COMPARISON OF THE INFECTIVITY OF INFLUENZA VIRUSES IN TWO HOST SYSTEMS: THE ALLANTOIS OF INTACT EGGS AND SURVIVING ALLANTOIS-ON-SHELL

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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 *precision* of the method, not to its *sensitivity*. 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 study we shall compare the sensitivity of the orthodox test in whole eggs with the sensitivity 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.

Materials and methods have been described in detail in the first paper of this series (Fazekas de St Groth and White, 1958*a*); the ten strains of influenza virus are the same as used in an earlier study (Fazekas de St Groth & Graham, 1954).

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

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https://doi.org/10.1017/S002217240003802X Published online by Cambridge University Press

only if the rate of multiplication of each strain in this system is known. Such experiments have been done, and will be published separately (White, 1959). 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 hr.; A-prime and B-strains attain their maximum by 72 hr. 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 2 or 3 days, while tests using the other viruses were invariably left for 3 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 1.

Table 1. *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 6×6 mm.^{2} bit of tissue. (One haemagglutinating unit is the amount of virus which causes partial agglutination of 10^7 fowl red blood cells.)

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 more than 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 3 days' incubation, and compared the titres given by the original and secondary test. In this way as little as five infective units could be detected, that is, the presence of infection was assessed by a criterion about a million times as sensitive as the standard test by haemagglutination (Table 2).

Whereas all cups positive at 3 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 & Henle (1953) for the behaviour of influenza viruses in the intact allantois.

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 fifty to sixty eggs (five to six replicates per dilution) and two or three trays of surviving tissue (sixteen to twenty-four 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

Table 2. *Subinoculation of infectivity tests*

(At the end of a standard infectivity test about 0·1 ml. of medium was transferred to the corresponding cups of a fresh tray.)

	Median infectivity titre*		
Strain	Standard test	Subinoculation	Difference
PR 8	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
FM 1	6·15	6·21	0·06
LEE	6·82	6·98	0·16
BON	5·10	5·19	0·09
		Mean difference	0·08

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

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 3 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 2 years. The only exception is the BON strain, known to behave atypically in several respects (Fazekas de St Groth & White, 1958*b*). In Table 3 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 twenty-four samples of BON virus were titrated,

a dozen each in two sessions. Samples of other strains tested at the same time (twenty-four 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 five replicates per dilution. The test in trays used twofold steps and eight 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

Table 3. *Comparative assay of infectivity in eggs and trays*

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

Strain	Number of comparisons	Tray/egg ratio*	± standard 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 five to six eggs and sixteen to twenty-four bits of allantois-on-shell at each level; the number of such comparisons is shown in the second column.

Table 4. *Comparative assay of infectivity in eggs and trays*

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

Strain	Number of comparisons	Tray/egg ratio*	± standard 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 five eggs at each level, and in twofold steps with eight bits of allantois-on-shell at each level; the number of such comparisons is shown in the second column.

the entries of Table 4 have about the same average error as those of Table 3. 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 $-0{\cdot}14$. This difference, however, was found in that particular set of titrations which gave an average difference of $-0{\cdot}25$ for fully infective virus which was disregarded in the construction of Table 3. For the same reason we have not included the results with the corresponding incomplete forms in Table 4.

As the average tray/egg ratios for infective 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

Table 5. *The degree of egg-to-egg variation in titrations of fully or partly infective virus*

Moran's test applied to the data of Tables 3 and 4.

	Average M-value		
Strain	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

material is the same as used for Tables 3 and 4; the method is Moran's test (1954*a*, *b*), applied earlier to the same problem in allantoic titrations (Fazekas de St Groth, 1955).

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 5, 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 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 paper (Fazekas de St Groth & White, 1958*b*). 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 ($r = +0{\cdot}73$; $P = 0{\cdot}01$) between the position of a particular strain in the tray/egg gradient (Tables 3 and 4) and the slope of its response curve. The same trend is evident if the data of the previous paper are analysed in the same way ($r = +0{\cdot}58$; $P \sim 0{\cdot}06$). 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 ten to twenty eggs only were compared.

The immediate consequences of this result will be discussed at the end of this paper; an experimental study of whether the connexion is based on casual relationship or represents two manifestations of some other mechanism, will be published separately. The investigation of another suggestive relationship, the correlation between the tray/egg gradient and the gradient of incomplete virus production (Fazekas de St Groth & Graham, 1954), which gives $r = -0{\cdot}65$ ($0{\cdot}05 > P > 0{\cdot}01$), is similarly postponed. The rest of this paper will deal with factors, mostly mentioned by other workers, which could cause differences like those appearing in Tables 3, 4 and 5. 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.

Toxic effect of plastic trays. 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 & 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, as would be the case if U.V.-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 (Fazekas de St Groth & White, 1958*a*), 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 4 hr. later. Then bits of tissue are added to each cup, and the infectivity end-points read 3 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 intracellular multiplication. In our experience the rate of inactivation at any temperature greatly depends on the medium in which the infective particles are suspended, and there are major differences in the behaviour of different strains.

A systematic study of this problem is under way, and a preliminary report is being prepared (Fazekas de St Groth, Graham & Stahl, 1959). For purposes of the present paper a smaller test was designed on the strains SW, CAM, FM1 and LEE. The two A-prime strains were included because they are closely 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, eight replicates per step), one set up as soon as the dilutions were made, the second after 4 hr. preincubation at 36° C. Table 6 gives the results.

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 3). Although the agreement is close even quantitatively, we do not wish to stress this aspect, as the end-points carry an error of about $\pm 0{\cdot}15$ $\log_{10}$ units, and are thus

Table 6. *Thermal inactivation in Standard Medium*

4 hr. at 36° C.

	Length of preincubation		
Strain	Nil	4 hr.	Difference
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.

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 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 were added, and the medium removed after a further 30 min.; the titre of these fluids shows the amount of virus *not taken in* by the cells.

The results (Table 7) 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 end-points were $10^{6\cdot28}$ and $10^{7\cdot20}$ for LEE and SW respectively. If the bit was removed at 135 min., and a fresh one added in its stead, $10^{5\cdot33}$ and $10^{6\cdot16}$ 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 only.

Effect of allantoic fluid. 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

Table 7. *Adsorption and viropexis in bits of allantois-on-shell*

	Titre of medium		
		After 135 min.	
Strain	Initially	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).

Standard Medium. However, as has already been shown (Fazekas de St Groth & White, 1958*a*), 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 furthest 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 1/1000 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 suffice: it revealed the situation at the time when 50–70 % of the inoculated particles had been bound irreversibly to cells.

Sets of whole eggs were inoculated allantoically with several known doses of virus, and after 2 hr. incubation they were 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 3 days' further incubation under standard conditions. One of the

Table 8. *The effect of subdividing the allantois after infection*

	Inoculum ($\log_{10}$ ID_{50})		Number of bits infected			
					Expected*	
Strain	Tray units	Egg units	Observed	Mean	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 $ID_{50} = 0{\cdot}693$ 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.

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 show whether the bits which received virus while still in the whole egg behaved like the native or the surviving allantois (Table 8).

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 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 8. To settle this question experimentally, we inoculated a known dose of virus into the allantoic cavity, and cut up the eggs 120 min. 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 9).

Table 9. *The distribution of infective particles on allantoic inoculation*

	Strip										
Zone	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
Airspace third	X	1	.	.	.	1	.	1	.	.	3
Airspace 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 hr. 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.

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. Similarly, there is no sign of syste*matic deviation along the meridional lines centring on the point of allantoic inoculation ($\chi^2_{(1)} = 0{\cdot}67$; $P = 0{\cdot}43$). 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 end-points in whole eggs.

https://doi.org/10.1017/S002217240003802X Published online by Cambridge University Press

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, end-points 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. More important, in our view, 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; within any of the strains it can be produced at will by choosing or creating appropriate environmental conditions. The next paper of the series will contain most of the technical information needed, and the application of these methods to the study of the critical step which governs susceptibility to infection will be resumed in later papers.

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.

https://doi.org/10.1017/S002217240003802X Published online by Cambridge University Press

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.

REFERENCES

FAZEKAS DE ST GROTH, S. (1955). *J. Hyg., Camb.*, **53**, 276.
FAZEKAS DE ST GROTH, S. & GRAHAM, D. M. (1954). *Brit. J. exp. Path.* **35**, 60.
FAZEKAS DE ST GROTH, S., GRAHAM, D. M. & STAHL, A. (1959). In preparation.
FAZEKAS DE ST GROTH, S. & WHITE, D. O. (1958*a*). *J. Hyg., Camb.*, **56**, 151.
FAZEKAS DE ST GROTH, S. & WHITE, D. O. (1958*b*). *J. Hyg., Camb.*, **56**, 523.
HORSFALL, F. L. (1954). *J. exp. Med.* **100**, 135.
HORSFALL, F. L. (1955). *J. exp. Med.* **102**, 441.
HORVÁTH, S. (1954). *Acta Microbiol. Ac. Sci. Hung.* **1**, 481.
LIU, O. C. & HENLE, W. (1953). *J. exp. Med.* **97**, 889.
MORAN, P. A. P. (1954*a*). *J. Hyg., Camb.*, **52**, 189.
MORAN, P. A. P. (1954*b*). *J. Hyg., Camb.*, **52**, 444.
RIGHTSEL, W. A., SCHULTZ, P., MUETHING, D. & MCLEAN, I. W. (1956). *J. Immunol.* **76**, 464.
WHITE, D. O. (1959). In preparation.

(*MS. received for publication* 17. IV. 58)