QUANTITATIVE ASPECTS OF THE MULTIPLICATION OF INFLUENZA A VIRUS IN THE MOUSE LUNG

Relation between the Degree of Viral Multiplication and the Extent of Pneumonia

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Little is known of the process of multiplication of viruses which are pathogenic for animals. As a consequence, the mechanism by which such agents increase in infected cells is largely conjectural. In contrast to the abundant data available on the quantitative features of multiplication of certain bacterial viruses, there is but little comparable information relating to animal viruses.

Recently, with a few animal viruses, quantitative studies have been undertaken and the results obtained serve to illuminate certain aspects of the multiplication process. It has been shown that with influenza viruses (1) or mumps virus (2) in the chick embryo, as too with pneumonia virus of mice (PVM) in the mouse (3), there occurs, after inoculation, a latent period during which the amount of virus does not increase. Dependent upon the virus employed, the duration of this period ranges from 6 hours with influenza A virus (1) to 24 hours with mumps virus (2). After the latent period, there occurs an interval during which the amount of virus in infected tissue increases relatively rapidly. The duration of this incremental period ranges from 2 hours with influenza A virus (1) to about 15 hours with PVM (3). Taken together, the latent period and the incremental period are considered to constitute a single cycle of multiplication. This is succeeded by other similar discrete cycles (1–3). The yield of virus per cycle ranges from about a 16-fold increase in titer with PVM (3) to approximately a 50-fold increase with influenza viruses (1). It has not been shown that these values represent the order of magnitude of the yield of virus per infected cell.

Quantitative studies with PVM (4) demonstrated that the virus increased in amount in the mouse lung, during the period of progressive multiplication, at a constant rate regardless of the quantity of virus inoculated. Moreover, it was shown that the extent of pneumonia resulting from this infection increased at a constant rate which also was independent of the quantity of virus inoculated. Although the two rates were different, that concerned with the development of lung lesions being the slower, it was demonstrated that the extent of pneumonia was a function of the ratio of the rates and the amount of virus present at a given time during the incremental period.

In previous communications (5–8) the possibility has been raised that PVM and influenza A virus may not utilize identical cellular metabolic systems for multiplication in the mouse lung. This hypothesis was developed when it was found that a chemical substance, the capsular polysaccharide of Friedländer bacillus, which interrupts the multiplication of PVM (5, 8), has no effect upon the multiplication of influenza A virus (6). The postulate gained support from the finding that the two viruses are capable of concurrent multiplication in the mouse lung, *i.e.*, do not show reciprocal interference (7).

It appears possible that, if a difference in the multiplication processes of these agents existed, it might be demonstrated directly by utilizing influenza A virus in quantitative studies in the mouse lung closely similar to those carried out with PVM (4). The results obtained in such studies are the subject of this communication. They indicate that influenza A virus increases in amount at a constant rate in the mouse lung; that the rate is independent of the quantity of virus inoculated; and that the rate of increase is markedly different from that of PVM. The results also indicate that the extent of pneumonia induced with influenza A virus in the mouse increases at a constant rate which is not dependent on the amount of virus inoculated; and that this rate also is different from that obtained with PVM.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was used throughout this study. This strain has been through innumerable mouse lung passages and is sufficiently pathogenic for the mouse so that intranasal inoculation of a 10^{-5} dilution leads to fatal disease associated with extensive pneumonia. A single suspension of infected mouse lungs, stored at −65°C. (9), was employed. This was prepared from the lungs of mice which had been inoculated 3 days previously with a 10^{-3} dilution of the PR8 strain.

Mice.—Three to 4 week old albino Swiss mice of The Rockefeller Institute strain were used. Mice were inoculated intranasally under light ether anesthesia, and each received an inoculum of 0.05 cc. A group of 6 mice was used for each variable in all experiments.

Infectivity Titrations.—These were carried out as previously described (10) by intranasal inoculation of 10-fold dilutions of infected mouse lung suspension. The 50 per cent maximum score end-point (10) was determined 12 days after inoculation. The infectivity titers are expressed in terms of the dilution of ground lung itself, *i.e.*, a 10 per cent suspension = 10^{-1}.

Hemagglutination Titrations.—Ten per cent suspensions were prepared by grinding infected lungs in saline for 3 minutes in a modified Waring blendor. Mixtures of equal volumes of lung suspension and so-called receptor-destroying enzyme (RDE), obtained from culture filtrates of *Vibrio cholerae* (11), were incubated at room temperature for 16 to 18 hours, and then at 37°C. for 2 hours after which they were centrifuged at 7,000 *g* for 10 minutes. Twofold dilutions of the supernates were made in 2.5 per cent sodium citrate, and to each an equal volume of 0.25 per cent chicken RBC was added. Readings were made after the mixtures had stood 1 hour at room temperature. The titers are expressed as the reciprocal of the final dilution of the lung suspension which showed the usual 2+ hemagglutination end-point.

Scoring of Lung Lesions.—Lungs were examined at autopsy and the extent of pneumonia estimated as described previously (10). The lung lesion score is expressed as the ratio of the total observed score to the maximum possible score for the group (*i.e.*, a score of 30 for a group of 6 mice).

Calculations.—These were carried out precisely as in the investigation on the rates of multiplication and lesion development with PVM (4). The quantity of virus inoculated was calculated from the infectivity and hemagglutination titers of the inoculum, taking into account the dilution and the volume of the inoculum relative to the volume used in each titration. The number of hemagglutinating units of virus per lung was computed from the hemagglutination titer of the suspension, taking into account the volumes employed. To obtain a figure for the maximum amount of pneumonia comparable to the maximum number of hemagglutinating units of virus per lung, the lesion score was multiplied by a factor of 10 as in the previous study (4).

EXPERIMENTAL

The rate of increase in viral concentration and the rate of increase in the amount of pneumonia were studied simultaneously in mouse lungs infected with the PR8 strain of influenza A virus. The design of the experiments was identical with that used in studies on pneumonia virus of mice (PVM) in the same host species (4). The amount of virus inoculated was varied over a wide range. At frequent intervals after inoculation, both the viral concentration and the amount of pneumonia were determined in groups of mice. Repeated observations were made over periods sufficiently long to assure that, with each amount of virus inoculated, maximal values for both viral concentration and extent of pneumonia were reached. The concentration of virus was measured by the hemagglutination procedure because of its relative precision and because it has been demonstrated that with influenza viruses hemagglutination is caused by the virus itself (12).

A large number of mice were inoculated intranasally with dilutions of a single suspension of mouse lungs infected with the PR8 strain. The dilutions ranged from 10^{-1} to 10^{-6}. The suspension used for the preparation of inocula had an M.S.50 infectivity titer of $10^{-6.4}$ and a hemagglutination titer of 4096. Groups of 6 mice each were killed at intervals of a few hours after inoculation. The lungs were examined immediately and the lesions scored as described above. The lungs were then stored at −28°C. until the completion of the experiment. Mice which died were autopsied as soon as possible and their lungs were stored at −28°C. until the other mice in the same group were killed. The time after inoculation was taken as the average time that all mice in a group survived. Suspensions were prepared of the lungs of each group and treated with RDE as described above. Hemagglutination titrations were then carried out in duplicate. Two complete experiments were carried out with each amount of virus inoculated. The results presented represent the geometric means of the hemagglutination titers observed at each time interval in the two experiments and the arithmetic means of the two lung lesion scores observed at the same intervals.

The results of these experiments are presented in Table I. A variation of 1,000-fold, *i.e.* from 10^{-1} to 10^{-4}, in the amount of PR8 inoculated did not markedly affect the quantity of virus that was formed in the lungs. With such inocula the maximal values ranged from 3.28 to 3.82 log units per lung. With smaller inocula, *i.e.* 10^{-5} or 10^{-6}, the maximal amounts of virus formed were of the order of 1/10th these values. The smaller the inoculum, the longer was the time required before maximal viral concentration developed.

TABLE I

Amounts of Virus and of Pneumonia in the Mouse Lung after Infection with PR8

Virus inoculum 0.05 cc. intranasal			Time after inoculation	Amount of virus in lung		Amount of pneumonia		
Dilution	M.S.50 doses	Hemagglut. units		Hemagglut. titer 10 per cent suspension	Units per lung	Lesion	Score	Lesion score (× 10) Maximum = 10^3 per lung
	log	*log*	*hrs.*	*geometric mean*	*log*	*L/M**	*per cent*	*log*
10^{-1}	5.4	2.4	4	64	2.08	0/30	0	<1.00
			6	256	2.68	0/30	0	<1.00
			8	256	2.68	0/30	0	<1.00
			10	768	3.16	0/30	0	<1.00
			12	512	2.98	0/30	0	<1.00
			14	1024	3.28	0/30	0	<1.00
			18	1024	3.28	0/30	0	<1.00
			24	2048	3.58	0/30	0	<1.00
			30	1780	3.52	2/30	7	1.85
			36	891	3.22	2/30	7	1.85
			48	548	3.01	3/30	10	2.00
			68	192	2.55	21/30	70	2.85
			82	96	2.25	26/30	87	2.94
10^{-2}	4.4	1.4	6	16	1.47	0/30	0	<1.00
			10	64	2.08	0/30	0	<1.00
			12	47	1.94	0/30	0	<1.00
			14	128	2.38	0/30	0	<1.00
			16	128	2.38	0/30	0	<1.00
			18	316	2.77	0/30	0	<1.00
			24	724	3.13	0/30	0	<1.00
			30	1024	3.28	0/30	0	<1.00
			36	1445	3.43	0/30	0	<1.00
			48	1000	3.27	0/30	0	<1.00
			72	1776	3.52	14/30	47	2.67
			79	2048	3.58	30/30	100	3.00
			84	1024	3.28	30/30	100	3.00
10^{-3}	3.4	0.4	10	8	1.17	0/30	0	<1.00
			12	6	1.05	0/30	0	<1.00
			14	16	1.47	0/30	0	<1.00
			16	32	1.78	0/30	0	<1.00
			18	96	2.25	0/30	0	<1.00
			20	96	2.25	0/30	0	<1.00
			24	632	3.07	0/30	0	<1.00
			30	2048	3.58	0/30	0	<1.00
			36	1777	3.52	0/30	0	<1.00
			48	1777	3.52	0/30	0	<1.00
			72	3559	3.82	7/30	23	2.36
			86	1777	3.52	30/30	100	3.00
			92	2048	3.58	24/30	80	2.90

TABLE I—*Concluded*

Virus inoculum 0.05 cc. intranasal			Time after inoculation	Amount of virus in lung		Amount of pneumonia		
Dilution	M.S.50 doses	Hemagglut. units		Hemagglut. titer 10 per cent suspension	Units per lung	Lesion	Score	Lesion score (× 10) Maximum = 10^3 per lung
	log	*log*	*hrs.*	*geometric mean*	*log*	*L/M**	*per cent*	*lo*
10^{-4}	2.4	−0.6	24	4	0.87	0/30	0	<1.00
			30	256	2.68	0/30	0	<1.00
			36	1024	3.28	0/30	0	<1.00
			48	1024	3.28	0/30	0	<1.00
			72	768	3.16	0/30	0	<1.00
			96	768	3.16	14/30	47	2.67
			114	128	2.38	23/30	77	2.89
			122	24	1.65	28/30	93	2.97
10^{-5}	1.4	−1.6	24	0	<0.27	0/30	0	<1.00
			30	0	<0.27	0/30	0	<1.00
			36	0	<0.27	0/30	0	<1.00
			48	12	1.35	0/30	0	<1.00
			72	13	1.78	0/30	0	<1.00
			96	64	2.08	4/30	13	2.11
			120	128	2.38	11/30	37	2.57
			140	32	1.78	21/30	70	2.85
			150	16	1.47	24/30	80	2.90
			152	16	1.47	30/30	100	3.00
10^{-6}	0.4	−2.6	24	0	<0.27	0/30	0	<1.00
			30	0	<0.27	0/30	0	<1.00
			36	0	<0.27	0/30	0	<1.00
			48	0	<0.27	0/30	0	<1.00
			72	16	1.47	0/30	0	<1.00
			96	48	1.95	0/30	0	<1.00
			120	64	2.08	3/30	10	2.00
			144	8	1.17	7/30	23	2.36
			168	0	<0.27	15/30	50	2.70
			192	0	<0.27	11/30	37	2.57

* L = lesion score; M = maximum possible score.

The extent of the pulmonary lesions which developed also was not markedly affected by the amount of PR8 inoculated over a range of 10,000-fold variation, *i.e.*, from 10^{-1} to 10^{-5}. In all cases almost complete pulmonary consolidation was induced. When a very small inoculum was employed, *i.e.* 10^{-6}, which corresponded to only 2.5 M.S.50 doses, the maximal lesion score was 50 per cent, indicating that, on the average, only half of each lung was affected and all mice survived. The smaller the inoculum, the longer was the time required before pneumonia of maximal degree developed.

When the amount of virus observed at each interval is plotted against the

time after inoculation, as in Fig. 1, a family of curves emerges. With the larger inocula, *i.e.* 10^{-1} to 10^{-4}, the slopes of the curves are similar during the incremental period indicating that the rate of multiplication of the virus was

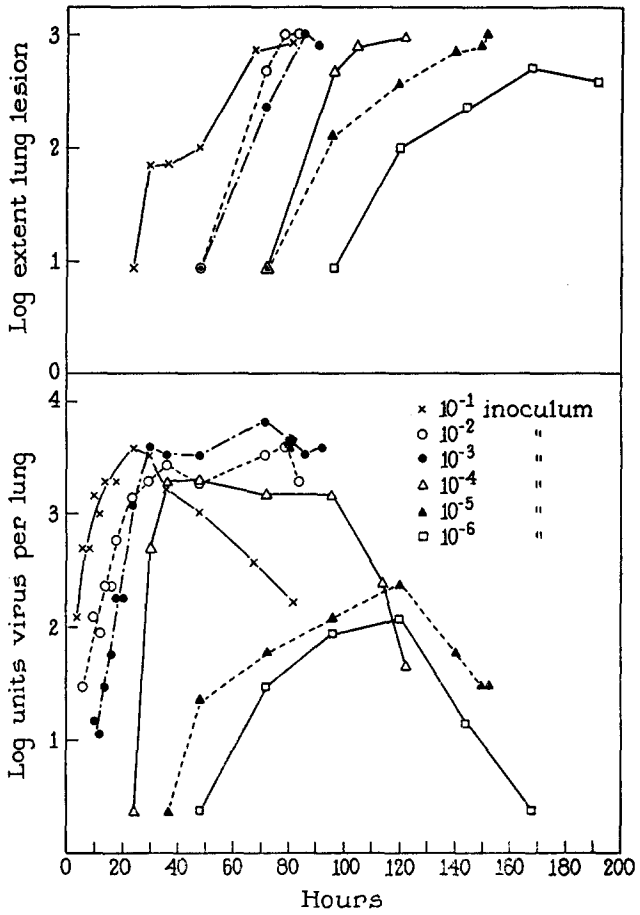

FIG. 1. Changes in the amount of virus and in the amount of pneumonia in the mouse lung as related to time after inoculation of varying quantities of PR8. Each experimental point was determined by the results obtained in 2 groups of 6 mice each. In each case the amounts of pneumonia and of virus were determined in the same lungs.

approximately constant and independent of the amount of virus given. With small inocula, *i.e.* 10^{-5} or 10^{-6}, the slopes of the curves are much less steep and, as was indicated above, the greatest quantity of virus formed was markedly diminished.

It appears that the mouse lung supports the multiplication of PR8 to an

extent represented by a hemagglutination titer of about 2048 which may be considered as the limiting viral concentration obtainable under these experimental conditions. When this limiting quantity was reached, a concentration plateau appeared, the duration of which depended on the amount of virus inoculated. In mice which survived longer than 100 hours, the plateau was followed by a decremental period. The factors which are responsible for the plateau and the decremental period are not clear, but that factors other than specific antibodies are involved seems probable (13).

The finding that inoculation of very small amounts of PR8, *i.e.* a dilution of 10^{-5} or 10^{-6}, did not lead to the development of maximal amounts of virus is consistent with the results obtained under closely similar conditions with PVM (4) as also with those found with influenza A viruses by Taylor (14) as well as Fazekas de St. Groth and Donnelley (15). Because the highest viral levels found with such inocula did not develop until 120 hours and maximal levels observed with larger inocula did not persist longer than 96 hours, it seems probable that unknown factors associated with the decremental period contributed to the result.

When the extent of the lung lesions is plotted against time after inoculation, another family of curves develops as is shown in Fig. 1. The slopes of the lines obtained with inocula of 10^{-1} to 10^{-4} are similar to each other, but are less steep than those of the corresponding curves which illustrate the rate of increase in virus. The slopes of the lines obtained with small inocula, *i.e.* 10^{-5} and 10^{-6}, are somewhat different from the others and appear not to be less steep than those of the corresponding viral curves. It appears then that with PR8, as with PVM (4), lesions developed in the mouse lung at a rate slower than that of the increase in virus whenever more than 25 M.S.50 doses of virus was inoculated.

If the rate of multiplication of PR8 in the mouse lung is constant and independent of the amount of virus inoculated, it would be expected that during the incremental period the experimental points should approach a straight line when the quantity of virus found divided by the amount inoculated is plotted against time. That this prediction is valid is demonstrated in Fig. 2. With inocula of 10^{-1} to 10^{-4} there is no evidence of systematic deviation from a straight line. However, as was to be expected from the curves shown in Fig. 1, inocula of 10^{-5} or 10^{-6} did not yield comparable values. The earliest times that virus was demonstrable after inoculation with these small amounts were 48 and 72 hours, respectively. It is probable that the true incremental period had been passed through during these relatively long intervals and that the hemagglutination procedure was not sufficiently sensitive to reveal the early changes in the amount of virus.

If the rate of development of lung lesions after inoculation with PR8 is constant and independent of the amount of virus given, it would be anticipated

that, during the developmental period, the observed values should lie near a straight line if the extent of lesions divided by the amount of virus inoculated is plotted against time. A test of this prediction is presented in Fig. 3. Except for the values obtained with an inoculum of 10^{-1}, there is no systematic deviation of the experimental points from one line despite the great variation in the amount of virus inoculated.

In a quantitative study with PVM in the mouse lung (4) it was demonstrated that the important variables could be equated by the expression:

$$x = f \cdot i \cdot e^{kt} \tag{1}$$

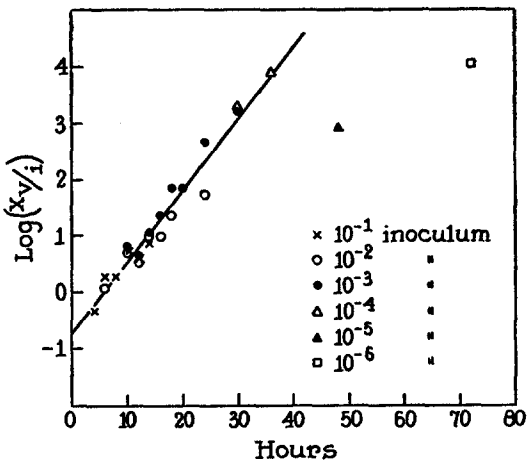

FIG. 2. Linear relation between the amount of virus (x_v) per mouse lung divided by the amount of PR8 inoculated (i) and time after inoculation. Experimental points obtained during the incremental period are plotted. The slope of the line, *cf.* equation 2, indicates that the amount of virus increased at a rate of 1,100-fold per day.

in which x = the observed value, i = amount of virus inoculated, t = time, and both f and k = constants, and that this equation provided a means for predicting either the amount of virus or the extent of lung lesions during the incremental period. From the data shown in Figs. 2 and 3, it becomes apparent that the same equation is applicable with PR8 in the mouse lung, for in the form:

$$\log\left(\frac{x}{i}\right) - \log f = 0.434 \cdot k \cdot t \tag{2}$$

the equation requires that a straight line be obtained when log (x/i) is plotted against t. Under these circumstances log f = intercept on the y axis, and $0.434 \cdot k$ = slope of the line. From the line drawn through the experimental points shown in Fig. 2, in which x = amount of virus per lung (x_v), it is found that $f = 0.178$ and $k = 7.01$ when the time is converted to days as in the previous study (4). From the line drawn through the observed values shown in Fig. 3, in which x = amount of pneumonia per lung (x_l), it is found that $f = 0.178$ and

$k = 2.14$ with time in days. The finding that $f = 0.178$ in both instances indicates that approximately 18 per cent of the virus inoculated was successful in initiating a progressive infection. The slopes of the lines show that the amount of virus increased at a rate of 1,100-fold per day; that the amount of pneumonia increased at a rate of 8.5-fold per day. It appears, therefore, that the amount of virus increased 132 times more rapidly than the amount of pneumonia. The ratio between the two values of k (2.14/7.01) = 0.305, and, as was previously demonstrated with PVM (4), either the amount of PR8 (x_v) or the amount of pneumonia (x_l) induced with this agent can be computed, during the incremental period, from the equation

$$\frac{\log x_v}{t_v} \cdot 0.305 = \frac{\log x_l}{t_l} \tag{3}$$

in which t_v = time of determination of viral titer and t_l = time of observation of extent of pneumonia.

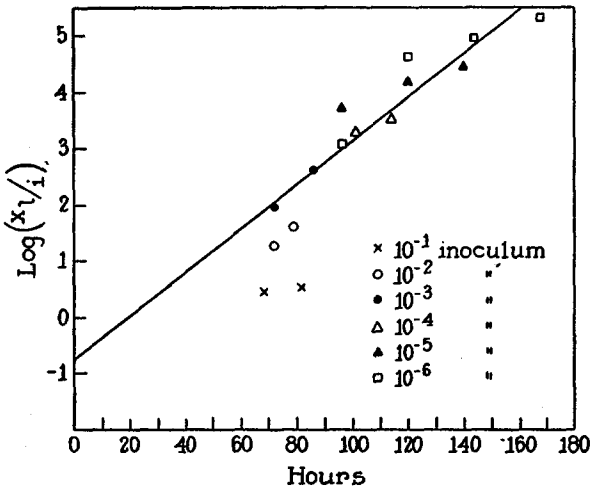

FIG. 3. Linear relation between the amount of pneumonia (x_l) per mouse lung divided by the amount of PR8 inoculated (i) and time after inoculation. Experimental points obtained during the developmental period are plotted. The slope of the line, *cf.* equation 2, indicates that the amount of pneumonia increased at a rate of 8.5-fold per day.

DISCUSSION

The results obtained in this investigation provide direct evidence that the multiplication process of influenza A virus, PR8 strain, in the mouse lung is different from that of pneumonia virus of mice, PVM, (4) in the same tissue. Although there is, as yet, very little information regarding the cellular metabolic processes which are associated with the multiplication of either PR8 or PVM in the lung of the mouse, the finding that certain measurable features of the incremental period are strikingly dissimilar provides additional support for the hypothesis which led to the present study. In this connection, it should be pointed out that Ackermann (16) recently obtained data from which he adduced

that the citric acid cycle was essential for the multiplication of PR8 in the mouse lung. The relation of reactions of the Krebs cycle to the multiplication of PVM has not yet been determined.

It appears evident that the equations which were developed to relate the controllable variables and to test the predictability of experimental findings with PVM (4) serve to relate the variables examined in the PR8 mouse lung system and make feasible the prediction of observed results. It should be emphasized that the chief function utilized, *i.e.* equation 1, in this and the preceding study (4), was developed from theoretical considerations on the assumption that infection of an host cell with a virus leads to the development of a relatively constant number of new viral particles which on release from that cell can each infect another host cell and repeat the developmental cycle. The evidence obtained in this investigation provides support for the validity of this assumption with respect to the multiplication of PR8 in the mouse lung.

From the data obtained in the present study and that which preceded it (4), the following quantitative differences in the processes of multiplication of PR8 and PVM in the mouse lung emerge: (*a*) with PR8 the inoculated virus appears to be 60 times more efficient than PVM in initiating a progressive infection; (*b*) with PR8 the amount of virus increases about 139 times more rapidly than PVM; (*c*) pneumonia induced with PR8 increases approximately 1.8 times faster than that induced with PVM; and (*d*) with PR8 the amount of virus increases about 130 times more rapidly than the amount of pneumonia while with PVM the ratio of the same two rates is 1.7.

In a recent communication (17) on the effects of Newcastle disease virus in the mouse lung, the hypothesis was developed that factors other than viral multiplication *per se* may contribute to the induction of pulmonary lesions. The findings obtained in the present study appear to afford independent support for this concept. The fact that the rate of increase in the amount of PR8 is so markedly different from that of PVM (4), whereas the rate of development of pneumonia induced with PR8 is not greatly different from that induced with PVM, suggests that the multiplication process is not the only factor upon which the appearance of lesions depends.

SUMMARY

Influenza A virus, PR8 strain, increases in amount in the infected mouse lung at a relatively constant rate. When more than 25 M.S.50 doses of virus is inoculated, the rate of multiplication appears to be independent of the amount of virus introduced; has a value of 1,100-fold increase per day. The rate of increase in the pulmonary lesions induced by infection of the mouse lung with PR8 also appears to be relatively constant and independent of the amount of virus inoculated; has a value of 8.5-fold increase per day.

The essential variables in the PR8-mouse lung system appear to be equated satisfactorily by functions which were derived previously (4) during a similar quantitative investigation on pneumonia virus of mice (PVM). Evidence in support of the hypothesis that the processes of multiplication of PR8 and PVM are different in the mouse lung is presented.

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