Centrifugal Sedimentation of Virus Particles for Electron Microscopic Counting

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Centrifuge cells with conical chambers were provided by using special inserts for the stainless-steel tubes that fit the Spinco SW-39 rotor. Particulate material, centrifuged in these cells, was collected on carbon-coated glass discs. These discs were exposed to OsO_4 vapor, dehydrated in graded alcohols, air-dried, and metal-shadowed. The metal-shadowed carbon film was floated from the glass, mounted on a grid, and examined. A knowledge of cell geometry and microscope magnification allowed correlation of the number of particles observed to a volume of the original suspension. A precision of $\pm 6\%$ at the 95% confidence level was attained when counting approximately 100 particles per $10,000 \times$ field. Applications and advantages of the method are discussed.

Electron microscopic enumeration techniques for viruses and other small particles (200 nm or less) have included the spray droplet method of Backus and Williams (1), the agar filtration method of Kellenberger and Arber (4), and the loop drop method of Watson et al. (14). The papers of Crane (2), Overman and Tamm (5), Sharp (7), and Smith and Benyesh-Melnick (13) describe centrifugation of suspended particles directly onto filmed electron microscope grids. It has been observed that films deform or tear during centrifugation, and dispersion is not uniform since particles are found in greater concentration at the depressed centers of grid squares (13). Sharp (6) described centrifugal collection of virus particles on collodion-coated glass. Galasso and Sharp (3), Sharp (7, 8), Sharp and Beard (9), Sharp and Buckingham (10), Sharp and Overman (11), and Smith and Benyesh-Melnick (12) have used the surface of agar to receive centrifuged virus particles. Sedimented virus particles were stripped from the agar by coating the surface with a film of collodion which, after drying, could be floated free. Stripping by collodion film recovered most but not all of the virus particles. Smith and Benyesh-Melnick (12) stated that 93% of polyoma virus was recovered by the first collodion film, and used a correction factor of 1.07 to account for this partial recovery. Sharp (7) experienced difficulty in quantitatively recovering meningopneumonitis virus from agar surfaces.

An improved method of particle collection from liquid suspension has been developed in our laboratories. The technique and its applications will be described, and advantages of the method will be discussed.

MATERIALS AND METHODS

Conical sector cells could have been designed to fit any swinging bucket centrifuge, but for our purposes the Spinco model L centrifuge (Beckman-Spinco Div., Beckman Instruments Inc., Palo Alto, Calif.) and the Spinco SW39 swinging bucket rotor (Beckman Instruments Inc.) most nearly fulfilled the requirements for speed, ruggedness, and small volume. Spinco stainless-steel centrifuge tubes (Beckman Instruments Inc.) for the SW39 rotor were the containers for the special cells.

These stainless-steel cells were designed in three parts as shown in Fig. 1 and 2A. A hemispherical base, C, with a flat-topped boss supported a cylindrical body, B, whose lumen was a conic frustum. A pin, D, was used to couple the base and cylindrical body together to form the cell. A threaded handle, A, was screwed into the top of B after centrifugation to facilitate removal of the cell from the centrifuge tube. The cylindrical body was so designed that the interior walls corresponded to radii of the rotor when rotated at high speed. This prevented wall effects or turbulence which would interfere with even sedimentation.

Particles were collected on carbon-coated glass discs prepared as follows. Discs (8.0 mm) of no. 3 thickness cover glass (special order, Corning Glass Works, Corning, N.Y.) were soaked in acetone for several hours, rinsed in three changes of absolute alcohol, drained on filter paper, and air-dried in a dust-free environment. The dry discs were coated with carbon in a vacuum evaporator and stored in a dust-free container until used.

Cells were assembled as follows. A base was set upon a support pin on the holder (Fig. 2B), a carbon-coated glass disc was placed on the boss of this base, carbon film uppermost, a cylindrical body was carefully lowered over the boss, and the coupling pin was inserted (Fig. 2C). The assembled cell was lowered into a stainless-steel centrifuge tube which was placed in a

FIG. 1. Cell components. The conical sector portion, B, is coupled to the hemispherical base plug, C, by means of the pin, D. The handle, A, is screwed into B to facilitate removal of the assembled cell at completion of centrifugation.

rotor bucket. A 1-ml amount of particle suspension was added to each cell through the top vent. After capping, the buckets were assembled to the rotor.

The time and speed of centrifuging were dependent on the size and density of the suspended particles. Calculated times and speeds for complete sedimentation of 100-nm virus particles were 41.5 min at 10,000 rev/min or 10.4 min at 20,000 rev/min. To insure complete particle recovery, the actual running time was 150% of the calculated sedimentation time. Because of the extra weight of the cells, all centrifugations were made at 20,000 rev/min or less to avoid exceeding the safety factor of the rotor.

After centrifuging, the buckets were opened and the cells were removed and placed upright on the holder. The pins were removed and the bodies were lifted from the bases. The glass discs were placed in a petri plate containing a few drops of 2% OsO_4 solution. After 15 min of exposure to OsO_4 vapors, the discs were briefly immersed in distilled water, followed by 5-min exposures to 50%, 95%, and absolute ethanol. The dehydrated discs were air-dried and shadowed with platinum in a vacuum evaporator.

FIG. 2. Conical sector cell showing components and assembly. (A) Components disassembled. (B) Hemispherical base set upon pin of holder. Other parts not assembled. (C) Assembled cell on holder.

After shadowing, each disc was scratched along perpendicular diameters to divide the film into quadrants. The disc was immersed (1 to 5 min) in a 25% solution of potassium hydroxide, removed, drained, and again carefully lowered at an acute angle into the potassium hydroxide solution. This caused the film to float onto the surface of the liquid from whence it was lifted on a fine mesh screen and transferred successively to the surface of water, 0.2 N hydrochloric acid, and two more water baths. The washed film was picked up on a 200-mesh copper grid, drained, air-dried, and examined in the electron microscope. The portion of the quadrant nearest the center of the disc was chosen for particle counting since in this area there is a greater chance of even distribution.

From a knowledge of cell geometry and height of fluid in the cell, it was possible to calculate a factor which related the number of particles per field to the number of particles per milliliter of original suspension. The calculation and use of this factor are described in Appendix 1.

Counts were made either directly from the electron microscope screen or from photographic plates of the various fields. The first method was faster, but the second method gave more certainty of identification when working with particles of various sizes and shapes.

FIG. 3. Representative electron microscope fields showing uniform dispersion of particles. (A) *Vaccinia* virus, (B) adenovirus 6, (C) influenza virus (PR-8), (D) coxsackievirus A-21, (E) vesicular stomatitis virus, (F) polystyrene latex spheres. Mark on each picture represents 1 μ m.

RESULTS

Typical microscopic fields (Fig. 3) showed that this method can be used to collect and count particles of differing size and shape whether viral or nonviral. Assay of supernatant fluids, after centrifuging viral preparations, showed less than 0.1% of the original biological activity (TCID₅₀, plaque-forming units, or hemagglutinating units) remained un sedimented.

The precision of the method was demonstrated by loading the three cells with equal amounts of the same dilution of virus. After centrifuging, fixing, drying, and shadowing, the films were stripped as described. Portions of two opposite quadrants from each of the three discs were mounted on grids and five randomly chosen fields were counted for each grid. The results (Tables 1 and 2) showed that the average of five randomly selected fields provides a reliable estimate of the number of particles per field and therefore particles per unit volume.

A statistical analysis of a larger series of samples (5 counts/grid $\times$ 2 grids/cell $\times$ 3 cells/centrifugation $\times$ 1 centrifugation/day $\times$ 5 days =

TABLE 1. *Counts of purified PR-8 influenza virus^a*

Cell 1		Cell 2		Cell 3	
Grid 1	Grid 2	Grid 1	Grid 2	Grid 1	Grid 2
99	97	103	104	103	98
100	101	97	95	103	94
103	93	101	97	100	104
103	93	97	95	99	99
102	96	103	105	98	104

^a Average values are as follows. Cell 1: grid 1, 101.4; grid 2, 96.0. Cell 2: grid 1, 101.2; grid 2, 99.2. Cell 3: grid 1, 100.6; grid 2, 99.8. Overall average is 99.5.

TABLE 2. *Counts of purified herpes febrilis virus^a*

Cell 1		Cell 2		Cell 3	
Grid 1	Grid 2	Grid 1	Grid 2	Grid 1	Grid 2
91	85	88	86	94	91
93	95	96	87	96	85
89	104	87	90	98	90
91	94	94	97	94	85
92	97	94	90	85	92

^a Average values are as follows. Cell 1: grid 1, 91.2; grid 2, 95.0. Cell 2: grid 1, 91.8; grid 2, 90.0. Cell 3: grid 1, 93.4; grid 2, 88.6. Overall average is 91.7.

150 counts) indicated that, when counting approximately 100 particles/field at 10,000 magnification, the precision of five counts was $\pm 6\%$ at the 95% confidence level.

A series of centrifuge runs was performed on a stock of purified PR-8 influenza virus. The concentration of this stock virus was adjusted to give counts of approximately 100 particles per 10,000 $\times$ field when completely sedimented onto the carbon-coated glass discs. All operations were performed as indicated earlier, except the duration of the centrifuge runs was 5, 10, 15, 20, 30, 40, 50, or 60 min. Fresh portions of virus stock were used for each centrifuge run. The loaded rotor was accelerated rapidly, and, when it reached 10,000 rev/min, a stopwatch was started. At the conclusion of the allotted time, the power was turned off, and the rotor was allowed to coast to a stop. Only the time at 10,000 rev/min was counted, so each of the indicated times has an inherent error factor combining the forces experienced during acceleration and deceleration. Two grids were prepared from each of the three discs representing a given time. Five counts were made on each grid. In Table 3, the values in the columns headed cell 1, cell 2, and cell 3 are the average of 10 counts.

A sedimentation time of 38.6 min was calculated for PR-8 influenza virus. This would give the theoretical sedimentation plot shown in Fig. 4. The actual values (Table 3) are also plotted for comparison. The difference between the two lines is due to the gravitational forces experienced during acceleration and deceleration. The straight line plot of values from 5 to 30 min and the constancy of the values from 40 to 60 min indicate that no loss of particles from the carbon film is experienced during manipulations subsequent to centrifuging.

TABLE 3. *Counts of purified PR-8 influenza virus with various times of centrifuging at 10,000 rev/min*

Duration of run	Cell 1	Cell 2	Cell 3	Total	Avg
<i>min</i>					
5	17 ^a	20 ^a	19 ^a	56	19
10	34	31	31	96	32
15	49	50	48	147	49
20	64	63	64	191	64
30	92	89	89	270	90
40	100	100	101	301	100
50	98	98	99	295	98
60	101	101	100	302	101

^a Values in columns headed cell 1, cell 2, and cell 3 are the average of 10 counts (5 counts $\times$ 2 grids).

FIG. 4. Plot of particles of PR-8 influenza virus counted per 10,000 $\times$ field against time of centrifugation at 10,000 rev/min. Theoretical sedimentation plot drawn for comparison.

DISCUSSION

The described technique presents a method of collecting particles with a minimum of physical manipulation. Only pipetting, mixing, and centrifuging are involved. Forces of shearing and impinging are not experienced. Suspensions may be kept at optimum pH and osmolality until the particles are collected on the carbon-coated disc. We believe that particle aggregation seen on the grid represents the aggregation present in the suspension before centrifuging.

Analysis of supernatant fluids indicates that, with this method, sedimentation is 99.9% complete. The reproducibility of results and the straight line plot of experimental values in Fig. 4 indicate that all particles which are sedimented appear on the final film. If this is not valid, one must accept the loss of a constant and reproducible fraction of particles during preparation and mounting of the film independent of the concentration of particles in the original suspension.

The fields shown in Fig. 3 were obtained with relatively pure virus preparations, but this does not imply that the method cannot be used to evaluate nonpurified virus suspensions. We have routinely performed counts on cell lysates after infection with adenovirus, vaccinia virus, vesicular stomatitis virus, influenza virus, coxsackievirus A-21, bacterial viruses, and *Streptomyces* sp. viruses.

The presence of high concentrations of solute does not interfere with particle enumeration by this method. Bacteria prepared in media containing high concentrations of solute have been successfully examined and show no artifacts due to salt crystals. Viruses purified by cesium chloride, tartrate, or sucrose gradients were easily collected and counted.

A significant advantage of this technique is the possibility of examining and counting virus stocks containing relatively few particles. Preliminary calculations indicated that significant counts could be obtained from preparations containing as few as 10^6 particles per ml if the particles could be recognized at 2,000 $\times$. Experimental studies with dilute suspensions of vaccinia virus verified these calculations.

We present this as a precise and reliable technique for counting virus particles and thereby determining the multiplicity of particles per infectious unit.

APPENDIX 1

We desire to know the number of particles per cubic centimeter of the particle suspension which is loaded into the centrifuge cell. By examination, the number of particles per photographic plate can be determined. The factor F , which relates these two quantities, may be expressed as

$$F = \frac{\text{particles/cm}^3}{\text{particles/plate}} \\ = \text{plate/cm}^3$$

Thus F has the dimensions of number of plates and volume. Since we can only count the particles on one plate at a time, F relates the particles on one plate to the minute volume of suspension which originally contained these particles.

By using the following information

$$\begin{aligned} \text{area of photographic plate} &= 5.9 \times 6.8 \text{ cm} \\ &= 39.44 \text{ cm}^2 \end{aligned}$$

$$\text{height of liquid in cell} = 2.26 \text{ cm}$$

$$\text{magnification} = 10,000$$

$$\text{taper correction (Appendix 2)} = 0.869$$

we find that the volume containing the particles that will appear on one photographic plate is

$$\begin{aligned} V &= \frac{(39.44 \text{ cm}^2)(2.26 \text{ cm})(0.869)}{(10,000)^2} \\ &= 7.74 \times 10^{-7} \text{ cm}^3 \end{aligned}$$

Therefore

$$\begin{aligned} F &= 1 \text{ plate} / 7.74 \times 10^{-7} \text{ cm}^3 \\ &= 1.29 \times 10^6 \text{ plate/cm}^3 \end{aligned}$$

As a sample calculation, assume a count of 105 particles on a given plate at 10,000 magnification:

$$\begin{aligned} \text{count/cm}^3 &= (105 \text{ particles/plate}) \\ &\quad (1.29 \times 10^6 \text{ plate/cm}^3) \\ &= 1.35 \times 10^8 \text{ particles/cm}^3 \end{aligned}$$

Similar factors can be calculated for other combinations of liquid height, plate area, and magnification.

APPENDIX 2

A taper correction must be used because particles in a given rotational plane do not follow parallel paths on their way to the carbon-coated glass disc, but instead follow diverging paths. The path of each particle is a section of a radius in a given rotational plane.

To calculate the taper correction, assume a wedge such that $AJ = DJ = CK = BK = 8.65$ cm. This corresponds to a wedge in the centrifugal force field where the plane A, B, C, D corresponds to the surface of the carbon-coated glass disc, and JK is the center of rotation of the rotor. Also $EJ = HJ = GK = FK = 6.39$ cm. This wedge may be compared in the same way. The plane E, F, G, H corresponds to the surface of the liquid in the cell and JK is the center of rotation of the rotor. Therefore, the liquid depth is $EA = FB = GC = HD = 2.26$ cm. We may define the following volumes: V_w = volume of the wedge whose corners are A, B, C, D, J, and K; V_x = volume of the wedge whose corners are E, F, G, H, J, and K; V_s = volume of the wedge sector whose corners are A, B, C, D, E, F, G, H; V_r = volume of a rectangular solid whose base is A, B, C, D and whose height is 2.26 cm.

As the area whose corners are A, B, C, and D becomes extremely small and approaches the limit, the perpendicular distance between the planes ABCD and EFGH is nearly equal to the radial distance. Then when $AB = BC = CD = DA =$ unit distance we may write:

$$\begin{aligned} V_s &= V_w - V_x \\ &= \frac{(1)(1)(8.65)}{2} - \frac{(6.39/8.65)(1)(6.39)}{2} \\ &= 4.325 - 2.361 \\ &= 1.964 \end{aligned}$$

Also:

$$\begin{aligned} V_r &= (1)(1)(2.26) \\ &= 2.26 \end{aligned}$$

The taper correction is the ratio of V_s to V_r . This correction is the fraction of particles contained in a

volume, V_r , which will appear on the base A, B, C, D after centrifugation. It may be calculated as follows:

$$\begin{aligned} \text{taper correction} &= \frac{V_s}{V_r} \\ &= \frac{1.964}{2.26} \\ &= 0.869 \end{aligned}$$

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