INFLUENZA IN SURVIVING CELLS

- A Study in Host-Virus Interaction -

by

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STATEMENT

The experiments described in Chapter I were carried out in close collaboration with Dr. S. Fazekas de St.Groth. Those of Chapters II and III were conducted by myself along general lines suggested by Dr. Fazekas. Chapter IV was predominantly mine and Chapters V and VI entirely mine.

The work reported in Chapter I will appear shortly in Volume 56 of the Journal of Hygiene.

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TABLE OF CONTENTS

	page
INTRODUCTION	1.
CHAPTER I. An improved assay for the infectivity of influenza viruses	3.
CHAPTER II. The dose-response relationship between influenza viruses and the surviving allantois	37.
CHAPTER III. Comparison of the infectivity of influenza viruses in two host systems	68.
CHAPTER IV. Variation of host resistance to influenza viruses	99.
CHAPTER V. The mechanism of natural resistance to influenza infection	128.
CHAPTER VI. The parameters of multiplication in the surviving allantois	149.
GENERAL SUMMARY	174.
APPENDIX: MATERIALS AND METHODS	178.
REFERENCES	184.

I N T R O D U C T I O N

By 1955, when this work was begun, our knowledge of influenza had increased considerably since the virus had first been grown successfully in a laboratory host twenty-two years earlier (Smith, Andrewes and Laidlaw, 1933). Physically and chemically viruses were well characterized, and antigenic studies had been taken some distance. With the discovery of haemagglutination (Hirst, 1941; McClelland and Hare, 1941) and, in consequence, of a manageable technique for the titration of infectivity, quantitative studies had been made possible. As a result, influenza had come to be the most studied of all animal viruses, and the influenza virus-allantoic cell system to serve as a model for investigations of the mechanism of viral reproduction. Some of the stages of the multiplication cycle, notably those of adsorption and release, were quite well understood. But the all important "dark phase" of the intracellular cycle was still a complete mystery, and a number of the basic problems connected with multiplication, among them incomplete virus formation, interference, and a variety of genetic interactions, remained unsolved, in spite of having been recognised and studied in great detail.

Not the least of the difficulties to be overcome was the absence of a technique for the assay of

infectivity in which the measurement of this property was not confounded with an unknown factor of host resistance. A titration technique that solved this problem would, by its very nature, open up avenues for the study of the mechanism of susceptibility differences between hosts.

It was with this in mind that the present study was undertaken.

CHAPTER I

AN IMPROVED ASSAY FOR THE INFECTIVITY OF INFLUENZA VIRUSES

INTRODUCTION

The standard tests for infectivity of influenza viruses in eggs or mice have two major shortcomings. First, the host systems are known to be inhomogeneous, i.e., the response does not depend solely on the dose; and second, this variation from host to host cannot be assessed independently, since a single test only can be made on any one egg or mouse. Thus the two probabilities - the presence of an infective unit in the inoculum and the success of a particular virus-host interaction - are confounded, so that we estimate, not the number of infective units, but an unknown function of this number. Valid comparisons of infectivity can still be made as long as one is satisfied with a relative answer, and does not wish to enquire into the nature of host resistance.

In principle, the method could be improved in either of two ways: if a new, uniformly susceptible host be found, the problem of variability would vanish; or, if means were devised to allow replicate tests on the same host, the variability could be estimated independently. The first of these is the high ideal of virology. Even if it were realized, the problem of inter-host variation would be eliminated rather than solved. The second approach appears more practical since there are some promising precedents.

There are two encouraging indications that influenza is not impossibly exacting in its growth requirements. Lahelle and Horsfall (1949) demonstrated that influenza multiplies satisfactorily in the allantoic cavity of eggs previously chilled to 4°C, and Bernkopf (1949) developed the technique of growing the virus in de-embryonated eggs. A year previously Weller and Enders (1948) had opened up new avenues in demonstrating production of haemagglutinin by influenza in Maitland-type tissue culture. There followed in rapid succession quite a number of techniques for the assay of influenza in suspensions of allantoic membrane. The methods varied in such details as medium, container and agitation. In some instances the allantois was minced (Gajdusek, 1953; Horváth, 1954; Burr, Campbell, Morgan and Nagler, 1954); in others the membrane was divided into larger fragments (Fulton and Armitage, 1951; Ackermann, 1951; Tamm, Folkers and Horsfall, 1953; Wunder, Brandon and Brinton, 1954). But, though each technique presented its own special advantage, not one was comparable in sensitivity with the intact allantois.

In attempting to overcome this limitation, we built largely on the pioneering work of Fulton and Armitage (1951). Their experiments showed that fragments of the chorioallantois could be maintained in a simple

medium; that these fragments could be infected by influenza viruses; and that infection could be readily recognized by haemagglutination. However, their test was 20-80 times less sensitive than the standard infectivity test in eggs. The task of our study, then, was to learn the reasons for this lack of susceptibility and, if feasible, to remove its causes.

BASIC EXPERIMENTS

I. Fulton and Armitage (1951), as well as Tamm, Folkers and Horsfall (1953) stripped the chorio-allantois from mid-term eggs, cut it into conveniently sized squares, and exposed these to infective virus. In the meantime it had been observed (Fulton and Isaacs, 1953) that the chorion does not support the continued multiplication of influenza viruses, that is, virus coming into contact with one surface of the fragments will be as good as lost, thus lowering the sensitivity of the test. In the first experiment therefore we compared free fragments of the chorio-allantois with similar fragments which had not been stripped from the shell, so that only their allantoic surface was exposed to the inoculum.

The technical details were the same as in the experiments of Fulton and Armitage: the test was set up in plastic trays and incubated, with continuous shaking, at 35°C for 64 hours. The results are shown in Table 1.

The test gave much higher infectivity titres when done on membranes left attached to the shell. This improvement, however, may not have come wholly from keeping the chorion covered.

The two preparations were found to differ in hydrogen-ion concentration. The pH of the medium, set

TABLE 1

Susceptibility to infection of the surviving chorioallantois

Effect of stripping the membrane from the shell

Experiment*		Membrane		Difference
		stripped	attached	
A	(15)	4.0	7.4	3.4
B	(10)	4.0	7.5	3.5
C	(10)	6.3	7.8	1.5
D	(10)	5.5	8.3	2.8

Mean difference : 2.8 $\log_{10}$ units

* The figure in brackets gives the number of parallel rows of 3.16-fold dilutions. The titres are in $\log_{10}$ units.

initially at 7.20, dropped overnight to about 6.3 in all cups with stripped membranes, and rose to about 7.8 in all cups with membranes-on-shell. At the end of the experiment the respective values were 5.05 and 7.40. Clearly, in the first case the buffer of the medium could not cope with the organic acids produced; in the second the shell contributed to the buffering by its CO_3-HCO_3 system. This point is of particular interest in view of recent observations (Fauconnier, 1953; 1954; 1955) indicating that eggs with an allantoic pH below 6 will not support the multiplication of influenza viruses, although their susceptibility can be restored by adding earth carbonates.

Also, the stripped squares of membrane curled up almost as soon as prepared, usually with their allantoic side in. This would tend to lower the chance of allantoic infection, especially since these small rolls float from one side of the cup to the other when shaken, and there can be but little movement of fluid from inside the roll. Again, the free fragments were in much poorer condition by the end of the experiment than those left on the shell; indeed, in the latter we could see no change.

II. Shifting pH, exposure of the chorion, ineffective rinsing, trauma and limited survival - any

or all of these factors might have made the difference. Their contributions, alone or in concert, were further analysed in the next experiment.

As a control group, pieces of the chorio-allantois were prepared according to Fulton and Armitage, as described above. The second group was made up of similar-sized squares cut from the floor of the air-space. Here the shell membrane was left attached to and covering the outer surface, so that the contribution of the allantoic lining could be assessed separately. The third group consisted of minced chorio-allantois: a square of the size used in the other groups was cut into 20 to 30 small pieces. Thus the area remained the same, but since these smaller pieces did not roll up, their two sides were equally exposed to the inoculum and the rinsing away of metabolites was not impeded by the dead volume of fluid trapped within the scroll of membrane. The fourth, fifth and sixth groups tested the buffering effect of the shell: to pieces of membrane prepared as for groups I, II, III, the corresponding bit of shell was added. The seventh group combined all these functional differences from the control group by leaving the chorio-allantois attached to the shell.

The results in Table 2 show that loss of infective virus on contact with the chorion is not the

TABLE 2

Susceptibility to infection of the surviving chorioallantois.

Contribution of various physical factors.

Experiment*	Stripped I	Air-space II	Minced III	Stripped + shell IV	Air-space + shell V	Minced + shell VI	Attached VII
A (10)	4.0	4.6	5.4	5.4	6.6	6.6	7.4
B (10)	5.2	4.7	5.6	7.0	7.1	6.0	7.7
C (10)	5.5	< 6.0	< 6.0	7.2		7.1	8.3
D (10)	< 4.5	5.0	4.5	7.3	7.7	8.3	8.7
Mean titre	~ 4.7	~ 4.8	~ 5.2	6.7	7.1	7.0	7.9

* The figure in brackets gives the number of parallel rows of 3.16-fold dilutions. The titres are in $\log_{10}$ units.

main contributor to the lower sensitivity of the method of Fulton and Armitage (_cf._ groups I and II). Mincing the membranes somewhat increased the susceptibility (see groups I and III) but not sufficiently to equal the titres seen in group VII. Neither does the mere adding of a separate bit of egg shell (groups IV, V and VI) make all the difference, although it had the expected effect of stabilizing the hydrogen-ion concentration; the pH of the last four groups was about 7.5 after two days' incubation, whereas in groups I, II, and III it dropped uniformly to a value as low as 5.2. Thus, while this more detailed comparison failed to show up any one factor as solely responsible, it is clear that their combined effect raised sensitivity several hundredfold. Even with this gain, however, the technique did not yet equal the sensitivity of _in ovo_ titrations.

III. Another factor that could alter the susceptibility to infection is the type and rate of agitation. Fulton and Armitage showed that shaking of the cultures was essential for viral multiplication, although aeration was not. The effect of agitation may therefore be due to the ready removal of metabolites from the cellular surface. Efficient rinsing can be assured by letting the air-water interface pass over the

membrane, or by increasing the inertia of the tissue fragment. The former is achieved by substituting rotation or vigorous horizontal shaking for the gentle tilting movement of the machine designed by Fulton and Armitage. The latter is automatically fulfilled by leaving the membrane attached to the shell, a firm support practically wedged to the bottom of the culture cups.

In one experiment stripped membranes were agitated either in a tilting machine, as designed by Fulton and Armitage, or in a horizontal shaker working at 120 oscillations per minute and 8 cm thrust (Kantorowicz, 1951). The respective mean titres were 5.83 and 6.45 ID_{50} respectively, proving that more vigorous shaking was definitely superior. It should be remembered in this connection that Horváth (1954), who used rolling instead of tilting, demonstrated a fourfold increase in sensitivity with a technique closely related to that of Fulton and Armitage. Several comparative tests were made of the effects of shaking and rolling, using attached membranes. The mean difference was found to be 0.09 ID_{50}, with an error of 0.11 - clearly insignificant. These two methods of agitation were used as alternatives in all experiments to follow.

DEVELOPMENT OF TECHNIQUE

Improvements of design

The use of membranes-on-shell and optimal shaking of the cultures brought the sensitivity of the method within 0.6 log units of *in ovo* titrations. To narrow this gap further we next looked to the medium. However, since even the best we could hope for was a relatively small change, the accuracy of the infectivity titrations had to be improved first. With this in mind we tried to get rid of uncontrollable variables. Instead of allotting bits of host tissue at random, as had been the practice in earlier experiments, the squares from each egg were cut into separate Petri dishes and then distributed orthogonally over the trays. This arrangement allowed assessment and hence elimination of any variation between eggs, as well as direct measurement of the irreducible error, *viz.*, variation within eggs.

To narrow the fiducial range of the means, dilutions of infective virus were closely spaced (two-fold steps), and more replicates (usually 10 or 20) per dilution were used, that is, altogether 100 to 200 bits of membrane for each of the treatments.

In all but sighting experiments the simple contrasts were replaced by more economical factorial designs; and tests separated in time were made comparable

by using the same seed, a single allantoic fluid culture grown from a small dose of virus, ampouled and stored on solid CO_2. This uniform test inoculum was the BEL strain of influenza A (Burnet and Bull, 1943), a virus particularly suited to quantitative work by virtue of its steep dose-response curve in allantoic infectivity tests (Fazekas de St.Groth, 1955).

<u>The medium</u>

We compared first a number of simple synthetic media such as those of Weller and Enders (1948), Fulton and Armitage (1951), Eaton (1952), and Wunder, Brandon and Brinton (1954), which have been used already for similar purposes, as well as the standard diluent used for infectivity tests in this laboratory, an isotonic saline containing 0.8 p.c. NaCl, 0.06 p.c. $CaCl_2.6H_2O$, 0.017 p.c. $MgCl_2.6H_2O$, 0.5 p.c. gelatine, and buffered at pH 7.2 with M/50 Tris-(hydroxymethyl)-aminomethane - HCl (TRIS - HCl).

<u>Diluent</u>. At one stage of these experiments we tried to make up media in simple distilled water, and this resulted in a twentyfold drop in susceptibility. The cause of this difference remains unknown; the remedy is the use of either glass-distilled water or distilled water passed through a deionizing column (Bio-Deminrolit, a mixed bed resin produced by the Permutit Company Ltd.,

London). The latter served as a source of water for all media used in this study.

Hydrogen ion concentration. The sighting experiments came out, on the whole, in favour of media based on Hanks' (1948) balanced salt solution with the addition of a small quantity of protein. It also appeared that a higher concentration of calcium ions than the physiological increased susceptibility. This point has been made already by Fulton and Armitage, but their use of phosphate buffer precluded the appropriate practical changes owing to the low solubility of calcium phosphates. For this reason we employed TRIS-buffer (M/100, pH 7.2) in the next set of experiments. In the course of these, and some further experiments designed to test the effect of pH during viral multiplication, we found that irrespective of the pH set by the buffer, the medium invariably shifted to pH 7.5 within the hour, due to the great buffering capacity of egg shell. Since any method that leaves the membranes attached to the shell implies a pH of 7.5 for the medium, an attempt to set the hydrogen ion concentration at another value is futile, and the incorporation of a buffer in the medium superfluous. The use of egg shell as buffering agent is not unprecedented, even in virology, as Daniels, Eaton and Perry (1952) have already employed it in

cultures of minced chick embryo tissue.

Ions. After orientation through a number of smaller tests, a single comprehensive factorial experiment (omitting quadruple interactions) was performed on 5400 bits of surviving allantois. The basic medium contained gelatine (0.5 p.c.), glucose (0.1 p.c.), $NaHCO_3$ (0.014 p.c.), phenol red (0.00025 p.c.) and chloramphenicol (0.025 p.c.). The factorial variables were NaCl (0.4, 0.6, 0.9, 1.2 p.c.), KCl (0.005, 0.02, 0.06, 0.12 p.c.), $CaCl_2$(0,0.02, 0.08, 0.20, 0.50, 0.80 p.c.), $MgCl_2$ (0,0.0005, 0.005, 0.02, 0.05, 0.15 p.c.). Where the combination of ions was insufficient to bring the osmotic pressure into the physiological range, the appropriate amount of glucose was added to ensure isotonicity.

All combinations were tested over ten doubling dilutions of BEL virus, with eight replicates per step (i.e. on 80 bits of membrane), and the results read after two days' incubation.

Significantly lower titres were obtained only with the lowest sodium (0.4 p.c. NaCl) and the highest magnesium (0.15 p.c. $MgCl_2$) concentrations. The optimal combination of these cations lay around the region of 0.8 p.c. NaCl, 0.06 p.c. KCl, 0.08 p.c. $CaCl_2$, 0.005 $MgCl_2$. The medium used in subsequent experiments when

testing other factors contained the inorganic salts either in the above quantities, or in slightly different ones, but always within the optimum range defined in this test.

Contrary to the experience of other workers with surviving tissues, we found that the ionic constitution of the medium is of secondary importance when the tissues are left attached to the shell. Similarly, provision of anions, apart from chloride, is unnecessary as the requirements of the tissue are covered, presumably, by the amount released from the shell.

Glucose. The basic medium here contained the salts in concentrations given above, together with 0.5 p.c. gelatine and 0.025 p.c. chloramphenicol. Glucose was tested at the levels of 0, 0.01, 0.05, 0.10, 0.20 and 0.50 p.c. The media were then sterilized by autoclaving.

Highest infectivity titres were obtained in the range 0.01 to 0.05 p.c. Consequently 0.03 p.c. was chosen as the standard concentration of glucose for all subsequently prepared media.

Protein. When testing for protective colloids, we restricted our interest to such as will stand autoclaving and are readily available. Of these gelatine was found satisfactory, and when incorporated into the

basic medium gave maximum titres in the range 0.05 to 1.0 p.c. In the absence of gelatine the titres dropped by half, and were rather more variable. Casein, peptone or tryptose, in comparable concentrations, were found unsatisfactory. Hence the standard medium contained 0.2 p.c. of gelatine.

It is worth mentioning that most commercial brands of gelatine contain considerable quantities of sulphite and sulphate. Such were found to be unsuited for our particular purposes. The preparation "Fine Leaf Gelatine E" marketed by Soc. des Produits Chimiques Coignet, Belgium, proved entirely satisfactory.

Indicator. The incorporation of an indicator in the medium is useful for judging its pH both initially and at the end of the test. We used phenol red, at first in the concentration recommended for tissue culture work, *i.e.*, 0.002 p.c. (5.0 ml/litre of a 0.4 p.c. solution). This amount does not affect the susceptibility of the system; however, if the tissues are kept in it for 24 hours prior to infection, the titres then obtained are consistently lower.

This loss of susceptibility can be overcome by reducing the concentration to one-eighth, 0.00025 (*i.e.*, 25.0 ml/litre of a 0.01 p.c. stock solution of phenol red). At this level the change of colour is still

striking.

Antibiotics. The orthodox combination of antibiotics (penicillin 100 U/ml and streptomycin 100 ug/ml) fell short of the ideal in two ways. First, the shells of our eggs often carried bacteria resistant to both; and second, neither antibiotic withstands autoclaving, and thus they must be added to the medium separately just before use. Tests on these two antibiotics, as well as on aureomycin, terramycin and chloramphenicol, showed that while none of them influenced the infectivity titre up to 400 ug/ml, chloramphenicol answered all our demands by coping with the usual contaminants, and retaining its antibiotic power after autoclaving. Thus it could be added to the medium before sterilization, in quantities of 100 ug/ml, a concentration antibiotically effective.

Comparison of media. Finally, the medium emerging as optimal from the above tests (to be referred to henceforth as Standard Medium) was compared with the starting material, the Modified Glucosol of Fulton and Armitage, as well as with two natural media, normal allantoic and amniotic fluid (Table 3).

Clearly, the Standard Medium is the equal of normal amniotic fluid, commonly regarded as the perfect medium for tissue cultures, and significantly superior to

TABLE 3

Susceptibility to infection of the surviving chorioallantois

Comparison of media

Medium	Mean infectivity titre* ± standard error
Modified Glucosol	7.53 ± 0.10
Normal allantoic fluid	7.31 ± 0.03
Normal amniotic fluid	7.85 ± 0.10
"Standard Medium"	7.91 ± 0.07

* Titres in $\log_{10}$ units.

normal allantoic fluid, the natural ambient of these cells, and to the medium of Fulton and Armitage.

<u>Trays</u>

<u>Size</u>. The earliest tests were performed in the 25 x 25 x 2.5 cm perspex trays described by Fulton and Armitage. Although these are wholly adequate for tests on a smaller scale, their bulk and the need for sealing them individually makes their routine use cumbersome. We have therefore preferred the handy smaller plastic trays[1], now widely used for haemagglutinin titrations.

<u>Spacing</u>. One cannot abstract from the literature firm directives on the need of aeration, as Fulton and Armitage deem it unnecessary, while Horváth (1954) finds it essential. Our experience agrees with the latter since, when the trays were placed directly on top of each other, the pH dropped below 5.5 and the virus did not grow to haemagglutinating level. This is actually to be expected from the figures of Daniels, Eaton and Perry (1952), as they found that one gramme of chorioallantoic tissue consumes 250 µl of O_2 per hour, and the supply we have allowed would run out within two days. By separating the trays with the aid of spacing frames the volume

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[1] Obtained from Prestware Ltd., Lombard St., London.

of air available to each bit of membrane can be increased over sixfold, and this is more than sufficient for the period of incubation. The spacing frames are made by bending a strip of 66.5 x 2.5 x 0.3 cm perspex round one of the trays (or a suitable mould) and sticking two 14 x 1.5 x 0.3 cm pieces inside the frame (see Plate 3).

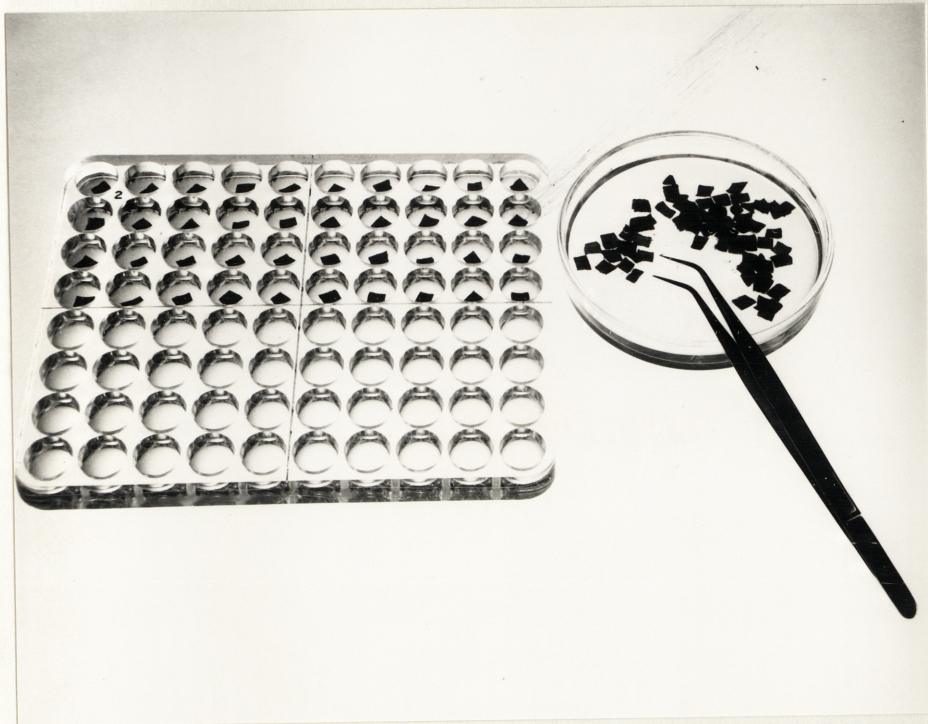
Mounting. Batteries of plastic trays separated by spacing frames can be built up and placed on the shaking machine as a unit.

To prevent evaporation of the medium, the trays have to be enclosed hermetically. After experimenting, unsuccessfully, with different types of boxes, we finally found the most efficient and convenient way of sealing the trays by wrapping them in thin polythene sheets, the ends of which were fastened with cellotape. A battery of four trays needs a sheet 44 x 92 cm in size.

Since some of the fluid inside the envelope evaporates to saturate the enclosed air, and this evaporation occurs mainly from the marginal rows of cups, the batteries were first surrounded by a strip (15 x 80 cm) of lint moistened in lukewarm water (see Plate 3).

Aluminium supports to take two of these batteries (*i.e.*, 8 trays) were specially made, and fitted on a horizontal shaker (Kantorowicz, 1951) working in a constant temperature room, set at 36°C (Plate 4).

Plate 1. Preparation of host tissue. (Step 3 of standard technique.)



<u>Plate 2</u>. Delivery of host tissue into plastic trays. (Step 4 of standard technique.)

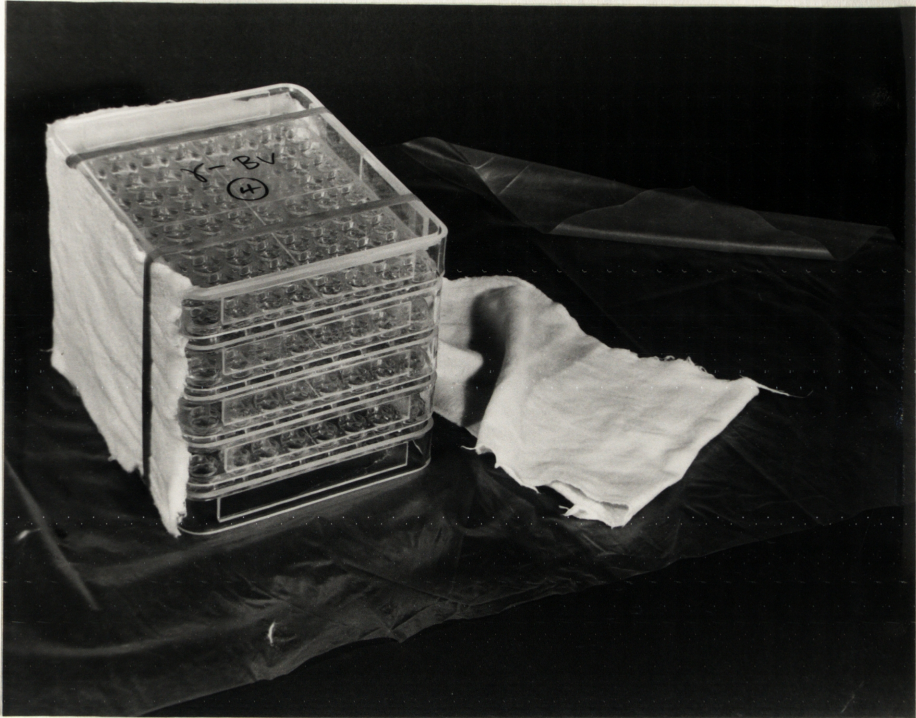


Plate 3. Assembly of infected trays into a battery of four. The trays are separated by spacing frames, and half wrapped in a strip of moist lint. The outer wrapping (polythene sheet) is also visible. (Step 6 of standard technique.)

Plate 4. Batteries of trays mounted on shaking machine. (Step 7 of standard technique.) The large trays on the lower rung are of the type designed by Fulton and Armitage (1951).

<u>Cleaning</u>. The usual method of cleaning perspex trays by immersion into 1 to 10 p.c. HCl was found unsatisfactory for two reasons. First, the residues of protein left on the trays were coagulated into a film lining the cups; this can be removed only by applying abrasive to each cup separately. Second, some mold spores resistant to acid survived the procedure and contaminated the medium in subsequent tests. The alternative method of sterilizing by ultraviolet radiation is efficient as far as decontamination goes, but acrylic plastics tend to polymerize under this treatment, develop fine cracks and liberate toxic substances (which are removable by rinsing in ethanol).

The method finally adopted makes use of the proteolytic action of NaOH. The trays are immersed in a bath of 4 p.c. NaOH for an hour or two, then rinsed thoroughly under the tap in running water. Finally they are mounted in the spacing frames and dried, face down, in an incubator at 36°C, where they can be stored ready for use. The antibiotic in the Standard Medium could easily cope with the small number of bacteria which might be present in the tap water. (At first a rinse with sterile distilled water was included after washing of the trays, but this was found superfluous.)

Setting up of test

Temperature. In the early tests no effort was spared to keep all reagents at 35°C and perform all operations at the same temperature. This of course is inconvenient, and experiments were conducted in parallel to see what difference it makes if the test is set up at room temperature and neither equipment nor medium are pre-warmed. Altogether five experiments were done, using 2880 bits of tissue. To our surprise, the test at room temperature proved superior by a small though consistent margin (difference between means 0.09 $\log_{10}$ units, error ± 0.037). The likely explanation for this finding is the lower rate of heat inactivation of the inoculum at room temperature during the period beginning with inoculation and ending with the infection of the host tissue. Whatever the true reason, the test can be set up wholly at room temperature without any loss of sensitivity.

Delivery of medium. We used a pipetting machine which was first rinsed by circulating through it boiling tap water for 15 minutes.

With the type of tray used, the volume of medium can be varied between 0.2 and 0.5 ml. When comparing infectivity titres obtained in various volumes no significant difference was found. As a rule 0.3 ml of Standard Medium was delivered into each cup, a volume

which covers the membrane and does not spill over even when vigorously shaken.

Host tissue. Embryonated eggs were opened with scissors at the albumen end, below the reflection of the chorio-allantois. The embryo was tipped out, and the membrane adhering to the shell rinsed twice with Standard Medium. In those few cases where the membrane lifted off, the egg was discarded.

Parallel strips (about 0.6 cm wide) running from the albumen end to the air-space were cut first, and these were then cut crosswise into squares and dropped into a Petri dish half filled with Standard Medium. (Hesitant, mincing use of the scissors will crack the shell; however, this is not detrimental to the outcome of the test.) One of these small squares of membrane-on-shell was then transferred with a pair of forceps into each cup of the plastic trays (Plates 1 and 2).

We have varied the size of host tissue experimentally, and found that, whereas the final yield of virus varied in direct proportion to the surface area, the susceptibility to infection was the same for all sizes.

When testing whether orientation of the bits of tissue had any effect on the final infectivity titres, we found a trivial difference in favour of membranes facing upwards. This, fortunately, is the position they naturally

tend to take up in the cups.

All the experiments in this study were made on eggs incubated for 11 days at 38.4°C. There is an important correlation between age and susceptibility. The detailed findings relating to this phenomenon are postponed till Chapter IV.

Inoculation. Dilutions of the material to be tested were made up in tubes of Standard Medium kept in an ice-bath, usually while an assistant prepared and distributed the host tissue in the trays. Calibrated sterile Pasteur pipettes were used to deliver the inoculum as a drop of 0.025 ml. If the inoculum was dropped into the cups an hour before the tissue, the mean titres were about twofold lower than when the inoculum was added last, presumably due to heat inactivation. Also, placing bits of tissue into cups already containing virus requires great care if splashing and carry-over on the forceps is to be avoided. For these reasons inoculation always followed distribution of tissue.

If for any reason the prepared trays cannot be inoculated immediately, the sensitivity of the method does not necessarily suffer. After cutting up, the bits of tissue may be left in either the Petri dishes or the trays for at least two hours, either on the bench or in an incubator. If the trays are agitated at 35°C, the

sensitivity to infection does not drop more than 0.1 $\log_{10}$ unit in the first 24 hours. Such stability of the host tissue allows interruption of a set of titrations whenever convenient. Indeed, samples from a time series, such as growth experiments, can be titrated on tissue derived from the same host.

Incubation. After inoculation the trays are mounted, as described above, and placed on the shaking machine. The temperature of incubation makes no difference to the titres obtained as long as it lies within the range of 34 to 37°C.

The degree of agitation was tested on the reciprocating shaker run at 120 oscillations per minute, with the thrust varied between 2 and 10 cm. This was not reflected in any change of susceptibility to infection. Since other combinations of frequency and amplitude can give the same degree of agitation, these results can be extrapolated to other rates of shaking. The finding of Fulton and Armitage, that shaking was essential only during the first day of incubation, was confirmed.

When testing whether three days' incubation gives higher titres than two, the average rise was found to be only 0.04 log units, not significant. This behaviour characterizes BEL and other fast growing influenza strains; some, as will be shown later, multiply more

slowly and hence have to be incubated for three days.

<u>Reading</u>. When the trays are taken down, the medium is clear and uniformly pink, due to the pH change; turbidity caused by bacterial and fungal contamination is obvious when present. Since using chloramphenicol as sole antibiotic, we have had no bacterial contamination in several thousand tests. On odd occasions, and then always only on the third day of incubation, some of the cups showed fungal growth. These cups were marked and ignored, on principle, when computing infectivity titres, although their reactions did not differ from the rest of the cups receiving that particular dilution of virus.

The bits of tissue were picked out with a pair of fine forceps, moving from higher dilution to lower. At first the forceps were sterilized by dipping into boiling water between each cup. Later, after extensive control tests had demonstrated that the volume of fluid carried on the forceps is never sufficient to cause false positive reaction in an otherwise negative cup, this practice was abandoned. (The maximal yield of a small square of tissue is about 25 haemagglutinating units against a standard drop of 10 p.c. red cells; and the volume of fluid adhering to the forceps is about a hundredth of the medium in one of the cups.)

When the host tissue has been removed, a

standard drop (0.025 ml) of a 10 p.c. fowl red cell suspension is added to each cup, the trays well shaken and left standing for half an hour at room temperature. The pattern of settled cells is read as positive (complete agglutination) or negative (no agglutination). Intermediate patterns rarely occur, and then only with slowly growing strains; they are read as positive.

CONCLUSIONS: THE ASSAY OF INFECTIVITY

1. Prepare "Standard Medium" (SM):

NaCl	8.0 g
KCl	0.6 g
$CaCl_2$	0.8 g
$MgCl_2$	0.05 g
Glucose	0.3 g
Gelatine (acid free)	2.0 g
Chloramphenicol	0.1 g
Phenol Red (25 ml of 0.01 p.c. stock solution)	0.0025 g
H_2O (glass distilled, or deionized)	to 1,000 ml

Adjust pH with normal NaOH to about 7 (yellowish orange colour of indicator). Sterilize by autoclaving at 115°C for 30 minutes.

2. Deliver 0.3 ml of SM into each cup of clean plastic tray.

3. De-embryonate 11-day eggs at the sharp end, rinse membrane twice with SM, cut shell into 6 x 6 mm squares into Petri dish half-filled with SM.

4. Transfer one square of tissue-on-shell into each cup of prepared trays.

5. Inoculate cups by adding an 0.025 ml drop of dilutions prepared from test material.

6. Build battery of 4 trays separated by spacers and covered by blank on top, surround it with strip of moistened lint, wrap in polythene sheet.

7. Mount pairs of batteries in metal supports, place on

horizontal shaker working in warm room, incubate for 48 hours at 35°C.

8. Remove trays, pick out bits of tissue with fine forceps, add one drop of 10 p.c. fowl red cells to each cup, shake.
9. Read test after 30 minutes: positive haemmagglutination = infection.
10. Clean trays and spacers by immersing in 4 p.c. NaOH for an hour, thoroughly rinse in running tap water, fit trays in spacers, dry in incubator at 35°C.

SUMMARY

A new method has been developed for assaying the infectivity of influenza viruses.

Surviving allantoic epithelium serves as host-tissue, in the form of small squares of membrane left attached to the egg-shell.

The medium needed to maintain this tissue is simple, can be sterilized by autoclaving, and keeps indefinitely.

The test is set up in transparent plastic trays, and the results can be read with the naked eye after two days.

One 11-day egg yields up to a hundred bits of tissue, each the equal in sensitivity of a whole egg.

CHAPTER II

THE DOSE-RESPONSE RELATIONSHIP BETWEEN INFLUENZA VIRUSES AND THE SURVIVING ALLANTOIS

INTRODUCTION

While a number of tissues will serve as host for the multiplication of influenza viruses, none yet known is uniformly susceptible to infection. The variation from host to host of needs lowers the accuracy of assays, but this is of no great moment as the loss can be made good in practice by doing a larger number of tests. The challenging aspect is the existence, side by side, in a set of seemingly identical host organisms of some highly susceptible to infection and others naturally resistant.

It was with an investigation of this phenomenon in mind that we set out to improve the method of Fulton and Armitage (1951), so that it might afford experimental means for the study of the nature and distribution of host-resistance to infection. Once the allantoic lining of the developing chick, the most commonly used host system for influenza viruses, can be divided into small fragments and maintained *in vitro* without loss of susceptibility, the way is open to alter environmental conditions at will and observe their effect on the response of the tissue. The first stage of this work was to define the basic dose-response relationship against which natural and experimental variation may then be evaluated. This chapter therefore deals with the infective behaviour of ten influenza strains maintained under

optimal conditions in surviving bits of the allantois.

EXPERIMENTS

Variation within eggs

Susceptibility to infection. The first set of experiments served to determine whether or not different regions of the allantois were equally infectible. Since systematic variation is most likely to occur along the axial gradients of development, squares of membrane-on-shell were collected from five zones of each egg: the albumen end, the albumen third, the centre, the airspace third and the airspace end. Bits from 24 eggs were distributed in separate plastic trays containing 0.30 ml of Standard Medium (SM) per cup. A series of twofold dilutions of the BEL (A) strain was used as infective inoculum, and the test was read after 48 hours' incubation at 36°C. The titres were worked out by the simplified computation (Fazekas de St. Groth, 1955) based on the method of Reed and Muench (1938). Table 4 gives the results.

Clearly, the mean infectivity titres of the five groups are statistically indistinguishable. The same conclusion was reached by an analysis of variance where, after elimination of any effect the difference between eggs might have contributed, we obtained the variance ratio of 1.40 with 4 and 92 degrees of freedom. Such a value would occur purely by chance once in every

TABLE 4

Susceptibility to infection of different regions of the allantois

Region	Infectivity titre	±	standard error
Albumen end	7.78	±	0.14
Albumen third	7.92	±	0.18
Centre	7.92	±	0.16
Airspace third	7.89	±	0.20
Airspace end	7.85	±	0.20
Mean	7.87	±	0.077

Each of the titres, given in log_{10} units/0.025 ml, is based on replicates from twenty-four eggs.

four trials.

Whereas these results suggest that the <u>average</u> susceptibility of bits does not vary from one region to another, they do not answer the more stringent question whether the probability of infection is the same for every single piece. Since any deviation from uniformity will flatten the dose-response curve (Moran, 1954a), variations of this kind may be detected and evaluated by appropriate statistical tests. The elegant method of Moran (1954a, 1954b) was designed for this particular purpose, and found to be more powerful than other parametric methods (Armitage and Spicer, 1956). We applied Moran's test to infectivity titrations on the BEL strain, done during the development of the technique (Fazekas de St. Groth and White, 1958), as well as to further tests performed on nine other strains of influenza virus in a comparative study of the intact and surviving allantois (Chapter III).

The results of Table 5, based on a large volume of experimental material, suggest no significant differences in susceptibility between bits of allantois-on-shell derived from any one egg. This holds for all ten strains of influenza virus tested.

<u>The yield of haemagglutinating virus</u>. It does not follow from their uniform response to infective virus

TABLE 5

Distribution of susceptibility to infection within 11-day eggs

Strain	M-values	$\bar{M}$
WSE (A)	-1.4,-1.4,-0.7,-0.4,0.0,0.0, +0.4,+0.4,+0.4,+0.7,+0.7,+1.0	+0.03
PR8 (A)	-2.1,-1.4,-1.0,-1.0,-0.7,-0.7, -0.4,0.0,+0.4,+0.7,+0.7,+1.4	-0.38
MEL (A)	-2.4,-1.8,-1.8,-0.4,0.0,0.0, +0.4,+0.4,+0.7,+0.7,+1.4,+1.8	-0.09
BEL (A)	-1.4,-1.0,-0.7,-0.7,-0.4,-0.4, 0.0,0.0,0.0,+0.7,+0.7,+1.0	-0.18
CAM (A')	-2.8,-2.4,-2.1,-1.8,-0.7,-0.4, 0.0,+0.4,+0.7,+0.7,+1.0,+1.8	-0.38
FM1 (A')	-1.0,-1.0,-0.7,-0.4,-0.4,-0.4, 0.0,+0.4,+0.4,+0.7,+0.7,+1.0	-0.06
LEE (B)	-2.1,-0.7,-0.7,-0.4,-0.4,-0.4, -0.2,+0.1,+0.4,+0.7,+0.9,+1.0	-0.10
BON (B)	-1.8,-1.4,-1.0,-0.4,-0.4,0.0, +0.7,+0.7,+1.0,+1.4,+1.4,+1.4	+0.15
HUT (B)	-1.0,-0.4,-0.4,-0.4,0.0,0.0, +0.4,+0.4,+0.4,+0.4,+0.7,+1.0	+0.09
SW (swine)	-1.4,-1.0,-0.7,-0.7,-0.4,-0.4, -0.4,0.0,+0.4,+0.7,+0.7,+0.7	-0.20

Each of the M-values (Moran, 1954a; 1954b) is based on infectivity tests performed on bits of 11-day eggs with twofold dilutions of the test viruses and five replicates per dilution.

that bits derived from the same egg will behave uniformly when tested by other criteria of virus action. Indeed, equal susceptibility to infection, as demonstrated in the previous section, shows only the absence of gross qualitative differences within eggs. In the next set of experiments therefore we looked for quantitative variation, and chose to base our comparisons on the final stage of the intracellular cycle, namely the number of virus particles produced by a standard area of the allantois, and the rate at which these are released from cells. Both can be tested in the same experiment if a nearly saturating dose of virus is used as infective inoculum, and the medium is sampled near the peak of the first cycle of multiplication, and then again when the titres have reached their maximum.

The comparison of regions was made on the same material as used above for infectivity tests. The medium was sampled 7 and 24 hours after infection with about 10^6 ID_{50} of BEL virus (Table 6).

As in the case of susceptibility to infection, the regional differences within any one egg were insignificant, as regards both the rate of production and final yields. Similar experiments were done on strains WSE, PR8, CAM and SW. The findings were consistently negative, that is, no variation in yields beyond that due

TABLE 6

Yield of haemagglutinating virus from different regions of the allantois

Region	Yield at 7 hours	Yield at 24 hours
Albumen end	1.23 ± 0.06	2.30 ± 0.06
Albumen third	1.11 ± 0.08	2.21 ± 0.10
Centre	1.19 ± 0.06	2.21 ± 0.09
Airspace third	1.18 ± 0.05	2.18 ± 0.06
Airspace end	1.08 ± 0.05	2.17 ± 0.08
Mean	1.160 ± 0.028	2.211 ± 0.037

The yields ± standard errors are given in log_{10} units; each is based on 20 replicates.

to chance could be detected when bits from the same egg were compared. Since these particular experiments also tested inter-egg variation, they will be dealt with in detail under that heading.

Hereafter, bits from all regions of the allantois of a single egg were assumed to behave in the same way, and were used indiscriminately.

Variation between eggs.

The early hope that bits coming from one egg would behave uniformly was fulfilled by the findings of the previous sections. The magnitude and nature of variation between eggs remained to be determined. Once again, susceptibility to infection and final yield of virus particles were treated separately, but the tests were done on material from the same eggs, so that, should significant variation be found, the proper analysis of correlation between the two aspects of virus-host interaction could be performed.

Susceptibility to infection. Experiments under this heading were usually done on groups of ten to twenty eggs per strain of virus, i.e., on about 400 - 800 units of host tissue. The bits coming from each egg were distributed orthogonally over the trays, giving up to twenty independent inter-egg comparisons with five replicate rows per egg. Due to the systematic arrangement of host

tissue, the variation between eggs could be estimated separately and compared with the appropriate experimental error term (<u>viz</u>., variation within eggs) and also with the theoretically defined variance of quantal infectivity tests. The infective inoculum came from ampouled seed virus which was stored at -70°C. Although this should ensure the uniformity of the challenging inoculum, and thus allow pooling of results obtained on different days, the Tables of this section are based on tests done on the same day, using a single set of dilutions made up from one ampoule of seed virus. This precaution was taken to avoid the confounding of inter-egg variation, which we set out to determine, with possible variation due to other causes such as time, reagents or operators.

Since viruses belonging to type B are characterized by a slower rate of multiplication than A-strains, tests of this set were incubated for 72 hours at 36°C after infection. A summary of the analysis of variance computed from the results is given in Table 7.

Considering the experimental variances first, the results prove that susceptibility to infection varies from egg to egg. In absolute terms this variation is small. If, say, only a hundred bits of host tissue had been used to test each strain of virus, the variance ratios would not have reached even the lowest level of

TABLE 7

Variation of susceptibility to infection between bits of allantois-on-shell

Strain	Source of variation and (degrees of freedom)	Mean square	Variance ratio	Significance	
				experimental	theoretical*
WSE (A)	Between (18)	0.3774	3.57	$P < 0.001$	$P < 0.001$
	Within (76)	0.1058			
PR8 (A)	Between (19)	0.5064	5.07	$P < 0.001$	$P < 0.001$
	Within (80)	0.0999			
MEL (A)	Between (19)	0.1785	1.43	$P \sim 0.15$	$P \sim 0.008$
	Within (80)	0.1249			
BEL (A)	Between (15)	0.2200	1.74	$P \sim 0.08$	$P \sim 0.003$
	Within (64)	0.1266			
CAM (A')	Between (12)	0.5678	6.22	$P < 0.001$	$P < 0.001$
	Within (52)	0.0912			

Strain	Source of variation and (degrees of freedom)	Mean square	Variance ratio	Significance	
				experimental	theoretical*
FM1 (A')	Between (19)	0.2299	2.28	P~0.01	P<0.001
	Within (80)	0.1010			
LEE (B)	Between (8)	0.3275	3.35	P~0.007	P<0.001
	Within (36)	0.0979			
BON (B)	Between (17)	0.7589	4.40	P<0.001	—
	Within (72)	0.1723			
HUT (B)	Between (13)	0.2616	2.81	P~0.007	P<0.001
	Within (56)	0.0932			
SW (S)	Between (19)	0.1339	1.34	P~0.20	P~0.07
	Within (80)	0.0997			

significance ($P = 0.05$) with any of the strains. With the number of test subjects used in our experiment, seven strains are seen to vary significantly; two, MEL and BEL, are placed in the 10%-region; and only for one, SW, could the eggs be said to behave homogeneously.

If the theoretical variance[1], 0.09, is to be

---

[1] The variance of quantal dilution assays is

$$V = d^2 \sum \frac{p_i \cdot q_i}{n_i} , \tag{1}$$

where d is the log dilution step, n_i the number of subjects tested at level i, and p_i, q_i the expected proportion responding or failing to respond at that level. An unbiassed estimator of V is

$$S^2 = d^2 \sum \left(\frac{r_i}{n_i}\right)\left(\frac{n_i - r_i}{n_i}\right)\left(\frac{1}{n_i - 1}\right), \tag{2}$$

where r_i is the observed number of responses out of n_i. (This formula ignores the contribution of the periodic component (Irwin, 1937), which is for all practical purposes negligible at closely spaced doses and small n.) If the number of subjects is the same at each level, Eq.2 may be written

$$S^2 = \frac{d^2}{n} \cdot \frac{\sum[r_i(n - r_i)]}{n(n-1)} . \tag{3}$$

Now, for tests where the proportion of non-responders is given by the zero term of the Poisson distribution, the second part of the numerator in Eq. 3 equals Moran's statistic T.

In the case of twofold dilutions the expectation of T is $n(n - 1)$, and hence for this particular case the variance formula reduces to d^2/n. Thus, in $\log_{10}$ units, the theoretical variance of a test in a single row of twofold dilutions is 0.09. This value has been used to calculate the last column of Table 7.

used to evaluate inter-egg variation, the first question that must be answered is whether the experimental error (i.e. variation within eggs) is significantly higher than this minimum. The appropriate tests show that in the case of nine strains the observed difference would occur by chance in one quarter or more of all trials ($P \gg 0.25$); with the tenth strain, BON, the variation within bits of the same egg is significantly higher than the theoretical ($P = 0.02$).

The overall experimental variance within eggs is 0.1125, based on 676 degrees of freedom; its ratio to the theoretical minimum would be obtained only in 3% of the cases by chance, and is therefore just significant. Again, it is the BON strain which contributes overwhelmingly to this result. If only the other nine strains are considered, the pooled variance becomes 0.1054, with 604 degrees of freedom; the variance ratio so obtained is not significant ($P \sim 0.16$).

Thus, at least for the nine strains whose response curve within any one egg does not differ from the theoretical, the distribution of susceptibility between eggs may be assessed against the theoretical variance. Since the latter has an infinite number of degrees of freedom, the probabilities listed in the last column of Table 7 were derived from χ^2-tests rather than the less

extensively tabulated variance ratios. MEL and BEL now become significant, and SW so close to the conventional level of significance that the whole set of strains may be taken to show some variation between eggs.

With the finding of these small but consistent differences in the susceptibility of eggs, it was of interest to determine whether an egg which showed a certain degree of resistance to infection by a particular virus would react in the same way to other strains. The answer to this question was sought in the following experiment. A number of eggs was cut into 80 bits each, and 40 of these were used in an infectivity test with one strain of virus, 40 with another. The pairs CAM-LEE and BON-HUT were compared in such tests. The obtained correlation coefficients, 0.717 and 0.685, are based on 24 and 20 degrees of freedom respectively. Both are highly significant ($P < 0.001$), and show that the distribution of susceptibility does not depend on the virus used in the test, but is a characteristic of the eggs alone.

This demonstration, taken together with the fact that - excepting BON - the variation about the mean level of infectivity is the same for all strains, allows the pooling of observations and the plotting of a common distribution of host resistance between eggs. Indeed, in absolute value, the variance associated with the BON

strain hardly differs from the others, and therefore the histogram of Fig. 1 shows the distribution of susceptibility for the homoscedastic group in black, with the frequencies found for BON superimposed in white.

The normal curve has been fitted to the observed frequencies at 0.10 log (i.e. 0.44σ) intervals, as shown in Fig. 1. The small frequencies at the tail ends were pooled to give minimum totals of 5. According to the results the distribution of susceptibility between eggs does not differ from the normal ($\chi^2(7) = 8.17$; $P \sim 0.35$), and the apparent negative skewness is not significant. If the BON strain is included in the distribution, the fit becomes rather worse.

So far the experiments were designed to give the most informative comparisons of variation within and between eggs. And the span of challenging doses was chosen with an eye on the M-test, that is, to cover the whole range of responses from all positive to all negative. With the finding of small differences between eggs we wished to assess the significance of these independently and, if possible, more accurately. The appropriate design here ignores the tails of the dose-response curve and allocates correspondingly larger numbers of test subjects to groups which give the most information. In response curves based on the Poisson distribution this

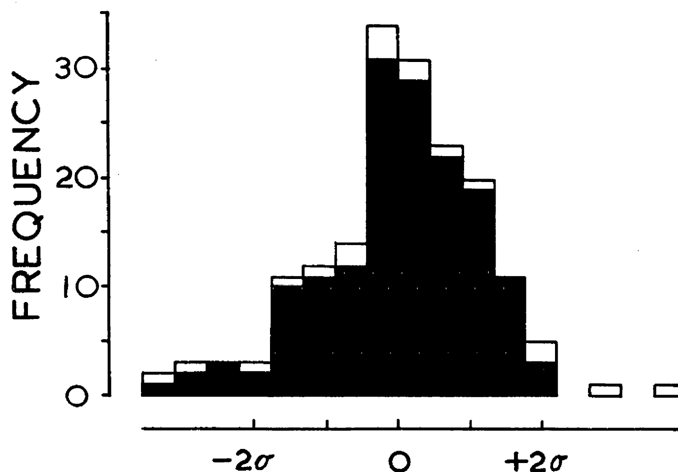


Figure 1. Variation of susceptibility between eggs.

(The histogram shows the distribution of log infectivity titres about their common mean. The tests were done on bits of allantois-on-shell from 169 eggs. The black columns represent strains WSE, PR8, MEL, BEL, CAM, FM1, LEE, HUT and SW; the white columns show the behaviour of the slightly aberrant BON strain. Each column contains infectivity titres covering a range of 0.10 $\log_{10}$ units.)

region lies in the range of doses giving between 60 and 90% "takes". In the next experiment therefore only three doses of virus were used, in steps twofold apart. Each of these was inoculated into 180 cups containing bits from 12 eggs. The results were analysed by the maximum likelihood fitting of the negative exponential.

The data of Table 8 were obtained with the BEL strain, one of the three which failed to show inter-egg variation on the internal evidence of the smaller experiments described above. Here, by increasing the number of replicates per level to 15 and by restricting the range of doses to the region of maximum information, the evidence for variation between eggs becomes unequivocal.

The same conclusion is reached when the slopes of the loglog curves (Mather, 1949) are examined. The estimated slopes for each of the twelve eggs were compatible with the slope defined by the Poisson distribution, that is, there was no sign of inhomogeneity among tissue derived from any one egg. The overall slope, on the other hand, was found to be significantly flatter.

The yield of haemagglutinating virus. These tests were done on the same eggs which have already served for the comparison of host-resistance to infection (see Table 7). Five carefully marked squares of allantois-on-shell were cut from each, and infected with about

TABLE 8

Variation of susceptibility between eggs

The figures show the number of infected bits of membrane-on-shell out of a total of 15.

Dilution ($\log_{10}$)	Egg 1	2	3	4	5	6	7	8	9	10	11	12	Total
7.42	15	12	13	15	11	15	15	15	14	12	15	15	167
7.72	8	10	14	15	5	13	11	15	13	11	11	14	140
8.02	6	9	10	12	3	6	7	10	10	10	10	6	99

Analysis of variance

Source of variation	Degrees of freedom	Mean square	Variance ratio	Significance
Between eggs	11	0.4926	10.72	$P < 0.001$
Within eggs	12	0.0459		

10^4 ID_{50} of one of the viruses. After 24 hours' incubation the medium was removed and tested for haemagglutinin against fowl red cells. Although in the actual titrations twofold dilutions were used, and hence the results read in $\log_2$ units, the entries of both Table 9 and Figure 2 have been transformed to $\log_{10}$ units. Thus the variances of the two kinds of test (infectivity and haemagglutinin) are directly comparable, and the distributions are plotted on the same scale.

Unlike in the assay of infectivity, where the inherent limitations of the method set an upper bound to the accuracy, the error of haemagglutinin titrations is relatively small and affords a more powerful criterion for the evaluation of egg-to-egg variation. For this reason the answer is clear-cut: the eggs are seen to vary significantly, all variance ratios being well beyond the 0.1% level of probability (Table 9). Just as an egg's resistance to infection does not depend primarily on the strain of virus used, so it was found that the yield of haemagglutinin was equally independent.

When the variances of all strains were compared, BON once again occupied a position apart from the other nine. In this instance however the discrepance was so marked (χ^2 in Barlett's test including BON 59.29, $P < 0.001$; without BON 6.92, $P \sim 0.5$) that we had to omit

TABLE 9

Variation of virus yield between bits of allantois-on-shell

Strain	Source of variation and (degrees of freedom)	Mean square	Variance ratio	Significance
WSE (A)	Between (18)	0.3315	8.85	$P < 0.001$
	Within (76)	0.0375		
PR8 (A)	Between (19)	0.1448	19.32	$P < 0.001$
	Within (80)	0.0075		
MEL (A)	Between (19)	0.1292	3.73	$P < 0.001$
	Within (80)	0.0346		
BEL (A)	Between (11)	0.1418	21.42	$P < 0.001$
	Within (48)	0.0066		
CAM (A')	Between (12)	0.2562	8.55	$P < 0.001$
	Within (52)	0.0300		

Strain	Source of variation and (degrees of freedom)	Mean square	Variance ratio	Significance
LEE (B)	Between (8)	0.2183	7.81	$P < 0.001$
	Within (36)	0.0279		
BON (B)	Between (17)	1.6003	35.42	$P < 0.001$
	Within (72)	0.0452		
HUT (B)	Between (13)	0.1417	5.22	$P < 0.001$
	Within (56)	0.0271		
SW (S)	Between (19)	0.1834	13.54	$P < 0.001$
	Within (80)	0.0135		

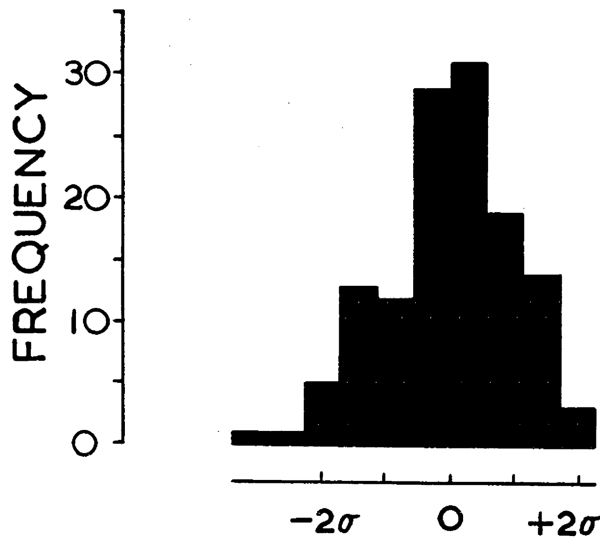


Figure 2. Variation of yield between eggs.

(The histogram shows the distribution of log haemagglutinin titres about their common mean. The tests were done on bits of allantois-on-shell from 145 eggs. The strains tested were WSE, PR8, MEL, BEL, CAM, FM1, LEE, HUT and SW. Each column contains haemagglutinin yields covering a range of 0.10 $\log_{10}$ units.)

this strain when the distribution of haemagglutinin yields was plotted in Figure 2.

It can be seen that the scatter is smaller than in Figure 1, that is, haemagglutinin yields vary less from egg to egg than does susceptibility to infection. The data are well fitted by the normal curve ($\chi^2_{(5)}$=4.48).

<u>Correlation between susceptibility and yield of virus</u>. Since both infectibility and the number of virus particles produced per unit area were found to vary from egg to egg, it became mandatory to find out whether these two manifestations of virus-cell interaction were linked or not. To this end the relevant data, examined separately above, were combined in an analysis of co-variance. The results, best summarized by the correlation coefficients (Table 10), show that variation in susceptibility is independent of variation in virus yield. This is evident both at the level of individual strains and in the pooled estimate of correlation, neither of which even approaches significance.

<u>The accuracy of infectivity tests</u>

The mere fact that such extensive tests as the above were needed to demonstrate significant variation in the behaviour of the host system would indicate that the accuracy of the tests is not much below the theoretical maximum attainable in quantal assays of infectivity. If

TABLE 10

Correlation between susceptibility and yield of virus

Strain	Number of tests	Correlation coefficient
WSE	19	+0.05
PR8	20	+0.20
MEL	20	0.00
BEL	12	+0.33
CAM	13	-0.15
LEE	9	+0.22
BON	18	+0.33
HUT	14	+0.28
SW	16	-0.50
	Mean	+0.078

the overall variance for a single row of twofold dilutions is calculated from Table 7 it comes to 0.158 in $\log_{10}$ units. Exclusion of the aberrant BON strain would lower this value by only about 10% (to 0.143); we have adopted the higher figure as an estimate of experimental variation. The standard error of a test with _n_ replicate bits per dilution is given by the familiar formula $\sqrt{0.158/n}$. If _d_-fold instead of twofold dilutions are used, the error will be approximately $\sqrt{d/2}$ times the above.

This basic error is increased if separate sets of dilutions are made up and tested in parallel, or if tests are performed by different operators, or at different times. Several experiments were run to check the contribution of these factors, and it was found that in the hands of reasonably trained workers the additional variation did not amount to much. Numerically, the increase of the variance due to different reagents, operators or times of performance came to 0.019, and 0.000 and 0.017 respectively. Thus while the basic error in a test with, say, eight replicates per twofold dilution would be ±0.140, it would rise to ±0.149 if different reagents were used, to ±0.150 if also done by different operators, and to ±0.156 if done on different days as well. Under the latter, the least favourable, conditions a difference

of 0.43 $\log_{10}$ units between endpoints would be significant at the 95% level of confidence. Such tests would use 80 bits of tissue derived from a single egg. Since 118 eggs would be needed to achieve the same accuracy in allantoic infectivity tests, or about 60 mice in intranasal tests - provided the virus is mouse-adapted - the economy of the technique needs little emphasis.

DISCUSSION

The experimental definition of the dose-response relationship between influenza viruses and the surviving allantois raises three points for discussion. First, the nature of interaction between host and parasite which would lead to an empirical curve like the one observed. Second, ways in which the component mechanisms could be separated and studied independently. And, third, the practical aspects, that is the value and limitations of the technique as a tool in virus research.

The theory of dilution assays, developed largely in response to the demands of bacterial tests, was based on two fundamental assumptions: (1) that the sample is taken at random from a homogeneous population, and (2) that each sample which contains at least one infective unit will register as positive at the time of scoring. If these postulates are fulfilled, the proportion of negative responses is an estimator of the zero term of a Poisson distribution whose mean is the number of infective units per inoculum. If only a fraction of organisms is infective, or if their probability of infection is distributed, the theory still holds. In the latter cases it will estimate the mean infectivity per sample, but the dose-response curve remains Poissonian. Nothing in the experience of bacterial population

sampling contradicted this theory - as long as tests were done *in vitro*. However, in similar tests in animals or, especially, when the theory was applied to viruses, striking discrepancies were observed. The reason is a third postulate implicit in the theory, namely that units of the assay system be of constant susceptibility. This postulate is automatically fulfilled in the case of synthetic media incubated under controlled conditions; it will hold rarely in living systems of assay, and only under exceptional circumstances in the assay of viruses, where multiplication depends on a more intimate biological interaction of host and parasite.

For an intelligent evaluation of viral infectivity the distribution of host resistance must be known, as it is an integral part of host-virus interaction. Methods have been proposed for the estimation of infectivity even in the absence of such knowledge (Moran, 1955; Armitage and Spicer, 1956), but they are admittedly no more than statistical makeshifts. The data presented above do not solve the problem, although they may be useful in limiting the area of admissible guesses. Armitage and Spicer have pointed out that Moran's test will detect variation in host resistance only if the average susceptibility is 0.25 or lower, or if its distribution shows extreme positive skewness. Since in practice the M-test

demonstrates significant deviations from the Poissonian model (Fazekas de St. Groth, 1955), and since the observed distribution (cf. Fig. 1) of susceptibility is, if at all, negatively skew, it follows that the average susceptibility must be low. On the other hand, since the ratio of infective to electron microscopically visible virus would fall within the range of 1/6 to 1/12 for the strains we were using (Donald and Isaacs, 1954), the average susceptibility cannot be lower than 0.08 to 0.15. This almost amounts to saying that under optimal conditions each visible virus particle is potentially infective, and that the outcome of a virus-cell encounter depends only on the behaviour of the cell. We may go even further and, as there was no detectable variation among bits derived from any one egg, suggest that of the eighteen hypothetical cell-virus interactions considered by Fazekas de St. Groth and Moran (1955) only Hypothesis XII fits the experimental facts. This - Eq.15 of the quoted paper - is a comparatively simple relationship, and should serve as first approximation in the study of natural resistance to virus infection.

The variation from egg to egg is much smaller when the effect is tested on surviving bits. This observation tends to incriminate the medium, since the difference between allantoic fluid and SM may be regarded

as the major difference between tests done _in ovo_ and _in perspice_ (_sit venia verbo!_). It should be mentioned here that on occasion when, due to inadequate rinsing of glassware, traces of detergent found their way into the medium, the infectivity titres dropped by as much as tenfold for some strains and the dose-response curve flattened to a degree found only in tests on whole eggs. There are also other means, some natural and some artificial, of altering host resistance or enhancing its variability. Since all of these treatments are without any direct effect on the virus, methods for the study of host resistance may be based on each of them and will be developed in Chapter IV.

As a method of assay the bits of allantois-on-shell are most naturally compared with the intact allantois of chick embryos. In respect to sensitivity, _i.e._ the smallest number of virus particles detectable, the two are on the whole equal. At the level of individual strains of influenza virus there are characteristic differences, and these will be evaluated and their mechanism studied in the next chapter. The accuracy of the bit-technique is consistently higher than of allantoic titrations, due to the steeper slope of the dose-response curve. This statement holds for the comparison of a single square of allantois-on-shell with a whole

egg. If the amount of information derived by the two techniques from one egg is considered, the difference is of the order of hundredfold in favour of the surviving tissue. Indeed, although the infectivity test is quantal, its power matches that of quantitative pock- or plaque-counting methods, as the 80-100 units of host tissue will provide a more precise estimate of infective units than do countable numbers of pocks on one membrane or plaques on one layer of cells.

To date only strains adapted to allantoic growth have been tested in this system, and it is therefore not known whether viruses obtained directly from cases of human influenza would grow equally well or grow at all. Neither is the system fully susceptible to infection by adapted strains: with the exception of one virus (SW), all other strains show lower infectivity than the maximum that could be expected from the number of characteristic particles visible under the electron microscope. Our method shares this shortcoming with all systems of cells known to support the multiplication of influenza viruses, but is perhaps unique in the sense that the two components of virus-cell interaction, the chance of an infective particle being present and the chance that the cell it meets is susceptible, can be studied separately.

SUMMARY

It is shown that bits of allantois-on-shell cut from any one egg do not differ either in susceptibility to infection by influenza viruses or in yield of haemagglutinin. For this reason the dose-response curve within any egg is Poissonian.

Bits cut from different eggs vary both in susceptibility and in haemagglutinin yield. Unlike in whole eggs however, the scatter is so small that it can be demonstrated only by refined tests done on a large number of subjects. For practical purposes the dose-response curve may still be taken as Poissonian.

Susceptibility and yield vary independently of one another.

The data are used to define the accuracy of infectivity tests in bits of allantois-on-shell; the expected errors are given both for straight replication and for tests done with different reagents, by different operators, or at different times.

C H A P T E R III

COMPARISON OF THE INFECTIVITY OF INFLUENZA VIRUSES IN TWO HOST SYSTEMS

INTRODUCTION

If the infectivity of influenza viruses is tested in the allantois of mid-term chick embryos, the response always shows signs of variation between eggs. There is as yet no way of telling in advance which of the eggs will be more and which less susceptible to infection, and no treatment is known to reduce or eliminate the variation. By using surviving bits of allantois-on-shell instead of whole eggs, it was possible to arrange experimental conditions so that the response of the host system became practically uniform. Since under these conditions the source of additional error vanishes and the residual variation approaches the theoretical minimum set by the nature of quantal infectivity tests, we shall call these conditions optimal. The adjective refers of course only to the <u>precision</u> of the method, not to its <u>sensitivity</u>. These two may vary independently. The first, a statistical property, is a measure of information yielded by an elementary unit of the assay system; the second, a biological feature, defines the smallest functional unit discernible. This, the infective unit, will equal the smallest physical unit, the virus particle, only if the system is of maximal sensitivity. In this chapter the sensitivity of the orthodox test in whole eggs is compared with that of the test in bits of the allantois kept

under optimal conditions.

Strictly speaking, the two methods are incommensurable, as the underlying dose-response curves are not of the same shape. However, if the point of median infectivity (ID_{50}) is adopted as operational criterion, one may make comparisons which will be meaningful in practice.

EXPERIMENTS

The scoring of infectivity for various strains of virus

Before comparing the two methods of assay, we had to determine whether the conditions found optimal for the BEL strain in earlier work would give the best results with other influenza viruses.

Length of incubation. The right time of harvesting can be determined accurately only if the rate of multiplication of each strain in this system is known. Such experiments have been done, but will be postponed till Chapter VI. The practical answer has been obtained more simply, by harvesting replicate infectivity tests at various times after inoculation. The results were on the whole what one would have expected from the behaviour of these viruses in the allantoic cavity. Type A strains all reach peak titres by 48 hours; A-prime and B strains attain their maximum by 72 hours only, the rise over the last day being between 0.2 and 0.4 $\log_{10}$ units. Accordingly, tests with A-strains were taken down either at two or three days, while tests using the other viruses were invariably left for three days.

Detection of the infective unit. The second question to be answered was whether the test for the presence of infection as practised on the BEL strain was sensitive enough for the rest of influenza viruses. The

experiment here imitated the least favourable conditions likely to occur in practice, namely a lag of several hours between cutting up and infecting the bits of tissue, part of which period was spent at room temperature. The haemagglutinin yields obtained under these conditions with the ten test strains are shown in Table 11.

Although some of the yields are fairly low (CAM, FM1, BON, HUT), all of them are more than enough to give complete agglutination with one drop of 5-10% fowl cells. The margin of safety is over twofold for even the lowest-yielding strain, and considerably wider for the rest.

To test this conclusion by the most sensitive method available, we subinoculated samples from each cup of an infectivity assay after three days' incubation, and compared the titres given by the original and secondary test (Table 12).

Whereas all cups positive at three days in the first test yielded virus on subinoculation, only a negligible minority of the negative cups was found to contain any infective virus. From the practical point of view the rises are quite insignificant, and only serve to confirm the phenomenon first described by Liu and Henle (1953) for the behaviour of influenza viruses in the intact allantois.

TABLE 11

Yield of haemagglutinin by surviving bits of the allantois kept under suboptimal conditions

Strain	WSE	PR8	MEL	BEL	CAM	FM1	LEE	BON	HUT	SW
Yield*	15	24	22	63	9	12	18	5	14	83

Each figure gives the mean yield of 50-100 bits of tissue.

* Number of haemagglutinating units present in the growth medium of a $6 \times 6\ mm^2$ bit of tissue. (One haemagglutinating unit is the amount of virus which causes partial agglutination of 10^7 fowl red blood cells.)

TABLE 12

Subinoculation of infectivity tests

Strain	Median infectivity titre*		Difference
	standard test	subinoculation	
PR8	5.88	5.95	0.07
MEL	7.24	7.29	0.05
BEL	7.12	7.14	0.02
CAM	6.26	6.37	0.11
FM1	6.15	6.21	0.06
LEE	6.82	6.98	0.16
BON	5.10	5.19	0.09
		Mean difference	0.08

At the end of a standard infectivity test about 0.1 ml of medium was transferred to the corresponding cups of a fresh tray.

* All titres are in $\log_{10}$ units.

Comparative tests of infectivity in eggs and trays

During development of the technique assays were always done by inoculating eggs and trays from the same set of virus dilutions. The infective seed, grown from small inocula and harvested at the peak of multiplication, was characterized by a high infectivity-to-haemagglutinin ratio (ID/HA ratio). Later, in experimental work unrelated to the theme of this study, several hundred titrations were performed also on influenza viruses of intermediate infectivity, some obtained by the method of von Magnus, others by various other means. The uniting feature of this group was their lowered ID/HA ratio. These latter tests will be treated separately, partly because of the abnormal characteristics of the viruses and partly because many of the comparisons are not based on simultaneous testing. The majority was first titrated in eggs, and a duplicate ampoule of snap-frozen virus tested later in trays, after a variable period of storage at -70°C.

Titration of fully infective virus. Usually ten twofold dilutions were inoculated into groups of 50-60 eggs (5-6 replicates per dilution) and two or three trays of surviving tissue (16-24 replicates per dilution). Thus the accuracy of the egg titrations was never more than half that of the tray tests, leading to a rather

large joint variance. The relative sensitivity of the two host systems was determined by subtracting the log ID_{50} observed in eggs ("egg titre") from the log ID_{50} observed in surviving bits of tissue ("tray titre"). The log differences, "tray/egg ratios", given in Table 13 are weighted means, where each experimental value has been multiplied by the reciprocal of its variance.

The tray/egg ratios spread nearly symmetrically on both sides of unity - there are as many strains to which the surviving allantois is more susceptible as the other way round. The extremes, set a hundredfold apart, are significantly different not only from each other but also from the central group of viruses. More precise separation of the strains is not justified in view of the large errors, although within this looser grouping they have maintained their positions well in tests done over the past two years. The only exception is the BON strain, known to behave atypically in several respects. In Table 13 it is shown to be least infective for bits. In our experience this corresponds to the general behaviour of the strain, although in one series of tests the average difference came to -0.25 only, which would be significantly different from the -1.13 listed above. In that particular series 24 samples of BON virus were titrated, a dozen each in two sessions. Samples of other

TABLE 13

Comparative assay of infectivity in eggs and trays

— Fully infective virus (ID/HA > 10^6) —

Strain	Number of comparisons	Tray/egg ratio*	± st. error
WSE (A)	12	+ 0.29	± 0.09
PR8 (A)	10	+ 0.28	± 0.05
MEL (A)	8	+ 0.54	± 0.10
BEL (A)	16	- 0.22	± 0.03
CAM (A')	8	+ 0.40	± 0.10
FM1 (A')	9	- 0.35	± 0.20
LEE (B)	11	- 0.51	± 0.09
BON (B)	6	- 1.13	± 0.20
HUT (B)	8	- 0.26	± 0.11
SW (S)	9	+ 0.92	± 0.08

* The tray/egg ratios, expressed in $\log_{10}$ units, give the mean (log "tray titre" - log "egg titre").

The assays were done in twofold steps with 5-6 eggs and 16-24 bits of allantois-on-shell at each level; the number of such comparisons is shown in the second column.

strains tested at the same time (24 in each session) gave the expected tray/egg ratios.

Titration of partly infective virus. The infectivity tests in eggs were done in 3.16-fold steps, with 5 replicates per dilution. The test in trays used twofold steps and 8 replicates per dilution. The loss of accuracy due to the wider spacing in whole eggs was made up by titrating a larger number of samples, so that the entries of Table 14 have about the same average error as those of Table 13. The infectivity-to-haemagglutinin ratio of this group was lower by 10 to 10,000-fold than that of fully infective virus.

As may be seen, there is good agreement on the whole between the tray/egg ratios obtained with the two types of seed. The biggest discrepancies, HUT and CAM, are not significant in terms of the error attached to these estimates. The only significant difference would have been between the two forms of BON virus, since it gave here a log ratio of -0.14. However, this difference was found in that particular set of titrations which gave an average difference of -0.25 for fully infective virus, disregarded in the construction of Table 13. For the same reason we have not included the results with the corresponding incomplete forms in Table 14.

As the average tray/egg ratios for infective

TABLE 14

Comparative assay of infectivity in eggs and trays

— Partly infective virus (ID/HA $< 10^5$) —

Strain	Number of comparisons	Tray/egg ratio*	± st. error
WSE (A)	14	+ 0.23	± 0.05
PR8 (A)	17	+ 0.28	± 0.05
MEL (A)	18	+ 0.51	± 0.05
BEL (A)	18	- 0.20	± 0.05
CAM (A')	18	+ 0.19	± 0.11
FM1 (A')	17	- 0.35	± 0.11
LEE (B)	18	- 0.42	± 0.10
HUT (B)	18	+ 0.18	± 0.10
SW (S)	18	+ 0.84	± 0.07

* The tray/egg ratios, expressed in $\log_{10}$ units, give the mean (log "tray titre" - log "egg titre").

The assays were done in 3.16-fold steps with 5 eggs at each level, and in twofold steps with 8 bits of allantois-on-shell at each level; the number of such comparisons is shown in the second column.

and incomplete forms of the same strain are statistically indistinguishable, the precaution of treating them separately is rendered unnecessary, and a pooled estimate will be used to fix the position of influenza strains in this gradient.

Variation of host resistance. The identity of infective behaviour in these two forms of virus can be further tested by comparing their response curves. The material is the same as used for Tables 13 and 14; the method is Moran's test (1954a; 1954b), applied earlier to the same problem in allantoic titrations (Fazekas de St. Groth, 1955) (Table 15).

The M-values obtained from titrations of fully and partly infective virus are closely similar, and there is no hint of a difference between these two forms, except perhaps with the strain HUT. However, as may be seen from the last column of Table 15, the tests on fully infective virus carry rather less weight in this case than with any other strain, and contribute little to the mean. The reason is that many of the original titrations did not extend from all-positive to all-negative and, since Moran's test is valid only if it covers the whole range of non-zero products, these were omitted, with a consequent drop in the accuracy of the mean.

Deviations from the Poissonian dose-response

TABLE 15

The degree of egg-to-egg variation in titrations of fully or partly infective virus

— Moran's test applied to the data of Tables 13 and 14 —

Strain	Average M-value		
	fully infective virus	partly infective virus	weighted mean
WSE (A)	0.82	0.81	0.82
PR8 (A)	0.33	0.77	0.60
MEL (A)	0.03	- 0.25	- 0.16
BEL (A)	0.54	0.68	0.61
CAM (A')	0.34	0.27	0.29
FM1 (A')	0.61	0.85	0.76
LEE (B)	0.57	0.69	0.64
BON (B)	1.01	1.12	1.08
HUT (B)	0.22	1.19	0.89
SW (S)	0.22	0.16	0.18

curve are definitely smaller in surviving bits of tissue than in whole eggs, a conclusion reached already on different materials and by different methods in the previous chapter. The difference in the behaviour of strains is once more evident: SW and MEL, for instance, are strikingly different from BON and HUT. Generally, the A-strains have a steeper dose-response curve than B-strains, but the scatter within each group is too wide and the overlap between groups too extensive to allow the drawing of a line of separation between types. On the other hand, we found significant correlation ($r = +0.73$; $P = 0.01$) between the position of a particular strain in the tray/egg gradient (Tables 13 and 14) and the slope of its response curve. The same trend is evident if the data of the previous chapter are analysed in the same way ($r = +0.58$; $P \sim 0.06$). Of the two sets of tests the former is the more powerful: each mean is based on the response of at least 250 eggs, while in the latter 10-20 eggs only were compared.

The immediate consequences of this result will be discussed at the end of this chapter. An experimental study of whether the connection is based on causal relationship or represents two manifestations of some other mechanism, should be done. An investigation of another suggestive relationship, the correlation between the

tray/egg gradient and the gradient of incomplete virus production (Fazekas de St. Groth and Graham, 1954), which gives r = -0.65 (0.05>P>0.01), might also prove fruitful, but both are beyond the scope of this thesis. The rest of this chapter will deal with factors, mostly mentioned by other workers, which could cause differences like those appearing in Tables 13, 14 and 15. Most of these tests were done on strains SW and LEE, representing the two ends of the tray/egg gradient. Although BON occupies a position even more extreme than LEE, the latter was preferred since BON is known to give aberrant and atypical reactions in almost every test connected with infectivity.

<u>Toxic effect of plastic trays</u>. Horváth (1954) mentions that glass vessels are preferable for the maintenance of surviving tissues, as plastic trays do not behave like inert containers. Similar was the experience of Rightsel, Schultz, Muething and McLean (1956) with polio viruses in tissue culture: vinyl plastics were found harmless but other plastics oozed some virucidal substance. We can confirm the findings of these authors, provided the trays have been exposed to ultraviolet radiation for prolonged periods. This would be the case if UV-light were used for sterilization. Even here however the toxic substances can be removed by a rinse in alcohol.

Methanol is preferable, not only because it is more volatile but also on account of its greater fungicidal power. If normal NaOH is used for cleaning the toxic effect is absent. This has been our experience over several years, and can be demonstrated specifically in the following experiment: dilutions of virus are made up in glass (pyrex) test tubes, and some trays are filled with these immediately, some four hours later. Then bits of tissue are added to each cup, and the infectivity endpoints read three days later. The same readings were obtained whether the inoculum was preincubated in glass or plastic. This behaviour is uniformly characteristic of all strains tested.

Thermal inactivation. Horsfall (1954; 1955) has repeatedly stressed how rapidly the infectivity of influenza viruses drops when exposed to temperatures optimal for their intra-cellular multiplication. A small test was designed on the strains SW, CAM, FM1 and LEE. The two A-prime strains were included because they are very similar in most respects, but widely separated in the tray/egg gradient. The experiment consisted of two parallel tests of infectivity (0.5 log dilutions, 8 replicates per step), one set up as soon as the dilutions were made, the second after 4 hours' preincubation at 36°C. Table 16 gives the results.

TABLE 16

Thermal inactivation in Standard Medium

— 4 hours at 36°C —

Strain	Length of preincubation		Difference
	nil	4 hours	
SW	6.81	6.43	0.38
CAM	5.83	5.25	0.58
FM1	6.59	5.85	0.74
LEE	7.46	6.50	0.96

Both infectivity titres and differences are in $\log_{10}$ units.

Thermal inactivation is evident with each of the strains tested, and this is what one should expect. The degree of inactivation gives the series SW < CAM < FM1 < LEE, which is exactly the same as the gradient of tray/egg ratios (*cf*. Table 13). Although the agreement is close even quantitatively, this aspect should not be stressed, as the endpoints carry an error of about 0.15 $\log_{10}$ units, and are thus insufficient to support more than a qualitative statement. On the face of it, then, thermal inactivation could be the mechanism underlying the observed differences of the tray-egg gradient.

Adsorption and viropexis. If differential inactivation by heat caused the differences between titres obtained in trays or eggs, it should be possible to show that the average virus particle spends a longer time outside the host cell in trays than it does in eggs, and that those particular strains which are at the bottom of the tray/egg gradient undergo adsorption and/or viropexis much more slowly than those at the top. This specific question has been tested on strains SW and LEE.

Various doses of Virus were added to the growth medium of several bits of tissue, and after 135 minutes (*i.e.*, about 0.9 of the 50% viropexis time, VT_{50}, for SW virus in whole eggs, and about 1.6 VT_{50} for LEE) the supernatant was removed from some bits; this sample

gives the amount of virus not adsorbed. To other cups 100 units of RDE was added, and the medium removed after a further 30 minutes; the titre of these fluids shows the amount of virus still outside the cells, that is not viropexed.

The results (Table 17) are the same as would have been found in an experiment on whole eggs: the two strains are adsorbed to about the same extent, but the uptake of LEE is much faster than that of SW. In this respect there is no difference in the behaviour of the whole allantois and of surviving bits, and hence thermal inactivation cannot be invoked to account for the observed differences of the tray/egg gradient.

Since this is a conclusion of consequence, and the objection might be made that the conditions of the test were those of saturation or near saturation, a similar experiment was done in which each cup of an infectivity test was treated in the manner described above. The straight infectivity endpoints were $10^{6.28}$ and $10^{7.20}$ for LEE and SW respectively. If the bit was removed at 135 minutes, and a fresh one added in its stead, $10^{5.33}$ and $10^{6.16}$ ID_{50} respectively were recorded. The differences, of the same order as above, show that the average time the virus particle spends in the medium is the same whether the dose is subsaturating or a single particle

TABLE 17

Adsorption and viropexis in bits of allantois-on-shell

Strain	Titre of medium				
	initially	after 135 min.			
		without RDE		with RDE	
LEE	7.80	7.45	(21%)	7.45	(21%)
	6.80	5.90	(46%)	5.85	(48%)
	5.80	4.00	(71%)	4.55	(58%)
	4.80	2.60	(78%)	3.00	(71%)
SW	7.70	6.85	(44%)	7.00	(38%)
	6.60	5.65	(48%)	5.85	(40%)
	5.40	4.30	(53%)	4.60	(43%)
	4.30	1.80	(82%)	3.50	(43%)

The titres were determined in haemagglutinin tests, and are given in $\log_2$ units. The figures in parentheses show the percentage of virus adsorbed (initial - residue without RDE), or viropexed (initial - residue with RDE).

only.

<u>Effect of allantoic fluid</u>. The major difference between the two assay systems is, at least initially, not the state of the host cells but rather the constitution of the medium. The effect of allantoic fluid should therefore be tested by setting up bits of allantois-on-shell in parallel trays, one containing allantoic fluid, the other Standard Medium. However, as has already been shown (Chapter I), allantoic fluid is the least satisfactory of all media tested, and its effect on the virus would always be confounded with its effect on the host tissue. For this reason we exposed various doses of virus to undiluted allantoic fluid for a limited time, usually two hours, and then transferred the bits of tissue to SM. Even in these tests the infectivity titres were always lower than on direct inoculation in SM, but the gradient observed was in no way related to the tray/egg gradient. Actually SW virus, which gives relatively lowest titres in the whole allantois, was found to be least affected by the presence of allantoic fluid, whereas other strains widely separated in the gradient gave approximately the same response. The only conclusion that may be drawn from these tests is a negative one, namely that the mere presence of normal allantoic fluid cannot account for the differences between egg and tray

titres. That allantoic fluid has no differential effect on the two strains farthest apart in the tray/egg gradient can also be shown by incubating large doses of SW and LEE with allantoic fluid *in vitro*, and then testing for infectivity. Here the allantoic fluid is diluted at least 1 in 1000 by the time it comes into contact with the host tissue. In such tests the two viruses gave much the same titres whether preincubated in allantoic fluid or SM.

The intracellular stage of multiplication. All findings so far would suggest that the difference in susceptibility between the intact allantois and surviving bits of it arises at a point in time after the virus has entered its host cell. Although this is the least known phase of the multiplication cycle, it is quite feasible to test at what particular stage the cells stop behaving as in whole eggs and start giving the response of surviving bits. For purposes of the present study a single experiment of this kind will do: it tested what the situation was at the time when 50-70% of the inoculated particles have been bound irreversibly to cells.

Sets of whole eggs were inoculated allantoically with several known doses of virus, and after two hours' incubation opened, rinsed and cut into bits of allantois-on-shell. Control eggs were cut up in the same

way, and then infected with the corresponding dose of virus. The proportion of infected bits was determined after three days' further incubation under standard conditions. One of the two strains of virus used, SW, was known to give titres eight times lower in whole eggs than in trays, the other strain, LEE, three times higher. These differences are of an order that should allow one to tell whether the bits which received virus while still in the whole egg behaved like the native or the surviving allantois (Table 18).

The answer is clear-cut: the membranes infected _in ovo_ and then cut up responded as if they had been infected in trays. In the case of SW virus the rise, 1.14 $\log_{10}$ units, is incompatible with the hypothesis of whole egg-type behaviour (_i.e._ zero rise). With LEE virus the results are equally definite at the higher level, the drop of 0.42 being significantly different from zero. The lower dose here gave an answer which is compatible with both hypotheses, and thus not decisive. This is partly due to the small difference between the alternatives, and partly to the weakness of binomial estimators in the region of low probabilities. However, when this half of the experiment was repeated with as closely similar reagents as we could manage, the findings supported only the hypothesis that eggs cut up at two

TABLE 18

The effect of subdividing the allantois after infection

Strain	Inoculum ($\log_{10}$ ID_{50}) tray units	egg units	Number of bits infected: observed	mean	expected* for bits	for eggs
LEE	1.76	2.23	12, 18, 5, 12, 17, 6	11.7	10.4	30.7
	†(1.16	1.63	6, 8, 10, 1, 9, 9	7.2	2.9	8.6
	†(1.12	1.59	4, 3, 3, 1, 0, 4	2.5	2.7	8.2
SW	1.74	0.86	16, 5, 13, 27, 23, 19	17.2	10.0	1.3
	1.14	0.26	9, 6, 5, 4, 4, 7	5.8	2.8	0.4

* The expected values were calculated on the assumption that 1 ID_{50} = 0.693 infective units, and that the inoculum is randomly distributed over the whole of the allantoic lining.

† Because of the equivocal answer obtained in the first experiment, the test was repeated with similar reagents.

hours after infection behaved as if they were infected as bits. When the results of the replicate tests are pooled, their mean has an abnormally large variance, but is compatible only with one of the hypotheses, like the rest of the results.

The slightly higher than theoretical values obtained with SW virus, if not regarded as random errors, could give rise to the objection that the inoculum was unevenly distributed, most of it attaching to the parietal allantois. Although this in itself could not account for the titres rising in the case of SW and dropping for LEE, taken together with the differential heat inactivation demonstrated earlier, it might lead to results like those of Table 18. To settle this question experimentally, we inoculated a known dose of virus into the allantoic cavity, and cut up the eggs 120 minutes later. As its place of origin was noted for each bit, we could test not only whether the whole of the parietal allantois got more than its share of the inoculum, but also whether the distribution over different regions was homogeneous or not (Table 19).

If the inoculated infective particles were distributed at random, 15.85 out of 300 squares should have been infected on the average. The observed number, 15, is well within the limits of chance variation.

TABLE 19

The distribution of infective particles on allantoic inoculation

Zone	Strip 1	2	3	4	5	6	7	8	9	10	Total
Albumen end	1	1					1	1			4
Albumen third		1	1			1					3
Centre	1			1							2
Air space third	X	1				1		1			3
Air space end	1									2	3
Total	3	3	1	1	0	2	1	2	0	2	15

Six eggs were each inoculated allantoically with 0.05 ml of saline containing 10 ID_{50} of LEE virus. Two hours later the shell with the chorioallantois attached was cut into 10 meridional lunes (strips) and each of these into 5 squares, according to zones. The table shows how many of the bits in each region produced haemagglutinin by 72 hours. Since 37.5% of the total allantois was represented by these bits, the expected total of takes over the six eggs was 15.85.

The point of inoculation is marked by an X.

Similarly, there is no sign of systematic deviation along the meridional lines centering on the point of allantoic inoculation ($X^2_{(1)} = 0.67$; $P = 0.43$). These results justify the conclusion that virus introduced into the allantoic fluid reaches the cellular wall in a random manner, and statistical models based on this assumption are valid.

DISCUSSION

By and large the test in surviving bits of allantois-on-shell is about as sensitive as the orthodox test of infectivity in the whole allantois. This average behaviour is made up of a characteristic and well reproducible pattern at the level of strains. There are some viruses to which the surviving tissue is more sensitive; a few run to the same titre in both host systems; others give higher endpoints in whole eggs. If the ten representative strains of influenza virus are arranged in the order of their relative titres in bits and eggs, the gradient SW (+0.88), MEL (+0.51), CAM (+0.31), PR8 (+0.28), WSE (+0.25), HUT (-0.02), BEL (-0.22), FM1 (-0.35), LEE (-0.47), BON (-1.02) is obtained. The figures in parentheses give the difference, in $\log_{10}$ units, of the estimated ID_{50} for the two kinds of test. Since these factors were found to be constant for each strain, and the same whether preparations of high or low ID/HA ratio were tested, they allow the transformation of the median infective dose for bits into the median infective dose for whole eggs. In this sense the two methods of assay may be regarded as equivalent. It must not be forgotten however that the dose-response relationship is not the same for the two, and therefore these transforming factors are valid only for the ID_{50}. For the same

reason, although the average sensitivity of the two tests is the same, endpoints obtained in surviving bits are always of greater precision.

With two of the strains, SW and MEL, the slope of the dose-response curves is what should be expected if the outcome of the test depended only on the presence or absence of an infective unit in the inoculum. At the same time the sensitivity of our technique for these two strains rises 0.88 and 0.51 log units respectively above that of allantoic inoculations, and certainly to within twofold of the maximum attained only if each elementary particle visible under the electron microscope is also an infective unit. Probably more important is the fact that those strains which do not come near maximal infectivity are also marked by a flatter than Poissonian response curve or, in other words, fall short of the ideal only by their failure to overcome host resistance. In this respect the surviving allantois differs from other tissues capable of supporting the growth of influenza viruses. It seems to be the least complicated system in which the basic problem of susceptibility to infection can be studied, and the only one where this property of the same host is naturally graded over a hundredfold range.

Under the uniform conditions maintained

throughout the present experiments the variation occurs between different strains of influenza virus: it will be shown in the next chapter that within any of the strains it can be produced at will by choosing or creating appropriate environmental conditions.

SUMMARY

It is shown that the assay for infectivity in bits of allantois-on-shell, which has been standardized on the BEL strain, is optimal also for nine representative strains of influenza virus tested.

On the average, the sensitivity of the technique is the same as of orthodox allantoic infectivity tests; its precision is always higher. The relative sensitivity of the two tests varies from strain to strain, the log tray-egg differences being SW (+0.88), MEL (+0.51), CAM (+0.31), PR8 (+0.28), WSE (+0.25), HUT (-0.02), BEL (-0.22), FM1 (-0.35), LEE (-0.47), BON (-1.02). This gradient is the same for fully infective and incomplete forms of influenza virus.

The tray-egg gradient of susceptibility is negatively correlated with the variation in host resistance.

The differences in susceptibility are not due to any effect of the plastic trays, to thermal inactivation of the virus, to differences in adsorption or viropexis in the two test systems or to the effect of allantoic fluid on the virus particle. The critical step has been shown to occur during the intracellular stage of multiplication.

C H A P T E R   IV

VARIATION OF HOST RESISTANCE TO INFLUENZA VIRUSES

INTRODUCTION

Whether influenza viruses are titrated in the allantois of whole eggs or in surviving bits of membrane-on-shell, variation from host to host is always present. In whole eggs the effect is readily demonstrable, but difficult to investigate as one test only can be made on any one egg. In surviving bits the experimental approach would present no difficulty, were it not for the fact that under optimal conditions of maintenance the egg to egg variation is minimal and requires prohibitively large tests for its demonstration. We have noticed however that under conditions which were below optimal, not only did the sensitivity of the technique drop but variation between eggs increased greatly. Indeed, often this was the first sign of suboptimal conditions. Such a combination of variable host resistance with the possibility of doing repeated tests on the same material should allow the study of what makes one set of cells more susceptible to infection than another.

The first stage of this work is concerned with general technique: ways of exaggerating inter-host variation and means of measuring it.

EXPERIMENTS

Age and susceptibility to infection

When the technique of titrating influenza viruses in the surviving allantois was being developed, the age of the tissue was treated as one of the variables. The connection between the degree and variation of susceptibility was first recognized there, but no experimental details were given apart from the recommendation that 11-day eggs be used for best results. These early tests, all done on the BEL strain, have since been repeated a number of times and also extended to other strains of virus.

On consecutive days batches of eggs were placed in an incubator running at 38.4°C, and then cut into bits of allantois-on-shell to be tested for susceptibility. Originally the age groups ranged from 8 to 20 days of incubation, but later the experiments were restricted to cover the span of 10 to 18 days only. Younger eggs are not suited to our technique; partly because the chorio-allantois is not yet fully developed and only few 6 x 6 mm squares can be cut from it, and partly because the membrane does not adhere firmly enough to the shell and will float off almost every time. It has been shown earlier that stripped membranes are always less susceptible to infection than those attached to the shell. As eggs get

older they contain less and less allantoic fluid and the endothelial surface of the membrane is usually covered with a crust of urates, difficult to remove by simple rinsing.

The bits, bathed in Standard Medium (SM), were infected with graded doses of BEL virus, set out in a series of twofold dilutions. Usually eight bits from the same egg received each dose, with ten eggs per age group. Since the seed varied in potency over the months, the experiments were first analysed individually. After it was found that the slope of the regression lines (age versus susceptibility, fitted by least squares) did not differ significantly from experiment to experiment, the information was pooled and a common regression line fitted (Figure 3).

Susceptibility to infection is seen to decrease gradually with the age of the host tissue. The deviations from linearity are insignificant ($\chi^2_{(7)} = 5.55$; $P \sim 0.6$), and the log slope of the line comes to -0.114, with an error of $\pm$0.014 log units.

Of the other strains it is sufficient to show that the two extremes, SW and LEE, are characterized by the same age-susceptibility relationship as BEL (Table 20).

The slopes, fitted by the method of least squares, are not different from that determined more

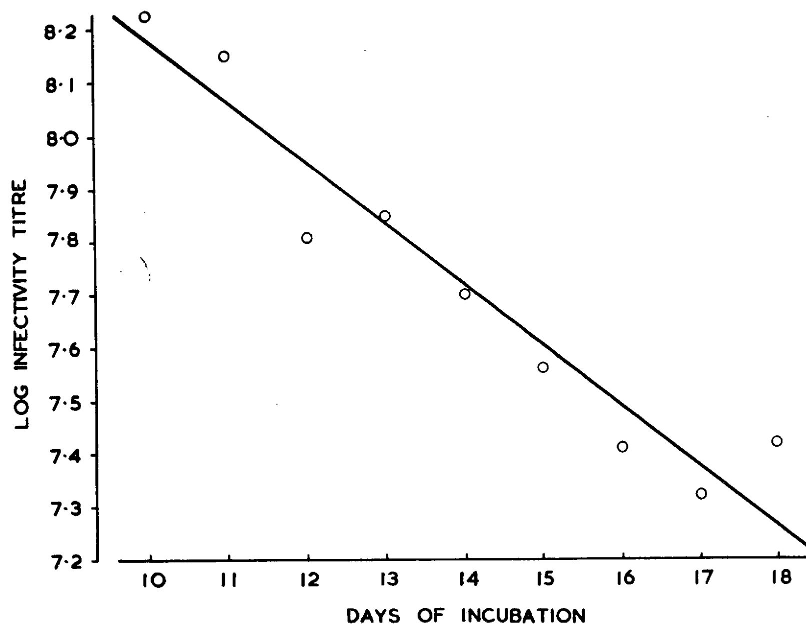


Figure 3. Decrease of susceptibility with age of incubation.

(Embryonated eggs were cut into bits of allantois-on-shell after a period of incubation at 38.4°C shown on the abscissa. Each set of bits was challenged with the same doses of BEL virus. Several experiments of this kind were pooled by adjusting the observed infectivity titres to a common mean.)

TABLE 20

Decrease of susceptibility with age of incubation

Strain	Days of incubation										Slope ± st. error
	10	11	12	13	14	15	16	17	18	19	
LEE		7.00	6.98	6.34	6.78	6.64	6.39	6.06	5.66		- 0.17 ± 0.037
SW	7.70	7.64	7.41	7.59	7.29	7.10	7.05	6.90	6.97	6.94	- 0.10 ± 0.012

The titres expressed in log_{10} units, are means based on surviving bits from ten eggs.

accurately on BEL virus. This latter estimate may therefore be taken as representative of influenza strains titrated in this system.

Although 10-day eggs are the most sensitive and, by extrapolation, younger ones perhaps even more so, for purposes of routine titrations 11-day eggs are preferred. This is the earliest age at which the chorioallantois does not slip off the shell when the bits are cut by the technique we have proposed. If, on occasion, eggs older than eleven days have to be used, the observed infectivity titres may be standardized by adding 0.1 log units for each day over eleven.

<u>Variation of host resistance within age groups</u>

The decline of susceptibility with age may reflect either some uniform change involving all eggs; this process may be imagined as the shifting of a distribution curve along the abscissa - the mean changes but not the variance. Or it may be that only some of the eggs have become less susceptible; such a process would be like increasing the variance of the distribution - if the same area is to be covered, the whole curve must become flatter and its mean must also shift. The statistical simile in this case goes beyond being a mere mental aid: it represents the simplest method of deciding the problem.

When the variation between bits from 11-day eggs was studied under optimal conditions of maintenance (Chapter II), it was found that deviations from the Poissonian model were of such minor degree that Moran's test failed to detect them. If the same test, when applied to older eggs, gives the same answer, it would prove that the drop in susceptibility is characteristic of each and every egg. If, on the other hand, the M-values become significantly higher than zero, an increase in the variance would be demonstrated, and with it a variable lowering of susceptibility.

In the experiment 40 bits were cut from 6-8 eggs at each of eight ages of incubation. They were orthogonally distributed over trays and inoculated with twofold dilutions of SW virus (5 replicates at each dose level). The mean infectivity titres fell, as expected, by about 0.1 $\log_{10}$ units per day. M-values were determined for each individual egg, and their mean is a measure of variation in susceptibility between bits from the same egg, i.e., of intra-egg variation. The overall M-value, calculated by pooling the results within an age group, measures inter-egg variation. These are the points plotted in Figure 4.

All M-values referring to individual eggs fall within the normal range, and indicate that no significant

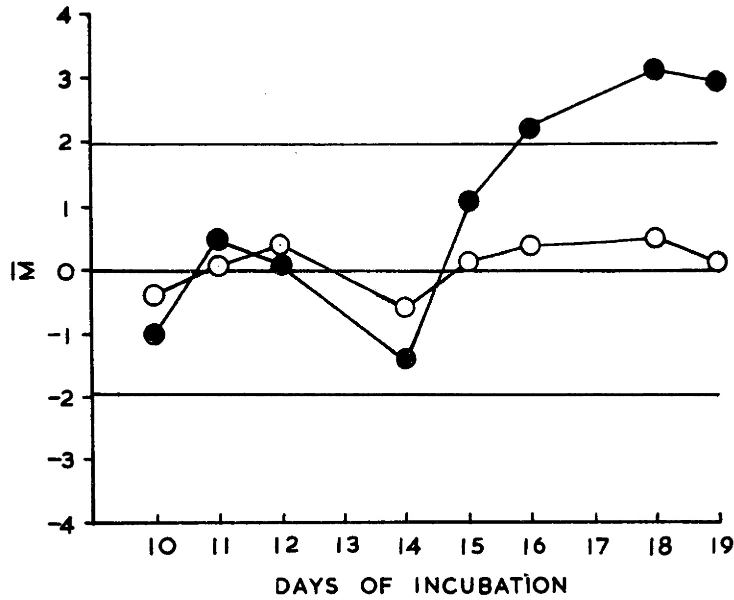


Figure 4. The effect of age on intra- and inter-egg variation of susceptibility.

(The abscissa gives the age of the embryo at the time when bits of allantois-on-shell were cut. The mean M-values calculated for individual eggs are shown by circles, the overall M-value for each age by dots.)

variation occurs between bits of the same egg, even an old one. The line connecting these points runs parallel to the abscissa, showing no trend whatever. The overall M-values on the other hand drift upwards with age until they reach uniformly significant deviations at and beyond the sixteenth day of incubation. This can mean only the presence of gross variation between eggs at these ages.

In order to obtain an independent and more precise estimate of the extent to which host resistance varies with age, bits were cut from twenty 11-day eggs and twenty 16-day eggs. In each of these sets a complete titration was performed on the SW virus. Mean infectivity titres were determined by the standard method, and an analysis of variance calculated to contrast age, variation between eggs and variation within eggs. Whereas, once again, the intra-egg (error) terms were similar and both indistinguishable from the theoretical variance of the Poisson distribution, the inter-egg variance of the 16-day group was four times higher ($P < 0.001$) than that of the younger group.

Hydrogen-ion concentration and susceptibility

Fauconnier (1953; 1954) has shown that inocula of 1000 ID_{50} of the PR8 (A) strain fail to multiply in the allantois of eggs whose allantoic fluid has a pH

below 5.8-6.0. The finding raises several interesting questions, two of which have immediate bearing on the problem of host resistance. First, in view of the progressive fall of allantoic pH with age, can the age gradient in bits be explained by this effect alone? And second, what influence have pH differences on routine titrations of influenza viruses in the allantois of young eggs?

<u>Relationship of initial pH and susceptibility of surviving bits</u>. A number of 15-day eggs were dipped, and the pH of the allantoic fluids determined, using a pH meter, with an accuracy of ± 0.05 units. Separate infectivity tests were performed on bits of each egg, by inoculating them from the same series of dilutions of BEL virus. The results, together with those of a similar test performed on 18-day bits, are given in Figure 5.

Clearly, there is no difference in susceptibility to infection once the allantois has been transferred to Standard Medium in which the pH settles round 7.5 within the hour. Whatever the effect of acid allantoic fluid, it does no irreparable harm to the cells of the allantois. It is equally certain that in this system the rise of host resistance with age has nothing to do with hydrogen-ion concentration.

In whole eggs therefore one must suppose two

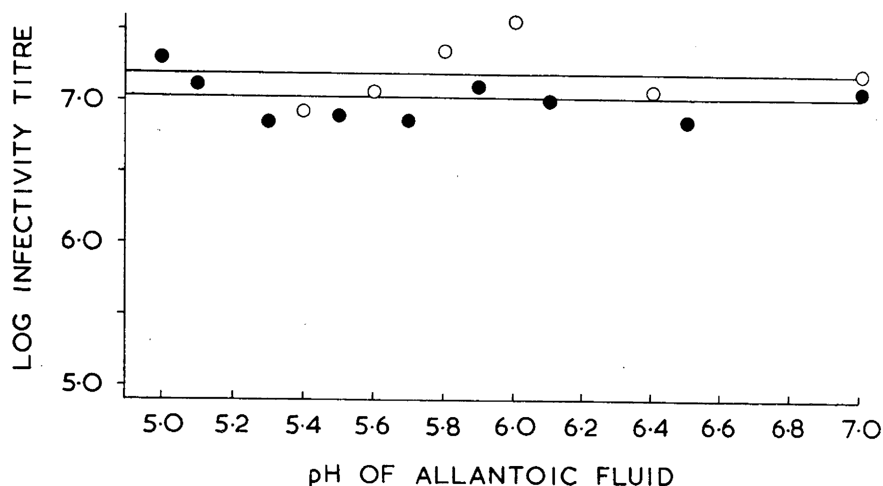


Figure 5. The influence of hydrogen-ion concentration on the susceptibility of the surviving allantois.

(The abscissa shows the pH of the allantoic fluid bathing the tissue *in ovo*; for the infectivity tests all bits were transferred to Standard Medium of pH 7.5. The dots show the average behaviour of 18-day eggs, the circles of 15-day eggs, when challenged with dilutions of BEL virus.)

overlapping mechanisms, each lowering susceptibility. Thus, if the effect of pH has been extracted, the contribution of age should become demonstrable as the residual factor. That this is so, is strongly suggested by the fact that slope of the age-susceptibility curve in whole eggs is considerably steeper than that found in surviving bits.

Allantoic pH and susceptibility of whole eggs. As a preliminary test, Fauconnier's basic experiment was repeated and expanded to define quantitatively the susceptibility corresponding to various hydrogen-ion concentrations. A large batch of 15-day eggs was sorted into groups of about a dozen according to the pH of their allantoic fluid. A complete infectivity test was performed on each of these groups by inoculating them from a single dilution series of the BEL strain. The regression of infectivity titre on allantoic pH is plotted in Figure 6.

The findings are in complete accord with Fauconnier's results on the PR8 strain, and provide a good estimate of the rate at which susceptibility diminishes over the critical range of pH.

The mean pH of allantoic fluid in our 11- and 12-day eggs is as low as 6.8 ± 0.7 and 6.4 ± 0.6, respectively. At each age level the pH is distributed

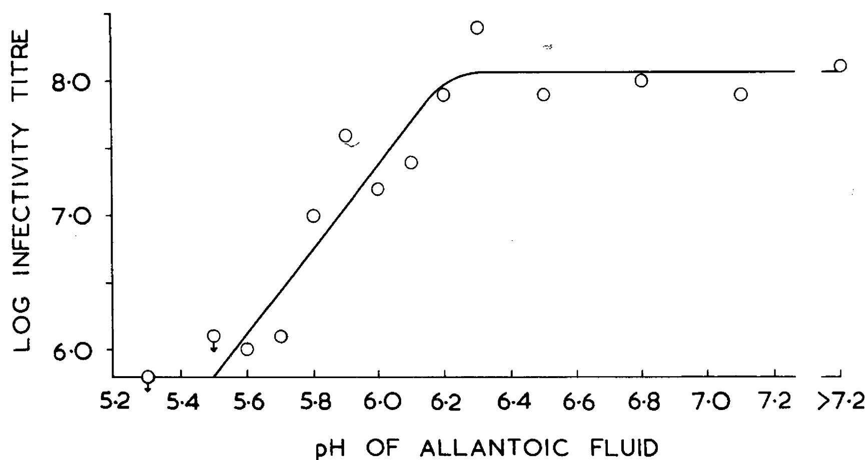


Figure 6. The influence of allantoic pH on susceptibility of eggs.

(15 day eggs, grouped according to pH of allantoic fluid shown on the abscissa were challenged with dilutions of BEL virus.)

approximately normally with, if anything, a slight positive skewness. A not insignificant proportion even of young eggs then have hydrogen-ion concentrations falling within the critical zone, and it may be anticipated that allantoic fluid pH would have some effect on the outcome of any routine infectivity test _in ovo_.

To test this idea in a simple way, we made a practice for some time of determining the pH of all negative allantoic fluids at the end of routine infectivity titrations in 11-day eggs. The negative eggs were divided into two groups: those below the endpoint, presumably negative because they failed to receive an infective particle; and those above the endpoint, negative for some other reason. The actual pH readings are of course those of 14-day eggs, and do not represent the pH obtaining at the moment of infection. The latter can be estimated though with fair accuracy from the regression of pH with age. Two typical titrations of this kind are shown in Table 21.

In both cases the difference between the mean pH of the two groups is highly significant. The negative eggs below the endpoint have higher average pH, and show the normal scatter for eggs of that age. The negatives above the endpoint are of uniformly low pH, which is also reflected in the smaller variance. Clearly, the latter

TABLE 21

Hydrogen-ion concentration of allantoic fluid and susceptibility to infection

Strain		Hydrogen-ion concentration of allantoic fluid	mean	variance
WSE	above endpoint	5.5, 5.5, 5.6, 5.7, 5.7, 5.8, 5.9, 5.9, 6.0, 6.1, 6.2, 7.4	5.94	0.26
	below endpoint	5.8, 6.2, 6.3, 6.6, 7.3, 7.6, 7.6, 7.7, 8.0, 8.5	7.16	0.78
PR8	above endpoint	5.1, 5.4, 5.4, 5.5, 5.5, 5.5, 5.5, 5.6, 5.6, 5.7, 5.7, 5.7, 5.8, 5.9, 6.1, 6.2, 6.5	5.69	0.11
	below endpoint	5.4, 5.5, 5.6, 5.6, 5.6, 5.7, 5.9, 6.3, 6.3, 6.3, 6.3, 6.5, 6.8, 6.9, 7.1	6.12	0.30

The pH of all negative allantoic fluids was determined at the conclusion of infectivity tests done on 11-day eggs.

represent a sub-population of the former, drawn from the acid end of the normal pH-distribution. If eggs like the second experimental group are used for routine titrations of infectivity, the dose-response curve will approach an asymptote set below unity, as envisaged by Gard (1953).

Temperature of pre-incubation and susceptibility

An incidental observation led to another method by which host resistance can be increased. If eggs are incubated, even from eleven days onwards, at 35°C rather than 38°C, the rise of host resistance with age is much more rapid. Whereas, for example, bits from 18-day eggs incubated at 38.4°C throughout have been shown to be about 0.7 log units less susceptible than at 11 days, bits from eggs which have been kept at 35°C between 11 and 18 days show a drop of 1.0-1.6 log units over that period. These differences are of an order readily detected by our infectivity test. Advantage is taken of this finding in the next chapter, where, in a study of the mechanism of host resistance, eggs varying considerably in susceptibility to infection are required.

Enhancement of host resistance

The medium we have developed in answer to the practical demands of infectivity tests is a minimal mixture of four simple salts, gelatine and glucose, and

therefore can scarcely be expected to do more than maintain the allantoic cells in a viable but non-multiplying state for a short period of time. It has already been shown that this period is adequate for the appearance of haemagglutinin even in membranes infected with the slowest-growing strains (Chapter III). But if quantitative studies of multiplication are contemplated, it is important to know whether the susceptibility of the membrane drops at all on maintenance in SM, and if so, at what rate. Twenty-four hours were taken as a reasonable test period. The first set of eggs was cut up and placed in trays one day, and shaken overnight at 36°C; the second set was cut up next day when both were infected from the same dilution series of the ten virus strains. Both sets were then incubated for a further three days before reading the results. Several experiments of this kind were done on each strain.

The results of Table 22 reveal little or no loss of susceptibility for the strains MEL, BEL, CAM, SW, but deterioration between two- to sevenfold when the other viruses served as indicator. That these figures do not reflect the true loss of susceptibility at 24 hours is suggested by the fact that some cups negative at four days can be shown to contain multiplying virus: if the medium is subinoculated into fresh bits, these will

TABLE 22

The effect on susceptibility of prolonged maintenance in artificial medium

Strain	Drop in susceptibility			
	test 1	test 2	test 3	mean ± st. error
WSE	0.57	0.60		0.58 ± 0.015
PR8	0.69	0.53	0.68	0.63 ± 0.052
MEL	0.01	0.03		0.02 ± 0.010
BEL	0.06	+0.02	0.16	0.07 ± 0.052
CAM	0.20	+0.20	0.00	0.00 ± 0.115
FM1	0.14	0.44	0.43	0.34 ± 0.098
LEE	0.49	0.41	0.84	0.58 ± 0.132
BON	0.97	0.80	0.90	0.89 ± 0.016
HUT	0.28	+0.13	0.75	0.30 ± 0.254
SW	0.06	0.22	+0.09	0.06 ± 0.090

The same seed of the viruses shown was used to infect bits of allantois-on-shell, half of which had been freshly prepared, half 24 hours earlier and shaken in the meantime at 36°C. The figures give, in $\log_{10}$ units, the difference between the two infectivity titres.

produce haemagglutinin within a day.

The matter can be decided directly if the same experiment is done in a slightly different way. Bits pre-shaken for 24 hours were infected with about 10^4 ID_{50}, and their medium was titrated 16 hours later for its haemagglutinin content. Here one is testing in effect the number of cells capable of yielding virus (Table 23).

The drop in total yield of haemagglutinin is much the same as the apparent drop of susceptibility was in Table 22. When considering the small differences between the two tests, it should be remembered that the data of Table 23 refer to the point in time 36 hours after exposure to SM, while those of Table 22 to a point at least 12 hours later. In these terms one effect of maintenance in an artificial medium can be imagined as a gradual loss of cells capable of yielding virus. That the effect is selective and varies quantitatively from strain to strain, is shown by the fact that BEL, CAM and SW fall once more into a separate group, closest to the controls.

If the membranes are not shaken during the period of preliminary incubation, the deterioration is greater and rather more erratic.

TABLE 23

The effect on yield of prolonged maintenance in artificial medium

Strain	Yield		
	fresh bits	pre-shaken bits	difference
PR8	1.20	0.72	-0.48
BEL	1.75	1.46	-0.29
CAM	1.09	0.85	-0.24
FM1	0.87	0.39	-0.48
LEE	1.89	1.30	-0.59
BON	1.54	1.03	-0.51
SW	1.78	1.59	-0.19

The same seed of the viruses shown was used to infect bits of allantois-on-shell, half of which had been freshly prepared, half 24 hours earlier and shaken in the meantime at 36°C. The figures give, in $\log_{10}$ units, the number of agglutinating doses yielded per bit.

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<u>Variation of enhanced host resistance</u>

In view of the finding on old eggs that inter-egg variation in susceptibility increased while the average susceptibility dropped, an experiment was designed to test whether the same phenomenon occurred with pre-shaken bits.

Twenty 11-day eggs were cut up, and half of the bits inoculated with various doses of the PR8 strain. The rest of the bits was first shaken for 24 hours at 36°C, and then inoculated with an identical series of dilutions made up from a sister ampoule of PR8 seed. The mean titre of the two groups differed by 0.67 log units; the extent of variation can be estimated by comparing the variances attached to these means (Table 24).

The test done on fresh bits has a variance characteristic of optimal conditions, and is indistinguishable from the theoretical value. The pre-shaken group has a variance more than twice as high ($0.01 > P > 0.001$). It is clear then that whether susceptibility is decreased by ageing or the capacity to yield by unfavourable conditions, the phenomenon is always accompanied by an increased variability in the behaviour of eggs. The value of these findings lies in their prospective use in the study of host resistance.

TABLE 24

The variation of susceptibility after prolonged maintenance in artificial medium

Treatment of host tissue	Egg																				Mean	Variance
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
fresh	6.66	7.15	6.57	7.06	6.79	6.90	6.85	6.32	6.89	6.92	6.36	6.85	6.70	7.09	6.00	6.55	6.97	7.09	6.36	6.77	6.74	0.09
pre-shaken	5.50	6.55	5.72	6.30	6.18	5.50	6.40	5.65	5.80	5.80	6.00	6.20	6.32	5.80	5.72	6.10	6.10	7.40	5.60	6.70	6.07	0.22

The effect of deficient and unfavourable media

Inter-egg variation may be further enhanced if bits are pre-shaken in deficient media. Daniels, Eaton and Perry (1952) have observed, and we could readily confirm, that the chorioallantis is equally susceptible to infection by influenza viruses whether glucose is included in the medium or not. If however the membranes are pre-incubated for 24 hours in such a defective medium, they no longer support multiplication as readily as controls pre-shaken in an adequate medium. We have observed very considerable variability between eggs as regards their deterioration under these conditions, presumably because the cells vary markedly in their endogenous supply of glucose.

It is interesting to note that membranes from old eggs, already somewhat resistant to infection, do not deteriorate as much on pre-shaking as do younger ones, indicating that the two effects are not simply additive.

The system of influenza virus and allantois-on-shell is exquisitely sensitive to traces of detergent, much more so than the same viruses in whole eggs. We have on occasion encountered difficulty when inadequate rinsing of glassware left minute quantities of detergent either in the bottles of SM or in the test tubes used for making up the dilution series. When the effect was

tested experimentally, we found that concentrations as low as 10^{-9} were sufficient to lower the endpoints by a factor of ten. It is remarkable that, as with the effect of pre-shaking, there is a striking difference between the behaviour of, say, MEL virus on the one hand, and LEE on the other. The former proved itself much more resistant to detergents. As in the previous section, the loss of susceptibility was always accompanied by increased variation between eggs, and in cases of gross deterioration also by signs of variation between bits derived from the same egg.

DISCUSSION

When this series of studies in host resistance was mapped out, the route seemed quite clear. First a practical method was needed to allow replicate infectivity tests on the same host and then, provided that intra-host variation was small in terms of variation from host to host, the mechanism of the latter could have been investigated directly. As it turned out, solution of the first problem all but eliminated the second. While for purposes of routine titrations nothing more could be asked, such uniformity of response bars the way to the understanding of what makes one egg susceptible and the next resistant. It became our paradoxical task therefore to put back variation into the system of assay developed at the beginning of these studies. The simplest way of doing this was to trace back the steps which led to optimal conditions of maintenance, and stop at a point where variation was not only present but also readily measurable by available techniques.

Older chick embryos are less susceptible to infection than young ones, and Fauconnier's fundamental work has linked this phenomenon with the pH of allantoic fluid. We fully confirmed his findings, but also isolated a second factor working towards the same end, one which is independent of hydrogen-ion concentration and

persists even after bringing the tissue into a medium of controlled, high pH.

The mechanism by which acid environment lowers the susceptibility of the host tissue is still unknown. Some unpublished experiments have shown that the infectivity of influenza viruses does not deteriorate more rapidly in the presence of acid than of alkaline allantoic fluid when incubated at 37°C *in vitro*. In the allantoic cavity therefore the fluid can scarcely be considered to harm the virus itself. Nor, it has been established, has it any permanent deleterious effect on the cells, for the susceptibility of membranes in trays is in no way correlated with the pH of the allantoic fluid that once bathed them.

Whatever the mechanism, the susceptibility of intact eggs drops rapidly as the pH of allantoic fluid falls below 6. And since a proportion even of 11- or 12-day eggs - routinely used for infectivity titrations - have hydrogen-ion concentrations of this order, this factor must have considerable bearing on the flattening of the dose-response curve in allantoic titrations of infectivity. Whether it is the sole factor could be determined only by more extensive investigations of the kind done earlier by Fazekas de St. Groth (1955), by combining these with an analysis of covariance on

susceptibility and pH. It may be worth noting that, while the regression of our allantoic pH values on age has the same slope as Fauconnier's, the absolute values at each age level are consistently lower by about 0.5-1.0 pH units than his. A possible explanation would be that, for one reason or another, the biological age of our eggs may be higher than that of Fauconnier's eggs at the same physical age.

Apart from the natural decrease in susceptibility with age, all other methods altering host resistance affect the various strains differentially. For certain types of experiment therefore, where uniformity of the host tissue is required, the strains MEL, BEL, CAM, SW are to be preferred as they are less likely to suffer from uncontrollable changes in the environment. On the other hand, the sensitive strains, of which LEE is a good example, should be used where variation is to be exaggerated. Indeed, we have relied on this strain as indicator whenever it has been suspected that conditions of the test have fallen below the optimum defined in the first chapter. It may seem in retrospect that LEE would have been a more suitable test strain than BEL during development of the technique. However, since a drop in susceptibility is always accompanied by increased inter-egg variation, and under the optimum conditions defined for

BEL, the LEE strain was found not to vary either, the technique may be taken to be optimal for both.

SUMMARY

Methods are described for altering the susceptibility of the surviving allantois to infection by influenza viruses.

Under natural conditions susceptibility is a linear function of age between 10 and 18 days of incubation. The drop is 0.11 $\log_{10}$ units per day for all strains of influenza tested.

In whole eggs this decline is masked by the effect of acid allantoic fluid which prevents infection when the pH falls below 6. The effect is not directed against the virus particle, nor does it do permanent damage to the cell, as surviving tissues are equally susceptible whatever the pH of the allantoic fluid that bathed them *in ovo*.

Surviving membranes can be made less susceptible by incubating the eggs at 35° rather than 38°C; by maintenance for 24 hours in vitro; by use of deficient or inappropriate medium. These methods lower susceptibility more for some strains than for others.

All treatments which lower susceptibility also increase its variation from egg to egg.

C H A P T E R   V

THE MECHANISM OF NATURAL RESISTANCE
TO INFLUENZA INFECTION

INTRODUCTION

From the recognition that eggs are not uniformly susceptible to infection by influenza virus follows the challenge to investigate the mechanism of host resistance. The allantois-on-shell system would appear to provide a unique opportunity for such a study. Ironically however, it was found that the very uniformity of response of the isolated allantois maintained under optimal conditions tended to invalidate the obvious advantages of the technique for an investigation in which eggs differing considerably in susceptibility would be required. For this reason the preceding chapter was devoted to a search for ways of increasing the variability between eggs. Now that it is possible to select at will membranes differing by tenfold or more in their response to infection, the way is open for the type of study envisaged.

It has been shown that differences in the composition of the bathing allantoic fluid are in themselves responsible for substantial differences in the susceptibility of the allantois of intact eggs. By maintaining the allantois in a defined medium however, we have been able to eliminate such environmental factors from the picture. Whatever differences between individual membranes may remain must be at the more basic

cellular level.

EXPERIMENTS

The relative susceptibility of an egg may depend simply on some property of the egg alone, or may be a more complex function of host-virus interaction. This will depend on whether or not virus particles are homogeneous in their behaviour towards cells. An attempt was first made to determine this issue.

The virus

The working hypothesis was adopted that a given population of influenza virus consists of a mixture of particles varying in "infectivity" between the limits of 0 and 1; and that the observed differences between eggs reflect not simple differences in "susceptibility", but an interaction function of the susceptibility of the egg and the infectivity of the virus. In effect, a completely susceptible egg would be infectible by every virus particle; a more resistant egg only by a particle from the more virulent fraction of the population. The susceptibility of an egg could then be defined in terms of the minimum infectivity required of a particle in order that successful infection should occur.

Though such a hypothesis cannot easily be proved, powerful evidence would be obtained if a highly infective variant could be isolated from a highly resistant host infected at limit dilution. An attempt to

do this was made in the following experiment.

Bits were cut from 18-day eggs that had been incubated from 11 days onwards at 35°C. The membranes of such eggs have been demonstrated to differ from 11-day membranes by more than tenfold in susceptibility to infection. The trays were inoculated with a series of dilutions of SW, and after 40 hours' incubation each cup dipped for haemagglutinin. Amongst the large number of replicates one positive cup was found well beyond (> 1 log) the infectivity endpoint. The contents of this cup was assumed to have arisen from a singlevirus particle. Some of it was ampouled, and the rest used as starting material for another dilution series, which was in turn inoculated into a further group of 18-day bits. The process was repeated for 4 consecutive passages. At the conclusion of the seriesampouled samples of the 4 clones, together with the original seed, were titrated for infectivity both in 11-day bits and in bits from 18-day eggs. It was expected that, had a highly infective particle been selected from an original heterogeneous population at the first or at a subsequent passage, the new clone would consist of particles capable of multiplying equally readily in 11- or 18-day membranes.

The results, shown in Table 25, indicate that no such variant has been selected. It does not follow

TABLE 25

Comparative titration in susceptible and resistant bits of virus grown at limit dilution in 18-day membranes

Inoculum	Infectivity titre ($\log_{10}$)		
	in 11-day bits	in 18-day bits	difference*
Original	7.15	5.49	1.66
1st passage	5.16	4.04	1.12
2nd passage	5.05	3.47	1.58
3rd passage	4.26	3.20	1.06
4th passage	4.96	3.79	1.17

* The standard error of the difference between two end-points each determined on 8 replicates, 3.16-fold dilution steps, is 0.25.

that a virus population is homogeneous as regards infectivity, but simply that, if infectivity does vary from particle to particle, it is not a heritable property.

The host

Whether the relative susceptibility of an egg depends on the egg alone or is a function of host-virus interaction, the egg clearly plays a dominant part. For this reason the investigation was directed towards elucidating the role of the host.

The following served as a working model.

Assume that an egg shows a certain susceptibility, s, because only a fraction, s, of its cells are capable of supporting multiplication, the remainder being uninfectible. Let b represent the "burst size" of each infectible cell, and C the total number of cells in the allantois (or piece of membrane-on-shell).

Then the total final yield of virus from an infected membrane is given by the equation

$$Y = C.b.s.$$

And if two eggs of differing susceptibility are compared, their final yields will be related by the equation

$$\frac{Y_1}{Y_2} = \frac{C_1.b_1.s_1}{C_2.b_2.s_2}.$$

Now the number of cells in the allantois of different eggs (and similarly, in pieces of membrane-on-shell of constant size cut from different eggs) contain practically identical numbers of cells (Cairns and Fazekas de St. Groth, 1957). The equation therefore becomes to a good approximation

$$\frac{Y_1}{Y_2} = \frac{b_1 . s_1}{b_2 . s_2} .$$

If there is no interegg variation in burst size, the equation reduces still further to

$$\frac{Y_1}{Y_2} = \frac{s_1}{s_2} ,$$

and there will be an exact correspondence between susceptibility and yield.

If, on the other hand, b varies from egg to egg, but independently of s, the yields of the two eggs should still be related linearly to their susceptibilities, although the individual points will show a greater scatter around the best-fitting 45° straight line.

The hypothesis was tested in the following way.

Each of twenty 11-day eggs was cut into 45 pieces. Forty of the pieces served as host tissue for an infectivity test in which they received a twofold dilution series of PR8. The remaining 5 bits received about 10^4 ID_{50} of PR8, and their supernatants were

titrated for their total content of haemagglutinin after 40 hours.

An analysis of variance on each set of data revealed clearcut evidence of highly significant ($P < 0.001$) interegg variation between both the infectivity endpoints and the final haemagglutinin yields. The next step was to determine whether a relationship existed between the two properties. The mean infectivity titre and haemagglutinin yield for each egg were compared in a product-moment correlation test. The resulting correlation coefficient, +0.24, is far from significant.

This type of experiment was repeated on a total of 9 other strains of influenza, and the results have already been given in Table 10. In each case unequivocal evidence of interegg variation in both susceptibility and yield was obtained. Yet correlation coefficients of +0.05, +0.20, 0.00, +0.33, -0.15, +0.22, +0.33, +0.28 and -0.50 with a mean of +0.078 indicated that the two properties vary quite independently of each other.

The working hypothesis was promptly discarded. The most plausible alternative is also a simple one.

Assume that all cells of all eggs are potentially infectible, but that each cell of an egg of susceptibility, _S_, has only a certain chance, _S_, of being infected by a single infective virus particle.

The corollary to such a hypothesis would be that, since all cells will yield under conditions of infection by a large enough number of particles, eggs differing markedly in susceptibility would nevertheless yield equal numbers of particles.

Preliminary experiments always indicated a direct linear relationship between an egg's susceptibility to infection (as determined by an infectivity test in its bits) and its first-cycle yield of haemagglutinin from a subsaturating inoculum (taking 6½ or 7 hours as the latest time it is safe to assume that no second-cycle progeny have appeared). The latter property was used in the next experiment as a measure of susceptibility, using two groups of eggs known to differ markedly in this respect.

The entire procedure was carried out at 36°C. Bits of regular size were cut from 11- and 18-day eggs. Replicate pieces from each egg were inoculated with SW at each of 10 twofold dilution steps around the equivalence point. After allowing 2 hours for most of the inoculum to be taken up, 100 units of RDE were added to each cup and allowed to act for half an hour. The supernatant was then removed and the membranes rinsed twice before fresh SM was added. Shaking was then continued until the experiment was terminated 6½ hours after inoculation. The

contents of each cup was titrated for haemagglutinin in ice-cold citrate. The results are plotted in Figure 7.

All curves show the expected 45° slope in the region where the inoculum is subsaturating. The inflection point of each curve may be taken as the point of equivalence, where the average ratio of infective particles to susceptible host units equals one. This point provides a measure of the susceptibility of the egg. The maximum yield of the egg is given by the level at which the curve flattens out. In accordance with previous experience, the 18-day membranes are seen to be considerably more resistant to infection, and the variability between eggs is greater within this less susceptible group. Indeed one 18-day egg is so resistant that it has not reached maximum yield even with the most concentrated inoculum used. Among the remaining six eggs there is very little difference in yield size, and clearly no systematic difference between the 11- and the 18-day eggs. The results then give strong support to the second hypothesis.

The same conclusion was reached in an experiment using membranes from eggs covering a wide age range.

Susceptibility and final yield (at 24 hours from a large inoculum) were determined on bits from groups of six eggs at each age level. The mean value of each is plotted against age in Figure 8.

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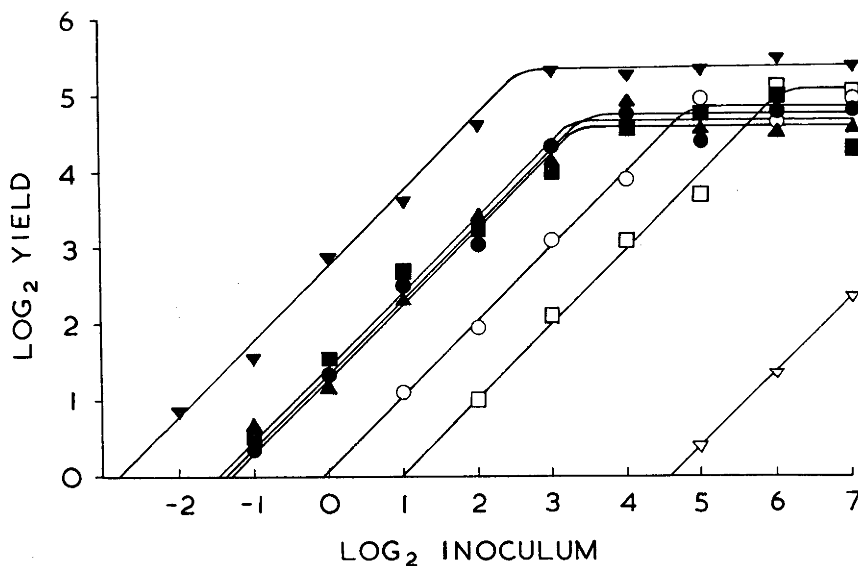

Figure 7. The relationship of inoculum size to first-cycle yield of haemagglutinin in susceptible and resistant membranes.

(Pieces of membrane-on-shell from four 11-day and three 18-day eggs were inoculated with a two-fold dilution series of SW spanning the equivalence point. The yield of haemagglutinin at 6½ hours is plotted at each dose level. The results from 11-day membranes are depicted by closed symbols, those from 18-day membranes by open symbols.)

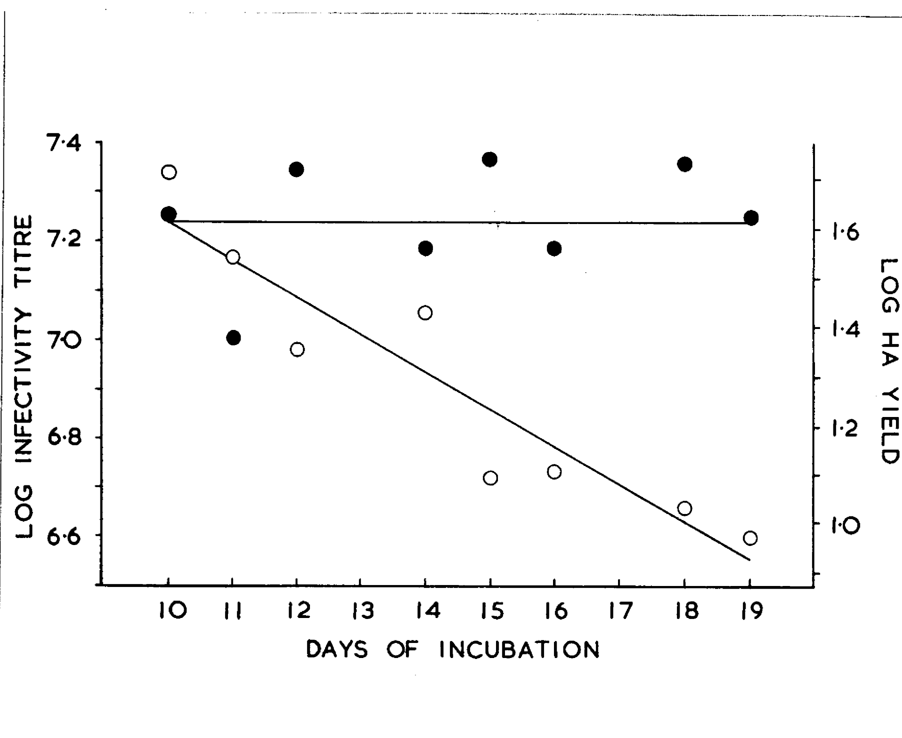

Figure 8. The relationship of susceptibility and yield to age of incubation.

(Bits from eggs of the various ages given on the abscissa were used as host tissue in an infectivity titration for the determination of their susceptibility to infection. Replicate bits from the same eggs were inoculated with a much larger dose and their final yield of haemagglutinin titrated 24 hours later. Mean yields at each age level are given by dots, mean infectivity titres by circles.)

As expected, susceptibility diminishes linearly with age, but the yields show no such trend.

<u>The fate of the non-multiplying fraction of the inoculum</u>

We have then a situation where each cell in an egg of susceptibility, $\underline{s}$, has a probability, $\underline{s}$, of being infected by a single virus particle. It would be of considerable interest to know the fate of the proportion, $1-\underline{s}$, of particles that fails to infect. Do they succeed in entering the cell in which they fail to multiply? Are they adsorbed but not taken in by the cell? Or are they not adsorbed at all? The answer can be found by measuring the extent of adsorption and viropexis under comparable conditions in eggs differing widely in susceptibility to infection.

Pieces of constant size were prepared at 36°C from 11- and 18-day eggs and each infected with a dose of SW virus equivalent to 10^4 ID_{50} in 11-day bits. After 2 hours' shaking the supernatant was removed from half the cups and titrated for its infective virus content. The replicate cups received at 2 hours 40 units of RDE, which was allowed to act for a further half hour before the supernatant was removed from these cups too and titrated for infectivity. In this instance, replicate dilution series were made in the presence and in the absence of anti-RDE rabbit serum (inactivated 56°C, 30

minutes).

The results in Table 26 give the total number of ID_{50} remaining in the supernatant at 2 hours. In the case of the group involving RDE, results were identical in the presence or absence of anti-RDE, because the initial dilution factor in making up the series for inoculation was such that less than one unit of RDE was finally present in each cup of the tray. The two figures were pooled in the table.

It is clear that both adsorption and viropexis are quite as efficient in 18-day membranes as in the more susceptible 11-day group. Indeed, it seems that both may be somewhat better in the older eggs, for whereas in the 11-day group 96% of the inoculum has been adsorbed and 73% viropexed at 2 hours, the corresponding figures for 18-day bits are 98% and 89%.

The results then indicate that in a partially resistant egg the large majority of infective virus particles actually enter cells yet fail to give rise to new infective particles.

TABLE 26

Uptake of virus by susceptible and resistant membranes

Stage of uptake measured	Age of membranes (in days)	Titre of supernatant after 2 hours ($\log_{10}$)	mean	Mean uptake (percent)
Adsorption (without RDE)	11	2.45,2.50,2.98,2.50	2.61	96%
	18	2.05,2.18,2.35,2.20	2.20	98%
Viropexis (with RDE)	11	3.06,3.50,3.58,3.57	3.43	73%
	18	2.90,2.89,3.13,3.21	3.03	89%

Bits of membrane-on-shell were inoculated with 10^4 ID_{50} (as measured in 11-day bits). After 2 hours' incubation half the supernatants were titrated for their content of infective virus. RDE was added to the remaining cups and allowed to act for half an hour before these fluids too were titrated for infectivity.

DISCUSSION

The following picture has emerged. Eggs differ in susceptibility to infection because each cell has only a given chance of being infected by even a fully infective virus particle. When an inoculum, either initially or after several cycles of multiplication, is large enough to ensure a multiplicity of infection of greater than $\frac{1}{s}$, then every cell in the egg will be infected and will yield maximally, with the result that susceptible and resistant eggs produce equal numbers of progeny. The many potentially infective particles which fail to initiate continuing multiplication in a resistant egg succeed not only in being adsorbed to the cell surface, but also in penetrating the cell wall.

One can only speculate on the reason for their failure to develop further.

The possibility that these particles may in fact undergo an abortive single cycle of multiplication with the production of non-infective haemagglutinin is ruled out by the results depicted in Figure 7, where it is clear that the first-cycle yield of haemagglutinin provides quite a faithful picture of true susceptibility differences between eggs.

It could be that the unsuccessful particles fail to find their way from the inside of the cell wall

to the site of multiplication.

Perhaps a more likely explanation would be that each cell has, not one, but a number of sites at which virus multiplication can occur. In a partially resistant cell only a proportion of these sites would be capable of supporting multiplication, though all would be able to hold or modify a virus particle.

Interesting in this regard are the findings of several other authors on a variety of viruses. Watson (1952) demonstrated multiple foci of fluorescence within each cell infected with mumps virus and stained by Coons' technique. It must be said however, that no such picture was obtained with influenza virus either by Watson herself or by other workers. Arising out of their finding that a high proportion of herpes simplex virus was found to be intimately bound to the mitochondria of infected liver cells, Ackermann and Kurtz (1952) proposed that the mitochondria may represent the site of viral synthesis. In a study of the growth of ectromelia virus in mouse liver, Nossal and de Burgh (1953, 1954) suggested the possibility of two cycles of multiplication within a single cell. (Schlesinger and Karr (1956a, b) have discussed the same proposition as applied to the reproduction of influenza in the allantoic cavity, but their evidence has lost its significance following a recent

paper by Cairns (1956) on the distribution of the inhibitor of haemagglutinin in the chorioallantoic membrane.)

The existence of multiple sites of multiplication within each cell would have important implications in interference studies. The experimental evidence leading to the conclusion that a single inactivated particle is capable of preventing multiplication in one cell could be equally well interpreted to suggest that a single particle is capable of preventing multiplication at one site in one cell.

There is an interesting parallel between the findings of this chapter and those of Cairns (1957) in his recent paper on "starting delay". Cairns has shown that an infecting particle pauses for a variable period at some time following viropexis before proceeding to multiply. The duration of this delay is randomly determined and has a half-time of about 8 hours. The delay shown by a particular cell can be overcome completely by increasing the multiplicity of infection. There is a basic similarity between this situation and the one encountered in the case of the particle that fails to infect at all. Indeed it may be more than a convenience to think of the fruitless virus-cell encounter as having an infinite starting delay.

The failure to detect genetically-stable differences in infectivity between virus particles does not of course exclude the possibility that non-heritable variation occurs. Indeed influenza has earned itself such a reputation for both lability and variability that one comes to expect such lack of uniformity as the rule rather than the exception. Recently it has been shown that populations of influenza virus can be separated into fractions differing in enzymic activity and a number of other properties, and that these differences are non-heritable (Smith and Cohen, 1956; Cohen and Smith, 1957).

SUMMARY

Eggs differ in susceptibility, not because they contain different proportions of susceptible cells, but because each cell in a particular egg has only a given chance of being infected by a fully infective virus particle. Under conditions of multiple infection, following either an overwhelming initial inoculum or several cycles of multiplication from a smaller inoculum, all cells in all eggs are infected and yield maximally.

The large majority of potentially infective virus particles fail to infect the average egg. These particles are adsorbed and taken in by the cell, but, once inside a partially resistant cell, fail to develop normally.

No heritable differences in infectivity could be detected in a population of virus particles studied.

C H A P T E R VI

THE PARAMETERS OF MULTIPLICATION IN THE SURVIVING ALLANTOIS

INTRODUCTION

In the allantoic cavity of the chick embryo kinetic aspects of the multiplication of influenza have been extensively studied. The allantois-on-shell system presents definite advantages over the conventional host for the investigation of certain special aspects of reproduction. One cannot get far, however, without first defining in quantitative terms the important parameters of multiplication in the new system. This chapter then is devoted first to the demonstration of similarities and differences between the quantitative features of multiplication in whole eggs and tissue fragments, and, second to an investigation of some aspects of multiplication to the study of which the allantois-on-shell system is especially suited.

EXPERIMENTS

Adsorption

Investigations in whole eggs have invariably revealed that, regardless of the size of the inoculum, a constant proportion becomes adsorbed to the cells of the allantois (Henle, 1949; Cairns and Edney, 1952). This in itself seems a most surprising finding, for, if the process were to follow the usual physical laws, adsorption should be more efficient from more dilute inocula. The explanation may lie in the partition of the virus between the cells and the haemagglutinin inhibitor of the allantoic fluid.

In the allantois-on-shell system, where the medium contains no such inhibitor, adsorption is found to be more complete from dilute inocula (Table 17). At the level of the single infecting particle about 90% of an inoculum of either SW or LEE is adsorbed in 2 hours (see page 86).

Viropexis

The same table reveals that virus is taken into the cell with the same efficiency in the isolated as in the intact allantois, and that similar strain differences occur. Fazekas de St. Groth (personal communication), working with whole eggs, has found a 50% viropexis time of 2½ hours for SW and 1½ hours for LEE. In trays, the

uptake at $2\frac{1}{4}$ hours of 43% of the most dilute SW inoculum and 71% of LEE shows a good correspondence.

Latent period

Progeny particles are released from the cell almost as soon as they are detectable in their finished form, but remain adsorbed to the outer surface of the cell for a further hour or so before appearing in the allantoic fluid (Cairns and Mason, 1953). For this reason the apparent latent period can be shortened by treating the membrane with RDE (Liu and Henle, 1951). Under these conditions new virus from a massive inoculum may begin to appear in the allantoic fluid as early as 3 hours after infection. In the isolated allantois the interval has been found to be exactly the same.

Starting delay. By contrast, when a single particle infects a single cell, the average latent period becomes longer and more variable. In a masterly study of this phenomenon Cairns (1957) has found that, at some stage after penetrating the cell wall, an infecting particle hesitates for a variable time before proceeding to multiply, so delaying the release of progeny by a corresponding period. Cairns has formulated a mathematical model, which requires that the extent of the delay is randomly determined and is not a characteristic of the particular egg. Following infection of a batch of eggs

at limit dilution, Cairns determined the time of earliest release of progeny by extrapolation backwards from the experimentally determined time of appearance of haemagglutinin, on the assumption that growth, once initiated, occurs at the same rate from egg to egg. The membrane-on-shell technique has certain unique advantages that enable some of these things to be examined by a somewhat different method.

A number of bits from several eggs were inoculated with such a dose that only a small minority would receive an infecting particle. At intervals 0.05 ml was removed from the supernatant of each cup and subinoculated into a fresh one. Subinoculated trays were then shaken for a further 3 days and spot-tested for haemagglutinin at the end of that time. The experiment does not detect the appearance of the very first progeny particle, for only one-tenth of the supernatant is transferred on each occasion and, in any case, a high proportion of the virus released will be readsorbed to cells immediately. It tests, however, the time of appearance of a certain fixed, small number of particles, and is clearly not seriously confounded with growth rate. The number of bits showing evidence of infection in each two-hourly interval is depicted in the histogram of Figure 9.

The distribution is fitted well by the negative

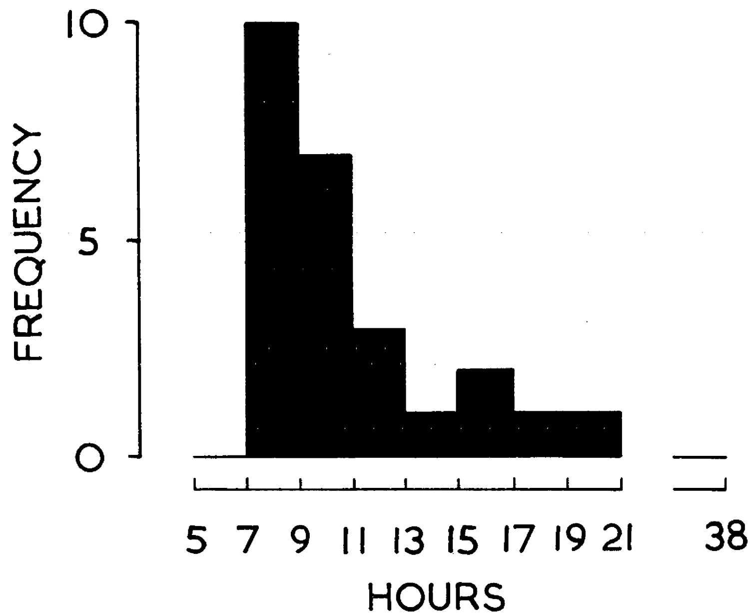

Figure 9. The distribution of "starting delay".

(Bits of allantois-on-shell were infected at limit dilution. At intervals of 2 hours o.05 ml of the supernatant was subinoculated into fresh cups and incubated for a further 3 days. The histogram shows the time of first appearance of infective virus in the 25 bits eventually showing evidence of infection.)

exponential and, in this respect, corresponds closely with Cairns' model. Taking the 7-9 hour interval as the earliest possible time of appearance of the number of progeny measured by this technique, "starting delay" has a half-time in trays of about 2 hours, compared with the 8 hours given by Cairns' data. Bits from any one egg showed no consistent tendency towards a long or a short delay. Eggs of two different ages, differing in mean susceptibility by 0.5 $\log_{10}$ units had identical delays. A group of 11-day bits showed a mean time of appearance of infective virus of 11.5 ± 1.00 hours and a corresponding group of 14-day bits 10.4 ± 0.98 hours.

Release of new virus

The differential response. Arising out of his starting delay model, Cairns proposed that the peak of release from infected cells should occur earliest from the largest inocula. This is a synchronizing effect, resulting from the fact that, as each cell is infected with a larger and larger number of particles, the chance of that cell receiving an early starter will be increased. Cairns has confirmed his hypothesis experimentally in de-embryonated eggs.

The surviving allantois is eminently suited to study of the "differential response" for two reasons. Firstly, membranes-on-shell can be rinsed more efficiently

than the de-embryonated egg, either by removal of the whole of the supernatant by suction or by transfer of the bit itself to a cup of fresh fluid. Secondly, replication within each egg enables one to extract this variable from the results.

Bits from 11-day eggs were inoculated with heavy doses of BEL 3.16-fold apart. After 2 hours' incubation RDE was added to each cup and allowed to act for half an hour. The supernatant was replaced by fresh medium at 2½ hours. At hourly intervals from 3 hours onwards the whole contents of each cup was removed and titrated for haemagglutinin. The differential responses of three eggs at three dose levels are plotted in Figure 10.

The figure confirms Cairns' contention that the peak of release occurs earlier from larger inocula. The sets of curves are similar in this respect, but differ in height, reflecting differences in burst size between the three eggs.

The experiment had been terminated at 7 hours. Other experiments, however, have been taken to 36 hours, and have confirmed the finding (Henle, Liu and Finter, 1954) that virus continues to be released from heavily infected cells for many hours at an almost undiminished rate. This type of picture is incompatible with the

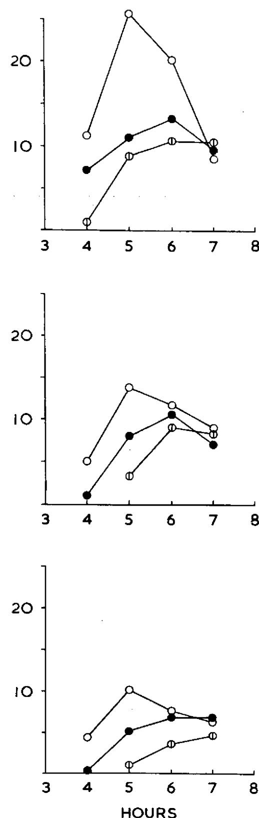

Figure 10. Differential yields of haemagglutinin.

(Pieces of allantois-on-shell were infected with a series of three heavy inocula of BEL spaced 3.16-fold apart. The membranes were treated after 2 hours with RDE, then rinsed. At hourly intervals from 3 hours onwards the whole of the supernatant was removed and titrated for its content of haemagglutinin. The three separate figures represent bits of allantois-on-shell from three different eggs. The differential yields from the largest inocula are shown by open circles, from the middle inoculum by dots, and from the smallest inoculum by halved circles.)

exponential type of release from single cells first described by Cairns (1952a) and later by Lwoff, Dulbecco, Vogt and Lwoff (1955) using another virus-cell system. In view of the finding that the allantois consists of more than a single layer of cells (Cairns and Fazekas de St. Groth, 1957), it has been suggested that the "continuous release" type of picture may in fact reflect the occurrence of a second cycle of multiplication in a deep layer of cells. Implicit in this idea is the assumption that influenza has the ability to spread otherwise than through the allantoic fluid. This can be put to the test using the allantois-on-shell system. Cairns and Fazekas have shown that the second layer of cells develops slowly, until by the fourteenth day it contains as many cells as the superficial layer. It seemed likely that still older eggs may have more than two layers of endothelial cells. Now, because of the distinction in this respect between young and old eggs, it should be possible, if spread to deeper cells occurs, to find a difference between the two in late release of virus following a saturating inoculum.

Eleven- and eighteen-day bits were infected with an overwhelming inoculum and treated with RDE at 2 hours. The yield of haemagglutinin was determined in half the bits at 7 hours ("first-cycle" yield) and half at 24

hours ("final" yield).

The yields, shown in the top half of Table 27, are seen to be identical at both points.

The same thing may be tested more critically by measuring only the late differential yield from the two age groups.

In a second experiment the whole of the supernatant was discarded at 20 hours, and the 20-24 hour differential yield from both groups determined.

The results for 11- and 18-day membranes, given in the bottom half of Table 27, are once again indistinguishable.

It could be, however, that just as infecting virus may have difficulty in reaching a deep layer of cells, so may second cycle progeny from such a layer have difficulty in escaping again into the allantoic fluid. The virus content of 11- and 18-day membranes was therefore measured at a late stage of multiplication in the following way.

De-embryonated eggs were infected with a massive inoculum of SW and turned for 24 hours. At this time they were treated with a heavy dose of RDE and very thoroughly rinsed. The membranes were then ground and incubated (some in the presence of more RDE) for a few hours to destroy the chorioallantoic inhibitor. The

TABLE 27

Early and late yield of haemagglutinin from young and old membranes

Experiment	Age of membranes (in days)	Log_{10} haemagglutinin yield ± st. error		
		7 hours	24 hours	20-24 hours
1	11	1.07 ± 0.03	1.83 ± 0.05	
	18	1.12 ± 0.04	1.81 ± 0.04	
2	11	1.33 ± 0.05		0.60 ± 0.05
	18	1.29 ± 0.04		0.63 ± 0.05

Bits of membrane-on-shell were inoculated with a massive dose of SW. After 2 hours they were treated with RDE and rinsed thoroughly. The total haemagglutinin content of the supernatant was titrated at 7 hours, 24 hours or for the interval between 20-24 hours.

preparations were then titrated for their content of haemagglutinin in ice-cold citrate-saline.

Once again, no difference was detected between the two age groups.

The findings strongly suggest that influenza is incapable of contiguous spread to the underlayer of the allantois.

Cellular yield. Since, given a sufficiently high multiplicity, all cells of the superficial layer are infectible (Chapter V), measurement of the total final output of haemagglutinin from a membrane will provide a thoroughly satisfactory estimate of the yield of the average allantoic cell.

In a small experiment, bits of approximately 6 mm x 6 mm were cut by eye and infected with either 10 or 10^5 ID_{50} of 10 strains of influenza. The total haemagglutinin content of the supernatant was titrated at 42 hours. The higher inoculum in each case was used to ensure that multiplication would be complete before uninfected cells began to die; the smaller dose was included as a control in case the larger one, giving a multiplicity of infection near unity, happened to contain such a proportion of heat-inactivated material as to cause appreciable interference (Burnet, Lind and Stevens, 1954). Since, however, no differences were in fact found

between the pairs, they were pooled in the results of Table 28.

The mean yields are consistently somewhat lower than one would have expected from experience with whole eggs. The gradient of strain differences is much the same in the two systems. Type A strains (and SW) constitute a clear-cut group about fourfold above the A-primes. Among the type B strains, LEE falls with the higher yielding group, BON and HUT with the lower.

<u>Growth rate</u>

If an egg is infected with a sufficiently small dose of virus, a number of cycles of multiplication will take place. However, the resulting growth curve will not show a series of discrete steps for two good reasons. Firstly, different cells begin to pour out their progeny after different delays, and secondly, release of virus is, in any case, a gradual process. Indeed, such a graph becomes very quickly logarithmic in form, and the slope of the graph on a log plot provides the best measure of growth rate.

Initially, however, a rough estimate of relative growth rates in the isolated and intact allantois was obtained by measuring the time of appearance of haemagglutinin following equivalent inocula.

Whole eggs and bits of membrane-on-shell in

TABLE 28

Cellular yield in the allantois-on-shell system

Strain	Log_{10} HA units per bit (1.2×10^5 cells)
WSE (A)	1.41 ± 0.09
PR8 (A)	1.61 ± 0.06
MEL (A)	1.63 ± 0.15
BEL (A)	1.65 ± 0.11
CAM (A')	1.06 ± 0.04
FM1 (A')	1.13 ± 0.09
LEE (B)	1.68 ± 0.06
BON (B)	0.89 ± 0.09
HUT (B)	1.00 ± 0.07
SW (Swine)	1.90 ± 0.06

Bits of allantois-on-shell measuring about 6 mm x 6 mm were inoculated with the 10 strains listed, and incubated for 42 hours.

The number of cells in the superficial layer of the allantois of a 6 mm x 6 mm eleven-day bit is approximately 1.2×10^5 (Cairns and Fazekas de St.Groth, 1957).

0.7 ml of SM were inoculated with 10^3 infective doses - the eggs with 10^3 "egg ID_{50}", the trays with 10^3 "tray ID_{50}" - of a number of strains. A volume of 0.05 ml was removed at intervals from all supernatants and tested for the ability to agglutinate a standard drop of 5% red cells. These results will have a considerable inherent error, largely due to slight differences between strains of inoculum size, when the titre of the preparation used may not have been known exactly in advance. They will be sufficient, however, to give a guide both to the relative growth rates of various strains and to the relative growth rates in the two host systems (Table 29).

The results show a consistent strain gradient, whether tested in eggs or trays. The A, A^1 and B strains fall into three clear cut groups in decreasing order of growth rate. Distinctions between individual members of these groups would not be justified on this data. In the isolated allantois growth of virus appears to be consistently slower by a small margin. The gap becomes greater, when it is remembered that 2-3 hours should be added to the figures in the "allantois-on-shell" column to equate the two sets of readings (the volume of the bathing fluid being ten times higher in eggs than in trays).

To express growth rate in absolute terms, the slope of the growth curve must be determined. This has

TABLE 29

Comparative growth rates in whole eggs and bits of allantois-on-shell

— Time of appearance of haemagglutinin —

Strain	Time of appearance of haemagglutinin (hours)	
	Eggs	Allantois-on-shell
PR8 (A)	18	20
MEL (A)	16	18
BEL (A)	15	15
CAM (A')	20	25
FM1 (A')	21	26
LEE (B)	27	29
BON (B)	29	33
SW (Swine)	16	18

Whole eggs and bits of allantois-on-shell were inoculated with equivalent doses (10^3 ID_{50}). At intervals of 1½ hours, 0.05 ml was removed from all supernatants and tested for the ability to agglutinate a standard drop of 5% red cells.

been done experimentally by titrating the total infective content of the supernatant at three intervals 4 hours apart, then fitting a straight line to the data. In such an experiment the inoculum size is irrelevant, for, once multiplication has become logarithmic, the slope will be constant for any one strain. Table 30 gives the experimentally determined growth rates in eggs and trays, following an inoculum of about 10^3 ID_{50} of the BEL strain.

Since the difference between the means is significant at the 5% level, it is apparent that growth is somewhat slower in the isolated than in the intact allantois. This is doubtless a direct reflection of the lower cellular yield.

Growth rate and susceptibility. Consider the expected growth rates of influenza in bits of allantois-on-shell of differing susceptibility to infection.

Let b equal the "burst size" of each cell in a particular egg, and s the susceptibility to infection of that egg.

Then, of the b progeny liberated from each infected cell, only a proportion, $b.s$, should be effective in initiating a second cycle, and only $(b.s)^{n-1}$ the nth cycle. Therefore, the growth curves of two eggs differing considerably in susceptibility, but having the same burst size, should quickly diverge. If one takes

TABLE 30

Comparative growth rates in whole eggs and bits of allantois-on-shell

— Slope of the growth curves —

	Slope (log_{10} per hour)	mean ± st. error
Eggs	0.65,0.56,0.54,0.52,0.51,0.50, 0.48,0.44,0.42,0.35,0.32	0.48 ± 0.029
Trays	0.49,0.35,0.30,0.29	0.36 ± 0.046

Eleven whole eggs and four bits of allantois-on-shell (from four separate eggs) were inoculated with about 10^3 ID_{50} of BEL. At intervals from 10 hours onwards all supernatants were sampled and titrated for their content of infective virus. To each set of data a straight line was fitted by the method of least squares.

bits of membrane-on-shell from eggs of, say, 11 and 18 days' incubation, where burst size is identical (Chapter V), but susceptibility differs by about eightfold, the difference in the slopes of their growth curves should be gross.

Pieces from eggs of 11, 14, 16 and 18 days' incubation were infected with about 100 ID_{50} of the SW strain. The supernatants were titrated for their content of infective virus at 16, 20 and 24 hours. When mean titres for each age group were determined at each point, the results were found to give in each case a straight line on a log plot. Since the height of individual lines varied according to the effective size of the inoculum, all lines were adjusted to pivot around a single point. The slopes of the four sets of data were left as experimentally determined (Figure 11).

Control measurements showed that the eggs decreased in susceptibility with age, as expected, at the rate of 0.1 $\log_{10}$ per day. Final yields from bits of all ages were, as usual, identical. But, quite unexpectedly, the growth rates in membranes of all ages were also found to be indistinguishable.

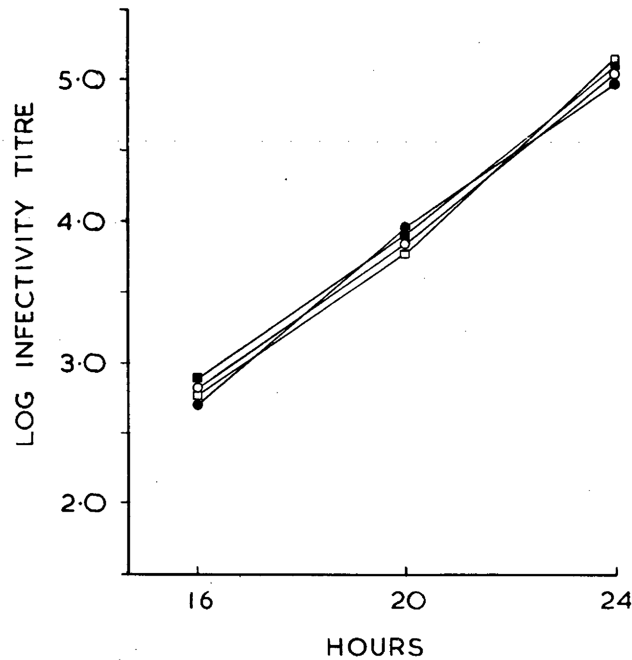

Figure 11. Growth rate in membranes of different susceptibility.

(Pieces of allantois-on-shell derived from eggs of four different ages were infected with about 100 ID_{50} of SW. The supernatants were titrated for their content of infective virus at 16, 20 and 24 hours. Titres from 11-day membranes are given by open circles, 14-day by open squares, 16-day by closed squares, and 18-day by closed circles. All results have been adjusted to pivot about a common mean.)

DISCUSSION

Clearly, this finding fails to fit the model. It leads one to think that, once infection has been initiated, all cells are uniformly completely susceptible to infection. Yet, it does not seem conceivable that infected cells could be capable of exerting some obscure remote control over cells as yet uninfected. There is a more likely explanation. Perhaps virus particles do in fact vary in infectivity, but newly produced particles are, for the brief time they are in transit from one cell to another, all fully infective. This same "fully-infective" virus must then degenerate soon after removal from the system in which it is multiplying, for virus harvested with every precaution during the exponential phase of multiplication will, when used as seed for a new system, never infect young and old membranes with equal facility. The virus population must by this time be assumed to have become heterogeneous with respect to infectivity. (As demonstrated in the previous chapter, however, such differences in infectivity would not be heritable.) Our concept of susceptibility differences would then be expanded to include the idea that a relatively resistant egg is infectible by only the most highly infective of particles, but a fully susceptible egg by any particle.

The feasibility of the concept that a population of influenza particles may show a spectrum of infectivity has already been discussed (page 147) in connection with the findings of Smith and Cohen on the heterogeneity of populations in respect of other properties.

Whatever the true explanation of the experiment, it does serve to highlight the distinction to be drawn between the first and subsequent cycles of multiplication. Cairns (1952b) has listed evidence calling for such a distinction. He points out that the first cycle of multiplication of influenza in the allantoic cavity takes longer than any subsequent cycle (Fazekas de St. Groth and Cairns, 1952), and that alterations in temperature of incubation can give rise to changes in the length of the first but not later cycles (von Magnus, 1951). Cairns' (1951) own finding that unadapted strains of influenza undergo only a single cycle of multiplication in mouse brain was the first of a variety of seemingly unrelated observations bearing on the same problem. Both in the chorion (Fulton and Isaacs, 1953) and in He La cells (Henle, Girardi and Henle, 1955) it appears that, regardless of inoculum size, growth of influenza ceases after one cycle. In the allantois preparations of "incomplete" virus reveal themselves to be heterogeneous

mixtures, containing a proportion of particles incapable of progressing beyond the first cycle of multiplication (Burnet, Lind and Stevens, 1955; Graham, 1955).

SUMMARY

All the known parameters of multiplication have been studied in the surviving allantois and found to be of a magnitude comparable with their counterparts in whole eggs. Adsorption and viropexis occur with equal efficiency in the two systems, the latent period is the same, and release of new virus follows a similar pattern. Burst size is, however, a little lower and growth a little slower than *in ovo*.

"Starting delay", as tested by the time of appearance of progeny from a single infected cell, has the same negative exponential distribution, but a half-time of only two hours.

Growth rate is identical in bits of grossly different susceptibility to infection.

There is some evidence that influenza is not capable of contiguous spread in the allantois.

G E N E R A L S U M M A R Y

A new technique has been developed for assaying the infectivity of influenza viruses. The host tissue consists of squares of allantois-on-shell maintained in a simple synthetic medium in plastic trays.

The method represents an economy in money, time and effort. One worker and an assistant can handle in a day up to 50 trays, in every respect the equivalent of 5000 eggs in the orthodox techniques. The sensitivity of the isolated allantois is, on the average, that of the intact egg. Because the dose-response curve is invariably steeper than in whole eggs, endpoints are estimated with greater precision in trays, 7 bits yielding as much information as 10 eggs.

All the known parameters of multiplication have been studied in bits of allantois-on-shell and shown to be comparable with their counterparts in whole eggs. Adsorption and viropexis occur with equal efficiency in the two systems, the latent period is of the same order, and release follows a similar pattern. Burst size and growth rate are somewhat lower in the isolated allantois.

Certain features of multiplication to which the technique is especially suited have been studied. "Starting delay" was found to have a half-time of 2 hours

and to be a chance phenomenon. There was every indication that influenza is incapable of contiguous spread in the allantois.

A study has been made of host resistance. Pieces from different regions of an egg behave uniformly in their response to infection. In consequence, the dose-response curve in bits from any one egg is the theoretical Poissonian curve. Different eggs, however, show different susceptibilities to infection. The extent of these differences between eggs is, however, much less when tested in the surviving than in the intact allantois, for the dose-response curve in trays, even using bits from a number of eggs, approximates to the Poissonian model. The wider scatter in whole eggs is largely due to the high proportion of acid allantoic fluids, even amongst the young eggs routinely used in infectivity tests.

The extent of the variation between bits of allantois-on-shell from different eggs can be increased by any procedure that lowers the average susceptibility of the tissue. This can be done artificially by incubating eggs at 35° rather than 38°C, by shaking membranes for some time before inoculation, or by the use of deficient media. There is also a natural decline of susceptibility with age of incubation at the rate of

0.1 $\log_{10}$ per day. This factor is quite distinct from the transient environmental effect of acid allantoic fluid in intact eggs. The susceptibility of the isolated allantois varies also as tested by different strains of influenza. There is a well-marked gradient, varying over a hundredfold range, from SW, giving titres tenfold higher in bits than in whole eggs, to BON, giving titres tenfold lower. The critical step in the determination of whether a particle behaves as if in the intact or the surviving allantois occurs some time after viropexis.

A study of the mechanism of host resistance has revealed the following picture. Eggs differ in susceptibility, not because they possess different proportions of susceptible cells, but because each cell in a particular egg has only a given chance of being infected by a fully-infective virus particle. Under conditions of multiple infection, following either an overwhelming initial inoculum or several cycles of multiplication from a smaller inoculum, all cells in all eggs are infected and yield maximally. The majority of potentially infective virus particles fail to infect the average egg. These particles are adsorbed and taken in, but, once inside the cell, fail to develop fully. Evidence was obtained that, while individual particles from a "standard" virus population may vary in "infectivity",

such differences are not heritable.

A P P E N D I X

MATERIALS AND METHODS

Diluents

Saline: 0.85% sodium chloride in distilled water.

Calcium magnesium saline: 0.85% sodium chloride, 0.0079% magnesium chloride, 0.0028% calcium chloride, dissolved in distilled water, adjusted to pH 7.2 with 0.02M borate buffer.

Citrate saline: 0.45% sodium chloride, 2.0% sodium citrate, 0.0063% citric acid, dissolved in distilled water, adjusted to pH 7.2 with 0.025M Tris buffer.

Gelatin saline: 0.5% gelatin in Ca Mg saline.

Red blood cells

Fowls were bled from a vein straight into citrate saline. The blood was centrifuged and washed three times, then stored at 4°C.

For use in haemagglutinin titrations cells were always made up within a day of use. When required only for the "spot-testing" of infectivity titrations, cells up to a week old were often employed. Under these circumstances, however, suspensions were always made up in either Ca Mg saline or citrate saline. This was in view of the finding that suspensions in saline began after a few days to show spontaneous agglutination with SM.

Receptor-destroying enzyme

Growth. The 4Z strain of Vibrio cholerae was

grown for 16 hours in Revai peptone broth adjusted to pH 7.2. The organisms were inactivated, after adjustment to pH 6, for 30 minutes at 55°C, according to the recommendation of Burnet and Stone (1947a), then centrifuged to remove the sediment.

These crude extracts had a number of disadvantages.

(1) They were of low titre.

(2) In low dilution, they sometimes themselves agglutinated red blood cells.

(3) Also in low dilution, they digested chorioallantoic membrane, possibly due to the presence of the "desquamating enzyme", which has been shown to destroy intestinal epithelium (Burnet and Stone, 1947b).

All these drawbacks were overcome by concentrating and purifying the enzyme by the RBC adsorption-elution technique of Burnet and Stone (1947a).

On occasions when RDE of particularly high titre was required, we used material prepared by a new technique (Ffrench and Ada, 1957), and kindly made available by Mr. G. Ada of Melbourne.

<u>Titration</u>. RDE was titrated by the method of Burnet and Stone (1947a). Crude preparations had a titre of 200, eluates variably higher, and Ada's stock

2000 per 0.25 ml.

Anti-RDE

An antiserum to RDE was prepared, essentially according to Burnet (1949), but with the aid of Freund's (1956) adjuvant.

The serum had a titre of 400 against 4 units of RDE.

Eggs

Eggs from white Leghorn fowls were incubated at 38°C and 55-65% relative humidity, and mechanically turned twice a day. Normally, they were candled for use at 11 days.

If left, our eggs tend to hatch at 19½-20 days, rather than 21.

Virus strains

The following egg-adapted strains of influenza were used.

Type A:

WSE substrain of WS, the prototype of influenza A (Smith, Andrewes and Laidlaw, 1933), adapted to grow and produce pocks on the chorioallantois (Burnet, 1936).

PR8 (Francis, 1934).

MEL (Burnet, 1935).

BEL (Burnet and Bull, 1943).

Type A-prime:

CAM (Anderson, 1947).

FM1 prototype of influenza A-prime (Rasmussen, Stokes and Smadel, 1948).

Type B:

LEE prototype of influenza B (Francis, 1940).

BON (Beveridge, Burnet and Williams, 1944).

HUT (Burnet, Stone and Anderson, 1946).

Swine influenza:

SW Swine-15 (Shope, 1931).

Growth. Eleven-day eggs were inoculated allantoically by the method given by Beveridge and Burnet (1946) with 10^2-10^4 ID_{50} contained in about 0.1 ml of gelatin saline or "Standard Medium". After 30 hours' incubation the eggs were chilled and their allantoic fluids harvested aseptically.

Storage. The material was distributed in small ampoules in 0.2 ml aliquots, snap-frozen, and stored in a dry-ice cabinet until immediately before use.

Titration of infectivity in the allantoic cavity

Logarithmic series of dilutions were made up in ice-cold gelatin saline or SM, and standard volumes inoculated allantoically into groups of 10- to 12-day eggs. After 48-72 hours' incubation at 35°C the allantoic fluids were sampled using a small, all-glass version of

Henle and Henle's (1944) "harvesting pipette", and spot-tested for the presence of haemagglutinin (Hirst, 1942). Endpoints were calculated, unless otherwise specified, by the method of Reed and Muench (1938). Infectivity titres in the text are given as $\log_{10}$ ID_{50}.

Titration of infectivity in the surviving allantois

The method is summarized at the end of Chapter I.

De-embryonated egg technique

Eggs were de-embryonated through a hole 1¼" in diameter at the albumen end and rinsed thoroughly before adding 4-5 ml of SM to each. The eggs were then sealed with aluminium milk bottle caps (stuck on with paraffin wax) and fitted in groups of up to 36 into wooden holders. The holders were placed on a rotating machine, situated in a warm-room, which rolled the eggs through 360° twelve times per minute with their long axes inclined at 17° to the horizontal. Eggs were normally given an hour or so to become stabilized at $36^{\circ}C$ before receiving the inoculum through a small hole in the cap.

Titration of haemagglutinin

Volumes of 0.25 ml of diluent were delivered from an automatic pipetting machine into each cup of transparent plastic trays (obtained from Prestware Ltd., Lombard St., London, and cast off a die owned by the Medical Research Council of Great Britain). Routinely,

normal saline was used as diluent. However, Ca Mg saline was employed instead in titrations of RDE, and ice-cold citrate saline where the action of contaminating RDE was to be inhibited. In the latter case, comparisons of titres with any obtained at room temperature were not valid (Miller and Stanley, 1944). Serial twofold dilutions were made with the aid of Takátsy's (1955) spiral loop. To each cup was added 0.025 ml of a 5% fowl red cell suspension as a single drop from a calibrated pipette. Trays were shaken, and the test read by the pattern of settled cells after 35 minutes' standing at room temperature.

Partial ("one-plus") agglutination was taken as the end-point. Titres were divided by two in order to convert the results to standard HA units per 0.25 ml. The technique, with an error of 8% per single titration, was found to be somewhat more accurate in our hands than the conventional method (Salk, 1944).

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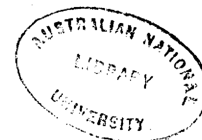

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