# THE RELATIONSHIP OF PEANUT STUNT VIRUS TO CUCUMBER MOSAIC VIRUS AND ASPERMY VIRUSES OF TOMATO AND CHRYSANTHEMUM.

by

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# INTRODUCTION

The product of heritable change in a plant virus has been referred to as a strain (35). Strains of a plant virus should be distinguishable, as a group, from other plant viruses by their natural vector and behavior in it, and by at least some of their physiochemical properties such as the type and amount of nucleic acid, sedimentation coefficients, arrangement of protein subunits and particle morphology. Finally, serological cross reactions should only occur among members of the group (21).

The existence of strains within a plant virus group most plausibly occurred by mutation and host adaption in the evolution from a common stock. Although partially reflecting the desire to put knowledge into an orderly fashion, the grouping of plant viruses should be of practical value in the areas of comparative virology, virus identification, origins of viruses and nomenclature (21).

Peanut stunt virus (PSV) was first isolated in 1966 in Virginia where it caused a destructive disease of peanut (Arachis hypogaea L.) (40). The non-persistent manner of aphid transmission and particle morphology suggested that it belonged to a group of small icosahedral viruses, the cucumber mosaic virus (CMV) group, uniquely

distinct in these properties from all other known plant viruses.

Prior to the discovery of PSV, tomato aspermy virus and numerous chrysanthemum viruses were discovered in England and the United States. Again, because of their non-persistent means of aphid transmission, these viruses were thought possibly to be related to CMV.

Despite numerous investigations, the possibility that these viruses make-up a distinct plant virus group remains undecided because:

1. Cross protection and conventional serological techniques seem to be inadequate to determine these relationships.
2. The viruses have been difficult to purify, are unstable and poorly immunogenic.
3. On occasions, investigators have used poorly defined or improperly identified viruses.

Using cucumber mosaic virus, peanut stunt virus and aspermy-type viruses from tomato and chrysanthemum, this study was undertaken to: 1) attempt to produce high-titered antisera against PSV and CMV, 2) study the electrophoretic migration of the viruses as a possible means of strain differentiation, and 3) reevaluate the immunological relationships among these viruses using conventional and non-conventional serological techniques.

## LITERATURE REVIEW

The occurrence of cucumber mosaic virus (CMV) in the United States was first reported by Doolittle (17) and Jagger (30) in 1916. Price in 1934 presented evidence showing that variants of CMV, differentiated on the basis of symptomatology, could be obtained from CMV-infected plants and arose by a natural mutation process (47). In 1952, Sill and Walker (55) described work with an isolate of CMV from spinach which was later characterized as the American-type culture (AC-127) of CMV, strain Y (2). This isolate has been utilized in numerous investigations, (33, 36, 44, 51, 52, 57).

Tomato aspermy virus (TAV) was first isolated in England from tomato (Lycopersicon esculentum Mill.) in 1949 by Blencowe and Caldwell (7). Chrysanthemums (Chrysanthemum indicum L.) growing nearby the infected tomatoes were suggested to be the source of the virus. As a result, numerous aspermy-type viruses derived from chrysanthemum (CV) have since been reported (12, 23, 24, 26, 27, 36, 45) and frequently referred to as TAV (23, 24, 27, 45).

Peanut stunt virus (PSV) was first described by Miller and Troutman, who isolated the virus on a farm in Virginia (40). Subsequently, a virus discovered in

the state of Washington has been reported as a western strain of PSV (PSV-W) (42).

CMV (63), TAV (7), CV (27) and PSV(29) are transmitted by aphids in a non-persistent manner. The viruses are readily sap transmissible.

CMV and PSV are small, icosahedral viruses with particle diameters of 28-30 mμ (44, 51, 59) and 25-30 mμ (43, 56), respectively. A diameter of 23-25 mμ has been reported for CV (L-strain) (37). The ribonucleic acid (RNA) content is 18% (Q strain) (20) to 18.5% (Y strain) (33) for CMV and 16% for PSV-W (43). The sedimentation coefficients of CMV and PSV are 92-101s (20, 33, 57, 61) and 97-107s (43, 56), respectively. CMV particles are constructed of 180 protein subunits in pentamer-hexamer clusters (18), each subunit having approximately 287 amino acids and a molecular weight of 32,000. (61).

A frequent problem with CMV, attributed to both its instability and tendency to aggregate (51), has been obtaining purified virus. The poor immunogenicity of CMV-Y has been suggested to reflect upon the propensity of the virus to degrade when mildly heated or exposed to low salt concentrations (52). In the first purification procedure outlined for CMV-Y, infected tobacco tissue was homogenized in high molarity (0.5 M) potassium phosphate buffer and clarified with 8.5% n-butanol (57).

The virus was concentrated using acid precipitation and differential centrifugation. van Regenmortel, unsuccessful with above procedure, was able to purify an isolate of CMV from South Africa (CMV-S) using density gradient electrophoresis (59). Scott reported that application of either of the above procedures for the purification of CMV-Y from tobacco was unsatisfactory (51). Suitable levels of infectivity could be obtained only when tissue was homogenized in high molarity (0.5 M) sodium citrate or potassium phosphate buffers containing thioglycollic acid (51). In doing so, however, undesirable aggregation of the virus resulted causing reduced infectivity and loss of virus with differential centrifugation. Aggregation in the chloroform-clarified homogenate, prior to the initial high speed centrifugation, could be overcome by dialysis on low molarity (0.005M) sodium borate buffer. Scanning patterns of purified virus using density-gradient centrifugation revealed major and minor (faster) sedimenting components (52). Other researchers have reported success with this procedure for the purification of CMV-Y (33, 36).

Grogan et al. (24) developed a procedure for the purification of CV which has also been reported to be successful for TAV-B (Blencowe isolate) (36). Clarification of the homogenate was achieved by preferential

precipitation of host components by acidification. The virus was concentrated by differential centrifugation.

Purification procedures for PSV (56) and PSV-W (43) from cowpea (Vigna sinensis (Torner) Savi) have utilized differential centrifugation following chloroform-butanol clarification. In each instance, infectivity was associated with a particle sedimenting at approximately 100s in sucrose density gradient columns which has not yet been reported for CMV, TAV or CV. The fewer purification difficulties with PSV suggest that it is a more stable virus.

Early evidence for or against the relatedness of CMV and TAV was based upon cross protection. Such protection assumes that following virus infection of a plant cell, further infection of that same cell with a related virus cannot occur (49). Blencowe and Caldwell reported that TAV-B did not cross protect against two strains of CMV (7). Hollings subsequently confirmed this report with three strains of TAV (27). Based on their cross protection studies, Govier (22), Graham (23) and Hitchborn (26) considered CMV and TAV related. Such contradictions can be reconciled when one considers that TAV-B has since been shown to be serologically related to CMV while at least CV isolates are not (36). Since TAV and CV can each infect chrysanthemum, the exact identity of the chrysanthemum-isolated viruses used by Graham (23),

Hitchborn (26) and Hollings (27) cannot be known. Although the same virus (TAV-B) was used by Blencowe and Caldwell (7) and Govier (22), they arrived at different conclusions. This apparent contradiction could be due to individual interpretation since cross protection is not an all-or-nothing phenomenon (49) or to climatic differences (23).

In his review on plant virus classification, Gibbs states that "the sequence of nucleotides in the nucleic acid of a virus is perhaps the only functional basic character of a virus. If this is so, a classification based on the nucleotide sequence should be our ultimate aim." (21). Homologous nucleotide sequences in certain portions of different viral nucleic acids would then be the fundamental basis for relatedness. Unfortunately, the nucleotide sequence of any viral genome remains unknown. Operationally more feasible would be the establishment of relatedness based on similar primary structures (amino acid sequences) of different viral coat proteins which could be directly correlated to similar nucleotide sequences assuming the universality of the genetic code. However, only the amino acid sequence of the protein of tobacco mosaic virus (TMV) has been determined (3, 58).

By 1966 85 antisera were already reported to have been prepared against plant viruses (60). The development of gel-diffusion serological techniques have greatly facilitated investigations concerned with relationships

of plant viruses and practically replaced the free-liquid precipitation reactions (60). From the point of view of practicality, similar chemical structure as based on immunological tests offer specificity, second only to structural chemical analyses, and utility (35).

Grogan et al. reported that CMV and TAV were unrelated based on their agar-gel double-diffusion studies, although both TAV isolates used were from chrysanthemum (24). Lawson showed that TAV-B possessed some antigenic determinants common to the Y and Imperial strains of CMV (36). CMV-Y antiserum produced both low and high molecular weight precipitin zones in diffusion against TAV-B. Spur formation between the high molecular weight precipitin zones of each virus indicated serological non-identity. In the reciprocal reaction only low molecular weight precipitin line of CMV-Y formed. The low molecular weight precipitin line of CMV-Y has been reported to represent a product of virus degradation combined with antibody whereas the high molecular weight precipitin line will be formed in the reaction of antibody with undegraded virus (52). Lawson also reported that CV antisera, prepared against two isolates of the aspermy-type virus from chrysanthemum, reacted weakly with the high molecular weight antigen of TAV-B. While a reciprocal reaction was not observed in gel diffusion, that TAV-B

antiserum reacted with CV could be detected in microprecipitin tests. CMV-Y or CMV-I did not react with any of the CV antisera; the same was true in reciprocal tests. The three CV isolates proved to be serologically identical against any of their own antisera. As a result of these findings, Lawson considered TAV-B to be a strain of CMV. He also suggested that the three isolates of CV (and those like them) be considered a separate group identified by a lack of serological relatedness to CMV but showing some serological relationship to TAV-B. In 1969, Mink presented evidence that PSV-W possessed some antigenic determinants in common to certain strains of CMV, to TAV-B and CV (41). He suggested that CV (L strain) was sufficiently similar serologically to PSV-W to consider them strains. No conclusions could be reached concerning the relationships of PSV-W to CMV and TAV-B.

# MATERIALS AND METHODS

## Cultural Conditions

The isolate of CMV was obtained from J. L. Troutman and was characteristic of the Y strain described by American Type Culture Collection (2). The PSV isolate used was described by Miller and Troutman (40). Isolates of the aspermy-type viruses, TAV-B, the British type culture as described by Blencowe and Caldwell (7), and CV-L were obtained from R. H. Lawson (36). The viruses were maintained in either Nicotiana tabacum L. var. Vesta 5 or N. tabacum L. var. Xanthi NN.

Inoculum was prepared by grinding infected leaf tissue in 0.1M or 0.01M sodium phosphate buffer, pH 7.7, using a mortar and pestle. The pestle was used to apply the buffer extract to tobacco in the 3-5 leaf stage, previously dusted with 600-mesh carborundum. For infectivity assays using V. sinensis var. Early Ramshorn, inoculum was applied with a cheesecloth pad. Following all inoculations, leaves were rinsed with water. All plants for maintaining the viruses, for host range studies, and for infectivity assays were grown from seed in a greenhouse in a mixture of steam sterilized soil and expanded shale (Weblite).

## Purification

Slightly different methods were used to purify each of the viruses from leaves of Vesta 5 tobacco, following initial grinding in a Waring Blendor in the appropriate buffer. All low speed centrifugations were at 8-10°C for 10 minutes at 10,000 rpm in a Lourdes centrifuge (Model A-2).

Tissue infected with CMV for 8-10 days was ground in 0.1M sodium phosphate buffer, pH 7.7 with 0.2% (w/v) sodium diethyldithiocarbamate (DIECA) at the rate of 1 gm tissue: 2.0-2.5 ml buffer. The tissue homogenate was strained through 4 layers of cheesecloth and then combined while stirring with a volume of chloroform equal to the tissue weight (51). Following additional stirring for approximately 2 minutes, the emulsion was broken by low speed centrifugation and the aqueous phase was pipetted off the lower organic layer. Virus was concentrated from the aqueous phase by centrifugation at 50,000 rpm for 45-50 minutes in the Type 65 rotor or at 30,000 rpm for 2.5-3.0 hours in the Type 30 rotor in a Beckman L2-65B ultracentrifuge. Pellets were resuspended in 0.005M sodium borate buffer, pH 9.0 (51). Insoluble material was removed by an additional low speed centrifugation. The preparation was kept cold in ice throughout the procedure.

TAV-B and CV-L infected tobacco leaf tissue,

harvested 10-16 days after inoculation, was ground in 0.1M phosphate buffer with 0.2% DIECA in the proportions of 1 gm: 2 ml. All suceeding steps were identical to those used for CMV, except that the pellets were resuspended in 0.01M sodium phosphate buffer, pH 7.7.

PSV was purified according to Tolin (56) from tobacco leaves or primary leaves of cowpea harvested 11-12 days or 7-8 days after inoculation, respectively. Extraction was achieved by grinding in 0.05M sodium phosphate buffer, pH 7.7, with 0.2% DIECA, followed by clarification with a mixture of chloroform and 1-butanol (1:1) in the proportions 1 gm: 1.25-1.50 ml: 1 ml, respectively. The remaining steps were exactly the same as those outlined for TAV-B and CV-L.

Antisera were prepared for CMV and PSV. Both viruses were purified as described, except that pellets were resuspended in buffer containing 0.2% formaldehyde (HCHO) in an attempt to increase stability (28). An additional high speed centrifugation in the Type 65 rotor for 45 minutes at 50,000 rpm further concentrated the virus, which was again resuspended in buffer with 0.2% HCHO.

## Analysis and Properties of Purified Virus

### Centrifugation

Partially purified virus preparations were separated by sucrose density-gradient centrifugation (8) and

analyzed with the ISCO Model D Density Gradient Fractionator and Ultraviolet Analyzer (9). Density-gradient columns were prepared in cellulose nitrate tubes for the SW 27 rotor (1" x 3 1/2")or for the SW 65 rotor (1/2" x 2") by layering 6, 9, 9 and 9 ml or 4 x 1.1 ml of 100, 200, 300 and 400 mg sucrose/ml of buffer, respectively. After preparation, gradients for the SW 27 rotor were allowed to stand at 6°C for at least 12 hours before use; gradients for the SW 65 rotor were stored for at least 6 hours prior to use. Gradients not used within 1 week were discarded.

The SW 27 rotor gradients were analyzed at 254 mμ in the range of 0-1.0 absorbancy units to give a scanning pattern of absorbancy versus depth. A flow rate of 3 1/3 ml/minute was used. Absorbance could be increased by a factor of 3 by positioning inserts in flow cell horizontally. The SW 65 rotor gradients were analyzed in the same manner except that an absorbancy range of 0-0.75 (0-0.25 range, horizontal inserts) and a flow rate of 0.5 ml/minute were used. Samples above, at and below the virus zone were collected manually during the fractionation process for infectivity assays. Further virus purification was achieved by concentrating the virus from collected zones by centrifugation in the Type 65 rotor (50,000 rpm for 90 minutes).

The ultraviolet absorption spectrum and the

specific absorbance at 260 mμ ($A_{260}$) of the virus preparations and gradient-collected virus were determined with a Unicam (Model SP 800A) spectrophotometer. Absorbancy of a 1 mg/ml suspension at 260 mu was taken to be 5.0 (33).

Gel electrophoresis

Polyacrylamide gel electrophoresis (46) was performed in 2.5 and 3.75% separating gels made from stock solutions as prescribed in the chemical formulations of Canalco. When polymerization of the separating and stacking gels was complete in 5 mm (i.d.) glass tubes about 60 mm in length, the virus samples were introduced into the sample gel or layered directly onto the stacking gel. In the latter case, a 0.1 ml of 0.01M phosphate or 0.005M borate buffered 10% sucrose solution was first placed onto the stacking gel to which the sample was added and mixed. About 25 - 100 μl samples were inserted into the disc gel apparatus (Canalco Model 12 system) and each reservoir was filled with one liter of buffer. The power supply (Model 21, Arthur H. Thomas Co., Philadelphia, Pa.) was then immediately connected, anode to the lower reservoir. Electrophoresis was conducted at 6°C for 3-5 hours at a constant current of 2.5 mA per tube. The gels were removed from the glass tubes after completion of electrophoresis and stained with 0.5% aniline black in 7% acetic acid for at

least 1.5 hours. Gels were destained electrophoretically and stored in 7% acetic acid.

## Serology

### Antisera preparation

Two rabbits each received 3 weekly hindleg, intramuscular injections of freshly purified, formaldehyde-treated, virus emulsified with an equal volume of Freund's complete adjuvant. The total amount of each virus injected was approximately 17 mg. Prior to the initial injection, 7-10 ml of normal serum was taken from each rabbit. The rabbits immunized against PSV were bled on the second and third week after the final injection. CMV immunized rabbits were bled on the second week after the final injection only. Normal and immune sera were obtained by cardiac puncture using either a 10 or 50 ml sterilized syringe with a 2", 18-gauge needle (5). When injecting virus or taking blood samples, the rabbits were first swabbed in the area of needle insertion with 70% ethyl alcohol containing 1% (v/v) iodine. Sterile blood samples were stored overnight at 6°C. On the following day, the serum was pipetted from the clot and centrifuged for 15 minutes at 3,000 rpm in a conical centrifuge tube. The serum was removed carefully and dispensed into small test tubes or ampoules in 1-5 ml volumes. A 0.1% (w/v) solution of sodium azide was added to the serum samples to give a

final concentration of 0.001% per ml. Some serum samples were combined with an equal volume of glycerin while the rest were frozen. All sera were kept at -20°C until used. Anti-TAV and anti-CV-L sera were obtained from R. H. Lawson (36).

Conventional serological tests

Microprecipitin tests were performed in disposable plastic petri dishes (15 x 100mm) according to the procedures outlined by Ball (4). PSV, purified through two high speed centrifugations, was serially diluted from 1.0-0.03 mg/ml in 0.01M neutral phosphate buffer. Normal and immune sera were serially diluted from 1/2 to 1/64 in neutral 0.01M phosphate buffered 0.85% NaCl (PBS). The 0.01M phosphate buffer and PBS served as antigen and serum controls, respectively. Normal tobacco antigen extracted in 0.05M phosphate buffer, pH 7.7, was centrifuged at low speed and used to detect healthy reaction. Using a 0.025 ml per drop disposable micropipette, one drop of virus and serum were combined in appropriate squares drawn as an 8 x 8 pattern with a wax pencil. Each dish was flooded with mineral oil until all the drops were completely covered. Dishes were stored at 6°C for 4 hours and then read under a dissecting scope with dark field illumination. They were checked through 48 hours incubation for any changes.

Agar-gel double-diffusion tests were conducted in disposable plastic petri dishes (15 x 100mm) containing 14 ml of 0.7% Ionagar (Consolidated Laboratories, Inc., Chicago Heights, Illinois) with 0.01% sodium azide (4). Eight wells punctured with a No. 4 cork borer were symmetrically spaced 4mm apart around a central well punctured with a No. 5 cork borer in freshly prepared plates. Virus concentrations were determined spectrophotometrically, with an estimated 10% correction for non-viral absorbance at 260 mμ. The central wells were charged with 0.1 ml of virus. Six peripheral wells were filled with 0.075 ml of serial 2-fold dilution of antisera. The remaining two wells were charged similarly with PBS and normal serum diluted 1/4. Various well arrangements were also utilized for additional serological tests. Plates were checked daily for formation of precipitin lines.

Density-gradient serology

A slightly modified procedure from that described by Ball and Brakke was used in the density-gradient serology investigations (6). Using partially purified virus, virus-serum mixtures were incubated for short periods of time. Centrifugation time was reduced by using the SW 65 rotor.

The anti-CMV and anti-PSV sera used throughout the density-gradient serology experiments were titrated against their homologous virus. Serial 2-fold dilutions of the anti-CMV and anti-PSV sera were combined with equal volumes (0.25 ml) of virus preparations diluted to 1.0-2.4 $A_{260}$ units, and mixed thoroughly. From the virus-serum mixtures, 0.4 ml aliquots were layered onto SW 65 gradients after incubation at 6°C for 15 minutes (CMV) and 25 minutes (PSV). The gradients were centrifuged for 25 minutes at 60,000 rpm. Normal serum (1/8) and buffer controls were also included. The virus peak areas were measured planimetrically (Model L 30 AB, Los Angeles Scientific Instrument Co., Inc., Los Angeles, California) and converted to µg of virus per gradient tube.

The anti-CMV and anti-PSV sera and their respective normal sera were diluted 1:4 in 0.01M phosphate buffer, pH 7.7, in tests against TAV-B, CV-L, and PSV. The sera were diluted in 0.005M borate buffer, pH 9.0, for reactions against CMV. Virus preparations, diluted to the previously described absorbancy levels, were combined with an equal volume (0.25 ml) of sera. After thorough mixing, the solutions were incubated at 6°C for 15 minutes with CMV, for 20 minutes with TAV-B, and for 25 minutes with CV-L and PSV. Buffer controls consisted of combining 0.25 ml virus with the same volume of resuspending

buffer. Following incubation, 0.4 ml was layered onto gradients and centrifuged. In cross reactions with the anti-TAV-B and anti-CV-L sera, a 1/8 dilution of serum was mixed with virus. In these reactions, normal sera were not available.

Normal Vesta 5 tobacco antigen, as well as TMV from Vesta 5, were purified in a similar manner as that used for the viruses. Normal antigen preparations were incubated with anti-CMV and anti-PSV sera and centrifuged for 25 minutes.

# RESULTS

## Host Reactions

The symptomatology of the four viruses wer compared on *N. tabacum* var. Vesta 5 (tobacco), *V. sinensis* var. Early Ramshorn (cowpea), *Zinnia elegans* L. (zinnia), *Cucumis sativus* L. var. Improved Chicago Pickling (cucumber) and *Chenopodium amaranticolor* (Coste & Reyn.) and are summarized in Table I. Vesta 5 tobacco was infected systemically by each virus. Initial symptoms appeared in 4-5 days as diffuse chlorotic lesions on the inoculated leaves. Plants infected with CMV expressed severe vein clearing in the new growth followed by a brilliant yellow mosaic pattern. The systemic symptoms induced by TAV-B and CV-L were less severe and appeared in 8-9 days as a mild chlorotic mottle. Tobacco infected with PSV showed the least severe systemic symptoms, with a faint chlorotic mottle.

In cowpea, only PSV could be recovered from the trifoliate leaves following inoculation of primary leaves. The primary leaves, 5-7 days after inoculation, developed chlorotic and irregular necrotic spots, followed by vein clearing in the emerging trifoliate leaves. Necrotic lesions developed in 1-2 days on cowpea primary leaves inoculated with CMV and became progressively larger in the next 2-3 days. The pinpoint necrotic lesions

Table I. Reactions on tobacco, cowpea, zinnia, cucumber and C. amaranticolor produced by peanut stunt virus (PSV), chrysanthemum virus (CV-L), tomato aspermy virus (TAV-B) and cucumber mosaic virus (CMV).

| HOST | HOST REACTION TO VIRUSES: PSV | CV-L | TAV-B | CMV |
|---|---|---|---|---|
| Tobacco | L[a] -chlorotic lesion<br>S[b] -faint mottle | L -chlorotic lesion<br>S -mottle | L -chlorotic lesion<br>S -mottle | L -chlorotic lesion<br>S -mosaic |
| Cowpea | L -necrotic, chlorotic lesions<br>S -N.R.[c] | L -necrotic lesion<br>S -N.R. | L -necrotic<br>S -N.R. | L -necrotic lesion[d]<br>S -N.R. |
| Zinnia | L -N.R.<br>S -increased stem growth | L -N.R.<br>S -stunted growth | L -N.R.<br>S -secondary stem growth[e] | L -N.R.<br>S -mosaic |
| Cucumber | L, S -N.R. | L, S -N.R. | L, S -N.R. | L -N.R.<br>S -mosaic, stunted growth |
| C. amaranticolor | L -chlorotic lesion<br>S -N.R. | L -chlorotic lesion<br>S -N.R. | L -chlorotic lesion<br>S -N.R. | L -chlorotic lesion<br>S -N.R. |

[a]Local reaction on inoculated leaf
[b]Systemic reaction
[c]No reaction
[d]Expanding lesion
[e]No virus recovered on cowpea

produced by CV-L and TAV-B on primary leaves appeared in 2-3 days and did not expand (Fig. 1).

Zinnia inoculated in the four-leaf stage with CMV developed a mosaic pattern in new growth (Fig. 2-A). Inoculation with PSV stimulated stem elongation (Fig. 3) and leaf curling (Fig. 2-C) but caused no mosaic. CV-L induced stunting (Fig. 3) and some leaf curl (Fig. 2-B) but no mosaic. Zinnia inoculated with TAV-B failed to show symptoms (Fig. 2-D) other than an increase in secondary stem growth, which was also characteristic of PSV and CMV-infected plants (Fig. 3). Infection of cowpea was not achieved using tissue from three separate TAV-B inoculated zinnia. The other viruses were recovered on cowpea.

Cucumber inoculated at the cotyledonary stage with TAV-B, CV-L and PSV failed to develop symptoms. None could be recovered on cowpea from cucumber 2-3 weeks after inoculation. CMV infection initiated mosaic and stunting after 12 days.

On C. amaranticolor, all four viruses induced pinpoint chlorotic lesions 1-2 days after inoculation.

## Analysis and Properties of Purified Virus

### Centrifugation

Consistent analyses were obtained when 0.25-0.50 $A_{260}$ units of purified virus in 0.2-0.5 ml was layered

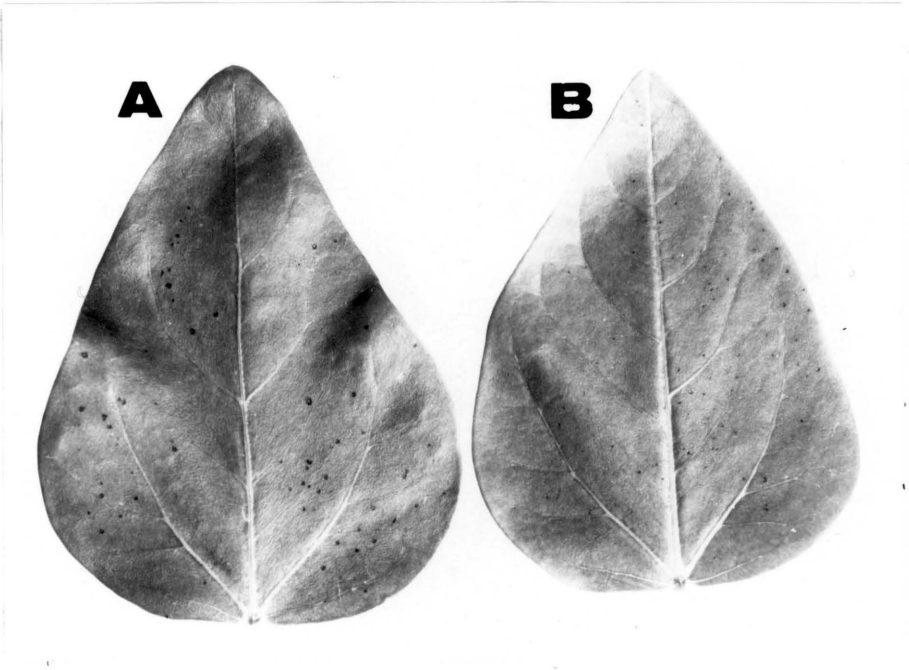



Figure 1. Local lesions induced by CMV (A) and TAV-B (B) on cowpea four days after inoculation.

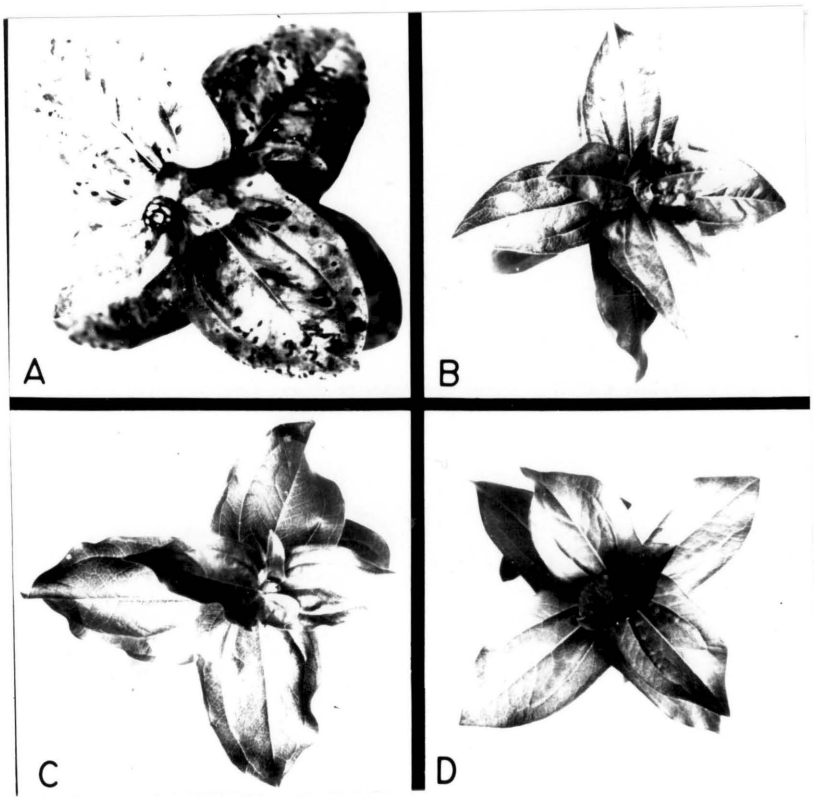



Figure 2. Foliar symptoms on zinnia 17 days after inoculation with CMV (A), CV-L (B), PSV (C) and TAV-B (D).


Figure 3. Growth effects of the four viruses on zinnia 17 days after inoculation. H=healthy control.

onto SW65 gradients and centrifuged at 60,000 rpm for 20 minutes. Alternatively, 4-8 $A_{260}$ units in 2 ml was layered onto SW27 gradients and centrifuged for 3 hours at 27,000 rpm. All of the virus preparations contained a component sedimenting to a depth of 2.2-2.4 cm in SW65 gradients (Fig. 4) or to 3.2-3.6 in SW27 gradients, as well as some slowly sedimenting components. Healthy tobacco, processed in the same manner as infected tissue and layered onto gradients in equivalent $A_{260}$ units, contained only slowly sedimenting components (Fig. 5).

CV-L and PSV did not aggregate and preparations were useful for further tests for at least one week. In contrast, preparations of CMV and TAV-B had a strong tendancy to aggregate during and after purification, as determined by the appearence of a faster sedimenting zone in scanning patterns. The increased centrifugation time required when using the Type 30 rotor frequently seemed to enhance the aggregation. Several attempts to purify CMV according to Scott were unsuccessful (51).

The ultraviolet absorption spectrum of the virus collected zones from gradients showed a maximum absorbance at 258-260 mμ. The minimum for CMV and for TAV-B, CV-L and PSV was 235-236 mμ and 240-242 mμ, respectively (Fig. 6). The $A_{260/280}$ ratios were 1.90 for CMV, 1.86-1.88 for TAV-B and CV-L and 1.73 for PSV.

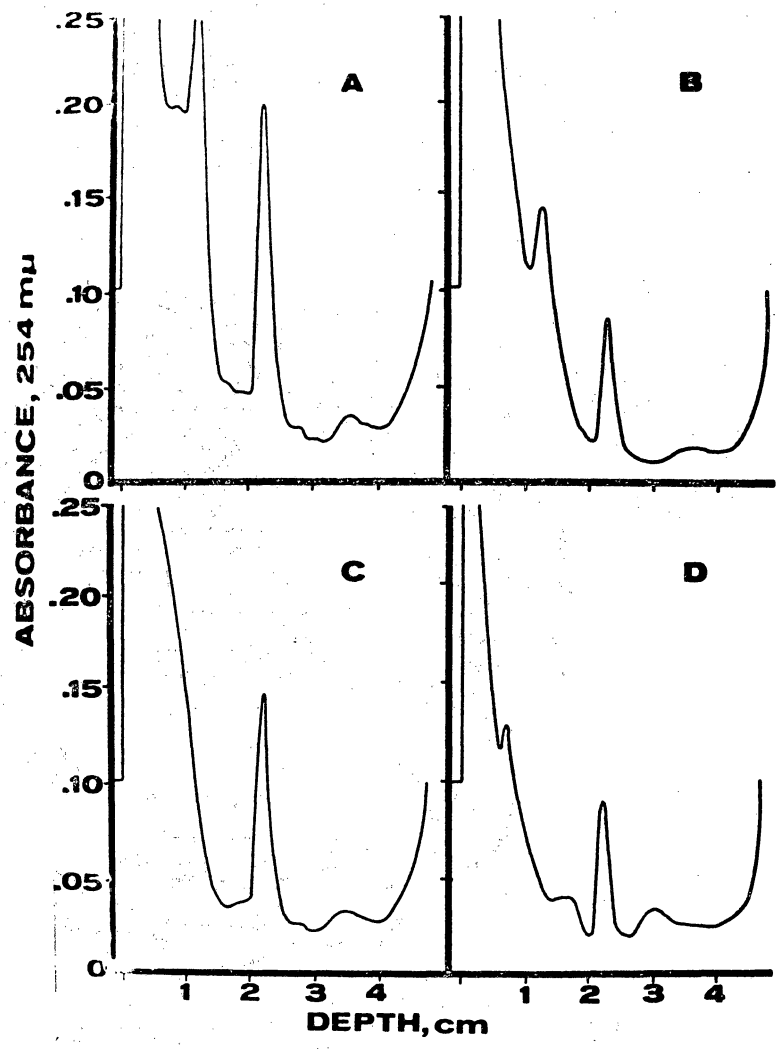



Figure 4. Photometric scanning patterns of sucrose density gradient columns prepared in 0.01M phosphate buffer, pH 7.7 (A, B, C,) or 0.005M borate buffer, pH 9.0 (D). Virus preparations ($A_{260}$=0.3) centrifuged for 20 minutes at 60,000 rpm. A=PSV; B=CV-L; C=TAV-B; D=CMV.

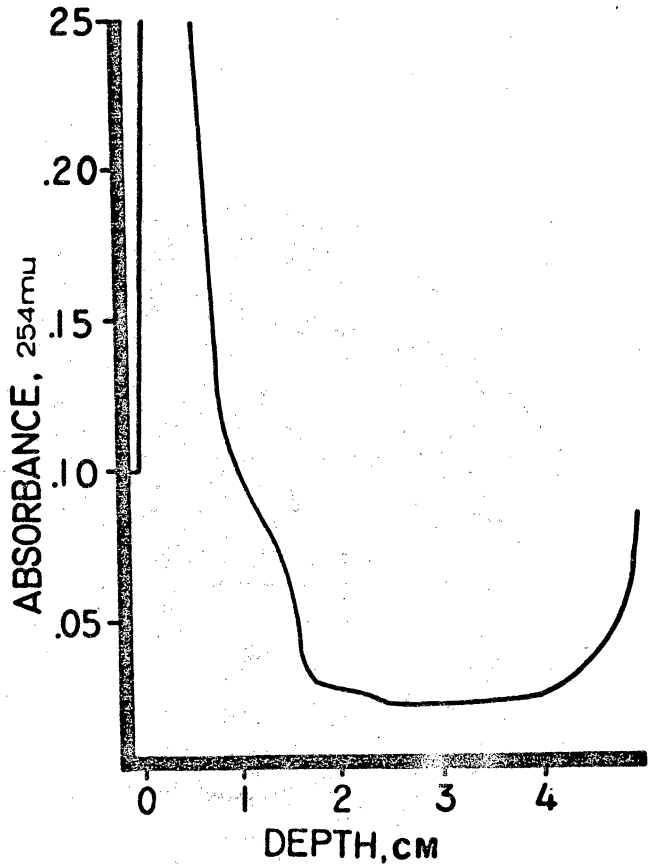



Figure 5. Scanning pattern of normal tobacco extract ($A_{260}$=0.3) layered onto sucrose gradient prepared in 0.01M phosphate buffer, pH 7.7 and centrifuged for 20 minutes at 60,000 rpm.

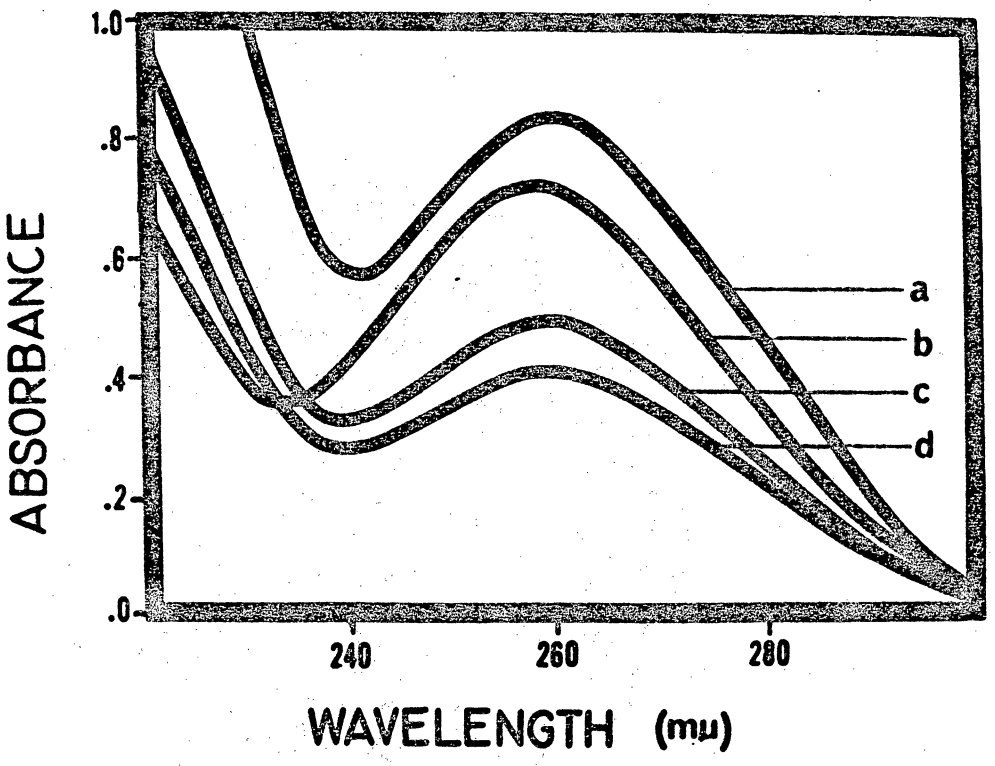



Figure 6. Ultraviolet absorption spectra of PSV (a), CMV (b), TAV-B (c) and CV-L (d) collected from sucrose gradients.

For infectivity assays of CMV, TAV-B and CV-L, 1-2 ml of a virus preparation ($A_{260}$=15) was layered onto SW27 gradients, centrifuged and scanned ($A_{254}$=0-3). Sequential 2 ml samples were taken manually through the virus zone. Four cowpea half-leaves were inoculated with each sample in an incomplete block design (48). With TAV-B and CV-L infectivity was closely correlated with the zone sedimenting at the same rate as PSV (Fig. 7). No infectivity could be associated with the CMV zone in two attempts, although unfractionated preparations were typically infectious. For example, purified extracts of CMV infected tobacco gave an average of 19 lesions at a 1:10 (w:v) dilution.

CMV and PSV prepared for immunization by HCHO treatment sedimented at the same rate as untreated virus. In addition slower sedimenting zones were also present (Fig. 8). To determine if any stability differences existed between HCHO-treated PSV and untreated virus samples of each were layered onto gradients prepared in 0.01M phosphate buffer, pH 7.7, and water, and centrifuged. The control and treated PSV suspensions were obtained from the same tissue batches by resuspending half of the pellets in 0.01M phosphate buffer containing 0.2% HCHO and other half was 0.01M phosphate buffer alone.

The typical sedimenting zone was absent only in

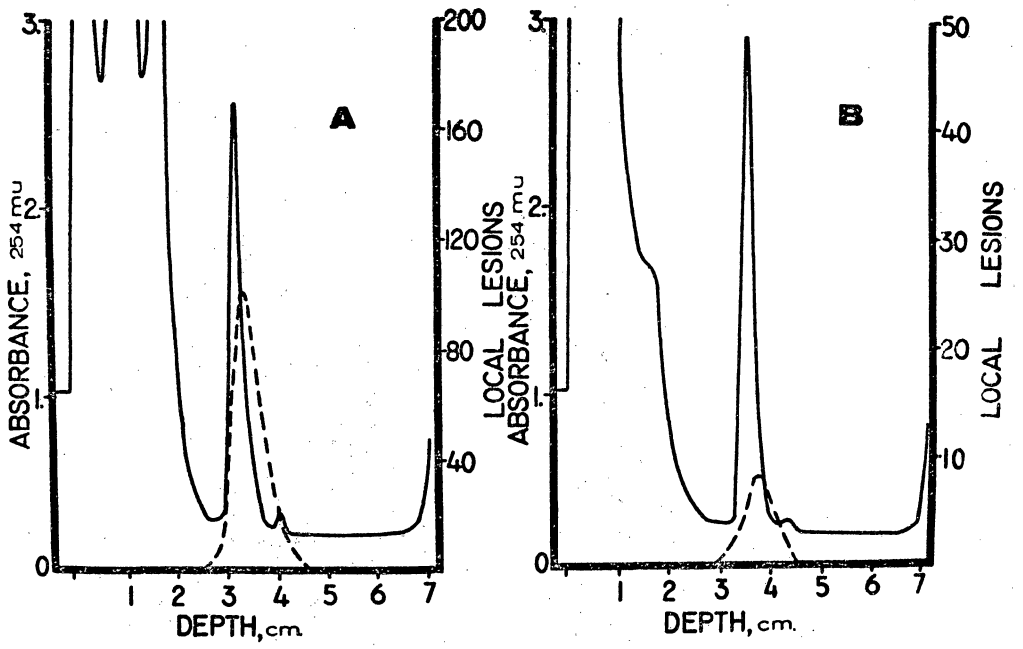



Figure 7. Scanning patterns (solid line) of CV-L (A) and TAV-B (B) in sucrose gradients prepared in 0.01M phosphate buffer, pH 7.7. Gradients layered with virus preparations ($A_{260}$=15) and centrifuged for 3 hours in the SW27 rotor at 27,000 rpm. Infectivity (dotted line) is an average of the number of lesions on four cowpea half-leaves.

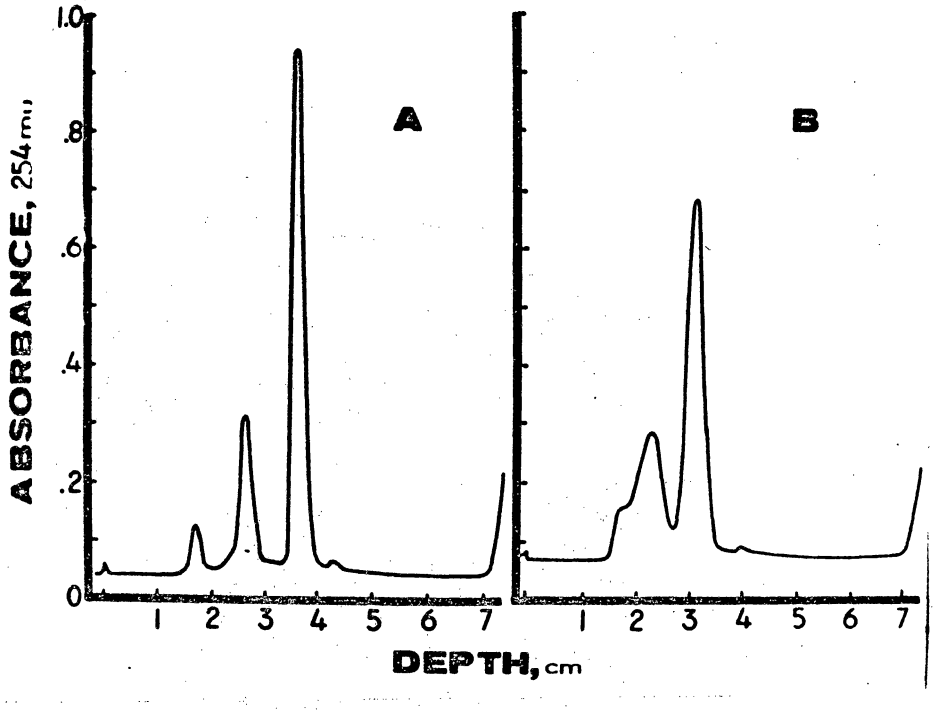



Figure 8. Scanning patterns of formaldehyde-treated PSV (A) and CMV (B) preparations layered onto SW27 gradients prepared in 0.01M phosphate buffer, pH 7.7, and 0.005M borate buffer, pH 9.0, respectively. Gradients centrifuged for 3 hours at 27,000 rpm. (PSV at $A_{260}$=5.2; CMV at $A_{260}$=5.6)

the water gradient with the control sample (Fig. 9). This gradient, however, possessed a slower sedimenting zone conceivably a virus degradation product, which was also present in both phosphate gradients.

Gel electrophoresis

Virus for polyacrylamide gel electrophoresis was purified through two high speed centrifugations or collected from gradient zones and concentrated by centrifugation.

Tris-glycine buffer (ionic strength=0.19), pH 8.2-8.4, as prescribed by Davis, was initially used in electrophoresis experiments (15). Using this buffer, no electrophoretic migration of the virus moieties occurred in the 2.5 and 3.75% separating gels with samples containing up to 400 µg of virus. Sample and stacking gels, however, were highly stained indicating that the virus had only slight or no mobility in the buffer.

When a 0.10 ionic strength glycine-NaCl buffer, pH 9.5, was used, some electrophoretic migration of the viruses in the 2.5% separating gel was achieved (39). A stained band just within the separating gel was consistently observed, as well as some more rapidly moving components (Fig. 10-A). Since CMV has been shown to degrade (33) and precipitate (52) in the presence of

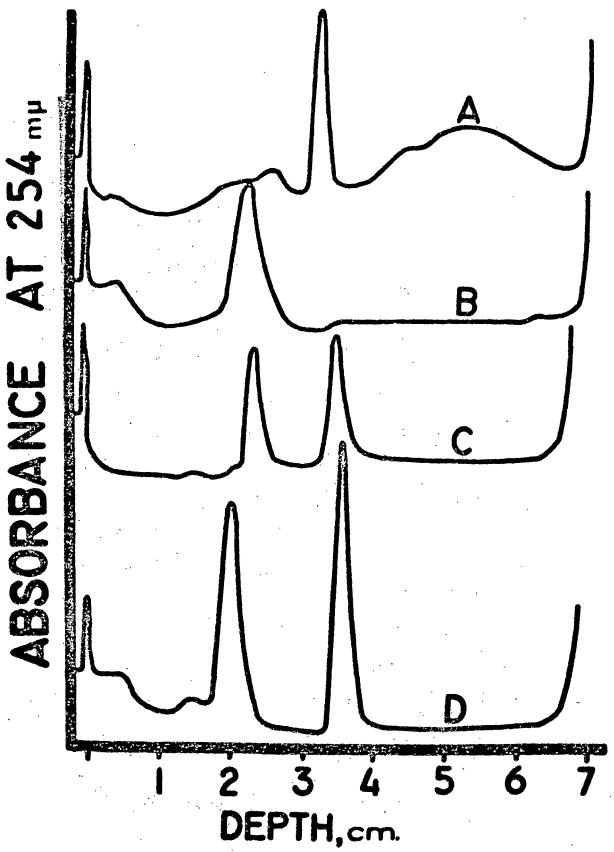
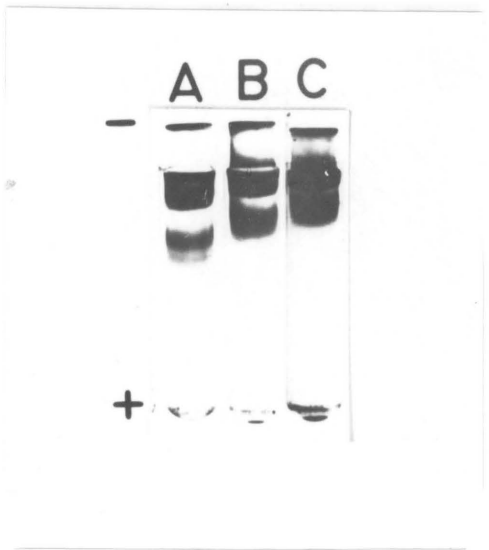



Figure 9. Scanning patterns of untreated (B, D) and formaldehyde-treated (A, C) PSV ($A_{260}$=3) layered onto sucrose gradients prepared in water (A, B) and 0.01M phosphate buffer, pH 7.7 (C, D). Gradients centrifuged for 3 hours in the SW27 rotor at 27,000 rpm.

A.




B.

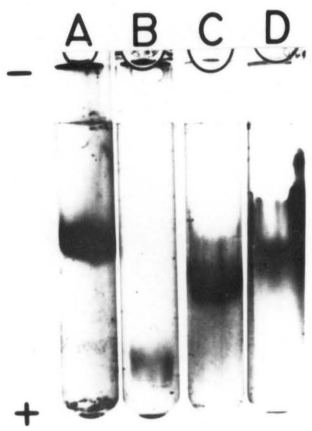



Figure 10. Results of large-pore acrylamide gel electrophoresis. Migration is from top to bottom. A. Electrophoretic patterns of PSV (200µg) after 3 (C), 4 (B), and 5 (A) hours using the glycine-NaCl buffer, pH 9.5. B. Electrophoretic patterns of gradient-fractionated TAV-B (A), CMV (B), PSV (C) and CV-L (D) after 5 hours using the tris-glycine buffer, pH 9.0. (TAV-B=70µg; CMV=40µg; PSV=80µg; CV-L=60µg)

chloride salts, further use of this buffer seemed undesirable.

The most rapid and satisfactory migration of virus moieties occurred using a 0.019 ionic strength tris-glycine buffer, pH 9.0 (54). Each virus appeared to move as a single component. The migration of CMV was more rapid than the other viruses. PSV migrated only slightly faster than CV-L and TAV-B. A problem frequently encountered with the use of large pore acrylamide gels was the inadequate polymerization leading to band streaking (Fig. 10-B).

Attempts at degrading the virus and comparing migration of protein subunits were not successful. Virus degraded by high salt concentrations resulted in fragments still possessing peak absorbance at 260 mμ, suggesting nucleic acid contamination.

## Serology

The immune sera from the rabbits injected with PSV shall be referred to as 901 or 902 followed by the numerals I or II to indicate serum obtained from the first or second bleeding, respectively. Since the rabbits immunized against CMV were bled only once, their immune sera will be referred to as 903 or 904.

Microprecipitin tests

Non-specific precipitation of PSV occurred in PBS and at all normal serum dilutions at virus concentrations of 0.25-1.0 mg/ml. This would suggest, as reported for CMV-Y, that PSV is rapidly precipitated in the presence of low salt concentrations (52). Normal tobacco antigen reacted with first bled sera out to the 1/8 dilution. At 0.06 mg virus/ml, three of the immune sera reacted out to the 1/16 dilution and the fourth to the 1/8 dilution.

The presence of normal tobacco antibodies in the immune sera in addition to non-specific precipitation of the virus, indicated the microprecipitin tests were unsuitable for PSV. Microprecipition tests were not performed with CMV.

Gel diffusion

Gel diffusion tests with PSV were conducted in Ionagar prepared with water, 0.01M phosphate buffer (pH 7.7) or PBS. PSV, purified through two high speed centrifugations, was diluted to concentrations of 0.90, 0.45 and 0.225 mg/ml. Only the 901-II and 902-II antisera were used.

At all virus concentrations, a single precipition line was formed in each Ionagar media. It extended to the 1/32 dilution in water agar, to the 1/16 dilution

in phosphate buffered agar and to the 1/4 dilution in PBS agar.

Normal tobacco antigen concentrated 10-fold by a single high speed centrifugation following chloroform clarification, formed the same precipitin line as described above for each Ionagar media. The low speed, unconcentrated preparation of normal tobacco antigen also formed a diffuse precipition line close to the antiserum wells at 1/2 through 1/8 dilutions.

In further tests with different well arrangements, a precipitin line, not present in the normal tobacco antigen, was formed in water agar at PSV concentrations of 5.4 and 1.8 mg/ml against each antiserum diluted 1/4 (Fig. 11-A). It was positioned closer to the virus well than two healthy precipitin lines and did not form at 0.9 mg virus/ml.

PSV collected from gradients and concentrated to 1.0 mg/ml by centrifugation, formed a single curved precipitin line in tests against the 902-II antiserum at a 1/4 dilution (Fig. 11-B). It was only faintly visible against a 1/8 dilution. No deflection of the line occurred toward the normal tobacco antigen precipitation lines. A strain of PSV has also been reported to form only a single precipitin line to the high molecular weight (virus) antigen (43).

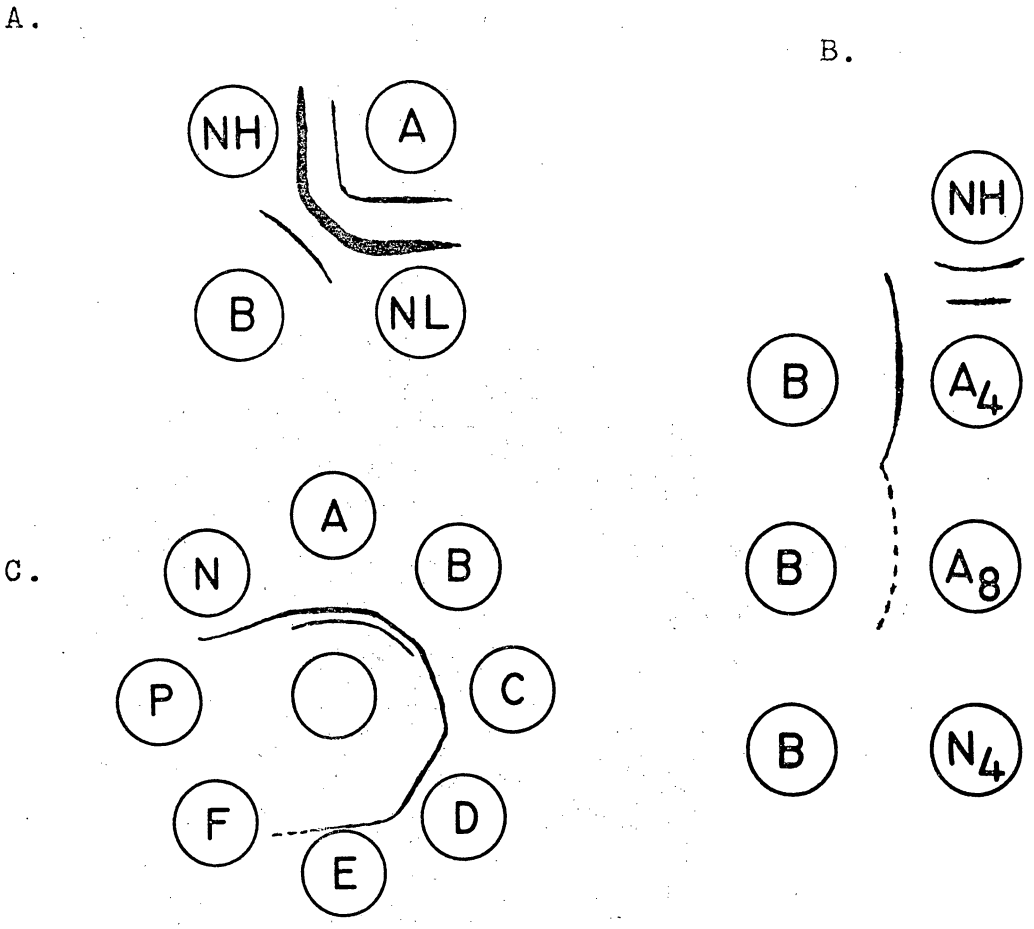



Figure 11. Diagrams of results of gel diffusion tests with PSV and CMV. NH, normal, high speed concentrated tobacco antigen; NL, normal low speed clarified tobacco antigen. A. A, PSV antiserum diluted 1/4; B, PSV=5.4 mg/ml. B. $A_4$, $A_8$, 902-II antiserum diluted 1/4 and 1/8 respectively; B, gradient-fractionated PSV (1.0 mg/ml). C. Peripheral wells charged with serial two-fold dilutions of CMV antiserum from 1/2(A) through 1/64(F); P, PBS; N, normal serum diluted 1/2.; CMV=1.8 mg/ml.

Gel diffusion tests with CMV at 1.8, 0.9 and 0.45 mg/ml in 0.005 M borate buffer, pH 9.0, were performed in water Ionagar only (52). At 1.8 mg/ml CMV preparations reacted with both antisera to form a precipitin line, closer to the antigen well than healthy reaction, at the 1/2 and 1/4 dilutions (Fig. 11-C). The same precipitin line also formed with CMV at 0.9 mg/ml in reaction against 904 antiserum, but not with 903. Neither antiserum formed this precipitin line with 0.45 mg/ml CMV. Healthy reaction, was present at each virus concentration.

At the virus concentrations used, neither the anti-PSV or anti-CMV sera could be shown to react appreciably beyond the 1/4 dilution. The possibility of demonstrating cross reaction in gel diffusion tests, therefore, seemed remote.

Density gradient serology

The analysis of antibody-antigen reactions by density-gradient centrifugation has been shown to be an extremely sensitive test primarily because serological reactions which are not visible in conventional techniques can be detected (6). As shown by Ball and Brakke, the end point of microprecipitin tests does not indicate the end of a serological reaction but only the point at which precipitate does not scatter enough light (6).

Since density gradient serology allows for direct detection of virus, it also has greater specificity than either the microprecipitin or gel diffusion tests in which a virus-antibody reaction is assumed by the formation of precipitate. Because our sera did not favor visual observation of precipitation, possessed normal tobacco antibodies, and precipitated non-specifically the viruses being studies, further investigation using density-gradient serology seemed desirable.

Analysis of centrifuged incubation mixtures of PSV preparations and antiserum dilutions revealed complete removal of 6.1 µg virus at the 1/8 dilution and nearly no removal of virus at the 1/128 dilution. Scanning patterns of CMV preparations incubated with dilutions of its homologous antiserum showed nearly complete removal at the 1/8 dilution and practically no removal at the 1/64 dilution. The percentage of non-specific loss of PSV and CMV when reacted against normal serum at 1/8 was 14.8% and 29.9%, respectively. A nearly horizontal straight line has been reported when the area under the virus peak was plotted against normal serum dilution (6). If this is assumed correct, the minimum dilution endpoint for the anti-PSV and anti-CMV sera would be 1/64 and 1/16, respectively (Fig. 12).

Incubation times chosen for each virus were essentially expedient. The incubation of CMV and TAV-B for

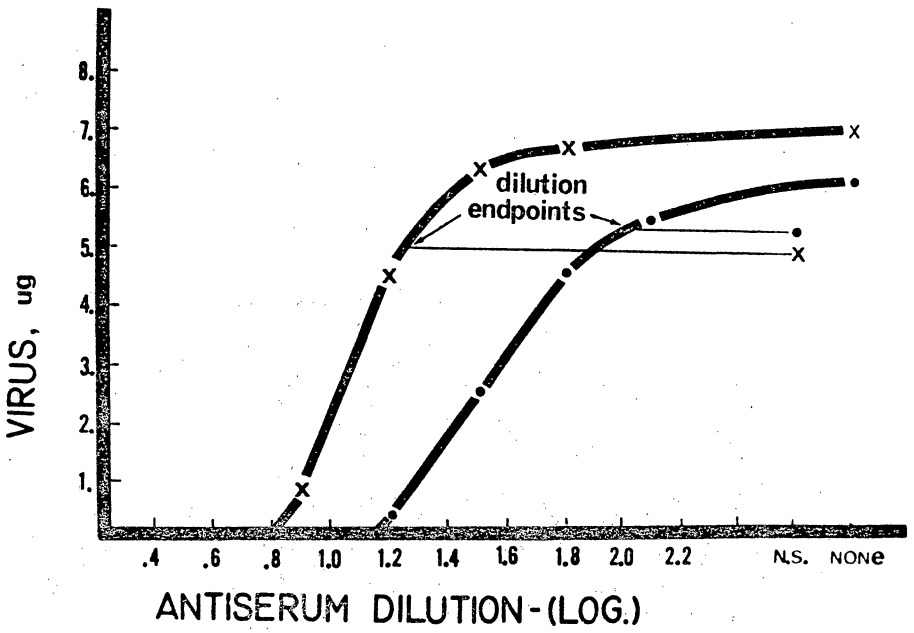



Figure 12. Micrograms of virus in scanning patterns of centrifuged density-gradient columns layered with CMV-antiserum mixtures (—X—) and PSV-antiserum mixtures (—●—). Four-tenths of a milliliter layered onto sucrose gradients prepared in 0.005M borate buffer, pH 9.0, (CMV) and 0.01M phosphate buffer, pH 7.7 (PSV). Gradients centrifuged at 60,000 rpm for 25 minutes. (N.S. and none are μg of virus in normal serum (1/8) and buffer controls, respectively.) Antiserum dilutions reported as the logarithm of the reciprocal titer.

longer periods than reported was observed occasionally to be unsatisfactory because of the instability of the viruses. All four viruses were reacted against the four antisera. Measuring peak areas planimetrically, 2.7-11.7 μg of virus was detected in buffer control gradients. Assuming an $A_{260}$ of 1.0 mg/ml to be 3.24, approximately 6.6 μg TMV was detected in buffer control gradients (10).

All the antisera completely removed their homologous viruses. The anti-CMV serum partially removed in a specific manner both PSV and TAV-B but failed to remove CV-L. When the concentration of CV-L was increased, no specific loss could again be detected (Table II). Against the anti-PSV serum, CMV was completely removed. CV-L and TAV-B were specifically, but not completely removed (Table III). CV-L was partially removed in reaction against the anti-TAV-B serum while CMV remained unreacted. PSV was only partially removed (Table IV). The anti-CV-L serum completely removed TAV-B and PSV, while CMV failed to react with the antiserum (Table V).

When TMV was incubated with anti-PSV serum, no decrease in unreacted virus occurred in comparison to normal serum. However, a small amount of virus was specifically removed by the anti-CMV serum. Non-specific loss of the virus did occur in comparison to buffer

Table II. Results of density gradient analysis of precipitin reaction with anti-CMV serum. Virus incubated in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0(CMV). Normal serum (N.S.) and antiserum (A.S.) diluted 1:4 in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Four-tenths of a milliliter layered onto gradients and centrifuged for 25 minutes at 60,000 rpm.

| VIRUS | INCUBATION MIXTURE: BUFFER | N.S. | A.S. | NON SPECIFIC LOSS (%) | SPECIFIC LOSS (%) |
|---|---|---|---|---|---|
| PSV | 7.0[a] | 6.8 | 2.3 | 2.9[b] | 64.3[c] |
| TAV-B | 11.5 | 7.7 | 0 | 33.0 | 67.0 |
| CV-L | 4.7 | 4.1<br>6.3 | 4.1<br>6.3 | 12.8 | 0<br>0 |
| CMV | 2.7 | 2.7 | 0 | 0 | 100.0 |

[a] µg virus per gradient tube

[b] % non-specific loss

$$= \frac{\mu g \text{ in buffer gradient} - \mu g \text{ in N.S. gradient}}{\mu g \text{ in buffer gradient}} \times 100$$

[c] % specific loss

$$= \frac{\mu g \text{ in N.S. gradient} - \mu g \text{ in antiserum gradient}}{\mu g \text{ in buffer gradient}} \times 100$$

Table III. Results of density gradient analysis of precipitin reaction with anti-PSV serum. Virus incubated in 0.01M phosphate buffer pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Normal serum (N.S.) and antiserum (A.S.) diluted 1:4 in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Four-tenths of a milliliter layered onto gradients and centrifuged for 25 minutes at 60,000 rpm.

| VIRUS | INCUBATION MIXTURE: BUFFER | N.S. | A.S. | NON SPECIFIC LOSS (%) | SPECIFIC LOSS (%) |
|---|---|---|---|---|---|
| PSV | 2.7[a] | 2.7 | 0.0 | 0.0[b] | 100.0[c] |
| TAV-B | 11.5 | 5.9 | 1.1 | 48.7 | 41.7 |
| CV-L | 3.8<br>3.8 | 3.4<br>4.0 | 2.0<br>1.2 | 10.5<br>+ | 36.8<br>68.4 |
| CMV | 6.1 | 5.4 | 0 | 11.5 | 88.5 |

[a] µg virus per gradient tube

[b] % non-specific loss

$$= \frac{\text{µg in buffer gradient} - \text{µg in N.S. gradient}}{\text{ug in buffer gradient}} \times 100$$

[c] % specific loss

$$= \frac{\text{µg in N.S. gradient} - \text{µg in antiserum gradient}}{\text{µg in buffer gradient}} \times 100$$

Table IV. Results of density gradient analysis of precipitin reaction with anti-TAV-B serum. Virus incubated in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Antiserum diluted 1:8 in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Four-tenths of a milliliter layered onto gradients and centrifuged for 25 minutes at 60,000 rpm.

| VIRUS INCUBATED IN: | VIRUS: PSV | TAV-B | CV-L | CMV |
|---|---|---|---|---|
| BUFFER | 7.0[a] | 4.5 | 4.1 | 11.7 |
| ANTI-TAV-B SERUM | 3.6 | 0 | 2.7 | 11.7 |

[a] µg virus per gradient tube

Table V. Results of density gradient analysis of precipitin reaction with anti-CV-L serum. Virus incubated in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Antiserum diluted 1:8 in 0.01M phosphate buffer, pH 7.7 (PSV, TAV-B, CV-L) or in 0.005M borate buffer, pH 9.0 (CMV). Four-tenths of a milliliter layered onto gradients and centrifuged for 25 minutes at 60,000 rpm.

| VIRUS INCUBATED IN: | VIRUS: PSV | TAV-B | CV-L | CMV |
|---|---|---|---|---|
| BUFFER | 7.0[a] | 4.5 | 4.1 | 11.7 |
| ANTI-CV-L SERUM | 0 | 0 | 0 | 11.7 |

[a] µg of virus per gradient tube

Table VI. Results of density gradient analysis of precipitin reaction with anti-PSV serum (A) and anti-CMV serum (B) against TMV. Virus incubated in 0.01M phosphate buffer, pH 7.7. Normal sera (N.S.) and antisera (A.S.) diluted 1:4 in 0.01M phosphate buffer, pH 7.7. Four-tenths of a milliliter layered onto sucrose gradient and centrifuged for 20 minutes at 60,000 rpm.

| VIRUS | INCUBATION MIXTURE: BUFFER | N.S. | A.S. | NON SPECIFIC LOSS (%) | SPECIFIC LOSS (%) |
|---|---|---|---|---|---|
| TMV | 6.6[a] | 5.2 | 5.2(A) | 21.2[b] | 0[c] |
| TMV | 6.6 | 5.2 | 4.8(B) | 21.2 | 6.1 |

[a] µg virus per gradient tube

[b] % non-specific loss

$$= \frac{\text{µg in buffer gradient} - \text{µg in N.S. gradient}}{\text{µg in buffer gradient}} \times 100$$

[c] % specific loss

$$= \frac{\text{µg in N.S. gradient} - \text{µg in antiserum gradient}}{\text{µg in buffer gradient}} \times 100$$

controls. Scanning patterns of normal tobacco antigen mixed with anti-PSV and anti-CMV sera contained no absorbing components which coincided with the virus.

## DISCUSSION

Of the characters used in this study to classify plant viruses, host range, symptomatology, and electrophoretic migration are useful for distinguishing strains, whereas serological reactions are most useful for grouping viruses (21).

The differentiation of the viruses studied here based on host range and symptomatology indicated that some plants are highly useful for this purpose while others are not. The reactions of zinnia and C. amaranticolor represent the extreme cases.

Reconstitution studies with broad bean mosaic, brome mosaic, and cowpea chlorotic mottle viruses (25) and with cucumber virus 4 (32) have shown that the nucleic acid alone determines the capacity of a plant virus to infect and produce symptoms in a host. The variation in reactions induced in certain hosts in this study indicates that each virus is a unique genetic system. If each virus is related but at the same time unique, these variations may be because of either specific differences in certain nucleotide sequences or dissimilar metabolic effects.

Since the sedimentation coefficients of CMV (20, 33, 57, 61) and PSV (43, 56) are about 100s and since TAV-B and CV-L sedimented at about the same rate as CMV and

PSV in sucrose gradients, the sedimentation coefficients of TAV-B and CV-L should also be about 100s. Viruses of similar morphology to CMV with sedimentation coefficients above 100s are typically stable in salt solutions while those with values below 100s are distinct from CMV sufficiently in this property as well as in their nucleic acid content (21). Having equal sedimentation coefficients offers additional evidence that these viruses are related. That many plant viruses morphologically similar to CMV exist, has been suggested to indicate that there are only a limited number of ways to construct a virus (14).

Infectious virus was recovered from CV-L and TAV-B gradient zones, but not from zones of CMV. The inability to fractionate infectious CMV from gradients may suggest that sucrose has a deleterious effect on the virus. Sucrose, however, has been shown to increase the number of lesions produced by a strain of CMV on cowpea (16). While PSV, CV-L and TAV-B from zones all had absorption spectrum minima at 240-242 mμ and were infectious, the minimum for CMV was 235-236 mμ. This change in the absorption of CMV probably indicates alterations in the viral protein or nucleic acid leading to a low specific infectivity not detectable at the virus concentration used. The $A_{260/280}$ ratios reported are all higher than

would be expected for viruses with 18-20% nucleic acid content. Values in the range of 1.56-1.66 have been previously reported for PSV-W (43) and CMV (33).

The procedures developed for purification also illustrated differences between the viruses. PSV and CV-L were stable and non-aggregating in 0.01M phosphate buffer, pH 7.7, whereas TAV-B was frequently observed to aggregate in the same buffer. Differences in the tendencies of viruses to aggregate in a particular buffer should reflect basic differences in the amino acid compositions of their coat proteins.

The electrophoretic mobility of a virus is determined almost entirely by the nature and number of charged groups at its surface (19, 50). Because the viruses have about the same diameters, the resistance encountered by each during electrophoresis in the acrylamide gels was assumed to be the same. The greater mobility of the virus in the 0.019 ionic strength buffer can be explained by the fact that the total effective charge of a virus will be greater in buffers of decreasing ionic strength (13). The poor mobility of the viruses in the other buffers was probably caused by a reduced charge and degradation. The electrophoretic migration of CMV was distinct suggesting differences in the amino acid composition of its coat protein from the other viruses. Each virus

moved as a single electrophoretic component. This is in contrast to the electrophoretic heterogeneity displayed by bean pod mottle and broad bean mottle viruses in acrylamide gels (53).

The weak immunological response evoked not only by CMV and PSV, but also by TAV and CV, has been reported on numerous occasions (20, 24, 36, 43, 52). In spite of attempts to stabilize CMV and PSV, these viruses still proved to be poor immunogens. This is in contrast to other plant viruses of similar morphology which have been shown to be highly antigenic. Immune sera produced against five isolates of cowpea mosaic virus had titers of 8,192 to 32,768 in gel diffusion tests (1). Antisera to turnip yellow mosaic virus had titers of over 4,000 (38).

Despite the low-titered character of our sera and aggregational tendencies of CMV and TAV-B, analysis of serological cross reactions using density gradient centrifugation seems very promising. This technique provides a sensitive analysis of the tube precipitin test with only unreacted virus sedimenting at a typical rate (6). Only the serological cross reactions of the virions and not their degradation products were thus detectable.

As reported previously by Lawson (36) and Grogan et al. (24) and confirmed here, CMV and CV-L possess no

common antigenic determinants. In addition to the TMV controls, this finding illustrates the specificity of the test. While CMV and CV-L share no common antigenic determinants, both of their antisera cross reacted with TAV-B. In gel diffusion tests, Mink reported that CMV-Y and a strain of PSV reacted with antiserum to another isolate of CMV in a manner indicating serological non-identity (41). This fact caused reservations in interpreting the relationships between the viruses. It seems highly probable that considerable antigenic variation exists among the members of this plant virus group. It should be expected, therefore, that different antigenic groups present on one virus may also be present on other member viruses, although these determinants need not be the same. Although CMV and CV-L are serologically unrelated, they have different antigenic determinants common to TAV-B.

The terms 'epitope' and 'cryptotope' were introduced to indicate antigenic determinants that are on the surface of an antigen and hidden or masked within the antigen, respectively (31). It is logical to assume that the cryptotopes of a virus are exposed during the immune response since degradation of the virus should take place. For a cryptotope to be detected in serological tests antigen breakdown must also occur. In reciprocal tests, TAV-V antiserum reacted with CV-L but not with

CMV. TAV-B antiserum has been reported to react only with the low molecular weight antigen of CMV-Y (36). This was suggested to be due to complete virus degradation, since TAV-B and CMV-Y in diffusion against the CMV-Y antiserum developed fused high molecular weight precipitin lines with spur formation indicating common epitopes. The results here showed that no reaction occurred between CMV and TAV-B antiserum. From this it is concluded that our isolate of CMV, at least serologically, is different from the Y strain used by Lawson. A possible explanation of our results is that the common antigenic determinant is buried with CMV and on the surface of TAV-B.

PSV antiserum cross reacted with all the viruses. The same was true in reciprocal tests. PSV, therefore, is the only virus of the four studied here which has epitopes common to the other three viruses.

The term 'serotype' was introduced by Kassanis to indicate viruses that have only a few epitopes in common and as such are distantly related serologically (34). In contrast, strains of a virus were thought to share all or most of their epitopes. This distinction of serotypes and strains, however, does not appear to be generally valid (62). With a large number of animals, both the homologous and heterologous titer and time of appearence were

quite variable. Differences in the amount of specific absorption reported here, for example, should not be expected to reflect differences in the degree of serological relationship. It was suggested by van Regenmortel and von Wechmar that the term 'serotype' should be used to distinguish viruses which are serologically distinct whether or not the relationship appears to be close or remote; strains of a virus should be serologically indistinguishable but differ in some other property (62).

All of the viruses studied here have been shown to be distinct on the basis of host range and symptomatology. Accepting the definitions of van Regenmortel and von Wechmar and applying them to the results of the density gradient serology study it is proposed that:

1. Viruses which reciprocally cross react are strains. (PSV and TAV; CV-L and PSV; CV-L and TAV-B; PSV and CMV)
2. Viruses which cross react only one way are serotypes. (TAV-B and CMV)
3. Viruses which do not cross react are serologically unrelated (CMV and CV-L)

Positive serological relationships between viruses have been proposed to reflect upon specific amino acid sequences in common which are vital to the continued existence of the viruses and are thus genetically

preserved during evolution (11, 60). If the viruses of this group are of common ancestry, the predecessor of this group could be speculated to resemble most closely PSV, since it is the only virus showing the proposed strain relationship to the other three viruses. Although the most recently discovered of the four viruses, this possibility is reasonable. In 1934 Price showed a derivative of CMV which infected cowpea systemically and in a manner resembling PSV (47). It has been shown with a large number of cowpeas, that mutants of CMV can be obtained which infect cowpea systemically also (64). The discovery of a strain of PSV in the state of Washington in 1967 probably indicates that the virus is geographically widespread (42).

A major disadvantage of serological tests is that they reflect similarities between viruses only through the very small number of nucleotides that code for the amino acids in the antigenic groups. It has been estimated, at a maximum, that only 4% of the nucleotides of TMV code for the amino acids in its antigenic groups (21). It should not be surprising, therefore, to find many other properties of CMV and CV-L which are the same. The viruses could even prove to be serologically related when high-titered antisera can be produced.

The electrophoretic mobility of TAV-B, CV-L and

PSV in acrylamide gels, as mentioned previously, resembled each other. The results of the serological study indicate strain relationships among each of these viruses. In contrast, CMV besides being electrophoretically distinct was also serologically distinct from TAV-B and CV-L.

The adoption of a type strain for this virus group is essential. Favored would be one which behaved serologically like the PSV used here and would show cross reactivity to all members of the group. Viruses showing serological relationship to the type strain would continue to be members of the group even though they may be serologically unrelated. This is justified because serology reflects upon the similarities or dissimilarities of viruses by comparing only a very small portion of their nucleic acids.

## SUMMARY

The host range and symptomatology, sedimentation rate, electrophoretic migration and serological reactions of four small icosahedral plant viruses were compared as a basis for strain differentiation. Although some hosts showed no or only partial differentiation of the viruses, the reaction of zinnia distinguished each virus as a unique genetic system.

Scanning patterns of centrifuged sucrose gradients showed that each virus sedimented at approximately the same rate and indicated similar size, shape, density. Virus isolated from gradients of TAV-B, CV-L and PSV was infectious while the CMV zone was not. All virus samples from gradients had high $A_{260/280}$ ratios.

In large-pore acrylamide gels, each virus appeared to move as a single component in the 0.019 ionic strength buffer. The distinctive electrophoretic migration of CMV was believed to reflect basic differences in the amino acid composition of its coat protein from the other viruses. That TAV-B, CV-L and PSV migrated at a similar rate suggested that the amino acid composition of their coat proteins may be similar.

Formaldehyde-treated PSV and CMV, in spite of some evidence for stabilization, evoked only a weak immunological response in animals. Microprecipitin tests with PSV were shown to be unsatisfactory due to the presense of normal tobacco antibodies and non-specific virus precipitation. In gel diffusion tests, each virus reacted with its homologous serum out to the 1/4 dilution only.

Analysis of serological cross reactions between each virus was achieved using density-gradient centrifugation. PSV was the only virus which showed reciprocal cross reactions with each of the other viruses. CMV and CV-L were considered serologically unrelated while a serotype relationship was proposed between CMV and TAV-B, Assuming the validity of the phylogenetic approach to plant virus classification, the common stock of these viruses was postulated to be most like PSV. Because of the apparent complexity of this virus group, the strict requirement for serological cross reactivity between all its members does not seem of value. Serological relationship of all members to a type strain was suggested as an alternative criterion.

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# THE RELATIONSHIP OF PEANUT STUNT VIRUS TO CUCUMBER MOSAIC VIRUS AND ASPERMY VIRUSES OF TOMATO AND CHRYSANTHEMUM.

by

John W. Groelke

## Abstract

Host range and symptomatology of peanut stunt virus (PSV), cucumber mosaic virus (CMV), the Blencowe isolate of tomato aspermy virus (TAV-B) and a chrysanthemum virus (CV-L) varies on selected hosts. In sucrose density-gradient centrifugation each virus has a sedimentation rate of approximately 100s. Purified preparations of PSV and CV-L are homogenous and stable while CMV and TAV-B aggregate and lose infectivity. In acrylamide gel electrophoresis, all the viruses move as a single component with CMV migrating faster than the other three. Formaldehyde-stabilization of PSV and CMV did not increase the titer of immune sera. Classical microprecipitin and gel diffusion tests were unsatisfactory because of non-specific precipitation and antibodies to normal host antigens. Analysis of incubated homologous and heterologous virus-antibody mixtures by density-gradient

centrifugation detected specific precipitation with surface antigenic sites on the virions. In reciprocal tests, CMV and CV-L show no serological relationship. CV-L and TAV-B react reciprocally and are strains. CMV antiserum reacts with TAV-B, but not conversely. PSV reacts reciprocally with the other three viruses, and thus is related at the strain level to all three. Of the viruses compared, PSV appears to be most like the hypothetical parent strain of the CMV group, since it possesses antigenic sites in common to at least three members of the group.