Completely Dispersed Suspensions of Vaccinia Virus Particles.* (29506)

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The reaction of a culture of cells to inoculation with a suspension of virus particles is critically dependent upon the relative numbers of particles and cells. Counting of both is necessary if quantitative evaluation of the result is to be made. The ratio thus established is usually called the multiplicity but this number represents the average number of virus particles reaching each cell *only* when there is complete dispersion of both cells and virus particles. Substantial aggregation is usually present in preparations of vaccinia virus(1,2,3) as well as in those of other viruses(7,8) and much of it usually remains even after such dispersive treatments as the application of sonic waves.

The present paper describes the production of suspensions of vaccinia virus particles without any aggregation. It provides also, some data on the stability of the dispersed state. This is in terms of reaggregation rates which allow estimation of the useful life of these suspensions.

Materials and methods. The vaccinia virus (WR mouse neurotropic strain from the American Type Culture Collection), its adaptation to and its culture in Earle's L cells have been fully described elsewhere(1). Stock virus was prepared from sealed slant cultures 72 hours after infection. About one million cells in one ml of complete growth medium were treated for 3 minutes with 9 KC sonic waves, as described in the reference above, to release and partially disperse the virus. The particles were counted and found to be 1.1×10^{10} VP/ml. The preparation was then frozen at $-20°$ for 3 days awaiting the experiment.

Particle counting was done by sedimenting a dilute suspension of virus in a specially constructed centrifuge rotor (Type SU, Ivan Sorvall, Inc., Norwalk, Conn.) upon an agar surface from which pseudoreplicas were made for study in the electron microscope(5). Total particle counts were made and the numbers of groups in pairs, triplets, etc. were recorded as well. All dilutions of the stock virus were made with 0.005 M phosphate buffer at pH 7.3. Allowances were made for the chance sedimentation of one free particle upon another. Thus, if the virus were perfectly dispersed a few coincidences of this kind would occur in the pictures such that the observed percentage (f) of free particles can be predicted in terms of the fraction (p) of the total picture area occupied by particles, as follows:

$$\mathrm{Log_e}\ (100/f) = 4p$$

Sharp and Buckingham(6) *Equation 1*

Experimental results. Stock virus was thawed in a water bath at 21°C, agitated vigorously with a capillary pipette and rubber squeeze bulb, then diluted to a concentration of 3×10^7VP/ml, sonic treated 3 minutes and sedimented for preliminary examination of general particle appearance and observation of residual cell debris. Such pictures (Fig. 1) are not optimum for aggregation analysis because there are so many particles present (440 on the $2'' \times 2''$ picture of which this is a part) that the chance superposition of particles is in excess of 10%. They are, however, suitable to portray the general nature of the unpurified virus with which this work was done.

Stock virus was diluted to 1.5×10^9, 1.5×10^8, 1.5×10^7 and 3.0×10^6 VP/ml and each dilution was treated separately 3 minutes with sonic waves. A part of each of the first 2 samples was diluted further to 1.5×10^7 VP/ml and samples of all 4 were sedimented immediately for electron micrography. The remainder of all 4 samples was

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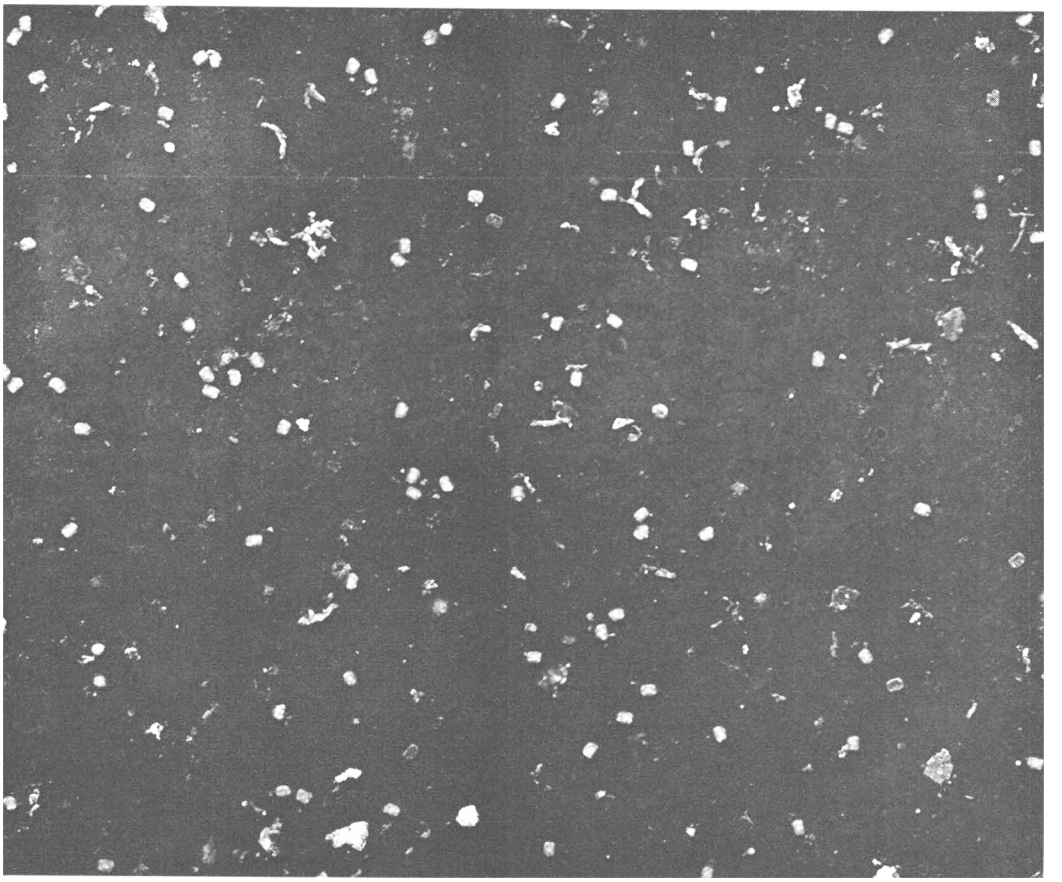

FIG. 1. Unpurified vaccinia virus particles treated with 9 KC sonic waves at a concentration of 3×10^7 VP/ml. This picture shows the relative quantities of virus and debris. Pictures for analysis (Table I) were made at ½ this concentration to reduce the frequency of mechanical coincidences (one particle falling by chance upon another).

kept at 21°C for 1 hour when fresh dilutions of the first 2 were made again and more pictures were taken. After 3 hours similar dilutions were made again from the sonic treated material and a final set of micrographs was taken of all 4 samples. In this way the samples that were sonic treated and held at 1.5×10^9, 1.5×10^8 and 1.5×10^7 were all examined at the same final dilution. Five pictures were made of random fields from each of these 3 preparations. Twenty-five pictures were made of the 3×10^6 VP/ml sample at zero time and 30 at 3 hours.

The collected data in Table I show first that there is little variation among the total particle counts on the 9 samples examined at 1.5×10^7 VP/ml. The average count per picture was 227 particles with a standard error of 11.7. This shows that no significant number of particles was lost or for any reason did not appear in the pictures of the more aggregated preparations.

Virus treated with sonic waves at the high concentrations has more aggregation than that at lower concentrations. Dilution to the same concentration for examination does not obliterate these differences. Reaggregation is also much more rapid at high than it is at low concentrations. The percentages of single particles and of active units (AU)† in the Table are simply the uncorrected counts of particle images in the pictures. A more coherent view of the results can be obtained

† The count of Active Units (1) is the sum of all single particles, pairs, triplets, etc., that are seen in the pictures.

from the graphs on which the data are percentages of single particles, corrected as shown in *Materials and methods,* for superposition. In the first of these (Fig. 2) it is clear that preparations sonic treated at 1.5 $\times$ 10^8 VP/ml or less are not only well dispersed but quite stable. At 1.5 $\times$ 10^9 VP/ml reaggregation is rapid, reducing the percentage of singles from an initial value of 80% to 35% in 3 hours. Sonic treatment at 3 $\times$ 10^6 VP/ml has reduced the aggregation to zero. This result is clearly predicted also by the other data plotted on the graph of Fig. 3 in which the percentage of free particles at zero time for each sample is plotted against the log of the concentration at which it was sonic treated. The result can be expressed as a simple log function of the concentration

$$f = 100 - K \log c/c_o \qquad \textit{Equation 2}$$

The constants of the graph are K = 8.50 and C_o (the extropolated point of maximum

TABLE I. Residual Aggregation Among Unpurified Vaccinia Virus Particles Treated at Several Levels of Concentration, with 9 KC Sonic Waves. Singles and AU refer to the images of particles on pictures. The dispersion attained during treatment as well as that retained at a later time are clearly dependent on concentration.

Virus concentration VP/ml			Virus particles counted									
During sonic treatment	Diluted for electron microscope	Elapsed time (hr)	VP per picture*	Total	No. of groups of 2	3	4	5	6 to 10	11 to 22	% Singles (images)	% AU† (images)
1.5×10^9	1.5×10^7	0	242	1212	111	16	5	2	0	0	75	86
1.5×10^9	1.5×10^7	1	221	1103	114	54	14	10	18	2	41	60
1.5×10^9	1.5×10^7	3	223	1114	86	50	26	15	16	10	33	51
1.5×10^8	1.5×10^7	0	221	1106	63	8	2	1	1	0	83	90
1.5×10^8	1.5×10^7	1	236	1176	70	12	5	1	0	0	83	90
1.5×10^8	1.5×10^7	3	239	1183	103	19	2	1	0	0	77	87
1.5×10^7	1.5×10^7	0	203	1015	45	1	0	0	0	0	91	95
1.5×10^7	1.5×10^7	1	226	1129	54	11	0	0	0	0	88	93
1.5×10^7	1.5×10^7	3	233	1165	63	6	2	0	0	0	87	93
3.0×10^6	3.0×10^6	0	41	1241	7	0	0	0	0	0	99	99
3.0×10^6	3.0×10^6	3	48	1232	17	2	0	0	0	0	97	98

* Avg count per picture (1st 9) 227, $\sigma = 11.7$.

† AU = sum of all groups, including singles.

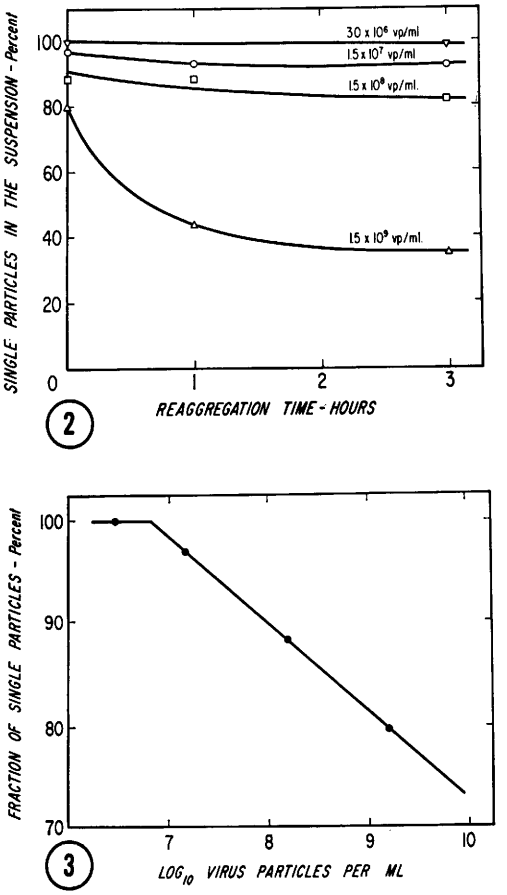

FIG. 2. Reaggregation of vaccinia virus particles after sonic treatment at several concentrations. Percentages of single particles are those of Table I, corrected for mechanical coincidence.

FIG. 3. The fraction of single particles in the virus suspension after a given treatment with 9 KC sonic waves is a linear function of the $\log_{10}$ of the virus particle concentration at time of treatment. Complete dispersion is indicated at 6.3 $\times$ 10^6 and was observed at 6.0 $\times$ 10^6 VP/ml.

concentration which allows 100% dispersion) is 6.31×10^6 VP/ml. The actual dilution at which 100% dispersion was observed (Fig. 3) was 3×10^6 VP/ml.

Discussion. These results provide the first direct evidence that vaccinia virus can be prepared in completely dispersed suspension. It is often assumed that virus preparations are completely dispersed if the plot of the logarithms of the surviving fraction of infectivity forms a straight line with time of irradiation with ultraviolet or X-rays. It can be shown, however, that this criterion is not sufficiently critical to provide evidence that all plaques from such suspensions are the result of uncomplicated single particle action.

The 3 minute time chosen for the sonic wave treatment of samples is not only much less than the time of treatment which produces changes in the titer of well dispersed preparations of this virus(4) but it is such that essentially all the dispersive effect that can be wrought at a given concentration will have been achieved. Thus, each of the points on Fig. 3 probably represents a close approach to equilibrium, at that concentration, between the dispersive forces of the sonic waves and those of reaggregation. The degree of dispersion can be expressed, as we have shown, as a simple log function of virus particle concentration at 6.31×10^6 VP/ml and less. Our observation of 100% free particles was made very near this value (3×10^6 VP/ml) and the dispersion was quite stable for 3 hours (Fig. 2). This period can presumably be greatly increased by addition of protein and/or lowering the temperature (1) thus providing time during which this dispersed virus may be titrated or otherwise used for experimental purposes. One such purpose is evident. Since aggregation is common in virus preparations it is clearly necessary to use only completely dispersed preparations for plaque purification of virus strains. Other uses are in all experiments in which the multiplicity of infection is a critical influence and this covers a large part of laboratory virology.

Demonstration of complete dispersion of a virus preparation constitutes a test of both dispersive measures and analytical procedure. The sedimentation method of virus particle preparation for the count by electron microscopy is peculiarly effective in this work because the particles are sedimented directly from solution and thus they take up positions characteristic of the liquid dispersion, from which they came. Removal of supernatant fluid and drying of the sample obviously do not alter this pattern or such effects would have been revealed by these data. It is possible that methods involving the deposit of virus particles from drops of fluid, either during dialysis through a collodion film or during drying, would produce altered patterns of deposit, micrographs of which would not yield the maximum dispersion of the suspension under study. Only the actual demonstration of complete dispersion as well as the orderly approach to this condition can dispel this doubt regarding the preparation method used for microscopy.

Summary. Analysis by means of the electron microscope of vaccinia virus released from infected L cells and dispersed by sonic waves has shown the percentage of single particles to be a linear function of the log concentration in the region 1.5×10^9 to 1.5×10^7 VP/ml. The extrapolated concentration at which the sonic waves should produce complete dispersion (all single particles) is 6.3×10^6 VP/ml. Actual measurements made at 6.0×10^6 VP/ml showed that it had indeed been achieved. Reaggregation at this concentration was barely perceptible but at the higher concentrations it was quite rapid. Completely dispersed virus is essential in experiments on multiplicity dependent phenomena such as interference and multiplicity reactivation.

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Digital Blood Flow and Distensibility During Immersion in Cold Water.* (29507)

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The decreased distensibility of fingers immersed in ice water has been ascribed to increased venomotor tone(1). However, Edwards and Burton(2) found that there is an increase in volume of fingers immersed in ice water and have suggested that reduction in distensibility may be due to increased venous filling. In an attempt to determine the interrelationships between finger volume, digital distensibility, blood flow and venous pressure, studies have been made at different local and environmental temperatures.

Methods. The experiments were performed on 2 subjects with bath temperatures (T_w) at 5, 9.5, 20 and 38.5°C and room temperature (T_r) of 12, 20 and 28°C. The subjects lay on a cot with the right hand placed in a 27 l water tank. The tank was raised so that when the hand was in position the tips of the fingers were about 15 cm above heart level.

Changes in finger volume were determined by a mercury-in-rubber strain gauge(3,4) compensated for temperature changes(5). The strain gauge was applied to the tip of the middle finger and changes in circumference recorded on a Sanborn polygraph. A cuff wrapped around the root of the finger was intermittently inflated and the final increase in volume at each of 7 cuff pressures observed. Regression equations of change in volume on cuff pressures between 20-50 mm Hg were calculated by the method of least squares. The slopes of the regression lines were taken as indices of distensibility and the calculated cuff pressures at which no changes in finger volume would take place were taken as venous pressure. Blood flow was estimated by observing the rate of change in finger volume immediately after inflation of the cuff to 70 mm Hg.

Results. Distensibility was positively related to both T_w and T_r. However, distensibility appeared to be limited to a maximum at T_w 40°C and a minimum at T_w 5°C so that the effects of changes in T_r were greatest at intermediate bath temperatures (Fig. 1A). Blood flow through the fingertips was minimum at T_w 10 to 20°C and was reduced at all bath temperatures by a reduction in T_r (Fig. 1B). Below T_w 10°C flow increased. Thus an increase in blood flow was associated with an increase in distensibility when T_w was 20 and 40°C but below 20°C an increase in flow was associated with a decrease in distensibility.

Finger volume after 20 minutes' immersion varied inversely with T_w (Fig. 2). The relationship between distensibility, T_r and finger volume is shown in Fig. 3. While distensibility was lower at larger finger volumes, reducing the room temperature resulted in a reduction in distensibility.

Distensibility was inversely related to the calculated venous pressure (Fig. 4). The correlation coefficient was —.88 ($n = 12$) and the relationship between the two did not appear to be altered by changes in T_r.

Discussion and summary. The increased blood flow through the finger during immer-

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