Particle Numbers Associated with Mechanical and Aphid Transmission of Some Plant Viruses

H. L. Walker and T. P. Pirone

Department of Plant Pathology, University of Kentucky, Lexington 40506.
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ABSTRACT

Manual inoculation of leaves of *Chenopodium amaranticolor* resulted in the production of one local lesion for every 10^7 cucumber mosaic virus (CMV) or tobacco etch virus (TEV) particles, for every 3×10^6 cowpea chlorotic mottle virus particles, and for every 10^5 tobacco mosaic virus particles in the inoculum. Systemic infection of tobacco plants with CMV was obtained with manually applied inoculum which contained 10^7 particles, but not with inoculum which contained 10^6 particles. Aphids could consistently transmit, to tobacco plants, CMV acquired from solutions which contained 7×10^{12} virus particles/ml and occasionally from solutions which contained 7×10^{11} particles/ml. The volume of liquid associated with the stylets of an aphid was determined to be between 0.6 and 4.2×10^{-10} ml. Based on a CMV concentration of 7×10^{12} particles/ml, about 1,000 virus particles would be present in this volume. A method is described for purification of TEV which yields unaggregated virus suitable for particle counts.

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Steere (12) has reported that one local lesion can be obtained for every 50,000 TMV particles in an inoculum. This value was obtained by manual inoculation of a virus suspension to leaves of a local lesion host. The purpose of the first part of our study was to determine the ratio of lesions to particles of stylet-borne (nonpersistent) viruses applied manually to a local lesion host, and to compare this value with that obtained for nonaphid-transmitted viruses. Cucumber mosaic virus (CMV) and tobacco etch virus (TEV) were chosen as representative isometric and flexous rod stylet-borne viruses. These were compared with tobacco mosaic virus (TMV) and cowpea chlorotic mottle virus (CCMV), the latter a nonaphid-transmitted virus morphologically similar to CMV.

The second part of this study compares the number CMV particles required for manual inoculation of a tobacco plant with the number required for aphid transmission.

MATERIALS AND METHODS.—*Purification.*—A common strain of TMV was purified from tobacco (*Nicotiana tabacum* L. 'Samsun nn') by differential centrifugation. CCMV, obtained from C. W. Kuhn, was propagated in cowpeas [*Vigna sinensis* (Torner) Savi 'Early Ramshorn'], harvested about 2 weeks after inoculation and purified according to the procedure of Bancroft et al. (2). The Wisconsin 102 strain of CMV was propagated in Havana 425 tobacco, harvested 7-10 days after inoculation, and purified according to the procedures of Scott (10) or Takanami & Tomaru (13).

A severe strain of TEV was propagated in Havana 425 tobacco. About 200 g of systemically infected leaves were harvested 10-15 days after inoculation and chilled to 4 C; all subsequent procedures were carried out at 2-5 C. The tissue was homogenized in a Waring Blendor in cold 0.5 M potassium phosphate buffer, pH 7.0, which contained 0.5% mercaptoethanol, in the proportion of 130 ml buffer/100 g tissue. The homogenate was strained through two layers of cheesecloth, and 9% (v/v) *n*-butanol added slowly with constant stirring to the filtered homogenate. The mixture was allowed to incubate 10-15 min, then centrifuged at 12,000 *g* for 10 min. The supernatant fluid was strained through two layers of Miracloth, and 200 ml were layered onto a 10 × 100-cm column packed with Sephadex G-50 (medium) which had been equilibrated with 0.02 M sodium borate buffer (pH 8.2). The virus was eluted from the column with 0.02 M borate buffer (pH 8.2) and centrifuged at 78,000 *g* for 1.5 hr. The pellets were resuspended in 0.02 M borate buffer (pH 8.2), and the low and high speed centrifugations repeated. The yield was 60-100 mg virus/kg tissue.

Particle counting.—Samples of polystyrene latex (PSL), Run No. LS-057-A (0.264 μ diam) and Run No. LS-063-A (0.557 μ diam) were obtained from Dow Chemical Company. Dilution standards were prepared according to Bahr et al. (1). A mixture consisting of 0.05 ml PSL standard, 0.30 ml 0.1% bovine serum albumin, 0.64 ml 0.2% ammonium bicarbonate, pH 7.8, and 0.01 ml of appropriately diluted virus was sprayed onto collodion-coated, carbon-reinforced grids with an EFFA spray apparatus (E. F. Fullam, Inc.). The grids were shadowed with platinum-palladium alloy and observed with a JEM 7A electron microscope.

Two to three grids were examined for each sample, and 10 to 12 droplets of 10-15 μ diam were photographed. The ratio of virus particles to PSL was determined from the prints, and the virus concentration was calculated.

Infectivity assay.—To determine the average number of virus particles required to produce a local lesion, *Chenopodium amaranticolor* Coste & Reyn. was used for all four viruses. This plant was the most sensitive of a number of local lesion hosts tested for CMV, CCMV, and TEV, and was similar to Havana 425 tobacco in its sensitivity to TMV. The top four fully expanded leaves were dusted with Carborundum, and 20 μliters of a suspension which contained a known number of virus particles were applied with the index finger to each half-leaf. With the exception of TMV, all preparations were assayed as soon as possible after purification, to minimize loss of infectivity due to aging. Each virus was suspended in the buffer which was found, in preliminary tests, to result in maximum lesion production. When viruses other than TMV were assayed, the opposite half-leaf was inoculated with a standard suspension of TMV, in order to provide a direct comparison of TMV with each virus. This also gave an indication of the variation in susceptibility of plants from experiment to experiment.

Aphid transmission.—Rearing and handling of aphids, acquisition of virus by aphids through membranes, and inoculation feedings of aphids on test plants were as described by Normand & Pirone (8).

RESULTS.—*Standardization of PSL.*—Dry weight determinations of nine 1-ml aliquots of a 1:10 dilution of 0.264 μ PSL gave a value of 3.55 ± 0.02 mg/ml. This agreed with the value of 3.5% solids supplied by the manufacturer. From this average weight, a specific gravity for PSL of 1.05 g/cm^3 and a particle volume of 9.64×10^{-15} cm^3, the standard PSL suspension was calculated to contain 3.5×10^{11} particles/ml. Standardization of a duplicate suspension gave a value of 3.48×10^{11} particles/ml. Similar determinations for the 0.557 μ PSL gave a value of 9.5 ± 0.15 mg/ml for a particle concentration of 9.9×10^{10} particles/ml in the standard suspension.

The accuracy of the spray-droplet technique was tested by a mixing of the two sizes of PSL in known proportions, based on dry weight. The mixture was sprayed onto EM grids and the numbers of each particle per droplet were counted. Using the 0.264 μ PSL as a standard, the concentration of the 0.557 μ PSL was calculated to be 11.2×10^{10} particles/ml, a value which differed by 13.5% from the theoretical value of 9.9×10^{10} calculated by dry weight. This variation is well within the range expected (7, 11, 14).

Standardization of TMV preparations.—Particle

counts of a stock preparation of purified TMV yielded values of $3.7 \times 10^{10}(\pm 0.52 \times 10^{10})$ and 3.6×10^{10} ($\pm 0.42 \times 10^{10}$) virus particles/ml in two experiments. The number of TMV particles in this preparation was also determined spectrophometrically. Using the value of 3.24 for the absorbance of a 1 mg/ml solution of TMV at 260 nm (3), the concentration was determined to be 3.7×10^{10} particles/ml.

Particles of TMV per local lesion.—TMV was diluted in 0.01 M potassium phosphate buffer (pH 7.0) for assays. The average number of TMV particles per local lesion ranged from 5.9×10^4 to 2.87×10^5 in 11 experiments. The average value for all experiments was 1.64×10^5 particles/lesion (Table 1).

Particles of CMV per local lesion.—Two methods of purification were used for CMV. Virus purified by Scott's method (10) was always aggregated, but aggregation was reduced and particle counts could be made when such virus was diluted in buffer containing 0.001 M EDTA. Excessive EDTA in the spray mixture produced undesirable deposits in the microdroplets. Best results were obtained when the virus was first diluted in 0.2% ammonium bicarbonate, containing 0.001 M EDTA, at pH 7.8. An aliquot of this preparation was then transferred to the spray mixture which contained no EDTA. Dilutions of CMV for all infectivity assays were made in 0.005 M sodium borate buffer, pH 9.0.

In two experiments with CMV prepared by Scott's method (10), with the virus treated with EDTA prior to spraying, the average number of CMV particles per local lesion was 1.6×10^7 and 4.0×10^7 (Table 1).

CMV purified by the method of Takanami & Tomaru (13) was less aggregated than that purified by the other methods, and it also had a higher specific infectivity. In two experiments, the average number of CMV particles per local lesion was 1.1×10^7 and 9.4×10^6 (Table 1).

Particles of CCMV per local lesion.—CCMV was diluted in 0.01 M sodium acetate buffer, pH 5.0, for infectivity assays. In two experiments, the average number of virus particles per local lesion was 3.16×10^6 and 3.13×10^6 (Table 1).

Preparation of nonaggregated TEV.—Neither of two purification procedures (5, 9) developed for TEV yielded virus suitable for particle counting. The addition of urea (5) produced preparations with a minimum of lateral aggregation, but end-to-end aggregation often involved 50-60% of the particles. The procedure described in MATERIALS AND METHODS yielded preparations in which less than 10% of the particles were aggregated. When the aggregates were twice the length of single particles, they were counted as two particles; if less, they were counted as one particle. "Short" particles were not counted.

Particles of TEV per local lesion.—TEV was diluted for assay in 0.02 M borate buffer, pH 8.2. In two experiments with purified TEV, the average number of virus particles per local lesion was 8.5×10^6 and 1.4×10^7 (Table 1).

Since the TEV particle can be detected, by electron microscopy, in relatively impure preparations, we compared the specific infectivity of virus in clarified sap extracts with that of purified virus. Three to 4 g of infected tobacco leaves were chilled and triturated in a mortar with 3-4 ml of cold 0.02 M borate buffer, pH 8.2. The extract was strained through cheesecloth and centrifuged at 3,000 *g* for 5 min. The supernatant fluid was removed and immediately diluted 1:10 in the same buffer and assayed on *C. amaranticolor*. Twenty to 50 μliters of extract was then added to the spray mixture containing PSL, and particle counts were made in the

TABLE 1. Number of virus particles in inoculum per lesion produced on *Chenopodium amaranticolor*

Virus	No. particles in inoculum ($\times 10^8$)	Standard error (% of mean)	Avg lesion no. per half-leaf	No. particles per lesion ($\times 10^6$)	TMV[f] particles per lesion ($\times 10^5$)
CMV[a]	14.3	12.9	89	16.1	0.59
CMV[a]	14.3	8.5	36	39.7	2.54
CMV[b]	23.2	4.1	209	11.1	2.87
CMV[b]	20.1	7.6	215	9.35	1.40
TEV[c]	4.48	11.5	53	8.45	1.50
TEV[c]	12.1	6.2	84	14.4	1.56
TEV[d]	8.12	13.8	174	4.67	1.39
TEV[d]	10.1	9.2	100	10.1	1.28
TEV[d]	10.7	10.3	100	10.7	1.23
CCMV[e]	13.8	13.5	437	3.16	1.45
CCMV[e]	2.97	12.2	95	3.13	2.27

[a] CMV = cucumber mosaic virus. Purified by method of Scott (10).
[b] Purified by method of Takanami & Tomaru (13).
[c] TEV = tobacco etch virus. Purified preparation.
[d] Tissue extract.
[e] CCMV = cowpea chlorotic mottle virus. Purified by method of Bancroft et al. (2).
[f] TMV = tobacco mosaic virus.

usual way. The average number of particles per local lesion for virus prepared in this manner was similar to that for purified virus. In three experiments, the average number of particles per lesion was 4.7×10^6, 1.0×10^7, and 1.1×10^7 (Table 1).

Number of CMV particles required for systemic infection.—The third- or fourth-youngest leaf of a Havana 425 tobacco plant in the 5- to 6-leaf stage was inoculated with 2.5 μliters of purified CMV. In these, as well as in the aphid transmission studies, the virus was purified by the method of Takanami & Tomaru (13), but EDTA was not used in the purification process. EDTA was added to the aliquots used for particle counting, but the virus used in transmission studies had not been treated with EDTA. In four experiments, 11/24, 1/23, 3/24, and 4/24 plants became infected when rubbed with inoculum which contained 1×10^7 particles. No infection was obtained with inoculum which contained 1×10^6 or fewer particles.

Number of CMV particles involved in aphid transmission.—Aphids (*Myzus persicae* Sulz.) were allowed to probe solutions which contained a known number of CMV particles per ml. The aphids were then transferred to Havana 425 tobacco plants (one aphid/plant) to test for virus transmission. The results (Table 2) show that aphids could consistently transmit virus acquired from solutions which contained 7×10^{12} particles/ml, and occasionally from solutions which contained 7×10^{11} particles/ml.

The CMV involved in the transmission process is that which is associated with the stylets. Thus, an attempt was made to estimate the volume of liquid which is associated with the stylets. Aphids were allowed to probe through a Parafilm membrane into ^3H_2O (specific radioactivity 1 c/ml). Membranes, probing time and sucrose concentration were the same as those used in the virus transmission tests. Individual aphids were then removed from the membrane and either anesthetized with CO_2 or handled without anesthesia. Their stylets were then either removed from the proboscis and cut off as near to the head as possible, or cut off along with the proboscis. The mouthparts were then placed in Bray's solution (4). The amount of ^3H_2O associated with the stylets was calculated by comparing the counts (determined by liquid scintillation) with those produced by known volumes of the ^3H_2O.

The volume of ^3H_2O associated with the stylets was in the range of 0.6 to 4.2×10^{-10} ml. (Table 3). The number of virus particles from a suspension containing 7×10^{12} particles/ml (Table 2) which would be contained in this volume would be 420-2,940 or, roughly, 1,000 particles.

DISCUSSION.—Sharp (11) reported that a standard error of about 16% of the mean is typical for particle counts by the spray technique. The standard errors in our tests ranged from 4.1 to 13.8%. The concentration of TMV determined by particle counting agreed well with that determined by spectrophotometry. Therefore, the particle counts reported in this paper are believed to be as representative of the true particle concentrations as is possible with the present methods of assay.

Although the data for particle concentrations are quite accurate, the number of particles per lesion for a given virus were often found to vary 2- to 3-fold in repeated experiments. This variation could be a reflection of differences in specific infectivity of different virus preparations, but more probably resulted from differences in susceptibility of leaves of the test plants in the different experiments. Evidence for this is the fact that the number of particles per

TABLE 2. Transmission of cucumber mosaic virus acquired by *Myzus persicae* from solutions containing known numbers of virus particles

	Number of virus particles/ml[a]			
	7×10^{13}	7×10^{12}	7×10^{11}	7×10^{10}
Experiment	Transmission			
1	9/24[b]	6/24	0/24	0/24[c]
2	4/24	0/24	1/24	0/24
3	5/24	1/24	0/24	0/24
4	4/24	1/24	0/24	0/24
5	5/24	6/24	0/36	0/36
6	2/12	5/24	2/24	0/24
	4/12[d]	3/24[d]	0/24[d]	0/24[d]
	4/12[e]	2/24[e]	1/24[e]	0/24[e]
7	2/12	2/24	0/24	0/24

[a] Virus was suspended in 5.0 mM sodium borate buffer (pH 9.0), unless otherwise noted.
[b] Numerator: plants infected; denominator: number of test plants. A single aphid was placed on each test plant.
[c] No transmission was obtained from solutions which contained 7×10^{10} or fewer particles in any experiment.
[d] Virus was suspended in distilled water.
[e] Virus was suspended in 5.0 mM sodium borate pH 8.2.

TABLE 3. Volume of ^3H_2O associated with the mouthparts of aphids which probed through a Parafilm membrane into ^3H_2O[a]

Aphid treatment	Volume (ml × 10^{-10})
Mouthparts removed from anesthetized aphids	
Stylets ensheathed in proboscis	0.6
Stylets only	1.3
Proboscis only	0.6
Stylets only	1.3
Stylets (exposed) + proboscis	0.7
Stylets (exposed) + proboscis	1.0
Stylets (exposed) + proboscis	1.7
Mouthparts removed from nonanesthetized aphids	
Stylets ensheathed in proboscis	0.8
Stylets ensheathed in proboscis	1.5
Stylets ensheathed in proboscis	2.6
Stylets ensheathed in proboscis	4.2
Stylets (exposed) + proboscis	3.7
Stylets (exposed) + proboscis	3.5
Stylets (exposed) + proboscis	2.7

[a] Individual aphids were allowed to probe for 30 sec into ^3H_2O (specific radioactivity 1 c/ml) which contained 5% sucrose. The aphids were then either (i) anesthetized with CO_2 and their mouthparts amputated; or (ii) held down with a camel's-hair brush and their mouthparts amputated. The mouthparts were placed in Bray's solution and the amount of ^3H_2O determined by liquid scintillation counting. Counting efficiency for ^3H was 50%.

lesion with the standard TMV preparation varied over the course of the experiments (Table 1). Decrease of infectivity of the TMV standard with time was not a factor, because the results tabulated in Table 1, in chronological order, show no evidence of a progressive decline. Despite these variations, it is obvious that the number of TMV particles per local lesion is far fewer than the number for the other viruses. The values obtained with the stylet-borne CMV and TEV were similar, when the best CMV preparations are considered. A local lesion was produced per every 10^7 particles inoculated. The value for CCMV was somewhat lower than that for CMV and TEV, and considerably higher than that for TMV.

The method by which the ratios of lesions to characteristic particles were determined in this study does not provide the minimum lesion:particle ratio. Using Steere's method (12), in which a much larger volume (500 μliters) was applied to as many leaves as possible, we obtained values of one lesion for every 25,000-50,000 TMV particles in the inoculum. This represents an efficiency some 2- to 4-fold greater than that obtained by the method presented here. However, the method used in this study was chosen because we wished to obtain comparative ratios for the different viruses, using a reasonable number of test plants.

Infection of tobacco, a systemic host of CMV, required the presence of about 10^7 CMV particles in a manually applied inoculum. No infection was obtained when inoculum which contained 10^6 or less particles was applied. Although this value is similar to the average value of 10^7 particles/observed local lesion, determined with *C. amaranticolor*, it must be borne in mind that they are not directly comparable, due to the possibility of multiple infections of the systemic host.

Aphids could transmit CMV consistently from suspensions which contained 7×10^{12} particles/ml, and occasionally from suspensions which contained 7×10^{11} particles/ml. Transmission was not obtained from suspensions which contained 7×10^{10} particles/ml or less. The volume of liquid associated with the stylets was in the order of 10^{-10} ml as determined by the ^3H_2O experiments. The average values obtained with nonanesthetized aphids were somewhat higher than those for anesthetized aphids, although the individual values overlap. The experimentally determined values agree well with the volume of the food canal within the stylets of *Myzus persicae*, which we calculated from the data of Forbes (6). Using 425 μ as the average length of the stylet bundle of a fully grown nymph, and 0.9 μ as the average diameter of the food canal (range 0.6 to 1.2 μ), and considering the food canal as a cylinder, a value of 225 μ^3 or 2.5×10^{-10} cc was obtained. The number of CMV particles in a volume of 10^{-10} ml would be in the order of 10^3 if the virus is acquired from a suspension of 7×10^{12} particles/ml, and 10^2 from a suspension of 7×10^{11} particles/ml. These values are several orders of magnitude lower than the 10^7 particles required for successful manual inoculation.

The indirect manner used to determine the number of particles associated with aphid stylets does not preclude the possibility that virus particles may be selectively absorbed to the stylets. If such is the case, the number would be higher than that calculated on the basis of volume. We have made a number of attempts to determine whether CMV particles are accumulated on the stylets. The techniques used included manual inoculation to *C. amaranticolor* of stylets (and the virus thereon) excised from aphids which had probed 7×10^{12} particles/ml CMV suspensions; attempted elution of virus from such stylets, followed by infectivity assay or electron microscopic examination of the eluate; and electron microscopic examination of replicas of stylets from aphids which had probed virus suspensions. In no case were we able to detect infectivity or virus particles. However, as there is no way to determine whether high concentrations of virus particles could be detected by these methods if they were present, these experiments cannot be regarded as at all conclusive.

If selective absorption to stylets does occur, it seems unlikely that within 30 sec of probing it could provide for the 10^4- to 10^5-fold increase needed to bring the CMV particle concentration up to the value of 10^7 required for manual inoculation.

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