Purification of Citrus Crinkly-Leaf Virus

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In 1963, Dauthy and Bové (3) reported preparation, from infected citrus leaves, of a partially purified isolate of citrus crinkly-leaf virus (CCLV) that proved infectious when mechanically inoculated to citrus and to *Vigna sinensis* vars. Blackeye and Black. Later, they (4) found that such preparations obtained from CCLV-infected lemon leaves contained pseudospherical particles, whereas similar preparations made from healthy lemon leaves did not contain such particles. Only preparations containing these particles caused infection when mechanically inoculated to test plants. However, the degree of purity of the preparations used was not high, and consequently, the diameter of the particles, when estimated from low-magnification micrographs of negatively stained preparations, was found to be rather low.

In the meantime, Corbett (personal communciation), working with citrus infectious variegation virus (CIVV), found the disease to be associated with pseudospherical particles of a diameter of 27 to 33 mμ when measured on micrographs from shadowed preparations. This paper reports recent progress in the purification of CCLV, and presents evidence that CCLV and CIVV are strains of the same virus.

Materials and Methods

VIRUS STRAINS.—The citrus crinkly-leaf virus used was strain 81-A-65 of J. M. Wallace; strain 63-1 (from California) was also used. In the case of citrus infectious variegation virus, strain 1234, test 9, from T. J. Grant, was used. The viruses were multiplied in Lisbon and Eureka lemon leaves. As reported by Grant and Corbett (6), we found that the virus concentration was lower in old leaves than in young ones. Thus, only young, tender lemon leaves were used in this investigation.

INFECTIVITY TEST.—*V. sinensis*, var. Blackeye No. 5, from the Lyng Seed Company, Modesto, California, was used for testing infectivity. Pale green chlorotic lesions on the inoculated primary leaves (cotyledons), followed by systemic symptoms on the trifoliate leaves were produced by CCLV. Young lemon seedlings were also used as test plants. Inoculation of the purified preparations was made with a glass spatula in the presence of Carborundum.

PURIFICATION OF CCLV.—Young lemon leaves were cut into small pieces with scissors, and homogenized at 4°C in a Waring Blendor in the presence of the following buffer: sodium phosphate, 0.02 M (pH 8.0) + magnesium sulfate, 0.005 M + bentonite at the final concentration of 1 mg/ml of homogenate. The bentonite came from B.K.H. Co., San Francisco, and was prepared according to Fraenkel-Conrat *et al.* (5). For 100 g of leaves, 250 ml of buffer was used. The homogenate was squeezed by hand through 4 layers of cheesecloth, and the resultant filtrate (pH 6.8) was shaken for 30 min at 4°C, with a mixture of 100 ml of cold primary butanol and 100 ml of cold chloroform. Centrifugation of the emulsion at 1,000 g for 30 min yielded an upper aqueous phase that was recovered, filtered over glass-wool, and left at 4°C for 15 to 16 hr. The precipitate that settled out during this period was removed by centrifugation (7,000 g for 30 min at 2°C.)

After high-speed centrifugation in the cold (30,000 rpm for 150 min in rotor No. 30 of the Spinco model L centrifuge), the supernatant yielded pellets that were resuspended in 2 ml of phosphate-buffer 0.02 M (pH 8.0), containing magnesium sulfate at the concentration of 0.005 M. The

suspension was clarified by low-speed centrifugation (2,000 g for 15 min). Bentonite was mixed with the supernatant to the final concentration of 0.5 mg/ml, then eliminated by a 15 min centrifugation at 15,000 g. A last cycle of high- and low-speed centrifugation in the cold [120 min at 40,000 rpm, pellets resuspended in phosphate-KCl buffer (see below), and 15 min at 2,000 g] yielded the fraction used in the following step.

FIRST SUCROSE DENSITY-GRADIENT CENTRIFUGATION.—The method of Brakke (1, 2) was used. Solutions containing, respectively, 100, 200, 300, and 400 mg of sucrose per ml were prepared with the following phosphate-KCl buffer: K_2HPO_4, 0.005 M; KH_2PO_4, 0.0005 M; and KCl, 0.01 M (final pH 7.0). The gradients were prepared by successively layering in a centrifuge tube (for the SW 25 rotor of the Spinco model L centrifuge) 7 ml of the 40 per cent solution, 7 ml of the 30 per cent solution, 7 ml of the 20 per cent solution, and 4 ml of the 10 per cent sucrose-solution. The gradients were kept at 4°C for 15 hr before use. One ml of the suspension obtained after the last cycle of high- and low-speed centrifugation was added on top of the gradient and centrifuged 3 hr at 24,000 rpm.

DIALYSIS.—In preparations obtained from CCLV-infected leaves, 3 light-scattering zones were obtained. The lowest one, zone III, located between 3.9 and 4.4 cm from the top of the tube, shows 2 sub-zones close together. Zone III was recovered with a hypodermic syringe, and dialyzed overnight against tris-acetate buffer, 0.01 M (pH 7.0), containing NaCl at the concentration of 0.1 M. The dialyzate was infectious when mechanically inoculated into test plants.

The virus in the dialyzate was sedimented by centrifugation (120 min at 40,000 rpm). The pellets were resuspended in a small volume of phosphate-KCl buffer, and the suspension was clarified by low-speed centrifugation (15 min at 3,000 g). The supernatant was used in the following step.

SECOND SUCROSE DENSITY-GRADIENT CENTRIFUGATION.—Gradients were prepared with solutions containing, respectively, 150, 200, 250, 300, and 350 mg of sucrose per ml. The sucrose solutions were made up with the same buffer used for the first gradients. Five ml of each sucrose solution was layered in the SW 25 tubes, in such order that the sucrose concentration decreased from the bottom of the tube to the top. Gradients were prepared 2 hr before use. One ml of the supernatant obtained in the preceding step was added on top of the gradients; the tubes were then centrifuged for 120 min at 25,000 rpm.

After centrifugation, the gradient showed 2 clearly separated zones.

The zones were recovered separately and dialyzed overnight against 0.01 M tris-acetate buffer, pH 7.0, containing 0.1 M NaCl. The particles in the respective dialyzates were sedimented by a last high-speed centrifugation (2 hr at 40,000 rpm), and resuspended in dialyzing buffer.

ULTRACENTRIFUGATION.—Sedimentation coefficients were determined with a Beckman analytical ultracentrifuge, equipped with UV-optics, and run at 20,410 rpm. Photographs were taken every 4 min.

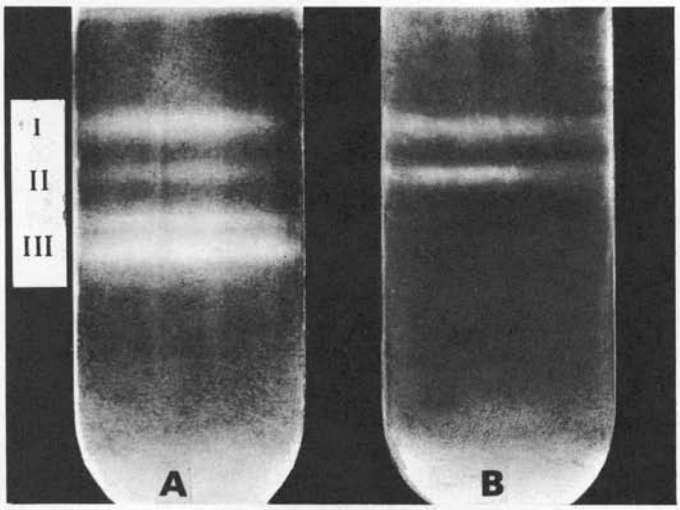

FIGURE 1. *Sucrose density-gradient centrifugation of preparation from: A. CCLV-infected lemon leaves, and B. Healthy lemon leaves.*

ELECTRON MICROSCOPY.—Collodion-carbon films were used on the grids of a Siemens 7 hole diaphragm. The virus preparations on the grids were fixed with 10 per cent glutar-dialdehyde, rinsed with water, and negatively stained with 1 per cent uranyl-acetate. They were examined with a Siemens electron microscope Elmiskop "1."

Results

DENSITY-GRADIENT CENTRIFUGATION.—With preparations from CCLV-infected lemon leaves, the first density-gradient centrifugation yielded 3 zones (Fig. 1,A), zones I, II, III, respectively located between 2.7 and 3.0 cm, 3.3 and 3.5 cm, and 3.9 and 4.4 cm from the top of the tube. With preparations from healthy leaves, only the 2 upper zones were obtained, zone I and zone II (Fig. 1,B). Zone III always revealed 2 subzones located close together. When zone III was centrifuged through the

second sucrose density-gradient the 2 sub-zones, IIIA and IIIB, became clearly separated. Sub-zone IIIA was located between 3.6 and 3.8 cm from the top of the tube, whereas sub-zone IIIB was located between 4.0 and 4.3 cm.

INFECTIVITY.—Zone III of the first sucrose-gradient centrifugation produced numerous chlorotic local lesions when rubbed on cotyledons of Blackeye cowpea No. 5 (Fig. 2,B); later, the trifoliate leaves of these plants produced systemic symptoms. No lesions or other symptoms were obtained with zone I (Fig. 2,A) or Zone II (Fig. 2,C).

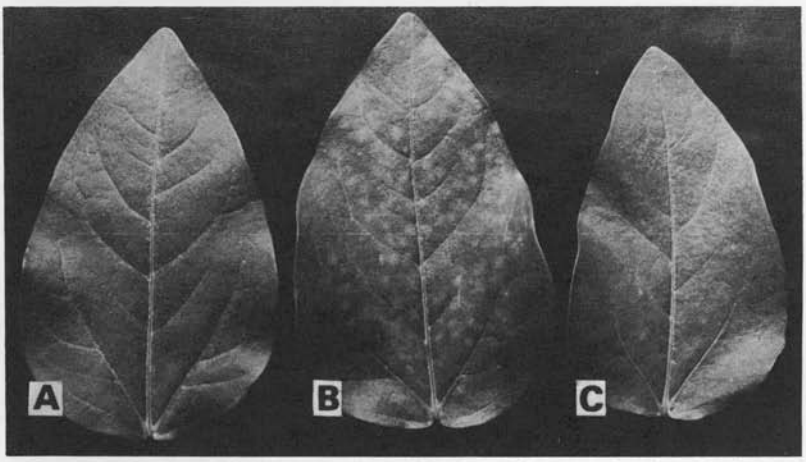

FIGURE 2. *Leaves* of Vigna sinensis *var. Blackeye No. 5 inoculated with: A. Zone I. B. Zone III, note chlorotic local lesions. C. Zone II.*

Young leaves of Lisbon and Eureka lemon seedlings were also mechanically inoculated with zone III. Vein-flecking symptoms, very similar to those of psorosis, often appeared first; later, typical symptoms of citrus crinkly-leaf virus were obtained (circular clear spots and crinkled leaves).

Each of the 2 sub-zones, IIIA and IIIB, obtained after the second density gradient centrifugation, was also infectious when inoculated into cowpeas or Eureka lemon seedlings.

ELECTRON MICROSCOPY.—After dialysis and concentration, the material in zone III was examined in the electron microscope and numerous pseudospherical particles were seen (Fig. 3,A). Their diameter ranged from 25 to 30 mμ. The preparation also contained some particles of much smaller diameter, and a few long, thin filaments (Fig. 3,B). The small particles and the filaments are contamination from zones I and II, as

shown by electron microscopy. Practically all the small particles and filaments could be removed by centrifuging zone III through the second sucrose gradient (Fig. 3,C). An electron micrograph of material from sub-zone IIIA is represented by Figure 3,D. Particles in sub-zone IIIB

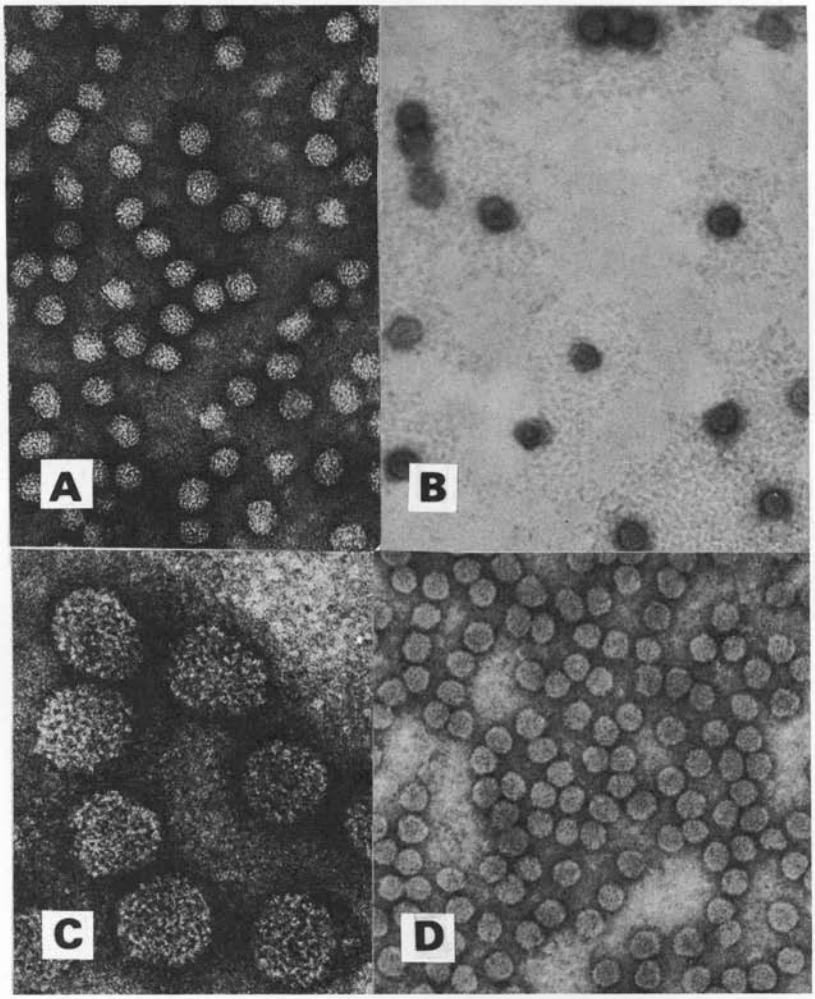

FIGURE 3. *Electron micrographs of components of zone III from CCLV-infected lemon leaves after sedimentation. A. After resuspension in water; magnification, 120,000. B. After resuspension in tris-buffer; magnification, 120,000. C. After a second sucrose density-gradient centrifugation and resuspension in water, magnification, 400,000. D. Components of zone IIIA resuspended in tris-buffer, magnification, 120,000.*

appear slightly larger in diameter than those in sub-zone IIIA. During the course of this investigation we found that the particles of zone III were more stable when resuspended in buffer instead of water. This instability may explain the distortion and breakage of some of the particles shown in the electron micrographs (Fig. 3,A,C).

ABSORPTION SPECTRUM.—The absorption spectrum of either zone IIIA or IIIB after the sucrose has been dialyzed out, is shown in Figure 4

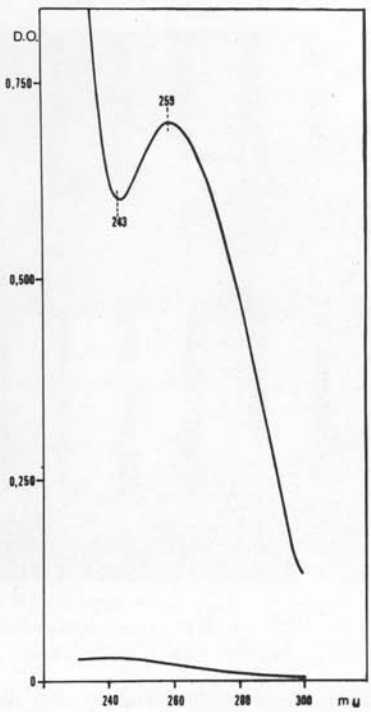

FIGURE 4. *Absorption spectrum of zone IIIA from CCLV-infected lemon leaves, D.O.: optical density, wavelength in mμ. Zone IIIB yields the same spectrum.*

and is typical of a nucleoprotein. The ratio OD_{Max}/OD_{Min} equals 1.2, and $OD_{280m\mu}/OD_{260m\mu}$ amounts to 0.65 (OD = optical density).

SEDIMENTATION COEFFICIENT.—Figure 5 illustrates the sedimentation patterns of the components of zone IIIA (Fig. 5,A) and of zone IIIB (Fig. 5,B) after dialysis and concentration. The sedimentation coefficient of the particles in zone IIIA is 95 S, whereas the particles in zone IIIB have a sedimentation coefficient of 108 S.

COMPARISON BETWEEN CCLV AND CIVV.—The methods described have been applied to preparations from young Lisbon and Eureka lemon leaves infected with CIVV. Sucrose density centrifugation of such preparations yielded exactly the same 3 zones, located at the same positions, as those obtained with CCLV-infected material. As with CCLV, zone III proved to be infectious and can be separated into 2 sub-zones.

Under electron microscope examination, the CIVV preparation also showed pseudospherical particles of 25-30 mμ in diameter. Moreover,

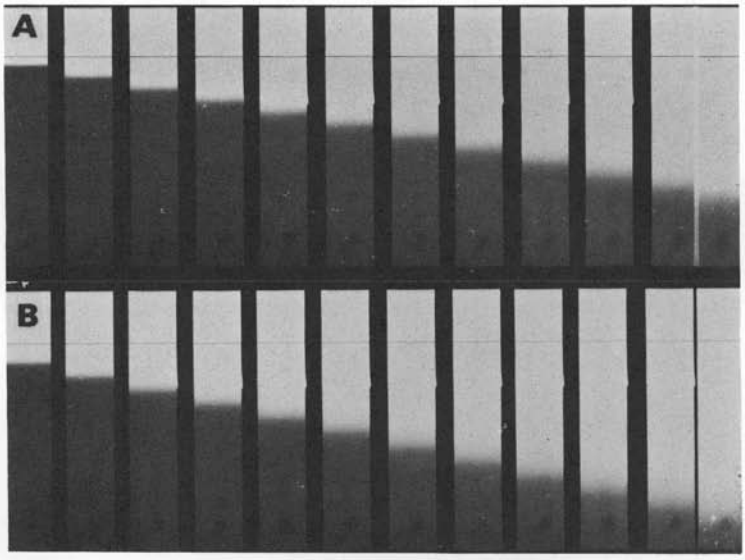

FIGURE 5. *Sedimentation pattern of particles from CCLV-infected lemon leaves. A. In zone IIIB. B. In zone IIIA. The particle suspensions subjected to analytical ultracentrifugation had optical densities of 0.77 at 260 mμ.*

the absorption spectrum of such a preparation was identical with that of CCLV.

PURIFICATION OF CCLV FROM *V. sinensis*.—Citrus crinkly-leaf virus from mechanically infected cowpea seedlings (*V. sinensis* var. Blackeye No. 5) was also purified. The results obtained were essentially the same as when the virus from infected lemon leaves was purified. Three zones, I, II, and III were obtained, and zone III was divided into sub-zones IIIA and IIIB; infectivity was associated with zone III only. When examined in the electron microscope after the second density-gradient centrifugation, the virus particles were identical to those of similar preparations from CCLV-infected lemon leaves.