INTERFERENCE BETWEEN THE INFLUENZA VIRUSES

I. The Effect of Active Virus upon the Multiplication of Influenza Viruses in the Chick Embryo*

By JAMES E. ZIEGLER, Jr., M.D.,

Lieutenant, Medical Corps, United States Naval Reserve

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

Lieutenant Commander, Medical Corps, United States Naval Reserve

(*From the United States Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, January 14, 1944)

It has been observed in certain instances that the presence in an organism of one virus prevents or inhibits the multiplication of another virus in that organism. This phenomenon has been called "interference".

The initial observations on the interference phenomenon were made in virus diseases of plants. In 1929, McKinney (1) observed that plants infected by tobacco mosaic virus resisted infection with a variant strain of this virus. Thung (2), in 1931, was apparently the first to study the phenomenon in detail in plants. Numerous examples of interference between plant viruses have been reported subsequently. A review of the literature concerning the interference phenomenon in virus diseases of plants was published by Price (3).

The first observation concerning interference between animal viruses was made in 1935 by Hoskins (4) who showed that in monkeys a neurotropic strain of yellow fever virus produced interference with infection by a viscerotropic strain of the same virus. Findlay and MacCallum (5) subsequently demonstrated the interfering effect of Rift Valley fever virus upon infection by yellow fever virus in the monkey and the less marked reciprocal effect in mice. The influence of infection of the monkey with the virus of lymphocytic choriomeningitis upon subsequent infection by poliomyelitis virus was studied by Dalldorf and coworkers (6, 7), and in a later paper Dalldorf (8) presented evidence suggesting that the effect was to a certain extent reciprocal. Andrewes (9) described a local tissue interference between virus III and the fibroma virus of Shope in the rabbit. Jungeblut and Sanders (10, 11) have reported the occurrence of interference in the monkey between their murine virus and poliomyelitis virus. Andrewes (12) has recently shown that in tissue culture, interference between a neurotropic strain and pneumotropic strains of influenza A virus was demonstrable.

Delbrück and Luria (13) have extended the range of observed instances of interference in their studies of the phenomenon as it occurred between one strain of *Escherichia coli* bacteriophage and another strain active against the same bacterium.

---

*The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views and opinions which are expressed in this paper.

The present paper reports observations on the effects of the presence in the chick embryo of influenza A virus, influenza B virus, or swine influenza virus upon the multiplication of either of the other two viruses.

Methods

Viruses.—The PR8 strain (14) of influenza A virus, the Lee strain (15) of influenza B virus, and strain 1976 of swine influenza virus (16) were used in this study. For convenience, these viruses will be termed PR8 virus, Lee virus, and swine virus, respectively.

Each of the three viruses had been passed numerous times in mice, and subsequently the PR8 and Lee viruses had been through many passages in the chick embryo. The swine virus had been passed only a few times in embryos.

Pools of allantoic fluids from infected chick embryos were used as the source of virus in each experiment. Pools containing active virus were stored at −72°C. in a CO_2 cabinet (17).

Virus Titrations.—The quantity of active virus present in an infected allantoic fluid or chick embryo suspension was determined by titration in chick embryos as described by Hirst (18). The highest dilution which produced, after 48 hours' incubation of the inoculated embryos, allantoic fluids with the property of chick red blood cell agglutination, was considered to contain 1 embryo infective dose. This quantity of virus will be termed 1 E.I.D.

Chick Embryos.—Chick embryos were incubated at 38°C. for 7 to 11 days before inoculation. In each experiment all embryos were of the same age. Inoculation was carried out by the intraallantoic route as described by Hirst (19). Reinoculation was done through the paraffin seal over the site of the first injection. The volume of the inoculum was always 0.1 cc. When dilutions were used they were made in sterile broth.

Allantoic Fluids.—After inoculation with active virus, embryos were incubated usually for 48 hours, and the allantoic fluids then removed. In certain experiments the incubation period was varied; such variations are stated below. At the end of the incubation period, the eggs were usually cooled at 4°C., a method suggested by Rickard (20) for obtaining allantoic fluids free from blood. In some instances, when the only information sought was the presence or absence of RBC agglutination, the eggs were not cooled, and the allantoic fluids were removed along with a small amount of blood from the embryonic vessels.

Tests for the Presence of Virus.—Three methods were used to establish the presence of influenza virus in allantoic fluids. These methods were: (1) chicken RBC agglutination (21), (2) subinoculation of chick embryos followed after an appropriate interval by method 1, and (3) serial passage in chick embryos followed after an appropriate interval in each passage by method 1. It can readily be demonstrated that the third technique is more delicate than the second and, in fact, is probably as delicate as any test for the detection of small amounts of influenza virus. It has been shown that the first test is far less delicate than the second; the difference in sensitivity is of the order of 1 to 1000 or more (22).

It has been found that agglutination of the embryonic RBC present in allantoic

fluids removed from embryos which were not cooled gave virus titration end points comparable to those obtained when washed chicken RBC were added to fluids removed free of blood. This modification of method 1 has proved useful as a rapid method of determining the presence or absence of RBC agglutination.

Identification of Virus.—Specific antisera were used to identify the virus or viruses present in infected allantoic fluids. Hyperimmune ferret sera[1] against either PR8 virus or Lee virus were used in some experiments. In others, immune rabbit sera against either of these viruses or swine virus were used. The degree to which nonspecific inhibition of RBC agglutination by these antisera occurred in the presence of the heterologous viruses was determined. None of the immune sera, in the dilutions used in the experiments described below, was capable of inhibiting RBC agglutination by 2 or more units of either heterologous virus. The rabbit antisera against PR8, Lee, and swine viruses when tested against 8 units of the homologous virus had RBC agglutination inhibition titers of 1:1024, 1:1024, and 1:256, respectively. The ferret antisera against PR8 and Lee viruses, when tested in the same manner, both showed RBC agglutination inhibition titers of 1:6400.

Allantoic fluids removed without contaminating blood were usually tested undiluted, or diluted with an equal volume of saline solution. Aliquots were then mixed with the desired antisera previously diluted with saline solution. To this mixture was added an equal volume of a 1.5 per cent suspension of washed chicken RBC in saline solution. The presence or absence of RBC agglutination was noted after the mixtures had stood at room temperature for 1 hour. Inhibition of agglutination by a given antiserum was taken as evidence that the homologous virus was present in the allantoic fluid tested.

EXPERIMENTAL

Reciprocal Interference in the Chick Embryo between Active Influenza A and Influenza B Viruses.—Experiments were carried out to determine whether or not chick embryos infected by influenza A or influenza B virus could also be infected subsequently with the other virus.

Each of one group of embryos was inoculated as described above with 10 E.I.D. of PR8 virus. A second group of embryos was inoculated with 10 E.I.D. of Lee virus. After 24 hours' incubation the first group was reinoculated with 10 E.I.D. of Lee virus, while the second group received 10 E.I.D. of PR8 virus. At the same time, 2 groups of embryos inoculated 24 hours previously with sterile broth were reinoculated with equal quantities of either one virus or the other. After 48 hours' additional incubation the embryos were cooled at 4°C., and the allantoic fluids were removed individually. The presence of influenza virus in each allantoic fluid was determined by the RBC agglutination method. Identification of the virus or viruses present in each fluid was carried out with specific ferret antisera. For this purpose, the inhibition of RBC agglutination by the homologous antiserum was employed as described above.

[1] Obtained through the courtesy of Dr. George K. Hirst of the International Health Division of The Rockefeller Foundation.

The results of this experiment are presented in Table I. It will be seen that in all instances but one the allantoic fluids removed from embryos inoculated initially with PR8 virus and 24 hours later with Lee virus were found to agglutinate RBC in the presence of anti-Lee serum but not in the presence of anti-PR8 serum. One allantoic fluid produced agglutination when mixed with either antiserum. Similarly, with a single exception, the allantoic fluids from eggs first inoculated with Lee virus and subsequently with PR8 virus, produced RBC agglutination in the presence of anti-PR8 serum but not in the presence of anti-Lee serum. The exception was an allantoic fluid which produced RBC agglutination in the presence of either antiserum.

TABLE I

Results of RBC Agglutination Tests with Allantoic Fluids from Chick Embryos Inoculated Initially with PR8 or Lee Virus and Subsequently with the Heterologous Virus

Initial inoculum	Second inoculum 24 hrs. after first inoculation	RBC agglutination (48 hrs. after second inoculation)		
		Allantoic fluid 1:4	Allantoic fluid 1:8 + anti-PR8 ferret serum 1:128	Allantoic fluid 1:8 + anti-Lee ferret serum 1:128
*10 E.I.D.**	*10 E.I.D.*			
PR8	Lee	10/10‡	1/10§	10/10
Lee	PR8	6/6	6/6	1/6
Broth	Lee	4/4	4/4	0/4
"	PR8	5/6	0/6	5/6

* E.I.D. indicates embryo infective doses.

‡ The numerator represents the number of allantoic fluids which produced agglutination. The denominator represents the number of embryos inoculated.

§ The inhibition of RBC agglutination by a specific antiserum indicates that all the agglutination which occurs in the absence of this serum is produced by the homologous virus. In this example, 9 of the 10 allantoic fluids contained only PR8 virus in RBC agglutinating concentration.

These results indicate that, with the exceptions noted, influenza B virus did not multiply to the extent necessary to produce RBC agglutinating allantoic fluids when it was introduced into embryos previously infected with influenza A virus. Similarly, influenza A virus when introduced into embryos previously infected with influenza B virus did not increase sufficiently to cause RBC agglutination by the allantoic fluids of these embryos. This evidence indicates that infection of the chick embryo by either virus led, under the conditions of this experiment, to interference with the multiplication of the other virus in the same embryo.

Reciprocal Interference in the Chick Embryo between Active Influenza A, Influenza B, and Swine Influenza Viruses.—The preceding experiment suggested that infection of the chick embryo by influenza A or influenza B virus might interfere with the multiplication of swine influenza virus in the same embryo.

Experiments were carried out to test this possibility and the possibility that, as in the preceding experiment, the effect might be reciprocal.

Each of 3 groups of chick embryos was inoculated with approximately 10 E.I.D. of either PR8, Lee, or swine virus. After 24 hours' incubation, embryos which had received either PR8 or Lee virus were reinoculated with 10 E.I.D. of swine virus, while some of the embryos originally inoculated with swine virus were reinoculated with either 10^2 or 10^3 E.I.D. of PR8 virus, and the others were reinoculated with either

TABLE II

Results of RBC Agglutination Tests with Allantoic Fluids from Chick Embryos Initially Inoculated with PR8, Lee, or Swine Virus and Subsequently Reinoculated with One of the Heterologous Viruses

Initial inoculum	Second inoculum 24 hrs. after first inoculation	RBC agglutination (48 hrs. after second inoculation)			
		Allantoic fluid 1:2	Allantoic fluid 1:4 + anti-PR8 rabbit serum 1:32	Allantoic fluid 1:4 + anti-Lee rabbit serum 1:32	Allantoic fluid 1:4 + anti-swine rabbit serum 1:8
E.I.D.	*E.I.D.*				
PR8 10	Swine 10	4/4*	0/4	—	4/4
Lee 10	" 10	4/4	—	0/4	4/4
None	" 10	4/4	4/4	4/4	0/4
Swine <10	PR8 10^2	3/3	2/3	—	1/3
" <10	" 10^3	3/3	3/3	—	0/3
None	" 10	3/3	0/3	3/3	3/3
Swine <10	Lee 10	2/2	—	1/2	1/2
" <10	" 10^2	2/2	—	2/2	0/2
" <10	" 10^3	2/2	—	2/2	0/2
None	" 10	4/4	4/4	0/4	4/4

* The numerator represents the number of allantoic fluids which produced agglutination. The denominator denotes the number of embryos inoculated.

10, 10^2, or 10^3 E.I.D. of Lee virus. At the same time 3 groups of normal embryos were inoculated with 10 E.I.D. of either PR8, Lee, or swine virus. After 48 hours' additional incubation, the embryos were cooled at 4°C. and the allantoic fluids removed individually. The presence of RBC agglutination and the identification of the virus were determined as described previously; specific rabbit antisera against each of the 3 viruses were used in the RBC agglutination inhibition tests.

The results of this experiment are shown in Table II. Allantoic fluids removed from embryos inoculated initially with either PR8 or Lee virus and subsequently reinoculated with swine virus agglutinated RBC in the presence of anti-swine virus serum, but did not produce agglutination when mixed with antiserum homologous to the virus used in the first inoculation. Similarly, with but two exceptions, the RBC agglutination produced by fluids from em-

bryos first inoculated with swine virus and subsequently with varying amounts of PR8 or Lee virus was completely inhibited in the presence of anti-swine virus serum, but was not inhibited when these fluids were mixed with antiserum homologous to the virus used in the second inoculation. Two allantoic fluids produced RBC agglutination in the presence of anti-swine virus serum but not in the presence of antiserum homologous to the second virus inoculated.

These results indicate that when swine influenza virus was inoculated in embryos previously infected with PR8 or Lee virus, it did not multiply to the degree associated with RBC agglutination. The same order of interference was noted when eggs previously inoculated with swine virus were reinoculated with PR8 or Lee virus; neither of the subsequently introduced viruses increased to RBC agglutinating levels. In the two instances in which no agglutination attributable to swine virus was produced by allantoic fluids from embryos into which it was first introduced, the virus contained in the second inoculum was found to be present.

This evidence indicates that infection by any one of the 3 influenza viruses used, resulted, under the conditions described, in interference with the multiplication of either of the other 2 viruses.

The Effect of Small Initial Inocula upon the Multiplication of Large Secondary Inocula.—The previous experiments have dealt with the effect of small amounts of one virus upon the multiplication of equally small amounts of another virus inoculated subsequently. The extent of the effect of small initial inocula upon increasingly large inocula of another virus was investigated.

The procedures used were similar to those described above. Embryos which received an initial inoculum of 10 E.I.D. of PR8 virus were reinoculated after 24 hours' incubation with either 10, 10^2, or 10^3 E.I.D. of Lee virus. Embryos inoculated with 10^2 E.I.D. of PR8 virus were reinoculated after 24 hours with either 10^4, 10^5, or 10^6 E.I.D. of Lee virus. Embryos which received 10 E.I.D. of Lee virus were reinoculated after the same interval with either 10, 10^2, 10^3, or 10^4 E.I.D. of PR8, and other embryos initially inoculated with 10^2 E.I.D. of Lee virus received a second inoculation of either 10^3, 10^4, 10^5, or 10^6 E.I.D. of PR8 virus. At the time these embryos received the second inoculum groups of normal embryos were given either 10, 10^4, or 10^6 E.I.D. of PR8 virus while others received either 10 or 10^6 E.I.D. of Lee virus. After 48 hours' incubation, the embryos were cooled at 4°C. and the allantoic fluids removed individually. Tests for the presence of RBC agglutination and for the identification of virus were carried out as in the preceding experiments.

The results of these experiments appear in Table III. Allantoic fluids removed from embryos initially inoculated with 10 E.I.D. of PR8 virus, and subsequently with either 10, 10^2, or 10^3 E.I.D. of Lee virus, in all cases produced RBC agglutination in the presence of anti-Lee serum, and their RBC agglutinating property was completely inhibited by anti-PR8 serum. A similar result was obtained when allantoic fluids from embryos inoculated first with 10^2

E.I.D. of PR8 virus and subsequently with either 10^4, 10^5, or 10^6 E.I.D. of Lee virus were tested in the same manner.

Embryos which received an initial inoculum of 10 E.I.D. of Lee virus, and subsequently were reinoculated with either 10, 10^2, 10^3, or 10^4 E.I.D. of PR8 virus yielded, with but two exceptions, allantoic fluids which produced RBC

TABLE III

Results of RBC Agglutination Tests with Allantoic Fluids from Chick Embryos Initially Infected with Small Inocula of PR8 or Lee Virus and Subsequently Inoculated with Larger Amounts of the Heterologous Virus

Initial inoculum	Second inoculum 24 hrs. after first inoculation	RBC agglutination (48 hrs. after second inoculation)		
		Allantoic fluid 1:2	Allantoic fluid 1:8 + anti-PR8 rabbit serum 1:32	Allantoic fluid 1:8 + anti-Lee rabbit serum 1:32
E.I.D.	*E.I.D.*			
PR8 10	Lee 10	6/6*	0/6	6/6
" 10	" 10^2	6/6	0/6	6/6
" 10	" 10^3	3/3	0/3	3/3
" 10^2	" 10^4	5/5	0/5	5/5
" 10^2	" 10^5	4/4	0/4	4/4
" 10^2	" 10^6	4/4	0/4	4/4
Lee 10	PR8 10	8/8	8/8	1/8
" 10	" 10^2	8/8	8/8	0/8
" 10	" 10^3	3/3	3/3	0/3
" 10	" 10^4	3/3	3/3	1/3
" 10^2	" 10^4	5/5	5/5	0/5
" 10^2	" 10^5	5/5	5/5	2/5
" 10^2	" 10^6	4/4	4/4	3/4
None	Lee 10	4/4	—	0/4
"	" 10^6	6/6	—	0/6
"	PR8 10	4/4	0/4	—
"	" 10^4	4/4	0/4	—
"	" 10^6	5/5	0/5	—

* The numerator represents the number of allantoic fluids which produced agglutination. The denominator denotes the number of embryos inoculated.

agglutination in the presence of anti-PR8 serum, but not when mixed with anti-Lee serum. The two exceptions were fluids which gave strong RBC agglutination in the presence of anti-PR8 serum and also gave weak but definite RBC agglutination in the presence of anti-Lee serum. The group of embryos given 10^2 E.I.D. of Lee virus as an initial inoculum, and reinoculated with 10^4 E.I.D. of PR8 virus, gave fluids which produced no RBC agglutination in the presence of anti-Lee serum but produced RBC agglutination when mixed with anti-PR8

serum. Similar results were obtained with only 3 of 5 allantoic fluids from embryos which had received an identical initial inoculum and a subsequent inoculation of 10^5 E.I.D. of PR8 virus. The remaining 2 allantoic fluids produced RBC agglutination in the presence of either antiserum. Agglutination in the presence of either antiserum was also produced by 3 of 4 allantoic fluids from embryos which received 10^2 E.I.D. of Lee virus as the initial and 10^6 E.I.D. of PR8 virus as the second inoculum. One embryo in this group yielded allantoic fluid which gave RBC agglutination when mixed with anti-PR8 serum, but not when mixed with anti-Lee serum.

These results indicate that infection of the embryo with small inocula of PR8 virus caused interference with the multiplication of Lee virus irrespective of the size of the inoculum of the latter virus. On the other hand, infection of the embryo with small inocula of Lee virus consistently resulted in interference with the multiplication of PR8 virus only when the size of the inoculum of the latter virus did not exceed that of the former by more than 100 times. When larger PR8 virus inocula were used, increasing numbers of allantoic fluids were found to contain both viruses. This evidence suggests that the resistance to infection with Lee virus which was induced by infection by PR8 virus was more complete than was the case when the order of inoculation was reversed.

The Effect of Time Interval between Inoculations upon the Interference Phenomenon.—With the demonstration of reciprocal interference between these strains of influenza A, influenza B, and swine influenza viruses, it seemed clear that the dominant infective rôle was assumed by that virus which first reached the embryo. Experiments were planned to determine how soon after an initial inoculation of active virus this dominance was established.

Two groups of embryos were inoculated; each embryo of one group received 10 E.I.D. of PR8 virus, and each embryo of the other group was given 10 E.I.D. of Lee virus. One-fourth of the group which received PR8 virus was also inoculated simultaneously with 10 E.I.D. of Lee virus, and equal numbers of the remainder of the group were reinoculated with 10 or 10^2 E.I.D. of Lee virus either 4, 8, or 12 hours after the original inoculation. The group of embryos originially inoculated with Lee virus was reinoculated with similar amounts of PR8 virus at identical time intervals. Each group of embryos was incubated for 48 hours following reinoculation. The embryos were then cooled, after which the allantoic fluids were removed and tested as in previous experiments.

The results are presented graphically in Fig. 1. When 10 E.I.D. of PR8 virus and 10 E.I.D. of Lee virus were inoculated simultaneously into embryos, the allantoic fluids from these embryos produced RBC agglutination in the presence of either anti-PR8 serum or anti-Lee serum. When 4 hours elapsed between the original inoculation with PR8 virus and reinoculation with 10^2 E.I.D. of Lee virus, 4 of 6 embryos yielded allantoic fluids which produced

RBC agglutination in both antisera. The other 2 allantoic fluids produced RBC agglutination which was completely inhibited, in one case by anti-PR8 serum, and in the other by anti-Lee serum. When reinoculation with Lee virus was done 8 hours after the original inoculation with PR8 virus, all 5 allantoic fluids showed RBC agglutination; in 4 this property was completely inhibited by anti-PR8 serum but not by anti-Lee serum, while in 1 it occurred in the presence of either antiserum. When an interval of 12 hours elapsed between inoculations, all of the embryos yielded allantoic fluids which produced RBC agglutination that was inhibited completely by anti-PR8 serum but not by anti-Lee serum.

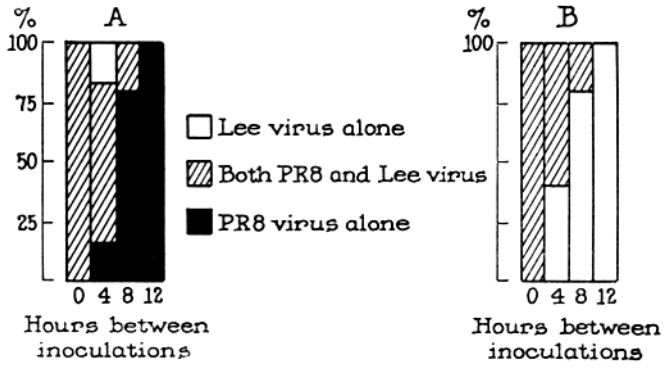

FIG. 1. Results of RBC agglutination inhibition tests with specific rabbit antisera and allantoic fluids from embryos inoculated with both PR8 and Lee viruses. A, initial inoculum PR8 virus 10 E.I.D.; second inoculum, Lee virus 10 or 10^2 E.I.D. B, initial inoculum, Lee virus 10 E.I.D.; second inoculum, PR8 virus 10 or 10^2 E.I.D.

Similar results were obtained with embryos which received 10 E.I.D. of Lee virus followed at various intervals by PR8 virus. When the Lee virus was followed 4 hours later by PR8 virus, 3 of 5 of the fluids gave RBC agglutination in both antisera, and the agglutination produced by the other 2 fluids was completely inhibited in the presence of anti-Lee serum. When 8 hours elapsed between the initial Lee virus inoculation and subsequent reinoculation with PR8 virus, only 1 of 5 of the allantoic fluids produced agglutination in the presence of either antiserum. The 4 other fluids produced agglutination which was completely inhibited by anti-Lee serum but not by anti-PR8 serum, and this was also true of all the allantoic fluids from the embryos in which the interval between the initial Lee inoculation and the subsequent PR8 inoculation was 12 hours.

These results indicate that during the first 4 hours after inoculation the first virus was unable or able only occasionally to prevent the multiplication to RBC agglutinating levels of the second virus. However, an interval of 12 hours

between inoculations was sufficient to produce inhibition of multiplication of the second virus in all instances. These results also suggest that there was a relationship between the extent of multiplication of the first virus and the degree of interference which resulted from its presence in the embryo.

In order to discover whether any significant relationship existed between the development of interference and the curve of virus multiplication, the virus titer of allantoic fluid pools and embryo suspensions from embryos inoculated with either PR8 or Lee virus alone was determined after various periods of incubation.

Two groups of embryos were inoculated; each embryo of one group received 10^2 E.I.D. of PR8 virus, and each embryo of the other group was given 10^2 E.I.D. of Lee virus. The embryos were then incubated, and 4 from each group were removed from the incubator 4, 8, 12, 16, and 24 hours, respectively, after inoculation. After cooling, the allantoic fluid and embryo were removed separately from each egg. The allantoic fluids from each group of 4 eggs were pooled, as were the embryos. The latter were then ground in sterile broth. Serial tenfold dilutions of the allantoic pools were prepared, and 2 embryos were inoculated with each dilution. The embryo suspensions were tested in an identical manner. The embryo-infectivity end points were determined as described above. The experiment with Lee virus was repeated because of the unexpected results obtained; the results of the second experiment were essentially similar to those of the first.

The results are shown graphically in Fig. 2. Following inoculation with PR8 virus the multiplication of virus followed a rapidly rising curve, and the virus titer of both the embryos and the allantoic fluids increased at similar rates. The multiplication of Lee virus appeared to follow a significantly different curve. The virus titer of the allantoic fluids increased in a manner similar to that observed in the case of PR8 virus although the increase in Lee virus was much less rapid. Moreover, in the case of the embryos no virus was demonstrable in 50 per cent suspensions up to and including 16 hours after inoculation. At 24 hours, however, the virus titer of the embryo pool was as high as the titer of the allantoic fluid pool.

It was shown in the previous experiments that interference was demonstrable with either virus in all instances when the interval between the 2 inoculations was 12 hours and that when shorter intervals were used the phenomenon occurred with less frequency. The results of the present experiment suggest that the development of interference may not be a simple function of the concentration of virus either in the allantoic fluid or in the infected embryo, since marked differences in the curves of virus multiplication were found with these 2 agents.

It has already been shown that the interference produced by the presence of PR8 virus in the embryo was more effective against subsequent large inocula of the Lee virus than was the reciprocal interference by Lee virus with large

inocula of PR8 virus. It will be shown in the accompanying paper (23) that PR8 virus was inhibited under various conditions with somewhat less ease than was Lee virus. These results suggest that the behavior of PR8 virus in this respect may be a reflection of its more rapid rate of multiplication in the chick embryo.

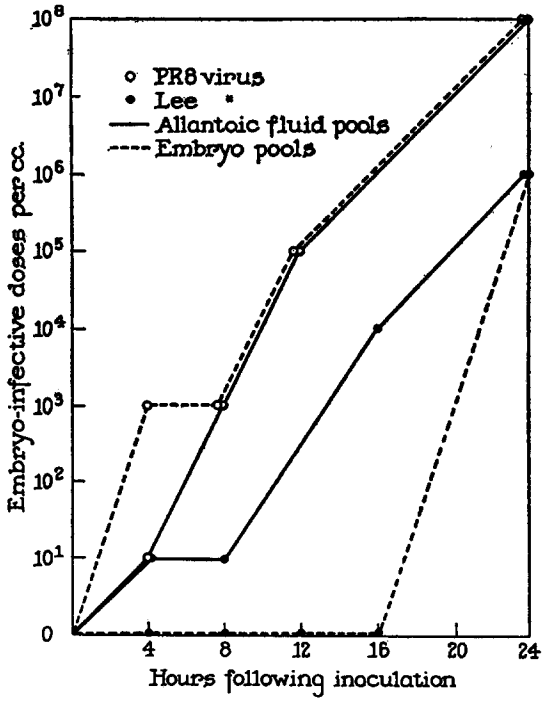

FIG. 2. Embryo infectivity titers of allantoic fluid pools and embryo pools at various intervals after infection with either PR8 or Lee virus.

Attempts to Demonstrate the Second Virus Inoculated in Embryos Previously Infected with Influenza Virus.—The RBC agglutination method as an indicator of influenza virus multiplication has the disadvantage that results are positive only when the virus is present in relatively high titer. Failure to demonstrate RBC agglutination after inoculation with influenza virus may indicate merely that insufficient virus was produced to cause agglutination, and cannot be taken as evidence that no multiplication whatsoever occurred. It seemed possible that in the system under study an embryo previously infected by one influenza virus might still support some multiplication by a different influenza virus even though it did so to a degree significantly less than would a normal embryo. Since it could not be expected that the direct application of the RBC agglutina-

tion method would provide information on this point, experiments were designed to test the possibility in other ways.

TABLE IV

Results of RBC Agglutination Tests with Allantoic Fluids from Chick Embryos Subinoculated with Fluids from Embryos Inoculated with Both PR8 and Lee Virus

Original embryos						Subinoculated embryos			
			RBC agglutination (48 hrs. after second inoculation)				RBC agglutination after 48 hrs.' incubation		
Embryo No.	Initial inoculum	Second inoculum 24 hrs. after first inoculation	Allantoic fluid 1:2	Allantoic fluid 1:4 + anti-PR8 serum 1:32	Allantoic fluid 1:4 + anti-Lee serum 1:32	Inoculum: Allantoic fluids from original embryos — Embryo No.	Allantoic fluid 1:2	Allantoic fluid 1:4 + anti-PR8 serum 1:32	Allantoic fluid 1:4 + anti-Lee serum 1:32
	E.I.D.	*E.I.D.*							
1	PR8 10	Lee 10^2	++++	0	++++	1 } Allantoic fluid 1:4	++++	0	++++
2	" 10	" 10^2	++++	0	++++	2 }	++++	0	++++
3	" 10	" 10^2	++++	0	++++	3 }	++++	0	++++
						1 } Allantoic fluid 1:8 + anti-PR8 rabbit serum 1:16	0	−	−
						2 }	0	−	−
						3 }	0	−	−
4	PR8 10	Lee 10	++++	0	++++	4 } Allantoic fluid 2:3 + anti-PR8 ferret serum 1:3	±*	±	0
5	" 10	" 10	++++	0	++++	5 }	+*	+	0
6	" 10	" 10^2	++++	0	++++	6 }	0	−	−
7	" 10	" 10^3	++++	0	++++	7 }	0	−	−
8	Lee 10	PR8 10^2	++++	++++	0	8 } Allantoic fluid 1:4	++++	++++	0
9	" 10	" 10^2	++++	++++	0	9 }	++++	++++	0
						8 } Allantoic fluid 1:8 + anti-Lee rabbit serum 1:16	0	−	−
						9 }	++++	+++	0
10	Lee 10	PR8 10	++++	++++	0	10 } Allantoic fluid 2:3 + anti-Lee ferret serum 1:3	+*	±	0
11	" 10	" 10^2	++++	++++	0	11 }	±*	±	±
12	" 10	" 10^2	++++	++++	0	12 }	0	−	−
13	" 10	" 10^3	++++	++++	0	13 }	+	+	0

* These allantoic fluids tested after 72 hours' incubation.

Two groups of allantoic fluids from infected embryos were tested by subinoculation of normal embryos. One group of allantoic fluids obtained from embryos inoculated initially with 10 E.I.D. of PR8 virus and subsequently with 10^2 E.I.D. of Lee virus showed only the presence of PR8 virus when tested by the RBC agglutination inhibition method. The other group of allantoic fluids, obtained from embryos inoculated initially with 10 E.I.D. of Lee virus and subsequently with 10^2 E.I.D. of PR8 virus, showed only the presence of Lee virus when tested in a similar manner. The individual allantoic fluids from both groups were diluted 1:4 in saline solution, and 0.1

cc. of each was then inoculated in normal embryos. Additional normal embryos were inoculated with the same allantoic fluids after specific rabbit antiserum had been added. Anti-PR8 serum was used with the first, and anti-Lee serum with the second group of allantoic fluids. After 48 hours' incubation of the subinoculated embryos the presence of influenza virus and the identity of the virus were determined as described above.

In another experiment allantoic fluids were obtained from embryos inoculated initially with 10 E.I.D. of either PR8 virus or Lee virus and subsequently with from 10 to 10^3 E.I.D. of the heterologous virus, respectively. The individual allantoic fluids from embryos inoculated initially with PR8 virus were mixed with anti-PR8 ferret serum, while the allantoic fluids from embryos inoculated initially with Lee

TABLE V

Results of RBC Agglutination Tests with Allantoic Fluids from Chick Embryos Inoculated with Small Amounts of PR8 Virus and Reinoculated at Varying Intervals with Very Large Amounts of Lee Virus

Initial inoculum	Interval between initial inoculation and reinoculation with Lee 10^6 E.I D.	RBC agglutination (48 hrs. after first inoculation)		
		Allantoic fluid 1:2	Allantoic fluid 1:4 + anti-PR8 rabbit serum 1:32	Allantoic fluid 1:4 + anti-Lee rabbit serum 1:32
10^2 E.I.D.	*hrs.*			
PR8	0	6/6*	6/6	0/6
"	4	6/6	6/6	0/6
"	8	6/6	6/6	0/6
"	Not reinoculated	6/6	0/6	6/6

* The numerator represents the number of allantoic fluids which produced agglutination. The denominator represents the number of embryos inoculated.

virus were mixed with anti-Lee ferret serum. The mixtures were then inoculated in normal embryos which were subsequently treated as described above.

The results of these tests are shown in Table IV. It will be observed that when subinoculation of normal embryos was carried out, unequivocal evidence of the presence of the second virus in the original allantoic fluids was not obtained. In those instances in which the subinoculated embryos were incubated for 72 rather than for 48 hours, slight RBC agglutination of doubtful significance was noted. If this was in fact caused by the presence of the second virus, it seems apparent that in the original allantoic fluid it could have been present only in a concentration approaching the limits of detectability.

The Effect upon the Interference Phenomenon of Variations in the Time and the Quantity of the Second Inoculum.—The effect of a very large quantity of Lee virus inoculated simultaneously with, or at various intervals after, infection of the embryo with a small quantity of PR8 virus was studied.

A number of groups of embryos were each inoculated with 10^2 E.I.D. of PR8 virus. Group 1 was immediately reinoculated with 10^6 E.I.D. of Lee virus. Group 2 was

reinoculated with the same quantity of Lee virus 4 hours after the initial inoculation, and group 3 was treated similarly 8 hours after the initial inoculation. Group 4 was not reinoculated and served as controls for the initial RP8 virus inoculation. The embryos were incubated for 48 hours from the initial inoculation, and the allantoic fluids were tested in the usual manner.

The results of this experiment are shown in Table V. It will be seen that when very large amounts of Lee virus were introduced simultaneously with, or as long as 8 hours after, infection of the embryo with a small quantity of PR8 virus, evidence of Lee virus alone was obtained by the RBC agglutination inhibition tests of the allantoic fluids obtained 48 hours after the initial inoculation.

These results indicate that the usual direction of interference between the influenza viruses can be reversed, provided that certain limits of time and quantity are exceeded.

DISCUSSION

The phenomenon of interference between virus infections has interested many investigators. Numerous examples of interference between plant viruses have been encountered, and in many instances these have been carefully studied. Only a few instances of interference between animal viruses have been described, and in most of these the nature of the host-virus system has made difficult a detailed study of the phenomenon. Despite the numerous investigations which have been made, many questions relating to the mechanisms responsible for interference remain unanswered.

Infection of the chick embryo by the influenza viruses appears to offer an experimental system for a study of interference, which possesses many advantages. Among these are the rapidity with which inoculated influenza virus ordinarily multiplies, the regularity with which even small virus inocula reach full titer in the normal embryo, and the further fact that in the great majority of instances the embryos contain neither extraneous viruses nor contaminating microorganisms. In addition, the extra-embryonic fluids from infected eggs contain large quantities of influenza virus relatively free of the foreign materials present in tissue suspensions. Simple and rapid *in vitro* methods are available for the serological identification of the viruses in these fluids, and combined *in vivo* and *in vitro* techniques for the detection of small quantities of virus can be applied to them.

The finding that reciprocal interference occurs between the strains of influenza A, influenza B, and swine influenza viruses used in this study is of considerable interest, since it constitutes additional evidence that the interference phenomenon can, under certain conditions, be demonstrated between antigenically distinct viruses. It is well established that influenza B virus shares

no demonstrable common antigen either with influenza A or swine influenza virus, yet interference between any 2 of these 3 agents has been shown to occur.

Andrewes (12) studied the interference phenomenon in chick embryo tissue culture and demonstrated its occurrence between different strains of influenza A virus. Recently Henle and Henle (24) published a preliminary report of experiments with influenza viruses in chick embryos which supports the available evidence that interference is demonstrable with these agents.

The fact that interference can occur between agents which, so far as is known, are antigenically unrelated, notably between the viruses of Rift Valley fever and yellow fever, and those of lymphocytic choriomeningitis and poliomyelitis, has led most investigators to think that the phenomenon is not a manifestation of specific immunity. All attempts to demonstrate antiviral substances as the mediators of interference have yielded negative results. Further evidence against the supposition that interference may be a manifestation of immunity is afforded by the rapidity with which the conditions necessary for interference may be produced. In the present study, the infection of embryos with very small quantities of one virus led in from 8 to 12 hours to a state which caused interference with the multiplication of another virus. The same state could be produced, as well, on the introduction of a very large amount of one virus simultaneously with a small amount of another. In the system under study the total observation period was rarely more than 72 hours, so it seems very unlikely that the interference demonstrated was the result of any of the common immunological mechanisms.

Under the conditions of the experiments described, any one of the three virus strains employed when first introduced in the embryo regularly interfered with the multiplication of either of the other two viruses inoculated somewhat later, provided certain limits of time and quantity were not exceeded. This finding suggests that the basic mechanisms responsible for the production of interference were in each instance similar.

The available evidence does not indicate that interference resulted from the interaction of one virus with another, nor did the presence in the chick embryo of multiplying virus *per se* interfere with the multiplication of another virus. It seems reasonable to think that the state responsible for interference was induced by an alteration in the susceptible tissues of the chick embryo which followed the establishment of the initial virus infection. This alteration may be effected in a few hours and results in almost complete insusceptibility to infection by another virus which ordinarily multiplies in the tissues involved. The insusceptibility induced is, however, not an absolute effect. It varies somewhat in degree and may to a certain extent be overcome when large secondary virus inocula are employed. For example, when an embryo infected by Lee virus is reinoculated after 24 hours with a very large quantity of PR8 virus, inhibition of PR8 virus, if it occurs, is slight. Equally large amounts of Lee

virus inoculated after 24 hours appear, however, to be inhibited in an embryo previously inoculated with PR8 virus. This might be interpreted as indicating multiple infection of single cells, infection of different cells by the two viruses, or the result of the different rates of multiplication of these agents. The fact that the effect does not appear to be reciprocal under these conditions lends weight to this last hypothesis.

The reversal of the direction of interference, in which the multiplication of the initially inoculated virus is inhibited by the simultaneous or later addition of very large amounts of another virus, would seem to offer evidence of the quantitative nature of interference. The occurrence of this reversal indicates that the effect produced by a small amount of virus in from 8 to 12 hours, may be attained very rapidly with a large amount of virus. It also suggests that between two viruses involved in interference there may be competition for a substance which exists in the tissues of the embryo in a certain fixed amount. According to this hypothesis, when interference is produced by small amounts of virus, the initially inoculated virus would make unavailable through multiplication more or less of this substance. If the virus were in the more rapid phase of multiplication, it would have a decided advantage over small amounts of a secondarily inoculated virus in competing for the remainder of the hypothetical substance for which both viruses appear to have a common affinity. If, on the other hand, a very large quantity of another virus were introduced into an embryo already infected, the advantage might be shifted in the direction of the second virus.

The observation of Hirst (19) that chicken RBC to which influenza virus had been adsorbed and subsequently eluted were no longer agglutinable by the same or a different influenza virus bears a certain analogy to the interference phenomenon. In this instance, the erythrocytes appear to be sufficiently altered by temporary contact with one virus to cause them to be incapable subsequently of adsorbing another virus. It seems possible that there may be more than a superficial similarity between this alteration in cell surface reactivity and the change in tissue susceptibility which results in interference.

CONCLUSIONS

Reciprocal interference between influenza A, influenza B, and swine influenza viruses has been demonstrated in the chick embryo. Certain temporal and quantitative factors which influence the production of interference in this host-virus system have been studied. The implications of these observations in relation to the mechanism by which interference is produced are discussed.

BIBLIOGRAPHY

1. McKinney, H. H., *J. Agric. Research*, 1929, **39**, 557.
2. Thung, T. H., *Handel. Nederl. Ind. Natuurwetenschap., Congr. 6de*, 1931, 450, cited by Price (3).

3. Price , W. C., *Quart. Rev. Biol.*, 1940, **15,** 338.
4. Hoskins, M., *Am. J. Trop. Med.*, 1935, **15,** 675.
5. Findlay, G. M., and MacCallum, F. O., *J. Path. and Bact.*, 1937, **44,** 405.
6. Dalldorf, G., and Douglass, M., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39,** 294.
7. Dalldorf, G., Douglass, M., and Robinson, H. E., *J. Exp. Med.*, 1938, **67,** 333.
8. Dalldorf, G., *J. Immunol.*, 1939, **37,** 245.
9. Andrewes, C. H., *J. Path. and Bact.*, 1940, **50,** 227.
10. Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72,** 407.
11. Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1942, **76,** 127.
12. Andrewes, C. H., *Brit. J. Exp. Path.*, 1942, **23,** 214.
13. Delbrück, M., and Luria, S. E., *Arch. Biochem.*, 1942, **1,** 111.
14. Francis, T., Jr., *Science*, 1934, **80,** 457.
15. Francis, T., Jr., *Science*, 1940, **92,** 405.
16. Shope, R. E., *J. Exp. Med.*, 1935, **62,** 561.
17. Horsfall, F. L., Jr., *J. Bact.*, 1940, **40,** 559.
18. Hirst, G. K., *J. Immunol.*, 1942, **45,** 285.
19. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.
20. Rickard, E. R., personal communication.
21. Hirst, G. K., *Science*, 1941, **94,** 22.
22. Hirst, G. K., *J. Exp. Med.*, 1942, **75,** 49.
23. Ziegler, J. E., Jr., Lavin, G. I., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **79,** 379.
24. Henle, W., and Henle, G., *Science*, 1943, **98,** 87.