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Influenza Virus Multiplication in the Chick Chorio-allantoic Membrane

By F. FULTON

The London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1

AND A. ISAACS

The National Institute for Medical Research, Mill Hill, London, N.W. 7

SUMMARY: A description is given of techniques used for studying influenza virus infection of the allantoic and chorionic surfaces of the chick chorio-allantoic membrane. It was possible to infect one surface of the membrane at a time and to show that the two layers behaved independently and reacted differently. In contrast to the allantoic layer, the chorionic cells appeared to support only a single cycle of multiplication. This conclusion is based particularly on the small rise of infectivity which occurs following chorionic inoculation of virus, and on the proportion between the amount of virus inoculated and the yield of soluble antigen. Some evidence is presented which suggests that the elementary bodies are less readily released from chorionic cells than from the allantoic cells.

In a preliminary communication (Isaacs & Fulton, 1958), it has been shown that there is an essential difference in response of chorionic and allantoic cells of the chick chorio-allantoic membrane to infection with influenza A virus. The purpose of this paper is to present a detailed analysis of this difference and to describe the special techniques devised to study the problem.

MATERIALS AND METHODS

THE DESIGN OF APPARATUS FOR CULTIVATING INFLUENZA VIRUS IN EXCISED CHORIO-ALLANTOIC MEMBRANE

Preliminary experiments *in ovo* had shown that influenza virus A inoculated on to the chorionic surface of the chorio-allantoic membrane appeared to undergo a development which could best be explained by supposing that virus multiplication was strictly confined to the surface inoculated, and that the virus only went through one cycle of multiplication, after which the process came to a stop, presumably because of the failure of the cells first infected to liberate new virus. This contrasted notably with the sequence of events which followed inoculation of the virus into the allantoic cavity. As is well known, the allantoic cells become infected, and if the inoculum is not sufficient to saturate all the available cells immediately, the first cycle of multiplication in the infected cells is followed by the liberation into the allantoic cavity of new virus, which is then adsorbed on to still undamaged cells to repeat the process. In this way virus multiplication in the allantoic cells proceeds by a number of cycles until all the available cells have been exhausted.

It was immediately obvious that the different behaviour of the chorionic cells could be due simply to the different physiological conditions on their side

of the membrane and need not reflect any fundamental difference in the response of the cells on the two sides to infection. To resolve this problem it was necessary to infect the two sides of the membrane under comparable conditions.

A method of titrating influenza virus in small pieces of excised chorio-allantoic membrane has already been described (Fulton & Armitage, 1951; Fulton, 1952). In this technique the pieces of membrane float in a simple salt solution contained in cavities drilled in a thick block of Perspex; virus is added to the solution and is at liberty to enter cells on either side of the membrane. Two new pieces of apparatus were therefore designed to allow infection to occur on one or other side or on both sides simultaneously under identical physiological conditions.

*Apparatus permitting infection of one side of the membrane
or of both sides simultaneously*

The device which will be referred to as a doublet is constructed of Perspex and is illustrated in Pl. 1. A piece of excised chorio-allantoic membrane is held as a diaphragm between two identical chambers which can be filled with fluid containing virus. The exact dimensions are not of critical importance, but in the model shown the internal diameter of the end chambers is 19 mm. The membrane is held firmly between the two thin Perspex plates which are drilled with four central holes of diameter 6.85 mm. These holes permit fluid to flow freely to the surface of the membrane, but the membrane is supported and prevented from billowing into the adjacent chamber. The central component is a piece of polythene 0.25 mm. thick which prevents the membrane being crushed between the two drilled plates. The inner face of each end chamber is fitted with a circular rubber washer which fits into a circular groove. The washer lies 1 mm. above the surface of the inner face plate and, when assembled, provides an effective fluid seal between the end chambers and the drilled plates.

To assemble the apparatus, the end chamber which holds the four bolts is laid flat on the bench with the inner face plate uppermost. This chamber is engraved with an A and will be on the allantoic side of the membrane. One of the drilled plates is now fitted into position over the bolts, and on it is laid flat a piece of chorio-allantoic membrane with the chorionic side uppermost. It is important to use membranes from 15- to 16-day embryos, as before this time they are too fragile and are liable to be torn and perforated. With a little practice it is easy, by inspection, to distinguish the two sides of the membrane. When the membrane is being removed from the egg a small piece of shell membrane is left adherent to the chorionic side, and as an additional precaution, the allantoic side may be recognized by observing that the large blood vessels are very superficial on this surface.

Before being placed in position on the drilled plate, the excised membrane is freed of shell membrane, washed in glucosol (Parker, 1938) and cut to a suitable size. When in position it is immediately covered with the polythene component, the other drilled plate and finally the second end-chamber which

is engraved with a C. The components are then sealed together by means of nuts which screw down on the four bolts. As with all apparatus made of this plastic the tolerances of the holes which fit over the bolts should be generous, to allow for the high coefficient of thermal expansion. It will be observed from the photograph that each end-chamber is pierced laterally by a small hole; since in assembling the apparatus it is important that the holes in the two end-chambers should lie in the same plane, all the Perspex components are notched along one face so that they may be correctly aligned. After assembly, the apparatus is laid horizontal on the bench with the end-chamber holes uppermost. Fluid with or without virus may now be introduced into the chambers through the small holes by means of a fine Pasteur pipette. The volume normally introduced is 5 ml. and, after filling, the chamber is sealed by closing the small holes with a piece of Sellotape (Adhesive Tapes Ltd., Boreham Wood).

The apparatus is now placed in an incubator at 37° for 24 or 48 hr. and kept in constant motion on a rocking machine of the tilting tray type, the tilt being in the long axis of the apparatus. The rocking machine has already been illustrated in an earlier paper (Fulton & Armitage, 1951); in this application, however, it is geared down to give only six sweeps/min. The fluid used in the end-chambers is the modified glucosol with penicillin, and streptomycin also described in the earlier paper. At the end of the experiment the fluid is removed by pipette and assayed for haemagglutinin.

Before use, the apparatus is sterilized by immersing it for half an hour in 10 % HCl. It is then washed in sterile distilled water and dried in an incubator maintained at 37°. The components lie on sterile blotting-paper, and precautions are taken to avoid dust falling on them while drying. They are stored in a sterile tin.

Apparatus permitting infection of one side of the membrane only

The device which will be referred to as a unit, was also made of Perspex, and is illustrated in Pl. 2.

The base plate containing the four bolts is 78 mm. square. To assemble it, the base plate is covered with a rubber mat 3 mm. thick. Over the central part of the mat is placed a 40 mm. square of filter-paper which has been soaked in glucosol; over it is laid a slightly larger square of sterile cellophan also soaked in glucosol. A piece of chorio-allantoic membrane is now laid out flat on the cellophan. As with the doublet, it is removed from a chick embryo 15–16 days old, washed in glucosol, carefully orientated and then cut to an appropriate size. On it is placed the contact plate 6 mm. thick and drilled centrally with four holes of diameter 14·8 mm., and over this the spacer plate and the central circular cover to prevent dust falling on the membrane. The components are brought into firm apposition by tightening thumb screws on the four corner bolts. In this way four cells are created which have for a floor a piece of chorio-allantoic membrane, arranged with either side uppermost. The use of four small holes in the central area rather than one large hole was the result of bitter experience; for it proved very difficult to avoid, over so large an area,

a small tear which would completely nullify an experiment. With four cells it was usual to obtain at least three in which, at the end of the experiment, the membrane on the floor was seen to be intact.

For certain experiments a nine-hole face plate was used, each hole having a diameter of 8.7 mm. After assembly the cells are filled with an appropriate amount of modified glucosol containing antibiotics and the virus inoculum. In the unit with the four-hole face plate each cell contains 0.25 ml.; in the unit with the nine-hole face plate each cell contains 0.20 ml. The top disk is sealed by running hot wax around its rim, and the unit is rocked for 24 or 48 hr. in an incubator maintained at 37°. The rocking machine is geared to provide forty-five sweeps/min. At the end of the experiment the fluid in each cell is removed and assayed for haemagglutinin; also the intact membranes forming the floors of the cells are cut out with a sharp knife, pooled, ground in a micro Ten Broeck grinder and assayed for complement-fixing antigens.

In preparing the unit for an experiment it is not necessary to sterilize the base-plate, the rubber mat or the filter-paper. The cellophan is sterilized by boiling in distilled water. The other components are soaked for half an hour in 10 % HCl, rinsed in sterile distilled water and then dried in an incubator maintained at 37°.

THE ASSAY OF COMPLEMENT-FIXING ANTIGENS

Suspensions of chorio-allantoic membrane infected with influenza virus contain a mixture of two apparently distinct complement-fixing antigens; the elementary body antigen which is strain specific, and an antigen of much smaller particle size, often referred to as the soluble antigen which is only group specific (Hoyle & Fairbrother, 1937).

In this study it was desired to assay each component of the mixture separately. A common method (Hoyle, 1950) is to use a specific immune ferret serum for the measurement of the elementary body antigen, since such sera often contain only traces of antibody capable of reacting with soluble antigen. For the assay of soluble antigen, human convalescent serum is used which contains large amounts of antibody capable of reacting with the soluble antigen. By choosing sera from patients infected with a strain of influenza virus remote antigenically from the strain in the mixture being assayed, it is possible to employ dilutions of the human serum which react strongly with the soluble antigen and not measurably with the elementary body.

In the preliminary experiments *in ovo* which are reported here, only the complement-fixing soluble antigen was measured and the assay was made with a suitable batch of human convalescent serum using a modification of the technique described by Hoyle (1948).

For later experiments and for all the experiments made *in vitro* a more precise method for the assay of complement-fixing soluble antigen was used and the elementary body antigen was also measured. For the assay of soluble antigen the collection of suitable human sera is clearly a matter of some practical difficulty, and one of us (Fulton, 1949) has suggested an alternative method which employs animal convalescent sera. In that paper ferret sera were used which had been specially selected for high content of antibody

reacting with soluble antigen. In the present work a more convenient method is used; Ananthanaryan (to be published) has demonstrated that guinea-pigs infected intranasally with a large dose of purified influenza virus elementary bodies regularly develop in 14 days a high antibody titre to both elementary body and soluble antigen, though the strains differ in their power to evoke antibodies for the soluble antigen. In our experience the Barratt (BAR) strain of A' virus provokes higher titres of antibody for the soluble antigen than the PR8 strain of A virus given in comparable dose, so that by adjustment of the infecting dose sera possessing the desired characteristics may be prepared. For all the complement-fixation tests with the specific guinea-pig sera the plate technique was used (Fulton & Dumbell, 1949). The apparatus described in that paper has been simplified by using Perspex plates ruled with only 100 squares in 10 rows of 10 columns, and spacing the complement dilutions in the columns at log 0.2 intervals. To facilitate removal of the film of condensation which develops on the plates after removal from the cold and from the hot box, a light breeze from a fan is allowed to play over the plates in the frame before the drop of sensitized blood is added and before the results are read.

In the experiments to be described it was necessary to assay membrane suspensions containing elementary bodies of the PR8 strain of influenza A virus and also the associated soluble antigen. Accordingly, two different lots of guinea-pig serum were prepared.

Guinea-pig serum for assay of PR8 soluble antigen

Fulton & Dumbell (1949) have shown that whereas convalescent mouse antiserum prepared by infection with the PR8 strain contains a large number of antibodies capable of reacting in a complement-fixation test with BAR elementary bodies, the converse is not so. Guinea-pigs behave in the same way, so that if they are infected intranasally with the BAR strain of influenza A' virus they develop high titres of antibody for the BAR elementary bodies, but the sera do not usually fix complement with the PR8 elementary bodies.

Eleven guinea-pigs, 3 months old, were infected intranasally with BAR elementary bodies purified by one cycle of adsorption and elution on chick erythrocytes. Each animal received 768 units of haemagglutinin contained in 0.5 ml. After 14 days they were bled from the heart and separately assayed for complement-fixing antibodies with standardized BAR and PR8 elementary body suspensions. These antigens are standardized to fix 1.2 log units of complement in the presence of excess homologous guinea-pig serum.

It was desirable that the sera should have a high titre when mixed with the BAR elementary body suspension and a relatively low titre with the PR8 elementary body suspension. Of the eleven sera, nine possessed these characteristics, and they were pooled and dried *in vacuo*. A sample of the pooled dried serum was then reconstituted and assayed again for antibodies reacting with the standardized BAR and PR8 elementary body suspensions, and with influenza A soluble antigen. To avoid any danger of cross-reactions with the homologous elementary body antibody, the soluble antigen was prepared from chorio-allantoic membranes infected with the PR8 strain.

Membrane suspensions (20 % v/v) were adsorbed with chick erythrocytes until there was no demonstrable haemagglutinin; they were then centrifuged at 20,000 g for 2 hr.

The results of these titrations are shown in Fig. 1 *a*. At dilutions of 1/10 and greater there was no reaction with the standardized PR8 elementary body

Fig. 1. Influenza virus complement-fixing antibodies in convalescent sera: log units of complement fixed at selected serum dilutions. (*a*) Guinea-pig immune serum (BAR strain). Solid line: BAR elementary body antigen; broken line: soluble antigen. (*b*) Guinea-pig immune serum (PR8 strain). Solid line: PR8 elementary body antigen; dot-dash line: BAR elementary body antigen; broken line: soluble antigen. (*c*) Human serum. Solid line: PR8 elementary body antigen; broken line: soluble antigen.

suspension or with less concentrated suspensions. The points on the curve of fixation with the BAR elementary body suspension represent the log units fixed with the standardized antigen. With the soluble antigen there is a strongly marked zone of inhibition in the region of antigen excess; these zones are illustrated in Fig. 2. Accordingly, a three-dimensional test had to be set up and the points on the curve of fixation with soluble antigen represent the log units of complement fixed maximally at selected serum concentrations.

For the specific assay of PR8 soluble antigen the serum is used at a constant dilution of 1/10, and the antigen titre is defined as the highest initial dilution which will fix 1·7 log units of complement.

Fig. 2. Complement fixation of PR8 soluble antigen with BAR immune guinea-pig serum diluted 1/10. In each row the cross indicates the column in which the 50 % end-point was observed.

Guinea-pig serum for assay of PR8 elementary body antigen

Ten guinea-pigs, 8 months old, were infected intranasally with PR8 elementary bodies purified by one cycle of adsorption and elution on chick erythrocytes. Each animal received 512 units of haemagglutinin contained in 0·5 ml. After 14 days they were bled from the heart and the sera were separately assayed for complement-fixing antibodies with standardized PR8 and BAR elementary body suspensions. It was desirable that the sera should have high titres when mixed with the PR8 suspension and a relatively low titre when mixed with the BAR suspension. Of the ten sera, six possessed these characteristics and were pooled and dried *in vacuo*. A sample of the pooled dried serum was reconstituted and then assayed again with the standardized PR8 and BAR elementary body suspensions, and with a preparation of BAR soluble antigen. The results of these titrations are shown in Fig. 1*b*. Again the points on the curves of fixation with the two elementary body suspensions represent log units fixed with the standardized antigens. The points on the curve of fixation with the heterologous soluble antigen represent the log units fixed maximally at the selected serum concentrations. For specific assay of PR8 elementary body antigen the serum is used at a dilution of 1/60. The antigen titre is defined as the highest dilution which will fix 1·7 log units of complement.

Comparison of the two methods of assaying PR8 soluble antigen

The new method of assaying soluble antigen was compared with the conventional technique. A suitable batch of human serum was obtained, inactivated at 56° for 30 min., and absorbed with 5 % sheep erythrocytes. The serum was then tested using the plate technique with standardized PR8

elementary body antigen, and in a three-dimensional experiment with PR8 soluble antigen. The result is shown in Fig. 1c.

Again, in the case of the curve of fixation with soluble antigen the points represent maximal fixations as there is, in dilutions of serum close to the antibody titre, a well-marked zone of inhibition in the region of antigen excess. With standardized PR8 elementary body antigen the antibody titre is 1/64; with the PR8 soluble antigen the antibody titre is approximately 1/1280. At any fixed serum level from 1/80 to 1/1280 the titre of the PR8 soluble antigen preparation was 1/256. Assayed by the guinea-pig serum pool the same soluble antigen preparation also had a titre of 1/256. Thus the two methods are comparable when used with the plate technique. It is, however, impossible to compare directly the titres of an antigen preparation measured with the plate technique and measured with the conventional tube technique at a single complement level, because the conditions of primary fixation are quite different.

MISCELLANEOUS METHODS AND MATERIAL

Strains. Two virus strains were used. The PR8 strain of influenza A virus, and the Barratt (BAR) strain of influenza A' virus/1947.

Assays of haemagglutinin were made by a pattern test, the unit (A.D.) being the least amount of virus suspension in 0.25 ml. of diluent which, when mixed with an equal volume of 0.5 % chick erythrocytes, caused partial agglutination of the cells.

Membrane suspensions were prepared in one of two ways: (a) by grinding in a mortar or Ten Broeck grinder with glass powder; (b) by freezing in a bath of alcohol and solid carbon dioxide and thawing slowly, and then repeating the process twice.

Incubation with receptor-destroying enzyme. Before assay of haemagglutinin, all the membrane suspensions were incubated with receptor-destroying enzyme for a sufficient time to destroy inhibitor (Burnet & Stone, 1947).

Infectivity titrations for the experiments *in vitro* were carried out by the tissue suspension method (Fulton & Armitage, 1951). For the experiments made *in ovo*, infectivity titrations were made by conventional methods in eggs.

RESULTS

Experiments carried out in vitro

Assays of haemagglutinin

Using a unit with a nine-hole face plate, a piece of chorio-allantoic membrane was arranged with its allantoic side uppermost. The cells were filled with 0.2 ml. of virus dilution in modified glucosol. The inoculum was $10^{4.3}$ I.D.50's of BAR A' virus. At this dilution no haemagglutinin is initially demonstrable. After rocking the unit for 48 hr. the fluid in each of the nine cells was separately assayed for haemagglutinin. In two such experiments haemagglutinin was found in sixteen of the eighteen cells in titres up to 1/100.

When the experiments were repeated with the chorionic surface uppermost, in seventeen of the cells there was no demonstrable haemagglutinin; in one of

the cells haemagglutinin was present with a titre of 1/10, but in this cell the membrane forming the floor was obviously rent, thus exposing the allantoic side to infection.

Using a doublet with 5 ml. of modified glucosol in each of the end-chambers, BAR virus added in a concentration of $10^{4.3}$ I.D.50's to the allantoic side gave readily demonstrable haemagglutinin in that chamber after 24 or 48 hr., but not in the opposite chamber. When virus was added to the fluid in the chamber on the chorionic side, haemagglutinin was not demonstrable in either chamber after 48 hr. If virus was placed in both chambers, haemagglutinin was demonstrable after 24 or 48 hr. on the allantoic side only.

Fig. 3. Complement-fixing antigens in excised chorio-allantoic membrane: log units of complement fixed at selected antigen dilutions. (a) Membrane infected on the allantoic surface. (b) Membrane infected on the chorionic surface. Solid line: PR8 elementary body antigen; broken line: PR8 soluble antigen.

These results were confirmed on many occasions, and were unaffected even if the sensitivity of the virus assay was enhanced by placing a few pieces of chorio-allantoic membrane floating in the uninoculated fluid on the chorionic side. If amounts of virus too small to be detected by haemagglutination had been released by the chorionic cells, multiplication of the virus would have occurred in the pieces of membrane, and the threshold of the assay would have been attained. From these experiments it is concluded that virus elementary bodies are released into the fluid from the allantoic side in large numbers, but that very little, if any, virus is released from the chorionic side. Virus multiplication seems to be confined to the surface inoculated.

Assay of complement-fixing antigens

The experiments were made with a unit having a four-hole face plate, and the time allowed for virus multiplication was 24 hr. With an inoculum of $10^{4.3}$ I.D.50's of BAR A' virus, no virus antigens were demonstrable in the cells whose floor was the chorionic surface of the membrane. When the floor of the cell was the allantoic surface, however, virus antigens in high titre were found. An actual titration is illustrated in Fig. 3a, where the elementary body antigen has a titre of 1/80, and the soluble antigen a titre of 1/40.

With the chorionic surface uppermost it was possible to demonstrate virus antigens in the membrane after 24 hr. if the virus inoculum was increased to $10^{6.8}$ I.D.50's of BAR A' virus. An actual titration is illustrated in Fig. 3*b*. Both types of antigen were found, the elementary body antigen having a titre of 1/48 and the soluble antigen a titre of 1/60. With these large inocula, there was, of course, demonstrable haemagglutinin in the fluid in the cell at the start of the experiment. But the virus antigens in the membrane do not represent merely seed virus adsorbed to the surface of the membrane, for virus was not found in membranes assayed after incubation for 1 hr., and virus antigens were found after 24 hr. if the inoculum was removed from the cells after 1 hr. and replaced by virus-free diluent.

From these experiments it is concluded that both kinds of virus antigen appear in membranes infected on their chorionic surface, but that a large proportion of the cells must be infected initially if the antigens are to be detected by the relatively insensitive method of assay by complement fixation. This confirms the hypothesis that only a single cycle of virus multiplication is occurring.

Experiments carried out in ovo

The results of inoculating PR8 virus on the chick chorion *in ovo* have been previously described (Isaacs & Fulton, 1953). The main findings from this work are:

(i) Following inoculation on the chorion, PR8 virus did not normally penetrate the chorio-allantoic membrane, but was confined in its growth to the chorionic layer.

(ii) When a small dose of PR8 virus (100 I.D.50's) was inoculated on the chorion a limited increase in infectivity occurred, so that the amount of infective virus increased 10- to 100-fold. No soluble antigen or haemagglutinin was detected in these eggs.

(iii) Following inoculation of a large dose of virus on the chorion there was a rapid production of soluble antigen and, in addition, small amounts of haemagglutinin were detected. The amount of soluble antigen produced was directly related to the amount of virus inoculated, as is shown in Fig. 4. It will also be seen from Fig. 4 that within the limits observed a tenfold increase in the amount of inoculated virus led to a twofold increase in the titre of soluble antigen.

(iv) When a large dose of virus was inoculated on the chorion, the titre of soluble antigen was first detectable after 3 hr. and was high at 24 hr. By 48-72 hr. a considerable decline in titre had occurred. However, this decline in titre could be prevented by giving repeated (twice daily) chorionic inoculations of virus. The results of an experiment which illustrate this are shown in Table 1.

In these experiments only the soluble antigen in the membranes was assayed by complement fixation; virus elementary bodies were measured by assay of haemagglutinin after treatment of the membrane suspensions with receptor-destroying enzyme. The facts that only traces of haemagglutinin were detected in membranes following chorionic inoculation of virus *in ovo*,

whereas elementary bodies were readily detected in similar experiments *in vitro* were in apparent contradiction. A number of investigations were carried out on the relative sensitivities of the different techniques used in the

Fig. 4. The relation between the virus inoculum and the titre of complement-fixing soluble antigen which develops when eggs are inoculated on the chorion with dilutions of a PR 8 virus suspension containing $10^{7.8}$ I.D. 50's and 2.5×10^8 units of haemagglutinin.

Table 1. *Titres of soluble antigen in membranes from thirty eggs at intervals after chorionic inoculation*

Soluble antigen titre at time (hr.) shown after a single injection				Titre after repeated injections	
6	24	48	72		
240	640	40	80	1280	
320	640	320	40	640	
20	1280	640	10	640	
120	40	10	160	640	
160	960	50	140	320	
320	960	640	20	640	
G.M.	145	513	126	50	640

Inoculum: 0.5 ml. of PR 8 virus containing 1280 units of haemagglutinin.

Membranes from individual eggs were frozen and thawed and titrated for soluble antigen with human serum.

G.M. = geometric mean.

preparation of membrane suspensions in the experiments; *in vitro* the membrane suspensions had been made by grinding them in a Ten Broeck grinder, whereas for the experiments *in ovo* the membrane suspensions had been prepared by freezing and thawing. In eggs injected intra-allantoically, similar yields of elementary body and soluble antigen were obtained from membranes

treated either by grinding or by freezing and thawing. In eggs infected on the chorion, both techniques released the same amount of soluble antigen, but more elementary bodies were released by grinding. An experiment illustrating this is shown in Table 2.

Table 2. *Titres of specific antigens in membranes 24 hr. after chorionic infection*

Egg no.	Area	Treatment	Haemag- glutinin units O = < 2	Complement-fixing antigen titre O = < 6	
				EB*	S.A.†
1	Dropped	Ground	32	7	28
		Frozen	3	0	34
	Residue	Ground	0	0	0
2	Dropped	Ground	24	7	28
		Frozen	0	0	17
	Residue	Ground	4	0	0
3	Dropped	Ground	48	10	28
		Frozen	0	0	17
	Residue	Ground	0	0	0
4	Dropped	Ground	16	0	28
		Frozen	0	0	17
	Residue	Ground	2	0	0

Inoculum; 0.5 ml. of PR8 virus containing 1280 units of haemagglutinin.

Comparison of 'dropped' and residue areas, and of freezing or grinding of 'dropped' area. The complement-fixing antigens were assayed with guinea-pig sera.

* Elementary body antigen.

† Soluble antigen.

In this experiment the two techniques were applied to halves of the 'dropped' portion of the same membranes. It was essential to use the 'dropped' portion only for this comparison since multiplication of virus was largely confined to this part. Provided that the membranes are ground and treated with receptor-destroying enzyme, the assay of elementary bodies by the measurement of haemagglutinin is about as sensitive a method of detecting virus multiplication in the chorionic cells as assay of complement-fixing soluble antigen. The assay of elementary bodies by complement fixation was slightly less sensitive.

DISCUSSION

There are a number of ways in which multiplication of influenza virus expresses itself differently in the chorionic and the allantoic cells. Most strikingly, the chorionic cells seem to support a single cycle of multiplication only. The evidence for this is as follows. First, only a small rise in infectivity occurs after chorionic inoculation. Secondly, after a large inoculum the titre of soluble antigen rises rapidly and may be nearly maximal after 6 hr. After 48 hr. the titre has declined but this fall in titre can be prevented by re-inoculating with more virus. Thirdly, multiplication of virus is largely restricted to the area which is inoculated, that is, to the 'dropped' area. And finally, there is a

F. FULTON & A. ISAACS—VIRUS MULTIPLICATION IN THE CHICK CHORION. PLATE 1

F. FULTON & A. ISAACS—VIRUS MULTIPLICATION IN THE CHICK CHORION. PLATE 2

proportion between the amount of virus inoculated and the yield of soluble antigen. In all these respects the chorionic cells behave differently from the allantoic but show a suggestive parallel to the mouse brain in its reaction to unadapted virus (Schlesinger, 1950; Cairns, 1951). The difference between the yield of elementary bodies from chorionic cells, depending on whether freezing and thawing or grinding is used, suggests that the elementary bodies are not readily released from the chorionic cells. As a working hypothesis, we visualize the elementary bodies in nests within the chorionic cells and surrounded by soluble antigen which is much more readily released by freezing and thawing.

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EXPLANATION OF PLATES

PLATE 1

A doublet: apparatus permitting infection of one side of the chorio-allantoic membrane or of both sides simultaneously.

PLATE 2

A unit with four-hole face plate: apparatus permitting infection of only one side of the chorio-allantoic membrane.

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