# Improved Purification of Two Potato Carlaviruses

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## ABSTRACT

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Electron microscopic examinations of potato virus M (PVM), and potato virus S (PVS), purified by procedures employing clarification with organic solvents, revealed that virus preparations were unsuitable for characterization studies because of extensive fragmentation of the particles. Considerably higher virus yields and percentages of intact particles were obtained by a new purification scheme that entailed sap clarification with calcium phosphate, polyethylene glycol precipitation, two cycles of differential centrifugation using sucrose cushions, rate zonal centrifugation in sucrose, and equilibrium centrifugation in $Cs_2SO_4$. The estimated yield of PVM, obtained from equilibrium density gradient zones, was $46 \pm 9$ $\mu$g of virus per gram of tissue, whereas that of PVS was $31 \pm 6$ $\mu$g of virus per gram of tissue. Purified PVM or PVS behaved as homogeneous systems in sedimentation-velocity experiments.

Potato viruses M and S, members of the carlavirus group, are two of the most common viruses of potato (*Solanum tuberosum* L.) (14,20,21). The symptoms induced by these viruses in potato range from very mild to severe depending on virus strain and potato cultivar; yield losses up to 10–20% have been reported (6,12,20,21). Potato virus M (PVM) has been distinguished from potato virus S (PVS) by symptomatology, host range, and serological properties (6). Since no information is available on the physicochemical properties of PVM and PVS, experiments were undertaken to characterize these two carlaviruses. However, in repeated trials using the published purification procedures for these two potato viruses (13,19), it was not possible to obtain workable amounts of intact virus particles. Examination with an electron microscope and analytical ultracentrifugation studies revealed that the final virus preparations using these techniques contained high percentages of fragmented particles that were unsuitable for characterization studies.

This paper describes a purification scheme that resulted in increased yields of potato viruses M and S with no detectable contamination, and higher proportions of intact virions. Characterization of these two potato carlaviruses will be published in a subsequent report.

## MATERIALS AND METHODS

**Viruses and host plants.** Potato virus M (ATCC/PV 104) was propagated in tomato (*Lycopersicum esculentum* L., 'Rutgers') whereas potato virus S (ATCC/PV 103) was propagated in *Nicotiana clevelandii* A. Gray. Tomato seedlings at the four-leaf stage, and *N. clevelandii* seedlings at the six-leaf stage were mechanically inoculated with PVM and PVS, respectively. Titer of PVM or PVS in infected plants was monitored by the enzyme-linked immunosorbent assay (ELISA) as described by Clark and Adams (3) and Tavantzis (16). Locally and systemically infected leaves were harvested 4 wk after inoculation. Infected tissue containing high virus titer was frozen with liquid nitrogen and stored at −20 C until purification. Infectivity of purified PVM or PVS preparations was tested by mechanical inoculation of french bean (*Phaseolus vulgaris* L., 'Red Kidney').

**Virus purification.** The extraction procedure for legume carlaviruses described by Veerisetty and Brakke (18) was used with some modifications. All steps in the procedure were performed at 4 C or on ice. Buffers were boiled and glassware was sterilized before use. Infected leaf tissue was homogenized (1:3, tissue weight:volume solution) with a large mortar and pestle or in a Waring Blendor with 0.165 M disodium phosphate, 0.018 M trisodium citrate buffer (pH 9) containing 0.1% sodium diethyldithiocarbamate (DIECA), 0.5 mM ethylenediamine tetraacetate (EDTA), and 0.5% 2-mercaptoethanol (ME). The homogenate was filtered through a layer of Miracloth (Calbiochem, San Diego, CA 92112), and centrifuged at 9,500 *g* for 10 min. The supernatant was clarified with calcium phosphate as described by Brakke (2). Calcium phosphate was formed in situ by slow and simultaneous addition to the supernatant of one-twentieth volume of 0.2 M $Na_2HPO_4$ and one-hundredth volume of 1.0 M $CaCl_2$ with constant stirring for 15–20 min. Following clarification of the extract by low-speed centrifugation (9,500 *g*, 10 min), the supernatant was adjusted to 6% (w/v) polyethylene glycol (PEG) 6000 MW, and then stirred slowly for 2.5 hr. The PEG-precipitated virus was collected by centrifuging at 5,000 *g* for 20 min. The pellet was resuspended in 0.0165 M disodium phosphate, 0.0018 M trisodium citrate (DPTC) buffer (pH 9) in approximately one-third the original volume of the sap containing 1 mM dithiothreitol (DTT) instead of 0.5% ME and stirred slowly for 2 hr. This preparation was clarified by centrifuging at 9,500 *g* for 10 min, and the resulting virus suspension was layered on a pad of 10 ml of 20% (w/v) sucrose in DPTC buffer and centrifuged at 105,000 *g* for 4 hr in an IEC SB 110 swing-out rotor. Pellets were resuspended in DPTC buffer, clarified, and the resulting extract was layered on a pad of 10 ml of 20% (w/v) sucrose in buffer and centrifuged at 105,000 *g* for 4 hr. The high-speed pellets were resuspended in buffer (20 $\mu$l/g of tissue) without DIECA or ME, stored overnight at 4 C, and then clarified by low-speed centrifugation. One-milliliter aliquots containing 5–10 $A_{260\ nm}$ units were layered onto 15–35% (w/v) sucrose gradients and centrifuged at 105,000 *g* for 2.5 hr. For rate-zonal centrifugation, sucrose density gradient columns were prepared 24 hr before centrifuging by layering 5, 9, 8, 9, and 5 ml of 150, 200, 250, 300, and 350 mg of sucrose per milliliter of DPTC buffer. The gradients were fractionated with an ISCO density gradient fractionator equipped with a UV analyzer. Fractions containing virus particles (see results, Fig. 1) were pooled, adjusted to 6% (w/v) PEG and 0.3 M

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NaCl, and stirred slowly for 2 hr. Following low-speed centrifugation, the virus pellet was resuspended in 3–5 ml of DPTC buffer. Aliquots (1 ml) containing 2–3 $A_{260\ nm}$ units were layered onto 10–50% (w/w) $Cs_2SO_4$ gradients in DPTC buffer and centrifuged until equilibrium (105,000 *g*, 18 hr) as previously described (15). Following fractionation, the purified virus solution was dialyzed overnight against DPTC buffer to remove the $Cs_2SO_4$. Prior to adapting the purification scheme described here, previously reported procedures (13,19) were used that included homogenization with 0.5 M borate buffer (pH 8.2), clarification of leaf tissue homogenate with organic solvents (eg, chloroform, carbon tetrachloride or ether), and virus concentration with PEG precipitation, differential, and rate-zonal centrifugation.

**Electron microscopy.** Purified virus preparations were examined with a Philips model 201 electron microscope to assess the integrity of virus particles. Sample aliquots (10-$\mu$l) were absorbed for 30–60 sec to carbon-backed grids, coated with 0.3% formvar, and the virus particles were negatively stained with 1% (w/v) sodium phosphotungstate (pH 7.0) and 0.015% (w/v) octadecanol for 15 sec. Excess stain solution was removed with blotting paper and grids were examined at an accelerating voltage of 60 kV.

**Purity criteria.** The purity of the virus preparations was tested by equilibrium density gradient centrifugation; boundary analysis in sedimentation-velocity experiments with a Beckman model E analytical ultracentrifuge, following the procedures of van Holde and Weischet (5); and analysis of the virus-associated proteins and nucleic acids by polyacrylamide gel electrophoresis by using the methods of Tavantzis et al (15).

**Determination of virus yield.** Virus concentration of the final preparations was estimated from the absorbance at 260 nm from spectra obtained with a Cary model 15 spectrophotometer. An arbitrary value of extinction coefficient ($E^{0.1\%}_{260\ nm} = 2.8$) corrected for light scattering was used. Extinction coefficient values used for other carlaviruses ranged from 2.1 to 3.0 (11,17,18).

## RESULTS

**Preliminary purification of the two potato carlaviruses.** Published purification schemes (see Materials and Methods) resulted in the loss of large amounts of virus due to aggregation in each purification step. The final virus preparations were not suitable for characterization studies because of the very small number of intact particles present (Fig. 2). Similar results were obtained when the same procedures were employed for purification of potato virus S (PVS) from infected leaf tissue of *N. clevelandii* (Fig. 3). These methods caused major or total losses of PVM or PVS infectivity. Electron microscopy showed that intact or fragmented particles of purified preparations of PVM or PVS were flexuous, whereas particles from crude extracts were straight or slightly curved. The $A_{260}/A_{280\ nm}$ ratio of the final PVM or PVS preparation was 1.60 ± 0.05, significantly higher than those reported for other carlaviruses (11,17,18).

Purification experiments with 0.1 M tris-HCl buffer (pH 9) containing 0.5% 2-mercaptoethanol (ME) and 0.1% DIECA yielded no virus. In subsequent experiments, PVM purified by the DPTC buffer (pH 9.0) was irreversibly precipitated following dialysis against 0.1 or 0.05 M tris-HCl buffer (pH 9). Unless otherwise indicated, subsequent virus extractions were with 0.165 M disodium phosphate, 0.018 M trisodium citrate buffer (pH 9) containing 0.5% ME, 0.1% DIECA, and 0.5 mM EDTA, and the extract was clarified with calcium phosphate.

**Purification of potato virus M.** Extractions from PVM-infected tomato leaf tissue frozen with liquid nitrogen prior to storage at −20 C resulted in higher yields of purified virus than extractions from fresh tissue. Extracts obtained by use of a mortar and pestle gave virus preparations with a slightly higher proportion of intact particles, but lower total amounts of virus than those obtained by homogenization with a Waring Blendor for 2 min at 30-sec intervals. Clarification of the homogenate with calcium phosphate removed much of the contaminating host material. A second cycle of differential centrifugation was necessary for complete removal of green pigment from the extract. The addition of 0.1% DIECA to the homogenization buffer considerably enhanced the infectivity of the extract, whereas the use of 1 mM DTT and 0.5 mM EDTA improved virus recovery following resuspension of PEG precipitates or high-speed centrifugation pellets. Virus preparations obtained from two cycles of differential centrifugation and subjected to sucrose density-gradient

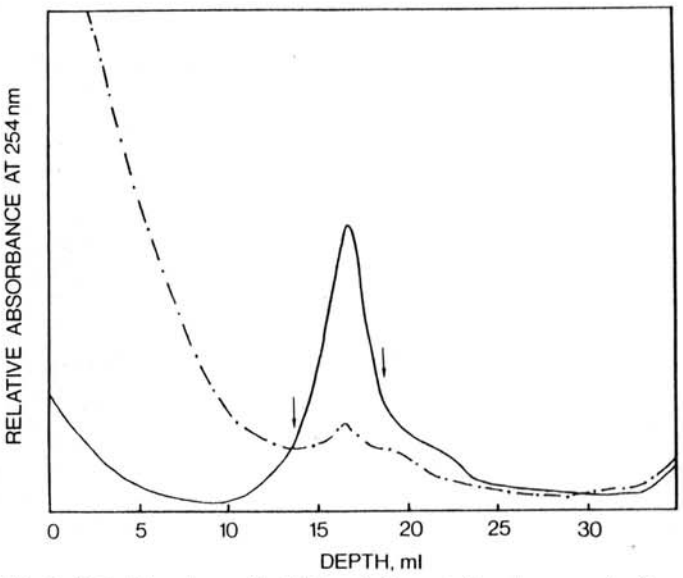


**Fig. 1.** Optical density profile (254 nm) of a centrifuged sucrose density-gradient column containing partially purified potato virus M (PVM) preparations. One-milliliter aliquots of partially purified PVM (5–10 $A_{260\ nm}$ units) were layered on 15–35% (w/v) sucrose gradients and centrifuged (105,000 *g*, 2.5 hr) at 4 C. Potato virus M sediments at the position of the absorbance peak 16.5 ± 0.5 ml below the meniscus. Aliquots of the same batch of PVM-infected tomato tissue were extracted with 0.165 M disodium phosphate, 0.018 M trisodium citrate buffer (pH 9) (——), or 0.5 M borate buffer (pH 8.2) (—·—), but otherwise they were subjected to the same purification procedure as described in this paper. Arrows show the portion of the virus peak used for further purification.

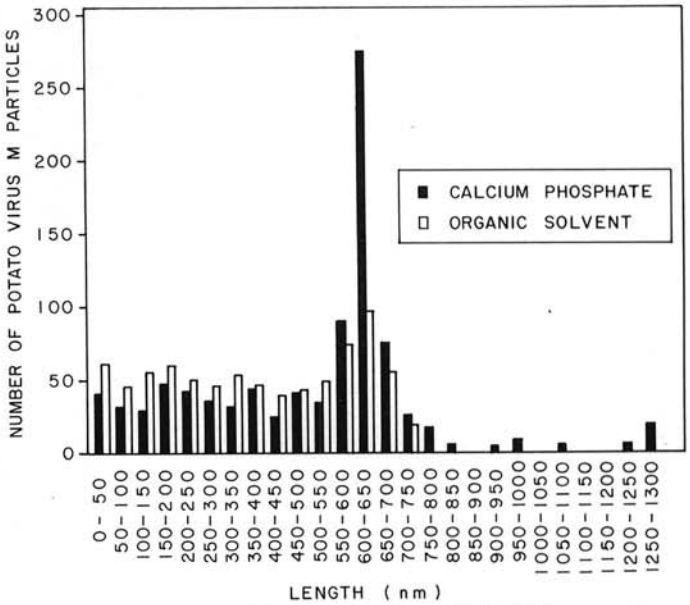


**Fig. 2.** Size distribution of virus particles in purified PVM preparations. Aliquots of the same batches of PVM-infected tomato tissue were extracted with 0.165 M disodium phosphate, 0.018 M trisodium citrate buffer (pH 9), and clarified either by treatment with peroxide-free ether and carbon tetrachloride or by calcium phosphate precipitation (see Materials and Methods), but otherwise they were subjected to the same purification procedure as described in this paper.

centrifugation exhibited a single sedimenting zone 16.5 ± 0.5 ml below the meniscus (Fig. 1). Electron microscopy, ELISA ($A_{405\ nm}$ ranged from 1.85 to 2.17), and infectivity tests showed that fractions corresponding to this peak contained infectious PVM particles. Numbers of local lesions per half-leaf (LL/HL) ranged from 16 to 23 and were directly proportional to the UV absorbance of the virus peak. A trace of low-molecular-weight, UV-absorbing host material was present near the meniscus in virus samples prepared in DPTC buffer, but a large amount of host material occurred in virus samples prepared with 0.5 M borate buffer (pH 8.2) (Fig. 1). Approximately 80% of the total absorbance of a given virus sample, layered onto a sucrose density-gradient column, could be accounted for in the peak containing the PVM particles. There was a low level of infectivity (4–7 LL/HL) associated with the "shoulder" that occurred at 20–24 ml, which contained slightly aggregated virus particles. A small amount of aggregated virus was pelleted during sucrose density-gradient centrifugation. Aggregation was partially reversed when pellets from density gradient tubes were suspended in buffer containing 1 mM DTT and 0.5 mM EDTA. Equilibrium centrifugation of PVM in a 34% (w/w) solution of CsCl resulted in total loss of the virus due to irreversible aggregation. However, PVM preparations, obtained from peak fractions of sucrose gradients and layered onto 10–50% (w/w) preformed gradients of $Cs_2SO_4$, exhibited a single density component (Fig. 4) containing infectious virus particles (35–41 LL/HL).

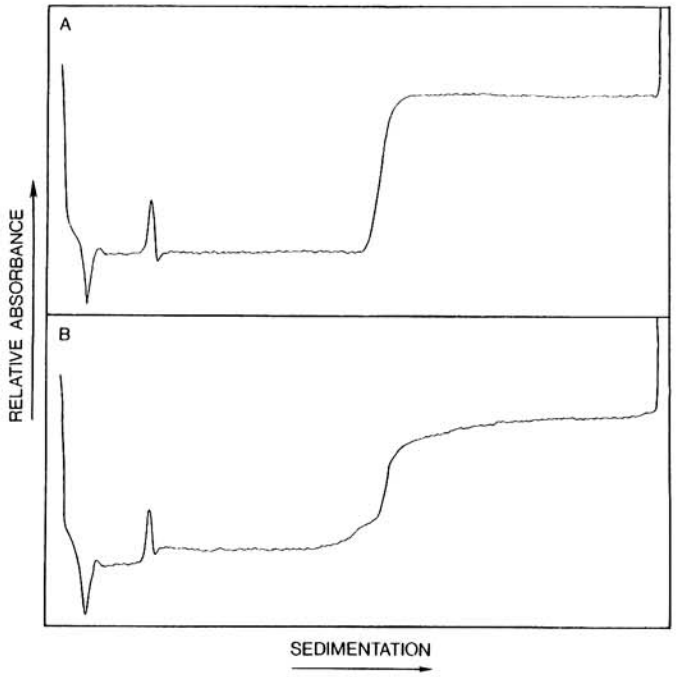


**Fig. 3.** Moving boundary sedimentation of purified potato virus M (0.1 mg/ml). Typical scanner traces (18,000 rpm; T = 20.0 C; λ = 280; 30-nm cells) following 25 min of centrifugation. **A,** Purified PVM prepared as described in this paper. **B,** Virus samples were obtained by extraction with 0.5 M buffer (pH 8.2), clarification with chloroform (1:1, v/v), two cycles of polyethylene glycol precipitation, and differential and rate-zonal centrifugation in 0.05 M borate (pH 8.2) as previously described (13).

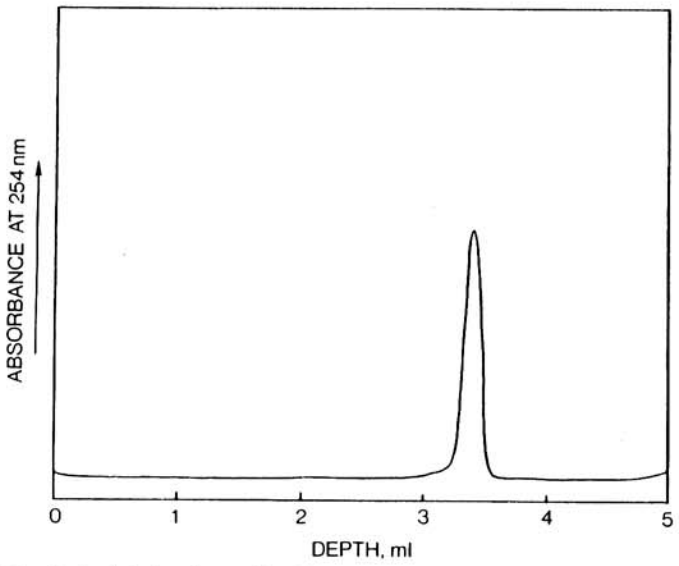


**Fig. 4.** Optical density profile (254 nm) of the sucrose gradient-purified PVM following equilibrium density-gradient centrifugation in $Cs_2SO_4$. One milliliter of a PVM preparation (2–3 $A_{260\ nm}$ units), obtained from peak fractions of sucrose gradients as noted in Fig. 1, was layered on a 10–50% (w/w) gradient of $Cs_2SO_4$ and centrifuged to equilibrium (105,000 *g*, 18 hr) at 4 C. Sedimentation was from left to right.

**Properties of purified preparations of PVM.** Purified PVM preparations contained 44% (319 of 726) intact particles, measuring 645 ± 10 nm × 13 nm (Figs. 2 and 5), which was comparable to the 41% (42 of 102) of intact particles present in leaf dip preparations. Fragmentation of particles appeared to be random and, in part, due to manipulations, ie, drying and staining, required for electron microscopy. The estimated yield of purified PVM, obtained from equilibrium density-gradient zones, was 46 ± 9 μg of virus per gram of tissue, based on the assumption that 2.8 $A_{260\ nm}$ units are equivalent to 1 mg/ml of virus. The absorbance spectrum of purified virus was typical of nucleoprotein. A sample prepared similarly from healthy tomato leaf tissue showed no absorbance in the ultraviolet range. The purified PVM spectrum showed minimum and maximum absorption at 245 and 260 nm, respectively. The $A_{260}/A_{280\ nm}$ ratio was 1.21 ± 0.02 corrected for light scattering. A nucleic acid content of 5–6% by weight was calculated from the absorbance ratio value using the methods of Layne (7) and Paul (10).

**Evaluation of purity.** Purified virus obtained by sucrose density-gradient centrifugation always produced a single UV absorbing peak in equilibrium $Cs_2SO_4$ density-gradient columns (Fig. 4). Healthy tomato leaf tissue, subjected to similar purification, did not give any UV-absorbing peaks in sucrose or $Cs_2SO_4$ density gradients. In addition, the moving boundary, observed in sedimentation-velocity experiments with microgram quantities of purified PVM, corresponded to a homogeneous component (Fig. 3A). In contrast, the moving boundary of PVM, purified as

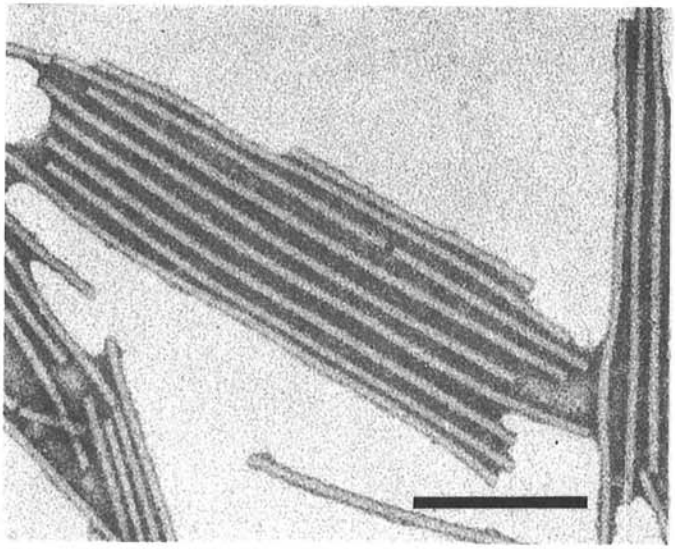

**Fig. 5.** Electron micrograph of a purified PVM specimen negatively stained with 1% sodium phosphotungstate (pH 7.0). The bar represents 200 nm.

previously described (19), was characteristic of a polydisperse system because of particle fragmentation or aggregation (Fig. 3B). Analysis of the virion-associated proteins on 12.6% polyacrylamide slab gels resolved a single polypeptide band. Nucleic acid, isolated from purified PVM virions, migrated as a single component on 2.4% polyacrylamide-agarose gels.

**Purification of potato virus S.** The procedure adapted for isolation of PVM gave approximately $31 \pm 6$ μg of PVS per gram of tissue assuming an extinction coefficient of $E_{260\ nm}^{0.1\%} = 2.8$. When clarification with organic solvents (1:1, v/v) was substituted for treatment of the crude extract with calcium phosphate, PVS yield was reduced to $5.4 \pm 0.5$ μg of virus per gram of tissue, and particle breakdown was considerably higher (Fig. 6). Carbon tetrachloride, chloroform, or peroxide-free ether, in combination or alone, were the clarifying agents used in these experiments. The $A_{260}/A_{280\ nm}$ ratio of purified PVS solutions was $1.20 \pm 0.02$. Purified virus preparations contained 51% (296 of 581) of intact particles measuring $645 \pm 10$ nm × 13 nm (Fig. 6). Potato virus S behaved as a homogeneous component in sedimentation-velocity experiments. A single protein and a nucleic acid component were resolved by electrophoretic analysis on polyacrylamide gels.

## DISCUSSION

The most serious problem in the isolation of PVM and PVS by following published purification schemes (13,19) was the failure to obtain preparations with appreciable amounts of intact virus particles. Thus, the resulting virus samples may be suitable for production of specific antisera but not for characterization studies. Another major problem was the loss of large amounts of virus during the subsequent steps of purification, especially following treatment of the crude extracts with varying volumes (1:12 to 1:1) of carbon tetrachloride, chloroform, or ether. Potato virus S appeared to be more sensitive than PVM to organic solvent treatment. Aliquots of crude extracts obtained from PVS-infected plants and clarified by one or two organic solvents but otherwise subjected to the purification procedure described here, yielded approximately one-quarter as much virus as was obtained from the corresponding aliquots clarified with calcium phosphate. The consistently high $A_{260}/A_{280\ nm}$ ratios, ie, $1.60 \pm 0.05$, of virus suspensions obtained by sucrose density-gradient centrifugation indicated the presence of nonvirion nucleic acids.

The purification procedure described here consistently yielded 40–50 mg of virus per kilogram of leaf tissue. Inoculation of tomato seedlings at the four-true-leaf stage and *N. clevelandii* seedlings at the six-leaf-stage with PVM and PVS, respectively, resulted in higher virus yields than those obtained by inoculating older plants for the purpose of harvesting more tissue. Virus titer, as measured by ELISA (Table 1), reached a maximum 3–4 wk postinoculation, and then declined at a relatively slow rate. The qualitative and quantitative improvement of the virus preparations obtained in this study was shown to be due to a combination of factors such as the extraction buffer, the mild clarification of the plant sap with calcium phosphate, and the sucrose cushions used for pelleting the virus by high-speed centrifugations. The calcium phosphate clarification method was not as effective as the organic solvent treatment in removing plant pigments. However, the PEG precipitation and the two cycles of differential centrifugation resulted in a colorless preparation, highly enriched with virus. The extraction buffer, 0.165 M disodium phosphate, 0.018 M trisodium citrate (DPTC) (pH 9), which is the same as that used by Veerisetty and Brakke (18) for purification of some legume carlaviruses, improved yields of PVM and PVS while providing partially purified preparations containing much less host material. Analytical ultracentrifugation indicated that DPTC-purified virus appears to be a homogeneous component (Fig. 3). However, electron microscopy showed that final virus suspensions contained 44 and 51% intact particles of PVM and PVS, respectively (Figs. 2 and 6). This discrepancy could be attributed to a certain degree of particle fragmentation during preparations of virus specimens for examination with the electron microscope. Although both viruses were successfully extracted in DPTC buffer containing DIECA, EDTA, and ME, this buffer and various additives could not prevent or reverse particle aggregation in subsequent purification steps. However, addition of dithiothreitol (DTT) in the buffer prevented and partially reversed virus aggregation. Dithiothreitol seems to be superior to mercaptoethanol in protecting sulfhydryl groups because it is less likely to be oxidized by air and because of its capacity to break disulfide bonds (4,9). Thus, it appears that the capsid protein subunits of PVM and PVS may contain a substantial number of SH groups. Virus aggregation during purification could be attributed, in part, to the conversion of these SH groups into disulfide bonds. The procedure described here was also successfully employed for purification of potato virus X, and potato virus Y (S. M. Tavantzis, *unpublished*), members of the potexvirus and potyvirus groups, respectively (8). This procedure may prove to be an effective approach for purification of other filamentous viruses as well.

The two potato carlaviruses, PVM and PVS, are still exclusively known by their biological properties. However, biological properties can only be studied by employing test plants and vectors, which all vary widely with experimental conditions and among individual plants and/or insects. But the identity of a virus cannot be well established in this way, even if conditions of research are standardized to allow comparison of results between laboratories (1). Determination of the physicochemical properties of PVM and

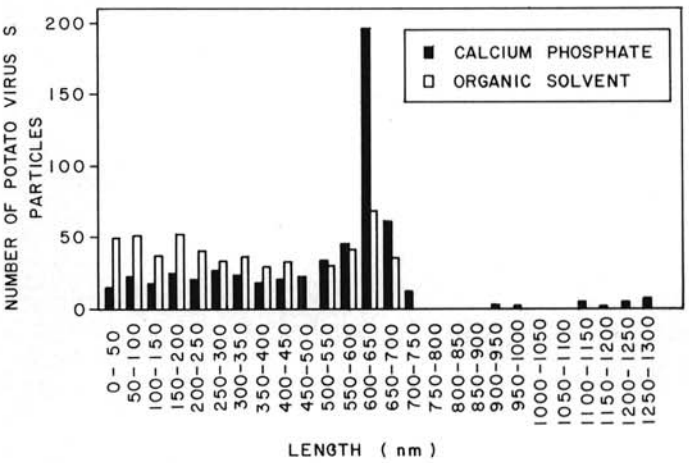


**Fig. 6.** Size distribution of PVS particles in purified preparations. Aliquots of the same batches of leaf tissue of *Nicotiana clevelandii* infected with PVS were extracted with 0.165 M disodium phosphate, 0.018 M trisodium citrate buffer (pH 9), and clarified either by treatment with peroxide-free ether and carbon tetrachloride or by calcium phosphate precipitation (see Materials and Methods), but otherwise they were subjected to the same purification procedure as described in this paper.

TABLE 1. Effect of time after inoculation on PVM titer, as measured by ELISA, in tomato leaves

| Postinoculation time (wk) | Absorbance[a] (405 nm) |
|---|---|
| 1 | 0.077 |
| 2 | 0.213 |
| 3 | 0.351 |
| 4 | 0.506 |
| 5 | 0.477 |
| 6 | 0.441 |
| 7 | 0.395 |
| 8 | 0.356 |
| Healthy | 0.021 |

[a] Means of two replications, five plants, three wells per sample in duplicate ELISA microplates. Virus extracts were prepared by homogenizing PVM-infected tomato tissue in 10 volumes of phosphate-buffered saline (0.02 M phosphate and 0.15 M NaCl [pH 7.4], containing 2% (w/v) polyvinyl pyrrolidone (MW 10,000), 0.05% (v/v) Tween-20, and 0.1% mercaptoethanol) and clarifying by centrifugation at 9,500 *g* for 10 min.

PVS will greatly improve our knowledge of these potato viruses including how to control them. Data on the characterization of PVM and PVS will be reported in a subsequent paper.

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