Particle Counting of Polyoma Virus.* (26642)

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There is a need for combined physical and biological quantitation of polyoma virus, particularly as we wish to determine the variations in the ratio of the number of physical virus particles to the number of infectious units in polyoma-infected cell cultures during various phases of the growth cycle. Polyoma virus is often extensively aggregated when observed in the electron microscope. This feature may frustrate accurate estimation of total virus mass by biological titrations alone. The present study describes a method for counting polyoma virus particles, and shows a correlation between physical and biological measurements on similar preparations.

Materials and methods. A plaque-purified polyoma virus strain (obtained from Dr. R. Dulbecco, California Inst. of Technology) was used. The strain was maintained by serial passages in primary or secondary Swiss mouse embryo cultures, according to the methods described from Dulbecco's laboratory(1,2). With passage, the virus maintained its cytopathogenic activity in mouse embryo cultures, its ability to agglutinate guinea pig red blood cells, and its capacity to be neutralized by specific polyoma antiserum (obtained from Dr. B. Eddy, Nat. Inst. of Health).

The plaque assay described by Dulbecco and Freeman(1) was employed with several modifications. A 2-hour adsorption period at 37°C was used and the virus inoculum was in a volume of 0.5 ml, since using smaller amounts of fluid caused drying of the center portion of the monolayers. The overlay medium contained 5% horse or fetal bovine serum.

Guinea pig red blood cells, collected in Alsever's solution, washed 3-4 times in veronal buffer (pH 7.3) and kept at 4°C up to 4 days, were used for the hemagglutination (HA) test. They were washed once more before use. Serial 2-fold dilutions of virus were made in veronal buffer (pH 7.3) in transparent plastic panels (Linbro); 0.5 ml of 0.5% red cell suspension was added to 0.5 ml of the virus dilutions and the panels kept overnight at 4°C. The HA titers were expressed as the reciprocal of the last dilution of virus eliciting complete agglutination.

The basic technic used for virus particle counting was that described by Sharp(3) and Smith and Sharp(4) for vaccinia virus. Some modifications were made to facilitate the counting of the much smaller polyoma virus (45 mμ). Centrifugation for counting was done in the Spinco SW-25.1 swinging bucket rotor at 25,000 rpm. Maximum centrifugal force achieved with the SW-25.1 rotor is 90,000 × G, which gives rapid sedimentation of polyoma virus from a short column of fluid. Flat bottomed lucite chambers were made for the cups so that flat agar plugs could be inserted and removed easily. Fig. 1 shows the components used in this procedure. The lucite adapter (A) contains a flat bottomed cylindrical chamber 19 mm deep and 19 mm in diameter. Into this chamber slips a snugly fitting lucite insert (B) into which a pre-cut agar disc (C) of approximately 13 mm diameter has been placed. Agar is cut with an ordinary cork hole borer. A small air escape hole is cut on one edge of the agar disc. The parts are assembled wet so that all spaces between components are filled with distilled water. One ml of the appropriately diluted virus suspension is placed in the chamber, centrifuged for a sufficient time (approximately 20 minutes), the supernatant fluid carefully removed by suction, the lucite insert removed with a tapered lucite rod (D), the agar block lifted out with a curved spatula (E) and placed on a slide to dry. The remaining steps of preparing the pseudoreplica for examination in the electron microscope are identical to those previously described(3).

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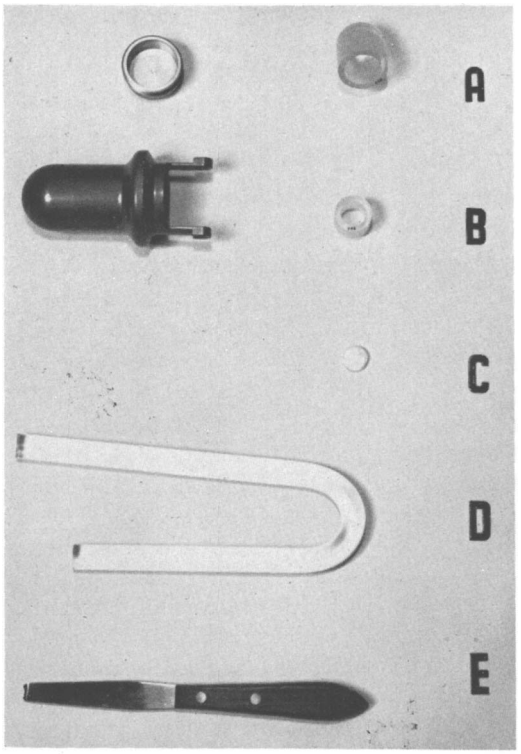

FIG. 1. Apparatus used in polyoma virus counting.

Calculation of number of particles of virus per ml of original fluid depends upon the following considerations. 1. Approximately 93% of the virus is recovered on the first pseudoreplica. A correction factor of 1.07 is used. 2. The chambers used in this work were cylindrical rather than sectoral in shape. At the zone of virus sedimentation, therefore, there is a slight radial spread of virus particles moving to the flat agar receiving surface. This effect produces a dilution of approximately 7%. A correction factor of 1.07 is used. (The short column of virus suspension minimizes this effect. However, sectoral chambers are being designed to eliminate this consideration.) 3. At intervals a calibrated carbon replica grating (obtained from Ernest F. Fullam, Inc.) is photographed at the magnification routinely employed for particle counting (4,600) and projected on the gridded screen used for counting. Projection magnification is approximately 20 ×. The area examined on each preparation is thus calculated directly. The area usually examined per micrograph is 50 to 70 μ^2. A total of 5 micrographs are examined for a single calculation.

Purified crystalline trypsin (Carworth) and chymotrypsin, delta (Nutritional Biochemicals Corp.) were used. Virus-enzyme mixtures were incubated at 37°C for 20 minutes. After enzyme treatment specimens were treated with sonic vibration for 30 seconds in a manner described previously(4). Filtered saline was used as a diluent.

Electron microscopy was done with an RCA Model EMU-3-C. Low speed centrifugation was done in an International horizontal centrifuge at 165 × G for 10 minutes in conical centrifuge tubes.

Results. Various concentrations of enzyme mixture were used to clarify crude virus suspensions for counting. Some reciprocity between the effects of time and enzyme concentration was noted. Treatment for prolonged periods of time resulted in a loss of countable virus particles. Final enzyme concentrations of 0.01-0.033% were found satisfactory for treatment at 37°C for 20 minutes.

Fig. 2 shows fields of polyoma virus typical of those counted. The particles were quite uniform in size and morphology. Aggregation was one of the notable features in these preparations, however. Some preparations showed numerous doughnut shaped forms, apparently empty membranes (see arrow, Fig. 2). In many preparations filamentous structures were a prominent feature (Fig. 3). These forms were not counted in this study.

Experiments were done to determine whether the particle counting methods as described above are valid for quantitating polyoma virus. Fig. 4 shows the results of one of these experiments. Serial dilutions of treated virus were made and each dilution counted. The average number of particles per field is plotted against relative concentration. The straight line drawn through these points goes through the origin.

The precision of the method was studied by making repeated counts on the same specimens. Many of these determinations were made on different days. The results are shown in Tables I and II. Average deviation

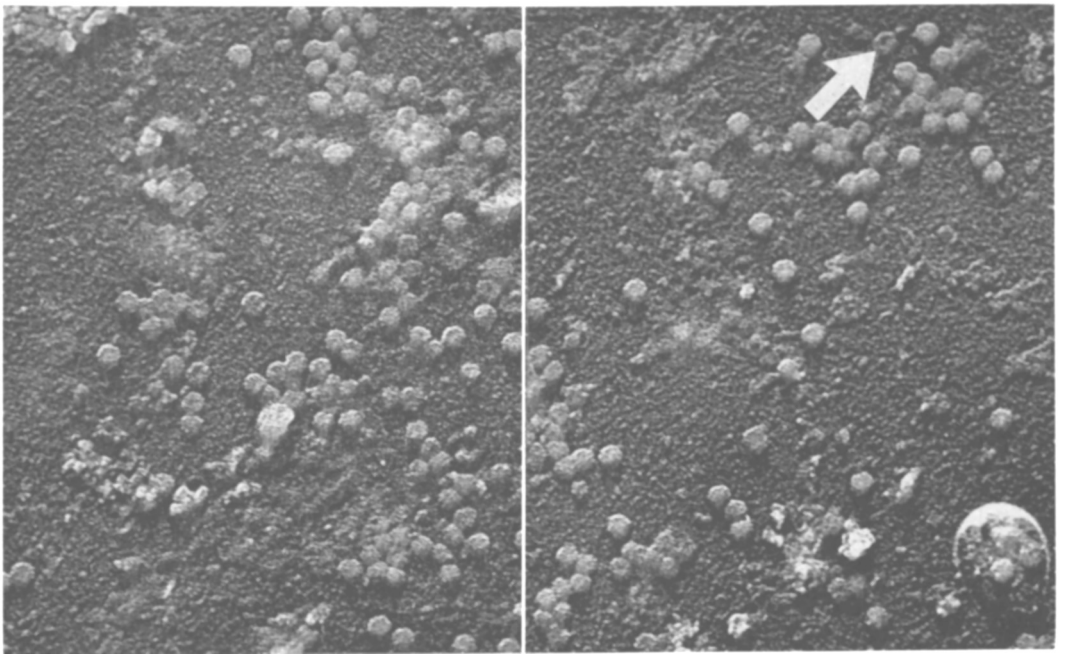

FIG. 2. Typical fields of polyoma virus examined for particle counting (66,000 ×). Arrow indicates doughnut form.

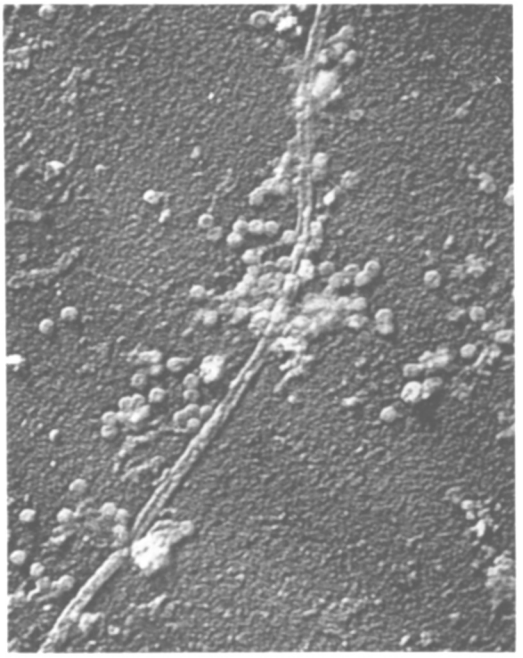

FIG. 3. Field showing filamentous structure found in many polyoma preparations (44,000 ×).

was 16 and 17% for 2 different specimens.

We are particularly interested in combining biological and physical methods in the analysis of virus-cell interaction phenomena, considering especially the cytocidal and integrated characteristics of this virus. One of the first efforts in this direction was to correlate virus characteristics using virus suspensions harvested at different intervals during a growth curve experiment with secondary mouse embryo cells infected with a high virus multiplicity.

The samples described in Table III were taken 4-8 days after infection. They contained both intra- and extracellular virus obtained by 4 cycles of freezing and thawing of the infected cultures without centrifuga-

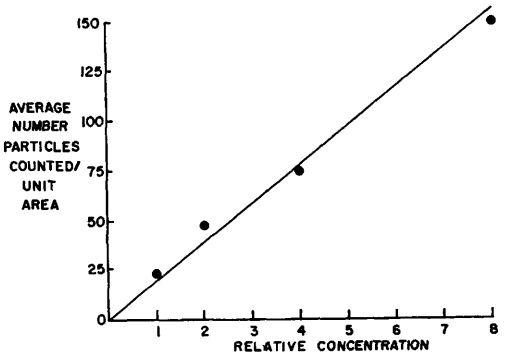

FIG. 4. Relationship between serial dilution and particle counts of polyoma virus.

TABLE I. Precision of Polyoma Particle Counting; Virus Not Pre-centrifuged at Low Speed.

No.	Avg No. particles/unit area	Calculated No. particles/ml ($\times 10^9$)	% deviation from avg
1	211	5.0	21
2	304	7.2	14
3	318	7.5	19
4	261	6.2	2
5	259	6.1	3
6	379	8.9	41
7	204	4.8	24
8	236	5.6	11
9	266	6.3	0
10	213	5.0	21
Avg	265	6.3	16

tion of the cell debris prior to examination. The average particle:plaque forming unit (PFU) ratio was 200. An average of 7×10^7 particles and 4×10^5 PFU was equivalent to one hemagglutination unit. Table IV

TABLE II. Precision of Polyoma Particle Counting; Virus Pre-centrifuged at Low Speed.

No.	Avg No. particles/unit area	Calculated No. particles/ml ($\times 10^9$)	% deviation from avg
1	160	3.8	19
2	174	4.1	12
3	179	4.2	10
4	246	5.8	24
5	230	5.4	16
6	228	5.4	21
7	154	3.7	17
Avg	196	4.6	17

describes the same characteristics of samples containing extracellular virus alone, obtained by low speed centrifugation of supernatant fluids to remove cell-associated virus and large virus aggregates. The particle:PFU ratio was much lower, averaging 38. An average of 5×10^6 particles and 1.4×10^5 PFU was equivalent to one hemagglutination unit. These values are 5-10 fold less than those found for whole, uncentrifuged virus suspensions.

Similar experiments were done also with other supernatant fluids obtained from infected cultures when CPE was extensive and a large number of cells had come free from the glass. Counts and titrations were made on this and on supernatant fluids after low speed centrifugation. Table V shows the results of 2 experiments. Low speed centrifugation removed 83-92% of the total number of virus particles, the majority of which were obviously in large aggregates. Fortunately the enzyme and sonic treatment of these uncentrifuged suspensions reduces these aggregates to sizes sufficiently small for counting. It was not uncommon to see aggregates of 50-100 particles on microscopic examination of these preparations, however. It is apparent, then, that the infectivity and hemagglutination titers of these preparations were not significantly reduced by low speed centrifugation, although the particle concentration was greatly reduced.

Discussion. A relatively simple, precise technic for counting polyoma virus particles in tissue culture fluids heavily contaminated with cell debris is described. At least 3 morphologic types of structures were observed: (a) spherical, electron dense particles, (b) flat, electron transparent "doughnut" forms, and (c) filaments. We have consistently observed filamentous structures in high titer material. The diameter of the filaments is very nearly that of the spherical particles. Occasionally a filament was seen with internal structure, apparently segmenting into spherical particles. We agree with Howatson and Almeida(5) that these are

TABLE III. Particle Count—Infectivity—Hemagglutination Activity Relationships; Uncentrifuged Virus Suspensions.

Sample No.	Particles/ml ($\times 10^{10}$)	PFU/ml ($\times 10^{8}$)	HA units/ml ($\times 10^2$)	Particles/PFU	Particles/HA unit ($\times 10^7$)	PFU/HA unit ($\times 10^5$)
1-4	4.1	2.3	8	180	5	3
2-5	4.7	3.3	9.6	140	5	3
3-6	5.3	3.8	8	140	7	5
4-8	4.8	1.4	4	340	12	4
Avg	—	—	—	200	7	4

TABLE IV. Particle Count—Infectivity—Hemagglutination Activity Relationships; Supernatant Virus after Low Speed Centrifugation.

Sample No.	Particles/ml ($\times 10^9$)	PFU/ml ($\times 10^7$)	HA units/ml ($\times 10^2$)	Particles/PFU	Particles/HA unit ($\times 10^6$)	PFU/HA unit ($\times 10^4$)
1- 7	1.1	2.0	1.6	55	7	12
2- 8	2.4	6.4	8.0	38	3	8
3-10	3.6	12	8.0	30	5	15
4-12	4.4	15	8.0	30	6	19
Avg	—	—	—	38	5	14

TABLE V. Particle Count — Infectivity — Hemagglutination Activity Relationships Using Whole, Uncentrifuged Virus Suspensions and Their Supernatants after Low Speed Centrifugation.

Sample No.	Preparation	Particles/ml ($\times 10^9$)	PFU/ml ($\times 10^7$)	HA units/ml ($\times 10^3$)	Particles/PFU	Particles/HA unit ($\times 10^6$)
216	Whole	63	15	3.8	420	20
	Supernatant	4.7	10	2.6	47	2
217	Whole	24	12	5.1	200	5
	Supernatant	4.1	11	5.1	37	.8

probably virus or virus related structures because we have never seen them in cultures not infected with polyoma virus.

There was a consistent correlation between particle counts and biological titrations of similar materials. Extensively aggregated, unsedimented materials were characterized by high particle:infectivity and high particle:hemagglutinating unit ratios, as would be expected. With virus suspensions from which gross aggregates were removed by low speed centrifugation, much lower ratios were obtained. Experiments are under way to find ways to de-aggregate polyoma virus without causing a loss in biological activity. Apparently large aggregates play no detectable role in the infectivity and hemagglutination activity of polyoma virus suspensions although as much as 92% of the virus may be in this form. Since infectivity measurements do not detect 90% of virus mass in such preparations, particle counting is probably a more dependable measure of total virus production.

Efforts are being made to increase the sensitivity of particle counting so that dependable counts may be made when the virus is in very low concentrations relative to cell debris. We are continuing the study of this virus-cell relationship with both biological and physical methods in the hope that new information can be obtained.

Summary. A method is described for quantitative counting of polyoma virus particles with a precision of about 16%. The method involves sedimentation of virus upon agar in the swinging-bucket ultracentrifuge rotor, pseudoreplication, and electron microscopy of the particles. The particle counts were correlated quantitatively with biological activity. Aggregation is an outstanding feature of this strain of polyoma virus. When this was minimized, a particle count to infectivity ratio of 38:1 was obtained. Approximately 5×10^6 particles were equivalent to 1 hemagglutinating unit.

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