III. DEVELOPMENT OF INFECTIVITY, HEMAGGLUTINATION, AND COMPLEMENT FIXATION ACTIVITIES DURING THE FIRST INFECTIOUS CYCLE

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Certain of the experiments reported in the preceding paper suggested that propagation of influenza virus in or on the cells lining the allantoic sac of the chick embryo can be divided into two major phases. According to this suggestion, the first phase would correspond to the production of some incomplete non-infectious forms or building blocks of the virus which, in the second phase, are converted or combined into fully active virus (1). It seemed possible that the material produced in the earlier phase might be endowed with some attributes of influenza virus other than infectivity. Consequently, attempts were made to study the development of hemagglutinins and of complement-fixing antigens. In order to be able to measure these properties during the early period after infection, i.e. during the first cycle, it was necessary to employ infective doses in the order of $10^8$ ID$_{50}$ of active virus.

It has been found in previous experiments that the selection of the seed is of prime importance (2). If the inocula were prepared under adverse conditions such as permitted the accumulation of inactive virus in the allantoic fluids, passage of these without dilution was found to result in partial interference with the propagation of the active virus by the non-infective material. On the other hand, if the allantoic fluids intended for seed were harvested when the infectivity just reached its peak, or shortly before that time, they could be used undiluted for passage to new chick embryos without the apparent intervention of interference. It is obvious that the present study required seed prepared under the latter conditions.

The experiments to be reported in this paper were conducted exclusively with the PR8 strain of influenza A virus. They show that production of both the virus (600S) and soluble (30S) complement fixation antigens (3) in the tissues can be demonstrated prior to the development of hemagglutinins which, in turn, precede the increase in infectivity. It will also be shown that the titer of hemagglutinins may increase in the allantoic fluid without a concomitant rise in infectivity and that under certain conditions suspensions of allantoic mem-

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brane may show marked titers of infectivity without showing a corresponding increase in development of hemagglutinins.

Methods and Materials

Seed was prepared by injecting 10-day-old chick embryos with 0.2 ml. of allantoic fluid containing the PR8 strain of influenza A diluted in broth to 10⁻⁷. After incubation of the eggs for 48 hours at 36-37°C., they were chilled and the allantoic fluids harvested aseptically. The seed thus obtained was stored at 4°C. for not more than 5 days before use. These allantoic fluids contained between 10⁹⁻¹⁰ and 10⁷⁻¹⁰ ID₅₀ per ml. at the day of the experiment, and agglutinated chicken red cells in dilution 1:2048 to 1:8192.

The technics used for the growth experiments and the methods employed for the preparation of inactive, interfering virus have been described in detail in the preceding papers (1, 4). In some of the experiments of the present studies, immune serum was injected subsequent to infection of the eggs. The sera were prepared as follows, according to the technic described by Hudson, Sigel, and Markham (5). Infectious allantoic fluid was injected intramuscularly (1 ml) and intraabdominally (3 ml) into adult chickens. They were bled 10 to 14 days later and the sera tested for their ability to inhibit hemagglutination. Those sera which in dilution 1:64 neutralized 256 or more units of hemagglutinin of PR8 or Lee virus, respectively, were pooled and used for the tests. They were injected allantoically in 0.2 ml. amounts either undiluted or diluted, 1:10, in buffered saline solution.

The methods employed for the assay of infectivity have been published (1, 4). Hemagglutination was measured by the pattern test. To 0.4 ml. of each of the serial twofold dilutions of the allantoic fluids or membrane suspensions, 0.2 ml. of a 1 per cent suspension of washed, fresh chicken erythrocytes was added. After shaking, the tubes were incubated at 4°C. until the red cells had settled, and the degree of agglutination was read according to the pattern formed at the bottom of the tubes (6). The initial dilution of the test material giving the last definitely positive reading (±) was taken as the end-point. In many instances, the hemagglutination test was run in duplicate or repeated a second time. In these cases, the average values were computed for inclusion in the figures and tables. With certain of the preparations, the test tubes were shaken and read again after settling of the red cells.

The complement fixation technic employed has been fully described (3). When a concentrated preparation of the virus or 600S antigens was desired, the allantoic fluids or the suspensions of allantoic membranes were subjected to high speed centrifugation at 20,000 R.P.M. for 20 minutes, and the sediments were resuspended in a small volume of buffered saline solution to effect an 8- to 16-fold concentration of the antigen. The supernatant fluids were tested for the presence of 30S or soluble antigen. No attempts were made to concentrate this fraction. To differentiate further between the two antigenically distinct fractions, two types of sera were employed in all tests: a human influenza A convalescent serum containing high levels of antibodies against both the virus and soluble antigens, and a human postvaccination serum which reacted with the virus but not with the soluble antigen. The last initial dilution of the preparations giving complete fixation of complement in the presence of antibody was considered as the end-point. Influenza B convalescent sera were used for control purposes.

EXPERIMENTAL

Divergence in the Titers of Infectivity, Hemagglutination, and Complement-Fixing Activity

In the first experiment to be reported, 12-day-old chick embryos were infected with 0.2 ml. of undiluted infectious allantoic fluid containing 10⁸⁻⁴ ID₅₀ of PR8
virus. The allantoic fluids and membranes from groups of six eggs each were harvested at hourly intervals beginning 1 hour after infection, and tested for infectivity, hemagglutinating capacity, and complement-fixing activity in the presence of specific human sera. The results are recorded in Fig. 1. On the left side are shown the data of the allantoic fluid series, on the right those obtained with the membrane preparations. In the allantoic fluid series, the constant period of infectivity amounted to 6 hours. Thereafter, the virus titer began to rise slowly. The allantoic fluids of eggs incubated for 10 to 24 hours yielded infectivity levels in excess of $10^{9.8}$ ID$_{50}$ per ml., which indicated that the seed did not contain significant amounts of interfering agents (2).

Hemagglutination tests with the same fluids showed that in agreement with earlier observations (7), some of the injected hemagglutinins remained unadsorbed, yielding a titer of about 1:12. The constant period before hemagglutination was observed to increase extended over 3 hours only, in contrast to the 6 hours before a rise in infectivity occurred. Following the 3 hour constant period, a slight but definite increase had occurred by the 4th hour, as determined by duplicate and repeat tests, and in the next 2 hours, the titer rose about 40-fold, whereas the infectivity remained unaltered. This unexpected result requires further study. At present, only this much may be said: The early increase of the hemagglutinins in the allantoic fluid requires both the living

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**Fig. 1.** The development of infectivity, hemagglutinating, and complement-fixing activities in allantoic fluids and membranes following infection with $10^{8.9}$ ID$_{50}$ of active virus.

ID$_{50}$ = 50 per cent infectivity doses per ml. Hem = hemagglutinin titer. CFV = complement-fixing titer of virus antigen; conc. = after eightfold concentration. CFS = complement-fixing titer of soluble antigen.
host and fully active virus, since neither the injection into living chick embryos of similar amounts of virus inactivated by ultraviolet irradiation, nor the inoculation of comparable concentrations of active virus into eggs which had been killed just prior to the test by intravenous injection of small amounts of normal allantoic fluid, led to a change in the titer of the non-adsorbed hemagglutinins during a 24 hour period of incubation.

The complement fixation tests with the straight allantoic fluids as antigens became positive after 6 hours. However, upon concentration by high speed centrifugation, a rise in the antigen titer (600S or virus antigen) became ap-

\[\text{TABLE I}\]

\textbf{The Formation of Soluble and Virus Complement-Fixing Antigens}

<table>
<thead>
<tr>
<th>Material</th>
<th>Serum</th>
<th>Time after injection, hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Allantoic fluid</strong></td>
<td><strong>Original</strong></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><strong>Supernate</strong></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><strong>Sediment conc. 16X</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Supernate</strong></td>
<td><strong>Postvaccination</strong></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><strong>Convalescent</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Sediment conc. 16X</strong></td>
<td><strong>Postvaccination</strong></td>
<td>±</td>
</tr>
<tr>
<td></td>
<td><strong>Convalescent</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Allantoic membrane</strong></td>
<td><strong>Original</strong></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><strong>Supernate</strong></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><strong>Sediment conc. 8X</strong></td>
<td>0</td>
</tr>
</tbody>
</table>

* Contains antibodies against the virus or 600S antigen only.
† Contains antibodies against the virus and soluble or 30S antigens.

parent at 4 hours concurrently with the development of hemagglutinins. The supernatant fluids were uniformly negative. Thus, no soluble or 30S antigen became demonstrable in the allantoic fluids during the period under test. Results of these titrations are included in Table I.

Tests with the suspensions of allantoic membranes are shown in the right-hand section of Fig. 1. The infectivity showed a constant period of 5 hours instead of the usual 4, as observed with more dilute inocula of virus (1). The titer rose sharply by the 6th hour, but only a slight additional increase was noted thereafter. The hemagglutination tests remained negative for 3 hours.
The 4 hour suspension gave irregular patterns of agglutination in the initial reading of the test. Upon shaking up of the test tubes and resettling of the cells, the titer increased somewhat and the usual patterns were observed. The titers continued to rise in the ensuing hours, although at a gradually decreasing rate.

The complement fixation test with the membrane preparations remained negative in the first 2 hours; in the 3rd hour it became positive, and increased in strength up to the 5th hour. No significant rise in titer was recorded thereafter. Both the virus and soluble antigens developed simultaneously, as shown in detail in Table I. Concentration of the virus antigen by high speed centrifugation increased the titer approximately eightfold, but did not lead to an earlier demonstration of antigen in contrast to the findings in the allantoic fluid.

This experiment showed the divergence between development of infectivity and hemagglutinating property, since the ratio between the infectivity and hemagglutinin titers changes during the experimental period. The number of ID₅₀ corresponding to 1 hemagglutinating unit for the various preparations of both the allantoic fluids and the membranes in the first 3 hours after infection amounts to about 10⁷, but it subsequently decreases to about 10⁶ ID₅₀ by the 6th hour.

All test employing undiluted allantoic fluid, i.e. between 10⁹.⁰ and 10⁹.⁶ ID₅₀ per dose, gave, in general, the same results as those recorded in Fig. 1. The periods elapsing before any changes in the various reactivities became measurable have been recorded in Table II. It is obvious that with the complexity of the experimental procedures and the technics used, minor variations may be expected. However, in two instances, the discrepancies appeared more pronounced. The constant period of infectivity in the membranes of Experiment 2 extended only over 3 hours. No explanation can be given for this exceptional result. The other irregularity concerns the complement fixation tests in Experiment 4, with concentrated allantoic fluid antigens which remained negative for 5 hours instead of 3. However, as can be seen in the membrane preparations of the same series, the appearance of both positive hemagglutination and complement fixation reactions was likewise somewhat delayed.

Dilution of the seed virus in tenfold steps led to the results shown in Fig. 2. This figure represents the results of several experiments employing each of the individual dilutions. As far as the allantoic fluid series are concerned, the infectivity showed constant periods of 5 to 6 hours, varying in the individual tests within this range. The levels obtained during the constant periods were spaced apart in conformity with the dilution of the seed. Hemagglutinins increased measurably during the experimental period only in those groups of eggs injected with undiluted or tenfold diluted seed virus. In each instance, the increase was noted before the infectivity commenced to rise. Finally,
TABLE II

Constant Periods in Allantoic Fluid and Membrane as Measured by Infectivity, Hemagglutination, and Complement Fixation

<table>
<thead>
<tr>
<th>IDu injected</th>
<th>Allantoic fluid</th>
<th>Allantoic membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infectivity</td>
<td>Hemagglutination</td>
</tr>
<tr>
<td></td>
<td>hrs.</td>
<td>hrs.</td>
</tr>
<tr>
<td>$10^9.00$</td>
<td>6</td>
<td>3-4</td>
</tr>
<tr>
<td>$10^9.20$</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>$10^9.26$</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$10^9.20$</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$10^9.10$</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>$10^9.03$</td>
<td>5-6</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 2. The effect of dilution of the seed virus upon development of the various properties of influenza virus in allantoic fluids and membranes.
complement fixation antigen became demonstrable under these conditions only when undiluted seed was employed.

The results of infectivity titrations with the suspensions of allantoic membranes showed that after injection of undiluted seed, a constant period of 5 hours was observed. If one-tenth or less than that amount was injected, it extended regularly over 4 hours. After the constant period, the titer rose to similar heights in 6 hours in the case of the $10^0$ and $10^{-1}$ inocula, and the results in the $10^{-2}$ series approached that level. However, when the seed was diluted further, the resulting curves ran roughly parallel. In the hemagglutination tests, positive results were obtained during the experimental period of 6 hours only when undiluted seed or 10- and 100-fold dilutions thereof were injected. The test became positive at the 4th, 5th, and 6th hours, in the order of the decreasing dilutions used for the inoculation. The titer obtained at the end of the experimental period with the $10^{-1}$ inoculum did not quite reach the level observed in the tests with undiluted seed, as was the case in the infectivity titrations. The development of positive complement fixation reactions with centrifugally concentrated materials (virus antigen), likewise, showed spacing at hourly intervals according to the dilution of seed virus used. However, the test regularly became positive 1 hour prior to the hemagglutination reaction. Again, the titers obtained in 5 to 6 hours following injection of $10^0$ or $10^{-1}$ dilutions of the seed were not identical, but the difference noted was less than that which might have been expected, considering the dilution factor of the seed. With seed diluted more than 100-fold, only the infectivity remained measurable, whereas the other properties no longer reached detectable levels of activity during the experimental period.

These observations seem to indicate that the $10^{-1}$ inoculum, or about $10^3$ ID$_{50}$, causes infection of all the susceptible cells, since ten times the dose of seed virus does not increase the yield of virus in the membranes. The data of the hemagglutination and complement fixation tests, on the other hand, are not quite in agreement with this conclusion. However, discrepancies between the infectivity and the other properties studied have been noted also in the allantoic fluid series, as outlined above, which indicate a certain independence of the various activities of each other.

**The Effect of Homologous and Heterologous Irradiated Virus upon the Growth Curves**

As has been pointed out in the preceding publication (1), a secondary injection of virus inactivated by ultraviolet irradiation following the infection of the eggs produced marked inhibition of propagation of virus in the tissues in case the homologous irradiated agent was used, and only a relatively slight effect in the case of the heterologous type. The inhibition by homologous irradiated virus was the more pronounced, the smaller the dose of active virus used for infection. On the other hand, when about $10^0$ ID$_{50}$ of active virus was used for
infection, the secondary injection of homologous inhibitor failed to produce a distinct inhibitory effect; the constant period was only slightly prolonged, and the infectivity levels reached were of the order of those attained in the groups of eggs treated with the heterologous irradiated agent. The results of hemagglutination and complement fixation tests at this level of infecting dose, likewise, were closely similar, regardless of whether homologous or heterologous irradiated virus was injected following infection. They were slightly inferior to the levels obtained in the control groups not treated with irradiated virus.

The Effect of Immune Serum upon the Growth Curves

It is generally accepted that antibody cannot reach virus particles harbored by the host cells. In the type of experiments reported here, it was considered possible that immune serum injected into the allantoic cavity of the infected eggs would neutralize a large part of the non-adsorbed seed virus, and also part of any seed virus that might be superficially adsorbed onto the membranes and not be participating in propagation.

In preliminary experiments, it was established that antibodies against influenza virus are not adsorbed onto the membranes of normal chick embryos, nor are they resorbed into the bloodstream to a measurable extent during experimental periods extending over 6 to 8 hours. When normal eggs were injected by the allantoic route with immune serum, and the membranes harvested by the usual technic several hours later, it could be shown that the resulting tissue suspensions were devoid of any neutralizing activity as far as infectivity was concerned, and inhibition of hemagglutination did not exceed that exerted by suspension of normal membranes.

Injection of anti-PR8 sera into eggs one-half hour following infection with about 10⁹ ID₅₀ of PR8 virus failed to neutralize all the free, non-adsorbed virus in the allantoic fluid, even when 0.2 ml. of undiluted serum was employed. As seen in Fig. 3, which summarizes the composite results of four experiments of this kind, the infectivity decreased by more than 99 per cent, and only a slight rise was noted in the 6th hour of incubation. The hemagglutination and complement fixation reactions remained negative throughout the experimental period.

The tests with the allantoic membranes showed a decrease in infectivity to approximately 0.1 per cent of that of the controls in the first 3 hours. The titer then increased very sharply, and by the 5th hour, matched the levels noted in the groups not treated with serum. Thus, evidence has been obtained that a large part of the adsorbed seed virus can be neutralized without affecting significantly the ultimate yield of virus in the tissues. With regard to the hemagglutination tests, the serum prevented apparently the development of this activity from reaching detectable levels in the membranes during the entire period under study, in spite of the fact that the infectivity titers in both
groups attained similar heights at the end of the 6 hour incubation. High speed centrifugation of these materials and resuspension of the sediments in one-eighth of the original volume, likewise, failed to reveal hemagglutinins. No explanation can be given at present for this paradoxical finding. As to the complement fixation tests, only the 600S or virus-antigen was measured in these series after concentration by high speed centrifugation. It developed in gradually increasing amounts, but its appearance was somewhat delayed and the titers were reduced as compared with the controls. If one-tenth the amount of homologous immune serum was injected following infection, the general appearance of the growth curves in the tissues was similar to that described for the tests with undiluted serum, except for the fact that under these conditions, small amounts of hemagglutinin became demonstrable at the end of the 6 hour incubation period. Using undiluted anti-Lee instead of anti-PR8 serum, the results resembled those of the control series except for slight reductions in the various activities in the allantoic fluids, caused most likely by non-specific

Fig. 3. The effect of injection of homologous antisera subsequent to infection with about $10^9$ ID$_{50}$ upon the development of the various properties of influenza virus in allantoic fluids and membranes.
inhibitors present in the serum (8). The allantoic membrane titrations were not affected by this property of the serum.

Similar experiments were conducted with more dilute seed and undiluted homologous immune serum. The essential results are recorded in Fig. 4. In this figure, the infectivity titers obtained during the constant periods in the allantoic fluids and membranes with and without secondary administration of serum are plotted according to the dilution of seed virus used for the primary injection. It also includes a comparison of the results recorded with membranes at the end of the experimental period (6 to 8 hours after infection).

Fig. 4. The effect of homologous immune serum upon the amount of virus in chick embryos infected with decreasing amounts of virus as measured: (a) in the allantoic fluid, (b) the allantoic membrane during the constant periods, and (c) in the tissue at the end of the experimental period.

It can be seen that in the allantoic fluids, injection of serum resulted in a similar rate of reduction in infectivity when undiluted or tenfold diluted seed virus was injected. With further dilution of the inoculum, most or all of the virus activity was neutralized.

The reduction of infectivity in the membrane during the constant period, which never was less than 3 hours, proceeded at a similar rate, regardless of the dilution of the seed. This difference in the neutralization of free and adsorbed virus may be comparable to the difference between the rates of adsorption of virus onto the allantoic sac and onto red cells, which has been discussed in a preceding paper (4). The non-adsorbed virus and the antibodies are intermixed in the same menstrum, allantoic fluid, and therefore, the chances of their
colliding are greater, whereas neutralization of the adsorbed virus can take place only at the fluid-tissue interface.

Inspection of the data obtained with membranes at the end of the experimental period shows that only in the case of undiluted seed, both control and serum groups reached similar levels of infectivity; i.e., the virus grew out to the same extent in conjunction with the host cells in both series. With tenfold dilution of the seed, only a slight reduction was noted, but on further decrease in the dose \(10^{-3}\) or less, the titers attained in the serum groups amounted to less than 1 per cent of those encountered in the controls. Essentially similar results were obtained if one-tenth of the amount of serum were used in these experiments. However, only with dilution of the seed to \(10^{-4}\) or less, was all of the non-adsorbed virus neutralized in the allantoic fluid.

**DISCUSSION**

Studies on the propagation of influenza virus in the allantoic membrane, using large doses of seed virus, require particular precautions. It has been shown in the past that, under certain conditions, apparently enough inactive virus may accumulate in the allantoic fluids of the infected eggs to cause interference with the multiplication of the active virus on passage (2). For example, if eggs are seeded with large amounts of virus and permitted to incubate for extended periods of time, or if the harvested allantoic fluids are stored under adverse conditions, or frozen and thawed repeatedly, one finds that upon subculture in eggs dilute seed will produce a larger yield of virus than a more concentrated inoculum. The conditions can be reproduced by partially inactivating optimally grown seed by ultraviolet irradiation. On the other hand, if allantoic fluid is harvested at the time the infectivity has just reached its peak, i.e., 48 hours after inoculation of seed diluted to \(10^{-5}\) to \(10^{-4}\), for instance, then passage of the undiluted allantoic fluid will result in infectivity titers equal to those obtained in subcultures of dilute seed.

Subsequent studies by von Magnus (9) have shown that factors other than inactivation of virus may play a role in the interfering activity of certain seeds. Serial passage of undiluted allantoic fluid at 18 to 24 hour intervals, starting with optimally produced seed, will lead after two to three transfers to inferior results, comparable to those described in the above paragraph. Although this type of experiment does not exclude the possibility of inactivation of some of the non-adsorbed seed virus during the incubation period, interpretation of the data is complicated by the demonstration in such allantoic fluids of particles smaller in size than the active virus, which apparently are endowed with hemagglutinating, and possibly interfering activities (10). It has been suggested that these particles constitute "precursors" of the virus.

In the preceding paper (1), indirect evidence has been presented which may be taken to indicate that the formation of virus occurs in at least two phases;
possibly during the first, apparently non-infectious, immature forms or building blocks of virus are produced which, in the second phase, are converted or combined into fully active virus. The data recorded in the present study seem to afford more direct evidence for this suggestion.

The growth curve experiments with suspensions of allantoic membranes, using $10^9 \text{ID}_{50}$ of virus as inoculum, show that the various properties of influenza virus become detectable or reveal increments in the following order: The complement-fixing activity against both the virus or $60\text{S}$ and the soluble or $30\text{S}$ antigens reaches measurable levels in 3 hours, the hemagglutinating capacity in 4 hours, and infectivity begins to increase in 5 to 6 hours. Concentration of the active materials by high speed centrifugation fails to change the time relationships: there still remain 2 hours during which no record can be obtained concerning the activities of the virus in the tissues. It must be emphasized, however, that both the complement fixation and the hemmagglutination reactions require accumulation of relatively large quantities of the active materials before they become measurable. These experiments indicate then that the hypothetical "immature forms" or building blocks of the virus are already endowed with complement-fixing and hemagglutinating activities.\(^1\)

Similar differences between the infectivity, on the one hand, and the hemagglutinating and complement-fixing capacities, on the other, are noted in the curves obtained with the allantoic fluids. A distinct rise in hemagglutinins accompanied by complement-fixing activity occurs regularly 4 hours after infection; i.e., 1 to 2 hours before any changes in infectivity become apparent. Although there is no proof that the data obtained with the membranes and fluids are based upon the same basic phenomenon, it seems likely that they are.

The non-infectious hemagglutinating and complement-fixing materials are considered, at present, as immature virus particles. However, the available evidence is insufficient, as yet, to regard this problem as closed. The experiments presented by Gard and von Magnus (10), and those recorded in the preceding paper (1) favor the explanation just outlined, but the possibility that this material derives from the injected seed virus has not been definitely refuted. It is improbable that the early increase in hemagglutinins in the allantoic fluids is caused by elution or breakdown of the seed virus, since in either case there should be concurrent changes in the infectivity. Furthermore, only injection of fully active virus produces these results, whereas injection of irradiated virus, in which the elution mechanism apparently is unaltered (11), does not lead to changes in the titer of residual non-adsorbed hemagglutinins (7). On the other hand, the fact that injection of immune serum after infection appears to prevent early formation of hemagglutinins in the membranes in spite of the development of infectivity titers comparable to those of the controls not treated with

\(^1\) Recent experiments indicated that the interfering capacity of these fluids after dialysis and ultraviolet irradiation increased simultaneously with the hemagglutinins.
serum, may be taken as support for the second alternative. However, these latter data may be based on other principles, possibly of a serological nature which will be discussed further below.

Another point concerning the constant period of infectivity in the tissue requires discussion. It has been suggested that some of the seed virus is “superficially adsorbed” onto the tissue; i.e., it probably is not participating in the propagative process (4). Indeed, much of the adsorbed seed virus remains accessible to the action of neutralizing antibody. Injection of specific immune sera following infection reduces the infectivity of the membranes by a factor of about 1000 and shortens the constant periods, but never to less than 3 hours. The “superficially adsorbed” virus sets a base line beyond which propagation of the “infecting virus” must proceed in order to become demonstrable as an increment in titer. The presented evidence suggests that with an inoculum of 10⁴ ID₉₀ of active virus, the maximal number of host cells becomes infected, since with larger doses the ultimate yield of virus in the tissue at the end of the experimental period does not increase. Thus, the base line in the case of an inoculum of 10⁴ ID₉₀ is approximately tenfold higher and, as a result, the constant period extends over 5 hours instead of 4.

The use of immune serum in these experiments has possible disadvantages. It is conceivable that multivalent antibodies attached to what is considered superficially adsorbed virus may retain free specific antibody groupings if the amount of seed virus is insufficient for their saturation. Upon emulsification of the tissues, these free groupings may then interact with some of the virus thus liberated which was previously inaccessible to the antibodies. Indeed, the increasing difference in the infectivity titers of the membranes in the control and serum groups at the end of the first cycle with the decrease in the amount of seed virus used for infection strongly suggests that such a carry-over of antibody activity may occur. The presented data offer then only a qualitative answer, namely, that some of the seed virus is superficially adsorbed onto the tissue, but they do not reflect what quantity of the seed virus found in membrane suspensions during the constant period falls into that category. Furthermore, it remains to be investigated whether the phenomenon of partial reactivation of neutral virus-antibody mixtures by dilution, as described by Taylor (12), may not affect the interpretation of some of the recorded data since the materials were highly diluted for the infectivity titration and by injection in the allantoic cavity. A difference between the reactivation of the infective and hemagglutinating properties could conceivably contribute toward an explanation of the paradoxical finding of a high increase in infectivity in the apparent absence of development of hemagglutinins in the tissue when immune serum is injected into the allantoic cavity after infection. From these various considerations it is obvious that the recorded experiments have established certain facts, but their explanation must await further experimentation.
SUMMARY

In agreement with earlier observations the infectivity titer in the allantoic fluids of chick embryos injected with influenza A virus remains constant for 5 to 6 hours before an increase in this activity can be noted. In contrast, the titers of hemagglutinin and complement-fixing antigen (virus antigen) have already begun to rise after 3 hours. The origin of the hemagglutinating and complement-fixing but non-infectious material is still obscure.

In the allantoic membrane development of both the soluble and virus antigens can be demonstrated after the 2nd hour of incubation and 1 hour prior to an increase in hemagglutinins and 2 hours prior to a rise in infectivity. Thus there remain the first 2 hours during which no record of virus activity in the tissues can be obtained.

Similar relationships are noted on dilution of the seed both in the allantoic fluids and membranes as long as the various properties reach measurable levels during the experimental period of one infectious cycle.

Injection of high titered immune serum following infection with about 10^9 ID_{50} reduces the amount of demonstrable seed virus in the allantoic fluid and membrane without significantly affecting propagation of the agent in the tissues as measured by infectivity titrations. The production of hemagglutinins appears markedly reduced under these conditions whereas formation of complement-fixing antigen is only slightly delayed and decreased.

No definite explanations for the various discrepancies between the infectivity and hemagglutination can be given at present.

Note Added to Proof.—In a recent paper which was received only after submission of the three foregoing manuscripts for publication, Hoyle (13) reported several observations in agreement with some of those recorded in the present studies. He was unable to detect more than 1 per cent of the adsorbed seed virus in the tissues of the allantoic sac of chick embryos as measured by hemagglutination and infectivity tests (see Paper I of this series (4)), and demonstrated that the development of one of the complement-fixing antigens, the soluble antigen, preceded the formation of hemagglutinin in the allantoic membrane, a point discussed, among others, in the above paper.

BIBLIOGRAPHY