PURIFICATION AND ELECTRON MICROSCOPY OF THE TOMATO SPOTTED WILT VIRUS

by

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D E C L A R A T I O N

I HEREBY CERTIFY THAT THIS RESEARCH IS THE RESULT OF MY OWN INVESTIGATION.

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C O N T E N T S

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"So will I compass Thine altar, O Lord:
That I may publish with the voice of thanksgiving,
And tell of all Thy wondrous works."

Psalm 26, v. 6b-7

CHAPTER 1

TSWV EXTRACTION FROM LEAF AND ROOT TISSUES

INTRODUCTION

The successful purification of a virus in quantity and without physical or chemical damage to the particles opens the way to a clearer understanding of many facets of the nature and functioning of the virus concerned. In particular, the elucidation of replication processes is greatly facilitated by accurate information concerning the nucleic acid, protein and other constituents of the virus and their organization in the virion. Identification and classification is also ultimately dependent upon detailed information of viral structure and composition.

Amongst the plant viruses not yet purified in a stable, active form is that of tomato spotted wilt (TSWV). Although known to be a relatively large, RNA-containing, membrane bound virus (Best, 1968), accurate information regarding structure and composition is limited. It is unrelated to any other recorded plant virus (Harrison, Finch, Gibbs, Hollings, Shepherd, Valenta and Wetter, 1971).

Both the world-wide distribution and very extensive host range of TSWV attest to a particularly successful physiologic foothold in the plant kingdom. It is the only virus characteristically transmitted by Thrips species (Messieha, 1969). Acquisition is by the larval stage only, rendering the adult viruliferous (Bald and Samuel, 1931). Despite such apparent yellows virus-type vector association the virus in plants is not phloem restricted (Ie, 1964).

Much is known of the *in vitro* behaviour of TSWV owing to the fact that it can be quantitatively bioassayed by mechanical inoculation. Suitable hosts for this purpose include *Nicotiana tabacum* L. var. Blue Pryor (Best and Samuel, 1936a), *N. glutinosa* L. (Best and Samuel, 1936b), Turkish tobacco (Black, Brakke and Vatter, 1952), *N. clevelandii* Gray (Milne, 1970) and *Petunia hybrida* Hort. (Milne, 1960; Selman and Milne, 1961).

In crude plant extract standing at room temperature and exposed to the atmosphere TSWV is inactivated in approximately two to five hours (Bald and Samuel, 1934). This is attributable mainly to oxidative inactivation (Best, 1937a). In addition, pH environments below about 6 or above 10 (Best and Samuel, 1936a; Best, 1966) and temperatures approaching 42°C inactivate TSWV rapidly and irreversibly (Best, 1946). Both these latter phenomena are of considerable theoretical importance, imposing also restrictions during purification studies.

A major advance was achieved when Best and Samuel (1936b) established that reductants such as cysteine and Na_2SO_3 prolong the viability of the virus *in vitro*. This discovery has subsequently provided the foundation for all TSWV extraction studies, the first being by Black *et al.* in 1952 (Table 1). Metal shadowing of infective preparations revealed the presence of oblate ellipsoid particles 90 to 120 nm in diameter.

TABLE 1. Methods, virus isolates and propagating hosts used in reported TSWV extraction studies from infected leaf material

Author	Method[a]	Propagating host	Isolate	
			Origin	Type[b] & Strain
Black, Brakke & Vatter (1952)	Fig. 1	N. rustica	N. America	?
" (1963)	" "	"	"	? (2 isolates)
van Kammen, Henstra & Ie (1966)	" "	"	Nether-lands	I[c]
Gumpf & Weathers (1972)	" "	Datura stramonium	N. America	I
Mohamed, Randles & Francki (1973)	" "	"	Australia	S[d]
Best (1958)	?	?	"	?
Best & Palk (1964)	Fig. 2	N. glutinosa	"	M (E)
Best & Katekar (1964)	" "	"	"	M (E)
Jennings & Best (1964)	" "	"	"	M (E)
Best (1966)	" "	"	"	M (E)
Best & Hariharasubramanian (1967)	" "	"	"	(i) M (E); (ii) S (R_1)
Tsakiridis & Gooding (1972)	Fig. 3	B-21 tobacco	Greece	M
Joubert (1973)[e]	Fig. 4	N. glutinosa	S. Africa	(i) M; (ii) S or I

[a] Method groupings refer to Figures 1 - 4, ignoring minor modifications

[b] Symptom type on tomato: S = severe, I = intermediate and M = mild, based on the strain grouping made by Best (1968). Where necessary, the reported symptoms on tomato and N. glutinosa have been used to locate the probable type.

c The strain used also in the present study

d Refer: Mohamed and Randles (1972)

e See also Joubert, Hahn, von Wechmar and van Regenmortel (1974)

Correlation of infectivity with such particles was subsequently established by Black, Brakke and Vatter (1963). The extraction procedure applied by these authors to systemically infected N. rustica L. leaf material (Fig. 1) included homogenization and clarification in 0,1 M phosphate buffer, pH 7 ('solvent 4'), followed by high speed and rate zonal sucrose gradient centrifugations. A reducing agent, 0,01 M Na_2SO_3, was provided throughout the extraction. Initial homogenization in the high molarity phosphate buffer was found to intensify virus aggregation so that after initial low speed centrifugation almost all infectivity was associated with the pellet (P_1). Resuspension of this pellet in 0,01 M Na_2SO_3 effectively redispersed the virus which, when again centrifuged at low speed, remained in the clarified supernatant (S_2). With respect to the concluding stages of the procedure the authors reported that, due to the instability of TSWV, equilibrium zonal centrifugation or zone electrophoresis "could be used with fair success most of the time" but little or no infectivity remained when both were applied. However, if such treatment was omitted contaminating host material presisted in the virus preparation.

Particles similar to those observed by Black et al. (1952) were briefly reported by Best in 1958. When apparently undeformed, such particles measured about 80 nm in diameter.

FIGURE 1. PURIFICATION PROCEDURE FOR TSWV IN SYSTEMICALLY INFECTED *Nicotiana rustica* LEAF TISSUE (BLACK *et al.*, 1952; BLACK, L.M. Personal communication)

(x) g[a] leaf material + (x) ml 'solvent 4'[b]; homogenize, strain

Clarification: Centrifuge 3-5 000 rpm, 20-30 min

- Supernatant (S_1), discard
- Pellet (P_1): resuspend in (x) ml 0,01 M Na_2SO_3, stand 30 - 60 min

Centrifuge 3-5 000 rpm, 30 min

- Pellet (P_2), discard
- Supernatant (S_2)

High speed centrifugation: 13 000 rpm (ca. 22 000*g*), 45 min, Servall Model SS-2 centrifuge

- Supernatant (S_3), discard
- Pellet (P_3): resuspend in $\frac{(x)}{10} - \frac{(x)}{20}$ ml 0,01 M Na_2SO_3[c], stand 30 - 60 min

Rate zonal sucrose gradient centrifugation: Layer 1 ml on 0-25,5% gradient containing 0,01 M Na_2SO_3, Servall Model SS-2 swinging bucket rotor

Centrifuge 10 000 rpm (9 500 - 16 200*g*), 120 min[d]

Virus zone (Z): For further purification apply either (i) or (ii) below

Either (i) Equilibrium zonal sucrose density gradient centrifugation: Layer 5 ml of virus zone on 30 - 60% gradient containing 0,01 M Na_2SO_3, Servall Model SS-2 angle rotor

Centrifuge 17 500 rpm (ca. 50 000*g*), 90 min[e]

Virus zone (EZ)

Or (ii) Zone electrophoresis:

Virus zone

[a] (x) g = starting weight of tissue; (x)ml an equivalent volume (w/v)

[b] 'Solvent 4' = 0,033 M KH_2PO_4, 0,067 M K_2HPO_4, containing 0,01 M Na_2SO_3 (pH 7)

[c] Modified by the omission of Tween-80 (see also Black *et al.*, 1963)

[d] Alternatively, 0-40% sucrose gradient, Spinco SW 25.1 rotor, 25 000 rpm (max. 90 000*g*), 25 min

[e] Alternatively, 30 - 60% sucrose gradient, Spinco SW 39 rotor

(Notations S_{1-3}, P_{1-3}, Z and EZ introduced by present author)

No preparative details were given.

The work on TSWV purification presented in this chapter was performed during the period 1960 - 1962 at the Laboratorium voor Virologie, Wageningen, the Netherlands. It sought firstly, to corroborate the results obtained by Black *et al.* (1952) and secondly, to establish the cause of the variability in virion size. In the latter regard consideration was given to the alternative possibilities that differences in virion size and/or structural integrity arise *in vivo* or are an artefact of extraction.

No indication was given by Black *et al.* (1952) that the isolate used in their purification studies comprised a pure strain. To examine, therefore, the possibility that differences in particle sizes, reported by these authors, might be attributable to strain heterogeneity, the described procedure was applied by the present author to a pure strain of the virus isolated in the Netherlands.

Means of shortening the procedure were sought as the extraction procedure used by Black *et al.* (1952) clearly exploits to the limit the *in vitro* longevity of the virus and structural deterioration could be expected to occur during such comparatively prolonged treatments. In particular, the use of selective adsorbants was extensively investigated.

Shortly after terminating the investigations presented in this chapter a second report on TSWV extraction by Black *et al.* (1963) appeared, confirming and amplifying their

findings published 11 years previously. However, no improvement in extraction procedure was reported, the primary hindrance being the instability of the virus. Of the subsequent investigators some have followed the method used by Black et al. (1952, 1963) whilst others, finding it unsatisfactory, have sought alternative procedures. Details of these studies are listed in Table 1.

In the experience of Best and co-workers (Best and Katekar, 1964; Best and Palk, 1964; Best, 1966, 1968) each of three Australian strains in N. glutinosa failed to aggregate satisfactorily when homogenized in 'solvent 4' (Fig. 1). These authors therefore devised an alternative clarifying step, homogenizing infected N. glutinosa leaf tissue in a phosphate-based buffer, termed 'solution B' (Fig. 2). In this environment the virus was retained almost entirely in the first supernatant.

Joubert (1973), unsuccessfully applying the procedure used by Black et al. (1963) to a South African isolate, reported 76% (av) infectivity for the resuspended P_1 pellet (Fig. 1) but only 36% infectivity for the ensuing S_2 supernatant. Likewise, Tsakiridis and Gooding (1972) resuspended the P_1 pellet in 'solution B' rather than in 0,01 M Na_2SO_3 (Fig. 3).

Arising out of the problem of severe, early virus loss the use of calcium phosphate chromatography for clarification was successfully investigated by Joubert (1973). In preparation for layering on the brushite column (Fig. 4), the crude N. glutinosa homogenate, containing 0,02 M sodium phosphate buffer, pH 7,2 and 0,01 M Na_2SO_3, was centrifuged

FIGURE 2. PURIFICATION PROCEDURE FOR TSWV IN SYSTEMICALLY INFECTED *Nicotiana glutinosa* LEAF TISSUE (BEST and PALK, 1964[a]; BEST, 1966, 1968)

60 g leaf material + 300 ml 'solution B'[b]; homogenize, strain

Clarification: Centrifuge 10 000*g*, 8 min

- Pellet, discard
- Supernatant

Differential centrifugation: 25 000 rpm, 30 min, Spinco No. 30 rotor

- Supernatant, discard
- Pellet: resuspend in 7,5 ml 'solution B'

Centrifuge 10 000*g*, 8 min

- Pellet, discard
- Supernatant

Rate zonal sucrose gradient centrifugation: Layer 2 ml on each of three 0-40% gradients containing 'solution B', Spinco SW 25 rotor

Centrifuge 24 000 rpm, 60 min

Virus zone: remove, pool

High speed centrifugation: 40 000 rpm, 60 min, Spinco No. 40 rotor

- Supernatant, discard
- Pellet: resuspend in 2,5 ml 'solution B'

Rate zonal sucrose gradient centrifugation: Layer on 0-40% gradient containing 'solution B', Spinco SW 25 rotor

Centrifuge 24 000 rpm, 60 min

Contiguous virus zones, upper (Cy2a) and lower (Cy2b): remove together

High speed centrifugation: 40 000 rpm, 20 min, Spinco No. 40 rotor

- Supernatant, discard
- Pellet: resuspend in 1 ml 'solution B'

Repeat high speed centrifugation:

- Supernatant, discard
- Purified virus in resuspended pellet

[a] Best and Palk (1964) employed a variation of this procedure: after the first rate zonal sucrose gradient centrifugation the virus was pelleted two or three times by high speed centrifugation to remove the sucrose. In some extractions electrophoresis was subsequently applied.

[b] 'Solution B' = 0,01 M sodium or potassium phosphate buffer, pH7 + 0,01 M Na_2SO_3 + 0,07 M Na_2SO_4 + 1 x 10^{-4} M ethylenediaminetetra-acetate (Na salt)

FIGURE 3. PURIFICATION PROCEDURE FOR TSWV IN SYSTEMICALLY INFECTED Nicotiana tabacum LEAF TISSUE (TSAKIRIDIS and GOODING, 1972)

160 g leaf material + 160 ml 0,1 M potassium phosphate buffer, pH7 + 0,01 M Na_2SO_3; homogenize, strain

Clarification: Centrifuge 4 000 g, 30 min

- Supernatant, discard
- Pellet: resuspend in 160 ml 'solution B'[a]

Add 240 ml Freon 114; homogenize 40 sec

Centrifuge 4 000g, 15 min

Aqueous phase

Add 80 ml Freon 114; homogenize 20 sec

Centrifuge 4 000g, 10 min

Aqueous phase

High speed centrifugation: max. 74 000g, 30 min

- Supernatant, discard
- Pellet: resuspend in 6 ml 'solution B'

Rate zonal sucrose gradient centrifugation: Layer on three 0-40% gradients containing 'solution B', Spinco SW 25 rotor

Centrifuge max. 83 000g, 60 min

Visible zone: remove, pool, dilute to 36 ml with 'solution B'

High speed centrifugation: max. 100 000g, 40 min

- Supernatant, discard
- Purified virus in pellet resuspended in distilled water (**FP**)

[a] See Fig. 2 for composition of 'solution B'

(Notation FP introduced by present author)

FIGURE 4. PURIFICATION PROCEDURE FOR TSWV IN SYSTEMICALLY INFECTED *Nicotiana glutinosa* LEAF MATERIAL (JOUBERT, 1973; JOUBERT *et al.*, 1974)

100g leaf material + 350 ml 0,02 M sodium phosphate buffer, pH7,2 + 0,01 M Na_2SO_3; homogenize, strain

Clarification: Centrifuge 10 000*g*, 10 min

- Pellet, discard
- Supernatant

Layer on calcium phosphate (brushite) column; elute with abovementioned phosphate buffer + sulphite

Eluate (400 ml)

High speed centrifugation: 25 000 rpm, 40 min, Spinco No. 30 rotor

- Supernatant, discard
- Pellet (**CP**): resuspend in 6 ml phosphate buffer + sulphite

Polyethylene glycol precipitation: Add 0,36 ml 25% PEG(MW 6 000) and 0,12 ml 25% NaCl; mix thoroughly

Centrifuge 10 000*g*, 5 min

Pellet (**PP**): resuspend in 6 ml distilled water

Rate zonal sucrose gradient centrifugation: Layer on 0-40% gradients containing phosphate buffer + sulphite, Spinco SW 25.2 rotor

Centrifuge 20 000 rpm, 60 min

Virus zone: remove

High speed centrifugation: 35 000 rpm, 35 min, Spinco No. 40 rotor

- Supernatant, discard
- Pellet (**RP**): resuspend in 0,2 ml phosphate buffer + sulphite

Stabilization: Add 0,2 ml 1% glutaraldehyde in 0,01 M phosphate buffer + sulphite

Sucrose gradient zone electrophoresis: Layer on 50% sucrose in 0-50% gradient in 0,02 M phosphate buffer, pH7,2 + 0,01 M Na_2SO_3

Electrophorese 16 h

Virus zone: remove

High speed centrifugation: 35 000 rpm, 35 min, Spinco No. 40 rotor

- Supernatant, discard
- Purified virus in resuspended pellet (**EP**)

(Notations CP, PP, etc., introduced by present author)

at low speed for 10 min. Although no evidence is presented regarding virus distribution between the resultant initial supernatant and pellet, high virus yields at later stages of the procedure clearly indicate satisfactory virus retention in the supernatant.

In the same manner that calcium phosphate chromatography was introduced by Joubert (1973) to supplement a modified form of the Best and Palk (1964) clarification step, Freon 114 treatment (Fig. 3) was used by Tsakiridis and Gooding ing (1972) to supplement a modification of the clarification method used by Black et al. (1952, 1963). In part these further developments were prompted by lack of success with both the method developed by Black et al. (1963) as well as that introduced by Best (1966).

The continuing lack of procedural unanimity amongst investigators attempting to purify TSWV, serves to emphasize the many difficulties that remain unresolved. As noted by Best (1968) and Joubert (1973) strain differences may be of significance. Tsakiridis and Gooding (1972) have suggested that choice of host tissue may also be an important factor.

The present investigation explored alternative clarification possibilities not examined in the abovementioned reports. These included the use of kaolin, ferric hydroxide gel or neutral calcium glycerophosphate as adsorbants. Strain specific responses were sought in similar tests employing hydrated calcium phosphate (Fulton, 1957) or activated charcoal. In addition, the possibilities

were tested of achieving clarification or of releasing stable, infectious viral components by the use of organic solvents. Throughout the study attention was given to the merits of alternative host tissues as virus source. Briefly, the feasibility of enhancing TSWV yield from tomato roots by the synergistic action of TMV, a conveniently separable virus, was also examined.

Highly favourable results were obtained on applying neutral calcium glycerophosphate clarification to extract of tomato roots systemically infected with TSWV and TMV. This has been briefly reported (Martin, 1964). Recent additional studies on this system are presented in Chapter 2.

MATERIALS AND METHODS

All plants needed for virus propagation, host range and infectivity studies were grown in steam pasteurized soil in a glasshouse maintained at approximately 20 - 23°C. Humidity was not controlled. Limited supplemental fluorescent lighting was provided during winter, ensuring also a minimum 12 h day length.

Tissue homogenization was effected using a mortar and pestle. All inoculations were made mechanically by rubbing the forefinger over plant tissue lightly dusted with 600 mesh carborundum powder.

Unless otherwise stated the electron microscope used was a Philips EM 100.

Selection and Identification of a Netherlands TSWV strain

A pure strain of TSWV was sought that would induce clearly recognizable systemic symptoms in tomato and necrotic local lesions in N. glutinosa; the latter to facilitate assay of virus infectivity. Study of a pure strain rather than a mixture of strains, was recognized as desirable both to promote reproducibility of results and to eliminate strain heterogeneity as a factor complicating interpretation.

The following selection procedure yielded a suitable strain from Campanula isophylla Moretti, a common host of TSWV in the Netherlands (Noordam, 1952):

Inoculation of N. glutinosa from TSWV-infected C. isophylla resulted in a number of scattered chlorotic or necrotic lesions. Six successive clonal transfers of single, necrotic lesions were made on N. glutinosa plants at approximately weekly intervals, after which each clone

was inoculated to Lycopersicon esculentum Mill. var. Potentate. When systemic symptoms appeared, one etch-inducing clone (Best, 1950, 1961) was selected for inoculation at limit dilution to N. glutinosa. From one such N. glutinosa plant bearing only a single necrotic lesion, a pure strain of TSWV was established.

This clone, termed Netherlands etch (Net) strain, was subsequently maintained in the TMV-resistant species Tropaeolum majus L. It was also propagated for prolonged periods in L. esculentum var. Moneymaker and for brief periods in N. rustica and N. tabacum var. White Burley.

Thrips transmissibility of this isolate was not tested. However, to confirm its identity as TSWV, in vitro thermal inactivation, longevity and host range studies were performed. For this purpose strained extract of systemically infected N. rustica leaves was diluted with an equal volume of neutral 0,01 M sodium phosphate buffer containing 0,01 M Na_2SO_3. Inactivation was assessed by inoculation to N. glutinosa. The host range tested included Capsicum annum L., Datura stramonium L., Petunia hybrida Hort., Solanum capsicastrum Link, Tropaeolum majus L., Dahlia pinnata Cav., Zinnia elegans Jacq., Sinningia (Gloxinia) hybrida Hort. var. Kaiser Wilhelm, Gomphrena globosa L., Chenopodium amaranticolor Coste & Reyn., Impatiens holstii Engler & Warb., Phaseolus vulgaris L. var. Manteiga and Cucumis sativus L. var. Gele tros.

For comparison the same range of species was inoculated with a Thrips-transmissible TSWV isolate (Milne, 1960) kindly supplied by Dr. T.A. Hill of Wye College, Kent, England

In this thesis this isolate is termed the Wye clone.

Unless otherwise stated, studies reported in this chapter relate to the Net strain of TSWV.

Extraction from Leaves

Virus source and preparatory techniques

Systemically infected leaf tissue of N. rustica, White Burley and tomato required for virus extraction was harvested on the eighth to tenth day after inoculation of the lower leaves. Inoculated leaves of N. rustica and White Burley to be extracted were picked three to four days after inoculation. Leaves were kept moist in a plastic bag at 2-5°C until ground in the appropriate buffer or Na_2SO_3. At most, three days separated harvesting of the leaves and their extraction. Homogenization and all subsequent steps involving clarification or purification were performed in a cold room at about 2°C. Unless otherwise stated, a Servall SS-34 rotor was used for low or high speed centrifugations.

Gradients were prepared by layering followed by storage for 24 h in the coldroom. Rate zonal density gradients comprised 0,01 M Na_2SO_3 containing 100, 200, 300 or 400 g sucrose/l in the proportions 1,4, 2,8, 2,8 and 1,4 ml in Spinco SW 39 rotor tubes or 7, 7, 7 and 4 ml in SW 25 rotor tubes. Equilibrium zonal density gradients comprised 7, 7, 7 and 4 ml layers, respectively, of 300, 400, 500 and 600 g sucrose/l 0,01M Na_2SO_3. After centrifugation visible zones were removed by means of a 5 ml syringe having the needle tip bent at a right angle. It was not possible in all cases to recover precisely the visible zone.

Sucrose gradient material to be shadowed for viewing in the electron microscope was placed on formvar coated grids and subjected to a glycerin wash, as devised by A.E. Vatter (Black et al., 1963). Ten minutes after applying the specimen drop to the grid the latter was inverted and floated for 10 min on 15% glycerin in 0,01 M Na_2SO_3. On removing the mounts from the glycerin the excess liquid was drawn off with filter paper, the preparations allowed to dry overnight at room temperature, shadowed with carbon/platinum at an angle of 30 degrees and examined in the electron microscope.

Infectivity assay

Infectivity levels during extraction were determined by inoculation to one half of each of 3 to 6 young, detached N. glutinosa leaves. Opposite half-leaves were inoculated with a suitable standard, usually the strained, starting homogenate at a low dilution with buffer. Inoculated leaves were briefly rinsed with tap water and placed on moist filter paper in petri dishes. Lesions were counted four days later, after incubation at approximately 20°C under continuous illumination of about 38 000 ergs/sec/cm^2. In most experiments the strained extract diluted with one to three volumes (w/v) of buffer or distilled water, induced 60 - 120 local lesions per N. glutinosa half-leaf.

Net strain extraction

In order to obtain Net strain TSWV particles for comparison with those reported by Black et al. (1952) two extraction experiments following the procedure set out in

Fig. 1, were conducted on systemically infected N. rustica leaves. In this method homogenization is performed in 'solvent 4'. In Experiment 1 the full procedure was applied, including equilibrium zonal centrifugation. Experiment 2 was terminated on conclusion of the rate zonal centrifugation step. Healthy N. rustica material was extracted concurrently in the first study. Initial stages of the procedure were applied also to systemically infected leaf tissue of the tomato variety Moneymaker.

Preliminary tests showed that addition of Tween-80 to the Na_2SO_3 solution used to resuspend the P_1 pellet did not enhance virus recovery. The abovementioned extraction experiments were, therefore, performed omitting the use of Tween-80, as shown in Fig. 1 (see footnote [c]). In 1963 Black et al. reported the same modification.

Studies on clarification

Attempts to improve the level of clarification obtainable by initial low speed centrifugation (Fig. 1) included the use of alternative solvents other than 'solvent 4', adsorbants and organic solvents.

Alternative solvents

Mixed samples of inoculated and systemically infected N. rustica leaf tissue were homogenized in an equivalent volume (w/v) of (i) 'solvent 4', (ii) 0,01 M sodium phosphate buffer, pH 7 containing 0,01 M Na_2SO_3, (iii) 0,16 M Na_2SO_3, (iv) 0,48 M Na_2SO_3 or (v) 0,96 M Na_2SO_3. In each instance the infectivity of the S_1, P_1 and S_2 extracts (Fig. 1) was determined in order to detect virus aggregation or dispersion.

Freezing of infected tissue was investigated as a means of enhancing cell disruption and possibly influencing the aggregation behaviour of TSWV in crude homogenate. Preliminary tests showed that highly infectious homogenates were obtained when systemically infected N. rustica or White Burley tobacco leaves kept for 26 h at -10°C, were partly thawed and macerated in one volume (w/v) of 0,01 M phosphate buffer, pH7 containing 0,01 M Na_2SO_3. A study was therefore performed in which systemically infected N. rustica leaf material frozen for 24 h at -10°C was clarified by low speed centrifugation. At homogenization one volume (w/v) of either 'solvent 4' or 0,96 M Na_2SO_3 was added.

In another study infected unfrozen tissue was homogenized in sucrose-containing phosphate buffer in an attempt to restrict virus sedimentation during early low speed centrifugation. Homogenization of systemically infected N. rustica leaf tissue was performed in two volumes (w/v) of 0,05 M sodium phosphate buffer, pH 7,5 containing 0,01 M Na_2SO_3 and 20% sucrose. The strained homogenate was centrifuged at 3 000 rpm for 15 min and the resultant, pelleted material washed in three volumes of buffer + sulphite + sucrose and recentrifuged. First and second centrifugation supernatants were pooled and inoculated. The final, washed pellets were resuspended in three volumes (w/v) of the complete buffer solution and similarly inoculated.

Adsorbants

Hydrated calcium phosphate (Fulton, 1959), activated

charcoal (Corbett, 1961), kaolin and $Fe(OH)_3$ gel were independently tested as clarifying agents. In the case of activated charcoal the adsorbant was present during grinding of the tissue. In most other studies the tissue was first ground with 1% powdered Na_2SO_3, strained and made up to volume (w/v) with distilled water prior to adding buffer and/or adsorbant. Alternatively, the adsorbant was applied to partially clarified, S_2 extract (Fig. 1) with a view to enhancing clarification. One ml aliquots of extract, each representing 1g tissue, were normally used in the tests. Unless otherwise stated, adsorption was allowed to proceed for 15 min after which time the adsorbant was removed by centrifugation at 3 000 rpm for 10 min. Further details regarding host tissues used and adsorbant applications are given below.

Hydrated calcium phosphate

Hydrated calcium phosphate (HCP) gel was prepared according to the method described by Fulton (1959) and used to clarify White Burley inoculated leaf homogenate diluted with selected phosphate buffers or distilled water. The phosphate buffers tested included the following: 0,025 M, pH 8; 0,0025 M - 0,1 M, pH 7,7. Various ratios of leaf mass : buffer : HCP (ml) were used. In all cases added gel was thoroughly mixed with the diluted extract and then removed by centrifugation. The resultant clarified supernatants were inoculated to determine the level of infectivity retained.

HCP was also applied to partially clarified tomato

S_2 extract (Fig. 1) prepared from systemically infected tissue. Aliquots were diluted with distilled water or Na_2SO_3 solution, after which HCP was added at the rate of 0,26 ml HCP/g starting leaf material:

S_2 diluted with distilled water at 1:3, 1:1 or 1:0,03,

S2 diluted 1:1 with Na_2SO_3 of molarity 0,01, 0,02, 0,04, 0,06, 0,08 or 0,1.

After centrifugation the supernatants were tested for infectivity.

Activated charcoal

Inoculated White Burley leaf tissue was ground as separate 1 g samples in the presence of increasing amounts of activated charcoal (British Drug Houses) to a maximum of 0,03 g charcoal (Table 11). Each homogenate was then diluted with 3 ml distilled water. The pH of such diluted extracts was approximately 6. After gentle stirring for 2 min the charcoal was removed by centrifugation. Supernatants were inoculated to N. glutinosa either directly or after dilution with an equal volume of 0,05 M phosphate buffer, pH 7,5.

Kaolin

A stock suspension of kaolin was prepared as follows: 10g Dixie kaolinite (R.T. van der Bilt, New York) was washed five times in 300 ml volumes of 1 N NaCl by successive suspension, shaking and centrifuging (2 500 rpm for 10 min). It was then washed twice in similar volumes of 10^{-5} N NaOH. The final pellets were resuspended in 10^{-5} N NaOH to yield a 10% kaolin stock suspension.

Kaolin was recovered from the stock suspension by pelleting at 3 000 rpm for one min. Generally, aliquots of 0,15 ml stock suspension, representing 15 mg kaolin, were used. The kaolin pellet was resuspended in 1 ml of 0,1 M sodium citrate / KOH buffer, pH 9,5 and briefly shaken to ensure dispersion of the clay. Prepared plant extract, as indicated below, was then quickly added and, after mild shaking for five sec, centrifuged at 3 000 rpm for 10 min. The supernatant, comprising the clarified plant extract, was tested for infectivity.

The plant extracts to which 1 ml resuspended kaolin was applied were the following:

(i) 1 ml sap of heavily inoculated N. glutinosa leaves: previously diluted with distilled water to yield 1 ml extract per 1 g starting tissue;

(ii) 1 ml partially clarified tomato S_2 extract (Fig. 1) prepared from systemically infected tissue, 1 ml corresponding to 1 g starting tissue;

(iii) 0,5 ml sap, diluted with distilled water as in (i) above, derived from a mixed sample of inoculated and systemically infected N. rustica leaf tissue. At homogenization, 0,5% Na_2SO_3 by weight was added. Clarification of this material required double the amount of kaolin per ml of citrate buffer.

The N. glutinosa leaves were extracted four days after inoculation. Throughout this period the leaves floated on distilled water in petri dishes kept at approximately 20°C under continuous illumination of about 38 000 ergs/sec/cm^2. The tomato and N. rustica

leaves were harvested from the glasshouse nine days after inoculation.

The alternative possibility of selectively eluting virus adsorbed to kaolin was also tested. A similar method was used, with some success, by Nakagawa and Akashi (1954) for extracting influenza virus. Four 1 ml aliquots of strained homogenate of White Burley inoculated leaves were each applied directly to a kaolin pellet derived from 0,6 ml stock suspension. After thorough resuspension, centrifugation at 3 000 rpm for 1 min served to sediment the adsorbed kaolin, which was then resuspended in 1 ml of 10^{-5}, 10^{-4}, 10^{-3} or 10^{-2}N NaOH for 5 min to induce virus elution. Eluates were recovered as supernatants after successive centrifugations at 3 000 and 10 000 rpm for 2 min each and immediately assayed for infectivity on N. glutinosa

Ferric hydroxide

A stock suspension of $Fe(OH)_3$ gel was prepared as follows : concentrated ammonia solution was added dropwise to 60 g $FeCl_3$ dissolved in 300 ml distilled water, until a persisting precipitate formed. Sufficient I N HCl was then introduced to redissolve the precipitate. A further 700 ml distilled water was added and the sol dialysed against running tap water to flocculate the $Fe(OH)_3$. Finally the gel was dialysed against distilled water. Prior to the removal of aliquots for use in clarification the suspension was gently shaken to ensure uniform dispersion of the gel.

Selective adsorption of leaf extract to $Fe(OH)_3$ gel was attempted at approximately pH 6 and pH 9 : (i) at *ca.*pH 6, to clarify crude extract by applying $Fe(OH)_3$ gel pelleted from 8 ml stock suspension per g systemically infected tomato leaf tissue; (ii) at *ca.* pH 9, employing 27 ml $Fe(OH)_3$ stock suspension adjusted to pH 9,7, in order to enhance the clarity of a 12 ml S_2 (Fig. 1) *N. rustica* extract, previously adjusted to pH 9. Amendment of pH was done with 20% ammonia solution. The *N. rustica* extract originated in this latter study from 12 g mixed inoculated and systemically infected leaves. Residual virus levels after clarification were tested by inoculation to *N. glutinosa*.

Selective elution of virus adsorbed to $Fe(OH)_3$ gel was also attempted, using 0,002 M KOH. Initial virus adsorption to the gel was effected from crude leaf sap of a mixed sample of inoculated and systemically infected *N. rustica* leaves. Sap and $Fe(OH)_3$ were mixed at the rate of 1 g leaf tissue per 6, 8 or 12 ml uncentrifuged $Fe(OH)_3$ stock suspension, previously adjusted to pH 9,7. The pellets obtained on subsequent centrifugation at 3 500 rpm for 3 min were each resuspended in two volumes of 0,002 M KOH and subjected to low speed centrifugation. All low speed supernatants were inoculated to *N. glutinosa*, to assess the (i) level of virus initially left unadsorbed by the gel and (ii) efficiency of virus elution from the gel.

Organic solvents

Chloroform, n-butanol or ether and carbon tetrachloride were applied to 5 - 8 ml aliquots of buffered extracts of White Burley leaf tissue.

Chloroform extraction (Schneider, 1953) of inoculated leaf tissue was performed in 0,05 M phosphate buffer, pH 8 containing 0,01 M sodium diethylenedithiocarbamate and 0,02 M sodium thioglycolate (Fulton, 1959). The ratio of leaf tissue (g) : buffer (ml) : chloroform (ml) : n-amyl alcohol (ml) ranged between 1,5:15:4:2 and 1,5:15:1,2:0,6. Thorough mixing was achieved by gentle shaking for 5 min after which the suspension was centrifuged at 3 000 rpm for 5 min. The aqueous top layer was removed in each case and centrifuged at 3 000 rpm for 30 min yielding a yellow supernatant which was inoculated to N. glutinosa.

In studies using n-butanol (Tomlinson, Shepherd and Walker, 1959), sap of systemically infected tissue was diluted with four volumes of 0,05 M phosphate buffer, pH 7,8 containing 0,01 M Na_2SO_3 and mixed with cooled (0,5°C) butanol equivalent to 2,5%, 4,75%, 7% and 9% of the final volume. The respective treatments were each mixed by intermittent stirring during a period of 45 min and then centrifuged at 3 300 rpm for 10 min. The resultant aqueous supernatants were dialysed for 90 min against distilled water and inoculated. Certain of the corresponding pellets were resuspended in $\frac{1}{8}$th volume of the phosphate buffer + sulphite solution and similarly inoculated.

Extraction using ether and carbon tetrachloride (Wetter, 1960) was performed on sap of systemically infected tissue, diluted 1:7 with 0,01 M phosphate buffer, pH 7 containing 0,01 M Na_2SO_3. The buffered extract was gently shaken with an equal volume of peroxide-free ether for 7 min and then centrifuged at 10 000 rpm for 4 min. The lower, water phase was removed and mixed with an equal volume of carbon tetrachloride. After gentle shaking for 7 min the emulsion was broken as before, yielding an almost clear supernatant. The undiluted supernatant and a 1:5 dilution with phosphate buffer+sulphite solution, were both inoculated for infectivity assay.

Extraction from Tomato Roots

Virus content in roots

The roots of three young Potentate tomato plants, inoculated two weeks previously at four weeks of age, were washed free of soil, dried by blotting and homogenized together in a mortar. The homogenate was strained and centrifuged at 2 500 rpm for 10 min. Serial ten-fold dilutions with distilled water were inoculated to both N. glutinosa and Petunia.

A more detailed examination of the distribution of virus in systemically infected tomato roots was performed in two experiments using the variety Moneymaker. Individual plants were grown, without aeration, in Knop-1865 solution (Hewitt, 1952) contained initially in 500 ml and, later, 1 L erlenmeyer flasks, wrapped in aluminium foil to exclude light. The plants were transferred to fresh

nutrient solution at weekly intervals. Inoculation was performed at 4 - 5 weeks after planting and roots were harvested 12 days later.

In Experiment 1, 18 terminal root portions of 7 cm length were taken from a single plant and divided into three groups of six roots each. The six roots of a group were then segmented identically as follows:

Group 1: four 1-mm segments, 0-1, 1-2, 2-3 and 3-4 mm from the tip,

Group 2: four 2-mm segments, 0-2, 2-4, 4-6 and 6-8 mm from the tip,

Group 3: two 1-cm segments, 0-1 cm and 4-5 cm from the tip.

Within each group the six segments from the same position on different roots, e.g., 0-1 mm, were pooled for homogenization and inoculation. Each set was ground in 0,2 ml 0,01 M Na_2SO_3 and inoculated to four half-leaves of N. glutinosa. The respective dilution factors, based on an ascertained average mass of 0,0043 g / 6 cm root length, were: Group 1 1 : 500, Group 2 1 : 250 and Group 3 1 : 50.

Two plants were used in Experiment 2. They were of a later planting but were otherwise similarly cultured, inoculated and harvested. Root segments, assayed on three half-leaves of N. glutinosa, were prepared as follows:

(i) six roots per plant were cut between 0 - 8 mm from the tip to yield four consecutive 2-mm segments each. Corresponding segments from different roots were pooled as before and ground in 0,2 ml 0,01 M Na_2SO_3, the dilution

factor being approximately 1 : 125.

(ii) three roots per plant were each cut between 0 - 4 cm from the tip to yield four consecutive 1-cm segments. Each set of segments was diluted with 0,5 ml 0,01 M Na_2SO_3 to maintain the dilution factor at approximately 1 : 125.

In each experiment the N. glutinosa half-leaves serving as control were inoculated with extract prepared from the remaining, unused roots, at a dilution of 1 : 20 with 0,01 M Na_2SO_3. An 'average control half-leaf count' was determined for each experiment, to serve as the standard for correcting test half-leaf totals on a uniform basis.

Virus source and preparatory techniques

Young plants of the tomato variety Moneymaker served as the virus source in all root extraction studies reported in this chapter. At approximately four weeks old, concentrated TSWV inoculum was applied to all leaves and the stems. For this purpose, top leaves of tomato plants developing acute etch symptoms were homogenized in a limited volume of 0,01 M Na_2SO_3. In two experiments the tomato source plants were simultaneously inoculated with TSWV and TMV. The TSWV inoculum was supplemented in these instances with TMV to a final concentration of approximately 0,05 mg TMV/ml.

Ten to twelve days after inoculation the stems of infected plants were cut off at 3 cm above soil level and the roots carefully washed in tap water. Excess

water was removed from the cleaned roots by blotting between paper hand towels. After weighing, the roots were kept for not more than two hours in a moist atmosphere at 1 - 5°C.

Homogenization was performed in a mortar in the presence of 1% Na_2SO_3 and small amounts of distilled water and washed sand. The crude homogenate was strained and the volume of the resultant extract made equivalent to the starting weight of root tissue by addition of distilled water. This form of root extract, referred to in this thesis as 'standard root extract', served as the starting material for certain of the initial clarification studies with adsorbants and for the subsequent virus purification studies.

Unless otherwise stated, centrifugation was performed using a Servall SS-34 rotor. Preparation of rate zonal sucrose gradients and of specimen material from gradients for shadowing and electron microscopy were as described for leaf extraction studies. In one experiment negative staining was also performed after glycerin wash. The 1% phosphotungstic acid staining solution used contained 0,03% bovine serum albumin but was not neutralized. Viewing was performed immediately after staining in a Siemens Elmiskop I electron microscope.

Infectivity assay

Infectivity assay was performed by inoculation to N. glutinosa as described under leaf extractions. In the second of the two studies in which TMV was co-inoculated, detached leaves of Petunia hybrida var.

nana compacta 'Celestial dieprose' were also used for assay.

Studies on clarification and purification

Adsorbants

The response of virus in tomato roots to clarifying treatment with HCP, activated charcoal, $FE(OH)_3$ and calcium glycerophosphate was examined. Kaolin was not tested. Unless otherwise stated, added adsorbants were removed after 15 min by low speed centrifugation at 3 - 5 000 rpm for 10 - 5 min and the resultant supernants inoculated to N. glutinosa for infectivity assay.

To make provision for possible major, strain-specific differences in virus response certain of the tests, as indicated below, were performed on roots infected with the Wye clone.

Hydrated calcium phosphate

Ratios of 1 g tomato root tissue : 0,8 ml phosphate buffer : 0,45, 0,9 and 1,2 ml HCP (Fulton, 1959) were tested using phosphate buffers of 0,01 and 0,05 M, pH 7 and 0,06, 0,4, 0,6 and 0,8 M, pH 8. In a test on the Wye clone, phosphate buffers of 0,0005 M and 0,05 M, pH 7 were used, at ratios of 1 : 0,8 : 0,9 or 1,2.

Activated charcoal

Aliquots of root homogenate were mixed with activated charcoal (British Drug Houses) at ratios of 0,001 - 0,03 g/g root tissue. After 30 min each sample was diluted with two volumes of 0,0005 M phosphate buffer, pH 7 + 0,001 M Na_2SO_3 and centrifuged at 2 000 rpm for

1 min.

Ferric hydroxide

Clarification of infected tomato root extract by means of $Fe(OH)_3$ gel was performed in the presence of phosphate buffers, pH 6 and 7, borate buffer pH 9 or KCl solution, as detailed below:

(a) extract containing Net virus, diluted with an equal volume of either 0,05 M phosphate buffer, pH 7 + 0,01 M Na_2SO_3 or 0,2 or 0,05 M KCl + 0,01 M Na_2SO_3;

(b) extract containing Wye virus, diluted with half volumes of either phosphate buffer, pH 6 at 0,05 or 0,005 M, or borate buffer, pH 9 at 0,05 or 0,0005 M.

In (a) above, gel pellets obtained from 0,4, 0,8, 1,2 and 1,6 ml $Fe(OH)_3$ stock suspension were applied per 1 g host tissue. In (b) the gel sedimented from 1,6 ml stock suspension was used per 1 g host tissue, each such gel pellet being first resuspended in 0,5 ml of the respective buffer before applying it to the extract.

Calcium glycerophosphate (Neutral)

Neutral calcium glycerophosphate, hereafter referred to as CGP(N), comprises both the highly soluble α-isomer and the sparingly soluble β-isomer in undetermined ratio. The solubility of CGP(N) (E. Merck, Darmstadt) is about 1 part salt in 40 parts cold water. It has the formula $CH_2OH \cdot CH(OH) \cdot CH_2 \cdot O \cdot PO:O_2$ Ca.

In clarification studies with both Net strain and Wye clone, CGP(N) was applied to root homogenate at levels of 0,005 - 0,24 g / g root tissue, in the presence

of approximately two volumes 0,0005 M phosphate buffer, pH 7 + 0,01 M Na_2SO_3. After standing for 30 min and occasionally stirred, the CGP(N) was removed by low speed centrifugation at approximately 3 700 rpm for 10 min. Further removal or dissolution of residual colloidal CGP(N) was effected, respectively, by additional centrifugation or mild acidification with ascorbic acid, as indicated in the text.

The effect of applying CGP(N) in phosphate buffers of higher molarity was also briefly tested.

Based on results obtained in the abovementioned studies with CGP(N) extensive further studies were performed with this clarifying agent. Experimental details concerning these studies are given with the results.

RESULTS

Virus Isolate

The symptoms induced by the Netherlands etch (Net) isolate in the two principal host species used for differentiating TSWV strains were as follows :

Lycopersicon esculentum var. Potentate: Approximately 12 days after light inoculation of young plants at the 5-leaf stage, the first systemic symptoms appeared in expanding leaflets of the leaf immediately above the highest inoculated leaf. These symptoms comprised localized clearing of veins and adjacent tissue and were followed after a few days by white ring-spot and vein etching (Fig. 5). Subsequent new growth was strongly mottled.

FIGURE 5. Initial systemic ring-spot (R) and vein etch (E) induced by Net strain of TSWV in Potentate tomato

Nicotiana glutinosa: Necrotic local lesions appeared on leaves of half-grown plants three to four days after inoculation and expanded fairly rapidly to form large pigmented, zonate lesions, characteristic for the tomato spotted wilt virus. Subsequent systemic leaf necrosis was briefly severe, after which the new growth showed slight chlorosis. As a rule, flowering was not prevented. In the case of plants inoculated when very young, however, the chlorosis was sufficiently severe to bring growth to a standstill.

The Net strain may therefore be regarded as falling within the broad spectrum of TSWV strains of intermediate severity (Table 2). The symptoms produced on N. tabacum var. White Burley were also typical of intermediate-type strains on tobacco (Norris, 1946; Best and Gallus, 1953; Best, 1961).

Longevity in vitro and thermal inactivation point of the Net strain were characteristic for TSWV. After 24 h at room temperature only one lesion was initiated on six half-leaves of N. glutinosa, whereas 580 lesions were produced by the control extract kept at 1°C. Heating the extract to 46°C for 10 min reduced the total lesion count on six half-leaves from 633 to 36. Infectivity was destroyed completely at 48° C.

The host range agreed essentially with that reported for TSWV (Table 3). A parallel host range study performed on the Wye clone of TSWV, described by Milne (1960) as being of intermediate virulence (Table 2), revealed agreement between the two isolates regarding all hosts listed in Table 3, including Cucumis sativus L.

TABLE 2. Characteristic symptoms of TSWV strains reported by Norris (1946), Best and Gallus (1953) and Best (1901)

SYMPTOMS	Severe				Intermediate						Mild	
On tomato[a]												
Primary: necrosis	+											
Systemic:												
Necrosis: apex, stem	+											
" : leaf	+	+		+					+′			
Yellow blotch: leaf		+	+			+	+′					
Bronzing: leaf				+				+′				
Etch: leaf[b]					+	+′	+′	+′	$+_w$	$+_y$		
Purpling: veins, petiole or stem, or diffuse			+				+	+				
Yellowing: leaf			+					+	+	+		
Mottle: persistent							+	+	+	+	+′	
" : transient											+′	+′
On *N. glutinosa*												
Primary:												
Necrosis	+	+	+	+	+	+	+					
Ring-spot								+	+			
Yellow blotch										+	+	+
Systemic			+	+	+	+	+	+	+	+	+	+
STRAINS												
Natural: Norris (1946)	TB						N	R			M	VM
Best & Gallus (1953)	A	B	D						C_1	C_2		E
Recombinant: Best (1901)				R_1	R_2	R_3						

[a] Symptoms on var. Kondine Red (Norris, 1946) or Dwarf Champion (Best and Gallus, 1953; Best, 1961). Severity rating based on grouping by Best (1953, 1968)

[b] C_1 and C_2 induce white ($+_w$) and yellow ($+_y$) etch symptoms, respectively.

+′ An alternative response, or one not always manifest

TABLE 3. Host range of TSWV Net strain isolated from *Campanula isophylla* Moretti

Host	Local symptoms	Systemic symptoms	Reference
Capsicum annuum L.	+	+	(a)
Datura stramonium L.	+	+	(a,b)
Petunia hybrida Hort.	+	-	(a,b)
Solanum capsicastrum Link	+	+	(a,b)
Tropaeolum majus L.	-	+	(b)
Sinningia (*Gloxinia*) *hybrida* Hort. var. Kaiser Wilhelm	+	+	(c)
Dahlia pinnata Cav.	-	-	(b)
Zinnia elegans Jacq.	-	+	(a)
Impatiens holstii Engler & Warb.	+	+	(d)
Phaseolus vulgaris L. var Manteiga	+	-	(e)
Chenopodium amaranticolor Coste & Reyn.	+	-	(f)
Gomphrena globosa L.	+	+	(f)
Cucumis sativus L. var. Gele tros	+	$+^{t}$	(g)

(a) Smith (1932)

(b) Norris (1946)

(c) Noordam (1943)

(d) de Bruin-Brink, Maas Geesteranus and Noordam (1953)

(e) Costa (1957)

(f) Hollings (1959)

(g) Infection of an unspecified variety by the same virus strain (Ie, 1970, 1971). t = transiently systemic

The distinctive symptoms on C. sativus are described here to provide more detail than is given by Ie (1970, 1971). Four days after inoculation of cotyledons of the variety Gele tros local lesions developed, consisting of a white spot of less than 1 mm diameter, generally surrounded by a feint chlorotic ring (Fig. 6a). During the next three days the necrotic, white centre in some cases expanded to the outer edge of the chlorotic ring, attaining a maximum diameter of approximately 3 mm. There was often no further change in the lesions, although some developed a limited, lightly chlorotic halo about the necrotic spot. In general no systemic spread of the virus took place, but rarely the first true leaf developed a few small spots and occasional halo-spots (Fig. 6b). These were shown by mechanical inoculation to Petunia, to contain TSWV. The second true leaf was without symptoms and was not infective.

Maintained in nasturtium by serial transfer the Net strain proved highly stable over a period of two years.

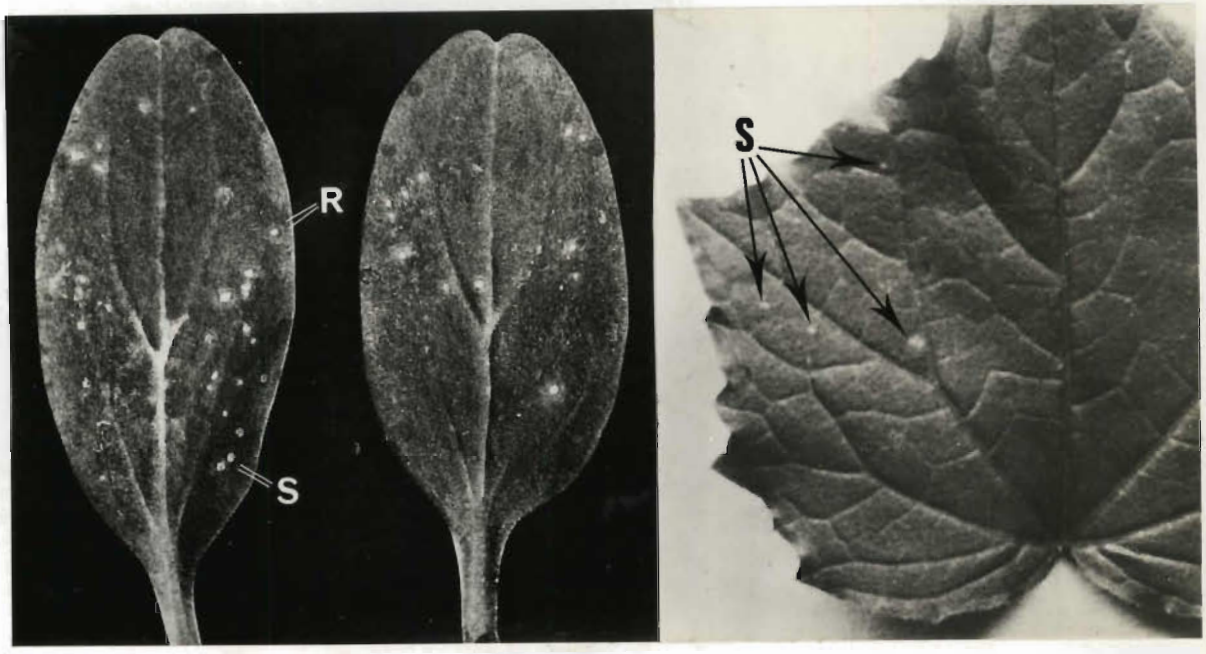


FIGURE 6. Net strain-induced symptoms on Cucumis sativus var. Gele tros

a. Spot (S) and ring-spot (R) lesions on inoculated cotyledons

b. Systemic infection spot lesions on the first true leaf

Extraction from Leaves

Extraction from Nicotiana rustica

Applied to systemically infected N. rustica leaf tissue extract, rate zonal centrifugation according to the procedure developed by Black et al. (1952), at stage Z (Fig. 1) yielded partially purified Net strain TSWV. In both Experiment 1 and 2 a visible zone was clearly discernible in the density gradient centrifuge tube, located 2,2 to 2,4 cm (Experiment 1), or 2,3 to 2,6 cm (Experiment 2), below the meniscus. In a concurrent extraction of healthy tissue no such zone was obtained.

Electron microscopy of material from the visible zone revealed numerous partly flattened particles (Fig. 7) closely resembling those which Black et al (1963) correlated with infectivity. Non-viral material present included ribosomes, Fraction 1, membranous structures, wall fibres and amorphous aggregates. Virus particle diameters ranged from 72 to 120 nm, the majority being between 96 and 112 nm. It was therefore concluded, that within the range 70 nm to 120 nm, approximately, particle size diversity observed in electron micrographs is not due to strain mixture. Infectivity was not associated exclusively with the visible zones (Table 4) and TSWV-type particles were readily discernible also in the adjacent layers.

Diseased and healthy tissue extracts subjected to a final equilibrium zonal centrifugation step in Experiment 1 each produced two visible 2-4 mm zones, at depths of

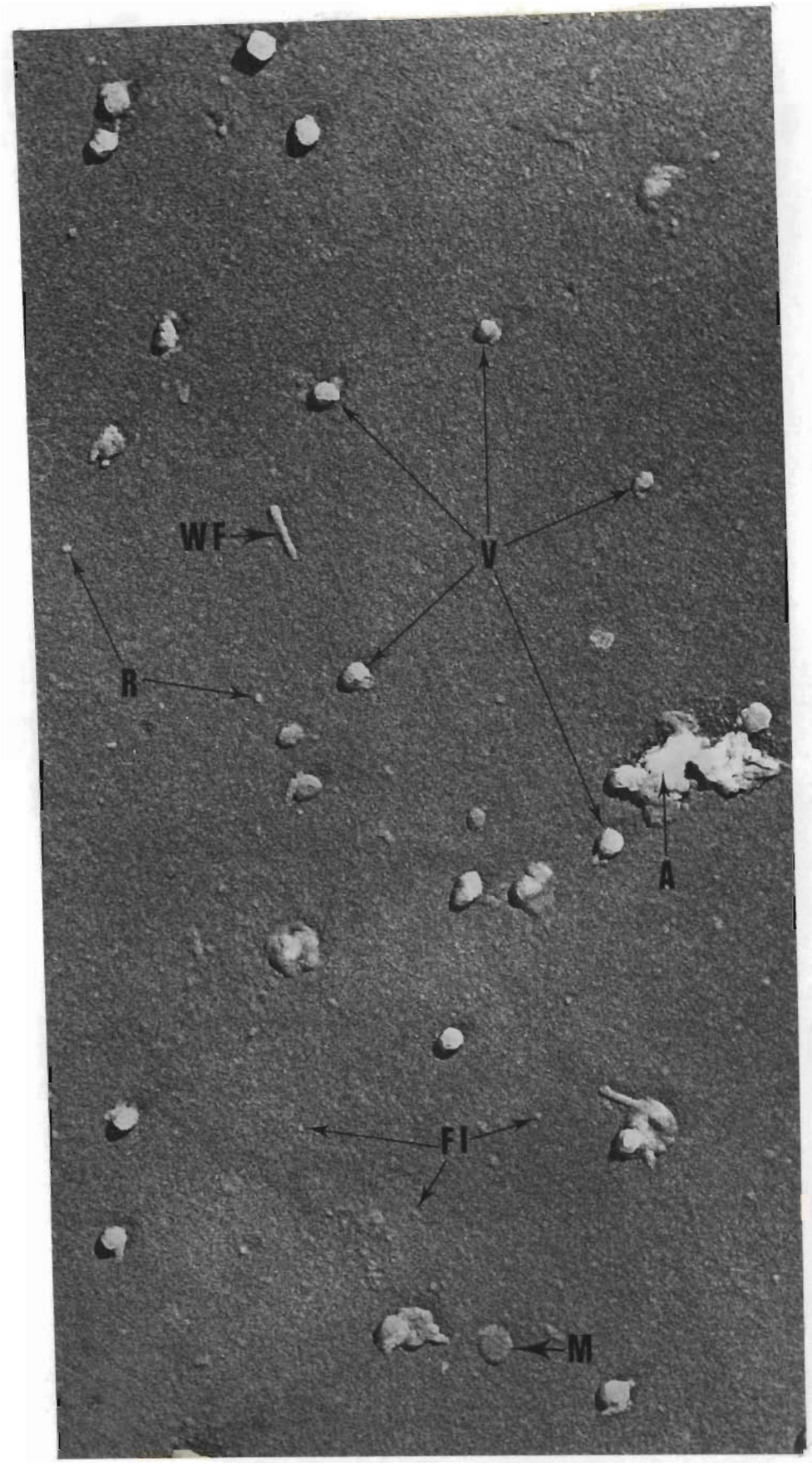


FIGURE 7. Undiluted, glycerin-washed material from the rate zonal gradient visible zone obtained according to the method used by Black et al. (1952) from Nicotiana rustica leaves systemically infected with Net strain of TSWV

V = TSWV-type particle; Fl = Fraction 1; R = ribosome; M = membranous structure; WF = wall fibre; A = amorphous material

TABLE 4. TSWV Net strain infectivity assayed during extraction from systemically infected Nicotiana rustica leaf material homogenized in 'solvent 4'

Extraction step[a]	Lesions as percentage of Control[b]	
	Experiment 1	Experiment 2
S_1 (discarded)	104[c]	
P_2 (")	128	
S_2		153
$S_2 \times \frac{1}{10}$	100	100
S_3 (discarded)	99	35
P_3	105	187
Rate zonal gradient		
0-6 mm above visible zone (discarded)	14	109
Z visible zone, 6 mm	52	243
0-6 mm below visible zone (discarded)	86	164
6-12 mm " (discarded)		103
Equilibrium zonal gradient		
upper visible zone	1	
EZ lower visible zone	67	

[a] See Fig. 1

[b] Control inoculum: S_2 diluted to $\frac{1}{10}$ with 'solvent 4', italicized in the above tabulated results. In Experiments 1 and 2, respectively, this inoculum produced 134 and 74 lesions (av) per N. glutinosa half-leaf.

[c] Within each experiment the volume (ml) of extract at the stages P_2, S_2 and S_3 was constant, approximating the starting weight (g) of tissue (Exp. 1, 25g; Exp.2, 20g). S_1 was twice this volume, P_3 was $\frac{1}{10}$th (Exp. 1) and $\frac{1}{6}$th (Exp. 2). Z Zones were each of 3 ml volume,

approximately 1,5 cm and 2,7 cm. The lower zone from diseased tissue produced 195 lesions on three N. glutinosa leaves and the upper zone only 8 lesions (Table 4), whilst both zones from healthy tissue were non-infectious. In appearance, the infectious zone was wider and less green than the corresponding zone from healthy tissue.

Electron microscopy of material taken from the infectious zone gave unsatisfactory results as the grids, examined after glycerin wash, were almost completely clear and contained no virus particles. Similarly prepared grids of the comparable lower zone material from healthy tissue contained significant amounts of membrane and wall fibre fragments (Fig. 8). In addition, a limited number of Fraction 1 particles were present, together with numerous very small, unidentified particles. There is a striking resemblance between TMV particles and certain of the wall fibre fragments indicated in Fig. 8. However, the preceding clarified P_3 extract was also non-infective and no TMV or other zone was present in the rate zonal sucrose gradient.

The clarification method used by Black et al. (1952) provides for immediate low speed centrifugation of the tissue extract after homogenization in 'solvent 4' (Fig. 1). In order to promote aggregation in the abovementioned two experiments, however, the homogenate in 'solvent 4' was left to stand for either 15 min (Experiment 1) or one h (Experiment 2) before centrifugation. Appreciable virus nevertheless remained in the S_1 supernatant (Table 4) which, according to the procedure being followed,

was discarded. Further losses accompanied the discarding of the P_2 pellet and S_3 supernatant.

FIGURE 8. Equilibrium gradient lower zone material obtained from healthy Nicotiana rustica leaves extracted according to the method used by Black et al. (1952). Fl=Fraction 1; M=membranous structure; A=amorphous material; WF=wall fibre; S=small, unidentified particles. (X 30 000)

Attempted extraction from tomato

An attempt was made to apply the method used by Black et al. (1952) to systemically infected leaf tissue of tomato. Twenty-five g infected young leaf tissue of the tomato variety Moneymaker was used.

The infectivity of the discarded, S_1 supernatant was moderate (Table 5). Redispersion of the P_1 pellet in 0,01 M Na_2SO_3 followed by low speed centrifugation

at 5 000 rpm for 30 min yielded a very dense, dark green supernatant which was, therefore, further centrifuged at 10 000 rpm for 10 min. The resultant, highly infectious S_2 supernatant was still dark green. On rate zonal gradient centrifugation no visible zone developed; instead the gradient appeared progressively greener towards the base. In view of this result the gradient was not fractionated.

TABLE 5. Comparative TSWV infectivity at S_1 and S_2 stages during clarification of systemically infected tomato leaf homogenate

Extraction step	Infectivity [a]
S_1	
$S_1 \times \frac{1}{30}$ [b]	12
S_2	
$S_2 \times \frac{1}{2}$	100

[a] Expressed as a percentage of the $S_2 \times \frac{1}{2}$ infectivity (italicized), which amounted to 161 lesions (av) per N. glutinosa half-leaf

[b] Dilutions made with 0,01 M Na_2SO_3

Clarification

Alternative solvents

Significant loss of virus in the discarded S_1 and P_2 fractions (Table 4) was confirmed in other experiments in which systemically infected N. rustica leaf tissue was homogenized in 'solvent 4'.

When 'solvent 4' or 0,01M potassium phosphate buffer, each containing 0,01M Na_2SO_3, was homogenized with a mixed sample of inoculated and systemically infected N. rustica leaves the S_1 supernatant likewise displayed considerable infectivity (Table 6). Because of the negligible effect on S_1 infectivity produced by a 10-fold dilution in these experiments, it is difficult to assess the significance of the lower S_1 infectivity using 0,01M buffer. The S_2 supernatants were equally green in colour.

Table 7 shows the results of comparable clarification studies in which phosphate buffer was replaced by an equal volume of 0,16, 0,48 or 0,96 M Na_2SO_3 solution. S_2 infectivity was apparently not significantly affected. S_1 infectivity of the 0,16 M Na_2SO_3 treatment showed no response to dilution, whilst at higher salt concentrations infectivity increased on dilution.

Clarification improved with increasing Na_2SO_3 application, the 0,96 M Na_2SO_3 level of treatment yielding a very light green S_2 supernatant. The slightly darker supernatant derived from the 0,48 M Na_2SO_3 treatment approximated those produced by the previous two phosphate buffer treatments (Table 6).

TABLE 6. Comparative TSWV infectivity assayed during clarification of Nicotiana rustica leaf extract in 'solvent 4' [a] or 0,01M potassium phosphate buffer [b], pH7

Clarification step	Infectivity[c]	
	'solvent 4'	0,01M Buffer
S_1 (discarded)	103	44
$S_1 \times \frac{1}{10}$ [d]	91	48
P_1	96	70
S_2	100	90

[a] See Fig. 1. Both leaf tissue samples included inoculated and systemically infected leaves.

[b] Containing 0,01M Na_2SO_3

[c] Expressed as a percentage of the S_2 'solvent 4' infectivity (italicized)

[d] Diluted with 0,005 M Na_2SO_3

TABLE 7. Comparative TSWV infectivity assayed during clarification of Nicotiana rustica leaf extract[a] in Na_2SO_3 solution

Clarification step	Infectivity[b]		
	0,16 M Na_2SO_3	0,48 M Na_2SO_3	0,96 M Na_2SO_3
S_1 (discarded)	62	40	22
$S_1 \times \frac{1}{10}$ [c]	60	87	74
P_1	177	115	107
S_2	86	122	99

[a] See Fig. 1. Each leaf tissue sample comprised inoculated and systemically infected leaves.

[b] Expressed as a percentage of the S_2 'solvent 4' infectivity italicized in Table 6

[c] Diluted with 0,005 M Na_2SO_3

Freezing of infected tissue at -10°C for 24 h prior to homogenization reduced infectivity (Table 8). Frozen and unfrozen tissue homogenized in high molar Na_2SO_3 yielded equally light green S_2 preparations. These were noticeably clearer than the corresponding 'solvent 4' extracts.

TABLE 8. Comparative TSWV infectivity levels during clarification of Nicotiana rustica extract prepared from systemically infected unfrozen or frozen tissue[a]

Clarification step	Infectivity[b]			
	'solvent 4'		0,96 M Na_2SO_3	
	Unfrozen	Frozen	Unfrozen	Frozen
S_1 (discarded)	54	20	6	0
$S_1 \times \frac{1}{10}$[c]	28	16	34	8
S_2	*100*	36	85	58
$S_2 \times \frac{1}{30}$	8	6	9	1

[a] See Fig. 1. Tissue frozen at -10°C for 24 h immediately prior to homogenization

[b] Expressed as a percentage of the 'solvent 4' S_2 infectivity (italicized)

[c] Diluted with 0,005 M Na_2SO_3

A final modification sought to increase retention of virus in the clarified, supernatant fraction by both homogenizing the tissue and washing the first low speed pellets in buffered sucrose solutions. The buffer used for both steps comprised 0,05 M sodium phosphate, pH7,5, 0,01 M Na_2SO_3 and 200g sucrose/L.

The results (Table 9) indicate that sucrose addition did not promote increased virus retention in the pooled low speed supernatants, S_1'. This is seen in the high infectivity of the pellet P_1'.

TABLE 9. Comparative TSWV infectivity assayed during clarification of systemically infected Nicotiana rustica homogenate prepared in 0,05 M sodium phosphate buffer, pH7,5, containing 0,01 M Na_2SO_3 and 20% sucrose

Clarification step	Infectivity[a]
Homogenate	
Homogenate x $\frac{1}{5}$	114
" x $\frac{1}{50}$	63
" x $\frac{1}{500}$	1
S_1'[b]	*100*
P_1'	251

[a] Expressed as a percentage of the S_1' infectivity (italicized)

[b] S_1' volume (ml) approximately 6x the starting weight (g) of tissue; P_1' volume approximately 3x

Adsorbants

Hydrated calcium phosphate

Homogenate of inoculated White Burley leaves clarified by the addition of 0,025 M phosphate buffer, pH 8 and HCP in the proportion 1(g):1,5(ml):0,6(ml) experienced complete loss of virus by adsorption to the HCP. At ratios of 1:3:2 or 1:3:3 no significant improvement was observed when phosphate buffers, pH 7,7 between 0,1 M and 0,0025 M were used, or when distilled water replaced buffer (Table 10). Virus loss tended to diminish slightly when dilute buffer was used.

Distilled water enhanced clarification by HCP. At 1:3:3 ratio the clarified extracts were all light yellow and equally clear whilst at 1:3:2 all, except that prepared with distilled water, were greenish-yellow.

Virus in S_2 (Fig. 1) tomato leaf extract diluted with 0,1 - 0,01 M Na_2SO_3 or distilled water became completely adsorbed on adding HCP at 0,26 ml/g starting leaf material.

Activated charcoal

A comparatively severe reduction in infectivity occurred even at low rates of added activated charcoal (Table 11). Application of an equal volume of 0,05 M phosphate buffer to suspensions treated with 0,003 - 0,01 g charcoal induced chlorophyll aggregation and markedly reduced infectivity.

TABLE 10. Relative infectivity of White Burley inoculated leaf extracts treated with HCP in the presence of 0,1 - 0,0025 M phosphate buffer, pH 7,7 or distilled water

Buffer or distilled water	Infectivity [a]	
	1:3:3 [b]	1:3:2
No HCP		
0,025 M	100	100
With HCP		
0,1 M	1	
0,05	3	6
0,025	2	2
0,0125	3	6
0,005	4	14
0,0025	4	15
Distilled water		12

[a] Expressed as a percentage, in each experiment, of the infectivity (italicized) of the appropriate crude homogenate mixed with three volumes of 0,025 M buffer

[b] Ratio of plant tissue (g) : buffer or distilled water (ml) : HCP (ml)

TABLE 11. Relative infectivity of White Burley inoculated leaf extracts either (i) undiluted or (ii) diluted with 1 volume of 0,05 M phosphate buffer, pH 7,5 after treatment with activated charcoal

Activated charcoal (g/g tissue)	Undiluted		Diluted	
	Infectivity[a]	Clarity	Infectivity[a]	Clarity
Nil	100	dark green	84	dark green
0,001	78	" "	51	" "
0,003	65	lighter green	10	chlorophyll aggregated
0,005	46	"	10	
0,01	25	light green	2	
0,03	4	clear	1	clear

[a] Expressed as a percentage of the infectivity of unclarified, unbuffered extract (italicized)

Kaolin

Washed kaolin freshly resuspended in 0,1 M sodium citrate/KOH buffer, pH 9,5 adsorbed selectively to host material in crude sap. On removing the kaolin by low speed centrifugation the clarified supernatant usually retained a high level of infectivity.

Five such tests were performed on extracts of inoculated N. glutinosa leaves, applying 15 mg kaolin/g tissue. The infectivity levels of the clarified supernatants, expressed as percentages of the respective, untreated extract diluted

1:1 with 0,05 M phosphate buffer, pH7 were 90%, 55%, 100%, 119% and 75%. In the lastmentioned test, reducing the amount of kaolin by $\frac{1}{6}$th or $\frac{1}{3}$rd caused no change in supernatant infectivity. Clarification at the lowest kaolin level was, however, unsatisfactory.

Two further experiments on similar material tested the effect of lowering the pH of the 0,1 M citrate buffer in which the kaolin was resuspended before adding it to sap. In Experiment 1 severe virus loss occurred at all pH levels tested: in Experiment 2 infectivity of the pH 9,5 supernatant was superior (Table 12). Clarification was enhanced at lower pH levels in both studies.

TABLE 12. Relative infectivity of *N. glutinosa* sap clarified with kaolin[a] suspended in 0,1 M citrate buffers of differing pH

Buffer pH	Infectivity[b]	
	Experiment 1	Experiment 2
9,5	11	75
8,5	17	
7,5	41	30
6,5	<10	34
5,8	<10	

[a] 15 mg kaolin/g tissue

[b] Expressed as percentages, in each experiment, of the comparable infectivity of crude extract diluted 1:1 with 0,05 M phosphate buffer, pH 7 containing 0,01 M Na_2SO_3

N. rustica sap prepared from a mixed sample of inoculated and systemically infected leaves homogenized in the presence of 0,5% by weight of Na_2SO_3, displayed high infectivity after clarification by 30 mg kaolin per g tissue. In each of five replications, using aliquots of the same extract, approximately 100% infectivity was displayed by the milky, amber clarified extract after removal of the kaolin.

In a limited, unsuccessful study, kaolin was applied to enhance clarification of a highly infectious S_2 (Fig. 1) tomato leaf extract derived from systemically infected tissue. Citrate buffered kaolin, added at the rate used for clarifying *N. glutinosa* crude sap, rendered the extract water clear and reduced the assayed infectivity from 160 (av) lesions / half leaf to zero.

Attempted elution of TSWV maximally adsorbed to kaolin from homogenate of inoculated White Burley tissue proved unsatisfactory. On resuspending the adsorbed kaolin, only 10^{-2} N NaOH effected significant, but non-selective, release of the virus (Table 13). The original four kaolin-treated extracts were non-infectious, confirming satisfactory initial adsorption of virus by the kaolin.

TABLE 13. Eluates obtained by NaOH washing of kaolin which had been used to adsorb infective White Burley extract

NaOH eluant	Eluate	
	Clarity	Infectivity[a]
10^{-2} N	very green	57
10^{-3}	light green	7
10^{-4}	very light green	0
10^{-5}	" " "	0

[a] Expressed as a percentage of the infectivity of the crude extract diluted 1:1 with 0,05 M phosphate buffer, pH 7 containing 0,01 M Na_2SO_3

Ferric hydroxide

The gel state of the $Fe(OH)_3$ in the stock suspension persisted between pH 6 and 10, approximately, indicating a zero point of charge at about pH 8. According to Summer (1963), below this point the gel becomes predominantly positively charged, and above it, negatively charged. In tests conducted at pH 7, stock suspension gel agglutinated with the negatively charged colloids montmorillonite, Rose Bengal and HCP but not with the basic stain Neutral Red.

Clarification of crude tomato leaf extract at about pH 6 and of N. rustica S_2 extract (Fig. 1) at about pH 9, by the application of 8 ml and 2,25 ml, respectively, of stock suspension $Fe(OH)_3$ per g leaf tissue, was accompanied

by severely reduced infectivity. Likewise, in the preliminary phase of the elution study, application of 8 or 12 ml stock suspension $Fe(OH)_3$ pH 9,7 per g *N. rustica* tissue achieved much virus adsorption (Table 14). Elution of the $Fe(OH)_3$ with two volumes of 0,002 M KOH effected limited release of the virus.

TABLE 14. Relative infectivity of (i) *N. rustica* extracts from mixed inoculated and systemically infected leaf tissue adsorbed with $Fe(OH)_3$ and (ii) 0,002 M KOH eluates derived from the adsorbed $Fe(OH)_3$

$Fe(OH)_3$ stock suspension (ml/g tissue)	Adsorbed extract		Eluate	
	Infectivity[a]	Clarity	Infectivity[a]	Clarity
6	64	dark green	64	orange-brown
8	37	light green	30	yellow-brown
12	23	light pink	33	dark yellow

[a] Expressed as a percentage of the infectivity of the crude extract

Organic solvents

Extracts treated with chloroform or ether and carbon tetrachloride were non-infective. At low levels of chloroform, clarification was more effective in 0,05 M phosphate buffer, pH 8 than in 0,0125 M buffer. However, such improvement was accompanied by greater virus loss.

n-Butanol-treated extracts showed decreasing infectivity with increasing solvent concentration (Table 15). At the 7% butanol level clarification was moderate and virus loss severe.

TABLE 15 Relative infectivity of supernatants and precipitates from low speed centrifugation of n-butanol treated, dialysed White Burley leaf extracts[a]

n-Butanol (%)	Supernatant		Precipitate
	Infectivity[b]	Clarity	Infectivity[b]
0	83	green	154[c]
2,5	83	"	
4,75	60	"	151
7	5	light green	
9	0	clear	0

[a] Centrifuged 3 000 rpm for 10 min; dialysed 90 min against distilled water

[b] Expressed as a percentage of the infectivity of the uncentrifuged, buffered extract

[c] Precipitate infectivity is not to be compared directly with that of the supernatants as the latter were inoculated at greater (8x) dilution.

The inactivating effect of n-butanol was confirmed in other experiments in which dialysis was avoided. The clarified supernatants were, in such studies, either diluted with six volumes of distilled water before inoculation, or centrifuged at 26 000 rpm for 45 min and the resuspended pellets inoculated.

Extraction from Tomato Roots

Virus content in roots

Extract of tomato roots, var. Potentate, harvested 12 days after inoculation of the leaves and stems, caused the following lesion numbers (av)/ test leaf when serially diluted with distilled water:

	N. glutinosa	Petunia
10^{-1}	178	15
10^{-2}	34	6
10^{-3}	2	1
10^{-4}	0	0

Infectivity of this order on N. glutinosa indicated a sufficiently high content of TSWV in tomato roots to warrant the use of such tissue as virus source in purification studies.

The dilution response observed on Petunia supports evidence reported by Milne (1960) of the occurrence in tomato roots of a factor(s) which, at low dilution, inhibits TSWV infection of Petunia.

Inoculation of N. glutinosa with extracts of short segments taken from the terminal 5 cm of systemically infected roots of the variety Moneymaker revealed a high

concentration of virus throughout most of this tissue (Table 16). Experiment 1 yielded evidence of reduced virus content in the 2-mm tip portion.

TABLE 16. Number of lesions / N. glutinosa half-leaf produced by 0,1-, 0,2-and 1-cm segments taken near the root tip of systemically infected tomato roots, var. Moneymaker

Exp. No.	Dilution with 0,01 M Na_2SO_3	Segment distance (cm) from root tip									
		0 - 1							1 - 2	2 - 3	3 - 4	4 - 5
		0-0,1	0,1-0,2	0,2-0,3	0,3-0,4	0,4-0,6	0,6-0,8	0,8-1,0				
1	1 : 500	1	4	13	12							
	1 : 250	2		50		37	48					
	1 : 50	152										129
2	1 : 125	97		106		60	64					
	1 : 125	113							86	79	90	

Clarification

Adsorbants

Hydrated calcium phosphate

In tests with both Net strain and Wye clone infectivity fell to approximately 50% or less on applying 0,9 or 1,2 ml HCP/g tomato root tissue (Table 17).

TABLE 17. Relative infectivity of tomato root extracts treated with HCP in the presence of 0,8 - 0,0005 M phosphate buffer, pH 7 - 8[a]

Virus isolate	Buffer		HCP (ml/g root tissue)		
	Molarity	pH	0,45	0,9	1,2
Net	0,8	8			26[b]
	0,6	"			2
	0,4	"			2
	0,06	"			2
	0,05	7	103	54	
	0,01	"			2
Wye	0,05	"		15	16
	0,0005	"		3	0

[a] The ratio of root mass : buffer was 1 g : 0,8 ml

[b] Expressed as a percentage of the infectivity of the appropriate crude homogenate diluted with 2 volumes (Net strain) or 4 volumes (Wye clone) of 0,01 M phosphate buffer, pH 7 containing 0,01 M Na_2SO_3

At 0,45 ml HCP/g tissue, clarification was limited, whilst at the two higher application rates appreciable clarification was achieved, becoming progressively slightly less, however, with increasing phosphate buffer molarity. When the HCP pellets from the 0,06 - 0,8 M studies were individually restored to volume with 0,8 M

phosphate buffer, pH 8 and recentrifuged, each eluate displayed approximately 43% relative infectivity. The level of clarification was uniform and a little inferior to that of the 0,8 M initial, clarified extract displaying 26% relative infectivity, as shown in Table 17.

Activated charcoal

Tomato root extracts treated with activated charcoal at 0,02 and 0,03 g / g tissue displayed little or no infectivity (Table 18). Clarification was satisfactory at and above the 0,02 g charcoal level.

TABLE 18. Relative infectivity of tomato root extract after treatment with activated charcoal

Activated charcoal (g/g tissue)	Infectivity[a]
0,001	100
0,005	100
0,02	5
0,03	0

[a] Expressed as a percentage of the infectivity of the untreated root extract similarly diluted

Ferric hydroxide

At all levels of $Fe(OH)_3$ gel applied to Net strain-containing root extract in an equal volume of phosphate buffer pH 7 or KCl solution, virus loss by adsorption to the gel was severe (Table 19). Effective clarification of root extract was achieved using gel sedimented from 1,2 or 1,6 ml stock suspension.

TABLE 19. Relative infectivity of extracts of tomato roots infected with Net or Wye isolate, assayed after clarifying treatment with $Fe(OH)_3$ gel

Virus isolate	Buffer or salt			$Fe(OH)_3$			
		Molarity	pH	0,4[a]	0,8	1,2	1,6
Net	Phosphate (+ 0,01 M Na_2SO_3)	0,05	7	15[b]	0	0	0
	KCl (+ 0,01 M	0,2	8,8	27	0	0	0
	Na_2SO_3)	0,05	8,9	33	0	0	0
Wye	Phosphate	0,05	6				1
		0,005	"				1
	Borate	0,05	9				31
		0,0005	"				20

[a] Volume of $Fe(OH)_3$ stock suspension the gel from which was used with 1 g root tissue

[b] Infectivity expressed as a percentage of the appropriate crude extract diluted with one volume 0,05 M phosphate buffer, pH7 + 0,01 M Na_2SO_3 (Net strain) or two volumes 0,01 M phosphate buffer, pH7,5 + 0,01 M Na_2SO_3 (Wye clone)

In the Wye clone study, therefore, the 1,6 ml level was applied in order to ensure adequate clarification. This was achieved, the final supernatants being very slighly milky and nearly colourless. However, at pH 6 the virus was almost completely eliminated by the gel, whilst at pH 9, using borate, only limited infectivity persisted (Table 19).

Calcium glycerophosphate (Neutral)

Treatment of Net strain-infected root homogenate with a wide range of CGP(N) levels, viz., 0,005-0,24g CGP(N)/g root tissue, in the presence of two volumes of 0,0005 M phosphate buffer, pH 7 + 0,01 M Na_2SO_3, caused no apparent reduction of infectivity. The extracts were assayed after removing the CGP(N) by low speed centrifugation. Appreciable clarification occurred when 0,02g or more CGP(N)/g tissue was used.

Table 20 shows in more detail the high relative infectivity of clarified supernatants obtained after applying 0,08 - 0,24 g CGP(N)/g root tissue in the presence of 0,0005 M phosphate buffer, pH 7. When the pellet from 0,16 g CGP(N)/g tissue, applied in 0,0005 M buffer, was eluted with a similar volume of 0,8 M phosphate buffer, pH 8 the eluate displayed only 32% infectivity (Table 20). Although this latter result may not be interpreted as directly confirming that obtained by supernatant assay, viz., that a high proportion of virus is retained in the clarified supernatant, it also does not contradict it.

Raising the molarity of the phosphate buffer to 0,005 or 0,05, pH 7 during clarification with 0,16 g CGP(N)/g tissue reduced the infectivity of the clarified supernatant to 13 and 8%, respectively (Table 20). At the higher buffer concentration the extract was marginally

TABLE 20. Relative infectivity of tomato root extracts clarified with CGP(N) in phosphate buffer and assayed after low speed centrifugation[a]

CGP(N)g/g root tissue	Phosphate buffer (2,5ml/g tissue)		Infectivity	
	Molarity	pH	Supernatant	Pellet[b]
0,08	0,0005	7	112[c]	
0,16	"	"	107	32
	"	"	133	
	0,005	"	13	
	0,05	"	8	
	0,8	8	55	55
0,24	0,0005	7	136	

[a] 3 500 rpm for 10 min

[b] Pellets restored to volume with 0,8 M phosphate buffer, pH 8, centrifuged at 3 500 rpm for 10 min and the resultant supernatant assayed

[c] Expressed as a percentage of the infectivity of crude extract diluted at the same rate with 0,01 M Na_2SO_3

clearer than those prepared with 0,0005 or 0,005 M buffer, which showed comparable clarification. Infectivity increased to 55% when the phosphate buffer was changed to 0,8 M, pH 8. Eluate of the corresponding pellet, restored to volume with 0,8 M buffer, likewise displayed 55% relative infectivity.

In tests with Wye clone at the higher levels of CGP(N), viz., 0,16-0,24 g/g tissue, similar results were obtained. Applied in two volumes of 0,0005 M phosphate buffer containing 0,01 M Na_2SO_3, satisfactory clarification was achieved without reduction of infectivity.

Additional low and intermediate speed centrifugation of extract treated with 0,08 or 0,16 g CGP(N) in the presence of 0,0005 M phosphate buffer, pH 7 caused only limited reduction of infectivity. Net strain-infected root extract treated at 0,08 g CGP(N) and centrifuged for 10 min at 3 000 rpm and then 15 min each at 5 000 rpm and 10 000 rpm displayed 78% of the starting infectivity. Wye clone studies confirmed this result. In one experiment Wye clone infectivity of approximately 60% was recorded for extract clarified at 0,16 g CGP(N) and centrifuged at 3 500 rpm for 25 min and 10 000 rpm for 3 min. A second experiment yielded 98% infectivity after centrifugation at 4 500 rpm and 10 000 rpm for 10 min each. After further centrifugation of the supernatant at 35 000 rpm for 3 min in a Spinco SW 39 rotor and addition of a half volume of 0,05 M borate, pH 9 infectivity was 106%.

Virus purification

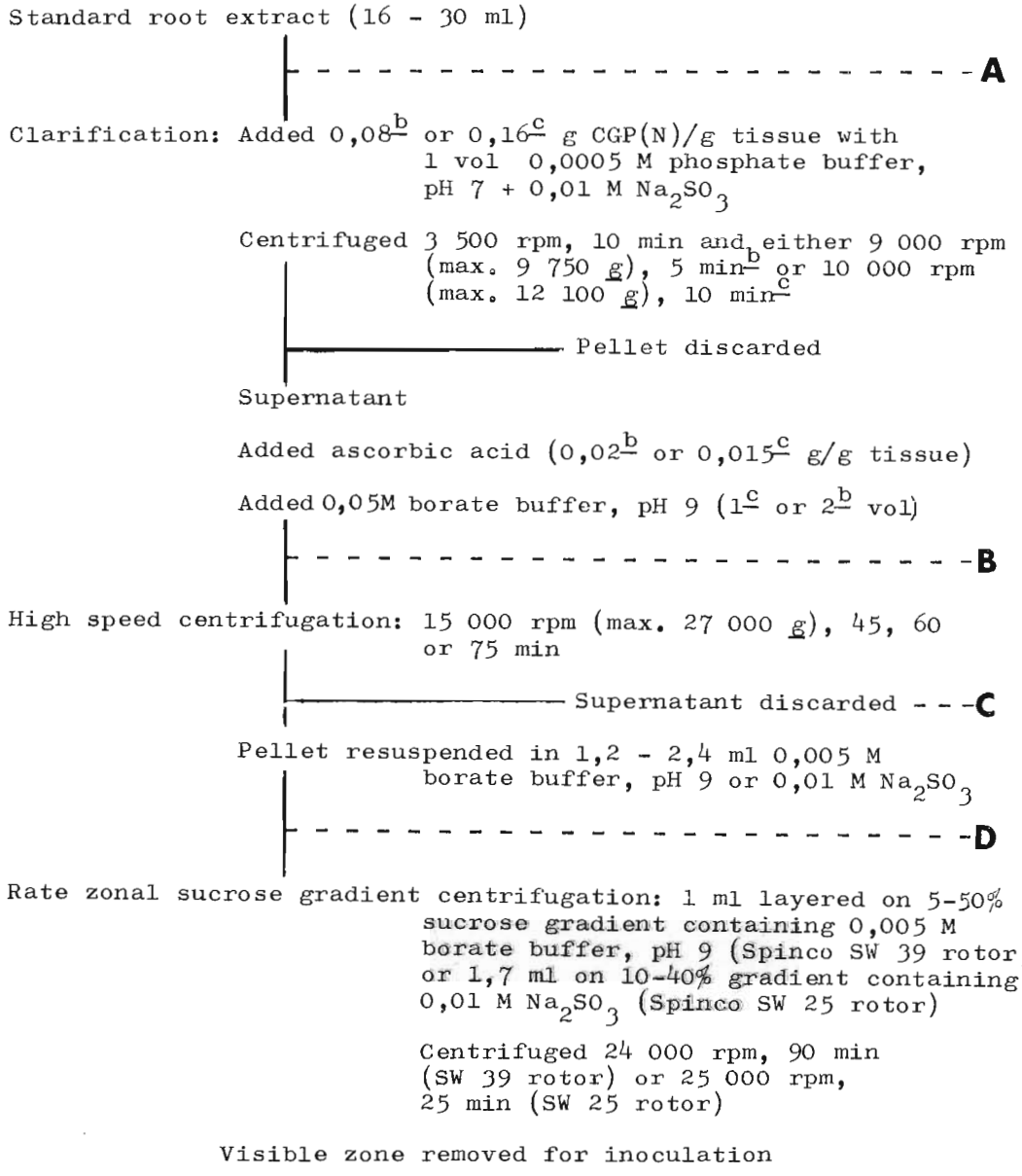
Preparatory extractions

Preliminary purification studies incorporating clarification with CGP(N) were performed on both Net strain and Wye clone of the virus (Fig. 9). In view of the instability of TSWV particles the number of subsequent steps was initially kept to a minimum. Thus only one intervening high speed centrifugation was included preparatory to rate zonal sucrose density gradient centrifugation. The aim of these studies was restricted to determining for both isolates the quality of sucrose gradient separation attainable by such minimal purification and the associated, retained infectivity.

Four such preliminary experiments were performed (Table 21), one on Net strain-infected root material (Exp. N1) and three on roots infected with Wye clone (Exp. W1-3). Infectivity was assayed at two or more of the stages indicated in Fig. 9 as A, B and D. In one instance the infectivity of the discarded high speed supernatant, C, was tested. Concurrently with Experiment W3 healthy tomato roots were also extracted (Exp. H1).

The infectivity of the four starting extracts, shown against A in Table 21, appears comparable, notwithstanding the fact that the root material came from different plant groups grown in succession over a period of a few weeks. No significance can be attached to the apparent correlation between dilution and infectivity, as in previous studies it was found that such limited dilution of crude extract does not predictably alter infectivity.

FIGURE 9. PRELIMINARY PROCEDURES USED IN EXPERIMENTS N1 AND W1 - 3 FOR EXTRACTING NET STRAIN OR WYE CLONE TSWV FROM INFECTED TOMATO ROOTS[a]



[a] See Table 21 for additional details. Infectivity was assayed at selected stages, indicated A - D

[b] Net strain extraction (Exp. N1)

[c] Wye clone extractions (Exp. W1 - 3)

TABLE 21. Root extract infectivity assayed at three stages during preparation for sucrose gradient centrifugation (Fig. 9)

Extraction stage	Net strain : 0.08 g CGP(N)/g tissue[a]		Wye clone : 0,16g CGP(N)/g tissue					
	Exp. N1		Exp. W1			Exp. W2	Exp. W3	
A	94[b]	Standard root extract diluted with 1 vol buffer[c]	45	Standard root extract diluted with 3 vol buffer	53	Standard root extract diluted with 2 vol buffer	103	Standard root extract diluted with 1 vol buffer
B	96	diluted with 2 vol 0,05 M borate buffer[d]	73	diluted with 1 vol 0,05 M borate buffer		(diluted with 1 vol 0,05 M borate buffer)	107	diluted with 1 vol 0,05 M borate buffer
C	13	after 15 000 rpm, 45 min		(15 000 rpm, 75 min)		(15 000 rpm, 60 min)		(15 000 rpm, 45 min)
D	289	in $\frac{1}{20}$ vol 0,005 M borate buffer; 1 ml layered SW 39 gradient	392	in $\frac{1}{15}$ vol 0,005 M borate buffer; not layered	247	in $\frac{1}{15}$ vol 0,005 M borate buffer; 1 ml layered SW 39 gradient	198	in $\frac{1}{8}$ vol 0,01 M Na_2SO_3; 1,7 ml layered SW 25 gradient

[a] starting mass of tissue : Exp. N1, 30g; Exp. W1, 21g; Exp. W2, 19g; Exp. W3, 16g

[b] local lesions/half-leaf, averaged for three N. glutinosa half-leaves; corrected against control within each experiment

[c] 0,01 M phosphate buffer, pH 7 + 0,01 M Na_2SO_3

[d] boric acid-borax buffer, pH 9

All three Wye clone extracts at stage B were highly clarified, displaying only a trace of milkiness when viewed in transmitted light. The pH of these extracts was approximately 7,2, having been briefly 5,8 before addition of the borate buffer. By comparison the Net strain B extract was slightly less clear, possibly due to the milder clarifying centrifugation (Fig. 9). A larger amount of ascorbic acid was applied to this extract (Fig. 9), the pH falling to 5,6, after which a double volume of borate buffer was quickly introduced to return the pH to above neutrality.

In all experiments extracts at stage D (Table 21) were lightly milky in transmitted light. Those of W1 and W2, both concentrated 15-fold with respect to the standard root extract, were similar in appearance. N1, which had been concentrated 20-fold was comparatively denser whilst W3, concentrated only 8-fold was clearer. The higher infectivity of the W1 final extract compared with that of W2 may be due in part to prolonging the 15 000 rpm centrifugation to 75 min. However, in both N1 and W3 studies appreciable virus was recovered by 45 min centrifugation. Similarly, assay of the discarded N1 supernatant, C, revealed that a comparatively limited proportion of virus remained unsedimented after 45 min.

Rate zonal centrifugation of the clarified N1 and W2 extracts using a Spinco SW 39 rotor at 29 000 rpm for 90 min did not produce distinct virus zones (Table 22).

Instead, lying between 1 and 3 cm below the meniscus in both gradients there occurred a broad, milky band that increased slightly in intensity with depth. Fractions removed from the lower half of the band in each tube showed high infectivity. When the two W2 gradient fractions, made up as 1:9 dilutions with distilled water as used for assay, were dialysed for 4 h against distilled water at 2°C all infectivity disappeared.

In preparation for rate zonal sucrose gradient centrifugation in a Spinco SW 25 rotor the final pellet in Experiment W3 was resuspended in 2 ml 0,01 M Na_2SO_3 (Table 21). Centrifugation at 25 000 rpm for 25 min resulted in a moderately distinct 5 mm-wide light scattering zone at 2,4-2,9 cm below the meniscus (Table 23). This zone was highly infectious, but infectivity was not restricted to it. The gradient containing fractionated healthy root extract (H1) was lightly milky between 2,4 - 3,4 cm.

At this stage the following conclusions were drawn:

(i) CGP(N) applied at 0,16g/g root tissue in 0,0005 M phosphate buffer, pH7 affords a safe and effective means of clarifying tomato root extract containing either Net strain or Wye clone (Table 20; Fig. 9 and Table 21);

(ii) centrifugation at 10 000 rpm (max. 12 100 g) for 10 min to remove undissolved CGP(N), followed by ascorbic acid application (Best, 1939) to dissolve residual

CGP(N) by reducing pH to 5,8, and even 5,6, have no clearly harmful effect on infectivity (Fig. 9 and Table 21 - Stage B):

(iii) gradient overloading was probably a major contributory factor in the unsatisfactory separations encountered in studies N1 and W2 using a Spinco SW 39 rotor and, to a lesser extent, in W3 using a Spinco SW 25 rotor;

(iv) the presence of a slightly milky layer in the H1 gradient tube at the same depth as virus in Exp. W3 (Table 23) indicates that the relatively simple procedure employed is unlikely to yield pure virus;

(v) sufficient infectivity is retained at the termination of the procedure to permit insertion of differential centrifugation prior to sucrose gradient rate zonal centrifugation. The anticipated effect of this would be to reduce both the level of non-virus material in the layered extract and the risk of gradient overloading.

These considerations were taken into account when devising a series of three further experiments that concluded the first phase of this investigation.

TABLE 22. Appearance and infectivity of SW 39 rotor sucrose gradient fractions derived from clarified extract of tomato roots infected with Net (Exp. N1) or Wye (Exp. W2) isolates

Depth below meniscus (cm)	Appearance in tube: N1 and W2	Infectivity[a]			
		N1 gradient		W2 gradient	
		lesions per half-leaf	% D	lesions per half-leaf	% D
0-1,0	clear				
1,0-1,5	light milkiness,				
1,5-2,0	increasing	3	1		
2,0-2,5	with depth	139	47	126	25
2,5-3,0		168	63	105	33
3,0-4,0	brownish, not milky				
pellet	brownish, present in N1 only				

[a] (i) Gradient fractions diluted 1:1 with 0,005 M borate buffer, pH 9 (Exp. N1) or 1:9 with distilled water (Exp. W2) and each assayed on three N. glutinosa half-leaves

(ii) Infectivity of the diluted gradient fractions expressed both directly as local lesions/half-leaf and, after correcting for the control half-leaf count, as percentages of the infectivity of the respective undiluted D extracts used for layering (Table 21)

TABLE 23. Appearance and infectivity of SW 25 rotor sucrose gradient fractions derived from clarified extract of healthy (Exp. H1) or Wye strain-infected (Exp. W3) tomato roots

Depth below meniscus (cm)	H1 gradient	W3 gradient		
	Appearance	Appearance	Infectivity[a]	
			lesions per half-leaf	% D
0-2,0	clear	clear	-	-
2,0-2,4		very slightly milky	35	35
2,4-2,9	slightly milky	milky zone	48	50
2,9-3,4	throughout	very slightly milky	39	28
3,4-3,9	almost clear	almost clear	38	19
3,9-4,4			22	16
4,4-5,8			-	-
pellet	very small	small	-	-

[a] (i) Exp. W3 gradient fractions diluted 1:9 with 0,01 M Na_2SO_3 and each assayed on three N. glutinosa half-leaves

(ii) Infectivity of the diluted gradient fractions expressed both directly as local lesions/half-leaf and, after correction against control half-leaf counts, as percentages of the infectivity of the undiluted D extract used for layering (Table 21)

(iii) - = not tested

Purification studies

The procedure adopted (Fig. 10) provided for initial clarification of the root extract using low speed centrifugation and CGP(N), after which two cycles of differential centrifugation preceded rate zonal sucrose gradient centrifugation. To enhance elimination of CGP(N) the level of ascorbic acid applied was doubled. This was immediately followed by dilution with three volumes of 0,05 M Na_2SO_3 instead of one volume 0,05 M borate buffer.

In these studies the effect of concurrent infection by both Net strain TSWV and TMV on TSWV extractability was also examined. From preliminary observations it was known that dual infection with these viruses initiated moderate to severe necrosis of inoculated leaves at approximately seven days after inoculation, followed rapidly by severe apical necrosis.

In two studies, conducted approximately one month apart, tomato roots were harvested from six-week-old plants systemically infected with both Net strain and TMV as a result of simultaneous inoculation 12 days previously (Table 24, Exp. N2 and N4). During the interval between these two studies a comparable extraction was performed on roots from an identical group of plants inoculated with Net strain alone (Table 24, Exp. N3). The plants experiencing dual infection underwent extensive necrosis of inoculated and systemically infected leaves. The roots of these plants, washed from soil, displayed numerous discrete brown lesions and an overall brownness, apparently

FIGURE 10. PROCEDURE USED IN EXPERIMENTS N2 - 4 FOR EXTRACTING NET STRAIN TSWV FROM TOMATO ROOTS INFECTED WITH THIS VIRUS ALONE OR IN COMBINATION WITH TMV[a]

Standard root extract (11,5 - 31,5 ml)

Clarification: Centrifuged 3 000 rpm, 5 min

- Pellet resuspended in starting volume 0,0005 M phosphate buffer, pH7 and centrifuged 3 000 rpm, 5 min
- Supernatants pooled
- Pellet discarded

Added 0,16 g CGP(N)/g tissue, stood 10 min with occasional stirring

Centrifuged 4 000 rpm (max. 1 980 *g*), 5 min and 10 000 rpm (max. 12 100 *g*), 15 min

- Pellet discarded
- Supernatant

Added ascorbic acid (0,03g/g tissue)

Added 0,05 M Na_2SO_3 (3 vol)

Differential centrifugation 1: 16 000 rpm (max. 30 900 *g*), 45 min

- Supernatant discarded
- Pellet, resuspended in starting volume 0,01 M Na_2SO_3 or 0,01 M Na_2SO_3 + 0,01 M $MgCl_2$[b]

Centrifuged 4 000 rpm, 10 min

- Pellet discarded
- Supernatant

Differential centrifugation 2: high speed centrifugation, pellet resuspension (in 0,8 - 2,5 ml 0,01 M Na_2SO_3[b]), low speed centrifugation

Supernatant

Rate zonal sucrose gradient centrifugation: 1,5 - 2 ml (Spinco SW 25 rotor) or 0,7 ml (SW 39 rotor) layered on 0-40% sucrose gradient containing 0,01 M Na_2SO_3[b]

Centrifuged 25 000 rpm, 30 min (SW 25 rotor) or 24 000 rpm, 90 min (SW 39 rotor)

Visible zone removed for inoculation and electron microscopy

[a] See Table 24 for additional details

[b] 0,01M Na_2SO_3 + 0,01 M $MgCl_2$ used in 'TSWV alone' extraction (Exp. N3)

TABLE 24. Additional details relating Experiments N2 - N4 to the extraction procedure shown in Figure 10.

Detail	TSWV alone	TSWV + TMV	
	Exp. N3	Exp. N2	Exp. N4
Root mass (g)	19,5	31,5	11,5
Final volume of extract (ml)	2,0	3,5	0,8
Concentration factor	9,75	9,0	14,4
Zonal centrifugation:			
Layered extract (ml)	1,5	2,0	0,7
Rotor type	SW 25	SW 25	SW 39
Operation	25 000 rpm 30 min	25 000 rpm 30 min	24 000 rpm 90 min

undergoing collapse. Macroscopically they were significantly poorer in quality than roots infected with Net strain alone, which were considerably less discoloured.

In both extractions from roots infected concurrently with Net strain and TMV, viz., Exp. N2 and N4, the final step of rate zonal sucrose gradient centrifugation (Fig.10) yielded an approximately 1 cm-broad, light-scattering region (Fig.11) well separated from the clearly discernible TMV zone (T_2, T_4).

Excellent recovery of almost pure, infective, TSWV-type particles was achieved in the N2 study. Electron microscopy of material from zone Z_3 (Fig. 11), viewed after 1:10 dilution with 0,01 M Na_2SO_3 and a glycerin wash

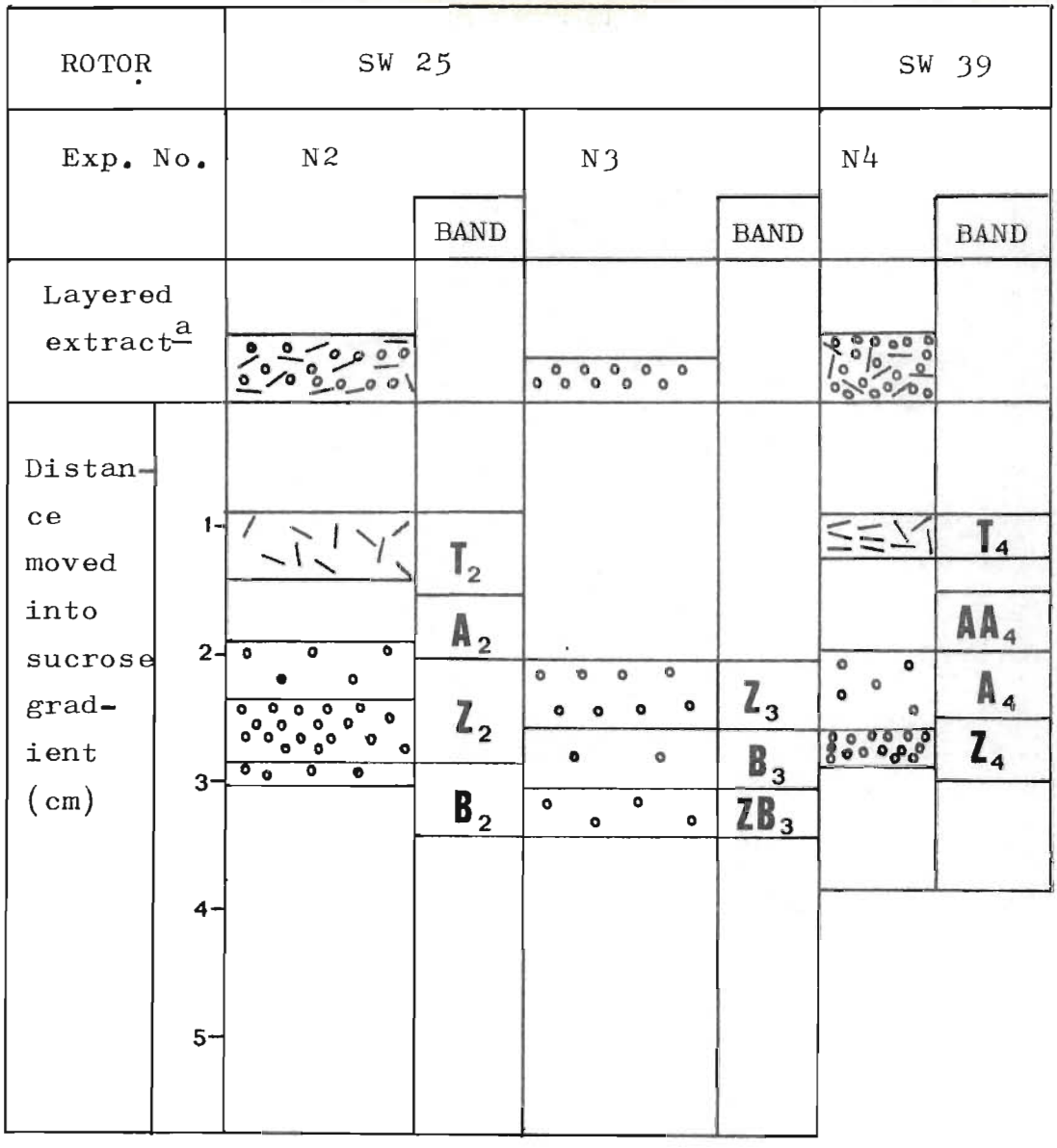


FIGURE 11. Visible bands in gradients layered with differentially centrifuged extract of tomato roots infected with Net strain TSWV, either alone (Exp. N3) or together with TMV (Exp. N2 and N4)

[a] \ \ \ = TMV, °°° = TSWV, indicating both band composition and light-scattering intensity of visible bands.

revealed numerous, comparatively uniform, partially flattened particles (Fig. 12). Evidence was found of a limited amount of contaminating host material (Fig. 13). No structures resembling TSWV-type particles were observed in the TMV zone (Fig. 14). Conversely, when examining the Z_2 zone TMV particles were very rarely encountered.

Measurement of 231 TSWV-type particles derived from this zone and located near the centres of 16 electron micrograph fields, indicated diameters of between about 100 nm (Fig. 15a) and 150 nm (Fig. 15b). Measurements were corrected within each field against polystyrene particles of diameter 264 nm, sprayed on the grids. The range of measured TSWV-type particle diameters showed a reasonably symmetrical unimodal distribution (Fig. 16). Transformed to spheres by a graphical method, the extreme 100 and 150 nm particles approximated 75 - 85 nm diameter.

The two sets of particles shown in Fig. 15a and b were derived almost simultaneously and identically from the same sucrose gradient zone, Z_2. Those seen in (b) apparently underwent greater flattening during air-drying on the grid. Occasionally comparable disparity was encountered also between particles in close proximity (Fig. 15 insets). However, it is estimated that, as spheres, the diameter of each of the two particles shown in the left inset would be approximately 83 nm. Particles recovered from the bands A_2 and B_2 immediately above and below the main zone are shown in Figs. 17a and b, respectively.

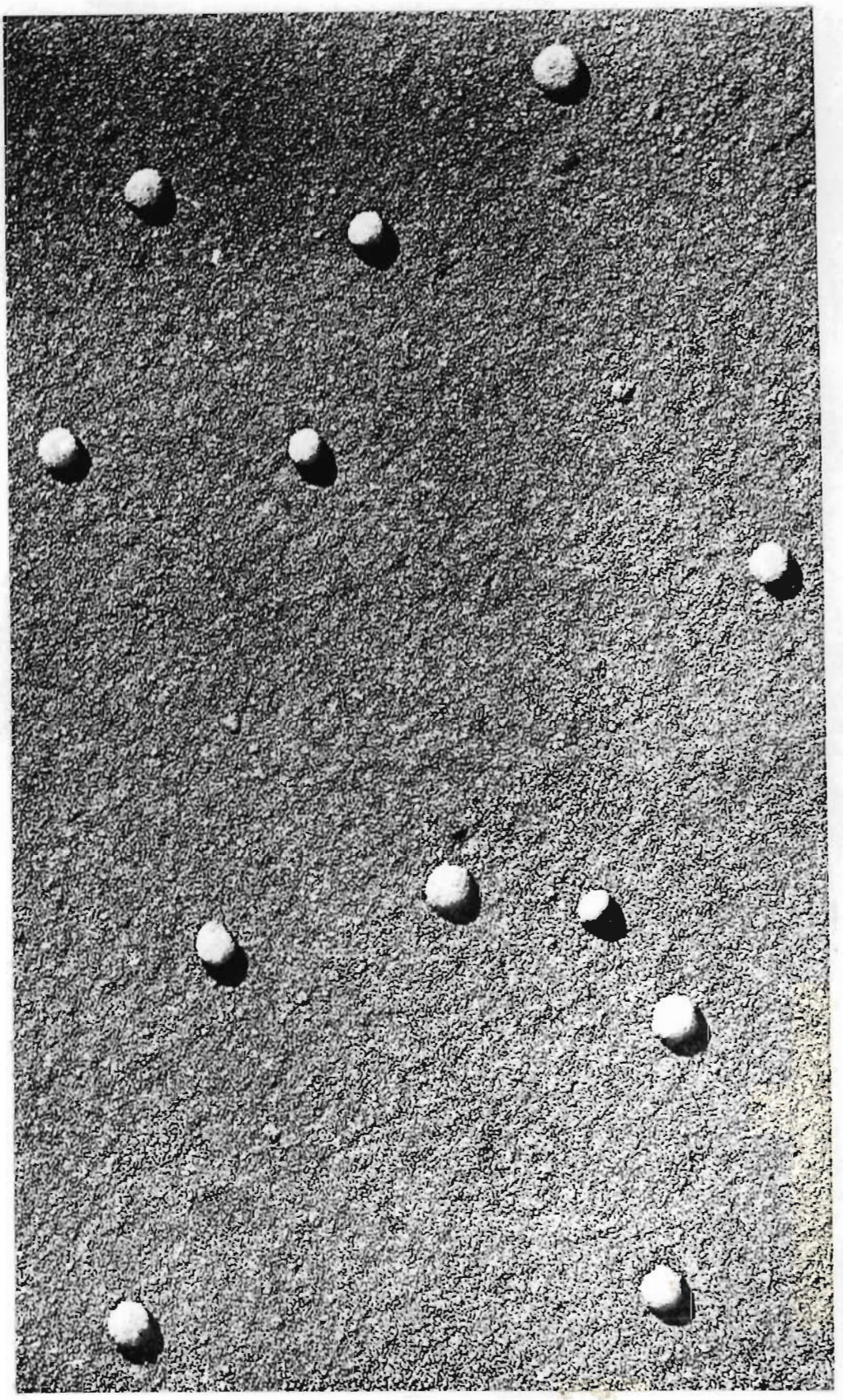


FIGURE 12. Experiment N2: TSWV-type particles present in the visible zone, Z_2 (see Fig. 11), viewed after 1:10 dilution with 0,01 M Na_2SO_3 and glycerin wash. Extraction was performed on tomato roots infected with TSWV and TMV, using CGP (N) clarification followed by differential and rate zonal sucrose gradient centrifugation (X60 000)

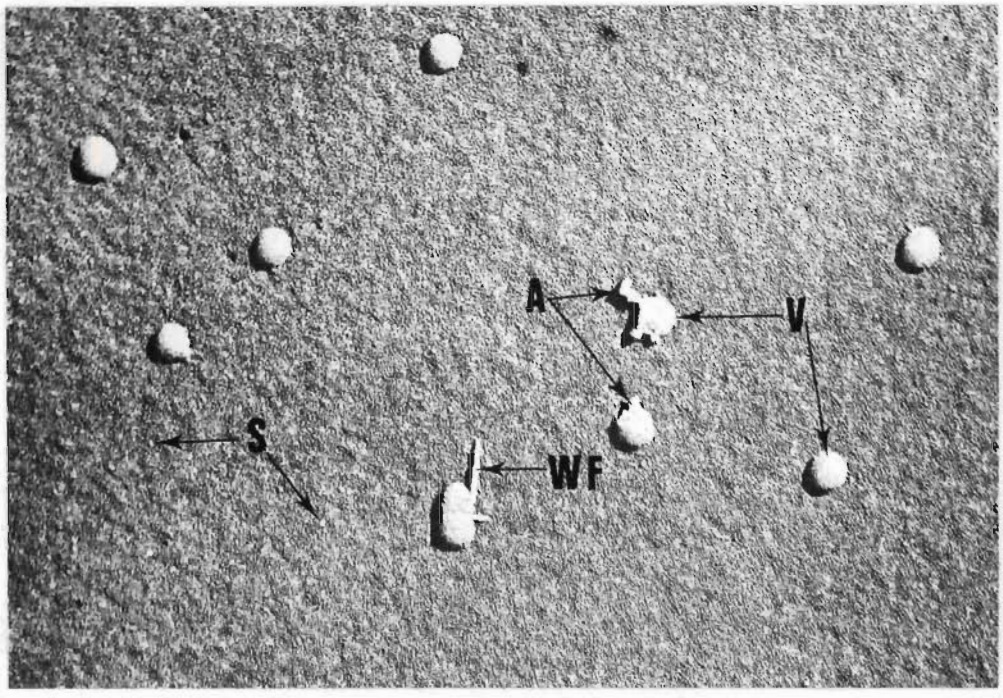
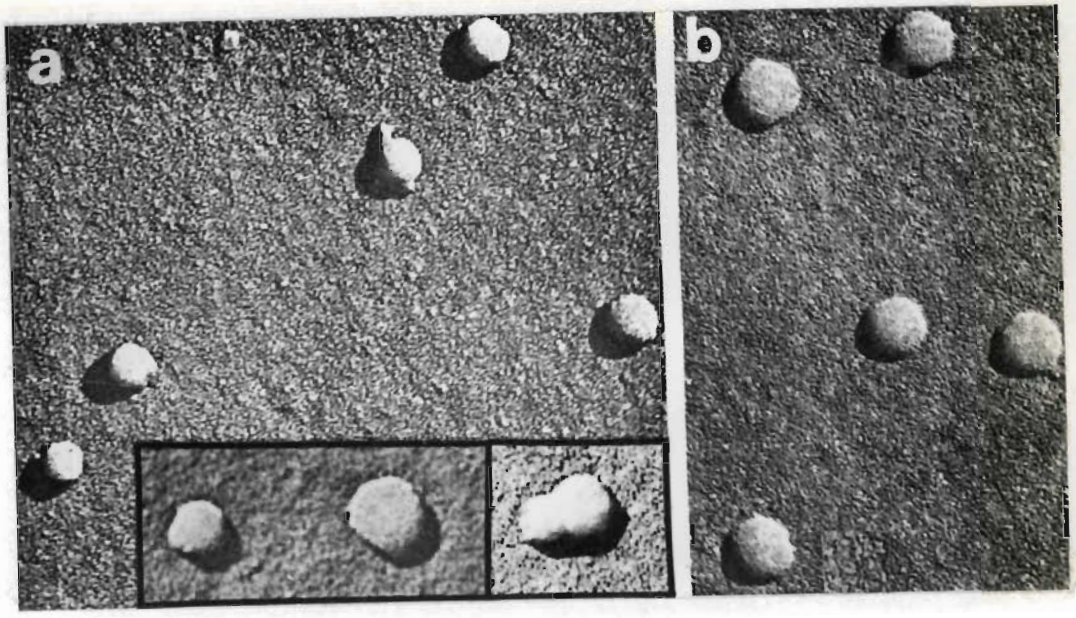


FIGURE 13. Experiment N2: Host material found as contaminant in the virus zone Z_2 (see Fig. 11), prepared as for Fig. 12. V = TSWV-type particle; WF = wall fibre; A = amorphous material; S = spherical particles of uncertain origin. (X 30 000)

FIGURE 14. Experiment N2: Undiluted material from the TMV zone (see Fig. 11). T = TMV. (X30 000)



FIGURES 15a and b. Experiment N2: Two groups of particles on different grids prepared from the Z_2 zone (see Fig. 11).

Insets: Two pairs of TSWV-type particles, each pair illustrating the marked difference in appearance encountered also between particles in close proximity. (X 60 000)

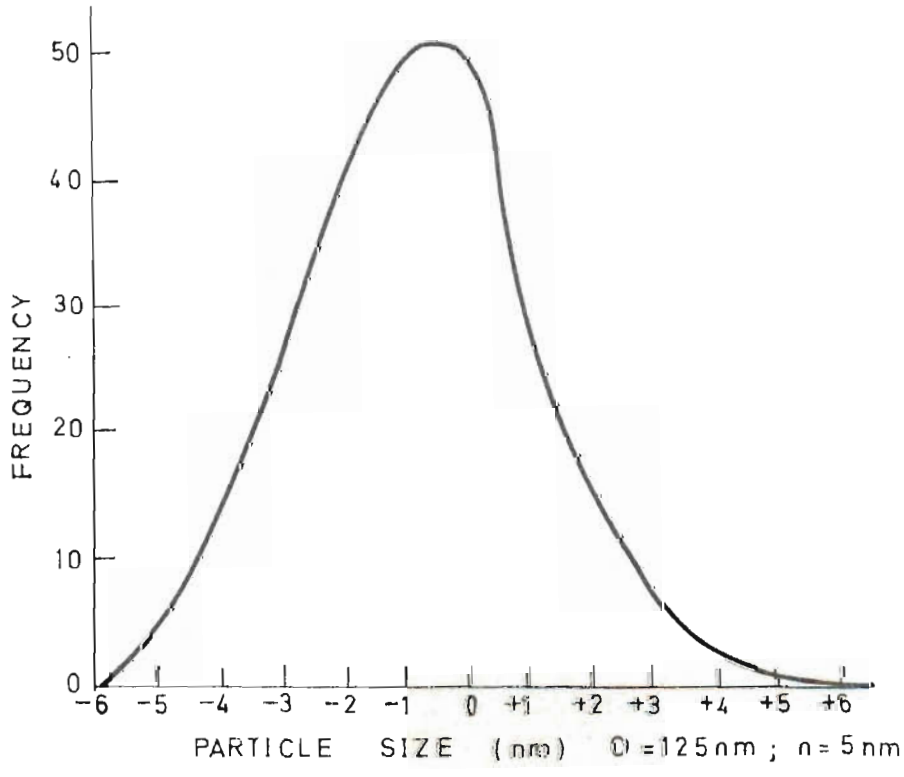


FIGURE 16. Experiment N2: Distribution of 231 TSWV-type particle diameters observed in 16 electron micrograph fields

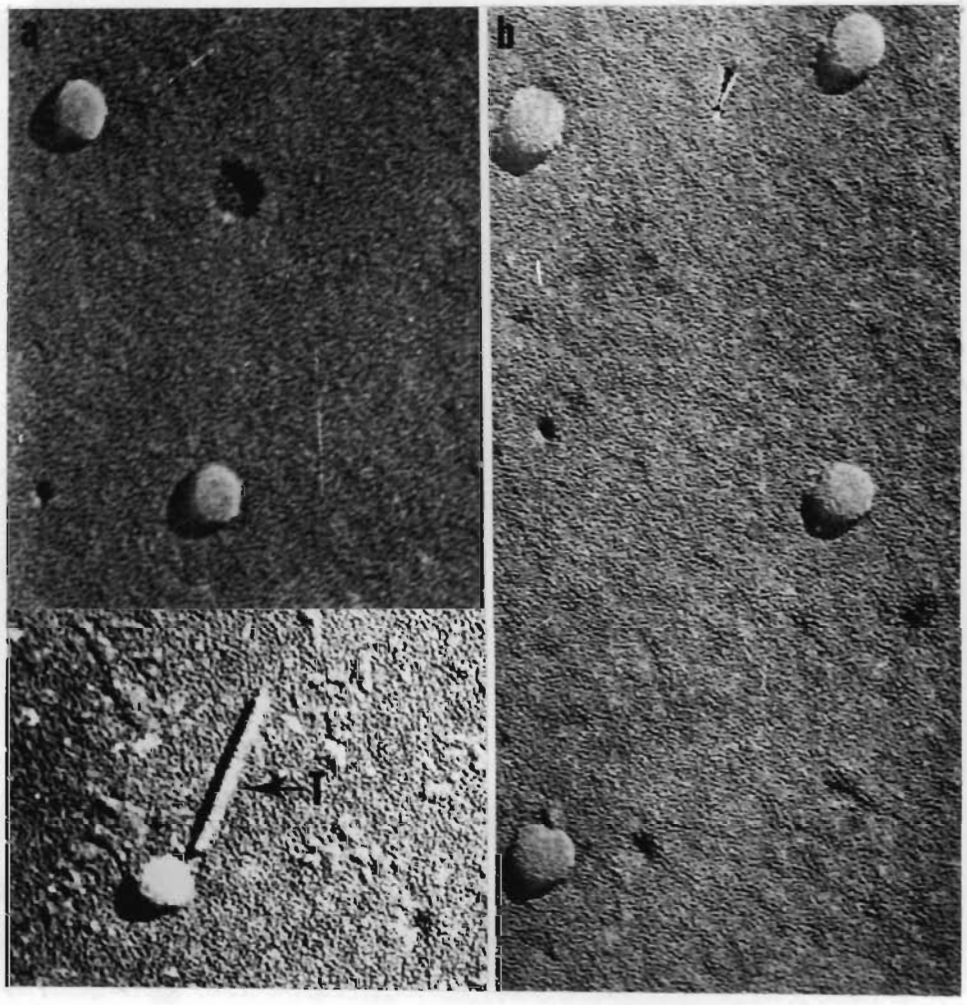
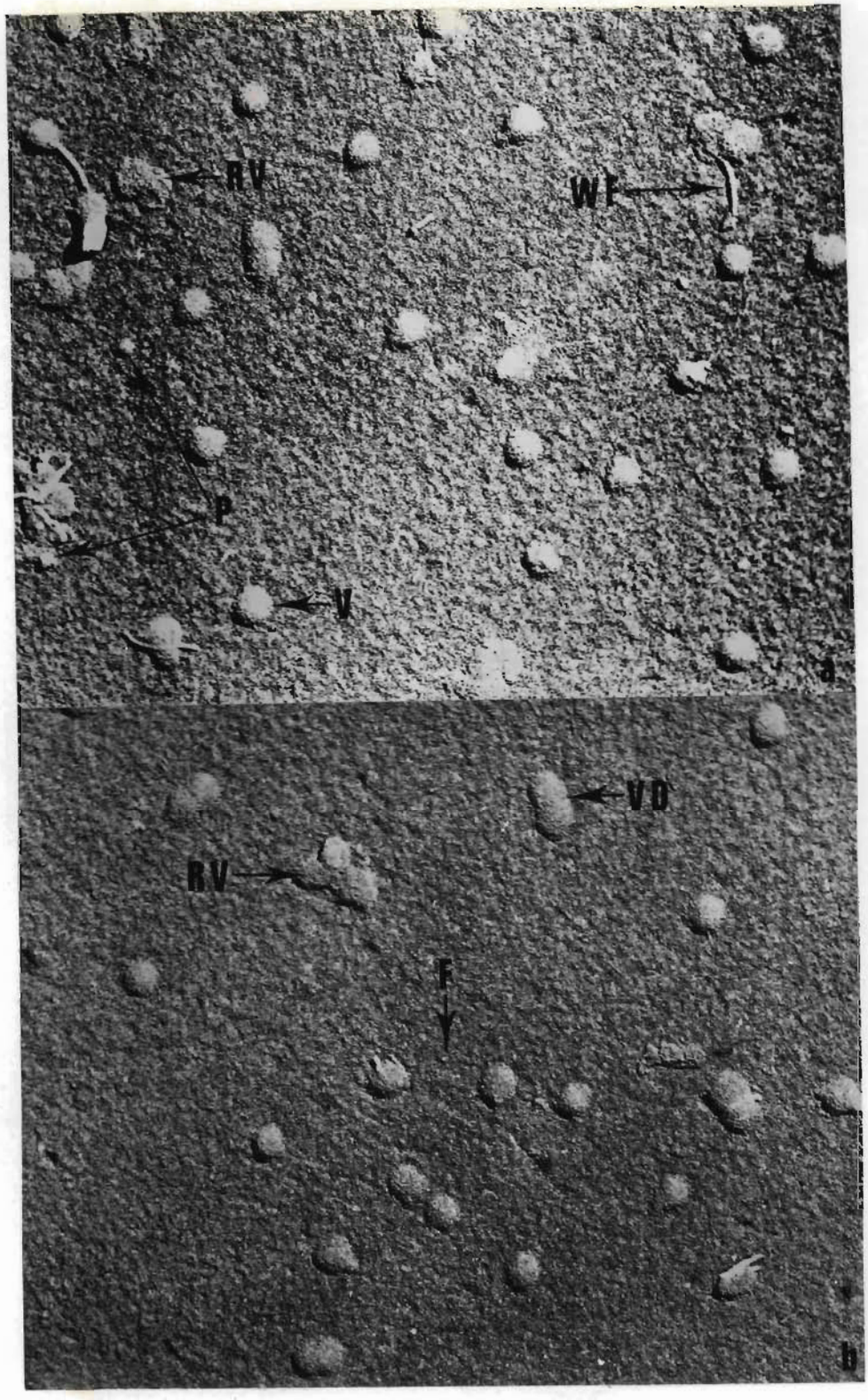


FIGURE 17. Experiment N2: TSWV-type particles from band A_2(a) or B_2(b) (see Fig.11). T = TMV (x 60 000)

The ratio of particle numbers per photographic field for the A_2, Z_2 and B_2 bands at 1:10 dilution was approximately 5:40:10. Inoculation of six N. glutinosa half-leaves with Z_2 and B_2 aliquots diluted 1:10 with 0,01 M Na_2SO_3 resulted, respectively, in 111 and 64 TSWV-type local lesions/half-leaf. The A_2 band was not assessed owing to its proximity to the TMV zone.

In the N4 experiment, employing a Spinco SW 39 rotor for the gradient centrifugation (Fig. 11), all TSWV-type particles observed in material taken from the lower zone, Z_4, showed appreciable flattening. Two fields of this material, after 1:10 dilution and glycerin wash, are presented in Figs. 18a and b. There is limited evidence also of virus rupturing. Undiluted AA_4 material after glycerin wash is shown in Fig. 19 to illustrate the range of particles encountered in the region between the TSWV and TMV light-scattering zones. The Z_4 band contained, in very low concentration, particles of approximately 50 nm diameter not encountered in the earlier extractions.

Viewed at 1:10 dilution the number of TSWV-type particles present per Z_4 field (Figs. 18a and b) was greater than that per Z_2 field (Figs 12 and 13) at equivalent magnification. This was possibly due in part to the use of a smaller gradient (Table 24) and also to the fact that the gradient material removed for electron microscopy was more strictly limited to the brightest light-scattering region. Inoculation of Z_4 material at 1:10 dilution with 0,01 M Na_2SO_3 resulted in 30 lesions/half-leaf on Petunia. The crude extract inoculated to Petunia after similar



FIGURES 18 a and b. Experiment N4: Two fields of material from the same light-scattering TSWV zone, Z4, developed in a Spinco SW 39 gradient from extract of TSWV + TMV-infected roots (see Fig. 11).
V = TSWV-type particle; VD = TSWV dimer; RV = possibly ruptured TSWV-type particle; P = particle approximately 50 nm in diameter; F = FI-type particle; WF = wall fibre (or possible TMV). (X 30 000)

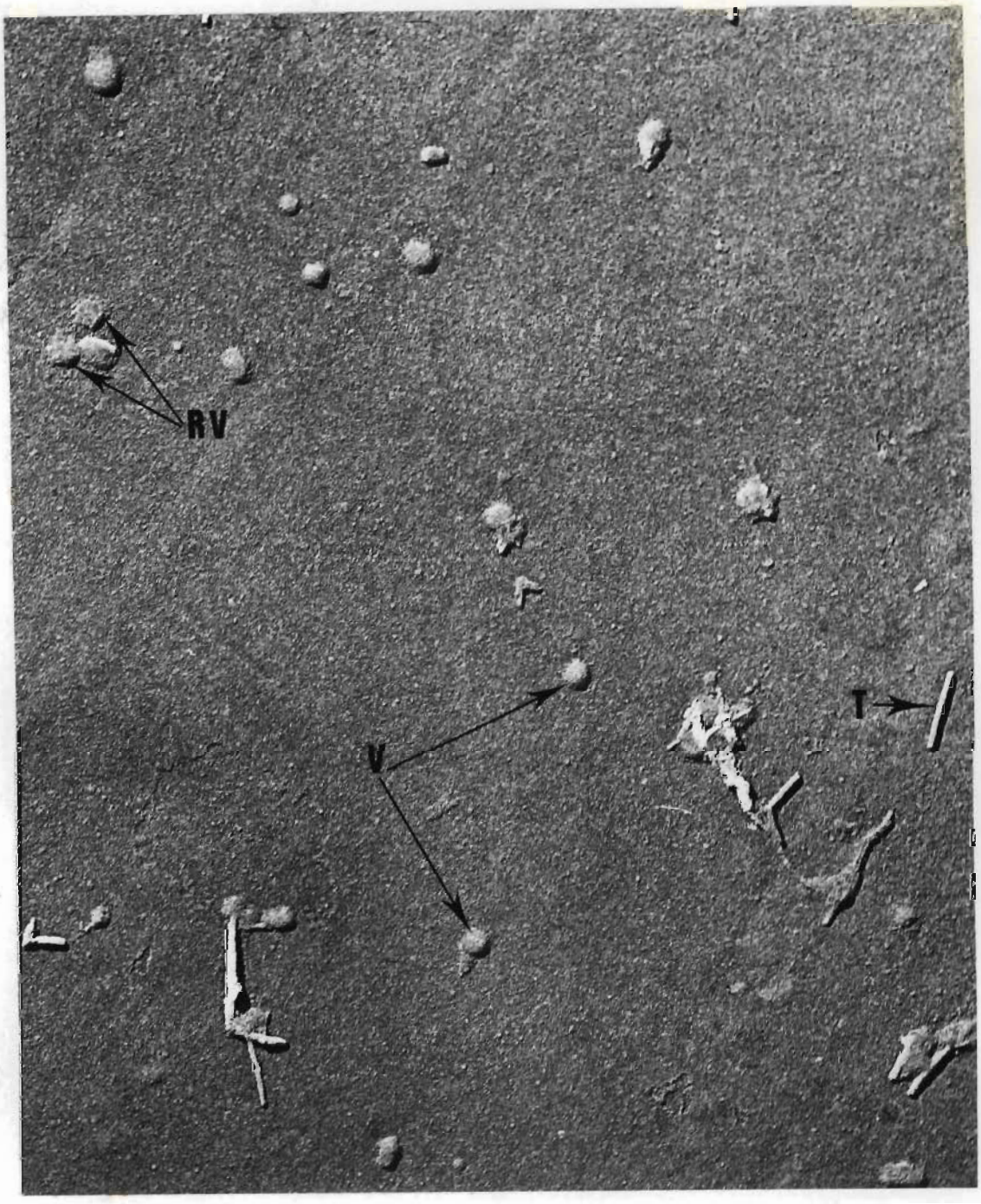


FIGURE 19. Experiment N4: Undiluted, glycerin-washed gradient material taken at AA4 between the TSWV and TMV light scattering zones (see Fig. 11). V = TSWV-type particle; RV = possibly ruptured TSWV-type particles; T = TMV. (X 30 000)

dilution initiated 5 lesions/half-leaf. In comparing these results with Experiment N2 it must be noted that the leaves of Petunia are smaller in size than N. glutinosa, yield characteristically fewer lesions in response to finger-applied TSWV inoculum (Milne, 1960) and are sensitive to the inhibitor(s) present in tomato roots (Milne, 1960).

The result of the final rate zonal centrifugation in Experiment N3, utilizing extract of roots infected with TSWV alone, differed significantly from the results obtained in Experiments N2 and N4. In the lower half of the SW 25 rotor tube two 5 mm zones occurred (Fig. 11, Z_3 and ZB_3), separated by a less dense 5 mm band, B_3. The intensity of light-scattering by the two zones was similar and appreciably less than that of the TSWV zone in either the N2 or N4 study.

Electron microscopy of undiluted material of zone Z_3, viewed after glycerin wash, revealed a heterogeneous mixture of structures. Rounded, TSWV-type particles, approximately 90 to 180 nm in diameter, were present in low numbers (Figs. 20, 21). A small proportion of similar, tailed structures were also present. Spherical particles of approximately 50 nm diameter, resembling those observed in Experiment N4, were again encountered (Fig. 20 and inset), together with numerous membranous structures of uncertain, although possibly viral, origin. Identical membranous structures were also seen in glycerin washed, 1:10 0,01 M Na_2SO_3 dilutions prepared from the same zone (Fig. 22), indicating that they did not arise in the undiluted preparations from virion disruption due to

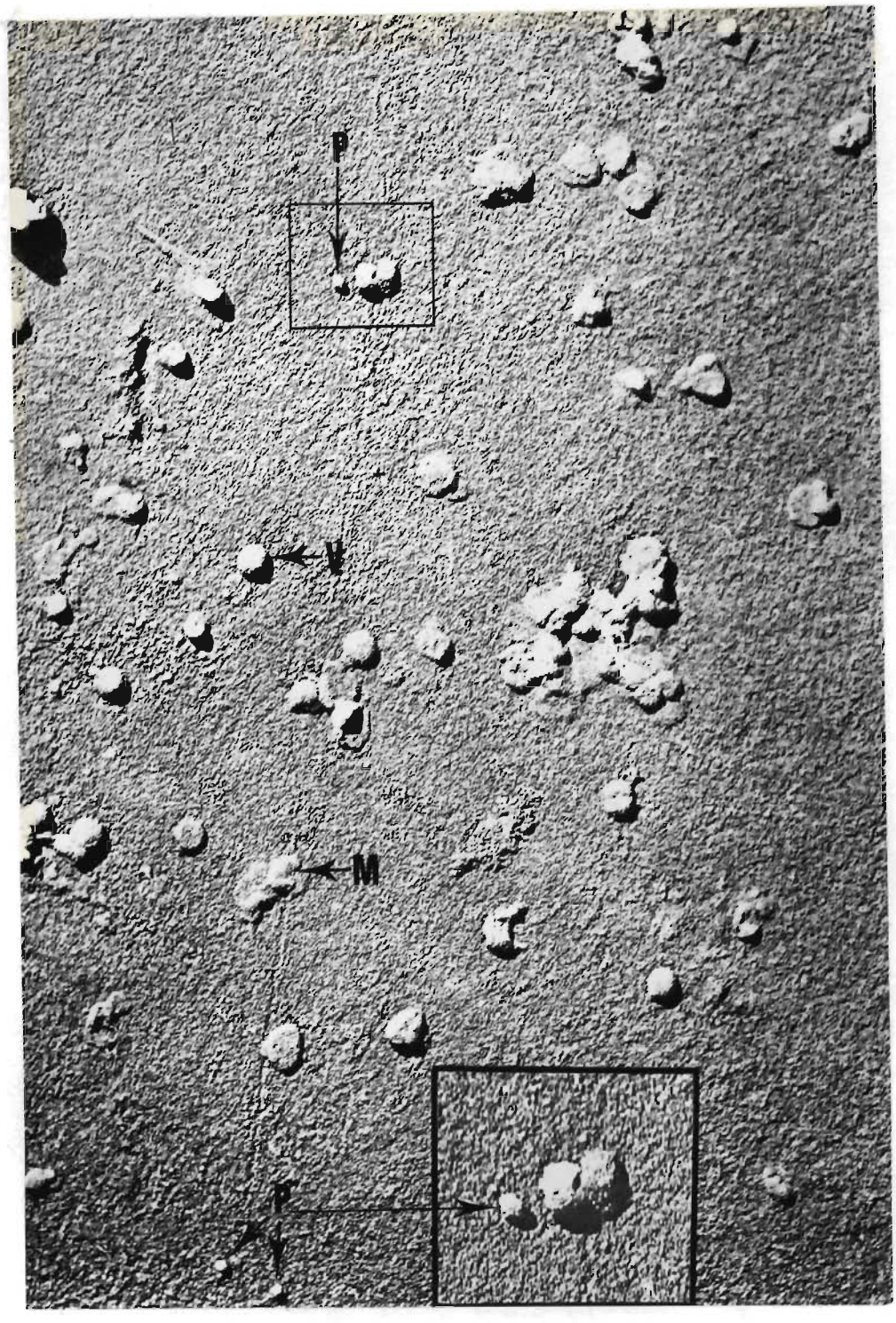


FIGURE 20. Experiment N3: Undiluted, glycerin washed material of the zone Z_3 (see Fig.11). V= TSWV-type particle; M=membranous structure; P=particles approximately 50 nm in diameter. (X 30 000)

Inset: Detail from Fig. 20, showing a ca. 50 nm particle near an almost unflattened, 90 nm, TSWV-type particle. (X 60 000)

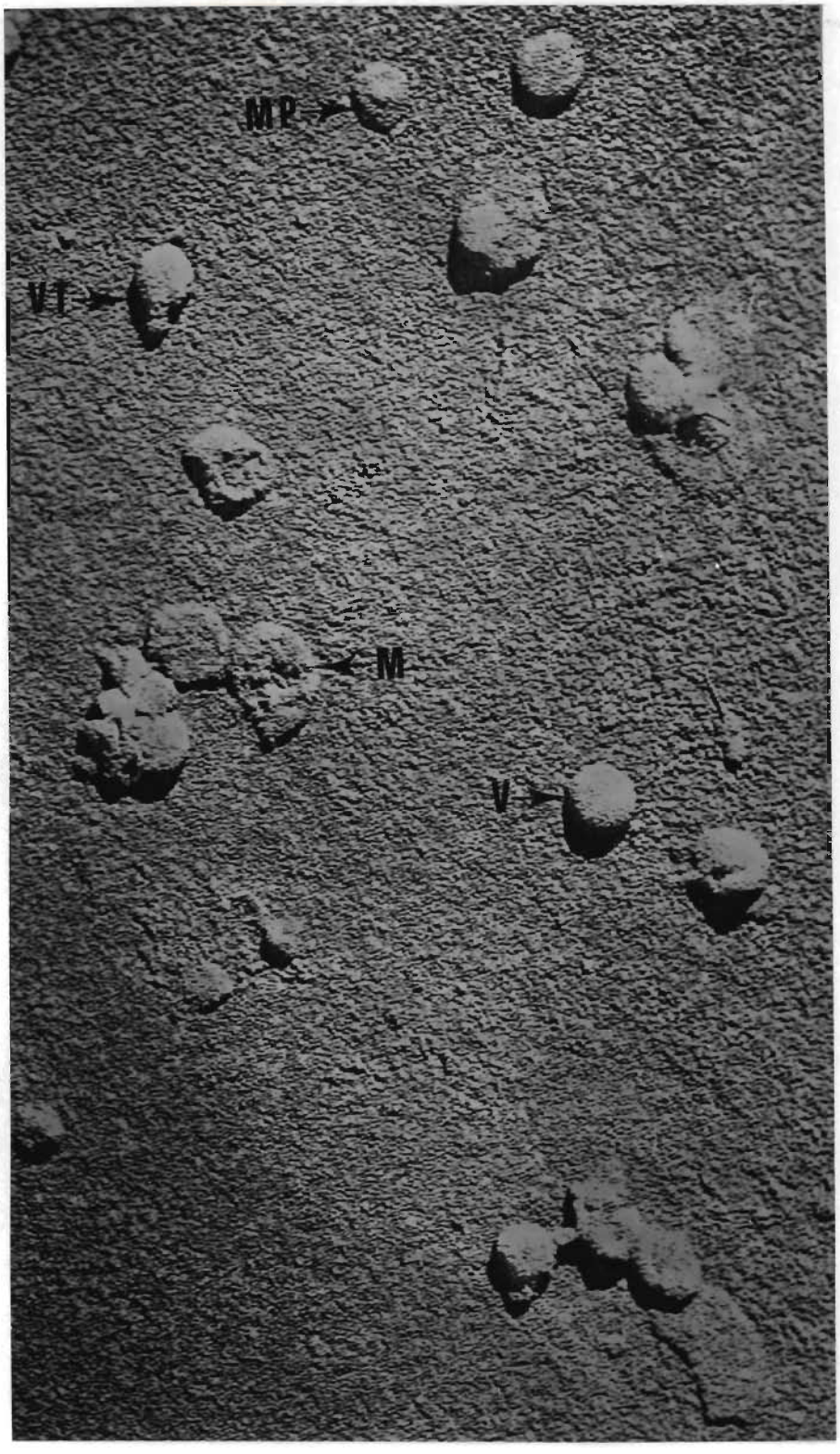


FIGURE 21. Experiment N3: Z_3 material similar to that shown in Fig. 20, at X 60 000. V = TSWV-type particle; VT = tailed TSWV-type particle; M = membranous structures, some of which appear to be derived from TSWV-type particles. One (MP) contains, or overlies, an approximately 50 nm particle.

FIGURE 22. Experiment N3: Membranous structures present in Z_3 gradient material after 1:10 dilution with 0,1 M Na_2SO_3 and glycerin wash (see Fig.11). (X 60 000)

excessive residual sucrose on the grid. Compared in the electron microscope, the bottom zone, ZB_3, was quantitatively poorer than the upper zone but qualitatively indistinguishable (Fig. 23).

Inoculation of N. glutinosa with Z_3, B_3 and ZB_3 material, undiluted, caused only 6, 1 and 0 lesions, respectively. By comparison, the extract used for layering, diluted with an equal volume of 0,01 M Na_2SO_3, initiated 171 lesions/half-leaf. The starting standard root extract, similarly diluted, yielded 185 lesions and, diluted 1:9, 172 lesions/half-leaf.

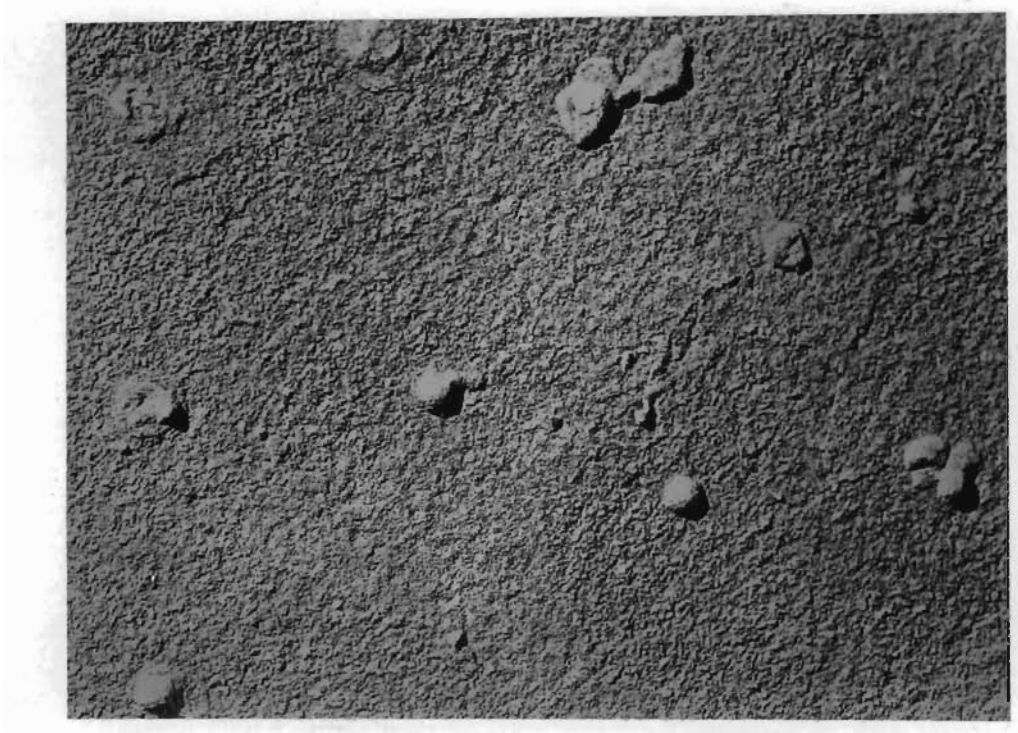


FIGURE 23. Experiment N3: Undiluted, glycerin washed ZB_3 gradient material (see Fig. 11). (X 30 000)

A group of negatively stained TSWV-type particles from the upper zone, A_3, is shown in Fig. 24. Similar particles, in lower concentration and mixed with larger membranous structures, were also present in the lower zone, AB.

Particles 1 and 2, converted to a round outline, would display diameters of approximately 120 - 130 nm. Each contains appreciable internal component, but its detailed structure is not resolvable. A possible manifestation of strand conformation is indicated (arrows).

Of particular interest concerning particle 1 is the contour of the limiting membrane. Where it is in con-

tact with the internal component it tends to bulge slightly outwards. Between such regions the membrane tends to be straight or to indent slightly. Particle 2 displays the same features.

In both these particles, and also particle 3, there appears to be extensive, if not complete continuity of the internal component. Particle 5, the smallest, is approximately 105 - 110 nm in diameter. The integrity of the particle membrane was apparently adequate to preclude stain entry. Particle 4, on the other hand, appears to have retained only two small fragments of the internal component, located at diametrically opposite points of the particle. The material seen at 'x' and 'y' may be fragments of internal component released from virions.

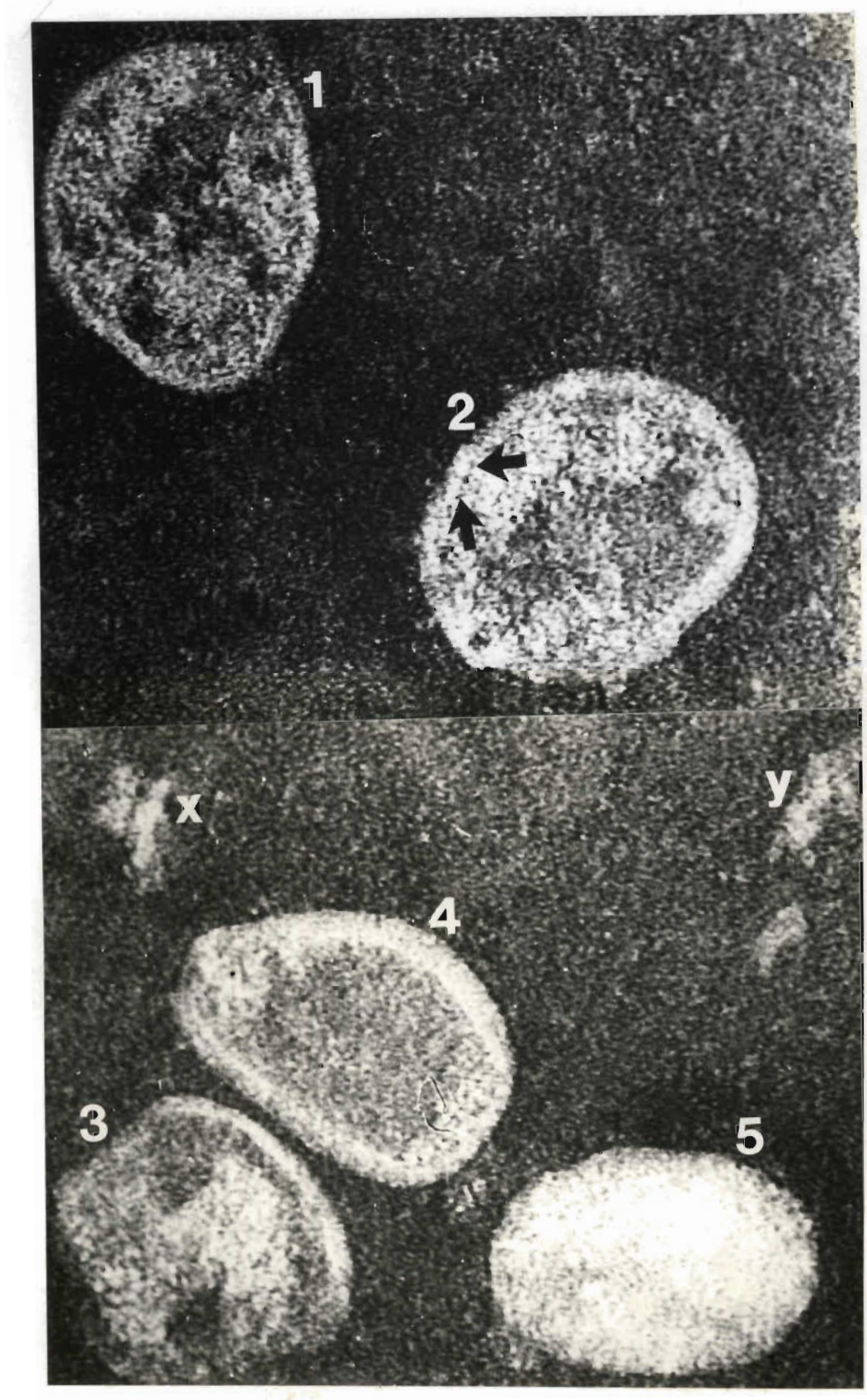


FIGURE 24. Experiment N3: Five TSWV-type particles from zone Z_3, negatively stained with 1% PTA (see Fig. 11). Internal component is discernable within particles 1, 2, 3 and 4 and possibly as released pieces at 'x' and 'y'. An indication of strand conformation in the internal component is arrowed at particle 2. (X 400 000)

DISCUSSION

Identification of the Net strain of TSWV isolated from Campanula isophylla was based initially on symptoms produced on this host and on tomato and Nicotiana glutinosa. Confirmation of its identity as a strain of TSWV was obtained in thermal inactivation, longevity in vitro and host range studies. Mechanical transmissibility of the isolate was regarded also as a significant corroborative diagnostic property (Gibbs, 1969).

Thrips transmissibility was not tested by the present author. However, the same isolate was shown (Ie, personal communication) to be not transmissible by Thrips tabaci. Non-transmissibility has been reported also for two other isolates of TSWV (Samuel, Bald and Pittman, 1930; Jones, 1939).

The white 1 - 3 mm local lesions produced on cotyledons of Cucumis sativus by both Net strain and the Wye clone were clear and relatively numerous. This host has been recognized (Ie, 1970) as a useful indicator of TSWV. Ie (1971) has subsequently reported on ultrastructural aspects of the virus/host inter-relationship.

In previous strain differentiation studies on TSWV (Norris, 1946; Best and Gallus, 1953; Best 1961) the tomato varieties Potentate and Moneymaker, employed in the present study, were not included and therefore the Net isolate cannot be compared directly with the strains described by these authors. Nevertheless, as the Net strain induced etch leaf symptoms followed by mottling on both above-mentioned tomato varieties,an 'intermediate' severity rating

appears warranted.

The observed TSWV strain stability in nasturtium, noted also by Bald (1962), is significant in view of the cyclic alternation in symptom severity displayed by this host on infection with TSWV.

Although strain stability in tomato is also appreciable (Best, 1961), in the present study two strain departures did occur on this host. In autumn of the second year a single tomato plant developed symptoms typical of the TB (or A) strain (Table 2). One transfer of this severe form was attempted and proved successful. A second change occurred in mid-winter. Symptoms of vein and petiole purpling replaced the typical etch symptoms on tomato, whilst on N. glutinosa a mixture of chlorotic and necrotic lesions were produced instead of the uniformly necrotic lesions typical of Net strain. Selman (1941) reported that a low lime content in the soil tends to promote vein purpling in tomatoes infected with TSWV. However, analysis of the soil used by the present author revealed no lime deficiency.

From the above reports and findings it appears that environmental conditions may either bring about a change merely in symptom expression or mediate a selective effect on certain strains. Norris (1946) has drawn attention to the importance of strain ratio, temperature and light as interacting factors responsible for TSWV symptom variation under natural conditions.

Extraction of Net strain-infected N. rustica leaves, using the procedure presented in Fig. 1, yielded at the

rate·zonal gradient stage (Z) large, spherical-type particles, approximately 70-120 nm in diameter (Fig. 7). These closely resembled the particles reported by Black et al. (1952, 1963). In both Experiment 1 and 2 such particles were present in the clearly visible, light-scattering gradient zones found at a depth similar to that reported by Black et al. (1963). Inoculation from the visible zones demonstrated the appreciable infectivity of the component material (Table 4). Adjacent regions were also infectious and contained similar TSWV-type particles.

These results, confirmed in a subsequent study by van Kammen et al. (1966) using the same virus isolate, support the conclusion drawn by Black et al. (1952, 1963) and Best (1958) that such particles represent the TSWV virion recoverable from leaf tissue. Neither a comparable zone nor TSWV-type particles were recovered from healthy N. rustica leaf tissue similarly extracted by the present author and also by Black et al. in 1963.

With regard to TSWV distribution in the rate zonal sucrose gradient Black et al. (1963) noted that when working with "unusually high concentration of virus" the region below the visible zone also contained appreciable virus. Separation from contaminating host material is likewise strongly influenced by concentration factors. However, in the experience of all workers applying this method (Black et al., 1963; van Kammen et al., 1966; Mohamed et al., 1973), the visible zone after rate zonal centrifugation contained contaminating host material.

The nature of these impurities was not specified by Black et al. (1963) but can be seen in Fig. 6 of their report to include small, spherical particles of two sizes principally, as well as aggregated material difficult to identify. van Kammen et al. (1966) mention contaminating levels of chloroplast and endoplasmic reticulum (ER) fragments sufficient to preclude the making of reliable chemical analyses of virus membrane composition. Similarly, the purified virus preparations obtained by Mohamed et al. (1973) included host membranous material and were pale green in colour.

In both last-mentioned studies the virus zone was subjected to a final high speed centrifugation. This could be expected to remove most, but probably not all, of the smaller, spherical material recognizable in shadowed preparations taken directly from the virus zone (Black et al., 1963). In the present study, employing C/Pt shadowing of the grids, ribosomes, Fraction I and cell wall fibres were discerned in samples from the virus zone (Fig. 7). Membranous structures and aggregated material were also present. No evidence was found of the 50 nm particles seen by Best and Palk (1964) and Best (1966) in high concentration in negatively stained virus preparations extracted from N. glutinosa leaf material according to the method given in Fig. 2.

It is not known what caused the grids prepared from the most infective, lower, equilibrium gradient zone of diseased tissue extract (Table 4) to be almost completely clear. As morphological instability of the virus is known to increase during purification (Black et al., 1963; Best

and Palk, 1964) disintegration may have occurred during preparation of the grids. This included a glycerol wash, followed by drying at room temperature (1h) and 70°C (overnight). However, virions from the rate zonal gradient mounted on grids at the same time were not destroyed by this treatment (Fig. 7).

Of the healthy leaf extract it was anticipated that no zone would be found after equilibrium zonal gradient centrifugation. This step was performed with a sample taken from the preceding, zoneless, rate zonal gradient at a depth corresponding to the virus zone in the infected tissue gradient. However, two widely separated zones were produced, which matched in depth the two zones obtained from the infected host extract. The lower zone was narrower and slightly greener than the corresponding infectious zone. As shown in Fig. 8, it appeared to contain the full range of contaminating materials encountered in the virus zone of the preceding rate zonal gradient (Fig. 7).

According to Black (1955), studies by Brakke have shown that rate zonal centrifugation effectively promotes the separation of larger plant viruses, such as TSWV, from chloroplast fragments. During equilibrium zonal centrifugation both these structural types tend to band together. In the present study the very slight greenness of both lower equilibrium gradient zones, more noticeably that derived from healthy tissue extract, point to the presence of chloroplast fragments in the zone. The upper zones in these gradients presumably comprised low density material such as the large mitochondrial-type structures found by Brakke (Black, 1955) to be readily separable from

TSWV by equilibrium zonal, but not rate zonal, centrifugation.

Black et al. (1963) provide few details concerning the results obtained from equilibrium zonal gradient centrifugation of TSWV. No mention is made of the presence of either a single zone or, as in this study, two zones, nor is the depth of the virus zone given. Thus the purified virus zone recovered in their studies may not have corresponded with the lower, most infective zone derived in the present study. However, this is thought unlikely. If the lower zone had contained a significant proportion of non-viral material this would have been readily detected by electron microscopy, as in Fig. 7.

Contrary to the experience of Black et al. (1952, 1963) with two North American isolates, Net strain in crude N. rustica leaf sap did not undergo almost complete aggregation on adding one volume of 'solvent 4'. Despite the significant S_1-stage virus losses, however, the clarified, S_2 extracts showed high infectivity (Table 4). The further successful partial purification of the virus from N. rustica under these circumstances (Fig. 7) indicates that the procedure allows for considerable variation in the starting level of virus. In the opinion of Black et al. (1963), a "comparatively high concentration" of virus in N. rustica is sufficient to achieve satisfactory results at the rate zonal centrifugation stage.

In this investigation the same procedure proved unsatisfactory, however, when applied to systemically infected tomato leaf tissue having a high virus content

(Table 5). Commencing with a weight of tissue identical to that of N. rustica used in Experiment 1 (Table 4) very severe overloading of the rate zonal sucrose gradient resulted. This occurred despite additional centrifugation of the resuspended P_1 pellet, at 10 000 rpm for 10 min, to reduce the density of the S_2 supernatant. So unsatisfactory was the rate zonal centrifugation result that, even in the absence of a confirmatory experiment, it may be taken as evidence that host type significantly affects the clarification attainable by this method.

All reported unsuccessful attempts to extract TSWV using this method have apparently been made on hosts other than N. rustica (Best and Palk, 1964; Best, 1968; Tsakiridis & Gooding, 1972; Joubert et al., 1974). The evidence from the present study, however, indicates that for the strains concerned the method might also prove effective if extraction were attempted from N. rustica. Recently, Gumpf & Weathers (1972) and Mohamed et al. (1973) successfully applied the procedure to Datura stramonium leaf tisue (Table 1). Initial dilution of the sap was performed with 1,5 or 4 volumes of 'solvent 4', respectively, instead of 1 volume (Fig. 1). No information was presented to explain this modification, either in quantitative terms of S_2 infectivity or otherwise. Clearly it is contrary to the observation made by Best (1968) that addition of more than 1 volume of 'solvent 4', which ensures that the pH of the homogenate is raised to pH 6,6 - 7,6, enhances virus retention in the supernatant. Application of 4 volumes of 'solvent 4' was tested in two N. rustica experiments

in the present study but no change in Net strain aggregation resulted.

Other modifications tested with a view to promoting virus aggregation of Net strain in crude N. rustica extract were also unsuccessful. These included the homogenization of mixed samples of inoculated and systemically infected leaves, as opposed to systemically infected leaves alone, in 'solvent 4' or 0,01 M potassium phosphate buffer, pH7 (Table 6), or concentrated Na_2SO_3 (Table 7). Alternatively, systemically infected leaf tissue was frozen for 24 h before being homogenized in 'solvent 4' or concentrated Na_2SO_3 (Table 8). Selected results from the various clarification studies on unfrozen N. rustica tissue, presented above in Tables 4, 6, 7 and 8, have been consolidated in Table 25. It can be seen that the distribution of virus between the S_1 and S_2 supernatants remained relatively constant. Caution is needed in interpreting individual results as different inhibitory and/or stimulatory factors probably played a rôle in the various inoculations, e.g., natural inhibitors (Milne, 1960), electrolyte concentration and saturation effects at high inoculum levels. Nevertheless, in none was the initial aggregation of the virus almost complete.

Little is known concerning the basis of the reversible aggregation induced by 'solvent 4'. The apparent insensitivity of Net strain virions to large increases in salt concentration (Table 7) raises the question of the nature of the virus-containing aggregates reaching the first pellet, P_1. Net isolate is known to occur in vivo

TABLE 25. Infectivity of selected supernatants and resuspended pellets obtained during clarification of *N. rustica* leaf homogenate containing phosphate buffer pH 7 or Na_2SO_3 (consolidated from Tables 4, 6, 7 and 8)

	Diluent[a]								
	Phosphate buffer + 0,01 M Na_2SO_3					Na_2SO_3 (molarity)			
	0,01 M	0,1 M ('solvent 4')				0,16	0,48	0,96	0,96
Table No. / Inoculum	6	4	4	6	8	7	7	7	8
		Exp. 1	Exp. 2						
S_1 (discarded)	44[b]	104		103	54	62	40	22	6
$S_1 \times \frac{1}{10}$	48			91	28	60	87	74	34
P_1	70			96		177	115	107	
P_2 (discarded)		128							
S_2	90		153	100	100	86	122	99	85
$S_2 \times \frac{1}{10}$		100	100						
$S_2 \times \frac{1}{30}$					8				9

[a] Ratio of tissue (g) to diluent (ml), 1:1

[b] In Tables 4, 6 and 8 infectivity is expressed as a percentage of the italicized, S_2 result bearing the same Table No. The values in Table 7 are given as percentages of the italicized, 'solvent 4' S_2 result in Table 6.

within ER lumina (Ie, 1964, 1971). Such structures are probably severely ruptured during homogenization, but rapidly sedimenting forms might persist. In the present study enhanced cell rupturing, induced by prior freezing, did not improve the proportion of virus remaining in the first supernatant, S_1 (Table 8).

As discussed by Best & Palk (1964), anomalous sedimentation behaviour during clarification could arise if TSWV particles are not all of uniform size. In this event the method of clarification used could determine the size of the particle isolated. Extracting mild strain E from N. glutinosa leaf tissue (Table 2), according to the procedure shown in Fig. 2, these authors recovered predominantly ca. 50 nm particles in high concentration, together with a low proportion of ca. 90 nm particles. The smaller particles, referred to by the authors as virus "cores" or "elementary" bodies, occurred singly to some extent but were seen mostly as dumbbell-shaped pairs. Viewing was performed after positive staining with 2% uranyl acetate, which revealed a lightly staining common membrane linking and encapsulating the terminal "cores". In electron micrograph fields displaying plentiful capsular material, as many as eight of the smaller particles were linked in series. Often, two to four were recognized within a single 90 nm particle; occasionally this number rose to six or eight.

Although Fig. 3 presented by Best & Palk (1964) appears to indicate that certain extractions of strain E

yielded almost exclusively particles of the larger type, Best (1966) noted that the paired, 50 nm particles occurred "abundantly and regularly" in a very large number of preparations of this strain. Other strains extracted by the same procedure likewise yielded particles of both size groups, but, unlike strain E, 90 nm particles predominated (Best, 1966; Best and Waite, 1966). Best and Palk (1964) recognized that the smaller particles, or "cores", might either originate *in vivo* or arise *in vitro* by disruption of larger structures. In the latter connection attention was drawn particularly to the possible significance of EDTA in 'solution B' (Fig. 2). Previously, Best (1962a) had found that EDTA added to crude extract had an enhancing effect on TSWV infectivity at 10^{-4} M but depressed infectivity at 10^{-2} M.

Significant contributions towards an understanding of the possible origin of the 50 nm particles have recently been made by Tsakiridis and Gooding (1972), Joubert (1973) and Joubert *et al.* (1974), who established that partially purified TSWV virions of mild or severe strains undergo marked changes in morphology and staining properties if pH or composition of the suspending medium and stain is altered. Their findings are summarised in Table 2b. At pH 3 or 4 the virions were roughly spherical. Unstained, i.e., stain excluding, partly stained and densely stained, i.e. stain filled, particles occurred, all having approximately the same diameter. At pH 5 the form was similarly spherical. Assessing PTA stain penetration at this pH has proved difficult, however. Whereas Joubert (1973) concluded

TABLE 26. EFFECT OF SOLVENT COMPOSITION AND pH ON THE MORPHOLOGY AND STAINING OF PARTIALLY PURIFIED TSWV, UNFIXED (+) OR FIXED WITH 0,1% GLUTERALDEHYDE (F), AS REPORTED BY TSAKIRIDIS AND GOODING (1972)[a], JOUBERT (1973)[b] AND JOUBERT et al. (1974)[c]

pH	Extraction stage					Stain penetration of particles[c]	(i) Solvent and (ii) stain				Fig. No. in reference [a], [b] or [c] above
	Fig. 3	Fig. 4					(i) Distilled water (ii) 2% PTA at specified pH	(i) Phosphate + sulphite, pH 7,2 (ii) Neutral 2% PTA	(i) 'Solution B', pH 7 (ii) Neutral 2% PTA	(i) Phosphate +sulphite, pH 7,2 (ii) 2% uranyl acetate, pH 4,1	
	FP	CP	PP	RP	EP						
3				+		Most virions penetrated	S				[b] 34A
4	+					" "	S				[a] 2B
				+		" "					[b] 33
					F	Most not penetrated				S	31B
5				+		Uncertain	?				[b] 34B (see [c])
6				+		Not penetrated					[b] 34C
7	+					" "					[a] 2A
		+				" "					[b] 28C
		+				Rarely penetrated		S			28A
		+				Not penetrated					28B
			+			" "					29A
			F			" "					29B
				+		" "					30D
				+		" "		(concentrated)			30A
				+		" "		(dilute)			30B
				+		" "					30C
					F	Many penetrated		S			31A & 32
8				+		Not penetrated					34D

○ = No stain penetration

S = Partially filled with stain; internal component discernible

● = Completely filled with stain; presence of internal component uncertain

that no penetration occurs, the converse opinion, that the particles are partly filled with stain, has subsequently been expressed (Joubert et al., 1974). No explanation was presented for either viewpoint. At pH 6 to 9, the highest pH tested, PTA stain did not penetrate unfixed virions, which frequently appeared irregular in shape due to bud-like and/or tail-like protuberances (Table 26).

Joubert (1973) noted that at pH 6 such distortion was maximal. However, as indicated in Table 26, distortion of comparable RP-stage virions resuspended in distilled water and stained at pH 7 was only marginally less severe. Resuspension in phosphate-sulphite buffer, pH 7,2 reduced the degree of distortion slightly. Significantly, PTA-stained virions in 'solution B' showed a marked tendency to assume dumbbell-shaped forms at both early (CP) and later (RP) stages of extraction (Fig. 4). Particles at the RP stage when resuspended in distilled water and stained at pH 8 displayed limited tail-type deformation. From the published micrographs the impression is gained that the effect of this treatment resembled that obtained at pH 7 in 'solution B'.

On grounds of the abovementioned results Joubert (1973) concluded that the TSWV virion is probably spherical and that the dumbbell-shaped morphology previously encountered by Best (1966) in certain purified preparations is an artefact. It would appear that Best had earlier come to the same conclusion. Thus, in his comprehensive review on the virus Best (1968) describes TSWV as a

representation given of strain E dumbbell-shaped structures in Fig. 13 shows the two polar particles linked not only by a common membrane but also by nucleic acid or nucleoprotein. According to this view the internal component is to be regarded as one unit and its polarization into two terminal bodies as an artefact.

An alternative possibility not referred to by Best (1968) is that each polar body derives initially from a discrete virion. Mohamed et al. (1973) noted the marked morphological resemblance between 60 nm (mean) particles which they obtained by detergent (Nonidet) treatment of TSWV virions and the 55 nm (= 50 nm) "cores" observed as single particles or dumbbell-shaped pairs by Best & Palk (1964). Mohamed et al. (1973) therefore suggested that the 55 nm particles prepared by Best & Palk may have lost both the outermost layer of surface projections and an underlying outer membrane during purification. It appears implicit in this proposal that the dumbbell structure is to be interpreted as the partial reseparation, in a membrane depleted form, of virions that underwent pairing and a degree of membrane fusion during purification. Further confirmatory evidence for this model is wanting. Also, the negligible infectivity of the Nonidet-treated preparations contrasts sharply with the high level of infectivity reported by Best & Palk (1964).

The dumbbell-shaped structures obtained by Joubert (1973), on the other hand, appear to have arisen from single virus particles. The size range of a number of the component, terminal bodies also corresponds reasonably

well with that of the 50 nm "elementary" bodies reported by Best and Palk.

However, account must also be taken of the reference by Best (1968) to paired, 80 - 96 nm spiked structures making up the dumbbell, the average diameter of the internal, terminal cores being 54 nm. Such spiked particles are clearly individually comparable in size to the entire, 68 - 102 nm spiked virions reported by van Kammen et al. (1966). Dumbbells of this type possibly differ from those reported earlier by Best and Palk (1964) and Best (1966) in being complete dimers.

In the present study TSWV-infected N. rustica leaves, extracted according to the method reported by Black et al. (1952, 1963), yielded neither 50 nm or dumbbell-shaped particles. The sustained high proportion of virus sedimented by low speed centrifugation even after adding 20% sucrose to the crude homogenate (Table 9) affords evidence of gross virus grouping, aggregation or adsorption to larger cellular components rather than a difference in virus particle size.

The accumulated evidence from purification studies of TSWV in leaf material thus affords no conclusive indication that any strain comprises, in part or wholly, 50 nm-type virions or sub-virions warranting special modification of extraction procedures. Using a method (Fig. 4) that avoids early loss of such smaller particles Joubert (1973) recovered only large TSWV virions resembling those described by Black et al. (1963). Significantly, the study included extraction from tissues infected with a

mild strain of the virus. Furthermore, evidence has now become available to allay any suspicion that all Australian strains may possess morphological uniqueness associated with their failure to aggregate in 'solvent 4' (Best and Palk,1964; Best, 1966). Having tested various extraction procedures for use with an Australian isolate (Francki, Randles and Mohamed, 1970) the method finally adopted by Mohamed et al. (1973) was that developed by Black et al. (1963) involving the 'solvent 4' clarification step.

Published dip preparation micrographs of TSWV freshly released from leaf cells of various hosts show only particles 80 nm or larger (Kitajima, Costa & Carvalho, 1963; Milne, 1970; Gumpf and Weathers, 1972; Joubert, 1973). A few severely distorted particles are shown by Joubert (1973 - see Fig. 27) in a stained preparation, but no correlation with strain type was suggested.

In situ studies of TSWV-infected leaf tissue have to date revealed approximately 80 nm-type particles but not 50 nm particles or dumbbell structures (Ie, 1964, 1971; Kitajima, 1965; Milne and de Zoeten, 1967; Francki and Grivell, 1970; Francki et al., 1970; Milne, 1970). Ie (1971) observed also 30 nm particles in infected cells of cucumber cotyledons, but not in other hosts studied. Apparently in none of the abovementioned studies was a mild strain investigated that could be equated with strain E, used by Best & Palk (1964) and Best (1966).

Within leaf tissues of tomato infected by either of two different, undefined TSWV strains Milne (1970) observed both "mature", 70 - 80 nm particles and "enveloped",

"virus-like", 100 nm particles. The former were individually bounded by a unit membrane and collectively enclosed in large numbers within interconnecting cisternae. "Enveloped" particles, on the other hand, displayed two concentric membranes and were found only in recently infected cells. It was concluded that "enveloped" particles originate by budding of parallel membranes occurring within areas of viroplasm and are subsequently transformed into "mature" particles. Milne suggested that the dumbbell particles reported by Best (1968) of the Australian strain E might have been budding "enveloped" particles in the process of formation. However, it seems unlikely that particles in this transitory state would repeatedly have been extracted in such overwhelming proportions relative to "mature" particles. On the other hand, strain-specific qualities could introduce significant in vivo phenomena. For example, in the abovementioned study on tomato by Milne (1970), exhaustive examination failed to locate virus particles in diseased tissue infected with a Wye isolate.

Nevertheless, the findings of the present and other studies discussed above confirm almost unanimously the preliminary reports by Black et al. (1952) and Best (1958) that the TSWV virion in leaf tissue is a spherical, non-rigid particle, approximately 80 nm or larger in diameter. In general appearance TSWV virions resemble those of influenza virus. At the time of this investigation influenza virus particles were known to be bounded by a membrane layer (Horne, Waterson, Wildy and Farnham, 1960).

It was therefore anticipated that TSWV virions would be similarly bounded and that organic solvents would cause particle disruption. However, the possibility could not be excluded that removal of an outer lipid layer might release an infectious, comparatively stable internal component. The results obtained independently with chloroform, n-butanol or ether and carbon tetrachloride showed that TSWV infectivity is destroyed, even at solvent levels affording only limited clarification. There is, accordingly, no evidence of a released infectious internal component. Subsequently it was found by Mohamed et al. (1973) that incomplete removal of the particle membrane by Nonidet detergent also destroys infectivity. The procedure given in Fig. 2 has been found to yield at least three marginally infective bands of light material when applied to extract of strain E-infected tissue (Best, 1964, 1966, 1967). However, the relationship, if any, of these materials to whole virus is unknown (Best, 1968).

The reported successful phenol extraction of TSWV nucleic acid (Best, 1962b, 1963) appears to have been reassessed as no mention of this finding occurs in the subsequent comprehensive review of TSWV by the same author (Best, 1968). In preliminary studies the present author was not able to gain infective TSWV nucleic acid by phenol extraction using the method developed by Gierer & Schramm (1956).

Attempts to curtail drastically the time needed to extract whole TSWV from leaf material by selective adsorption of host material, were in part successful. The

severe reduction observed in Net strain infectivity on adding HCP (Table 10) or activated charcoal (Table 11) to leaf extract are in agreement with the reported results concerning other isolates (Fulton, 1957; Joubert, 1973). Both these adsorbants also failed selectively to adsorb host material in root homogenates (Tables 17, 18). It appears unlikely, therefore, that either will prove suitable for clarifying extensively any strain of TSWV.

In contrast, unbuffered leaf extracts of N. glutinosa and N. rustica usually displayed 75 - 100% of the original infectivity when clarified by the addition of 15 or 30 mg sodium kaolinite per g tissue, respectively. When the kaolin level was increased to 60 mg/g tissue and applied to White Burley tobacco leaf extract all virus was adsorbed. Likewise, kaolin at 15 mg/g tissue adsorbed all virus from previously partially clarified sap of tomato leaf tissue. In the latter instance the enhanced TSWV adsorption is probably attributable to the improved purity of the virus: a similar effect has been noted regarding bentonite adsorption of wild cucumber mosaic virus (Dunn and Hitchborn, 1965).

The value of bentonite as a selective clarifying agent was demonstrated by Dunn and Hitchborn (1965) in studies covering a range of small isometric and filamentous viruses. A number of such viruses tested by these authors did not adsorb to bentonite used at 20 - 25 mg/g tissue in the presence of phosphate buffered 10^{-3} M or higher Mg^{++}. Under these conditions 18 S protein (Fraction I), ribosomes and green material adsorbed readily. However, tobacco rattle virus and the bean form of TMV, unlike other strains

of that virus, became adsorbed together with the plant components even at 10^{-4} M Mg^{++}. In preliminary studies at Wageningen, application of the related clay montmorillonite, at 12 mg/g White Burley leaf tissue, eliminated all infectivity. Subsequently, Joubert (1973) reported that as little as 3 mg bentonite/g leaf tissue reduced infectivity significantly.

Incomplete clarification using a low level of bentonite has, however, proved very beneficial in the extraction of certain viruses. Initial application of approximately 6 mg of predominantly sodium bentonite per g tissue followed by further smaller controlled applications, greatly facilitates the purification of some apple viruses (Lister, Bancroft and Nadakavukaren, 1965; de Sequeira and Lister, 1969; Maat and Vink, 1971). Considerable amounts of host material, including Fraction 1 protein and phytoferritin, are not eliminated by such treatment, necessitating further clarification by acidification or polythylene glycol (de Sequeira and Lister, 1969). Similarly, magnesium bentonite at about 6 mg/g tissue has proved useful for initial, partial clarification of leaf extract containing 50 nm membrane bound particles of carrot mottle virus (Murant, Goold, Roberts and Cathro, 1969). A limited amount of green material persisted after bentonite treatment and could not be eliminated by further bentonite application without losing most of the infectivity. Calcium phosphate (brushite) chromatography was therefore applied to effect further clarification. The authors noted that vesicular structures of different sizes, thought to be cell membrane fragments, were not eliminated by such two-step clarification.

In the present study, levels of kaolin or montmorillonite less than 15 mg and 12 mg/g tissue, respectively were not seriously considered because complete, one-step clarification, of the type subsequently achieved with other viruses by Dunn and Hitchborn (1965), was sought. This approach was necessitated by the rapid inactivation of the virus in vitro. The comparative success achieved with clarifying levels of kaolin led to similar testing of $Fe(OH)_3$ gel. Kaolin, unlike montmorillonite and bentonite, displays little planar negative charge (Marshall, 1949). Its comparatively limited exchange capacity is due predominately to accidental edge charges, comprising unsatisfied positive and negative valencies in the ratio 1:1. $Fe(OH)_3$ is similarly amphoteric, become predominantly positively charged under acid conditions (Sumner, 1963). However, $Fe(OH)_3$ proved consistantly unsatisfactory in a wide range of tests. When applied to leaf or root homogenate at levels affording satisfactory visual clarification within the pH range 6 to 9, the virus adsorbed strongly to the gel and was not readily eluted by alkali. Apparently $Fe(OH)_3$ has not been tested by other workers as an adsorbant for use in virus clarification.

Thus, of the adsorbants tested in this study as clarifying agents for leaf extract containing TSWV, kaolin appears to offer most promise. Depending on tissue type the amount of kaolin required varied between

15 and 30 mg/g tissue. At still lower application rates kaolin may be found to cause consistantly negligible virus loss and thus usefully serve as the first stage of a compound clarification operation. In the more recently formulated TSWV extraction procedures (Tsakiridis and Gooding 1972; Joubert, 1973) extension of the clarification phase to include two or more complementary clarifying steps has been favoured. The extent to which this can be done is largely dependent on the stability and longevity of the virus concerned. Significantly, the *in vitro* longevity of carrot mottle virus in *Nicotiana clevelandii* leaf extract at 5°C is prolonged by initial, limited bentonite application (Murrant *et al.*, 1969). Should this effect be attainable also in the case of TSWV using kaolin, a major factor hindering the extraction of the virus may have been overcome. However, in more recent tests, bentonite, at 0,04 mg/g root tissue, did not prolong infectivity. The appreciable infectivity of *N. rustica* leaf extract clarified with high molar Na_2SO_3, shown in Table 25, also appears to warrant further investigation.

The use of root tissue as virus source was explored as a means of avoiding chloroplast material and thereby also shortening the extraction procedure. TSWV evidently may attain high concentrations in host roots. In this study appreciable infectivity was displayed by extracts of both entire tomato root systems and segments of the terminal 5 cm root tip (Table 16). At the time that the study at Wageningen was terminating the results of which were

published almost one and a half years later (Martin, 1964). Best (1962a) reported that the infectivity of homogenized N. glutinosa roots was almost two-thirds that of the corresponding systemically infected leaf tissues. In vivo root studies have proved confirmatory (Ie, 1964; Kitajima, 1965).

Ford, in 1964, described the use of pea root tissue for purifying two rod-shaped viruses, viz., clover yellow mosaic virus and Idaho pea streak virus. Although initial infectivity of the root extract was only 40 - 70% that of the leaf extract, equal or greater amounts of purified virus were recovered from roots. In addition, the presence of relatively less host material after root extract clarification made it possible to omit one cycle of differential centrifugation. Browning of root extracts was less than that of leaf extracts, indicative possibly of a weaker phenol oxidizing system in roots. More recently, maize rough dwarf virus, which comprises approximately 70 nm isometric particles, has been purified from maize roots (Wetter, Luisoni, Conti and Lovisolo, 1969).

The severe TSWV loss experienced during root extract clarification with HCP prompted the testing of CGP(N), another relatively insoluble form of calcium phosphate. At no apparent virus loss, CGP(N) afforded marked clarification of root extracts containing either Net strain or Wye clone TSWV, provided less than 0,005 M phosphate buffer was used (Table 20).

After extensive preliminary testing the procedure

set out in Fig. 10 was derived for extracting TSWV Net strain from tomato roots. The method was used comparatively on roots infected with only Net strain or also with TMV. The latter virus displayed synergistic action in tomato, resulting in extensive necrosis of leaves and root deterioration. By using roots in this severely pathological state it was hoped largely to avoid a major contaminant comprising co-sedimenting vesicular structures that may, according to Hodge, Martin and Morton (1956), derive from physically ruptured endoplasmic reticulum. Elimination of other potential contaminants, e.g., inhibitors, may also occur in such tissue (Adomako, Owusu and Oduro, 1974).

Two purification experiments conducted with doubly infected material each yielded a clearly visible TSWV zone in the lower half of the sucrose gradient tube. This zone was well separated from the lighter, TMV zone and contained negligible TMV. Electron microscopy revealed the limited presence of fine particles but no host membrane components (Figs. 12, 13, 15, 17, 18). Contamination was more evident (Fig. 18a) when rate zonal separation was performed in the smaller, Spinco SW 39 rotor (Exp. N4 : Table 24 and Fig. 11). Virus dimer formation was particularly noticeable also in this experiment in which the clarified extract was applied to a smaller gradient (Fig. 18b).

In contrast extract prepared from roots infected with TSWV alone yield two zones approximately 5 mm apart, in the lower half of the gradient tube (Fig. 11, Exp. N2). Both zones, especially the lower, showed little infectivity, although the concentrated extract applied

gradient had been highly infective. Electron microscopy revealed relatively few TSWV-type virus particles, the lower zone being slightly the poorer (Figs. 20, 21, 23). In addition, a considerable amount of apparently collapsed vesicular material was present. However, noting the severe decline in infectivity caused by the centrifugation it may be correct to conclude that a large proportion of these structures comprise collapsed virus particles.

The sucrose gradient used in this experiment differed from those of experiments N2 and N4 in that it contained 0,01M $MgCl_2$ (Fig. 10), found by Brakke (1956) to stabilize potato yellow dwarf virus. However, M $MgCl_2$ has subsequently been shown to enhance the inactivation of a wide range of membrane bound animal viruses heated to 50°C for 15 min (Wallis, Melnick and Rapp, 1965). By contrast, M $MgSO_4$ exerted a markedly stabilizing effect. It is possibly significant in this connection that 'solution B' (Fig. 2), a buffer specifically designed for use in TSWV extraction, contains 0,07 M Na_2SO_4. This medium was found to minimise TSWV inactivation or loss during pellet redispersion following high speed centrifugation (Best and Palk, 1964; Best 1966). In terms of infectivity, 0,2 or 0,1M Na_2SO_4, in the presence of 0,01 M Na_2SO_3, were also highly beneficial (Best, 1966). Chlorides and phosphates however, did not reduce virus loss. Although Best (1962c) noted no enhancement of TSWV infactivity on adding "Mg at 10^{-3} molar concentration" the above findings, taken together, afford an indication that $MgSO_4$ may have a stabilizing effect on the virus.

An alternative explanation for the combined occurrence of low infectivity and apparently ruptured particles in Experiment N3 (Table 24) is that TSWV purified from roots is highly unstable in the absence of a protective factor such as TMV. Attention is given to this question in Chapter 2, in which is presented subsequent findings relating to root extraction studies on a South African isolate.

In all experiments with tomato roots reported in this chapter the initial extract was consistently highly infectious, as little as 11g of roots proving more than adequate to complete a purification study (Tables 21, 24). Care was taken to use only highly infectious inoculum when infecting plants from which the roots were later to be harvested for TSWV extraction. No attempt was made to compare TSWV levels in singly and doubly infected roots. Subsequently, Limonade (T., personal communication)working with the same strain, found that co-inoculation with TMV caused no change in the pattern and levels of increasing TSWV infectivity displayed by the systemically invaded, younger leaf tissues. In the present study the high TSWV concentrations encountered in doubly-infected roots affords indirect evidence that the same relationship may apply in root tissue.

The investigations reported in this chapter were directed principally at purifying the virus as mildly and rapidly as possible. The highly abbreviated procedure developed for extracting Net strain from tomato roots requires a minimum of about four and a half h. Infectious

particles prepared in this manner (Fig. 12) closely resembled those derived from leaves of N. rustica (Fig. 7) using the procedure developed by Black et al. (1952). Observed diameters of the partially flattened particles measured approximately 100 - 150 nm (Figs. 12, 13, 15), 150 - 185 nm (Fig. 18) and 125 - 165 nm (Figs. 20, 21) when extracted from roots with the aid of CGP(N). Equivalent spherical diameters for the 100 - 150 nm particles were estimated at 80 $\pm$ 5 nm. This estimate is in general agreement with the reported in situ diameters of TSWV particles in roots of tomato and other hosts, viz., 70 nm (Ie, 1964), 50 - 80 nm (Martin, 1964) and 100 nm (av) Kitajima, 1965). Thus TSWV particles produced in root tissues appear to correspond in vivo and in vitro with those developing in leaves.

Lack of internal measurement standards undoubtedly contributed to the overall size variations. Variability within the same micrograph may partly reflect differences in the physiological condition of individual virions having in mind that the particles observed in this study were all of one strain. In this connection, particle maturation and ageing may be significant: Hoyle (1954) noted that influenza virus particles released into allantoic fluid contract in size, possibly due to water loss. That differences in shadowed TSWV particle diameters are not due solely to surface tension effects is clearly seen when examining certain particles occurring in close proximity (Fig. 15a).

The relationship of the ca. 50 nm particles, observed in low concentration in two root extractions, to those recovered from leaf tissues (Best & Palk, 1964; Best, 1966) and to whole virions is uncertain. Examination of Fig. 7 presented by Black et al. (1963), also reveals such particles. However, in the present study, and apparently also that by Black et al. (1963), they were encountered together with distorted or ruptured TSWV particles, implying that they may derive from the larger particles. This conclusion is in agreement with the findings reported by Joubert (1973). It appears correct, therefore, that TSWV extraction procedures should be directed at the recovery of particles approximately 80 nm in diameter in a stable, infectious state.

PTA staining of certain viruses has been found to result in disruption of the virions (Mackenzie and Haselkorn, 1972; Milne, 1972; Milne, Conti and Lisa, 1973). Thus atypical, stained TSWV forms (Best and Palk, 1964; Best, 1966; van Kammen et al., 1966; Tsakiridis and Gooding, 1972; Joubert, 1973) cannot be regarded as showing also the form of the particle in the absence of the stain, unless this is confirmed by other methods of preparation. Likewise, the complete absence of "enveloped" TSWV particles (Milne, 1970) in negatively stained, purified preparations in this and other extraction studies may not be entirely reliable.

The observed tendency for the negatively stained particle membrane to indent or flatten where not in contact with the internal component (Fig. 24) may explain the

occasional presence, in shadowed preparations, of virions with flat sides or corners (Fig. 15a). In many TSWV particles sectioned in situ the internal component is to be seen similarly periferally located (Milne, 1970; Ie, 1971). However, numerous other particles in the same micrographs are almost, or completely, filled with the densely staining internal component. Furthermore, both conditions are encountered also in "enveloped" particles (Milne, 1970), thought to be recently formed, maturing virions.

That this range of structural types should be due to plane of sectioning seems unlikely in a particle of such limited size. It is also difficult to accommodate either extreme form as a transitory stage leading to the other, since both extremes occur in "mature" and "enveloped" particles (Milne, 1970). A possible explanation is that TSWV virions are not all uniform with respect to the amount of internal component present. Such a condition would also, as one possibility, contribute to the diffuse gradient boundaries reported for TSWV by Black, Brakke and Vatter (1963).

CHAPTER 2

FURTHER STUDIES ON TSWV EXTRACTION FROM ROOTS

INTRODUCTION

The results reported in Chapter 1 demonstrated the potential of tomato roots as a source of TSWV for purification studies on the virus. Application of CGP(N) followed by ascorbic acid yielded a highly infective, density gradient TSWV zone, approaching purity. The testing of CGP(N) arose from a search for a suitably selective, phosphate-based adsorbant that would facilitate shortening of the time necessary to effect TSWV purification.

Certain aspects of the extraction procedure could not, in the time available at Wageningen, receive adequate study. Principally, these concerned :

(i) the mode of action of CGP(N) as a clarifying agent;

(ii) the synergistic contribution of TMV to clarification and/or level of extractible TSWV;

(iii) the observed gradient double band and low yield of TSWV extracted in $MgCl_2$.

Access recently to controlled environment growth cabinets made it possible to resume studies on the virus, giving attention, specifically, to the issues listed above. In this work an isolate from Dahlia was used. To achieve maximal standardization of host root environment, hydroponic culture was adopted almost exclusively.

MATERIALS AND METHODS

Virus Isolate

TSWV was mechanically transmitted from *Dahlia* to *Nicotiana glutinosa* and passaged twice on the latter species, from isolated single lesions, before inoculation to tomato, var. Homestead. Thereafter the clone was maintained in this tomato variety by mechanical transmission at approximately 12-day intervals. Such plants, and other younger plants to be used for this purpose, were kept in isolation, receiving 12 h daily illumination in a nursery portion of a laboratory maintained at 23 - 25°C.

Thermal inactivation temperature, longevity *in vitro* and host range studies were performed to confirm the identity of the isolate. The hosts used were *Petunia hybrida* var. Rose of Heaven, *Tropaeolum majus*, *Cucumis sativus* and *Gomphrena globosa*. The operation of an inhibitor from infected tomato roots, limiting infectivity for *Petunia* (Milne, 1960), was tested in dilution studies.

Plant Material and Virus Propagation

The host used in all extraction studies was tomato, var. Homestead. Seed was germinated in peat and the seedlings, when approximately three weeks old, were transferred to Hoaglands nutrient solution in 2 750 ml black plastic beakers, 15 cm in internal diameter. Two or three seedlings were supported by cotton wool in 2,5 cm holes cut in a 2-cm thick white polystyrene cover over

each beaker. Aeration was provided during 10 min of each h and the nutrient level was maintained daily.

Seedlings not to be used as source plants for TSWV were moved from the nursery area in the laboratory to a glasshouse permanently covered with plastic mesh providing 30% shading. Air-conditioning units were set at maximum cooling. The maximum (av) and minimum (av) glasshouse temperatures were as follows :
January-March, 28,5°C and 23,8°C; April-June, 27,1°C and 21,8°C; July-September, 27,5°C and 23,2°C; October-December, 29,4°C and 25,1°C.

Suitably uniform plants were inoculated with TSWV approximately four to five weeks after initial transfer of the seedlings to nutrient solution. Characteristically, at this age, the first young flower buds were forming. Pots containing one or more plants bearing an open flower were discarded.

TSWV inoculation was performed mechanically, as described in the previous chapter. All inoculum comprised the nursery-maintained clone of the virus. The inoculum was prepared from systemically infected, apical leaf material in the acute phase of infection, ground in a limited volume of 0,01 M Na_2SO_3. Homogenization was performed rapidly with a chilled mortar and pestle and the homogenate was immediately subdivided into suitable smaller volumes and placed in ice until used. Prior to inoculation, the lowest, smaller leaves on each plant were removed. The stem and upper surface of all half-grown and larger leaves were then dusted with 400 mesh carborundum powder and inoculated, without subsequent washing.

At each occasion when a group of healthy plants was inoculated for later use in TSWV extraction, limited test inoculations with the same inoculum were made to Petunia and N. tabacum var. Samsun NN to establish both the presence of adequate TSWV and absence of TMV. For the latter purpose an aliquot of the inoculum was kept for 24 h at room temperature before inoculation to Samsun NN. In addition, two apical leaflets, less than half-grown, were picked from each healthy plant immediately prior to inoculation, pooled and used as inoculum on Samsun NN, to confirm that the inoculated plants were free of TMV.

Studies on the effects of dual infection with TSWV and TMV were performed using TMV at a final concentration of approximately 0,05 or 0,0005 mg TMV/ml of inoculum, as required. In certain experiments the TMV was added to the TSWV inoculum and applied uniformly, whilst in others it was applied either before or after the TSWV as a full inoculation or by touching only every third leaflet. The latter practice is referred to in this thesis as 'spot-inoculation'.

Immediately following inoculation with either or both viruses the inoculated plants were placed in a Conviron EF7H portable, controlled environment cabinet. One such cabinet was continuously available for use during this phase of the investigation, whilst a second was available at irregular intervals. In the planning of experiments involving, concurrently, TSWV - and TSWV + TMV-inoculated plants it was therefore necessary to remain within this restriction, and also that of keeping

TMV-inoculated plants separately in another cabinet, away from those infected only with TSWV.

Unless otherwise stated, TSWV incubation in host plants proceeded at 23°C and 70% minimum humidity. Normally, full illumination of approximately 100 000 ergs/sec/cm^2 at pot level, was provided for 12 h daily, together with continuous aeration of the roots. At inoculation, nutrient solution was almost completely replaced by fresh solution and thereafter the level was restored twice daily.

The formulation of Hoaglands nutrient solution used almost exclusively in this study, was as follows:

Salt	Stock solution	Volume in 1 litre nutrient solution
KH_2PO_4	1 M	1 ml
$Ca(NO_3)_2$	1 M	5 ml
KNO_3	1 M	5 ml
$MgSO_4$	1 M	2 ml
$MnSO_4.H_2O$	9,1 m M	1 ml
H_3BO_3	46,2 m M	
$ZnSO_4.7H_2O$	0,7om M	
$CuSO_4.5H_2O$	0,32m M	
$Na_2MoO_4.2H_2O$	0,11m M	
$FeSO_4.7H_2O$	5,57g /L	5 ml
Na_2EDTA	7,45g /L	

Briefly, two alternative nutrient solutions lacking EDTA were also used to test for an effect by EDTA on extractable TSWV levels in tomato roots. EDTA has been shown

to enhance TMV replication in excised tomato roots cultured aseptically in White's solution (Brants, Graafland and Kerling, 1962). One of the alternative nutrient solutions, used in the present study was based on a medium described by Hewitt (1952), with the omission of $Ga(NO_2)_3$. As amended, the medium is referred to in this thesis as Long Ashton solution, having the following composition:

Salt	Wt in 10 L nutrient solution	
KNO_3	0,02	g
$Ca(NO_3)_2$(Anhydrous)	6,56	g
$NaH_2PO_4.2H_2O$	2,08	g
$MgSO_4.7H_2O$	3,69	g
Ferric citrate	0,245	g
$MnSO_4.H_2O$	22,3	mg
$CuSO_4.5H_2O$	24,0	mg
$ZnSO_4.7H_2O$	29,0	mg
H_3BO_3	18,6	mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	0,35	mg
$Al_2SO_3.12H_2O$	1,86	mg
$CoSO_4.7H_2O$	0,23	mg
$NiSO_4.7H_2O$	0,28	mg

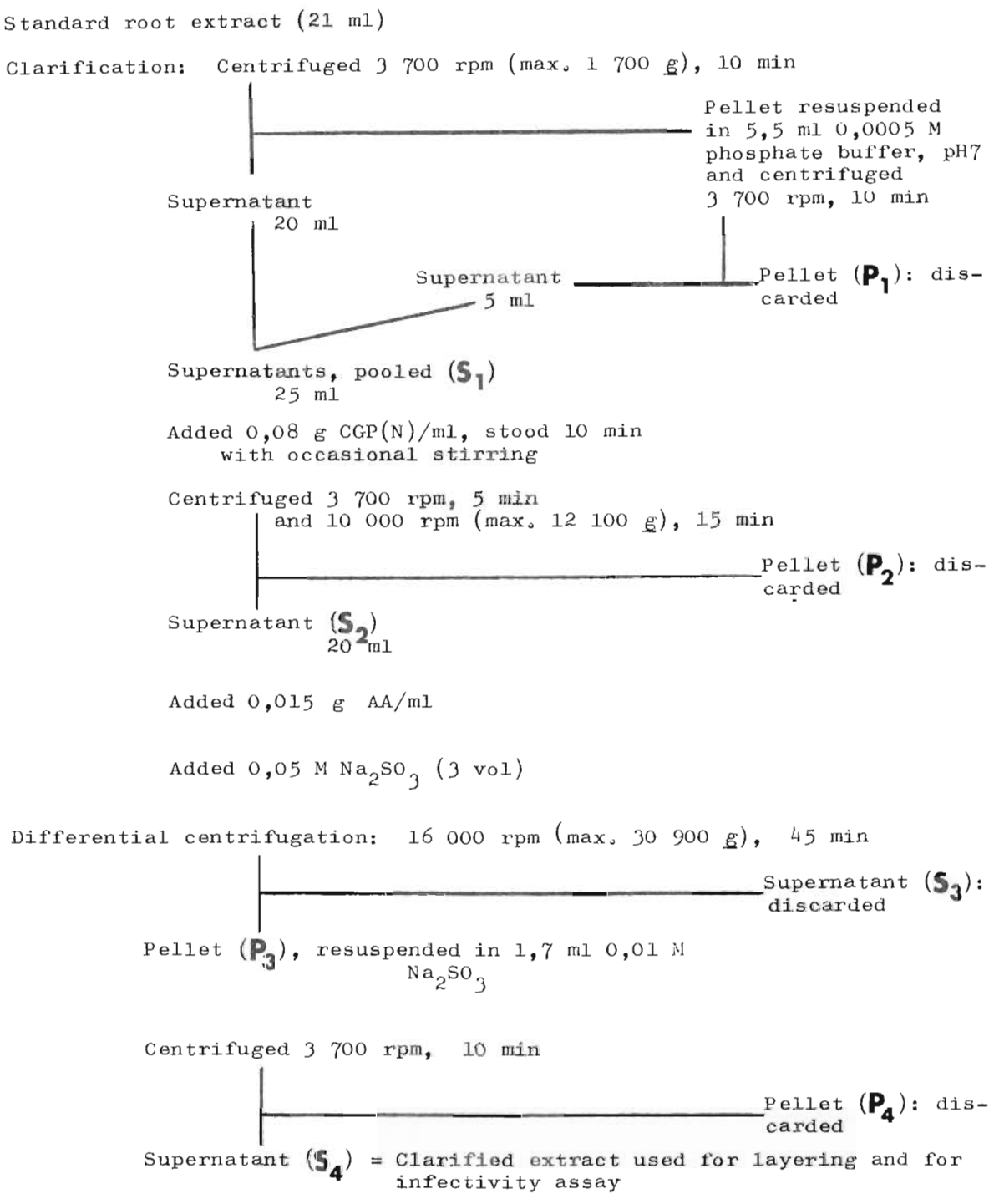
The other solution comprised the abovementioned Hoaglands modified only by omitting ferrous sulphate and EDTA and substituting 24,5 mg ferric citrate/L. The latter component was added as required to the nutrient solution immediately before use.

Virus Extraction

As the aim of the studies presented in this chapter was not, in the first instance, to seek maximal TSWV purification but to investigate specific aspects of the extraction method reported in Chapter 1, a shortened, slightly modified version of the procedure given in Fig. 19 was adopted for routine use. The amended procedure, which is set out in Fig. 25, included only one cycle of differential centrifugation. In a range of studies on root tissues infected only with TSWV or also with TMV, clarification and TSWV yield attained at the S_4 stage by the abovementioned standard procedure (Fig. 25) were compared with that obtained when using alternative levels of CGP(N) and ascorbic acid (AA), soluble calcium glycerophosphate (CGP(S)) or sodium glycerophosphate (NaGP). CGP(S) differs from CGP(N) only in that it contains, in addition, 14% citric acid, which increases the solubility of this formulation to approximately twice that of CGP(N). Added as CGP(S) at the rate of 0,08 g/ml extract (Fig. 25) the calcium glycerophosphate dissolves completely and, in contrast to CGP(N) addition, no insoluble, potentially adsorptive, colloidal particles of the salt persist. NaGP likewise dissolves completely when applied to extract at the abovementioned rate.

Homogenization and all subsequent steps prior to the concluding determination of UV absorbance were performed in a cold room at approximately $3^{o}C$. Quoted centrifugation

FIGURE 25. SHORTENED PROCEDURE USED IN EXTRACTION STUDIES ON A SOUTH AFRICAN TSWV ISOLATE IN TOMATO ROOTS



rates other than that of the rate zonal sucrose gradient centrifugation, refer to the Servall SS-34 rotor, which was used initially for all extractions. Later, either a Beckman JA-21 or Servall SM-24 rotor, each having smaller tube volumes, was used instead. The amended centrifugation routine applicable to these rotors, was as follows:

Servall SS-34	-	Beckman JA-21 Servall SM-24
3 700 rpm	-	unchanged
10 000 rpm, 15 min	-	13 000 rpm, 12 min
16 000 rpm, 45 min	-	17 000 rpm, 36 min

Sucrose gradients for rate zonal centrifugation (Fig. 25) were prepared in a double well perspex unit by loading 12,3 ml 0,01 M Na_2SO_3 in the rear well and 12,3 ml 40% sucrose - containing 0,01 M Na_2SO_3 in the leading well. Equilibrium zonal gradients were similarly prepared by mixing 30% and 60% sucrose solutions containing 0,01 M Na_2SO_3. The Spinco SW 25 rotor centrifuge tubes were supported in ice during preparation and the gradients were used directly. In one experiment 4% PEG (MW 6 000) was added to the 40% sucrose component used to make the gradient. Rarely, a gradient of 0-50% glycerol in 0,01 M Na_2SO_3 was used instead of a sucrose gradient. To re-examine the effect of $MgCl_2$ on TSWV stability during purification (see Chapter 1) an experiment was performed in which, commencing at the P_3 stage (Fig. 25), all virus suspensions and the rate zonal gradient, contained 0,01 M $MgCl_2$. In addition, the procedure was lengthened by the

inclusion of a second cycle of high and low speed centrifugation before rate zonal centrifugation.

In two experiments in which glycerol gradients were used, material from the TSWV virus zone was examined by electron microscopy. Specimens were supported on collodion-coated grids and shadowed at an angle of 30° with gold/palladium. Viewing was performed in a Hitachi HU-11E electron microscope. In one experiment virus zone material taken from a sucrose gradient was placed on carbon-coated collodion grids, where it was fixed by adding an equal drop of 6% glutaraldehyde in 0,05M Na cacodylate buffer, pH 7 and dehydrated by critical-point drying. The procedure followed was that developed by Phil and Bahr (1970), employing the following solvent sequence:

(i) grids passed through 30, 60, 95 and 99,5% ethylene glycol, 1 min each,

(ii) grids immersed in Cellosolve (= ethylene glycol monoethyl ether = 2 - ethoxy-ethanol), 2 min,

(iii) Cellosolve exchanged with liquid CO_2 in the critical-point drying bomb preparatory to critical-point drying.

The grids were then shadowed and viewed as described above.

Gradient absorbance profiles at 254 nm wavelength, uncorrected for light scattering, were obtained after gradient centrifugation by draining the liquid through the base of the tube and past a LKB Uvicord 4700 unit fitted to a Metrohm Labograph E 478 recorder. Flow rate was regulated by a LKB Varioperpex peristaltic pump.

RESULTS

Virus Isolate

The South African isolate was biologically purified by single lesion transfer on *N. glutinosa* to an extent that would not justify it being regarded as a pure strain. It is accordingly referred to as a South African clone of TSWV.

The thermal inactivation and *in vitro* longevity characteristics of the clone were typical of TSWV. Likewise the responses induced in *Petunia*, *Tropaeolum majus*, *Cucumis sativus* and *Gomphrena globosa* matched those listed previously for Net strain (Table 3). Sensitivity to the inhibitor in infected tomato roots when inoculated to *Petunia* was confirmed.

Inoculated leaves of tomato var. Homestead remained symptomless at 23°- 25°C. Systemic vein-clearing appeared on the sixth day after inoculation, followed within two days by moderately severe, 1-2 mm necrotic flecking of leaflets on young leaves nearing maturity. Younger leaf tissues tended to be slightly chlorotic, whilst subsequent new growth was symptomless.

On inoculated leaves of half-grown *N. glutinosa* the clone produced pigmented, necrotic lesions typical of TSWV. Systemic foliar necrosis developed during a brief, acute stage, after which normal growth was resumed.

On the basis of the abovementioned symptoms on tomato and *N. glutinosa* the clone may be regarded as predominantly severe in character (see Table 2).

Healthy Roots

The S_1 supernatant (Fig. 25) obtained after initial low speed centrifugation of healthy tomato roots was brown and milky in appearance and completely opaque. Addition of 0,08g CGP(N)/ml followed by centrifugation at 3 700 and 10 000 rpm, produced a light, wine-coloured, partially translucent supernatant (S_2) which cleared to a translucent, very faintly yellow liquid when mildly acidified with AA. Comparable extract prepared without addition of CGP(N) and AA was milky, brown and almost opaque.

Evidence of the improved clarification gained by CGP(N) and AA treatment was detected also on passing rate zonal gradients containing treated or untreated healthy root extracts, through a Uvicord 4700 operating at 254 nm wavelength. Examples of such gradient profiles are shown in Fig. 26. These were recorded, as indicated in Fig. 25, after layering and centrifugation of the respective S_4-stage extracts. Gradients of root extracts derived without addition of CGP(N) and AA consistently yielded the saddle-type profile shown in Fig. 26. The sharp increase in absorption observed near the tops of gradients bearing extracts clarified by CGP(N) and AA application is partly due to residual AA, which absorbs strongly at 254 nm. Exact reproducibility of peak heights and positions proved unattainable.

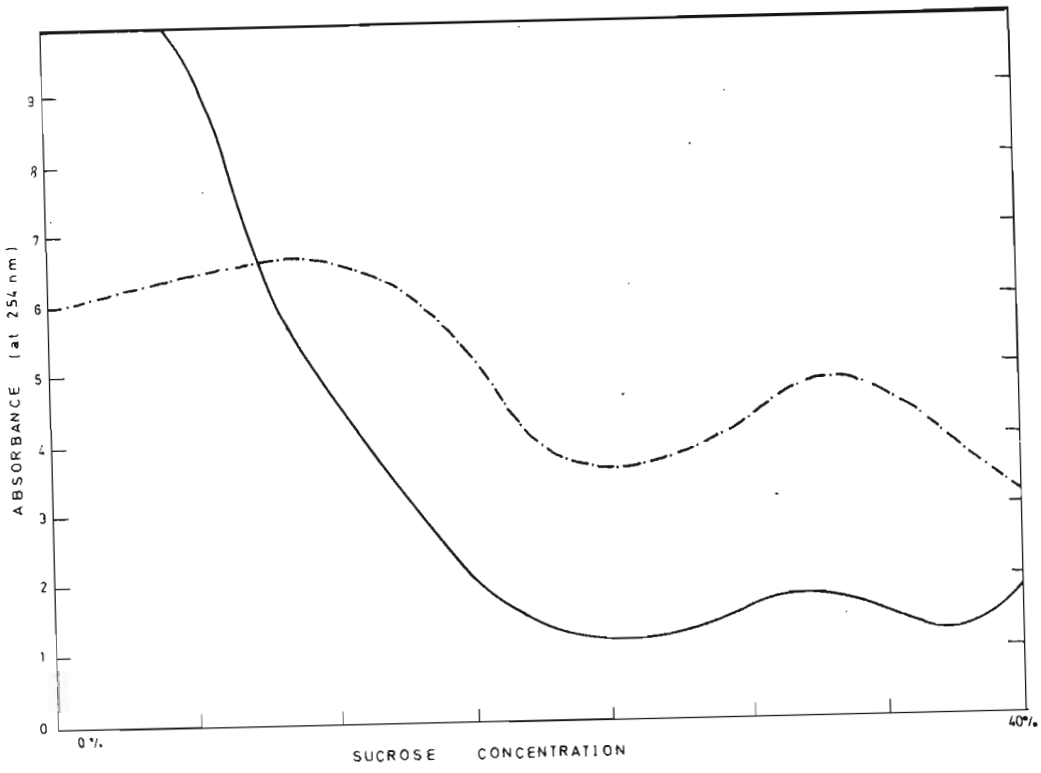


FIGURE 26. Gradient profiles of S_4-stage extracts (see Fig. 25) from healthy tomato roots: standard CGP(N) and AA clarification (———); clarified by centrifugation, omitting CGP(N) and AA (-•-•-)

TSWV-infected Roots

Gradient profiles of S_4-stage root extracts derived from plants infected for 8, 10 or 13 days and clarified without addition of CGP(N) and AA are shown in Fig. 27a. The profile at 8 days after inoculation shows a resemblance to that of healthy material similarly extracted (Fig. 26). At later harvestings the peak observed in the lower half of the tube tended to steepen, especially at its upper

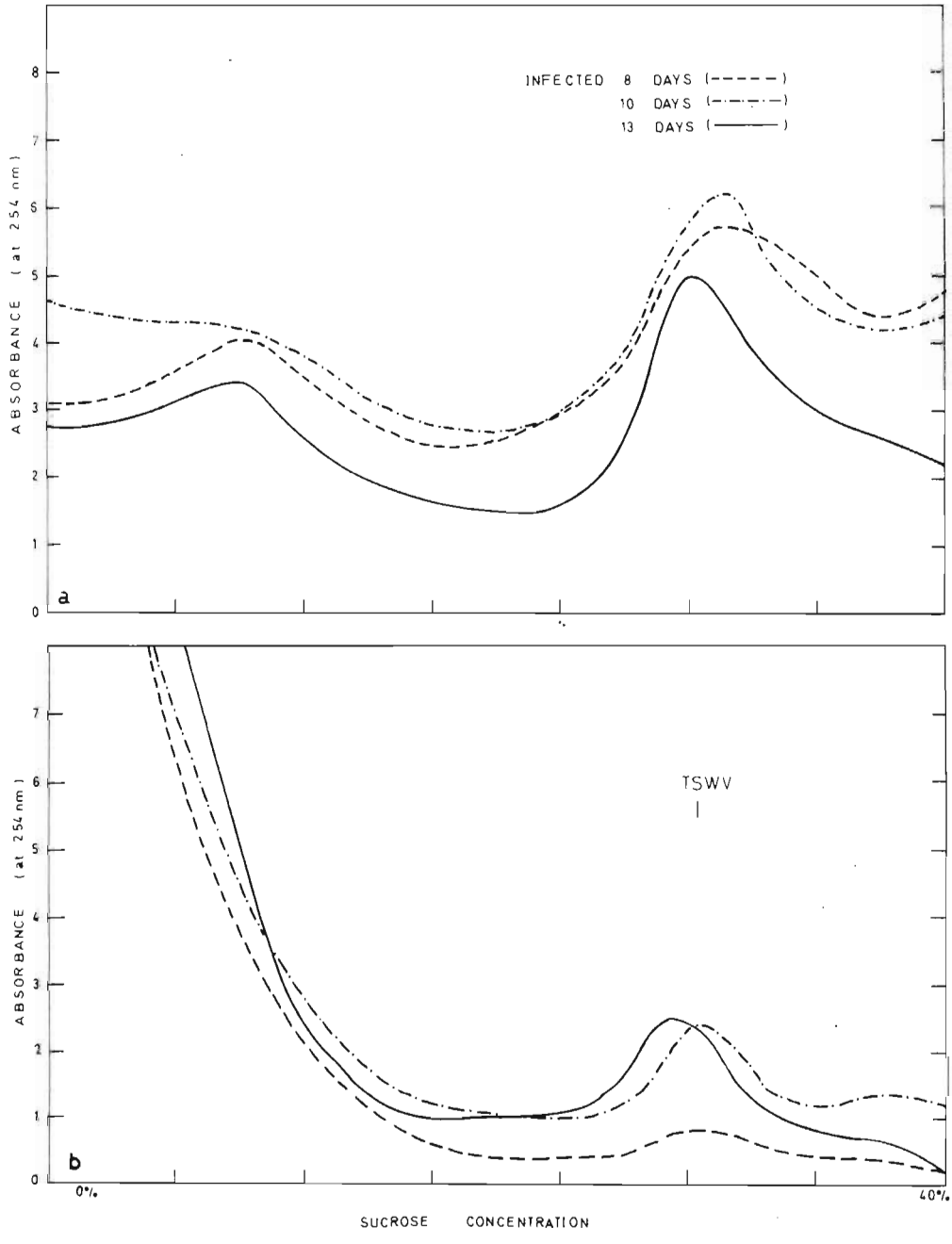


FIGURE 27. Gradient profiles of S_4-stage root extracts from 8-, 10- or 13- day TSWV-infected plants: a. clarified by centrifugation, omitting CGP(N) and AA; b. standard CGP(N) and AA clarification (TSWV incubation: 23°C; 12 h daily illumination)

boundary, due to increasing presence of virus (Fig. 27a). In contrast, corresponding extracts clarified with CGP(N) and AA, especially those of the 10th day and later, produced a distinct virus peak (Fig. 27b). Comparison of profiles in Fig. 27 reveals that CGP(N) and AA treatment reduced considerably the amount of non-viral material present at the depth of the virus in the gradient. A further example of the contribution of virus in CGP(N)/AA-treated extract to absorbance at the depth of the TSWV zone is given in Fig. 28. In the relevant experiment, a healthy root sample was extracted simultaneously with a TSWV-infected sample taken on the 14th day after inoculation.

A comparative extraction of roots cultured for 12 days after TSWV inoculation, in either Hoaglands solution or in Long Ashton nutrient medium lacking EDTA (Hewitt, 1952), showed the former to have no obviously detrimental effect on the level of recoverable TSWV. The relevant gradient profiles and S_4 infectivity assay results are presented in Fig. 29 and Table 27, respectively. In further experiments, substitution of a modified Hoaglands solution, from which ferrous sulphate and EDTA were omitted and ferric citrate was added daily as Fe^{+++} source, had no effect on extractable TSWV. Accordingly, Hoaglands solution was retained as the root nutrient medium in all subsequent clarification studies reported in this chapter.

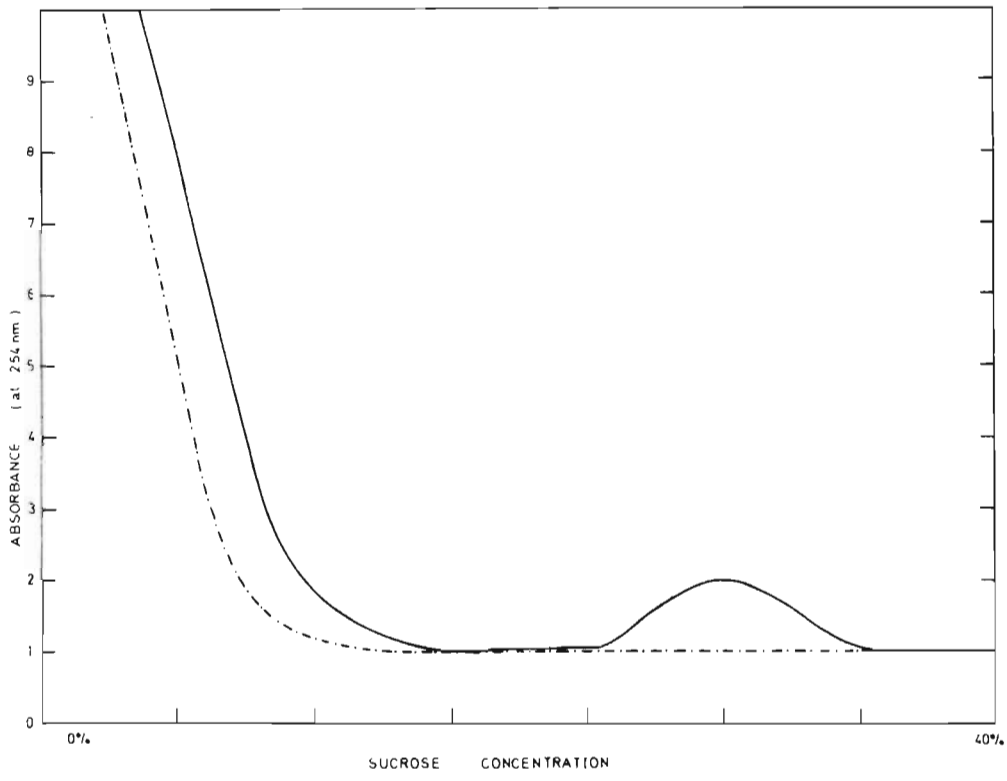


FIGURE 28. Gradient profiles of S_4 root extracts from healthy (-.-.-) and TSWV-infected plants (———) (TSWV incubation: 14 days at 23°C; 12 h daily illumination)

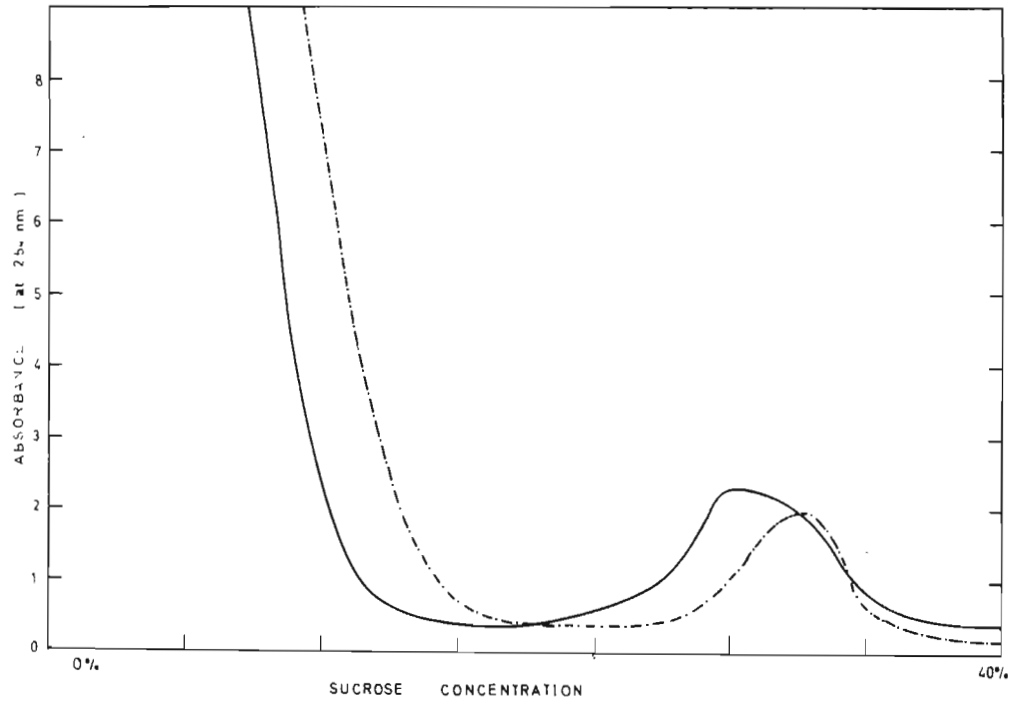


FIGURE 29. Gradient profiles of S_4 root extracts from TSWV-infected plants incubated with roots in (i) Hoaglands solution (————) or (ii) Long Ashton solution (-•-◦-◦-) (TSWV incubation: 12 days at 23°C; 12 h daily illumination)

TABLE 27. TSWV infectivity of diluted S_4 extracts (see Fig. 25) from attached roots kept in Hoagland or Long Ashton nutrient solution during 12-day TSWV incubation

S_4 dilution with 0,01M Na_2SO_3	Infectivity[b]	
	Hoagland	Long Ashton
1 : 14[a]	$\frac{1501*}{1225*} = \underline{100\%}$	$\frac{697}{1100*} = 52\%$
1 : 135	$\frac{567}{972*} = 49\%$	$\frac{230}{2200*} = 9\%$

[a] S_4 dilution at 1 : 14 to equate the volume to that of the S_1 supernatant

[b] Lesion totals on nine half-leaves of Petunia. Denominator: control. Infectivity expressed also as a percentage of the infectivity of the 1 : 14 diluted S_4 extract of Hoagland-incubated roots (italicized). Identical inocula are indicated by an asterisk.

Virus peak size in Uvicord profiles of CGP(N) and AA-clarified extracts was found to be influenced by the level of residual non-viral material present, the latter reflected in the height above the base line of portions of the curve adjacent to the virus peak. Thus in Fig. 27b the TSWV peak obtained on the 13th day after inoculation would probably have been slightly smaller had the level of clarification matched that of the 8th day material.

The pH of S_2 extract after standard treatment with 0,08 g CGP(N) and 0,015 g AA/ml, as set out in Fig. 25, was approximately 5,9. Extract similarly centrifuged but

not treated with either reagent was approximately neutral. Application of 0,009 g AA/ml to extract untreated with CGP(N) likewise reduced the pH to 5,9 but effected negligible clarification (Fig. 30). On the other hand,

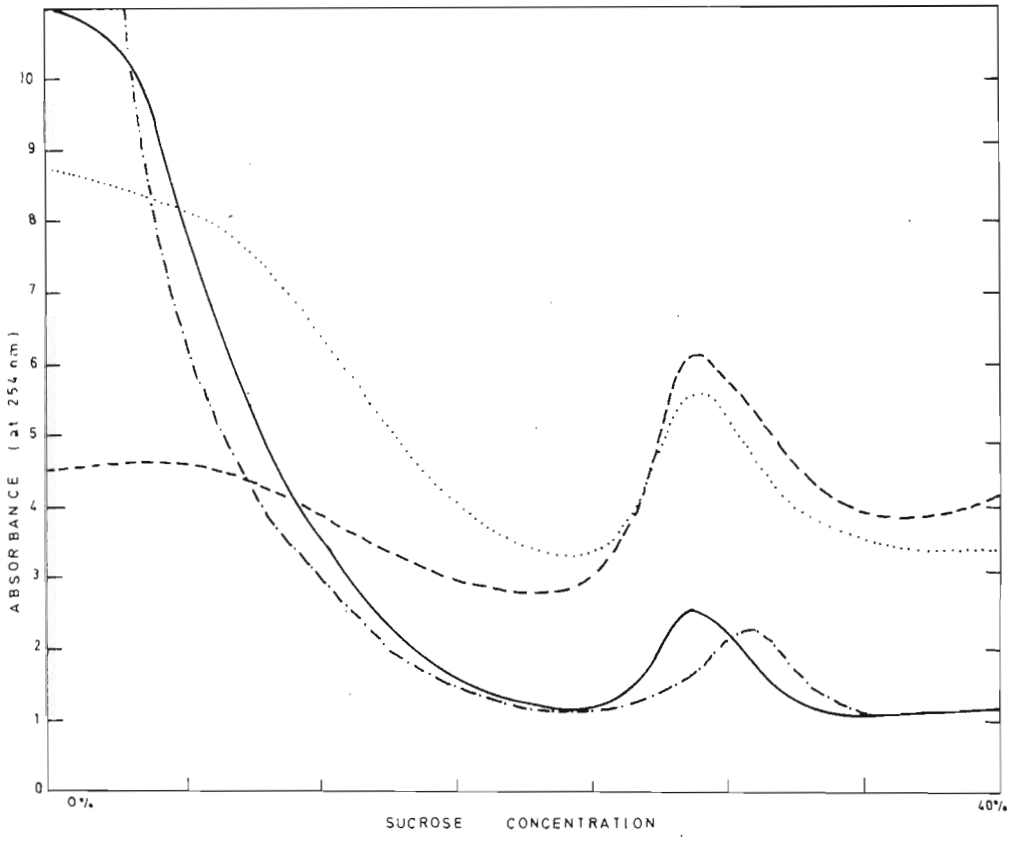


FIGURE 30. Gradient profiles of S_4-stage root extracts from TSWV-infected plants, clarified (i) without CGP(N) and AA (-------), (ii) with 0,009 g AA/ml (·····), (iii) with 0,04 g CGP(N)/ml and 0,011 g AA/ml (-·-·-), or (iv) with 0,08 g CGP(N)/ml and 0,015 g AA/ml (———) (TSWV incubation: 10 days at 23°C; 12 h daily illumination)

CGP(N) at 0,04 g/ml followed by 0,011g AA/ml lowered the pH to 5,9 and afforded clarification equal to that attained with the standard CGP(N) and AA application. As shown in Table 28, infectivity was considerably enhanced as a result of either 0,04 or 0,08 g CGP(N)/ml treatment, possibly due to inhibitor removal by the CGP(N).

TABLE 28. TSWV infectivity of S_4-stage root extracts clarified with or without CGP(N) and/or AA

Exp. No.	Days between inoculation and harvest	Infectivity of crude root homogenate[a]	Infectivity[a] (i)[b] No CGP(N) No AA	(ii) No CGP(N) 0,009g AA/ml	(iii) 0,04g CGP(N) 0,011g AA/ml	(iv) 0,08g CGP(N) 0,015g AA/ml
1a	8	16*/21*	33/11* = 6%			55/3* = 100%
1b	8	17*/19*				112/4*
2	10	6*/18*	31/8* = 15%	12/7*	144/3*	237/9* = 100%
3	11		449/695* = 38%			242*/142* = 100%

[a] Local lesion totals on six half-leaves of Petunia. Numerator: crude homogenate or clarified S_4 extract, the latter diluted 1:14 with 0,01 M Na_2SO_3 to equate the volume to that of the S_1 supernatant. Denominator: control. Identical inocula within an experiment are indicated by an asterisk.

[b] Infectivity is expressed, in this column, also as a percentage of the infectivity of the corresponding standard treatment extract in column (iv)(italicized)

Clarification effected by the standard procedure (Fig. 25) was also superior to that obtained when, after 0,04 g or 0,08 g CGP(N)/ml, the level of AA added, viz., 0,005 g or 0,01g/ml, respectively, was sufficient only to reduce the pH to 6,25. Fig. 31 shows the three gradient profiles recorded in this experiment. Infectivity of the S_4 extracts was not assayed but the profile of the standard treatment gradient is seen to include the expected virus peak.

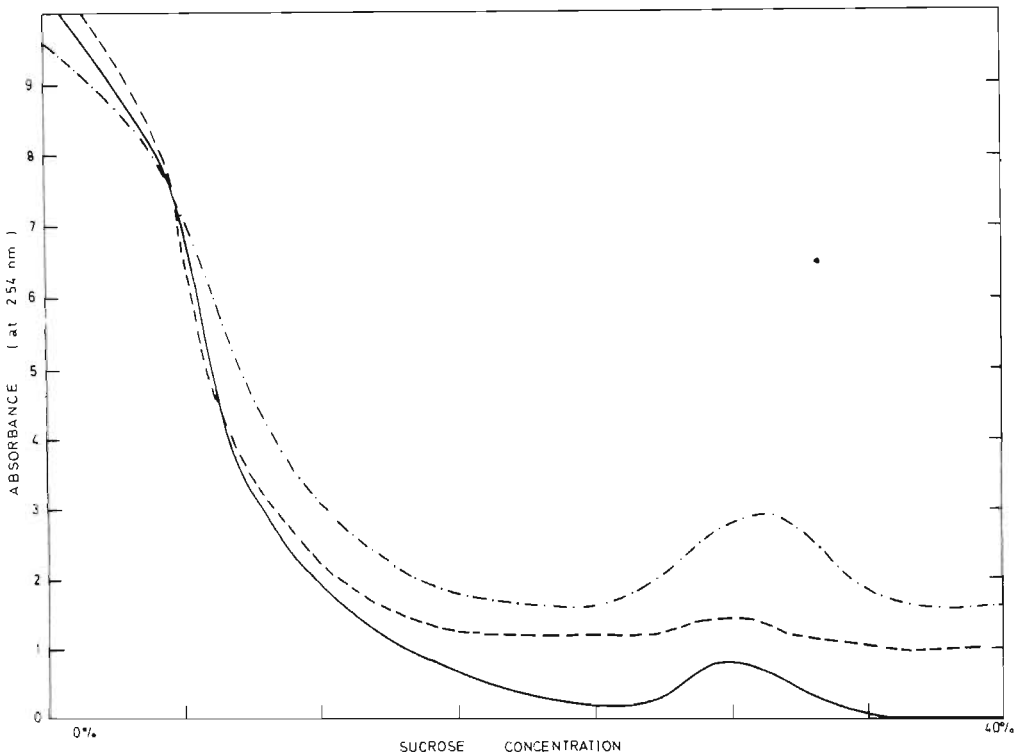


FIGURE 31. Gradient profiles of S_4-stage extracts from TSWV-infected plants clarified with (i) 0,04 g CGP(N)/ml and 0,005 g AA/ml (-·-·-), (ii) 0,08 g CGP(N)/ml and 0,01g AA/ml (-----) or (iii) 0,08g CGP(N)/ml and 0,015g AA/ml (———) (TSWV incubation:

Conversely, clarification and infectivity were unchanged relative to that obtained using the standard 0,015g AA/ml treatment, after the level of AA had been increased to 0,02g/ml.

Dilution of extract with 1 vol 0,05 M Na_2SO_3 instead of 3 vol, immediately after AA addition caused likewise no significant change in either clarification or infectivity. Complete omission of the Na_2SO_3 dilution step had also little effect, resulting in slightly superior clarification (Fig. 32) but reduced infectivity (Table 29).

TABLE 29. Effect of omitting dilution with 1 volume 0,05 M Na_2SO_3 immediately after standard CGP(N) and AA application, on TSWV infectivity at S_4 and Z root extract stages (see Fig. 25)

Extraction stage (Fig.25)	Diluted with 1 vol 0,05 M Na_2SO_3 (+) or Not diluted (-)	Infectivity[a]		
		Control	Test	Test % Control
S_4	+	75*	79*	105
	-	60*	47	78
Z	+	34*	60	176
	-	107*	171	160

[a] Local lesion totals on six half-leaves of Petunia. S_4 and Z extracts diluted 1:14 and 1:4, respectively, with 0,01 M Na_2SO_3 to equate the volume to that of the S_1 supernatant. Identical inocula are indicated by an asterisk.

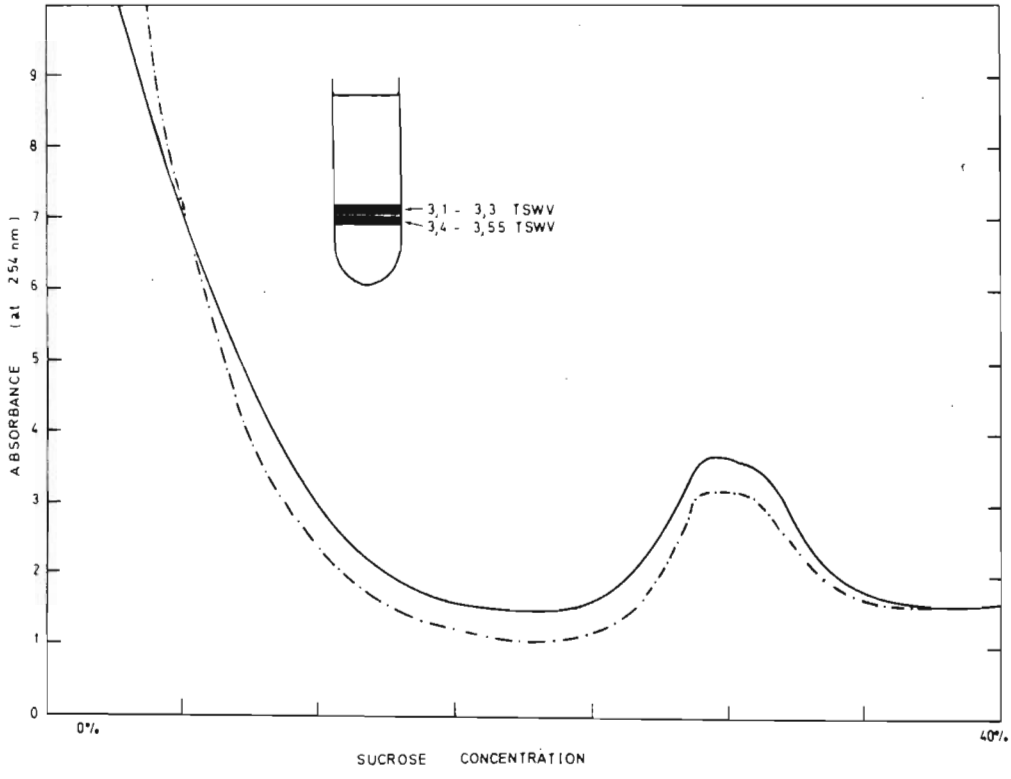


FIGURE 32. Gradient profiles of S_4-stage root extracts (i) undiluted (-·-·-) or (ii) diluted with 1 vol 0,05 M Na_2SO_3 (———) after standard CGP(N) and AA application (TSWV incubation: 10 days at 23°C; continuous, half-intensity illumination)

A verified consequence of not diluting the extract with Na_2SO_3 immediately following AA addition is that, until resuspended after the first high speed centrifugation, the virus remains at the relatively low pH of 5,9. In this experiment, as in most others reported in this

chapter, examination of the gradient tubes in the dark under a pencil-beam of light, revealed two adjacent light-scattering, TSWV bands in each tube. The depth and width of these bands was identical in the two tubes, as indicated in the gradient tube representation in Fig. 32. The occurrence of paired bands was revealed also by a shouldered form of virus peak in the gradient profile. The abovementioned results presented in Fig. 32 and Table 29 were obtained using roots of plants subjected to continuous, half-intensity lighting throughout the 10-day incubation period.

One or more washings of the final low speed pellet, P_4, with small volumes of 0,01 M Na_2SO_3, whether after earlier CGP(N) and AA treatment or not, was found to be not justifiable, recovering negligible additional virus.

Gradient profiles obtained in two experiments in which CGP(S) or NaGP were substituted for CGP(N) as clarifying agent, are shown in Fig. 33a and b. Clarification obtained with 0,08 g CGP(S)/ml extract was macroscopically comparable to that achieved with equivalent CGP(N), the gradient in each CGP(S) extraction likewise displaying paired TSWV bands. During clarification, addition of the CGP(S) brought the pH of the extract to 6,3, which was about 0,6 lower than when equivalent CGP(N) was used. In Experiment 1 only 0,008g AA/ml, and in Experiment 2 0,012 g AA/ml, was needed further to reduce the pH to 5,9. Both such CGP(S) - clarified extracts used for layering were highly infectious (Table 30). In contrast, NaGP at 0,08 g/ml was significantly less effective. Application of the NaGP raised the pH of the

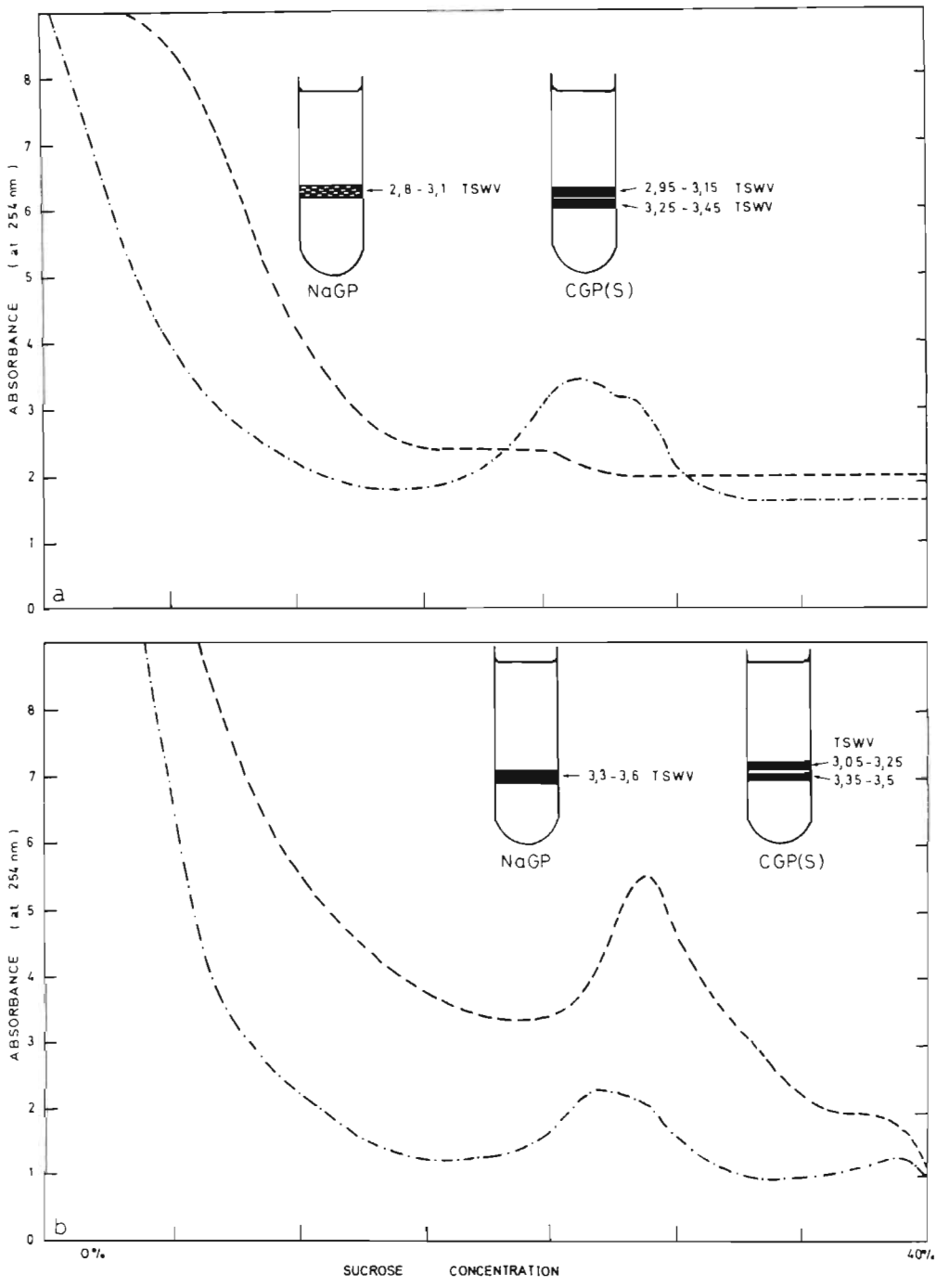


FIGURE 33. Gradient profiles of S_4-stage root extracts from TSWV-infected plants, clarified with CGP(S) (-.-.-.)or NaGP (-----) at 0,08 g/ml extract

a. Experiment 1: Extracted on 9th day after TSWV inoculation; 0,0085g and 0,03 g AA/ml, respectively, added to CGP(S)- and NaGP-treated extracts

b. Experiment 2: Extracted on 12th day after TSWV inoculation; 0,012 g AA/ml added both to CGP(S)- and NaGP-treated extracts (TSWV incubation: 23°C; 12 h daily illumination)

extract in Experiment 1 to 8,3, subsequent addition of as much as 0,03 g AA/ml reducing it only to 6,2. The resultant S_4-stage extract was almost non-infective (Table 30) and no distinct virus zone was discernable in the gradient (Fig. 33a). When, in Experiment 2, AA addition was limited to 0,012 g/ml a single virus zone was produced in the gradient (Fig. 33b) and infectivity was partly restored (Table 30).

TABLE 30. TSWV infectivity of S_4-stage root extracts after clarification with (i) 0,08 g CGP(S)/ml and 0,0085 g or 0,012 g AA/ml, or (ii) 0,08 g NaGP/ml and 0,03 g or 0,012 g AA/ml or (iii) omitting CGP(S), NaGP and AA

Exp. No.	AA g/ml extract	Infectivity[a]		
		CGP(S)	NaGP	Without CGP (S) or NaGP
1	0,0085	$\frac{433*}{462*}$ = 100%		
	0,03		$\frac{9}{673*}$ = 1,5%	
2	0,012	$\frac{852*}{586*}$ = 100%	$\frac{172}{328*}$ = 36%	
	nil			$\frac{122}{450*}$ = 19%

[a] Lesion totals on nine half-leaves of Petunia. Numerator: clarified extract diluted 1:14 with 0,01 M Na_2SO_3 to equate volume to that of the S_1 supernatant. Denominator: control. Identical inocula within an experiment are indicated by an asterisk. Infectivity expressed also, in each experiment, as a percentage of the infectivity of the corresponding CGP(S) treatment (italicized)

Dual Infection with TSWV and TMV

Nine days after inoculation with a concentrated, crude suspension of TSWV supplemented with approximately 0,05 mg/ml TMV, tomato plants maintained at 23°C and receiving 12 h daily lighting, displayed marked localized necrotic deterioration of upper, and total necrotic collapse of lower, inoculated leaves. By the 11th day at least 50% of inoculated tissue had been destroyed. The identical TSWV inoculum without added TMV induced neither necrosis nor chlorosis of the inoculated leaves.

In contrast, systemic symptoms were not intensified as a result of dual virus inoculation: inoculation with one or both viruses induced moderately severe, 1-2 mm, necrotic flecking. Roots infected with both viruses, however, were markedly browner and more flaccid than were roots containing TSWV alone.

Sucrose gradient profiles derived after standard clarification (Fig. 25) of root extracts from plants concurrently inoculated with both viruses are shown in Fig. 34. The extractions were performed immediately after harvesting on the 9th, 11th and 12th days following inoculation. Assay of the S_4 extracts prepared on the 11th and 12th days, diluted 1:14 with 0,01 M Na_2SO_3, each resulted in 10-24 lesions per Petunia half-leaf. The extract of the 9th day was not assayed. Similar results were obtained when homogenization and extraction were performed after overnight storage of the severed root systems in a saturated atmosphere at 2-5°C.

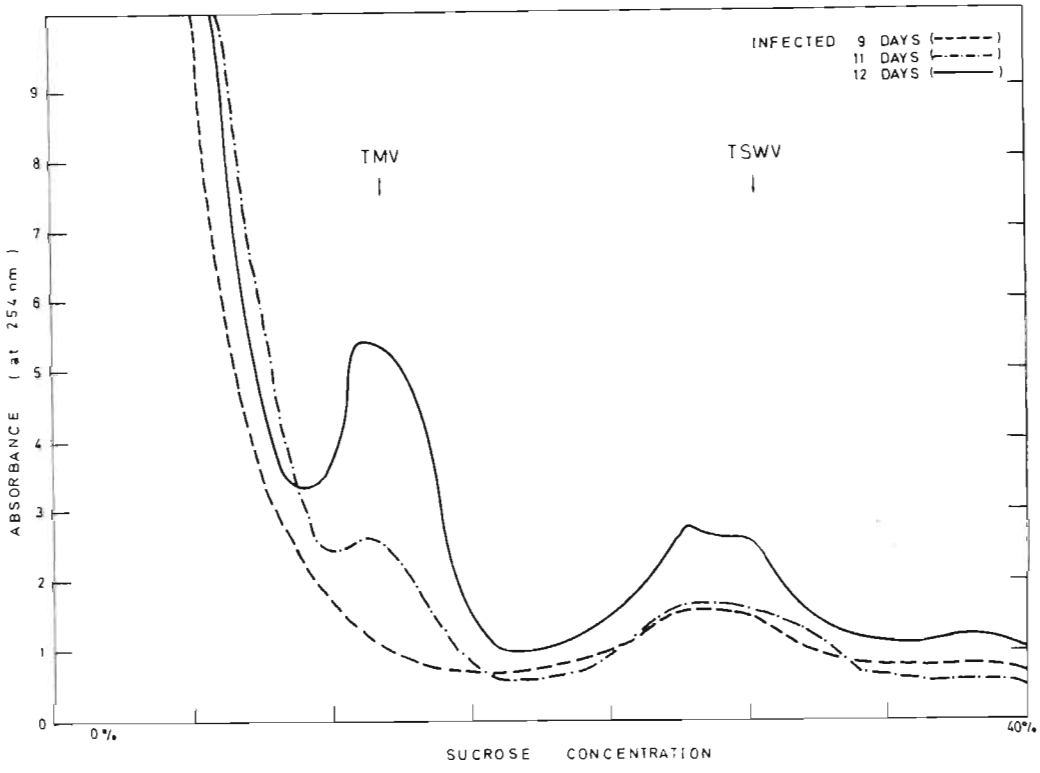


FIGURE 34. Gradient profiles of S_4 root extracts from plants inoculated 9, 11 or 12 days previously with TSWV + 0,05 mg TMV/ml (Incubation: 23°C; 12 h daily illumination)

At 20°C and 12 h daily illumination symptom development on the aerial parts of plants concurrently inoculated with concentrated TSWV and 0,05 mg TMV/ml was considerably milder than at 23°C. At the lower temperature plants infected with both viruses were not recognizably different from those without TMV. However, harvested on the 13th day, root systems infected also

with TMV were, as at 23°C, noticeably browner and poorer in condition than those infected with TSWV alone. In particular, the former displayed many brown, 2-4 mm lesions on the young, aerial roots originating from stem bases in the humid atmosphere prevailing between the polystyrene cover and nutrient liquid surface.

The infectivity of S_4 root extracts prepared from such TSWV- or TSWV + TMV- infected plants harvested on the 13th day, is compared in Table 31. Corresponding gradient profiles are given in Fig. 35. The extract

TABLE 31. TSWV infectivity of clarified S_4 root extracts from plants inoculated 13 days previously with either TSWV alone or TSWV + 0,05 mg TMV/ml

Extract dilution[a]	S_4 infectivity[b]	
	TSWV	TSWV + TMV
(i) 1:14	$\frac{915*}{721*}$ = 100%	$\frac{362}{341*}$ = 84%
(ii) 1:119	$\frac{166}{924*}$ = 14%	$\frac{228}{633*}$ = 28%

[a] S_4 extracts diluted with 0,01 M Na_2SO_3 at 1:14 to equate the volume to that of the S_1 supernatant

[b] TSWV lesion totals on nine half-leaves of *Petunia*. Denominator: control. Infectivity expressed also as percentages of the infectivity of the italicized, TSWV extract. Identical inocula are indicated by an asterisk.

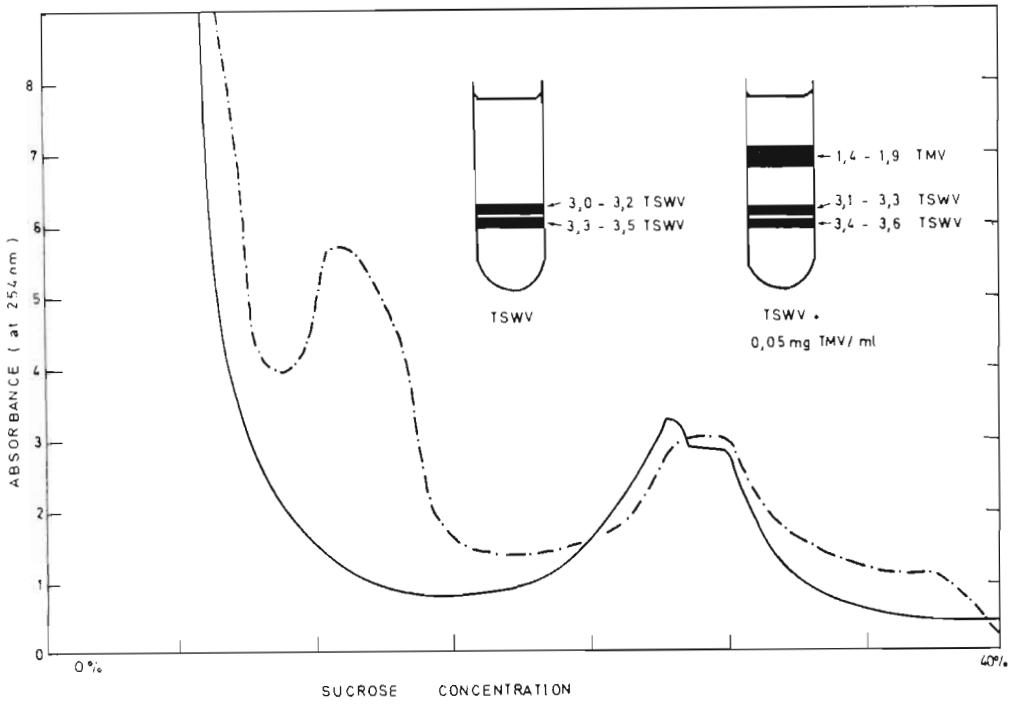


FIGURE 35. Gradient profiles of S_4 root extracts from plants inoculated 13 days previously with TSWV alone or TSWV + 0,05 mg TMV/ml (TSWV incubation: 20 °C; 12 h daily illumination)

lacking TMV yielded a slightly more pronounced TSWV upper zone, which finding was confirmed in profiles obtained from duplicate gradients. Visual inspection of the gradients in a beam of light revealed two adjacent TSWV bands in each, more distinctly separated in tubes lacking TMV.

In Fig. 35 it can be seen that profile height above the base line in regions adjacent to the TSWV peak is less in the case of extract from roots infected only with TSWV.

The general similarity of the peaks obtained from singly and doubly infected extracts appears not to be contradicted by the infectivity assay results obtained with the S_4 material used for layering (Table 31). These results also reveal an inhibitory effect operating to reduce the TSWV infectivity of the more concentrated of the two inocula containing TMV. That this is probably due to the TMV was established in other studies. For example: crude root homogenate containing TSWV and producing 43 lesions/half-leaf (= 100%) on Petunia showed only 55% and 45% infectivity, respectively, when supplemented with 0,33 or 1 mg TMV/ml (final concentration). This effect, which superimposes on that caused by the natural TSWV inhibitor present in tomato roots (Milne, 1960), complicates the comparative interpretation of lesion counts in the TSWV/TMV/tomato roots/Petunia system. A study in which purified TMV was included in the 0,01 M Na_2SO_3 used to resuspend a P_3-stage pellet (Fig. 25) indicated that approximately 1 mg TMV gives rise, in the ensuing rate zonal gradient, to a visible, approximately 2 mm, TMV zone.

In Fig. 36 are presented the profiles obtained when TMV infection at 23°C was initiated either one or two weeks prior to TSWV inoculation. TMV inoculation was performed on both occasions by spot-inoculating every third leaflet on a plant with a suspension of either (i) 0,05 mg TMV/ml or (ii) 0,0005 mg TMV/ml, containing carborundum. At the subsequent TSWV inoculation all leaflets and stems were uniformly inoculated, after which

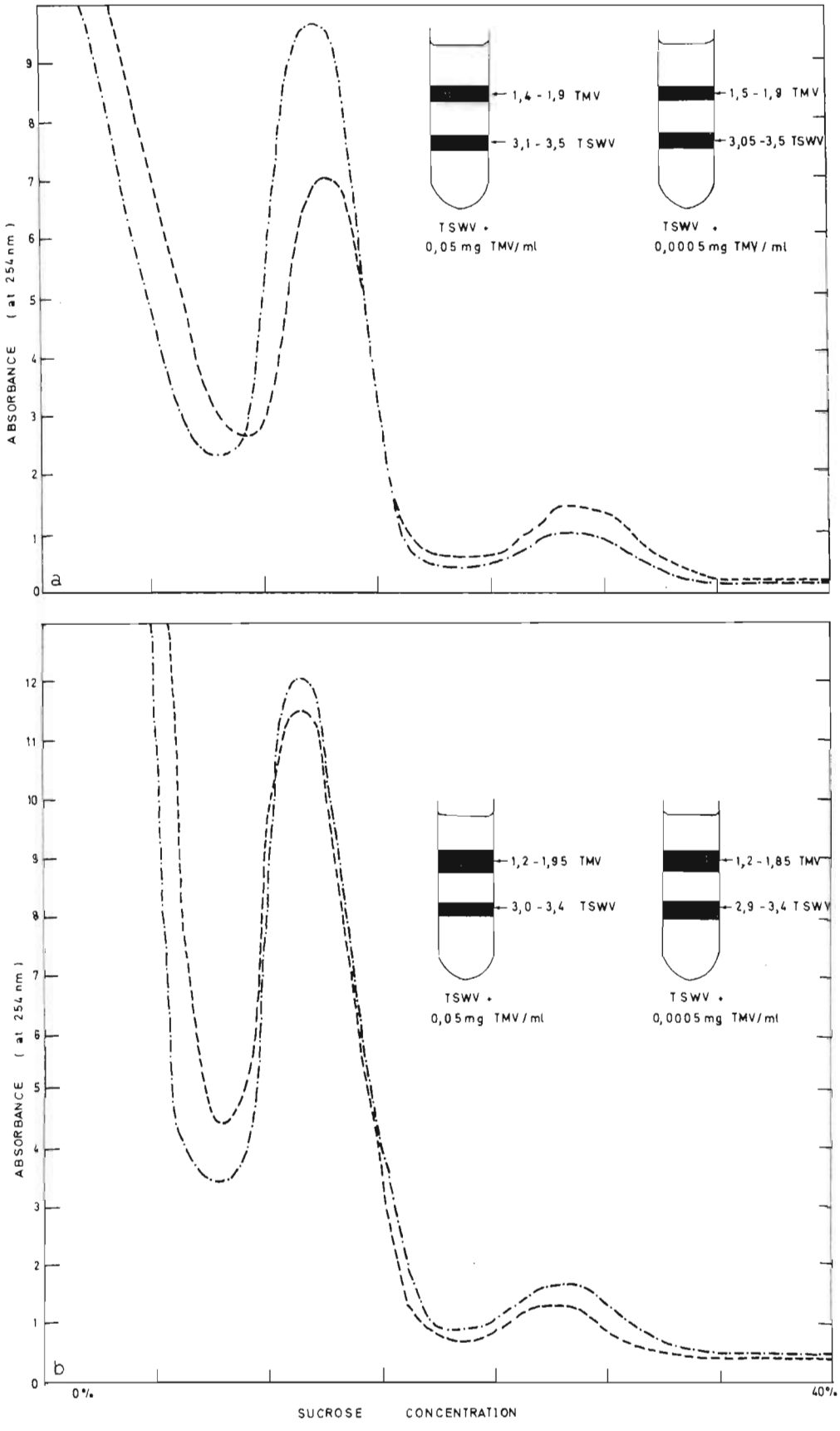


FIGURE 36. Gradient profiles of S_4 root extracts from plants inoculated with TMV at 0,05 mg/ml (-•-•-•-) or 0,0005 mg/ml (- - -) prior to TSWV inoculation and incubation for 11 days:

a. TMV inoculation 2 weeks previously

b. TMV inoculation 1 week previously

incubation at 23°C continued for a further 11 days. Comparison of the gradient profiles in Fig. 36 with each other and with that for the 11th day after simultaneous inoculation with 0,05 mg TMV/ml and TSWV (Fig. 34) affords no evidence that either level of clarification or TSWV yield is increased by prior TMV inoculation. Infectivity assay on Petunia (Table 32) revealed only a possible tendency for reduced TSWV lesion counts from roots of plants more heavily inoculated with TMV two weeks previously. The most severe foliar symptom development after the TSWV inoculation was observed in plants to which the concentrated TMV inoculum had been applied one week previously (Table 32). Also noteworthy in Fig. 36 are :

(i) in no gradient was a double-banded TSWV zone observed, but the shouldered form of the peak persisted;

(ii) recovery of TMV was appreciably greater from plants infected for only one week prior to TSWV inoculation.

In a similar experiment, plants heavily inoculated with TSWV were subsequently spot-inoculated with 0,0005 mg TMV/ml on the sixth day of TSWV incubation at 23°C. Plants infected with both viruses were then immediately transferred to 26°C, whilst others not inoculated with TMV remained at 23°C. All plants received 12 h daily illumination. S_4 extract from roots of the reinoculated plants gave rise to distinctively sharp, paired TSWV zones in the sucrose gradient (Fig. 37a, (iii)) and displayed greater TSWV infectivity (Table 33, Exp. 1d). In contrast, extensive inoculation of all leaves with TMV (0,0005 mg TMV/ml) on the sixth day and continuing incubation at 23°C, resulted in only limited S_4 infectivity (Table 33, Exp. 2). The gradient profile for this

TABLE 32. Effect of TMV infection for either 1 or 2 weeks prior to TSWV inoculation on (i) symptom development at 23°C with 12 h daily illumination and (ii) TSWV infectivity of 11th day S_4 root extract

Interval between TMV and TSWV inoculations (weeks)	TMV inoculum (mg/ml)	Symptom development		S_4 infectivity[b]
		Leaf necrosis[a]	Roots	
2	0,05	I: moderate; 7 S: moderate	Uniformly light brown lacking discrete lesions	$\frac{633}{844*}$ = 72%
	0,0005	I: slight; 8 S: moderate	"	$\frac{613*}{595*}$ = 100%
1	0,05	I: moderate; 8 S: very severe	"	$\frac{492}{453*}$ = 106%
	0,0005	I: slight; 8 S: moderate	"	$\frac{377}{368*}$ = 99%

[a] I = inoculated leaves (localized necrosis); day of first appearance after TSWV inoculation

S = systemically infected leaves (interveinal necrosis)

[b] TSWV lesion totals on nine half-leaves of Petunia. Numerator: S_4 extracts assayed after 1:14 dilution with 0,01 M Na_2SO_3 to equate the volume to that of the S_1 supernatant. Infectivity expressed also as a percentage of the infectivity of the S_4 extract shown as 100% (italicized). Identical inocula are indicated by an asterisk.

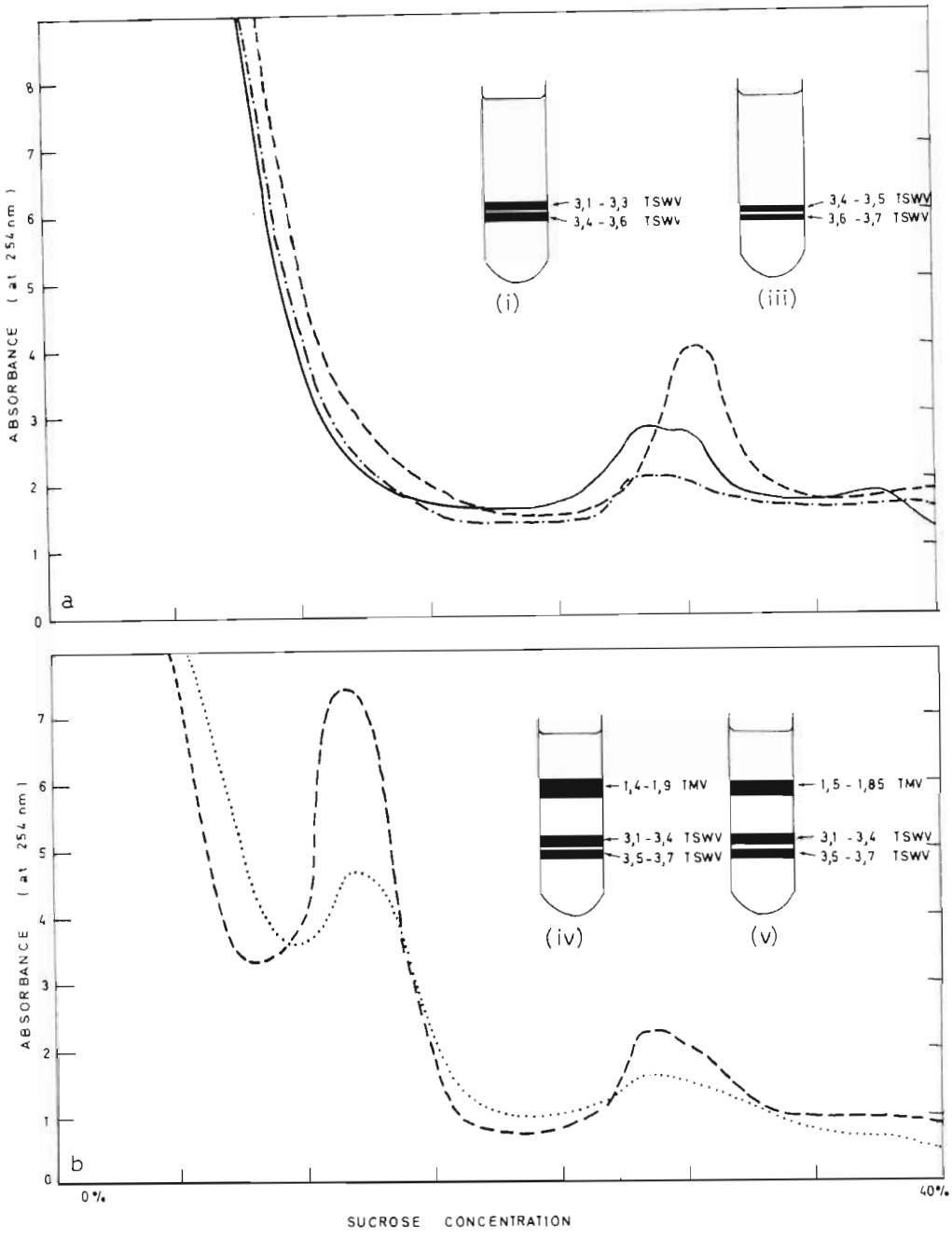


FIGURE 37. Gradient profiles of S_1 root extracts from TSWV-infected plants subsequently inoculated with TMV:

a. TSWV inoculation followed by (i) incubation at 23 °C for 12 days (———), (ii) incubation at 23 °C for 12 days, 0,0005 mg TMV/ml inoculated to all leaves on the sixth day (-·-·-·), (iii) incubation at 23 °C for six days, spot inoculation with 0,0005 mg TMV/ml on the sixth day, further incubation at 26 °C for six days (– – – –);

b. TSWV inoculation followed immediately by spot-inoculation with (iv) 0,05 (– – –) or (v) 0,0005 mg TMV/ml (·····); incubation at 23 °C for six days followed by 26 °C for six days.

TABLE 33. Effect of delayed TMV inoculation on TSWV infectivity of S_4 root extracts prepared 12 days after TSWV inoculation

Exp. No.	Inoculation[a]		Incubation temp. (°C)[b]	S_4 infectivity[c]
	Day	Inoculum		
1a	1	TSWV	23	$\frac{766}{426*}$ = <u>100%</u>
b	1	TSWV; 0,05 mg TMV/ml	23;26	$\frac{229}{327*}$ = 39%
c	1	TSWV; 0,0005 mg TMV/ml	23;26	$\frac{592}{473*}$ = 70%
d	1	TSWV		
	6	0,0005 mg TMV/ml	23;26	$\frac{1341}{461*}$ = 162%
2	1	TSWV		
	6	0,0005 mg TMV/ml	23	$\frac{198}{808}$ = 20%

[a] TSWV (Exp. 1 and 2), TMV (Exp. 2): complete inoculation, all leaves and stems. TMV (Exp. 1): spot-inoculation of each third leaflet only

[b] In Exp. 1b-d plants were incubated for six days at 23°C followed by six days at 26°C. All plants in both experiments received 12 h daily illumination

[c] TSWV lesion totals on nine half-leaves of <u>Petunia</u> Numerator: S_4 extract assayed after 1:14 dilution with 0,01 M Na_2SO_3 to equate volume to that of the S_1 supernatant. Denominator: control. Identical inocula are indicated by an asterisk. Infectivity in Exp. 1a-d expressed also as a percentage of the infectivity of the italicized, TSWV extract. Similarly in Exp. 2 infectivity was 20% of that shown by the corresponding S_4 extract of roots infected with TSWV only.

extraction similarly reflects a low TSWV content (Fig. 37a, (ii)). No TMV zone was detected in either gradient derived from plants inoculated with TMV on the sixth day. Profile heights adjacent to the TSWV peak in each gradient were almost identical, indicating that a similar level of clarification was attained in the two extractions.

Spot-inoculation with 0,05 or 0,0005 mg TMV/ml three h after normal TSWV inoculation, followed by successive incubation at 23°C and 26°C, each for six days, did not significantly enhance TSWV peak size or reduce the level of non-viral material present (Fig. 37b). The comparatively diminished TSWV infectivity displayed by the S_4 inocula from roots containing both viruses (Table 33, Exp. 1b and 1c) probably reflects differences in inhibitory effects depending on the specific TMV level present (Fig. 37b).

Continuous illumination during TSWV incubation at 23°C changed the response of inoculated leaves to infection. Whereas leaves experiencing a daily dark period of approximately 12 h remained symptomless, as is characteristic of almost all TSWV strains (Table 2), under continuous lighting chlorotic blotching and scattered necrosis was noticeable on the fourth day. By the 12th day most inoculated leaves were dead. Systemic symptoms, in particular chlorosis, were slightly intensified. A schedule of foliar symptom development, which was only marginally more severe in plants fully inoculated also with 0,05 mg TMV/ml three h after inoculation, is given in Table 34. Roots infected with both viruses did not

TABLE 34. Foliar symptoms associated with TSWV or TSWV and TMV[a] infection at 23°C under continuous illumination

Incubation period (days)	Foliar symptoms[b]	
	TSWV	TSWV and TMV
1 - 2	-	-
3	I: feint chlorotic blotches	I: as for TSWV
4	I: chlorotic blotches and scattered necrosis S: epinasty	I:) as for TSWV S:)
5 - 7	I: intensified chlorosis and increased scattered necrosis S: epinasty; 5-12cm leaves rolled and curled downwards, slightly chlorotic	I:) as for TSWV S:)
8 - 9	I: lower leaves dead; upper show marked necrotic flecking and chlorosis S: tops reduced, crumpled, drooping, with scattered 1-2 mm necrosis	I:) necrosis slightly more extensive than on corresponding TSWV plants S:)
12	I: most leaves dead; a few upper inoculated leaflets yellow with necrotic flecks S: very youngest leaves epinasic and chlorotic but mostly not necrotic; scattered to severe bronzing and necrosis on older leaves	I: all leaves dead S: slightly more severe than TSWV

[a] Complete inoculation with 0,05 mg TMV/ml, three h after TSWV inoculation

[b] I = inoculated leaves; S = systemic infection

differ in appearance, being markedly discoloured, without discrete lesions. Results of S_4 infectivity assays performed on the 8th, 10th and 12th days after inoculation are given in Table 35a. Zone distribution in the corresponding centrifuged gradient tubes is presented in Table 35b, whilst in Fig. 38 the gradient profiles of the 10th day extraction are shown. The combined evidence from infectivity assay and UV absorbance is that extractible TSWV was comparatively reduced in roots infected with both viruses.

The results of an experiment comparing the TSWV infectivity of gradient virus zone (Z) material (Fig. 25) derived from plants inoculated with only TSWV or, two days later, with 0,05 mg TMV/ml also, are given in Table 36. Plants infected only with TSWV received continuous illumination during the incubation period, whereas the plants infected with both viruses were illuminated for 12 hr daily. Incubation was at 23° C for 10 days. Assays on N. glutinosa and Petunia both showed the virus zone material from singly infected roots to be slightly the more infective. No TMV-type lesions developed on the N. glutinosa leaves used for either inoculation. In the gradient containing no TMV the TSWV zone comprised the usual adjacent narrow bands, whilst the other tube contained a single, broader TSWV band.

The experiment also examined TSWV infectivity at other stages of extraction. These results, using Petunia as local lesion host, are set out in Table 37. The three

TABLE 35. TSWV recovery from roots of plants continuously illuminated at 23°C after inoculation with TSWV or TSWV and TMV[a]

a. TSWV infectivity of S_4 extract

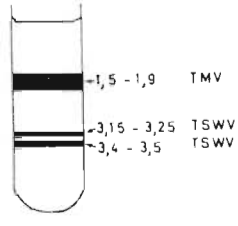
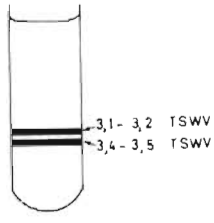
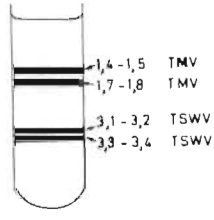
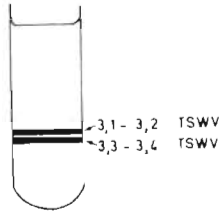
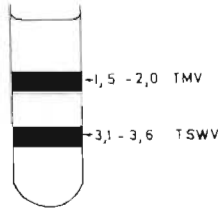
Interval before extraction (days)	S_4 infectivity[b]		
	Dilution with 0,01 M Na_2SO_3	TSWV	TSWV + TMV
8	1 : 14		62
10	1 : 14	$\frac{380}{210}$ = 100%	$\frac{74}{66}$ = 62%
	1 :135	$\frac{56}{143}$ = 22%	$\frac{30}{216}$ = 8%
12	1 : 14	$\frac{159}{115}$ = 100%	$\frac{71}{133}$ = 39%
	1 :135	$\frac{44}{100}$ = 32%	$\frac{0}{64}$ = < 1%

[a] = Full inoculation with 0,05 mg TMV/ml three h after TSWV inoculation

[b] = TSWV lesion totals on nine half-leaves of *Petunia*. Denominator: control, common only to treatments of the same day. Infectivity expressed also as a percentage of the infectivity of the italicized TSWV extract of the same day

TABLE 35 (cont.)

b. Gradient appearance

Interval before extraction (days)	Gradient appearance	
	TSWV	TSWV + TMV
8		
10		
12		

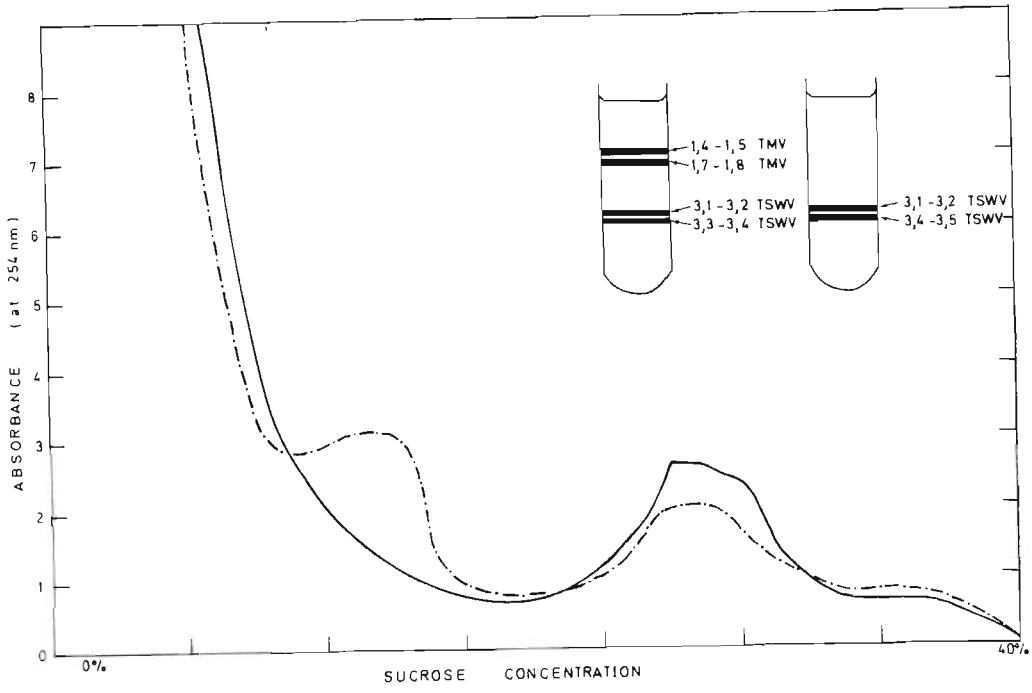


FIGURE 38. Gradient profiles of S_4 root extracts from plants illuminated continuously after inoculation: inoculated only with TSWV (———); fully inoculated three h later also with 0,05 mg TMV/ml (-•-•-) (TSWV and TMV incubation : 10 days at 23°C)

stages designated 'standard root extract', 'S_4 (1:14)' and 'Z(1:7)', were at approximately equivalent dilution. The infectivity levels recorded reflect clearly the inhibitory effect of tomato root inhibitor and TMV. In the results derived from roots infected only with TSWV it is evident that comparatively little of the TSWV infectivity present in the S_4 extract was recovered in the gradient zone. The apparently converse result obtained when extracting TSWV from extract containing

also TMV, shows clearly the inhibitory effect of the TMV. The infectivity of this extract at the S_4 stage is of the same order as that displayed by roots of plants simultaneously inoculated with both viruses, presented at the start of this section in relation to Fig. 34.

TABLE 36. TSWV infectivity of sucrose gradient virus zone, Z, (Fig. 25) derived from roots infected only with TSWV or also with TMV[a] at 23°C

Assay host	Infectivity of[b] TSWV virus zone	
	TSWV	TSWV and TMV
N. glutinosa	$\frac{204}{200}$ = 100%	$\frac{105}{163}$ = 63%
Petunia	$\frac{206}{39}$ = 100%	$\frac{86}{28}$ = 55%

[a] Full inoculation with 0,05 mg TMV/ml two days after TSWV inoculation

[b] TSWV lesion totals on six half-leaves Denominator: control, which differed for the two hosts. Zone infectivities compared also as percentages on each host

TABLE 37. Comparative TSWV infectivity of extracts from roots infected only with TSWV or also with TMV[a], assayed at successive stages during TSWV extraction

Extraction stage (Fig. 25)	Infectivity[b]		
	TSWV[c]	TSWV + TMV[d]	
		(i)	(ii)
Standard root extract	$\frac{180}{65}$ = 48%	$\frac{31}{41}$ = <1%	129%
" " (1:9)	$\frac{115}{29}$ = 69%	$\frac{13}{116}$ = <1%	19%
S_4 (1:14)	$\frac{172}{30}$ = 100%	$\frac{24}{41}$ = <1%	100%
" " (1:19)	$\frac{76}{41}$ = 32%	$\frac{3}{59}$ = <1%	9%
Z	$\frac{206}{39}$ = 92%	$\frac{86}{28}$ = 54%	525%
" (1:7)	$\frac{24}{15}$ = 2%	$\frac{40}{41}$ = <1%	42%

[a] Full inoculation with 0,05 mg TMV/ml two days after TSWV inoculation

[b] TSWV lesion totals on six half-leaves of Petunia Denominator: control, a 1:1 mixture of S_4 (1:14) from TSWV and TSWV + TMV extractions

[c] TSWV infectivity expressed also as a percentage of the S_4 (1:14) infectivity (italicized).

[d] Column (i): TSWV infectivity expressed also as a percentage of the infectivity (italicized) of the abovementioned TSWV S_4 (1:14) extract

Column (ii): TSWV infectivity expressed as a percentage of the infectivity (italicized) displayed by this extract at S_4 (1:14) inoculation

Banding Within TSWV Zone

The observation that sucrose gradient centrifugation of extract from TSWV-infected roots clarified with CGP(N) or CGP(S) in the absence of TMV, invariably gives rise to two adjacent bands of approximately equal width in the TSWV zone, led to studies seeking to determine the cause. Two alternative possibilities were considered:

(i) the double band is an artefact produced by calcium glycerophosphate application, or

(ii) the double band is not an artefact, but (a) its presence or absence depends upon the interval between inoculation and extraction and/or (b) in the absence of clarification by calcium glycerophosphate, gradient overloading results in inferior resolution and the formation of only a single band.

Recovery of a typical double band from layered S_4-stage extract of roots harvested on the 12th day and clarified only by centrifugation (Fig. 39) afforded evidence that the double band is not an artefact of calcium glycerophosphate clarification. Likewise, delaying root harvesting and extraction until the 20th day after TSWV inoculation, by which time the recoverable level of TSWV had fallen significantly, produced no change in the ratio of the two bands to each other (Fig. 39). On the other hand, approximately 1 mg TMV added at the P_3 stage caused a slight deterioration of double band resolution. Significantly higher concentrations of TMV in the S_4 extract used for layering on the gradient invariably resulted in the occurrence of an undivided TSWV light-scattering zone.

The effect of reducing the duration of gradient centrifugation was also examined. A centrifugation time of 25 min was applied to approximate the operation at 25 000 rpm for 25 min used by Black et al. (1952, 1963) and also in the purification experiments (N2-4) reported in Chapter 1. In none of these earlier studies was adjacent TSWV bands encountered. Centrifuged for 25 min, clarified S_4 extract derived from 8 ml standard root extract (Fig. 25), gave rise to clearly discernible, paired TSWV bands at depths of 2,2 - 2,3 and 2,4 - 2,6 mm, respectively.

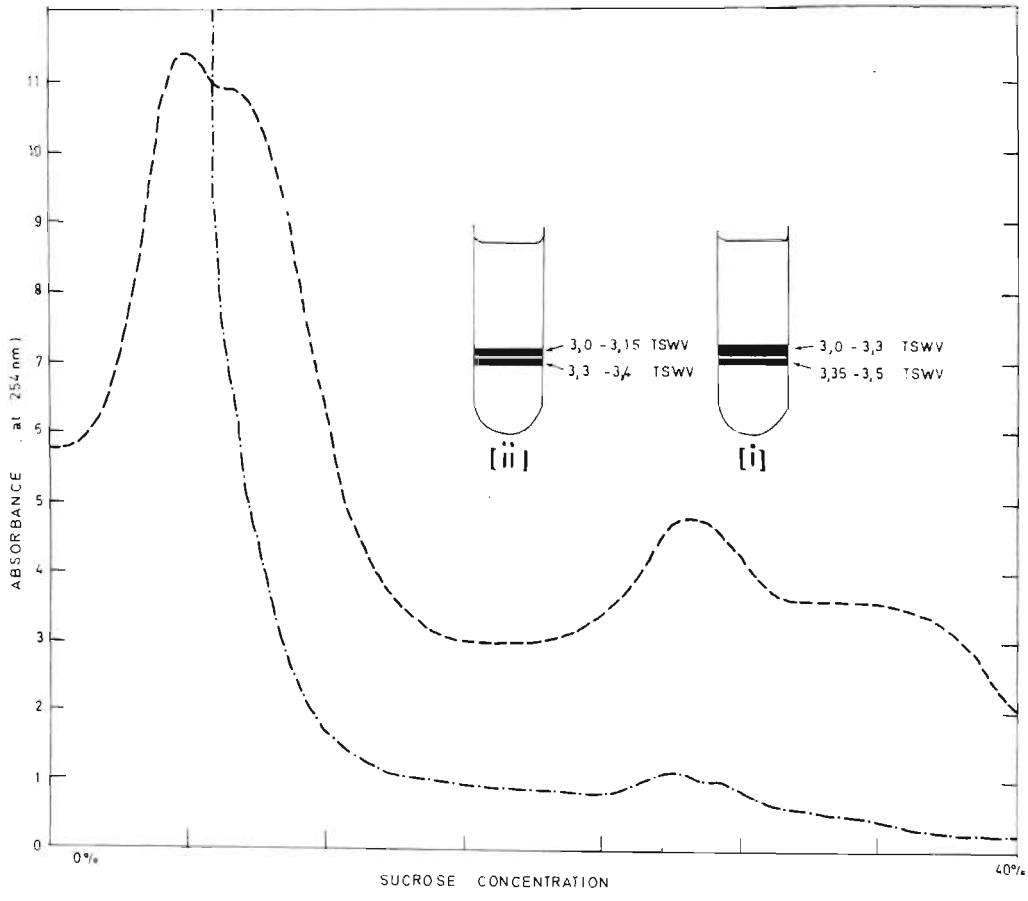


FIGURE 39. Gradient profiles of S_4-stage root extracts: (i) clarified only by centrifugation 12 days after inoculation (------); (ii) standard CGP(N)/AA clarification (Fig. 25) 20 days after inoculation (-·-·-)(TSWV incubation: (i) 23°C; continuous illumination, (ii) 23°C; 12 h daily illumination)

Also examined was the possibility of a relationship between the double bands recovered in recent studies and the more widely separated bands observed in Experiment N3 of Chapter 1 (see Fig. 11). In that study, 0,01 M $MgCl_2$ was included in the virus solvent and the sucrose gradient. However, in a similar test on the South African clone, use of 0,01 M Na_2SO_3 containing 0,01 M $MgCl_2$, to resuspend the P_3-stage extract (Fig. 25) and subsequently, throughout a second cycle of high and low speed centrifugation and rate zonal sucrose gradient centrifugation, resulted in the usual pair of adjacent TSWV bands. Inclusion of $MgCl_2$ therefore failed to reproduce a wide separation of bands encountered when this solvent was previously used at Wageningen. In the more recent study, aliquots of 0,1 ml were removed from the gradient immediately above the upper band and below the lower band, for infectivity assay. When diluted 1:20 with 0,01 M Na_2SO_3 the two were moderately and equally infective, producing approximately 15 lesions/half-leaf on Petunia Similar infectivity was displayed by the standard root extract when diluted 1:20. No enhancement of infectivity was observed when the abovementioned diluted upper and lower band samples were mixed in equal proportions and inoculated to Petunia.

Band infectivities were further examined by gradient fractionation after standard root extraction (Fig. 25). In one experiment, results of which were presented in part in Table 35, the TSWV zone obtained from roots extracted eight days after inoculation with TSWV and TMV was fractionated and the fractions assayed for infectivity on

Petunia. Fig. 40 shows the comparative infectivity of each fraction. The result reflects a slight superiority in the infectivity of the lower band material. Inoculation of Samsun NN to detect residual, contaminating TMV in the pooled fractions proved negative. The latter inoculation was made after the fractions had been kept 24 h at room temperature.

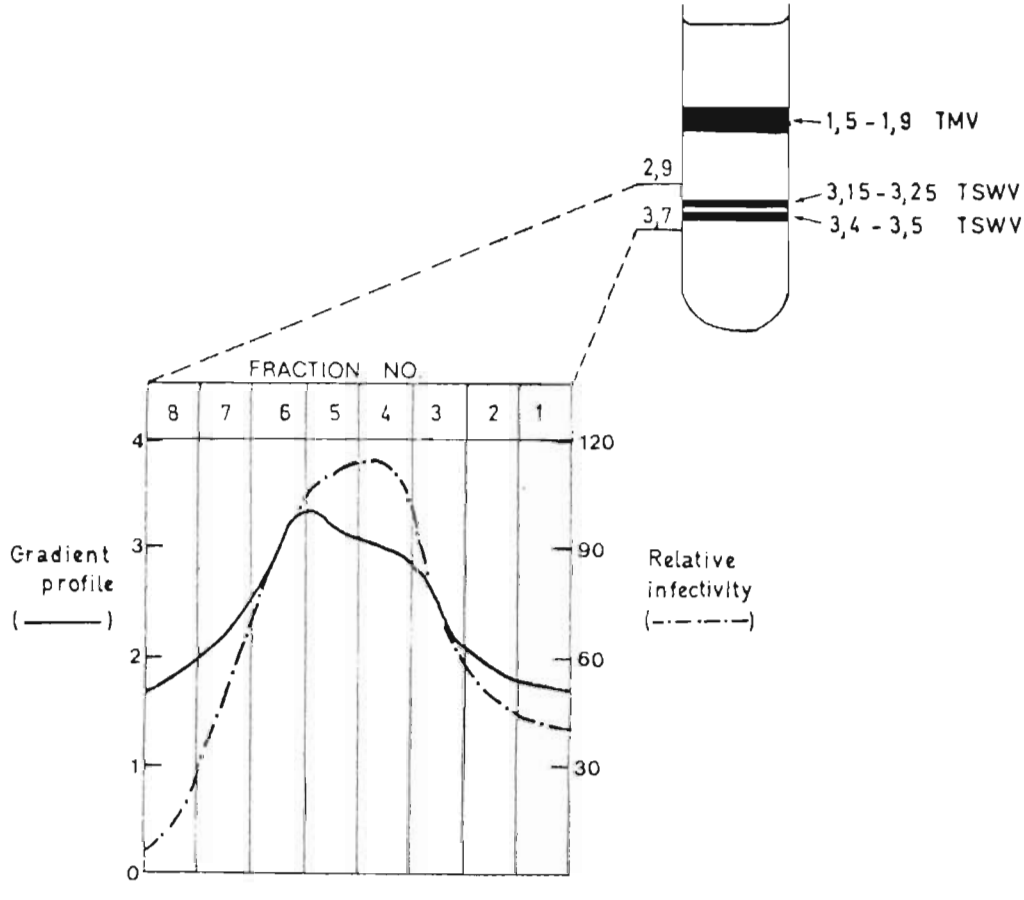


FIGURE 40. Infectivity of fractions making up the double band TSWV zone shown in Table 35b, day 8. Each fraction diluted 1:4 with 0,01 M Na_2SO_3 for inoculation to six half-leaves of Petunia. Lesion totals corrected against common control treatment applied to opposite half-leaves.

The abovementioned result was confirmed in a second study on extract of roots harvested on the 13th day after TSWV inoculation (Table 38). In the latter instance, 1 ml aliquots were carefully extracted with a syringe from the deeper half of the lower band or from the higher half of the upper band. As complete bands were not removed, lesion totals reflect only 'sample', not 'band', infectivity levels. Of uncertain significance is the limited infection enhancement produced when upper and lower band materials were mixed at the higher dilution.

TABLE 38. TSWV infectivity of outer margin material from upper and lower bands and mixtures of both, inoculated at various dilutions

Dilution[a]	Upper	Lower	Upper + Lower
1:4	$\frac{148}{308}$[b] = *100%*	$\frac{76}{253}$ = *63%*	
1:14	$\frac{120}{642}$ = *39%*	$\frac{114}{470}$ = *64%*	$\frac{87}{568}$ = *32%*
1:119	$\frac{0}{391}$ = *0%*	$\frac{0}{292}$ = *0%*	$\frac{4}{347}$ = *2%*

[a] Diluted with 0,01 M Na_2SO_3

[b] Lesion totals on nine half-leaves of *Petunia*. Denominator: control, comprising the S_4 extract, as used for layering, diluted 1:14 with Na_2SO_3 to equate its volume with that of the S_1 supernatant (Fig. 25). Infectivity expressed also as a percentage of the infectivity (italicized) of the 'upper 1:4' inoculum.

Electron micrographs of material from the upper and lower band samples, fixed when on the grids, by dilution with 6% glutaraldehyde in 0,05 M Na cacodylate buffer, pH7

and then dehydrated by critical-point drying, are shown in Fig. 41a and b, respectively. Each band includes TSWV-type particles as well as numerous clavate particles and a few that are more dumbbell-like. A rare example of a particularly well-preserved virion from the lower band is shown in Fig. 42. It measures approximately 70 nm in horizontal diameter and is estimated, from shadow length, to be about 80 nm high. The other two particles appear to have volumes similar to that of the spherical particle, despite their different forms. The nature of the plentiful particulate material and aggregates, covering a wide range of sizes, is not known.

An attempt was made to derive two bands from the single, wide, TSWV band produced when TMV is present in high concentration. The plants required as virus source were inoculated with homogenate of young leaves in the acute stage of dual infection with TSWV and TMV. Eleven days later the roots were harvested and two 15 g quantities of root were extracted identically. The procedure applied was that shown in Fig. 25, modified for 15 ml standard root extract. In addition, the rate zonal gradient centrifugation was shortened to 25 min duration. At this stage the two tubes displayed a TMV band and a single, 6,5 or 7 mm thick, TSWV band. The upper half of the TSWV band in both tubes was recovered, pooled and diluted to 12 ml with 0,01 M Na_2SO_3. The remaining lower halves of the bands were similarly removed, pooled and diluted, after which the virus in each suspension was pelleted by centrifugation in a Servall SM-24 rotor at 17 000 for 36 min.

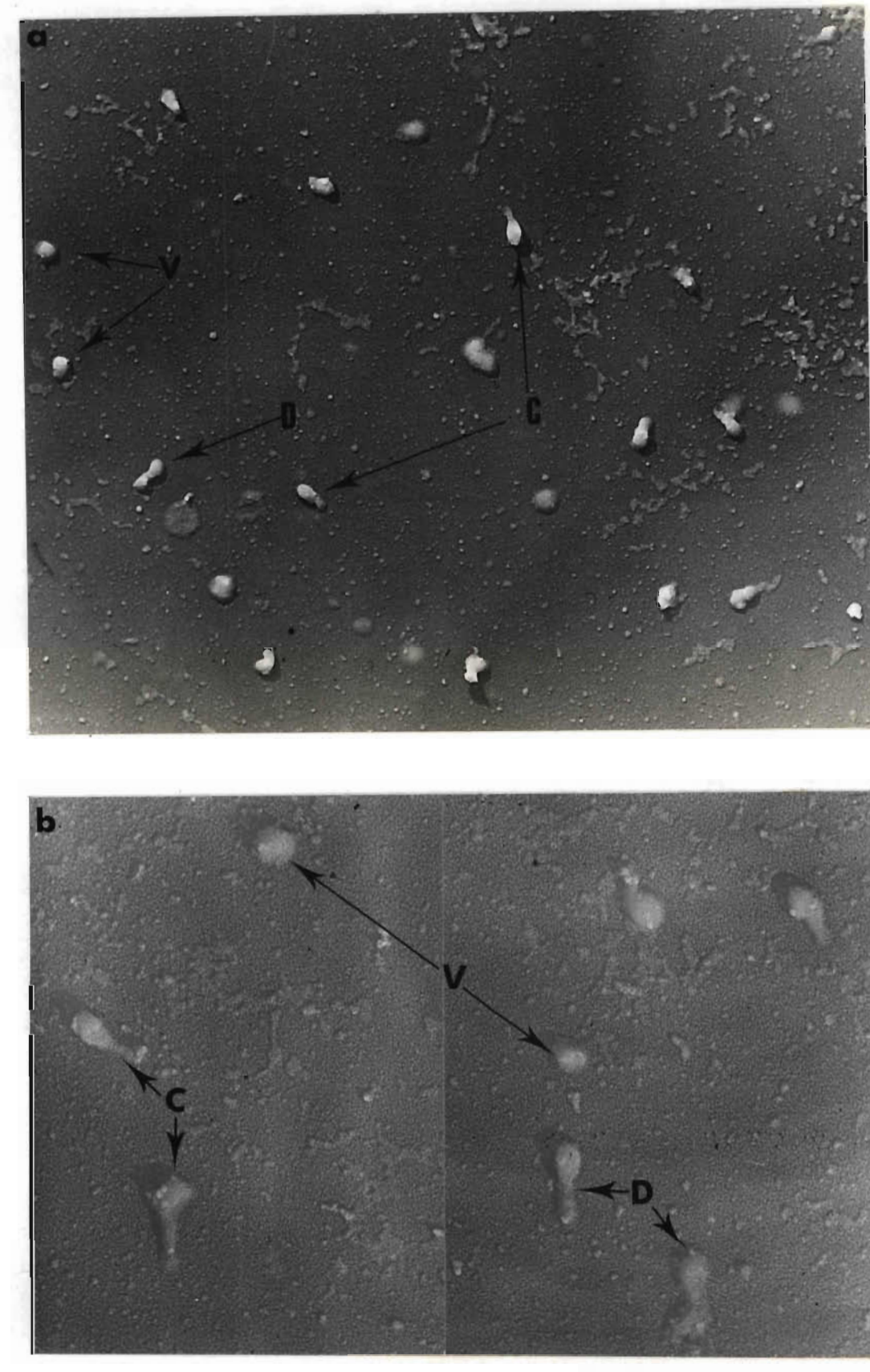


FIGURE 41. TSWV particles from a. upper and b. lower bands after fixation with 6% glutaraldehyde in 0,05 M Na cacodylate buffer, pH7 and critical-point drying. V= TSWV-type particle; C=clavate particle; D=dumbbell-type structure (a. X 36 000; b. X 73 200)

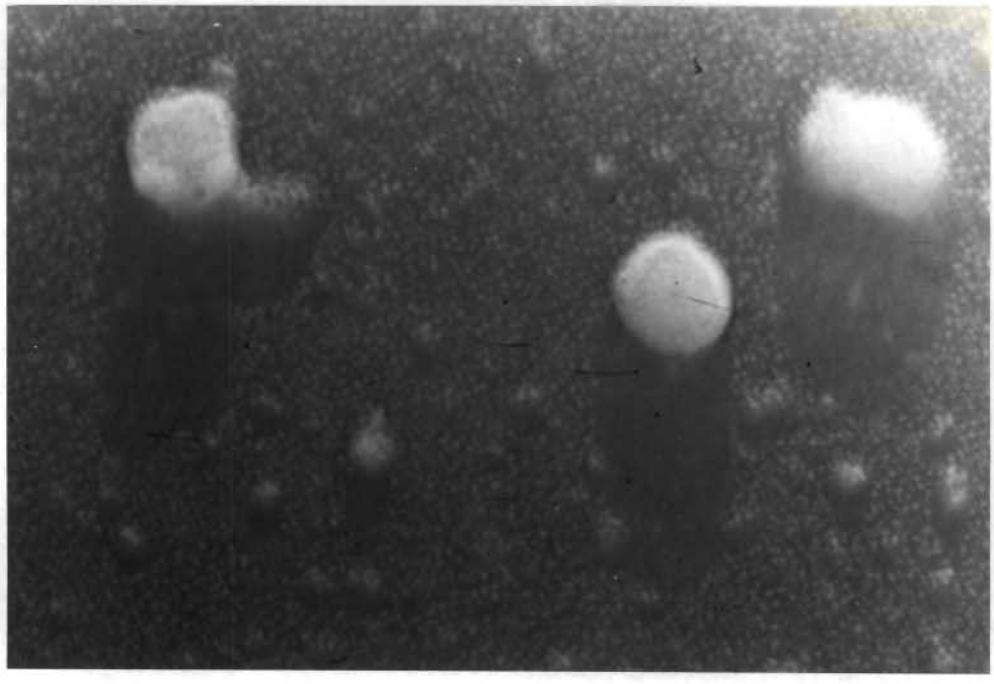


FIGURE 42. TSWV particles from the lower band, after fixation with 6% glutaraldehyde in 0,05 M Na cacodylate buffer, pH7, critical point-drying and shadowing at 30°. (X 240 000)

Each of the two pellets was resuspended in 1,5 ml 0,01 M Na_2SO_3. Of these suspensions, 1,2 ml 'upper half' and 'lower half' were independently layered on a 0-40% sucrose gradient, containing 0,01 M Na_2SO_3, in a Spinco SW 25 rotor tube. Centrifugation for 45 min at 23 000 rpm produced in each tube a clear, single, TSWV band, with no trace of a second, minor TSWV band. The depth of the two bands did not, however, differ significantly, being 3,25 - 3,55 mm and 3,3 - 3,7 mm in the tubes containing 'upper half' and 'lower half' material, respectively. Neither tube contained a visible TMV band.

For the purpose of testing for possible enhancement of infectivity when material of the two bands is mixed, the

topmost 2 ml was recovered from the 'upper half' band of the second gradient centrifugation, whilst from the other tube containing 'lower half' material, 2 ml was taken at the lowest part of the virus zone. Table 39 shows the various test combinations of these virus suspensions inoculated to Petunia and their respective levels of infectivity. Tested at the same dilution, viz., 1:4, as the original 'upper half' and 'lower half' suspensions (Treatments 1 and 2) all three combination inocula (Treatments 3a, 4 and 5a) showed increased infectivity. Testings of two combinations at 1:8 dilution (Treatments 3b and 5b) yielded confirmatory evidence.

TABLE 39. Comparative TSWV infectivity of 'upper half' and 'lower half' sucrose gradient TSWV bands inoculated alone and in combination

Treatment No	Inoculum composition			Infectivity		
	'upper half' (ml)	'lower half' (ml)	0,01 M Na_2SO_3 (ml)	Lesion totals[a]	% of 'upper half' infectivity	% of 'lower half' infectivity
1	0,4	-	1,6	$\frac{166}{127}$	100%	(129%)
2	-	0,4	1,6	$\frac{93}{92}$	(78%)	100%
3a	0,3	0,1	1,6	$\frac{194}{74}$	203%	(259%)
b	"	"	3,2	$\frac{331}{148}$	173%	(221%)
4	0,2	0,2	1,6	$\frac{278}{159}$	135%	173%
5a	0,1	0,3	1,6	$\frac{408}{143}$	(220%)	282%
b	"	"	3,2	$\frac{229}{163}$	(101%)	139%

[a] Lesion totals on eight (Treatments 1 and 2) or nine (other treatments) half-leaves of Petunia. Denominator: control, common to all treatments

In a second study, 0,7 ml aliquots were removed from upper (depth 2,1 - 2,3 mm) and lower (depth 2,4 - 2,6 mm) bands after rate zonal gradient centrifugation of S_4 extract of TSWV-infected roots and layered separately on 30 - 60% sucrose gradients for equilibrium centrifugation at 21 000 rpm for 2,5 h. This resulted in a light scattering band at 1,7 - 1,9 in the tube containing upper band material, and at 1,8 - 2,1 mm in the other tube. A third tube that had been layered with 0,7 ml from a single-band, TSWV zone, derived from the identical S_4 extract to which had been added approximately 18 mg TMV, displayed a single zone at 1,7 - 1,9 mm depth. Removal of each zone and inoculation, either alone or in various combinations, produced no lesions. In contrast, the commencing standard root extract from roots infected only with TSWV, showed high infectivity.

In an attempt to increase the extent of separation of the two bands the following three modifications to the concluding standard extraction procedure (Fig. 25) were tested separately:

(i) substitution of rate zonal gradient centrifugation by equilibrium zonal sucrose gradient centrifugation,

(ii) rate zonal gradient centrifugation in glycerol,

(iii) rate zonal centrifugation in 0-40% sucrose containing a 0-4% PEG gradient, similarly increasing with depth.

S_4 extract (Fig. 25) prepared from roots infected with TSWV, was layered on a 30-60% sucrose gradient containing 0,01 M Na_2SO_3 and centrifuged at 14 000 rpm for 2 h, 3 h or 4,5 h. At each time interval the TSWV zone was clearly

visible as only a single band, at depths of 1,5 - 1,8 mm, 1,9 - 2,25 mm and 2,5 - 3,05 mm, respectively. It is possible, however, that the TSWV zone may have resolved into discrete bands had centrifugation been continued, although at 4,5 h there was no evidence to indicate this. Inoculation of an undiluted sample from the virus zone after 4,5 h centrifugation showed the virus to be still highly infective.

Separation of the two bands of the TSWV zone was unchanged in 10 - 50% glycerol gradients centrifuged at 23 000 rpm for 30 or 40 min. Fig. 43 shows the shouldered profiles obtained under these conditions, when the level of TMV present was not excessive. Electron microscopy of material taken directly from the complete TSWV zone after centrifugation for 30 min showed single particles and small virus aggregates (Fig. 44). Occasionally, ca. 50 nm particles were encountered. A considerable portion of each grid square contained virus particles in high concentration, located often in an enveloping, agranular material (Fig. 45). In localized areas were observed fine filaments of considerable length having a thickness of approximately 6 nm where not at right angles to the direction of shadowing (Fig. 46). In the absence of a significant amount of any other contaminating material in these areas it is possible that the threads may bear some relationship to the virus. In some fields membrane fragments were seen. In another study, upper and lower bands recovered after centrifuging S_4 extract of TSWV-infected roots for 45 min at 23 000 rpm in a 0-60% glycerol

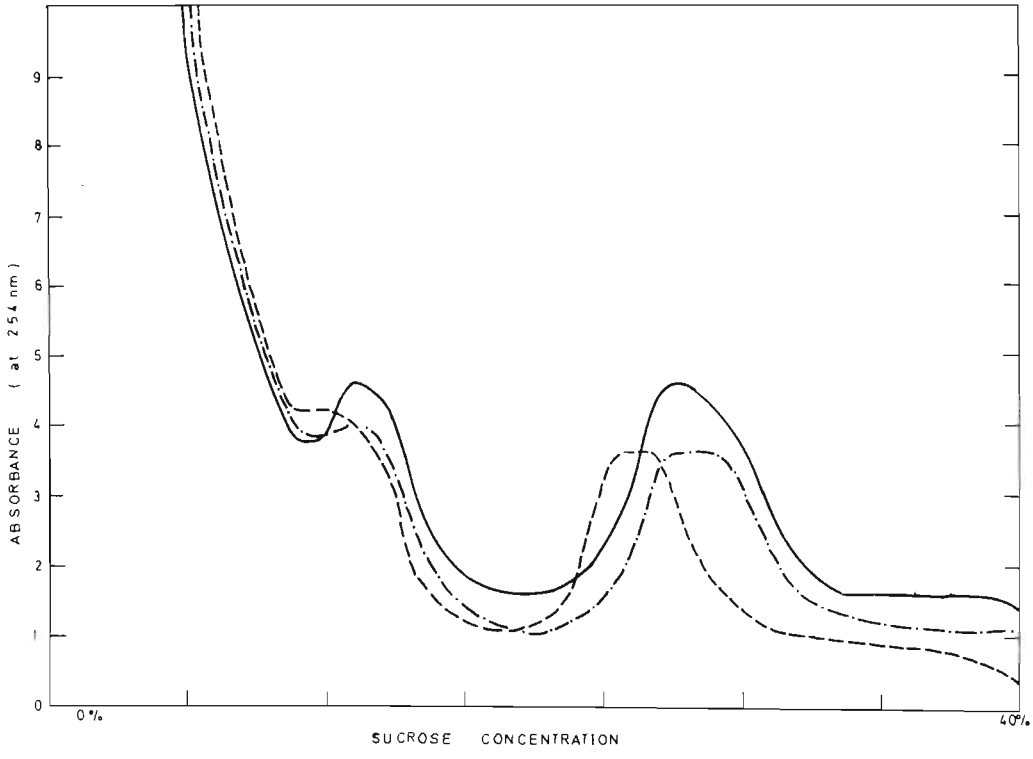


FIGURE 43. Gradient profiles of S_4 extract, containing a low level of TMV, centrifuged for 45 min in 0-40% sucrose (——), or in 0-50% glycerol for 30 min (----) or 40 min (-·-·-)

gradient were examined separately after 1:4 dilution with 0,01 M Na_2SO_3. No difference was discernible in the virus particles of the two bands. Both bands also contained appreciable amounts of membranous material, as shown in Fig. 47, prepared from the bottom band.

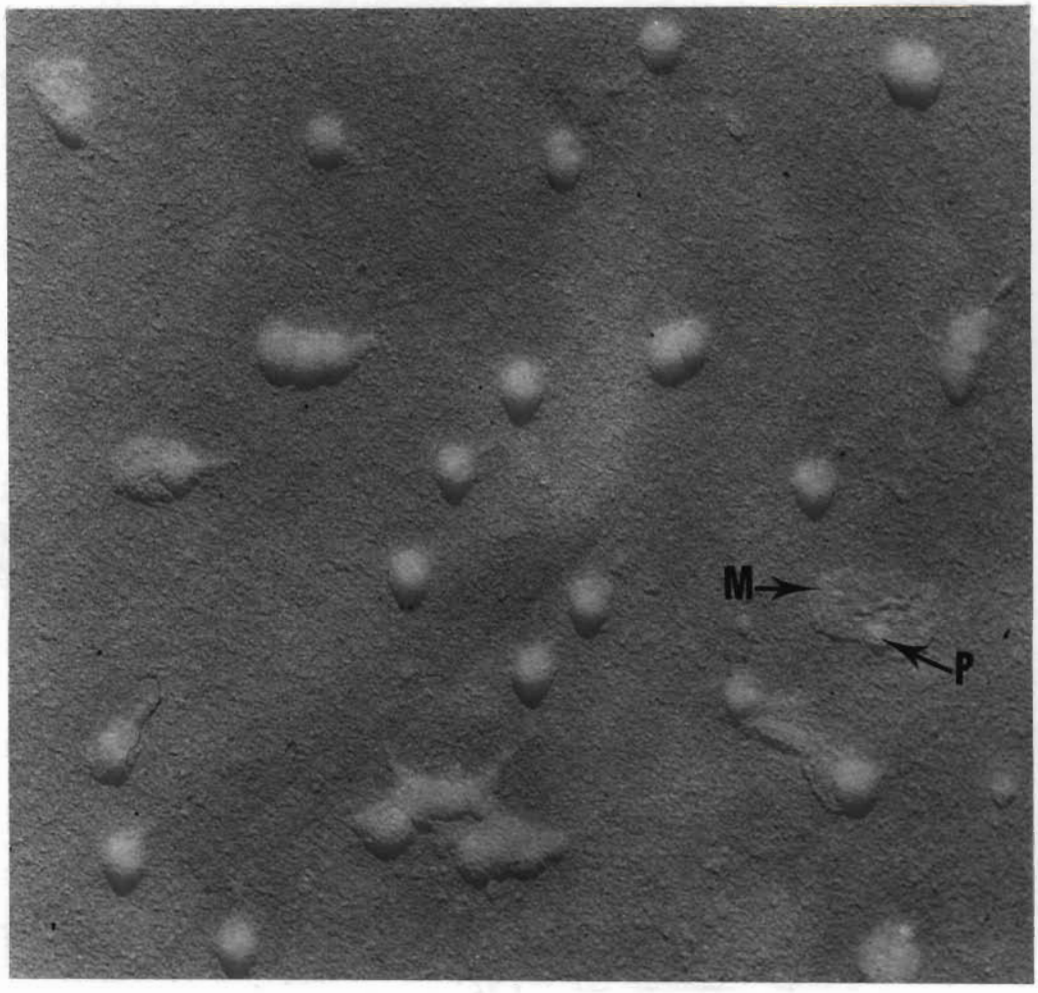


FIGURE 44. Single TSWV particles and small aggregates of virions recovered directly from a glycerol gradient virus zone. P = ca. 50 nm particle; M = membranous structure (X 60 000)

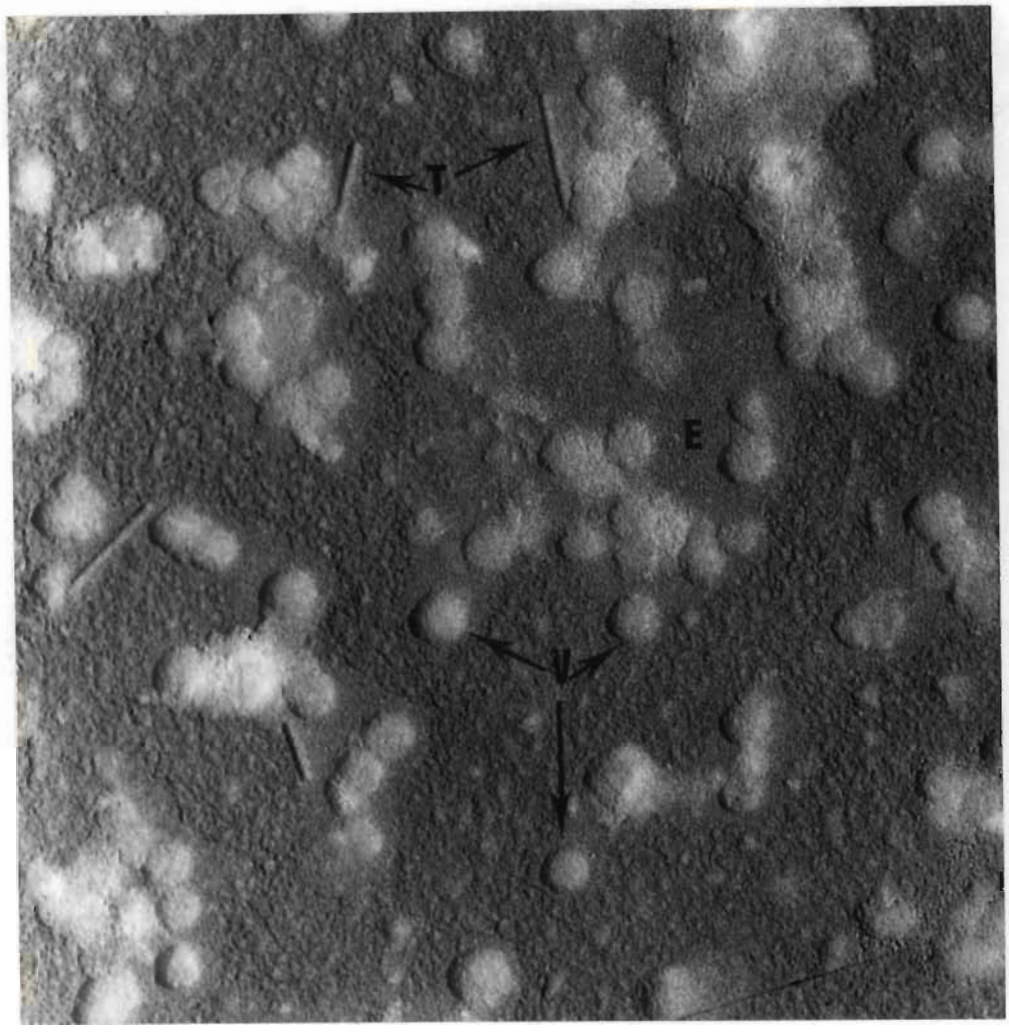


FIGURE 45. TSWV particles, some enveloped in agranular material, recovered directly from a glycerol gradient virus zone. V = TSWV particles; T = TMV; E = enveloping, agranular material. (X 60 000)

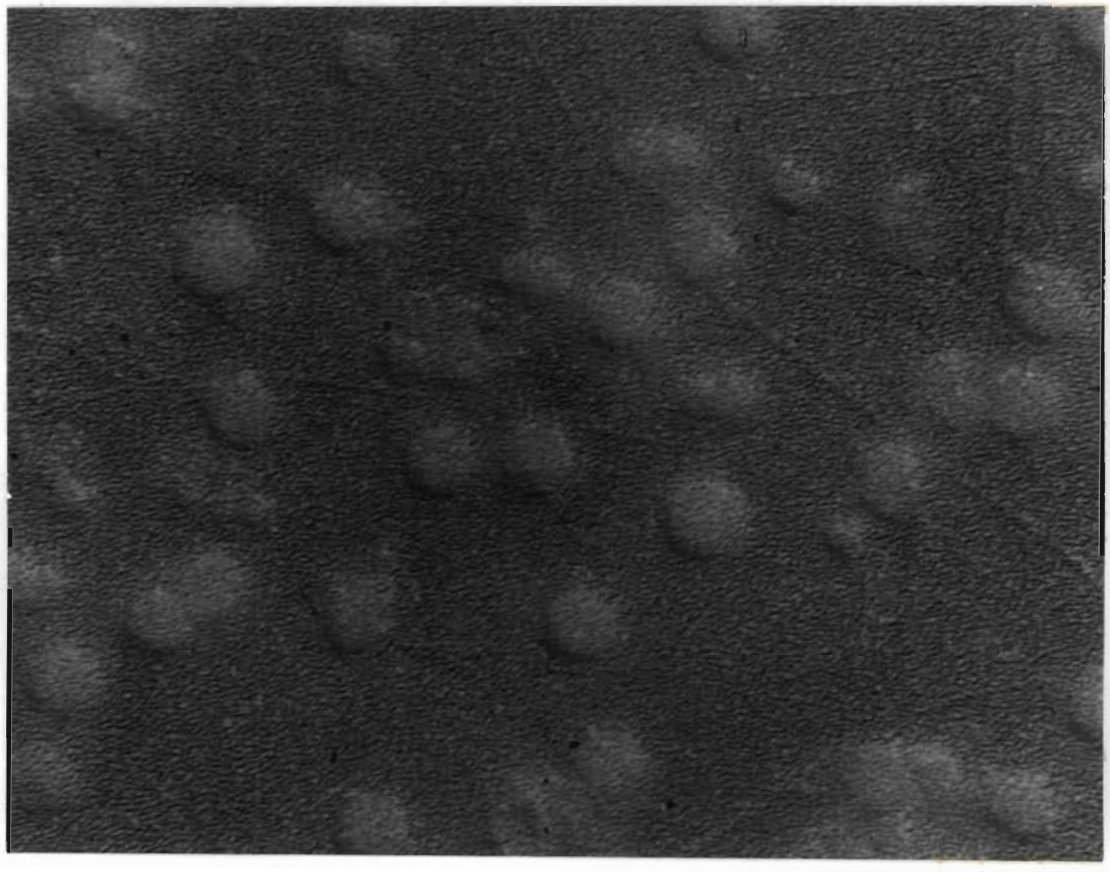


FIGURE 46. Filaments present in an adjacent view of the grid bearing the material shown in Fig. 45. (X 96 000)

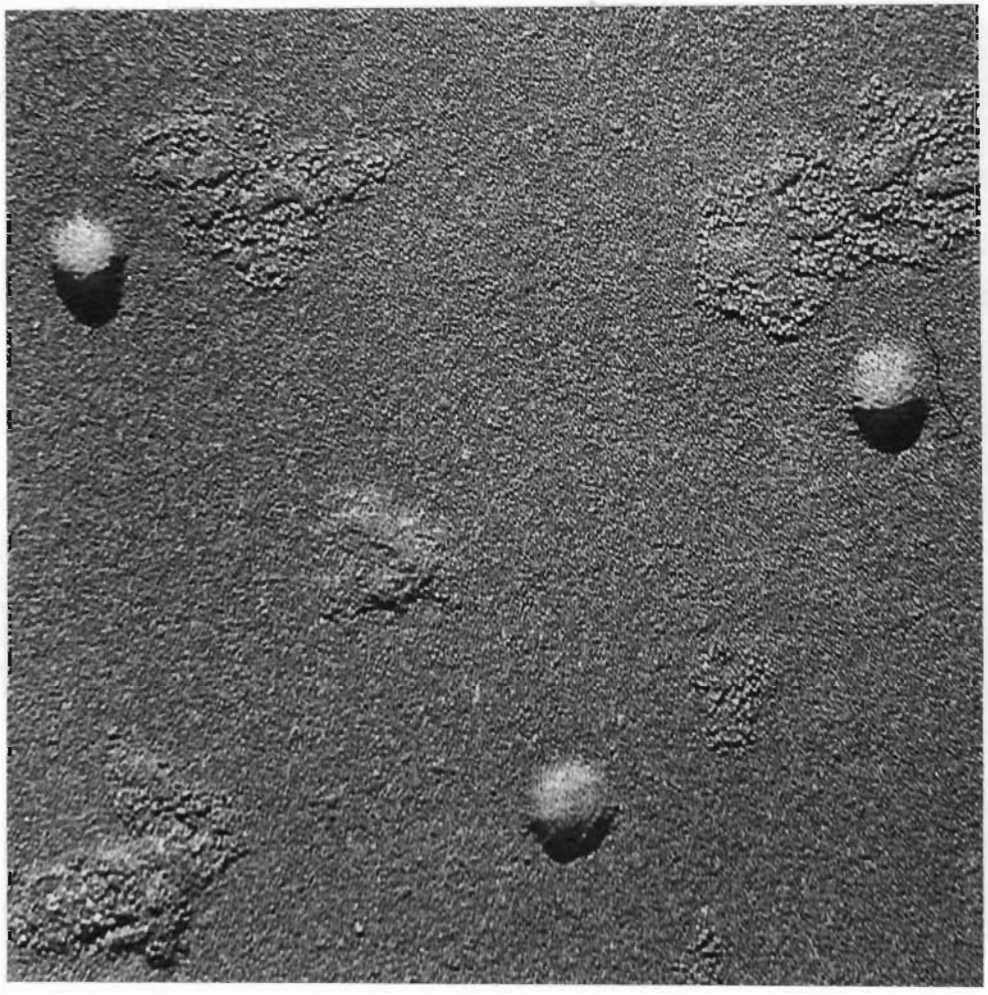
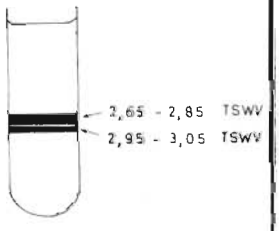
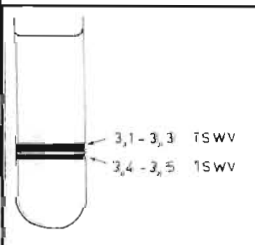
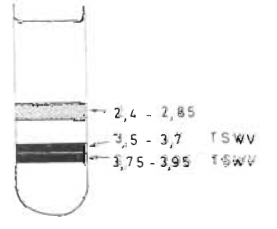
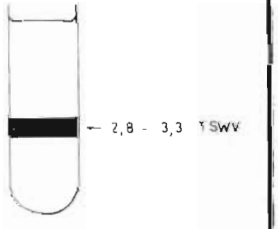
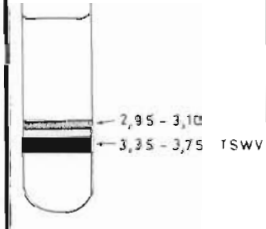
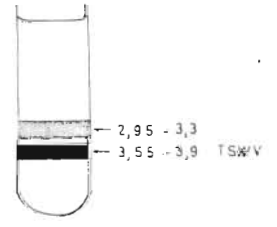
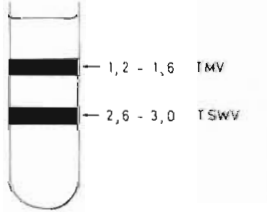
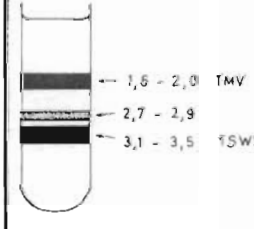
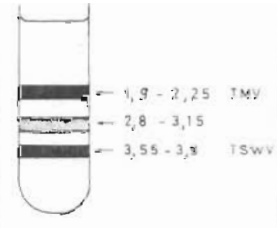


FIGURE 47. TSWV particles and membranous material obtained directly from the lower band in a 0 - 60% glycerol gradient. (X 96 000)

The third alternative gradient form investigated, viz., 0-40% sucrose + 0-4% PEG, in 0,01 M Na_2SO_3, caused no improvement in band separation when S_4 extract was centrifuged at 23 000 rpm for 45 min. In initiating the use of this gradient it was anticipated that the lower band might precipitate preferentially when it encountered, in the increasing PEG gradient, a suitable concentration of PEG. The effect of prolonging centrifugation to gain deeper penetration of the bands into the gradient was therefore investigated. This was applied as two short centrifugations of 10 min each at 23 000 rpm, allowing gradient tube inspection after each period. In addition, acceleration and deceleration were each of seven min duration. The S_4-stage extracts used for layering were derived from roots of plants inoculated 13 days previously with TSWV alone or also with approximately 0,05 mg TMV/ml. During incubation the plants were kept at 20°C, receiving 12 h daily illumination, and were therefore comparable to those from which the results shown in Table 31 and Fig. 35 were obtained. The experiment also included NaGP-treated extracts in an attempt to detect the factor responsible for inhibiting TSWV infectivity for Petunia. Previous studies (see Table 30) had indicated that this factor is probably removed by CGP(N) or (S) clarification of root extract (Fig. 25), but not by identical NaGP treatment.

The results of this experiment are shown in Table 40. There is no evidence of a progressive, PEG-induced effect on the TSWV bands. In both gradients prepared from NaGP-

TABLE 40. Bands produced in gradients comprising 0 - 40% sucrose + 0-4% PEG layered with S_4-stage extract of roots infected with TSWV or TSWV + TMV and treated during clarification with CGP(N) or NaGP

Virus/ treatment	Progressive centrifugation time[a]		
	45 min	+ 10 min	+ 10 min
TSWV/ CGP(N)			
TSWV/ NaGP			
TSWV + TMV/ NaGP			

[a] Centrifuged at 23 000 rpm

treated extract a new zone having a brownish appearance developed after the first additional centrifugation lasting 10 min. However, a similar zone appeared in the CGP(N)-treated tube after further centrifugation, making it unlikely that this zone comprises the inhibitory material. This was confirmed in inoculation studies. Aliquots of both the CGP(N)-clarified S_4 extract and the virus zone material from the same treatment, were equally infective after dilution, at two levels, with either 0,01 M Na_2SO_3 or material from the new zone. In both tubes containing NaGP-treated extract the TSWV zone retained a single band form. The infectivity of the band was, however, not established.

DISCUSSION

The action of CGP(N) as clarifying agent has, in this chapter, been shown not to require the presence of undissolved, colloidal CGP(N). The ability of CGP(S) to effect comparable clarification indicates that the dissolved calcium glycerophosphate clarifies by promoting aggregation of host components.

Comparison of the profiles shown in Fig. 26 relating to healthy tomato root extracts clarified with or without addition of CGP(N) and AA (Fig. 25), appears to indicate merely a quantitative change in the material present in the lower half of the gradient tube. However, comparison with the profile for the CGP(N)/AA- clarified healthy root extract in Fig. 28, reveals also a qualitative effect.

Whether or not virus aggregation is also enhanced by CGP(N) and CGP(S) was not conclusively shown. The very high infectivity of the CGP(N)/AA-clarified S_4 extract reported in Table 27 (Hoaglands) and Fig. 29,(i) provides indirect evidence that the induced aggregation is partially specific for host components. Furthermore, in numerous experiments absolute TSWV infectivity for Petunia increased following standard clarification (Fig. 25), presumably due to removal of the tomato root inhibitor. However, such infectivity increase could also reflect a qualitative change in individual virions, e.g., an effect on membrane structure, or disaggregation.

Notwithstanding careful attention to the setting and operation of the Uvicord detector and recorder system, profile heights in regions adjacent to the TSWV peak, reflecting level of clarification, varied for comparable

material extracted at different times. The profiles recorded in Fig. 30, (iv) and 31, (iii), both derived from extracts prepared by standard CGP(N) and AA treatment ten days after TSWV inoculation, demonstrate this variability. The reason for the superior clarification of the latter extract is not known. In addition, no evidence was found to indicate that the level of clarification changes directionally as TSWV incubation is prolonged. For example, profiles obtained by standard extraction of TSWV-infected roots 10 (Fig. 30, (iv)) and 14 (Fig. 28) days after inoculation revealed comparable clarification, in both cases inferior to that achieved in the 12th-day extraction shown in Fig. 29, (i).

NaGP application produced significantly poorer clarification than was obtained using CGP(N), unless it was followed by addition of a comparatively high level of ascorbic acid (Fig. 33). However, the effect of the latter was to reduce both S_4-stage infectivity (Table 30) and the light-scattering intensity and absorbance of the TSWV zone (Fig. 33a and Table 30). The results of Experiment 2 shown in Fig. 33b appear to indicate that the tomato root inhibitory factor is not eliminated by NaGP treatment.

The clarification studies reported in Chapter 1, using soil-grown, Moneymaker variety tomato roots infected with Net strain or Wye clone, noted persisting high levels of TSWV infectivity after clarification with CGP(N) and AA (Figs. 20, 21). These studies were performed using, as assay host, N. glutinosa which is not affected by the TSWV inhibitor in tomato roots (see Fig. 20). In the later

studies, using Homestead tomato roots grown in Hoaglands nutrient solution, clarified, S_4 extracts (Fig. 25) were likewise highly infectious in certain experiments (Tables 27, 31, 33). Thus the potential usefulness of CGP(N) and AA, as also CGP(S), is not dependent on host variety or TSWV strain. At 23°C, with 12 h daily illumination, incubation for approximately 12 days appears to be a prerequisite for high S_4 virus yield following TSWV inoculation. Likewise, incubation for 13 days at 20°C with 12 h daily illumination facilitated appreciable S_4 virus recovery (Fig. 35 and Table 3). The lower infectivity levels noted in other experiments involving 23°C incubation and 12 h daily illumination, are attributable to shorter incubation times of 10 days or less (Tables 28, 37). Exceptionally, the 9th and 12th day S_4-stage extracts clarified by CGP(S) application, both showed moderately high infectivity (Table 30). Poor virus recovery was obtained when roots of plants inoculated 20 days previously were extracted (Fig. 39). At 23°C continuous lighting after TSWV inoculation enhanced symptom development in the form of severe chlorosis and necrosis of inoculated leaves. However, the yield of TSWV on the 12th day (Tables 29, 35a) was inferior to that from plants receiving the normal 12 h illumination.

In extraction studies on roots infected with both TSWV and TMV, assay of TSWV proved largely impossible at the S_4 stage due to the inhibitory effect exerted by TMV on TSWV lesion initiation in Petunia. However, gradient profiles indicated that the level of TSWV recoverable 12 days after concurrent TSWV + 0,05 mg TMV/ml inoculation was not markedly different to that obtained when TSWV

alone was present (Figs. 29, 34, 37). Other inoculation regimes exploring the possibility of synergistically increasing the TSWV content in root extract were, with one exception, unsuccessful. When TSWV inoculation was followed after six days at 23°C by spot-inoculation with 0,0005 mg TMV/ml and transfer to 26°C, a significant increase occurred in both S_4 TSWV infectivity (Table 33, Exp. 1d) and TSWV peak size (Fig. 37a,(iii)). In contrast, full inoculation on the sixth day with TMV of the same concentration, followed by resumed incubation at 23°C, produced the opposite effect (Fig. 33, Exp. 2 and Fig. 37a,(ii)). Although leaf injury may play a rôle in the latter regime, both these results are at this stage unexplained. Application of TMV on the sixth day was regarded as a possible means of weakening host resistance at a time when TSWV multiplication and translocation could be expected to be approaching their most active phase.

Continuous illumination of plants during dual virus incubation resulted in further slight intensification of symptoms (Table 34). The difficulty experienced in interpreting TSWV infectivity results on Petunia during such studies because of TMV presence, is apparent in Tables 35a and 37. With respect to Table 35a it is unlikely that the inhibitory effect of the TMV was significantly reduced by the 1:135 dilution. The appreciably smaller TSWV peak produced by extract of roots infected with both viruses, shown in Fig. 38, and the lower infectivity of TSWV zone material derived from roots infected with both viruses (Tables 36, 37) may be indicative that TSWV yield cannot be enhanced by exploiting means that intensify foliar

symptoms. It must be noted, however, that in both these experiments full delayed TMV inoculation was performed. This sequence was noted above (Table 33, Exp. 2 and Fig. 37a, (ii)) to depress TSWV yield in plants receiving 12 h daily illumination.

The possibility, mentioned in Chapter 1, that the more severe pathological condition produced in roots by dual infection could result in reduced levels of contaminating membranous materials in TSWV preparations, finds no supporting evidence in the more detailed studies conducted on the South African clone. Comparison of gradient profiles prepared simultaneously from singly and doubly infected roots, shown both in Fig. 35 and 38, reveals at least equal clarification of extract derived from roots infected with TSWV alone. The failure of the clarification steps preceding rate zonal centrifugation (Fig. 25) to eliminate membrane fragments is seen in the sample from a glycerol gradient TSWV zone, presented in Fig. 47. However, it is not known what proportion of the membrane fragments derived from virus partices. Furthermore, the severe pathological state induced by the synergistic action of Net strain TSWV and TMV could not be fully reproduced with the South African clone. Roots of dual inoculated plants grown in soil at 23°C likewise failed to develop discrete lesions. Nevertheless, the occurrence of lesions on aerial roots of plants incubated at 20°C after inoculation with both viruses, may be an indication that absence of more extensive lesion development on roots was mainly due to cultural factors. The comparatively lower yield of virus from roots incubated in Long Ashton solution (Fig. 29 and Table 27) may indicate

an effect by the nutrient solution on either virus production during incubation or the nature of aggregates formed during extraction.

The cause of banding within the TSWV zone, in both sucrose and glycerol gradients, has not been resolved. In the second phase of this study, using the South African clone, upper and lower light-scattering bands were observed to be often of equal width (Fig. 33, day 9 without TMV; Fig. 35, day 13 with or without TMV; Fig. 37a, day 12 with or without TMV; Table 35b, days 10 and 12 without TMV and days 8 and 10 with TMV). There was nevertheless also a marked tendency for the upper band to be wider than the lower band (Fig. 32, day 10 without TMV; Fig. 37b, day 12 with TMV; Fig. 39, days 12 and 20 without TMV). Furthermore, in one experiment in which bands of equal width were recovered, the upper was considerably more distinct (Fig. 35, day 13 without TMV). Conversely, in profiles of the single band TSWV peaks observed at the highest TMV levels there was a tendency for the lower region of the band to be the more prominent (Fig. 36b, day 11). These opposing tendencies appear to afford a partial explanation for the band forms previously observed in the final Net strain experiments (Fig. 11): (i) in the absence of TMV two TSWV bands were present, the upper being more prominent (Exp. N3); (ii) when TMV was present and, in addition, a greater mass of starting tissue (Exp. N2) or a smaller gradient (Exp. N4) was used (Table 24), only a single wider band developed that was more dense in the lower half. In view of the failure of $MgCl_2$ to increase separation of the adjacent bands in a study on the South African clone, it appears unlikely that the wider separation of the Net strain bands in Experiment N3 (Fig. 11) was caused by the $MgCl_2$. There is also no

evidence supporting the possibility mentioned in Chapter 1, that TMV may stabilize TSWV virions during extraction, thereby preventing loss of infectivity and the transformation of the single band into two bands. On the contrary, the occurrence of paired TSWV bands in a number of experiments in which a significant level of TMV was present (Figs. 35, 37b; Table 35b) would seem to indicate that the TMV has no marked effect of the type suggested. Further evidence for this conclusion may be found in (i) the persistence of the shouldered form of TSWV peak in profiles obtained at the highest TMV levels, when only a single, broad TSWV peak arises and (ii) the inferior infectivity, noted in Table 36, of the TSWV zone recovered from doubly infected roots.

Best and Palk (1964), Best (1968) and Tsakiridis and Gooding (1972) previously reported paired bands within the TSWV zone after sucrose gradient centrifugation. Other investigators noted only a single zone. Using the method presented in Fig. 2, Best and Palk (1964) and Best (1968) observed two adjacent bands in gradients of the second rate zonal centrifugation. The lower band showed higher specific infectivity whilst the upper band contained the greater concentration of empty shells and membranous material (Best and Palk, 1964). Tsakiridis and Gooding (1972), applying the relatively shorter extraction procedure set out in Fig. 3, noted similar adjacent bands after the first, and only, sucrose gradient centrifugation. The latter authors did not attempt to determine the infectivity of the individual bands. They estimated that final virus recovery, after applying Freon 114 to promote clarification, was about 10% of that originally present. It appears

unlikely that gradient overloading was a significant factor in this extraction. Similarly, in the studies by Best and Palk (1964) and Best (1968), a double, TSWV band was manifest when the estimated amount of virus had fallen to 18% (av) of that originally present. The use, in the present study, of only 21 g root tissue for each extraction probably ensured that the amount of virus present was sufficiently small to avoid zone instability. On the other hand, when a high concentration of TMV was also present in the layered suspension only a single TSWV band was resolved.

There is at present insufficient evidence to show whether or not the band pairs produced by the three, in many respects dissimilar, extraction procedures arise for the same reason. Should this be the case, as seems likely, the conclusion would be warranted that banding is not due directly either to calcium glycerophosphate or AA, used in the present study, or to 'solvent B', used by Best and Palk (1964), Best (1968) and Tsakiridis and Gooding (1972). The occurrence, in the present study, of banding when phosphate buffer and 0,01 M Na_2SO_3 were used during clarification by centrifugation alone would seem to indicate that banding may also be observable using the procedures shown in Fig. 1 and 4, if the commencing amount of plant material is greatly reduced.

van Kammen et al. (1966) detected approximately 100% greater relative infectivity in lower than upper sample fractions of a single 1 cm-wide TSWV band obtained from leaf material according to the procedure shown in Fig. 1. In the present study a smaller difference in infectivity

was observed when upper and lower material from a single, wide band derived from doubly infected roots was compared (Table 39, treatment Nos. 1 and 2). In another experiment in which discrete upper and lower bands were compared (Fig. 40), greater infectivity of lower band material was again noted. This finding is in agreement with results obtained by Best and Palk (1964) and Best (1968) in studies on leaf extract. The results presented in Table 38, relating to 1 ml samples removed with a syringe from immediately above and below the brightest portions of upper and lower bands, respectively, indicate superior lower band infectivity at 1:14 dilution. In this experiment the relatively greater infectivity of the lower band sample on dilution could indicate that the band contains virus aggregates able to undergo dissociation. The occurrence of aggregation at lower levels of the TSWV zone is indicated also by the infectivity associated with fraction No. 1, shown in Fig. 40.

Paired bands attributable to differences in aggregation state, have been encountered in extractions of other viruses. In a study on maize rough dwarf virus, which comprises approximately 70 nm particles, Luisoni, Milne and Boccardo (1975) noted the presence of a distinct band, comprising 95% virions plus 5% dimers, below the major virus band. The extraction was performed on infected maize roots, using Freon 113 and sucrose density gradient centrifugation. The algal virus SM-1, having a diameter of approximately 88 nm, likewise displayed a tendency to form a distinct lower band, comprising aggregated particles, when extracted

by differential centrifugation and sucrose density gradient centrifugation (Safferman, Schneider, Steere, Morris and Diner, 1969). In the present study electron microscopy of material taken from a glycerol gradient TSWV zone showed evidence of virus aggregation (Fig. 44). The presence of an agranular, enveloping material associated with certain virus aggregates was also noted (Fig. 45). In one experiment the TMV zone also comprised two bands (Fig. 38).

It is also possible that TSWV comprises two component particles. However, of the numerous reported multicomponent plant viruses (Jaspars, 1974) none is membrane-bound. In the present study limited evidence for possible TSWV multicomponency was encountered in the enhanced infectivity of mixtures of 'upper half' and 'lower half' suspensions reported in Table 39. As this result still requires confirmation, discussion of its implications is unwarranted. In this regard it may be significant that (i) TSWV infectivity has been observed to decrease more rapidly at high dilutions than was theoretically expected (Best, 1937b) and (ii) TSWV strain recombination has been reported (Best, 1961).

A third possible explanation for the paired bands is that the lower band represents complete particles whilst the upper contains particles that are defective, either inherently or as a result of the extraction process. Tissue culture studies with many different animal viruses have revealed the production of defective, interfering particles which contain only a part of the viral genome (Huang and Baltimore, 1970). In the present study no evidence was found that the ratio of the two bands to each other, in the absence of TMV, diverged strikingly in

different experiments or was correlated with duration of infection.

After critical-point drying, electron microscopy (Figs. 41, 42) revealed the same range of particle forms, including spherical, tailed, clavate, dumbbell and brick-shaped, in preparations from both upper and lower gradient bands. Additional studies will be needed to determine whether this range of particle types correctly reflects the forms occurring in the bands. Furthermore, Polsen and Stannard (1970) have shown that the morphology of membrane-bound viruses can be adversely affected by syringe-type uptake, introducing severe shearing forces. Destructive surface tension effects associated with air drying of oncornaviruses were successfully eliminated by the application of critical-point drying together with glutaraldehyde fixation and uranyl acetate staining (de Harven, Beju, Evenson, Basu and Schidlovsky, 1973). Similarly, glutaraldehyde - fixed intracisternal A particles, purified from plasma cell tumour tissue, showed no pleomorphism when shadowed and viewed after dehydration in ethanol, containing 0,5% uranyl acetate, and critical-point drying in Freon 113 (Malech and Wivel, 1976). Joubert (1973), reporting the use of glutaraldehyde for TSWV stabilization prior to electrophoresis (Fig. 4), drew attention to the fact that during fixation plant proteins may become bound to the surface of the virion.

The filaments of approximately 6 nm diameter seen in Fig. 46 cannot be related with certainty to the TSWV particles present. However, their diameter is similar

to the arrowed element in Fig. 24, interpreted as a short, resolvable strand of the internal component. In contrast the filaments occasionally observed in association with shadowed TSWV particles by Joubert (1973) were either 15 or 25 nm in diameter.

Much attention was paid in this investigation to the clarification stage of TSWV extraction. Using roots as the virus source greatly promoted clarification, which was further enhanced by the application of CGP(N) and AA. The level of clarification achieved was superior to that gained using NaGP. However, the results presented in Table 40 concerning an experiment in which extracts were centrifuged on a sucrose + PEG gradient for 45 min followed by two additional 10 min periods, show that additional resolution can be accomplished in this way. Further testing of this system appears warranted, to examine the desirability of increasing the concentration gradient of PEG and salt and of prolonging centrifugation at lower speeds. In order to sharpen resolution, limitation of starting root material to less than about 8 g may be desirable. Comparison of extracts clarified only by centrifugation and extracts treated also with CGP(N), CGP(S) or NaGP could yield information regarding the nature of banding within the TSWV zone, observed when calcium glycerophosphate but not sodium glycerophosphate, is applied. It may also provide a means of isolating the inhibitory factor from tomato roots that is apparently removed by treatment with the calcium salt.

SUMMARY

The purification of tomato spotted wilt virus (TSWV) was investigated using, initially, systemically infected leaves as virus source and later tomato roots.

A Netherlands isolate of the virus was biologically purified by limit dilution to yield a pure strain, termed the Netherlands etch (Net) strain, of intermediate virulence on tomato. In the tomato varieties Potentate and Moneymaker the strain induced acute phase white etch and ringspot symptoms followed by persistent mottling of the new growth. The strain was further characterized by thermal inactivation, *in vitro* longevity and host range studies, in all respects conforming to the reported properties of TSWV. *Cucumis sativus* var. Gele tros was found to be a potentially useful indicator host for TSWV, producing 1 - 3 mm white spots and ringspots on inoculated leaves. Subsequent systemic spread was limited to the production of a few similar spots on the first true leaf.

Rate zonal sucrose gradient centrifugation of extract prepared from systemically infected *Nicotiana rustica* leaves, according to the method reported by Black, Brakke and Vatter (1952), yielded a single, 2 - 3 mm thick, infective, light-scattering band. Electron microscope examination of this material revealed partially flattened 72 - 120 nm, spherical-type particles similar to those observed by the abovementioned authors. It was concluded that variation in TSWV particle diameter within this range, as observed after air drying on the grid, is not due to strain mixture. Equilibrium sucrose gradient centrifugation of the material

comprising the abovementioned rate zonal gradient band resulted in two well-separated bands, the lower being infective. However, two similarly located, non-infective bands were also obtained from healthy leaf extract. Electron microscopy of material from the lower band obtained from healthy tissue revealed host constituents, whilst similarly prepared grids of infected material were almost clear, containing no virus.

Attempted extraction from systemically infected tomato leaves using the same method proved unsuccessful due to the inadequacy of the procedure to effect satisfactory clarification of the tomato leaf homogenate.

In an endeavour to control virus loss during clarification by low speed centrifugation of the crude N. rustica extract, alternative solvents for the 0,1 M phosphate buffer, pH7 containing 0,01 M Na_2SO_3 were substituted at homogenization. These included 0,16 M - 0,96 M Na_2SO_3 or 0,01 M phosphate buffer pH7 containing 0,01 M Na_2SO_3. None altered the aggregation pattern of the virus. However, 0,96 M Na_2SO_3 enhanced clarification. Freezing of infected tissue at -10°C for 24 h prior to extraction reduced infectivity.

Hydrated calcium phosphate gel, activated charcoal, kaolin and ferric hydroxide gel were independently tested as potentially selective adsorbants in an attempt to shorten the time required to extract the virus from leaf tissue of Nicotiana spp. The variable factors introduced included (i) buffer formulation, pH and dilution, (ii) leaf tissue type and host species used as virus source and (iii)

extract of N. rustica at 30 mg/g leaf tissue caused appreciable clarification without significant virus loss. The other adsorbants reduced infectivity significantly when applied at levels affording satisfactory clarification.

Application of the organic solvents chloroform, n-butanol or ether and carbon tetrachloride to crude sap destroyed infectivity, even at levels inadequate to effect satisfactory clarification. No infectious nucleic acid was recovered after treatment with water saturated phenol.

Inoculation studies with homogenates of terminal root segments and entire root systems of tomato infected with Net strain, established the presence of extractable levels of TSWV in tomato roots. The abovementioned adsorbants, except kaolin, were re-examined for use in clarifying TSWV-containing root extract but again proved unsatisfactory. Acceptable clarification accompanied by only limited virus loss was achieved with neutral calcium glycerophosphate, CGP(N), at ratios of 0,08 - 0,24 CGP(N)/ml extract diluted with 2 vol 0,0005 M phosphate buffer, pH7 containing 0,01 M Na_2SO_3. Selected comparable tests performed on a TSWV isolate obtained from Wye College, England gave confirmatory results.

A procedure based on initial clarification with CGP(N) was devised for extracting Net strain from tomato roots. Co-inoculation of the virus source plants with approximately 0,05 mg TMV/ml (final concentration) was performed to facilitate elimination of contaminating host membrane fragments. It had previously been observed that, whereas plants inoculated with TSWV alone developed no lesions on

inoculated leaves when incubated at 20-23°C with normal daily illumination, leaves inoculated with both viruses developed severe necrosis. This was followed by severe apical necrosis.

Roots harvested from such plants for TSWV extraction were noticeably browner and more flaccid than were roots infected with TSWV alone and were extensively marked with 2 mm necrotic spots. Crude extract diluted with 3 vol of the abovementioned phosphate buffer + sulphite solution was clarified with 0,16g CGP(N)/g tissue followed by intermediate speed centrifugation and addition of 0,03g ascorbic acid/g tissue to eliminate residual CGP(N). After two cycles of differential centrifugation 0-40% sucrose gradient rate zonal centrifugation was applied for 30 min at 25 000 rpm in a Spinco SW 25 rotor, or 90 min at 24 000 rpm in a SW39 rotor. A clear TSWV band, having considerable infectivity and well separated from an upper, TMV band, was recovered in both of two experiments conducted, one with each of the abovementioned rotors. The time required to complete the extraction procedure was approximately 4,5 h.

Electron microscopy of the TSWV bands revealed spherical-type particles of diameter 100-185 nm. The principal deformation observed was flattening. The estimated spherical, i.e., unflattened, diameter of the particles observed was 80 ± 5 nm. Use of the SW25 rotor achieved greater purity and the virus particles were, in general, less flattened than when the smaller rotor was used. A very low number of approximately 50 nm particles was encountered in the latter extraction. Employing the

larger rotor, a comparable extraction of roots infected only with TSWV yielded two less dense bands, 5 mm apart, having low TSWV infectivity. Electron microscopy of shadowed material from these bands revealed few TSWV-type spherical particles and numerous membranous structures probably derived from TSWV particles, and a few approximately 50 mm particles. Particles stained with 1% phosphotungstic acid containing 0,03% bovine serum albumin, appeared full, i.e., stain excluding, partly stained or densely stained. Structural details of the internal component could not clearly be resolved. Localized areas at which internal component and particle membrane were closely associated were seen to be more resistant to collapse, thus determining particle outline and producing deviations from circular form.

The abovementioned results were obtained at the Laboratorium voor Virologie, Wageningen, the Netherlands during the first phase of this investigation lasting from 1960 to 1962.

A second phase, employing a characterized, South African clone of TSWV displaying severe-type infection in tomato, was performed recently at the Faculty of Agriculture, University of Natal, Pietermaritzburg. Resumption of the study was made possible by access to one, and occasionally two, Conviron EF7H controlled environment cabinets. At 20 - 23°C and 12 h daily illumination the clone caused no symptoms on inoculated leaves of tomato var. Homestead. Systemic symptoms comprised moderately extensive, 1-2 mm interveinal necrosis, resumed growth being symptomless.

Extractions were performed on tomato roots of this variety grown in Hoaglands solution. Infectivity assays were performed on Petunia. In order to compare clarification levels achieved in different experiments and to confirm inoculation results, UV absorbance profiles of rate zonal sucrose gradients were recorded at 254 nm by means of a LKB Uvicord 4700.

Using roots infected with the South African clone, the clarifying action of CGP(N), applied at 0,08 g/ml to extract marginally diluted with 0,0005 M Na_2SO_3 containing 0,01 M Na_2SO_3, was examined in detail and compared with the clarification produced by soluble calcium glycerophosphate, CGP(S), and sodium glycerophosphate, NaGP. CGP(S), which is a formulation comprising the same isomers of calcium glycerophosphate as CGP(N) rendered more soluble by the addition of 14% citric acid, yielded comparable clarification. Unlike CGP(N) it dissolved completely when applied at the same rates, indicating that the clarifying action of CGP(N) is due to induced aggregation of root extract omponents and not significantly to adsorption by suspended, colloidal particles of CGP(N). There was evidence of selective removal of certain host components. No significant enhancement of TSWV aggregation was noted. In contrast to CGP(N) and CGP(S), NaGP afforded negligible clarification.

It was ascertained that host incubation for 12 days after TSWV inoculation was required to ensure a satisfactory yield of virus on extraction.

The previously observed synergism between TSWV and TMV

was re-examined and its effect on extractable TSWV in tomato roots determined under various conditions, including simultaneous, prior and delayed inoculation of 0,05 or 0,0005 mg TMV/ml. However, the only treatment to enhance TSWV recovery significantly was that in which 0,0005 mg TMV/ml was lightly inoculated to every third leaflet on the sixth day after full TSWV inoculation. Incubation in this instance was at 23°C for the six days prior to the TMV inoculation, and thereafter at 26°C for a further six days. Illumination was provided for 12 h daily. In certain of the abovementioned experiments the plants were incubated under continuous illumination but no increase in TSWV recovery resulted. In both singly and doubly infected plants continuous illumination after inoculation induced initial, severe local chlorosis and necrosis of the inoculated leaves, which then rapidly underwent complete collapse. Systemic leaf chlorosis and necrosis, as also root discoloration, were only slightly enhanced relative to that observed on plants receiving 12 h daily illumination. Thus, apart from the abovementioned late inoculation with TMV, all other attempts to exploit the synergistic action of the two viruses to increase the yield of TSWV on extraction proved unsuccessful. A strong, inhibitory effect by TMV on the infectivity of TSWV for Petunia complicated interpretation of results from assays performed prior to rate zonal gradient centrifugation. However, in view of the effective separation of TSWV from TMV achieved by rate zonal gradient centrifugation, reliance could be placed on gradient absorbance profiles to indicate levels of TSWV recovery.

Rate zonal sucrose gradient centrifugation of extract derived from roots infected only with TSWV and clarified with CGP(N) or CGP(S) followed by ascorbic acid consistently yielded a TSWV zone comprising two adjacent, approximately 2 mm thick, light-scattering bands about 1,0 mm apart. The presence of a high concentration of TMV in the TSWV suspension layered on the gradient suppressed this development, producing instead a single, broader TSWV band. Likewise, when NaGP was applied instead of CGP(N), affording little clarification, only a single band was recovered. Significantly, however, two adjacent bands were observed when a restricted volume of extract clarified only by centrifugation from roots infected with TSWV alone was subjected to rate zonal sucrose gradient centrifugation. In extensive studies with CGP(N)/AA-clarified extracts the ratio of the levels of the two bands obtained from TSWV-infected roots was found not to differ significantly between the eighth and twentieth days after inoculation.

Investigation of the relative infectivity of the two bands obtained after CGP(N) clarification by gradient fractionation and inoculation to Petunia showed the lower band to be slightly more infective than the upper band. Subsequent infectivity complementation studies, in which the infectivity of upper and lower band material was tested independently and in various combinations, showed in one experiment significant enhancement of infectivity after mixing, in another, slight enhancement and in a third, no enhancement. The complete disappearance of infectivity encountered in a final infectivity complementation experiment re-emphasized the limitation imposed on such studies

and their interpretation by the inherent instability of the virus.

Centrifugation on 0-50% glycerol gradients similarly yielded two adjacent bands. Material from the upper and lower bands, fixed with 6% gluteraldehyde in 0,05 M sodium cacodylate buffer, pH 7 and dehydrated by critical-point drying both included spherical, tailed, clavate, dumbbell and brick-shaped particles. More extensive studies employing other methods of fixation and dehydration are required to establish whether this range of particles occurs also in the gradient zones prior to their removal by syringe.

No clear evidence was gained regarding the morphology of the TSWV internal component. However, exceptional extra-viral filaments in a shadowed preparation and a possible strand segment seen within a negatively stained virion both measured approximately 6 nm in diameter.

The significance of the double band condition within the TSWV zone was not resolved in this investigation. However, it would appear to be due neither to CGP(N) or CGP(S), nor to 'solvent B' used by the two groups of workers who previously reported similar double band TSWV zones. The possibilities are discussed of the banding being due to aggregation, or to the occurrence of multipartite or defective particles.

The results of infectivity assays performed immediately after clarification indicate that CGP(N) application, but not NaGP, removes the tomato root factor that inhibits TSWV infectivity on Petunia. During an attempt to enhance

separation of the adjacent TSWV bands by prolonging centrifugation on a 0-40% sucrose + 0-4% polyethylene glycol gradient a brownish band emerged immediately above the TSWV band in the gradient containing NaGP-treated extract. However, as centrifugation was further prolonged, a similar band emerged also in the gradient containing CGP(N)-treated extract. Infectivity tests confirmed that this band does not contain the inhibitory factor. Based on this study consideration is given to the desirability of using very limited samples of concentrated but relatively unclarified suspensions in extraction studies, in order (i) to investigate the nature of the TSWV banding phenomenon and (ii) to identify the factor in tomato roots that selectively inhibits TSWV infectivity.

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