it is still unclear how the coat protein is synthesized. However, it is most probable that it does not involve the separate translation of individual cistrons on the genomic RNA as found in CarMV

(Salomon *et al.*, 1978). Studies with TBRV show that mature coat protein was not translated in the Marcu and Dudock cell-free system but was detected in infected tobacco protoplast (Fristch and Harrison, 1978). However, it is quite likely that such coat proteins may be derived from post-translational cleavage or translation of subgenomic mRNA. Fristch and Harrison (1978) suggested that the TBRV coat protein is synthesized by post-translational cleavage. However, it was not shown whether the largest products contained the coat protein amino acid sequence to substantiate this.

In TRSV, the absence of coat protein amino acid sequence in the apparently complete translational products of its genomic RNAs suggests that its synthesis by post-translational cleavage is unlikely. The following evidence and the discussion above support the suggestion that the coat protein may be translated from a subgenomic mRNA which is transcribed from the RNA-S as shown in Fig. 2. TRSV RNA-S, like TMV RNA, was not completely translated *in-vitro* and the coding capacity of the untranslated part of the RNA is sufficient for the coat protein. However, further investigations are necessary to substantiate this hypothesis. These should include (a) translation of total leaf RNA fractions extracted from tissues (Knowland *et al.*, 1975) at different times after inoculation with TRSV or (b) isolation and translation of polysomal RNA (Jackson and Larkins, 1976; Bol *et al.*, 1976) associated with TRSV infection. A comparison of the products obtained with products synthesized *in-vivo* using a protoplast system would also yield information on how the genes on the viral RNAs are expressed *in-vivo*.

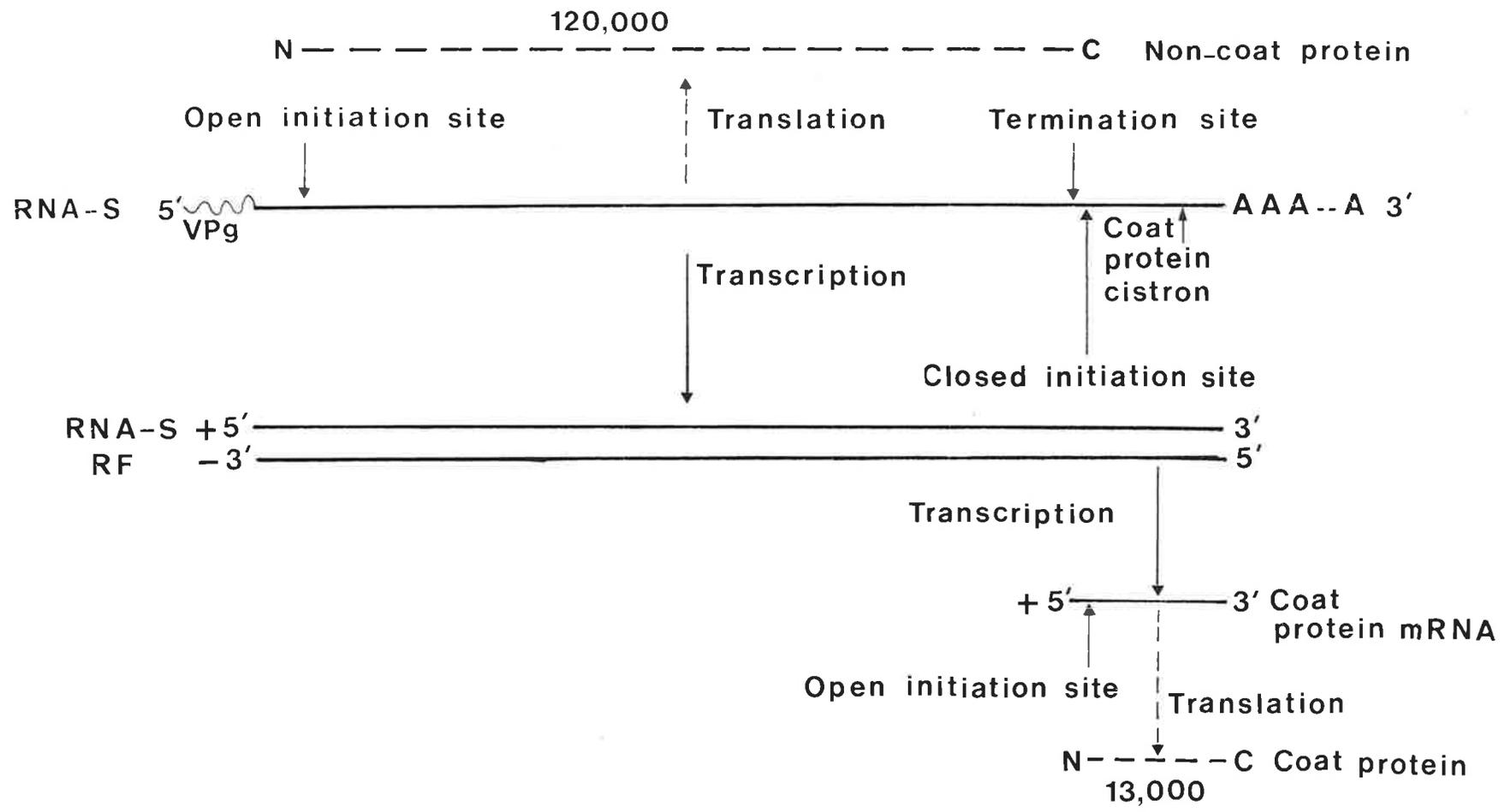
G. Function of the VPg in TRSV

VPgs are protein components recently discovered on certain viral RNAs and their functions in virus synthesis are still not fully understood. The VPg in TRSV is found to be not required for translation of the genomic RNA although the cap structure that it appears to replace,

Figure 2. A model for the translation of TRSV RNA-S and coat protein

The model suggests that the TRSV coat protein cistron on the RNA-S is not translated. During infection, a subgenomic mRNA coding for the coat protein is transcribed from the replicative form (RF).

Mature coat protein molecules are then translated from the new coat protein mRNA. The rest of RNA-S is translated into (various) non-coat polypeptides, the largest of which has a molecular weight of about 120,000.



may be involved in regulation of translation of the mRNAs (Both *et al.*, 1975a, 1975b; Muthukrishnan *et al.*, 1975; Roman *et al.*, 1976; Hickey *et al.*, 1976). It was also shown that the VPg of CpMV is not only unnecessary for translation *in-vitro* but in its absence, a higher translation activity could be obtained (Stanley *et al.*, 1978). Similarly, poliovirus mRNA is devoid of the VPg (Nomoto *et al.*, 1977b; Petterson *et al.*, 1977). Thus it appears that the VPgs in all these viruses may be involved in synthesis of viral RNAs and not synthesis of viral proteins.

It has been suggested that VPgs, like the 3'-CpCpA-OH nucleotide sequence (Lesiewicz and Dudock, 1978), are structures associated with RNAs which replicate without DNA as an intermediate (Daubert and Bruening, 1979); e.g. in poliovirus it has been suggested to function as primer for RNA synthesis, being attached and removed from the RNA very readily *in-vivo* (Ambros and Baltimore, 1978). However, only VPgs of the Nepoviruses, TRSV and TBRV and the Calicivirus, VEV, are required for infectivity (Harrison and Barker, 1978; Burroughs and Brown, 1978) while those in CpMV and Picornaviruses are not (Stanley *et al.*, 1978; Lee *et al.*, 1977; Hruby and Roberts, 1978). A major difference between Picornavirus and Calicivirus involves the translation of viral protein. Since the VPg is virus coded (Golini *et al.*, 1978; Daubert and Bruening, 1979), this may be a clue as to why in some viruses their VPgs are required for infectivity and in others not. In the Picornaviruses and CpMV the viral proteins appear to be synthesized by post-translational cleavage of the precursor polypeptide (Black and Brown, 1975/76; Sritkin and Agol, 1978; Pelham, 1978, 1979a). In VEV and possibly in TRSV, virus specific proteins are translated from subgenomic mRNAs as well as the genomic RNAs (Black *et al.*, 1978; Black and Brown, 1978; Ehresmann and Schaffer, 1977). Thus it is possible that the VPgs in VEV and TRSV are required for transcription of the subgenomic mRNAs,

and thus are required for infection. On the other hand, since lack of VPg did not affect translation, all the proteins required for virus synthesis would still be synthesized in the absence of the VPgs in the Picornaviruses and CpMV, thus the VPgs are not necessary for infection.

The data on the size of the VPg detected in TRSV is controversial and more work is required to clarify this aspect.

APPENDIXPAPERS PUBLISHED

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