Serological, Physical, and Chemical Properties of Strains of Cherry Leaf Roll Virus

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ABSTRACT

Serological tests with strains of cherry leaf roll virus isolated from cherry, rhubarb, golden elderberry, dogwood and elm showed that the dogwood and elm strains were closely related; the cherry strain more closely related to dogwood than to elm; and the rhubarb and golden elderberry strains more closely related to each other than to the other three strains. The five strains consisted of three viral components with sedimentation coefficients of approximately 52 S, 114 S, and 132 S. The top component was not infective, but the middle and bottom components were infective, serologically identical and had similar ultraviolet absorption spectra. The lysine content of the dogwood, elm and golden elderberry strains was significantly lower than that of the rhubarb and cherry strain. The molecular weight of the protein subunits of the complete virus of each strain was 55,000. The dogwood and elm strains moved towards the anode in agar electrophoresis at pH 6.5, whereas the rhubarb, golden elderberry and cherry strains moved to the cathode. Each strain produced two serologically distinct electrophoretic components. Two bands with molecular weights of 2.1 and 2.3 $\times 10^6$ daltons were formed when RNA of the golden elderberry strain was run in polyacrylamide gel electrophoresis. The base percentage composition of the middle and bottom components of golden elderberry strain was: G = 26.61; A = 21.41; C = 22.76; U = 29.22 and G = 26.55; A = 22.01; C = 22.32; U = 29.12, respectively.

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Additional key words: amino acid analysis, gel-diffusion, analytical ultracentrifuge.

Since cherry leaf roll virus (CLRV) was first isolated from cherry in England (3) the virus has been found in Europe in a range of species: *Sambucus nigra* L. (12, 14); *S. racemosa* L. (12); *Rheum rhaponticum* L. (rhubarb) (15); *Rubus procerus* Muell. (4); and *Rumex obtusifolius* L. (Walkey, *unpublished*). Later it was found that North American isolates of elm mosaic and golden elderberry viruses, from *S. nigra aurea* L. in Canada and *S. canadensis* L. in New York State, were serologically related to CLRV and could be considered strains of the same virus (8). A strain of CLRV has also been isolated from *Cornus florida* L. (dogwood) trees in Maryland (16).

Although CLRV resembled other members of the nepovirus group in many of its properties (4), it differed in that the sedimentation coefficient of the middle component (114 S) of the golden elderberry strain, was higher than those reported for tobacco ringspot virus [91 S (13)], arabis mosaic virus [93 S (10)] or raspberry ringspot virus [92 S (10)]. Preparations of both the middle and bottom (132 S) components of the golden elderberry strain were reported to be infective (6), whereas only the bottom component of the other viruses is infective.

The object of the present investigation was to study the serological relationships and the chemical and physical properties of strains of CLRV originating in Europe and North America and to investigate further the properties of the 114 S and 132 S components of the virus.

MATERIALS AND METHODS.—The following strains of CLRV were studied: cherry, type strain (provided by R. Cropley, East Malling Research Station, England); rhubarb (15); golden elderberry (6); elm mosaic virus and dogwood (provided by J. P. Fulton, University of Arkansas). All isolates were cultured in *Nicotiana clevelandii* Gray.

Various tobacco species were compared as propagation hosts of the virus, but *N. clevelandii* yielded higher virus concentrations than *N. tabacum* L. 'White Burley', 'Xanthi', 'Haronova', and 'Havana' or *N. rustica* L. The highest virus concentrations were obtained from *N. clevelandii* leaves harvested 12-14 days after inoculation. Very low yields of virus were obtained if the harvested leaves were deep frozen prior to virus purification. Several purification procedures were tested and relative yields of virus from each were compared in the Spinco Model E analytical centrifuge. The following method was the most satisfactory: infected *N. clevelandii* leaves were homogenized in 0.5 M citrate buffer, pH 6.5, mixed with chloroform in the ratio of 1:1:1(g:ml:ml). After centrifugation at 1,800 rpm (International SBV centrifuge) for 10 min, the aqueous phase was further clarified at 11,500 rpm (15,500 *g*) for 15 min. Polyethylene glycol 4,000 was then added to the aqueous phase (6 gm/1,000 ml) and the mixture stirred for 2 hr before being centrifuged at 11,500 rpm (15,500 *g*) for 20 min. The pellets containing the precipitated virus were retained and resuspended in 2.0 ml of 0.01 M Tris [tris (hydroxymethyl) aminomethane]-HCl buffer, pH 7.3. The resuspended pellets were pooled and allowed to stand overnight before centrifugation at 10,000 rpm (9,000 *g*) for 10 min. The aqueous phase was then centrifuged at 28,000 rpm for 2 hr in a Spinco No. 30 rotor after which the pellets were resuspended

in 0.01 M Tris buffer, pH 7.3, for at least 1 hr before the suspension was centrifuged at 10,000 rpm (9,000 *g*) for 10 min. The partially purified virus was removed in the aqueous phase and layered onto 10-40% sucrose gradients in 0.01 M Tris buffer and the gradients centrifuged at 23,000 rpm for 2 hr in a SW 25.1 rotor. Virus bands were removed with a hypodermic syringe and dialyzed overnight against 0.01 M Tris buffer. The dialyzate was concentrated by centrifugation at 36,000 rpm for 2 hr in the Spinco No. 40 rotor and the virus pellets resuspended in 0.01 M Tris buffer. All procedures were carried out at 3 C. Infectivity of virus preparations was assayed on *Chenopodium quinoa* Willd. using the half-leaf method.

Sedimentation coefficients of virus components separated in the gradients were determined in a Spinco Model E analytical centrifuge run at 35,600 rpm at 20 C, and photographs were taken at 4-min intervals.

Antisera were prepared in rabbits by three intramuscular injections of virus (1.0 mg in adjuvant) at approximately 10-day intervals. The rabbits were bled at weekly intervals, starting 10-14 days after the second injection.

Agar-gel diffusion tests were made on 8 X 2.5 cm glass slides using 2-mm layers of 0.9% agar prepared in distilled water and containing 0.5% sodium azide. Wells 4 mm in diam, spaced 5 mm between centres were cut in the agar for titre tests and wells 5 mm in diam spaced 7 mm between centres were used for spur precipitin line tests. Purified virus was used in all tests and antisera were diluted with 0.15 N saline. A Gelman electrophoresis apparatus with similar slides was used for agar electrophoresis. The agar and tray buffers were composed of citrate-phosphate-tris solution at the required pH. Virus preparations were placed in wells 3 mm in diam and the current was applied at 50 V for 150 min. The virus was located by placing homologous antiserum in parallel troughs on either side of the antiserum well and precipitin lines were allowed to develop overnight. When necessary to improve definition, precipitin lines in both immunological and agar electrophoresis tests were stained with 0.1 M DL-3,4-dihydroxyphenylalanine (DOPA) prepared in 0.1 M potassium phosphate buffer, pH 7.4 (11). The stain was applied to a square of filter paper laid on the gel surface above the precipitin line.

The amino acid composition was determined on virus preparations hydrolyzed in 6 M HCl at 107 C for 24 hr. After hydrolysis the excess HCl was removed by flash evaporation and samples were applied to a Beckman amino acid analyzer in citrate buffer, pH 2.2. The nucleotide composition was determined as described by Stace-Smith et al. (13).

Molecular weights of the protein subunits were determined by SDS polyacrylamide gel electrophoresis. Twenty to 30 µl of degraded virus were layered onto the gel and electrophoresis was carried out at 6 mA per gel for 3 hr at room temperature. Protein lines were developed by fixing the gels in 15% trichloroacetic acid, staining overnight in Coomassie blue (0.25% dissolved in 10% trichloroacetic acid), and destaining in 10% trichloroacetic acid.

Ribonucleic acid for molecular weight determination was prepared by the SDS/phenol method of Clark & Lister (2), except that the aqueous layer following phenol precipitation was washed three times with an equal volume of ether to remove remaining phenol. Ten to 20 µl of RNA preparation in 10% sucrose was layered on the 2.4% gel and the current passed for 150 min at 4 mA per tube. RNA bands were developed by staining in 0.02% toluidine blue O in 40% ethylene glycol monomethyl ether in the dark for 12 hr and destaining in distilled water.

RESULTS.—*Serology*.—The serological relationships of the five strains were studied in double gel-diffusion tests by titration with homologous and heterologous antisera (Table 1) and by spur precipitin lines (Fig. 1) formed when different strains in adjacent wells were reacted with various antisera diluted 1:16 and 1:128 (Table 2). All tests were repeated at least twice.

Single precipitin lines were formed in all reactions. The results (Tables 1 and 2) showed that the five strains were serologically related but not identical. In the titre tests (Table 1) the dogwood and elm strains showed a closer relationship to one another than to the other three strains, and the cherry strain had greater affinity with the dogwood than with the rhubarb or golden elderberry strains.

The spur reaction tests confirmed the close relationship of the elm and dogwood strains, but showed that they were not identical. Although their antigens formed confluent precipitin lines when

TABLE 1. Homologous and heterologous titres of strains of cherry leaf roll virus in double gel-diffusion tests

Antigen	Antiserum				
	Rhubarb	Golden elderberry	Cherry	Dogwood	Elm
Rhubarb	512[a]	128	128	16	16
Golden elderberry	512	256	128	128	128
Cherry	512	256	2,048	1,024	128
Dogwood	16	128	512	2,048	1,024
Elm	128	128	256	1,024	1,024

[a] Figures are reciprocals of antiserum titres.

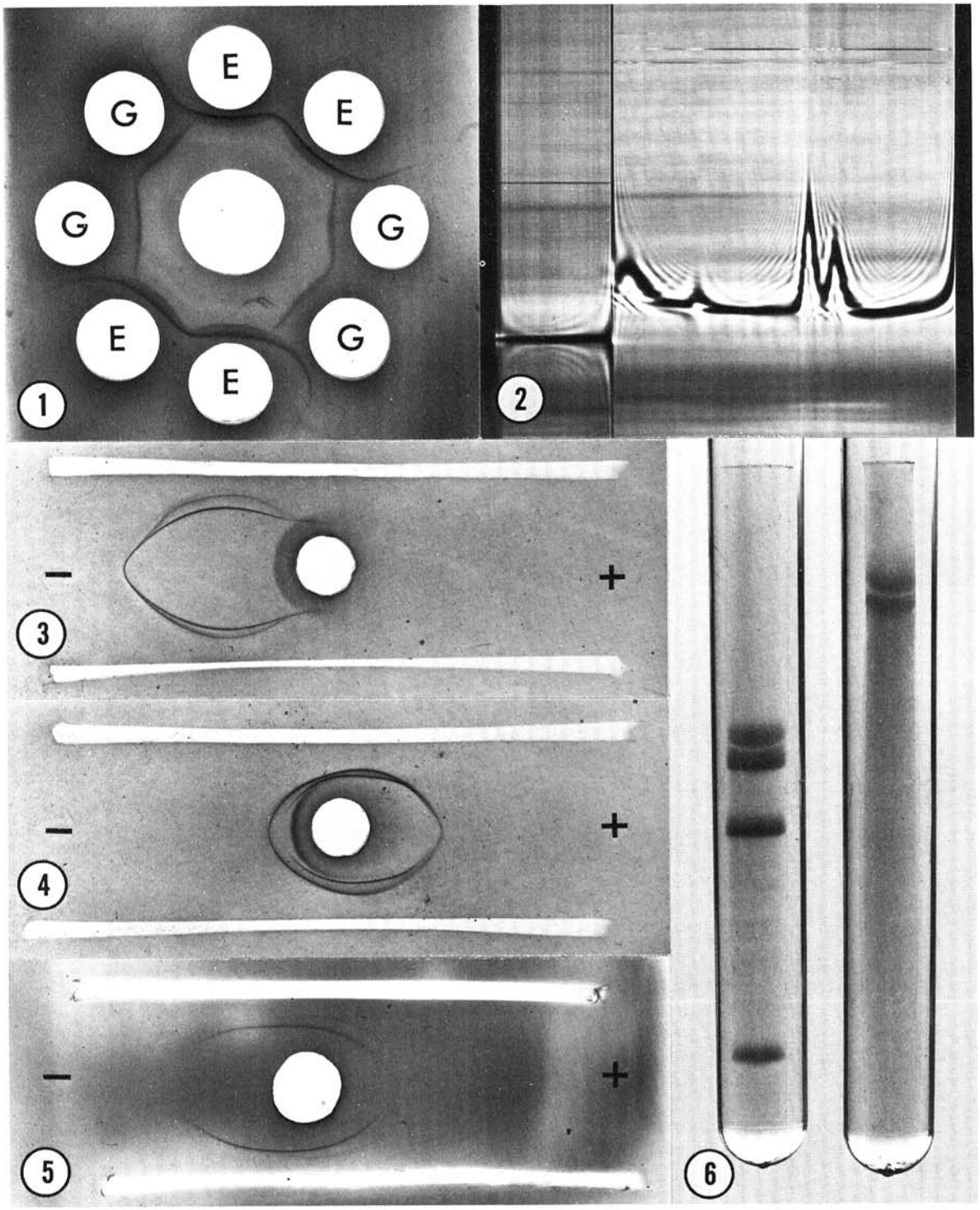

Fig. 1-6. 1) Gel-diffusion test showing spur precipitin lines formed when purified preparations of the elm (E) and golden elderberry (G) strains of cherry leaf roll virus are placed in adjacent wells and reacted against antiserum to the elm strain. 2) Analytical ultra-centrifuge photograph of preparation of the rhubarb strain showing relative proportion of the three virus components. Peak on left represents host constituents. Photograph taken with Schlieren optics 12 min after reaching speed of 35,600 rpm. 3) Precipitin lines developed in agar gel buffered at pH 6.5 showing the movement of the golden elderberry strain towards the cathode after electrophoresis. 4) Movement of dogwood strain towards the anode after electrophoresis is at pH 6.5. 5) Agar-gel slide showing the two serologically distinct, electrophoretic components of the bottom (132 S) component of the golden elderberry strain at pH 7.5. 6) Electrophoresis of the RNA in 5% polyacrylamide gel of the golden elderberry strain (right) and of the standard brome mosaic virus (left).

TABLE 2. Spur precipitin lines formed when antisera to strains of cherry leaf roll virus were reacted with homologous and heterologous antigens in double gel-diffusion tests

Antigen	Antiserum				
	Rhubarb	Golden elderberry	Cherry	Dogwood	Elm
Rhubarb × Cherry	R[a] +[b] C–[c]	R + C–	R – C+	Conf.[d]	R – C+
Rhubarb × Dogwood	R + D–	R + D–	R – D+	R – D+	R – D+
Rhubarb × Golden elderberry	R + G–	R – G+	R + G–	R + G–	R + G–
Rhubarb × Elm	R + E–	R + E–	R – E+	R – E+	R – E+
Cherry × Dogwood	Conf.	Conf.	Conf.	C – D+	C – D+
Cherry × Golden elderberry	C – G+	C – G+	C + G–	C + G–	C + G–
Cherry × Elm	C + E–	C + E–	C + E–	C – E+	C – E+
Dogwood × Golden elderberry	D – G+	D – G+	D + G–	D + G–	D + G–
Dogwood × Elm	Conf.	Conf.	Conf.	Conf.	Conf.
Golden elderberry × Elm	G + E–	G + E–	G + E–	G – E+	G – E+

[a] R = rhubarb strain; G = golden elderberry strain; C = cherry strain; D = dogwood strain, and E = elm strain.
[b] + designates the antigen whose precipitin line extends to form a spur.
[c] – designates the antigen with no spur.
[d] Conf. designates a confluent precipitin line.

reacted together against homologous and heterologous antisera, when dogwood or elm antigens were paired against cherry or golden elderberry antigens, the precipitin reactions were not identical. The reactions of the cherry strain indicated that it was more closely related to dogwood than to elm, whereas the rhubarb and golden elderberry strains had greater affinities with each other than with the other three strains.

Virus components.—The improved purification procedure used in the present study yielded three virus peaks when concentrated solutions of the five strains were examined by Schlieren optics in the analytical ultracentrifuge (Fig. 2). The sedimentation coefficients of the virus strains were similar to one another, approximately 52 S for the top component, 114 S for the middle component and 132 S for the bottom component. These three components would correspond to the top (T), middle (M), and bottom (B) components of other nepoviruses. When the material was layered on a sucrose gradient and centrifuged, three opalescent bands were observed which corresponded in intensity and position to the peaks observed in the analytical centrifuge.

Agar-gel diffusion tests showed the material from the 52 S band reacted specifically with homologous CLRV antiserum and electron microscopic examination showed it to consist of empty virus particles approximately 30 nm in diam. No infectivity was observed when samples of the 52 S bands were inoculated to *C. quinoa*, but both the 114 S and 132 S bands contained infective virus. The infectivity of these two bands was approximately equal. A single, confluent precipitin line was formed when the 114 S and 132 S components were reacted against their homologous antisera in gel-diffusion tests, indicating that they were serologically identical.

Ultraviolet absorption.—Absorption spectra were determined for purified preparations of the dogwood and golden elderberry strains of CLRV. Both the middle and bottom components had similar spectra which were typical of nucleoprotein, with minimum absorption at 240 nm and maximum absorption at 260 nm. Maximum absorption of the top component was between 270 and 275 nm, consistent with protein spectra. The 260-280 ratios of the middle and bottom components were 1.70 - 1.72 (cherry strain) and 1.77 - 1.76 (golden elderberry strain).

Amino acid composition.—The amino acid composition of the five CLRV strains based on analysis of one or more purified preparations of each strain is shown in Table 3. The data are presented as molar percentage of amino acid recovered following 24-hr hydrolysis of the proteins of each virus strain. The amino acid compositions of the five strains was similar, except that the lysine concentration in the rhubarb and cherry strains was significantly higher than in the other three strains.

In further analyses, no significant difference was detected in the amino acid composition of the middle and bottom components of the golden elderberry strain.

Protein subunit molecular weight.—The molecular weights of the protein subunits of the five virus strains were similar when determined by polyacrylamide gel electrophoresis. The average molecular weight of the subunits of the strains ranged from 54,200 to 55,700 (Table 4). No significant difference could be detected between the molecular weights of the protein subunits of the top, middle and bottom components of the golden elderberry strain of the virus.

Electrophoretic mobility.—The electrophoretic movement of purified preparations of the five virus

TABLE 3. Amino acid composition of cherry leaf roll virus strains

Amino acids	Molar percentage of amino acids recovered from a 24-hr hydrolysis				
	Rhubarb[a]	Cherry[b]	Golden elderberry[c]	Dogwood[d]	Elm[e]
Lys	5.45	5.58	3.12	3.74	3.52
His	2.02	1.83	1.97	1.64	1.97
Arg	5.62	6.07	5.17	5.44	5.10
Asp	8.71	9.57	8.22	7.74	8.31
Thr	5.85	6.32	6.86	7.72	6.58
Ser	6.17	6.84	6.74	7.85	7.03
Glu	9.23	9.92	7.94	9.00	8.41
Pro	6.47	5.33	7.42	6.33	5.94
Gly	9.53	9.00	7.63	8.92	9.88
Ala	9.46	8.53	11.79	10.11	11.29
Val	7.02	7.27	6.65	5.95	6.73
Met	2.81	2.65	2.61	2.21	2.45
Ilu	4.70	4.07	5.05	4.71	4.80
Leu	9.43	9.60	9.93	10.09	9.60
Tyr	3.03	2.76	2.77	2.59	2.66
Phe	4.50	4.65	6.13	5.95	5.70

[a-e] Average values of 2, 2, 4, 1, and 1 analyses, respectively.

strains was examined in buffered agar gel at pH 6.5. At the pH tested, the rhubarb strain moved 6 mm, golden elderberry 8 mm, and cherry 5 mm towards the cathode (Fig. 3). In contrast, the dogwood strain moved 2 mm and elm 4 mm towards the anode (Fig. 4). The movement towards the cathode was probably caused by electrophoretic endosmosis in the agar gel. In these tests, two serologically distinct precipitin lines were formed by the five strains. The same virus preparations, when reacted against their homologous antisera in agar-gel diffusion tests without electrophoresis, produced only a single precipitin line.

In further electrophoretic tests, the same two precipitin lines were found to occur in both the middle (114 S) and bottom (132 S) (Fig. 5) components. In agar-gel diffusion tests, the middle and bottom components produced only the single precipitin line. The results indicated that the two electrophoretic components do not correspond with the centrifugal components of the virus.

Nucleic acid properties.—Polyacrylamide gel electrophoresis of RNA separated from purified preparations of the golden elderberry strain, produced two bands in the gel (Fig. 6). The molecular weight of these two components was estimated by comparing their mobility with that of RNA from brome mosaic virus (9). Assuming a linear relationship between log molecular weight and distance moved (1), the molecular weight of the upper band was 2.3×10^6 daltons and the lower band 2.1×10^6 daltons. This is in good agreement with the 2.4×10^6 and 2.1×10^6 RNA components reported by Jones & Mayo (7).

TABLE 4. Molecular weights of the protein subunits of cherry leaf roll virus strains

Strain	Range of Mol. wt[a]	Mol. wt (av)
Rhubarb	54,100 - 56,300	55,140
Cherry	55,000 - 56,000	55,700
Dogwood	53,000 - 55,100	54,200
Elm	55,100 - 55,500	55,400
Golden Elderberry	53,700 - 54,500	54,200
top component		54,500
middle component		55,700
bottom component		54,400

[a] At least three samples of each strain were tested and three to four tubes of each sample were run per experiment.

The nucleotide composition of the middle and bottom component RNA's was determined for the golden elderberry strain. The average composition of recovered nucleotides from three analyses in moles percent were: middle component, G = 26.61; A = 21.41; C = 22.76; U = 29.22 and bottom component, G = 26.55; A = 22.01; C = 22.32; U = 29.12. Statistical analysis showed that the percentage adenylic acid in the bottom component was significantly higher than in the middle component (at the 5% level) and that the uridylic acid concentration in the middle component was significantly higher than in the bottom component (at the 5% level).

DISCUSSION.—The serological, physical and chemical properties of the five isolates studied confirmed that they are related to one another and should be considered strains of CLRV.

The occurrence of CLRV in a species of dogwood native to North America, as well as in native elm, strongly supports the suggestion (7) that CLRV occurs naturally in North America, rather than its being recently imported from Europe. Both the dogwood and elm strains were isolated from trees growing in the midwest of the United States and the similarity of their properties in the present studies strongly suggests a common origin. The two strains have a close serological relationship, their electrophoretic movement is similar, and amino acid

analysis showed the molar percentage of lysine in their proteins was significantly lower than that of the European strains studied. In contrast, the golden elderberry strain isolated from *S. nigra aurea* in British Columbia was not closely related to the dogwood and elm strains of the virus. Except for the low molar percentage of lysine in the protein, this isolate showed stronger affinity with the English rhubarb strain than with any other strain. The rhubarb strain has also been found to be closely related to CLRV isolated from *S. canadensis* in New York State (8). As the golden elderberry isolates were obtained from plants in commercial nurseries, it is possible that this strain could have originated in Europe and was imported into North America in nursery stock.

The dogwood strain of CLRV used in these studies appears to differ serologically from the virus isolated from dogwood in Maryland. Our dogwood isolate reacted almost to titre against the elm strain, whereas the dogwood isolate from Maryland reacted very weakly against the elm strain (16).

The sedimentation coefficients obtained for the various strains confirmed that CLRV is unique among nepoviruses in the relatively high S-value of its middle component. Both the middle and bottom components were found to be infective, when separated by two cycles on sucrose gradients from purified preparations of the rhubarb and golden elderberry strains. It must be recognized that two components that are as close in their sedimentation coefficients as the M and B components of CLRV are difficult to separate upon sucrose gradient centrifugation. Recent results of Jones & Mayo (7), indicating that both M and B components are necessary for maximum infectivity, would suggest that the separations that we achieved were incomplete.

Our results are at variance with a recent report by Ford et al. (5), not only on the sedimentation coefficients of the components but on the size of the virus particle. They characterized an isolate of elm mosaic and report a particle size of 19 nm and three viral components sedimenting at 45, 65 and 92 S. The Iowa isolate of elm mosaic was serologically related to Fulton's isolate of elm mosaic and there is well documented evidence that Fulton's elm mosaic is serologically related to CLRV (8) and, as such, would be expected to share many of the physical and chemical properties of CLRV. Our results reported in this paper demonstrate that five isolates of CLRV are very similar in their physical and chemical properties and are similar in most respects to the other viruses in the nepovirus group. The particle size and S-values reported for the Iowa isolate of elm mosaic are almost certainly in error. One possible explanation in the discrepancies in sedimentation coefficients is that Ford et al. (5) did not correct for temperature, solvent viscosity and virus concentration in calculating the S-values of their material, since their values were considerably lower than the previous reports (6, 7). However, a simple upward adjustment of their values would not completely explain the differences. The ratio of S-values for our T, M, and B components is 1:2.2:2.5, whereas their ratios are 1:1.4:2.0, respectively.

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