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Enumeration of VSV Particles and a Demonstration of Their Growth Kinetics by Electron Microscopy.* (31662)

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A knowledge of the physical particle count of a virus sample would be of great help in determining whether the variance in published results is due to host cell variation, virus adaptation, or amount of virus(1). Biological titer alone does not give sufficient information to properly quantitate and compare various results. The plaque forming unit (pfu) does not give the total number of virus particles (vp) in a given suspension nor does it give the number of infectious particles(2). Hackett(3) has expressed the need for meaningful particle counts of vesicular stomatitis virus (VSV) for a clearer understanding of homologous inhibition. In a study of the radiosensitivity of VSV, Pittman *et al* (4) have expressed the need for particle counts in understanding the effect of radiation on infected cells. It has been further suggested that the ratio of the actual number of virus particles to the number of biological units provides a more accurate estimation of the quantitative relationships between various virus samples(5). The present report is an attempt to answer the need for an accurate technique for particle counting of VSV and to demonstrate its application to certain growth properties of the virus.

Materials and methods. The cell line used throughout these studies was the Earle L strain originally obtained from Dr. W. Earle and kept in continuous passage. The cells were grown in Medium 199 containing 10%

horse serum, 400 units/ml of penicillin and 40 μ g streptomycin/ml.

The Indiana serotype of VSV was obtained from the American Type Culture Collection. It was adapted to growth in L cells and virus obtained from the 23-27th passage was used in these experiments.

The basic particle count technique is that described by Sharp(6) with minor modifications. Briefly, infected cell cultures were treated in a Raytheon sonic oscillator (Model S-102A) for 3 minutes in order to lyse the cells and disperse the virus to obtain homogenous samples. The crude cell lysates are diluted in phosphate buffered saline (PBS) and centrifuged onto an agar receiving surface at $17,600 \times g$ in a SU Sorvall counting rotor for 20 minutes. The agar block is removed and exposed to 2% osmium tetroxide vapors for 10 minutes. Pseudoreplicas are made and the collodion film with the virus is placed on stainless steel grids, shadowcast and examined by electron microscopy (RCA EMU 3 H). Repeated stripping of the agar block revealed that more than 99% of the virus was removed with the first pseudoreplica. The particle count is calculated according to the formula described by Sharp(6).

Crude cell lysates with as little as 1×10^7 vp/ml could be counted with no purification procedures. When the particle count is less than 2×10^8 vp/ml, cellular debris tends to obscure the picture. This background level of debris may be satisfactorily reduced by enzymatic treatment with trypsin

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TABLE I. Efficiency of Count Procedure for VSV.

| Sample | Dil | Avg #vp/unit area | vp/ml $\times 10^6$ | Diff from mean (r) | r ² | Standard error |
|--------|-------|----------------------|---------------------|-----------------------|----------------|---|
| 1 | 1:100 | 121 | 11.1 | .1 | .01 | $\sigma = \sqrt{\frac{\Sigma r^2}{N-1}}$ $\sigma = 7.9\% \text{ of mean}$ |
| | 1:100 | 125 | 11.5 | .3 | .09 | |
| | 1:100 | 118 | 10.9 | .3 | .09 | |
| | 1:200 | 65 | 12.0 | .8 | .64 | |
| | 1:200 | 51 | 9.4 | 1.8 | 3.24 | |
| | 1:200 | 62 | 11.4 | .2 | .04 | |
| | 1:200 | 65 | 12.0 | .8 | .64 | |
| | | | Avg = 11.2 | $\Sigma r^2 = 4.75$ | | |
| 2 | 1:100 | 77 | 7.08 | .27 | .07 | $\sigma = 7.8\% \text{ of mean}$ |
| | 1:100 | 79 | 7.27 | .46 | .21 | |
| | 1:100 | 66 | 6.07 | .74 | .55 | |
| | 1:100 | 74 | 6.81 | .00 | .00 | |
| | 1:200 | 41 | 7.54 | .73 | .53 | |
| | 1:200 | 34 | 6.26 | .55 | .30 | |
| | 1:200 | 36 | 6.62 | .19 | .04 | |
| | | | Avg = 6.81 | $\Sigma r^2 = 1.70$ | | |
| 3 | 1:100 | 13 | 1.19 | .06 | .0036 | $\sigma = 5.0\% \text{ of mean}$ |
| | 1:100 | 14 | 1.29 | .04 | .0016 | |
| | 1:200 | 6.6 | 1.21 | .04 | .0016 | |
| | 1:200 | 7.2 | 1.32 | .07 | .0049 | |
| | | | Avg = 1.25 | $\Sigma r^2 = .0117$ | | |

and chymotrypsin. A stock solution of 0.2% trypsin (Difco 1:250) and 0.2% chymotrypsin, delta (Nutritional Biochemicals Corp.) is stored frozen until required. A 2:1 dilution of cell lysate to enzyme was made and incubated in a 37°C water bath for 30 minutes, after which the sample was further diluted in PBS so that the lysate was diluted 1:10. This treatment had no effect on the morphology of the virus but greatly reduced the cell debris.

Results. Particle count. VSV has a typical bullet morphology and is readily distinguishable from the cell debris; therefore, no difficulties were encountered in identifying the particles. A portion of a count photograph is shown in Fig. 4B. The first attempt to determine the efficiency of the sedimentation count technique with this virus was to investigate the repeatability of the counts. Three samples with different amounts of virus were diluted 1:100 and 1:200 in phosphate buffered saline and processed for count as described above. Each dilution was repeated 2 to 4 times. The results, described in Table I, indicate a very high efficiency. In other samples, where over 500 vp/field were counted, a second stripping of the agar was made to determine if any vp were being left

behind on the agar block. The subsequent photographs revealed an average of less than 2 vp/field.

A further experiment was performed to determine whether the counts were consistent with dilution. Fig. 1 shows the results of one such experiment. The average number of particles is plotted against the relative concentration.

Growth curves. It was observed that the yield of virus varied greatly from passage to passage, from 100 to 15,000 vp/cell. To determine whether the yield was dependent upon the multiplicity of infection (m.o.i.) and whether consistent yields could be obtained when cultures were routinely inoculated with a known multiplicity of viral particles, a series of growth curves was performed. It had been observed that a more accurate zero time of infection with vaccinia virus could be obtained by inoculation of susceptible cells in a centrifugal field(7). However, since the adsorption and latent period of VSV is relatively short for an animal virus, being less than 2 hours in chick embryo, monkey and swine kidney, HeLa and L-cells(8), the host cells were inoculated both by sedimentation and frozen until counts were performed.

Using a range of multiplicities from 1 to

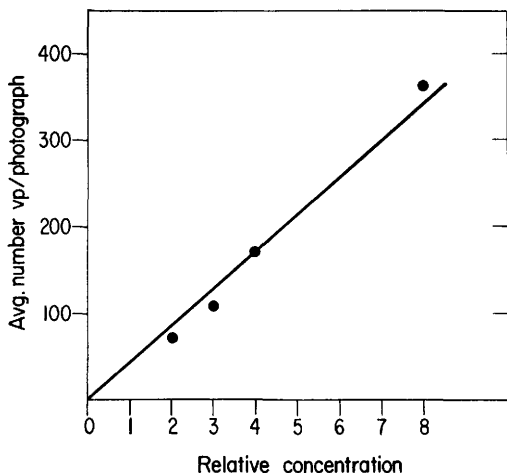


FIG. 1. Relationship between virus particle count and dilution of VSV. A total of 9,000 particles was counted, each point representing an average from 10-15 photographs for each of the 4 dilutions.

inoculation as described previously(7) spinning at $18,500 \times g$ for 15 minutes and kinetically. Four-day-old homogeneous monolayers were used in the kinetic inoculations. Representative bottles were harvested and the number of cells per monolayer determined. The medium was removed and the appropriate multiplicity of virus particles added in a 0.2 ml volume. After a 1-hour adsorption period the monolayers were washed and overlaid with media. At various intervals sample

bottles were removed from the incubator and 100 similar results were obtained by both techniques (Fig. 2 and 3). There is a reduced yield of virus with increasing m.o.i. Optimal yields are obtained when the inoculum is sufficient to infect all the cells with a minimal number of virus particles. There is less scatter of points with the sedimentation inoculation because all the cultures were set up from a pool of infected cells, whereas in the kinetic inoculation cultures there was some variation between monolayers. Viral growth became evident by the 4th hour of infection and reached a maximum between the 12th to 24th hour post infection depending on the m.o.i. The average time of peak virus production appears to be about the 19th hour post infection. Thus, by controlling the m.o.i., yields averaging 3,000 vp/cell within 24 hours of infection may be consistently obtained.

Virus replication. The results utilizing the particle count technique are in general agreement with those of Franklin(9), Wagner *et al*(10) and Pittman *et al*(8). Using biological titer as a measurement of viral presence these authors showed that most of the infectious virus is extracellular indicating rapid release. A comparison of the number of particles in the supernatant fluid to those associated with the cells supports this finding and contributes

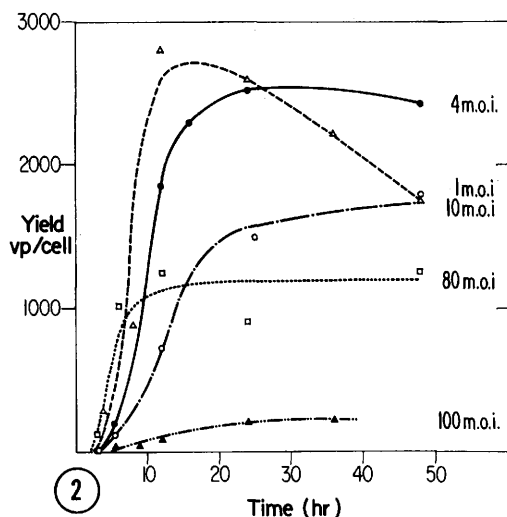


FIG. 2. Growth of VSV in L cells following sedimentation inoculation. The cells were infected with a range of 1 to 100 vp/cell.

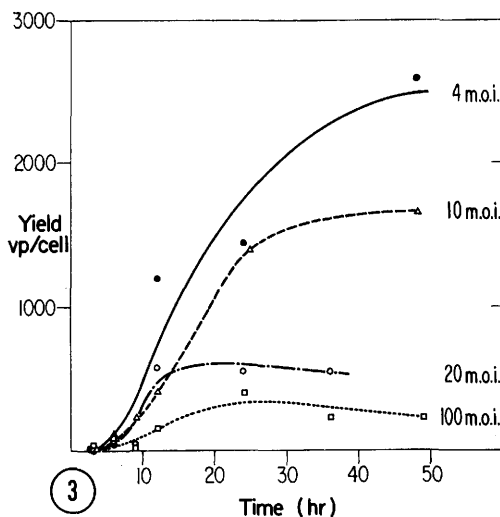


FIG. 3. Growth of VSV in L cells following kinetic inoculation. The cultures were infected with a range of 4 to 100 VP/cell.

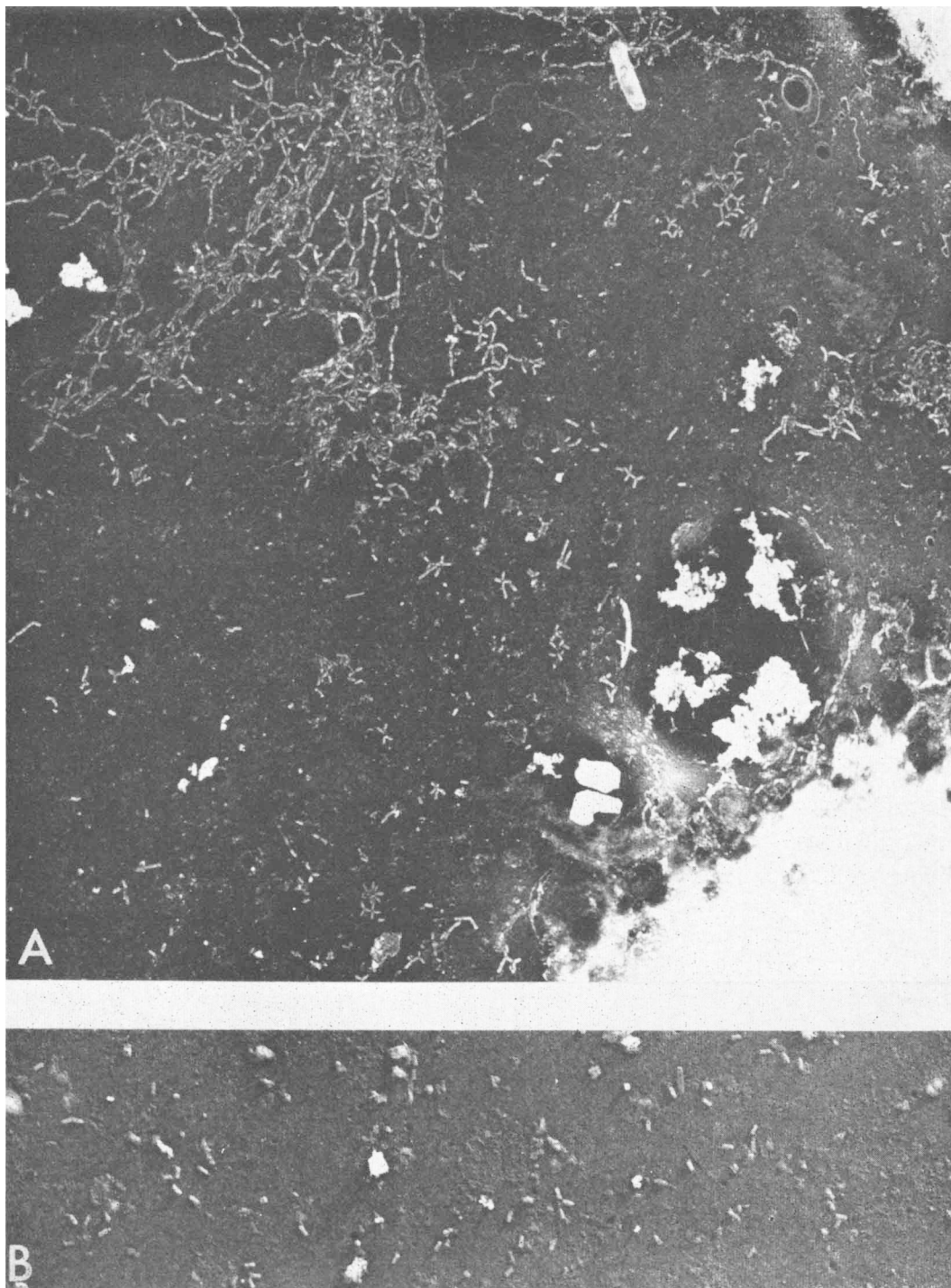


FIG. 4. A. Infected cell preparations with no dispersal treatment reveal long chains of virus particles connected by strands of cellular material. Upper and lower right corners show portions of the whole cells. 5970 \times . B. The virus is easily dispersed as shown in a portion of this photograph used for particle count. 7540 \times . These photographs are projected onto a 4 x 4 foot screen for increased magnification and ease of counting.

one further piece of information regarding VSV replication. It has been shown(5) that this technique lends itself well to a study of natural viral aggregation, unaffected by drying properties. A suspension of 20-hour VSV-infected cells was centrifuged onto an agar receiving surface with no prior dispersal techniques in order to determine normal state of virus aggregation. Electron micrographs revealed long chains of virus particles connected by a filamentous strand, Fig. 4A. It is still not firmly established whether the virus is produced at the membrane and the particles extruded individually(11), whether it is elaborated into cytoplasmic vacuoles(12,13), whether the virus is produced in granular areas separated from the cytoplasm(14), or whether the mode of replication varies with the host cell. In our experience it appears that the virus is released as it is produced, but it seems to form chains of virus particles connected by some cellular material. These chains are readily broken by any rough handling, such as freeze-thaw or sonic vibration. A typical count preparation is shown in Fig. 4B; routine treatment of 2-3 minutes in the Raytheon sonic oscillator produces well dispersed virus. These photographs are projected onto a 4×4 screen for counting with a final magnification of $37,000\times$. Because of the nature of the strands and the ease with which they are broken, they would not be easily seen in ultrathin sections.

Discussion. The sensitivity, precision and accuracy of the several virus particle counting methods have been sufficiently reviewed by Sharp(6). The major advantages of the sedimentation procedure are that it is possible to obtain accurate virus particle counts from unpurified suspensions with as little as 10^5 vp/ml with a large virus such as vaccinia and it provides information on the natural aggregation pattern of the virus. The data presented here demonstrate that this technique can be easily adapted to VSV and that the efficiency of this procedure is even greater than that reported for vaccinia(5). The results reported here indicate the standard error for the count procedure to be less than 8% of the mean as opposed to 16% for vaccinia. The use of both the physical

particle count technique and the biological titer technique in quantitating viruses would standardize the various virus samples as to quality (vp/biological unit), thereby facilitating the comparison and understanding the various results.

The growth curves, based on the physical yield of virus particles, described here indicate that the m.o.i. plays a major role in the production of VSV. Hackett(3,15) and Huang and Wagner(16) have reported that some VSV suspensions contain small non-infectious particles similar to the infectious particle in every respect except length. These particles interfere with and inhibit the replication of the virus. These authors have demonstrated by biological titer that undiluted passage of the virus results in decreased yield. The information presented here supports these findings on a physical basis and further indicates that consistent yields of virus can be obtained.

Fig. 2 presents data which show that the highest yield is obtained when the m.o.i. approximates 1 vp/cell (which according to Poisson distribution is the 4 m.o.i. curve). When not all the cells are infected there is optimum production over the first 12 hours, but the virus is rapidly adsorbed by the uninfected cells in great numbers as demonstrated by the 1 m.o.i. curve. These cells in turn produce very little virus, similar to the 100 m.o.i. curve. Since the best yield is obtained when the actual m.o.i. approximates 1, it appears that the virus particle to infectivity ratio is very low. This ratio for high quality virus is usually about 10(6). Work is currently in progress to examine the vp:pfu ratio taking into account the dispersion of the virus and the conditions of incubation in order to verify the high infectivity indicated here.

The study of viral aggregation by means of the sedimentation procedure is most useful in qualitative studies(5) and in this report has also provided additional information on the replication of VSV. The virus is released shortly after its completion but, as shown in Fig. 4A, it may be extruded in chains rather than individually. Sonic vibration in the Raytheon apparatus reveals that this

connecting strand is easily broken yielding fairly dispersed virus.

Summary. Growth curve studies indicate that the m.o.i. plays a major role in the replication and resultant quantity of VSV. Consistent high yields of the virus can be obtained when the m.o.i. is kept at approximately 1. Natural aggregation studies using the count technique indicate that the virus is extruded from the cells in loosely connected chains. The agar sedimentation procedure for counting virus particles can be readily adapted to vesicular stomatitis virus with high efficiency.

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A Comparison of Calcium Dependent Contraction in Glycerinated Uterine Smooth Muscle and Cardiac Muscle. (31663)

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Initially work in this laboratory showed that adenosine triphosphate (ATP) and magnesium (Mg) were necessary for contraction in glycerinated guinea pig uterine fibers(1). It was noted that in approximately 25% of freshly extracted fibers (4 days to 1 month) but not in older fibers calcium (Ca) produced a slight increase in tension following maximum contraction with ATP and Mg. It was suggested that this may be the result of the presence of a small amount of a labile relaxing factor which survived the extraction procedure. However, subsequent studies revealed that not only ATP and Mg were necessary for contraction but a small amount of Ca had to be present and that Ca contaminating the fibers was enough in most cases to produce maximum contraction in the presence of ATP and Mg. This suggested the possibility that

the Ca dependent contraction in uterine muscle may be the result of a factor(s) similar to that described in skeletal and cardiac muscle (3,4). This factor(s) which is probably protein in nature can be extracted from skeletal and cardiac muscle and is lost during extraction in glycerol in cardiac muscle(5,6). For these reasons a study was done to compare the effects of prolonged extraction in glycerol on the calcium dependent contraction in uterine smooth muscle and cardiac muscle.

In addition, it was noted that glycerinated uterine fibers from estrone pretreated guinea pigs developed tensions of approximately 100 g/cm². It has been reported that estrogen treatment not only increases the concentration of extractable actomyosin but also increases the synthesis of proteolytic enzymes in the lysosomes. Szent-Gyorgyi reported that