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

## X. An Experimental Analysis of the von Magnus Phenomenon

By NORMAN B. FINTER,‡ M.R.C.P., OSCAR C. LIU, M.D., and WERNER HENLE, M.D.

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

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It has been shown that upon inoculation of chick embryos by the allantoic route with large doses of influenza virus, the yields of the agent after 24 to 48 hours of incubation are usually less than those obtained following injection of smaller amounts of the same seed (1). These paradoxical results depended to some extent upon the preparation and handling of the inocula since the effect was exaggerated after partial inactivation of the seeds by ultraviolet light or, to a lesser degree, by heating at 56°C. (1). It was thought, therefore, that the phenomenon could be attributed to interference by inactivated virus, which had accumulated in the seeds during passage or on storage, with the propagation of the remaining infectious virus; *i.e.*, by what is referred to as "autointerference" (2). Subsequent studies on the interfering activity of virus rendered non-infectious by ultraviolet light (1, 2) or by heating to 56°C. (3, 4) were largely restricted to tests in which the interfering agent was injected 1 to 24 hours *prior* to the infectious challenge virus. The results were evaluated, as a rule, only by titrations of hemagglutinins in the yields obtained 1 to 2 days after challenge but when the infectivities were also determined, it was apparent that production of both infectious virus and hemagglutinins was inhibited to similar extents. Thus the term "interference" has been accepted to denote total or partial similtaneous inhibition of the development of *all* virus activities.

In this sense, interference cannot account entirely for the results obtained upon passage of undiluted infected allantoic fluids. It has been shown by von Magnus (5) that after inoculation of large doses of freshly prepared seeds relatively small amounts of infectious virus are produced, whereas the yields of hemagglutinins are of the usual order. This phenomenon becomes more pronounced when infected allantoic fluids are passed in series allantoically without dilution ("undiluted passage series"). In such a series near maximal titers of hemagglutinin are found in allantoic fluids harvested after 20 to 24 hours in each of the first three or four consecutive undiluted passages,

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‡ Research Fellow on study leave from Cambridge University. Present address: Public Health Laboratory, Northampton, England.

whereas successively smaller amounts of infective virus will generally be noted in the serial harvests. Thus the ratio between the infectivity and hemagglutinin titers ($ID_{50}$/HA ratio) decreases in such passage series from a maximum value of $10^6$ to $10^7$ (as found after inoculation of diluted seed virus) to values of $10^3$ or less in the harvests from the 3rd or 4th undiluted passage. With further serial transfers of undiluted allantoic fluids the yields of hemagglutinins usually also decrease significantly, formation of infectious virus may increase and with it a rise in the $ID_{50}$/HA ratio of the progeny is observed.

The decrease in the $ID_{50}$/HA ratios in an undiluted passage series is caused then largely by a reduction in the formation of infectious virus, whereas at least in the first few transfers, liberation of hemagglutinins is not reduced. This result has been interpreted to mean that under these conditions "incomplete" virus is formed; *i.e.*, virus material which possesses hemagglutinating activity and other attributes of the virus (6), but which is lacking in infectivity. As pointed out by Schlesinger (7), this may denote that the virus material obtained is either *developmentally* or *functionally* incomplete. The dominant virus components in such undiluted passage materials differ in some respects from those in standard preparations as determined by analytical centrifugation (8, 9), chemical fractionation (10), and electron microscopy (11). The appearance of "incomplete" forms of virus has been demonstrated also in the central nervous system upon intracerebral inoculation of mice with large amounts of non-neurotropic strains of influenza virus (12) or in tissue cultures of human cancer cells, strain HeLa (13).

The experiments to be presented here were designed to obtain more information on the following factors which may contribute to the von Magnus phenomenon.

(*a*) *The Role of Inactivated Virus Which May Have Accumulated in the Seeds.*—Attempts were made to obtain inocula under conditions which largely excluded accumulation of inactive virus. For this purpose the deembryonation technic (14, 15) was employed which permits the collection solely of the virus liberated during 1- or 2-hour intervals at given stages of the incubation period. Such seeds, derived from inoculation of dilute standard virus and consisting almost entirely of infectious virus, still gave evidence of the von Magnus effect. Horsfall (16), employing different technics, arrived at similar conclusions. On the other hand, partial inactivation of standard virus at 37°C. *in vitro* seemed to enhance the phenomenon. These results will be reported in the two papers to follow (17, 18).

(*b*) *The Quantitative Aspects of Host Cell-Virus Interrelationships.*—Cairns and Edney (19) had reported that some incomplete virus is formed when only one in one hundred cells are infected with standard virus. Using related technics the conclusion could not be confirmed and multiple infection of cells appears to be required in order to obtain non-infectious hemagglutinins.

(*c*) *The Interrelations between Infectivity, Hemagglutinin Concentrations, and $ID_{50}$/HA Ratios of the Inocula and the Corresponding Properties in the Progenies.*

—The results of over 50 undiluted passages of various types of seeds were available for such analyses. The data indicate that the progenies are not determined entirely by the relative proportions of infectious virus and non-infectious hemagglutinins in the seeds but that other factors come to the fore with an increase in the passage number.

## *Methods and Materials*

The technics used for inoculation of chick embryos (20), titration of infectivity (20) and of hemagglutinins (15, 22), as well as the methods used for obtaining growth curves in the intact chick embryo (21, 22) and for deembryonation (15) have been described in previous papers of this series.

*Virus Preparations and Passages.*—Only the PR8 strain of influenza A virus was used in these studies. The designation *Standard Virus* (ST)[1] has been given to preparations in which presumably the vast majority of virus particles were infectious, so that the $ID_{50}$/HA ratio was maximal. It is realized that in such viral suspension the presence of *some* non-infectious hemagglutinin, be it inactivated "complete" virus or "incomplete" virus, cannot entirely be excluded, although the technics used recently by Horsfall (16) tend to exclude the presence of large amounts of non-infectious virus. With this reservation, standard virus preparations, as used here, were allantoic fluids harvested after incubation periods of 24 to 48 hours from eggs inoculated allantoically with small amounts of virus ($10^2$ to $10^5$ $ID_{50}$); at the time of harvest, the infectivity titers in the allantoic fluids were still rising or had just reached their peak. With the particular titration technics employed, the $ID_{50}$/HA ratios of such fluids were of the order of $10^{6.5}$. In some instances, the eggs were deembryonated by the technic described (15) during the period of rapid increase in infectivity, and the virus liberated into the medium during 1 or 2 hours' incubation on the rotating machine was used as seed for further passages.

*Serial Passages of Undiluted Seeds.*—The first undiluted seed passage was initiated with standard virus obtained as described above. Groups of 11- to 13-day-old chick embryos were used and in different undiluted passage series, the amounts inoculated into the allantois varied from 0.2 to 1.0 ml., as did the period of subsequent incubation (20 to 48 hours). After incubation, the allantoic fluids were harvested and passed into further eggs without dilution, either at once or after storage at 4°C. overnight. This procedure was repeated for the desired number of passages. Sometimes the harvested allantoic fluids were stored for longer periods, either at 4°C. or at −70°C. before passage. Allantoic fluids harvested in the 1st, 2nd, etc., undiluted passages were designated as UP[2] 1, UP 2, etc.

In several experiments serial passages were made of undiluted media harvested from deembryonated eggs. In these experiments standard seed was inoculated into the allantois of a number of 14-day-old chick embryos which were then deembryonated 7 or 14 hours later. The pooled media collected after a 2 hour period of rotation were inoculated without dilution into further intact chick embryos which in turn were deembryonated at the 7th or 14th hour, and so on in series.

## EXPERIMENTAL

*Passage of Undiluted Seeds Obtained under Conditions Minimizing Inactivation*

*Passage Series.*—During the past several years, serial passages of undiluted

[1] ST, standard virus.

[2] UP, undiluted passage.

allantoic fluid infected with PR8 virus were carried out on numerous occasions for various experimental purposes. The results of these passages were on the whole in agreement with those published by von Magnus (5), Fazekas de St. Groth and Graham (23), and others, and, therefore, will not be presented here in detail. However, in none of these series $ID_{50}$/HA ratios lower than $10^{3.3}$ were observed (see below), in contrast to the data recorded by von Magnus, who noted ratios as low as $10^{1}$. Donald and Isaacs (24), likewise failed to obtain such low ratios. The conditions of passage employed in the individual series varied to some extent with respect to the incubation periods (20 to 48 hours), the volumes transferred (0.2 to 1.0 ml.), or the handling of the seeds between passages (immediate transfer or storage at 4 to −70°C. for periods from a few days to several months). Analysis of the data obtained under these various conditions indicated that the von Magnus effect was increased when long incubation periods were employed, when the volumes used for transfer were large, and when the seeds had been stored between passages under adverse circumstances; *i.e.*, at 4°C. for 2 to 3 weeks but not at −70°C. for several months. Thus, it became apparent that conditions favoring inactivation of "complete" virus, either *in ovo* or on storage, produced seeds which were more effective in inducing this phenomenon. These considerations suggested two experimental approaches to the problem. On the one hand, the effect of exposure of standard seeds of PR8 virus to 37°C. was studied and the results are recorded in the two papers to follow (17, 18). On the other hand, efforts were made to minimize inactivation of "complete" virus during passages by the use of short incubation periods *in ovo*, or by employing media for passage which were collected after 2 hours from eggs deembryonated at the 7th or 14th hour after infection. A representative experiment is described below.

A number of 14-day-old chick embryos were inoculated by the allantoic route with $10^{5.4}$ $ID_{50}$ of virus. After 14½ hours they were deembryonated. The pooled media harvested from these after 1 hour of rotation in the incubator had an $ID_{50}$/HA ratio of 7.2, indicating that very little inactivated virus was present. The pool was used as seed to infect a number of 12- and 14-day-old embryos in the evening of the day of harvest. The 14-day-old embryos were deembryonated on the next day, after 14 hours of incubation, and the allantoic fluids from the 12-day-old embryos were harvested after 22 hours. Also on this day more embryos of the same two batches were inoculated with the seed which had been stored overnight at 4°C. The older embryos were deembryonated after 7 hours of incubation and allantoic fluids were collected from the younger ones after 9 hours. The media from the 2 groups of deembryonated eggs were collected after 2 hours of further incubation at 37°C. on the rotating machine. Thus 4 different harvests were obtained, namely, A, 7th to 9th hour media from deembryonated eggs; B, 14th to 16th hour media from deembryonated eggs; C, 9th hour allantoic fluid; and D, 22nd hour allantoic fluid. After storage at 4°C. for 12 hours or less, each seed was inoculated without dilution in 0.4 ml. amounts into further 12- (seeds C and D) or 14-day-old embryos (seeds A and B). The allantoic fluids were collected again after 9 hours and 22 hours (series C and D, respectively), and the media 2 hours after deembryonation at 7 hours and 14 hours (series A and B, respectively). Two more passages were carried out according to this schedule.

The results of the 4 undiluted passage series are shown in Table I. Considering each series *individually*, the results are comparable to those recorded in the literature (5, 23). In the first 2 or 3 passages the yields of hemagglutinins did not differ significantly but decreasing amounts of infectious virus were found and the $ID_{50}$/HA ratios decreased correspondingly. In the deembryonation series, A, and the *in ovo* series, C, in which the seeds were harvested after the shorter incubation periods, the yields of hemagglutinin decreased by the 3rd or 4th passage to low or non-detectable levels. However, in these passages

TABLE I

*Undiluted Passages under Conditions Minimizing Accumulation of Inactivated Complete Virus*

| Starting seed | Conditions of passage series | | | | Assay | Results of passage series | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Series | Procedure | Harvests used for passage and assay | | | Passage No. | | | |
| | | | Material | Time | | 1 | 2 | 3 | 4 |
| | | | | *hrs.* | | | | | |
| Medium from eggs deembryonated 14½ hrs. after inoculation of $10^{5.4}$ $ID_{50}$ and collected 1 hr. later. | A | Deembryonated 7th hr. | Medium | 7–9 | $ID_{50}$/ml. | 8.4 | 7.7 | 6.0 | 5.9 |
| | | | | | HA/ml. | 1.9 | 1.9 | 0.7 | <0.4 |
| | | | | | $ID_{50}$/HA | 6.5 | 5.8 | 5.3 | >5.5 |
| | B | Deembryonated 14th hr. | Medium | 14–16 | $ID_{50}$/ml. | 8.2 | 7.4 | 7.2 | 7.6 |
| | | | | | HA/ml. | 2.0 | 2.1 | 1.9 | 1.9 |
| | | | | | $ID_{50}$/HA | 6.2 | 5.3 | 5.3 | 5.7 |
| $ID_{50}$/ml. 9.4<br>HA/ml. 2.2<br>$ID_{50}$/HA 7.2<br>Volume used in passages 0.4 ml. | C | *In ovo* | Allantoic fluid | 0–9 | $ID_{50}$/ml. | 9.0 | 7.7 | 6.7 | 5.1 |
| | | | | | HA/ml. | 2.5 | 2.4 | 2.2 | <0.4 |
| | | | | | $ID_{50}$/HA | 6.5 | 5.3 | 4.5 | >4.7 |
| | D | *In ovo* | Allantoic fluid | 0–22 | $ID_{50}$/ml. | 9.2 | 7.5 | 6.6 | 7.8 |
| | | | | | HA/ml. | 2.7 | 2.7 | 2.8 | 2.1 |
| | | | | | $ID_{50}$/HA | 6.5 | 4.8 | 3.8 | 5.7 |

parallel growth curve studies, to be described below, showed that after longer incubation hemagglutinins were released into the allantoic fluids, and that the $ID_{50}$/HA ratios had increased considerably.

In comparing the results of the 4 series with one another, it can be seen that in the 1st passages all the 4 harvests had similar $ID_{50}$/HA ratios which fell within the arbitrary range of standard virus, although they were lower than the ratio of the starting seed. The titers of infectious virus and hemagglutinins were lower in the media of the 2 deembryonation series (A and B) than in the allantoic fluids of the two *in ovo* groups (C and D). In subsequent passages the ratios decreased more strikingly with an increase in the total incubation period both in the deembryonation series (*i.e.*, B *vs.* A) and in the *in ovo* series (*i.e.*, D *vs.* C)

and the declines in the ratios were also more marked in the latter two groups than in the former. It is apparent then that the von Magnus phenomenon is still produced to some extent under conditions when only little inactive "complete" virus could have accumulated in the seeds. The effect is less striking the shorter the incubation periods *in ovo* and still less marked when the allantioc fluids containing residual non-adsorbed seed virus as well as virus liberated in the early period of incubation have been removed as a result of deembryonation. However, interpretation of these results is complicated by the fact that different total amounts of viral material were used as seed for corresponding undiluted passages in the 4 different series. This difference affects the results since, as will be shown below, certain relationships exist between the relative and total concentrations of infectious virus and hemagglutinin in the seed and the resultant titers in the progeny. By inference, these experimental results suggest again that accumulation of inactivated "complete" virus may contribute to the von Magnus phenomenon.

*Growth Curves with Undiluted Passage Seeds.*—In undiluted passage series the mere evaluation of infectivity and hemagglutinins in harvests obtained at an arbitrary time interval following injection of various seeds is not entirely satisfactory. In these experiments a large amount of virus material is injected as seed and of this a considerable percentage remains unabsorbed in the allantoic fluid of the injected eggs. It is thus often not possible to ascertain how much of the virus found in harvests at the end of an arbitrary incubation period has been formed during that period and how much of it represents residual seed. Satisfactory evidence of production and liberation of new virus material can, however, be obtained if the results of the passages are studied dynamically, that is in growth curves, as shown by von Magnus (5, 25).

The seeds of 3 of the passage series shown in Table I (A, C, and D) were used for *in ovo* growth curve experiments, which, in fact were conducted in parallel with these passages. After allantoic inoculation of the various seeds into groups of 12-day-old chick embryos the allantoic fluids were collected from representative numbers at successive intervals of 2 to 4 hours and assayed for infectivity and hemagglutinin concentration.

The results are presented in Fig. 1. No residual seed hemagglutinins were found in the early allantoic fluids of series A; low levels in series C; and some what greater amounts in series D. In all passages there was evidence of liberation of hemagglutinins, but in the 2nd, 3rd, and 4th passages of series A the titers rose to detectable levels after increasingly longer periods of incubation, whereas in series C and D, the titers were definitely elevated over the threshold levels by the 5th hour in the 2nd or 3rd passages and a delay in rise was noted only in the 4th. Some increases in infectivity were noted in all instances with the possible exception of the 2nd passage of series D (22nd hour allantoic fluid seed). This type of result will be discussed below. The rise in infectivity

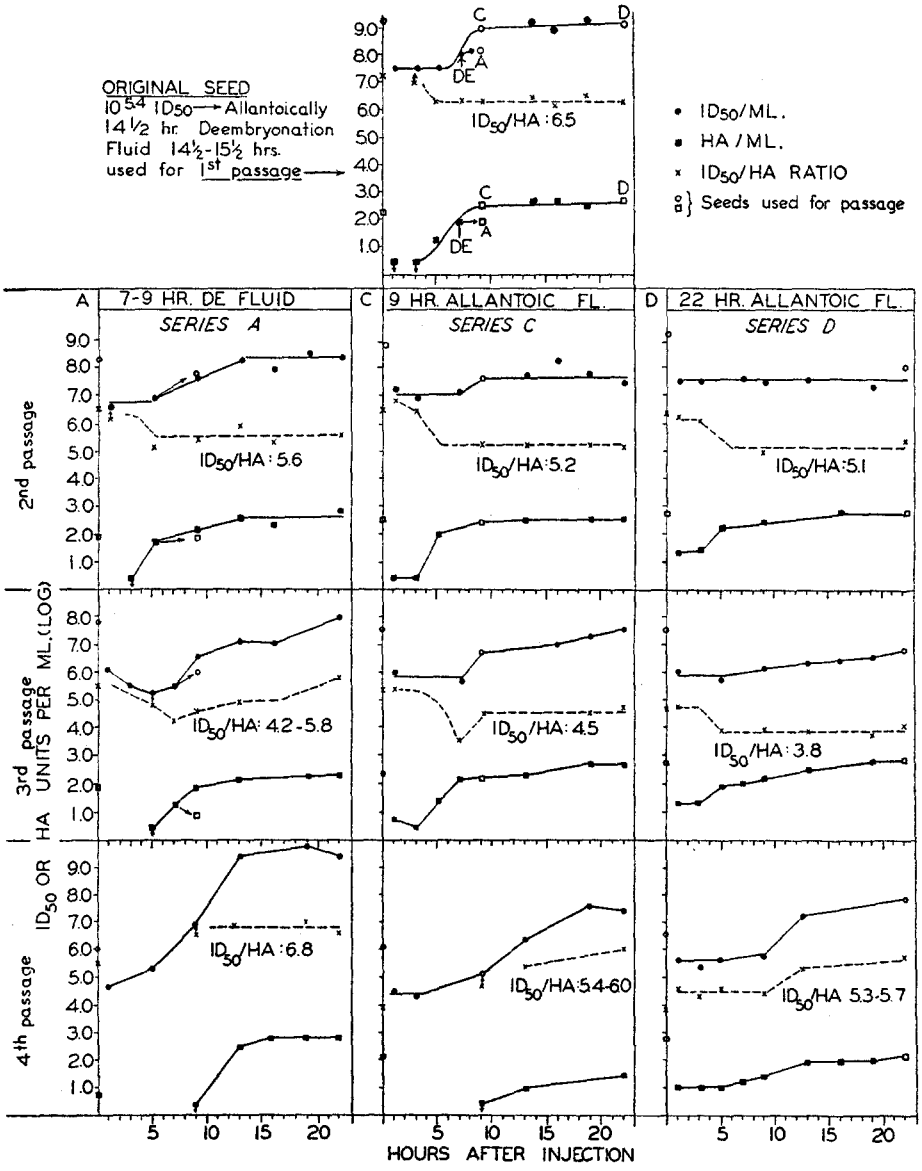


FIG. 1. Growth curves in intact chick embryos with seeds derived from the serial undiluted passages presented in Table I. Series A: 7 to 9 hour media from deembryonated eggs; Series C: 9th hour allantoic fluids; and Series D: 22nd hour allantoic fluids.

began usually between the 5th and 7th hour, but in the later passages of series D it occurred with some delay. The $ID_{50}$/HA ratios during the first 5 hours were the same as those of the inocula used (non-adsorbed seed). As soon as liberation of virus material became apparent the ratios changed. From the 1st to 3rd passages they decreased to a variable extent in all series but in the 4th, they generally increased again. This rise occurred in series C and D, in spite of the fact that the seeds leading to this effect revealed similar HA concentrations as the preceding ones. This point will be discussed later. In the 1st and 2nd passages the ratios remained constant at the new levels for the experimental period. In the 3rd passage this was again the case in series C and D but in series A the ratio, after an initial decrease to $10^{4.2}$, increased gradually to $10^{5.8}$. Such increases in the ratio seem to be apparent also in series C and D in the 4th passages. Thus it would seem that a greater proportion of infectious virus may be produced on occasion late in the incubation periods.

These results confirm the conclusions drawn from the passage data (Table I) and extend them in that it is more clearly seen that with seeds relatively free of inactivated virus (series A) the von Magnus effect still obtained but it was less pronounced and on continued passage the yields readily approached standard virus again. With the allantoic fluid seeds harvested after incubation for 9 hours and for 22 hours the von Magnus phenomenon is progressively more marked and there is less reversion in the later passages toward production of standard virus.

As has also been shown by von Magnus (5), evidence of liberation of infectious virus into the allantoic fluid can sometimes not be obtained by the *in ovo* growth curve technic even though considerable quantities of hemagglutinin are released (*e.g.*, series D, 2nd passage). This does not imply that no infectious virus is liberated, but rather that the amount released is too small to be detected in the presence of the high threshold level of non-adsorbed seed virus. This assumption has been verified by Bernkopf (14) using the deembryonation technic, and a similar experiment has been described fully elsewhere ((26), experiment 4).

### *The Relation of the Dose of Standard Seed to the Production of Non-Infectious Hemagglutinins*

Since the von Magnus phenomenon is seen only when a large inoculum of virus is used, it has been considered that multiple adsorption of virus particles by individual cells may be responsible for the change in composition of the progeny. However, Cairns and Edney (19) concluded from their experimental results that "incomplete" virus is formed when as few as 1 per cent of the available cells have been infected. In essentially related experiments this conclusion could not be confirmed.

Groups of five 13-day-old chick embryos each were inoculated with one of successively smaller amounts of a standard PR8 seed using 2-fold steps. After 1 hour of incubation the eggs

were deembryonated and a preparation of ultraviolet-irradiated influenza B virus (Lee strain) was added to the medium in order to block any remaining susceptible cells by induction of interference (27). After rotation of the eggs in the incubator for 1 hour, the suspension of interfering virus was decanted, the insides of the eggs were washed twice in the usual manner and fresh modified glucosol solution was added. At various intervals after further rotation aliquots of the media were removed from the eggs for assay.

As can be seen in Fig. 2, following inoculation of the larger amounts of virus similar quantities of hemagglutinin had been liberated at each time of

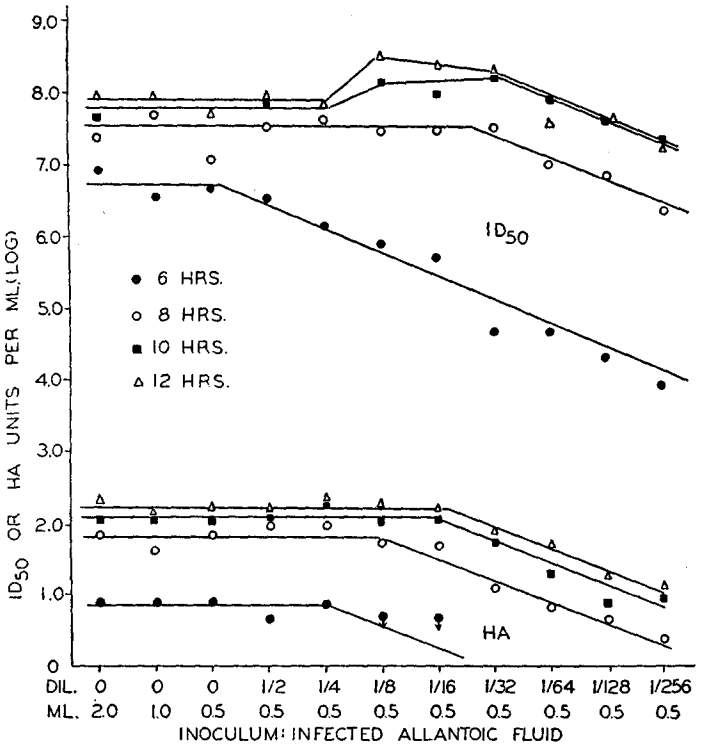


FIG. 2. Relation between the amounts of standard virus inoculated and the yields of infectious virus and hemagglutinins after incubation periods of 6 to 12 hours. (For technical details see text.)

sampling. The number of seed dilutions yielding the same amounts increased, however, to some extent with the period of incubation. These results indicate that there is an upper limit to the amount of hemagglutinin that may be derived from infected cells in a given time and that this limit may be reached only after some delay when the seed has been diluted to a certain level. With further dilution of the inoculum the yields at each time decreased roughly in 2-fold steps in accordance with the dilution steps of the seed. The liberation of infectious virus did not quite match this pattern. Whereas in 6 hours only the 3

largest inocula gave similar results, in 8 hours the first 8 amounts of the seed produced the same yields of infectious virus. On further dilution of the inoculum the yields decreased roughly in proportion to the amount of virus injected. By the 10th and 12th hours relatively little additional liberation of infective virus resulted in the first 5 groups, but a definite increase was seen in the remaining ones. The average $ID_{50}$/HA ratios in the yields derived from the 5 largest inocula (2.0 ml. undiluted to 0.5 ml. seed diluted 1:4) were $10^{5.6}$, regardless of the time of harvest. The average ratios obtained in the yields of the more dilute inocula (1:32 to 1:64) were $10^{6.4}$, or within the range of "standard" virus, again independent of the time of collection. With the intermediate dilutions of the seed (1:8 and 1:16) the ratios obtained in the yields fell between the 2 values, and they were the lower the earlier the harvests were made. This result appears to correspond to the late rise in the $ID_{50}$/HA ratios seen in some of the growth curves presented in Fig. 1, and may denote a delay in "completion" of the virus under certain conditions. It is evident then that non-infectious hemagglutinins appeared in the yield when more than 0.5 ml. of a 1:32 dilution of the seed were injected, or when slightly less than $10^8$ $ID_{50}$ were inoculated. Two similar experiments with standard virus were carried out using again a heterologous interfering virus preparation in one, and RDE in the other for the prevention of second infectious cycles. Both gave essentially similar results.

### *Interrelationships between the $ID_{50}$ and HA Units in the Inoculum and the Progeny Derived Therefrom*

During the course of these and other studies numerous infected allantoic fluids or media from deembryonated eggs were inoculated without dilution into chick embryos. When the results from all these passages were analyzed it became apparent that there exist certain relationships between the number of $ID_{50}$ and of HA units inoculated with the seed and the composition of the progeny collected 20 to 24 hours later. For purposes of analysis the inocula were divided into groups in which the seeds contained either similar numbers of $ID_{50}$ or of HA units, or revealed similar $ID_{50}$/HA ratios, within ranges of 0.5 to 0.7 $\log_{10}$ units. The actual ranges were chosen so that each group contained sufficient numbers of seeds for analysis.

*Yields from Passage of Seeds Containing Similar Concentrations of Infectious Virus but Different Amounts of Hemagglutinin.*—In Fig. 3, the yields of virus are plotted against the number of hemagglutinin units in the inocula. There are 4 different groups of seeds in each of which the number of $ID_{50}$ inoculated agreed within a narrow range. In this figure, and in Fig. 4, different symbols are used in order to identify the different types of seeds used; *i.e.*, standard, 1st, 2nd, or 3rd undiluted passages. It will be seen that the yields of infectious virus and the $ID_{50}$/HA ratio of the progenies in each group decreased as the

number of HA units inoculated increased. Liberation of hemagglutinins was of the same order with all seeds as long as $10^7$ or more $ID_{50}$ were injected. The $ID_{50}$ titers and the $ID_{50}$/HA ratios of the progenies can be fitted reasonably well to straight lines, all having about the same slopes. When the seeds contained less infectious virus ($10^{6.1}$ to $10^{6.8}$ $ID_{50}$) the yields of hemagglutinins were also reduced with an increase in the HA units in the inocula. In this case, the $ID_{50}$ values and the ratios of the progenies again seem to fall on straight lines but their slopes appear to be less steep than those obtained in the other groups. If the maximal possible value for the $ID_{50}$/HA ratio is taken to be approximately $10^7$, it will be seen that this value was reached after inoculation

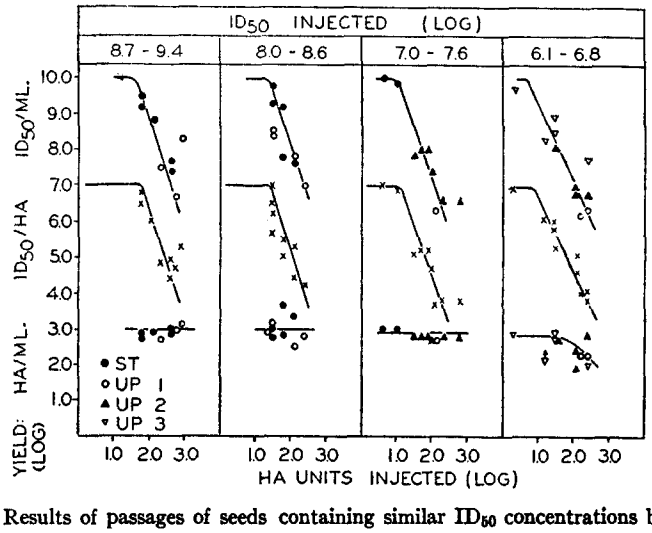


FIG. 3. Results of passages of seeds containing similar $ID_{50}$ concentrations but different amounts of hemagglutinins.

of increasingly smaller amounts of hemagglutinin as the number of $ID_{50}$ in the seeds decreased.

*Yields from Passage of Seeds Containing Similar Numbers of HA Units but Different Concentrations of Infectious Virus.*—In Fig. 4, the yields of virus are plotted against the number of $ID_{50}$ inoculated for 4 different groups of seeds, each representing one narrow range of hemagglutinin concentrations. In each group both the yield of infectious virus and the $ID_{50}$/HA ratio tended to be higher after inoculation of a larger number of $ID_{50}$ than after a smaller number. However, when less than a critical number of $ID_{50}$ was inoculated ($<10^7$ $ID_{50}$) the yields of infectious virus and the ratios increased again. The greater the number of HA units in the inoculum, the more pronounced were these changes, and conversely, no such effects were noted when less than 10 HA units were injected. Reference to the average level of non-adsorbed infectious virus of the

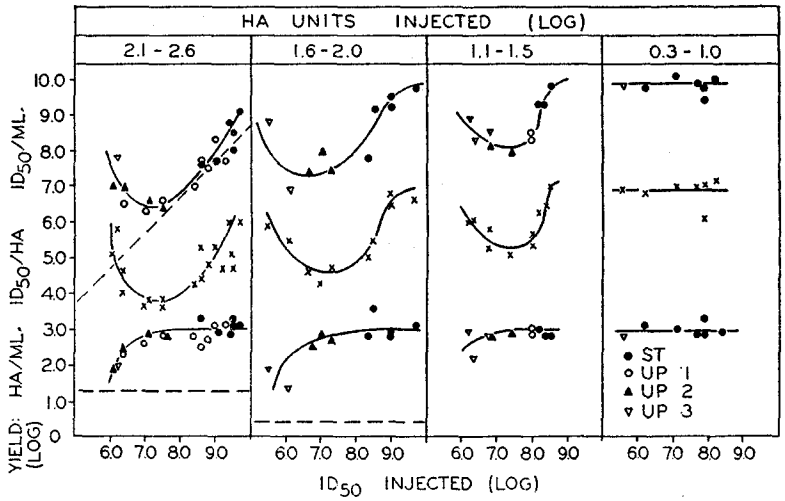


FIG. 4. Results of passages of seeds containing similar numbers of HA units but different amounts of infectious virus. The broken lines indicate the levels of non-adsorbed seed virus 2 hours following inoculation.

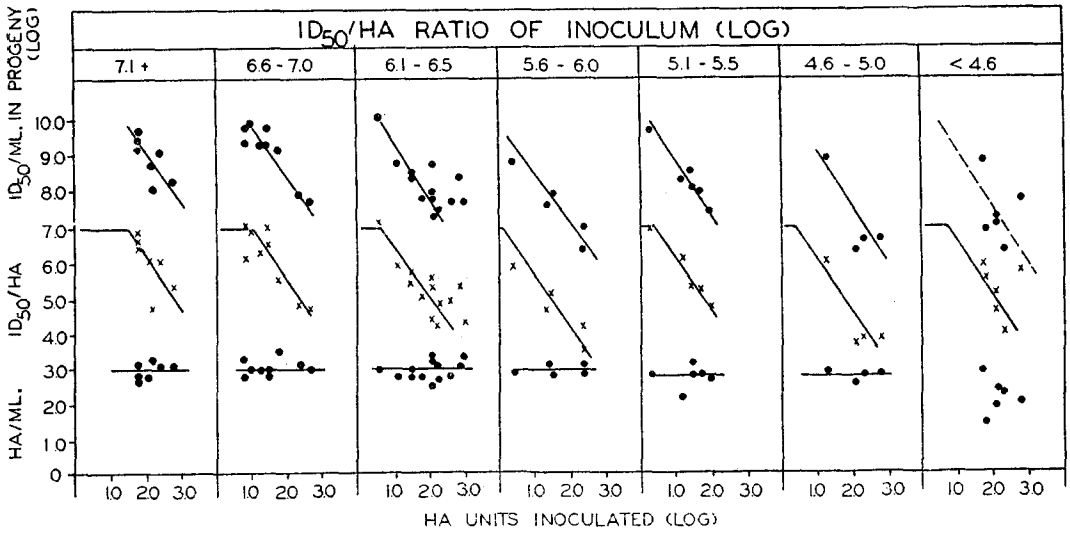


FIG. 5. Results of passages of seeds revealing similar $ID_{50}$/HA ratios but different amounts of hemagglutinins.

seed determined at 2 hours (broken line in the figure) indicates that significant increases occurred in all instances except in the groups injected with the largest amounts of hemagglutinin. In this case the 24 hour yields equalled the 2 hour levels in the middle range of seed infectivities. However, during the incubation period considerable inactivation of the residual seed virus must have occurred (16, 17) and thus enough infectious virus apparently was produced to maintain the 2 hour level. The yields of hemagglutinins were roughly of the same order as long as at least $10^7$ $ID_{50}$ were injected. When, however, the inoculum contained less infectious virus than this, smaller yields of hemagglutinins resulted as more and more HA units were injected. This critical number of seed $ID_{50}$ seems to correspond roughly to the number below which the yields of $ID_{50}$ and the $ID_{50}$/HA ratio of the progeny increase again. It will be seen from the figure that both these results were obtained mainly after inoculation of seeds of the 2nd or 3rd undiluted passages.

*Yields from Passages of Seeds Revealing Similar $ID_{50}$/HA Ratios but Containing Different Amounts of Hemagglutinin (or Infectious Virus).*—In Fig. 5, the yields of virus from 7 different groups of seeds with decreasing $ID_{50}$/HA ratios are plotted against the number of HA units inoculated. It will be seen that in 2 of the groups there are only a few seeds. Also, in some of the groups considerable irregularities are apparent, which are not surprising since in deriving $ID_{50}$/HA ratios the technical errors incident to both infectivity and HA titrations are combined. Nevertheless, the results seem to indicate that for seeds within a given range of $ID_{50}$/HA ratios, there are straight line relationships between the number of HA units (or correspondingly $ID_{50}$) inoculated and the yields of infectious virus and the ratios of the progeny. The hemagglutinin levels attained were of a similar order in all the groups although a slight over-all decline may be discerned as the ratio of the seed decreases. With a seed ratio of less than $10^{4.6}$ the yields of hemagglutinins decreased considerably. Straight lines have been fitted through the values for the ratios of the progenies in the 7 charts, all showing the same slopes. These reach the $10^7$ value ("maximum attainable" level) at points corresponding to inoculation of different amounts of hemagglutinin for seeds with different ratios. The higher the ratio of the seed the larger a number of HA units may be inoculated and still lead to a ratio of about $10^7$ in the progeny. However, as the seed $ID_{50}$/HA ratio falls progressively below a critical value ($10^{5.5}$) more hemagglutinin may again be inoculated and yet the progeny will have a ratio of $10^7$.

## DISCUSSION

The data presented confirm the results obtained by serial passages of undiluted allantoic fluids infected with influenza virus as reported by von Magnus (5, 25) and others. Production of hemagglutinins was unaffected for 2 or 3 serial transfers, but the yields of infectious virus were often considerably

reduced in the first, and regularly in the 2nd to 4th passages. These changes were reflected in the $ID_{50}$/HA ratios of the progenies, which fell from $10^6$ to $10^7$, characteristic of "Standard" virus preparations, to as low as $10^{3.3}$. The decreases in the $ID_{50}$/HA ratios in successive passages appeared to be influenced by the particular experimental conditions employed. They were somewhat more marked when the period of incubation in each passage was 48 hours instead of 20 to 24; when the volumes of seed inoculated were increased from 0.2 to 1.0 ml.; and when the seeds were stored between passages under unfavorable conditions. These observations suggested that the presence in the inocula of "complete" virus inactivated during incubation *in ovo* at 37°C. or on storage, might play a considerable part in the von Magnus phenomenon.

This suggestion raised the question whether a seed consisting entirely of infectious virus would be capable of inducing the von Magnus effect; *i.e.*, of yielding non-infectious hemagglutinins (NIHA).[3] If seeds are prepared by inoculation of small doses of virus and harvest during the period of rapid increase of virus in the allantoic fluid, accumulation of significant amounts of inactivated virus can be presumed to be excluded. This has been more firmly established recently by Horsfall (16). In the present study the precautions were carried even further by using for passage virus liberated during 2 hour intervals into the medium of eggs deembryonated shortly before or during the period in which the rate of maximal liberation of infectious virus had become established (26). The high $ID_{50}$/HA ratios obtained in such seeds indicated that little inactivated virus could have been present. Yet, both types of starting seeds on first passage lowered the $ID_{50}$/HA ratios to some extent, and on second transfers under the same conditions this effect became somewhat more pronounced, although the results did not approach those seen with the technic employed by von Magnus. Thus, it would seem that some NIHA is produced also by injection of fully infectious virus.

Efforts were made to define more clearly the conditions under which NIHA is formed. Here, two approaches were used. In the first, serial 2-fold dilutions of standard seeds with $ID_{50}$/HA ratios of about $10^{6.5}$ were inoculated allantoically and the infectious process was held to one cycle by subsequent induction of interference in remaining uninfected cells by inactivated heterotypic virus or by injection of RDE. NIHA production became apparent only when 0.5 ml. of the seeds were injected in dilutions not higher than 1:16 or 1:32. With more dilute inocula the progenies revealed standard $ID_{50}$/HA ratios. Taking the value of $10^{9.5}$ $ID_{50}$/ml. as the average for such standard seeds, it is apparent that the critical amount of infectious virus which just will produce some NIHA corresponds to about $10^8$ $ID_{50}$. The number of cells lining the allantoic cavity has been estimated to be of the same order (28). However, recent more

[3] NIHA, non-infectious hemagglutinins.

accurate determinations indicate that their number is smaller ($10^{7.4}$) (29, 30). It follows that NIHA production results when every cell has the opportunity to adsorb several virus particles. This result is contrary to the observations presented by Cairns and Edney (19), who concluded that NIHA formation becomes apparent when only one in one hundred cells has been infected. Since the $ID_{50}$/HA ratios of the progenies observed by these authors fell to levels as low as $10^4$, it is suggestive that the inocula they employed did not correspond to "Standard" virus seeds as used in the present study.

The other approach is based upon analyses of the results of over 50 undiluted passages of standard and UP seeds under similar experimental conditions. These offered the opportunity to evaluate the interrelationships between the $ID_{50}$ and HA titers of the seeds and their ratios, on the one hand, and the composition of the progenies derived therefrom, on the other. If the seeds are arranged in groups according to similar infectivities (Fig. 3), it is clear that as the HA concentration of the inocula increases the yields of infectious virus decrease in each group, the relationships being expressed by straight lines on logarithmic scales. The slopes of the lines are the same for the groups of seeds containing more than $10^7$ $ID_{50}$. Since in these cases the HA production was not affected, the $ID_{50}$/HA ratios decreased in parallel with the yields of infectious virus. When the seeds contained less than $10^7$ $ID_{50}$, the HA titers of the harvests also were reduced with an increase in the hemagglutinins injected. The slopes of the lines linking the yields of infectious virus and the $ID_{50}$/HA ratios in this group seem to be less steep than in the others, which is in keeping with the data presented in Fig. 4 to be discussed below. Using the $ID_{50}$/HA ratios of the seeds as basis for grouping (Fig. 5), similar straight line relationships on logarithmic scales are apparent in all groups between the HA units (or $ID_{50}$) injected and the infectivity titers and ratios of the progenies, all lines showing apparently similar slopes. However, with consecutive decreases in the seed ratios, the positions of the lines first appear to move successively to the left, but then they return again toward the right of the charts. The reasons for these changes become clear with the 3rd type of analysis, in which the seeds are grouped according to their HA titers. It seems to be apparent from Fig. 4 that the yields do not depend entirely upon the relative concentrations of $ID_{50}$ and HA units in the inocula but that other factors are also involved. For any given amount of seed HA greater than 10 units the yields of infectious virus (but not of hemagglutinin) decreased rapidly with a reduction in the number of $ID_{50}$ injected from $10^9$ to about $10^7$. With a further decrease in the seed $ID_{50}$, less hemagglutinins were found in the progenies; the infectivity titers rose, and thus also a considerable increase in the $ID_{50}$/HA ratios was noted. As a result, the yields of infectious virus and of the ratios fall on trough-shaped curves. It is seen that mainly seeds of the 2nd or 3rd undiluted passages were responsible for the decreased HA production and concomitant increases in yields of infec-

tious virus. It would thus seem probable that the non-infectious hemagglutinins in such seeds differ to some extent from those of earlier passages. With respect to this suggestion, it is of interest to note that differences have been reported in the sedimentation constants of the dominant virus components obtained in successive undiluted passages (8) as well as in their lipid content (10).

These analyses showed that as long as less than 10 HA units were injected, regardless of the type of seed employed, the progenies revealed $ID_{50}$/HA ratios in the range of "Standard" virus. It has been shown recently that the number of particles constituting one HA unit is the same for standard and undiluted passage seeds (24, 11) although they differ to some extent morphologically. With standard virus 10 HA units would correspond to approximately $10^{7.5}$ $ID_{50}$ and, most likely more. Thus upon inoculation of 10 or more HA units, conditions are established for multiple adsorption of virus particles per cell, a prerequisite for NIHA production, as discussed above. However, it also is apparent that within the limits discussed in the preceding paragraph, the greater the proportion of infectious virus in seeds of given HA levels, the smaller the yields of NIHA in the progeny.

The available evidence indicates then that multiple infection of cells with fully infectious virus may produce the von Magnus effect to some extent, but the phenomenon is considerably more pronounced if the seeds contain appreciable amounts of non-infectious hemagglutinins. These, as pointed out, may represent inactivated "complete" virus, accumulating in the seeds under various conditions of passage. However, the question remains whether all the NIHA found in undiluted passage seeds can be accounted for by inactivation of complete virus or whether other forms of NIHA may also be present. Several considerations favor the latter view. (*a*) The 24 hour harvest of an undiluted passage may contain, for example, $10^8$ HA units and $10^{6.5}$ $ID_{50}$. If it is assumed that at some stage the virus corresponding to all the hemagglutinins present had been infectious, the total number of $ID_{50}$ that might have been found would have reached at least $10^{9.5}$. Thus, in order to explain the titers actually observed on the basis of inactivation during the period of incubation at 36 to 37°C. loss of infectivity at the rate of more than 3 $\log_{10}$ units in less than 20 hours would have to be assumed, which is at least 100 times greater than the rate of inactivation observed *in vitro* with the strain of virus employed (1.1 $\log_{10}$ unit per day (17)). The rate of inactivation of extracellular virus *in ovo* does not seem to differ significantly from that observed *in vitro* (16, 17). (*b*) Growth curves obtained with undiluted standard virus or first and second undiluted passage seeds in chick embryos have shown that as soon as production of viral material in the allantoic membranes or its liberation into the allantoic fluid becomes detectable (after incubation of 3 to 4 and 5 to 6 hours, respectively) the $ID_{50}$/HA ratios of the harvests fall below those of the seeds (5, 25, 16, 31) indicating that NIHA is released without delay. This is even

more evident in differential growth curves in deembryonated eggs (15) in which release of low ratio material can be detected up to 2 hours earlier. Furthermore, once the maximal rate of liberation has been established, usually in 6 to 8 hours, the yields of $ID_{50}$ and HA units per 1 or 2 hour interval remain nearly constant for periods in excess of 24 hours (17). These results appear to be incompatible with the view that all NIHA found under these conditions represent complete virus which has been inactivated during the course of incubation. It is apparent rather that large amounts of NIHA of a different kind are released from the host cells in undiluted passages.

It is essential then to compare undiluted passage seeds with standard virus which has been inactivated at 37°C. *in vitro*. Although it has been found that such heated standard seeds can reproduce the von Magnus effect to some extent (32, 16), certain of the results also point to significant differences between undiluted passage and heated standard seeds. These will be discussed in the papers to follow (17, 18).

## SUMMARY

An analysis has been made of factors contributing to the von Magnus phenomenon; *i.e.*, the emergence of increasing quantities of non-infectious hemagglutinins (NIHA) in successive passages in the allantois of chick embryos of undiluted allantoic fluids infected with influenza virus.

Using the PR8 strain, the von Magnus phenomenon was pronounced when the serial seeds were obtained under conditions which permitted extensive inactivation of infectious virus during individual passages. Correspondingly, it was reduced but not abolished when precautions were taken to avoid accumulation of inactivated virus in the inocula. Thus, inactivated virus may be taken as a contributing factor.

Preparations of infectious virus obtained under conditions largely excluding the presence of inactivated virus were capable of yielding some NIHA on passage as long as sufficient amounts were injected to permit each host cell to adsorb several infectious virus particles. However, the fact remains that more NIHA was found in the harvests when the inocula contained a large proportion of non-infectious virus material.

Following injection of various types of seeds NIHA appeared in the allantoic fluids as soon as liberation of virus became detectable. This time relationship and the rates of release of non-infectious virus components seemed to exclude that the NIHA obtained consisted entirely of infectious virus which had been inactivated during incubation *in ovo*. It was apparent rather that NIHA other than that due to heat-inactivated virus was released.

Correlations between the infectivities and hemagglutinating capacities of over 50 standard and undiluted passage seeds and the compositions of the harvests derived therefrom on passage without dilution indicated that the

corresponding activities in the yields did not depend entirely upon the relative concentrations of infectious virus and non-infectious hemagglutinins in the inocula but that apparently different forms of NIHA were obtained in successive undiluted passages.

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