Serologically Specific Electron Microscopy in the Quantitative Measurement of Two Isometric Viruses

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ABSTRACT

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The serologically specific method for the electron microscopic detection and assay of viruses was tested for the quantitative measurement of two isometric viruses, cowpea mosaic (CpMV) and cauliflower mosaic (CaMV). For CpMV it was necessary to coat the grids with highly diluted antiserum and to use a low-salt medium to avoid aggregation and uneven distribution of virus on the grids. Under these conditions the number of CpMV particles adsorbed to the grids decreased linearly with dilution. The lowest detectable concentration was 0.01 μg/ml or 10^9 virions/ml, about the same threshold concentration required to produce infections on a local lesion host. In comparative trials for measurement of CpMV during multiplication in cowpea protoplasts, the serologically specific electron microscopic method and bioassays on a local lesion host gave essentially the same growth curves. For CaMV, aggregation and uneven distribution of virions on coated grids was not a serious problem, but the use of a low-salt medium reduced nonspecific adsorption of virus to carbon films. The sensitivity of the method for CaMV was similar to that for CpMV, but a linear relationship was not obtained between the number of CaMV virions adsorbed to serologically specific grids and dilutions of crude extracts of virus-infected plants. The number of CaMV virions adsorbed to the grids at low sap dilutions was less than expected, which indicated that the virus was not released readily from inclusion bodies under those conditions. Hence the method cannot be used for the quantitative measurement of CaMV in crude extracts of infected plants.

The counting of virus particles attached to grids, to which virus antibodies previously were adsorbed, is a technique established by Derrick (4) for quantitative analysis of rod-shaped viruses in crude extracts. Similar analyses of isometric virus particles have not been reported, although Derrick and Brlansky (5) recently stated that the method is useful for detecting several isometric viruses in plant extracts. From the authors' experience, nonspecific adsorption is a greater problem with isometric viruses and it is more difficult to distinguish cellular components from these viruses than from rod-shaped viruses.

The present investigation concerns the detection and quantitative measurement of two spherical viruses, cauliflower mosaic and cowpea mosaic viruses, by serologically specific electron microscopy. Conditions were developed which allowed specific adsorption of these viruses with relatively little nonspecific background material. Conditions also were developed that produced a uniform distribution of these viruses which ordinarily tend to aggregate on grids coated with antiserum. The method was used to determine the number of cowpea mosaic virus particles in extracts of protoplasts infected with the virus and the sensitivity of this method was compared with bioassays on the primary leaves of cowpea, a local lesion host. In addition, the method for the quantitative measurement of cauliflower mosaic, a virus for which no useful local lesion host is known, was evaluated.

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MATERIALS AND METHODS

Isolate Sb12 (1) of cowpea mosaic virus (CpMV-Sb12) was purified from cowpea plants according to the large-scale procedure described (3). An extinction coefficient of 8.0/mg/ml (1-cm light path) at 260 nm (9) was used to estimate virus concentration and the virion particle weights were calculated from the viral protein and RNA molecular weights and composition (6, 13). The cabbage B strain of cauliflower mosaic virus (CaMV-B) was purified according to Hull et al. (7). An extinction coefficient of 7.0/mg/ml (1-cm light path) at 260 nm (11) and a value of 22.8×10^6 daltons for the virion particle weight (7) was used to estimate numbers of virions per unit volume.

Antiserum to a mixture of middle- and bottom components of CpMV-Sb12 was prepared as described by Siler et al. (12). The antiserum titer, defined here as the highest dilution of antiserum that gave a visible

precipitate in tube precipitation tests using 0.01 mg virus/ml at 37 C (8), was 1/1,600. Control serum was obtained by pre-immunization bleeding on the day of first injection. Antiserum to CaMV (titer of 1:500) was prepared essentially by the same method as CpMV antiserum (1 mg/ml purified CaMV was injected five times at intervals of 10 days).

Copper electron microscope grids [74-μm (200-mesh)], coated with carbon films backed with Parlodion, were made serologically specific for CpMV or CaMV by floating them for 30 min at 25 C on solutions of the specific antiserum. The method used was essentially the same as described by Derrick (4) with some modifications. For routine experiments, CpMV antiserum was diluted 1:1,000 and CaMV antiserum was diluted 1:200 with Tris buffer (0.05 M, pH 7.5). After washing the grids by floating them on droplets of 0.05 M Tris, pH 7.5, to remove unadsorbed serum proteins, they were floated on 50-μliter drops of purified virus preparation or crude extracts of infected protoplasts for 2 hr at room temperature (approximately 25 C).

Extracts of CpMV-infected protoplasts were prepared and the protoplasts infected according to the method of Beier and Bruening (2). Protoplasts were isolated from primary leaves of cowpea, *Vigna sinensis* 'Blackeye No. 5' and were inoculated (10^6 protoplasts/ml) with purified CpMV-Sb12 at a concentration of 10 μg/ml in the presence of 2 μg/ml of poly-L-ornithine. For standard experiments, the protoplasts (about 8×10^6) were harvested 45 hr after inoculation and stored at −70 C. Thawed protoplasts were mixed with 3 ml of 0.05 M potassium phosphate buffer, pH 7.0, and were homogenized in a Brinkman Polytron homogenizer. The homogenate was centrifuged at low speed and the supernatant fluid, diluted 1/10 with Tris buffer (0.05 M, pH 7.5), was used for the immuno-specific procedure described above. After they had been floated on virus preparations or protoplast extracts, the grids were washed first by floating them on Tris buffer and then on glass distilled water for a few minutes. The washed grids were air-dried and shadowed with platinum-palladium (80:20) at an angle of 30 degrees or stained with uranyl acetate in 70% ethanol for 30 sec. The electron micrographs were taken at a magnification of about 8,000 (RCA Model EMU3H Electron Microscope). The electron micrographs were projected on a 56-cm square piece of white cardboard (divided into 36 × 36 unit squares) and the number of virus particles per unit area (28.4 μm^2) was determined. Usually three electron micrographs taken from different areas of a grid were made for each estimation. In several preliminary trials relatively little variation in number or distribution of virus was shown between grids.

RESULTS

The first experiments were carried out with CpMV-infected protoplasts which had been diluted 1:10 with tris-NaCl (0.05 M Tris, 0.9% NaCl) following the procedure described by Derrick (4). A 1:50 dilution of the CpMV-antiserum was used for coating the grids, which then were floated on drops of protoplast extracts for 2 hr at 37 C. The result can be seen in Fig. 1-A. Particles of various sizes and shapes were distributed over the entire grid and it seemed very unlikely that all of them were cowpea mosaic virions. However, on grids which had been coated with pre-immunization serum and treated as described above, these particles were not visible. Moreover, the irregular-sized particles were not observed when protoplast extracts diluted about 10-fold were used. An even distribution of TMV particles was attached to the grid coated with antiserum specific to TMV and incubated with 0.1 μg/ml TMV. Thus, it was reasonable to assume that the particles seen on grids specific to CpMV were in fact aggregates of cowpea mosaic virions.

Several procedures were tested to overcome the formation of aggregates. A more dilute antiserum (1:1,000) was used to reduce the number of CpMV-specific antibody sites on the grid; the aggregates were smaller but still heterogenous in size. A combination of a 1:1,000 dilution of CpMV antiserum and an incubation temperature of 25 C produced a better result; particles approximately the size of single CpMV-virions were visible on the grids.

The addition of salt, such as NaCl, although necessary for optimum antigen antibody reaction in tube precipitation tests (8), may not be necessary for the specific adsorption of virus particles to grids coated with antiserum, and may even promote aggregation. Thus, an experiment was done in the absence of salt, at room temperature, using highly diluted antiserum (1:1,000). Only a few 'aggregates' were visible, whereas most of the particles were evenly distributed and uniform in size and shape (Fig. 1-B). However, the question remained whether the absence of salt, while resulting in decreased aggregation of virus, might also reduce the number of

TABLE 1. Influence of NaCl concentration in the reaction mixture on the amount of cowpea mosaic (CpMV) or cauliflower mosaic virus (CaMV) particles attached to serologically specific or nonspecific grids

Virus name and reaction mixture NaCl supplement	No. virions attached to grid	
	Virus antiserum	Normal serum
Cowpea mosaic virus:		
0.9 % NaCl	1,780[a]	3
0.45% NaCl	1,620	0
without NaCl	2,560	0
Cauliflower mosaic virus:		
0.9 % NaCl	1,840	131
0.45% NaCl	2,080	60
without NaCl	1,890	14

[a]The numbers refer to virions attached to serologically specific grids per unit area of 28.4 μm. Each number represents an average of three unit areas taken from a single grid. The experiments were done with purified virus preparations diluted with 0.05 M Tris buffer, pH 7.5, with or without NaCl. Grids specific for CpMV or CaMV were floated on 50-μliter drops of virus preparation for 2 hr at 25 C. Control grids were obtained by coating them with unspecific serum. Further details are given in the text. The final concentrations were 1 μg/ml of CpMV (1×10^{11} particles/ml) and 10 μg/ml of CaMV or (2×10^{11} particles/ml). The dilutions of CpMV antiserum and CaMV antiserum were 1:1,000 and 1:200, respectively.

particles attached to the grid. As can be seen from the data in Table 1, about the same number of CaMV particles attach to grids in the absence of NaCl and more CpMV particles attached to serologically specific grids in the absence of NaCl than when NaCl was present. Another interesting effect correlated with the absence of added NaCl became obvious when CaMV particles had been adsorbed to grids coated with pre-immunization serum.

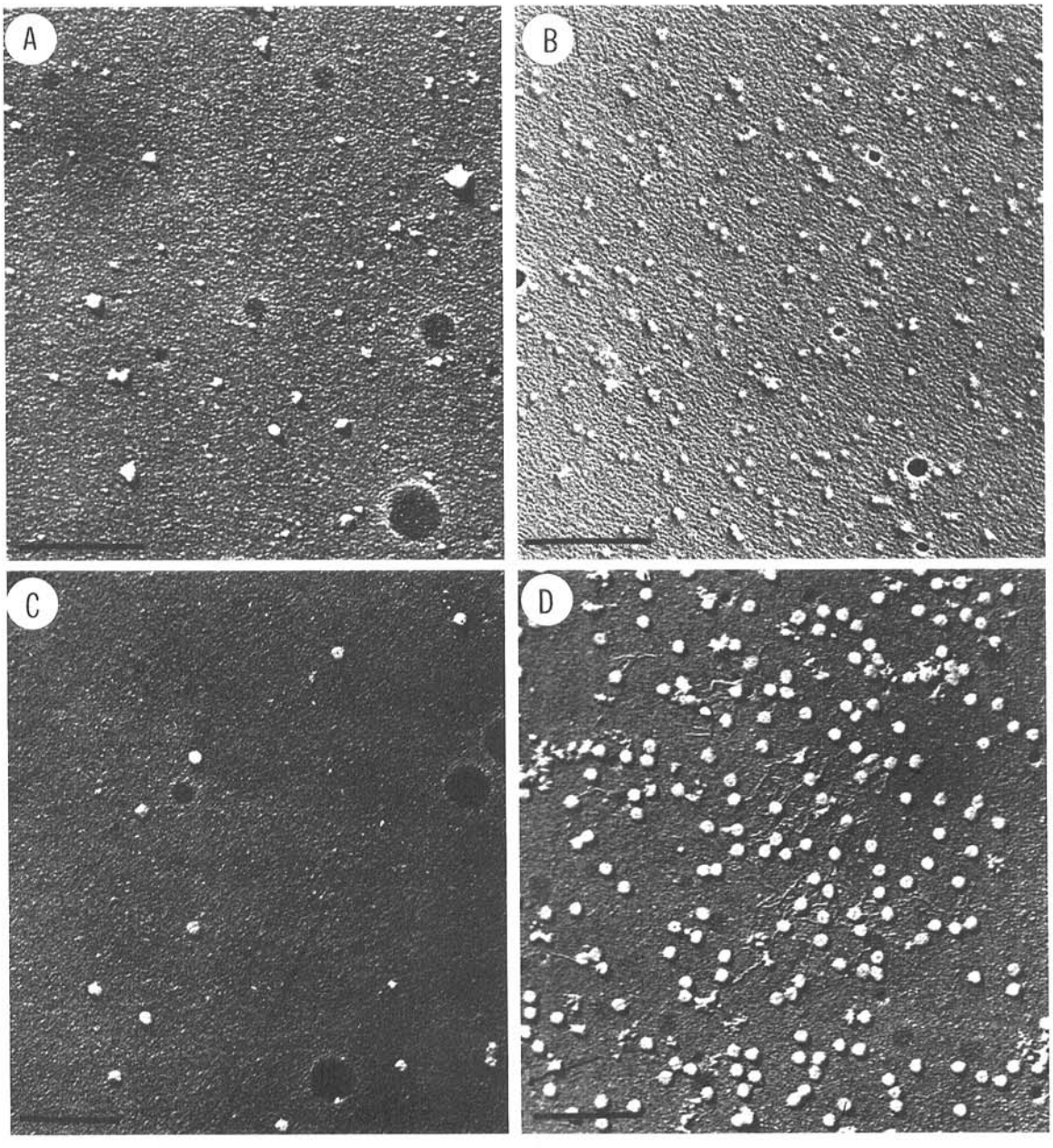

Fig. 1-(A to D) Electron micrographs of virus particles attached to serologically specific grids. **A)** Grids were coated with cowpea mosaic virus (CpMV)-antiserum diluted 1:50 with Tris buffer (0.05 M, pH 7.5). Extracts of CpMV-infected protoplasts were prepared as described in text. A 1:10 dilution of the CpMV extract was made with Tris-NaCl (0.05 M, Tris, 0.9% NaCl). The grids were floated on 50-μliter drops of CpMV extracts for 2 hr at 37 C. The bar represents 500 nm. **B)** The same dilution of CpMV extract was used, however, the dilution was made in 0.05 M Tris buffer alone, the CpMV-antiserum had been diluted 1:1,000, and the coated grids were floated on CpMV extracts at room temperature (approximately 25 C). The bar represents 500 nm. (C and D) A purified cauliflower mosaic virus (CaMV) preparation (3.0 mg/ml) was diluted 1:300 with Tris buffer (0.05 M, pH 7.5). Grids had been coated with either **C)** normal serum or **D)** with CaMV-antiserum. Dilutions (1:200) of the normal serum and antiserum were made with Tris buffer. Coated grids were floated on 50 μliter of CaMV for 2 hr at 25 C. The bars represent 500 nm.

In the presence of 0.9% NaCl the number of CaMV particles attached to serologically nonspecific grids was rather high, but in the absence of salt the number was reduced, indicating a higher specificity (Table 1-B, Fig. 1-C).

Particles of CaMV did not tend to form aggregates as often as did those of CpMV under the various conditions used. This could be related partly to the different particle sizes of these two viruses. The particle weight of CaMV is 22.8×10^6 daltons compared to an average particle weight of only 5.5×10^6 daltons for CpMV. Moreover, the diameter of CaMV particles is almost twice that of CpMV particles. It is, therefore, not unexpected that CaMV particles attached to grids appear more distinctive (Fig. 1-D) than CpMV particles at the same magnification. In addition, CpMV particles tended to be buried under the platinum-palladium shadow which resulted in less contrast. Generally there was no difference in the array or appearance of virions whether purified CpMV or a crude extract of CpMV-infected protoplasts had been used. In the latter case the background was very clear (Fig. 1-B) and nonspecific adsorption was very low on grids with normal serum.

Serologically specific electron microscopy was found to be a convenient and sensitive method for measuring the concentration of CpMV in partially purified solutions or crude extracts. After suitable conditions for the attachment of CpMV particles to serologically specific grids were established, serial dilutions of purified CpMV preparations or crude extracts of CpMV-infected protoplasts were made. The log of the number of CpMV particles decreased linearly with dilution (Fig. 2). The lowest concentration of CpMV particles detectable using serologically specific electron microscopy without added NaCl in the medium was 0.01 μg/ml or approximately 10^9 virions/ml (Fig. 2). Incubation times longer than 2 hr gave rise to a slightly higher number of particles attached to the grid at a given concentration. However, as a result of the longer incubation time, clusters of virus particles often were visible which made the accurate counting of virions difficult.

Table 2 shows a comparison of CpMV detection by immuno-specific electron microscopy compared with bioassays on a local lesion host and two other commonly used serological methods, tube precipitin tests, and immunodiffusion in gels.

It was impossible to determine the sensitivity of the serologically specific electron microscope method for CaMV in a manner similar to CpMV. A CaMV preparation diluted to a final concentration of 10 μg/ml (2×10^{11} particles/ml) resulted in the attachment of approximately 2,000 virions to a given area of a grid specific for CaMV (Table 1). The same number of CpMV virions per milliliter resulted in the attachment of approximately 4,000 virus particles to a grid specific for CpMV. Thus, the sensitivity of this assay seems to be similar for both viruses. For CaMV, the assay could be made more sensitive by increasing the reaction time from 2 hr to 5 hr; the occurrence of clusters of virions is not a problem with this virus.

The specificity of the assay is quite high. There were almost no CpMV particles attached to the grids coated with the control serum when the concentration of virus particles was 10^{11} CpMV particles/ml (Table 1) and there were about 135 times more CaMV particles attached to serologically specific grids than to control grids in the absence of NaCl in the reaction mixture (Table 1, Fig. 1-C).

The advantage of the technique described by Derrick (4) lies in its usefulness for the identification and quantitative analysis of viruses in crude leaf extracts rather than in a purified preparation. As a test system for

TABLE 2. Quantities of cowpea mosaic virus detected by local lesion assay and selected serological tests

Concentration of virus applied to grid[a] (μg/ml)	Presence and/or quantity of virus determined by:			
	Local lesion assay	Serologically specific grids[c]	Tube precipitin test[d]	Immunodiffusion test[e]
50	...	...	+	(+)
10	...	...	+	
5	...	...	+	
1	...	1,700	(+)	
0.5	105	800		
0.1	54	150		
0.05	23	75		
0.01	3	12		
0.005	1.25			
0.001	0.16			

[a]Calculated from the absorbance at 260 nm and an extinction coefficient of 8.0/mg/ml (1-cm light path).

[b]Lesions per half leaf on *Vigna sinensis* 'Chinese Red × Iron'.

[c]Number of particles attached to a unit area of serologically specific grids. Grids coated with CpMV-antiserum (1:1,000) were floated on 50-μliter drops of purified CpMV in 0.05 M Tris buffer, pH 7.5, at room temperature for 2 hr.

[d]For the tube precipitin test the symbol (+) indicates the dilution endpoint (further dilution did not result in any visible precipitation). The CpMV antiserum was diluted 1:50 in phosphate buffer, pH 7.0 (0.1 M, 0.9% NaCl) and incubated with an equal volume of diluted purified CpMV at 37 C overnight.

[e]For the immunodiffusion test and symbol (×) indicates the dilution endpoint using CpMV antiserum at 1:50 dilution in phosphate-saline (0.1 M, 0.9% NaCl) in 0.8% Ionagar (Difco) gels containing 0.4% NaCl and 0.02% sodium azide.

CpMV, the rate of virus multiplication in CpMV-infected protoplasts was determined by serologically specific electron microscopy and by local lesion assays on cowpea. The protoplasts were prepared and infected as described by Beier and Bruening (2). A comparison of the two assays revealed essentially the same growth curves (Fig. 3).

When serologically specific electron microscopy was tested for the diagnosis of CaMV in crude extracts of infected turnip plants, it was successful in the detection of three strains (cabbage B, CM4-184, and NY8153) but it failed in the detection of a fourth, which was designated 'Phatak'. The latter strain has very small inclusion bodies, with correspondingly less virus in infected plants.

In further tests with CaMV, the method was not satisfactory for quantitative measurement of the virus in crude extracts because a linear relationship was not shown between virus concentration and virus particles attached to serologically specific grids. The results of two trials with dilutions of crude saps from infected plants are shown in Fig. 4. It can be seen that less than the expected amount of virus is adsorbed to grids treated with low dilutions of sap (Fig. 4), implying that much of the virus was not available for attachment to the grids except at high dilutions of the sap. Moreover, with sap extracts the variation among the various dilutions is much greater than when a purified preparation of the virus is used (Fig. 4). When purified CaMV was added to extract of healthy plants followed by quantitative serologically specific electron microscopy, a linear relationship between numbers of particles and dilution was obtained (Fig. 4), hence the presence of plant material cannot be regarded as the cause of the problem.

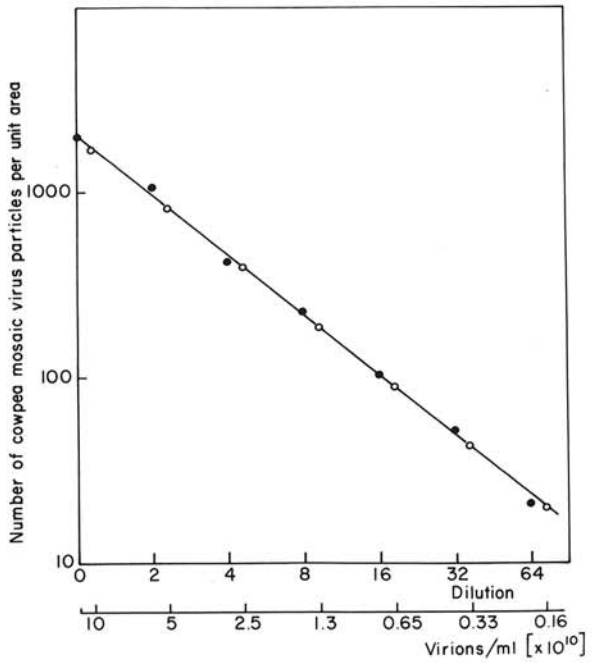

Fig. 2. Relationship between the logarithm of the number of cowpea mosaic virus (CpMV) particles attached to a unit area (28.4 μm^2) of serologically specific grids and dilution of a crude extract of CpMV-infected protoplasts. The open and closed circles represent the results of two separate experiments. The infected protoplasts were harvested 45 hr after inoculation. The initial protoplast extract was diluted 1:10 with Tris buffer (0.05 M, pH 7.5); the CpMV antiserum had been diluted 1:1,000, and the coated grids were floated on CpMV extracts at 25 C for 2 hr. The lowermost scale indicates the actual concentration of CpMV particles in the protoplast extract. These values were obtained from a second calibration curve in which a serial dilution was made with a purified CpMV-Sb preparation of known concentration.

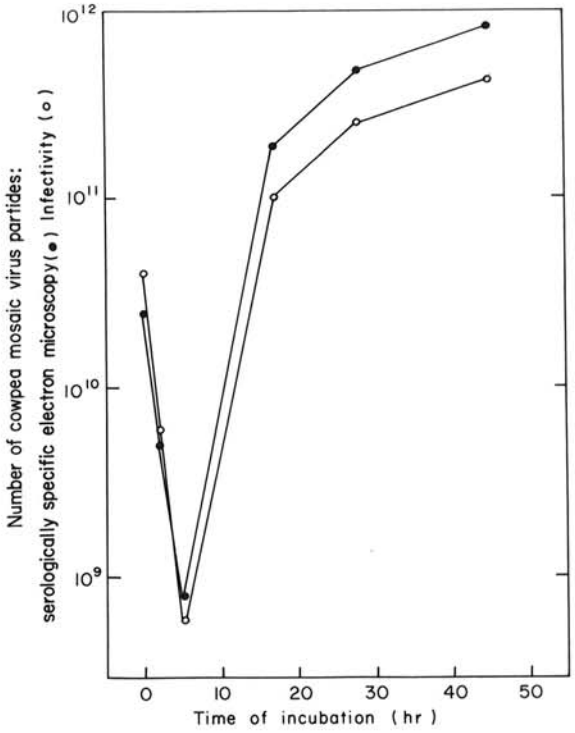

Fig. 3. The number of cowpea mosaic virus (CpMV) particles recovered from cowpea leaf protoplasts at various times after inoculation as shown by infectivity assays and by serologically specific electron microscopy. Protoplast extracts from infected protoplasts were prepared as described in the text. Aliquots of the same sample were used for either infectivity assays or serologically specific electron microscopy. For comparison, the results of both types of assay are expressed as virions per milliliter of extract. For infectivity assays the protoplast extract was inoculated on primary leaves of 7-day-old *Vigna sinensis* 'Chinese Red × Iron', and local lesions were counted after 4 days. A relationship between the average number of lesions per leaf and the virus concentration (virions per milliliter) in the protoplast extract was developed by assay of a dilution series of CpMV-Sb12 virus standards of known concentration on opposite leaves of the same plant. For serologically specific electron microscopy aliquots of protoplast extracts were diluted with Tris buffer (0.05 M, pH 7.5). Grids coated with CpMV antiserum were floated on drops of protoplast extracts at 25 C for 2 hr. A relationship between the average number of virus particles attached to a given area per grid and the virion concentration (virion/ml) in the protoplast extract was calculated from the calibration curve shown in Fig. 2.

DISCUSSION

Serologically specific electron microscopy is a sensitive method for determining the number of virions in crude leaf extracts with rod-shaped viruses like TMV and PVY (4) as well as for isometric viruses like CaMV and CpMV, as shown by this work. The lowest detectable concentration of TMV was 0.01 μg/ml or 2×10^8 particles/ml (4). For CpMV these values were 0.01 μg/ml or 1×10^9 particles/ml (Fig. 2). This method is thus much more sensitive than other types of assay based on antigen-antibody interaction. By means of the tube precipitation test (8) and the immunodiffusion test (10) the lowest concentrations of CpMV detectable were 1 μg/ml and 50 μg/ml, respectively (Table 2). Serologically specific electron microscopy is as sensitive as a local lesion assay and the results just as reproducible in this particular case. This was very obvious when the increase of infectivity in protoplasts after inoculation with CpMV was compared by means of a local lesion assay and serologically specific electron microscopy. The growth curves were similar (Fig. 3).

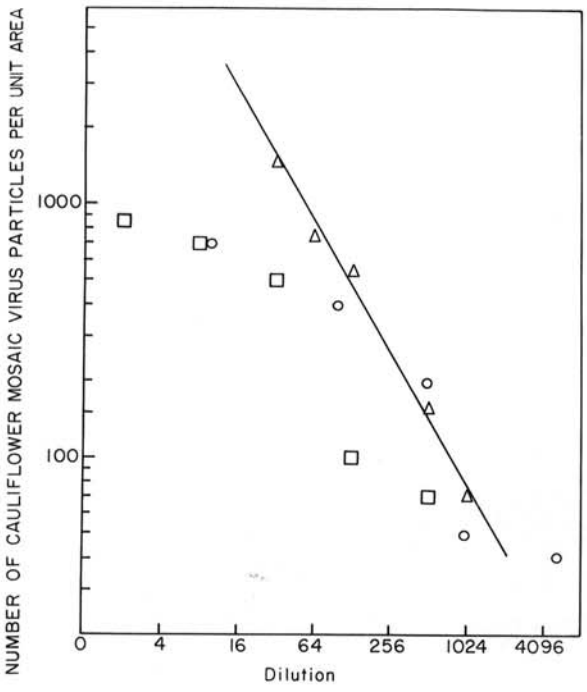

Fig. 4. Relationship between the logarithm of the number of virions per unit area of serologically specific grids and the dilution of a crude extract of cauliflower mosaic virus-infected plants. The open squares and open circules show the number of virions attached per unti area in two trials in which 1 g of infected turnip leaf (cabbage B strain) was homogenized in 1 ml of 0.05 M Tris, 0.5% NaCl, pH 7.5, followed by the indicated dilutions with the same buffer. Serologically specific grids were prepared by floating new grids for 30 min on antiserum diluted 1:30. The grids were stained with 1% uranyl acetate in 70% ethanol for 30 sec. The open triangles show the numbers of virions attached per unit area of serologically specific grids when 10 μliters of purified CaMV (at 3.75 mg/ml) was added to an extract consisting of 1 g of healthy turnip leaf homogenized in 1 ml of 0.05 M Tris, 0.5% NaCl followed by the dilutions indicated.

The salt concentration in the reaction mixtures during attachment of the viruses to serologically specific grids was found to be an important factor. The presence of 0.9% NaCl in the virus solution containing CpMV, for example, caused clumping of virus particles into irregular-sized aggregates in which individual virions could not be counted. Attachment in virus solutions without salt gave a well-distributed array of virus particles (Fig. 1-A,B) as well as on an improvement in the quantity of virus attached to the grid (Table 1).

A potential use of the immuno-specific electron microscope method may be in a virus system where no local lesion host is available or, if available, is lacking in sensitivity as is the case with CaMV. Unfortunately, the method is not quantitatively reliable with this particular virus. The virus is essentially occluded in infected cells since virtually all virions are embedded in the interior of the inclusion bodies. Very little virus occurs outside the inclusion body in undisturbed cells. The data in Fig. 4, in which less than the expected amount of virus appears on grids treated with low dilutions of sap, suggest that relatively little virus is released from the inclusion bodies until the extract is diluted more than about 50-fold. Even then, the great variability between treatments suggests that the method could not be used for quantitative measurement of CaMV in crude extracts.

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