STUDIES ON HOST-VIRUS INTERACTIONS IN THE CHICK EMBRYO-INFLUENZA VIRUS SYSTEM*

V. Simultaneous Serial Passage of the Agents of Influenza A and B in Relation to Variations in the Growth Cycle of Influenza B Virus

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(Received for publication, June 5, 1951)

It has been shown that influenza viruses of types A and B readily interfere with each other in the chick embryo. The interference phenomenon could be demonstrated when the two agents were fully active (1) as well as when one of the agents was inactivated by ultraviolet irradiation to the extent that it lost its propagating activity but not its interfering and hemagglutinating capacities (2–4). It is well established by now that in interference experiments with various viruses factors of dosage and timing have to be considered in order to obtain exclusion of one of the viruses. The available information on the interference phenomenon in general has been the subject of a recent review (5). For the present communication only interference between the two influenza viruses in fully active form has to be considered (1). It was shown that the interfering agent could be injected into the allantoic cavity prior to, simultaneously with, or subsequent to the heterologous virus to be excluded. Relatively little virus was sufficient to exclude the heterologous agent provided it was given far enough in advance to insure propagation of the interfering agent. When the two viruses were injected simultaneously, one had to be in excess in order to exclude the other. Finally, only large doses of virus of one type administered after injection of a small quantity of the other, were able to induce some interference with the latter within certain time limits; *i.e.*, the interfering injection had to be given within 8 to 12 hours after the virus to be excluded.

From this resumé it is clear that simultaneous growth of both influenza A and B viruses can be obtained on first subculture provided proper adjustments are made as to the relative concentrations of the agents and the timing of the injections. However, the situation appeared further complicated by the finding

* The work described in this paper has been supported by a grant-in-aid from the Public Health Service.

that the period of growth in conjunction with the host cells differed with the type of virus, as shown in one-step growth curves (6, 7). For influenza A virus periods of 5 to 6 hours elapsed before the new generations of virus were liberated from the infected tissues into the allantoic fluid, whereas for influenza B strains, 8 to 10 hours were required in the experiments cited. Thus, influenza A virus would be favored somewhat in interference experiments, which actually seemed to be the case in the conditions under which the tests were conducted (1).

From the available information it would have appeared rather unlikely that both influenza A and B viruses could be carried simultaneously through numerous passages in the chick embryo. Yet this has been actually accomplished by Sugg and Magill (8, 9). In the earlier experiments (8) the mixed strains were carried through 10 consecutive passages using dilutions of the seeds of 10^{-3}. In the later series (9) both viruses were still present after 52 transfers of undiluted seeds.

In the light of the seeming discrepancy between the above considerations and the results of simultaneous passage additional experiments appeared to be indicated for clarification of this problem. The results of these investigations are the subject of this paper. It was possible to confirm readily the observations of Sugg and Magill in that both influenza A and B viruses were carried simultaneously through 10 passages provided certain quantitative conditions were obtained. It was found, furthermore, that the cellular growth period in conjunction with the host cells for influenza B virus became markedly shorter when the dose of virus was increased, whereas with influenza A virus no such effect of large doses was noted in previous experiments (6, 10). It is evident, therefore, that when the concentration of influenza B virus in a mixture with A virus is sufficiently great, both agents have similar periods of growth in association with the host cells, resulting in an even chance for both to multiply. Thus an explanation has been furnished for the seemingly contradictory observations mentioned above.

Methods and Materials

Virus.—The PR8 strain of influenza A and the Lee strain of influenza B virus were used in these studies. For seed preparations infected allantoic fluids were diluted in sterile broth to 10^{-6} or 10^{-7} and 0.2 ml. amounts were injected allantoically into 10 to 11 day old chick embryos. After 48 hours of incubation at 36° to 37°C. the allantoic fluids were collected and assayed for infectivity and hemagglutinating capacity.

For combined passage the 2 viruses were mixed in the proportions indicated in the text and the mixtures were injected in varying dilutions, using 0.2 ml. amounts, into 8 to 10 chick embryos at the 10th to 11th day of development. The allantoic fluids were collected after incubation of the eggs at 36° to 37°C. for 24 hours and tested for the presence of the individual viruses by hemagglutination and, in a few instances also by infectivity tests, in the presence of rabbit immune sera against either the PR8 or the Lee strains. In a few preliminary tests the embryos injected with the mixtures were incubated for 48 hours.

The technic used to obtain growth curves has been fully described (10).

Infectivity Titrations.—The method employed for single strain infections has been discussed previously (10). In the case of mixed infections the virus preparations were mixed with rabbit immune sera against either PR8 or Lee viruses in such a manner that the final dilutions of virus amounted to 10^{-1}, 10^{-2}, and so forth, and those of the sera to 1:100 to 1:500. These concentrations of the sera were found sufficient to neutralize at least 10,000 ID_{50} of the homologous agents.

Hemagglutination Tests.—The technic used has been fully described in the preceding paper of this series (11). Typing of the virus was performed by mixing 0.2 ml. of serum, diluted 1:64 to 1:256 with 0.2 ml. amounts of serial dilutions of virus, and 0.2 ml. of a 1 per cent suspension of chick red cells. The tests were read in the usual manner after incubation at 4°C. for 75 to 90 minutes.

Rabbit Immune Sera.—In the production of the sera adjuvants were used according to established technics (12–14). Viral concentrates were mixed with Falba and mineral oil in a ratio of 1:1:4, and 4 ml. of this emulsion was injected subcutaneously between the shoulder blades. The antibody levels reached maximal heights in about 4 weeks and remained at high levels for several months thereafter. In the earlier experiments no effort was made to free the sera of non-specific inhibitor of hemagglutination. Later on, they were treated with sodium periodate according to Hirst (15).

Other technics are referred to in the text where indicated.

EXPERIMENTAL

Simultaneous Passage of Influenza A and B Virus

In preliminary experiments the PR8 and Lee strains were mixed in varying proportions and 0.2 ml. of the various mixtures were injected into each of a number of chick embryos. After incubation of the eggs at 37°C. for 24 hours the allantoic fluids were collected and assayed for (*a*) the presence of the 2 viruses; and (*b*) for their relative concentrations, both measured by hemagglutination tests in the presence of heterologous immune serum. In the first of these tests the mixtures consisted of equal volumes of PR8 10^{-1} and Lee 10^{-1}; PR8 10^{-1} and Lee 10^{-2}; and PR8 10^{-2} and Lee 10^{-1}. In each instance both viruses were found in the harvests. From the first mixture a few allantoic fluids were obtained in which the hemagglutinin titers of the PR8 and Lee viruses were equal, although in most of this group, as well as in those of the second, the PR8 titers exceeded that of the Lee virus. With the third mixture, the reverse was true, the Lee titers being significantly higher than those determined for PR8.

In the second preliminary experiment the mixtures were made by adding to one part each of undiluted allantoic fluid containing PR8 virus ($10^{9.9}$ ID_{50}/ml.) one, three, or five parts of Lee virus ($10^{9.0}$ ID_{50}/ml.). The mixtures were then injected into chick embryos in dilutions 10^{-1}, 10^{-3}, and 10^{-5} and the allantoic fluids collected from groups of eggs after incubation periods of 24 and 48 hours. A summary of the qualitative results is given in Table I. As can be seen when the mixed inocula were diluted 10^{-1} both PR8 and Lee viruses were found in the fluids collected after 24 and 48 hours. When the seeds were diluted 10^{-3} all fluids harvested in 24 hours contained measurable levels of PR8 virus but only

some of them revealed Lee virus. In 48 hours a few more fluids had become positive for Lee virus when the seed contained PR8 and Lee in ratios 1:1 and 1:3, and all in the 1:5 ratio series. Dilution of the mixtures to 10^{-5} resulted in 48 hours in the appearance of PR8 hemagglutinins only.

TABLE I

The Influence of Varying Ratios of PR8 to Lee Virus and of Dilution of These Mixtures upon the Propagation of the Agents in Chick Embryos

Seed		Incubation period	Hemagglutination test				Result
			No. of eggs positive in presence of				
Ratio PR8:Lee	Dilution of mixture		Anti-Lee serum (PR8)*	Anti-PR8 serum (Lee)	Anti-Lee and Anti-PR8	Saline	
		hrs.					
1:1	10^{-1}	24	9/9‡	9/9	0/9	9/9	PR8 + Lee
		48	9/9	9/9	0/9	9/9	PR8 + Lee
	10^{-3}	24	9/9	1/9	0/9	9/9	PR8 > Lee
		48	7/7	3/7	0/7	7/7	PR8 > Lee
	10^{-5}	48	9/9	0/9	0/9	9/9	PR8
1:3	10^{-1}	24	8/8	8/8	0/8	8/8	PR8 + Lee
		48	9/9	9/9	0/9	9/9	PR8 + Lee
	10^{-3}	24	8/8	5/8	0/8	8/8	PR8 > Lee
		48	8/8	6/8	0/8	8/8	PR8 > Lee
	10^{-5}	48	8/8	2§/8	1§/8	8/8	PR8
1:5	10^{-3}	24	9/9	5/9	0/9	9/9	PR8 > Lee
		48	9/9	9/9	0/9	9/9	PR8 + Lee
	10^{-5}	48	9/9	0/9	0/9	9/9	PR8

* indicates virus component determined.

‡ 9 out of 9 eggs gave positive hemagglutination.

§ Titer of 1:2, the anti-PR8 serum was not strong enough to neutralize completely the hemagglutinins present.

It is obvious from these results that materials for passage had to be chosen carefully as was done also by Sugg and Magill (8, 9). When allantoic fluids were selected with nearly equal hemagglutinin titers for PR8 and Lee, both viruses could be carried through as many as 9 serial passages without great difficulty in the present studies, but several attempts had to be made before evidence of Lee virus was obtained in the 10th transfer. The data of this pas-

sage series are presented in Table II. It can be seen that the fluids used for the passage series were diluted not more than 100-fold and frequently, particularly in the later passages, were used undiluted. In all passages in addition, several increasing dilutions of the seed were employed but in these cases the Lee component was readily lost. This is illustrated in Table III which offers some representative examples. This finding is somewhat contrary to the observations of Sugg and Magill (8) who passed their strains usually in dilution 10^{-3}. This discrepancy may partly be due to the fact that in the present series the infected eggs were incubated at 36° to 37°C. for 24 hours instead of at 35°C. for 55 to

TABLE II

Details of the Simultaneous Passage of PR8 and Lee Viruses

Passage No.	Egg No.	Hemagglutination titer in the presence of				Dilution used for subsequent passage
		Anti-Lee serum (PR8)	Anti-PR8 serum (Lee)	Anti-Lee + Anti-PR8	Normal serum	
0*						10^{-1}
1	4	1:48	1:32	<1:2	1:256	10^{-1}
2	4	1:192	1:48	<1:2	1:512	10^{-1}
3	3	1:16	1:12	<1:2	1:192	10^{-1}
4	3	1:24	1:24	<1:2	1:256	10^{-2}
5	1	1:24	1:24	<1:2	1:192	10^{-0}
6	5	1:24	1:8	<1:2	1:256	10^{0}
7	1	1:48	1:32	<1:2	1:512	10^{0}
8	5	1:96	1:48	<1:2	1:192	$10^{-0.5}$
9	8	1:48	1:32	<1:2	1:512	$10^{-0.5}$ and 10^{0}
10*a*	6	+	−	−	+	
10*b*	4	+	−	−	+	
10*c*	4	+	−	−	+	
10*d*	1-5	+	±	−	+	
10*e*	1-5	+	±	−	+	

* PR8 and Lee allantoic fluids mixed in equal proportions.

65 hours. Furthermore, the sera used in the above tests appeared to contain higher levels of non-specific inhibitory substances so that low concentrations of hemagglutinin may have been neutralized non-specifically. However, the hemagglutinin titers in the controls with normal rabbit serum were always substantially higher than could be expected from the estimated combined titers of PR8 and Lee hemagglutinins in the presence of anti-PR8 and anti-Lee sera. With the $NaIO_4$-treated sera (15), as used later on in growth curve experiments (see below), a similar discrepancy seemed to be apparent.

The hemagglutination test for the presence of virus is, of course, relatively insensitive, and substantial amounts of virus have to be present before it will become positive. If passage material, which upon 10-fold dilution had failed

TABLE III

Loss of the Lee Component upon Injection of Diluted Seed Derived from the PR8-Lee Passage Series

Seed			Number of eggs positive in presence of				Result
Passage No.	Egg No.	Dilution	Anti-Lee serum (PR8)	Anti-PR8 serum (Lee)	Anti-Lee + Anti-PR8	Normal serum	
1	4	10^{-1}	8/8	6/8	0/8	8/8	PR8 > Lee
		10^{-4}	6/6	0/6	0/6	6/6	PR8
4	3	10^{0}	4/9	9/9	4/9	9/9	PR8 < Lee
		10^{-1}	10/10	10/10	0/10	10/10	PR8 + Lee
		10^{-2}	9/9	7/9	0/9	9/9	PR8 > Lee
6	5	10^{0}	8/8	8/8	0/8	8/8	PR8 + Lee
		10^{-1}	7/8	0/8	0/8	8/8	PR8
		10^{-2}	8/8	0/8	0/8	8/8	PR8
9	1–5	10^{0}	10/10	4/10	0/10	10/10	PR8 > Lee
		$10^{-0.5}$	10/10	0/10	0/10	10/10	PR8

TABLE IV

Propagation of Lee Virus from Dilute Mixed Passage Seed after Neutralization of the PR8 Virus by Immune Serum

Experiment No.	Seed			Hemagglutination Test			
				Number of eggs positive in presence of			
	Passage No.	Dilution	Serum	Anti-Lee serum (PR8)	Anti-PR8 serum (Lee)	Normal serum	Result
1	6	10^{0}	None	5/5	2/5	5/5	PR8 > Lee
		10^{-1}	None	7/7	0/7	7/7	PR8
		10^{-2}	None	8/8	0/8	8/8	PR8
		10^{-2}	Anti-PR8	0/7	6/7	6/7	Lee
2	6	10^{0}	None	8/8	8/8	8/8	PR8 + Lee
		10^{-1}	None	7/8	0/8	7/8	PR8
		10^{-2}	None	8/8	0/8	8/8	PR8
		10^{-2}	Anti-PR8	0/8	8/8	8/8	Lee

to evoke evidence of propagation of Lee virus by the hemagglutination test, was mixed with anti-PR8 serum in dilution 1:100 and then injected into eggs, it was found (Table IV) that even on 100-fold dilution, growth of Lee virus was obtained.

Growth Curve Experiments

It proved possible then to confirm the findings of Sugg and Magill (8, 9) of the simultaneous passage in series of influenza A and B virus. However, the technic, as employed, did not permit an evaluation of the relative rates of development of the 2 viruses. By applying the growth curve method (6, 10) further data were obtained which are presented in Fig. 1 and 2.

Sixty 12 day old chick embryos were inoculated with 0.2 ml. of a mixture of undiluted allantoic fluids containing PR8 and Lee virus in a ratio of 1:3. Similar numbers of eggs were injected with PR8 virus diluted 4-fold in saline or with Lee virus to which one-fourth volume of saline had been added. Thus, in the single strain control series the same amounts of the agents were injected as were present in the mixed inoculum. The allantoic fluids and membranes from groups of 6 eggs each were collected at hourly intervals after inoculation and assayed for their hemagglutinin content either in the presence of saline, normal rabbit serum anti-PR8, anti-Lee, or a mixture of anti-PR8 and anti-Lee sera. All sera had been treated with $NaIO_4$, which substantially reduced their non-specific inhibition.

In Fig. 1 (upper charts) are compared the results of hemagglutinin titrations of allantoic fluids derived from the PR8 control series with the PR8 component of the combined PR8-Lee series; and the results of the Lee controls with the corresponding Lee titrations of the mixed series. It can be seen that there was no significant change in the constant periods. In the combined PR8-Lee series the quantities of hemagglutinins of each type liberated were markedly less than those released in the corresponding control series. The results with the membrane suspensions are given in the lower charts of Fig. 1. They are essentially similar except that hamagglutinins became measurable slightly later in the mixed virus series than in the single strain controls. This is in accordance with a decreased production of the individual viruses. In Fig. 2 the various data obtained in the mixed series are compared with each other. It can be seen that both the PR8 and Lee hemagglutinin curves obtained by titration in the presence of heterologous serum ran closely parallel. The total hemagglutinin assay in the presence of saline or normal serum gave titers substantially higher than one would expect from the calculated combined titers of the individual components. Whether the small amounts of non-specific inhibitor left in the various sera after treatment with $NaIO_4$ (Fig. 1) may account for this apparent discrepancy can not be decided at the present time. In other experiments the relative concentration of the two viruses in the seed was varied slightly to favor one or the other. The resulting curves were principally similar, except that whatever virus was in excess in the seed produced higher hemagglutination levels than the agent injected in smaller quantity. Essentially similar results were obtained in growth curves with allantoic fluid seed derived from the 5th passage of the combined transfer series.

Thus, it appears that both viruses may multiply independently in the allan-

toic tissue on mixed infection of chick embryos but to a less extent than when singly injected in similar concentration. The data presented indicated that the

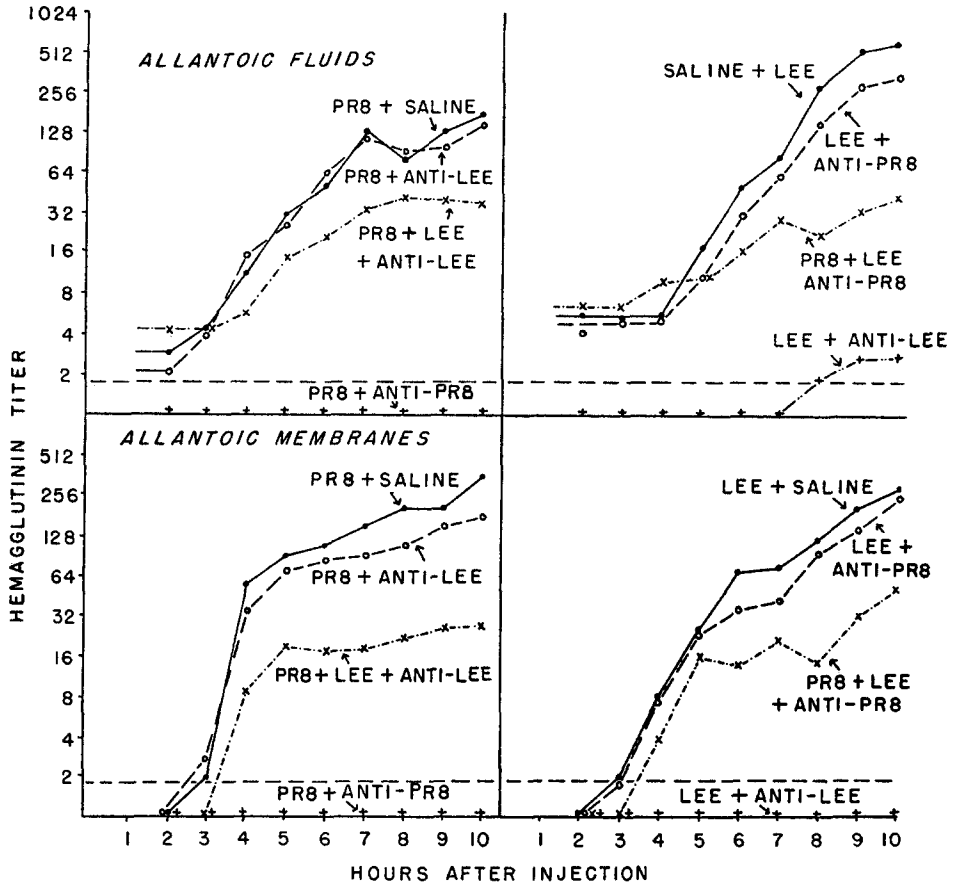

FIG. 1. Comparison of hemagglutinin curves in allantoic fluid and membrane obtained after injection of mixed PR8 and Lee viruses or single strains. PR8 + saline = inoculum PR8, assay of hemagglutinin titer in the presence of saline solution; PR8 + Lee + anti-Lee = inoculum PR8 + Lee mixture, assay of hemagglutinin titer in the presence of anti-Lee serum, *i.e.* the PR8 component; and so forth. The horizontal broken line indicates the starting dilution of the various preparations used.

constant periods, as measured by hemagglutination technic did not differ significantly for the 2 viruses under the conditions of these experiments. Furthermore, when allantoic fluids obtained from such mixed and individual growth curves were assayed for infectivity the following results were obtained. In the PR8 titrations, both of the control and of the mixed series (the latter

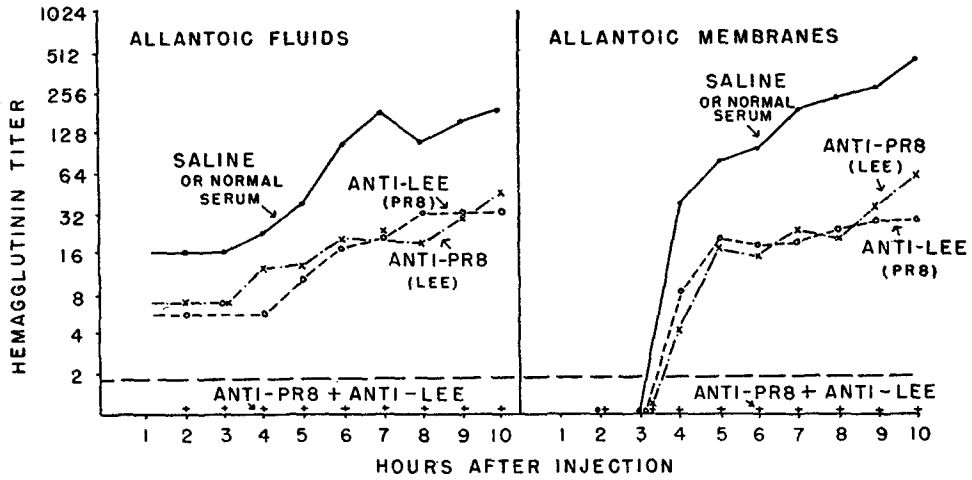

FIG. 2. Comparison of PR8 and Lee hemagglutinin curves in allantoic fluid and membrane obtained after injection of mixed PR8 and Lee seed as assayed in the presence of anti-PR8 or anti-Lee serum. The total hemagglutinin content is shown by titration in the presence of normal serum or saline solution.

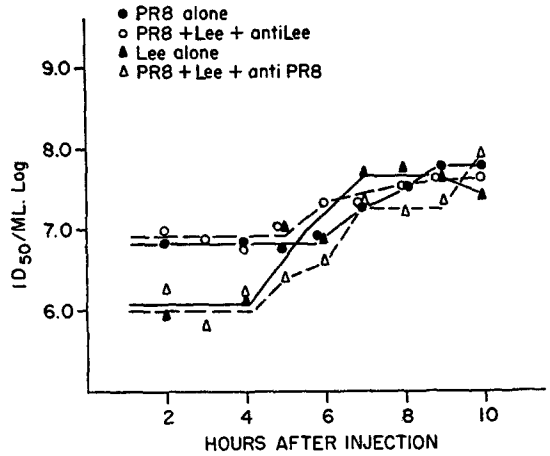

FIG. 3. Comparison of infectivity curves of PR8 and Lee viruses obtained in allantoic fluid after injection of mixed or single strains.

in the presence of anti-Lee serum), the infectivity in the allantoic fluids remained constant for 5 to 6 hours (non-adsorbed seed virus) before new generations of virus were liberated, in agreement with earlier reports (6, 7, 10). The constant periods as measured in the corresponding assays for Lee virus, on the other hand, were only 4 to 5 hours, in contrast to expectations. The con-

stant periods recorded previously were of the order of 8 to 10 hours (6, 7). A summary of 2 such experiments is given in Fig. 3.

The earlier experiments differed from those presented above in that vastly different amounts of seed virus were employed; *i.e.*, 10^4 to 10^5 ID_{50} of Lee virus as against 10^8 to 10^9 ID_{50} in the present series. That the amount of seed virus

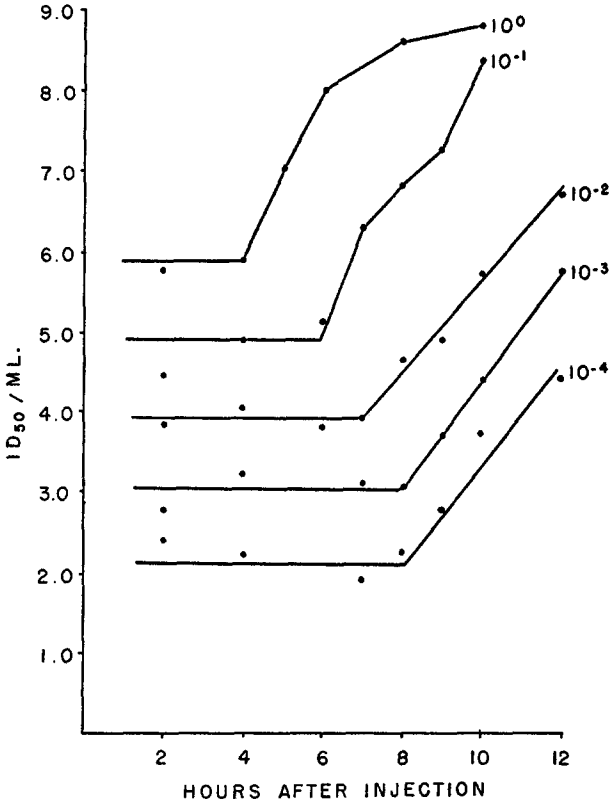

FIG. 4. The relation of the concentration of Lee virus in the seed to the extent of the constant periods of infectivity in the allantoic fluid.

injected exerts a marked influence on the extent of the constant period in the case of the Lee strain was demonstrated in the following experiments. Growth curves were obtained after injection of varying amounts of Lee virus and it was noted that the constant periods following injection of undiluted allantoic fluid were of the order of 4 to 5 hours. With an inoculum of 10^{-1} they increased to about 6 to 7 hours, with 10^{-2} to 7 to 9 hours and with higher dilutions of the seed they extended over 8 to 10 hours, as reported previously. The data of an experiment of this type are presented in Fig. 4.

The infectivity titrations of the membrane suspensions showed lesser variations of the constant periods. With concentrated seed they were of the order of 3 to 4 hours and increased with the dilution of the seed to 5 to 6 hours (Figs. 5 and 6). After the constant periods the virus titers rose rapidly and the virus increased at similar rates several hours in advance of liberation into the al-

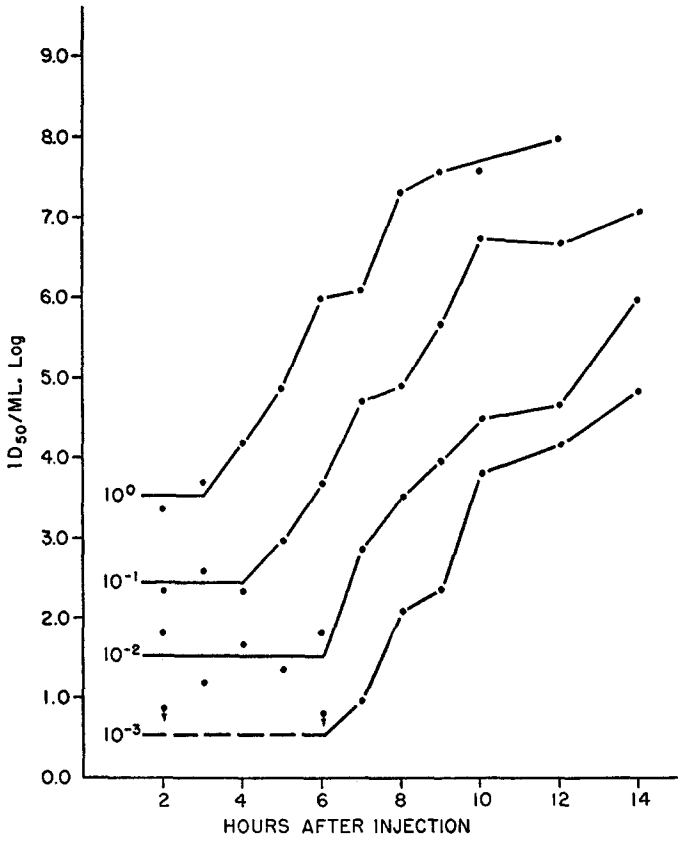

FIG. 5. The relation of the concentration of Lee virus in the seed to the extent of the constant periods of infectivity in the allantoic membranes, and the rate of development of new virus.

lantoic fluid. Whereas in most experiments the virus titer rose more or less steadily (Fig. 5) in other experiments (Fig. 6) with the more concentrated seed, it reached a peak in 7 to 8 hours and thereafter a distinct fall in titer was noted by the 10th hour. The latter type of curve would indicate that all susceptible cells had been infected and upon liberation the titers in the tissue decreased. However, this phenomenon has not been observed regularly and other explanations for this variability might have to be sought.

DISCUSSION

The data presented confirm the experiments reported by Sugg and Magill (8, 9) that under certain conditions both the PR8 strain of influenza A and the Lee strain of influenza B virus can be passed simultaneously in series through chick embryos. As mentioned in the introduction this fact seemed to contradict

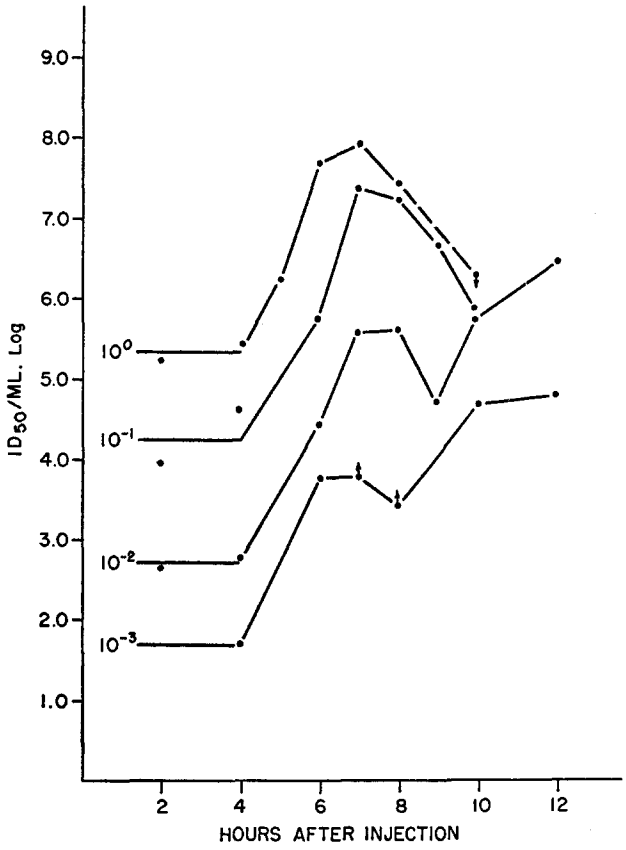

FIG. 6. The increase and subsequent decrease of virus in allantoic membranes as observed in occasional experiments.

somewhat the observations made on interference between the two viruses (1–4), particularly in view of the difference in the period required for the completion of the infectious cycle of the 2 agents noted previously (6, 7). However, the data reported above furnish a simple explanation for this discrepancy. Since the growth period of Lee virus decreases substantially when large quantities of seed are injected into the eggs it becomes evident that both viruses will have more or less an even chance, provided large enough concentrations of

both viruses are present in the seed. In agreement with this interpretation is the fact that the mixed seed never could be diluted very highly without loss of the Lee virus on subculture. In the present series the mixed passage materials could be diluted at most 100-fold and still yield both viruses as measured by hemagglutination tests. At this dilution, constant periods as short as 7 hours have been obtained for Lee virus on occasion. With higher dilutions of the Lee seed the constant periods were regularly of the order of 8 to 10 hours, substantially longer than those found in infections with PR8 virus (5 to 6 hours).

The data presented offer an intriguing problem. The mechanism of the shortened growth cycle and early liberation of Lee virus following injections of large concentrations of this agent is not understood at present and deserves further analysis.

SUMMARY

The combined passage of influenza A and B viruses in series, as reported by Sugg and Magill, has been confirmed. When the mixed passage materials were not too highly diluted both agents could be traced through 10 transfers.

Growth curve experiments revealed that both agents developed independently, as measured by hemagglutination-inhibition tests in the presence of specific immune sera against one or the other type. However, the hemagglutinin titers of the 2 viruses in the mixed series were always substantially lower than those recorded when the strains were used individually as seed in the same concentrations as were employed in the mixed series.

Assay of the infectivity titers of the individual strains in the presence of appropriate immune sera led to the demonstration that the time required for the growth cycle of influenza B virus varied with the dose of seed virus. With undiluted infected allantoic fluid as seed only 4 to 5 hours elapsed before new generations of virus were liberated. With increasing 10-fold dilution of the seed the constant period became increasingly longer until it stabilized at 8 to 10 hours. This finding offers an explanation for the seeming discrepancy between the observations on interference between the 2 viruses and the difference reported previously in the extent of their growth periods, on the one hand, and the fact that the 2 agents could be carried simultaneously in series through numerous passages in the chick embryo.

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