FORMATION OF NON-INFECTIOUS INFLUENZA VIRUS IN MOUSE LUNGS: ITS DEPENDENCE UPON EXTENSIVE PULMONARY CONSOLIDATION INITIATED BY THE VIRAL INOCULUM*

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Investigation of the pathogenesis of viral pneumonia in mice has revealed that certain strains of influenza viruses produce extensive pulmonary damage under circumstances in which viral multiplication appeared to be limited (1). To initiate cell damage, inoculation of a large number of viral particles was essential (1). Under these conditions, although little or no increase in titer of infectious virus could be detected, a marked accumulation of viral particles endowed with the capacity to hemagglutinate chicken erythrocytes was manifest (2).

Similar phenomena have been described in other host tissues (3-7). von Magnus has studied the development of non-infectious influenza virus in the allantoic sac of the chick embryo, and considers these viral particles to be incomplete viruses (4, 8-11). The production of similar non-infectious influenza viral forms in mouse brain (5), deembryonated eggs (6), and tissue culture (7) has also been described. Details of these studies have been reviewed recently (11-15).

Experimental data on bacterial (13, 16), insect (13, 17), and animal (13, 18) viruses suggest that viral synthesis is a series of developmental steps which lead to mature, infectious viral particles. Studies of the multiplication cycles of influenza viruses (19, 20) as well as of the formation of non-infectious influenza viral particles (4, 8, 9, 11) have led to the hypothesis that influenza viruses undergo similar stages of maturation of which the soluble complement-fixing antigen and hemagglutinin are detectable examples. This investigation was undertaken: (*a*) to study the details of formation of non-infectious influenza virus in the mouse lung in order to attempt to identify its role in the viral synthetic process and the mechanism of its development; and (*b*) to determine whether a causal relationship existed between production of pulmonary cell damage and formation of non-infectious viral particles.

It is the purpose of this paper to present evidence which indicates that development of non-infectious influenza virus in mouse lungs was closely correlated with and probably dependent upon host cell injury effected by the original virus inoculated; that cell damage was not a result of viral multiplication; and that the non-infectious virus formed could not categorically be termed a developmental stage in viral synthesis.

Materials and Methods

Viruses.—The influenza A viruses employed were: CAM[1] strain; 6 strains isolated at Western Reserve University in 1951 and designated as WRU 2-51, etc., by their assigned strain numbers; 2 strains isolated at the Hospital of the Rockefeller Institute, New York in 1950; FM1 strain; Rhodes strain;[2] and PR8 strain. In addition, the Lee strain of influenza B virus was used. In all experiments these viruses will be referred to by their strain designation. All viruses were maintained by inoculation of 0.1 ml. of a 10^{-4} dilution into the allantoic sac of 9 to 11 day old chick embryos (21). Viruses were stored as undiluted, infected allantoic fluid in sealed glass ampules at $-70°C$. (22).

Animals.—Three to 4 week old Webster strain albino Swiss mice obtained from Carworth Farms were used in all experiments. Light ether anesthesia was employed to prepare mice for intranasal inoculation, and an inoculum of 0.05 ml. per mouse was used. White Leghorn embryonated hen eggs obtained from a single dealer were incubated at 39°C. for 10 to 11 days before being used for propagation of viruses.

Preparation of Lung Suspensions.—Ten per cent suspensions were prepared in 0.85 per cent NaCl by grinding for 3 minutes with a teflon plastic tissue grinder (23) attached to a Sargent cone drive stirring motor. A small amount of sterile alundum was employed. This method of preparation yielded highly reproducible results (24).

Receptor-Destroying Enzyme (RDE).—The method for preparation of receptor-destroying enzyme was obtained from Dr. I. W. McLean, Jr., Parke-Davis and Co., Detroit. Flasks of beef brain-heart infusion broth were inoculated with *Vibrio cholera* and agitated in a mechanical shaker for 18 hours at room temperature. Cultures were then placed at 4°C. for 48 hours. After a preliminary centrifugation, the cultures were filtered through a Coors No. 3 porcelain candle. The culture filtrate, referred to as RDE, was stored at 4°C. The potency of the preparation was tested, as previously described (25), by its capacity to prevent hemagglutination of chicken red blood cells by PR8 virus.

Virus Titrations.—Infectivity. Mouse lung suspensions were centrifuged at 2000 R.P.M. for 2 minutes, and the supernates employed for the infectivity titration. Serial 0.5 log_{10} (1:3.16) dilutions of mouse lung suspension or infected allantoic fluid were prepared in tryptose phosphate broth which contained 500 units of crystalline penicillin G and 0.5 mg. of streptomycin per ml. Each dilution of material to be tested was inoculated into the allantoic sacs of four 10 to 11 day old chick embryos as previously described (21). The presence of virus in each allantoic fluid was determined by the hemagglutination reaction (26): to undiluted and 1:10 dilution of each fluid was added an equal volume of 1 per cent chicken erythrocytes and the pattern of red blood cells on the bottom of the tubes observed after 60 minutes at room temperature. Infectivity titrations were carried out on all lungs from a single experiment on the same day. Infectivity titers are expressed as the final dilution of

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[1] Obtained through the courtesy of Dr. John Y. Sugg, Cornell University Medical College, New York.

[2] Kindly supplied by Dr. Fred M. Davenport, School of Public Health, University of Michigan, Ann Arbor.

ground mouse lung (10 per cent suspension $= 10^{-1.0}$) or allantoic fluid which infected 50 per cent of the chick embryos (E.I.$_{50}$) (27).

Hemagglutination.—Equal volumes of uncentrifuged 10 per cent lung suspensions and RDE were mixed and incubated at room temperature for 16 to 18 hours, and then at 37°C. for 2 hours. After incubation, mixtures were centrifuged at 6590 g for 10 minutes. Serial twofold dilutions of the supernates were prepared in 2.5 per cent sodium citrate, and to each was added an equal volume (0.4 ml.) of a 0.5 per cent suspension of chicken red blood cells in 2.5 per cent sodium citrate. Titers are expressed as the reciprocal of the final dilution of ground mouse lung which showed the usual 2 plus end point.

Complement Fixation.—Soluble antigen was prepared by centrifugation of lung suspensions at 26,360 g for 60 minutes in the number 40 rotor of the model L Spinco preparative centrifuge. A standard complement fixation titration in which 2 units of commercial amboceptor and 2 units of complement were employed was carried out to determine the quantity of soluble antigen in lung suspensions (28). To serial twofold dilutions of soluble antigen prepared in 0.2 ml. of 0.85 per cent NaCl containing 0.01 per cent MgSO$_4$ was added 0.4 ml. of complement and 0.2 ml. of pooled human convalescent serum (2 units), respectively. After a period of incubation for 1 hour at 37°C., a 3 per cent suspension of sensitized sheep RBC was added, and a second period of incubation of 30 minutes at 37°C. was carried out. Titers were determined after the tubes remained at 4°C. overnight. The titer is expressed as the highest final dilution of ground mouse lung in the first phase which fixed complement completely. For convenience a 10 per cent lung suspension which contained no demonstrable hemagglutinin or soluble antigen is expressed as a titer of 1:10 or less (log -1.0 or less).

Lung Lesion Score.—Lungs were examined at autopsy and the lesions scored in terms of the estimated amount of consolidation present, $\pm$ to 5+ (29), and expressed as the ratio of the sum of the observed score to 30, the maximum possible score per group of 6 mice.

EXPERIMENTAL

Reproducibility of Infectivity Titrations of Mouse Lungs in Chick Embryos.— To compare viral infectivity and hemagglutination titration end points with confidence, a method of infectivity titration, *in ovo*, which had a degree of accuracy comparable to the hemagglutination titration, was essential. The degree of reproducibility of titrations carried out with serial 0.5 log$_{10}$(1:3.16) dilution decrements was determined to ascertain the adequacy of this titration technique.

Fifty mice were inoculated intranasally with 10^4 E.I.D. (embryo infectious doses) of PR8 virus and killed 2 days after initiation of infection. Lungs were removed aseptically and stored at -30°C. in an electrical deep freeze (30). Three different pools of infected lungs were employed for chick embryo infectivity titrations; 10 per cent suspensions were prepared from each lot of mouse lungs immediately before titrations were carried out.

The results of 22 individual infectivity titrations done on 3 separate 10 per cent suspensions of mouse lungs infected with PR8 virus were tabulated and the reproducibility of the end point was determined (31). The infectivity titers obtained from these titrations had a normal distribution. In Table I the essential data and the results of the appropriate computations are presented. It should be pointed out again that 3 different lots of infected mouse lung suspensions were employed for these titrations, and several series of titrations were

done from each pool. The observed variation of each series of titrations was considerably less than that noted for the entire group in Table I. The greatest deviation from the geometric mean of all titrations was log +0.45 and the mean deviation of the entire series of infectivity titrations was ±0.17. The standard deviation of the distribution of end points was 0.215. It can be demonstrated that with any 2 individual infectivity end points determined by the infectivity titration method described, a difference of log 0.61 or greater should occur by chance in not more than 5 per cent of the titrations.

These data indicate that the accuracy of the infectivity method employed is within the range usually considered for the hemagglutination titration method used. That is, a difference of 0.6 log between 2 infectivity titers or 0.6 log (fourfold) between 2 hemagglutination titers may be considered a significant difference.

TABLE I

Reproducibility of Chick Embryo Infectivity Titration End Points of PR8—Virus Infected Mouse Lungs

Preparations of mouse lungs titered No.	No. of titrations* in chick embryos‡	Infectivity titer of mouse lungs			Deviation from geometric mean			Standard deviation σ
		lowest	highest	geometric mean	least	greatest	mean	
3	22	log −8.42	log −9.25	log −8.80	log −0.03	log +0.45	log ±0.17 (1.5-fold)	0.215 (1.6-fold)

* 0.5 log₁₀ (1:3.16) dilution decrements were employed.

‡ 4 chick embryos per dilution.

Formation of Non-Infectious CAM Virus.—The CAM strain of influenza A virus, a so called influenza A prime type, because of its unique characteristics (32), has been employed in many of the experiments to be reported. This agent, although unadapted to mice, does effect extensive pulmonary consolidation upon initial inoculation of infected allantoic fluid into mouse lungs (32); these lesions, however, are not transferable on serial passage (32). Furthermore, during the development of extensive pulmonary consolidation subsequent to a large viral inoculum, little or no multiplication of infectious CAM virus can be detected (32, 33). To produce lesions a large quantity of virus is essential (32, 33). The similarity of these findings to those described for PR8 and Lee viruses in mouse brains (5) suggested that an increase in viral components other than infectious virus, *i.e.*, viral hemagglutinin and soluble complement-fixing antigen, may develop in mouse lungs infected with this agent. The following experiments were carried out to investigate this question.

Two series of 24 mice each were inoculated intranasally with $10^{7.8}$ and $10^{4.8}$ E.I.D. of CAM virus, respectively. Groups composed of 6 mice in each series were killed after inter-

vals of 2, 24, and 48 hours. The lungs were removed aseptically and stored at −30°C. until the experiment was completed. The fourth group of 6 mice in each series was observed for 7 days, and mice which survived were killed and the lung lesions scored. Ten per cent suspensions of lungs were prepared and infectivity, hemagglutination and complement-fixing soluble antigen titrations were carried out.
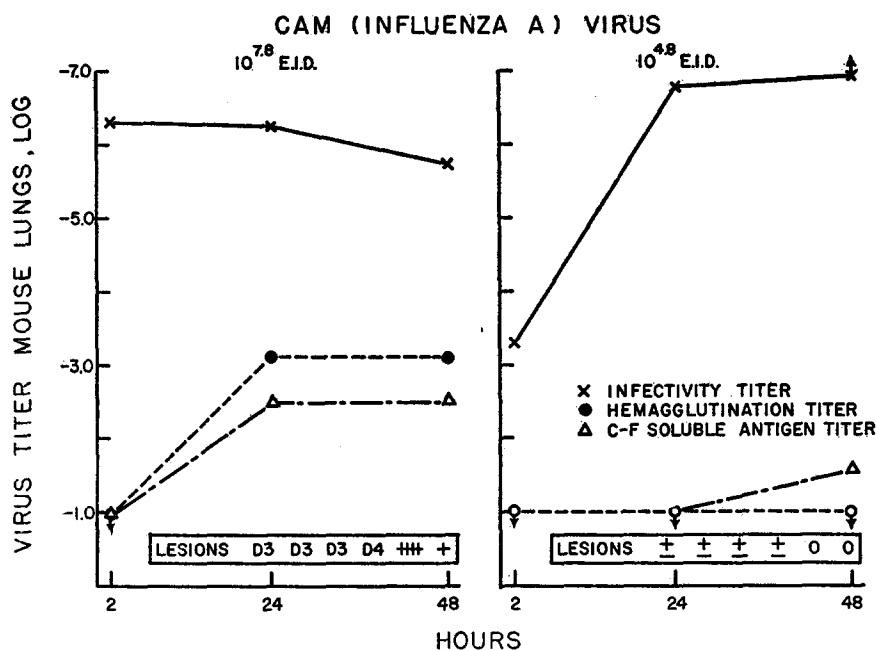
CAM (INFLUENZA A) VIRUS

Fig. 1. Comparison of the multiplication of CAM strain of influenza A virus in lungs of mice inoculated with undiluted ($10^{7.8}$ E.I.D.) or a 10^{-3} dilution ($10^{4.8}$ E.I.D.) of infected allantoic fluid. Viral multiplication was measured by infectivity, hemagglutination and soluble complement-fixing antigen titrations. Titers represent the final dilution of unground lung. Groups of 6 mice were used at each time interval. Lung lesions were scored on survivors 7 days after viral infection; death of a mouse is designated by "D," and day of death by numeral which follows (D3 = 3rd day).

The results of a typical experiment are presented graphically in Fig. 1. Following inoculation of $10^{7.8}$ E.I.D. of CAM virus no increase in infectious virus was detectable. Indeed, in this experiment as well as in each of 8 similar experiments an actual decrease in infectious virus occurred. Regardless of the apparent absence of viral synthesis as determined by infectivity titrations when $10^{7.8}$ E.I.D. of virus was inoculated, a marked increase in viral hemagglutinin and soluble antigen ensued. It will be noted that the large inoculum resulted in extensive pulmonary consolidation, and, indeed, death in 4 of the 6 mice in the group observed for 7 days. In sharp contrast, when 1000-fold less CAM virus was inoculated a marked increase in infectious virus occurred, and

a titer was attained which was significantly higher than any reached following infection with the larger inoculum. Despite the considerable propagation of infectious virus, only a low titer of soluble antigen was reached and no viral hemagglutinin could be detected. Moreover, very minimal pulmonary lesions developed in 7 days. The failure of the appearance of hemagglutinins after infection with $10^{4.8}$ E.I.D. of CAM virus could not be explained merely upon the failure of dissociation of virus from host tissue mucopolysaccharide: (a) a marked excess of RDE was employed to assist enzymatic inactivation of host tissue inhibitors; and (b) the enzymatic component of these viral particles would not reasonably be expected to differ from those whose propagation was initiated by the larger inoculum. These data indicate that infection with a large quantity of CAM virus results in formation of viral particles, the majority of which are incapable of the initiation of subsequent viral infection but able to hemagglutinate chicken erythrocytes. In addition, there develops soluble viral material which combines with specific antibodies.

Influences of Quantity of Virus Inoculated upon Multiplication of Lee and PR8 Viruses.—Since CAM virus is an unusual agent, it was essential to determine whether reduced synthesis of infectious virus and development of a non-infectious form resulted from inoculation of a large quantity of a mouse-adapted virus.

Experiments identical with those carried out with CAM virus were performed with mouse-adapted Lee and PR8 viruses. Infected allantoic fluid obtained from chick embryos inoculated with Lee- or PR8-infected mouse lung suspension was employed in order to infect with the maximum quantity of virus. With Lee virus $10^{6.5}$ E.I.D. was the highest concentration employed. With PR8, mice were inoculated with infected allantoic fluid containing $10^{7.2}$ E.I.D. per 0.05 ml., and tenfold concentrated infected allantoic fluid which had $10^{8.4}$ E.I.D. per mouse inoculum (0.05 ml.). To concentrate virus, infected allantoic fluid was centrifuged at 41,190 g for 60 minutes, and the sediment resuspended to one-tenth volume.

The results of 2 experiments carried out with Lee virus are summarized in Fig. 2. In mice inoculated with undiluted infected allantoic fluid marked suppression of synthesis of infectious virus was observed as compared with mice infected with 1000-fold less virus. Indeed, mice inoculated with $10^{6.5}$ E.I.D. of Lee virus had a titer of infectious virus less than 10 per cent of that demonstrated in lungs of mice which received the smaller inoculum. Despite the reduced propagation of infectious virus, however, hemagglutinin attained a titer which was significantly higher in mice given the greater quantity of virus.

The results of experiments carried out with PR8 virus similarly demonstrated the formation of non-infectious influenza virus in lungs from mice infected with a large inoculum. However, with PR8 the quantity of virus essential to reproduce this phenomenon was considerably greater than with Lee. With $10^{7.2}$ E.I.D. of PR8, development of non-infectious virus could not be detected but rather a suppression of viral synthesis as measured by either infectivity or

hemagglutination titrations was observed. When a concentrated inoculum which contained $10^{8.4}$ E.I.D. of virus was employed, however, the results were very similar to those with Lee virus (Fig. 2). The experiments done with Lee and PR8 viruses indicate that the development of non-infectious influenza virus in mouse lungs resulted from inoculation of large quantities of several different viruses, but the actual amount required to produce this phenomenon varied with each agent
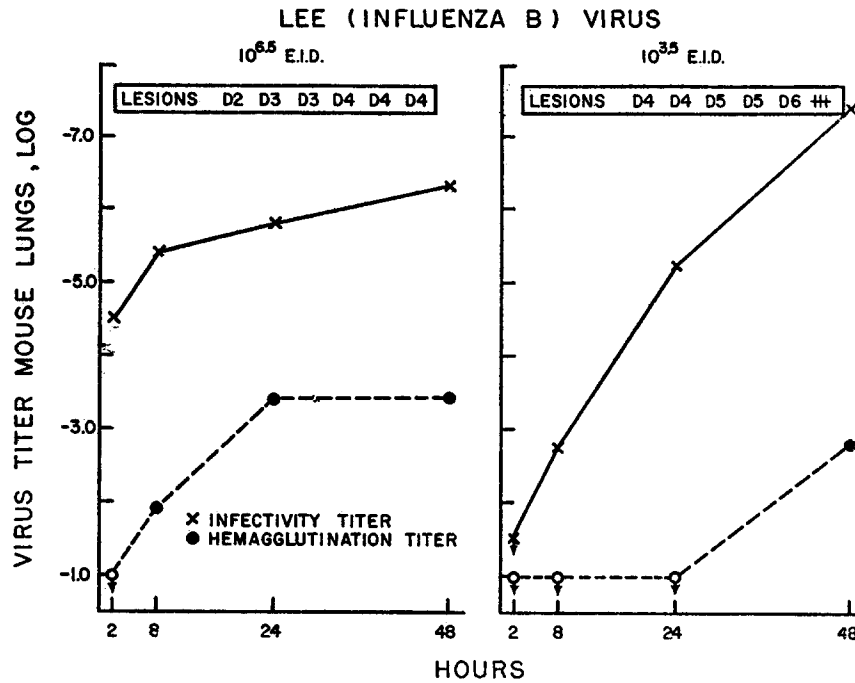

FIG. 2. Comparison of the multiplication of the Lee strain of influenza B virus in lungs of mice following intranasal inoculation of $10^{6.5}$ E.I.D. or $10^{3.5}$ E.I.D. Quantity of virus present in groups of 6 mice at each time interval was determined by infectivity and hemagglutination titrations.

Initial Cycle of Multiplication of CAM Virus Measured by Infectivity, Hemagglutination and Soluble Antigen Titrations.—To determine the temporal relationship of development of non-infectious virus to the change in concentration of infectious virus in the mouse lung, the titers of these components were measured during the period in which it was reasonable to assume the initial cycle of multiplication would occur.

Sixty mice were inoculated intranasally with $10^{8.0}$ E.I.D. of CAM virus, and groups of 6 mice each killed at frequent intervals. All infectivity, hemagglutination, and soluble complement-fixing antigen titrations were done on the same day.

The results obtained in 3 experiments, expressed as geometric means, are summarized in Fig. 3. It is clear that although a gradual but continual decrease of infectious virus occurred commencing 4 hours after initiation of infection there was a concomitant marked increase in specific soluble antigen and hemagglutinin. It is of interest that the rise in soluble antigen began one-half hour before the increase in hemagglutinin or decrease in infectious virus. During the initial cycle of multiplication of PR8 in mouse lungs a similar rise in soluble antigen was observed approximately one-half hour before the increase in hemagglutinin or infectious virus (34). These experiments indicate that non-
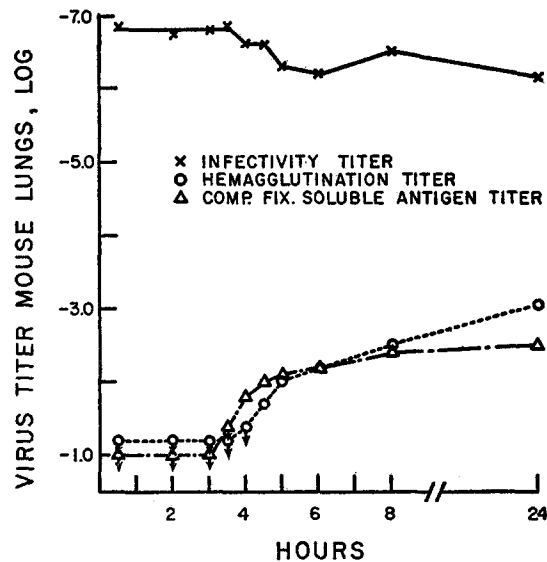

FIG. 3. The initial cycle of multiplication of CAM virus in lungs of mice infected intra-nasally with $10^{8.0}$ E.I.D. Groups of 6 mice were employed at each time interval.

infectious virus hemagglutinin and soluble antigen developed at the time which would be expected during the normal synthesis of virus. The reciprocal fall in infectious virus and rise in non-infectious components suggest 2 hypotheses: (a) under the conditions of infection in these experiments viral multiplication could not progress to formation of fully infectious viral particles but rather partial synthesis of virus was manifest by formation of "incomplete" or non-infectious virus (3, 5); or (b) propagation of virus proceeded as usual but the property of infectivity was rapidly inactivated. These experiments did not permit a choice of either hypothesis.

Effect of Quantity of Non-Adapted Influenza A Virus Inoculated on Multiplication of Virus in Mouse Lungs.—The development of non-infectious virus was demonstrated with an agent which produced extensive lesions in mouse lungs.

To determine whether host cell damage was a prerequisite for the formation of this viral form, experiments were carried out with influenza viruses which were unadapted to mouse lungs and unable to effect pulmonary consolidation.

Identical experiments were carried out with 10 strains of influenza A viruses, identified in section on Materials and Methods. The results with all of these
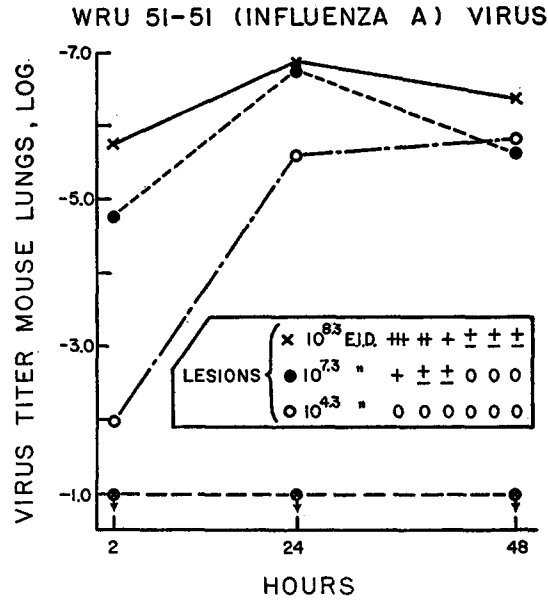
WRU 51-51 (INFLUENZA A) VIRUS

FIG. 4. Effect of the quantity of mouse-unadapted WRU 51-51 strain of influenza A virus inoculated intranasally upon subsequent viral multiplication and development of pulmonary consolidation. Groups of 6 mice were used, and lung lesions were scored 7 days after viral inoculation.

agents were similar, and therefore only data from experiments with one virus, WRU 51-51, will be presented.

Mice in 3 lots of 24 each were inoculated intranasally with $10^{8.3}$, $10^{7.3}$, and $10^{4.3}$ E.I.D., respectively, of WRU 51-51. The inocula were prepared from infected allantoic fluid concentrated 10-fold by high speed centrifugation; from this concentrate decimal serial dilutions were prepared. Groups of 6 mice from each lot were killed 2, 24, 48 hours and 7 days after inoculation. Lungs from mice in the first 3 groups killed in each lot were used for viral infectivity, hemagglutinin and soluble antigen titrations. Lungs from mice killed 7 days after infection were examined and pulmonary lesions scored.

The results of this experiment indicate (Fig. 4) that there was no interference with propagation of infectious virus even when a concentrated inoculum, $10^{8.3}$ E.I.D., was employed. Indeed, the actual viral titer obtained after infection with either $10^{7.3}$ or $10^{8.3}$ E.I.D. was higher than that in mice inoculated

with 1000–10,000 fold less virus. Not only was there no suppression of multiplication of infectious virus, but also there was no demonstrable development of non-infectious virus. In fact, no viral hemagglutinin or soluble antigen could be detected even in lungs of mice infected with the greatest quantity of virus. It is of importance to note that with WRU 51-51 virus, as well as the 9 other agents tested, despite the large quantity of virus inoculated and the significant degree of viral multiplication which occurred, no extensive lung lesions formed. These data are in sharp contrast to those presented from experiments with CAM virus (Fig. 1) and Lee virus (Fig. 2) in which even smaller viral inocula were employed. The comparison of the quantity of CAM, WRU 51-51, and the other 9 egg-adapted viruses inoculated into mice is valid although the titrations of these agents were carried out in chick embryos (35). It has been shown that quantitative relationships of concentration of viruses inoculated into mice may be determined in chick embryos when comparisons are made with egg-adapted influenza viruses (35). A striking correlation of marked pulmonary lesions with formation of non-infectious virus is inescapable. Indeed, even reduced synthesis of infectious virus by so called "auto-interference" (36) appeared to be correlated with lung damage.

Viral Multiplication Following Intranasal Inoculation of Mixture of Infectious and Heat-Inactivated CAM Virus.—The question presents itself of the relative importance of cell injury initiated by the viral inoculum and inoculation of inactive viral particles in diminishing production of infectious virus, as well as in effecting the development of non-infectious virus. To answer this question by direct means, experiments were carried out with infected allantoic fluid heated for short periods of time to permit inoculation of mice with a mixture of infectious and non-infectious virus.

Infected allantoic fluid which contained $10^{8.3}$ E.I.D. per 0.1 ml. of CAM virus was heated at 56°C., and aliquots removed at minute intervals up to 10 minutes. Each aliquot was inoculated intranasally into 6 mice and the degree of pulmonary consolidation determined after 7 days. That aliquot of allantoic fluid, heated for the shortest period, which produced lesions in less than 50 per cent of the total lung volume was selected for subsequent experiments. Incubation of infected allantoic fluid at 56°C. for 5 minutes fulfilled this condition. Heated allantoic fluid was titered in chick embryos to determine the titer of infectious virus present. This mixture of infectious and heat-inactivated virus was then employed in experiments designed as previously described.

The inoculum employed consisted of approximately 99.9 per cent non-infectious viral particles and 0.1 per cent ($10^{5.0}$ E.I.D.) infectious virus. It is clear from the results of 2 experiments summarized in Fig. 5 that non-infectious CAM virus did not develop under these circumstances. Furthermore, only minimal lung lesions formed in 2 of the 6 animals. Although the quantity of infectious virus synthesized in lungs of mice inoculated with the undiluted heated allantoic fluid was greater than that produced in mice which received

the 10^{-2} inoculum containing $10^{3.0}$ E.I.D. of infectious virus, it was not the maximum which might be anticipated in mice infected with $10^{5.0}$ E I.D. contained in an inoculum composed in large proportion of fully infectious viral particles (cf. Fig. 1).

These data suggest that although formation of non-infectious virus may be dependent upon damage of host cells, interference with synthesis of infectious
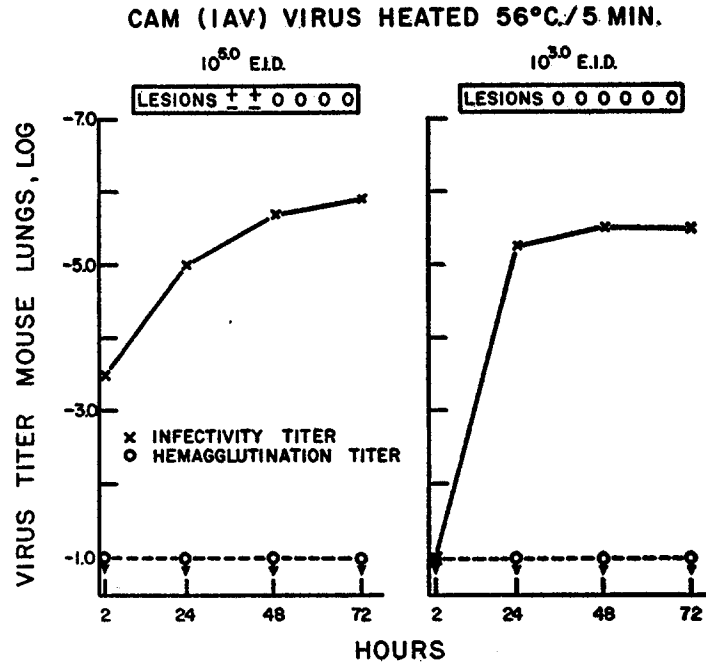

FIG. 5. Multiplication of CAM virus and lesion formation when mixtures of infectious and heat-inactivated virus were inoculated intranasally. Infected allantoic fluid was heated at 56°C/5 minutes. Undiluted or a 10^{-2} dilution of the heated, infected allantoic fluid was inoculated into groups of 30 mice. Viral multiplication was measured by infectivity and hemagglutination titrations. Lung lesions were scored 7 days after viral infection. Lungs from 6 mice were used for each end point.

virus in the mouse lung as in the chick embryo can be promoted by inactivated viral particles. This reduction in multiplication of infectious virus, however, was not comparable in magnitude to that which followed infection with large quantities of fully infectious CAM, Lee, or PR8 viruses.

Mouse Lung Passage of Non-Infectious and Infectious CAM Virus.—The production of non-infectious or "incomplete" influenza virus has been effected in the allantoic sac of the chick embryo by initial inoculation of a very large quantity of influenza A or B virus and subsequent serial passage of the undiluted infected allantoic fluid in the embryonated egg (3, 4, 8-12). von Magnus

has demonstrated that upon serial passage of mixtures of infectious and "incomplete" viral particles not only does synthesis of infectious virus decrease but also formation of "incomplete" virus increases. To compare the development of non-infectious influenza virus in the mouse lung and the allantoic sac of the chick embryo it was of interest to determine the effect of serial passage of undiluted 10 per cent mouse lung suspensions upon the further development

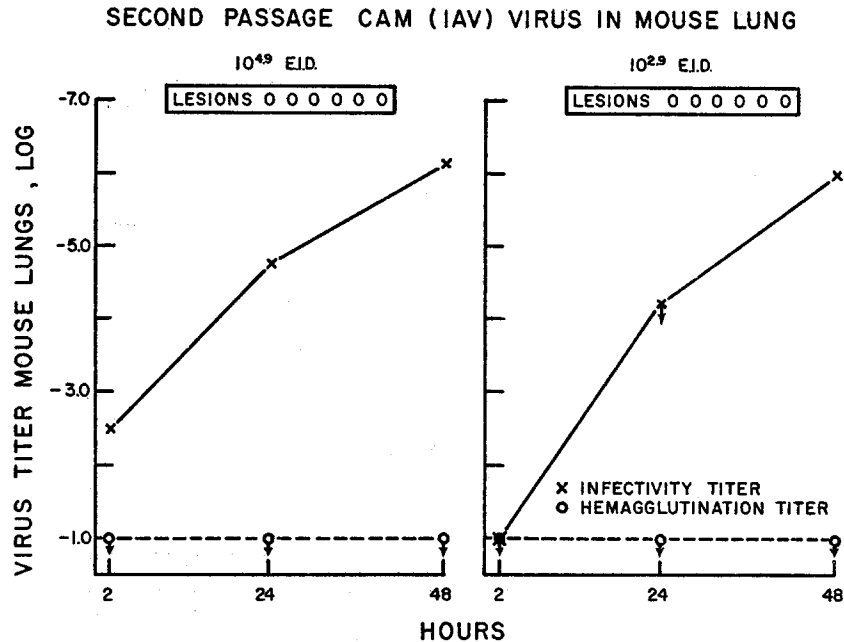SECOND PASSAGE CAM (IAV) VIRUS IN MOUSE LUNG

FIG. 6. Mouse passage of CAM virus in lung suspension containing high titer of non-infectious virus. Lungs for passage were obtained from mice infected with $10^{7.3}$ E.I.D. of CAM virus in allantoic fluid and killed 24 hours after viral inoculation. Viral multiplication and development of lung lesions were compared in mice inoculated with a 10 per cent or 0.1 per cent infected lung suspension. The 10 per cent suspension contained $10^{4.9}$ E.I.D. of infectious virus and $10^{2.11}$ units of hemagglutinating virus. Six mice were employed for each time interval.

or the fate of non-infectious influenza viral particles contained in the suspension.

Mice were inoculated intranasally with $10^{7.3}$ E.I.D. of CAM virus contained in undiluted infected allantoic fluid, and the experiment was carried out exactly as described previously. The results obtained were essentially the same as presented graphically in Fig. 1. The 10 per cent suspension of lungs obtained from mice killed 24 hours after infection had an infectivity titer of $10^{-5.2}$ E.I.$_{50}$ and a hemagglutination titer of 1:128. This suspension and a 10^{-2} dilution prepared from it were each inoculated into 24 mice. Groups of 6 mice from each inoculum series were killed after specified intervals; lung lesions scored and virus titrations of lung suspensions carried out.

The results of this experiment summarized in Fig. 6 indicate that under these conditions non-infectious virus did not multiply or even persist in a quantity that could be detected, notwithstanding the fact that the undiluted inoculum contained a large number of these particles. It is clear that serial passage of non-infectious virus in mouse lungs did not increase production of non-infectious particles. It is significant that no lung lesions developed with either viral inoculum. The multiplication of infectious virus, as in the previously described experiment in which a mixture of heat-inactivated and infectious virus was employed, occurred at a slower rate and yielded a smaller quantity of virus than would be predicted from previous experiments in which a comparable quantity of infectious virus was inoculated. This is further confirmation that the presence of inactive particles in an infecting inoculum does interfere with viral synthesis in mouse lungs (36).

Determination of Quantity of Total Antigenic Material in Lungs Infected with Undiluted and Diluted CAM Virus.—Intranasal inoculation of large quantities of certain strains of influenza viruses resulted in development of a high titer of viral material which agglutinated red blood cells and combined with specific antibody although there was little or no increase in infectious virus. This is in sharp contrast to the high titer of infectious virus produced without concurrent formation of viral hemagglutinins subsequent to infection with a smaller quantity of virus. These data present a qualitative comparison of the viral content of lungs infected with large or small quantities of virus, but do not reflect the total quantity of viral material synthesized in each group. The gross antigenic viral content may be ascertained by determination of the quantity of antibodies produced in mice following injection of dilutions of the infected material (37, 38).

Lung suspensions from mice killed 24 hours after intranasal inoculation of $10^{7.8}$ E.I.D. or $10^{4.8}$ E.I.D. of CAM virus were employed. The results of the experiment from which these suspensions were selected were similar to those summarized in Fig. 1. Lungs from mice infected with the large inoculum contained $10^{5.2}$ E.I.D. of infectious virus, $10^{3.11}$ viral hemagglutinating units, and $10^{2.5}$ units of soluble antigen. The suspension from mice infected with 1000-fold less virus included $10^{6.8}$ E.I.D. or greater of infectious virus and no detectable hemagglutinin or soluble antigen. To each lung suspension was added formalin to a final concentration of 1:2000, and the suspensions stored at 4°C. for 2 days. These formalinized materials were completely non-infectious when tested in chick embryos. Tenfold dilutions of these suspensions were prepared and each dilution injected intraperitoneally into 6 mice, 0.5 ml. per mouse. A second intraperitoneal injection of identically diluted suspension was given 1 week after the initial one. After an interval of 10 days all mice were challenged by intranasal inoculation of $10^{1.7}$ M.S.$_{50}$ (50 per cent maximum score end-point (29)) of a mouse-adapted influenza A virus, WRU 51-51, closely related antigenically to CAM virus. Mice were observed for a period of 10 days, all survivors were killed, and lung lesions scored. The results are expressed as the dilution of mouse lung injected intraperitoneally into mice in which a 50 per cent maximum score was produced by a standard challenge inoculum. This is termed the 50 per cent antigenic extinction titer.

The results of this experiment, presented in Table II, clearly indicate that the total content of viral antigen in lungs of mice 24 hours after infection with $10^{7.3}$ E.I.D. or $10^{4.8}$ E.I.D. of CAM virus was essentially identical despite the high titer of hemagglutinin and low titer of infectious virus in one suspension and the larger quantity of infectious material but absence of hemagglutinin in

TABLE II

Comparison of Total Quantity of Antigenic Viral Material in Mouse Lungs Containing Relatively Large Amounts of Either Non-Infectious or Infectious CAM Virus

Immunized with*				Inter-val	Intranasal inoculation, 0.05 ml.	Lung lesions‡							Anti-genic extinc-tion titer§
						Mouse No.						Score	
Virus	Dilu-tion mouse lung	Infec-tious virus‖	Non-infec-tious virus‖			1	2	3	4	5	6		
	E.I.D., log¶	units**, log		days	E.I.D.¶, log							T/M ‡‡	
CAM§§	$10^{-1.0}$	4.2	2.11	10	WRU(M)‖‖1.7	++	±	0	0	0	/†	2/25	
"	$10^{-2.0}$	3.2	1.11	"	"	++	+	±	±	0	0	4/30	
"	$10^{-3.0}$	2.2	0.11	"	"	+++	+++	++	+	±	/	9/25	
"	$10^{-4.0}$	1.2	0.01	"	"	D4††	D5	D6	D7	+++	++	25/30	$10^{-1.6}$
"	$10^{-5.0}$	0.2	0.001	"	"	D5	D6	D6	++	++	++	21/30	
"	$10^{-6.0}$	0.02	0.0001	"	"	D4	D5	D5	D5	D5	++	27/30	
"	$10^{-1.0}$	5.8	<0.60	"	"	+	±	0	0	0	0	1/30	
"	$10^{-2.0}$	4.8		"	"	+	±	±	0	0	0	2/30	
"	$10^{-3.0}$	3.8		"	"	+++	+	+	+	/	/	6/20	
"	$10^{-4.0}$	2.8		"	"	D7	D10	++	+	+	±	14/30	$10^{-1.9}$
"	$10^{-5.0}$	1.8		"	"	D5	D6	D7	D7	+++	/	23/25	
"	$10^{-6.0}$	0.8		"	"	D4	D5	D5	D5	D6	++	27/30	

* 2 injections, 0.5 ml. each intraperitoneally; 7 day interval between the 2 injections.
‡ Lung lesions of survivors scored after 10 days.
§ 50 per cent antigenic extinction titer.
‖ Contained in untreated mouse lung suspension.
¶ E.I.D. = 50 per cent embryo infectious doses.
** Hemagglutinating units.
‡‡ Numerator = total lesion score; denominator = maximum score possible.
§§ Inactivated with 1:2000 formalin for 2 days at 4°C.
‖‖ Mouse-adapted WRU 51-51 strain of influenza A virus.
†/ = mouse died during immunization; no evidence of viral infection.
†† Mouse died on 4th day with complete pulmonary consolidation.

the other. Similar titrations carried out with lung suspensions derived from experiments in which PR8 virus was used yielded identical results to those presented in Table II. These data imply that the mechanisms for viral synthesis were not reduced quantitatively by the large viral inoculum, but rather that this infecting dose altered the qualitative expression of the synthetic process.

Multiplication of a Recently Isolated Influenza A Virus Adapted to Mouse Lung. —From the experimental data presented it might be inferred that to initiate formation of non-infectious influenza virus in the mouse lung, the agent inoculated must have the capacity to injure host cells. The validity of this hypothesis

appeared to be susceptible to direct test: adaptation of an influenza virus to the mouse lung should yield an agent which, when inoculated in large quantities, could damage host cells extensively and concomitantly elicit development of non-infectious virus. To test this postulate the following experiments were conducted.

Serial passage of the WRU 51-51 strain of influenza A virus in mouse lung was initiated by intranasal inoculation of undiluted infected allantoic fluid. At 48 hour intervals after infection mice were killed, a sterile 10 per cent mouse lung suspension prepared, and passage continued with intranasal inoculation of a 10^{-2} dilution of lung suspension. After 13 serial passages this virus was adapted to the mouse lung as evidenced by the fact that infection with a 10^{-4} dilution of mouse lung suspension resulted in death of all mice within 10 days. A pool of allantoic fluid infected with this mouse-adapted strain was then prepared in the usual manner. The mouse-adapted WRU 51-51 was identified immunologically as closely related to the parent chick embryo-adapted virus if not identical with it. Groups of mice were inoculated intranasally with $10^{7.6}$ and $10^{3.6}$ E.I.D., respectively. Six mice from each group were killed 2, 24, and 48 hours after infection, and hemagglutination, soluble antigen complement fixation, and chick embryo infectivity titrations carried out on lung suspensions. Six mice from each group were observed up to 7 days and the extent of the lung lesions of the survivors at that time was determined.

The results of 2 experiments are summarized graphically in Fig. 7. It is clear that with inoculation of a large quantity of this agent, adapted to effect extensive host cell damage, there was a proportionately greater production of viral material which agglutinated chicken erythrocytes than of material which infected chick embryos. With this agent, as with Lee virus (cf. Fig. 2), not only were viral hemagglutinin and soluble antigen attained in relatively high titers following inoculation of a large quantity of virus, but also the actual titers reached were higher than those measured subsequent to infection with 1000-fold less virus. On the other hand, the amount of infectious virus synthesized was significantly greater following the smaller inoculum. It is important to recall that this mouse-adapted influenza A virus had as its parent an egg-adapted virus which when inoculated intranasally into mice did not produce significant pulmonary consolidation and did not instigate formation of non-infectious virus (cf. Fig. 4). These data lend support to the thesis that the initial damage of host cells by the viral inoculum is an essential condition for development of non-infectious virus in mouse lungs.

The Effect of Dilution of Viral Inoculum upon Non-Infectious Virus Production.—The hypothesis has been proposed that host-cell injury initiated by the infecting viral inoculum is essential in order that non-infectious virus form in mouse lungs. Examination of the data presented indicates that a large number of infectious viral particles were required to effect the extensive cell damage described. In terms of this hypothesis, the least quantity of virus able to produce extensive lesions should also be the smallest amount of virus which upon inoculation would result in significant production of non-infectious viral

particles. Experiments were designed to test the validity of the suggested postulate by the conditions proposed. CAM virus was ideally suited for these
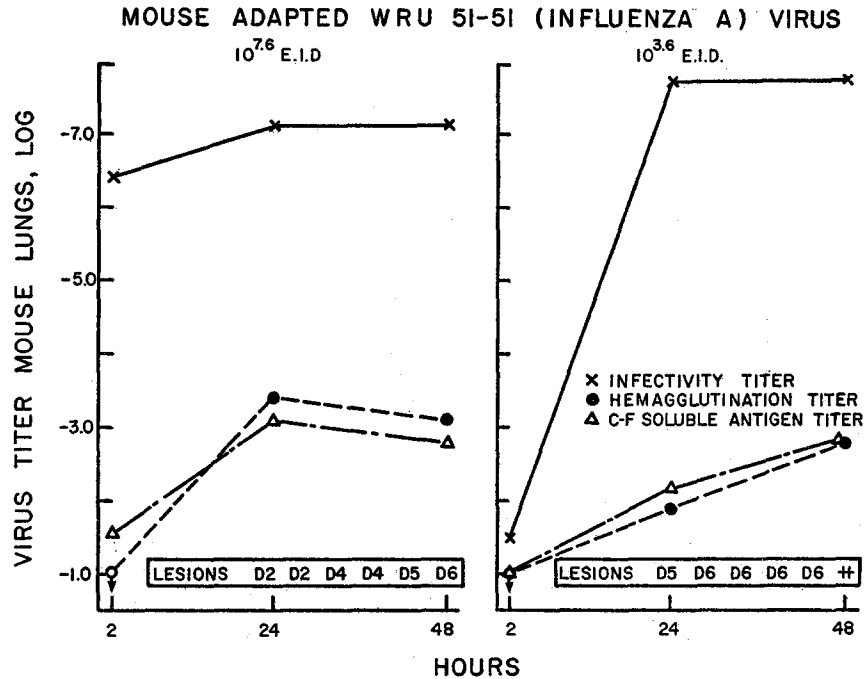

FIG. 7. Effect of mouse adaptation of WRU 51-51 strain of influenza A virus upon the characteristics of viral multiplication of the adapted strain in mouse lungs. Virus was passaged 13 times in mouse lungs and once in allantoic sacs of chick embryos before an undiluted and a 10^{-3} dilution of allantoic fluid containing $10^{7.6}$ E.I.D. and $10^{3.6}$ E.I.D. of infectious virus, respectively, were employed.

experiments for with inoculation of decreasing quantities of virus a corresponding diminution of lung damage is observed.

Twofold dilutions of allantoic fluid which initially contained $10^{8.0}$ E.I.D. of CAM virus per 0.1 ml. were prepared and each inoculated intranasally into 24 mice. Mice were killed at the customary times. Lung lesions were scored and lungs saved for hemagglutination and chick embryo infectivity titrations. Two identical experiments were done.

The geometric means of the results of these experiments are summarized in Fig. 8. To present clearly the quantitative relationships of the development of infectious and hemagglutinating viral particles not only are the titers of these viral components plotted but there is also presented the ratios of the infectivity to the hemagglutination titers. If the infectivity and hemagglutination titers increase proportionately, the ratio of the titers remains the same; if the hemag-
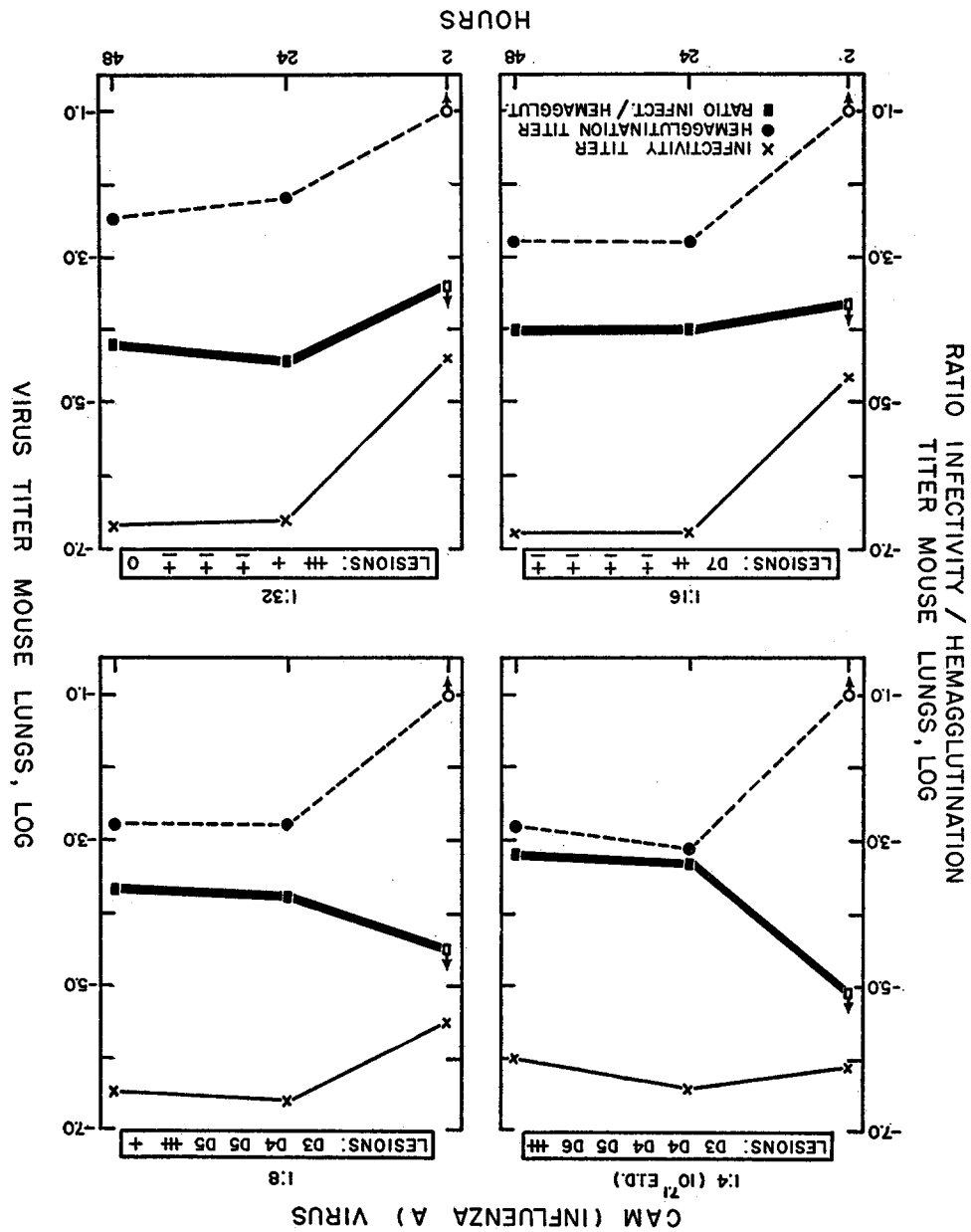
FIG. 8. Correlation of the extent of lung lesions and viral multiplication with the dilution of CAM virus infected allantoic fluid inoculated intranasally into mice. Quantity of virus in lungs was determined by infectivity and hemagglutination titrations. The ratio of infectivity titer to hemagglutination titer is indicated.

glutinin increase is proportionately greater than that of the infectious particles the ratio decreases; and conversely, if the infectivity titer rise is relatively greater than the hemagglutination titer increase, the ratio increases. For the purpose of brevity only the critical portions of these experiments are offered in this figure. As the viral inoculum became more dilute the pulmonary lesions which developed within 7 days diminished in extent and the mortality rate decreased. Concomitant with the diminution in severity of lung lesions alterations in the proportional development of infectious virus and viral hemagglutinins occurred. Indeed, at an inoculum dilution of 1:16 the slope of the line representing the ratio of the infectivity titer to the hemagglutination titer was practically horizontal, an indication that the quantitative development of these viral components was proportional. It is of interest that the 50 per cent maximum lesion score end point of these complete titrations was 1:13. That is, the quantity of virus which produced consolidation of 50 per cent of the total lung volume was essential to instigate development of non-infectious virus. Inoculation of that amount of virus which effected less cell damage did not result in reduced synthesis of infectious virus or detectable formation of non-infectious particles. These data further support the hypothesis that the development of non-infectious virus and the accompanying interference with synthesis of infectious virus is dependent upon injury of susceptible host cells.

DISCUSSION

Intranasal inoculation of a large quantity of certain strains of influenza viruses led to the development of specific viral hemagglutinins in high titer, whereas no increase in titer of infectious virus could be detected. The viral hemagglutinin was considered, therefore, to be non-infectious influenza virus. This phenomenon was closely correlated with extensive pulmonary consolidation following virus inoculation. Indeed, to observe formation of non-infectious virus, a viral inoculum was required which was of sufficient size to effect extensive cell damage rapidly. The data presented imply that this host cell injury was essential to the development of non-infectious viral particles. This in turn lends support to the hypothesis (1, 2) that influenza viruses can damage host cells in the mouse lung prior to or in the absence of significant viral multiplication.

Production of non-infectious influenza virus has been studied in other hosts (3–7) and particularly in the allantoic sac of the chick embryo. Certain distinct characteristics appear to differentiate these unusual phenomena of viral multiplication in the mouse lung and the embryonated egg: (a) whereas appearance of non-infectious virus may require at least 2 allantoic sac passages in the chick embryo, this viral component emerged on the initial passage in mouse lungs; (b) serial passage of non-infectious virus is possible in chick embryos, whereas in the murine host only fully infectious virus was demonstrable after the initial

passage; it must be emphasized that lung lesions did not develop upon inocula-
tion of lung suspension containing a high titer of non-infectious virus; (c) in
the chick embryo viral strains were employed which did not kill the host, in
contradistinction to the high mortality rate observed in mice; and finally
(d) the data obtained clearly indicate that the formation of non-infectious
virus in mouse lung was not dependent upon inoculation of inactive viral
particles, whereas multiplication of so called "incomplete" virus in chick
embryos is considered by von Magnus to be reliant upon interference by
non-infectious, "incomplete" viral particles (10-12). Others (39, 40) do not
agree with this concept. Detailed comparison of development of non-infectious
influenza virus in deembryonated eggs (6), mouse brain (5), or tissue culture (7)
with that observed in mouse lungs is not possible from the reported data. One
similarity in the phenomenon observed in each of these hosts, however, may
be noted: the virus employed was potentially able to effect host cell damage.
Moreover, in brains of mice as in lung, serial passage of incomplete virus was
unsuccessful.

In mouse lungs the appearance of viral hemagglutinins has been associated
with the adapted state of the agent (41). It has been pointed out that upon
adaptation to this host the influenza virus increases its capacity to inactivate
inhibitors of hemagglutination, and in this manner dissociate from host material
(42). The evidence presented above indicates that viral hemagglutinins may
develop in mouse lungs even though the strain employed is not adapted to that
animal. Under these circumstances the total quantity of virus synthesized is not
the controlling process which determines the appearance of hemagglutinins, but
rather it is the capacity of the inoculated virus to damage host cells which
appears essential for the demonstration of viral hemagglutinins. Therefore,
this form of viral virulence, the initiation of host cell damage, is not only
essential for formation of non-infectious virus, but also for the demonstration
of the property of hemagglutination of influenza viruses in mouse lungs.

If host cell injury is an intrinsic factor in development of noninfectious
influenza virus in mouse lung, how does this virus-induced cell damage effect
its profound influence on viral synthesis? The following hypotheses may be
considered:

1. A host cell which is severely injured when infected may be unable to
continue all processes essential for viral propagation. Therefore, materials
necessary to synthesize infectious particles are unavailable and incomplete, or
immature, non-infectious viral particles are produced. The hypothesis that the
chick embryo contains in limited quantity an "unknown substance" required
for synthesis of infectious viral particles was proposed by Cairns and Edney to
explain their data on the development of "incomplete" influenza virus (39).
Destruction of this hypothetical cell substance in mouse lung by severe damage
of the host cell would extend this hypothesis to interpret the evidence presented

above. Infection of the mouse lung with a large number of infectious viral particles, however, was not alone the essential factor in the development of non-infectious virus, as suggested (39, 40), but rather extensive damage of the host cells was required. There appear to be no data which might disprove this postulate. The evidence, however, does suggest other hypotheses which might be more satisfactory. Indeed, the instances in which a relatively high titer of infectious virus is produced without prior or concomitant appearance of hemagglutinin in the allantoic sac of chick embryos (43), as well as in mouse lungs, cast doubt upon the role of the hemagglutinin as an early, immature viral particle.

2. Cell damage may alter the host cells to such an extent that viral multiplication in them occurs at a rate appreciably slower than the rate at which infectious viral particles can be inactivated under ordinary conditions in the mouse lung. The net result observed would be an apparent absence of synthesis of infectious virus but a marked propagation of non-infectious particles. Horsfall has demonstrated this phenomenon to occur in the allantoic sac of the chick embryo when artificial mixtures prepared with heat-inactivated and fully infectious viral particles were inoculated (44). That this phenomenon is not operative in development of non-infectious virus in mouse lungs, however, appears evident when one considers the rate of formation of non-infectious virus. In the initial cycle of multiplication (Fig. 3), the rate of development of non-infectious CAM virus was the same as the incremental rate for infectious virus when smaller inocula were employed (24). Moreover, non-infectious CAM virus appeared at the same rate as did hemagglutinating or infectious PR8 virus in mouse lungs (34). The decrease in multiplication rate effected by a large quantity of inactive virus when mixed with infectious particles was observed in experiments in which CAM virus infected allantoic fluid was heated at 56°C. for 3 minutes and the mixture of infectious and non-infectious particles employed as the inoculum (Fig. 5); although the multiplication rate of infectious CAM virus was significantly reduced, non-infectious virus was not detected. Indeed, the failure to propagate non-infectious virus on second mouse lung passage although the inoculum contained a large proportion of non-infectious virus is strong evidence against this hypothesis.

3. Schlesinger has suggested (14) that formation of non-infectious influenza virus is the result of an aberrant multiplication cycle. Damage to the host cell could initiate such abnormal viral propagation. There is no experimental evidence available which disproves this hypothesis.

4. Infected host cells injured in consequence may release or activate cellular components which are rapidly able to convert newly formed infectious viral particles to non-infectious ones. Although direct evidence to support this hypothesis has not been obtained, data are available which suggest this to be a possible mechanism: (a) the concomitant decrease in infectious CAM virus

when non-infectious particles began to increase at 3.5 to 4 hours after viral inoculation (Fig. 3); and (b) the almost identical quantity of viral antigenic material in lungs in which a high titer of non-infectious virus was measured and in lungs in which infectious particles were the predominant viral component (Table II).

It has been postulated by von Magnus (4, 8–11) that "incomplete" or non-infectious influenza virus develops in the chick embryo as a result of multiple infection of host cells by infectious and "incomplete" viral particles. "Incomplete" particles interfere with the propagation of infectious virus and as a result "incomplete" rather than mature, infectious virus is synthesized. The findings presented do not suggest these events to be essential for the formation of non-infectious influenza virus in mouse lungs. The experimental data obtained do not permit a definite selection of one of the four mechanisms proposed to explain the role of host cell injury on development of non-infectious virus in mouse lungs. The evidence, however, does not favor the hypothesis that the non-infectious virus produced is an "incomplete" or "immature" viral precursor. Rather it is proposed that these data suggest that extensive host cell damage by the virus inoculated initiated an aberrant multiplication cycle (14) or effected rapid inactivation of infectious virus as it was synthesized.

SUMMARY

Formation of non-infectious virus—particles which hemagglutinate red blood cells and react with antibody to fix complement but do not infect the chick embryo or mouse—occurred when large quantities of certain strains of influenza viruses were inoculated intranasally into mice. Dependent upon the agent employed, $10^{6.5}$ to $10^{8.5}$ E.I.D. was essential to elicit this phenomenon. To accomplish this unusual multiplication it was essential to use a strain of virus which effected extensive pulmonary consolidation; strains of virus which did not produce marked lung lesions, even when as much as $10^{8.5}$ E.I.D. was inoculated, did not form non-infectious virus. The development of this viral form was directly dependent upon the extent of cell damage obtained: consolidation of more than 50 per cent of the lung volume was required. The majority of non-infectious particles developed during the initial cycle of viral multiplication, and concurrently with the formation of non-infectious virus there was a corresponding decrease in the number of infectious viral particles. Non-infectious virus could not be propagated on serial passage in mouse lungs: on second lung passage only fully infectious virus was detectable. The formation of the non-infectious viral form was not the result of interference with synthesis of infectious virus by inactivated virus in the inoculum; for inoculation of heated infected allantoic fluid which contained more than 99 per cent of non-infectious virus did not result in the development of new non-infectious virus. Although inoculation of a large quantity of virus resulted in infection which

yielded a relatively low titer of infectious and high titer of non-infectious virus, inoculation of a small quantity of the agent resulted in a high yield of infectious virus and no non-infectious that was detectable. In both instances the total quantity of antigenic viral material synthesized in the mouse lungs was the same. These data do not support the hypothesis that the non-infectious virus formed consisted of immature or incomplete viral particles, but suggest instead that non-infectious virus is inactivated virus or some aberrant form of the agent.

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