# AN IMPROVED ASSAY FOR THE INFECTIVITY OF INFLUENZA VIRUSES

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(With Plate 1)

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 inquire 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; and even if it were realized, the problem of interhost variation would not be solved, merely eliminated. The second approach appears more practical since there are promising precedents, and, if succesful, more informative since it would give a direct measure of interhost variation.

In exploring the latter, we built largely on the pioneering work of Fulton & Armitage (1951). Their experiments showed that fragments of the chorio-allantois 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 twenty to eighty 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 & Armitage (1951), as well as Tamm, Folkers & 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 & 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 & Armitage; the test was set up in plastic trays and incubated, with continuous shaking, at 35° C. for 64 hr. 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 initially at 7·20, dropped overnight to about 6·3 in all cups

Table 1. *Susceptibility to infection of the surviving chorio-allantois. Effect of stripping the membrane from the shell*

| | Membrane | | |
|---|---|---|---|
| Experiment* | Stripped | Attached | Difference |
| 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.

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 & 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 twenty to thirty 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–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.

Table 2. *Susceptibility to infection of the surviving chorio-allantois. Contribution of various physical factors*

| Expt.* | 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.

The results in Table 2 show that loss of infective virus on contact with the chorion is not the main contributor to the lower sensitivity of the method of Fulton & 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 2 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 100-fold. 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 & 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 & 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 & 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 $ID_{50}$, proving that more vigorous shaking was definitely superior. It should be remembered in this connexion that Horváth (1954), who used rolling instead of tilting, demonstrated a fourfold increase in sensitivity with a technique closely related to that of Fulton & 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 $\pm$ 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 $\log_{10}$ 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 (twofold steps), and more replicates (usually ten or twenty) per dilution were used, that is, altogether 100–200 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, Beveridge, Bull & Clark, 1942), a virus particularly suited to quantitative work by virtue of its steep dose-response curve in allantoic infectivity tests (Fazekas de St Groth, 1955).

### *The medium*

We compared first a number of simple synthetic media such as those of Weller & Enders (1948), Fulton & Armitage (1951), Eaton (1952) and Wunder, Brandon & 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 % NaCl, 0·06 % $CaCl_2.6H_2O$, 0·017 % $MgCl_2.6H_2O$, 0·5 % gelatine,

and buffered at pH 7·2 with M/50 Tris-(hydroxymethyl)-aminomethane—HCl (=TRIS—HCl).

*Diluent.* At one stage of these experiments we tried to make up media in simple distilled water, and this resulted in a 20-fold 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 & 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 & 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%), glucose (0·1%), $NaHCO_3$ (0·014%), phenol red (0·00025%) and chloramphenicol (0·025%). The factorial variables were NaCl (0·4, 0·6, 0·9, 1·2%), KCl (0·005, 0·02, 0·06, 0·12%), $CaCl_2$ (0, 0·02, 0·08, 0·20, 0·50, 0·80%), $MgCl_2$ (0, 0·0005, 0·005, 0·02, 0·05, 0·15%). 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 eighty bits of membrane), and the results read after 2 days' incubation.

Significantly lower titres were obtained only with the lowest sodium (0·4% NaCl) and the highest magnesium (0·15% $MgCl_2$) concentrations. The optimal combination of these cations lay around the region of 0·8% NaCl, 0·06% KCl, 0·08% $CaCl_2$, 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 % gelatine and 0·025 % chloramphenicol. Glucose was tested at the levels of 0, 0·01, 0·05, 0·10, 0·20 and 0·50 %. The media were then sterilized by autoclaving.

Highest infectivity titres were obtained in the range 0·01–0·05 %. Consequently, 0·03 % 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 would stand autoclaving and were readily available. Of these gelatine was found satisfactory, and when incorporated into the basic medium gave maximum titres in the range 0·05–1·0 %. 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 % 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 % (5·0 ml./l. of a 0·4 % solution). This amount does not affect the susceptibility of the system; however, if the tissues are kept in it for 24 hr. 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./l. of a 0·01 % 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 $\mu$g./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 $\mu$g./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 $\mu$g./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 & 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 normal allantoic fluid, the natural ambient of these cells, and to the medium of Fulton & Armitage.

Table 3. *Susceptibility to infection of the surviving chorio-allantois. 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.

*Trays*

*Size.* The earliest tests were performed in the 25 × 25 × 2·5 cm. Perspex trays described by Fulton & 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,* now widely used for haemagglutinin titrations.

*Spacing.* One cannot abstract from the literature firm directives on the need of aeration, as Fulton & 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 *et al.* (1952), as they found that 1 g. of chorio-allantoic tissue consumes 250 $\mu$l. of $O_2$ per hour, and under such conditions the supply would run out within 2 days. By separating the trays with the aid of spacing frames the volume 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 × 2·5 × 0·3 cm. Perspex round one of the trays (or a suitable mould) and sticking two 14 × 1·5 × 0·3 cm. pieces inside the frame (see Pl. 1, fig. 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 × 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 × 80 cm.) of lint moistened in lukewarm water (see Pl. 1, fig. 3).

* Obtained from Prestware Ltd., Lombard St, London.

Aluminium supports to take two of these batteries (i.e. eight trays) were specially made, and fitted on a horizontal shaker (Kantorowicz, 1951) working in a constant temperature room, set at 36° C. (Pl. 1, fig 4).

*Cleaning.* The usual method of cleaning Perspex trays by immersion into 1–10% 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. Secondly, some mould spores resistant to acid survived the procedure and contaminated the medium in subsequent tests. The alternative method of sterilizing by ultra-violet 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% 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 will 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 the trays, but this was found to be 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 prewarmed. 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 $\pm$ 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 min.

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 reflexion 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 (Pl. 1, figs. 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 which also varies from strain to strain of virus. The detailed findings relating to this phenomenon form the subject of a subsequent paper.

*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 2 hr., 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 hr. 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 34–37° 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 & Armitage, that shaking was essential only during the first day of incubation, was confirmed.

When testing whether 3 days' incubation gives higher titres than two, the average rise was found to be only 0·04 $\log_{10}$ units, not significant. This behaviour characterizes BEL and other fast growing influenza strains; some, as will be shown

in another paper, multiply more slowly and hence have to be incubated for 3 days.

*Reading.* 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 the 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 twenty-five haemagglutinating units against a standard drop of 10 % 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 % fowl red-cell suspension is added to each cup, the trays well shaken and left standing for $\frac{1}{2}$ hr. 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.

### *The dose-response relationship*

The new test, developed above, has been compared with the orthodox allantoic infectivity test using nine strains of influenza virus besides BEL. The most striking difference is the steepness of the dose-response curves with the new method, as against the varying degree of deviation from the Poissonian observed in intact eggs (Fazekas de St Groth, 1955). For this reason the simple comparison of the two methods of assay is not admissible and the data, throwing new light on the nature of interhost variation, will be analysed separately. It can be stated in anticipation that the new method is definitely more accurate than the orthodox, and that there are important and characteristic strain differences in sensitivity, some strains giving up to ten times higher titres than in eggs, some six times lower. The strain differences, again, are beyond the scope of a technical study.

## 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 % stock solution) | 0·0025 g. |
| $H_2O$ (glass distilled, or deionized) | to 1000 ml. |

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

(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 × 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 a 0·025 ml. drop of dilutions prepared from test material.

(6) Build battery of four 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 hr. at 35° C.

(8) Remove trays, pick out bits of tissue with fine forceps, add one drop of 10 % fowl red cells to each cup, shake.

(9) Read test after 30 min.; positive haemagglutination = infection.

(10) Clean trays and spacers by immersing in 4 % NaOH for an hour, thoroughly rinse in running tap-water, fit trays in spacers, dry in incubator at 35° C.

## SUMMARY

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

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

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

4. The test is set up in transparent plastic trays, and the results can be read with the naked eye after 2 days.

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

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## EXPLANATION OF PLATE

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

Fig. 2. Delivery of host tissue into plastic trays. (Step 4 of standard technique.)

Fig. 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.)

Fig. 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 & Armitage (1951).

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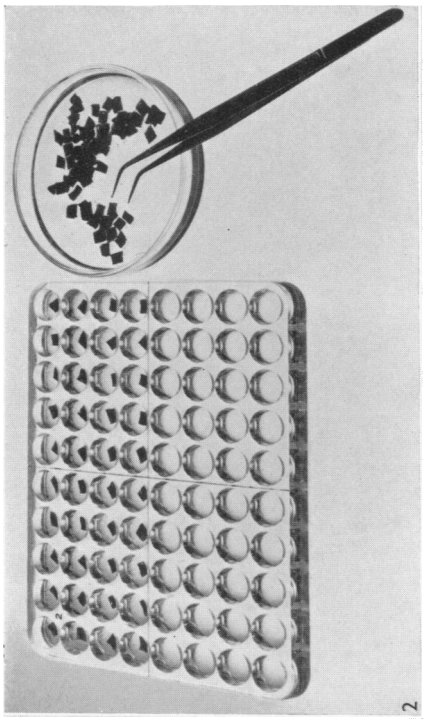

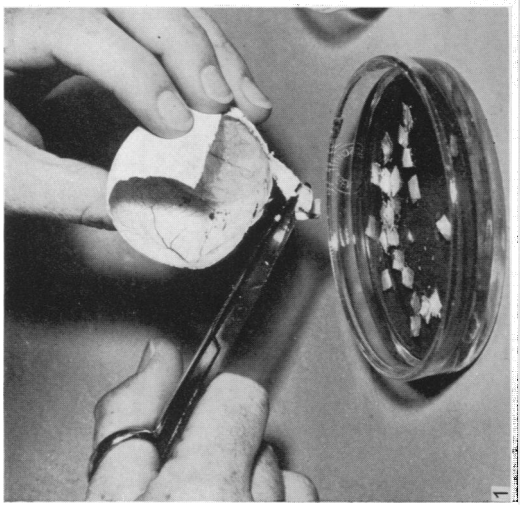

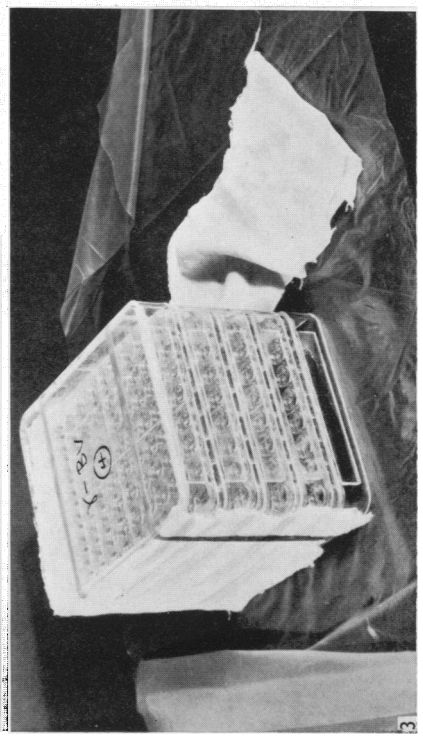

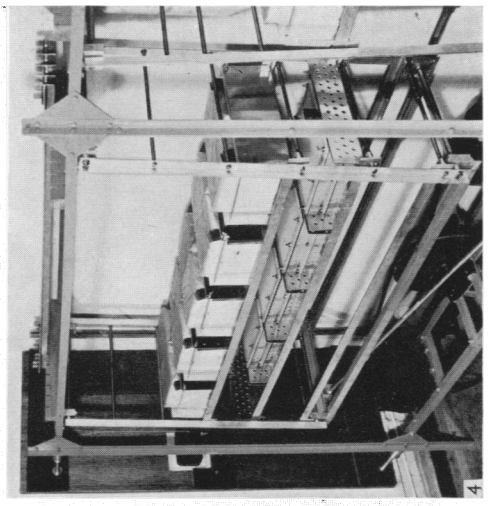

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