PURIFICATION AND BIOPHYSICAL PROPERTIES OF
TOMATO SPOTTED WILT VIRUS

J.J. Joubert

UNIVERSITY *of the*

WESTERN CAPE

Thesis presented for the degree of
Doctor of Philosophy (Agriculture)
at the University of Stellenbosch.
Promotor:
M. Barbara von Wechmar, Ph.D.(Agriculture).
December 1972.

CONTENTS

ACKNOWLEDGEMENTS

CHAPTER I

INTRODUCTION

The virus etiology of tobacco wilt, a disease of tobacco known in South Africa since 1905, was determined by Moore (1933). After establishing the relationship between this virus and its thrips vector, she realized that this virus was identical to, or a strain of the Australian tomato spotted wilt virus described by Samuel, Bald & Pittman, 1930.

Although the virus nature of this disease had already been established in 1930, the first attempt to obtain purified virus preparations was only reported twenty years later (Black, Brakke & Vatter 1952). A detailed description of the purification method appeared in a second paper when the authors also reported some bio-physical properties of the virus (Black et al., 1963).

Subsequent attempts by different laboratories to purify the tomato spotted wilt virus (TSWV) were greatly facilitated by the earlier work on the in vitro properties of the virus. To retain in-fectivity during the purification process it was essential to pre-vent oxidation of the virus (Bald & Samuel, 1934; Samuel, Best & Bald, 1935), to control the pH of the environment (Best & Samuel, 1936a; Best, 1966) and to work at a low temperature.

Best and Palk (1964), Best (1966) and Tsakiridis (1971) were unable to purify TSWV according to the method described by Black et al. (1963). Tsakiridis (1971), on the other hand found the me-thods of Best and Palk (1964) and Best (1966) to be unsatisfactory.

The lack of agreement between different workers regarding purification procedures may be caused by the use of different strains of the virus in different laboratories. Such a situation is encountered for instance when attempts are made to purify strains of a virus which differ in stability. With cucumber mosaic virus for example, the method suitable for purifying the stable S strain was not successful with the unstable Y strain (van Regenmortel, 1964).

In all the above mentioned purification procedures all the strains used, except that of Black et al. (1963), showed the same in vitro physical properties traditionally used as criteria to characterise TSWV (temperature inactivation point, longevity under controlled pH conditions).

On the other hand, the strains used by different workers reacted differently to the same buffer solutions. While the isolates of Black et al. (1952, 1963) could be precipitated by 0,1 M potassium phosphate buffer (pH 7,0) containing 0,01 M sodium sulphite, the isolates used by Best (1966) did not precipitate under these conditions.

It was thus decided to investigate the ability of different published purification procedures for South African isolates of TSWV.

The success of Martin's (1964) purification method, in which calcium glycerophosphate was used as an absorbent, seems uncertain since no infectivity tests were performed.

The differences in particle morphology, reported in different laboratories, also warranted further investigations. Shadowcasted

particles were either round, (Martin, 1964) or showed bud-like eva-
ginations (Black et al., 1963). In these shadowcasted preparations,
the particle varied between 85-150 nm in diameter. In osmium tetro-
xide fixed virus preparations the diameter of the virus particles
varied between 68-102 nm, mostly round to oval in shape while some
particles revealed tail-like structures. In the virus preparations
of Best and Palk (1964) and Best (1966) some virus particles were
dumbbell-shaped and are referred to as "twin" particles. These "twin"
particles consisted of two approximately 55 nm particles encased in
an unstained membrane.

In ultrathin sections of virus infected plant tissue, the virus
particles observed were more or less spherical in shape, the probable
particle size being between 70 and 100 nm (Ie, 1964 & 1971; Martin,
1964; Kitajima, 1965; Francki & Grivell, 1970; Milne, 1970). Smaller
55 nm particles (Best & Palk, 1964), dumbbell-shaped particles (Best
& Palk, 1964; Best, 1966) or particles with bud-like or tail-like
evaginations (Black et al., 1963; van Kammen, Henstra & Ie, 1966)
were never observed in sectioned material.

It thus seemed that the variation in particle shape in purified
virus preparations could be artifacts of the purification process.
This possibility was tested in the present work.

CHAPTER II.

LITERATURE REVIEW.

A. Occurrence of the disease.

Brittlebank (1919) was the first to notice and describe the disease "spotted wilt of tomato" in Australia, although he did not realize its virus etiology. Within ten years this disease was reported throughout Southern Australia as the most serious disease of tomatoes. Its virus nature was established in 1930 when the causative virus received its present name of tomato spotted wilt virus (Samuel, Bald & Pittman, 1930).

Reports of the disease also came from the United Kingdom (Smith, 1931 & 1932) and subsequently from other parts of Europe, South and North America, Asia and Africa (Best, 1968).

Lounsbury (1906) described a heavy infestation of tobacco wilt in the Kat River Valley (Eastern Province, South Africa) in 1905. This same disease was studied again by Moore (1933) in the same locality. By this time the farmers called the disease "Kromnek" or "Kat River Wilt" of tobacco. Moore showed that the thrips species _Frankliniella_ transmitted the causal agent. She realized that this disease was probably caused by a virus and she assumed that it was either identical to, or a strain of the Australian tomato spotted wilt virus. On further investigation she showed that the virus was easily transmitted to tomatoes by grafting pieces of Kromnek-infected tobacco plants onto healthy tomatoes, which then developed the characteristic tomato spotted wilt symptoms.

B.   Synonyms.

Tomato spotted wilt virus (TSWV) has received various names
at each new "discovery": "Kromnek" or "Kat River Wilt" of tobacco,
and the "Kat Valley" disease of tomatoes in South Africa (Moore,
1933); Hawaian "yellow spot" on pineapples (Parris, 1940; Sakimura,
1940); Tomato tip blight in Oregon (Norris, 1943); Cineraria streak
(Jones, 1944); Corcova, Vira-cabeca, Necrose do topo and Peste negra
in South America (Sakimura, 1947); Tomato bronzing, Makhorka tip
chlorosis and Lycopersicon virus 7 (Sakimura, 1962).

Lesser known synonyms were tomato virus 1 in Johnson's classi-
fication; Lycopersicum virus 3, Smith; Lethum australiense var.
typicum, Holmes; Ananas virus 1, Smith; and pineapple side rot virus
(see Smith, 1957).

The identity of all the above diseases was established by the
specific virus-vector relationships.

C.   Virus-vector relationships.

Pittman (1927) was the first to show that TSWV was transmitted
by Thrips tabaci Lind. In later years this finding was confirmed by
several other workers in various parts of the world (Sakimura, 1947).
The thrips vector is the only known means of transmission of TSWV
in nature.

The presently recognized vector species are Thrips tabaci,
Frankliniella schultzei (Tryb.), F.occidentalis (Perg.), and F.fusca
(Hinds) (Samuel et al., 1930, Gardner, Tompkins & Whipple, 1935;
Sakimura, 1962). All the other vector species mentioned in earlier
references are synonyms of one of the above four species (Sakimura,
1962).

An important discovery was made by Bald & Samuel (1931) when they showed that only insects which had fed on diseased plants during their larval stage, could transmit the virus as adults. There is a definite latent period of several days between acquisition of virus and transmission to healthy host plants. This finding, as well as the inability of adult insects to acquire the virus, was later confirmed (Linford, 1932; Smith, 1932; Moore, 1933; Bailey, 1935; Razvyazkina, 1953 and Sakimura, 1962).

Sometimes the larvae become infective if the latent period is completed before commencement of the pupal phase (Bald & Samuel, 1931; Razvyazkina, 1953 and Sakimura, 1963).

The minimum acquisition feeding time of 30 minutes for T.tabaci, is the only available information regarding the acquisition threshold of thrips vectors (Razvyazkina, 1953).

A minimum inoculation threshold of 5 minutes was recorded for T.tabaci. A prolonged feeding time gave an increase of infection from 4 percent with a 15 minute feeding period to 77 percent for a 4 day feeding period. Transmissions after 15 minute inoculation feedings were infrequent and erratic (Sakimura, 1963).

The latent period usually extends through the pre-pupal and pupal stages (average: 4 days) and the first transmission occurs from 0 to 4 days after emergence of the adult insect. The general average of the latent period observed for F.fusca was 9,3 days with a range of 4 to 12 days. For T.tabaci it was 10,7 days with a range of 4 to 18 days (Sakimura, 1963).

The retention period may extend over the entire life of the adult insect or for only a short period terminating a considerable

time before the insect's death. It may vary from 1 to 43 days for F.fusca and from 2 to 30 days for T.tabaci (Sakimura, 1963).

In some insects, infectivity is persistent for long periods while in others, it is short and sporadic with long non-infective periods between transmissions. The reason for this inconsistency seems to be a variation in the original amount of the virus acquired by different insects and the inability of the virus to multiply in the vectors (Sakimura, 1962).

Thrips usually suck through their mandibles from the mesophyll tissue after piercing the epidermal tissue. This type of feeding leaves silvery scars, usually not affecting vascular tissue. A shallow type of feeding is also recognised where penetration is limited to the epidermal tissues and/or a few adjacent mesophyll cells. Such feedings are usually limited to small areas without leaving silvery scars (Sakimura, 1962). Transmission of the virus is apparently adversely affected by extremely shallow and short feedings on the epidermal tissues (Sakimura, 1963). From these reports, it is not clear which host tissue contains the highest virus concentration for vector acquisition.

The tomato spotted wilt and tobacco ringspot viruses are the only confirmed thrips-borne virus diseases (Messieha, 1969). Three other cases need further clarification: the manihot mosaic disease in Brazil, the pistachio rosette disease in Russia (although their virus etiology has not been proven) and the sunflower mosaic virus in Argentinia, which apparently is transmitted by T.tabaci (Sakimura, 1962).

The important difference between nymphs and adults in their ability to acquire the virus, has not yet been explained. Day &

Irzykiewicz (1954) concluded from their gut-puncturing experiments, that this difference is not due to differences in permeability of the mid-gut wall. Neither could it be explained along the lines of differences in pH or redox potentials between the mid-gut contents of the nymphs and adult insects.

D.   Symptomatology.

One of the outstanding features of TSWV is the diversity of symptoms it can produce in infected plants. The symptoms may vary in individual plants and for any particular host, they vary with the age and nutrition level, as well as with environmental conditions, especially temperature. With the discovery of strains it became clear that much of this variation was due to differences in the proportion of strains present in any one plant (Best, 1968). Essentially, it is a stunting disease while wilting is not a characteristic feature. The plants usually survive throughout the season in a dwarfed condition.

A characteristic symptom on vigorously growing tomatoes is the appearance of bronze-coloured markings on the younger leaves usually followed by a cessation of growth. Affected plants usually remain alive for many months in a dwarfed condition. In early infections, fruiting is inhibited, while plants infected at a later stage show pale red or often yellow and white areas on the skin of ripe tomatoes (Samuel et al., 1930).

On tobacco, the most frequent symptom is cessation of growth. The apex is often bent over at right angles, giving rise to the South African name of "Kromnek", literally meaning "bent neck". Stem necrosis, accompanied by deep brown pigmentation on the side

towards which the apex is bent, is common in field tobacco. Systemically invaded tobacco leaves, usually become necrotic along the lateral veins on the lower half of the leaf, often on the one side only. A characteristic mottle effect due to the invasion of the lower half of the leaf may also occur (Samuel & Bald, 1933 & Moore, 1933).

E.   Host Range.

Best (1968) compiled a list of all of the susceptible host plants. Species of the Solanaceae and the Compositae represent most of the 160 dicotyledonous plants included in the list. Only 6 monocotyledonous hosts are attacked by the virus of which pineapples are the most important.

F.   Strains.

The work of Norris (1946) demonstrated that most of the variability of TSWV symptoms in any one host species, is due to the fact that the virus is present not as a single entity, but in the form of a complex of strains. This is the fundamental cause of variation in symptom expression although the environment plays a subsidiary role.

The principal technique used by Norris for strain separation, was the excision and successive transfer of selected pieces of tissue, mostly local lesions and sometimes component parts of a mottled leaf. Five strains were separated in this manner and Norris described the symptoms of these strains on sixteen host species. These symptoms vary from terminal necrosis to a barely distinguishable mottle on tomatoes.

Best and Gallus (1950, 1953) purified five strains from a

single thrips-induced lesion on a tomato plant. The strains were separated by the single-lesion-at-limit-dilution technique (SLLD-technique). This technique ensures that in a mixed isolate, only the strain present in the greatest amount would be left to form a lesion, the lesser components being eliminated through dilution.

The diagnostic symptoms by which they selected strains, were based on the rule that each symptom must be an invariable property of the strain and that it must be readily distinguishable from the symptoms of the other strains.

Only three plants viz. Lycopersicum esculentum (cv. Dwarf Champion), Nicotiana tabacum (cv. Blue Pryor) and N. glutinosa were used by them as indicator hosts.

According to the systemic symptoms, three main groups of TSWV strains were described: those producing severe necrosis along with the formation of pigments (strains A, B, D); those producing only a very mild surface necrosis unaccompanied by pigmentation (strains C_1, C_2) and those with neither a visible necrosis nor pigmentation (strain E).

G.   Cross-protection.

The protection of a plant by a mild strain of TSWV against a more severe strain has been demonstrated (Best, 1954). This phenomenon was not only effected after the mild strain C_1 was well established throughout the plant prior to a secondary infection, but also when a mixed inoculum of both strains C and A were applied simultaneously.

When determined quantitatively, it was found that the protection

of tomato plants by the mild strain E against the severe strain A was not complete.

H.   Genetic recombination.

The intermediate symptoms expressed in plants inoculated with two distinct strains of TSWV have been explained as a recombination of the genetic material of the two virus strains. According to this theory it should be possible, from plants infected with two strains of a virus, to recover new virus strains, which differ from the originally introduced virus strains but which possess some of the characteristics of each parental strain.

This theory was confirmed by Best & Gallus (1955) with strains A and E of TSWV. Plants exhibiting intermediate symptoms were used as a source of material for SLLD-tests and a new strain, combining some of the characteristics of each original strain was obtained.

In two recombination experiments between strains A and E, 9,8 percent and 21 percent of the primary lesions were variants of the parental strains. A mutation rate of 0 percent was determined for strain A and 0,24 percent for strain E in testing the possibility of the new strain being a mutant (Best, 1961 & 1965). Since the re-combination rate was 2 orders of magnitude higher than the apparent mutation rate, Best (1968) argued that the new variants could not be attributed to mutation and were most probably the result of gene-tic recombination.

I.   Quantitative determinations.

1) Biological assay. The amount of infective TSWV in two or more inocula has been compared with an accuracy of $\pm$ two

percent by the local lesion technique (Best, 1968).

2) <u>Electron-microscopic assay</u>. Virus particles are sufficiently characteristic to be counted on electron microscope grids but it is often difficult to distinguish damaged virus particles from intact ones. As there is no way to determine the relative proportions of infective to non-infective particles, it is impossible to relate particle counts to the concentration of infective virus.

3) <u>Spectrophotometric assay</u>. Since much of the virus disintegrates during preparation, the use of an empirical factor to convert OD_{260} to dry weight of virus is of doubtful value.

4) <u>Serological assay</u>. Serological end point titres can be used as a qualitative test to determine the presence of virus in a solution, but the accuracy is not as good as in the biological infectivity method.

J. <u>Reaction to the environment</u>.

1. <u>In vivo</u>.

a) <u>The effect of light</u>. A significant increase in the number of local lesions was obtained when plants were kept in the dark for 24 hours before and after inoculation respectively. Although bright artificial light inhibited the formation of local lesions, the appearance of local lesions was faster and the final number was higher on plants subjected to continuous light, compared to plants subjected to alternative light and dark periods (Best, 1935, 1936).

b) <u>The effect of temperature</u>. Local lesions not only appeared 10 hours earlier at 20°C than at 15°C but they increased in

size more rapidly while their total number was also 30 per-
cent higher (Best, 1936, 1939).

2. In vitro.

    a) The effect of oxidising and reducing agents. Addition of
    reducing agents to infective plant tissue macerated in dis-
    tilled water, prolonged the infectivity from less than 5
    hours to about 36 hours. Oxidising agents either immediately
    destroyed infectivity or accelerated inactivation (Bald &
    Samuel, 1934; Samuel, Best & Bald, 1935). At redox potentials
    above the critical value between E_h + 0,01 and E_h + 0,02,
    virus infectivity was instantaneously destroyed but below
    this critical redox potential it was preserved.

3. The effect of pH. TSWV was rapidly inactivated at and below
    a pH value of 5 and above a pH value of about 10. The optimal
    pH value for maintaining virus infectivity was found to be
    7 (Best, 1966; Best & Samuel, 1936a). The inactivating effect
    of some reducing agents has been ascribed to the fact that
    they lower the pH of the test media (Samuel et al., 1935).

4. The effect of temperature. Buffered virus suspensions, con-
    taining a reducing agent, were better preserved at 0^oC than
    at room temperature (Best & Samuel, 1936b).
    Bald & Samuel, (1931) determined a thermal inactivation tem-
    perature of 42^oC in crude plant juice but an inactivation
    temperature of 46 $\pm$ 1^oC was reported for plant juice buffered
    at pH 7 and containing a reducing agent (Best, 1946).

K.   Purification of TSWV.

The instability of TSWV was the biggest obstacle in purification

attempts and procedures requiring more than 8 to 10 hours seldom gave good results (Black, Brakke and Vatter, 1963).

To maintain maximum virus infectivity, the optimal in vitro conditions for the virus, such as pH 7, temperature (Best & Samuel, 1936b) and the presence of a suitable reducing agent (Bald & Samuel, 1934) were incorporated in all the reported purification schemes.

The main techniques for clarification and concentration of TSWV thus far used were differential and density gradient ultracentrifugation.

Black et al. (1963) stressed the importance of starting with an extract containing a comparatively high virus concentration, since much of the infectivity was lost when centrifugation was the main technique applied. The highest virus concentration was found in the systemically infected parts of those plants which had a high local lesion count on the inoculated leaves. The top portions of plants were used before the symptoms reached the acute phase.

They found that the virus was highly aggregated in 0,1 M potassium phosphate buffer containing 0,01 M sodium nitrate but that resuspension of the first low speed pellets (P$_1$) in 0,01 M sodium nitrate dispersed the virus again (Fig.1). Low ionic strength conditions proved to be an important factor in minimizing aggregation.

After gradient centrifugation, a white visible zone, diffuse and without sharp boundaries, was usually present in the gradient tube. Compared to the infectivity of samples taken from various depths in the density gradient tube, the white visible zone contained the highest infectivity. The infective zone contained roughly spherical particles varying considerably in shape.

Fig.1. <u>Purification of TSWV</u> (Black <u>et</u> <u>al</u>., 1963).

Leaf tissue was homogenized in an equal volume of 0,1 M phosphate buffer (pH 7) containing 0,01 M sodium sulphite ("Solvent 4").

Leaf tissue + buffer

Homogenise for 1 to 2 minutes
Squeeze through cheesecloth
Centrifuge for 20 to 30 minutes
at 3 000 to 5 000 rpm in an International type 1-SB centrifuge*

P_1 SL_1 (discard)

Resuspend in 0,01 M Na_2SO_3 and let it stand for 30 to 60 minutes
Centrifuge for 30 minutes at 3 000 to 5 000 rpm in an International type 1-SB centrifuge*

SL_2 P_2 (discard)

Centrifuge for 45 minutes at 13 000 rpm (ca. 22 000 g) in the Servall model SS-2 centrifuge*

P_3 SL_3 (discard)

Resuspend in 0,01 M Na_2SO_3
Layer onto 10 to 40% sucrose density gradient (in 0,01 M Na_2SO_3) after 30 to 60 minutes
Centrifuge for 25 minutes at 25 000 rpm in the Spinco SW 25-1 rotor
Locate and withdraw light scattering zones

Virus zone

Centrifuge for 60 minutes at 24 000 g*

(van Kammen <u>et</u> <u>al</u>., 1966)

Layer onto 30 to 60% sucrose density gradient in 0,01 M Na_2SO_3
Centrifuge for 90 minutes at 50 000 g in Spinco SW-39 rotor
Locate and withdraw light scattering zones

P_4 SL_4 (discard)   Virus zone

* Rotor type not specified

In an attempt to shorten the purification procedure to about 4,5 hours, Martin (1964) used the roots of systemically invaded tomato plants and calcium glycerophosphate as an absorbent (Fig.2). Martin, however, performed no infectivity tests and the success of his procedure seems uncertain. Similar to the finding of Black et al. (1963), Martin also observed a variability in shape as well as a considerable flattening of the particles in electron micrographs.

Best and Palk (1964) also employed differential and density gradient ultracentrifugation for purifying TSWV (Fig.3). After the first gradient centrifugation the virus was either subjected to electrophoresis or to a second cycle of gradient centrifugation. Virus particles, usually oval after the first density gradient, tended to distort with further purification. When the virus was subjected to either electrophoresis or a second step of gradient centrifugation dumbbell-shaped virus particles, connected by a distended envelope, could be observed.

Best & Palk (1964) and Best (1966) used a specially composed buffer, which minimized the loss of infectivity when the virus was concentrated by high speed centrifugation and resuspended under optimal conditions. This buffer referred to as "Solution B" contained 0,01 M sodium phosphate buffer at pH 7; 0,07 M sodium sulphate; 0,01 M sodium sulphite and 1×10^{-4} M disodiumethylene-diaminotetra-acetate (EDTA).

Best (1966) continued his experiments on virus purification employing this buffer in conjunction with differential and gradient centrifugation and found that 80 percent of the infectivity in the tube was concentrated in a single, sharply defined light scattering band. After the second gradient separation this band was again ob-

Fig.2. <u>Purification of TSWV</u> (Martin, 1964).

Root extract containing 1% Na_2SO_3 (w/w).

Centrifuge for 5 minutes at 3 000 rpm.

SL_1 P_1

Wash with 0,0005 M phosphate buffer (pH 7)

Centrifuge for 5 minutes at 3 000 rpm*

SL_2 P_2 (discard)

Pool SL_1 & SL_2

Add calcium glycerophosphate at a rate of 0,08 gm/ml

Centrifuge for 5 minutes at 2 000 g*

SL_3 P_3 (discard)

Centrifuge for 15 minutes at 13 000 g*

SL_4 P_4 (discard)

Add ascorbic acid (0,015 gm/ml) and dilute with 3 volumes 0,05 M Na_2SO_3

Centrifuge for 45 minutes at 30 000 g*

P_5 SL_5 (discard)

Resuspend in 0,01 M Na_2SO_3

Centrifuge for 10 minutes at 2 000 g*

SL_6 P_6 (discard)

Centrifuge for 45 minutes at 30 000 g*

P_7 SL_7 (discard)

Resuspend in 0,01 M Na_2SO_3

Centrifuge for 10 minutes at 2 000 g*

SL_8 P_8 (discard)

Layer onto 10 to 40% sucrose density gradient

Centrifuge for 30 minutes at 25 000 rpm in the Spinco SW 39 rotor

Locate and withdraw light scattering zones

Virus zone

* Rotor type not specified

Fig.3.   Purification of TSWV (Best, 1966; Best and Palk, 1964)

Leaf tissue was homogenised in a phosphate buffer ("Solution B") which contained 0,01 M sodium phosphate at pH 7; 0,07 M sodium sulphate; 0,01 M sodium sulphite and 1×10^{-4} M ethylenediamine-tetra-acetate (Na-salt).

60 gm of infective leaves + 300 ml solution B

Homogenise and squeeze through cheese-cloth
Centrifuge for 8 minutes at 10 000 g*

SL_1 P_1 (discard)

Centrifuge for 30 minutes at 25 000 rpm in the Spinco no. 30 rotor

P_2 SL_2 (discard)

Resuspend in solution B
Centrifuge for 8 minutes at 10 000 g

SL_3 P_3 (discard)

Layer onto 0 to 40% sucrose density gradient in solution B
Centrifuge for 60 minutes at 24 000 rpm in Spinco SW 25-1 rotor
Locate and withdraw virus light scattering zone

Virus zone

Centrifuge for 20 minutes at 40 000 rpm in Spinco no 40 rotor

P_4 SL_5 (discard)

Resuspend in solution B
Layer onto 0 to 40% sucrose density gradient in solution B
Centrifuge for 60 minutes at 24 000 rpm in Spinco SW 25-1 rotor
Locate and withdraw virus zone

* Rotor type not specified

Virus zone

Centrifuge for 20 minutes at 40 000
rpm in Spinco no. 40 rotor

P_5 SL_5(discard)

Resuspend in solution B
Centrifuge for 20 minutes at 40 000
rpm in Spinco no. 40 rotor

P_6 SL_6(discard)

UNIVERSITY *of the*

WESTERN CAPE

tained together with a diffuse second band, 1 mm wide, situated 0,5 to 1,0 mm above it. This lighter band had a much lower infectivity compared to the heavier band just below it. Grids prepared from this upper band showed a much greater concentration of apparently empty virus particles.

Working with the pure strain E, Best (1966) showed that it could be precipitated from solution in the presence of 25 percent ammonium sulphate. Less than half of the infectivity was recovered by this procedure. The dumbbell structures or "twin" particles occurred so abundantly and regularly in preparations of strain E, that they were regarded as characteristic of this strain. They occurred much less in the other strains examined.

The virus used by Black et al. (1963) was aggregated to such an extent by the 0,1 M phosphate buffer, that it precipitated during low speed centrifugation. It was readily resuspended in a solution of low ionic strength.

Contrary to this finding, Best & Palk (1964) and Best (1966), found no aggregation of the virus even with the same molar concentration of phosphate buffer or various other buffers. Seventy percent and often more than eighty percent of the virus infectivity was found in the supernatant after low speed centrifugation.

From the differences in behaviour of TSWV when purified by Black et al. (1963), Best and Palk (1964) and Best (1966), it seems likely that Black et al. were dealing with different strains of the virus.

Tsakiridis (1971) was unable to repeat the purification procedures of Black et al. (1963) and Best (1966), and he modified their methods by using Freon 114 (1,2-dichloro-1,1,2,2-tetrafluorethane)

Fig.4.   <u>Purification of TSWV</u> (Tsakiridis, 1971)

Both "Solvent 4" (Black <u>et</u> <u>al</u>., 1963) and "Solution B" (Best & Palk, 1964 and Best, 1966) were employed in this purification scheme.

160 gm infective leaf tissue + 160 ml "solvent 4"

Homogenise and squeeze through cheese-cloth

Centrifuge for 30 minutes at 4 000 g *

P_1 SL_1(discard)

Resuspend in 160 ml "solution B"

Add 240 ml Freon 114 and homogenise for 40 seconds

Centrifuge for 15 minutes at 4 000 g *

Aqueous phase

Add 80 ml Freon 114 and homogenise for 20 seconds

Centrifuge for 10 minutes at 4 000 g *

Aqueous phase

Centrifuge for 30 minutes at 74 000 g *

P_2 SL_2(discard)

Resuspend in 6 ml "solution B"

Layer onto 0 to 40 sucrose density gradient in "solution B"

Centrifuge for 60 minutes at 83 000 g *

Locate and withdraw light scattering virus zone

Virus zone

Centrifuge for 40 minutes at 100 000 g *

P_3 SL_3(discard)

Resuspend in distilled water

Final virus preparation

* Centrifugations were performed in either a Servall Model 2-B or a Spinco Model L2-65 centrifuge. Rotor types not specified.

as clarification agent (Fig.4).

L.   Virus morphology in purified preparations.

1) Shadowed virus particles.   From a study of virus prepara-
tions in which the diameter of virus particles ranged from
85 to 120 nm, Black et al. (1963) concluded that the most
probable shape and size of TSWV was a spherical particle of
85 nm diameter.

Martin (1964) who also examined shadowed preparations
reported larger particle diameters, ranging from 100 to 150
nm for spherical and flattened particles respectively.

Because of shadowing, no information about virus struc-
ture could be obtained although some of the particles re-
vealed "small buds" on one side.

2) Positively and negatively stained virus particles.   Best &
Palk (1964), in studying unshadowed virus preparations found
that the particle diameter varied according to the method
used for purification. Since the virus particles were most-
ly oval in shape, they reported a particle diameter corres-
ponding to that of a circle with the same area.

Virus pellets after a single gradient centrifugation
step (Fig.3) were fixed with osmium tetroxide, embedded in
methacrylate resin and sectioned at a thickness of about
30 nm. Sections, positively stained with either uranyl ace-
tate or lead hydroxide, contained practically only 57 nm
particles along with a few 90 nm particles and larger empty
shells. In drop preparations (i.e., when a drop of virus so-
lution was applied directly onto a grid) of parallel samples
subjected to either electrophoresis or another cycle of den-

sity gradient centrifugation, the virus particles varied much in shape and size. In such preparations the round to oval particles were predominantly 90 nm in diameter but dumbbell-shaped or "twin" particles were also found. These "twin" particles consisted of two approximately 55 nm particles encased by an unstained membrane. The membranous tube connecting the two particles varied much in length. The function of the empty shell, evident in both sectioned and negatively stained drop preparations, was probably that of an envelope which initially contained two to four virus particles. Negatively or positively stained virus particles never revealed bud-like structures like those evident in the shadowed preparations.

The existence of a virus membrane was clearly demonstrated in osmium tetroxide fixed preparations, negatively stained with potassium phosphotungstate (van Kammen, Henstra & Ie, 1966). In presumably empty or partially filled particles, penetrated by the stain, an approximately 5 nm wide unstained hydrophobic layer surrounded each particle. A 5 nm wide protein layer composed of visible subunits could be observed on the periphery of this white unstained layer; the presence of a similar protein layer on the inside of the white layer was suggested in many particles. Negative staining also revealed small membrane-encased evaginations. These structures are probably homologous to the "buds" in shadow-casted preparations. Particle diameters varied between 68 and 120 nm.

In positively stained dumbbell-shaped particles, the outer protein layer was seen as protuberances or spikes about 5 nm long (Best, 1966). The inner lightly stained

portion of the membrane was probably the seat of the lipid discovered in TSWV (Best and Katekar, 1964).

The morphology of TSWV has been diagramatically presented by Best (1968) and he concluded that it is in essence a pleomorphic myxovirus, although the inner structure of the virus particles was never revealed in purified preparations.

The above mentioned data regarding the shape and size of purified TSWV particles is summarised in Table I.

M.   <u>Virus morphology studied with the "dip" technique</u>.

Virus particles were always revealed in <u>in</u> <u>vitro</u> preparations when infected tissue was crushed and spread on glutaraldehyde (Milne, 1970). The diameter of such particles was 85 to 100 nm and no dumb-bell-shaped particles, tail-like evaginations or particles smaller than 55 nm were ever observed.

None of several stains revealed any inner structure while a pale ring, probably indicating the position of the membrane, could be seen. A mottled layer 6 to 7 nm thick, surrounded each particle and was probably homologous with the shell between the two membranes of sectioned enveloped particles (Milne, 1970).

N.   <u>Virus morphology in tissue sections</u>.

In ultrathin plant tissue sections the virus particles, round to oval in shape and about 70 nm in diameter, occurred in membrane bound clusters (Ie, 1964). Similar observations were made by Martin (1964) in sections prepared from virus infected tomato roots where the virus particles, varying from 50 to 80 nm, were also contained in a membraneous structure.

Electron dense structures in a primary lesion on a <u>N.glutinosa</u> leaf were described as virus particles which had budded off <u>in</u> <u>vivo</u> as extrusions from a membrane (Best & Palk, 1964).

As there was not always a discernible boundary between the membrane surrounding the virus clusters and clearly identifiable endoplasmic reticulum elements, it has been described as part of the endoplasmic reticulum (Kitajima, 1965). The virus containing membranes as well as the endoplasmic reticular elements were also

found as rough and smooth forms (Francki & Grivell, 1970) while virus particles were also found in the nuclear membrane which is known to be connected to the endoplasmic reticulum. This observation is further supported by the frequent occurrence of particles containing structures linked to the nuclear membrane. Virus particles were never found to be associated with other cell organelles or to occur free in the cytoplasm. The circular to elliptical virus particles varied from 80 to 120 nm in diameter in Kitajima's preparations; they are 10 to 50 nm larger than those reported by Ie (1964) and 30 to 40 nm larger than those reported by Martin (1964).

In the first of two papers Milne (1967) demonstrated a double membrane, which closely resembled an ordinary cytoplasmic membrane, around each virus particle. The similiarity between TSWV and influenza virus, an unstable lipid containing virus of about 85 nm, is revealed in his sections. Influenza virus particles acquire their outer lipid containing coats when the complete virus moves through the outer cell membrane into the external environment. Within this membrane is the characteristic "helical component" or nucleic acid coils of myxoviruses. However, to classify TSWV as a myxovirus one will have to wait that a similar internal "helical component" is revealed and that it can be shown that the viral lipid is acquired by extrusion through a cell membrane.

Milne (1970) investigated whether TSWV satisfied the above mentioned requirements by sequential sampling and sectioning of infected plant tissue. Mature virus particles were nearly spherical and usually 75 to 80 nm in diameter, the extremes being 70 to 100 nm. The mottled cores of mature particles were surrounded by a three

layered membrane, each layer being 2,5 nm thick. Complete virus particles, each surrounded by a densely staining coat always occurred in membrane bound vesicles.

Virus particles were always associated with the amorphous electron dense viroplasm, which probably consists of a ribonucleo-protein that is involved in the early stages of virus synthesis. Analogous structures were also demonstrated by Francki & Grivell (1970), Ie (1971) and Kitajima (1965). The mature virus particles associated with the viroplasm were not only surrounded by a shell of dense matter but also by another triple structure membrane giving rise to enveloped virus particles. These enveloped virus particles were probably derived by budding off from pairs of parallel mem-branes, 12,5 nm apart, which were often seen near the viroplasm and enveloped particles. This process of budding off is suggested by configurations of the parallel membranes frequently observed in ultrathin sections of infected leaf tissue. Finally, the outer mem-branes of a group of enveloped particles probably join up to form a cisterna and the inner membranes of these particles are released into it to form mature virus particles. Neither the helical ribo-nucleoprotein nor the budding off characteristic of myxoviruses at cell membranes have been observed. In conclusion Milne (1970) states that the above mentioned characteristics resemble those found in leukovirus infections rather than what is found in myxovirus infec-tions.

Numerous virus particles, roughly spherical and about 86 nm in diameter have been observed in mesophyll, epidermal and guard cells as well as in the vascular tissues of leaf tissue (Francki & Grivell, 1970). These authors found it difficult to establish the exact role of the endoplasmic reticulum, the densely staining granular material

TABLE II. The shape and size of TSWV particles in ultrathin tissue sections.

Extremes in particle size (nm)	Probable particle size (nm)	Particle shape	Author
47-77	57	Oval to round	Best & Palk, 1964.
--	70	More or less spherical	Ie, 1964.
50-80	--	Oval to spherical	Martin, 1964.
80-120	100	Spherical or elliptical	Kitajima, 1965.
--	85	Roughly spherical	Francki & Grivell, 1970.
70-100	75-80	Nearly spherical mature virus particles	Milne, 1970.
95-120	100	Nearly spherical enveloped particles	Milne, 1970.
70-90	--	--	Ie, 1971.

and the long filament observed during virus synthesis. During cell division, virus particles are seen scattered among the chromosome material, which will ensure that both daugther cells contain virus particles following the division of an infected cell.

The dark diffuse material found shortly after inoculation and which is probably analogous with the viroplasm described by Milne (1970), was found to be typically striated with a periodicity of 4 to 5 nm and locally concentrated into more dense striated spots (Ie, 1971). During treatment of thin sections with pronase, these locally denser spots in the dark diffuse masses were completely digested, leaving white spots 50 to 70 nm in diameter. This suggested that these dense spots/were proteinaceous. In systemically

infected <u>Tropaeolum</u> <u>majus</u> L. and <u>N</u>. <u>tabacum</u> cv. Samsun NN tissue the dark diffuse material was always surrounded by ribosomes, accompanied by osmiophylic bodies, probably of lipid-like nature. These lipid-like bodies may be interpreted as the centres from which the membraneous systems are initiated. In addition to the cytoplasmic inclusions, small particles varying from 28 to 33 nm in diameter were found in the cotyledons of cucumber seedlings. The presence of these particles is not understood and hard to explain; when they occurred in high concentrations the diffuse material was completely or nearly completely lacking (Ie, 1971). Dark diffuse material was never found in any older virus infected cells containing large quantities of mature virus clusters. Virus particles were always round and 70 to 90 nm in diameter.

All this data is summerized in Table II.

O.   <u>Serology</u>.

Although TSWV is extremely labile and has a half-life period of approximately 20 minutes at $35^{\circ}C$ (Best, 1946) it has the capacity to induce antibody production in rabbits (Best and Hariharasubramanian, 1967).

Using virus preparations, prepared according to the method of Best (1966) antisera against strains E and R_1 were obtained after a series of intramuscular and intravenous injections. With the precipitin reaction, end point titres of 1/512 and 1/256 were recorded respectively for strains E and R_1 in the homologous reactions. Heterologous end point titres were 1/64 for strain E with strain R_1 antiserum and 1/32 for strain R_1 with strain E antiserum. Undiluted clarified sap from infected plants reacted positively with

the antisera.

In comparing infectivity and serological titres at various stages of the purification process, it was found that the ratio of infectivity to the serological titre decreased as purification proceeded. This indicated that non-infective virus was serologically equivalent to infective virus.

Feldman and Boninsegna (1968) thought that heating plant sap (10 minutes at 70°C) might possibly give TSWV denatured protein a fixed configuration which might preserve its antigenic capacity for eliciting the formation of antibodies active against the virus. This was demonstrated in double diffusion Ouchterlony agar gel tests. The resulting serum not only reacted with normal plant components but an additional precipitin line, probably formed by TSWV, was formed with plant sap containing TSWV. The titres obtained were 1/6 and 1/10 respectively. A similar antiserum obtained with un-heated antigen failed to react with TSWV antigen.

An antiserum with an endpoint titre of 1/8 was obtained by Tsakiridis (1971) after intravenous injections of TSWV purified with Freon 114. The original virus complex as well as the virus strains subsequently isolated from this complex reacted with anti-serum prepared against a mild strain. Antibody-antigen reactions were assayed in agar-gel, using systemically infected leaf tissue as test antigen. An identical antigen was determined in the original virus complex and in the necrotic-, ringspot- and mild strains when these antigens were allowed to react with mild strain anti-serum. Fused precipitin lines proved the identity of the antigens. Additional precipitin lines diffusing more slowly were formed when mild strain antiserum was tested against antigens of the necrotic

strain and the original virus complex.

P.   Chemical composition of the virus.

1) Lipid content.   The finding in TSWV of virus-specific lipid
   was the first demonstration of the presence of lipid in a
   plant virus. The total lipid content varied between 17,8
   and 19,7 percent (Best and Katekar, 1964).

   Besides the lipid content, the virus was also found to
   contain about 7% of carbohydrates.

2) Nucleic acid.   Paper chromatograms of P^{32} labelled virus
   RNA demonstrated the presence of guanine, adenine, cytosine
   and uridine and thus confirmed TSWV as a RNA virus (Best
   and Symons, 1963).

   The RNA nature of this virus was also determined by
   positive orcinol and negative diphenyl amine reactions (van
   Kammen et al., 1966).

3) Amino acids.   A partial amino acid composition of strain E
   was determined by Jennings and Best (1964). The low percen-
   tage of nitrogen is largely accounted for by the 19% lipid
   and 7% carbohydrate found in the virus. The percentage re-
   covery of nitrogen as amino acids, indicates the presence
   in the virus of a significant amount of non-protein nitro-
   gen.

Q.   Physical properties.

1) Sedimentation constant.   An approximate $S_{20,w}$ value of
   3,07 times that of tobacco mosaic virus was determined by
   sucrose density gradient centrifugation. The density of

sucrose in which the virus will not sediment was determined
as 1,207 (Black et al., 1963). More reliable sedimentation
constants were determined by Best (1966) in an analytical
centrifuge. Virus obtained after one step of gradient cen-
trifugation (Fig.3) had $S_{20,w}$ values varying between 492
and 551 with a mean value of 524. In two runs with virus
obtained after two steps of gradient centrifugation $S_{20,w}$
values of 530 and 534 were obtained.

2) Electrophoretic mobility. The infective virus zone obtained
   after the first sucrose density gradient separation yielded
   3 peaks with electrophoretic mobilities of 0,6 x 10^{-5},
   3,5 x 10^{-5} and 5,3 x 10^{-5} cm^2 v^{-1} sec^{-1} respectively (Best,
   1968). No mention was made of the buffer, pH and ionic
   strength used.

3) Ultraviolet absorption curve. Due to the size of TSWV a
   correction for light scattering should be applied to optical
   density measurements in the ultraviolet range. Virus pre-
   parations usually yielded an absorption curve with a slo-
   ping shoulder (Best, 1966).

   The absorption curve for the highly infectious lower
virus band (Best, 1966) in the second density gradient tube
was typical for a nucleoprotein with the exception that it
had two maxima, one at 270 nm and one at 260 nm. The curve
for the upper less infective virus band resembled that of
a protein with little nucleic acid. This is in conformity
with previous observations that this band contains many
empty envelopes and disrupted membranes.

R.   Virus effects on the plant.

Best (1937 b,c) explained the rapid loss of infectivity and
the browning of TSWV-infected tissue homogenates when exposed to
air, by the action of a heat labile oxidase enzyme. This enzyme
catalysed the aerobic oxidation of catechol, quinol, and phenol to
their corresponding quinones which were probably responsible for
virus inactivation. Tyrosinase which is also found in infective
tissue could not be demonstrated with certainty in healthy plants.
Addition of reducing agents to homogenates prevented inactivation
of the virus and the browning of the homogenates.

The concentration of the amino acids and amides in TSWV-infec-
ted tomato plants was much higher than in control plants, the total
concentration in the leaves being three times higher and that in
the stems about 8 times higher (Selman, Brierly, Pegg and Hill,
1961). It was concluded that this accumulation indicated protein
hydrolysis or a partial failure of protein synthesis.

In seeking an explanation for the stunting effect of TSWV on
tomato plants, Grieve (1943) determined the auxin content of virus-
infected and healthy control plants. He concluded that one of the
actions of the virus on plant growth was caused by the destruction
of auxin probably by the oxidase in infected plants. (Best, 1937
b,c).

Oxygen uptake by homogenates prepared from TSWV-infected toma-
to plants was found to be higher than in those from healthy control
plants (Best, 1937a; Grieve, 1951) and infected plants had double
the output of carbon dioxide, although the respiratory quotient
was the same for healthy and diseased plants.

CHAPTER III

MATERIALS AND METHODS.

A. Virus source.

Two virus strains, propagated from single thrips-induced local lesions, were used throughout this work.

B. Virus propagation.

TSWV strains were propagated in $\underline{N}$. $\underline{glutinosa}$ plants, grown insect free in steam sterilized soil at 19-21°C in the absence of direct sunlight. The maximum light intensity on a clear summer day, never exceeded 11 000 foot candles as measured with a Lunasix photometer.

For inoculum, leaves showing systemic virus symptoms, were macerated with a mortar and pestle in 0,02 M phosphate buffer (pH 7,2) containing 0,01 M sodium sulphite (1 gm of tissue/2 ml buffer). The sap was expressed through cheesecloth, and after adding a small amount of celite, it was rubbed onto the fifth well developed leaf. At this stage, the fifth leaf was about 5 cm long.

Local lesions appeared 3 to 4 days after inoculation and systemic symptoms were visible after the 10th day. Approximately 48 hours after the expression of systemic symptoms, these leaves were harvested, weighed, and batches of 30 gm were stored in plastic containers in a liquid nitrogen refrigerator until required. Empty 250 ml plastic fruit juice containers were used for this purpose.

No additional pre-conditioning of plants before inoculation
and harvesting was found necessary (Best, 1966) when plants were
grown in fully shaded greenhouses.

C.   Virus assay.

The effects of various treatments on the infectivity of the
virus at different stages of the purification procedure, were de-
termined by counting the number of local lesions produced on the
inoculated leaves of N. tabacum cv. Turkish. Eight leaves on four
young plants were used in assaying the virus.

D.   In vitro properties in plant sap.

In all tests performed, systemically infected leaves of N.
glutinosa were used. Where pH control was necessary, the leaves
were macerated in 0,02 M phosphate buffer pH 7,2 at a ratio of
1 gm of leaves in 2 ml of buffer. The virus infectivity was assayed
on Turkish tobacco.

> (1) Longevity in extracted plant sap at room temperature
>   (Bald & Samuel, 1931):
>   Infective plant tissue macerated in distilled water (1:1
>   dilution), was assayed every 15 minutes for infectivity.
>
> (2) The effect of pH 7 buffer at room temperature (Best &
>   Samuel, 1936a):
>   Expressed sap in pH 7 phosphate buffer was kept at 19-20°C
>   and the virus activity assayed every 60 minutes.
>   When a sample of this plantsap was kept in crushed ice,
>   virus activity was assayed every 3 hours.

(3) <u>Virus infectivity in a pH 7 buffer containing a reducing agent and kept in crushed ice</u> (Best, 1968):

Infected tissue, macerated in 0,02 M phosphate buffer at pH 7 containing 0,01 M sodium sulphite and kept in crushed ice, was assayed every 8 hours for virus activity.

E. <u>Buffers</u>.

The following buffers were used at different stages of this work:

a) "<u>Solvent 4</u>" (Black <u>et al</u>., 1963): This phosphate buffer (pH 7) consisted of 0,033 M KH_2PO_4 + 0.067 M K_2HPO_4 to which was added 0,01 M Na_2SO_3.

b) "<u>Solution B</u>" (Best, 1966): This buffer consisted of 0,007 M Na_2HPO_4 + 0,003 M NaH_2PO_4 (pH 7) and contained 0,07 M Na_2SO_4 and 0,01 M Na_2SO_3 and 1 x 10^{-4} M ethylenediaminotetra-acetate (Na-salt).

c) <u>0,1 M phosphate buffer</u> (pH 7,2) was used as a stock solution and consisted of the following:

Solution A; 35,8 gm Na_2HPO_4.12 H_2O made up to 1000 ml with distilled water,

and

Solution B; 7,8 gm NaH_2PO_4.2H_2O made up to 500 ml with distilled water.

The 0,1 M phosphate buffer consisted of a 1000 ml of solution A and 430 ml of solution B.

The phosphate-sodium sulphite buffer referred to in this work as the phosphate-sulphite buffer consisted of a five-

fold dilution of this stock buffer (0,02 M) containing in addition 0,01 M sodium sulphite.

d) <u>0,1 M pH 8,6 Borate buffer</u>: This buffer contained the following substances:

0,035 M H_3BO_3; 0,0175 M NaOH; 0,0075 M HCl and 0,073 M NaCl (Polson & Deeks, 1962; Polson & Russel, 1967).

e) <u>0,5 M pH 6,9 Citrate buffer</u>:   300 ml of 0,5 M sodium citrate was adjusted to pH 6,9 with 10 ml 0,05 M citric acid.

f) <u>0,1 M Cacodylate buffer, pH 6,0</u>:   The pH of a solution consisting of 0,1 M KCl and 0,02 M cacodylic acid was adjusted to pH 6,0 with approximately 10 ml 1,0 M KOH (Incardona & Kaesberg, 1964).

F.   <u>Purification</u>.

1) <u>Temperature</u>: All work was done in a cold-room at 2° to 4^oC or in a laboratory where all the required glassware and other apparatus were kept in crushed ice. Pre-cooled rotors were used in centrifuges with temperature control set at 2^oC.

2) <u>Buffer</u>: Virus infected leaves where homogenized in the phosphate-sulphite buffer which satisfied the optimum <u>in</u> <u>vitro</u> requirements of TSWV.

3) <u>Clarification</u>: After homogenizing infected leaf-tissue for 2 minutes in a Waring Blendor in the above mentioned phosphate-sulphite buffer, the homogenate was centrifuged for 10 minutes at 10 000 rpm in the SS-34 rotor in a Sorvall SS-3 automatic superspeed centrifuge to remove plant matter.

Further clarification was attempted by using the following
procedures:

(a) Absorption to hydrated calcium phosphate (Fulton, 1959).

(b) Flocculation with 1% bentonite (Dunn and Hitchborn, 1965).

(c) Charcoal and celite clarification (Galvez, 1964; Steere,
1959; von Wechmar and van Regenmortel, 1970).

(d) Chromatography on calcium phosphate columns (Murant,
Goold, Roberts and Cathro, 1969). This method was final-
ly incorporated in a procedure for purifying TSWV.

(4) <u>Preparation of calcium phosphate columns</u>: Fractionation of
virus-containing extracts by chromatography on the brushite
form of calcium phosphate, is a technique which has been
used for purifying several viruses of vertebrates (Taverne,
Marshall and Fulton 1958) and a plant virus, carrot mottle
virus (Murant <u>et al</u>., 1969). The method of Taverne <u>et al</u>.
(1958), was used for preparing, packing and elution of the
columns.

With a two-channel LKB 4912A peristaltic pump, equal
volumes of 0,5 M $CaCl_2$ and 0,5 M $Na_2HPO_4.12H_2O$ were pumped
into a flask on a magnetic stirrer at a flowrate of 5 ml/
min. The precipitate was allowed to settle down and washed
five times by decantation with distilled water. It was
stored as a suspension in distilled water at $4^{\circ}C$ and never
used till 20 hours later.

Glass columns of about 4,5 cm in diameter and fitted
with a sintered glass plate at the bottom were used for
packing columns. They had a bed volume of about 200 ml. A
filter paper disc was layered on top of the column to avoid

disturbing the surface when adding materials to the column.

The virus-containing extract was run through this column, and the elution volume determined with a LKB Uvicord densitometer. As soon as a 10 percent transmission was recorded, the eluate was collected in a flask. A volume of 20 ml more than that of the virus extract initially put onto the column, was collected. Elution was carried out with the 0,02 M, pH 7,2 phosphate-sulphite buffer.

(5) Concentration:

    (a) Ultracentrifugation. The virus was concentrated by highspeed centrifugation in either the Spinco model L or L2-65 centrifuges, using the Spinco no 30 or no 40 rotors at speeds of 25 000 rpm and 35 000 rpm for 40 and 30 minutes respectively.

    (b) Precipitation. Precipitation of TSWV from solutions was achieved by adding polyethylene glycol (PEG, MW 6 000) and sodium chloride to the solutions. Both PEG and sodium chloride were used as 25 percent solutions (w/v) in distilled water.

During the earlier stages of purification, when the virus was in suspensions of 300-330 ml, PEG and sodium chloride were added to the suspensions to give final concentrations of 12 and 3 percent respectively. In the later stages of the purification scheme virus pellets were usually resuspended in 6 ml of buffer; to these suspensions PEG and sodium chloride were added to give final concentrations of 1,5 and 0,5 percent respectively. Virus containing precipitates were separated by

centrifugation at 10 000 rpm for 5 minutes in the Spinco no. 40 rotor and the pellets were resuspended in buffer or distilled water.

(6) Density gradient centrifugation:

Sucrose gradient columns for the Spinco SW 25-2 swinging bucket rotor were set up according to Brakke (1953, 1967). Sucrose solutions, containing 10, 20, 30 and 40 gm sucrose in 100 ml of the phosphate-sulphite buffer were layered onto each other in volumes of 6, 12, 12 and 10 ml respectively. Before use, the gradients were kept at $4^{O}C$ for 14-17 hours to equilibrate. Two millilitres of clarified virus extract was layered on each gradient.

After centrifugation for 60 minutes at 20 000 rpm any light-scattering bands were located by illuminating the tube by parallel light from a microscope lamp of which the light intensity could be varied. Any light-scattering bands were sampled by syphoning with a thin stainless-steel tube with a bent-up tip at a flow rate of 0,5 ml/minute.

(a) Ultra-violet absorption profiles.

When it was necessary to obtain ultra-violet absorption profiles of gradients, ribonuclease free sucrose (Miles-Seravac) was used to prepare the gradients. The gradients were pumped through a Beckman fraction recovery system and a LKB Uvicord densitometer at a flowrate of 0,5 ml/minute.

(b) Differences in light scattering zones after density gradient centrifugation.

After density gradient centrifugation, the tube contain-

ing the different light-scattering zones was illuminated from above with a high-intensity microscope lamp. This lamp was fitted with a blue filter transmitting light between 450 nm and 550 nm only. The gradient was photographed on Ilford FP4 film and developed for five minutes in ID_2 developer.

The negatives were scanned in a Photovolt recording electrophoresis densitometer. The resultant densitometer tracings (eg. Fig.8C) recorded light intensity of the different zones.

G.   Fixation of the virus.

The virus pellets obtained after concentrating the virus zone from sucrose density gradients were resuspended in the phosphate-sulphite buffer. An equal volume of 0,8-1 percent glutaraldehyde in 0,01 M phosphate buffer (pH 7,2) was added to this virus suspension.

As soon as the pH of the stock solution of glutaraldehyde fell below a pH of 5,0 it was treated with barium carbonate which was subsequently removed by low speed centrifugation (Glauert, 1965). This treatment removed any impurities formed by oxidation. It has been shown that oxidation products are responsible for poor fixation.

H.   Zone electrophoresis.

Electrophoretic separations are independent of size, shape and density of virus particles, which make them an ideal complement to differential and density gradient centrifugation. This technique has

been described in detail   (van Regenmortel 1964b, 1966; Polson and Russell, 1967).

Zone electrophoresis was performed in a sugar density gradient, prepared in either the pH 8,6 borate buffer, or the phosphate-sulphite buffer. Sodium sulphite was added to the borate buffer at a final concentration of 0,01 M.

Phenol red was used as a standard reference to indicate the progress of electrophoresis and to determine the R_{ϕ} values of the different components for the purpose of characterization (van Regenmortel, 1968).

I.   Physico-chemical techniques.

1) Spectrophotometric determinations: Ultra-violet spectra and absorbancies were determined with a Beckman Model DB spectrophotometer. When determining the absorbancies at 260 and 280 nm, corrections were made for light scattering, as described by Best (1966) and Bonhoeffer and Schachman (1960). To achieve this, the OD at wavelengths between 320 and 700 nm were plotted against wavelength on a log-log scale and this line extrapolated to 210 nm. The resultant values were subtracted from the observed absorbancies to obtain true absorbancies.

2) The extinction coefficient: Virus preparations, subjected to zone electrophoresis in phosphate-sulphite buffer and resuspended in distilled water, were used to calculate the extinction coefficient. The OD of virus solutions at 260 nm was determined in a 1 cm light path cuvette in a Beckman DB

spectrophotometer. A Sartorius five digital analytical balance was used for precise weight determinations.

With a Hamilton syringe calibrated to 0,05 ml, exact amounts of a virus solution of known OD_{260} were measured into small glass vials of known weight. These containers were kept for 20 hours at $65^{\circ}C$ and allowed to reach ambient temperature for 4 hours. After weighing, they were again heated for four hours, allowed to cool down and weighed again. If necessary, this was repeated until a constant dry weight was recorded.

The extinction coefficient was calculated from the OD_{260} and weight measurements.

3) Determination of the sedimentation coefficient: Sedimentation coefficients were determined in a Spinco Model E analytical ultra-centrifuge, equipped with electronic speed control. Using either the schlieren or UV optical systems, photographs of the sedimenting boundary were taken at regular time intervals.

The sedimentation coefficient of a particle can be defined as its migrational velocity under the influence of unit centrifugal field:

$$s = \frac{dx/dt}{\omega^2 x}$$

where dx/dt = rate of sedimentation (cm/sec).,

x = distance of boundary from axis of rotation, and

ω = angular velocity (rad./sec.).

A precooled An-D rotor and precooled 12 mm (2^O or 4°) single sector cells were used at a speed of 10 000 rpm for determining the S_{20} value of TSWV. The temperature was recorded during the run and the average rpm was determined from the revolution counter with a stop watch only when the calculated speed differed more than 0,2 percent from the set speed.

Schlieren plates were measured in a Nikon microcomparator and the UV photographs in a photovolt densitometer. For each photograph, the logarithm of the distance of the boundary to the axis of rotation was determined. The maximum ordinate of the schlieren peak, or the point on the boundary in the densitometer tracing corresponding to 50 percent of the plateau concentration, was used as the boundary position in these measurements. The logarithms of the distances were plotted against the time intervals and the slope was calculated.

The sedimentation coefficient was calculated from this slope using the equation:

$$s = \text{slope} \times \frac{2,303}{60\,\omega^2}$$

$$\text{where } \omega = \frac{2\pi\,\text{rpm}}{60}$$

Sedimentation coefficients are usually reported as $S_{20,w}$ values, which are the theoretical values that would have been obtained in distilled water at 20^OC, using the equation:

$$S_{20,w} = S_{obs} \, (\eta_t) / (\eta_{20})$$

where η_t = the viscosity of water at the temperature at
which the experiment was performed, and

η_{20} = the viscosity of distilled water at 20^OC.

A correction for the difference in the relative viscosity
of the solvent compared to that of water was not applied
since with the solvents used, it would have amounted to not
more than 1 percent.

A typical example of a calculation for determining the
S_{20} value of TSWV appears in Addendum I.

4) Determination of the electrophoretic mobility: In an elec-
tric field, charged particles will migrate towards the
electrode of opposite charge. The electrophoretic mobility
u, can be defined as the ratio of the velocity of the par-
ticles v, to the electric field strength E.

$$u = \frac{v}{E.}$$

As the net charge of a particle depends on the ionic
strength of the solvent, it is necessary to maintain constant
ionic conditions by means of a buffer when measuring the
mobility of a particle. The moving boundary method was used
to record the progress of electrophoretic migration in a
Tiselius cell of the Spinco Model H electrophoresis-diffu-
sion apparatus. The temperature of the waterbath was control-
led between $2,5^O$ and $2,6^O$C. After temperature equilibration,
the boundaries were moved into the field of view of the op-
tical system and electrophoresis was started. A current of
13 mA was usually employed. Schlieren peaks were photographed

at regular time intervals and the displacements of the peaks during these time intervals were measured in a Nikon micro-comparator.

The electrophoretic mobility was calculated from the following equation:

$$u = \Delta hAC/It \; cm^2 \; volt^{-1} \; sec^{-1}$$

where Δh = displacement of the peak (cm).

A = cross sectional area of the cell (0,75 cm^2)

C = conductivity of the solvent ($ohm^{-1}cm^{-1}$)

I = current (amps) and

t = time interval.

An example of a calculation of the electrophoretic mobility appears in Addendum II.

5) <u>Conductivity</u>: For the determination of the electrophoretic mobility of the particles, it is necessary to know the conductivity of the buffers.

A LKB conductolizer, modified by replacing the 2 000 ohm helipad and vernier with a decade resistance measuring from 0,1 ohm to 9 999,6 ohm, was used for measuring the resistance of the cells when filled with the various buffer solutions.

The cell constant (K) was determined by filling the cell with 0,1 M potassium chloride, which has a specific conductance (C) of 0,00715 $ohm^{-1} \; cm^{-1}$ at 0^oC and substituting the measured resistance (Rm) in the formula

$$K = RmC$$

The potassium chloride was then replaced with the

buffer used. After allowing sufficient time for temperature equilibration, the resistance of the cell was determined and the specific conductance determined by the formula:

$$C = \frac{K}{Rm}$$

where C = specific conductance of the buffer,

K = cell constant and

Rm = resistance of the cell filled with buffer at the specific temperature.

See Addendum III for a typical calculation.

J.   Serology.

(i) <u>Antiserum production</u>. Antisera were prepared in rabbits by intramuscular injections of virus preparations emulsified in Freund's incomplete adjuvant. Rabbits were given four injections, one week apart, and were bled at weekly intervals, the first blood being drawn one day before the fourth injection.

To preserve the antisera, 0,01 percent Merthiolate (Thiomersal) was added. Antisera were stored in a deepfreeze at -10°C until required. Titer determinations were made with twofold antiserum dilutions in 0,85 percent sodium chloride (saline) containing 0,01 percent Merthiolate.

(ii) <u>Double diffusion in plates</u> (Ouchterlony method). The Ouchterlony gel diffusion precipitin test was used as a qualitative test to detect the presence of virus in plantsap and partially purified preparations. The tests were performed in Petri dishes of 10 cm diameter containing 20 ml of 0,75 percent agar in buffered

saline containing Merthiolate (0,01%) as preservative.

Cutters were used to cut wells in the 4 mm thick agar layer (van Regenmortel, 1966).. Wells were either 6 or 4 mm in diameter and arranged hexagonally around a central well of the same size, the wells being 3 mm from each other and 5 mm from the central well.

The Ouchterlony gel diffusion precipitin test was also used to determine the titres of the different sera. Each of a series of twofold dilutions of antiserum was tested against a series of antigen dilutions. The titre of the serum was determined as the highest serum dilution at which a precipitin reaction still occurred.

(iii) Fraction 1 protein. Fraction 1 (F 1) protein, a common contaminant in plant virus preparations (van Regenmortel, 1966) was prepared from N. glutinosa leaves. The leaves were homogenised in 0,5 M citrate buffer (pH 6,9) and chloroform (van Regenmortel, 1964a). After breaking the chloroform emulsion by low speed centrifugation, the supernatant was filtered through cottonwool and the fraction 1 protein was concentrated by differential centrifugation at 40 000 rpm for 120 minutes and 15 000 rpm for 10 minutes in the Spinco no. 40 rotor. The pellets were resuspended in phosphate-sulphite buffer or 0,5 M citrate buffer.

This final protein preparation was used as test antigen in evaluating the TSWV antisera. All dilutions of F1 antigen were done with the above mentioned buffers.

K.   Electron microscopy.

Grids were examined in a Siemens Elmiskop 1 and photographed

on Ilford N40 plates.

Copper grids (200 mesh/inch), with carbon coated collodion
support films, were used in routine examinations of virus prepara-
tions. Grids were floated with the filmside downwards on virus
suspensions for 10 to 60 minutes and washed afterwards with a few
drops of a potassium phosphotungstate (PTA) solution. A two percent
PTA solution, adjusted to pH 7 with potassium hydroxide, was used
throughout this work. Grids were also prepared by the dip method in
distilled water (Brandes, 1964) and by the dip method with glutaral-
dehyde (Milne, 1970).

Carbon-collodion coated platinum grids were used when determining
the size of TSWV. Drops of virus solution were placed on the grids
and allowed to remain there for 10 to 60 minutes, when the remainder
was removed with filter paper. The grids were allowed to dry at room
temperature and were then shadowcasted in a Siemens UBG 500 shadow-
caster with tungstic oxide at a 30° angle. The size of the virus
particles was measured in a Nikon shadowgraph at 20 times magnifica-
tion. The magnification of the electron micrographs was approximately
4 600 times.

As it is difficult to determine the true magnification in the
Elmiskop 1 electron microscope or to reproduce this magnification
consistently over a period of time, it is essential to include in
the examination a virus of known mean diameter or length. Dimensions
can then be compared by parallel photography and measurements
(Brandes and Bercks, 1965). The inclusion of an internal calibration
standard such as a virus, is more reliable than the use of polysty-
rene latex spheres, which may shrink considerably under the electron

beam (Brandes, 1964; Brandes & Paul, 1957; van Regenmortel, Hahn & Fowle, 1964).

Tobacco mosaic virus (TMV), with a normal length of 300 nm (Brandes, 1964), was included as a standard. Grids were prepared as described above, and electron micrographs were taken with the same instrument magnification of approximately 4 600 times.

The calculation of normal dimensions, was performed according to the method outlined by Brandes (1964) and statistically checked to see whether the measurements corresponded to a normal frequency distribution. (See Addendum VI for a typical calculation.)
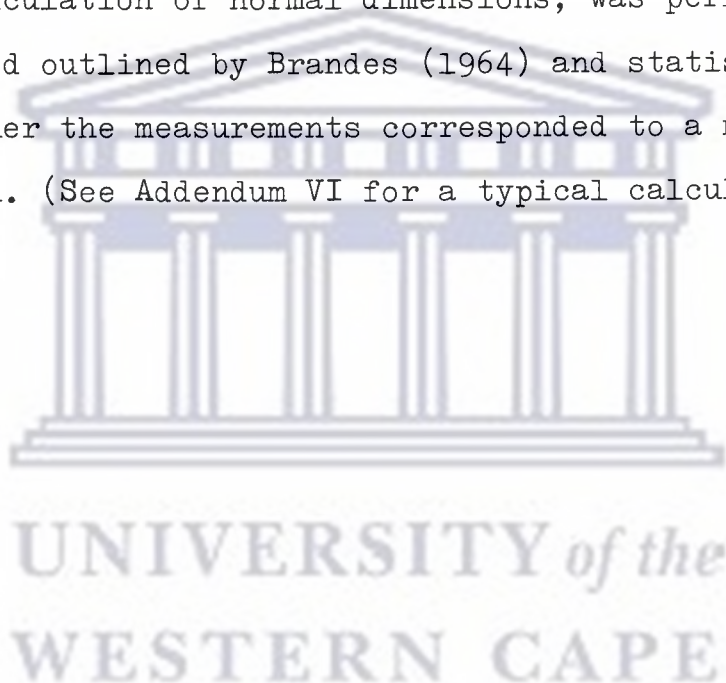
CHAPTER IV

RESULTS.

A.   Virus source.

Thrips tabaci, the most important vector of TSWV, was collected around tobacco fields on Datura stramonium L. plants, which are common weeds and is an important virus source in the Western Cape. The thrips which normally do not multiply on tobacco plants in nature, were released on young tobacco plants in insect proof cages in the greenhouse. Material from single thrips-induced local lesions was extracted and diluted by the local-lesion-at-limit-dilution technique. Separate virus strains were derived from different types of local lesions.

From TSWV infected tobacco plants collected at five different localities in the Stellenbosch district, only two virus strains were obtained. These strains always occurred together in the same plant. N. tabacum cv. Turkish and N. glutinosa were used as indicator hosts.

The different virus isolates were propagated by mechanical inoculation on Turkish tobacco in shaded greenhouses at 19-21°C.

B.   Virus strains.

1) The mild strain: No local lesion symptoms were expressed on the inoculated leaves of N. glutinosa plants kept in a greenhouse in the absence of direct sunlight. In mid-summer

with its higher light intensity or when the plants were grown in about 40 percent transmitted sunlight, the inoculated leaves reacted with ill-defined, faint and yellow chlorotic lesions.

Independently of temperature and light, systemic symptoms are evident five to seven days after inoculation. Vein clearing is the first visible systemic symptom. In the leaf directly above the inoculated one, vein clearing is usually limited to the basal half of the leaf while it is expressed over the entire younger top leaves. The leaves beneath the inoculated one, usually developed normally.

Shortly after vein clearing is fully developed, growth of the invaded leaves, petioles and growth-tip is arrested and plants may remain alive in a dwarfed condition for up to three months. Cessation of growth is accompanied by a crinkling of the leaves which is pronounced in the younger top leaves. After about five weeks, the interveinal areas of the systemically invaded leaves become yellow to white in colour, while the tissue next to the veins remains green.

N. tabacum cv. Turkish, reacted to the mild strain with yellowish necrotic ringspots. Each local lesion consisted of two to four rings with normal green tissue separating the rings. The central ring may later form a whitish necrotic spot. Systemically invaded leaves showed small white necrotic spots, vein-clearing and irregular necrotic line patterns and sometimes also necrotic rings. Cessation of growth and crinkling of the leaves was sometimes expressed and plants remained alive for up to three months, during

which period their development was retarded.

2) <u>The necrotic strain</u>: On <u>N</u>. <u>glutinosa</u>, purple necrotic ringspots appeared after four to five days. These ringspots usually spread to produce large necrotic areas. Systemic symptoms were vein-clearing followed by necrosis of the basal areas of the leaves, cessation of growth and the death of the plant after about six weeks due to the growth-tip and upper leaves becoming necrotic.

Circular necrotic lesions are expressed on the inoculated leaves of <u>N</u>. <u>tabacum</u> after four days. Spreading of these lesions causes the leaves to die off in about ten days. Systemic symptoms are essentially necrosis, usually appearing along the main vein first and later spreading rapidly to all the lateral veins and also into the interveinal areas. The necrotic areas along the veins, are sometimes confined to the lower half of the leaf and often on the one side only. The tops of such plants are usually bent over to one side while a brown necrotic streak appears on this side of the stem. The death of such plants is usually brought about in six to eight weeks.

C.   <u>Characterization of TSWV.</u>

The strains under study were characterized as TSWV according to the criteria, listed by Best (1968):

i) The virus isolates were derived from a single thrips induced lesion.

ii) Symptoms produced on tomato and tobacco correspondend to those described by Samuel <u>et</u> <u>al</u>. (1930) and Samuel & Bald (1933)

respectively.

iii) In extracted plantsap, infectivity was lost between 30 and 45 minutes in one, and between 45 and 60 minutes in two other tests. This is well within the range of less than 2 to 5 hours (Bald & Samuel, 1934).

iv) At neutral pH and at $20^{\circ}C$ the infectivity of an inoculum was lost after 5, 7 and 11 hours in three evaluations, respectively. This is within the 24 hour period characteristic for TSWV (Best, 1968). When kept in crushed ice, the infectivity in three parallel tests was lost after 8, 9 and 15 hours, which is within the 24 hour limit determined by Best (1966).

v) When sodium sulphite was added to an inoculum kept at $0^{\circ}C$ and neutral pH, infectivity was retained for 40 hours, 50 hours and 60 hours respectively in three infectivity assays. The minimum determined by Best (1966) was 24 hours.

vi) In three separate determinations, virus infectivity was completely lost after keeping an inoculum for 10 minutes at $46 \pm 0,5^{\circ}C$.

vii) When the pH of an inoculum was adjusted to 5,0 or less, complete loss of infectivity occurred within 2 minutes.
With each of the above in vitro determinations, parallel control inoculations with untreated plant sap were performed and in each case the starting material was found to be highly infective.

viii) An average sedimentation constant of 551 S was determined for partially purified virus preparations compared to an average of 532 S as reported by Best (1966).

ix) In the electron microscope, virus particles varied from 90 to

115 nm in diameter.

D.   Harvesting and storage of infected leaves.

In comparing the virus content of systemically invaded leaves,
it was found that the highest virus concentration occurred in leaves
36 to 48 hours after the onset of the systemic symptoms. This was
determined by inoculation of the different homogenates at a dilution
of 1:15. Successful purification depended largely on a relatively
high virus concentration in the infected leaf tissue.

Within a batch of plants, individual plants usually reached the
stage of optimal virus content in a period of 5 to 7 days after
inoculation. Leaves at the optimal stage were harvested each day,
weighed and added to previously harvested leaves in plastic contain-
ers in a liquid nitrogen refrigerator. This ensured that purification
could be done with material of a high virus content.

Leaves were stored in 30 gm batches in liquid nitrogen and
when required, the frozen leaves were first pulverised with an iron
rod in the plastic containers. Small quantities of these frozen leaf
pieces were rapidly stirred with 120 ml of appropriate buffer, pre-
heated to $37^{O}C$. By mixing small quantities at a time, ice formation
was prevented. The temperature of this pulp was usually between 1^{O}
and $4^{O}C$. At this stage a Waring Blendor was used to homogenize the
pulp.

E.   Purification.

1) Comparison of previously published purification procedures.

In order to obtain purified TSWV for biophysical studies, the purification methods of Best (1966), Black et al. (1963) and Martin (1964) were repeatedly tried. None of these methods ever led to the presence of a light scattering virus zone in the gradient tubes. Material obtained after sampling and pelleting of the zones where virus zones should have occurred was examined in the electron microscope and revealed some virus particles scattered among a high concentration of extraneous material.

The loss of virus brought about by the different treatments during purification, was determined with the local lesion technique. The amount of infective virus retained with each step was determined and compared with the initial amount of infective virus present in the homogenate. The amount of residual virus was expressed as a percentage of the lesions produced by the homogenate. In this way it could be determined why the above mentioned techniques were unsuccessful in purifying the South African strains.

With the method of Black et al. (1963), only 15 percent of the virus was retained in the sample layered onto the density gradient (see Fig.1). The loss of virus activity during purification is indicated in Table III.

The concentration of virus in pellet P_1, after homogenization and low speed centrifugation, is similar to that reported by Black and his co-workers (Fig.5). Best (1966) however, with the identical procedure, found that less than 20 percent of the infectivity was found in the pellet P_1. The most likely explanation could be differences in virus strains used. The greatest loss of virus was found when pellet P_1 was resuspended in 0,01 M Na_2SO_3 and subjected to low

TABLE III. Retained virus activity, as a percentage of the infectivity of the homogenate, when TSWV was purified according to the method of Black et al., (1963). (See Fig.5)

| Purification step | Percentage infectivity | | | | B** | C*** |
| | A* | | | | | |
	1	2	3	Average		
First pellet (P_1)	80	71	76	76	91	ca. 10
Second supernatant (SL_2)	27	50	33	36	71-90[a]	-
Third pellet (P_3)	16	21	9	15	-	-

* As determined during the course of this work.
** As determined by Black et al., 1963.
*** As determined by Best, 1966.
[a] As a percentage of the infectivity of that in SL_1

speed centrifugation in order to improve clarification. Only 36 percent of the virus infectivity was retained in supernatant SL_2 instead of 85 percent as reported by Black and co-workers. The virus in supernatant SL_2 was concentrated into pellet P_3 by high speed centrifugation. This pellet was resuspended in 0,01 M Na_2SO_3 and subjected to gradient centrifugation. The amount of virus left did not produce a visible virus containing zone in the density gradient. A dense white-green zone occurred throughout the entire density gradient and could have concealed a very diffuse virus band. Dispersal of the virus from pellet P_1 seemed to be most unsatisfactory and the most likely reason for failure of this method.

Next, the method of Best (1966) was also tested quantitatively to determine the loss of virus infectivity during purification (see

Fig.3). When working with the phosphate based buffer, "solution 4", Best (1966) reported that more than 70 percent of the virus infectivity was always found in supernatant SL_1 after homogenization and subsequent centrifugation (see Fig.3). This was confirmed with the South African isolates. The results are presented in Table IV.

TABLE IV.   Retained virus activity, as a percentage of the infectivity of the homogenate, when TSWV was purified according to the method of Best (1966) (See Fig.5).

Step	Percentage infectivity				
	A*				B**
	1	2	3	Average	
First supernatant (SL_1)	74	81	62	72	70-80
Second pellet (P_2)	53	69	58	60	-
Third supernatant (SL_3)	2	5	8	5	-

* As determined during the course of this work.
** As determined by Best (1966).

The virus in supernatant SL_2 was concentrated in pellet P_2 by highspeed centrifugation. The greatest loss in virus infectivity was found when the resuspended pellet P_2 was subjected to low speed centrifugation. Further clarification, after resuspension of pellet P_2 was necessary since a relatively high amount of plant material was present in pellet P_2. The centrifugal forces used by Best were much higher than those used by Black and co-workers and the centrifugation times were much shorter.

In further tests with both these procedures the solutions with
a high virus content were layered directly onto the density gradients.
Low speed centrifugation of the resuspended P_1 in the method of
Black and co-workers, and low speed centrifugation of the resuspen-
ded P_2 in the purification of Best (1966) was thus omitted. These
samples did not reveal any definite virus containing zones, but only
a dense green-white light scattering zone from the meniscus to the
bottom of the density gradient. This finding stressed the importance
and necessity of improving the clarification of the virus sample
prior to density gradient centrifugation.

Martin's (1964) method (see Fig.2) was not investigated as
thoroughly as the above mentioned methods. The virus infectivity of
the samples layered onto the density gradients was compared with
those of the homogenates. In both assays, only 2 and 7 percent of
the virus infectivity was retained after clarification with calcium
glycerophosphate and two cycles of differential centrifugation.

From these preliminary investigations, it was clear that low
speed centrifugation is unsatisfactory as a means of clarification
because of excessive virus loss, although it also appeared that some
form of clarification is essential.

It was decided therefore to compare different clarification
methods with respect to their ability to retain virus infectivity.

The different clarification agents tested were added to diffe-
rent steps during the purification scheme set out in Fig.6. By
assaying the virus infectivity at different stages during purifica-
tion it was possible to determine the loss of virus occurring with

Fig.6. The centrifugal procedure followed when different clarifica-
tion agents were tested with respect to their ability to
retain virus infectivity.

Infective leaf tissue + Buffer[*]

**Homogenise for 2 minutes

Squeeze through cheesecloth
Centrifuge for 10 minutes at
10 000 rpm in Sorval SS-34
rotor

SL_1*** P_1(discard).

Centrifuge for 40 minutes at
25 000 rpm in the Spinco no
30 rotor

P_2 SL_2(discard)

Resuspended in 6 ml buffer and
divided into 2 batches of 3 ml

Resuspended P_2 (3 ml batch).   Resuspended P_2 (3 ml batch)

Centrifuge for
10 minutes in
the Spinco no
40 rotor at 10 000
rpm

SL_3 P_3(discard).

The resuspended part of pellet P_2 and the supernatant SL_3 was
layered onto density gradients.

*   Infective leaf tissue was either macerated in Best's neutral
phosphate buffer (1966) at a concentration of 1:5 (w/v) or the
phosphate-sulphite buffer (pH 7,2) used in this work at a con-
centration of 1:3 (w/v).

**   Bentonite or hydrated calcium phosphate was added to the homo-
genate (see sections 2a and 2b on pages 61 and 62).

*** Supernatant SL_1 was clarified by either charcoal and celite fil-
tration or by chromatography on calcium phosphate columns (see
sections 2c and 2d on pages 63 and 65).

the different treatments. The infectivity at the different stages
of purification was compared to that of the homogenate.

The degree of clarification obtained with the different clari-
fication agents was usually determined after gradient centrifugation
of the different samples. After incorporation of the different cla-
rification agents into the purification scheme set out in Fig.6,
the light scattering components present in the density gradient were
compared to those present after purification by centrifugation alone.
Additional clarification of the resuspended pellet P_2, by low speed
centrifugation, was also tested in this way.

2) <u>A comparison of different clarification agents</u>.

a) <u>Bentonite flocculation</u>.   (Dunn & Hitchborn, 1965).
Different quantities of a bentonite solution were added
to the homogenate prior to low speed centrifugation. The bentonite
concentration was determined by weighing the residues after drying
known quantities of the bentonite solution. Bentonite was added on
a weight basis. The loss of virus with bentonite clarification is
set out in Table V.

Bentonite concentrations of 25 to 55 mg per 1 gm of leaf tissue
removed so much virus and plant material, that no pellets were vi-
sible after high speed centrifugation of supernatant SL_1. When con-
centrations of 12 to 13 mg bentonite were used for 1 gm of leaf
tissue, no virus zones were seen in gradient tubes after gradient
centrifugation of either the resuspended pellet P_2 or the third

TABLE V.   Retained TSWV infectivity, as a percentage of the homo-
genate after bentonite clarification. (See Fig.6).

mg of bentonite per 1 gm of leaf tissue	Percentage infectivity	
	SL$_1$	SL$_3$
Experiment I:		
55	0	0
27	0	0
13	7,6	2,3
6	27	1,6
3	35	2,9
Experiment II:		
50	0	0
25	0	0
12	13	0,5
6	37	2,3
3	41	1,6

supernatant SL$_3$. Clarification with the lower bentonite concentra-
tions was no better than that obtained by Best's procedure (see
Fig.3).

b) Absorption to hydrated calcium phosphate (Fulton, 1959).

Hydrated calcium phosphate (HCP) was thoroughly mixed
with the homogenate prior to low speed centrifugation. Various quan-
tities of HCP were added to the homogenate in order to determine the
most suitable ratio for clarification and maximum retention of virus
infectivity. The loss of virus with this purification method, is pre-
sented in Table VI.

TABLE VI. Retained TSWV infectivity as a percentage of the infectivity of the homogenate, after clarification with hydrated calcium phosphate. (See Fig.6).

Ratio of leaves: HCP: buffer (w/w/v)	Percentage infectivity		
	SL_1	P_2	SL_3
1:1:3	0	0	0
1:0,7:3	42	36	11
1:0,4:3	78	47	12

With the high concentrations of HCP, all the virus was removed with the first low speed centrifugation. With the two lower HCP concentrations, much of the virus was left in supernatant SL_1 but clarification was incomplete. A sample of the resuspended pellet P_2, examined in density gradient centrifugation, revealed no virus zones in the dense green-white zone which occurred from the top to the bottom of the tube. The virus content of supernatant SL_3 was too low to reveal a virus zone in gradient tubes.

c) <u>Charcoal and Celite clarification</u>.

This method of clarification which greatly simplifies the purification of tobacco mosaic virus (von Wechmar & van Regenmortel, 1970), was also applied to TSWV.

Various quantities of charcoal and celite, always in the same ratio, were mixed with supernatant SL_1 after homogenisation and low speed centrifugation (see Fig.6). The slurry was filtered by suction through a Buchner filter with a 6 mm celite pad. Concentration of the filtrate was achieved by high-speed centrifugation. Since these

high speed pellets (P_2) contained a large amount of plant material, one half of the resuspended pellets was subjected to low speed centrifugation in order to improve clarification. The virus content of both samples was compared by assaying virus infectivity and by comparing the resultant virus containing zones, if present.

TABLE VII. Retained TSWV infectivity, as a percentage of the homogenate, after clarification with charcoal and celite. (See Fig.6).

gm of charcoal and celite per 100 gm leaf tissue.		Percentage infectivity	
Charcoal	Celite	Filtrate	SL_3
5	5	8	0,2
7,5	7,5	43	1,9
7,5	7,5	38	0,3
8,0	8,0	90	2
9,0	9,0	10	1,4
10	10	80	7,4
10	10	24	0,2
10	10	50	30
10	10	30	4,5
12	12	5	1,6
12	12	0,2	0,1
15	15	0	0

From Table VII it is clear that all the virus was removed from supernatant SL_1 with the higher concentrations of the clarifying agents. In the four tests with 10 gm of charcoal and celite, the virus infectivity of the filtrate varied from 24 to 80 percent in comparison with that of the homogenate. These tests were done over

a period of four weeks, keeping the purification procedure as constant as possible.

When the resuspended pellets P_2, were directly subjected to density gradient centrifugation, it was sometimes possible to distinguish faint virus zones, 18 to 25 mm from the meniscus. After pelleting and a second cycle of gradient centrifugation, the zones still revealed extraneous components in the electron microscope. Very often, no virus could be seen in the electron microscope after the second density gradient centrifugation, although a relatively high concentration was observed after the first gradient separation.

The great variation in virus content of the different filtrates after the same treatment, demonstrates the unreliability of this method for purifying TSWV. Galvez (1964) demonstrated that the method used for removing the charcoal from the extracts greatly affects the final virus concentration. The diameter of the funnel used and the thickness of the pad formed by the charcoal had an important effect in holding the plant components and the virus particles.

> d) <u>Chromatography on calcium phosphate (brushite) columns</u>
> (Murant <u>et</u> <u>al</u>., 1969).
>
> The clarification of supernatant SL_1 (see Fig.6), after

homogenization and low speed centrifugation, was attempted by chromatography on calcium phosphate (brushite) columns.

The elution volume of the brushite columns was determined with phenol red and the supernatant was allowed to flow through these columns. After collecting the supernatant, the columns were eluted stepwise with 0,04, 0,06, 0,08 and 0,1 M phosphate buffers (pH 7,2)

containing 0,01 M sodium sulphite.

The virus activity of the different eluates was determined and compared to that of the homogenate. In the four preliminary experiments done, nearly all the virus infectivity was found in the first eluant, which actually was the buffer used for homogenizing the leaf tissue. As can be seen in Table VIII none of the virus was irreversibly adsorbed to the brushite column, while elution was sharp and did not take place over a range of different concentrations.

TABLE VIII.   Retained TSWV infectivity, as a percentage of the infectivity of the homogenate, after chromatography on calcium phosphate columns. (See Fig.6).

Experiment	Percentage infectivity		
	SL_1	0,02 M eluate	0,04 M eluate
1	89	82	1
2	79	66	4
3	66	54	2
4	83	63	1

The extent of clarification of supernatant SL_1 was quite remarkable and obvious when comparing the gold coloured virus containing eluant with the starting greenish supernatant. Most of the green plant material of the first supernatant was adsorbed onto the top half of the column and was not eluted with the different phosphate buffers tested.

Concentration of the virus containing eluant by high speed centrifugation, yielded soft jelly-like pellets of 7-9 mm diameter,

which were easily resuspended. When this virus suspension was compared with the corresponding virus suspensions obtained in previous clarification attempts, including the purification methods of Black et al. (1963) and Best (1966), it appeared that a greatly superior clarification had been achieved.

Gradient centrifugation of these virus solutions always revealed intense light scattering virus zones. Other diffuse light scattering zones, above and below the virus zones, were also visible.

With calcium phosphate clarification, an important goal was achieved. The first supernatant was clarified to such an extent, that it was possible to omit the step of low speed centrifugation preceeding gradient centrifugation. This is important because the preceeding experiments have shown that low speed centrifugation is the main step responsible for the loss of virus.

A brushite column with an elution volume of about 110 ml, was found to be sufficient for clarifying the supernatant of 30 gm of leaves homogenised in 90 ml of buffer. The precipitate formed by mixing 150 ml 0,5 M sodium phosphate with 150 ml 0,5 M calcium chloride, gave a column with such an elution volume.

F.   Purification by chromatography and gradient centrifugation.

The following procedure was adopted for TSWV purification using the 0,02 M phosphate-sulphite buffer (pH 7,2) throughout.

The slurry obtained by mixing 90 gm of leaf tissue with 330 ml of buffer, was homogenised for two minutes in a Waring Blender. The pulp was squeezed through cheesecloth and then centrifuged for 10

minutes at 10 000 rpm in the Sorvall SS-34 rotor. The resulting
greenish supernatant was added to a brushite column and eluted with
the same buffer used for homogenation. Elution was sharp and the
elution volume determined with a LKB-Uvicord densitometer (Fig.7).
This determination is more accurate and less time consuming than
the determination with phenol red (Taverne et al., 1958). As soon
as a 10 percent transmission was recorded, the eluate was collected.
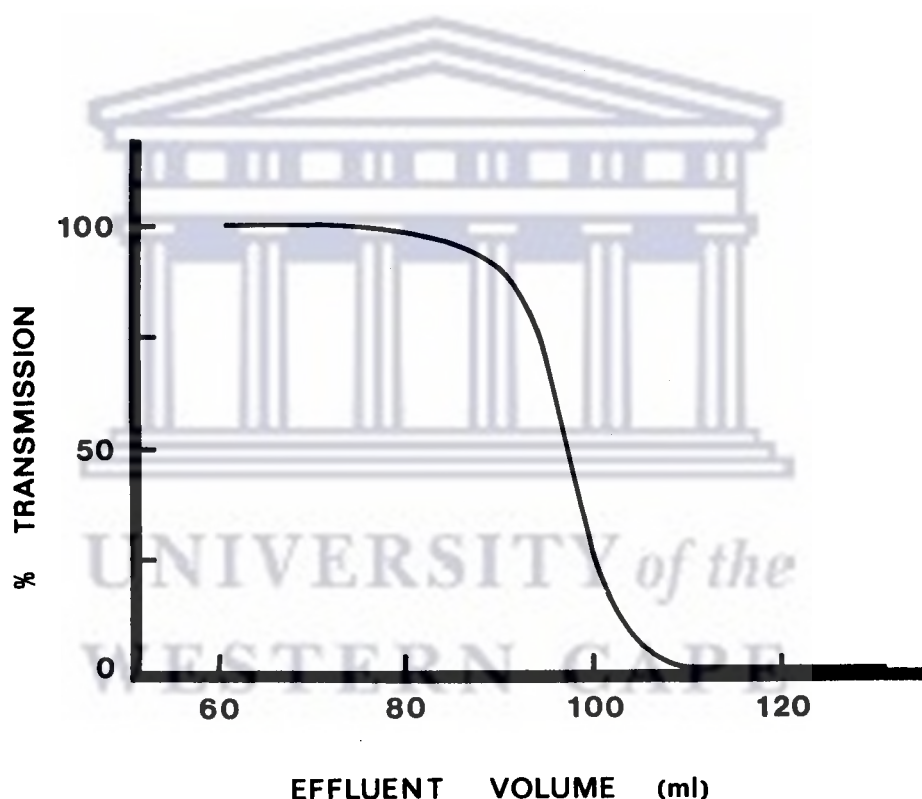
Fig.7. Transmission at a wavelength of 254 nm of the
virus containing supernatant eluted from calcium phos-
phate columns.

Most of the green material in the first supernatant SL_1 (see Fig.6)
was adsorbed to the column. None of this plant material was eluted
with different phosphate concentrations, a concentration of 0,1 M
phosphate being the highest concentration tested. The virus contain-
ing eluate was centrifuged for 40 minutes at 25 000 rpm in the Spinco
no 30 rotor and the large, 8 to 10 mm jelly-like pellets were resus-

pended in 6 ml phosphate sulphite buffer.

Two millilitre portions of the resuspended virus pellets were layered onto three tubes, each containing a 10-40 percent sucrose density gradient and centrifuged for 60 minutes at 20 000 rpm in the Spinco SW 25-2 rotor. The following light scattering zones (Fig.8A), as measured from the meniscus, could be distinguished in the density gradients:

i) a white zone, a, 2-8 mm from the meniscus. The upper part is usually more concentrated than the lower part, and

ii) a diffuse zone, b, 9-18 mm from the meniscus,

iii) the intense light scattering virus zone, c, 18-25 mm from the meniscus, and

iv) a diffuse 5 mm zone, d, directly beneath the virus zone.

The density gradients were slightly opalescent between the distinguishable zones and the bottom of the tubes. A yellow-greenish pellet was formed at the bottom of the tube.

Zone c contained up to 85 percent of the virus infectivity of the tube, the rest of the infectivity was confined to the lower half of the gradient. After sampling of the virus zone c, it was centrifuged for 35 minutes at 35 000 rpm in the Spinco no. 40 rotor. A slight greenish pellet, 5-6 mm in diameter was formed in the centrifuge tube. Electron microscopy of this zone revealed a high concentration of virus particles with some unidentified material scattered among the virus particles (Fig.9). The virus particles varied much in shape and their appearance differed from sample to sample.
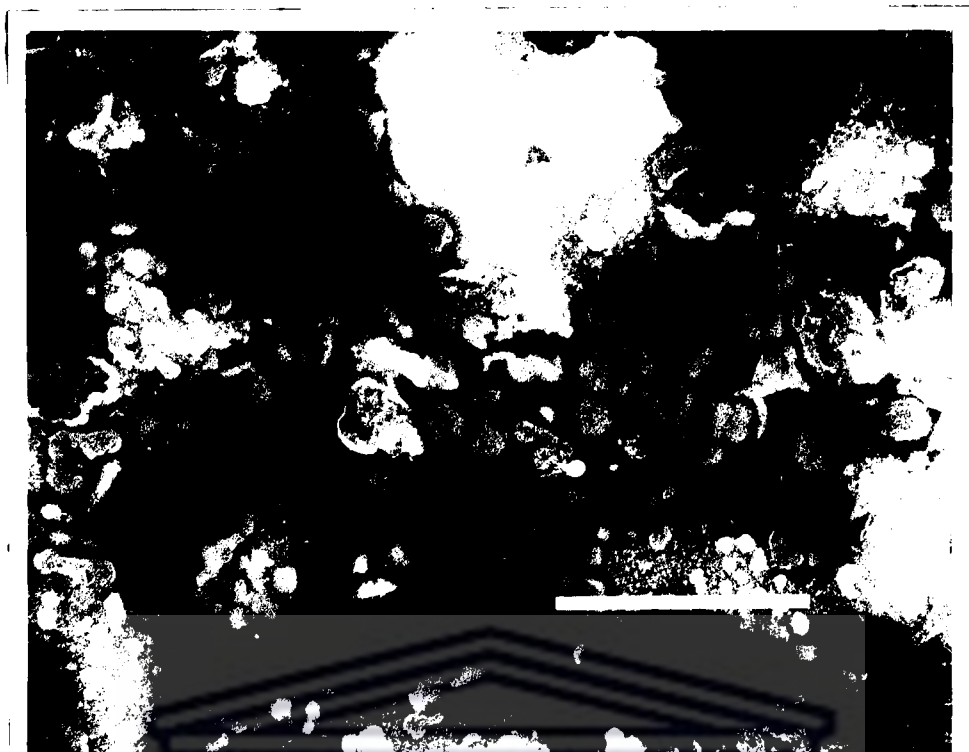
Fig.9. Virus particles of the 18 to 25 mm virus zone after calcium phosphate clarification and density gradient centrifugation, negatively stained with 2% neutral PTA. The bar represents 500 nm.

The light scattering intensities of the different zones in the density gradient (Fig.8A) are presented in Fig.8C. The height of the different peaks is also representative of the concentrations of the material in each zone.

The strong absorption of ultraviolet light by zone a, when compared to that of the other light scattering zones (Fig.8B), suggested the presence of a nucleic acid component in this zone since nucleic acid absorption is much stronger than that of proteins at similar concentrations. According to the intensity profiles, zone a contained much less material than the other light scattering zones.

In trying to separate the plant material from the virus, virus samples were subjected to a second step of gradient centrifugation. Pellets obtained from the resultant virus zones still revealed plant material among the virus particles in the electron microscope. The

virus pellets of the first gradient which were 5-6 mm in diameter, were reduced to 2-3 mm pellets without much further purification. Often, when using material relatively low in virus content, the virus pellet after the first step of gradient centrifugation disappeared with further gradient centrifugation and pelleting.

It appeared therefore that a second density gradient step was inefficient in removing the plant material and that it led to an important loss of virus.

1) <u>Zone electrophoresis</u>.

Virus samples, after a single step of gradient centrifugation, were also subjected to zone electrophoresis at 4°C. The borate buffer (pH 8,6) containing 0,01 M sodium sulphite and the phosphate-sulphite buffer (pH 7,2) were used at a current of 17 mA. In both cases, two light scattering zones, about 1 mm apart, could be distinguished after three hours. After another hour, the upper intense zone became more diffuse and vanished completely within two hours. The lower diffuse zone was not destroyed, but it contained no virus particles visible in the electron microscope and was not infective.

At this stage, it was realized, that the virus could only be purified by a relatively short purification procedure if no ways to preserve its infectivity could be found.

2) <u>Sedimentation coefficient</u>.

In comparing the light scattering intensities of the two zones obtained in zone electrophoresis, the relatively low concentration of extraneous material, as observed in the electron microscope, was confirmed. Although not homogeneous, the virus samples after a single

step of gradient centrifugation, were used for the determination
of sedimentation coefficients. The sedimentation rates were deter-
mined with schlieren optics, and in later experiments with both
schlieren and UV optics simultaneously (Table IX).

TABLE IX. $S_{20,w}$ values of TSWV preparations obtained by calcium
phosphate clarification and density gradient centrifu-
gation. Virus preparations were resuspended in 0,01 M
sodium sulphite.

Experiment	Optical system	
	Schlieren	UV
1	547	x
2	534	x
3	548	x
4	566	x
5	560	x
6	536	470
7	555	484
8	557	515
9	528	487
10	570	457
11	562	452
Average	551	477

x Not determined.

A standard deviation of $\pm$ 13 was determined for the $S_{20,w}$
values determined with schlieren optics. The sedimentation constant
thus is 551 $\pm$ 13.

Fig.10 shows a typical TSWV sedimentation peak obtained by using schlieren optics. Fig.11A shows a photograph obtained by using UV optics and Fig.11B shows the resulting densitometer tracing.

The slope of the sedimenting boundary and the baseline in the densitometer tracing (Fig.11B) indicate the presence of extraneous UV absorbing material in the TSWV preparation which may explain the lower S value obtained by this method. This was confirmed serologically (see Fig.17A) and in zone electrophoresis experiments (see Fig.13).

3) <u>Stabilizing of virus samples with glutaraldehyde</u>.

In order to employ longer and harsher purification techniques, virus containing preparations were fixed with glutaraldehyde, a common fixative used with tissue sections. After pelleting the density gradient virus zone, it was resuspended in 0,3 ml phosphate-sulphite buffer and to this virus suspension was added an equal volume of 0,8-1 percent glutaraldehyde in 0,01 M phosphate buffer (pH 7,2).

Virus samples fixed with glutaraldehyde, revealed only round to elliptical virus particles (Fig.12) in contrast to the pleomorphic particles of parallel unfixed virus samples (Fig.9).
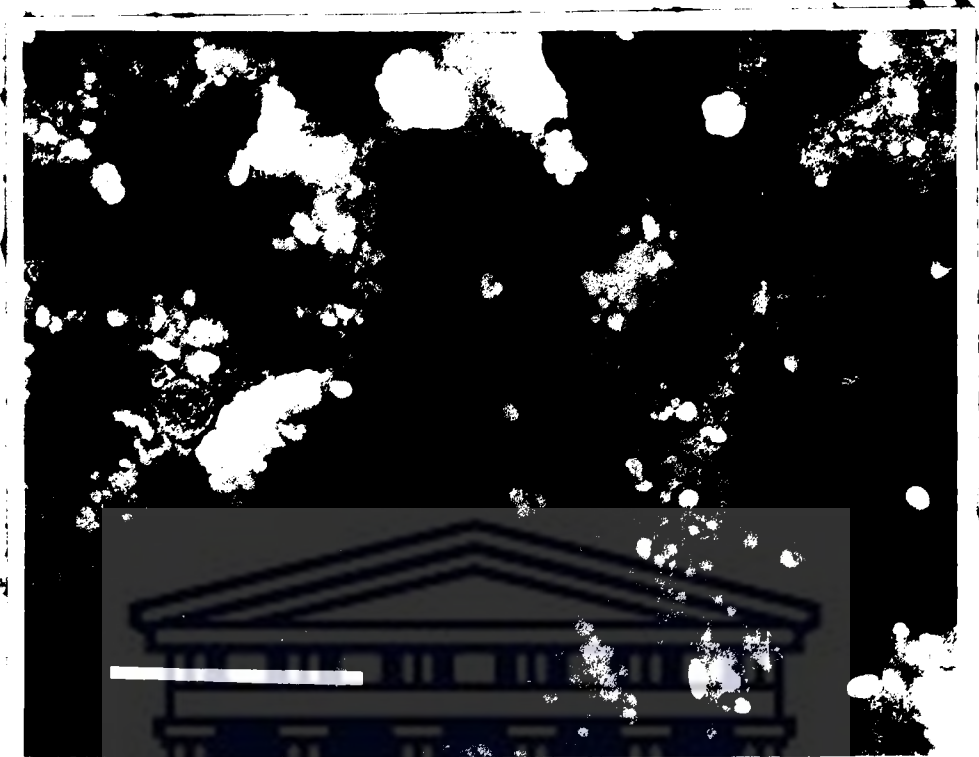
Fig.12. A virus preparation obtained by calcium phosphate clarification and gradient centrifugation, fixed with 0,4% glutaraldehyde and negatively stained with 2% neutral PTA. The bar represents 500 nm.

4) Zone electrophoresis of virus samples fixed with glutaraldehyde.

Glutaraldehyde fixed material from density gradient virus zones could be further purified by borate zone electrophoresis, a technique which had been shown to destroy unfixed virus samples after about 5 hours. After electrophoresis for 14-16 hours at 17-20 mA, two light scattering zones could be distinguished in the zone electrophoresis apparatus (Fig.13).

Fig.13. Virus zones in a borate zone electrophoresis
column. The virus preparation was obtained by calcium
phosphate clarification and gradient centrifugation
and was fixed with glutaraldehyde.

Examination of these zones in the electron microscope revealed
that the distinct upper zone contained practically only virus par-
ticles (Fig.14A). The diffuse lower zone consisted of large pleo-
morphic structures (Fig.14B). These structures probably are col-
lapsed, stain penetrated TSWV particles devoid of their nucleic
acid component. This is suggested by the size of these structures
and the presence of what could be an outer limiting membrane.

Occasionally a very diffuse zone could be distinguished direct-
ly above the intense light scattering virus zone. Prolongation of
electrophoresis to 20 or 22 hours did not completely separate this
very diffuse zone from the virus zone. The lower edge of this very
diffuse zone could never be distinguished since it was always masked

by the virus containing zone. The foreign material present among
the virus particles in Fig.14A is representative of the material
obtained after concentration of this zone by ultracentrifugation.
Although this diffuse zone could only occasionally be distinguished,
it was probably always present because foreign material was always
revealed in electron micrographs of material from the virus zone.

The R_ϕ-values corresponding to the zones observed in a series
of borate zone electrophoresis experiments are presented in Table X.
The ratio of the distance moved by the upper limit of the virus
zone to the distance moved by the phenol red has been termed the zone
electrophoretic R_ϕ of a virus (van Regenmortel, 1968).
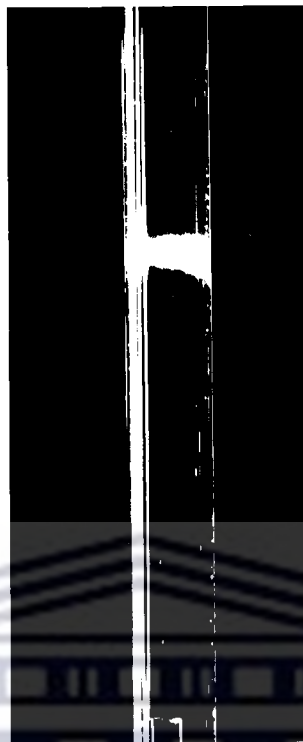
TABLE X. R_ϕ values of the light scattering zones in a borate
(pH 8,6) zone electrophoresis column. The virus prepara-
tion was obtained by calcium phosphate clarification,
gradient centrifugation and was subsequently fixed with
glutaraldehyde.

R_ϕ-values		
The occasionally present top zone	The distinct virus zone	The diffuse lower zone
*	0,67	0,42
*	0,67	0,43
*	0,66	0,42
*	0,67	0,38
0,75	0,65	0,38
*	0,62	0,41
*	0,64	0,46
0,74	0,64	0,36
0,77	0,68	0,42
0,75	0,65	0,42

* Not distinguishable; probably too diffuse.

Although foreign material was still distinguishable among the virus particles collected from the distinct virus zone, these samples were used to immunize a number of rabbits.

5) <u>Sedimentation coefficients of glutaraldehyde fixed virus preparations.</u>

After borate zone electrophoresis of gluteraldehyde fixed virus preparations, the virus containing zone was concentrated by ultracentrifugation and resuspended in distilled water. Sedimentation coefficients (Table XI) of the resuspended virus pellets were simultaneously determined with both the UV and schlieren optical systems.

The virus pellets were resuspended in distilled water in order to determine the dry weight of virus after investigating it spectrophotometrically.

TABLE XI. $S_{20,w}$ values of TSWV obtained by calcium phosphate clarification, gradient centrifugation, glutaraldehyde fixation and borate zone electrophoresis (pH 8,6). The virus was resuspended in distilled water.

Experiment	$S_{20,w}$ values	
	Schlieren optical system	UV optical system
1	610	605
2	617	601
3	718	685
Average	648	630

The $S_{20,w}$ values of virus samples obtained after glutaraldehyde fixation and zone electrophoresis were much higher compared to the

values obtained with unfixed material (see Table VIII). With UV-
optics, the average value was 630 S in comparison with the previous-
ly determined average of 472 S. With schlieren optics, an average
S value of 648 was obtained in comparison with the average of 551 S
obtained after gradient centrifugation only.

6) Immunization of rabbits with virus preparations obtained after
   borate zone electrophoresis.

Three rabbits, designated TSWV-Ab 1, TSWV-Ab 2 and TSWV-Ab 3,
were immunized with virus preparations obtained after calcium phos-
phate clarification, gradient centrifugation, glutaraldehyde fixa-
tion and borate zone electrophoresis. Some foreign matter could,
however, still be detected with the electron microscope in these
preparations (Fig.14A).

In parallel serological reactions, the sera from all the rabbits
reacted similarly to the different antigens tested and no differences
could be detected amongst them. Serum TSWV-Ab 1 was then used through-
out this part of the work. Serological tests were always performed
with a series of twofold dilutions of the sera and antigens under
investigation.

A single precipitin line was formed in the serological reaction
between a virus preparation purified by zone electrophoresis and the
homologous antiserum (Fig.15).

Fig.15. The precipitin pattern of a virus preparation
obtained by calcium phosphate clarification, gradient cen-
trifugation, glutaraldehyde fixation and borate zone
electrophoresis with a homologous antiserum in phosphate-
sulphite-saline agar pH 7,2. The central well was filled
with antiserum TSWV-Ab 1 diluted 1/16. Wells 1 to 8 con-
tained two-fold dilutions of the virus preparations.

The movement of TSWV particles into the agar was poor and pre-
cipitin lines were formed near the virus containing well. This poor
movement of virus particles into the agar layer is due to the low
virus concentrations present after zone electrophoresis and also to
the low diffusion coefficient of the large virus particles.

The ability of these sera to react with plant material was
tested with infective plant juice and with the virus preparations
obtained at different stages of purification. It was found that, be-
sides the virus precipitin lines, other precipitin lines were also
formed (Fig.16).

Virus precipitin lines were formed on the edges of wells 1 and
3 (see Fig.16) while an additional strong reaction occurred with an
antigen present in the concentrated virus preparation obtained after

calcium phosphate clarification. A third unidentified precipitin
line was also formed near the central well opposite the virus pre-
paration in well 1 (see Fig.16).
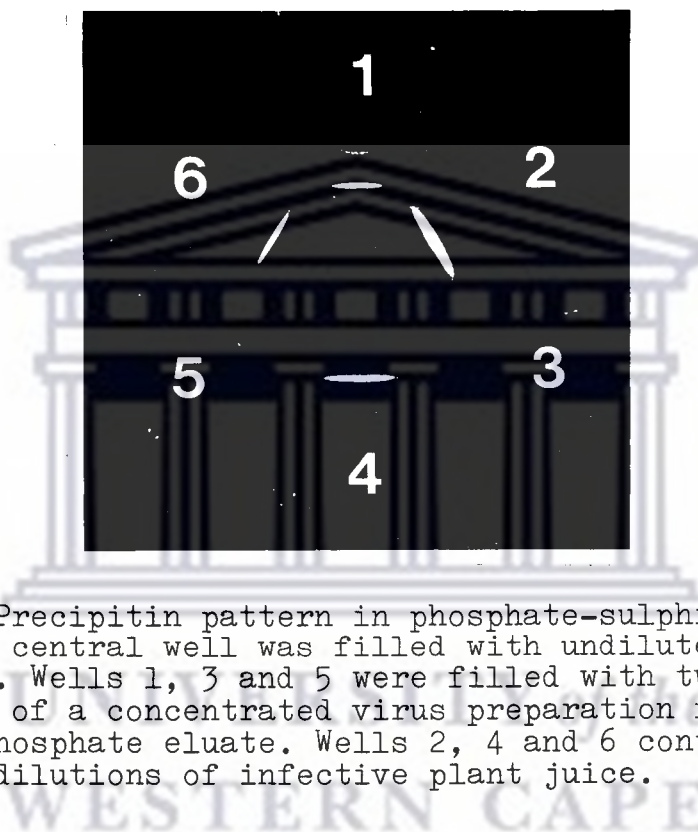
Fig.16.   Precipitin pattern in phosphate-sulphite-saline
agar. The central well was filled with undiluted serum
TSWV-Ab 1. Wells 1, 3 and 5 were filled with two-fold
dilutions of a concentrated virus preparation from the
calcium phosphate eluate. Wells 2, 4 and 6 contained
two-fold dilutions of infective plant juice.

The identity of the contaminating antigen still present after
calcium phosphate clarification was established as F 1 protein when
purified F 1 preparations were included as antigen in precipitin
tests (Fig.17). In phosphate-sulphite-saline agar (Fig.17A), two
contaminating antigens were demonstrated in the calcium phosphate
eluate and also in the virus zone after gradient centrifugation. With
a parallel reaction in cacodylate-saline agar (pH 6,3), no virus
precipitin lines were formed (Fig.17B) but the two contaminating
antigens present in partially purified virus preparations were clear-
ly demonstrated. These antigens were both identified as components

of F 1 protein also present in healthy plant juice. Both these fractions in purified F 1 protein and healthy plant juice were also demonstrated in pH 6,9 citrate-saline agar (Fig.17D) whereas only a single precipitin line appeared in a parallel reaction, in phosphate-sulphite-saline agar (Fig.17C).

The characteristic position of TSWV precipitin lines in agar plates, greatly facilitated the serological identification of this virus. Although the antisera also reacted with F 1 protein, these precipitin lines were formed well away from the virus precipitin lines and did not lead to any confusion.

These sera will obviously not be suitable for serological reactions in tubes, unless they could be freed from F 1 antibodies by cross absorption.

The titres and homogeneity of the sera obtained with different antigen preparations are presented in Table XVI.

7) Investigations of F 1 protein contamination of TSWV preparations.

In purifying TSWV, relatively short centrifugation times were used and this was not expected to allow the retention of molecules as small as F 1 protein with a sedimentation coefficient of about 18 Svedberg units. With gradient centrifugation, it should be possible to separate a 18 S component completely from TSWV particles with an S value of about 540. It was, however, stressed by van Regenmortel (1966) that F 1 protein can form different aggregates with higher S values which are likely to be partly responsible for the contamination of virus preparations prepared by differential ultracentrifugation.

A   B
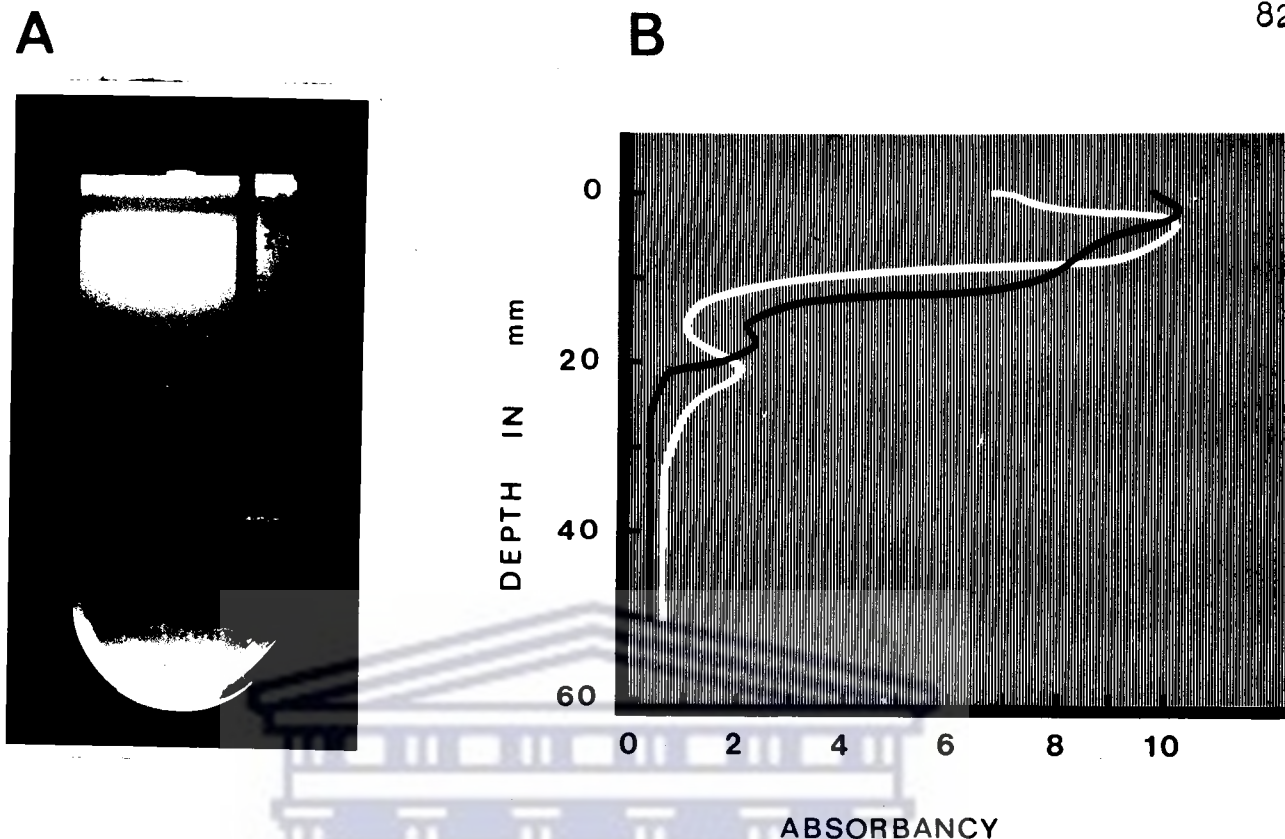
Fig.18. Gradient centrifugation of F 1 protein. A, Photograph
of the light scattering zone of F 1 protein in a sucrose gra-
dient. B. Optical density profile of the density gradient as
in A , superimposed on an optical density profile of a virus
containing density gradient as in Fig.8B. The white line re-
presents the virus solution and the black line a F 1 protein
solution.

When purified F 1 protein was subjected to gradient centrifuga-

tion a dense light scattering zone could be seen 1-11 mm from the

meniscus. The area directly beneath this zone was diffuse without

any light scattering zones(Fig.18A). It seemed therefore that a good

separation between F 1 and the virus particles which are concentrated

in a zone 18-25 mm below the meniscus should be possible.

In determining the optical density profiles of the gradient

columns, the exact position of F 1 protein was determined (Fig.18B),

With this more critical method 4 different zones could be distin-

guished, the two lower zones being found at the position where the

virus zone occurred. The presence of F 1 protein in the virus con-

taining zone was also confirmed serologically with serum TSWV-Ab 1

(Fig.17B).

Brakke (1960) demonstrated that the width of a light-scattering zone after gradient centrifugation depends on the amount of virus floated onto the gradient column. A light scattering zone of about 20 mm was observed after gradient centrifugation of a sample containing 10 mg TMV.

Since the considerable width of the F 1 protein zone caused its lower boundary to be present in the TSWV zone, F 1 protein contamination of TSWV may be attributed to the high concentration of F 1 protein present in the virus samples prior to gradient centrifugation.

Two F 1 protein fractions were detected serologically in the TSWV samples used for immunization (see Fig.17A). It has been shown before that F 1 protein, isolated by ultracentrifugation from wheat leaves, consists of two components which can be distinguished serologically (von Wechmar, 1967). Fractionation of the two antigenic forms of F 1 protein was achieved by chromatography on DEAE ion-exchange cellulose columns. It is also known that the UV absorption spectra of preparations containing F 1 protein vary according to the method of purification (van Regenmortel, 1964). When obtained by chloroform emulsification the UV absorption spectrum was that of a nucleoprotein but when the protein was obtained by clarification with hydrated calcium phosphate, the spectrum resembled that of a typical protein with a maximum absorption peak at 280 nm.

8) <u>Borate zone electrophoresis of F 1 protein.</u>

As the virus antisera also reacted with F 1 protein, this component should have the same or nearly the same electrophoretic mobility as TSWV in borate zone electrophoresis columns. F 1 protein ob-

tained by chloroform emulsification was also subjected to borate zone electrophoresis and in two experiments, R_ϕ values of 0,72 and 0,74 were determined. Another diffuse zone, with a R_ϕ value of 0,50 was always present in the lower part of the gradient column. F 1 protein was thus probably the zone occasionally distinguishable directly above the virus containing zone in borate zone electrophoresis. As it was never completely separated from the virus zone, some F 1 protein was thus contaminating the virus preparations used for immunization.

G. Purification by chromatography, PEG precipitation and gradient centrifugation.

1) PEG precipitation of crude plant sap.

The precipitation of virus by the addition of PEG and NaCl has been reported for tobacco mosaic virus, turnip yellow mosaic virus and turnip crinckle virus (Leberman, 1966; von Wechmar & van Regenmortel, 1970).

This rapid method of concentrating viruses was also tested for TSWV. After homogenising virus infected leaf material in the phosphate-sulphite buffer, the homogenate was centrifuged for 10 minutes at 10 000 rpm in the Sorvall SS-34 rotor and the supernatant devided into batches of 50 ml. To each batch were added different quantities of 25 percent solutions (w/v) of PEG and NaCl respectively. Final concentrations of PEG and NaCl were 12% - 9% and 6% - 3% respectively. The precipitates were separated by low speed centrifugation and were resuspended in 0,02 M phosphate-sulphite buffer. The virus content of the precipitates was roughly estimated by the presence or

absence of a light scattering zone in density gradient tubes.

Because of the large amount of plant material present in the PEG precipitates, resuspended precipitates were not suitable for gradient centrifugation and they were first given a low speed centrifugation. Both the resulting supernatants and pellets were subjected to gradient centrifugation in order to determine the distribution of virus between them. In comparing the light scattering of the virus zones, it was found that about one third of the virus was lost by low speed centrifugation.

It was found that with different batches of infective leaf material, different quantities of PEG and NaCl were needed to precipitate all the virus. With increasing amounts of PEG and NaCl, more F 1 protein was precipitated together with the virus.

The plant sap obtained after macerating different batches of infective leaf material may differ in its chemical composition since the plants were not propagated under absolutely constant conditions. Differences in salt concentration may thus be responsible for the varying amounts of TSWV present in PEG precipitates obtained at different times.

It appears therefore that ultracentrifugation is the best available method for concentrating TSWV.

2) <u>PEG precipitation of clarified sap</u>.

After PEG precipitation of TSWV from clarified plant sap, it was striking that only diffuse zones occurred above the virus zones after gradient centrifugation of some samples while no material could

be detected in the corresponding regions with other samples. The top regions of the density gradients have been shown to contain F 1 protein (Fig.18) and to be responsible for the contamination of virus preparations. It thus seemed that PEG precipitation could greatly reduce or even eliminate F 1 protein contamination of TSWV preparations.

The virus pellets obtained by concentrating clarified sap were resuspended in 6 ml phosphate-sulphite buffer. To this virus suspension were added 0,36 ml of a 25 percent PEG solution and 0,12 ml of a 25 percent NaCl solution to give final concentrations of 1,5 percent PEG and 0,5 percent NaCl respectively. The virus precipitate was separated by a 5 minute centrifugation at 10 000 rpm in the Spinco no 40 rotor and resuspended in 6 ml distilled water. Both the supernatant and the resuspended precipitate were then subjected to gradient centrifugation.

A virus zone could not be distinguished in the gradient column after gradient centrifugation of the supernatant. In the gradient tubes containing the resuspended PEG precipitate, a dense virus containing zone c, 19-25 mm from the meniscus was observed. A diffuse zone d, 5 mm in width, was present directly below the virus zone. The lower part of the tube, situated 30 mm below the meniscus was slightly opalescent (Fig.19).

Optical density profiles of the gradients at 254 nm, revealed a strong UV absorbing zone a, at the sample-sucrose interface (Fig.19B). These zones were not always evident with the naked eye and were not infective. The intensity of the light scattering zones

is presented in Fig.19C.

F 1 protein was serologically demonstrated in the virus suspensions prior to PEG precipitation and also in the supernatant after precipitation but not in the resuspended precipitate (Fig.20). It was therefore decided to include this differential precipitation of TSWV particles in the purification method. It was found to be of great importance for obtaining antigenically pure virus preparations.

Fig.20. Precipitin bands formed by virus containing suspensions obtained at different stages of the purification procedure. The central well was filled with undiluted serum TSWV-Ab 1. Wells 1 and 5 contained a preparation of the concentrated calcium phosphate eluate; wells 2 and 6 contained a preparation of the PEG virus precipitate; wells 3 and 7 contained a preparation of the supernatant after PEG precipitation of the virus and wells 4 and 8 contained plant juice in buffer (1:1). Phosphate-sulphite-saline agar was used.

3) Borate zone electrophoresis.

After calcium phosphate chromatography and subsequent precipitation with PEG, the virus zones in density gradients were still

found to contain residual contaminating material when examined by electron microscopy. This sample after glutaraldehyde fixation was then submitted to zone electrophoresis. The results showed that the contaminating material could be separated from the virus by this procedure. The $R_\emptyset$ values of the components agreed with those previously recorded. (See Table X & XII).

TABLE XII. $R_\emptyset$ values of components in borate zone electrophoresis. Preparations had been submitted to calcium phosphate clarification, PEG precipitation, density gradient centrifugation and glutaraldehyde fixation.

$R_\emptyset$-values		
Occasionally present zone	Distinct virus zone	Diffuse lower zone
x	0,69	0,43
x	0,69	0,42
x	0,62	0,39
x	0,65	0,39
0,78	0,67	0,40
0,75	0,64	0,46
0,75	0,67	0,46
0,75	0,68	0,43
0,73	0,62	0,36
0,75	0,65	0,41

x: Not distinguishable.

The lower boundary of the zone that was occasionally present was never observed since it was always masked by the distinct virus zone. Prolongation of electrophoresis did not separate this zone from the virus zone.

4) <u>Immunization of a rabbit with purified TSWV</u>.

Rabbit TSWV-Ab 4 was immunized with the virus preparation characterized by a R_ϕ value of 0,65 described in the previous section (Table XII).

No precipitin lines were formed when antiserum obtained from this rabbit was tested with healthy plant juice or purified F 1 protein preparations. The antigen preparations used to immunize this rabbit, however, contained part of the upper diffuse zone with R_ϕ value of 0,75. It seems therefore that this contaminant is not antigenic or that its concentration is too low to induce the formation of antibodies.

A similar top zone, with a R_ϕ value of 0,75 was present above the virus containing zone when PEG precipitation was omitted from the purification procedure (see Table X). The sera of rabbits TSWV-Ab 1, TSWV-Ab 2 and TSWV-Ab 3, immunized with antigens prepared in this way strongly reacted with F 1 protein.

It was thus evident that PEG precipitation successfully separated the F 1 protein antigens from TSWV and led to antigenically pure virus preparations.

The results obtained with serum TSWV-Ab 4 are presented in Table XVI. (See Discussion).

5) <u>Phosphate sulphite zone electrophoresis</u>.

The diffuse R_ϕ 0,75 zone above the virus zone was absent when electrophoresis was performed in the phosphate sulphite-buffer. With phosphate zone electrophoresis of glutaraldehyde fixed material,

two light scattering zones, 1 mm apart, could be distinguished 4-6
hours after the onset of electrophoresis. After 15-17 hours, only
a single light scattering zone, located centrally in the phenol red
band, was observed. Examination of this zone in the electron micros-
cope, showed that it contained virus. In five experiments, the fol-
lowing R_{ϕ} values were obtained: 1,00; 0,98; 1,03; 0,99 and 1,02 with
an average of 1,00. In order to detect any diffuse zones directly
above or below the virus zone, the phenol red indicator was omitted
in further experiments (Fig.21).

Fig.21. Virus zone on a phosphate-sulphite zone electrophoresis
column. The virus preparation was purified by calcium phosphate
clarification, PEG precipitation, density gradient centrifugation
and subsequently fixed with glutaraldehyde.

The R_{ϕ} 0,75 zone as well as the R_{ϕ} 0,41 zone were not visible
when electrophoresis was performed in phosphate-sulphite buffer. It

is probable that the material responsible for the formation of these zones was denatured and was represented by the greenish aggregates, which were observed in the lower part of the gradient column.

With this buffer, a rise in temperature was observed about 9 hours after the onset of electrophoresis in the lower part of the sucrose gradient and in the region of the capillary. This change in temperature may be ascribed to the lower conductivity of the 0,02 M phosphate-sulphite buffer compared to that of the 0,1 M borate buffer, and to the fact that the smallest cross section of the U-tube is found in the region of the capillary.

As the rise in temperature occurred late in electrophoresis and only in the regions of the $R_{\emptyset}$ 0,41 zone, it probably caused the denaturation and subsequent aggregation of the material present. Large floccules were observed in this region. Due to their higher electrophoretic mobility, the virus particles at this time had already moved away from this region. Virus preparations after phosphate-sulphite zone electrophoresis (Fig.22) were free of the foreign matter found in the electron micrographs obtained after borate zone electrophoresis (Fig.14A).
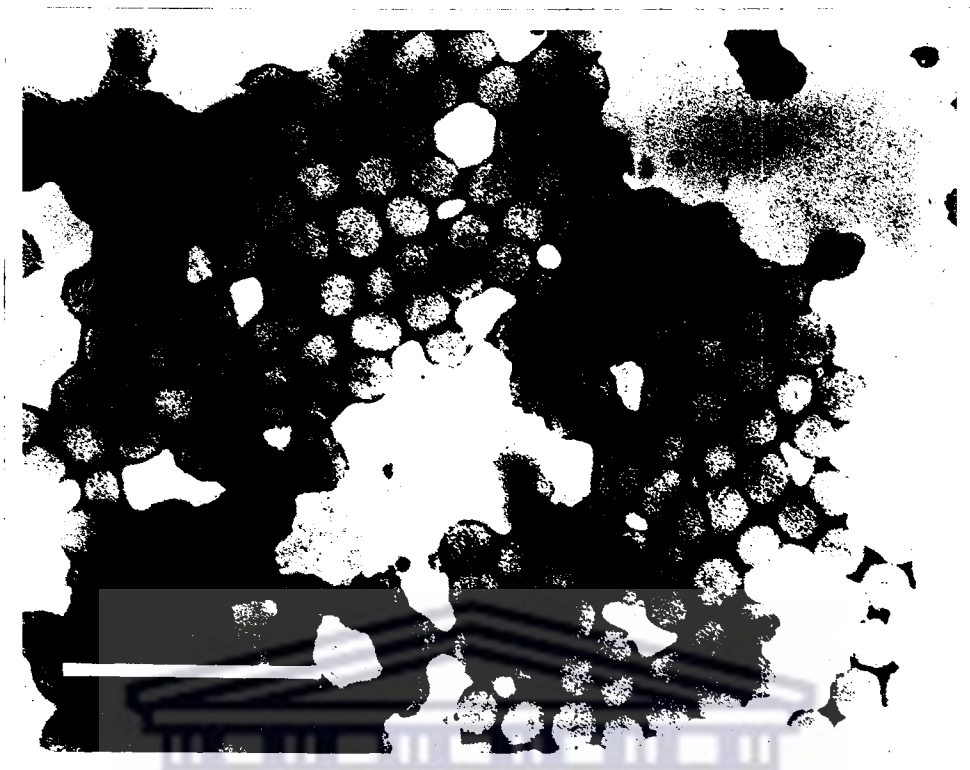
Fig.22. A glutaraldehyde fixed virus sample after calcium phosphate clarification, PEG precipitation, density gradient centrifugation and phosphate-sulphite zone electrophoresis (pH 7,2). The preparation was negatively stained with 2% neutral PTA. The bar represents 500 nm.

6) <u>Immunization of rabbits.</u>

Four rabbits, TSWV-Ab 5 to TSWV-Ab 8, were immunized with the virus preparation characterized by a $R_{\emptyset}$ value of 1,00 in phosphate-sulphite zone electrophoresis.

No precipitin lines were formed when the sera of these rabbits were tested against plant juice or purified F 1 protein.

The results obtained with these sera are presented in Table XVI. (See Discussion).

H. <u>The finally adopted purification procedure for TSWV.</u>

From the preceeding experimental work a purification procedure

Fig.23. <u>The procedure adopted for purification of TSWV.</u>

The 0,02 M phosphate buffer (pH 7,2), containing 0,01 M sodium sulphite, was used with this procedure.

90 gm infected leaves + 330 ml phosphate-sulphite buffer

Homogenise for 2 minutes and squeeze through cheesecloth

Centrifuge for 10 minutes at 10 000 rpm in the Sorvall SS-34 rotor

SL_1 P_1(discard)

Layer onto a calcium phosphate column and elute with the phosphate-sulphite buffer

Eluate

Centrifuge for 40 minutes at 25 000 rpm in the Spinco no 30 rotor

P_2 SL_2(discard)
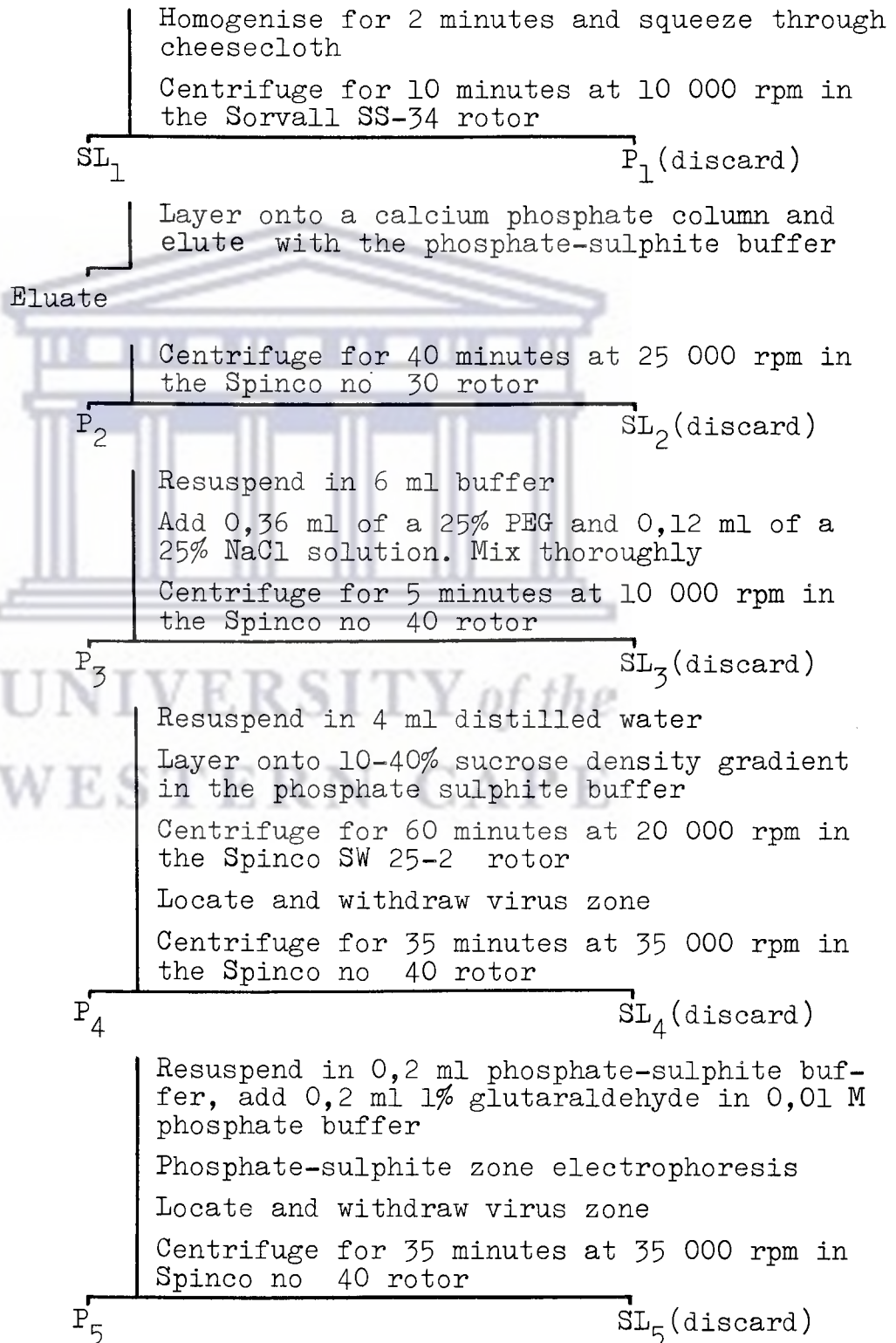
Resuspend in 6 ml buffer

Add 0,36 ml of a 25% PEG and 0,12 ml of a 25% NaCl solution. Mix thoroughly

Centrifuge for 5 minutes at 10 000 rpm in the Spinco no 40 rotor

P_3 SL_3(discard)

Resuspend in 4 ml distilled water

Layer onto 10-40% sucrose density gradient in the phosphate sulphite buffer

Centrifuge for 60 minutes at 20 000 rpm in the Spinco SW 25-2 rotor

Locate and withdraw virus zone

Centrifuge for 35 minutes at 35 000 rpm in the Spinco no 40 rotor

P_4 SL_4(discard)

Resuspend in 0,2 ml phosphate-sulphite buffer, add 0,2 ml 1% glutaraldehyde in 0,01 M phosphate buffer

Phosphate-sulphite zone electrophoresis

Locate and withdraw virus zone

Centrifuge for 35 minutes at 35 000 rpm in Spinco no 40 rotor

P_5 SL_5(discard)

Final virus preparation

for TSWV was finally developed (Fig.23). Compared to the previously published purification procedures the main improvements of this method lie in the use of calcium phosphate chromatography for clarification, a technique which retained up to 80 percent of the virus activity, and in the use of PEG precipitation which made it possible to separate TSWV successfully from solutions which contained a relatively high concentration of F 1 plant protein.

1) <u>Ultracentrifugal analysis.</u>

The homogeneity of the virus preparations obtained by the purification procedure outlined in Fig.23, was determined in the analytical centrifuge. Sedimentation coefficients were determined for the unfixed partially purified preparations before electrophoresis and again for the same samples after fixation and subsequent electrophoresis (Table XIII).

TABLE XIII. Sedimentation coefficients of TSWV in the 0,02 M phosphate-sulphite buffer. Left: unfixed TSWV prior to zone electrophoresis. Right: after fixation and zone electrophoresis.

$S_{20,w}$ value	
Before fixation and zone electrophoresis	After fixation and zone electrophoresis
537	592
524	576
528	582

2) <u>Ultraviolet absorption spectra.</u>

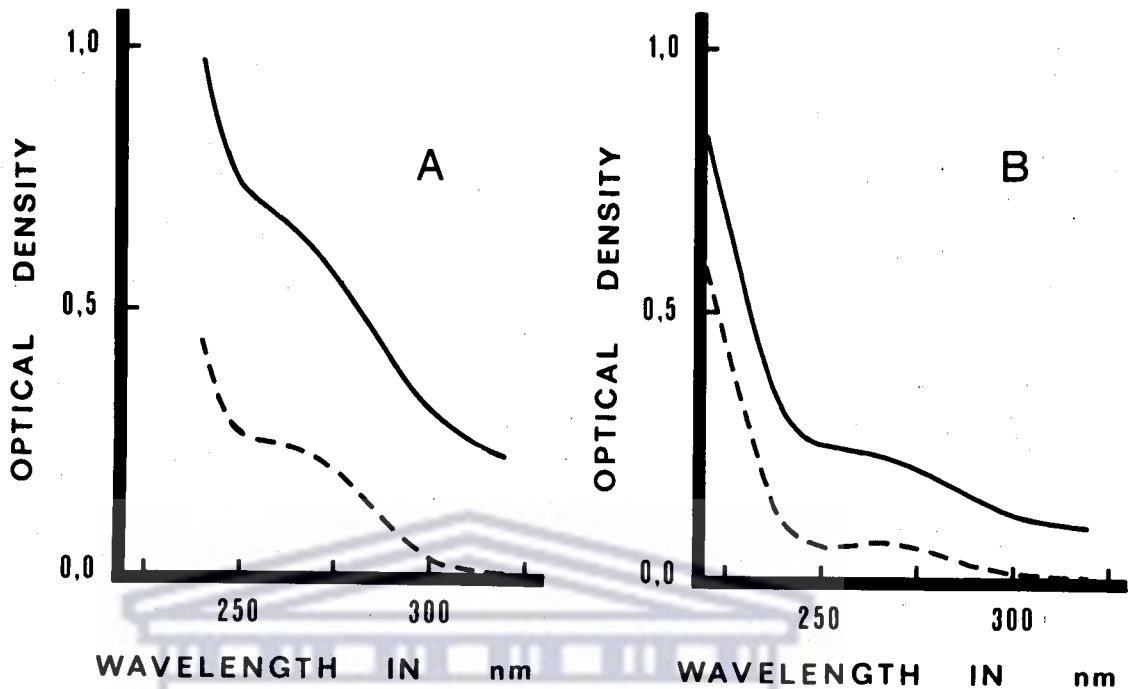When examined spectrophotometrically, the virus preparations

Fig.24A & B. Ultraviolet absorption spectra of virus preparations obtained by the purification method in Fig.23. A, at a virus concentration of 2,07mg/ml. B, at a virus concentration of 0,62 mg/ml. The solid line represents the uncorrected optical density values and the dotted line the corrected optical density values.
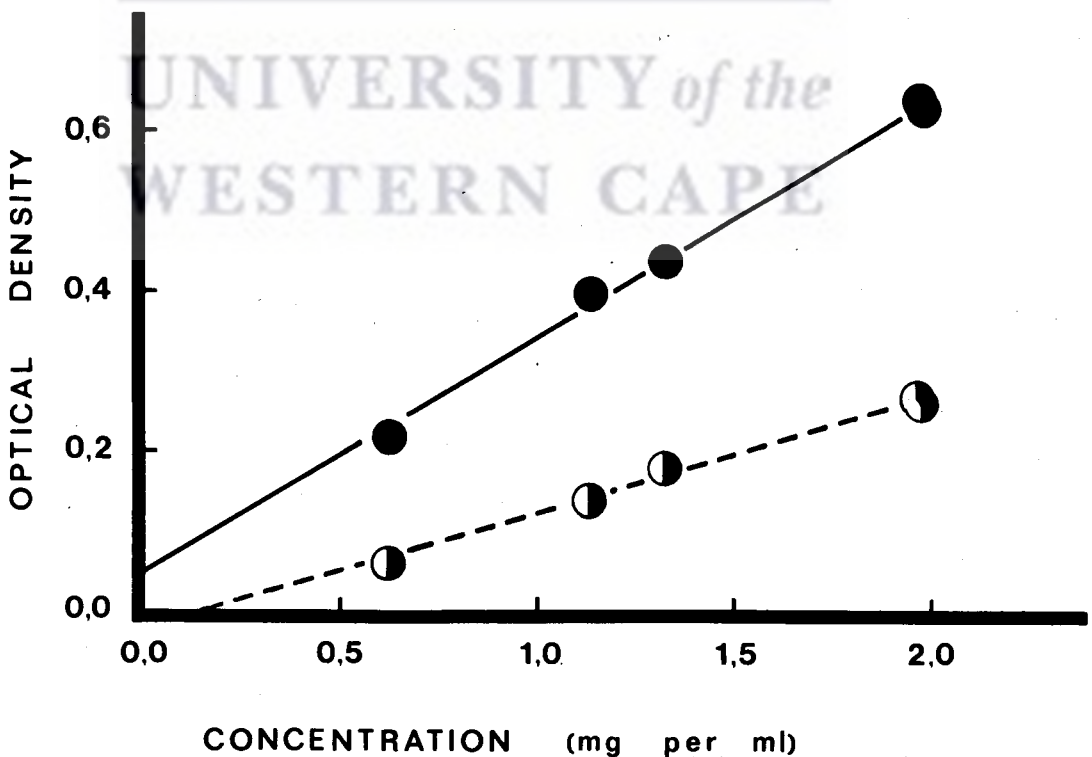

Fig.25. A plot of optical density versus concentration of virus preparations obtained by the purification method in Fig.23. The solid line represents the uncorrected optical density values and the dotted line the corrected optical density values.

obtained with the purification procedure set out in Fig.23 always exhibited UV absorption curves with sloping shoulders (Fig.24). The ratio of absorbancy at 280 nm to that at 260 nm always varied between 1,25 and 1,29 for the uncorrected light scattering curves (Table XIV).

After applying the correction for light scattering at virus concentrations of about 2 mg/ml, the corrected absorption curve still exhibited the sloping shoulder of the uncorrected absorption curve (Fig.24A). At virus concentrations of 0,6-1,3 mg/ml, more flattened absorption curves were obtained. The corrected absorption curves at virus concentrations of 0,5 to 0,7 mg/ml, however, resembled typical nucleoprotein curves (Fig.24B). In the case of corrected absorption curves, the 260/280 absorption ratio always varied between 1,44 and 1,50. This variation was independent of concentration. The 260/280 absorption ratio varied between 1,25 and 1,29 in the case of uncorrected absorption spectra.

As optical density is directly proportional to virus concentration, a plot of virus concentration against optical density gives a straight line with an intercept at 0. Similar plots of optical density at 260 nm against concentration, for both uncorrected and corrected optical density values, tended to give straight lines (Fig.25). The straight line, however, did not intercept at zero concentration which is probably due to experimental errors in the determination of virus concentration. Virus concentrations were determined by drying exact volumes of virus suspensions until a constant

weight was obtained.

An extinction coefficient ($E_{260}^{1 \, mg/ml}$) of 0,34 was determined for TSWV from the uncorrected optical density values in Table XIV. A value of $E_{260}^{1 \, mg/ml} = 0,12$ was determined from the corrected optical density values.

3) <u>The electrophoretic mobility</u>.

The partially purified virus preparation after density gradient centrifugation, contained enough virus to determine the electrophoretic mobility. After concentrating the virus zone, the pellet was resuspended in 0,2 ml of the appropriate buffer, to which was then added 0,2 ml of a 0,8 percent glutaraldehyde solution in the same buffer. With such virus preparations, diluted to 8 ml, a fringe shift of 4 to 6 fringes was usually obtained.

By electrophoresis in the Tiselius cell, the electrophoretic mobilities of both the components observed in zone electrophoresis, could be determined (Table XV).

TABLE XV. The electrophoretic mobilities of virus preparations after calcium phosphate clarification, PEG precipitation, density gradient centrifugation and glutaraldehyde fixation.

Buffer	pH	Electrophoretic mobility x $10^{-5} cm^2 volt^{-} sec^{-1}$	
		Plant component	Virus component
Phosphate[a]	7,0	11,46	19,83
Phosphate[b]	7,0	11,59	18,70
Phosphate[c]	7,5	12,40	19,35

[a] The 0,1 ionic strength buffer of Miller & Golder (1950).

[b & c] Phosphate-sodium sulphite buffer adjusted to pH 7,2 with either 4,0 M NaH_2PO_4 or 4,0 M Na_2HPO_4.

All virus activity was destroyed when attempts were made to resuspend virus pellets in buffers with pH-values of 6,5 and lower.

I.   Electronmicroscopy.

1) Dip preparations.

When leaf tissue surrounding local TSWV lesions was crushed and spread onto a drop of distilled water on an electron microscope grid, examination of these grids revealed virus particles of variable shape and size. This variability in shape and size was prominent in closely packed groups of virus particles (Fig.26A). Solitary particles were usually elliptical to round in shape and only occasionally did such particles reveal a tail-like structure. Virus particles were even and smooth in texture and tubular or filamentous forms were not observed.
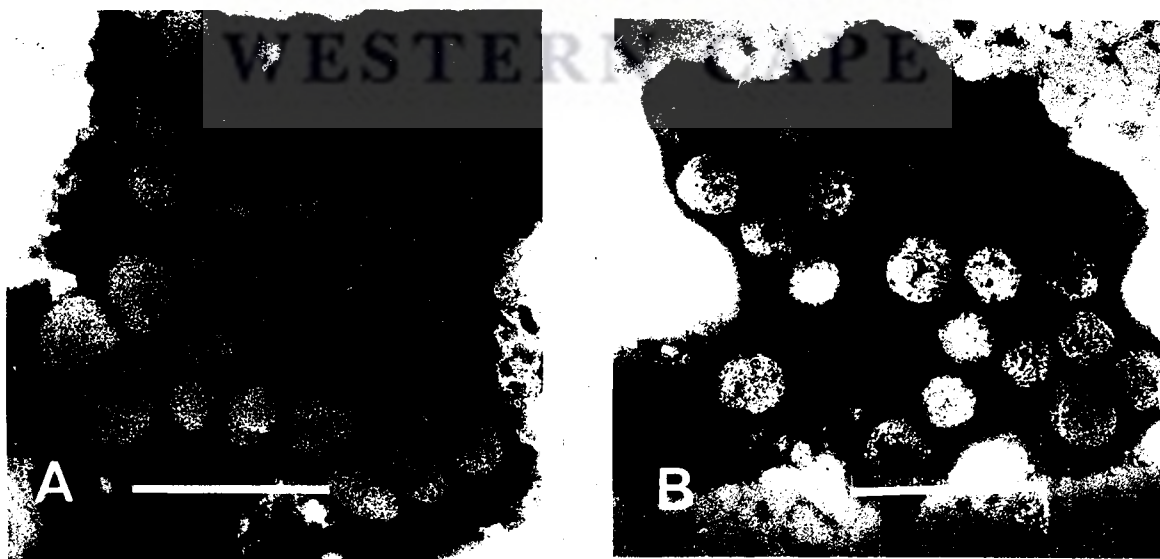

Fig.26. Dip preparations of virus particles negatively stained with 2% neutral phosphotungstic acid. A, a dip preparation spread onto distilled water. B, a dip preparation spread onto 1% glutaraldehyde in 0,01 M neutral phosphate buffer. The bars represent 250 nm.

In virus preparations from systemically invaded leaf tissue, crushed and then spread onto a 1% glutaraldehyde solution in phosphate buffer pH 7,0 virus particles were always round in shape (Fig.26B), The virus particles were uneven in texture. Virus particles never revealed any buds or tail-like structures.

Small rodlike protuberances or spikes could be observed on the surfaces of the virus particles with both the dip methods (Fig.26). The spikes were most prominent where neighbouring particles were closely packed together.

With both dip methods, clusters of virus particles encased in a membrane were occasionally observed (Fig.27).

Fig.27. Virus particles encased in a membrane, observed with the distilled water dip method. Negatively stained with 2% neutral phosphotungstic acid. The bar represents 250 nm.

2) <u>During purification</u>.

The effect of various solvents and different purification

treatments on the shape of the virus was determined in the electron microscope. The different virus containing pellets in the purification procedure (see Fig.23) were resuspended in distilled water, the phosphate-sulphite buffer (pH 7,2), or Best's phosphate buffer (pH 7), and then compared with each other. Part of each virus suspension was fixed with the glutaraldehyde fixative and compared to unfixed virus preparations.

The shape of the unfixed virus particles varied greatly in the different solvents (Fig.28, 29 & 30). Parallel fixed virus samples, at any stage of the purification procedure, only revealed round to elliptical virus particles. The fixed virus preparation after PEG precipitation is representative of such virus samples (Fig.29B).

When resuspended in Best's buffer the unfixed virus particles tended to be dumbbell-shaped (Fig.28B & 30C), a tendency sometimes revealed when they were resuspended in distilled water (Fig.29A).

In unfixed virus preparations particle shape was also influenced by the virus concentration of the sample. Virus particles were roughly spherical in the phosphate-sulphite buffer at high virus concentrations (Fig.30A).while the same sample at a $^1/5$ dilution in the same buffer revealed particles with many different forms (Fig.30B).

Fig.28. ..eparations of virus containing pellets after concen-
tration of the calcium phosphate eluate, negatively stained with
2% neutral phosphotungstic acid. A, resuspended in the phosphate-
sulphite buffer; B, resuspended in Best's phosphate buffer and C,
resuspended in distilled water. The bars represent 500 nm.

Fig.31. Virus sample after calcium phosphate clarification, PEG precipitation, gradient centrifugation, glutaraldehyde fixation and phosphate-sulphite zone electrophoresis (pH 7,2). The virus was resuspended in phosphate-sulphite buffer. A, negatively stained with 2% neutral phosphotungstic acid. B, positively stained with 2% uranyl acetate (pH 4,1). The bars represent 500 nm.

At every stage during purification, the virus particles were always homogeneous in texture and always resembled the particles revealed in distilled water dip preparations (Fig.26A). A different type of particle, probably empty and penetrated by the stain, occurred among the normal virus particles after zone electrophoresis (Fig.31A & B). The incidence of these presumably empty particles varied to a great extent in the different preparations examined. Relatively few of these particles were revealed when zone electrophoresis preparations were resuspended in buffer, but the incidence was high when resuspended in distilled water.

A white unstained ring, probably the lipid of the virus membrane, was always revealed on the periphery of the empty particles. The small external spikes or protuberances were also more clearly demonstrated in the empty particles than in the intact particles (Fig.32).
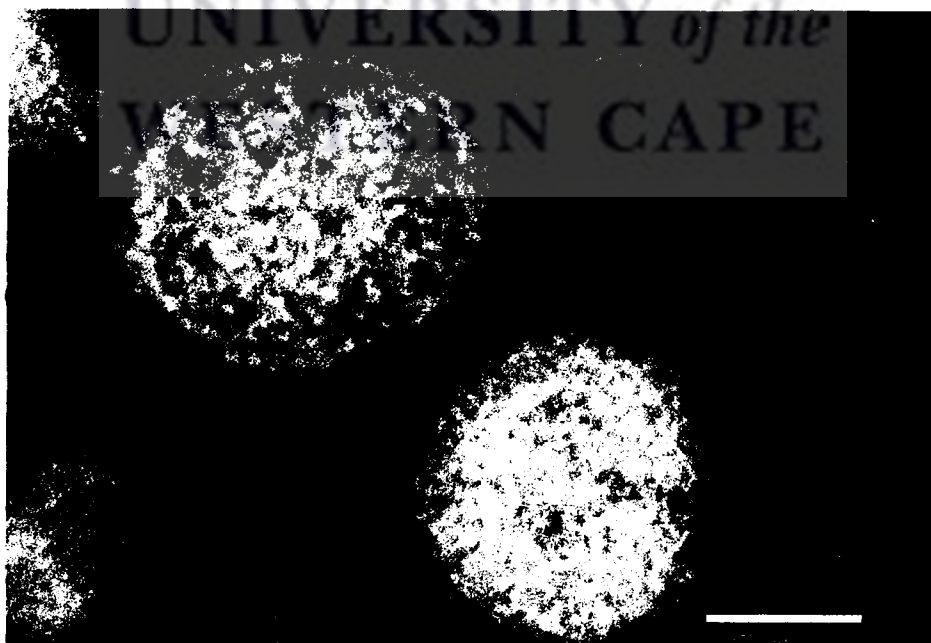

Fig.32. Virus sample after calcium phosphate clarification, PEG precipitation, gradient centrifugation, glutaraldehyde fixation and phosphate-sulphite zone electrophoresis (pH 7,2). The virus was resuspended in phosphate-sulphite buffer and negatively stained with 2% neutral phosphotungstic acid. The bar represents 30 nm.

3) <u>The effect of pH on particle shape</u>.

A most striking difference was revealed when unfixed virus
preparations, resuspended in either distilled water or phosphate-
sulphite buffer, were stained with neutral phosphotungstic acid
(PTA) and uranyl acetate (pH 4,1). When stained with uranyl acetate,
the virus particles were mostly round in shape and did not reveal
any tail-like or bud-like structures. Uranyl acetate stained prepa-
rations not only revealed intact negatively stained virus particles,
but also a great number of presumably empty particles, penetrated by
the stain (Fig.33).

Fig.33. A virus preparation after calcium phosphate clarification,
PEG precipitation and gradient centrifugation, stained with uranyl
acetate (pH 4,1). The bar represents 500 nm.

When stained with neutral PTA, particle shape varied to a great
extent (Fig.30A & B), but particles penetrated by the stain were not
revealed.

This effect of different stains at different pH values led to further investigations of the effect of pH on virus shape. Virus suspensions in distilled water were mixed with equal volumes of 2 percent PTA solutions at pH values from 3 to 9, the pH interval being 1.

At pH 3, the virus particles were mostly round in shape and penetrated by the stain. Intact particles were sometimes observed among the empty particles (Fig.34A). A characteristic of the virus particles at pH values of 4 and 5, was the "dimples" or invaginations filled with the stain (Fig.34B). Particles penetrated by the stain were never observed.

Virus particles were most distorted at pH 6. Tail-like and/or bud-like protuberances could be observed with all the particles (Fig.34C) while particles penetrated by the stain were never observed.

At the higher pH values, between pH 7 and 9, the virus particles varied much in shape but to a much lesser extent, than at pH 6. These preparations did not reveal the darker stained "dimples" or invaginations (Fig.34D).

4) <u>Viral ribonucleoprotein</u>.

In order to determine whether TSWV could be classified as a myxovirus, attempts were made to demonstrate the internal ribonucleoprotein component which is characteristic of myxo- and paramyxoviruses.

Careful examination of more than 200 TSWV preparations over a period of 2 years revealed only intact virus particles in which an internal component could not be seen. In contrast to this, an inter-

nal component has been visualized in some spontaneously disrupted influenza virus particles penetrated by PTA (Almeida & Waterson, 1970). Nucleocapsids have also been demonstrated in stain-penetrated parainfluenza viruses (Choppin & Stoeckenhuis, 1964).

Uniform filamentous structures with a diameter of approximately 12,5 nm, which approximated a nucleoprotein component, were associated with TSWV preparations resuspended in distilled water (Fig.30D). The filaments in this negatively stained preparation did not reveal a helical array of subunits as was demonstrated for the nucleocapsids of paramyxoviruses (Finch & Gibbs, 1970). However, a parallel shadowed sample did reveal a helical structure in filaments with a diameter of approximately 25 nm (Fig.35). These filaments had double the diameter of the filamentous structures in the negatively stained preparations (Fig.30D).

Fig.34. Unfixed virus preparations after calcium phosphate clari-
fication, PEG precipitation and gradient centrifugation. The virus
preparation was resuspended in distilled water. A, negatively
stained with PTA pH 3. B, negatively stained with PTA pH5. C, ne-
gatively stained with PTA pH 6. D, negatively stained with PTA
pH 8. The bars represent 500 nm.

Fig.35. Unfixed virus preparation after calcium phosphate clarification, PEG precipitation and gradient centrifugation. The preparation was shadowed at an angle of 25° with tungsten trioxide (WO_3). The bar represents 250 nm.

Several attempts to obtain similar filamentous structures associated with virus particles in distilled water, were unsuccessful. Exhaustive scanning of virus particles resuspended in distilled water, occasionally revealed some particles with tubular structures. These tubes, exhibiting no helical structure, were either approximately 25 nm (Fig.36A) or approximately 15 nm in diameter (Fig.36B)

Disrupting TSWV particles in several ways never revealed the existance of a ribonucleoprotein component in the electron microscope. Virus containing solutions were treated with peroxide free ether (Becker, 1963; Finch & Gibbs, 1970), sodium deoxycholate (Duesberg, 1969) or Tween 20.
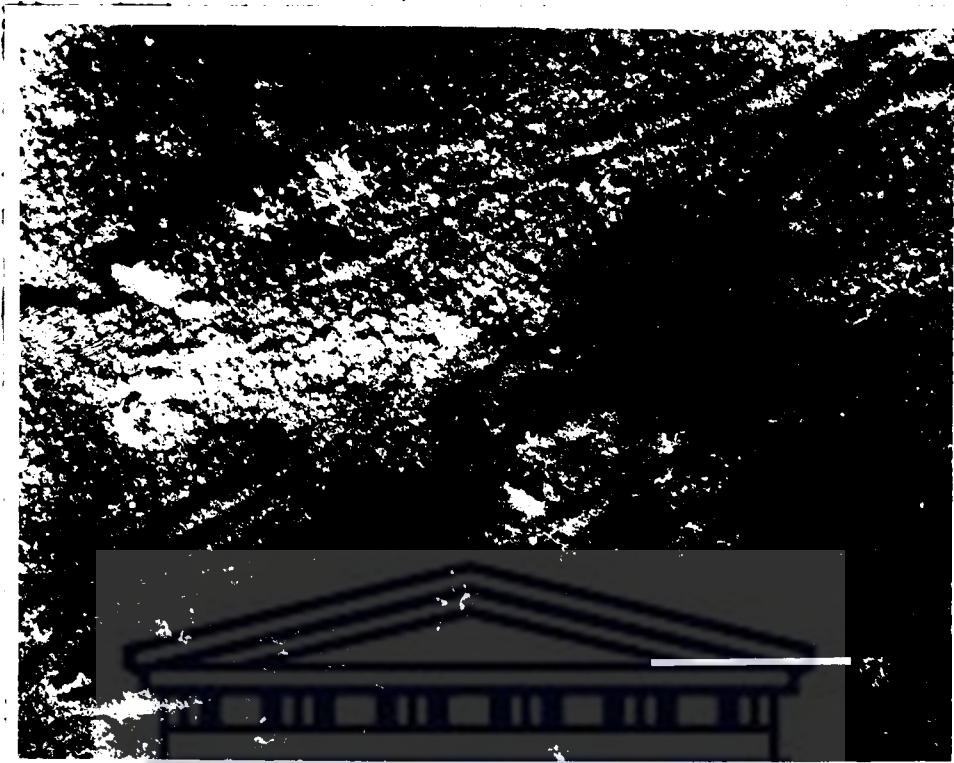
Fig.36A & B. Unfixed virus preparations after calcium phosphate
clarification, PEG precipitation and gradient centrifugation.
The preparation was resuspended in distilled water and shadow-
casted with tungsten trioxide (WO_3) at an angle of 25°. The bars
represent 250 nm.

5) <u>Size determinations</u>.

Preparations of TSWV which had been partially purified by density gradient centrifugation and fixed with glutaraldehyde (Fig.23), were used to determine the diameter of the virus particle. Since there is a slight variability in the shape of the particles when present in closely packed groups, only those occurring as single particles were used for size determinations (Fig.37). Typical examples of the calculations of the normal lengths and normal diameters of TMV and TSWV appear in Addenda IV and V.

Fig.37. A virus preparation after calcium phosphate clarification, PEG precipitation, gradient centrifugation and glutaraldehyde fixation. The virus was mixed with TMV and shadowcasted at an angle of 25° with tungstan trioxide. The bar represents 250 nm.

When measured against TMV, which has an accepted normal length of 300 nm (Brandes, 1964; van Regenmortel <u>et</u> <u>al</u>., 1964), the average

diameter of TSWV particles was determined as 111,7 nm. With the con-
fidence limits of both viruses taken into consideration, the diameters
of TSWV particles should not be less than 110,2 nm or more than
113,3 nm. All the TSWV particles measured were round in shape.

In determining the diameter of individual particles, the length
of the visible part of the particle along with its shadow, were simul-
taneously determined. From these measurements it was possible to de-
termine the height of the particle above the supporting film on the
grid (Addendum VI). The average ratio of the height to diameter of
ten randomly chosen virus particles was 0,478, which indicated a con-
siderable flattening of the particles.

In contrast to the circular appearance of the particle when
viewed from above, the ratio of particle height to width suggested
that the particles were elliptical in shape. As a result of the flat-
tening, the actual diameter of TSWV should be less than the 111,7 nm
reported above.

Corrected diameters for TSWV particles were determined when the
elliptical particles were transformed to spherical particles with
the same circumference (Marks, 1946). A typical transformation from
an elliptical to spherical particle is represented in Addendum VII.

The normal diameter for spherical particles was determined as
85,3 nm with extremes of 84,3 and 86,3 nm when the confidence limits
of both TMV and TSWV were taken into consideration. A frequency dis-
tribution diagram of the normal particle lengths and diameters for
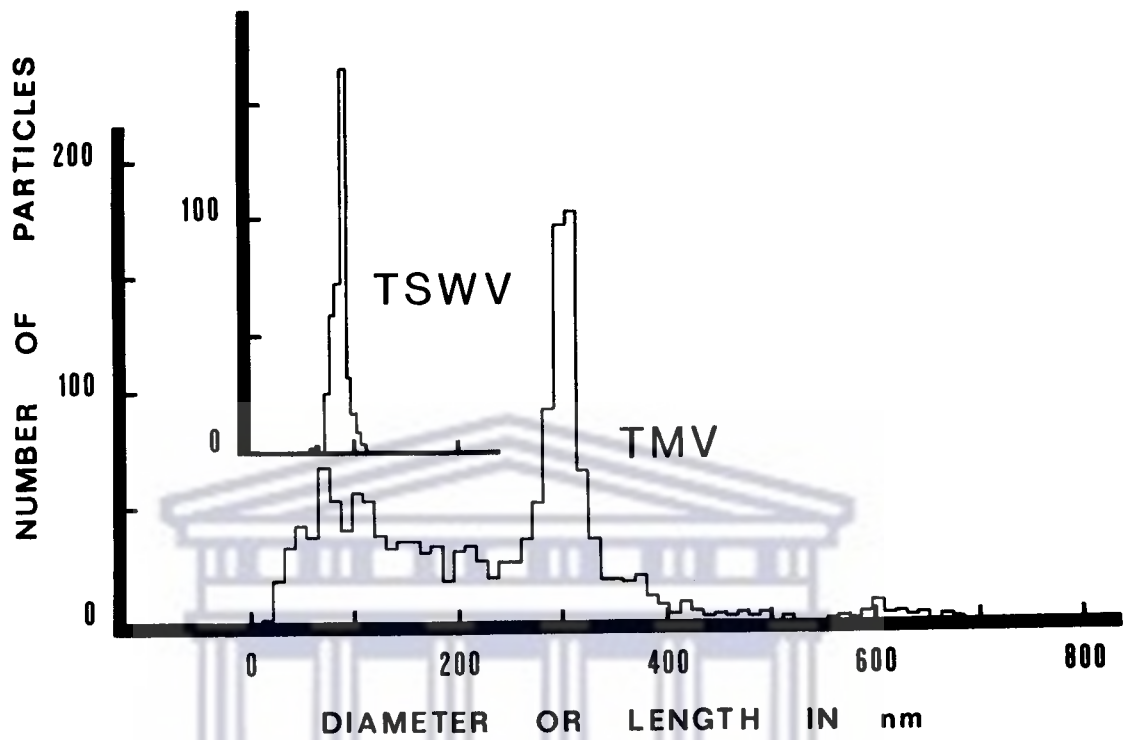TMV and TSWV is presented in Fig.38.

Fig.38. Frequency distribution diagrams of TSWV and TMV. The diameter of TSWV particles is that of an ellipse transformed to a spherical particle with the same circumference.

CHAPTER V

DISCUSSION.

In the past, one of the most important criteria for identifying the virus etiology of TSWV, was its transmission by thrips vectors. Messieha (1969) however, showed that tobacco ringspot virus (TRSV) could also be transmitted by T. tabaci. Acquisition and transmission of TRSV are performed only by the thrips larvae whereas TSWV is acquired by the larvae and transmitted by the adult insects. The larvae only rarely transmit TSWV. For identification purposes it is therefore essential to show that adult insects are capable of transmitting TSWV.

The diversity in symptom expression of TSWV strains is widely recognized (Norris, 1946; Best & Gallus, 1950 & 1953) and was confirmed with strains used in this work. TSWV infected tobacco plants collected in the Western Province over the past 4 years, were all infected with two different strains. Plants infected with a single strain of the virus were never found. Symptom description of TSWV infected tobacco (Moore, 1933; Samuel & Bald, 1933) has been a valuable initial criterium for detecting TSWV infected plants.

Different purification methods for TSWV have been found to be unsatisfactory for purifying all TSWV strains. Best and Palk (1964) and Best (1966) were unable to purify strain E with the method of Black et al. (1963), and both methods were unsatisfactory in the hands of Tsakiridis (1971). In purifying cucumber mosaic virus (CMV) strains it was also found that the different strains varied in stability and could not be satisfactorily purified with a single method

(van Regenmortel, 1967).

Regular infectivity assays during different purification steps have helped to determine the steps responsible for virus loss when virus was purified according to the methods of Black et al. (1963) and Best (1966).

With the purification method of Black and co-workers (see Fig.1) the virus particles were aggregated to such an extent after homogenization that they were precipitated by the subsequent low speed centrifugation (see Table III). Inadequate dispersal of the virus particles from these precipitates resulted in an important loss in virus infectivity during the subsequent low speed centrifugation.

No aggregation of the virus was evident when TSWV was purified according to the method of Best (1966; see Fig.3). More than 70 percent of the virus infectivity was usually found in the supernatant after homogenization and low speed centrifugation (see Table IV). The virus in the supernatant was concentrated into a pellet by high speed centrifugation. When this method was used the loss of virus infectivity seemed to be the result of inadequate dispersal of the virus from the high speed pellets since infectivity was easily lost during subsequent low speed centrifugation.

No adequate dispersal of TSWV particles from pellets was obtained after concentration of the particles by using high ionic strength buffers (Black et al., 1963) or ultracentrifugation (Best, 1966). The majority of the particles were lost after resuspension of the pellets and subsequent clarification by low speed centrifugation.

It was thus necessary to clarify virus solutions to such an

extent before concentrating the virus particles into pellets, that
clarification of the resuspended pellets by low speed centrifugation
could be omitted.

Excellent clarification of the virus containing supernatant
after homogenization and subsequent low speed centrifugation (see
Fig.23) was obtained with chromatography on calcium phosphate columns
(Taverne et al., 1958; Murant et al., 1969). The virus solutions were
clarified to such an extent that it was not necessary to use low speed
centrifugation for this purpose. The loss of virus varied only between
6 and 20 percent.

Further fractionation of these clarified virus suspensions was
obtained by density gradient centrifugation. Electron microscopic
examination of the virus in the light scattering zone revealed a high
concentration of unidentified material present among the virus par-
ticles. The same material was detected in the electron microscope
after a second step of gradient centrifugation. This is in contra-
diction with the results of Best (1966) who obtained highly purified
virus preparations with two successive steps of gradient centrifuga-
tion.

It seems likely that the variable results obtained with the
different purification methods is simply due to the fact that the
different strains that were used varied in their sensitivity and be-
haviour in the various buffer solutions. Each strain probably requires
its own set of conditions to maintain maximum infectivity.

Lengthy purification procedures, based on ultra- and gradient
centrifugation, also contributed to a great loss in virus. Often,

when using material relatively low in virus content, the virus
pellets after the first step of gradient centrifugation disappeared
with further subsequent gradient centrifugation and pelleting.

Since density gradient centrifugation was found to be inadequate
for the complete separation of TSWV from the contaminants present, it
became important to investigate separation methods other than those
based on size and density differences. Attempts were then made to
use zone electrophoresis in a sugar gradient, a technique extensively
used in van Regenmortel's laboratory (van Regenmortel, 1968) for the
routine purification of plant viruses.

Successful separation of the unidentified components present in
the TSWV solutions could however, not be accomplished with zone elec-
trophoresis. The virus activity in the initial sample was completely
destroyed 4-5 hours after the onset of electrophoresis. Successful
zone electrophoretic separation was achieved after stabilizing the
TSWV particles with glutaraldehyde.

Glutaraldehyde is known to be an efficient fixative for cellular
preparations which have to be examined in electron microscopy (Saba-
tini, Bensch & Barrnett, 1963). The cellular constituents are stabi-
lized to such an extent that they can withstand temperatures of up
to 60° for 4 days in preparing material for sectioning (Luft, 1961).
Glutaraldehyde reacts with the free amino groups of proteins and is
an efficient coupling agent for covalently linking enzymes to proteins
(Avrameas & Ternynck, 1969). The stabilization of protein molecules
with glutaraldehyde is most likely due to the fact that the free
amino groups in a protein molecule are crosslinked with each other.
It is thus possible that proteinaceous material can be coupled onto

TSWV where fixation is performed with partially purified TSWV preparations.

Zone electrophoresis was performed with the pH 8,6 borate buffer in order to compare the zone electrophoretic R_f values with those of other viruses (van Regenmortel, 1968). After electrophoresis (see Table X) the virus material appeared to be pure, as judged from electron micrographs (see Fig.14A). Three light scattering zones, each with a characteristic R_f value, could be observed after zone electrophoresis. The R_f 0,42 zone consisted of large pleomorphic structures which probably were collapsed, stain-penetrated virus particles devoid of their nucleic acid component. The virus particles in the R_f 0,65 zone were concentrated. Directly above the virus containing zone a diffuse zone with a R_f value of 0,75 was occasionally distinguished. No pellets were ever revealed after pelleting of the R_f 0,75 zone but material resembling that found among the virus particles in Fig.14A could however, occasionally be distinguished in the electron microscope. Material similar to that in the virus preparation in Fig.14A is observed in the position of the virus zone after gradient centrifugation of plant sap. This preparation had a zone electrophoretic R_f value of 0,70 - 0,75. The R_f 0,75 light scattering zone in borate zone electrophoresis is thus most likely plant material. The homogeneity of the R_f 0,65 samples was determined in the analytical ultracentrifuge when a single component was observed with the schlieren optical system. These preparations were used to immunize rabbits.

Double diffusion serological reactions with the resultant antisera however, showed that the virus material used for injecting the

rabbits could not have been completely pure. Precipitin lines were also formed against two antigens present in healthy plant juice. One of these antigens was identified as F 1 protein when serological precipitin reactions were performed in phosphate-sulphite-saline agar (pH 7,2) (see Fig.16 & 17A). The other unidentified antigen which appeared in cacodylate-saline agar is likely to be a serological sub-fraction of purified F 1 protein (see Fig.17B). This agrees with the known heterogeneity of purified F 1 protein which can be fractionated into two distinct components by ion exchange chromatography (von Wechmar, 1967).

In evaluating the TSWV antisera, the importance of performing serological precipitin reactions in different buffers at different pH values was clearly demonstrated.

As stressed by van Regenmortel (1966), the presence of antibodies to plant components in virus antisera is undesirable for it destroys the specificity of the serological method. Antibodies against plant antigens not only complicate the serological detection and diagnosis of virus infections but they also make the study of serological cross-reactions between viruses more difficult.

Serological tests showed that complete separation of F 1 protein from TSWV was not achieved with gradient centrifugation and borate zone electrophoresis. The presence of F 1 protein at the position to which TSWV sediments during gradient centrifugation was detectable when the gradients were scanned by UV absorption. Incomplete separation of the 18 S F 1 protein component from the 551 S TSWV component is due to the overloading of the density gradients with F 1 protein

still present in the virus samples.

The contamination of the zone electrophoretic virus sample with F 1 protein can be attributed to one or both of the following possibilities:

1) Fixation of the virus particles after gradient centrifugation with glutaraldehyde (see Fig.23) may link F 1 protein onto the virus particles. The coupling of leaf material onto TSWV particles is suggested by electron microscopy when dip preparations from TSWV infected leaves were prepared by the glutaraldehyde dip method of Milne (1970). TSWV particles prepared in this way always revealed dark electron dense spots on the surface of the virus particles (see Fig.26B). Similar dense spots were never revealed when parallel samples were prepared with buffer instead of glutaraldehyde (see Fig.26A).

2) If cross-linking of F 1 protein and TSWV does not occur, TSWV obtained by zone electrophoresis may be contaminated with F 1 protein in another way. Contamination of the virus preparations is possible when the contaminating material has a zone electrophoretic R_ϕ value similar to that of TSWV. F 1 protein from $\underline{N}$. $\underline{glutinosa}$ plants, obtained by chloroform emulsification consisted of two components with R_ϕ values of 0,72 - 0,74 and 0,50. The R_ϕ 0,72 component can thus be responsible for the contamination of TSWV samples obtained by borate zone electrophoresis. A similar R_ϕ 0,72 zone was however, obtained with healthy plant sap. The R_ϕ value of 0,51 for F 1 protein from $\underline{N}$. $\underline{glutinosa}$ is similar to that determined for $\underline{N}.\underline{tabacum}$

(van Regenmortel, 1966).

The contamination of TSWV samples with F 1 protein was prevented when the TSWV particles were precipitated with PEG prior to gradient centrifugation. The successful separation of TSWV and F 1 protein in this way was demonstrated serologically (see Fig.20). Antiserum to TSWV, obtained after PEG precipitation and borate zone electrophoresis, dit not contain antibodies to plant components (see Table XVI).

Although F 1 protein was successfully separated from the TSWV particles after PEG precipitation, a diffuse light scattering zone with a R_{ϕ} value of 0,75 was still present following electrophoresis. Unidentified R_{ϕ} 0,75 material, similar to that in Fig.14A, was present in the zone electrophoretic virus samples used to immunize rabbit TSWV-Ab 4. This material was not antigenic since no antibodies against it could be detected in the resultant antiserum to TSWV (see Table XVI).

The above mentioned results seem to indicate that the zone electrophoretic R_{ϕ} 0,75 component present in purified F 1 protein from N. glutinosa plants does not consist of F 1 protein but represents some other antigenic inactive component. The F 1 protein is thus probably concentrated in the R_{ϕ} 0,50 light scattering zone.

It also seems likely that the F 1 protein which is present in the TSWV sample after gradient centrifugation is linked to the TSWV particles with glutaraldehyde. This would explain why the resultant TSWV particles elicit an immune response to both F 1 protein and TSWV. If there is any residual F 1 protein present in the TSWV sample after PEG precipitation, it can be successfully separated from TSWV by gradient centrifugation because it is present in sufficiently low

concentration.

The diffuse light scattering zone (R_ϕ 0,72) which was present directly above the virus containing zone (R_ϕ 0,68) was never evident when zone electrophoresis was performed in the phosphate-sulphite buffer instead of the borate buffer. Electron microscopy of the virus particles contained in the R_ϕ 1,00 light scattering zone (see Fig.21), never revealed the presence of any foreign material in this zone (see Fig.22). On the other hand non-antigenic R_ϕ 0,72 material was always present in the virus samples after borate zone electrophoresis (see Fig.14A).

All the data regarding the different purification methods and the resultant antisera are presented in Table XVI.

It was not possible to detect TSWV serologically in infective plant tissue with any of the TSWV antisera. The presence of TSWV could also not be detected serologically during the initial steps of the purification scheme (see Fig.23) when the virus was diluted in a large quantity of buffer solution. Serological detection only became possible after a fifty fold concentration of the virus by PEG precipitation or ultracentrifugation. The failure to detect TSWV serologically in infective plant juice can thus be attributed to the low concentration of the antigen in infective plant tissue and during the early stages of purification. All the serological reactions were performed with the double diffusion technique. More refined methods such as the bentonite and latex tests were not tested.

The sedimentation coefficient of TSWV determined by Best (1966) varied between 492 and 551 S with a mean value of 524 S. Similar variations occurred in the sedimentation coefficients determined for the South African strains purified by a single step of gradient centrifugation (see Fig.23). The sedimentation coefficients varied between 528 and 570 S with a mean value of 551 ± 13 S. Heterogeneity of the samples was suggested by the assymmetry of the schlieren peak (see Fig.10) and by the slope of the sedimenting boundary and the baseline in the UV tracing (see Fig.11B). When determined with UV optics, the sedimentation coefficients varied between 452 and 515 S with an average of 477 S (see Table IX). These UV values represented an average sedimentation coefficient of the virus and of extraneous material present in the preparations.

Sedimentation velocity determinations were always performed with 0,25 ml of a virus suspension. The first photograph of the sedimenting boundary was usually taken at schlieren angles of 60-70°, 2 to 3 minutes after the desired speed was reached. By determining the areas under the schlieren peaks, it was possible to make a rough comparison of the virus content in the different samples. Attempts were made to determine the influence of virus concentrations on the sedimentation coefficients. This was only done when sedimentation velocity experiments were performed under identical conditions, but no correlation between virus concentration and sedimentation coefficient could be found.

The variations in sedimentation coefficients (see Table IX) may be attributed to a variation in the amount of sucrose present in the virus samples after gradient centrifugation and zone electrophoresis.

The presence of sucrose in the solvents would result in viscosity differences that would influence the sedimentation velocity of the virus particles. Due to the instability of TSWV, the virus pellets could not be freed of the sucrose present. Repeated washing and/or resuspending and pelleting of the virus pellets only contributed to a loss in virus.

The homogeneity of the virus samples used to determine the extinction coefficient of TSWV was determined according to the differences between sedimentation coefficients determined with schlieren and UV optics similtaneously (see Table XI). These dif£ ferences were always less than 5 percent which indicated the absence of extraneous material. For extinction coefficient determinations, virus pellets were resuspended in distilled water and the sedimentation coefficients obtained should not be regarded as reliable. When sedimentation coefficients are determined in distilled water a variation can be expected due to the charge effects (Svedberg & Pedersen, 1940). The primary object of these experiments was to determine the homogeneity of the virus samples.

Sedimentation coefficients of TSWV were greatly influenced by glutaraldehyde fixation. Sedimentation coefficients were determined for the unfixed, partially purified TSWV after a single step of gradient centrifugation and again for the same sample after fixation with glutaraldehyde and zone electrophoresis (see Table XIII). The sedimentation coefficients were about 10 percent higher after fixation and zone electrophoresis than before fixation and zone electrophoresis. The cross-linking of proteins occurring when virus is treated with glutaraldehyde may result in an increase in the weight

of the virus particles and a higher sedimentation constant.

·The different values of the sedimentation coefficients that have been obtained are compared in Table XVII.

A sedimentation coefficient of 551 ± 13 is suggested for TSWV. This average was obtained from measurements on particles obtained after relative short purification scheme and before the virus particles had been altered by the glutaraldehyde treatment.

A considerable loss of virus occurring during zone electrophoresis was evident from sedimentation velocity experiments. The area under the schlieren peak, after fixation and electrophoresis of TSWV, was less than 50 percent of the area under the schlieren peak when the sedimentation coefficient was determined prior to fixation and zone electrophoresis.

In spite of this considerable loss of virus during purification, virus yields of 6 to 8 mg. were obtained from 100 gm of leaf tissue, The virus yield was determined when the final virus preparation was dried to constant weight at 60°C. Tsakiridis (1971) purified 3 mg of virus from 100 gm of leaf tissue, the virus yield being determined by drying the final virus preparation to constant weight over anhydrous calcium sulphate. Best (1966) obtained 1 mg of virus from 100 gm of leaf tissue with his purification method. No mention was however, made of the method employed to determine the virus yield.

The UV absorption spectra for purified TSWV, when corrected for light scattering according to Bonhoeffer and Schachman (1960), differed markedly from those determined by Best (1966). The corrected absorption curves of Best always revealed two maxima, a major maximum

at 270 nm and another at 260 nm. Since the maximum virus yield reported by Best was 0,6 mg (Best, 1968) it can be assumed that his absorption curves were done with virus preparations containing 0,6 mg/ml virus or less. In the present work corrected UV spectra at virus concentrations of 0,6 mg/ml or less, were invariably those of a nucleoprotein with a single maximum at 260 nm (see Fig.24A). At higher virus concentrations (see Fig.24B) the corrected UV absorption curves still resembled the uncorrected absorption curves.

The extinction coefficients that have been determined can be regarded as only approximate values since the extrapolation of the straight line, in a plot of optical density against concentration, did not intercept at zero concentration. Reliable corrected optical density values are difficult to obtain because of the high degree of light scattering encountered in the UV absorption spectra of TSWV.

The instability of TSWV and the low yields obtained after purification made it impossible to determine the electrophoretic mobilities of this virus accurately. Dialysis to equilibrate the virus samples, which is essential in these determinations, destroyed infectivity to such an extent that the virus preparations were not suitable for electrophoretic determinations. Fixation of the virus particles also resulted in changing the surface charges on the virus particles and therefore their electrophoretic mobility. From the limited information obtained, it can be concluded that the isoelectric point of TSWV is at a pH value less than pH 7, a pH range at which virus activity was destroyed.

A certain degree of distortion of purified TSWV particles is suggested when particle diameters, as determined by different authors,

are compared with each other. The diameters of these particles, round
to oval in shape, varied between 62 and 150 nm (see Table I). An in-
ternal calibration standard of known dimensions was not included
when the above mentioned diameters were determined. The diameter of
TSWV particles was determined as 111,7 nm when measured against TMV
with an accepted normal length of 300 nm (Brandes, 1964; van Regen-
mortel et al., 1964). With the confidence limits of both viruses
taken into consideration, the diameters of TSWV particles should not
be less than 110,2 nm or more than 113,3 nm. These values are higher
than the probable particle diameters set out by the different authors
(see Table I).

Variations in diameter can be attributed to the flattening of
the virus particles, clearly demonstrated by Martin (1964) with
shadowed virus particles. The apparent spherical particles with long
shadows were 100 nm in diameter while the flattened particles with
short shadows were 150 nm in diameter. The flattening of the virus
particles was not evident in positively or negatively stained prepa-
rations and has thus not been taken into consideration in published
studies on stained material.

In determining the diameter of TSWV particles, the length of the
visible part of the particle along with the length of the particle
shadow were also determined in order to calculate the height of the
particle above the supporting film of the grid. The average ratio of
the height to the diameter of TSWV particles was 0,478 which indica-
ted a considerable flattening of the TSWV particles to form ellipsoids.

The most reliable information about the shape of TSWV particles
should be obtainable from ultrathin sections of infective tissue.

Since these particles are supported by the surrounding plant tissue they should be less vulnerable to distortion than those present in purified preparations. The shape of TSWV in ultrathin sections was described as roughly spherical to slightly oval (see Table II) with diameters ranging from 47 to 120 nm. These particles were not as flattened as those seen in drop preparations. Particle diameters were determined by transforming the ellipsoid virus particles to spherical particles with the same circumference. The diameter for spherical TSWV particles was determined as 85,3 nm with extremes of 84,3 and 86,3 nm when the confidence limits of both TMV and TSWV were taken into consideration.

The reality of TSWV particles with a diameter of 57 nm, as described by Best and Palk (1964), remains uncertain since several workers have been unable to confirm their existence. These particles were observed in ultrathin sections of purified virus preparations prepared by a single step of gradient centrifugation (see Fig.3). Such preparations have previously been shown to contain a relative high amount of plant components which could be misinterpreted for virus particles or viral components. It is possible that plant components were described as the 57 nm virus particles since these particles exhibited external structures not known to occur on TSWV particles. The structures described as large empty envelopes which contained four 57 nm virus particles, are also most probably plant components. The drop preparations of "purified" virus particles not only contained the 57 nm particles but also 88 nm particles which correspond to the TSWV particles isolated in different laboratories.

The infectivity tests performed by Best and Palk (1964) to estab-

lish the infectivity of the 57 nm and the 88 nm particles are meaning-
less since these particles always occurred together in their prepara-
tions. The relative proportions of the two particle types, however,
varied considerably with the extent of purification. No attempts were
made to separate the two particle types from each other since they
always occurred together at the same depth in the gradient tube.
Since infectivity could not be ascribed beyond doubt to the 57 nm
particles their status remains obscure.

In 1966 Best demonstrated that the predominant particles in pre-
parations of strain E were particles 50 nm in diameter which occurred
mainly in pairs. These twins or dumbbell-shaped particles occurred
abundantly and regularly in preparations of strain E and much less
frequently in the other strains in which 75 to 90 particles predomi-
nate. Best thus did not regard the dumbbell-shaped particles as arti-
facts of the preparatory procedure. This interpretation is, however,
not beyond criticism since artifacts can be produced regularly when
the same method of preparation is used at different times.

Since the South African strains also tend to form dumbbell-
shaped particles when suspended in Best's phosphate based buffer (see
Fig.28B)& 30), these particles can be regarded as a product of the
preparatory procedure and not as a property of any strain. It was
demonstrated that particle shape not only varied in the different
solvents used (see Fig.28, 29 & 30) but that shape also varied accor-
ding to the virus concentration (see Fig.30A & B).

Distortion of the virus particles was also induced by certain
pH values of the staining solutions used. The virus particles were

mostly round or doughnut shaped at pH values between 3 and 5 (see Fig.33, 34A &B). Parallel samples at these pH values were not infective. Particles were most distorted at pH 6 (see Fig.34C) while a variety of shapes were observed at pH values between 7 and 9. Parallel samples at these pH values were infective.

The differences in particle shape in the different solvents and with the different stains were not always clearcut but it clearly demonstrated that the variability in particle shape in purified preparations can be attributed to in vitro conditions and not to morphological characteristics of TSWV strains.

The variation in particle morphology in purified preparations may be attributed to artifacts which arise during preparation of the specimens for electron microscopy. TSWV particles are probably spherical in shape as observed in thin sections of infected plant cells, where the surrounding plant tissue should prevent or reduce a variation in particle morphology.

Variations in TSWV particle morphology are similar to that for lettuce necrotic yellows virus when purified virus preparations were stained with PTA at different pH values (Wolanski & Francki 1969).

The staining characteristics of the TSWV particles varied with the pH of the PTA solutions used. In the pH range of 3-6 the virus membrane stains more intensely than in the pH range from 7-9. This indicates a better penetration of the spikes, present on the virus membrane, by the staining solution.

The variation in shape of the virus particles and changes in the staining characteristics with changes in the pH of the staining

solutions may possibly be explained by osmotic and imbibition pheno-
mena (Wolanski & Francki, 1969). Alterations in the electric charges
on the viral envelope will result from the different ionic environ-
ments at different pH values. It has been established that proteins
swell at pH values away from the isoelectric point, probably due to
an isotropic expansion of the molecule caused by a coulombic repul-
sion of the charged groups. During expansion, the rearrangement in
spatial orientation of the folding of the peptide chains is such,
that a more random configuration is obtained.

A similar phenomenon may occur with the proteins of the viral
envelope where a slight swelling would be enough to alter the pene-
tration of the viral envelope. Both the physical swelling of each pro-
tein molecule and the effect of imbibition of water into the virus
particle will result in a morphological alteration of particle shape.
At pH values closer to the isoelectric point the envelope will be in
its most compact and ordered form. Some variation in shape from pre-
paration to preparation at the same pH values, may be due to differen-
ces in the rate of drying of the stained preparation on the microscope
grids. This will affect the time allowed for the osmotic and imbibition
phenomena to operate (Wolanski & Francki, 1969).

Since an internal nucleocapsid was not demonstrated beyond doubt
for TSWV particles, the classification of this virus as a myxovirus
remains uncertain. Since a filamentous structure with a helical array
of subunits, like the nucleocapsids of paramyxoviruses, was only once
observed during careful examination of more than 200 TSWV preparations,
it cannot be regarded as a nucleocapsid of TSWV. The diameter of these
structures was 25 nm which is 25 percent more than the diameter of the
nucleocapsids of paramyxoviruses (Finch & Gibbs, 1970).

CHAPTER VI

CONCLUSION.

The inability to purify TSWV by means of the different published purification methods may be ascribed to the inadequate clarification obtained by centrifugation. The amount of plant contaminants present in the virus containing pellets after high speed centrifugation necessitated low speed centrifugation. Because of insufficient dispersal of the virus particles from these high speed pellets, most of the virus was lost with low speed centrifugation.

Satisfactory clarification of plantsap was effected by chromatography on calcium phosphate columns with a negligible loss in virus infectivity. The considerable amount of F 1 protein present in the virus samples after clarification resulted in overloading of the density gradient and led to the contamination of the virus containing zone present below the F 1 zone. Stabilization of the virus particles with glutaraldehyde lead to a coupling of the F 1 protein onto TSWV particles, a complex which elicited antibody formation against both TSWV and F 1 protein. Linkage was stable enough to withstand separation by electrophoresis.

To prevent the formation of a TSWV-F 1 protein complex, the virus was separated from F 1 by PEG precipitation. The residual F 1 protein present could then be successfully separated from TSWV by gradient centrifugation. During subsequent fixation, no TSWV-F 1 complexes were formed, and antigenically pure TSWV was obtained after zone electrophoresis.

Fixation of the virus particles with glutaraldehyde did not prevent the loss of virus during purification. This was evident in the smaller schlieren peaks obtained after each successive purification step.

A considerable reduction in the virus pellets usually occurred with electrophoresis. Developing "mild" purification techniques may reduce virus loss. The possibility of serological detection of TSWV in plant juice should be further investigated with more sensitive methods than the double diffusion procedure used in this work. Preparation of TSWV for immunization should be investigated in greater detail in order to obtain antisera with higher titers.

The variation in sedimentation coefficients of TSWV warrants further investigation. Glutaraldehyde fixation of TSWV, when only partially purified, may link proteinaceous substances onto the virus particles and in that way influence sedimentation constants. The presence of variable amounts of residual sucrose in the virus preparations after gradient centrifugation may also have influenced the sedimentation velocity by increasing the viscosity.

The possibility of stabilizing purified TSWV in different buffers at different pH values and ionic strengths should be further investigated. Some buffers may facilitate the determination of certain biophysical properties like the electrophoretic mobility. The use of Raleigh optics would provide precise data for concentration determinations. The extinction coefficient and the dependence of sedimentation velocity on concentration could then be reinvestigated.

A study of the viral nucleic acids and the nucleoprotein will present valuable information regarding the possible classification of TSWV as a myxovirus. Sedimentation velocity studies and molecular weight determinations should indicate whether the nucleic acid component of TSWV is composed of different fragments as in influenza virus. Such a study is under progress.

It has been shown that virus morphology varies according to the pH and the type of buffer used. This explains the contradictory reports that were published on the morphology of this virus.

--ooo00OOO00ooo--
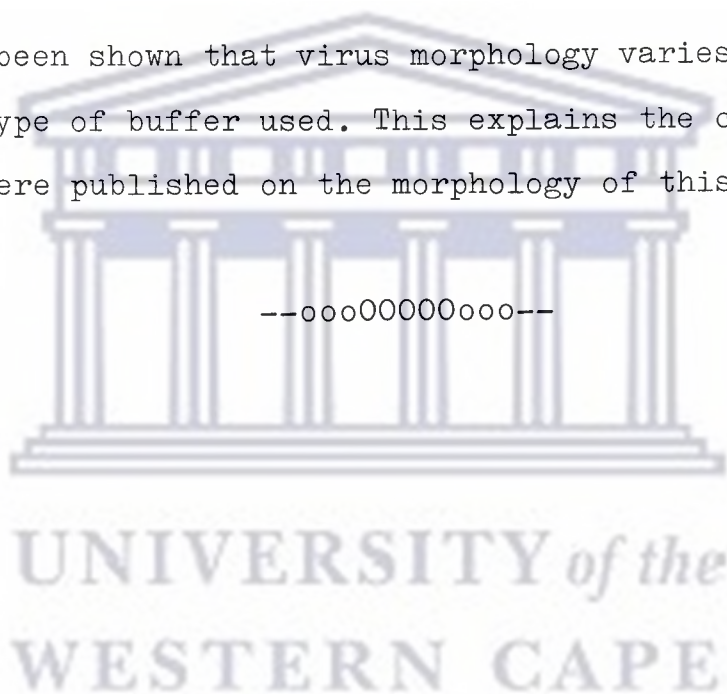
REFERENCES

ALMEIDA, J.D. & WATERSON, A.P. 1970. In: The biology of the large RNA viruses (R.D. Barry and B.W. Mahy, eds.), pp.27-51. Academic Press, New York and London.

AVRAMEAS, S. & TERNYNCK, T. 1969. The cross-linking of proteins with glutaraldehyde and it's use for the preparation of immunoadsorbents. Immunochemistry 6, 53-66.

BAILEY, S.A. 1935. Thrips as vectors of plant diseases. J. econ. Ent. 28, 856-863.

BALD, J.G. & SAMUEL, G. 1931. Investigations on spotted wilt of tomatoes. II. J. Coun. scient. ind. Res. Austr. 54, 1-24.

BALD, J.G. & SAMUEL, G. 1934. Some factors affecting the inactivation of the virus of tomato spotted wilt. Ann. appl. Biol. 21, 179-190.

BECKER, W.B. 1963. The morphology of the tern virus. Virology 20, 318-327.

BEST, R.J. 1935. The effect of environment on the production of primary lesions by plant viruses. J. Aust. Inst. agric. Sci. 1, 159-161.

BEST, R.J. 1936. The effect of light and temperature on the development of primary lesions of the viruses of tomato spotted wilt and tobacco mosaic. Aust. J. exp. Biol. med. Sci. 14, 223-239.

BEST, R.J. 1937a. The quantitative estimation of relative concentrations of the viruses of ordinary and yellow tobacco mosaics and of tomato spotted wilt by the primary lesion method. _Aust_. _J_. _exp_. _Biol_. _med_. _Sci_. 15, 65-79.

BEST, R.J. 1937b. On the presence of an "oxidase" in the juice expressed from tomato plants infected with the virus of tomato spotted wilt. _Aust_. _J_. _exp_. _Biol_. _med_. _Sci_. 15, 191-199.

BEST, R.J. 1937c. Plant viruses. The influence of recent knowledge on methods for their control. _J_. _Aust_. _Inst_. _agric_. _Sci_. 5, 162-169.

BEST, R.J. 1937d. _J_. _Proc_. _Aust_. _Chem_. _Inst_. 4, 375. (As reviewed by Best, (1968).)

BEST, R.J. 1946. Thermal inactivation of tomato spotted wilt virus. _Aust_. _J_. _exp_. _Biol_. _med_. _Sci_. 24, 21-25.

BEST, R.J. 1954. Cross protection by strains of tomato spotted wilt virus and a new theory to explain it. _Aust_. _J_. _biol_. _Sci_. 7, 415-424.

BEST, R.J. 1961. Recombination experiments with strains A and E of tomato spotted wilt virus. _Virology_ 15, 327-**339.**

BEST, R.J. 1965. Interactions between plant viruses in their hosts: antagonism between strains of plant viruses. _Enzymologia_ 29, 377-384.

BEST, R.J. 1966. Preparation and properties of tomato spotted wilt virus (strain E). _Enzymologia_ 31, 333-346.

BEST, R.J. 1968. Tomato spotted wilt virus. _Adv_. _Virus_ _Res_. 13, 65-146.

BEST, R.J. & GALLUS, H.P.C. 1950. Investigations on plant viruses. Tomato spotted wilt virus. Rep. Waite agric. Res. Inst. 1943-1947, 35-36.

BEST, R.J. & GALLUS, H.P.C. 1953. Strains of tomato spotted wilt virus. Aust. J. Sci. 15, 212-214.

BEST, R.J. & GALLUS, H.P.C. 1955. Further evidence for the transfer of character determinants (recombination) between strains of tomato spotted wilt virus. Enzymologia 17, 207-221.

BEST, R.J. & HARIHARASUBRAMANIAN, V. 1967. Serological studies on tomato spotted wilt virus (Strains E and R_1). Enzymologia 32, 128-132.

BEST, R.J. & KATEKAR, G.F. 1964. Lipid in a purified preparation of tomato spotted wilt virus. Nature, Lond. 203, 671-672.

BEST, R.J. & PALK, B.A. 1964. Electron microscopy of strain E of tomato spotted wilt virus and comments on its probable biosynthesis. Virology 23, 445-460.

BEST, R.J. & SAMUEL, G. 1936a. The reaction of the viruses of tomato spotted wilt virus and tobacco mosaic to the pH of media containing them. Ann. appl. Biol. 23, 509-537.

BEST, R.J. & SAMUEL, G. 1936b. The effect of various chemical treatments on the activity of the viruses of tomato spotted wilt virus and tobacco mosaic. Ann. appl. Biol. 23, 759-780.

BEST, R.J. & SYMONS, R.H. 1963. Chemistry of viruses. F. Incorporation of ^{32}P into the virus. Rep. Waite agric. Res. Inst. 1962-1963, 34-35.

BLACK, L.M., BRAKKE, M.K. & VATTER, A.E. 1952. Partial purifica-
tion and electron microscopy of tomato spotted wilt
virus. _Phytopathology_ 42, 3. (Abstract).

BLACK, L.M., BRAKKE, M.K. & VATTER, A.E. 1963. Purification and
electron microscopy of tomato spotted wilt virus.
Virology 20, 120-130.

BONHOEFFER, F. & SCHACHMAN, H.K. 1960. Studies on the organiza-
tion of nucleic acids within nucleoproteins. _Biochem._
biophys. _Res_. _Commun_. 2, 366-371.

BRAKKE, M.K. 1953. Zonal separation by density gradient centrifu-
gation. _Archs Biochem_. _Biophys_. 45, 275-290.

BRAKKE, M.K. 1960. Density gradient centrifugation and its appli-
cation to plant viruses. _Adv_. _Virus Res_. 7, 193-222.

BRAKKE, M.K. 1967. Density gradient centrifugation. _In_: Methods
in Virology (K. Maramorosch & H. Koprowski, eds.) Vol.
II, pp.93-118. Academic Press, New York and London.

BRANDES, J. 1964. _Mitt_. _Biol_. _Bundesanstalt Land- Forstwirt-_
schaft, Berlin-Dahlem 110, 1-130 (As reviewed by
Brandes & Bercks, 1965.)

BRANDES, J. & BERCKS, R. 1965. Gross morphology and serology as
a basis for classification of elongated plant viruses.
Adv. _Virus Res_. 11, 1-24.

BRANDES, J. & PAUL, H.L. 1957. Das Elektronenmikroskop als Hilfs-
mittel bei der Diagnose pflanzlicher Virosen. _Arch_.
Mikrobiol. 26, 358-368.

BRITTLEBANK, H.H. 1919. A new tomato disease - spotted wilt.
_J_. _Dep_. _Agric_. _Vict_. 27, 231-235.

CHOPPIN, P.W. & STOECKENHUIS, W. 1964. The morphology of SV_5
virus. _Virology_ 23, 195

DAY, M.F. & IRZYKIEWICZ, H. 1954. Physiological studies on thrips in relation to transmission of tomato spotted wilt virus. _Aust. J. Biol. Sci._ 7, 274-281.

DUESBERG, P.H. 1969. Distinct subunits of the ribonucleoprotein of influenza virus. _J. molec. Biol._ 42, 485-499.

DUNN, D.B. & HITCHBORN, J.H. 1965. The use of bentonite in the purification of plant viruses. _Virology_ 25, 171-192.

FELDMAN, J.M. & BONINSEGNA, J.A. 1968. Antiserum for tomato spotted wilt virus. _Nature, Lond._ 219, 183-184.

FINCH, J.T. & GIBBS, A.J. 1970. Observations on the structure of the nucleocapsids of some paramyxoviruses. _J. gen. Virol._ 6, 141-150.

FRANCKI, R.I.B. & GRIVELL, C.J. 1970. An electron microscope study of the distribution of tomato spotted wilt virus in systemically infected _Datura stramonium_ leaves. _Virology_ 42, 969-978.

FULTON, W.R. 1959. Purification of sour cherry necrotic ring-spot and prune dwarf viruses. _Virology_ 9, 522-535.

GALVEZ, G.E. 1964. Loss of virus by filtration through charcoal. _Virology_ 23, 307-312.

GARDNER, M.W., TOMPKINS, C.M. & WHIPPLE, O.C. 1935. Spotted wilt of trunk crops and ornamental plants. _Phytopathology_ 25, 17. (Abstract).

GLAUERT, A.M. 1965. The fixation and embedding of biological specimens. _In_ Techniques for electron microscopy (D. Kay, ed.), pp.166-212. Blackwell Scientific Publications, Oxford and Edinburgh.

GRIEVE, B.J. 1943. Studies on the physiology of host parasite relations. IV. Some effects of tomato spotted wilt on growth. Aust. J. exp. Biol. med. Sci. 21, 89-101.

GRIEVE, B.J. 1951. Rep. Aust. N.Z. Ass. Advmt. Sci. 28, 102. (As reviewed by Best, 1968.)

HOYLE, L., HORNE, R.W. & WATERSON, A.P. 1961. The structure and composition of the myxoviruses. II. Components released from the influenza virus by ether. Virology 13, 448-459.

IE, T.S. 1964. An electron microscope study of tomato spotted wilt virus in the plant cell. Neth. J. Plant Pathol. 70, 114-115.

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

INCARDONA, N.L. & KAESBERG, P. 1964. A pH-induced structural change in brome grass mosaic virus. Biophys. J. 4, 11-21.

JENNING, A.C. & BEST, R.J. 1964. Partial amino acid composition of tomato spotted wilt virus (strain E). Virology 24, 205-208.

JONES, L.K. 1944. Streak of mosaic and cineraria. Phytopathology 34, 941-953.

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

LAUFFER, M.A. 1944. The influence of concentration upon sedimentation rate of tobacco mosaic virus. J. Am. Chem. Soc. 66, 1195-1198.

LEBERMAN, R. 1966. The isolation of plant viruses by means of "simple" coacervates. <u>Virology</u> 30, 341-347.

LINFORD, M.B. 1932. Transmission of pineapple yellow-spot virus by <u>Thrips tabaci</u>. <u>Phytopathology</u> 22, 301-324.

LOUNSBURY, C.P. 1966. Tobacco wilt in Kat River Valley. <u>Agric. J. Cape G.H.</u> 28, 784-803.

LUFT, I.H. 1961. Improvements in epoxy embedding methods. <u>J. biophys. biochem. Cytol</u>. 9, 409-414.

MARKS, L.S. 1946. <u>In</u> Mechanical engineers handbook, pp.107 McGraw-Hill, London.

MARTIN, M.M. 1964. Purification and electron microscope studies of tomato spotted wilt virus (TSWV) from tomato roots. <u>Virology</u> 22, 645-649.

MESSIEHA, M. 1969. Transmission of tobacco ringspot virus by thrips. <u>Phytopathology</u> 59, 943-945.

MULLER, G.L. & GOLDER, R.H., 1950. Buffers of pH 2 to 12 for use in electrophoresis. <u>Arch. Biochem</u>. 29, 420-423.

MILNE, R.G. 1967. Plant viruses inside cells. <u>Science Progress</u>, <u>Oxford</u> 55, 203-

MILNE, R.G. 1970. An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stained preparations. <u>J. gen. Virol</u>. 6, 267-276.

MOORE, E.S. 1933. The Kromnek or Kat River disease of tobacco and tomato in the Eastern Province (South Africa). <u>U. of S. Afr. Dept. Agric. Forestry, Sci. Bull</u>. 123.

MURANT, A.F., GOOLD, R.A., ROBERTS, I.M. & CATHRO, J. 1969. Carrot mottle - a persistent aphid-borne virus with unusual properties and particles. <u>J. gen. Virol</u>. 4, 329-341.

NORRIS, D.O. 1943. Strains of spotted wilt virus and the iden-
tity of tomato tip blight virus with spotted wilt.
J. Coun. scient. ind. Res. Aust. 16, 91-92.

NORRIS, D.O. 1946. The strain complex and symptom variability
of tomato spotted wilt virus. J. Coun. scient. ind.
Res. Aust., Bull. 202.

PARRIS, G.K. 1946. Mechanical transmission of yellow-spot
virus. Evidence for identity with spotted wilt virus.
Phytopathology 30, 299-312.

POLSON, A. & DEEKS, D. 1962. Zone electrophoresis of entero
viruses. J. Hyg., Camb. 60, 217-234.

POLSON, A. & RUSSEL, B. 1967. Electrophoresis of viruses. In
Methods in virology (K. Maramorosch and H. Koprowski,
eds.) Vol.II, pp.391-426. Academic Press, New York
and London.

PITTMAN, H.H. 1927. Spotted wilt of tomatoes. J. Coun. scient.
ind. Res. Aust., Bull. 1.

RAZVYAZKINA, G.M. 1953. The importance of the tobacco thrips
in the development of outbreaks of tip chlorosis of
Makhorha. (In Russian). English abstract in: Rev.
appl. Ent. A42, 146.

SABATINI, D.D., BENSCH, K. & BARNETT, R.S. 1963. Cytochemistry
and electron microscopy. The preservation of cellular
ultrastructure and enzymatic activity by aldehyde
fixation. J. cell. Biol. 17, 19-29.

SAKIMURA, K. 1940. Evidence for the identity of the yellow-
spot virus with the spotted wilt virus. Experiments
with the vector, Thrips tabaci. Phytopathology 30,
281-299.

SAKIMURA, K. 1947. Thrips in relation to gall forming and
plant disease transmission: a review. Proc. Hawaii.
ent. Soc, 13, 59-96.

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

SAKIMURA, K. 1963. Frankliniella fusca, an additional vector
for the tomato spotted wilt virus, with notes of
Thrips tabaci, another vector. Phytopathology 53,
412-415.

SAMUEL, G. & BALD, J.G. 1933. Tomato spotted wilt on tobacco.
J. Dep. Agric. S. Aust. 37, 190-195.

SAMUEL, G., BALD, J.G. & PITTMANN, H.A. 1930. Investigations
on spotted wilt of tomatoes. J. Coun. scient. ind.
Res. Aust., Bull. 44.

SAMUEL, G., BEST, R.J. & BALD, J.G. 1935. Further studies on
quantitative methods with two viruses. Ann. appl,
Biol. 22, 508-524.

SELMAN, I.W., BRIERLY, M.R., PEGG, G.F. & HILL, T.A. 1961.
Changes in the free amino acids and amides in tomato
plants inoculated with tomato spotted wilt virus.
Ann. appl. Biol. 49, 601-615.

SMITH, K.M. 1931. Studies on potato virus diseases. VIII. On
a ringspot virus affecting solanaceous plant. Ann.
appl. Biol. 18, 1-15.

SMITH, K.M. 1932. Studies on plant virus diseases. XI. Further experiments with a ringspot virus: Its identification with spotted wilt of tomato. Ann. appl. Biol. 19, 305-330.

SMITH, K.M. 1957. Tomato spotted wilt virus. In: A text book of plant viruses, 2nd edition. Chruchill, London.

STREERE, R.L. 1959. The purification of plant viruses. Adv. Virus Res. 6, 3-73.

SVEDBERG, T. & PEDERSEN, K.O. 1940. The ultracentrifuge. Claredon Press, Oxford.

TAVERNE, J., MARSHALL, J.H. & FULTON, F. 1958. The purification and concentration of viruses and soluble antigens on calcium phosphate. J. gen. Microbiol. 19, 451-

TSAKIRIDIS, J.P. 1971. Biological and serological characterization of tomato spotted wilt virus in Greece. M.Sc. dissertation, North Carolina State University.

VAN KAMMEN, A., HENSTRA, S. & IE, T.S. 1966. Morphology of tomato spotted wilt virus. Virology 30, 574-577.

VAN REGENMORTEL, M.H.V. 1964a. Separation of an antigenic plant protein from preparations of plant viruses. Phytopathology, 54, 282-289.

VAN REGENMORTEL, M.H.V. 1964b. Purification of plant viruses by zone electrophoresis. Virology 23, 495-502.

VAN REGENMORTEL, M.H.V. 1966. Plant virus serology. Adv. Virus Res. 12, 207-271.

VAN REGENMORTEL, M.H.V. 1967. Biochemical and biophysical properties of cucumber mosaic virus. Virology 31, 391-396.

VAN REGENMORTEL, M.H.V. 1968.   Zone electrophoretic $R_\emptyset$ values
of plant viruses.   S. Afr. med. J. 42, 118.

VAN REGENMORTEL, M.H.V., HAHN, J.S. & FOWLE, L.G. 1964. Internal
calibration of electron micrographs with an orchid
virus.   S. Afr. J. agric. Sci. 7, 159-164.

VON WECHMAR, M.B. 1967.   A study of viruses affecting Graminae
in South Africa.   Ph.D. dissertation, University of
Stellenbosch.

VON WECHMAR, M.B. & VAN REGENMORTEL, M.H.V. 1970.   A simple pro-
cedure for purifying tobacco mosaic virus.   S. Afr.
med. J. 44, 151.

WOLANSKI, B.S. & FRANKCKI, R.I.B. 1969.   Structure of lettuce
necrotic yellows virus.   II.   Electron microscopic
studies on the effect of pH of phosphotungstic acid
stain on the morphology of the virus.   Virology 37,
437-447.

Addendum I.

A typical calculation of a sedimentation coefficient.

The readings below were obtained from a series of photographs taken during a sedimentation experiment, using schlieren optics. The photographs had been taken at 4 minute intervals and the distance from the boundary to the inner reference hole was measured directly off the photographic plate in a shadowgraph.

Calculation of the sedimentation coefficient of TSWV suspended in 0,02 M phosphate buffer (ph 7,2) containing 0,01 M sodium sulphite.

Measured distance on plate from boundary to inner reference hole (mm).	Distance in cell = mea-sured dis-tance magni-fication fac-tor (mm).	x = Actual distance from boun-dary to axis of rotation* (cm).	Logarithm of actual distance from boun-dary to axis of rotation
4,963	2,26	5,926	0,7727
6,025	2,74	5,974	0,7763
7,183	3,27	6,027	0,7801
8,304	3,79	6,079	0,7838
9,430	4,30	6,130	0,7875
10,600	4,83	6,183	0,7912
11,701	5,34	6,234	0,7948

* The distance from the inner reference hole to the axis of rotation = 5,70 cm.

Magnification factor = 2,191

rpm = 10 000 rpm

$$\frac{2,303}{60\,\omega^2} = 3,499 \times 10^{-8}$$

Temperature = $3,0^{\circ}$C.

$$\frac{\eta\,3,0^{\circ}}{\eta\,20^{\circ}} = 1,62$$

The figures in the last column were plotted versus the time interval in minutes and a straight line, with a slope of $92,5 \times 10^{-5}$, was obtained. This slope was then inserted in the equation.

$$s = \text{slope} \times \frac{2,303}{60^2}$$

$$= (92,5 \times 10^{-5})\,(3,499 \times 10^{-8})$$

$$= 323,6 \times 10^{-13} \text{ sec.}$$

$$S = 323,6$$

The correction to standard conditions in distilled water is:

$$S_{20,w} = S_{obs.}\,\frac{\eta\,3,0^{\circ}}{\eta\,20^{\circ}}$$

$$= 323,6 \times 1,62$$

$$= 524,2$$

Addendum II.

A typical electrophoretic mobility calculation.

The data presented were obtained with the virus in 0,02 M phosphate (pH 7,5) buffer containing 0,01 M sodium sulphite. The experiments were performed at 2,6°C.

Time in minutes (t)	Displacement after minutes in the descending channel in mm (Δh)	Electrophoretic mobility cm^2 $volt^{-1}sec^{-1}$ x 10^{-5}
0	0	0
3	2,248	19,627
3	2,212	19,312
3	2,375	20,736
Average	2,275	19,892

u, mobility = ΔhAC/It
  where A = the cross sectional area of the channel (0,75 cm^2)
       C = the specific conductance of the buffer
                                              (0,002724 ohm^{-1})
       t = time interval (3 min.)
       I = current (0,013)
       Δh = average displacement of the ascending and descending boundaries (0,2212 cm)

Then, u = $\dfrac{0,2214 \text{ x } 0,75 \text{ x } 0,002724}{3 \text{ x } 60 \text{ x } 0,013}$

       = 19,312 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}

<u>Addendum III</u>.

A typical calculation of the specific conductance of a buffer.

The resistance of the 0,02 M phosphate buffer (pH 7,2), containing 0,01 M sodium sulphite was determined with a LKB conductolyzer fitted with a decade resistance. An average of 4450,7 ohm was calculated from twelve values varying between 4448,9 and 4452,4 ohm.

The specific conductance was calculated from the equation

$$C = \frac{K}{Rm}$$

Where K = cell constant = 10,93, and

Rm = the resistance = 4450,7 ohm

$$C = \frac{10,93}{4450,7}$$

$$= 0,002456 \ ohm^{-1}$$

The resistance of each buffer was determined at the same temperature at which electrophoretic mobility experiments were performed.

Addendum IV.

Calculation of the normal length of tobacco mosaic virus (TMV) in arbitrary units for the use as a calibration standard in the calculation of the diameter of tomato spotted wilt virus.

Length of particles in units (x)	Frequency (f)	fx	fx^2
22	19	418	9 196
23	27	621	14 283
24	27	648	15 552
25	36	900	22 500
26	51	1 326	34 476
27	93	2 511	67 797
28	172	4 816	134 848
29	176	5 104	148 016
30	66	1 980	59 400
31	36	1 116	34 596
32	18	576	18 432
33	17	561	18 513
34	17	578	19 652
Total Σ	755	21 155	597 261

Arithmetic mean $= \dfrac{\Sigma fx}{\Sigma f} = \dfrac{21\ 155}{75} = 28,0198$

Variance $= \dfrac{\Sigma fx^2 - \dfrac{(\Sigma fx)^2}{\Sigma f}}{\Sigma f - 1}$

$\qquad = 5,9696$

Standard error $= \dfrac{Variance}{\Sigma f}$

$\qquad = \dfrac{5,9696}{755}$

$\qquad = 0,088\ 915$

95% confidence limit $= \pm\ 0,96 \times 0,088\ 915$

$\qquad = \pm\ 0,174\ 273$

Thus: $28,019\ 8 \pm 0,174\ 3$ units $= 300$ nm.

Addendum V.

Calculation of the diameter of TSWV.

Diameter of particles in arbitrary units (x)	Frequency (f)	fx	fx^2
8,0	7	56,0	448,00
8,5	4	34,0	289,00
9,0	42	378,0	3 402,00
9,5	24	228,0	2 166,00
10,0	113	1 130,0	11 300,00
10,5	88	924,0	9 702,00
11,0	133	1 463,0	16 093,00
11,5	37	425,5	4 893,25
12,0	27	324,0	3 888,00
12,5	3	37,5	468,75
13,0	1	13,0	169,00
Total	479	5 013,0	52 819,00

Arithmetic mean = 10,441 1

Variance   = 0,743 0

Standard error = 0,039 3

95% confidence limit = ± 0,039 3 x 1,96

                    = ± 0,077 1

28,019 8 units for TMV = 300 nm

thus, 10,441 1 units for TSWV = $\dfrac{300 \times 10,441\ 1}{28,019\ 8}$

                    = 111,789 8 nm

    When the confidence limits for both viruses are taken into consideration, the diameter of TSWV should not be less than 110,2 nm or more than 113,3 nm with an average of 111,7 nm.

Addendum VI.

Determination of the height of ten randomly chosen particles (in arbitrary units).

2a	a	a + x	x	2b	b	b/a
11,5	5,75	17,0	11,25	5,25	2,625	0,456
10,0	5,00	16,5	11,50	5,36	2,680	0,536
12,0	6,00	16,0	10,00	4,66	2,330	0,388
10,5	5,25	16,0	10,75	5,01	2,505	0,477
10,0	5,00	16,5	11,50	5,36	2,680	0,536
10,0	5,00	14,0	9,00	4,20	2,100	0,420
9,5	4,75	14,5	9,75	4,55	2,275	0,478
9,0	4,50	15,0	10,50	4,90	2,450	0,544
11,5	5,75	17,5	11,75	5,48	2,740	0,476
10,5	5,25	16,0	10,75	5,01	2,505	0,477
					Average	0,478

where 2a = the diameter of a particle,

a + x   = the distance from the tip of the shadow to the opposite side of the particular particle.

x   = the length of the shadow,

and 2b   = the height of the particle.

2a and a + x are measured on the electron micrograph.

2b is calculated from the formula:

$$2b = x.\text{tangens } 25^o$$

(the specimen grid was shadow casted at an angle of 25°)

The ratio of b/a should be unity for spherical particles. An average ratio of 0,478 indicates a considerable flattening of the particles.

Addendum VII.

The transformation of elliptical particles to spherical particles with the same circumference.

The circumference of an ellipse is calculated from the following formula (Marks, 1946):

$$\pi (a + b) K$$

where, a = half of the width of an ellipse,

b = half of the height of an ellipse,

$$K = \left[1 + \frac{1}{4} M^2 + \frac{1}{64} M^4 + \frac{1}{256} M^6 + \ldots\ldots \right], \text{ and}$$

$$M = (a - b)/(a + b)$$

For any value of M the corresponding value of K can be read from the following table:

For M = 0,1   0,2   0,3   0,4   0,5   0,6   0,7   0,8   0,9

K = 1,002 1,010 1,023 1,040 1,064 1,092 1,127 1,168 1,216

For M = 1,0

K = 1,273

The diameter of a spherical particle with the same circumference as an ellipse is calculated from the following:

$$2\pi r = \pi (a + b) K.$$
$$2r = (a + b) K.$$

The elliptical particles in Addendum VI were transformed to spherical particles as described above in order to obtain the corrected diameters.

The diameters of elliptical TSWV particles when transformed to spheres (in arbitrary units).

a	b	a - b	a + b	$\dfrac{a - b}{a + b}$ (M)	K	2r
5,75	2,65	3,125	8,395	0,373	1,035	8,67
5,00	2,680	2,320	7,680	0,302	1,025	7,87
6,00	2,330	3,670	8,300	0,441	1,049	8,74
5,25	2,505	2,745	7,755	0,354	1,031	8,00
5,00	2,680	2,320	7,680	0,302	1,025	7,87
5,00	2,100	2,400	7,100	0,408	1,042	7,40
4,75	2,275	2,475	7,025	0,352	1,031	7,24
4,50	2,450	2,050	6,950	0,295	1,022	7,10
5,75	2,740	3,010	8,490	0,355	1,031	8,75
5,25	2,505	2,745	7,755	0,354	1,031	8,00

For example:

$$2\pi r = \pi (a + b)\ K$$
$$2r = (a + b)\ K$$
$$= 8,395 \cdot 1,035$$
$$= 8,67$$

For the same particle in Addendum VI the width was determined as 11,5 and the height as 5,25 units.

With both the confidence limits for TMV and the corrected diameters for TSWV taken into consideration, the diameter of spherical TSWV particles should not be less than 84,3 nm or more than 86,3 nm with an average of 85,3 nm.