# PHYSICAL AND BIOLOGICAL OBSERVATIONS ON HERPESVIRUS

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ABSTRACT

SMITH, KENDALL O. (Baylor University College of Medicine, Houston, Texas). Physical and biological observations on herpesvirus. J. Bacteriol. **86**:999–1009. 1963.—The development of herpesvirus in human lung fibroblasts was studied by plaque assays and physical particle counts. Approximately 200 infectious units and 60,000 particles were produced by single cells during a single growth cycle. Production of physically recognizable particles preceded infectious virus particle production by about 5 to 6 hr, suggesting the occurrence of a maturation process during formation. Aggregation of particles in clusters and chains was observed in many cases. One of the mechanisms for this aggregation was the connection of particles by deoxyribonucleic acid strands. These strands appeared to connect some particles in a way that suggests a structural continuity between their cores.

Several reports describing the development of infectious herpesvirus in chick embryos and in tissue cultures have appeared during the past 4 years (Farnham and Newton, 1959; Hoggan and Roizman, 1959; Wheeler and Canby, 1959; Scott, McLeod, and Tokumaru, 1961). The classic negative staining work of Wildy, Russell, and Horne (1960) with herpesvirus made possible the simultaneous study of the morphological and the biological properties of this virus during its growth in tissue cultures. With this in mind, Smith and Melnick (1962*a*) developed a method for preparing and staining herpesvirus which permits quantitation of the particle numbers and morphological study of the virus in the same preparation. Soon after this, Watson (1962) described three other methods for counting negatively stained herpesvirus particles. He reported particle–plaque-forming unit (PFU) ratio determinations and growth curve data in which both particle counts and infectivity measurements were made. The report represented here is a further effort to combine recently developed quantitative physical techniques with the precise plaque assay method to study the morphological and biological changes herpesvirus undergoes during a one-step growth cycle. Some new observations are recorded concerning the mechanism of aggregation of this virus.

## MATERIALS AND METHODS

The herpesvirus strain used in this study was isolated about 1 year ago from a patient with severe generalized herpes, identified by a specific antiserum and designated VA-herpes. Virus was usually passed at either 34 or 35.5 C, harvested at 20 to 24 hr or after substantial cytopathic effect was observable, and then stored at −60 C in a Revco deep freeze.

Primary rabbit kidney cells and human embryonic lung cell lines were used for passage and growth of herpesvirus. Eagle's medium containing 20% calf serum was used to grow the cells, and Eagle's medium with 10% calf serum was used for virus dilution and for nutrient medium in infected-cell cultures. Prescription-bottle (1 oz) monolayers were used for both growth and titration of virus. Each bottle provided approximately 14 $cm^2$ of surface area for cell growth. Bottles were washed by the C and M (Calgon-metasilicate) method prior to use (Scherer, 1955).

The number of cells in replicate monolayers was determined by trypsinizing the cells in a measured volume of fluid and counting in a hemocytometer.

The gamma-globulin technique for herpes plaque assays, described by Roizman and Roane (1961), was used for virus infectivity titrations, except that much smaller volumes of virus were used for inoculation (0.10 to 0.20 ml rather than 1.0 ml), and adsorption was continued for 2 hr instead of 1 hr at 34 C. Bottles were rotated every 15 min during adsorption and overlaid with 0.5% pooled human gamma-globulin in Eagle's medium containing 10% calf serum.

The particle counting and staining procedure

TABLE 1. *Effect of sonic oscillation on the infectivity of herpesvirus*

| Time of treatment | No. of plaques per bottle* | Avg no. of plaques per bottle |
|---|---|---|
| *sec* | | |
| 0 | 24, 28 | 26 |
| 10 | 63, 73 | 68 |
| 30 | 99, 90 | 95 |
| 90 | 79, 92 | 86 |
| 240 | 83, 49 | 66 |

* Two bottles were used in each test.

TABLE 2. *Simultaneous titration of a single herpesvirus dilution in five different cell lines and 20-hr virus yields after high multiplicity inoculation*

| Cell line | Avg no. of plaques per bottle | | Virus yield per cell (PFU) |
|---|---|---|---|
| | Expt 1* | Expt 2* | |
| Human fibroblast #1 | 111 | 27 | 68 |
| Human fibroblast #2 | 124 | — | — |
| Human fibroblast #3 | 119 | — | — |
| Human fibroblast #4 | 108 | 35 | 88 |
| Rabbit kidney | 79 | 41 | 108 |
| Average | 106 | 34 | 88 |

* Different virus suspensions were used in these two experiments.

previously described in detail by Smith and Benyesh-Melnick (1961) and Smith and Melnick (1962*a*, *b*) was used. Potassium phosphotungstate (pH 7.0) and uranyl acetate were used on separate duplicate specimens for staining. Specimens were stained for 10 to 20 sec in 0.2% stain, carefully drained on absorbent paper, and air-dried. An RCA EMU-3F electron microscope was used exclusively.

A Raytheon sonic oscillator (9 kc, model S-102A) was used for sonic oscillation of virus suspensions prior to titration or counting (Smith and Sharp, 1960). Routinely, sonic treatment was for 20 to 30 sec.

## RESULTS

*Effect of sonic oscillation on the infectivity of herpesvirus.* Sonic wave treatment has been found to increase the infectivity of vaccinia (Galasso and Sharp, 1962) and influenza (Hannoun, Proudhomme, and Gustini, 1960) viruses significantly, probably by physical dispersion. We considered it desirable, therefore, in the earlier stages of this work to determine the exact sonic wave treatment conditions which would give the maximal infectivity in crude tissue culture suspensions of herpesvirus. Virus was harvested near the peak of the growth cycle, stored for a brief period at −60 C in a deep freeze, and thawed at 37 C; the cell debris was suspended by rapid pipetting, and 2.0 ml of this fluid were placed in a Lusteroid Spinco tube (0.5 by 2 in.). The tube was sealed with a rubber stopper, taped, immersed in about 25 ml of water contained in a Raytheon sonic oscillator cup, and sonically treated for various periods of time (Smith and Sharp, 1960). At 10, 30, and 90 sec, 0.30-ml volumes were removed for plaque titration, and the remainder was sonically treated for an additional 150 sec (Table 1). The titer was raised about fourfold by exposure to sonic waves for 20 to 30 sec; therefore, all specimens in the experiments to be described were treated in this way prior to titration or particle counting. It was clear that there was some physical deaggregation of the virus particles during the 20 sec of sonic oscillation, although aggregates of 50 to 100 particles were commonly encountered after treatment. The data in Table 1 suggest that sonic oscillation beyond 30 sec resulted in a slow inactivation of herpesvirus, so this was avoided in further work.

*Relative sensitivity of various cell lines to herpesvirus.* A continuous supply of cells which are equally susceptible to virus is desirable for quantitative work. Therefore, different stock lines of human lung fibroblasts which were in frozen stock and are presently being used in our laboratory were revived by passage and compared with primary rabbit kidney cells for their efficiency in forming VA-herpesvirus plaques on monolayers. The two central columns of Table 2 show the results of two such experiments. It is clear that there is no significant difference among the susceptibilities of the five different cell lines to this herpesvirus strain.

Three of the cell lines were then infected with identical high-concentration inocula of herpesvirus to give input multiplicities of 3 to 5. (Cells from replicate bottles were trypsinized and counted to make this calculation.) After 22 hr of incubation at 35.5 C, cells were frozen at −60 C, thawed at 37 C, and sonically treated; then the virus suspensions were titrated on human fibroblasts. The resulting yields per cell are recorded in the right-hand column of Table 2. It appears,

therefore, not only that the cell lines are equal in sensitivity to herpesvirus, but also that they produce approximately equal quantities of this virus.

*Particle-PFU ratio determinations.* The particle-PFU ratios of serial passages of the VA-herpes virus were determined. Harvests of virus were titrated and counted on separate occasions (Table 3). The average ratio for this strain of herpes was 370, and varied between 300 and 510 in different passages.

TABLE 3. *Particle-PFU ratios of different strain VA herpesvirus passages at 34 C*

| Passage no. | Particle count per ml | PFU/ml | Particles per PFU |
|---|---|---|---|
| 1 | $7.6 \times 10^9$ | $1.7 \times 10^7$ | 450 |
| 2 | $2.1 \times 10^{10}$ | $6.7 \times 10^7$ | 310 |
| 3 | $1.17 \times 10^{10}$ | $2.3 \times 10^7$ | 510 |
| 4 | $1.58 \times 10^{10}$ | $5.7 \times 10^7$ | 280 |
| 5 | $2.0 \times 10^{10}$ | $6.6 \times 10^7$ | 300 |
| Avg | — | — | 370 |

Emphasis should be placed at this point upon the fact that no low-speed "clarifying" centrifugation was done in any of these studies as a means of partial purification preliminary to particle counting (as was done by Watson, 1962). Thus, rather large aggregates of virus particles, an example of which is shown in Fig. 1, remained in the suspensions which we counted. An aggregate of 20 particles (Fig. 1) measured about 1 $\mu$ in diameter (bacterial size), and would be removed from suspension by low-speed centrifugation. Nearly all crude tissue culture-grown herpesvirus suspensions which we examined contained many large aggregates, some consisting of over 100 particles. Removal of such aggregates by low-speed centrifugation does not decrease the infectivity of suspensions appreciably, if at all, because an aggregate can almost certainly account for no more than one infectious unit. However, the loss of a substantial mass of countable virus by the use of low-speed centrifugation for partial purification results in a large underestimation of the total virus particle yield per cell and an alteration of the particle-PFU relationship in crude herpesvirus suspensions. If these parameters are desired, high priority should be given to the choice of counting methods which do not require low-speed clarifying centrifugation of the virus specimen.

*General morphological observations on crude herpesvirus.* Some of the outstanding morphological features observed with the herpes strains described in these studies are illustrated in Fig. 1 to 11. Figure 1, showing a medium sized aggregate of particles, illustrates the fact that particles commonly contain various amounts of deoxyribonucleic acid (DNA) in their cores. A slight excess of uranyl acetate was used for routine particle counting, because the viral protein coats can be seen to better advantage when uranium lodges between the capsomeres and within the holes of the capsomeres. This excess stain can be removed, if desired, by simply draining more thoroughly and dipping the preparations in distilled water. The state of aggregation varied to some degree from sample to sample, but conservatively over 80% of the virus particles from tissue culture suspensions examined were in aggregates of five or more.

Another of the more prominent features observed in crude virus suspensions was the aggregation of particles by interconnecting strands (Fig. 2 to 5). Filamentous structures of this kind have been seen in both tissue culture herpesvirus preparations and have been associated with particles taken directly from vesicular lesions of the skin and mucous membranes. These strands often connect particles which are several microns apart, thus making large networks of loosely aggregated virus particles. Application of proteolytic enzymes for periods sufficiently long to cause a gross reduction in particulate cell debris and a partial degradation of the protein capsids did not destroy these strands (0.1% trypsin, 0.1% chymotrypsin for 20 min at 37 C, followed by 0.1% pepsin at pH 2.0 for 15 min). However, the application of 0.005% crystalline deoxyribonuclease for 20 min at 37 C completely destroyed the connecting strands. Application of deoxyribonuclease after pepsin treatment resulted in a removal of the uranium-stainable cores (Smith and Melnick, 1962*a*). Many of the individual strands approximated 18 to 20 A in diameter, the theoretical value for DNA (Watson and Crick, 1953). The tentative conclusion, therefore, was that the strands were DNA molecules which were attached in some rather firm way to individual herpesvirus particles. Microscopy strongly suggested that this is one of the principal mechanisms of aggregation of the virus.

The possibility that this was cellular DNA rather than unassembled viral DNA was explored. Electron photomicrographs were ex-

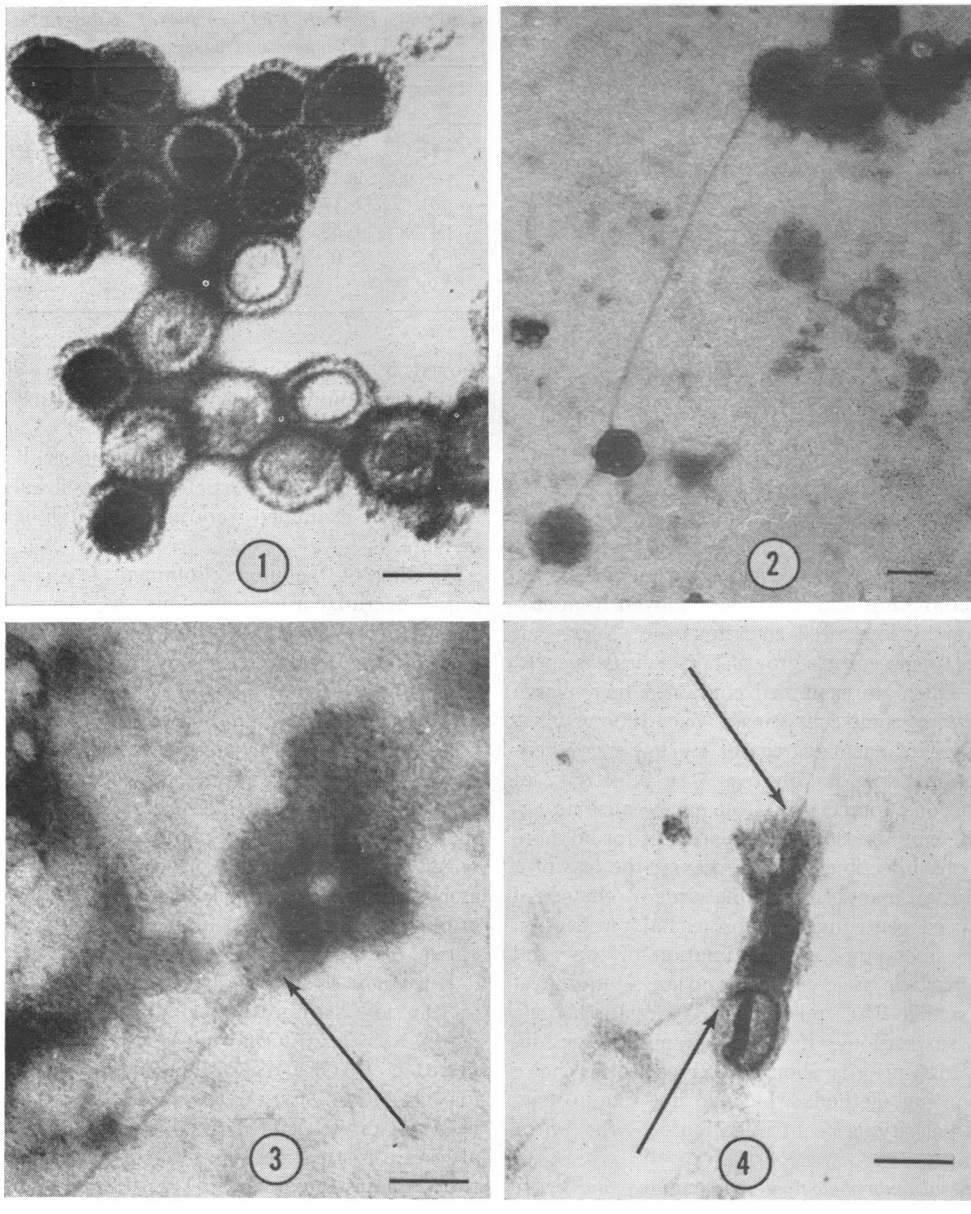


FIG. 1. *Herpesvirus (stained with uranyl acetate) showing both stained and unstained core areas. Marker on all micrographs equals 100 mμ.*

FIG. 2. *Herpesvirus (stained with uranyl acetate) showing several particles connected by a filamentous structure.*

FIG. 3. *Herpesvirus (stained with uranyl acetate) showing an apparent attachment of a filamentous structure to the incomplete core of one particle.*

FIG. 4. *Short chain of herpesvirus particles (stained with uranyl acetate) showing what appear to be DNA strands connected to the incomplete cores of two particles.*

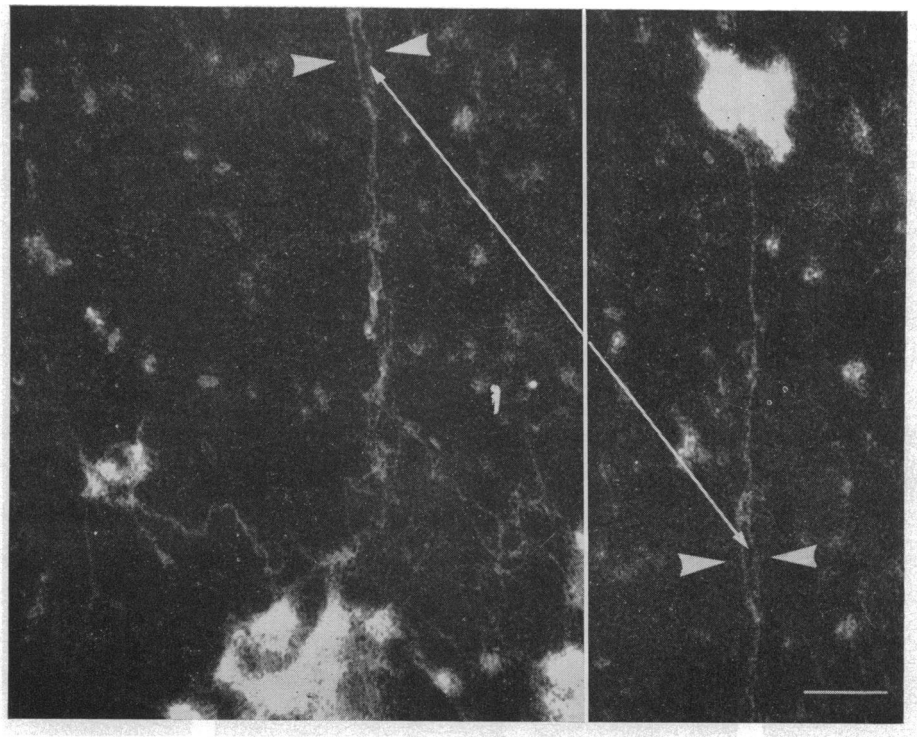

FIG. 5. *Herpesvirus, treated with proteolytic enzymes to digest the capsids, then stained with uranyl acetate to reveal the remaining filamentous structures (reference points show the same area on two prints of one large field, cut for convenience of demonstration).*

amined to determine whether the strands had any particular orientation to or connection with the cores of individual virus particles. Figures 3 and 4 show morphological evidence which suggests that filaments are attached to points within the incomplete cores of particles (see arrows). However, from these observations one could not rule out the possibility that the DNA strands simply run along the outside of and are somehow merely bound tightly to the protein capsids. To examine this possibility, virus protein capsids were almost completely digested by treatment for 0.5 hr with a 0.1% trypsin-chymotrypsin mixture, then with 0.1% pepsin for 1.5 hr. This digested material was diluted, sedimented upon agar, and pseudo-replicated as usual. Over 99% of the capsids were degraded beyond physical recognition by this procedure, yet the loosely bundled DNA cores remained. Many of these cores were connected to other cores through strands (Fig. 5). This is perhaps one of the strongest evidences suggesting the viral DNA nature of these connecting strands. Additional supporting evidence is the observation that nearly all strands ended in particles, and very few "loose ends" could be found in any of the routinely prepared virus specimens.

Other evidence for the connection of virus particle cores through strands of DNA is shown in Fig. 6. This photomicrograph illustrates the rather common arrangement of particles in short chains, like streptococci, with an obvious orientation of their incomplete cores along the long axis of the chain. This arrangement might be the result of a structural continuity between the cores of the individual particles, this being more readily seen in incomplete particles. Figure 7 shows a higher magnification micrograph which demonstrates to some extent the arrangement of the strands within the incomplete cores of individual particles. There appears to be a folding of the strands within the capsid, and there is a suggestion of orderliness. (The center arrow points to what seems to be a connecting filament between two particles.) Figure 8 demonstrates what appears to be an aggregate of DNA at one point along a long filament (see arrow). This structure

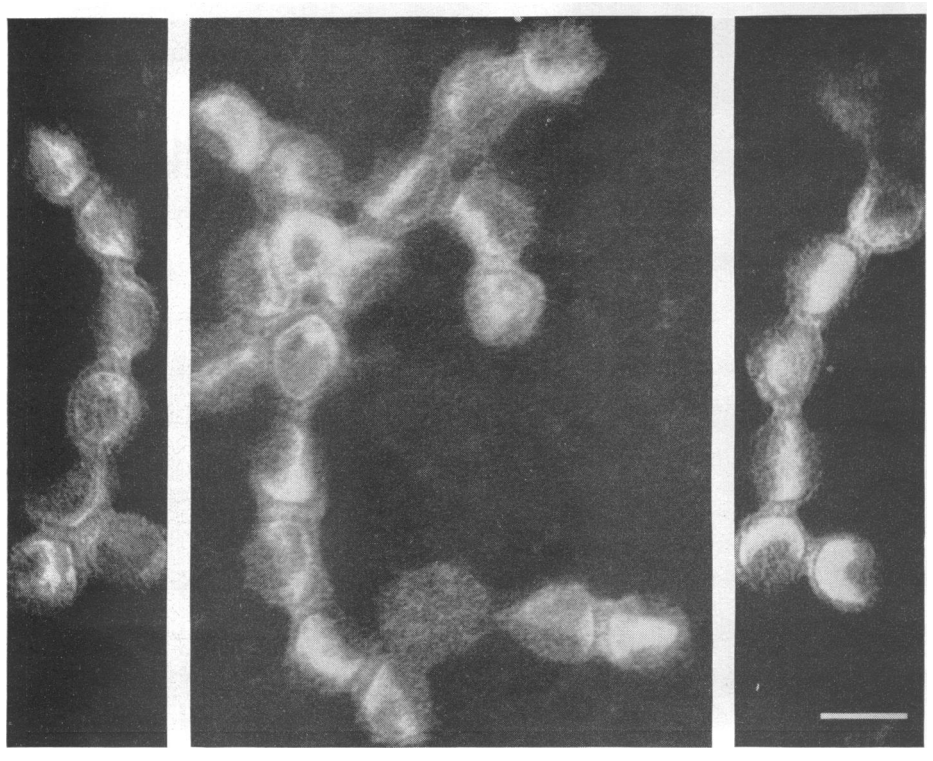

FIG. 6. *Herpesvirus aggregates (stained with uranyl acetate) showing chainlike arrangements of the particles.*

approximates the size of a virus core. (An incomplete but identifiable particle is seen above it.) Large and small aggregates or points of folding along DNA strands, such as this, were commonly encountered in our preparations. These structures may be the naked, more or less complete DNA cores around which protein subunits aggregate to form the capsid and eventually mature to be complete virus particles. It should be noted that nearly all the micrographs presented show multiple strandedness of the interconnecting filaments. Also, it was found that virus preparations which were purified by CsCl density gradient centrifugation showed aggregation by these strands. This indicates that this phenomenon is not an artifact produced by enzymatic degradation of particles.

Another of the more obvious features seen in crude tissue culture herpes preparations is the numerous enveloped forms (Wildy et al., 1960), as are shown in Fig. 9. Enveloped as well as naked particles were often connected by DNA strands. The percentage of enveloped forms in the total particle population usually was about 20 to 35%. Several experiments were done to determine the physical changes resulting from diethyl ether treatment of herpesvirus. In every case, the envelopes were almost completely destroyed or removed from the aqueous phase by brief treatment (5 min of shaking at room temperature). Infectivity dropped several log units or was completely destroyed, although neither the cores nor the capsids were substantially altered. This might suggest that the herpesvirus envelope plays some role in infectivity.

All herpesvirus populations which we examined contained particles with various degrees of fullness. The phosphotungstate negative stain and the uranyl acetate positive stain were extremely useful when applied separately for examination of herpesvirus structures. We found that particles not penetrated by phosphotungstate are often collapsed (Fig. 10, upper left), whereas partially or completely penetrated particles are better preserved. Enveloped forms, whether phosphotungstate-penetrated or not, were usually well

preserved but much of the fine structure was often hidden by the envelope. Specimens in which the majority of particles were stained intensely in the core area by uranyl acetate treatment showed predominantly unpenetrated particles when stained with phosphotungstate. The reverse was true in specimens in which particles stained poorly in their core areas with uranyl acetate (Smith and Melnick, 1962*a*). We found that negative staining gave excellent resolution of capsid substructure but was less desirable for revealing degrees of core completion. Uranyl acetate stained the capsid poorly but gave much more subtle resolution of core structures than did phosphotungstate. For these reasons, separate negative and positive stains were made of most specimens for morphological cross reference and counting.

*Particle counts and infectivity titrations during a single growth cycle.* A number of one-step growth curve experiments were done at temperatures ranging from 34 to 37 C. An effort was made in each of these experiments to use effective multiplicities of greater than four to infect nearly every cell and minimize the effects of secondary growth cycles. In one rather large experiment, virus growth at 34 and 37 C was compared. In this experiment, human lung fibroblasts were planted in bottles, allowed to grow to full monolayers (which required 3 days), selected for uniformity, carefully drained of nutrient fluid, and the cells from three bottles were counted. The average number of cells per bottle was $1.21 \times 10^6$. Recently passed herpesvirus was sonically treated, and 0.20-ml volumes of undiluted suspension were inoculated onto each of the monolayers. After 2 hr of incubation at 34 C, the inoculum was removed, bottles were washed two times with nutrient fluid, and 4.0 ml of nutrient fluid were replaced in each bottle. Half of the bottles were incubated at 34 C and half at 37 C. At appropriate times after inoculation, duplicate bottles were frozen at −60 C and stored at that temperature until titrated and counted at the end of the experiment. To titrate the inoculum and determine the precise

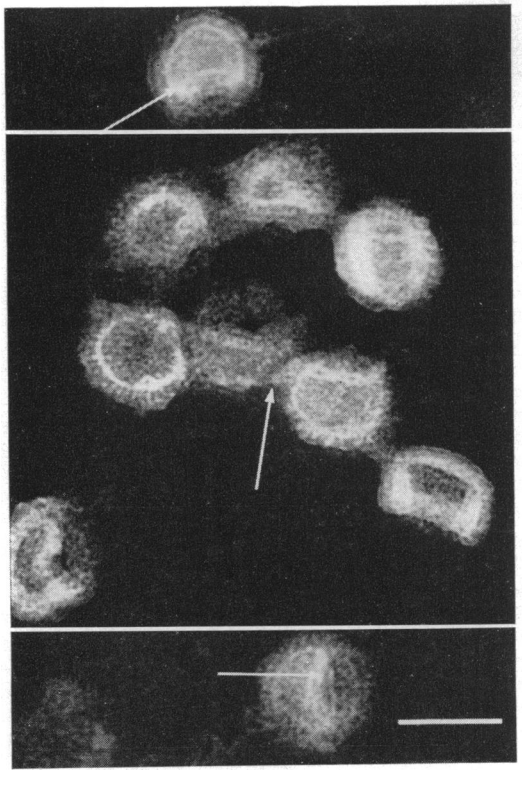

FIG. 7. *Herpesvirus (stained with uranyl acetate), showing arrangement of the filamentous structures in the core areas.*

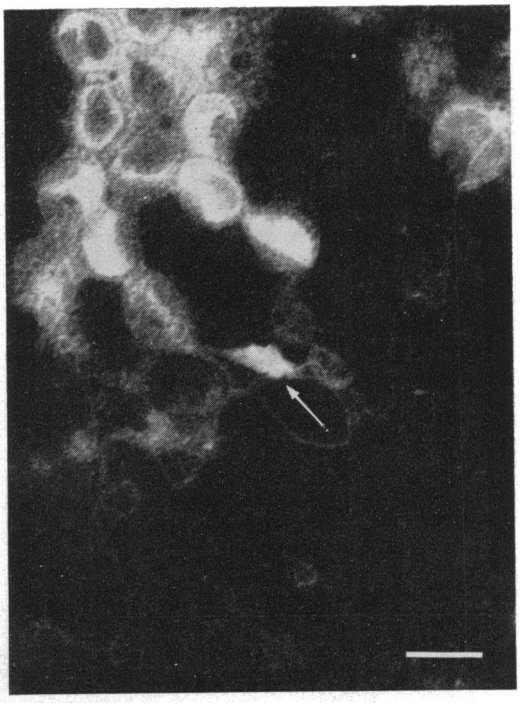

FIG. 8. *Herpesvirus (stained with uranyl acetate), showing loops and what appear to be naked cores along the length of long filamentous structures.*

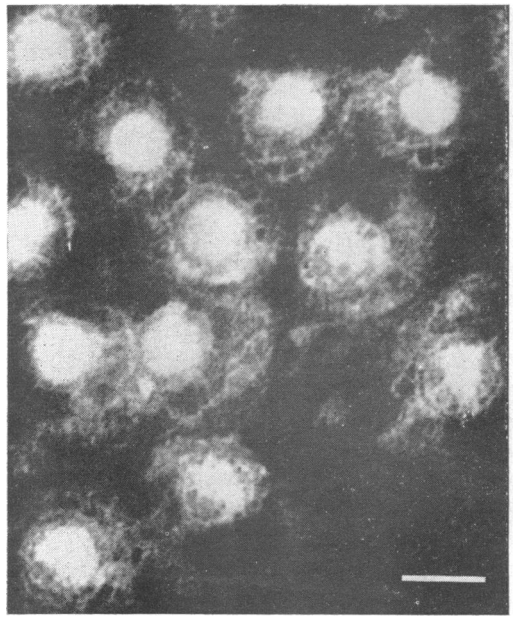

FIG. 9. *Herpesvirus (stained with uranyl acetate), showing enveloped forms.*

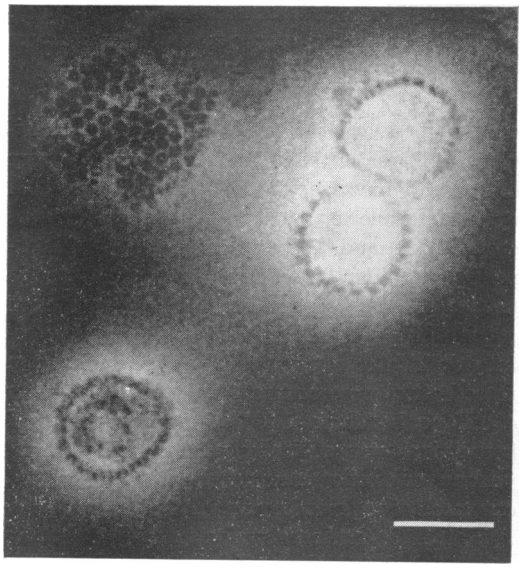

FIG. 10. *Herpesvirus (negatively stained with potassium phosphotungstate).*

*effective* multiplicity of infection, serial dilutions were made; 0.10- and 0.20-ml volumes were inoculated onto duplicate monolayers, and these were incubated with bottles in the main experiment. The inoculum titration was completed as described for plaque titration in Materials and Methods, with the following results. In the $10^{-5}$ dilution, an average of 84 plaques was counted in the bottles inoculated with 0.10 ml and 118 in the bottles inoculated with 0.20 ml. The *effective* multiplicity of infection was, therefore, $118 \times 10^5 \div 1.21 \times 10^6$, or almost exactly 10. The relative inefficiency of using larger volumes of inoculum is clearly demonstrated in the fact that

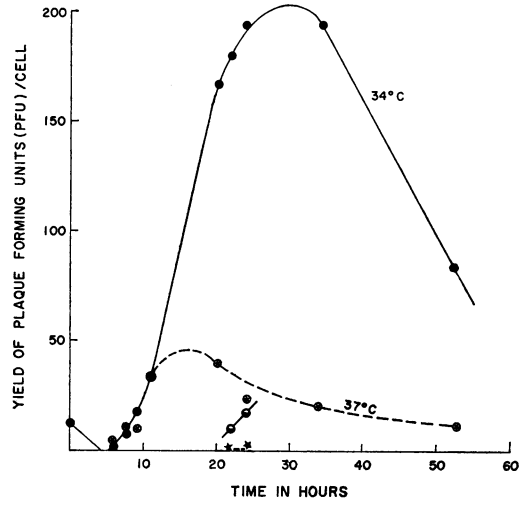


FIG. 11. *Growth of herpes simplex virus in human fibroblasts. Symbols:* ●, *total infectious virus at 34 C;* ◒, *extracellular infectious virus at 34 C;* ⊛, *total infectious virus at 37 C;* ★, *extracellular infectious virus at 37 C.*

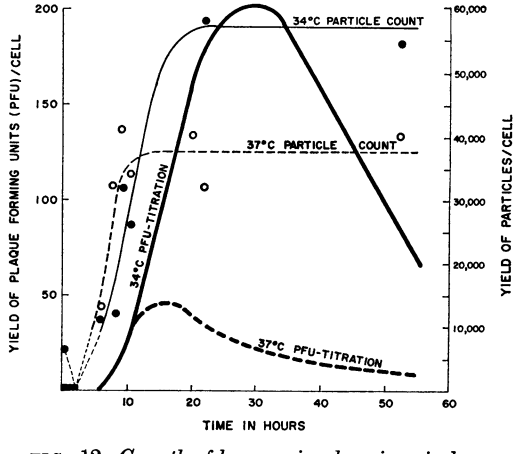


FIG. 12. *Growth of herpes simplex virus in human fibroblasts. Heavy lines indicate infectious virus;* ● *and* ○, *total particle counts.*

0.20-ml volumes, or twice the volume of virus suspension used for routine assay (0.10 ml), gave only 1.4 times more plaques after 2 hr of adsorption. Larger volumes were somewhat useful, however, in increasing effective multiplicities by a rather modest factor.

The results of this experiment, typical of others, are shown in Fig. 11 and 12. Based on past experience, samples were chosen to focus attention upon the earlier parts of the growth cycle, the peak yield period, and periods after thermal inactivation had substantially reduced infectivity. Figure 11 shows the results of infectivity titrations. (Zero time was designated as the time at which virus was introduced, not the end of adsorption.) The general aspects of the curves are somewhat similar to one-step growth curves of herpesvirus described by Hoggan and Roizman (1959). Early growth at 34 and 37 C was almost identical, the curves superimposing for the first 12 hr. However, the peak infectivity at 37 C was reached somewhat earlier than at 34 C, and the yield was markedly less. The period of infectious virus synthesis at 34 C extended over a period approximately twice as long and resulted in a peak infectious unit yield about four times as large as at 37 C.

Extracellular infectious virus was estimated at 22 and 24 hr by carefully removing 2.0 ml of supernatant fluid, centrifuging at 2000 $\times$ $g$ for 10 min in conical tubes to remove dislodged cells, and removing the top half of the fluid for titration. The two lower curves in Fig. 11 show the rather small amount detected. It is apparent that over 90% of the infectious virus is intracellular at or slightly after the peak of herpesvirus growth at either temperature. This is in good agreement with the basic observations of Hoggan and Roizman (1959).

Figure 12 shows the same infectivity curves in heavy lines (without points) and, for comparison, the data obtained from particle counts. Physical particle production (right scale) and infectious virus production (left scale) were plotted so that their peaks were approximately the same. It is clear that increases in countable virus particles occurred somewhat earlier and continued for a shorter period of time than did increases in the amounts of infectious virus. This is easily seen in the growth curves at 34 C, in which the particle count and infectivity curves are almost exactly parallel. Note that the particle count curves precede the infectivity curves in time by about 5 to 6 hr.

The particle-PFU ratio at the peak of infectivity at 34 C was about 300. It is seen in the graph that the yield of particles at 37 C was about 38,000 per cell, or two-thirds the yield at 34 C; yet the infectious virus yield was only one-fourth that at the lower temperature. The particle-PFU ratio at the peak of infectivity at 37 C was over 800. It is apparent from these data that the most meaningful particle-PFU ratios of herpesvirus would be obtained with virus grown at specified temperatures and harvested as near the peak of infectivity as possible. At points substantially earlier than this, the ratio would be high, possibly because of incomplete maturation of the partially formed but identifiable particles (800 at 10 hr, 34 C). At points substantially later, the ratio would be high because of thermal inactivation of the virus (over 800 at 53 hr, 34 C). As shown in Fig. 12, the particle concentrations remained almost constant for many hours after peak production, and after substantial thermal inactivation of the virus had taken place.

The morphology of the particles was scrutinized at various points during the growth cycle. At 6 hr, the particles were low in concentration, quite ragged in appearance, and rarely enveloped, and only occasionally were extra-particle DNA strands seen. At 7.5 hr, many particles were enveloped, and numerous DNA strands were seen. From this point on, the particle numbers increased rapidly; about the same fraction of particles were enveloped (25 to 30%), and large numbers of extra-particle strands were present in all specimens. Roughly the same quantity of extraviral DNA strands was seen in specimens taken at various periods until the peak of virus production. (Quantitation of these strands would be difficult.) It appears, then, that large excesses of unassembled DNA are present at all times after particle production begins. There seems to have been a slight shift toward a higher state of core completion during the latter part of the infectious virus production. Particles appeared morphologically most perfect after the peak of infectious virus had been reached. The state of fullness did not seem to change significantly during thermal inactivation, and particles were morphologically almost identical to those seen at the infectivity peak. The extent of particle core completion at

the peaks of the growth cycles at 34 and 37 C was approximately the same.

## DISCUSSION

Osterhourt and Tamm (1959) reported differences in the susceptibilities of human amnion cells from different embryos to herpesvirus. Our experience with human lung fibroblasts and rabbit kidney cells has been quite to the contrary, however. We found a remarkable consistency among the susceptibilities of four different human lung fibroblast lines, and the susceptibility of human fibroblasts was equal to that of rabbit kidney cells. This was extremely fortunate, since our experiments extended over several months and the use of one particular line was often inconvenient.

Production of infectious herpesvirus at 34 C was vastly superior to that at 37 C, which is in agreement with the observations of others (Farnham and Newton, 1959; Hoggan and Roizman, 1959). The physical requirements for virus production at 37 C seem to be sufficient, however, since the particle yield at that temperature was nearly two-thirds that at 34 C and the degree of core completion at the peak of 37 C virus growth was approximately that at the lower temperature.

The biological activity of virus grown at 37 C was very poor, only one in about 800 particles being infectious. One-step growth curves run at 35.5 C, not described here, gave peak yields intermediate between those obtained at 34 and 37 C. The reason for the inefficiency of infectious virus production at the higher temperatures remains unknown, but it does not appear to result from a quantitative inadequacy of structural components.

One of the mechanisms of herpesvirus aggregation seems to be connection of particles by DNA strands. Extremely long, loosely connected chains of particles are often seen, like beads on a string, and these sometimes stretch for several microns. The morphological observations indicate a structural continuity between the cores of different particles through these strands. We have made similar observations with SV-40 virus (Smith and Wallis, *in preparation*). Peters (1962) demonstrated the release of vaccinial DNA by enzymatic digestion. Noyes (1962) has recently described the release of filamentous structures from vaccinia virus (by ultrasonic degradation) which are thought to be DNA molecules. Kleinschmidt (1962) demonstrated viral and bacterial DNA strands microscopically by metal shadowing. These structures closely resemble those we have seen in herpesvirus preparations.

At all stages in the growth cycle after 7 hr, many DNA strands were apparent. Often loops, circles, and fairly large naked aggregates of DNA could be seen at intervals along these strands (Fig. 8). It is difficult to understand how DNA could be incorporated or folded into a protein capsid which had already formed completely; therefore, it seems likely that the aggregation or folding of DNA into a core takes place first, and then a protein capsid is formed around it by the assembly of capsomere subunits in a more or less orderly fashion. Alternatively, the folding may take place simultaneously with the formation of the capsid. This hypothesis is supported by the observations that the protein coats of the earliest countable particles were quite ragged in appearance, that there was a 5- to 6-hr lag between the appearance of physically recognizable particles and infectious virus, and that the physically most perfect protein capsids were seen late in the cycle.

One of the explanations for the large disparity between particle counts and infectivity titrations of herpesviruses may be the failure of capsids to form around sufficiently long strands of viral DNA so as to inclose a complete viral genome within the particle. However, even at the peak of infectivity in the one-step growth curves described in Fig. 11 and 12, only about one-fourth of the particles appeared to be complete in regard to their cores. Aggregation may cause an apparent lower quality, but certainly by no greater factor than 10 (probably 3 to 6). The rather high average particle-PFU ratio observed (370) for the VA strain of herpesvirus cannot be entirely explained, therefore, on the basis of particle aggregation or quantitative incompleteness of the cores. It is possible that some particles, although they contain what appears to be the near-maximal amount of core DNA, may not contain the proper segment of unbroken DNA which bears the entire message of the viral genome. Although the length of such a complete genome is presently unknown, it seems likely that single particles which have physically incomplete cores would be noninfectious. However, the beadlike linear arrangement of such incomplete particles may play a significant role in infection. Attachment to a cell and penetration of one particle in a chain could ultimately result in

the gradual incorporation of several connected incomplete particles. Each particle may contain only part of the genome, but several together might contribute all that is required for infection.

Comparative studies have been made of the particle-PFU ratios of VA herpesvirus, which has a rather high average (370), and another strain obtained through the kindness of Herbert Kaufman. The latter strain has a much lower average ratio. Differences in morphology, adsorptive properties, and biological characteristics were studied, and will be reported elsewhere. It should be mentioned here, however, that to date ratios of less than 30 were obtained only when the Kaufman strain was centrifuged to eliminate large cell debris and virus aggregates from suspension. This compares closely with results reported by Watson (1962) with precentrifuged herpesvirus suspensions. Large masses of virus are lost by this procedure (Smith and Benyesh-Melnick, 1961), however, and we feel that it should be carefully avoided whenever it is desirable to learn the total amount of virus produced per cell. The current trend toward expressing virus titrations in terms of yield per cell makes this even more essential.

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