A Purification Procedure for Enhancement of Citrus Tristeza Virus Yields and its Application to Other Phloem-Limited Viruses

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ABSTRACT

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An improved purification procedure for increased yield and recovery of intact particles of citrus tristeza virus (CTV) involves the use of polyethylene glycol *p*-isooctylphenyl ether in the extraction buffer, two polyethylene glycol precipitation steps, and centrifugation on a preformed step isopycnic cesium sulfate gradient. The quantity and quality of CTV was monitored throughout the purification procedure by enzyme-linked immunosorbent assay (ELISA) and serologically specific electron microscopy. The ELISA with antisera prepared against unfixed CTV was better at detection of short length CTV particles from cesium sulfate gradient zones than ELISA with antisera prepared against formaldehyde-fixed CTV. Use of this purification procedure yielded as high as 31% intact CTV particles in purified infectious preparations. The procedure was also used to assay for virus-like particles from *Nandina domestica* 'Nana-purpurea' and from grapevines affected with corky bark disease, both of which are suspected to be infected by phloem-limited closteroviruses.

Citrus tristeza virus (CTV), a phloem-limited closterovirus, is the most economically important virus affecting citrus (4,8). Tristeza was first recognized to be caused by a virus by Meneghini in 1946 (20). Progress has been made on purification of CTV that, in turn, has led to the development of several serological assays for detection of CTV in plants (6,16). A technique to transmit CTV mechanically by slash inoculations has been developed (14). However, detailed biochemical and serological characterization of CTV virions and components has been limited by lack of a procedure to obtain milligram amounts of highly purified virus. The purpose of this work was to develop a purification procedure that could provide milligram amounts of CTV for further use in the characterization of the properties of the viral coat protein.

We developed a quantitative assay for CTV using enzyme-linked immunosorbent assay (ELISA) and serologically specific electron microscopy (SSEM) that can be used to determine the quality of purified CTV (13). We report here a modified purification procedure that gives high yields of purified virus containing a high proportion of intact threadlike particles that are 1,800–2,000 nm long. This procedure is shown to be applicable for the extraction of phloem-limited viruses from *Nandina domestica* Thunb. 'Nana-purpurea' and from grapevines affected with corky bark. The value of using both ELISA and SSEM in devising purification schemes is discussed. A preliminary report of this procedure has been published (19).

MATERIALS AND METHODS

Virus isolates and propagation hosts. Several isolates of CTV that differ widely in their biological activities were used in this study. All CTV isolates were aphid transmitted before use and indexed free of other known citrus viruses. The T3 isolate, originally described by Grant and Higgins (17), causes severe stunting, vein-clearing, and chlorosis on citron (*Citrus medica* L.) seedling clone 'Arizona 861' and Mexican lime (*C. aurantifolia* (Christm.) Swing.). It also causes seedling yellows in Eureka lemon (*C. limon* (L.) Burm. f.) and sour orange (*C. aurantium* L.) seedlings and a decline of sweet orange (*C. sinensis* (L.) Osb.) on sour orange rootstock. The T4 isolate (16) produces moderate symptoms on Mexican lime but does not cause seedling yellows or decline of sweet orange on sour orange. The T26 and T30 isolates are mild Florida isolates originally obtained from naturally infected field sources. They produce mild vein clearing and stem pitting on Mexican lime and do not induce seedling yellows or decline of sweet orange on sour orange. *C. excelsa* Webster, citron clone 'Arizona 861,' and Palestine sweet lime (*C. limettioides* Tan.) were used as propagation hosts for CTV.

N. domestica 'Nana-purpurea' and seedlings of common *Nandina* were provided by N. A. Ahmed, University of Florida, Gainesville (1,2). St. George grapevines (*Vitis vinifera* L.), clones L7V9 and V. C. ABV10, healthy and with corky bark, respectively, were used. All plants were kept in a partially shaded, air-cooled glasshouse with temperatures ranging from 21 to 30 C.

Purification procedure for phloem-limited viruses. Bark and leaf tissue in 50- to 300-g lots were frozen in a large mortar on dry ice and pulverized, then transferred to another mortar at room temperature and homogenized in 0.10 M Tris-Cl buffer, pH 8.4, containing 0.1% (v/v) polyethylene glycol *p*-isooctylphenyl ether (PGIE) (Triton X-100) (extraction buffer). The final ratio of extraction buffer to fresh weight of the tissue was 5 ml/g. The extract was centrifuged at 10,000 *g* for 20 min, and then polyethylene glycol (PEG, MW of 6,000) and NaCl were added to the supernatant to a final concentration of 4 and 0.8% (w/v), respectively. The suspension was stirred for 1 hr at 4 C and the precipitate was collected by centrifuging at 10,000 *g* for 20 min. The pellet was resuspended in 0.04 M potassium phosphate buffer, pH 8.0, at a ratio of 1.3 ml/g of tissue. The suspension was stirred for 1 hr at 4 C and the resuspended pellet was centrifuged at 5,000 *g* for 10 min. The supernatant was collected, made to a final concentration of 5% PEG and 1% NaCl, and stirred for 1 hr in the cold. The mixture was centrifuged at 10,000 *g* for 15 min. The pellet

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was resuspended in 30 ml of 0.05 M Tris-Cl, pH 8.0, and stirred at 4 C for 1 hr. After centrifugation at 5,000 *g* for 10 min, 5 ml of the supernatant was layered on top of a cesium sulfate (Cs_2SO_4) preformed step isopycnic (PSI) gradient. The gradients were centrifuged overnight at 36,000 rpm at 4 C in a Beckman SW 41 rotor.

The virus zone was collected from the PSI gradients after centrifugation and dialyzed overnight against 0.05 M Tris-Cl buffer, pH 8.0, to obtain a more highly purified virus preparation. The virus preparation was centrifuged at 10,000 *g* for 10 min and the supernatant was adjusted to 22 ml with 0.05 M Tris-Cl buffer, pH 8.0, mixed with 17.0 ml of a 53% (wt/v) solution of Cs_2SO_4 in 0.05 M Tris-Cl buffer, pH 8.0, then sealed in a quick seal VTi50 centrifuge tube (Beckman Instruments, Palo Alto, CA). The tubes were centrifuged for 24 hr at 50,000 rpm at 4 C and then fractionated.

Cs_2SO_4 gradients. PSI gradients were made in Beckman SW41 centrifuge tubes (Beckman Instruments) by overlayering 2.0, 2.0, 2.0, and 1.0 ml of 8.83, 17.66, 26.5, and 35.33% (all w/w), respectively, of Cs_2SO_4 dissolved in 0.05 M Tris-Cl buffer, pH 8.0. Five milliliters of the virus preparation was overlayered onto the gradients before centrifugation for 15 hr at 36,000 rpm at 4 C in a Beckman SW 41 rotor.

Step Cs_2SO_4 gradients were made by diluting 5 ml of virus preparation with 4.4 ml of 0.015 M K_2HPO_4 solution and 4 ml of 53% (w/w) Cs_2SO_4 dissolved in 0.05 M Tris-Cl buffer, pH 8.0. This mixture was then layered onto 3.6 ml of the 53% Cs_2SO_4 solution in a Beckman SW 27.1 centrifuge tube as described by Gonsalves et al (16). The gradients were centrifuged at 19,000 rpm for 15 hr at 4 C in a Beckman SW 27.1 rotor.

ELISA. The ELISA was performed as previously described (5,13). Antisera prepared against unfixed CTV virus isolate T4 and against formalin-fixed CTV virus isolate T4 were used (11,16). Absorbance values (405 nm) were determined in a dual beam spectrophotometer after dilution of the substrate from ELISA plate wells with three parts distilled water. The blank contained unreacted *p*-nitrophenol phosphate diluted similarly. The absorbances were multiplied by dilution factors and are expressed as total A_{405nm} per gram of fresh weight.

SSEM. The SSEM assays were performed as previously described (10,11) with these changes. Antisera prepared against CTV isolate T4 unfixed virus (16) was diluted 1/500 with 0.02 M Tris buffer, pH 8.0, and used to coat parlodion coated grids. Grids were stained for 1 min on drops of 5% uranyl acetate in 50% ethanol, rinsed for 20 sec in 95% EtOH, and air dried. Grids were observed and micrographs taken (35-mm film) at a magnification of ×7,000. After development, the film was cut, mounted in slide holders, and projected, and images were drawn onto paper mounted on a wall. Particle lengths were measured by using a map measurer. A waffle grating (2,160 lines per millimeter, Ted Pella Inc., Tustin, CA) was photographed at the same magnification and used as a reference. For particle counts, all the virus particles on one negative were counted.

Mechanical transmission. Samples to be tested for infectivity from Cs_2SO_4 gradients were dialyzed for 15 hr at 4 C in potassium phosphate buffer, pH 8.0, containing 10% sucrose. The dialyzed preparation was inoculated to citron using the knife-cut procedure described by Garnsey et al (14) but 80 slashes were used per plant. Nine plants were inoculated with each preparation. All plants were assayed by ELISA 60 days after symptoms developed in control plants that had been graft-inoculated with CTV.

Gradient fractionation. After centrifugation, gradients were fractionated with an ISCO Model 640 fractionator and an ISCO UA-4 UV monitor. The virus-containing zone was collected, dialyzed overnight in 0.05 M Tris-Cl, pH 8.0, then centrifuged at 10,000 *g* for 10 min. The absorbance at 260 nm of the supernatant was determined. An extinction coefficient of 2 $(mg/ml)^{-1}$ cm^{-1} at 260 nm was assumed and is the value determined for another closterovirus, sugar beet yellows virus (7).

Buoyant density was determined by the method of Ifft et al (18) with refractive indices of 10 μl-samples taken from each fraction immediately after fractionation of the gradients.

TABLE 1. Effect of additives to the extraction buffer on amount of citrus tristeza virus (CTV) purified from 27-g samples of *Citrus excelsa* leaf midribs as estimated by enzyme-linked immunosorbent assay (ELISA) and absorbance at 260 nm

Additive[a]	ELISA[b] $A_{405\ nm}$	Gradient[c] zone $A_{260\ nm}$
Experiment I (T3 isolate)		
None	1,250 w	0.30 z
2.0% urea	175 v	0.04 y
0.38 mM OBG[d]	1,325 x	0.32 z
0.2% PVP[e]	3,550 z	0.46 z
0.1% PGIE[f]	2,150 y	0.38 z
Experiment II (T4 isolate)		
None	2,000 u	0.61 x
0.2% urea	2,725 w	0.85 xy
1.0 mM OBG	2,275 v	0.69 x
0.2% PVP	3,925 x	1.18 zy
1.0% PGIE	4,025 y	1.20 z
0.1% PGIE	4,450 z	1.39 z

[a] 0.1 M Tris-Cl buffer, pH 8.4, with the indicated additive.

[b] Expressed as total $A_{405\ nm}$ per gram of fresh weight. Samples for ELISA were taken immediately before layering the virus preparation onto preformed step isopycnic (PSI) gradients. Means of three different determinations. Healthy tissue had an ELISA value of 25. Values with different letters after them are significantly different as determined by Duncan's multiple range test, $P = 0.01$.

[c] Total A_{260} of CTV as determined after PSI gradients were centrifuged, fractionated, the virus zone collected, dialyzed against 0.05 M Tris-Cl buffer, pH 8.0, overnight, and low speed centrifugation. No absorbance or ELISA values were obtained from comparable gradient zones when extracts from healthy tissue were centrifuged. Values given having different letters beside them are significantly different as determined by Duncan's multiple range test, $P = 0.01$.

[d] OBG = octyl-β-O-glucopyranoside.

[e] PVP = polyvinyl pyrrolidone, 40,000 MW.

[f] PGIE = polyethylene glycol *p*-isooctylphenyl ether.

RESULTS

Effect of additives in the extraction buffer on CTV yield. The purification procedure described was used to determine if additives to the extraction buffer would result in greater yields of virus. Leaf midribs were cut from plants (*C. excelsa*) infected with either the T3 or T4 isolate of CTV. The midribs were thoroughly mixed, then divided into 27-g subsamples for virus purification. The leaf midribs were extracted either with Tris buffer (0.1 M Tris, pH 8.4) or Tris buffer plus one of the following: 2.0 and 0.2% urea, 0.38 and 1.0 mM octyl-β-O-glycopyranoside, 0.2% 40,000 MW polyvinyl pyrrolidone (PVP), or 1.0 and 0.1% PGIE. The final PEG pellet for each extraction was resuspended in 5.0 ml of 0.015 M potassium phosphate buffer, pH 8.0, centrifuged at 5,000 *g* for 10 min, filtered through a small pad of glass wool in a funnel, and layered onto a PSI gradient. The yield of each treatment was estimated from samples taken for ELISA immediately before the virus preparations were layered onto the PSI gradients and also by determination of total absorbance at 260 nm of the virus-containing zones collected from the PSI gradients. The results are summarized in Table 1. All additives except 2% urea increased virus yields as estimated by ELISA values. In experiment I, there was no increase in yield based on A_{260} values. In experiment II, however, PVP and PGIE additives resulted in greater yields based on A_{260} values. Urea at 0.2% had little effect on recovery of virus, compared to no additive; but at 2.0% concentration, virus yield was reduced. The membrane solubilizer, octyl-β-O-glycopyranoside (3), had no effect on yield.

Effect of additives and type of Cs_2SO_4 gradient. Leaf midribs were cut from T4-infected citron leaves, throughly mixed and divided into 30-g subsamples. Extraction buffers used were 0.1 M Tris, pH 8.4, or Tris buffer plus one of the following additives: 0.2% PVP, 0.1% PGIE, or 0.2% PVP plus 0.1% PGIE. The midribs were pulverized in dry ice, then extracted in the appropriate

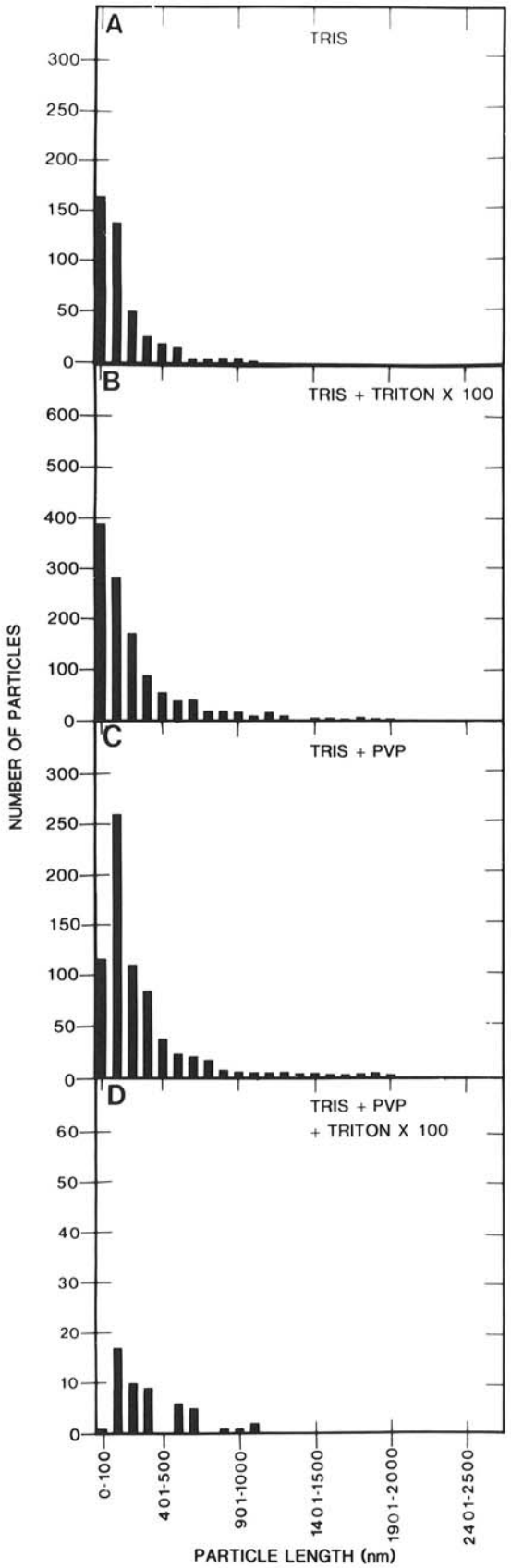

Fig. 1. Histograms of virus particle lengths after extraction in **A,** 0.1 M Tris-Cl buffer, pH 8.4; **B,** 0.1 M Tris-Cl buffer, pH 8.4, plus 0.1% polyethylene glycol *p*-isooctylphenyl ether; **C,** 0.1 M Tris-Cl buffer, pH 8.4, plus 0.2% polyvinyl pyrrolidone; and **D,** 0.1 M Tris-Cl buffer, pH 8.4, plus 0.2% polyvinyl pyrrolidone and 0.1% polyethylene glycol *p*-isooctylphenyl ether. This comparison was made after extraction in the indicated buffer and filtration through cheesecloth but before polyethylene glycol concentration and centrifugation.

extraction buffer. The virus in the extract was precipitated twice with PEG and resuspended as previously described. The final PEG pellet was resuspended in 10 ml of 0.01 M potassium phosphate buffer, pH 8.0, centrifuged at 5,000 *g* for 10 min, filtered through a small pad of glass wool in a funnel, then divided into two equal parts. One 5-ml part was layered onto a step Cs_2SO_4 gradient; the other 5-ml part was layered onto a PSI gradient.

The relative efficiency of the extraction buffer with and without additives to extract virus particles was quantified by ELISA and SSEM (Table 2). This comparison was made after homogenization and filtration through cheesecloth and before any centrifugation. As estimated by particle counts from SSEM micrographs, extraction buffer containing 0.1% PGIE was better than any of the other treatments, and extraction buffer containing 0.2% PVP plus 0.1% PGIE was worse than any other treatment (Table 2). As estimated by ELISA, the extraction buffer containing 0.2% PVP plus 0.1% PGIE resulted in greater absorbance at 405 nm than the other treatments, whereas the absorbance values for extraction buffer containing either 0.1% PGIE or 0.2% PVP were greater than those for the extraction buffer with no additives (Table 2). However, when histograms were prepared, the only treatments containing any intact (1,900–2,000 nm) virions were those that contained only 0.1% PGIE or 0.2% PVP (Fig. 1).

A comparison was made of the step gradients and the PSI gradients for each of the different extraction buffers after the PEG concentration steps (Fig. 2). The buoyant density of the step gradients (Fig. 2A) after centrifugation began at about 1.213 g/ml at the top and remained that density for about two-thirds of the way to the bottom of the tube where the density then increased rapidly. Host material collected on the surface of the tube and formed a thick layer that had to be removed before fractionation of the tube (16). Also, a considerable amount of host material concentrated near the CTV area of the gradient (Fig. 2A). The host material was so near the virus that it contaminated the virus whether the tubes were fractionated by side puncture or by a fractionator.

The PSI gradients (Fig. 2B) had a buoyant density of 1.000 g/ml at the top and, after the first few milliliters, the density increased linearly to the bottom of the tube. Plant material concentrated well above (5–7 mm) the CTV zone, and a clear area was usually apparent between the opalescent virus zone and the host material when the tubes were illuminated from below. UV-absorbing zones occurred below the virus zone in these gradients (Fig. 2B), but these were better separated from the virus than similar UV-absorbing zones in the step gradients (Fig. 2A).

Gradients were fractionated into 0.2-ml fractions. Aliquots of each fraction were removed for determination of buoyant density, for ELISA, and for SSEM. Results obtained from extraction buffer plus 0.1% PGIE are used for illustration (Figs. 2 and 3).

The gradient fractions were assayed by ELISA using two

TABLE 2. Effect of additives to the extraction buffer on amount of citrus tristeza virus (CTV) released as determined by serologically specific electron microscopy and enzyme-linked immunosorbent assay (ELISA)

Additive[a]	Particle[b] count	ELISA[c]
None	639 y	1,050 x
0.1% PGIE[d]	1,427 z	3,450 y
0.2% PVP[e]	553 y	3,150 y
0.2% PVP + 0.1% PGIE	121 x	5,700 z

[a] 0.1 M Tris-Cl buffer, pH 8.4, with the indicated additive.
[b] Average of three different determinations using serologically specific electron microscopy and antisera prepared against whole, unfixed CTV. Values given having different letters beside them are significantly different as determined by Duncan's multiple range test, $P = 0.01$.
[c] Total A_{405} per gram of fresh weight. Antisera were prepared against whole, unfixed CTV. Values given having different letters beside them are significantly different as determined by Duncan's multiple range test, $P = 0.01$.
[d] PGIE = polyethylene glycol *p*-isooctylphenyl ether.
[e] PVP = polyvinyl pyrrolidone, 40,000 MW.

different antisera. When antiserum made against formaldehyde-fixed CTV was used, one major peak of CTV with a slight skew to the least dense side was detected in either type of gradient. CTV antigen was detected throughout the upper 50 fractions of the step gradients (Fig. 2A) but not in the PSI gradients (Fig. 2B). When antiserum made against unfixed CTV was used for ELISA with the same fractions, the major peak of CTV reactivity in the PSI gradients was shifted to a greater density and a minor peak of CTV reactivity was detected (Fig. 2B). There were no noticeable differences in reactivity with the two antisera when the step gradients were analyzed.

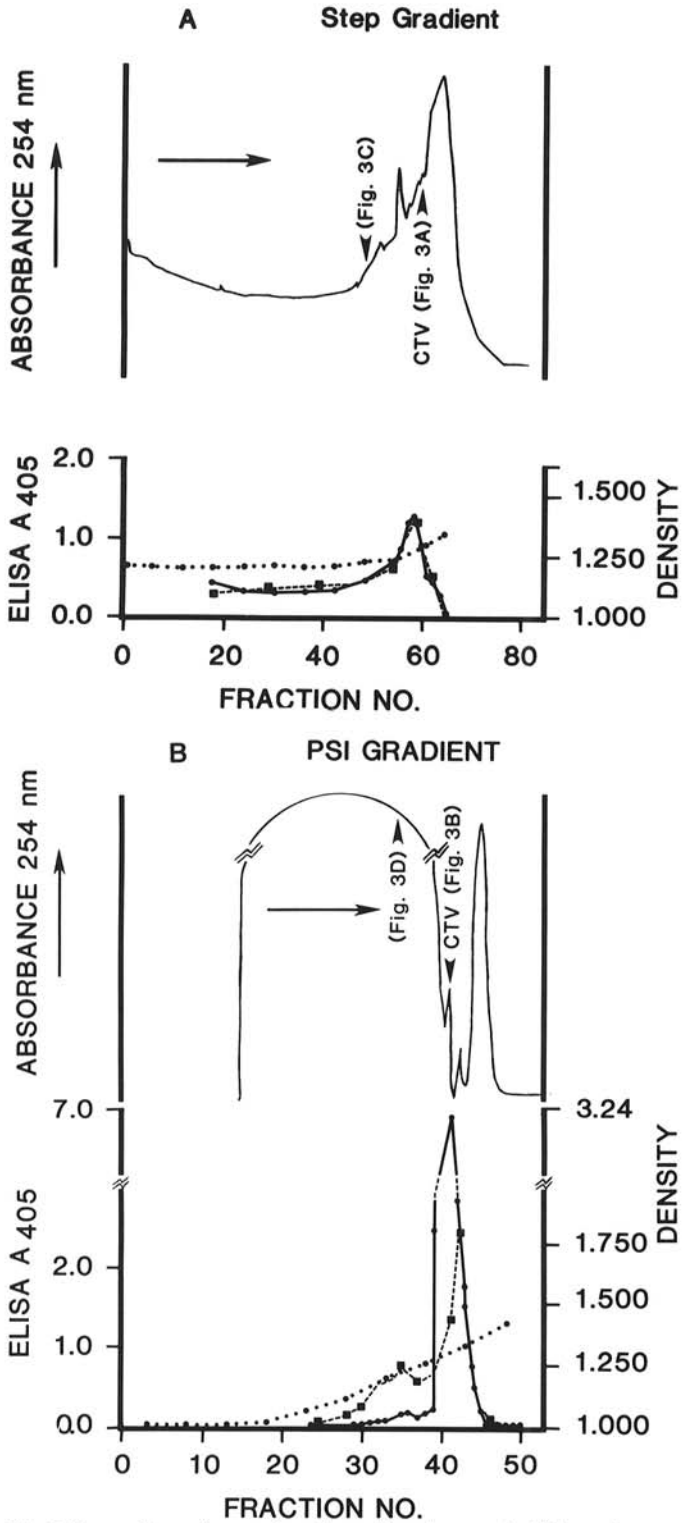

Fig. 2. Comparison of separations from either **A,** a step Cs_2SO_4 gradient or **B,** a preformed step isopycnic Cs_2SO_4 gradient. The relative absorbance at 254 nm, buoyant density (• · · • · · •), and values of enzyme-linked immunosorbent assay using antisera prepared against formaldehyde-fixed citrus tristeza virus (CTV) (•–•–•) or against unfixed CTV (□---□---□) are shown. The location of the major CTV zone is indicated (→), and fractions used for histograms in Figure 3 are indicated (3A, B, C, and D).

The fractions from the gradients were analyzed by SSEM for quantity and length of virus particles (Fig. 3). A histogram (Fig. 3B) of the major peak (arrow, Fig. 2B) from the PSI gradients indicated the intact CTV particles, 1,800–2,000 nm, as well as many shorter particles. A histogram (Fig. 3D) of the minor peak detected by ELISA using unfixed virus antisera showed mostly short particles with few intact particles. A histogram (Fig. 3A) of the lengths of particles detected in the fraction that gave highest ELISA values with the step gradients showed the shorter particles of various lengths with few intact particles, but a histogram (Fig. 3C) from two fractions above the ELISA peak did not show any intact particles.

Aliquots (0.1 ml) of the fractions with the most intact particles as represented in histograms (Fig. 3A and B) were dialyzed as described previously, diluted to 0.25-ml final volume, and used for infectivity tests. Infectivity was obtained from the CTV peaks from PSI gradients using extraction buffer without additive and with either 0.1% PGIE or 0.2% PVP as additives (Table 3). The CTV peak from step gradients was infectious only when the extraction buffer had either 0.1% PGIE or 0.2% PVP as an additive. None of the other fractions tested were infectious from either type of gradient.

Using the purification procedure reported by Gonsalves et al (16), the mean yield of four purifications of the T3 isolate from sweet lime was 0.22 ± 0.07 A_{260} per 100 g of bark tissue. For five purifications of the T4 isolate from sweet lime bark tissue, the mean was 0.26 ± 0.16 A_{260} per 100 g. Using the modified procedures described and comparable tissue harvested at the same time, the mean yield for T3 isolate from sweet lime from nine purifications was 0.88 ± 0.07 A_{260} per 100 g of bark tissue. For 10 purifications from sweet lime bark tissue of the T4 isolate, the mean was 0.42 ± 0.13 A_{260} per 100 g. The average A_{260nm}/A_{280nm} ratio was 1.12 ± 0.01. In a comparison of six purifications with the procedure reported here and six purifications with the procedure reported by Gonsalves et al (16) from bark tissue of *C. excelsa*, the average yields were 5.1 A_{260} and 0.65 A_{260} per 100 g, respectively, a significant difference ($P = 0.01$).

Application to other phloem-limited viruses. The purification procedure reported here was used with seedlings of common *N. domestica*, with plants of *N. domestica* 'Nana-purpurea', and with healthy and corky bark-affected St. George grapevines. With

TABLE 3. Infectivity of citrus tristeza virus after extraction with different buffers, collection from either preformed step isopycnic (PSI) or step gradients, and dialysis overnight in 0.015 M potassium phosphate buffer, pH 8.0, with 10% sucrose

Buffer[a]	Gradient	Intact[b] particles (%)	Infectivity[c]
Tris	PSI	19.1 y	1/9
Tris	Step	5.1 wv	0/9
Tris + 0.1% PGIE[d]	PSI	31.4 z	3/9
Tris + 0.1% PGIE	Step	3.2 wv	1/9
Tris + 0.2% PVP	PSI	12.7 x	2/9
Tris + 0.2% PVP	Step	6.8 w	1/9
Tris + 0.1% PGIE + 0.2% PVP	PSI	0.0 v	0/9
Tris + 0 .1% PGIE + 0.2% PVP	Step	0.0 v	0/9

[a] 0.1 M Tris-Cl, pH 8.4, plus the additive indicated.
[b] Number of virus particles from 1,800 to 2,000 nm long divided by total number of particle counted × 100. Means are from three replications. Values with different letters after them are significantly different as determined by Duncan's multiple range test, $P = 0.01$.
[c] Number of citrus plants infected with citrus tristeza virus shown by both enzyme-linked immunosorbent assay and symptoms/total number of plants slash-inoculated.
[d] PGIE = polyethylene glycol *p*-isooctylphenyl ether.

Nandina, the leaf petioles and leaves were used as purification tissue, and with grapevines, the bark from growth flushes 1–0.3 cm in diameter was used. PGIE was omitted in the extraction buffer for grapevines to prevent formation of a milky-colored suspension after homogenization of tissue.

No UV-absorbing bands from healthy grape or seedlings of common *Nandina* were apparent when the second set of Cs_2SO_4 gradients were scanned (Fig. 4). Two UV-absorbing zones were present in purifications from Nana-purpurea. The buoyant density of the top zone was 1.2600 g/ml and that of the bottom zone was 1.3500 g/ml. Purifications from grapevine with corky bark also had two UV-absorbing zones with buoyant densities of 1.2585 g/ml and 1.3590 g/ml for the top and bottom zones, respectively. Particles about 1,600–1,800 nm long were seen on examination of the top zones from both Nana-purpurea and grape with corky bark; with the transmission electron micrographs, spherical virus-like particles were seen from the bottom zones. Buoyant density of CTV purified at the same time was 1.2570 g/ml.

DISCUSSION

Numerous purification procedures previously reported for CTV (4) gave yields from Mexican lime bark tissue in the range of 0.04–0.75 mg/100 g of tissue. A simple purification procedure was recently reported (6) for recovery of full length CTV ssRNA, but yield was not reported and infectivity of the apparent intact CTV particles was not demonstrated. The purification procedure reported here results in significantly greater yields of highly purified CTV virions, compared with the method of Gonsalves et al (16). Yields of CTV purified by this procedure are as high as 2–3 mg/100 g of bark tissue of *C. excelsa*, and the preparation contains up to 31% intact particles (Table 3). The improvement of yield of the T3 isolate was especially noteworthy as it consistently outyielded the T4 isolate, whereas the method of Gonsalves et al (16) consistently gave lower yields of the T3 isolate than of the T4 isolate. Virus purified by this method has been used to produce high quality antisera (*unpublished*), has been demonstrated to be infectious (Table 3), and has been used as a source of CTV coat protein for polypeptede mapping after partial digestion with proteolytic enzymes (*unpublished*). This procedure is well suited for purifying from as much as 300 g of tissue.

The simultaneous use of ELISA and SSEM to monitor the improvement of a purification scheme illustrates the usefulness and limitations of each procedure. In Table 2, for example, use of ELISA alone would erroneously imply that the addition of both 0.2% PVP and 0.1% PGIE together would greatly improve the extraction of CTV particles. However, SSEM indicated that there were relatively few virus particles. We assume that virus particles were disrupted and that it was this antigen that was detected in the homogenate by ELISA. Use of both ELISA and SSEM permits an estimation of virus integrity and quantity from UV-absorbency zones from gradient scans.

The specificity of the ELISA system can be influenced by the way the injected antigen is prepared. In Figure 2B, for example, the use of antisera prepared to formaldehyde-fixed CTV indicated one virus-containing zone, whereas use of antisera prepared to unfixed

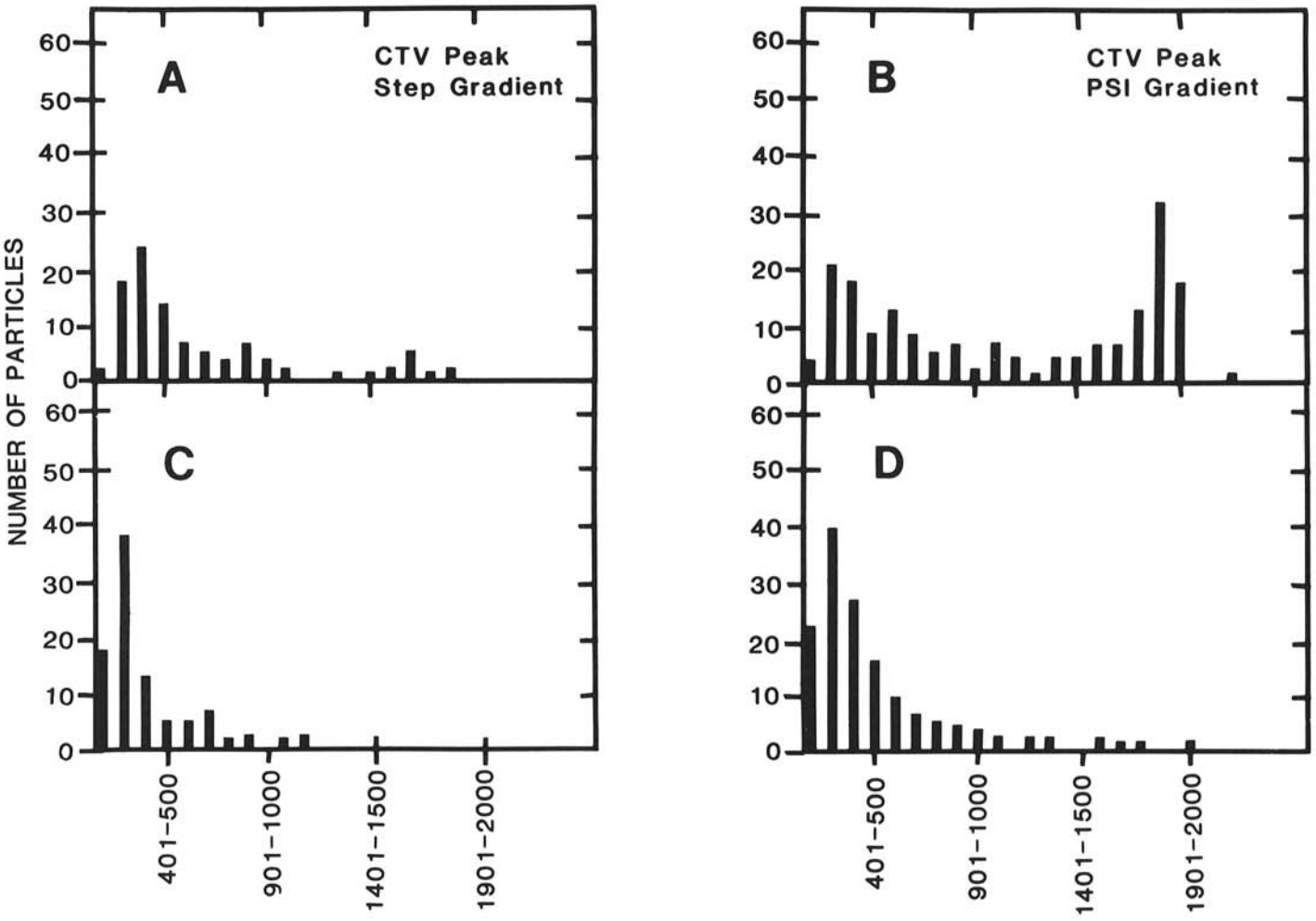

Fig. 3. Histograms of particle lengths of citrus tristeza virus (CTV) from **A**, CTV peak from step gradient; **B**, CTV peak from preformed step isopycnic gradient; **C**, area 10 fractions above CTV peak from step gradient; and **D**, area of minor CTV peak from preformed step isopycnic gradients. Arrows in Fig. 2 show locations of fractions used.

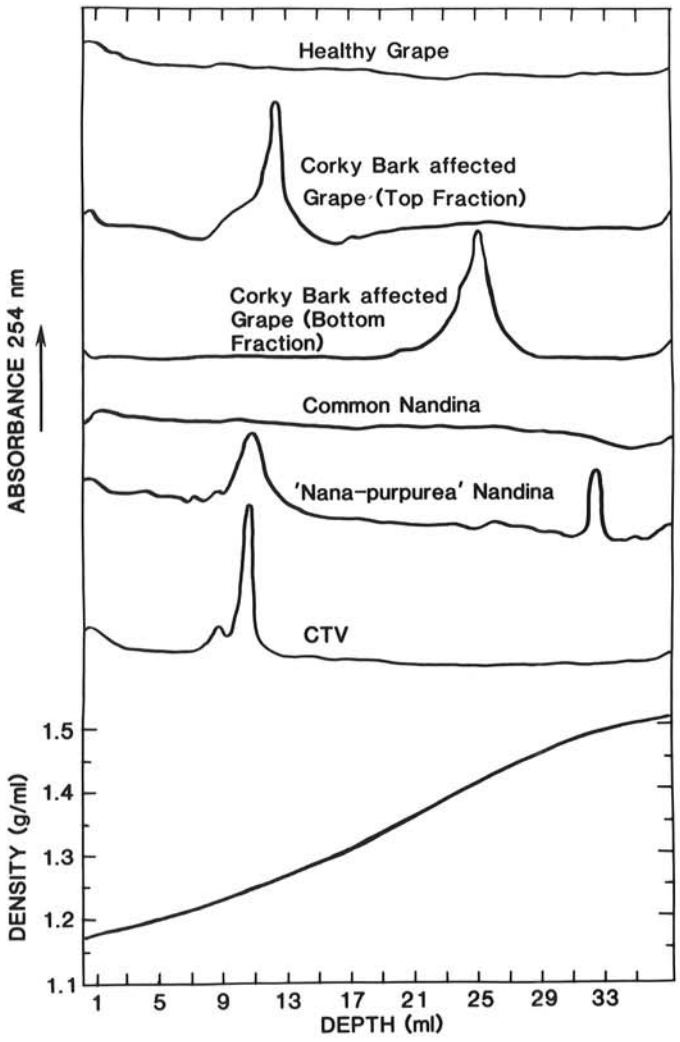

Fig. 4. Relative absorbance at 254 nm of the second set of Cs_2SO_4 preformed step isopycnic gradients after purification of healthy grape; corky bark affected grape, top fraction and bottom fraction (less dense and most dense ultraviolet-absorbing zones collected separately after centrifugation on preformed step isopycnic gradients); seedlings of common *Nandina*; *Nandina domestica* 'Nana-purpurea'; and *Citrus excelsa* infected with T3 isolate of citrus tristeza virus.

CTV indicated the presence of a minor peak that, on SSEM examination (Fig. 3D), was composed of short CTV particles. These populations of virus particles could be separated from each other by using the PSI gradients but not the step gradients. The antisera prepared to unfixed CTV must recognize an additional antigenic site(s) not recognized by antisera prepared to formaldehyde-fixed CTV. This observation confirms earlier work suggesting that there are at least two different antigenic sites for formaldehyde, unfixed, and sodium dodecyl sulfate-degraded CTV-specific antibodies (11).

Closteroviruses present special problems that must be considered in purification. The long, flexuous particles are subject to shearing and also tend to aggregate and to absorb to host membranes (4). Based on serology, CTV titer has been estimated to be as high as 200 μg/g of tissue (4). In purification, the first problem is how to extract the most virus particles yet retain integrity of the virion. Once the virus particles are extracted, the next problem is to purify and concentrate the intact particles without aggregation and shearing. When particle lengths were compared in crude homogenates, intact CTV particles were evident only when PVP or PGIE was added to the extraction buffer (Fig. 1). Although pulverizing frozen tissue releases a lot of virus, it does not appear to be the most efficient process for the extraction of intact virus particles (15).

The PSI gradients seem to be superior to the step gradients for concentrating the intact virus particles and separating them from the shorter length particles (Table 3, Fig. 3A and B). This is reflected in both the percentage of total particles that are near full length as determined by SSEM and by the infectivity of slash-inoculated preparations into indicator plants, probably the most sensitive method to estimate the presence of intact CTV virions. The infectivity rates were always better from PSI gradients than from step gradients.

When the purification procedure as described for CTV was applied to other viruses that are reportedly phloem limited, good results were obtained. The UV-absorbing zone with a buoyant density of 1.2600 g/ml from Nana-purpurea is close to what would be expected of the closterovirus-like Nandina stem pitting virus (1,2). The second UV-absorbing zone with a buoyant density of 1.3500 g/ml from Nana-purpurea was unexpected. However, previous examination of tissues from Nana-purpurea with the transmission electron microscope revealed spherical virus-like particles (1), which may be the cause of the more dense UV-absorbing zone. The application of the purification procedure to grapevines with corky bark resulted in two UV-absorbing zones, one with a buoyant density of 1.2585 g/ml and the other with a buoyant density of 1.3590 g/ml. The least dense UV zone is similar to what would be expected for a closterovirus such as the one previously reported in grape with corky bark (9,21). In this case, however, the purification was directly from grapevines rather than from tobacco. Spherical virus-like particles have also been reported from grapevines (12). The spherical virus-like particles detected in Nana-purpurea and grape with corky bark may not be strictly phloem limited as are the closteroviruses, but this procedure enables their detection and purification. Work has been initiated to better characterize these virus-like agents from *Nandina* and grapevines.

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