TABLE III.
Tests for Amounts of Virus in Local Tissue of Hind Feet of Hamsters at Intervals of 1, 7, and 8 Hr After Injection of MM Virus.
Tests at 7 hr. (Exp. I. Injections Feb. 2, feet frozen over night before tests for virus.)

	Hamster I.		Hamster II.	
Dilution of tissue	10^{-2}	10^{-3}	10^{-2}	10^{-3}
	2	6	3	3
	3	S	3	6
	4	S	S	S

	Tests at 1 hr. (Exp. II. Injections Mar. 12).					
	Hamster I. Right hind foot		Hamster I. Left hind foot		Hamster II. Right hind foot	
Date of test*	Mar. 20, '48		Mar. 23, '48		Mar. 15, '48	
Dilution of tissue	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
	3	S	3	S	4	S
	S	S	S	S	S	S
	S	S	S	S	S	S

	Tests at 8 hr. (Exp. II. Injections Mar. 12.)								
	Hamster II. Left hind foot			Hamster III. Right hind foot			Hamster III. Left hind foot		
Date of test*	Mar. 15, '48			Mar. 20, '48			Mar. 23, '48		
Dilution of tissue	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
	2	3	S	2	2	S	3	3	6
	2	4	S	3	3	S	3	4	S
	3	S	S	3	4	S	3	5	S

* Feet frozen until time of test.

feet of hamsters were tested at 1, 7 and 8 hours after injection of a 10^{-4} suspension of MM virus. The feet were frozen for 1 to 11 days before tests for virus were made. The results, as shown in Table III and in Fig. 1, indicate that even as early as 7 to 8 hours after injection there was considerable growth of virus in the inoculated feet.

Summary and conclusion. After inoculation into the pad of the hind foot of a hamster, MM virus increases in amount in the local tissue. Subsequent increases in virus in the blood and viscera are in turn followed by appearance of the virus in the central nervous system. It appears certain that the virus grows in the foot. Whether the growth occurs in subcutaneous tissue, muscles, or nerve endings was not determined in these experiments.

16510

The Mechanism of Egg-White Inhibition of Hemagglutination by Swine Influenza Virus.*

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Egg-white (EW) has been found capable of inhibiting hemagglutination by purified preparations of swine influenza virus and derivatives obtained by heating the virus or treating it with formaldehyde.[1] This capacity

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[1] Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 312.

of EW showed an interesting dependence on the history of the virus preparation. Thus, the EW inhibition titers for heated virus and for an aged formolized swine influenza vaccine averaged approximately 170,000 and 54,000, respectively, while the titers for highly infectious, untreated preparations, which we have designated SF, may be as low as 50 to 100.

Such a dependence of the inhibition titer on the character of the virus preparation was first described by Francis,[2] who found that the ordinarily low inhibition titer of normal serum for influenza virus B hemagglutination was greater by a factor up to 128, but more typically 32 to 64, if the virus preparation had been heated at 56°C for 30 minutes. This behavior, which has been observed also with other body fluids and tissue extracts and has recently been the subject of several extensive investigations,[3,4] stands in marked contrast with the considerably greater effects observed with EW.

In a previous report,[1] evidence has been presented that the mechanism of EW inhibition of hemagglutination by swine influenza virus involves combination between an EW component and virus. Further evidence on this point, as well as on the great disparity in inhibition titers for the active, untreated swine influenza virus and those for the vaccine, is provided in the present communication.

Materials and methods. The swine influenza virus was the egg-adapted strain which has been employed in other studies in this laboratory[5] and which came originally from Dr. R. E. Shope; it has been designated as Strain 15. The active, untreated virus (SF) used in the present work was obtained from a pool of chorio-allantoic fluid from 4,269 eggs infected with the agent and harvested as previously described.[6] Concentration of the virus was effected by spinning 27.8 l of the fluid in the modified Sharples centrifuge[6] at 47,000 g at a flow rate of 1.5 l per hour. The virus concentrate in a volume of 220 ml was spun in the angle centrifuge at 1,600 g for 10 minutes. The virus in a 90-ml portion of the supernatant fluid was then sedimented in the vacuum type centrifuge by spinning at 20,000 g for 1 hour. The large pellets, showing little opaque material, were dispersed in 240 ml of Ringer solution and centrifuged in the angle head at 1,600 g for 10 minutes. These manipulations were completed on 11/6/47 and the virus, stored at 0.234 mg N per ml at about 4°C, had a 50% endpoint infectious unit of $10^{-14.7}$ g N on 2/9/48.

The vaccine was a Sharples concentrate of swine influenza virus obtained as previously described[7] and treated with 0.05% formalin and 1:50,000 phenyl mercuric borate on 5/22/44. It had been stored at about 4°C in the interim.

Hemagglutinative capacity was determined by the method of Hirst,[8] and the test mixtures were compared visually with the standard red blood cell suspensions.[5] The diluent throughout was buffered saline which consisted of a solution of 0.81% sodium chloride and 0.005 M phosphate at pH 7.3. The inhibition titer is defined as the reciprocal of the dilution at which the standard 2-plus endpoint, signifying the inhibition of all but 1 hemagglutinating dose (HD), was obtained. Generally, 4 HD were added, and 3 HD were inhibited. Variations arose from experiment to experiment because of the difficulty in determining the hemagglutinating dose precisely.

The egg-white was separated from the yolk and forced through several layers of fine-meshed gauze supported on a wire screen in a 20-ml syringe. Further mixing of the white before sampling was accomplished by repeated pipetting.

Experimental. An experiment was carried out to determine the dependence of the inhibition titer on (a) the order in which the reagents EW, vaccine and chicken RBC were

2 Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

3 Burnet, F. M., *Lancet*, 1948, **254**, 7.

4 Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 315.

5 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

6 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

7 McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

8 Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

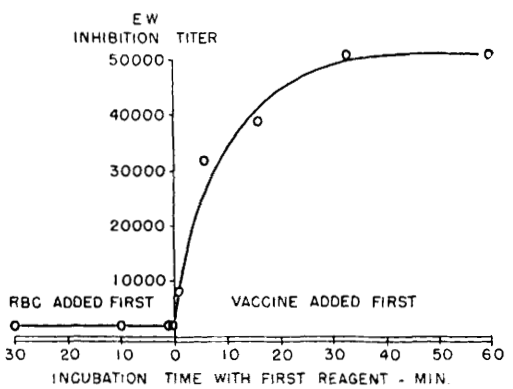

FIG. 1.

Inhibition titer as a function of the order of addition of chicken red blood cells and vaccine to egg-white (EW) dilutions and the time of incubation at 28°C with the reagent added first. The ordinate of each point denotes the reciprocal of the final dilution of EW at which the standard 2-plus agglutination endpoint was obtained in a titration involving constant RBC, constant vaccine and a set of final EW dilutions ranging from 1:500 to 1:512,000. The only variables among the titrations were the order of addition of RBC and vaccine to the EW dilutions and the elapsed time between the addition of these two reagents.

mixed, and (b) the time of incubation of EW with one of the reagents, vaccine or RBC, before the other reagent was added. Progressive 2-fold dilutions of EW were made with buffered saline, and 0.5-ml portions of each dilution were distributed in several tubes so as to provide several sets of dilutions. To each tube of some of the sets was added 1.0 ml of 2% RBC, and to each tube of other sets 0.5 ml of a vaccine dilution containing 8 HD per ml. After different periods of incubation at room temperature (28°C), the appropriate amount of the second reagent, RBC or vaccine, was added to bring all the sets to the same reagent composition. After an additional hour, the degree of agglutination was determined in the usual manner. Fig. 1, which summarizes the results, shows that the EW inhibition titer was experimentally invariant, at the level 2,000, with respect to the time of incubation of EW with RBC prior to the addition of vaccine. On the other hand, with extended incubation of EW with vaccine before the addition of RBC, the titer showed a progressive rise, attaining a maximum of 51,000 after about 30 minutes incubation. Moreover, when a fresh mixture of RBC and vaccine was quickly added to EW dilutions, the inhibition titer was only 500. These results provide strong evidence that EW is effective as inhibitor through obstructive combination with vaccine, and not with RBC. Accordingly, we may explain the several inhibition titers observed in this experiment in terms of the outcome of a mutually exclusive competition of EW and RBC for combining groups of the vaccine, a particular titer depending on which of these reagents is given the greater opportunity of reacting with vaccine.

A similar experiment in which EW dilutions were incubated at 26°C with 4 HD of active, untreated swine influenza virus (SF) for varying periods prior to the addition of RBC gave, however, a quite different result, the inhibition titer *decreasing* from a value of 80 after one minute of incubation to a value of 20 after about 15 minutes. At 22°C, a titer of 320 after one minute of incubation was followed by a titer of 160 after 15 minutes and a titer of 80 after one hour. This rapid decrease in titer, manifested by progressive emergence of the hemagglutinating activity of SF, indicated a capacity of SF to destroy or inactivate, but not merely to neutralize, the EW inhibitor (cf. [3,4,9–11]) and provided an explanation for the variation in titers encountered in preliminary experiments with SF when the time of incubation was not under control.

Evidence that the inhibitor effective against vaccine is likewise destroyed by SF and, accordingly, that the same EW component is responsible for inhibition of both SF and vaccine is provided in Table I. Several identical mixtures containing EW in a final concentration of 1:50 and 12 HD of SF per ml were incubated for varying periods at room temperature (25°C) and then diluted 2-fold serially to provide 2 parallel sets of dilutions, with 0.5 ml in each tube. To each tube of one set was added 0.5 ml of vaccine

[9] Friedewald, W. F., Miller, E. S., and Whatley, L. R., *J. Exp. Med.*, 1947, **86**, 65.

[10] de Burgh, P. M., Yu, P. C., Howe, C., and Bovarnick, M., *J. Exp. Med.*, 1948, **87**, 1.

[11] Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301.

TABLE I. Effect of Incubation of Egg-White (EW) with Untreated Swine Influenza Virus (SF) on the Egg-White Inhibitor for SF and for Vaccine.

Min. incubation before dilution	Titrated with	Reciprocal of final dilution of incubated mixture								
		8	16	32	64	128	256	512	1024	2048
Approx. 1	4 HD* vaccine	++	+±	0	0	±	+	+±	+±	±
		++	±	0	0	0	0	0	0	0
	Saline	++	±	0	0	0	0	0	0	0
35	4 HD vaccine	++	+	±	+	+	±	±	+	+
		++	++	+	+	++	++	++	++	++
		++	++	±	0	0	0	0	0	0
	Saline	++	++	++	+	++	+	+	+	+
64	4 HD vaccine	++	±	+	±	±	±	±	+	+
		++	++	+	+	++	++	++	++	++
		++	++	++	0	0	0	0	0	0
	Saline	++	++	++	+	++	+	+	+	+
125	4 HD vaccine	++	±	+	±	±	+	+	+	+
		++	++	+	+	++	++	++	++	++
		++	++	±	0	0	0	0	0	0
	Saline	++	++	++	+	++	+	+	+	+
EW control (no SF)	4 HD vaccine	0	0	0	0	0	+	+	+	±
		0	0	0	0	0	0	0	0	0
	Saline	0	0	0	0	0	0	0	0	0
SF control (no EW)	Saline	+	±	±	±	0	0	0	0	0
		+	+	+						
		+	+	+						

* Hemagglutinating doses.

(4 HD), and to each tube of the second set 0.5 ml of saline. After 30 to 40 minutes incubation, to allow maximal reaction of inhibitor with vaccine, 1.0 ml of 2% RBC was added, and the extent of agglutination was determined after one hour. The results (Table I) show progressive destruction of inhibitor for vaccine accompanied by progressive emergence of the hemagglutinating activity of SF. This result suggests, at the same time, the usefulness of vaccine for studying the inactivation of EW inhibitor by SF. In a comparable experiment with vaccine substituted for SF, a slight inactivation of the inhibitor was evident after 5 hours and 19 hours, but the effect was too small to be regarded as significant in the present state of the experiments.

Since the degree of purity of the ultracentrifugally concentrated virus preparation SF was not known, it was desired to obtain evidence on the nature of the SF constituent which was responsible for the inactivation of EW inhibitor. For this, we resorted to the use of convalescent (anti-influenza) swine serum. Such a serum, obtained from an animal after experimental intranasal infection with active virus in chorio-allantoic fluid,[12] may be regarded as the most specific reagent for this virus at present available, since the opportunity is minimized for the occurrence in it of antibodies directed against materials extraneous to the virus itself. Orienting experiments showed that this serum was capable of neutralizing the hemagglutinative activity of 3 HD of either SF or vaccine in a dilution of 1:2500. A normal swine serum, selected for a control, was approximately 50 to 100 times weaker in this respect.

A basic experiment involving the incubation of EW, in a dilution of 1:50, with the minimal quantity of SF (about 6 to 8 HD per ml of mixture) which caused inactivation of at least 90% of the inhibitor in one hour at room temperature, as determined with vaccine (Table I), was modified slightly to allow the study of the effect of convalescent serum. Mixtures of equal volumes of 1:200

[12] McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1947, **56**, 109.

TABLE II.

Effect of Convalescent (Anti-Influenza) Swine Serum on the Inactivation of Egg-White (EW) Inhibitor by Untreated Swine Influenza Virus (SF)

Initial mixture	After 10 min., room temp., add	After one more hr, add	Reciprocal of final dilution of mixture in titrations with 4 HD* vaccine							
			8	16	32	64	128	256	512	1024	2048
Saline (2 vols.)	EW	Saline	0	0	0	0	++	++	++++	++++	++++
Immune serum + saline	Saline	"	++	++	++	++	++	++	++++	++++	++++
Immune serum + SF	EW	"	+	+	++	++	+	+	+	++++	++++
Saline + SF	EW	Immune serum		0	0	0	0	+	++++	++++	++++
Normal serum + SF	EW	Saline	++	++	++	+	++	++	++++	++++	++++
			++	++	++	++	++	++	++++	++++	++++

* Hemagglutinating doses.

serum, 3:50 EW, 22 HD of SF per ml, and saline were prepared as indicated in Table II. After incubation was complete, the mixtures were diluted progressively with saline, and 0.5 ml of each dilution was incubated with 0.5 ml (4 HD) of vaccine for 30 to 40 minutes at room temperature before the addition of RBC. It was found (Table II) that the convalescent serum, added in the amount required to neutralize the hemagglutinative activity of the SF employed, was completely effective in suppressing the inhibitor-inactivating capacity of SF when the serum and SF were incubated together for ten minutes before the addition of EW. On the other hand, normal pig serum, tested in quantities up to 40 times the effective quantity of immune serum, was without demonstrable effect in suppressing inhibitor inactivation; and immune serum added to a previously incubated mixture of SF and EW was likewise ineffective. Accordingly, the suppression of inhibitor inactivation by immune serum may reasonably be ascribed to a combination between the specific antibody and the virus, and, in consequence, the capacity of SF to inactivate EW inhibitor must be ascribed to the virus particles themselves.

Discussion. The evidence from different kinds of experiments clearly indicates that the capacity of egg-white (EW) to inhibit hemagglutination by swine influenza virus rests on the capacity of an unidentified EW component to combine with virus and to obstruct in this way the agglutinative reaction of virus with RBC.

In the course of these experiments, it was found that the EW inhibitor was rapidly inactivated by small quantities of highly infectious virus (SF) but not by the non-infectious virus of an aged formolized vaccine. As EW was incubated with SF, the hemagglutinating activity of SF, initially depressed by EW, gradually emerged until it attained, within experimental error, its full, uninhibited level; and, at the same time, the amount of inhibitor titratable with vaccine was reduced.

To account for this inactivation of EW inhibitor by SF, one may advance two reasonable general hypotheses, an *enzymatic* hy-

pothesis which postulates a capacity of the virus particle, or, more specifically, of a given reactive patch of the virus surface, to inactivate *successive* molecules of inhibitor, inactivating one first, being released, inactivating a second, and so on; and a *stoichiometric* hypothesis which postulates an upper limit to the amount of inhibitor that can be inactivated by a single reactive patch of the virus, the limit being fixed by the number of inhibitor molecules that can attach *at one time* to the same reactive patch. For the moment we may neglect, since there is no evidence for it, the possibility of penetration of the virus surface by inhibitor molecules, and we may suppose that all of the phenomena must be accounted for in terms of reactions occurring at the surface of the virus particle.

The crucial observations that have to be explained by these hypotheses are (a) the inactivation of a large amount of inhibitor by a small amount of virus, and (b) the progressive emergence of the hemagglutinating activity of virus following an initial period of inhibition. Until the inhibitor has been isolated and weighed, its inactivation by virus must be discussed in terms of inhibitor activity. A calculation shows that one hemagglutinating dose of SF, corresponding to about $10^{-7.6}$ g of virus N (cf.[5]), is capable of inactivating in one hour at room temperature under the conditions of our experiments an amount of inhibitor which is able to suppress the hemagglutinating activity of approximately 100 hemagglutinating doses of vaccine. Since SF and vaccine have, within experimental error, the same hemagglutinating activity per unit weight of N, this is equivalent to saying that a given weight of SF is able to inactivate an amount of inhibitor effective against approximately 100 times that weight of vaccine.

To account for this disproportion by a stoichiometric hypothesis, one is led to suppose that SF is approximately 100 times as effective as vaccine in binding EW inhibitor. Then, to account for the emergence of SF from an initial inhibition, one must further suppose that the reactive patches of the virus, in stable combination with inhibitor, are dissociated from the rest of the virus particle and that the released minus-patch virus particle retains, or acquires anew, essentially full hemagglutinative activity. It is evident that the stoichiometric hypothesis is so complicated as to make its acceptance undesirable at the present time.

On the other hand, an enzymatic hypothesis offers a relatively simple explanation of the observed phenomena. According to such an hypothesis, the initial reaction of combination of virus with EW inhibitor is followed by destruction of the inhibitor and release of the unmodified virus, enabling it to combine with and destroy yet another molecule of inhibitor. As a consequence, the EW inhibition titer is low. This is true if the virus is of the highly infectious sort such as occurs in SF. If, however, the virus has been modified so that, while retaining its capacity to combine with RBC, specific antibody, and EW inhibitor, it has lost its capacity to destroy the EW inhibitor, the combination of such a modified virus with inhibitor is stable, and the EW inhibition titer is high. Thus, to account for the difference in inhibitor-inactivating capacities of SF and vaccine, one need postulate only a change in the inhibitor-binding patches of the virus, such that the loss of enzymatic capacity is not accompanied by the loss of combining capacity. The great disparity in EW inhibition titers for SF and those for vaccine may be attributed in part to a disparity in the inhibitor-inactivating capacities of SF and vaccine and in part to a second factor which is suggested by the experiment with vaccine recorded in Fig. 1. In this experiment it was found that the EW inhibition titer was greatly dependent on the order of mixing the three reagents vaccine, EW and RBC; in particular, the low titer, 2,000, was obtained when the 3 reagents were mixed simultaneously, in contrast with the high titer, 51,000, obtained when RBC were added to suitably incubated mixtures of EW and vaccine. On the assumption that the competition of EW and RBC for SF is similar to the competition of these reagents for vaccine and that the de-

struction of inhibitor by SF continues during the period of the titrations when RBC are present, we may suppose that, as virus is regenerated from complexes with inhibitor, a system prepared by adding RBC to a mixture of SF and EW progresses toward a system in which the 3 reagents have been mixed simultaneously; consequently, the inhibition titer will be lower than one might predict from a consideration of the amount of inhibitor destruction alone.

It will be seen that this interpretation of the relations among SF, vaccine, and EW inhibitor is in general accord with the explanation recently offered by Burnet[3] and by Hirst[4] for an analogous set of relations involving other viruses and normal serum as inhibitor.

Summary. Evidence has been presented that the mechanism of egg-white inhibition of hemagglutination of chicken red blood cells by swine influenza virus involves combination between an egg-white component and the virus, this combination obstructing the reaction of virus with red blood cells. Moreover, it has been found that the inhibitor is rapidly inactivated by highly infectious virus, with regeneration of the virus, but not by an aged formolized vaccine consisting of inactivated virus; and that the inactivation of inhibitor by virus is suppressed by convalescent (anti-influenza) swine serum. The results have been interpreted in terms of an enzymatic hypothesis of virus function in the phenomenon.

16511

Use of Formalin-Treated Red Cells for the Study of Influenza A Virus Hemagglutinating Activity.

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Human erythrocytes, treated with formalin, the writer has found, retain their ability to react with influenza A virus in a manner quite similar to that of untreated human erythrocytes. For certain types of experiments the use of such formalized red cells possesses distinct advantages over the use of normal red cells.

To prepare formalized red cells, equal volumes of a 50% (by volume) suspension of washed red cells in saline and formalin containing sodium chloride in a concentration of 0.85%, were mixed and allowed to stand for 2 to 3 days. If the formalin concentration of the mixture was between 10 and 50%, the cells became dark brown gradually over a period of 24 hours or so. The cells also became cohesive and very resistant to hemolytic agents. The stability of the cells thus treated was marked. Samples of formalized cells stored at 4°C have retained their activity undiminished for influenza A virus for almost two years now. Furthermore, the cells have retained the microscopic appearance and shape of fresh, normal, red cells.

Concentrations of formalin of less than 10% (3.6% formaldehyde) in the mixture failed to produce browning of the red cells over 2-3 days at 4°C usually, but did render the cells cohesive. Such cells hemolyzed more readily in hypotonic solutions than did the cells prepared with higher concentrations of formalin.

Before using the formalized red cells in reactions with influenza virus the uncombined formaldehyde was removed by repeated washing with saline or water. The cells were allowed to stand in contact with each wash fluid for a period of at least several hours to permit the establishment of an equilibrium between the free formaldehyde inside and outside the cells. Generally, 6 to 10 such