16822

# Enumeration of Virus Particles by Electron Micrography.*

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In the study of viruses or other particulate materials in aqueous suspension with the electron microscope, it is necessary to secure a thinly spread deposit of the particles on the collodion film. This collodion film is then studied by transmitted electrons either in light or dark field illumination, or the particles on the film may be metal shadowed by the method of Williams and Wyckoff[1] before study. In any case it is desirable that each kind of particle in the suspension be present on each area of the collodion forming one complete field in the electron microscope. It is desirable also that the aggregates, if any, of the particles seen in the microscope should not be produced in the procedure by which the particles were deposited on the film; that is, any aggregation observed should be representative of the state of dispersion of the particles present in the suspension under study. Furthermore, it is highly desirable that the number of particles per unit area of the collodion (per microscope field) be the same from place to place and equal to the number in a known volume of the suspension under study.

At present there is no technic fulfilling these conditions. The usual procedure consists of placing a drop of the virus suspension on the collodion film and leaving it for a few seconds. The fluid is then withdrawn with a fine pipette, and the residual, thin film is left to dry and to deposit its particles on the collodion. Although it is sometimes possible to obtain suspensions of animal viruses in water, this is not the general rule. Most of these materials do not disperse well in water, and other laboratory studies on such viruses are carried out in some suitable buffered salt solution. It is frequently desirable that electron micrographs be obtained with such salt suspensions for study of the state of the virus under these conditions for comparison with other data, those from the ultracentrifuge, for example. However, when the residue of salt solution containing virus dries on the collodion film, changes occur in salt concentration and, probably, in pH as well. The liquid film does not dry evenly, and as a result of a combination of these factors, it is clear that any particular distribution of deposited virus bodies or aggregates among them is likely to bear little resemblance to the state of dispersion of the particles in the suspension from which they came. In this paper† a method has been used in the study of formolized swine influenza virus which promises to correct some of the ills described above.

*Materials and methods.* The method is based on the use of a specially designed cell fitting into the rotor of the air-driven analytical ultracentrifuge. The cell, shown in the drawing of Fig. 1, is similar to the one used for sedimentation velocity studies but with some modifications for the present purpose. As seen in Fig. 1, the cell itself consists of 2 lucite windows held apart by a lucite block in which there is a space of about 1 ml volume with trapezoidal sides coinciding with radii of the rotor. In preparation for filling the cell, one lucite window is put in place with a thin rubber gasket to prevent leakage and locked in with threaded aluminum ring and spanner wrench. A piece of cover slip glass coated with collodion is inserted at the bottom of the cell and the cell filled with the virus sus-

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1 Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

† This work was reported at the meeting of the Electron Microscope Society of America held in Toronto, September 10, 1948.

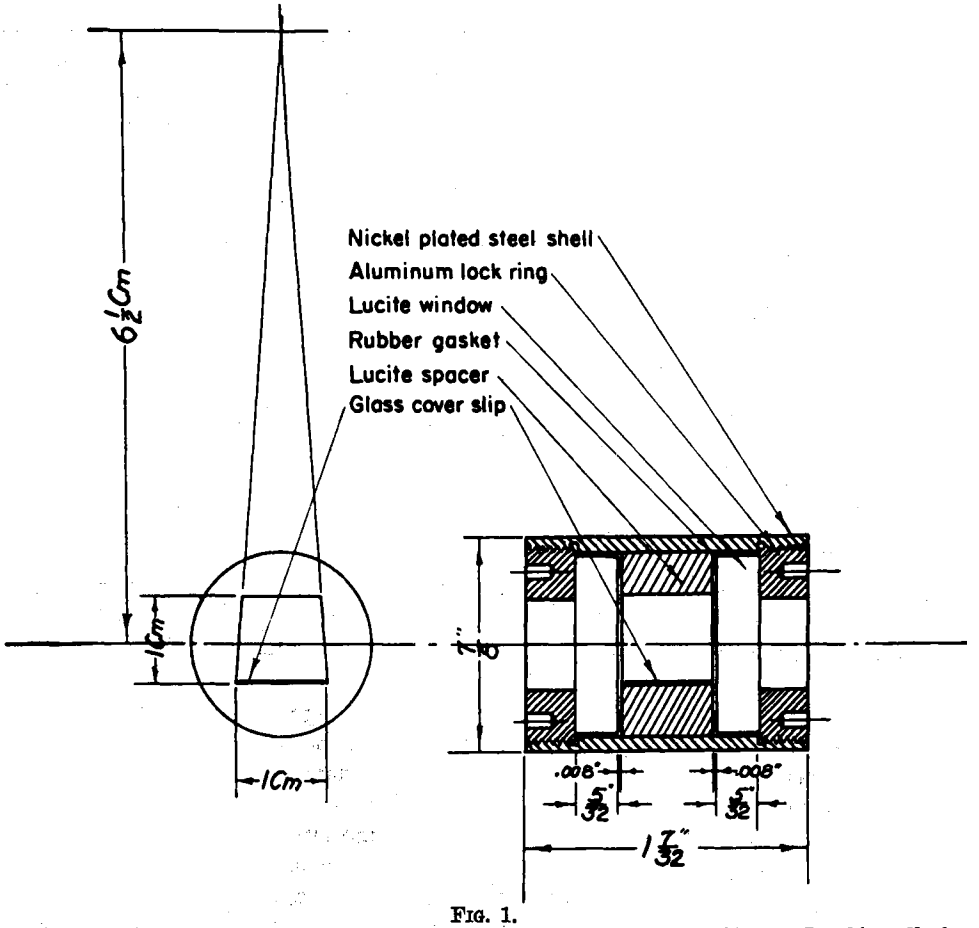


FIG. 1.

Construction details of the rotor cell for the analytical ultracentrifuge. In this cell the virus from about one ml fluid is sedimented onto the collodion-coated glass at the bottom. This glass is then removed; the collodion is stripped off; and the electron microscope is used to count and study the virus deposited upon it.

pension, after which the second window is placed and fastened with the corresponding aluminum ring. The cell is then inserted in the rotor of the analytical ultracentrifuge and, in the present experiment, spun at 16,500 g for 30 minutes; under such conditions the influenza virus particles should sediment, convection free, upon the collodion coating on the glass cover slip. After the run, the cell is opened and the cover slip carefully removed, dipped twice in distilled water to remove the salt and then dried. The film is scratched across with a needle and floated off the glass on a distilled water surface, The film, divided by the scratch, comes off in two pieces, on one of which is placed a standard 200 mesh electron microscope screen. The other piece is caught from below atop another such screen, this being the one with the virus particles on top for use in shadow casting.

Swine influenza virus was obtained from the chorio-allantoic fluid of virus infected chick embryos 4 years ago and formolized for the preparation of vaccines. The procedures for concentration and partial purification of the virus by ultracentrifugation have been described elsewhere.[2] This material was in Ringer solution in a stoppered tube stored in the refrigerator at 2 to 8°C.

*Experimental.* In the preliminary experi-

2 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

ments virus preparations for electron micrography were made in the usual manner. The suspension of formolized virus was diluted one to ten and prepared for the electron microscope by drying a small amount on the collodion film. Such preparations yielded approximately 25 to 100 virus particles per $cm^2$ in pictures taken at a magnification of 4,500 x. This was about the concentration of virus ordinarily employed for electron microscope study of the purified virus.

In order, now, to produce pictures of controlled deposition of virus, the procedure departed from the usual methods. A further dilution of the virus suspension 1-100 (total dilution 1-1000) was made with physiological saline solution and this was put into the rotor cell described above and spun for 30 minutes at 16,500 g. Such preparations yielded uniform distribution of virus particles, and pictures were taken with and without shadow casting. The procedure was repeated using 1-2,000, 1-4,000, 1-8,000, 1-16,000 and 1-32,000 dilutions of the same starting material. When the series was finished, repeat runs were made on freshly made 1-4,000, 1-8,000 and 1-16,000 dilutions to check the original data.

All of the electron micrographs were made at a magnification of 4,500 x, and a grid of fine lines one centimeter apart was projected with the original negatives so that the prints would have a counting standard of area independent of photographic enlargement. About a hundred pictures in all were made of various areas of the films produced. The counts per $cm^2$, as well as the total number of particles counted, and the statistical variation expressed as standard deviation ($\sigma$) are shown for the various dilutions and for the repeat runs in Table I. Strips of representative pictures of the chromium shadow-cast preparations are shown in Fig. 2.

Although this procedure is more difficult than simple drying of the material in the usual way, it yields counts agreeing well with the dilution of the starting suspension. Variation in the number of virus particles per unit area is small, and for this reason, one can get a good general impression of the preparation from examination of only a few fields. Furthermore, the counts obtained from repeat runs at a given dilution showed gratifying similarity.

*Calculations.* Some calculations have been made to determine the number of virus particles to be expected per unit area in these pictures. For this it was necessary to know the weight of virus per ml of the suspension used, the average unhydrated weight of the particles, and the magnification in the picture. The nitrogen content measured on purified swine influenza virus from this laboratory was 9%.[3] For the present work, a sample of the swine influenza vaccine was sedimented at 16,500 g for 30 minutes, and the nitrogen content of the sedimented material obtained by difference between Kjeldahl measurements on the whole material and on the supernate of this run. The resulting value multiplied by the factor 11.1 gave 1.85 mg virus per ml of the starting material. The 1/1,000 dilution thus contained 0.00185 mg virus per ml.

The average particle size, density and water content were taken from previously published data on the freshly purified virus.[4] In order to check the applicability of these data to the formolized virus used in the present experiments, 4 sedimentation velocity runs were made, 2 in physiological saline solution and 2 in a solution of bovine serum albumin in physiological saline (density 1.046). In the first pair of studies the sedimentation rates of the formolized virus corrected to water viscosity at 20°C were 671 and 677 x $10^{-13}$, and in the second pair the values were 379 and 377 x $10^{-13}$. When these values were plotted against the densities of the respective suspending media, 1.004 and 1.046, it was seen that the formolized virus had a size and density the same as the analogous properties reported for the freshly purified virus.[4] This is taken to mean that no great error will be made in using for particle radius, the value 58.5 m$\mu$; for wet density, 1.100; and for water content by weight, 39.2%.

[3] Taylor, A. R., *J. Biol. Chem.*, 1944, **153**, 675.

[4] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., *J. Biol. Chem.*, 1945, **159**, 29.

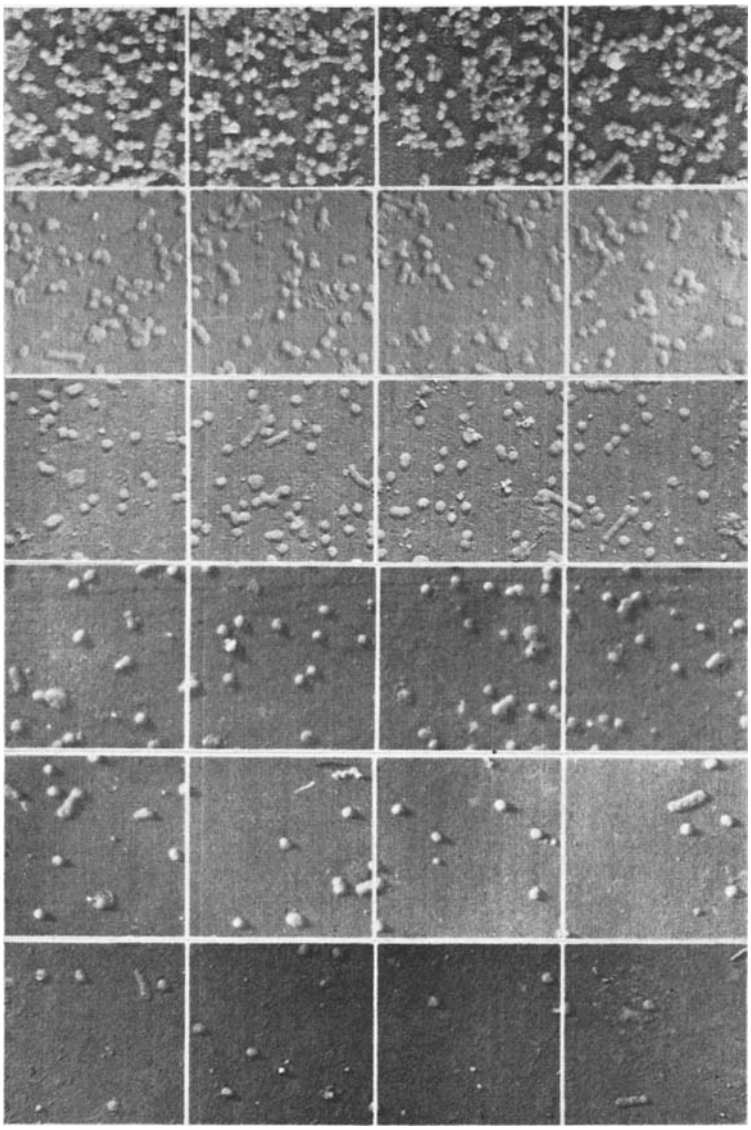

FIG. 2.

Influenza virus deposited upon a collodion membrane by sedimentation in the ultracentrifuge. These strips are representative of pictures taken of deposits from dilutions of 1-1,000, 1-2,000, 1-4,000, 1-8,000, 1-16,000, and 1-32,000 of the starting material. The data from the counts are given in Table I. The particles are shadow-cast with chromium at an angle of about 15°

At an electron magnification of 4,500 x, we can then calculate as follows:

$$\frac{4/3\ \pi\ r^3\ \rho\ (1-0.392) = \text{g dry wt per particle.}}{0.00000185 \text{ grams/ml}} = \frac{\text{particles per ml in the 1/1,000 dilution}}{4/3\ \pi\ r^3\ \rho\ (1-0.392)}$$

Equation 1.

where

$r$ = hydrated virus particle radius ($58.5 \times 10^{-7}$ cm)

1.100 = $\rho$ hydrated virus particle density.

$1.85 \times 10^{-6}$ = grams virus per ml in 1/1,000 dilution.

Because of the sector shape of the cell, the number of particles sedimented on unit area of its bottom is not simply the product of the area by the height but 7.1% less, an amount by which the trapezoidal sector area

TABLE I.

| Dilution | Total particles counted | | Particles observed per cm² | | Particles expected Equation 2 |
|---|---|---|---|---|---|
| | Run I | Run II | Run I | Run II | |
| 1/ 1,000 | 5,420 | — | 121 ± 10* | — | 151 |
| 1/ 2,000 | 3,418 | — | 61 ± 7 | — | 76 |
| 1/ 4,000 | 2,257 | 1,727 | 31 ± 6 | 34 ± 8 | 38 |
| 1/ 8,000 | 867 | 863 | 19 ± 5 | 16 ± 4 | 20 |
| 1/16,000 | 415 | 261 | 7 ± 2 | 7 ± 3 | 10 |
| 1/32,000 | 361 | — | 4 ± 2 | — | 5 |

* Variations are expressed as mean standard deviation. Standard deviation of the mean is of course much less and dependent on the number counted.

is less than that of a rectangle of the same height. The number of images per unit area of a micrograph at 4,500 x is then given by:

$$\frac{3 \times 1.85 \times 10^{-6} \times 0.929}{4\,\pi\, r^3\, 1.10\,(1-0.392)\,(4{,}500)^2} = 151$$

Equation 2.

The numbers expected for dilutions higher than 1/1,000 are correspondingly less. Clearly these calculated numbers are strongly dependent on accurate knowledge of particle size and electron magnification, for these enter the equation in third and second power respectively. The influence of the other variables is correspondingly less, and it can be judged from Equation 2.

*Discussion.* From the results shown in Fig. 2 and Table I, it is clear that this method makes possible the counting of the particles of swine influenza virus, yielding numbers closely proportional to the dilution factor of the starting material. Furthermore, agreement is quite good between these numbers and those predicted from calculations involving not only the physical characteristics of the virus particles but also the purity of the starting material. The observed numbers are lower by about 19% than the predicted ones as shown in Table I. Although error in several places may account for this, one source of error is immediately suspected. Not all of the sedimented virus particles might actually remain on the membrane through washing to appear in the final pictures.

The distribution of particles over the collodion membrane as seen in the electron microscope is exceedingly uniform and the pictures obtained in the process come from dilutions of virus 100 to 3,200 greater than those considered optimum for microscopy using standard technics. The advantages of such a method need not be discussed at length. If the results are proved by subsequent work to be strictly quantitative, the possible applications of the procedure are many, not the least of which should be to the study of viruses in preparations of such low virus content that too little of the agent would be present for purification and concentration, and in which the concentration is so low that the usual technics of preparation for microscopy would produce too few virus particles per field to be recognized.

For study of aggregation in suspension, this method should be useful not only for viruses but also for any other suspended material, such as bacteria in liquid media. For such large bodies as bacteria, any horizontal centrifuge carrying a suitable cell could be used for sedimentation of the particles directly on a microscope slide where, after removal from the centrifuge, they could be studied with a light microscope. It would be necessary to calculate the number of pairs, triplets, etc. to be expected from chance sedimentation of two or more particles on the same area of the slide; but when this number is subtracted, the excess would be characteristic of the liquid suspension. The author is not aware of any other satisfactory method of studying this problem.

*Summary.* A method has been devised for counting virus particles in a suspension by means of the electron microscope. Studies on formolized purified swine influenza virus showed that the particles could be sedimented with a uniform distribution on a collodion membrane in the ultracentrifuge as revealed by electron micrographs of the particles on the

membrane. The findings showed a close correlation with the dilution factor and with the number of particles calculated from chemical and physical data obtained with the virus.

## 16823

## Electronmicroscopy of Cells from Tissue Cultures Infected with Vaccine Virus.

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One of the obvious needs in electronmicroscopy is a satisfactory method for good visualization of intact cells to provide information on their internal structure and the presence of virus particles in infected preparations. Progress in this field has been made in the use of specially prepared histological sections[1] and in the studies of individual avian cells cultured on plastic membranes.[2,3] The present report describes recent observations on the intracellular growth of vaccine virus observed by electronmicroscopy with a modification of the plastic membrane technic.

*Materials and methods.* The technic for cultivation of mammalian epithelial cells on plastic membranes and their preparation for visualization by the electron microscope has been described in detail elsewhere.[3-5] In brief, this is as follows: .02 cc of a 0.6% solution of "Formvar" (Shawinigan Falls Products Co., Shawinigan Falls, Quebec, Canada) in ethylene chloride is dropped into a petri dish full to overflowing with chilled Ringer's solution. A cover slip held by forceps is inserted under the membrane covering the fluid in the petri dish and the cover slip is raised through the membrane; this provides a smooth non-adherent plastic coating with a distinct growth-differentiating effect; epithelial growth is enhanced and fibroblasts are inhibited.[2,3] Six such cover slips with the plastic membrane facing upward are placed on a specially prepared glass slide. Small tissue explants are placed on each of the 6 plastic membranes. Special nutrient fluid (plasma-extract supernate, embryonic extract and Tyrode) is added to each culture and maintained in place by a small glass ring sealed with vaseline. The 6 cover slips with their membranes and cultures are covered with a petri dish and infected at the start or after growth has taken place. The nutrient medium is changed at 3-4 day intervals if indicated. Strict asepsis is observed throughout the tissue culture procedure. At an appropriate time, usually 3-5 days for vaccinia, the cover slips with the plastic membrane to which are now attached the growing tissue cells are lifted from the culture chamber. A cover slip with the plastic surface facing up is placed in a petri dish containing Ringer's solution; under these conditions the membrane detaches itself completely from the cover slip and floats on the surface. The floating membrane in the petri dish is examined under low power with an ordinary microscope and the desired fields are selected. These are fixed in the center of the metallic mount by a modification[4] of the usual technic employed in electronmicroscopy.

In the present work, minute explants (weighing about 0.1 mg) prepared from the renal cortex of adult rabbits were used. Cultures were inoculated with a washed suspension of neurovaccinia elementary bodies. Elec-

1 Richards, A. G., Jr., Anderson, T. F., and Hance, R. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 148.

2 Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

3 Wirth, J., and Barski, G., *Ann. Inst. Pasteur*, 1947, **73**, 987.

4 Wirth, J., *C. R. Acad. Sci.*, 1947, **225**, 899.

5 Wirth, J., in: Levaditi, C., and Lépine, P., *Traité des Ultravirus des Maladies Humaines*, 2nd edition, Paris, 1948 (Maloine éd.), page 1683.