

# Factors Affecting Manual Transmission, Purification, and Particle Lengths of Wheat Spindle Streak Mosaic Virus

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Contribution No. 689 from the Cell Biology Research Institute and Contribution No. 254 from the Ottawa Research Station.

The authors thank P. L. Sherwood for technical assistance, and M. K. Brakke, Plant Pathology Department, University of Nebraska, for an isolate of wheat soil-borne mosaic virus and for suggestions regarding virus purification.

Accepted for publication 22 December 1970.

## ABSTRACT

Wheat spindle streak mosaic virus (WSSMV) was transmitted manually from diseased wheat plants collected in the field in early May or grown in infective soil in a growth room at 6-12 C. Juice from diseased plants had a low infectivity titer, and lost most infectivity within 1 hr at 10 C. Initial infectivity was not increased by various buffers or sodium sulphite solution with bentonite. Inoculum was still infectious after 1 hr if prepared by grinding diseased leaves in phosphate buffers at pH 9, 8, and 7 but not at lower pH levels. Virus in leaf pieces was inactivated by heating at 47.5 C for 10 min. Highest percentage infection was usually accomplished by preparing a wad of diseased leaves, then rubbing it on wet emery paper just before rubbing it on the leaves of each test plant.

Particles observed in dip preparations from dis-

eased wheat leaves were  $12.8 \times 190$ -1,975 nm, with peaks in numbers of particles of 275-300 and 600-625 nm. Preparations from differential centrifugation of juice extracted from infected wheat leaves in 0.5 M sodium borate at pH 9.0 and then emulsified with one-third volume of a 1:1 mixture of *n*-butanol and chloroform, and the final pellet resuspended in phosphate buffers, contained particles 90-1,000 nm long. Peaks in numbers of particles occurred at lengths ranging from 125-175 nm at pH 10 to 275-300 nm at pH 5. None of the preparations was infectious. The particles flocculated at pH 8.0 or lower. The results indicate that infectivity is associated with particles more than 1,000 nm long, but these are fragile, and hence break and lose infectivity quickly in expressed plant juice. It appears that no intact particles are retained through the procedure used to purify the virus. *Phytopathology* 61:569-574.

Wheat spindle streak mosaic, which is widespread in areas of Ontario where winter wheat is commonly grown, is caused by a soil-borne virus that requires 30-90 days' incubation at 5-15 C (5). The virus has been transmitted manually many times, but the results have been variable. Slender particles were found in preparations from diseased wheat plants, but the lengths were so variable that the normal length of the particles was questionable.

Experiments were done to determine factors affecting manual transmissibility of wheat spindle streak mosaic virus (WSSMV), and the preparation of concentrates of virus particles.

**MATERIALS AND METHODS.**—For some tests, WSSMV was obtained from naturally diseased wheat from the field; but the normal sources were Kent wheat plants that became diseased by growing in naturally infective soil in a growth room, or plants infected by manual transmission from such plants. Inoculum was prepared from fresh leaves with clear mosaic symptoms by grinding 1 g leaves with 4 ml water. Initially, inoculations were done by the leaf rub method. Later, the artist's airbrush method was used (8). For this, 2 mg of 600 mesh Carborundum was added to each 4 ml of inoculum, then sprayed on the plants with a Paasche Model H airbrush at an air pressure of 21 kg/cm<sup>2</sup>. The pressure was controlled by a pressure valve on a portable air pressure tank. The nozzle of the airbrush was held

about 3 cm from the leaves. A third method of inoculation, a leaf tissue rub method (10), involved folding diseased leaves to form a wad, sometimes held together by a rubber band, or by knotting the leaves. The wad was rubbed alternately on fine wet emery paper dusted with 600-mesh Carborundum powder, then on the leaves of a test plant backed by a flat surface. This method minimized the time between the freeing of juice from the diseased leaves and applying it to the test plant.

Most inoculations were done on Kent wheat grown in the greenhouse to the 2-3 leaf stage but transferred to the growth room 1 day before inoculation. There were usually 12-18 plants grown in each 5-inch clay pot. The inoculated plants were kept in the growth room at 6-12 C with 1,000 to 1,500 ft-c of cool-white fluorescent light for 12 hr/day. Some plants showed symptoms after 1 month, but final notes were not recorded until 3 months after inoculation.

The following procedure for partial purification yielded preparations with an abundance of particles. Fresh leaves (30 g) were immersed in 0.5 M sodium borate at pH 9.0 (30 ml) and ground with a mortar and pestle or with a small food grinder. The juice was squeezed through sheer Dacron or nylon cloth which is nonabsorbent. An additional half volume of the buffer (15 ml) was mixed with the pulp, and the liquid squeezed from this was added to the first extract. One volume of a 1:1 mixture of *n*-butanol and chloroform

(6) at a 5 C temp was added slowly to two volumes of the leaf extract while stirring rapidly. Stirring was continued for 15 min, then the emulsion was centrifuged at about 3,000 g for 15 min. The top aqueous phase was drawn off and recentrifuged at about 3,000 g for further clarification. The supernatant was centrifuged in the No. 30 rotor of the Spinco Model L centrifuge at 28,000 rpm for 90 min. The precipitate was resuspended in 12 ml of 0.01 M sodium tetraborate at pH 9.0, then centrifuged at about 3,000 g for 10 min. The supernatant was centrifuged in the No. 40 rotor at 38,000 rpm for 90 min. The precipitate was usually resuspended in 1.5 ml of 0.85% saline in 0.01 M phosphate buffer at pH 7.0 (PBS). Sometimes the precipitate was resuspended in water, then mixed with equal portions of various buffers or other solutions to be tested as suspending media.

The presence of particles in the preparations was determined by placing droplets of dilutions of the preparations on Formvar-coated grids, blotting off the excess, dialyzing on distilled water to remove salts, shadowing with palladium, then examining with an electron microscope.

For attempts to develop an antiserum, a 1-ml portion of a preparation suspended in PBS was emulsified with an equal volume of Freund's incomplete adjuvant; and half of the emulsion was injected into each thigh of a rabbit. After four such injections at weekly intervals, serum samples were tested by the microprecipitin test against concentrates of particles.

Particles were measured in shadowed dip preparations from young leaves showing pronounced symptoms and in preparations from concentrates resuspended in different buffers. For width measurements, particles were negatively stained with 1 or 2% potassium phosphotungstate at pH 7. The particles were measured from photographs taken on a Siemens-Elmskop I electron microscope.

**RESULTS.—Factors affecting manual transmission.—Sources of inoculum.**—WSSMV was transmitted repeatedly by the leaf rub method from diseased plants collected from the field or grown in infective soil in the growth room, but inoculations were successful only from plants that had not recently experienced daily mean temperatures much above 15 C for prolonged periods. Inoculations usually failed from plants obtained in the field after warm weather in late May and early June, unless the plants were transplanted and grown at 6-12 C for several weeks before transmission was attempted. When diseased plants were kept continuously at 6-12 C, about equal transmission was obtained with inoculum prepared from old leaves with clear symptoms and from younger leaves, and from plants at all stages of development before maturity. However, there was considerable variability in the percentage infection in different pots of plants inoculated with the same preparation by the leaf rub method.

Infective inoculum could be prepared from excised diseased leaves kept at 5 C as long as they remained turgid and fresh, but not after etiolation or decomposition began. In a storage test, leaf pieces placed over  $\text{CaCl}_2$  retained only a trace of infectivity after 2 days

at 5 C and -15 C, and were not infective after 7 days at either temperature.

**Infectivity and stability of inoculum.**—Tests involving the use of inoculum at different times after preparation showed that infectivity diminished rapidly. In some tests, 30% to 60% of the test plants developed symptoms if they were rubbed with inoculum within 5 min after preparation from fresh leaves. Inoculations done with the same preparations after 30-60 min usually failed. However, in one test, inoculum prepared and kept at 10 C infected 48% of the test plants inoculated within 1 min and 13% after 4 hr, but none after 7 hr.

Initial tests involving the infectivity of dilutions of inoculum resulted in no infection at any dilution, probably because of rapid loss of infectivity while the dilutions were being prepared. In a test in which the inoculum was prepared at about 10 C and the test plants were inoculated within 10 min after extraction from the source plants, the percentage infection of 20-25 test plants inoculated with each dilution was 30% for  $\frac{1}{4}$ , 12% for  $\frac{1}{16}$ , 7% for  $\frac{1}{64}$ , and 0.0% for  $\frac{1}{256}$  or higher dilutions of the juice.

These and other results indicate that low initial infectivity and rapid loss of infectivity of WSSMV in expressed juice are major causes of variable results from infectivity tests.

**Heat inactivation.**—In initial tests on heat inactivation, all samples of juice, including the nonheated control, lost infectivity during the time involved in extraction, treatment, and inoculation. To avoid uncontrolled inactivation of the virus in expressed juice, further heat inactivation tests were done on 1-g samples of leaves cut into 1-cm lengths and immersed in 4 ml of water. The samples were heated for 10 min at temperatures ranging from 35 to 65 C, then cooled to 10 C. The control was kept at 10 C. After grinding in the water in which they were immersed + 2 mg Celite, each preparation was immediately rubbed on the leaves of about 30 test plants. In one test, 3 to 10% infection occurred with inoculum from leaf pieces exposed to temperatures up to 47.5 C, but not at 50 C or higher. In another test, 13 to 20% infection occurred with inoculum from leaf pieces exposed to temperatures up to 45 C, but not at 47.5 C.

**Effects of supplements on infectivity of inoculum.**—To test the effects of phosphate buffers at different pH levels, 1-g samples of leaves with clear mosaic symptoms were ground with 4 ml of 0.5 M and 0.1 M potassium phosphate buffers ranging from pH 4.5 to pH 9.0. Two mg of Celite were added to each preparation. After 1 hr at 20 C, each preparation was rubbed on the leaves of about 20 Kent wheat plants. No infection resulted from inoculum prepared from either the 0.5 M or 0.1 M buffers at pH 4.5, 5.0, or 6.0, but at pH 7 infection was 0 and 7%, at pH 8 it was 6% and 11%, and at pH 9 it was 14% and 23%, respectively. These results indicate a stabilizing effect of the buffer at pH 9, with preference for 0.1 M buffer.

Other supplements which have been used to increase infectivity (9) were tested for effects on infectivity of WSSMV. One g of leaves from diseased plants was ground in a mortar with 10 ml of water or solutions

including additions; then the preparation was immediately rubbed on the leaves of about 30 test plants. Inoculum prepared with water without supplements infected 62% of the test plants. The addition of 5 mg of Celite before grinding resulted in 58% infection, and after grinding, 45% infection. The addition of 5 mg of 600 mesh Carborundum before and after grinding resulted in 30% and 65% infection, respectively. These results did not indicate any significant improvement in infectivity by the use of these abrasives. Similarly, the use of 0.1 and 0.5 M sodium borate (pH 9.0), 0.1 M borax (pH 9.2), 0.5 M  $K_2HPO_4$  including 0.1% bentonite, or 0.1%  $K_2SO_3$  including 0.1% bentonite, all tested with 5 mg Celite added before as compared with after grinding, did not increase the infectivity of inoculum over that prepared with water as the only supplement to the diseased leaves.

*Stability of infectivity in relation to method of inoculation.*—Since rapid loss of infectivity of inoculum was evident in experiments done by the leaf rub method, and supplements caused no substantial improvements in initial infectivity, other methods of inoculation were tested.

The artist's airbrush spray method of inoculation usually caused equal or slightly higher infection than the leaf rub method. In addition, plants could be inoculated more quickly, thus reducing the loss of infectivity of inoculum during delays before application.

The leaf tissue rub method was developed to further reduce the time between the release of juice on the surface of diseased leaf tissue and its application to test plants. This more direct method of inoculation usually produced a higher percentage infection and less variable results than either of the other methods.

In one test comparing the normal leaf rub, the artist's airbrush, and the leaf tissue rub methods of inoculation, infection was 48%, 57%, and 85%, respectively, among ca. 30 plants inoculated by each method. In another, it was 1%, 12%, and 44%, respectively, among about 200 plants inoculated by each method.

*Age of test plants.*—To determine if age of test plants

affected susceptibility, Kent wheat plants ranging from the one-leaf to tillering stages (6 to 30 days after seeding) were inoculated with WSSMV by the leaf rub, artist's airbrush spray, and leaf tissue rub methods. Plants of the same age in two pots were inoculated by each method. One pot from each pair was placed in a series so that plants were inoculated in order from the youngest to the oldest. In the other series, plants were inoculated in order from oldest to youngest. The percentages of plants infected from the leaf rub and artist's airbrush spray methods were so low and variable (0 to 12%) that no meaningful comparisons could be made of the susceptibilities of plants at different ages. WSSM developed in some plants of all ages inoculated by the leaf tissue rub method. Infection varied from 18% for plants inoculated in the first leaf stage (6 days after seeding) to 67% for plants with 5 leaves (21 days after seeding). There was 28% infection in the oldest plants inoculated, which were in the tillering stage 30 days after seeding. Differences between the two pots for some treatments were as great as differences between treatments; therefore, age of plants did not appear to be a major cause of variations in infection from manual inoculations. Similar results with respect to age of test plants were obtained in other experiments.

*Treatment of test plants before and after inoculation.*—Test plants grown in the greenhouse appeared to be as susceptible if inoculated before being placed in the cool growth room (6-12 C) as when inoculated after 24 hr or 14 days in the growth room. If plants were inoculated in warm conditions (20-25 C), infection was not reduced if they were kept for 2 hr, but was eliminated after 24 hr or longer at the warm temperature before removal to the cool growth room for incubation of the virus.

Although it was reported that a 6-day dark treatment, starting 6 days after inoculation, caused a substantial increase in percentage of plants developing symptoms of wheat (soil-borne) mosaic (4), similar treatments in three separate tests did not increase the percentage of

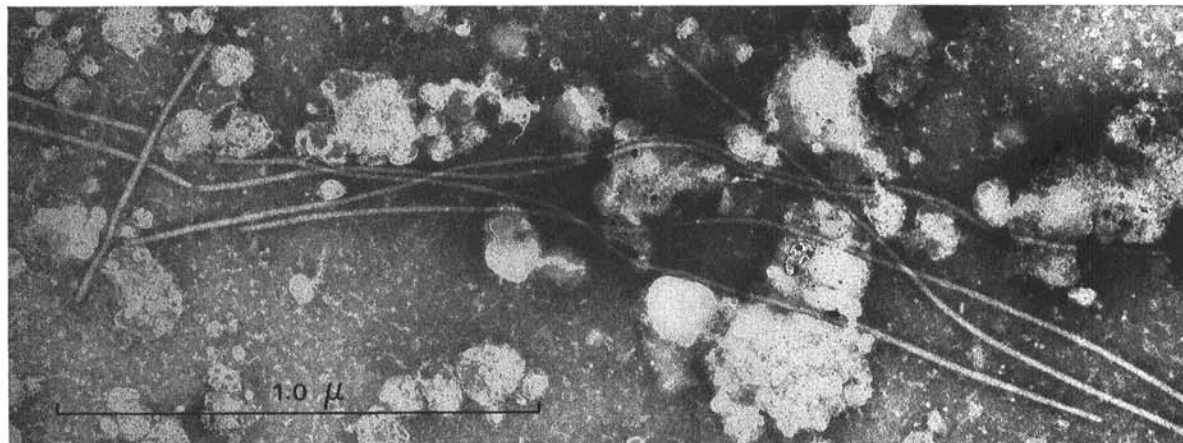


Fig. 1. Threadlike particles in a negatively stained leaf dip preparation from wheat infected with wheat spindle streak mosaic virus.

plants that developed symptoms after inoculation with WSSMV.

*Particles from diseased plants.—Measurements in leaf dip preparations.*—Particles were observed with difficulty in leaf dip preparations from wheat with WSSM symptoms. They were usually sparsely distributed but sometimes were found in clumps, and were often partly obscured by plant constituents (Fig. 1). The average measurement for thickness of particles in preparations negatively stained with phosphotungstic acid was 12.8 nm. The lengths measured in shadowed preparations ranged from 190 to about 2,000 nm, with peaks in frequency occurring at 275 nm and about 625 nm (Fig. 2).

Comparative examinations of leaf dip preparations from wheat infected with wheat soil-borne mosaic virus (WMV) from Nebraska showed that particles of the latter virus were abundant, clearly distinguishable, stiff rods about 26 nm in diam. They ranged from 87 to 625 nm long, but most were 100 to 200 nm long. The normal length was 161 nm, but a minor peak in frequency occurred at about 300 nm. These observations and measurements confirm that the particles associated with WSSMV are distinctly different from the particles of WMV.

*Effects of pH on aggregation of particles in concentrates.*—Initial attempts to purify the virus from plant juice extracted without buffers, or with buffers near neutrality, failed regardless of the nature of further treatments before differential centrifugation. None of these methods yielded concentrates of particles until the leaves were ground in alkaline buffers, which probably prevented aggregation of virus particles in the crude juice (2).

An abundance of particles was observed in concentrates from juice prepared by grinding WSSMV-infected leaves in 0.5 M sodium borate buffer at pH 9.0, then clarifying by the *n*-butanol-chloroform method. After the final precipitate was resuspended in PBS, it contained particles which varied from 87 to 1,000 nm in length. There was a major peak in frequency of particles at about 300 nm and a minor peak at about 540 nm. Attempts to infect wheat with WSSMV by manual inoculation with the concentrates failed.

After a rabbit had been injected 4 times at weekly intervals with 1 ml of concentrate from 30 g of leaves emulsified with 1 ml of Freund's incomplete adjuvant, serum samples were tested for reaction against twofold dilutions of concentrates of particles from diseased plants. In precipitin tests, bulky, flocculent precipitates formed in the lower dilutions of the concentrates mixed with any of a range of dilutions of serum from injected rabbits. However, the precipitates were identical when the same dilutions of the concentrates were mixed with normal serum or with 0.85% saline solution. Precipitates that were resuspended and examined with the electron microscope were found to consist of particles like those found in the original concentrates. No flocculent precipitates formed in concentrates similarly prepared from healthy wheat plants. It therefore appeared that at pH 7, the particles flocculated spontaneously

within 30 to 60 min when incubated at 40 C or left for longer periods at room temperature.

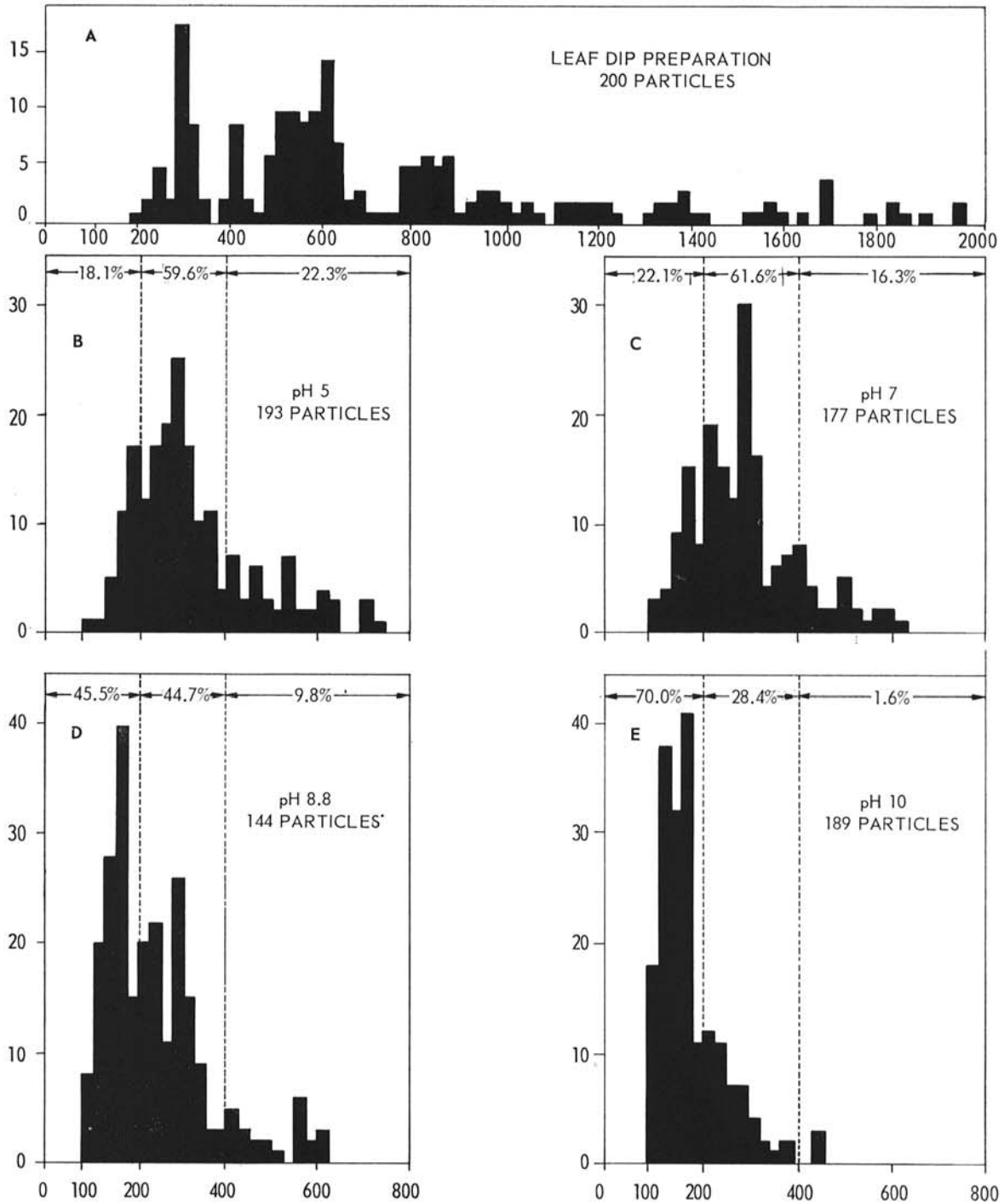
Flocculation did not occur in suspensions of particles mixed with rabbit serum, saline solution, water, or neutral buffers if they contained 0.5 M sucrose, 0.5 M glycerin, or 0.2% Igepon detergent. Furthermore, flocculation did not occur if the particles were suspended in 0.01 M phosphate or 0.2 M borate buffers at pH 9.0 or 10.0, but as pH was adjusted downward from 8.4, the rate and density of flocculation increased to a maximum at about pH 5 to 3.5.

*Effects of pH on lengths of particles in concentrates.*—Particles that were resuspended in 0.01 M phosphate buffers at pH 5.0, 7.0, 8.8, and 10.0 were measured to determine the effects of pH on length. As the pH of the suspending medium was decreased from 10 to 5, the max length of particles increased from about 460 to 740 nm, the numbers of particles more than 200 nm long increased from 30 to 82%, and the most frequent length increased from 125-175 to 275-300 nm (Fig. 2). None of these preparations was infectious.

**DISCUSSION.**—Even when comparable sources of infected plants were used for inoculum, and when wheat was inoculated and grown under similar conditions, manual inoculations for transmission of WSSMV yielded variable results because of a rapid loss of infectivity of the virus in the inoculum. Inoculation procedures that could be done most quickly after the extraction of the juice from the source plants caused the highest percentage infection; hence the leaf tissue rub method, which involves rubbing the freshly abraded surface of a wad of diseased leaves directly on the leaves of test plants, produced the most consistently high percentage infection.

WSSMV appears to be very sensitive to pH. Although the percentage infection from freshly extracted juice was not increased by buffers, inoculum retained infectivity longer at pH 9.0. Also, particles were retained during differential centrifugation of juice; and particles in partially purified preparations did not aggregate as quickly in phosphate buffer at pH 9 as at successively lower pH levels.

Measurements from leaf dip preparations showed particles about 13  $\mu$  in diam and peaks of particle length distribution at 275-300  $\mu$  and ca. 600 nm. These measurements correspond closely with measurements reported for wheat yellow mosaic virus in Japan (3). However, particles in the leaf dip preparations varied from 190-1975 nm in length, indicating that fragmentation and/or aggregation must have occurred. The partially purified preparations contained only particles less than 1,000 nm long, including many less than 190 nm. These preparations were not infectious; hence it appears that infection is associated with the longer particles which are not retained during the purification procedures. Since the positions of the peaks of particle length distribution in the partially purified preparations were different for each pH level of the suspending medium, there is no assurance that the peaks in length distribution in the leaf dip preparations are reliable



**Fig. 2.** Distribution of lengths of particles from wheat infected with wheat spindle streak mosaic virus; **A**) leaf dip preparation; **B, C, D, E**) concentrates of particles resuspended in 0.01 M phosphate buffers at pH 5, 7, 8.8, and 10, respectively.

indicators of the lengths of infectious particles. Infectivity could be associated with extremely long particles like those of citrus tristeza or beet yellows viruses (1,

7). In any case, the instability of infectivity of WSSMV inoculum appears to be related to fragility and aggregation of the virus particles.

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