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NOTES

Concentration of Herpesviruses

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A method has been developed for the concentration of herpesviruses by negative pressure ultrafiltration through dialysis tubing. The procedure results in high titer concentrates of unaggregated, morphologically intact viral particles with a virtually quantitative recovery of infectivity.

Many of the herpesviruses do not replicate to high titers in culture (2, 9), and for most experimental purposes some form of concentration is necessary. We report on a method for the production of high titer concentrates of two herpesviruses, strain HF herpes simplex virus (HSV) and strain AD-169 cytomegalovirus (CMV). Such concentrates are suitable for use as viral inocula, or, if combined with appropriate purification procedures, for

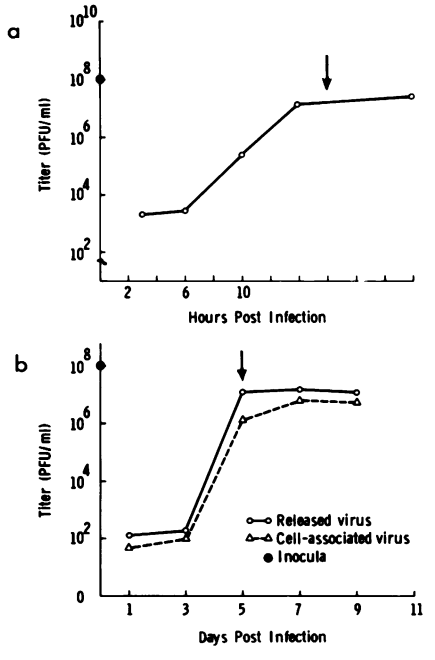

FIG. 1. *Growth curves for HSV (a) and CMV (b). Monolayers were infected with pretitered concentrated inocula of cell-free virus and, after adsorption, they were incubated with 1 ml of medium per 10^5 cells. For assay of cell-associated CMV, 2×10^5 cells were homogenized in 2 ml of medium. The arrows designate the times at which cultures were harvested for concentration.*

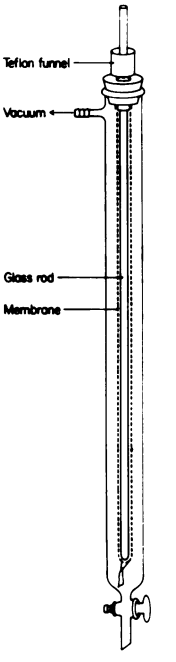

FIG. 2. *Concentration apparatus. The membrane is Visking cellophane dialysis tubing, size 8, which can withstand the vacuum (40 mm of mercury) without additional support. The tubing is knotted at one end. The other is stretched by hydraulic pressure and fitted over the bottom of the hollow teflon plug. The glass rod is used to maintain a high surface-to-volume ratio and therefore a high flow rate (approximately 15 ml/hr), during the later stages of concentration. Approximately 1/10 true size.*

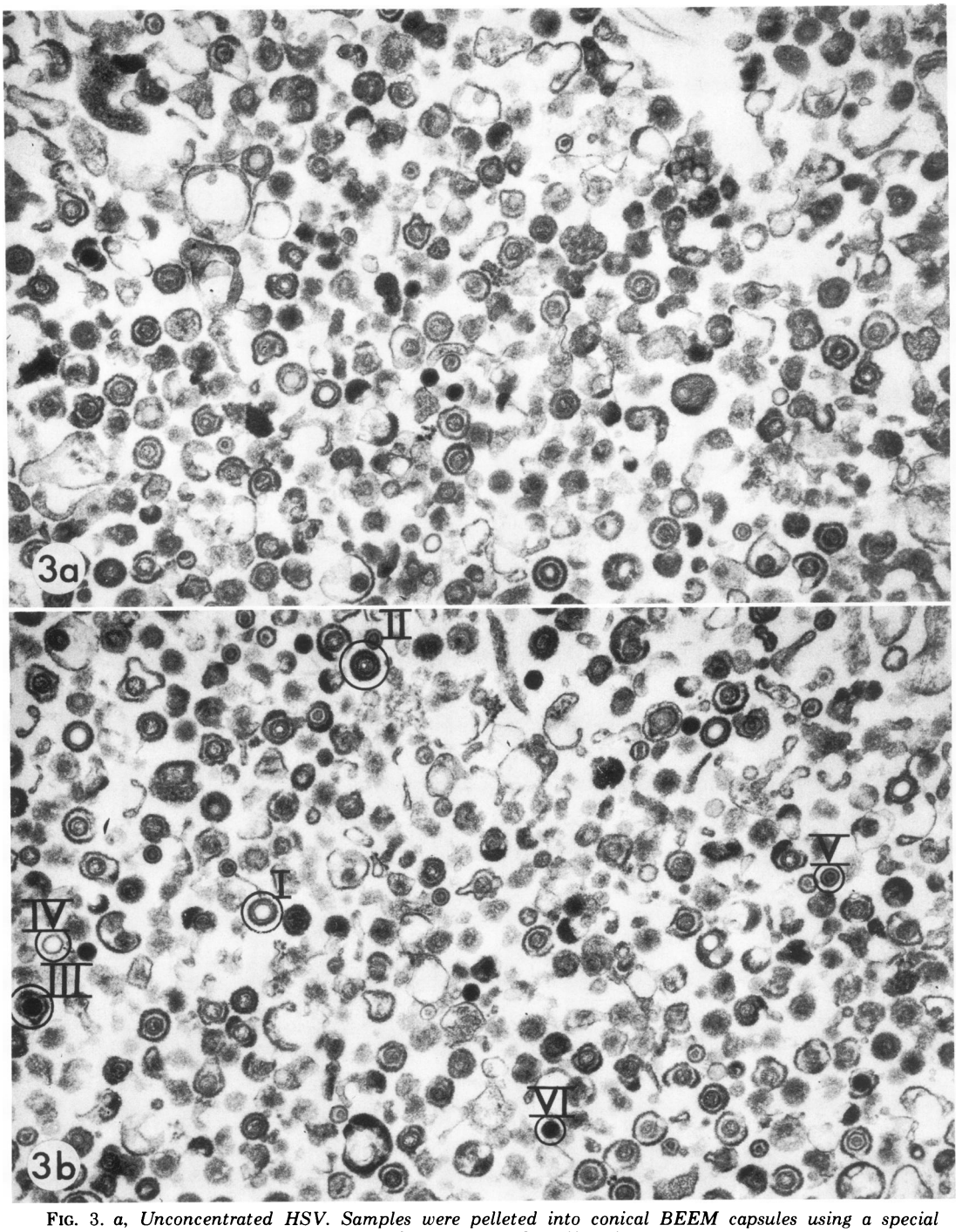

FIG. 3. *a, Unconcentrated HSV. Samples were pelleted into conical BEEM capsules using a special adaptor (BEEM, Inc., Bronx, N.Y.) for the SW27.1 rotor. Centrifugation was at 29,300 × g at the mid-point of the bucket, for 2 hr. Pellets were fixed with glutaraldehyde and osmium tetroxide by standard techniques and examined by electron microscopy. 26,400×. b, The same sample as Fig. 3a following 20-fold concentration. It is evident that the morphology of the concentrated virions is very similar to that of the unconcentrated sample. The Roman numerals refer to the six basic particle types used in the statistical comparisons. They are as follows: enveloped particles with "empty" (I), intermediate (II), or dense (III) capsid interiors, and the corresponding unenveloped forms with "empty" (IV), intermediate (V), or dense (VI) capsid interiors. 26,400×.*

biochemical and serological characterization of the virus. Unlike many other techniques (1-3, 5-8), this method avoids chemical treatment, aggregation, or morphological damage to the virus and gives a high recovery of infectivity.

Both herpesviruses were grown in monolayers of WI-38 cells, which were harvested either at 16 hr (HSV) or 5 days (CMV) postinfection. At these times the titers, as determined by plaque assay (11, 12), approached plateau levels (Fig. 1). Infected cells were scraped off the culture flasks, pelleted, and resuspended at density of 10^6 (HSV) or 3×10^6 (CMV) cells/ml in minimal essential medium without serum. For HSV the bicarbonate concentration was 7.7 mM, whereas for CMV, bicarbonate was omitted due to its inactivating effect (9, 10). Cells were disrupted either by homogenization or by three cycles of freezing and thawing. The suspension was clarified by low-speed centrifugation and filtered through two prewashed Millipore membranes with pore sizes of 0.8 and then 0.45 μm. This clarified suspension contained viruses, membrane fragments, and ribosomes, but was free of mitochondria, nuclei, and other large cellular debris.

The unconcentrated suspension was placed in the membrane sac of the apparatus shown in Fig. 2. This apparatus was originally developed (4) and applied to protein concentration by Lyman Craig. For large volumes, it may be equipped with an auxiliary reservoir.

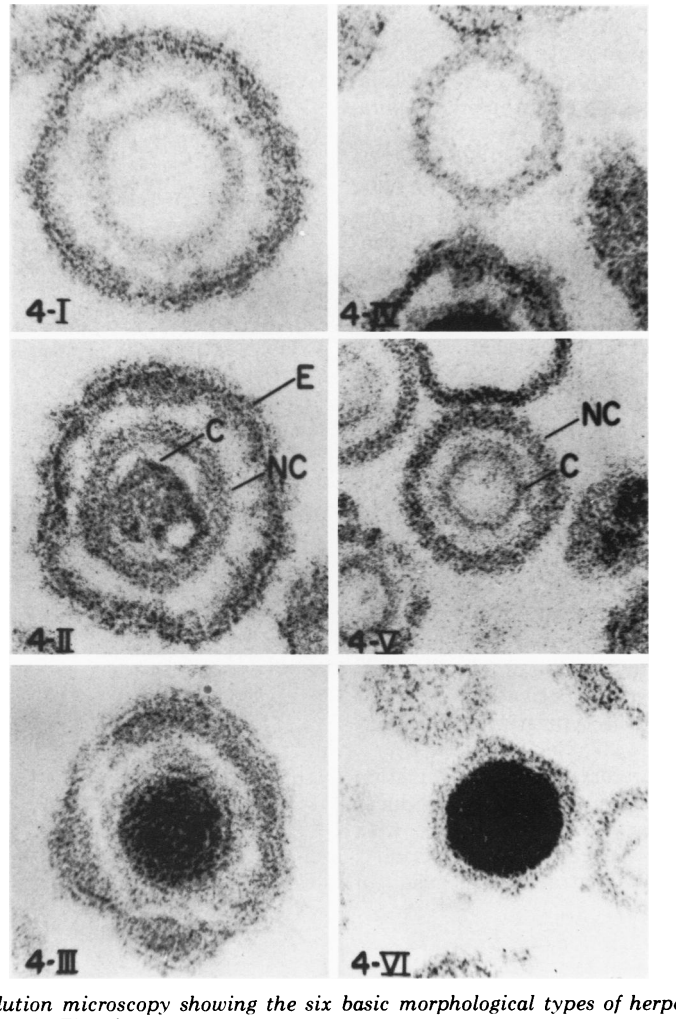

FIG. 4. *High resolution microscopy showing the six basic morphological types of herpesvirus corresponding to those illustrated in Fig. 3b. 137,000×.*

Concentration separates the original volume into two fractions: the ultrafiltrate, i.e., that which has passed through the membrane; and the concentrated retentate which remains within the sac. Due to the small pore size of the membrane, herpesviruses are completely retained and thus gradually concentrated as the volume within the sac falls.

The efficiency of concentration was judged by four criteria. (i) Plaque assay: no infectivity was detected in the ultrafiltrate, whereas the recovery of infectivity in the concentrated retentate was virtually quantitative. After 20-fold concentration, the recovery of HSV averaged 99%, and that of CMV averaged 85 to 90%. Similar results were achieved over a 10 to 60-fold range of concentration.

(ii) Negative staining: as expected from the high recovery of infectivity, negative staining of the concentrated retentates with 2% aqueous sodium phosphotungstate (*p*H 6.0) showed little aggregation of the particles. No particles were observed in negatively stained preparations of the ultrafiltrates.

(iii) Thin sections of viral pellets: Fig. 3a shows an unconcentrated suspension of HSV and Fig. 3b shows the same sample after concentration. The morphology of the concentrated viral particles appears unchanged as a result of this procedure.

(iv) Statistical comparison: a variety of morphological types are present in such herpesvirus preparations. In fact, at least six distinct forms, illustrated in Fig. 3b and 4 can be defined. Particles (500) in unconcentrated suspensions and in the corresponding retentates of HSV were counted and classified into one of these six types. The resulting frequencies were analyzed as a multinomial population with six classes, by using the chi square test. No significant differences between the two viral populations emerged even when the rejection region, for a hypothesis of identity, was set at the very high value of 0.5. This implies that there was no selective disruption or adsorption of any viral type during the concentration.

We conclude, therefore, that concentration by negative pressure ultrafiltration produces high titer preparations of herpesviruses with virtually quantitative recovery of infectivity, and that such concentrates, because of their morphological integrity and freedom from aggregation, are ideally suited for electron microscopy observation of viral structure, for quantitative studies, and for investigations requiring high multiplicities of infection.

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LITERATURE CITED

1. Abodeely, R. A., L. A. Lawson, and C. C. Randall. 1970. Morphology and entry of enveloped and deenveloped equine abortion (herpes) virus. J. Virol. **5**:513-523.
2. Chambers, R. W., J. A. Rose, A. S. Rabson, H. E. Bond, and W. T. Hall. 1971. Propagation and purification of high-titer human cytomegalovirus. Appl. Microbiol. **22**:914-918.
3. Cline, G. B., C. E. Nunley, and N. G. Anderson. 1966. Improved continuous flow centrifugation with banding. Nature (London) **212**:487-489.
4. Craig, L. C. 1965. Differential dialysis, p. 35-74. *In* C. N. Reilly (ed.), Advances in analytical chemistry and instrumentation, vol. 4. Interscience Publishers, New York.
5. Dales, S., and H. Silverberg. 1969. Viropexis of herpes simplex virus by HeLa cells. Virology **37**:475-480.
6. McCombs, R. M. 1969. Concentration and purification of herpes-viruses (simplex, cytomegalo, and EB) in a zonal centrifuge. Appl. Microbiol. **17**:636-638.
7. Norcross, C., J. F. McCrea, and S. Angerer. 1963. Purification of herpes simplex virus with fluorocarbon and potassium tartrate density gradients. Virology **21**:522-525.
8. Roizman, B., and P. G. Spear. 1968. Preparation of herpes simplex virus of high titer. J. Virol. **2**:83-84.
9. Vonka, V., and M. Benyesh-Melnick. 1966. Interactions of human cytomegalovirus with human fibroblasts. J. Bacteriol. **91**:213-220.
10. Vonka, V., and M. Benyesh-Melnick. 1966. Thermoinactivation of human cytomegalovirus. J. Bacteriol. **91**:221-226.
11. Wentworth, B. B., and L. French. 1969. Plaque assay of *Herpesvirus hominis* on human embryonic fibroblasts. Proc. Soc. Exp. Biol. Med. **131**:588-592.
12. Wentworth, B. B., and L. French. 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. **135**:253-258.