REACTIONS BETWEEN INFLUENZA VIRUS AND A COMPONENT OF ALLANTOIC FLUID

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It is well known that there are present in certain tissues of various normal animal species components which are capable of inhibiting hemagglutination by influenza viruses (1–10). Relatively little attention has been given to a component of this kind which is present in the allantoic fluid of normal chick embryos. Svedmyr (7) previously reported upon such a component and studied some of its properties. In view of the fact that, at the present time, most work on influenza viruses is carried out with material obtained from the allantoic sac of the chick embryo, it seemed important to study in detail this inhibitor and the reactions between it and influenza viruses. In the present communication evidence will be presented regarding its nature. Evidence also will be presented to show that it combines with the virus particle, and that following combination the complex dissociates partially, but not completely. In addition, it will be shown that after dissociation the inhibitor is altered, *i.e.* it is no longer capable of combining with additional influenza virus, and therefore cannot be demonstrated directly by the available techniques.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was used. The virus, which previously had been passed many times through chick embryos and mice, was cultivated in the allantoic sac of White Leghorn chick embryos which had been incubated at 39°C. for 10 to 12 days. After inoculation with approximately 10^5 E.I.D. of virus the eggs were incubated at 35°C. for 48 hours, and then chilled at 4°C. overnight before the allantoic fluids were harvested. The infected allantoic fluids employed in hemagglutination experiments were sterile pools obtained from groups of 8 to 10 eggs and were stored at 4°C. Infected fluids which were used for the inoculation of additional eggs were diluted with nine parts of sterile normal horse serum (previously heated at 56°C. for 30 minutes) and stored in nitrocellulose tubes at −70°C.

Normal Allantoic Fluid.—For the most part the normal allantoic fluid used was removed from chick embryos which had been incubated at 39°C. for 14 days. Following incubation the eggs were chilled at 4°C. overnight and the allantoic fluid from groups of 8 to 10 eggs was pooled. Each pool of normal fluid was stored at 4°C. until used. Frequently, a precipitate formed in the allantoic fluid upon storage at 4°C.; this was removed by centrifugation and discarded.

Virus Hemagglutination Titrations.—Serial twofold dilutions of infected allantoic fluid were made in 0.85 per cent NaCl solution buffered at pH 7.2 (0.01 M phosphate). To 0.4 cc. of each dilution in 10 × 75 mm. Pyrex tubes was added 0.4 cc. of a 0.5 per cent suspension of washed chicken RBC in buffered saline. Readings were made after the tubes had stood 1 hour at room temperature. The end point was taken as the highest dilution at which definite (2+) agglutination of the RBC occurred.

EXPERIMENTAL

Increase in the Inhibitor in Extraembryonic Fluids with Age.—Before undertaking a detailed study of the component in normal allantoic or amniotic fluid which inhibits hemagglutination by influenza virus, it was first necessary to determine the age of the embryo at which the fluids contained most inhibitor.

Groups of 10 to 20 normal embryonated eggs were incubated at 39°C. for periods from 7 to 14 days. After incubation the allantoic and amniotic fluids from each group of eggs were pooled separately and each pool was tested for its ability to inhibit hemagglutination with the PR8 strain. Two methods were used: With Method I serial twofold dilutions of a single virus suspension were made in each allantoic or amniotic fluid pool, and the hemagglutination titers obtained were compared with the titer of the virus diluted in buffered saline. With Method II serial twofold dilutions of each pool of allantoic or amniotic fluid were made in buffered saline; to each dilution was added a suspension of heated virus (56°C. for 1 hour) diluted so as to give a final concentration of 4 hemagglutinating units. The titer of the inhibitor was taken as the highest dilution of fluid which prevented definite (1+) hemagglutination.

The results of the experiments with allantoic fluid are presented in Fig. 1 *A*. The curve obtained by Method I is plotted as the difference between the logarithms of the hemagglutination titers obtained when the virus was diluted in normal allantoic fluid as compared to buffered saline. The curve obtained by Method II is plotted as the logarithm of the highest dilution of normal allantoic fluid which prevented hemagglutination by 4 units of virus. It will be seen that both methods gave similar results, although the curve obtained by Method II has a slightly greater slope. The results demonstrate that the concentration of inhibitor in normal allantoic fluid increases progressively with increasing age of the embryo. In general these results are in agreement with the findings of Svedmyr (7) who employed a technique analogous to Method I. However, the present results do not indicate that the concentration of inhibitor is relatively constant after the 11th or 12th day, as was previously suggested (7).

The results of experiments with amniotic fluid are shown in Fig. 1 *B* in which the plotting procedure is identical with that employed in Fig. 1 *A*. Here again, the slope obtained by Method II is greater than that obtained by Method I. It will be noted that there is almost no demonstrable inhibitor in amniotic fluids from normal eggs less than 10 days of age, but that thereafter the concentration of inhibitor increases rapidly with increasing age of the embryo until a peak is reached at 13 days. The inhibitor concentration as determined by Method II at this time is greater than that of allantoic fluid from 14 day embryos. These findings are similar to those of Svedmyr (7) insofar as they may be compared.

Determination of Inhibitor Concentration.—The methods employed to determine the concentration of inhibitor in normal allantoic fluid were of two main types: (*a*) inhibition of decreasing quantities of virus by a constant quantity

of inhibitor, and (*b*) inhibition of a constant quantity of virus by decreasing quantities of inhibitor. They are presented in detail below.

Decreasing Virus versus Constant Inhibitor.—In the early experiments an infected allantoic fluid pool was diluted serially in normal allantoic fluid and the hemagglutination titer obtained in the usual manner was compared with the titer obtained after dilution of the same pool in

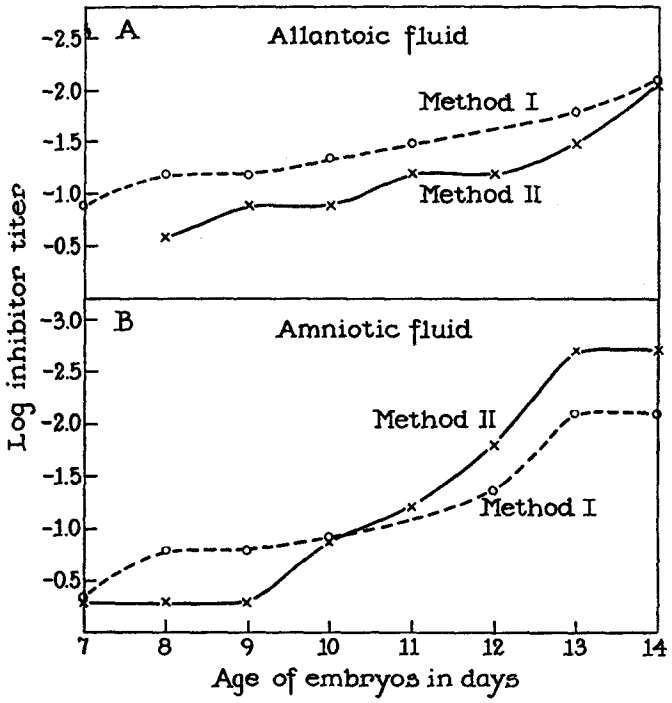

FIG. 1. Increase in inhibitor concentration in extraembryonic fluids with age. Graph A represents results obtained with normal allantoic fluid. Graph B shows the results obtained with normal amniotic fluid. The concentration of inhibitor was determined by two methods: In Method I the virus was diluted in allantoic or amniotic fluid and the log of the difference in titer from a control titration in saline is shown; in Method II dilutions of the fluids were tested against 4 units of heated virus and the log of the highest dilution which inhibited hemagglutination is shown.

buffered saline. In this procedure it is evident that the concentration of normal allantoic fluid increases slightly but progressively with each dilution of the infected pool. In order to control this variable the following procedure was employed.

Serial twofold dilutions of an infected pool were made in buffered saline. To 0.2 cc. of each dilution were added 0.2 cc. of normal allantoic fluid from 14 day old embryos and 0.4 cc. of a 0.5 per cent suspension of washed chicken RBC. The hemagglutination titer obtained was compared with that obtained in a similar titration in which buffered saline was substituted for normal allantoic fluid. The number of hemagglutinating units of virus inhibited was taken as the ratio of the titer in saline to the titer in the presence of normal allantoic fluid.

In Table I the results obtained in a number of experiments carried out by this method are summarized. It will be seen that between 1 and 24 hemagglutinating units of virus were inhibited by normal allantoic fluid when different pools of virus were employed. However, in each instance the quantity of virus inhibited was directly related to the titer of the virus pool used. That is, in the presence of a constant concentration of normal allantoic fluid an almost constant hemagglutination titer is obtained regardless of the level of the original virus titer. Therefore, in order to determine differences in the concentration of inhibitor, it is important to use a virus pool with the highest possible titer.

TABLE I

Relation between Hemagglutination Titer and Quantity of Virus Inhibited by a Constant Amount of Normal Allantoic Fluid

Infected* allantoic fluid pool	Hemagglutination titer‡		Virus inhibited
	In buffered saline	In normal allantoic fluid§	No. of hemagglutinating units
A	256	128	1
B	512	128	2
C	1024	128	4
D	2048	128	8
E	2048	64	16
F	6144	128	24

* Allantoic fluids harvested 48 hours after inoculation of PR8.

‡ Expressed as the reciprocal.

§ Obtained from 14 day embryos.

Constant Virus versus Decreasing Inhibitor.—In this procedure serial twofold dilutions of normal allantoic fluid from 14 day embryos were made in buffered saline. To 0.4 cc. of each dilution were added 0.2 cc. of virus, diluted so as to give a final concentration of 4 hemagglutinating units, and 0.2 cc. of a 1 per cent suspension of washed chicken RBC. The titer of the inhibitor was taken as the highest dilution of normal allantoic fluid which prevented definite (1+) hemagglutination.

The results of a typical experiment are shown in Table II. It is seen that normal allantoic fluid inhibited hemagglutination only when it was diluted eightfold or less. Of numerous fluids tested none could be diluted more than 1:8 and still cause inhibition under these conditions.

Constant Heated Virus versus Decreasing Inhibitor.—It was shown by Francis (5) that influenza B virus, when heated at 56°C., lost very little of its hemagglutinating capacity, but gave much higher hemagglutination inhibition titers with normal sera than did equivalent amounts of unheated virus. It seemed possible that similar results might be obtained with normal allantoic fluid, and

consequently titrations were carried out in a manner identical with that described above except that 4 hemagglutinating units of heated virus (56°C. for 1 hour) was used.

The results of a typical experiment with heated virus are also shown in Table II. It will be seen that 4 units of heated virus was inhibited by a much higher dilution of normal allantoic fluid than was an equal amount of unheated virus. In Table III the results of a number of experiments carried out with different

TABLE II

Inhibition Titer of Normal Allantoic Fluid with Infectious and Heated Virus

Infected allantoic fluid		Dilution of normal allantoic fluid*										Inhibition titer
Treatment	Hemagglutinating units per tube	2	4	8	16	32	64	128	256	512	1024	
None	4	0	0	0	1	2	3	4	4	4	4	8
Heated‡	4	0	0	0	0	0	0	0	0	2	4	256

* Expressed as the reciprocal.

‡ 56°C. for 1 hour before dilution.

TABLE III

Relation between Hemagglutination Titer of Virus Pool and Inhibition Titer Obtained with Normal Allantoic Fluid

Infected allantoic fluid pool	Hemagglutination titer	Inhibition titer of normal allantoic fluid*
1	1024	32
2	2048	64
3	2048	128
4	4096	128
5	8192	128
6	8192	256

* Determined with 4 units of virus after heating.

pools of heated virus are summarized. In each instance the titer of inhibitor represents the highest dilution of normal allantoic fluid which inhibited 4 units of heated virus. Because the various virus pools had different titers, the dilution employed varied from one pool to another. It will be seen that 4 units of heated virus, when derived from a high titer pool, was inhibited by a considerably higher dilution of normal allantoic fluid than an equal number of units of heated virus derived from a pool of lower titer. It appears, therefore, that the titer of inhibitor in normal allantoic fluid is influenced by the hemagglutination titer of the virus suspension employed to determine it.

Centrifugation of Inhibitor.—The behavior of the inhibitor in normal allantoic

fluid towards influenza virus suggested that it might be a single component and of moderately large size. This impression was strengthened when it was found not to dialyze through cellophane membranes. To obtain information as to the size of the inhibitor relative to that of influenza virus, the effect of high gravitational fields on the inhibitor and on influenza virus was compared. Svedmyr (7) found that the inhibitor was sedimented at 27,000 R.P.M.

Nitrocellulose tubes containing 20 cc. of either normal allantoic fluid or allantoic fluid from embryos infected with PR8 were centrifuged at 15,000 R.P.M. for varying periods in a high speed vacuum apparatus (11). Both normal and infected fluids were centrifuged simultaneously. The centrifuge head was similar to that previously described (12) but was almost twice as large; the diameter = 30.1 cm. After centrifugation the top 10 cc. of each supernate was removed carefully, the remainder of the supernate was then withdrawn, and the sediment was resuspended in a quantity of buffered saline equal to the original volume. The inhibitor titer of the normal fluid fractions was determined as described above against 4 units of heated virus and the hemagglutination titer of the infected fluid fractions was determined in the usual manner.

The results are presented in Fig. 2 in which the upper portion (*A*) shows the titers obtained with the top 10 cc. of the supernates and the lower portion (*B*) shows those obtained with the resuspended sediments. Both the inhibitor and hemagglutination titers are plotted as logarithms. It will be noted that the titer of the inhibitor in the supernates decreased at a much slower rate than did that of the virus and, in addition, that with the resuspended sediments the virus titer increased at a faster rate than did the inhibitor titer. These results indicate that the inhibitor in normal allantoic fluid is significantly smaller than the virus particle (PR8) but show that some sedimentation of the inhibitor is obtained even at a speed of 15,000 R.P.M.

Heat Stability of the Inhibitor.—The effect of heat upon the inhibitor was determined in the following manner:

Specimens of normal allantoic fluid which had been dialyzed overnight against buffered saline (pH 7.2) were heated in a water bath at temperatures of 70 or 100°C. for varying periods. After this treatment the inhibitor concentration of each specimen was determined against 4 units of heated virus as described above. The allantoic fluid, which was almost water-clear before heating, developed only a very slight turbidity even when heated at 100°C. for 1 hour.

The results obtained are shown in Table IV. A twofold decrease in the inhibitor titer occurred after 45 minutes at 70°C. but no further drop in titer was found after 60 minutes. On heating at 100°C. the inhibitor titer decreased somewhat more rapidly but approximately 25 per cent of the inhibitor remained demonstrable even after 1 hour. It is evident that the inhibitor is very stable to heat. Svedmyr (7) showed previously that the inhibitor was not completely destroyed by similar heating.

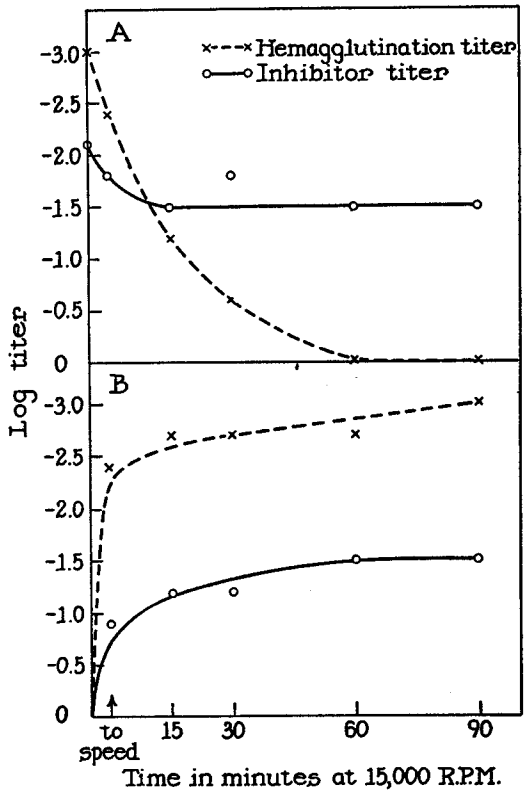

FIG. 2. Effect of high speed centrifugation on the inhibitor as compared with the virus. Aliquots of normal allantoic fluid and infected allantoic fluid were centrifuged simultaneously. Both the hemagglutination titer with infected fluid and the inhibitor titer with normal fluid of the top 10 cc. of the supernates, as shown in Graph A, and of the resuspended sediments, as shown in Graph B, are plotted against the time of centrifugation.

TABLE IV

Effect of Heating on Inhibitor

Treatment of normal allantoic fluid		Inhibition titer*	Treatment of normal allantoic fluid		Inhibition titer*
°C.	*min.*		°C.	*min.*	
—	—	128	—	—	128
70	15	128	100	15	128
70	30	128	100	30	64
70	45	64	100	45	64
70	60	64	100	60	32

* Determined against 4 hemagglutination units of heated virus.

Effect of pH on the Inhibitor.—The pH stability range of the inhibitor was determined in the following manner:

Normal allantoic fluid which had been dialyzed overnight against buffered saline was divided into aliquots. The pH of each aliquot was adjusted to the desired level by the dropwise addition of either 1 N HCl or 1 N NaOH. The specimens adjusted to pH 2.6 and 3.3 became slightly cloudy but no easily sedimentable precipitate formed; the fluid remained clear at the various other pH levels. After 1 hour at room temperature or 30 minutes at 70°C. the inhibitor titer of each specimen was determined against 4 units of heated virus.

The results are summarized in Table V. It will be seen that after 1 hour at room temperature the inhibitor was demonstrable in undiminished concentration at pH levels from 10.7 to 6.3, but at lower pH levels the titer decreased

TABLE V

Stability of Inhibitor at Different pH Levels

Normal allantoic fluid adjusted to‡	Inhibition titer*	
	After 60 min. at room temperature	After 30 min. at 70°C.
pH		
2.6	16	2
3.3	16	2
4.6	32	16
5.3	32	32
6.3	64	64
7.0	64	64
9.8	64	32
10.0	64	32
10.7	64	16

* Determined against 4 hemagglutination units of heated virus.

‡ With 1 N HCl or 1 N NaOH.

progressively. After heating at 70°C. the inhibitor titer remained constant only at pH 7.0 and 6.3 and was reduced on both the alkaline and acid sides.

Effect of Temperature on Inhibition.—The effect of temperature on the inhibition of influenza virus (PR8) by normal allantoic fluid was studied by the constant inhibitor-decreasing virus technique described above. Identical titrations were carried out simultaneously at 4°C., room temperature (22–26°C.), and 37°C. with the same pools of virus.

The results of several such experiments are summarized in Table VI. The results of a similar experiment carried out with heated virus are also shown. It will be noted that the quantity of virus inhibited at 4°C. was from four- to eightfold greater than the quantity inhibited at room temperature; that the quantities of virus inhibited at room temperature and at 37°C. were almost identical; that when heated virus was employed there was very little difference

between the quantity inhibited at 4°C. and the quantity inhibited at room temperature; and that the quantity of heated virus inhibited at room temperature was the same as the quantity of infectious virus inhibited at 4°C. These results show that the inhibition of hemagglutination by normal allantoic fluid can be increased by lowering the temperature to 4°C., and that the same increase in inhibition can be obtained at room temperature or 4°C. by the use of heated virus.

Action of Enzymes and Periodate on the Inhibitor.—Evidence as to the nature of the inhibitor was sought by studying the effect of different purified enzymes upon its capacity to inhibit hemagglutination. It was found that crystalline trypsin[1] in low concentration failed to alter the inhibitor titer. However, when allantoic fluid which had been dialyzed against buffered saline (pH 7.2)

TABLE VI

Effect of Temperature on Quantity of Virus Inhibited by a Constant Amount of Normal Allantoic Fluid

Infected allantoic fluid pool	Hemagglutination titer*			
	In buffer saline	In normal allantoic fluid		
		4°C.	22–26°C.	37°C.
A	256	32	128	256
B	1024	32	128	128
C	2048	16	128	128
B heated‡	1024	16	32	—

* Expressed as the reciprocal.

‡ 56°C. for 1 hour.

was treated with a relatively high concentration of crystalline trypsin (1.25 mg. per cc.) for 3 hours at 37°C., there was almost complete destruction of inhibitor activity as is shown in Table VII. It is well established that egg white contains a trypsin inhibitor in very high concentration (13), and it seems probable that a similar substance may be present in allantoic fluid. If this were true, it would provide an explanation for the failure of trypsin in low concentration to destroy inhibitor activity.

The effect of crystalline ribonuclease and highly purified desoxyribonuclease on the inhibitor was also determined.

An equal volume of crystalline ribonuclease[2] (1.0 mg. per cc.) or desoxyribonuclease[2] (1.0 mg. per cc. in 0.03 M $MgSO_4$) was added to dialyzed allantoic fluid and the mixtures were

[1] Obtained through the courtesy of Dr. M. Kunitz of The Rockefeller Institute, Princeton, New Jersey.

[2] These enzymes were kindly provided by Dr. M. McCarty of the Hospital of The Rockefeller Institute, New York.

incubated at 37°C. for 4 hours. Following this the inhibitor titer of each mixture was determined against 4 units of heated PR8.

The results obtained are also shown in Table VII. It will be noted that neither enzyme had any apparent effect upon the inhibitor titer.

Burnet (8) showed that several mucinous substances inhibit hemagglutination by influenza viruses. This prompted the treatment of allantoic fluid with several hyaluronidase preparations.

Four preparations were used: a pneumococcal hyaluronidase[2] (containing 1500 viscosity units per mg.) prepared from a type II R (D39R) culture by the method of Meyer *et al.* (14);

TABLE VII

Effect of Enzymes and Periodate on Inhibitor

Treatment of normal allantoic fluid			Inhibitor titer	
Material	Concentration	Time	After treatment	Control not treated
	mg./cc.	*hrs.*		
Pneumococcal hyaluronidase	0.001	1	<2	256
Pneumococcal hyaluronidase (heated)*	0.001	1	256	256
Crystalline trypsin	1.25	3	4	128
Crystalline ribonuclease	0.5	4	128	128
Desoxyribonuclease‡	0.5	4	128	128
	per cent			
Streptococcal extract	10.0	1	64	64
Leech extract	10.0	1	64	64
Testicular extract	10.0	1	64	64
$LiIO_4$	0.025 M	2	<4	64

* 70°C. for 5 minutes in buffered saline.

‡ In 0.03 M $MgSO_4$.

a streptococcal extract[2] (approximately 100 viscosity units per cc.) prepared from the supernate of a culture of group A, type 4 hemolytic streptococci; a crude bull testicular extract[2] (570 viscosity units per cc.) prepared by the method of Kass and Seastone (15); and a leech extract[3] (approximately 500 viscosity units per cc.) prepared by the method of Claude (16). The pneumococcal hyaluronidase was dissolved in distilled water and added to dialyzed normal allantoic fluid to yield a concentration of 0.001 mg. per cc.; the streptococcal, testicular, and leech extracts were added to specimens of allantoic fluid to yield a concentration of 10 per cent. The fluids were incubated in a water bath at 37°C. for 1 hour and then at 70°C. for 5 minutes in order to inactivate the various enzymes. After treatment the inhibitor titer of each mixture was determined against 4 units of heated PR8.

The results of these experiments are presented in Table VII. It will be seen that there was no demonstrable inhibitor in allantoic fluid after treatment

[3] Kindly provided by Dr. S. Rothbard of the Hospital of The Rockefeller Institute, New York.

with pneumococcal hyaluronidase, whereas the inhibitor titer was undiminished in the fluids treated with streptococcal, testicular, or leech extracts. This raised the possibility that the component of the pneumococcal preparation responsible for the destruction of the inhibitor might be something other than hyaluronidase. The preparation did contain a hemolysin but had no proteolytic activity against gelatin or casein.[4] The ability of the pneumococcal enzyme to destroy the inhibitor was completely inactivated after heating for 5 minutes at 70°C. in the presence of allantoic fluid, buffered saline, or phosphate buffer (pH 7.1, μ 0.5); the hyaluronidase activity as determined by the viscosimetric method was also destroyed under these conditions.[4]

Meyer *et al.* (17, 18) compared hyaluronidases of different origin by both viscosimetric and reductometric methods and found that pneumococcal hyaluronidase hydrolyzed the substrate to almost 100 per cent of the theoretical amount, but that testicular and leech hyaluronidases, both of which contained more activity than the pneumococcal preparations in viscosimetric tests, hydrolyzed the substrate to only 50 and 40 per cent, respectively. They concluded that hyaluronidases were mixtures of at least two enzymes, one attacking the long chain molecules, the other hydrolyzing the aldobionic acid units. This could provide an explanation for the failure of the streptococcal, testicular, and leech extracts to destroy inhibitor activity. The very low concentration of pneumococcal hyaluronidase required to destroy inhibitor activity supports the idea that one of the hyaluronidase enzymes was responsible for the destruction of inhibitor activity.

Hirst (10) showed that sodium periodate destroys the virus receptors of red blood cells and also the hemagglutination inhibitor in normal rabbit serum. He suggested that this effect was due to the alteration of a polysaccharide by periodate. The effect of periodate on the inhibitor in allantoic fluid was therefore studied.

One volume of 0.1 M $LiIO_4$ was added to 3 volumes of normal allantoic fluid. After 2 hours at room temperature 2 volumes of 7.5 per cent glucose was added to inactivate the remaining periodate, and the inhibitor titer was then determined against 4 units of heated PR8.

The results of this experiment are shown in Table VII. It will be seen that insofar as could be determined the inhibitor was completely inactivated by lithium periodate.

It appears that three substances have been found, each of which is capable of inactivating the inhibitor in normal allantoic fluid. These are: crystalline trypsin, pneumococcal hyaluronidase, and lithium periodate. The destruction of inhibitor activity by crystalline trypsin suggests that at least part of the inhibitor is protein, while inactivation by pneumococcal hyaluronidase as well as periodate suggests that the inhibitor contains carbohydrate. The available

[4] These determinations were kindly carried out by Dr. M. McCarty of the Hospital of The Rockefeller Institute, New York.

evidence is consistent with the idea that the inhibitor is, in all probability, a mucoprotein.

Combination between Inhibitor and Virus.—It seemed possible that the inhibitor might prevent hemagglutination in either one of two ways: it could block the virus receptors on the RBC, or it could combine with the virus itself. In order to test the first possibility, chicken RBC were suspended in normal allantoic fluid for varying periods, then sedimented and resuspended in buffered saline. Such treated RBC were found to be agglutinated by both infectious and heated virus equally as well as untreated RBC. These results indicate that the inhibitor did not combine with virus receptors on the RBC. In order to determine whether the inhibitor combined with the virus itself, the following experiment was carried out.

Serial twofold dilutions of normal allantoic fluid were made in duplicate in buffered saline. To each dilution were added 4 units of infectious PR8 and sufficient RBC to yield a concentration of 0.25 per cent. To similar duplicate dilutions of normal allantoic fluid were added 4 units of heated virus and 0.25 per cent RBC. The inhibitor titer with one series of dilutions containing infectious virus and one containing heated virus was determined in the usual manner. In the case of the other two series the RBC were sedimented immediately after mixing by centrifugation at 2,500 R.P.M. for 2 minutes, the supernate from each tube was discarded, and the cells were resuspended in a quantity of buffered saline equal to the original volume. In every instance the inhibitor titer was determined after 1 hour at room temperature.

The results obtained from this experiment are shown in Table VIII. It will be noted that the inhibitor titer determined against 4 units of infectious virus in the usual manner was very low but that with the duplicate titration, in which the RBC were sedimented immediately and resuspended in saline, the apparent inhibitor titer was 16-fold higher. This indicates that the virus was not sedimented with the RBC in those tubes which contained sufficient inhibitor. When heated virus was used, the inhibitor titer was high in the case of both titrations. The results of this experiment suggest that the inhibitor combines with the virus itself and thereby prevents adsorption of the virus by RBC; that infectious virus is eluted rapidly although incompletely from the inhibitor and is then capable of uniting with RBC; and that heated virus is almost completely incapable of dissocatiing from the inhibitor just as it is incapable of dissociating from RBC (19). It seems apparent that once the virus-inhibitor reaction has reached equilibrium RBC are incapable of causing the virus to dissociate from inhibitor. If this were not the case, inhibition of hemagglutination in the presence of normal allantoic fluid would not be demonstrable.

The Rate of the Reaction between Virus and Inhibitor.—It was suggested by the results obtained in the preceding experiment that influenza virus combined with inhibitor and then rapidly although incompletely dissociated from it. In order to obtain additional information concerning the reaction between virus and inhibitor, the following experiment was carried out.

Serial twofold dilutions from 1:2 to 1:16 of virus were made in undiluted normal allantoic fluid and immediately placed in a water bath at 37°C. Aliquots of each dilution mixture were removed after varying times and immediately heated at 56°C. for 1 hour in order to stop the reaction between virus and inhibitor. The hemagglutination titer of each aliquot was then determined in the usual manner. The inhibitor titer of each aliquot against 4 units of heated virus was also determined after the aliquot had been heated additionally at 70°C. for 5 minutes in order to completely eliminate the hemagglutinating activity of the virus originally present in it.

The results of this experiment are presented graphically in Fig. 3 in which the upper portion (*A*) shows the virus hemagglutination titers and the lower

TABLE VIII

Evidence for Combination between Inhibitor and Virus

Mixture				Centrifugation*	Dilution of normal allantoic fluid										Inhibitor titer
PR8-infected allantoic fluid		Normal allantoic fluid	RBC												
Treatment	Hemagglutinating units per tube				2	4	8	16	32	64	128	256	512	1024	2048	
			per cent	R.P.M.												
None	4	Indicated dilution	0.25	None	0	0	0	1	2	3	4	4	4	4	4	8
	4	Indicated dilution	0.25	2500	0	0	0	0	0	0	0	1	4	4	4	128
Heated‡	4	Indicated dilution	0.25	None	0	0	0	0	0	0	0	0	2	4	4	256
	4	Indicated dilution	0.25	2500	0	0	0	0	0	0	0	0	0	2	4	512

* Mixture centrifuged immediately after preparation. Sedimented RBC resuspended to volume in buffered saline.

‡ Undiluted fluid heated at 56°C. for 1 hour.

portion (*B*) the inhibitor titers plotted as logarithms against the time of incubation at 37°C. It will be noted that, when the mixtures of virus in normal allantoic fluid were heated at 56°C. for 1 hour immediately after preparation, hemagglutination was demonstrable only in the mixture with the lowest dilution of virus, *i.e.* 1:2, and then only in very low titer. However, when the mixtures were incubated at 37°C. for varying times before heating at 56°C., the hemagglutination titer of each mixture increased progressively with time. The rate of increase in titer became slower as the ratio of virus to normal allantoic fluid decreased, and the maximum titer reached with each mixture was progressively lower than would have been expected in terms of the quantity of virus present in the mixture. The inhibitor titers determined with the same

mixtures showed that the quantity of demonstrable inhibitor decreased progressively as the hemagglutination titer increased, and the inhibitor completely disappeared from each mixture at the same time that the hemagglutination titer reached a maximum level except with the mixture which originally con-

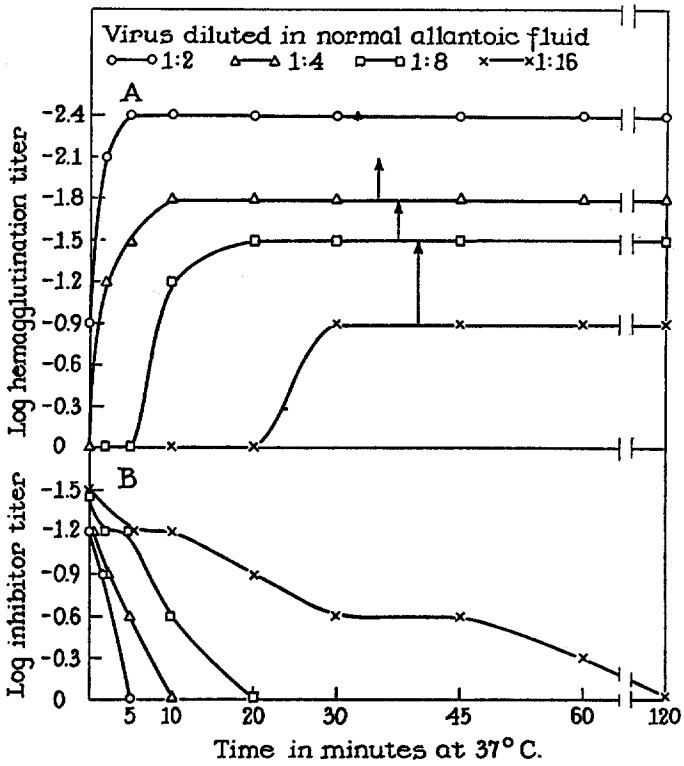

FIG. 3. The rate of the reaction between virus and inhibitor. Serial twofold dilutions of virus in allantoic fluid were incubated at 37°C. Aliquots were removed after varying periods and immediately heated at 56°C. for 1 hour. The hemagglutination titer of each aliquot is shown in Graph A. The arrows above each curve indicate the level which the hemagglutination titer should have reached if all virus had been released from combination with inhibitor. The inhibitor titer against 4 units of heated virus, after each aliquot had been heated additionally at 70°C. for 5 minutes, is shown in Graph B.

tained a 1:16 dilution of virus. In this mixture there was still a small amount of demonstrable inhibitor present when the hemagglutination titer reached a maximum level, but even in this instance the inhibitor titer decreased on further incubation. These findings provide strong evidence that the virus combines with the inhibitor and then dissociates partially from it. They also indicate that combination between the virus and the inhibitor takes place very rapidly,

and that upon completion of the reaction the inhibitor is no longer demonstrable. Furthermore, the results of this experiment indicate that the quantity of virus which is capable of dissociating from inhibitor is dependent upon the ratio of virus to inhibitor; the lower the ratio, the less virus is released.

Quantitative Relation between Free and Combined Virus.—Additional information concerning the effect of the virus-inhibitor ratio on the quantity of virus capable of remaining combined with inhibitor was obtained in the following manner:

Serial twofold dilutions of infectious virus were made in buffered saline and to each dilution was added an equal quantity of normal allantoic fluid. Thus, the inhibitor concentration in the resulting mixtures remained constant while the virus concentration progressively decreased. The mixtures were held at room temperature for 30 minutes or longer so as to reach equilibrium. In certain experiments the mixtures were held at 37°C. for as long as 24 hours. The hemagglutination titer of each mixture then was determined in the usual manner. The quantity of virus combined with inhibitor, *i.e.* the quantity of virus incapable of dissociating from inhibitor, in each mixture was taken as the difference between the total quantity of virus added to the mixture and the quantity of free virus demonstrable by hemagglutination.

The results obtained in three separate experiments are plotted graphically in Fig. 4: curve I was fitted to points which show the logarithm of the quantity of virus combined with inhibitor plotted against the logarithm of the total quantity of virus added; curve II was fitted to points which show the logarithm of the quantity of free virus plotted against the logarithm of the total quantity of virus added. It will be noted that as the total amount of virus in the mixtures decreased, *i.e.* as the ratio of virus to inhibitor decreased, relatively less free virus was demonstrable, and a higher proportion of the total virus remained combined with inhibitor. Under these circumstances it would be expected that curves I and II should diverge from each other as, it will be seen, they do. Thus it appears that in the presence of a constant concentration of inhibitor the quantity of virus which remains combined at equilibrium is a function of the total quantity of virus present in the mixture. In this connection it is of importance to recall that with infected allantoic fluids which showed various hemagglutination titer levels, *i.e.* free virus, the quantity of virus which combined with a constant amount of added inhibitor was directly related to the free virus level (*cf.* Table II). It appears that the reaction between influenza virus and inhibitor can be expressed in terms of the usual equilibrium equation:

$$V + I \leftrightarrows VI \qquad (1)$$

in which VI represents virus (V) combined with inhibitor (I). It should, however, be emphasized that the inhibitor is altered by the virus as is the case also with the inhibitor present in normal serum (19). It was found that incubation of mixtures of virus and inhibitor at 37°C. for periods as long as 24 hours did not lead to any consistent alteration in the equilibrium; no additional

free virus was released from combination after the first few minutes of the reaction.

Inhibitor in Egg White.—Numerous attempts were made to concentrate and purify the inhibitor in normal allantoic fluid by a number of procedures with

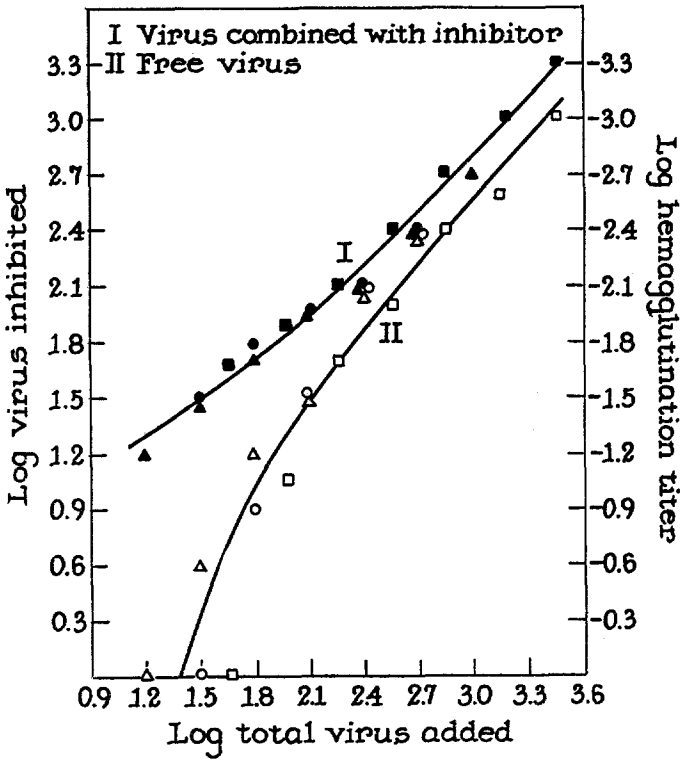

FIG. 4. The equilibrium between virus and inhibitor. Equal portions of undiluted allantoic fluid were added to serial twofold dilutions of virus. After 30 minutes at room temperature the quantity of free virus in each mixture was determined by the hemagglutination technique. The quantity of virus combined with inhibitor, curve I, was determined from the difference between the total quantity of virus added and the quantity of free virus found, curve II.

very little success. This was partially attributable to the very low concentration of the inhibitor and therefore a richer source derived from the egg was sought. It was found that egg white in very high dilution was capable of inhibiting hemagglutination: a 0.25 per cent solution of egg white in saline had approximately the same inhibitor activity as undiluted normal allantoic fluid. The inhibitor in egg white was found to react with influenza virus in the same manner as does that in allantoic fluid, and its properties, *i.e.* heat stability, pH stability, sedimentation in the centrifuge, and inactivation by enzymes, were

closely similar to those of the inhibitor in allantoic fluid. Therefore, it appeared that the two substances were very closely related if not identical.

Purification of the inhibitor present in egg white was undertaken.

Ovalbumin, ovomucoid A (ovomucin), and lysozyme, substances fairly readily separated from egg white, were found to have little or no inhibitor activity. However, it was found that most of the inhibitor could be sedimented with the precipitate which formed when egg white was diluted with 3 to 4 volumes of distilled water, or when a solution of egg white in 1 per cent saline was brought to 0.3 saturation with ammonium sulfate. Slight purification could be obtained with either method by redissolving the precipitate in 1 per cent saline adjusted to pH 8.0 and reprecipitating several times as before. However, with each additional precipitation the material became increasingly more insoluble.

The most highly purified preparation of the inhibitor was obtained when egg white was fractionated by a cold-alcohol precipitation method adapted from that devised by Cohn *et al.* (20) for fractionating serum proteins. By this method most of the inhibitor activity was found in the fraction which was precipitated at −3°C., pH 7.9, and an ethanol concentration of 25 per cent. This fraction was extremely difficult to put into solution from the dried state even with violent stirring. It was found that the final material in a concentration of about 0.06 mg. per cc. in 1 per cent saline inhibited the same quantity of heated virus as does normal allantoic fluid. On this basis it can be computed that 1 unit of heated virus should be inhibited by approximately 0.5 μg. of the purified material. Chemical analyses on this fraction were not made but it was found to give both a positive Molisch reaction for carbohydrate and a positive biuret reaction for protein.

Inhibitor in Infected Allantoic Fluid.—On numerous occasions attempts were made to demonstrate the presence of inhibitor in infected allantoic fluid after the virus had been either inactivated or removed. The virus was inactivated by either heat or treatment with alkaline pH; *i.e.*, 10 or more; it was removed from the allantoic fluid by either centrifugation or cold-alcohol precipitation. On no occasion was it possible to demonstrate any inhibitor activity whatsoever in infected fluid. In view of the results of the various experiments described above, it would not be expected that inhibitor could be demonstrated in infected allantoic fluid. Because of the prolonged opportunity for contact with virus during the course of multiplication, it would be anticipated that some of the inhibitor should be combined with virus while the remainder, although dissociated from combination with virus, should be altered as a result of viral action. In neither instance could present methods demonstrate the presence of the component.

Bound Virus in Infected Allantoic Fluid.—It is evident from the results of preceding experiments that virus bound to inhibitor is unable to agglutinate RBC. There is, however, no evidence that bound virus is incapable of adsorption to RBC. Nonetheless, it seemed possible that, if part of the virus in

infected allantoic fluid actually were bound to inhibitor, the free virus in the fluid might be separated from the bound virus by adsorption onto and elution from RBC. If such a separation were accomplished, then free virus alone should react differently with added fresh inhibitor than the original infected fluid, *i.e.* more free virus should be bound by fresh inhibitor in the absence of bound virus than in its presence because, as is shown above, the total quantity of virus which combines with inhibitor is directly related to the ratio of virus to inhibitor. However, it was found that virus which had been adsorbed onto and eluted from RBC reacted with fresh inhibitor in a manner quantitatively identical to that of the original infected fluid. It would appear, therefore, that adsorption onto and elution from RBC does not provide evidence of a separation of free from bound virus.

It was found also that there was no significant reduction in the embryo infectivity titer of virus which had been serially diluted in normal allantoic fluid so as to provide a great excess of inhibitor; the 50 per cent embryo infectivity titer was $10^{-8.0}$, whereas that of the control was $10^{-8.5}$. This indicates that virus bound to inhibitor is equally as infectious for the embryo as is free virus. It has been shown (21) that when influenza virus is adsorbed onto RBC from infected allantoic fluid, the extent of the decrease in the hemagglutination titer is closely paralleled by a decrease in the infectivity titer. This evidence suggests that bound virus is adsorbed onto RBC to an extent similar to that of free virus. If this were not the case, the hemagglutination titer, which is a measure of free virus, should be decreased by RBC adsorption to a greater extent than the infectivity titer.

DISCUSSION

It appears evident from the results obtained in this study that there is in normal allantoic fluid a component which is capable of combining with influenza virus and that virus which is combined with the component is incapable of causing hemagglutination. When normal allantoic fluid is added to infected allantoic fluid, the inhibiting component present in the former fluid promptly combines with the free virus present in the latter fluid. This reaction reaches equilibrium in a short period of time and the relative proportions of the components which enter into it then remain constant so long as their concentrations are not changed by the addition of more inhibitor or more virus. Present evidence indicates clearly that although some of the virus dissociates from the inhibiting component after combination not all of the virus is released from combination and a relatively large proportion remains in stable union with the component. As a result of experiments in which the concentrations of virus and inhibitor were varied with respect to each other, it was found that the quantity of virus which remains combined with a constant amount of inhibitor and obviously also the quantity which dissociates from inhibitor are

directly related although not strictly proportional to the quantity of virus present in the system.

It is apparent that there is a striking difference between the virus-inhibitor reaction and the virus-erythrocyte reaction. The latter reaction appears to proceed to completion and all or almost all the virus dissociates from RBC (21). The former reaction appears not to go to completion but instead reaches a fixed equilibrium. Over the range of variables studied the smallest proportion of virus which remained combined with inhibitor was at least 50 per cent and the largest 100 per cent. Following partial dissociation of the virus-inhibitor complexes, the inhibitor which is released is so altered that it can no longer be demonstrated directly by available techniques. In this respect, the reaction is analogous to the virus-erythrocyte reaction. It is well established that on dissociation of the latter combination the RBC also are altered and do not again combine with the virus (21). It follows directly from these considerations that whatever quantity of the inhibiting component may be free in infected allantoic fluid would be expected to be similarly altered and not demonstrable as a consequence. It will be recalled that all attempts to demonstrate the presence of unaltered inhibitor in infected fluid were unsuccessful. It is, of course, possible that infected fluid is actually devoid of the inhibiting component and that all which was present at the time infection was initiated was not only altered as a result of contact with the virus but actually was destroyed or otherwise removed from the allantoic fluid during the course of infection. This seems a forced and unlikely assumption, particularly because there is evidence which indicates that virus combined with inhibitor is present in infected fluid. In the light of the quantitative relationship between inhibitor concentration and virus concentration it can be shown that, if inhibitor remains present and in constant concentration in infected allantoic fluid, proportionately more virus should be combined in fluids of low virus concentration than in fluids of high virus concentration. Therefore, the addition of fresh inhibitor to infected fluids should lead to the binding, *i.e.* inhibition, of less free virus in fluids of low virus concentration than in fluids of high virus concentration. It was found that such a relationship can be demonstrated.

The evidence obtained in this study makes it appear highly probable that allantoic fluid infected with influenza virus contains at least three components which are in equilibrium: (1) free virus (which is capable of causing hemagglutination), (2) altered inhibitor (which is no longer capable of combining with virus), and (3) virus combined with inhibitor. Under these circumstances the addition of normal allantoic fluid to infected allantoic fluid serves merely to upset an existing equilibrium which then promptly becomes reestablished at a different level. The increased inhibitor concentration of the system should lead to an increase in the amount of bound virus at the expense of free virus. The evidence indicates that this occurs. It is important to point out that virus

combined with inhibitor appears to be equally as infectious for the chick embryo as free virus.

Many of the characteristics of the hemagglutination reaction with influenza virus suggest that erythrocytes are agglutinated as a direct result of their combination with virus particles and not because of secondary alterations produced in the red blood cells. Indeed, Heinmets (22) in studies on the RBC-virus reaction with the electron microscope obtained evidence which suggests that virus particles form linkages between erythrocytes and thereby cause them to agglutinate. There appear to be adequate reasons for thinking that the virus is "divalent" or possibly "multivalent" with respect to red blood cells; *i.e.*, a single virus particle can combine with more than one erythrocyte simultaneously. If it is assumed for purposes of simplification that the virus is merely divalent, *i.e.* can combine with but two erythrocytes simultaneously, then it is probable that both "valences" should be free or uncombined if hemagglutination is to occur. In terms of this hypothesis, if one valence were combined with inhibitor while the other remained free, hemagglutination should not occur even though the virus should still be capable of adsorption onto and elution from RBC. It seems likely that such a situation may acutally exist in infected allantoic fluid and that some of the virus which is combined with inhibitor can also unite with erythrocytes. That RBC can adsorb virus particles without becoming agglutinated has been shown recently (22). In view of these considerations it would appear that present procedures for the purification of influenza viruses probably do not permit of a complete separation of virus particles from combined inhibitor. In this connection, it is of interest that Cohen (23) found that highly purified influenza virus preparations obtained from infected allantoic fluid contained a considerable amount of antigenic material characteristic of the host. Knight (24) obtained similar results with highly purified preparations from both allantoic fluid and mouse lung suspensions and expressed the opinion that host material was incorporated into the virus particle itself. The results of the present study suggest that the host component, *i.e.* inhibitor, is bound to the surface of the virus particle as a result of a definite reaction and is, in all probability, not an essential constituent of the virus.

On the basis of chemical analyses Knight (25) concluded that the virus particles contain a polysaccharide in addition to other substances. He also found an appreciable amount of glucosamine in sedimentable material from normal allantoic fluid and suggested that the amount of host material in purified virus-containing particles could be ascertained from their glucosamine content. Inasmuch as the results obtained in the present study indicate that the inhibitor present in allantoic fluid is probably a mucoprotein which is capable of forming a stable combination with the virus, it would appear that a simple

explanation may be offered for some of the results previously obtained in immunological and chemical studies on purified preparations.

The presence of virus-inhibitor complexes in infected allantoic fluid may have consequences of both practical and theoretical significance. Mention has been made of the difficulties of separating free virus from bound virus either in the centrifuge or by adsorption and elution from RBC. Because hemagglutination titrations serve to measure free virus, but not virus bound by inhibitor, they may not reflect accurately the total concentration of virus in an infected allantoic fluid. Moreover, if different strains of virus react in quantitatively different manners with inhibitor, an inconstant ratio between infectivity and hemagglutination titers might be anticipated. It is well known that freshly recovered strains may give very peculiar and irregular results both in hemagglutination and in hemagglutination-inhibition titrations in the presence of immune serum. It seems probable that certain of the unusual reactions obtained with such strains may be attributable to the presence of virus-inhibitor complexes.

SUMMARY

Evidence is presented which shows that there is a component present in normal allantoic fluid, probably mucoprotein in nature, capable of combining with influenza A virus (PR8), and that following combination between this component and the virus only partial dissociation of the complex occurs. Evidence is also presented which strongly suggests that the component is present in virus-infected allantoic fluid in which it is in part combined with the virus and in part free although altered by viral action. The probability that the component is present as well in highly purified preparations of influenza virus, and its effect upon various reactions obtained with this agent are discussed.

Addendum.—In a recent paper Lanni and Beard[5] reported that egg white is highly effective in inhibiting hemagglutination by heated swine influenza virus and suggested that this capacity is attributable to a component which combines with the virus.

BIBLIOGRAPHY

1. Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.
2. Hirst, G. K., *J. Exp. Med.*, 1943, **78**, 99.
3. Burnet, F. M., and Beveridge, W. I. B., *Australian J. Exp. Biol. and Med. Sc.*, 1945, **23**, 187.
4. McCrea, J. F., *Australian J. Exp. Biol. and Med. Sc.*, 1946, **24**, 283.
5. Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

[5] Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 312.

6. Friedewald, W. F., Miller, E. S., and Whatley, L. R., *J. Exp. Med.*, 1947, **86**, 65.
7. Svedmyr, A., *Ark. Kemi, Mineral. och Geol.*, 1947, **24**, 1.
8. Burnet, F. M., *Australian J. Sc.*, 1947, **10**, 21.
9. de Burgh, P. M., Yu, P., Howe, C., and Bovarnick, M., *J. Exp. Med.*, 1948, **87**, 1.
10. Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301.
11. Blum, J., unpublished data.
12. Pickels, E. G., *Rev. Scient. Instr.*, 1942, **13**, 93.
13. Lineweaver, H., and Murray, C. W., *J. Biol. Chem.*, 1947, **171**, 565.
14. Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.*, 1940 **71**, 137.
15. Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.
16. Claude, A., *J. Exp. Med.*, 1937, **66**, 353.
17. Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.
18. Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.
19. Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 315.
20. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.
21. Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.
22. Heinmets, F., *J. Bact.*, 1948, **55**, 823.
23. Cohen, S. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 356.
24. Knight, C. A., *J. Exp. Med.*, 1946, **83**, 281.
25. Knight, C. A., *J. Exp. Med.*, 1947, **85**, 99.