riboflavin was essentially the same, while xanthine oxidase on diet D was almost 3 times as high as on diet A. Similarly a comparison of the liver supernatant fractions, which contained all the xanthine oxidase(5), showed approximately a 3-fold difference between diets C and A in riboflavin content but only a 30% difference in xanthine oxidase activity. Hence, xanthine oxidase did not constitute a major fraction of the total riboflavin present in either liver or intestine, nor was it affected by diet in a manner characteristic of other flavin constituents.

Summary. 1. Weanling rats fed diets deficient in protein or riboflavin for 2 to 4 weeks had liver riboflavin levels that were reduced approximately 40% and 55% respectively; the corresponding reduction in intestinal riboflavin was approximately 15% and 55%. About 60% of the riboflavin in normal liver was in the sedimentable particles (nuclei plus mitochondria plus microsomes), and the above loss of riboflavin was proportionately the same from both the particulate and supernatant fractions. 2. The incorporation of soy flour in the diet as a source of the unidentified xanthine oxidase factor had no effect on the total riboflavin content of the liver or intestine, but did increase the liver and intestinal xanthine oxidases 30% and 170% respectively. The amount of riboflavin bound in xanthine oxidase represents only a very small fraction of the total riboflavin present. Liver xanthine oxidase was decreased only 25% by a riboflavin deficient diet.

1. Muntwyler, E., Seifter, S., and Harkness, D. M., *J. Biol. Chem.*, 1950, v184, 181.

2. Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, 1948, v173, 345.

3. Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v192, 49.

4. Strong, F. M., *Biol. Symposia,* 1947, v12, 143.

5. Meikleham, V., Wells, I. C., Richert, D. A., and Westerfeld, W. W., *J. Biol. Chem.*, 1951, v192, 651.

6. Westerfeld, W. W., and Richert, D. A., *J. Biol. Chem.*, 1951, v192, 35.

7. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, v140, 725.

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Growth of Newcastle Disease Virus in the Embryonated Egg. (19571)

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(Introduced by C. Phillip Miller.)

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There have been many approaches to the study of the factors involved in the multiplication of viruses. The early investigators were obliged to study virus propagation in terms of the reaction that followed the introduction of the agent into animals, isolated organs, or tissues. The growing interest in the phenomenon of virus propagation has given rise to many new avenues of approach and numerous ingenious technics. Although a large body of data have been collected, it appears that much basic information regarding the activity of the virus particle is still lacking. Especially is this true in regard to the mechanism of the development of the infectious and the hemagglutinating properties of some viruses. While considerable data regarding the influenza virus have been accumulated(1-5) the literature revealed but limited information on the growth and behavior of the Newcastle disease virus. However, some of its physical, immunological, and infectious properties have been described (6-10).

Since Burnet has reported a similarity between the viruses of Newcastle disease and influenza(11) this study was undertaken to apply to the Newcastle disease virus some concepts of host-virus interplay that had been described for influenza. This was ac-

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complished by investigating the production of the infective and hemagglutinative properties of Newcastle disease virus.

Materials and procedures. Newcastle disease virus. The CRE strain of Newcastle disease virus, isolated from a fatal case of Newcastle disease in a chicken, was obtained through Dr. William L. Ingalls, Department of Veterinary Pathology, Ohio State University. The virus had been subjected to many passages through embryonated eggs. The dilutions of virus used for inoculating fertile eggs were made in sterile brain heart infusion broth (Difco, pH 7.4). *Irradiated Virus.* The procedure followed for the preparation of the irradiated viruses was that described by Henle and Henle(12) for the preparation of ultraviolet inactivated influenza virus. *Eggs.* The Wayne strain of Leghorn eggs incubated at 37°C was used throughout this study. The age of the embryos used will be indicated in the experimental section to follow. *Inoculation procedure.* After candling each egg for viability, a small portion of the egg shell over the embryo was removed. The shell membrane was then pierced with a tuberculin syringe fitted with a No. 27 gauge needle. The inoculation was made into the allantoic sac. Sterile paraffin was used to seal the site of injection. *Harvesting procedure.* To obtain allantoic fluid, the blunt end of the egg was removed to expose the shell membrane, which was pierced with a blunt forcep. With a deliberate thrust, the allantoic sac was punctured. A sterile pipette or syringe and needle made it possible to aspirate the allantoic fluid. Care was taken to avoid rupture of the amniotic sac, the yolk sac, and blood vessels. It was possible to obtain clear allantoic fluid by this technic without previously chilling the eggs. *Erythrocyte suspension.* Hemagglutination tests were made with chicken erythrocytes that had been washed 3 times with phosphate buffered saline (pH 7.4). A 0.5% suspension of washed cells was prepared in buffered saline (pH 7.4). Freshly drawn red blood cells were used in each experiment. *Hemagglutination tests.* The hemagglutination test employed was a modification of the technic described by Salk(13). Titrations were made in round-bottom tubes measuring 7 x 75 mm. Doubling dilutions of allantoic fluid were prepared in buffered saline (pH 7.4) in such a manner as to leave 0.5 ml of the diluted virus in each tube. To each tube was added 0.5 ml of 0.5% washed chicken red blood cells. The titrations were incubated at room temperature for 1 hr and then read according to the pattern of the sedimented cells. The incubation time of 1 hr appeared to give the most satisfactory results. If allowed to stand longer, the pattern of the cells became indistinct and difficult to read. Diffuse agglutination patterns were interpreted as positive hemagglutinations while the appearance of a well defined "button" was considered as negative.

Experimental. A. *Growth of Newcastle disease virus as measured by infectivity titers.* Embryonated eggs, 12 days old, were inoculated via the allantoic sac with 0.2 ml of allantoic fluid containing approximately 50 LD_{50} of virus as determined by fertile egg titration. The dilution of this inoculum by the allantoic fluid contained in the egg was not determined. At intervals of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 24 hours after infection 5 eggs were opened and 1.0 ml of allantoic fluid was removed from each egg and pooled. Doubling dilutions of each of the infected fluids were later inoculated into sixteen 10-day-old fertile eggs (4 eggs per each dilution) and their LD_{50} titers were determined by the method of Reed and Muench(14). Six inoculated eggs were allowed to incubate for a longer period and none of these died before 48 hours. Fig. 1 is a graphic presentation of the data obtained from two independent determinations.

It can be seen that between one and 4 hours after inoculation there was a relatively constant quantity of virus present in the allantoic fluid. Beginning at 4 hr there was a very sudden rise in the virus content which appeared to reach its maximum at 6 hr, after which there was a slackening in the output but not an arrest of virus production. The virus titer continued to rise slowly, and reached another heightened period between ten and twelve hours. A very definite leveling off of detectable virus became evident at 16 hr and was apparently sustained for the re-

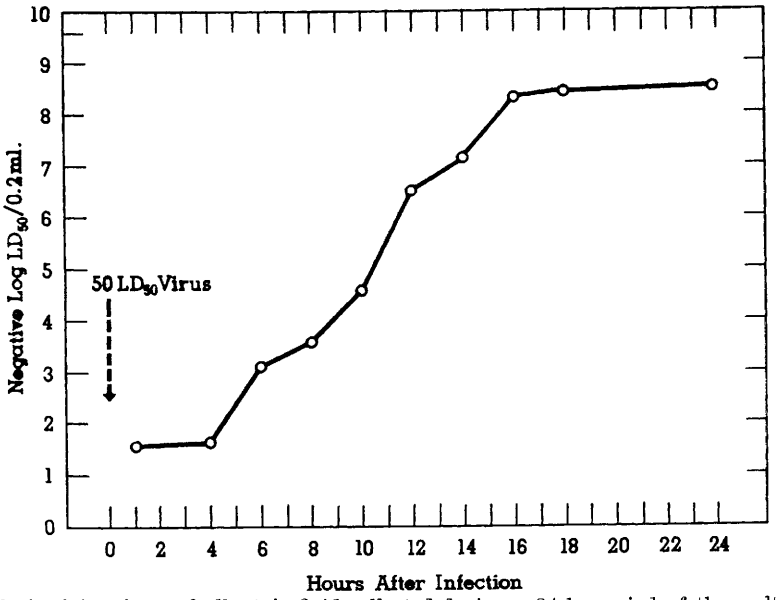

FIG. 1. Infectivity titers of allantoic fluid collected during a 24 hr period of the multiplication of Newcastle disease virus.

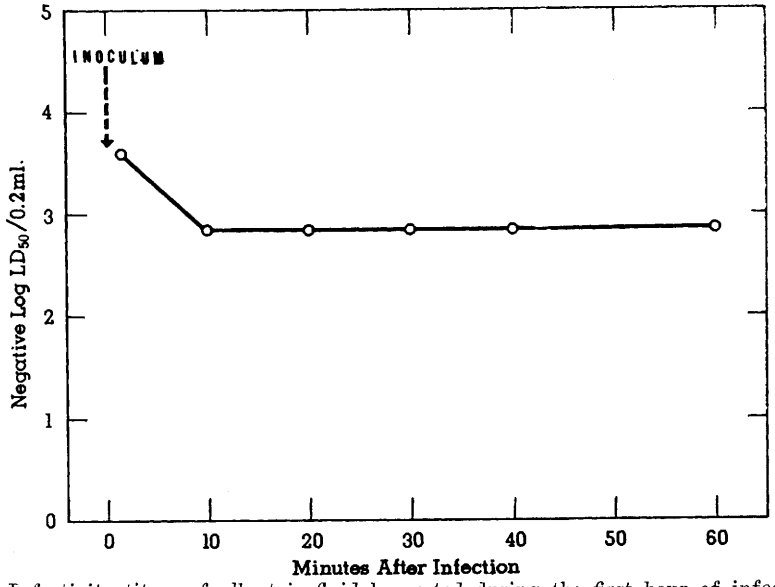

FIG. 2. Infectivity titers of allantoic fluid harvested during the first hour of infection with Newcastle disease virus.

mainder of the 24-hr period.

It was then decided to examine the growth activity of the virus immediately after its introduction into the egg. For this, 12-day-old, fertile eggs were inoculated with 0.2 cc of a 1/100 dilution of virus. This inoculum had been found to produce uniformly good infectivity. Fig. 2 contains the data obtained from the titrations of samples taken immediately and at 10 minute intervals. An examination of these data shows that a decrease in the virus content of the allantoic fluid takes place within 10 minutes after it has been introduced into the egg. The remaining 50

TABLE I. Infectivity and Hemagglutination Titers of Allantoic Fluid Collected During 1-24 Hr of Growth Period.

Sample (hr)	Infectivity titers*	Hemagglutination titer							
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
1	1.6	—							
2	1.5	—							
	2.5	—							
4	1.5	—							
	2	—							
6	3	—							
	3.5	—							
8	3.2	—							
	4	—							
10	4.3	—							
	5	—							
12	6	+							
	7	—							
14	6	+	+						
	8	+	+	+	+				
16	8.3	+	+	+	+				
	8.6	+	+	+	+	+	+		
18	8	+	+	+	+				
24	8.4	+	+	+	+	+	+	+	+
	8.6	+	+	+	+	+	+	+	+

* Infectivity titers expressed as negative logs.

minutes are marked by a stationary quantity of virus in the fluid.

B. *Detection of hemagglutinins during growth period of Newcastle disease virus.* Pooled samples of infected allantoic fluid harvested for infectivity titers were tested for their capacity to agglutinate washed chicken erythrocytes. Table I shows the results obtained in 2 determinations when doubling dilutions of these fluids were mixed with 0.5% red blood cells. Included in this table are the infectivity titers of these samples. It was not possible to demonstrate hemagglutination earlier than 12 hr following inoculation of virus into the egg. The first appearance of hemagglutination was followed by increased titers in the subsequent samples. Tests were not carried out beyond the 24-hour period. Similar determinations were made on samples of allantoic fluid collected every 10 minutes during the first hour of infection. None of the samples tested gave titers above 1:2 and in the 60 minute sample the presence of hemagglutination in the dilutions of 1:2 was doubtful. This reaction was not checked for non-specific hemagglutination.

C. *Effect of irradiated virus on multiplication of active Newcastle disease virus.* Twelve-day-old embryonated eggs, infected with 10 LD_{50} of Newcastle disease virus, were inoculated with 0.5 ml of undiluted allantoic fluid containing 250 LD_{50} of Newcastle disease virus that had been inactivated by exposure to ultra-violet irradiation for 9 minutes. The inactivated virus suspension was introduced into the egg 1 hr after the infective dose had been administered. At intervals of 2, 4, 6, 8, 10, and 12 hr following the infective dose, 5 eggs were opened and 1.0 ml of allantoic fluid from each egg was removed and pooled. A titration of viable virus was done by preparing dilutions of the pooled samples and inoculating 0.5 ml of the dilutions into four 10-day-old fertile eggs. The data for this experiment are presented in graphic form in Fig. 3. When this figure is compared with the growth curve of this same virus (Fig. 1) it can be seen that the addition of irradiated virus to embryonated eggs already infected with viable virus has the ability of altering the usual course of events. A step-like growth pattern was observed suggesting abrupt inter-

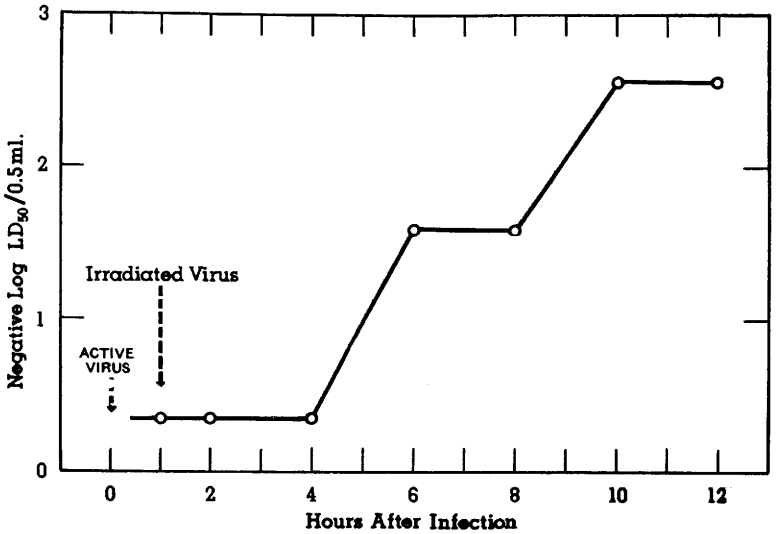

FIG. 3. Effect of homologous irradiated virus on multiplication of active Newcastle disease virus.

ruptions in growth.

Discussion. The findings in the foregoing experiments appear to indicate that Newcastle disease virus inoculated into the allantoic sac of the embryonated egg was adsorbed onto susceptible embryonic tissue before it began to proliferate. The introduction of a quantity of virus into the allantoic sac is followed by a static period of approximately 4 hr during which time a constant amount of virus can be detected in the allantoic fluid. Within 10 minutes after its introduction approximately 87% of the infective dose disappeared from the allantoic fluid (Fig. 2). It appears therefore that an equilibrium between virus in the allantoic fluid and virus in the cells of the chorio-allantoic membrane had been established during the first 10 minutes and maintained for the remainder of this 4 hr period. Similar findings have been reported by Stulberg, Schapira and Todd in tissue culture studies(15).

The constancy in quantity of virus recoverable from the allantoic fluid during the first 4 hr of infection would seem to indicate that the virus activity in progress is occurring within susceptible host cells, and does not influence the infectivity titers of allantoic fluids examined during this period. Presumably the virus present in the allantoic fluid during this phase of apparent quiesence represents virus that had been prevented from entering susceptible cells.

At the conclusion of the 4th hr of infection, there is a very marked increase in the quantity of virus in the allantoic fluid. Stulberg and his co-workers observed this increase to occur between 9 and 11 hr(15).

Employing the technic for producing an interference phenomenon, an attempt was made to block the advent of a second growth cycle. While the results as presented in Fig. 3 are equivocal, they are suggestive of a growth cycle of approximately 4 hr duration. Assuming the first growth cycle to end 4 hr after its initiation, the marked increase of virus in the allantoic fluid that follows may be the result of the elution of multiplied virus from chorioallantoic cells. The plateau in the curve between 6 and 8 hr can then be thought to be a period of maximum adsorption of active virus onto susceptible cells during the second growth cycle. The inactivated virus presumably reduced the number of potentially susceptible cells and thus there was a limitation of adsorption as suggested in Fig. 3. Most significant, however, is the rise in virus titer at the 8th hr after the proposed "first burst."

The inability to demonstrate hemagglutinating titers compatible with infectivity titers during the early phase of growth may be ex-

plained by either assuming that a hemagglutinating unit of virus had not yet been accumulated or by the alternate hypothesis offered by Henle and Henle(4) who suggest that the formation of influenza virus proceeds through a development of immature forms or building block. The contentions of these workers can be extended to the present study since it can be seen that while the infectivity titers of the 16th, 18th and 24th hr samples remain stationary, the hemagglutinating titers continue to rise. This paradox almost bespeaks a maturation process that endows the virus with its hemagglutination property, and is an event which lags behind the development of its infectivity.

Summary. 1. Data are presented to support the contention that the growth of Newcastle disease virus in the developing chick embryo occurs in cycles of 4 hr. The apparent 4-hr latent period in the production of virus following the inoculation of an infective dose does not correspond to the lag phase in the multiplication of bacterial cells, but rather indicates an intercellular development of virus particles that cannot be determined by any of the present technics for measuring viral activity. The maximum adsorption of virus onto susceptible cells occurs within 10 minutes after its introduction into the embryonated egg. 2. The existence of an unequal development of infective and hemagglutinating titers during the growth of Newcastle disease virus is described and an attempted explanation for this discrepancy is made on the hypothesis that the hemagglutination unit results from a maturation of the infectious particles and cannot therefore be detected early in its growth.

1. Blumenthal, H. W., Greif, D., Pinkerton, H., and DeWitt, R., *J. Exp. Med.*, 1950, v91, 321.
2. Henle, W., *J. Exp. Med.*, 1949, v90, 1.
3. ———, *J. Exp. Med.*, 1949, v90, 13.
4. Henle, W., and Henle, G., *J. Exp. Med.*, 1949, v90, 23.
5. Hoyle, L., *Brit. J. Exp. Path.*, 1948, v29, 390.
6. Bang, F. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v63, 5.
7. ———, *J. Exp. Med.*, 1948, v88, 233.
8. Burnet, F. M., and Ferry, J. D., *Brit. J. Exp. Path.*, 1934, v15, 56.
9. Cunha, R., Weil, M. L., Beard, D., Taylor, A. A., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1947, v55, 69.
10. Doyle, T. M., *J. Comp. Path. and Therap.*, 1927, v40, 144.
11. Burnett, F. M., *Aust. J. Exp. Biol. Med. Sci.*, 1942, v20, 81.
12. Henle, W., and Henle, G., *Am. J. Med. Sci.*, 1944, v207, 717.
13. Salk, J. E., *J. Immunol.*, 1944, v49, 87.
14. Reed, L. S., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
15. Stulberg, C. J., Schapira, R., and Todd, H. C., Abstracts of papers M1:68, Soc. of Amer. Bacteriologists, 1950, 50th General Meeting.

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Response of the Baby Chick to West Nile Virus. (19572)

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The virus employed in this study was the B 956 strain of West Nile virus furnished by the American Type Culture Collection in Washington, D. C. This virus was obtained by the above institution from Dr. K. C. Smithburn(1) of the Rockefeller Foundation Laboratories in New York, who had isolated this virus strain from a patient in Uganda, Africa and had passed this virus 25 times in Swiss albino mice by intracerebral inoculation. This virus has been cultivated in chick embryos by other investigators(2). At this laboratory the above-mentioned virus was passed 4 additional times in mice by the intracerebral route before initiating the present study.

Methods and results. Three-week-old Swiss albino mice (Webster strain) were employed.