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

XIII. Some Aspects of Non-Infectious Virus Production

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(Received for publication, February 15, 1956)

It is well established (1–8) that appreciable quantities of non-infectious hemagglutinins (NIHA) are obtained in the chick embryo following inoculation of large quantities of standard influenza virus; *i.e.*, of seeds amongst which the vast majority of virus particles are infectious. In such seeds the ratio between infective doses and hemagglutinating units (ID_{50}/HA ratio) exceeds $10^{6.0}$. Analysis of the conditions for NIHA production revealed that multiple adsorption of seed virus onto individual cells is required. NIHA production is considerably increased, if the seeds contain high proportions of non-infectious virus; *i.e.*, either "incomplete virus" as obtained in undiluted passage series according to von Magnus as cited in references 8–11 or standard virus inactivated at 37°C. (6, 7, 12–14). Inoculation of any one of these types of seeds in high dilution will yield progenies almost free of NIHA. This has been considered a result discrepant with those mentioned above since, depending on the dilution of the seeds after one, two or more infectious cycles each cell will gradually have the opportunity to adsorb numerous virus particles and thus conditions for NIHA production should ultimately be established. In order to obtain more information on this apparent discrepancy, a number of experimental approaches have now been used which concerned the initial adsorption period prior to deembryonation, the effects of prolonged incubation of the eggs after deembryonation and of sudden secondary exposures of the cells to large quantities of virus, and the role of cell receptors in the production of NIHA. The results are recorded below.

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

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Methods and Materials

Virus Preparations.—The PR8 strain of influenza A was used throughout for infection of 11- to 14-day-old chick embryos. *Standard seeds* (ST) were prepared by allantoic inoculation of about 10^4 ID_{50} and the allantoic fluids were collected after further incubation of the eggs at 36 to 37°C. for 24 hours; *i.e.*, when the infectivity titers were still rising or just had reached their peaks (7, 8). Under these conditions the yields consisted almost entirely of infectious virus and revealed ID_{50}/HA ratios in the order of $10^{6.5}$. *Heated standard seeds* (Δ ST) were derived from standard seeds by exposure to 37°C. *in vitro* for several days according to the technic described (12). *Undiluted passage seeds* (UP) were produced essentially by the method of von Magnus (9) as recorded elsewhere (8). The incubation periods employed for the consecutive passages of undiluted infected allantoic fluids were restricted to 24 hours. *Interfering virus preparations* consisted of standard seeds of the homologous virus (PR8) or the heterotypic Lee strain of influenza B which, following dialysis against 20 volumes of phosphate buffered saline solution, had been irradiated in Petri dishes on a mechanical rocker by ultraviolet light for 3 to 4 minutes by the method described previously (15). In order to assure complete inactivation the virus material was transferred to fresh Petri dishes after the first 90 to 120 seconds of irradiation.

Growth Curves.—The technics employed for growth curves *in ovo* and in deembryonated eggs (4) have been fully described (2) and (16), respectively.

Receptor-Destroying Enzyme (*RDE*).—This enzyme was kindly supplied by Dr. Richard Haas of the Behring Werke, Marburg, Germany. The preparations contained approximately 2560 units per ml.

Other technical details are described in the text.

EXPERIMENTAL

The Effect of Variation in the Initial Adsorption Period Prior to Deembryonation.—The sooner eggs are deembryonated following infection the less is the opportunity afforded for adsorption of the seed virus onto the allantoic membrane. When undiluted standard seed was employed and deembryonation was performed after 5 and 60 minutes respectively, the differential yields obtained at hourly exchanges of the media revealed considerable differences (Table I). In the 5 minute series release of HA became apparent only by the 7th hour; *i.e.*, 1 hour later than when deembryonation was delayed for 60 minutes. By the time liberation had achieved constant rates it was seen that more than 10 times as much infectious virus but only about 2 times the quantity of HA was obtained per hour on the average from the eggs deembryonated 5 minutes after infection as compared to the 60 minute group. Correspondingly, the ID_{50}/HA ratios in the yields differed by a factor of about 10 in the two groups. It is evident that mainly infectious virus production had been reduced by the prolonged adsorption period. Since the non-adsorbed seed virus was largely removed by the deembryonation procedure this effect may be ascribed either to the entry of several virus particles into, or to multiple adsorption of virus particles onto, increasingly more host cells during the longer adsorption period. When dilute inocula (10^{-4}) were used no such differences were apparent.

Changes in the ID_{50}/HA Ratios of the Virus Material Liberated into the Media

of Deembryonated Eggs on Prolonged Incubation.—In previous experiments concerning differential growth curves in deembryonated eggs, the emphasis has

TABLE I

Hourly Yields of Virus Material from Eggs Deembryonated after 5 and 60 Minutes, Respectively

Inoculum	Time of harvest	DE, 5 min.			DE, 60 min.		
		ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA
	hr.	*log*	*log*	*log*	*log*	*log*	*log*
10^0 0.4	5	—	<0.7	—	4.0	<0.7	—
	6	—	<0.7	—	5.1	0.9	4.2
	7	6.3	0.7	5.6	5.6	1.2	4.4
	11	7.3	1.5	5.8	5.5	1.2	4.3
	12	6.8	1.5	5.3	5.8	1.2	4.6
	24*	6.6	1.3	5.3	5.2	1.0	4.2
	26	6.7	1.3	5.4	5.8	1.3	4.5
10^4 0.4	12	—	<0.7	—	3.8	<0.7	—
	24*	7.4	1.0	6.4	7.8	1.3	6.5
	26	7.3	1.3	6.0	7.5	1.3	6.2
	28	—	1.3	—	7.3	1.2	6.1

* Average hourly yields calculated from cumulative results from the 12th to 24th hour.

TABLE II

Decrease in ID_{50}/HA Ratios of Differential Yields on Prolonged Incubation

Experiment No.	Inoculum		Time of DE	Period of observation	ID_{50}/HA ratios of differential yields		
	Dil.	Vol.			Initial*	Final*	Decrease
		ml.	*min.*	*hrs.*	*log*	*log*	*log*
1	10^0	0.5	5	5–36	5.5	5.0	0.5
	10^{-3}	0.5	5	13–36	6.2	5.4	0.8
2	10^0	0.4	60	6–27	4.3	4.5	+0.2
	10^{-4}	0.4	60	24–34	6.5	6.0	0.5
3	10^0	0.2	5	8–40	5.1	4.7	0.4
4	10^0	0.2	30	6–36	6.3	5.8	0.5

* Mean of first or last 3 harvests, respectively.

been placed on the fact that liberation of progeny from the infected cells proceeded at nearly constant rates for periods in excess of 30 hours (18). Although this in general is true, the liberation of infectious virus may show, on occasion, an over-all slight decline, whereas the release of hemagglutinin often is less affected. As a result, the ID_{50}/HA ratios may decline slightly over the long

experimental periods. This is evident from the data summarized in Table II. It is seen that after inoculation of undiluted standard virus the ratios of the 2 hourly yields may ultimately decrease by as much as half a $\log_{10}$ unit during 30 or more hours of observation. However, in Experiment 2, in which deembryonation was not performed before 60 minutes following infection and the period of observation extended only over 24 hours there was no such decline. In the two instances in which diluted seeds were employed similar decreases in the ratios were noted. These results suggest that with prolongation of incubation the yield of NIHA increases, following infection with large inocula, and some NIHA is released in time even after inoculation of diluted standard seeds.

The Effect of Delayed Deembryonation Following Infection with Dilute Standard Virus on the ID_{50}/HA Ratios of the Yields.—The following experiment was carried out in an attempt to test more thoroughly the above suggestion that some NIHA is ultimately produced after dilute inocula.

A sufficient number of 11-day-old chick embryos was divided into 5 groups. These were injected with 0.2 ml. of a standard seed diluted to 10^{-5}, 60, 48, 32, 24, or 12 hours prior to deembryonation. The allantoic fluids were saved. The media were then exchanged at 2 hour intervals for 10 hours. The yields of infectious virus and of hemagglutinins in the exchange media as well as in the allantoic fluids were assayed in the usual manner.

The results are shown in Fig. 1. The data on the allantoic fluids obtained at the time of deembryonation of the 5 groups agree with past experience; that is, the concentration of infectious virus reached its peak in about 36 hours and thereafter showed a slight decline. The hemagglutinin curve, on the other hand, continued to rise slowly up to the 60th hour. In accordance with these results the ID_{50}/HA ratios decreased from $10^{6.7}$ to $10^{5.3}$. This result may be explained to a large extent by inactivation of liberated infectious virus accumulating in the allantoic fluid on prolonged incubation *in ovo* (7, 12), the rate of inactivation exceeding somewhat the release of new infectious virus late in the experimental period. However, the results obtained on deembryonation suggest that this might not be the only explanation. The 5 exchange media of the groups deembryonated after 12 and 24 hours gave closely similar results. The yields contained on the average $10^{8.2}$ and $10^{8.4}$ ID_{50} ml., respectively, and $10^{1.9}$ and $10^{2.0}$ HA units; *i.e.*, average ID_{50}/HA ratios of $10^{6.3}$ and $10^{6.4}$ were found. In the groups deembryonated successively later (36, 48, and 60 hours), the average yields of infectious virus decreased to $10^{7.3}$, $10^{7.1}$, and $10^{5.7}$ ID_{50}, respectively, and those of hemagglutinins to $10^{1.5}$, $10^{1.2}$, and $<10^{0.7}$ units. The corresponding ID_{50}/HA ratios were thus $10^{5.8}$, $10^{5.9}$, and more than $10^{5.0}$. It is apparent that the ratios of the virus material released late in the incubation decreased by about half a log. However, during the period of most active production and release, the first 30 to 36 hours (18), there was no significant deviation from the ratio compatible with standard virus.

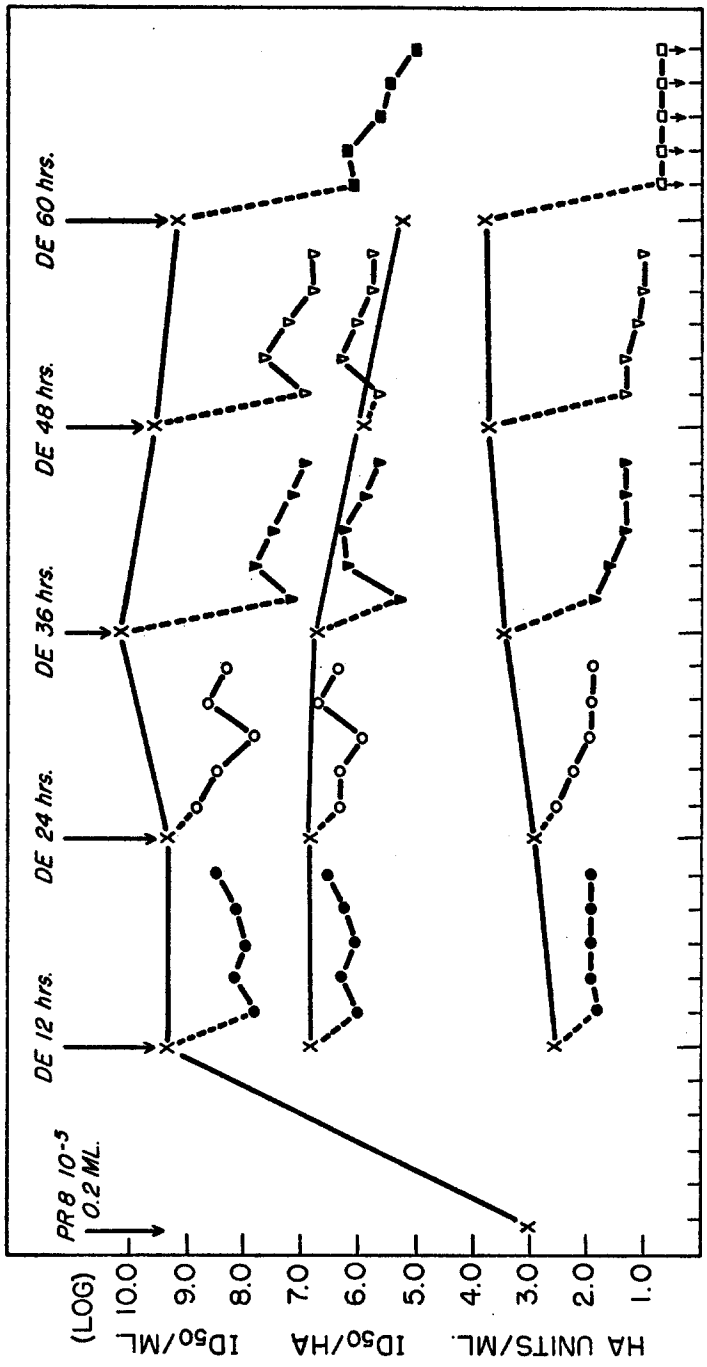

FIG. 1. The effect of delayed deembryonation following infection with dilute standard virus on the yields obtained in differential growth curves.

It is generally assumed that after inoculation of a dose of virus as employed here (10^4 ID_{50}) the infectious process proceeds sufficiently rapidly so that certainly by 24 hours all available cells are infected. Yet, in the subsequent 12 hours when presumably each cell had the opportunity to adsorb numerous additional virus particles no release of NIHA became apparent. After the 36th hour the concentrations of virus materials decrease in the membranes, presumably, because production falls off and the release into the medium likewise decreases rapidly (19). It is conceivable that under these conditions the rate of liberation is decreased and that thus some inactivation may occur of finished virus prior to its liberation or that as yet incomplete virus materials are released. In any event, the results presented in the above two sections show that liberation of NIHA after dilute inocula is minimal and occurs at most at the very end of the active period of viral multiplication.

The Effect of Additional Adsorption of Virus on the ID_{50}/HA Ratio of the Progeny.—It is evident from the above experiments that conditions yielding NIHA do not readily develop following inoculation of dilute inocula of virus, even though ultimately multiple infection of cells must occur. This may have several reasons: (*a*) the virus which will become available for additional adsorption onto individual cells during the incubation period will be fully infectious under the conditions under which it is produced, and multiple infection with fully infectious virus produces the von Magnus effect only to a relatively small extent (8); and (*b*) there is no "burst" phenomenon which would lead to sudden overloading of remaining susceptible cells. On the contrary, a rather slow saturation is expected since all cells, whether infected initially by the seed or by the progenies of the first or subsequent cycles, will release virus at nearly constant rates for long periods of time (18). It was of interest, therefore, to determine what effect sudden addition of large amounts of virus would have upon the yields in the intact chick embryo as well as in deembryonated eggs.

In the first experiment to be reported a sufficient number of 10-day-old chick embryos were infected with approximately 10^6 ID_{50} of standard virus (ID_{50}/HA ratio of $10^{6.3}$); *i.e.*, the virus-cell ratio was less than 1. Individual groups of these eggs were then injected after increasingly later intervals with 0.5 ml. of a 4th undiluted passage (UP) seed ($10^{6.6}$ ID_{50}, $10^{3.0}$ HA units). The allantoic fluids were collected 24 hours after the initial inoculation and the ID_{50} and HA levels were determined.

The results are shown in Table III. As can be seen, up to the 6th hour following infection with dilute standard virus, a second injection of UP seed still influenced the results and the virus materials obtained were indistinguishable from the virus material derived from inoculation of the UP seed alone. When the UP virus was injected at the 12th hour or later it had no influence on the initial infection and standard type virus was released. Since the initial dose of standard virus was relatively large it is reasonable to assume that by the 12th hour all cells were infected. At that stage the conditions had changed sufficiently so that production of NIHA could no longer be established.

The above experiment indicated that conditions for NIHA production can be established only within a relatively short period of time after infection; that is, when either sufficient numbers of uninfected cells are still available or when infection of cells by standard virus particles has not gone beyond the initial stages. In order to analyze the situation further, attempts were made to infect nearly all the available cells initially and to expose them subsequently after deembryonation at various times to excessive amounts of active or ultraviolet-inactivated homologous or heterotypic virus. Accordingly the following experiment was performed:—

TABLE III

Injection of UP Virus Following Infection with Dilute Standard Virus

First injection	Second injection		Harvest			
	Material	Time	Time	ID_{50}/ml.	HA/ml.	ID_{50}/HA
		hrs.	*hrs.*	*log*	*log*	*log*
	None	—	1	<4.0	<0.4	—
		—	24	9.8	3.6	6.2
PR8 standard 10^{-3}, 0.2 ml. ID_{50}/HA $10^{6.3}$	PR8 UP 4 10^{0}, 0.5 ID_{50}/HA $10^{3.6}$	0	24	7.6	2.9	4.7
		1	24	7.8	3.0	4.8
		3	24	7.2	3.0	4.2
		6	24	7.8	2.9	4.9
		12	24	9.7	3.3	6.4
		24	24	9.8	3.6	6.2
None		0	24	7.5	2.8	4.7

A sufficient number of 15-day-old chick embryos was inoculated with 0.2 ml. of standard seed diluted 1:3 and deembryonation was performed 10 minutes later. Under these conditions enough virus was injected to allow several virus particles per cell. However, the results to be discussed indicate that not every cell was infected initially because of the early deembryonation. The eggs were then divided into 4 major groups. The first served as control, the second was exposed to the various virus preparations during the 2 to 4 hour period; the third during the 4 to 6 hour interval; and the fourth from the 12th to 14th hour. Groups 2 to 4 were then divided into 4 subgroups each. To the eggs of group (*a*) were added $10^{4.5}$ HA units of standard PR8 virus (6 ml. dialyzed allantoic fluid plus 4 ml. of glucosol); to group (*b*) the same preparation but inactivated by ultraviolet light; to group (*c*) $10^{4.5}$ HA units of standard active influenza B virus (Lee); and to group (*d*) irradiated Lee virus. The media were exchanged every 2 hours and their activities analyzed in the usual manner.

The results are summarized in Fig. 2. On the left, the data concerning the effects of additional exposure to active or irradiated PR8 virus are given, and on the right the corresponding values obtained on addition of the Lee preparation. In the control group, liberation of hemagglutinins was noted not before the 10th hour which indicates that not all cells were initially infected since

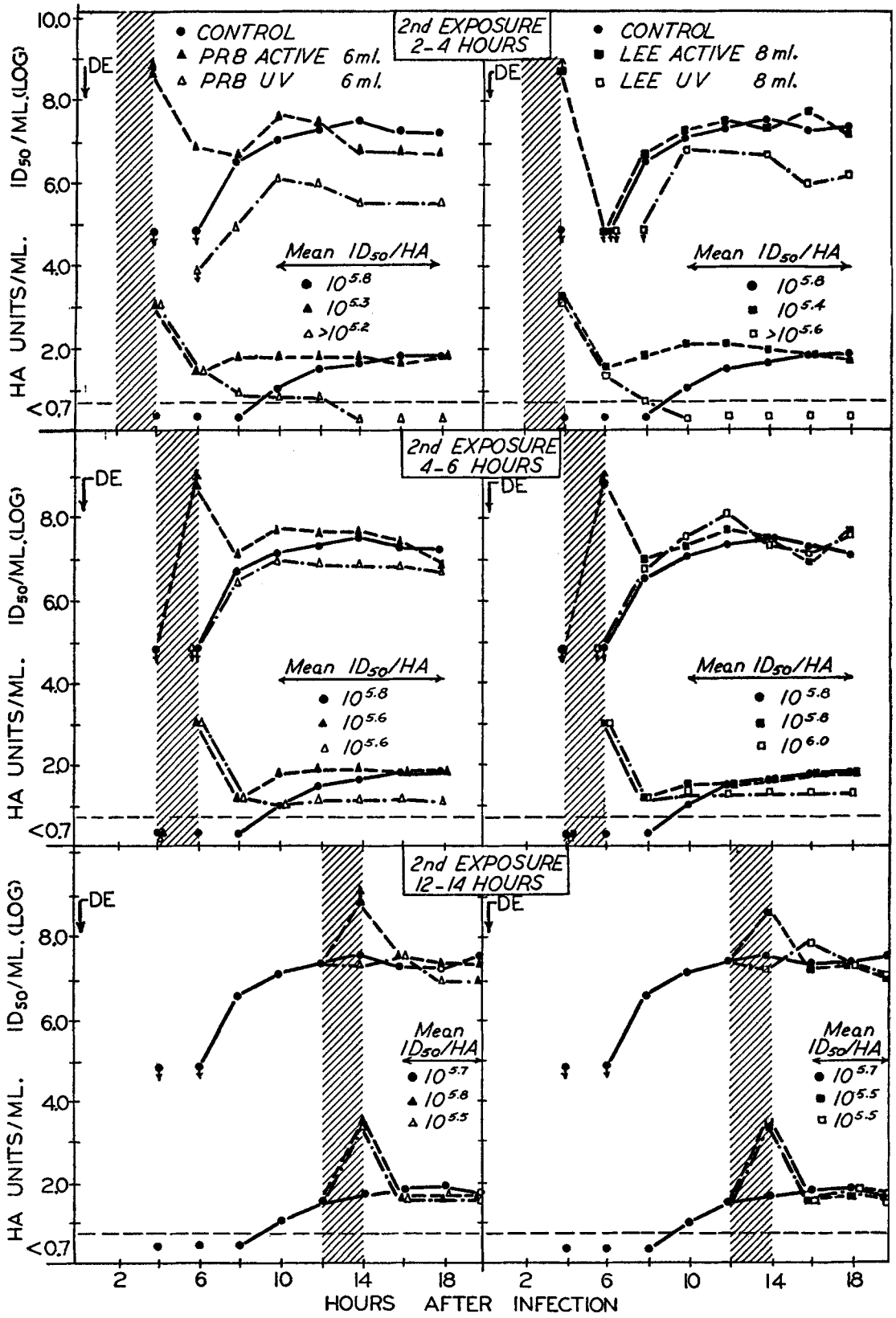

FIG. 2. The effect of addition of large amounts (6 to 8 ml.) of homologous or heterotypic, active or ultraviolet-irradiated virus to the media of deembryonated eggs for 2 hour periods at various times after infection with a near saturation inoculum of standard virus (0.2 ml. $10^{-0.5}$). Deembryonation was performed 10 minutes after primary infection.

undiluted inocula and later deembryonation usually yields HA by the 6th hour. A rise in infectivity was noted by the 8th hour and the initial ID_{50}/HA ratio was slightly greater than $10^{6.0}$, but it subsequently decreased gradually to $10^{5.6}$ in agreement with the data recorded above. In the groups additionally exposed to virus materials from the 2nd to 4th hour, several results are apparent. Superinfection with *active* PR8 or Lee virus led to earlier release of HA and the titers were higher up to the 12th or 14th hours than in the controls. In the influenza B series the early yields of hemagglutinins were identified as Lee components in that they were neutralized by specific anti-Lee sera but not by anti-PR8. The infectivity levels after the 6th hour were not significantly different from those of the controls. Thus, the ID_{50}/HA ratios in the yields were reduced mainly in correspondence to the greater HA levels by at most 0.5 $\log_{10}$ units on the average. The addition of *irradiated virus* preparations at this time was still capable of inducing interference to the extent that only small or non-detectable quantities of HA were released and the ID_{50} levels attained were lower than those measured in the controls or in the groups exposed to additional infectious virus. If the second exposure was postponed to the 4- to 6-hour period, the same general results were still apparent although to a reduced degree. Addition of the various virus preparations to the media from the 12th to 14th hour was without noticeable effect.

These results again denote that conditions for significant production of NIHA can be established only within a very short period of time following a near saturation inoculum of standard virus, as mentioned above. Sudden additional exposure of the cells to numerous infectious virus particles 2 hours after infection increases somewhat the yield of NIHA; at 4 hours only a minor change and at 12 hours no effect is seen. Since liberation of HA in the controls was delayed it is likely that many uninfected cells were still available by the time of the second exposure at 2 hours and to some extent even at 4 hours. This suggestion is supported by the facts that (*a*) in the active influenza B virus series the HA component released early could be typed as Lee virus and there was no evidence of X virus production (20); and (*b*) interference could still be induced by irradiated virus. With respect to the latter effect it is evident from the data that interference by inactivated PR8 virus based on the infectivity data was somewhat more pronounced than by the corresponding Lee preparation. This result appears to be related to the depressor effect of irradiated homologous virus previously described (21), which presumably is attained by interaction of the inactivated virus with the already established infectious process within the cell. It would appear from these data that after infection by standard virus is well under way, additional overloading of the cells with virus does not alter the composition of the yields.

In another experiment a large amount of standard virus was injected (0.4 ml. of 10^0) and deembryonation was performed after 60 minutes. At this time,

0.5 ml. of ultraviolet inactivated PR8 or Lee virus ($10^{3.4}$ HA units) was added to the medium for a half-hour period. The results are shown in Fig. 3. In the control series release of hemagglutinins became apparent by the 6th hour indicating that initially presumably all cells were infected. In the group exposed additionally to irradiated Lee virus the titers were somewhat higher and in that

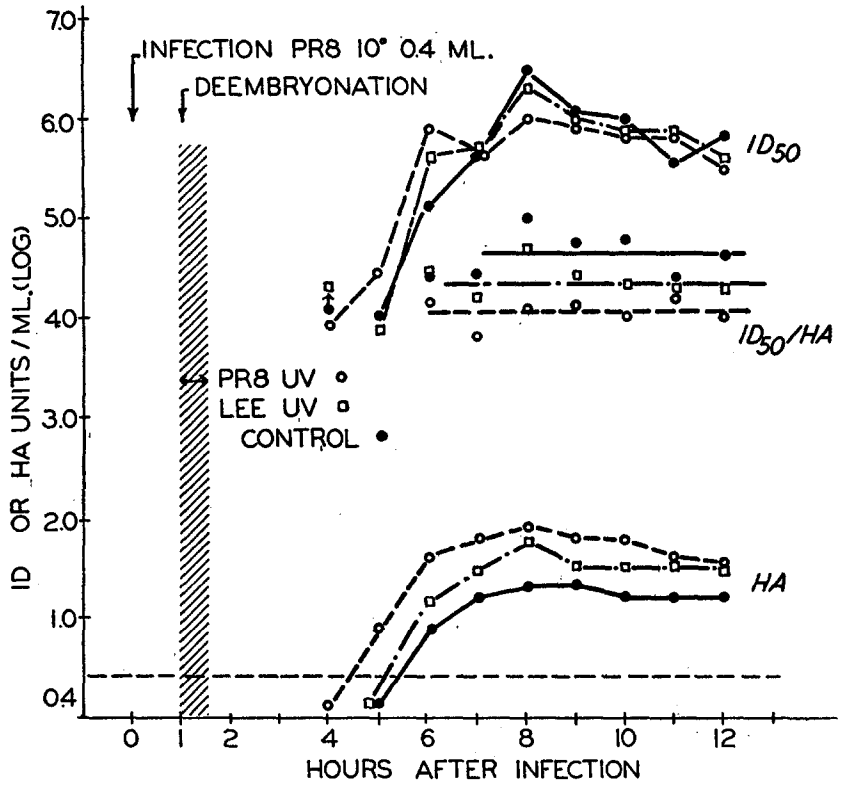

FIG. 3. The effect of addition of homologous or heterotypic ultraviolet inactivated virus (0.5 ml.) to the media of deembryonated eggs for a 30 minute period beginning at the time of deembryonation or 1 hour after infection with a saturation inoculum of standard virus (0.4 ml. 10^9).

treated with inactivated PR8, release was apparent 2 hours earlier and the yields were about 4 times those of the controls. The infectivity levels in the irradiated PR8 group also rose earlier but then in subsequent harvests did not differ significantly from the other two groups. As a result, the ID_{50}/HA ratios were highest in the control series and lowest in the group exposed additionally to irradiated PR8 virus, the difference being 0.6 $\log_{10}$ units. Principally similar results were obtained in a parallel test in which deembryona-

tion was performed after 10 minutes instead of 60. These results do not appear to tally entirely with those shown in Fig. 2 in that appreciable interference was noted there by the irradiated virus added after 2 hours. However, the infecting dose in the present experiment was about 6 times larger than in the preceding one and presumably all cells had been infected initially. Furthermore, the doses of irradiated virus differed ($10^{3.4}$ HA units instead of $10^{4.5}$) and they were administered earlier (after 1 hour instead of 2) and for a shorter period (½ hour instead of 2). It is possible that in the present experiment the irradiated viruses, although inactivated to the point that no residual infectivity was left, were still capable of possibly partial multiplicity reactivation (22, 13), whereas in the previous test inactivation had been more extensive, or with the large dose of irradiated virus interference became the prominent feature.

Although these experiments leave several questions open, the primary intent of these tests was to determine whether late overloading of infected cells with virus particles does result in increased NIHA production and release. The data do not support this suggestion and it is evident rather that conditions for NIHA production can be established only during or shortly after infection of the cells. Furthermore, the early addition of infectious or irradiated standard virus, as a rule, produces only relatively small decreases in the ID_{50}/HA ratios of the progenies which are not comparable to those seen following inoculation of large amounts of undiluted passage seeds (9, 10, 23) or standard seeds heated at 37°C. (12, 13).

The Role of Receptor Destruction in the Yield of NIHA.—The above experiments showed that conditions for NIHA production can be established only early in the infectious process and under conditions when each cell may adsorb rapidly several virus particles. The question arose to what extent destruction of external cell receptors may influence NIHA production.

(*a*) *Experiments with RDE.*—Native or heated standard and undiluted passage seeds appear to possess similar receptor-destroying activity per HA unit as shown by adsorption onto and elution from red cells or by destruction of inhibitors of hemagglutination (19, 24, 25). This indicates that this capacity is not related to any significant degree to NIHA production, since the relative yields of these components obtained following injection of the various types of seeds differ considerably, even when similar total amounts of HA are injected (8, 12). By the use of receptor-destroying enzyme (RDE) of cultures of *Vibrio cholerae*, this point could be more firmly established. In previous studies it was noted that RDE, given after infection with small doses of virus, prevented additional adsorption of virus onto the tissue and thus the liberated virus remained free leading to a steeper rise in ID_{50} titers of extracellular virus (18), but the infection could be held to the cells initially infected by the seed (one-step growth curves) and no detectable levels of HA were released (16, 18).

In differential growth curves employing large inocula the addition of RDE (250 units) to the media did not affect significantly the rate and period of liberation of infectious virus and of hemagglutinins and thus the ID_{50}/HA ratios

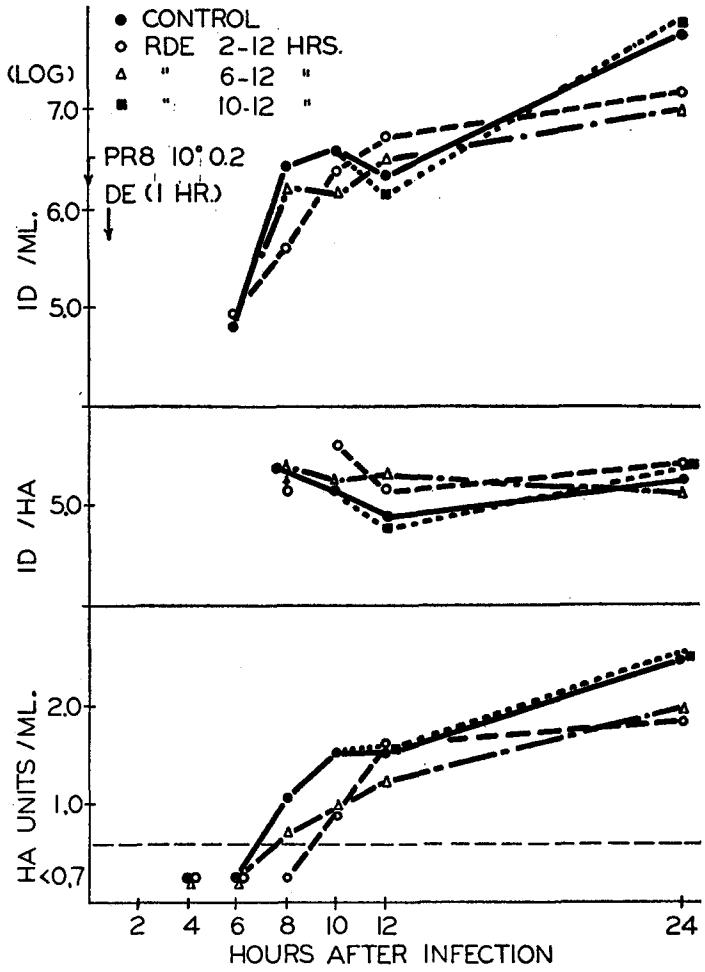

FIG. 4. The effect of addition of RDE (2500 units) to the media of deembryonated eggs at various times after infection with a near saturation inoculum of standard virus and deembryonation (1 hour).

in the yields were not significantly different from those of the controls (18). It seemed to be essential, however, to reexamine this question by using greater concentrations of RDE at different times during the incubation period.

A sufficient number of 15-day-old embryos was injected with 0.2 ml. of undiluted standard seed and deembryonated after 1 hour of incubation. The eggs were then divided into 4

groups: the first served as control; the second series received media containing 10 per cent RDE or about 2500 units per egg at each 2 hourly exchange period beginning 2 hours after infection. In the third group RDE was added at the 6th hour; and to the 4th only at the 10th hour. From the 12th hour on all eggs received medium without RDE and the yields were permitted to accumulate to the 24th hour.

The results are shown in Fig. 4. As can be seen, treatment with RDE from the 2nd hour on had a delaying effect on the liberation of HA and ID_{50} and lower levels were attained subsequently during the cumulative period from 12 to 24 hours. Even when RDE was given only after 6 hours there was a similar though less marked effect. Addition of RDE at the 10th hour was without effect. The ID_{50}/HA ratios were slightly higher initially in the groups receiving RDE by the 2nd or 6th hour, the differences being at most 0.5 $\log_{10}$ units.

In a second similar experiment not only the media but also the membranes were studied for virus activity. RDE was present in the media from the 2nd to 12th hours. Again a delay in liberation of ID_{50} and HA was seen and the ID_{50}/HA ratio was higher by 0.6 $\log_{10}$ units on the average as compared to the controls. Hemagglutinins were detectable in the membranes 2 hours earlier in the RDE series, but the levels attained were lower in subsequent harvests than in the controls. The ID_{50}/HA ratios of the treated and control groups differed to about the same extent as in the media; *i.e.*, by 0.7 $\log_{10}$ units. Treatment of the membranes removed from representative eggs in the control series with RDE *in vitro* for 90 minutes at 37°C. before washing and emulsification did not alter the ID_{50} and HA titers significantly as compared with the results obtained with control membranes not exposed to RDE *in vitro*.

These data can best be interpreted by the assumption that not all cells were infected or saturated with virus initially and that the addition of RDE 2 and 6 hours after injection of the eggs was still capable of halting the spread of infection to some extent. This is supported by the delay in release of HA and ID_{50} and the lower levels of these activities attained after the cumulative periods. The fact that the ID_{50}/HA ratios were, if anything, higher than those of the controls, points to a significant difference between the effects of the addition after infection of RDE and of large amounts of virus since the latter led to somewhat lower ID_{50}/HA ratios in the liberated virus material. These effects were seen only early after infection but again, late in the incubation period, no changes were noted.

(*b*) *Experiment with Periodate.*—It has been reported (26) that on treatment of the allantoic cavity with carefully adjusted concentrations of periodate the cell receptors are modified but that infection with influenza virus can be affected through the modified receptors after the periodate had been "neutralized" by glycerol. Under these conditions, infection even with dilute standard virus was said to result in the release of large quantities of NIHA in that the ID_{50}/HA ratios of the progenies were about 100 times lower than those

of the controls (27). In preliminary tests periodate and glycerol were mixed *in vitro* and virus was added 10 to 15 minutes later. After incubation of the mixtures at 37°C. for half an hour, appreciable losses in infectivity became apparent. Furthermore, undiluted passage of such partially inactivated preparations produced, like heat-inactivated virus, progenies with ID_{50}/HA ratios

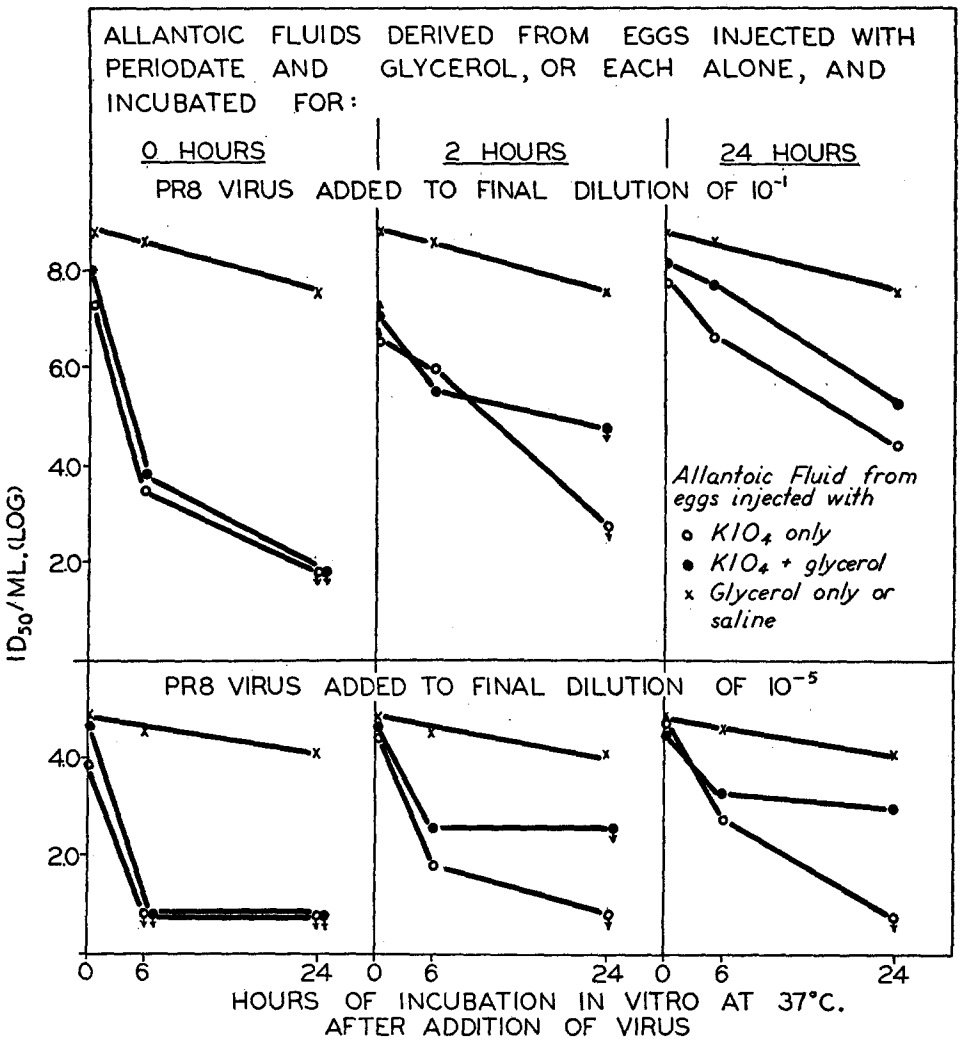

FIG. 5. Inactivation *in vitro* at 37°C. of standard virus diluted 10^{-1} or 10^{-5} in allantoic fluids collected after 0 to 24 hours of incubation following injection of KIO_4, KIO_4 + glycerol, or glycerol alone.

lower than those seen in the yields derived from untreated inocula. This may denote that either the periodate was not neutralized rapidly or that products of interaction between periodate and glycerol, among them formaldehyde, were not without effect, as has been previously assumed (27). It remained to be shown, however, that the same situation obtains also in the allantoic cavity of the chick embryo where the chemicals or their reaction products may be resorbed or bound to the tissues. Accordingly the following experiment was carried out.

Adequate numbers of 11-day-old chick embryos were divided into 4 groups. The first was injected with 1 ml. of 0.01 M KIO_4; the second with the same amount of periodate followed ½ hour later by 0.1 ml. of 0.3 M glycerol; the third with this dose of glycerol only; and the fourth with 1 ml. of saline. The allantoic fluids from some of the eggs were collected immediately after completion of the injections, and from others after incubation at 37°C. for 2 and 24 hours, respectively. To these fluids PR8 virus was then added to final dilutions of 10^{-1} or 10^{-5}. Aliquots were removed immediately or after incubation *in vitro* at 37°C. for 6 and 24 hours. These samples were assayed within a few minutes after collection for infectivity by the usual technic.

The results of this experiment are shown in Fig. 5. The rates of inactivation of virus *in vitro* at 37°C. in the 3 pools of allantoic fluids derived from eggs injected either with glycerol only or with saline were of a similar order. The data for each virus level were combined, therefore, and the means are presented in the figure. The rates of inactivation were compatible with those previously presented (12). On the other hand, the inactivation of virus in pools of allantoic fluid obtained from eggs injected with periodate or periodate and glycerol differed significantly. The most striking loss in infectivity was recorded when the fluids had been collected immediately after injection of the chemicals. With a delay in harvest of the allantoic fluids the inactivating capacity decreased. However, the allantoic fluids collected 24 hours after injection of periodate and glycerol still reduced the infectivity of the added virus to a considerable extent in 6 to 24 hours at 37°C. Thus, the "incomplete" virus derived from periodate-treated allantoic membranes following infection with dilute standard virus (27) may well represent complete virus which had been rendered non-infectious after its release by inactivating substances, be they residual periodate or products of the interaction between periodate and glycerol.

DISCUSSION

The available evidence supports the view that the formation of incomplete virus results only under conditions of multiple infection of host cells (6–9, 28). The present data indicate that such multiple infection must be achieved within a short period of time in order to induce production of incomplete virus. Adsorption of seed virus onto the allantoic membrane following allantoic injection requires some time. Although 70 per cent or more may be adsorbed at the

end of 1 hour (5, 17, 29), additional adsorption seems to occur on further incubation (7, 14). Correspondingly, the yield of incomplete virus is greater when the adsorption period prior to deembryonation extends over 1 hour instead of 10 minutes. Superinfection with large doses of infectious virus after a primary near saturation inoculum of standard seed may change the composition of the progeny only when applied within 2, or at most 4 hours after the initial infection. At these times, an appreciable number of uninfected cells may still have been available and, indeed, the fact that upon superinfection with heterotypic virus the progenies contained mainly the latter agent, supports this view. It is also possible that when the primary infectious process has reached only its initial stage the superinfecting virus may still be capable of influencing the composition of the progeny. After the primary infection is well established; *i.e.*, when production and liberation of progenies have reached nearly constant rates, additional exposure of the cells to excessive amounts of standard or incomplete virus is without significant effect. It is to be noted, however, that on prolonged incubation of infected deembryonated eggs the differential yields show a gradual though small decline in the ID_{50}/HA ratios. In the light of the above data it is unlikely that these changes are due to additional adsorption of liberated virus.

These observations may explain why on infection of chick embryos with dilute standard seed little or no non-infectious hemagglutinins are released when all cells ultimately have been multiply infected. On injection of a small dose of infectious virus a few cells only will be infected initially. The progeny derived therefrom will invade as yet uninfected cells but also may be adsorbed in part onto cells which already harbor virus. The progenies from the second set of cells may again be distributed in part between infected and uninfected cells. Thus, multiple infection should occur not only in the remaining cells at the time of the last cycle but also gradually in cells infected during preceding cycles. Since liberation of progenies from infected cells appears to be not a rapid process but proceeds at low, nearly constant rates for prolonged periods of time (18), multiple infection of cells may be accomplished only at a slow rate so that sudden, nearly simultaneous overloading does not occur and thus NIHA formation can no longer be induced in most of the cells. Yet, as mentioned above, on prolonged incubation *in ovo* or in deembryonated eggs, some NIHA may appear in the yield, particularly after the most active period of propagation has passed. In that case, one may assume that inactivation of complete virus may occur at 37°C. in the tissue prior to its release or that terminal changes in the cells may lead to premature liberation of as yet incompleted virus.

This interpretation of the data would imply that there exists a limited time interval between primary and subsequent infections of cells in which conditions for NIHA formation can still be established. It is not possible with the available

host system and technics to define this interval further or to establish this suggestion more firmly. The multiplicity of infection required for NIHA formation is also known only within rough limits. According to Horsfall (7, 14) 3 to 5 virus particles are needed. It is conceivable, however, that with different types of seeds the relationships may vary significantly. With large inocula of standard virus, when the majority of virus particles are infectious, incomplete virus production is less pronounced than with heated standard or with undiluted passage seeds (8, 28). Since following infection with dilute standard virus the progenies becoming available for multiple infection of cells are almost all infective, NIHA production, if it occurs (see above), would be expected to be of a low order.

It has been considered that with a high multiplicity of infection the virus may destroy cell receptors and that this change in some way may be related to incomplete virus production. This notion seemed to derive support from the experiments of Fazekas de St. Groth and Graham (27), who reported that after treatment of the cell receptors with potassium periodate (KIO_4) and subsequent neutralization of the chemical by glycerol, the cells yielded incomplete virus even after infection with dilute standard virus. The data presented here indicate that after injection of periodate and glycerol the allantoic fluids contain inactivating substances which are still noticeable after incubation for 24 hours. Thus, the production of incomplete virus under these conditions may be questioned since any infectious virus that might be released from the cells would be exposed to these inactivating agents during the remainder of the incubation period.

Experiments with RDE, likewise, failed to indicate that loss of the cell receptors influenced incomplete virus formation. If large amounts of RDE were added early after a near saturation inoculum it delayed liberation of virus material, the yield was slightly reduced and its ID_{50}/HA ratio was somewhat higher than that seen in the controls. Addition of RDE later in the infectious process was without effect. These results seem to denote that the multiplicity of infection can be reduced early after inoculation and thus the progenies obtained under these conditions resemble those seen when a slightly smaller dose of seed is employed without subsequent addition of RDE. This interpretation is compatible with the results of previous studies with RDE when it was shown that by external cell receptor destruction a previously established infectious process could be held to one cycle; *i.e.*, one-step growth curves were obtained (16). If the RDE was removed after a short interval, the cell receptors apparently regenerated (30) and ultimately standard virus was released (16).

Although ultraviolet inactivated virus retains its receptor-destroying activity (31), the effects of addition of such preparations after infection differed in several aspects from those obtained with RDE. Exposure of cells to excessive amounts of irradiated virus from the 2nd to 4th hour after a near saturation

inoculum of standard seed resulted in significant interference (Fig. 2) and, since little or no detectable hemagglutinins were released, the ID_{50}/HA ratio of the progenies could not be determined. Later exposures to such quantities of irradiated virus were without effect. In this respect it must be recalled that destruction of cell receptors is not a prerequisite for interference in that absorption of challenge virus onto cells in which interference has been induced can readily be detected (*cf.* reference 32). The fact that in this experiment the homologous irradiated virus was somewhat more effective than the heterotypic Lee strain agrees with previous studies (21, 33) in which injection of irradiated heterotypic virus within 1 to 3 hours following a small dose of standard seed led to one-step growth curves comparable to those obtained by the use of RDE (16), whereas homologous irradiated virus largely suppressed the production of infectious progenies under these conditions. These results suggested that at least irradiated homologous viruses and, therefore, presumably also the corresponding infectious agents are still capable of entering cells 1 to 3 hours following infection. In the second experiment of this kind (Fig. 3), a saturation inoculum of standard seed was employed and considerably smaller doses of irradiated virus were added earlier after infection (1 hour) and for a shorter period of time (30 minutes). Under these conditions interference was not apparent, possibly because all cells were infected initially and the dose of irradiated virus may have been too small to induce still significant effects. It would seem, however, that the inactive viruses were still capable of entering the cells since the progenies derived therefrom differed from those of the control series, in that they revealed lower ID_{50}/HA ratios. This is in contrast to the effects of RDE, which tended to increase the ratios of the yields. The changes in the ID_{50}/HA ratios were accounted for by an increased yield of hemagglutinins, the irradiated homologous virus being somewhat more effective in this respect than the heterotypic agent. The production of infectious virus was not correspondingly increased, on the contrary, if anything, it was reduced. In addition, release of ID_{50} and HA was noted somewhat earlier in the series employing ultraviolet PR8 than in the ultraviolet Lee or control groups. Related observations were reported recently by Cairns (34) who compared these results with those previously cited in support of the occurrence of multiplicity reactivation (22). It was suggested that the presence of large quantities of inactivated, enzymatically active virus in the system by destruction of the cell receptors would prevent adsorption of any liberated virus thus leading to earlier and quantitative detection of the progenies. However, some of the present and previous data do not seem to be entirely compatible with this view. (*a*) The addition of RDE following infection, as pointed out, yielded results different in several respects from those obtained with irradiated virus; (*b*) the increased HA production following exposure of infected cells to irradiated virus was not accompanied by a correspondingly greater yield of infectious progeny as seen in multiplicity reactivation (22); (*c*) the irradiated

virus produced this result only when added early after infection; (*d*) the irradiated virus was present only for a short period of time, yet the increased HA release continued for the remainder of the experimental period; and (*e*) multiplicity reactivation was seen only when the infectivity of the seed had been reduced up to 10,000-fold but not when exposure to ultraviolet or heat had been more extensive. Yet, the present data as well as Cairn's were obtained with virus rendered completely non-infectious. These various points do not entirely exclude the possibility that the presence of excessive amounts of enzymatically active virus may prevent adsorption of progenies to some extent, but the fact remains that no evidence has been obtained as yet which shows conclusively that receptor destruction by RDE or inactivated virus *per se* is capable of changing the composition of progenies in terms of the proportions of infectious and non-infectious virus particles.

The available information together with the above considerations strongly suggests that incomplete virus formation is an intracellular process resulting from invasion of cells within a limited period of time by several virus particles. The multiplicity required and the influence of increasing multiplicity have not as yet been firmly established. If all the virus particles are infectious the yield of NIHA is relatively small. However, on entrance of an increasingly greater proportion of inactivated standard or incomplete virus the yields will contain, within limits, increasingly more incomplete virus.

SUMMARY

Certain aspects of the formation of non-infectious hemagglutinins (NIHA) in the chick embryo infected with influenza virus have been analyzed.

It was shown by the use of combined *in ovo*-deembryonation technics that little or no NIHA is released following infection with small doses of standard virus during the most active and constant growth periods of the virus extending to about the 36th hour of incubation in spite of the fact that multiple infection of cells must have taken place in the latter half of that period. A slight decrease in the ID_{50}/HA ratios of the yields obtained after the 36th hour, coinciding with the falling off of virus production and release may possibly be explained in terms of inactivation of completed virus or leakage of as yet incompleted virus from damaged cells.

Exposure of the entodermal cells of the allantois of eggs deembryonated shortly after injection of saturation or near saturation inocula of standard seed to large quantities of infectious virus added to the media at various times after infection and not extending over more than 2 hours resulted in a decrease of the ID_{50}/HA ratios of the progenies only during the first 2 or possibly 4 hours after the primary inoculation. Later addition did not influence the yields. As discussed, such sudden and heavy exposures of cells are not expected to occur during the infectious process induced by small inocula of standard seed.

The possible role of destruction of cell receptors in NIHA production has

been analyzed in several ways. The addition of receptor-destroying enzyme (RDE) to the media of deembryonated eggs after near saturation inocula of standard seeds, if anything, increased the ID_{50}/HA ratios of the progenies, and that only when added during the first few hours following infection, presumably by reducing the changes for high multiplicity of infection of cells. In contrast, ultraviolet-inactivated virus, which retains its enzymatic activity, lowered, if anything, the ID_{50}/HA ratios of the progenies, when present in the media of deembryonated eggs from the 2nd to 4th or possibly 6th hour after infection. Excessive amounts of irradiated virus may still cause some degree of interference under these conditions. Later addition of irradiated viruses were without effect with respect to NIHA production or interference. In attempts to alter the cell receptors prior to infection by potassium periodate (KIO_4), it was noted that the addition of glycerol led to the appearance and partial retention for at least 24 hours of substances in the allantoic fluids which were capable of inactivating considerable proportions of standard virus. These data indicate that destruction of external cell receptors plays little if any role in NIHA production.

The implications of these findings are discussed.

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