JOURNAL OF VIROLOGY, Mar. 1970, p. 275-281
Vol. 5, No. 3

Spectrum of Physical Properties Among the Virions of a Whole Population of Vaccinia Virus Particles

D. G. SHARP AND PETER M. McGUIRE

Laboratory of Biophysics, Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Received for publication 17 December 1969

Populations of virions released from cultures of L cells infected with vaccinia virus are composed of particles which differ substantially from each other in sedimentation rate and buoyant density. Clumps of two and three virions sediment enough faster than single particles so that fractions containing only singles and others with predominantly pairs can be isolated. The observed velocity range for single particles is much greater than that attributable to diffusion and convection in the centrifuge. Plaquing efficiency is three times higher in a small fraction of the slowest-moving virions than in the major part of the population, even though no size difference can be seen in the electron microscope. Isopycnic densities in potassium tartrate range from 1.15 to 1.23, enough to account for the observed range in velocities. Centrifugation was done in the BXIV zonal rotor at very low virion concentration (less than 10^8 per ml at any point in the spectrum). Virus count and state of aggregation were determined by electron microscopy.

The chemical composition and physical characteristics of vaccinia virus have been determined many times since the early penetrating experiments of Hoagland et al. (4) and Pickels and Smadel (7). The measurements have since been improved; perhaps the purity of the isolated virus has been improved, but one feature of the method has not changed. The process still involves purifying the virus, removing all extraneous material, soluble and particulate, of host or other origin, and then determining the characteristics of this purified fraction. The characteristics found are usually reported as those of vaccinia virus. This is satisfactory only if all of the virions, including those lost in purification, are alike. If they are not all alike, our present data are, at best, averages which give no indication of the range of variability within the population and, at worst, they may be derived from a purified fraction that could be a major part, but might not necessarily include all significant parts, of the original population. The latter possibility becomes increasingly probable as purification methods tend to become more selective, particularly those involving isopycnic or sedimentation velocity banding in a gradient of density.

There is a need for physical and chemical characterization of whole populations of virions. It is quite unlikely that these populations will be composed of identical particles, particularly if they have been passed many generations without cloning. Within such populations, there is doubtless a spectrum of properties, possibly genetic markers, more probably differences traceable to host influences but nevertheless interesting as the spectrum may reveal changes in the composition of populations in response to changes in environment, host, etc.

In this paper are described experiments with the BXIV zonal centrifuge rotor of Anderson (1) and the electron microscope used as a virion counter and means of determining the exact extent of virion aggregation. The Anderson rotor provides a large volume in which all the virions in crude lysates of poxvirus-infected cells can be banded without any concentrating or selective purifying pretreatment and in dilution sufficiently great to minimize the ever-present particle aggregation (6). This paper presents the method, some spectra, and data showing that maximum plaquing efficiency is present in a minor part of the velocity spectrum. Isopycnic density spectra are presented as well. Effects of cloning, continued passage, change of host, etc., upon the physical spectra will be presented in subsequent papers.

MATERIALS AND METHODS

Virus. The virus used was WR (mouse neurotropic) strain of vaccinia carried in this laboratory through 162 passages in L cell cultures. Its plaquing efficiency on L cell monolayers is high; 10% to 20% of the

virions produce plaques, and no evidence of synergistic plaque formation (3) has been observed with it.

Infected cell cultures, incubated 96 to 120 hr to maximum virion yield, were scraped from the glass, treated with 20-KHz sound waves to release the virus from the cells, and frozen at −20 C until used.

Zonal analysis. The BXIV zonal centrifuge rotor was brought up to 1,800 rev/min empty and held at this speed while gradient and virus samples were introduced through tygon tubing of one-eighth inch (3.2 cm, internal diameter) via the running seals. Slightly convex gradients were produced with reagent-grade sucrose, in 650-ml quantities, in 0.005 M phosphate buffer (*p*H 7.0). These were pumped into the rotor, entering at the periphery, dilute end first, until the entire volume was filled. Virus was introduced (15-ml volume containing about 10^{10} virions) by means of a syringe and needle, placing it in the innermost position. In the experiments with 10 to 30% sucrose gradients, the virus was suspended in 5% sucrose made up with phosphate-buffered saline. In later experiments with 15% to 30% gradients, the virus was suspended in 15% sucrose made up with 0.005 M phosphate buffer, *p*H 7.0. A 50-ml volume of buffer was injected afterward, displacing a total 65-ml volume of the denser part of the gradient out through the tubing through which it came in and advancing the virus to occupy a band 1.6 mm thick at the 2.7-cm rotor radius. The running seal with connecting tubing was then removed, and a vacuum-tight cap was put over the opening in the rotor. The centrifuge was evacuated, and the rotor speed was increased smoothly to the desired maximum and held constant until deceleration. Some of the experiments were made with the Spinco model L centrifuge; with it, the rotor was allowed to coast, without brake, down to 1,800 rev/min, when air was admitted to the rotor chamber. In later work with the Sorvall ultracentrifuge, in which there is no gear train to provide frictional drag, a small amount of reverse pressure had to be applied to its fluid drive to decelerate the rotor to unloading speed in about 6 min. Running seal and feed lines were reattached, and the whole contents of the rotor were displaced through the innermost connecting tubing, by pumping in a denser piston fluid through the peripheral connection. Twenty-six bottles (25 ml each) were collected in series in this unloading operation. Densities were determined by 1-ml pycnometer. Virion counts and aggregation analyses were made on suitably diluted samples by the agar pseudoreplica technique of Sharp (6, 8) with the use of the electron microscope. Most methods of preparing suspended virions for electron microscopy will induce some aggregation, which cannot be tolerated here. Sucrose concentrations of 3% (w/v) or less do not interfere with the virion-counting technique, so a maximum dilution of 10 × was needed only at the 30% end of the gradient. Low-count samples were sometimes dialyzed to avoid dilution before counting. The number of single particles and the number of groups in each size category were counted in each of the samples.

Suitable dilutions of the samples were made directly into growth medium 199 and titrated for plaque formation upon monolayers of L cells.

Isopycnic banding of the virus was done in the same way as the velocity experiments, except that the denser gradients required were prepared with potassium tartrate and the centrifuging speed and time were increased.

RESULTS

In a sucrose gradient of 10 to 30% (w/w), optimal conditions for observation of the separate sedimentation rates of single particles, pairs, and triplets were obtained (Fig. 1) by operating the centrifuge 30 min at 10,000 rev/min. A few aggregates of larger size were seen but, of the few that were present in the starting sample, most had doubtless sedimented beyond the region available for observation. The three maxima are clearly observed. There were only single particles in the slowest part of the singles region and even at the peak there were 88% singles. One bottle at the 450-ml level contained 90% pairs (Fig. 2). There is, nevertheless, a broad spreading apparent in all the peaks. This might be due to instability of the aggregates, convection and mixing in the machine (possibly during the unloading of the rotor), or to a physical heterogeneity among the virions.

Inasmuch as separated samples did not change in degree or mode of aggregation, singles in these dilute samples did not quickly aggregate, nor pairs quickly disintegrate into singles, we decided to reduce the steepness of the sucrose gradient further and examine the effect upon the spread of the singles peak only.

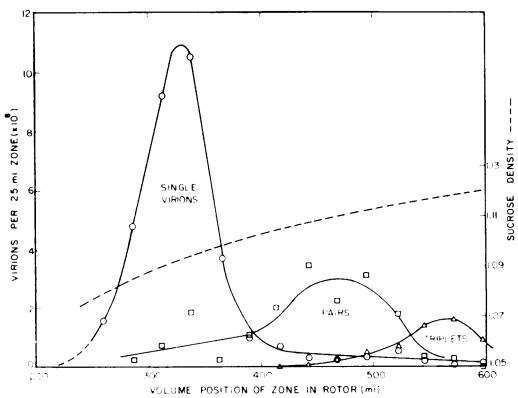

FIG. 1. *Sedimentation velocity spectrum of an unpurified vaccinia virus population at low resolution, showing separate peaks consisting of single virions, aggregated pairs, and triplets moving from left to right in a sucrose gradient. To calculate the number of pairs or triplets, divide the ordinate value by 2 or by 3. The BXIV zonal rotor was held at 10,000 rev/min for 30 min with acceleration and deceleration schedule such that* $\sum\omega^2\Delta t = 2.72 \times 10^9$ *for the whole run. Fluid temperature was 26 C.*

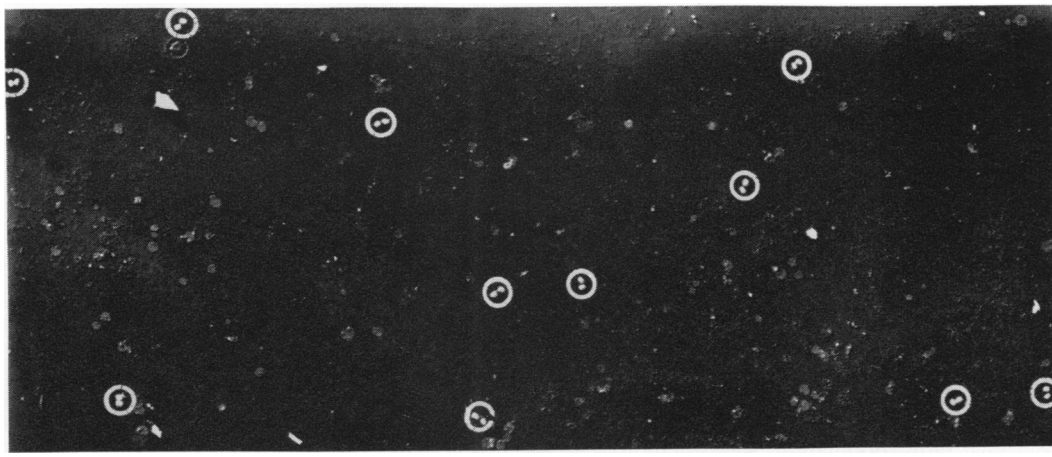

FIG. 2. *Part of one of the count pictures taken from the zone of maximum pairs (Fig. 1). This suspension was prepared for the electron microscope about 1 hr after the separation was made in the gradient, showing the stability of this selected fraction of aggregates. Ninety per cent of the virions in this zone were in pairs.*

In a 15 to 30% (w/w) sucrose gradient with rotor operating at 12,600 rev/min for 20 min, single virions were found in all samples taken from 150 to 600 ml. The distribution was broad and single-peaked at the 325-ml level (Fig. 3), with no evidence of smaller secondary peaks. The spread of the distribution was greater than it was in the previous 10 to 30% gradient experiment. We have been unable to detect any difference in virion size in the electron micrographs used for counting. Particles from the slower and faster zones of the spectrum look alike. Evidence that the spread was not due to mechanical mixing came from isopycnic banding of polystyrene particles obtained from the Dow Chemical Company (Midland, Mich.). These particles, of uniform density (1.05) and average size (1.17 μm diameter), were banded in a 9 to 19% (w/w) sucrose gradient, adjusted in position to produce the band at approximately the same position in the rotor as that where the virus maximum was found (325 ml). When this band was pumped out of the rotor and the particles were counted in a Petroff-Hauser counting chamber with the light microscope, all of them were found in three adjacent bottles. The maximum width of this band is shown as a dotted line on Fig. 3, indicating the maximum contribution of convective mixing associated with unloading a sharp band of material from the BXIV zonal rotor.

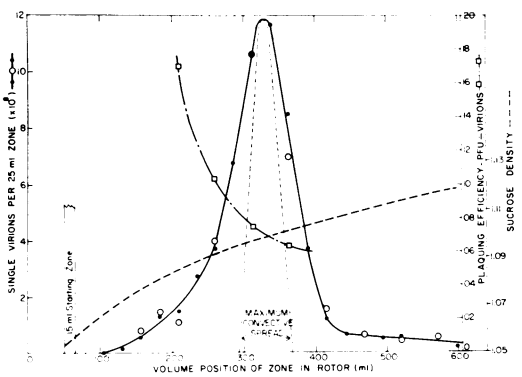

FIG. 3. *Sedimentation velocity spectrum of the same vaccinia virus population as that of Fig. 1 but at higher resolution. Data from two runs are included to show the degree of reproducibility obtained. Electron micrographic counts of single virions show much greater spread than that of polystyrene latex reference particles (dotted peak). Plaquing efficiency of the slower-moving virions is substantially greater than that of those in the peak zone.*

Sedimentation of the virus in 15 to 30% sucrose at 12,600 rev/min for 30 min advanced the peak of single particles to the 450-ml zone, providing the additional data necessary for computation of virion size and density. The spectrum remained continuous; there was no evidence of splitting into subpeaks.

Plaque titrations of the virus in all zones of the gradient (Fig. 3) have shown a recovery of 80 to 100% of the input plaque-forming units (PFU). Some loss would be expected, of course, from the few rapidly sedimenting groups of larger size. Special attention was given to those zones containing essentially all single particles. Beginning at the left, at the point where the virion count reaches significant magnitude, and extending somewhat beyond the principal peak, where the number of pairs begins to increase, the ratios of PFU to virus particles (VP) have been deter-

mined carefully. The efficiency of plaque formation in this spectral region is shown in Fig. 3, where it may be seen that the slower-moving particles are about three times more efficient than those moving in or on the faster side of the major peak. Six experiments of this kind have given similar results.

The sucrose concentration increases from left to right in Fig. 1 and 3, but this has no effect upon the plaque titer of the virus in these experiments. Control experiments involved suspension of virus in 30% sucrose for the same interval of time as that of the centrifuge run, and then it was diluted and titrated. There was no detectable effect of sucrose on the plaquing efficiency of this virus.

Buoyant density or isopycnic banding of the vaccinia virus population was attempted in a sucrose gradient. After 19 hr of spinning at 30,000 rev/min only 15% of the virus remained in the density region $1.17 < \rho < 1.24$. Countable virus was first detected at $\rho = 1.20$. Increasing numbers were found at increasing density levels but the peak obviously lay well beyond $\rho = 1.24$. The input sample, 15 ml, contained a total of 9.5×10^9 virions, of which 69% were single particles. Individual samples ranged from 61 to 83% singles in the region $1.217 < \rho < 1.24$, but there was no tendency for this factor to decline as the count increased toward the 1.24 level. In this respect the centripetal end of the nearly isopycnic density distribution, which is revealed in this experiment, is quite different from those of the foregoing velocity spectra, in which the slower-moving units are all single virions.

Inasmuch as the mean isopycnic density of the population lies beyond $\rho = 1.24$, and the density of the heaviest particles certainly further, a display of the complete density spectrum of this vaccinia virus in sucrose seems impractical. The precipitous rise in viscosity with increasing sucrose concentrations beyond 50% would seem to preclude it.

Suspension of the virus for several hours at room temperature in 40% (w/w) potassium tartrate solution had no effect on its plaque titer in L cells. In preformed gradients [15% to 45% (w/w)] of this salt, a single virus band was found with peak at $\rho = 1.20$ after 19 hr at 30,000 rev/min. In gradients of less steepness (20 to 35%), virus was found at all levels between the extremes of $\rho = 1.15$ and 1.22. The centroid of the distribution lay at 1.20 but twin maxima were present at $\rho = 1.190$ and $\rho = 1.206$ (Fig. 4). The twin peaks were not clearly separated at the base line. There was no visible difference in the appearance of the individual virions in the two regions, and the virion aggregation was heavy in both regions. Only about 30% of the particles

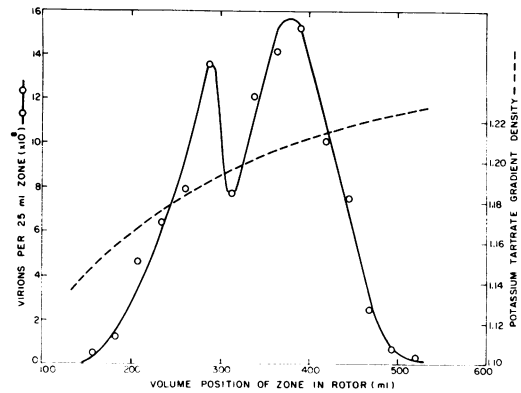

FIG. 4. *Density spectrum of vaccinia virus population in a very shallow gradient of potassium tartrate. Heavy virion aggregation was found in both zones of the bimodal distribution, although the concentration nowhere was over 10^8 virions per ml, less than 1 μg per ml. The rotor was operated 19 hr at 30,000 rev/min.*

were present as singles except at the extreme edges of the distribution (both high and low density), in which the few particles seen were singles. Aggregation analysis of the input suspension revealed 72% singles.

Figures 5 and 6 show the nature of the particle aggregation in the regions of the dense and less dense peaks, respectively. Although the group size and frequencies were similar, there was definitely less extraneous material associated with (included in) the groups in the denser peak.

The virions of highest plaquing efficiency in the velocity spectrum are few in number; nevertheless attempts were made to gather and concentrate a sufficient quantity of them for independent velocity and banding density measurements. Unequivocal results have not yet been obtained. They await further evaluation of some changes that we have observed in virus that has been exposed to concentrated sucrose solutions. When a virion population has been separated in a velocity spectrum such as that of Fig. 3, fractions cut from this spectrum must be dialyzed before they may be rerun to determine their individual behavior. When virions from such dialyzed fractions have been rerun, their final positions have not been the same as they were in the initial run nor were they found in the position of the original maximum, as they would be if the apparent velocity heterogeneity were an artifact caused by diffusion, mechanical mixing, etc. The velocity spectrum of the whole virion population was changed when the virus was suspended for 2 hr in 30% sucrose solution. After dialysis at 4 C for 16 hr (sucrose concentration reduced to about 1%), this virus was rerun in the 15 to 30% gradi-

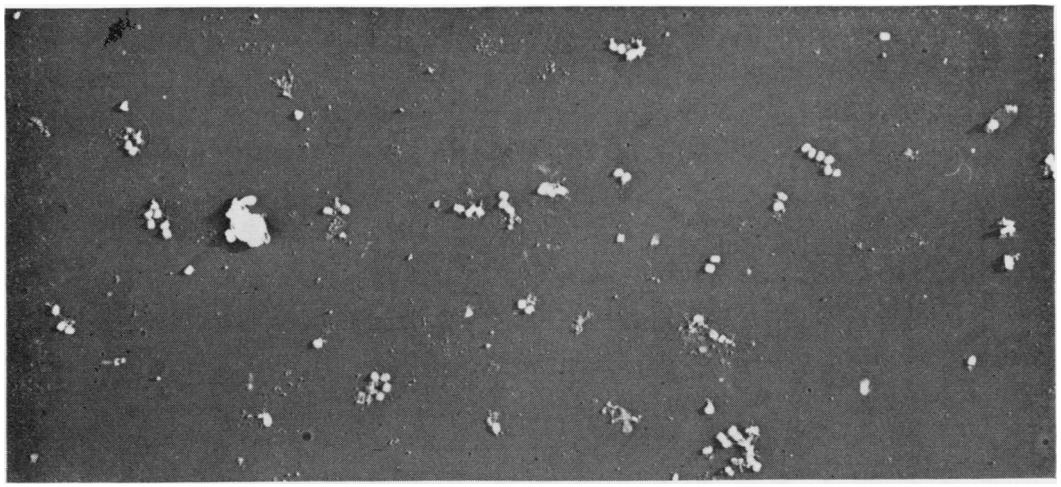

FIG. 5. *Aggregated but relatively clean virus suspension from the major peak zone of Fig. 4, ρ = 1.206.*

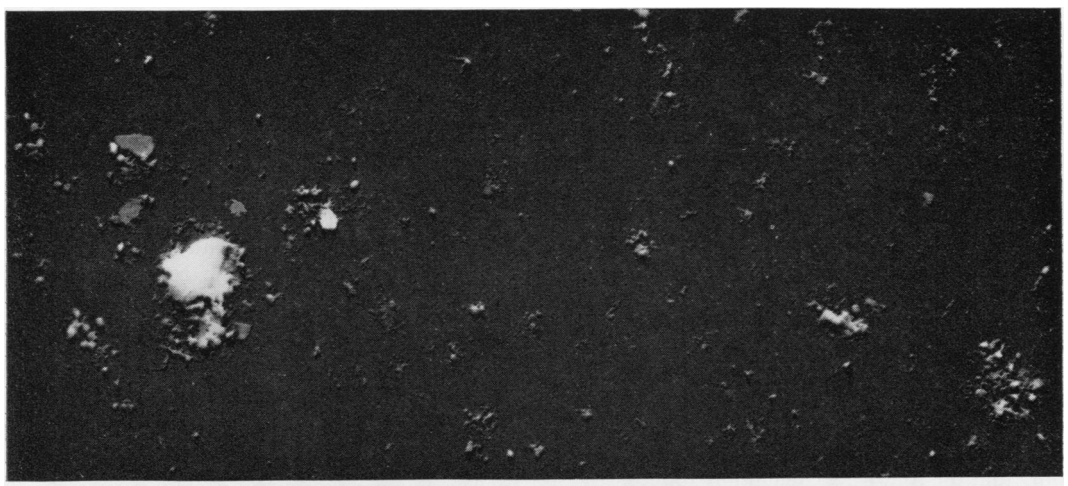

FIG. 6. *In the minor peak zone (ρ = 1.190) of Fig. 4, the virions were mostly associated in groups with extraneous material, probably of host origin.*

ent for comparison with Fig. 3. The peak moved further to the right (to the 375-ml zone), and the left (slow) side of the spectrum curve was much steeper, indicating a change in the proportion of fast to slow virions.

Calculation of virion size and density from sedimentation velocity measurements in a sucrose gradient. If a spherical particle moved radially in a centrifugal field through a uniform fluid of density ρ_s, its velocity s_v at any distance R from the axis is proportional to R. The difference between its density and that of the fluid ($\rho_v - \rho_s$) and its radius r are assumed to remain unchanged as it moves. From two or more measurements of the time required for movement through different distances, one can calculate the product $r^2(\rho_v - \rho_s)$ but neither ρ_v nor r can be extracted unless one of them is determined independently.

If two or more such sedimentations are made in a gradient of density (and viscosity), it should be possible to calculate, from the times and corresponding distances, both the radius and the density of the sedimenting particle. The sedimentation velocity (S_v) at distance R in the gradient is:

$$S_v = \frac{2}{9}\omega^2 r^2 \left[\frac{(\rho_r - \rho_s)\, R}{\eta_s}\right] \qquad (1)$$

In this equation ω is the angular speed of the rotor, η_s and ρ_s are the viscosity and the density of the gradient solution at the point R. The time required for the particle to move through the increment of distance ΔR will be:

$$\Delta t = \frac{\Delta R}{S_v} \quad (2)$$

and the total time for sedimentation from starting position R_o to final position R, will be:

$$T_m = \sum_{R_0}^{R} \frac{\Delta R}{S_v} \quad (3)$$

A zonal centrifuge run consists of acceleration, constant high speed, deceleration, and unloading at a much lower constant speed. The value T_m in equation 3 can be closely approximated by taking frequent rotor speed measurements during the entire process and dividing the sum $\sum\omega^2\Delta t$ by $(\omega_{max})^2$, the square of the constant high speed part of the run. In the present work, unloading speed was one-seventh of the maximum speed, and the total contribution of acceleration, decelerations, and unloading times to T_m was 6 min of a total of 26. For these low speeds, it was necessary to compare electric pulse frequency from the centrifuge drive with alternating-current line frequency by using an oscilloscope. Tachometer indications could not be read to the necessary accuracy.

There are virions of many velocities in the population (Fig. 3), but it seems probable that those that form the maximum of the concentration peak at 26 min are the same as those that appear in the peak of a similar run of 36-min duration. This is the only ready means of identifying a part of the population, and it is to this part only that the following calculation applies.

Values of S_v were calculated for radial increments of 2 mm by using equation 1 and a range of trial values of ρ_v and r. Corresponding values of T_m from equation 3 were then calculated and compared with the experimental values. The interpolation to determine from these tables the combination of best fit (ρ_v = 1.205 and r = 1.12×10^{-5} cm) was done graphically. In subsequent work we expect to program this process for digital computer using several gradients that prove to be most useful.

DISCUSSION

Some virus preparations contain intact or complete particles as well as empty "ghosts." Others like those from vesicular stomatitis may contain two physically different kinds of virions of similar composition (9). In both of these cases, the different components of the population appear as discrete bands in zone velocity or isopycnic banding experiments in appropriate density gradients. Still other viruses, particularly those of the myxo group, exhibit rapidly broadening continuous bands characteristic of their heterogeneity (2). This behavior would be anticipated from observation of their pleomorphic character in the electron microscope. Viruses of the pox group were among the first to be examined by electron microscopy. They have been often studied in this way and among the large viruses they are regarded as highly homogeneous with respect to size and shape. Joklik (5) states that his carefully purified rabbit poxvirus is homogeneous when banded in a 25 to 40% sucrose gradient. The vaccinia virus that we have examined appears quite homogeneous in the electron microscope, but it is definitely heterogeneous when banded in sucrose. A broad velocity spectrum is observed.

Virion counting and aggregation analysis have shown that in the very dilute preparations examined here, the small clumps (pairs and triplets) move out ahead of the single particles as expected. Their relative numbers are therefore not in a state of sensitive dynamic equilibrium which is disturbed when the mixture is separated in this way. Dilute fractions of pairs remain as pairs. The broad spectrum of velocities seen among the single virions is definitely not due to diffusion or mechanical mixing in the centrifuge. It is therefore necessary to attribute it to a spectrum of densities in the virion population. Isopycnic banding of the same virus population in a potassium tartrate gradient reveals such a density heterogeneity, which presumably exists in sucrose as well. This suggests heterogeneity of virion composition. Experiments now in progress with double-labeled virus are expected to yield values of deoxyribonucleic acid per virion and protein per virion.

Dahlberg and Simon (2) have interpreted velocity heterogeneity in populations of Newcastle disease virus in terms of size only. They do not speak of density in their discussion but they propose the large virions as multiploid. We must consider virion density, but as yet we have not succeeded in an independent measurement of the density of the fast- and slow-moving particles from the sucrose gradients. It is the slow-moving ones that have the greater plaque-forming efficiency, but direct experimental evidence that they sediment slower because they are less dense is lacking. It does, however, seem probable, and it may mean that the slow virions are different in composition but this is not absolutely necessary. The population may be quite uniform in both size and composition and still have a broad distribution in sedimentation velocities in a hyper-

tonic environment. If we consider a suspension of spherical virions all of the same diameter and density but differing slightly from each other in rigidity of structure, they may differ from each other in permeability to the solute used to produce the osmotic pressure (and the gradient) or, if they exclude the solute molecules, in their physical reaction to the resulting pressure. Under these conditions, they will tend to shrink, probably to different degrees, and, although this shrinkage may be small, its effect upon sedimentation rate could be great indeed. Consider the variables influencing sedimentation rate s. These are particle radius r and density difference between virion and solution $(\rho_v - \rho_s)$, which influences s in the following manner:

$$s = kr^2(\rho_v - \rho_s) \quad (4)$$

We have assumed that the virions at the peak of the sedimenting band in a short run are the same ones that form the peak after a longer run in the same gradient. Insofar as this criterion of recognition is acceptable, both $\rho_v = 1.205$ and $r = 1.12 \times 10^{-5}$ cm can be calculated for the virus in our sucrose gradient. A small shrinkage of the virion in response to osmotic stress would influence this very little through change in r^2 as it appears in equation 4, but ρ_v = virion weight divided by $\frac{4}{3}\pi r^3$, so the small difference between ρ_v and ρ_s is exceedingly sensitive to changes in r. In fact, a shrinkage of 2% in particle radius in a solution of density 1.08 would increase its sedimentation rate by over 50%. According to this concept, it could be the virions that resist the osmotic forces, which do not shrink or shrink the least, that are the least dense in sucrose; these are the ones that sediment slowest and are also the most efficient plaque producers.

The physical heterogeneity of the unpurified virion population examined here stands in sharp contrast to the homogeneous purified preparations described by Joklik (5). If genetic heterogeneity is the cause of the broad velocity spectrum, careful cloning should narrow it. This is being investigated. On the other hand, if homogeneous preparations are obtained from heterogeneous starting populations by careful purification, one must apply purification with great care indeed, lest he inadvertently lose a significant minor component, such as the few virions of highest plaquing efficiency.

ACKNOWLEDGMENTS

We acknowledge with thanks the expert technical assistance of William D. Fyfe, electron microscopist.

This investigation was supported by Public Health Service grant RG-5177 from the Division of Research Grants, National Institutes of Health; by the donation by the Ivan Sorvall, Inc., Norwalk, Conn., of a fluid-drive ultracentrifuge; by contract with the Atomic Energy Commission which provided the BXIV zonal rotor and gradient-making equipment; and by Public Health Service training grant GM-01138 from the National Institute of General Medical Sciences.

ERRATUM

In a preliminary presentation of a part of these data at the 27th Annual Meeting of the Electron Microscopy Society of America, speed and time were stated in the abstract as 15,000 rev/min and 20 min. They should have been as they are in this paper, 10,000 rev/min and 30 min.

LITERATURE CITED

1. Anderson, N. G. 1966. The development of zonal centrifuges. National Cancer Institute Monograph 21. U.S. Government Printing Office, Washington, D. C.
2. Dahlberg, J. E., and E. H. Simon. 1969. Physical and genetic studies of Newcastle disease virus: Evidence for multiploid particles. Virology 38:666–678.
3. Dalton, D., K. S. Kim, and D. G. Sharp. 1967. Multiplicity activation of vaccinia virus in L cells. Proc. Nat. Acad. Sci. U.S.A. 58:1758–1761.
4. Hoagland, C. L., J. E. Smadel, and T. M. Rivers. 1940. Constituents of elementary bodies of vaccinia. I. Certain basic analyses and observations on lipid components of viruses. J. Exp. Med. 71:737–750.
5. Joklik, W. K. 1962. The purification of four strains of poxvirus. Virology 18:9–18.
6. Kim, K. S., and D. G. Sharp. 1966. Electron microscopic observation on the nature of vaccinia virus particle aggregation. J. Immunol. 97:197–202.
7. Pickels, E. G., and J. E. Smadel. 1938. Ultracentrifugal studies on the elementary bodies of vaccinia virus. J. Exp. Med. 68:583–606.
8. Sharp, D. G. 1960. Sedimentation counting of particles via electron microscopy, p. 542–548. Verh. 4th Int. Kongr. Elektronenmikroskopie. Berlin, 1958.
9. Wagner, R. R., T. A. Schnaitman, and R. M. Snyder. 1969. Structural proteins of vesicular stomatitis viruses. J. Virol. 3:395–403.