ON THE REPRODUCTION OF INFLUENZA VIRUS

Quantitative Studies with Procedures Which Enumerate Infective and Hemagglutinating Virus Particles

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After infection with influenza virus the amount of the agent increases at a rapid rate and may reach relatively high levels. Disease develops, after inocula of moderate or small size, only if the virus multiplies; and it appears when the amount of the agent is large. Whether it is the process of reproduction, the presence of a large amount of the virus, or both that lead to disease is not yet clear.

On the premise that if more could be learned about reproduction of the virus the mechanism of disease production might be better understood, the reproductive process has been studied in this laboratory for some years. To study reproduction, it is necessary to know something about the parents as well as the offspring. It is desirable to be able to count the number of both and to identify certain characters. To this end, procedures have been developed which make possible the enumeration of both infective and hemagglutinating influenza virus particles. These techniques have been employed in an investigation on the reproductive process. Previous reports from this laboratory (1, 2) have summarized briefly certain of the results secured in this study.

Many features of the multiplication of influenza virus have been extensively investigated during recent years. Because procedures which permitted precise enumeration of both infective and hemagglutinating particles were not available, it was necessary to employ very large conversion factors in efforts to correlate the results of infectivity and hemagglutination titrations. The need for such conversion factors became evident with the initial work of Hirst (3) and has been emphasized by the more recent studies of von Magnus (4–8), Hoyle (9, 10), Henle and coworkers (11–16), Cairns and coworkers (17, 18), and Fazekas de St. Groth and coworkers (19–21). The ratio between infectivity and hemagglutination titration end points, *e.g.*, EID_{50}/HA ratio, has been reported (5, 9, 13, 20, 21) to have a value of about 10^6 to 10^7 depending somewhat on the technique employed. However, it was not demonstrated that the preparations used contained only infective virus particles. On the basis of evidence obtained in this study, it is probable that a considerable proportion of non-infective hemagglutinating particles was present.

As a result of the extensive work of von Magnus (4–8) and Gard and coworkers

(22, 23) the idea that so called "incomplete" virus is formed after very large inocula has gained wide acceptance. The basis of the "incomplete" virus concept was a marked discrepancy between infectivity and hemagglutination titration end points which resulted in values for the ID_{50}/HA ratio much lower than those commonly obtained. The basic observations were confirmed recently by Cairns and Edney (17) and extended to other strains by Fazekas de St. Groth and Graham (21). Because of the lack of precise enumeration procedures, it has not been feasible previously to describe results in other than relative terms, and a quantitative evaluation of the bearing of non-infective hemagglutinating particles on the reproductive process has not been possible.

This paper presents in full a number of the findings which have been obtained in a quantitative study on the reproduction of influenza virus. It will be shown that influenza A virus (PR8) remains infective only for a short time under physiologic conditions; that the half-life of infective particles at 35°C. in allantoic fluid is no longer than 2½ hours. It will be demonstrated that the instability of the infective property can lead to the accumulation of large numbers of non-infective hemagglutinating particles and that such naturally inactivated particles can affect the reproductive process in a number of ways. The results of kinetic studies on adsorption, accumulation, and decay of infective particles *in vivo* are presented. It will be shown that under most experimental conditions, reproduction results in the appearance of the same number of infective and hemagglutinating particles; *i.e.*, only complete or mature particles are produced. On loading the host cell system with large inocula unusual alterations in the reproductive process develop and, in addition to infective particles, there also appear hemagglutinating particles which are non-infective. It will be demonstrated that naturally inactivated particles in adequate number can cause alterations in the reproductive process which are such as might lead to the appearance of non-infective particles.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was employed. This strain has been through very many passages in the chick embryo. During the past 10 years it has been maintained by occasional passage in the allantoic sac. Between passages infective allantoic fluid has been stored at −60°C. Preparations of seed virus for this study were obtained as follows: Infective allantoic fluid was diluted in sterile broth at 4°C. and 0.2 ml. was injected into the allantoic cavity of 10 day old chick embryos. After incubation at 35°C., the eggs were chilled for 1 hour at −26°C. in a mechanical refrigerator. Allantoic fluid was then removed, pooled, and immediately stored either at 4°C. or at −60°C. Antimicrobial substances were not added either to the original inocula or to seed virus pools. Allantoic fluid which yielded bacteria on culture was discarded.

Embryonated Eggs.—Fertile white Leghorn eggs which had been incubated at 38°C. for 10 days were employed. Groups of 5 to 8 eggs were used, each egg was inoculated into the allantoic cavity with 0.2 ml. After inoculation, eggs were held at 35°C. in a humidified incubator. Allantoic fluid was removed after the desired period of incubation. All available fluid was removed from each egg in a group at one time and the fluids were pooled and

aliquots were immediately stored either at 4°C. or at −60°C. in the absence of CO_2 gas (24). Antimicrobial substances were not added to the experimental pools. All which yielded bacteria on culture were discarded. The concentration of hemagglutinating particles was determined on aliquots stored at 4°C. for no more than 7 days. The concentration of infective particles was determined on aliquots stored at −60°C. which were thawed but once. The quantity of allantoic fluid obtained 1 to 2 days after inoculation ranged from 4 to 5 ml. per egg.

Erythrocytes.—Blood was taken from three or more Rhode Island Red roosters, pooled, and mixed with acid-citrate-dextrose solution (25). The erythrocytes were collected on the same day and washed three times in the centrifuge with buffered saline. Suspensions containing about 10^7 RBC per ml. in 0.14 M NaCl, buffered at pH 7.1 with 0.01 M phosphate, were stored at 4°C. not longer than 5 days.

Enumeration of Hemagglutinating Virus Particles.—The number of hemagglutinating particles was computed from the number of RBC that sedimented at an increased rate under standardized conditions. The details of the procedure and the specially designed apparatus employed will appear in another communication (26). In brief, a photometric apparatus was arranged so that the concentration of RBC at a fixed level in the test tube could be determined from the amount of light scattered. Pyrex test tubes with an inside diameter of 11 mm. were employed. Each tube was selected by optical standardization with saline and RBC suspension. The light beam was 9 mm. in diameter and its center crossed the tube 21.5 mm. below the meniscus, and 11.5 mm. from the bottom. Each tube contained 3.0 ml. of RBC suspension (5×10^6 RBC per ml.) in buffered saline along with the desired dilution of virus. Virus dilutions were prepared in buffered saline by transferring 1.5 ml. to 1.5 ml. of diluent in series. After mixing virus dilutions with RBC suspension, a number of tubes were placed promptly in the apparatus and not disturbed thereafter. The virus-RBC reaction occurred *in situ* at 22–24°C. By means of a mechanically operated carrier each tube could be moved into and out of the light path without disturbing the sedimenting RBC. Measurements of RBC concentration at the level of the light beam were made at 10 minute intervals, beginning usually at 90 minutes and ending at 140 minutes. With small amounts of virus, the decrease in the RBC concentration in the light path is linear during this period, and all the data can be used in computing a precise value for the concentration at 120 minutes. In the usual manner (27), the exact virus dilution which would cause 35 per cent more of the RBC, than in the control tubes, to sediment through the light path in 120 minutes was computed. The number of hemagglutinating virus particles was taken as equal to one-half the number of rapidly sedimenting RBC, on the basis, that under the conditions stated, each virus particle would be expected to react on the average with only two RBC during collisions and would tend to form an erythrocyte doublet. Support for the validity of this idea was provided recently by the independent work of Levine, Puck, and Sagik (28).

Enumeration of Infective Virus Particles.—The number of infective particles was computed from the 50 per cent infectivity end point on titration in the allantoic sac of 10 day old chick embryos. For infectivity titrations, virus preparations were frozen at −60°C. immediately after harvest, and stored promptly at −60°C. in the absence of CO_2 gas. Aliquots were thawed but once and then discarded. A series of 3.16-fold dilutions was made by transfer of 2.0 ml. into 4.32 ml. of sterile broth at 4°C. A separate pipette was used at each step in the series. A group of 4 to 6 embryos was inoculated with each dilution; 0.2 ml. was injected into the allantoic cavity. Eggs were then incubated at 35°C. for at least 48 hours. The allantoic fluid from each was removed after chilling at −26°C., as described above, and dilutions were tested with 0.25 per cent RBC for hemagglutinating virus. The 50 per cent end point was determined by the method of Reed and Muench (29). On the basis of the Poisson distribution, this end point was taken as equivalent to the injection of 0.69 infective particle per egg.

Computation of the Number of Non-Infective Hemagglutinating Particles.—The number of non-infective (N) particles was taken as equal to the difference between the number of hemagglutinating (H) and the number of infective (I) particles, in accord with the relation: [H] = [I] + [N]. Support for the idea that this relation is valid is given in the experimental section.

Precision of Enumeration of Hemagglutinating Particles.—A series of 30 measurements of the precision of the procedure in simultaneous duplicate or replicate determinations gave a standard deviation of ±15.8 per cent for the estimates of the number of hemagglutinating particles in various virus preparations.

Precision of Enumeration of Infective Particles.—A series of 34 measurements of the precision of the technique in simultaneous duplicate or replicate determinations yielded a standard deviation of ±40.9 per cent for the estimates of the number of infective particles in various virus preparations.

TABLE I

Results of Typical Replicate Determinations of the Number of Particles of Influenza A Virus (PR8)

Replicate No.*	Hemagglutinating particles (H) per ml.	Infective particles (I) per ml.	Ratio I/H
	× 10^9	× 10^9	
1	3.80	1.10	0.29
2	2.88	1.10	0.38
3	3.23	0.62	0.19
4	4.36	1.32	0.30
5	4.90	0.76	0.15
Mean‡	3.83 ± 0.63	0.98 ± 0.23	0.26 ± 0.07

* The allantoic fluid pool employed was obtained 48 hours after inoculation of 3 × 10^2 infective virus particles per egg.

‡ ± = mean deviation from the mean.

EXPERIMENTAL

Enumeration of Particles of Influenza A Virus (PR8).—The results of a typical series of replicate determinations of the number of virus particles in infected allantoic fluid are shown in Table I. The allantoic fluid pool used was collected 48 hours after inoculation of only 3 × 10^2 infective virus particles. The number of hemagglutinating particles (H) found per milliliter of fluid was, on the average, approximately four times greater than the number of infective particles (I); the ratio of infective to hemagglutinating particles (I/H) had an average value of 0.26. As is demonstrated in later sections, an I/H ratio of this value is to be expected after incubation of infected eggs for 48 hours and can be attributed to the marked instability of the infective property of influenza virus particles at 35°C. On the basis of the relation, [H] = [I] + [N], described above, the number of non-infective

hemagglutinating particles (N) in this allantoic fluid pool was 2.85×10^9 per ml., or 74 per cent of the total number of hemagglutinating particles. Because the quantity of allantoic fluid obtained from each egg averaged 5.0 ml., the total number of virus particles (H) produced per egg in 48 hours was about 1.91×10^{10}.

Rate of Inactivation of Infective Particles.—When infected allantoic fluid

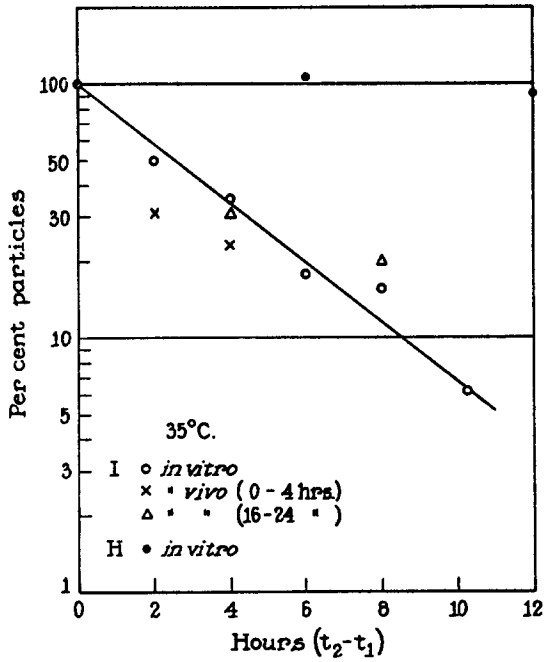

FIG. 1. Rate of spontaneous inactivation of infective (I) particles of influenza A virus (PR8) in allantoic fluid at 35°C. Each point is the mean of results obtained in 3 to 12 experiments. From 0 to 4 hours *in vivo*, the results represent the difference between the number of hemagglutinating particles and the number of infective particles lost during the interval. From 16 to 24 hours *in vivo*, the results represent the difference between the number of hemagglutinating particles and the number of infective particles gained during the interval. The interval between the time of the first (t_1) and that of the second (t_2) measurement is given on the abscissa.

was held at 35°C. *in vitro*, the number of infective particles decreased in an exponential manner as time increased. The results of a number of experiments on the spontaneous inactivation of infective virus particles are shown in Fig. 1. Each point is the mean of results obtained in 3 to 12 experiments. Different infected allantoic fluids, with virus concentrations varying over a range of 10,000-fold were employed. Regardless of the number of infective particles present initially, the proportion of particles that lost infectivity was

nearly constant during each 2 hour interval. After only 8.5 hours at 35°C., approximately 90 per cent of the originally infective particles had become non-infective. Throughout this period the number of hemagglutinating particles remained unchanged.

Computations from these data show that the time required to inactivate 50 per cent of infective particles was 147 minutes; *i.e.*, the half-life of I particles in allantoic fluid at 35°C. *in vitro* was approximately 2.5 hours. As is demonstrated in later sections, the half-life of infective particles in the allantoic fluid of the living embryo at 35°C. appears to be identical with that found *in vitro*. Lauffer *et al.* (30) previously determined the rate of inactiva-

TABLE II

Effect of Time of Incubation on the Number of Hemagglutinating and Infective Particles of Influenza A Virus (PR8) in Allantoic Fluid

Time after inoculation*	Hemagglutinating particles (H) per ml.	Infective particles (I) per ml.	Ratio I/H	Non-infective particles (N)‡ per ml.
hrs.	$\times 10^9$	$\times 10^9$		$\times 10^9$
16	0.002	0.002	1.00	0.00
20	0.135	0.112	0.83	0.02
22	1.36	1.32	0.97	0.04
24	1.74	1.82	1.04	0.00
26	1.35	1.20	0.89	0.15
28	2.40	1.20	0.50	1.20
48	7.59	1.59	0.21	6.00
72	4.48	0.64	0.14	3.84

* 3×10^2 infective particles inoculated.

‡ Computed from the relation: [N] = [H] − [I].

tion of infective influenza A virus *in vitro* at somewhat higher temperatures. Their data indicate that the half-life becomes even shorter than that given above as the temperature is increased. The results of some preliminary experiments indicate that the half-life of influenza B virus (Lee) is no longer than 2.5 hours at 35°C. *in vitro*.

Production of Fully Infective Virus Preparations.—When a small number of infective particles was inoculated and allantoic fluid was harvested at various intervals thereafter, it was found that the proportion of particles that were infective decreased as the period of incubation was increased. The results of some typical experiments are given in Table II. The values given are means of results obtained in 2 to 6 experiments at each time interval. After inoculation of only 3×10^2 infective particles, allantoic fluids with an I/H ratio of 0.8 or more were obtained at 16 to 26 hours. Such preparations were considered to be fully infective for, within the probable errors of the

enumeration procedures, they contained the same number of infective and hemagglutinating particles. However, after 28 hours the I/H ratio decreased progressively until at 48 and 72 hours the values were only 0.21 and 0.14, respectively. These values indicate that at 48 hours only 21 per cent, while at 72 hours only 14 per cent, of the hemagglutinating particles were still infective.

The progressive decrease in the I/H ratio with increasing time after inoculation can be taken as an indication of the rate at which originally infective particles were inactivated and converted to non-infective (N) hemagglutinating particles *in vivo*. The number of N particles present at each interval was computed, as described above, and is given in Table II. After 26 hours, the number of hemagglutinating particles continued to increase and in fact the production of such particles during the period 26 to 48 hours was far larger than that during the interval 0 to 26 hours. The accumulation of N particles was especially marked after 26 hours. Data obtained in other experiments with much larger inocula, which are included in Fig. 1, show that the rate of late inactivation *in vivo* corresponded with that obtained *in vitro*. As can be seen, the data obtained *in vivo* 16 to 24 hours after inoculation coincide well with those described above on the inactivation of infective particles *in vitro*.

Levine *et al.* (28) found recently, with similar enumeration procedures, an average value of 0.33 for the I/H ratio at 48 hours. In addition, they demonstrated that particle counts with the electron microscope agreed well with hemagglutinating particle measurements. Donald and Isaacs (31) have found that the number of EID_{50} was about 0.1 the particle count obtained with the electron microscope when allantoic fluid was harvested at about 30 hours.

In Table III are shown some typical results of experiments with inocula of various size and composition which yielded fully infective virus preparations. The larger the number of infective particles inoculated the shorter was the period of incubation which gave a yield that was fully infective. Additional data bearing on this relation are given in later sections.

Decrease in Concentration of Virus Particles after Inoculation.—After inoculation into the allantoic fluid of embryonated eggs, the number of virus particles decreased progressively from 0 to 4 hours. When fully infective virus inocula, *i.e.* $I/H \simeq 1.0$, were used and the concentrations of infective and hemagglutinating particles were determined at intervals, results like those shown in Fig. 2 were obtained. The decrease in the number of infective particles when 3×10^7 or fewer were inoculated, was nearly linear on a logarithmic scale from 0 to 4 hours. The lowest concentration of I particles was found regularly at 4 hours, when the number of particles inoculated was not greater than 3×10^7. After this interval, an increase in the number of I particles occurred which also was nearly linear on the same scale. It should

be noted that at 8 hours, the increase in the number of I particles was sufficient to reestablish the concentration at the time of inoculation.

In contrast, when 3×10^8 infective particles were inoculated, the concentration of I particles decreased but little after 2 hours and then remained almost constant until 8 hours. The first increase in I particle concentration, after inocula of this size, occurred at 10 hours. With inocula containing 3×10^7 to 2×10^9 particles, the rate of decrease in the concentration of H particles was slower than that for I particles.

In Fig. 3, the decrease in the concentration of infective particles with

TABLE III

Production of Fully Infective Preparations of Influenza A Virus (PR8)

Inoculum		Incubation	Allantoic fluid		
Infective particles	Non-infective particles (N)		Hemagglutinating particles (H)	Infective particles (I)	Ratio I/H
No. injected	No. injected		per ml.	per ml.	
		hrs.	$\times 10^8$	$\times 10^8$	
3×10^2	0	22	14.7	16.2	1.10
"	0	22	12.7	11.0	0.87
"	0	24	19.1	21.4	1.12
"	0	24	15.5	15.8	1.02
"	0	26	12.6	13.5	1.07
"	0	26	14.4	11.0	0.76
3×10^4	3×10^5	16	3.2	3.4	1.06
3×10^6	0	10	0.08	0.06	0.75
"	0	12	3.3	2.5	0.76

increasing time after inoculation was corrected for the amount of inactivation which occurred during the intervals employed. This was done on the basis that the rate of inactivation of I particles in allantoic fluid *in vivo* was equal to that found *in vitro*. As is shown in Fig. 1, the increasing difference between the number of H particles and the number of I particles lost in the interval, 0 to 4 hours, was closely similar to that expected if the rates of inactivation *in vivo* and *in vitro* were in fact equal.

Under these circumstances, the proportion of inoculated virus particles that disappeared from the allantoic fluid from 0 to 4 hours was almost independent of the number inoculated over a range of 1,000-fold. In addition, this proportion was nearly constant at each interval whether measured by infective or by hemagglutinating particles. Regardless of the number of particles injected, about 21 per cent remained present in the allantoic fluid at 2 hours. At 4

hours, about 5 per cent remained present when 3×10^7 or fewer particles were injected. In these experiments with fully infective virus inocula, no evidence for a constant concentration or plateau period was found when the number of particles inoculated was 3×10^7 or less. On theoretical grounds, it would not be expected that a plateau period should have occurred. In this

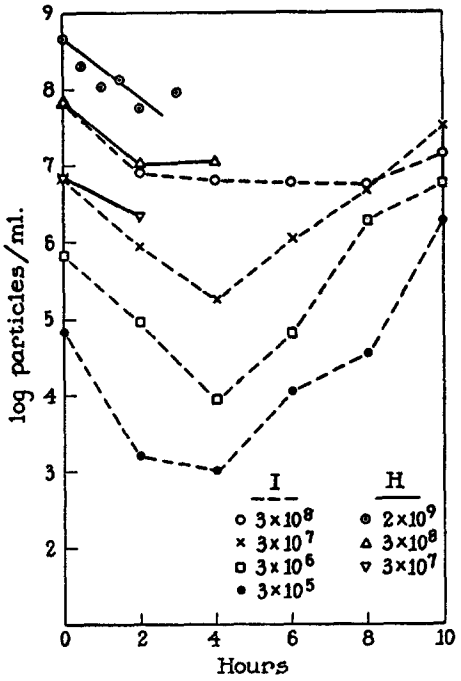

FIG. 2. Changes in the concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid with time after inoculation of fully infective preparations, *i.e.* I/H ratio $\simeq$ 1.0, of influenza A virus (PR8). Results obtained during both the decrease period, 0 to 4 hours, and the early increase period, 4 to 10 hours, after inocula of various size are shown.

regard, these findings are dissimilar to those reported previously by Henle and Henle (13) and Henle and Liu (16).

The difference in the number of virus particles injected and the number found in the allantoic fluid during the interval, 0 to 4 hours, may be taken as a measure of the number of particles adsorbed by the allantoic membrane. When the number of infective particles which had become inactivated at each interval was taken into account, it became evident that hemagglutinating and infective particles were adsorbed at the same rate and to the same extent (*cf.* Fig. 3). In these experiments, the mean value for the maximum number of particles adsorbed after the largest inocula employed was 1.6×10^9. Compu-

tations (13, 17, 20) based largely on indirect evidence have indicated that the number of cells lining the allantoic membrane may be about 10^8. If this value is assumed to be correct, then with the largest inocula used in this study the maximum number of particles adsorbed per cell was about 16. As is indicated in later sections, the number of allantoic cells may not be as large as has been thought previously. Cairns and Edney (17) previously reported that, as

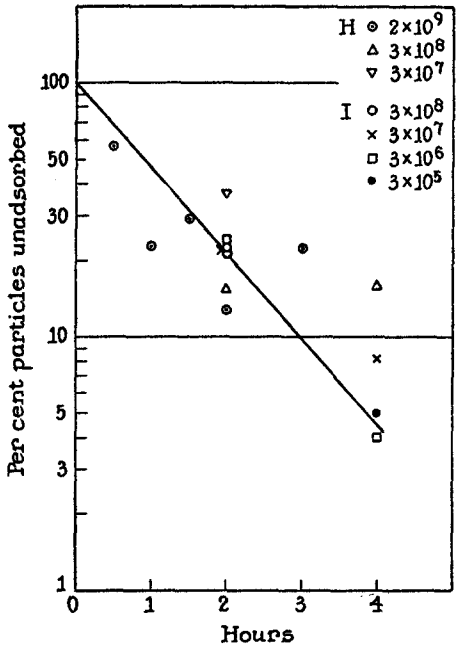

FIG. 3. Rate of decrease in concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid after inoculation of fully infective preparations, *i.e.* I/H ratio $\simeq$ 1.0, of influenza A virus (PR8). Measurements of infective particle concentration were corrected for inactivation on the basis of a half-life for I particles of 147 minutes at 35°C. in allantoic fluid.

measured by hemagglutinating units, the proportion of virus adsorbed was independent of the size of the inoculum and amounted only to about 50 per cent of that injected. On the basis of their computations they estimated that about 30 particles per cell could be adsorbed by the allantoic membrane.

Because of the slow rate of adsorption of particles by the allantoic membrane, a considerable proportion of the infective particles inoculated became inactivated, *i.e.* non-infective, during the adsorptive period. Of the unadsorbed particles in the allantoic fluid, 42 per cent became non-infective in 2 hours and 66 per cent in 4 hours, on the basis of a half-life for I particles of approximately 2.5 hours. Therefore, to measure adsorption precisely by

means of infective particles, it was necessary to correct the data in terms of the proportion of I particles which were inactivated concurrently. In previous studies (16, 32) on the adsorption of infective virus as measured by ID_{50} doses, the effect of spontaneous inactivation of I particles during the long adsorptive period was not taken into account.

Rate of Increase in Concentration of Virus Particles.—When fully infective virus inocula were employed the rate at which the concentration of virus particles increased in the allantoic fluid was nearly constant on a logarithmic

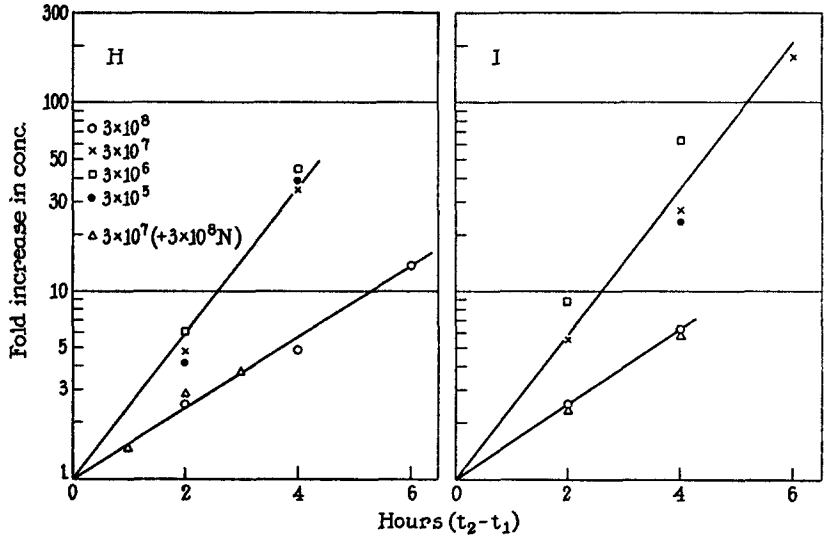

FIG. 4. Rate of increase in concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid after inoculation of fully infective preparations, *i.e.* I/H ratio $\simeq$ 1.0, of influenza A virus (PR8). N indicates non-infective particles, spontaneously inactivated at 35°C. *in vivo*. The interval between the time of the first (t_1) and that of the second (t_2) measurement is given on the abscissa.

scale for some hours after the period of decreasing concentration was completed, so long as the number of particles inoculated did not exceed 3×10^7. As is shown in Fig. 2, the point of minimum virus concentration was regularly 4 hours with inocula of this size. Thereafter, the number of infective and hemagglutinating particles increased at a rapid rate.

As is shown in Fig. 4, the rate of increase was the same, whether determined by enumeration of infective or hemagglutinating particles, after inoculation of 3×10^5 to 3×10^7 infective particles. With inocula of this size or less, the time required to double the concentration of virus particles during the logarithmic increase period was 46 minutes. During this period, the rate of increase remained constant for at least 6 hours.

When larger inocula were used the rate of increase was reduced. Thus, inoculation of 3×10^8 infective particles led to a rate of increase only one-half that obtained with smaller inocula. As is shown in Fig. 4, the time to double the virus concentration was then 92 minutes, whether determined by infective or hemagglutinating particle enumeration. A closely similar reduction in the rate of increase was found also when an equally large number of non-infective particles was present in the infective inoculum. As is demonstrated in Fig. 4, injection of 3×10^8 non-infective (N) particles (spontaneously inactivated at 35°C. *in vivo*) along with 3×10^7 infective particles did not lead to a rate of increase corresponding to that expected with this number of I particles. Under these conditions, the rate of increase was identical with that found after inoculation of 3×10^8 I particles; the time to double the virus concentration was 92 minutes as indicated by measurements with both hemagglutinating and infective particles. Smaller numbers of non-infective particles, *i.e.* 3×10^7 or less, when injected along with I particles had no effect on the rate of virus increase as measured by either procedure.

Thus, it appears that there is an upper limit to the number of virus particles, either infective or non-infective, which can be injected without markedly affecting the rate of increase in concentration of newly formed virus particles. When more than this number was injected the rate of increase was diminished relative to that found with smaller inocula.

The rate at which the concentration of virus particles increased after inocula of 3×10^7 or less infective particles, corresponds well with the findings reported previously by Ziegler and Horsfall (33), Henle *et al.* (32), and von Magnus (6) with this virus. Computations with their data indicate that when 10^4 or less EID_{50} were inoculated, the rate of virus increase observed in the allantoic fluid during the logarithmic period was equivalent to a doubling time of about 40 minutes. Other studies from this laboratory indicate that the rate of increase is markedly dependent upon the host cell system employed. Thus, with the same virus in the mouse lung, the doubling time was found to be approximately 140 minutes (34). In the allantoic sac, other strains of influenza A virus, *i.e.* FM1 (26) and DSP (10), have shown comparable rates of increase. However, strains of influenza B virus, *i.e.* Lee and MB, exhibit definitely slower rates of increase and the doubling time appears to be 60 minutes (26, 33).

Virus Reproduction after Inoculation of Fully Infective Virus.—Inoculation of fully infective virus preparations, *i.e.* those with an I/H ratio $\simeq$ 1.0, led to the production and release of fully infective virus when the number of particles inoculated was not greater than 3×10^6. The results of experiments in which 3×10^5 and 3×10^6 infective particles were inoculated are shown in Fig. 5. From the first appearance of new virus particles, which occurred constantly at the first measurement made after 4 hours, the I/H ratio had a

value approaching 1.0 at each interval analyzed up to 12 or 14 hours. This indicates that all hemagglutinating particles were infective when they appeared; *i.e.*, that non-infective hemagglutinating particles were not produced in measurable amount. After 12 or 14 hours, the number of hemagglutinating particles increased more rapidly than did the number of infective particles as might be expected because of inactivation resulting from the short half-life of I particles. After either inoculum, the number of non-infective particles accumulated rapidly after 14 hours. In both cases the proportion of N particles present at 22 hours was greater than 66 per cent of the total, although almost none were found at 12 hours.

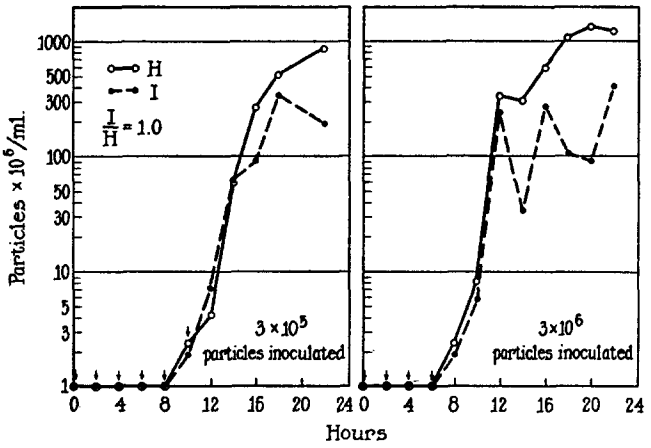

FIG. 5. Concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid at intervals after inoculation of fully infective preparations, *i.e.* I/H ratio $\simeq$ 1.0, of influenza A virus (PR8). The number of particles inoculated per egg was 3×10^5 and 3×10^6, respectively. Arrows indicate values lower than those plotted.

On inoculation of 3×10^7 or more infective particles, unaccompanied by non-infective particles, the new virus particles which emerged into the allantoic fluid were not all infective. The results of experiments in which 3×10^7 and 3×10^8 infective particles were inoculated are shown in Fig. 6. After either of these large inocula, the new virus particles, released during the logarithmic increase period, had an I/H ratio constantly less than 0.1, indicating that less than 10 per cent of the hemagglutinating particles present were infective. The rate of increase in virus concentration after inoculation of 3×10^7 I particles was closely similar to that obtained with 3×10^6 or 3×10^5 particles (*cf.* Fig. 5). However, a much slower rate of increase was found on inoculation of 3×10^8 I particles, as was also shown above (*cf.* Fig. 4). After 12 hours, the I/H ratio became even lower in both cases which indicates that non-infective particles were accumulating. By 20 hours, the proportion

of N particles present was greater than 96 per cent of the total in both experiments.

The total number of hemagglutinating particles produced in 20 hours was approximately the same, *i.e.* about 10^9 per ml., regardless of the number of infective particles inoculated over a range of 1,000-fold. However, the number of infective particles found in the yield after these inocula was inversely related to the number of I particles inoculated and was considerably reduced when this number was 3×10^7 or more. The maximum number of I particles found after inoculation of 3×10^7 such particles, was approximately 10^8 per ml.; after 3×10^8 only about 10^7 per ml. It is not possible to compare

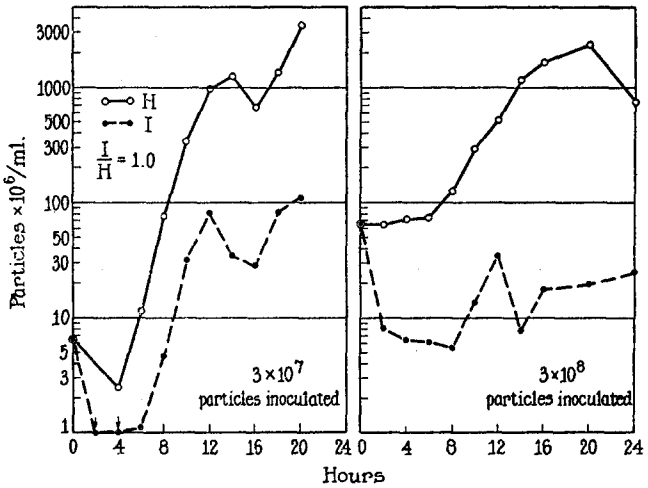

FIG. 6. Concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid at intervals after inoculation of fully infective preparations, *i.e.* I/H ratio $\simeq$ 1.0, of influenza A virus (PR8). The number of particles inoculated per egg was 3×10^7 and 3×10^8, respectively. Arrows indicate values lower than those plotted.

these results directly with those secured by other workers for, unfortunately, similar experiments with fully infective virus inocula appear not to have been carried out previously.

Production of Partially Infective Virus Preparations.—When incubation was continued for 48 hours or more after inoculation of a small number of infective particles only a moderate proportion of the particles in the yield remained infective (*cf.* Table II). Similarly, inoculation of a considerably larger number of particles followed by incubation for 20 to 24 hours also led to the development of only partially infective preparations. The results of a number of typical experiments which yielded partially infective virus preparations are shown in Table IV. These allantoic fluid pools contained from 2 to

22 per cent infective virus particles and were similar to those used as inocula in the experiments described in the section which follows.

Virus Reproduction after Inoculation of Partially Infective Virus.—Inoculation of from 10^5 to 10^6 infective particles usually led to the production, at 24 hours or later, of virus preparations with I/H ratios of 0.1 or less. Moreover, as was shown above (*cf.* Table II), inoculation of only 3×10^2 infective particles yielded, at 48 hours, virus preparations which had I/H ratios of no more than 0.2. Such ratios indicate that a large proportion of the virus particles were non-infective. As was shown above, these low ratios can be attributed to the spontaneous inactivation *in vivo* of initially infective particles.

TABLE IV

Production of Partially Infective Preparations of Influenza A Virus (PR8)

Inoculum		Incubation	Allantoic fluid		
Infective particles	Non-infective particles (N)		Hemagglutinating particles (H)	Infective particles (I)	Ratio I/H
No. injected	No. injected		per ml.	per ml.	
		hrs.	$\times 10^9$	$\times 10^9$	
3×10^5	3×10^6	24	1.82	0.16	0.09
"	"	24	3.30	0.06	0.02
"	"	24	1.90	0.27	0.14
3×10^6	0	20	1.33	0.09	0.07
3×10^2	0	48	11.7	1.10	0.09
"	0	48	10.9	1.86	0.17
"	0	48	6.0	1.35	0.22

Preparations of this kind have been commonly employed as seed virus material by various workers (6, 11, 16, 17, 21). The finding that such preparations have an I/H ratio no greater than 0.2 indicates that no more than 20 per cent of the hemagglutinating particles in the seed virus were infective. As is demonstrated below, the presence of a sufficient number of non-infective particles in an infective inoculum can cause marked alterations in the reproductive process.

In Figs. 7 and 8 are shown the results obtained at 16 and 20 hours, respectively, after inoculation of varying amounts of fully infective virus preparations, *i.e.* I/H ratio $\simeq$ 1.0, and of 10 per cent infective virus preparations, *i.e.* I/H ratio $\simeq$ 0.1. The 10 per cent infective preparations were obtained 24 hours after inoculation of 10^6 I particles. Each point shown is the mean of results secured in 2 separate experiments. Both the number of infective particles and non-infective particles present in each inoculum are given. As

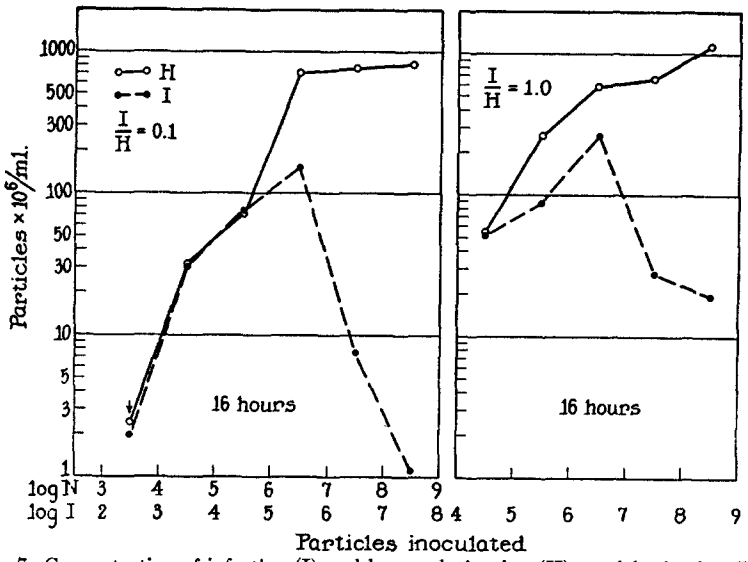

FIG. 7. Concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid at 16 hours after inoculation of varying amounts of influenza A virus (PR8). Both partially infective preparations (I/H ratio $\simeq$ 0.1) which contained 10 times more non-infective (N) than infective particles, and fully infective preparations (I/H ratio $\simeq$ 1.0) which contained only infective particles were used as inocula. Arrows indicate values lower than those plotted.

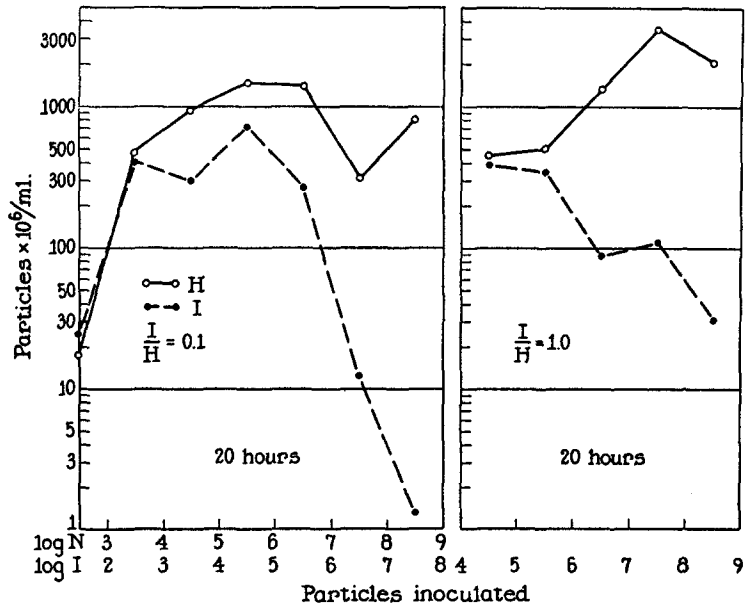

FIG. 8. Concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid at 20 hours after inoculation of varying amounts of influenza A virus (PR8). Both partially infective preparations (I/H ratio $\simeq$ 0.1) and fully infective preparations (I/H ratio $\simeq$ 1.0) which contained only infective particles were used as inocula.

shown in Fig. 8, the presence of 3×10^7 or more non-infective particles in the infective inocula reduced the yield of hemagglutinating particles, at 20 hours, to an average of 23 per cent of that obtained with the same inocula of fully infective virus. The reduction in the yield of infective particles under the same conditions was far more marked and was evident both at 16 hours (*cf.* Fig. 7) and 20 hours (*cf.* Fig. 8). When 3×10^7 or more N particles were injected simultaneously with 0.1 as many I particles, the yield of infective particles, at both 16 and 20 hours, was only about 5 per cent of that obtained when comparable amounts of fully infective virus alone were inoculated.

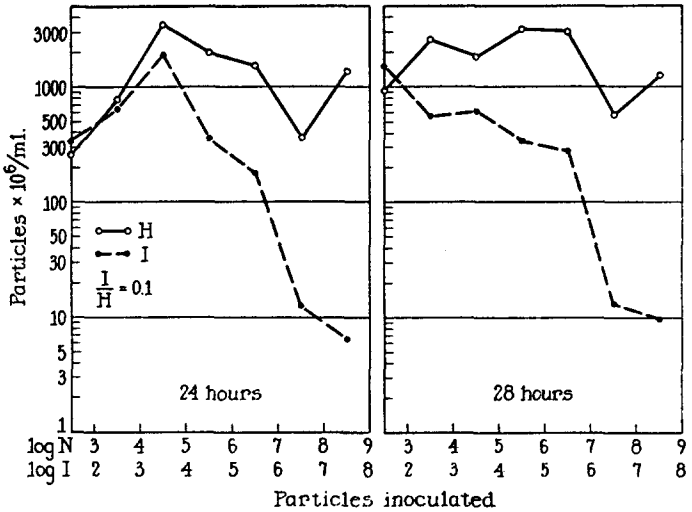

FIG. 9. Concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid at 24 and 28 hours, respectively, after inoculation of varying amounts of influenza A virus (PR8). Partially infective preparations (I/H ratio $\simeq$ 0.1), which contained 10 times more non-infective (N) than infective particles, were used as inocula.

It should be emphasized that, even as early as 16 hours, fully infective yields of virus were obtained only if the infective inocula contained no more than 3×10^5 N particles. Moreover, at 20 hours, fully infective virus yields were obtained with 10 per cent infective inocula, only when no more than 3×10^2 I particles were inoculated.

In Fig. 9 are shown the results obtained at 24 and 28 hours, respectively, after inoculation of varying quantities of 10 per cent infective virus preparations, *i.e.* I/H ratio $\simeq$ 0.1. As in Figs. 7 and 8, each point is the mean of results obtained in 2 separate experiments. The findings show that, regardless of the duration of the reproductive process, the presence of 3×10^7 or more N particles in the infective inocula led to a persistent reduction in the yield of hemagglutinating particles. They show also that, under the same condi-

tions, the yield of infective particles was much more strikingly diminished; *i.e.*, no more than 5 per cent of that obtained when 3×10^6 or less N particles were present in the infective inocula.

It will be noted that as time was increased, inocula which resulted in the production of fully infective yields contained progressively smaller numbers of infective particles. Thus, although the inoculation of 3×10^4 I particles

TABLE V

Effect of Mixtures of Non-Infective and Infective Particles of Influenza A Virus (PR8) on the Proportion of Infective Particles in the Yield

Inoculum			Incubation	Virus yield*		
Infective particles	Non-infective particles (N)			Hemagglutinating particles (H)	Infective particles (I)	Infective particles
No. injected	Inactivated at	No. injected		No./ml.	No./ml.	Per cent
			hrs.			
3×10^5	—	0	16	2.6×10^8	8.9×10^7	34.0
3×10^5	56°C., 60 min.	1×10^8	16	3.3×10^8	8.3×10^7	25.0
1×10^5	56°C., 30 min.	3×10^8	16	8.3×10^7	6.1×10^7	73.0
3×10^5	—	0	20	5.1×10^8	3.4×10^8	66.0
3×10^5	56°C., 60 min.	1×10^8	20	2.5×10^9	3.4×10^8	13.0
1×10^5	56°C., 30 min.	3×10^8	20	1.5×10^9	3.4×10^8	22.0
1×10^5	35°C., 48 hrs.	2×10^8	16	2.9×10^7	7.4×10^5	2.5
1×10^5	35°C., 48 hrs.	2×10^8	20	6.6×10^7	1.6×10^6	2.4
3×10^7	—	0	16	6.8×10^8	2.9×10^7	4.3
1×10^7	22°C., 4 days	6×10^8	16	1.2×10^9	7.1×10^6	0.6
3×10^7	—	0	20	3.4×10^9	1.1×10^8	3.2
1×10^7	22°C., 4 days	6×10^8	20	2.5×10^9	6.2×10^6	0.2
3×10^4	—	0	24	7.4×10^7	7.1×10^7	96.0
1×10^4	22°C., 8 days	1×10^9	24	9.5×10^7	8.3×10^5	0.9

* In allantoic fluid.

gave yields with an I/H ratio of about 1.0 at 16 hours (*cf.* Fig. 7), at 28 hours such a yield was obtained only after the inoculation of 3×10^1 I particles (*cf.* Fig. 9).

It appears obvious from the results shown in Figs. 7 to 9 that there is a definite limit to the number of non-infective particles which can be included in infective inocula without seriously altering the reproductive process. As few as 3×10^7 N particles reduced the total yield of new virus particles to about 30 per cent and also markedly diminished the number which were in-

fective at each of the time intervals studied. Moreover, the effects produced by including N particles in the inocula, were quantitatively greater as regards diminution of the yield of I particles than those produced by comparable numbers of I particles in the absence of preformed N particles.

Virus Reproduction after Inoculation of Mixtures of Non-Infective and Infective Virus.—As was demonstrated above, the non-infective particles in partially infective virus inocula can markedly affect the reproductive process when present in sufficient number. That the addition of non-infective particles to fully infective virus preparations also can lead to similar alterations in virus reproduction is shown below. Infective influenza virus particles can be made non-infective in a variety of ways without diminishing their hemagglutinating activity. One of the simplest means is thermal inactivation (3, 35). The rate of inactivation has been shown to be markedly dependent on temperature (30). In the present study the temperature used to cause inactivation was found to be an important variable and affected the capacity of the non-infective particles to produce alterations in the reproductive process.

The results of some typical experiments with mixtures of non-infective and infective particles are shown in Table V. The mixtures were prepared promptly after various thermal inactivating procedures and were used as inocula in the usual manner. Non-infective particles prepared at 56°C., for either 30 or 60 minutes, did not cause a striking effect on the yield of hemagglutinating or infective virus particles. Even when as many as 3×10^8 such particles were added to inocula containing 10^5 infective particles, both the amount of virus produced and the proportion that was infective were not diminished. However, non-infective particles prepared at 35°C. (48 hours) or at 22°C. (4 or 8 days) did cause definite reductions in the number of infective particles found in the yield. Addition of 10^8 or more non-infective particles, inactivated at these lower temperatures, to inocula containing 10^4 to 10^7 infective particles regularly caused a reduction in the proportion of I particles obtained as compared with control inocula containing similar numbers of infective particles. The smaller the number of infective particles included in the mixture the more marked was the reduction caused by the non-infective particles in the proportion of I particles found in the yield.

The results of a series of experiments with fully infective virus inocula as well as with mixtures of such virus and non-infective particles prepared at 56°C. (30 or 60 minutes) are shown in Fig. 10. Each point is the mean of the results of 4 to 10 experiments in each of which the yields of hemagglutinating and infective virus particles were analyzed at 16 and 20 hours, respectively. As was shown above (*cf.* Figs. 7 and 8), the inoculation of a sufficiently large number of infective particles alone caused reduction in the proportion of I particles found in the yield at fixed intervals. From the results shown in Fig. 10, it is clear that when 3×10^7 or more I particles, but no N particles, were

inoculated there was an inverse relation between the number inoculated and the proportion of I particles found in the yield. Only when less than 10^6 infective particles were inoculated did the yield contain 50 per cent or more I particles, at the late intervals studied. It should be emphasized that a con-

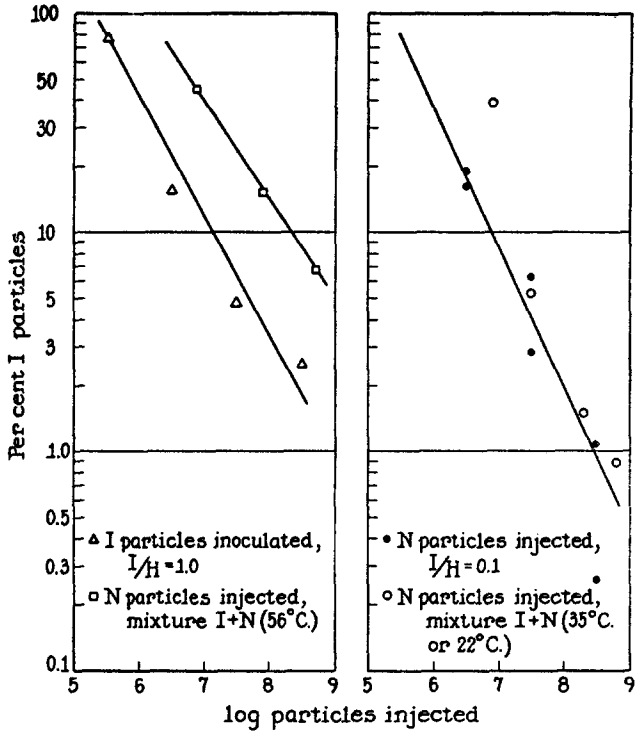

FIG. 10. Average proportion of infective (I) particles in the allantoic fluid at 16 and 20 hours after inoculation of mixtures of infective and non-infective (N) particles of influenza A virus (PR8). Non-infective particles were prepared by inactivating infective particles *in vitro* at 56°C. for 30 or 60 minutes, 35°C. for 48 hours, or 22°C. for either 4 or 8 days. For comparison, the average results obtained at the same intervals after inoculation of fully infective preparations (I/H ratio $\simeq$ 1.0) and partially infective preparations (I/H ratio $\simeq$ 0.1) are also shown. The partially infective preparations used, contained 10 times more non-infective (N) particles, spontaneously inactivated in the allantoic fluid *in vivo*, than infective particles.

siderable proportion of the infective particles inoculated became non-infective through inactivation during the long adsorptive period (*cf.* Fig. 1). Because of the short half-life of infective particles at 35°C., virus that was fully infective at the time of inoculation did not retain infectivity in undiminished degree in allantoic fluid *in vivo* during the adsorptive period.

It is also evident, from the results shown in Fig. 10, that inoculation of mixtures containing varying numbers of non-infective particles, inactivated

at 56°C., and infective particles did not diminish the proportion of I particles in the yield to the same extent as did the inoculation of large numbers of infective particles alone. In these experiments, relatively small numbers of infective particles, *i.e.* 3×10^5 or less, were included in the mixtures, so that the effect of the I particles themselves would be insignificant in extent. As is shown by comparison of these results with those given in Figs. 7 and 8, the proportion of I particles found in the yields was not significantly affected by the addition of large numbers of 56°C.-inactivated particles to the infective inocula. Mixtures containing as many as 5×10^8 N particles (56°C.) did not decrease the yield of I particles when compared with similar infective inocula.

The results of a series of experiments with partially infective virus preparations, *i.e.* I/H ratio $\simeq 0.1$, as well as with mixtures of fully infective virus and non-infective particles prepared under milder conditions, *i.e.* 35°C. for 48 hours or 22°C. for 4 or 8 days, are also shown in Fig. 10. Each point is the mean of the results of 4 to 10 experiments in each of which the yields of virus were analyzed for hemagglutinating and infective particles at 16 and 20 hours, respectively. With the 10 per cent infective inocula, there was an inverse relation between the number of non-infective (N) particles contained in the inocula and the proportion of I particles found in the yields. This relation was clearly demonstrable when the number of N particles present in the inocula was 3×10^7 or more.

Moreover, the inoculation of mixtures of non-infective particles, inactivated at either 35° or 22°C., and infective particles produced results closely similar to those secured with 10 per cent infective virus inocula. As is shown in Fig. 10, with such mixtures there was also an inverse relation between the number of non-infective (N) particles contained in the inocula and the proportion of I particles found in the yield. As in the case of 10 per cent infective inocula, this relation was demonstrable when the number of N particles in the inocula was 3×10^7 or greater. There was no correlation between the number of infective (I) particles inoculated with these mixtures and the proportion of I particles found in the yields. As example, some mixtures which contained more than 10^8 N particles (inactivated at 22°C.) also contained only 10^4 I particles and yet the yield contained only about 1 per cent I particles. The results of an experiment with such a mixture are shown in Table V.

That large amounts of non-infective virus in infective inocula led to a reduced ID_{50}/HA ratio was clearly demonstrated previously by von Magnus (6, 8, 36) and was affirmed by the recent results of Fazekas de St. Groth and Graham (21). It seems clear that, in the present study, the quantitative effects produced by the addition of non-infective (N) particles, inactivated at 35° or 22°C., to infective inocula were marked. Furthermore, the effects produced by the addition of particles inactivated at either temperature *in vitro* corresponded closely with those produced by non-infective particles which had become inactivated *in vivo* in partially infective preparations. Thus, it is evi-

dent that the presence in infective inocula of a sufficient number of non-infective (N) particles, *i.e.* 3×10^7 or more, can alter the reproductive process and lead to the appearance of a much reduced proportion of infective virus particles.

DISCUSSION

The instability of infective influenza A virus particles causes them to lose the infective property rapidly at temperatures used for the incubation of embryonated eggs. The demonstration that the half-life of infective particles is only 2.5 hours at 35°C. in allantoic fluid, both *in vitro* and *in vivo*, introduces a previously unrecognized factor in studies on reproduction of the virus. The importance of this factor became clear when precise enumeration procedures for both infective and hemagglutinating particles were developed in this laboratory and employed in investigations on the reproductive process. Many of the apparent discrepancies between infectivity and hemagglutination titers which are abundant in the literature can be resolved on the basis of the brief half-life of infective particles.

It has not been possible with any procedure to separate infective from hemagglutinating particles. There is a large body of evidence indicating that infective particles constantly possess the hemagglutinating property. However, the reverse is not true, and hemagglutinating particles may or may not possess the infective property. The available evidence makes it probable that when the number of infective particles is equal to the number of hemagglutinating particles, these two properties reside in the same virus particles. When there are fewer infective (I) than hemagglutinating (H) particles, the difference can be accounted for by hemagglutinating particles which are non-infective (N). The quantitative relation between the concentrations of these particles may be formulated by the equation: $[H] = [I] + [N]$. Through the use of enumeration procedures for both infective and hemagglutinating particles, it has been possible to test the validity of this relation under a wide variety of conditions. The quantitative data obtained indicate that the relation fits the findings and provides a means for determining the number of non-infective particles.

The results reported by Levine *et al.* (28), which appeared while this investigation was in progress, provide strong support for the idea that the number of hemagglutinating particles is equal to the total number of virus particles. Their particle counts with the electron microscope were in good agreement with the number of hemagglutinating particles determined by a procedure similar to that used in this investigation. The fact that preparations were obtained regularly, in the present study, which had similar numbers of infective and hemagglutinating particles indicates that nearly all newly formed particles are infective when certain experimental conditions are fulfilled. The

major variables bearing on the production of fully infective influenza A virus preparations were the size and composition of the inoculum and the duration of incubation of infected eggs. Fully infective preparations were constantly obtained, during the period of logarithmic increase in virus concentration, when the inoculum contained any number of infective particles up to 3×10^6 and no more than 10^6 non-infective particles (spontaneously inactivated *in vivo*). Under these conditions, only infective particles were found in the allantoic fluid until the concentration of particles reached approximately 10^8 per ml. Thereafter, as the logarithmic rate of increase diminished, non-infective particles accumulated in increasing concentration. Only with very small inocula, *i.e.* about 10^2 infective particles, was it possible to obtain infective particles in a concentration as high as 10^9 per ml. In this case too, non-infective particles accumulated rapidly after the logarithmic rate of increase in virus concentration diminished.

During the logarithmic period, the rate of increase in virus concentration was so rapid when the doubling time equalled 46 minutes, that inactivation resulting from the short half-life of infective particles was not detected. The proportion of particles which became non-infective during this interval was too small to be demonstrated by the enumeration procedures used. However, during later periods, when the rate of increase slowed down and the doubling time approached or exceeded the half-life of infective particles, accumulation of non-infective particles was readily demonstrated. That the non-infective particles which appeared after the logarithmic increase period were derived from infective particles which became inactivated *in vivo* seems probable. The rate of their appearance corresponded with that expected from inactivation of infective particles with a half-life of but 2.5 hours.

In contrast to the wide variety of conditions which could be used and regularly resulted in the reproduction only of infective particles, there was but one condition which led to the emergence of non-infective particles. Such particles appeared during the logarithmic increase period only when the inoculum contained 3×10^7 or more infective particles or 3×10^7 or more non-infective particles (inactivated either *in vivo* or *in vitro* at 35° or 22°C.). After such relatively large inocula, only a fraction of the new particles which appeared in the allantoic fluid were infective regardless of the time of assay. This finding is in accord with the results reported previously by von Magnus (6–8). When this one condition was fulfilled, the rate of increase in the concentration of non-infective particles during the logarithmic period was almost identical with that of infective particles. This points against the possibility that these non-infective particles appeared as a result of inactivation of infective particles after they had reached the allantoic fluid. Whether they were inactivated after reproduction while still in the allantoic cells themselves or were actually produced as non-infective particles, as has been suggested by

von Magnus (6–8, 36), remains an open question. So far, it has not been possible to determine the rate of inactivation of infective particles inside infected cells.

If it is assumed that the number of cells in the allantoic membrane is about 10^8, as has been suggested (13, 17, 20), an explanation is required for the finding that only 3×10^7 non-infective particles can markedly alter the reproductive process. These numbers give a particle-cell ratio of 0.3 which indicates that there was only one particle present per three cells. It seems unlikely that a single non-infective particle could affect more than one cell. However, if previous estimates were too large and the number of cells is actually about 10^7, the present findings can be resolved in terms of a straightforward hypothesis. Then the particle-cell ratio would have a value of approximately 3.0 and there would be enough non-infective particles to give about one per cell in terms of the Poisson distribution. On the basis that the number of cells was of the order of 10^8 (20), Cairns and Edney (17) computed that a reduced ID_{50}/HA ratio was obtained in the yield when the particle-cell ratio was as low as about 0.02. Even if it is assumed that the number of cells is this large, the present findings both with infective and with non-infective particles are not in accord with such an interpretation.

That the well ordered kinetics of the reproductive process are altered by too large an initial load of virus particles is even more clear from the results obtained after inoculation of 3×10^8 particles. This number gives a particle-cell ratio of about 30 if the number of cells is taken to be 10^7. After inocula of this size, the following alterations were regularly observed: The interval before new particles were demonstrable was increased by a factor of about 2. The rate of increase in particle concentration was decreased by the same amount. The proportion of infective particles which emerged was decreased by a factor of 20 or more. Either infective particles or non-infective particles inactivated *in vivo* or *in vitro* (at 35° or 22°C.), caused each of these alterations in the reproductive process when the number injected was 3×10^8 or more. It is obvious that when the rate of increase is diminished, the more nearly the time to double the virus concentration approaches the half-life of infective particles the smaller will be the proportion of infective particles found.

SUMMARY

Procedures which make possible the enumeration of both infective and hemagglutinating influenza A virus particles have been developed and used in a quantitative investigation on the reproduction of the agent. Infective particles were found to be highly unstable and their half-life was only 147 minutes in allantoic fluid at 35°C. both *in vitro* and *in vivo*. The instability of infective particles provides an explanation for the rapid accumulation of

non-infective particles which retained the hemagglutinating property. The number of non-infective (N) particles was determined from the difference between the number of hemagglutinating (H) particles and the number of infective (I) particles as indicated by the relation: [N] = [H] − [I].

When the half-life of infective particles was taken into account, both infective and hemagglutinating particles were found to disappear from the allantoic fluid; *i.e.*, were adsorbed by the allantoic membrane, at the same logarithmic rate after inoculation. Inoculation of any number of particles up to 3×10^7 was followed by a constant and progressive decrease in the proportion of unadsorbed particles from 0 to 4 hours. Approximately 20 per cent of particles were unadsorbed at 2 hours and about 5 per cent at 4 hours. Inoculation of 3×10^8 or more particles led to a larger proportion of unadsorbed particles at 4 hours. The maximum number of particles adsorbed was computed to be about 1.6×10^9.

The concentration of both infective and hemagglutinating particles increased rapidly in the allantoic fluid after 4 hours when any number of infective particles up to 3×10^7 was inoculated. With such inocula, the rate of increase during the logarithmic period was constant and the time to double the concentration of infective or hemagglutinating particles was 46 minutes. With larger inocula, *i.e.* 3×10^8 particles, the concentrations of infective and hemagglutinating particles did not increase until after 8 hours and the rate of increase was much slower. The time to double the concentration of either then became 92 minutes.

The number of infective particles was approximately equal to the number of hemagglutinating particles during the logarithmic increase period when any number of infective particles up to 3×10^6 was inoculated and no more than 10^6 non-infective particles were included in the inoculum. This finding was taken to indicate that all or almost all particles produced and released under these conditions were infective. That such particles became inactivated rapidly and led to the accumulation of an increasing number of non-infective particles after the logarithmic period can be explained by the short half-life of infective particles.

The number of infective particles was no larger than one-tenth the number of hemagglutinating particles during the logarithmic increase period after 3×10^7 or more infective particles had been inoculated or when smaller inocula were used which also contained 3×10^7 or more non-infective particles. Non-infective particles prepared *in vitro* at 35° or 22°C. were as effective as those which accumulated *in vivo* in diminishing the proportion of infective particles in the yield. The extent of the reduction in the proportion of infective particles was directly related to the number of non-infective particles included in the inoculum. The yield of hemagglutinating particles was diminished when the inoculum contained 3×10^7 or more non-infective particles.

The rate of increase was reduced so that the time to double the concentration became 92 minutes when the inoculum contained 3×10^8 non-infective particles.

It appears from these findings that the single condition which will lead to the emergence of non-infective particles during the logarithmic period is a high initial particle-cell ratio. Because non-infective particles are equally as effective as infective particles in producing this result, it seems probable that the appearance of non-infective but hemagglutinating particles is not a necessary accompaniment of the reproductive process.

Addendum.—After this manuscript was accepted for publication an independent investigation on the number of the allantoic cells was undertaken in this laboratory. The results of this study by Tyrrell, Tamm, Forssman, and Horsfall will be reported shortly in another communication. In summary, it was found by direct counting procedures that the allantoic sac of the 10 day old chick embryo contained 1.8×10^7 (standard deviation 0.1×10^7) allantoic cells.

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