

Counting Infectious Viral Particles in Tissue Culture

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Virologists look upon the research carried out with the bacteriophages of the T group (1) as establishing a pattern to be followed in the study of the kinetics of infection and genetics of other viruses. However, the lack of a precise method for the estimation of viral growth and the separation of the progeny of single organisms has been the most important stumbling block in the application of the results of bacteriophage research to animal viruses. Two attempts to develop such quantitative animal virus techniques will be described.

The first, a method developed by Dulbecco (2), is a faithful reproduction of the seeded agar plate used in the study of bacterial viruses. The virus must be highly destructive of host cells. The agent of Western equine encephalomyelitis was found to be the most suitable. The host cells are the chick embryo "fibroblasts," separated from other tissue elements by treatment with trypsin and repeated centrifugation. These cells, derived from all parts of decapitated 9-day-old chick embryos, do not form pure cultures, of course, but are uniformly susceptible to infection with many of the cyto-pathogenic viruses. Under appropriate conditions of cultivation, they will readily form a single sheet of cells covering the entire floor of a culture flask.

The Petri plates, inadequate for tissue culture work, are replaced by 80 mm. Carrel flasks. Single sheets of "fibroblasts" are formed therein after 48 hours' incubation of the cellular inoculum at 37° C. Uniform distribution and absorption of the virus to the cells is achieved by temporarily removing the nutrient fluids and covering the cells with a small volume (0.5 ml.) of viral inoculum. The virus is maintained in the immediate vicinity of the original site of cellular penetration by the inclusion of agar in the medium added after inoculation. After 3 days of additional incubation, clearings of lysed cells can readily be detected. They are analogous to the "plaques" produced by bacteriophages.

Several separate experiments indicate that the number of clearings is inversely proportional to the dilution of the virus. Therefore, it can be assumed that each clearing is produced by the progeny of a single virus particle. The results obtained by this method agree with those of routine chick embryo titration.

A second quantitative animal virus technique has been developed by Weiss and Huang (9). This method is quite different from Dulbecco's for it involves the microscopic counting of infected cells.

The virus chosen for study was the agent of feline pneumonitis, which is large enough to be detected in ordinary light microscopy and forms characteristic inclusion bodies (7). Unlike the virus of Western equine encephalomyelitis, however, it has a high degree of cellular specificity and will grow well, outside its normal host, only in the entodermal cells of the yolk sac.

The characteristics of the cells of the yolk sac cultured *in vitro* have been studied extensively by Grodzinski (3) and Thomas (6). Their

observations greatly facilitated the search for the area of the chick embryo which forms the largest colonies of entodermal cells *in vitro*. This area was finally located in a narrow band of the avascular membrane, just peripheral to the sinus terminalis of the 4-day-old embryo. Cleanly cut and properly embedded explants from this area, 2 mm. in diameter, give rise, in 4 days, to single layers of cells covering an area of approximately 1 cm². The cells of these colonies are typically large and vacuolated, containing white and yellow yolk, and, as far as it can be judged morphologically, are all of the same type. A mixture of one part chicken serum and three parts Hanks' balanced salt solution (4) is an adequate nutrient. The cultures thus prepared are ready for inoculation. The viral suspension is added and the cultures shaken for 4 hours.

A feline pneumonitis virus particle which successfully invades a cell develops in it for approximately 48 hours before lysing it and infecting other cells. The infected cells are readily recognized by ordinary microscopy. The viral inclusions resemble those described in the intact chick embryo (7, 8) and are readily distinguished from other viruses of the same group, such as the agent of murine pneumonitis virus. Cultures fixed and stained approximately 30 hours after inoculation, before any of the cells have lysed, reveal well-developed inclusion bodies which can be counted and serve as a gauge of the original inoculum.

Unlike the agent of Western equine encephalomyelitis, feline pneumonitis is not very virulent. Moulder and Gogolak (5) estimated that approximately 100 particles are necessary to initiate infection in the chick embryo. In tissue culture many factors affect the efficiency of infection. For example, the infected cell counts vary significantly in different embryos as well as in different areas of the same embryo. The composition and volume of the nutrient medium and the handling of the culture after inoculation also influences the results. Shaking the cultures gently for 4 hours increases the infected cell count. Most of the factors affecting the infected cell counts are probably still unknown, and are possibly responsible for the fairly large deviation from the mean still encountered in this procedure.

In spite of the above mentioned difficulties, the infected cell count method of estimation of the size of a viral inoculum, under the best conditions, is as sensitive as the routine chick embryo titration and considerably more accurate. Counts carried out in quadruplicate are usually able to reveal 3-fold and often 2-fold differences. It is also apparent that the counts are inversely proportional to the dilution of the virus and, therefore, indicate that a single successful particle is sufficient to start infection of a cell.

The details of the two methods described above are not comparable. Fibroblast cultures are obviously as unsatisfactory for feline pneumonitis virus as entodermal cell cultures for Western equine encephalomyelitis. Each method is adapted to a particular type of virus: the first for highly virulent organisms and the second for those which form inclusions. They greatly simplify the study of the relationship of these viruses to their host cells and offer the possibility of extending to animal viruses research previously confined to bacteriophages.

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