REPRODUCTION OF INFLUENZA VIRUSES

Quantitative Investigations with Particle Enumeration Procedures on the Dynamics of Influenza A and B Virus Reproduction

By FRANK L. HORSFALL, Jr., M.D.

(From The Rockefeller Institute for Medical Research)

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On the premise that virus reproduction is the central problem in virus disease, the reproductive process has been investigated in this laboratory for some time. This premise is based on the concept that in nature virus induced lesions are not due merely to the presence of the agent but develop only after reproduction occurs. In earlier reports (1–3), the results of kinetic studies on the reproduction of pneumonia virus of mice (PVM) and influenza A virus (PR8) in the mouse lung as well as the associated development of lung lesions were described. More recently (4–6), the results of studies on the reproduction of influenza and Newcastle disease viruses in the allantoic sac of the chicken embryo were summarized. The recent development of procedures which yield counts of influenza virus particles (7, 8) made possible precise quantitative studies based on two independent properties of the particle. An investigation which employed both the unstable infective property and the stable hemagglutinating property of influenza A virus for particle enumeration has been reported in detail (8).

The demonstration (9) that the number of allantoic cells is 1.8×10^7, a value considerably smaller than earlier estimates (*cf.* reference 10), provided a basis for relating the number of virus particles and the number of available cells. This relation has been utilized in the present study to derive quantitative information on the kinetics of reproduction of influenza A and B viruses at the cell level. The results of numerous earlier studies on the multiplication of influenza viruses were reviewed in the preceding communication (8).

This report describes the results of an investigation on the reproduction of influenza B virus (Lee) in the allantoic membrane of the intact chicken embryo. It also describes the results of further studies on the reproduction of influenza A virus (PR8) in the same host tissue. The allantoic membrane is unusually favorable for studies of virus reproduction because the allantoic cells represent a natural monolayer of flat epithelial cells, the inner surfaces of which are in direct contact with the allantoic fluid. It will be shown that the infective property of Lee virus particles is even more unstable than that

of PR8 virus particles (8). It will be demonstrated that this instability leads to the rapid accumulation of non-infective particles which retain the hemagglutinating property and that this accumulation is even more extensive than with PR8 (8). The results of studies with various particle-cell ratios on adsorption, appearance time, accumulation, maximal yield, and inactivation of influenza virus particles *in vivo* are given. It will be shown that with particle-cell ratios below one the rate of appearance of new particles is constant on a logarithmic scale during early intervals and that the numbers of particles measured by the infective and the hemagglutinating property correspond closely. It will also be shown that with both Lee and PR8 virus, particle-cell ratios of about 3 or more can diminish the rate of reproduction, decrease the yield of new virus particles, and decrease the proportion of infective particles in the yield. It will be demonstrated that non-infective particles can cause similar alterations in the reproductive process at comparably high particle-cell ratios.

Materials and Methods

Viruses.—The Lee strain of influenza B virus and the PR8 strain of influenza A virus were used. Both had many passages in the allantoic sac. Seed virus pools were prepared exactly as in the preceding study (8). In brief, 10 day old chick embryos were inoculated into the allantoic cavity with virus diluted in cold broth. After incubation at 35°C. the eggs were chilled in air at −26°C. Pools of allantoic fluid were immediately frozen and stored at −60°C. in the absence of CO_2 gas (11). No antimicrobial substances were used at any stage. Pools which contained bacteria were discarded.

Embryonated Eggs.—White Leghorn eggs were used, as in the preceding study (8). They were incubated at 38°C. for 10 days. Groups of 5 to 10 eggs were employed. Each egg was given 0.2 ml. of diluted virus into the allantoic cavity and then held at 35°C. All allantoic fluid was removed after the eggs were chilled in air at −26°C. On the average, 5 ml. of fluid per egg was obtained. The fluids from each group were pooled, immediately frozen, and stored at −60°C., as above. No antimicrobial substances were used. Pools containing bacteria were discarded. For measurements, aliquots were thawed once and then discarded.

Number of Allantoic Membrane Cells.—The number of cells lining the allantoic cavity of the 10 day old chick embryo has been shown to be 1.8×10^7 (standard deviation = 0.1×10^7) (9). This value was used for computation of all virus particle–allantoic cell ratios.

Erythrocytes.—Rhode Island Red roosters were used, as in the preceding study (8). Blood from three or more was mixed with acid-citrate-dextrose solution (12) and pooled. A rooster was not bled oftener than once in 5 weeks. Erythrocytes were washed 3 times with buffered saline on the day they were obtained and stored at 4°C. at about 10^7 RBC per ml. in 0.14 M NaCl, buffered at pH 7.1 with 0.01 M phosphate. Suspensions were not stored longer than 4 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 carefully standardized conditions in the presence of diluted virus. The details of the procedure used and the photometric apparatus employed were described in a previous paper (8). The following features of the technique may be emphasized: The RBC concentration is determined from the amount of light scattered, not by reduction in light transmission. A concentration of 5×10^6 RBC per ml. is used in the reacting mixtures. The reaction tubes remain in the

vibration-free photometric apparatus throughout the observation period of 140 minutes. They are moved through the light beam mechanically at intervals of 10 minutes without disturbing the sedimenting RBC. The number of hemagglutinating particles is taken as equal to one-half the number of rapidly sedimenting RBC on the basis that with low virus particle–RBC ratios each particle reacts with only 2 RBC (7, 8).

Enumeration of Infective Virus Particles.—The number of infective particles was computed from the 50 per cent infectivity end point on 0.5 log titration in the allantoic cavity of 10 day old chick embryos. The details of the procedure are described in a previous paper (8). Inoculated eggs were incubated at 35°C. for at least 72 hours with Lee virus and for at least 48 hours with PR8 virus. Recent work in this laboratory (13) demonstrated that Lee virus infectivity end points were 3.6-fold higher after 72 as compared with 48 hours incubation. Additional incubation to 96 hours did not increase the end points. With PR8 virus, however, incubation for either 72 or 96 hours did not regularly increase the end point over that found after 48 hours incubation. Liu and Henle (14) have reported on the effects of the period of incubation on the infectivity end point with these two viruses. The production of hemagglutinating virus was taken to indicate infection of the allantoic membrane. On the basis of the Poisson distribution, the 50 per cent end point was taken as equivalent to the injection of 0.69 infective particle per egg.

Computation of Number of Non-Infective Hemagglutinating Particles.—As in the preceding study (8), the number of non-infective (N) particles wes determined from the relation: [N] = [H] − [I], when H = hemagglutinating particles, and I = infective particles.

Precision of Enumeration of Hemagglutinating Particles.—With Lee virus, a series of 23 measurements of the precision of the photometric procedure in simultaneous replicates gave a standard deviation of 17.6 per cent for estimates of the number of hemagglutinating particles. This compares well with the standard deviation of 15.8 per cent found previously (8) with PR8 virus.

Precision of Enumeration of Infective Particles.—With Lee virus, a series of 24 measurements of the precision of the titration procedure in non-simultaneous replicates gave a standard deviation of 43 per cent for estimates of the number of infective particles. This agrees with the standard deviation of 41 per cent reported earlier (8) with PR8 virus.

EXPERIMENTAL

Enumeration of Particles of Influenza B Virus (Lee).—The results of a typical series of non-simultaneous duplicate determinations of the number of Lee virus particles in allantoic fluid pools are shown in Table I. Each pool was collected 28 hours after inoculation of 2×10^4 infective particles per egg. The number of infective particles (I) was, on the average, 63 per cent of the number of hemagglutinating particles (H). On the basis that [N] = [H] − [I], as described above, the number of non-infective particles (N) was, on the average, 0.66×10^9 per ml.; *i.e.*, 37 per cent of the number of hemagglutinating particles. Thus, at 28 hours there was in the individual allantoic fluid a total of about 8.8×10^9 virus particles (H) of which more than half were infective. Such a high proportion of infective particles was obtained only when the number of virus particles inoculated was less than 1 per allantoic cell and the period of incubation was short. As shown below, the infective property of Lee virus at 35°C. is even more unstable than that of PR8 virus (8).

Rate of Inactivation of Infective Particles.—On holding allantoic fluid *in vitro* at 35°C., the number of infective particles decreased rapidly. As shown in Fig. 1, the number of hemagglutinating particles did not change appreciably during 12 hours, but 90 per cent of the infective particles were inactivated in 4.8 hours. This occurred regardless of the initial concentration or proportion of infective particles over a wide range. The proportion of particles that lost infectivity was constant per unit of time during the interval

TABLE I

Results of Duplicate Determinations of the Number of Particles of Influenza B Virus (Lee)

Allantoic fluid pool*	Hemagglutinating particles (H) per ml.	Infective particles (I) per ml.	Per cent infective particles‡
	× 10^9	× 10^9	
1	2.14	1.62	75.0
	1.51	1.58	105.0
2	3.80	2.40	63.0
	2.88	1.10	38.0
3	1.20	0.79	66.0
	0.69	0.44	64.0
4	1.00	0.45	45.0
	0.98	0.51	52.0
Mean (Pools 1 to 4).....	1.77	1.11	63.0

* Each pool contained allantoic fluid from 5 eggs. All pools were collected 28 hours after inoculation of 2 × 10^4 infective virus particles per egg. The inoculum had an I/H ratio of 0.2.

‡ Calculated on basis that $\frac{\text{I per ml.}}{\text{H per ml.}} \times 100 =$ per cent I. All duplicate determinations were carried out on different days.

studied, indicating that inactivation corresponded with first order reaction kinetics.

It was computed that the time needed to inactivate 50 per cent of infective particles of Lee virus was only 85 minutes; *i. e.*, the half-life of infective particles in allantoic fluid at 35°C. *in vitro* was about 1.5 hours. As is also demonstrated in Fig. 1, the half-life of infective particles in the allantoic fluid of the intact embryo at 35°C. seemed to be identical with that *in vitro*. In the earlier study (8), it was shown that the half-life of infective particles of PR8 virus under the same conditions *in vitro* or *in vivo* was 147 minutes. Thus, the infective property of both influenza viruses is strikingly unstable at 35°C. and Lee virus is even less stable than PR8.

Time of Incubation and Yield.—Because of the instability of the infective property, it was expected that the proportion of particles that were infective would decrease as the period of incubation was increased. As shown in Table II, this occurred even after small inocula. When 5×10^4 infective particles were inoculated, fluids containing more than 50 per cent of infective particles were not obtained later than 24 hours. At 32 and 48 hours, the proportion of infective particles decreased to 26 and 7 per cent, respectively. Despite the

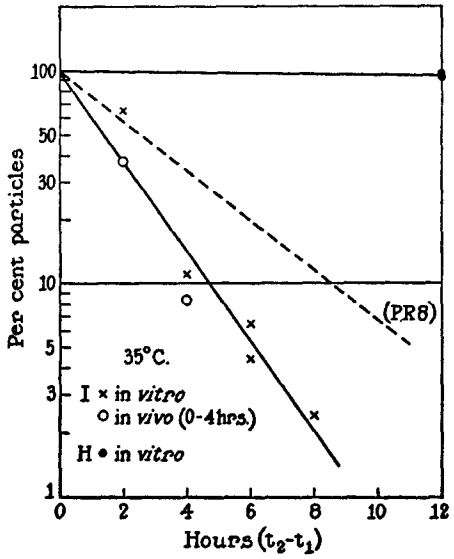

FIG. 1. Rate of spontaneous inactivation of infective particles (I) of influenza B virus (Lee) in allantoic fluid at 35°C. Each point is the mean of results obtained in two or more experiments. The results secured *in vivo* represent the difference between the decrease in the number of hemagglutinating particles (H) and the decrease in the number of infective particles during the same intervals in the intact embryo. The interval between the time of the first (t_1) and that of the second (t_2) measurement is given on the abscissa. The curve for inactivation of PR8 virus is identical with that reported previously (8).

high concentration of hemagglutinating particles which was attained, *i.e.* 7.9×10^9 per ml., the concentration of particles which were infective did not reach 10^9 per ml. at any interval. As shown below, regardless of the number of particles inoculated or the time of incubation, it was possible only in rare instances to obtain as many as 10^9 infective particles of Lee virus per ml. As demonstrated previously (8), somewhat higher concentrations of infective particles of PR8 virus can be secured regularly.

The progressive decrease in the proportion of infective particles with increasing time of incubation can be attributed to spontaneous inactivation of newly formed particles *in vivo*. Such inactivation leads to the rapid accu-

mulation of non-infective particles (N) in the allantoic fluid. On the basis that [N] = [H] − [I], the number of non-infective particles was computed and is shown in Table II. The concentration of non-infective particles approached 10^9 per ml. at 28 hours and was 7.3×10^9 at 48 hours. Comparable accumulation of non-infective particles was shown previously with PR8 virus (8).

Highly Infective Preparations.—Allantoic fluids containing relatively high concentrations of Lee virus and a large proportion of infective particles were obtained only if the inoculum was small and the time of incubation was short. The results of representative experiments which yielded highly infective prep-

TABLE II

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

Time after inoculation*	Hemagglutinating particles (H) per ml.	Infective particles (I) per ml.	Per cent infective particles	Non-infective particles (N)‡ per ml.
hrs.	× 10^9	× 10^9		× 10^9
16	<0.001	0.001	>100.0	0.00
20	0.006	0.008	>100.0	0.00
24	0.091	0.050	55.0	0.04
28	1.17	0.34	29.0	0.83
32	1.76	0.46	26.0	1.30
40	4.78	0.66	14.0	4.12
48	7.91	0.56	7.0	7.35

Values are the means of results obtained in two experiments at each interval.

* 5×10^4 infective particles inoculated.

‡ Computed on basis that [N] = [H] − [I].

arations are shown in Tables I and III. With inocula of 0.01 or less infective particle per cell and sufficiently short periods of incubation, it was possible to secure yields in the neighborhood of 10^9 per ml. with about 50 per cent of the particles infective. As shown below, the larger the number of infective particles inoculated, the shorter was the optimum period of incubation for a highly infective preparation. In the preceding study (8), a similar relation was demonstrated for PR8 virus.

Changes in the Concentration after Inoculation.—During the first 4 hours after inoculation into the allantoic cavity, the number of virus particles in the fluid decreased progressively. As shown in Fig. 2, the decrease in either infective (I) or hemagglutinating (H) particles was almost linear on a logarithmic scale from 0 to 4 hours if the inoculum contained 1.5×10^7 or less virus particles, *i.e.* 0.8 or less per cell. The rate of decrease in concentration during the first 2 hours was nearly independent of the number of particles inoculated. However, after inoculation of 1×10^8 or more particles, little

decrease in concentration of hemagglutinating particles occurred after 2 hours although the number of infective particles continued to diminish until 4 hours in every experiment. On the basis of the very short half-life of infective particles at 35°C. (*cf.* Fig. 1), this was expected. Similar results were obtained with PR8 virus in the preceding study (8).

The lowest concentration of infective particles was usually found at 4 hours regardless of the number of particles inoculated. Thereafter, both hemagglutinating and infective particles increased rapidly in concentration, and in every case the increase was nearly linear on a logarithmic scale from 4 to 10 hours. With all inocula except that containing only 1.5×10^5 infective

TABLE III

Highly Infective Preparations of Influenza B Virus (Lee)

Inoculum				Incubation	Virus yield*		
Infective particles		Non-infective particles (N)			Hemagglutinating particles (H) per ml.	Infective particles (I) per ml.	Per cent infective particles
No.	Particles per cell‡	No.	Particles per cell‡				
				hrs.	× 10^8	× 10^8	
2×10^4	0.001	7×10^4	0.004	28	24.0	16.2	67
"	"	"	"	28	9.5	6.0	63
"	"	"	"	28	33.1	16.2	49
"	"	"	"	28	8.7	4.3	49
2×10^5	0.01	7×10^5	0.04	22	3.8	4.2	110
"	"	"	"	22	5.1	2.5	49
2×10^6	0.1	7×10^6	0.4	12	0.50	0.11	22
"	"	"	"	12	0.41	0.11	27

* In allantoic fluid pools.

‡ Computed on the basis that the number of cells lining the allantoic membrane = 1.8×10^7 (9).

particles, the increase in the number of particles re-established the zero time concentration by 6 hours.

Adsorption by Allantoic Membrane.—The difference in the number of hemagglutinating particles injected and the number found in the allantoic fluid during the interval, 0 to 3 hours, can be taken as a measure of the number of virus particles adsorbed by the allantoic membrane. The hemagglutinating property is so stable that no inactivation occurs during this period at 35°C. (*cf.* Fig. 1). However, the infective property is rapidly inactivated, and on the basis of a half-life of 85 minutes, 77 per cent of unadsorbed particles become non-infective in 3 hours. Therefore, to measure adsorption by infective particle enumeration, it was necessary to correct the data for inactivation during the intervals studied. As shown in Fig. 3, when this was done, the rate of adsorption, whether measured with hemagglutinating or infective particles,

was nearly independent of the number of particles inoculated over a range of 10,000-fold. About 50 per cent of the injected particles were adsorbed in 72 minutes. This value corresponds fairly well with that computed from the results of the preceding study (8) with PR8 virus; 50 per cent of injected particles were adsorbed in 54 minutes. Thus, during an adsorption period of 3 or more hours, at least 82 per cent of the particles were adsorbed.

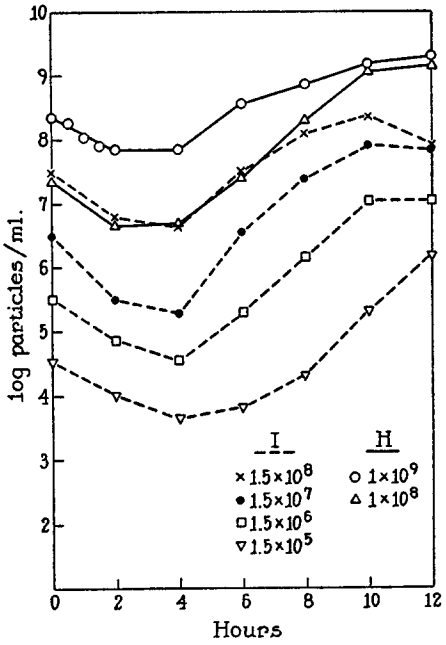

FIG. 2. Changes in concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid after inoculation of various amounts of influenza B virus (Lee). Each point is the mean of results obtained in two or more experiments. Two virus preparations were used as inocula; their I/H ratios were 0.15 and 0.2, respectively. Inoculated eggs were held at 35°C. for the time indicated. The number of I or H particles inoculated at zero time is shown.

With the largest inocula employed, the mean value for the maximum number of Lee virus particles adsorbed was 0.8×10^9. On the basis that the number of cells lining the allantoic membrane is 1.8×10^7 (9), it was computed that one allantoic cell can adsorb at least 44 Lee virus particles. From the data of the preceding study (8), it was computed that one allantoic cell can adsorb about 89 PR8 virus particles. Some support for the validity of these values was obtained on recomputation of the earlier adsorption data of Henle (15), Cairns and Edney (16), and Liu and Henle (17) in terms of the new count (9) of the number of allantoic cells and the half-life of infective particles at 35°C.

Rate of Increase in Concentration.—After inoculation of highly infective preparations (*cf.* Table III), the rate of increase in concentration of Lee virus particles in the allantoic fluid was almost constant on a logarithmic scale for a number of hours. As shown in Fig. 4, the rate of increase was not affected by the number of particles inoculated over a range of 300-fold but was definitely diminished after inoculation of 2×10^8 or more particles. For some hours after the adsorption period, the rate of increase was the same whether

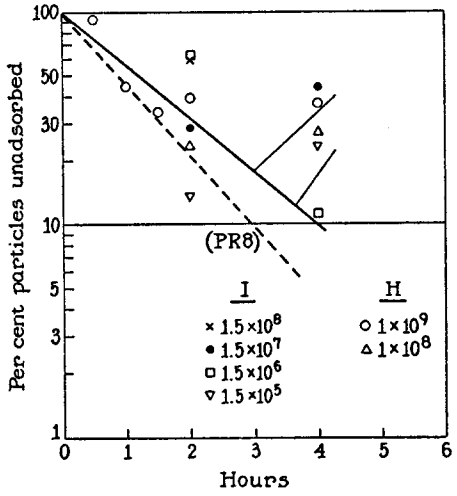

FIG. 3. Rate of decrease in concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid after inoculation of various amounts of influenza B virus (Lee). Each point is the mean of results obtained in two or more experiments. Two virus preparations were used as inocula; their I/H ratios were 0.15 and 0.2, respectively. Inoculated eggs were held at 35°C. for the time indicated. Measurements of infective particle concentration were corrected for inactivation on the basis of a half-life of 85 minutes at 35°C. The increase lines inserted at 3 and 3.6 hours represent these determined experimentally after inoculation of 1.5×10^8 and 1.5×10^7 I particles, respectively. The curve for adsorption of PR8 virus is identical with that reported previously (8).

it was determined by measurement of the concentration of hemagglutinating or infective particles. With inocula containing 1×10^8 or less particles, *i.e.* 5 or less particles per cell, the time to double the concentration of particles during the logarithmic increase period was 43 minutes. This doubling time remained constant for at least 6 hours.

With inocula containing 3×10^8 or more particles, the rate of increase was definitely reduced, as is shown in Fig. 4. Whether measured with hemagglutinating or infective particles, the doubling time was extended to 65 minutes after such large inocula, *i.e.*, 15 or more particles per cell, and did not become shorter regardless of the period of observation. A comparable increase in

the doubling time occurred when a large number of non-infective (N) particles, *i.e.* 2×10^8, were added to a small inoculum of infective particles, *i.e.* 2×10^5. The non-infective particles were prepared by holding infective allantoic fluid at 22°C. for 8 days *in vitro*. This procedure regularly yielded

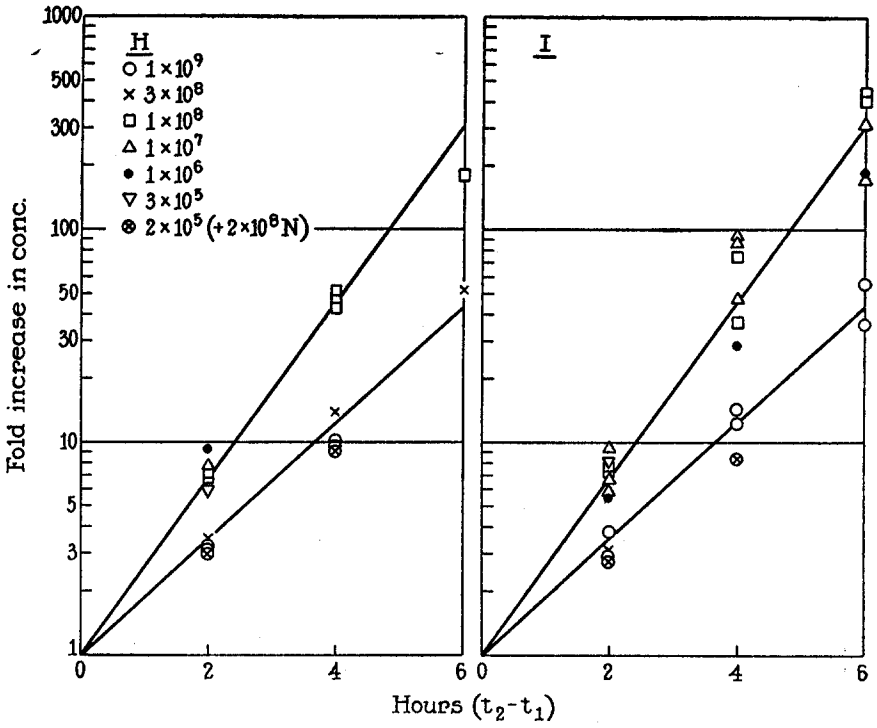

FIG. 4. Rate of increase in concentration of infective (I) and hemagglutinating (H) particles in the allantoic fluid after inoculation of various amounts of influenza B virus (Lee). Each point is the mean of results obtained in two or more experiments. Three virus preparations were used as inocula; their I/H ratios were 0.3, 0.5, and 0.6, respectively. Inoculated eggs were held at 35°C. for the duration of the experiments. N indicates non-infective particles, inactivated *in vitro* at 22°C. for 8 days, which were added to infective inocula as indicated. The interval between the time of the first (t_1) and the time of the second (t_2) measurement is given on the abscissa.

preparations with more than 99.9 per cent of non-infective particles. As is clear from Fig. 4, such non-infective particles, at a concentration of about 10 per cell, were as effective as a similar number of infective particles in increasing the doubling time. Smaller numbers of non-infective particles, *i.e.* 1 per cell, had no effect on the doubling time when added to relatively small infective inocula.

In the preceding study (8), closely similar results were obtained with PR8 virus. With inocula of about 2 particles or less per cell, the doubling time was 46 minutes. But with about 16 particles per cell, whether infective or non-infective (inactivated at 35°C.), the doubling time was increased to 92 minutes. It was surprising that the rate of increase in concentration of Lee virus was as rapid as that for PR8 virus. Earlier work in this laboratory (8, 18) had raised the possibility that the rate of increase for Lee virus was slower than that for PR8. However, computations with the data of Liu and Henle (19) indicate that the rates they encountered were comparable to those found in this study.

TABLE IV

Effect of Large Inocula on Rate of Production of Influenza A and B Viruses

Virus	Inoculum	Incubation	Hemagglutinating particles (H)*	
	Infective particles per cell		No. per cell	Yield per cell per hr.‡
		hrs.		
Influenza B (Lee)	8.3	4	20	—
		6	102	41
		8	198	48
		10	421	111
"	0.8	4	0.4	—
		6	7	3
		8	52	23
		10	320	134
Influenza A (PR8)	16.6	6	21	—
		8	34	7
		10	82	24
		12	142	30
"	1.6	6	3	—
		8	21	9
		10	92	36
		12	272	90

* Values are the means of results obtained in two experiments at each interval.

‡ Mean yield per hour during each 2 hour interval.

As shown in Table IV, the effect of large inocula on the rate of production of both viruses is also evident when the number of particles which appear per cell per hour is determined. After inocula containing about 8 to 16 infective particles per cell, the early yield increased only from 41 to 111 par-

ticles per cell per hour, *i.e.* about 3-fold, with Lee and from 7 to 30, *i.e.* about 4-fold, with PR8. In contrast, after inocula containing about one particle per cell, the early yield with Lee increased from 3 to 134 particles per cell per hour, *i.e.* about 45-fold, and with PR8 from 9 to 90, *i.e.* 10-fold.

Thus, with both Lee and PR8 there appears to be an upper limit to the number of particles that can be inoculated without causing a reduction in the rate of increase. This number seems to be in the neighborhood of 2 to 5 particles per cell, and almost certainly is less than 10 particles per cell. When more than this number of particles are injected, the dynamics of the repro-

TABLE V

Appearance Time of Influenza A and B Viruses

Virus	Inoculum	Time before new virus particles appeared in allantoic fluid*		
	Infective particles per cell	Hemagglutinating particles (H)	Infective particles (I)‡	Ratio, minutes H/I
		min.	*min.*	
Influenza B (Lee)	8.3	188	174	1.08
"	0.8	214	224	0.96
"	0.08	246	235	1.05
"	0.008	331	303	1.09
Influenza A (PR8)	16.6	144	293	0.49
"	1.6	173	257	0.67
"	0.16	198	216	0.92
"	0.016	216	201	1.02

* Computed from intercept of adsorption and increase curves.

‡ Corrected for inactivation during adsorptive period on basis that half-life of Lee = 85 minutes; PR8 = 147 minutes.

ductive process appear to be altered, and the rate at which new particles accumulate in the allantoic fluid is reduced. Additional effects on the maximal yield of virus particles and on the proportion that retain the infective property are described in later sections.

Appearance Time.—Because both the decrease in concentration of PR8 (8) and Lee virus particles during the adsorptive period (*cf.* Fig. 3) and the increase in concentration (8) for some hours thereafter (*cf.* Fig. 4) are linear on a logarithmic scale, an estimation of the appearance time can be obtained from the intercept of corresponding curves. When curves for adsorption of infective particles are employed, it is necessary to correct for inactivation in terms of the half-life of the virus used. The results of such computations for both Lee and PR8 viruses are shown in Table V. The data for PR8 were

taken from the preceding study (8). With both viruses, the time before hemagglutinating particles appeared in the allantoic fluid increased as the number of particles inoculated was decreased. With Lee virus, the time before infective particles appeared was almost identical with the time of appearance of hemagglutinating particles, regardless of the number of particles per cell inoculated. With PR8 virus, on the other hand, inocula containing more than one particle per cell led to relative delay in the appearance of infective particles. However, PR8 inocula which contained 0.2 or less particles per cell gave H/I time ratios which approached unity as did all Lee inocula.

Thus, it appears that with Lee virus infective and hemagglutinating particles emerge at the same time. No evidence was obtained that non-infective hemagglutinating particles appear before infective particles regardless of the size of the inoculum. On the other hand, with PR8 virus infective and hemagglutinating particles emerge at the same time only if the inoculum contains 0.2 particles per cell or less. The appearance of infective particles seems to occur later than hemagglutinating particles after large PR8 inocula of 2 or more particles per cell. In this special case, non-infective hemagglutinating particles apparently do emerge into the allantoic fluid before infective particles. This is in accord with earlier findings of Liu and Henle (17), Henle and Henle (20), and Hoyle (21) with PR8 and DSP strains of influenza A virus.

Appearance times computed as described above represent the time from inoculation to the first appearance of new particles in the allantoic fluid. Thus, they represent the sum of the "latent period" in the membrane and the escape period, *i.e.*, time required for new particles to escape from the cells of the membrane. Cairns and Mason (22) and Tamm and Tyrrell (23) have obtained evidence that the escape period for both PR8 and Lee viruses is about 1 hour. On this basis, the "latent period" in the membrane *per se* would be about 60 minutes shorter than the values given in Table IV.

Liu and Henle (19) reported that the time of appearance of infective Lee virus in both the allantoic membrane and fluid was inversely related to the size of the inoculum. However, the duration of the so called constant periods they found was considerably longer than the intervals computed in this study. Appearance times obtained with inocula well below one particle per cell probably do not accurately reflect the course of events at the cell level because successive cycles of reproduction in a series of cells may then occur. Very large inocula containing about 10 particles per cell probably also should be considered suspect for, as shown above and in the preceding study (8), they cause marked alterations in the kinetics of the reproductive process. When about one particle per cell is inoculated, the average "latent period" in the allantoic membrane, *i.e.*, time of appearance in allantoic fluid minus escape period, appears to be between 150 and 160 minutes for both Lee and PR8

viruses. These intervals are somewhat shorter than those computed from the titration data of Liu and Henle (19) and Henle and Liu (24) on the allantoic membrane *per se*.

Reproduction after Inoculation of Various Quantities.—Inoculation of varying numbers of Lee virus particles of which a constant proportion, *i.e.* 20

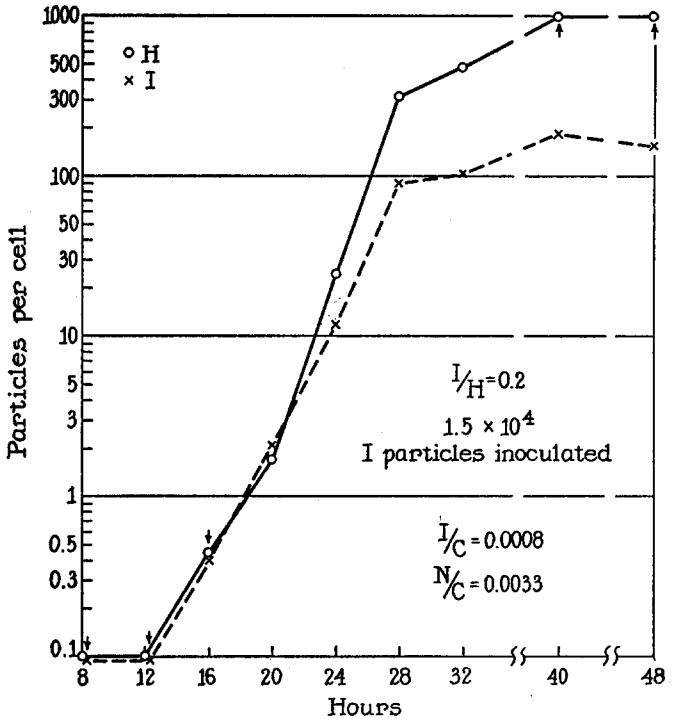

FIG. 5. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at intervals after inoculations of 1.5×10^4 I particles of influenza B virus (Lee). The inoculum had an I/H ratio of 0.2. I/C = infective particles inoculated per cell. N/C = non-infective particles inoculated per cell. Arrows indicate values either lower or higher than those plotted. Each point is the mean of results obtained in two or more experiments.

per cent, were infective yielded results shown in Figs. 5 to 9. Only infective particles were released initially into the allantoic fluid when the total number of particles inoculated, *i.e.* infective plus non-infective, was 0.04 per cell or less (*cf.* Figs. 5 and 6). Even when the number inoculated was 0.4 per cell, about 30 per cent of the particles in the early yields were infective (*cf.* Fig. 7). However, when the total number of particles inoculated was 4 or more per cell, only about 13 per cent of particles in the early yields were infective (*cf* Figs. 8 and 9).

In all instances, as was expected from the short half-life of infective particles, the proportion of infective particles decreased progressively as the period of incubation was increased. With any inoculum larger than 1.5×10^4 infective particles, this decrease was striking and led at 40 and 48 hours to yields which contained on the average only 1.2 and 0.2 per cent, respectively, of infective particles.

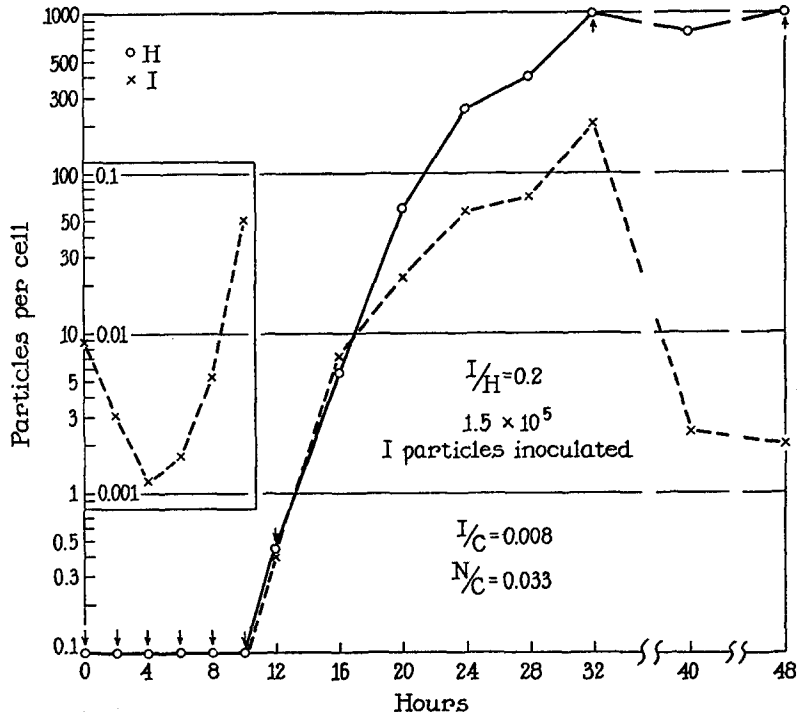

FIG. 6. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at intervals after inoculation of 1.5×10^5 I particles of influenza B virus (Lee). I/C = infective particles inoculated per cell. N/C = non-infective particles inoculated per cell. Arrows indicate values either lower or higher than those plotted. Each point is the mean of results obtained in two or more experiments.

Inoculation of 1.5×10^5 or less infective particles, *i.e.*, a total of 0.04 or less particle per cell, yielded results shown in Figs. 5 and 6. The numbers of infective and hemagglutinating particles were almost identical until 20 hours with the smaller inoculum and 16 hours with the larger. This indicates that nearly all hemagglutinating particles were infective when they appeared early in the allantoic fluid and that non-infective particles were not produced in advance of infective particles. After 20 hours the number of hemagglutinating particles increased more rapidly than the number of infective particles.

This reflects accumulation of non-infective particles which represented more than 70 and 80 per cent, respectively, of the yields at 28 hours after the smaller and larger inocula. With prolonged incubation, accumulation of non-infective particles became more marked. Comparable inocula of PR8 virus yielded similar results in the preceding study (8).

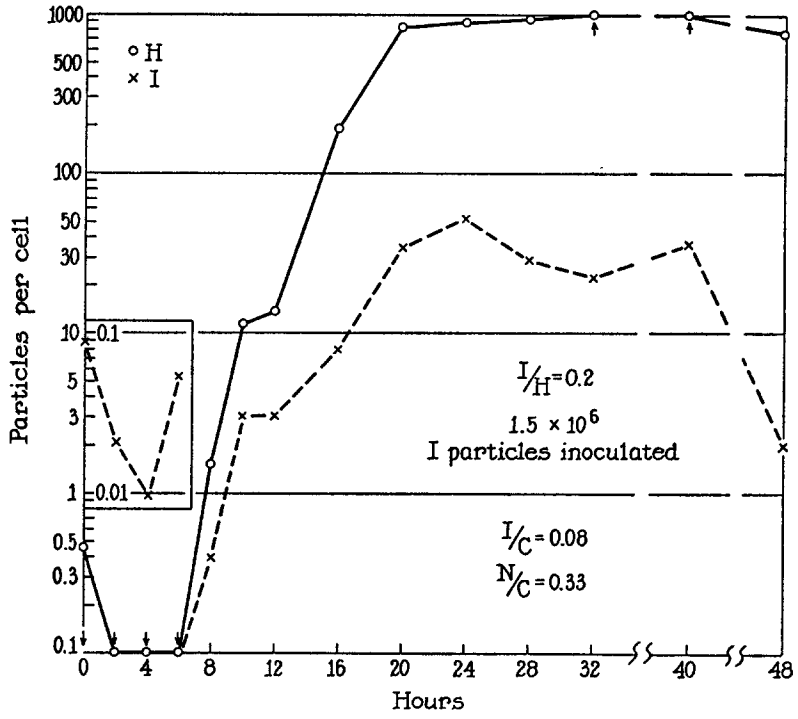

FIG. 7. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at intervals after inoculation of 1.5×10^6 I particles of influenza B virus (Lee). I/C = infective particles inoculated per cell. N/C = non-infective particles inoculated per cell. Arrows indicate values either lower or higher than those plotted. Each point is the mean of results obtained in two or more experiments.

Inoculation of 1.5×10^6 infective particles, *i.e.*, a total of 0.4 particle per cell, led to results shown in Fig. 7. The number of infective particles was about one-fourth the number of hemagglutinating particles even at 8 and 10 hours. This indicates that within 4 hours after the virus concentration had begun to increase, about 75 per cent of particles were non-infective. After 12 hours the number of hemagglutinating particles increased much more rapidly than the number of infective particles. By 16 and 20 hours the difference between these numbers indicates that non-infective particles had accumulated

to the extent of about 95 per cent. At these same intervals and a comparable inoculum, similar results were obtained previously (8) with PR8 virus.

Inoculation of 1.5×10^7 or more infective particles, *i.e.*, a total of 4 or more particles per cell, yielded results shown in Figs. 8 and 9. The number of infective particles was about one-tenth the number of hemagglutinating

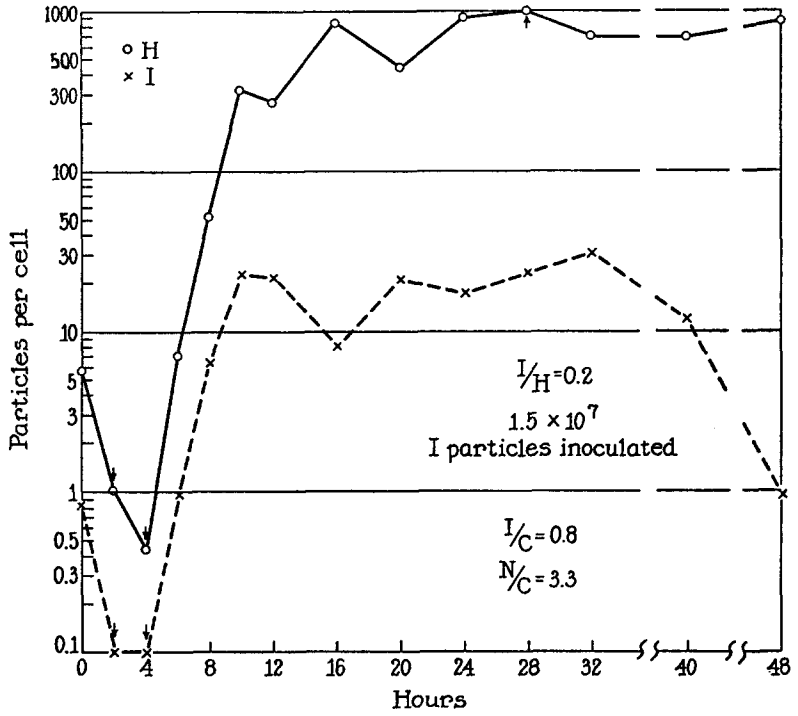

FIG. 8. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at intervals after inoculation of 1.5×10^7 I particles of influenza B virus (Lee). I/C = infective particles inoculated per cell. N/C = non-infective particles inoculated per cell. Arrows indicate values either lower or higher than those plotted. Each point is the mean of results obtained in two or more experiments.

particles at 6 and 8 hours. On the basis that the "latent period" in the membrane was 2.5 hours, as indicated above, it appears that about 90 per cent of particles were non-infective within 3.5 hours after the end of this period. This represents more rapid inactivation than can be explained on the basis of a half-life of 85 minutes. However, it must be emphasized that there is no information on the half-life of infective particles in the membrane *per se*. After 10 hours with either large inoculum, the numbers of infective particles did not increase much at any period, and the accumulation of non-infective par-

ticles became progressively more striking as incubation was prolonged. Comparable large inocula of PR8 virus were shown to yield similar results in the preceding study (8), although the proportion of non-infective particles which appeared after large inocula was even greater.

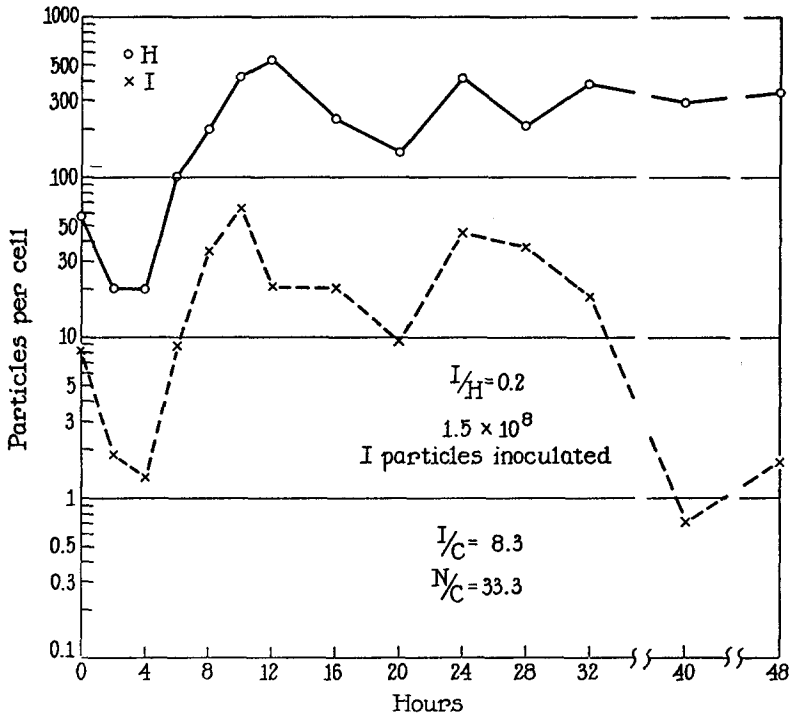

FIG. 9. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at intervals after inoculation of 1.5×10^8 I particles of influenza B virus (Lee). I/C = infective particles inoculated per cell. N/C = non-infective particles inoculated per cell. Each point is the mean of results obtained in two or more experiments.

Partially Infective Preparations.—Preparations which contained between 90 and 99 per cent non-infective particles were easily obtained *in vivo*. The two variables which affected the proportion of infective particles in the fluid were the total number of particles inoculated and the period of incubation. The proportion of infective particles obtained was inversely related to both. The results of a number of experiments with inocula of widely different size are shown in Table VI.

After inocula which provided a total of 5 or more particles per cell, only a small percentage of the new particles were infective even at 12 or 16 hours.

TABLE VI

Partially Infective Preparations of Influenza B Virus (Lee)

Inoculum				Incubation	Virus yield*		
Infective particles		Non-infective particles (N)‡			Hemagglutinating particles (H) per ml.	Infective particles (I) per ml.	Per cent infective particles
No.	Particles per cell	No.	Particles per cell				
				hrs.	× 10^8	× 10^8	
$1\frac{5}{8} \times 10^8$	5.5	1×10^9	55.0	12	21.4	1.4	6.5
$1\frac{5}{8} \times 10^7$	0.5	3×10^8	16.0	12	5.2	0.25	4.8
$2\frac{7}{8} \times 10^8$	11.0	7×10^8	39.0	16	8.3	0.72	8.7
$2\frac{5}{8} \times 10^7$	1.1	7×10^7	3.9	16	30.2	0.29	0.9
$2\frac{5}{8} \times 10^5$	0.01	7×10^5	0.04	32	60.2	3.5	5.8
$2\frac{5}{8} \times 10^4$	0.001	7×10^4	0.004	32	15.8	1.9	12.0
$2\frac{5}{8} \times 10^6$	0.11	7×10^6	0.4	48	2.8	0.07	2.5
$2\frac{5}{8} \times 10^5$	0.01	7×10^5	0.04	48	53.7	0.76	1.4

* In allantoic fluid pools.

‡ Inactivated at 35°C. *in vivo*.

TABLE VII

Yield of Influenza A and B Virus Particles per Cell

Virus	Inoculum	Maximal yield of virus particles*			
	Infective particles per cell	Hrs. of incubation	Hemagglutinating particles per cell	Infective particles per cell	Per cent infective particles
		range	*mean*	*mean*	
Influenza B (Lee)	8.3	24–32	337	33	9.8
"	0.8	24–32	948	23	2.4
"	0.08	24–32	977	37	3.8
"	0.008	28–32	931	141	15.1
"	0.0008	40–48	1494	168	11.2
Influenza A (PR8)	16.6	16–20	388	3	0.8
"	1.6	20–28	441	11	2.5
"	0.16	24–28	555	77	13.9
"	0.016	28–32	942	115	12.2
"	0.0016	32–40	712	167	23.4

* In allantoic fluid pools.

Similarly, with inocula of 0.05 or less particle per cell, only a small percentage of the new particles were infective at 32 or 48 hours. As is indicated above (*cf.* Table III), highly infective preparations of Lee virus can be obtained only under carefully controlled conditions. In the preceding study (8), the

same variables were shown to affect the production of partially infective preparations of PR8 virus.

Yield of Virus Particles per Cell.—With both Lee and PR8 viruses, large inocula, *i.e.*, about 10 infective particles per cell, led to the production of a smaller number of new virus particles than did inocula of about 0.1 or less

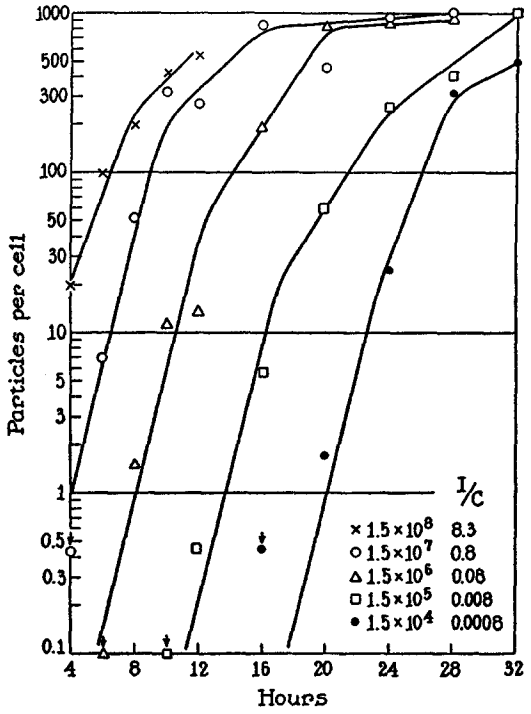

FIG. 10. Rate and extent of increase in the number of hemagglutinating (H) particles per cell after inoculation of various amounts of influenza B virus (Lee). I/C = infective particles inoculated per cell. The early segment of each curve has a slope equal to that shown in Fig. 4 for a comparable inoculum.

particle per cell. As shown in Table VII, large inocula diminished the maximal yield measured by either hemagglutinating or infective particles. With Lee virus, an inoculum of 8 particles per cell gave a maximal yield of hemagglutinating particles that was only about 35 per cent of the yield obtained after smaller inocula. With PR8 virus, an inoculum of 16 particles per cell gave a maximal yield of hemagglutinating particles that was only about 50 per cent of the average yield obtained after smaller inocula. With PR8, but not with Lee virus, an inoculum of about one particle per cell also led to a reduction in the maximal yield.

The intervals at which maximal yields were found tended to increase with both viruses as the number of particles inoculated was decreased. This is illustrated in Figs. 5 to 9 and was evident in the preceding study on PR8 (8). With both viruses, the largest yield of infective particles was found, regard-

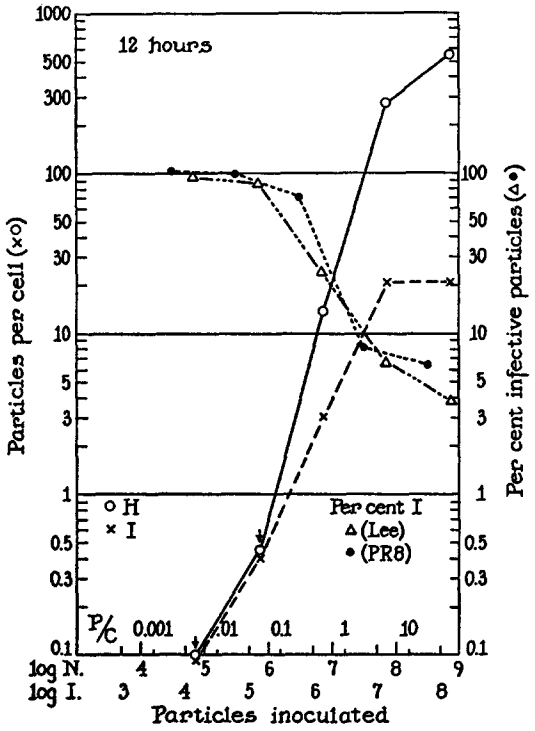

FIG. 11. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at 12 hours after inoculation of varying amounts of influenza B virus (Lee). N = non-infective particles. P/C = infective (I) plus non-infective (N) particles inoculated per cell. Per cent infective particles $= \frac{\text{I per cell}}{\text{H per cell}} \times 100$. Arrows indicate values lower than those plotted. Data for PR8 virus from preceding study (8).

less of the size of the inoculum, during the same interval that the maximal yield of hemagglutinating particles occurred. Neither with Lee nor with PR8 virus was it possible to obtain yields of more than 100 infective particles per cell unless small inocula, *i.e.*, about 0.01 particle or less per cell, were used. In fact, there appeared to be an inverse relation between the size of the inoculum and maximal yield of infective particles. As shown in Table VII, the proportion of particles that were infective was never very large at the intervals when maximal yields were obtained. Because of the short half-life of

infective particles of either virus and the time needed to reach maximal virus concentrations, this finding was not surprising. With inocula of about 0.2 particle or less per cell, Lee virus yielded between 900 and 1400 hemagglutinating particles per cell; PR8 virus, between 500 and 900. These values are

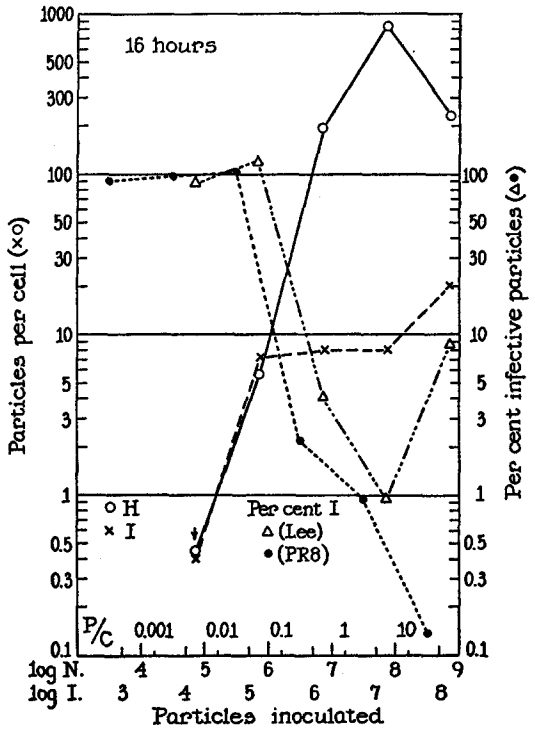

FIG. 12. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at 16 hours after inoculation of varying amounts of influenza B virus (Lee). N = non-infective particles. P/C = infective (I) plus non-infective (N) particles inoculated per cell. Per cent infective particles = $\frac{\text{I per cell}}{\text{H per cell}} \times 100$. Arrow indicates value lower than that plotted. Data for PR8 virus from preceding study (8).

similar to those reported recently from this laboratory (25) with chick embryo lung cultures and the WS strain of influenza A virus.

In Fig. 10, a series of curves demonstrating the rate and extent of increase in the number of hemagglutinating particles of Lee virus after inocula of various size are shown. The early segment of each curve has a slope that corresponds with the rates of increase shown in Fig. 4, *i.e.*, with the inoculum which gave a particle-cell ratio of 8.3, the slope of the early segment is equivalent to a doubling time of 65 minutes; with all the other inocula to a doubling

time of 43 minutes. The later segments of each curve were drawn to fit the data as closely as possible. It is evident that, regardless of the number of infective particles inoculated, the rate of increase diminished after the number of particles produced was 20 per cell or more. However, the larger the

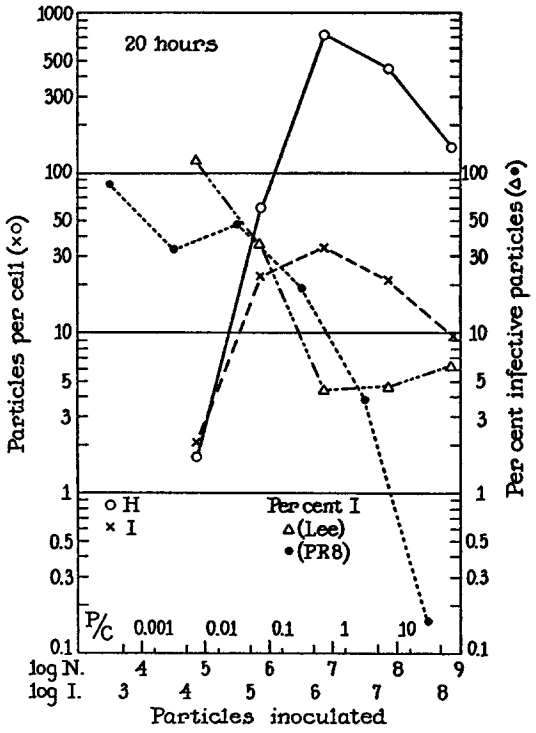

FIG. 13. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at 20 hours after inoculation of varying amounts of influenza B virus (Lee). N = non-infective particles. P/C = infective (I) plus non-infective (N) particles inoculated per cell. Per cent infective particles = $\frac{\text{I per cell}}{\text{H per cell}} \times 100$. Data for PR8 virus from preceding study (8).

inoculum, the greater was the number of particles produced before the rate of increase changed. With inocula which gave about one or more infective particles per cell, the change in rate occurred only after some 200 particles per cell had been produced. It is noteworthy that all inocula, excepting that which gave a particle-cell ratio of 8.3, led to the production of about the same number of particles (*cf.* Table VII); *i.e.*, approximately 1000 per cell. However, as expected, the time required to reach a high yield was inversely related to the size of the inoculum. Although there were constant 10-fold

decrements in the number of particles inoculated, the increment in time to reach a constant yield, *e.g.* 20 particles per cell, was not constant and appeared to increase progressively as the inoculum was decreased. This can be explained on the basis that successive cycles of adsorption, reproduction, and release occurred after the smaller inocula.

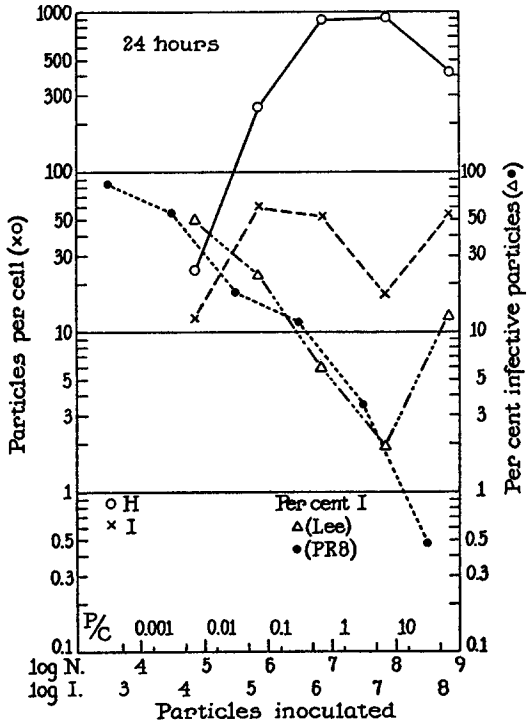

FIG. 14. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at 24 hours after inoculation of varying amounts of influenza B virus (Lee). N = non-infective particles. P/C = infective (I) plus non-infective (N) particles inoculated per cell. Per cent infective particles = $\frac{\text{I per cell}}{\text{H per cell}} \times 100$. Data for PR8 virus from preceding study (8).

Effect of Inocula of Different Size on the Yield at Constant Intervals.—The various particles per cell-time curves shown above in Figs. 5 to 9 demonstrated that inocula of different size resulted in curves which were dissimilar particularly as regards infective particles. The effect of the number of particles inoculated on the proportion of infective particles in the yield became more obvious when the data obtained at constant time intervals were analyzed. Such analyses are shown in Figs. 11 to 15. Both the number of hem-

agglutinating particles and the number of infective particles found at fixed intervals, as well as the proportion of infective particles, are given. For comparison, the proportion of infective particles found at the same time intervals in the preceding study (8) on PR8 are included.

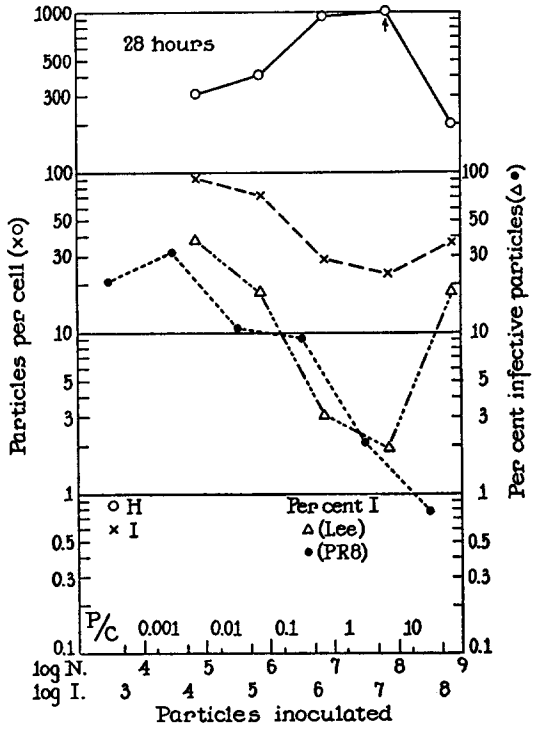

FIG. 15. Number of infective (I) and hemagglutinating (H) particles per allantoic membrane cell in the allantoic fluid at 28 hours after inoculation of varying amounts of influenza B virus (Lee). N = non-infective particles. P/C = infective (I) plus non-infective (N) particles inoculated per cell. Per cent infective particles = $\frac{\text{I per cell}}{\text{H per cell}} \times 100$. Arrow indicates value higher than that plotted. Data for PR8 virus from preceding study (8).

As shown in Fig. 11, the proportion of Lee virus particles that were infective was 6 per cent or less even at 12 hours if the inoculum contained a total of 6×10^7 or more particles; *i.e.*, infective plus non-infective particles. This number is equivalent to about 3 particles per cell. Highly infective yields, *i.e.* 50 per cent or more infective particles, were obtained only if the inoculum contained 0.03 particle per cell or less. At 16 hours, (*cf.* Fig. 12) the proportion of infective particles ranged from 9 to 0.9 per cent if the inoculum contained 6×10^6 or more particles; *i.e.*, about 0.3 particle per cell. Even at this

early interval, highly infective yields were secured only if the inoculum contained 0.03 particle per cell or less.

As shown in Figs. 13 and 14, increasing the time interval to 20 or 24 hours led to a progressive decrease in the number of particles that could be inoculated if highly infective yields were to be obtained. Thus, at both periods such yields were secured only if the inoculum contained no more than 0.003

TABLE VIII

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

Inoculum				Incubation	Virus yield§		
Infective particles	Non-infective particles (N)		Total particles per cell		Hemagglutinating particles (H)	Infective particles (I)	Per cent infective particles
No.	Inactivated at	No.			per ml.	per ml.	
				hrs.	$\times 10^8$	$\times 10^8$	
2×10^5	35°C., 28 hrs.*	1×10^5	0.02	16	0.05	0.06	>100.0
"	22°C., 8 days‡	6×10^8	33.0	16	0.04	0.007	17.5
2×10^5	35°C., 28 hrs.	1×10^5	0.02	20	1.86	1.20	64.4
"	22°C., 8 days	6×10^8	33.0	20	0.55	0.039	7.1
"	22°C., 8 days	2×10^8	11.0	20	0.40	0.029	7.2
"	22°C., 8 days	7×10^7	3.9	20	0.40	0.035	8.7
2×10^5	35°C., 28 hrs.	1×10^5	0.02	24	25.1	7.58	30.2
"	22°C., 8 days	6×10^8	33.0	24	1.1	0.003	0.3
2×10^4	35°C., 28 hrs.	1×10^4	0.002	24	8.7	4.6	52.8
"	22°C., 8 days	6×10^8	33.0	24	1.2	0.018	1.5

* Spontaneously inactivated *in vivo* and present in seed preparation.

‡ Inactivated *in vitro* and added to diluted seed virus.

§ In allantoic fluid pools.

particle per cell. Moreover, at both periods, the proportion of infective particles ranged from 12 to 2 per cent if the inoculum contained 0.3 or more particle per cell. The results obtained at 28 hours are shown in Fig. 15 and serve to confirm the relations observed at earlier intervals between size of the inoculum and proportion of infective particles in the yield.

The data for PR8 virus (8), shown in Figs. 11 to 15, correspond very closely with those for Lee virus except when very large inocula were used; *i.e.*, 20 particles or more per cell. In the intervals from 16 to 28 hours, the proportion of infective particles after such inocula was considerably smaller with PR8 than with Lee virus. No adequate explanation for this difference has been found. It is apparent, however, that the relation between the number of

particles per cell inoculated and the production of yields with a high proportion of infective particles was almost identical with both Lee and PR8 viruses at each time interval examined.

Effect of Mixtures of Non-Infective and Infective Particles on the Yield.—From the results shown in Figs. 11 to 15, it is evident that with both Lee and PR8

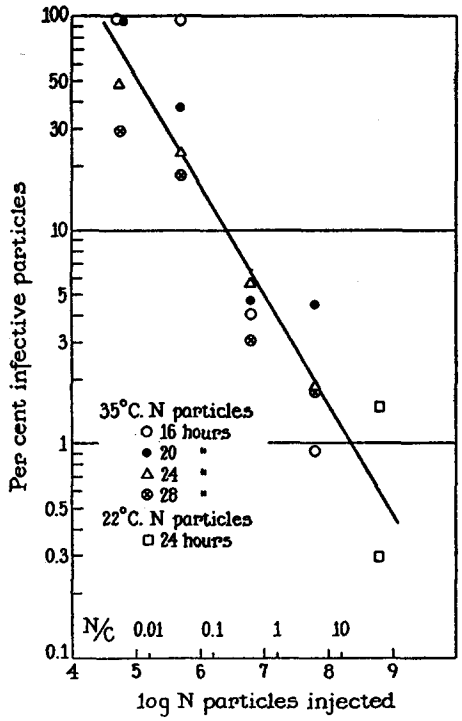

FIG. 16. Proportion of infective (I) particles in the allantoic fluid at indicated intervals after inoculation of mixtures of infective and non-infective (N) particles of influenza B virus (Lee). Non-infective particles were inactivated either at 35°C. for 28 hours *in vivo* or at 22°C. for 8 days *in vitro*. N/C = non-infective particles injected per cell.

viruses the inoculation of 0.3 or more particle per cell led to yields containing only a small proportion of infective particles regardless of the time interval. Because the number of infective and non-infective particles in the inocula used varied in an identical manner, it was not possible from these data to determine the relative effect of either variety of particle separately on the reproductive process.

The use of prepared mixtures of infective and non-infective particles, in which the number of non-infective particles was much larger than the number of infective particles, provided information on this issue. The results of exper-

iments with such mixtures are shown in Table VIII. The mixtures were prepared by adding non-infective particles, inactivated at 22°C. for 8 days *in vitro* (8), to diluted seed virus preparations which contained 65 per cent of infective particles. Addition of 7×10^7 or more of such non-infective particles, *i.e.* about 4 or more per cell, to inocula containing only 2×10^4 or 2×10^5 infective particles caused a reduction in the number of hemagglutinating particles produced and in the proportion that were infective, regardless of the time interval at which the yield was analyzed. On the average, the reduction in the number of hemagglutinating particles was 70 per cent as compared with controls which received the same infective inocula but only small numbers of non-infective particles. The reduction in the proportion of infective particles was even more marked. On the average, the control yields contained 62 per cent infective particles while the yields after mixtures contained only 7 per cent. The smaller the number of infective particles in the mixture, the more marked was the reduction in both the number of hemagglutinating particles and in the proportion that were infective.

The results of a number of experiments with infective inocula of various size which contained different numbers of non-infective particles inactivated at either 35°C. (28 hours *in vivo*) or 22°C. (8 days *in vitro*) are given in Fig. 16. Regardless of the interval at which the yield was analyzed, the proportion of infective particles appeared to be inversely related to the number of non-infective particles included in the inoculum. On a logarithmic scale this relation was approximately linear. After infective inocula which also contained 0.3 non-infective particle per cell, the proportion of infective particles in the yield ranged from 3 to 6 per cent. When the number of non-infective particles in the inoculum was 3 or more per cell, the proportion of infective particles in the yield ranged from 0.3 to 5 per cent. It should be emphasized that non-infective particles arising from spontaneous inactivation at 35°C. *in vivo* appeared to be equally as effective in this regard as particles inactivated at 22°C. *in vitro*.

In the preceding study (8), an almost identical relation between the number of non-infective particles included in infective inocula and the proportion of infective particles in the yield was demonstrated with PR8 virus. As with Lee virus, inactivation at either 35°C. *in vivo* or 22°C. *in vitro* gave non-infective particles which were equally as effective as infective particles in producing this result. It is noteworthy that non-infective particles inactivated at 56°C. had no such effect (8).

DISCUSSION

The most striking findings to emerge from this investigation are the effects of the particle-cell ratio on the dynamics of the reproductive process of influenza viruses. With influenza B virus, when the particle-cell ratio was rel-

atively low, *i.e.* 0.3 or less, the dynamics of reproduction had remarkably constant features. Thus, at ratios ranging from 0.3 to 0.003, the doubling time did not vary appreciably, the number of particles produced was nearly constant, and the proportion of infective particles in early yields was regularly high. Only the appearance time showed indications of increasing as the ratio was reduced. This apparent increase, noted previously (19), can be explained on the basis of successive cell cycles of adsorption, reproduction, and release. Clearly, as the ratio is diminished, the number of successive cycles needed to infect all cells increases.

In contrast, whenever the particle-cell ratio was high, *i.e.* 3 or more, alterations in the dynamics of virus reproduction became apparent. At ratios of 8 or more, the alterations were marked and were evidenced by a decrease in the appearance time, an increase in the doubling time, a decrease in the number of particles produced, and a decrease in the proportion of infective particles in early yields.

Analysis of the data reported in the preceding study (8) on influenza A virus revealed an almost identical relation between the particle-cell ratio and the dynamics of the reproductive process with that agent. With low ratios, *i.e.* 0.3 or less, the various parameters of reproduction, excepting only the appearance time, were almost constant. A ratio of 3 or more, however, produced alterations in the process. When the ratio was 10 or more, each of the changes noted above became apparent.

With both influenza viruses, large numbers of non-infective particles inactivated at 35°C. or 22°C. (8) were as effective as infective particles in causing alterations in the dynamics of reproduction. At comparable high ratios, *i.e.* 3 or more non-infective particles per cell, the doubling time was increased, the number of particles produced was decreased, and the proportion of infective particles was decreased. Thus, it appears that there is a critical ratio which, if exceeded with either infective or non-infective particles, leads to definite changes in influenza virus reproduction.

As an explanation for this critical particle-cell ratio, the following hypothesis is put forward: Influenza virus particles possess intrinsic toxicity and can damage cells even when they do not reproduce (*cf.* references 26 and 27). Damage to cells of the allantoic membrane occurs when about 3 particles per cell are adsorbed. The larger the number of particles adsorbed, the greater is the resulting cell damage. The damage is reflected in a decrease in the efficiency of virus reproduction. The decrease in the efficiency of the process appears as: (*a*) reduction in the rate of reproduction, as evidenced by increase in the doubling time, (*b*) reduction in the capacity to support reproduction, as evidenced by decrease in the yield of new particles, and (*c*) reduction in the proportion of particles that are infective after release from the cells.

As first shown by von Magnus (28, 29) and confirmed by others (16, 30),

very large inocula of influenza virus commonly lead to marked discrepancies between infectivity and hemagglutination titration end points on the yield. Not only do low ID_{50}/HA ratios appear after large inocula in the allantoic sac, in which the phenomenon has been most studied (*cf.* reference 8), but also they have been demonstrated in the mouse brain (31), mouse lung (32), and in tissue culture (33, 34) after large inocula. These unusual discrepancies led to development of the "incomplete" virus concept which holds that non-infective hemagglutinating particles are an immature form, *i.e.* precursors, of mature infective particles (35). Schlesinger (36) obtained evidence which casts some doubt on this concept and suggested that non-infective particles may be degenerated products of complete virus particles. Moreover, Ginsberg (32) demonstrated a correlation between the development of lung lesions in the mouse and the early appearance of non-infective particles and raised the possibility that cell damage was responsible for the lack of the infective property. Recently, Henle *et al.* (34) found that non-infective particles appeared in HeLa cell cultures only if cytopathogenic effects were produced and proposed that the "incomplete" reproductive cycle might be related to the toxic effects of large inocula. In the preceding study on the reproduction of influenza A virus in the allantoic sac (8), it was shown that the single condition which leads to the appearance of non-infective particles during the early increase period is a high particle-cell ratio.

The very large inocula used in earlier studies almost certainly yielded particle-cell ratios considerably in excess of the critical value described above and therefore would be expected to produce the unusual alterations in the dynamics of reproduction demonstrated in this and the preceding study (8). Because of the striking instability of the infective property of influenza viruses, it is not surprising that there is such rapid accumulation of non-infective particles nor that this should become even more impressive when the rate of reproduction is decreased. As has been shown, the rate of reproduction is markedly decreased by either infective or non-infective particles at ratios of 8–10 or more per cell. Therefore, it is suggested that the cell damage resulting from high particle-cell ratios, the alterations produced in the reproductive process by such damage, and the instability of the infective property of influenza viruses provide an adequate explanation for the discrepancies observed previously by other workers.

SUMMARY

Influenza A and B virus reproduction in the allantoic membrane of the intact chicken embryo was studied quantitatively with particle enumeration procedures. Virus particles were enumerated on the basis of two independent properties; capacity to infect and to cause hemagglutination. The infective property of influenza B virus (Lee) was even more unstable than

that of influenza A virus (PR8). Inactivation occurred at a constant logarithmic rate which was independent of the concentration of particles and corresponded with first order reaction kinetics. In allantoic fluid at 35°C. either *in vitro* or *in vivo*, Lee virus had a half-life for infectivity of only 85 minutes. In contrast, the hemagglutinating property, like that of PR8, was relatively stable and was not appreciably affected by 12 hours at 35°C.

On the basis that the number of non-infective particles is equal to the number of hemagglutinating particles minus the number of infective particles and that the number of cells lining the allnatoic membrane is 1.8×10^7, the effects of various particle-cell ratios on the reproductive process were analyzed.

Adsorption of infective and non-infective Lee particles occurred at the same logarithmic rate, *i.e.* about 50 per cent in 72 minutes, and the rate was nearly independent of the particle-cell ratio up to a value of 55. The adsorption capacity of an allantoic cell was at least 44 Lee or 89 PR8 particles.

The interval before new particles appeared in the allantoic fluid increased as the particle-cell ratio was decreased with both Lee and PR8. At ratios of 0.2 or less, the appearance time for infective particles was nearly identical to that for hemagglutinating particles with both viruses. At ratios of about 1.0, the "latent period" in the allantoic membrane *per se* was computed to be 150 to 160 minutes for both Lee and PR8.

The number of particles, both infective and hemagglutinating, increased at a constant logarithmic rate for 6 hours or more after the adsorptive period. With Lee virus, at a particle-cell ratio of 5 or less, the doubling time was constant and had a value of 43 minutes. The dynamics of the logarithmic increase period suggest that reproduction corresponds to an autocatalytic reaction in which the rate is proportional to the amount of material produced. When the particle-cell ratio was increased to 10 or more, either with infective or non-infective (inactivated at 35°C. or 22°C.) particles, the doubling time increased to 65 minutes. Comparable effects from high ratios were found with PR8.

Non-infective particles accumulated at a rapid rate after the interval of constant logarithmic increase regardless of the particle-cell ratio. This accumulation was even more striking with Lee than with PR8 as was expected because of the shorter half-life of the infective property. With both viruses at particle-cell ratios of 4 or more, a large proportion of the particles were non-infective within a few hours after new particles appeared.

At particle-cell ratios of 0.2 or less, the maximal yield was relatively constant, *i.e.*, about 900 to 1400 hemagglutinating particles per cell with Lee and 500 to 900 with PR8. However, even with very low ratios, *i.e.* 0.001 or less, it was not possible to obtain more than about 160 infective particles per cell with either virus regardless of the interval. As was expected, the lower

the ratio, the longer was the interval before maximal yields were produced. At ratios of about 10, the maximal yield was reduced by 50 per cent or more with both viruses. Comparable reductions in yield were obtained whether the high particle-cell ratio was due to infective or non-infective (inactivated at 35°C. or 22°C.) particles.

These findings indicate that there is a critical particle-cell ratio above which alterations appear in the dynamics of reproduction of influenza viruses. This ratio has a value of approximately 3. The observed alterations in the reproductive process are discussed in relation to the hypothesis that adsorption of 3 or more infective or non-infective particles per cell induces cell damage.

Addendum.—After this manuscript was completed, the reports of Finter, Liu, and Henle (37) and Paucker and Henle (38, 39) on the multiplication of influenza A virus (PR8) in the allantoic sac were published. Certain of their data appear to provide additional support for the concept, developed in the preceding paper (8) and extended in the present communication, that non-infective influenza virus particles emerge from the cells only when the total number of particles introduced, *i.e.* infective and non-infective particles, is larger than the number of allantoic cells.

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