Number of Virus Particles in Insects and Plants Infected with Wound Tumor Virus

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Received for publication 21 December 1967

A new procedure for counting virus particles was employed to measure the concentration of wound tumor virus in purified virus preparations, in plant tumors, and in the insect vector. Partially purified wound tumor virus was used to establish the quantitative features of the method. A 1-g amount of plant tumor tissue contained an average of 5×10^{10} virus particles and 1 g of insect tissue contained 2×10^{10} particles.

Wound tumor virus (WTV) has been the subject of intensive studies since its discovery by Black and Oman in 1941 (3). But the work was complicated by the lack of a simple quantitative assay. The only infectivity test available was an indirect assay which was time-consuming and inaccurate. This assay involved the injection of virus-free leafhoppers, followed in several weeks by a test of their ability to transmit the virus to susceptible plants (8). Reddy and Black (9) improved the accuracy of the infectivity test by determining the relative concentrations of WTV from the results of serological tests with extracts from injected insects. However, for routine investigations, the method is rather laborious and permits only indirect measurements of virus concentrations. Recently, Gamez and Black (7) reported on the application to WTV of the particle-counting technique of Backus and Williams (1, 2). In our work we used a new procedure, developed by Strohmaier (11), to determine the number of virus particles in WTV-infected insects and plants.

MATERIALS AND METHODS

Virus source. For virus acquisition, second or third instar nymphs of the clover leafhopper *Agallia constricta* Van Duzee were confined in plastic cages to WTV-infected red clover (*Trifolium incarnatum* L.). After 1 week, the leafhoppers were transferred to healthy clover plants where they were kept for 3 to 5 weeks. The insects, which had moulted to adults by that time, were collected and stored at -20°C . Root tumors were harvested from a clone of sweet clover, *Melilotus officinalis* L., which was systemically infected with WTV (4). According to the procedure of Black (5), a 10% (w/v) suspension was prepared by grinding the root tumors in a solution of 0.055 M K_2HPO_4 and 0.01 M Na_2SO_3 . Virus-exposed insects were macerated with a TenBroeck grinder in 0.85%

NaCl to give a 10% (w/v) suspension of insect tissue. After removal of debris by filtration, the suspensions were centrifuged at $4,000 \times g$ at 4°C . The supernatant fluids were homogenized with half their volume of the fluorocarbon Genetron 113. After recentrifugation, the aqueous phase was collected.

Virus purification. One liter of a 10% (w/v) suspension of crude extracts from root tumors was concentrated to 200 ml by dialysis under reduced pressure. The procedure of Overall and Wright (6) was employed. After low-speed centrifugation to remove debris, most of the virus was pelleted from the supernatant fluid by centrifugation at $48,200 \times g$ for 2 hr. The virus pellet was resuspended in a solution of 0.1 M glycine and 0.01 M MgCl_2 , which was then floated on the top of a sucrose gradient (5). After centrifugation at $73,000 \times g$ for 35 min, the visible virus zone was removed and dialyzed for 10 hr against the glycine buffer.

Virus particle counting. The enumeration of WTV particles was carried out according to the method described by Strohmaier (11). This procedure combined the separation of particles by density gradient centrifugation with the sedimentation of the virus on grids for examination under the electron microscope. It was performed in the type of gradient centrifuge tube described previously (11, Fig. 1B).

Density gradient centrifugation. Formvar-coated nickel grids, supported by beads of mercury, were placed into two of the cavities of the bottom section of the centrifuge tube (11, Fig. 1Ab). The other two cavities were left empty. A gradient of D_2O - H_2O was prepared, which permitted WTV, with a density of about 1.35 g/cm^3 , to sediment at a uniform speed. The gradient, consisting of 0.1 ml each of 39, 54, 68, and 80% D_2O , was filled in the respective sections of the centrifuge tube. The sections of the tube were aligned so that the holes formed a channel which was closed off by the two cavities lacking nickel grids (position I). After a preliminary centrifugation to sediment debris, the bottom section was rotated until the gradient was positioned over the grid containing cavities (position II). The centrifugation was con-

FIG. 1. WTV deposited on coated nickel grids by centrifugation in Strohmaier's gradient cell; partially purified, stained with 2% uranyl acetate. $\times 30,000$.

tinued until the virus zone was sedimented on the grids. The optimal time of centrifugation was calculated (11) by using a sedimentation coefficient of 550S. In position I, the virus zone moved to the lower segments after 33 min at $58,000 \times g$; after 33 min in position II, it was sedimented on the grids.

Electron microscopy. After centrifugation, the grids were removed, stained with 2% uranyl acetate (pH 4.5) or shadowed with chromium, and examined in a Siemens Elmiskop I electron microscope at a magnification of $10,000 \times$. Between 200 and 400 particles per electron micrograph were counted in virus-exposed insects and root tumor preparations. The number of particles per gram of tissue was calculated by using the formula of Sharp (10).

RESULTS

Partially purified WTV was used to test the quantitative features of the method. Preliminary results were unsatisfactory because of virus aggregation. The aggregates could not be prevented from forming, even by sonic vibration, but they could be dispersed if relatively low concentrations of the material were treated with Genetron 113 (Fig. 1). An experiment was carried out in duplicate to study the relationship

FIG. 2. Relationship between serial dilution and particle count of two samples of partially purified wound tumor virus. The linear regression was computed from mean particles per unit area. The horizontal lines indicate the standard error of the mean.

between serial dilution of partially purified WTV and number of particles. For each sample, four different concentrations were used and four counts were made of each concentration. The mean particle count of the two samples and the

FIG. 3. *WTV deposited on coated nickel grids by centrifugation in Strohmaier's gradient cell. (A) 2.5% (w/v) suspension of root tumors shadowed with chromium. (B) 10% (w/v) suspension of virus-exposed insects shadowed with chromium. $\times 30,000$.*

standard error of the mean are shown in Fig. 2. The linear regression of mean particles per unit area was computed. The slopes were 1.92 in one experiment and 2.02 in the other, as compared to a theoretical value of 2.00. A statistical analysis showed that the slopes obtained in the two experiments were not significantly different from the theoretical line.

Crude virus extract from root tumors was used to determine the effect of centrifugation time on the number of particles. Virus concentrations obtained after centrifuging for 33 min in position I and 33 min in position II were compared with those obtained after 25 min in position I and 45 min in position II. The results did not differ significantly. However, the distinction between virus particles and other particles of similar size and shape was sometimes difficult. To estimate the resulting error, different preparations of root tumors and infected insects were examined and duplicate counts were made. In one case, only those particles exhibiting the characteristic size and shape of WTV were counted (VP) and, in the other, particles resembling WTV but differing slightly from the characteristic size and shape were included (VP + VLP). The ratio VP:(VP + VLP) of the different preparations varied from 1:1.32 to 1:2.18. The same preparations were used to check whether the distribution of the particles on the grid, as shown in the micrograph, was random. A counting grid with 12 equal squares, 6.0×6.0 cm, was placed on the micrograph and the number of particles in each square was determined. A random distribution of particles was found when aggregation was absent. In the presence of aggregates, which were observed in several micrographs, a statistically significant value could be obtained only by counting the particles in a large area. Preparations with 200 to 400 particles per micrograph were selected (Fig. 3A) and four counts were made of each preparation. The number of WTV particles per gram of tumor tissue was calculated for two samples and found to be 5.4×10^{10} and 4.8×10^{10} .

Preparations of virus-exposed insects contained less virus and more impurities than the plant tumor tissue. Therefore, two additional centrifugations of the former were necessary. In the first two centrifugations, the virus was sedimented in the cavities, then resuspended, and recentrifuged as previously described (Fig. 3B). The number of virus particles per gram of insect tissue was 1.7×10^{10} and 1.9×10^{10} in two samples.

DISCUSSION

By using the spray-droplet procedure of Backus and Williams (1, 2), Gamez and Black (7) esti-

mated the number of virus particles per gram of root tumor and insect tissue as approximately 1×10^{12} . With our method, we obtained values of approximately 5×10^{10} per gram of root tumor tissue and 2×10^{10} per gram of insect tissue. This discrepancy may be due to a difference in the virus concentration in the initial material or to the different methods employed. Since we did not observe a significant loss of virus during our procedure, it appears likely that the discrepancy was caused by differences in the original material. However, the possibility that this discrepancy may be due to the different methods cannot be excluded on the basis of these experiments.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant no. CA07453 from the National Cancer Institute.

We are indebted to D. C. McCune for the statistical analysis and to June Castaldo for technical assistance.

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