Predictability of Quantitation of Small Virions by Electron Microscopy (36225)

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Since the limit of detection of viral particles with UV adsorption is 10^{10} particles/ml and that of routinely employed electron microscopy is 10^7–10^8 particles/ml, it is necessary to devise ways of concentrating low-titer preparations of small virions severalfold if they are to be counted. It would be advantageous if this concentration could be achieved directly in the counting procedure.

Backus and Williams (1) reported the limit of detection of the spray droplet technique to be 10^9–10^{10} particles/ml, while Sharp (2), using a special centrifuge rotor cell, obtained high sensitivity from suspensions of concentrations as low as 10^6 virus particles/ml, as did Mathews and Buthala (3) and Smith *et al.* (4, 5) using other sedimentation methods. Review articles by Sharp (6, 7) and Isaacs (8) indicate that much of the work to date has been done with virus particles larger than 400 Å. The work reported here is concerned with virions 250 Å in diameter.

Materials and Methods. The sensitivity of electron microscope observations can be increased if the virus particles in a given volume of sample can be sedimented onto one microscope grid.

In order to sediment all the particles in a given volume of fluid onto the surface of one 3.3-mm grid, special centrifuge tubes were designed to fit the SW 39 rotor bucket of a Spinco centrifuge. A variety of shapes were considered; the ideal was assumed to be an inverted cone with an opening below the constriction to funnel the particles and still distribute them evenly over the small area without ring formation. However, experiments showed that for small particles this is not generally necessary. Tubes without a constriction gave better total particle recovery and were therefore used for counting. A centrifuge tube, holder for assembling the tube, and a snap-off tool for removing the cap are shown in Fig. 1. The dimensions of the commercially fabricated[3] nylon tube are: top, 9 mm i.d.; bottom, 3 mm i.d.; length, 49 mm. A depression is molded in the bottom of the tube to hold the specimen screen. The holder for the tube serves a dual purpose: The upper part is movable and serves as a vise. The tube is placed in the spring-loaded holder with the top of the tube down; a grid is then placed in the depression, and a cap placed on the bottom of the tube and sealed by using the vise. The snap-off tool is used for removing the cap after centrifugation. The tubes are disposable, thus avoiding the possibility of cross-contamination by succeeding samples.

In our studies a 400 mesh Formvar carbon-coated copper specimen grid, made hydrophilic by the technique of Stephens (9), in which the grids are exposed to a 2-kV discharge for 1 min at a pressure of 50 μ, was placed in the tube as described above. The tube containing the grid and approximately 1 ml of virus solution was placed in an SW 39 rotor bucket. Water was added to the area between the tube and the bucket to a level just below the top of the nylon tube, with no

[1] Supported by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission.

[2] Operated for the U.S. Atomic Energy Commission by Union Carbide Corporation Nuclear Division.

[3] Fabricated by United-Carr, Knoxville, Tennessee.

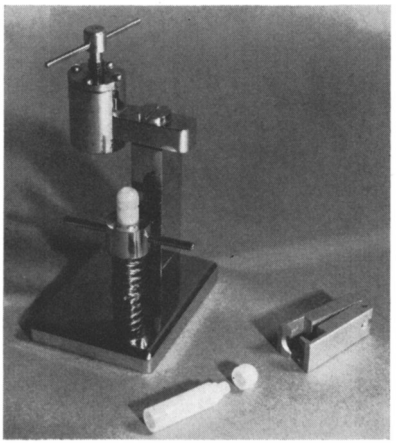

FIG. 1. Equipment used in studies of polio virus, Qβ bacteriophage, and polystyrene latex spheres.

leakage of solution from the tube observed. After centrifugation the tube was removed from the rotor bucket and the liquid withdrawn. The cap was removed from the tube by means of the snap-off tool; the grid was removed with forceps. In some instances the grid was washed with distilled water prior to shadowing with a heavy metal or negative staining with phosphotungstic acid (PTA), pH 7.0. All calculations were performed using the relation from ref. (6),

$$N = \frac{NcM^2}{AhK},$$

where A is the area of the electron microscope plate used, M is the microscope magnification, h is the estimated height of the liquid in the tube, and $K = 1$.

Results. Polio vaccine. Polio vaccine[4] was used as a model system for evaluating the counting techniques; the material was purified and concentrated by Dr. N. G. Anderson of this laboratory. The particle titer was 4×10^{12}/ml by light absorbance (Anderson) and 6×10^{11}/ml by sedimentation counting (Reimer) in Tyrode buffer solution. Dilutions based on the value 6×10^{11}/ml of polio virus, using water as the diluent, were prepared and centrifuged as described at 39,000 rpm for 90 min in an L-2

[4] Provided by Dr. Charles Reimer, National Center for Disease Control, formerly with Eli-Lilly.

TABLE I. Particle Counting Using Nylon Taper Tubes.

Material	No. particles in solution	Particles/sample	Fields counted	Calculated no. of particles/ml	Confidence limit .05 level
Latex	6×10^6	27	4	1.04×10^6	$\pm 0.37 \times 10^6$
	6×10^7	540	4	1.69×10^7	$\pm 0.22 \times 10^7$
	6×10^8	5596	5	0.86×10^8	$\pm 0.49 \times 10^8$
Polio	6×10^6	194	15	0.99×10^6	$\pm 0.49 \times 10^6$
	6×10^7	3183	3	8.27×10^7	NA[a]
	6×10^9	11276	1	8.5×10^8	NA
Qβ	6×10^5	88	17	1.22×10^6	$\pm 0.15 \times 10^6$
		708	55	3.00×10^6	$\pm 6.7 \times 10^6$
		303	26	2.78×10^6	$\pm 5.3 \times 10^6$
	3×10^6	44	10	1.06×10^6	$\pm 0.76 \times 10^6$
	6×10^6	4223	55	3×10^6	$\pm 6.7 \times 10^6$
		581	29	4.9×10^6	$\pm 8.7 \times 10^6$
	6×10^7	3219	18	0.42×10^8	$\pm 6.2 \times 10^7$
		16565	27	1.47×10^8	$\pm 1.44 \times 10^8$
		17103	30	1.37×10^8	$\pm 1.06 \times 10^8$
	3×10^8	9638	7	3.25×10^8	$\pm 1.42 \times 10^8$
	6×10^8	1×10^5	8	3.29×10^9	$\pm 2.22 \times 10^9$
		2×10^5	7	6.6×10^9	$\pm 6.7 \times 10^9$
	6×10^9	1×10^5	3	8.35×10^9	$\pm 3.8 \times 10^9$
	3×10^{10}	3.6×10^4	6	1.42×10^{10}	$\pm 4.9 \times 10^{10}$

[a] NA: not available.

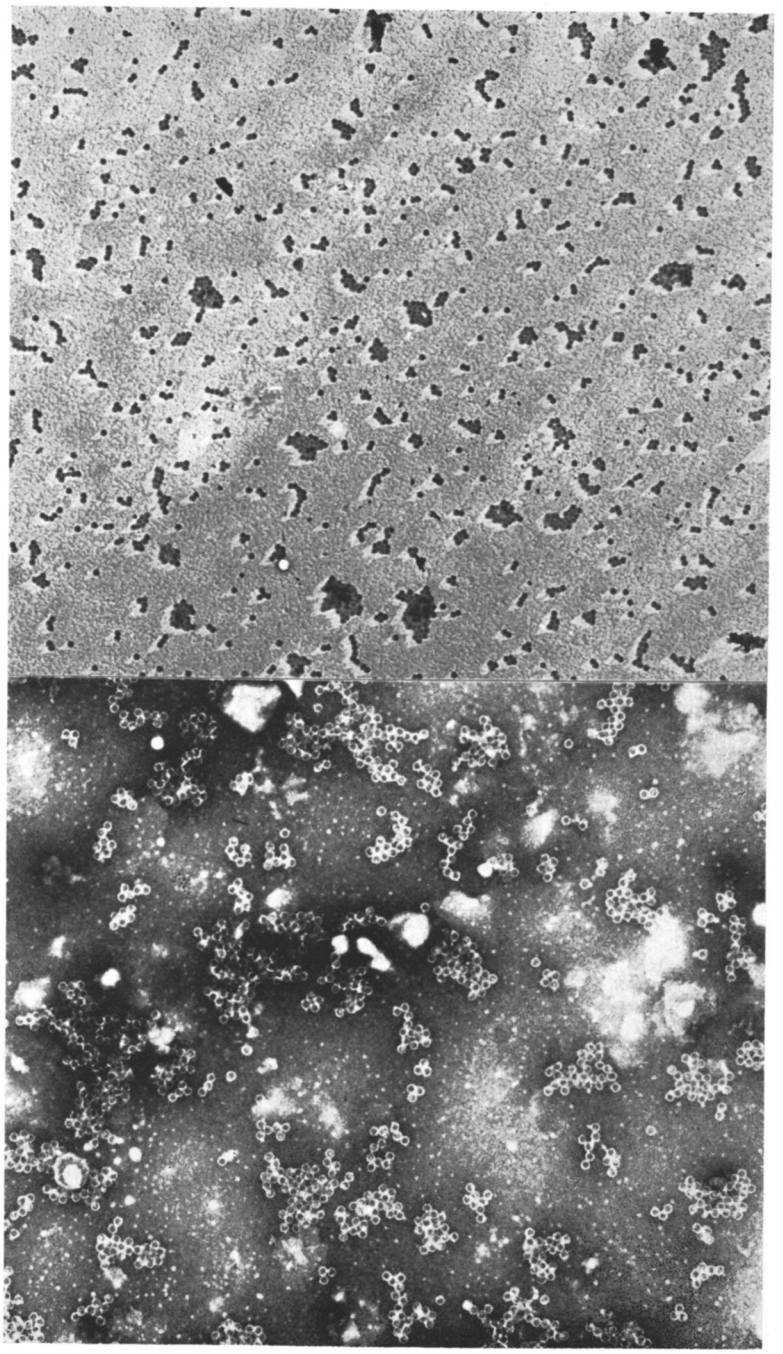

FIG. 2. Representative micrographs for polio vaccine (top), 32,000×, and Qβ bacteriophage (bottom), 64,000×.

Spinco centrifuge with the brake off. After centrifugation the grids were shadowed with Pt–Pd and examined with an RCA-EMU-3G electron microscope. Pictures of several areas were made at 3000 diameters and the plates used for counting. Results are given in Table

I. A representative electron micrograph is shown in Fig. 2 (top).

Qβ bacteriophage. Qβ bacteriophage[5] was also used in evaluating the method. The concentration of Qβ was determined by mixing with a known concentration of polystyrene latex spheres[6] and counting the number of virus particles and latex spheres in several drops. From the ratio of spheres to particles, one is able to calculate the concentration of particles in a solution. The concentration of Qβ in the stock solution was calculated to be 6×10^{14} particles/ml. Various dilutions of Qβ from 10^5–10^9 were prepared using 0.005 *M* Tris buffer, pH 7.0, at 25°, containing 10^{-4} *M* Mg^{2+} and centrifuged at 39,000 rpm for 90 min. After centrifugation the grid was removed from the tube and negatively stained with PTA, pH 7.0. Grids so prepared were examined using the Siemens Elmiskop 1A. Pictures made at 7800 diameters of 12–15 fields selected at random were used for counting. Results are given in Table I. A representative electron micrograph is shown in Fig. 2 (bottom).

Latex spheres. Polystyrene latex spheres obtained from Dow Chemical Company were used in a few experiments during the evaluation of the counting technique. Latex spheres 0.312 μ in diameter, 6×10^{12} particles/ml, were diluted with distilled water to concentrations of 10^6, 10^7, and 10^8 particles/ml. These diluted solutions were spun at 5000 rpm for 5 min. After centrifugation the grids were examined in the electron microscope without being shadowed or stained. The results are given in Table I.

Discussion. From the data presented in Table I, it seems reasonable to conclude that one can count 250-Å particles in a solution of 10^6 particles/ml if all particles from 1 ml of solution are spun onto a specimen grid. The somewhat high count on some samples is attributable either to uneven spreading of the virus on the grid or to aggregates already in the solution. For example, in a 10^6 particles/ml solution it is not unusual to find on one photographic plate an aggregate made up of 10 or 12 virus particles. Polio virus appeared to spread somewhat better than Qβ. There was no evidence that virus stuck to the nylon edges of the tubes or collected at the edge of the grid. Latex spheres collected near the edge of the grid in a circular configuration, probably due to the size of the latex, as shown by the light micrograph in Fig. 3.

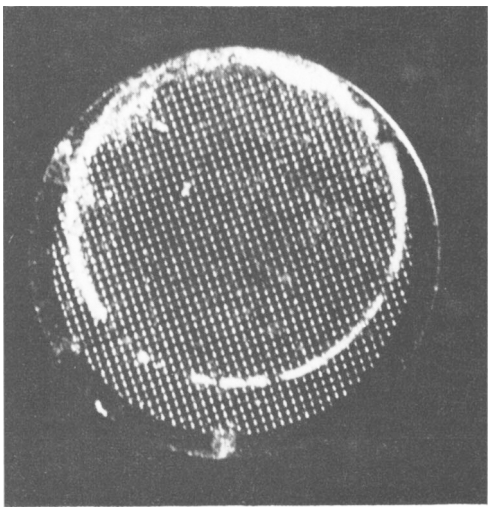

FIG. 3. Light micrograph of polystyrene latex spheres.

A 12-tube rotor designed in this laboratory for operation at 30,000 rpm was evaluated during the course of this study. The partially disassembled rotor with an insert necessary to seal the tube at the top is shown in Fig. 4. Qβ particles collected at the edge of the grid when this rotor was used but did not when sedimented in an SW 39 rotor. A presumptive explanation of the difference is that there are resonances in the swinging bucket assembly not present in a solid rotor which result in mixing during centrifugation, yielding a broadly distributed sediment.

Completeness of recovery of polio virus cannot be ascertained very easily at this time because of (a) uncertainty as to whether the true titer of the virus concentrate was 4×10^{12} or 6×10^{11} particles/ml, and (b) the limited number of areas which were counted. The data indicate loss of particles for all concentrations of latex; particle loss was

[5] Provided by Dr. L. R. Overby, Abbott Research Laboratories.

[6] Provided by Dr. E. Kellenberger, University of Geneva.

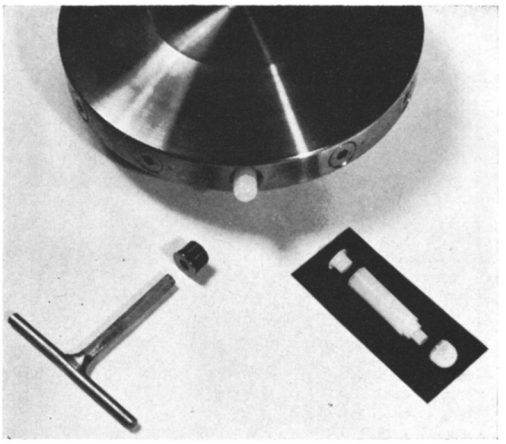

FIG. 4. Partially disassembled 12-tube rotor showing the tube-sealing device.

shown for Qβ in the concentration range of 10^6 and 10^8, with an apparent gain for concentrations of 10^5 and 10^7. For polio, best agreement was for concentration of 10^7 particles. Best accuracy was obtained for particle concentrations in the range of 10^6 particles.

Analysis of the data [7] gave standard deviations based on particle counts for Qβ phage as shown in Table II. Had the particles been evenly distributed (no clumping), one would have expected $\bar{X} = 1.075$ (SD). The mean number of counts ($\bar{X}$) was almost equal to the standard deviation (SD), the actual linear regression being $\bar{X} = 1.075\ (SD)^{1.1}$, indicative of highly variable counts. The standard deviation was somewhat smaller in relation to the mean for higher concentrations, as reflected by the exponent 1.1 in the above expression, and with higher counts one found several clumps of particles present in each counting area, tending to lessen the clumping effect. Ensuing statistical analysis resulted in the following counting procedure.

Visual examination of the data in Table II showed that it followed some sort of geometric pattern. A modified geometric distribution was found for which with increasing area counted, the probability of getting zero count decreased quadratically with zero. Which is to say that if the count has many areas with no particles, doubling of the areas counted will reduce the number of zero counts quadratically, and the variance will be smaller in relation to the mean. A statistically optimum procedure is one in which counts are made of areas taken randomly regardless of the presence or absence of particles. The choice of area size should be such that the number of particle counts per area averages 20–30. The relation

$$\bar{C} = \bar{X} \pm 2\ \text{SE}$$

can be used to establish an approximate 95% confidence interval. From this relationship, 100 areas need to be counted for a mean with an 80% confidence interval, 400 areas for a 90% confidence interval, 1600 areas for a 95% confidence interval, and 40,000 areas for a 99% confidence interval.

These results lead to the conclusion that for particles in the size range treated above one could, using manual methods, achieve the

TABLE II. Standard Deviations for Qβ Bacteriophage.

Concentration	Mean number of counts	Estimated standard deviation	Number of sample
6×10^5	4.4	6.1	5
	5	3.2	5
	4.9	3.9	7
3×10^6	4.6	1.8	9
6×10^6	14.3	14.9	4
	15.9	32.2	8
	0.3	0.8	6
	5.1	2.5	7
	5.7	4.6	7
6×10^7	79.6	82.0	8
	188.3	153.7	6
	91	163.5	5
	70.4	74.0	5
	68.8	29.2	6
	337	182	4
	59.5	16.9	4
	113	72.6	5
	231	153	6
3×10^8	1377	310	7
6×10^9	317.5	59.8	4
	551	75.6	3
$(3 \times 10^{10}$	60316	9716	2)

[7] The statistical analyses were done by Drs. Kim O. Bowman and David Hoel of the Oak Ridge National Laboratory Mathematics Panel.

90% confidence level with a reasonable expenditure of effort; but work at the 95% level would require an inordinate effort. A second conclusion is that it would be possible to count small viruses with foreknown precision if modern computerized flying spot scanners were used. With such instrumentation one could count rapidly and to a precision higher than 95%. The technique using the counting tubes described here allows one to detect and count small virus concentrations as low as 6 $\times$ 10^6/ml and possibly the high side of 10^5/ml with predictable precision.

Summary. Numerous studies of sedimentation techniques for counting virus particles using electron microscopy have shown an ultimate practical limit of detection of the order of 10^6 particles/ml. This report described (a) counting experiments using disposable molded nylon centrifuge tubes designed to concentrate and sediment small virus particles onto one microscope specimen grid and (b) a statistically derived method for determining the number of fields and particles that need to be counted for a desired precision.

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