THE MULTIPLICATION OF INFLUENZA VIRUSES IN THE FERTILE EGG

A REPORT TO THE MEDICAL RESEARCH COUNCIL

By L. HOYLE

From the Public Health Laboratory, Northampton

(With Plates 3 and 4, and 5 Figures in the Text)

Hoyle & Fairbrother (1937) showed that tissues infected with influenza virus contained two distinct particles, the infective virus elementary body, and a smaller particle, the soluble antigen, which could be demonstrated by complement-fixation tests.

It was at first thought that the soluble antigen was either produced by the multiplying elementary body, or was a product of its disintegration. However, as a result of a study of the multiplication of influenza virus in the chorio-allantoic membrane of the fertile egg it became evident that neither of these views was in accord with observed facts, and in 1948 the author (Hoyle, 1948) advanced an entirely new concept of the mechanism of the virus multiplication. It was found that complement-fixing soluble antigen appeared in extracts of the chorio-allantoic membrane, and rapidly increased in amount, some time before any increase in infectivity. Also virus inoculated to the allantoic sac was taken up by the cells lining the sac but appeared to undergo some change so that it could not be detected in extracts of the chorio-allantoic membrane by red-cell agglutination or by infectivity tests. It was suggested that the virus existed in two forms, an extracellular infective phase, the elementary body, and an intracellular multiplying phase which was probably identical with the soluble antigen. It was supposed that virus elementary bodies entered the cells of the chorio-allantoic membrane and there became disintegrated, liberating particles of soluble antigen which then multiplied in the cells. At a later stage infective elementary bodies were reformed by a process of aggregation of the previously formed soluble antigen.

Henle (1949*a, b*) and Henle & Henle (1949) in a similar investigation obtained closely comparable results. They found that virus taken up by the cells of the chorio-allantoic membrane could not be recovered from the cells for 5–6 hr. but that during this time complement-fixing antigen appeared in the membrane. However, Henle showed that not only did the complement-fixing properties appear before the development of infectivity, but also that the membrane extracts developed the ability to agglutinate red blood cells before any increase in infectivity. There was, therefore, a stage in the life cycle of the virus at which particles could be detected which possessed red cell agglutinating properties but were not infective. Such particles had been previously detected in the allantoic fluid in eggs inoculated with a large dose of virus by Gard & von Magnus (1946), who

regarded them as a precursor of the infective virus. Henle suggested that virus was built up in the cells from 'non-infectious immature forms or building blocks which are later converted into fully active virus'. These immature forms were believed to possess both complement-fixing and agglutinating properties.

The present paper describes further studies of the virus life cycle in which the observations of the author are brought into line with those of Henle and Gard & von Magnus. A study has also been made of the morphology of the extracellular phase of the virus and of the method of its excretion from the cell. A much more complete picture of the process of virus multiplication can now be given.

Before considering the life cycle in detail stage by stage, it is necessary to consider the properties of the virus elementary body and certain evidence bearing on its probable structure.

PROPERTIES OF THE ELEMENTARY BODY

If eggs are inoculated by the allantoic route with a moderate dose of virus (e.g. 5–10 million infective doses) and are incubated for 18–20 hr., the allantoic fluid is found to contain a large amount of highly infective virus elementary bodies. These can be partially purified and concentrated by adsorption on fowl or guinea-pig red blood cells and subsequent elution of the washed cells into a smaller volume of saline. Such a concentrated elementary body preparation has a number of properties by which the presence of virus can be detected and its amount measured.

(1) *The fluid is infective to eggs.* This infectivity can be measured by inoculating eggs by the allantoic route with decimal dilutions, incubating 48 hr. and determining the presence of virus by the ability of the allantoic fluid to agglutinate red cells. This test requires a large number of eggs and for many purposes a simpler method is needed. It has been found that the amount of complement-fixing soluble antigen produced after 6 hr. incubation in the chorio-allantoic membrane of eggs inoculated by the allantoic route depends on the number of cells which have been infected, and hence is approximately related to the number of infecting doses in the inoculum. If eggs are inoculated with 1 ml. of the fluid to be tested and with 1 ml. of a 1:100 dilution, and the content of soluble antigen in the chorio-allantoic membranes measured after 6 hr. incubation a fairly accurate measure of the infectivity of the fluid can be made.

(2) *The fluid agglutinates red blood cells.* The agglutinin titre can be conveniently measured by the Salk test (Salk, 1944). The author uses guinea-pig red cells in a dilution of 1:300.

(3) *The elementary body contains antigens which react with antibodies in convalescent sera.* These antigens are most conveniently measured by the complement-fixation test. Two types of antigen can be detected, a *group antigen* present in all strains of influenza virus A and probably identical with the soluble antigen of Hoyle & Fairbrother (1937), and *specific antigens* which vary from strain to strain. Suitable sera for the measurement of these antigens are readily obtained. Most of the work described in this paper has been done with the D.S.P. strain of virus isolated in 1943. A serum reacting with the specific antigen of D.S.P. virus was obtained by infecting a ferret. The ferret convalescent serum reacted with D.S.P.

elementary bodies to a titre of 1:128 and only reacted with a preparation of soluble antigen to a titre of 1:2. It was used in a dilution of 1:30 to measure specific antigen. Sera produced in experimental animals often contain very little antibody to soluble antigen in contrast to human convalescent sera which usually contain large amounts of such antibody. The group antigen was measured by titration with human convalescent serum from the 1949 epidemic. This epidemic was due to a strain of A prime type which was not closely related to D.S.P. virus, so that most of the convalescent sera from this outbreak reacted to high titre only with the group antigen and to a much lower titre against the specific antigens of D.S.P. virus. The particular batch of pooled 1949 convalescent serum used reacted with soluble antigen to a titre of 1:128, while chess-board experiments of the type described by Hoyle (1945) showed that its titre against specific D.S.P. antigen was only 1:20. It was used in a dilution of 1:30. The complement-fixation tests described in this paper were all done by the micro method of Hoyle (1945).

QUANTITATIVE RELATIONSHIPS BETWEEN THE PROPERTIES OF THE ELEMENTARY BODY

The elementary body can thus be detected and its amount measured by four different tests. These tests are very unequal in sensitiveness. Different preparations of elementary bodies may show slight differences, but in general the following quantitative relations hold good. An elementary body preparation containing 1000 million infecting doses per ml. will give an agglutinin titre of 1:1000, a specific complement-fixing antigen titre of 1:2, and a group complement-fixing antigen titre of 1:2. In the author's previous work (Hoyle, 1948, 1949), it was found convenient to express the agglutinating power of a fluid in terms of agglutinating units per ml., the unit being the smallest amount giving complete agglutination in the Salk test. The complement-fixing power was similarly expressed in units per ml., the unit being the amount giving 50% fixation in the micro complement-fixation test. In the case of the elementary body 1 complement-fixing unit would be equal to 25 agglutinin units or 25 million infecting doses. These units are also used in this paper.

STRUCTURE OF THE ELEMENTARY BODY

Wiener, Henle & Henle (1946) showed that soluble antigen could be liberated from the elementary body by sonic disruption. A simpler method of disruption of the elementary body is afforded by treatment with ether. Andrewes & Horstmann (1949) showed that many viruses could be inactivated by treatment with 20% ether.

A concentrated preparation of D.S.P. elementary bodies prepared from infected allantoic fluid by adsorption and elution from red blood cells was divided into two parts, and one shaken with 20% ether and then incubated for 3 hr. at 37° C. to remove the ether. The original and treated preparations were then tested for content of agglutinin and of specific and group complement-fixing antigen. The preparations were then adsorbed with red blood cells and the supernatant fluids again tested. The following results were obtained.

The original elementary body preparation had an agglutinin titre of 8192. After treatment with ether the agglutinin titre was increased to 16,384. All the agglutinin in both preparations was removed by adsorption with red blood cells. The serological results are shown in Table 1. When titrated with a human serum reacting with group antigen the original preparation reacted to a titre of 1:16, and the antigen was removed by adsorption with red cells. After ether treatment the preparation still gave the same titre of 1:16, but a large part of the antigen was no longer adsorbed by red cells. Ether treatment therefore appears to liberate soluble antigen from the elementary body.

Table 1. *Titration of complement-fixing antigens in elementary Body Suspensions before and after treatment with Ether*

Serum	Antigen	Antigen control	Antigen dilution					
			1:1	1:2	1:4	1:8	1:16	1:32
Group human serum control + + + +	(1) Original elementary body preparation	+ + + +	0	0	0	0	++	++++
	(2) Preparation (1) after adsorption with red blood cells	+ + + +	+++	++++	++++	++++	++++	++++
	(3) Preparation (1) after ether treatment	+ + + +	0	0	0	0	++	++++
	(4) Preparation (3) after adsorption with red blood cells	+ + + +	0	0	0	0	++++	++++
	Preparation 1		0	0	0	0	+	++++
Specific ferret serum control + + + +	Preparation 2		+	++++	++++	++++	++++	++++
	Preparation 3		+	+	++	++	++++	++++
	Preparation 4		++++	++++	++++	++++	++++	++++

++++ = complete haemolysis.
+, ++, +++ = intermediate degrees of haemolysis.
0 = no haemolysis, i.e. complete fixation of complement.

When titrated with specific ferret serum rather different results were obtained. The specific antigen of the original preparation was removed by adsorption on red cells. After treatment with ether the antigen titre appeared to be reduced and the complement fixation was of an incomplete nature, many tubes showing partial fixation. Adsorption on red cells removed the antigen. It appeared that treatment with ether reduced the antigenicity of the specific antigen so that incomplete fixation of complement occurred. Such an effect might be due to a great reduction in particle size of the antigen.

Treatment with ether appeared to disrupt the elementary body into smaller units and to dissociate the combination of specific and group antigens so that these appeared as separate units, the specific antigen adsorbed on red cells and the group antigen not so adsorbed. This conclusion was confirmed by the results of filtration of these preparations through gradocol membranes of various pore sizes. Owing to the low sensitivity of the agglutination and complement-fixation tests an accurate measurement of the size of the various units was not possible, but the following results were obtained.

The agglutinin content of the original elementary body preparation was reduced

to 0.4 % filtration through a membrane of A.P.D. 0.38μ , and none could be detected in a filtrate through a membrane of A.P.D. 0.2μ .

After treatment with ether 10 % of the group or soluble antigen could be detected in a 0.38μ filtrate, and the antigen was just detectable in a 0.2μ filtrate. None was found in a 0.12μ filtrate. The soluble antigen was therefore appreciably smaller than the elementary body.

After ether treatment 20 % of the agglutinin passed a 0.38μ filter, 1 % passed a 0.2μ filter, and the agglutinin was detectable in a 0.12μ filtrate. It was retained by a 0.06μ filter. The agglutinin after ether treatment appeared therefore to be much smaller than the elementary body and was probably smaller than the soluble antigen.

It may be tentatively suggested that the elementary body consists of an aggregate of group and specific antigen particles enclosed in an ether soluble envelope, possibly of a lipid nature. Removal of this envelope results in dissociation of the aggregate. The agglutinin titre increases because of the release of specific antigen (which is probably identical with the agglutinin since both are adsorbed by red cells) from combination with the group antigen. It is to be noted that disruption of the elementary body by ether does not result in any increase in antigen titre in complement-fixation tests, the titre of group or soluble antigen remains unchanged, while that of specific antigen shows a decrease, this decrease being probably due to a great reduction in particle size of the antigen. It appears that antibody molecules can gain access to all the antigen in the intact elementary body, which might suggest that the lipid envelope is in the form of a network permeable to antibody.

Having considered the properties and possible structure of the infective elementary body we can now pass to a study of the life cycle stage by stage.

UNION OF VIRUS WITH THE CELL

When virus is introduced into the allantoic sac the greater part is taken up by the cells of the chorio-allantoic membrane. The union is very rapid, most of the virus being taken up by the cells within a few minutes, but is not complete. In the author's previous experiments (Hoyle, 1948) about 90 % of the virus united with the cells. This percentage is constant over a fairly wide range of inocula but with very large inocula, 1000 agglutinin units or more, the percentage uniting with the cells becomes less than 90 %. Henle & Henle (1949) found a somewhat lower percentage union, 70 %, with the cells using small inocula. Two factors account for the incomplete nature of the union with the cell. The first is the presence of a substance resembling Francis inhibitor (Francis, 1947) in normal allantoic fluid. This inhibitor has been studied by Hardy & Horsfall (1948) and by Svedmyr (1948*a, b*; 1949*a, b, c*). The inhibitor is probably derived from the cell walls. With small inocula the effect of this inhibitor in preventing union of virus with the cell might be considerable, but with large doses the inhibitor is so rapidly destroyed by the virus that it would have little effect. The presence of this inhibitor may account for the different percentages of union obtained by the author and by Henle. The second factor is that the union of virus and cell is probably inherently of a reversible nature so that an equilibrium is set up depending on the relative concentration

of the two elements. The union of virus with the chorio-allantoic membrane is very similar to its union with red cells. If a series of dilutions of virus is adsorbed with a constant amount of red cells it is found that over a wide range a constant percentage of the virus is adsorbed. A similar relation is found if dilutions of virus are adsorbed with minced chorio-allantoic membrane *in vitro* (Text-fig. 1). This percentage law does not hold for very small or very large amounts of virus.

Text-fig. 1. *Adsorption of virus by red cells and by minced chorio-allantoic membrane in vitro.* 1 ml. quantities of dilutions of D.S.P. allantoic fluid were adsorbed for 10 min. at 37° C. with (a) 100 million guinea-pig red cells, (b) minced chorio-allantoic membrane of one egg. Salk titres measured before and after adsorption. O—O, R.B.C.; x---x, C.A.M.

The initial union of virus with the cells is quite independent of the cell nutrition; thus it occurs equally as well *in vitro* as *in vivo*. Nor is it necessary that the virus should be viable. Virus inactivated with formaldehyde is adsorbed by the cells in the same way as live virus. The union of virus with the cells is entirely prevented by neutralizing serum.

EFFECT OF NEUTRALIZING SERUM

Three groups of three 12-day-old fertile eggs were inoculated by the allantoic route with a large dose of D.S.P. virus as below:

Group A: inoculated with 0.3 ml. D.S.P. 18 hr. allantoic fluid.

Group B: inoculated with 0.3 ml. D.S.P. allantoic fluid and 30 min. later with 1.0 ml. of human convalescent serum containing neutralizing antibody to D.S.P. virus.

Group C: inoculated with 1.0 ml. serum and 30 min. later with 0.3 ml. of D.S.P. allantoic fluid.

The eggs were incubated for 6 hr., the chorio-allantoic membranes removed, suspended in 1 ml. saline, frozen and thawed three times, incubated at 37° C. for 6 hr. to allow elution of virus from cell receptors, centrifuged, and the pooled supernatants tested for soluble antigen by titration with human convalescent serum, and for agglutinin by the Salk test:

Group A (control) C.F. Titre 1:14 Salk titre 4096

Group B (serum after virus) C.F. Titre 1:24 Salk titre 2048

Group C (serum before virus) C.F. Titre Nil Salk titre Nil

The growth of virus was completely prevented by the previous inoculation of neutralizing serum, but serum added 30 min. after the virus was without effect. Neutralizing serum therefore prevents the initial union of virus and cell.

PENETRATION OF VIRUS TO THE INTERIOR OF THE CELL

Hirst (1942) showed that the virus contained an enzyme-like substance which united with receptor substances in the red blood cell and subsequently destroyed the receptor, the virus being then released or eluted from combination with the cell. Hirst (1943) also showed that similar phenomena occurred in excised ferret lung; virus introduced intratracheally was first adsorbed and then subsequently eluted. Burnet, McCrea & Stone (1946) showed that the cells of the chorio-allantoic membrane behaved in the same way, and Fazekas de St Groth (1948*a, b*) extended the observation to mouse lung. The virus is therefore equipped with an enzyme-like mechanism capable of uniting with, and destroying, certain receptors in the cell wall. These are probably mucoprotein in nature (Burnet, 1948). This receptor-destroying enzyme would clearly enable the virus to penetrate the mucoprotein layer of the cell wall and so gain access to the interior.

Evidence that such penetration does in fact occur is afforded by the experiments with neutralizing serum described above. The addition of neutralizing serum 30 min. after inoculation with virus fails to prevent or modify the subsequent growth of virus in cases where the original inoculum was large enough to infect all the cells. It is clear, therefore, that within 30 min. the virus has penetrated to a point at which it is inaccessible to neutralizing serum.

The chorio-allantoic membrane 1 hr. after infection is almost unable to adsorb antibody from an agglutinin-inhibiting serum. An egg was inoculated by the allantoic route with 1280 agglutinin units of virus. After 1 hr. the membrane was removed. The residual allantoic fluid contained 400 agglutinin units so that 880 units had been taken up by the cells. The membrane was washed in saline and used to adsorb 1 ml. of an agglutinin-inhibiting serum containing 128 units of antibody. The titre of the serum after adsorption was 96 so that only 32 units of antibody had been adsorbed. It appeared, therefore, that the major part of the agglutinin was inaccessible to the antibody.

DISINTEGRATION OF THE VIRUS

Hoyle (1948) showed that extracts of the chorio-allantoic membrane made 1–3 hr. after inoculation contained no virus demonstrable by the red cell agglutination test, and even by the highly sensitive infectivity test less than 1 % of the virus taken up by the cells could be recovered. These observations were confirmed by Henle & Henle (1949). The chorio-allantoic membrane of the living egg appears to be able to take up and destroy between 500 and 1000 agglutinin units of virus. The destruction takes place very rapidly, a trace of virus may be demonstrable 30 min. after inoculation, but by 1 hr. no virus can be demonstrable by agglutination tests in membrane extracts. Infective virus does not reappear in the membrane until 5–6 hr. after inoculation. However, between 2 and 5 hr. after inoculation non-infective particles of smaller size than the elementary body can be demonstrated in the membrane by complement-fixation tests (Hoyle, 1948), and between 3 and 5 hr. non-infective units can be demonstrated by the red cell agglutination test (Henle & Henle, 1949). Such particles could be derived from the elementary body if the latter had undergone disintegration.

The maximum destruction of virus is produced by the intact membrane in the living egg, but appreciable amounts of virus may be destroyed *in vitro*.

1 ml. quantities of dilutions of D.S.P. 20 hr. allantoic fluid were adsorbed with the washed chorio-allantoic membrane of one egg. After 15 min. at 37° C. the membranes were centrifuged off and the amount of virus taken up measured by determining the content of residual virus in the supernatant fluids. The membranes were washed, suspended in 1 ml. of saline containing 0.08 % sodium azide, frozen and thawed three times, and incubated for 6 hr. at 37° C. to allow of elution of virus from cell receptors. The fluids were then centrifuged and the virus content of the supernatants measured by the Salk test. Results are shown in Table 2.

Table 2. *Adsorption and destruction of virus by chorio-allantoic membrane in vitro*

Amount of virus agglutinin units	Amount taken up by membrane	Amount recovered in membrane extract
16	16 (100 %)	Nil (0 %)
32	30 (96 %)	Nil (0 %)
64	56 (87 %)	Nil (0 %)
128	96 (75 %)	2 (2 %)
256	192 (75 %)	16 (9.3 %)
512	384 (75 %)	64 (16.6 %)
1024	768 (75 %)	256 (33.3 %)
2048	1536 (75 %)	768 (50 %)

With large amounts of virus a constant percentage was adsorbed by the membrane. With smaller amounts the percentage adsorbed increased. With amounts of virus of less than 100 agglutinin units no virus could be recovered from the membrane by freezing and thawing and subsequent elution, but with larger amounts an increasing percentage was recovered, reaching 50 % with 1500 units taken up by the membrane.

The destruction of virus appears to be effected by some constituent of the cell

stroma. Thus if the chorio-allantoic membrane is disintegrated by freezing and thawing and the stroma centrifuged off, the supernatant fluid is unable to destroy virus, but the resuspended stroma destroys virus in the same way as the whole membrane.

It appears that virus introduced into the allantoic sac penetrates to the interior of the cells of the chorio-allantoic membrane and then unites with some part of the cell stroma. This union is not an adsorption of virus similar to that occurring with red cells, since virus cannot be recovered from the membrane by any process of elution however prolonged. At a later stage, however, particles smaller than the elementary body can be recovered from the membrane so that it seems probable that the virus elementary body undergoes disintegration into smaller units.

INTRACELLULAR MULTIPLICATION OF THE SOLUBLE ANTIGEN

Between 1 and 2 hr. after inoculation, no evidence of the presence of virus in the chorio-allantoic membrane can be obtained, the virus apparently being firmly linked to some constituent of the cell stroma and probably undergoing disintegration. If eggs are inoculated with a large dose of virus (500–1000 agglutinin units) so as to infect all the available cells, the first evidence of growth of virus which can be obtained is the appearance and rapid increase in amount of complement-fixing soluble antigen in extracts of the membrane. This antigen is first detectable at about 2 hr. and increases logarithmically between 2 and 4 hr., doubling itself about every 20 min. Both the rate of increase and the total amount produced are remarkably constant (Text-fig. 2). After 4 hr. the rate of increase falls off. This

Text-fig. 2. Production of complement-fixing soluble antigen in the chorio-allantoic membrane of eggs inoculated with a large dose of D.S.P. virus (0.4 ml. of undiluted infective allantoic fluid). The figure shows the results of seven experiments.

multiplication of soluble antigen is entirely dependent on the nutrition of the cells. Thus if eggs are inoculated with virus, incubated for 1 hr. to allow infection of the cells and penetration of virus to the interior, and the chorio-allantoic membranes

removed, suspended in saline, and the incubation continued, no production of soluble antigen occurs. If, however, the membranes are suspended in Tyrode's solution a small production of antigen may occur, but much less than occurs in the intact egg. The production of soluble antigen is therefore dependent not merely upon the viability of the cells but on a continued supply of nutrient material to them.

Certain dyes are able greatly to retard the production of soluble antigen in the membrane, especially the violet dyes of the triphenylmethane group (Hoyle, 1949). Addition of 0.1 mg. of Crystal Violet or Dahlia Violet to the allantoic fluid 30 min. after inoculation of a large dose of virus retards and reduces the production of soluble antigen, and the possibility was suggested that the effect was due to interference with the metabolism of the infected cell. Proflavine in amounts of 1.0 mg. has a similar effect (Text-fig. 3). As with the triphenylmethane dyes the effect

Text-fig. 3. Effect of addition of 1.0 mg. of proflavine to the allantoic fluid of eggs 30 min. after inoculation on the production of complement-fixing soluble antigen in the chorio-allantoic membrane of eggs inoculated with 0.4 ml. of undiluted D.S.P. allantoic fluid. ○—○, control; ×---×, proflavine.

appears to be due to interference with the cell metabolism, since the dye in the concentration used has no effect *in vitro* either on the red cell agglutinating power of the virus or on the antigenicity of the soluble antigen.

The amount of soluble antigen produced appears to be dependent on the number of cells infected. Thus the amount produced is roughly dependent on the size of the inoculum where this is less than 200 million infective doses, but over this amount no appreciable increase in production occurs. (The allantoic sac of a 12-day egg is lined by approximately 100–200 million cells.)

Treatment of the virus with certain antiseptics prevents the intracellular multiplication of soluble antigen. Formaldehyde is the best example. Eggs were inoculated with virus which had been treated for 30 min. with 1:1000 formaldehyde. It was found that the formolized virus was taken up by the cells in the same

way as live virus and appeared to penetrate to the interior in the same way since less than 1 % could be recovered from the membrane, and the membrane adsorbed very little antibody from an agglutinating-inhibiting serum. But no intracellular production of soluble antigen occurred in eggs inoculated with formalized virus.

With untreated virus, 4 hr. after inoculation the amount of soluble antigen present in the membrane is about fifty times the amount present in the original inoculum, and by 6 hr. the multiplication has reached 100-fold. Thus in a typical experiment an egg was inoculated with 500 agglutinin units of virus. By titration of the virus suspension with human convalescent serum reacting only with the soluble antigen fraction this amount of virus was shown to contain twenty complement-fixing units of soluble antigen, and the same figure was obtained by titration of the virus suspension after disintegration of the elementary body with ether. After 4 hr. incubation an extract of the chorio-allantoic membrane contained 1000 units of soluble antigen. A 50-fold increase had therefore occurred.

It is clear from these experiments that the first sign of multiplication of influenza virus in the infected egg is an increase in complement-fixing soluble antigen occurring between 2 and 4 hr. after inoculation. It is to be noted that there is no comparable increase in infectivity of the membrane extract. If the infectivity increased in the same way as the soluble antigen content, at 4 hr. the membrane should contain some 25,000 million infecting doses of virus. In fact, only about 1 million infecting doses can be demonstrated in the membrane at 4 hr., an amount less than 1 % of that originally inoculated. It seems evident that the soluble antigen is itself a multiplying unit and not a product of the multiplication of infective virus in the membrane.

INTRACELLULAR PRODUCTION OF AGGLUTININ

When eggs are inoculated with a large dose of virus, extracts of the chorio-allantoic membrane develop the ability to agglutinate red cells between 3 and 4 hr. after inoculation. Text-fig. 4 illustrates the results of seven such experiments. The eggs were inoculated with 0.4 ml. of D.S.P. 20 hr. allantoic fluid, and the membrane extracts were made by suspending the membrane in 1 ml. of saline containing 0.08 % sodium azide, freezing and thawing four times and then incubating at 37° C. for 6 hr. to allow elution of virus. The fluid was then centrifuged and the agglutinin content measured by the Salk test. It will be seen that while the time of appearance of agglutinin was fairly consistent there was great variation in the amount produced, in striking contrast to the relatively constant production of soluble antigen in the same experiments (Text-fig. 2). The appearance of the agglutinin is usually very sudden, a sharp almost vertical rise occurring between 3 and 4 hr. after inoculation. This vertical rise is followed by a slower increase in agglutinin production which is not very apparent in studies of the membrane extracts, since excretion of agglutinin into the allantoic fluid commences at about 5 hr. The further increase in agglutinin production is more readily followed by measuring the rise of agglutinin titre in the allantoic fluid.

It has been shown above, that between 2 and 4 hr. there occurred a 50-fold increase in the amount of soluble antigen as compared with the amount in the

original inoculum. There is no evidence of any comparable increase in agglutinin between 2 and 4 hr. Owing to variation in titre of the 20 hr. D.S.P. allantoic fluids used as inocula, in the experiments of Text-fig. 4, the actual inocula varied between 200 and 1600 agglutinin units, while the yield of agglutinin at 4 hr. varied from 50 to 6400 units. It seemed possible that the sudden vertical increase in agglutinin titre between 3 and 4 hr. might represent the release of agglutinin derived from the original inoculum from combination with the cell stroma, and that a real increase in agglutinin only occurred at a later stage. It was found that when the original inoculum was less than 500 agglutinin units the yield of agglutinin at 4 hr. was

Text-fig. 4. Results of seven experiments on the production of agglutinin in the chorio-allantoic membrane of eggs inoculated with a large dose of D.S.P. virus (0.4 ml. undiluted infected allantoic fluid).

slightly less than the original inoculum and the subsequent rate of increase was rather slow. When the inoculum was 1000 agglutinin units or more the yield of agglutinin at 4 hr. was usually slightly greater than the inoculum and the subsequent increase was rapid. The production of soluble antigen always precedes the appearance of agglutinin, and it is possible that it is the multiplication of the soluble antigen which results in the release of the agglutinin from its combination with the cell stroma. If the production of soluble antigen is retarded by treatment with crystal violet there is a corresponding retardation in the appearance of agglutinin.

THE PRECURSOR OF GARD AND VON MAGNUS

The sudden rise in agglutinin titre of the membrane extract between 3 and 4 hr. is not accompanied by any increase in infectivity. Eggs were inoculated with 0.4 ml. of D.S.P. allantoic fluid. Membrane extracts were prepared at 4 hr. and at 6 hr. and the agglutinin titre and infectivity measured with the following results.

Original inoculum: agglutinin titre 2560 (infectivity 1000 million infective doses per ml.).

4 hr. membrane extract: agglutinin titre 5120 (infectivity 1 million infective doses per ml.).

6 hr. membrane extract: agglutinin titre 10240 (infectivity 100 million infective doses per ml.).

The agglutinin which appears in the membrane at 4 hr. is clearly not infective virus. Henle & Henle (1949) showed that the appearance of agglutinin in eggs inoculated with a large dose of virus preceded any increase in infectivity. Gard & von Magnus (1946) had shown that non-infective agglutinating particles smaller in size than the infective virus could be found in the allantoic fluid of eggs inoculated with a large dose of virus, and that production of these particles probably preceded production of infective virus. The author's observations confirm these findings, and it seems probable that the agglutinin which appears in the membrane extract at 4 hr. is similar to the precursor of Gard & von Magnus.

This agglutinin can be adsorbed and eluted from red blood cells in the same way as infective virus. A preparation of agglutinin from a 4 hr. membrane extract was made by adsorption and elution from red cells, and a similar preparation of elementary bodies was made from a 20 hr. allantoic fluid. The serological properties of these two preparations were compared by titration with specific ferret serum and with a human convalescent serum reacting with soluble antigen. The results are shown in Table 3. The precursor contained both specific antigen and group

Table 3. *Serological properties of elementary body and precursor*

	Salk titre	Complement fixation titres	
		Specific ferret serum	Group human serum
Elementary body preparation	8192	1:20	1:16
Precursor preparation	4096	1:14	1:6

(soluble) antigen, but the amount of soluble antigen was only about half as great in relation to specific antigen or agglutinin as in the case of the elementary body. This finding was confirmed by the results of disintegration of the precursor with ether. It was found that the precursor could be disrupted by ether treatment in the same way as the elementary body, but that a smaller amount of soluble antigen was released than in the case of the elementary body.

INTRACELLULAR PRODUCTION OF SPECIFIC COMPLEMENT-FIXING ANTIGEN

The production of specific complement-fixing antigen in the membrane runs closely parallel to the production of agglutinin, the antigen appearing between 3 and 4 hr. after inoculation. The amount of specific antigen produced at 4 hr. is considerably less than the amount of soluble antigen, indicating that only slight multiplication of the specific antigen has occurred during this time. The whole of the specific antigen in a 4 hr. membrane extract is removed by adsorption with red cells, and it seems probable that the antigen is identical with the agglutinin. Text-fig. 5 shows the intracellular production of agglutinin, soluble antigen and specific antigen in eggs inoculated with 1000 agglutinin units of virus.

Text-fig. 5. Production of agglutinin, specific complement-fixing antigen, and soluble antigen in the chorio-allantoic membrane of eggs inoculated with 1000 agglutinin units of D.S.P. virus. □---□, agglutinin; ×---×, specific antigen; ○—○, soluble antigen.

STATE OF VIRUS IN THE MEMBRANE AT 4-5 HR.

The experiments described above indicate that the conditions in the membrane at 4-5 hr. are somewhat complex. A membrane extract at this time shows all the properties of the virus elementary body except that the infectivity is extremely low. The extracts agglutinate red cells and contain both specific and soluble complement-fixing antigen. However, these properties are carried by particles of smaller size than the elementary body. These particles are of two kinds, one adsorbed by red blood cells and one not adsorbed. The first, analogous to the von Magnus precursor, agglutinates red cells, contains all the specific antigen and a small amount of soluble antigen, while the second consists of soluble antigen alone. In relation to the original inoculum there has been a considerable increase in soluble antigen and a much smaller increase in agglutinin and specific antigen.

PRODUCTION OF INFECTIVE VIRUS

When eggs are inoculated with a large dose of virus, e.g. 500 million infective doses, the infectivity of extracts of the chorio-allantoic membrane 1 hr. after inoculation is less than 1 % of that which would be expected in view of the amount of virus taken up by the cells, and is usually of the order of 1 million infecting doses. The infectivity remains at this low level for about 4 hr., but between 5 and 6 hr. after inoculation a rise in infectivity occurs. In a typical experiment the infectivity rose from 1 to 100 million infecting doses between 4 and 6 hr. However, membrane extracts never at any stage attain a high infectivity; the main appearance of infective virus is in the allantoic fluid. The infectivity of the allantoic fluid increases rapidly from 6 hr. onward, and the fluid may come to contain a total of 20,000 million infecting doses or more. There is a corresponding rise in the agglutinin titre of the allantoic fluid. The results strongly suggest that infective virus is not really produced in the cell at all, but that the infectivity is acquired at the moment of passage of virus through the cell wall. Not only does the infectivity appear to be acquired at the moment of excretion, but the main production of agglutinin appears to occur at this time.

MORPHOLOGY OF INFECTIVE VIRUS

If an infected allantoic fluid is adsorbed with red blood cells and the virus eluted the eluate shows on examination by dark-background microscopy large numbers of spherical particles, but also occasionally filaments of various lengths are seen. These filaments were described by Mosley & Wyckoff (1946). They have been studied by Heinmets (1948) and by Chu, Dawson & Elford (1949) using the electron microscope. The method of examination devised by Dawson & Elford (1949), where the particles are examined in the electron microscope after adsorption on red cell ghosts, has proved particularly valuable. It seems highly probable from the work of these authors that both the spherical particles and the filaments are forms of the virus. Most of the standard laboratory strains of virus show few filamentous forms, but strains of A prime type isolated in 1949 show very large numbers of filaments. Heinmets (1948) regarded the filamentous forms as aggregates formed from elementary bodies during storage, but Chu *et al.* (1949) regard them as probably a stage in the multiplication of the virus.

The author has studied the problem using the dark-background microscope. The D.S.P. strain of virus used in the work described above shows few filamentous forms, but a strain Burch isolated in 1949 showed very large numbers of filaments. Pl. 3, fig. 1 shows the appearances seen in an eluate of Burch allantoic fluid by dark-background microscopy. The fluid shows spherical particles, thin rather rigid filaments, thicker flexible filaments, drumstick forms, chains of spherical particles and composite forms of various types. It seemed important to determine whether the filaments were found in extracts of the chorio-allantoic membrane or whether they were only found in the allantoic fluid. The Burch strain of virus was found to behave in the same way as the D.S.P. virus in eggs, production of soluble antigen, agglutinin, and infective virus occurred in the same way as with the D.S.P. strain.

Membrane extracts were prepared in the usual way at various stages of incubation, and the agglutinating particles adsorbed on red cells and eluted, concentrating the preparations where necessary in order to obtain fluids of agglutinin titres of the order of 1-4000. Similar preparations were made from allantoic fluids. On examination by dark background it was found that while filamentous forms could be found in the allantoic fluids at all stages of incubation, and were very numerous in the case of the Burch strain, no filaments were ever seen in extracts of the chorio-allantoic membrane.

The possibility was considered that the absence of filamentous forms in membrane extracts might be due to their destruction by the process of freezing and thawing used in making extracts. An allantoic fluid eluate of the Burch strain showing numerous filaments was frozen and thawed three times and again examined. Numerous filaments were still present, though the average length was shorter than in the original fluid. The process of freezing and thawing caused some disintegration of the longer forms, but did not cause total destruction of the filaments. It is unlikely therefore that the absence of filaments in membrane extracts can be due to destruction in the process of making the extracts.

The filamentous forms are completely destroyed by treatment with ether. An allantoic fluid eluate of Burch strain was shaken with 20 % ether, and immediately examined. No filamentous forms were seen, and the spherical particles appeared to be greatly reduced in number, and in some cases appeared swollen as if undergoing disintegration.

It is evident that a remarkable change in the structure and properties of the virus must occur at the time of excretion from the cell. A clue to the nature of this change was afforded by a study of the behaviour of the cell at this time when examined by the dark-background microscope.

EXCRETION OF VIRUS FROM THE CELL

Examination of normal chorio-allantoic membrane by dark-background microscopy

A normal 12-day fertile egg was opened, the allantoic fluid removed, and the embryo and yolk sac turned out leaving the chorio-allantoic membrane in the shell. Scrapings were then taken from the internal surface of the membrane with a platinum loop and the detached portions of lining membrane suspended in a drop of allantoic fluid and immediately examined in the dark-background microscope. It was found that the detached pieces of lining membrane showed a number of transparent cytoplasmic protrusions along the edge. As the preparation warmed up on the stage of the microscope these balloon-like protrusions increased in numbers. Many of them became detached from the cell and floated away as spherical globules of various sizes into the allantoic fluid. In other cases the protrusions remained attached to the cells by a fine filament, often of great length. Tubular protrusions were also formed and these tended to increase in length and become narrower and were often seen to segment giving an appearance resembling a chain of beads. All types of protrusions were liable to break away from the cells and float away into the allantoic fluid. Pl. 3, fig. 2 shows the appearances after 30 min. examination.

It seemed probable that osmotic effects were responsible for these phenomena. The rising temperature during examination caused an increase in the internal osmotic pressure in the cells, and this resulted in the ballooning of the cell membrane and the formation of tubular processes. However, it seemed unlikely that the effects were entirely an artefact, since spherical globules of various sizes could be found in allantoic fluid from intact eggs and also occasionally fine filaments were seen. It is probable that the phenomena seen under dark-background examination represent an exaggeration of a process which might occur normally in the intact egg. It seemed that there was a rather delicate balance between the internal osmotic pressure of the cell and the elasticity of the cell membrane, and that disturbances of this balance led to the formation of cytoplasmic protrusions of various types.

Appearance of the cells in infected eggs

Very similar appearances were seen in eggs inoculated with virus, especially if the examination was made at the time when the agglutinin titre of the allantoic fluid was rising. Eggs were inoculated with 0.1 ml. of infected allantoic fluid diluted 1:100 and examined at 18–24 hr. With both Burch and D.S.P. strains of virus the lining membrane of the allantoic sac showed under dark-background examination cytoplasmic protrusions similar to those seen in normal eggs. Long tubular processes were common and these were seen to break away from the cells. As soon as continuity with the cell was broken the tubular processes began to shrink, and in many cases they segmented to give an appearance resembling a string of beads. The shrinking process continued and the ultimate result was the formation of a filament of the type found free in the allantoic fluid of infected eggs. It was often possible to follow under the microscope the complete series of changes whereby a tubular cytoplasmic protrusion became converted into a filamentous form of virus. With the D.S.P. strain of virus there was a greater tendency for the filaments to fragment than with the Burch strain, so that a greater number of spherical particles were produced.

These changes were undoubtedly occurring in the intact egg, since desquamated cells could be found in the allantoic fluid which showed all types of cytoplasmic protrusion and all stages in the conversion of a tubular protrusion into a virus filament could be seen. Plate 4, fig. 1 shows the appearance of cytoplasmic protrusions from a piece of detached lining membrane in an egg infected with Burch virus, and Pl. 4, fig. 2 shows desquamated cells found in the allantoic fluid in an egg inoculated with D.S.P. virus. With D.S.P. virus the changes occurred more rapidly and sometimes in the allantoic fluid only cells in the final stage could be found. Such cells appeared to consist of little but a nucleus with four or five filaments remaining attached to it.

It seemed probable that the normal balance between the osmotic pressure of the cell and the strength of the cell membrane was disturbed in the infected egg as a result of attack by the virus on the cell wall. This resulted in a weakening of the cell walls with the formation of balloon-like or tubular protrusions. On becoming detached from the cell these protrusions began to shrink, probably as a result of

the surrounding membrane becoming more permeable. The elementary bodies and filamentous forms of virus in the allantoic fluid would thus consist of a mass of virus protein enclosed within remains of the cell membrane, the shape of the final particle being a result of the shape of the original cell protrusion.

DISCUSSION

The experiments described in this paper enable us to formulate a fairly complete picture of the life cycle of the influenza virus, though the picture may require modification in some of its details as a result of further work.

The infective elementary body consists of an aggregate of soluble antigen and specific antigens enclosed in a lipoidal envelope. The specific antigens are probably identical with the agglutinin and are capable of uniting with receptor substances of a mucoprotein nature in the wall of the host cell. The complex is also capable of destroying the mucoprotein of the cell wall so that the virus is able to penetrate to the interior. On penetration the virus combines with some substance in the cell stroma and disintegrates into its component units. It is not clear how this disintegration occurs. It may be that the disintegration is a result of passage through the cell wall. This might occur if the lipid envelope of the elementary body became absorbed into the lipid part of the cell wall from which it was probably originally derived. Or disintegration may occur inside the cell. In a recent paper on multiplication of the animal viruses, Bauer (1949) has suggested that viruses may unite with and multiply in association with the chromidia.

The first detectable evidence of intracellular multiplication of the virus is the logarithmic increase in soluble antigen which occurs between 2 and 4 hr. The soluble antigen must be itself a self-reproducing unit. A 50-fold increase in its amount occurs between 2 and 4 hr. A corresponding increase in infective virus would result in the membrane containing at 4 hr. some 25,000 million infecting doses. In actual fact, only about 1 million infecting doses can be demonstrated, and it is therefore clear that the increase of soluble antigen is not a result of a parallel multiplication of infective virus. The soluble antigen is probably the fundamental nucleoprotein of the influenza virus. Its properties are those of a protein, its iso-electric point is the same as that of the elementary body (Bourdillon & Lennette, 1940), and serological evidence suggests that it accounts for the major bulk of the elementary body. The production of soluble antigen is dependent on the adequate nutrition of the host cell and is retarded by dyes such as crystal violet and proflavine which probably act by interfering with cell nutrition.

In extracts of infected tissue the soluble antigen always appears as a particle of smaller size than the elementary body. Centrifugation of such extracts at speeds sufficient to sediment the elementary body produces little reduction in complement-fixing titre. There seems no reason to suppose that the particle size is not the same in the infected cell. It is of course possible that in the infected cell the multiplying virus is localized into some form of inclusion body, but there is no evidence for this and, if it was so, it would have to be assumed that the inclusion body became disrupted when making extracts. The balance of evidence would indicate that the

soluble antigen is the fundamental multiplying nucleoprotein of the influenza virus and that it is to be regarded as a self-reproducing macro-molecule.

The multiplication of soluble antigen between 2 and 4 hr. is not paralleled by any comparable increase in specific antigens or agglutinin. Between 3 and 4 hr. after inoculation there is a sudden appearance of agglutinin and specific antigen, but the amount produced is approximately equal to the amount present in the original inoculum. There are two possibilities. The first is that the specific antigens like the soluble antigen are self-reproducing and that they multiply simultaneously with the soluble antigen, but owing to their affinity for cell receptors they are more difficult to recover in membrane extracts and are therefore detected at a later stage and in smaller amount. But if this were so, while one might expect difficulty in detecting small amounts of agglutinin and specific antigen, once the amount present was sufficient to destroy the cell receptors there seems no reason why the whole amount should not be recovered. In fact, the amount recovered at 4 hr. is closely comparable to the amount present in the original inoculum. It is unlikely, therefore, that any appreciable increase in agglutinin and specific antigen occurs in the first 4 hr. It seems that the agglutinin which appears between 3 and 4 hr. is derived by the release of agglutinin present in the original inoculum from combination with cell stroma, and this release appears to be dependent on the previous multiplication of soluble antigen. The main increase in agglutinin and specific antigen occurs at a later stage coincident with excretion of virus from the cell.

When the agglutinin present in the original inoculum has been released from combination with the cell stroma it may be expected to combine with the soluble antigen and the complex so formed would be able to attack the cell wall. Further production of specific antigen and agglutinin occurs during this attack, and it is possible that the specific antigen of the original inoculum acts as a pattern molecule causing the synthesis of more specific antigen molecules of the same type. In this way the strain would retain its serological identity on passage. Serological studies of the specific antigens after destruction of the elementary body by ether suggests that they have a particle size smaller than the soluble antigen, and their ability to give complement fixation is much reduced in the absence of soluble antigen. The possibility has to be considered that they may be polysaccharide haptens and that they are synthesized by the soluble antigen-specific antigen complex from the mucoprotein of the cell wall.

The further stages in the cycle appear to depend on the nature and vigour of the attack on the cell wall. Observations of the normal lining cells of the allantoic sac in the dark-background microscope indicate the existence of a delicate balance between the osmotic pressure inside the cells and the mechanical strength of the cell membrane. If this balance is disturbed cytoplasmic protrusions may occur, and portions of cytoplasm enclosed in cell membrane may become detached from the cell. The inhibitor found in normal allantoic fluid may be derived from the cells in this way. In the infected egg similar changes may be expected to occur following the weakening of the cell membrane resulting from the attack of the virus. If detached portions of lining membrane from infected eggs are examined in the early stages of excretion of virus many spherical and tubular protrusions of

cytoplasm can be seen. These protrusions presumably contain aggregating virus. On becoming detached from the cell they shrink, partly as a result of the increasing permeability of the membrane due to the continuing attack of the virus, and also because of the increasing aggregation of the virus protein. The final result is the formation of spherical elementary bodies and filaments of various types. The filaments appear to consist of masses of aggregated virus protein linked by tubes of lipid material derived from the cell wall. The variety of the particles encountered in infected allantoic fluid appears to be a reflexion of the varied nature of the attack on the cell wall and the aggregation of the virus units. With the 1949 strains of virus the attack on the cell wall appears to proceed rather slowly so that there is time for the formation of long tubular cytoplasmic protrusions, and these tend to remain intact and become converted into filaments. With the D.S.P. virus the attack on the cell wall is more rapid, and the tendency to fragmentation of the cytoplasmic protrusions is greater so that spherical elementary bodies predominate. With very large doses of virus the cell wall may be so rapidly destroyed that particles are liberated prematurely before much aggregation has occurred. Under these conditions the precursor of Gard & von Magnus appears in the allantoic fluid.

Infectivity appears to be acquired as a result of passage through the cell wall. The intracellular forms of virus—soluble antigen and precursor—are almost non-infective. The soluble antigen is the fundamental multiplying unit. It probably has to compete with other self-reproducing nucleoproteins in the cell, and the competition may only be successful if the initial amount of virus nucleoprotein introduced into the cell is sufficiently large. This may explain why the precursor of Gard & von Magnus is much less infective than the elementary body. The extra-cellular phase of the virus is to be regarded as a packet of aggregated virus protein enclosed in a lipid envelope derived from the wall of the host cell, and infectivity may well be a function of the size of the packet. The morphology of the extra-cellular forms of the virus does not appear to depend on any inherent characteristic of the virus protein but is a result of the way in which the virus is excreted from the host cell.

REFERENCES

- ANDREWES, C. H. & HORSTMANN, D. M. (1949). *J. gen. Microbiol.* **3**, 290.
 BAUER, D. J. (1949). *Nature, Lond.*, **164**, 767.
 BOURDILLON, J. & LENNETTE, E. H. (1940). *J. exp. Med.* **72**, 11.
 BURNET, F. M. (1948). *Lancet*, **1**, 7.
 BURNET, F. M., MCCREA, J. F. & STONE, J. D. (1946). *Brit. J. exp. Path.* **27**, 228.
 CHU, C. M., DAWSON, I. M. & ELFORD, W. J. (1949). *Lancet*, **1**, 602.
 DAWSON, I. M. & ELFORD, W. J. (1949). *Nature, Lond.*, **163**, 63.
 FAZEKAS DE ST GROTH, S. (1948a). *Austr. J. exp. Biol. Med. Sci.* **26**, 30.
 FAZEKAS DE ST GROTH, S. (1948b). *Aust. J. exp. Biol. Med. Sci.* **26**, 271.
 FRANCIS, T. (1947). *J. exp. Med.* **85**, 1.
 GARD, S. & VON MAGNUS, P. (1946). *Ark. Kemi, Min. Geol.* **24** B, no. 8, 1.
 HARDY, P. H. & HORSFALL, F. L. (1948). *J. exp. Med.* **88**, 463.
 HEINMETS, F. (1948). *J. Bact.* **55**, 823.
 HENLE, W. (1949a). *J. exp. Med.* **90**, 1.
 HENLE, W. (1949b). *J. exp. Med.* **90**, 13.
 HENLE, W. & HENLE, G. (1949). *J. exp. Med.* **90**, 23.
 HIRST, G. K. (1942). *J. exp. Med.* **76**, 195.

Fig. 1.

Fig. 2.

Fig. 1.

Fig. 2.

- HIRST, G. K. (1943). *J. exp. Med.* **87**, 99.
HOYLE, L. (1945). *J. Hyg., Camb.*, **44**, 170.
HOYLE, L. (1948). *Brit. J. exp. Path.* **30**, 123.
HOYLE, L. (1949). *Brit. J. exp. Path.* **29**, 390.
HOYLE, L. & FAIRBROTHER, R. W. (1937). *J. Hyg., Camb.*, **37**, 512.
MOSLEY, V. M. & WYCKOFF, R. W. G. (1946). *Nature, Lond.*, **157**, 263.
SALK, J. E. (1944). *J. Immunol.* **49**, 87.
SVEDMYR, A. (1948*a*). *Brit. J. exp. Path.* **29**, 295.
SVEDMYR, A. (1948*b*). *Brit. J. exp. Path.* **29**, 309.
SVEDMYR, A. (1949*a*). *Brit. J. exp. Path.* **30**, 237.
SVEDMYR, A. (1949*b*). *Brit. J. exp. Path.* **30**, 248.
SVEDMYR, A. (1949*c*). *Brit. J. exp. Path.* **30**, 254.
WIENER, M., HENLE, W. & HENLE, G. (1946). *J. exp. Med.* **83**, 259.

EXPLANATION OF PLATES

PLATE 3

Fig. 1. Morphology of virus in infected allantoic fluid (Burch strain 1949). A, elementary bodies; B, thin rigid filaments; C, flexible filaments; D, drumstick forms; E-G, composite forms.

Fig. 2. Production of cytoplasmic protrusions from the edge of a piece of detached lining membrane from the allantoic sac of a normal 12-day fertile egg under examination in the dark-background microscope.

PLATE 4

Fig. 1. Cytoplasmic protrusions from the edge of a piece of detached lining membrane from the allantoic sac of an egg inoculated 24 hr. previously with a small dose of Burch virus. Dark background.

Fig. 2. Appearances under dark-background microscopy of desquamated cells found in the allantoic fluid of an egg inoculated 24 hr. previously with a small dose of D.S.P. virus.

(*MS. received for publication 3. VI. 50.*)