Purification and Properties of Citrus-Leaf-Rugose Virus

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ABSTRACT

The name "citrus-leaf-rugose virus" (CLRV) is proposed for a mechanically transmitted citrus virus which was discovered in Florida and provisionally described earlier as "crinkly-leaf-type virus." CLRV was transmitted to numerous citrus and herbaceous hosts. CLRV isolates differed in their stunting effects on grapefruit (*Citrus paradisi* 'Duncan') seedlings, and strain interference between isolates was shown. CLRV symptoms differed from those caused by citrus variegation virus (CVV) in citrus and herbaceous hosts. Cross-protection between CLRV and CVV was shown in citron (*C. medica* 'Etrog'). CLRV was readily purified from young citrus-leaf tissue by use of calcium phosphate gel clarification and differential and density-gradient centrifugation. Three to 5 mg of purified virus were obtained from 100 g of citrus-leaf tissue. CLRV particles were isometric and averaged 28 nm in diameter. Purified virus had an absorption minimum at 244 nm, a maximum at 260 nm, and a 260/280 ratio of 1.4 to 1.45. The thermal inactivation point was near 60 C. Longevity in vitro was 1 to 2 days at room temperature in crude extracts. Purified CLRV was stable for several months at 4 C. Antisera were prepared to purified CLRV, which reacted to purified virus and to extracts from CLRV-infected citrus and herbaceous hosts. CLRV antiserum reacted heterologously to CVV, but not to tobacco streak or cowpea mosaic viruses.

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A newly discovered citrus virus was reported in Florida several years ago (7). Leaf-flecking symptoms produced by this mechanically transmissible virus in lemon [*Citrus limon* (L.) Burm. f. 'Eureka'] were similar to those described for citrus crinkly leaf virus (CCLV) elsewhere (15), and the virus was provisionally designated as a crinkly-leaf-type virus (CLTV). This name has been used in several publications (4, 9, 10).

CLTV was recovered from orange (*C. sinensis* (L.) Osbeck), grapefruit (*C. paradisi* Macf.), tangelo (*C. paradisi* × *C. reticulata* Blanco), and mandarin (*C. reticulata*) trees in the field. It was transmitted to other citrus plants, including 'Etrog' citron, *C. excelsa* Wester, lime (*C. aurantifolia* (Christm.) Swingle 'Mexican'), and Eureka lemon. Noteworthy symptoms, other than the leaf-flecking in Eureka lemon, were rugose or pucker symptoms in Mexican lime and severe stunting in grapefruit seedlings (7). CLTV was transmitted also to some herbaceous plants, including *Petunia axillaris* (Lam.) BSP, tobacco (*Nicotiana tabacum* L. 'Turkish'), bean (*Phaseolus vulgaris* L.), and cowpea [*Vigna unguiculata* (L.) Walp.]. Differences in symptoms produced by CCLV and CLTV were noted (7). Subsequently, CLTV was shown to react heterologously with antiserum to citrus variegation virus (CVV) (8).

The differences in biological and serological properties between CLTV and CVV or CCLV reported previously, and in this paper, suggest that a clear distinction should be made between these viruses. The name citrus leaf rugose virus (CLRV) is proposed for CLTV and is used here.

This paper presents further information on the citrus and herbaceous host range of CLRV, the existence of strains and strain interference, the purification of the virus in quantity directly from citrus tissue, some properties of the virus, and preparation of a specific antiserum. Evidence is presented also for cross-protection between CLRV and CVV, and the heterologous serological relationship between these viruses is confirmed.

MATERIALS AND METHODS.—*Plants.*—All plants were grown in steam-sterilized potting soil and kept in an air-cooled, partially shaded greenhouse. Air temperatures ranged from 20 to 29 C, depending on the season, and light intensity at midday ranged from 9,000 to 20,000 lux. When greenhouse temperatures exceeded 27 C, plants inoculated for assay or increase purposes were held in an air-conditioned chamber (24 ± 2 C) constructed of clear Mylar. Supplemental light was supplied in winter by Gro-Lux wide-spectrum lights to give a 16-hour photoperiod (1.6W/m^2 bench area).

Eureka lemon, Etrog citron, and *C. excelsa* plants were propagated as cuttings from virus-free seedlings. All other plants were grown from seed.

Virus sources.—The source of CLRV (ATCC No. PV 195) used in most tests was obtained originally from a Robertson navel orange tree infected with several citrus viruses (7). It had been transmitted serially by mechanical inoculation from orange to Eureka lemon to Turkish tobacco to Mexican lime and back to Eureka lemon. The last plant was free of other detectable citrus viruses and became the donor source of CLRV.

Two other isolates of CLRV were used: CLRV-3, a mechanically transmitted isolate from a seedling Orlando

tangelo tree, and CLRV-4, a mechanically transmitted isolate from a Changsha mandarin tree. The isolate of citrus variegation virus (F-CVV) used was our Florida isolate, ATCC No. PV196.

Transmission.—Graft inoculations were made by leaf-piece (10) or chip-bud techniques. Mechanical inoculations were made by conventional leaf-inoculation procedures. Inocula were prepared in cold, 0.05 M neutral potassium phosphate buffer, and applied with sterile cotton swabs to leaves dusted with 500-mesh Carborundum.

Virus properties in tissue extracts.—Some properties of CLRV were measured in extracts of infected tissue made with neutral, 0.05 M potassium phosphate. Red Kidney bean was used as the assay host. Tissue extracts used in thermal inactivation studies were filtered through glass wool and a 1.2 μm Millipore filter and were loaded into thin-walled, glass capillary tubes. The tubes were fire-sealed at one end and heated in a water bath for 10 minutes at the indicated temperature. Controls were virus extracts prepared the same way and stored on ice until assayed.

Purification.—CLRV was purified by differential and density-gradient centrifugation after clarification with hydrated calcium phosphate gel (HCP) (5, 8). Leaf tissue was added (1g:3ml, w/v) to a solution of 0.01 M sodium diethyldithiocarbamate, 0.02 M sodium thioglycolate, and 0.02 M potassium phosphate, pH 7.4. A small amount of Dow Antifoam A was added, and the ingredients were homogenized with a Sorvall Omnimixer (100 volts for 60 seconds). The homogenate was expressed through cheesecloth, and the residue re-extracted with a small volume of buffer. All solutions were kept below 10 C.

The filtrates were centrifuged at 2,200 *g* for 12 minutes. The supernatant was thoroughly mixed with calcium phosphate gel [prepared according to Fulton (5), and centrifuged at 2,700 *g* to a paste-like consistency]. The gel was added at a rate of 6 ml per 10 g of tissue. The gel-extract mixture was centrifuged for 15 minutes at 2,200 *g*; the supernatant was removed and centrifuged for 90 minutes at 78,000 *g*. The high-speed pellets were resuspended with a buffer (RB) of 0.005 M potassium phosphate and 0.005 M $MgCl_2$ (pH 7.2). The resuspended pellets were given another cycle of differential centrifugation (10 minutes at 6,000 *g* + 60 minutes at 150,000 *g*). The resuspended final high-speed pellet was subjected to rate-zonal, density-gradient centrifugation for 3 or 3.5 hours at 25,000 RPM in a Spinco SW25.1 rotor.

Linear sucrose-gradient tubes were prepared with a Beckman gradient former. Sucrose concentration was normally 110 to 390 mg/ml in neutral, 0.02 M potassium phosphate buffer.

After centrifugation, gradient tubes were observed with a top light for light-scattering zones. They were then scanned at 254 nm and fractionated with an ISCO density-gradient fractionator.

Virus zones from the gradient tubes were dialyzed overnight against neutral, 0.02 M potassium phosphate buffer and concentrated by centrifugation.

The same procedure was used to obtain purified preparations of CVV and to process healthy tissue for identification of host components.

Sedimentation coefficients.—Sedimentation coefficients were estimated by the method of Brakke (1) and by analysis in a Spinco Model E analytical centrifuge with Schlieren optics. The analytical centrifuge runs were made at 35,600 RPM and 20 C in an An-D rotor. Exposure interval was 4 minutes.

Ultraviolet absorption.—Purified virus preparations were filtered through a 0.45 μm filter before ultraviolet (UV) absorption was determined. Absorption at 320 nm was nominal and no correction was made for light scattering.

The extinction coefficient was calculated with a purified preparation of CLRV dialyzed against H_2O, and given two additional cycles of high-speed centrifugation to remove traces of sucrose. The final pellet was resuspended in glass-distilled water and filtered. Dry weight was measured after samples had been dried for 72 hours at 102 C. Further drying for 24 hours at 114 C did not change the dry weight.

Electron microscopy.—Purified virus was mixed 1:1 with a solution of 2% potassium phosphotungstate and 0.25% bovine serum albumin (pH 6.7) or with a 1.0% solution of unbuffered uranyl acetate. The stained virus preparation was placed on grids covered with carbon-coated formvar membranes and examined in a Phillips Model 200 electron microscope. Magnification was determined from a diffraction grating (21,600 lines/cm) photographed at the same instrument magnification.

Serology.—Rabbits were immunized by intramuscular and intravenous injections of purified virus. Virus preparations injected intramuscularly were emulsified 1:1 with Freund's incomplete adjuvant. Rabbits were bled from the ear, and the serum fraction was preserved by addition of sodium azide (0.02%) or glycerol (50%).

Agar gel double-diffusion tests were conducted in 100 × 15 mm plastic petri dishes loaded with 12 ml of agar solution. The solution contained 0.75% Ionagar No. 2, or 0.75% Epiagar (Colab Laboratories, Inc., Glenwood, Illinois), and 0.02% sodium azide. Wells in the agar were cut with an Auto-Gel® punch (Grafar Corp., Detroit, Michigan).

Plant extracts for gel diffusion tests were obtained by expressing sap from leaf tissue with a hand press, or by grinding tissue in a buffer containing 0.02 M sodium phosphate and 0.02 M sodium sulfite, pH 8.0.

RESULTS.—*Citrus hosts.*—Besides citrus hosts previously described, CLRV was readily graft-transmitted to Etrog citron, citrange (*C. sinensis* × *P. trifoliata* (L.) Raf. 'Morton'), lime (*C. reticulata* var. *austera* Swingle 'Rangpur'), rough lemon (*C. jambhiri* Lush.), citrange cultivar 'Rusk', sour orange (*C. aurantium* L.), *C. reticulata* hybrid 'Temple', trifoliate orange (*P. trifoliata*), and Valencia orange.

Most of these plants were symptomless as young plants under glasshouse conditions. The leaf-flecking in Eureka lemon, the rugose symptoms in Mexican lime (Fig. 1-D), and the severe stunting in grapefruit reported earlier (7) were verified again in this study. Two field isolates (CLRV-3 and CLRV-4) did not cause marked stunting in Duncan grapefruit seedlings, but did cause typical symptoms in Mexican lime, Eureka lemon, and herbaceous hosts.

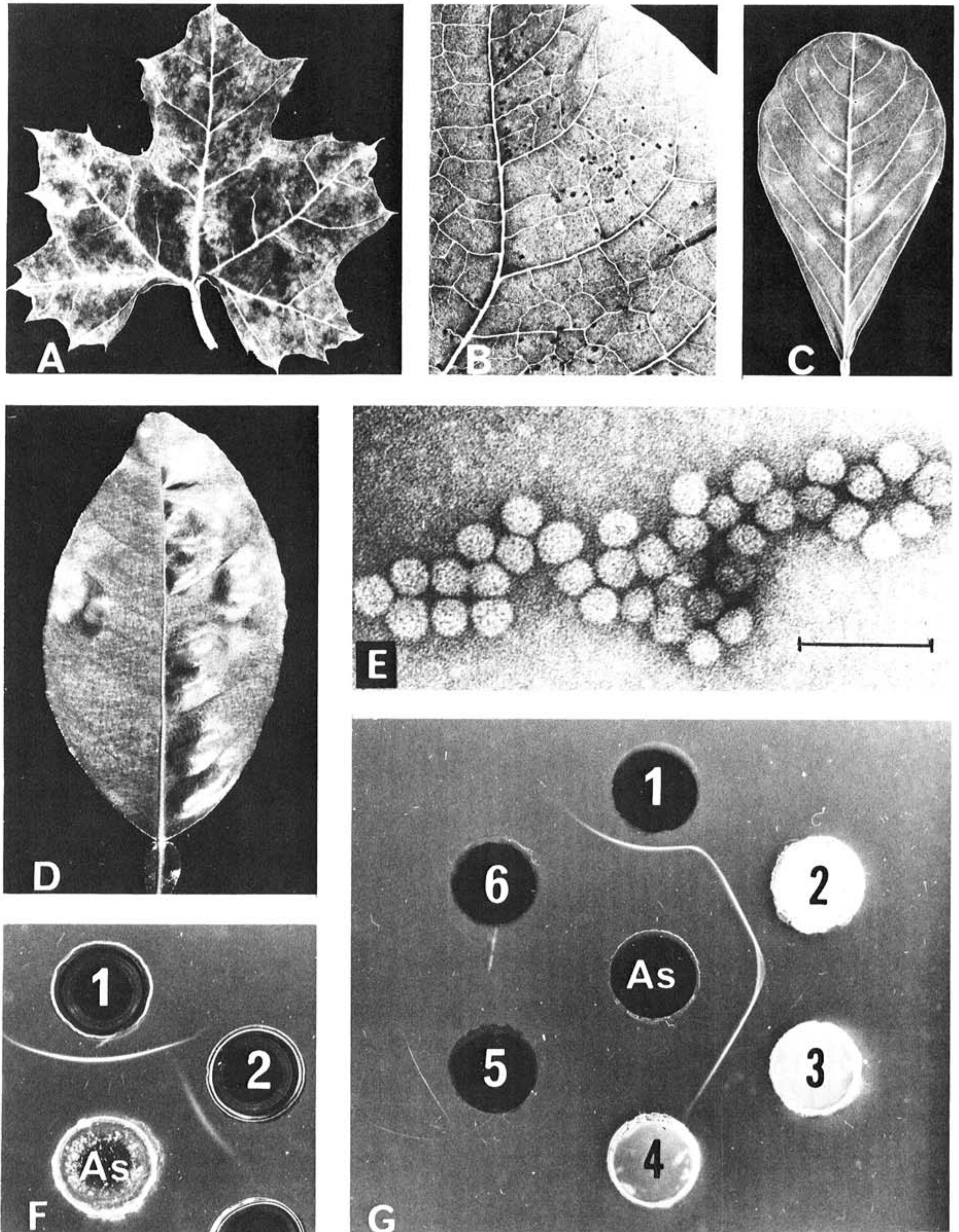

Fig. 1-(A to G). Symptoms, particle morphology, and serological reactions of citrus-leaf-rugose virus (CLRV). **A)** Mosaic symptom in *Momordica balsamina;* **B)** small (1 mm), necrotic local lesions on inoculated primary leaf of Red Kidney bean; **C)** local lesions on inoculated leaf of *Crotalaria spectabilis;* **D)** rugose symptom on leaf of Mexican lime; **E)** CLRV particles stained in uranyl acetate (scale bar = 100 mm); and **F)** reaction of CLRV antiserum (As) to purified CLRV (Well 1) and to purified citrus variegation virus (Well 2). Although the CLRV antiserum had not shown healthy reaction (See G-4), it was absorbed (intragel) with healthy *Citrus excelsa* extract. **G)** Reaction of CLRV antiserum (As) to different antigens. Well 1 contained CLRV purified from *C. excelsa;* well 2 - purified CLRV plus extract from healthy *C. excelsa* in buffer; well 3 - extract from CLRV-infected *C. excelsa* in buffer; well 4 - healthy *C. excelsa* extract alone; and wells 5 and 6 - two buffers used for virus preparation and for plant extracts. Well spacing, 7mm; picture taken at 6 days.

In contrast to CCLV or CVV, there was little or no deformation of the leaves of Eureka lemon plants infected with CLRV. A chlorotic mottle was observed infrequently in scattered leaves of sweet orange, Etrog citron, and *C. excelsa*.

CLRV was readily transmitted mechanically to Eureka lemon, Mexican lime, *C. excelsa*, Etrog citron, Duncan grapefruit, and sweet orange from citrus or noncitrus donor plants. Virus titer in succulent, young citrus tissue was quite high (dilution end point of 10^{-4}), but decreased rapidly as leaves matured under warm conditions.

Noncitrus hosts of CLRV.—The virus was readily transmitted mechanically from various citrus plants to noncitrus hosts. Small, necrotic local lesions, usually 1 mm or less in diameter, were formed on the inoculated leaves of *Crotalaria spectabilis* Roth., *Phaseolus vulgaris* L. 'Red Kidney' (Fig. 1-B), 'Bountiful', 'Tennessee Greenpod', 'White Halfrunner', and 'Richgreen'; and *Vigna unguiculata* 'Early Ramshorn', 'Ladyfinger Round', and 'Black Local'. Lesions on leaves of *C. spectabilis* were surrounded by a chlorotic halo (Fig. 1-C). Under our conditions, Red Kidney bean was the best local-lesion host tested. Lesion size and definition were best when plants were grown before inoculation under light that caused slight etiolation, and when the primary leaves were inoculated just before full expansion. Lesions, sometimes visible 48 hours after inoculation, were well-defined in 4 to 5 days. Fewer and smaller local lesions were produced on plants grown under more intense light or inoculated after the primary leaves had fully expanded. No systemic symptoms were observed on these local-lesion hosts, and sap inoculations from noninoculated leaves to Red Kidney bean were negative.

A systemic mottle was observed in leaves of *Chenopodium quinoa* Willd.; *Cucumis sativus* L. 'MR-17' and 'National Pickling'; and *Momordica balsamina* L. (Fig. 1-A). However, these symptoms were either erratic or poorly defined. CLRV was recovered from systemically infected tissue on assay to Red Kidney bean.

The following were all symptomless hosts of CLRV under our conditions: *Gomphrena globosa* L.; *Nicotiana clevelandii* Gray × *N. glutinosa* L. hybrid (2), *N. longiflora* Cav, *N. megalosiphon* Heurck & Muell-Arg., *N. rustica* L., *N. tabacum* L. 'Turkish' and 'Havana 425', *Petunia axillaris* (Lam.) BSP, *P. hybrida* Vilm 'Burpee Blue'. Extracts of young leaf tissue harvested from systemically infected *N. tabacum* and petunia plants 10 to 20 days after inoculation were usually infectious at dilutions of 10^{-3}.

No symptoms were observed in *Capsicum annuum* L. 'California Wonder', *Cassia occidentalis* L., *Chenopodium album* L., *Cucurbita maxima* Dcne. 'Buttercup', *Cucurbita pepo* L. 'Small Sugar', *Datura stramonium* L., *Dolichos biflorous* L., *D. lablab* L., *Lycopersicon esculentum* Mill. 'Homestead-24', *Phaseolus lunatus* L. 'Fordhook', *Pisum sativum* L. 'Wando', and *Sesamum indicum* L. 'White'. Assays from these plants on Red Kidney bean were negative.

Cross-protection studies.—A cross-protection test was set up with CLRV-3 and CLRV-4, which did not cause appreciable stunting in grapefruit, and with the standard isolate which did.

Three groups of 14 seedlings each received the following treatments: (i) no "protecting" inoculation; (ii) graft inoculation with CLRV-3; and (iii) graft inoculation with CLRV-4. After plants receiving "protecting" isolates had become systemically infected (shown by bioassay), half of the plants in each group were challenged by graft inoculation with the standard isolate. Healthy plants, plants inoculated with CLRV-3, and plants inoculated with CLRV-4, which had been challenged by the standard CLRV isolate, grew an average of 21.0, 72.6, and 74.0 cm, respectively, in 159 days after challenge inoculation. In the last 80 days of that period, growth was 2.2, 39.9, and 40.9 cm, respectively, indicating cessation of growth in the unprotected plants. Unchallenged plants of the same three series grew an average of 70.9, 74.7, and 66.4 cm, respectively, in the 159 days.

Several attempts were made to test cross-protection between CVV and CLRV. Sweet orange seedlings and Eureka lemon cuttings systemically infected with CVV were challenged by graft inoculation with CLRV. CLRV was recovered by bioassay 3 months later from new growth, indicating a lack of protection. However, when CLRV was used to protect Eureka lemon cuttings, only three of six challenged by graft inoculation with CVV showed symptoms of CVV infection. Later, a test was run with Etrog citron cuttings, which show only an occasional small leaf pucker when infected with CLRV, but show strong mosaic and leaf distortion symptoms when infected with CVV. All healthy citron plants inoculated mechanically, or by grafting with CVV, showed symptoms in 3 to 6 weeks. Two of five citron plants infected with CLRV and challenged with CVV by graft inoculation showed irregularly distributed CVV symptoms on some leaves 4 months after inoculation, and the others showed none. The five citron plants infected with CLRV and challenged twice by mechanical inoculation with CVV remained symptomless after 4 months.

Properties of CLRV in leaf extracts.—Extracts of young Etrog citron leaves diluted 1:100 in buffer were infectious after 24-hours incubation at room temperature

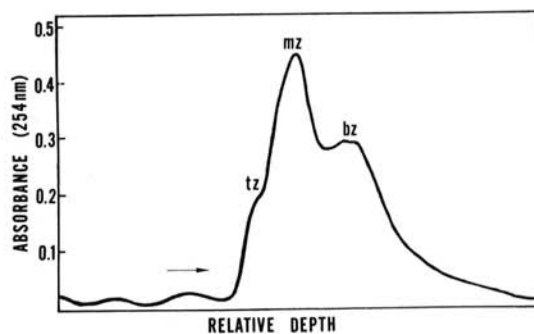

Fig. 2. Tracing of the absorbance profile of a partially-purified citrus leaf rugose virus (CLRV) preparation centrifuged on a sucrose gradient. Sucrose concentration varied linearly from 110 mg/ml to 390 mg/ml. Centrifugation time was 3.5 hours at 25,000 RPM in Spinco SW. 25.1 rotor. Tube was scanned at 254 nm with an ISCO fractionator equipped with 5-mm flow cell. Top, middle, and bottom peaks are indicated. Smaller peaks toward meniscus (ordinate) are host components. Arrow indicates direction of sedimentation.

(25 C) and sometimes after 48 hours. Lesion counts per leaf averaged 400 or more for assays during the first 5 hours and dropped to about 100 after 24 hours. These extracts stored at 0 to4C remained highly infectious after 48 hours. Extracts from frozen tissue (−20 C) produced 35 to 40% fewer lesions than extracts from comparable fresh tissue. Infectivity of extracts made at a one-tenth dilution was eliminated or greatly reduced by heating for 10 min at 60 C. Occasionally a few lesions were produced by extracts heated at 60 C, but none was produced by extracts heated at higher temperatures.

Aliquots of a dilute solution of purified CLRV (OD_{260} = 0.10) were incubated at 50, 60, and 70 C for 10 min, by procedures described for crude extracts. Lesion counts averaged 60, 3, and 0 per half leaf, respectively, whereas assay of untreated virus on opposite half leaves produced 250 to 400 lesions.

Centrifugation at 10,000 *g* for 15 minutes removed infectivity from extracts adjusted to pH 4.4, but not from extracts adjusted to pH 5.0.

Test for seed transmission.—Seeds were harvested from a seedling Orlando tangelo tree in the field that was infected with CLRV, presumably by natural means. Sixty-four seedlings were grown and assayed serologically for CLRV. All were negative, whereas experimentally infected plants reacted positively.

Purification.—The purification schedule described has been used successfully in two laboratories. The same schedule was used successfully to purify CLRV from petunia, tobacco, *C. excelsa,* Eureka lemon, and Etrog citron plants. Hydrated calcium phosphate gel treatment provided good clarification of citrus extracts. The first high-speed pellets were clear to slightly amber and resuspended rapidly. The small amount of host material and gel in a halo around the virus-containing pellet was largely eliminated in the second cycle of differential centrifugation.

Density-gradient tubes loaded with 3-5 OD_{260} units of the resuspended second high-speed pellet and centrifuged 3.5 hours at 24,000 RPM contained several light-scattering zones. One was a faint zone about 1 cm below the meniscus, also observed in preparations from healthy plants. Three closely spaced zones were observed 1.9 to 2.6 cm from the meniscus, which were not present in extracts from healthy plants. The relative amount of light-scattering in these zones was consistent in many runs. The top zone (TZ) was always present in lowest concentration and was visible sometimes only faintly. The middle zone (MZ) was in the highest concentration and occupied a relatively narrow band. The bottom zone (BZ) was broader than either TZ or MZ and appeared intermediate in concentration. It sometimes appeared to contain two components.

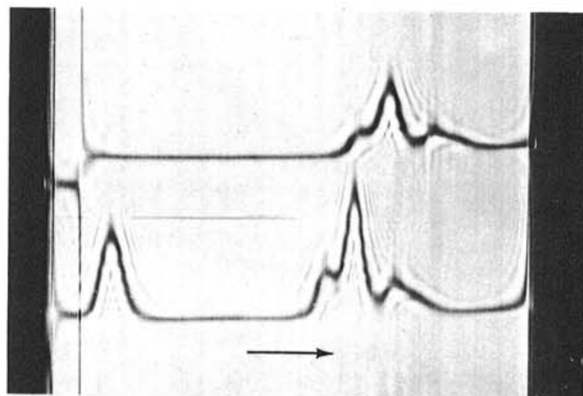

Fig. 3. Schlieren patterns of citrus leaf rugose virus (CLRV) during centrifugation in An-D rotor of Spinco Model E analytical centrifuge. Upper pattern is purified preparation after density-gradient centrifugation. Lower pattern is partially purified preparation before density-gradient centrifugation. Host component (far left) is in higher concentration than normal in most partially purified preparations. Frame was taken after 20 min centrifugation at 35,600 RPM at 20 C.

Besides the light-scattering zones, a light-amber zone, visible against a white background, was sometimes present in the area just above TZ. This amber zone, present also in extracts from healthy plants, may have been phytoferritin (3).

The UV absorbency profiles of centrifuged CLRV gradients revealed essentially the same pattern as did visual observation (Fig. 2). In the 3-hour gradients, the virus zones were not well separated. TZ appeared as a shoulder on the upper side of the MZ peak, and BZ appeared as a broad plateau on the lower side. Longer centrifugation times improved the separation somewhat, especially of MZ and BZ.

Water-clear pellets were obtained when the combined virus zones in the gradient tubes were removed, dialyzed, and concentrated by high-speed centrifugation. Commonly, 15-25 OD_{260} units of purified virus were obtained from 100 g of young leaf tissue of *C. excelsa* or Etrog citron.

Purified preparations of CLRV, diluted to an OD_{260} of 0.01, produced 300 to 500 local lesions per half leaf of Red Kidney bean. Purified CLRV induced typical symptoms on Mexican lime, Eureka lemon, and Duncan grapefruit plants. Filtered, purified preparations of CLRV were highly infectious after 6 months of storage at 4 C in RB.

UV absorption.—Purified preparations of CLRV showed a typical nucleoprotein absorption curve in the UV range. The maximum absorption was at 260 to 261 nm, and the minimum was at 243 to 245 nm. The maximum/minimum ratio was 1.23 to 1.27, and the 260/280 ratio was 1.40 to 1.46. The UV absorbency profile for MZ and BZ components was similar. The absorbance of a 1 mg/ml solution of CLRV in a 1 cm light path was 5.3 at 260 nm.

Relative infectivity of CLRV components.—To compare the relative infectivity of the virus zones observed in density-gradient tubes, small samples of MZ and BZ were carefully removed through the side of the tube with a small syringe. The MZ sample was collected from the upper side of the MZ and presumably also contained some TZ. The sample of BZ was collected from the lower size of the BZ zone. The samples were diluted to the same absorbency at 260 nm and assayed for infectivity on opposite half leaves. At an OD_{260} of 0.015, BZ yielded 269 local lesions per half-leaf (10 leaves in two tests), whereas MZ yielded 0.9.

Small samples of the various CLRV zones were collected also from density gradients with the ISCO fractionator. These samples were diluted 10- or 100-fold,

without equalizing absorbancy, and assayed. Lesion counts from BZ were 20 to 100 times those from MZ, although MZ was in higher concentration. No lesions were produced by TZ. In one test, MZ and BZ samples were mixed and compared with the separate components at the same dilution. Lesion counts averaged 0, 49, and 63 for MZ, BZ, and the mixture, respectively.

Sedimentation constants.—The approximate S_{20} for CLRV was first determined by comparison with southern bean mosaic virus (SBMV). Both the MZ and BZ of CLRV sedimented more slowly than SBMV. S_{20} values of 92 and 104 were estimated for MZ and BZ, respectively.

Subsequently, purified preparations of CLRV were examined in an analytical centrifuge. The Schlieren pattern (Fig. 3) showed three distinct peaks comparable to those of TZ, MZ, and BZ in sucrose gradients. A shoulder on the lower edge of the BZ peak also suggested the presence of a fourth, faster-sedimenting component. The S_{20} values calculated from the analytical centrifuge runs were 79, 89, and 98 for TZ, MZ, and BZ, respectively. The shoulder on the BZ component was estimated at 106 S. Values were similar for virus suspended in RB or 0.075 M KCl plus 0.016 M Tris, pH 7.4.

Particle size.—Particles in negatively stained, bulk-purified preparations were generally isometric (Fig. 1-E) and averaged 28 nm for the 275 particles measured. Diameter of individual particles often varied as much as 3 nm from the mean, and some particles were distorted. Particles stained in uranyl acetate were better preserved than those stained in potassium phosphotungstate. Addition of formaldehyde (3%) before staining increased CLRV stability in potassium phosphotungstate.

Serology.—Several antisera were prepared to purified preparations of the standard isolate of CLRV. The virus preparations injected contained all zones. None of these sera was of remarkably high titer, although 6 to 10 mg of virus had been injected over a period of 4-8 weeks. Best results were obtained when rabbits were given one or two initial injections, followed by booster injections when antibody production in response to previous injections began decreasing.

The initial injections yielded an antiserum with a dilution end point of 1:1 to 1:16. Antibody titer decreased after several weeks, and a booster injection of 1 or more mg of virus was given. Antiserum with an end point of 1:128 to 1:512 was obtained 7 to 10 days after the booster shot.

With the exception of several bleedings from one rabbit, the antisera obtained usually did not react visibly with healthy plant antigens in agar diffusion tests.

Reaction of CLRV antiserum to several CLRV antigen sources is shown in Fig. 1-G. Ordinarily, a single precipitin line formed in gel diffusion plates. Antigen movement through the agar varied with the source. Precipitin lines formed as a reaction between purified virus and CLRV antisera curved back toward the antigen well. Lines formed against virus from crude extracts of infected plants were straight and closer to the serum well, indicating dissociation and more rapid movement of the antigen. Purified virus mixed with sap from healthy plants also produced a single, straight line. Apparently, only a single antigen-antibody system was involved, because no spurs were observed at the junction of "fast" and "slow" precipitin lines (Fig. 1-G). The antisera to the standard CLRV isolate reacted homologously to CLRV-3 and CLRV-4.

The MZ and BZ components from density gradients were not serologically distinguishable. MZ and BZ fractions, used in the infectivity comparison assays, were placed in adjacent wells in agar gel plates and tested against CLRV antiserum. A single precipitin line formed to each component, and the MZ and BZ lines fused with no evidence of spurs.

Precipitin zones were visible at lower reactant concentrations in agar with only 0.02% NaN_3 added than agar which also contained 0.85% NaCl, or 0.85% NaCl plus 0.05 M potassium phosphate buffer, pH 7.1.

The CLRV antisera reacted well with extracts of young, succulent CLRV-infected citrus tissue, as reported earlier (9). Extracts made with a 1:10 or 1:20 ratio of tissue to buffer yielded strong lines when tested against sera diluted 1:4 or 1:8. Extracts of young citrus leaves prepared at a 1:50 ratio reacted clearly when tested against properly diluted sera. Purified CLRV, frozen, lyophilized, or stored at 4 C for 11 months reacted like freshly purified virus in gel diffusion tests.

Antisera to CLRV reacted heterologously to purified preparations of the Florida isolate of CVV in agar gel diffusion tests (Fig. 1-F). A distinct spur formed at the junction of homologous and heterologous precipitin lines, and the heterologous titer was four- to eightfold less. CLRV antiserum reacted to extracts from citrus and herbaceous hosts infected with CVV in some, but not all, tests. Cross-reaction was best shown with a purified antigen source adjusted to an optimum concentration.

CLRV antiserum reacted weakly with California isolates of CCLV and CVV (P. R. Desjardins, *personal communication*). Positive reactions in gel plates were obtained with extracts from lemon leaves infected with CCLV and CVV and with extracts from bean and cucumber infected with CVV. Again, comparable extracts from healthy plants gave no reaction.

Several tests were conducted to see if CLRV was serologically related to tobacco streak (TSV) and cowpea mosaic (CPMV) group viruses, which have some similar properties. Purified CLRV at an OD_{260} of 1.0 did not react in gel diffusion plates with antisera to TSV-B and TSV-C (kindly provided by R. W. Fulton) at twofold serum dilutions from 1:4 to 1:128. Purified TSV-B and TSV-M (also obtained from R. W. Fulton) at an OD_{260} of 1.0 failed to react with CLRV antiserum diluted from 1:4 to 1:128. In the same tests, strong homologous reactions were observed at 1:4 and 1:8 serum dilutions.

Antisera to broadbean true mosaic virus, cowpea mosaic virus, and squash mosaic virus (kindly provided by R. J. Shepherd) did not react with purified CLRV. Several virus concentrations from OD_{260} of 0.25 to 2.0 were tested against fourfold serum dilutions up to 1:1024. Cowpea mosaic virus and its antiserum (kindly provided by C. L. Niblett) also failed to react with CLRV and its antiserum in reciprocal tests. Strong homologous reactions occurred with the reactant concentrations used.

DISCUSSION.—The properties of CLRV reported in this study are similar to several other multicomponent, isometric plant viruses. The 260/280 ratio for CLRV is, perhaps, lower than normally expected for small,

isometric viruses, but similar to CVV (8, 14) and CCLV (16).

CLRV has other properties similar to those of CVV and the closely related CCLV, including particle size and the presence of several virus components with similar sedimentation coefficients (3, 8, 14, 16). The reaction of CLRV antiserum to CVV confirms the reciprocal reaction observed previously between CLRV and CVV antiserum (8). Results of the cross-protection tests between CVV and CLRV suggest that protection occurs in at least some hosts. Protection was shown best, because of the striking differences in symptoms, when CLRV-infected citron plants were challenged with CVV. In the initial tests, when CVV apparently failed to protect against CLRV, symptoms could not be used to measure protection, and results had to be based on virus assay. Also, the CLRV challenge inoculation was made by grafting, a severe procedure that puts protected plants under continuous challenge from virus produced in the graft tissue. Although protection was observed in CLRV-infected citron plants graft-challenged with CVV, the protection was less complete than against challenge by mechanical inoculation.

Although CLRV and CVV are apparently related, they are easily distinguished. Serological differences in the virus coat-proteins are indicated by the reciprocal heterologous reaction between CLRV and CVV. The UV absorption profile of CLRV in sucrose gradients differs from that of CVV (8). The TZ component of CLRV is not apparent in CVV gradients (8), and the relative concentration of the MZ component is higher for CLRV. As measured by yield, infectivity assay or serological assay, CLRV occurs in greater concentrations than CVV in comparable citrus hosts. One of the unusual features of CLRV is that it occurs in relatively high concentrations in citrus and can be readily purified in quantity from citrus.

CLRV and CVV differ markedly in biological properties. CLRV and CVV share many common hosts, but cause different symptoms. CVV does not produce the rugose symptoms in Mexican lime caused by CLRV, and CLRV does not cause the distortion and severe variegation patterns in Eureka lemon associated with CVV infection (15). CVV causes a severe mosaic and leaf distortion in Etrog citron, whereas CLRV causes only an occasional pucker. CVV causes variable chlorotic to necrotic local lesions on primary leaves of cowpea, followed by a systemic mottle or mosaic (11, 13), but CLRV causes only small necrotic local lesions and no systemic infection. CVV produces a brilliant systemic veinbanding symptom in Red Kidney bean (6, 13) and no local symptoms, whereas CLRV causes only necrotic local lesions. CCLV also causes systemic infections in bean and cowpea (13). Because of these differences, it seems desirable to distinguish CLRV from CVV and CCLV as proposed. The name chosen reflects the distinctive leaf symptom observed in Mexican lime.

Citrus viruses in citrus hosts have been considered subjects difficult to study. However, CLRV was mechanically transmitted from and to citrus hosts without difficulty, was reasonably stable in extracts from citrus leaves, and multiplied to concentrations that allowed use of citrus as an increase host. Inhibitors of virus infection have been described in citrus (11, 12), but these were not a factor in the present study, since extracts of CLRV-infected citrus were highly infectious, even when diluted 100-fold. As noted in a similar study with CVV (8), there were advantages to using citrus plants as increase hosts: (i) virus titer was at least as high as in the herbaceous hosts tested, (ii) citrus increase hosts provided repeated crops of tissue, and (iii) the chance of picking up a contaminating virus in a herbaceous increase host was eliminated.

Vigorous citrus cultivars that put out frequent, succulent flushes of growth were the most desirable hosts for virus increase. *Citrus excelsa*, a lime relative, and Etrog citron grew vigorously, even when infected with CLRV, and supported high titers of CLRV in new growth. These plants grew best under relatively warm conditions (26-32 C) and required careful watering and fertilizing to grow well for long periods in greenhouse containers. CLRV titer in citrus leaves dropped rapidly as leaves matured under warm conditions, and were harvested frequently. Fresh leaf tissue was often stored at 4 C for 5 to 7 days before use.

The ease of obtaining purified CLRV and the availability of rapid bioassay and serological assay procedures should encourage other studies with this virus.

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