Purification of Curly Top Virus

David L. Mumford

Plant Pathologist, USDA Agricultural Research Service, Crops Research Laboratory, Logan, Utah 84322.

Cooperative investigations of the USDA Agricultural Research Service, U.S. Department of Agriculture; the Beet Sugar Development Foundation; and the Utah Agricultural Experiment Station.

The author thanks George Cochran and John Chidester, Utah State University, for assistance with procedures and equipment and Fred Hayes for the electron micrograph.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Accepted for publication 9 July 1973.

ABSTRACT

Curly top virus was purified from 'Turkish' tobacco. Extracts were clarified with chloroform and butanol. The virus was concentrated, either by precipitation with polyethylene glycol and NaCl, or by ultrafiltration. Clarified virus was partially purified on sucrose-density gradients, then further purified by gel chromatography, using agarose. Purified preparations had max ultraviolet-light absorption at 260 nm and min absorption at 240 nm and contained isometric particles ca. 20 nm in diameter. The virus was followed through the purification procedure by a plant-infectivity bioassay in which the insect vector fed on the infectious preparations.

Phytopathology 64:136-139

Additional key words: density-gradient centrifugation, gel chromatography, electron microscopy, sugarbeet.

There are few published reports of attempts to purify curly top virus (CTV). A brief report (12) in 1956 indicated that rod-shaped particles had been observed in partially purified extracts from sugarbeet leaves. Bennett mentioned (2) unpublished results in which small spherical bodies were observed when phloem exudate was passed through membranes having a pore size of about 25 nm. The absence of an efficient method of mechanically transmitting CTV and the lack of any known local lesion host have discouraged attempts at purification.

Utilizing a plant-infectivity bioassay accomplished by allowing the insect vector *Circulifer tenellus* Baker to feed on infectious solutions, the virus was followed through a purification procedure that is believed to have yielded nearly pure virus particles. A description of this procedure and an electron micrograph of the resulting particles are presented here.

METHODS AND RESULTS.— A virulent isolate (66-10) of CTV (7) was used throughout this investigation. The virus was maintained in a susceptible cultivar of sugar-

beet *Beta vulgaris* L. 'US 33'. The source plant for virus extraction was tobacco *Nicotiana tabacum* L. 'Turkish'. Tobacco was chosen instead of sugarbeet because it was suspected that sugarbeet contained many substances that were potential inhibitors during virus extraction. Initial tests indicated that crude extracts from infected tobacco were more infective than similar extracts from either roots or leaves of sugarbeet.

Eight- to 9-wk-old tobacco plants were inoculated by caging five to ten viruliferous beet leafhoppers on each plant for 5 days. These leafhoppers had acquired CTV by feeding on infected sugarbeets for 7 days. Fourteen to 21 days after inoculation, the entire top half of each plant, which showed severe symptoms, was harvested and extracted.

Bioassay.— The bioassay for CTV consisted of allowing leafhopper vectors to feed on virus preparations (3), and using these leafhoppers for inoculations in a plant-infectivity test. Preparations to be assayed were diluted 10^{-1}, 10^{-2}, and 10^{-3} in 10% sucrose solution containing 0.007 M Na citrate (4). About 0.3 ml of each dilution was placed between two layers of Parafilm (9) stretched over a small cage containing 8 to 12 nonviruliferous leafhoppers. The leafhoppers were allowed to feed on the preparations through the membrane overnight under fluorescent light. These leafhoppers were then used to inoculate 17- to 18-day-old seedlings of highly susceptible inbred EL 31 sugarbeets. Two leafhoppers were caged for 6 days on a cotyledon of each of four seedlings per dilution. Beginning 5 days after inoculation, the number of seedlings showing symptoms was determined periodically, with the last determination being at 14 days. An infectivity index was calculated for each preparation, based on the time required for symptoms to appear and the number of plants infected at each of the dilutions (8). An illustration of how the infectivity index was obtained is shown in Table 1.

Extraction, clarification, and concentration.— Figure 1 outlines the procedures used to purify CTV. All procedures were done at room temperature except centrifugation, which was done at 5 C. The virus was extracted by homogenizing diseased tobacco tissue with 0.01 M phosphate buffer (KH_2PO_4-Na_2HPO_4) (pH 7.0) containing 0.01 M Na_2SO_3 and 0.001 M ethylenediaminetetraacetic acid (EDTA) at the rate of 2 ml/g of tissue. Pulp and coarse plant fragments were removed by squeezing homogenate through four layers of cheesecloth and centrifuging the filtrate at 5,000 rpm for 15 min. All low-speed centrifugations were done in a Sorvall SS-3 by using a type GSA rotor.

TABLE 1. Determination of the infectivity index of a virus preparation based on time of infection and dilution of virus

Dilution	Infectivity: Days after inoculation 5	7	11	14	Sum [a]	Multiplex [b]	Product
10^{-1}	1 [c]	2	4	4	11	1	11
10^{-2}	0	1	2	3	6	2	12
10^{-3}	0	0	1	1	2	3	6
						Infectivity Index	29

[a] Sum of all plants showing symptoms at all dates for each dilution.

[b] Negative log of the dilution.

[c] Number of seedlings infected of four inoculated.

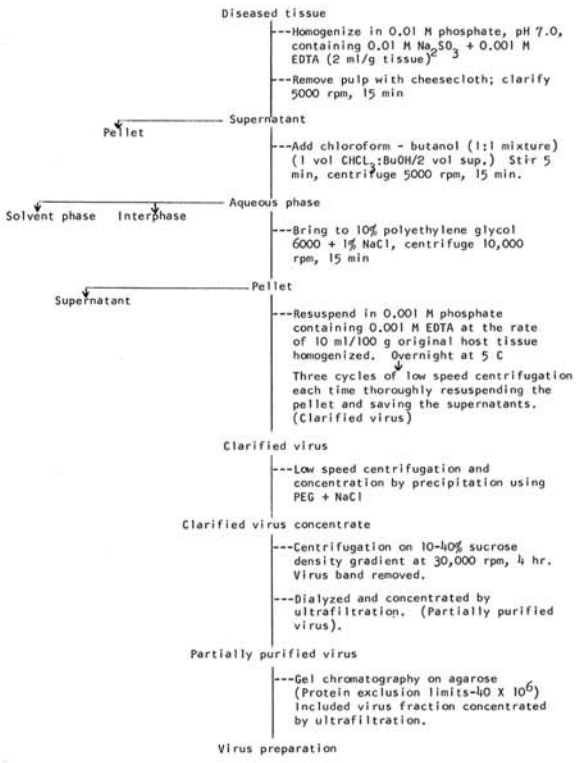

Fig. 1. Procedure for purification of curly top virus from infested tobacco tissue

One volume of a mixture of cold chloroform: butanol (1:1, v/v) was slowly added, with vigorous stirring, to two volumes of extracted juice (10). The mixture was stirred an additional 5 min, then centrifuged at 5,000 rpm for 15 min. A clear brown aqueous phase containing most of the infectivity was collected from the top of each tube. The virus was precipitated by bringing the aqueous phase to 10% with polyethylene glycol 6,000 (PEG) and 1% with NaCl (6) and centrifuging at 10,000 rpm for 15 min. All detectable infectivity was present in the pellet. The pellet was resuspended in 0.001 M phosphate buffer, containing 0.001 M EDTA, at the rate of 10 ml/100 g of original host tissue. After sitting overnight at 5 C, the preparation had accumulated considerable sediment, so it was centrifuged at 5,000 rpm for 15 min. The supernatant was saved, and the pellet was washed three times by thorough resuspension in 0.001 M phosphate-EDTA buffer and low-speed centrifugation. The three supernatants containing virus recovered by the washings were combined with the original, and the preparation was designated "clarified virus." The preparation could be stored at 5 C for several wk without detectable loss of infectivity. Before using this preparation on density gradients, it was centrifuged at low speed to remove sediment and concentrated by re-precipitation, using the same conc of PEG+NaCl used earlier. The final volume was adjusted to 2 ml/100 g of original host tissue.

Density-gradient centrifugation.— Sucrose gradients

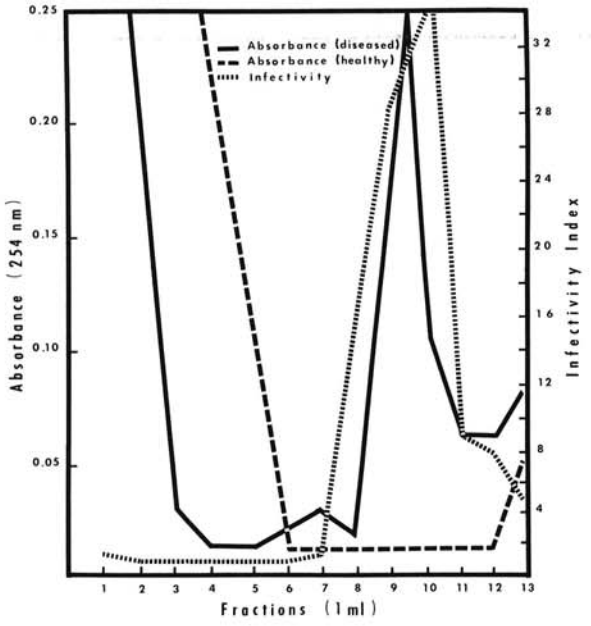

Fig. 2. Differences between absorbance profiles from density-gradient centrifugation of preparations from healthy and diseased tissue. Note association of infectivity with absorbance from diseased preparation.

were prepared in 0.001 M phosphate buffer containing 0.001 M EDTA. A 10 to 40% gradient was formed in 14-ml tubes 96 mm long, using a gravity-flow gradient former. One ml of clarified virus concentrate was layered on each gradient immediately after forming and centrifuged at 30,000 rpm for 4 hr at a temperature of 10 C. Density-gradient centrifugation was done in an International B-50 ultracentrifuge, using a type SB-283 swinging-bucket rotor.

After centrifugation, gradient-tube contents were fractionated into 1-ml fractions through an ultraviolet analyzer at a wave length of 254 nm. Ultraviolet-light absorbance profiles showed two zones of absorbance in diseased preparations that were not present in comparable healthy preparations (Fig. 2). Highest infectivity was associated with the lower zone, which was 6 cm below the meniscus, and indicated the virus was concentrated in this zone. Fractions containing the virus zone from several (usually six) density-gradient tubes were combined. Sucrose was removed and the virus was concentrated, using an Amicon ultrafiltration cell with an XM 100 Diaflo membrane. The preparation was usually reduced to a 2-ml sample, which was purified by gel chromatography. The preparation at this stage was designated partially purified virus.

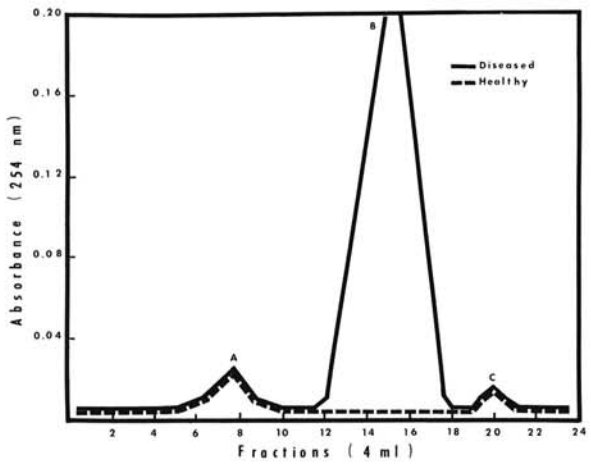

Fig. 3. Differences between absorbance profiles as healthy and diseased preparations were eluted from gel columns. Most infectivity was associated with peak B from the diseased preparation.

Gel chromatography.— Two ml of partially purified virus was allowed to flow through a 2.5×34 cm agarose-gel column (Sepharose 2B, approximate exclusion limits, 40×10^6 MW; Pharmacia Fine Chemicals) and eluted with 0.001 M phosphate buffer (pH 7.0). The void volume of the column was determined by using blue dextran 2,000 at a flow rate of 14 ml/h regulated by a peristaltic pump. Effluent from the column was monitored continuously with an ultraviolet analyzer at a wave-length of 254 nm. Chromatography elution profiles of preparations from diseased tissue had three absorbance peaks (A, B, C), while those from healthy tissue had only two (A, C) (Fig. 3). Peak A from the diseased preparation was excluded from the gel, and some infectivity (infectivity index of 3) was associated with this peak. Highest infectivity (infectivity index of 29), however, was associated with peak B, representing particles that had been included in the gel and eluted after the void volume. A similar peak was not present in preparations from healthy tissue (Fig. 3). A small amount of infectivity (infectivity index of 2) was also associated with peak C from diseased preparations.

Electron microscopy.— Those fractions eluted from the gel column that included the major peak B from diseased preparations were combined and concentrated by ultrafiltration. Comparable fractions from healthy preparations were also combined and concentrated. Both kinds of preparations were stained with 0.5% uranyl acetate (pH 4.8) and examined with a Zeiss EM 952 electron microscope. Isometric particles 18 to 22 nm in diam were observed (Fig. 4) in fractions from diseased preparations, but not in fractions from healthy preparations.

DISCUSSION.— Many different methods were tried during attempts to purify CTV. Only in the method presented, was it possible to get the virus to form a distinct zone during density-gradient centrifugation. In all other attempts, the virus was distributed through the density-gradient tube. Even in the procedure presented here, the partially purified virus obtained from density-gradient centrifugation was not sufficiently pure to permit satisfactory examination with the electron microscope.

Agarose-gel chromatography separated the virus from two other ultraviolet-absorbing components. This procedure was believed to yield nearly pure particles of CTV; however, some difficulty was still encountered in obtaining electron micrographs. The possibility that a breakdown material from the virus was interfering with microscope observation is postulated.

Particles of CTV are similar in shape but smaller in size than two other leafhopper-transmitted viruses. Oat blue dwarf virus was reported to be a small spherical particle

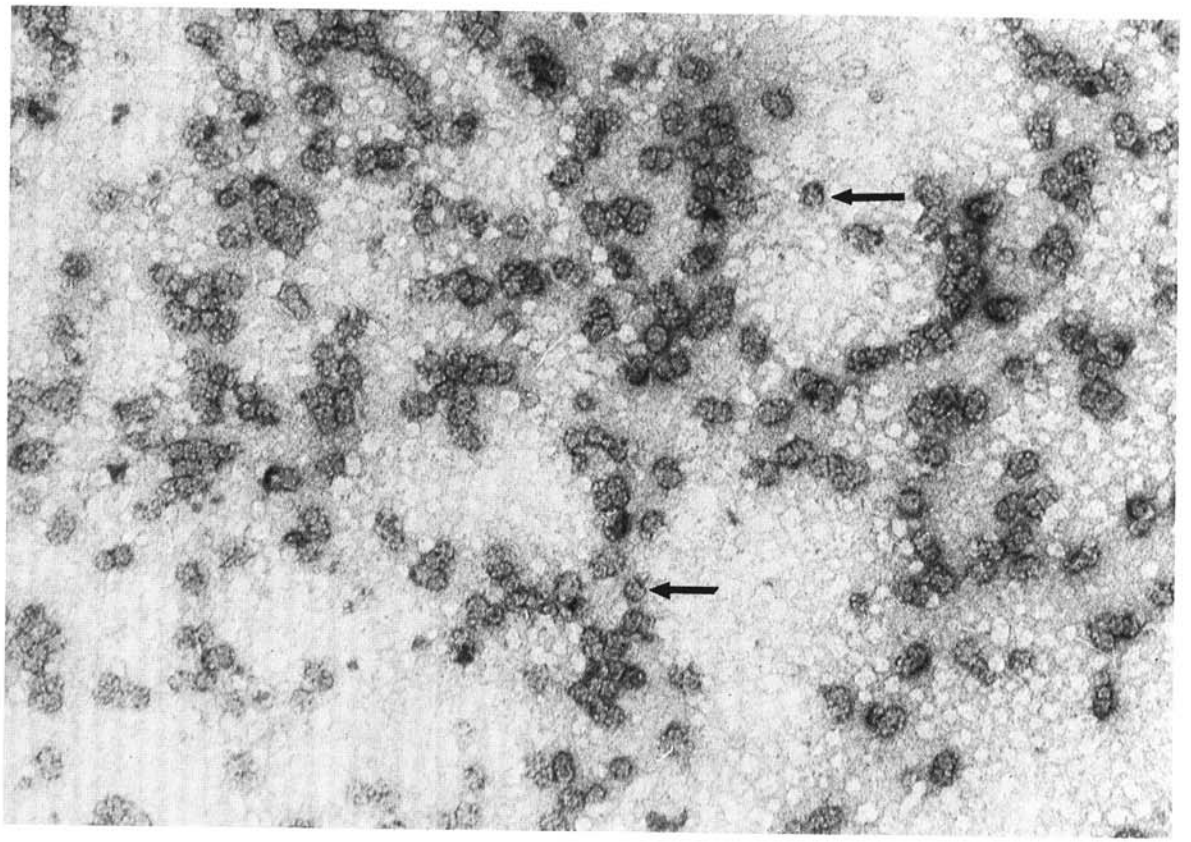

Fig. 4. Electron micrograph of curly top virus particles (18 to 22 nm in diam). Most particles are in groups of two or more. Single particles are indicated by arrows.

28 to 30 nm in diam (1), and rice tungro virus was reported to be 30 to 33 nm in diam (5). The surface structure of CTV particles appeared to have "knobs" (Fig. 4) similar to those noted by Steere (11) on particles of turnip yellow mosaic virus examined in crystals by the frozen-replica method.

LITERATURE CITED

1. BANTTARI, E. E., and R. J. ZEYEN. 1969. Chromatographic purification of the oat blue dwarf virus. Phytopathology 59:183-186.
2. BENNETT, C. W. 1971. The curly top disease of sugarbeet and other plants. Monograph No. 7. The American Phytopathological Society, St. Paul, Minn. 81 p.
3. CARTER, W. 1927. A technic for use with homopterous vectors of plant disease, with special reference to the sugar-beet leaf hopper, Eutettix tenellus (Baker). J. Agr. Res. 34:449-451.
4. FIFE, J. M. 1938. Effect of sodium citrate on release of curly top virus from alcoholic precipitate of plant juice. Phytopathology 28:561-574.
5. GALVEZ, G. E. 1967. The purification of virus-like particles from rice tungro virus-infected plants. Virology 33:357-359.
6. HEBERT, T. T. 1963. Precipitation of plant virus by polyethylene glycol. Phytopathology 53:362.
7. MUMFORD, D. L., and W. E. PEAY. 1970. Curly top epidemic in western Idaho. J. Amer. Soc. Sugar Beet Technol. 16:185-187.
8. RAYMER, W. B., and T. O. DIENER. 1969. Potato spindle tuber virus: a plant virus with properties of a free nucleic acid. 1. Assay, extraction, and concentration. Virology 37:343-350.
9. ROCHOW, W. F. 1960. Transmission of barley yellow dwarf virus acquired from liquid extracts by aphids feeding through membranes. Virology 12:223-232.
10. STEERE, R. L. 1956. Purification and properties of tobacco ringspot virus. Phytopathology 46:60-69.
11. STEERE, R. L. 1957. Electron microscopy of structural detail in frozen biological specimens. J. Biophys. Biochem. Cytol. 3:45-60.
12. THORNBERRY, H. H., and D. D. HICKMAN. 1956. Partial purification of curly-top virus. Phytopathology 46:29 (Abstr.)