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

IV. The Role of Inhibitors of Hemagglutination in the Evaluation of Viral Multiplication

By OSCAR C. LIU, M.D., AND WERNER HENLE, M.D.

(From the Division of Virology, the Department of Public Health and Preventive Medicine, School of Medicine, University of Pennsylvania and The Children's Hospital of Philadelphia, Philadelphia)

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Facts have been presented recently which indicate that propagation of influenza virus in the allantoic membrane of the chick embryo occurs in stages. They were derived from investigation along two lines: (*a*) the inhibitory effect upon viral multiplication of secondary injections of homologous virus inactivated by ultraviolet light; and (*b*) the order of appearance in the infected tissues of various properties associated with influenza virus. In the first series of experiments evidence has been presented that propagation of the virus occurs in two steps at least. The homologous irradiated virus appears to effect the first stage, the production of presumably incomplete, non-infectious virus, which subsequently, in the second step, is converted into infectious virus. This conversion apparently is not inhibited by the homologous inactivated virus (1). In the second approach the first growth cycle following injection of large doses of active seed virus was analyzed. It appeared that the formation of both the soluble and virus-bound complement-fixing antigens preceded that of the hemagglutinating capacity (2, 3), and this, in turn, the increase in infectivity (3), thus suggesting 3 major steps in virus multiplication. The data published by Schlesinger (4) on the development of "incomplete" influenza virus in the brain of mice are in line with this interpretation. These observations require further analysis in the light of the numerous reports concerning substances interfering with hemagglutination.

Inhibitors of hemagglutination, also referred to as receptor substances, have been found in extracts of red cells (5–7) and animal tissues (4, 5, 8, 9), in normal animal sera (10–12), tears (13), saliva (14), urine (15), mucin derived from various animal sources (16), egg white (17–19), and also in allantoic fluid (19–22) and membranes (20). These inhibitors seem to be devoid of any significant effect upon the infectivity

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of the virus (5, 8, 18, 19, 23). All indications at present point to a mucoprotein nature of these substances (6, 19, 24–26). They are affected by periodate (24) and trypsin (19) and resist, in many instances, heating at 70°C. or higher (5, 8, 19, 21). The virus apparently possesses enzyme activities which permit destruction of red cell receptors and inhibitors (27). This property of the virus can largely be destroyed by heating of virus to 50 to 56°C., depending on the strain, without seriously affecting the hemagglutinating activity. An enzyme of similar nature has been found in culture filtrates of *V. cholerae* (28) which has been called receptor-destroying enzyme or RDE.

The inhibitor in normal allantoic fluid has been studied extensively by Svedmyr (21, 23, 26) and Hardy and Horsfall (19), mainly by the inhibition of hemagglutination technic. The inhibitor increases in quantity from the 9th to 15th day of embryonic development. It is only partially sedimentable in the high speed centrifuge. It combines with the virus which thus is prevented from being adsorbed onto red blood cells. Upon incubation at 37°C. the inhibitor is gradually destroyed by virus action and as a result hemagglutinins become free and measurable again. Similarly, *in vivo*, the inhibitor in allantoic fluid decreases gradually after infection. But the inhibitor is never completely destroyed and it has been reported that some inhibitor remains permanently attached to the virus (19).

It is obvious from this resumé (more detailed reviews on this subject have been published recently (29, 30)) that inhibitors of hemagglutination in allantoic fluid as well as in membrane must be considered in analyses of various phases of the host-virus interactions in the chick embryo–influenza virus system. In the studies to be reported the inhibitory properties of these materials derived from *in vivo*, or growth curve, experiments (1, 3) were compared with those obtained from *in vitro* tests in which virus was mixed with normal allantoic fluid or membrane suspension and incubated at 37°C. for various periods of time. In both types of experiments virus was used in the infective state as well as after inactivation by ultraviolet light so that it no longer was able to increase in quantity but retained its interfering and hemagglutinating capacities (31). Three major problems were explored. The first concerned the effect of inhibitor upon the hemagglutinin titer of the active or irradiated virus added to the systems, the changes occurring during the experimental periods, and the results of various manipulations. In the second set of experiments attempts were made to determine changes in the concentration of inhibitor as a result of virus action. The various materials derived from *in vivo* and *in vitro* experiments were heated to 70°C. in order to destroy the hemagglutinins present, and thereafter, assayed for inhibition of hemagglutination against test virus preparations. Finally, the third approach involved the adsorption onto red cells of hemagglutinins from the various materials and the subsequent elution therefrom.

Methods and Materials

Virus.—The PR8 strain of influenza A virus was used throughout these studies. The virus was prepared by inoculation of 10 to 12 day old chick embryos by the allantoic route with

0.2 ml. of infected allantoic fluid diluted in beef heart infusion broth to 10^{-6} or 10^{-7}. After incubation of the infected eggs for 48 hours at 36 to 37°C. the allantoic fluids were collected. For *in vivo* or *in vitro* experiments, in which seed was required both in the infective state and inactivated by ultraviolet irradiation, the allantoic fluids were dialyzed for 16 to 36 hours at 4°C. against 20 volumes of phosphate buffered saline solution at pH 7.0. Portions of the dialyzed fluids were irradiated by ultraviolet light for a period sufficient to destroy the reproducing capacity of the virus but not the interfering and hemagglutinating activities as previously described (31). In the hemagglutination-inhibition tests the allantoic fluids were used native or after heating to 56°C. for 30 minutes (12).

In Vivo Experiments (Growth Curves).—These were conducted, as previously described (3), by injecting 0.2 to 0.5 ml. of undiluted seed virus into adequate numbers of 13 to 14 day old chick embryos. Allantoic fluids and membranes were collected from groups of 4 to 6 eggs at hourly intervals and the suspensions of membranes were prepared, sometimes after storage for a few days at −20°C., according to the established technics (1, 3). Aliquots of the various materials were (*a*) left untreated; (*b*) heated at 56°C. for 30 minutes to inactivate their eluting and inhibitor-destroying activities; and (*c*) heated at 70°C. for 30 minutes to destroy their hemagglutinating activity but not their inhibitory properties. The latter preparations were cleared of precipitates by centrifugation which did not lower their inhibitory activity. In all experiments allantoic fluids and membranes were collected from uninoculated eggs for control purposes and treated in a manner identical with that used for the infected preparations. All preparations were stored at 4°C. for short periods of time until the various tests could be performed, usually not longer than 24 hours.

In Vitro Experiments.—Allantoic fluids and membranes were collected from 13 to 14 day old normal embryos and the tissues were suspended in saline solution in a manner similar to that used for the infected allantoic membranes. Active or irradiated virus was added to aliquots of these materials in proportions similar to those encountered in the *in vivo* experiments; *i.e.*, 12 ml. aliquots of normal fluid or membrane suspension were mixed with 0.5 to 1 ml. of virus preparation. The mixtures were incubated in a water bath at 37°C. and samples were removed after 0, 5, and 30 minutes, and after 1, 2, 4 and 18 hours. As in the *in vivo* experiments aliquots were (*a*) left untreated, (*b*) heated to 56°C., and (*c*) to 70°C. for 30 minutes. For purposes of control virus was added to saline solutions instead of normal allantoic fluid or membrane suspension, and saline solution was mixed with normal allantoic fluid or membrane suspension instead of virus. These preparations were kept under the same conditions as the experimental preparations.

Hemagglutination Test.—Serial 2-fold dilutions of the preparations were made in saline solution, using 0.4 ml. volumes, and 0.2 ml. of a 1 per cent suspension of thrice washed chicken red cells was added. The test mixtures were incubated in the cold room at 4°C. for 1 to 1½ hours and read according to the pattern formed by the red cells at the bottom of the tubes. The last tube giving partial but definite agglutination (±) was considered the endpoint which is expressed as the reciprocal of that dilution. In case the last reacting dilution gave + agglutination the ± endpoint was interpolated halfway between this and the next higher dilution.

Hemagglutination-Inhibition Tests.—Multiple series of 2-fold dilutions of native or heated test virus (56°C. for 30 minutes) were prepared, using 0.2 ml. volumes. To individual rows of the test virus dilutions was added 0.2 ml. of one of the materials heated at 70°C. for 30 minutes. Subsequently 0.2 ml. of a 1 per cent suspension of chicken red cells was added to each tube and the test was incubated at 4°C. and read according to the same criteria as were used for the hemagglutination test. The difference in the titer of the test virus in saline and that obtained in the presence of the materials heated to 70°C. indicates the degree of inhibition. It is expressed as the fraction of the control virus titer.

Adsorption-Elution Curves.—Equal parts of virus preparation and a 3 per cent suspension of chicken red cells, both prechilled, were mixed, incubated in an ice bath, and samples were

removed after ½ and 1 hour. At that time the mixture was transferred to a water bath at 37°C. and further samples were removed after ½, 1, 2, and 4 hours. The samples were immediately centrifuged, the two removed from the ice bath in chilled centrifuge cups, at 2,000 R.P.M. for a few minutes in order to remove the red cells and the supernates were saved for determination of the residual hemagglutinin titers. Samples of the virus preparations diluted 2-fold in saline solution served for determination of the original titer. In a few tests, the virus—red cell mixtures were incubated immediately at 37°C. for adsorption elution curves. In that case samples were taken after 5 minutes, ½, 1, 2, and 4 hours.

Receptor-Destroying enzyme of V. cholerae Culture Filtrates (RDE)[1].—The 35A3 strain of *V. cholerae* was grown in tryptose broth for 16 to 18 hours at 37°C. The culture was cleared by centrifugation and the supernate passed through a Berkefeld N filter. The filtrate was tested for its activity according to the method of Burnet (28). It was added in 1/2 to 1/10 volumes to the various virus preparations and incubated at 37°C. for 2 hours. The addition of calcium ions was found unnecessary. Hemagglutination tests were then performed in 2 per cent citrate saline. Control tests were conducted with virus preparations to which uninoculated tryptose broth had been added.

Periodate Treatment.—Four parts of the various preparations were mixed with one part of 0.1 M $NaIO_4$ and incubated for 30 minutes at 37°C. Thereafter the periodate was neutralized by one part of 40 per cent glucose. For control purposes saline solution was used as a substitute for the periodate.

EXPERIMENTAL

1. Influence of Inhibitor upon Hemagglutinin Titers

(*a*) *In Vivo Experiments.*—The allantoic fluids and membrane suspensions collected after varying periods of incubation following injection of active or irradiated seed virus were tested for their hemagglutinating capacity before and after heating to 56°C. for 30 minutes. The results are shown in the left-hand charts of Fig. 1. As can be seen the hemagglutinin titers in the native allantoic fluids following injection of active seed showed the type of curve previously reported (3); a constant level of residual, non-adsorbed seed hemagglutinins for 3 hours, followed by a marked rise in activity. When the allantoic fluids were heated to 56°C. the curve was similar except that the titers were 2 to 4 times lower. Such decreases in titer were noted also with the seed virus upon heating. The hemagglutinin titers in fluids obtained after injection of irradiated virus remained constant for the period of the experiment (non-adsorbed seed) and the difference between the heated and non-heated harvests was about 4-fold. In other experiments a slight decrease in titer became apparent at the 24 hour interval.

Considering the membrane suspensions from the active seed series no hemagglutination was found for 3 hours in agreement with previous observations (3). Thereafter a rapid increase occurred. When the suspensions were heated to 56°C. the latent period extended over 5 hours and the difference between the titers in the native and heated preparations thereafter decreased only slowly from 32- to 4-fold. Following injection of irradiated virus no hemagglutinins

[1] The RDE preparations were kindly furnished by Dr. Maria Wiener Kirber.

appeared in the membranes. The discrepancy between the fluids and membranes in the effect of heat can possibly be explained on the basis of the presence of

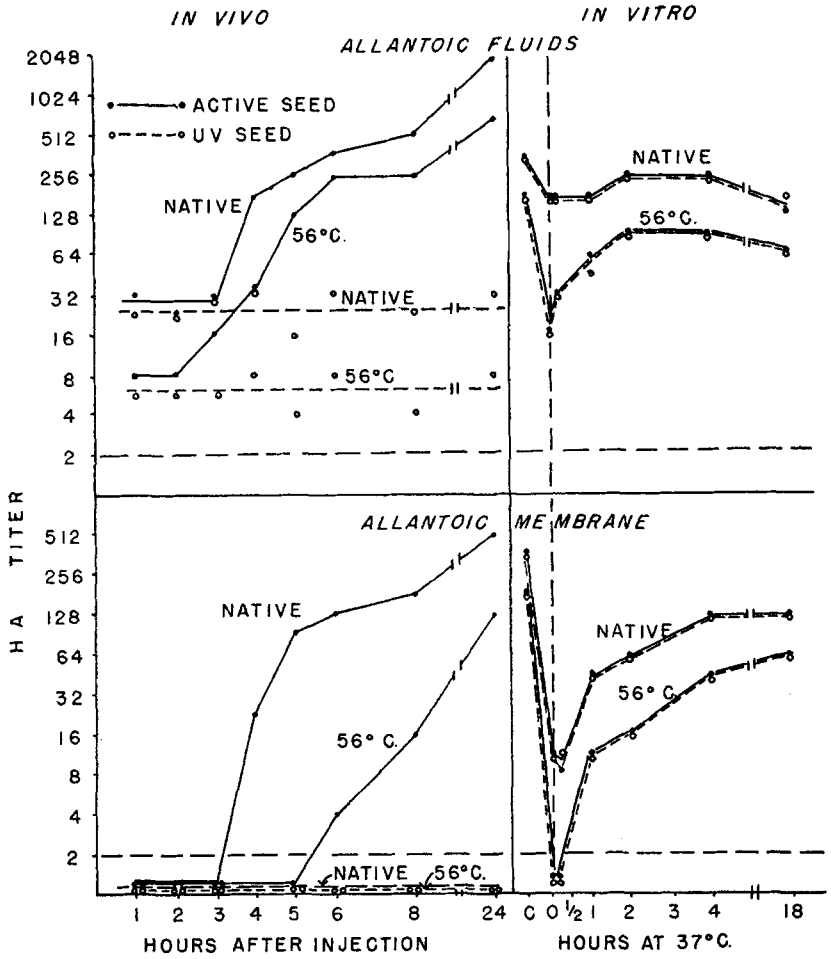

FIG. 1. Hemagglutinin titers (HA) of allantoic fluid and membrane suspensions (heated to 56°C. or native) derived from *in vivo* and *in vitro* experiments with active or irradiated (UV) seed after various hours of incubation. The broken horizontal lines in this and subsequent figures denote the starting dilution of the materials under test. C = virus control in saline solution.

greater concentrations of inhibitor in the earlier harvests of membrane, heated virus being more susceptible to its action (12). Furthermore, in the unheated preparations, the active virus may destroy additional inhibitor during the period elapsing between the preparation of the suspension and the hemagglutination

test. Finally, hemagglutinins in the early harvests may possibly be more susceptible to heat.

It had been noted previously in growth curve experiments with active virus that membrane preparations of the 3 to 6 hour harvests, which were originally negative or showed low titers, revealed some hemagglutination activity or an increase in titer if the test after the first reading was shaken and the red cells allowed to settle again (3), or when the membrane suspensions were retested after several days of storage at 4°C. This indicated the destruction of inhibitor by the active virus present in the preparations. The interaction of inhibitor in the earlier membrane harvests could be shown more clearly by the

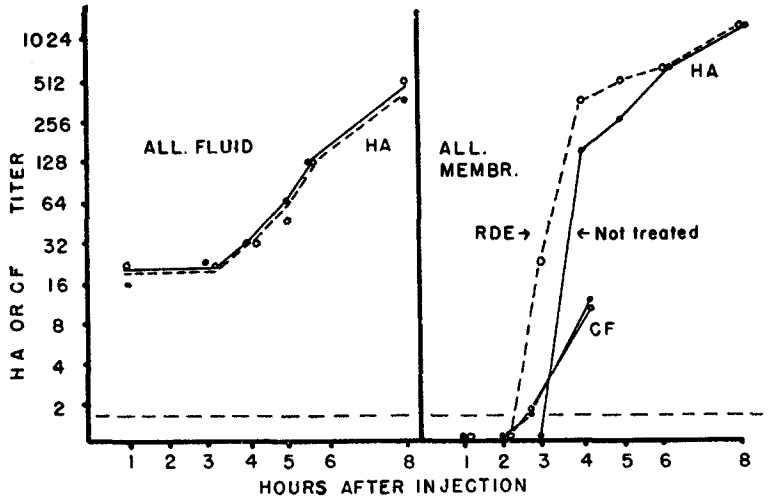

FIG. 2. The effect of RDE upon the hemagglutinin titers (HA) of allantoic fluids and the hemagglutinin and complement-fixing antigen titers (CF) of allantoic membrane suspensions derived from an *in vivo* experiment.

effect of RDE upon the hemagglutinin titers. An experiment to illustrate this point is shown in Fig. 2. Aliquots of the various allantoic fluids and membrane suspensions were treated with RDE and the hemagglutinin titers of the treated preparations were compared with those obtained with the control materials. There was no significant difference in the hemagglutinin titers in the fluids but a marked change was noted in the membrane suspensions. The latent period was reduced to 2 hours by RDE and up to the 5th hour the titers in the treated group were higher than in the non-treated preparations. Thereafter the results were identical. Complement fixation tests with the membrane suspensions did not show such a change in the time relationships nor in the titers.

(*b*) *In Vitro Experiments.*—The above experiments were complicated by the fact that with the active seed the amount of virus, of course, increased during the experimental period, and consequently it can be assumed that inhibitor will be destroyed more and more rapidly. Furthermore, additional inhibitor may be formed by the host tissues. Although in the *in vitro* tests the reagents are constant these experiments are nevertheless not strictly comparable to the conditions encountered in growth curves since *in vitro* all the inhibitor in the normal membrane suspension is accessible to the virus, whereas *in vivo* it can reach presumably only the inhibitor incorporated in the entodermal layer. The results of an experiment of this kind are presented in the right-hand charts of Fig. 1.

As can be seen, there were no significant differences between the results obtained with active and irradiated virus when added to the normal materials. The hemagglutinin titers in the normal fluid-virus mixtures showed some evidence of inhibition when compared with the saline-virus control. They were lowest immediately after mixing, increased thereafter over a period of 2 hours and they stayed constant for the next 2 hours. A decrease was noted in 18 hours, possibly as a result of hemagglutinin destruction over this period of time at 37°C. This was not noted in other experiments. In the samples heated to 56°C. the titers were generally lower than in the native preparations and the difference in titer was greatest in the 0 and 5 minute samples; *i.e.* they showed a 6- to 10-fold decrease, whereas later on it was reduced to about 4-fold. The hemagglutinin titer in the unheated as well as in the heated preparations never quite reached the corresponding levels of virus diluted in saline solution, which stayed constant over the period of incubation.

In the membrane series the hemagglutinin titers of the unheated preparations were considerably lower in the early samples and only in 4 hours was the maximal titer reached, which, in turn, was lower than that obtained in saline. The heated materials gave titers one-quarter that of the non-heated ones except in the 0 and 5 minute preparations where the difference was somewhat greater than 6-fold.

2. *Destruction of Inhibitor by Virus Action as Measured by Hemagglutininationation-Inhibition Tests*

(*a*) *In Vivo Experiments.*—It was considered that changes in the content of inhibitor, particularly in the infected allantoic membrane, would offer an approach to the determination of virus activity in the early hours after infection. Consequently, hemagglutination-inhibition tests were performed according to the technic described in the section on methods and materials with preparations derived from growth curve experiments and heated to 70°C. immediately after harvest. An experiment of this type is shown in the left-hand side of Fig. 3. Normal allantoic fluid obtained from eggs of the same batch

as was used for the growth curve reduced the hemagglutinin titer to 1/12 to 1/32 of that of the controls of native or heated test virus (30 minutes, 56°C.) in saline solution, respectively. Relatively little inhibitor was left after 1 hour of incubation both in the active and irradiated seed virus series. The inhibitor gradually decreased further over the next 4 hour period but the hemagglutinin

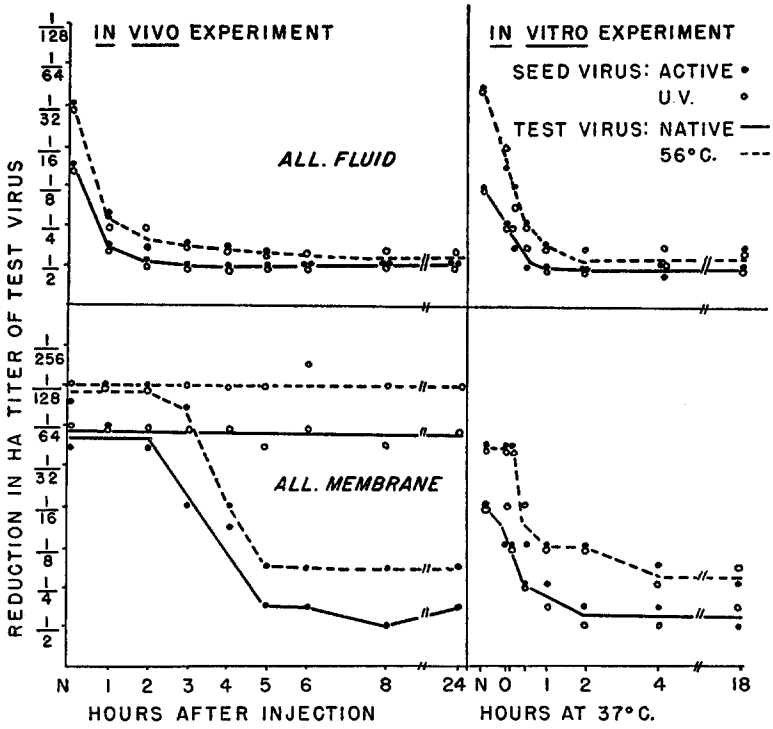

FIG. 3. Reduction in hemagglutinin titer (HA) of test virus (native or heated to 56°C.) by the inhibitory effect of allantoic fluid and membrane suspensions (heated to 70°C.) derived from *in vivo* and *in vitro* experiments with active or irradiated seed after various hours of incubation. N = normal allantoic fluid or membrane suspension prior to injection or addition of seed virus.

titer never reached the level of the saline control as was noted also by others (19).

In the membrane preparations of the active seed series inhibition remained on the normal membrane level for 2 hours. At the time when complement-fixation antigens and hemagglutinins (after RDE treatment) became measurable, inhibition began to decrease rapidly and reached the lowest level in 5 hours. The hemagglutinin titer of the test virus at this stage still was only one-third to one-sixth that of the saline control depending on whether native

or heated vest virus was employed. With the irradiated seed no change occurred in the inhibiting activity of the membranes. Treatment of representative preparations with RDE removed almost completely the inhibiting effect.

(*b*) *In Vitro Experiments.*—Inhibition tests with materials prepared by *in vitro* incubation of normal allantoic fluid and membrane suspension with active or irradiated virus are shown in the right-hand side of Fig. 3. Both the active and irradiated virus decreased the inhibitory activity of the normal materials at similar rates. In the fluids the loss is rather rapid and even the 0 hour samples, *i.e.* removed and heated to 70°C. immediately after mixing of normal fluid and virus, showed already distinctly lower inhibitory effects as compared to the normal fluid-saline controls. After 1 hour at 37°C. there was no significant additional decrease, but the titer of the test virus was still only one-half to one-third that of the virus control in saline, when native or heated test viruses were employed, respectively.

In the membrane series the results were essentially similar, except that maximal loss of inhibition was attained only in 2 to 4 hours at 37°C. and that the difference in hemagglutinin titers of native and heated test viruses was more marked than in the corresponding fluid preparations. Here again, treatment of representative preparations with RDE removed almost completely the inhibitory activity both in fluids and membranes.

3. Adsorption-Elution Curves

(*a*) *In Vivo Experiments.*—Study of adsorption-elution curves obtained with hemagglutinins in allantoic fluids and membranes of growth curve experiments revealed that the degree of adsorption of hemagglutinins onto red cells at 0°C. depended on the time of harvest of the virus materials. This is shown graphically in Fig. 4. Fluids collected 2 to 4 hours after injection of active virus (F2 and F4) showed only relatively little adsorption at 0°C., the hemagglutinin titers decreasing from 12 to 4, and 48 to 16, respectively. The increasingly later harvests showed increasingly larger degrees of adsorption. When the virus–red cell mixtures were subsequently placed at 37°C. the 2 to 6 hour harvests showed distinct additional adsorption of hemagglutinins and elution was apparent only after 1 hour at 37°C. In the 8 and 24 hour preparations elution became noticeable immediately on transfer of the mixtures to 37°C. yielding adsorption-elution curves which resembled those reported previously by Hirst (27). The results with the corresponding membrane suspensions were essentially similar.

When adsorption-elution curves were carried out entirely at 37°C. (Fig. 5) it can be seen that adsorption in the early harvest of allantoic fluid was complete within 5 minutes in that the hemagglutination tests became negative in the supernates. In the late harvest (F24) adsorption and elution overlapped so that no marked decrease in hemagglutinins was recorded, as has been noted previously (27). The latter phenomenon was also observed with membrane

suspension (M8), whereas in the early harvest (M4) only an increased speed but not an increased degree of adsorption was noted.

If the various materials were heated to 56°C. for 30 minutes the inhibitor-destroying and the eluting capacities of the virus were largely eliminated, and consequently, the extent of adsorption onto red cells could be measured more accurately. However, heating affects the hemagglutinin titer, particularly of the earlier harvests, as has been shown in the first experimental section of this paper. Thus, only a qualitative answer can be obtained from an experiment such as recorded in Fig. 6. The degree of adsorption of heated hemagglutinins

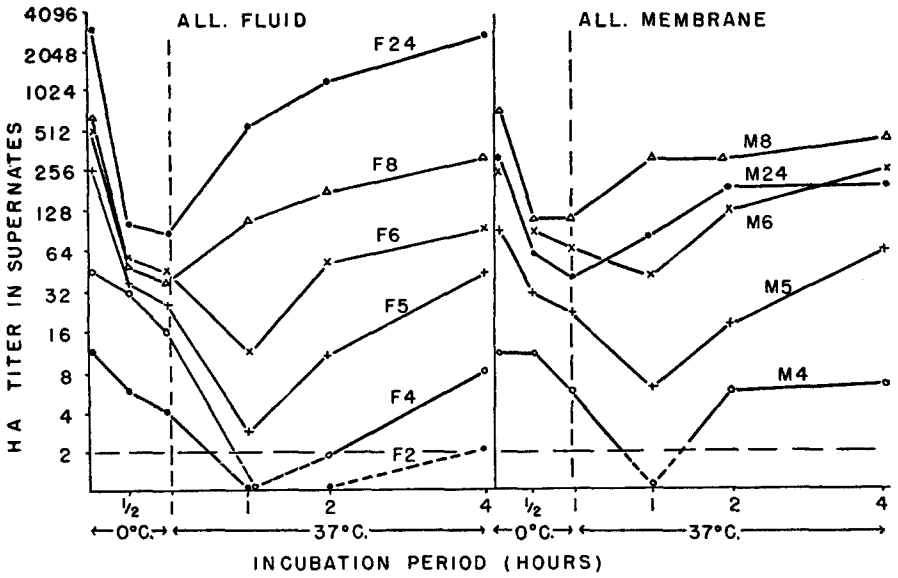

FIG. 4. Adsorption-elution curves obtained with allantoic fluid and membrane suspensions derived from an *in vivo* experiment with active seed virus. F = allantoic fluid; M = allantoic membrane suspension; the number following F or M indicates the hour of collection.

onto red cells at 0°C., again varied with the incubation period of the test material. When the mixtures of virus and erythrocytes were transferred to 37°C. additional adsorption occurred in *all* preparations. It is apparent then, that the increased adsorption of virus at 37°C. is only partly due to destruction of inhibitor and caused to a large extent by an increased rate of adsorption at that temperature. No significant elution from the red cells was noted with the heated virus.

When irradiated virus was used as seed for *in vivo* experiments no changes in hemagglutinin titer (residual non-adsorbed seed) occurred in the allantoic fluids, as shown above. In the adsorption-elution curves prepared from these fluids the ratio of the number of red cells to hemagglutinins in the mixtures

remained constant. It was found that the degree of adsorption of hemagglutination at 0°C. increased somewhat with the time of intra-allantoic incubation

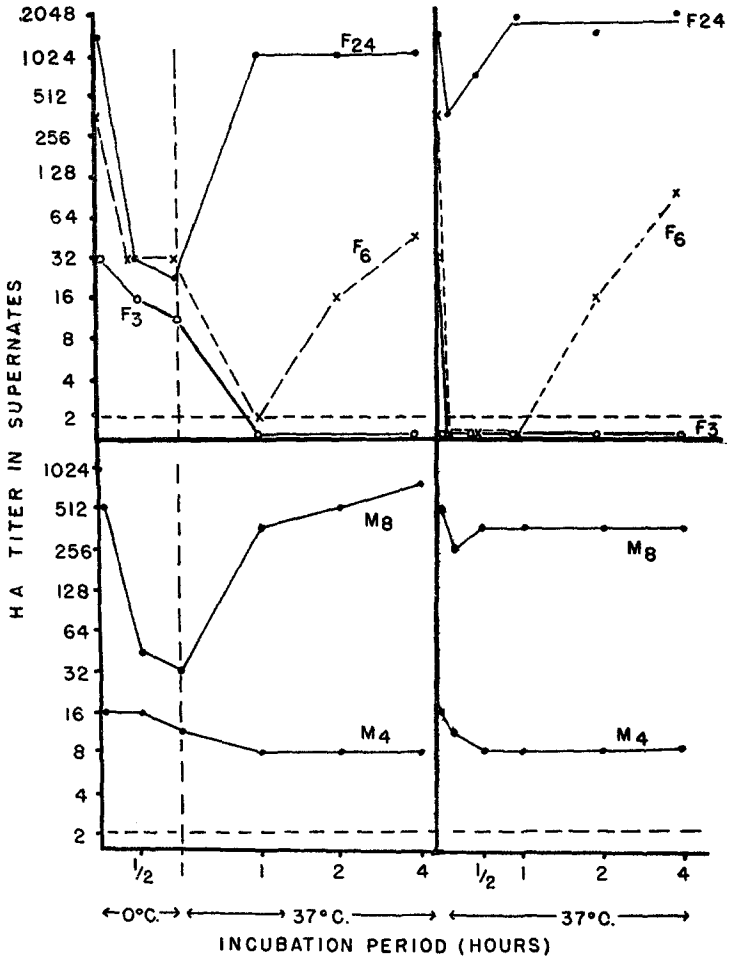

FIG. 5. Comparison of adsorption-elution curves obtained by incubation of mixtures of allantoic fluid or membrane suspension from an *in vivo* experiment (active seed) and red cells at 0°C. followed by 37°C. (left-hand charts) and at 37°C. only (right-hand charts).

of the test materials, thus indicating a gradual loss of inhibitor. On transfer of the virus–red cell mixtures to 37°C. adsorption increased to the extent that no hemagglutinins were measurable in the supernates after 1 hour at this temperature and only thereafter did elution become evident. These results resemble those obtained in the *in vitro* experiments to be discussed below.

It has not been possible to separate the hemagglutinating component from the inhibitor of adsorption by high speed centrifugation in agreement with observations by others using the hemagglutination inhibition test for assay (19, 20). The virus was sedimented from allantoic fluids at 20,000 R.P.M. for 20 minutes and the pellets were resuspended in buffered saline solution to correspond to the original volumes. Adsorption-elution curves obtained with these materials resembled those presented in Fig. 4, derived from the original virus preparations.

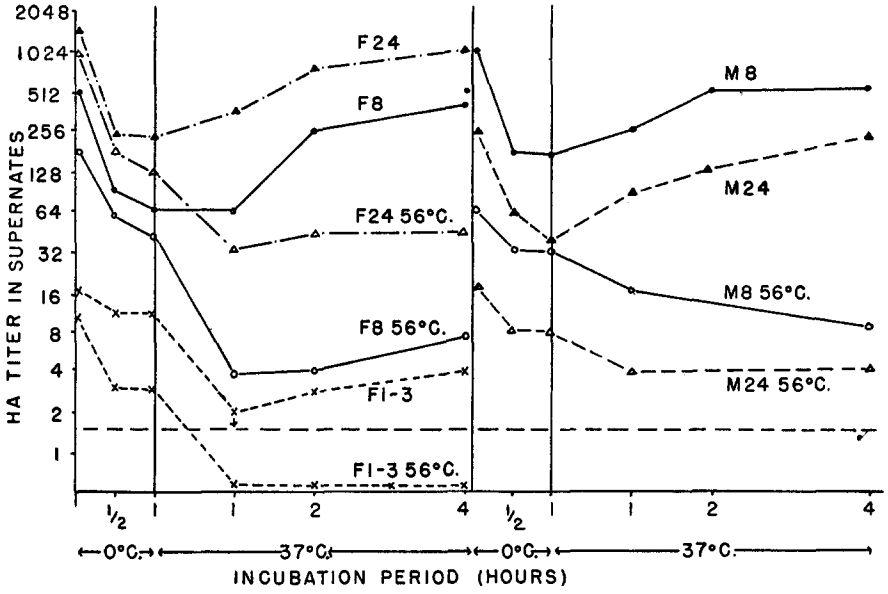

FIG. 6. Comparison of adsorption-elution curves with allantoic fluids and membrane suspensions derived from an *in vivo* experiment (active seed) before and after heating to 56°C.

When the allantoic fluids and membranes after harvest were incubated at 37°C. for 2 hours or treated with RDE, the adsorption-elution curves subsequently obtained showed more extensive or almost complete adsorption of hemagglutinins at 0°C. Likewise, treatment of the materials with sodium periodate increased the rate of adsorption at 0°C. On the other hand, the eluting property of the virus was somewhat affected by $NaIO_4$ treatment as shown in Fig. 7.

(*b*) *In Vitro Experiments.*—Adsorption-elution curves with mixtures of native (or irradiated) virus and normal allantoic fluid or membrane suspension incubated at 37°C. for various periods of time are shown in Fig. 8. The degree of adsorption onto red cells at 0°C. increased with the time of prior incubation of

the mixtures at 37°C. But even after 18 hours at 37°C. adsorption was not as marked as with virus in saline solution. On transfer of the red cell virus mixtures to 37°C. additional adsorption occurred where it was low at 0°C., whereas in the 18 hour sample elution immediately gained the upper hand. After 1 hour at 37°C. all samples showed evidence of elution.

The inhibition of adsorption by normal allantoic fluid was studied further without prior incubation with virus but after various manipulations. In the experiment shown in Fig. 9, virus was mixed with normal fluid or saline and adsorption-elution curves were set up directly. One curve was obtained by

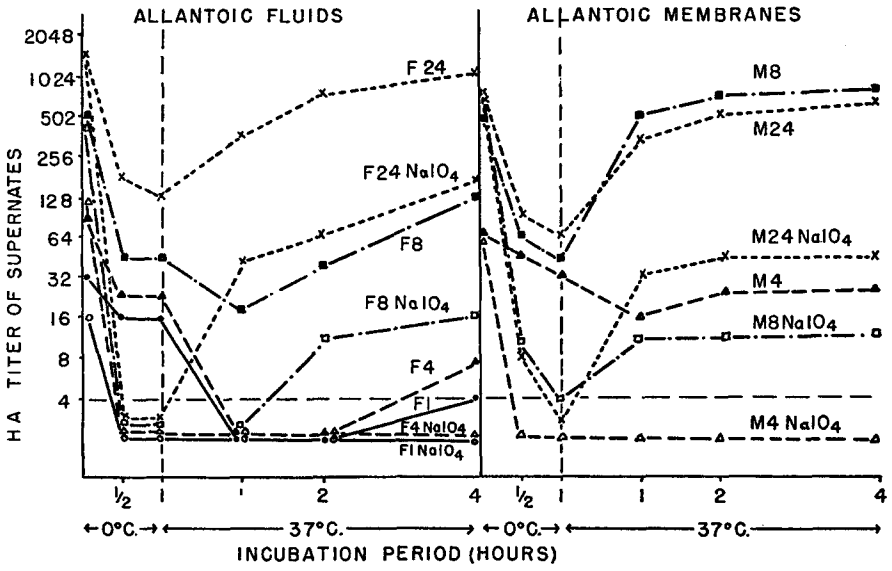

FIG. 7. The effect of periodate treatment of allantoic fluids and membrane suspensions from an *in vivo* experiment (active seed) upon adsorption-elution curves.

incubating the red cell–virus mixtures first at 0°C. for 1 hour and subsequently at 37°C., the other by incubating at 37°C. only. It is seen that at 0°C. there was a striking difference in adsorption, as in the previous tests, between the virus in normal fluid and in saline, whereas at 37°C. both curves were essentially similar.

The type of curve depended on the relative proportions of virus to normal fluid; with increasing concentrations of virus the relative adsorption at 0°C. increased, and so it did with increasing dilution of normal fluid. When the latter was diluted 25-fold the adsorption-elution curves were identical with those obtained with virus diluted in saline solution.

The inhibitor of adsorption in normal fluid was only partially sedimentable

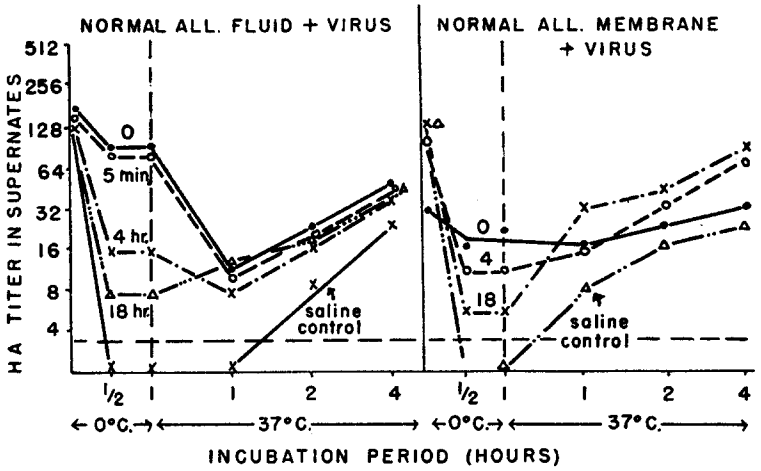

FIG. 8. Adsorption-elution with materials derived from an *in vitro* experiment; *i.e.*, with mixtures of active or irradiated virus and normal allantoic fluid or membrane suspension incubated for various periods of time at 37°C. Saline control = mixture of active or irradiated virus with saline solution.

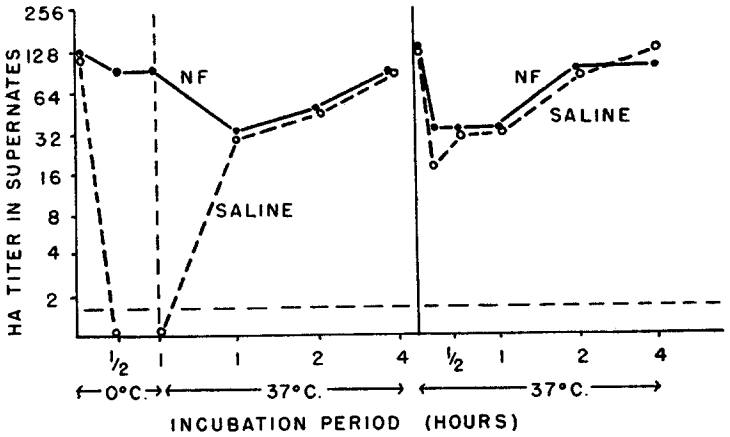

FIG. 9. Comparison of adsorption-elution curves obtained with mixtures of virus and normal allantoic fluid (NF) or saline solution immediately after preparation. Left chart: incubation at 0°C. followed by 37°C.; right chart: incubation at 37°C. only.

in the high speed centrifuge at 20,000 R.P.M. for 20 minutes as seen in Fig. 10. Both the supernate and sediment inhibited adsorption of virus onto red cells at 0°C. but when transferred to 37°C. adsorption was rapid before elution gained the upper hand.

The inhibitor of adsorption in normal allantoic fluid was largely destroyed

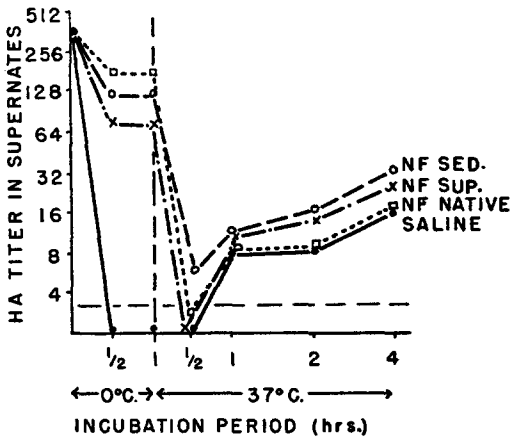

FIG. 10. Partial sedimentation of the inhibitor of adsorption in normal allantoic fluid by high speed centrifugation (20 minutes at 20,000 R.P.M.). Adsorption-elution curves with mixtures of active virus and (*a*) normal allantoic fluid, (*b*) high speed supernate, (*c*) high speed sediment, and (*d*) saline solution.

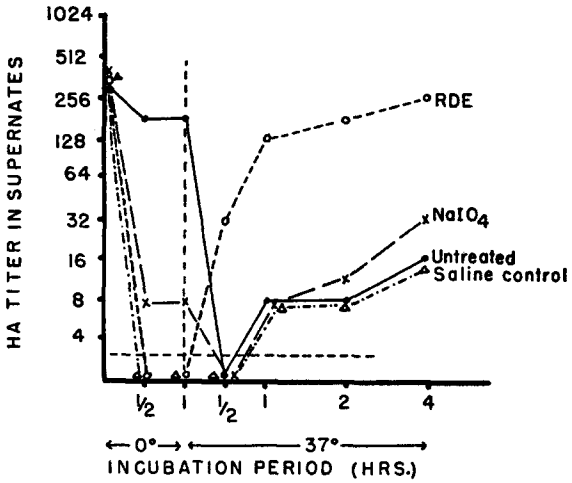

FIG. 11. Loss of the inhibitor of adsorption in normal allantoic fluid by treatment with RDE or periodate. Adsorption-elution curves with mixtures of active virus and (*a*) normal allantoic fluid untreated; (*b*) treated with RDE; (*c*) treated with $NaIO_4$; and (*d*) saline solution.

by RDE or periodate as seen in Fig. 11. It was largely removed by Seitz filtration but was resistant to heating at 70°C. for 30 minutes.

DISCUSSION

The data presented clearly indicate that, depending upon the technic employed, inhibitors of hemagglutination may interfere with the proper evaluation of viral multiplication in the allantoic cavity of the chick embryo, on the one hand, and offer further insight into the virus activities in the host, on the other. The various approaches used will be discussed in the same order as used for presentation of the data.

Influence of Inhibitor on Hemagglutination.—The experiments described in this first section indicate that inhibitor of hemagglutination found in allantoic fluid may somewhat reduce the hemagglutinin titers in the *in vivo* and *in vitro* experiments in the very early harvests but after 1 to 2 hours of incubation at 37°C. it is largely destroyed by virus action and the titers obtained will correspond more closely to the actual amount of hemagglutinins present. Therefore, the inhibitor in fluid does not seem to affect seriously the general interpretation of the results of growth curves. It is realized, however, that the technics of assay used are rather insensitive and that more accurate methods of titration may yet reveal residual inhibitory effects.

In the membrane suspensions more inhibitor appears to be present and, consequently, reductions in hemagglutinin titers persist over longer periods of incubation. In addition one would expect that the *in vivo* experiments are complicated by the unavailability to the seed and growing viruses of a large portion of the inhibitor, which will be brought into contact with the virus only upon emulsification of the tissue. As a result the data concerning the hemagglutinin content of the membranes obtained from growth curve experiments are significantly influenced by inhibitor, particularly in the early harvests, and only after 5 hours of incubation, or even later, will the value obtained correspond more closely to the actual titer. If the inhibitor in the tissue suspensions is treated with RDE hemagglutination becomes measurable after a latent period of only 2 instead of 3 hours; *i.e.*, by the 3rd instead of by the 4th hour. This result shows that the complement-fixing antigens and hemagglutinins appear at about the same time, and not the former prior to the latter, as previously indicated (2, 3). The earlier results obviously were obscured by the interaction of inhibitor. On the other hand, infectivity, which has been reported to be unaffected by inhibitor (5, 8, 18, 19, 23), increases in the tissue only after about 4 hours of incubation. Preliminary and as yet unpublished data obtained in growth curves with and without injection of RDE into the infected eggs failed to show a change in the time relationships with regard to infectivity and its titers. Thus, the hypothesis of stagewise development of influenza virus in the allantoic membrane (1–3) is still supported by the available data.

Under the conditions of these experiments irradiation of the seed virus by ultraviolet light does not affect the inhibitor-destroying property as is evident from the *in vitro* experiments, and the results with the allantoic fluid in the *in*

vivo series. Since no hemagglutinins develop in the membranes following injection of irradiated virus the inhibitor-destroying activity could not be measured by this technic.

In comparing the hemagglutination results of the unheated preparations of both types of experiments with those obtained with the materials after heating to 56°C. it is apparent that some inhibitor is destroyed by the active virus in the period elapsing between the harvest and preparation of the tissue suspensions on the one hand, and the reading of the hemagglutination tests, on the other.

Destruction of Inhibitor by Virus Action.—The main point of the experiments presented in the second section is the fact that the inhibitor in allantoic fluid, both *in vitro* and *in vivo*, is rapidly decreased by active or irradiated virus. The inhibitor in membrane is decreased *in vivo* measurably only by active but not by irradiated virus whereas *in vitro* both agents are equally effective in lowering the inhibitory activity. The loss of inhibitor in membrane *in vivo* becomes apparent at the time the complement-fixing antigens and hemagglutinins appear; *i.e.*, after 2 hours of incubation. *In vitro*, both the active and irradiated virus preparations have caused maximal decrease in inhibition by that time. Schlesinger, who studied the "incomplete cycle" of influenza virus multiplication in the central nervous system of mice, likewise found that loss of inhibitor became apparent only at the time hemagglutinins made their appearance (32). In order to explain these observations concerning the membrane series one may consider several possibilities.

Although it is known that about 70 per cent of the seed virus, be it infective or irradiated, is adsorbed onto the allantoic membranes *in vivo* (33), it may be that only a small fraction of that amount actually "enters" host cells. This quantity may be insufficient to destroy significant amounts of inhibitor. In case infective seed is used virus material increases in time and at that point inhibitor begins to decrease. Irradiated virus, as used in these studies does not multiply.

The assumption that only a small amount of the seed virus "enters" host cells appears to be contradicted by other observations. Only about 1 per cent of the seed calculated to be adsorbed can actually be demonstrated in membrane suspensions (2, 33). The question which of the fractions, the one found or the missing portion, is the one involved in propagation has not been answered convincingly although it was demonstrated that the former can be largely neutralized by specific antibodies injected into the allantoic cavity without markedly affecting propagation (3). This would indicate that the "missing" virus is removed from the action of antibody, whereas the small fraction of seed which can be recovered is superficially adsorbed and not responsible for propagation. Similarly, the interfering action of irradiated virus can not be neutralized by antibodies injected subsequently (34) and the fact that "mul-

tiplicity reactivation" may occur (35), likewise, would seem to indicate "entrance" of irradiated virus into the cells. If these assumptions are true and a relatively large proportion of both iradiated and infective virus "enters" the cells there should be some effect upon inhibitor early after injection in the light of their equal inhibitor-destroying activity *in vitro*.

It is possible that the delay in effect is based upon the quantitative aspects of the reaction. After maximal destruction of inhibitor has been achieved by infective virus the residual inhibitory activities in membrane suspensions, both of the *in vivo* and *in vitro* series are distinctly larger than in the corresponding fluid series. In both instances the residual amount of inhibitor can be largely removed by RDE. This indicates that the reaction may reach an equilibrium, as has been reported by Hardy and Horsfall (19). Depending upon the relative concentrations of virus and inhibitor more or less of the latter will be destroyed. The reaction in membrane suspensions, having greater inhibitory activity, may come to a standstill at a lower level than the reaction in the fluid with less inhibitor. When the balance is upset by increase in virus material more inhibitor will be destroyed.

In view of the relatively large amount of infective and irradiated virus involved in these experiments it is questionable whether this explanation suffices. Some thought may be given therefore to the following hypothesis. It has been postulated in studies of bacteriophage (36) and the same suggestion has been made recently with regard to influenza virus (37) that the agent entering the host cell loses its identity, it is "disassembled", and, indeed, as has been pointed out, it can not be demonstrated by conventional means. This one might assume, may also lead to loss of enzyme activity and only when virus material is formed and built up to certain levels does this property reappear and consequently inhibitor may be destroyed only then. This would presumably happen only in the case of active seed virus, whereas with irradiated virus (as used here) no such development occurs.

Adsorption-Elution Curves.—In the interpretation of the data presented in the 3rd section one has to consider a number of factors: adsorption is prevented by inhibitor at 0°C.; at 37°C. inhibitor is destroyed readily by virus action; also, at 37°C., adsorption is more extensive; the rate of elution is more rapid at this temperature; and, finally, in some of the experiments the ratio of red cells to hemagglutinin units varies and with it the number of adsorption-elution cycles prior to final liberation of the hemagglutinins. Thus the experimental conditions are rather complex. However, the 0°C. section of the curves appears little influenced by most of the factors mentioned and that is the most revealing one in relation to inhibitor.

The inhibitor of adsorption is gradually rendered ineffective by virus action at 37°C. *in vivo* and *in vitro*. Virus inactivated by ultraviolet light is as effective as the native agent except that it does not destroy inhibitor in membranes *in*

vivo. It is inactivated by RDE or periodate; it is resistant to heating at 70°C. for 30 minutes; it is only partially sedimentable at 20,000 R.P.M. in the high speed centrifuge; and its effect is rapidly lost upon dilution. In every respect tested this inhibitor behaves like that measured by hemagglutination inhibition tests as reported by others (19, 21, 23). It appears then, that the adsorption-elution curve technic affords only another test for the determination of inhibitor activity. However, in comparing the results of the first two technics discussed above with those considered in this section, it is evident that the adsorption-elution curve technic offers a somewhat more sensitive method of assay.

Whether inhibition of adsorption plays a role in infection of the allantoic tissue is difficult to evaluate. First of all the inhibition of adsorption onto red cells is shown most strikingly at 0°C., whereas at 37°C. with the amount of virus employed, the effect is scarcely demonstrable. Judging from the tests employing the hemagglutination-inhibition technic, the inhibitor in allantoic fluid is mostly destroyed after 1 hour of incubation at 37°C. However, with smaller concentrations of seed it would be reasonable to assume that a longer period of time elapses before the reaction reaches equilibrium (19). This consideration may explain the fact that the constant periods of infectivity in the allantoic fluids, covering the time from injection of the seed until liberation of new generations of virus, may be somewhat shorter (5 hours) when large doses are injected (3) as compared to the results with more dilute inocula when the period of infectivity extended usually over 6 hours (38, 39). The striking variations in constant periods with influenza B virus, which range from 4 to 9 hours, depending upon the concentration of the seed (40), have not been correlated as yet with the effect of inhibitor.

SUMMARY

The role of inhibitors of hemagglutination in the evaluation of host-virus interactions in the chick embryo–influenza virus system has been analyzed. Comparisons were made between materials (allantoic fluids and membrane suspensions) derived from *in vivo* (growth curve) experiments at hourly intervals after inoculation, and from *in vitro* tests in which normal allantoic fluids and membrane suspensions were incubated with virus at 37°C. for various periods of time. In both instances large amounts of virus were added to the systems, resulting in comparable concentrations of the agent. The seeds employed were either fully active or irradiated by ultraviolet light to the extent that the virus lost its capacity to increase but kept its interfering and hemagglutinating properties. The various materials were assayed for (*a*) the hemagglutinating titers of the virus present in the systems before and after heating to 56°C.; (*b*) the concentration of inhibitor in the materials at various stages of incubation after heating to 70°C. for 30 minutes as measured by the hemagglutination-

inhibition reaction with native or heated test virus (30 minutes 56°C.); and (*c*) the degree of adsorption of the hemagglutinins present in the materials onto chicken red cells at 0°C. and their subsequent elution at 37°C. The effects of receptor-destroying enzyme (RDE), treatment with sodium periodate, or high speed centrifugation on the inhibitory activities were studied in some of the tests.

The essential results which indicate certain sources of error in the evaluation of host-virus interactions as well as means for studying virus activity at the early stages of the infectious process, were as follows:

1. Though some inhibitory effects on hemagglutination were noticeable in the allantoic fluid during the 1st hour after inoculation they were, as a rule, no longer apparent after this interval, and treatment with RDE did not increase the hemagglutinin titers. Thus, the interpretation of growth curve data concerning allantoic fluids hardly seems to be affected by inhibitor. On the other hand, striking effects were noted with the membrane suspensions of growth curve experiments in that RDE shortened the latent period to 2 hours and the titers in the first few positive samples (4 to 5 hours) increased, whereas in later harvests no such effect was noted. Under these conditions complement-fixation antigens and hemagglutinins made their appearance in the tissues simultaneously and not as previously reported the former prior to the latter. However, the infectivity showed increments only several hours after these two activities had become measurable. Thus the hypothesis of the stagewise development of influenza virus is still supported by these data.

2. Using the inhibition of hemagglutination technic it was found that the inhibitor in allantoic fluid rapidly decreased as a result of the action of active and irradiated virus, but destruction was never complete. In the membranes of the *in vivo* series only active seed led to loss of inhibitor, again without complete destruction, beginning at the time complement-fixing antigen and hemagglutinins became measurable. Irradiated seed was without effect *in vivo* whereas, in the *in vitro* tests it equalled the activity of the active virus. The implications of this difference in the effectiveness of active and irradiated seed *in vivo* with regard to the understanding of the mode of viral multiplication are discussed.

3. Although many factors may influence the shape of adsorption-elution curves it is felt that at 0°C. the extent of adsorption is directly related to the amount of inhibitor present in the systems. In the early hours after inoculation the degree of adsorption was relatively small but it increased gradually with the time of incubation. The inhibitor of adsorption was destroyed by RDE and $NaIO_4$ and was only partially sedimentable by high speed centrifugation. In every respect studied its properties corresponded with the findings obtained with inhibitors in the hemagglutination-inhibition technic. Although the difference in the rapidity of inhibitor destruction as measured by the various technics might suggest a multiplicity of inhibitors it is felt that it rather denotes a greater sensitivity of the adsorption technic as compared to the others.

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