Investigations on the Purification of Citrus Variegation Virus

G. P. MARTELLI, G. MAJORANA, and M. RUSSO

PURIFICATION of sap-transmissible viruses is being recognized as an important aspect of the study of citrus viruses. To date, the causal agents of crinkly-leaf (1) and tatter-leaf (13) diseases have been investigated in some detail. Similar studies on citrus variegation virus (CVV) are scant, although this virus is mechanically transmissable, with equal facility, to herbaceous hosts (2, 3, 5, 6), where it may attain appreciable concentration (10, 11). Therefore, purification and comparison of CVV isolates from different geographic areas was undertaken. The results of these studies are reported here.

Methods and Results

VIRUS SOURCES.—In these trials, 2 isolates of CVV originating from sour orange stocks growing in different localities of southern Italy (Gorgono and Fonoli) and the original California isolate of CVV (supplied by J. M. Wallace) were used.

HOST PLANTS.—For the purpose of virus purification, the plants *Phaseolus vulgaris* L., *Vigna sinensis* (L.) Endl., and *Tithonia speciosa* Hook. were all used as virus multiplication hosts. However, French beans (*P. vulgaris*) were used more frequently because of their rapid growth and ability to support fair multiplication of virus (dilution end point of infectivity in crude sap of 10^{-3} to 10^{-4}).

PURIFICATION PROCEDURE.—In early trials, a modification of Steere's (14) chloroform-butanol method yielded infectious preparations of the virus that were contaminated by normal cell components (10). To obtain cleaner preparations, butanol, Mg-activated bentonite, calcium phosphate, acidification, and ethyl alcohol were used as a clarifying agents; freezing and thawing procedures were used for the same purpose. Various buffers (phosphate, citrate, borate) were used as homogenizing media at different molarities.

Several purification schemes caused almost complete precipitation of green material. However, the end result was still unsatisfactory because the final virus preparations were either non-infectious or were contaminated with varying amounts of unwanted substances (e.g., fraction 1 protein). The contaminants were usually detected spectrophotometrically and/or serologically. Thus far, a procedure similar to that devised by

Scott (12) for purification of cucumber mosaic virus has given the best results.

Varying amounts (usually 250-300 g) of frozen infected tissues were ground in a blendor at room temperature in the presence of cold citrate buffer (0.5 M, pH 6.5) and chloroform in the relative proportions of 1:1:1 (w:v:v). A reducing agent, 0.1 to 1.0 g of sodium salt of ascorbic or thioglycolic acid per 100 g of material, was added to the mixture before homogenizing. To avoid an increase of temperature during grinding, this operation was performed in the shortest possible time, and the emulsified homogenate was immediately transferred to an ice bath.

The emulsion was broken by centrifuging in a refrigerated "Superspeed 40" MSE ultracentrifuge for 10-15 min at 13,000 g. The clear supernatant was then subjected to 2 or 3 alternate cycles of high- and low-speed centrifugation. Low-speed runs were usually made at 10,000-15,000 g, and high-speed centrifugations were made mostly in a Spinco "model L" ultracentrifuge for 2 hr at 78,000 g or for 50 min at 150,000 g. The pellets resulting from the first high-speed run occasionally contained traces of green pigments, but those from subsequent centrifugations were usually clear. The pellets were always resuspended in a small volume (2-3 ml) of 0.02 M phosphate buffer of pH 7.2.

Further separation of the virus from normal plant constituents was attempted on sucrose density gradient columns. These were prepared by layering 7 ml each of 10, 20, and 30 per cent and 4 ml of 40 per cent sucrose solutions in 0.02 M phosphate buffer in 1 x 3 in. cellulose centrifuge tubes. Aliquots not exceeding 1 ml of concentrated virus suspension were layered on top of the sucrose columns which were then centrifuged 2.5 hr in a SW 25.1 Spinco rotor at 24,000 rpm. After centrifugation, 2 well-defined, very close, opalescent bands were consistently observed at depths of 21 and 24 mm from the meniscus of the tube. The distance from the meniscus and between the bands varied slightly in successive experiments.

Similar bands, in the same position, were obtained with all CVV-infected tissues processed. None were obtained with CVV-free tissues. Usually very little or no opalescence was observed either above or below the bands. The opalescent layers were recovered by puncturing the side of the tube, but due to their closeness, it was practically impossible to avoid a certain amount of mixing during extraction.

After removal from the density gradient tubes, the bands were diluted 1:3 with phosphate buffer or dialized overnight against the same buffered solution and then concentrated by centrifugation.

INFECTIVITY.—Plants of *P. vulgaris* and *V. sinensis*, mechanically inoculated with material from the light-scattering bands, always developed typical CVV symptoms. This suggests that both bands contain infectious material. However, the dilution end point of these preparations was surprisingly low (usually between 10^{-3} and 10^{-4}) and seldom exceeded the values of infectivity found in crude non-concentrated sap. Purified preparations, mixed 1:1 in glycerin and kept in a freezer, retained their infectivity for at least 2 months.

HOMOGENEITY TESTS.—In preliminary experiments significant differences in the composition of density gradient bands were not detected. Therefore, tests for evaluating the homogeneity of virus preparations were performed on the material resulting from both bands collected together.

SPECTROPHOTOMETRY TESTS.—Ultraviolet light absorption spectra of the density gradient bands determined with a Beckman DB spectrophotometer invariably indicated that they are composed mainly of nucleoproteins (E max = 260 mμ and E min = 243 mμ). The ratio of optical density at 280 mμ to optical density at 260 mμ varied from 0.72 to 0.77. This value would correspond to an approximate nucleic acid content of 8-9 per cent (9).

ELECTRON MICROSCOPE EXAMINATIONS.—Virus preparations, shadowed with gold-palladium or negatively stained in phosphotungstic acid (PTA), were observed under a Hitachi HU-11B electron microscope. A large number of spherical particles with poorly resolved outline and shape were seen on metal-shadowed grids (Fig. 1,A).

The negatively stained preparations also contained isometric particles (Fig. 1,B,C). Of these, a few that appeared satisfactorily preserved exhibited an hexagonal contour. The majority of the particles were in various stages of disruption, and a large amount of amorphous material believed to originate from degradation of the particles was scattered on the grids. Fixing virus preparations with 2 per cent neutral formaldehyde prior to mounting in PTA did not decrease significantly either the extent or severity of disruption. Particles believed intact were approximately 26 mμ in diameter. This figure, however, is only tentative since the lack of uniformity in the particle size, due to degradation, made measurements exceedingly difficult.

The virus preparations appeared essentially homogenous, and extraneous particles reminiscent of protein fraction 1 were rarely detected. This was true of both California and Italian isolates of CVV.

SEROLOGICAL TESTS.—Outcherlony's agar double diffusion test was

used to detect "healthy" antigens in final virus preparations. Material from the density gradient bands was allowed to react against serum prepared from rabbits immunized to healthy bean plants. However, no visible precipitin lines were observed.

Purified preparations of an Italian isolate of CVV were administered to

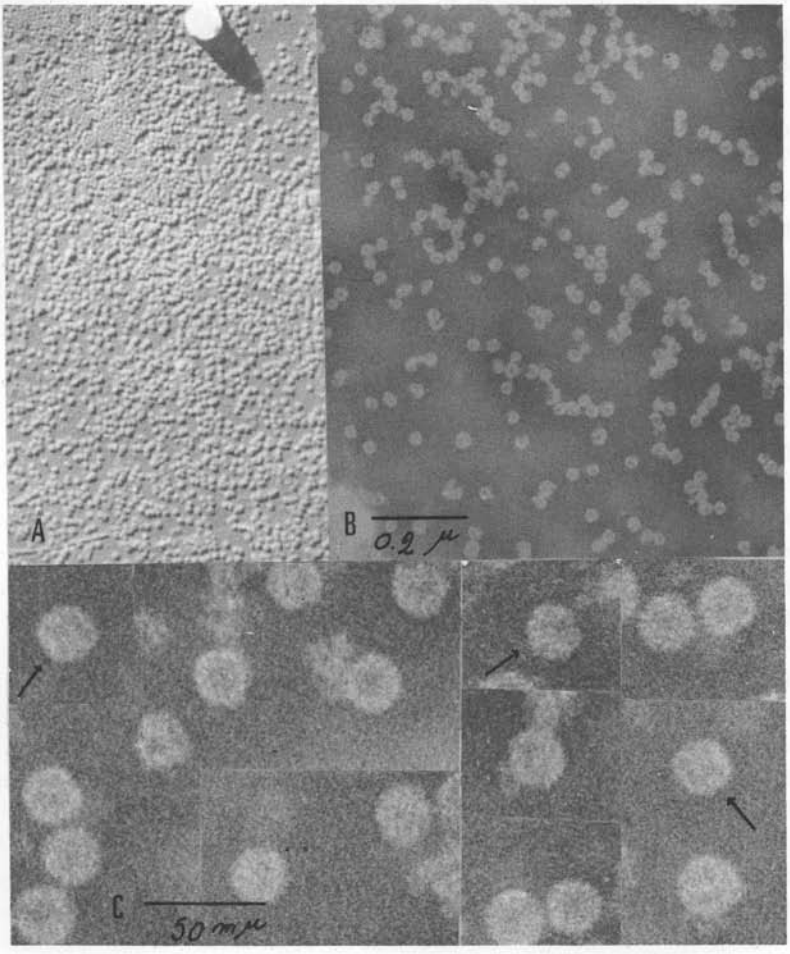

FIGURE 1. *A. Electron micrograph of purified CVV preparation shadowed with gold-palladium. Polystyrene-latex 357 mμ in diameter. B. Same as in A., but negatively stained with phosphotungstic acid. Note the homogeneity of the preparation and the great number of particles in various stages of disruption. C. Selected particles from a field similiar to B. Particles are better preserved and show some indication of hexagonal contour (arrows).*

a rabbit in a series of subcutaneous, intramuscular, and intravenous injections (15). However, this first attempt to prepare a serum against CVV yielded unsatisfactory results. The anti-CVV serum had a titer to the virus not exceeding 1:16 and also reacted strongly with sap of healthy herbaceous host plants.

The similar behavior during purification of CVV isolates from California and Italy indicates that they are not essentially different irrespective of their different geographic origin.

The electron miscroscope studies suggest that the purification procedure used increases considerably the ratio of virus particles to host components. Although such results are encouraging, they are not entirely satisfactory because final preparations have low infectivity, low nucleic acid content, and contain many disrupted particles as well as some normal plant constituents.

Certain aspects of these results merit further comment. For example, the infective light-scattering bands of density gradient columns are always well defined and relatively wide. However, in our experience with other plant viruses, bands smaller than those obtained with CVV often possess higher infective power. Hence, the low infectivity of the CVV preparations may be explained in one of two ways: the virus becomes largely inactivated during extraction and concentration, or massive quantities of non-virus material are present in the density gradient bands.

Serological and electron microscope evidence do not support this latter possibility. In fact, the bands do not react to any visible extent with anti-healthy serum. Furthermore, they are composed almost entirely of spherical bodies that retain the general appearance and size of virus particles even though degraded somewhat.

It is known that PTA can disrupt some viruses (7). However, its negative action may be counterbalanced in some cases by fixing virus preparations prior to negative staining (4). In these studies, formaldehyde-treated CVV suspensions contained an appreciable number of broken particles. This is not conclusive evidence that PTA does not degrade CVV, and it suggests that the virus particles were damaged to a certain extent before exposure to PTA.

If many virus particles are degraded during purification, their nucleic acid is likely to be inactivated or to come out of the protein coat and thus be left behind during centrifugation. Such degraded virus preparations should therefore contain materials with different sedimentation rates and form more than one band in density gradient columns. Also,

the nucleic acid content would be lower than that expected in preparations of a spherical virus (8). Because of its behavior during purification, CVV seems rather fragile. This idea is in agreement with previous studies (6, 11) which suggest that the virus is unstable.

The ability of CVV antisera to react with healthy antigens was unexpected and disturbing because the serological and electron microscope checks indicated negligible contamination of the virus suspensions. The more critical and gentle purification procedures now under trial, may reduce certain shortcomings of the present method.

ACKNOWLEDGMENTS.—This work was supported by the Consiglio Nazionale delle Ricerche, Roma, under the program of the "Gruppo di lavoro del C.N.R. per le virosi—Sottogruppo epidemiologia e fisiopatologia nei rapporti con i virus." The authors are indebted to Dr. J. M. Wallace, Department of Plant Pathology, University of California, Riverside, for kindly supplying the California isolate of CVV, and to Mr. N. Greco for his valuable technical assistance.

Literature Cited

1. DAUTHY, D., and BOVÉ, J. M. 1964. Isolement du virus de la frisolée des citrus (crinkly leaf virus). I. Note preliminaire. Fruits 19: 187-193.
2. DESJARDINS, P. R., and WALLACE, J. M. 1962. Cucumber, an additional herbaceous host of the infectious variegation strains of citrus psorosis virus. Plant Disease Reptr. 46: 414-416.
3. DESJARDINS, P. R., and WALLACE, J. M. 1966. Host reactions of citrus infectious variegation virus indicating wide diversity of virus strains. Phytopathology 56: 347-348.
4. GIBBS, A. J., NIXON, H. L., and WOODS, R. D. 1963. Properties of purified preparations of Lucerne mosaic virus. Virology 19: 441-449.
5. GRANT, T. J., and CORBETT, M. K. 1960. Mechanical transmission of the infectious variegation virus of citrus. Nature (London) 188: 519-520.
6. GRANT, T. J., and CORBETT, M. K. 1961. Mechanical transmission of infectious variegation virus in citrus and non-citrus hosts, p. 197-204. *In* W. C. Price [ed.], Proc. 2nd Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
7. HORNE, R. W., and WILDY, P. 1963. Virus structure revealed by negative staining. Adv. Virus Res. 10: 101-170.
8. KNIGHT, C. A. 1964. Structural biochemistry of plant viruses, p. 292-314. *In* M. K. Corbett and H. D. Sisler [eds.], Plant Virology. Univ. Florida Press, Gainesville.
9. LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 447-454. *In* S. P. Colowick and N. O. Kapland [eds.], Methods in Enzymology. Vol. III. Academic Press, New York.
10. MAJORANA, G., and MARTELLI, G. P. 1965. Il Faglio quale ospite erbaceo del virus della "Variegatura infettiva" degli Agrumi. Phytopath. Medit. 4: 170-172.
11. MAJORANA, G., and MARTELLI, G. P. 1968. A comparison of citrus variegation and citrus crinkly-leaf (CCLV) virus isolates from Italy and California, p.

273. *In* J. F. L. Childs [ed.], Proc. 4th Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
12. Scott, H. 1963. Purification of cucumber mosaic virus. Virology 20: 103-106.
13. Semancik, J. S., and Weathers, L. 1965. Partial purification of a mechanically transmissible virus associated with tatter leaf of citrus. Phytopathology 55: 1354-1358.
14. Steere, R. L. 1956. Purification and properties of tobacco ringspot virus. Phytopathology 46: 60-69.
15. Wetter, C. 1965. Serology in virus disease diagnosis. Ann. Rev. Phytopat. 3: 19-42.