Partial Purification of the Pea Seed-Borne Mosaic Virus

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ABSTRACT

The pea seed-borne mosaic virus (PSbMV) was partially purified using a system of high molarity buffer, chloroform, and low-speed centrifugation for initial clarification of infected pea extracts prior to polyethylene glycol (PEG-MW 6,000) precipitation and two cycles of differential ultracentrifugation for the concentration of particles. Final extracts were infectious to pea when diluted to an OD_{260} of 1×10^{-5}. Electron micrographs of shadowed preparations showed high concentrations of virus particles in the partially purified product. Although repeated attempts to eliminate additional host contaminants were unsuccessful, the final preparations appeared to be suitable for use in antiserum preparation.

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Additional key words: clarification, virus concentration, aggregation.

The pea seed-borne mosaic virus (PSbMV) (10, 21) [apparently synonymous with the pea leaf-rolling virus (14), pea leaf-rolling mosaic virus (1, 15), and the pea fizzletop virus (8)] is a flexuous rod-shaped virus that is transmitted both mechanically, by several species of aphids (7, 11), and by seeds (10, 13, 14, 22). Electron micrographs of leaf dip preparations (3) by a number of researchers (1, 10, 15, 21) showed this virus to be 650-750 nm in length. These and other properties such as a dilution end point between 10^{-3} and 10^{-4}, thermal inactivation point between 50 and 60 C when heated for 10 min, and survival in vitro at 20-24 C for 1-4 days were in agreement between researchers (10, 13, 15). The physical properties of the Wisconsin isolate of PSbMV were in agreement with the above.

These studies demonstrated that the PSbMV had physical properties which corresponded to the properties of a number of mechanically transmitted, nonpersistent, aphid-transmitted, flexuous rod-shaped viruses. These viruses generally occurred in their hosts in such low concentration that the purification resulted in only low yields of virus. Recently, techniques have been developed to overcome the problems of low yield, instability, and particle aggregation, which have previously hindered purification of these viruses (5, 6, 10, 16, 18, 19, 23). Initial attempts to use these techniques for the purification of PSbMV resulted in partially purified preparations with complete loss of infectivity or infectivity no higher than untreated sap from infected plants.

This study describes methods used to develop a purification scheme for PSbMV that resulted in highly infective virus preparations which contained sufficient quantities of virus to be used in antiserum preparation.

MATERIALS AND METHODS.—The isolate of PSbMV used in this study was obtained from a single infected pea, *Pisum sativum* L., seedling, grown from an infected seed. This isolate was maintained by periodic mechanical transfer to seedlings of pea cultivar 'Dark Skin Perfection' (DSP). Virus for purification was increased in the DSP cultivar because this cultivar is highly susceptible to PSbMV and resistant to bean virus 2 (BV2). Seeds of DSP were planted in steamed compost soil and watered sparingly with tap water. Seedlings were grown in a greenhouse air temp of 22-26 C under a 16-hr day length supplemented with fluorescent light providing an intensity of 3,228 lx (300 ft-c) to the top leaves of 2.54-cm (1-inch) high seedlings. They were inoculated mechanically by rubbing at least two pairs of leaflets dusted with 600-mesh Carborundum. Inoculum consisted of approximately 1 g of infected tissue ground in 3 ml of 0.03 M potassium monobasic - sodium dibasic phosphate buffer, pH 8.0. Inoculated seedlings were maintained under the above conditions until harvested for purification studies.

The DSP cultivar was used throughout this study as an assay host to follow the progress of the purification scheme. Extracts after treatment were serially diluted in buffer and used to inoculate two leaflet pairs per seedling of at least 10 DSP seedlings per dilution. Seedlings were inoculated 12 days after planting and were maintained in the above greenhouse conditions both before and after inoculation. Each experiment was generally repeated three times.

Centrifugation for the initial purification studies was conducted on a Sorvall Superspeed Angle Centrifuge Type SS-IA (10 min - 3,100 rpm). When amounts of tissue used exceeded 70 g per treatment, centrifugation was conducted on an International centrifuge Model UV in 250-ml bottles (3,000 rpm, 15 min). Centrifugation after PEG precipitation was done on the Sorvall RC-2 refrigerated centrifuge SS-34 rotor (15 min at 8,000 rpm). Centrifugation of resuspended pellets after PEG precipitation and centrifugation was similarly executed (10 min at 8,000 rpm). High speed centrifugation was in No. 30 (120 min at 30,000 rpm) and No. 40 (90 min at 36,000 rpm) rotors of a Spinco Model L ultracentrifuge. Initial high speed pellets were resuspended overnight on a shaker in 5 ml 0.05 M sodium borate buffer and pellets from the second

high speed centrifugation were resuspended overnight in 2.5 ml 0.05 M sodium borate buffer. Centrifugation after pellet resuspension was in a Spinco ultracentrifuge (15 min at 8,000 rpm) using No. 30 and No. 40 rotors.

RESULTS.–*Period of optimum incubation.*–To determine the time after inoculation when virus infectivity was highest and the possible influence of temperature on virus concentration, infected plants incubated at 28, 24, 20, and 16 C were sampled 4, 8, 12, and 16 days after inoculation. The slurry resulting from triturating 3 g of tissue in glass-distilled water (30 ml) was strained through cheesecloth and diluted further in glass-distilled water before it was used to inoculate DSP seedlings.

Infectivity was high after 8 days at each of the four temperatures (Table 1). There was no significant increase in infectivity at 24 C or above, after 8 days incubation. There was also no significant difference in infectivity of extracts from peas harvested 8 days after inoculation grown at 24 and 28 C. Maximum infectivity at 20 and 16 C, however, was reached in 12 days. Seedlings grown at 24 C, were more vigorous and grew faster than seedlings grown at 28 C. Therefore, because time was an important consideration, seedlings grown at 24 C were harvested 8 days after inoculation in all subsequent experiments. Symptoms at this time corresponded to the end of the vein-clearing stage.

Low speed centrifugation.–Couch & Gold (4) reported that lettuce mosaic virus was lost when centrifuged briefly at low speeds. Preliminary experiments with PSbMV suggested similar behavior. Tissue was triturated and filtered as before. The virus preparation was then centrifuged at three speeds corresponding to 3,100 rpm, 7,200 rpm, and 10,600 rpm. Supernatant solutions were further diluted in glass-distilled water and assayed as before. Some virus appeared to be lost regardless of speed of centrifugation (Table 2). The greatest amount of virus, however, was lost at the highest speed of centrifugation. Initial low-speed centrifugation for subsequent experiments was generally conducted at 3,100 rpm or below.

Homogenizing buffer and method of extraction.–Further preliminary studies compared the infectivity of extracts after grinding tissue in four diluents. Either 0.05 M sodium borate buffer, pH 8.2, or 0.05 M potassium monobasic - sodium dibasic phosphate buffer, pH 7.0, were equally satisfactory and superior to Tris[tris(hydroxymethyl) amino methane]-HCl (Tris-HCl) buffer, pH 8.0, or glass-distilled water for extracting virus. In addition, the Waring Blendor was the most efficient method for grinding infected tissue. Grinding infected tissue with other methods such as the mortar and pestle, Omnimixer, or meat grinder resulted in unacceptable loss of virus during subsequent clarification.

TABLE 1. Optimum incubation period and temperature for pea seed-borne mosaic virus multiplication in pea

Days after inoculation	28 C	24 C	20 C	16 C
	Percent plants infected[a,b,c]			
4	74,63 j	80,75 j	25,14 k	9,6 k
8	i 93,82 j	i 91,81 j	88,64 j	88,62 j
12	i 93,93 j	i 96,93 j	i 100,92 j	i 96,69 j
16	i 90,83 j	i 97,80 j	i 100,88 j	i 97,88 j

[a] Averages for three trials. First number, 1/10 diluted inoculum; second number, 1/50 diluted inoculum. Minimum of 10 plants used in each assay.

[b] Within a column of treatment means at each temperature, those preceded by the same letter do not differ significantly from each other (5% probability level).

[c] Within each rank of treatment means (days after inoculation), those followed by the same letter do not differ significantly from each other (5% probability level) using Duncan's new multiple range test.

TABLE 2. Influence of initial low speed centrifugation (10 min) on pea seed-borne mosaic virus infectivity in supernatant

Speed of centrifugation	Dilution 1/10	1/50	1/100	1/1,000
	Percent plants infected[a,b]			
Uncentrifuged	100 i	83 i	65 ij	25 i
3,100 rpm (1,230 *g*)	98 ij	86 i	75 i	19 i
7,200 rpm (6,620 *g*)	86 j	50 j	41 j	16 i
10,600 rpm (14,350 *g*)	69	28 j	9	2

[a] Averages for three trials. Minimum of 10 plants used in each assay.

[b] Within a column of dilution means, those followed by the same letter do not differ significantly from each other (5% probability level) using Duncan's new multiple range test.

TABLE 3. Methods used for preliminary clarification of pea seed-borne mosaic virus-infected pea extracts

Treatment	Dilution 1/10	1/50	1/100	1/1,000
	Percent plants infected[a,b]			
Effect of freezing				
Frozen 5 hours, –20 C	91 i	77 i	62 ij	10 i
Frozen 30 min, dry ice	89 i	75 i	40 i	9 i
Not frozen	100 i	94 i	78 j	35
Effect of Na_2HPO_4				
0.05 M sodium borate buffer	100 i	94 i	79	36
0.3 M Na_2HPO_4	97 i	100 i	100 i	58 i
0.1 M Na_2HPO_4	100 i	100 i	100 i	48 i
0.05 M sodium borate buffer plus 0.1 M Na_2HPO_4	100 i	100 i	97 i	54 i

[a] Averages for three trials. Minimum of 10 plants used in each assay.

[b] Within a column of dilution means, those followed by the same letter do not differ significantly from each other (5% probability level) using Duncan's new multiple range test.

Clarification and virus concentration.—De Sequeira & Lister (6), working with filamentous apple viruses, reported that the best yields were obtained when the extracts were well clarified. Therefore, a series of experiments was undertaken to find an adequate clarification technique that would yield highly infectious virus preparations. Freezing tissue was evaluated as a method of clarification. Infected tissue (20 g) was frozen for 30 min in dry ice, for 5 hr at −20 C, or left unfrozen. Tissue was then ground in a Waring Blendor at the rate of 1 g tissue/9 ml 0.05 M sodium borate buffer at pH 8.2, filtered through cheesecloth, and centrifuged 10 min at 3,100 rpm. The supernatant solution was further diluted in 0.05 M sodium borate buffer and assayed as before. Although freezing and low speed centrifugation aided in clarification, there was a significant difference in infectivity between frozen and nonfrozen tissue treatments particularly at the 1:1,000 dilution (Table 3). We did observe an over-all trend in loss of infectivity from the frozen tissue treatments.

De Sequeira & Lister (6) found that controlled additions of bentonite enhanced clarification without undue loss of filamentous apple viruses. Therefore, bentonite prepared according to de Sequeira & Lister was tested extensively for clarification of PSbMV-infected pea extracts. There was an unacceptable loss of virus at rates of bentonite addition necessary to clarify pea extracts adequately. A single addition of bentonite generally resulted in less virus loss than two successive additions of bentonite interspersed with a low speed centrifugation.

Organic solvents are widely used to purify plant viruses. Inouye (10) reported that of the organic solvents tested in the purification of PSbMV (carbon tetrachloride-ether combination, butanol, and chloroform) all except chloroform destroyed the infectivity of PSbMV-infected extracts. We further tested the use of chloroform, butanol (8.5%), and a butanol-chloroform (1:1, v/v) mixture for their clarification efficiency and effect on infectivity of PSbMV. Infected tissue (20 g) was ground in 0.05 M sodium borate buffer, pH 8.2, centrifuged for 10 min at 3,100 rpm, treated with solvent in a 1:1 (ml solvent/ml supernatant fraction) ratio, incubated as described by Shepard & Grogan (18), and centrifuged at 8,000 rpm for 10 min. The supernatant solution was collected and given a single cycle of high speed centrifugation (2 hr at 30,000 rpm). Pellets were resuspended and assay dilutions made in 0.01 M potassium monobasic - sodium dibasic phosphate buffer, pH 7.2. Controls consisted of infected tissue ground in buffer and centrifuged at low speed only. Chloroform was the only organic solvent tested that provided adequate clarification without loss of virus. The 1:1 butanol-chloroform mixture and 8.5% butanol treatments eliminated all infectivity from the resuspended high speed pellet when tested at the 1/50 dilution or higher.

Na_2HPO_4 has also been used for preliminary extraction and clarification (20). PSbMV-infected tissue (20 g) was ground in Na_2HPO_4 0.1 M or 0.3 M at the rate of 1 g tissue/9 ml buffer. An additional treatment consisted of grinding tissue in 0.05 M sodium borate buffer plus 0.1 M Na_2HPO_4. The control consisted of grinding the tissue in 0.05 M sodium borate buffer at pH 8.2. Extracts were centrifuged 10 min at 3,100 rpm. Supernatant solutions were diluted in 0.05 M sodium borate buffer, pH 8.2, and assayed for infectivity. The use of Na_2HPO_4 resulted in well-clarified extracts which statistically contained more virus than extracts treated with sodium borate buffer alone at the 1:100 and 1:1,000 dilutions (Table 3).

Polyethylene glycol (PEG) has been reported useful in the purification of many viruses including members of the PVY group (5, 9, 12). Using a modification of the technique used by de Sequeira & Lister, infected tissue (20 g) was homogenized in 0.05 M sodium borate buffer, pH 8.2, and centrifuged as before. NaCl and PEG (MW 6,000 - Union Carbide Corp.) were added with stirring, to the supernatant liquid to final concentrations of 0.02 M or 0.2 M and 4%, respectively. Approximately 15 min after addition of PEG, the solution turned from clear to cloudy. After 1 hr incubation at 4 C, the mixture was centrifuged at 8,000 rpm for 10 min and pellets resuspended in 2 ml of 0.05 M sodium borate buffer, pH 8.2, for 2 hr at 4 C. Centrifugation was repeated and the supernatant fractions saved. Resuspension of pellets and centrifugation was repeated. Supernatant fractions from the two low speed centrifugations were combined and diluted in 0.05 M sodium borate buffer, pH 8.2, for infectivity assays. Polyethylene glycol (4%) and NaCl were effective in concentrating virus into a small volume and in maintaining infectivity at a level only slightly below the untreated control (Table 4). The use of 0.02 M NaCl resulted in significantly greater amounts of virus in the final extracts than 0.2 M NaCl in all but the 1:1,000 dilution.

A subsequent experiment combined the bentonite clarification and PEG-NaCl virus precipitation methods. After extraction in 0.05 M sodium borate buffer, pH 8.2, a small quantity of bentonite (28 mg/100 ml extract) was added. The solution was incubated for 1 hr at 4 C and centrifuged at low speed. Sodium chloride (0.02 M) and PEG at three concentrations (4, 6, 8%) were added to the supernatant fractions. Solutions were incubated and centrifuged at low speed. Both supernatant fractions and pellets from a single low speed centrifugation resuspended in sodium borate buffer were assayed for infectivity. The greatest amount of virus was concentrated in the pellet resulting from centrifugation of each of the three treatments (Table 4). Supernatant solutions contained a similar amount of virus regardless of the concentration of PEG added. A greater efficiency of virus extraction was not obtained with PEG concentrations higher than 4%.

We next wanted to determine whether increasing the amount of tissue and coupling the best methods of clarification would increase the infectivity of the final extracts. Seventy grams of infected tissue was

ground in 0.05 M sodium borate buffer plus 0.1 M Na_2HPO_4 and 0.01 M EDTA at pH 7.5 (1 g tissue/3 ml buffer), and centrifuged at low speed. Supernatant fractions were then treated with either the PEG-NaCl virus precipitation method alone, chloroform alone, chloroform clarification prior to the PEG-NaCl method, or the PEG-NaCl method prior to the chloroform clarification. After the various treatments and centrifugation, collected supernatant fractions were given two cycles of differential ultracentrifugation with pellets resuspended in 0.05 M sodium borate buffer at pH 7.5. Of the combined methods tested, the chloroform clarification prior to the PEG-NaCl virus precipitation resulted in the highest infectivity (Table 5). The amount of virus in final extracts using this series of treatments appeared to be much higher than any of the other methods used.

Several purification schemes for the purification of rod-shaped viruses have utilized a higher molarity buffer (0.5 M) in the extraction-clarification process (5, 18, 19, 23). In addition, Scott (17) has employed the simple procedure of grinding CMV-infected tissue, buffer, and organic solvent together in a blender in a 1:1:1 (v/v) ratio. Using this method of homogenization and 120 g of tissue, we compared three purification methods using either 0.5 M sodium borate buffer at pH 7.5, 0.5 M potassium monobasic - sodium dibasic phosphate buffer at pH 7.5, or 0.05 M sodium borate buffer plus 0.1 M Na_2HPO_4 and 0.01 M EDTA at pH 7.5 as the extraction medium. After extraction clarification with chloroform, and low speed centrifugation, supernatant fractions were collected through a pad of glass wool and given one cycle of high speed centrifugation (2 hr at 30,000 rpm). Pellets were resuspended in 0.05 M sodium borate buffer at pH 7.5. The maintenance of high infectivity in the resuspended pellet solutions of all

TABLE 4. Use of polyethylene glycol for pea seed-borne mosaic virus concentration from virus-infected pea extracts

Treatment	Dilution 1/10	1/50	1/100	1/1,000
	Percent plants infected[a,c]			
Use of Polyethylene Glycol (PEG) MW 6,000				
No PEG				
Low speed centrifugation only	100 i	87 i	71 i	39 i
4% PEG[b] plus 0.02 M NaCl	94 i	67 i	55 i	21 i
4% PEG[b] plus 0.2 M NaCl	59	26	18	15 i
Single Addition of Bentonite Followed by the Addition of Three Concentrations of PEG[b]				
Bentonite, NaCl, 4% PEG[b] Pellet	90 i	52 i	30 i	12 i
Bentonite, NaCl, 6% PEG[b] Pellet	94 i	47 i	21 i	6 i
Bentonite, NaCl, 8% PEG[b] Pellet	63 i	46 i	29 i	4 i

[a] Averages for three trials. Minimum of 10 plants used in each assay.

[b] PEG MW 6,000 was used.

[c] Within a column of dilution means, those followed by the same letter do not differ significantly from each other (5% probability level) using Duncan's new multiple range test.

TABLE 5. Selection of techniques which resulted in well clarified final extracts containing high concentrations of pea seed-borne mosaic virus

	Dilution 1/10	1/50	1/100	1/1,000
	Percent plants infected[a]			
Preliminary Extraction Procedures				
PEG (4%) & 0.02 M NaCl alone	93	50	20	8
Chloroform alone	100	92	78	10
$CHCl_3$ PEG (4%) (MW 6,000), & 0.02 M NaCl precip.	100	100	100	100
PEG (4%) & 0.02 M NaCl precip. followed by chloroform addn.	100	64	25	8
Influence of High Molarity Buffer on Infectivity				
0.05 M sodium borate buffer, 0.01 M Na_2HPO_4, and 0.01 M EDTA, pH 7.5	100	100	100	70
0.5 M potassium monobasic-sodium dibasic phosphate buffer, pH 7.5	100	100	100	100
0.5 M sodium borate buffer, pH 7.5	100	100	100	100

[a] Averages for a single trial. Minimum of 10 plants used in each assay.

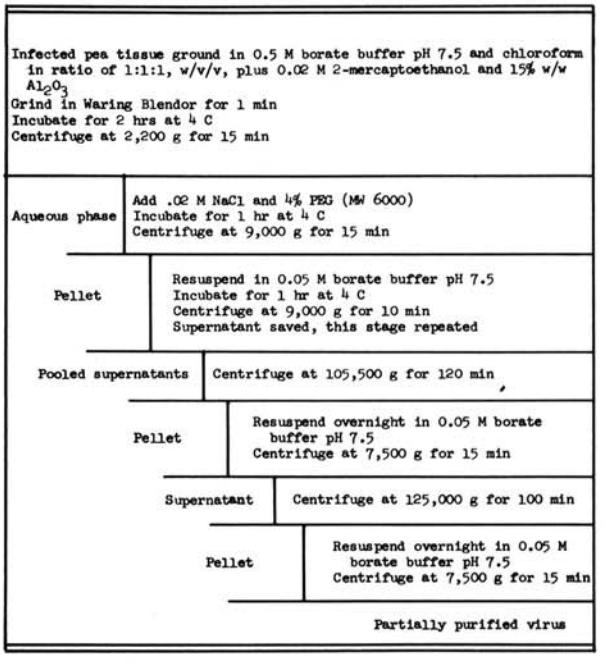

Fig. 1. Scheme for partial purification of pea seed-borne mosaic virus.

treatments was readily apparent (Table 5). The high-speed pellets from the 0.5 M potassium monobasic - sodium dibasic phosphate buffer treatment were more difficult to resuspend than the pellets from the 0.5 M or 0.05 M sodium borate buffer treatments. Since borate buffer has been reported by Tomlinson (23) to reduce particle aggregation, 0.5 M sodium borate buffer was used routinely in all further purifications.

This improved technique of extraction and clarification was then coupled with the PEG-NaCl precipitation technique and the extracts treated as previously stated. To detect differences between treatments, it was necessary to extend the serial dilution series to 1/100,000. The addition of the PEG-NaCl precipitation technique to the high molarity buffer method resulted in slightly higher amounts of virus in the final extract (55% of seedlings infected at 1/10,000 dilution) than the high molarity buffer alone (20% of seedlings infected at 1/10,000 dilution). Electron micrographs of the partially purified preparations confirmed the results of the infectivity assay.

Infected pea extracts, partially purified by the procedure shown in Fig. 1, were colorless to faint yellow and opalescent. Inoculum with a calculated OD_{260} of 1×10^{-5} was infectious for pea in infectivity studies replicated eight times (Fig. 2). Ultraviolet light absorption curves (Fig. 3) resembled curves of rod-shaped viruses with a 260/280 ratio of

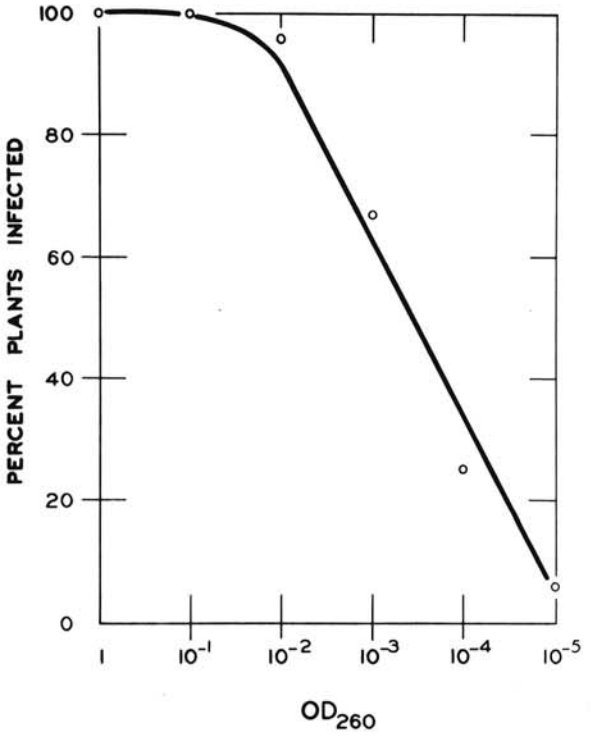

Fig. 2. Influence of dilution on infectivity of pea seed-borne mosaic virus in partially purified extracts. Average data from eight separate trials.

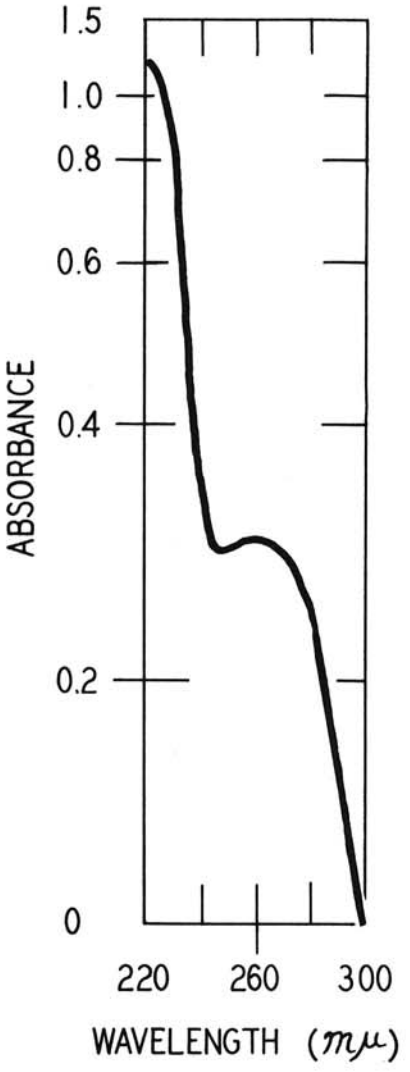

Fig. 3. Nucleoprotein absorption spectrum of partially purified pea seed-borne mosaic virus.

1.15 - 1.25 and a prominent minimum at 240 nm. The OD_{260} of partially purified extracts from 120 g starting material ranged from 125-200 units.

Electron micrographs (shadowed preparations) showed high concentrations of virus particles in addition to varying amounts of contaminants apparently of host origin (Fig. 4). Attempts were made to further purify these preparations using rate zonal sucrose density gradient centrifugation in 10-40% sucrose gradients dissolved in 0.05 M sodium borate buffer at pH 7.5 prepared according to Brakke (2). Virus preparations (1 ml–OD_{260} of 10) were layered onto gradients and centrifuged 2 hr in a SW 25.1 swinging bucket rotor at 25,000 rpm. Gradients were fractionated in an ISCO fractionator and samples collected. Normally four bands were detected with infectivity associated with only the bottom two bands and a small transparent pellet (Fig. 5). After

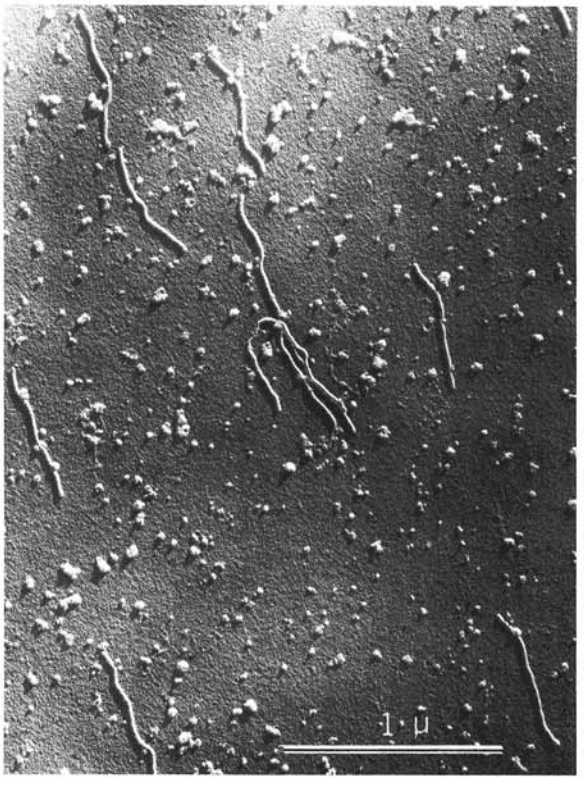

Fig. 4. Electron micrograph of partially purified pea seed-borne mosaic virus.

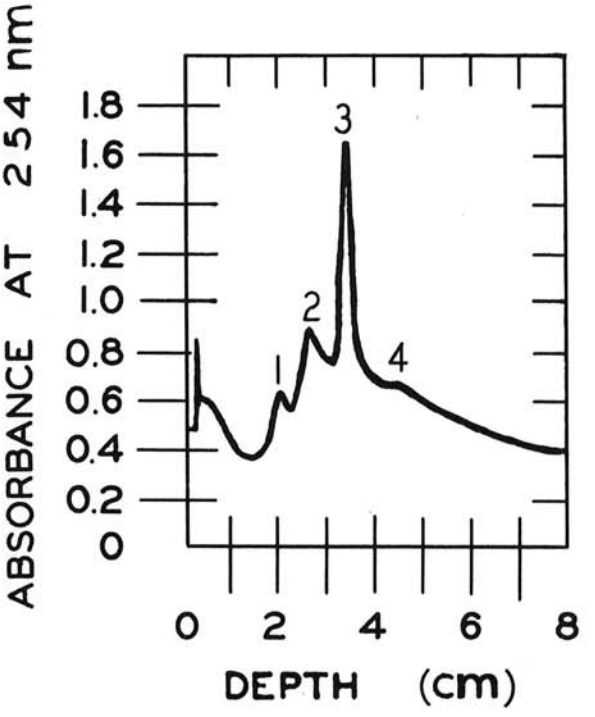

Fig. 5. Profile of a density-gradient tube from a 2-hr centrifugation of a partially purified extract of pea seed-borne mosaic virus.

dialysis against 0.005 M sodium borate buffer at pH 7.5, material was concentrated 10-fold using a collodion bag apparatus under vacuum. Electron micrographs showed particles associated with the bottom two bands and the pellet. Particles were not separated from contaminating globular material using the sucrose density gradient method as described.

DISCUSSION.—The scheme utilized to partially purify PSbMV appeared to be a successful method of concentrating a filamentous rod-shaped virus which existed in low concentrations in pea. Electron micrographs of leaf dip preparations and extracts from preliminary purification methods demonstrated varying degrees of particle aggregation. The problem of aggregation appeared to be greatly reduced using this final purification scheme.

The grinding of infected tissue in the presence of both high molarity buffer and chloroform resulted in a greater extraction efficiency than when each was used separately. Although this was not well understood, the presence of organic solvent in the extraction medium may have prevented an irreversible binding of virus to host material.

The precipitation of virus with PEG was an easy and efficient method of purification which permitted the elimination of large amounts of soluble host material early in the purification schedule. Addition of EDTA to low molarity (0.05 M) sodium borate buffer reduced the pH to approximately 7.5. Additional experimentation showed the amount of virus recovered after PEG precipitation at the lowered pH was appreciably greater than at pH 8.2.

The purification scheme presented may be valuable in the purification of rod-shaped viruses which previously were purified only in amounts unsuitable for further study.

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