Properties of a Strain of Tobacco Mosaic Virus Isolated from White Ash Trees

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ABSTRACT

The properties and identity of a virus isolated from leaves of white ash trees showing chlorotic ring spots and line pattern foliar symptoms have been determined. This virus has a dilution end point of 10^{-6} - 10^{-7} and a thermal death point of 97 C in crude sap of infected *Chenopodium* and tobacco leaves, and produced symptoms on 26 out of 45 hosts tested.

Electron microscopy of clarified sap of naturally infected ash leaves, and of purified virus preparation from inoculated tobacco, revealed that the virus particles were rodshaped and similar in size to those of tobacco mosaic virus (TMV). On sucrose density-gradient, the purified virus separated into one band which upon UV-monitoring gave a single infectious peak. Antiserum to the ash virus was produced which reacted positively with its homologous antigen and with two strains of TMV with a titer of 1:4,096.

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Of the virus and virus-like disorders reported to affect ash (12), there is no information as to the nature of the causal agents of ash infectious variegation (12), ash necrotic leaf curl (3), and ash leaf marbling (3). Ash chloro-necrotic leaf spotting has been associated with tobacco necrosis virus (2), and ash witches; broom (10) [which has now been reported to be widespread in New York (6) and Massachusetts (11)] has been associated with a mycoplasma (6).

In addition to these, a new disease of white ash was described from New York and was shown to be caused by a strain of tobacco ringspot virus (5). Similar virus-like symptoms were observed on white ash trees in Massachusetts in the late sixties, and a more systematic survey in 1972 showed that a disease causing a mosaic line pattern on white ash leaves was quite widespread in central and western Massachusetts (7). Initial attempts to transmit the virus and determine its properties, suggested that this virus was different from the one described by Hibben and Bozarth (5). It was, therefore, considered desirable to attempt to identify the apparently viral causal agent of the disease in white ash. A report on the transmission and partial host range of this virus is appearing elsewhere (8). This paper reports the identification and properties of the virus isolated from white ash trees.

MATERIALS AND METHODS. — Mechanical transmission attempts on various herbaceous and woody hosts were made by macerating one part of infected leaf tissue in four parts of water or 0.01 M pH 7.0 phosphate buffer. Systemically infected *Chenopodium amaranticolor* and *Nicotiana tabacum* '211' were used as sources of inoculum. *C. amaranticolor*, *N. glutinosa*, and *N. tabacum* cultivars 'Samsun NN' and 'Havana 425' were used as local lesion assay hosts.

Herbaceous host range and symptomatology. — Forty-five selected plant species were inoculated by rubbing Carborundum-dusted leaves with infectious sap diluted (1:4, v/v either in neutral phosphate buffer or in water. Plants were judged susceptible whenever they produced local lesions on inoculated leaves and/or some type of systemic symptoms within 21 days after inoculation. Inoculated plants not showing symptoms were checked for latent infection by back-inoculation to known susceptible plants. Since results of initial experiments indicated that this virus may be a strain of tobacco mosaic virus, a comparative host range study of the ash virus and two other strains of TMV was conducted. One of these strains was collected from a tobacco field near Amherst and is designated herein as 'field TMV'. The other TMV strain was supplied by R. F. Bozarth of Boyce Thompson Institute, originally obtained from F. O. Holmes and is herein designated 'Holmes TMV.'

Properties in crude sap. — Dilution end point, thermal inactivation point, and longevity in vitrowere determined using crude sap of systemically infected leaves of *C. amaranticolor* or *N. tabacum* '211', and bioassaying on local lesion assay hosts.

Properties of purified virus. — Several virus purification procedures were tested. The one finally selected was a combination of the procedures of Francki (4) and Steere (13). Sucrose density gradients were prepared by successive layering of 2.5 ml each of 10, 20, 30, and 40% sucrose solutions in 14-ml centrifuge tubes. One ml of purified virus sample was then layered onto the gradient and centrifuged at 44,000 *g* for 2 h at 4 C. Gradients were passed through an ISCO UA 2 ultraviolet monitor connected to an ISCO density gradient fractionator. Identical procedures were followed when using crude sap of tobacco leaves infected with 'field TMV', 'Holmes TMV', or the ash virus.

For serological assays, both purified ash virus from tobacco and virus infected crude sap from ash leaves, were tested by both the microprecipitin and the Ouchterlony agar gel double-diffusion methods (1). The ash virus was tested against antisera of the following viruses: (i) homologous antiserum prepared against the ash virus itself, and (ii) antisera PVAS-1, PVAS-31, and PVAS-52 of three different strains of TMV obtained from ATCC. Similar tests were carried out with both infected

TABLE 1. Host range and types of symptoms produced by susceptible plants inoculated mechanically with the ash isolate of tobacco mosaic virus

Species tested [a]	Symptoms [b]	
	On inoculated leaves	Systemic
Amaranthaceae		
Gomphrena globosa	Chl., L.L.	Chlorosis, occasional killing of apical meristem
Apocynaceae		
Vinca rosea 'Dwarf rosea'	-	-
Chenopodiaceae		
Beta vulgaris 'Baby Canning'	L.L.	Killing of plants
Chenopodium amaranticolor	Chl., L.L.	Mottle, ring-like pattern
C. quinoa	Chl., L.L.	Chl. rings, mottle
Compositae		
Zinnia elegans 'Exquisite'	L.L.	-
Cucurbitaceae		
Cucumis sativus 'National Pickling'	-	-
Cucumis sativus 'Marketer'	-	-
Cucurbita moschata 'Butternut'		
C. pepo 'Zucchini Hybrid'	-	-
C. pepo 'Early Gold Summer Crookneck'	-	-
C. pepo 'Early Prolific Straightneck'	-	-
Leguminosae		
Glycine max	-	-
Medicago sativa	-	-
Phaseolus olimensis 'Burpee's Fordhook'	-	-
Phaseolus vulgaris 'Pinto'	L.L.	-
Phaseolus vulgaris 'Scotia'	-	-
Phaseolus vulgaris 'Red Kidney'	-	-
Phaseolus vulgaris 'Harvester'	-	-
Phaseolus vulgaris 'Tendercrop'	-	-
Phaseolus vulgaris 'Topcrop'	-	-
Phaseolus vulgaris 'Garden Bean'	-	-
Vigna sinensis 'Early Ramshorn'	-	-
Oleaceae		
Fraxinus americana	Chl., L.L.	Chl. spots
F. pennsylvanica	Chl., L.L. & Chl. concentric rings	Local systemic rings
F. excelsior		
Solanaceae		
Capsicum frutescens 'Burpee's Hybrid'	L.L.	-
Datura stramonium	-	-
Nicotiana glutinosa	L.L.	-
N. rustica 'Pricilla'	L.L.	-
N. rustica 'Brasilia'	Necrotic rings	Stem necrosis
N. sylvestris	L.L.	Mosaic
Nicotiana tabacum 'Bel-W3'	L.L.	Stem necrosis
Nicotiana tabacum 'Bel-W4'	-	Mosaic
Nicotiana tabacum 'Havana 38'	-	Mosaic
Nicotiana tabacum 'Havana 425'	L.L.	Stem necrosis
Nicotiana tabacum '211'	-	Mosaic
Nicotiana tabacum 'J.W. Broadleaf'	-	Mosaic
Nicotiana tabacum 'K-1'	-	Mosaic
Nicotiana tabacum 'Samsun NN'	L.L.	Occasional killing of plants
Nicotiana tabacum 'T-48'	-	Mosaic
Nicotiana tabacum 'Turkish'	-	Mosaic
Petunia hybrida 'Little Giant'	L.L.	
Solanum melongena 'Super-hybrid'	L.L.	Stem necrosis

[a] Four plants of each species were inoculated in each experiment. The experiment was repeated at least 10 times.

[b] Chl. L.L. = chlorotic local lesions. L.L. = necrotic local lesions.

crude sap and purified preparations of 'field TMV' and 'Holmes TMV.'

Electron microscopy.—Virus suspensions obtained from the sucrose density-gradient centrifugation, and from clarified sap of infected ash leaves from the field, were negatively stained with 1% phosphotungstic acid (PTA), placed on carbon-coated Formvar grids, and observed with a Philips 200 electron microscope.

RESULTS.—*Host Range.*—In herbaceous host range studies, the ash isolate of TMV was capable of infecting 26 of 45 species tested (Table 1). In most cases, the virus from ash produced reactions similar to those produced by both the "field" and "Holmes" TMV strains. This, in part, supports the view that the ash virus is a strain of TMV. But certain differences were also evident from the comparative host range study. The isolate from ash differed from 'field TMV' in that the former produced local lesions on 'Pinto' beans and systemic symptoms on eggplant while 'field TMV' did not. Furthermore, the ash virus differed from both 'field TMV' and 'Holmes TMV' isolates in that it did not infect *Datura stramonium*, while the other two induced local lesions on that host.

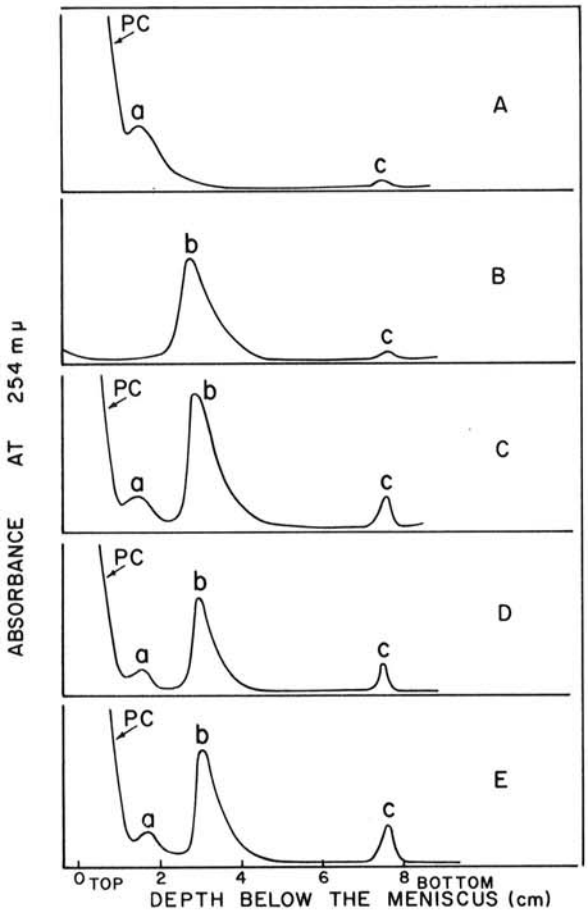

Fig. 1 (A to E). Comparative photometric scanning tracings of sucrose density-gradient tubes on which 1 ml of clarified healthy or infected tobacco sap or purified virus had been layered and centrifuged at 44,000 *g* for 2 hours. **A)** Healthy tobacco sap. **B)** Purified preparation of the ash isolate of TMV obtained from tobacco. (C), (D), and (E): Clarified sap from tobacco infected with **C)** the virus from ash; **D)** 'Holmes TMV'; **E)** 'field TMV.' Legend: PC = Top plant component. (a) = Another plant component, probably ribosomes. (b) = The virus peak. (c) = Bottom component, probably nuclear materials plus some aggregated or adsorbed virus. Peak (b) contained most of the infectivity, with some infectivity in peak (c).

Properties in crude sap.—Sap extracted from infected tobacco and *C. amaranticolor* leaves remained infective when heated to 96 C and 97 C, respectively, when diluted to 10^{-7} and when incubated at room temperature for at least 13 months. In crude sap of infected ash leaves from the field, the virus was still infective after 9 months at room temperature.

Properties of purified virus.—The ultraviolet 260/280 absorbancy ratio of the purified ash virus isolate following sucrose density gradient centrifugation was 1.23. Virus concentrations of up to 20 mg/ml were routinely obtained. During the rate-zonal sucrose density-gradient centrifugation, the ash virus isolate was concentrated into a single opalescent band about 3 cm below the meniscus and it gave only one distinct peak when the gradients were fractionated and monitored with the ultraviolet anlayzer (Fig. 1-b). Fig. 1 also illustrates the comparative photometric density gradient scanning of the ash virus isolate and the other two TMV strains. These results indicate that the virus from ash has the same sedimentation properties as the 'field TMV' and 'Holmes TMV' isolates.

Serology.—In microprecipitin tests, all three ATCC antisera against TMV and the antiserum prepared against the ash virus isolate reacted positively in high titers (1:1,024 - 1:4,096) to the ash virus antigen. The results were positive with both purified ash virus or virus in clarified sap of infected ash leaves, although in the latter case the titer was only 1:256. Using the agar gel double-diffusion method, positive reaction bands were obtained which indicated that the ash virus isolate is serologically related to tobacco mosaic virus.

Electron microscopy.—Fig. 2 and 3 show electron micrographs of the ash isolate of TMV from clarified sap of naturally infected ash leaves and of a purified virus preparation from *Chenopodium amaranticolor*, respectively. The electron micrographs show clearly that the particles of the virus causing the disease in white ash are rod-shaped. Although considerable fracturing and end-to-end aggregation was evident and did not allow determination of the average size of the particles, a few of the particles have lengths corresponding exactly to the length of TMV particles.

DISCUSSION.—The foliar symptoms of naturally infected white ash trees in the field initially suggested that the disease was caused by the same virus identified as an ash strain of tobacco ringspot virus (TRSV) (6). For this reason, initial experiments were directed towards isolating TRSV. The results described above, however, indicate that the ash virus isolated in Massachusetts has properties which are quite different from those of the ash strain of TRSV found in New York. Based on the properties of the virus described herein, there appears to be adequate evidence that this virus is a strain of tobacco mosaic virus.

The properties of the virus in crude ash sap and the characteristic local lesions on *Nicotiana glutinosa* first

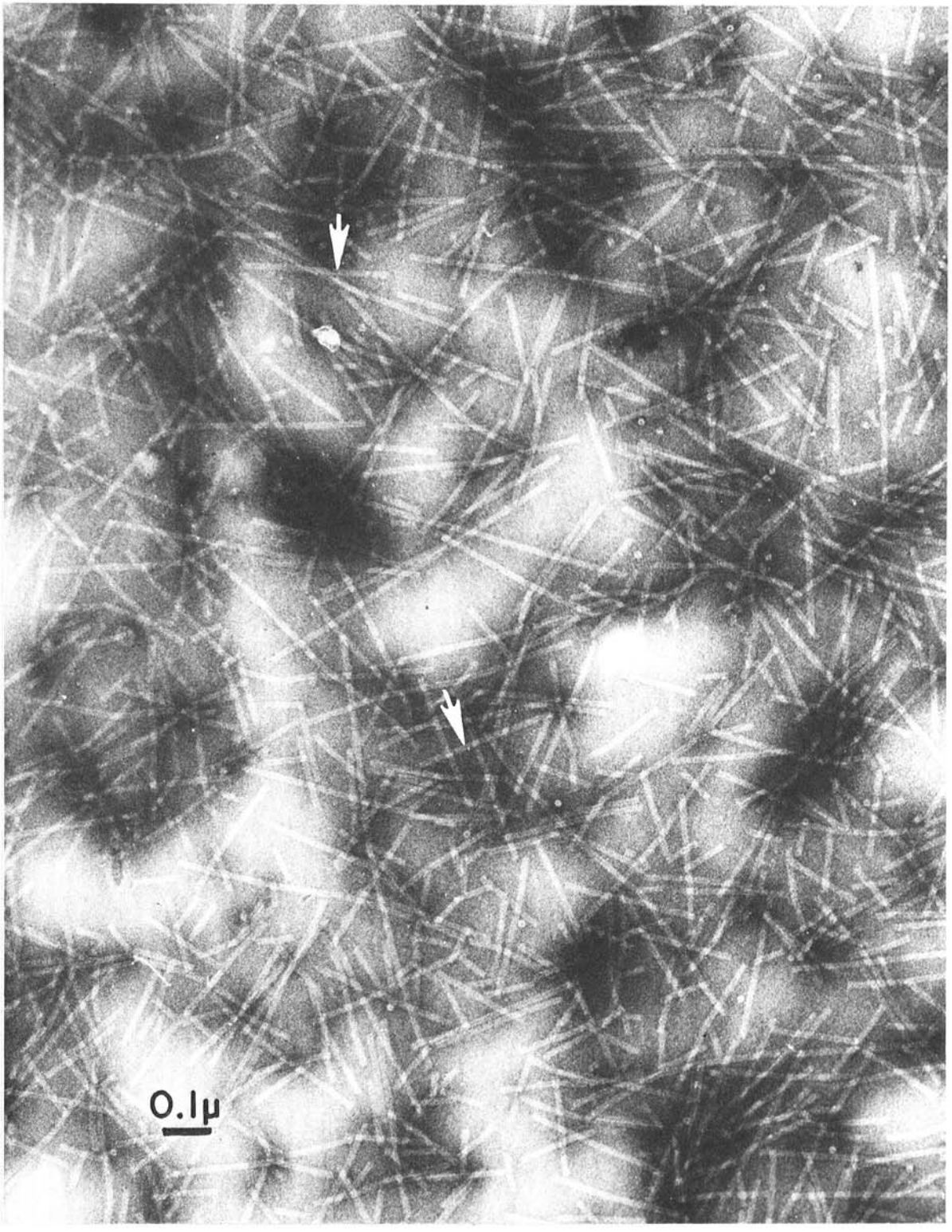

Fig. 2. Electron micrograph of negatively stained partially clarified sap from infected ash leaves revealing rod-shaped particles. Arrows point to particles similar in size to tobacco mosaic virus particles (×81,920).

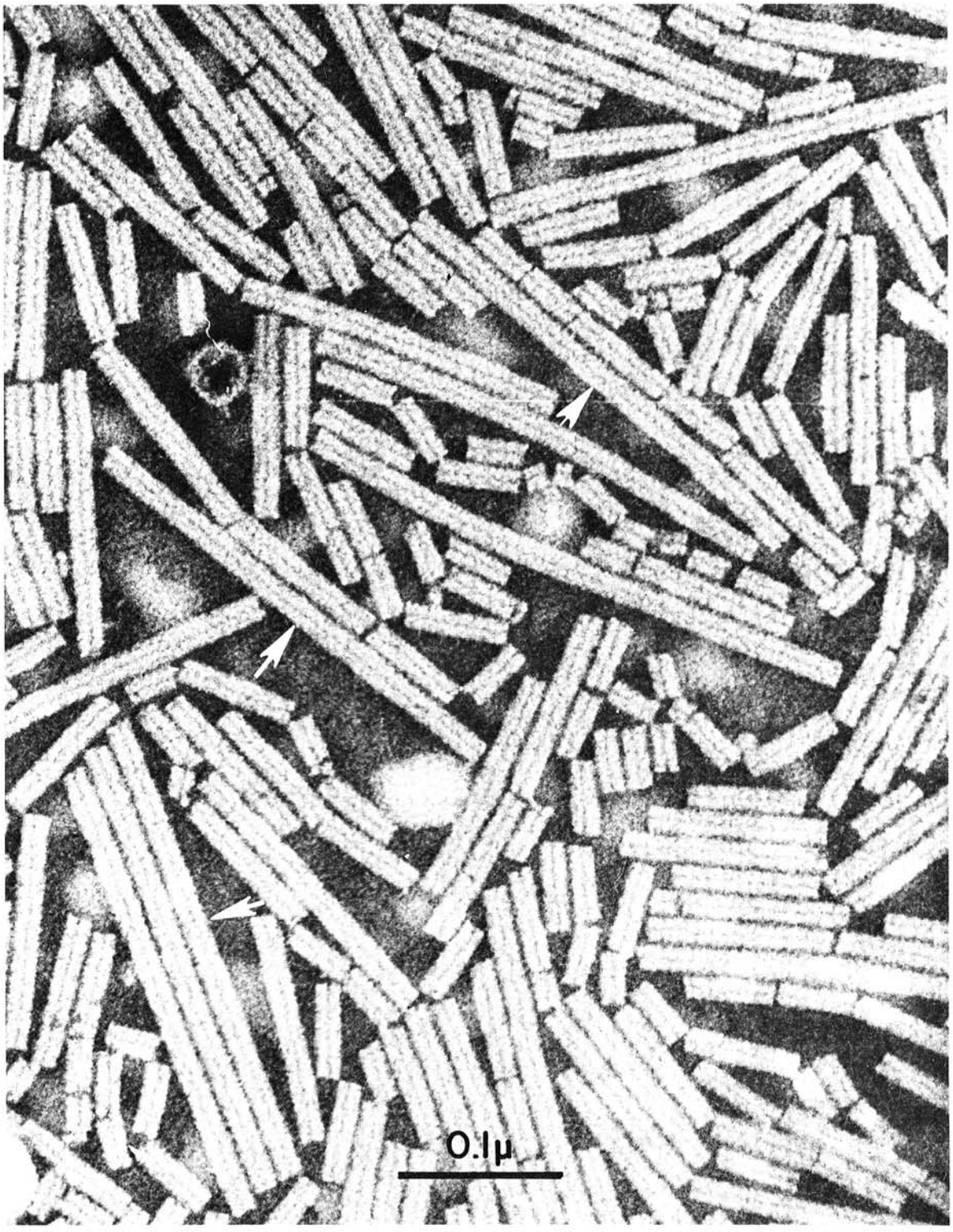

Fig. 3. Electron micrograph of a purified preparation of the ash isolate of TMV obtained from systemically infected *Chenopodium amaranticolor*. Arrows point to particles with lengths equal to the TMV particles (×241,260).

suggested that the virus from ash is a strain of tobacco mosaic virus. From that point on, extraordinary precautions were taken to ascertain that this was not a contaminant. Mortars and pestles were scrupulously washed and sterilized and hands were washed with soap twice in warm water before each mechanical transmission experiment. Numerous mechanical transmission experiments were performed with sap from infected ash leaves inoculated on *Chenopodium*; even the symptomatic ash leaves used for inoculum were rinsed with water to ascertain that no contaminating material was present on the surfaces of the leaves; in all cases the results were positive and identical. Partially clarified leaf sap from infected ash trees in the forest was taken for electron microscopy along with purified virus preparations from *C. amaranticolor* and *N. tabacum* '211'; all preparations contained similar rod-shaped virus particles. Furthermore, partially clarified sap of infected ash leaves was tested against TMV antisera along with sap of *Chenopodium* and *Nicotiana* infected with the virus from ash. The antigen from all these sources reacted positively and similarly with the TMV antisera. The results from electron microscopy, and from serology with this virus, were taken as positive and conclusive evidence that the virus from ash is an isolate of TMV and that the virus transmitted to and studied in *Chenopodium* and *Nicotiana* was not a contaminant.

Comparative host-range experiments showed mostly similarities and a few differences in the host reactions caused by these different isolates of TMV. It is apparent that the ash isolate of TMV produced different results from the other TMV strains in several of the hosts inoculated. For example, the ash isolate failed to infect *D. stramonium*, while the two other strains of TMV infected it.

Once it became apparent that the virus from ash was an isolate of TMV, purification procedures used for TMV (4,13) were employed to purify the ash isolate of TMV. It soon became evident, however, that the ash isolate has a propensity for fragmentation and end-to-end aggregation. These properties of the virus have made some phases of its characterization more difficult. Fig. 3 is an electron micrograph of purified virus prepared from *C. amaranticolor* and the degree of fragmentation of the virus is evident. The amount of aggregation and fragmentation was not reduced even when *N. tabacum* '211' was used as a source of virus for purification. However, the ultraviolet readings of purified preparations were as expected for TMV, and the 260/280 ratio was similar to that of purified common tobacco mosaic virus.

It would, therefore, appear that from the host-range results, the properties of the virus in crude sap, the electron microscopical observations, and the results of serological tests that the virus isolated from white ash tree leaves is a strain of tobacco mosaic virus. Although TMV had been isolated from other trees (9), this is the first case in which TMV has been found to be associated with and to cause disease symptoms on a tree.

LITERATURE CITED

1. BALL, E. M. 1961. Serological tests for the identification of plant viruses. Amer. Phytopathol. Soc., Ithaca, New York. 16 p.
2. CASALICHO, G. 1965. Chloro-necrotic leaf spotting of ash (Fraxinus excelsior). Monti e Boshi 16:39-46.
3. CIFFERI, R. A., C. CORTE, and D. RUI. 1961. Two viruses of Fraxinus: "necrotic leaf curl" and "leaf marbling". Riv. Pathol. Vegetale Ser. I: 111:241-250.
4. FRANCKI, R. I. B. 1966. Some factors affecting particle length and distribution in tobacco mosaic virus preparation. Virology 30:388-395.
5. HIBBEN, C. R., and R. F. BOZARTH. 1972. Identification of an ash strain of tobacco ringspot virus. Phytopathology 62:1023-1029.
6. HIBBEN, C. R., and B. WOLANSKI. 1971. Dodder transmission of a mycoplasma from ash witches' broom. Phytopathology 61:151-156.
7. LANA, A. O. and G. N. AGRIOS. 1973. A disease of white ash caused by tobacco mosaic virus. Phytopathology 63:203 (Abstr.).
8. LANA, A. O., and G. N. AGRIOS. 1974. Transmission of a mosaic disease of white ash to woody and herbaceous hosts. Plant Dis. Rep. 58:536-540.
9. NIENHAUS, F., and C. E. YARWOOD. 1972. Transmission of a virus from oak leaves fractionated with Sephadex. Phytopathology 62:313-315.
10. PLAKIDAS, A. G. 1949. Witches' broom, a graft transmissible disease of Arizona ash - Fraxinus berlanderi. Phytopathology 39:498.
11. SCHALL, R. A., and G. N. AGRIOS. 1973. Graft transmission of ash witches' broom to ash. Phytopathology 63:206-207 (Abstr.).
12. SELISKAR, C. E. 1964. Virus and virus-like disorders of forest trees. In FAO/IUFRO Symposium on Internationally Dangerous Forest Diseases and Insects, Vol. I, Meeting No. V. FAO(Food Agric. Organ., U.N.), Rome. 44p.
13. STEERE, R. L. 1963 Tobacco mosaic virus-purifying and sorting of associated particles according to length. Science 140:1089-1090.