Multiplication of Newcastle Disease Virus in Chick Embryo Tissue Culture. (23430)

ALEXANDER KOHN AND ROBERT GOLDWASSER
(Introduced by A. L. Olitzki)
Israeli Institute for Biological Research, Ness-Ziona, Israel

Various results have been reported in studies of the quantitative aspects of multiplication of Newcastle disease virus (NDV) in chick embryos and in tissue cultures. For example, the latent period of virus growth in the chick embryo has been reported to be as short as 3 hours or as long as 15 hours(1-3). In tissue cultures of trypsinized chick embryo cells a latent period of 3 to 4 hours was found by one author(4) and a 24-hour period by another, working both with chick embryo and adult chicken lung tissues(5). In view of these discrepancies, it was felt that additional investigations were warranted.

Materials and methods. The glassware used for growth of the tissue cultures was heated in a muffle furnace to 500°C for one hour. The necessity of maintaining separate stocks of glassware for these purposes or the use of special detergents or acids in cleaning was thus obviated. *Virus.* The strain of NDV used was isolated in Israel and designated HP(6). Infected allantoic fluids from the 54-64th passage of the virus in embryonated eggs were used in all experiments here described. *Two media* were used: *M* Hank's balanced salt solution—100 ml, lactalbumin hydrolysate—0.5 g. *M-1* Hank's balanced salt solution 97 ml, lactalbumin hydrolysate—0.5 g, calf serum (from calves aged 6 months to one year)—2 ml. Each solution contained, in addition penicillin (100 units/ml) and streptomycin (50 μg/ml). Medium M-1 was used for growing of tissue cultures. When it was desired to maintain the cells without allowing them to multiply, medium M-1 was replaced by medium M. *Virus titration.* Virus was titrated in the allantoic sac of 9- to 11-day-old embryonated eggs. After infection eggs were incubated 72 hours, deaths were noted and all eggs tested for appearance of hemagglutinins (HA). Only those showing HA activity were considered as having been infected. Titers were calculated in accordance with the method of Reed and Muench(7) and were expressed as log (ELD_{50}) per 0.1 ml of virus suspension. *Preparation of tissue cultures.* Chick embryos were prepared in a manner similar to that described by Dulbecco(8). Nine or 10-day-old embryos were aseptically removed from the eggs and their heads and legs cut off. The embryos were then pressed through a 24-mesh stainless steel wire grid in the bottom of a 50 ml syringe into a vessel containing Earle's balanced salts solution. After 10 minutes (to allow the pulp to settle), the supernatant was sucked off and replaced with 0.2% trypsin in Earle's solution. The suspension was incubated in a water bath at 37°C for 20 minutes, dispersed with the aid of an automatic pipette, and filtered through an Evans filter(9). The filtrate was centrifuged at 3000 rpm for 3 minutes, the supernatant discarded, and the sedimented cells were washed once with Hank's salts solution. The concentration of the final cell suspension was determined in a hemocytometer. M-1 medium was added, to give a final concentration of $1x10^6$ cells per ml, and the suspensions were distributed in 10 ml amounts in sterile, 70 mm Petri dishes. Incubation was carried out at 37°C in an atmosphere containing 4% CO_2.

Results. Tissue cultures, prepared as above, were placed in the incubator. After 48 hours, the supernatant fluids were removed from 3 of the cultures and the cell layer was rinsed with warm phosphate buffered saline. Five ml of a trypsin in Earle's solution was added to each Petri dish, and the dishes were incubated at 37°C for 10 minutes. The cells thus removed from the surface of the glass were concentrated by centrifugation and counted in a hemacytometer. In 3 such experiments the cell count was found to average from 3 to $5x10^6$ cells per plate, and the maximum variations in any one experiment

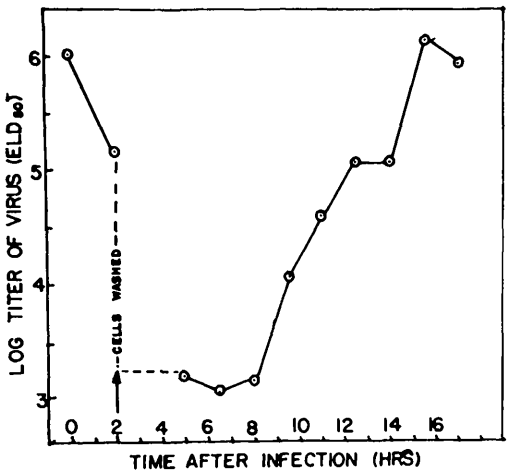

FIG. 1. Multiplication of NDV in tissue culture (chick fibroblasts).

were not larger than 30%.

The supernatant fluids from the remaining plates (usually 5) were removed and replaced with 1 ml of a suspension of NDV in M medium. The amount of virus added was one ELD_{50} to each 10-15 cells in the culture. After a period of time at 37°C to allow for adsorption, the supernatant fluids were removed, the plates were washed repeatedly with fresh medium, and finally 10 ml of medium M was added to each plate. The cultures were returned to the incubator and at various intervals of time, aliquots were taken from each culture, pooled and titrated for infectivity in chick embryos. Control plates showed degeneration of cells only after 4 days of incubation, while in monolayers infected with NDV, destruction of cells was already evident after 3 days of incubation. No attempt was made to correlate the appearance of virus in the medium with the morphological changes in single, virus-releasing cells(11).

The results of 2 such experiments are shown in Fig. 1 and 2. In the experiment represented by Fig. 1, the multiplicity of infection was 0.3, the time allowed for adsorption of virus 2 hours and the average number of cells per plate 3.3×10^6. The virus titer in the supernatant fluids of the cultures remained constant at low level until the sampling performed 9½ hours after infection. This titer probably represented the residual virus remaining after washing of the cultures following infection. The 9½ hour sample showed approximately a 10-fold rise in titer, which continued to rise steadily until 12½ hours after infection. A further rise in titer was detected in the 15½ hour sample.

In the experiment represented by Fig. 2, the multiplicity of infection was 0.07, the time allowed for adsorption 2 hours, and the average number of cells per culture was 3.8×10^6. Samplings were performed at longer intervals than in the former experiment, and were continued until 114 hours after infection. Virus release began 6-16 hours after infection and continued until the 34th hour after inoculation. After that time the virus titer remained more or less constant until the termination of the experiment. The average yield of chick embryo LD_{50} per cell in this experiment was calculated to be approximately 300.

Discussion. The results described above are in good agreement with those of Stulberg, Shapira and Todd(10), who, working with chick embryo fragments embedded in plasma, found that the latent period in the growth cycle of NDV lasted between 9 and 11 hours. However they differ widely from those reported by Levine and Sagik(4), who found that the latent period lasted 3 to 4 hours. Furthermore, the latter authors obtained an average yield of 19-37 virus particles per infected cell, whereas in the experiments described above, the average yield approximated 300 infective particles of NDV per cell in the culture. While it may be that the differences in yield might be due to strain differences in the virus, it seems more likely

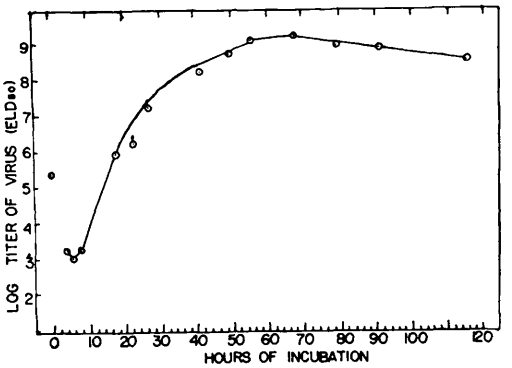

FIG. 2. Growth curve of NDV in tissue culture.

to be indicative of suboptimal conditions in the tissue culture system used in the above mentioned investigations.

It is possible that the reasons for these discrepancies are connected with physiological differences between "attached" and "unattached" cells. Observations made in this laboratory indicate that these differences may be important at least in the case of chick embryo fibroblasts. When cell suspensions were prepared with the aid of 0.25% trypsin, either from chick embryos or from monolayers, the cells failed to attach to the walls of the glass vessels in the absence of serum, retained their globular form, and did not show any obvious metabolic activities such as production of acid in the presence of glucose. In the presence of small amounts of either horse or calf serum (0.1%-0.2%) the same cells attached themselves to the glass walls, took on the typical shape of fibroblasts, and caused acidification of the medium in the presence of glucose, even after removal of the serum by means of washing, and addition of medium from which serum was excluded.

In the system described by Levine and Sagik, although serum was present in the media, other conditions were such that the cells remained in suspensions, *i.e.* in "unattached" form during the period of virus multiplication. It might be that these conditions led to a shortened latent period and lowered virus yield.

Summary and conclusions. The multiplication of cytopathogenic strain of Newcastle disease virus in monolayers of chick embryo cells was studied. The latent period in the growth cycle of this virus was found to last 8-9½ hours, followed by a period of rise lasting 3-4½ hours. The average yield of virus per cell in the tissue culture was found to be approximately 300.

1. Horsfall, F. L., Jr., *Science*, 1956, v123, 674.
2. Nadel, M. K., and Eisenstark, A., *J. Bact.*, 1955, v69, 173.
3. Gordon, L., Birkeland, J. M., and Dodd, M. C., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 205.
4. Levine, S., and Sagik, B. P., *Virology*, 1956, v2, 57.
5. Jones, A. L., *Brit. Abstr. Med. Sci.*, 1956, v3, 33.
6. Kohn, A., *Am. J. Vet. Res.*, 1955, v16, 450.
7. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
8. Dulbecco, R., *Proc. Nat. Acad. Sci., N. Y.*, 1952, v38, 747.
9. Evans, V., Earle, W. R., Sandford, K. K., and Shannon, J. E., *J. Nat. Cancer Inst.*, 1951, v11, 907.
10. Stulberg, G. S., Shapira, R., and Todd, H. C., *Bact. Proc.*, 1950, p68.
11. Lwoff, A., Dulbecco, R., Vogt, M., and Lwoff, M., *Virology*, 1955, v1, 128.

Received June 20, 1957. P.S.E.B.M., 1957, v96.

A Method for Continuous Intravenous Infusion of Dogs Confined to Cages.* (23431)

PAUL H. JORDAN, JR., AND BERNARD F. SAND (Introduced by Edward R. Woodward)
Surgical Service, Wadsworth General Hospital, Veterans Administration Center, and the Department of Surgery, University of California Medical Center, Los Angeles

The availability of intravenous fat emulsions satisfactory for clinical use has necessitated reevaluation of the efficacy of complete parenteral alimentation. The ideal conditions for investigation of this problem in dogs required that normal animals, unrestrained but confined to metabolic cages, receive intravenous fluids at a constant rate throughout each 24 hour period of study. To fulfill these conditions, it was necessary to devise a method for continuous administration of fluids which would protect the intravenous tubing against mechanical injury resulting from the dog's activity. The swivel developed by Jacobs(1) for the continuous infusion of dogs confined to a room was unsuitable for our work; how-

* This work was supported by U. S. Public Health Service Grant.