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

XIV. The Relation between Tissue-Bound and Liberated Virus Materials under Various Conditions of Infection

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Past studies of this series have been concerned with the development of non-infectious hemagglutinins during the process of replication of influenza virus under a variety of conditions. In growth curves in the intact chick embryo various properties of the virus appear in the allantoic membrane or increase therein differentially; *i.e.*, following a latent period of about 2 hours, the soluble complement fixation antigen (S) can be measured first, followed shortly by the development of the virus or V antigen and of hemagglutinins (HA), and a rise in infectivity is detected last (1). Similar observations have been made by Hoyle (2, 3). These results were interpreted in terms of a step-wise development of the virus in that components with S, V, and HA activities were formed first and these were converted subsequently into infectious virus. However, these experiments were carried out under conditions when every cell lining the allantoic cavity was probably infected with more than one seed virus particle and subsequent studies (4–10) revealed that in that case the final yields in the allantoic fluids always contain an appreciable proportion of non-infectious hemagglutinins (NIHA); in other words, according to the above hypothesis, one would have to assume that a considerable number of the non-infectious particles are never converted into infectious virus.

The yield of NIHA is considerably increased upon serial passage of undiluted infected allantoic fluids, as was shown first by von Magnus (10–12) and subsequently confirmed by others (7, 9, 13, 14). If such passages are carried out under conditions minimizing inactivation of virus during the incubation periods *in ovo* at 37°C. the von Magnus effect is less pronounced than when the seeds used for transfer are likely to contain appreciable quantities of heat-inactivated virus (7–9). This experience suggested that inactive virus in the seed may play a significant role in the phenom-

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enon. Indeed, standard seeds of virus, exposed *in vitro* to 37°C. for long periods of time are capable of reproducing the von Magnus effect to some extent in that such heated preparations will yield on passage considerable quantities of NIHA (7, 8, 10, 15–17). Yet such heated standard (ΔST) virus and undiluted passage (UP) seeds do not behave alike in every respect. When the two types of seeds were compared on the basis of similar infectivity and HA titers, the yields derived from the UP inocula contained up to 100 times more NIHA than those obtained from the corresponding ΔST virus (15). These and other results show that the NIHA components in UP fluids are different from heat-inactivated complete virus and probably represent immature or incomplete virus particles. Furthermore, evidence has been presented that different degrees of incompleteness may be discerned in consecutive undiluted passages (9), as was suggested also by von Magnus (10).

The above studies, with one exception (6), have shown that production and liberation of incomplete virus is clearly related to adsorption of several virus particles per cell (7–11, 14, 16) and that the composition of the progeny is dependent upon the relative concentrations of infectious, heat-inactivated complete and incomplete virus particles in the seeds. Furthermore, it seems to be essential that the cells are exposed within a narrow period of time to large numbers of virus particles in order to yield NIHA (18). A slow saturation of the cells, such as may be expected to occur in consecutive infectious cycles after infection of the allantois with small doses of standard virus, does not lead to ultimate release of significant amounts of NIHA, although under these conditions a non-infectious hemagglutinating component appears in the infected tissues which is less readily sedimentable by high speed centrifugation than infectious virus (19).

It would appear then that a number of incomplete forms of virus are obtained under a variety of conditions aside from heat-inactivated complete virus. It has been suggested repeatedly (1–3, 5, 10–12), although questioned by others (8, 17), that these incomplete forms represent intermediary stages in virus development. In the present study an effort has been made to shed more light on the role of the incomplete forms in the infectious cycle, especially by correlation of their concentrations in infected tissues with those in the virus materials released therefrom. None of the results are in conflict with the above suggestion, yet they fail to prove finally that the incomplete forms are in fact intermediary components in virus development.

Methods and Materials

Virus.—The PR8 strain of influenza A virus was used throughout these experiments. *Standard virus*, in which presumably the vast majority of virus particles were infectious, was produced by allantoic inoculation of chick embryos with small doses of virus (10^3: to 10^4 ID_{50}) and the allantoic fluids were collected after incubation of the eggs at 36 to 37°C. for 24 hours, when the infectivity titers were still rising or just had reached their peak (8, 9). For some experiments the standard seeds were heated prior to inoculation at 37°C. for several days according to the technic described (15). Undiluted passage seeds (11) were prepared as previously reported (9).

Growth Curves in Intact Chick Embryos.—This technic has been fully recorded (1).

Differential Growth Curve Technic in Deembryonated Eggs.—The deembryonation technics

(5) as used in this laboratory have been reported previously (20). In the present study not only the media but also the allantoic membranes were employed for analysis of virus activities. The required number of 14- to 15-day-old chick embryos were inoculated each with 0.2 to 0.5 ml. of a given type of seed without dilution or diluted up to 10^{-6}. After incubation for various periods at 37°C. the eggs were deembryonated by the usual technic and placed on the rotating machine. In some experiments the medium contained initially receptor-destroying enzyme (RDE) to a concentration of 1 per cent or 250 to 500 units per egg, in order to remove superficially adsorbed seed virus. After deembryonation the media were exchanged every 2 hours for periods up to 36 hours by decanting and addition of fresh glucosol. All operations were carried out in the warm room. Aliquots of the media, thus collected, were diluted 10^{-1} in 10 per cent horse serum broth and stored in flame-sealed ampules in the dry ice chest. At each interval 3 eggs were removed, the membranes were washed twice with glucosol in the shell, and twice more after their removal from the shells in Petri dishes. Following draining, they were placed in the freezer at −20°C. The following day the membranes were emulsified in chilled Waring blendors, using 5 to 10 ml. of cold glucosol per membrane. After clarification by low speed centrifugation aliquots of the supernates were placed in ampules and stored at dry ice temperature. The frozen aliquots, both of the media and membrane suspensions, were used for infectivity titrations, which, in most instances, were completed within 2 weeks. The remainder of the samples were kept at 4°C. and employed within a few days for complement-fixation and hemagglutination tests. The tissue preparations for the latter were, as a rule, treated with RDE as described (21) in order to inactivate residual inhibitor of hemagglutination, which is present in appreciable amounts only in early harvests whereas at the time production and liberation of virus reach maximal levels the membrane suspensions are free of significant concentrations of this component.

Assays for Virus Activities.—The technics of *infectivity* (22) and *hemagglutinin* titrations have been fully described (20). For the *complement fixation* tests two types of sera were employed, both produced in the guinea pig. For detection of the virus or V antigen (strain-specific antigen), sera were used which had been obtained by intraperitoneal injection of ultraviolet-inactivated PR8 virus. Only sera free of antibody to the soluble antigen and host components were selected and pooled. Sera for the determination of soluble or S antigen (type-specific antigen) were prepared in the following manner (23). The $L_3$47 strain was inoculated in dilutions 10^0 to 10^{-4} into guinea pigs by the intranasal route under light ether anesthesia. Two to 3 weeks later the animals received an intraperitoneal booster injection of 1 ml. of S antigen derived from the allantoic membrane of chick embryos infected with the $L_3$47 strain. These membrane suspensions had been largely freed of virus particles by centrifugation at 25,000 R.P.M. for 30 minutes and the supernates in addition had been absorbed twice with large amounts of washed chicken red cells. Under these conditions the guinea pigs developed high titers of anti-S and also considerable levels of anti-$L_3$47 V, which, however, failed to cross-react with PR8 V antigen (23). The sera were standardized by optimal titration technic and the dilution of serum which gave the highest antigen titers was employed in the analyses presented here.

For the complement-fixation test, serial 2-fold dilutions of the various virus preparations were made in triplicate, using 0.1 ml. per tube. To the first set was added (0.1 ml. amounts) diluted anti-V serum, to the second anti-S and to the third saline. Thereafter, 0.2 ml. of guinea pig complement was added to each tube, diluted sufficiently so that each aliquot contained about 2 hemolytic units. Following incubation at 4°C. overnight, 0.2 ml. of sensitized sheep cells (1 per cent) were added and the test was read after 30 minutes of further incubation at 37°C. in the usual manner. The antigen dilution leading to a 3 or 4+ fixation of complement was taken as the endpoint and the results are expressed as antigen units per milliliter.

All values presented for the tissue preparations (ID_{50}, HA, CFV, and CFS) were cor-

rected, when needed, to give the number of units per milliliter for suspensions of one membrane per 10 ml. of buffered saline solution, in order to permit direct correlation of the results with those of the media amounting to 10 ml. per egg. The total present in the system thus can be obtained simply by increasing the values recorded by a factor of 10.

EXPERIMENTAL

Relation between liberated Progeny and Virus Material in the Tissue

Previous studies in deembryonated eggs were largely restricted to the release of virus into the medium, using the cumulative or differential growth curve technics (9, 15, 20, 24). The latter method offered the opportunity to correlate the hourly or 2 hourly yields in the medium with the amounts of virus materials present at the onset of each period in the tissues. These experiments were carried out as described in the section on methods.

Results with Undiluted Standard Seeds.—Fig. 1 shows the data of an early test with undiluted standard seed which was restricted to infectivity and hemagglutinin assays. This experiment has been summarized in a previous report (7). On the left side the results of titrations of the individual *media* are given, whereas on the right the calculated cumulative curves are plotted (dots). These results agreed with those previously reported (24); *i.e.*, liberation of ID_{50} and HA became apparent after 4 hours of incubation and the rates of liberation remained at high levels up to the termination of the experiment in 36 hours. The average ID_{50}/HA ratio in the yields was $10^{5.7}$. The *membrane* titers (circles), which obviously are cumulative only, are plotted on the right hand chart. The ID_{50} and HA curves rose simultaneously and at nearly similar rates, then a plateau was reached, which was maintained up to about the 22nd hour in case of the HA activity and up to the 32nd hour in that of infectivity. Thus, it appears that the hemagglutinin level fell off prior to the ID_{50} titers. This difference was not seen regularly and often both levels remained high for the total period of 36 hours. The mean ID_{50}/HA ratio in the tissue suspensions was $10^{4.2}$; *i.e.*, 1.5 $\log_{10}$ units lower than that of the liberated virus.

It is obvious from the data that the membranes contained considerably more non-infectious hemagglutinins than the yields derived therefrom in the media. It is also apparent from the right-hand chart that the hemagglutinins were accumulating in the tissues prior to the time when release could first be detected, the interval being of the order of 2 hours. With respect to infectious virus, the time of increase in the membrane coincided with that of first liberation. Thus it would seem that as soon as infectious virus is completed, it is released from the cells. This suggestion is further emphasized when the titers in the tissue suspensions at the onset of a given period are compared to the quantities of infectious virus and hemagglutinins released therefrom into the medium in the ensuing 2 hours. The individual points of the membrane curves were transferred therefore to the left-hand chart and the attached arrows are directed

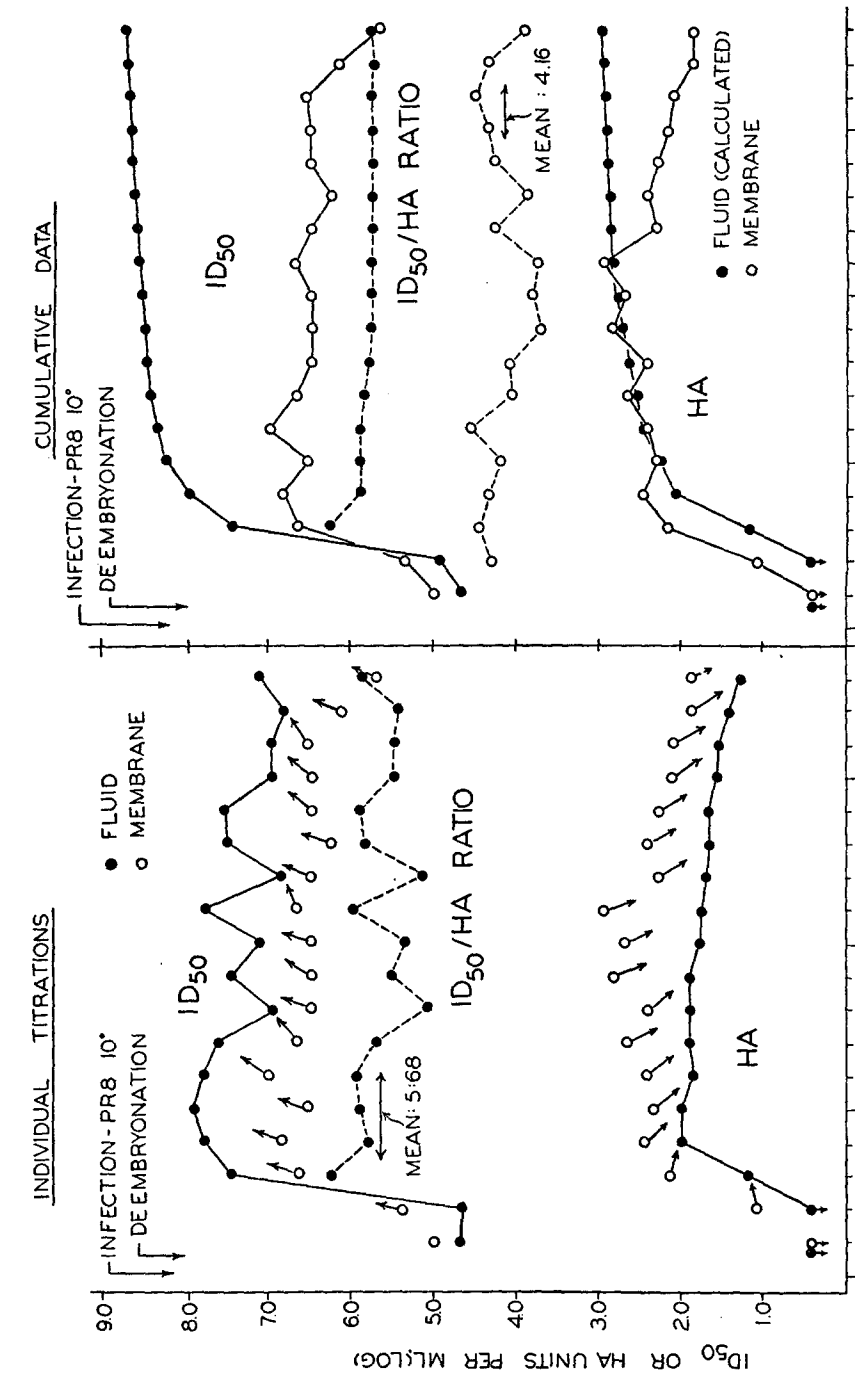

FIG. 1. The 2 hourly yields of ID_{50} and HA units in the media of deembryonated eggs in relation to the respective titers in the allantoic membranes. Inoculum: undiluted standard seed.

toward the respective yields. It can be seen that regularly more infectious virus (about 8×) was released in each 2 hour period than was initially present in the tissue, since the ID_{50} titers of the media on the average fell 0.9 $\log_{10}$ units *above* those of the corresponding membrane suspensions. In contrast, only a fraction of the hemagglutinins (about ¼) was liberated under those conditions, as the titers of the media fell on the average 0.6 $\log_{10}$ units *below* those of the tissue.

In Fig. 2, the combined data of 5 similar experiments with undiluted standard seeds are plotted in which, in addition to ID_{50} and HA assays, also the virus (V) and soluble (S) complement-fixing antigens were titrated. It can be seen that the results of the infectivity and hemagglutination tests were principally similar to those described above. With respect to the complement-fixation assays only the membrane suspensions gave evidence of activity. The S antigen titers rose somewhat earlier (1–3, 25) and were generally slightly higher than those of the V component. That there was no detectable S antigen in the media was not surprising since this component even in the allantoic fluid of intact chick embryos under cumulative conditions is usually not present in measurable quantities within 24 hours of incubation (2, 26). The failure to find V antigen in the media is not as readily explained. The ratio between HA and CFV activity in the membranes was of the order of $10^{0.5}$. Although the HA levels in the media amounted to only ⅓ to ¼ of those found in the tissue, one would have nevertheless expected measurable quantities of V antigen in the fluids if the same HA/CFV ratio would apply. Actually, this ratio in the fluids was found to be greater and exceeded certainly $10^{1.0}$ and $10^{1.2}$ in some tests. Thus, the membranes contained at least 3 to 5 times more V antigen than could be accounted for by the HA titer. It is suggestive, therefore, that the tissues contain, aside from the S antigen (which does not react with anti-V), 2 antigens which react with anti-V sera, one of which is devoid of HA activity. Although this suggestion needs to be more firmly established, it is of interest to note that Brand recently presented data on the existence of a non-hemagglutinating V component in allantoic fluids infected with A prime strains (27).

Seeds Producing Large Amounts of NIHA.—Similar experiments were carried out with seeds known to produce large quantities of non-infectious hemagglutinins; *i.e., standard seeds heated at 37°C. in vitro* for several days (15) and *undiluted passage seeds* (11). Only an experiment with heated standard seed is presented here in detail as an example.

The seed had been heated for 48 hours at 37°C. Its infectivity titer had been reduced about 100-fold to $10^{7.2}$ per ml. but the HA titer had remained unchanged ($10^{3.2}$ units/ml.). The media contained 1 per cent RDE throughout the experiment. They were exchanged at 2 hourly intervals from the 3rd to 19th hour and then the yields were permitted to accumulate to the 31st hour. The value obtained after this cumulative period was divided by 6, the number of 2 hour intervals covered, in order to obtain the average yields during this time. Membranes were collected on 6 occasions during the total experimental period.

The results, shown in Fig. 3, are in principal agreemnet with those shown above, even though considerable quantities of non-infectious hemagglutinins were produced by the heated seed. Again, almost 10 times more infectious virus was released in a given 2 hour interval than was present in the tissue at onset

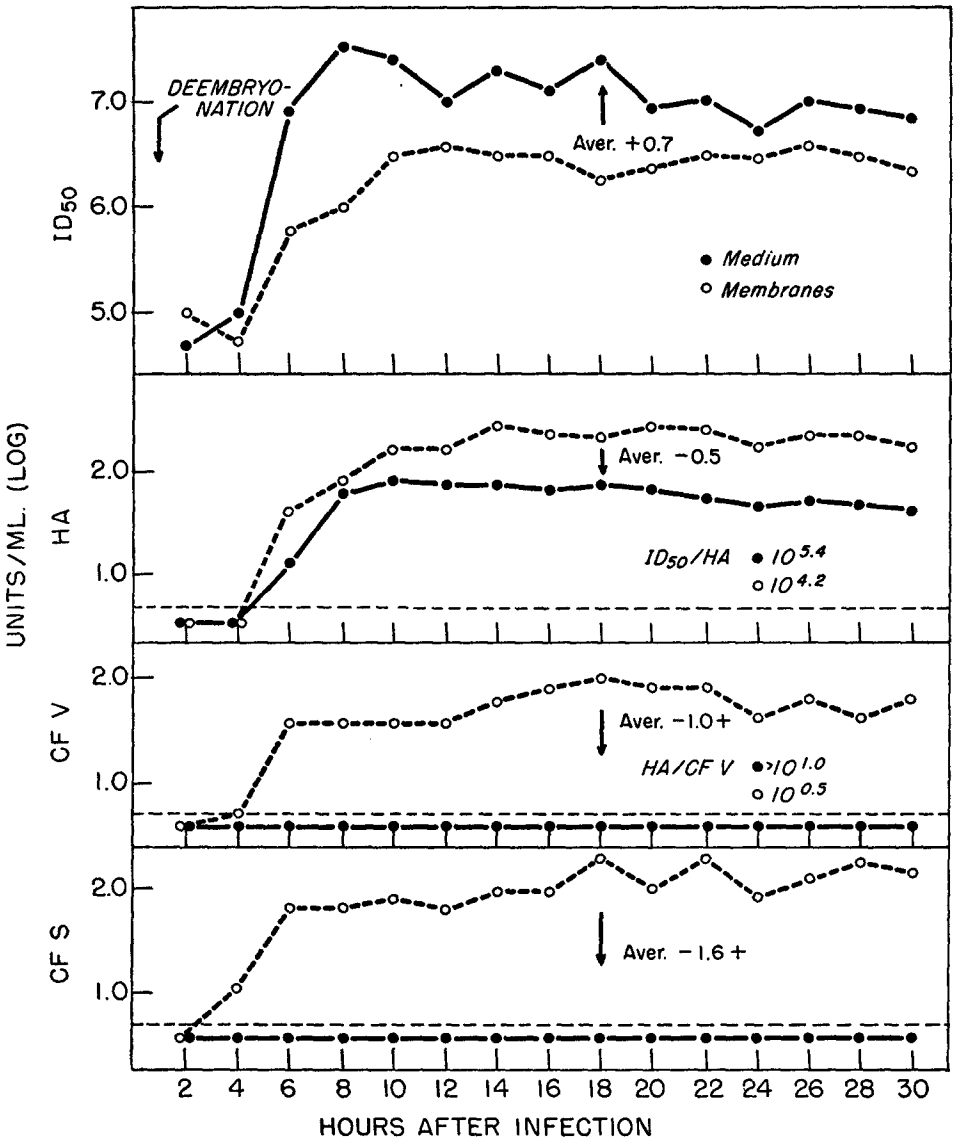

FIG. 2. The 2 hourly yields of ID_{50}, HA, CFV, and CFS units in the media of deembryonated eggs in relation to the respective titers in the allantoic membranes. Inoculum: undiluted standard seed (summary of 5 separate experiments).

of that period, but only ¼ of the HA units was liberated. In accordance, the average ID_{50}/HA ratio in the membranes was 1.4 $\log_{10}$ units below that of the media; *i.e.*, $10^{2.8}$ *vs.* $10^{4.2}$. The titers of the complement-fixing antigens in the tissue, particularly those of the S component, were somewhat lower than in the experiments with standard seeds. Yet, the media should have given just

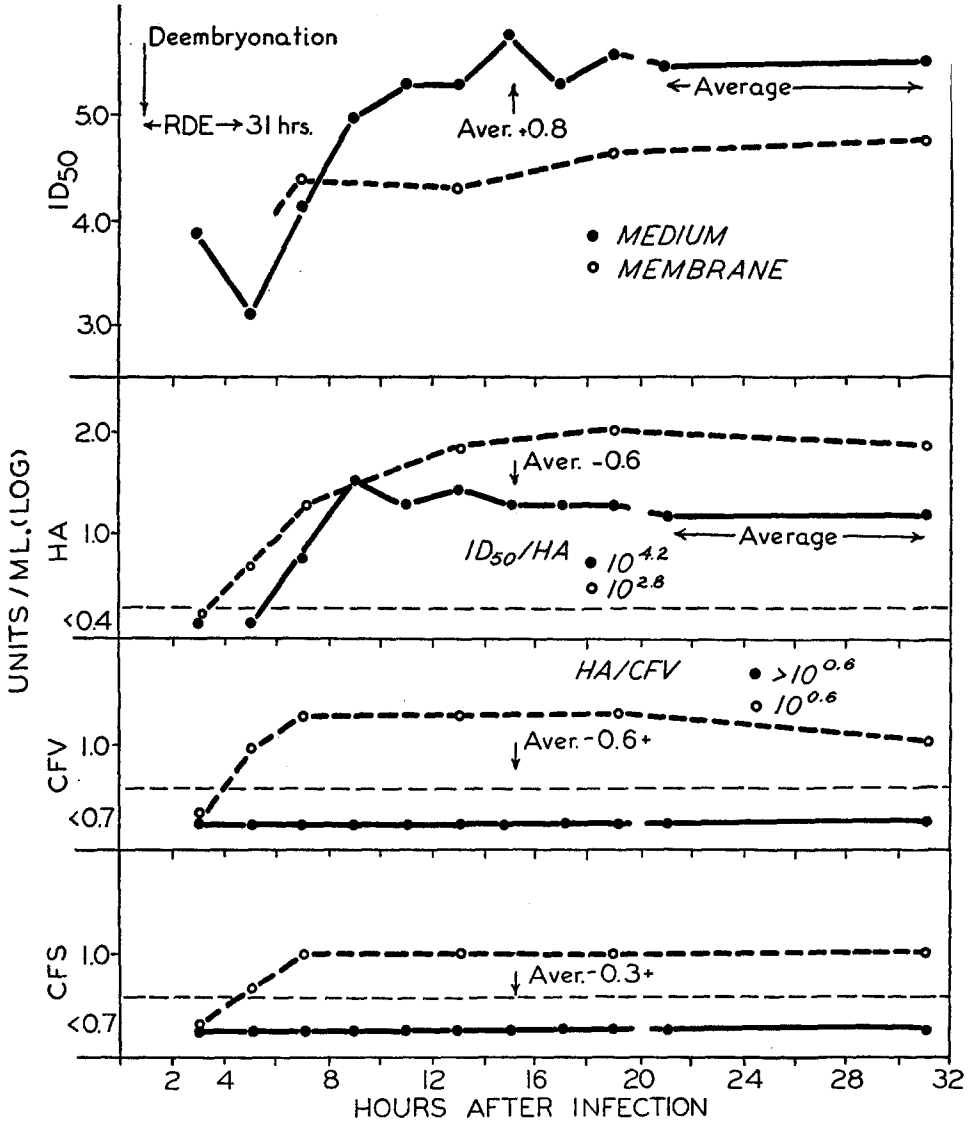

FIG. 3. The 2 hourly yields of ID_{50}, HA, CFV, and CFS units in the media of deembryonated eggs in relation to the respective titers in the allantoic membranes. Inoculum: undiluted standard seed heated *in vitro* at 37°C. for 48 hours.

detectable CFV levels on the basis of HA/CFV ratios in the tissue of $10^{0.6}$, which, however, was not observed in confirmation of the preceding experiments.

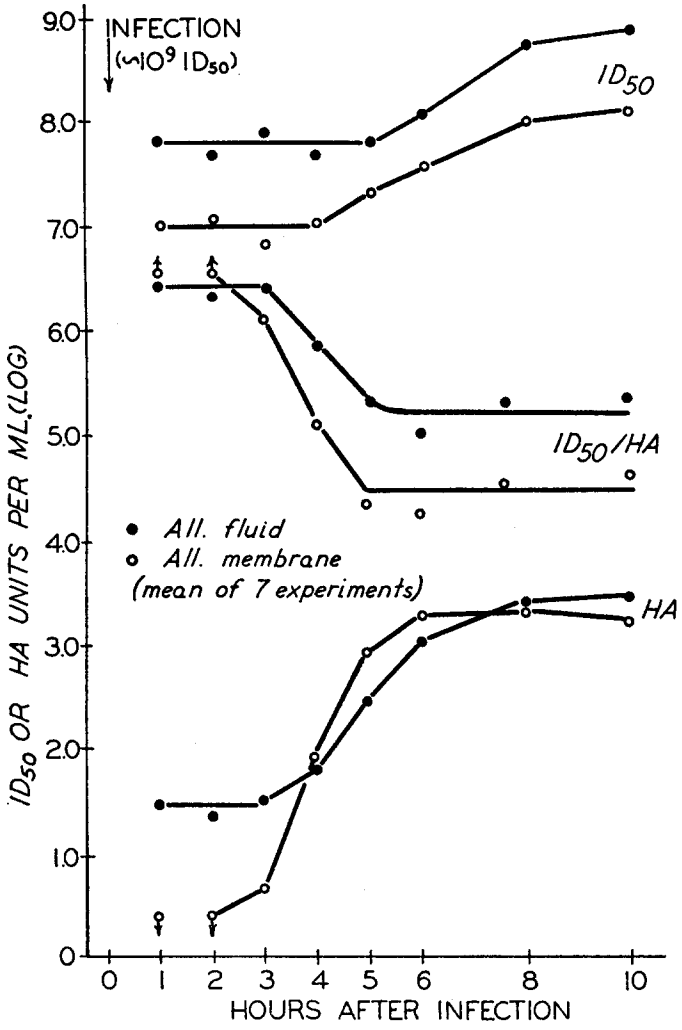

FIG. 4. Comparison of the ID_{50}/HA ratios in the allantoic fluids and membranes of intact chick embryos following injection of undiluted standard seed. (Summary of 7 experiments.)

Results obtained with 2nd undiluted passage seeds in 2 experiments were in essential agreement in that the discrepancies in relative quantities of infectious virus and of hemagglutinins released were responsible for more than 10-fold differences in the ID_{50}/HA ratios of tissue-bound ($10^{0.5}$ and $10^{1.2}$) and liberated virus materials ($10^{1.7}$ and $10^{2.4}$).

Results in Intact Chick Embryos.—The discrepancy between ID_{50}/HA ratios in liberated and tissue-bound virus materials is not restricted to the deembryonated egg system. That similar differences obtain also in the intact egg between allantoic fluid and membrane has been indicated in a previous report (15) both under conditions of infection with undiluted standard virus or with the same seed heated at 37°C. for 5 days. A review of older experiments concerning *in ovo* growth curves following injection of undiluted standard seeds confirmed this observation. A summary of 7 such experiments is shown in Fig. 4, presenting

TABLE I

Differences in ID_{50}/HA Ratios of Liberated and Tissue-Bound Virus Following Infection with Dilute Standard Seed

Inoculum PR8 standard	Test	*In Ovo*, 24 hrs.			DE, 2 hr. results (28 to 38 hrs.)				
					Medium		Membrane		
		(A) Fluid	(B) Membrane	Difference (A) − (B)	(A) Average	Range	(B) Average	Range	Difference (A) − (B)
		log	*log*	*log*	*log*	*log*	*log*	*log*	*log*
10^{-2}	ID_{50}	9.6	8.5		7.7	7.5–7.9	7.5	7.4–7.8	
	HA	3.3	2.9		1.8	1.6–2.1	2.4	2.2–2.5	
	ID_{50}/HA	6.3	5.6	0.7	5.9	5.7–6.0	5.1	5.0–5.3	0.8
10^{-4}	ID_{50}	9.8	8.6		8.0	7.8–8.3	7.6	7.5–7.7	
	HA	3.3	3.1		1.8	1.6–2.1	2.5	2.4–2.5	
	ID_{50}/HA	6.5	5.5	1.0	6.2	5.8–6.4	5.1	5.0–5.2	1.1
10^{-5}	ID_{50}	10.2	8.0		8.0	7.6–8.6	7.7	7.4–8.2	
	HA	3.6	2.6		1.9	1.7–2.2	2.5	2.1–2.7	
	ID_{50}/HA	6.6	5.4	1.2	6.1	5.8–6.5	5.2	4.7–5.6	0.9
10^{-6}	ID_{50}	9.8	8.5		7.8	7.3–8.1	7.6	7.4–7.8	
	HA	3.6	2.9		1.8	1.7–2.1	2.5	2.3–2.7	
	ID_{50}/HA	6.2	5.6	0.6	6.0	5.8–6.2	5.1	4.9–5.5	0.9

the mean values of ID_{50} and HA titers obtained. It is apparent from the figure that the discrepancy between the 2 ratios in the intact chick embryo is somewhat less striking than in deembryonated eggs; *i.e.*, of the order of 0.7 $\log_{10}$ units in the average instead of 1.2 to 1.5. This difference presumably is a reflection of the experimental conditions; in the *in ovo* experiments the yields accumulate in the allantoic fluids during the experimental periods and some of the free infectious virus unquestionably will be inactivated with prolongation of the incubation period at 37°C., thus lowering the ratios in the allantoic fluids. In the deembryonated eggs the liberated virus is removed at frequent intervals before extensive inactivation may become apparent.

Results Obtained with Dilute Standard Seeds.—The discrepancies between

the ID_{50}/HA ratios in the allantoic fluids and media of deembryonated eggs, on the one side, and in the tissue suspensions, on the other, were seen not only after inoculation of large quantities of seed but also after injection of dilute standard virus. This is demonstrated by the following experiment.

Groups of 24 13-day-old chick embryos were injected with 0.5 ml. of standard virus diluted to 10^{-2}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively. After 24 hours of incubation the eggs were deembryonated, the allantoic fluids were saved, and the membranes of 3 in each group were harvested at this time. To each of the remaining deembryonated eggs 10 ml. of medium was added which then was exchanged at 2 hour intervals of incubation on the rotating machine. At the end of every 2 hour period, 3 eggs were removed from each group for harvest of the membranes. The ID_{50} and HA titers in the allantoic fluid, media, and tissue suspensions were determined in the usual way.

The results of this experiment are summarized in Table I. It is seen that the ID_{50}/HA ratios in the tissues in every instance were lower than those found in the liberated progeny, the differences ranging from 0.6 to 1.2 $\log_{10}$ units in the *in ovo* part of the experiment, and from 0.9 to 1.1 in the deembryonated series. Again, only ¼ to ⅕ of the hemagglutinins present in the membranes was released in any given interval. However, at most, 2½ times the quantities of infectious virus found in the tissues were liberated during the same intervals. Thus, the differences in ratios of membranes and media were somewhat less striking than in the preceding experiments employing large inocula. It should be noted that the infectivity titers of the membranes under the latter conditions were considerably lower than in the present test. It is also to be noted that the experiment was restricted to a relatively late period (28 to 38 hours after infection) and it is possible that earlier in the infectious process somewhat greater amounts of infectious virus were released.

Attempts to Evaluate the Role of NIHA in the Infectious Process

The data presented thus far indicate that under the various conditions described the infected membranes always contain more non-infectious hemagglutinin than appears in the yield derived therefrom. The data also denote that once infectious virus is produced in conjunction with the tissues it is liberated rapidly, as was suggested previously by Hoyle (3). The question remains whether the "excess" hemagglutinins found in the tissues are in any way participating in the infectious process or whether they represent a stable quantity retained on or in the cells. This problem was approached in a number of different ways, which will be related below.

Extracellular vs. Intracellular Hemagglutinin.—The question whether the HA components are on or in the cells has been approached previously (18) by the addition of RDE to the medium of deembryonated eggs. Only when this agent is employed very early after infection can an effect be discerned, but later in the infectious process it does not alter the composition of the liberated as well

as of the tissue-bound virus materials. This, to some extent, may be inferred also from the experiment shown in Fig. 3, when RDE was present in the medium throughout the period of observation, yet the results did not differ significantly from those obtained in the absence of RDE (see Fig. 1 for example). Furthermore, treatment of intact membranes from infected eggs with RDE after their collection did not lower the HA or ID_{50} titers to a significant extent (18). Thus it would appear that the hemagglutinins are beyond the reach of RDE and, therefore, located most likely within the confines of the cells.

Inhibitor Destruction in the Tissues Following Infection with Various Types of Seeds.—Both normal allantoic fluid and membrane suspensions contain inhibitors of hemagglutination, also referred to as receptor substance (21, 28–30). The media of deembryonated eggs *per se* are free of inhibitory activity, but they may become contaminated with small amounts of allantoic fluid or albumen left in the deembryonated eggs. Furthermore, some receptor substance may be shed into the medium from the tissue during incubation. In the course of infection the inhibitors are rapidly destroyed by virus action. In the allantoic fluid, non-adsorbed seed hemagglutinin levels of 1:32, for instance, inactivate the inhibitor within 1 hour (21). The inhibitor in the membranes starts to decrease as soon as HA activity becomes detectable and is reduced in proportion to the increase in hemagglutinins, but, even when the titer has reached levels of 1:256, some slight inhibitory activity might still be apparent (21, 30), or it may be partly regenerated (31). Since no information as to inhibitor destruction during incomplete virus production was at hand, the following type of experiment was carried out.

Groups of 13- to 14-day-old embryonated eggs were inoculated with 0.5 ml. of undiluted seeds which had been exposed to 37°C. for varying lengths of time. For comparison, native as well as ultraviolet irradiated standard seeds were included in the tests. The technic which has been described elsewhere (21) consisted essentially of collecting chorioallantoic membranes from groups of 4 to 5 eggs at hourly intervals up to the 6th hour, as well as 8 and 24 hours after injection. Allantoic fluids were sampled at 24 hours in order to determine the ID_{50}/HA ratio of the progenies. Suspensions of the membranes were prepared in the usual manner and each preparation was subdivided into (*a*) an untreated portion for the determination of hemagglutinin activity and (*b*) a portion which was heated at 70°C. for 30 minutes (in order to destroy the HA activity) for assay of the inhibitor (which remains intact) by hemagglutination-inhibition tests, using a technic previously described (21).

Fig. 5 shows typical results obtained with 3 different types of seeds. The native standard virus and the heated seed exposed to 37°C. for 4 days yielded progenies in 24 hours with ID_{50}/HA ratios of $10^{5.6}$ and $10^{3.8}$, respectively. The standard seed which had been irradiated by ultraviolet light for 3 minutes had lost its reproductive capacity. On the left-hand side of the chart, the percentage of membrane inhibitor content (taking the activity exhibited shortly after infection as 100 per cent) and, on the right-hand side, the number of HA

units produced are plotted against time after infection. It can be seen that in the series inoculated with standard virus the inhibitor activity began to fall off between 2 to 3 hours after infection, at which time HA was detected first in the membrane. Subsequently, the inhibitor content declined steeply while the HA titer rose rapidly. Little further loss in inhibitor activity was seen after the 6th hour, when near maximal HA titers had been reached in the membrane. Similar results were obtained when heated seed was used as inoculum except that the onset of inhibitor destruction was somewhat delayed in accordance

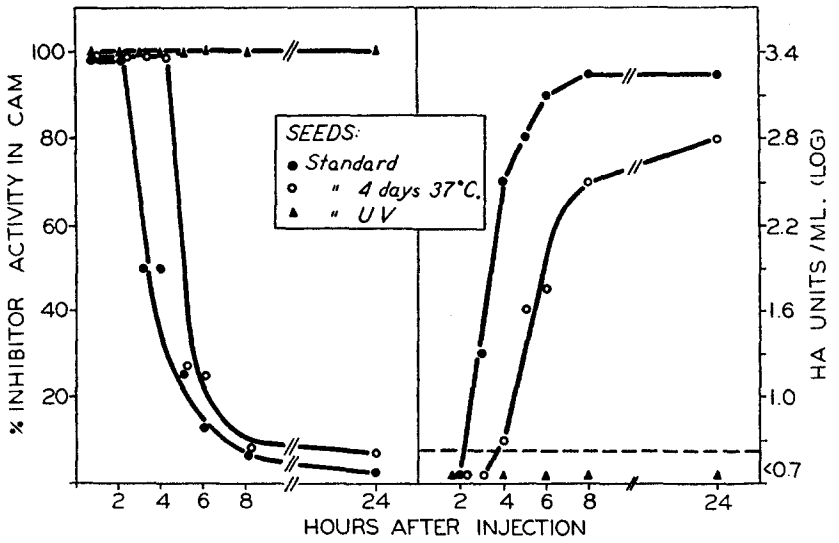

FIG. 5. The inactivation of inhibitor of hemagglutination in the allantoic membrane of chick embryos following injection with undiluted standard seed, native or heated *in vitro* at 37°C. for 4 days.

with a later rise in HA titer. Irradiated virus, on the other hand, did not affect the membrane inhibitor content and no hemagglutinins were produced.

It is apparent then that the production of infectious and of incomplete viruses is accompanied by nearly complete destruction of inhibitor of hemagglutination in the membranes. The discrepancy between the ID_{50}/HA ratios of the allantoic fluids (or media) and the tissue suspensions thus cannot be based on inhibitor activity. On the contrary, since more inhibitor is present in the membranes and its destruction requires greater virus concentrations the discrepancy, if anything, may actually be larger than measured by the technics employed.

The Effect of Incubation at 4°C.—It was thought that if the infectious process could be stopped by some manipulation a difference in response of infectious virus production and the NIHA levels might indicate two different processes,

or, in turn, similar responses would favor the view that both are part of one process, the NIHA being an intermediary stage in development. As a first approach attempts were made to interrupt the process merely by incubating

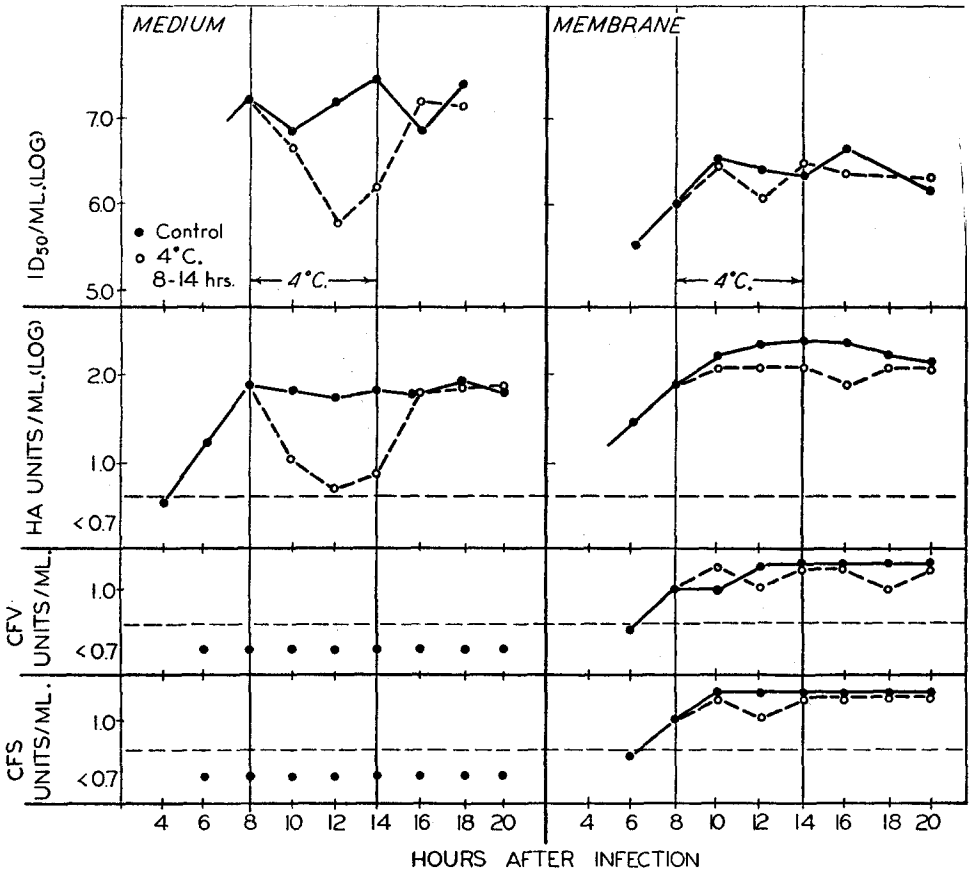

FIG. 6. The effect of cooling to 4°C. on the ID_{50}, HA, CFV, and CFS titers of the allantoic membranes and the 2 hourly yields derived therefrom in deembryonated eggs inoculated with undiluted standard seed.

the deembryonated eggs at 4°C. for a short time during the period of active production and liberation.

Duplicate sets of eggs were infected with 0.4 ml. of undiluted standard virus and deembryonated 30 minutes later in the usual manner. The media were exchanged at 2 hour intervals and by the 8th hour, when production and liberation were expected to have reached their peaks, one set was transferred from the 37°C. incubator to the 4°C. cold room. After 3 further exchanges (6 hours at 4°C.) the eggs were returned to the hot room and further

exchanges of media made there. The media and membranes obtained at the various intervals were assayed in the usual manner.

The results of this experiment are shown in Fig. 6. As can be seen, release of both ID_{50} and HA units into the media fell off rapidly on transfer of the eggs to 4°C. As soon as the preparations were returned to 37°C., liberation resumed at the level of the controls which had been maintained at 37°C. all the time. In the tissues only minor changes were observed which presumably were due to the facts that (*a*) production had not quite reached its maximal rate at the time of chilling and thus further increases were prevented (HA) and (*b*) that some significant liberation still occurred in the first 2 hour period at 4°C., reducing the membrane titers. Thus the virus material in the tissue remained essentially stationary until the temperature was raised again.

The Effect of Enzyme Inhibitors.—The above experiment did not provide the desired answer since at 4°C. production as well as liberation stopped and the *status quo* was maintained. It was thought next that this problem might be solved by search for enzyme inhibitors which would be capable, on addition to the medium of deembryonated eggs, to interrupt temporarily production of virus materials in the tissues without destroying the cells, so that production could be resumed on removal of these substances. It was found that potassium cyanide in 0.01 to 0.001 M concentration was suitable for this purpose.

In preliminary differential growth curve experiments cyanide was added for 2 hour periods to the medium at increasingly longer intervals following infection and deembryonation and the effects were evaluated in terms of liberation of ID_{50} and HA units into the media. The results of one experiment are shown in Fig. 7. It can be seen that when cyanide was present in the medium for 2 hours during the first 6 hours after infection, liberation of infectious virus and of detectable quantities of hemagglutinins was delayed for 4 and 6 hours, respectively, as compared to the controls. The ID_{50} titers of the treated and untreated preparations reached ultimately comparable levels. With respect to the HA titers the results were similar except that the presence of cyanide during the very early period (1 to 3 hours) apparently had a somewhat more pronounced effect in that the titers in the treated group on recovery did not quite reach those of the controls. If the cyanide was added during the period of increasing liberation (6 to 8 hours) the curves of both ID_{50} and HA release remained on a plateau and resumed their ascent slowly after removal of the cyanide until they reached the control levels. In case liberation had attained maximal levels (8 to 10 hours or later) release of both activities fell off immediately but with the removal of cyanide increased liberation became gradually apparent and again the control levels were reached in time. In other experiments cyanide was added also at later periods (16 to 18 or 20 to 22 hours) with similar, although somewhat less striking results.

With the information thus obtained experiments were set up, including ex-

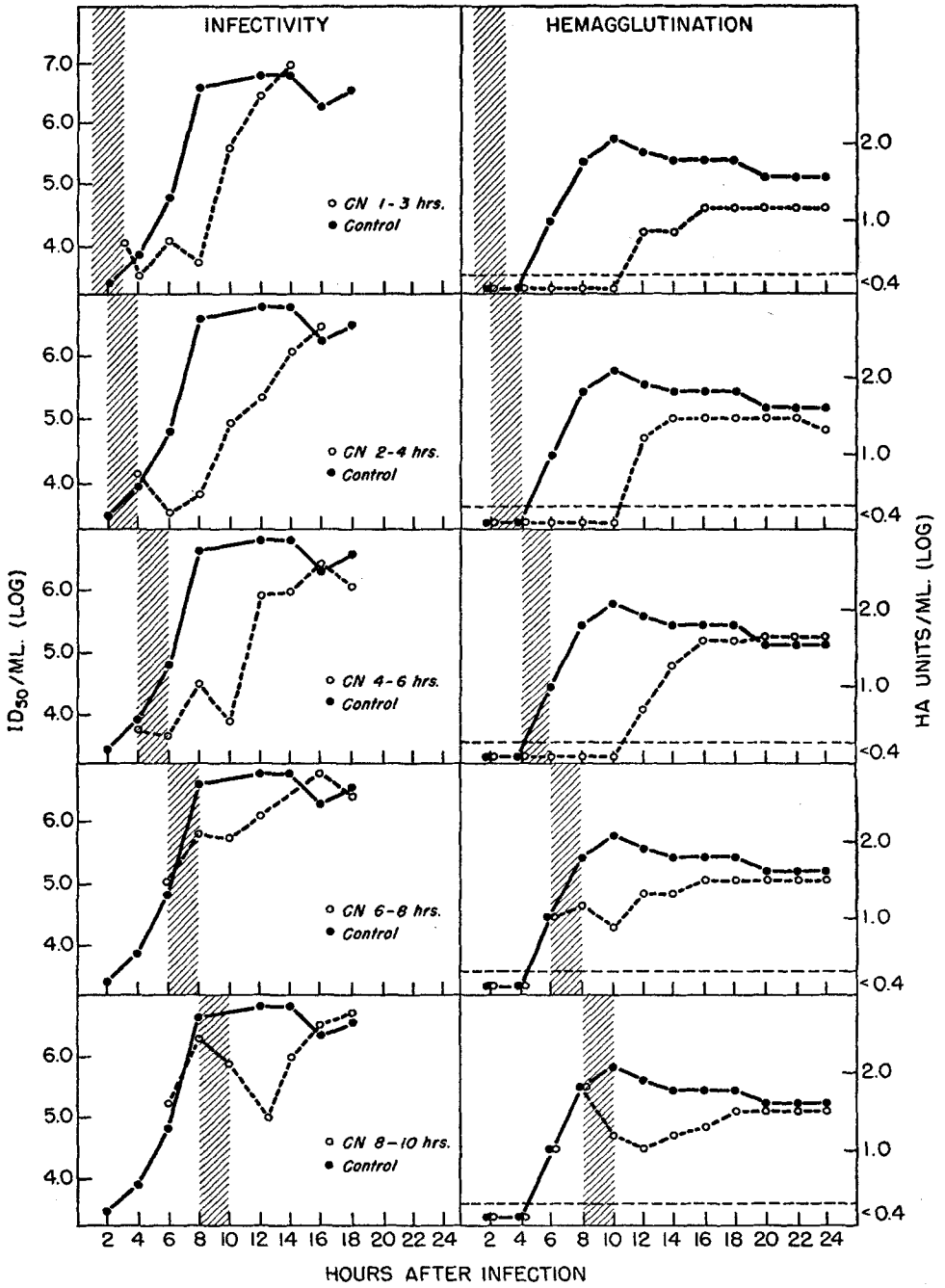

FIG. 7. The transitory depression in the 2 hourly yields of ID_{50} and HA units resulting from the addition of potassium cyanide to the media of deembryonated eggs for 2 hour periods at varying intervals after infection with undiluted standard seed.

amination of the membranes as well. The cyanide was added from the 10th to 12th hour in order to assure that the infectious process had reached maximal production and liberation. It can be seen in Fig. 8 that as soon as the chemical was added the titers of infectious virus and hemagglutinins fell within the mem-

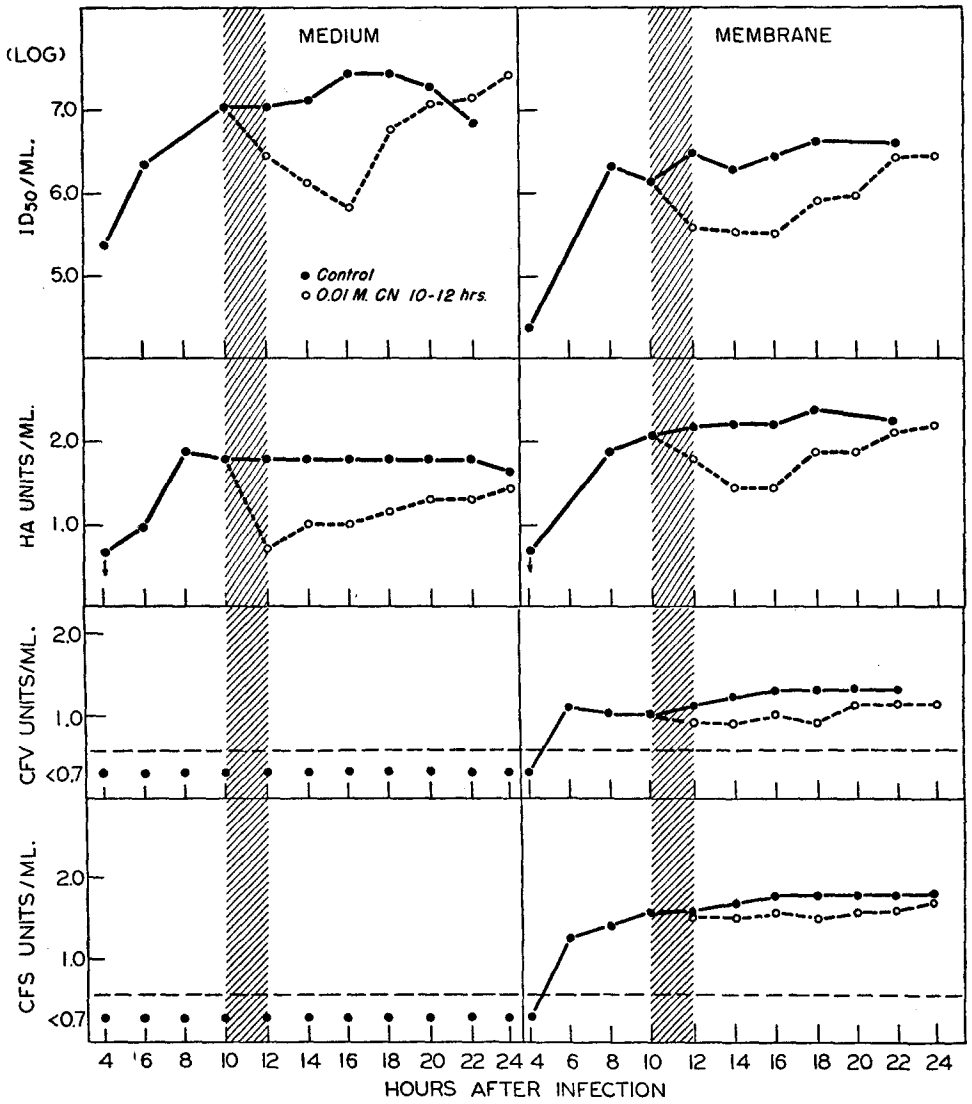

FIG. 8. The transitory depression by potassium cyanide of the ID_{50}, HA, CFV, and CSF titers of the allantoic membranes and the 2 hourly yields derived therefrom in deembryonated eggs inoculated with undiluted standard seed.

branes and the fluids and after removal all titers rose again reaching ultimately the levels of the controls. The ID_{50}/HA ratios of the membranes or the fluids did not change significantly during cyanide-induced depression in titers. The CFV and CFS titers appeared to be somewhat less affected. Thus, one can conclude that the non-infectious ("excess") hamagglutinins in the tissues represent not a stable population which is "just sitting there," but are part of a dynamic process.

DISCUSSION

In the present study it has been shown that in the process of multiplication of influenza virus non-infectious hemagglutinins appear in relatively greater quantities in the infected allantoic membrane than in the virus material released therefrom. This is evident from the ID_{50}/HA ratios which were always significantly lower in the tissues than in the allantoic fluids of intact chick embryos or in the media of deembryonated eggs. The differences in ratios were of a similar order regardless of whether the inocula yielded small (ST seed) or considerable quantities of NIHA (ΔST or UP seeds). Correlation of assays derived from differential growth curve experiments in deembryonated eggs revealed that the differences in the ID_{50}/HA ratios of tissue-bound and extracellular virus materials were essentially due to the fact that in a given 2 hour interval up to 10 times the amount of infectious virus was released which was present in the membranes at the onset of the period, but only about ¼ of the hemagglutinin was liberated. This situation obtained not only under conditions when non-infectious hemagglutinins are produced in appreciable concentration, but also to some extent when dilute standard inocula were employed which lead to liberation of progenies consisting almost entirely of infectious virus. Thus, even under standard conditions of influenza virus multiplication, some NIHA is found in the tissues, but none is released as such. This has been demonstrated recently also by Granoff (19).

It was conceivable that the relatively larger quantities of NIHA in the tissues represented, at least in part, a stable population not directly related to the growth processes. The experiments with cyanide clearly excluded this possibility in that as soon as the inhibitor was added both the ID_{50} and HA levels in the tissues decreased and their liberation was reduced correspondingly. When the cyanide was subsequently removed both activities increased again gradually in parallel in the tissue and liberation was resumed without significant changes in the ID_{50}/HA ratios. Thus, one can conclude that the NIHA forms part of a dynamic process.

As has been pointed out in the introduction, the NIHA obtained under the various conditions described differs in some aspects from heat-inactivated standard virus (15) and various forms of incomplete virus may be discerned (9, 10, 19). This is further emphasized by the results of studies on the com-

ponents obtained in consecutive undiluted passages employing physical, chemical, or biological technics. The sedimentation constants of the predominant components obtained decrease progressively (32, 33), but heating of standard virus at 56°C. does not change its sedimentation rate (32). Electronmicroscopy of incomplete virus revealed particles different in some aspects from standard virus (10, 34). The lipid content appears to increase on consecutive passages (35) and the concentration of ribonucleic acid decreases (36). The amount of soluble (S) antigen released from elementary bodies by the ether technic of Hoyle (37) is reduced with the decrease in the ID_{50}/HA ratio of the progeny and less is found in virus particles derived from the allantoic membrane than those obtained from the fluids (38). The progenies of heated standard virus have not as yet been studied to any great extent and compared with undiluted passage seeds except that on further undiluted passage they yield results similar to those of UP series (15) and that the content of soluble antigen also of these progenies decreases with a decrease in the ID_{50}/HA ratio (38). Finally, the NIHA observed in the tissues following infection with dilute standard virus appears to be smaller in size or less dense than fully infectious virus since the two can be separated readily by centrifugation at 20,000 R.P.M. for 20 minutes (19). These various observations clearly show that incomplete virus is not a single entity.

In answer to the question whether the incomplete forms of virus represent altered seed virus or are actually produced in the chick embryos the following points can be made, which in part have been considered previously (16). (*a*) The HA concentration in the yields may increase more than 100-fold over that of the seed (8–11, 16) and there is no evidence that the seed virus particles disintegrate into HA units sufficiently small and numerous to account for increases in activity of that magnitude; (*b*) the cyanide experiments showed that the NIHA forms part of a dynamic process; (*c*) the incomplete forms appear in the tissues and are released therefrom after latent periods comparable to those seen under standard conditions (9, 11, 15) and once production and liberation of the virus materials have reached their peaks, they continue at nearly constant rates and in unchanging relative concentrations of ID_{50} and HA units for periods in excess of 24 to 30 hours (15, 24); (*d*) as shown above, concurrent with the appearance of incomplete virus inhibitor of hemagglutination in the tissues decreases rapidly, as was noted previously on infection with standard virus (21, 30, 31), whereas ultraviolet-inactivated virus has not such effect although it is enzymatically active; and, finally, (*e*) P^{32} is incorporated not only into the NIHA components derived from HeLa cells (39), but also into those obtained from infected chick embryos at a rate similar to that seen in standard virus reproduction (40).

These various observations clearly favor the conclusion that the hemagglutinins are actually produced in the allantoic membranes. However, one may

consider that a more complex situation is involved in that initially complete virus is released which subsequently is immediately adsorbed onto already infected cells (since saturation inocula were mostly employed) and that this additionally adsorbed virus is then rapidly inactivated and changed. It will be recalled that entry of infectious virus into normal cells leads to rapid disappearance of all virus activity (2, 22). In the already infected cell this eclipse phenomenon may not necessarily occur but only partial inactivation and alteration of the additionally adsorbed virus. This possibility would seem to be excluded, however, by the early appearance of NIHA in the first infectious cycle, the constant rate of its production and release, referred to above, and finally by the fact that addition of large amounts of infectious or inactivated virus to the medium of deembryonated eggs after virus production is well under way has no effect upon the composition of the virus materials in the tissues or on the progeny liberated into the medium (18).

It also has been considered that inactivation of infectious progeny may occur rapidly in the infected cells under conditions of overwhelming infection prior to its liberation, particularly since the rate of reproduction appears to be reduced (8, 17). There are several points against this view. The inactivation rates of extracellular PR8 virus at 37°C. *in vitro* or in the allantoic fluid of infected chick embryos differs according to the lines employed. Horsfall reported a half-life of about 2½ hours (8), whereas it was found to be closer to 7 in this laboratory (15), which does not support the inactivation theory as readily. The total production of hemagglutinins following injection of native or heated standard virus or of undiluted passage seeds may be of a similar order; yet the ID_{50}/HA ratios of the progenies may show differences of more than 3 $\log_{10}$ units, even in the very first hours of measurable activity. To explain these differences on the basis of heat inactivation would require rates far in excess of those seen with extracellular virus. Aside from these considerations, the fact remains that the NIHA components obtained differ in various properties from heat-inactivated virus, as discussed above.

It appears from these observations that the incomplete virus is produced as such and does not represent complete virus which has been inactivated subsequent to its production. It would be the simplest assumption to consider the various forms of incomplete virus as intermediary stages in the development of infectious virus. After inoculation of dilute standard seed NIHA is found only in the tissues but none (19) or only questionable quantities (18) are released as such. This would strongly suggest an intermediary stage (19). With undiluted inocula of standard virus the HA titers rise in the tissues 1 to 2 hours prior to detectable liberation of hemagglutinins into the medium of deembryonated eggs as was noted previously also in experiments in the intact chick embryo (1–3). Increases in ID_{50} levels in the membranes are observed nearly simultaneously with the first evidence of release of infectious virus into the media. On prolonged incubation in deembryonated eggs a reduction in HA

activity was noted in some experiments in the tissues prior to a decrease in ID_{50} titers (Fig. 1). These data may be interpreted to mean that non-infectious hemagglutinating components are formed first and then converted into infectious virus shortly before or during liberation as has been suggested by Hoyle (3) and, correspondingly, HA production falls off earlier, while conversion into infectious virus still goes on, so that a nearly constant ID_{50} level is maintained for a few hours longer. However, under the conditions of overwhelming infection with standard virus NIHA is found not only in the tissues but some of it is relased as such from the cells in addition to infectious virus. Injection of undiluted passages or partially heat-inactivated standard seeds leads to liberation of large quantities of NIHA and, therefore, one may not as readily accept the suggestion that these incomplete particles are also precursors of mature virus. It will be recalled, however, that the tissues always contain greater proportions of NIHA than the liberated progenies, the ID_{50}/HA ratios being lower in the former, and that the cyanide experiments strongly indicated that the non-infectious components were part of a dynamic process. It is suggestive, therefore, that some of the NIHA of the tissues is not shed as such but possibly only after conversion into infectious virus. As pointed out, the release of incomplete virus is restricted to situations in which the host cells had the opportunity to adsorb several virus particles within a narrow period of time (18), be they all fully infectious, or partly non-infectious, or even possibly only non-infectious HA components (14, 16). Although under conditions of multiple exposure the infectious process conceivably may take an altogether aberrant course; a possibly simpler assumption would be that reproduction is stunted at some stages, and completion of virus cannot be attained for as yet undetermined reasons.

A number of hypotheses have been advanced which could account for such interruptions of the infectious process. (*a*) It has been suggested (6) that the production of intermediary components may exceed the capacity to complete the virus particles; *i.e.*, certain metabolites, needed for completion, are in short supply and produced at too slow a rate to keep up with the demand. In that case, one might expect that late in the incubation period such metabolites would gradually become available and, indeed, in several growth curve experiments a late unexplained rise in the ID_{50}/HA ratios of the progenies has been observed (9, 11). (*b*) It has been proposed (11, 12) that on rapid overloading of a cell with virus some of the seed components may interfere with a late stage in virus development. In this regard, it is of interest to point out that the injection of large quantities of homologous ultraviolet-inactivated virus within a few hours following infection with dilute standard seed may prevent or decrease the production of infectious virus in the membrane (41, 42). (*c*) It has been considered (17, 43) that on multiple adsorption of virus particles the cells are damaged and thus no longer capable of supporting complete infectious cycles or, at least, the rate of reproduction of infectious virus is

reduced (8, 17). In support of this hypothesis, reference has been made to the toxic properties of influenza virus (44, 45). This relationship must be considered with some reservation, however, since it has been the common experience that virus preparations derived from dilute standard seeds reveal the greatest toxicity, yet being composed almost entirely of infectious particles, would produce relatively little incomplete virus. Conversely, virus preparations obtained by infection with large inocula are distinctly less toxic but are expected to produce appreciably more incomplete virus. Although these various hypotheses would seem to account for interruption of the infectious process possibly at different stages of development (depending upon the types of seed employed), they would not necessarily explain premature release of the various forms of incomplete virus from the cells unless liberation is conditioned on the pressure of the total population of virus particles, regardless of their developmental stage and not only on that of infectious virus. If release is achieved by damage to the cell wall and overwhelming infection leads more rapidly to damage of the cells (hypothesis (*c*)), premature leakage of virus materials may result. As pointed out, there are objections to the assumption that the toxic activity of the virus is the damaging factor, but one may possibly consider the receptor-destroying enzyme activity of the virus in this respect, which appears to be of similar order in standard inocula, whether native or heated at 37°C., and in undiluted passage seeds (29, 46). The inactivation by the enzyme of internal or external mucoprotein inhibitors of the cells conceivably could expedite egress of virus materials at various stages of development.

It has not been possible to arrive at a conclusive answer as to the role of incomplete forms of virus in the infectious process. Whether all or only some of them represent intermediary stages, or possibly aberrant forms of virus development cannot be decided at the present stage. The data presented do not contradict the assumption that they are intermediary stages but they also fail to prove that they are. Nothing short of completion of these particles by some manipulation would ultimately prove the point. The fact that cortisone appears to reduce incomplete virus production (47) may be a step in that direction. It is conceivable that this hormone prevents more extensive damage to the cell, thus permitting completion of virus.

SUMMARY

Studies have been reported concerning the relationships between virus materials found in the allantoic membranes and media of eggs deembryonated after injection of Standard (ST), heat-inactivated (37°C.) standard (ΔST), and undiluted passage (UP) seeds.

It was found that the membranes always contained relatively more noninfectious hemagglutinins (NIHA) than the media and, correspondingly, the ratios between infectious virus and hemagglutinin units (ID_{50}/HA) in the

tissues were up to 1.5 $\log_{10}$ units lower than in the liberated progeny. These differences were seen not only following inoculation of undiluted ST, ΔST, and UP seeds, the progenies of which always contain considerable proportions of NIHA, but also when dilute ST inocula were employed which lead to the liberation of only infectious virus. Essentially similar differences in the ID_{50}/HA ratios were observed also in the allantoic membranes and fluids obtained from growth curve experiments in the intact chick embryo employing the various types of seeds.

In correlating the liberated virus materials in the media of deembryonated eggs to those in the membranes it was noted that in any given 2 hour interval during the phase of nearly constant production and release up to 10 times the quantity of infectious virus was shed as was present in the tissues at the onset of that period. In contrast, only about ¼ of the hemagglutinins were released during the same time. The viral (V) and soluble (S) complement-fixing antigens were found in the tissues but no detectable quantities were released during any 2 hour interval.

The NIHA in the membranes apparently is located within the cells since it could not be released by the action of RDE. Intracellular inhibitors of hemagglutination were readily inactivated following inoculation of undiluted ST, ΔST, or UP seeds but not when ultraviolet-inactivated virus was used. The inhibitor activity decreased in proportion to the hemagglutinins produced.

Transfer of infected deembryonated eggs to the cold room after production and liberation of progeny were well under way immediately halted further release but in the tissues the *status quo* was maintained and release was resumed on return to the 37°C. incubator. The addition of potassium cyanide to the medium of deembryonated eggs at 37°C. during the period of nearly constant production and release of virus material reduced immediately and to comparable extents the ID_{50} and HA titers in the tissues and liberation decreased in proportion. On removal of the cyanide 2 hours later, both titers in the tissues gradually returned to those of the untreated control eggs with a corresponding increase in liberation. The ID_{50}/HA ratios were not affected by these manipulations. It is concluded that the NIHA in the membranes forms part of a dynamic process.

An attempt has been made in the discussion to integrate the present results with previous observations concerning the formation of incomplete forms of virus and their nature and role in the infectious process.

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