suspension cultures at pH 7.4, it was found that the cells had to adjust the medium to a lower pH value before they could begin logarithmic growth. For medium containing 20% serum, this level was pH 7. The pH then remained rather constant during the remainder of the logarithmic growth phase and dropped more rapidly as the cells entered the stationary phase. This phenomenon of adjustment of environmental pH by bacterial populations has been adequately described by Gale(12). The relationship of this adjustment to duration of the lag period was illustrated by the fact that no lag occurred in cultures started at pH 7. It was further noted that one of the possible roles of serum in the medium was to act as a buffering agent. In this regard it was of interest to note that the level at which the pH stabilized during logarithmic growth was directly proportional to the serum concentration.

Summary. The agitated culture has been used in studying the growth cycle of strain L (Earle) mouse fibroblasts. From data available, it is seen that mammalian cells growing *in vitro* display a growth pattern quite similar to that of other microbiological populations. Some points which have been studied and can now be evaluated are the following: 1) the lag period for these cells appeared to be eliminated completely when log-phase cells were used as inoculum in media equilibrated to pH 7, 2) growth rate appeared to be independent of the inoculum size and also independent of serum concentration, 3) generation time for these connective tissue cells, under conditions studied, was approximately 40 hours; 2 generations could usually be observed in these cultures and saturation concentration in some instances reached 2,000,000 cells per ml, 4) steady-state cultivation of tissue cells has been approached. The results indicated that populations could be maintained in such a manner, and this problem will be investigated.

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Equivalence between Vaccinia Particles Counted by Electron Microscopy and Infectious Units of the Virus. (22621)

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In earlier studies on the relation of the number of vaccinia virus particles to the infectious unit variable results were obtained (1-3). The lowest average ratio of particles to infectious units heretofore reported is 4.2:1(3).

Three features of experiments relating number of virus particles to the infectious unit are most important. Clearly, the method of virus titration to obtain the number of infectious units must be highly sensitive and reproducible. Burnet showed that infectivity of vaccinia virus suspensions could be determined by counting the pocks produced by the virus on the chorioallantoic membrane of the embryonated egg(4). Such titrations have the advantage of expressing infectivity in terms of pock-forming units rather than re-

quiring the calculation of a 50% end point. Moreover, conditions have been defined under which such titrations give precise results(5). Secondly, inactivation of virus particles during preparatory procedures should be avoided. This is readily achieved with vaccinia virus because the infectivity of this virus is relatively stable. Thirdly, it is mandatory to distinguish between the virus particles *per se* and fragments of tissue which might be confused with the virus. The electron microscope provides magnification sufficient for positive identification of the characteristic brick-like shape of vaccinia virus and minimizes the possibility of confusing the virus with tissue debris. The present paper reports results obtained when virus particle counts made in the electron microscope were compared to infectivity titrations made on the chorioallantoic membrane of the embryonated egg.

Materials and methods. Virus. Vaccinia virus, initially a calf lymph preparation, obtained from the New York City Department of Health, was passed on the chorioallantoic membrane of the embryonated egg for a total of 8 passages. Passages were made by the inoculation of 0.2 ml containing approximately 1×10^4 pock-forming units (PFU) of stock virus onto the chorioallantoic membrane of 12-day-old eggs. After 3 days of incubation, the infected membranes were removed, weighed, and buffered saline containing 5% inactivated normal horse serum was added to make a 10% suspension. Membranes were homogenized in a blendor and the suspension cleared in an International horizontal centrifuge at 1500 rpm for 10 minutes. The opalescent supernate was stored at —60°C.

Vaccinia virus titrations. The method used for titrations of vaccinia virus has been described previously(5,6). In brief, quarter-log dilutions of stock virus were inoculated onto the chorioallantoic membrane of 12-day-old embryonated eggs and pocks were counted after 3 days of incubation. Inocula were diluted so that 3-20 pocks would be produced per membrane over a 10-fold dilution range. In the calculation of the virus titer, the pock counts at each dilution were averaged for groups of 5 membranes. These numbers were then multiplied by their dilution factors, and this figure was converted to PFUs per ml. The standard deviation of pock counts with this technic was approximately 20%.

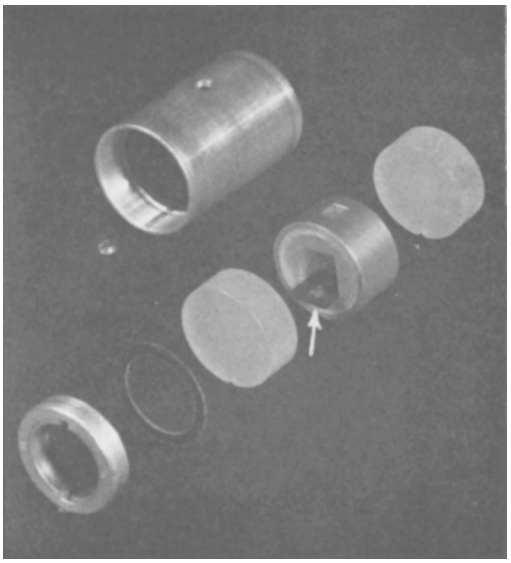

FIG. 1. Centrifugation cell as proposed by Sharp (7) and built by the Specialized Instruments Corp. The present modification includes a stainless steel slide which fits in the trapezoidal chamber and to which are affixed the collodion-coated copper grids (arrow).

Polystyrene latex (PSL). The PSL used in all the following experiments was from Dow Chemical Co., Lot LS-057A. This suspension had a dry weight of 10.1%, particle density of 1.052 and in 577 visual measurements had a diameter of 264 mμ with a standard deviation of 2.3%. The original suspension was diluted 1:20 in double distilled water to make stock suspension No. 1. Over a period of 5 months, 9 dry weight determinations by a micro-gravimetric method gave an average of 5.32 mg/ml. Stock No. 2 represented a 1:100 dilution of No. 1, and Stock No. 3 represented a 1:10,000 dilution of No. 1. On the basis of the dry weight determinations, Stock PSL No. 3 contained 5.25×10^7 particles/ml.

Electron microscope preparations. A modification of the method described by Sharp(7) was used. Special cells designed to fit in a 4-hole analytical rotor of a Spinco Model L centrifuge were employed. The essential parts of the cell are shown in Fig. 1 and are similar to those described by Sharp except

TABLE I. Recovery of PSL in Modified Centrifugation Cell.

Centrifugation run	Enumeration of PSL* by electron microscopy: Fields counted	Mean count per field†	PSL per ml
1	12	56.7	4.8×10^7
2	15	58.5	4.9 "
3	12	61.5	5.1 "
4	21	55.5	4.7 "

Mean No. of PSL particles/ml by count = 4.9×10^7

No. of PSL particles/ml determined from dry wt = 5.25×10^7

% recovery (dry wt = 100%) = 93.3%

* PSL = Polystyrene latex.

† Counts adjusted to an area of uniform size (see text).

for the bottom metal slide on which collodion-coated electron microscope grids are affixed with glue.* Fluid to be examined was injected into the assembled cells. The precise final volume in the cell was determined gravimetrically. The particles were sedimented directly onto the collodion-coated wire mesh. After the run the grids were removed from the slide, shadowed, and examined in the electron microscope.

Results. Before undertaking studies with the virus, it was important to determine whether, with the cell modification described, accurate particle counts could be obtained after sedimentation. Calculations indicated that sedimentation of PSL would be completed after centrifugation at 6100 g for 23.4 minutes. However, for additional certainty runs were made at 15,750 g for 45 minutes.

In Table I are presented the results of 4 runs with PSL stock suspension No. 3. In each run 12-21 fields were photographed at random at the approximate magnification of 10,000. The precise magnification was determined for each photograph on the basis of the value of 264 mμ for the diameter of PSL particles, and the area of the field photographed was then calculated. It is apparent from the recovery values of PSL sedimented directly onto the collodion-coated screen that the method employed was as accurate as the methods used by Sharp(8). In the present study recovery was 4.9×10^7 by count with a suspension which contained 5.25×10^7 particles per ml by dry weight. Many fields were examined on the microscope screen to determine distribution of the PSL particles. Distribution appeared to be uniform in all areas. Analyzed statistically, the variation in the individual counts was indicated by the standard deviations of 14, 20, 16, and 54% for runs 1-4 respectively.

Since these results indicated that this simplified method of making electron microscope preparations gave reproducible results with the PSL particles, experiments were designed to adapt this to vaccinia virus suspensions. The 2 stock vaccinia preparations (1 and 2) were titered for infectivity on the chorioallantoic membrane of embryonated eggs. The results are presented in Table II.

Various methods were used in attempts to purify the virus, but although considerable purification could be accomplished as far as obtaining the virus free of debris, loss of virus was great enough to make the methods impractical for present purposes. The following procedure was therefore adopted. The opalescent 10% suspension was diluted 1:100 by quantitively pipetting 1 ml into a 100 ml flask to which was added 1 ml of PSL Stock No. 2 and double distilled water to a volume of 100 ml. This suspension was then used to fill the chambers of the centrifugation cells without further treatment. These preparations contained debris, but due primarily to the unusual shape of vaccinia virus, counts were possible. Moreover, losses of virus particles and inactivation of infectivity were avoided. As with the PSL, the virus preparations were centrifuged at 15,750 g for 45 minutes, a force

TABLE II. Titrations of Vaccinia Virus Preparations on Chorioallantoic Membrane of Embryonated Eggs.

Vaccinia virus	PFU's/ml*	Mean
Prep. 1	3.3×10^9	3.2×10^9
	3.5 "	
	2.7 "	
Prep. 2	1.4 "	1.4×10^9
	2.0 "	
	.8 "	

* Pock-forming units.

* Scotch masking tape No. 202 was placed in xylol. The solution of adhesive material thus obtained was used.

TABLE III. Numerical Relation between Pock-Forming Units and Virus Particles.

Vaccinia virus	Enumeration of vaccinia virus particles by electron microscopy: Fields counted	Mean count per field	Particles per ml	PFU's/ml* by egg titration	Ratio, PFU's/particles
Prep. 1	20	36.4	2.9×10^9	3.2×10^9	1.1
Prep. 2					
Exp. 1	16	13.0	1.0 "	1.4 "	1.4
" 2	23	8.5	.7 "	1.4 "	2.0
				Mean ratio, PFU's/particles = 1.5	

* Pock-forming units per ml.

20 times greater than that needed to sediment the virus, calculated on the basis of a sedimentation constant of 4910 S. PSL was included for precise determination of magnification.

Table III gives the results of these experiments. In all experiments counts were made on photographs rather than on the electron microscope screen. After the grid was placed in the microscope, the first field brought into focus was photographed and thereafter one field of each presenting square was photographed. Concentration of virus particles in the suspensions used was determined on the basis of the number seen per area of the grid and the volume of the fluid in the cell measured gravimetrically (Table III). Concentration of virus particles was also determined on the basis of the ratio of virus to PSL particles in the fields examined and the known number of PSL particles per ml ascertained gravimetrically. The results obtained with the 2 procedures were in good agreement.

It is apparent from the results obtained that the ratio of infectious units to virus particles was very nearly 1:1. Preparation 1

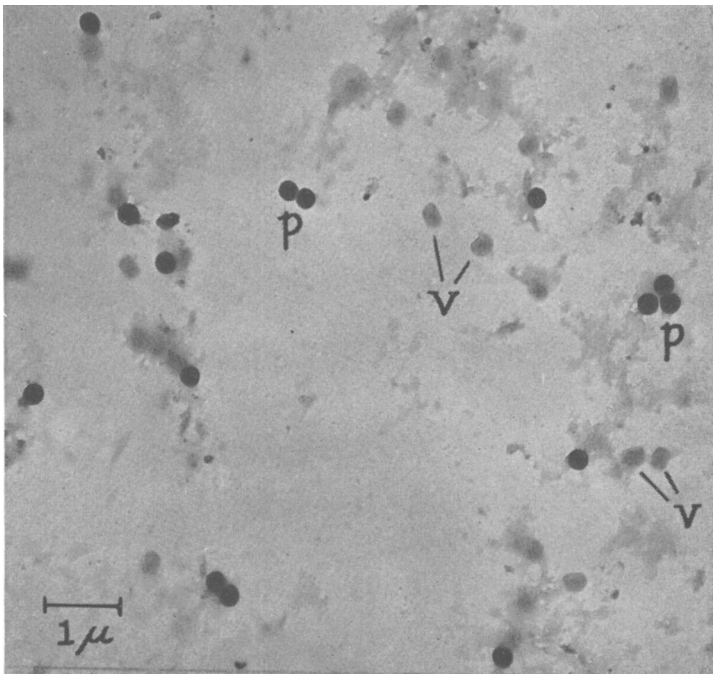

FIG. 2. Vaccinia virus stock diluted 1:100 with distilled water to which PSL was added as an internal control of magnification. The preparation was lightly shadowed with chromium. This field is representative of those obtained with Prep. 2, Exp. 2. P = PSL; V = vaccinia virus.

contained more virus than Preparation 2, both by particle counts and infectivity titrations.

As would be expected, there was considerable variation in the amount of debris from field to field. Fig. 2 is a representative field obtained with Preparation 2. In this experiment virus particles were specially well delineated. Fields containing considerable amounts of debris still left the particles discernible in most instances in this as well as other experiments. No large clumps of virus were found although in some instances groups of 2 or 3 particles were present. No particular association of the particles with debris was noted, and no adsorption of the virus onto the polystyrene balls was observed. Statistical analysis of counts with Preparation 1 showed a standard deviation of 39%. Runs with Preparation 2 gave standard deviations of 23 and 31% respectively for the 2 experiments.

Discussion. The present studies indicate that under the conditions described a single vaccinia virus particle initiates infection on the ectodermal surface of the chorioallantoic membrane of embryonated chicken eggs. There is every probability that under various experimental conditions the ratio of virus particles to infectious unit can change, as indicated by Parker(9). For example, use of a rabbit strain of vaccinia titered for infectivity on the chorioallantoic membrane of the egg may give much higher particle/infectious unit ratios than would the fully egg-adapted strain used in the present experiments. The advantage of the present modification of Sharp's method is mainly that of simplicity and speed. Only a few minutes are required to mount the coated grids on the metal slide, insert this into the cell and make the run. At the end of the run, the grids are ready for shadowing and examination. There is some question as to whether experiments of the type done with vaccinia can be adapted to other animal viruses unless considerable purification of the virus is accomplished first. The shape and density of vaccinia make it difficult to confuse vaccinia virus particles with normal tissue components and make the particles distinguishable even when they are enmeshed in tissue debris. Experiments in this laboratory with influenza viruses have demonstrated the difficulty of working with unpurified suspensions when the virus particle in question is a sphere without other distinguishing characteristics. Work with purified virus suspensions utilizing the "spray-gun" technic has yielded information on the relationship between infectious units and virus particles with bacteriophage(10), influenza virus(11), and poliomyelitis virus(12).

Summary. 1. Studies of the relation between the number of infectious or pock-forming units of vaccinia virus and the number of virus particles indicate a ratio of 1.5:1 under conditions described. 2. A modified centrifugation cell is described with which virus suspensions can be concentrated and the particles then counted by electron microscopy. The virus particles are sedimented directly onto coated electron microscope grids which when removed from the centrifugation cell are ready for shadow-casting and examination. The precision of this method is comparable to that obtained by other technics.

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