Purification and Characterization of a Virus Associated with the Grapevine Leafroll Disease

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ABSTRACT

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Grape leafroll virus (GLRV) which is associated with the leafroll disease in grapevine, and also is transmissible to herbaceous plants, was purified from *Nicotiana glutinosa* and *Datura metel* and characterized. Purification involved a combination of solvent extraction, polyethylene glycol precipitation, differential centrifugation, and equilibrium density-gradient centrifugation in CsCl. Flexuous, rod-shaped, infectious virus particles were found in the purified preparation. They were heterogeneous in size due to fragility and aggregation. The sedimentation coefficient of the n normal-size particle (13 × 790 nm) was about 150S. The virus formed a single band at 1.32 g/cm^3 upon CsCl equilibrium centrifugation. Viral RNA extracted from purified GLRV formed a single, though somewhat diffuse, band upon agarose-acrylamide slab-gel electrophoresis. The molecular weight of GLRV-RNA was estimated as 2.9 - 3.5 × 10^6. Infective GLRV-RNA could be eluted out of polyacrylamide gels. A single protein band, 31,000 daltons in molecular weight, was detected upon polyacrylamide gel electrophoresis of purified GLRV in the presence of sodium dodecyl sulfate or urea. No virus particles were found in ultrathin sections of GLRV-infected tissues of either grapevine, *N. glutinosa, or D. metel.* In dip preparations, however, virus particles were readily observed in the sap of the above herbaceous plants, but not in grapevines. A variety of inclusion bodies and abnormal structures were observed in ultrathin sections of *N. glutinosa* and *D. metel.* These included pinwheeles, bundles of filaments, arrays of crystalline matter, and giant mitochondria. Grape leafroll virus is classified in the potyvirus group.

The leafroll disease of grapevines is a major world-wide viticultural problem (7). The causative agent is graft-transmissible, and recently was transmitted to herbaceous plants by inoculating them with phenol extracts of infected grapevine leaves (14). We also were able to transmit the causative agent back to grapevine, re-extract it, and successfully re-inoculate *Nicotiana glutinosa* L. Hence, Koch's postulates have been satisfied and the virus from *N. glutinosa* is apparently the causative agent of the leafroll disease. However, as discussed below, we would rather refer to it at the present time as a "leafroll-associated virus".

In the present paper, we report the purification of the grapevine leafroll virus (GLRV) and the characterization of some of its physical and chemical properties. Ultrastructural changes specific to GLRV-infected tissues also are described.

MATERIALS AND METHODS

Virus propagation.—The same grape leafroll isolate (14) that originally was transmitted to *N. glutinosa* by phenol extraction from a GLRV-infected grapevine, was used throughout this study. Towards the end, however, the California leafroll isolate White Emperor was also purified by us. Grape leafroll virus was propagated in either *N. glutinosa* or *Datura metel* L. in an insect-proof, temperature-controlled greenhouse (22-24 C), but no other environmental conditions were controlled. Virus purified from old infections of *N. glutinosa* contained a band of host impurities upon CsCl gradient centrifugation. Hence, all starting materials were from young *N. glutinosa*, or from *D. metel* (irrespective of age).

Electron microscopy.—Grape leafroll virus, at different stages of purification, was mounted on collodion-filmed grids and negatively stained with 2% potassium phosphotungstate at pH 7.0. Samples from CsCl gradients were placed on the grids and washed by several passages through double-distilled water.

Ultrathin sections were made from the elongation zone of roots (1) and from the blades of mature leaves. Fixation was done in 0.1 M cacodylate buffer pH 7.0 containing 5% glutaraldehyde for 3 hr and postifixation in 1% OsO_4 in 0.1 M phosphate buffer pH 7.0. Dehydration was carried out by a series of increasing concentrations of ethanol (30-100%) and finally by propylene oxide. Epon-embedded material was sectioned (5,000-7,000 nm) in a LKB Ultratome, using glass knives. Thin sections were mounted on collodion-filmed grids and stained with uranyl acetate and lead citrate.

Virus characterization.—Since GLRV suspensions exhibited an intense light-scattering in the ultraviolet (UV) range, virus particles were disrupted by alkali prior

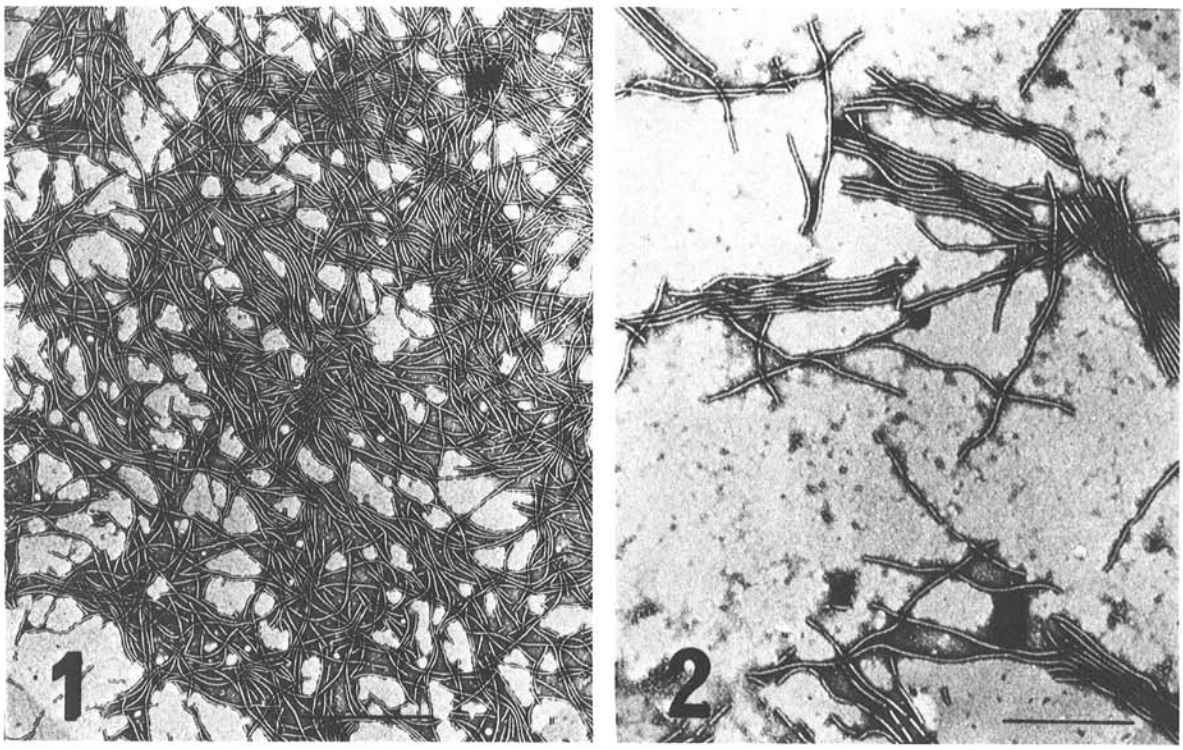


Fig. 1-2. Electron micrographs of purified grape leafroll virus preparations. **1)** General view (bar = 0.1 μm). **2)** Fragility and aggregation of GLRV (bar = 0.05 μm).

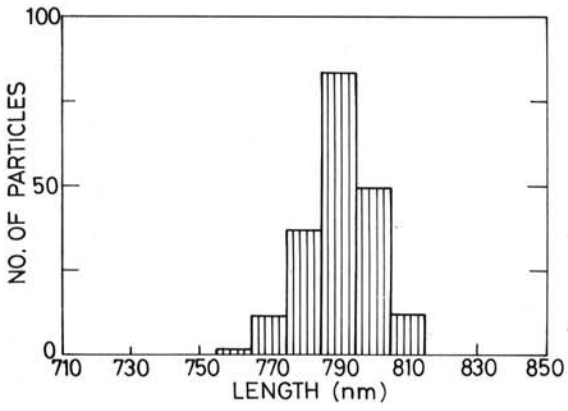


Fig. 3. Statistical distribution of lengths of purified grape leafroll virus particles.

to spectral analysis. Virus suspensions were brought to 0.01 M with NaOH, and then either diluted at least one hundred times, or dialyzed against water. Spectra of similarly-treated GLRV and TMV were compared.

Ribonucleic acid was determined by orcinol (2) and also was estimated by phosphorus analyses. Samples of 10-100 μliters of virus suspension were pyrolyzed on a strong flame with 10% magnesium nitrate. Pyrophosphates were hydrolyzed in 0.5 N HCl at 100 C, and phosphate was determined with 0.42% ammonium molybdate: 10% ascorbic acid (6:1, v/v). Samples of TMV were similarly analyzed to give a standard curve. Protein was determined according to Lowry et al. (11). Grape leafroll virus banded at about the center of the tube when centrifuged to equilibrium in 36% (w/v) CsCl. However, when a band of host origin appeared, it was easier to separate in 33% CsCl in which GLRV banded at the lower quarter of the tube. Density of fractions was determined by weighing micropipetted 100-μliter samples.

Polyacrylamide gel electrophoresis was performed according to Loening (10). Ribonucleic acid was electrophoresed on 3% gels and proteins on 7.5% gels. Agarose-acrylamide (0.5%-1.7%) slab-gel electrophoresis was performed, and gels were stained with "Stains All", according to Dahlberg et al. (3). Urea and sodium dodecyl sulfate (SDS) gels were stained with Coomassie blue according to the procedure of Fairbanks et al. (6).

RESULTS

Initial purification of grape leafroll virus.—Leaves of *N. glutinosa* or *D. metel* were homogenized in a Waring Blendor in 0.1 M ethylenediaminetetraacetic acid pH 8.0 (2 ml per gram of leaf tissue), squeezed through cheesecloth, and centrifuged at 8,000 *g* for 15 min. The supernatant fluid was shaken with 0.2 volumes of CCl_4, centrifuged at low speed, and the resultant upper layer was collected. The CCl_4 treatment was repeated, after

which polyethylene glycol 6000 and NaCl were added to the clarified sap to make it 5% and 0.125 M, respectively. A precipitate was allowed to form during 1 hr in an ice bath. The precipitate was collected by centrifugation at 12,000 *g* for 20 min, resuspended in water, and centrifuged again as before. This procedure was repeated twice, and the two supernatant fluids were pooled and spun at 100,000 *g* for 90 min. The resultant pellet was resuspended in water overnight at 4C, centrifuged at 10,000 *g* for 10 min, and the slightly turbid supernatant fluid served as a purified GLRV preparation.

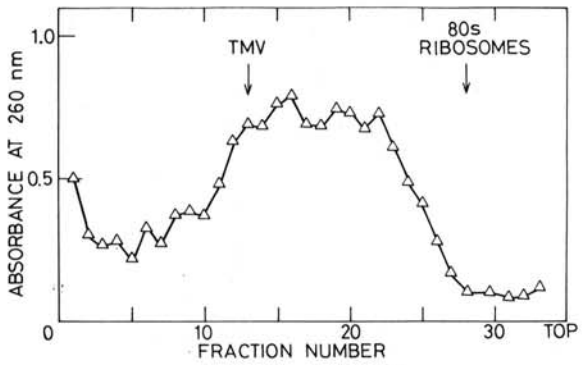


Fig. 4. Ultraviolet absorption pattern of a sucrose density-gradient centrifugation of purified GLRV. Arrows indicate the peak positions of tobacco mosaic virus (TMV) (180 S) and of locust oocyte ribosomes (80 S) that were centrifuged in sister tubes and served as markers. Grape leafroll virus infectivity could be obtained from about the center of the peak and all the way down to the bottom.

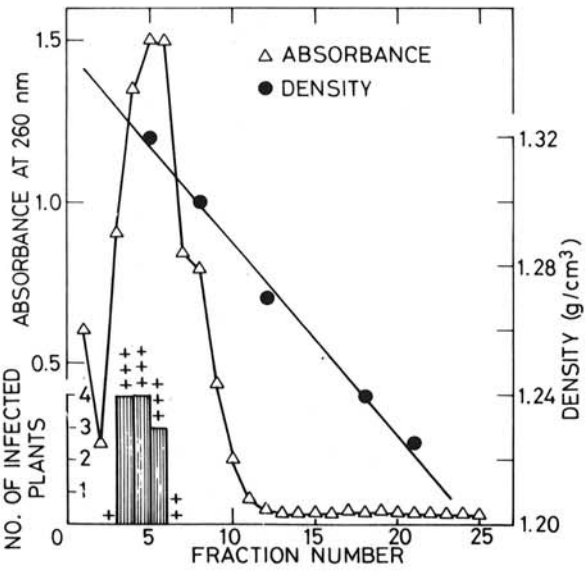


Fig. 5. Distribution patterns of ultraviolet absorption, infectivity, and particle count in fractions from CsCl equilibrium density gradient of grape leafroll virus (GLRV). A small sample of every fraction was negatively stained and the number of GLRV particles was estimated therein. The number of plus signs indicates the relative abundance of virus particles. Another portion of every fraction was measured at 260 nm and then inoculated onto four *Nicotiana glutinosa* plants to check its infectivity.

Further purification and characterization.—Purified GLRV was infectious. Electron micrographs revealed a mass of flexuous rods (Fig. 1), many of them broken, and a tendency of the particles to form aggregates (Fig. 2). The mean particle-size of GLRV is 13 × 790 nm (Fig. 3).

As expected from its wide size-distribution, GLRV demonstrated a polydisperse UV-absorbing pattern upon velocity-gradient centrifugation in 15-30% sucrose (Spinco L-5, rotor 41T; 45 min; 40,000 rpm). Infectivity was detected from approximately the center of the peak downward in the gradient. If the center of the UV zone was considered to be the position of the normal-length GLRV, then with the aid of the internal markers (180 S TMV and 80 S ribosomes from locust oocytes), the sedimentation coefficient of GLRV was estimated to be 150 S (Fig. 4).

Purified GLRV was suspended in 33% CsCl and centrifuged for 24 hr at 40,000 rpm (Spinco L-5, rotor 50 Ti). A visible opalescent band was observed at a density 1.32 g/cm^3. The following distribution patterns were found to coincide: (i) UV absorbancy, (ii) GLRV infectivity, and (iii) the count of virus particles observed by electron microscopy (Fig. 5). Some sharper, more intense lines could sometimes be observed within the

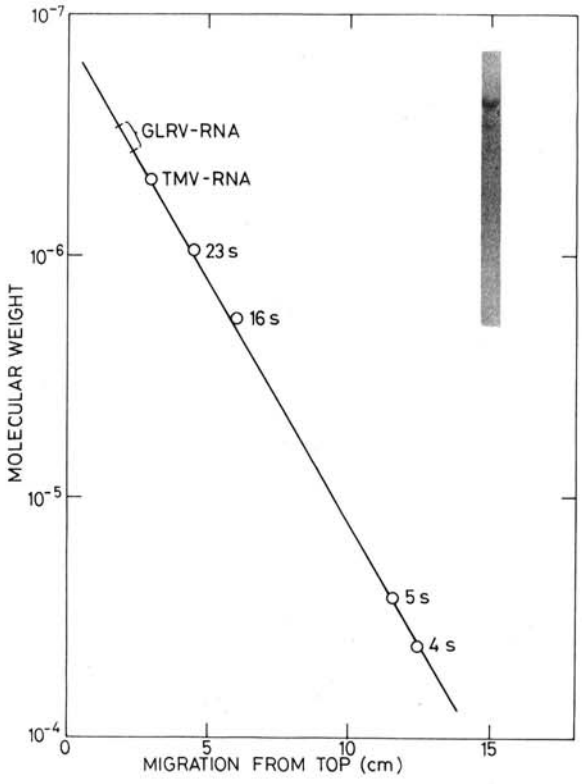


Fig. 6. Molecular weight determination for grape leafroll virus-RNA by agarose-acrylamide slab-gel electrophoresis. Molecular weight markers were ribonucleic acid (RNA) species extracted from *Escherichia coli* and from tobacco mosaic virus (TMV). The inset represents the homogeneity of the grape leafroll virus-RNA.

limits of the GLRV band. It seems as though there is some variation not only in particle size, but also in the particle composition. This is supported by the slight variation in size exhibited by GLRV-RNA (see below).

The nucleic acid of GLRV is RNA since virus preparations reacted positively with orcinol and negatively with diphenylamine. In addition, GLRV-RNA that had been electrophoresed on gel slabs, was stained by "Stains All" in a manner typical for single-stranded RNA.

The UV spectrum of alkali-disrupted GLRV was very much the same as that of similarly treated TMV (A260/280 = 1.15), indicating about 5% RNA in GLRV. The amounts of RNA and protein in GLRV also were estimated by the orcinol reaction, by its phosphorus content, and by the Lowry reaction for proteins. In each test, TMV of similar range of absorbancy served as standards. The GLRV composition was found to resemble TMV in each of the above tests (4.8 - 5.5% RNA).

Virus components.—Infectious RNA was extracted from purified GLRV with phenol. When GLRV-RNA was electrophoresed on agarose-acrylamide slab gels, a single, though somewhat wide, band was observed (Fig. 6). Ribonucleic acid markers were electrophoresed on adjacent slabs, and providing that the log-linear proportion of the distance of migration with molecular weight still holds at this range, the molecular weight of GLRV-RNA is 2.9 - 3.5 $\times$ 10^6 daltons. The wide zone could represent size distribution due to some heterogeneity in virus composition, but no distinct bands could be observed within the zone. In another experiment, GLRV-RNA was electrophoresed on 3% polyacrylamide gel, after which the gel was scanned at 260 nm. When the UV-absorbing zone, which migrated much slower than a marker of TMV-RNA, was sliced out and the RNA eluted, it was found to be infectious. Hence, the material was positively identified as GLRV-RNA.

Cesium chloride-banded GLRV was brought to 7 M

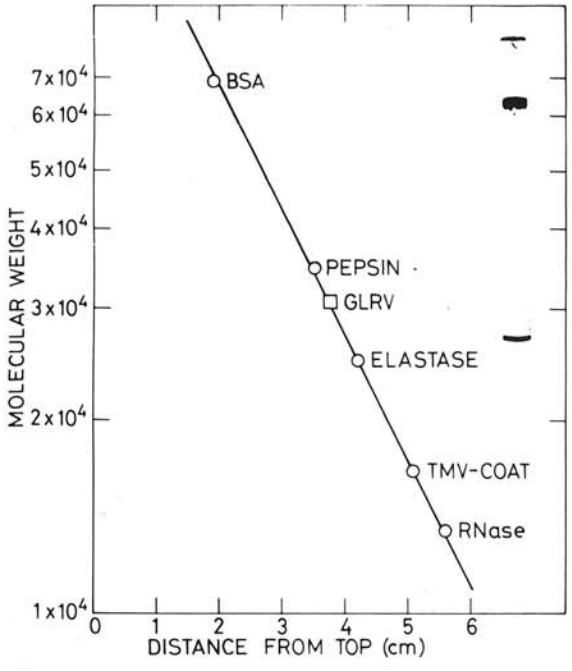
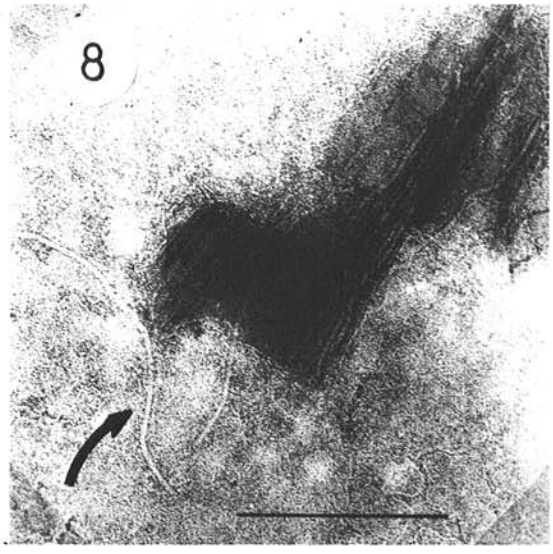


Fig. 7. Molecular weight determination for grape leafroll virus protein by sodium dodecyl sulfate-polyacrylamide gels. The inset represents the homogeneity of the virus protein in urea-polyacrylamide gel.



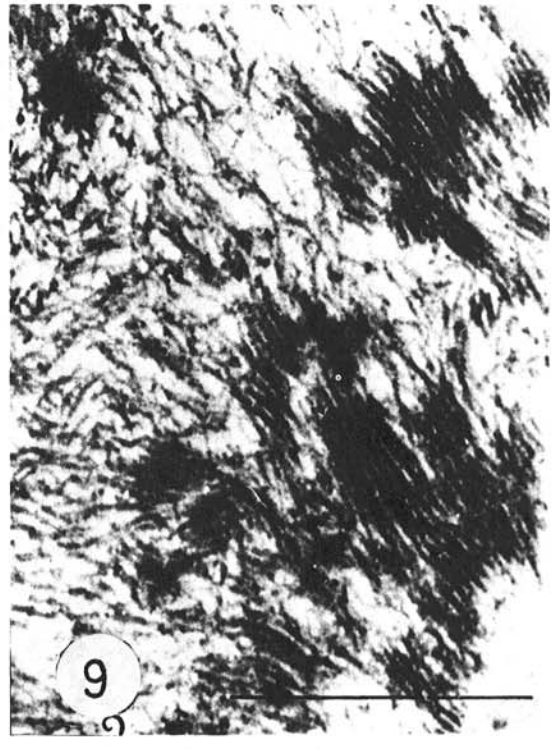


Fig. 8-9. Grape leafroll virus (GLRV). **8)** Negatively-stained dip preparation from leaf of *Nicotiana glutinosa* infected with GLRV. **9)** An ultrathin section through a sieve tube in the secondary phloem of GLRV-infected grapevine cane (courtesy of Dr. T. A. Shalla). Bar = 0.05 μm.

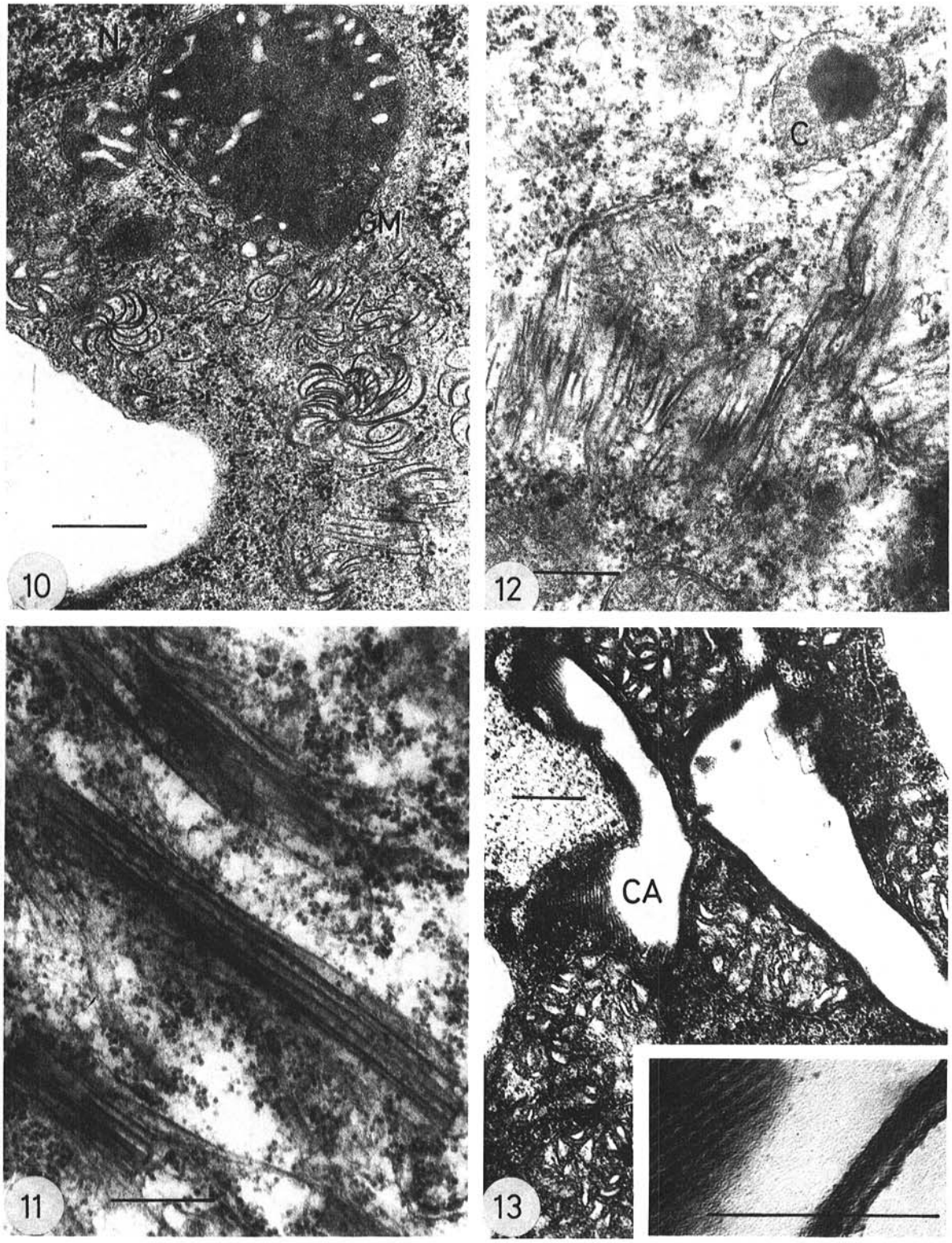


Fig. 10-13. Ultrathin sections through GLRV-infected tissues. **10)** Pinwheels (PW) and giant mitochondria (GM) in the elongated zone of *Nicotiana glutinosa* root. N = nucleus. **11)** Fascicular assemblages in a root cell of *Datura metel*. **12)** Similar to Fig. 12. Note crystal (C). **13)** *D. metel* root cell showing deformed mitochondria and vacuoles containing arrays of crystalline matter (CA and inset). Bars in all these micrographs represent 0.05 μm.

with urea and electrophoresed in 7.5% gels containing 7 M urea. A single proteinaceous band was observed. The same procedure was repeated with SDS-disrupted GLRV in a gel system containing 0.2% SDS throughout. In the SDS system, beside a major band that comprised over 90% of the protein, a minor band of heavier material also was detected. The addition of 10 mM mercaptoethanol did not change the above patterns. Owing to the homogeneity of the urea-prepared protein, the minor SDS-protein band was considered to be an aggregate. The molecular weight of the major GLRV-protein was determined from SDS-mercaptoethanol gels as 31,000 daltons (Fig. 7).

On the basis of 5% RNA of a molecular weight of 3.5×10^6 in GLRV, the virus particle is 7×10^7 daltons, and is coated by about 2,100 identical proteinaceous subunits.

Cytopathology of grape leafroll virus (GLRV)-infected tissue.—We were not able to detect any virus-like structure in thin sections of grapevines, *N. glutinosa*, or *D. metel.* However, we did find particles of virus shape and size in leaf dips from the above herbaceous plants (Fig. 8), but not from grapevines. Shalla and Goheen (*personal communication*) did, however, find particles resembling the presently reported ones in an ultrathin section of GLRV-infected grapevine cane, though only in one out of hundreds of sections examined (Fig. 9).

Various types of inclusions and other cytopathological manifestations were readily observed in GLRV-infected tissues of *N. glutinosa* and *D. metel.* These included pinwheels and giant mitochondria (Fig. 10), bundles of filaments (Fig. 11, 12), and arrays of crystalline matter (Fig. 13). None of these inclusions was detected in infected grapevine tissues.

DISCUSSION

The description of the GLRV particle, based on the above analytical data, is of a flexuous rod, measuring 13×790 nm, and weighing 70×10^6 daltons. Its single-stranded RNA molecule weighs 3.5×10^6 daltons and its approximately 2,100 identical protein subunits (31,000 daltons each) are arranged in helical symmetry as suggested by the cross-banding of the particles. The above data correspond well with information available for other members of the potyvirus group (4, 8, 9, 12, 13). The virus characteristics and the cytopathology of GLRV-infected tissue, clearly place this virus in the PVY group as reviewed by Edwardson (5).

Our inability to detect GLRV particles in grapevines could be attributed to high dilution, or to a very specific localization of the virus in this particular host. Another possibility to be taken into future consideration is that GLRV seldom appears as a mature virus particle in grapevine. A major obstacle in exploring this possibility is the present difficulty encountered in extracting RNA suitable for analytical studies from grapevine tissues which are particularly rich in polysaccharides and other mucilaginous substances.

Ribonucleic acid extracted from GLRV-infected *N. glutinosa* leaves could infect Mission grapevine indicator and typical leafroll symptoms developed. Furthermore, it was possible to extract the above infected Mission leaves with phenol and with that extract to successfully re-infect *N. glutinosa* with GLRV. However, since we could not find any other physical evidence for the presence of this virus in grapevines [beside the single finding of Shalla and Goheen (*personal communication*) as reported above], we prefer, at this stage, to refer to it as a "leafroll-associated virus".

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