STUDIES ON HOST-VIRUS INTERACTIONS IN THE CHICK EMBRYO–INFLUENZA VIRUS SYSTEM*

VII. Data Concerning the Significance of Infectivity Titration End-Points and the Separation of Clones at Limiting Dilutions

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(Received for publication, March 11, 1953)

The technics for determination of the 50 per cent infectivity end-points of individual preparations of influenza virus in the allantoic cavity of chick embryos are well established. The available data indicate that the values obtained by these means are reproducible within narrow limits, depending upon the scale of dilutions employed in the titrations as well as upon the number of embryos injected with each dilution (1, 2). Such titrations, which determine the number of infectious units in a given material, have been satisfactory as a means of controlling the potency of viral preparations intended for production of vaccines and other applied problems. In the evaluation of the mechanism of multiplication of influenza virus it is essential not only to determine the number of infectious units present in the infected materials at any particular stage of the developmental cycle but to translate the results into the number of infectious virus particles present. The question as to how many virus particles constitute one ID_{50} has not been resolved in a satisfactory manner as yet. Calculations based upon results of various studies with purified preparations of influenza virus have indicated that in the order of 10 virus particles represent one ID_{50} (3). However, it must be realized that in these experiments some of the virus particles may have lost their infectious property either during cultivation or the subsequent purification procedures. Furthermore, not every virus particle injected into the allantoic cavity will be adsorbed onto the entodermal cells of the allantois (4–6). In addition, not every adsorbed particle seems to "enter" the host cell and thus may not induce the reproductive process; *i.e.*, some virus particles apparently remain "superficially adsorbed" (7, 8). These considerations strongly suggest that "effective adsorption" of one infectious virus particle will initiate the infectious process which, after several cycles of adsorption, intracellular multiplication, and liberation, will result in the appear-

* The work described in this paper has been supported by a grant-in-aid from the National Institutes of Health, Public Health Service.

ance of detectable levels of hemagglutinins in the allantoic fluids of the injected embryos. The considerations also indicate that more than one virus particle will have to be injected allantoically in order to obtain this result. In line with this interpretation is the experience previously reported (2) that after injection of a seed preparation in limiting dilutions, *i.e.* in dilutions leading to infection of only a percentage of the injected embryos, the allantoic fluids of the infected eggs show high titers of hemagglutinins whereas the pooled negative fluids were found to harbor no virus as determined by passage to new chick embryos. This "all or none" response indicated that the yield obtained under these conditions represented a clone of virus obtained from "effective" adsorption of a single virus particle. Consequently the technic of passage in limiting dilutions has been used in order to isolate variants or mutants from natural populations of virus (9) or "recombinant" types of virus after mixed infections of host cells with 2 related but biologically distinct agents (10, 11).

In view of the implications of the various experiments referred to above a study of the quantitative aspects of infectivity titrations and the efficacy of separation of artifically mixed strains of virus by the limiting dilution technic seemed to be warranted. The results of these efforts, to be reported here, indicate that the conditions are not as clear-cut as previously assumed and great care has to be exercised in the interpretation of data got by employing the technic of limiting dilutions for isolation of pure strains of virus.

Methods and Materials

Viruses.—The PR8 strain of influenza A and the Lee strain of influenza B were used in these experiments. The seed viruses were prepared by inoculation of 11-day-old chick embryos by the allantoic route with 0.2 ml. of infected allantoic fluid in dilution 10^{-5} to 10^{-6}. After further incubation of the eggs for 48 hours at 36-37°C. the allantoic fluids were collected and the concentration of virus in the pools determined by infectivity titrations. For this purpose 10 chick embryos were each inoculated with 0.5 ml. of one of the 10-fold dilutions of the virus preparations and after further incubation of the embryos for 72 hours at 36-37°C. the allantoic fluids were harvested individually and tested for the presence of hemagglutinins. The 50 per cent infectivity end-point, based upon the results of the hemagglutination test, was calculated according to the method of Reed and Muench. The PR8 seeds contained between $10^{9.5}$ and $10^{10.3}$ ID_{50}/ml., the Lee preparations between $10^{8.5}$ and $10^{8.9}$ ID_{50}/ml.

Mixed Infections.—The PR8 and Lee seeds were mixed in such a manner that the mixtures contained approximately equal numbers of ID_{50} of each virus. In earlier experiments one mixture was prepared and this was subsequently diluted in 2-fold steps in the neighborhood of the predetermined 50 per cent infectivity end-point. Later on, when the results of mixed seed titrations were to be compared with those of the individual strains the virus preparations were diluted separately in 2-fold steps around the predetermined 50 per cent infectivity end-point and then mixed in equal volumes with the corresponding dilutions of the heterologous virus or with sterile broth. In the various experiments 15 to 30 11-day-old chick embryos were each inoculated allantoically with 0.5 ml. of one of the dilutions of either the mixed seed or the single strains. The inoculations were made through a hole drilled into the shell at the side of the egg, using a ¼ inch needle in order to assure that the total inoculum was placed into the allantoic cavity. The holes were sealed with nail polish, and the eggs were incubated further

at 36-37°C. for 72 hours with the exception of one experiment, to be described in the text, in which incubation was continued up to 96 hours. After chilling of the eggs in chipped ice the allantoic fluids were harvested individually. In earlier experiments separate pipettes were used to remove samples of each fluid and the forceps employed was carefully rinsed in alcohol and flamed after each harvest. However, in the light of the results obtained, it was thought possible that not all virus adhering to the forceps was destroyed. In order to avoid the use of a forceps, separate syringes and needles were employed for harvest of each individual allantoic fluid in later experiments. Since this change in procedure did not markedly affect the results to be described, the data have been combined for presentation. The individual fluids were distributed in the following manner: (*a*) 0.4 ml. into one tube; (*b*) 0.1 ml. into another tube; and (*c*) 2 to 3 ml. into a 3rd tube. To the tubes of series (*a*), 0.2 ml. of a 1 per cent suspension of chicken cells was added in order to test for the presence of hemagglutinins. These tubes were incubated at 4°C. until the red cells had settled and the results were evaluated in terms of the pattern of the red cells in the bottom of the tubes. In those cases in which positive results were obtained, 1 ml. of saline solution was added to the corresponding tubes of series (*b*), and these were then employed for the determination of the type of virus present by hemagglutination-inhibition tests. For this purpose, 0.2 ml. amounts each were distributed into 5 tubes. To the first was added 0.2 ml. of saline solution, to the second 0.2 ml. of dilute anti-PR8 serum, to the third 0.2 ml. of dilute anti-Lee, to the fourth 0.2 ml. of a mixture of anti-PR8 and anti-Lee and to the fifth 0.2 ml. of normal serum. The last control was not always included. The sera were derived from hyperimmunized rabbits and had been freed largely of non-specific inhibitor of hemagglutination by absorption with ultraviolet-inactivated, antigenically distinct virus of the homologous type in case of the anti-PR8 serum, or with similarly treated swine influenza virus in case of the anti-Lee serum. After removal of the virus by high-speed centrifugation the sera were heated to 60°C. for 20 minutes and used in dilution 1:16 to 1:64. After permitting the virus-serum mixtures to stand at room temperature for 30 to 60 minutes, 0.2 ml. amounts of a 1 per cent suspension of chicken red cells were added to all tubes and the degree of agglutination determined after further incubation of the test at 4°C. for 90 minutes. All tubes of series (*c*) were quick frozen in a dry ice–alcohol bath and then stored at —20°C. until the typing had been completed and embryos were available for passage. After rapid thawing, fluids revealing the presence of PR8 hemagglutinins were mixed with dilute, non-absorbed anti-PR8 serum (0.2 ml. fluid + 1 ml. anti-PR8 1:50); those exhibiting Lee agglutinins were mixed with non-absorbed anti-Lee in similar concentrations; and the negative fluids were passed without addition of any serum. The allantoic fluid–serum mixtures were incubated at 37°C. for 2 hours prior to inoculation of 0.2 ml. amounts into 5 chick embryos each. After incubation of the embryos at 36-37°C. for 72 hours the allantoic fluids were collected individually and tested for the presence of hemagglutinins. In case positive results were obtained the type of virus present was established by hemagglutination-inhibition tests according to the technic outlined above.

A few additional technical details are given in the tests now to be described.

EXPERIMENTAL

A number of titrations, using 2-fold steps in dilutions, were carried out in chick embryos with seeds consisting of mixtures of PR8 and Lee virus in equal concentrations according to preliminary infectivity assays of the individual preparations of the strains. The allantoic fluids of the inoculated eggs were collected separately after an incubation period of 72 hours and tested for the presence of hemagglutinins and, those found, were typed by hemagglutination-inhibition tests. In each instance the 50 per cent infectivity end-point based

upon all positive fluids regardless of type was calculated according to Reed and Muench and the distribution of "takes" in per cent was then plotted in relation to the number of ID_{50} injected. Since the data thus obtained in individual experiments were closely comparable, the results of 3 such tests, which were accompanied by similar titrations of the individual strains, were combined for this presentation. Furthermore, since the 50 per cent infectivity end-points did not vary by more than 0.05 $\log_{10}$ unit in the individual tests no corrections for this slight variation in titer appeared to be necessary. The curves shown in Fig. 1 represent the percentages of allantoic fluids positive within the range of about $\frac{1}{32}$ to approximately 32 ID_{50} of *mixed* seed virus injected. The individual points are based on the results obtained with 60 to 70 embryos in the middle range ($\frac{1}{4}$ to 8 ID_{50}) and on 30 to 40 in the remainder. Curve A reveals the percentage of all infections regardless of the type of virus found. It can be seen that an occasional infection occurred after injection of as little as $\frac{1}{32}$ ID_{50} and, on the other hand, not every embryo inoculated with 4 ID_{50} of mixed seed became infected and in other instances as seen in Fig. 2, not even after injection of 8 ID_{50}. Curve B represents the percentage of embryos revealing PR8 hemagglutinins. As one would expect from the composition of the seed ($\frac{1}{2}$ PR8), the curve, although similar in shape to curve A, intersected the 50 per cent infectivity level at about the 2 ID_{50} (mixed seed) mark. Again in accordance with the attempted equal mixture of the seed viruses the percentage curve of Lee infections (curve C) initially ran closely parallel with the PR8 curve; it reached a peak after inoculation of 2 ID_{50} of mixed seed, where slightly less than 50 per cent of the embryos gave direct evidence of infection with Lee virus. Thereafter it fell again and with an inoculum of 32 ID_{50} only 7 per cent of the eggs revealed Lee hemagglutinins. The failure to detect Lee virus on direct examination in fluids obtained after injection of the larger doses of the mixed seed is explainable on the basis of interference of the PR8 strain with influenza B virus (12). In the dilutions of seed used the growth cycle of influenza B is markedly longer than that of influenza A virus (6, 7) and, therefore, the latter is increasing more rapidly in quantity and thus has the opportunity to infect the majority of the remaining susceptible cells to the exclusion of the Lee virus in later cycles. Finally, curve D in Fig. 1 indicates the percentage of allantoic fluids which revealed dual infections on direct examination by hemagglutination-inhibition tests. The percentage was always low but even at the $\frac{1}{2}$ ID_{50} level one fluid was found to possess both hemagglutinins.

These experiments show then that on the whole the distribution of PR8 and Lee fluids was of the order to be expected. At the lower levels of infection the positive fluids were nearly equally distributed between PR8 and Lee and dual infections were uncommon. With an increase in the mixed inoculum the PR8 strain gained the upper hand resulting in partial interference with the propagation of the Lee strain.

A comparison of the percentage curves as obtained by single infection or by mixed infection but analysed for the individual components showed no significant differences as far as the PR8 strain is concerned. In this case the ID_{50} values used are based on the preliminary titrations of the *single* strains. As seen in Fig. 2 the individual points fitted closely to one curve. The points obtained in titrations of the Lee strain alone, likewise fell onto this curve. On the other hand, the rise in the influenza B curve derived after mixed infections prior to

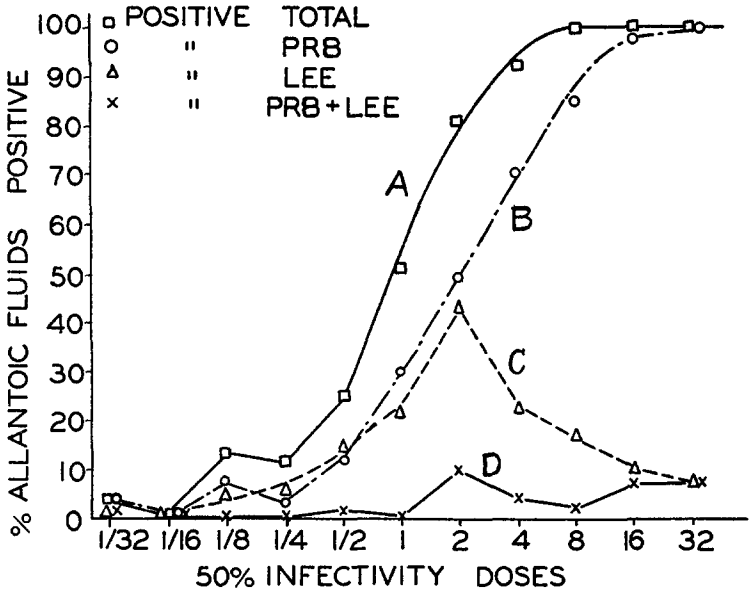

FIG. 1. The percentage of allantoic fluids revealing hemagglutinins 72 hours after inoculation of varying doses of mixed seed ($\frac{1}{32}$ to 32 ID_{50} in terms of the combined viruses). Curves A, total positive; B, positive for influenza A (PR8); C, positive for influenza B (Lee); and D, positive for influenza A and B.

reaching its peak and subsequent decline, referred to above, appeared to be somewhat slower, indicating that interference may be operating in some of the inoculated eggs already at the stage at which only $\frac{1}{2}$ to 1 ID_{50} of each virus was injected, or 1 to 2 ID_{50} of the mixed seed.

The various allantoic fluids collected individually in the titration experiments both of mixed or single seeds were passed separately to new embryos by the allantoic route under the following conditions. The negative fluids were injected without serum into 5 embryos each. If the allantoic fluids harvested after 72 hours revealed hemagglutinins, their type was determined. Fluids of the mixed infection series, revealing PR8 hemagglutinins, were passed after mixing with anti-PR8 serum, and the Lee fluids with anti-Lee serum. In case

hemagglutinins became detectable on passage under these conditions they also were typed by hemagglutination-inhibition tests. The over-all results of these tests are presented in Fig. 3. In section A the per cent infectivity curves are compared which were obtained with mixed seed before and after passage regardless of the type of virus found; *i.e.*, the increase in the percentage of positive results was due to passage of originally negative fluids. It is seen that even after injection of ¼ to ⅛ ID_{50} of *mixed* seed, fluids were obtained which on direct examination failed to show hemagglutinins but which yielded virus on

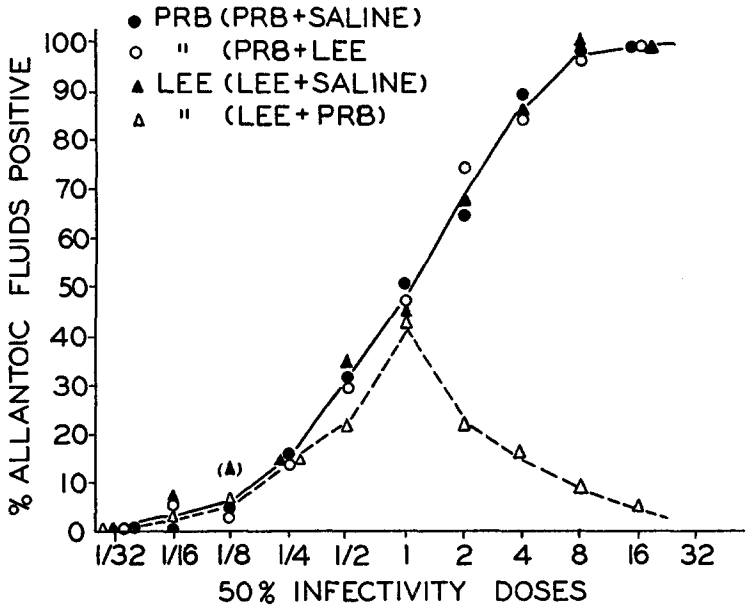

FIG. 2. The percentage of allantoic fluids revealing influenza A or B hemagglutinins 72 hours after inoculation of varying doses of mixed or single seeds (1/32 to 16 ID_{50} in terms of the individual strains).

passage. Section B summarizes the data obtained with the PR8 virus on passage of fluids derived from single strain or mixed seed infections. In the former the increased percentage of positive results was based upon passage of originally negative fluids, whereas in the latter case it was due in part to passage of negative fluids, in part to passage in the presence of anti-Lee serum of those originally yielding Lee hemagglutinins only. It can be seen that the increases were of the same order in both instances and, therefore, the results after single and mixed infections are practically indistinguishable. In section C the corresponding data for influenza B virus are given. The differences between the curves obtained by direct examination of single and mixed infections have been referred to above. After passage under the various conditions the initial curves ran

closely parallel but in the region of 1 to 4 ID_{50} (in terms of the Lee component) the curve obtained in the mixed seed series showed an "indentation" which was similar in all 3 individual experiments from which the figure was computed.

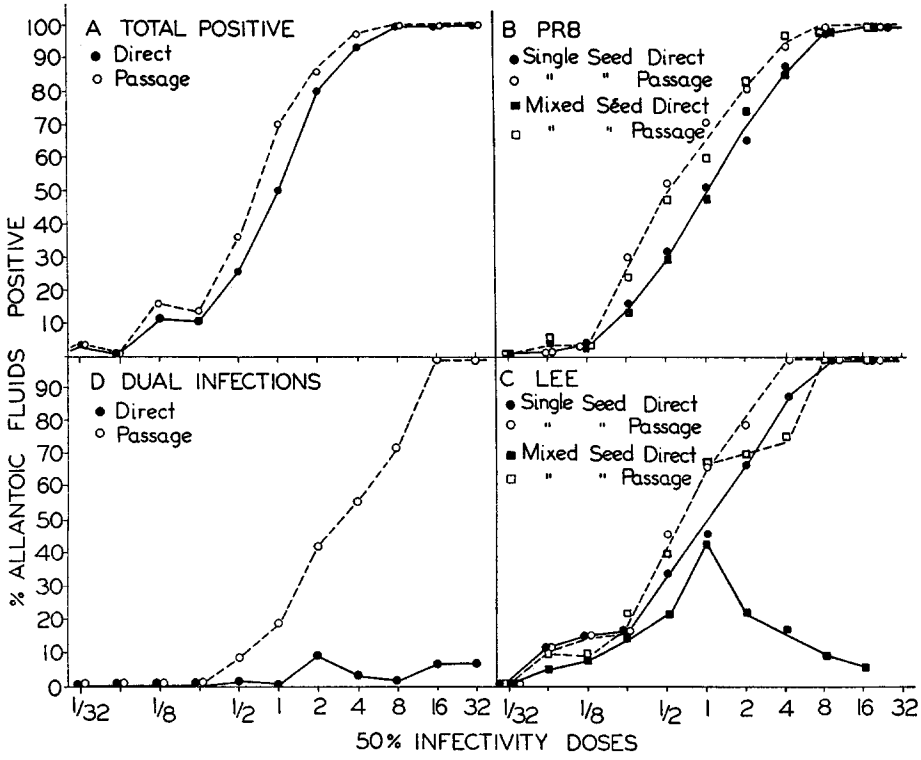

FIG. 3. Comparison of the percentage of allantoic fluids revealing the presence of virus 72 hours after inoculation with varying doses of mixed or single seeds, (*a*) on direct examination by hemagglutination; and (*b*) on additional passage (PR8 fluids in the presence of anti-PR8; Lee fluids in the presence of anti-Lee; and negative fluids without serum). In section A (total infections) and section D (dual infections) the ID_{50} values are expressed in terms of the mixed seed. In sections B and C (PR8 or Lee, respectively) the ID_{50} values are based on the individual strains.

It would seem that in the region where the interference effect became marked on direct examination, demonstration of the "excluded" virus by passage was not regularly successful. It is possible that whatever quantity of Lee virus had been produced in the early infectious cycles had become inactivated on prolonged incubation as employed for the original infectivity titrations and thus escaped detection. It is also conceivable that some of the infectivity might have been lost on storage of the fluids before passages were feasible, although this

consideration should hold for all preparations. Section D shows the curves of dual infections as determined by direct examination and following passage. The striking increase in the percentage of dual infections was largely due to the detection of the Lee virus in fluids originally showing only PR8 hemagglutinins in the preparations derived from inoculation of the mixed seed in greater concentration although in some instances Lee fluids yielded PR8 virus on passage under the appropriate conditions. The dent in the Lee curve referred to in section C was also reflected to some extent in the dual infection curve after passage.

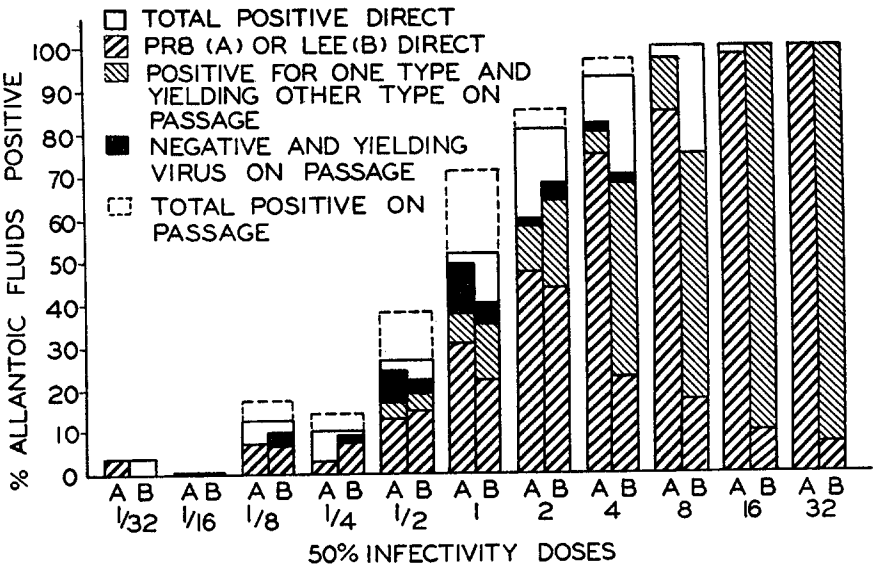

FIG. 4. Analysis of the data presented in Fig. 3 as to the percentages of fluids yielding influenza A or B viruses on direct examination by hemagglutination or indirectly on passage.

The data obtained on passage of the fluids collected from the mixed seed titrations are further analyzed in Fig. 4. The individual columns represent the percentage of allantoic fluids totally positive on direct examination at a given ID_{50} level of *mixed* seed. Each column is divided longitudinally to indicate on the left the percentage positive for influenza A and on the right that positive for influenza B. The heavy cross-hatched parts indicate the percentages positive upon direct examination. The thin cross-hatched sections represent the percentages of fluids which yielded evidence for one virus upon direct examination and which contained the other agent as shown by passage in the presence of the appropriate immune sera. Such "conversions" occurred even at the $\frac{1}{2}$ ID_{50} level. The finding that a fluid yielded Lee virus on direct examination and the PR8 strain only after passage was less common than the reverse, which

occurred with high frequency in the range where interference was noted. The solid black sections give the percentages of fluids originally negative but which induced viral multiplication on passage.

These results were in many respects unexpected. It had been thought that incubation of injected embryos for 72 hours certainly would permit completion of the infectious process. Yet at this stage some allantoic fluids were collected, which contained virus as shown by passage, but in insufficient concentration to cause hemagglutination on direct examination. This problem was further analyzed in the following experiment using only the PR8 strain as seed.

Thirty 10-day-old embryos were each injected allantoically with 0.5 ml. of virus diluted in 2-fold steps from $10^{-8.6}$ to $10^{-10.1}$. After incubation of the eggs for 72 hours 1 ml. of allantoic fluid was withdrawn from each embryo by separate needle and syringe. The opening in the shell was sealed and the eggs were incubated further for a total of 96 hours when again aliquots of the allantoic fluids were collected separately by individual pipettes. The 72 hour fluids were studied in the following ways: (*a*) by qualitative hemagglutination test; (*b*) the positive fluids were titrated for total content of hemagglutinins; and (*c*) the negative fluids were passed to fresh chick embryos, using 5 eggs per sample, to determine whether or not virus was present at levels not detectable by hemagglutination. The 96 hour fluids were (*a*) tested for the presence of hemagglutinins and (*b*) when found their concentration was determined.

The results are presented graphically in Fig. 5, which contains a separate chart for every dilution of seed originally injected. Each dot represents the results obtained with one fluid; the hemagglutinin titer observed after 72 hours is found on the abscissa, that noted in 96 hours on the ordinate. As can be seen if the hemagglutination test was positive in 72 hours the titers with one exception were high. The exceptional fluid was found in the $10^{-9.5}$ series with a titer of 1:12. On further incubation some of the titers increased slightly, others showed significant declines. In the technic used a 2-fold difference in titer must be considered insignificant and most of the rises fell into this category. On the other hand, some of the decreases in titer were in excess of one 2-fold step and these occurred mainly in the series of embryos injected with the larger quantities of seed virus. Such decreases in hemagglutinin titer on prolonged incubation have been observed previously (5). A total of 3 embryos were found which yielded negative fluids in 72 hours but high titers in 96. The exceptional fluid mentioned above with a titer of 1:12 in 72 hours remained at this level for the next 24 hours. The majority of negative fluids at 72 hours failed to reveal the presence of virus on passage. However, in 11 instances virus grew out on passage. Three of these had shown hemagglutinins by the 96th hour but the other 8 still failed to show measurable hemagglutination at that time. These fluids are represented in the charts by open circles and it can be seen that they were found in the groups injected with the last 4 dilutions of seed virus. These results indicate that (*a*) when hemagglutinins are found in 72 hours they are present, as a rule, in high titers. Experiments with Lee virus were similar in

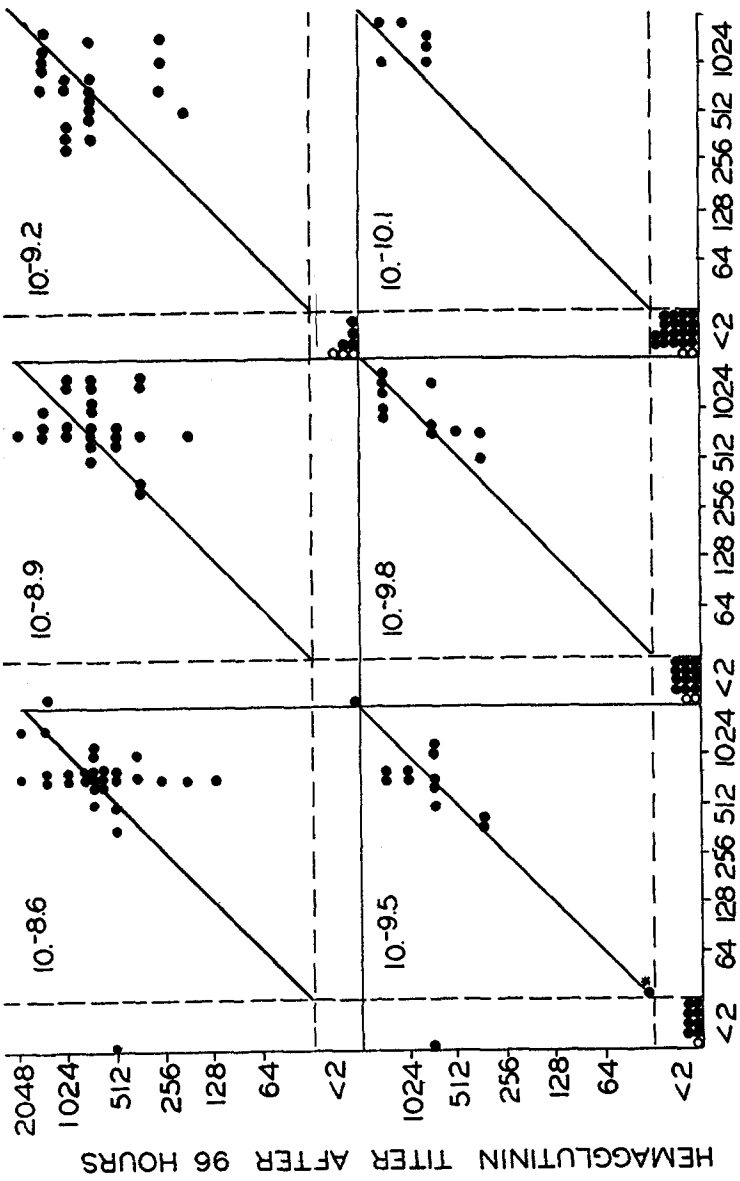

FIG. 5. Comparison of hemagglutinin titers obtained 72 hours after injection of varying doses of influenza A viruses with those found 96 hours after infection in the same embryos. The open circles denote allantoic fluids which failed to cause hemagglutination at 72 and 96 hours yet yielded virus on passage of the 72 hour sample.

nature except that a greater variation in levels was observed at the 72 hour period and titers as low as 1:8 were observed. (*b*) In a few instances hemagglutinins may become detectable in 96 hours only; and (*c*) a few allantoic fluids may harbor virus at 72 hours and yet no hemagglutinins can be detected in 96 hours. It is obvious from these results as well as those of the mixed seed series that infection of the allantoic cavity by influenza virus is not regularly an "all or none" response as previously assumed and that on occasion, some other factors play a role in the success of infection.

DISCUSSION

The data presented offer a problem in interpretation. Previous observations have indicated that the first infectious cycle of influenza A virus, *i.e.*, from adsorption of the agent onto the tissue up to liberation of 50 per cent of the new generations of virus requires in the order of 8 hours (4, 13), and up to 10 to 12 hours in the case of influenza B virus, depending on the concentration of virus injected (14). Subsequent cycles possibly may be shorter (13). Furthermore, it has been estimated that in one cycle between 50 and 100 ID_{50} of virus are liberated from the infected tissue into the allantoic fluid for every ID_{50} adsorbed onto susceptible cells (4, 6, 13). According to these observations an incubation period of 72 hours after inoculation of a minimal infectious dose should permit the completion of a sufficient number of infectious cycles, each involving 50 to 100 times more host cells than the preceding one, to result in liberation of detectable concentrations of hemagglutinins into the allantoic fluid, if not exhaustion of all the available susceptible host cells. Indeed, in the majority of embryos infected with such small doses of virus this appears to be the case and their allantoic fluids show high hemagglutinin titers 72 hours after infection. Yet, in the titrations of influenza virus near the 50 per cent infectivity end-point, completion of the infectious process in a few chick embryos was found to be delayed considerably, in that hemagglutinins on occasion became detectable in the allantoic fluid only on the 4th day of incubation. Even more confusing is the observation that on occasion allantoic fluids were obtained after incubation periods of 72 hours which failed to agglutinate red cells but revealed the presence of virus on passage and yet, on incubation of the same embryos for an additional 24 hours, still no demonstrable levels of hemagglutinins had developed. Titrations of mixed seeds composed of approximately equal numbers of ID_{50} of PR8 and Lee virus gave in many respects similar results. The fact that many of the allantoic fluids collected from such titrations revealed on direct examination evidence only of the presence of influenza A virus but on passage with anti-A serum revealed evidence also of the presence of the B strain is readily explainable on the basis of interference by the more rapidly multiplying PR8 virus with the more slowly propagating Lee strain (4, 12). However, the reverse, the presence of small amounts of influenza A virus

in fluids showing on direct examination only B-hemagglutinins, cannot be explained on this basis and, therefore, must be considered in the same light as the data referred to above obtained in single strain titrations. In evaluating these results factors concerning the technic, the viruses, and the host have to be reviewed.

On the technical side contamination of the allantoic fluids during harvest was suspected but in later experiments appeared to be definitely excluded since separate needles and syringes were employed for harvest of individual allantoic fluids. The possibility that the inocula on occasion were delivered not into the allantoic cavity but for instance into the yolk sac with a resulting delay in allantoic infection seems to be unlikely in view of the fact that injections were made from the side of the eggs, employing ¼ inch needles.

As to the virus, it was thought that the agents demonstrable on passage only may represent non-adsorbed seed virus but this suggestion can be disregarded on account of the quantitative aspects. Only a total of 1 ml. or less of the harvested allantoic fluid was used for passage, and yet several, if not all the 5 embryos injected became infected. Similar results were also obtained when the materials were diluted 10-fold prior to passage. The possibility of selection of slow growing variants appears to be excluded by the results of the passages.

Finally, host factors must be considered in the interpretation of these results. The possibility of a factor of host resistance has been suggested previously by Fazekas and Cairns (13) in an attempt to explain discrepancies between percentage infectivity curves actually observed within a narrow range of inocula of influenza A strains (0.01 to 30 ID_{50}) and a theoretical curve based on mathematical considerations. Normal allantoic fluid contains an inhibitor of hemagglutination which is largely destroyed by virus action but part of the inhibitor may remain permanently attached to the virus (15). There is no evidence that this inhibitor *prevents* infection (15–17) although it may conceivably *delay* adsorption. The inhibitor concentration in allantoic fluid may vary to some extent and thus in embryos with larger amounts of inhibitor a delay in propagation conceivably could result. However, the experiments with mixed seed in which on occasion influenza B virus was dominantly present but the A strain was demonstrated only on passage could not be explained by this possible effect of inhibitor. Individual embryos on the whole do not seem to show any differences in supporting viral multiplication since large inocula uniformly induce infection. It would seem to be more likely that individual entodermal cells lining the allantoic cavity may differ in their capacity to support viral multiplication. Thus, one could visualize that in certain cells "entry" of the virus, or the intracellular events leading to multiplication, or liberation of the new generations of virus may be delayed or may proceed at a slower rate. If that were the case, such cells may be referred to as "inefficient" for the purpose of this discussion, whereas those producing virus at the usual time and rate may be called "efficient" cells. This assumption could well explain the instances in which

influenza B virus outgrew the A strain. If on mixed infection a PR8 virus particle is adsorbed onto an "inefficient" cell, whereas a Lee particle enters an "efficient" cell the latter gains the advantage in time and quantity to infect additional susceptible cells, ultimately to the exclusion of the A strain. However, this view would not support the "delayed" or "arrested" propagation observed on occasion in embryos infected with small doses of a single strain.

As will be apparent, there is no generally satisfactory explanation for all the observed phenomena. Whatever the ultimate explanation may be, the data presented will have to be taken into account in considering the significance of infectivity titrations as well as of attempts at isolation of clones of virus derived from single virus particles.

In infectivity titrations of influenza virus in the allantoic cavity a 72 hour incubation period is employed as a rule. Yet, as shown in the present study, the infectious process may not go to completion in that period in every individual embryo used. By extending the incubation period to 96 hours a few additional allantoic fluids may show evidence of infection by the hemagglutination test. However, the increase in titer is relatively small, at most it is raised by 0.3 $\log_{10}$ unit and, therefore, the over-all results of virus titrations may not be significantly affected, particularly when comparisons are made between different preparations, with which a similar increase may be expected in all. On the other hand, when the quantitative aspects of the infectious process are under study, these results must be considered in the final evaluation.

It has been assumed in the past that the yield of virus in allantoic fluid injected with limiting dilutions of seed virus (in which only a percentage of the embryos show evidence of infection by hemagglutination tests) represents a clone derived from adsorption of one virus particle. Consequently this technic has been employed to separate naturally occurring variants or mutants, or "recombinant" types of virus after mixed infections. Although this may have been accomplished in some of the recorded studies (9–11), the technic is not absolutely safe as is indicated by the data presented. Attempts made in this laboratory to obtain mutual interchanges of properties between strains of influenza A virus by the methods employed in multiplicity reactivation (18) and subsequent separation of the yields by the limiting dilution technic indicated originally some success but on further passages the "recombinants" turned out to be mixtures of the "parent strains." These results actually lead to the analyses presented here.

SUMMARY

Equal mixtures of influenza A (PR8) and B (Lee) viruses, based on predetermined ID_{50} values of the individual preparations, were titrated in closely spaced steps near the 50 per cent infectivity end-point. Typing of the hemagglutinins found in the allantoic fluids after incubation of the eggs for 72 hours showed an approximately equal distribution between types A and B, when less

than 2 ID_{50} of the mixed seed had been injected. With larger inocula influenza A became dominant because it reproduces at a faster rate than the influenza B virus. While this result agreed with expectations, it was found that passage of the allantoic fluids revealing influenza A agglutinins in mixture with anti-A serum, and of B agglutinins with anti-B, yielded the heterologous virus in many instances, even when only one-half of an ID_{50} of mixed seed had been administered in the original titration. "Negative" fluids obtained from embryos injected with as little as one-eighth of an ID_{50} upon passage yielded on occasion virus of one or the other type.

Similar closely spaced titrations near the 50 per cent end-point of single strains (PR8) indicated that if hemagglutinins were found after incubation of 72 hours they were of high titer, as a rule. However, in some embryos hemagglutinins became detectable only between the 3rd and 4th days of incubation. In addition, negative allantoic fluids removed from embryos 72 hours after injection yielded on occasion virus on passage, yet no hemagglutinins were found in some of these eggs after an additional incubation period of 24 hours, or a total of 96 hours.

None of the possible explanations for these various observations, which have been discussed in detail, is completely satisfactory. However, the data indicate that in infectivity titrations the ID_{50} end-point obtained at 72 hours, or even after 96 hours, does not reflect the total amount of virus present in the material titrated. The data also denote that separation of variants, or mutants or "genetic recombinants" by the limiting dilution technic, although possible, does not represent an absolutely safe procedure.

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