and reinjected a few minutes later without difficulty. The newborn mouse easily tolerates the removal of .025 ml of blood or the injection of .05 ml of cell suspension.

There are several advantages that this technic provides over the intravenous route. One person can perform the operations without the aid of an assistant, and the inexperienced injector can become quite proficient after the first few attempts. Because of the size of the heart and the transparency of the skin, no magnification is required as with intravenous injections. There are no post-injection hematomas, and there is no need to apply pressure after withdrawal of the needle.

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Enumeration of Vaccinia Virus Particles in Crude Extracts of Infected Tissues by Electron Microscopy.* (24366)

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A previous report indicated that accurate counts of vaccinia virus particles could be made in unpurified suspensions by diluting the crude 10% extract 1:100 and sedimenting the virus out of the diluted sample onto a collodion coated grid. Counts were then made in the electron microscope(1). It was felt that, even in the presence of much non-viral debris, vaccinia particles could be discerned clearly enough for quantitative determinations. Other studies, however, have questioned the validity of this general procedure(2). The question is whether there is some dilution range of the crude extract in which replicate counts of virus are feasible and in which counts are linear with dilution. The present paper analyzes a large number of vaccinia virus counts performed on crude 10% extracts of virus-infected chorioallantoic membrane and rabbit skin tissues utilizing count-dilution curves and demonstrates that accurate and reproducible virus particle counts are obtainable in these non-purified suspensions.

Materials and methods. Virus and virus extracts. Vaccinia virus was obtained from North Carolina Dept. of Health as a calf lymph suspension and maintained in this laboratory by passage on chorioallantoic membrane of 12-day-old embryonated eggs and on skin of rabbits. Egg membranes were removed 66 hr after inoculation and areas showing confluent vaccinia lesions were cut out and extracted in glass tissue grinder with 5% horse serum in normal saline. The extract was then centrifuged at 1600 rpm for 5 minutes. The supernate was removed, divided in aliquot parts and stored at –60°C. The egg membrane virus used was the 5th egg passage of the original calf lymph suspension. Rabbit skin passages were begun with this 5th passage egg membrane virus and harvests made as described by Parker and Rivers(3). After the pulp was extracted to 10% by weight with 5% horse serum in saline in glass tissue grinder, this extract was then centrifuged at 2600 rpm for 10 minutes. The supernate was then removed, divided into aliquot samples and stored at –60°C. *Virus particle counts.* Tubes containing the 10% vaccinia virus extracts were processed for counting immediately after thawing and thawed samples were never refrozen. If a small precipitate was present in the thawed sample, the tube was

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shaken by hand to disperse it but no further centrifugation was done. Particle counts were made by agar sedimentation method of Sharp(4,5) and the diluted samples were sedimented in specially designed Servall-Type SU counting rotor in a Servall refrigerated centrifuge. Fig. 1 shows this 8-cell rotor with details of one of the cells shown in exploded form. Diluent for adjusting the crude virus suspension to concentrations convenient for counting was 0.005 M phosphate buffer, pH 7.0. In experiments where dilution greater than 1:300 was necessary bovine serum albumin was added to make a 0.1% solution. Electron micrographs were made from lightly shadowed pseudoreplicas with an RCA model EMU3 electron microscope. All photographs were made at magnification of 1708x on glass plates after the field had been chosen by randomly selecting the center portion of each grid for the photograph. Negatives were then projected at a 6x magnification onto ruled white cardboard for particle counting. Each datum represents average number of virus particles in 5 such negatives. The number, N, of virus particles/ml is calculated from the radial height (h = 1.3 cm) of the trapezoidal cell chamber, thickness, t, of agar, magnification (M) and area (A) of negative projected for the count, and number (n) of particles counted in that area. The following equation was used: $N = \frac{n M^2}{0.91 A(h-t)}$. Spray droplet counts were made by the method of Backus and Williams(6). Semi-purification of vaccinia virus for spray experiments was accomplished as follows: Vaccinia virus infected egg chorioallantoic membranes weighing 1.5 g were ground and extracted to 20% by weight. This extract was then centrifuged at 1600 rpm for 5 minutes, the supernate removed and stored at 4°C for 12 hours. After this interval the material was again centrifuged at 1600 rpm for 5 minutes and the supernate removed. The supernate was then centrifuged at 11,000 g for 30 minutes in an angle centrifuge and the pellet resuspended in saline containing 5% horse serum. After resuspension a final centrifugation was done at 1000 rpm for 1 minute and the supernate removed for immediate use. Polystyrene latex (Dow lot 580G) was added to give 6.09 x 10^9 latex spheres per ml.

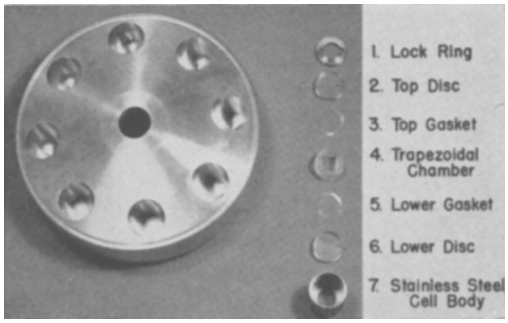
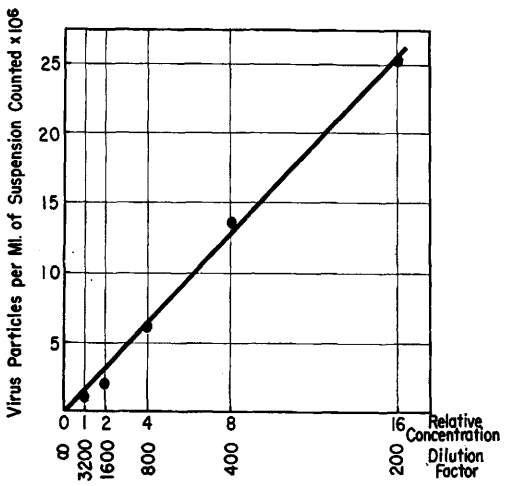


FIG. 1. Special virus-counting centrifuge rotor showing one of the cells with parts arranged in order of assembly. Eight cells (8 counts) can be run at one time and a spun steel cover (not shown) reduces air turbulance when rotor operates in a standard refrigerated centrifuge.

Results. Relation of virus particle counts to dilution of sample. Two experiments were performed to learn the dependence of count upon dilution of crude virus extract and to establish, for these particular materials, optimal dilution for virus particle counting. The results of a series of crude egg membrane extract and crude rabbit skin extract are shown in Figs. 2 and 3 respectively. The egg membrane extract which had less non-viral debris of the two, gave countable pictures in dilution as concentrated as 1:200 but not at 1:100. Linearity of the count plotted against dilution factor was good. Dilution of 1:300 was considered optimal for vaccinia virus particle counting of chorioallantoic membrane material extracted to 10% as described in *Materials and methods*. A small portion of one of the particle count photographs is shown in Fig. 4.

Similar results were obtained with 10% extracts of the scraped pulp from vaccinia virus-infected rabbit skin except that somewhat greater dilution was necessary before foreign material and virus particles were dispersed sufficiently for reliable and reproducible counting. The dilution range of this crude extract, (Fig. 3), is 1:400 to 1:2000 and in this range the counts are linear with dilution. The dilution value 1:666 (ref. Fig. 3) was considered to be optimal for vaccinia virus counts of rabbit skin extracts. Fig. 5 shows



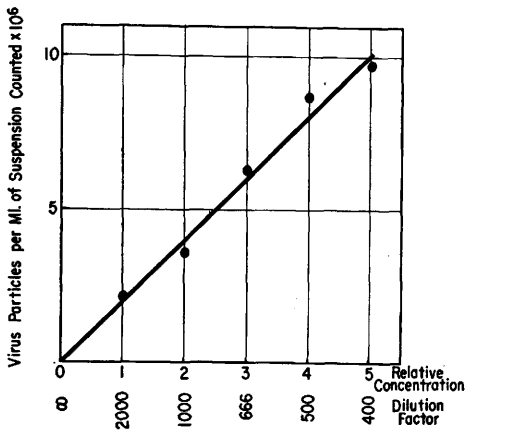


FIG. 2 (top). Dependence of agar sedimentation count of vaccinia virus particles on dilution of a 10% extract of infected chorioallantoic membrane.

FIG. 3 (bottom). Dependence of agar sedimentation count of vaccinia virus on dilution of a 10% extract of scraped pulp from virus-infected rabbit skin. Point at dilution 1:666 is average of the 7 counts shown in Table II.

a small portion of a field counted at this particular dilution.

It is important to note that the photographs show neither the best nor the worst portions of the many fields counted. They do depict, however, an average representation of amount of debris present in most samples and illustrate that this particular virus can be distinguished in fields containing a large amount of non-viral material. The debris in both egg membrane and rabbit skin extracts bears no relationship to the distribution of the virus particles, as previously reported by Overman and Tamm(1), and there was no evidence of virus adsorption onto any of the particulate material. Some viral aggregation occurred but individual particles could be counted in each group and the groups were small and numerous enough to be well distributed over each set of 5 pictures. In some early runs, uncountable preparations appeared to result from allowing thawed extracts to remain at 4°C for prolonged periods before diluting and counting. This practice was discontinued and all counts thereafter were made immediately after the extract was thawed.

Replicate virus particle counts on egg membrane and rabbit skin extracts. Using the data obtained in the previous experiments for optimal counting dilutions of egg membrane and rabbit skin extracts, experiments were designed to determine degree to which replicate counts on aliquot samples of the same 10%

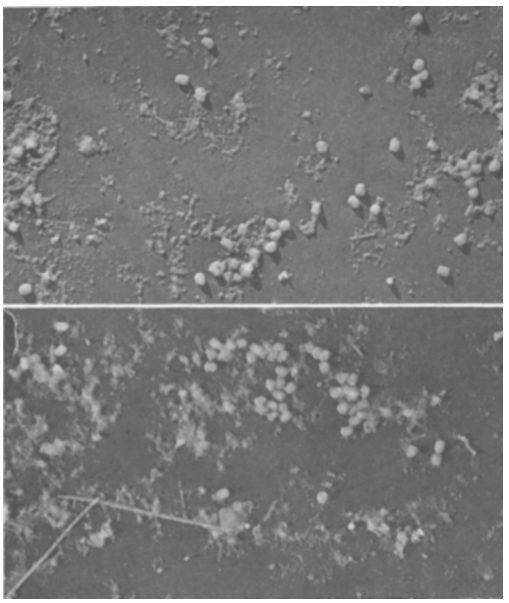


FIG. 4. (top). Vaccinia virus (unpurified). One-ninth of the area of a single electron micrograph of 10% suspension of chorioallantoic membrane taken at 1708 × magnification and photographically enlarged to 10,000 ×. Typical foreign material and particle groupings are visible. Each count in the tabulated data is total count from 45 times the area shown here. Sample size was 1:300 ml and the calculated count for the 10% suspension was 7.6×10^9 per ml.

FIG. 5 (bottom). Vaccinia virus (unpurified). 10% rabbit skin suspension, 1:666 ml sample was used and calculated count was 4.2×10^9 per ml of the 10% suspension. Other data the same as for Fig. 4.

TABLE I. Vaccinia Virus Particle Counts in Dilutions of Aliquot Samples of 10% Extracts of Virus-Infected Chorioallantoic Membranes.

10% membrane extract, 5th passage			
1st series		2nd series	
Particles per photo at 1:300 dilution*	Calculated particles per ml of 10% ext.	Particles per photo at 1:300 dilution*	Calculated particles per ml of 10% ext.
192	6.2×10^9	229	8.1×10^9
142	6.0	197	7.5
157	6.6	182	6.9
157	6.6	222	8.4
192	8.1	189	7.2
185	7.8	173	6.6
149	6.3	206	7.8
157	6.6	172	6.3
221	9.3	218	8.1
171	7.2	232	8.7
185	7.8	207	7.8
Mean	$= 7.14 \times 10^9$	Mean	$= 7.6 \times 10^9$
Stand. dev.	$= 1.0 \times 10^9$	Stand. dev.	$= 0.84 \times 10^9$
	$= 14\%$		$= 11\%$

* Avg No. of particles in 5 photographs.

crude extract could be expected to vary. Each count represents a separate centrifugation cell run. Results of replicate counts on egg membrane passaged virus are presented in Table I. Using different lots of calf lymph virus as starting material 2 series of passages were made at different times and, for the present study, the selection of 5th passage of each series was purely arbitrary. Variation among aliquot samples of 5th passage of the first series ranged from 6.0 to 9.3 x 10^9 particles/ml in the 10% extract and in the 5th passage of the second series the counts ranged from 6.3 to 8.7 x 10^9 particles/ml. Standard deviations of the 2 series were 14% and 11%. It is apparent, therefore, that not only is the reproducibility of the counts quite good but also that in 2 different passage series total virus particle counts were remarkably similar. As an additional check on reproducibility of the method another tube of 5th passage material from each series was thawed and counted about 1 month after above experiments had been completed. The second count of series 1, 5th passage, gave 7.6 x 10^9 particles as contrasted to 7.1 x 10^9 for the 11 replicate counts. With material from series 2, 5th passage, the repeat count was 7.0 x 10^9 as compared with 7.6 x 10^9 for the replicate counts.

Since rabbit skin material appeared to contain more debris than egg membrane extracts it was felt that the standard deviation in replicate counting of this material might be somewhat greater. The results of 7 replicate counts on vaccinia virus rabbit skin pulp extracted to 10% are presented in Table II. Interestingly, the mean of these counts of 4.2 x 10^9 had a standard deviation of only 10%. This may mean that although certain extracts contain more debris than others, this factor merely limits the countable dilution range and does not *per se* make the total count at optimal dilution less reproducible.

Comparison of spray and sedimentation methods for particle counting of vaccinia virus. To check the overall accuracy of agar sedimentation counting method for vaccinia virus, a preparation was made suitable for counting also by the spray droplet method. For such a study it was necessary to concentrate and semi-purify a vaccinia virus suspension to contain 10^{10} virus particles/ml or greater for spray gun experiments. This was done as described in *Materials and methods.* The resulting virus particle counts by the 2 methods are summarized in Table III. The results obtained on the same material by these 2 different procedures are in good agreement.

Discussion. As previously reported(1), vaccinia virus particle counts can be made directly on 10% extracts of the infected tissues by adjusting dilution of the extract to a point which gives minimum non-viral debris yet retaining sufficient numbers of virus particles for significant counts. The present method utilized pseudoreplicas of the sedimented virus

TABLE II. Vaccinia Virus Particle Counts in Dilutions of Aliquot Samples of a 10% Extract of Virus-Infected Rabbit Skin.

Particles/photo at 1:666 dilution of 10% ext.*	Calculated particles/ml of 10% ext.
53	4.5×10^9
49	4.1
41	3.5
51	4.3
49	4.0
56	4.7
53	4.5
Mean	$= 4.2 \times 10^9$
Stand. dev.	$= 10.0\%$

* Avg No. of particles in 5 photographs.

TABLE III. Comparison of Vaccinia Virus Particle Counts by Agar Sedimentation and Spray Droplet Methods on the Same Concentrate.

	Method	
	Agar sedimentation	Spray droplet
Dilution of virus concentrate	2313	4.05
Latex particles/ml mixture		6.09×10^9
Total virus particles counted	4358*	833†
Ratio: Virus/latex		4.1
Calculated count for concentrate	1.04×10^{11}	1.01×10^{11}

* Fifteen micrographs at a magnification of 1708× were counted with an avg of 291 particles per photograph.

† Fifteen of the largest droplets with an avg of 555 particles/droplet.

rather than the direct sedimentation described previously but this was a matter of convenience and our other experiments with the direct method show it to be a reliable and reproducible technic.

Certain minimum conditions are required for vaccinia virus counting of the type described and certainly one of these is familiarity with the morphologic variation exhibited by this virus and well described by Peters(7). A major part of the foreign material present in these extracts does not sediment and is eliminated with the supernate. The extraneous particulate matter which does appear on the collodion replicate may obscure virus if it is too thick so that degree of dilution for counting is critical. However, non-viral material in properly diluted samples is readily distinguished from the virus.

It is not possible to show pictures adequately illustrating the problems encountered in these studies. To portray 200 virus particles/photograph from suspension containing only about 5×10^9 particles/ml it is necessary to operate the electron microscope at very low power. These negatives are then enlarged by projecting them on ruled white cardboard making an area of projection approximately 1 ft. square. Thus, for reasons of space, the photographs which illustrate this paper represent only small portions of the areas actually counted. In more recent studies the negatives have been projected on a screen as one would project any slide or photographic transparency.

Finally, it is important to emphasize that the data here described apply to only one virus, vaccinia. Both authors have attempted particle counts of various viruses in unpurified and semi-purified suspensions and have encountered difficulties associated with ease of recognition of the particular virus particle in its particular environment.

Summary. Vaccinia virus particles have been counted accurately and reproducibly in dilutions of 10% extracts of infected egg chorioallantoic membrane and rabbit skin by the agar sedimentation method. Standard deviation of these counts ranged from 10 to 14%. Comparison with concentrated, purified vaccinia virus, of counting by sedimentation and spray droplet methods gave results which are in excellent agreement. Various factors important in the accurate counting of vaccinia virus in crude tissue extracts are discussed.

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