

OTHER VIRUS DISEASES OF CITRUS

Association of Particle Size with Sedimentation Velocity of the Nucleoprotein Components of Citrus Variegation and Citrus Leaf Rugose Viruses*

Dennis Gonsalves and S. M. Garnsey

Citrus leaf rugose virus (CLRV) and citrus variegation virus (CVV) have been purified and shown to have a number of similar properties (Garnsey, 1974, 1975). One such property is the sedimentation heterogeneity of both the nucleoprotein and nucleic acid components (Rana *et al.*, 1974; Gonsalves and Garnsey, 1975b).

Until recently, centrifugal heterogeneity of spherical viruses in sucrose gradients was generally attributed to differences in particle density, and not size. Lister *et al.* (1972) showed, however, that the multicomponent tobacco streak virus (TSV) was composed of particles that grouped into three different size classes, all with the same density. Because particle

size corresponded positively to sedimentation rate, particle size heterogeneity was indicated as the source of centrifugal heterogeneity.

Garnsey (1975) reported that, although purified preparations of CLRV had a mean diameter of 28 nm, particles with diameters up to 3 nm from the mean were observed. This observation prompted a study of the particle size of the centrifugal components of CLRV and CVV.

Evidence is presented that populations of CLRV and CVV particles fall into distinct size classes which correlate to their centrifugal properties.

MATERIALS AND METHODS

Virus isolates and purification. The CLRV (ATCC PV 195) and CVV (ATCC PV 196) isolates used in this work have been described previously (Garnsey, 1974, 1975). Both viruses were increased in *Citrus excelsa* Wester, Eureka lemon, and Etrog citron and purified from young leaves as described previously (Garnsey, 1975). Preparations were suspended in a pH 7.2 buffer containing 0.005 M K_2HPO_4 and 0.002 M $MgCl_2$ (hereafter designated as PM buffer).

Centrifugation analysis of CLRV and CVV. Rate zonal and analytical centrifugation of CLRV and CVV preparations were done as described previously (Garnsey, 1975; Gonsalves and Garnsey, 1975b).

Separation of nucleoprotein components. The CLRV and CVV nucleoprotein components were purified by three successive cycles of sucrose density-gradient centrifugation. Initially, 5 OD 260 nm units of virus were layered on each of six linear gradients of 5 to 30 per cent (wt./vol.) sucrose dissolved in PM buffer. The tubes were centrifuged at 27,000 rpm for 4.5 hours at 6°C in a SW 27.1 rotor. The respective nucleoprotein component zones were detected with an ISCO UA 4 analyzer and collected with an ISCO 640 fractionator, pooled, diluted about threefold with PM buffer, then pelleted by centrifugation at 45,000 rpm for 2.5 hours, at 6°C in a Beckman 60 Ti rotor. The resuspended components were

*Florida Agricultural Experiment Station, Journal Series Paper No. 6059

further purified by a second centrifugation cycle. The fractionated components were dialyzed against PM buffer but not concentrated by high speed centrifugation. For the third centrifugation cycle, ca. 1 OD 260 nm unit of component (except CLRV-NP 4, for which ca. 0.5 unit was used) was layered on a 5 to 20 per cent sucrose (in PM buffer) gradient and centrifuged at 27,000 rpm for 3.5 hours at 6°C in an SW 27.1 rotor. The components were collected as before and used directly for electron microscopy or for infectivity assays.

Electron microscopy. Virus suspensions diluted to ca. 0.3 OD 260 nm units were mixed 1:1 with egg albumin (0.25 per cent) and placed on grids covered with carbon-coated membranes for several minutes and the excess suspension drawn off. A drop of distilled water was placed on the grid and drawn off to remove sucrose. The grid was flooded

with 0.5 per cent uranyl acetate pH 4.7. Excess stain was removed immediately. Grids were examined and photographed with a Philips 201 electron microscope. Diameter measurements of intact particles were taken by projecting the negatives against a white paper at a fixed distance. Two diameter measurements were then taken at right angles for each particle. About a hundred particles were measured. All intact particles in a projected area were measured. Comparisons of measurements were made from negatives taken in a single work session. Instrument magnification was calculated from photographs of a carbon replica of a diffraction grating (21,600 lines/cm) taken at same magnification.

Infectivity assays. Black Local cowpeas were used for infectivity assays of CVV. Inoculation methods have been described (Gonsalves and Garnsey, 1975b).

RESULTS

Rate zonal and analytical centrifugation. CLRV and CVV separated into four components when centrifuged in sucrose gradients with a narrow range of concentration. These were designated as NP 1 to 4 in order of decreasing sedimentation velocities (Gonsalves and Garnsey, 1975b). A major difference between the CLRV and CVV preparations was the very small amounts of NP 4 in CVV preparations (fig. 1). In fact, NP 4 of CVV was not detected by Schlieren optics in analytical centrifugation runs (fig. 2). Also, NP's 1 and 2 of CVV separated slightly more in sucrose gradients than those of CLRV (fig. 1). Throughout this and other work (Garnsey, 1974, 1975; Gonsalves and Garnsey, 1974, 1975a, 1975b, 1975c), the component ratios of CVV and CLRV have remained fairly constant and the amounts of CVV-NP 4 have always been very low. The sedimentation coefficients for CLRV-NP's 4, 3, 2, and 1 were calculated as 79.1s, 88.2s, 97.9s, and 104s, respectively, by analytical centrifugation. Those for CVV-NP's 3, 2, and 1 were 86.8s, 95.9s, and 106s, respectively. Coefficients for both viruses were determined simultaneously.

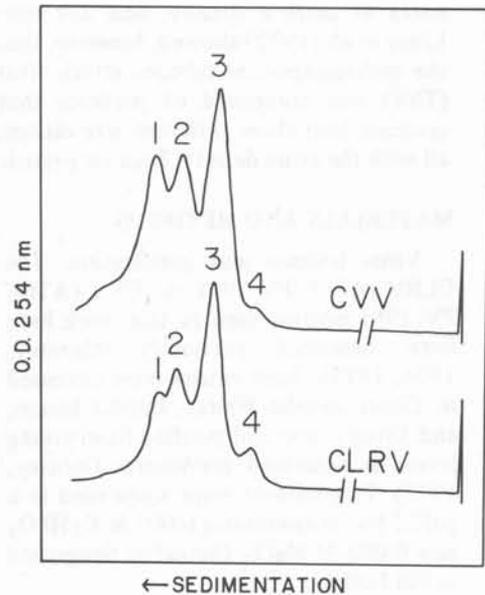


Fig. 1. Sedimentation patterns of CVV and CLRV in 20 to 50 per cent sucrose gradients visualized by UV absorption. Data from Gonsalves and Garnsey (1975b).

Electron microscopy. Histograms of particle diameter measurements from unfractionated CVV and CLRV preparations did not show well-defined size

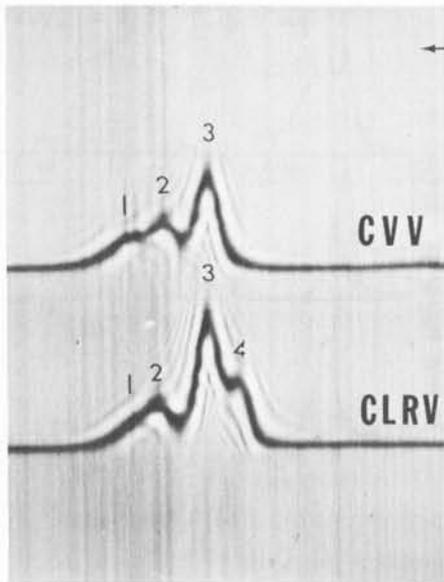


Fig. 2. Sedimentation patterns of CVV and CLRV in the analytical centrifuge visualized by Schlieren optics. Picture taken after 12 minutes at 35,000 rpm. Virus concentration about 20 OD 260 nm units in Tris buffer.

classes (figs. 3A, 4A). The mean for all measured particles was 29.8, and 28.3 nm for CVV and CLRV preparations. The particle diameters ranged from about 26 to 35 nm for CVV, and 24 to 35 nm for CLRV preparations.

When measurements were taken of particles from purified, individual components of CLRV and CVV however, the existence of well-defined classes was observed between the components. Purified component preparations showed little, if any, contamination when analyzed spectrophotometrically in sucrose gradients (figs. 3A, 4A). Histograms constructed from CLRV component particles measurements showed modal diameters of about 24.8 ± 1 , 26.3 ± 0.5 , 31.3 ± 0.5 and 32.2 ± 0.5 nm for NP's 4, 3, 2, and 1, respectively (fig. 3B). The mean diameters for all measured particles of CLRV NP's 4, 3, 2, and 1 were 24.5, 26.4, 31.2, and 32.2 nm, respectively. Similar histograms of CVV component particle measurements showed modal diameters of about 27.8 ± 0.5 , 30.7 ± 0.5 , and 32.6 ± 0.5 nm for NP's 3, 2, and 1, respectively

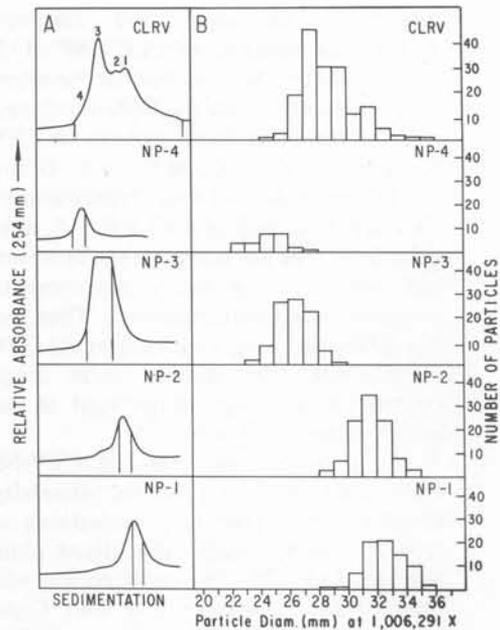


Fig. 3. (A) Migration of unfractionated CLRV and CLRV components purified by three centrifugation cycles in sucrose density gradients; and (B) histograms of diameter measurements of particles from unfractionated CLRV and purified CLRV components observed in (A). Material collected from within the hash marks (A) was used for particle measurements.

(fig. 4B). CVV-NP4 was in such small amounts that enough material could not be collected for analysis. Mean diameters of all measured particles of CVV-NP's 3, 2, and 1 were 27.6, 30.8, and 32.4 nm, respectively.

Most particles of CLRV- and CVV-NP's 3, 2, and 1 particles remained intact when negatively stained with uranyl acetate at pH 4.7 (figs. 5A, B). Observations indicated, however, that a large population of the observed CLRV-NP 4 particles was disrupted in uranyl acetate (fig. 5A). It was also noted that some particles were slightly oval shaped, probably due to distortion. Occasionally, bacilliform particles were observed.

Infectivity of CVV components. Previous infectivity studies with separated nucleoprotein components of CLRV indicated that NP 4 and NP 3 were not

infectious (Gonsalves and Garnsey, 1974). Combinations of CLRV NP's 1+2, and NP's 1+2+3 were infectious, however, with the latter having the highest infectivity. On the other hand, reports on CVV (Garnsey, 1974; Corbett and Grant, 1967) have indicated that preparations of CVV-NP 3, as well as CVV-NP 1+2, were infectious. The NP components, however, had not been rigorously separated to minimize cross-contamination. Thus, we did infectivity assays with separated CVV components that showed little cross-contamination when centrifuged in sucrose gradients (fig. 4A).

In an initial test, purified CVV-NP components were assayed for infectivity singly and in all possible combinations on separate plants. Singly inoculated components and NP's 2+3 combination had little, if any, infectivity (fig. 6A). Combinations of NP's 1+2, 1+3 and 1+2+3 showed significant infectivity, with the three-component combinations having about twice the infectivity of the two-component combinations (fig. 6A). It was noted however, that lesion numbers produced by a single inoculum varied from plant to plant, thus obscuring close comparison between the infectious preparations. Direct infectivity comparisons (using opposite leaves of single plants) of NP's 1+2+3 versus NP's 1+2, and versus NP's 1+3 showed that the three component combination was about four times

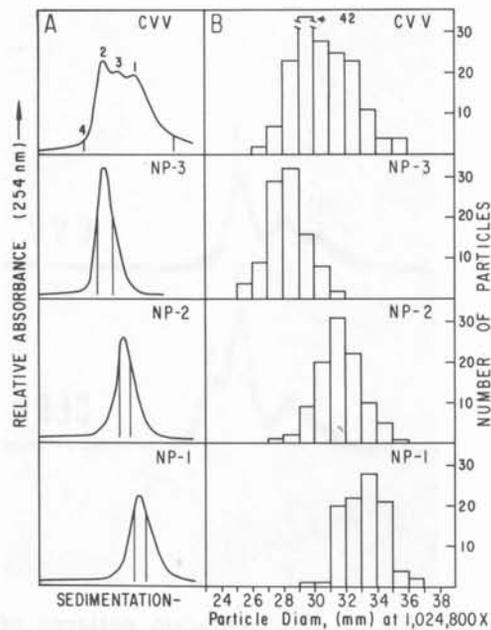


Fig. 4. (A) Migration of unfractionated CVV and of CVV components purified by three centrifugation cycles in sucrose density gradients; and (B) histograms of diameter measurements from unfractionated CLRV and of purified components observed in (A). Material collected from within the hash marks was used for particle measurements and infectivity assay.

more infectious than the two-component combinations.

DISCUSSION

Our data clearly show that particles comprising the centrifugal components of CLRV and CVV increase in size with increasing sedimentation velocities. These data, coupled with the observation that formalinized components of CLRV have similar densities (Lister, Gonsalves and Garnsey, unpublished), support the contention that centrifugal heterogeneity of CLRV and CVV is due to differences in particle size rather than differences in densities.

It is not too surprising that histograms of particle measurements from unfractionated virus preparations (figs. 3, 4) did not reveal the size classes associated with

the centrifugal components. Because the size differences between the means of adjacent components were no more than 15 per cent, these size classes would be obscured in a heterogeneous population. However, one would expect a broad distribution in particle diameters. This was, in fact, observed in the histograms from unfractionated virus preparations.

Actual particle sizes may not be exact, because magnifications were not exhaustively calibrated to an internal size standard. This, however, does not affect the relative size differences between particles, because all procedures were comparable.

Like CLRV (Gonsalves and Garnsey,

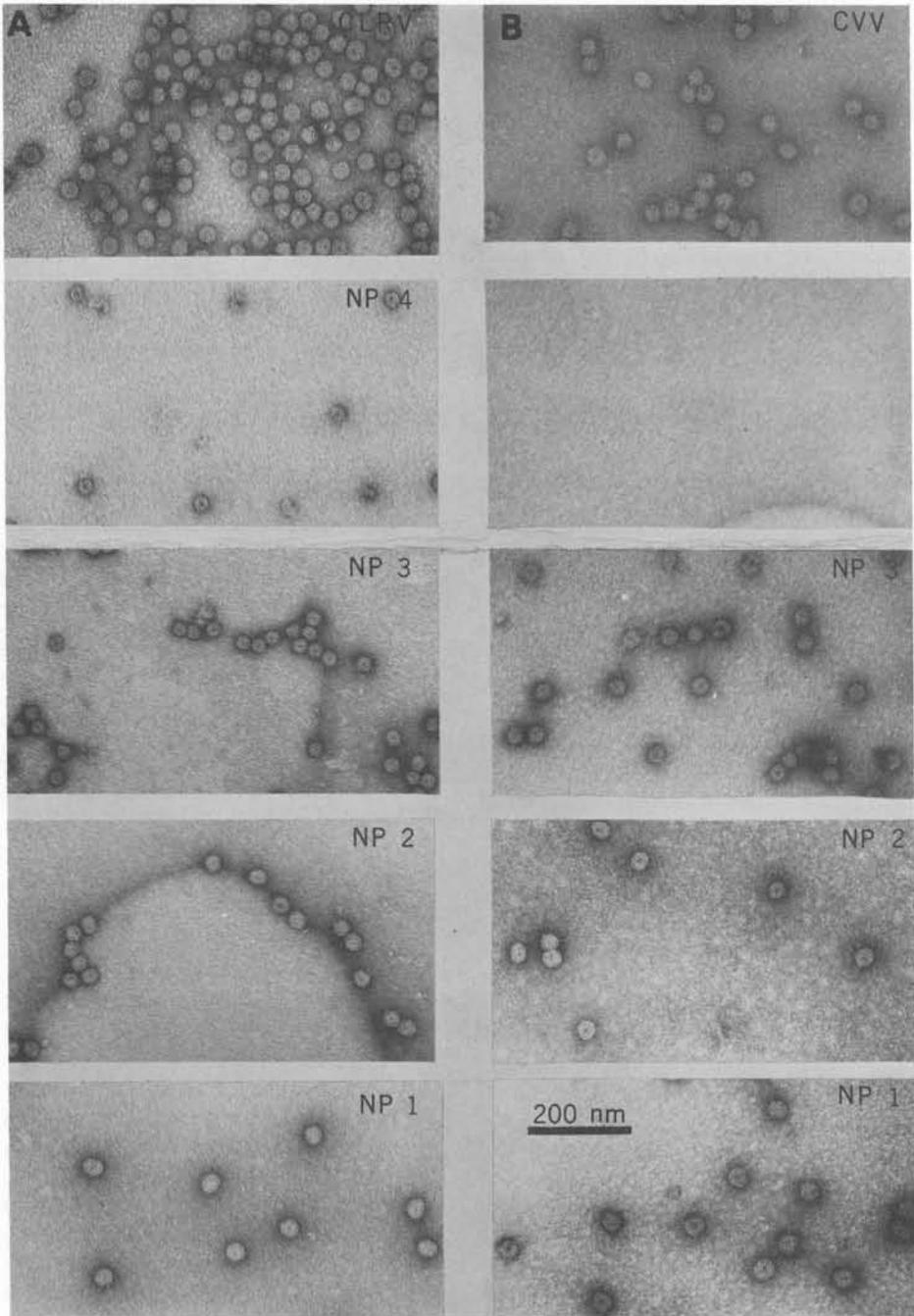


Fig. 5. Electron micrographs of purified components (NP's 1, 2, 3, and 4) of (A) CLRV and (B) CVV. CVV-NP 4 was not in sufficient amounts to be analyzed. The photograph in the NP-4 position for CVV shows background of a typical negatively strained grid area.

1974), CVV-NP's 1, 2, and 3 probably contain RNA's 1, 2, and 3, respectively (Gonsalves and Garnsey, 1975b). Unlike CLRV, however, CVV-NP 4 is barely detectable in purified preparations and would not account for the amount of RNA 4 and 4a present in nucleic acid preparations of CVV. Thus, RNA's 4 and 4a must be present in NP's 3, 2, or 1, more likely the latter two. RNA's 1 and 2 have molecular weights of about 1×10^6 daltons (d); RNA 3 equals ca. 0.7×10^6 d, and RNA's 4 and 4a equal about 0.3×10^6 d (Gonsalves and Garnsey, 1975b). Because a combination of either RNA's 3+4, or 3+4a would have a mass of ca. 1×10^6 d, such aggregates could be housed in particles that sediment in the NP 1 and/or 2 region.

We showed earlier (Gonsalves and Garnsey, 1975b) that nucleic acid preparations containing CVV-RNA's 1+2+3 were not infectious, but could be activated by adding either coat protein or a mixture of RNA 4 and 4a. Based on the precedent set for CLRV (Gonsalves and Garnsey, 1975a), we suggested that each of the three heaviest RNA's (1, 2, and 3) of CVV were required for infectivity. In this work, combinations of NP's 1+2+3 were highly infectious, but combinations of NP's 1+2, and NP's 1+3 were also infectious. Although cross-contamination of particles undoubtedly accounted for some of the infectivity of the two-component combinations, additional factors probably contributed to the infectivity, especially that of the NP's 1+2 combination. If one assumes that NP 1 is composed of particles containing not only RNA 1, but also RNA's 3+4 and 3+4a, the infectivity of NP's 1+2 become understandable, because they would have the necessary RNA's (1, 2, and 3) plus coat protein. The infectivity of NP's 1+3 was more likely due to traces of contamination with NP 2 particles. Indeed, the histogram of purified NP 1 showed that 19 per cent of the particles fell into the modal diameter of NP 2 particles. Other workers (Garnsey, 1974; Corbett and Grant, 1967) also reported that the NP's 1+2 zones were infectious. The infectivity obtained by these workers may also have

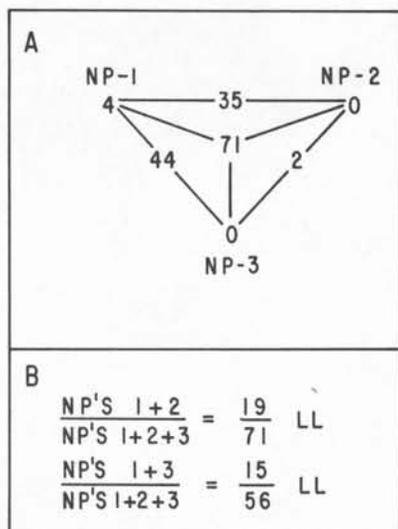


Fig. 6. Infectivity assay of purified CVV components (1, 2, and 3). (A) Infectivities of single components at vertices, of two components at sides, and of three components in middle of triangle. Average number of lesions on 10 leaves. (B) Infectivities of NP's 1+2+3 compared against NP's 1+2, and against NP's 1+3. Opposite leaf comparisons were made. Average number of lesions on eight leaves.

been due to the above stated reasons.

Lister *et al.* (1972) suggested that Tulare apple mosaic virus and the ILAR viruses (Fulton, 1968) would be candidates for a group of viruses, typified by TSV, in which centrifugal components arise from differences in size rather than in density. Recent evidence (Lister, Gonsalves and Garnsey, unpublished) indicates that CLRV is serologically related to Tulare apple mosaic virus. Furthermore, TSV protein can activate preparations of CVV- and CLRV-RNA's 1+2+3 (Gonsalves and Garnsey, 1975c). Other workers have also noted that CVV has properties similar to the ILAR viruses (Rana *et al.*, 1974), and that CLRV has centrifugal components similar to TSV (Garnsey, 1975). Data presented here further supports inclusion of CLRV and CVV in the ILAR group.

ACKNOWLEDGEMENTS

We are grateful to Mr. Rodney Whidden and Mrs. Carmen Henderson (USDA, Orlando), and Mr. Horace Lasater (University of Florida, Lake Alfred) for tech-

nical assistance, and to Dr. Ernest Hiebert (University of Florida, Gainesville) for making the analytical centrifugation runs.

LITERATURE CITED

- CORBETT, M.K., and T.J. GRANT
1967. Purification of citrus variegation virus. *Phytopathology* 57: 137-43.
- FULTON, R.W.
1968. Relationships among the ringspot viruses of *Prunus*. Sonderdruck aus Tagungsberichte no. 97, 123-38.
- GARNSEY, S.M.
1974. Purification and serology of a Florida isolate of citrus variegation virus, p. 169-75. In L.G. Weathers and M. Cohen, (eds.), Proc. 6th Conf. Intern. Organization Citrus Virol., Univ. California Div. Agr. Sci., Berkeley.
- GARNSEY, S.M.
1975. Purification and properties of citrus-leaf-rugose virus. *Phytopathology* 65: 50-57.
- GONSALVES, D., and S.M. GARNSEY
1974. Infectivity of the multiple nucleoprotein and RNA components of citrus leaf rugose virus. *Virology* 61: 343-53.
- GONSALVES, D., and S.M. GARNSEY
1975a. Functional equivalence of an RNA component and coat protein infectivity of citrus leaf rugose virus. *Virology* 64: 23-31.
- GONSALVES, D., and S.M. GARNSEY
1975b. Nucleic acid components of citrus variegation virus and their activation by coat protein. *Virology* 67: 311-18.
- GONSALVES, D., and S.M. GARNSEY
1975c. Infectivity of heterologous RNA-protein mixtures from alfalfa mosaic, citrus leaf rugose, citrus variegation, and tobacco streak viruses. *Virology* 67: 319-26.
- LISTER, R.M., S.A. GHABRIEL, and K.N. SAKSENA
1972. Evidence that particle size heterogeneity is the cause of centrifugal heterogeneity in tobacco streak virus. *Virology* 49: 290-99.
- RANA, G.L., A. QUACQUARELLI, and G.P. MARTELLI
1974. Some properties of citrus variegation virus, p. 165-68. In L.G. Weathers and M. Cohen, (eds.), Proc. 6th Conf. Intern. Organization Citrus Virol., Univ. California Div. Agr. Sci., Berkeley.