THE DEPENDENCE OF THE PATHOLOGICAL LESION UPON THE MULTIPLICATION OF PNEUMONIA VIRUS OF MICE (PVM)

KINETIC RELATION BETWEEN THE DEGREE OF VIRAL MULTIPLICATION AND THE EXTENT OF PNEUMONIA

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Infection of the mouse lung with pneumonia virus of mice (PVM) provides one of the few acute respiratory diseases, induced with a virus in its *natural host*, suitable for intensive study as an experimental model (1–3). Not only is the lung the sole organ of the mouse infectible by the agent, but also the gross pathological alterations which develop in the mouse lung are readily measurable (2, 4). In addition, the concentration of the virus is determinable by hemagglutination *in vitro* with a relatively simple and reasonably precise procedure (5–7).

In earlier studies (1, 2, 7) with PVM, there were indications that the amount of pneumonia which developed was related to the quantity of virus inoculated. Moreover, the available evidence (2, 7–9) suggested that the amount of virus formed also was related to the quantity of virus inoculated. These considerations led to the hypothesis that a causal relation exists between the extent of viral multiplication and the degree of pathological alteration. Experiments were undertaken to test this postulate as directly as feasible.

Evidence obtained in the present study indicates that the extent of the pneumonic lesion in the mouse is dependent upon the degree of multiplication of PVM. Correlations bearing on the kinetics of this relationship are presented. It is shown that both the rate of viral multiplication and the rate of increase in the extent of the lesion are independent of the amount of virus inoculated and remain constant until limiting amounts of virus or pneumonia have developed. It is shown also that the concentration of virus as well as the extent of the pneumonic lesion can be computed at any time during the first 8 days after inoculation when the quantity of virus inoculated is known. Moreover, it is shown that during the period when viral multiplication is under way the amount of pneumonia which will develop is predictable from the viral concentration measured at any time during the incremental period.

Materials and Methods

Mice.—Albino Swiss mice of the Rockefeller Institute strain, 3 to 5 weeks of age, were employed. They were handled in a manner identical with that described previously (2). Groups of 6 mice were used for each variable in all experiments.

Virus.—Pneumonia virus of mice (PVM), strain 15 (1), was used. Suspensions of mouse lungs infected with the virus were prepared and stored exactly as in earlier studies (2, 7); dilutions were made in sterile broth containing 10 per cent normal horse serum. Inoculation was by the intranasal route; 0.05 cc. per mouse under light ether anesthesia.

Infectivity Titrations.—These were carried out with serial tenfold dilutions of infected lung suspensions precisely as in previous studies (2, 7). The 50 per cent maximum score end point (M.S.50) was employed (2, 4). Infectivity titers are expressed in terms of the degree to which the ground lung itself was diluted, *i.e.*, a 10 per cent suspension = 10^{-1}.

Hemagglutination Titrations.—These were carried out in duplicate with serial twofold dilutions of infected lung suspensions exactly as described previously (7). Fresh suspensions of mouse RBC were used as routine; the final RBC concentration in the mixtures was 0.4 per cent. To obtain hemagglutination, 10 per cent lung suspensions in saline were heated at 70°C. for 30 minutes and then were centrifuged at 7,000 *g* for 10 minutes. The usual 2+ hemagglutination pattern end point was employed. Titers are expressed in terms of the dilution of the heated 10 per cent suspension and therefore are tenfold lower than corresponding infectivity titers. All titers presented are the geometric means of two or more titrations.

Mouse Lung Lesions.—The extent of gross pneumonia was estimated by careful inspection of each lobe of the lungs immediately after autopsy. The proportion of the total lung volume showing gross pneumonia was determined as described in earlier studies (2) and is expressed as per cent of the maximum score (4).

Calculations.—The quantity of virus given each mouse in an inoculum of 0.05 cc. was calculated from the infectivity titer of the inoculum as well as from the hemagglutination titer, taking into account the dilution and volume of the inoculum relative to the volume used in the two titrations, *i.e.*, 0.05 cc. with infectivity and 0.4 cc. with hemagglutination. The quantity of virus present per lung was calculated from the mean hemagglutination titer of a heated 10 per cent suspension of all the lungs in a group, taking into account the volumes employed. In order to obtain a figure for the maximum amount of pneumonia, *i.e.*, 100 per cent, of about the same magnitude as that for the maximum number of hemagglutinating units of virus per lung, all lung lesion scores were multiplied by a factor of 10 which yields a maximum score value of 1,000.

EXPERIMENTAL

A study was made of the rate of change in viral concentration and in extent of pneumonia in the mouse lung after infection with pneumonia virus of mice (PVM). The amount of virus used to initiate infection was varied over the widest possible range. Both viral concentration and amount of pneumonia in each group of mice were determined at regular intervals until all animals had either died of the disease or recovered from it. Earlier studies (2, 7–9) showed that hemagglutination with PVM is caused by the viral particle *per se* and that the concentration of virus is determinable with somewhat greater precision by hemagglutination titrations than by infectivity titrations.

A 10 per cent infected mouse lung suspension employed for the preparation of inocula had an M.S.50 infectivity titer of $10^{-4.8}$ and a hemagglutination titer of 1:1,000; tenfold dilutions to 10^{-5} were used as inocula. Groups of 6 mice were killed at intervals after inoculation. Mice which died were autopsied promptly and the amount of pneumonia determined; the lungs were removed and stored at −70°C. until they could be added to the lungs of the

remaining mice in the group. The time which elapsed between inoculation and death or autopsy of the animals was taken as the mean survival time for each group. The concentration of virus, as indicated by the hemagglutination titer, and the amount of pneumonia were determined as described above.

The results of one experiment are presented in detail in Table I. The time required for maximal viral concentration to develop was longer the smaller the quantity of virus inoculated. However, varying the amount of virus inoculated by a factor of 1,000 *i.e.*, from 10^{-1} to 10^{-4}, had no effect upon the total amount of virus formed; in each instance, $10^{3.58}$ hemagglutinating units per lung developed. Only when the amount of virus inoculated was exceedingly small, *i.e.*, a dilution of 10^{-5} corresponding to 0.2 of an M.S.50 dose, was the maximal viral concentration significantly diminished. It is noteworthy that in this case all animals survived throughout the observation period. Irrespective of the quantity of virus inoculated, a progressive decrease in viral concentration occurred in all groups which survived for 9 days or more. The longer the period of survival after this time, the more marked was the decrease in the amount of virus present. This evidence confirms earlier results (7).

As is also shown in Table I, the time required for maximal pneumonia to develop was longer the smaller the quantity of virus inoculated. But varying the amount of virus inoculated by a factor of 100, *i.e.*, from 10^{-1} to 10^{-3}, had no effect upon the extent of the lung lesion; in each instance 100 per cent pneumonia developed. When small inocula were employed, *i.e.*, dilutions of either 10^{-4} or 10^{-5} corresponding to 2 and 0.2 M.S.50 doses, respectively, the amount of pneumonia which developed was diminished and with the highest dilution all animals survived.

When these results are plotted against time, as illustrated in Fig. 1, it becomes apparent that the rate of increase in viral concentration, as related to the quantity of virus inoculated, can be described by a family of nearly parallel lines with similar slopes. In short, the rate of formation of new viral particles appears to be constant, or nearly so, and independent of the amount of virus inoculated. In like manner, the rate of decrease in viral concentration after the 9th day also appears to be relatively constant.

As can also be seen from Fig. 1, the rate of increase in the extent of pneumonia, as related to the amount of virus inoculated, can be described by another family of approximately parallel lines with similar slopes. It is evident that these slopes are less steep than those of the viral concentration rate lines. It appears, then, that the rate of extension of the lung lesion is also nearly constant and independent of the amount of virus inoculated. It will be noted that when small inocula were used, *i.e.*, 10^{-4} or 10^{-5}, the lesions progressed only to submaximal values and then ceased to extend. In both instances the lesion plateaus were associated with decreasing viral concentration.

Wide variations in the amount of virus inoculated seemed not to affect the

TABLE I

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

Viral inoculum 0.05 cc. intranasal			Time after inoculation	D/T*	Amount of virus in lung		Amount of pneumonia	
Dilution	M.S.50 doses	Hemagglutinating units			Hemagglutination titer 10 per cent suspension	Units per lung	Lesion score	Lesion score (× 10) maximum = 10^8 per lung
log	*log*	*log*	*days*		*mean*	*log*	*per cent*	*log*
−1.0	3.3	2.1	2	0/6	2	0.87	0	<1.00
			4	0/6	192	2.85	7	1.84
			6	1/6	1024	3.58	57	2.75
			6.1	6/6	1024	3.58	100	3.00
			7.0	6/6	890	3.52	100	3.00
			7.4	6/6	1024	3.58	100	3.00
−2.0	2.3	1.1	2	0/6	0	<0.57	0	<1.00
			4	0/6	128	2.68	3	1.47
			6	0/6	1024	3.58	50	2.69
			6.8	5/6	1024	3.58	97	2.98
			6.8	6/6	890	3.52	100	3.00
			7.4	5/5	768	3.45	100	3.00
			7.6	5/6	768	3.45	90	2.95
−3.0	1.3	0.1	2	0/6	0	<0.57	0	<1.00
			4	0/6	16	1.77	0	<1.00
			6	0/6	512	3.28	10	2.00
			7.6	3/6	1024	3.58	70	2.84
			8.8	6/6	1024	3.58	100	3.00
			9.8	6/6	128	2.68	100	3.00
−4.0	0.3	−0.9	2	0/6	0	<0.57	0	<1.00
			4	0/6	0	<0.57	0	<1.00
			6	0/6	192	2.85	0	<1.00
			8	0/6	1024	3.58	20	2.30
			9.1	3/6	256	2.98	73	2.86
			11.5	4/6	0	<0.57	73	2.86
			12.7	3/5	12	1.65	63	2.79
−5.0	−0.7	−1.9	2	0/6	0	<0.57	0	<1.00
			4	0/6	0	<0.57	0	<1.00
			6	0/6	16	1.77	0	<1.00
			8	0/6	192	2.85	3	1.47
			10	0/5	96	2.55	12	2.08
			13	0/5	0	<0.57	16	2.20
			15	0/6	0	<0.57	16	2.20

* D = mice which died; T = mice in group.

rate of either viral multiplication or extension of the lesion, both of which progressed geometrically, but did affect the degree of both as well as the time required for the development of maximal values. If the assumption is made that PVM multiplies in a manner analogous to the multiplication of bacterial viruses (10); that infection of a cell leads to the formation of a relatively constant number of new viral particles which when released can infect

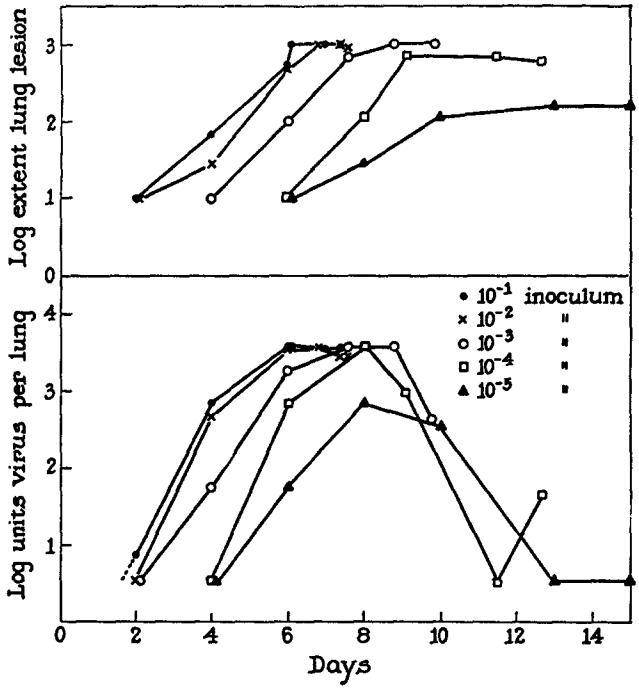

FIG. 1. Changes in the concentration of virus and in the amount of pneumonia in the mouse lung as time passes after inoculation of varying quantities of PVM. Each experimental point was determined by the results obtained in a group of 6 mice. In each case the amounts of pneumonia and of virus were ascertained in the same lungs.

other cells and then repeat the cycle, it would be expected that results similar to those shown in Fig. 1 would be obtained. The larger the number of viral particles inoculated, the greater the number of cells infected and, given a constant rate of multiplication, the shorter would be the time required to infect all available susceptible cells, *i.e.*, to reach limiting viral concentration. Were this postulate valid, it would be anticipated that all the experimental points obtained during the incremental period should fall close to one line if the observed viral concentration divided by the amount of virus inoculated were plotted against time. A test of the prediction is shown in Fig. 2. The data em-

ployed include not only those shown in Table I but also those obtained in a number of similar experiments carried out over a long period of time. There appears to be no systematic deviation of the experimental values obtained during the incremental period from one line even though the amount of virus inoculated was varied by 10,000-fold.

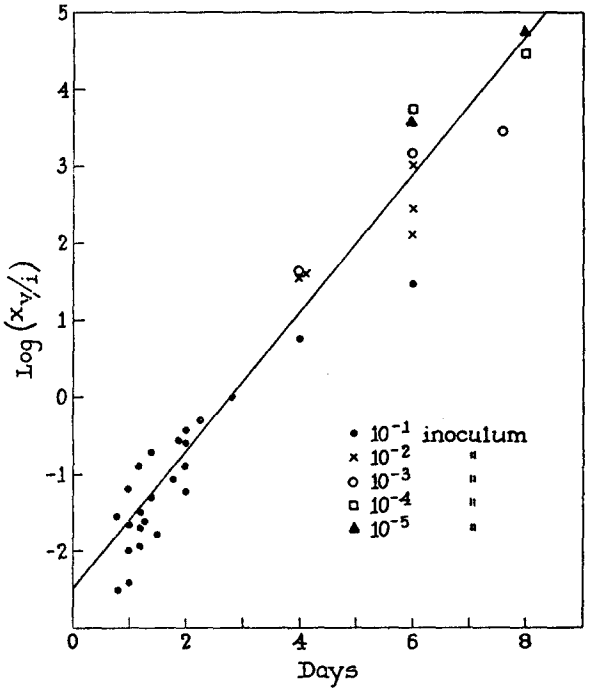

FIG. 2. Linear relation between amount of virus (x_v) per mouse lung divided by quantity of virus inoculated (i) and time after inoculation of varying quantities of PVM. Experimental points obtained during the incremental period, but not after limiting concentrations of virus were attained, are plotted. The slope of the line shown, *cf.* equation 2, indicates that, irrespective of the amount inoculated, viral concentration increased at a rate of 7.9-fold per day until limiting concentrations developed.

If the further assumption is made that infected cells are damaged as a result of supporting viral multiplication, it would be expected that gross evidence of such damage, *i.e.*, lung lesions, should increase geometrically. Moreover, the number of viral particles inoculated would affect the extension of the lesions in a manner closely similar to its effect upon viral concentration. Under these circumstances it would be anticipated that the experimental points obtained during the incremental period also should fall near to one line if the observed extent of lesions divided by the amount of virus inoculated were

plotted against time. A test of this prediction is shown in Fig. 3. The data employed were all obtained in the same experiments as those used to provide data for Fig. 2. In this case, too, there is no systematic deviation of the observed values found during the incremental period from one line despite the large variation in the quantity of virus inoculated.

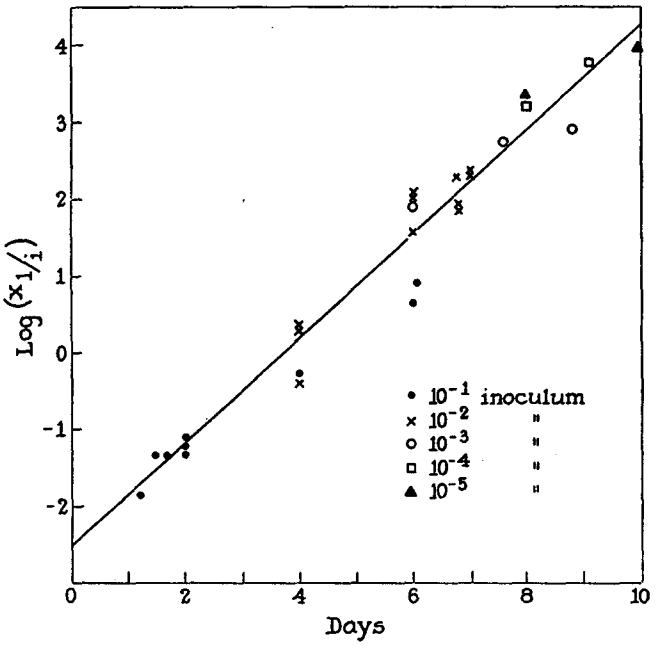

FIG. 3. Linear relation between amount of pneumonia (x_t) per mouse lung divided by quantity of virus inoculated (i) and time after inoculation of varying quantities of PVM. Experimental points obtained during the incremental period, but not after maximal amounts of pneumonia had developed, are plotted. The slope of the line shown, *cf.* equation 2, indicates that, irrespective of the amount of virus inoculated, the extent of pneumonia increased at a rate of 4.7-fold per day until limiting amounts developed.

From theoretical considerations it can be shown that the expression:—

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

in which x = the observed value, i = amount of virus inoculated, t = time, and both f and k = constants, equates the essential variables in a system such as that postulated for the multiplication of PVM. In the form:—

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

the equation predicts that a straight line will be obtained if log (x/i) is plotted against t; that log f = intercept on y axis and $0.434 \cdot k$ = slope. This is precisely the manipu-

lation which was utilized in the graphs shown in Figs. 2 and 3. When x = amount of virus per lung (x_v), the line shown in Fig. 2 indicates that $f = 0.003$ and $k = 2.07$. When x = amount of pneumonia per lung (x_l), the line shown in Fig. 3 indicates that $f = 0.003$ and $k = 1.55$. That $f = 0.003$ in both cases indicates that only 0.3 per cent of the virus inoculated was successful in initiating a progressive infection. The values of k indicate that viral concentration increased at a rate of 7.9-fold per day; that the extent of pneumonia increased at a rate of 4.7-fold per day, *i.e.*, viral concentration increased 1.7 times more rapidly than the extent of pneumonia. It appears that, during the incremental period, equation 2 predicts the extent of pneumonia as closely as it predicts viral concentration and in the two cases only the value of k is different. The ratio between the two values of k (1.55/2.07) = 0.75 and it can be shown that either the amount of virus (x_v) or the amount of penemonia (x_l) can be computed, as it approaches a limiting value during the incremental period, from the function:—

$$\frac{\log x_v}{t_v} \cdot 0.75 = \frac{\log x_l}{t_l} \tag{3}$$

in which t_v = time of determination of viral concentration (x_v) and t_l = time of determination of extent of pneumonia (x_l).

The degree of correspondence between the observed extent of pneumonia and the amount of pneumonia computed on the basis of the viral concentration by means of equation 3 is shown in Table II. Irrespective of the amount of virus inoculated, the extent of the pneumonic lesion can be predicted with considerable precision, during the incremental period, from the viral concentration measured either earlier or later during the same period. The mean deviation of the computed from the observed values for the amount of pneumonia is ± 10.5 per cent. This finding provides strong support for the postulate that the amount of pneumonia is dependent upon the amount of viral multiplication.

As is shown in the accompanying paper (11), the latent period for PVM, during which no increase in viral concentration is demonstrable, is approximately 15 hours. When a large number of viral particles is inoculated, the latent period is followed by a rapid increase in viral concentration which is of the order of 16-fold. A single cycle of multiplication appears to require about 24 to 30 hours. The results of the present study correspond fairly closely with these findings; the computed increase in viral concentration, given above, is 7.9-fold per day. The fact that the increment resulting from a single cycle of viral multiplication which requires approximately 27 hours is not widely dissimilar from the daily increment in concentration during progressive multiplication indicates that the constant f (*i.e.*, 0.003) applies only to inoculated viral particles and not to those released from infected cells after each cycle of multiplication. Dividing the per day increment found during progressive multiplication (*i.e.*, 7.9-fold) by the single cycle increment (*i.e.*, 16-fold)

gives a value of 0.49 which probably represents a fair estimate of the intercycle infective efficiency of the viral particles formed during each cycle.

From the data shown in Table I, it is clear that the longest time required to reach maximal limiting viral concentration was 8 days, *i.e.*, with an inoculum of 10^{-4}. During this period approximately 7 successive cycles of multiplication

TABLE II

Comparison between the Observed and Computed Values for the Amount of Pneumonia after Infection with PVM

Viral inoculum dilution	Amount of virus		Amount of pneumonia		
	Time (t_v) after inoculation	Hemagglutinating units per lung	Time (t_1) after inoculation	Observed	Computed equation 3
log	*days*	*log*	*days*	*per cent*	*per cent*
−1.0	4	2.85	4	7	14
	6	3.58	4	7	6
	6	3.58	6	57	48
	4	2.85	6.1	100	>100
	6	3.58	7	100	>100
−2.0	6	3.58	4	3	6
	6	3.58	6	50	48
	6	3.58	6.8	97	100
−3.0	6	3.28	6	10	29
	7.6	3.58	7.6	70	49
	7.6	3.58	8.8	100	>100
−4.0	6	2.85	6	0	14
	8	3.58	6	0	10
	8	3.58	8	20	47
	8	3.58	9.1	73	100
−5.0	6	1.77	6	0	2
	8	2.85	8	3	14
	8	2.85	10	12	46

should have occurred. If all inoculated viral particles as well as all particles formed during each cycle were successful in infecting susceptible cells, it would be expected that the total increment in viral concentration should have been $16^7 = 10^{8.4}$. It is obvious from the results shown in Table I that no such large increment developed. However, if only 0.3 per cent of the inoculated particles initiated infection, as the constant f (*i.e.*, 0.003) indicates, and nearly all the particles formed per cycle were successful in carrying on the infection, then it can be computed that the observed increment should not have been greater

than 10^5. This corresponds fairly well with the experimental results which indicate that the increment was in fact $10^{4.48}$ (*cf.* Table I, inoculum = 10^{-4}).

As is evident from the results shown in Table I, there occurred after the period of geometric increase in viral concentration an interval, before the death of the mouse, during which the quantity of virus in the lung remained fixed at maximal or nearly maximal levels. During this plateau period, there was no change in viral concentration. The larger the amount of virus inoculated, the shorter was the time until the concentration plateau was reached. With inocula which were not large enough to lead to uniformly fatal disease (*i.e.*, 10^{-4} and 10^{-5}) such viral concentration plateaus were not clearly evident. In these instances progressive decrease in the quantity of virus in the lung occurred after the 9th day.

The results presented in Table I show also that after the period of geometric increase in the extent of the lung lesion there occurred an interval, prior to the death of the mouse, during which the amount of pneumonia did not change. These periods were of shorter duration with the larger inocula than the corresponding viral concentration plateaus as would be expected from the different slopes of the increment lines for the two variables shown in Figs. 2 and 3, respectively. With dilute inocula, *i.e.*, 10^{-4} and 10^{-5}, the lesion plateaus persisted until 12.7 and 15 days, respectively. Decrease in the amount of pneumonia, corresponding to the decrease in viral concentration, did not occur during the period covered in these experiments. However, if recovered mice are held for still longer periods, progressive resolution of the lesion does occur (12).

DISCUSSION

The close correspondence between the observed and the computed data obtained in this study provides evidence in support of the hypothesis which led to the investigation. The finding that, with PVM, the extent of pneumonia in the mouse lung is predictable from the viral concentration determined at any time during the first 8 days of the infection indicates that the pathological lesion is dependent upon viral multiplication. If cells infected with PVM are each able to support the formation of a relatively constant number of new viral particles in a fixed interval of time, it would be anticipated that the rate of multiplication should be independent of the quantity of virus inoculated. The evidence supports the conclusion that this is the fact. Because the number of susceptible cells in the lung obviously is limited, it would be expected that the total number of new viral particles formed could not exceed a limiting value and therefore that the viral concentration would eventually reach, but not go beyond, a fixed maximal level irrespective of the quantity of virus inoculated. That this occurred in all instances but one is evident from the data recorded. In the case of the single exception, the amount of virus inoculated,

i.e., 10^{-5}, was so small that maximal viral concentration should not have developed until 9.1 days. Inasmuch as maximal levels attained with larger inocula did not in any case persist longer than 8.8 days and usually began to decrease even earlier, *i.e.*, 8 days, it appears that in this instance the factors responsible for the decremental period became operative before the maximal level could be reached.

What the factors are which lead to a progressive decrease in viral concentrations after the 8th day is both puzzling and important. Although the present study sheds no new light on them, earlier work (7) indicates that the development of circulating antibodies is not an adequate explanation. That proteolytic enzymes in the lung may contribute to the rapid disappearance of the virus seems possible because of the fact that such enzymes readily eliminate PVM (7). This view is sustained by results presented in the accompanying paper (11) which show that both non-infective virus and virus which does not multiply owing to the presence of Friedländer bacillus polysaccharide disappear from the mouse lung within 2 days. In this connection, it may be pointed out that the plateau periods for viral concentration observed in this study did not in any case persist longer than 1.4 days and, on the average, lasted only 1.1 days. Full elucidation of the factors responsible for the decremental period would contribute to a better understanding of the recovery process.

Whatever the explanation for the decline in viral concentration during the 2nd week, it is clear that once the decremental period has begun there is no further extension of the pneumonic lesion. The amount of pneumonia appears to be determined directly by the degree of viral multiplication. When multiplication ceases, pneumonia does not increase beyond a predictable limit and subsequently (12) undergoes gradual resolution. In the light of these findings it would be expected that a substance capable of inhibiting viral multiplication during the incremental period should also inhibit extension of the pathological lesion. Results presented in the accompanying paper (12) indicate that this is the case.

Grateful acknowledgment is made of the counsel given by Dr. Vincent P. Dole in the derivation of the equations.

SUMMARY

The rate of multiplication of PVM in the mouse lung is relatively constant, averaging 7.9-fold per day with but slight variations, irrespective of the amount of virus inoculated. Similarly, the rate of increase in the amount of pneumonia is relatively constant, averaging 4.7-fold per day, even though the quantity of virus inoculated is varied over a wide range. It follows that viral multiplication proceeds 1.7 times more rapidly than does extension of the

pathological lesion, both reaching limiting maximal values in periods which are predictable from the amount of virus inoculated. From the concentration of virus determined at any time during the incremental period, the amount of pneumonia present earlier or later in the incremental period can be computed with considerable precision. The results support the postulate that the extent of the pathological lesion is dependent upon the degree of viral multiplication.

BIBLIOGRAPHY

1. Horsfall, F. L., Jr., and Hahn, R. G., *J. Exp. Med.*, 1940, **71,** 391.
2. Horsfall, F. L., Jr., and Curnen, E. C., *J. Exp. Med.*, 1946, **83,** 25.
3. Horsfall, F. L., Jr., and Curnen, E. C., *J. Exp. Med.*, 1946, **83,** 43.
4. Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70,** 209.
5. Mills, K. C., and Dochez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57,** 140.
6. Mills, K. C., and Dochez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60,** 141.
7. Curnen, E. C., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1946, **83,** 105.
8. Curnen, E. C., Pickels, E. G., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1947, **85,** 23.
9. Curnen, E. C., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1947, **85,** 39.
10. Delbrück, M., *Biol. Rev.*, 1946, **21,** 30.
11. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1951, **93,** 151.
12. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1951, **93,** 161.