Improved Purification Procedure for Peanut Stunt Virus, Incitant of Tephrosia Yellow Vein Disease

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Appreciation is extended to F. F. Smith for aphid transmission, Sandra Boatman for analytical ultracentrifugation, R. H. Lawson for electron microscopy, and T. T. Hebert for peanut stunt virus antiserum.

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ABSTRACT

Peanut stunt virus (PSV-T) was isolated from *Tephrosia vogelii* Hook. f., a legume species being studied because the leaves produce the insecticide rotenone. Leaflets on 5 to 30% of the compound leaves on 20% of the plants exhibited a bright chlorosis along the veins, epinasty, and leaflet distortion, but plants were not stunted. The virus infected most herbaceous species inoculated. The green peach aphid *Myzus persicae* transmitted PSV-T in greenhouse experiments. Thermal inactivation point was between 55 and 58 C, dilution end point 5×10^{-3} to 1×10^{-4}, and longevity in vitro at 24 C was 1 to 2 days. The virus was purified from *Vigna, Lycopersicon, Nicotiana, Tephrosia, Capsicum, Datura metel,* and *D. stramonium* plants in experiments designed to compare yields of virus. Yields ranged from 8 to 9 mg virus/10 g of *D. stramonium* leaf tissue in eight trials when sap was clarified with Mg-treated bentonite as compared with 0.05 to 0.15 mg/10 g of tissue using the traditional procedure of clarifying cowpea tissue with chloroform-butanol. The virus was serologically identical to the Eastern strain of peanut stunt virus in agar gel tests. Purified virus reacted with antisera to cucumber mosaic, chrysanthemum aspermy, and the Western strain of peanut stunt virus. Antiserum has been deposited with the American Type Culture Collection No. 62.

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Additional key words: serology, aphid transmission.

Tephrosia vogelii Hook. f., a legume, was grown and evaluated in the USA as a potential source of the insecticide rotenone (1). During 1969, about one-third of the plants in field plots at Glenn Dale, Md., exhibited bright yellow veins in a few leaves, suggesting virus infection. Similar symptoms appeared in about 5% of the plants during 1971 field trials. This paper describes the disease and the causal virus. It also outlines a procedure for purifying peanut stunt virus isolated from *Tephrosia* (PSV-T), and of a simple procedure for routine serological indexing of peanut (*Arachis hypogaea* L.) plants.

MATERIALS AND METHODS.—Type peanut stunt virus (PSV) was obtained from Sue Tolin (ATCC PV 164). Antiserum to PSV Eastern strain was obtained from T. T. Hebert, and to PSV Western strain from the ATCC No. 39. Other antisera were obtained from M. Hollings or produced in our laboratory.

Virus isolation and host range.—Virus from *Tephrosia* was isolated by trituration of leaves in 1% phosphate buffer pH 8.0 and inoculation of corundum-dusted leaves of *Phaseolus vulgaris* L. 'Pinto', *Vigna sinensis* (Torner) Savi 'Early Ramshorn', and *Nicotiana tabacum* L. 'Samsun'. We inoculated 3 to 10 plants of each additional herbaceous genus with infectious tobacco sap diluted with the same buffer. Plants were observed for 4 to 8 weeks. Inoculated plants that remained symptomless were back-indexed on Samsun tobacco or *Chenopodium quinoa* Willd.

Physical properties.—Crude juice from primary leaves of PSV-T-infected *N. tabacum* 'KY-35' tobacco was used for studying the thermal inactivation point, dilution end point, and longevity in vitro. Juice was diluted 1:10 with 0.025 M phosphate buffer pH 7.2 and held at 24 C to determine longevity in vitro; further diluted to determine dilution end point; or 1-ml aliquots of 1:10 dilution were heated for 10 min in 4-ml glass tubes submerged into a constant temperature water bath to determine thermal inactivation point. Plants were inoculated immediately after each treatment. Results were based on local lesion counts on KY-35 tobacco.

Purification.—Initially the virus was purified from *V. sinensis* 'Early Ramshorn' and from KY-35 tobacco following the procedure of Mink et al. (14). Later, Mg-treated bentonite was used to clarify cowpea juice (18). Virus yields were higher with this treatment than with chloroform-butanol. This led to a comparison of yields of purified virus using infected tissue from several plant species.

Fresh tissue was blended 2 min in 4.5 volumes (0.22 g/ml) of 0.025 M phosphate buffer pH 7.2 containing 0.02 M 2-mercaptoethanol. The slurry was pressed through cheesecloth, and the liquid centrifuged 10 min at 12,000 *g*. To the supernatant fluids, which ranged in color from clear yellow-green to dense green, Mg-treated bentonite was added prepared according to Dunn & Hitchborn (3), except that the final concentration was adjusted to 75 mg bentonite/ml.

The quantity needed to give a clear yellow supernatant upon recentrifugation ranged from 6 to 20 ml/100 g of fresh tissue depending on the plant species. Cowpea juice was clarified with 6 to 8 ml, whereas *Datura stramonium* L. and tomato sap required 18 to 20 ml of bentonite to obtain clear

low-speed supernatants. If supernatants were not free of green material, small additional amounts of bentonite were added, and after another 5 min, the low-speed centrifugation was repeated. When more bentonite than necessary was used to remove green material, virus was also removed when the mixture was centrifuged. The clear yellow supernatant fluid was then centrifuged 1.25 hr at 105,000 *g*. Pellets were resuspended in 0.02 M sodium citrate buffer pH 7.5, and held overnight at 4 C.

After clarifying the resuspended pellets at 8,000 *g* for 5 min, 1.0-ml aliquots of the resulting supernatant fluid were layered onto 10 to 40% sucrose gradients (18) in 0.02 M citrate buffer. Gradients were centrifuged at 98,000 *g* for 2 hr and analyzed, using an ISCO fractionator and ultraviolet light analyzer (Instrumentation Specialties Co., Lincoln, Neb.). Fractions were bioassayed on KY-35 tobacco and *Tephrosia* at 1:10 dilution with phosphate buffer. Virus was reconcentrated for serological experiments and electron microscopy by centrifugation at 105,000 *g* for 1.5 hr and resuspension of the pellets in 0.02 M citrate buffer.

Homogeneity of virus purified from *D. stramonium* was ascertained by electron microscopy, using 2% uranyl acetate stain, and by analytical ultracentrifugation. We approximated virus yields by reading a dilution of the purified virus in the spectrophotometer, multiplying absorbency at 260 nm by the dilution factor, and specific extinction coefficient (optical density-weight relationship) of 0.2 which assumed 18 to 20% ribonucleic acid in virus (10).

Serology.—A 5-lb. rabbit was injected intramuscularly with 3 to 4 mg of purified virus emulsified with incomplete adjuvant once a week. Virus in the last two of the six injections was formylated to a final concentration of 0.2%. We collected 20 ml of blood each week, beginning 1 week after the third injection. We determined virus antibody titer by using a 0.15 mg/ml preparation of purified virus, and host protein antibody titer by using healthy *D. stramonium* sap. Serological tests were performed using the gel double-diffusion technique. We used 0.75% Ionagar No. 2 in distilled water containing 0.04% NaN_3 with

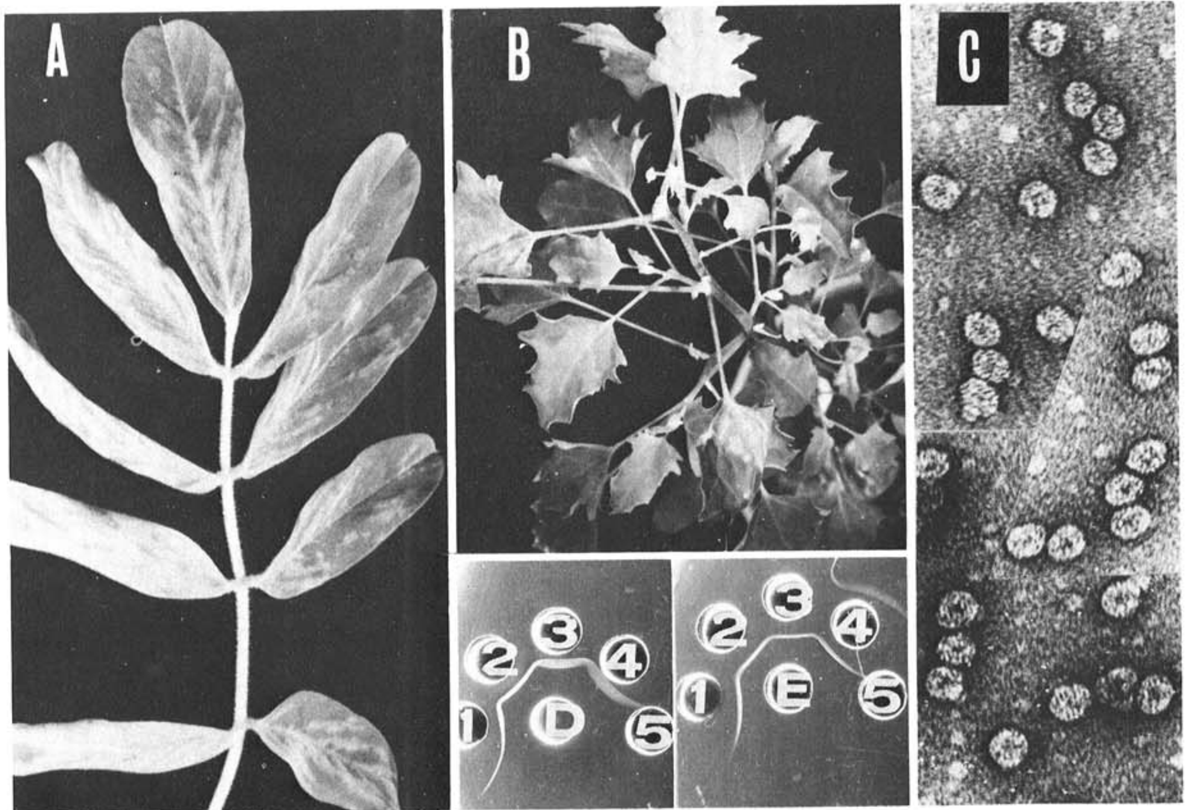

Fig. 1. A,B) Peanut stunt virus, *Tephrosia* isolate (PSV-T). **A)** Leaf from naturally infected *Tephrosia* plant. **B)** Systemic infection of *Chenopodium quinoa*. **C)** Particles of PSV-T purified from *Datura stramonium* and stained with 2% uranyl acetate. Some particles are flattened and distorted (× 182,500). **D)** Undiluted PSV-Eastern strain antiserum; and **E)** PSV-T antiserum diluted 1:16. Outer wells (1-4) contained concentrated (1, 2) and 1:8 dilution (3, 4) of virus purified from *D. stramonium*; 1, 3 = PSV-E; 2, 4 = PSV-T. Well 5 contained purified extract of healthy *Datura*. Absence of spur formation suggests that the two isolates are serologically identical.

wells 5 mm apart. Final readings were made after 2 days at 24 C.

During the identification phase, and to determine relationships to other viruses, purified virus was tested with antisera to 22 spherical viruses. They were: bean pod mottle, broad bean mottle, brome mosaic, cowpea chlorotic mottle, and cowpea mosaic; carnation ringspot, carnation Italian ringspot, and carnation mottle; tomato ringspot, blackringspot, and bushy stunt; strawberry latent; lettuce-, beet-, and raspberry ringspot; cherry leafroll; petunia asteroid mosaic; Pelargonium leaf curl; cucumber mosaic; squash mosaic; and chrysanthemum aspermy; peanut stunt virus (PSV) East (5) and West (14) strains.

Aphid transmission.–Green peach aphids, *Myzus persicae* (Sulzer), were used to transmit PSV-T from infected to healthy *Tephrosia* (19).

RESULTS.–*Symptoms and host range.*– Symptoms in field plants were noticed in August and were characterized by bright yellow chlorosis, usually along the veins of leaflets (Fig. 1-A). In some instances, leaflets were entirely chlorotic, distorted or showed epinasty. Affected plants were not stunted or malformed.

The experimental host range for our isolate of PSV was essentially the same as that reported for PSV-E (5, 11). The following plant species or varieties either have not been reported as experimental hosts of PSV-E or reacted differently to our isolate of PSV. Systemic symptoms (leaf mottling and distortion) developed in inoculated plants of *Apium graveolens* L., *Capsicum frutescens* L. 'Cayenne', *Chenopodium foetidum* Schrad., *C. quinoa* Willd., *Crotalaria juncea* L., *Datura metel* L., *D. stramonium* L., *Nicotiana clevelandii* Gray, *N. tabacum* L. 'KY-35' and 'Samsum', *Petunia hybrida* Vilm. 'Snowball', *Physalis peruviana* L., *Tephrosia vogelii* Hook. f., and *Vinca rosea* L. Chlorotic local lesions without systemic infection developed in: *Amaranthus caudatus* L., *Chenopodium amaranticolor* Coste & Reyn., *C. murale* L., and *Helianthus annuus* L. 'Mammoth Russian'. Local and systemic infection without symptoms occurred in: *Ageratum houstonianum* Mill. 'Blueball', *Beta vulgaris* L., *Celosia cristata* L., *Cynara scolymus* L., *Dianthus barbatus* L., *Lobelia erinus* L. 'Crystal Palace', *Nepeta cataria* L., *Phaseolus limensis* Macf., *Pisum sativum* L. 'Alaska', *Salpiglossis sinuata* Ruiz & Pav., and *Zinnia elegans* Jacq. 'Blaze'. No infection occurred in *Alyssum maritimum* Lam., *Antirrhinum majus* L. 'Mardi Gras', *Brassica rapa* L. 'Yellow Globe', *Callistephus hortensis* Nees, *Digitalis purpurea* L., *Momordica balsamina* L., *Solanum tuberosum* L. 'Saco', or *Vernonia anthelmintica* (L.) Willd. PSV-T incited a strap or wire leaf condition in *Torenia fournieri* Lind and wavy necrotic lines on inoculated leaves of Tabasco pepper plants (Fig. 2).

Our isolate differed from those in previous reports (5, 14) in that it incited systemic symptoms in *C. quinoa* (Fig. 1-B), and did not incite a clear-cut symptom in *Pisum sativum* L. 'Alaska' or *Vicia faba* L. To determine the possibility that the systemic symptoms in *C. quinoa* were incited by another virus, we inoculated *Tephrosia* and peanut with inoculum from systemically infected *C. quinoa*. These species responded with characteristic yellow veins and stunt symptoms, respectively, suggesting that we were not working with a mixture of viruses.

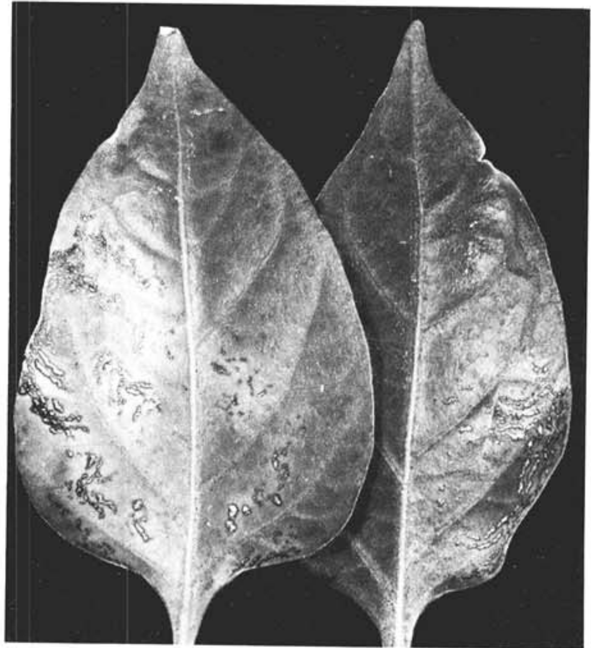

Fig. 2. Local necrosis in *Capsicum frutescens* 'Tabasco' 6 weeks after inoculation with peanut stunt virus.

Symptoms in *D. stramonium* and *D. metel* appeared after 3 weeks, at which time the virus had reached a high titer. Leaf symptoms were equally severe after 8 weeks, when plants were 22 to 26 inches tall.

Symptoms among five peanut cultivars varied considerably after 1 month when infected with PSV-T under winter greenhouse conditions. Cultivar Tennessee Red showed distinct necrotic local lesions. Response to systemic infection ranged from no symptoms (Virginia 56 R) to severe systemic mosaic (Starr) 1 month after inoculation. Cultivars NC-2, Virginia Bunch 67, and Tennessee Red showed a conspicuous dark-brown necrosis of the expanding tip leaves of each terminal. After 4 months, all cultivars were equally and severely stunted as compared with healthy control plants.

Tephrosia seedlings inoculated with purified PSV-T responded with systemic chlorosis like that observed in source plants (Fig 1-A). However, in contrast with infected field plants, greenhouse seedlings inoculated when 3 inches tall became severely stunted and chlorotic. Most plants were dead within 1 month after inoculation.

Physical properties.–The thermal inactivation point of PSV-T was between 55 and 58 C; dilution end point, between 5×10^{-3} and 1×10^{-4}; and longevity in vitro, between 1 and 2 days. PSV-T was transmitted by aphids to seven of ten *Tephrosia* plants in two experiments.

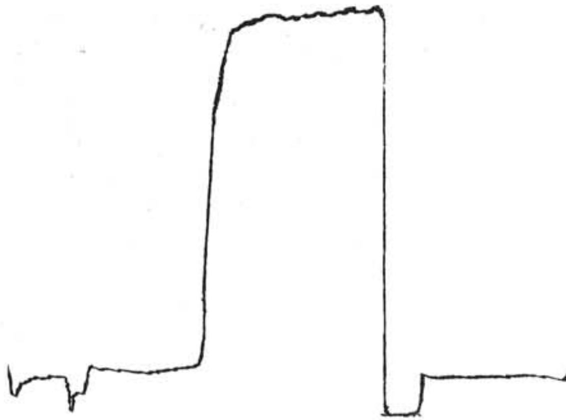

Fig. 3. Analytical ultracentrifuge ultraviolet optics pattern of purified PSV-T in 0.1 M buffer. Sedimentation left to right. Photoelectric scan made 20 min after attaining 23,150 rpm.

Purification.—The bentonite clarification procedure resulted in highly infectious purified virus upon density-gradient rate zonal centrifugation (Fig. 1-C, Fig. 3). Purified preparations produced an average max:min 260:240 nm absorbency ratio of 1.36 and 260:280 ratio of 1.81.

Yields of purified virus from *Vigna sinensis* and *D. stramonium* were considerably higher when bentonite rather than when chloroform-butanol was used. *D. stramonium* consistently yielded more virus than did *V. sinensis*, regardless of the purification procedure used. Yields of virus from species tested are given in Table 1. Yields as high as 9.2 mg virus/10 g of leaves were obtained using *D. stramonium* harvested at weekly intervals from 4 to 12 weeks after inoculation.

We compared the infectivity of PSV-T purified by each of the two procedures. From freshly purified preparations of 1 mg/ml, we diluted the virus in 0.025 M phosphate buffer to 100, 50, 10, 5, and 1 μg/ml. In duplicate experiments, both preparations incited 100 to 400 lesions/leaf in *C. quinoa* at 100, 50, and 10 μg/ml, 10 to 50 lesions/leaf at 5 μg/ml, and incited only one to five lesions/leaf at 1 μg/ml.

Yields of purified virus from each of seven species suggested large differences in virus titer among the source species (Table 1). We attempted to correlate purified virus yields with infectious virus titer by assaying extracted sap on cowpea half-leaves. However, we saw few or no lesions on leaves rubbed with some of the inocula, especially that from *Datura*. We assumed this was due to inhibitors which are known to occur in *Datura*.

Serology.—Antiserum collected from the first bleeding, performed 1 week after the third injection, had an antibody titer of 1:16, and it rose to 1:1,024 in the fourth bleeding. Undiluted antiserum did not produce a visible band in agar with healthy crude *D. stramonium* sap.

Antisera to PSV-T and to PSV-E reacted with each virus in adjacent wells forming a single continuous band without visible spur formations at any of the virus concentrations used (Fig. 1-D,E). In three experiments, the homologous and heterologous titers of PSV-T antiserum were the same.

Concentrated preparations of PSV-T also reacted with antisera to PSV-W, cucumber mosaic virus, and chrysanthemum aspermy virus when antisera were not diluted beyond 1:4 to 1:8. PSV-W antiserum diluted more than 1:4 did not react with PSV-T at any concentration up to 5 mg/ml. PSV-T did not react with any of the other antisera tested.

A series of experimental tests were conducted to develop a rapid and reliable method of serologically indexing diseased peanut plants for PSV. Triturated

TABLE 1. Comparison of species, clarification procedure, and time after inoculation on yield of purified peanut stunt virus, *Tephrosia* isolate

Plant species	Procedure	Weeks after inoculation	mg Virus/10 g
Datura stramonium L.	Chloroform-butanol	8	2.7-2.9[d]
D. stramonium L.	Bentonite	4-12	7.5-9.2[b,c,d]
D. stramonium L.	Bentonite	3	6.1-14.0
Vigna sinensis (Torner) Savi	Chloroform-butanol	1	0.05-0.23[d]
V. sinensis (Torner) Savi	Bentonite	8	1.5-1.6[d]
V. sinensis (Torner) Savi	Bentonite	1	0.2-0.3
Lycopersicon esculentum Mill. 'Rutgers'	Bentonite	7	0.35[a,b]
Capsicum frutescens L. 'California Wonder'	Bentonite	7	0.85[a,b]
Tephrosia vogelii Hook. f.	Bentonite	7	0.11[a,b]
Datura metel L.	Bentonite	8	3.4[a,c]
Nicotiana tabacum L. 'KY-35'	Bentonite	8	0.5[a,b]
N. tabacum L. 'KY-35'	Bentonite	2	2.5[a,c]

[a] Not replicated.
[b,c] Purified simultaneously: [b] 4 weeks after inoculation; [c] 4.5 weeks after inoculation.
[d] Range of yields of purified virus from nine trials.

leaf samples of some peanut cultivars were mucilaginous, and virus did not readily diffuse through the agar. Hence, we compared several variations in procedure. Leaflet and petiole samples of greenhouse-grown healthy and diseased plants of cultivars Va 56 R, Tennessee Red, and NC-2 were tested in agar plates with and without 0.5% sodium dodecylsulfate (SDS) or saline, and against dilutions of our PSV-T antiserum. The best result was obtained by triturating 0.3 g of immature leaves in 1 ml of water, using agar plates without SDS or saline, and using antiserum diluted 1:4 to 1:8. We observed a distinct sharp band with all diseased but not with healthy samples of the 3 cultivars within 8 to 24 hr.

DISCUSSION.—Although *Tephrosia* has been grown annually at the Glenn Dale station for the past 11 years, this disease was not observed until 1969. PSV is seed-transmitted (2, 11); whether this was the source of virus is unknown. Several reports deal with the sudden appearance in 1964 (12) and rapid spread of PSV in beans, peanuts, and tobacco in Virginia and North Carolina (4, 7, 12).

PSV-T appears to be identical to PSV-E based on host range, symptomology, and serological data. PSV-T incited systemic necrosis in three of five peanut cultivars inoculated with infectious cowpea sap in the greenhouse. Apparently, this symptom has not been reported before in the USA. Reddy et al. (15) in India and Frezzi (6) in Argentina described virus diseases of peanut which included necrosis of the growing point. We do not know whether the causal viruses are related to PSV.

The high yields of purified virus are attributable to both host virus titer and the bentonite clarification procedure. Several reports describe purification of PSV (14, 16, 17), and all involve cowpea as the source of virus and a chloroform-butanol clarification procedure. None gave the yield of purified virus; however, they are known to be low (T. T. Hebert, Sue Tolin, *personal communications*). At no time after inoculation did PSV-E or PSV-T approach the titer in cowpea that it eventually did in *D. stramonium*.

All purification experiments were designed to compare virus yields. Consequently, we usually blended very small samples of leaves; i.e., 8 to 10 g in large volumes of buffer, usually 45 ml. This ratio probably contributed to the high yields of virus which were indeed free of plant materials, based upon analysis in the analytical ultracentrifuge or the electron microscope.

The difference between our 260:280 ratio of 1.81 and that of Mink's 1.6 (14) may be the result of two different purification procedures, or possibly the difference between PSV-W and PSV-T.

PSV is a member of the Cucumovirus group (9) which includes cucumber mosaic (CMV) and tomato aspermy virus (TAV). Chrysanthemum aspermy (CAV) (8), PSV-E (8), and PSV-W (13) are each serologically related to several members of this group. PSV-T reacted with antisera to CMV, CAV, PSV-E, and PSV-W. Furthermore, after electrophoresis, PSV-T RNA is multicomponent (Sandra Boatman and J. M. Kaper, *personal communication*) and similar to that observed for CMV RNA (10).

Apparently no one has succeeded in obtaining a serological reaction between PSV-E and its homologous antiserum diluted beyond 1:32 (T. T. Hebert, Sue Tolin, *personal communications*). In gel double-diffusion tests with purified PSV-T, we obtained distinct single bands within 24 hr with Hebert's PSV-E antiserum diluted 1:128 and with PSV-T antiserum diluted 1:1,024, but only with appropriate concentrations of virus for each dilution of antiserum. Virus concentrations above or below the optimum for each antiserum dilution frequently failed to produce a visible band.

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