Purification, Electron Microscopy, and Serology of the Dogwood Ringspot Strain of Cherry Leafroll Virus

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ABSTRACT

A virus was mechanically transmitted from petals of two naturally infected *Cornus florida* trees with chlorotic ringspot leaf symptoms to *Chenopodium quinoa* and then to plants in 28 of 40 genera. The virus, named dogwood ringspot (DRSV) strain of cherry leafroll virus (CLRV), incited chlorotic ringspot and vein chlorosis in mechanically inoculated dogwood seedlings. We obtained partially purified preparations of the virus by blending fresh tissue in buffer and clarifying the sap with bentonite or chloroform. Resuspended virus pellets of DRSV or CLRV alone always produced two bands 2 mm apart in sucrose gradients centrifuged 2 hr at 98,000 *g*. Particles of DRSV were consistently smaller than those of CLRV in all buffers tested. Particle diameters varied as much as 2 to 3 nm in populations of particles of each virus, but averaged 24 to 25 nm for DRSV and 28 to 30 nm for CLRV each in Tris [tris (hydroxymethyl) amino methane] buffer. Extracted RNA of DRSV and CLRV was infectious in concentrations as low as 2.5 μg/ml. An immunized rabbit produced antiserum with an antibody titer of 1:512. DRSV is serologically related to CLRV and golden elderberry virus, and distantly related to elm mosaic virus. DRSV and its antiserum have been deposited with the American Type Culture Collection as No. PV 142 and PV-AS-70, respectively.

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Additional key words: ribonucleic acid, host range, properties.

A disorder observed among dogwood trees (*Cornus florida* L.) was characterized by terminal dieback, dead flower spurs, and mild chlorosis of leaf veins near the midrib. Tobacco ringspot virus was isolated from three of seven affected trees (20). Another virus was isolated from two other trees which showed chlorotic ringspots on a few leaves. Host range, electron microscopy, and serological studies of the second virus are described here.

MATERIALS AND METHODS.–Cherry leafroll virus (CLRV) isolate CH125 (3) was kindly provided by R. Cropley in England. Antisera studied and homologous titers in gel diffusion tests (name of donor in parentheses) were cherry leafroll-216, 1:1,024 (D. Z. Maat); cherry leafroll-CH125, 1:512 (R. Stace-Smith); golden elderberry (GEV), 1:512 (R. Stace-Smith); and elm mosaic, 1:64 (J. P. Fulton). Other antisera used in identifying the dogwood virus were obtained from the American Type Culture Collection, or produced in our laboratory, or provided by M. Hollings and B. Kassanis (England), R. Bercks and C. Wetter (Germany), R. Fulton, R. Shepherd, and W. Hewitt (USA). Our source of pancreatic ribonuclease and activated charcoal and the centrifuges, spectrophotometers, and gradient fractionators used were as described earlier (19).

Dogwood ringspot virus (DRSV) was isolated from 25-year-old trees planted at the Glenn Dale Hospital. The white bract tissue of the flowers was triturated in 0.025 M phosphate buffer pH 7.5, and the juice rubbed onto 6- to 8-week-old plants of *Chenopodium quinoa* Willd. and *Nicotiana tabacum* L. 'Samsun'. An experimental host range and physical properties were determined by previously described procedures (19). All assays were made on *C. quinoa*. The DRSV cultures were renewed 4 times during the 2-year period of the study from fresh or frozen dogwood flowers from a single tree.

Purification.–DRSV and CLRV were extracted from Samsun tobacco 10 to 20 days after its inoculation, and from *C. quinoa* after 6 to 10 days by several different clarification procedures. Our initial procedure involved blending fresh tissue in a mixture of neutral 0.02 M sodium citrate buffer (1:1.5 to 2.0, w:v) containing 0.02 M 2-mercaptoethanol and 0.1 M $MgSO_4$ (5 ml/100 g of tissue). The liquid of the resulting slurry was expressed through cheesecloth, adjusted to pH 5.5 with acetic acid, and centrifuged first at 12,000-15,000 *g* for 10 min, then at 100,000 *g* for 1.25 hr. Each pellet was resuspended in 1 ml of 0.02 M sodium citrate buffer.

In a second procedure, we used activated charcoal (Merck 18351) and bentonite, prepared according to Dunn & Hitchborn (5). Tobacco or *C. quinoa* tissue was blended as described above. The liquid was expressed through cheesecloth and mixed with 4.0 g of powdered charcoal/100 ml of expressed liquid. This preparation was left for 15 min, then centrifuged at 5,000 *g* for 10 min. Three ml of the bentonite suspension was added to 100 ml of the resulting light-green supernatant liquid, and the mixture was centrifuged at 5,000 *g* for 10 min. Additional bentonite was added when centrifugation of the mixture did not yield a supernatant liquid free of

green color. The clear, infectious supernatant liquid was then centrifuged at 100,000 *g*, and the pellet resuspended as described above.

We also obtained clarified tobacco or *C. quinoa* sap by blending the tissue in 0.025 M phosphate buffer (1:2, w:v) for 1 min, then adding cold chloroform (1:0.25, w:v), blending this misture for 1 min and finally centrifuging the resulting slurry at 5,000 *g*. The clear yellow supernatant was then centrifuged at 100,000 *g* for 1.25 hr, the pellets were taken up in 0.02 M sodium citrate buffer, and this preparation was held overnight at 4 C.

Virus in resuspended high-speed pellets from each of the above procedures was further purified by density gradient, rate zonal centrifugation. One to 2 ml of the clarified preparation were layered onto each 10 to 40% sucrose gradient (19) in 0.02 M neutral sodium citrate buffer and centrifuged 1.5 hr at 98,000 *g*. Gradients were fractionated and monitored with an ISCO system (Instrumentation Specialties Co., Lincoln, Nebr.). Sucrose was removed from the fractions by centrifugation at 100,000 *g* followed by dialysis in 0.02 M citrate buffer.

Serology.—A rabbit was injected with virus intramuscularly once a week for 9 weeks. One to 2 mg of partially purified virus in 1 ml of buffer were emulsified with an equal volume of Freund's incomplete adjuvant for each injection. The rabbit was bled weekly after the fourth injection.

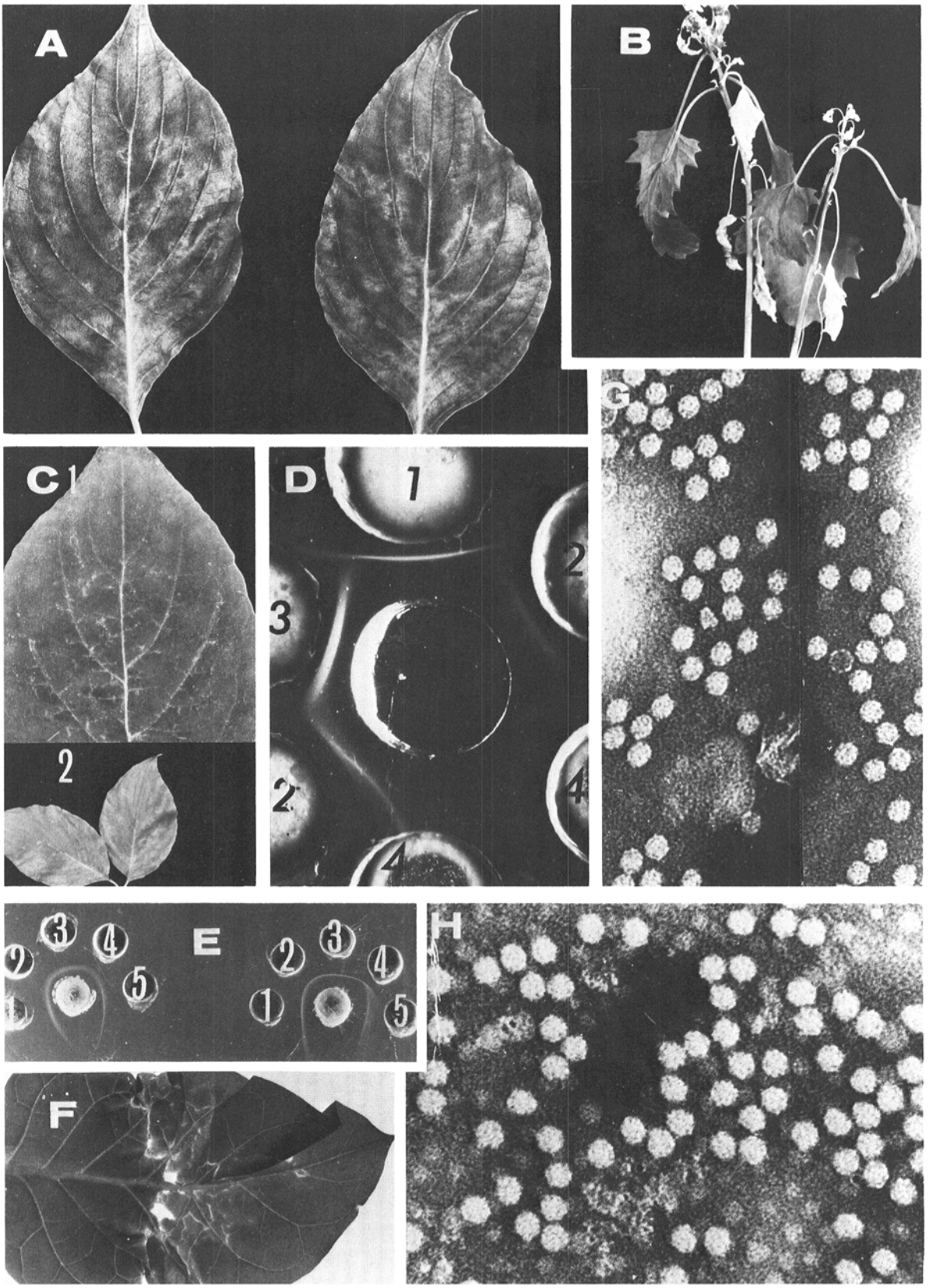
Purified dogwood virus was tested against antisera to 23 spherical viruses by the gel double-diffusion technique using 0.75% Ionagar No. 2 prepared in saline solution with 0.04% sodium azide as the preservative. The dogwood virus was placed in center wells and antisera in peripheral wells. The antisera tested were for apple mosaic, arabis mosaic, bean pod mottle, beet ringspot strain of tomato blackring, brome mosaic, carnation mottle and ringspot, cherry leafroll, cowpea mosaic and chlorotic mottle, cucumber mosaic, cucumber necrosis, grape fanleaf, *Prunus* necrotic ringspot, southern bean mosaic, sowbane mosaic, strawberry latent ringspot, raspberry ringspot, tobacco necrosis and ringspot, tomato ringspot and blackring, and turnip yellow mosaic viruses. We confirmed identity of the dogwood virus by sending purified noninfectious virus and its antiserum to R. Cropley, East Malling, England (1) (Fig 1-D); and antiserum to D. Z. Maat, Wageningen, The Netherlands (11).

Electron microscopy.—Critical comparisons of the particle diameters of the DRSV and CLRV were made using purified reconcentrated preparations from sucrose gradients. Samples in 0.02 M phosphate, 0.02 M citrate, and 0.02 M Tris [tris (hydroxymethyl) amino methane] buffers, each at pH 7.0, were placed on Formvar-coated grids and stained with 2% phosphotungstic acid adjusted to pH 7.1 with KOH. Forty to 50 virus particles of each preparation were measured in magnifications calibrated with 312 and 500 nm polystyrene latex particles.

RESULTS.—*DRSV source trees.*—A few leaves on 25-year-old trees exhibited faint chlorotic ringspots or arcs (Fig. 1-A). They usually were adjacent to the primary veins. Other leaves showed a mild mottle. The ringspot symptom disappeared by early summer each year, and during midsummer, leaves of affected trees showed upward leaf rolling similar to CLRV-infected cherries as illustrated by Posnette & Cropley (13).

Host range, symptoms, and physical properties.—The symptoms incited by DRSV in herbaceous hosts were similar to those caused by GEV (8), and usually less severe than the symptoms incited by CLRV (2, 4, 15) in several hosts. The following plant species or cultivars either have not been reported as experimental hosts of CLRV or GEV, or they reacted differently to our DRSV strain of CLRV. Systemic symptoms (chlorosis, oak leaf pattern, or mottle) developed in inoculated plants of *Chenopodium foetidum* Schrad., *Glycine max* (L.) Merr. 'Kanrich', *Helianthus annuus* L. 'Mammoth Russian', *Phaseolus vulgaris* L. (five cultivars), *Physalis peruviana* L., and *Torenia fournieri* Lind. Systemic necrotic lesions, ringspots, death of the growing point, or wilt occurred in *C. quinoa, Coleus blumei* Benth., *Nepeta cataria* L., *Pisum sativum* L. 'Alaska', and *Sesamum indicum* L. Species which reacted with necrotic or chlorotic local lesions but without systemic infection were *Beta vulgaris* L., *Capsicum frutescens* L. 'Tabasco', *Cucumis sativus* L. 'Improved Long Green', *Momordica balsamina* L., and *Zinnia elegans* Jacq. 'Blaze'. Local and systemic infection without symptoms occurred in: *Antirrhinum majus* L. 'Mardi Gras', *Apium graveolens* L., *Celosia cristata* L., *Digitalis purpurea* L., *Lobelia erinus* L., *Petunia hybrida* Vilm., *Plantago virginica* L., *Nicotiana glutinosa* L., *N. repanda* Willd. ex Lehm., *N. rustica* L., *Salpiglossis sinuata* Ruiz & Pav., and *Tagetes minuta* L. No infection occurred in *Ageratum houstonianum* Mill. 'Blueball', *Alyssum maritimum* (L.) Lam., *Brassica rapa* L. 'Yellow Globe', *Callistephus chinensis* (L.) Nees, *Crotalaria mucronata* Desv., *Cynara scolymus* L., *Cynoglossum grande* Douglas 'Firmament', *Datura metel* L., *Dioscorea composita*

Fig. 1. A,B,C) Symptoms induced by a dogwood ringspot virus. **A)** Leaves from naturally infected dogwood trees. **B)** Systemic infection of *Chenopodium quinoa* showing apical necrosis. **C)** Systemically infected leaves from mechanically inoculated dogwood seedling showing chlorotic flecking of veins and ringspots. **D,E)** Agar double diffusion serology. **D)** Center well contains dogwood ringspot virus (DRSV) antiserum and peripheral wells contain *C. quinoa* sap with (1) DRSV; (2) cherry leafroll virus (CLRV) Dutch-216; (3) CLRV-English-CH125; (4) sap only (experiment performed by R. Cropley). **E)** Center wells contain DRSV (left) top component, (right) bottom component. The antisera, each diluted 1:8, are: (1) golden elderberry; (2) CLR-CH125; (3) DRS; (4) elm mosaic 1:2 dilution; (5) CLR-216. **F)** *Nicotiana tabacum* 'Samsun' systemically infected with DRSV. **G,H)** DRSV and CLRV particles, respectively, in 0.02 M phosphate buffer and stained with 2% phosphotungstic acid (X 182,000).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Hemsl., *Hibiscus rosa-sinensis* L., *Pelargonium zonale* (L.) L'Her., *Salix wimmeriana* Grenier & Godron, and *Vinca rosea* L. The most distinctive symptoms occurred in *C. quinoa,* with necrotic ringspots 1 mm in diam after 2 to 4 days, and systemic apical necrosis after 6 to 8 days (Fig. 1-B). *Helianthus annuus* L. had bright yellow ringspots and oak-leaf patterns, and varieties of *Phaseolus vulgaris* L. developed systemic mosaics. DRSV incited local and systemic necrotic etched rings and oak-leaf patterns in Samsun and KY-35 tobacco (Fig. 1-F).

Of 15 dogwood seedlings mechanically inoculated over a 6-month period with purified virus, six developed chlorotic flecks along the veins of a few leaves after several months (Fig. 1-C, 1). Leaves on two of the same seedlings exhibited an occasional chlorotic ringspot (Fig. 1-C,2) similar to that seen on the virus source trees. These rings appeared about 3 months after inoculation, and disappeared after another 3 months. DRSV was isolated after repeated attempts by mechanical transmission from immature leaves of four of the infected seedlings. None of four *Prunus cerasus* L. seedlings mechanically inoculated in the greenhouse showed virus symptoms and virus could not be isolated from these plants.

We also inoculated 20 4-inch-tall Montmorency and Bing cherry trees in the field, using chips from the dogwood seedlings infected as a result of mechanical transmission of the virus and 10 additional trees with chips taken directly from the infected source dogwood trees. None appeared diseased after 10 months; however, the incubation period may be several years.

Sap extracted from tobacco remained infectious when heated to 53 C for 10 min, incubated at room temperature for 24 hr, or diluted to 1:1,000 and then assayed on *C. quinoa*; however, heating to 56 C, incubation at room temperature for 48 hr, and a dilution of 1:2,000 inactivated the preparations.

Purification.—Each of the procedures resulted in highly infectious virus preparations which incited lesions in *C. quinoa* when diluted as much as 1:20,000. However, none resulted in yields greater than 4 to 6 mg virus/100 g of tissue. Upon fractionating and monitoring sucrose gradients, we always observed two components for each of the viruses. When centrifuged 2.5 hr in 10 to 40% sucrose gradients, these components appeared as distinct peaks, but not completely separated. Each component of DRSV gave a typical nucleoprotein spectrum with a minimum absorption at 240 nm and maximum absorption at 260 nm. Spectra of five preparations of the top component were essentially identical with the spectra of the bottom component, and each had a 260:280 ratio of 1.77. The analytical ultracentrifuge pattern of DRSV in Fig. 2 is typical of the relative amount and sedimentation rates of these components of each virus.

Each of the components incited necrotic ringspots followed by systemic necrosis when bioassayed in *C. quinoa* (Fig. 1-B). The same reactions were obtained when a 0.5-ml fraction representing the trailing edge of the slower sedimenting component and a similar volume representing the leading edge of the more rapidly sedimenting component were assayed. Repeated attempts were made to establish and propagate the two components separately. However, virus purified from these attempted separations always produced a typical double peak upon analysis in sucrose gradients.

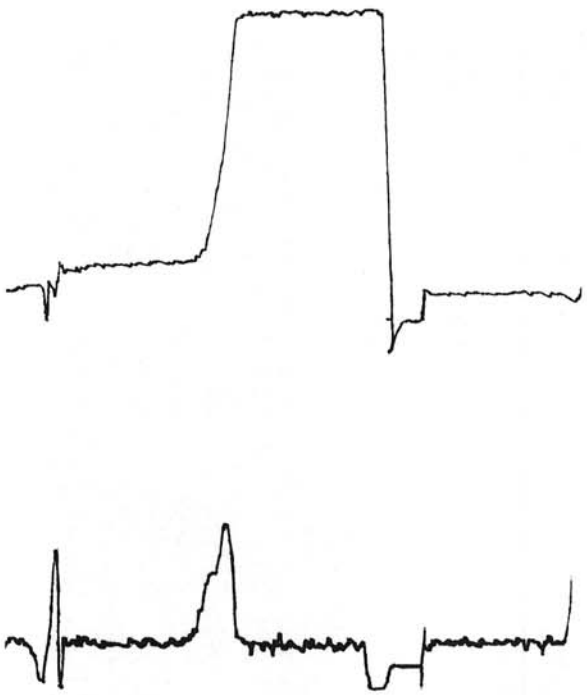

Fig. 2. Analytical ultracentrifuge ultraviolet optics pattern of dogwood ringspot virus in 0.2 M phosphate buffer at 0 C. Sedimentation left to right. Photoelectric scan made 20 min after attaining 23,150 rpm. Lower tracing is a derivative pattern of concentration versus radial distance tracing in upper tracing.

Serology.—A rabbit was immunized with the combined DRSV components. Antisera had a titer of 1:512 when the animal was bled 2 weeks after the final injection; antiserum diluted 1:3 did not produce a band with crude sap of healthy tobacco or *C. quinoa*. In tests using partially purified DRSV at 7, 3.5, and 0.5 mg/ml, antisera titers were: DRSV, 512; CLRV (Dutch & British Columbia sources), 128; golden elderberry, 128; and elm mosaic, 4. In tests conducted by D. Maat using two sources of CLRV in *C. quinoa* sap, DRSV appeared to be less closely related to CLRV since the homologous titer of CLRV was 1:1,024 and the heterologous titer with DRSV antiserum was 1:64 (Table 1). CLRV, elm mosaic, and golden elderberry antisera each reacted with the partially separated DRSV components (Fig. 1-E). DRSV did not react with any of the other antisera tested.

Electron microscopy.—One hundred particles were measured of DRSV and of CLRV when each was suspended in each of three buffers.

A mixture of DRSV components in any of three

TABLE 1. Reciprocal titers of antisera to dogwood ringspot and cherry leafroll viruses (CLRV) against the English and Dutch cultures of cherry leafroll virus

Virus	Antisera[a]		
	CLRV 125 (England)	Eckelrade 216 (Dutch)	Dogwood ringspot (USA)
CLRV 125[b]	8	1,024	64
Eckelrade 216[b]	8	1,024	64
Dogwood ringspot[c]		64	512
Healthy sap[a]	0	0	0

[a] Tested by the agar double diffusion technique.
[b] Determinations made by D. Maat, Wageningen, The Netherlands, using sap from infected *Chenopodium quinoa*.
[c] Purified virus.

buffers showed a consistently smaller average particle diameter than that of CLRV particles in the same three buffers (Fig 1-G). Many particles in 0.02 M phosphate, citrate, or Tris buffer measured 23 nm in diam, with the largest particles measuring 25 nm.

The mixed components of CLRV also ranged in particle diameter (Fig 1-H). Most of the particles resuspended in citrate buffer were 25 nm, those in phosphate were 26 to 28 nm, and those in Tris buffer were 28 to 30 nm in diam. A range of 3 nm in diam was often observed on the same grid regardless of the buffer used. Many particles did not show a distinct hexagonal profile. None of the particles in phosphate or citrate buffer was as large as 30 nm.

Ribonucleic acid.—RNA was extracted from DRSV by a previously described procedure (19). Yields were 25 to 35% of total calculated RNA in intact virus. It was infectious in concentrations as low as 2.5 μg/ml when assayed on *C. quinoa*. No infectivity remained when the RNA was treated 1 hr at 0 C with 1×10^{-7} M pancreatic ribonuclease.

DISCUSSION.—It is not surprising that a strain of CLRV was found in dogwood trees in the USA. Since the cherry leafroll virus disease was first described in England (13), it has been reported from Holland (11, 12), Yugoslavia (16), and Germany (6, 10). The virus has been identified from *Sambucus racemosa* (14, 15) and *Rumex obtusifolius* L. (17) as well as from sweet and sour cherry. Serologically distinct strains of CLRV have been identified from *S. nigra* L. in Canada (8) and Scotland (9), and from *S. canadensis* L. (9) and *Ulmus americana* L. (7) in the USA. J. P. Fulton isolated virus from dogwood trees in Arkansas *(personal communication)*; however, we do not know whether his isolate is related to DRSV. The origin of our virus source trees could not be determined. Dogwood is native to this region.

Most CRLV particles were larger than those of DRSV in PO_4 buffer. Walkey & Webb (18) reported a particle diameter of 27 nm for CLRV, whereas Cropley & Tomlinson (4) reported a particle diameter of ca. 30 nm. However, in their electron photomicrograph of virus stained with phosphotungstate, particles ranged in size from 24 to 30 nm in diam (4).

Our results confirm those of Cropley's *(personal communication)* in that CLRV particles vary in diameter depending on the method of preparing samples for electron microscopy. Furthermore, a range of particle sizes is usually present on the same grid. CLRV suspended in Tris buffer had a consistently larger particle diameter than did those in phosphate or citrate buffers at comparable concentrations.

These results emphasize the importance of comparison of particle sizes of different virus strains under the same conditions and with the same electron microscope. Although the particle diameters we report for CLRV and DRSV may not be absolute values, the measurements do reflect size differences among particles in populations of these two viruses. Reported differences in particle size may result from differences in staining techniques, in buffers, and in electron microscope magnifications.

Hansen & Stace-Smith (8) and Jones & Murant (9) reported a serological relationship between GEV and CLRV. In our tests, DRSV was related equally to GEV and CLRV, and was more distantly related to elm mosaic virus.

The similarity of host ranges and symptoms incited by DRSV and GEV is impressive. Both viruses incited rings and arcs in the leaves of their original hosts. In fact, DRSV appears to be more like GEV (8), and one called S-3 (9), both from North America, than like CLRV in Europe (2, 4). Other properties of DRSV are also the same as those reported for GEV (8) except for particle diameter. However, they apparently are not serologically identical if the homologous titer of GEV antiserum is 1:512, because GEV and DRSV antisera produced visible bands against DRSV in a common well in three experiments when dilutions of the GEV and DRSV antisera did not exceed 1:128 and 1:512, respectively. There are also many minor differences between DRSV and GEV in host range and symptomology.

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