# Enumeration of Virus Particles in Ultrathin Sectioned Pellets (35081)

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The methods of counting virus particles by electron microscopy were reviewed by Sharp (1). These methods involve placing the particles on an electron transparent membrane or making a replica or pseudoreplica of a surface containing the particles. The main problems encountered when using these techniques are distinguishing the virus particles from the cellular debris, and preventing aggregation of the particles. We reasoned that these problems might be solved by pelleting the virus and cutting ultrathin sections. Studies to be reported here support this contention.

*Methods.* Adenovirus type 2 was treated in a sonic oscillator and filtered through Celite (2). An aliquot was prepared for ultrathin-section electron microscopy by pelleting, fixing, and embedding in a BEEM bottleneck capsule (3). The pellet was cut from the tip of the bottleneck block (Fig. 1A), rotated 90° and embedded with the same resin mixture in a silicon rubber mold, thus forming a flat block (Fig. 1B). Ultrathin sections from the flat block were examined in the electron microscope in order to sample the distribution of the virus and measure the height of the pellet, Hp.

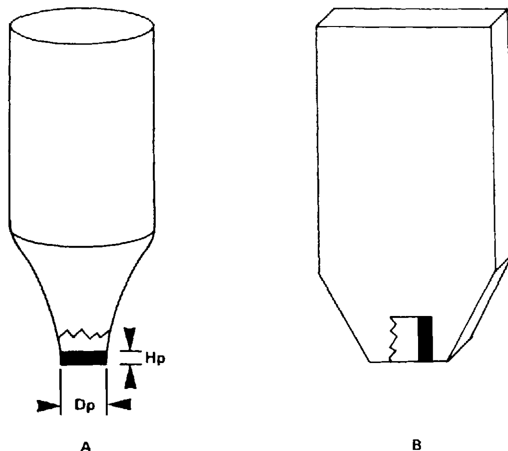


FIG. 1. Schematic diagram of bottleneck (A) and flat (B) blocks containing virus pellet. $Hp$ is the pellet height and $Dp$ is the pellet diameter.

The diameter of the pellet, D$p$, was determined by two methods: (i) measuring the i.d. of the bottleneck capsule, and (ii) measuring the o.d. of the pellet with a micrometer disc. The value of Dp was $8.0 \times 10^{-2}$ cm as measured by both methods. The volume of the cylindrical pellet, $Vp$, is calculated from the equation

$$Vp = \pi \left( \frac{Dp}{2} \right)^2 Hp.$$

The virus particles were counted in a circular area of the electron micrograph(s) of the pellet (Fig. 2A). The volume of the disc in which the virus particles were actually counted, $Vd$, is given by the formula

$$Vd = \pi \left( \frac{Dc}{2} \right)^2 S,$$

where $Dc$ is the diameter of the circular area in which the virus particles are counted and $S$ is the section thickness. (See Appendix 1 for a determination of section thickness.) The total number of virus particles, $T$, in the entire pellet is

$$T = \frac{N\,Vp}{Vd}$$

where $N$ is the average number of particles counted per field. The concentration of virus in the original sample is

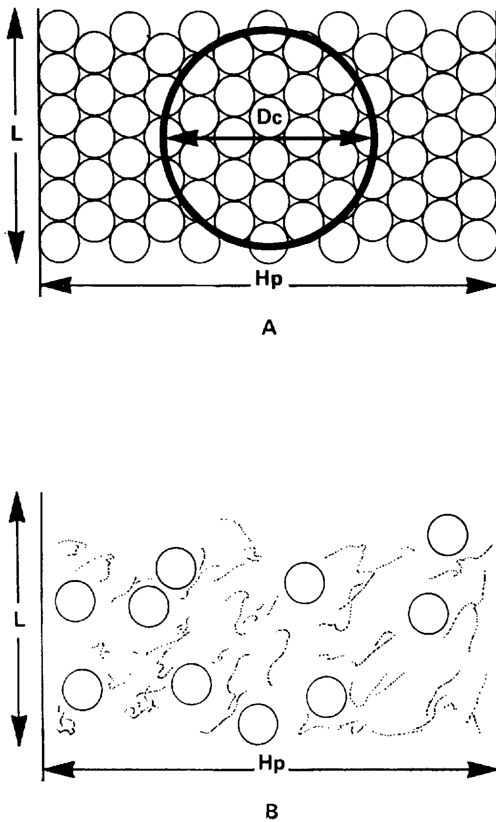


FIG. 2. Schematic diagram showing the distribution of purified virus (A) and virus plus debris (B). The particles are counted in a circular area of diameter $Dc$ or a rectangular area $L \times Hp$.

$$C = \frac{TR}{V} = \frac{NDp^2}{Dc^2}\frac{Hp}{S}\frac{R}{V},$$

where $R$ is the reciprocal dilution and $V$ is the volume of the viral material pelleted.

Since the virus particles may not be uniformly distributed throughout the height of the pellet when debris is present in the sample, $N$ must represent the average value of the number of particles present throughout $Hp$. Where maldistribution exists, it would be more accurate to count the particles present in an area $L \times Hp$ (Fig. 2B). The concentration is then

$$C = \frac{\pi\,(Dp/2)^2\,NR}{LSV}.$$

Note that $Hp$ does not appear in this equation, thus eliminating the necessity for a precise determination of this term.

*Results.* Serial dilutions of adenovirus type 2 were prepared as described for examination in the electron microscope. For large pellets it was necessary to photograph overlapping fields in order to sample one continuous strip from the top to the bottom of the pellet. Figure 3 shows the dose–response relationship between the virus dilution and $Hp$. It can be seen that $Hp$ is proportional to the concentration of virus. Table I shows the distribution of the virus throughout the height of these three pellets and the concentration of adenovirus type 2 as determined by this method. These values are in excellent agreement with the value of $1.5 \times 10^9$ particles/ml as determined by sedimentation on agar (4).

The precision of this technique was measured at the lower limit of sensitivity for adenovirus. Nine identical samples of infectious canine hepatitis virus were prepared as described. In each sample, the particles in a given area ($L \times Hp$) were counted. The average value and standard deviation for the

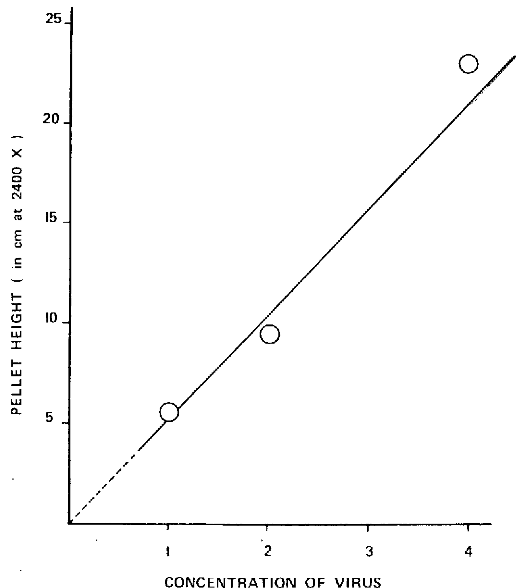


FIG. 3. A comparison of the height of the virus pellet, $Hp$, for varying dilutions of adenovirus type 2.

TABLE I. Distribution and Concentration of Virus Particles in the Pellet for Varying Dilutions of Adenovirus Type 2.

| Reciprocal dilution | Micrograph no.[a] | Particle (count/field) | $N$ (av count/field) | Conc (particles/ml) |
|---|---|---|---|---|
| Undiluted | 1 | 68 | | |
| | 2 | 260 | | |
| | 3 | 254 | 147 | $1.7 \times 10^9$ |
| | 4 | 75 | | |
| | 5 | 76 | | |
| 2 | 1 | 178 | 155 | $1.4 \times 10^9$ |
| | 2 | 132 | | |
| 4 | 1 | 127 | 127 | $1.4 \times 10^9$ |

[a] The number of electron micrographs required to measure the height of the pellet, $Hp$, at 2400×.

nine samples were 43 ± 5 adenovirus particles. The concentration of adenovirus was calculated to be $5 \times 10^6$ particles/ml. Counting particles over a larger area or increasing the number of particles in the sample should increase the precision.

Inconsistent results were obtained when using purified T-4 phage.[1] Such samples neither packed tightly nor contained large amounts of debris to act as an embedding matrix. In an effort to obtain precise and accurate values using such materials, two modifications of the technique were made. First the machined Lucite holders, which were slightly imperfect in regard to capsule fit, were replaced with holders which were formed around the BEEM capsules by using a liquid casting plastic (Clear Cast by American Handicrafts Co.). This was done in order to obtain a better fit of the BEEM capsule in the holder, and thereby prevent harmful distortion of the BEEM capsule under high centrifugal force. After the virus had been pelleted, 0.3–0.6 ml of a 0.03% solution of washed sheep erythrocytes were added. The samples were then centrifuged at low speed in order to cap the virus pellet with a thin layer of erythrocytes. These cells acted as a protective plug to prevent the virus pellet from being physically disturbed during the many fluid changes associated with fixation, dehydration, and embedding. Using these modifications, excellent quantitation of the purified T-4 phage was obtained. The appearance of a pellet is shown in Fig. 4.

[1] This material was kindly supplied by Dr. David M. Shapiro, Dept. of Biochemistry, University of Texas Medical School at San Antonio.

*Discussion.* One of the advantages of counting viruses by this technique is that large amounts of cellular debris in the sample do not interfere with particle identification. Viruses which are pleomorphic and therefore difficult to recognize by negative staining may be more easily recognized when sectioned since characteristic outer membranes and internal structures can be visualized clearly. Indeed, some debris in the sample is desirable since it gives the pellet greater size and coherence. This procedure also allows the analysis of the sample purity since the ratio of the volume of virus to the total volume of the pellet can be calculated. Thus various virus purification methods can be quantitatively evaluated by this method. The precision appears to be satisfactory and particle counts compare closely with those obtained by another method.

Only objects which are clearly recognizable as virus particles should be counted. Size, shape and the characteristic outer membranes, capsid and core can be used as criteria for particle recognition. Figure 5 shows different diagrammatic sections of equal length, each having a thickness $S$, through a pellet of purified virus. Each section cuts a varying number of particles; but if the above criteria are used for particle recognition, the

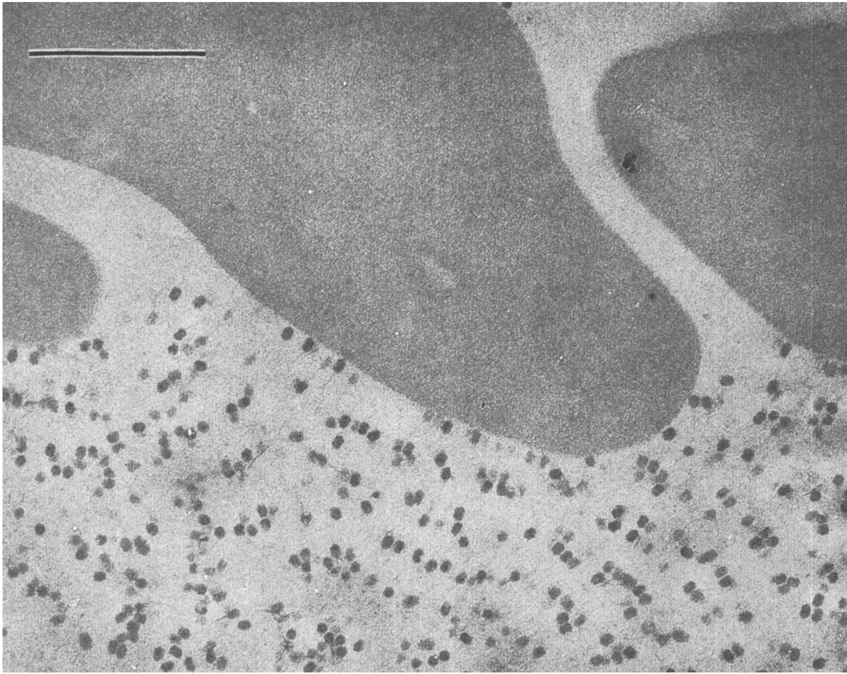

FIG. 4. Thin section of a pellet of purified T-4 phage that was capped with sheep erythrocytes. The bar equals 1 $\mu$.

counts will be about the same in each case. The largest error will result in counting purified viruses in crystalline array as illustrated. In this case, electron images may be of value in determining the plane in which the crystal was cut, and thus aid in calculating the number of particles present. Pelleting purified virus lessens the adverse effects of particle aggregation since such a pellet tends to become one uniform aggregate.

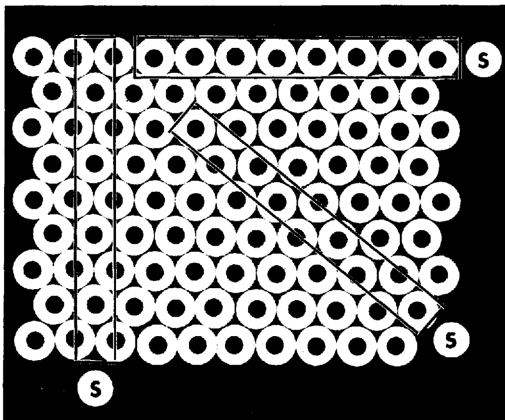


FIG. 5. Schematic diagram illustrating an array of virus particles. Different sections of equal length and thickness $S$ may cut varying numbers of particles.

Perhaps the biggest disadvantage of this counting technique is the time required to fix, dehydrate, embed, and section the virus. Approximately 5 days are needed to prepare the sample as compared to less than 1 day when using other particle counting techniques. Most of this time is needed for curing the Epon. There are several other minor difficulties. Since the distance between the grid supports is 80 $\mu$ when using 200 mesh copper grids, the maximum pellet height (excluding the erythrocyte "cap") must be less than 80 $\mu$ in order to view the entire pellet height without obstruction. This can be achieved by serially diluting the virus and selecting only appropriate pellet sizes for quantitation. Also, only 10–20% of the handmade bottleneck capsules purchased from BEEM were judged sufficiently uniform and free of flaws for use in quantitation. (The remaining capsules were used for nonquantitative work.)

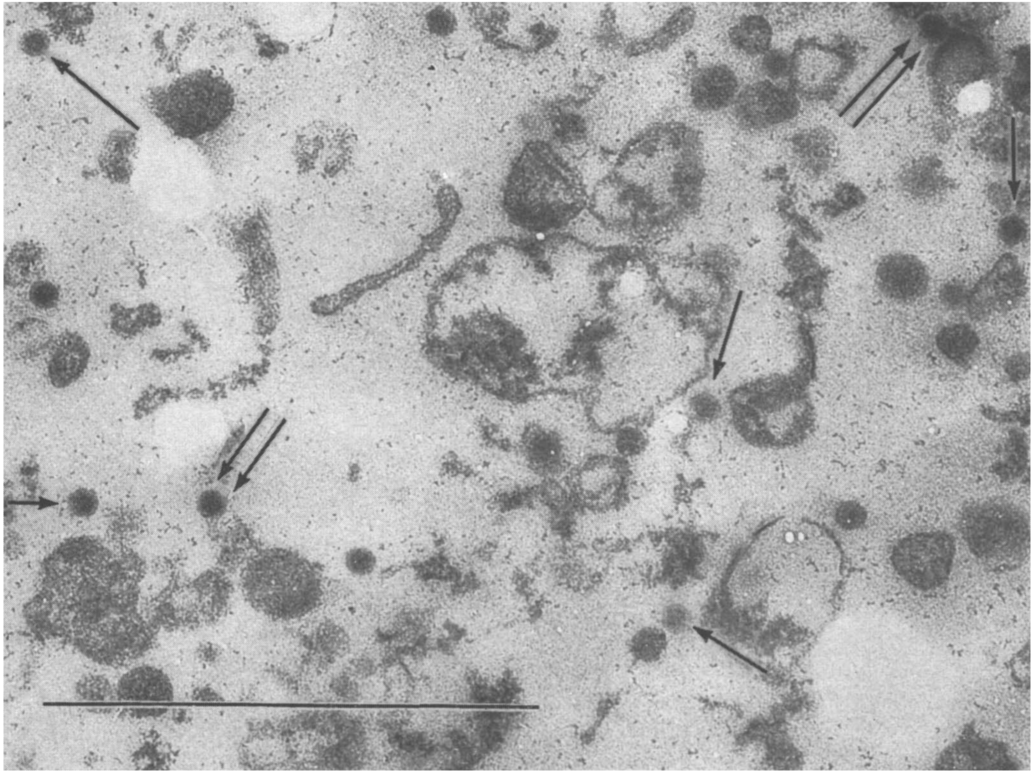

FIG. 6. Thin section of a pellet of partially purified adenovirus type 2. Single arrow shows particle clearly sectioned. Double arrow shows particle which was not sectioned. The bar equals 1 $\mu$.

Finally, the maximum amount of supernatant fluid must be removed to assure proper fixation, dehydration, and embedding, yet the pellet must not be disturbed. By holding the capsule in front of a high intensity lamp, the fluid level may be observed, and the maximum amount of fluid may be removed with a small capillary pipette.

*Summary.* A method is described which permits a quantitative estimate of sample purity and virus particle concentration by ultrathin-section electron microscopy.

*Appendix I.* The relationship of interference color to section thickness was described by Peachey (5). This scale allows the estimation of section thickness to within 10 or 20 m$\mu$. The section thickness may be calculated with higher precision by the following considerations. Assuming that a cubic-symmetry virus (such as adenovirus) is spherical in shape, the probability ($P$) of sectioning a particle is equal to the particle diameter ($Dv$) divided by the section thickness ($S$). $P$ can be determined from the electron micrograph(s) as the ratio of the number of sectioned particles ($Ns$) to the total number of particles counted ($Nt$). Thus

$$S = \frac{(Dv)\ (Nt)}{Ns} = Dv,$$

when most of the particles are sectioned (Fig. 6).

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