Discovery and Characterization of Elm Mosaic Virus in Iowa

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ABSTRACT

A virus was isolated from leaves of Moline elm exhibiting ringspot mosaic symptoms and enations. It was serologically identical to type strain elm mosaic virus (EMV). This is the first report of EMV in Iowa. This isolate of EMV was examined with an electron microscope in shadowed and negatively stained preparations, and was seen associated with necrotic ringspots in fixed preparations of inoculated *Chenopodium quinoa* leaves. The particle is a polyhedron of 19-nm diam. New hosts of EMV are reported. Density gradient and analytical ultracentrifugation revealed that EMV was composed of particles of three densities: top, middle, and bottom components of $S_{20,w}$ 45, 65, and 92, respectively. Infectivity was associated only with middle and bottom components. The buoyant density of the major bottom component was 1.3328 g/cm^3.

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Three Moline elm trees (*Ulmus americana* L.) in Harrison County, Iowa, were observed in 1967 in a condition of decline. Leaf symptoms were suggestive of a viral disease. The syndrome included sparse foliage; small leaves, with some deformed, similar to those observed by Swingle et al. (15); enations on the lower leaf surface lined up in a row on either side of the midvein and between the secondary veins (Fig. 1); and mosaic and chlorotic ringspot patterns. Reviews (4, 10, 11) and preliminary reports (7, 9) have been made. An unknown virus was isolated from Moline elms showing such symptoms in 1967. This study was undertaken to identify the causal agent and determine its basic properties.

MATERIALS AND METHODS.—The virus was maintained in the greenhouse by the grafting of healthy elm seedlings with virus-infected budwood and by mechanical transfers to elm seedlings and herbaceous plants. We made mechanical transmissions by grinding virus-infected elm leaves in 0.02 M $NaSO_4$ buffer, pH 7.0, and inoculating the cotyledons of *Vigna sinensis* Torner (Savi) 'Early Ramshorn'. Subsequent transmissions were made with 0.01 M phosphate buffer, pH 7.0, to *Chenopodium quinoa* Willd. All infectivity assays for virus were made on *C. quinoa*.

Green peach aphids (*Myzus persicae* Sulz.) were given 5- and 20-min acquisition feedings on systemically infected *C. quinoa*. Test feedings on *C. quinoa* were 10 min.

RESULTS.—*Symptoms and host range.*—The enations appear to be a characteristic interaction of the Moline elm and the virus. American elm seedlings expressed the mosaic and ringspot symptoms after mechanical inoculation with the virus, but enations were never reproduced in this host under experimental conditions. We were unable, however, to obtain Moline seedlings to prove that the enations were virus-induced. The infected trees were checked 3 consecutive years, and the enations, plus mosaic and ringspot, were always present.

New hosts and their respective symptoms are listed in Table 1.

The Iowa elm virus (EMV) produced numerous local lesions on *C. quinoa* and became systemic, killing some plants within 15-20 days by causing necrosis of terminal and axillary growing points. Necrotic ringspot symptoms on inoculated leaves of Samsun NN or Turkish tobacco (Fig. 2-a) formed erratically, much the same as those induced by tomato ringspot virus (TomRSV). True chlorotic rings are expressed on systemically infected *Nicotiana clevelandii* L. (Fig. 2-b). Symptoms induced by the Iowa EMV were indistinguishable from those induced by TomRSV or tobacco ringspot virus (TRSV) in systemically infected *Cucumis sativus* L.

Cross-protection tests.—We made these tests by inoculating *C. quinoa* and *Nicotiana tabacum* L. 'Samsun NN' plants with the virus, then challenge-inoculating after 12-15 days with TomRSV or TRSV. Reciprocal cha nges were made on Samsun NN plants infected with TomRSV or TRSV.

Plants of *C. quinoa* systemically infected with the Iowa EMV were not infected with TomRSV or TRSV when challenged 12-15 days later. But, tobacco plants systemically infected with the Iowa isolate developed local lesions when challenge-inoculated with either TRSV or TomRSV. Tobacco systemically infected with TomRSV protected against infection when challenge-inoculated with the Iowa EMV. This one-way protection agrees with reports by others (4, 17) for EMV.

Transmission studies.—No aphid transmission of the virus could be demonstrated. Moreover, there was no evidence of seed transmission of the virus in 5,227 *C. quinoa* seedlings grown from recovered, systemically infected plants. However, pollen of *C. quinoa* was infective.

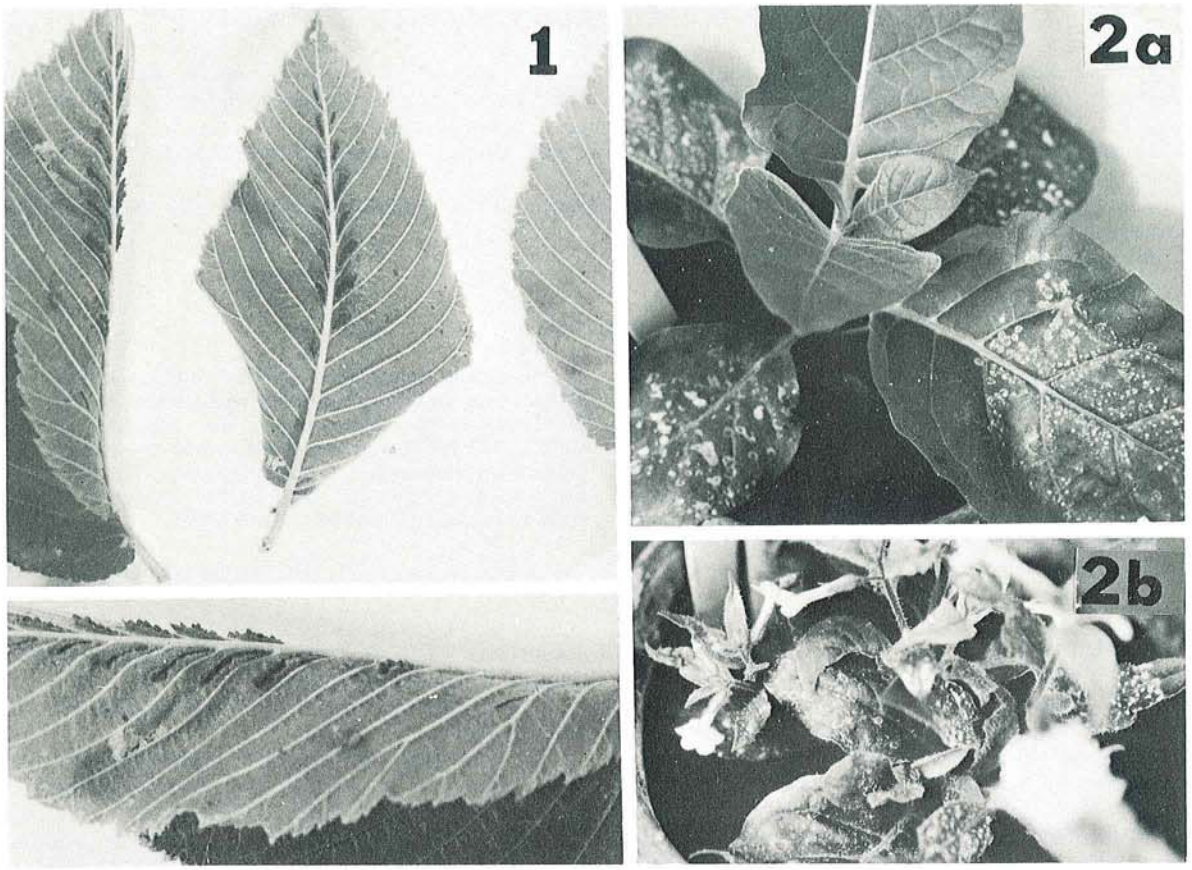

Fig. 1-2. 1) Enations on the lower surface of leaves from Moline elm from which elm mosaic virus was isolated. **2)** Chlorotic and necrotic ringspot symptoms induced by elm mosaic virus on (a) *Nicotiana tabacum* 'NN' and (b) *N. clevelandii.*

Physical properties.—We determined physical properties of the virus in crude sap by grinding infected *Cucumis sativus* L. 'National Pickling' in 0.01 M phosphate buffer, pH 7.0. We made longevity in vitro (LIV) tests by holding expressed sap at 25 C and assaying at 4-hr intervals for 4 days. We determined thermal inactivation (TIP) by heating 1.0-ml samples of clarified sap (diluted 1/10 with phosphate buffer) at 5-C intervals for 10 min and assaying for virus on *C. quinoa*. We determined dilution end point (DEP) by making 10-fold serial dilutions of the expressed sap in buffer and inoculating to *C. quinoa*.

Virus infectivity in vitro was maintained up to 10 days at 25 C. Infectivity was maintained at least 14 days in frozen leaves from *C. quinoa*, but not from *V. sinensis*. The virus was inactivated after heating for 10 min at 62 C, but not after heating for 10 min at 60 C. The DEP was between 10^{-4} and 10^{-5} when assayed on *C. quinoa.*

Purification.—We made initial attempts to purify the virus by the method of Stace-Smith (13), which resulted in poor virus recovery. The preparations lost infectivity when the clarified sap was frozen. The final pellet was still infective when we omitted this step. The pellet was quite small, with an average yield of 1 mg protein/kg infected host tissue, and infectivity was low. Ammonium sulfate treatment also decreased infectivity and reduced yield. We made subsequent purifications (34 total) by grinding infected tissue in 0.01 M phosphate buffer, pH 7.0, or 0.5 M borate buffer, pH 6.9 (w/v), and expressing through gauze. The expressed sap was mixed (v/v) with a mixture of equal parts of cold chloroform and n-butanol, stirred for 30 min at 4 C, and clarified at 5,000 *g* for 20 min. Chloroform-butanol (14) decreased final infectivity least of all clarification treatments. The clear amber supernatant fluid was pipetted off, and the pellet and chlorophyllous liquid were discarded. Further purification was accomplished by differential centrifugation. The final pellet was suspended in either 0.02 M borate buffer, pH 8.2, for storage and density gradient analysis, or in distilled water for determination of sedimentation coefficients. Purified virus was more stable in 0.02 M borate buffer, pH 8.2, than in either 0.01 M phosphate buffer (pH 7.0) or 0.5 M borate buffer (pH 6.9). With borate buffer on the final pellet, infectivity was retained more than 30 days at 4 C, whereas with other treatments, infectivity was lost in 10-15 days.

TABLE 1. Host range of elm mosaic virus (EMV) isolated from Moline elm in Iowa

Species inoculated	Symptoms[a]	
	Local	Systemic
Amarantaceae		
Gomphrena globosa L.[b]	–	–
Apocynaceae		
Vinca rosea L.	M	NS, NSt, PD
Chenopodiaceae		
Chenopodium alba L.	–	–
C. amaranticolor Coste & Reyn.[b]	NNL, CLL	N
C. quinoa Willd.	NNL	+, D
Compositae		
Helianthus annuus L.[b]	+	+
Cucurbitaceae		
**Cucumis sativus* L.[c] 'National Pickling' & 'Ohio MR-17'	CLL	CL, N
Cucurbita maxima L.	CLL	
C. pepo L.[b]	–	–
Labiatae		
Ocymum basilicum L.	–	–
Leguminosae		
**Phaseolus vulgaris* L.[c] 'Bountiful'	+	NSt, PD
'Scotia'	+	NS, NSt, M
**Pisum sativum* L.[c] 'Perfected Wales'	+	+
Vicia faba L. var. Minor	–	Mos (1 plant)
**Vigna sinensis* Endl.[c] 'Early Ramshorn'	NS, VN	VN, CS
Scrophulariacea		
Antirrhinum majus L.[b]	+	+

[a] Plants were inoculated with expressed *Chenopodium quinoa* sap in phosphate buffer. Abbreviations are: CL = chlorotic lesions; CLL = chlorotic local lesions; CR = chlorotic rings; CS = chlorotic spots; D = premature death; M = mottle; Mos = mosaic; N = general necrosis; NLL = necrotic local lesions; NR = necrotic rings; NS = necrotic spots; NSt = necrotic stipple; PD = premature defoliation; VN = vein necrosis; + = symptomless carrier; and – = noninfected. The presence of virus was confirmed by reindexing on *C. quinoa* and *Vigna sinensis*.

[b] EMV is distinctly different from tomato ringspot virus, i.e., serologically (4); thus, all hosts without an asterisk are new. For those who consider EMV related to tomato ringspot virus (17), these hosts are not new (8).

[c] *These hosts have been reported previously.

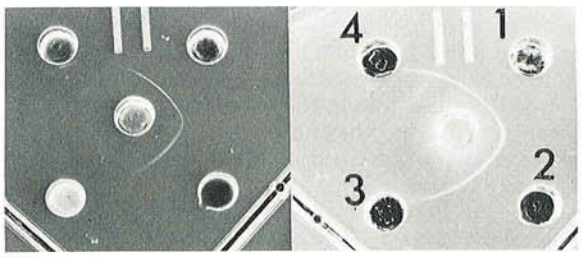

Fig. 3. Immunodiffusion test of the purified Iowa isolate of elm mosaic virus (EMV) in the center well reacted against antisera specific for **1)** Fulton's (Wisc.) type EMV; **2)** Iowa EMV; **3)** tomato ringspot virus; and **4)** tobacco ringspot virus; (Left) 1 day, and (Right) 1 week after preparation.

Purifications were made from the following hosts: *N. tabacum* 'Samsun NN', 'Wisconsin 38', and 'Turkish'; *V. sinensis*; *C. sativus* 'National Pickling' and 'Ohio MR-17'; and *C. quinoa*. The best host was *C. quinoa*, with yields of 100-150 mg/kg of tissue; other hosts yielded 5-10 mg/kg of infected tissue.

Density-gradient centrifugation.—Particle density was determined by density-gradient centrifugation with either sucrose or cesium chloride gradients. Sucrose gradients consisted of 1.0-ml layers each of 10, 20, 30, and 40% sucrose in phosphate buffer. The sucrose gradient was allowed to equilibrate for 24 hr. Then, purified virus (0.5 ml) was layered on it and centrifuged for 90 min at 204,000 *g* in a Beckman SW50 rotor. Cesium chloride gradient tubes were prepared from 3 M CsCl (optical grade) and centrifuged at 204,000 *g* for 18-24 hr. Gradient densities were standardized by Micule density markers (Microspheres, Inc., Palo Alto, Calif.). Gradients were fractionated on an ISCO ultraviolet density-gradient fractionator. Fractions from each peak were assayed for infectivity after dialysis for 24 hr against phosphate buffer.

Cesium chloride gradients of virus purified from *C. quinoa* leaves that had been suspended in 0.02 M borate, pH 8.2, yielded three distinct light-scattering zones (Fig. 4-a). Two additional minor peaks often were observed below the major bottom component. Although found in rather low concentrations, slight infectivity was always associated with them. The density of the bottom component of Iowa EMV was 1.3328 g/cm^3, determined with the aid of density gradient Micules.

Virus infectivity was assayed from fractions collected from sucrose gradients. Infectious nucleic acid was associated with both middle and bottom components, but never with the top component. Virus suspended in 0.01 M sodium ethylenediaminetetraacetic acid (EDTA), pH 7.0, showed a single band on sucrose gradients, but was not infective.

The ultraviolet absorption spectrum of the virus (Fig. 4-b), A_{260}:A_{240} ratio = 2.80 ± 0.05, was typical for spherical viruses containing high amounts of nucleic acid. The bottom component demonstrated a nucleoprotein spectrum, with a shift to a minimum absorption at 260 nm. The A_{260}:A_{240} ratio for the middle component varied from 2.65 to 2.75.

Analytical ultracentrifugation.—Sedimentation properties of the Iowa EMV components were measured by centrifugation of the purified virus in a Spinco Model E analytical ultracentrifuge, using an An-D rotor with Schlieren optics at a 60-degree interference angle. Exposures were taken at 4-min intervals at a rotor speed of 31,410 rpm. Sedimentation coefficients were determined by the graphic method described by Markham (6).

Virus preparations were analyzed by Schlieren optics to compare purification methods and to determine sedimentation coefficients for the virus components. Purified virus from *C. quinoa* yielded three peaks: 45S, 65S, and 92S (Fig. 4-c). These

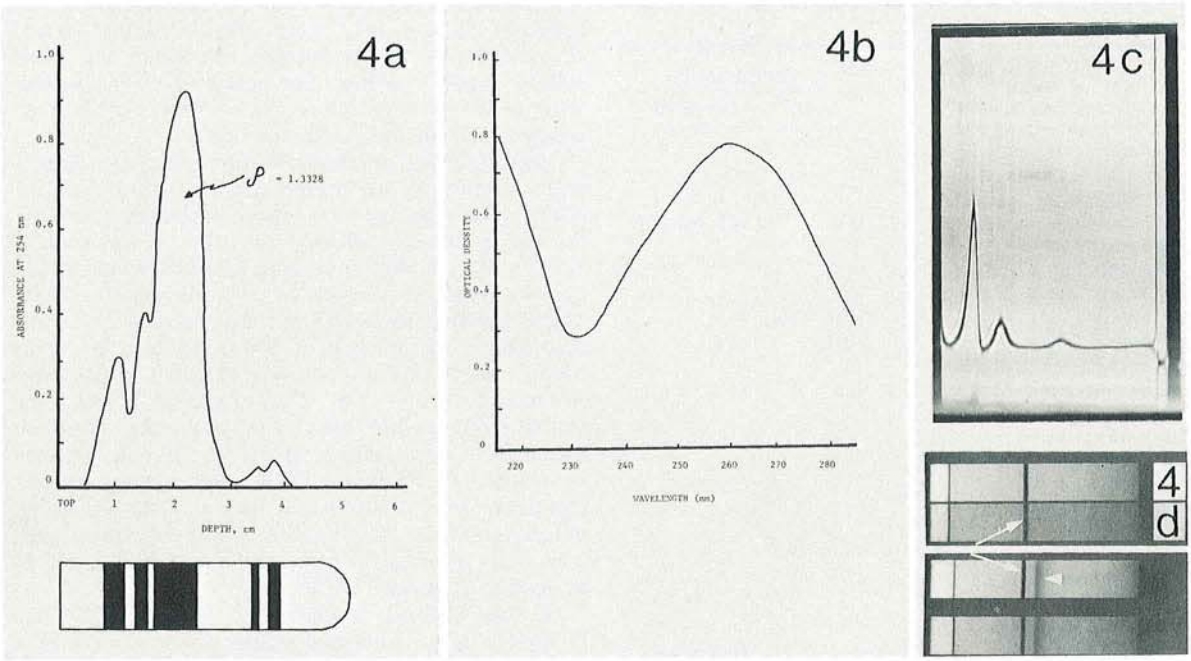

Fig. 4. Ultraviolet absorbance scans of purified elm mosaic virus (EMV) preparations showing **a)** its multicomponent nature after equilibrium density gradient centrifugation 18 hr in 3 M CsCl and collection by an ISCO fractionator; **b)** the typical nucleoprotein absorption spectrum; **c)** a Schlieren pattern at 31,420 rpm of purified elm mosaic virus at room temperature in 0.02 M borate buffer, pH 8.2; and **d)** a buoyant density determination of EMV (wedge) in CsCl including a tobacco mosaic virus marker (arrow).

components were comparable to the top, middle, and bottom components observed in density gradient analyses.

Buoyant density determinations were made in the Spinco Model E analytical ultracentrifuge with an ultraviolet optical system. Negatives were developed and then scanned with a Joyce automatic recording microdensitometer. The sample of optical-grade 3 M CsCl-virus mixture was equilibrated with the aid of a refractometer to a density of 1.333 g/cm^3, the approximate density of the infectious component of the virus.

The buoyant density of the virus was 1.3326 g/cm^3, according to microdensitometer tracings of ultraviolet absorption photographs.

Serology.—Antiserum to the Iowa EMV isolate was produced by intravenous injections to rabbits with 0.5 ml purified virus at 2-day intervals for 2 weeks. Intravenous boosters of 0.5 ml purified virus were given occasionally to maintain the titer. A subcutaneous injection containing 1.0 ml purified virus in 1.0 ml Freund's incomplete adjuvant was given 2 weeks before collection of blood by cardiac puncture.

Serological tests were conducted by the microprecipitin test described by Ball (1). Twofold serial dilutions of virus and antiserum were applied dropwise on plastic plates, covered with mineral oil, and incubated overnight at 4 C. Controls consisted of saline and normal rabbit serum.

Immunodiffusion tests were made in plates containing 0.5% Ionagar No. 2 and 0.2% NaN_3. The central well contained purified Iowa elm virus, and, in the outer wells, were placed antisera specific for the following: TomRSV, TRSV, Iowa EMV, type EMV (4), normal rabbit serum, and saline control. Other antisera tested by immunodiffusion were rose mosaic virus, line pattern mosaic virus, raspberry ringspot virus, necrotic ringspot virus, bromegrass mosaic virus, and cucumber mosaic virus.

Immunodiffusion tests showed that, when the Iowa EMV was reacted with its homologous antiserum and the Wisconsin EMV antiserum, the reaction was homologous. No reaction occurred between the purified Iowa EMV and TomRSV antiserum (Fig. 3). The Iowa EMV did not react with any other antisera tested.

Purified Iowa EMV was subjected to electrophoresis in 0.02 M phosphate buffered 1% Noble agar on LKB 6800-A immunoelectrophoresis equipment at 250 v-DC for 45 min, along with TomRSV, and antiserum for either virus was added to a center trough when the run was completed. Reaction slides were processed as described earlier (12).

The electrophoretic relationships and the conclusions drawn were the same as those for immunodiffusion, that Iowa EMV reacts in a homologous manner with EMV antiserum.

Electron microscopy.—Purified preparations of the virus were either shadowed or negatively stained for observation with the electron microscope. We

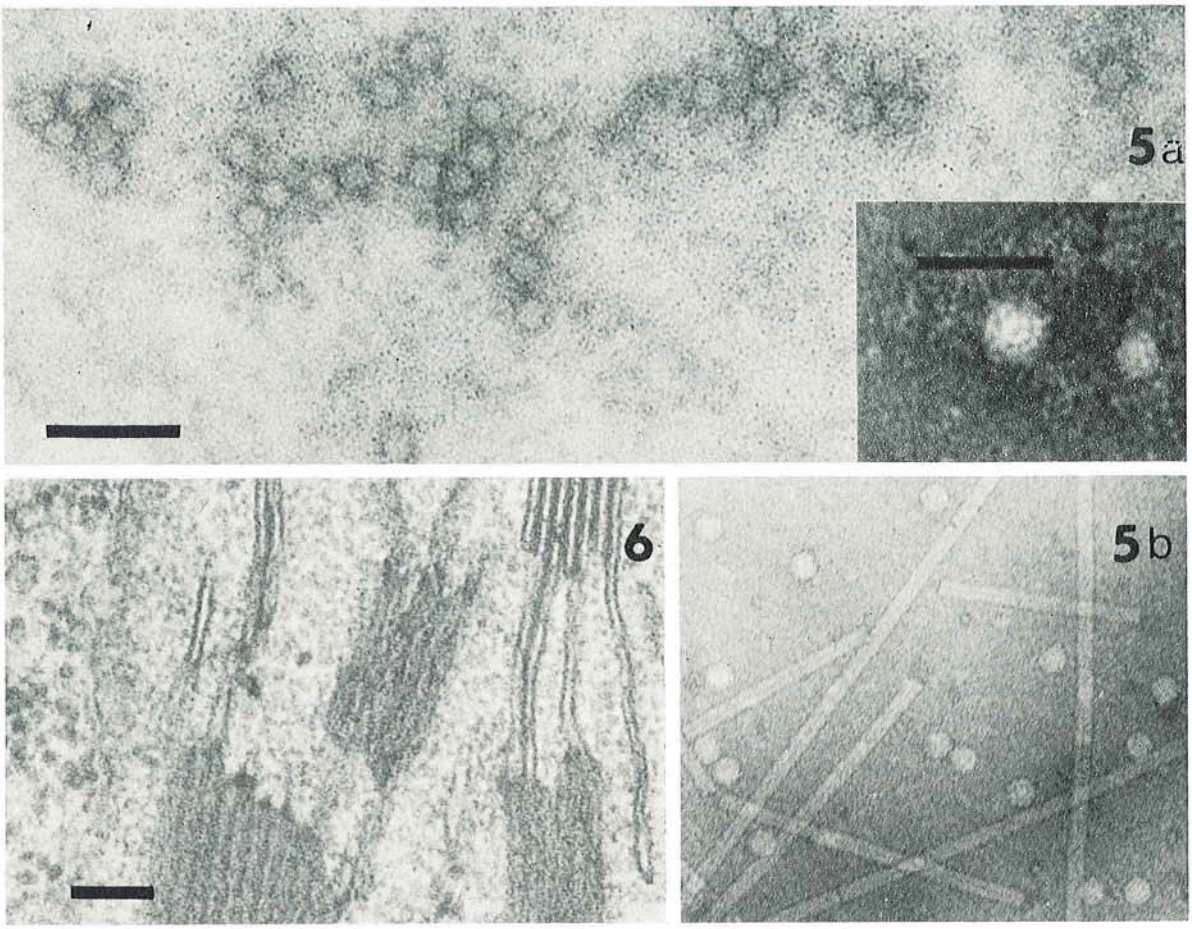

Fig. 5-6. 5) (a) Negative stain preparation of elm mosaic virus (EMV) (bar = 100 nm). Inset shows some detail of the EM virion and fraction 1 protein (bar = 50 nm). (b) Negative stain preparations of EMV with TMV as an internal standard. **6)** Cross section of a local lesion in a *Chenopodium quinoa*-inoculated leaf showing the virus particles distributed in the cytoplasm (bar = 100 nm).

negatively stained grids by atomizing a mixture of purified virus (8 drops), 2% phosphotungstic acid (8 drops), and sometimes 1% bovine serum albumin (1-2 drops), onto Formvar-coated grids. We made shadowed preparations by applying a thin film of virus suspension to a Formvar-coated grid, drying, and shadowing the preparations with platinum-palladium (80:20) at a 20 degree angle. Measurements were made on 350 particles from four different purifications. The instrument magnification was determined by using a carbon grating replica grid of 54,800 lines/inch obtained from E. F. Fullam Co. One exposure of each of three areas was made, and three measurements from each exposure were averaged to determine the final magnification. In 1½ years' time, the variation in the instrument magnification varied less than 3% when checked at several intervals. These preparations showed that the Iowa EMV is a polyhedral particle, having a mean diam of 19 (range 17-21) nm (Fig. 5-a). When TMV was used as an internal standard (assuming a mean diam of 15 nm), the EMV particles averaged 20.5 nm diam (Fig. 5-b).

Local lesions from inoculated, infected *C. quinoa* leaves were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 hr, postfixed with cacodylate-buffered 1% OsO_4 for 2 hr, stained during ethanol dehydration with uranyl acetate (5), and embedded in araldite-epon for sectioning. Thin sections were cut with a diamond knife on an LKB-3 ultramicrotome, floated onto Formvar-coated grids, stained with uranyl acetate and Reynolds lead citrate, and observed with a Hitachi HU 11C (or occasionally with an RCA EMU-3F) electron microscope.

These sections of local lesion areas demonstrated that the virus particles are not distinct aggregates, but are rather loosely organized in the cytoplasm (Fig. 6). The size correlates closely with those measured from purified preparations.

DISCUSSION.—We conclude from our host-range, physical-property, serology, and cross-protection tests that the Iowa EMV is elm mosaic virus.

Based on density-gradient centrifugation and

analyses of buoyant density and Schlieren data, EMV probably is a multicomponent virus similar to TRSV. Iowa EMV seemed quite unstable as we searched for optimum conditions for purification and storage. When EMV was centrifuged in sucrose gradients in the presence of 0.01 M EDTA, pH 7.6, a single broad, noninfective band appeared above the usual position for the two infectious components, rather than the usual three components. This is additional evidence for its instability, although EDTA does have a detrimental effect on infectivity and structure of a relatively stable spherical virus under certain conditions (18). The position of this broad band above the normal infective component bands suggests that loss of infectivity was associated with structural changes of the virus particles.

No surveys were made to determine the extent of EMV in elms throughout Iowa. Since it is pollen- and seed-transmitted (2), it may be widespread. It is of interest that pollen, but not seed of *C. quinoa*, transmitted EMV.

The enations, quite commonly virus-induced in various hosts, are the only part of the symptom syndrome of EMV on elm not described previously. Unfortunately, the Moline elm had a number of undesirable horticultural traits and is no longer available commercially. It does not grow "true" from seed, and must be vegetatively reproduced. We were unable to obtain seedlings of this cultivar to prove that the enations were EMV induced; no other EMV-infected plant responded by enation production.

The necrotic, stem-collapse symptom of *V. sinensis* is especially diagnostic. It occurs specifically at the juncture and immediately below (for about 1 cm) the attachment of the petioles of the two primary leaves. Varney & Moore (16) mention stem necrosis of cowpeas, but they did not elaborate on the observable peculiarity of the particular location or its possible diagnostic value. Although we did not test other *V. sinensis* cultivars, this reaction may be limited to only one or a few.

We observed the ringspot symptom primarily in the acute stage of the disease in elm seedlings, and the mosaic symptom was characteristic of the chronic stage. Schmelzer et al. (11) discussed these as caused by two separate viruses.

Symptomless infections seem to be common for EMV in other hosts (Table 1) in addition to those reported in *N. glutinosa* and tomato (16). Fulton & Fulton (4) refer to nematode acquisition of EMV from Alaska peas, yet we were unable to infect Perfected Wales pea (Table 1). Many *Prunus* species, but few herbaceous hosts, have been reported susceptible to EMV (3). We were unable to infect *C. pepo, G. globosa, D. stramonium,* and *D. metel* with EMV, which McLean (8) lists as susceptible to tomato ringspot virus. Based on these host range results, plus only one-way protection with, and serological unrelatedness to, TomRSV, we concluded that the virus we isolated from diseased Moline elm in Iowa was the causal agent of the observed symptoms, and that the virus was identical to the type EMV.

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