The Composition of a Population of Vaccinia Virions (36192)

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The large size and characteristic shape of vaccinia virions make them relatively easy to recognize and count by electron microscopy, even in the midst of substantial quantities of cellular debris. The fact that accurate particle counts of this kind can be made at concentrations as low as 10^6 virions/ml has permitted their use in locating the position and distribution of virions in rate-zonal sedimentation experiments. The movement of single virions can be studied by this means, quite apart from the effects of aggregation that tend to interfere with the interpretation of experimental data taken at the much greater virion concentrations necessary for optical observations by either absorption, scattering, or refraction of light.

These things have been demonstrated in our earlier publication (1) along with the observation that the few slowest-sedimenting virions in the broad single-peaked sedimentation spectrum were more efficient in plaque production than those of the majority that sedimented faster. In view of the significance attached by others to the function of incomplete (2) and deficient (3) interfering virions of other species, we have attempted the difficult task of determining the distribution of DNA, protein, and lipid per virion in the sedimentation spectrum of unpurified vaccinia virus preparations. In this way, we hope to include all minority elements of the virion population that might otherwise be lost through over-zealous purification. The apparent physical heterogeneity of the populations might thus be interpreted in terms of composition and/or structure and possibly later, in terms of phenotypic or genotypic factors.

Materials and Methods. Vaccinia virus. WR (mouse neurotropic) strain from the American Type Culture Collection has been passed 162 times in Earle's L cells at a multiplicity of approximately 50 virions/cell.

Sedimentation velocity analyses of dilute virion suspensions in the BXIV zonal centrifuge rotor (4) have been made by counting radioactive responses from labeled virions and by counting virions by electron microscopy as previously described (1).

Labeling of virus and L cells was done in bottles containing confluent monolayers of about 20 million cells. Growth medium was removed from the cultures, they were washed with phosphate buffered saline (PBS) and allowed to sit at room temperature for 1 hr. Virus stock was diluted with PBS to contain 10^9 virions/0.5 ml; and then treated with 20-kHz waves for 2 min to reduce aggregation. A 0.5 ml quantity of the dilute virus was added to each bottle, giving an input multiplicity of about 50 virions/cell. Following a 2 hr adsorption period, 15 ml of prewarmed growth medium containing 50 μCi of each radioactive isotope was added. Maximum yields of virus were harvested after 120 hr at 37°. Sham-infected cells were labeled the same way except that there was no virus in the 0.5 ml of PBS added.

Isotopes from New England Nuclear Corp. were L-amino acid-^{14}C (U) mixture for viral protein, choline-methyl-^{3}H chloride (sp act, 100 mCi/mmole) for viral lipid and thymidine methyl-^{3}H (sp act, 6.7 Ci/mmole) for viral nucleic acid.

Preparation of samples for radioactive counting. Samples of labeled virus from the zonal rotor were diluted 1:10 with PBS and passed through Millipore filters of mean pore size 0.22 μ. After passage of 9/10 of the sample, 10 ml of PBS were added to the remaining fluid to purge the filter.

Filter discs were removed, dried overnight, and placed in scintillation vials containing 10 ml of Fluor solution No. 1, Packard Tech. Bull. No. 1 or 15.2 g of PPO + 0.38 g of

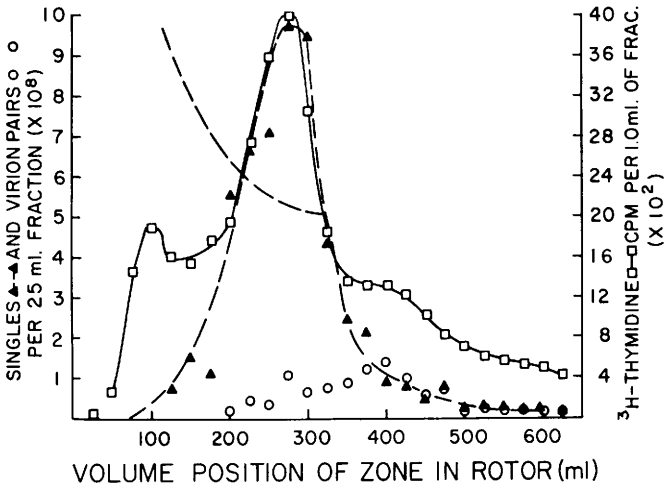

FIG. 1. Sedimentation velocity spectrum of a labeled virion population (no purification) released by 20-kHz waves from infected L cells: Thymidine-^{3}H counts are shown with virion counts for singles and adhering pairs superimposed. Sedimentation was from left to right in a 15–30% (w/w) sucrose gradient in the BXIV zonal rotor. (upper curve without points) shows plaquing efficiency in the region of principally single virions.

POPOP/gallon. A Packard Tri-Carb scintillation counter was used, standardized for simultaneous (pulse-height) analysis of ^{3}H and ^{14}C counts. Virtually no ^{3}H counts were found in the ^{14}C channel while 7% of the ^{14}C counts appeared in the ^{3}H channel. A quench series containing 0–3% sucrose showed that no quench correction was necessary for residual sucrose from centrifuge samples.

Experiments and Results. A 15 ml band of the infected lysate from dual labeled cell cultures was set up at the 75 ml position (Fig. 1) of the zonal rotor. There were approximately 10^{10} virions in this band and its radial thickness was 1.7 mm. After a slow acceleration to 12,600 rpm, and 20 min at that speed, the rotor was slowed to 1800 rpm for unloading. Equivalent integrated time at 12,600 rpm was 26 min. Single virions were found at all volume levels from 100 to

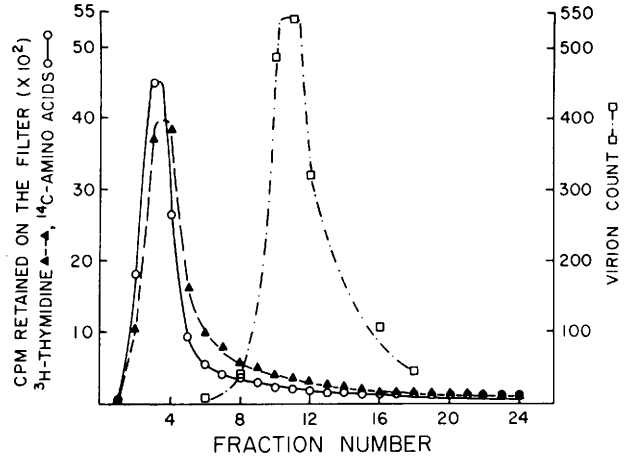

FIG. 2. Control, sedimentation velocity spectrum of thymidine-^{3}H, and ^{14}C label from sham-labeled L cells: Unlabeled virions were added and their concentration was determined as in Fig. 1 by counting in the electron microscope. Apparently there is no label attached to the sedimenting virus.

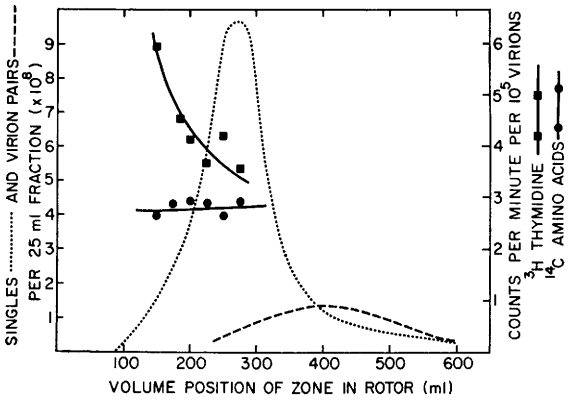

FIG. 3. Composition of pox virions as a function of sedimentation velocity: In the region of mostly single virions, the ^{14}C counts per virion remain essentially constant but the thymidine-^{3}H counts per virion change much as did the plaquing efficiency shown in Fig. 1.

600 ml, forming a smooth curve with single peak at the 280 ml level. Virion pairs were much fewer in number coming to peak concentration at the 400 ml level. Aggregates of 3 or more virions were negligible in number. Many previous analyses of this kind show that this is a characteristic velocity spectrum of such populations.

The ^{3}H count from thymidine measured in the same fractions has a major peak at about the same place as that for single virions. There is a shoulder corresponding to the peak of virion pairs (sedimenting faster) and a small sharp peak just to the right of the starting position. Essentially the same features appear in the curve of ^{14}C counts from the same fractions and in the similar but separate experiment on a virion population labeled with choline-^{3}H. While the most prominent feature in the distribution of all three labels is the major peak in the region of that for virion count, the distribution of labeled host material has a single peak (Fig. 2) corresponding to the small secondary one of Fig. 1. When the extracts of sham-infected cells were centrifuged to obtain the above control curves, unlabeled virions were added in the same numbers that were present in the infected lysate of Fig. 1. These virion counts are shown in Fig. 2. Apparently there is no nonspecific coating or other attachment of labeled host material to these cold virions as no discontinuity appears in the cpm curves in the region of the virus particle peak.

The spectral distribution of label *per virion* is now calculated by subtracting at each point a number of cpm that come from labeled nonviral material. This number is different at each point but if we consider that the ^{3}H count of Fig. 1 shows just the virus with a nonviral curve of the same shape (not necessarily the same size) as that of Fig. 2 superimposed, we have a rational means for making the correction. By measuring the heights h and H of the stationary peaks of Figs. 1 and 2 and multiplying each ordinate of Fig. 2 by the ratio h/H, the result is the set of quantities to be subtracted. The result is a substantially constant ratio of cpm/virion for ^{14}C in the region (Fig. 3) occupied mainly by single virions. The ^{3}H count from thymidine in the same experimental fractions indicated that the average DNA content of the slowest sedimenting virions was about twice as great as that of the major part of the population in the peak region. In a separate experiment choline-^{3}H label per virion was, like the ^{14}C in the above experiment, constant in the same spectral region.

A different method for determining DNA per virion in the fractions from the spectrum was based on the finding that treatment of lysates of sham-infected cells with DNAse permitted practically all of the ^{3}H count to pass the filter. So spectral fractions from lysates of infected cells were treated for 3 hr with 50 μg/ml of DNAse at 37°. The slow peak of ^{3}H counts (Fig. 1) was removed; as

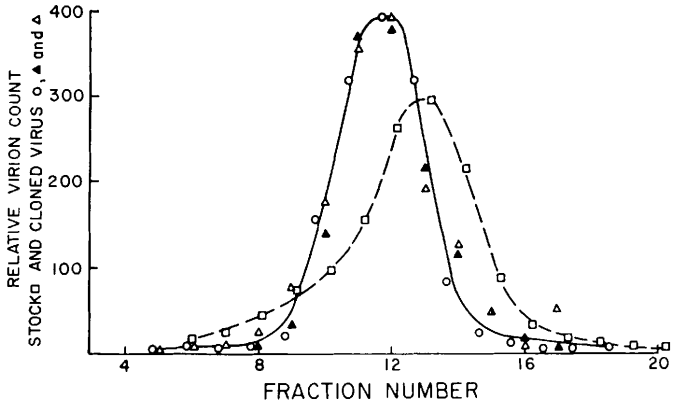

FIG. 4. The sedimentation velocity spectrum of three subpopulations selected from the original population by cloning under conditions providing a high probability that each is the progeny of a single virion: Physical heterogeneity is less and the peak sedimentation rate of these subpopulations is shifted in the direction of greater plaquing efficiency (Fig. 1) and greater DNA per virion (Fig. 3).

would be expected, the slow peak for ^{14}C was not affected. DNAse-resistant ^{3}H counts found on the filter were presumed to come from intact virions. When these were divided by the number of virions in the fraction the DNA/virion ratios were the same as those obtained by the subtraction method (Fig. 3). Similar use of protease and lipase was thought to be impractical because of the probable susceptibility of viral coat components to these enzymes.

Cloned subpopulations were prepared from the original population with especial care: (i) to prevent selection of plaques arising from virion aggregates; and (ii) to minimize the number of generations involved in production of enough virus for sedimentation velocity analysis (about 10^{10} virions). A few single virions will pass through a 0.45μ filter (Millipore). Monolayer plates inoculated with about 20 such particles produced widely separated plaques and one of the clones was obtained in this way. Two others came from the slowest sedimenting fraction of a spectrum such as that of Fig. 1 (125 ml level) in which only single particles were present. Infected cells and loose virions gathered by aspirating the plaque areas were diluted to 2 ml and treated with 20-kHz waves, then sedimented in flatbottomed centrifuge tubes upon small monolayers of L cells to achieve maximum input multiplicity. Each culture contained about a million cells. They all produced enough virus to inoculate one more passage in L cells at $M = 10$, producing sufficient virions for SV analysis (Fig. 4). Slight differences in these three spectra are considered insignificant at this time. The curve drawn through one set of the data points fits well for all three and it is definitely displaced to the left (slower sedimentation) of that for the whole population from which they came. Its width at half-height divided by its maximum height is less than that of the whole population showing reduced physical heterogeneity in each of the three cloned populations. There is, nevertheless, a substantial amount remaining.

Discussion. The sedimentation velocity (SV) spectrum of a double labeled virion population shows ^{14}C protein label and ^{3}H nucleic acid label peaking essentially together with the virion count of electron microscopy. The choline-^{3}H label, in separate experiments, did the same. In all three cases, the SV spectra of labeled material from uninfected cells produced a major peak at the starting position which extended, continuously decreasing in height, into the region where the virions would appear in infected material (Fig. 2). When corrections were made for this by subtraction, and the remaining ^{14}C (protein) and ^{3}H (choline) counts were divided by the virion counts in those fractions, the resulting specific activities of both these components

were the same in all fractions (Fig. 3). The same process applied to the ^3H (DNA) counts indicated a richer DNA content per virion for those few in the trailing edge of the SV spectrum. When further experimentation revealed the disappearance of the nonsedimenting DNA peak after DNAse treatment of labeled but not infected cells, this too showed that DNAse-treated ^3H counts per virion were the same as those obtained by the subtraction method. The virions of highest DNA content are few, but they appear in the same SV spectral region where virions of highest plaquing efficiency were found in earlier work (1).

Three freshly cloned populations produced slightly sharper SV peaks of virion count than those of the parent population, and these were displaced to the left (slower) by an amount substantially greater than the observed variation between them (Fig. 4), suggesting a selection of more efficient plaque forming virions. Further work will be required to determine whether the physical heterogeneity of the parent virion population is due in part to genetic heterogeneity. At this point it seems more likely that there may be virions of several, perhaps many, degrees of incompleteness in these populations. It is unusual, but not unique, to find dense virions sedimenting slower than those of lesser density (5).

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