Purification and Properties of Pea Seed-Borne Mosaic Virus

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ABSTRACT

Pea seed-borne mosaic virus (PSbMV) was purified from roots and leaves of systemically infected *Pisum sativum* '447'. Of several procedures investigated, best results were obtained by grinding root tissue in 0.01 M sodium diethyldithiocarbamate (NaDIECA) + 0.01 M cysteine or leaf tissue in NaDIECA + cysteine containing 0.01 M ethylenediaminetetraacetic acid (EDTA) and clarifying with one-half volume of chloroform. Clarified suspensions were concd by one cycle of differential ultracentrifugation, and further purified by centrifugation on columns containing 30% sucrose, 4% polyethylene glycol MW 6000, and 0.12 M NaCl. Precipitated virus was suspended in 2% sucrose containing 0.1% Igepon T73 at pH 7.0. This procedure yielded a single, highly infectious, visible zone 24-26 mm below the meniscus of rate sucrose density-gradient tubes: healthy tissue handled similarly produced no visible zones.

The virus had a sedimentation coefficient of 148 ± 1 S and contained 5.3% ribonucleic acid with a base ratio of adenine 44.0%, guanine 22.8%, cytidylic acid 17.6%, and uridylic acid 15.6%. The protein coat contained aspartic acid and glutamic acids in greatest amounts. Elongated flexuous rods with a modal length of 770 nm were found in preparations fixed with 3.5% glutaraldehyde.

All properties of PSbMV were similar to those reported for other viruses in the potato virus Y group.

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Additional key words: aggregation, virus properties.

In 1968, a seed-borne virus of peas (*Pisum sativum* L.) was reported in Washington (16) for which the name "pea seed-borne mosaic virus" (PSbMV) was adopted (15) because of its relationship to a similar virus reported from Japan (10). The biological and morphological properties of PSbMV indicate that it is a member of the potato virus Y group. It is a flexuous, rod-shaped particle within the 730-790 nm range (15), has a moderately restricted host range (1), is aphid-transmitted in a stylet-borne manner (6), and produces pinwheel inclusions in leaf cells (8). However, pinwheels are rarely found in pea root cells (8).

Preliminary attempts to purify PSbMV from leaf tissue indicated that this virus, like many other viruses in the potato virus Y group, had a strong tendency to aggregate. However, we found PSbMV could be purified readily from pea root tissue. Prior to the report by Stevenson and Hagedorn (23) we found that PSbMV could also be purified from pea leaf tissue if ethylenediaminetetraacetic acid disodium salt (EDTA) was included in the initial extraction step (11). This report presents procedures based on this technique, and a novel use of polyethylene glycol (PEG) precipitation that we found useful in preparing highly purified PSbMV.

MATERIALS AND METHODS.—*Virus source and assay.*—The PSbMV isolate (W-1) (ATCC isolate PV 184) was obtained from infected pea seed and maintained in *Pisum sativum* '447'. Plants used for purification were grown in sand-filled, 10.2-cm (4-inch) diam clay pots in a 22-28 C greenhouse supplemented with fluorescent light at an intensity of 10^4 lx for a 16-h photoperiod. Plants were inoculated at the second-node stage, and leaf or root tissue was harvested 20-30 days later.

Local lesion assays were made on *Chenopodium amaranticolor* Coste & Reyn. grown in a 22-28 C greenhouse at 4,300 lx with a 16-h photoperiod. Assay plants were placed in the dark two days before inoculation and moved to a 25-C growth chamber under 10^4 lx intensity, 20-h photoperiod after inoculation. Treatments to be assayed were applied to one-half of each of four leaves and compared with a standard virus preparation applied to each opposite half-leaf.

Partial purification.—The ability of nine clarification procedures to remove host components and yet retain infectivity was compared using homogenates of pea leaf or root tissue. In general, 18 g of infected tissue were ground in 180 ml of 0.01 M sodium diethyldithiocarbamate (NaDIECA) + 0.01 M cysteine·HCl (14) for 2 min in a Waring Blendor, the juice expressed through cheesecloth and aliquots clarified by various treatments listed below. After treatment the preparations were centrifuged 30 min at 3,000 *g* and assayed.

Different methods of concentrating the virus were tested. Clarified juice was concd by two cycles of differential ultracentrifugation using a Spinco model L preparatory ultracentrifuge or by one cycle of differential ultracentrifugation and one cycle of precipitation with polyethylene glycol and sucrose. Polyethylene glycol-sucrose tubes were prepared with 10 ml of 30% sucrose containing 4% PEG (MW 6,000) and 0.12 M sodium chloride. Twenty ml samples were layered on PEG-sucrose tubes and immediately centrifuged for 2 h at 24,000 rpm in a SW 25.1 rotor. The supernatant containing most of the host materials was discarded and the infectious pellets resuspended overnight in 2.5 ml of 2% sucrose containing 0.1% Igepon T73 at pH 7.0.

Density-gradient tubes.—Rate sucrose density-gradient tubes were prepared according to Brakke's method (2) by layering 7, 7, 7, and 4 ml of solutions containing 40, 30, 20, and 10 g sucrose made up to 100 ml with distilled water at pH 7.0, respectively, into SW 25.1 centrifuge tubes. Equilibrium density-gradient tubes were prepared by layering 4, 4, 4, and 4 ml of solutions containing 60, 50, 40, and 30 g sucrose made up to 100 ml with distilled water pH 7.0, respectively. All density-gradient tubes were stored for 18 h at 4 C before use.

Virus properties.—Phosphorus analyses were made according to the method of Nakamura (18) using solutions containing approximately 2 mg/ml PSbMV. Base ratios were determined by the paper chromatographic technique of Smith and Markham (20). Amino acid composition of PSbMV was determined in a Beckman 121 amino acid analyser using the acid hydrolysis procedure described by Moore and Stein (17). The sedimentation coefficient in pH 7.0 phosphate-sucrose gradients was determined by the method described by Brakke (3) using tobacco mosaic virus (TMV) as the reference. A value of 185 S was used for TMV (12).

Serology.—Antiserum against PSbMV was obtained by intravenous injections of a rabbit with a virus preparation from equilibrium density-gradient tubes. The 2-ml intravenous injections (0.1 mg/ml of virus) were administered five times at 2-day intervals.

Microslides were prepared by a modification of the technique reported by McCrum et al. (13). Templates made of 3-mm-thick Plexiglas having wells (2 mm in diam) positioned 6 mm from the center well and 5 mm from adjacent outer wells were secured directly to 76 × 25-mm glass microscope slides by taping the ends with plastic electrician's tape. A 0.2% agar solution, made by adding equal volumes of 0.4% sodium azide in distilled water and 0.4% Ionagar No. 2 in distilled water [autoclaved 15 min at 1.05 kg-force/cm^2 (15 pounds pressure)], was applied slowly between the template and microscope slide, so that the wells remained free of agar. The microslide was placed in a freezer (−28 C) for 3 min to quickly solidify the agar solution. The microslide was then placed in a petri dish and surrounded with mineral oil until the oil level was just below the upper edge of the template. The wells were filled with distilled H_2O, pH 7.0, which was removed before adding the test solutions and serum. After reactants were added the microslide was completely covered with mineral oil and allowed to set at room temp for 48 h.

Electron microscopy.—Samples were removed from the visible, infectious zone in the equilibrium density-gradient tubes and the sucrose removed by centrifugation in a No. 30 rotor at 29,000 rpm for 2.5 h. The pellet was resuspended in 2 ml of distilled water, pH 7.0, and mixed with an equal volume of 3.5% glutaraldehyde pH 7.0 and stored for 30 min at room temp. The fixed virus was applied to Formvar-coated grids, negatively stained with 2% phosphotungstic acid (PTA) pH 7.0 and examined in a Philips model 300 electron microscope.

RESULTS.—*General virus properties.*—Although juice from pea leaf or root tissue was highly infectious when initially prepared, no infectivity was found in juice from pea leaf tissue stored for 24 h at room temp. Juice from infected pea root tissue remained infectious for 96 h, the longest time tested (Table 1). Juice from leaf tissue was infectious at 24 h when diluted 10^{-1} or 10^{-2} and remained infectious up to 96 h. These results suggested the presence of an inhibitor or inactivating substance present in pea leaf tissue.

TABLE 1. Effect of time on infectivity of pea seed-borne mosaic virus in crude juice from pea leaf or root tissue stored at room temp

	Time			
Treatment[a]	24 h	48 h	74 h	96 h
Leaf juice	0/122[b]	0/136	0/114	0/91
10^{-1} dil. of leaf juice[c]	22/162	47/201	6/160	1/197
10^{-2} dil. of leaf juice[c]	25/62	22/78	6/76	30/88
Root juice	37/84	30/59	36/90	27/48

[a]Pea seed-borne mosaic virus-infected pea leaf or root tissue was triturated, squeezed through cheesecloth, and the juice stored in Parafilm-covered tubes.

[b]Total local lesions on three half-leaves of *Chenopodium amaranticolor*. Numerators are lesion numbers produced by the treatment, denominators are lesion numbers produced on the opposite half of each leaf by freshly prepared control.

[c]Leaf juice was diluted with distilled water adjusted to pH 7.

TABLE 2. The effect of clarification treatments on the infectivity of PSbMV from pea leaf and root tissue

	Color[a]		Local lesions/half-leaf[b]	
Treatment[a]	leaf	root	leaf	root
8.5% *n*-Butanol (24)[c]	Y	LY	1/253	3/688
Charcoal (5)	LG	Gr	350/428	745/876
Chloroform (19)	LY	LY	361/366	123/125
Chloroform + Butanol (22)	LY	LY	4/272	5/473
Chloroform + Igepon T73[d]	LY	LY	228/392	561/618
Ether-CCl_4 (25)	LG	LY	107/211	718/772
Freezing[e]	LG	LY	6/267	57/678
Heat 40 C[f]	G	LY	93/168	229/503
pH 5.0[g]	LY	LY	10/203	779/802

[a]Numbers in parentheses following treatments are literature citations in which the designated clarification techniques are described.

[b]Color of the supernatant after low-speed centrifugation. Y = yellow, LY = light yellow, LG = light green, G = green, Gr = gray. Numbers in parentheses are literature citations for the given treatment techniques.

[c]Numerators are local lesions produced by the treatment and the denominators are local lesions produced on the opposite half of the *Chenopodium amaranticolor* leaf by the nontreated control.

[d]Dialized for 24 h against 0.01 M NaDIECA + 0.01 M cysteine·HCl before assay.

[e]Igepon T73 (0.1%, w/v) was added and dissolved. Half-volume of chloroform added and stirred for 30 min.

[f]Homogenate was stored at −28 C for 2.5 h.

[g]Crude plant sap was heated to 40 C for 1.0 h.

[h]The pH was adjusted to 5.0 with 10% acetic acid (with stirring) during a period of 30 min.

The dilution end point of PSbMV was between 10^{-3} and 10^{-4} using either root or leaf tissue. Traces of infectivity remained in samples heated 10 min at 55 C, but no infectivity was obtained with samples heated 10 min at 60 C.

Effect of clarification treatments.—Although chloroform-butanol or 8.5% butanol effectively clarified pea leaf and root extracts, these treatments eliminated nearly all infectivity (Table 2). In experiments not reported we found this was probably due to direct inactivation of virus since noninfectious, serologically active, flexuous rods could be recovered from density-gradient tubes layered with butanol-clarified extracts.

Chloroform, chloroform + Igepon T73, and charcoal treatments clarified both leaf and root extracts and had little effect on infectivity levels. Ether-carbon tetrachloride or pH 5.0 clarification had little effect on infectivity of root extracts, but greatly reduced infectivity of leaf extracts (Table 2).

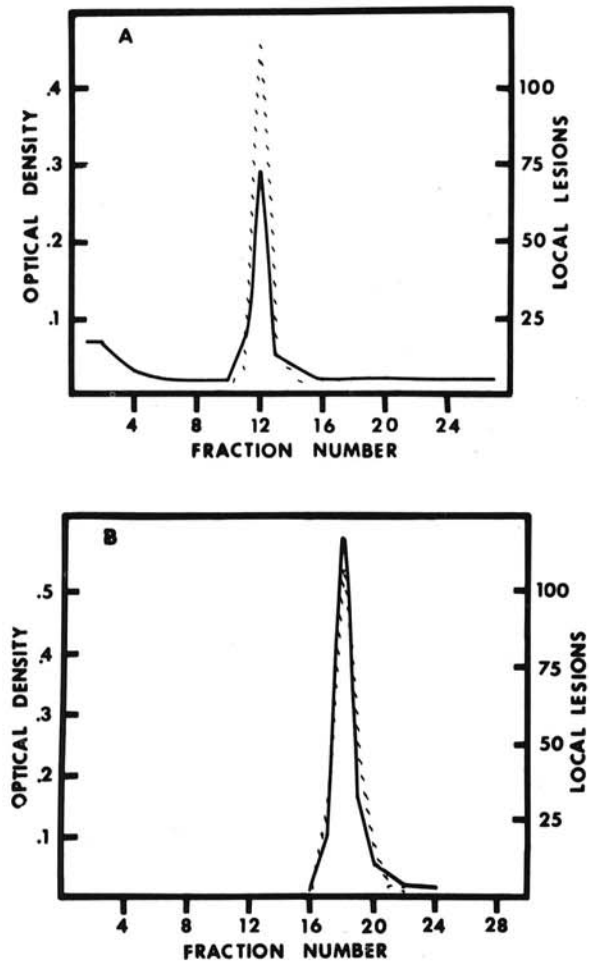

Fig. 1-(A-B). Association of ultraviolet absorbance at 260 nm (solid line) and pea seed-borne mosaic virus infectivity (broken line) from density-gradient tubes. Local lesions are an average of four half-leaves of *Chenopodium amaranticolor*. **A)** Rate density-gradient tube. **B)** Equilibrium density-gradient tube.

Purification.—The effectiveness of clarification and concn procedures was estimated by (i) the appearance of an infectious, visible zone in density-gradient tubes; (ii) the OD 260 and 260/280 ratios of fractions removed from the visible zone region; and (iii) infectivity levels.

No discrete visible zones were observed in tubes layered with any of the clarified leaf extract treatments when the tissue was ground in the presence of NaDIECA + cysteine·HCl, or 0.01 M neutral phosphate buffer. However, low levels of infectivity were recovered from some of these tubes in the region 24 to 26 mm below the meniscus. Of these, chloroform clarification resulted in the highest levels of infectivity, but tubes contained heavy opalescence throughout.

A distinct visible zone 24 to 26 mm below the meniscus was obtained with all clarification procedures of root tissue extracts. However, if treatments were given two cycles of differential centrifugation the resulting density-gradient tubes contained considerable opalescence throughout. If clarified preparations were given one cycle of differential centrifugation and one cycle of PEG-sucrose precipitation, most of the nonspecific opalescence was eliminated. Similar results were obtained from leaf tissue homogenized in 0.01 M NaDIECA + 0.01 M cysteine·HCl + 0.01 M EDTA adjusted to pH 7.0 with 4 M NaOH.

Maximum yields of purified virus containing the least detectable amounts of host components were obtained by homogenizing 18 g of freshly harvested tissue in 180 ml of freshly prepared NaDIECA + cysteine·HCl (for root tissue) or NaDIECA-cysteine·HCl + EDTA (for leaf tissue) for 2 min in a Waring Blendor. Juice expressed through cheesecloth was incubated at 30 C for 1 h and subsequently emulsified 30 min at 30 C with one-half volume of chloroform using an overhead stirrer. The emulsion was broken by centrifuging at 3,000 *g* for 30 min. The upper phase was removed and centrifuged in a No. 30 rotor at 29,000 rpm for 1.5 h. Pellets were suspended overnight in 4 ml of cold distilled water, adjusted to pH 7.0 with 0.005 M NaOH. The resuspension was centrifuged 15 min in a No. 40 rotor at 10,000 rpm, and the supernatant layered onto PEG-sucrose tubes and centrifuged 2 h at 24,000 rpm in a SW 25.1 rotor. The supernatant was discarded and each pellet resuspended overnight in 2.5 ml of 2% sucrose containing 0.1% Igepon T73 at pH 7.0. After centrifugation in a No. 40 rotor at 15,000 rpm, the supernatants from three runs (nine tubes) were combined, and the PEG-sucrose cycle was repeated. The final pellets were resuspended overnight in 2 ml of 2% sucrose plus 0.1% Igepon T73, centrifuged 10 min in a No. 40 rotor at 15,000 rpm, layered on rate sucrose density-gradient tubes, and centrifuged 2 h at 24,000 rpm. This procedure yielded a single, highly infectious, visible zone 24 to 26 mm below the meniscus, whereas healthy tissue handled similarly produced no visible zones.

Association of infectivity with visible density-gradient zone.—Rate density-gradient tubes were fractionated, using an ISCO density-gradient fractionator. Each 1 ml fraction was diluted with distilled water adjusted to pH 7.0 and concd by ultracentrifugation in a No. 40 rotor at 39,000 rpm for 1 h. The pellets were resuspended in 1 ml of distilled water pH 7.0 and assayed for infectivity. Infectivity was associated with the visible zone (Fig. 1-A).

Fractions 11 through 14 from two rate tubes were combined, layered on equilibrium density-gradient tubes and centrifuged 16 h at 24,000 rpm. A single visible zone was located 39 to 41 mm below the tube meniscus. One-ml fractions were removed and concd into 1 ml of distilled water (pH 7.0) by ultracentrifugation. The infectivity was associated with the visible zone (Fig. 1-B).

Properties of the virus.—Infectious material removed from the 24-26 mm region of rate density-gradient tubes and concd in 0.01 M neutral phosphate buffer by differential ultracentrifugation, had ultraviolet absorption spectra typical of nucleoproteins with a low nucleic acid content. Solutions with low light scattering above 320 nm had 260:280 ratios between 1.14 and 1.18, suggesting a nucleic acid content of approximately 5.5%. Phosphorus analysis of five whole virus preparations (2 mg/ml) indicated a phosphorus content of $0.48 \pm 0.01\%$. Assuming phosphorus constitutes 9% of the wt of the nucleic acid, the nucleic acid content of the virus was $5.3 \pm 1\%$.

Paper chromatography and spectrophotometric analysis of acid hydrolysates of whole virus indicated the presence of only the four nitrogen bases commonly found in ribonucleic acid. The average value of two nitrogen-base ratio determinations for PSbMV was: adenine, 44.0%; guanine, 22.8%; cytidylic acid, 17.6%; and uridylic acid, 15.6%.

A sedimentation coefficient of 148 ± 1 S was calculated for PSbMV using Brakke's method (3).

Methods used for amino acid analysis resulted in the destruction of cysteine and tryptophan. The composition and relative molar ratios of the remaining amino acids are shown in Table 3. The acidic amino acids aspartic and glutamic acid occurred in about twice the concn of the basic amino acids.

TABLE 3. Amino acid composition of pea seed-borne mosaic virus protein

Amino acid	Relative molar[a] ratio	
	24-h hydrolysis[b]	48-h hydrolysis[b]
Alanine	3.8	3.3
Arginine	2.3	2.5
Aspartic acid	5.2	4.7
Cysteine	---	---
Glutamic acid	5.2	4.9
Glycine	2.8	2.7
Histidine	1.0	1.0
Isoleucine	1.6	1.8
Leucine	2.3	2.2
Lysine	1.5	1.5
Methionine	2.1	1.9
Phenylalanine	1.1	1.1
Proline	1.7	1.4
Serine	2.6	2.2
Threonine	2.1	2.0
Tryptophan	---	---
Tyrosine	1.3	1.2
Valine	2.5	2.4

[a]Based on histidine concn = 1.0.
[b]Average of two analyses.

Elongated flexuous rods were consistently found in preparations from infectious visible density-gradient zones. When fixed with 3.5% glutaraldehyde, and negatively stained with PTA, these particles had a modal length of 770 nm (Fig. 2-A). In contrast, a modal length of

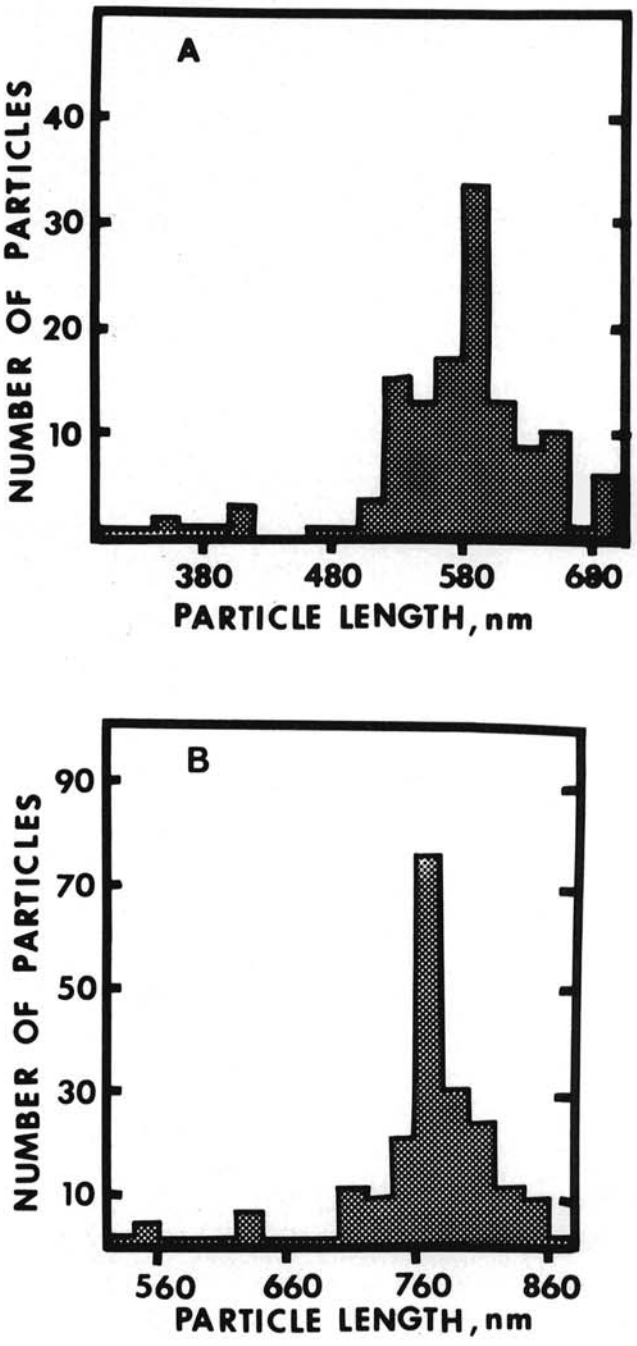

Fig. 2-(A, B). Particle-length distribution of pea seed-borne mosaic virus. **A)** Pea leaf dip preparation fixed with 2% Formalin and negatively stained with phosphotungstic acid (PTA). **B)** Purified virus preparation fixed with 3.5% glutaraldehyde and stained with PTA.

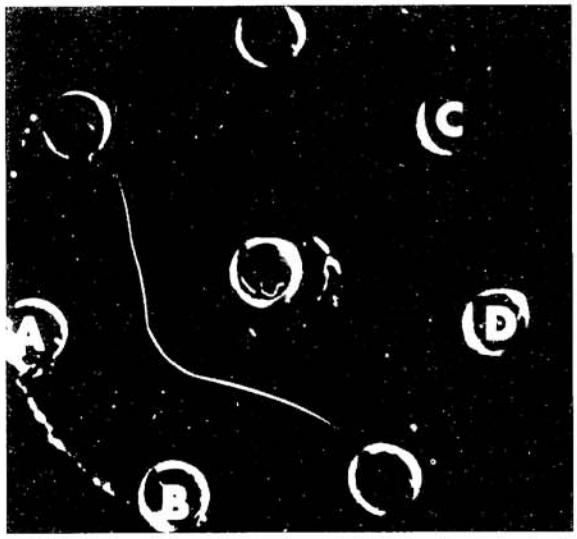

Fig. 3-(A-D). Precipitin lines observed in gel diffusion microslide. Middle well contained pea seed-borne mosaic virus antiserum. **A)** Pea seed-borne mosaic virus purified from root tissue. **B)** Pea seed-borne mosaic virus purified from leaf tissue. **C)** Healthy pea leaf tissue. **D)** Healthy pea root tissue.

590 nm was obtained with standard leaf dip techniques using 2% Formalin as the fixative (Fig. 2-B). Preliminary results suggest that preparatory treatments such as clarification, concn and fixation have a marked effect on the length of PSbMV particles observed in the electron microscope.

Serology.—Antiserum produced against purified PSbMV (0.1 mg/ml) had a homologous titer of 1/1,024 in microprecipitin tests. Virus purified from both root or leaf tissue reacted with PSbMV-antiserum in gel diffusion microslide tests (Fig. 3). No reactions were observed with preparations from healthy leaf or root tissue in either test.

DISCUSSION.—Polyethylene glycol-sucrose centrifugation appeared to be a critical step in the purification of PSbMV from either root or leaf tissue. Sedimenting the virus into 30% sucrose containing 4% PEG and 0.12 M NaCl where it precipitated and was pelleted while most of the contaminating host material remained in solution was more effective than the standard PEG precipitation technique (7). In addition to removing contaminants, this procedure effectively concd the virus. Preparations concd in this manner were highly infectious compared to those concd by differential ultracentrifugation, and were less likely to produce the broken particles often observed in the electron microscope (Knesek and Hampton, *unpublished*). We have found this procedure to be useful for purifying other viruses in the PVY group.

Four viruses in the potato virus Y group have now been purified and partially characterized by biochemical and biophysical methods. In addition to PSbMV, there are potato virus Y (21), tobacco etch virus (4), and turnip mosaic virus (9). Each virus sediments as a single infectious component, with a sedimentation coefficient near 150 S. Each contains approximately 5% ribonucleic acid which appears to be rich in adenine relative to the other nitrogen bases. In this regard, PSbMV appears to contain an unusually high proportion of adenine. In addition, these viruses contain proteins in which the two most prominent amino acids are aspartic and glutamic acids.

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