THE EXPERIMENTAL PRODUCTION OF COMBINATION FORMS OF VIRUS

I. Occurrence of Combination Forms after Simultaneous Inoculation of the Allantoic Sac with Two Distinct Strains of Influenza Virus

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The elegant and convincing demonstration by Hershey and Rotman of genetic interchange between two bacterial viruses infecting the same host cell (1) has stimulated interest in the demonstration of similar recombinations between animal viruses. A first attempt in this direction has been that of Burnet and his colleagues (2–5) and their findings will be briefly considered. A description of representative experiments by these authors with the Mel and NWS strains (4) will be used as a means of summarizing their approach to the problem of demonstrating an exchange of genetic characters between influenza viruses.

The Mel and NWS strains behave differently in a number of *in vitro* tests and, in addition, only NWS is neurotropic in mice. Both viruses were inoculated together in high concentrations into mice by the intracerebral route and the animals were sacrificed after 4 days. A brain emulsion was titered in the allantoic sac and the serological type of the predominating virus was determined for the individual allantoic fluids, harvested after 2 days' incubation. Both Mel and NWS type fluids were found. Certain of the Mel type fluids were tested *in vitro* in a variety of ways and the results were compared with those of similar tests on the parent viruses. These "markers" involved such phenomena as the type of pattern formed when red cells were agglutinated, the hemagglutination titer achieved in the allantoic sac following infection, and the degree of inactivation of the hemagglutinin at 56°C. In these tests, the Mel type fluids from eggs inoculated with mouse brain had some characters like the original Mel, some like NWS, two were intermediate between these strains and two were "unique" in that they were unlike the markers of either parent form. The authors described no attempts to determine whether this peculiar pattern of characters persisted on passage of the strains. In addition, they found that these same Mel fluids, after a single passage at limiting dilutions, were neurotropic for mice in varying degrees.

The presence of markers from both parent types in single allantoic fluids is in itself

unconvincing evidence that genetic interchange has taken place unless it can be further demonstrated that the new pattern of characters can be transmitted without change from egg to egg at limiting dilutions. Furthermore, proof that neurotropism has been transferred from NWS to Mel requires that the fluid being tested be free from parent NWS. In our own experience, a single passage of a mixture of viruses at the limiting dilution provides a very poor guarantee of strain purity. The acceptance of these new Mel strains as inherently neurotropic must await evidence that the serological character and degree of mouse pathogenicity remain constant throughout several passages at limiting dilutions.

The work just reviewed, while suggestive of some kind of virus interaction in the infected host, has not seemed to us to provide a method by which stable recombinant strains can be readily and reproducibly isolated, purified, and studied in detail. In our earliest attempts to obtain evidence of virus recombination, we were impressed by the wide variations commonly occurring in many of the *in vitro* markers which were used, so we decided to confine our attention to the most stable characters available as markers; namely, the presence of specific virus antigens.

The following account reports successful efforts to obtain virus strains containing antigens derived from each of two parent strains as a result of introducing these latter simultaneously into the allantoic cavity. The maintenance of the new strains by serial passage in the chick embryo will be dealt with in a succeeding paper.

Methods

Pairs of antigenically different strains of influenza A virus were employed for which monotypic sera could be readily prepared by the simple method of reciprocal absorption. One of the main advantages of the specific antigenic complex is its stability (6, 7). Although minor quantitative variations in the antigenic pattern of a virus may occur with laboratory manipulation, there is no evidence thus far that qualitative changes take place in the type-specific antigens of influenza virus, even after adaptation to the mouse through prolonged passage.

Two markers per strain are needed for a demonstration of genetic interchange, but it was assumed on hypothetical grounds that the specific antigenic complex of each virus might serve as a multiple marker. If the complex were made up of several antigens, a recombinant form containing part of the specific elements of each of two parents might occur. The hemagglutinin of such a form might be inhibited by specific antisera against both parents, or the virus might be neutralized by both antisera. Double inhibition or neutralization would be evidence that antigens from two parents were present on a single particle. Were such a strain discovered, efforts would be made to obtain it free from parent forms.

Virus Strains.—The strains of influenza virus used in this work were as follows: (*a*) WSN (W),[1] a neurotropic form of strain WS which was originally isolated by Francis and Moore (8) and was obtained from Dr. T. Francis, Jr.; (*b*) Melbourne (M) was obtained in 1948 from Dr. F. M. Burnet; (*c*) FM-1 (F) is an A′ strain and was obtained from Dr. J. E. Smadel.

Production of Seed Virus.—Virus for the tests on double infection was made from dilute seed (10^{-6}) and was usually prepared on the day of the experiment. Inoculations (0.1 ml.) were made into the allantoic sac (AS) of 11-day-old chick embryos and after 40 hours' incubation they were chilled at 4°C. and the coarse debris removed from the allantoic fluid (AF) by low speed centrifugation. A concentrate was prepared by centrifugation at 21,000 R.P.M. for 1 hour in the No. 21 rotor of a Spinco centrifuge. The sediment was resuspended in glucosol[2] at $\frac{1}{100}$th the original volume and stored if necessary at 4°C.

Preparation of Antisera.—Rabbits were inoculated intravenously with 2 ml. of allantoic fluid on 3 successive days. After a 2 week interval, another series of three injections were given and the animals were bled 2 weeks later. If a rabbit yielded satisfactory sera the process was repeated at intervals of 2 or more months.

The sera were tested for homologous and heterologous hemagglutination inhibition (HAI) titer against the pair of viruses to be used in subsequent tests, and those with the highest homologous titer, if accompanied by a low heterologous level, were selected for use. Absorption of sera showing considerable crossing antibody was costly and unsatisfactory. Each serum was given a preliminary treatment with RDE (6) and was then absorbed at a dilution of 1:4 with one-half the original serum volume of 100 times concentrated virus of heterologous type. The virus was added slowly, incubated for several hours at 37°C., centrifuged at 13,000 R.P.M. for 1 hour and the supernatant was heated at 65°C. for 30 minutes after the addition of sodium citrate. The serum was retested for heterologous and homologous antibody. With a good serum, a single absorption was sufficient to remove the heterologous antibody to a level at which no detectable inhibition of four hemagglutinin (HA) units occurred at a final serum dilution of 1:16. It was preferred, but not always possible, to work only with absorbed sera which had homologous titers of 1:2000 or better.

Hemagglutinin and Hemagglutination Inhibition Titrations.—HA titrations were carried out by the usual pattern method, using twofold dilutions of virus in saline. Each tube contained 0.5 ml. of virus dilution and 0.5 ml. of 0.5 per cent chicken red blood cells (final concentration 0.25 per cent). The tests were read after 45 minutes and were graded as + (full agglutination), ± (partial agglutination), and − (no agglutination). The titers were recorded as the reciprocal of the final dilution of allantoic fluid at the point where a ± reaction occurred, interpolating between two dilutions when there was no ± reaction.

HAI tests were performed by making twofold dilutions of serum in saline (0.25 ml. per tube) to which was added four HA units of virus (0.25 ml.) and 0.5 ml. of 0.5 per cent red

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[1] *Abbreviations.*—The following letters are used as strain designations throughout: F for FM-1, M for Melbourne, and W for WSN. HA stands for hemagglutinin and HAI for hemagglutination inhibition. AF for allantoic fluid and AS for allantoic sac. The letter X is used to designate combination forms from two virus strains.

[2] Glucosol was used as the diluent for all virus that was inoculated into eggs. It was modified from the original (9) by the addition of antibiotics:—

Solution A.—NaCl 8.0 gm., $CaCl_2$ 0.2 gm., $MgCl_2 16H_2O$ 0.5 gm., and glucose 1.0 gm., diluted to 1 liter with distilled water and autoclaved.

Solution B.—Na_2HPO_4 9.47 gm., and KH_2PO_4 9.08 gm. per liter of solution; sterilized by heating to 100°C. on 3 successive days. Before use, one volume of solution A was added to one volume of B and penicillin and streptomycin were added to a final concentration of 1000 units per ml.

cells. The end-points were expressed as the reciprocal of the final serum dilution giving a ± reaction.

Virus Titrations in Ovo.—Serial tenfold dilutions of virus were made in glucosol. The inoculum was 0.1 ml. per egg. After 40 hours, the eggs were chilled and the allantoic fluid tested for HA at a dilution of 1:2. End-points (ID_{50}) were calculated in the usual manner.

EXPERIMENTAL

The Reactions of Artificial Mixtures of Virus Strains in the Hemagglutination Inhibition Test

The hemagglutination inhibition test will detect no more than one strain in an artificially prepared mixture of two antigenically distinct viruses. This is the basic observation on which the present series of papers is founded. In this respect, the HAI test is very different from the complement fixation reaction with which both components of a two-virus mixture can be readily detected (10). Failure of the HAI test system to demonstrate the presence of both constituents is due to the fact that it reveals antigens through inhibition of the test reaction by antibody. In the complement fixation test, the fundamental reaction, absorption of complement, is promoted by antibody.

The behavior of virus mixtures in HAI tests was investigated in some detail because of their importance in subsequent work. The limitations of the HAI test can be readily demonstrated with two completely unrelated viruses (influenza A and B) but the more complicated case of two related but specific strains of influenza A was chosen since it was felt that it would be more instructive in subsequent combination studies.

Mel and WSN were the strains selected. Previous studies have shown that WS (from which WSN was derived) and Mel are influenza A viruses of distinct types (6). M and W allantoic fluids were carefully tested for HA titer, and mixtures of the two were prepared covering a wide range of M/W HA ratios. Four units of each of these mixtures were used in HAI tests against various dilutions of Mel and WSN antisera. The first results, shown on the left in Table I, were obtained with sera in their original unabsorbed state and are of little interest except to illustrate how important it was to have reagents which are strictly specific in the kind of test we were going to employ. These two sera were fairly strain-specific even in the crude state, the heterologous titer being of the order of 1 to 3 per cent of the homologous titer (lines 1 and 9). In mixtures in which one virus made up 75 per cent or more of the total, the predominant strain was readily detected (lines 2, 3, 7, and 8). At the point of equivalence the HAI titer was high with both sera, in seeming contradiction of our first statement that only one serum would inhibit a virus mixture. However, after the sera had been rendered specific through reciprocal absorption procedures, the expected limitation was found, as can be seen from the rest of Table I. In this part of the experiment, there was good inhibition with one serum and none with the other when either virus made up 75 per cent of the total. Over the range of near virus equivalence (lines 4, 5, and 6), no inhibition was detected with either antiserum, the reason for this being that when one virus component was inhibited by serum the effect was masked by the full agglutination given by the remaining 1.5 to 2 units of the other component. As would be expected, the use of eight units of these mixtures (last columns of Table I) merely widened the zone of masked inhibition.

With this much certain, the working hypothesis was formulated on which most of our subsequent tests have been based. It can be stated as follows: Since HAI tests with specific sera and artificial mixtures of two viruses show inhibition with the predominant virus, or, in the case of near equivalence, with neither virus, then a preparation which shows inhibition with both sera contains a combination form as the major component, consisting of virus in which all or part of the specific antigenic complex of both parent types are contained in a single discrete unit. In other words, a double inhibition would indicate some kind of interaction between the two virus strains. This hy-

TABLE I

HA Inhibition Titrations of a Series of Mel and WSN Mixtures Tested with Unabsorbed and Absorbed Sera

	Mixture containing HA units		Unabsorbed sera HA inhibition titer		Absorbed sera HA inhibition titer		Absorbed sera tested against total of 8 HA units	
No.	Mel	WSN	Mel	WSN	Mel	WSN	Mel	WSN
1	4.0	0	25,000	256	3,000	<32	1,500	<32
2	3.5	0.5	25,000	256	3,000	<32	1,500	<32
3	3.0	1.0	16,000	512	1,000	<32	<32	<32
4	2.5	1.5	12,000	1,000	<32	<32	<32	<32
5	2.0	2.0	6,000	3,000	<32	<32	<32	<32
6	1.5	2.5	3,000	4,000	<32	<32	<32	<32
7	1.0	3.0	512	6,000	<32	1,500	<32	<32
8	0.5	3.5	512	8,000	<32	1,500	<32	512
9	0	4.0	256	8,000	<32	2,500	<32	1,500

Strains WSN and Mel were mixed *in vitro* to give the proportions of HA units shown in the first two columns. Four HA units of each of these mixtures were titrated against falling twofold dilutions of immune rabbit serum. The reciprocal of the highest dilution of antiserum giving agglutination against the various sera are shown in columns 3 to 6. The last two columns show the inhibition titers against a total of eight HA units of the same mixtures.

pothesis has proved useful and subsequent experiments have tended to confirm it. The main questions in the beginning were whether exchange of antigens took place at all, and, if they did, would such combination forms be inhibited by both parent sera or possibly by neither. To make many observations on a wide scale, a simple, reproducible test was necessary which could be used with a minimum of effort.

All material in which virus combinations were suspected was first tested for HA titer and then four units was used in HAI tests against a single dilution of two specific antisera. Inhibition with a single serum was taken to indicate the predominant parent type of virus present. Inhibition with neither serum was indicative of the presence of both parent types in nearly equivalent amounts, and this reaction was seldom seen. Inhibition with both sera was taken as evidence that a combination form of virus predominated. When the absorbed

sera had homologous titers of 1/2000 they were usually used at a final dilution of 1:128 or 1:256. Controls were run in every test with parent viruses to insure the absence of crossing antibody or normal inhibitor in the sera. This test was used as the first means of typing infected fluids. It is a fairly obvious fact, but should be borne in mind, that the test shows only the predominant type of virus present and does not rule out the occurrence of lesser amounts of other types in a given fluid.

The question whether doubly inhibited virus forms could be induced under experimental conditions was soon answered by a test performed for another purpose.

0.1 ml. of a 10^0 dilution of the M and W strains was inoculated simultaneously into the allantoic cavity of several chick embryos. After 9 hours' incubation, the chorioallantoic membranes were removed, washed, and ground in glucosol. Fifty embryos were inoculated with the limiting infective dilution (10^{-7}) of this membrane suspension. Thirty eggs yielded allantoic fluids containing detectable HA. Of these, six were of type W and 24 were of type M. All of the M fluids were injected into mice intracerebrally at dilutions 10^{-1} and 10^{-2}. The lower dilution of six fluids killed mice within the usual incubation period for strain W. Subculture of these mouse brains in the allantoic sac yielded W fluids in every case, thus showing clearly the inadequacy of a single limiting dilution passage in establishing strain purity. In one of these six groups all the mice that received the 10^{-2} dilution of the M fluid survived. One animal was sacrificed at 4 days and the limiting infective dilution of its brain for the allantoic sac was found to be 10^{-1}. Of four chick embryos, positive as a result of inoculation, one gave an M fluid and two gave W fluids. The fourth contained a virus hemagglutinin which was readily inhibited by both M and W antisera, indicating the predominance of a combination form. This new type of virus was carried through seven serial egg passages but was eventually lost, so no further details will be given. However, the fact that combination forms existed gave encouragement in the search for their production by other means. A detailed description of this search will be limited to one technique by which we have been able to produce such forms at will.

The Effect of Simultaneous Inoculation of Influenza A Strains FM-1 and WSN into the Allantoic Sac

Preliminary tests involving double infection of the allantoic sac with influenza A virus strains FM-1 and WSN, showed that combination viruses could be readily produced, but the yield of such forms was affected by a wide variety of factors. The following experiment was performed to examine the effect of varying the proportion and the total amounts of strains FM-1 (F) and WSN (W) in the inoculum. Two incubation times were employed and the type of virus produced in the chorioallantoic membranes was compared with that occurring in the corresponding fluids.

Strains FM-1 and WSN were chosen for this work since they represent distinct virus types within the influenza A group. Freshly prepared seed virus of each type was concentrated 100 times by centrifugation at high speed and then diluted in tenfold steps. Various pairs of these dilutions were combined in equal volume and injected into four to nine embryos (0.1 ml. each). After 20 hours, some of the embryos were chilled and the membranes and allantoic fluids were harvested. The remaining eggs were incubated for 48 hours. All

fluids and membrane suspensions were tested for the serological type of the predominating virus by the simple method described in a previous section.

TABLE II

Simultaneous Inoculation of the Allantoic Sac with Mixtures of Influenza A Strains WSN (W) and FM-1 (F)

Duration of incubation	Source of virus treated	ID_{50} of F virus inoced./egg	ID_{50} of W virus inoced./egg				
			$10^{8.9}$ (10^{2})*	$10^{7.9}$ (10^{1})	$10^{6.9}$ (10^{0})	$10^{5.9}$ (10^{-1})	$10^{4.9}$ (10^{-2})
hrs.							
20	Fluid	$10^{9.7}$ (10^{2})*	W W W W W	W X F F F	F F F F F		
	Membrane		W W W W W	F – F F F	F F F F F		
48	Fluid		W W W X	F F F F	F F F –		
	Membrane		X – – –	F – – –	F F – F		
20	Fluid	$10^{8.7}$ (10^{1})	W W W W F	W W X X	F F F F –		
	Membrane		W W W W F	W X X X	F F F F –		
48	Fluid		W W W	X X X –	W F F F	F F F F	F F F F
	Membrane		W – –	– – – –	– W F F		
20	Fluid	$10^{7.7}$ (10^{0})	W W W W W	W W W X X	X F F F F		
	Membrane		W W W W –	W X X X W	X X F F–		
48	Fluid		W W W W	W W W W	X X F F	F F F F	F F F F
	Membrane		W W W W	W W – –	– – – –	– – – –	
48	Fluid	$10^{6.7}$ (10^{-1})	W W W W	W W W W	W W W W	W X F F	W F F –
48	Fluid	$10^{5.7}$ (10^{-2})*		W W W –	W W W F	W X F F	X X X –

F, W, and X indicate the predominant type of virus found in the allantoic fluid or membrane suspension after the indicated incubation period.

–, indicates that the HA titer was below detectability.

* Figure in parenthesis indicates dilution of inoculum in relation to original allantoic fluid.

Table II shows the number of ID_{50} of each virus in the various inocula and the subsequent yield of the three serological virus types. In following several of the columns down vertically it will be seen that with constant W and falling F inoculum the type of virus found shifted from F to W. The combination or X forms appeared mainly in the region of transition. Twenty-two embryos yielded either fluids or membranes in which the X form predominated. All but one of these came from eggs in which the F/W ID_{50} ratio of the inoculum was either 6.0 or 0.6. X forms were produced throughout a wide range

of inoculum size. In a number of eggs the membrane HA titer was below detectability, especially after 48 hours. In only five eggs was X virus found in the membrane and not in the fluid, and subsequent tests have been confined to the latter. In that part of the experiment in which the incubation time was varied, 21 per cent of the eggs yielded X virus after 20 hours and 23 per cent after 48 hours.

The Effect of Simultaneous Inoculation of Influenza Strains WSN and Melbourne into the Allantoic Sac

A number of pairs of influenza strains were tested in a manner similar to that described in the preceding section. All of them yielded combination forms. The results of simultaneous inoculation of strains WSN (W) and Mel (M) are given (Table III) because they illustrate a few points not found with F and W and because these strains were used for the passage studies described in the succeeding paper. The experiment was set up in the same manner as the previous test, except that M virus was concentrated only 10 times instead of 100 and the range of dilutions employed was wider than before.

It will be seen that the highest percentage of X fluids occurred in eggs that were given large inocula, and this was typical of our general experience in many such tests. Again the combination virus appeared over a wide range of dilutions. With the large inocula, X virus occurred mainly when the W/M ID_{50} ratio was 1.0 while with more dilute inocula the optimum ratio for X production shifted to 100. This difference is probably correlated with the fact that M virus grows at a much faster rate than W virus, and hence with dilute inocula 100 times more W was needed for the two agents to be present in nearly equivalent amounts by the time multiple infection of cells was taking place in the allantoic sac. Out of a number of M-W experiments similar to this one, no test failed to produce some X forms, and one test yielded a much higher percentage of X fluids than the one shown in Table III.

Double infection was also tested with tissue cultures, in which whole allantoic membranes were incubated in glucosol plus bovine serum ultrafiltrate. The tissues were washed 1 hour after adding the inoculum, and after 24 hours' incubation some membranes and suspending fluids yielded virus which was predominantly a combination type. The distribution of X forms was similar to that found with double infection of the allantoic sac.

Estimation of the Amount of Combination Virus in Allantoic Fluids

The two-tube serum test for the quick typing of virus in allantoic fluids yields no information concerning the quantitative relationships of the various constituents which cause hemagglutination in our materials. In dealing with a simple artificial mixture of two strains, the HA titer of each virus can be determined readily by making dilutions for the HAI test in two spe-

TABLE III

Simultaneous Infection of the Allantoic Sac with Influenza A Strains WSN (W) and Melbourne (M)

ID_{50} of M virus inoced/egg	Duration of incubation	ID_{50} of W virus inoculated/egg						
		$10^{9.0}$ (10^{2})*	$10^{7.0}$ (10^{1})	$10^{6.0}$ (10^{0})	$10^{5.0}$ (10^{-1})	$10^{4.0}$ (10^{-2})	$10^{3.0}$ (10^{-3})	$10^{2.0}$ (10^{-4})
	hrs.							
$10^{8.0}$	24	X X X X X X	X X X M	M M M M M M	M M M			
(10^{1})	48	W W W X X X	X X X X X X	M M M M M	M M M			
$10^{7.0}$	24	W W W W W X	X X X X X X	M M M M M	M M M	M M M		
(10^{0})	48	W W W W M	X X X M M M	M M M	M M M	M M M		
$10^{6.0}$	24	W W W W M	W X X	X X X X X	M M M M M	M M M	M M M	
(10^{-1})	48	W W W W	W W X X M	M M M M M	M M M	M M M	M M M	
$10^{5.0}$	24	W W W	W W X X M	W X X M M	X M M M M	M M M M M M	M M M	
(10^{-2})	48	W W W	W W W W W	X X M M	M M M M M	M M M	M M M	
$10^{4.0}$	24	W W W M	W W W W M	W W X X	X X X M M	M M	M M M	M M M
(10^{-3})	48	W W W W W	W W W M M	W W X M	M M M M M	M M M	M M M	M M M
$10^{3.0}$	24		W W W	W W W	W X X M M	X X M M M	M M M M M M	M M M
(10^{-4})	48		W W W W M	W W X	W W X X M	M M M M M M	M M M	M M M
$10^{2.0}$	24			W W W W W	W W W W M	X X M M M	M M M	M M M
(10^{-5})	48			W W W W M	W W W W W W	W W W X M	M M M M M M	M M M

The symbols W, M, and X indicate the predominant type of virus found in the allantoic fluids of the infected eggs after 24 and 48 hours.

* The figures in parenthesis denote the dilution of inoculum used (0.05 ml./vol.) expressed in relation to the original AF. 10^{2} indicates that the original AF was concentrated 100 times.

cific immune sera. Fluids from eggs that received an inoculation of two viruses might contain three virus forms, M, W, and X. Titration of such a fluid in M serum would give the HA titer of the W virus present, since both the M and X hemagglutinin would be inhibited and correspondingly titration in W serum would give the M titer since the W and again the X hemagglutinin would be inhibited. The difference between the total HA titer minus the M plus W titer represents the amount of hemagglutinin inhibited by both sera and hence is the X titer. Such a titer discrepancy was found by Liu and Henle in passing mixtures of Lee and PR8 in the chick embryo (11), but they attributed the result, in part at least to non-specific inhibitory effects of their

TABLE IV

Titrations of the Parent and X Virus Content of Allantoic Fluids from Doubly Infected Embryos

No.	Dilution of AF in infecting inoculum		HA titer in saline*	HA titer in W serum (M virus)	HA titer in M serum (W virus)	HA titer discrepancy (X virus)	X titer per cent of total titer
	M	W					
1	10^{2}	10^{2}	512	<16	64	432	84
2	10^{0}	10^{0}	512	64	<16	432	84
3	10^{0}	10^{1}	256	64	<16	176	69
4	10^{-4}	10^{-1}	512	24	256	232	45
5	Pool of X fluids		3000	196	256	2548	84
6	M control		2000	2000	<16	0	0
7	W control		1024	<16	1024	0	0

Twofold dilutions of allantoic fluids were made in saline (0.25 ml.) to which were added in 0.25 ml. amounts either saline or absorbed serum (final dilution 1:128) and, after mixing, 0.5 per cent cells were added (0.5 ml.) and the end-points read as the dilution of serum giving a plus-minus agglutination.

* Normal rabbit serum from which the non-specific inhibitor was completely removed was also used in place of saline with similar results.

sera. This brings out two limitations of the method of determining X titer by means of the titer discrepancy. The first is that non-specific inhibiting effects (normal serum inhibitor and crossing antibody) must be removed from a serum so completely that even the ± agglutination at the end-point of a titration is not affected. This is not difficult to do but may require that a serum which is otherwise satisfactory be re-treated with RDE or further absorbed with virus. The second limitation is that a discrepancy of 50 per cent or less of the total titer is usually not significant, a fact imposed by the in herent error of about one tube in the pattern type of HA test.

Individual and pooled X fluids from the experiment shown in Table III were tested for the HA titer of M, W, and X by diluting them in saline and in absorbed M and W sera. The latter had homologous HAI titers of 1:2000 or

more and were used at a final dilution of 1:128. Normal serum, treated with RDE and absorbed with concentrated virus, was at times used as a diluent for determining the total HA titer, but since it gave the same results as a saline diluent its use was abandoned. As will be seen from the control results with M and W virus alone (Table IV), each antiserum was completely specific in this test and gave no titer deficiency. In three fluids from eggs that received mixed inocula and contained predominantly X virus by the preliminary test, there was a titer discrepancy of 84 per cent. While this value was found repeatedly in other tests it was never exceeded. It was also commonly found that X fluids from eggs that had received relatively dilute inocula (No. 4, Table IV) showed titer discrepancies which were of borderline significance.

TABLE V

Comparison of HA Inhibition Titer of Absorbed Sera against Homologous Virus with Titer against X Virus

4 HA units of virus	HAI titer of absorbed sera	
	M	W
M	1024	<16
W	<16	128
X	512	128
$\frac{X}{\text{Homologous}}$ HAI ratio.....	0.5	1.0

Four HA units of each virus were tested against falling twofold dilutions of absorbed rabbit serum. The X allantoic fluid was one in which the combined titer of the parent viruses, M and W, was 16 per cent of the total HA content by the type of test shown in Table IV.

Demonstration of the Efficiency of Inhibition of the X Hemagglutinin

Another test of a quantitative nature consists of a comparison of the HAI titer of a specific serum in inhibiting its homologous hemagglutinin to that in inhibiting the X hemagglutinin. The fluid chosen for this test contained virus which was 84 per cent X as determined by the titer deficiency calculation. Hence, four HA units of this virus contained about one-half HA unit of M plus W virus, which was insufficient to interfere with or mask the serum inhibition of X virus. Four HA units of M, W, and this X virus were mixed with falling twofold dilutions of absorbed M and W antisera in a standard type of HAI test. The HAI titers of the sera are shown in Table V, from which it will be seen that the W serum had the same titer against W virus as against X virus and only twice as much M serum was needed to inhibit the X hemagglutinin as the M hemagglutinin. This may be expressed as an

X/W HAI titer ratio of 1.0 and an X/M HAI ratio of 0.5. The difference in ratio between the two sera is within the limits of error of the titration method.

We did not have the foresight to perform this test on a large number of X fluids but subsequent experience with passage X virus has indicated that this result was probably typical. The high efficiency of M and W sera in inhibiting the X hemagglutinin was surprising since it was anticipated that a serum which contained antibodies for only part of the specific antigens on a particle would not inhibit hemagglutination very readily.

SUMMARY

The fact that the hemagglutination inhibition test will not detect more than one component in an artificial mixture of two antigenically distinct strains of virus was demonstrated experimentally. With this proved, the inference seemed justified that the inhibition of the hemagglutinin of a virus suspension by each of two antisera, specific for differing strains of virus, would be an indication that some of the specific antigenic components from each of these viruses were both present in single particles of the virus suspension.

Two pair of influenza A strains were used to simultaneously inoculate the allantoic sac, and some embryos thus infected yielded virus which had its hemagglutinin inhibited by sera specific against each of the parent forms. Such doubly inhibitable hemagglutinin contributed as much as 84 per cent of the total hemagglutinin in certain fluids. Each of the specific antisera inhibited the hemagglutinin of the combination form very nearly as well as it inhibited its own hemagglutinin.

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