Partial Purification of Viruslike Particles Associated with the Citrus Tristeza Disease

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ABSTRACT

Threadlike particles associated with the tristeza disease of citrus were partially purified, employing a combination of gentle grinding, precipitation by polyethylene glycol and differential centrifugation, and using electron microscopy as an assay. Particles in negatively stained dip preparations from leaves or stem bark exhibited a normal length of 200 mμ and a width of 10-11 mμ. Sedimentation constants of purified preparations, determined in sucrose density gradients, ranged from 105 to 131 S, with a calculated value of 140 S (±10) at zero depth. In purified preparations, normal length particles amounted to 26%, whereas variations in the procedure resulted in fragmentation of almost all particles. In different tissues of four citrus varieties, the highest concentrations of particles were always found in the stem bark. Bark from Key limes was the best source for extraction. In Key lime leaves, particle concentration was correlated with symptom intensity. Phytopathology 60:75-78.

Only a few citrus viruses have thus far been purified and characterized, and these could be transmitted mechanically to citrus or noncitrus hosts. Corbett & Grant (5) purified citrus variegation virus, and proved that infectivity is associated with 30-mμ isometric particles. Tatter leaf virus, which can also be mechanically transmitted, has been purified, and infectivity shown to be caused by 650 × 19-mμ particles (11).

In various species and varieties of citrus, Kitajima et al. (8) found that infection by tristeza is always associated with threadlike particles approximately 10-12 mμ × 2,000 mμ. Similar particles were not detected in the sap from noninfected plants. These results have been confirmed (4, 10), but attempts to isolate and purify these particles gave only partial and inconsistent success (10).

In this paper we report on the partial purification of these threadlike particles, using electron microscopy as an assay method during the purification procedure, and some of their properties.

MATERIALS AND METHODS.—The strain of tristeza virus used originated from a graft-infected *Citrus sinensis* (L.) Osbeck Shamouti Sweet orange found in a citrus collection in Miqweh Yisra'el. This strain induces vein-clearing, yellowing, and cupping on *C. aurantifolia* (Christm.) Swing. Key lime and *C. macrophylla* Wester, sometimes killing the new growth, and later causes pitting and striation of the stem beneath the bark. These symptoms seem to be identical to those caused by the T_3 strain (7).

Virus for purification studies was increased in plants of Key lime grown in a screenhouse in plastic pots containing a 3-kg mixture of sandy loam and peat, or directly in the soil surrounded by the screenhouse. The plants were inoculated by side-grafting with a bud, and 2 weeks later were pruned to force new growth. Healthy noninoculated plants were grown similarly for control purposes. Leaves, bark, or roots were harvested 2-6 months after inoculation, when leaf symptoms on inoculated plants were most pronounced.

Samples for electron microscopy were prepared by the dip method directly from infected tissue into a drop of neutralized 1% phosphotungstic acid (PTA) on Formvar carbon-coated grids (3). After 1-2 min, excess fluid was removed with a small piece of filter paper. Specimens obtained during the purification procedure were scanned by placing a single drop on the Formvar carbon-coated grid for 2-5 min. After that, excess fluid was removed and a drop of 2% neutralized PTA was placed on the grid for 0.5 min, after which excess fluid was removed as before. Observations and electron micrographs were taken with either an RCA EMU-2 or JEM-7A scope.

For quantitative estimates on the number of particles per volume, a modified procedure based on a method described for T_4 phage was used (1). The negative staining-spray droplet technique (9) was found unsatisfactory, because shearing forces produced by a nebulizer caused fragmentation of particles. Mixtures of 0.1 ml 1% bovine albumin and 0.1 ml 2% PTA were added to 0.5 ml of the virus suspension, and after thorough mixing, a drop was placed for 2 min on a 400-mesh grid. The number of particles was counted at low magnification (×8,000) in 10 grid openings distributed at random on the grid, using different concentrations of tobacco mosaic virus (TMV) as a reference.

RESULTS.—*Partial purification.*—In preliminary experiments using the dip method, different tissues, i.e., stem bark, leaves, and roots from tristeza-infected Key lime, Shamouti, and *C. aurantium* L. Sour orange were examined to determine those that contain high numbers of the threadlike particles. In dip preparations made from stem bark of Key lime, comparatively high numbers of particles, 1-2/grid opening, were consistently found, whereas from Key lime leaves only 1-2 particles/10 grid openings were found. In dip prepara-

tions from the bark of Shamouti and Sour orange, particles were seldom observed, and none were found in dips made from Shamouti and Sour orange leaves. Therefore, stem bark from Key limes was used as a source during the development of the purification procedure. Different methods of mechanical grinding and homogenization, using a Waring Blendor, "Virtis" homogenizer, or "Ultra-Turrax", generally gave extracts with only few particles of normal length. A high proportion (95%) of particles was broken, apparently due to shearing forces produced during homogenization. However, when the tissue was ground with the aid of a mortar and pestle, the number of particles of normal length was relatively high, 40-50%.

The procedure adopted for extracting and concentrating the particles employed a combination of differential centrifugation and precipitation with polyethylene glycol (PEG) (6). Stem bark from tristeza-infected Key limes was peeled when leaf symptoms were pronounced, and stored at −18 C. Aliquots of 25-30 g were ground in a mortar in the presence of liquid air, and the powder was thawed by the addition of 0.05 M Tris[tris (hydroxymethyl) amino methane]-HCl buffer pH 7.4 (1 g/2.5 ml). The liquid was expressed through cheesecloth, and the pulp was again extracted with the same buffer. The two extracts were combined and centrifuged, first for 10 min at 5,000 rpm (4,000 *g*) and, after removing the pellet, again for 5 min at 7,000 rpm (8,000 *g*). Four g of PEG (mol wt 6,000) and 4 ml of NaCl 20% were then added by stirring to 100 ml of the greenish-yellow supernatant. After the PEG dissolved, the suspension was centrifuged for 15 min at 10,000 rpm (16,000 *g*). All centrifugations were conducted in a GSA Rotor Sorvall RC 2-B centrifuge at 4 C.

The supernatants were discarded and the pellets resuspended in 25 ml 0.04 M sodium phosphate buffer, pH 8.2/100 ml of original extract. After centrifugation for 10 min at 6,500 rpm (5,000 *g*), the supernatant was further purified by ultracentrifugation at 29,000 rpm for 90 min in a No. 30 Spinco rotor. The sedimented material was resuspended in the phosphate buffer, in one-tenth of the original volume, by gently stirring it overnight. All steps were conducted at 4 C. After an additional centrifugation for 10 min at 6,000 rpm (5,000 *g*), the supernatant was ultracentrifuged for 3 hr at 29,000 rpm. The resulting pellets were resuspended by stirring for 8-12 hr using double-distilled water, 0.02 of the original volume, as a suspending medium. The final suspension was centrifuged for 10 min at 6,000 rpm (5,000 *g*).

The number of particles increased markedly during the purification procedure, from a few, if any, in each grid opening in the first extraction medium, to an average of 10 after the first ultracentrifugation, and to a very high, uncountable number at the last step (Fig. 1-B).

In three experiments, starting with 25 g bark each, the number of threadlike particles after the final step of the procedure was approximately comparable to the number of TMV particles in a solution containing 30 μg/ml TMV. About 50 particles/grid opening were counted if the concentrated tristeza solution was diluted one-tenth, compared with a similar count of TMV particles in a 3-μg/ml solution. Caution, however, should be applied, when considering these data, as two viruses may be adsorbed differently by the Formvar film.

When Key lime leaves were used as a source, better results, based on the number of particles, were obtained if 0.05 M Tris/HCl buffer pH 8.4 was used for extracting instead of the pH 7.4 buffer used for bark extractions.

The addition of reducing agents such as thioglycollic acid, mercaptoethanol or sodium diethyl-dithiocarbamate, and the use of charcoal or other buffers, such as phosphate pH 6.0-8.2, glycine pH 6.0, or Tris/phosphate pH 7.0-9.0 during the purification procedure, did not increase yield, but more often resulted in a substantial loss (40-80%) of the threadlike particles.

Threadlike particles were not seen in negatively stained leaf or bark dip preparations from healthy Key limes or Shamouti oranges, nor in samples from healthy trees that were "concentrated" by the above outlined procedure.

Particle morphology.—Normal length determination of the threadlike particles (Fig. 1-A) was made from samples prepared by the dip method, because apparently this method did not cause extensive fragmentation of particles. One hundred particles from leaf and bark dips of tristeza-infected Key limes were measured at a magnification of ×50,000, using TMV as a standard; the distribution of particle length is shown in Fig. 2-A. Fifty-seven per cent were in the range of 1,900-2,100 mμ, with a most frequent length of 2,000 mμ. These data are in agreement with normal length measurements made by Kitajima et al. (8).

In partially purified preparations the number of normal particles decreased (Fig. 2-B). Out of 184 particles measured, 47 (25.5%) were in the 1,900-2,100 mμ range. However, if mechanical grinding or homogenization was employed during the extraction procedure, fewer than 5% of the particles were in the 1,900-2,100 mμ range, and more than 60% were shorter than 500 mμ.

The width of the particles in negatively stained preparation was 10-11 mμ. Measurements were made from dip preparations, using TMV (15 mμ) as a reference, at a magnification of ×50,000.

Rate of sedimentation of the threadlike particles in sucrose density gradients.—Brakke's method (2) was used to estimate the sedimentation rate of the threadlike particles in sucrose density gradients. Standard curves were established using PEG-purified TMV (6) with a sedimentation rate of 187 S as a reference. One ml of virus suspension was layered on each column. The depth of TMV was measured directly after illumination, while the tristeza-containing tubes were fractionated into 1-ml samples, dialyzed overnight against 0.04 M phosphate buffer pH 8.2, and screened with the electron microscope in order to find the fraction that contained the highest concentration of normal length particles. About 60% of the normal length particles were found in one fraction, the remainder being distributed over two to three fractions above and below. The sedimentation rate changed slightly with depth, ranging from 105 to 131 S (Fig. 3). A ratio of 0.746

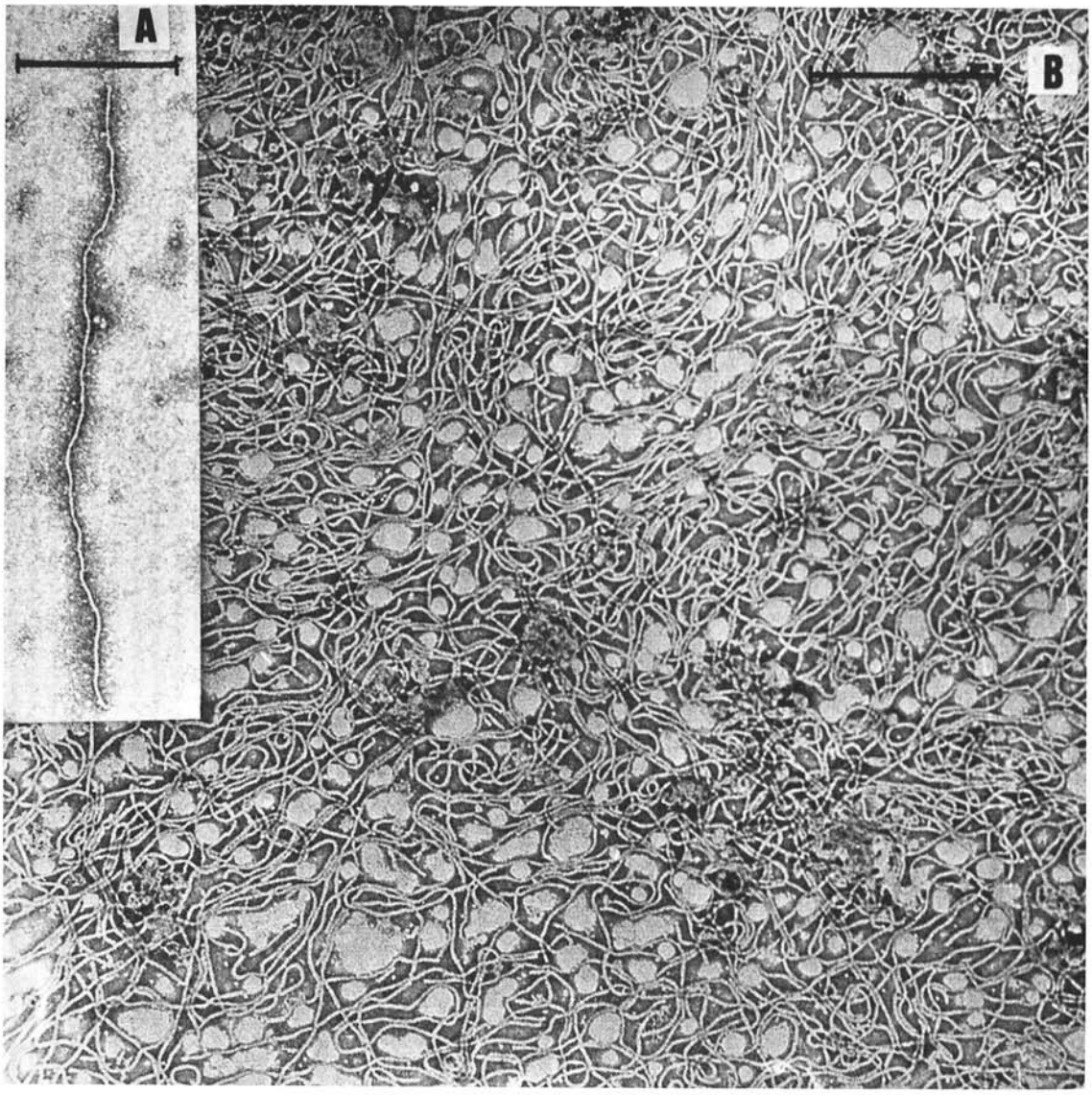

Fig. 1. Electron micrograph of threadlike particles from tristeza-infected Key lime bark, negatively stained with phosphotungstic acid. **A)** Dip preparation. **B)** Partially purified preparation. The scale represents 0.5 μ.

at zero depth yielded a calculated value of 140 S (± 10).

Particle content in different citrus species and tissues.—Dip and partially purified preparations were made from the following sources: leaves; stem bark; root bark; rootlets; and leaves from Key limes; Shamouti orange; *C. aurantium* L. (Sour orange); and *C. macrophylla*. The highest number of particles, in both dip and partially purified preparations, was found in the stem bark of Key limes, while rootlets and root bark, of all the species tested, contained few if any detectable particles (Table 1). In Key lime leaves, the number of particles was correlated with intensity of symptoms. In Shamouti and Sour oranges, particles were seldom found in dip preparations made from the different tissues, but could be detected regularly in partially purified samples from stem bark.

Discussion.—Methods for purifying and concentrating the threadlike particles associated with the tristeza disease encounter several difficulties. The relatively low concentration of particles and their restriction to phloem cells (10) necessitates using larger quantities

Table 1. Relative concentration of threadlike particles in partially purified preparation from various citrus sources

Source plant	Stem bark	Root bark	Root-lets	Leaves[a] 1	Leaves[a] 2
Key lime	++++	±	−	++	±
C. macrophylla	+++	−	−	++	±
Shamouti	+	−	−		−
Sour orange	+	−	−		±

[a] 1 = leaves bearing strong symptoms; 2 = leaves with no or weak symptoms. ++ = about 10 particles/grid opening in partially purified preparation from 25-g tissue; each additional + represents approximately a fivefold increase.

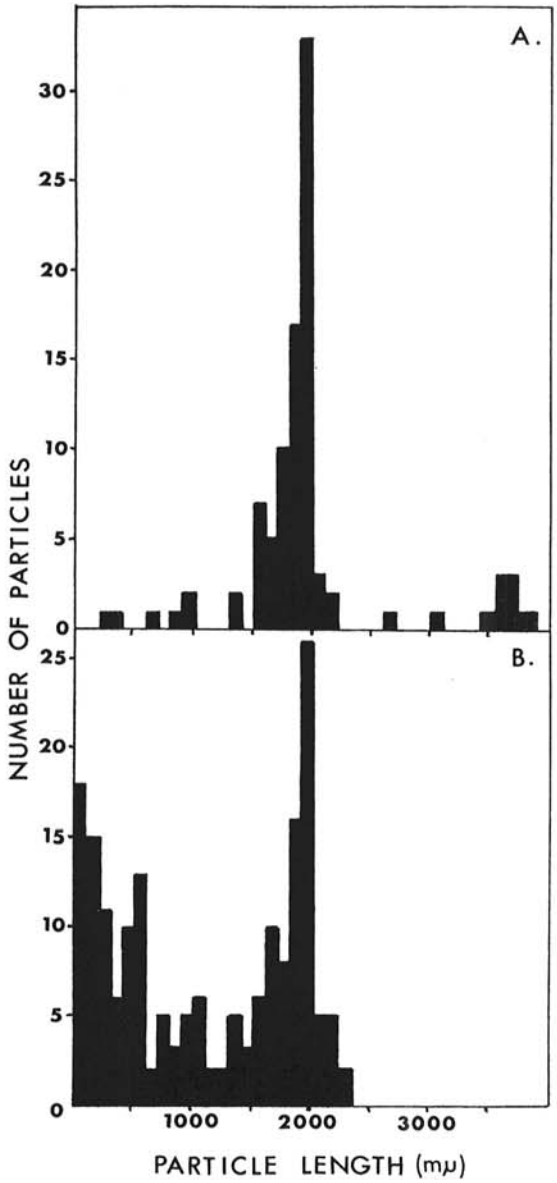

Fig. 2. Distribution of length of viruslike particles associated with tristeza from stem bark of Key limes. **A)** Dip preparations. **B)** Partially purified preparation.

of tissues rich in phloem layers (25 g bark yielded only μg). It is, therefore, not surprising that stem bark, with a relatively high content of phloem cells, proved a better source than leaves. Purification from stem bark was further facilitated because the amount of chloroplast debris in the homogenate was low. Procedures for purification of the threadlike particles have also to overcome their tendency for aggregation and fragmentation. The combination of comparatively gentle grinding, precipitation with PEG, a minimum of high-speed centrifugations, and prolonged stirring for resuspension, resulted in preparations with a relatively high concentration of normal-length particles. Variations from the outlined procedure employing mechanical

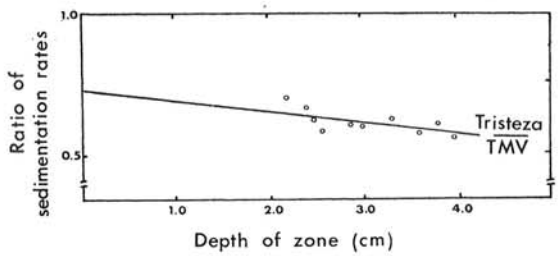

Fig. 3. Ratios of the sedimentation rates of the threadlike particles from tristeza-infected Key lime bark to tobacco mosaic virus.

homogenization and additional cycles of differential centrifugation yielded low concentration of particles, almost all fragmented. A similar sensitivity to fragmentation has been observed with the filamentous particles of beet yellows virus (12).

So far, typical particles were always found in concentrated samples prepared from stem bark of four species of citrus infected with tristeza.

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