

pH of the Chorio-allantoic Fluid of Chick Embryos Infected with Influenza Virus B.*

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Studies have been reported¹ on the rate of increase and the amounts of the influenza virus B (Lee strain) in the chorio-allantoic fluid of virus-infected chick embryos incubated at 35, 37, and 39°C. The increase in virus judged on the basis both of infectivity and hemagglutinative activity was greatest at 35°C and reached levels but little lower at 37°C. At 39°C the conditions were entirely unsuitable for the propagation of influenza virus B in chick embryos. While the temperature of incubation *per se* may influence the increase in influenza virus B in the chorio-allantoic fluid, it is possible that certain other factors, such as those concerned with the metabolism of the chick embryos, may be of great if not chief significance. Romanoff and Hayward² have shown that with incubation of normal chick embryos at 37.5°C, the pH

of the chorio-allantoic fluid progressively changes from a reaction of high alkalinity, pH 8, on about the 10th day to a reaction of medium acidity, pH 5 to 6, on about the 14th day. The total period of incubation of the fertile egg at which these low pH levels are reached is dependent on the stage of development of the embryo, which in turn varies considerably with the temperature of incubation. In the studies¹ on the influence of the temperature of incubation on virus propagation, an investigation was made also of the pH changes occurring in the chorio-allantoic fluid of the same embryos. The findings revealed definite relations between the increase of the virus and the pH of the chorio-allantoic fluid which suggest a probable influence of pH on the amounts of virus demonstrable. The results of these studies on the relation of pH to the increase of influenza virus B (Lee strain) in the chorio-allantoic fluid of virus-infected embryos incubated for various intervals at 35, 37, and 39°C are described in the present paper.

Methods and Materials. The data were collected on the same embryos utilized in the work previously reported.¹ Eggs for the experiments were obtained from a commercial hatchery on the morning of the 11th day of incubation at 37.5°C. The eggs were inoculated into the chorio-allantoic sac at room temperature with 0.05 ml of a 10⁻³ dilution in hormone broth of stock virus. The openings ground in the shells for inoculation were sealed with paraffin, and the eggs were then incubated at the desired temperature which did not vary more than $\pm 0.25^\circ\text{C}$. The humidity was kept at a high level by means of open pans of water placed on the floor of the incubator.

At intervals the chorio-allantoic fluids of 10 embryos were drawn off individually into test tubes. The volumes of the fluids from

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¹ McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 305.

² Romanoff, A. L., and Hayward, F. W., *Biol. Bull.*, 1943, **84**, 141.

TABLE I.
pH, Total Volume and Total Virus Yield (Red Blood Cell Agglutinative Activity) of Chorio-allantoic Fluid from Chick Embryos Incubated at 39, 37 and 35°C for Various Periods After Inoculation with Influenza Virus B (Lee Strain).

Time of Incubation Hr	Incubation Temp. °C	pH										Pooled aliquots	Total volume, ml	Total virus, gamma
		Individual embryo fluids												
		1	2	3	4	5	6	7	8	9	10			
6	39	6.7	6.5	7.9	7.2	8.2	7.3	6.6	7.4	8.3	6.5	7.3	66.9	0
	37	7.4	7.9	8.2	7.5	7.9	7.1	7.9	7.3	7.1	7.3	7.6	65.9	0
	35	—	—	—	—	—	—	—	—	—	—	7.4	66.9	0
12	39	6.5	6.7	7.6	7.1	7.8	7.8	7.5	7.8	7.9	6.4	7.5	64.5	0
	37	6.1	6.5	6.7	6.9	6.5	7.3	7.2	6.4	6.2	6.3	6.6	60.2	0
	35	—	—	—	—	—	—	—	—	—	—	7.4	57.9	0
18	39	6.5	6.7	6.7	6.5	6.6	6.9	7.2	6.8	7.1	7.0	6.8	63.0	0
	37	7.5	7.4	6.7	7.4	7.1	7.9	7.0	7.7	7.7	7.2	7.3	70.6	0
	35	—	—	—	—	—	—	—	—	—	—	7.5	51.9	16.5
24	39	7.2	6.6	7.2	7.3	7.0	7.5	6.9	7.4	6.2	6.5	7.4	63.6	0
	37	7.5	6.6	7.9	7.5	7.3	7.4	8.0	7.4	7.3	7.3	7.4	71.6	342
	35	—	—	—	—	—	—	—	—	—	—	7.6	60.3	1,450
36	39	6.3	6.9	6.3	7.3	6.5	6.0	6.9	6.7	6.6	6.3	6.7	65.5	21.5
	37	6.4	7.0	7.3	7.7	7.5	7.3	7.3	7.4	6.1	7.2	7.3	78.5	3,440
	35	—	—	—	—	—	—	—	—	—	—	7.7	56.5	4,390
48	39	6.6	6.5	7.0	6.5	7.6	6.3	5.8	6.6	6.1	6.5	6.7	59.1	51.4
	37	7.5	7.4	7.4	7.7	7.4	5.8	7.3	7.4	7.4	7.7	7.3	103.0	3,540
	35	—	—	—	—	—	—	—	—	—	—	7.7	76.7	5,380
60	39	6.6	6.0	5.8	5.7	6.2	6.4	6.5	6.3	6.7	6.5	6.4	53.6	0
	37	7.1	7.4	6.5	7.3	7.6	7.5	7.5	7.4	7.5	7.5	7.3	97.0	2,330
	35	—	—	—	—	—	—	—	—	—	—	7.9	86.0	5,920

the individual eggs were determined by weighing the tubes before and after the fluid was drawn into them. Aliquots of each group of 10 fluids were prepared by combining 1/10 of the volume of each respective fluid. Titrations were made of the hemagglutinative activity of each individual and each aliquot fluid for estimates of virus quantity. The method for converting the titration results, obtained in terms of titer, to the values representing absolute quantities of virus have been described.¹ Each aliquot fluid was titrated also for infectivity for chick embryos.

Measurements were made with the glass electrode of the pH of the individual fluids obtained after incubation of the eggs at 39 and 37°C and of the respective pooled aliquots of the fluids taken after incubation at 39, 37, and 35°C for the periods of 6, 12, 18, 24, 36, 48, and 60 hours of incubation. The individual fluids of the experiment at 35°C were not examined.

Results. The results are given in detail in Table I. The numbers assigned to the individual embryos in this table are the same as those given in Tables I (35°C), II (37°C),

and III (39°C) previously reported¹ so that a direct comparison can be made between those results and the present findings with each individual embryo shown in Table I. A direct comparison of the findings with the aliquot fluids, likewise can be made for the same reason. In Fig. 1 are shown the trends of the changes in pH of the chorio-allantoic fluid with respect to the period of incubation at the various temperatures. For comparison there is shown also in Fig. 1 the curve representing the changes in the pH of the chorio-allantoic fluid of normal embryos incubated at 37.5°C as determined by Romanoff and Hayward.²

It will be noted in Table I that the pH of the individual fluids of the embryos incubated at 39°C showed a progressive decrease into the acid range after 24 hours' incubation. The pH of the aliquot fluid at 24 hours was 7.3, but the values for all of the aliquot fluids of the later periods were below pH 7.0, as shown in Fig. 1. The virus quantity indicated by hemagglutinative capacity was measurable only in the groups of embryos incubated for 36 and 48 hours. A low degree of virus infec-

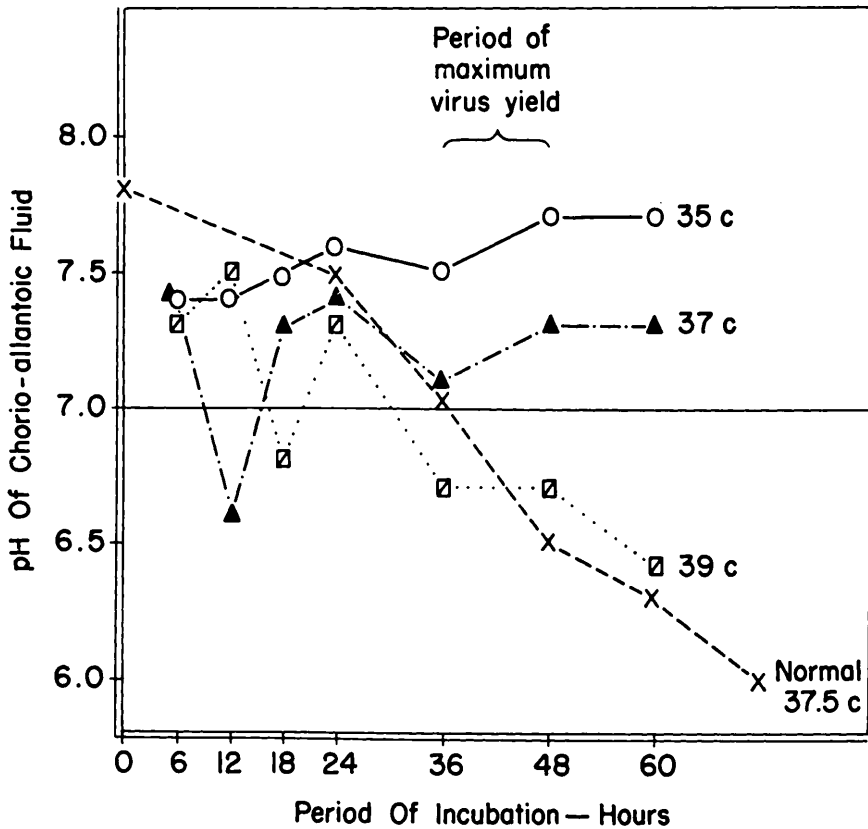


FIG. 1.

The relation of the pH of the chorio-allantoic fluid of embryos infected with influenza virus B (Lee strain) to the time of incubation at 35, 37, and 39°C after inoculation with the virus. The curve (broken line) characterized by the symbol (x) was constructed from the data of Romanoff and Hayward.² This curve indicates the changes which occur in the pH of the chorio-allantoic fluid during incubation at 37.5°C of normal chick embryos of the same stage of development as those infected with virus.

tivity for chick embryos was detectable¹ at all periods, reaching the highest value at 24 to 36 hours. The pH of the aliquot fluids from embryos incubated for 12 hours at 37°C was in the acid range as shown in Fig. 1. During the later periods of incubation, however, the pH was maintained above 7.0. The hemagglutinative capacity at this temperature of incubation was greatest at 36 to 48 hours, Table I, and was about 100 times that observed during the same incubation intervals at 39°C. The highest degree of virus infectivity observed¹ for these fluids was also reached at 36 to 48 hours and was likewise of the order of 100 times that observed for the corresponding groups incubated at 39°C. At the 35°C incubation temperature the pH of the aliquot fluids of the groups of 10 embryos

showed no tendency to become acid. On the contrary, there was a tendency toward a general increase in alkalinity throughout the experiment. A steady increase in hemagglutinative capacity was observed throughout the 60-hour incubation period, and the greatest rate of increase occurred during the 18- to 36-hour period. The highest levels of infectivity,¹ which were reached at 36 and 48 hours, were 6 to 7 times higher than those observed at 37°C.

Discussion. The present observations reveal a striking relation between the pH of the chorio-allantoic fluid of chick embryos infected with influenza virus B (Lee strain) and the increase of the virus in the fluid. Though the significance of the relationship is not entirely clear, certain findings suggest that

the pH of the chorio-allantoic fluid may be a principal factor limiting the increase or yield of the virus: (1) The pH range optimum for stability³ of the influenza virus B is at or above 7.9, and, judging from loss of hemagglutinative capacity, the virus is rapidly inactivated and perhaps degraded even at 4°C, in the acid pH range. An acid reaction of the chorio-allantoic fluid is reached in 18-30 hours and maintained thereafter in embryos incubated at 39°C, while an alkaline reaction is maintained throughout in the fluids of embryos incubated at the lower temperatures. It is noteworthy that the definite acid reaction at 39°C is attained in the period before the occurrence of massive increase in virus. (2) In contrast with the behavior of influenza virus B, the influenza virus A (PR8 strain) reaches high titers⁴ in the fluid of embryos incubated at 39°C. In further contrast is the observation that the pH range of optimum stability³ of the influenza virus A is 6.5 to 7.0, which is nearly 2 pH increments below the optimum range for influenza virus B. The acid reaction developed in the fluid at 39°C would thus not be expected to influence greatly the increase of the influenza virus A. (3) The temperature differential between 37 and 39°C is relatively small with respect to direct influence on the virus in comparison with the pH differential, as shown in Fig. 1, which is great in view of the sensitivity of the influenza virus B to hydrogen ion concentration in this range. (4) There is evidence⁵ that while influenza virus B as such is demonstrable only in small quantity by hemagglutinative and infectivity tests in the chorio-allantoic fluid of embryos incubated at 39°C, much may be formed only to be inactivated and partially broken down on exposure to the adverse pH of the chorio-allantoic fluid. This possibility has already

been discussed⁵ on the basis of sedimentation velocity and electron micrographic studies of material obtained from the fluid of embryos in which attempts were made to propagate influenza virus B at 39°C.

It is of interest to note the apparent influence of infection of the embryo with the virus on the hydrogen ion concentration of the chorio-allantoic fluids. This is especially well illustrated in the experiment in which incubation of the embryos was carried out at 37°C following inoculation with the virus. It is seen, Fig. 1, that an alkaline reaction was maintained throughout the later periods of the experiment, whereas the pH of the fluid of normal embryos of a comparable stage of development at 37.5°C becomes progressively more acid in similar periods. In the virus-infected embryos incubated at 35°C the pH of the chorio-allantoic fluid became progressively more alkaline. Only small effect was observed at 39°C. It appears likely that the establishment of massive infection of the embryo with virus leads to alteration of the processes of the excretion of acid metabolites with which is associated the change in the reaction of the chorio-allantoic fluid of normal embryos.

Summary. Influenza virus B (Lee strain) increases to small degree in the chorio-allantoic fluid of 11-day-old chick embryos incubated for 60 hours at 39°C after inoculation with the virus. Increase was great at 37°C and still greater at 35°C. The pH of the chorio-allantoic fluid of the same embryos descended into the acid region at 39°C; at 37°C the pH remained essentially above 7.0; and at 35°C the reaction was maintained well in the alkaline region. These relations suggest the possibility that the extent of increase of the virus in the chorio-allantoic fluid may be influenced more by the pH of the fluid at the various temperatures than by the temperature *per se*.

³ Miller, G. L., *J. Exp. Med.*, 1944, **80**, 507.

⁴ Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

⁵ Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, Dorothy, Beard, J. W., Feller, A. E., and Dingle, John H., *J. Immunol.*, 1944, **48**, 129.