THE GROWTH CYCLE OF INFLUENZA VIRUS A. A STUDY OF THE RELATIONS BETWEEN VIRUS, SOLUBLE ANTIGEN AND HOST CELL IN FERTILE EGGS INOCULATED WITH INFLUENZA VIRUS.

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Received for publication August 4, 1948.

THE work described in this paper was undertaken in an attempt to throw some light on the mechanism of multiplication of influenza virus in the infected cell, and on the nature and origin of the soluble antigen.

Hoyle and Fairbrother (1937) showed that extracts of tissues infected with influenza virus contained two distinct particles, the infective virus which was readily sedimented in the high-speed centrifuge, and a smaller particle, the soluble antigen, which was largely responsible for the complement-fixing properties of such extracts. Hirst (1941) showed that extracts containing influenza virus would agglutinate red blood cells, and that the agglutinin was indentical with the infective particle. The infective virus was adsorbed by the red cell, and subsequently eluted without apparent loss, and no multiplication of virus occurred. The infective virus has a particle size of 100 mμ, while the soluble antigen has a particle size of about 10 mμ (Henle and Wiener, 1944). Both particles will react in complement fixation tests, but the fixation differs in character (Hoyle, 1945 ; Wiener, Henle and Henle, 1946). The fixation due to the infective particle shows partial strain specificity, while that due to the soluble antigen does not. The soluble antigen is not adsorbed by red blood cells.

Evidence is brought forward in this paper to show that the influenza virus exists in two forms with distinct properties, an extra-cellular infective form which is able to penetrate through cell walls and agglutinate red blood cells, and an intracellular multiplying form which is non-infective, does not agglutinate red blood cells, and is possibly identical with the soluble antigen.

Most of the work has been done with the D.S.P. strain of influenza virus A. This strain was isolated from man by egg inoculation in 1943, and has since been maintained by passage in the allantoic sac. It is now highly adapted to growth in that situation and is very consistent in its behaviour. It is therefore a very suitable strain for quantitative studies.

Distribution of virus and soluble antigen in the infected egg.

Twelve day old fertile eggs were inoculated to the allantoic sac with 0·1 ml. of a 1 : 10 dilution of allantoic fluid from an egg infected 18 hours previously with D.S.P. Virus. This inoculum contained about 10 million infecting doses, and since the allantoic sac of a 12-day egg is lined by between 100 and 200 million

* A Report to the Medical Research Council.

cells, was sufficient to infect some 5–10 per cent of the cells. The eggs were incubated at 35° C. and examined after different intervals of time. The whole of the allantoic fluid of the egg was collected, and an extract was prepared from the chorio-allantoic membrane by grinding the membrane with sand and suspending in 5 ml. of saline. Sodium azide was then added in a concentration of 0·08 per cent to the allantoic fluids and chorio-allantoic extracts, and the crude extracts incubated for 6 hours at 37° C. to allow of elution of virus from the cells present. The fluids and extracts were then centrifuged and the virus content of the supernatants measured by the Salk modification of the Hirst test (Salk, 1944). Samples of the fluids were then adsorbed with fowl red cells to remove virus, and the content of soluble antigen determined by the complement fixation test using the micro method of Hoyle (1945). The results were expressed in agglutinin units and

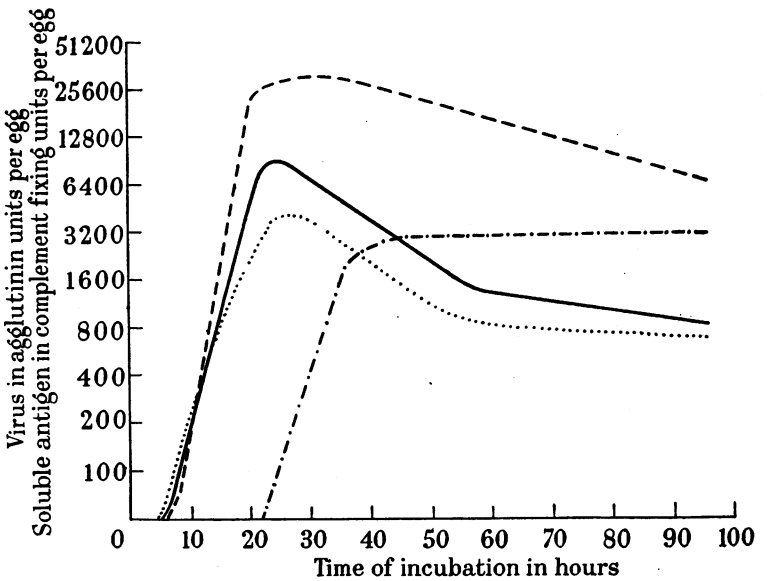

FIG. 1.—Distribution of virus and soluble antigen in eggs infected with D.S.P. virus.

———— = Virus in chorio-allantoic membrane
– – – – = Virus in allantoic fluid
. . . . . = Soluble antigen in chorio-allantoic membrane
— · — · — · = Soluble antigen in allantoic fluid

complement fixing units per egg, the agglutinin unit being the smallest amount of virus giving complete agglutination in the Salk test, while the complement fixing unit was the smallest amount of antigen giving 50 per cent fixation in the micro complement fixation test.

A number of such experiments were done and the virus and soluble antigen content of allantoic fluid and chorio-allantoic membrane measured at different times of incubation. Two or three eggs were sampled at each interval of time. The results of different experiments proved closely comparable, and Fig. 1 represents the average result of several experiments.

It will be seen that the growth of virus passes through four stages.

(1) *A lag phase.*—For a period of about 6 hours no virus can be demonstrated in the egg. Soluble antigen, however, is detected at about 5 hours in the membrane.

(2) *Phase of active increase.*—Virus appears in the membrane at about 6 hours, and between 6 and 24 hours virus and soluble antigen rapidly increase in amount. The appearance of soluble antigen precedes the appearance of virus and at first it increases more rapidly. Active increase of both particles ceases at 24 hours.

(3) *Phase of excretion.*—Virus and soluble antigen are released from the membrane and appear in the allantoic fluid. The behaviour of the two substances is, however, very different. Excretion of virus commences almost at once, virus being usually detectable in the allantoic fluid at 7 hours. The rate of excretion of virus is greater than the rate of production, so that the virus content of the allantoic fluid increases more rapidly than that of the membrane, and by 24 hours the virus content of the allantoic fluid is considerably greater than that of the membrane. The content further increases to about 30 hours as a result of completion of excretion from the membrane after active growth has ceased. By contrast the soluble antigen is excreted much more slowly and is usually not detectable in the allantoic fluid until 20 hours. The antigen content of the allantoic fluid then increases to about 40 hours, there being a corresponding fall in the antigen content of the membrane. It appears that while the virus is actively excreted the soluble antigen is much more intimately associated with the cells, and possibly only appears in the allantoic fluid as a result of cell destruction. Considerable cell destruction occurs between 18 and 30 hours and degenerate cells can be found in the allantoic fluid after 18 hours.

(4) *Phase of destruction.*—After about 40 hours' incubation the virus content of the allantoic fluid shows a slow, steady, logarithmic decline, presumably as a result of destruction of virus. The soluble antigen is, however, more resistant and no change in amount was seen over a period of 4 days.

Nature of the virus multiplication.

A consideration of Fig. 1 might suggest that the increase of virus between 6 and 24 hours was a result of logarithmic multiplication in the cells of the chorio-allantoic membrane followed by excretion into the allantoic fluid. However, it is improbable that multiplication occurs by any process comparable to binary fission. The apparent logarithmic increase is a result of taking the average figures for several eggs. If the virus content of the allantoic fluid is measured at intervals in individual eggs it is found that the increase does not proceed regularly, but rather takes place in a series of steps. Virus appears at about 6 hours, increases rapidly for about 3 hours, and then remains stationary for 2–3 hours, when a further step-like increase occurs. Fig. 2 shows a typical result.

The duration of the lag phase is to some extent dependent on the amount of inoculum. Eggs were inoculated with serial 10-fold dilutions of infected allantoic fluid and the duration of the lag phase determined by sampling the allantoic fluid at hourly intervals. The results are shown in Table I. The lag phase remained constant at about 6 hours with inocula of 100-million and 10-million infecting doses. When, however, the inoculum was reduced to 1-million doses, the duration of the lag phase was doubled.

It seems probable that multiplication occurs in a succession of intracellular cycles, each cycle lasting about 6 hours. After completion of the first cycle, virus

TABLE I.—*Effect of Size of Inoculum on Duration of Lag Phase.*

Inoculum.	Egg.	Presence of Virus in allantoic fluid. 4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.	10 hrs.	11 hrs.	12 hrs.	13 hrs.
100,000,000 M.I.D.	1	—	—	+	+	+	+	+	+	+	+
	2	—	—	—	+	+	+	+	+	+	+
10,000,000 M.I.D.	1	—	—	—	+	+	+	+	+	+	+
	2	—	—	—	+	+	+	+	+	+	+
1,000,000 M.I.D.	1	—	—	—	—	—	—	—	—	+	+
	2	—	—	—	—	—	—	—	—	+	+
	3	—	—	—	—	—	—	—	—	—	+

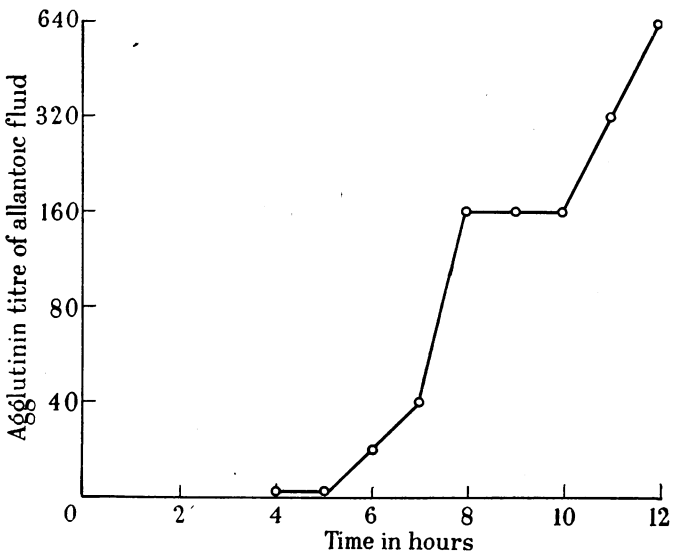

FIG. 2.—Showing step-like increase in virus content of allantoic fluid of egg inoculated with D.S.P. virus.

is released into the allantoic fluid and then infects further cells, and a second cycle occurs. If the inoculum is small, virus may not be demonstrable in the allantoic fluid until the second cycle is completed, and in such a case the duration of the lag phase will be doubled.

Observations similar to these were made by Henle, Henle and Rosenberg (1947), who showed that PR8 virus had a characteristic lag phase of 6 hours, while the Lee virus showed a lag phase of 9 hours. An ingenious direct measurement of the amount of virus multiplication in a single cycle was made by these authors. Eggs were inoculated with a small dose of virus, the amount taken up by the membrane was measured, and the infection of further cells was prevented by the addition of a large amount of heterologous irradiated virus. A single step-like increase of the virus content of the allantoic fluid occurrred, and it was found that an average multiplication of 63-fold occurred with PR8 virus.

It appeared, therefore, that the lag phase represented the duration of the first cycle of intracellular growth of the virus, and a special study was made of the behaviour of the virus in the first six hours after inoculation.

Union of virus with the cell.

When influenza virus is introduced into the allantoic sac of a 12-day egg, about 90 per cent of the virus is rapidly taken up by the cells lining the sac. It is probable that the initial union of virus with the cell is similar in nature to the adsorption of virus by red blood cells. Thus, if the chorio-allantoic membrane is removed from an egg, minced, and suspended in saline containing 0·08 per cent sodium azide to arrest cell metabolism, then such a membrane suspension behaves similarly to red blood cells in that it will adsorb virus and the virus is subsequently eluted. The elution process tends to be less complete than with the red cell. Thus, in a typical experiment, the minced chorio-allantoic membrane of one egg adsorbed 85 per cent of the virus from 1 c.c. of allantoic fluid with a Salk titre of 256, and 60 per cent of the adsorbed virus was eluted in three hours at 37° C. One hundred million red cells adsorbed 90 per cent of virus from the same fluid, and the whole of the adsorbed virus was eluted in 3 hours at 37° C. The chorio-allantoic membrane, like the red cell, does not adsorb soluble antigen.

The living chorio-allantoic membrane in the intact egg behaves very differently. When virus is introduced into the allantoic sac it is adsorbed by the membrane and is not subsequently eluted until 6 hours later, when the amount which appears is much greater than the original inoculum. Thus, in one experiment, an egg inoculated with 100 agglutinin units of virus showed no demonstrable virus in the allantoic fluid at 6 hours, while at 8 hours the fluid contained 1600 agglutinin units.

State of virus during the lag phase.

During the lag phase *no virus could be demonstrated in the chorio-allantoic membrane by the Salk test*, even when the original inoculum was so large that virus should have been easily demonstrable. Thus, in one experiment, an egg was inoculated to the allantoic sac with 0·5 ml. of allantoic fluid with a Salk titre of 1024. The egg was incubated 3 hours and the allantoic fluid and chorio-allantoic membrane removed. The allantoic fluid had a volume of 10 ml. and gave a Salk titre of 4, so that it contained less than 10 per cent of the inoculated virus. It therefore appeared that 90 per cent of the inoculated virus had been taken up by the cells of the chorio-allantoic membrane. The membrane was ground with sand and 2 ml. of azide saline, and incubated 8 hours to allow elution of virus from the cells. The supernatant fluid was then tested by the Salk test. No trace of virus could be detected.

A large number of experiments have been done on these lines and in no case could virus be demonstrated by the Salk test in the chorio-allantoic membrane during the lag phase, even total destruction of the cells by freezing and thawing failed to liberate any detectable amount of virus.

It seemed evident from these experiments that in the living chorio-allantoic membrane during the lag phase the virus was present in some form which did not agglutinate red blood cells. Attempts were therefore made to demonstrate the presence of virus by infectivity tests.

A 12-day egg was inoculated to the allantoic sac with 0·1 ml. of a 1 : 10 dilution of D.S.P. allantoic fluid with a Salk titre of 512. This corresponded to an inoculum of about 5-million infecting doses. The egg was incubated 3 hours, the chorio-allantoic membrane removed and washed rapidly in six changes of sterile saline to remove allantoic fluid. The membrane was then suspended in 10 ml. of saline and the cells destroyed by freezing and thawing 4 times. The fluid was then lightly centrifuged to remove debris, and the infectivity of the supernatant tested by inoculating eggs with serial 20-fold dilutions. In order to control the possible loss of virus by adsorption on cell debris after the freezing and thawing, a control test was done in which a normal chorio-allantoic membrane was suspended in 10 ml. of saline, frozen and thawed 4 times, and then an amount of virus equal to the test inoculum was added After standing for 3 hours the supernatant was tested for infectivity. The results are shown in Table II. It

TABLE II.—*Infectivity of Chorio-allantoic Membrane Extracts During the Lag Phase.*

Test Fluid.—Washed chorio-allantoic membrane from an egg inoculated 3 hours previously with 5 million M.I.D. of DSP virus. Membrane suspended in 10 ml. saline, frozen and thawed 4 times, fluid centrifuged.

Control Fluid.—Normal chorio-allantoic membrane suspended in 10 ml. saline, frozen and thawed 4 times, 5 million M.I.D. of DSP virus added, allowed to stand 3 hours, fluid centrifuged.

	Inoculum.	No. of Eggs Used.	No. of Eggs Infected.	Result.
Control Fluid.	0·1 ml. of 1 : 1 dilution	2	2	Infective in 1 : 8000 dilution
	0·1 ml. of 1 : 20 ,,	4	4	
	0·1 ml. of 1 : 400 ,,	4	4	
	0·1 ml. of 1 : 8000 ,,	6	4	
Test Fluid.	0·1 ml. of 1 : 1 dilution	4	3	Infective in 1 : 20 dilution
	0·1 ml. of 1 : 20 ,,	6	5	
	0·1 ml. of 1 : 400 ,,	5	0	
	0·1 ml. of 1 : 8000 ,,	2	0	

will be seen that the infectivity of the control fluid was of the order expected, since of 6 eggs inoculated with 0·1 ml. of a 1 : 8000 dilution (corresponding to 6 infecting doses) 4 became infected. By contrast the test fluid from the chorio-allantoic membrane after 3 hours' incubation was much less infective than expected, its infectivity being only 1 four-hundredth of the control.

It appeared that during the lag phase the greater part of the virus present in the chorio-allantoic membrane, was present in a non-infective form.

Production of soluble antigen during the lag phase.

The experiments of Fig. 1 showed that soluble antigen appeared in the chorio-allantoic membrane towards the end of the lag phase, preceding the appearance of infective virus by about 1–2 hours.

It seemed reasonable to suppose that if eggs were inoculated with a very large dose of virus so as to infect all the available cells, then the whole production of virus and soluble antigen might be concentrated into a single intracellular cycle and that a single step-like increase might occur. Eggs were inoculated to the

allantoic sac with 0·5 ml. of D.S.P. allantoic fluid with a Salk titre of 1024. Eggs were sampled at hourly intervals, the chorio-allantoic membranes being removed, washed with saline to remove allantoic fluid, suspended in 1 ml. of azide saline, and the cells disintegrated by freezing and thawing 4 times. The suspensions were then allowed to stand at room temperature over-night to allow elution of adsorbed virus from tissue debris, and were then centrifuged and the supernatants tested for virus and soluble antigen, The results of two such experiments are shown in Fig. 3. In these experiments with very heavy inocula the lag phase was shorter than usual. The concentration of soluble antigen showed the expected single step increase, a rapid and considerable production of soluble antigen occurring between 2 and 4 hours after inoculation. But it was found that the production of infective virus was very largely suppressed, the production being

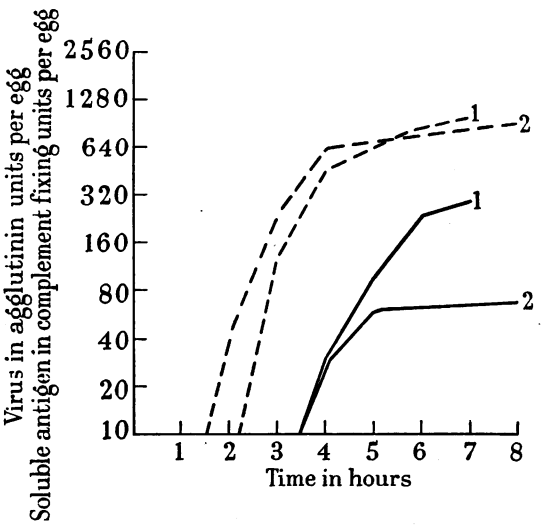

FIG. 3.—Production of virus and soluble antigen in chorio-allantoic membrane in eggs inoculated with a very large dose of virus (500 million M.I.D.).

Virus in chorio-allantoic membrane:
Experiment 1 = ————1
Experiment 2 = ————2

Soluble antigen in chorio-allantoic membrane:
Experiment 1 = - - - - 1
Experiment 2 = - - - - 2

very much less than expected and in some experiments being almost negligible. The suppression of virus multiplication when eggs are inoculated with a large dose of virus has been noted previously (Henle and Chambers, 1941 ; Miller, 1944). It appears that when eggs are inoculated with a very large dose of virus there occurs a rapid and considerable production of soluble antigen between 2 and 4 hours after inoculation, but there is often no comparable production of infective virus. It is possible that under these conditions the cells become infected with more than one particle of virus, and an unusually rapid intracellular growth occurs which makes excessive demands on the cell metabolism, with the result that the virus is often unable to complete its normal cycle.

Adsorption of antibody by infected chorio-allantoic membrane.

After 18 hours' incubation, the chorio-allantoic membrane of an infected egg contains large amounts of both virus and soluble antigen. Such an infected membrane was washed thoroughly in saline to remove allantoic fluid, cut into small pieces, and suspended in 0·4 ml. of saline. A normal uninfected membrane was similarly treated and to each suspension 0·1 ml. of a human convalescent serum containing both agglutinin-inhibiting and complement-fixing antibody was added. After standing 10 minutes, the fluids were centifuged and the antibody contents of the supernatants measured. It was found that the infected membrane adsorbed 75 per cent of the agglutinin-inhibiting antibody from the serum, but the complement-fixing antibody was unaffected.

This result probably means that the location of the infective virus differed from that of the soluble antigen. The infective virus was probably either extracellular or present on the cell surface, so that it was able to unite with antibody, while the soluble antigen was intracellular and inaccessible to the antibody.

DISCUSSION.

There can be little doubt that when influenza virus is introduced into the allantoic sac of the fertile egg, multiplication of the virus occurs in the cells lining the sac. This multiplication appears to occur in a series of intracellular cycles, each cycle lasting about 6 hours. At the end of each cycle infective virus is liberated from the cells into the allantoic fluid when it infects further cells and a further cycle commences. When virus is introduced into the allantoic sac, about 90 per cent is immediately taken up by the cells of the chorio-allantoic membrane. However, the virus appears to undergo some change in the cells so that it cannot be demonstrated by the usual methods. Extracts of the membrane made 3 hours after inoculation fail to agglutinate red cells, and although infective virus can be demonstrated by the highly sensitive infectivity test, the amount present appears to be less than 1 per cent of that expected. It seems that the major part of the virus becomes converted into a non-infective form which does not agglutinate red cells. In this intracellular phase, multiplication of the virus occurs and after some 6 hours, infective virus re-appears in the cells and is almost at once excreted into theallantoic fluid. The failure to demonstrate virus in the chorio-allantoic membrane during the lag phase might be due to its being firmly and irreversibly adsorbed by some cell constituent, but this is unlikely, since no such irreversible adsorption can be demonstrated *in vitro*. Thus if a chorio-allantoic membrane is removed from an egg, minced, and suspended in azide saline, the suspension does not irreversibly adsorb virus, nor does a suspension of chorio-allantoic membrane after freezing and thawing. Such suspensions behave as red blood cells, in that a temporary adsorption of virus is followed by elution in about 3–4 hours. It is much more probable that the virus becomes converted in the living membrane into an intracellular form, the properties of which differ from that of the extracellular infective virus. By analogy with the larger viruses such as vaccinia, psittacosis, etc., it might be expected that this intracellular phase would be a phase of large particle size, comparable to the inclusion bodies which occur in cells infected with these viruses. However, no inclusion bodies have ever been demonstrated in influenza. There must be a close relationship between the intracellular phase and the soluble antigen, and it is possible that the two are identical. The soluble antigen is probably nucleo-

protein in nature. It is antigenic, precipitated by acetic acid (Hoyle and Fairbrother, 1937), and by half saturation with ammonium sulphate (author's unpublished observation). Its isoelectric point is the same as that of the infective virus and it has an identical electrophoretic mobility (Bourdillon and Lennette, 1940). The infective virus can be shown by serological methods to contain soluble antigen (Hoyle, 1945) and Wiener, Henle and Henle (1946) showed that soluble antigen is liberated from the infective particle by sonic disruption. The soluble antigen thus appears to consist of virus protein. The fact that the maximum production of soluble antigen occurs in the earliest stages of growth of the virus in the egg would indicate that it is not merely a product of disintegration of the infective particles, as has often been suggested. The soluble antigen is non-infective, does not agglutinate red cells, appears in the chorio-allantoic membrane during the lag phase and rapidly increases in amount. It can be released from the cells by grinding with sand or by freezing and thawing and it appears in the allantoic fluid of infected eggs after about 20 hours, when cell destruction is taking place. The properties of the soluble antigen are in fact those which would be expected of the intracellular phase, and it seems reasonable to suppose that the two may be identical.

If this conception is correct, then the soluble antigen would represent the influenza virus reduced to its minimal particle size, and might be compared to a self-reproducing protein molecule of large size. It is of significance that the soluble antigens of all strains of influenza virus A appear to be serologically identical (Hoyle, 1945). By contrast the infective red cell agglutinating virus elementary body would be a more complex form of the virus. It contains soluble antigen, certain antigens responsible for the strain specificity seen in virus neutralization tests, and an enzyme mechanism enabling it to penetrate cell walls (Burnet, 1948). In fact, the infective particle appears to be essentially an extra-cellular phase of the virus, specially equipped to penetrate from one cell to another. It is only encountered in association with cells when it is either penetrating into the cell or in process of being excreted out of the cell. The soluble antigen is essentially intracellular and only appears in an extracellular position when the cells are destroyed.

It is probable that the production of the extracellular infective phase of the virus depends on the survival and active metabolism of the host cell. Thus when eggs are inoculated with very large doses of virus a very rapid production of soluble antigen occurs, indicating a very rapid intracellular growth, but in many cases the resulting yield of infective virus is much reduced and may even be negligible. It is possible that the very rapid intracellular growth of the virus so exhausts the metabolic power of the cell that it becomes unable to provide materials which may be necessary for the production of the extracellular infective particle. Even under optimal conditions it is probable that the yield of infective virus represents only a small part of the total production of virus protein. Thus, of the total of some 3600 units of complement-fixing antigen produced in an egg after 36 hours' incubation, only about 600 units represent soluble antigen associated with the infective particle, the remaining 3000 units being present as free soluble antigen.

In this connection some observations of Gard and Von Magnus (1946) are of interest. They found that in eggs inoculated with a large dose of virus the allantoic fluid contained two particles sedimenting at different rates in the ultracentrifuge. Both particles agglutinated red cells but it seemed probable that

only the larger particle was infective. They considered that the smaller particle, of size approximately half that of the infective particle, represented an incompletely developed form of the virus, and they refer to it as a precursor. They considered that this incomplete virus was not a disintegration product of the fully infective form, since it was produced in very large amounts and in the initial stages of growth. It is possible that this precursor of Gard and Von Magnus represents a stage in the virus life cycle immediately preceding production of the fully infective virus.

It is evident that tissues infected with influenza virus contain particles of virus protein of different sizes and with different properties. The hypothesis is advanced in this paper that these represent stages in a cycle of development. The alternative view might be that they represent disintegration products of the infective elementary body. But it is difficult to believe that a highly egg-adapted strain of virus would undergo disintegration on a scale sufficient to account for the very considerable production of soluble antigen which occurs in the earliest stages of growth, nor is it easy to understand why soluble antigen should be demonstrable in the chorio-allantoic membrane before infective virus can be demonstrated, especially as the soluble antigen can only be demonstrated by the complement fixation test which is much less sensitive than the red cell agglutination test.

SUMMARY.

A study has been made of the growth-cycle of influenza virus in the allantoic sac of the fertile egg. It is shown that the growth occurs in a series of intracellular cycles. Infective, red cell agglutinating virus inoculated into the allantoic sac is taken up by the cells lining the sac and becomes converted into an intracellular form which is non-infective, and does not agglutinate red cells. This form multiplies for a period of 6 hours when the infective form of the virus again appears, is immediately excreted into the allantoic fluid, infects further cells and the cycle is repeated.

The influenza virus thus exists in two forms, an extracellular infective form which agglutinates red blood cells and is able to penetrate cell membranes, and an intracellular multiplying form which is non-infective and does not agglutinate red cells.

The soluble antigen is produced in the chorio-allantoic membrane in large amounts in the earliest stages of growth of virus in the egg at a time when the intracellular phase of the virus is presumably multiplying, and it is possible that the soluble antigen is identical with the intracellular phase of the virus.

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