Properties of the Nucleoprotein Associated with Maize White Line Mosaic in Wisconsin

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ABSTRACT

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A disease called maize white line mosaic was found on corn in Wisconsin in 1979. The disease was associated with isometric particles and seemed to be soilborne. Mechanical transmission from corn to corn and to other herbaceous hosts was not successful. A purification method is described for the presumed causal agent. The etiological agent, called maize white line mosaic virus (MWLMV), was approximately 35 nm in diameter, consisted of a single nucleoprotein component, had a sedimentation coefficient of 117 S, a buoyant density of 1.353 g/cm^3, contained ssRNA with a molecular weight of 1.25×10^6, and its coat protein had a molecular weight of approximately 32,000. The extinction coefficient ($E_1^{0.1\%}_{cm}$) of the virus at 260 nm was 3.9. The base percent composition of the nucleic acid was: U = 24.3%, G = 30%, A = 19.7%, and C = 26%. An antiserum was produced against the virus and used for identification of the pathogen in corn and other hosts in Wisconsin and for demonstrating its relatedness to a similar virus found in Ohio and Vermont.

During routine surveys for the presence of maize dwarf mosaic (MDM) in sweet corn in Wisconsin, one of us (SMS) found plants with symptoms strikingly different from those of MDM. Plants were severely stunted and leaves showed a bright mosaic consisting of white to yellow and yellow-green chlorotic rectangular to linear (1–2 cm) patches and stripes (Fig. 1). In contrast to plants infected with MDM, symptoms were not confined to the whorl leaves, but were present on all leaves and persisted after pollination.

Leaf dip preparations for electron microscopy showed a constant association of large icosahedral particles with plants exhibiting the symptoms. Plants with the symptoms were found in 17 of 18 sweet corn fields within an 8.05-km (5-mile) radius of the field where the first plants were observed. Although the overall incidence of infected plants was low, it was noticed that they seemed to be more prevalent along the edges of fields and in low lying areas. The disease was found in sweet corn in northeastern Wisconsin in Sheboygan, Manitowoc, and Kewanee counties and in field corn in southeastern Wisconsin in Racine County. All of these counties border on Lake Michigan.

A similar disease, tentatively named white line mosaic, was observed in corn during the 1979 growing season in New York (1), Ohio (D. T. Gordon, *personal communication*), and Vermont (A. R. Gotlieb, *personal communication*).

The potential threat of this "new" disease in corn prompted a study of the properties of the particles associated with the disease and of their transmission characteristics. For the purposes of this paper, the etiological agent will be presumed to be the nucleoprotein particle consistently associated with the disease and will be further referred to as a virus.

MATERIALS AND METHODS

Source of tissue. Infected plants (*Zea mays* L.) and plant tissues were collected from several sweet corn fields in Wisconsin and stored at 3 – 4 C or at −20 C. *Setaria viridis* L. plants with suggestive symptoms were potted and maintained in the greenhouse.

Transmission. Mechanical inoculation of sweet corn seedlings was attempted by using suspensions of highly concentrated partially purified virus and several different inoculation methods: corundum-dusted leaves and roots of seedlings were rubbed with virus suspensions, leaves were infiltrated with suspensions and then pin pricked in the water-soaked areas, and whorls were inoculated by means of pin pricks through droplets of virus suspensions. Inoculations of the following herbaceous hosts also were attempted: *Gomphrena globosa* L., *Vinca rosea* L., *Zinnia elegans* Jacq., *Cucumis sativus* L., *Cassia occidentalis* L., *Pisum sativum* L., *Phaseolis vulgaris* L. 'Bountiful,' *P. vulgaris* L. 'Pinto,' *Vigna sinensis* Torner (Savi), *V. cylindrica* L. (Skeel), *Datura stramonium* L., *Nicotiana tabacum* L. 'Xanthi nc,' *N. tabacum* L. 'Havana 38,' and *Petunia hybrida* Vilm.

Seeds of sweet corn cultivar Stylepak were planted in a greenhouse in pots containing a layer of field soil, which had been collected from around roots of infected plants, sandwiched between layers of a steamed sand-compost mixture. Later, soil was collected from six production fields in which infected plants had been found. Where infected plants were still present, soil was taken from around roots of these plants. In harvested fields, soil was taken at random in areas where infected plants had been present. These large amounts of soil containing roots were roughly screened, placed in pots, and seeded with Stylepak sweet corn. Ears from four infected plants in the field were dried and the harvested seed was planted in steamed soil in the greenhouse to check for seed transmission.

Virus purification. Virus-infected tissues were collected in sweet corn fields in Sheboygan County and frozen at −20 C. One kilogram of tissue was triturated in a Waring blender in 1 L of extraction buffer (0.02 M phosphate buffer, pH 7.2, containing 0.02 M DIECA [diethyldithiocarbamate] and 0.02 M mercaptoethanol). The triturate was pressed through four layers of cheesecloth and clarified by mixing with one-fourth volume of chloroform and butanol (1:1) followed by centrifugation for 10 min at 10,000 *g*. The supernatant was then centrifuged for 90 min at 78,000 *g*. The pellet was resuspended in 0.02 M phosphate buffer (pH 7.2) and the suspension was centrifuged for 5 min at 8,000 *g*. The supernatant fluid was then layered over a 20% sucrose cushion (5 ml in Spinco #40 rotor tubes) and centrifuged for 2 hr at 105,500 *g*. The pellets were resuspended and aliquots of 0.5 ml containing 4.6 mg of virus were layered on continuous sucrose density gradients (10–40% sucrose w/v) and centrifuged for 3 hr at 82,000 *g*. The gradients and the 20% sucrose cushion were prepared in the resuspension buffer.

Virus properties. The sedimentation coefficient of MWLM-associated particles was determined in a Spinco model-E analytical centrifuge at 20 C in 0.02 M phosphate buffer (pH 7.2). When the centrifuge reached maximum speed (25,980 rpm), photographs

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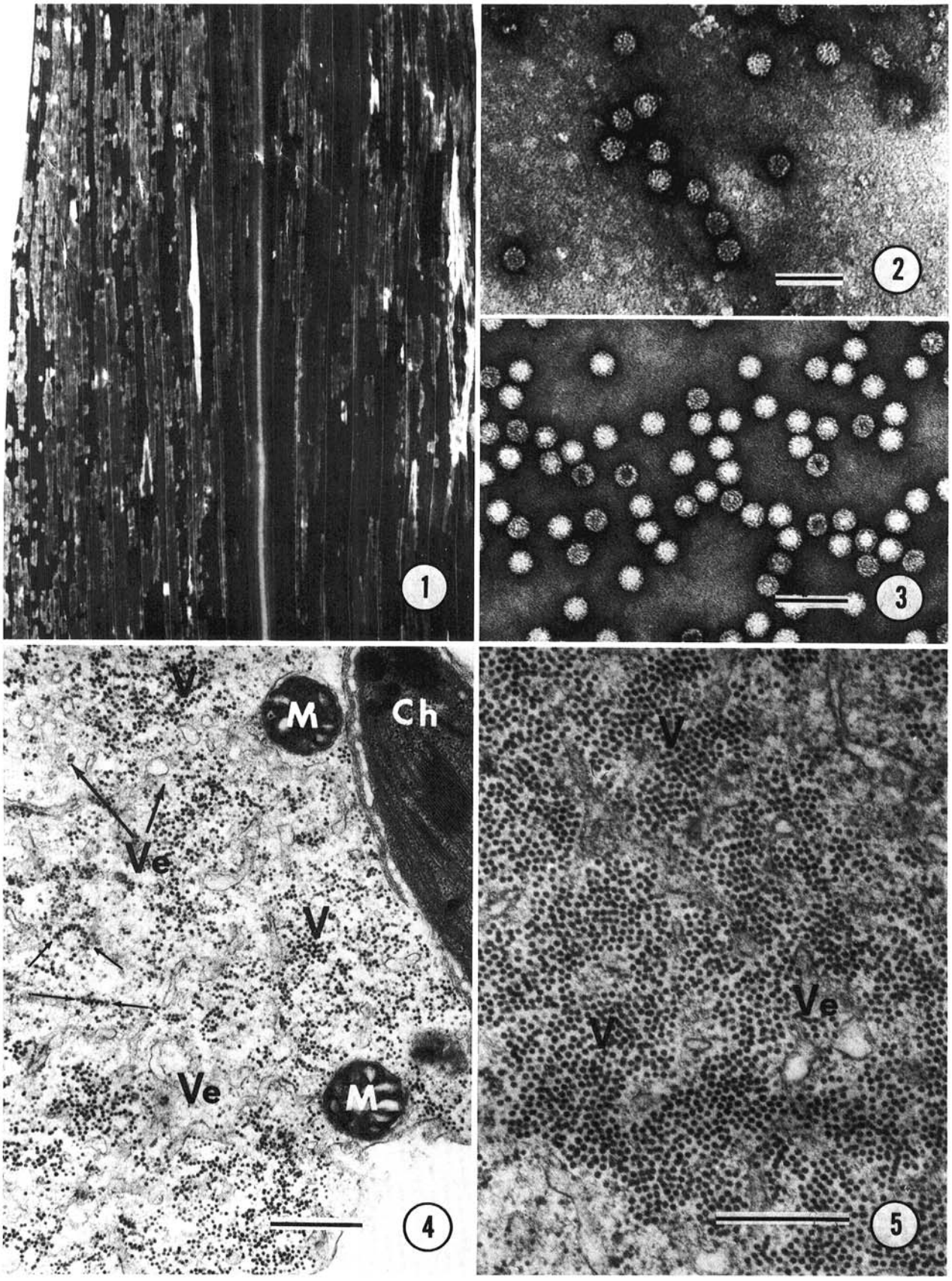

Figs. 1–5. Symptoms and signs of maize white line mosaic (MWLM). **1,** Leaf of field-grown sweet corn showing symptoms of MWLM infection. **2,** Dip preparation from sweet corn leaves showing MWLM virus particles. Note image clarity of the particles (35–36 nm in diameter) which were stained with uranyl acetate. (Bar is 100 nm). **3,** MWLM virus particles (35 nm in diameter) as seen in a purified preparation after density gradient centrifugation and staining with uranyl acetate. (Bar is 100 nm). **4,** MWLM-associated particles in the cytoplasm of mesophyll cells of sweet corn leaves. Note vesiculation (Ve and large arrows). Also note the spiral arrangement of particles (between small arrows). (Ch = chloroplast, M = mitochondrion). (Bar is 500 nm). **5,** MWLM-associated particles (V) in an inclusion in the cytoplasm of mesophyll cells of sweet corn leaves. Note areas of regular arrangement of particles in this inclusion and vesicles (Ve). (Bar is 500 nm).

were taken via UV optics at 4 min intervals. Two particle preparations were examined.

Virus recovered from sucrose density gradients (by ISCO density gradient fractionator) was mixed with CsCl (mean $\rho = 1.4$ g/cm^3) and centrifuged at 73,450 *g* for 48 hr in a Spinco SW 39 rotor. Gradients were fractionated (4 drops per fraction) and the density of each fraction was determined by refractometry and plotted against its absorbance at 260 nm (Cary 15 recording spectrophotometer). In a second experiment, the common U_1 strain of tobacco mosaic virus (TMV) was used as an internal standard ($\rho_{25} = 1.325$ g/cm^3) (9) in a CsCl gradient (mean $\rho_{25} = 1.34$ g/cm^3) also containing MWLMV. These gradients were centrifuged to equilibrium (20 hr) and photographed via Schlieren optics. The buoyant density of MWLMV was then determined in relation to the known density of TMV.

Viral nucleic acid was prepared by vigorously mixing 2 ml of virus suspension (3.9 mg/ml) with 2 ml of buffer (0.1 M tris pH 8.0, 0.5 M NaCl, 0.1% 8-hydroxyquinoline, and 0.5% SDS) saturated with phenol containing 10% meta-cresol. Phases were separated by low speed centrifugation. The water phase was re-extracted with phenol and separated by centrifugation. The aqueous solution was extracted twice with ethyl ether to remove the phenol and then the ether was removed by passing nitrogen through the aqueous solution. The nucleic acid was precipitated by the addition of two volumes of ice-cold ethanol. After centrifugation and drying, pellets were dissolved in 0.003 M tris-HCl (pH 8.5) and RNA yields were determined by UV absorbance with 25 used as the extinction coefficient for RNA.

Base composition of virion RNA was determined by using a modification of the methods of Uziel et al (10). Two-hundred and fifty μg of RNA were dissolved in 1 ml of 0.003 M tris-HCl (pH 8.5). Fifty μl of RNase A (2 mg/ml) and 5 μl of RNase T (1.83×10^5 units per milliliter) were added and the solution was held at room temperature for 20 min. Forty μl of snake venom phosphodiesterase (from *Crotalus adamanteus,* Sigma) was added and the solution was again incubated for 20 min at room temperature. Finally, 40 μl of alkaline phosphatase (12 mg/ml, Sigma) was added and the solution was allowed to stand at room temperature overnight.

Nucleosides were separated on a 0.9×20-cm column containing Aminex Q-155 cation exchange resin (Bio-Rad Laboratories, Richmond, CA 94804). The column was operated at 50 C with a flow rate of 12 ml/hr. The sample was applied to the column with air pressure and eluted with 0.4 M ammonium formate (pH 4.65). Fractions (1-ml) were collected and the ultraviolet absorption spectrum of each fraction was measured on a Cary 15 recording spectrophotometer. Nucleosides were identified by comparison to known spectra.

Viral coat protein was prepared by disrupting virus in the disruption buffer described by Hull and Lane (4). Gel electrophoresis was performed according to Maizel (5). The protein standards used for molecular weight determination were bovine serum albumin (mol wt 66,200), chick ovalbumin (mol wt 45,000), bovine trypsinogen (mol wt 25,400) and bovine β-lactoglobulin (mol wt 18,000).

The molecular weight of the viral RNA was estimated by gel electrophoresis (6) with brome mosaic virus RNAs utilized as standards (1.09×10^6, 0.99×10^6, 0.75×10^6, and 0.28×10^6 for RNAs 1,2,3, and 4, respectively).

The extinction coefficient ($E_{1\,cm}^{0.1\%}$) was determined by relating the $A_{260\,nm}$ of measured volumes of purified virus suspensions to the dry weight of their contents. Aliquots of buffer and of buffered virus suspension of known optical density were pipetted into different weighing bottles (10 each). The solutions were then dried and the weight of virus per milliliter of suspension was determined and averaged.

Electron microscopy. Dip preparations were made from field samples and negatively stained with magnesium uranyl acetate pH 4.0–7.0. Purified virus, after density gradient centrifugation, was dissolved in 0.02 M phosphate buffer (pH 7.2) and prepared for electron microscopy. Appropriate dilutions of the preparations were made in distilled water. Virus also was fixed in 0.5% glutaraldehyde in 0.01 M sodium acetate (pH 6) and then diluted in water for electron microscopic observation. Virus recovered from CsCl gradients was also viewed with the electron microscope.

Tissues showing symptoms of MWLM were collected from field-grown sweet corn, killed, fixed, and prepared for electron microscopy as described by de Zoeten and Gaard (2). Sections were viewed in a JEM 7 electron microscope.

Serology. Two rabbits were immunized by two intramuscular injections 1 wk apart. Antigen (6 mg per injection) was emulsified with complete adjuvant for the first injection and with incomplete adjuvant for the second one. A third and final injection (6 mg antigen in incomplete adjuvant) was administered 3 wk after the second injection and rabbits were exsanguinated 1 wk later. Serum titers were determined in microprecipitin tests. Ouchterlony gel diffusion tests were made as described by Powell et al (7).

RESULTS

Identification of the disease in field surveys was primarily on the basis of the distinctive symptoms. The presence of maize white line mosaic virus (MWLMV) in a number of plants was confirmed by electron microscopy, and a few specimens among those frozen earlier were later confirmed by serology to be virus-infected. In some plants, electron microscopy revealed both icosahedral particles and flexuous rods of which the latter were indicative of MDMV.

Results of all mechanical transmission attempts were negative, including the inoculations of a range of herbaceous hosts.

In initial soil transmission tests, at least one infected plant developed in each of four pots after two consecutive plantings. An overall infection of approximately 7% of the seedlings was observed. *Nicotiana tabacum* 'Xanthi nc' planted in the same pots remained virus-free as ascertained by serology. In a larger soil test, 0.8% of the seedlings became infected in the first planting. Infection was confirmed by serological tests. Stylepak sweet corn seed used in these tests did not contain the virus as determined by the absence of disease symptoms when seed was planted in sterilized soil.

Two of three *Setaria viridis* L. plants from a field in Sheboygan County were found to be virus-infected as determined by agar gel diffusion serology. Seeds from infected sweet corn plants did not produce infected seedlings (0 of 200).

Dip preparations prepared for electron microscopy from both corn and *Setaria* leaves with distinct disease symptoms showed the presence of many icosahedral particles with a diameter of approximately 35–36 nm (Fig. 2). The method employed for virus purification yielded 200–400 mg of virus per kilogram of plant tissue. In density gradients, a single sedimenting virus band was observed. In fresh preparations from both healthy and virus-infected plants, a component with a sedimentation coefficient of 44S was observed, but it disappeared from the preparations during storage for approximately 17 days at 4 C. In density gradient centrifugation, the UV absorbance of the material at the meniscus increased with time after preparation at the expense of the 44S peak. The A_{260}/A_{280} of purified virus preparations (E_{max} = 257.7 nm, E_{min} = 238.2 nm) after density gradient centrifugation was approximately 1.7. The extinction coefficient ($E_{1\,cm}^{0.1\%}$) at 260 nm was determined to be 3.9. The mean diameter of the virus after sucrose density gradient centrifugation was approximately 35 nm (Fig. 3) and the sedimentation coefficient was 117 S. The buoyant density of the virus was approximately 1.36 g/cm^3 when determined directly by fractionation of CsCl gradients that were subjected to equilibrium centrifugation. When the common U_1 strain of TMV ($\rho_{25} = 1.325$ g/cm^3) was used as an internal standard in equilibrium centrifugation of MWLMV in a Spinco Model E analytical centrifuge, the buoyant density of MWLMV was 1.353 g/cm^3 which indicates a particle RNA content of 20.88% (8). The buoyant density determination was done at pH 6 in .05 M sodium acetate after fixation in 0.5% glutaraldehyde. Fixation had no appreciable effect on buoyant density (R. W. Fulton, *personal communication*).

A single viral nucleic acid band with a molecular weight of approximately 1.25×10^6 was detected by gel electrophoresis. The

molecular weight of the protein subunit was found to be approximately 32,000. Viral nucleic acid was completely digested by the combination of RNases used in the determination of base composition (under both high and low salt conditions) which indicated that the viral nucleic acid was single-stranded. The base percent composition was: U = 24.3%, G = 30%, A = 19.7%, and C = 26%.

Electron microscopy of thin sections of infected sweet corn leaves revealed large concentrations of the virus in virtually all tissues including xylem elements. The size of the virus particle in tissues ranged from 24–28 nm with a dominant size of ~25 nm. The virus was easily recognizable whether present singly or in large aggregates (Figs. 4 and 5), because of its dark-staining characteristics. Although some regularity in virus arrangement could be seen occasionally, paracrystalline virus structures were not observed. Virus was seen only in the cytoplasm. Vesiculation of the cytoplasm and increased amounts of endoplasmic reticulum often was observed in areas where virus could be visualized. Large polyribosomes and/or a spiral arrangement of virus particles seemed to be a common feature of these areas. Three types of virus image were seen in tissue sections: a dark, heavily stained image; a lightly stained image; and (occasionally) images of particles that seemed to have a more lightly stained center.

Two antisera prepared to the Wisconsin isolate of MWLMV had titers of 1,024 and 2,048, respectively. The antisera provided by D. T. Gordon against an Ohio isolate of presumably the same virus from sweet corn reacted in gel diffusion with all of our virus isolates from the field as well as those recovered from plants apparently infected from the soil in greenhouse tests. A homologous reaction was obtained when an antiserum provided by A. Gotlieb prepared against the same or a similar virus mainly in field corn in Vermont was tested against our isolates and the Vermont isolate (no spur formation). Cross absorption tests conducted with our antisera and with those from Ohio and Vermont against our virus isolates and the Vermont isolate from field corn showed the Vermont and Wisconsin isolates to be identical. This could not be ascertained for the Ohio or New York isolates since they were not available for testing.

All Wisconsin sweet corn virus isolates and the isolate from *Setaria viridis* L. appeared to be identical, including the virus reported in field corn in Racine County.

DISCUSSION

The symptomatology of MWLMV in sweet corn is very characteristic. However, the inability at the present time to transmit this virus mechanically to either corn or other hosts prevents the establishment of an unequivocal relationship between the isolated nucleoprotein particles and the disease. Circumstantial evidence, however, of consistent association of characteristic 35-nm diameter virus particles with plants showing MWLM symptoms, and experiments showing the possible soil borne nature of this virus do point to a causal relationship between the particle (virus) and the disease.

The particles are present in unusually high concentrations in the tissues and have a rather unusual size (35 nm) in negatively stained preparations, when compared with other icosahedral plant RNA-viruses. Whether virus was viewed following fixation in glutaraldehyde at low or high pH or whether it was viewed unfixed had little effect on the size determinations. Virus particle diameters ranged from 34 nm at pH 4 to 36 nm at pH 7.2 in negatively stained dip preparations.

In view of the large size of this virus, the sedimentation coefficient 117 S is rather low. However, the low buoyant density 1.353 g/cm^3 indicates a low nucleic acid content which seems to fit this finding. It is clear from the RNase digestion studies that MWLMV is an ssRNA virus. The low molecular weight of 1.25 $\times 10^6$ estimated for the RNA of MWLMV confirmed the expectations, based on buoyant density measurements, that the RNA content of the particle is rather low.

It is curious that size measurements of the particle in situ should differ by 20 to 35% from those measured in negatively stained preparations. The center-to-center distance measured in semi-crystalline particle aggregates, is 28 nm. Since it is mainly the nucleic acid that determines the outline of the virus particles in situ, we feel that the size discrepancy, although larger than normal for icosahedral plant viruses measured in situ, is due to the low nucleic acid content of this virus.

Cytological observations of the virus in tissue sections did not give any indication as to the possible relationships to viruses in other plant virus groups. No inclusions characteristic of those in the comovirus, tymovirus, or nepovirus groups were observed, nor were the inclusions reminiscent of those in any other virus groups. Structures containing proliferated endoplasmic reticulum and vesicles suggestive of a virus replication complex could be identified (3).

The different particle images presented in sections are probably due to the fact that some particles are exposed in sections and others are completely embedded and, thus, are not reached by the stain applied after sectioning.

It has been shown by cross absorption of sera that the Vermont virus and the one from Wisconsin are identical. Furthermore, no spur formation was observed in agar gel diffusion between isolates from two geographically different areas, from the different areas within Wisconsin, or from different infected plants in soil transmission tests; these results indicate a relatively low variability among isolates. Similar results were obtained for the antiserum prepared to an Ohio isolate of the virus. Whether the Ohio and New York isolates are identical to the Vermont and Wisconsin isolates could not be established unequivocally because virus from these areas was not available. In intensive testing against sera to other corn viruses (1) it was observed that MWLMV reacts only with antiserum against an Italian corn virus referred to as maize-stripe mosaic virus (D. T. Gordon, *personal communication*). The low variability that was observed among isolates of MWLMV in serological tests may find its basis in the rather small amount of RNA in this virus.

Only one alternate host (*Setaria viridis* L.) has been found, but next season more extensive surveys will be made as part of a study of the epidemiology of this previously unknown virus in the United States. Furthermore, attempts are being made to determine the agent responsible for soilborne transmission of this virus. The antiserum now available will be useful in these studies.

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