# Purification of Curly Top Virus

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ABSTRACT

Curly top virus was partially purified from charcoal-clarified homogenates of frozen bean (*phaseolus vulgaris* L. 'Bountiful') stem tissue by differential and density-gradient ultracentrifugation. In rate sucrose density-gradient tubes, infectivity was associated with a visible band located 14-17 mm below the tube meniscus. The infectious band contained isometric particles having about 20% nucleic acid content and a sedimentation coefficient of 82 S. Traces of contaminating host materials were detected by cellulose acetate strip electrophoresis.

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Curly top virus (CTV) infects a wide range of plants (6), is known to remain infectious for long periods in tissue extracts(5), and can withstand exposure to several organic chemicals (1). Despite its relative stability in vitro, there are few published reports of attempts to purify CTV. Consequently, little is known about the properties of the infectious particle. A brief report in 1956 (7) indicated that rod-shaped particles were observed in partially purified extracts from CTV-infected sugarbeet leaves. In contrast, Bennett (2) mentioned unpublished results in which small spherical bodies were observed when phloem extracts from CTV-infected plants were passed through membranes having a 25-nm pore size.

The absence of an efficient method for transmitting CTV by mechanical techniques, combined with the lack of a known lesion assay host, has apparently discouraged most workers from attempting detailed purification studies. In preliminary studies we found that infectious CTV preparations could be obtained with any of several standard clarification procedures. However, each procedure left considerable amounts of normal plant components in the solutions, many of which were difficult to separate from the virus. This report describes a procedure by which we obtained highly infectious CTV preparations that contained only small amounts of host components.

MATERIALS AND METHODS.— Seed of *Phaseolus vulgaris* L. 'Bountiful' were planted in greenhouse flats and exposed 24 hr to viruliferous leafhoppers (*Circulifer tenellus* Baker) as the germinating seedlings emerged from the soil. Seven to 10 days later when the young trifoliolate leaves began to show severe epinasty and die, the plants were harvested and the primary leaves were discarded. The stems and petioles were frozen until used.

Forty-g samples of frozen tissue were homogenized in a blender with eight volumes of distilled water, the juice expressed through cheesecloth and 12% (wt/tissue wt) activated charcoal (Mallinckrodt) added. The mixture was centrifuged 20 min at 20,000 rpm in a number 30 rotor using a Beckman model L preparatory ultracentrifuge. The infectious supernatant fraction was concentrated into 2-ml distilled water by two cycles of differential ultracentrifugation (39,000 rpm for 1 hr and 10,000 rpm for 10 min in a number 40 rotor). Concentrated samples were layered onto rate sucrose density-gradient tubes (3) prepared 18 hr earlier using 4, 7, 7, and 7 ml of 10, 20, 30, and 40 percent sucrose, respectively. The samples were ultracentrifuged 2 h at 24,000 rpm in an SW 25.1 rotor. Homogeneity of final preparations was examined by cellulose acetate electrophoresis using a Shandon electrophoresis unit and Titan III cellulose acetate strips.

Infectivity assays were made using the insect vector. Solutions to be assayed were diluted up to $10^{-3}$ (depending upon the experiment) with 3% sucrose in distilled water. Each sample was assayed at 3-4 dilutions. Approximately 0.3 ml of each dilution was placed between two layers of Parafilm (4) stretched over small cages containing 10-20 nonviruliferous leafhoppers. The insects were allowed to feed through the membrane overnight under fluorescent light and then used to inoculate young sugarbeet seedlings. For experiments designed to associate infectivity with visible density-gradient zones, the number of seedlings showing symptoms was determined periodically for each dilution and an infectivity index calculated. The numbers of plants expressing symptoms at each observation date were combined for a given dilution and the sum multiplied by the negative logrithm of the dilution. The infectivity index represented the sum of all dilutions.

Fractions from density gradients layered with bean stem tissue were concentrated into distilled water by centrifugation, applied to Formvar-coated grids, negatively stained with 1% phosphotungstic acid, pH 7.0, and examined in a Hitachi HU-125 E-1 electron microscope.

TABLE 1. Relative infectivity of samples removed by side puncture from visible zone regions of a rate density-gradient tube containing a preparation from infected bean tissue

| Sample depth (mm) | Relative infectivity[a] |
|---|---|
| 8-12 | 0 |
| 14-18 | 128 |
| 20-25 | 1 |

[a] Reciprocal of highest infectious dilution.

RESULTS.— In every experiment density-gradient tubes layered with preparations from infected tissue contained a faint, noninfectious zone 10-13 mm below the tube meniscus and a bright zone 14-18 mm below the meniscus. This bright zone contained most of the infectivity found in the tube (Table 1). In some experiments a faint, noninfectious zone was present 21-23 mm below the meniscus.

Preparations from healthy tissue produced no visible density-gradient zones in some experiments but in other experiments produced two or three visible zones located in nearly the same positions as those obtained with infected tissue preparations. In these latter experiments, the middle zone from healthy tissue always appeared to be slightly higher (13-15 mm) and less intense than the comparable zone from infected tissue (Fig. 1A). The presence or absence of visible zones in control tubes seemed to be related to the batch of healthy tissue used. Some batches rarely produced these zones, whereas other batches invariably did. We did not examine growth conditions that may influence clarification of bean stem tissue.

In experiments where no zones were observed in the control tubes, the infectious 14-17 mm zone obtained from infected tissue contained material with an ultraviolet (UV) absorption spectrum typical of a nucleoprotein with about 20% nucleic acid (260/280 ratios = 1.68-1.70). On the other hand, the absorption spectra of the top and bottom zones appeared more typical of plant ribosomes (260/280 ratios = 1.9 or above) indicating that ribosomes were consistently more difficult to remove from infected tissue extracts than from healthy extracts. In experiments where zones were visible in control tubes, the absorption spectra of all zones in both tubes appeared typical of ribosomes. In these cases, ribosomal contamination was largely removed when the density-gradient tubes were fractionated into 1-ml fractions, each fraction diluted 12-fold with distilled water and reconcentrated into 1 ml distilled water by differential ultracentrifugation. No UV-absorbing material remained in fractions from density-gradient tubes containing healthy tissue preparations (Fig. 1B). Fractions 6-10 from the meniscus of tubes layered with CTV contained substantial amounts of UV light absorbing material and essentially all

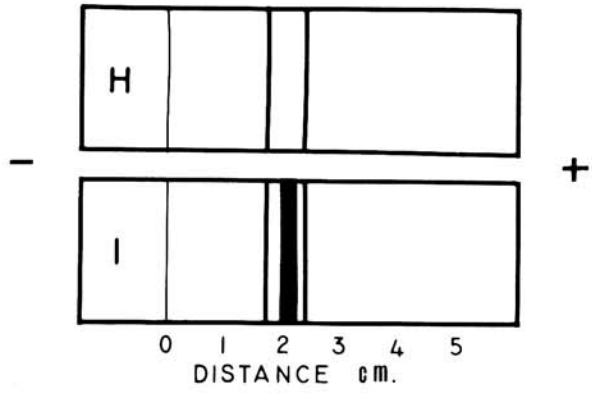


**Fig. 2.** Diagram of the migration patterns of reconcentrated density-gradient fraction 8 from healthy (H) and infected (I) bean stem tissue electrophoresed 10 min on cellulose acetate strips at 2 ma/strip in 0.05 ionic strength Electra HR buffer, pH 8.8.

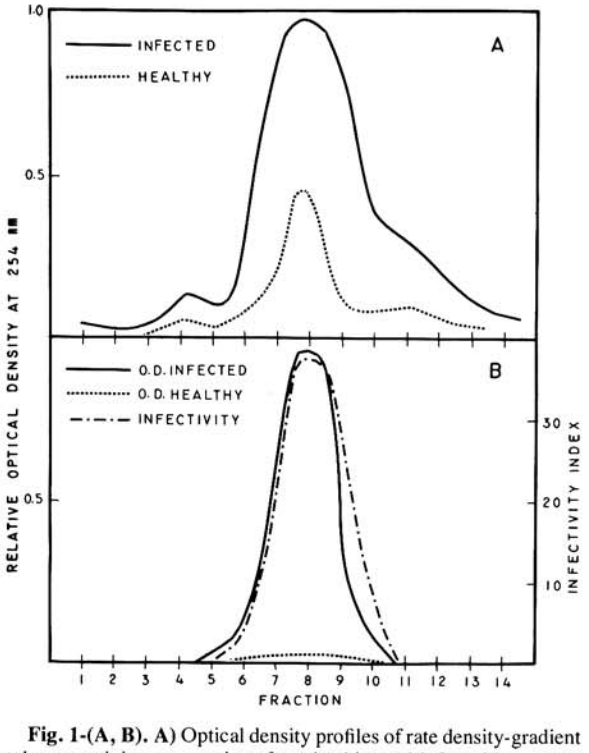


**Fig. 1-(A, B). A)** Optical density profiles of rate density-gradient tubes containing preparations from healthy and infected bean stem tissue. Sedimentation is from left to right. **B)** Association between optical density and infectivity in reconstituted density-gradient fractions.

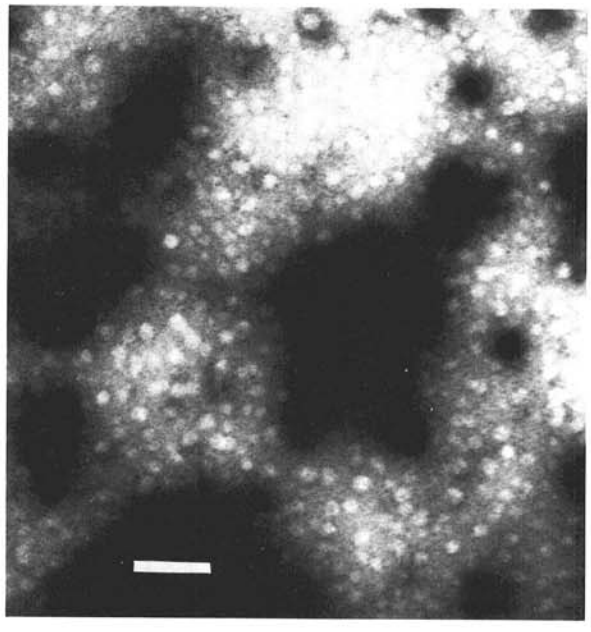

**Fig. 3.** Electron micrograph of reconcentrated fraction 8 from a density-gradient tube containing purified curly top virus negatively stained with 1% phosphotungstic acid. Bar represents 0.1 μm.

of the infectivity (Fig. 1B). The infectivity of fractions 6-10 was proportional to the UV absorbance present. The 260/280 ratio of reconcentrated fractions 7-8 was 1.68-1.70 suggesting little contamination by plant ribosomes.

The electrophoretic homogeneity of fraction 8 from control and virus tubes was examined by applying 100 $\mu$l of each fraction to Titan III cellulose acetate strips. The strips were electrophoresed at 2 ma/strip for 10 min in Electra HR buffer, pH 8.8 at 0.05 ionic strength and stained with Ponceau S stain. The results indicate that even though traces of plant ribosome components remained in both samples (Fig. 2) the preparation from infected tissue contained a major component not found in healthy tissue.

Isometric particles ca. 28 nm in diameter were observed in fractions 7, 8, and 9 reconcentrated from density-gradient tubes containing infected tissue preparations but were not observed in similar healthy tissue preparations (Fig. 3).

A sedimentation coefficient of 82 S was estimated for purified CTV in rate sucrose density-gradient tubes using peanut stunt virus (98 S) as the standard.

DISCUSSION.— We found no evidence to support the contention that CTV may be a rod-shaped virus. Our results indicate that CTV is a small isometric particle containing nearly 20% nucleic acid based on the 260/280 ratio. The sedimentation coefficient of 82 S that was estimated for CTV is very close to that of the 80 S plant ribosome fraction which makes it difficult to isolate totally pure virus by density-gradient procedures alone. In addition ribosomes from infected tissue appeared more difficult to remove than those from healthy tissue. Additional separations by electrophoresis or molecular sizing on agarose columns may be both useful and necessary to remove residual amounts of ribosomal contamination.

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