VARIATION OF HOST RESISTANCE TO INFLUENZA VIRUSES IN THE ALLANTOIS

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(With 4 figures in the Text)

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. Unlike previous papers of the series, this one deals with the reactions of both whole eggs and surviving bits. Otherwise the materials and methods are the same as used and described earlier.

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 connexion between the degree and variation of susceptibility was first recognized there, but no experimental details were given in the final report (Fazekas de St Groth & White, 1958*a*) 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 10–18 days only. Younger eggs

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https://doi.org/10.1017/S002217240001994X Published online by Cambridge University Press

are not suited to our technique; partly because at that age the chorioallantois is not yet fully developed and only few 6 × 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 allantoic fluid and the endothelial surface of the membrane is usually covered with a crust of urates, difficult to remove by simple rinsing.

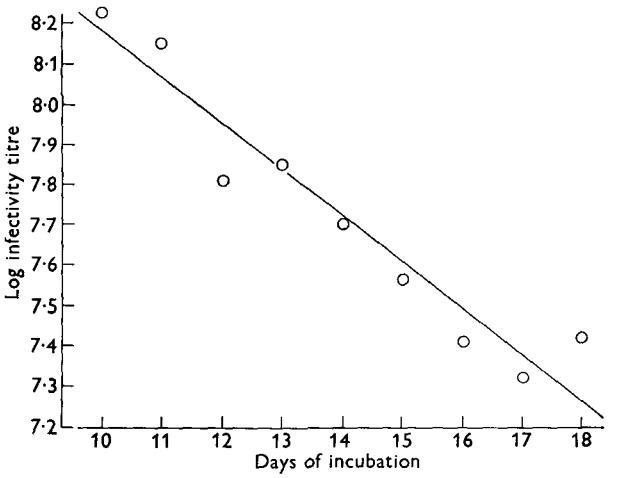

Fig. 1. Decrease of susceptibility with age of incubation. (Embryonated eggs were cut into bits of allantois-on-shell after a period of incubation at 38·4° C. shown on the abscissa. Each set of bits was challenged with the same doses of BEL virus. Several experiments of this kind were pooled by adjusting the observed infectivity titres to a common mean.)

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 (Fig. 1).

Susceptibility to infection is seen to decrease gradually with the age of the host tissue. The deviations from linearity are insignificant ($\chi^2_{(7)} = 5{\cdot}55$; $P \sim 0{\cdot}6$), and the log slope of the line comes to $-0{\cdot}114$, with an error of $\pm 0{\cdot}014$ 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 1).

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

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eggs older than 11 days have to be used, the observed infectivity titres may be standardized by adding 0·1 log units for each day over 11.

Variation of host resistance within age groups

The decline of susceptibility with age may reflect 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 resolving the problem.

Table 1. *Decrease of susceptibility with age of incubation*

	Days of incubation										
Strain	10	11	12	13	14	15	16	17	18	19	Slope ± S.E.
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.

When the variation between bits from 11-day eggs was studied under optimal conditions of maintenance (Fazekas de St Groth & White, 1958*b*), we 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 forty bits were cut from six to eight eggs at each of eight ages of incubation. They were orthogonally distributed over trays and inoculated with twofold dilutions of SW virus (five 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 Fig. 2.

All M-values referring to individual eggs fall within the normal range, and indicate that no significant 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 ($P \sim 0{\cdot}001$) than that of the younger group.

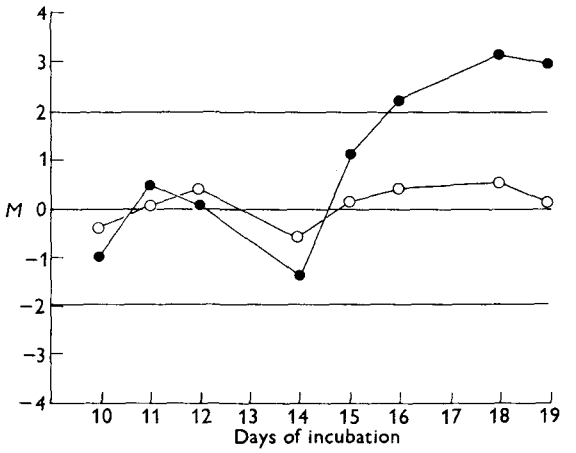

Fig. 2. The effect of age on intra- and inter-egg variation of susceptibility. (The abscissa gives the age of the embryo at the time when bits of allantois-on-shell were cut. The mean M-values calculated for individual eggs are shown by circles, the overall M-value for each age by dots.)

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 younger eggs?

Relationship of initial pH *and susceptibility of surviving bits.* A number of 15-day eggs were dipped, and the pH of the allantoic fluids determined, using a pH meter, with an accuracy of $\pm 0{\cdot}05$ 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 Fig. 3.

Clearly, there is no difference in susceptibility to infection once the allantois has been transferred to SM, 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 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.

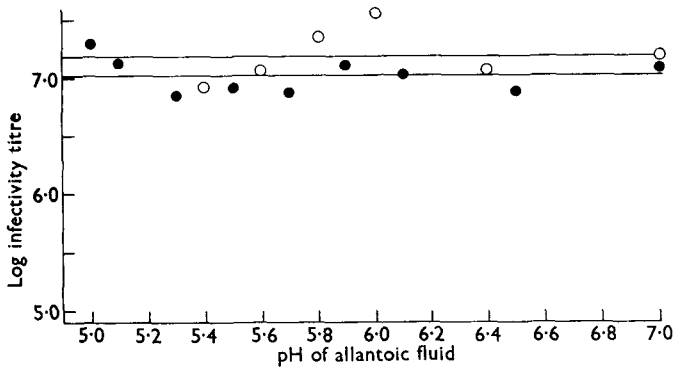

Fig. 3. The influence of hydrogen-ion concentration on the susceptibility of the surviving allantois. (The abscissa shows the pH of the allantoic fluid bathing the tissue *in ovo*; for the infectivity tests all bits were transferred to Standard Medium of pH 7·5. The dots show the average behaviour of 18-day eggs, the circles of 15-day eggs, when challenged with dilutions of BEL virus.)

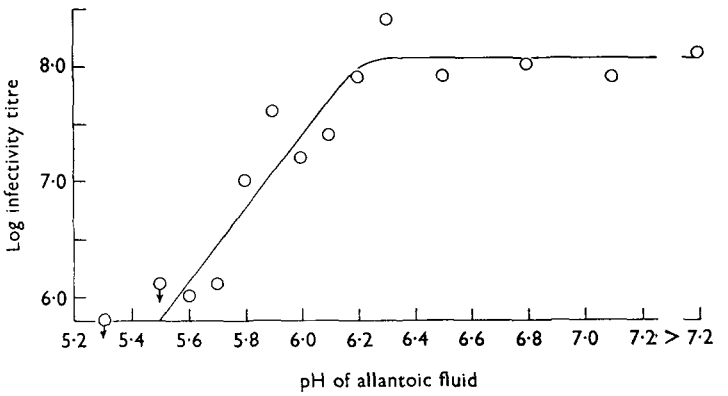

Fig. 4. The influence of allantoic pH on susceptibility of eggs. (15 day eggs, grouped according to pH of allantoic fluid shown on the abscissa were challenged with dilutions of BEL virus.)

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 Fig. 4.

The findings are in complete accord with Fauconnier's results on the PR 8 strain, and provide a good estimate of the rate at which susceptibility diminishes over the critical range of pH.

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The mean pH of allantoic fluid in our 11- and 12-day eggs is as low as $6{\cdot}8 \pm 0{\cdot}7$ and $6{\cdot}4 \pm 0{\cdot}6$, respectively. At each age level the pH is distributed 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 end-point, presumably negative because they failed to receive an infective particle; and those above the end-point, 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 2.

Table 2. *Hydrogen-ion concentration of allantoic fluid and susceptibility to infection*

Strain		Hydrogen-ion concentration of allantoic fluid									Mean	Variance
WSE	Above end-point	5·5, 6·1,	5·5, 6·2,	5·6, 7·4	5·7,	5·7,	5·8,	5·9,	5·9,	6·0,	5·94	0·26
	Below end-point	5·8, 8·5	6·2,	6·3,	6·6,	7·3,	7·6,	7·6,	7·7,	8·0,	7·16	0·78
PR8	Above end-point	5·1, 5·7,	5·4, 5·7,	5·4, 5·7,	5·5, 5·8,	5·5, 5·9,	5·5, 6·1,	5·5, 6·2,	5·6, 6·5	5·6,	5·69	0·11
	Below end-point	5·4, 6·3,	5·5, 6·3,	5·6, 6·5,	5·6, 6·8,	5·6, 6·9,	5·7, 7·1	5·9,	6·3,	6·3,	6·12	0·30

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

In both cases the difference between the mean pH of the two groups is highly significant. The negative eggs below the end-point have higher average pH, and show the normal scatter for eggs of that age. The negatives above the end-point are of uniformly low pH, which is also reflected in the smaller variance. Clearly, the latter represent a subpopulation 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 11 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 another paper (White, 1959) 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 (Fazekas de St Groth & White, 1958*c*). 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 was taken as a reasonable test period. The first set of eggs was cut up and placed in trays for one day,

Table 3. *The effect on susceptibility of prolonged maintenance in artificial medium*

	Drop in susceptibility			
	Test			
Strain	1	2	3	Mean ± S.E.
WSE	0·57	0·60		0·58 ± 0·015
PR 8	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
FM 1	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 hr. earlier and shaken in the meantime at 36° C. The figures give, in $\log_{10}$ units, the difference between the two infectivity titres.

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 3 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 hr. is suggested by the fact that some cups negative at 4 days can be shown to contain multiplying virus: if the medium is subinoculated into fresh bits, these will 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 hr. were infected with about 10^4 ID_{50}, and their medium was titrated 16 hr. later for its haemagglutinin content. Here one is testing in effect the number of cells capable of yielding virus (Table 4).

Table 4. *The effect on yield of prolonged maintenance in artificial medium*

	Yield		
Strain	Fresh bits	Pre-shaken bits	Difference
PR 8	1·20	0·72	−0·48
BEL	1·75	1·46	−0·29
CAM	1·09	0·85	−0·24
FM 1	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 hr. earlier and shaken in the meantime at 36° C. The figures give, in $\log_{10}$ units, the number of agglutinating doses yielded per bit.

The drop in total yield of haemagglutinin is much the same as the apparent drop of susceptibility was in Table 3. When considering the small differences between the two tests, it should be remembered that the data of Table 4 refer to the point in time 36 hr. after exposure to SM, while those of Table 3 to a point at least 12 hr. 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.

Variation of enhanced host resistance

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 PR 8 strain. The rest of the bits were first shaken for 24 hr. at 36° C., and then inoculated with an identical series of dilutions made up from a sister ampoule of PR 8 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 5).

The test done on fresh bits has a variance characteristic of optimal conditions, and is indistinguishable from the theoretical value (Fazekas de St Groth & White, 1958*b*, *c*). The pre-shaken group has a variance more than twice as high ($0{\cdot}01 > P > 0{\cdot}001$). It is clear then that whether susceptibility is decreased

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Table 5. *The variation of susceptibility after prolonged maintenance in artificial medium*

	Treatment of host tissue			Treatment of host tissue	
Egg	Fresh	Pre-shaken	Egg	Fresh	Pre-shaken
1	6·66	5·50	11	6·36	6·00
2	7·15	6·55	12	6·85	6·20
3	6·57	5·72	13	6·70	6·32
4	7·06	6·30	14	7·09	5·80
5	6·79	6·18	15	6·00	5·72
6	6·90	5·50	16	6·55	6·10
7	6·85	6·40	17	6·97	6·10
8	6·32	5·65	18	7·09	7·40
9	6·89	5·80	19	6·36	5·60
10	6·92	5·80	20	6·77	6·70
			Mean	6·74	6·07
			Variance	0·09	0·22

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.

The effect of deficient and unfavourable media

Inter-egg variation may be further enhanced if bits are pre-shaken in deficient media. Daniels, Eaton & Perry (1952) have observed, and we could readily confirm, that the chorioallantois 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 hr. 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. Thus, while glucose-free media would not be the method of choice when artificially lowering susceptibility, they furnish the means for increasing variation between eggs.

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 end-points 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 (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 paper of the series. 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

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

2. Under natural conditions susceptibility is a linear function of age between 10 and 18 days of incubation. The drop is 0·11 $\log_{10}$ unit per day for all strains of influenza tested.

3. 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*.

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

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

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(*MS. received for publication* 9. iv. 58)