# STUDIES ON NEWCASTLE DISEASE VIRUS

## II. Behavior of the Virus in the Embryo

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A study of the growth and behavior of an individual virus in the developing chick embryo has two important aspects. The biologist who is endeavoring to understand the comparative pathology of various experimental infections must have detailed information on this point. The chemist or commercial immunologist who uses the egg more as a test tube needs to know under what conditions he can obtain the maximum titer of virus. Although a large proportion of the same basic information is useful to both groups, it is more from the point of view of comparative pathology that these data on the virus of Newcastle disease have been collected and will be analyzed.

### *Methods*

The general methods used are those reported in the first paper in this series. Most of the measurements have been made by titrations of embryo infectivity, since it is not certain that the activity of the virus to agglutinate red cells is inseparable from such infectivity.

### *Distribution of the Virus in the Embryo*

Burnet (1) demonstrated in the first studies of embryo infections with Newcastle virus that the final concentration in the allantoic fluid was higher if the embryo was inoculated in the allantoic sac, and higher in the amniotic fluid if this sac was inoculated. Since then it has clearly been shown that route of inoculation plays a large rôle in the amount of virus obtained from different parts of the embryo (2). Since this is so, any comparison of the distribution of the different viruses in the embryo must take into account the route of inoculation. Our comparison presented in Table I is useful in demonstrating that, following either inoculation of the chorioallantoic membrane or into the allantoic sac, there is about 100 times as much virus in the allantoic fluid as is present in the embryo. There is however a high concentration of virus in the embryo following such inoculations. Newcastle virus then may be placed between the encephalitis groups of viruses and the influenza group. Following membrane inoculation, the viruses of Eastern and Western encephalitis (3) and of Venezuelan encephalomyelitis (4) attain the highest titer in the embryo and chorio-

allantoic membrane. West Nile virus localizes in membrane and embryo regardless of route of inoculation (5). Japanese B encephalitis also attains a higher titer in the embryo when inoculated by yolk sac (6). Newcastle virus is like these encephalitic viruses in that it kills the embryo and has a high titer in the embryo when inoculated by membrane or allantoically. However, the concentration is not as high as that of the allantoic fluid, and Newcastle virus thus corresponds to the influenza group of viruses which, following inoculation into the allantoic sac, multiply rapidly and attain their highest concentration there. To summarize, we may say that Newcastle virus resembles the encephalitis group in its ability to spread throughout the developing egg and to attain a high concentration in the embryo, but that it resembles influenza virus in its high concentration within the allantoic fluid before death.

The distribution of a virus cannot be considered separately from the growth rate. Two factors known to have an effect on the growth rate in other embryo

TABLE I

*Distribution of Newcastle Virus in 11-Day-Old Developing Eggs*

| Route of inoculation | Allantoic fluid | Amniotic fluid | 10 per cent embryo |
|---|---|---|---|
| Allantoic sac | $10^{-9.5}$ | | $10^{-7.2}$ |
| " " | $10^{-9.2}$ | $10^{-8.2}$ | |
| " " | $10^{-9.5}$ | | $10^{-6.7}$ |
| Membrane | $10^{-7.8}$ | $10^{-5.5}$ | $10^{-6.2}$ |
| " | $10^{-9.5}$ | | $10^{-6.7}$ |

infections, (1) size of inoculum and (2) temperature of inoculation, have been studied in this disease.

It has been shown that inoculation of concentrated suspensions of influenza virus, of either A or B strain, will produce a lower final titer of virus in the allantoic fluid than will the inoculation of a more dilute suspension. This is apparently part of the large problem of interference by dead virus (7). Such an effect has not been found in the case of Japanese B encephalitis (6). Present studies on Newcastle virus fail to show any effect of the size of inoculum on the amount of virus finally obtained (Table II). Indeed the larger inoculum produced a larger amount of virus earlier in the course of the infection, and in consequence killed the embryo earlier. Following minimal inocula the same high titer was obtained later and death was delayed.

### *Effect of Temperature on Rate of Growth*

The effect of temperature of incubation on the growth of a number of viruses has been recently summarized (8) and it has been pointed out that "chick embryos are more susceptible when incubated at 35 to 37°C. than at 39°C."

This generalization does not hold for Newcastle virus as shown in Table III. Indeed such an effect is not to be expected, for this virus must multiply in a

TABLE II

*Effect of Size of Inoculum on Yield of Virus Incubated at 37° C.*

| Experiment No. | Time of incubation | 50 per cent end-point of allantoic fluid | | | |
|---|---|---|---|---|---|
| | | Inoculum | | | |
| | | $10^{-0}$ | $10^{-2}$ | $10^{-4}$ | $10^{-7}$ |
| | *hrs.* | | | | |
| | 6 | $10^{-5.4}$ | $10^{-3.4}$ | $10^{-2.4}$ | |
| 1 | 24 | $10^{-8.3}$ | $10^{-8.0}$ | $10^{-7.3}$ | |
| | 43 | $10^{-9.3}$ | $10^{-9.3}$ | $10^{-8.0}$ | |
| | $11\frac{1}{2}$ | | | $10^{-4.8}$ | $10^{-1.8}$ |
| 2 | 22 | | | $10^{-8.0}$ | $10^{-5.3}$ |
| | 46 | | | $10^{-8.5}$ | $10^{-9.2}$ |
| | 71 | | | | $10^{-9.5}$ |

TABLE III

*Effect of Temperature of Incubation on the Yield of Newcastle Virus from the Allantoic Fluid of 11-Day-Old Embryos Inoculated into the Allantoic Sac*

| Experiment No. | Dilution of virus inoculated | Time of incubation | 35°C. | | 37°C. | | 39°C. | | 40°C. | | 41°C. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Embryo infectivity | Red cell titer | Embryo infectivity | Red cell titer | Embryo infectivity | Red cell titer | Embryo infectivity | Red cell titer | Embryo infectivity | Red cell titer |
| | | *hrs.* | | | | | | | | | | |
| 1 | $10^{-2}$ | 48 | $10^{-8.5}$ | 1/800 | $10^{-9.5}$ | 1/800 | $10^{-9.7}$ | 1/1600 | | | | |
| 2 | $10^{-2}$ | 40 | $10^{-8.7}$ | 1/6400 | | | $10^{-8.7}$ | 1/6400 | | | $10^{-9+}$ | 1/6400 |
| 3 | $10^{-1}$ | 41 | $10^{-8.5}$ | | $10^{-8.8}$ | | | | $10^{-8.7}$ | | | |
| 4 | $10^{-3}$ | 48 | $10^{-8.5}$ | | | | | | | | $10^{-9.4}$ | |
| 5 | $10^{-4}$ | $8\frac{1}{2}$ | $10^{-3.4}$ | | | | | | $10^{-5.0}$ | | | |
| | | $18\frac{1}{2}$ | $10^{-6.5}$ | | | | | | $10^{-8.0}$ | | | |
| | | 26 | $10^{-7.6}$ | | | | | | $10^{-8.6}$ | | | |

host (chicken) which normally has a rectal temperature of 40 to 41°C. Furthermore, studies of the influence of temperature on the yield of virus obtained in the influenza-encephalitis group of viruses have usually not been related to time. Encephalitis virus will grow better at 37° than at 42°C., but initial growth rates seem to be about the same (3). Vesicular stomatitis grows better at 35–36°C.

than at 39–40°C., but again initial growth rates in both 7- and 10-day embryos are about the same (9). The virus of influenza B does better at 35°C. than at either 37° or 39°C., but the initial growth rates at 35° and 37°C. are very similar (10). This suggests that the relation of temperature of incubation to the growth of viruses in the embryo is not a simple problem of optimum temperature of incubation but that complicated host-parasite relations may play a large rôle (8).

Further data on the effect of temperature of incubation on the growth rate of Newcastle virus are presented in Chart 1. It may be noted that growth at

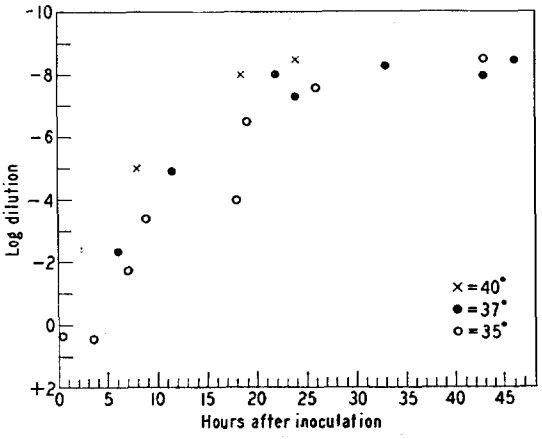


CHART 1. The relation of temperature of incubation to the growth rate of Newcastle virus in the allantoic sac of 11-day-old chicken embryos. All embryos were inoculated with a $10^{-4}$ dilution of freshly harvested allantoic fluid. Each point represents the titer obtained by pooling several embryos. The chart is a composite, but individual experiments (Table III, Experiment 5) demonstrate clearly the effect of higher temperatures. The titers below a log dilution $10^{-0}$ were obtained by inoculating 10 drops of the undiluted fluid to be tested ($10^{+1}$ dilution).

40°C. is more rapid than at 37°C. and this in general more rapid than at 35°C., but that the final titer is about the same (Table III). As with other embryo infections (3, 4, 9–11), and some other animal virus infections initial growth through a logarithmic (12, 13) phase occurs without pathological changes, and the highest titer of virus may be obtained before pathological changes set in.

In the succeeding paper certain attempts to separate the red cell–agglutinating activity of Newcastle virus from the embryo infectivity are presented. It may be worth giving here a detailed record of the rise in red cell titer in the allantoic fluid and its relation to the infectious titer of the fluid. Henle and Henle (7) have shown in the case of influenza infections that correlations between the content of active virus in allantoic fluid and hemagglutinin titer may exist only during the stage of rapid increase of the active virus (logarithmic

phase of growth) but not after the active virus titer has reached its peak and started to decrease. Presumably this is related to loss of embryo infectivity (death) without loss of hemagglutinin activity. In comparing these results with those for the Newcastle virus we must remember that embryos infected with influenza virus frequently live for some days after inoculation and after the maximum titer of virus is obtained in the allantoic fluid. This is not equally true of Newcastle virus, for death occurs sooner after its maximum titer has been obtained. It is also more stable than influenza virus. Therefore, the relation of embryo infectivity to hemagglutinin activity after death would not be dependent upon peculiar host-parasite relations but merely would mirror the persistence of the more stable hemagglutinin characteristics as contrasted with embryo infectivity. Hence we cannot present any significant data on Newcastle virus after 48 hours (with dilute inoculum 72 hours).

Table IV presents a summary of our data on the relation of these two characteristics during the logarithmic phase of growth, following the inoculation of different concentrations of virus. When the known error in the method of determining embryo infectivity is considered (only three embryos were used in each dilution in this test) it is seen that we can conclude only that there is a concomitant increase in red cell–agglutinating activity and in embryo infectivity, and that the geometric rate of increase of hemagglutins ceases at about the same time as does the embryo infectivity (20 to 25 hours) (Chart 1). Thus there is in our study no evidence of a separation of the two functions.

The question of a latent period before the logarithmic phase of growth begins was considered. The data in Chart 1 are inadequate to a decision. The embryos were inoculated with $\frac{1}{20}$ cc. of a $10^{-4}$ dilution of allantoic fluid, and this means that each embryo received about 50,000 $LD_{50}$ doses. This was diluted by a factor of $\frac{1}{20}$ cc. in 50 cc. (volume of egg) or $\frac{1}{1000}$, which means that a drop of test inoculum from a recently inoculated embryo would contain a maximum of 50 $LD_{50}$ infectious units. These calculations take no account of the probable fixation of the virus on the tissue. It follows that although in the few hours after inoculation of the virus into the allantoic sac we did not recover the "expected" amount (see Table V), we cannot conclude that there was a latent period before its proliferation. Any virus formed may have been fixed in the tissues.

### *Effect of Age of Embryo*

In the preceding paper, it was noted that there was relatively little effect of age on the susceptibility to the virus. This, however, would not mean that the same total amount of virus was obtainable from allantoic fluid of eggs of different ages. The point was tested by the inoculation of 10-, 12-, and 14-day-old embryos into the allantoic sac (Table VI). The fluid was harvested from two to five embryos of each age, and titrations of red cell agglutination indi-

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*Rate of Increase of Virus in Allantoic*

| Time | Egg No. | Undiluted allantoic fluid: Red cell agglutinin of harvested allantoic fluid 1/10 | 1/100 | 1/200 | 1/400 | 1/800 | 1/1600 | 1/3200 | 1/6400 | 1/12800 | Embryo infectivity of pool | Egg No. | Red cell agglutini 1/10 | 1/100 | 1/200 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *hrs.* | | | | | | | | | | | | | | | |
| | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 0 | 0 |
| 6 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $10^{-5.2}$ | 2 | 0 | 0 | 0 |
| | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 3 | 0 | 0 | 0 |
| | 1 | 0 | + | ++ | +++ | ++ | + | 0 | | | | 1 | +++ | 0 | 0 |
| 17 | 2 | 0 | +++ | +++ | + | 0 | 0 | 0 | | | | 2 | + | 0 | 0 |
| | 3 | 0 | +++ | +++ | ++ | 0 | 0 | 0 | | | | 3 | + | 0 | 0 |
| | 1 | 0 | 0 | +++ | +++ | +++ | + | 0 | | | | 1 | 0 | +++ | +++ |
| 24 | 2 | 0 | 0 | +++ | +++ | +++ | ++ | 0 | | | $10^{-5.5}$ | 2 | 0 | ++ | ++ |
| | 3 | 0 | 0 | +++ | +++ | +++ | ++ | 0 | | | | 3 | 0 | ++ | +++ |
| | 1 | 0 | 0 | ++ | +++ | +++ | ++ | 0 | 0 | 0 | | 1 | 0 | + | +++ |
| 32 | 2 | 0 | 0 | +++ | +++ | + | 0 | 0 | 0 | 0 | | 2 | 0 | 0 | +++ |
| | 3 | 0 | 0 | +++ | +++ | +++ | +++ | 0 | 0 | 0 | | 3 | 0 | 0 | +++ |
| | 4 | 0 | +++ | +++ | +++ | +++ | ++ | 0 | 0 | 0 | | | | | |
| | 1 | 0 | + | ++ | +++ | +++ | ++ | 0 | 0 | 0 | | 1 | +++ | +++ | +++ |
| 43 | 2 | + | +++ | +++ | +++ | +++ | +++ | ++ | 0 | 0 | $10^{-6.2}$ | 2 | 0 | 0 | +++ |
| | 3 | +++ | +++ | +++ | +++ | +++ | +++ | + | 0 | 0 | | 3 | 0 | 0 | +++ |
| | 4 | 0 | +++ | ++ | 0 | 0 | 0 | 0 | 0 | 0 | | | | | |
| | 1 | +++ | +++ | +++ | +++ | +++ | + | 0 | 0 | 0 | | 1 | +++ | h | h |
| 52 | 2 | − | − | +++ | +++ | +++ | +++ | 0 | 0 | 0 | | 2 | +++ | + | + |
| | | | | | | | | | | | | 3 | 0 | 0 | ++ |
| 64 | | | | | | | | | | | | 1 | ++ | ++ | +++ |
| | | | | | | | | | | | | 2 | 0 | 0 | +++ |
| | | | | | | | | | | | | 3 | ++ | ++ | +++ |

BLE IV

*Fluid from Various Amounts of Inoculum*

| 1/100 dilution | | | | | | | 1/10,000 dilution | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| in of harvested allantoic fluid | | | | | | Embryo infectivity of pool | Egg No. | Red cell agglutinin of harvested allantoic fluid | | | | | | | | Embryo infectivity of pool |
| 1/400 | 1/800 | 1/1600 | 1/3200 | 1/6400 | 1/12800 | | | 1/10 | 1/100 | 1/200 | 1/400 | 1/800 | 1/1600 | 1/3200 | 1/6400 | 1/12800 | |
| 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 0 | 0 | 0 | 0 | 0 | 0 | $10^{-5.5}$ | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $10^{-5.5}$ |
| 0 | 0 | 0 | 0 | 0 | 0 | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 0 | 0 | 0 | 0 | 0 | 0 | | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 0 | 0 | 0 | 0 | 0 | 0 | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| ++ | + | 0 | 0 | | | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| ++ | + | 0 | 0 | | | $10^{-5.0}$ | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $10^{-7.4}$ |
| ++ | +++ | ++ | 0 | | | | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| ++ | +++ | 0 | 0 | 0 | 0 | | 1 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| ++ | +++ | + | 0 | 0 | 0 | | 2 | +++ | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| ++ | ++ | 0 | 0 | 0 | 0 | | 3 | 0 | ++ | +++ | +++ | +++ | 0 | 0 | 0 | 0 | |
| ++ | +++ | ++ | 0 | 0 | 0 | | 1 | +++ | +++ | +++ | +++ | +++ | + | + | 0 | 0 | |
| ++ | +++ | +++ | ++ | 0 | 0 | $10^{-5.5}$ | 2 | +++ | +++ | +++ | +++ | ++ | + | 0 | 0 | 0 | $10^{-8.0}$ |
| ++ | +++ | +++ | + | 0 | 0 | | | | | | | | | | | | |
| h | h | +++ | +++ | ++ | 0 | | 1 | 0 | 0 | ++ | +++ | +++ | ++ | 0 | 0 | 0 | |
| +++ | +++ | +++ | + | 0 | 0 | | 2 | 0 | 0 | +++ | +++ | ++ | 0 | 0 | 0 | 0 | |
| +++ | +++ | + | + | 0 | 0 | | | | | | | | | | | | |
| +++ | +++ | + | 0 | 0 | 0 | | 1 | 0 | 0 | 0 | +++ | +++ | +++ | + | 0 | 0 | |
| +++ | +++ | +++ | + | 0 | 0 | | 2 | 0 | 0 | 0 | + | + | ++ | + | 0 | 0 | |
| +++ | +++ | +++ | 0 | 0 | 0 | | 3 | 0 | 0 | 0 | + | ++ | +++ | + | 0 | 0 | |

TABLE V

*Growth of Virus after Inoculation of a $10^{-4}$ Dilution of Allantoic Fluid at 35° C.*

| Time | Titer |
|---|---|
| *hrs.* | |
| 1½ | $10^{+0.3}$ |
| 3¾ | $10^{>0}$ |
| 7 | $10^{-1.7}$ |
| 18 | $10^{-4.0}$ |

TABLE VI

*Effect of Age of Embryo on Titer of Virus Obtained from Allantoic Fluid*

| 10 days | | 12 days | | 14 days | |
|---|---|---|---|---|---|
| Embryo infectivity | Red cell titer | Embryo infectivity | Red cell titer | Embryo infectivity | Red cell titer |
| $10^{-9.0}$ | 1/4000 | $10^{-9.0}$ | 1/2000 | $10^{-9.0}$ | 1/1000 |

cated that there was a slight decrease in the amount of virus obtainable. This was not great enough to be picked up on infectivity measurements.

## SUMMARY

The virus of Newcastle disease of chickens resembles those of the encephalitis group in its ability to spread throughout the developing egg and embryo, but it is similar to influenza virus in the high concentration of it found in the allantoic fluid before death. No effect of the size of the inoculum on the final titer of virus in the allantoic fluid was detected. Good growth occurred at temperatures from 35° to 41°C., apparently more rapid at 40°C. than at 35°C. No appreciable development of virus capable of agglutinating red cells but of low embryo infectivity was found. Although virus multiplication was not immediately perceptible after inoculation, this cannot on present evidence be attributed to a real lag phase.

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