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THE UNIVERSITY OF ALBERTA

ISOLATION AND CHARACTERIZATION OF TOBACCO STUNT VIRUS

by

SAAD ASAD MASRI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

A series of experiments were conducted to determine optimal conditions for extracting tobacco stunt virus (TSV) from infected tomato leaves (Lycopersicon esculentum Mill. cv Trip-L) in different buffer systems. For virus purification the sap was extracted in glycine buffer pH 7.6, ontaining 0.01 M Na_2SO_3 and 0.002 M $MgCl_2$. The extract, mixed with 4 mg bentonite/g of leaf and clarified by centrifugation, was treated with 2% Triton X-100. TSV, precipitated with 8% polyethylene glycol 6000, was subjected to a cycle of differential centrifugation. The estimated average yield was 3 mg of virus/100 g of tomato leaf tissue. TSV particles were rod-shaped, 20 nm in diameter and 200-375 nm in length when negatively stained with 2% uranyl acetate and examined in the electron microscope.

RNA isolated from TSV by extraction with phenol-chloroform was separated into three bands with apparent molecular weights of 4.2×10^6, 1.8×10^6 and 1.2×10^6 when subjected to 6% polyacrylamide gel electrophoresis. The estimation of the molecular weight of the three species of TSV-RNA by electron microscopy was 3.9×10^6, 1.9×10^6 and 1.4×10^6, respectively.

The mixture of all three fractions of TSV-RNA from sucrose density gradient centrifugation was infectious when tested on Chenopodium amaranticolor Coste & Ryne. TSV-RNAs were resistant to RNase at high salt concentrations, and sensitive at low salt concentrations. Total TSV-RNA had a Tm of 75°C in 0.1 x SSC and 62°C in 0.01 x SSC.

These observations suggest that TSV-RNA is at least mosty double stranded.

In ultrastructural investigations of TSV-infected cells of C. amaranticolor, aggregates of TSV particles, arranged in a side-by-side fashion, were found in the cytoplasm of leaf mesophyll cells.

TSV-antisera, with titers of up to 1/512 in homologous reactions in a ring precipitin test, were produced in rabbits by intramuscular injections of purified TSV emulsified in Freund's complete adjuvant. In serological tests with lettuce big-vein virus (LBVV), which resembles TSV in many features, TSV-antiserum reacted positively with LBVV antigen up to a dilution of 1/256, in the ring precipitin assay, indicating a close serological relationship between the two viruses. Based on these studies it is suggested that TSV is a representative of a previously unrecognized virus group, and that LBVV is another possible member.

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LIST OF ABBREVIATIONS

antibody	(Ab)
base pairs	(bp)
β-mercaptoethanol	(BME)
bovine serum albumin	(BSA)
complementary deoxyribonucleic acid	(cDNA)
daltons	(Da)
deoxynucleotide triphosphate	(dNTP)
deoxyribonuclease	(DNase)
dithiothreitol	(DTT)
double-stranded	(ds)
electron microscope	(EM)
enzyme-linked immunosorbent assay	(ELISA)
ethylenediaminetetraacetate	(EDTA)
kilodalton	(KD)
messenger ribonucleic acid	(mRNA)
molecular weight	(MW)
N,N,N',N'-tetramethylethylenediamine	(TEMED)
phosphate buffered saline	(PBS)
polyacrylamide gel electrophoresis	(PAGE)
polyethylene glycol	(PEG)
ribonuclease	(RNase)

ribonucleic acid	(RNA)
ribosomal ribonucleic acid	(rRNA)
single-stranded	(ss)
sodium dodecyl sulfate	(SDS)

CHAPTER I

GENERAL INTRODUCTION

Tobacco stunt virus (TSV) is one of the classical soil-borne viruses that is transmitted by a chytrid fungus, Olpidium brassicae (Wor.) Dang., and causes severe stunting in certain cultivars of tobacco plants (Nicotiana tabacum L.). The association between the fungus and the virus has been well studied (Hidaka, 1960; Hidaka et al., 1962, Hiruki, 1964, 1965, 1975; Alderson, 1975). However, our understanding of the nature of infectious TSV has been limited, mainly due to its instability in plant sap, although the virus infectivity remains viable in its fungal vector for as long as 20 years (Hiruki, 1987). The infectivity appears to be maintained as long as the fungus is viable since virus infectivity has been recovered from the same stock of the fungus when resting spores were allowed to germinate to infect tobacco and tomato seedlings.

In this study, the virus isolate was obtained from TSV-carrying resting sporangia of O. brassicae. The O. brassicae resting sporangia preparation from a stock studied previously (Hiruki, 1964) served as source material.

Earlier studies (Hiruki, 1964, 1965, 1975) showed that the addition of some chelating agents, particularly 1-phenylthiosemicarbazide (1-PTC) or 4-PTC (Hiruki, 1975), can stabilize the virus to allow mechanical transmission.

It was shown also that Chenopodium amaranticolor Coste & Reyn. is a suitable local lesion host for TSV (Hiruki, 1964). More recently, the occurrence of virus-like particles was reported in thin sections of leaf mesophyll cells of TSV-infected tobacco plants (Kuwata and Kubo, 1981). However, the properties of the virus have not been studied in detail.

The objectives of this study were:

1. To evaluate different buffer systems and select a buffer suitable for the extraction of TSV;
2. To develop a practical method for purification of TSV; and
3. To investigate physical, chemical and serological properties of purified TSV.
4. To study the relationship between the virus and its vector.
5. To study the properties of the nucleic acid isolated from the virus.

CHAPTER II

LITERATURE REVIEW

A. Soil-Borne Viruses

A virus is soil borne if it infects plants via the roots (Hiruki and Teakle, 1987). This could occur by mechanical penetration through wounds (a typical example is tobacco mosaic virus (TMV)), or through a motile biological vector. The vectors that transmit viruses in soil are nematodes and fungi (Cadman, 1963a; Grogan and Campbell, 1966; Hiruki and Teakle, 1987). This review will deal with fungus transmitted viruses.

B. Fungus Transmitted Viruses

Two known classes of virus-transmitting fungi are Chytridiomycetes, which includes Olpidium spp. and Plasmodiomycetes, which contains Polymyxa spp. and Spongospora subterranea.

The virus-transmitting fungi may carry viruses externally or internally. The externally carried viruses can be eliminated by air drying for 30 days or by mild acid treatment for 1 hr (Campbell and Fry, 1966). Viruses that are carried internally by fungus zoospores can persist in the resting spores for long periods of time. For example, TSV can persist as long as 20 years (Hiruki, 1987). In the

resting spores of O. brassicae, lettuce big-vein virus can resist air-drying for a month, or mild acid treatment for 1 hr (Campbell and Fry, 1966). The internally carried viruses can be divided into three groups.

There are 17 virus and virus-like agents that can be transmitted by fungi belonging to 4 groups (Table 1).

1. Isometric particles

The viruses in this group include tobacco necrosis virus (TNV), cucumber necrosis virus (CNV) and satellite viruses of TNV.

TNV, first described by Smith and Bald (1935), has a wide host range with a world-wide distribution. Virus particles are normally restricted to the infected roots, but in some cases the virus can spread systemically causing a severe disease in tulips (Augusta disease; Mowat, 1970) and French beans (Behncken, 1968). It can infect certain plant species without inducing visible symptoms (Gama et al., 1982). The TNV virion is a small icosahedral particle, 30 nm in diameter, which contains a single-stranded RNA genome of molecular weight ranging from 1.3-1.6 x 10^6 (Uyemoto, 1981). There are several strains of TNV which are related or unrelated serologically (Uyemoto and Grogan, 1969), and more than seven serotypes of TNV are known (Uyemoto et al., 1968). In nature, TNV occurs as a stable virus but may, in some cases, be found as an unstable variant where the coat protein is present in a soluble form (Babos and Kassanis, 1962; Kassanis and Welkie, 1963). TNV, in its stable form, was shown to be

Table 1. Fungus transmitted viruses and their vectors.

PARTICLE SHAPE (group)	VIRUS	VECTOR	VIRUS-VECTOR RELATION
Isometric	Cucumber necrosis	*Olpidium radicale*	Nonpersistent
	Satellite	*O. brassicae*	Nonpersistent
	Tobacco necrosis	*O. brassicae*	Nonpersistent
Straight tubular	Soil-borne wheat mosaic	*Polymyxa graminis*	Persistent
	Potato mop-top	*Spongospora subterranea*	Persistent
Soil-borne wheat mosaic virus	Broadbean necrosis	?(*Polymyxa* spp.)	Persistent(?)
	Beet necrotic yellow vein	*P. betae*	Persistent
	Oat golden stripe	*P. graminis*	Persistent(?)
	Rice stripe necrosis	*P. graminis*	Persistent(?)
	Peanut clump	*P. graminis*	Persistent(?)
	Hypochoeris mosaic	-	-
	Nicotiana velutina	-	-
Tobacco stunt virus	Tobacco stunt	*O. brassicae*	Persistent
	Lettuce big vein	*O. brassicae*	Persistent
Filamentous	Barley yellow mosaic	*P. graminis*	Persistent
Barley yellow mosaic virus	Oat mosaic	*P. graminis*	Persistent
	Rice necrosis mosaic	*P. graminis*	Persistent
	Wheat spindle streak mosaic	*P. graminis*	Persistent
	Wheat yellow mosaic	*P. graminis*(?)	Persistent(?)

Note: This is a revision of the table prepared by Hiruki and Teakle (1987).

transmitted by O. brassicae zoospores (Teakle, 1960). Teakle and Gold (1963) found that TNV was not inactivated by adding antisera to the mixture of the zoospores and TNV, suggesting that TNV was carried internally by the zoospores. However, this result was refuted by Kassanis and Macfarlane (1964) when they found that the virus can be inactivated by properly adding concentrated TNV antiserum to the zoospore suspension. This conclusion was supported by other workers (Campbell and Fry, 1966) who showed that numerous TNV particles were found to be adsorbed to the outer membrane of the zoospores when the O. brassicae zoospores were fixed and negatively stained. On the other hand, only a few particles were found adsorbed to the surface of nontransmitting strains of O. brassicae (Temmink et al., 1970). The virus particles adsorbed to the axonemal sheath were considered to be more important in virus transmission, because the flagellum appeared to be retracted into the cytoplasm along with the adsorbed virus particles (Temmink and Campbell, 1969b).

CNV shares certain similar physical properties with TNV but is serologically unrelated to TNV (Tremaine, 1972). While CNV is not transmitted by O. brassicae it is readily transmitted by O. cucurbitacearum (Dias, 1970a; 1970b). Lange and Insunza (1977) reclassified this fungus as O. radicale. Even though CNV-RNA was transmitted mechanically to the leaves of cucumber plants, the fungus failed to transmit free CNV-RNA to the roots of the cucumber plants. Therefore, it was concluded that the coat protein was an essential factor to the binding of the virus to the plasma membrane of the zoospores

(Stobbe et al., 1982). Salt concentrations high enough to destroy the zoospores inhibit the transmission of CNV, yet the addition of some chemicals like $NaNO_3$ will reduce the transmission of CNV by O. radicale zoospores without inhibiting the mechanical transmission of CNV and without inhibiting zoospore infection of cucumber roots (Stobbs et al., 1982).

The satellite viruses are also carried externally and transmitted by O. brassicae zoospores (Teakle, 1962; Kassanis, 1964). They are small icosahedral particles of 17-18 nm in diameter and originally were believed to be defective TNV particles (Bawden and Pirie, 1950). However, Kassanis and Nixon (1960) and Kassanis (1962) established that these particles are serologically unrelated to TNV or CNV but depend upon them for their multiplication due to insufficient coding capacity in their RNA genome. Because of its genetic dependence on TNV, this small virus was designated as TNV satellite virus (SV) (Kassanis, 1962). There have been three SV serotypes isolated in the U.S.A., designated as A, B, and C (Uyemoto et al., 1968). The European strains were grouped by Kassanis and Macfarlane (1968) as SV_1, SV_2 and SV_3.

SV can only be activated by TNV (Kassanis and Nixon, 1961). Activation requires proper matching between TNV and its SV in an unknown mechanism. Since the unstable form of TNV can activate SV, it follows that the RNA, not the coat protein, is responsible for the activation process (Babos and Kassanis, 1962). SV, like TNV, is selectively transmitted by the fungus vector (Kassanis and Macfarlane, 1964).

2. Straight tubular particles

There are three viruses belonging to this group, namely beet necrotic yellow vein virus (BNYVV) which causes rhizomania disease of sugar beets and is transmitted by P. betae (Tamada et al., 1972), soil-borne wheat mosaic virus (SWMV) which is transmitted by P. graminis, and potato mop-top virus which is transmitted by S. subterranea.

Rhizomania disease was first reported in Italy by Canova (1959). The disease was first thought to be caused by a combination of two viruses, namely a TNV strain as a contaminant associated with P. betae. Tamada (1970) isolated a rod-shaped virus from rhizomania infected sugar beets in Japan. The disease was later found in France (Putz and Vuittenez, 1974) and in Germany (Tamada, 1975).

Tamada (1975) proved that the disease is caused by a rod-shaped virus with particles of different sizes which is transmitted by P. betae. The Japanese isolate of the virus consisted of particles of three different sizes which were 65-105 nm, 270 nm and 390 nm in length and 20 nm in width (Tamada, 1975). The French isolate contained particles of four distinct lengths of 85 nm, 100 nm, 265 nm and 390 nm (Putz, 1977). Recent studies have shown that all isolates contain four RNA segments. RNA_1 with 7100 bases, RNA_2 of 4800 bases, RNA_3 of a size that ranges between 1500 and 1880 bases, and RNA_4 of 1150 to 1400 bases (Richards et al., 1985, Bouzoubaa, et al., 1985; Ziegler et al., 1985). Based on northern blot hybridization experiments with cDNA, it was found that the virus from root extracts

contained uniform RNA patterns, while the virus extracted from leaves of sugar beets of mechanically infected leaves of C. quinoa contain RNA of different sizes (Koenig et. al, 1986). Based on nucleotide sequence analysis it was found that RNA_3 and RNA_4 have extensive nucleotide homology (Bouzoubaa et al., 1986).

In the precipitin-ring test, antiserum against BNYVV with a titer of 1/1,024 did not react with SWMV, tobacco rattle virus or TMV (Tamada and Baba, 1973). Putz (1977) found that the antiserum against the French isolate reacted strongly with the Japanese isolate. There was no serological reaction between BNYVV and SWMV, potato mop-top virus or TMV (Putz, 1977). Based on other serological, morphological and biological properties, the BNYVV is believed to be distantly related to TMV and has been classified as a member of the tobamovirus group (Harrison, 1977). Virus-like particles occur in the cytoplasm of P. betae zoospores, and virus infectivity persists in the resting spores of P. betae after air drying (Tamada, 1979). However, this is not supported by recent investigation (Langenberg and Giunchedi, 1982). Recently there is another study showing virus particles in the resting zoospores (Abe, 1987).

SWMV, another example of a classical soil-borne virus, was studied initially by McKinney and co-worker (1957) who suggested the necessity of a soil-borne vector for its transmission. The virus was later shown to be transmitted by P. graminis (Estes and Brakke, 1966). Virus transmission was not prevented by the addition of concentrated antisera to the zoospore suspension. It was also found that the virus was not affected by treating the viruliferous resting

spores with 0.1 N NaOH or with 0.1 N HCl for 1 hr (Rao and Brakke, 1969). The virus is rod-shaped with a width of 20 nm and two distinct lengths of 110-160 nm and 300 nm (Brakke, 1971b; Gumpf, 1971). A Japanese strain was found to have short rods of about 110-150 nm and the long ones 300 nm (Tsuchizaki et al., 1972). The nucleic acid consists of bipartite, single-stranded RNA genomes of molecular mass of 1.84×10^6 and 0.95×10^6 daltons contained in the long and short particles respectively. Various lengths found in the smaller segment were due to deletion mutation during successive mechanical transmission; this results in a virus that causes severe symptoms in infected plants (Shirako and Brakke, 1984a, 1984b). Using a cell-free translation system, the smaller RNA was found to contain the coding sequence for the virus coat protein of Mw 19,700 (Hsu and Brakke, 1985; Shirako and Ehara, 1986).

The two particles are required for infection (Hibino et al., 1981). In complementation tests of four isolates, the short particles controlled particle length, serotype, coat protein, and inclusion bodies while the long particles controlled infectivity and virus concentration (Tsuchizaki et al., 1975). Based on serological evidence, this virus is distantly related to TMV and potato mop-top virus (Kassanis and White, 1971; Powell, 1976; Randles et al., 1976).

Potato mop-top is another rod-shaped soil-borne virus and is transmitted by S. subterranea. This virus is carried internally by the zoospores of the fungus (Jones and Harrison, 1969). Once inside the resting spores, the virus can withstand air drying for more than

a year (Jones and Harrison, 1969). The virus is rod-shaped with a width of 20 nm and two distinct lengths of 125 nm and 275 nm. Kassanis and White (1977) found that the virus is serologically related to TMV and SWMV. Based on these findings it was classified as a member of the tobamovirus group (Van Regenmortel, 1981).

3. Filamentous particles

This group includes five other viruses that are carried internally by zoospores of the fungus P. graminis. They are wheat yellow mosaic virus (WYMV), barley yellow mosaic virus (BYMV), rice necrosis mosaic virus (RNMV), wheat spindle streak virus (WSSV) and oat mosaic virus (OMV). These viruses are filamentous particles ranging from 275-1000 nm and are known to produce pinwheel inclusions (Hollings and Brunt, 1981, Hibino et al., 1981). Based on the occurrence of the inclusion bodies, Harrison (1977) classified this group of viruses as potyviruses. However, Hollings and Brunt (1981) rejected this classification because of the differences in morphological and serological properties.

WYMV, BYMV and RNMV occur only in Japan and share a common antigen (Inouye, 1968, 1974; Hibino et al., 1981). These viruses are transmitted by P. graminis (Inouye and Fujii, 1977, Kusaba and Nakata, 1975; Inouye and Saito, 1975). They are flexuous particles of two predominant lengths of 275 and 550 nm, and a width of 11-14 nm. No reports have been published regarding the properties of their nucleic acid or coat protein.

OMV is found in North America and England (Catherall and Hayes, 1970). It is a flexuous particle of 650 x 11 nm (Usugi et al., 1981). The virus is a very poor antigen and serologically related to all the flexuous viruses that are transmitted by P. graminis. The OMV has not yet been further characterized.

WSSMV is also transmitted by P. graminis and is found only in North America (Barr and Slykhuis, 1969; Slykhuis, 1970; Wiese and Hooper, 1971; Brakke, 1971b; Hooper and Weise, 1972). A similar virus, WYMV, which is found in Japan, possesses morphological and serological characteristics similar to those of WSSMV. The particles are flexuous rods 700 x 15 nm (Hebert and Panzio, 1975; Hooper and Wiese, 1972). The virus was found to be serologically distantly related to the other soil-borne viruses that produce pinwheel inclusions (Usugi et al., 1981).

4. Unclassified virus-like agents

Viruses of this group are carried internally by zoospores of fungi are the disease-causing agents of lettuce big vein (LBV) and tobacco stunt (TSV). LBV was believed once to be caused by some toxin released by O. brassicae (Fry, 1958; Grogan et al., 1958). However, this claim was later rejected and it was suggested that the disease was caused by an infectious, grafttransmissible agent which produced virus-like symptoms (Campbell, 1962; Campbell and Grogan, 1963; Campbell, 1980). Campbell (1962) demonstrated that the virus-like agent was carried within the resting spores of O. brassicae on

the basis of its resistance to strong chemical treatments. Also, air-drying of contaminated soil for up to 5 months did not prevent the transmission of the LBV agent (Lim et al., 1970; Campbell, 1979). It was later shown that air-drying for 39 months did not affect the transmission of the agent (Campbell and Fry, 1966). This virus-like agent was also found to be carried internally by zoospores, since washing of the viruliferous zoospores did not prevent the transmission of the virus-like agent (Campbell and Grogan, 1964).

Haeske (1958) and Chod et al. (1976) reported rod-shaped virus-like particles in the roots of infected plants. Recently, similar virus-like particles were found in negatively-stained leaf-dip preparations obtained from infected lettuce leaves (Kuwata et al., 1983).

Tobacco stunt disease was first discovered in Japan in 1943 (Nakamura and Tsumagari, 1943), and by 1956 the disease was widespread, causing severe damage to tobacco seedlings (Hidaka et al., 1956). The disease was reported to be soil-borne as it did not occur after steam-sterilization of infested soil (Hidaka et al., 1956). The causal agent in the infested soil was reported to withstand air drying and oxygen treatment for 40 days (Hidaka et al., 1956). This suggested that the virus is present inside a resistant vector. The presumptive vector was believed to be a fungus since infectivity was sensitive to certain fungicides (Hidaka et al., 1956). Furthermore, there was a good correlation between the occurrence of the disease and the presence of O. brassicae in the infested soil (Hidaka, 1960). The disease agent was also transmitted to tobacco seedlings by means

of a suspension of zoospores from resting sporangia of O. brassicae obtained from stunt-infested soil, proving the association between TSV and its vector (Hiruki, 1965; Hidaka and Tagawa, 1965).

The causal agent of the disease was believed to be a spherical virus 25 nm in diameter (Hidaka, 1954). However, this finding was later refuted and the virus was suggested to be 18 nm in diameter and to be transmitted by grafting only (Hidaka et al., 1956). TSV was found to be unrelated to TNV on the basis of host range, serology, symptomology and cross protection tests (Hiruki, 1975). The disease was suggested to be caused by a virus with a loose protein coat that encapsidates the viral RNA (Alderson, 1975). In addition to transmission by O. brassicae vector, the virus could be mechanically transmitted if some chelating agents were added to the extracting buffer (Hiruki, 1964). C. amaranticolor is a suitable local lesion host (Hiruki, 1964) and has been used as an assay host (Hiruki, 1964, 1965, 1967, 1975, Hiruki et al., 1975, Alderson, 1975).

The acquisition of TSV by zoospores of O. brassicae occurs only in vivo (Hiruki, 1965). Attempts to transmit TSV by mixing O. brassicae zoospores with sap suspension, obtained by grinding infected leaves in phosphate buffer containing 1-PTC, failed (Hiruki, 1964).

Alderson (1975) found that 200-400 zoospores/ml of water were necessary for inoculation, whereas Hidaka and Tagawa (1965) reported 800 spores/ml were needed for successful transmission. When the zoospores penetrated the host cell, their cytoplasm became separated

from the host cytoplasm by a single membrane. This stage of infection is probably the time when acquisition of the virus could take place (Alderson, 1975). However, this observation has not been confirmed.

In nature, the occurrence of TSV has been reported on tobacco plants only. The severity of the disease varied with the variety of tobacco (Hidaka et al., 1956). Under experimental conditions, it was possible to transmit TSV using O. brassicae zoospores to thirty-five plant species in thirteen families (Hiruki, 1967). By mechanical transmission, it was possible to extend the host range to forty-one species in nine families (Hiruki, 1975).

Antiserum against partially purified TSV was obtained with a final titer of 1/1024 in the ring-precipitin test (Hiruki, 1975). Kiriyama (1975) was able to isolated antibody against TSV using ascitic fluid in mice with a final titer of 1/218. It was also possible to sero-diagnose naturally infected plants (Kiriyama, 1975). Infectious nucleic acid was produced from TSV-infected plants (Hiruki et al., 1974). The nucleic acid was believed to be RNA as it was selectively susceptible to ribonucleases, and it was stabilized by adding yeast RNA to the phosphate buffer prior to TSV extraction (Alderson, 1975).

C. Isolation of Unstable Plant Viruses

There are a number of plant viruses that are difficult to

purify. This is particularly so with those that are filamentous or rod-shaped particles. In some cases the difficulty is believed to be due, in part, to a low concentration in the plant tissue or to the loss of virus particles caused by aggregation (Till and Shepherd, 1967; Bar-Joseph and Loebenstein, 1970; Clark and Lister, 1971).

To improve the yield and the quality of virus preparations in purification of plant viruses, several methods have been developed. First, for virus extraction, a mortar and pestle was found to be useful for gentle extraction of virus from small quantities of leaf tissue (Pierpoint, 1966). It is also possible to use glass tissue homogenizers for this purpose (Steere, 1959). For larger quantities of leaf tissue, however, meat grinders of various types are used. In recent years the Waring blender has been preferred for extracting virus from leaf tissue because of ease of operation, sterilization and its efficient extracting power. However, it was found that some viruses, filamentous viruses in particular, were sheared during extraction in a blender (Bar-Joseph and Hull, 1974; Till and Shepherd, 1967). At the early stage of extraction many plant enzymes are usually liberated from lysozymes of plant cells, some of which may inactivate plant viruses (Steere, 1959). To prevent oxidation, a variety of reducing and chelating agents have been incorporated into extracting media (Pierpoint, 1966; Steere, 1959; Cornuet, 1960; Hiruki, 1964, 1975). To clarify the sap, several methods have been used successfully. Organic solvents have been employed as a means of clarifying plant sap and to precipitate some plant organelles and

ribosomes (Steere, 1956). However, certain plant viruses, membrane-bound viruses in particular, are not stable in organic solvents like the tomato spotted wilt virus (Luisoni et al., 1979; Best, 1968). For some viruses, it is possible to use other additives in order to clarify the plant sap prior to separation of virus particles by differential centrifugation. For example, Dunn and Hitchborn (1965) used magnesium bentonite during virus purification to precipitate ribosomes, 18 S protein particles and other green material like chlorophyl. This method has been used successfully by other workers to purify certain unstable plant viruses (Lister et al., 1965; de Sequira and Lister, 1969; Lister and Hadidi, 1971; Uyemoto and Gilmer, 1971). Benn and Murant (1979) found that the addition of Mg^{++} to the bentonite reduces infectivity of some viruses. Bentonite degraded certain stable plant viruses such as TMV and brome mosaic virus (BMV) by selective adsorption of viral coat protein (Brakke, 1971). Bentonite, with or without Mg^{++}, was also found to precipitate TSV (Alderson, 1975). The amount of bentonite needed to clarify plant sap varies with each preparation (Bar-Joseph et al., 1979).

Following clarification, plant viruses can be concentrated by a number of methods. Ammonium sulphate was used to precipitate plant viruses (Bawden and Pirie, 1942). The procedure was found to be time consuming, and other plant proteins may be precipitated along with the viruses (Markham, 1959). Centrifugation at high speed has been used extensively to precipitate different viruses (Stanley and Wyckoff, 1937; Sharp, 1953; Steere, 1959). The high speed centrifu-

gation produces physical shearing of rod-shaped viruses as well as insoluble aggregates (Long et al., 1976; Polson and Stannard, 1970; Tremaine et al., 1976). During resuspension of the pelleted virus, more breakage of virus particles may occur, resulting in additional losses in infectious virus yield (McNaughton and Matthews, 1971).

Polyethylene glycol (PEG), which is a linear polymer and chemically inert detergent, can be used to concentrate plant viruses. It was found that PEG 2400 at 20% (w/v) can precipitate ultraviolet-absorbing material such as nucleo-protein (McClendon, 1954). PEG 6000, along with sodium chloride, has been used extensively for purification of plant viruses (Hebert, 1963). The amount of PEG needed depends upon the virus that is to be precipitated. For example, Steere (1959) found that spherical viruses were precipitated using 4% while filamentous plant viruses required 6% (w/v) PEG 6000. Other workers found that 6% PEG gave 90% recovery of squash mosaic virus, a spherical virus, while at 4% there was only 70% recovery (Lastra and Munz, 1969). The concentration of PEG required for maximum virus precipitation depends upon the concentration of NaCl (Hebert, 1963).

Some plant viruses will form aggregates during precipitation or centrifugation. If these are firmly bound together, the infectivity of the virus preparation can be reduced substantially (Tremaine et al., 1976). Certain detergents, such as Triton X-100 (ethylphenoxy polyethoxyethanol), have been used for isolating polysomes (Kiho, 1968), TMV (Nozu and Yamaura, 1971), and plum pox virus (Van Oosten, 1972). In a study on the barley yellow mosaic necrosis virus Inouye

and Saito (1972) found that the concentration of Triton X-100 can be as low as 0.2% when used in conjunction with high speed centrifugation. In this procedure the pelleted virus was easily resuspended, and the yield and infectivity of the virus remained relatively high as a result of reduced aggregation of virus particles.

D. Plant viruses with double stranded RNA

The only characterized plant or animal viruses that contain double stranded RNA (dsRNA) are reoviridae (Matthews, 1982). These viruses have icosahedral shells which are 60-80 nm in diameter. Their dsRNA consists of ten to twelve segments, and an RNA-dependent polymerase is an integral component of the virion (Ikegami and Francki, 1976). This enzyme functions as a transcriptase that transcribes the ds genome segments into single-stranded messenger RNAs (Joklik, 1981). The plant virus members of this family differ from the animal reoviruses in their ability to multiply within their insect vectors, the number of segments comprising their RNA genomes, and certain details of virus particle structure (Joklik, 1981). These viruses belong to two major subgroups.

In the first subgroup, there are two viruses, namely wound tumor virus (WTV) and rice dwarf virus (RDV). Both viruses have a twelve segment RNA genome, and both are transmitted by leafhoppers (Black, 1964; Black and Knight, 1970). RDV is the only reo-like plant virus which is not tumorigenic (Shikata and Maramorosch, 1969).

In the second group, there are nine known viruses which are characterized by having ten segments in their RNA genome (Table 2). These include maize rough dwarf virus (MRDV), rice black-streak dwarf virus (RBSDV), sugarcane Fiji disease virus (SFDV), oat sterile disease virus (OSDV), pangola stunt virus (PSV), Lolium enation disease virus (LEDV), rice ragged stunt virus (RRSV), rice gall dwarf virus (RGDV) and cereal tillering disease virus (CTDV). All of these viruses are transmitted by and capable of multiplying in the plant hoppers (delphacids) (Milne and Luisoni, 1977b; Boccordo et al., 1979).

Even though there are differences between the two subgroups of reo-like plant viruses, they do exhibit some common properties. All such viruses have a wide host range infecting monocotyledonous plants, with the exception of WTV which infects dicotyledenous plants (Black, 1964).

The in vitro properties, such as longevity, thermal inactivation point and dilution end point, have not been studied in detail for some viruses. Available data, however, show that the longevity of RBDV is only a few hours at room temperature and 7 days at 4°C (Shikata, 1974). MRDV in frozen leaves kept at -20°C remained undiminished in infectivity for at least 10 years (Milne and Luisoni, 1977a). The survival of the antigenic components of the same virus was also found to be unimpaired by various conditions such as heating, freezing, or pH changes (Wetter and Luisoni, 1969). It was found, however, that there were some morphological changes under the conditions mentioned above such as dissociation of the outer membrane

Table 2. Plant reoviruses and their vectors.

SUB-GROUP	VIRUSES	PARTICLE SIZE	NO. OF RNA SEGMENTS	VECTORS
I	Wound tumor virus	70 nm	12	Agallia constricta A. quadripunctata Agalliopsis novella
	Rice dwarf virus	75 nm	12	Nephotettix cincticeps N. nigripictus Recillia dorsalis
II	Sugarcane Fiji disease virus	70 nm	10	Perkinsiella saccharicida P. vastatrix
	Rice ragged stunt virus	63 nm	10	Nilaparvata lugens
	Rice black-streak dwarf virus	70 nm	10	Laodelphax striatellus Unkanodes albifascia U. sapporona
	Maize rough dwarf virus	70 nm	10	L. striatellus Delphacodes propinqua Javesella pellucida Sogatella vibrix
	Cereal tillering disease virus	65 nm	10	L. striatellus Dicranotropis hamata
	Pangola stunt virus	65 nm	10	S. furcifera
	Oat sterile disease virus	65 nm	10	J. pellucida J. dubia J. obscurella D. hamata
	Rice gall drawf virus	70 nm	10	J. dubia J. discolor J. pellucida
	Lolium enation disease virus	70 nm	10	Unknown

of the virion producing B-spiked cores (Milne and Luisoni, 1977b). RBSDV was also found to be stable in frozen leaves, and infectivity remained for 7 days in rice sap at 4°C (Shikata, 1981; Kitagawa and Shikata, 1973). The thermal inactivation point was found to be 55-60°C for WTV and MRDV (Milne, 1977), whereas it was 60°C for RBSDV (Shikata et al., 1976). The dilution end point of RBSDV was 10^{-4} in a sap preparation from rice leaves and 10^{-5} in plant hopper extract (Shikata et al., 1976).

The polypeptides of the capsid proteins of group II plant reoviruses have been studied in detail. It was found that the viral core contains three protein components ranging in molecular weight from 50,000 to 160,000 (Shikata, 1981). The capsomere (subunit visible in the electron microscope) contains two polypeptides ranging in molecular weight from 36,000 to 96,000, while the amorphous outer layer contains two polypeptides ranging in molecular weight from 96,000 to 152,000 (Shikata, 1981; Nakata et al., 1978; Milne and Luisoni, 1977a).

RNA from WTV was found to have a base composition in which guanine and cytosine were in equal amounts and together comprised 44% of the total number of nucleotides (Black and Markham, 1963; Tomita and Rich, 1964). The same base composition was found in RNA extracted from RDV (Miura et al., 1966). The RNA genomes of both viruses comprised twelve segments, with a combined molecular weight of 16-16.6 x 10^6 daltons. These segments contained three genomes of the same molecular weights (Reddy et al., 1974). In detailed studies on WTV-

RNA, some of the RNA segments were not required for the multiplication of the virus inside the plant host (Reddy and Black, 1974). When infected clover plants were propagated from cuttings for several generations, the virus lost its ability to multiply inside the leafhopper vector. It was found that RNA5 and RNA7 were missing from such mutants (Redolf et al., 1973; Ikegami and Francki, 1975; Reddy et al., 1975; Reddy et al., 1976).

In the second subgroup, the RNA genome is divided into ten segments. RBSDV and MRDV have eight segments of the same molecular weight and FDV and MRDV have three segments of the same molecular weight (Redolfi et al., 1973; Ikegami and Francki, 1975; Reddy et al., 1975; Boccardo et al., 1978). The RNA was found to be double stranded on the basis of the thermal denaturation kinetics, resistance to RNase at high salt concentration, and a buoyant density of 1.60 g/ml in Cs_2SO_4 (Ikegami and Francki, 1975).

Serologically, reo-like plant viruses possess two kinds of antigens. The first is the dsRNA which elicits the production of antibodies of low titer and broad specificity (Francki and Jackson, 1972; Stoller, 1973; Moffitt and Lister, 1975). The second antigen is the coat protein. The titer of antisera produced against the coat protein can range between 1/512 to 1/8192 in a precipitin-ring test. The test usually results in higher titers than a gel-diffusion test (Milne and Luisoni, 1977a). There is no serological cross-reaction between the capsid proteins of WTA and RDV (Shikata, 1981). MRDV, RBSDV, PSV, and CTDV are serologically related in both the inner capsid and the B-spikes, whereas FDV did not react with them (Milne

and Luisoni, 1977b). It was found that in subgroup II there are at least three serological groups (Milne and Luisoni, 1977b).

To date there have not been described plant viruses with a rod-shaped morphology which contain ds RNA.

CHAPTER III

GENERAL MATERIALS AND METHODS

A. Virus

TSV infection was established by inoculating seedlings of N. tabacum cv Bright Yellow or L. esculentum cv Trip-L with O. brassicae which was originally obtained from a single isolate from tobacco infected with TSV. TSV-free O. brassicae was originally obtained by culturing a field isolate of O. brassicae on roots of cowpea, Vigna unguiculata (L.) Walp cv Early Ramshorn, for several generations (Hiruki, 1965).

B. Olpidium brassicae

Initial infection of tobacco seedlings with O. brassicae was established by sowing Bright Yellow tobacco seeds onto moistened quartz sand that had been mixed with resting spores of the fungus from the air-dried tobacco roots. Zoosporangia were formed approximately 3 to 4 weeks later at 18°C. Further inoculation with O. brassicae was done by dipping roots of up to 50 tobacco or tomato seedlings less than a month-old into a 100 ml suspension of O. brassicae zoospores (25,000 spores/ml) for 2 hr. The plants were watered with Hoagland solution (Hoagland and Arnon, 1950) or with

Hyponex (10 g/l) and kept incubated at 18°C ± 1°C until harvest.

To isolate O. brassicae zoospores for virus extraction, tobacco plants were removed from sand and the roots were washed in cold running water. The clean roots were immersed in water for 10 minutes at 20°C. The resulting zoospore suspension was passed through Whatman No. 4 filter paper to remove sand particles or plant debris. The suspension was then centrifuged at 5,000 g for 10 min (Sorvall RC-2B) with GSA rotor at 4°C. The pelleted zoospores were suspended in 0.01 M Tris-HCl (pH 7.5), containing 0.01 M Na_2SO_3, or in 0.05 M glycine (pH 7.5), containing 0.02 M Na_2SO_3, and frozen by dipping the test tube into acetone cooled by dry ice. The frozen material was then stored at -60°C. The roots of the plants were placed in Hoagland solution for 2 hr immediately after zoospore isolation. They were then washed in running water for 10 min, wrapped in wet cheesecloth, placed in 15 cm Petri plates, and covered with plastic bags. The plants were incubated at 20°C and zoospores were obtained from the same plants at daily intervals for 21 days.

C. Host Plants for Virus Propagation

Tobacco and tomato plants were grown in plastic trays (20 cm x 12 cm x 6 cm deep) containing acid-washed and steam-sterilized quartz sand. The plants inoculated with resting spores of O. brassicae that were known to carry TSV (hereafter referred to as TSV-Olpidium) were

kept at 18°C in temperature-controlled growth cabinets. After 2 weeks, the seedlings were transplanted into sterilized plastic pots 12 cm in diameter containing an autoclaved soil mixture (3 parts loam:2 parts peat:1 part sand) in a greenhouse at 18°C ± 2°C. In the summer months, the walls and roof were painted with 5% white Oarland shading compound to reduce heat and light intensity.

Although TSV-infected Bright Yellow tobacco produced typical stunt symptoms, it was rather difficult to maintain infected tobacco seedlings for an extended period of time due to severe stem and leaf necrosis. In contrast, tomato plants infected with TSV developed only veinclearing, chlorosis and yellow mottling (Plate 1). They were therefore more suitable as a virus propagation host than tobacco plants. Infected tomato plants were maintained by renewing them by cuttings or by sap-inoculation of young seedlings. Hyponex solution (5 g/l) was applied to the potted plants twice a week. Leaves were harvested repeatedly for TSV extraction when fully matured.

D. Assay Plants

Seeds of C. amaranticolor, a useful local lesion host for TSV (Hiruki, 1964, 1965, 1968, 1975), were germinated according to the method described by Alderson (1975). Seeds were washed in running water at room temperature for 24 hr, and germinated on wet filter paper in a petri plate for 3 days. Germinated seeds were transplanted into plastic trays containing the autoclaved soil-mix and

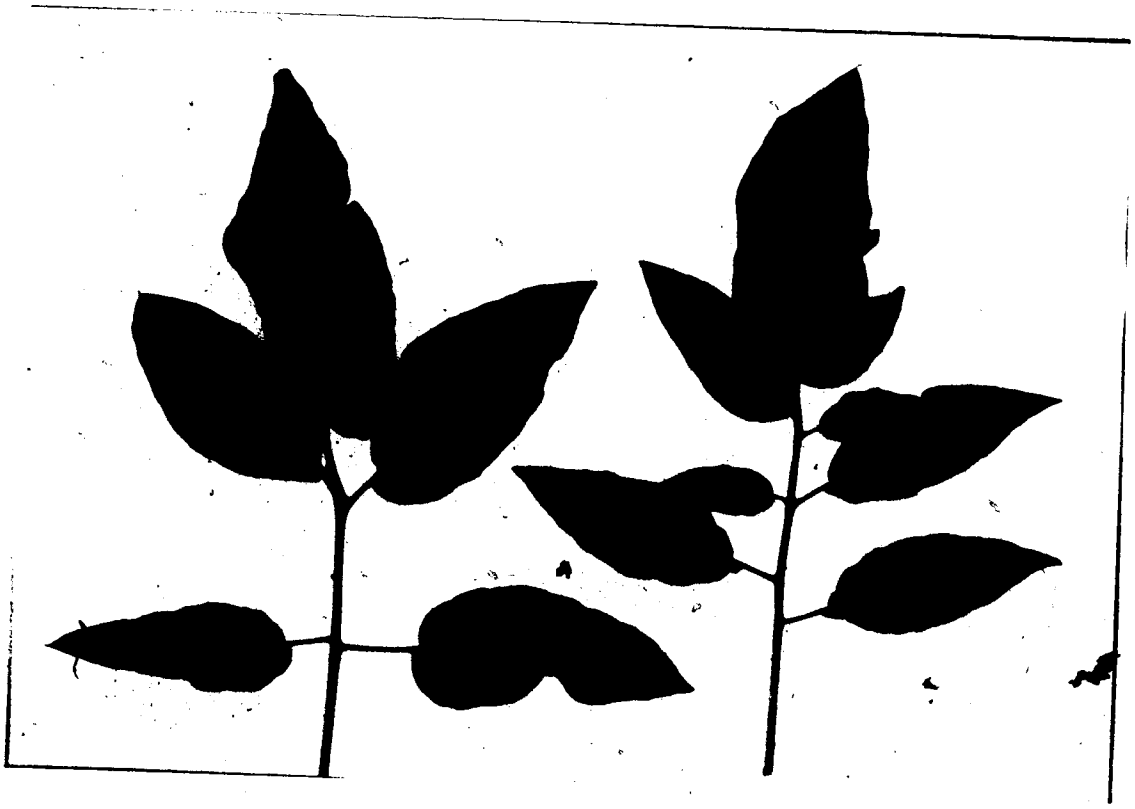

Plate 1. Systemic symptoms produced on a tomato leaf after root inoculation of seedlings with Opidium brassicae carrying tobacco stunt virus.

Left: Leaf from uninoculated plant.

Right: Leaf from inoculated plant.

grown in a greenhouse at 23°C ± 2°C with supplementary fluorescent and incandescent light, providing a daily 16 hr light period and a light intensity of about 20,000 lux. After 10 days the seedlings were transplanted into 12 cm autoclaved clay pots containing the same soil mix and grown under the same conditions until they reached the 4-6 leaf stage, which was approximately 25 days after transplanting. Plants were inoculated with a cotton swab dipped in inoculum by gentle and uniform stroking of leaves that had been uniformly dusted with Carborundum (600 mesh). Local lesions developed on inoculated leaves of C. amaranticolor in 7-14 days. (Plate 2).

Tomato seedlings were grown in autoclaved soil mix in a greenhouse at 20°C ± 2°C. Six week-old plants were inoculated with sap extracted from TSV-infected leaves. Three weeks later, the infected plants showed typical TSV symptoms.

For bioassay on tobacco, 4 week-old Bright Yellow tobacco plants were used.

Primary leaves of Red Kidney beans (Phaseolus vulgaris L.) were used to test for tobacco necrosis virus (TNV) contamination. Bean seeds were planted in University of California soil mix (hereafter referred to as U.C. mix) (Baker, 1957) in 12 cm autoclaved clay pots and grown at 25°C. Seven to 10-day-old seedlings with two primary leaves were selected and inoculated.

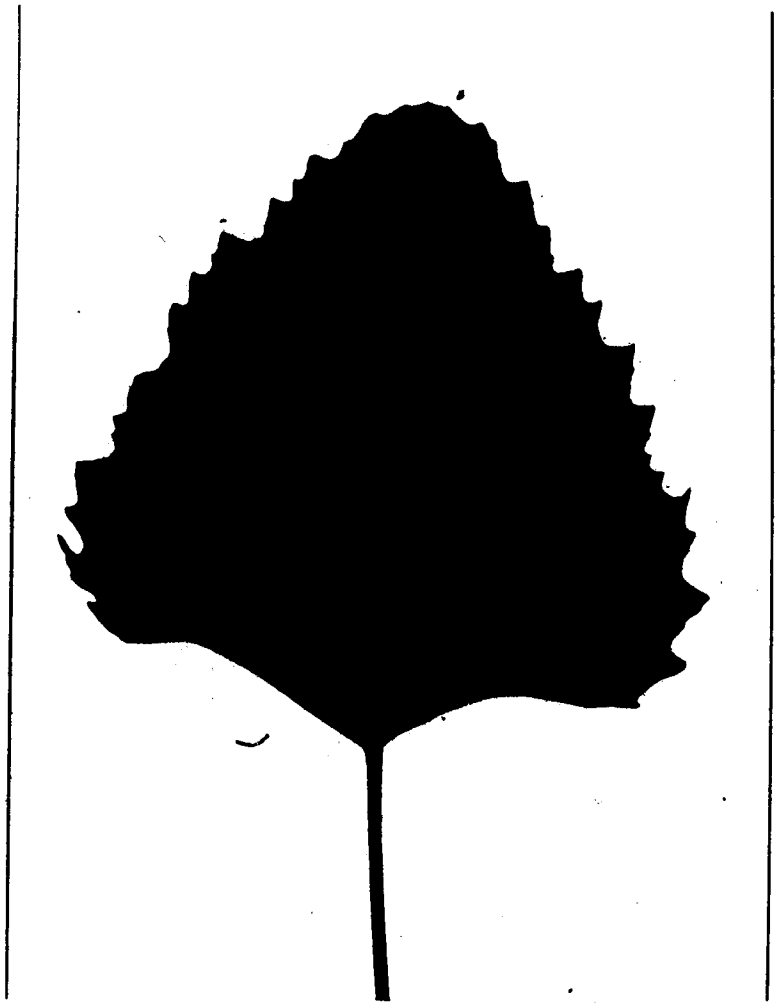

Plate 2. Local lesions on a Chenopodium amaranticolor leaf 14 days after inoculation with purified virus preparation from tomato leaves infected with tobacco stunt virus.

CHAPTER IV

INFECTIVITY OF TSV IN LEAF SAP

A. Introduction

Infectious particles of TSV have not been isolated or characterized since the first comprehensive description of the disease (Hidaka, et al., 1956).* This may well be due to the instability of TSV in crude plant extracts, and/or to unsuitable buffer systems used during the course of purification. In order to improve the buffer system, systematic studies are required of the composition of extracting buffer, the molarity and pH of the buffer solution, and buffer additives such as antioxidants and chelating agents.

B. Materials and Methods

1. Extracting buffer

Systemically infected tomato leaves showing typical TSV symptoms were harvested and cut into small pieces (about 2 mm^2). They were mixed, divided into 0.5 g portions and chilled at -60°C for 1 hr. The plant material was ground in 0.5 ml and 1 ml each of the

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* During preparation of this thesis, a manuscript describing TSV was published by Kuwata and Kubo (1986).

different buffers, with or without additives, in a chilled mortar. The extract was applied to C. amaranticolor leaves for bioassay.

2. Buffer additives

Different chelating agents and anti-oxidants were singly added to the extraction buffer in an attempt to stabilize TSV infectivity. These additives included:

a. Chelating agents: 10 mM ethylenediaminetetraacetate (EDTA, disodium salt), 10 mM sodium diethyl-dithiocarbamate (sodium DIECA), and 10 mM 4-phenyl-thiosemicarbazide (4-PTC).

b. Reducing agents: 1% sodium thioglycolate, 0.5% 2-mercapto-ethanol, 0.01 M Na_2SO_3 and 0.01 M sodium cyanide.

c. Nuclease inhibitors: Leaf samples collected and weighed as above were chilled and ground in 0.01 M Tris-HCl buffer pH 7.6 containing 0.01 M Na_2SO_3, and different nuclease inhibitors, namely heparin, disodium salt (Sigma) at a concentration of 150 units/ml, purified bentonite at a concentration of 4 mg/ml, and a combination of bentonite and heparin (4 mg bentonite and 150 units heparin/ml of buffer). The material was then applied to C. amaranticolor leaves to test TSV infectivity. In some experiments, the virus was precipitated with 8% PEG 6000, or centrifuged at 100,000 g for 90 min. The pelleted material was then suspended in Tris-HCl buffer and applied to C. amaranticolor leaves.

The above mentioned agents were added individually to the extracting buffer immediately before use. Buffers without additives

were used as a control. The final pH of the inocula were adjusted to 7.6 prior to applying them to C. amaranticolor.

3. Buffer molarity

Tomato leaves showing TSV symptoms were sliced and weighed as before. The samples were then chilled at -60°C for 1 hr, then ground in 0.5 ml Tris-HCl buffer pH 7.6, with different molarity ranging from 0.001 M to 0.5 M, containing 0.01 M Na_2SO_3. The extract was then applied to C. amaranticolor leaves for infectivity assay.

4. Buffer pH

Tomato leaves with TSV symptoms were sliced and weighed as before. The samples were then ground in 0.5 ml of 0.01 M Tris-HCl buffer containing 0.01 M Na_2SO_3. The buffer pH tested ranged from 5.0 to 9.0. The sap samples were tested on C. amaranticolor.

5. Recovery of TSV infectivity

Local lesions from C. amaranticolor leaves were cut, and divided into 3 portions of 0.5 g each. 0.5 ml 0.01 M Tris-HCl buffer, pH 7.6 containing 0.0. M Na_2SO_3 was added to each portion, then 1.5 mg of purified bentonite was added to the first portion before grinding in a mortar kept on ice at 4°C. The sap extracts obtained from the remaining two portions were centrifuged at 7,000 g for 10 min. To the extract from the second portion, 1.5 mg of bentonite was added. The third portion, containing no bentonite, served as a control. The

portions were centrifuged again at 7,000 g for 10 min. The sap extract from each portion was then applied to C. amaranticolor leaves by the half-leaf method and to four-week old tobacco plants to test infectivity.

6. Effect of Triton X-100

TSV-infected leaves were harvested and chilled as before. Two g of leaves were ground in a chilled mortar in 0.01 M Tris-HCl buffer pH 7.6 containing Na_2SO_3. The sap was then squeezed through a double layer of cheesecloth and centrifuged at 8,000 g for 10 min. A 20% Triton X-100 solution in the same buffer was added to the sap to give final concentrations of 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8%. The solutions were stirred on ice for 10 min. The mixture of sap and Triton X-100 was then centrifuged at 8,000 g for 90 min (Spinco L5-75, Rotor 30). The pelleted material was resuspended in Tris-HCl buffer and kept at 4°C for 30 min. The material was then centrifuged at 8,000 g for 10 min. The supernatant liquid was collected and applied to C. amaranticolor leaves for infectivity test.

C. Results

1. Effect of extracting buffers on the extraction of TSV from leaf tissue

The infectivity of TSV was greatly affected by the kind of buffer solution used at the time of sap extraction. The infectivity

of the leaf extract in 0.01 M phosphate buffer (pH 7.6), was lower than that in 0.01 M Tris-HCl (pH 7.6), while those in 0.01 M citrate buffer (pH 7.6) and in water were not infectious at all (Fig. 1). The TSV infectivity was not greatly affected when the ratio of the volume of the extracting buffers to the weight of the leaf sample increased fourfold (Fig. 2).

2. Effect of buffer additives on the extraction of TSV from leaf tissue

a. Chelating agents: The addition of some chelating agents such as 4-phenylthiosemicarbazide (4-PTC) to Tris-HCl buffer (Fig. 3) and phosphate buffer (Fig. 4) significantly stabilized TSV infectivity. The use of 4-PTC did not stabilize virus infectivity in citrate buffer.

b. Reducing agents: Sodium thioglycolate moderately stabilized TSV infectivity only with Tris-HCl buffer (Fig. 3). On the other hand, Na_2SO_3 and 4-PTC were very effective in stabilizing TSV infectivity in both buffers (Figs. 3 and 4). Mercaptoethanol and sodium cyanide did not have any positive effect on TSV infectivity (Figs. 3 and 4).

c. Nuclease inhibitors: Heparin appeared to be an effective nuclease inhibitor to stabilize TSV infectivity. However, when heparin was removed by PEG-6000 precipitation or differential centrifugation, TSV infectivity was only partially recovered.

Bentonite alone appeared to stabilize TSV infectivity when it

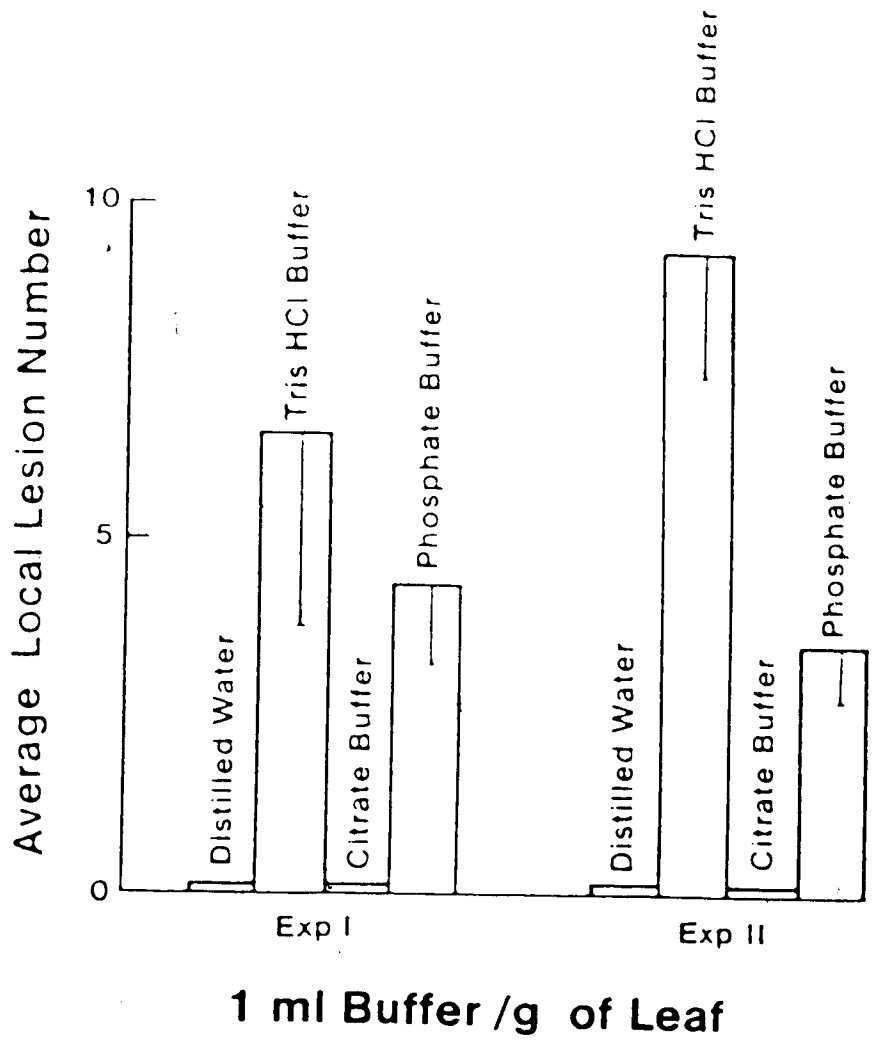


Fig. 1. Effect of different buffers, of 0.01 M and pH 7.6, added at a ratio of 1 ml/g of tomato leaf tissue, on tobacco stunt virus infectivity as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

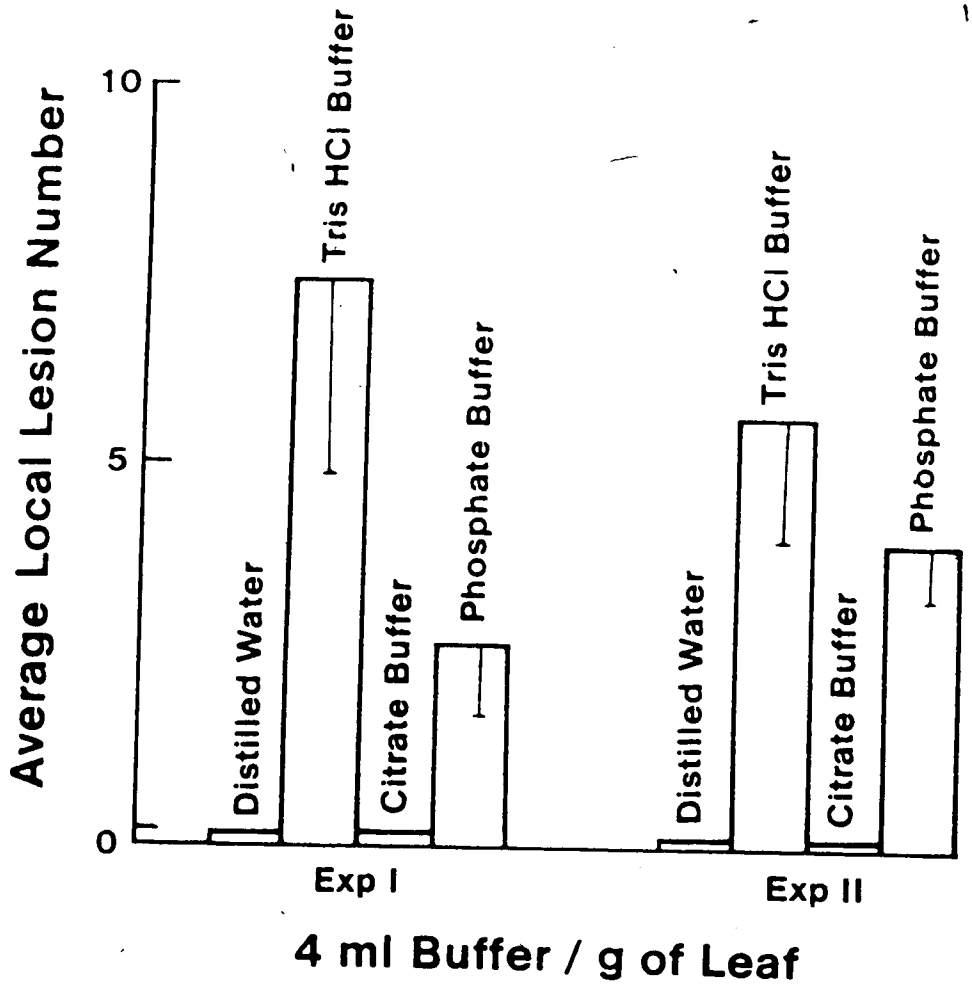


Fig. 2. Effect of different buffers, of 0.01 M and pH 7.6, added at a ratio of 4 ml/g of tomato leaf tissue, on tobacco stunt virus infectivity as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

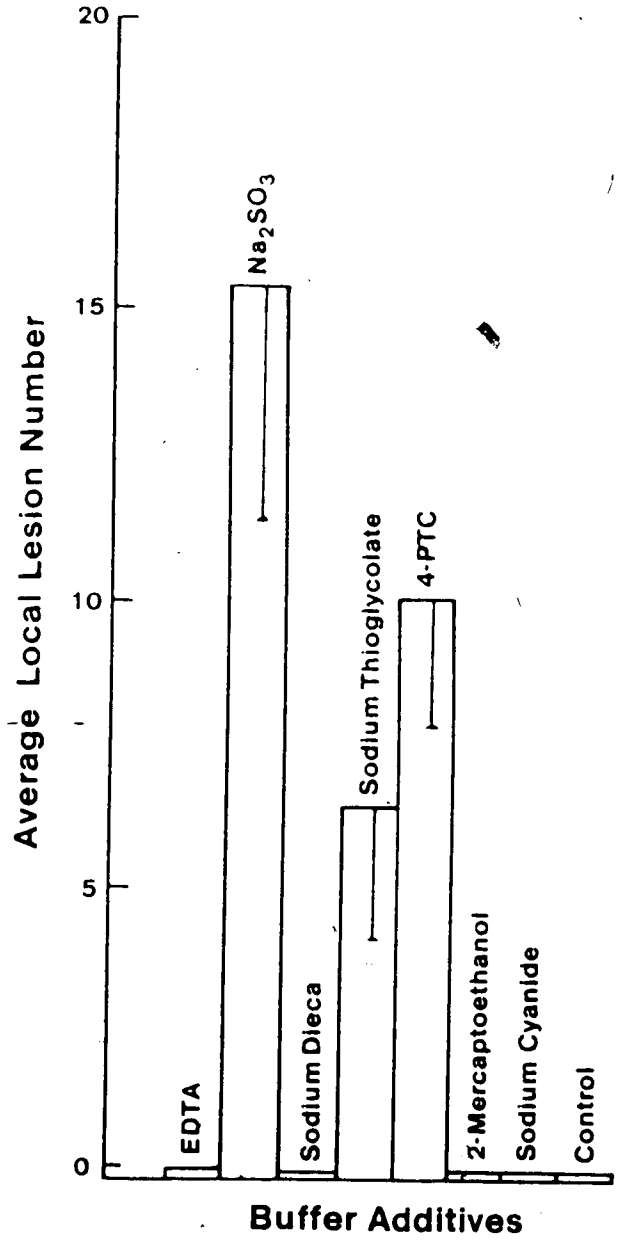


Fig. 3. Effect of different additives in 0.01 M Tris-HCl buffer pH 7.6 on tobacco stunt virus infectivity as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

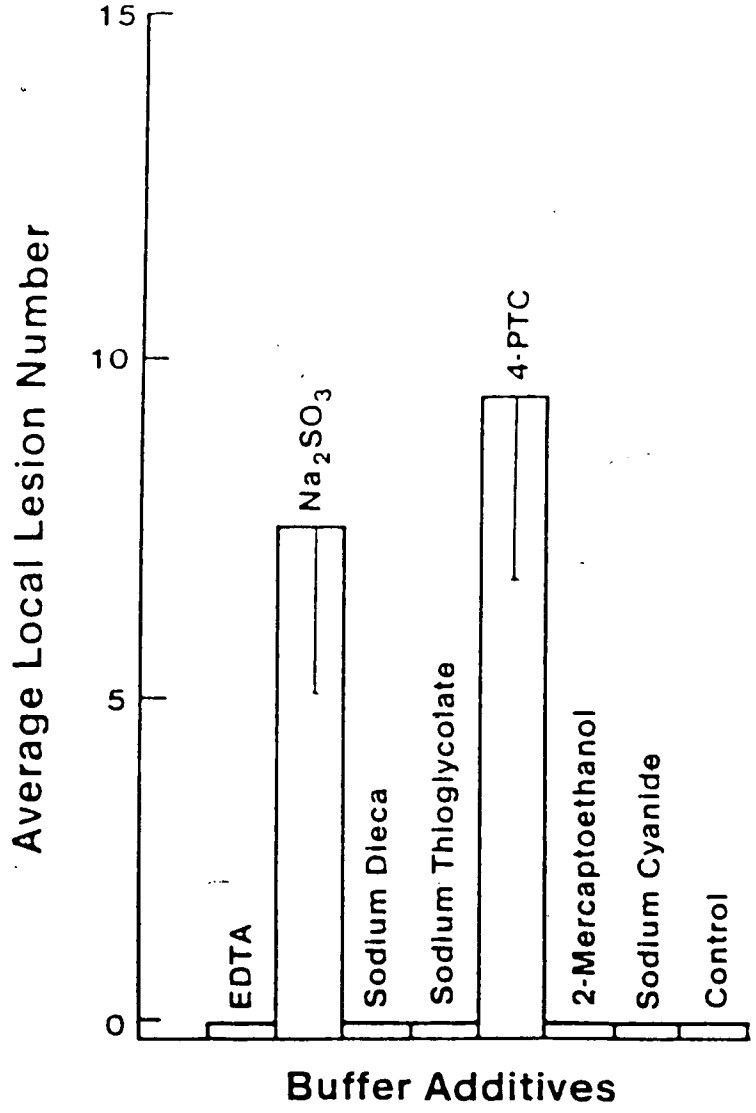


Fig. 4. Effect of different additives in 0.01 M phosphate buffer pH 7.6 on tobacco stunt virus infectivity as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

was added to leaf tissue at a ratio of 4 ml/g prior to grinding. When applied to tomato sap it precipitated green host material, and a pale yellowish supernatant liquid was obtained after low speed centrifugation. Heparin alone or in combination with bentonite did not significantly stabilize TSV infectivity (Figs. 5, 6 and 7).

3. Effect of buffer molarity on the extraction of TSV from leaf tissue

The highest infectivity was obtained when 0.01 M Tris-HCl (pH 7.6) was used as an extracting buffer. At lower buffer concentrations TSV infectivity was gradually reduced (Fig. 8). At a buffer molarity range over 0.01 M, the higher the buffer molarity the lower the TSV infectivity, and infectivity was abolished at 0.5 M (Fig. 8).

4. Effect of buffer pH on the extraction of TSV from leaf tissue

The highest TSV infectivity was obtained at a pH range of 7.0 to 8.0. Lowering buffer pH reduced TSV infectivity and it was lost at pH 6.0 and lower. At the same time, at a buffer pH exceeding 8.0, TSV infectivity was lowered, and it was completely lost at a pH of 9.0 (Fig. 9).

5. Recovery of TSV infectivity

When bentonite was added to the extraction buffer immediately before grinding the C. amaranticolor leaf bearing local lesions 11 to 14 days after inoculation, there was good recovery of TSV infectivity

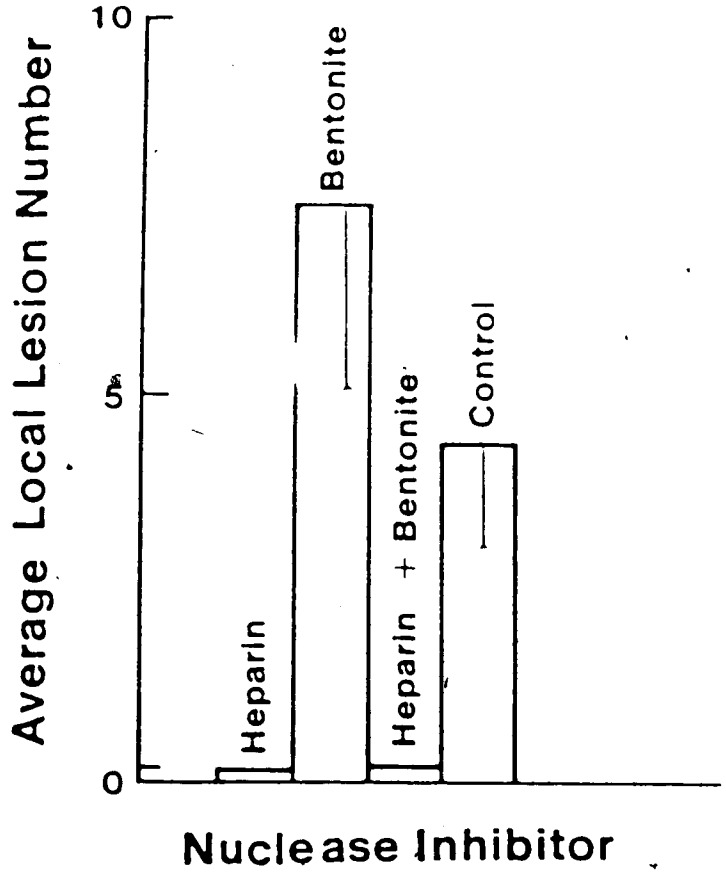


Fig. 5. Effect of nuclease inhibitors added to 0.01 M Tris-HCl buffer pH 7.6 on tobacco stunt virus infectivity in crude tomato sap as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

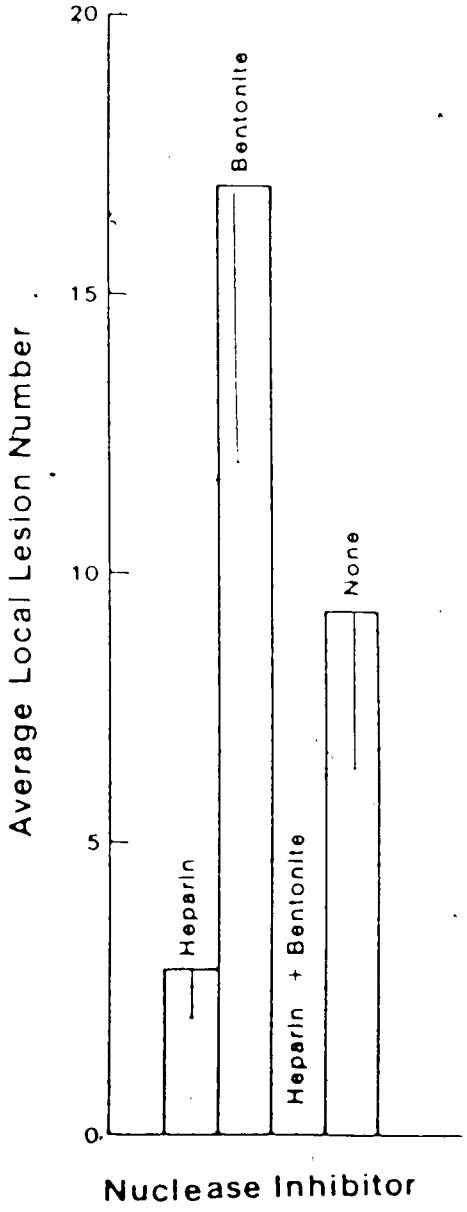


Fig. 6. Effect of nuclease inhibitors added to Tris-HCl buffer pH 7.6 on tobacco stunt virus infectivity after polyethylene-glycol precipitation as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

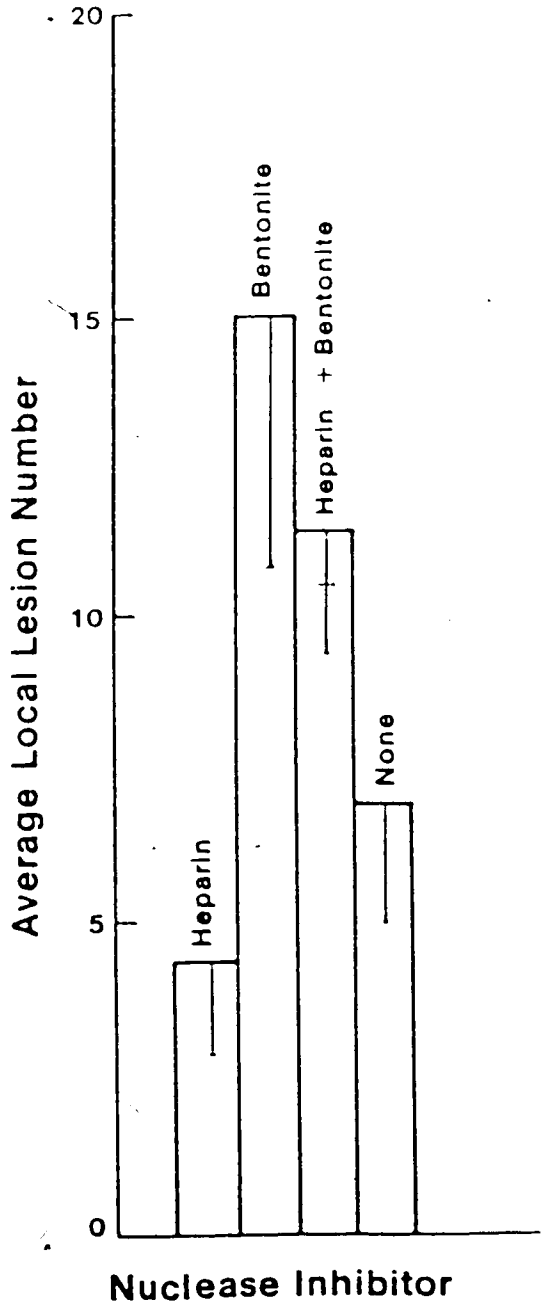


Fig. 7. Effect of nuclease inhibitors added to 0.01 M Tris-HCl buffer pH 7.6 on tobacco stunt virus infectivity after differential centrifugation as determined by the half-leaf method on Chenopodium amaranticolor. The bars represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

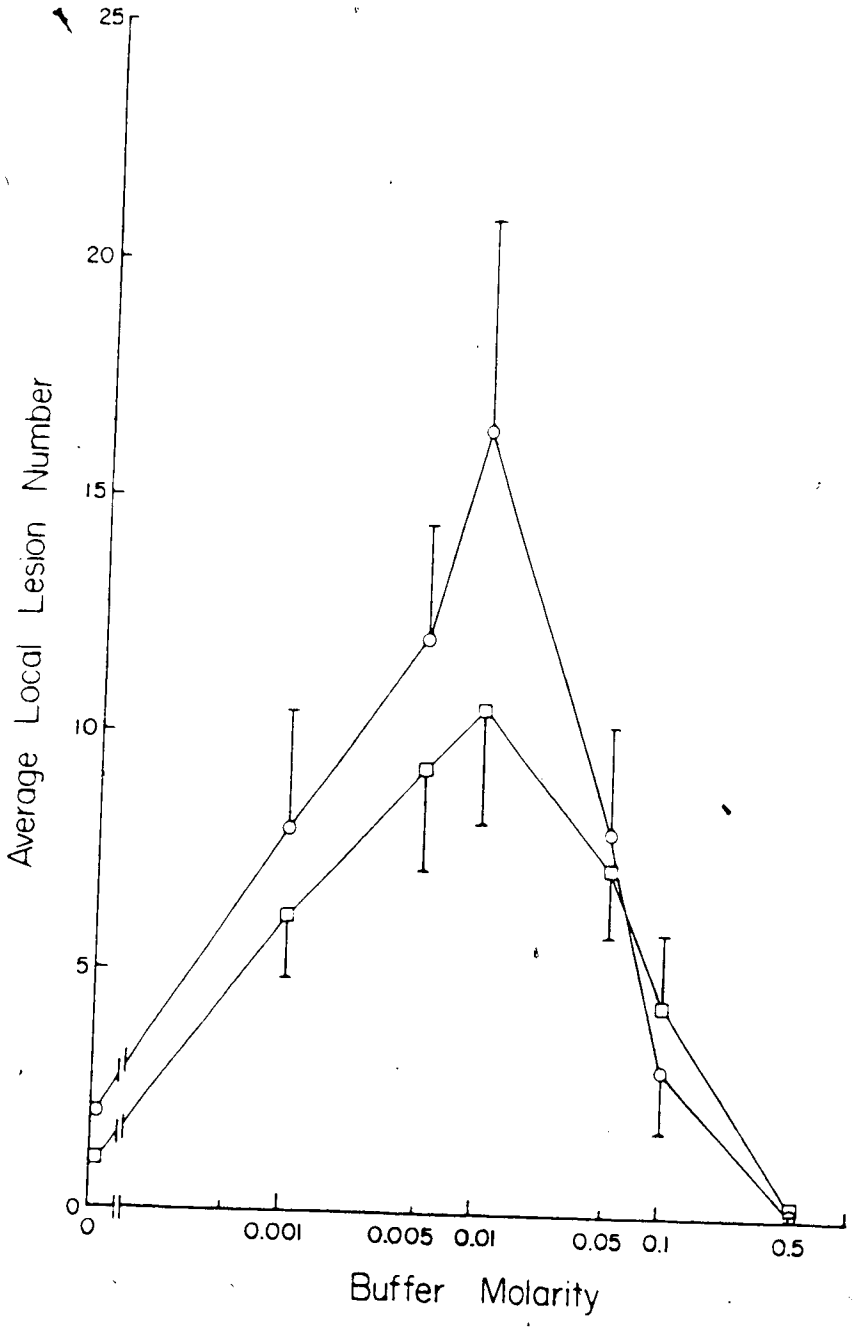


Fig. 8. **Effect of Tris-HCl buffer molarity, pH 7.6, on the infectivity of tobacco stunt virus as measured by the half-leaf method on Chenopodium amaranticolor. The open circles and open squares represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.**

(o-o - Experiment one. □-□ - Experiment two.)

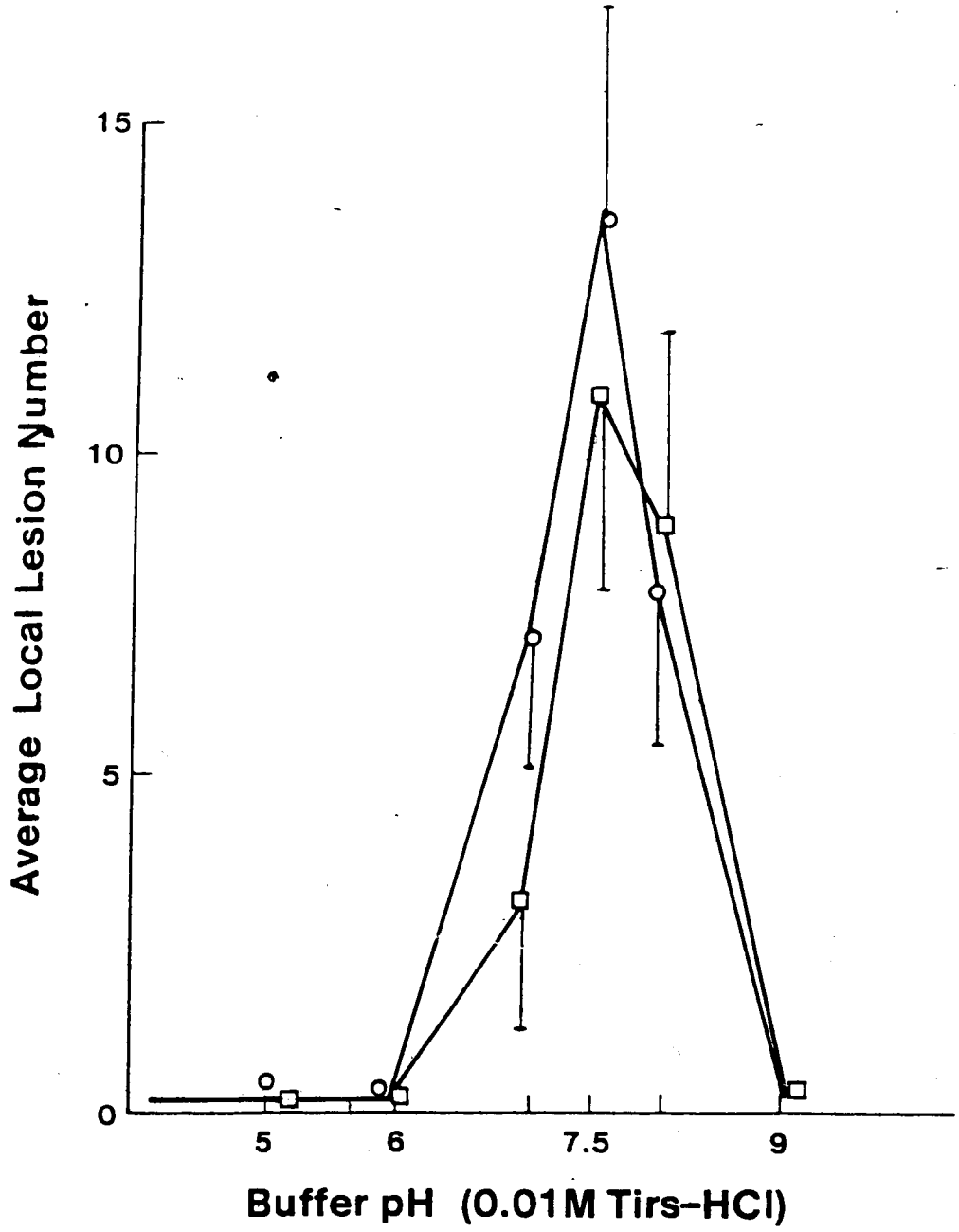


Fig. 9. Effect of buffer pH on the infectivity of tobacco stunt virus as measured by the half-leaf method on Chenopodium amaranticolor. The figures represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

(o-o - Experiment one. □-□ - Experiment two.)

on C. amaranticolor. Over 50% of the inoculated tobacco plants showed typical TSV symptoms 41 days after inoculation. TSV infectivity was also recovered from local lesions when the extracted sap was further clarified by adding bentonite. No local lesions appeared on C. amaranticolor and no stunt symptoms developed on tobacco plants when bentonite was not used.

6. Effect of Triton X-100

When Triton X-100 was introduced to plant sap, TSV infectivity was increased with the increase of Triton concentrations to reach a maximum level at 3% (Fig. 10). With the increase of Triton concentration above 3%, the infectivity of TSV preparations decreased to zero at levels of 7% - 8% (Fig. 10).

D. Discussion

Plant virus infectivity is usually measured by the number of local lesions produced on a test plant, or by the number of plants that show typical disease symptoms. To transmit the virus mechanically to a test plant, the plant tissue should be ground, and the virus particles should be released in an active form. The activity of the released virus in the plant sap is greatly affected by the buffer system, buffer pH and buffer additives. The buffer system affects the virus activity either directly or indirectly. For example, some virus inhibitors can be precipitated by different

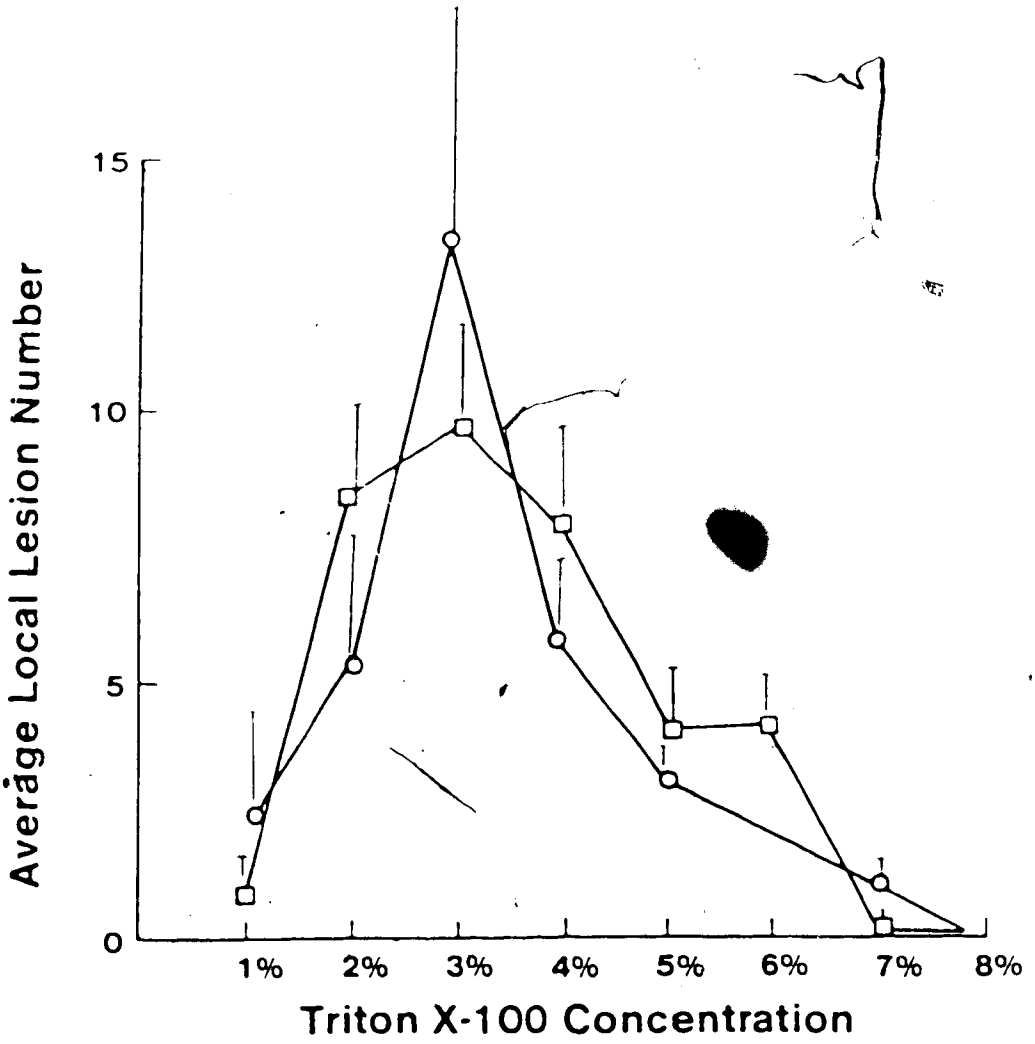


Fig. 10. Effect of Triton X-100 on tobacco stunt virus infectivity as determined by the half-leaf method on Chenopodium amaranticolor. The figures represent average local lesions from 8 half-leaves. Vertical lines represent a half of standard deviation for 8 replicates.

(o-o - Experiment one. □-□ - Experiment two.)

buffers such as Tris-HCl or phosphate buffer. The results of this study suggest that a presumptive inhibitor(s) is released with the virus in plant sap (Fig. 1 & 2). When the virus was diluted, the number of local lesions were not reduced proportionately, indicating a dilution of the inhibitor(s) (Simon et al., 1963). Since the stability of the virus in plant sap is influenced by this presumptive inhibitor(s), a method of preventing its action is essential. Assuming that this inhibitor is enzymic in nature, the removal of the metal ions that act as co-enzymes should greatly reduce the enzymic activity of this inhibitor which, in turn, increases the activity of the virus. For example, 4-PTC is a specific chelating agent for the Cu^{++} ions, which could be needed for the activity of the inhibitor(s). However, EDTA, which is a strong chelating agent, precipitates more metal ions, including some cations needed to hold the virus particles together. This result agrees with previous findings of other workers (Hiruki, 1964; Alderson, 1975). On the other hand, some anti-oxidants such as sodium sulfite can prevent the action of some phenolic compounds, thus preventing their action on the virus particles. In this study the addition of Na_2SO_3 greatly stabilized the infectivity of TSV in both Tris-HCl buffer as well as phosphate buffer. Kuwata and Kubo (1986) independently reached the same conclusion, but it was not in agreement with the finding of Alderson (1975), who used a relatively high concentration of Na_2SO_3 (0.1 M) in preparing TSV inoculum.

The infectivity of TSV was also found to be affected by the

buffer pH. This is not a surprising fact since some stable plant viruses like bromoviruses are greatly affected by the buffer pH. Such virus particles will swell and become sensitive to RNase at pH higher than 6.0 (Lane, 1974). On the other hand, some unstable plant viruses like closteroviruses can withstand changes in pH ranging from 4.0 - 8.0, as long as other conditions such as molarity and anti-oxidants are kept unchanged (Bar-Joseph et al., 1979).

TSV infectivity was also stabilized by the addition of Triton X-100. The effect of the Triton is not on the virus itself, but could be as the result of enhancemed release of the virus from plant cells. Therefore, in the presence of Triton, cell organelles will rupture, releasing virus particles and preventing adsorption of virus particles to cell debris. However, Triton at higher concentrations could be toxic to plant cells resulting in the death of the inoculated leaf (Fig. 10).

TSV infectivity was stabilized by the addition of bentonite. Bentonite was also effective in recovering TSV infectivity from local lesions that were produced on the leaves of C. amaranticolor; the infectivity from such local lesions could not be recovered in the absence of bentonite. C. amaranticolor is known to contain inhibitors that prevent or reduce the infectivity of plant viruses (Simon et al., 1963; Brakke, 1971a). This may mean bentonite is capable of precipitating such an inhibitor. When bentonite was added, the sap was clarified to yield a supernatant liquid slightly yellowish in color and made further purification of TSV much easier. The abrasive

effect of bentonite was not a factor in obtaining high local lesion numbers on assay plants, since its removal by centrifugation did not affect infectivity.

The results obtained in this study are improvements over the previous work (Alderson, 1975), in which the infectivity was reduced after the addition of 20 mg/g bentonite/g of leaf tissue. While the exact mechanism of virus stabilization by addition of bentonite has yet to be studied, the extent of the effectiveness of bentonite in stabilizing TSV infectivity could be influenced by the source, or by the amount of bentonite used in combination with the kind of buffer system used. In this chapter, a buffer system, namely 0.01 M Tris-HCl pH 7.6 containing 0.01 M Na_2SO_3, was found suitable to stabilize TSV particles, which could be used to further purify the virus.

CHAPTER V

PURIFICATION OF TSV

A. Introduction

Tobacco stunt virus was shown to be mechanically transmitted if certain chelating agents such as 1-PTC and 4-PTC were added to phosphate buffer (Hiruki, 1964, 1975). Further attempts to isolate infectious virus particles in sufficient quantity for characterization of the virus were not successful, probably due to its instability *in vitro* (Alderson, 1975). In the present study, attempts were made to develop procedures for purification of the virus. These included precipitation of the virus with PEG 6000, differential centrifugation, or a combination of both.

B. Materials and Methods

1. Purification of TSV using polyethylene glycol precipitation

All experiments were done in a cold room at 4°C. Infected leaves from tomato or tobacco plants were chilled for at least 2 hr at -60°C before use. The leaves were then subjected to extraction in a Waring blender with 2 ml 0.01 M Tris-HCl (pH 7.6) containing 0.01 M Na_2SO_3/g of frozen leaves. In some experiments, leaf tissue was

ground with a chilled mortar and pestle. The sap was then squeezed through a double layer of cheesecloth. The pulp was reground in 1 ml buffer/g of original leaf material. The sap was squeezed through cheesecloth and combined with the original sap, then centrifuged at 8,000 g for 10 min in a Sorvall RC2-B centrifuge. The supernatant was then stirred on ice and 4 mg bentonite/g of leaf was added slowly, according to the method previously described (Benn and Murant, 1979). The mixture was then centrifuged for 5 min at 8,000 g. The virus in the supernatant liquid was precipitated by adding 8% PEG 6000 (Fisher Scientific, New Jersey). The solution was stirred until the PEG was completely dissolved, then it was left in the cold room for 30-45 min before being centrifuged at 10,000 g for 20 min. The pelleted material was resuspended in Tris-HCl buffer in 1% of the original volume, recentrifuged, and the supernatant was collected. The infectivity of both the supernatant and pelleted material was tested on C. amaranticolor leaves after each centrifugation step.

2. Purification of TSV using differential centrifugation

TSV-infected leaves were harvested, chilled and subjected to extraction in a Waring blender. The sap, clarified with bentonite as described above, was subjected to centrifugation at 8,000 g, followed by centrifugation at 100,000 g for 90 min (Spinco L5-65, Rotor 30). The pelleted material was resuspended in 1% of the original volume of buffer, and centrifuged at 8,000 g for 10 min. The pellet and supernatant from this experiment were used as inocula for bioassay on

C. amaranticolor leaves. The final supernatant, which contained the virus, was also used for electron microscopic observations.

3. Purification of TSV using ultrafiltration

Bentonite-clarified sap obtained from TSV-infected plants was filtered through a 1.2 um Millipore filter using a 50 ml glass syringe. The clarified sap was then concentrated using a Millipore immersible CS 30 ultrafiltration unit. The filtration was terminated when the final concentration reached 1% of the original volume.

4. Effect of buffer systems on the purification of TSV from leaf tissue

Twenty g-portions of TSV-infected tomato leaves were harvested, chilled and extracted in a Waring blender using 60 ml of different buffers, namely 0.01 M sodium acetate, 0.01 M sodium citrate, 0.01 M glycine-NaOH, 0.01 M sodium phosphate and 0.01 M potassium phosphate. Na_2SO_3 was added to all buffers to a final molarity of 0.01 M and the pH of each buffer was adjusted to 7.6. Tris-HCl (0.01 M, pH 7.6) buffer was used as a control. Sap was clarified by mixing with bentonite, then centrifuged. Triton X-100 was added to a final concentration of 0.2% and this solution was centrifuged at 100,000 g for 90 min. The pelleted material was suspended in the different buffers to a volume of 1% of the original volume used. The material was then centrifuged at 6,000 g for 10 min. The supernatant liquid was applied to the leaves of C. amaranticolor for infectivity test.

A similar experiment was done for comparison by using different buffers supplemented with 0.002 M $MgCl_2$.

5. Bentonite - Triton X-100 - PEG treatments of infectious sap and differential centrifugation

Twenty g of tomato leaves showing typical symptoms were harvested, chilled and extracted in a Waring blender with 60 ml 0.01 M Tris-HCl buffer or 0.05 M glycine buffer containing 0.002 M $MgCl_2$ and 0.01 M Na_2SO_3 (GMS buffer). The sap was clarified by treatment with bentonite, then 0.2% Triton X-100 was added. PEG 6000 was added to a final concentration of 8% and the mixture was stirred for 30 min at 4°C. After centrifugation at 12,000 g for 15 min, the pelleted material was collected, resuspended in the same original buffer, and then centrifuged at 7,000 g for 5 min to remove insoluble host material. The supernatant liquid was centrifuged at 100,000 g for 90 min in the presence of 0.1% Triton X-100. The virus was then resuspended in 0.6 ml Tris-HCl buffer, diluted five times or 10 times, and applied to C. amaranticolor leaves for infectivity test.

6. Ultraviolet absorption spectrum of purified TSV preparation

For the determination of UV absorption spectrum the virus was scanned at UV wavelengths ranging between 220 and 310 nm using a Beckman DU-8 spectrophotometer with a wavelength scan module and a built-in recorder. Blank samples containing comparable buffer solutions were also scanned at the same wavelength.

C. Results

1. Polyethylene glycol precipitation method

PEG at a concentration of 8.0% precipitated tobacco stunt virus. The virus suspension from pelleted material was relatively more infectious than the sap extracts from tomato or tobacco leaves (Table 3).

2. Differential centrifugation

TSV with relatively high infectivity was isolated by differential centrifugation when the sap from tomato leaves was clarified initially with bentonite. In contrast, TSV infectivity was lost when unclarified sap was subjected to differential centrifugation (Table 3). The final infective preparation contained rod-shaped virus-like particles upon electron microscopic examination (Plate 3).

3. Ultrafiltration

TSV infectivity was concentrated from sap preparations of young tobacco leaves clarified with bentonite. It was not possible to concentrate TSV in sap, as estimated by the infectivity test, without bentonite clarification. This infectivity was lower than that of TSV preparation concentrated by PEG (Table 3).

4. Effect of bentonite and Triton X-100

The partially purified virus preparation from tomato that had

Table 3. Effect of different methods of virus concentration on the infectivity of tobacco stunt virus from tomato and tobacco leaf sap samples as determined by the half-leaf method on Chenopodium amaranticolor.

METHOD OF VIRUS CONCENTRATION	TOMATO		TOBACCO
	Exp. I	Exp. II	Exp. III
PEG	8	11	10
Bentonite Differential Centrifugation	0	0	6
Bentonite Ultrafiltration	-	2	3
Unclarified Sap	1	2	2

Note: In this experiment ~200 g of leaf tissue were homogenized in 600 ml of buffer then divided into 4 equal parts. The final preparation was suspended in 1.5 ml of buffer. The figures represent average lesion numbers on eight half-leaves of C. amaranticolor. The concentration factor is 100 times.

Plate 3. Electron micrograph of a purified preparation of tobacco stunt virus extracted from infected tomato leaves, negatively stained with 2% uranyl acetate. Bar represents 100 nm.

been treated with bentonite and Triton X-100 had a slightly yellowish color, whereas preparations from untreated sap remained dark green in color. The infectivity of the preparation treated with bentonite and Triton X-100 in combination was significantly higher than that of the preparations that had been subjected to treatment with bentonite alone or that of the preparations treated with Triton X-100 alone (Table 4).

When TSV was precipitated with 8% PEG in the presence of Tris-HCl followed by one cycle of differential centrifugation, TSV infectivity was lost. However, in the presence of GMS buffer, TSV infectivity was retained (Tables 5 & 6), and the final TSV suspension had a white fluorescent appearance.

5. Effect of different buffers

When different buffer systems were compared for their effects on TSV infectivity, the highest infectivity of partially purified TSV was obtained in the presence of 0.05 M Tris-HCl (pH 7.6). Only a medium degree of stabilizing effect on TSV infectivity was shown by 0.05 M glycine buffer alone. Although TSV infectivity was stabilized in replicated experiments with 0.05 M potassium phosphate, sodium acetate and sodium citrate, their effect was not as high as that of Tris-HCl or glycine buffer with 0.002 M $MgCl_2$. TSV infectivity was reduced greatly in 0.05 M sodium phosphate (Tables 5 & 6).

When 0.05 M Tris-HCl, glycine and phosphate (Na_2HPO_4-KH_2PO_4)

Table 4. Effect of bentonite and Triton X-100 on the infectivity of tobacco stunt virus from tomato leaf sap as determined by the half-leaf method on Chenopodium amaranticolor.

TREATMENT	PEG		DIFFERENTIAL CENTRIFUGATION		PEG FOLLOWED BY DIFFERENTIAL CENTRIFUGATION	
	EXPERIMENT		EXPERIMENT		EXPERIMENT	
	I	II	I	II	I	II
Bentonite	9.8	7.0	9.2	16.9	0	0
Triton X-100	7	6	4	7	0	0
Bentonite + Triton X-100	179	97	176	152	0	0

Note: In each of these experiments, 20 g of infected leaf tissues were homogenized in 60 ml of buffer. The final virus preparation in each treatment was suspended in 0.75 ml of buffer. The figures represent average lesion numbers on eight half-leaves of C. amaranticolor. The concentration factor is 90 times.

Table 5. Effect of $MgCl_2$ on the infectivity of tobacco stunt virus using different buffers as determined by the half-leaf method on Chenopodium amaranticolor.

BUFFER	WITH 0.002 M $MgCl_2$		WITHOUT $MgCl_2$	
	Exp. I	Exp. II	Exp. I	Exp. II
0.05 M Tris-HCl	191	149	180	152
0.05 M glycine	167	132	79	67
0.05 M phosphate (Na_2HPO_4-KH_2PO_4)	59	30	22	35

Note: In each of these experiments, 20 g of infected leaf tissues were homogenized in 60 ml of buffer. The final virus preparation in each treatment was suspended in 0.75 ml of buffer. The figures represent average lesion numbers on twelve half-leaves of C. amaranticolor. pH of each buffer was adjusted to 7.6. The concentration factor is 90 times.

Table 6. Effect of different buffers (0.01 M, pH 7.6) on the infectivity of tobacco stunt virus as determined by the half-leaf method on Chenopodium amaranticolor.

BUFFER SYSTEM	EXPERIMENT I	EXPERIMENT II
0.01 M Tris-HCl	131	146
0.05 M Tris-HCl	56	29
0.05 M potassium phosphate	22	16
0.05 M sodium phosphate	7	8
0.05 M glycine	95	76
0.05 M sodium acetate	33	46
0.05 M sodium citrate	29	49

Note: In each of these experiments, 20 g of infected leaf tissues were homogenized in 60 ml of buffer. The final virus preparation in each treatment was suspended in 0.75 ml of buffer. The figures represent average lesion numbers on twelve half-leaves of C. amaranticolor. The concentration factor is 90 times.

were supplemented with 0.002 M $MgCl_2$, significant stabilization of TSV infectivity was observed with glycine buffer but not with Tris-HCl nor with phosphate buffer (Table 5). When $MgCl_2$ was combined in certain buffer system there was a significant stabilization of TSV infectivity, as shown in the case of 0.05 M glycine buffer (Table 7).

6. Ultraviolet absorption spectrum of purified TSV preparations

The UV absorption spectrum of TSV preparations were typical of nucleoprotein with 260 to 280 ratio of 1.35 - 1.45, indicating a protein ratio of about 90 - 95% (Fig. 11).

D. Discussion

Several different approaches have been examined in this study in an attempt to devise a reliable purification procedure for TSV. The infectivity of TSV was affected by the method of purification as shown in the Results section. The most satisfactory result was obtained when Triton X-100 was added in combination with bentonite to the sap samples to be clarified. The infectivity of the treated sap samples was significantly higher than that of other sap preparations treated with bentonite or Triton X-100 alone (Table 4). When bentonite alone was added to the sap prior to low-speed centrifugation, the treated sap was still yellowish which suggested that the removal of host material in the sap preparations was not satisfactory. While it

Table 7. Effect of different buffers of 0.01 M on the infectivity of tobacco stunt virus obtained by PEG precipitation followed by one cycle of differential centrifugation as determined by the half-leaf method on Chenopodium amaranticolor.

TREATMENT	GLYCINE-$MgCl_2$ BUFFER		TRIS-HCl BUFFER	
	Exp. I	Exp. II	Exp. I	Exp. II
PEG	191	176	172	163
PEG + Differential Centrifugation	201	222	0	0

Note: In each of these experiments, 20 g of infected leaf tissues were homogenized in 60 ml of buffer. The final virus preparation in each treatment was suspended in 0.75 ml of buffer. The figures represent average lesion numbers from twelve half-leaves of C. amaranticolor. The concentration factor is approximately 90 times.

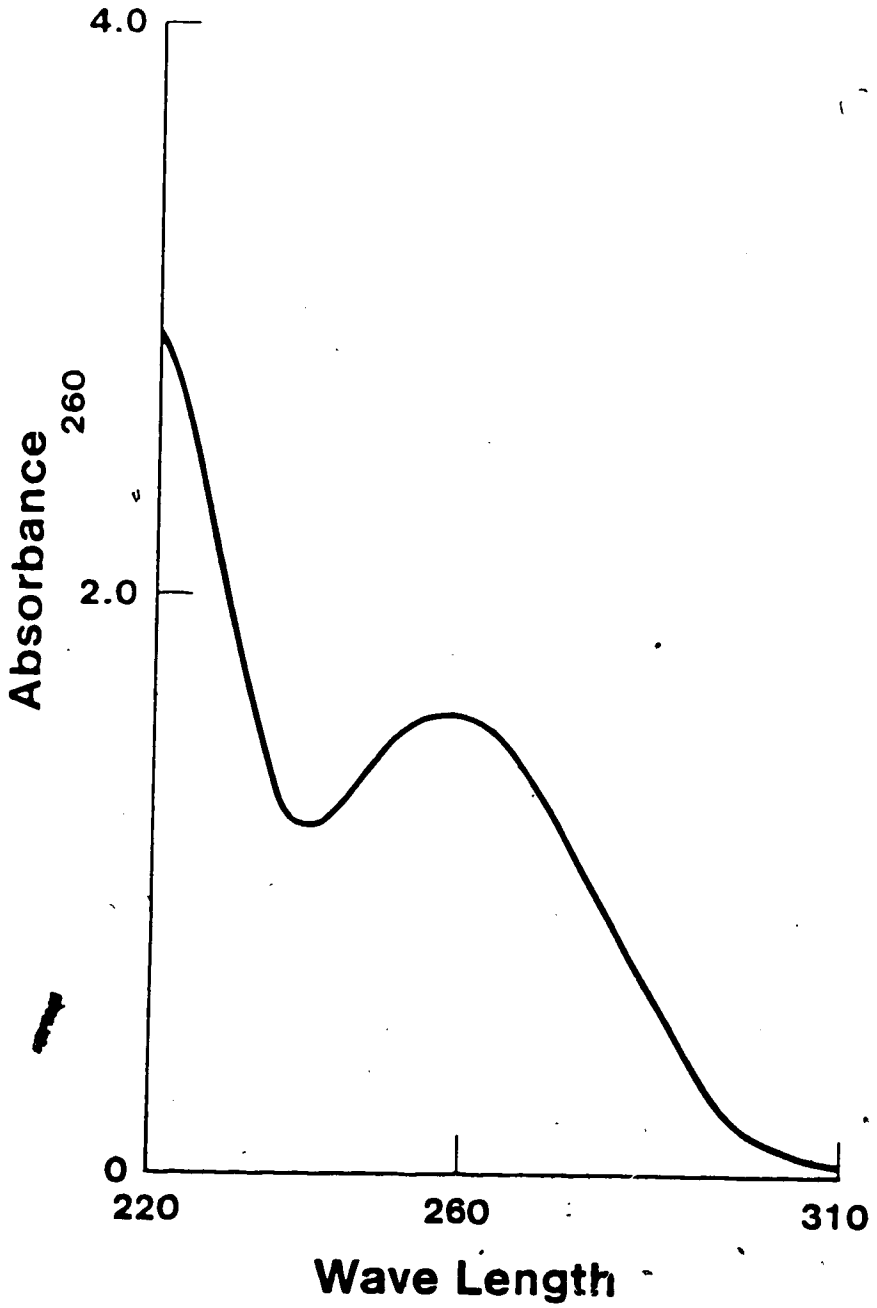


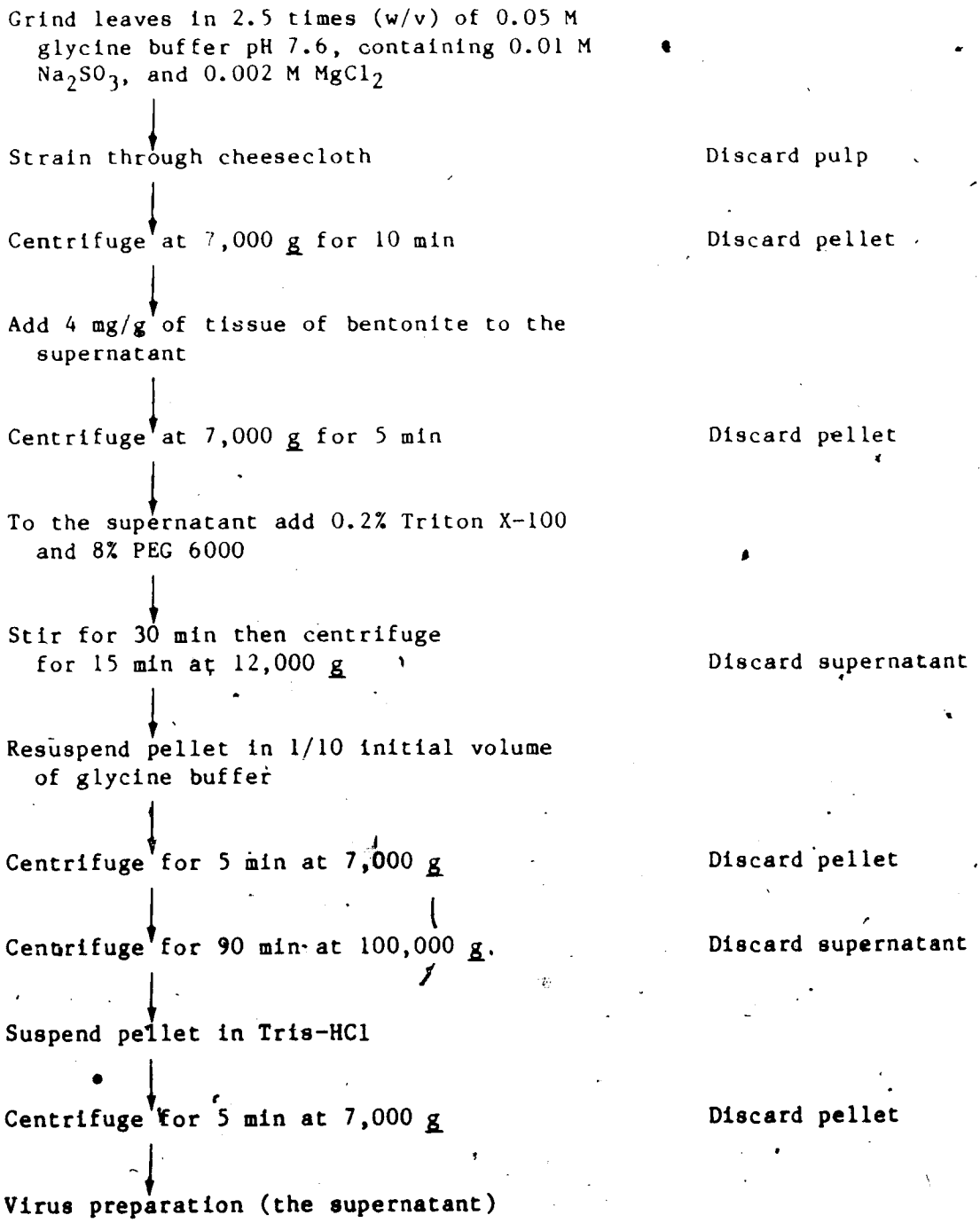
Fig. 11. UV absorbance spectrum of a partially purified preparation of tobacco stunt virus.

is known that the purification of certain closteroviruses was successful when plant sap was clarified with bentonite prior to virus concentration (de Sequira and Lister, 1969; Lister and Hadidi, 1971; Randles et al., 1976; Benn and Murant, 1979), there are also other reports concerning degradation of certain viruses caused by the bentonite treatment of extracted plant sap (Brakke and Van Pell, 1969; Brakke, 1971a). Bentonite was previously reported to inhibit TSV infectivity when it was present in the extracted sap (Alderson, 1975). This inhibition may have resulted from the inadequate amount of bentonite used, an inappropriate time of bentonite introduction into plant sap, or the kind of buffer system used in suspending bentonite. Triton X-100, an anionic detergent, was used to isolate TMV free of host materials (Nozu and Yamaura, 1971). This procedure was later found to be useful in purifying relatively unstable elongated viruses such as plum pox virus (Van Oosten, 1972). More recently, Triton X-100 was incorporated with phosphate buffer for extraction of virus-like, rod-shaped particles from big-vein diseased lettuce leaves (Kuwata et al., 1983). In later work, Kuwata and Kubo (1986) found that phosphate buffer was not adequate for TSV purification. However, Triton X-100 has not been used in combination with other detergents such as PEG or clay such as bentonite. Organic solvents such as chloroform are often used to clarify plant sap prior to virus concentration (Steere, 1959). However, the sap preparation containing TSV that was treated with chloroform was found to be not infectious and the UV absorbance spectrum of the final preparation was not

typical of a nucleoprotein, suggesting that the infectious entity of tobacco stunt was lost by the treatment (Kiriyama, 1975). It should be emphasized that the component, pH, and concentration of buffer system should be carefully selected for mixing with bentonite and Triton X-100. For example, when glycine buffer is present, infectious TSV preparations were obtained from the sap by clarifying with bentonite and Triton X-100, by precipitating TSV with PEG, and then subjecting the preparation to one cycle of differential centrifugation. However, in the presence of Tris-HCl buffer instead of GMS buffer, infectious preparations were obtained only when PEG or differential centrifugation alone was used. In contrast, the infectivity of the final preparations was lost when PEG precipitation of TSV was followed by a cycle of differential centrifugation (Table 7). Although the reason for this drastic loss of TSV infectivity was not determined, it could be due to breakdown of virus particles during purification. Once TSV was purified, however, it appeared that the purified particles were more stable in Tris-HCl buffer than in phosphate buffer (Table 5). This was also reported independently by Kuwata and Kubo, 1986). The actual mechanisms involved in the high recovery of TSV particles from sap preparations subjected to a combination of bentonite and Triton X-100 treatments is not known. However, it appears reasonable to assume that bentonite precipitated some presumptive inhibitor(s) which prevented transmission of TSV, while Triton X-100 disrupted cell organelles and prevented virus absorption to cell debris, thus permitting the final TSV preparations

to retain high infectivity. Thus, for purification of TSV, a flow chart is devised (Fig. 12).

Fig. 12. Flow chart of the purification procedure of tobacco stunt virus.



CHAPTER VI

CHARACTERIZATION OF TSV*

A. Introduction

Recently, virus-like particles were observed in thin sections of tobacco leaf tissue infected with TSV (Kuwata and Kubo, 1981). However, evidence that these particles are infectious TSV is lacking and there is no information available about the properties of these virus-like particles.

In this study, attempts were made to study in vitro properties, physical, and chemical properties as well as serological properties of TSV particles isolated by the procedures in Chapter V.

B. Materials and Methods

1. *In vitro* properties

a. TSV infectivity as compared to virus concentration. For the determination of specific infectivity, the purified virus suspension containing about 6 mg/ml of TSV, as determined by the protein content, was diluted to 2 mg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml and 10 µg/ml, and each diluted sample was then

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* During preparation of this thesis, a manuscript describing TSV was published by Kuwata and Kubo (1986).

inoculated onto C. amaranticolor using the half-leaf method.

b. Longevity of purified virus. The virus preparation at a concentration of 3 mg/ml was divided into three portions. The first was kept at room temperature (about 20°C), the second at 4°C and the third at -60°C. Sampling for the infectivity test, except for the samples kept at -60°C, was done at 2 hr intervals for 8 hr, then at 12 hr intervals for 10 days. For those incubated at -60°C sampling was done at 24-hr intervals.

c. Longevity of TSV in different buffers. The virus preparation was divided into two portions. The first was suspended in 0.01 M Tris-HCl, 0.01 M Na_2SO_3, pH 7.6, the second was suspended in 0.01 M phosphate buffer pH 7.6. Sampling for the infectivity test was done at room temperature at time intervals of 0, 30, 60, 90 and 120 min.

d. Thermal inactivation point. The final virus suspension in 0.01 Tris-HCl 0.01 M Na_2SO_3 was divided into 0.3 ml portions, which were placed in a water bath at various temperatures ranging from 20°C to 90°C with a 5°C difference.

The suspension was maintained at the specified temperature for 10 min then cooled in an ice bath until inoculation. A virus suspension that had been kept at 4°C was used as a control.

2. Physical properties

a. Determination of virus concentration and correlation of absorbance. The purified virus preparation was boiled in 1% SDS, 0.01 M mercaptoethanol and 0.1 M Tris-HCl buffer for 3 min. A

dialysed suspension was then treated with Bradford reagent whereupon 0.1 ml aliquots of sample at different concentrations were added to 2 ml of freshly prepared Bradford reagent and then the optical densities of mixtures were read at 605 nm. The protein concentration of the virus was then calculated using a standard curve for freshly prepared bovine serum albumin (Bradford, 1976).

b. UV absorption spectrum. The purified TSV preparations were subjected to sucrose gradient centrifugation. The fractions from the infectious peaks were collected and dialysed, and the final samples were scanned at a wavelength range between 220 - 330 nm using a Beckman DU-8 spectrophotometer equipped with an automatic scanner module.

c. Virus size and shape. Leaf-dip preparations were made from TSV-infected tomato leaves, tobacco leaves, and from local lesions developed on leaves of C. amaranticolor. The freshly excised leaves were transferred to a microscope slide and cut into pieces about 0.5 mm x 0.5 mm with a razor blade in the presence of Tris-HCl buffer containing 0.01 M Na_2SO_3. Carbon-coated 300 mesh grids were then floated on the sap for 1 min. Excess sap was then rinsed off with distilled water. A 2% uranyl acetate solution was then applied to the grids. The excess stain was removed by blotting against the edge of a filter paper. After air drying, the grids were examined with a Philips EM-200 or a Philips EM-201 transmission electron microscope at 60 or 80 KV. At the same time, samples of O. brassicae zoospores associated wih TSV were obtained by grinding a concentrated suspension (10^7 zoospores/ml of water) in a mortar and pestle and were

negatively stained. TSV-free O. brassicae zoospores were used as a control. Partially purified preparations of TSV as well as highly purified preparations obtained from linear sucrose gradient fractions were similarly stained with 2% uranyl acetate. The sucrose was removed by washing the grid several times with distilled water before staining.

d. Density gradient centrifugation. Linear Ficoll or sucrose gradients of 5-20% were prepared in Beckman SW28-1 tubes using an ISCO density gradient former. Based on protein content of 95%, two hundred ug of purified TSV in 0.2 ml was loaded on top of each tube and centrifuged at 100,000 g (Beckman SW28-1 Rotor) for 2 hr at 4°C. The tubes were scanned with an ISCO model D5 UV monitor by forcing the Ficoll out of the top of the tube by injecting 50% sucrose through the bottom of the tube. Fractions of 0.9 ml each were collected in separate tubes and centrifuged at 120,000 g for 1 hr, the pelleted material was resuspended in 0.1 ml Tris-HCl buffer, then inoculated onto C. amaranticolor with a glass spatula using the half-leaf method. In some experiments only the UV absorbing fractions were collected for subsequent use.

e. Equilibrium centrifugation. Cesium sulfate and cesium chloride gradients were prepared by layering 4 ml of each solution of the following concentrations in a centrifuge tube: 50%, 40%, 30% and 20%. A suspension containing 100 mg of purified virus obtained from the Ficoll gradient was added to the centrifuge tubes, and these were centrifuged for 18 hr at 100,000 g at 4°C using a Spinco SW 28-1 rotor. The tubes were removed and the contents were fractionated

using an ISCO UV-monitor. The material from UV absorbing peaks was pooled, dialysed against 0.05 M GMS buffer for 4 hr at 4°C and scanned with a Beckman DU-8 spectrophotometer and then inoculated onto C. amaranticolor using the half-leaf method. The material recovered from the peak fractions was also analysed for protein and nucleic acid contents by polyacrylamide gel electrophoresis. The density of cesium sulphate, from the UV absorbing peaks, was determined by a refractometer.

3. Chemical properties

a. Extraction of TSV nucleic acid. Purified TSV was suspended in 2X SSC (0.3 M NaCl, 0.03 M NaAc, 0.001 M EDTA, pH 6.8). SDS was added to a final concentration of 1%, and bentonite to a concentration of 0.3%. The virus suspension was shaken in an equal volume of water-saturated, redistilled phenol, and centrifuged at 7,000 g for 10 min. The aqueous phase was removed and shaken again with an equal volume of phenol-chloroform. The nucleic acid in the aqueous layer, separated by centrifugation, was re-extracted with an equal volume of chloroform for 2 min. Following centrifugation, the aqueous phase was removed and the nucleic acid was precipitated by adding two volumes of ethanol and 1/20 volume of 4 M sodium acetate. The nucleic acid was collected by centrifugation at 10,000 g for 10 min, dissolved in sterile water, and stored under alcohol.

b. Polyacrylamide gel electrophoresis. TSV-RNA was analysed by electrophoresis on 5% polyacrylamide gels using Tris-borate buffer, pH 8.3 (Peacock and Dingman, 1968) in an Aquebogue Model 100

apparatus (Aquebogue, NY) under nondenaturing conditions. One to two micrograms of RNA was loaded in each well and then run for 3 hr at 100 volts. The molecular weights were estimated using Hind III fragments of phage DNA as markers, and were based on the assumption that the three TSV RNA species are double-stranded in nature, the evidence for which is given below.

c. Digestion of TSV nucleic acid with ribonuclease. TSV nucleic acid was resuspended in either sterile 0.001 x SSC or in 2 x SSC at a concentration of 1 mg/ml. RNase A (BRL, Garthersburg, MD) which was boiled for 1 min was added to a final concentration of 1 µg/ml and the mixture was incubated at 37°C for 15 min. Reovirus RNA (Dearing strain), provided by Dr. P. Lee (University of Calgary), was used as control. The enzyme was removed by phenol-chloroform extraction, and the aqueous phase was subjected to polyacrylamide gel electrophoresis at 100 V for 4 hr in Tris-borate EDTA buffer, pH 8.3.

To study the kinetics of the action of RNase on TSV nucleic acid, samples of the RNA-RNase mixtures were removed at 2 min intervals and the absorbance at 260 nm (A_{260}) was measured in a Bausch and Lomb 2000 UV spectrophotometer. Reovirus RNA and bacterial ribosomal RNA (rRNA) were used as controls.

d. Electron microscopy of TSV-RNA. RNA from purified TSV were prepared for electron microscopy according to the method of Murant et al. (1981). TSV-RNA at a concentration of 200 µg/ml was denatured by heating at 50°C for 1 hr in 1 M glyoxal, 50% (w/v) dimethylsulphoxide (DMSO), 10 mM sodium phosphate buffer, pH 7.0. Glyoxylated RNA was

diluted 10-20 fold with 1 M Tris-HCl, 100 mM EDTA, pH 8.5. This RNA, at a concentration of 10-20 µg/ml, was diluted 50 fold in a solution 50% (w/v) formamide, 20 mM Tris-HCl, 2 mM EDTA (pH 8.5), containing 40 ug cytochrome C /ml, and spread onto a hypophase containing 20% formamide, 1 mM Tris, 100 mM EDTA, pH 8.5. The RNA was picked up on parlodion films attached to copper grids, stained for 30 sec with 0.05 mM uranyl acetate in ethanol, then rinsed in 90% ethanol, air-dried and rotary shadowed with platinum at an angle of 8°. The grids were examined and photographed in a Philips EM420 electron microscope operated at 100 kV. p-Neo-DNA plasmid (5245 bases) (Pharmacia; Uppsala, Sweden) was used as internal control. This DNA was added to the mixture just prior to spreading. The RNA molecules were traced and measured on photographic prints using a Hewlett Packard digitizer.

e. Melting behavior. Samples of TSV nucleic acid at a concentration of 50 µg/ml in 0.01 x SSC, and 0.1 x SSC were placed in a quartz cuvette in a Varian UV spectrophotometer equipped with a heating cell assembly. The cuvette was heated at a rate of 1°C/min and the absorbance at 260 nm was measured and plotted as a function of time of incubation.

f. Base composition. TSV-RNA and reovirus-RNA (control) were subjected to chromatography on a 25 ml column of Sephadex 100. The RNA preparations were then boiled in 1 N HCl for 1 hr, cooled to room temperature and freeze-dried. The ribonucleotides were separated by thin-layer electrophoresis in 0.25 M ammonium acetate buffer, pH 3.5

according to the method of Bieleski (1965). The nucleotides were detected by spraying the plate with 0.1 M rhodamine B in 60% ethanol. The areas representing different nucleotides were removed, dissolved in water, and their absorbance values at 260 nm were measured in a Bausch and Lomb 2000 UV spectrophotometer.

g. Structural protein. TSV was suspended in distilled water at a concentration of 2-4 mg/ml. Two volumes of dissociating buffer consisting of 0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol were added, and the solution was boiled for 3-5 min. The protein was electrophoresed in 10% acrylamide gels for 4 hr at 35 mAmp using 1.92 M glycine, 0.025 M Tris and 1% SDS (pH 8.3) as a running buffer (Laemmli, 1970). The gels were removed and stained with 0.02% Coomassie brillfant blue dissolved in 20% methanol and 7% acetic acid (v/v). Destaining was done by shaking the gel gently in a solution of 20% methanol, 7% acetic acid in water. The markers used to estimate the molecular weights were phosphorylase B (MW 92,500), bovine serum albumin (MW 68,200), ovalbumin (MW 43,000), carbonic anhydrase (MW 21,500), and soybean trypsin inhibitor (MW 16,000) (BioRad Laboratories; Richmond, CA).

h. Synthesis of cDNA. cDNA was synthesized according to Maniatis et al. (1982). TSV-RNA, which was gel purified, was denatured by incubating with 1 µl of 100 mM methyl mercury hydroxide for 10 min at room temperature. The mercury ions were quenched by the addition of 2 µl of 700 mM 2-mercaptoethanol at room temperature for 5 min. The reaction mixture was completed by the addition of 10

µl of 1 mg pd(N) random primer/ml (Pharmacia), 5 µl of 1 M Tris-HCl (pH 8.3), 7 µl of 1 M KCl, 2 µl of 250 mM $MgCl_2$, 2.5 µl of 20 mM dATP, 20 mM dGTP, 20 mM TTP, and 10 µl of ^{32}P dCTP (100 mCi) or ^{35}S dCTP (100 mCi). Forty units of reverse-transcriptase (Life Science, St. Petersburg, FL) were added and the mixture was incubated at 42°C for 1 hr. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH 8.0). The RNA template was digested by the addition of 25 ul of 150 mM NaOH. The mixture was incubated at 68°C for 1 hr, then neutralized by the addition of 25 µl of 150 mM HCl. The free nucleotides were removed by chromatography on Sephadex G-50.

i. Electroblotting of RNA. RNA preparations were electrophoresed on 6% acrylamide gels for 4 hr at 100 V. The gels were stained with 0.1 mM ethidium bromide. The RNAs were denatured by soaking the gel in 0.05 M NaOH for 30 min. The gels were neutralized by soaking in 1 M Tris-HCl, pH 8.0, and the RNAs transferred to nylon-base nitrocellulose paper (Hybond-N, Amersham, Oakville, Ontario) by electroblotting in a Bio-Rad electroblotter overnight at 120 V. The nitrocellulose membranes were baked at 80°C for 2 hr, then the RNAs were hybridized with ^{32}P-labelled cDNA, or ^{35}S-labelled cDNA, according to the method of Maniatis et al. (1982). The membranes were treated with a prehybridization fluid consisting of 6 x SSC, 0.5% SDS, 50 x Denhardt's solution, 100 mg/ml denatured salmon sperm DNA. Prehybridization was done at 42°C for 2 hr. The prehybridization fluid was removed and replaced by a hybridization solution consisting of 6 x SSC, ^{32}P-labelled denatured cDNA, 50 x Denhardt's solution,

and 0.5% SDS. The hydridization was done overnight at 42°C. The membranes were washed in 2 x SSC and 0.5% SDS at room temperature for 5 min, followed by washing with 0.1 x SSC and 0.5% SDS for 2 hr at 68°C, and air-drying at room temperature. A Kodak XR-5 X-ray film was placed on the membranes in a metal cassette and stored at -70°C for exposure.

j. Viral genome in *Olpidium* zoospores. To isolate O. brassicae zoospores, the roots of infected tobacco plants were immersed in water for 10 min at 20°C. The resulting zoospore suspension was passed through Whatman No. 4 filter paper to remove sand particles or soil debris. The suspension was centrifuged at 5000 g for 10 min (Sorvall RC-2B) at 4°C. The pelleted zoospores were suspended in 0.01 M Tris-HCl, pH 7.5. The zoospore suspension was centrifuged at 12,000 g for 1 min, and resuspended in 1% SDS, 15% glycerol and 0.01% bromophenol blue. Under these conditions the zoospores will lyse releasing the nucleic acid. The resuspended material was electrophoresed on 6% polyacrylamide gel at 100 V for 4 hr. Zoospores from virus-free plants were isolated and treated the same way as a control. The gels were transblotted into a nylon-base membrane and probed with cDNA produced from TSV-RNA.

k. *In vitro* translation of TSV-RNA. In vitro translation of TSV-RNA was done in a sterile wheat germ extract (BRL-8107 SA/SB). The RNA was denatured by the addition of methylmercury hydroxide to a final concentration of 1 mM. Mercury ions were removed by Sephadex-G50 chromatography. Undenatured RNA was used as a control. This RNA

was added to a mixture containing 2.0 µl of 500 mM potassium acetate (pH 7.5), 0.9 µl of 20 mM magnesium acetate (pH 7.5), 3 µl reaction mixture (BRL 8102), 10 µl wheat germ extract (BRL 8108), containing 5 µCi ^{35}S-methionine (Amersham). The mixture was incubated for 60 min at 25°C. The translated protein was analysed by electrophoresis on 10% SDS-polyacrylamide gels (Laemmli, 1970). Reovirus-RNA was used as a control.

4. TSV in leaf cells

For transmission electron microscopy, the leaf tissues from infected tobacco and tomato, as well as local lesions produced on C. amaranticolor 10-11 days after infection, were cut into 2 mm squares and fixed for 4 hr in 0.01 M Tris-HCl buffer containing 0.01 M Na_2SO_3, 2% glutaraldehyde and 2% formaldehyde (pH 7.0). The tissues were then washed overnight at 4°C in 0.01 M Tris-HCl (pH 7.5) followed by three washes of distilled water at 4°C. Postfixation was done at room temperature in 2% aqueous osmium tetroxide followed by three changes of distilled water for 15 min each. The tissues were dehydrated in an ethanol series of 70%, 80%, 90%, 95%, and 100% followed by two changes of propylene oxide of 15 min each. The tissues were transferred into a mixed solution (1:1) of propylene oxide and Araldite mixture (53% Araldite; 45% dodecyl succinic anhydride and 2% DMP-30), and left at room temperature for 24 hr. The tissues were transferred into 100% Araldite and incubated in rubber molds at room temperature for 12 hr followed by 36 hr at 60°C.

The tissues were sectioned on a Reichert OMV2 ultramicrotome using a diamond knife. The sections were collected on carbon-coated grids and were stained in 2% uranyl acetate for 2 hr and post stained in 0.2% aqueous lead citrate pH 13.0 for 1 hr. The sections were examined with a Philips EM200 or EM201 at 60 KV.

5. Serology

Five mg of purified TSV, suspended in 0.5 ml Tris-HCl (pH 7.6) were emulsified in an equal amount of Freund's complete adjuvant (BBL-L-Bioquest), then the mixture was injected intramuscularly twice at weekly intervals into a New Zealand white rabbit. Thereafter weekly intravenous injections were repeated for 5 weeks. The rabbit was bled at weekly intervals for determination of anti-TSV antibody titers. Final bleeding was done 2 weeks after the last injection.

LBVV preparations for use as antigen were purified by the method described in Chapter V.

TSV specific gamma globulin, precipitated by ammonium sulfate, was dialysed against 0.02 M phosphate buffered saline, pH 7.0 (PBS) at 4°C for 4 hr with four changes.

6. Precipitin-ring test

In this study three serological procedures, namely precipitin-ring test, agar gel double-diffusion test and enzyme-linked immunosorbent assay (ELISA), were employed. The antiserum titer was determined by precipitin-ring test carried out in 3 mm diameter micro-

tubes. Antiserum was diluted with 0.85% saline containing 7.5% sucrose. Onto 0.2 ml of antiserum in the test tubes the same volume of antigen was gently layered. The tubes were incubated in a water bath at 37°C and the results were observed after 10 min, 15 min, 30 min, 1 hr and 2 hr. Sap from healthy plants was used as a control. Normal rabbit serum was also used as a control.

7. Agar gel-double diffusion test

Ion-agar at a concentration of 0.7% was autoclaved and poured into a glass petri plate in which a well former module was placed in the center of the plate. Three hundred ul of the diluted antisera (1/128) were placed in the outside wells, while the antigen was placed in the center well. In other experiments SDS was added to the virus preparation to a final concentration of 1% before placing in the center well. The plates were kept at room temperature for 24 hr, then the reaction was photographed.

8. ELISA

Two mg alkaline phosphatase were dissolved and conjugated to 1 ml of the TSV specific globulin, at a concentration of 1 mg/ml (Clark and Adams, 1977). Different concentrations of globulin ranging from 1-30 µg/ml were used to coat the plates. Virus preparations from different sources were used as a source for antigen, namely tomato leaves with or without TSV symptoms, TSV local lesions from C. amaranticolor and inoculated tobacco leaves as well as healthy

controls from the above mentioned plants. The optimum concentration of the globulin as well as the conjugate was then established and used for all tests.

D. Results

1. *In vitro* properties

a. TSV infectivity as compared to virus concentrations. Under these experimental conditions, the minimum amount of TSV concentration needed to initiate an infection was 50 µg/ml (Fig. 13). The highest specific infectivity was achieved at 200 µg/ml, with a slight decrease at 400 ug and 2000 ug, respectively.

b. Longevity of purified TSV. Longevity of purified TSV at room temperature (20°C) was about 8 hr when suspended in 0.01 M Tris-HCl in 0.01 M Na_2SO_3, pH 7.6. No infectivity was found in a preparation kept at room temperature for 12 hr. At 4°C, TSV infectivity was drastically reduced after 8 hours and gradually diminished to zero after 10 days. At -60°C, TSV infectivity was lost after 5 days (Table 8).

When a purified TSV preparation was suspended in sodium phosphate buffer, there was a slight, gradual decrease in infectivity and a complete loss of infectivity after 2 hr (Table 9).

c. Thermal inactivation point. The minimum temperature required to inactivate TSV was found to be between 55-60°C for 10 min (Table 10).

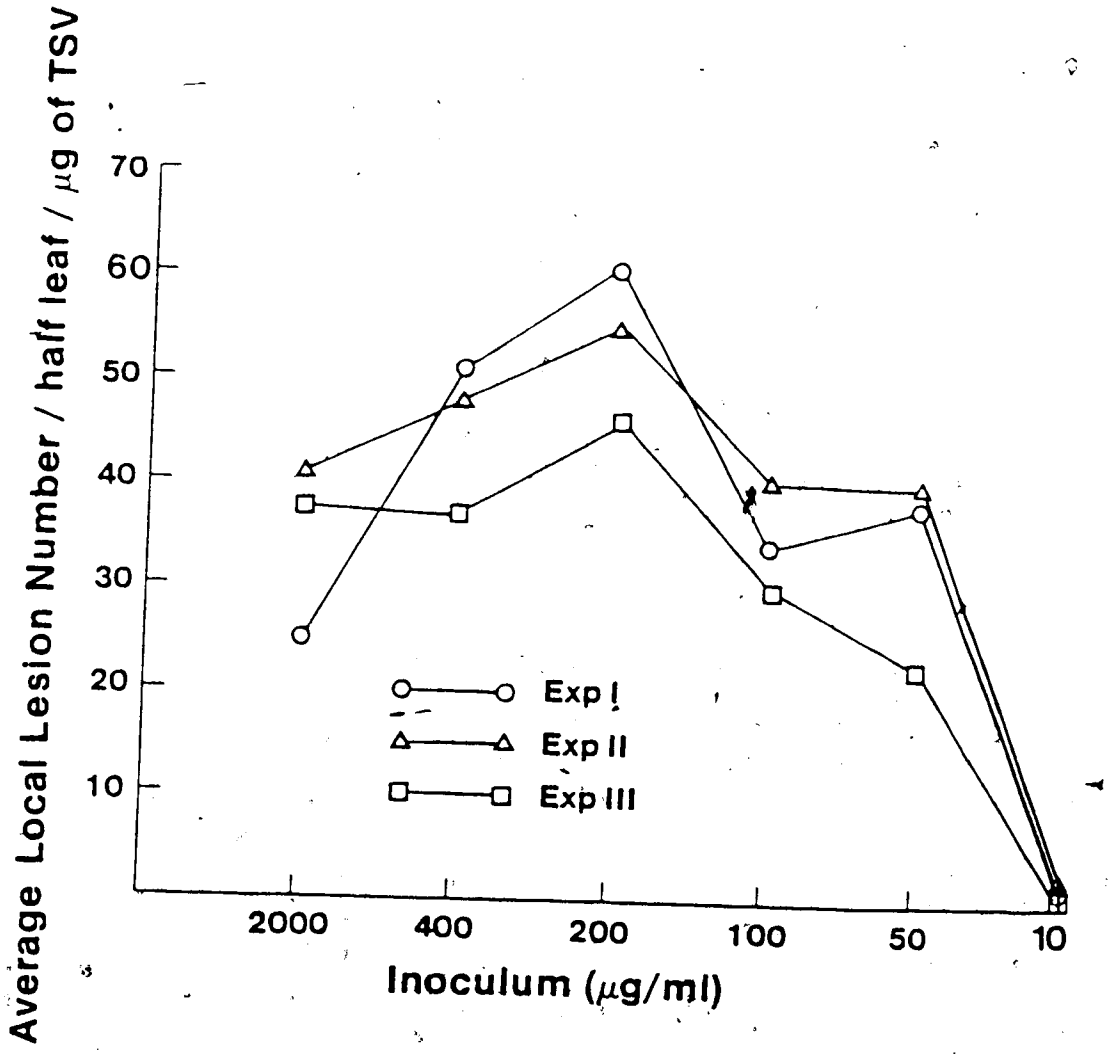

Fig. 13. The relationship of purified tobacco stunt virus concentration on the number of local lesions on Chenopodium amaranticolor as determined by the half-leaf method. Figures represent average local lesion on 8 half-leaves.

Table 8. Longevity of purified tobacco stunt virus at different temperatures as determined by the half-leaf method on *Chenopodium amaranticolor*.

TIME (hr)	TEMPERATURE (°C)					
	20°C		4°C		-60°C	
	Expt I	Expt II	Expt I	Expt II	Expt I	Expt II
0	193	139	193	139	193	139
2	72	133	145	137	N/T*	N/T
8	21	32	113	96	N/T	N/T
12	0	0	62	59	60	39
24	0	0	31	20	N/T	N/T
36	0	0	19	17	58	41
48	0	0	1	14	N/T	N/T
72	0	0	10	15	31	22
96	0	0	12	9	6	2
120	0	0	11	12	0	0
144	0	0	9	11	0	0
168	0	0	5	6	0	0
212	0	0	3	12	0	0
235	0	0	0	6	0	0

Note: The figures represent average local lesions on 8 half-leaves of *C. amaranticolor*.

* N/T, not tested.

Table 9. Longevity at room temperature of purified tobacco stunt virus suspended in different buffers as determined by the half-leaf method on Chenopodium amaranticolor.

SUSPENSION BUFFER	EXPT. I			EXPT. II		
	0 hr	1 hr	2 hr	0 hr	1 hr	2 hr
0.01 M Tris-HCl pH 7.6	193	117	72	139	122	89
0.01 M sodium phosphate pH 7.6	140	72	2	133	45	0

Note: The figures represent average local lesions of 8 half-leaves of C. amaranticolor.

Table 10. Thermal inactivation point of purified tobacco stunt virus in Tris-HCl buffer (pH 7.6) as determined by the half-leaf method on Chenopodium amaranticolor.

TREATMENT (°C)	EXPERIMENT		
	I	II	III
30	37	41	22
35	31	19	16
40	22	26	18
45	11	19	7
50	8	10	9
55	4	2	1
60	0	0	0
65	0	0	0

Note: The figures represent average local lesions on 8 half-leaves of C. amaranticolor.

2. Physical properties

a. UV absorption spectrum. UV absorption spectrum from purified virus preparations was typical of a nucleoprotein with a maximum absorbance at 261 nm and a minimum absorbance at 244 nm. The 260 to 280 ratio was found to be 1.35 - 1.45 when the virus was prepared in Tris-HCl buffer pH 7.6, and 1.50 - 1.65 when it was prepared in phosphate buffer pH 7.6 (Fig. 14), indicating breakdown of virus particles.

b. Virus size and shape. From electron microscope observations, TSV was found to be a rod-shaped virus with a width of 20 nm, and a length that ranges between 50 and 500 nm when 200 particles were measured on negatives using TMV as an internal standard (Fig. 15). Similar particles were found in leaf dip preparations from leaf tissues of tobacco and tomato, and an extract sample from O. brassicae zoospores that were known to be associated with TSV infectivity (Plates 3, 4 & 5).

c. Density gradient centrifugation. After sucrose or Ficoll gradient centrifugation, TSV was found to produce four UV-absorbing bands, a large one about 3 cm from the meniscus, and three small bands in the lower region (Fig. 16). The infectivity was associated with the band represented by fraction numbers 6 to 9.

d. Equilibrium centrifugation. TSV formed three bands upon scanning with UV light after cesium sulfate equilibrium centrifugation of 1.17 g/ml, 1.370 g/ml and 1.610 g/ml (Fig. 17). Virus particles were not detected by EM in the top band, nor was any nucleic

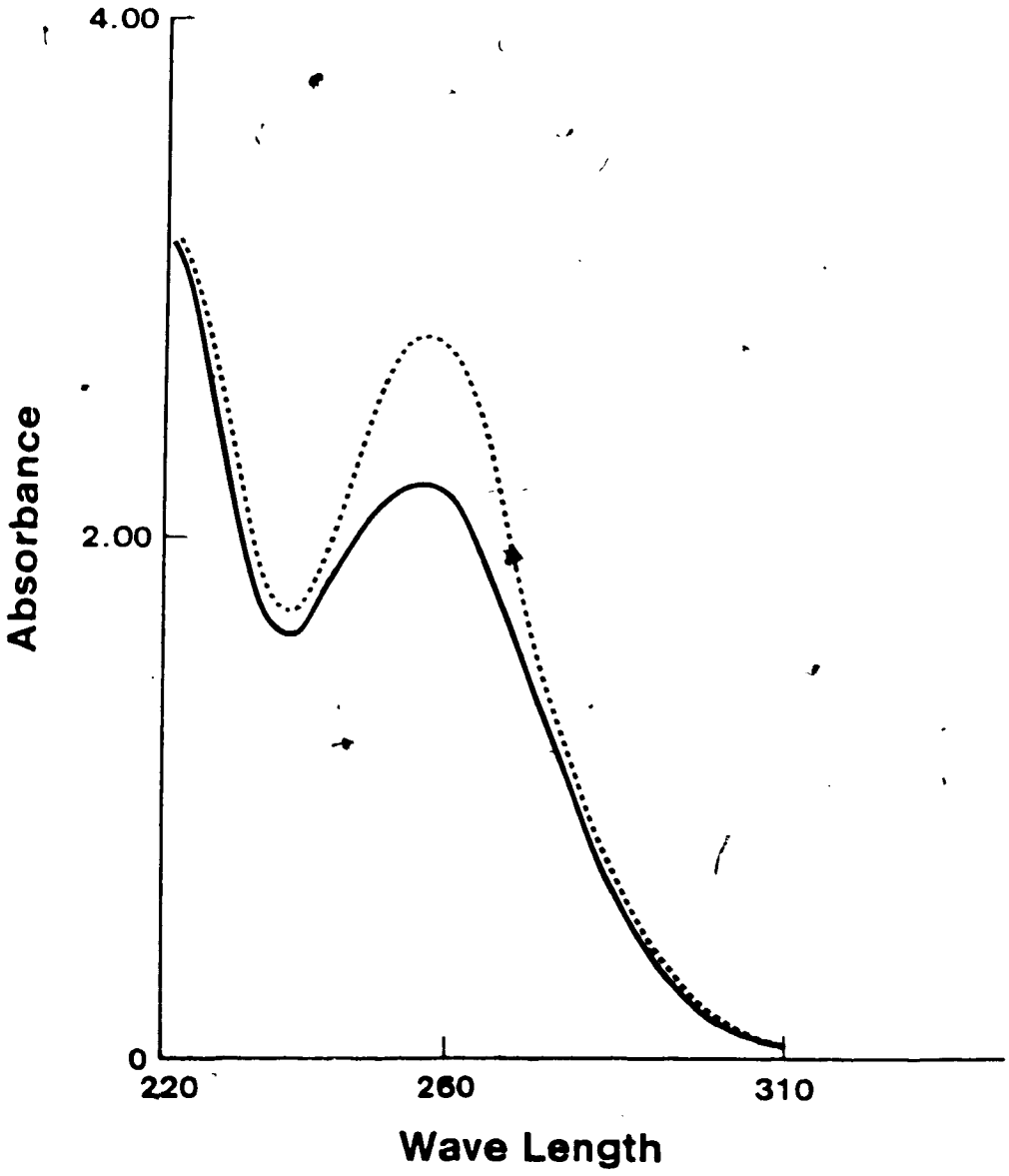

Fig. 14. UV absorbance spectrum of a purified preparation of tobacco stunt virus extracted with different buffers.

The solid line represents preparations done in Tris-HCl buffer. The broken line represents preparations done in phosphate buffer.

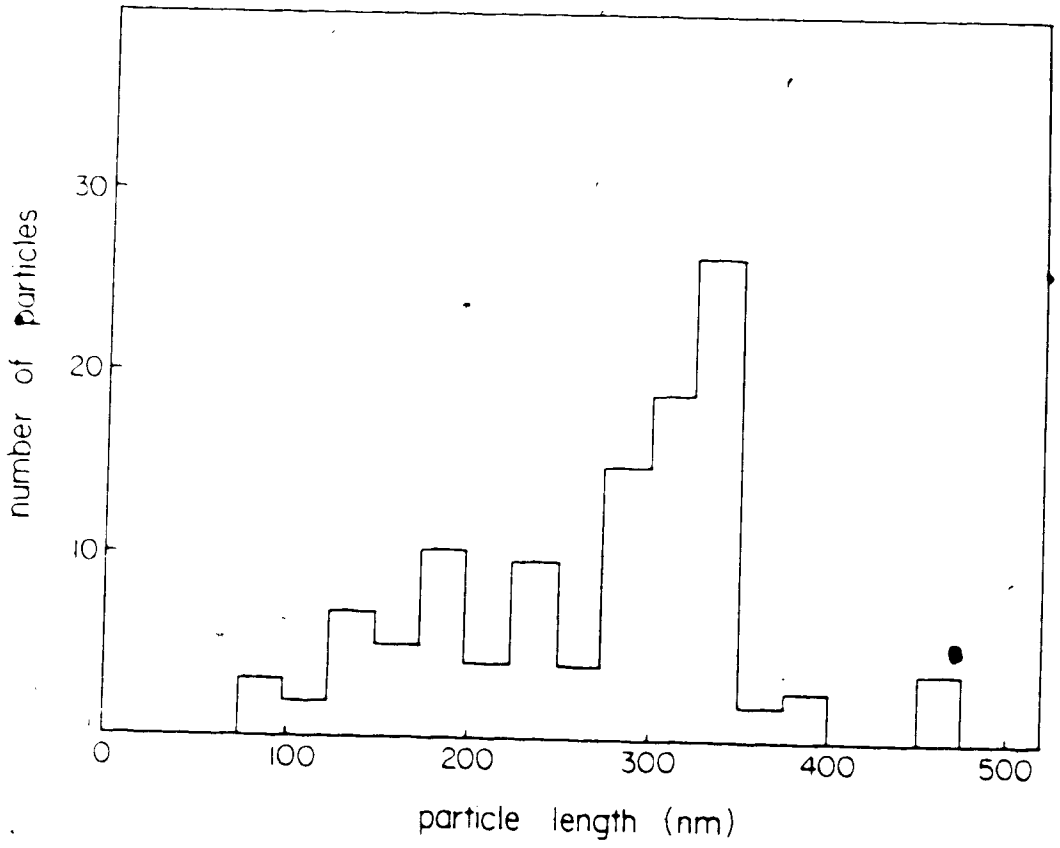

Fig. 15. Length distribution of virus particles from leaf-sap preparations of tomato leaves infected with tobacco stunt virus. Measurements were made on the film negative using an inverted microscope.

Plate 4. Electron micrograph of a leaf-dip preparation from tomato leaf tissue infected with tobacco stunt virus, negatively stained with 2% uranyl acetate. Bar represents 100 nm.

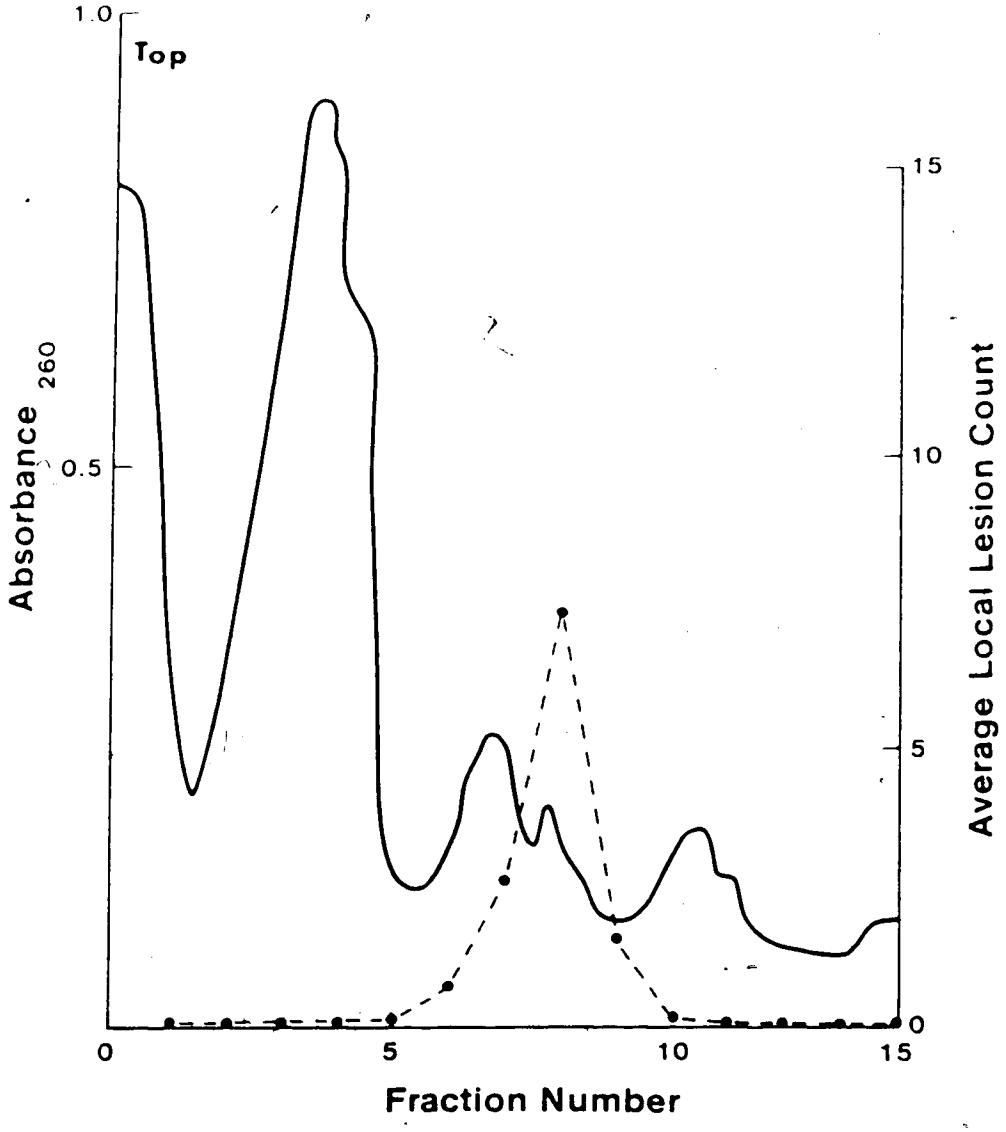

Fig. 16. Relationship between UV absorption spectrum and the infectivity of tobacco stunt virus (dotted line), after sucrose density gradient centrifugation on 5-20% sucrose gradient. Sedimentation is from left to right.

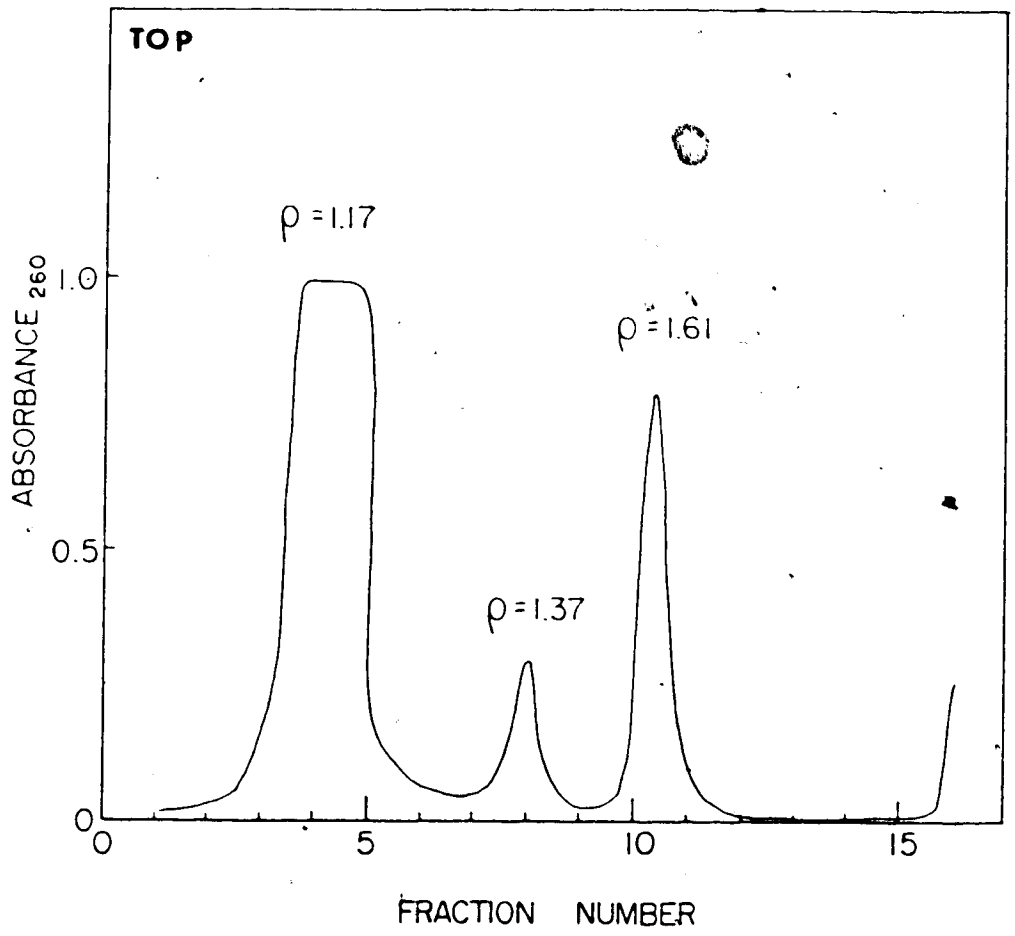

Fig. 17. UV absorption spectrum after cesium sulfate density gradiant centrifugation. Sedimentation is from left to right.

ρ = 1.17 represents coat protein.

ρ = 1.39 represents TSV particles.

ρ = 1.61 represents TSV-RNA.

acid detected by gel electrophoresis. However, TSV coat protein was associated with the top band when analysed on polyacrylamide gels. No infectivity was detected from any of the materials recovered from the three bands when tested on C. amaranticolor. However, virus-like aggregates were associated with the second band (Plate 6). It had coat protein of 51,000 daltons. The third band contained three RNA species of different molecular weights as observed when analysed on polyacrylamide gel. On cesium chloride, a top band was formed near the miniscus band and a precipitate at the bottom of the tube.

3. Chemical properties

a. Gel electrophoresis of TSV-RNA. Upon polyacrylamide gel electrophoresis, the nucleic acid extracted from TSV preparation that was subjected to sucrose gradient, separated into three segments with estimated molecular weights, 4.2×10^6, 1.8×10^6 and 1.2×10^6, respectively (Plate 7).

b. Effect of ribonuclease. The sensitivity of TSV nucleic acid to RNase A was monitored by the increase in hyperchromicity. At a low salt concentration (0.001 x SSC) there was a 20% increase in hyperchromicity. This increase was linear for up to 10 min after incubation, then leveled off (Fig. 18). This profile is similar to those obtained using bacterial ribosomal RNA or reovirus RNA under similar conditions. At a higher salt concentration (2 x SSC), TSV nucleic acid showed an increase in hyperchromicity of only 5% (Fig. 19); as compared to ribosomal RNA which showed a 25% increase in

Plate 5. Electron micrograph of a preparation of tobacco stunt virus from viruliferous zoospores of Olpidium brassicae, negatively stained with 2% uranyl acetate. Bar represents 100 nm.

Plate 6. Electron micrograph of a preparation of tobacco stunt virus obtained from cesium sulfate equilibrium centrifugation, and stained with 2% uranyl acetate. Bar represents 100 nm.

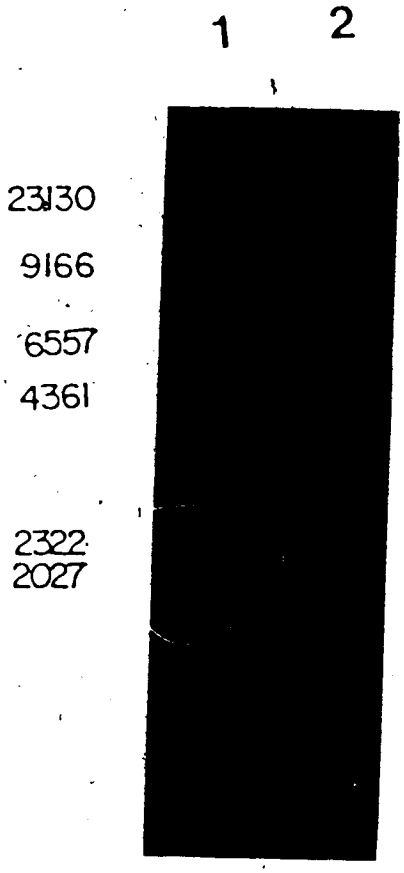

Plate 7. Polyacrylamide gel electrophoresis of tobacco stunt virus RNA. Electrophoresis was carried on 5% acrylamide gels for 3 hr at 100 V in buffer. The gels were stained with 5 µg/ml ethidium bromide for 5 min.

Lane 1, λ DNA cut with Hind III. Numbers at the left indicate base pair.

Lane 2, Tobacco stunt virus RNA

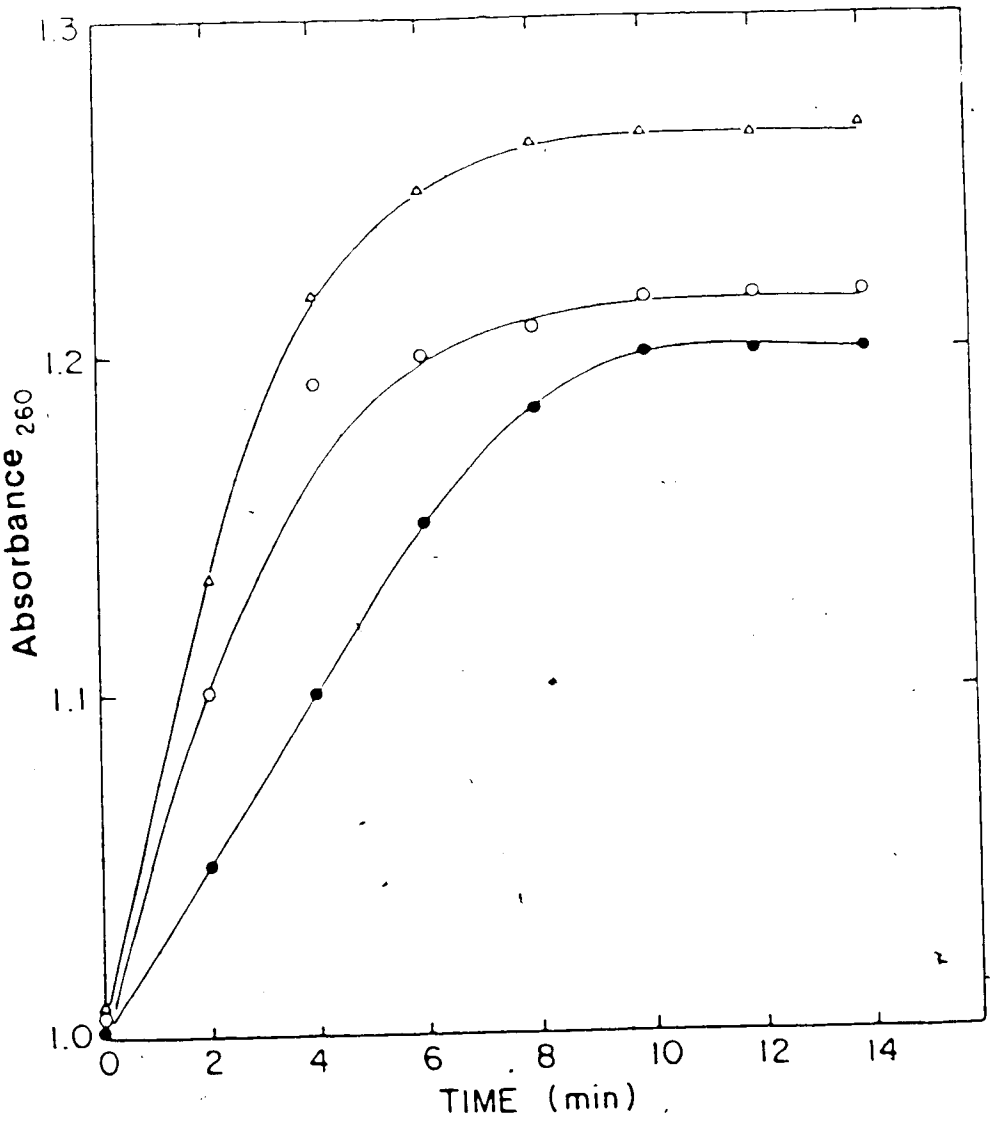

Fig. 18. The effect of a low salt concentration on the digestion of tobacco stunt virus RNA with RNase A. One milligram of TSV RNA (●—●), ribosomal RNA (△-△) or reovirus RNAs (○—○) in 0.001 x SSC were incubated at 37°C with 1 μg RNase A/ml, and the absorbance were monitored at 2 min intervals.

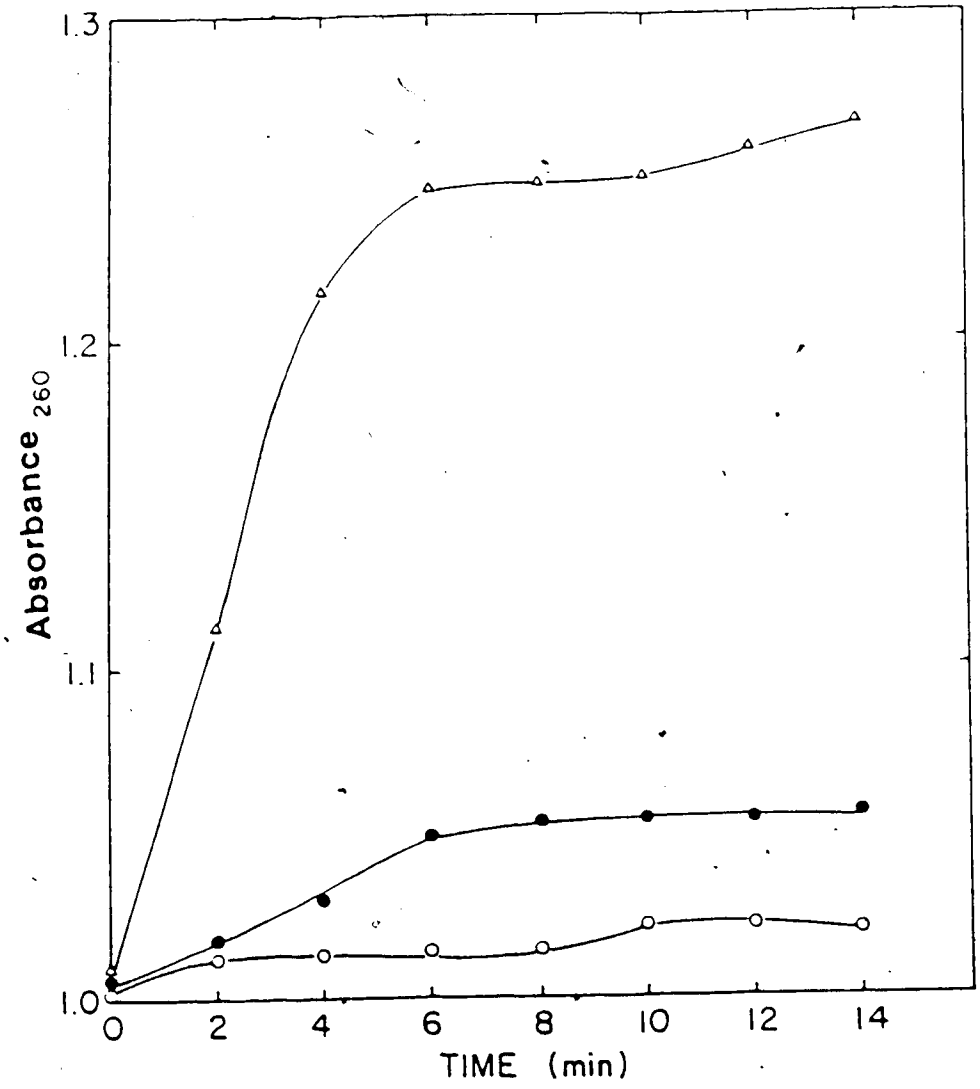

Fig. 19. The effect of a high salt concentration on the digestion of tobacco stunt virus RNA with RNase A. One milligram of TSV RNA (●–●), ribosomal RNA (△–△) or reovirus RNAs (o–o) in 2 x SSC were incubated at 37°C with 1 μg RNase A/ml, and the absorbance was monitored at 2 min intervals.

hyperchromicity. Reovirus RNA showed a 2% increase under this condition.

When the products of RNase digestion were analyzed by gel electrophoresis, TSV nucleic acid and reovirus RNA were completely digested under low salt conditions (0.001 x SSC) incubated at room temperature for 30 min. At higher salt concentrations (2 x SSC), the three TSV nucleic acid species appeared to exhibit differential susceptibilities to RNase A: the largest species was totally resistant, whereas the two smaller species were only partially resistant to RNase A (Plate 8, lane 3). Reovirus RNA was totally resistant to RNase A at high salt concentration.

RNase III, which is specific for double-stranded RNA, completely digested TSV nucleic acid as well as reovirus RNA in the two salt concentrations tested (Plate 8). TSV nucleic acid was not affected by DNase, nor was reovirus RNA (Plate 8).

Taken together, these results indicate that the TSV nucleic acid is similar to reovirus RNA in terms of RNase and RNase III sensitivity. Hence, TSV nucleic acid is most likely double-stranded RNA.

c. Melting curve. TSV RNA in 0.01 x SSC exhibited a melting profile with a Tm value of about 62°C with a maximum hyperchromicity increase of about 20% (Fig. 20). At a higher salt concentration (0.1 x SSC) the melting point was located at 75°C (Fig. 21). At 1 x SSC, the melting point was over 100°C (not shown). The melting points of reovirus RNA, under the same conditions, were 75°C, 85°C, and over

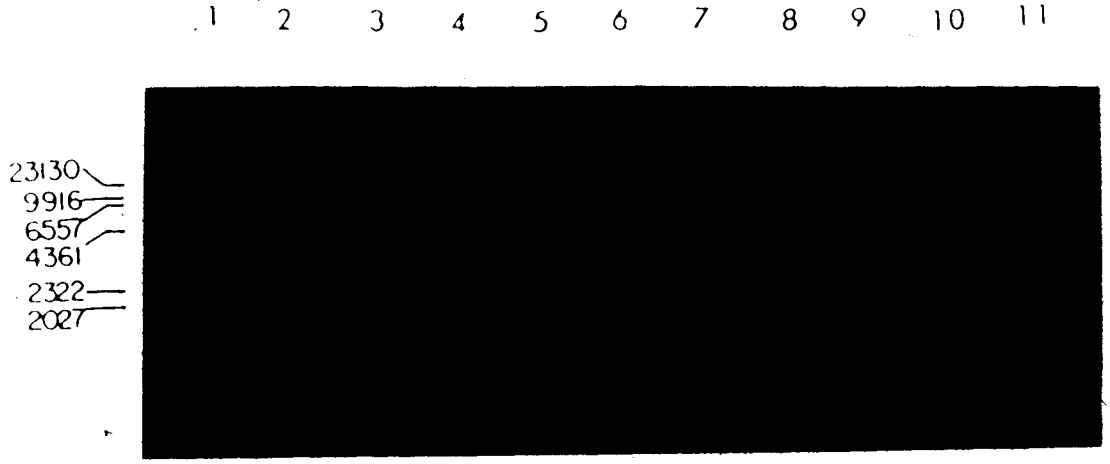

Plate 8. Polyacrylamide gel electrophoresis of TSV RNA treated with RNase at different salt concentrations. Electrophoresis was in 6% PAGE for 4 hr at 200 V. Bands were visualized by ethidium bromide.

Lane 1 - λ DNA cut with Hind III as marker. The numbers on the left indicate base pairs.

Lane 2 - Untreated TSV-RNA in Tris-EDTA buffer.

Lane 3 - TSV-RNA treated with 1 ug RNase A/ml in 2 x SSC for 30 min at 37°C.

Lane 4 - TSV-RNA treated with 1 ug RNase A/ml in 0.001 x SSC for 30 min at 37°C.

Lane 5 - TSV-RNA treated with RNase III for 30 min at 38°C.

Lane 6 - TSV-RNA treated with DNase in Tris-EDTA buffer for 30 min at 37°C.

Lane 7 - Untreated reovirus RNA (buffer) in Tris-EDTA.

Lane 8 - Reovirus RNA treated with 1 ug RNase A/ml in 2 x SSC for 30 min at 37°C.

Lane 9 - Reovirus RNA treated with 1 ug RNAse A/ml in 0.001 x SSC for 30 min at 37°C.

Lane 10 - Reovirus RNA treated with RNase III for 30 min at 37°C.

Lane 11 - Reovirus RNA treated with DNase in Tris-EDTA buffer for 30 min at 37°C.

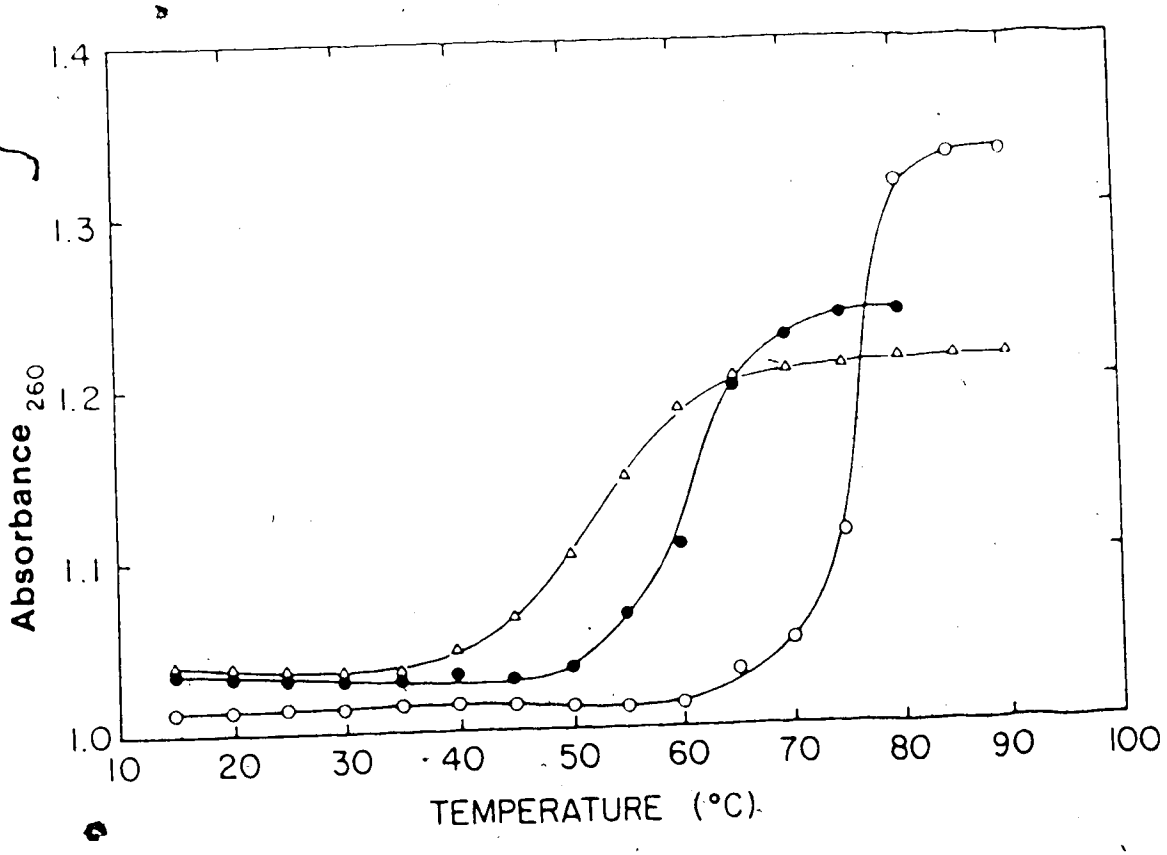

Fig. 20. Denaturation curve of tobacco stunt virus-RNA (●–●), ribosomal RNA (△–△) and reovirus RNAs (o–o) in 0.01 x SSC.

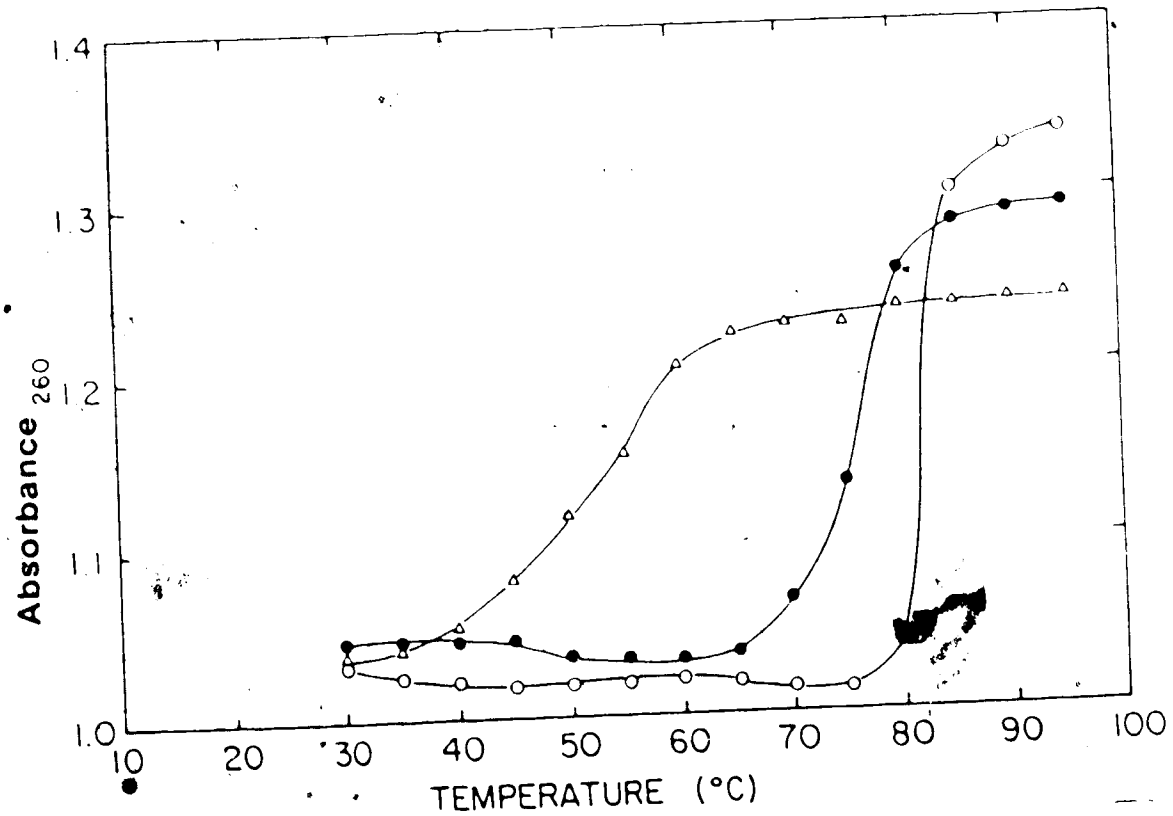

Fig. 21. Denaturation curve of tobacco stunt virus RNA in 0.1 x SSC (●–●), ribosomal RNA (Δ–Δ) and reovirus RNA (o–o).

100°C, respectively, with a maximum hyperchromicity of 32% (Figs. 20 and 21). This suggests that TSV-RNA is not fully double-stranded or it contains extensive mismatching.

d. Electron microscopy. As revealed by electron microscopy, TSV-RNA contained several fragments ranging in size between 1,000-5,700 base pairs with three major species corresponding to 5,685, 2,680, and 2,064 base pairs, respectively (Plate 9, Fig. 22). Assuming that the TSV-RNA is a double-stranded molecule, the estimated molecular weights are 3.9×10^6, 1.9×10^6, and 1.4×10^6, respectively.

e. Base composition. Analyses of total TSV nucleic acid indicated that guanosine and cytosine occur in an equal amount having a G/C ratio = 1.02 and with a G + C content of 55.2%. There were also equal amounts of adenosine and uracil (Table 11).

f. Molecular relationship of TSV-RNA segments. Segments of TSV-RNA were found to be closely related to each other. When cDNA was synthesized from the large component of 5,685 base pairs and used as a probe, it was found that the three bands with 5,685, 2,680, and 2,064 base pairs reacted with the same intensity (Plate 10). The large segment reacted with the same intensity when the middle component or the small component were used as probes. The small and the middle components bore little homology with each other (Plate 10). Thus, one can conclude that the smaller RNA species are breakdowns product of the larger RNA molecule, or they could be closely related to each other.

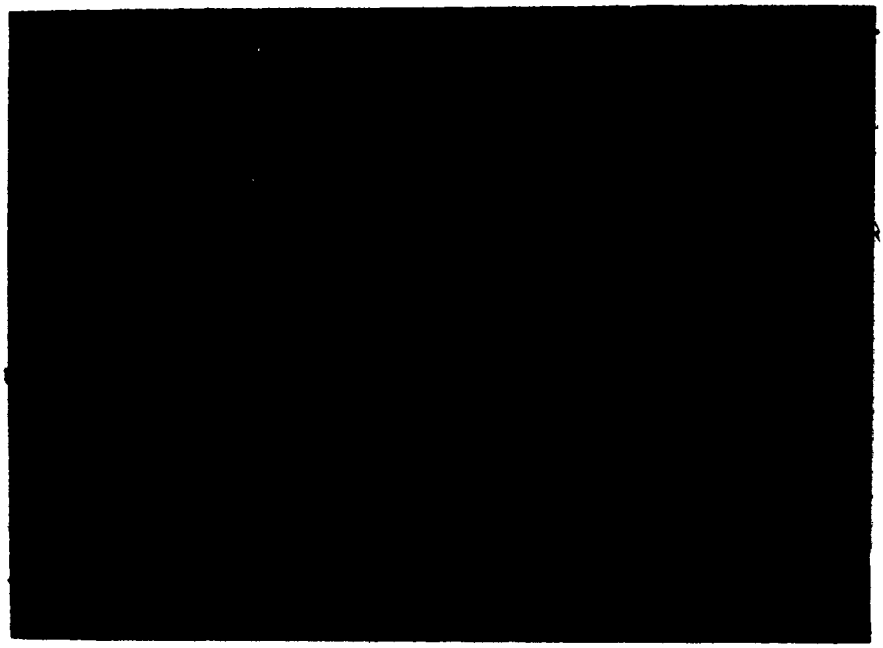

Plate 9. Electron micrograph of TSV RNA stained in 5 x 10^{-5} M uranyl acetate, and rotary shadowed with platinum. Bar represents 1 µm.

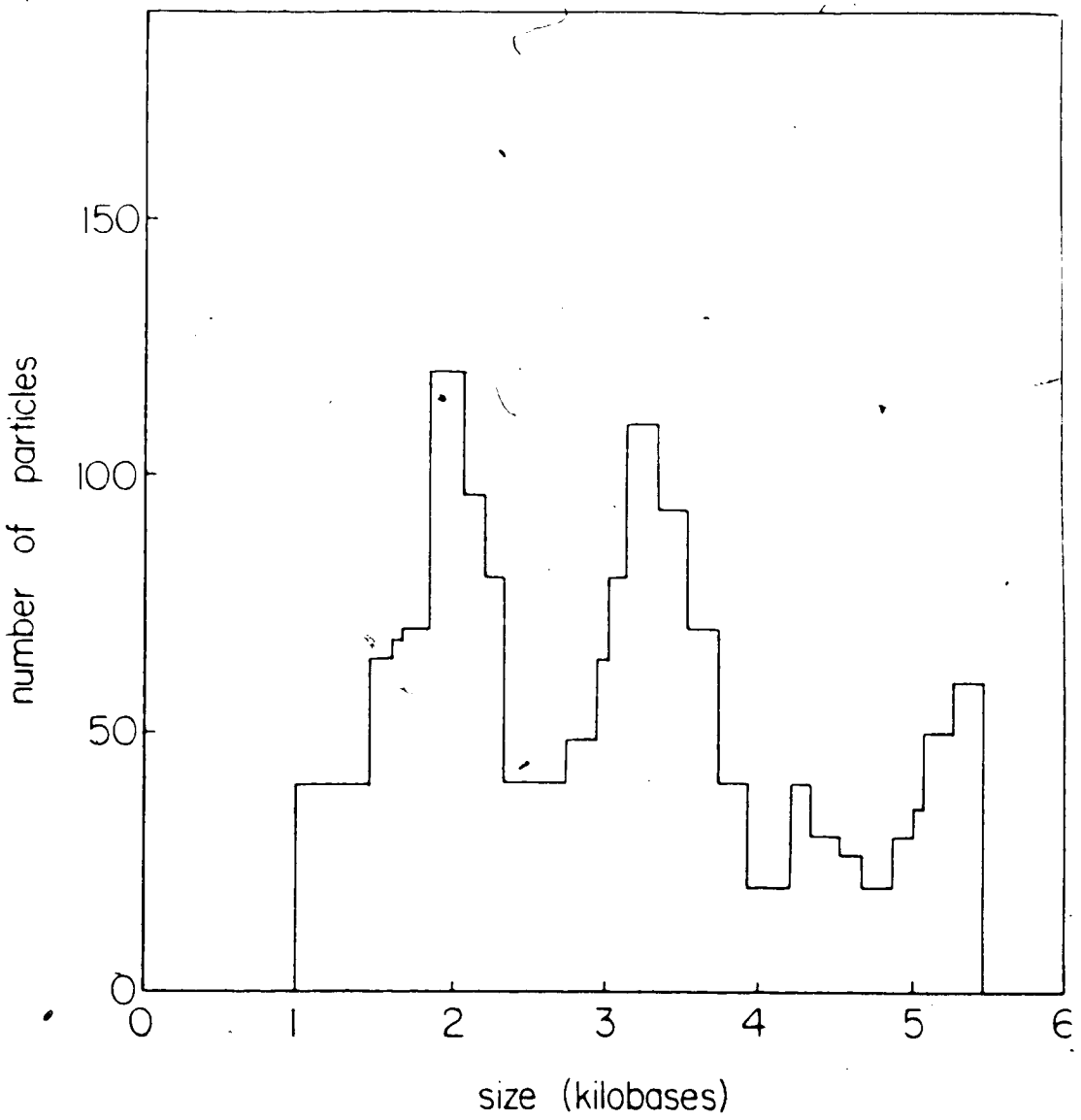

Fig. 22. Length distribution of TSV-RNA measured from electron micrographs. Neo-plasmid was used as an internal control.

Table 11. Base composition of tobacco stunt virus RNA.

	PERCENTAGE OF NUCLEOTIDE			
	Guanine	Adenosine	Cytosine	Uracil
TSV-RNA				
A	27.9	23.1	26.8	22.2
B	27.2	23.0	27.5	22.3
Average	27.51	23.05	27.65	22.25
Reovirus-RNA				
A	21.3	27.9	22.0	28.8
B	21.2	27.8	22.1	28.7
Average	21.25	22.85	22.05	28.75

A, Experiment one.

B, Experiment two.

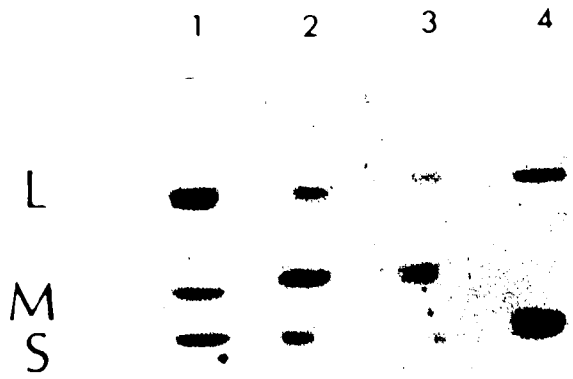

Plate 10. The molecular relationship of the different TSV-RNA segments. Total TSV-RNA was resolved on 6% polyacrylamide gel and transferred to nitrocellular paper. Transblotted RNA was hybridized to ^{35}S-labelled cDNA derived from the different RNA segments.

Lane 1, RNA hybridized to cDNA from total TSV RNA.

Lane 2, RNA hybridized to cDNA from the large fragment.

Lane 3, RNA hybridized to cDNA from the medium fragment.

Lane 4, RNA hybridized to cDNA from the small fragment.

g. Viral genome in *O. brassicae*. The large segment of 5685 base pair of TSV-RNA was found to be present in Olpidium zoospores. In addition, a small molecular weight nucleic acid of about 0.4 K daltons was found in both TSV-carrying Olpidium as well as TSV-free Olpidium (Plate 11). This small nucleic acid was not found in TSV-RNA preparation.

h. Structural protein. The structural protein of TSV migrated as a single band in 10% SDS-polyacrylamide gels (Plate 12). The estimated molecular weight of this protein was 51,000 based on the estimation method of Weber & Osborn (1972).

i. *In vitro* translation product. Under the conditions discussed in the Materials and Methods, denatured TSV-RNA directed the synthesis of three major polypeptides of molecular mass of 68 KD, 51 KD, and 28 KD respectively. The large RNA segment directed the synthesis of two polypeptides, 68 KD and 51 KD, while the small RNA directs the synthesis of a 28 KD polypeptide, and the medium-sized RNA did not direct the synthesis any polypeptide. When the translation products were immunoprecipitated with TSV-antibody, followed by analysis on SDS-PAGE, the 51 KD and the 28 KD products were immunoprecipitable (Plate 13).

4. TSV in leaf cells

Virus-like particles measuring between 250-350 nm in length were found in thin sections of leaf tissue bearing local lesions incited by TSV on C. amaranticolor. These particles were found in bands

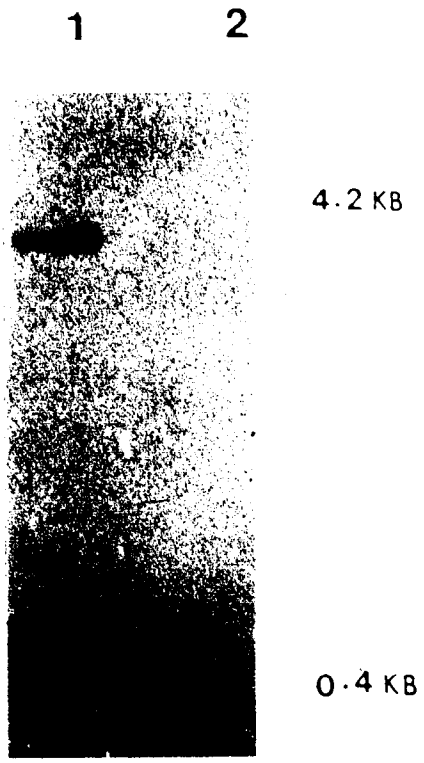

Plate 11. Identification of TSV-RNA from Olpidium nucleic acid by molecular hybridization. Total nucleic acid from Olpidium zoospores were resolved on 6% polyacrylamide gel and transferred to nitrocellulose paper. Transblotted RNA was hybridized to cDNA derived from total TSV-RNA. λ DNA was used as a marker; the λ DNA bands were marked prior to x-ray.

Lane 1, Hybridization to nucleic acid from TSV-carrying zoospores.

Lane 2, Hybridization to nucleic acid from healthy zoospores.

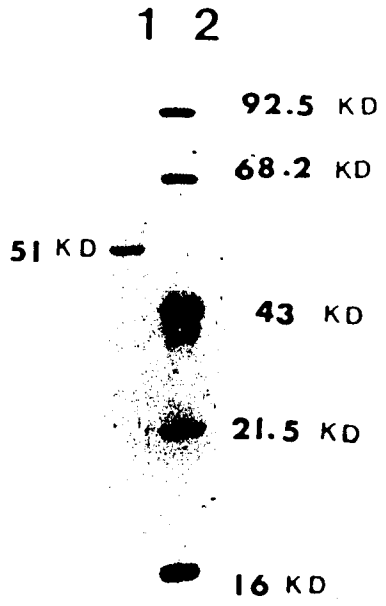

Plate 12. SDS polyacrylamide gel electrophoresis of the coat protein of tobacco stunt virus. Reference bands on the right lane are shown from top to bottom: phosphorylase B (92.5 KD), bovine serum albumin (68.2 KD), ovalbumin (43 KD), carbonic anhydrase (21.5 KD), and soybean trypsin inhibitor (16 KD).

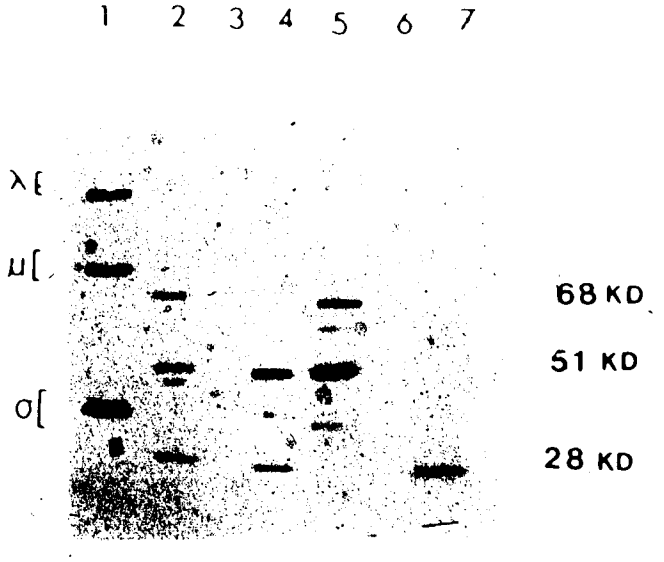

Plate 13. Fluorogram of SDS-polyacrylamide gel electrophoresis of tobacco stunt virus RNA translation products in wheat germ extracts. The gel was dried and X-ray film was placed on top and exposed overnight at -70°C.

Lane 1, Reovirus protein marker.

Lane 2, Translation product of denatured TSV-RNA.

Lane 3, Translation product of undenatured TSV-RNA.

Lane 4, RIp analysis of TSV-RNA translation product.

Lane 5, Translation product of the large RNA segment.

Lane 6, Translation product of the medium RNA segment.

Lane 7, Translation product of the small RNA segment.

formed by side to side aggregation of rod-shaped particles in the cytoplasm and mostly in regular arrangements (Plate 14).

5. Serology

There was a linear increase in antibody production that reached a maximum titer of 1/512 after 6 weeks (Fig. 23). In the ring precipitin test the minimum amount of TSV needed to produce positive reaction was about 0.3 mg/ml. It was not possible to detect the virus from the crude sap using this test. The final titer from the agar gel double diffusion test was 1/256 (Plate 15). In the double diffusion test, there was no reaction to sap extracted from healthy tomato, tobacco and C. amaranticolor plants. It was also possible to obtain a positive reaction to LBVV to a final titer of 1/256, in both the ring precipitin test and the agar gel double diffusion test.

Using ELISA, it was possible to obtain a positive reaction to TSV at a concentration of up to 50 µg/ml. Positive reactions were also obtained to crude sap from systemically infected tomato or tobacco or local lesions from C. amaranticolor. The optimum conjugate concentration to produce positive color was 6 ug/ml. When the conjugate concentration exceeded 15 µg/ml, some non-specific coloration occurred.

D. Discussion

In classical virology, certain physical properties were used for preliminary classifications of a given virus. These include thermal

Plate 14. Electron micrographs of a thin section of a leaf tissue bearing a local lesion of Chenopodium amaranticolor after inoculation with tobacco stunt virus. Arrays of virus-like particles are shown in the cytoplasm of a mesophyll cell. Bar represents 100 nm.

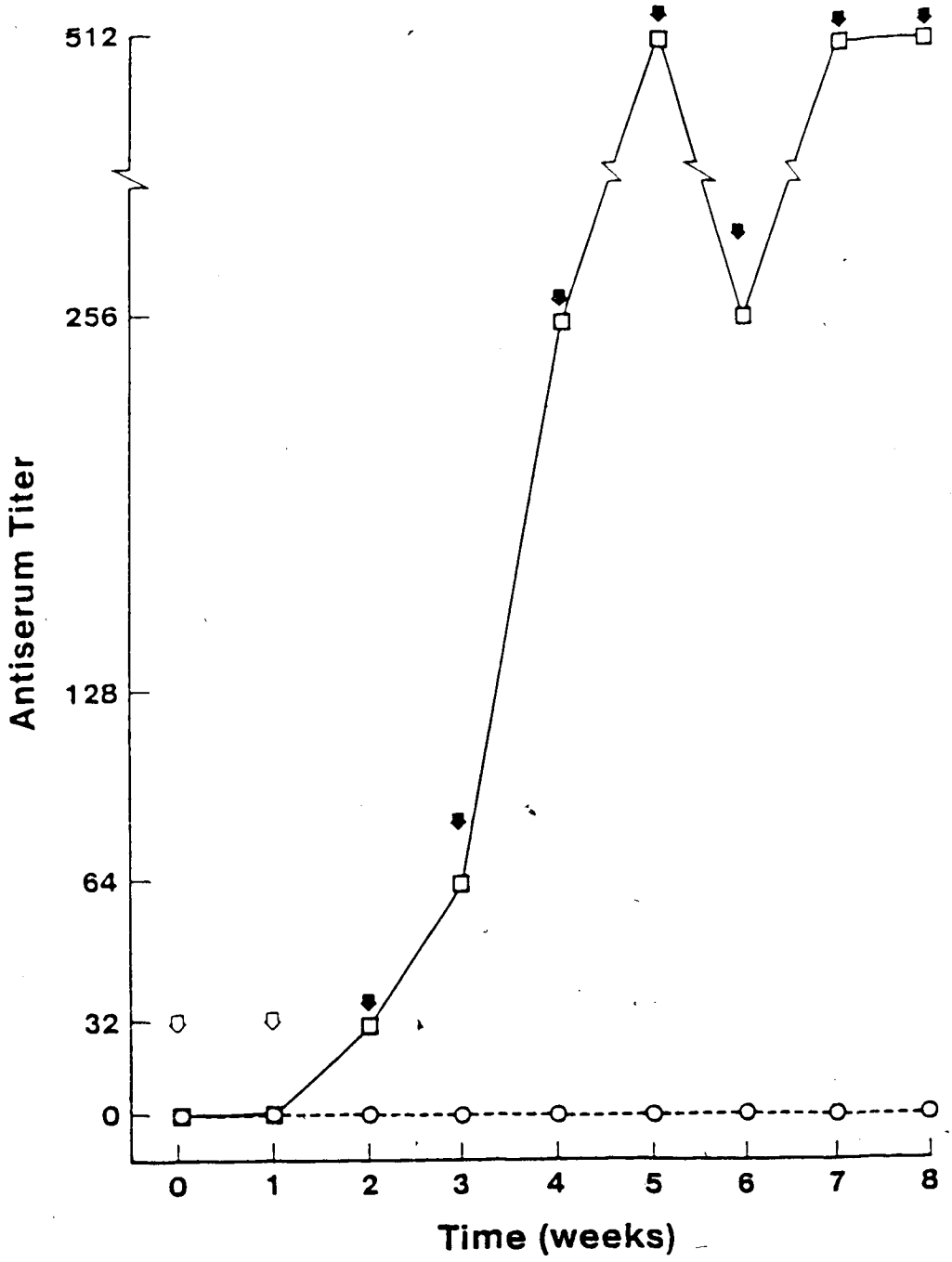

Fig. 23. The increases in antibody production in rabbits injected with tobacco stunt virus. The reaction was done by the precipitin-ring test. Open arrows indicate intramuscular injection, closed arrows indicate intravenous injection.

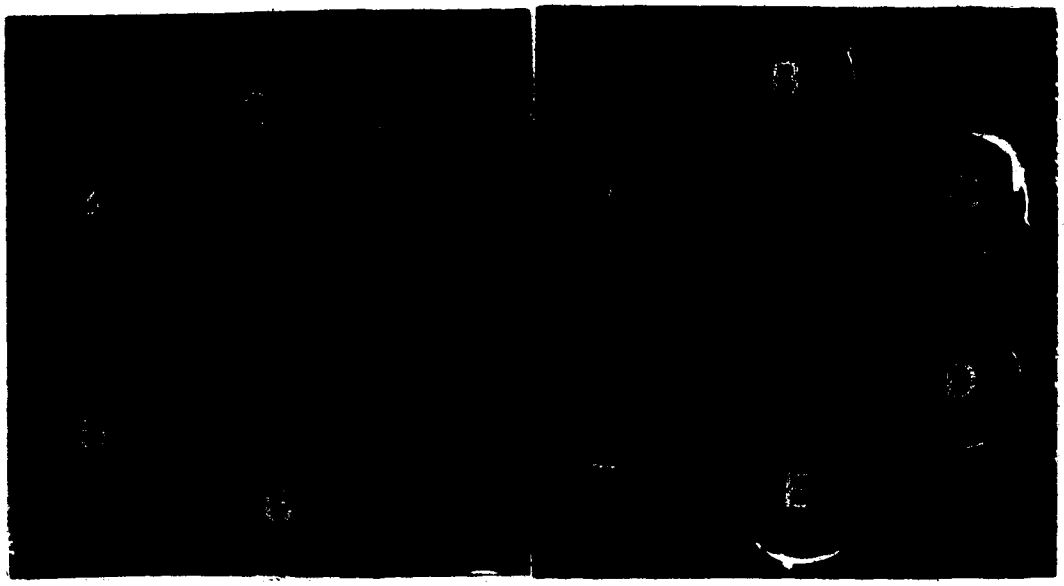

Plate 15. Reaction of tobacco stunt virus (TSV) antigen to TSV antibody in agar double-diffusion test.

Left, the TSV antigen was placed in the central well and the antibody is diluted 1/8, 1/10, 1/35, 1/64, 1/128 and 1/356, in wells numbered 1-6, respectively.

Right, the antibody against TSV was placed in the central well with lettuce big vein virus in well A, sap from TSV infected tomato plant in well B, TSV in well C, sap from healthy tomato in well D, purified preparation from healthy lettuce in well E and purified preparation from healthy tomato in well F.

inactivation point, dilution end point, longevity of the purified virus at room temperature, virus size and shape and sedimentation coefficient. In this study, all the above properties were investigated and were used as bases for further chemical analysis. The thermal inactivation point of purified TSV was found to be 60°C. This result was slightly lower than the previously reported result of 75-80°C (Hiruki, 1975) and higher than the thermal inactivation point in plant sap which was believed to be 35°C (Alderson, 1975). The fluctuation in the results could be due to many factors such as impurities, or the extraction buffer used. This also could be due to the time of the year the experiments were conducted, since the concentration of phenolic compounds in the plants varied according to seasons, being very low in early spring and very high in late summer (Francki, 1982). The dilution end point of TSV was found to be 10^{-3} which is consistent with previously published results (Hiruki, 1975). The longevity of TSV in vitro depends upon the kind of buffer used to suspend the virus. For example, in Tris-HCl buffer, the longevity of the virus at room temperature was 6 hr, whereas in sodium phosphate buffer, there was a gradual decrease in infectivity and complete inactivation after 2 hr (Table 10). This result could be due to the breakdown of virus particles, resulting in the release of the virus nucleic acid, thus exposing the virus RNA to the action of RNase, which results in a significant increase in UV absorbance (Fig. 14).

With regard to the specific infectivity, the minimum of 50 µg/ml

of purified TSV is required to initiate infection on C. amaranticolor (Fig. 11). On the other hand, other plant viruses require lower amounts. For example, TMV requires 1 µg/ml to start an infection in the form of local lesions (Wildman, 1959). It follows then, that the efficiency of infecting C. amaranticolor leaves is much lower than that of TMV. The highest efficiency in assaying TSV on C. amaranticolor, under the conditions of the present study, was obtained by using 200 µg/ml as inoculum. A possible reason for the lower infection efficiency of TSV with the increase of inoculum may be explained by the limited number of infectible sites on assay leaves (Siegel and Zaitlin, 1964) or by the presence of degradation products of TSV that interfere in the inocula of higher concentrations.

The virus, which is rod-shaped, appears to be of different lengths, with a majority of about 300-350 nm. However, in thin sections, the virus appears to be of one size of 250-350 nm, which is consistent with results obtained by other workers (Kuwata and Kubo, 1986). This indicates that shearing of virus particles occurs during purification which is consistent with results produced from unstable viruses (Bar-Joseph et al., 1979).

Upon electrophoretic analysis on polyacrylamide gels, the TSV-RNA preparations were found to contain three major species designated as large (4.2×10^6 daltons), medium (1.8×10^6 daltons), and small (1.2×10^6 daltons). Whereas the sizes derived from electron microscopy gave slightly different molecular weight, namely 3.9×10^6 daltons for the large species, 1.9×10^6 daltons for the medium

species, and 1.4 x 10^6 daltons for the small species. The slight difference in molecular weight could be due to secondary structure of the double-stranded RNA. The large RNA segment is homologous in composition to the middle and the small segments (Plate 10). However, the middle component is slightly homologous to the small segment indicating different composition which could be a result of a breakdown occurring in the large segment resulting in a production of the middle and small segments. The presence of a large segment of a similar molecular weight in TSV-carrying Olpidium zoospores (Plate 11) is further evidence of this hypothesis. When the virus was purified in 0.3 M phosphate buffer, pH 6.5, Kuwata and Kubo (1986) found rod-shaped virus-like particles 200-400 nm in length. The virus contained two molecules of RNA with molecular weights of 4.5 x 10^6 and 4.2 x 10^6, respectively. Since the RNA species are resistant to RNase A in high salt concentrations, they concluded that the RNA was double-stranded in nature. In the present study, TSV-RNAs were found to be double-stranded in nature by the different criteria tested, such as their relative resistance to degradation by RNase A at high salt concentration (0.1 x SSC), degradation at low salt concentration (0.001 x SSC), degradation by RNase III which is specific for double-stranded RNA, base pairing, and melting behavior. TSV-RNA exhibits a sharp melting profile with a Tm of 62°C. This melting temperature is saltdependent, and increases to about 75°C in 0.1 x SSC (Figs. 5 and 6).

The structural protein of TSV was of one species having a molecular mass of 51,000 daltons. This is the largest of all rod-shaped plant virus coat proteins that are known so far; for example, the molecular weight of the coat protein of TMV is 17,500 (Knight, 1975). While the molecular weight of TSV-coat protein appears to be unusually high, it is not a dimer because it could not be dissociated further by boiling in SDS-mercaptoethanol or by addition of 10 M urea prior to boiling. This fact legitimately puts TSV in a separate class of plant viruses, although it was suggested earlier by Kuwata and Kubo (1981), based on morphological studies, that TSV resembles viruses belonging to the tobamoviruses such as potato mop-top (Harrison and Jones, 1970).

The presence of dsRNA in plant viruses is not unique, for example, several plant viruses belonging to the reovirus family have been studied (Milne and Lesemann, 1978; Shikata, 1981). TSV is a unique virus because of the presence of dsRNA in an elongated virion. This dsRNA is different than some dsRNA species that were found in several healthy plants (Dodds and Bar-Joseph, 1984), because TSV dsRNA occurs only in infected plants or in TSV-carrying Olpidium zoospores. This dsRNA is infectious and codes for a coat protein that envelopes the virus particles.

The large RNA segment is about 4.2×10^6 in size. This, therefore, could theoretically code for a polypeptide of about 160 KD. The polypeptides that are synthesized from wheat germ extracts represent only about 75% of the coding capacity of the large RNA segment.

The 68 KD polypeptide is not due to the lack of a suppressor of tRNA species that promotes the readthrough of a UAG codon (Beier et al., 1984) because it cannot be precipitated by TSV antibody. The size of the middle segment is 1.8 KB with a coding capacity of a polypeptide of about 70 KD. The inability of this RNA segment to produce polypeptide In vitro is not caused by an endogenous RNase contamination due to the fact that a powerful RNase inhibitor was included in the reaction mixture. Therefore, the inability of this RNA to direct protein synthesis could be the lack of AUG initiation codon or it is not an efficient template for protein synthesis (Atabekov and Morozov, 1979). The polypeptide of about 28 KD that is synthesized by the small RNA segment is obviously part of the virus structural protein, because it can be recognized by the antibody produced against purified TSV particles.

The 68 KD polypeptide that is translated from the total TSV-RNA is clearly non-structural, and could have some enzymic function, since the assembly of dsRNA viruses need some enzymes such as RNA polymerase which are specific for ds RNA and which are lacking in normal cells (McCrae and Joklik, 1978). These results, combined with hybridization experiments, strongly sugest that TSV-RNA exist in nature as a single nucleic acid that breaks down at a specific point, producing 3 RNA segments. Based on this, a tentative map of the TSV genome is devised (Fig. 24).

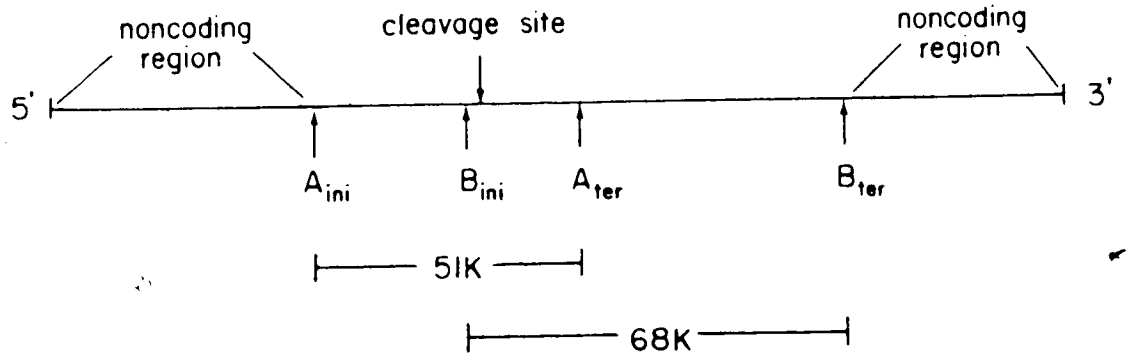

Fig. 24. Theoretical mapping of the TSV-RNA genome.

A_{ini} . The initiation signal for the coat protein.

B_{ini} . The initiation signal for the 68 KD protein.

A_{ter} . The termination signal for the coat protein.

B_{ter} . The termination signal for the 68 KD protein.

While there is little reliable information available concerning the relative merits of different immunization procedures, Kiriyama (1975) reported the production of TSV antiserum in ascitic fluids of mice using a partially purified TSV preparation. The homologous titer of TSV antiserum using the ring precipitin test was 1/512 when unabsorbed and 1/128 when absorbed by healthy plant sap. The antiserum did not react with TMV, cucumber mosaic virus, alfalfa mosaic virus, and tobacco leaf curl virus. Hiruki (1975), using the ring precipitin test, independently reported a homologous titer of 1/1024 of TSV-antiserum produced in rabbits by an intramuscular injection followed by 5 weekly intraveinal injections of purified TSV preparation.

In this study, using an injection similar to that in Hiruki's previous study (1975), a homologous titer of 1/512 was obtained from all of three rabbits used when using ring precipitin test, and 1/256 in agar double diffusion test.

In serological studies, the antibody produced against TSV reacted strongly with LBVV with a final titer of 1/256.

On the basis of information presented, it is possible to conclude that TSV is a rod-shaped virus containing double-stranded RNA encapsidated by a coat protein of an unusually high molecular weight and further study is required to determine the segmented nature of TSV-RNA. Since these characteristics are not found in any other plant virus, it is suggested that TSV is a type member of a new group of plant viruses, to which LBVV also belongs.

CHAPTER VII

GENERAL DISCUSSION

When an unknown virus disease is investigated, a general approach is to see if the causal virus (or viruses) is comparable with any that has been reported. Usually this is done by electron microscopic examination of virus particles or by serology or by both methods, and most recently cDNA hybridization of viral genome (Masri and Hiruki, 1987; Hiruki and Masri, 1987). None of these procedures gave rise to much progress in the investigation of TSV in spite of repeated attempts in the past 40 years since its first discovery in 1943. This difficulty is mainly due to the fact that the virus is usually unstable both *in vivo* (Hidaka and Hiruki, 1958) and *in vitro* (Hiruki, 1975), and as discussed in Chapter VI the infection efficiency of TSV is extremely low compared to most plant viruses. The identity of the disease-causing agent as a virus was, therefore, once much in doubt and it was referred to as tobacco stunt agent (Hidaka *et al.*, 1975; Alderson, 1975; Kuwata and Kubo, 1981). As indicated in Chapter IV, there are many factors that influence the stability of TSV *in vitro*. In the study of any unstable virus these individual factors and their combined effects on virus infectivity must be worked out, as done in this study, before virus purification is attempted. In the previous studies, Hiruki (1964, 1975) reported the stabilizing effect of chelating agents on TSV infectivity. Such a

stabilizing effect could be improved further by selecting a buffer system suitable for preservation of TSV infectivity as shown in Chapter IV and V.

The addition of Na_2SO_3 to Tris-HCl buffer or certain additives to glycine buffer had a desired effect on the stabilization of TSV infectivity. On the basis of these findings, therefore, it was possible to develop a suitable purification procedure later coupled with sucrose and cesium density gradient centrifugation to obtain purified TSV preparations for the study of physical and chemical properties as described in Chapter V.

The major achievements of the present investigation on the properties of TSV are the following. First, successful visualization of TSV particles by electron microscopy in the leaf tissue of C. amaranticolor bearing local lesions caused by the virus to Bright Yellow tobacco which later developed typical symptoms of tobacco stunt. The proof of TSV infectivity was reproducible in the transfer inoculation experiments from C. amaranticolor. Second, elongated virus particles measuring 200-375 nm in length were isolated by the procedure developed in this study and its infectivity as the causal agent of the tobacco stunt disease was demonstrated satisfactorily. Third, a single species coat protein of purified TSV with a molecular mass of 51,000 daltons was isolated. A comparable study on LBVV showed the LBVV also contains the coat protein with a similar molecular mass. Fourth, it was shown that TSV contains dsRNA, which is probably segmented. The occurrence of dsRNA in a rod-shaped plant

virus has not been reported and thus should be considered as a new feature. Fifth, TSV proved to be a moderate immunogen and production of TSV antiserum with a titer of 1/512 was achieved. Serological studies indicated that TSV and LBVV are serologically closely related.

Even though TSV particles were found in the extract preparations of viruliferous O. brassicae zoospores, the relationship between the virus and its vector is not completely understood. Thorough investigation should be initiated to study the relationship between the nucleic acids of both the virus and its vector.

The relationship between the different RNA segments can be better understood if cloning and sequencing of the cDNA can be achieved.

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