Characterization of a defective form of tomato spotted wilt virus

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CHARACTERIZATION OF A DEFECTIVE FORM OF TOMATO SPOTTED WILT VIRUS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
hoogleraar in de veeteeltwetenschap,
in het openbaar te verdedigen
op vrijdag 5 maart 1982
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

ISN- 15744 06-03

Aan mijn ouders

STELLINGEN

1 Het tomatebronsvlekkenvirus (TSWV) heeft een positief-strengig RNA als genoom.
Dit proefschrift.

2 De argumenten die Sakimura aanvoert om aan te tonen dat het tomatebronsvlekkenvirus zich niet vermenigvuldigt in thripsen die als vector van het virus optreden, zijn niet steekhoudend.
Sakimura, S. (1962). In: Biological transmission of disease agents. Ed. K. Maramarosch, Academic Press, New York, pp. 33-40.
Sakimura, S. (1963). Phytopathology 53, 412-415.

3 De mogelijkheid dat de voor cap-analogen ongevoelige initiatieplaats voor de eiwitsynthese op het 42S RNA van Semliki Forest virus door een specifieke RNase-activiteit ontstaan is, wordt door Van Steeg et al. te lichtvaardig van de hand gewezen.
Van Steeg, H., Pranger, M.H., Van der Zeijst, B.A.M., Benne, R. & Voorma, H. (1979). FEBSLetters 108, 292-298.

4 De overeenstemming in grootte tussen het chloroplast-DNA van de algen *Acetabularia cliftonii* en *A. mediterranea* en het genoom-DNA van *Escherichia coli* is pas dan een argument voor de endosymbionttheorie, als gebleken is dat dit chloroplast-DNA prokaryotisch van karakter is en veel meer genen bevat dan het 10 tot 20 maal kleinere chloroplast-DNA van alle andere tot nu toe onderzochte algen.
Padmanabhan, U. & Green, B.R. (1978). Biochim. et Biophys. Acta 521, 67-73.

5 De conclusie dat tomato bushy stunt virus een replicatie-strategie heeft die afwijkt van die van andere virussen uit de Tombusvirusgroep, is onvoldoende gefundeerd.
Henriques, M.-I.C. & Morris, T.J. (1979). Virology 99, 66-74.

6 De conclusie van Zawirska dat *Thrips tabaci* type 'tabaci' wél en *T. tabaci* type 'communis' niet als vector van het tomatebronsvlekkenvirus optreedt is uitsluitend gebaseerd op epidemiologische gegevens en heeft slechts dan geldigheid als de resultaten van overdrachtsexperimenten ermee in overeenstemming zijn.

Zawirska, I. (1976). Arch. Phytopathol. u. Pflanzenschutz 12, 411-422.

7 De biologische betekenis van de remming van de ethyleenproduktie door licht in tarweplanten die een tekort aan water hebben kan hierin liggen dat de planten alleen die bladeren laten vallen die ook 's nachts verwelkt blijven.

Wright, S.T.C. (1981). Planta 153, 172-180.

8 Relevant populatie-genetisch onderzoek is alleen mogelijk als de oecologie van de te onderzoeken soort(en) voldoende bekend is.

9 Het beleid van de natuurbeschermingsorganisaties, zoals dat tot uiting komt in het aankopen van landschappelijk waardevolle gebieden die daarna tot reservaat worden verklaard, gaat uit van een tegenstelling tussen natuur en landbouw en legitimeert zo de voortschrijdende schaalvergroting en intensivering in de landbouw die het milieu ernstig aantasten.

10 De Rijks Psychologische Dienst is een ondoorzichtige bedreiging voor de sollicitant.

11 De verschillende verklaringen voor de slechte economische toestand van ons land wekken de indruk dat economie een goochelen met getallen is, waarbij de politieke opvattingen van de goochelaar bepalend zijn voor de uitkomst.

F.N. Verkleij
Characterization of a defective form of tomato spotted wilt virus
Wageningen, 5 maart 1982.

VOORWOORD

Nu dit proefschrift voltooid is, wil ik iedereen bedanken die bij het tot stand komen betrokken is geweest.

Professor Van der Want voor het vertrouwen dat hij in mij gesteld heeft, zijn voortdurende belangstelling en de zorgvuldigheid waarmee hij het manuscript gelezen en gecorrigeerd heeft.

Dick Peters was de begeleider van het onderzoek. Dick, ik heb het erg gewaardeerd dat ik altijd bij je terecht kon, hoe vaak de telefoon ook ging, en je hulp bij het schrijven van het proefschrift heb ik bijzonder op prijs gesteld.

Magda Usmany heeft een belangrijk aandeel gehad in de praktische werkzaamheden. Beste Magda, het is niet toevallig dat de periode waarin de meeste resultaten van het proefschrift vergaard zijn, namelijk de laatste driekwart jaar, samenviel met de periode waarin jij bij mij kwam werken. Ik heb het erg leuk gevonden om met je te werken en ik ben je erkentelijk voor de voorbeeldige nauwgezetheid waarmee jij de proeven uitvoerde.

Het aardigste van mijn verblijf op virologie vond ik de begeleiding van studenten bij hun doctoraalonderzoek of onderzoek als onderdeel van de analistenopleiding (STOVA). Met plezier denk ik terug aan de periode van samenwerking met jullie: Kolja van Beek, Marijke Bossink, Francine Govers, Marcel Hakkaart, Jos van der Lubbe, Petra de Vries en Titus de Vries. Dr. Bob Coutts die te gast was op virologie, bedank ik voor zijn aandeel in de transcriptase-experimenten en vele discussies. Met Harry Flore deelde ik behalve de kamer ook het bestaan van promotie-assistent. Ik heb het altijd erg gezellig gevonden en wil je in ieder geval bedanken voor het uitvoeren van een aantal transcriptase-experimenten.

Ir. T.S. Ie heeft voor het onderzoek een aantal isolaten van TSWV aan mij beschikbaar gesteld en mij verder een paar elektronen-microscopische foto's gegeven die in Hoofdstuk 1 afgebeeld zijn. Joop Groenewegen en Dick Lohuis hebben mij geholpen met de elektronen-

microscoop, Wout Rozeboom was altijd bereid om allerlei technische problemen op te lossen, Karel Boekhorst heeft de figuren getekend en Dick Lohuis en de heren Brangert, Eimers en Bakker hebben de fotografie verzorgd. De talloze tabaksplanten die voor het onderzoek zijn gebruikt, zijn gekweekt en verzorgd door Gijs Looijen, Han Spekman en Henk Huberts. Veel waardering heb ik voor de bereidwillige medewerking die ik van allen ondervonden heb.

Dr. A.E. Steele bedank ik voor het corrigeren van de tekst op het Engels en mevrouw Korte van de afdeling Tekstverwerking voor het typen van het proefschrift.

Verder wil ik alle overige medewerkers van de vakgroep Virologie bedanken voor de prettige tijd.

De laatste woorden zijn voor Ineke, die het allemaal van zo nabij heeft meegemaakt.

CONTENTS

ABBREVIATIONS

A 260	absorbance at 260 nm
A 405	absorbance at 405 nm
AlMV	alfalfa mosaic virus
ATP	adenosine-5'-triphosphate
Ci	Curie
CCMV	cowpea chlorotic mottle virus
cpm	counts per minute
CPMV	cowpea mosaic virus
DI	defective interfering
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double stranded ribonucleic acid
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis (2-aminoethylether) tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fig.	Figure
G	glycosylated membrane proteins of TSWV
g	force of gravity
GTP	guanosine-5'-triphosphate
h	hour(s)
HEPES	2-(4-(2-hydroxyethyl)-1-piperazynyl) ethane sulphonic acid
M	molar
min	minute(s)
mol. wt.	molecular weight
mRNA	messenger ribonucleic acid
N	nucleocapsid protein of TSWV
NP-40	Nonidet P 40
oligo-(dT)	oligodeoxythymidylic acid
PBS	phosphate buffered saline (140 mM-NaCl, 2.6 mM-KCl, 1.4 mM-KH_2PO_4, 8 mM-Na_2HPO_4).
PBS-TDS	10 mM-sodium phosphate buffer (pH 7.2), 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate
PEMV	pea enation mosaic virus
poly-(A)	polyriboadenylic acid
rev/min	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
S	sedimentation coefficient in Svedberg units
SDS	sodium dodecyl sulphate
SSC	standard saline citrate (0.15 M-NaCl, 0.015 M-Na-citrate pH 7.2)
SSPE	subacute sclerosing panencephalitis
ssRNA	single stranded ribonucleic acid
SYNV	Sonchus yellow net virus
TCA	trichloro-acetic acid
TMV	tobacco mosaic virus
Tris	tris(hydroxymethyl)-aminomethane
tRNA	transfer ribonucleic acid
TSWV	tomato spotted wilt virus
UMP	uridine-5'-monophosphate
VSV	vesicular stomatitis virus
WTV	wound tumor virus

1 INTRODUCTION

1.1 TOMATO SPOTTED WILT VIRUS (TSWV)

Host range and distribution

TSWV is distributed world-wide and may cause serious diseases in certain important crops (Best, 1968; Francki & Hatta, 1981). The virus has a very wide host range. A list compiled by Best (1968) includes 157 dicotyledon and 6 monocotyledon species in 29 and 5 families, respectively. The most important agricultural plants affected by TSWV are tomato, tobacco, pepper, potato and groundnut (Francki & Hatta, 1981).

Transmission

TSWV is the only virus known to be transmitted by thrips (Francki & Hatta, 1981). Sakimura (1962) has reviewed transmission of this virus. The thrips species known to be vectors of TSWV are *Thrips tabaci* Lind., *Frankliniella schultzei* (Trybom), *F. occidentalis* (Pergande) and *F. fusca* (Hinds). According to Sakimura (1962, 1963) only thrips larvae can acquire virus. Although a protective mechanism against destruction of the virus may exist exclusively in the larval midgut, experiments to demonstrate differences between the midgut of larvae and adults of *T. tabaci* were unsuccessful (Day & Irzykiewicz, 1954, cited by Sakimura, 1962).

Data on the transmission of TSWV are limited. The minimum feeding time of *T. tabaci* in Russia to acquire virus was 30 min, and the inoculation threshold was 5 min (Razvyazkina, 1953, cited by Sakimura, 1962). Sakimura (1963) observed in Hawai that *T. tabaci* transmitted virus after an inoculation feeding period of 15 min, but the inoculation threshold was not determined in these experiments. An increase in transmission occurred when feeding times were

prolonged. After four days of feeding the frequency of infection was 77%.

The latent periods in *T. tabaci* and *F. fusca* range from 4 to 18 days and extend through the prepupal and pupal periods. The first transmission occurs 0 to 6 days after emergence (Sakimura, 1963). The virus is retained throughout its life, while the transmission by the different insects shows an erratic pattern. The existence of a long latent period and the retention of the infectivity over the whole life-span of the adult indicates that TSWV is persistent in its vector. Paliwal (1976) could serologically demonstrate the presence of TSWV in homogenates of thrips *(F. fusca)* transmitting the virus. However, an electron microscopic study on tissues of similar insects failed to detect TSWV (Paliwal, 1979).

No sufficiently reliable data are available to decide whether TSWV multiplies in its vector. The great variability in retention and latent period suggested to Sakimura (1962, 1963), that the original amount of virus acquired by the insects determines the amount of virus being transmitted. Further, transovarial transmission could not be demonstrated in *F. schultzei* (Samuel et al., 1930). These observations prompted Sakimura (1962, 1963) to conclude that TSWV does not multiply in its vector.

Properties of virus particles

Information on the structure of TSWV particles has recently been compiled by Francki & Hatta (1981). Electron microscopic pictures of purified TSWV particles and of ultrathin sections of infected leaves show spherical particles about 85 nm in diameter with an envelope the surface of which is covered with a layer of projections (Ie, 1964, 1971; Van Kammen et al., 1966; Mohamed et al., 1973).

Four major and three minor proteins were detected by electrophoresis of purified TSWV particles on SDS-polyacrylamide gels. (Tas et al., 1977a). The molecular weights of the major proteins, referred to as proteins 1 to 4, were estimated to be 27, 52, 58 and 78 x 10^3. The protein profile obtained after gel electrophoresis corresponded very well with that obtained by Mohamed et al. (1973); instead of proteins 2 and 3, however, they found only one protein.

Iodination experiments demonstrated that protein 1 is located inside the viral envelope. Protein 4 is the most exposed protein and is probably located on the envelope. The locations of proteins 2 and 3 in the virus are less clear (Tas et al., 1977a). Treatment of virus particles with the non-ionic detergent Non-idet P40 (NP-40) and subsequent centrifugation on sucrose gradients released an infectious fraction which contained predominantly protein 1, indicating that this protein is the nucleocapsid protein (Tas et al., 1977a; Van den Hurk et al., 1977). Recently, Mohamed (1981) identified three different sized nucleocapsids in TSWV preparations, by centrifugation of dissociated virus particles on sucrose gradients.

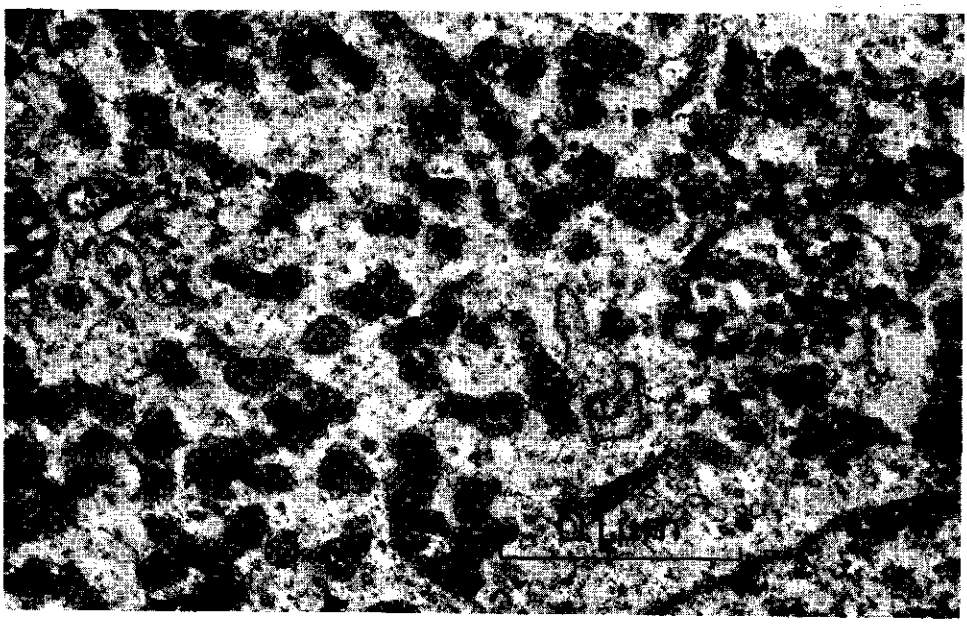
Tas et al. (1977a) demonstrated that proteins 2, 3 and 4 are glycosylated, whereas Mohamed et al. (1973) detected carbohydrate in all the major proteins.

Van den Hurk et al. (1977) showed that the genome of TSWV consists of single stranded RNA which is segmented; by electrophoresis on polyacrylamide gels three major segments of molecular weight of 2.1, 1.7 and 1.3 x 10^6 and one minor segment of mol. wt. 1.9 x 10^6 were found. The RNA was not infectious, suggesting that the RNA of TSWV is of a negative strand.

A defective form of TSWV

The existence of a defective form of TSWV has recently been described by Ie (1982). TSWV particles are present in the cytoplasm of plant cells. Electron microscopic studies showed that they occur in clusters within dilated cisternae of the rough endoplasmic reticulum (Fig. 1A,B). In addition to the virus particles, dark, amorphous, diffuse masses were observed in the cytoplasm (Ie, 1971; Kitajima, 1965; Milne, 1970). These masses, containing locally electron dense structures with a lattice periodicity of 5 nm, are not bounded by a membrane but are scattered throughout the cytoplasm amidst ribosomes (Fig. 1A,C). Since these structures were Pronase sensitive, they must contain protein (Ie, 1971).

Initially it was assumed that these amorphous masses were an early stage in the assembly process of the virus particles. The more dense spots in these masses were thought to represent the naked and somewhat coiled nucleoprotein capsid of the virus; in a further stage of development the nucleocapsids would be surrounded



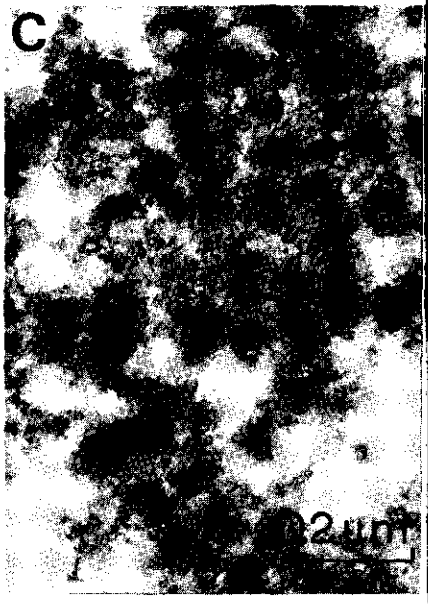


Fig. 1.A. Mesophyl cells of Nicotiana rustica infected with an isolate of TSWV from dahlia. The cytoplasm contains clusters of enveloped particles within the dilated cisternae of the endoplasmic reticulum and a complex of amorphous masses.
B. Detail of enveloped TSWV particles.
C. Detail of a complex of amorphous masses, showing a lattice periodicity of 5 nm.

by a membrane to yield typical TSWV particles (Ie, 1971). This assumption was the more attractive, as in cells infected with enveloped animal viruses, such as myxo- and paramyxoviruses, comparable structures were observed and identified as nucleocapsids (Choppin & Compans, 1975; Compans & Dimmock, 1969; Oyanagi et al., 1971; Robbins et al., 1980).

However, more recent observations indicated that this assumption was based upon incomplete information. In the course of many mechanical transmissions, Ie (1982) observed that in the cytoplasm of cells from plants infected with two isolates of TSWV, the characteristic TSWV particles had completely disappeared, whereas the amorphous masses occurred in abundance. It appeared, therefore, that these isolates, originally of the normal particle type of TSWV, had been converted to another type, which produced only dense amorphous masses. The isolates of the amorphous type were as infectious as the isolates of the normal particle type and had the same thermal inactivation point and host range.

Ie (1982) has advanced the hypothesis that the amorphous type is a defective form of TSWV, and may occur with most isolates of the normal virus. On repeated transfer by sap inoculation the defective form tends to build up at the expense of normal virus particles, until finally the latter completely disappears. Since the defective form is found after mechanical transmission of the virus, it may be assumed that the virus requires a membrane to be transmitted by thrips. In this way, thrips will act as a sieve that prevents the defective form from occurring in the field.

1.2 DEFECTIVE VIRUSES

As pointed out in the former section, the isolates of TSWV producing only amorphous masses may be regarded as defective forms. Many defective viruses are described in the literature. In this section I will present information on three different types of defective viruses. These viruses are defective because they have lost a characteristic property of the wild type virus. In this survey the biochemical features of the defective viruses will be discussed to clarify the molecular basis of the particular defect.

Defective interfering particles

The first type to be considered are the defective interfering (DI) particles of animal viruses. Huang & Baltimore (1970) listed the following properties of these particles: 1. they contain normal structural proteins; 2. they contain only a part of the viral genome; 3. they are not infectious by themselves, but can only multiply in the presence of homologous normal virus (standard virus); 4. and finally, they interfere with the multiplication of the standard virus. For almost every group of animal viruses DI particles have been found (Huang & Baltimore, 1977). Although a similar phenomenon among plant viruses has never clearly been observed, there are no arguments against the existence of DI particles among plant viruses.

DI particles are generated in most viral systems when cultures are serially infected with undiluted inocula. An important consequence of interference by a DI particle is that the number of DI particles increases at the expense of the standard virus. After multiple passages of an inoculum of standard virus and DI particles, the latter predominate in the progeny.

The nucleic acid of DI particles of RNA viruses must fulfill at least two demands. It must be replicated by a viral replicase and encapsidated by viral structural proteins. Analysis of the RNA of DI particles might reveal how these demands affect the size and composition of the RNA.

The RNA strands of many DI particles appear to be internal deletions of the RNA of the standard virus, whereby the 5' and 3' termini have been conserved. This situation has been found for the RNA of DI particles of poliovirus, which belongs to the picornaviruses (Lundquist et al., 1979; Nomoto et al., 1979; Villa-Komaroff et al., 1975), of Semliki Forest virus (Kennedy, 1976; Pettersson, 1981; Stark & Kennedy, 1978) and of Sindbis virus (Dohner et al., 1979; Guild & Stollar, 1977), which viruses belong to the togaviruses. The RNA of DI particles of influenza virus (myxoviruses) is composed of sequences from one of the three polymerase genes and has conserved the 5' and 3' termini (Davis & Nayak, 1979; Davis et al., 1980; Nakajima et al. 1979; Nayak, 1980).

The generation of these DI particle RNAs, which have deleted internal portions of the standard viral RNA and have conserved the 5'

nd 3' termini, can be explained by assuming that during the replication the viral RNA replicase leaves the template RNA at one point nd attaches again to it at a more distal point. This jump may be facilitated when these two points are close to each other by looping out of the intervening sequences (Bruton et al., 1976; Guild & Stollar, 1977; Kennedy, 1976).

The more complicated structure of the RNA of DI particles of esicular stomatitis virus (VSV; rhabdoviruses) has been studied intensively. The first class of DI particles of VSV contains an RNA hat is homologous to the 5' portion of the standard viral genome; he size of the molecules ranges from 10 to 50% of the VSV RNA (Schnitzlein & Reichmann, 1976; Stamminger & Lazzarini, 1974). The NA molecules of this class contain at the 3' terminus a 45 to 48 ucleotide sequence which is exactly complementary to the 5' terinal sequence (Keene et al., 1979; Perrault & Leavitt, 1977; Schubert et al., 1979). Only the 45 to 48 nucleotide long 3' terminal portion of the genome of these DI particles can be transcribed. he nucleotide sequence of this transcription product is the same s the 45 to 48 nucleotide sequence at the 5' terminus of the RNA f the DI particles (Semler et al., 1978; Schubert et al., 1978). he existence of exact complementary terminal sequences in these DI article RNAs suggests that they are generated by a copy-back mechanism wherein the replicase detaches from the template and resumes synthesis by copying the 5' terminal sequence of the nascent strand. This mechanism was originally proposed by Leppert et al. (1977) to explain the origin of the complementary terminal sequences in the RNA of DI particles of Sendai virus, another negative stranded RNA virus (paramyxoviruses).

A second class of DI particles of VSV contains an RNA that is omologous to the 5' portion of the VSV genome and is covalently inked to the complement of this RNA. Under non-denaturing conditions this DI particle RNA exists as a hairpin structure in which virtually all nucleotides are paired (Lazzarini et al., 1975; Perrault, 1976). A peculiarity of this DI particle is its ability to induce the synthesis of interferon (Marcus & Sekellick, 1977).

The third class of DI particles of VSV contains a genome representing the 3' portion of the VSV genome, coding for the N, NS, M and G proteins (Schnitzlein & Reichmann, 1976; Stamminger &

Lazzarini, 1974). The RNA of this DI particle has also retained a small part of the 5' portion of the standard viral genome and thus might be considered as a true internal deletion mutant (Epstein et al., 1980; Perrault & Semler, 1979). It is fully capable of *in vitro* and *in vivo* transcription of its genome (Colonno et al., 1977; Johnson & Lazzarini, 1977).

The emphasis on the 5' and 3' termini of the RNA of the DI particles is prompted by the idea that the terminal sequences of viral genomic RNAs are involved in the initiation of RNA replication. The RNA of all DI particles analysed thus far have conserved the 5' and 3' terminal nucleotide sequences of the standard viral RNA.

The RNA molecules of the first and second class of DI particles of VSV are exceptional, because they cannot be considered as internal deletions. Notwithstanding, Keene et al. (1977, 1978, 1979) demonstrated that the 5' and 3' termini of the VSV RNA are complementary for about 20 nucleotides. The authors suggest that these sequences, which are highly conserved in VSV, are involved in the initiation of the viral RNA replication of both the positive as well as the negative strand. One may argue that the enlarged complementarity in terminal nucleotide sequences in the DI particle RNAs gives a replicative advantage over the standard viral RNA, by an increased affinity for replicase molecules.

In addition to replication, another requirement of DI particle RNA is the minimal size needed for encapsidation. The minimal size required differs among the various viruses. The RNA of DI particles of Semliki Forest virus and Sindbis virus appears to be 18 to 20S, which is 15% of the standard viral RNA (Guild & Stollar, 1977; Stark & Kennedy, 1978). For the RNA of DI particles of poliovirus the minimal size is much larger: 85% of the standard viral RNA is conserved, only the sequences coding for the capsid proteins are deleted (Cole & Baltimore, 1973a; Nomoto et al., 1979). The RNA of DI particles of VSV have retained 10 to 50% of the 5' portion of the genome of the standard virus or 50% of the 3' portion (Schnitzlein & Reichmann, 1976; Stamminger & Lazzarini, 1974).

The most intriguing property of DI particles is their ability to increase in number at the expense of those of the standard virus.

ost likely, the mechanism of increase is based upon a preferential eplication of their RNA. A preferential translation of the DI particle RNA is not likely. Thus far, investigation have shown that nly the RNA of DI particles of poliovirus can act as mRNA (Villaomaroff et al., 1975). An advantage of DI particles in transcription can also be ruled out. All the DI particles of VSV the genomes f which have conserved the 5' portion of the VSV genome, have lost he ability to transcribe their RNA (Emerson et al., 1977; eichmann et al., 1974). Palma et al. (1974) and Cole & Baltimore (1973b, c) found positive evidence that the interference of DI particles occurs in the replication of the RNA of VSV and poliovirus sing mutants of VSV which are temperature sensitive in transcription or replicaton and using cycloheximide which selectively inhibits the replication of poliovirus.

The exact nature of the preferential replication is still a matter of speculation. Huang & Baltimore (1977) and Perrault et al. (1978) suggest that the replicative advantage of the DI particle NA is due to an increase in the affinity of the initiation sites or replicase molecules. When the amount of replicase molecules is imited, more DI particle RNA molecules than standard viral RNA olecules may be synthesized in a given period of time, since the ormer are smaller than the latter. Eventually, this may lead to reponderance of DI particle RNA over standard viral RNA.

The biological significance of the interference by DI particles ight be that it serves as a protective mechanism of the host, as riginally proposed by Huang & Baltimore (1970).

There is some evidence that DI particles are involved in the establishment of persistent infections (Huang & Baltimore, 1970, 1977). Such an involvement has been demonstrated for DI particles of VSV (Friedman & Ramseur, 1979; Holland et al., 1979), of Semliki Forest virus (Meinkoth & Kennedy, 1980) and of measles virus, a member of the paramyxoviruses (Rima et al., 1977), to mention a few examples.

Non-vector transmissible plant viruses

Isolates of wound tumor virus (WTV) and pea enation mosaic virus (PEMV) are examples of plant viruses which have lost their trans-

missibility by their vectors, due to a loss of their genetic information.

WTV belongs to the reoviruses. These viruses are spherical particles 75 to 85 nm in diameter, composed of two concentric protein shells. The genome consists of ten to twelve unique length double stranded (ds) RNA segments (Joklik, 1974). Reddy & Black (1973) identified twelve dsRNA segments in WTV particles. Electrophoresis on SDS-polyacrylamide gels of WTV particles revealed seven polypeptides (Reddy & MacLeod, 1976). These seven polypeptides were also synthesized in cultured leafhopper cells infected with WTV and in cell-free extracts directed by *in vitro* synthesized mRNA. Moreover, five other, presumably non-structural proteins were synthesized in both systems. It is probably that each of the twelve polypeptides is a primary gene product of a dsRNA segment (Nuss & Peterson, 1980).

WTV is transmitted by leafhoppers. The virus multiplies in its vector (Black & Brakke, 1952; Reddy & Black, 1972) and in cultured insect vector cells (Black, 1969; Reddy & Black, 1972). WTV, which multiplies also in plant hosts, produces tumours on the roots and stems of clover (*Medicago* spp.). Since the virus cannot be transmitted through inoculation of sap of infected plants, infection must occur by means of viruliferous leafhoppers. The virus can also be maintained and propagated in cuttings of clover plants (Black, 1970).

Black and his coworkers observed that several WTV isolates, which were maintained by successive cutting of infected sweet clover plants, either completely or partially lost their ability to be transmitted by the vector (Black, 1969; Liu et al., 1973). This loss of transmissibility was accompanied by a corresponding loss in relative specific infectivity in cultured leafhopper cells (Liu et al., 1973; Reddy & Black, 1969, 1974).

This indicates that two types of WTV isolates exist. One type, isolated in the field, is able to multiply in its vector as well as in its hosts plants. The other type, generated in the laboratory, is only able to multiply in hosts plants.

The genomes of those isolates which had lost the ability to multiply in leafhoppers or cultured leafhopper cells could be discerned from the wild-type genome. Reddy & Black (1974) found evi-

dence that the genomes of these isolates were partly deficient in one or more segments. The deficiencies were found in the segments 1, 2, 5 and 7. Further selection of these isolates resulted finally in genomes which had completely lost segment 2 or 5 (Reddy & Black, 1977). Analysis of the polypeptides of purified particles of the isolates which had lost segment 2 showed that a 131 000 mol. wt. polypeptide was lacking. This result indicates that segment 2 codes for the 131 000 mol. wt. polypeptide. The same conclusion was reached from results of *in vitro* translation studies (Nuss & Peterson, 1980).

The fusion of virus particles with lysosomes is an essential part of the infection cycle of reoviruses. The outer protein shell is removed in these organelles. This uncoating process can also be achieved *in vitro* by chymotryptic proteolysis, releasing subviral particles which are infectious as complete particles (Silverstein et al., 1976). A similar treatment of WTV particles removed the previously mentioned 131 000 mol. wt. polypeptide and a 96 000 mol. wt. polypeptide. The subviral particles generated were infectious in cultured leafhopper cells (Reddy & Macleod, 1976). It would be of considerable interest to know whether or not the subviral particles generated by the enzymic removal of the 96 000 mol. wt. polypeptide from those WTV particles which have lost segment 2 and, consequently, the 131 000 mol. wt. polypeptide, are infectious.

Pea enation mosaic virus (PEMV) is a two-component virus. It is transmitted by aphids in the circulative manner (Hull, 1981). After repeated manual transmissions isolates can lose their ability to be transmitted by aphids. Some isolates lose this property after only three or four mechanical passages (Tsai & Bath, 1974). Electrophoresis on polyacrylamide gels of aphid and non-aphid transmissible isolates indicated that the latter isolates had lost a minor protein (Hull, 1976). Serological analysis supported this finding. Antisera prepared against a non-aphid transmissible isolate contained a single antibody population. Those against a aphid transmissible isolate contained two antibody populations, one of which cross-reacted with non-transmissible virus and one which was specific to the transmissible isolate (Clarke & Bath, 1976). Adam et al. (1979) suggested that a deletion in RNA 1, the largest of the two RNA segments in PEMV, is the cause of the loss of aphid transmissibility.

Subacute sclerosing panencephalitis (SSPE) virus, a defective measles virus

Subacute sclerosing panencephalitis (SSPE) is a rare and slowly progressive disease of the central nervous system which occurs primarily in children. Several findings indicate that measles virus is involved in the aetiology of this disease. Patients with SSPE have elevated titers of measles virus antibodies in their blood and cerebrospinal fluid and have measles antigens detectable by immunofluorescence in their brain nerve cells (Johnson et al., 1978; Ter Meulen & Hall, 1978).

Additional evidence for the involvement of measles virus emerged when infectious measles-like virus (referred to as SSPE virus) could be isolated from brain cells of SSPE patients which were co-cultivated or fused with continuous cell lines, as HeLa or Vero cells (Horta-Barbosa et al., 1969). Electron microscopic examination of the co-cultivated or fused cells revealed tubular structures resembling nucleocapsids of measles virus (Katz et al., 1969; Oyanagi et al., 1971) and which were similar to the structures found in brain cells from patients with SSPE (Tellez-Nagel & Harter, 1966).

During an investigation of the role of the virus in the disease the biological and biochemical properties of SSPE virus strains and measles virus strains were compared. No essential differences could be demonstrated between the proteins of SSPE virus strains and measles virus strains. The M (matrix) protein of some SSPE virus strains could be discerned from that of measles virus strains by means of gel electrophoresis and serology (Hall et al., 1978a, b; Ter Meulen et al., 1978; Schluederberg et al., 1974; Wechsler & Fields, 1978; Wechsler et al., 1978). However, fingerprints failed to show characteristic differences between M proteins of several measles and SSPE virus strains (Hall et al., 1979).

The differences between the genomic RNA of SSPE virus and measles virus are small as well. Hall & Ter Meulen (1976) found evidence in an hybridization study that the genomic RNA of three SSPE virus strains contained 10% unique sequences in addition to the complete nucleotide sequences of the genome of the two measles virus strains investigated. These authors suggested this finding

could account for the observed small differences in size between the putative mRNA for the M protein of the SSPE virus strains and measles virus strains (Hall et al., 1978a, b), as well as for the seemingly larger molecular weight of the M protein of the SSPE virus strains studied (Schluederberg et al., 1974; Wechsler & Fields, 1978).

Sera of patients with SSPE have a low antibody titer to M protein and high titers to all other proteins of measles virus, whereas sera from patients with atypical measles contain antibodies to all measles or SSPE viral proteins (Hall et al., 1979; Machamer et al., 1981; Wechsler et al., 1979). The findings indicated that a reduced synthesis of M protein occurs in patients with SSPE. This hypothesis was supported by the observation that only SSPE virus strains which produce infectious virus particles by budding through the plasma membrane synthesize M protein, whereas SSPE virus strains which remain solely cell-associated and do not show any budding activity do not synthesize any M protein (Eron et al., 1978; Hall & Choppin, 1979; Lin & Thormar, 1980; Machamer et al., 1981; Ramsey et al., 1978). The amount of the other viral proteins synthesized differed for the various cell-associated strains. The strain IP-3 produced mainly nucleocapsid protein and a small amount of a protein with a mobility slightly larger than the glycoprotein of measles virus (Eron et al., 1978; Ramsey et al., 1978).

The results with the cell-associated strains of SSPE virus became still more significant when it was found that only these strains were able to cause SSPE-like persistent infections in young ferrets (Thormar et al., 1978) and rhesus monkeys (Albrecht et al., 1977).

The M protein of paramyxoviruses, to which measles virus belongs, is necessary for the production of infectious virus particles in the budding process (Choppin & Compans, 1975). Evidently, the lack of M protein synthesis and the resulting loss of the ability to produce infectious virus particles by budding is crucial in the conversion of normal measles virus into cell-associated neurovirulent SSPE virus. Since virus with functional M protein can be rescued from brain cells by co-cultivation with permissive cells, the host cell must play a major role in this conversion.

As in cells infected with normal measles virus, a 50S genomic RNA could be identified in cells infected with the cell-associated SSPE virus strain IP-3 (Ramsey et al., 1978). Thus the proposed defect in the M protein synthesis may occur either at the level of the transcription of the mRNA for the M protein or at the level of the translation of this mRNA.

1.3 SCOPE OF THE PRESENT INVESTIGATION

Problems

All isolates of TSWV forming in plant cells solely amorphous masses can be considered as defective forms of this virus, because they do not produce normal enveloped particles. In order to elucidate the nature of the defect the protein and nucleic acid composition of the defective and normal form were studied. Since the structure of the infectious agent of the defective form is not known, it was necessary to study the structural viral proteins and viral RNA present in plants infected with either the normal or defective form.

The other problem concerns the nature of the electron dense amorphous masses by which the defective form of TSWV is defined by electron microscopy. In spite of the disappearance of normal TSWV particles in the course of many sap transmissions, inoculations are successful by an infectious agent of unknown structure. This structure has the same thermal inactivation point and host range as normal TSWV particles (Ie, 1982). Thus, the question to be solved is whether the amorphous masses represent or are composed of the infectious agent of the defective form.

Approaches

The first problem concerns the possible biochemical differences or similarities between the normal and defective forms of TSWV.

In view of a defect in the genome of TSWV, it seemed useful to know exactly the number of RNA segments in normal TSWV particles and the coding function of each individual RNA segment. Experiments designed to determine the number of RNA segments and their coding function are described in Chapter 2.

The occurrence of viral structural proteins in infected plants was studied by serological means. Antibodies were raised against the nucleocapsid protein and against a fraction containing the membrane proteins of TSWV. Sap from leaves infected with either the normal or defective forms were tested in an enzyme-linked immunosorbent assay (ELISA). The results of these experiments are described in Chapter 3.

The RNAs which were synthesized during infection with a normal or defective form of TSWV were analysed with respect to their translation capacity and hybridization with viral RNA.

Polyribosomes isolated from plants infected with either form were used as a source of mRNA; *in vitro* translation of the polyribosomes and analysis of the translation products would enable demonstration of possible differences between the viral mRNAs from plants infected with the normal or defective form (Chapter 4).

In Chapter 5 experiments are described in which the individual RNA segments of normal TSWV particles were hybridized with dsRNA isolated from plants infected with either a normal or defective form. In this way it would be possible to examine whether all nucleotide sequences of the normal form were also present in the defective form.

In Chapter 6 the nature of the electron dense amorphous masses is investigated. An infectious nucleoprotein rich fraction was isolated and examined by electron microscopy. In addition, the protein and RNA composition of this fraction were analysed by gel electrophoresis.

Chapter 2 of this thesis has been submitted for publication in the Journal of General Virology with Petra de Vries and Dick Peters as co-authors.

2 TSWV RNA IS A POSITIVE STRAND

2.1 INTRODUCTION

Tomato spotted wilt virus (TSWV) is an isometric membrane bounded virus which measures 70 to 80 nm in diameter. The virus consists of four major and three minor high molecular weight proteins. One of the major proteins is a glycoprotein with a mol. wt. of 78 000 and is located on the surface; another major protein with a mol. wt. of 27 000 is associated with the nucleocapsid (Tas et al., 1977a).

Van den Hurk et al. (1977) have demonstrated that the genome consists of single-stranded RNA which is segmented. Three major and one minor segment were found by electrophoresis on polyacrylamide gels.

RNA extracted from virus is non-infectious (Van den Hurk et al., 1977). The presence of several RNA segments and an envelope pointed to a resemblance with negative-stranded RNA viruses, such as the orthomyxoviruses (Compans and Choppin, 1975) and the bunyaviruses (Obijeski & Murphy, 1977). For these reasons Van den Hurk et al. (1977) proposed a negative polarity of TSWV RNA. In a review, Atabekov & Morozov (1979) came to the same conclusion.

In this study we present evidence that TSWV contains a positive stranded RNA in three segments.

2.2 MATERIALS AND METHODS

Purification of TSWV and extraction of TSWV RNA. The purification of TSWV from systemically infected *Nicotania rustica* leaves was as described by Tas et al. (1977b) with some modifications. The tobacco plants were inoculated with an isolate from Yugoslavia, kindly supplied by Dr. Bužančić in 1979, and maintained in tomato plants propagated by cuttings.

Leaves were ground in 0.1 M-Na-phosphate buffer, pH 7.0, 0.01 M-Na_2SO_3 for 5 to 10 sec in a Waring Blendor at low speed and the homogenate was centrifuged for 10 min at 10 000 g. The pellet was resuspended in resuspension buffer (0.01 M-Na-phosphate buffer, pH 7.0, 0.01 M-Na_2SO_3) and allowed to stand with gentle stirring for 1 h. The suspension was clarified by a centrifugation at 10 000 *g* and the supernatant was centrifuged for 20 min at 100 000 *g*. The virus pellet was resuspended in a small volume of resuspension buffer and incubated with antiserum against healthy leaf material for 1 hr at 4°C. After removal of the precipitate the suspension was subjected to a rate zonal centrifugation in a 3 to 30% linear sucrose gradient in resuspension buffer for 45 min at 23 000 rev/min in a Beckman SW 27 rotor. The virus in the opalescent band was concentrated by further centrifugation.

The extraction of RNA from purified virions was done exactly as described by Van den Hurk et al. (1977). A good virus preparation from 250 g leaves yielded approx. 50 µg TSWV RNA. Tobacco mosaic virus (TMV) RNA was extracted by the same procedure. RNA of cowpea mosaic virus (CPMV) was extracted according to the method of Reijnders et al. (1973).

[^{125}I]-labelling of TSWV RNA. TSWV RNA was labelled with ^{125}I as described by Milner & Jackson (1979). The specific activity was approx. 2.4 x 10^6 cpm/µg; 40 - 50% of the radioactivity was precipitable by trichloro-acetic acid and 90% of the trichloro-acetic acid precipitable material was RNase sensitive. The labelled RNA sedimentated in a sucrose gradient at 5S.

Methylmercury-agarose gel electrophoresis. TSWV RNA preparations were analysed on horizontal 1% agarose gels (20 x 20 cm) containing 5 mM-methylmercury in electrophoresis buffer according to the method of Bailey & Davidson (1976). Routinely, 2 to 4 µg TSWV RNA was dissolved in 25 µl electrophoresis buffer containing 10 mM-CH_3HgOH and 10% glycerol.

After electrophoresis for 4 h at 150 V, and room temperature, gels were stained with a solution of 5 µg/ml ethidium bromide in 0.5 M-NH_4-acetate and the bands were visualized under ultraviolet light.

Fractionation of TSWV RNA on sucrose gradients. Isokinetic sucrose gradients were prepared by mixing 12 ml of 33% (w/v) sucrose dissolved in 10 mM-NaCl, 10 mM-Tris-HCl pH 7.5, 0.1% SDS with 12.3 ml of 5% (w/v) sucrose solution in the same buffer, as described by Grivell et al. (1971). Immediately before application of the sample approx. 100 µg RNA was incubated for 1 min at 95°C and cooled quickly to 0°C. The gradients were centrifuged in a Beckman SW 41 rotor for 16 h at 28 000 rev/min at 5°C. Absorbance profiles at 254 nm were recorded by means of a Uvicord spectrophotometer (L.K.B. Sweden). The RNA in the fractions was precipitated with 2.5 volumes of absolute ethanol, 0.1 volume of 3 M-Na-acetate pH 5.5 and *E. coli* tRNA (Boehringer) as carrier in a final concentration of 25 µg/ml. After incubation overnight at -20°C, the RNA was collected by centrifugation, washed three times with 70% ethanol, dried and dissolved in 25 µl double distilled water.

Oligo-(dT)-cellulose chromatography. Twenty-five µg TSWV RNA and CPMV RNA were analysed for the presence of poly-adenylated sequences by chromatography over oligo-(dT)-cellulose according to the method of Aviv & Leder (1972). A batch of 0.125 g oligo-(dT)-cellulose (Type T-3; Collaborative Research Inc., Mass., USA) was washed three times with 0.1 N-NaOH and poured into a 2 ml syringe. The high salt buffer contained 0.5 M-NaCl, 10 mM-Tris-HCl, pH 7.4, 1 mM-Na_3EDTA and 0.5% SDS. The low salt buffer had the same composition, but NaCl was omitted. Fractions of 0.5 ml were collected at an elution rate of 1 ml per 3 min. The optical density at 260 nm was measured on a Zeiss spectrophotometer.

Preparation of the wheat germ cell-free extract and the mRNA-dependent rabbit reticulocyte lysate. Wheat germ extracts were prepared exactly as described by Marcu & Dudock (1974). Before the extraction, the wheat germ (General Mills, Vallejo, Cal.) were floated in a mixture of cyclohexane and carbontetrachloride (75 ml : 250 ml) for 1 min. The rabbit reticulocyte lysate was prepared according to the method of Hunt & Jackson (1974). The rabbit was made anaemic by daily injection of 0.8 ml per kg weight of 1.25% (w/v) acetylphenylhydrazine over a period of four successive days. Eight days after the first injection the rabbit was bled, 5000 units heparin

in 1 ml double distilled water were added to prevent coagulation. The reticulocyte lysate was made mRNA dependent by the method of Pelham & Jackson (1976). To supplement this lysate with eukaryotic tRNAs, tRNAs from wheat germ, prepared as described by Van Tol & Van Vloten-Doting (1979), were added in a concentration of 50 µg/ml.

In vitro *translation of TSWV RNA.* The reaction mixture (25 µl) to translate TSWV and TMV RNA in the wheat germ cell-free extract contained 10 µl of extract, 2.5 mM-ATP (Na-salt), 225 µM-GTP (Na-salt), 7 mM-creatine phosphate, 10 µg/ml creatine phosphate kinase, 35 µM-amino acids excepting methionine, 20 mM-Hepes-KOH, pH 7.6, 0.2 mM-spermidine, 110 mM-K-acetate, 3.25 mM-Mg-acetate and 75 µg/ml TSWV RNA or 60 µg/ml TMV RNA, 1 µl [^{35}S]-methionine (about 7 mCi/ml, 1000 Ci/mmol, Amersham) was added. After incubation at 30°C for 1 h, samples of 2.5 µl of the reaction mixture were assayed for radioactivity by precipitation with hot trichloro-acetic acid (Moorman et al., 1976).

The reaction mixture (50 µl) to translate TSWV RNA and TMV RNA in the mRNA-dependent rabbit reticulocyte lysate contained 40 µl messenger-dependent lysate, 2.5 µg TSWV RNA or 1.5 µg TMV RNA and 1 µl [^{35}S]-methionine. The concentrations of $MgCl_2$ and KCl were 2.4 mM and 84 mM respectively. The reaction mixture was incubated for 1 h at 30°C and the incorporation of radioactivity into acid precipitable material was assayed as described by Stuik (1979).

Immunoprecipitation of translation products in the wheat germ cell-free system. To the rest of a wheat germ reaction mixture (22.5 µl) 10 µl 5x PBS-TDS (1x PBS-TDS is 10 mM-Na-phosphate buffer pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% Na-deoxycholate and 0.1% SDS), 5 µl 100 mM-Na_3EDTA pH 7.0 and 12.5 µl distilled water were added. The dissociated ribosomes were removed by a centrifugation at 100 000 *g* for 20 min in a Beckman air-fuge. The supernatant was divided into two samples of equal size. To one sample 5 µl of an immunoglobulin solution from anti-TSWV serum was added, to the other sample 5 µl of an immunoglobulin solution from pre-immune serum. The immunoglobin solutions were prepared from these sera by ammonium sulphate precipitation. To both samples 10 µl 5x PBS-TDS and distilled water were added to give a final reaction volume of 50 µl.

The mixtures were incubated overnight at 4°C. In order to precipitate the reaction products, 10 mg protein A-sepharose (Pharmacia), preswollen in a solution of 10 mM-Na-phosphate buffer pH 7.2 and 0.9% NaCl and washed with PBS-TDS, was added and incubated for 45 min at room temperature. After pelleting the sepharose spheres at 8000 *g* in an Eppendorf centrifuge, the pellets were washed three times with PBS-TDS and finally resuspended in Laemmli sample buffer for electrophoresis on polyacrylamide gels (Laemmli, 1970).

Gel electrophoresis and fluorography. The *in vitro* translation products or immunoprecipitates of the translation products were analysed on 10% polyacrylamide gels (14 cm long and 1.5 mm thick), according to the method of Laemmli (1970). Before drying on Whatman 3 MM paper the gels were routinely processed for fluorography (Bonner & Laskey, 1974). Bands were visualized by exposure of the dried gel to X-ray film (Kodak, RP Royal-X-Omat) at -80°C.

As molecular weight markers β-galactosidase (116 000 mol. wt.), phosphorylase A (94 000 mol. wt.), bovine serum albumine (68 000 mol. wt.), ovalbumin (43 000 mol. wt.), DNase (31 000 mol. wt.), trypsin (23 000 mol. wt.) and RNase (13 000 mol. wt.) were used. The proteins of purified TSWV particles were labelled with ^{125}I as described by Greenwood et al. (1963).

Preparation of polyribosomes and polyribosomal RNA. Polyribosomes were prepared according to the procedure of Jackson & Larkins (1976) from healthy or systemically TSWV-infected plants. The ribosome fraction sedimenting at a greater rate than 100S was collected and RNA was extracted by phenol-SDS.

Hybridization. Hybridization of [^{125}I]-labelled TSWV RNA with great excess of polyribosomal RNA was done as described by Milner & Jackson (1979).

Transcriptase assays. Purified TSWV and *Sonchus* yellow net virus (SYNV) particles were assayed for transcriptase activity essentially as described by Francki & Randles (1972). Nucleocapsid extracts from TSWV-infected plants were prepared and assayed for transcriptase activity as described by Francki & Peters (1978).

2.3 RESULTS

TSWV contains three RNA segments.

When RNA extracted from purified virions was analysed on a 1% agarose gel containing 5mM-methylmercury, three bands were detected (Fig. 1). The mol. wt. of the RNA segments corresponding to the

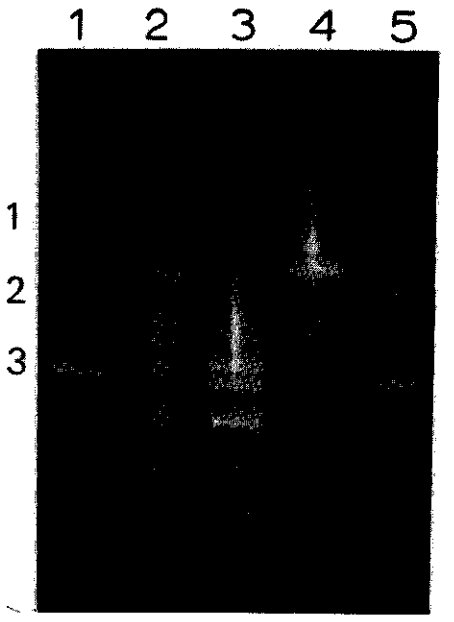


Fig. 1. Analysis of TSWV RNA by electrophoresis on a 1% agarose gel containing 5 mM-CH_3HgOH. Lane 1: TSWV RNA, 2: CPMV RNA, 3: CCMV RNA, 4: TMV RNA, 5: TSWV RNA.

bands observed was estimated to be 2.7 (± 0.05; 7800 bases), 1.7 (± 0.04; 5000 bases) and 1.1 (± 0.01; 3200 bases) x 10^6 mol. wt., using the RNAs of CPMV, TMV and RNA 1 and RNA 2 of cowpea chlorotic mottle virus (CCMV) as markers (Reijnders et al., 1974). These values, calculated by linear regression from five experiments, agree with those found by Van den Hurk et al. (1977), except that an RNA segment of mol. wt. 1.9 x 10^6, which occurred in minor amounts in their preparations, has never been observed in our extracts.

The RNA of TSWV was also analysed on an isokinetic sucrose gradient. Invariably, three bands were obtained (Fig. 2). A small shoulder was always observed sedimenting slower than the slowest

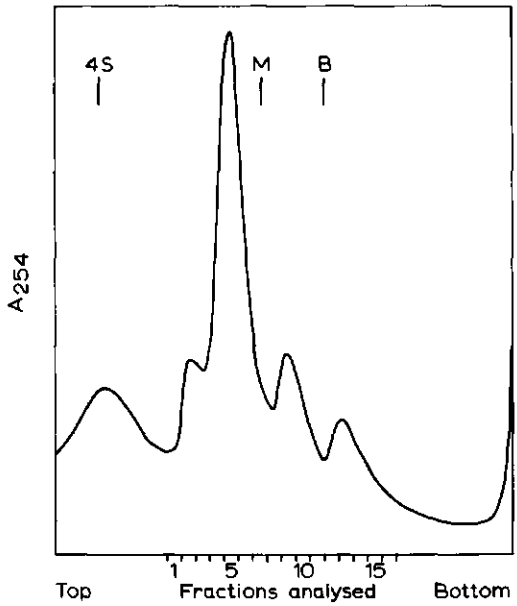


Fig. 2. Absorption profile of TSWV RNA centrifuged on a 5.0 to 33% isokinetic sucrose gradient. tRNA of wheat germs (4S) and the RNA of the middle (M) and bottom (B) component of CPMV were used as markers. The fractions 1 to 16 were used in translation studies the results of which are shown in Fig. 6.

band. Upon analysis of these bands on methylmercury agarose gels, no RNA segements other than the three mentioned above could be found. Some material was found at the top of the gradient, which is probably due to degraded RNA of host origin.

TSWV RNA contains no poly-(A).

Fig 3. shows that TSWV RNA, when eluted with high salt buffer, was not retained by the oligo-(dT)-cellulose column, in contrast to CPMV RNA which contains a poly-adenylate sequence (El Manna & Bruening, 1973). So it can be concluded that TSWV RNA does not contain poly-(A) sequences larger than 25 nucleotides (Groot et al., 1974). In this respect TSWV resembles the RNA of most plant viruses (Atabekov & Morozov, 1979).

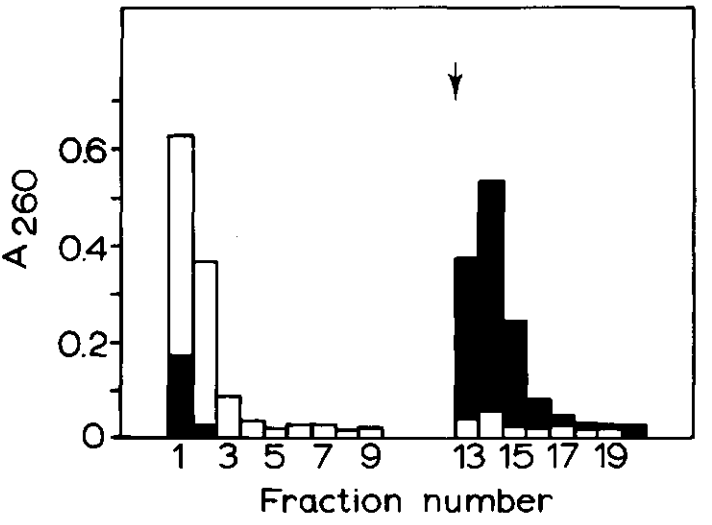


Fig. 3. Chromatography of TSWV RNA (white) and CPMV RNA (black) on an oligo-(dT)-cellulose column. Arrow indicates the shift from high salt to low salt buffer.

In vitro translation of TSWV RNA.

TSWV RNA directed the incorporation of [^{35}S]-methionine into hot acid precipitable material in a wheat germ cell-free system and a mRNA-dependent rabbit reticulocyte lysate. The optimal conditions for translation in the wheat germ cell-free system were 3.25 mM-Mg-acetate, 110 mM-K-acetate, 0.2 mM spermidine and 75 µg/ml RNA (Fig. 4). Strikingly, translation in this system was absolutely dependent on spermidine, a dependence which has also been found for the translation of CPMV RNA (Davies et al., 1977).

Analysis of the translation products by gel electrophoresis.

The translation products of TSWV RNA synthesized in both systems were analysed by polyacrylamide gel electrophoresis. Fluorography of the gels revealed a major polypeptide, synthesized in both systems, with a mol. wt. of 60 000 (Fig. 5A and 5B, lane 3). Another major polypeptide synthesized in both systems had approx. the same mol. wt. as protein 1, the nucleocapsid protein (Fig. 5A and Fig.

5B, lane 1 and 3). In the wheat germ cell-free system two minor high mol. wt. polypeptides were also synthesized, whose molecular

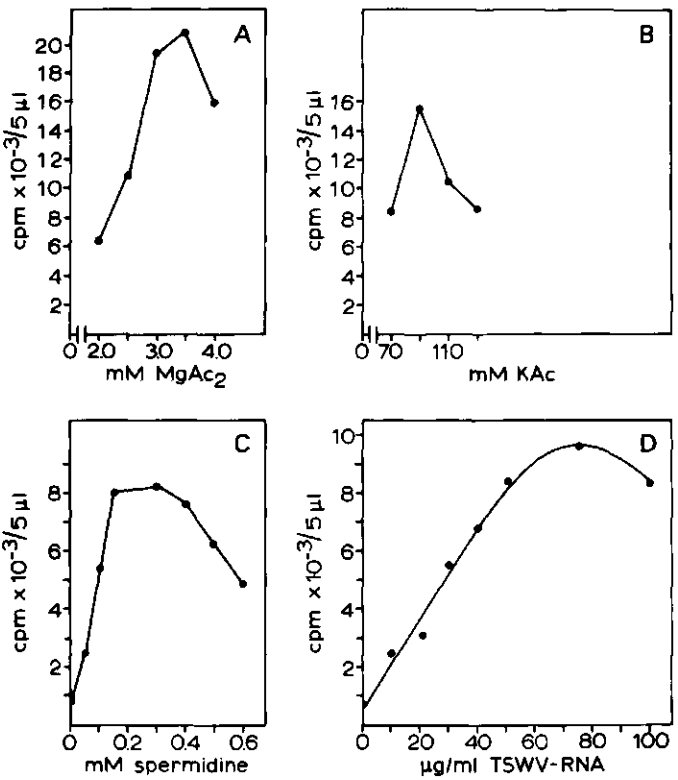
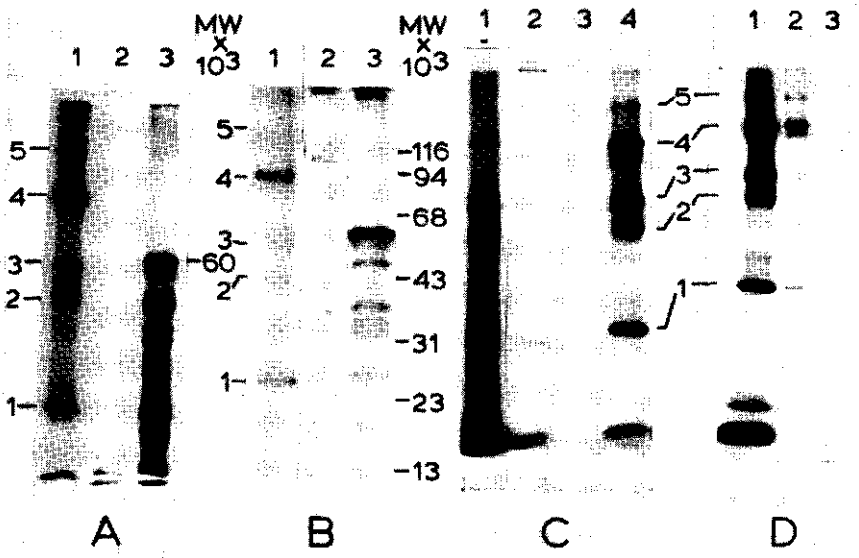


Fig. 4 Optimalization of the in vitro translation of TSWV RNA in the cell-free system of wheat germ for the concentration of A. Mg-acetate. B. K-acetate. C. spermidine. D. TSWV RNA. The conditions in the separate experiments were: A. 110 mM-K-acetate, 0.4 mM-spermidine, 60 µg/ml RNA. B. 3.25 mM-Mg-acetate, 0.4 mM-spermidine, 60 µg/ml RNA. C. 110 mM-K-acetate, 3.25 mM-Mg-acetate, 30 µg/ml RNA. D. 110 mM-K-acetate, 3.25 mM-Mg-acetate, 0.2 mM-spermidine.

weights corresponded to those of protein 4 and 5 of TSWV (Fig. 5A lane 1 and 3). In addition to the polypeptides mentioned, many other polypeptides were synthesized in the wheat germ system, the identity of which is unknown.

Translation of TMV RNA in these systems resulted primarily in products which were essentially the same as those reported by Knowland (1974), Pelham & Jackson (1976) and Davies et al. (1977).



ig. 5. Analysis of the in vitro translation products of TSWV RNA.
. Gel electrophoresis of the translation products made in the wheat germ cell-ree system. Lane 1: [^{125}I]-TSWV proteins, 2: endogenous translation products, 3: SWV RNA translation products.
. Gel electrophoresis of the translation products made in the mRNA-dependent abbit reticulocyte lysate. Lane 1: [^{125}I]-TSWV proteins, 2: endogenous transla-ion products, 3: TSWV RNA translation products.
. Gel electrophoresis of the immunoprecipitates of the translation products made n the wheat germ cell-free system under the direction of TSWV RNA. Lane 1: TSWV NA translation products, 2: immunoprecipitate with anti-TSWV serum, 3: immuno-recipitate with pre-immune serum, 4: [^{125}I]-TSWV proteins.
. Gel electrophoresis of the immunoprecipitates of [^{125}I]-TSWV proteins. Lane 1: ^{125}I]-TSWV proteins, 2: immunoprecipitate with anti-TSWV serum, 3: immunopreci-itate with pre-immune serum.

nalysis of the translation products by immunoprecipitation.

An immunoglobulin preparation made from antiserum raised against SWV precipitated only, in small amounts, one polypeptide from the SWV RNA directed translation products; namely that which had ap-rox. the same mol. wt. as the nucleocapsid protein (Fig. 5C). How-ever, the same immunoglobulin preparation was capable precipitating ll the proteins of [^{125}I]-labelled TSWV (Fig. 5D). This result ight indicate that of the structural proteins of TSWV only the nu-cleocapsid protein was synthesized *in vitro*. Alternatively, it is ossible that other structural proteins were synthesized too, but

could not be recognized by the antibodies owing to the absence of glycosylation and phosphorylation in the cell-free systems. The importance of sugar groups for the recognition by antibodies is well documentated for the membrane proteins of Semliki Forest virus (Kaluza et al., 1980).

In vitro *translation of fractionated TSWV RNA.*

To get more information about the genetic content of the individual RNA segments, TSWV RNA, fractionated on a sucrose gradient (Fig. 2), was analysed on a methylmercury-agarose gel and translated in the wheat germ cell-free system. The fractions which contained RNA 1 or RNA 2 (fractions 8 to 11 and 12 to 15 resp.) appeared to be contaminated with RNA 3. Gel electrophoresis and autoradiography of the translation products showed that the RNA in all fractions gave rise to the synthesis of the nucleocapsid protein; the RNA from the fractions containing only RNA 3 (fraction 1 to 7) supported also the synthesis of the 60 000 mol. wt. polypeptide and other polypeptides of intermediate mol. wt. (Fig. 6). This result means that RNA 3 contains information for the 60 000 mol. wt. polypeptide and the nucleocapsid protein, and this accounts for 60% of the coding capacity of RNA 3. The possible unique information content of RNA 1 and RNA 2 could not be ascertained, because the fractions in question were contaminated with RNA 3. Moreover, the RNA in these fractions (8 to 15) did not give rise to the synthesis of unique proteins when compared to the proteins made under the direction of RNA 3.

TSWV RNA does not hybridize with polyribosomal RNA from infected plants.

The results described above show that TSWV has a positive-stranded RNA. This means that the mRNA of TSWV should have the same polarity and one should expect that no hybridization occurs between it and TSWV RNA.

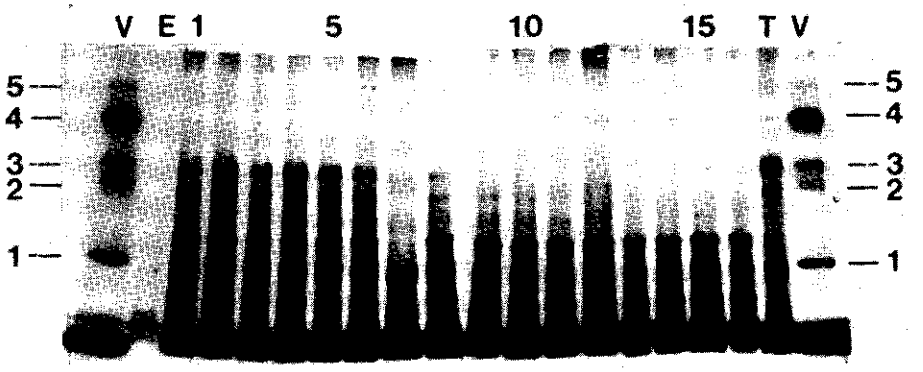


Fig. 6. Gel electrophoresis of the translation products made under the direction of the RNA in the fractions of the sucrose gradient (Fig. 2). V: [^{125}I]-TSWV proteins. 1 to 16: translation products of the RNA in the fractions represented in Fig. 2. T: translation products of complete TSWV RNA, E: endogenous translation products.

Table 1. Hybridization of [^{125}I]-TSWV RNA with polyribosomal RNA from healthy (H) or infected (I) plants after 0 h, 1 h 30 min and 24 h of incubation. Values (cpm) corrected for self-annealing (169 cpm).

[^{125}I]-TSWV RNA hybridized with	Values found after incubation for					
	0 h		1 h 30 min		24 h	
	+RNase	-RNase	+RNase	-RNase	+RNase	-RNase
polyribosomal RNA (H)	<0	1441	<0	1663	44	1840
polyribosomal RNA (I)	75	1287	30	1529	92	1602

To test this assumption, [^{125}I]-labelled TSWV RNA was hybridized with RNA extracted from the polyribosomal fraction of tobacco plants infected with TSWV. As seen in Table 1, no significant hybridization was obtained, indicating that TSWV RNA and its mRNA have the same polarity.

Table 2. Transcriptase activity associated with purified TSWV and SYNV particles (A) and nucleocapsid extracts (73 000 *g* pellets) from plants infected with TSWV and SYNV or from uninfected plants with and without actinomycine D (AMD) in the reaction mixtures (B).

A. Virus	[^{3}H]-UMP incorporation (cpm) after incubation for 0 h	1 h	24 h
no virus	50	56	61
TSWV	65	56	90
SYNV	45	400	15 000

B.	[^{3}H]-UMP incorporation (cpm)* +AMD	-AMD
nucleocapsid extracts from TSWV infected plants	<0	492
nucleocapsid extracts from SYNV infected plants	2500	9000
an identical extract from uninfected plants	<0	78

*values corrected for incorporation at 0 h.

TSWV does not show transcriptase activity.

Negative-stranded RNA viruses possess virus-associated transcriptases, which are able to synthesize a positive stranded RNA from their templates. Such an activity has been demonstrated *in vitro* in the particles of lettuce necrotic yellows virus, after dissolution with a non-ionic detergent (Francki & Randles, 1972; Toriyama & Peters, 1980). This activity could also be demonstrated in nucleocapsid fractions from leaves infected with this virus (Francki & Peters, 1978).

Application of these procedures, used to transcribe viral RNA either from purified TSWV preparations or nucleocapsid extracts from plant material did not result in any significant incorporation of [^{3}H]-UMP into acid precipitable product (Table 2). We conclude from

these experiments that TSWV does not contain transcriptase activity and this result supports the conclusion that TSWV has a positive-stranded RNA.

2.4 DISCUSSION

We present evidence that TSWV contains a positive-stranded RNA. TSWV RNA directed the *in vitro* synthesis of several proteins, one of which could be identified as the nucleocapsid protein. [^{125}I]-labelled TSWV RNA did not hybridize with polyribosomal RNA from infected leaves, and furthermore no transcriptase activity could be demonstrated in purified TSWV particles or in nucleocapsid preparations extracted from infected leaves.

Van den Hurk et al. (1977) have suggested that TSWV RNA has a negative polarity because they observed that it was non-infectious. It is possible that even though it has positive polarity, TSWV RNA is non-infectious because of the loss of a viral protein which is needed to initiate the infection; this has been demonstrated for the genomic RNAs of alfalfa mosaic virus (Van Vloten-Doting & Jaspars, 1977). However, the failure to detect infectivity of RNA preparations may perhaps also be due to the use of inocula in which the RNA concentration was too low. Since the specific infectivity and the RNA content of the virus are low, and since it can be expected that the three RNA segments are required for infection, inocula with a high RNA content may be necessary to answer the question whether the viral RNA by itself is infectious.

Analysis of TSWV RNA on a methylmercury-agarose gel revealed the presence of three RNA segments in the virus. We never observed the minor segment of 1.9 x 10^6 mol. wt. and the component which also could be stained with Coomassie-Brillant Blue, found by Van den Hurk et al. (1977). However, as heating TSWV RNA prior to electrophoresis resulted in the disappearance of the minor RNA segment of 1.9 x 10^6 mol. wt. (Fig. 5 in Van den Hurk et al., 1977), we think this RNA segment is the result of secondary structures in the RNA. Possibly, methylmercury is a more stringent denaturing agent than 3 M-urea at 60°C, which was used by Van den Hurk et al. (1977). The finding that the isolate used by Van den Hurk et al. (1977) and our isolate contain essentially the same RNA segments, suggests that the presence of three RNA segments is typical for TSWV.

The result of the *in vitro* translation of the RNA from the sucrose gradient fractions showed that almost all of the polypeptides made in both cell-free systems are in fact synthesized under the direction of RNA 3. This is not surprising, because RNA 3 is the major RNA of TSWV, and it is known that smaller RNAs are good competitors of larger RNAs in *in vitro* translation (Van Tol & Van Vloten-Doting, 1979; Pyne & Hall, 1979). It is possible that the two minor large polypeptides, made in the wheat germ cell-free systems, are products of TSWV RNA 1 and RNA 2 (Fig. 5A, lane 3).

RNA 1 can code for a polypeptide with mol. wt. of approx. 300 x 10^3 and the sum of the mol. wts. of the virus proteins 1 to 5 (Tas et al., 1977a) is also 300 x 10^3, so that this RNA would be sufficient to code for all the structural proteins. One might, however, expect more viral coded proteins to be needed for the multiplication of TSWV (for example, a replicase). In short, it is unlikely that all the genetic information is contained in RNA 1, so that the other RNAs will contain unique sequences too.

The results presented in this study stress the unique character of TSWV. Among the plant viruses, TSWV is the only enveloped virus with a positive-stranded segmented RNA genome. Carrot mottle virus is enveloped and has probably a positive-stranded RNA, but may consist of one RNA species (Murant et al., 1969; Halk et al., 1979). Also among the animal viruses TSWV has no counterpart. The togaviruses are enveloped but contain one positive-stranded RNA segment. The bunyaviruses are enveloped and contain three RNA segments, however, with a negative polarity (Obijeski & Murphy, 1977).

3 SEROLOGICAL ANALYSIS OF TSWV AND ITS DEFECTIVE FORMS

3.1 INTRODUCTION

In Chapter 1 I mentioned the occurrence of electron dense amorphous masses in cells infected with TSWV and suggested that these masses are formed by aggregation of nucleocapsids of a defective form of TSWV which do not mature in complete particles. The identification and analysis of the viral proteins in infected plants may reveal the defect which hinders the formation of complete particles.

The enzyme-linked immunosorbent assay (ELISA) may be sufficiently sensitive to undertake this study. Antibodies were raised against a membrane protein fraction and the nucleocapsid protein of TSWV. These antibodies were then used to investigate the proteins present in sap from plants infected with either normal or defective forms by means of ELISA.

In this Chapter I describe the purification of a fraction containing the membrane proteins and the nucleocapsid protein. I have used antisera raised against these purified proteins in experiments to examine the presence of these proteins in plants infected with normal or defective forms.

3.2 MATERIALS AND METHODS

Isolates of TSWV used. The experiments described in this Chapter were carried out mainly with two isolates of TSWV. One isolate, TSWV-Y, was kindly supplied by dr. Bužančić (Yugoslavia) in 1979. Electron microscopy of ultrathin sections of infected cells revealed that this isolate for the most part produced normal enveloped particles. The electron dense amorphous masses, typical for the defective form, were scarcely present. Furthermore, one could readily purify TSWV from tobacco plants infected with this isolate (see also Chapter 2).

The other isolate, TSWV-P, which originated in Poland, nowadays forms no complete particles, but only amorphous masses in infected cells. Originally, approx. fifteen years ago, it produced mainly normal particles. Repeated mechanical transfers probably caused a complete conversion to the defective form, for in ultrathin sections only electron dense amorphous masses can be observed. The origin of this isolate has been described by Ie (1982).

Purification of the viral membrane protein fraction and nucleocapsid protein. TSWV was purified from leaves infected with TSWV-Y as described in Chapter 2. To dissociate the virus, a sufficient amount of the non-ionic detergent Nonidet P40 (NP-40) was added to a final concentration of 2% to 0.25 ml of a suspension of approx. 3 mg TSWV as estimated by its protein content. The nucleocapsid protein was separated from the viral membrane proteins by centrifugation of the dissociated virus suspension through a sucrose gradient. The suspension was applied on to four 10 to 50% linear sucrose gradients in 0.01 M-Na-phosphate buffer pH 7.0, containing 0.01 M-Na_2SO_3, 0.5 M-NaCl and 0.5% NP-40, and centrifuged in a Beckman SW 41 rotor at 40 000 rev/min for 5.5 h at 4°C. Sixteen fractions were collected from each gradient; the corresponding fractions of the four gradients were pooled and their protein composition was analysed on a 10% polyacrylamide gel according to Laemmli (1970). For this purpose the proteins from one-sixth volume of the pooled fractions were precipitated with ethanol.

The fractions of the sucrose gradient containing either the nucleocapsid protein or the membrane proteins were pooled, dialysed against 0.01 M-Na-phosphate buffer containing 0.01 M-Na_2SO_3 and 0.1% NP-40, reduced in volume by placing the dialysis bags in solid sucrose and the fractions were finally lyophilized.

Preparation of antibodies. The lyophilized proteins were dissolved in 0.5 ml demineralized water, mixed with an equal volume of Freund complete adjuvant and injected subcutaneously in guinea pigs. As determined by electrophoresis of the purified nucleocapsid protein and the membrane protein fraction, the amount of protein injected was about 75 µg.

Two weeks later the animals were again injected with a similar sample containing the nucleocapsid protein and the membrane protein fraction, respectively.

The animals were bled two weeks after the last injection and the immunoglobulins were prepared by ammonium sulphate precipitation from the sera.

The immunoglobulins directed against the membrane protein fraction of TSWV will be designated as anti-G immunoglobulins and those against the nucleocapsid protein as anti-N immunoglobulins.

ELISA. The double antibody sandwich method of ELISA was carried out essentially as described by Clark & Adams (1977). The wells in the polystyrene Micro-elisa plates (M 129A, Dynatech Laboratories Ltd., Billingshurst, Sussex, UK) were coated with 200 µl immunoglobulin solution in concentrations as indicated in the legends for the figures, and incubated for 20 h at 4 °C. After washing the plates with tapwater, 200 µl of the samples to be tested were added to the wells. These samples were made by homogenizing leaf disks of 6 mm in diameter in 300 µl PBS containing 0.05% Tween 20 with a small sized Elvejhem Potter tube. Duplicate samples were obtained and, if necessary, they were diluted in the PBS-Tween solution. The plates were incubated overnight at 4 °C. After four washes in tap water 200 µl of a solution of immunoglobulins conjugated with alkaline phosphatase were added in the concentrations indicated in the legends for the figures. After incubation for 4 h at 37 °C 300 µl of a freshly prepared solution of substrate, namely 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine pH 9.8 (Sigma), was added to each well. After incubation at room temperature for 2 h, the colour intensity of each well was measured at 405 nm in an automatic spectrophotometer (Titertek Multiskan). The colour intensity of the wells containing only substrate was automatically subtracted from all photometer values.

3.3 RESULTS

Purification of the viral membrane protein fraction and the nucleocapsid protein

The separation of the nucleocapsid protein from the membrane proteins was achieved by centrifugation of dissociated virus particles through sucrose gradients. Fig. 1A illustrates the distribution of the proteins over the gradient. The fractions 1 to 4 and 8 to 14 were pooled and analysed by electrophoresis on a polyacrylamide gel. Fig. 1B shows that the fractions with the membrane proteins and the nucleocapsid protein were not contaminated with each other.

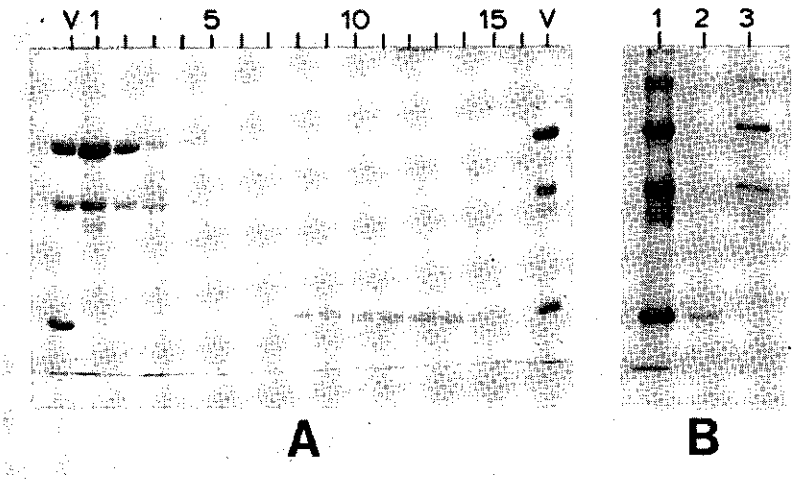


Fig. 1.A. Analysis of the viral proteins present in the fractions of the sucrose gradient by polyacrylamide gel electrophoresis. V: structural proteins of TSWV. 1 to 16: proteins present in the corresponding fractions of the gradient. B. Gel electrophoresis of TSWV (lane 1), purified nucleocapsid protein (lane 2), purified membrane protein fraction (lane 3).

The specificity of the antisera

The immunoglobulins, prepared from the antisera against the membrane protein fraction (anti-G immunoglobulins) and against the nucleocapsid protein (anti-N immunoglobulins) of TSWV did not show cross-reactions in heterologous combinations. As can be seen in Fig. 2, the anti-G immunoglobulins reacted only with the fractions of the gradient containing membrane proteins, and the anti-N immunoglobulins only with those containing nucleocapsid protein.

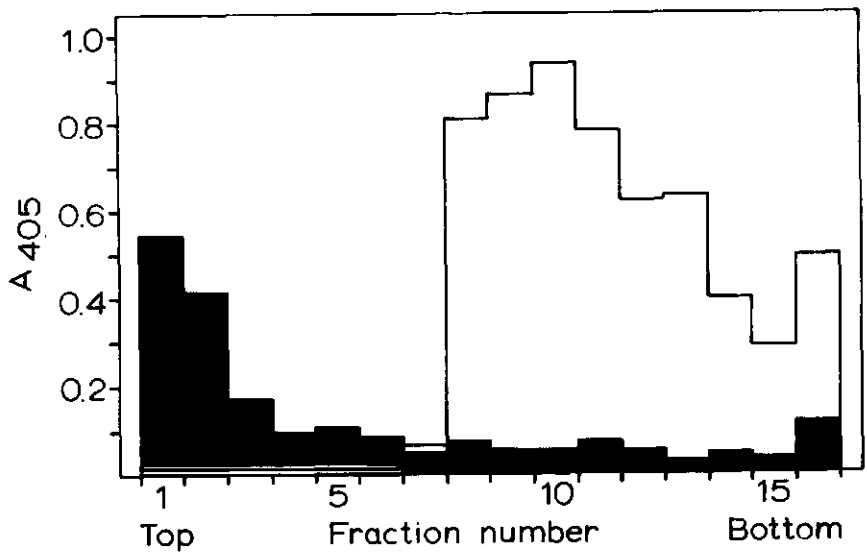


Fig. 2. Analysis by ELISA of the fractions sampled after centrifugation of NP-40 dissociated TSWV in a linear sucrose gradient (Fig. 1A). The black area shows the reaction in which the anti-G immunoglobulins were used in a coating and conjugate concentration of 10 and 4 µg/ml; fractions undiluted. The white area shows the reaction in which the anti-N immunoglobulins were used in a coating and conjugate concentration of 2 µg/ml; fractions 10-fold diluted. The numbers of the fractions are as in Fig. 1A.

Higher absorbances were obtained in the reaction between the nucleocapsid protein and its antiserum than between the viral membrane proteins and the antiserum against the membrane protein fraction. To obtain comparable readings, a concentration of 10 µg/ml and 4 µg/ml anti-G immunoglobulins were used to coat the plates and as conjugate, respectively, while the fractions to be tested were applied undiluted. These concentrations of anti-N immunoglobulins were 2 µg/ml and the fractions were diluted 10-fold. This result indicates that the titer of the anti-N serum was much higher than that of the anti-G serum. It is also possible that the presence of NP-40 in the fractions of the sucrose gradient negatively affected the antigenic properties of the viral membrane proteins.

Optimalization of ELISA to detect viral proteins in infected leaf sap

The reaction of sap from TSWV-infected plants with the anti-G immunoglobulins in ELISA was thoroughly investigated using different sap dilutions and conjugate concentrations. Fig. 3A shows that a 25-fold dilution of sap from leaves infected with the isolate TSWV-Y gives a distinct ELISA absorbance at a conjugate concentration of 10 µg/ml. The sensitivity of reaction decreased when lower conjugate concentrations were used (Fig. 3B and C). However, the

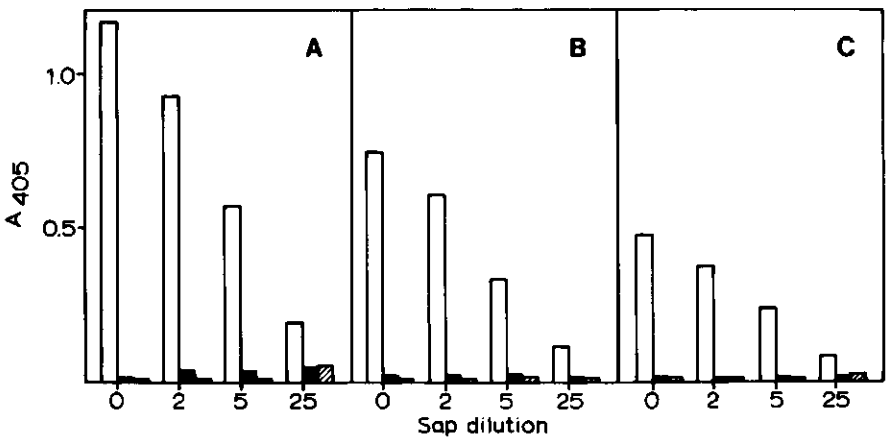


Fig. 3. Optimalization of ELISA with the anti-G immunoglobulins and sap from leaves infected with TSWV-Y (▭) and TSWV-P (■), and with sap from healthy leaves (▨). Conjugate concentration (A) 10 µg/ml, (B) 4 µg/ml, (C) 2 µg/ml. Coating concentration 10 µg/ml.

ELISA absorbances indicated that at 5- and 2-fold dilution of sap, and at a conjugate concentration of 4 µg/ml the reaction was sufficiently discriminative to demonstrate the presence of viral membrane proteins in sap from plants infected with TSWV-Y.

The reaction with the anti-N immunoglobulins and sap from infected plants have not been studied in detail. In the studies completed we confirmed our conclusion that the anti-N immunoglobulins gave stronger reactions than was obtained in reactions with anti-G immunoglobulins. At coating and conjugate concentrations of 2 µg/ml clear, positive reactions occurred with sap diluted 25-fold.

The detection of TSWV-antigens in leaves infected with TSWV-P, a defective isolate

Nucleocapsid protein was detected in sap from leaves infected with TSWV-Y (normal form) and TSWV-P (defective form) in ELISA. Absorbances of 1.0 and 0.95 were obtained in 2-fold dilutions. Equally positive reactions were still found at 32-fold dilution of these samples. Higher dilutions were not tested (Fig. 4A). These results

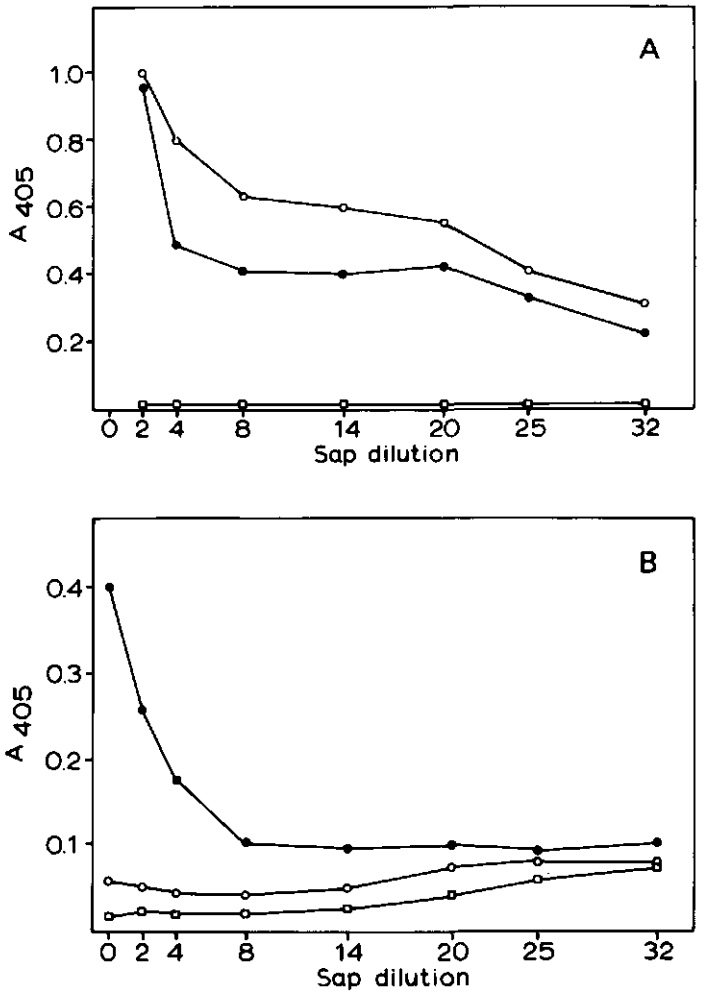


Fig. 4.A. ELISA tests with sap from leaves infected with TSWV-Y (●-●), and with TSWV-P (o-o) and from healthy leaves (□-□). A. with anti-N immunoglobulins in a coating and conjugate concentration of 5 and 2 µg/ml. B. with anti-G immunoglobulins in a coating and conjugate concentration of 10 and 4 µg/ml.

demonstrate that the nucleocapsid protein can be detected in plants infected with the normal and the defective form, and that its concentration in plants infected with either form is approx. the same.

The anti-G immunoglobulins gave only a positive reaction in ELISA with sap from leaves infected with TSWV-Y (normal form). The reaction with sap from leaves infected with TSWV-P (defective form) could be compared with the background readings, obtained with sap from healthy leaves (Fig. 4B). The results presented in Fig. 3A demonstrate that the anti-G immunoglobulins, used in a concentration of 10 µg/ml to coat the wells and a conjugate concentration of 10 µg/ml failed to give positive reactions with undiluted sap from leaves infected with the defective form. The results of these experiments indicate that the viral membrane proteins are not present in leaves infected with TSWV-P.

In these experiments represented in Fig. 4B, the anti-G immunoglobulins did not give a positive reaction with sap from leaves infected with the normal form after a 8-fold dilution, while the concentration of the immunoglobulins used to coat the wells and the conjugate concentration were 10 µg/ml and 4 µg/ml. Thus it appears, as has been already noted, that the titer of the anti-G serum was lower than that of the anti-N serum. Since no detergent was used in this experiment, the possibility that the antigenicity of the viral membrane proteins has been negatively affected by NP-40 can be ruled out.

The possibility cannot be excluded that the viral membrane proteins, responsible for ELISA reaction, were synthesized in an early stage of infection and disappeared in the course of infection. To examine the possibility of the disappearance of these proteins a number of plants infected with TSWV-P were tested over a period from 9 to 16 days after inoculation; in the beginning of this period the leaves start being infected systemically; at the end they become necrotic. No evidence of the occurrence of viral membrane proteins in sap from these plants was found (Fig. 5A), whereas the anti-N immunoglobulins gave positive reactions (Fig. 5B). This result indicates that no detectable amounts of viral membrane proteins were synthesized during the entire course of infection with TSWV-P.

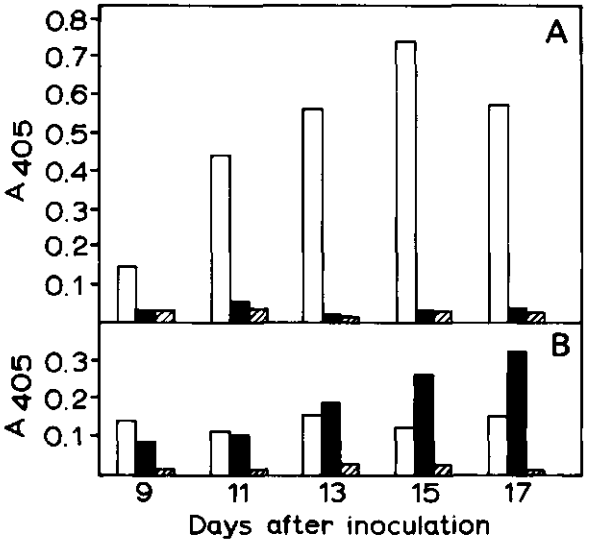


Fig. 5. ELISA tests with sap from leaves infected with TSWV-Y (▭) and TSWV-P (▬) on several days after inoculation. ELISA tests with sap from healthy leaves (▨) of the same age as the infected leaves served as controls. A. with anti-G immunoglobulins in a coating and conjugate concentration of 10 µg/ml; sap undiluted. B. with anti-N immunoglobulins in a coating and conjugate of 2 µg/ml; sap diluted 20-fold.

The detection of TSWV-antigens in leaves infected with other isolates

The results described above indicate that no viral membrane proteins occur in leaves infected with the defective isolate TSWV-P. One may argue that the antigenic determinants of the membrane proteins of the normal form (TSWV-Y) are so different from those of the defective form (TSWV-P), that the anti-G immunoglobulins directed against the membrane proteins of TSWV-Y do not recognize the membrane proteins of TSWV-P. I will give evidence that does not support this interpretation.

Most of the isolates of TSWV available to us proved to consist of normal particles as could be verified by electron microscopy. However, three of the isolates which were maintained for several years in the glasshouse, occur in the defective form.

Table 1. ELISA tests with sap from leaves infected with normal or defective isolates of TSWV, as verified by electron microscopy of ultrathin sections. The isolates were propagated in *N. rustica*, except isolate 'SNN' which was propagated in *N. tabacum* 'Samsun NN' and isolate 'T. majus', which was propagated in *Tropaeolum majus*. Anti-G immunoglobulins were used in a coating and conjugate concentration of 10 and 4 µg/ml. Anti-N immunoglobulins were used in a coating and conjugate concentration of 2 µg/ml. Leaf sap in all tests was 2-fold diluted.

isolate	form	anti-N	anti-G
TSWV-12	defective	0.90	0.04
TSWV-SNN	defective	0.93	0.04
TSWV-P	defective	0.89	0.04
TSWV-B 21	normal	0.39	1.36
TSWV-Thrips	normal	0.63	1.24
TSWV-Amerine	normal	0.68	0.54
TSWV-T. majus	normal	0.91	1.05
TSWV-Y	normal	0.34	0.66
N. rustica (not infected)		0.01	0.04

Leaves infected with these isolates were tested with ELISA. Table 1 gives a summary of these results. The isolates designated as 'normal' gave positive reactions with the anti-G immunoglobulins. In contrast, the isolates designated as 'defective' did not react with these immunoglobulins. Sap from all plants infected with the normal or defective isolates reacted with the anti-N immunoglobulins.

3.4 DISCUSSION

This study was started to investigate the protein composition of the defective form in infected leaves by means of a serological analysis. ELISA has proven to be a sensitive method to assess the presence of minute quantities of antigens in sap (Clark & Adams, 1977).

The nucleocapsid protein could be separated from the other viral proteins by centrifugation of a dissociated virus suspension through a sucrose gradient. In a similar way, Mohamed et al. (1973) have also separated these proteins from each other. In a recent publication, Mohamed (1981) has examined in more detail the nucleocapsid composition of TSWV. The absorption profile of the sucrose gradient which he used to separate the nucleocapsid from the membrane proteins, points to three different sized nucleocapsids. I was not able to find a similar distribution of nucleocapsids over the gradient, although the broad distribution of the nucleocapsids in the lower half of the gradient (Fig. 1A) suggests a heterogeneity in size of the nucleocapsids. Thus, I tend to conclude that the results of Mohamed are compatible with my findings.

Antibodies were raised against the nucleocapsid protein and a membrane protein fraction of TSWV. The latter contained mainly protein 2, 3 and 4. Attempts to separate protein 2 + 3 from protein 4 were unsuccessful. Preparative SDS-polyacrylamide gel electrophoresis of the proteins of TSWV resulted in a good separation, but ELISA tests with sera raised against these proteins failed.

I do not know to which proteins of the membrane protein fraction antibodies were produced. As protein 3 and 4 are predominant in this fraction (Fig. 1A), one would expect that antibodies occur to both proteins in the serum obtained. Therefore, both proteins do not occur in sap from leaves infected with isolates of the defective form. However, one cannot exclude that antibodies were produced mainly or only against one of these proteins. The anti-G immunoglobulins contain at least antibodies against protein 4, since these immunoglobulins reacted very well with purified and not dissociated TSWV particles; from the work of Tas et al. (1977a) it is known that protein 4 is exposed to the outside (spike protein). Furthermore, as will be discussed in Chapter 6, I obtained no any evidence that this protein and protein 3 too were present in extracts from plants infected with the defective form.

The results of ELISA tests on leaf sap from plants infected with several normal and defective isolates favour the hypothesis that in plants infected with defective isolates the viral membrane proteins are absent. It is unlikely that the anti-G immunoglobulins recognize the viral membrane proteins in plants infected with isolates of the

normal form of TSWV and not those infected with isolates of the defective form.

The presence of NP-40 in some samples might have complicated the results with the anti-G immunoglobulins. The anti-G immunoglobulins reacted very well with purified and not dissociated TSWV particles. Addition of Triton X-100 or NP-40 to the samples inhibited strongly the reaction: a reduction of 50% occurred in samples containing NP-40 in a concentration of 0.05%; a reduction of 95% occurred when this concentration was 0.5%. NP-40 did not assert its influence by inhibiting some reactions in the ELISA assay proper, since the reaction of the anti-N immunoglobulins was greatly enhanced by the addition of NP-40. This was probably because removal of the viral envelope exposed nucleocapsids. Therefore, the effect of NP-40 is probably due to its influence on the conformation of the viral membrane proteins in such a way that they are no longer recognized by the anti-G immunoglobulins as antigens.

This explanation is left with the discrepancy that the anti-G immunoglobulins were obtained by injecting guinea pigs with a viral membrane protein fraction which did contain NP-40. Apparently, in the guinea pig the effect of NP-40 on the conformation of the viral membrane proteins was annulled, since the immunoglobulins reacted badly with NP-40-treated TSWV suspensions.

In conclusion, the results of tests discussed in this Chapter give strong evidence that no viral membrane proteins are synthesized in cells infected with the defective forms of TSWV.

IN VITRO TRANSLATION OF POLYRIBOSOMES FROM LEAVES INFECTED WITH THE DEFECTIVE FORM

.1 INTRODUCTION

In the previous chapter evidence is presented that no viral membrane proteins could be detected in leaves infected with defective forms of TSWV. A likely explanation is that these proteins have not been synthesized during infection, perhaps due to a loss of genetic information for these proteins. This loss of genetic information can be studied by *in vitro* translation of polyribosomes from leaves infected with the defective form. Analysis of the translation products might reveal if the mRNAs coding for the viral membrane proteins are among the mRNAs in het polyribosomes isolated.

.2 MATERIALS AND METHODS

Isolates of TSWV used. The isolate TSWV-B21, forming normal particles, and the isolate TSWV-SNN, an isolate that is defective, were used. For this study the isolates were propagated in *N. glutinosa*.

Isolation of polyribosomes. Polyribosomes from leaves of healthy *N. glutinosa* plants or from leaves either primarily or systemically infected with TSWV-B21 or TSWV-SNN were prepared as described by Jackson & Larkins (1976).

Briefly, 5 g of leaves from which the midribs were removed were ground with a pestle and a mortar with 50 ml of high salt extraction buffer containing 200 mM-Tris-HCl, pH 9.0, 400 mM-KCl, 200 mM-sucrose, 35 mM-$MgCl_2$ and 25 mM-EGTA. The homogenate was centrifuged at 15 500 rev/min in a Sorvall SS 34 rotor. Triton-X-100 was added to the supernatant in a final concentration of 1%. The suspension was applied on to a 3 ml cushion of 1.75 M-sucrose in 40 mM-Tris-HCl, pH 9.0, 200 mM-KCl, 30 mM-$MgCl_2$ and 5 mM-EGTA, and centrifuged

for 2 h at 50 000 rev/min in a Beckman R 50 Ti rotor. The pellets were resuspended in approx. 200 µl double distilled water to obtain a concentration of 25 units at 260 nm per ml and this suspension then stored in liquid nitrogen. The yield was approx. 1 A_{260} unit per g leaf. The translation activity of the polyribosomes was not significantly reduced by repeated thawing of the samples.

The composition of the polyribosomes was sometimes checked by analysis on a sucrose gradient. The pellets were then resuspended in 200 µl resuspension buffer containing 40 mM-Tris-HCl, pH 8.5, 200 mM-KCl, 30 mM-$MgCl_2$ and 5 mM-EGTA. An amount of 5 A_{260} units of polyribosomes were applied on to a 15 to 33.5% isokinetic sucrose gradient, made in resuspension buffer (Noll, 1967; Van der Zeijst & Bloemers, 1976) and were centrifuged for 75 min at 41 000 rev/min in a Beckman SW 41 rotor. The absorption profile of the gradient was recorded by means of a Uvicord spectrophotometer.

In vitro *translation.* Translation of the polyribosomes was carried out in cell-free extract of wheat germ, prepared as described in Chapter 2. The reaction mixture of 50 µl contained 20 µl of the wheat germ extract and 0.25 A_{260} units of polyribosomes. The Mg-acetate, K-acetate and spermidine concentrations were 2.5 mM, 110 mM and 0.4 mM, respectively. As radioactive aminoacid was added 1 µl of [^{35}S]-methionine (7.7 mCi/ml, 1020 Ci/mmol, Amersham). Further reaction conditions were the same as used for the translation of TSWV RNA, as described in Chapter 2.

Gel electrophoresis. *In vitro* translation products or immunoprecipitates of these products with anti-TSWV immunoglobulins, obtained in a procedure which has been described in details in Chapter 2, were analysed by electrophoresis on 7.5 to 15% linear polyacrylamide gels (14 cm long, 1.5 mm thick) according to the method of Laemmli (1970).

Infectivity tests. The infectivity of virus within infected leaves of *N. glutinosa* was determined by inoculating detached leaves of *Petunia hybrida* cv. Pink Beauty with sap from infected leaves.

4.3 RESULTS

The development of infection

The aim of this study was to analyse the polypeptides synthesized under the regime of polyribosomes isolated from plants infected with the normal and defective forms of TSWV. One might expect that viral mRNA occurs in its highest concentration at the moment that the virus rapidly multiplies.

To get information on the time the virus multiplies, I studied the infectivity of primarily inoculated *N. glutinosa* leaves. The infectivity of these leaves was tested on *Petunia hybrida* leaves. The results, not shown in here, indicated that the first virus could be detected approx. 75 h after inoculation and that after 2 days the virus titer did not increase anymore. The normal form as well as the defective form gave the same growth response. On the basis of these results I isolated polyribosomes from *N. glutinosa* leaves infected 75 to 120 h before, in order to study their capacity to synthesize viral membrane proteins.

Polyribosomes from healthy and infected leaves

A typical polyribosome profile obtained in an isokinetic sucrose gradient, is shown in Fig. 1. The position of the 40S and 60S subunits in the gradient was identified by dissociation of the ribosomes by 50 mM-EDTA added before the centrifugation. The position of the 80S monosome peak has been identified after treating a polyribosome suspension with 10 µg/ml RNase A before centrifugation; this mild treatment degrades the mRNA molecules and produces free ribosomes.

No significant differences could be detected between the polyribosome profiles from leaves infected with the normal and defective form, irrespective of the age of the infection. Furthermore, the polyribosome profiles from healthy and infected leaves were the same.

The profile presented in Fig. 1 shows that no degradation has occurred during the isolation procedure. This implies that the polyribosomes should be active in the translation system.

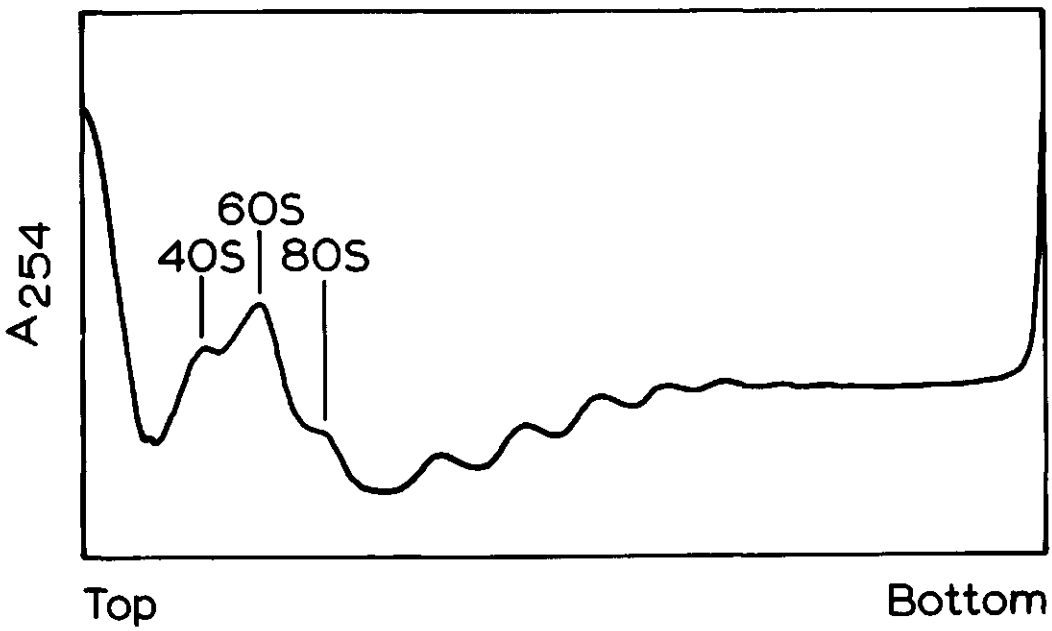


Fig. 1. A 254 nm absorption profile of a sucrose gradient with a polyribosome extract from N. glutinosa leaves isolated one day after inoculation with TSWV-B21. The extract was centrifuged through a 15 to 33.5% isokinetic sucrose gradient for 75 min at 41 000 rev/min in a Beckman SW 41 rotor.

Analysis of the translation products

The proteins, synthesized under the direction of polyribosomes, were analysed by electrophoresis on a 7.5 to 15% polyacrylamide gel. Fluorography of the gel (Fig. 2) shows that the polyribosomes isolated from leaves 60 h after inoculation with TSWV-B21 (normal form; lane 1) and from leaves 45 h after inoculation with TSWV-SNN (defective form; lane 5) did not give rise to the synthesis of proteins which were not synthesized under the direction of polyribosomes from healthy leaves (lane 4).

Later in the infection the polyribosomes from TSWV-B21 and TSWV-SNN infected leaves supported the synthesis of two unique polypeptides (lane 2 and 6). According to the molecular weight markers one

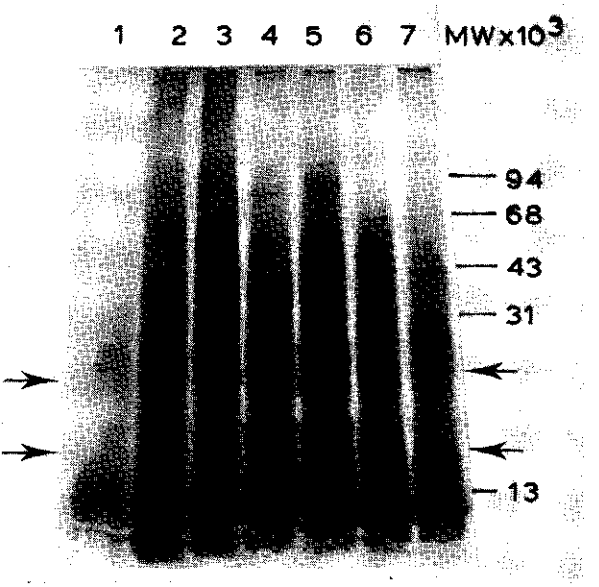


Fig. 2. Electrophoresis on a 7.5 to 15% linear polyacrylamide gel of the translation products synthesized under the direction of polyribosomes isolated from N. glutinosa leaves 60 h (lane 1), 84 h (lane 2) and systemically infected with TSWV-B21 (lane 3); healthy (lane 4); 45 h (lane 5), 93 h (lane 6) and systemically infected with TSWV-SNN (lane 7). Molecular weight markers are as described in Chapter 2. Arrows indicate positions of unique polypeptides.

protein had a mol. wt. of approx. 22 000 and comigrated with the nucleocapsid protein of TSWV. The other protein had a mol. wt. of approx. 14 000. These proteins were also synthesized under the direction of polyribosomes isolated from systemically infected leaves (lane 3 and 7).

In order to identify in another way virus specific proteins among the translation products, these products were incubated with immunoglobulins against TSWV particles. Upon analysis of the immunoprecipitates by gel electrophoresis, no any protein could be detected.

4.4 DISCUSSION

This study was initiated with the objective to examine the presence of mRNAs coding for viral membrane proteins in leaves infected

with the defective form. We do not known in which form the mRNA coding for these proteins is present within the infected cells. The synthesis of the coat protein of TMV in an infected cell proceeds via a subgenomic mRNA (Beachy & Zaitlin, 1975); the same mechanism applies for the synthesis of the coat protein of AlMV (Bol et al., 1976). The coat protein of CPMV is synthesized in another way. The two RNAs of the virus are translated into two large precursor polypeptides which are processed to several proteins, one of them being the coat protein (Stuik, 1979).

Apart from the lack of knowledge which mechanism applies for the synthesis of the membrane proteins of TSWV, the approach of studying possible losses of genetic information of the defective form by *in vitro* translation of polyribosomes and analysis of the translation products, has clearly failed. Even in leaves infected with the normal form I did not get evidence for the presence of mRNAs coding for the membrane proteins. Only the presence of a putative messenger activity for the nucleocapsid protein could be demonstrated in leaves infected with both forms.

It is not likely that this failure is due to the isolation procedure of polyribosomes. The polyribosome profile gives no indication of mRNA degradation. The use of high salt (400 mM-KCl) and Triton X-100 can be regarded as a guarantee that the membrane bound polyribosomes, which probably contain mRNAs coding for membrane bound proteins, were solubilized.

The amount of viral mRNA in a preparation of mRNA from infected plants is relatively low, as compared with the amount of viral mRNA in synchronically infected animal cells. Milner & Jackson (1979) have calculated by means of hybridization studies, that in leaves infected with SYNV, a negative stranded plant rhabdovirus, 1.5 to 4.6% of the poly-(A) containing mRNA is of viral origin. Of course, I admit that these values do not apply to the mRNAs of TSWV infected leaves, but I am convinced that they are indicative of what one could expect.

Nevertheless, in spite of its probably low concentration, the presence of the viral mRNA coding for the nucleocapsid protein could readily be demonstrated, for the nucleocapsid protein was visible among the translation products. Apparently, the corresponding concentration of mRNA coding for the viral membrane proteins was

much lower, because the polypeptide pattern of translation products synthesized under the direction of polyribosomes from healthy and infected leaves was exactly the same except for the nucleocapsid protein and a protein with a mol. wt. of 14 000.

In Chapter 2 I argued that the relative low amounts of RNA 1 and RNA 2 in TSWV particles may be the cause that no viral membrane proteins are synthesized in the *in vitro* translation of TSWV RNA. RNA 3, which contains information for the nucleocapsid protein, is the major RNA segment and acts perhaps also as a competitor for RNA 1 and RNA 2 in the *in vitro* translation. One might imagine that the same situation applies for the mRNAs associated with the polyribosomes.

Supposing that the viral mRNAs coding for the membrane proteins were present and were also with fidelity translated in the wheat germ cell-free system, the failure to detect these proteins can also be attributed to the quality of the antisera.

From Chapter 2 it is clear that the antiserum against TSWV is rather weak, as it had great difficulty to precipitate the nucleocapsid protein from the translation products which were synthesized under the direction of TSWV RNA alone. It was not possible to precipitate any other polypeptide. So it is probable that this antiserum was not able to precipitate the nucleocapsid protein out of a pool of translation products which were for the greatest part synthesized under the direction of host mRNAs. If this is the correct interpretation of the failure to precipitate the nucleocapsid protein by anti-TSWV serum, it is likely that this holds also for the viral membrane proteins. These, as discussed, may occur in even lower amounts as the nucleocapsid protein.

5 HYBRIDIZATION OF [^{32}P]-LABELLED TSWV RNA SEGMENTS WITH dsRNA FROM PLANTS INFECTED WITH THE NORMAL AND DEFECTIVE FORM

5.1 INTRODUCTION

In Chapter 3 I presented evidence that in cells infected with isolates of the defective form no viral membrane proteins are synthesized; especially protein 4, the most exposed protein (Tas et al. 1977a), is probably not synthesized. The genomes of these isolates may have lost the information coding for the membrane proteins or an indispensable part of it.

The replication of viral single stranded (ss) RNA proceeds through the synthesis of complementary strands in infected cells. It is generally believed that replicative intermediates play a role in the replication of viral RNA. They are thought to be composed of minus strands which are partly covered by nascent plus strands (Ralph, 1969). Although many investigators hold the view that double stranded (ds) RNA structures do not really exist in the cell and are mainly artefacts of the isolation procedure, dsRNA has proven to be useful in the elucidation of base sequence homology between RNA segments of multi-component viruses. Also, dsRNA has been widely used in the study of base sequence homology between different virus strains containing ssRNA as their genome.

Two different sized dsRNA species could be isolated from plants infected with CPMV a two-component virus, suggesting that for each viral RNA segment a corresponding dsRNA species exists (Van Griensven & Van Kammen, 1969; Van Griensven et al., 1973). The labelled viral RNA segments hybridized independently and without competition with dsRNA, indicating that the RNA segments have not the same base sequences (Van Kammen, 1971).

Bol et al. (1975) have carried out a comparable study on the genome of AlMV, a three-component virus. By analysis on polyacrylamide gels, they could demonstrate the presence of four dsRNA spe-

cies in a distribution similar of that of the viral RNA segments. Competition hybridization experiments indicated that the viral RNAs 1, 2 and 3 contain unique base sequences, and that RNA 4 is completely homologous with RNA 3. In their report they also present evidence that extensive homology exists between the base sequences in the genomes of four different AlMV strains.

Palomar et al. (1977) have studied the genome of several strains of barley stripe mosaic virus. Depending on the strain, two, three or four viral RNA segments were detected by polyacrylamide gel electrophoresis. Heterologous hybridization between labelled single stranded viral RNA with dsRNA revealed that the genomes of all the strains studied contain the same amount of genetic information in spite of differences in the number of RNA segments. Also, an important finding was that the number of dsRNA species was always equal to the number of viral RNA segments.

The examples presented above suggest that a study of the dsRNA isolated from plants infected with the normal and defective form of TSWV may reveal differences in the genomes of the two forms. Analysis of the purified dsRNA by gel electrophoresis may directly demonstrate this difference. Hybridization experiments between the labelled three RNA segments of normal TSWV particles and dsRNA from plants infected with the defective form may elucidate whether or not the genome of the defective form has lost base sequences present in the three RNA segments.

5.2 MATERIALS AND METHODS

Isolates of TSWV used. In this study the isolate TSWV-Y, forming normal particles, and the isolate TSWV-P, a defective isolate, were used. The isolates were propagated in *N. rustica*.

Separation of the RNA segments. The purification of TSWV from *N. rustica* plants systemically infected with isolate TSWV-Y and the extraction of its RNA with phenol-SDS are described in Chapter 2. The three RNA segments were separated by electrophoresis on an agarose gel containing methylmercury according to the method of Bailey & Davidson (1976). An amount of 24 µg TSWV RNA was dissolved in 150 µl electrophoresis buffer containing 50 mM-boric acid, 5 mM-NaB_4O_7. 10 H_2O, 10 mM-Na_2SO_4 and 1 mM-EDTA; to the buffer were further added

10 mM-CH_3HgOH and 10% glycerol. The RNA solution was divided in six equal parts and electrophoresed in a horizontal 1% agarose slabgel (20 x 20 cm) containing 5 mM-CH_3HgOH in electrophoresis buffer during 4 h at 150 V and at room temperature. After electrophoresis the gel was stained for 30 min with a solution of 5 µg/ml ethidium-bromide and 1% β-mercapto-ethanol. The bands containing RNA were excised under ultraviolet illumination, wrapped in parafilm and stored at -20 °C.

The RNA was recovered from the agarose slices by the freeze-squeeze method (Thuring et al., 1975). Briefly, the frozen agarose slices were pulverized and the extruded solution containing RNA was saved. In order to increase the yield of RNA, the agarose slices were mixed with 200 µl demineralized water and quickly frozen with dry ice. The frozen agarose slices were again pulverized, the extruded solution was saved. The agarose slices were centrifuged for 10 min at 10 000 *g* and the supernatant was brought together with the already extruded solutions. The pooled solutions were dialysed against demineralized water and lyophilized.

Labelling of the TSWV RNA segments with ^{32}P. The RNAs were labelled *in vitro* at the 5' end with γ-[^{32}P]-ATP and T_4-polynucleotide kinase, essentially as described by Goldbach et al. (1978). The lyophilized RNA was dissolved in 50 µl kinase buffer containing 15 mM-Tris-HCl pH 7.6, 10 mM-Mg acetate and 10 mM β-mercapto-ethanol, agarose residues were removed by centrifuging the solution through glasswool (Davies & Verduin, 1980). To 25 µl of the cleared solution approx. 0.75 µl T_4-polynucleotide kinase (Boehringer) was added. After dissolving 40 µCi γ-[^{32}P]-ATP, which was dried in a stream of air, the reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding SDS to a final concentration of 0.5%.

The labelled RNA was recovered by passage of the reaction mixture over Sephadex G-50 with 1 x SSC as elution buffer. The volume of the pooled fractions containing the labelled RNA was approx. 0.5 ml. An amount of approx. 10 000 cpm per 10 µl was incorporated, 75 to 90% of which could be precipitated with TCA. These RNA preparations were directly used for hybridization studies.

Isolation of dsRNA. Leaves of *N. rustica*, systemically infected with TSWV-Y (normal form) or TSWV-P (defective form) were harvested 12 to 14 days after inoculation. Healthy leaves of the same age were also harvested. The leaves were stored after removal of the midribs at -80 °C. An amount of 100 g frozen leaf tissue was crushed with a pestle and a mortar, mixed with 100 ml buffer containing 0.1 M-glycine, 0.1 M-NaCl, 0.01 M-EDTA and 0.01 M-Na_2HPO_4 pH 9.5 and put in a Waring Blendor. After addition of 200 ml of water saturated phenol containing 10% (w/v) m-cresol and 0.1% (w/v) 8-hydroxy-quinoline, 100 ml of a chloroform-isoamylalcohol mixture (24:1), 10 ml 20% (w/v) SDS, 5 ml 20% (w/v) para-aminosalicylic acid, 4 ml β-mercapto-ethanol, 3 ml diethylpyrocarbonate and 3 ml 2-butanol, the leaf tissue was immediately blended at low speed for 1 min.

After a low speed centrifugation the water layer (about 150 ml) was collected and extracted with one volume of 2.5 M-KH_2PO_4, pH 8.1, and one volume of methoxy-ethanol, to remove polysaccharides (Ralph & Bellamy, 1964). The nucleic acid in the water layer (about 350 ml) obtained after a second low speed centrifugation, was precipitated with 0.33% (w/v) cetyl-trimethylammoniumbromide (Assink, 1973). The precipitate was collected by a centrifugation at 20 000 *g* for 20 min and washed three times with 70% ethanol containing 0.1 M-Na-acetate to converse the nucleic acid from a cetyl-trimethylammonium salt in a sodium salt. The precipitate was dried in a stream of air and dissolved in 5 ml 1 x SSC. The solution was dialysed overnight against 1 x SSC.

The insoluble material was removed by a centrifugation for 10 min at 10 000 *g*. The yield of nucleid acid in this phase of the isolation was approx. 40 mg per 100 g leaf tissue. After the addition of Mg-acetate to a concentration of 5 mM and DNase I (Sigma, DN-EP) to a concentration of 10 µg per mg nucleic acid the solution was incubated for 30 min at 25 °C and subsequently dialysed against 1 x SSC. To degrade ssRNA the solution was incubated with a mixture of RNase A (10 µg/mg RNA) and RNase T_1 (1 µg/mg RNA) for 30 min at 37 °C. To remove the DNase and RNase, SDS to a concentration of 0.5% and Pronase E twice of the amount of RNase and DNase used, were added. This suspension was incubated for 30 min at 25 °C and extracted with a phenol-cresol-hydroxyquinoline mixture. The nucleic acid

(dsRNA) was precipitated from the water layer with ethanol, 200 µg tRNA from *E. coli* (Boehringer, RNase free) was added as carrier.

The precipitated RNA was washed three times with 70% ethanol and finally dissolved in 300 µl 2 x SSC. This preparation of dsRNA was used in the hybridization experiments.

When this RNA was analysed on 1% agarose gels containing methylmercury only one band comigrating with tRNA was visible.

Hybridization. [^{32}P]-labelled TSWV RNA, dsRNA and 2 x SSC were mixed in a total volume of 110 µl in a 1.5 ml Eppendorf tube. Each tube contained 5000 to 7000 TCA precipitable cpm. The tubes were carefully closed and heated in a pressure cooker for 5 min at 120 °C. When the pressure inside the cooker had been reduced, the tubes were quickly transferred to an incubator at 68 °C. The hybridization mixtures used to measure the aspecific absorption were placed immediately at -20 °C. After an incubation overnight, each mixture was divided in two equal parts, mostly containing 50 µl. To each sample was added 25 µl *E. coli* tRNA (10 mg/ml in 2 x SSC). To one of these samples 25 µl of a solution of RNase A and RNase T_1 (40 µg/ml and 4 µg/ml respectively, in 2 x SSC) was added, to the other sample 25 µl 2 x SSC was added. The samples were incubated for 30 min at 37 °C. To precipitate the undegraded RNA 5 µl yeast RNA (10 mg/ml) and 300 µl 10% cold TCA were added. After an incubation for at least 30 min on ice, the TCA precipitable RNA was transferred to glass microfibre paper (GF/C, Whatman), washed subsequently with 20 ml cold 5% TCA, 10 ml cold 0.5% TCA, 10 ml cold ethanol and 10 ml aceton. The disks were dried and transferred into 7 ml Lupo-luma (Lumac, Amsterdam, The Netherlands). The radioactivity was counted in a Packard liquid scintillation spectrophotometer.

Every hybridization experiment was carried out in duplicate. All values were corrected for the aspecific absorption on the filter, on an average of approx. 50 cpm. Hybridization of the [^{32}P]-TSWV RNAs without dsRNA (self-annealing) amounted to an average of 30 cpm, without subtraction of the cpm originating from the aspecific absorption.

.3 RESULTS AND DISCUSSION

As depicted in Fig. 1 (A, B & C), the three [^{32}P]-TSWV RNA segents hybridized with dsRNA isolated from plants infected with SWV-Y (normal form) to a value of approx. 30%; they did not hybrize with dsRNA from healthy plants. [^{32}P]-TSWV RNA 1 and 3 hybridzed with dsRNA from plants infected with TSWV-P (defective form) o values which were comparable with those obtained in the hybridiation with dsRNA from plants infected with TSWV-Y (Fig. 1A & 1C); ^{32}P]-TSWV RNA 2 hybridized to a significant lower value with dsRNA rom plants infected with TSWV-P (Fig. 1B). These results indicate hat the genome of TSWV-P is composed of the same RNA segments 1 nd 3 as the genome of TSWV-Y, but not of the RNA segment 2, a part f which seems to be lost. However, because of the limited amount f experimental data and the incomplete knowledge of the genome of SWV, this conclusion must necessarily be tentative.

When hybridized with saturating amounts of dsRNA isolated from lants infected with TSWV-Y, one would expect that the three [^{32}P]-SWV RNA segments, extracted from purified TSWV-Y, would reach a ybridization value of 100%. However, the maximum value reached was 5% (Fig. 1C). One might imagine many reasons for this low value. or example, the dsRNA preparation did not contain all the base sequences complementary to those present in the viral RNA segments. nother possibility is, that the dsRNA molecules and maybe the ^{32}P]-RNAs too, have endured so many scissions, that they were too mall to form stable duplex molecules which were RNase resistant. specially the heating of the hybridization mixtures at 120 °C may ave drastically affected the size of the RNA molecules. Wengler et l. (1977) have shown that 19S dsRNA and 42S ssRNA of alphaviruses re degraded to molecules of the size of 4S by heating them 15 min t 120 °C.

Nevertheless, the most likely reason for this low hybridization lateau I think is that the dsRNA preparations are contaminated with egraded viral ssRNA. Bol et al. (1975) found a hybridization value f 45% when intact [^{3}H]-labelled AlMV RNA was hybridized with intact irus specific dsRNA; by extrapolating the results to infinite dsRNA oncentration a value of 60% was obtained. These authors have separated the degraded viral ssRNA as much as possible from the dsRNA by

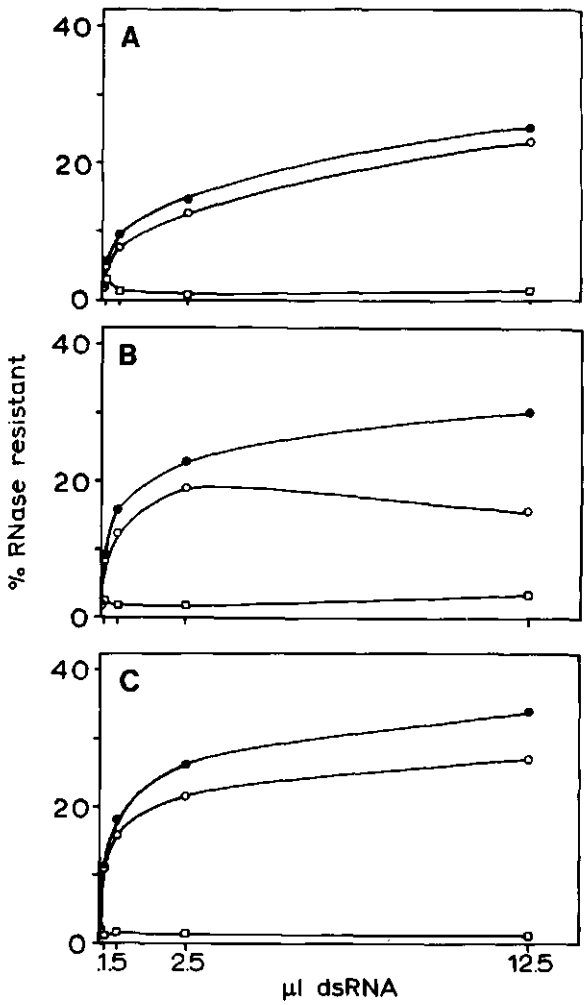


Fig. 1. Hybridization of [^{32}P]-TSWV-RNA segments with dsRNA isolated from plants infected with TSWV-Y (●-●) and TSWV-P (o-o) and with dsRNA from healthy plants (□-□). A. RNA 1. B. RNA 2. C. RNA 3.

a centrifugation over a sucrose gradient. Since I have omitted such a step in the purification of the dsRNA, one may assume that the dsRNA preparations were contaminated with degraded single stranded TSWV RNA, which competed with the labelled TSWV RNA in the hybridization.

As I have noted in the Material and Methods section, analysis of he dsRNA by electrophoresis on a methylmercury-agarose gel showed nly tRNA which was added as carrier. It is not probable that in-ected plants contained such small amounts of virus specific dsRNA, hat no detectable amounts could be purified from 100 g leaf mate-ial. Staining with ethidium-bromide is sensitive enough to detect .075 µg dsRNA in one band (Moussa, 1980). Bol et al. (1975) iso-ated from 1 kg tobacco leaves infected with AlMV 40 µg virus spe-ific dsRNA; Assink (1973) obtained 600 µg dsRNA from 1 kg cowpea eaves infected with CPMV. It is therefore more likely that the sRNA isolated from TSWV infected plants was not homogeneous, but onsisted of a polydisperse population of molecules. In this context he observation of Rezaian & Francki (1973) is of interest. They ere not able to purify high molecular weight dsRNA from plants in-ected with tobacco ringspot virus, although they applied a proce-ure which did not include the use of RNase. The small sized dsRNA hich they isolated hybridized nonetheless with viral ssRNA.

Another complicating factor in the experiments was that the pu-ity of the TSWV RNA segments could not be checked, since the RNAs, hen analysed on methylmercury-agarose gels, appeared to be de-raded. The rather rough method of recovery of the RNA from the garose slices, originally set up for the extraction of DNA, was robably responsible for that degradation. However, the gel system f agarose containing methylmercury as denaturing agent is used by any investigators to achieve pure RNA preparations. For example, elham (1979) has separated on a preparative agarose gel containing ethylmercury the RNA segments of tobacco rattle virus.

We do not know if some sequence homology exists between the RNA egments of TSWV. With this lack of knowledge in mind, the conclu-ions from the hybridization experiments now can be stated more recisely. The genome of TSWV-P (defective form) contains most, if ot all of the unique nucleotide sequences present in RNA 1 and RNA of TSWV-Y (normal form). It does not contain all the sequences hich are present in RNA 2 of TSWV-Y and has probably lost a part f RNA 2. The defective form has lost the ability to synthesize embrane proteins. As a consequence, the hypothesis seems valid hat the information for the membrane proteins is on RNA 2.

6 PURIFICATION OF NUCLEOCAPSIDS FROM LEAVES INFECTED WITH DEFECTIVE FORMS AND ANALYSIS OF THEIR RNA

6.1 INTRODUCTION

In Chapter 3 I indicated that in leaves infected with defective forms, the membrane proteins of TSWV could not be detected by means of ELISA. Hybridization studies (Chapter 5) showed that the base sequences of RNA 1 and RNA 3 and partly of RNA 2 of normal TSWV particles (TSWV-Y) could be detected in leaves infected with the isolate P, a defective form of TSWV.

Since no viral membrane proteins could be detected in leaves infected with defective forms, it can be assumed that the infectious component of these forms consists of naked nucleocapsids. Purification of these nucleocapsids from infected leaves and analysis of their RNA should reveal the RNA composition of the defective forms of TSWV.

6.2 MATERIALS AND METHODS

Isolates of TSWV used. In this study three isolates of the defective form, TSWV-P, TSWV-SNN and TSWV-12, were used. Each of the three isolates were propagated in *N. rustica*.

Purification of nucleocapsids. Systemically infected leaves were harvested 12 to 14 days after inoculation and midribs were removed. An amount of 25 g leaf material was homogenized in 100 ml 0.1 M-Tris-HCl, pH 8.0, containing 0.01 M-Na_2SO_3 and the homogenate was centrifuged for 10 min at 10 000 *g*. The supernatant was centrifuged for 30 min at 110 000 *g* and the pellet thus obtained was resuspended in a small volume of buffer of 0.01 M-Tris-HCl, pH 8.0, containing 0.01 M-Na_2SO_3. The suspension was subjected to a rate zonal centrifugation on a 1 to 20% linear sucrose gradient made in resus-

pension buffer for 30 min at 27 000 rev/min in a Beckman SW 27 rotor. Occasionally, the absorption profile of the gradient at 254 nm was recorded by means of a Uvicord spectrophotometer and fractions were collected. Mostly frequently, however, the light scattering band on the top of the gradient was collected and one-tenth volume of 2 M-NaCl and one-twenth volume of 20% NP-40 were added. This suspension was subjected to a second rate zonal centrifugation on a 25 to 55% linear sucrose gradient made in resuspension buffer containing 0.5 M-NaCl, for 16 h at 23 000 rev/min in a Beckman SW 27 rotor. The absorption profile of the gradient at 254 nm was recorded and fractions were collected. When not immediately used, these fractions were stored at -20 °C.

ELISA. The fractions of the sucrose gradients were assayed on the presence of nucleocapsid protein and membrane proteins of TSWV by means of ELISA. The procedure of ELISA has been previously described in details (Chapter 3). An amount of 200 µl was analysed of each fraction. The coating and the conjugate concentration of the anti-N immunoglobulins was 2 µg/ml. These concentrations were 10 µg/ml and 4 µg/ml for the anti-G immunoglobulins. All tests were done in duplicate.

SDS-polyacrylamide gel electrophoresis. The protein composition of each fraction of the sucrose gradients was determined by analysis of samples of 25 µl on 10% polyacrylamide slabgels according to the method of Laemmli (1970).

Infectivity tests. The infectivity of the fractions was analysed by local lesion assay on *Petunia hybrida* cv. Pink Beauty. Four half detached *Petunia* leaves were each inoculated with one droplet of the fractions to be tested. The leaves were incubated on moist filter paper in Petri dishes at 18 °C in continuous light. Local lesions were counted after the third day of inoculation.

Electron microscopy. The 10 000 *g* and 110 000 *g* pellets and the fractions of the sucrose gradients containing nucleocapsid material were examined by electron microscopy. After the addition of egg-white the nucleocapsid fractions were centrifuged for 2 h at 45 000

rev/min in a Beckman SW 50 rotor. The procedures of fixation, embedding and ultra-sectioning of the pellets were essentially as described by Ie (1971). The sections were examined with a Siemens Elmiskop-101 electron microscope.

Extraction and analysis of RNA. The fractions containing the nucleocapsid material were centrifuged during 2 h at 40 000 rev/min in a Beckman SW 41 rotor and the pellets were resuspended in 1 ml resuspension buffer. The RNA was extracted from this suspension by means of phenol-SDS. The RNA was precipitated by the addition of 2.5 volumes of absolute ethanol and 0.1 volume of 3 M-Na-acetate, pH 5.5. After an incubation overnight at -20 °C, the RNA was concentrated by centrifugation, washed three times with 70% ethanol, dried and dissolved in a small volume of double distilled water. The RNA was analysed by electrophoresis on a 1% agarose gel containing 10 mM-methylmercury as described by Bailey & Davidson (1976).

6.3 RESULTS

Infectivity of fractions from leaf homogenates

The infectivity of purified fractions of leaf homogenates from plants infected with TSWV-P, a defective form, might indicate how the nucleocapsids are distributed over the different fractions.

Table 1. Infectivity of different fractions of homogenates of leaves infected with TSWV-P as measured by the number of local lesions on detached leaves of *Petunia hybrida* cv. Pink Beauty.

Fraction of the homogenate	Experiment 1	2	3	4
10 000 *g* pellet	20	14	7	1
110 000 *g* pellet	NT*	37	51	76
supernatant of 10 000 *g* centrifugation	62	48	22	NT
supernatant of 110 000 *g* centrifugation	NT	5	30	13

*NT: not tested.

Table 1 shows the infectivity of different fractions obtained in four experiments. Most of the infectivity was found in the 110 000 *g* pellet and the 10 000 *g* supernatant. The purification of the nucleocapsids was continued from the 110 000 *g* pellet.

Electron microscopic examination of the different fractions from leaf homogenates

The 10 000 *g* and 110 000 *g* pellets of leaf homogenates containing the nucleocapsids of the defective form were analysed by electron microscopic examination of ultrathin sections of these pellets. The 10 000 *g* pellets contained among various fragments of cell organelles great clusters of the typical electron dense amorphous masses (Fig. 1A). These were similar in structure and density as the clusters seen in ultrathin sections of leaves infected with the defective form (Fig. 1A in Chapter 1). These clusters were, although less extensive, also observed in the 110 000 *g* pellets (Fig. 1B).

If it is assumed that the electron dense amorphous masses are formed by the infectious agent of the defective form, the presence of more electron dense masses in the 10 000 *g* pellets than in the 110 000 *g* pellets contradicts in some degree the infectivity of these two fractions. The 110 000 *g* pellets were more infectious than the 10 000 *g* pellets (Table 1). The low infectivity of the 10 000 *g* pellets, in spite of the presence of clusters of the electron dense amorphous masses, might be explained by the contaminating fragments of the cell which inhibit the infectivity of the nucleocapsids.

Analysis of the sucrose gradients

The 110 000 *g* pellets were subjected to two subsequent sucrose gradient centrifugations. The result of the first centrifugation, on a 1 to 20% sucrose gradient, is depicted in Fig. 2A. Most of the ultraviolet light absorbing material, which was highly infectious, was found on the top of the gradient. When assayed with ELISA, the fractions of the sucrose gradient did not show a significant reaction with the anti-G immunoglobulins, but a strong reaction with the anti-N immunoglobulins; although all fractions reacted with the

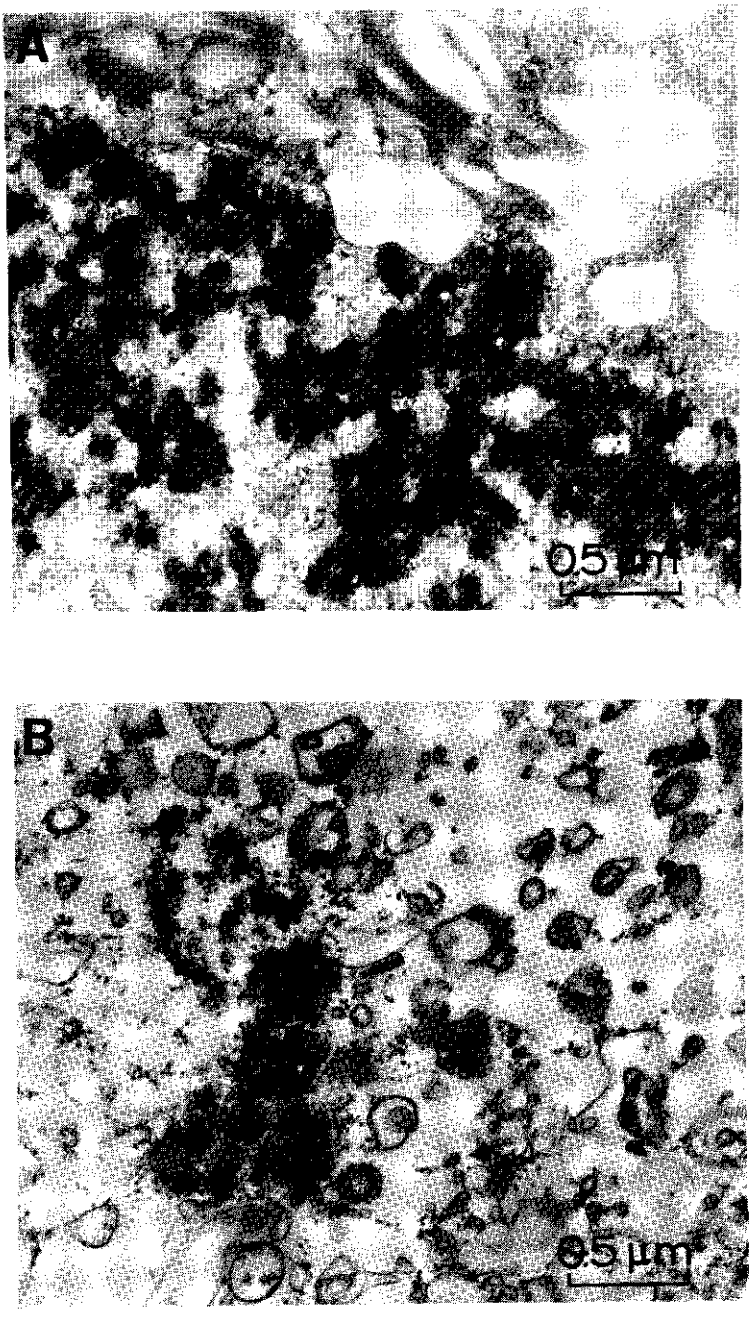


Fig. 1. Electron micrographs of 10 000 g pellet (A) and 110 000 g pellet (B) of homogenate of leaves infected with TSWV-P.

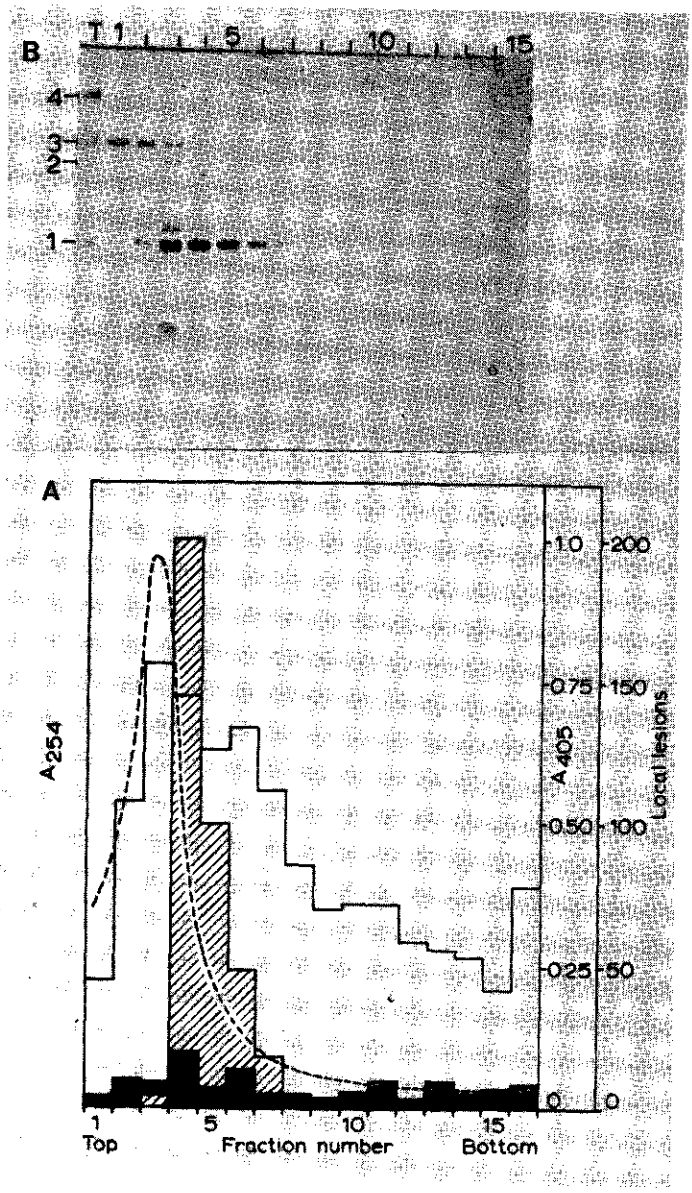


Fig. 2. Analysis of 110 000 g pellet of homogenate of leaves infected with TSWV-P on a 1 to 20% linear sucrose gradient. (A). Broken line: absorption profile at 254 nm of the gradient. White area under the solid line: absorption at 405 nm in ELISA in which anti-N immunoglobulins were used. Black area: absorption at 405 nm in ELISA in which anti-G immunoglobulins were used. Hatched area: infectivity (local lesions). (B). Gel electrophoresis of the fractions of Fig. 2A. Lane T: proteins of complete TSWV particles. Lane 1 to 15: fractions of the gradient.

anti-N immunoglobulins, those containing the ultraviolet light absorbing and infectious material did show the strongest reaction.

The fractions were also analysed by electrophoresis on polyacrylamide gels (Fig. 2B). The fractions containing material of which it was thought to consist of nucleocapsids, did contain considerable amounts of the nucleocapsid protein. From this figure it can also be concluded that the purification procedure followed so far, has resulted in a rather pure preparation of infectious nucleocapsids.

To acquire a preparation of nucleocapsids of still greater purity, the ultraviolet light absorbing and infectious fraction of the 1 to 20% sucrose gradient was subjected to an overnight centrifugation in a 25 to 55% sucrose gradient, containing 0.5 M-NaCl, after the addition of 0.1% NP-40 to the nucleocapsid suspension. The result of this centrifugation is depicted in Fig. 3A. The fractions containing the ultraviolet light absorbing material in the lower part of the gradient (fraction 9 to 14) did not show a reaction with the anti-G immunoglobulins in ELISA but a strong reaction with the anti-N immunoglobulins. Furthermore, by analysis on polyacrylamide gels, these fractions did contain huge amounts of nucleocapsid protein and relatively few contaminating protein (Fig. 3B). When tested for infectivity, none of the fractions of the 25 to 55% sucrose gradient appeared to be infectious.

The fractions containing nucleocapsids in both gradients were also studied by electron microscopic examination of ultrathin sections made after pelleting of these fractions. Fig. 4A is an electron micrograph of a nucleocapsid fraction of the 25 to 55% sucrose gradient. Clusters of the electron dense amorphous masses, typical for the defective form of TSWV, were visible, and the material appeared to be rather pure. The resemblance in structure, as seen in the electron micrographs, of the purified nucleocapsids and the electron dense amorphous masses suggests that these masses are indeed aggregates of nucleocapsids. In a picture at a greater magnification (Fig. 4B) one might consider the lightly coloured parts of the structures as nucleocapsids which are partly unrolled, while the darkly coloured parts can be considered as more tight aggregates of nucleocapsids.

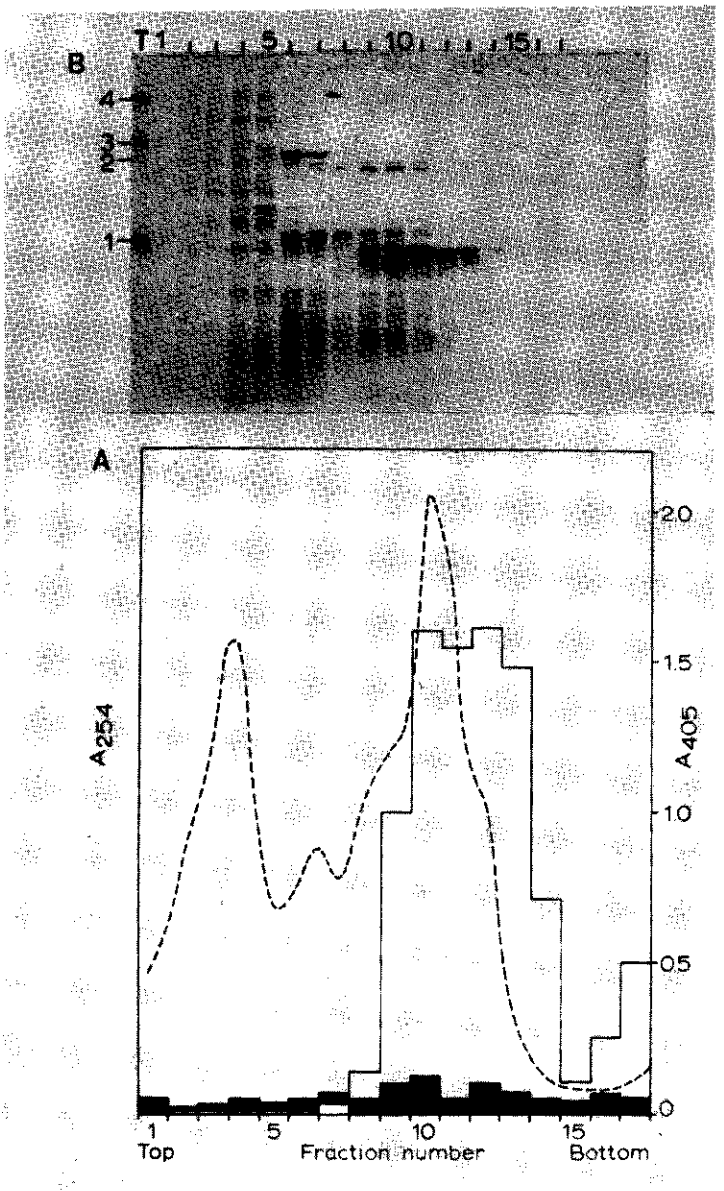


Fig. 3. Analysis of fractions 2 to 7 from the 1 to 20% sucrose gradient (Fig. 2A) after centrifugation on a 25 to 55% linear sucrose gradient. (A). Absorption profile at 254 nm of the gradient and reaction in ELISA of the fractions, according to the legends for Fig. 2A. (B). Gel electrophoresis of the fractions of Fig. 3A. Lane T: proteins of complete TSWV particles. Lane 1 to 17: fractions of the gradient.

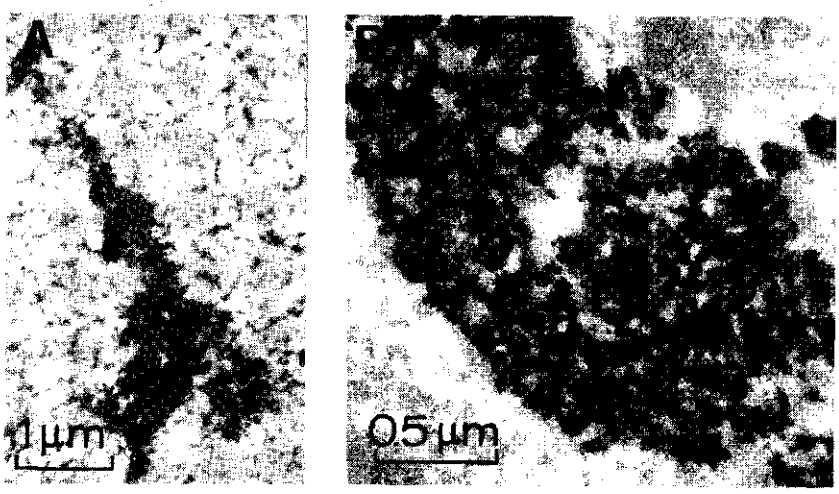


Fig. 4.(A). Electron micrograph of the pellet obtained by centrifuging fraction 10 to 12 of the 25 to 55% sucrose gradient represented in Fig. 3. (B). Electron micrograph of the pellet obtained by centrifuging fraction 2 to 7 of the 1 to 20% sucrose gradient represented in Fig. 2.

RNA composition of the nucleocapsids

The RNA extracted from the purified nucleocapsids was analysed by electrophoresis on methylmercury-agarose gels. The RNA from nucleocapsids of TSWV-P, a defective form, appeared to have three RNA segments like normal particles (TSWV-Y). RNA 1 and RNA 3 seemed to have the same size, whereas RNA 2 was smaller; this new RNA segment had a mol. wt. of approx. 1.4×10^6, when the RNAs of TSWV-Y and CPMV (not present in this gel) were used as markers (Fig. 5 lane 1, 2 and 3.

The RNA composition of the nucleocapsids of the two other isolates of the defective form was also analysed by gel electrophoresis. RNA 1 of the nucleocapsids from both isolates had the same mol. wt. as RNA 1 of normal particles, while changes were observed for RNA 2 and RNA 3 (Fig. 5 lane 4 and 5).

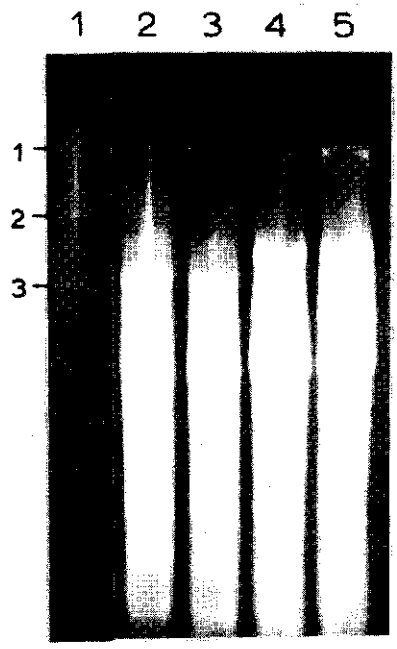


Fig. 5. Gel electrophoresis of RNA extracted from TSWV-Y particles and from nucleocapsids of defective isolates. Lane 1: TSWV-Y RNA. 2. TSWV-P RNA from nucleocapsids of 1 to 20% sucrose gradient. 3. TSWV-P RNA from nucleocapsids of 25 to 55% sucrose gradient. 4. TSWV-SNN RNA from nucleocapsids of 1 to 20% sucrose gradient. 5. TSWV-12 RNA from nucleocapsids of 1 to 20% sucrose gradient.

RNA 2 of the nucleocapsids of the isolate SNN (lane 4) appeared to be smaller than RNA 2 of the normal particles (lane 1), but also smaller than RNA 2 of isolate P (lane 2 and 3). A mol. wt. of 1.2 x 10^6 was found. Moreover, this segment occurred in relative high amounts. A third RNA segment with the same mol. wt. as that of RNA 3 of normal particles could only be observed in small amounts.

The RNA composition of the nucleocapsids of the isolate 12 (lane 5) resembled that of the isolate P (lane 2 and 3). RNA 2 had a lower mol. wt., approx. 1.3 x 10^6.

In all the RNA preparations of the purified nucleocapsids a smear of lower mol. wt. RNA was visible. Probably this material is degraded viral RNA and contaminating ribosomal RNA.

6.4 DISCUSSION

The electron micrographs of purified and infectious nucleocapsids isolated from leaves infected with defective forms of TSWV suggest that the electron dense amorphous masses are aggregates of nucleocapsids and represent the infectious agent of the defective forms. The structures seen in the electron micrographs resemble the nucleocapsids which are present in cells infected with measles virus (Robbins et al., 1980) and other paramyxoviruses (Choppin & Compans, 1975). Recently, Mohamed (1981) examined nucleocapsids from purified normal TSWV particles in the electron microscope. He observed threadlike structures, some of which appeared to be coiled. Similar structures might be identified in our preparations (Fig. 4B).

The purified nucleocapsids were no longer infectious after an overnight centrifugation. The infectious nucleocapsid fraction did not contain other proteins than the nucleocapsid fraction of the second gradient which was no longer infectious (Fig. 2B and 3B). Therefore, this loss of infectivity cannot be ascribed to the loss of an essential protein. Also, RNase activity is not likely to be the cause of the loss of infectivity, because the RNA composition of infectious and non-infectious nucleocapsids were exactly the same (Fig. 5 lane 3 and 4). It seems likely that the addition of NP-40 is responsible for the inactivation of the nucleocapsids in the second gradient.

The RNA of the normal form of TSWV is composed of three segments. Although one might expect that the normal form contains three nucleocapsids of different size too, we have not observed a distinct heterogeneity in size in the analysis of the sucrose gradients used to separate the nucleocapsid from the membrane proteins of dissociated virus particles (Chapter 3). However, as already mentioned, Mohamed (1981) recently demonstrated the presence of three different sized nucleocapsids in normal TSWV particles.

The absorption profiles of the sucrose gradients used to purify the nucleocapsids from the defective forms of TSWV give no evidence for the presence of different sized nucleocapsids. Therefore, correlation of the differences in RNA composition between the normal and defective forms with differences in nucleocapsid composition between the forms is not possible.

The nucleocapsids purified from the three defective isolates of TSWV studied contained three RNA segments. Two of them, RNA 1 and RNA 3, appeared to have the same mol. wt. as RNA 1 and RNA 3 of normal TSWV particles. RNA 2 in the nucleocapsids of the defective isolates was smaller than RNA 2 of normal TSWV particles. It is tempting to assume that RNA 1 and RNA 3 are the same segments as can be found in normal particles of TSWV and that RNA 2 is a part of RNA 2 of normal particles. This assumption is reinforced by the results of the hybridization experiments (Chapter 5), which have shown that in plants infected with the defective isolate TSWV-P, all the nucleotide sequences of RNA 1 and RNA 3 and a part of the nucleotide sequences of RNA 2 of TSWV-Y (normal form) can be detected.

7 GENERAL DISCUSSION

Objectives

The aim of this investigation was to elucidate the nature of the defect in TSWV giving rise to the defective form, by analysis of virus specific proteins and RNA in infected plants.

The experiments described in Chapter 3 show that no viral membrane proteins could be detected in plants infected with defective isolates. Nucleocapsid protein could readily be demonstrated in plants infected with either normal or defective isolates. These findings indicate that the defective isolates are able to direct the synthesis of the nucleocapsid protein but not that of the membrane proteins.

In order to analyse the genetic information of the defective form, polyribosomes from infected plants were allowed to complete nascent polypeptide chains *in vitro* and the translation products were analysed by gel electrophoresis. As described in Chapter 4, only the synthesis of the viral nucleocapsid protein, directed by polyribosomes from plants infected with isolates of either forms, could be demonstrated. The synthesis of other virus specific proteins could not be detected. Thus, the approach described in this chapter was unsuccessful.

The results of the hybridization of the individual TSWV RNA segments with dsRNA indicate that the defective isolate studied has conserved the nucleotide sequences present in RNA 1 and RNA 3 of the normal isolate, but has lost a part of the sequences in RNA 2 (Chapter 5).

I have further purified an infectious fraction from plants infected with several defective isolates (Chapter 6). This fraction contained structures which resembled the characteristic electron dense amorphous masses in infected cells. Analysis by gel electrophoresis revealed the occurrence of rather homogeneous nucleocapsid

protein in this fraction. These results indicate that the amorphous masses are aggregates of nucleocapsids, as originally suggested by Ie (1971).

Analysis of the RNA in the purified infectious fraction by gel electrophoresis showed that all the defective isolates studied contained RNA 1 and RNA 3 with the same mol. wt. as those of normal TSWV particles. However, RNA 2 was much smaller, a defect already indicated by the results of the hybridization experiments.

In conclusion, this study attributes to the understanding of the nature of the defect in TSWV, giving rise to the defective form. The defective form has lost the ability to direct the synthesis of membrane proteins and in its genome it has lost a part of RNA 2 of the normal form.

Genes on the TSWV RNA segments

The RNA composition of the genomes of the defective isolates allows me to make some speculations on the genetic information present in the three RNA segments of normal TSWV particles.

In discussing the *in vitro* translation experiments (Chapter 2), I suggested that all the unique genetic information is not restricted to RNA 1, but that the other RNA segments also contain unique sequences. Since the defective isolates have lost a great part of RNA 2 and at the same time the ability to direct the synthesis of membrane proteins, it is possible that RNA 2 contains the genetic information for these proteins.

The *in vitro* translation experiments have shown that RNA 3 contains information for the nucleocapsid protein. Excluding the possibility that the nucleotide sequence coding for this protein is also present in the other RNA segments, this result suggests that the defective isolates which form infectious nucleocapsids should contain RNA 3. Although the relative amounts of this RNA segment differ greatly, this argument seems to be valid for the defective isolates studied (Chapter 6, Fig. 5).

All the three defective isolates have conserved RNA 1 of the normal form. If the information for the viral structural proteins is on RNA 2 and RNA 3, as outlined above, RNA 1 may contain information indispensible for the multiplication of TSWV, whether or not

defective. In view of the positive strandness of the RNA of TSWV, a replicase could be a likely candidate to be encoded for by this RNA.

The bunyaviruses resemble TSWV in many respects. These animal viruses are enveloped spherical particles which measure approx. 100 nm in diameter. They are formed by budding from the Golgi system and the endoplasmic reticulum, and not from the plasma membrane as is the case for most enveloped animal viruses. We may expect that enveloped plant viruses (TSWV, rhabdoviruses) are formed in a similar way. The bunyaviruses are biologically transmitted by insects (mosquitoes). Their genomes are composed of three RNA segments with unique lengths; the molecular weights of these segments lie within the range of those of TSWV (Obijeski & Murphy, 1977).

In addition to the above mentioned properties TSWV resembles also the bunyaviruses when the genetic information of TSWV is distributed over its three RNA segments as I have described. It has been found that RNA S (small) of the bunyaviruses codes for the nucleocapsid protein, RNA M (middle) for the two membrane proteins and RNA L (large), presumably, for the virus associated transcriptase (Obijeski & Murphy, 1977).

The essential element is that the bunyaviruses do contain negative stranded RNA. In this respect TSWV seems to differ from the bunyaviruses, as presented in this thesis. However, this difference has such a great impact on the mode of viral RNA replication and transcription that it is ill-advised to consider TSWV as a plant bunyavirus.

Comparison of the defective form with other defective viruses

The defective form of TSWV cannot be regarded as a defective interfering particle (DI particle), since it is infectious and does not contain all the viral structural proteins. However, the defective form possesses a characteristic property of DI particles, namely their ability to increase in number of particles at the expense of the number of standard virus particles. After many passages of the normal form by mechanical inoculation, the electron dense amorphous masses, initially in minute amounts present, eventually outnumber the normal enveloped particles. The rate of conversion from

he normal form to the defective form is probably host-dependent. Ie personal communication) has observed that the number of complete articles decreases drastically and that the amorphous masses occur bundantly after passage of the normal virus through a series of ucumber plants. This process can take years in tobacco plants.

As has been described in Chapter 1, the molecular mechanism of he increase in number of DI particles at the expense of the number f standard virus particles is based upon a preferential replication f the DI particle RNA. Higher affinity sites for replicase mole- ules on the DI particle RNA and the relative small size of the DI article RNA may account for its replicative advantage.

The mechanism by which the defective form of TSWV supersedes the ormal form is not known. Perhaps particles of the defective form nucleocapsids) spread at a greater rate in the tissues of the lant than normal enveloped particles, because utilization of the ranslation machinery of the cells for the synthesis of the viral embrane proteins and assembly of nucleocapsids into mature virus articles are not required. This process may finally result in the omplete disappearance of the normal form of TSWV.

In this context it is remarkable that during the conversion of he normal form of TSWV to the defective form, normally enveloped SWV particles are found in close contact with the amorphous masses n a single cell (Ie, 1971). One might imagine that the membrane roteins coded by the normal form may also be used for assembling ucleocapsids of the defective form into enveloped particles. pparently, such a completion does not occur. It is possible that he synthesis and assembly of normal and defective virus particles ccur in different compartments of the cell. Alternatively, the mem- rane proteins produced by the normal form cannot be used by the de- fective form for assembling nucleocapsids of the latter in complete articles.

Depending on the virus-cell system electron micrographs of cells infected with paramyxoviruses sometimes show great aggregates of nucleocapsids (Choppin & Compans, 1975). In a later stage of devel- opment, the nucleocapsids mature in enveloped particles by budding from the plasma membrane.

The nucleocapsids of the measles-like SSPE virus cannot mature in enveloped particles because the M protein is not synthesized. As a consequence, spread of this defective virus to other cells can only occur by means of its strong cell-fusion activity, by which great syncytiae are formed.

The defective form of TSWV resembles SSPE virus in that it is composed of aggregates of nucleocapsids and is able to multiply without help of the normal enveloped virus.

It is not likely that membrane bounded plant viruses, as TSWV, spread from cell to cell by budding from the plasma membrane. Thus, the failure of the defective form to produce normal enveloped particles will not affect the spread of the virus. This in contrast with SSPE virus, which has lost the ability to spread by budding from the plasma membrane; it can spread in tissues only by its strong cell-fusion activity.

As has been suggested by Ie (1982), it is unlikely that species of thrips transmit the defective form of TSWV, since the viral envelope would have disappeared during evolution. If this hypothesis is true, the two forms of TSWV are comparable with the two types of wound tumor virus and pea enation mosaic virus. A consequence of the hypothesis is that in the field thrips may serve as a natural sieve preventing the degeneration of the normal form of TSWV to the defective form.

In this view, the very nature of the defect in TSWV supports the assignment of a crucial role to the viral envelope in the transmission of TSWV by its vector. The study of Beaty et al. (1981) also illustrates the importance of the viral envelope for virus transmission. These authors investigated bunyaviruses, the genomes of which are reassortments between the RNA segments of two bunyaviruses, snowshoe hare (SSH) and La Crosse (LAC). LAC virus is easily transmitted by its natural mosquito vector, *Aedes triseriatus;* SSH cannot be transmitted by this mosquito species. It appears that reassortment viruses, which contain RNA L and S of LAC virus but RNA M of SSH virus can no longer be transmitted. Since RNA M codes for the viral membrane proteins, their results suggest that these proteins are essential for the transmission of bunyaviruses. The authors propose that the viral membrane proteins determine the maturation of

virus particles from the plasma membrane of the salivary gland cells of the mosquitoes.

If this situation also prevails for TSWV and its vector, it is easy to understand why the defective form of the virus would not be transmitted by thrips. Also, the very absence of a viral envelope may cause a rapid degeneration of the naked nucleocapsids in the midgut of the insect by which transmission will be made impossible.

SUMMARY

The work described in this thesis was aimed at the elucidation of the nature of a defective form of TSWV which does not form complete particles during infection.

Properties of TSWV and the existence of a defective form of this virus are described in Chapter 1. A survey of the literature on three different types of defective viruses with properties significant for the understanding of the defect in TSWV is given also in this chapter. The purposes of the study are presented at the end of this chapter.

In view of a defect in the genome of TSWV, experiments were performed to determine the exact number of RNA segments in normal TSWV particles and the coding function of each individual RNA segment (Chapter 2). Electrophoresis of TSWV RNA on denaturing agarose gels revealed three segments with mol. wts. of 2.7, 1.7 and 1.1 x 10^6, to which will be referred as RNA 1, 2 and 3.

TSWV RNA acted as messenger in a cell-free system of wheat germ and in a mRNA-dependent rabbit reticulocyte lysate, indicating that it is of a positive strand. Analysis of the *in vitro* translation products by gel electrophoresis revealed that two major polypeptides were synthesized in both systems. One could be indentified by immunoprecipitation as the nucleocapsid protein; the identity of the other polypeptide, having a mol. wt. of 60 000, is unknown.

The positive strandness of TSWV RNA could be confirmed by other experiments. TSWV RNA labelled with ^{125}I did not hybridize with polyribosomal RNA from infected leaves and transcriptase activity could not be detected in purified preparations of TSWV or in nucleocapsid extracts from infected leaves.

In vitro translation of the RNA of TSWV, fractionated on sucrose gradients, and analysis of the translation products revealed that the nucleocapsid protein and a protein with a mol. wt. of 60 000 is encoded for by RNA 3. The coding functions of the two other RNA segments could not be determined.

The viral proteins present in plants infected with defective isolates were analysed by serological means (Chapter 3). Antibodies were raised against the nucleocapsid protein and against a fraction containing the membrane proteins of TSWV. Sap from leaves infected with isolates either of the normal or the defective form were tested in ELISA. Antibodies directed against the nucleocapsid protein reacted with sap from leaves infected with isolates of both forms. Antibodies directed against the membrane proteins reacted only with sap from leaves infected with normal isolates and not at all with sap from leaves infected with defective isolates. These results indicate that the defective form does not direct the synthesis of membrane proteins and consists solely of nucleocapsids.

Chapter 4 and 5 describe the analysis of the RNAs which are synthesized in leaves upon infection with either the normal or defective form of TSWV. Polyribosomes were isolated from infected plants at different times of infection. They were allowed to complete nascent polypeptide chains in an *in vitro* translation system and the synthesized polypeptides were analysed by polyacrylamide gel electrophoresis. Synthesis of the nucleocapsid protein in the course of infection with isolates of both forms could be detected. However, synthesis of the other viral structural proteins or proteins other than the polypeptides synthesized under direction of polyribosomes from healthy plants, could not be observed (Chapter 4).

The RNAs synthesized upon infection were also analysed by hybridization (Chapter 5). The three individual TSWV RNA segments were labelled with ^{32}P and hybridized with dsRNA isolated from plants infected with isolates either of the normal or defective form. RNA 1 and RNA 3 hybridized to the same extent with dsRNA from plants infected with isolates of both forms. RNA 2 hybridized to a significant lower value with dsRNA from plants infected with the defective isolate. This result indicates that in plants infected with the defective isolate the genetic information of RNA 1 and RNA 3 of normal TSWV particles is present, but the information of RNA 2 is only partly present. Since the defective form does not direct the synthesis of membrane proteins, RNA 2 contains possibly the information for the membrane proteins.

Chapter 6 describes experiments on the nature of the electron dense amorphous masses, by which the defective form is defined electron microscopically. An infectious nucleoprotein-rich fraction was purified from plants infected with several defective isolates. Analysis by ELISA and polyacrylamide gel electrophoresis revealed the presence of nucleocapsid protein of TSWV in this fraction. No other viral structural proteins were found. Electron microscopic examination of this fraction demonstrated structures resembling the electron dense amorphous masses. As this fraction was infectious it is concluded that the characteristic amorphous masses are composed of nucleocapsids. Analysis by gel electrophoresis of RNA extracted from the fraction revealed that the three defective isolates of TSWV studied have an RNA 1 and RNA 3 with the same mobility as those of normal TSWV particles. RNA 2 was much smaller, indicating that RNA 2 of the normal form has lost a fragment. The same conclusion was also obtained in experiments in which the viral RNA segments were hybridized with dsRNA (Chapter 5).

The results of this study are discussed in Chapter 7. The nature of the defect in TSWV giving rise to the defective form is defined and the significance of the results in view of the coding functions of the three RNA segments of TSWV is discussed. Also, the defective form of TSWV is compared with the defective forms of other viruses and the possible role of the viral envelope in the transmission of TSWV by thrips is discussed.

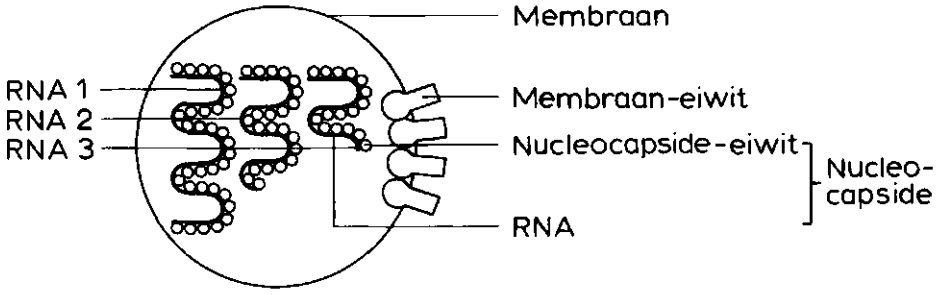
SAMENVATTING

In deze Nederlandse samenvatting zal ik proberen de inhoud van dit proefschrift uit te leggen op een manier die ook voor niet-ingewijden te begrijpen is.

Het bronsvlekkenvirus van de tomaat (TSWV, een afkorting van de Engelse naam tomato spotted wilt virus) kan behalve tomateplanten vele andere planten, waaronder tabak, aardappel en veel siergewassen, ziek maken. Wanneer tomateplanten geïnfecteerd zijn met TSWV verschijnen op de bladeren bronskleurige vlekken, op welk ziektebeeld de Nederlandse naam wijst. In het veld wordt TSWV verspreid door insekten, namelijk thripsen, die vaak onweersbeestjes worden genoemd. In het laboratorium kunnen wij het virus kunstmatig overbrengen door sap van geïnfecteerde planten op gezonde planten uit te smeren.

Wanneer wij cellen van planten die geïnfecteerd zijn met TSWV, bekijken met een elektronen-microscoop zien wij behalve allerlei structuren van de cel ook een groot aantal bolvormige deeltjes, die aan de buitenkant uitsteeksels bezitten (Hoofdstuk 1, Fig. 1A, B). Deze deeltjes zijn TSWV-deeltjes. De uitsteeksels zijn eiwitten, die op een membraan zijn ingeplant en daarom *membraan-eiwitten* worden genoemd. Binnenin de bolletjes zit het belangrijkste deel van het virus, namelijk de drager van de erfelijke eigenschappen, die ervoor zorgt dat het virus zich kan vermenigvuldigen. Bij veel plantevirussen, en ook bij TSWV, bestaat deze drager uit ribonucleinezuur, afgekort RNA. Wij zouden het RNA kunnen vergelijken met ponsbanden van een computer, waarin informatie is opgeslagen. Het bijzondere van TSWV is dat die informatie in enige afzonderlijke stukken RNA van verschillende lengte is opgeslagen. Het vrij kwetsbare RNA is in de bolvormige deeltjes beschermd door het *nucleocapside-eiwit*, dat dicht tegen het RNA aanligt. Het geheel van RNA en nucleocapside-eiwit wordt *nucleocapside* genoemd. Samenvattend kan men de *normale* TSWV-deeltjes beschouwen als bolletjes met aan de buiten-

kant de membraan-eiwitten en binnenin het RNA dat nauw omgeven is door het nucleocapside-eiwit. In de tekening is de structuur van het virus schematisch weergegeven.



Schematische, sterk vereenvoudigde voorstelling van de structuur van TSWV

Het bestaan van een *defecte* vorm van TSWV is aan het licht gekomen toen men cellen van geïnfecteerde planten verder ging bestuderen met de elektronen-microscoop. Men zag namelijk behalve de vertrouwde bolvormige TSWV-deeltjes ook nog donkere, *amorfe* (vormloze) *massa's*, die niet te zien waren in cellen van gezonde planten (Hoofdstuk 1, Fig. 1A, C). Aanvankelijk dacht men dat deze amorfe massa's een voorstadium in de ontwikkeling van de normale virusdeeltjes waren. Zij werden beschouwd als grote samenklonteringen van nucleocapsiden, dus van virus-RNA met het daaraan vastzittend nucleocapside-eiwit. In een later stadium van de ontwikkeling van het virus zouden losse nucleocapsiden omgeven worden door membranen met de daaruit stekende membraan-eiwitten. Zo zouden normale virus-deeltjes ontstaan. Maar aan deze opvatting ging men twijfelen toen men vond dat in de cellen van planten die geïnfecteerd waren met bepaalde stammen van TSWV alleen nog maar die amorfe massa's te zien waren en helemaal niet meer de vertrouwde bolvormige deeltjes. Sindsdien is men gaan spreken van een *defecte vorm* van TSWV. In de cellen van planten geïnfecteerd met deze vorm worden geen normale deeltjes meer gemaakt, maar uitsluitend amorfe massa's.

In het promotieonderzoek heb ik onderzocht welke verschillen er bestaan tussen de eiwitten en het RNA van de normale en defecte vorm

van TSWV. De resultaten van het onderzoek staan in dit proefschrift beschreven.

Hoofdstuk 2 gaat alleen over de normale vorm van TSWV. Ik heb gevonden dat TSWV drie stukken RNA van verschillende lengte bezit en verder heb ik geprobeerd vast te stellen welke informatie elk stuk afzonderlijk bevat. Dat laatste onderdeel is gedeeltelijk gelukt; ik heb namelijk gevonden dat het kleinste RNA (RNA 3) informatie bevat die nodig is voor de aanmaak van het nucleocapside-eiwit.

In hoofdstuk 3 staat beschreven welke eiwitten in de defecte vorm voorkomen. De volgende methode heb ik toegepast. Ik heb normale virus-deeltjes uit elkaar laten vallen en de membraan-eiwitten gescheiden van het nucleocapside-eiwit. Deze twee eiwitten heb ik afzonderlijk ingespoten in cavia's. Deze dieren maken als reactie op de inspuiting antilichamen die heel specifiek gericht zijn tegen het ingespoten eiwit. Ik heb wat bloed van de cavia's afgetapt en daaruit de antilichamen gehaald. Met deze antilichamen heb ik vervolgens bladeren die geïnfecteerd waren met òf de normale òf de defecte vorm getoetst op de aanwezigheid van de membraan-eiwitten en het nucleocapside-eiwit. Uit het onderzoek kwam naar voren dat de antilichamen tegen het nucleocapside-eiwit even sterk reageerden met sap van bladeren die geïnfecteerd waren met de normale vorm of met de defecte vorm. Dit betekent dat het nucleocapside-eiwit steeds aanwezig is in bladeren, of die nu geïnfecteerd zijn met de normale of met de defecte vorm. De antilichamen tegen de membraaneiwitten reageerden daarentegen uitsluitend met sap van bladeren die geïnfecteerd waren met de normale vorm en niet met sap van bladeren die geïnfecteerd waren met de defecte vorm. Ik heb uit deze resultaten geconcludeerd dat de defecte vorm wel het nucleocapside-eiwit bezit, maar niet de membraan-eiwitten.

Hoofdstuk 4 en 5 beschrijven hoe het RNA van de defecte vorm is samengesteld. Met een speciale techniek (hybridisatie), uiteengezet in hoofdstuk 5, heb ik aanwijzingen gevonden dat de defecte vorm een gedeelte van de informatie die ligt op RNA 2 van de normale vorm, verloren heeft. De informatie die ligt op zowel RNA 1 als RNA 3 van de normale vorm heeft de defecte vorm echter wel behouden.

Hoofdstuk 6 gaat over de amorfe massa's die karakteristiek zijn voor de defecte vorm. Ik heb nucleocapsiden gezuiverd uit planten

die geïnfecteerd waren met de defecte vorm. Het bijzondere was dat deze nucleocapsiden ook nog infectieus waren, dat wil zeggen, ik kon daarmee planten even goed ziek maken als met het complete virus. Wanneer ik deze nucleocapsiden onder de elektronenmicroscoop bekeek, zag ik structuren die sprekend leken op de amorfe massa's. Dit wijst erop dat de amorfe massa's inderdaad uit de nucleocapsiden van TSWV bestaan. Onderzoek naar de RNA-samenstelling in deze nucleocapsiden liet zien dat RNA 1 en RNA 3 even groot waren als RNA 1 en RNA 3 van normale TSWV-deeltjes, maar dat RNA 2 veel kleiner was. Dit resultaat had ik al op een andere manier gevonden, die beschreven is in hoofdstuk 5.

In hoofdstuk 7 bespreek ik de resultaten van het hele onderzoek in onderlinge samenhang. Ik geef aan waarom geen complete deeltjes te vinden zijn in cellen die met de defecte vorm zijn geïnfecteerd. Dit komt omdat de defecte vorm het vermogen verloren heeft om membraan-eiwitten te laten maken. De oorzaak van dat verlies ligt waarschijnlijk daarin dat een gedeelte van RNA 2 verloren is gegaan. Tenslotte bespreek ik waarom de defecte vorm nog niet gevonden is in planten die langs natuurlijke weg in het veld geïnfecteerd werden, maar uitsluitend in planten die in het laboratorium geïnfecteerd zijn. Waarschijnlijk komt dit omdat thripsen alleen maar de normale, complete vorm kunnen overbrengen en niet de defecte vorm.

REFERENCES

Adam, G., Sander, E. & Shepherd, R.J. (1979). Structural differences between pea enation mosaic virus strains affecting transmissibility by *Acyrthosiphon pisum* (Harris). Virology 92, 1-14.

Albrecht, P., Burnstein, T., Klutch, M.J., Hicks, J.T. & Ennis, F.A. (1977). Subacute sclerosing panencephalitis: experimental infection in primates. Science 195, 64-66.

Assink, A.M. (1973). Localization of the RNA replication of cowpea mosaic virus. Doctoral Thesis, Agricultural University, Wageningen, The Netherlands.

Atabekov, J.G. & Morozov, S. YU. (1979). Translation of plant virus messenger RNAs. Advances in Virus Research 25, 1-91.

Aviv, H. & Leder, P. (1972). Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proceedings of the National Academy of Science of the United States of America 69, 1408-1412.

Bailey, J.M. & Davidson, N (1976). Methylmercury as a reversible denaturing agent for gel electrophoresis. Analytical Biochemistry 70, 75-85.

Beachy, R.N. & Zaitlin, M. (1975). Replication of tobacco mosaic virus. VI. Replicative intermediate and TMV-RNA-related RNAs associated with polyribosomes. Virology 63, 84-97.

Beaty, B.J. Holterman, M., Tabachnik, W., Shope, R.E., Rohzon, E.J. & Bishop, D.H.L. (1981). Molecular basis of bunyavirus transmission by mosquitoes: role of the middle-sized RNA segment. Science 211, 1433-1435.

Best, R.J. (1968). Tomato spotted wilt virus. Advances in Virus Research 13, 65-146.

Black, L.M. (1969). Insect tissue cultures as tools in plant virus research. Annual Review of Phytopathology 7, 73-100.

Black, L.M. (1970). Wound tumor virus. CMI/AAB Descriptions of Plant Viruses No. 34. Commonwealth Mycological Institute, Kew.

Black, L.M. & Brakke, M.K. (1952). Multiplication of wound tumor virus in an insect vector. Phytopathology 42, 269-273.

Bol, J.F., Bakhuizen, C.E.G.C. & Rutgers, T. (1976). Composition and biosynthetic activity of polyribosomes associated with alfalfa mosaic virus infections. Virology 75, 1-17.

Bol, J.F., Brederode, F.T., Janze, G.C. & Rauh, D.K. (1975). Studies on sequence homology between the RNAs of alfalfa mosaic virus. Virology 65, 1-15.

Bonner, W.M. & Laskey, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. European Journal of Biochemistry 46, 83-88.

Bruton, C.J., Porter, A. & Kennedy, S.I.T. (1976). Defective-interfering particles of Semliki Forest virus: intracellular events during interference. Journal of General Virology 31, 397-416.

Choppin, P.W. & Compans, R.W. (1975). Reproduction of paramyxoviruses. In: Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R.R. (Plenum Press, New York) vol. 4, pp 95-178.

Clark, M.F. & Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34, 475-483.

Clarke, R.G. & Bath, J.E. (1976). Serological properties of aphid- and non aphid-transmissible pea enation mosaic virus isolates. Phytopathology 67, 1035-1040.

Cole, C.N. & Baltimore, D. (1973a). Defective interfering particles of poliovirus. II. Nature of the defect. Journal of Molecular Biology 76, 325-343.

Cole, C.N. & Baltimore, D. (1973b). Defective interfering particles of poliovirus. III. Interference and enrichment. Journal of Molecular Biology 76, 345-361.

Cole, C.N. & Baltimore, D. (1973c). Defective interfering particles of poliovirus. IV. Mechanisms of enrichment. Journal of Virology 12, 1414-1426.

Colonno, R.J., Lazzarini, R.A. Keene, J.D. & Banerjee, A.K. (1977). *In vitro* synthesis of messenger RNA by a defective interfering particle of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America 74, 1884-1888.

Compans, R.W. & Choppin, P.W. (1975). Reproduction of myxoviruses. In: Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R.R. (Plenum Press, New York) vol. 4, pp 179-252.

Compans, R.W. & Dimmock, N.J. (1969). An electron microscopic study of single-cycle infection of chick embryo fibroblasts by influenza virus. Virology 39, 499-515.

Davies, J.W., Aalbers, A.M.J., Stuik, E.J. & Van Kammen, A. (1977). Translation of cowpea mosaic virus RNA in a cell-free extract from wheat germ. FEBS Letters 77, 265-269.

Davies, J.W. & Verduin, B.J.M. (1979). *In vitro* synthesis of cowpea chlorotic mottle virus polypeptides. Journal of General Virology 44, 545-549.

Davis, A.R., Hiti, A.L. & Nayak, D.P. (1980). Influenza defective interfering viral RNA is formed by internal deletion of genomic RNA. Proceedings of the National Academy of Sciences of the United States of America 77, 215-219.

Davis, A.R. & Nayak, D.P. (1979). Sequence relationships among defective interfering influenza viral RNAs. Proceedings of the National Academy of Sciences of the United States of America 76, 3092-3096.

Dohner, D., Monroe, S., Weiss, B. & Schlesinger, S. (1979). Oligonucleotide mapping studies of standard and defective Sindbis virus RNA. Journal of Virology 29, 794-798.

El Manna, M.N. & Bruening, G. (1973). Polyadenylate sequences in the ribonucleic acids of cowpea mosaic virus. Virology 56, 198-206.

Emerson, S.U., Dierks, P.M. & Parsons, J.T. (1977). *In vitro* synthesis of a unique RNA species by a T particle of vesicular stomatitis virus. Journal of Virology 23, 708-716.

Epstein, D.A., Herman, R.C., Chien, I., Lazzarini, R.A. (1980). Defective interfering particle generated by internal deletion of the vesicular stomatitis virus genome. Journal of Virology 33, 818-829.

Eron, L., Sprague, J.A., Albrecht, P., Dunlap, R.C., Hicks, J.T. & Aulakh, G.S. (1978). Subacute sclerosing panencephalitis: an abortive infection by a measles-like virus. In: Negative strand Viruses and the Host Cell, eds. Mahy, B.W.J. & Barry, R.D. (Academic Press, London, New York, San Francisco) pp 157-167.

Francki, R.I.B. & Hatta, T. (1981). Tomato spotted wilt virus. In: Handbook of Plant Virus Infections and Comparative Diagnosis, ed. Kurstak, E. (Elsevier/North-Holland Biomedical Press, Amsterdam-New York-Oxford) pp 492-512.

Francki, R.I.B. & Peters, D. (1978). The interference of cytoplasmic membrane-bound material from plant cells with the detection of a plant rhabdovirus transcriptase. Journal of General Virology 41, 467-478.

Francki, R.I.B. & Randles, J.W. (1972). RNA-dependent RNA polymerase associated with particles of lettuce necrotic yellows virus. Virology 47, 270-275.

Friedman, R.M. & Ramseur, J.M. (1979). Mechanisms of persistent infections by cytopathic viruses in tissue culture. Archives of Virology 60, 83-103.

Goldbach, R.W., Borst, P., Bollen-de Boer, J.E. & Van Bruggen, E.F.J. (1978). The organization of ribosomal RNA genes in the mitochondrial DNA of *Tetrahymena pyriformis* strain ST. Biochimica et Biophysica Acta 521, 169-186.

Greenwood, F.C., Hunter, W.M. & Glover, J.S. (1963). The preparation of [^{131}I]-labelled human growth hormone of high specific radioactivity. Biochemical Journal 89, 114-123.

Grivell, L.A., Reijnders, L. & Borst, P. (1971). Isolation of yeast mitochondrial ribosomes highly active in protein synthesis. Biochimica et Biophysica Acta 247, 91-103.

Groot, G.S.P., Flavell, R.A., Van Ommen, G.J.B. & Grivell, L.A. (1974). Yeast mitochondrial RNA does not contain poly(A). Nature, London 252, 167-169.

Guild, G.M. Stollar, V. (1977). Defective interfering particles of Sindbis virus. V. Sequence relationships between SV_{STD} 42S RNA and intracellular defective viral RNAs. Virology 77, 175-188.

Halk, E.L., Robinson, D.J. & Murant, A.F. (1979). Molecular weight of the infective RNA from leaves infected with carrot mottle virus. Journal of General Virology 45, 383-388.

Hall, W.W. & Choppin, P.W. (1979). Evidence for lack of synthesis of the M polypeptide of measles virus in brain cells in subacute sclerosing panencephalitis. Virology 99, 443-447.

all, W.W., Kiessling, W.R. & Ter Meulen, V. (1978a). Biochemical comparison of measles and subacute sclerosing panencephalitis (SSPE) viruses.
In: Negative Strand Viruses and the Host Cell, eds. Mahy, B.W.J. & Barry, R.D. (Academic Press, London, New York, San Francisco) pp 143-156.

all, W.W., Kiessling, W. & Ter Meulen, V. (1978b). Membrane proteins of subacute sclerosing panencephalitis and measles viruses. Nature, London 272, 460-462.

all, W.W., Lamb, R.A. & Choppin, P.W. (1979). Measles and subacute sclerosing panencephalitis virus proteins: lack of antibodies to the M protein in patients with subacute sclerosing panencephalitis. Proceedings of the National Academy of Sciences of the United States of America 76, 2047-2051.

all, W.W. & Ter Meulen, V. (1976). RNA homology between subacute sclerosing panencephalitis and measles viruses. Nature, London 264, 474-477.

olland, J.J., Grabau, E.A., Jones, C.L. & Semler, B.L. (1979). Evolution of multiple genome mutations during long-term persistent infection by vesicular stomatitis virus. Cell 16, 495-504.

orta-Barbosa, L., Fuccillo, D.A., Sever, J.L. & Zeman, W. (1969). Subacute sclerosing panencephalitis: isolation of measles virus from a brain biopsy. Nature, London 221, 974.

uang, A.S. & Baltimore, D. (1970). Defective viral particles and viral disease processes. Nature, London 226, 325-327.

uang, A.S. & Baltimore, D. (1977). Defective interfering animal viruses. In: Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R.R. (Plenum Press, New York) vol. 10, pp 73-116.

ull, R. (1976). Particle differences related to aphid-transmissibility of a plant virus. Journal General Virology 34, 183-187.

ull, R. (1981). Pea enation mosaic virus. In: Handbook of Plant Virus Infections and Comparative Diagnosis, ed. Kurstak, E. (Elsevier/North-Holland Biomedical Press, Amsterdam-New York-Oxford) pp 239-256.

unt, T. & Jackson, R.J. (1974). The rabbit reticulocyte lysate as a system for studying mRNA. In: Modern Trends in Human Leukaemia, eds. Neth, R., Gallo, R.C., Spiegelman, S. & Stohlman, F. (J.F. Lehmanns Verlag, Munich) pp 300-307.

Ie, T.S. (1964). An electron microscope study of tomato spotted wilt virus in the plant cell. Netherlands Journal of Plant Pathology 70, 114-115.

Ie, T.S. (1971). Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. Virology 43, 468-479.

Ie, T.S. (1982). A sap-transmissible, defective form of tomato spotted wilt virus. Journal of General Virology, submitted.

Jackson, A.O. & Larkins, B.A. (1976). Influence of ionic strength, pH and chelation of divalent metals on isolation of polyribosomes from tobacco leaves. Plant Physiology 57, 5-10.

Johnson, K.P., Wolinsky, J.S. & Swoveland, P. (1978). Central nervous system infections (chronic). In: CRC Handbook Series in Clinical Laboratory Science, ed. Seligson, D. Section H: Virology and Rickettsiology Volume I Part 2, eds. Hsiung, G.-D. & Green, R.H. (CRC Press, Inc., West Palm Beach, Florida) pp 295-298.

Johnson, L.D. & Lazzarini, R.A. (1977). Replication of viral RNA by a defective interfering vesicular stomatitis virus particle in the absence of helper virus. Proceedings of the National Academy of Sciences of the United States of America 74, 4387-4391.

Joklik, W.K. (1974). Reproduction of reoviridae. In: Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R.R. (Plenum Press, New York) vol. 2, pp 231-334.

Kaluza, G., Rott, R. & Schwarz, R.T. (1980). Carbohydrate-induced conformational changes of Semliki Forest virus glycoprotein determine antigenicity. Virology 102, 286-299.

Katz, M., Oyanagi, S. & Koprowski, H. (1969). Subacute sclerosing panencephalitis: Structures resembling myxovirus nucleocapsids in cells cultured from brains. Nature, London 222, 888-890.

Keene, J.D., Rosenberg, M. & Lazzarini, R.A. (1977). Characterization of the 3' terminus of RNA isolated from vesicular stomatitis virus and from its defective interfering particles. Proceedings of the National Academy of Sciences of the United States of America 74, 1353-1357.

Keene, J.D., Schubert, M. & Lazzarini, R.A. (1979). Terminal sequences of vesicular stomatitis virus RNA are both complementary and conserved. Journal of Virology 32, 167-174.

Keene, J.D., Schubert, M., Lazzarini, R.A. & Rosenberg, M. (1978). Nucleotide sequence homology at the 3' termini of RNA from vesicular stomatitis virus and its defective interfering particles. Proceedings of the National Academy of Sciences of the United States of America 75, 3225-3229.

Kennedy, S.I.T. (1976). Sequence relationships between the genome and the intracellular RNA species of standard and defective-interfering Semliki Forest virus. Journal of Molecular Biology 108, 491-511.

Kitajima, E.W. (1965). Electron microscopy of viracabeca virus (Brazilian tomato spotted wilt virus) within the host cell. Virology 26, 89-99.

Knowland, J. (1974). Protein synthesis directed by the RNA from a plant virus in a normal animal cell. Genetics 78, 383-394.

Lazzarini, R.A., Weber, G.H., Johnson, L.D. & Stamminger, G.M. (1975). Covalently linked message and anti-message (genomic) RNA from a defective vesicular stomatitis virus particle. Journal of Molecular Biology 97, 289-307.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 277, 680-685.

Leppert, M., Kort, L. & Kolakofsky, D. (1977). Further characterization of Sendai virus DI-RNAs: a model for their generation. Cell 12, 539-552.

Lin, F.H. & Thormar, H. (1980). Absence of M protein in a cell-associated subacute sclerosing panencephalitis virus. Nature, London 285, 490-492.

Liu, H.-Y., Kimura, I. & Black, L.M. (1973). Specific infectivity of different wound tumor virus isolates. Virology 51, 320-326.

Lundquist, R.E., Sullivan, M. & Maizel, J.V., Jr. (1979). Characterization of a new isolate of poliovirus defective interfering particles. Cell 18, 759-769.

Machamer, C.E., Hayes, E.C. & Zweerink, H.J. (1981). Cells infected with a cell-associated subacute sclerosing panencephalitis virus do not express M protein. Virology 108, 515-520.

Marcu, K. & Dudock, B. (1974). Characterization of a highly efficient protein synthesizing system derived from commercial wheat germ. Nucleic Acids Research 1, 1385-1397.

Marcus, P.I. & Sekellick, M.J. (1977). Defective interfering particles with covalently linked (±) RNA induce interferon. Nature, London 266, 815-819.

Meinkoth, J. & Kennedy, S.I.T. (1980). Semliki Forest virus persistence in mouse L929 cells. Virology 100, 141-155.

Milne, R.G. (1970). An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. Journal of General Virology 6, 267-276.

Milner, J.J. & Jackson, A.O. (1979). Sequence complementarity of *Sonchus* yellow net virus RNA with RNA isolated from the polysomes of infected tobacco. Virology 97, 90-99.

Mohamed, N.A. (1981). Isolation and characterization of subviral structures from tomato spotted wilt virus. Journal of General Virology 53, 197-206.

Mohamed, N.A., Randles, J.W. & Francki, R.I.B. (1973). Protein composition of tomato spotted wilt virus. Virology 56, 12-21.

Moorman, A.F.M., Lamie, F. & Grivell, L.A. (1976). A coupled transcription-translation system derived from *Escherichia coli*: the use of immobilized deoxyribonuclease to eliminate endogenous DNA. FEBS Letters 71, 67-72.

Moussa, A.Y. (1980). The housefly virus contains double-stranded RNA. Virology 106, 173-176.

Murant, A.F., Goold, R.A., Roberts, I.M. & Cathro, J. (1969). Carrot mottle, a persistent aphid-borne virus with unusual properties and particles. Journal of General Virology 4, 329-341.

Nakajima, K., Ueda, M. & Sugiura, A. (1979). Origin of small RNA in von Magnus particles of influenza virus. Journal of Virology 29, 1142-1148.

Nayak, D.P. (1980). Defective interfering influenza viruses. Annual Review of Microbiology 34, 619-644.

Noll, H. (1967). Characterization of macromolecules by constant velocity sedimentation. Nature, London 215, 360-363.

Nomoto, A., Jacobson, A., Lee, Y.F., Dunn, J. & Wimmer, E. (1979). Defective interfering particles of poliovirus: mapping of the deletion and evidence that the deletions in the genomes of DI (1), (2) and (3) are located in the same region. Journal of Molecular Biology 128, 179-196.

Nuss, D.L. & Peterson, A.J. (1980). Expression of wound tumor virus gene products *in vivo* and *in vitro*. Journal of Virology 34, 532-541.

Obijeski, J.F. & Murphy, F.A. (1977). Bunyaviridae: recent biochemical developments. Journal of General Virology 37, 1-14.

Oyanagi, S., Ter Meulen, V., Katz, M. & Koprowski, H. (1971). Comparison of subacute sclerosing panencephalitis and measles viruses: an electron microscope study. Journal of Virology 7, 176-187.

Paliwal, Y.C. (1976). Some characteristics of the thrips vector relationship of tomato wilt spotted wilt virus in Canada. Canadian Journal of Botany 54, 402-405.

Paliwal, Y.C. (1979). Occurrence and localization of spherical viruslike particles in tissues of apparently healthy thrips, *Frankliniella fusca*, a vector of tomato spotted wilt virus. Journal of Invertebrate Pathology 33, 307-315.

Palma, E.L., Perlman, S.M. & Huang, A.S. (1974). Ribonucleic acid synthesis of vesicular stomatitis virus. VI. Correlation of defective particle RNA synthesis with standard RNA replication. Journal of Molecular Biology 85, 127-136.

Palomar, M.K., Brakke, M.K. & Jackson, A.O. (1977). Base sequence homology in the RNAs of barley stripe mosaic virus. Virology 77, 471-480.

Pelham, H.R.B. (1979). Translation of tobacco rattle virus RNAs *in vitro*: four proteins from three RNAs. Virology 97, 256-265.

Pelham, H.R.B. & Jackson, R.J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. European Journal of Biochemistry 67, 247-256.

Perrault, J. (1976). Cross-linked double-stranded RNA from a defective vesicular stomatitis virus particle. Virology 70, 360-371.

Perrault, J. & Leavitt, R.W. (1977). Inverted complementary terminal sequences in single-stranded RNAs and snap-back RNAs from vesicular stomatitis defective interfering particles. Journal of General Virology 38, 35-50.

Perrault, J. & Semler, B.L. (1979). Internal genome deletions in two distinct classes of defective interfering particles of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America 76, 6191-6195.

Perrault, J., Semler, B., Leavitt, R.W. & Holland, J.J. (1978). Inverted complementary terminal sequences in defective interfering particle RNAs of vesicular stomatitis virus and their possible role in autointerference. In: Negative Strand Viruses and the Host Cell, eds. Mahy, B.W.J. & Barry, R.D. (Academic Press, London, New York, San Francisco) pp 527-538.

Pettersson, R.F. (1981). 5'- Terminal nucleotide sequence of Semliki Forest virus 18S defective interfering RNA is heterogeneous and different from the genomic 42S RNA. Proceedings of the National Academy of Sciences of the United States of America 78, 115-119.

Pyne, J.W. & Hall, T.C. (1979). Efficient ribosome binding of brome mosaic virus (BMV) RNA 4 contributes to its ability to outcompete the other BMV RNAs for translation. Intervirology 11, 23-29.

Ralph, R.K. (1969). Double-stranded viral RNA. Advances in Virus Research 15, 61-158.

Ralph, R.K. & Bellamy, A.R. (1964). Isolation and purification of undegraded ribonucleic acids. Biochimica et Biophysica Acta 87, 9-16.

Ramsey, J.C., Eron, L., Sprague, J., Bensky, P., Jones, D., Dunlap, R. & Albrecht, P. (1978). Subacute sclerosing panencephalitis: an aberrant infection by a measles-like virus. In: Persistent Viruses, eds. Stevens, J.G., Todaro, G.J. & Fox, C.F. (Academic Press, New York, San Francisco, London) pp 635-641.

Reddy, D.V.R. & Black, L.M. (1969). Comparative infectivity of wound tumor isolates. Annual Review of Phytopathology 7, 87-92.

Reddy, D.V.R. & Black, L.M. (1972). Increase of wound tumor virus in leafhoppers as assayed on vector cell monolayers. Virology 50, 412-421.

Reddy, D.V.R. & Black, L.M. (1973). Electrophoretic separation of all components of the double-stranded RNA of wound tumor virus. Virology 54, 557-562.

Reddy, D.V.R. & Black, L.M. (1974). Deletion mutations of the genome segments of wound tumor virus. Virology 61, 458-473.

Reddy, D.V.R. & Black, L.M. (1977). Isolation and replication of mutant populations of wound tumor virions lacking certain genome segments. Virology 80, 336-346.

Reddy, D.V.R. & Macleod, R. (1976). Polypeptide components of wound tumor virus. Virology 70, 274-282.

Reichmann, M.E., Villareal, L.P., Kohne, D., Lesnaw, J. & Holland, J.J. (1974). RNA polymerase activity and poly(A) synthesizing activity in defective T particles of vesicular stomatitis virus. Virology 58, 246-249.

Reijnders, L., Aalbers, A.M.J. & Van Kammen, A. (1974). Molecular weights of plant viral RNAs determined by gel electrophoresis under denaturing conditions. Virology 60, 515-521.

Reijnders, L., Sloof, P., Sival, J. & Borst, P. (1973). Gel electrophoresis of RNA under denaturing conditions. Biochimica et Biophysica Acta 324, 320-333.

Rezaian, M.A. & Francki, R.I.B. (1973). Replication of tobacco ringspot virus. I. Detection of a low molecular weight double-stranded RNA from infected plants. Virology 56, 238-249.

Rima, B.K., Davidson, W.B. & Martin, S.J. (1977). The role of defective interfering particles in persistent infection of Vero cells by measles virus. Journal of General Virology 35, 89-97.

Robbins, S.J., Bussell, R.H. & Rapp, F. (1980). Isolation and partial characterization of two forms of cytoplasmic nucleocapsids from measles virus-infected cells. Journal of General Virology 47, 301-310.

Sakimura, K. (1962). The present status of thrips-borne viruses. In: Biological transmission of disease agents, ed. Maramarosch, K. (Academic Press, New York and London) pp 33-40.

Sakimura, K. (1963). *Frankliniella fusca*, an additional vector for the tomato spotted wilt virus, with notes on *Thrips tabaci*, another vector. Phytopathology 53, 412-415.

Samuel, G., Bald, J.G. & Pittman, H.A. (1930). Investigations on "spotted wilt" of tomatoes. Commonwealth of Australia, Council for Scientific and Industrial Research 44, 1-64.

Schluederberg, A., Chavanich, S., Lipman, M.B. & Carter, C. (1974). Comparative molecular weight estimates of measles and subacute sclerosing panencephalitis virus structural polypeptides by simultaneous electrophoresis in acrylamide gel slabs. Biochemical and Biophysical Research Communications 58, 647-651.

Schnitzlein, W.M. & Reichmann, M.E. (1976). The size and the cistronic origin of defective vesicular stomatitis virus particle RNAs in relation to homotypic and heterotypic interference. Journal of Molecular Biology 101, 307-325.

Schubert, M., Keene, J.D. & Lazzarini, R.A. (1979). A specific internal RNA polymerase recognition site of VSV RNA is involved in the generation of DI particles. Cell 18, 749-757.

Schubert, M., Keene, J.D., Lazzarini, R.A. & Emerson, S.U. (1978). The complete sequence of unique RNA species synthesized by a DI particle of VSV. Cell 15, 103-112.

Semler, B.L., Perrault, J., Abelson, J. & Holland, J.J. (1978). Sequence of an RNA templated by the 3'-OH RNA terminus of defective interfering particles of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America 75, 4704-4708.

Silverstein, S.C., Christman, J.K. & Acs, G. (1976). The reovirus replicative cycle. Annual Review of Biochemistry 45, 375-408.

Stamminger, G. & Lazzarini, R.A. (1974). Analysis of the RNA of defective VSV particles. Cell 3, 85-93.

Stark, C. & Kennedy, S.I.T. (1978). The generation and propagation of defective-interfering particles of Semliki Forest virus in different cell types. Virology 89, 285-299.

Stuik, E.J. (1979). *In vitro* translation of cowpea mosaic virus RNA. Doctoral Thesis, Agricultural University Wageningen, The Netherlands.

Tas, P.W.L., Boerjan, M.L. & Peters, P. (1977a). The structural proteins of tomato spotted wilt virus. Journal of General Virology 36, 267-279.

Tas, P.W.L., Boerjan, M.L. & Peters, D. (1977b). Purification and serological analysis of tomato spotted wilt virus. Netherlands Journal of Plant Pathology 83, 61-72.

Tellez-Nagel, I. & Harter, D.H. (1966). Subacute sclerosing leukoencephalitis: ultrastructure of intranuclear and intracytoplasmic inclusions. Science 154, 899-901.

Ter Meulen, V. & Hall, W.W. (1978). Review: slow virus infections of the nervous system: virological, immunological and pathogenetic considerations. Journal of General Virology 41, 1-25.

Ter Meulen, V., Hall, W.W. & Kreth, H.W. (1978). Pathogenetic aspects of subacute sclerosing panencephalitis. In: Persistent Viruses, eds. Stevens, J.G., Todaro, G.J. & Fox, C.F. (Academic Press, New York, San Francisco, London) pp 615-634.

Thormar, H., Mehta, P.D. & Brown, H.R. (1978). Comparison of wild-type and subacute sclerosing panencephalitis strains of measles virus. Journal of Experimental Medicine 148, 674-691.

Thuring, R.W.J., Sanders, J.P.M. & Borst, P. (1975). A freeze-squeeze method for recovering long DNA from agarose gel. Analytical Biochemistry 66, 213-220.

Toriyama, S. & Peters, D. (1980). *In vitro* synthesis of RNA by dissociated lettuce necrotic yellows virus particles. Journal of General Virology 50, 125-134.

Tsai, J.H. & Bath, J.E. (1974). The losses of transmissibility of two pea enation mosaic virus isolates by the pea aphid, *Acyrthosiphon pisum* (Harris). Proceedings of the American Phytopathological Society 1, 115-116.

Van den Hurk, J., Tas, P.W.L. & Peters, D. (1977). The ribonucleic acid of tomato spotted wilt virus. Journal of General Virology 36, 81-91.

Van der Zeijst, B.A.M. & Bloemers, H.P.J. (1976). 4698 isokinetic glycerol and sucrose gradients for density gradient centrifugation. In: Handbook of Biochemistry and Molecular Biology: Physical and Chemical Data Section, ed. Fasman, G.S. (Chem. Rub. Co., Cleveland) 3rd ed., Vol. 1, pp 426-519.

Van Griensven, L.J.L.D. & Van Kammen, A. (1969). The isolation of a ribonuclease-resistant RNA induced by cowpea mosaic virus: evidence for two double-stranded RNA components. Journal of General Virology 4, 423-428.

Van Griensven, L.J.L.D., Van Kammen, A. & Rezelman, G. (1973). Characterization of the double-stranded RNA isolated from cowpea mosaic virus-infected *Vigna* leaves. Journal of General Virology 18, 359-367.

Van Kammen, A. (1971). Cowpea mosaic virus, un virus au génome divisé. Physiologie végétale 9, 479-485.

Van Kammen, A., Henstra, S. & Ie, T.S. (1966). Morphology of tomato spotted wilt virus. Virology 30, 574-577.

Van Tol, R.G.L. & Van Vloten-Doting, L. (1979). Translation of alfalfa mosaic virus RNA 1 in the mRNA-dependent translation system from rabbit reticulocyte lysates. European Journal of Biochemistry 93, 461-468.

Van Vloten-Doting, L. & Jaspars, E.M.J. (1977). Plant covirus systems: three- component systems. In: Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R.R. (Plenum Press, New York) vol. 11, pp 1-53.

Villa-Komaroff, L., Guttman, N., Baltimore, D. & Lodish, H.F. (1975). Complete translation of poliovirus RNA in a eukaryotic cell-free system. Proceedings of the National Academy of Sciences of the United States of America 72, 4157-4161.

Wechsler, S.L. & Fields, B.N. (1978). Differences between intracellular polypeptides of measles and subacute sclerosing panencephalitis virus. Nature, London 272, 458-460.

Wechsler, S.L., Stallcup, K.C. & Fields, B.N. (1978). A comparison of the intracellular polypeptides of measles and subacute sclerosing panencephalitis virus. In: Negative Strand Viruses and the Host Cell, eds. Mahy, B.W.J. & Barry, R.D. (Academic Press, London, New York, San Francisco) pp 169-180.

Wechsler, S.L., Weiner, H.L. & Fields, B.N. (1979). Immune respons in subacute sclerosing panencephalitis: reduced antibody response to the matrix protein of measles virus. Journal of Immunology 123, 884-889.

Wengler, G., Wengler, G. & Filipe, A.R. (1977). A study of nucleotide sequence homology between the nucleic acids of different alphaviruses. Virology 78, 124-134.

CURRICULUM VITAE

Franciscus Nicolaas Verkleij werd op 9 oktober 1950 geboren in Hazerswoude. In 1969 behaalde hij het gymnasium β diploma en in hetzelfde jaar begon hij met de studie biologie aan de Universiteit van Amsterdam. In 1973 behaalde hij het kandidaatsexamen B4 (biologie met scheikunde als tweede hoofdvak) en in 1977 het doctoraalexamen, met als bijvaken aquatische oecologie (prof. J. Ringelberg) en moleculaire biologie (prof. P. Borst) en als hoofdvak plantenfysiologie (prof. D. Stegwee).

Van 16 april 1978 tot 16 april 1981 was hij als wetenschappelijk assistent werkzaam bij de vakgroep Virologie van de Landbouwhogeschool in Wageningen, in welke periode hij het onderzoek verrichtte waarvan dit proefschrift het resultaat is.