Purification and Serology of Peanut Mottle Virus

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Contribution 446 of the Department of Plant Pathology and Physiology.
The authors acknowledge the support of this work by the Virginia Agricultural Experiment Station and the Virginia Soybean Commission.
Accepted for publication 18 January 1983.

ABSTRACT

Tolin, S. A., and Ford, R. H. 1983. Purification and serology of peanut mottle virus. Phytopathology 73:899-903.

Peanut mottle virus (PMV) was purified from pea (*Pisum sativum* 'Little Marvel') by extraction in 0.1 M tris-HCl, pH 8.0, containing 0.05 M EDTA and 0.02 M Na_2SO_3, followed by clarification with 25% chloroform and precipitation with polyethylene glycol. Virus suspended in 0.01 M tris, pH 8.0, containing 0.5 M urea and 0.001 M EDTA sedimented primarily as a single component. Yields of 10–25 mg/kg of tissue were obtained. Both yield and infectivity were enhanced by Na_2SO_3 and EDTA. Specific antisera, with microprecipitin titers of 1/256, reacted with PMV-infected pea, soybean, and peanut from the greenhouse or field in agar immunodiffusion tests containing sodium dodecyl sulfate (SDS). Purified PMV at 0.2 mg/ml was completely degraded at SDS concentrations greater than 0.05% in 0.005 M tris-HCl, pH 8.0. PMV was not related serologically to bean yellow mosaic, clover yellow vein, or soybean mosaic viruses as shown by SDS gels or ELISA.

Peanut mottle virus (PMV), a potyvirus, was first described by Kuhn (11) and is known to occur worldwide in peanuts (*Arachis hypogaea* L.) (2). Additionally, it causes a severe disease of soybean (*Glycine max* (L.) Merr.) in the southeastern United States (5,6,12) and has recently been detected in clover and lupine in Georgia (4). PMV is widespread in the five peanut-producing counties in the southeastern part of Virginia (2,6) and causes a loss in yield (18). Several isolates of PMV have been obtained from both peanuts and soybeans. Purification of the virus was necessary before our isolates could be compared with previously reported strains (14) and an antiserum could be prepared for diagnostic purposes. Methods reported by Sun and Hebert (20) for a severe strain, and by Paguio and Kuhn (13) for a mild mottle strain, were initially employed, but both resulted in a low recovery of highly aggregated virus. This paper reports the development of a purification procedure that has been used successfully with three isolates of PMV, as well as other potyviruses, and includes the results of serological tests with PMV.

MATERIALS AND METHODS

Virus source. An isolate, designated PMV-H, obtained from a peanut plant growing in a commercial field near Holland, VA, and showing a mild mottle of young leaflets, was used for most of this study. It was maintained in a greenhouse on cultivar Virginia 56R peanut or pea (*Pisum sativum* L. 'Little Marvel'), or in leaf tissue desiccated over $CaCl_2$. Mechanical transfers from peanut to peanut were made by rubbing torn leaf pieces onto carborundum-dusted leaflets. Inoculum for pea and other hosts was prepared in 0.01 M phosphate buffer, pH 7.0 or 8.0.

The PMV-H isolate was first transferred four times between a local lesion host (*Phaseolus vulgaris* L. 'Topcrop'), using single lesions, and a systemic host, Little Marvel pea. Plants used as the source of virus for purification studies were grown in a greenhouse primarily during the winter and spring months. Inoculum consisting of infected pea sap in 0.01 M sodium phosphate (pH 8.0) was rubbed onto plants at the 2–4 leaf stage.

Virus assays. Infectivity, birefringence, electron microscopy, and density gradient centrifugation were used to assess the quantity and quality of virus in a preparation. Infectivity assays were conducted on Topcrop bean by inoculating half-leaves in an incomplete block design with 4–8 replications per treatment. Degree of aggregation of virus in sap extracted in various buffers was assessed by observing negatively stained particles in a transmission electron microscope, as well as sedimentation patterns in density gradients. Extracts were layered on linear 10–40% sucrose density gradients prepared in various buffers. The gradients were centrifuged 2 hr at 25,000 rpm in a Beckman SW27 rotor, or for 30 min at 60,000 rpm in a SW65 rotor, and were analyzed with an ISCO density gradient fractionator and UV analyzer. Samples for which birefringence was not evident had little or no virus when assayed by any of the above methods.

Virus purification. Systemically infected pea plants (except for roots) were harvested 11–14 days after inoculation and homogenized in a Waring blender with 2 ml of cold, freshly prepared buffer per gram of tissue. The extract was pressed through cheesecloth, stirred vigorously for 10 min with chloroform (1 ml/3 ml of extract), then centrifuged at low speed (10,000 *g* for 10 min) to break the emulsion. Desired amounts of polyethylene glycol 6000 (Matheson, Coleman and Bell, Norwood, OH 45212) (PEG) and NaCl were added while the clarified extract was being stirred. After an additional 30 min of stirring in the cold, precipitated virus was concentrated by centrifuging at 10,000 *g* for 10 min and was resuspended overnight in a volume of buffer equivalent to one-fifth of the initial volume of clarified extract. If a second PEG precipitation was used, resuspension was in 1/40 to 1/50 of the original volume.

A low-speed centrifugation (10,000 *g* for 10 min) followed each of the resuspension steps. Density-gradient centrifugation was carried out in linear sucrose gradients in either the SW27 or SW65 Beckman rotors, and centrifuged as described above. Zones from the preparative SW27 gradients were collected and diluted with an equal volume of buffer. Virus was pelleted by centrifuging for 1.5 hr at 160,000 *g*, and was resuspended in a volume of buffer to give a concentration of virus no greater than 3 mg/ml.

Specific buffers and additives are given in the Results. Virus yield was calculated spectrophotometrically using an extinction coefficient of 2.4 $(mg/ml)^{-1} \cdot cm^{-1}$ (15).

Serology. Antisera to PMV-H were prepared using virus purified through density gradients and diluted in phosphate-buffered saline (PBS). Two rabbits each received two intramuscular injections of approximately 1.5 mg of PMV emulsified with an equal volume of Freund's incomplete adjuvant accompanied by intravenous (IV) injections of the same amount of virus in PBS, 1 wk apart, followed 1 wk later by an IV injection of 2 mg of virus. Rabbits were bled 6

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wk after the initial injection, and sera, with 0.005–0.01% sodium azide added as a preservative, were stored frozen at −20 C.

Antibody titers were determined by microprecipitin tests with virus and antisera diluted in PBS. A modified gel-diffusion method for flexuous rod-shaped viruses was also used. For these tests, 14 ml of freshly prepared medium consisting of 0.6% Ionagar No. 2, 0.7% NaCl, 0.3% sodium dodecyl sulfate (SDS), and 0.1% NaN_3 (22) was placed in a 100-mm-diameter plastic petri dish. Antigens, either crude sap extracted with a mortar and pestle in a minimum amount of distilled water, or purified virus, were placed in any of six 4-mm-diameter peripheral wells symmetrically spaced 4 mm from a center well of the same size, into which the antiserum was placed. Plates were kept in a moist chamber at room temperature and observed after 24 and 48 hr.

Soybean mosaic virus and its antiserum were from this laboratory (8). Bean yellow mosaic virus and clover yellow vein virus and their antisera were obtained from O. W. Barnett (Clemson University, Clemson, SC 29631). Antiserum to PMV-S was provided by T. T. Hebert (North Carolina State University, Raleigh 27650) and to PMV-M2 by C. W. Kuhn (University of Georgia, Athens 30602).

RESULTS

Symptomatology. PMV-H and all other isolates of PMV induced a mild mottle on young leaves of Virginia 56R peanut typical of mild strains of PMV (14). A severe mosaic and line pattern in trifoliolate leaves and slight stunting were characteristic of plants of soybean cultivars Lee, Kanrich, Hood, and Essex infected with PMV-H, whereas some other isolates, including one designated PMV-B, caused mild symptoms. Soybean cultivars Dorman and York were not systemically infected by the PMV isolates. All PMV isolates infected both Alaska and Little Marvel pea and induced similar symptoms of bright yellow vein-banding and mosaic accompanied by leaf distortion and stunting. On Topcrop bean, PMV-H and other isolates induced typical, 0.5- to 1.5-mm-diameter reddish brown necrotic local lesions, which sometimes extended into veinal tissue causing vein necrosis and, in some cases, petiole and stem necrosis. Bean cultivars Kentucky Wonder, Kentucky Wonder Wax, and Monroe responded in a similar fashion. Systemic infection of these bean cultivars was not observed. Cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'Blackeye') was systemically infected and exhibited a very mild mottle.

Host and extraction buffer. Infectivity assays on Topcrop bean (Table 1) indicated that phosphate buffer, as utilized previously for PMV (13,20), was superior to distilled water or sodium citrate, which often is used for other potyviruses (3,21). Sodium sulfite, EDTA, or mercaptoethanol added to either water or citrate (9,19) did not increase infectivity, but when added to phosphate buffer infectivity was enhanced two- to threefold. This was particularly evident when Na_2SO_3 was included in treatments held at room temperature for 24 hr prior to inoculation. Although virus titer was essentially no different in Little Marvel pea than in Alaska pea at 18 days after inoculation, Little Marvel was used for all subsequent studies because of its preferred dwarf growth habit and insusceptibility to clover yellow vein virus. Only very low amounts of virus could be purified from Lee or Kanrich soybeans.

The suitability of including 0.01 M EDTA and 0.02 M Na_2SO_3 in the 0.1 M phosphate buffer (pH 8.0) used by Sun and Hebert (20) was also demonstrated by partial purification. Following clarification with 25% chloroform and one PEG precipitation (4% PEG, 0.5 M NaCl), pellets were resuspended in 0.02 M potassium phosphate (pH 8.0) containing 0.5 M urea and 0.02 M Na_2SO_3. Birefringence was evident in this preparation, but was not evident when the phosphate buffer for extraction contained sodium sulfite alone, sodium sulfite in combination with 0.001 M EDTA, or sodium sulfite with 0.01 M or 0.001 M $MgCl_2$. Samples receiving no clarification or the 8% *n*-butanol clarification and followed by PEG precipitation, or those receiving only high-speed centrifugation (2 hr at 100,000 *g*), were also not birefringent. Electron microscopy of the above samples confirmed that birefringent samples contained numerous individual virions, whereas in nonbirefringent samples, either no virions or clumps of highly aggregated particles were observed.

Efficacy of extraction buffers was also evaluated by analysis of the partially purified treatments by density-gradient centrifugation in either large (for the SW27 rotor) or small (for the SW65 rotor) tubes. Relative virus yield and degree of aggregation confirmed the results described above with additives to phosphate buffers. With citrate buffers, sedimentation profiles of PMV-H showed absorbing material over a wide range of the gradient. Citrate buffer

TABLE 1. Infectivity on Topcrop bean of peanut mottle virus-infected pea extracted in various solutions

Inoculum[a]	pH	Lesions on leaves inoculated	
		Immediately	After 24 hr
Alaska pea in			
distilled water	5.5	47[b]	...
+ 0.02 M Na_2SO_3	9.3	88	...
+ 0.01 M EDTA	4.6	2	...
+ both	7.1	6	...
0.5 M sodium citrate	8.6	0	...
+ 0.02 M Na_2SO_3	9.0	20	...
+ 0.01 M EDTA	7.0	0	...
+ both	7.6	1	...
0.05 M sodium citrate + 1% mercaptoethanol	7.2	1	...
+ 0.01 M EDTA	6.1	38	...
0.1 M NaH_2PO_4-Na_2HPO_4	8.0	44	6
+ 0.02 M Na_2SO_3	8.0	88	8
+ 0.01 M EDTA	8.0	152	132
+ both	8.0	136	80
Little Marvel pea in			
0.1 M NaH_2PO_4-Na_2HPO_4	8.0	60	38
+ 0.02 M Na_2SO_3	8.0	160	6
+ 0.01 M EDTA	8.0	172	120
+ both	8.0	108	180

[a] One gram of tissue ground in 5 ml of the designated solution, clarified with chloroform, centrifuged at 10,000 *g* for 10 min.
[b] Total lesions on four half-leaves.

consistently gave better results with PMV-B than with PMV-H.

In additional trials, extraction in 0.1 M tris-HCl, pH 8.0, containing 0.02 M Na_2SO_3 and 0.05 M EDTA resulted in yields of unaggregated virus higher than those obtained with phosphate buffer. Infectivity of virus extracted in tris was approximately equal to that of virus in phosphate buffer. Infected Little Marvel pea tissue ground in 0.05 M tris or phosphate buffer, pH 8.0, containing 0.05 M EDTA, produced a total of 481 and 428 local lesions, respectively, on eight half-leaves of Topcrop bean. Tris buffer was used for the final purification experiments with PMV-H, including preparation of virus for antiserum production.

Clarification and concentration. Density-gradient analysis of small volumes of samples also enabled a quick and effective means of comparing clarification and concentration methods. The procedure for treatment with 25% chloroform influenced the results. Shaking for 2–3 min gave a smaller and less clearly defined virus peak in comparison to stirring for 10 min. Emulsification in the blender was also not as effective in freeing virus from components sedimenting to the same depth as virus, and gave no improvement in yield. Results of typical tests of the effect of the pH of the phosphate extraction buffer in combination with clarification and concentration treatments are shown in Fig. 1. Chloroform treatment was 25% as before, and was compared with 8% (v/v) of a 1:1 mixture of chloroform and *n*-butanol, which was allowed to stand overnight in the cold. PEG precipitation was performed as above for the phosphate buffer. High-speed centrifugation for 1 hr at 100,000 *g* was followed by resuspension of pellets in 0.01 M phosphate at the appropriate pH. Volumes were adjusted so that the aliquots layered on gradients represented equivalent amounts of tissue extracts. Linear 10–40% gradients for the SW65 rotor were prepared in 0.01 M phosphate of the appropriate pH and also contained 0.02 M sodium sulfite and 0.001 M EDTA for improved resolution of virus zones. Results with phosphate buffer at pH 6.0 are not shown, since no virus was recovered with any of the treatments.

Overall, pH 8.0 was slightly better than pH 7.0. The virus suspension was not adequately clarified by chloroform treatment followed by high-speed centrifugation. When it was concentrated by PEG, however, the results were strikingly different. Sharp peaks of virus were evident and the pH 8.0 treatment resulted in less aggregation than did pH 7.0. Virus yield was reduced when clarification was by chloroform-butanol treatments as compared to the chloroform treatment alone. Centrifugation was generally better than PEG precipitation at pH 7.0 (Fig. 1A and C vs B and D), but at pH 8.0 there was little difference (Fig. 1E and G vs F and H) in virus yield. Virus was apparently irreversibly aggregated by PEG precipitation at pH 7.0. The method chosen for further study was chloroform clarification followed by PEG precipitation at pH 8.0, which allowed processing of large volumes of tissue extracts.

Purification. Critical steps in the purification process were revealed when large batches were purified. Grinding more than 200 gm in one batch often resulted in inadequate clarification and lower yields of virus per gram of tissue. Variations in PEG and salt concentration were tested, but those used by Sun and Hebert (20) appeared to be optimum. No more than a fivefold concentration could be made at the first PEG precipitation step. Additional concentration up to 10-fold with the second PEG precipitation usually resulted in a birefringent virus preparation suitable for preparative separation of a 2-ml aliquot on SW27 density gradients. However, a third PEG precipitation was sometimes used successfully to increase virus concentration. Slow resuspension of virus in the PEG pellets by gentle stirring in the cold for a few hours or overnight appeared to give higher yields than did rapid resuspension methods.

When tris buffer was used for extraction, PEG pellets were resuspended in 0.01 M tris, 0.5 M urea, and 0.001 M EDTA, pH 8.0. Decreasing the tris concentration to 0.005 M and pH to 7.2 caused a complete loss of soluble virus. Reducing urea concentration to 0.15 M or 0.35 M resulted in reduced virus purity. The EDTA concentration at this step was less critical, however, and its inclusion at 0.01 M or 0.001 M had little effect on yield. Substitution of 0.05 M $MgCl_2$ for EDTA at this step appeared to both disrupt and aggregate the virus.

In a typical preparative density gradient (Fig. 2A), the virus zone was well separated from slowly sedimenting materials, and showed little aggregation. Collection and concentration of virus from such zones results in purified virus having the sedimentation profile in Fig. 2B, showing an increased dimer peak. Final yields were 10–25 mg/kg of tissue.

Properties of purified PMV-H. Purified virus preparations gave an ultraviolet absorption spectrum typical of the potyviruses. The absorption maximum was 260 nm and the minimum was 246 nm, as previously reported for PMV strains (13,20). A definite inflection point at 290 nm indicated a high tryptophan content of the virus protein. A ratio of absorbance at 260 nm to that at 280 nm of 1.29, and a maximum-to-minimum ratio of 1.14, were calculated from values uncorrected for light scattering.

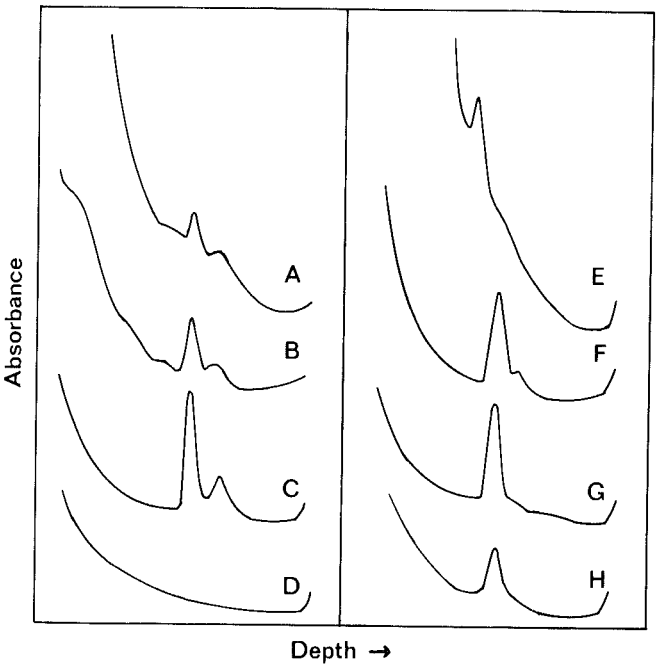

Fig. 1. Sedimentation profiles of peanut mottle virus purified in phosphate buffer comparing pH 7.0 (curves A–D) and pH 8.0 (curves E–H); 25% chloroform (curves A, B, E, and F) and 8% chloroform-butanol (curves C, D, G, and H); and concentration by high-speed centrifugation (curves A, C, E, and G) and polyethylene glycol precipitation (curves B, D, F, and H). Centrifugation was in linear 10–40% sucrose density gradients prepared in 0.01 M phosphate buffer at pH 7.0 (curves A–D) or pH 8.0 (curves E–H) containing 0.02 M Na_2SO_3 and 0.001 M EDTA, in a Beckman SW65 rotor at 60,000 rpm for 30 min.

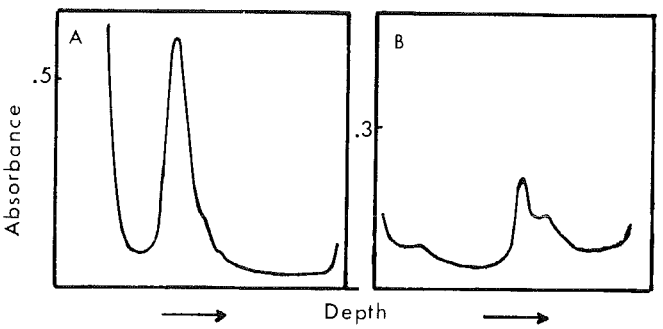

Fig. 2. Sedimentation profiles of peanut mottle virus purified by the tris buffer method. **A,** Virus purified by two precipitations with polyethylene glycol. **B,** Virus collected as in A and concentrated by high-speed centrifugation. Centrifugation was in linear 10–40% sucrose density gradients prepared in 0.005 M tris-HCl, pH 8.0, in a Beckman SW27 rotor at 25,000 rpm for 2 hr.

The sensitivity of PMV-H purified in tris buffer to SDS was tested by the methods of Boatman and Kaper (1), since SDS gel diffusion tests were effective with this virus. For the tests, 0.1 ml of virus at 0.2 mg/ml was combined with 0.1 ml of SDS (Matheson, Coleman and Bell, Norwood, OH 45212), and immediately layered onto SW65 density gradients prepared in tris buffer.

Sedimentation profiles (Fig. 3) indicated PMV-H was partially degraded by 0.005% and 0.01% SDS. At 0.05% SDS and at 0.5% SDS (*unpublished*), PMV-H was completely degraded to a slowly sedimenting component. Observation in the electron microscope of samples from this peak revealed no fragments of virus particles.

Serological tests. Antisera produced in both rabbits against PMV-H reacted in microprecipitin tests with 1 mg/ml of purified virus to a dilution of 1/256. In parallel tests, antiserum to PMV-S had a titer of 1/128 and to PMV-M2 a titer of 1/64. These virus strains were not available for reciprocal tests. In SDS-gel diffusion tests, undiluted antisera reacted with crude sap from infected pea, soybean, and peanut, but not with sap from healthy plants. Strong precipitin bands formed with sap from field samples of soybean and peanut. No spurs have been observed to form between PMV-H and PMV-B or any field isolates of PMV. Purified virus gave strong precipitin bands, with an optimum virus concentration of 0.2–0.5 mg/ml using 0.02 ml per well. Previous treatment of antigens with 0.1–1.0% SDS (16) did not improve either the intensity of the bands or the sensitivity of the test. Antisera could not be used, however, at a dilution greater than 1/2.

Best results were obtained with Ionagar No. 2, although several other agars, including Bacto-Agar, Difco agar, and agarose, were tested. Results nearly comparable to those with Ionagar No. 2 were obtained with purified agar (Code L-28; Oxoid Ltd., Baltimore, MD 21045, and London, England). Nonspecific precipitin bands could be reduced or eliminated by adjusting the NaCl concentration and by using only freshly prepared agar in the plates.

Anti-PMV-H antiserum did not react with soybean mosaic virus in either SDS-gel diffusion or microprecipitin tests. Additionally, no reaction was observed in gels or in enzyme-linked immunosorbent assays (ELISA) between anti-PMV-H and bean yellow mosaic virus and clover yellow vein virus. In reciprocal tests, PMV did not react with antisera to these other three legume viruses.

DISCUSSION

On the basis of host range, symptomatology, properties of purified virus, and serological reactions, certain viruses isolated from peanuts and soybeans in eastern Virginia have been identified as PMV. The purification procedure developed in this work confirms much of the work by Sun and Hebert (20) and Paguio and Kuhn (13) on properties and stability of PMV. Our work has confirmed that Little Marvel pea is a good host for the virus for purification and that sodium sulfite is important to maintain infectivity and limit aggregation. We found added benefit, however, from including EDTA and urea in the extraction buffer and for resuspension of virus following PEG precipitation. Either chloroform alone or a chloroform:butanol mixture can be used effectively for clarification of crude extracts, but resulting yield and extent of aggregation depend on pH and on the concentration method used. Best results were obtained by using tris buffer instead of phosphate. This method worked successfully for PMV-H, PMV-B, and a third strain of PMV isolated from Essex soybean, on which it caused severe symptoms. Soybean mosaic, potato Y, and maize dwarf mosaic viruses could also be purified by this procedure.

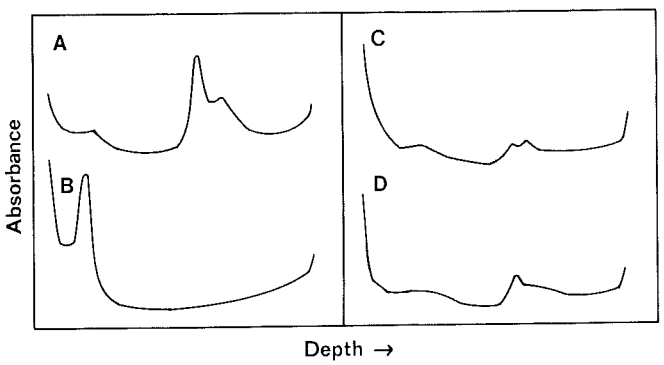

Fig. 3. Sedimentation profiles of peanut mottle virus treated with various concentrations of sodium dodecyl sulfate (SDS). Curve A, control; curve B, 0.05% SDS; curve C, 0.01% SDS; and curve D, 0.005% SDS. Centrifugation was in linear 10–40% sucrose density gradients prepared in 0.005 M tris-HCl, pH 8.0, in a Beckman SW65 rotor at 60,000 rpm for 30 min.

The use of SDS-containing agar gels for immunodiffusion tests with various modifications of the Gooding and Bing (7) method has had wide application (10,16,22). It has provided a rapid diagnostic test for PMV, since positive results are achieved with field samples of soybeans and peanuts. The mechanism by which SDS degrades or dissociates flexuous rod-shaped viruses is not known, but it undoubtedly occurs by a procedure that disrupts virion structure while retaining its antigenic reactivity with antibodies produced to intact viruses. With icosahedral viruses, Boatman and Kaper (1) have postulated that hydrophobic bonds between adjacent protein subunits as well as protein-nucleic acid bonds are affected by SDS. Since PMV-H is dissociated by SDS concentrations somewhat higher than those that affect the most sensitive icosahedral viruses, a different mechanism may be functioning for potyviruses. PMV seems to be somewhat unusual among potyviruses, however, because of its sensitivity to citrate and its requirement for sodium sulfite to maintain infectivity and reduce aggregation. All PMV strains may not be as sensitive to citrate, however, since extraction in citrate gave higher yields with PMV-B than with PMV-H.

Finally, Sun and Hebert (20) suggested that since plants of soybean cultivars Ogden, York, and Hood were not infected by PMV and were also resistant to SMV, there may be a relationship between the two viruses. However, PMV-H did not infect York, but it did infect Hood, the parent of York from which SMV-resistance was derived. In further studies with York, Roane et al (17) found that resistance to PMV and SMV is conditioned by closely linked, but separate, genes. This finding, together with our confirmation of lack of serological relationship, would substantiate the lack of relationship of PMV to SMV.

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