THE EXPERIMENTAL PRODUCTION OF COMBINATION FORMS OF VIRUS

II. A Study of Serial Passage in the Allantoic Sac of Agents That Combine the Antigens of Two Distinct Influenza A Strains*

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In the accompanying paper of this series (1), some experiments are presented in which the simultaneous inoculation of two distinct strains of influenza A virus into the allantoic sac resulted in the appearance of a new kind of virus containing some of the antigens from both strains. The present communication deals with the cultivation of this new form in the allantoic sac and tells of the recognition during serial passages of three varieties of it. One of these varieties was isolated in what appears to be pure culture and it is, so far as we are aware, the first instance in which antigens from two differing animal viruses have been combined in one stable unit.[1]

Methods

The accompanying paper (1) gives the details concerning the technique of hemagglutinin (HA)[2] and hemagglutination inhibition (HAI) titrations, the production of specific absorbed antisera and the performance of a two-tube serological typing test which was used throughout for the determination of the predominant kind of virus in allantoic fluids. It is important to keep in mind that this typing test disclosed only the predominant kind of virus (M, W, or X) in a suspension and did not exclude the presence of the others in lesser amounts.

The virus titrations *in ovo* used throughout the passage series were done in the usual manner (1). For 10^1 dilutions, 1 ml. of undiluted allantoic fluid was inoculated into the al-

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[1] The recombinant influenza strain isolated and described by Appleby (2) could be neutralized by antisera against both the parent types but since her reagents were not specific for the individual strains it is impossible to conclude that her organism contained antigens from two sources.

[2] HA stands for hemagglutination, HAI for hemagglutination inhibition, AS for allantoic sac, AF for allantoic fluid. M stands for strain Melbourne and W for WSN. X is used for the combination virus forms, a designation further subdivided in the text into X_1, X_2, X_3. P- indicates the passage number, X/M HAI titer ratio is the HAI titer of a specific anti-M serum against four HA units of X virus divided by the HAI titer of the same serum against four HA units of M virus. A ratio of one indicates a high efficiency of X virus inhibition.

lantoic sac. For all other dilutions a volume of 0.1 ml. was used. For neutralization tests *in ovo* triplicate sets of tenfold virus dilutions were prepared. To each tube of one set was added an equal volume of 1:8 normal rabbit serum, and corresponding amounts of absorbed anti-M and anti-W sera were added to the other sets. The inoculum per egg of these mixtures was 0.2 ml. All titrations *in ovo* were incubated for 40 hours before chilling and harvesting the allantoic fluid.

EXPERIMENTAL

It was obvious that not much progress would be made toward understanding the new combination virus unless it could be serially propagated in host cells, and all our efforts were bent in this direction for some time.

The general plan of attack was to induce X virus formation through the simultaneous inoculation of various amounts and proportions of M and W virus into the allantoic sac; and after incubation for 20 to 44 hours, the allantoic fluids from these eggs were typed. Those that yielded a doubly inhibitable hemagglutinin by the two-tube test (X fluids) were passed to new eggs (p-1) in dilutions covering the entire infective range of the suspension. X fluids from p-1 were passed to p-2 in dilutions, and so on in series.

A number of attempts were made along these lines, starting afresh each time, but the results were discouraging. X fluids from embryos that had been inoculated with M and W at a dilution higher than 10^{-1} gave rise only to M fluids in p-1. Where M and W were given at 10^{1} or 10^{2} the results were slightly better, in that occasional X fluids were found in the 10^{0} or 10^{-1} dilution of p-1 and sometimes p-2 but by the third transfer only M was found. The increasing predominance of M's can be understood from the fact that the time necessary for one ID_{50} of M virus to increase to full titer in the egg was of the order of 22 hours, while for W it was 30 hours.

It seemed possible that in preliminary tests the X form had been multiplying slowly but had been overwhelmed by the more vigorous parent strains, which regularly contaminated the original X fluids to the extent of 15 per cent or more (1). Since the X fluids from eggs given concentrated inocula in p-0 were the only ones that yielded X virus on passage it was decided to increase the size of the inoculum at each transfer by concentrating the agent from X fluids. As will be seen, this change was important, for it enabled the new virus to be successfully maintained throughout the early phases of serial passage in eggs.

The remainder of this paper deals essentially with a single experiment consisting of a passage series in the course of which three definite varieties of X virus were encountered. They will be termed X_1, X_2, and X_3. X_1 occurred in passages 1 through 10; X_2 from 10 through 32; and X_3 in passage 33 and beyond.

The Initial Formation of X Virus from M and W

The technique of inducing X virus formation by the inoculation of eggs with two strains has been covered in the accompanying paper. The proportions of M and W used for the present experiment are given in Fig. 1 (p-0).

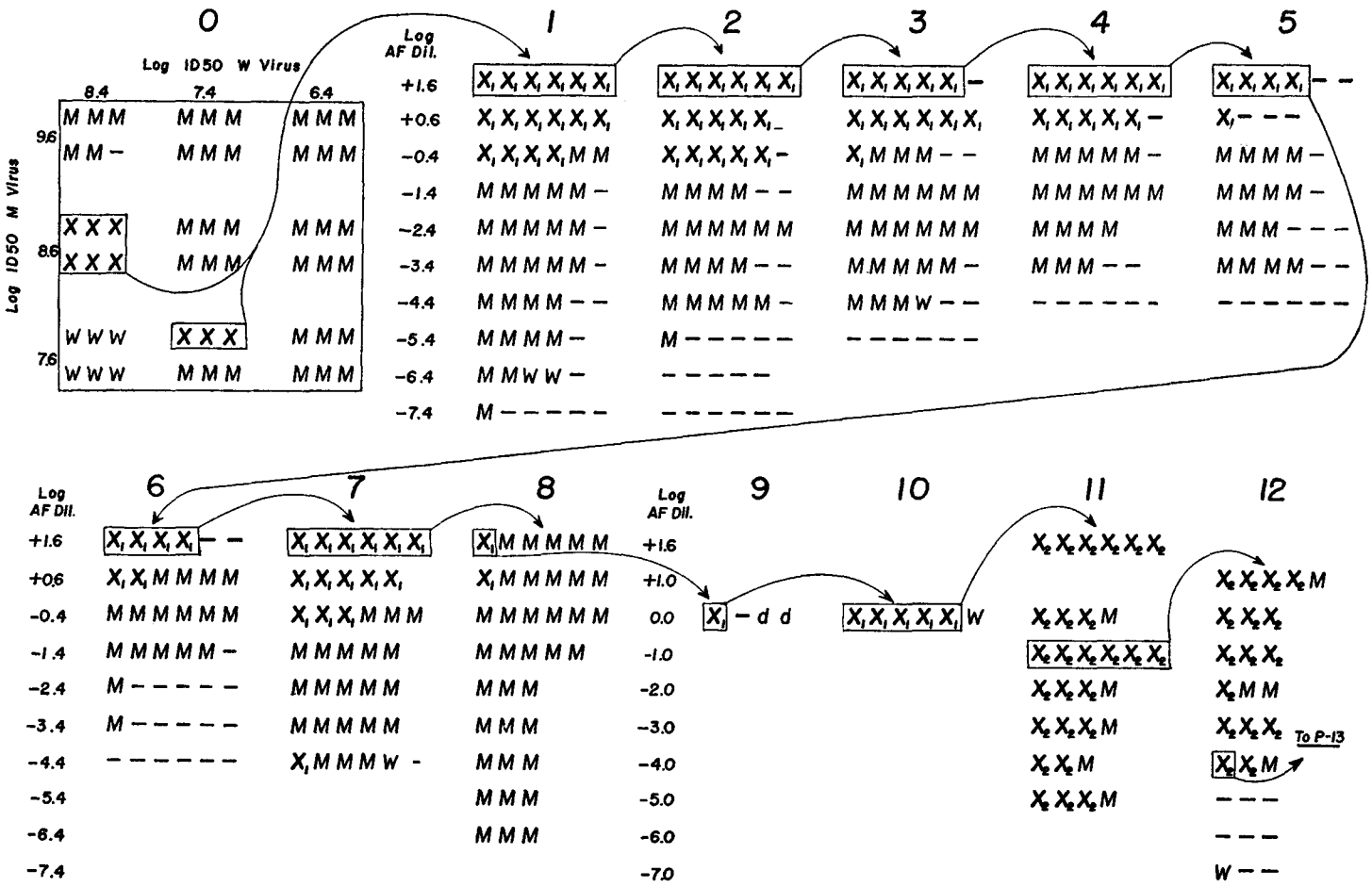

FIG. 1. The induction of X virus formation from M and W and the mode of passage of X_1 and X_2 through 12 serial transfers. The large numbers at the top of each column indicate the passage number. The letters X, X_1, X_2, M, and W indicate the predominant type of virus found in the fluid of an individual egg. — indicates no detectable hemagglutinin. d indicates death of the embryo before harvest. Under zero are given the number of ID_{50} of M and W virus used together for simultaneous inoculation. The letters boxed in indicate the fluids used for transfer of virus to the next passage, and the line of descent is indicated by arrows. Where more than one egg is included in a box the fluids were pooled and concentrated 40 times for passage. $10^{1.6}$ indicates the use of this concentrate. The pooled fluids from p-11 were filtered for passage and were not concentrated.

The allantoic fluids were harvested after 24 hours' incubation and the virus was typed in the usual way. There were nine fluids in which X virus predominated. They were pooled and the virus concentrated 40 times by centrifugation. This part of the experiment will be called p-0 since no passage of X virus was involved.

TABLE I

Tabulation of Titer Determinations from Fig. 1

	Passage No.												
	0		1	2	3	4	5	6	7	8	13	14	15
	M	W											
Log HA titer..	3.6	3.3	3.6	3.7	2.8	3.0	2.6	2.7	1.3	3.8	3.8	4.1	3.6
Total ID_{50} log.	−7.6	−6.4	−6.9	−4.9	−4.6	−3.6	−3.6	−2.0	−4.4	−6.9>	8.5	9.2	8.6
X ID_{50} log....	—	—	−0.6	−.9	0.0	+0.2	+1.2	+1.1	−0.2	1.6	—	—	—
Log total ID_{50} minus X ID_{50}.	—	—	6.3	4.0	4.6	3.8	4.8	3.1	4.2	8.5>	—	—	—
ID_{50}/HA ratio log.....	4.0	3.1	3.3	1.2	1.8	0.6	1.0	0.7	3.1	3.1>	4.7	5.1	5.0

The total virus and X ID_{50} titers were determined from data in Table II. The negative AF's below the end-point dilutions were arbitrarily excluded from the calculations since it was felt that many were negative owing to failure to inoculate the AS for technical reasons. The ID_{50} end-points were calculated for 0.1 ml. of AF and hence are 1 log lower than the usual expression of titer. Consequently, the ID_{50}/HA ratios are 1 log less than the conventional value. The ratios for the starting virus were unusually low and values of up to 5.5 for M and 5.0 for W have been obtained with these strains after repeated rapid passage from limiting dilutions.

The Maintenance of X_1 Virus through Ten Serial Passages in the Chorioallantoic Sac

X virus was started in serial passage in the chick embryo under the following conditions:

0.1 ml. of the 40 times concentrated X virus from p-0 was inoculated into the allantoic sac of each of six embryos ($10^{1.6}$ dilution of p-1 shown in Fig. 1). These eggs were incubated for 24 hours, the AF's harvested, typed, and the virus concentrated 40 times for use in p-2, etc. The line of virus passage was maintained exclusively through those eggs that received concentrated inocula and essentially the entire virus production of six embryos was used to inoculate another six at each transfer. This method was pursued for eight passages.

In addition, the passage virus was titrated in tenfold steps at each stage. The eggs receiving dilutions higher than $10^{1.6}$ were incubated for 40 hours, after which fluids were harrested.

The yield of types in individual eggs of the first eight passages is given in Fig. 1, and various quantitative aspects of the titrations are recorded in Ta-

ble I. In each passage, the total infectious titer and the titer of X virus was calculated in terms of ID_{50} per 0.1 ml. At all times, the X titer was extremely low in comparison with the total. The difference was more than a million-fold in p-1 and was about 10,000-fold from p-2 through p-7. The latter difference was maintained throughout a gradual drop and a sharp increase (p-7) in total ID_{50}. Two attempts to pass from X fluids that occurred with inocula smaller than $10^{1.6}$ ($10^{-0.4}$ and $10^{-4.4}$ in p-7) yielded only M fluids.

The vast majority of the eggs that received a $10^{-1.4}$ or higher dilution of virus yielded allantoic fluids which were M in type, the obvious reason for this being the rapid growth rate of this strain. P-4 was a typical titration in that only M fluids were found with an inoculum of $10^{-1.4}$ or less. When a small amount of anti-M serum (1:1000 final) was added to the dilutions used to inoculate p-4 (Fig. 2) some of the M's were suppressed and W fluids ap-

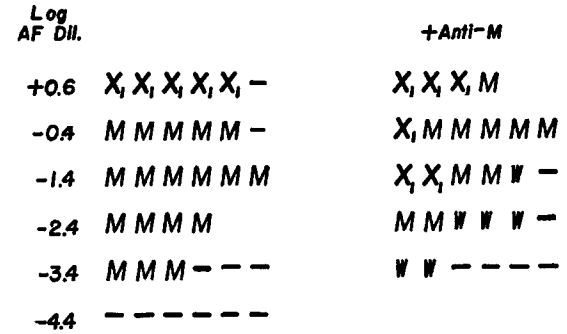

FIG. 2. Virus titration of p-4 in normal serum and in 1:1000 anti-M serum. See Fig. 1 for symbols.

peared at the limiting infective dilution. Apparently, the passage virus from p-3 contained M and W in almost identical titer. In p-1, p-3, and p-7, W fluids appeared at or near the limiting infective dilution. These facts indicated that W virus, although its growth rate was much lower than that of M virus, had been maintained at nearly the same concentration as M through seven serial passages in eggs. This was the first suggestion beside the serological evidence of double inhibition that there was some linkage between the two forms.

The course of the first seven passages gave no indication of any improvement in the ability of X virus to compete with the growth of M and W in the allantoic sac. The prospect of isolating X virus was further dimmed by the results of p-8 in which only one X fluid was found at $10^{1.6}$. This meant that the passage of concentrates had to be abandoned and on the basis of previous experience the complete loss of the strain appeared to be imminent. Hence, this fluid was passed at only a single dilution (10^{0}) into p-9. The p-9 eggs

were inadvertently left in the incubator for 72 instead of 24 hours. Whether this break in technique had anything to do with subsequent results will have to be determined in the future. Of four eggs inoculated, one was negative, two died, and the fourth yielded an X fluid. This was used for the 10th passage, which was again inoculated only at 10^0. After 48 hours, one egg yielded a W and five gave X fluids. The latter were concentrated for inoculation into p-11, which will be discussed in the next section. In view of subsequent events, the lack of titrations in p-9 and p-10 was most regrettable, but could not be corrected since the entire AF concentrate had been used up at each passage.

The Appearance and Behavior in Serial Passage of a Second Variety of Combination Virus (X_2)

The X_1 virus was able to induce the formation of X fluids in the first eight passages only when inoculated into eggs at very low dilution. A sharp change in the character of X_1 virus took place between p-8 and p-11. In p-11 (Fig. 1), X virus appeared for the first time as the predominant organism in the majority of fluids in a titration, including two in which the inoculum had been 10^{-5}. Pooled allantoic fluids from the 10^{-1} dilution of p-11 were filtered to eliminate a bacterial contaminant and the filtrate was inoculated into eggs in dilutions for p-12. Again, the X fluids appeared in profusion, including two at the limiting infective dilution. This striking and newly acquired capability of X virus was regularly manifest through many further passages and this form will be called X_2.

When X_2 first began to appear in very high dilutions it was felt that its isolation in pure form would take at most a few passages. The unexpected difficulties which were encountered testify to our failure to appreciate at this time the fundamental nature of the X_2 particle. Six early passages (p-13 to p-18) are shown in Fig. 3. An X_2 fluid occurred in the limiting dilution of each of the passages from 12 through 16. Each was used for transfer of virus in series, and in spite of this there was no consistent improvement in the yield of X fluids. M appeared as the predominant form in many dilutions while W, as in the first seven passages, occurred at or near the limiting dilution.

The results in p-16 are of special note. The virus for this passage had come through four, consecutive, limiting dilution transfers. The inoculum was diluted in twofold steps and at the end-point there were three positive eggs, one M, one W, and the third an X_2. These fluids were carefully tested by HAI (Table II) and by neutralization tests which showed that M and W were indistinguishable from the parent forms. HA titrations of the X_2 fluid showed that it contained 72 per cent X, 25 per cent M, and 3 per cent W virus. On passage, this fluid yielded all three types (p-17).

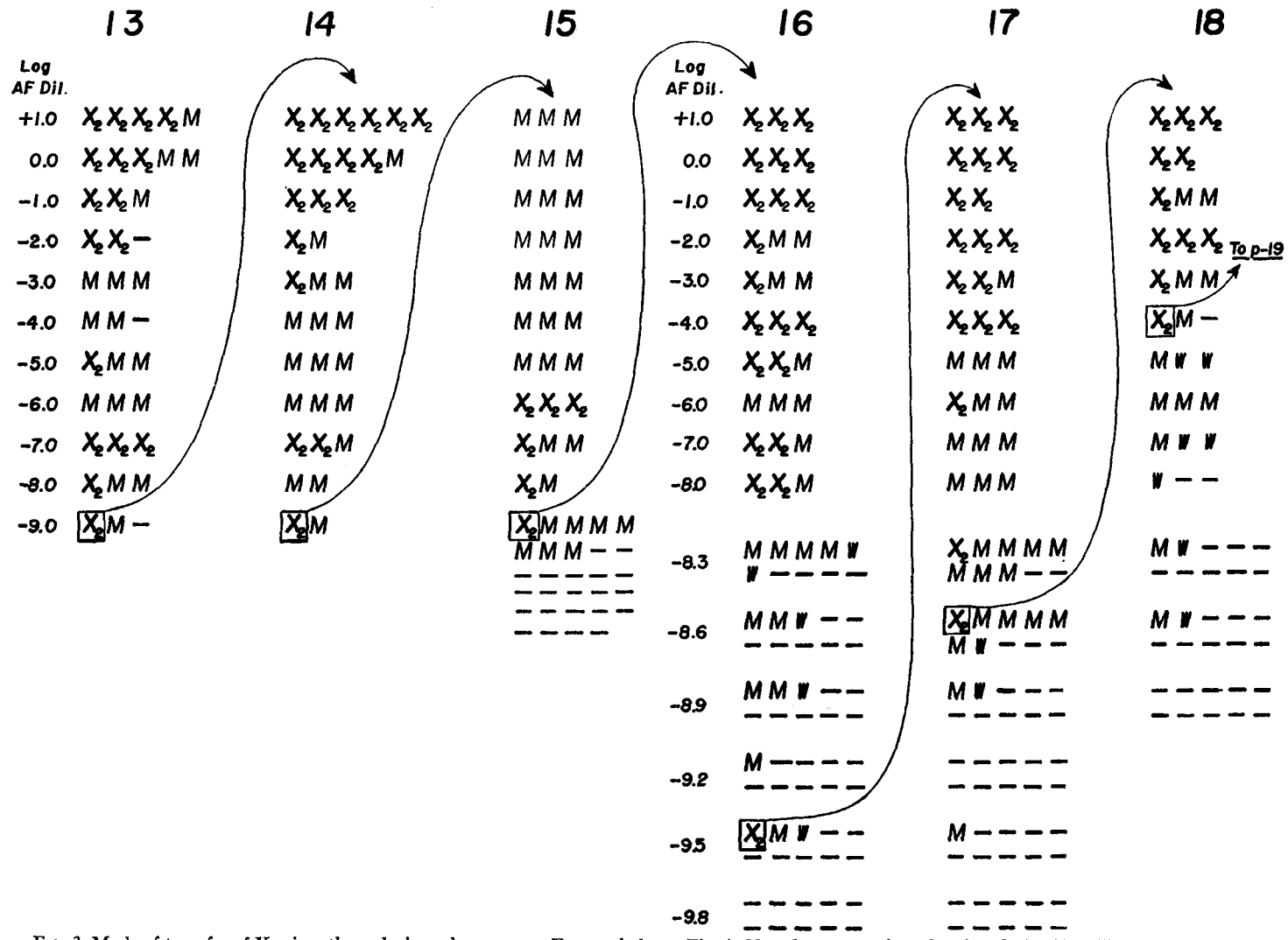

FIG. 3. Mode of transfer of X_2 virus through six early passages. For symbols see Fig. 1. 29 embryos were inoculated with the 10^{-9} dilution of the passage virus from p-14 and all are shown at the bottom of column 15.

It seemed quite unlikely that the three virus forms, M, W, and X, had all passed through four limiting dilution passages and appeared by chance with equal frequency in the final positive dilution of p-16, especially in view of the relatively slow growth rate of W and probably X_2 in relation to M. A more satisfactory explanation of this event was that the eggs of the final infective dilution of p-16 were inoculated with X_2 virus which in two cases reverted to M and W and in one instance reverted either so slowly or so late to M and W that a combination form predominated in the final yield. Data to support this interpretation will be given in another section.

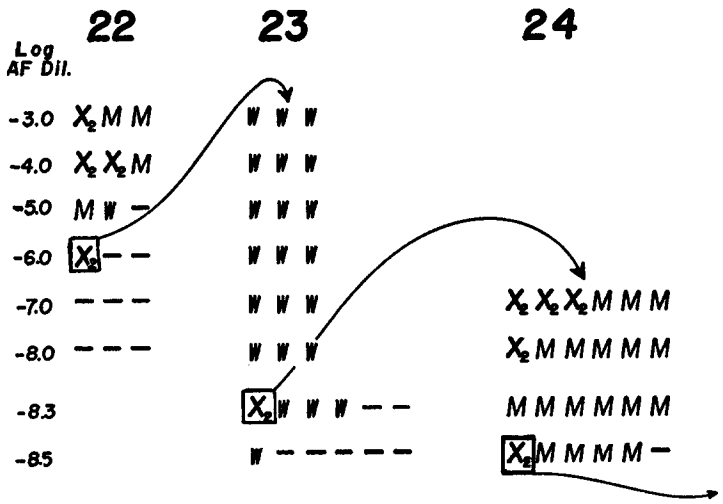

FIG. 4. A series of three serial passages of X_2 showing a marked shift in the yield of various types of fluids. See Fig. 1 for symbols.

Beginning with the 12th passage, X_2 virus was carried in several collateral lines, one from low, another from intermediate, but the main line was carried from high and usually the limiting infective dilution. Some of these lines ended in failure, yielding only M fluids, others were dropped because the results failed to improve with passage. Only the line passed from high dilutions will be discussed in detail. The total yield of X_2 fluids and their distribution within a titration varied enormously from passage to passage, and these changes followed no discernible pattern. An extreme example of this is shown in passages 22 to 24 (Fig. 4) in which an X fluid (p-22) from a titration with an average distribution of X, M, and W yielded fluids on transfer which were all W with one exception (p-23). Further passage from the lone X fluid of p-23 again gave the most common mixture of types with M occurring most frequently.

The passage titrations which have been described in detail give a somewhat

distorted impression of the average results with X_2 virus. In over 100 such titrations (main and collateral lines) the results can be briefly summarized as follows:—

Over 90 per cent of the eggs inoculated with AF dilutions 10^1, 10^0, and 10^{-1} yielded X_2 fluids. From 10^{-2} toward the higher dilutions, the percentage of X_2 fluids gradually decreased and in the last two positive dilutions was less than 25 per cent. When the virus was diluted in twofold steps the limiting dilution yielded a very low proportion of X fluids. W fluids occurred by and large only in the limiting dilution with an incidence of about 25 per cent.

Serological Studies on X_1 and X_2 in Vitro

As noted in the accompanying paper (1), the X fluids which occurred as the result of inoculation of two strains into the same egg had two outstanding serological characteristics: (*a*) They exhibited a titer deficiency; *i.e.*, the M plus the W HA titer failed to equal the total HA titer. Reasons were given for believing that this deficiency was the X titer. (*b*) Antisera specific for M and W were nearly as efficient in inhibiting X virus hemagglutination as they were in inhibiting the HA of their homologous strain.

Similar results were obtained when X_1 and X_2 fluids were tested for titer discrepancy. Pooled fluids from p-1 had discrepancies as high as 95 per cent (Table II). With X_2, the discrepancies were on the whole lower and varied between 60 and 93 per cent. M virus usually exceeded W virus in titer.

Information is lacking on the efficiency of inhibition of the X_1 hemagglutinin, but from unpublished data it is apparent that the X/M and X/W HAI titer ratios were at least 0.25 and were probably higher. Tests on X-2 virus from p-15 and p-16 are shown in Table III and in these the ratios varied in the main from 0.5 to 1.0. X_2 virus, like the X from p-0, was efficiently inhibited by both M and W antisera. Some of the M's and W's from high dilutions were tested in the same way and none showed any detectable inhibition with large amounts of heterologous antiserum.

Tests on X_2 Virus in Ovo

A virus that combines antigens from two parents should be subject to double neutralization *in ovo*. A number of attempts were made to determine the infective titer of M and W viruses in allantoic fluids by carrying out triple *in ovo* titrations in the presence of normal, anti-M, and anti-W sera. The technique is given under methods. The concentration of immune sera used was sufficient to neutralize at least one million ID_{50} of the homologous strain. Nevertheless the sera were so specific that at the same dilution they failed in repeated tests to neutralize even one ID_{50} of heterologous virus. This type of test corresponds to the determination of HA titers in a fluid by diluting in normal and immune sera (Table II). The number of ID_{50} of W virus was esti-

mated by diluting the virus in M serum, and the titer of W virus by diluting in M serum and the *in ovo* titer deficiency (total ID_{50} minus the M and W ID_{50}) should be the viable X titer. In artificial mixtures of two viruses there was no titer deficiency by this method.

HA titrations of the hemagglutinin of X_2 fluids had shown that the average suspension containing 75 per cent X_2 virus, and 25 per cent M and W.

TABLE II

Serological Tests Showing the Hemagglutinin Titer of M, W, and X Virus in Fluids from the Passage Series

Source		Total titer	M titer	W titer	Discrepancy (X titer)	Discrepancy
						per cent
p-1	$10^{1.6}$ (pool)	2000	32	64	1900	95
	$10^{0.6}$ (pool)	1024	48	16	960	94
	$10^{-1.6}$ (pool)	196	<16	<16	164	84>
p-16	$10^{-1.0}$	760	196	<16	548	72
	$10^{-2.5}$	4000	1000	128	2872	72
p-17	10^{0}	5000	380	16	5600	93
	$10^{-3.3}$	1500	196	256	1048	70
p-29	10^{0}	3000	1000	96	1900	63
	$10^{-7.0}$	3000	512	512	1976	66
p-27	$10^{-2.0}$	1500	196	96	1200	80
	$10^{-9.0}$	370	128	16	226	61
p-31	$10^{-6.0}$	750	96	380	284	38
Mel control		2000	2000	<32	—	—
W control		2000	<32	2000	—	—

Triplicate HA titrations were done in saline (or normal serum, RDE-treated) and in absorbed anti-W and anti-M sera. The failure of the last two values to add up to the total or saline titer is the titer discrepancy which is believed to be the X titer. The same sera were not used throughout and the M and W controls given here are only representative of many that were used.

A difference of this order of magnitude (3/1) was difficult to demonstrate conclusively with *in ovo* virus titrations because the inherent error of that method is so large. In a number of virus titrations on X_2 fluids, it was found that the W content was usually low, sometimes being 1 per cent or less of the total titer. The M titer was generally high and in a number of fluids was one-half to one-fourth of the total titer. The deficiency or viable X_2 titer was therefore 50 to 75 per cent of the total, a value not inconsistent with the *in vitro* results.

The foregoing tests were done with X_2 fluids from eggs that had received

virus diluted to 10^{-6} to 10^{-8}. When X_2 fluids from the 10^1 dilution were tested for neutralization the results were more striking. One such test is shown in Fig. 5. The inoculum came from the 10^1 dilution of p-20. When diluted in normal serum this suspension infected eggs through a dilution of 10^{-9}. The limiting infectious dilution of M and W for this preparation, however, was 10^{-6}. Hence, it may be concluded that dilutions 10^{-7}, 10^{-8}, and 10^{-9} contained only X virus yet they yielded all three types of fluids. Some of the M's and W's from these high dilution were investigated further and found to be

TABLE III

Serological Tests on X, M, and W Fluids for Titer Equivalence in HAI Tests

Passage No.	From egg receiving dilution	Predominant virus type in fluid	HAI titer anti-M	HAI titer anti-W
15	$10^{-1.0}$	X	2000	1500
	$10^{-4.0}$	X	1000	375
	$10^{-9.0}$	X	2000	2000
	Controls	M	2000	<32
		W	<32	4000
16	$10^{-8.9}$	W	<16	8000
	$10^{-9.5}$	W	<16	8000
	$10^{-8.9}$	M	2000	<16
	$10^{-9.1}$	M	2000	<16
	$10^{-9.5}$	M	2000	<16
	$10^{-9.5}$	X	1000	2000
	Controls	M	2000	<16
		W	<16	4000
31	$10^{-6.0}$	X	64	500
	Controls	M	32,000	<16
		W	<16	1500

HAI tests were performed in the usual fashion with four HA units of virus. Each passage was tested separately with its own controls.

serologically like the parent types; *i.e.*, they were neutralized and the HA was inhibited by one serum only. This is clear evidence that the X_2 virus reverted to original types since M and W fluids were found in dilutions 100 and 1000 times beyond the limiting infective titer of M and W. The reversion was mainly to W. Other fluids were found in which the reversion was mainly to M, signifying that the X_2 form in 10^1 dilutions was not uniform.

The Emergence and Isolation of X_3 Virus

One of the objectives of repeated passage of X_2 virus was to foster the appearance of a form in which the rate of reversion to parent types was very

low, preferably zero. It was felt that the best opportunity for this was passage from high dilution X fluids, in which presumably the reversion rate was low. However, there was no significant improvement in the yield of X fluids between the 11th and the 31st passage with the high dilution line.

It will be remembered that one of the characteristics of X_2 was the efficiency with which both M and W antisera inhibited its hemagglutinin and, as a consequence, the X_2 fluids always stood out sharply in the two-tube typing test since agglutination was completely absent with both sera. Beginning with the 25th passage, an increasing number of X fluids were found in which the inhibition with M serum was only partial. One of these fluids (10^{-6}

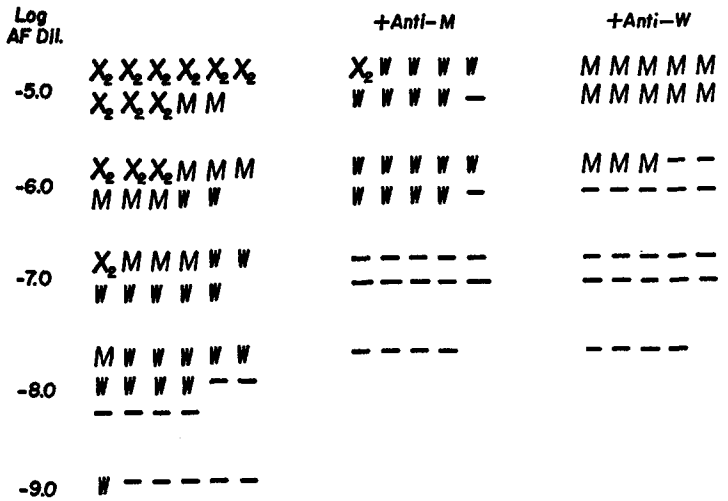

FIG. 5. Titration of passage 21 (collateral line) in normal and immune sera. The inoculum for this test came from a 10^1 X_2 fluid of p-20. Triplicate dilutions were prepared and sera (normal, anti-M, and anti-W) were added to all dilutions to a concentration (final) of 1:16.

dilution of p-31 Fig. 6) was examined by more detailed serological methods and the results are shown at the bottom of Tables II and III.

It can be seen from Table III that when this fluid was tested for inhibition of four HA units by W serum that the usual result was obtained in that it took only three times as much serum as was necessary for inhibition of four HA units of W virus. The M serum employed was quite potent and inhibited its own hemagglutinin at a dilution of 1:32,000 but a 1:64 dilution was necessary to completely inhibit four units of HA from this X fluid. These results were carefully controlled and the inhibition by M serum, though low in titer, was specific and was not due to serum inhibitors or to crossing antibody. This weak inhibition by M serum vitiated attempts to measure the HA titer of W virus in this fluid (Table II) and, hence, rendered the titer discrepancy calculation meaningless.

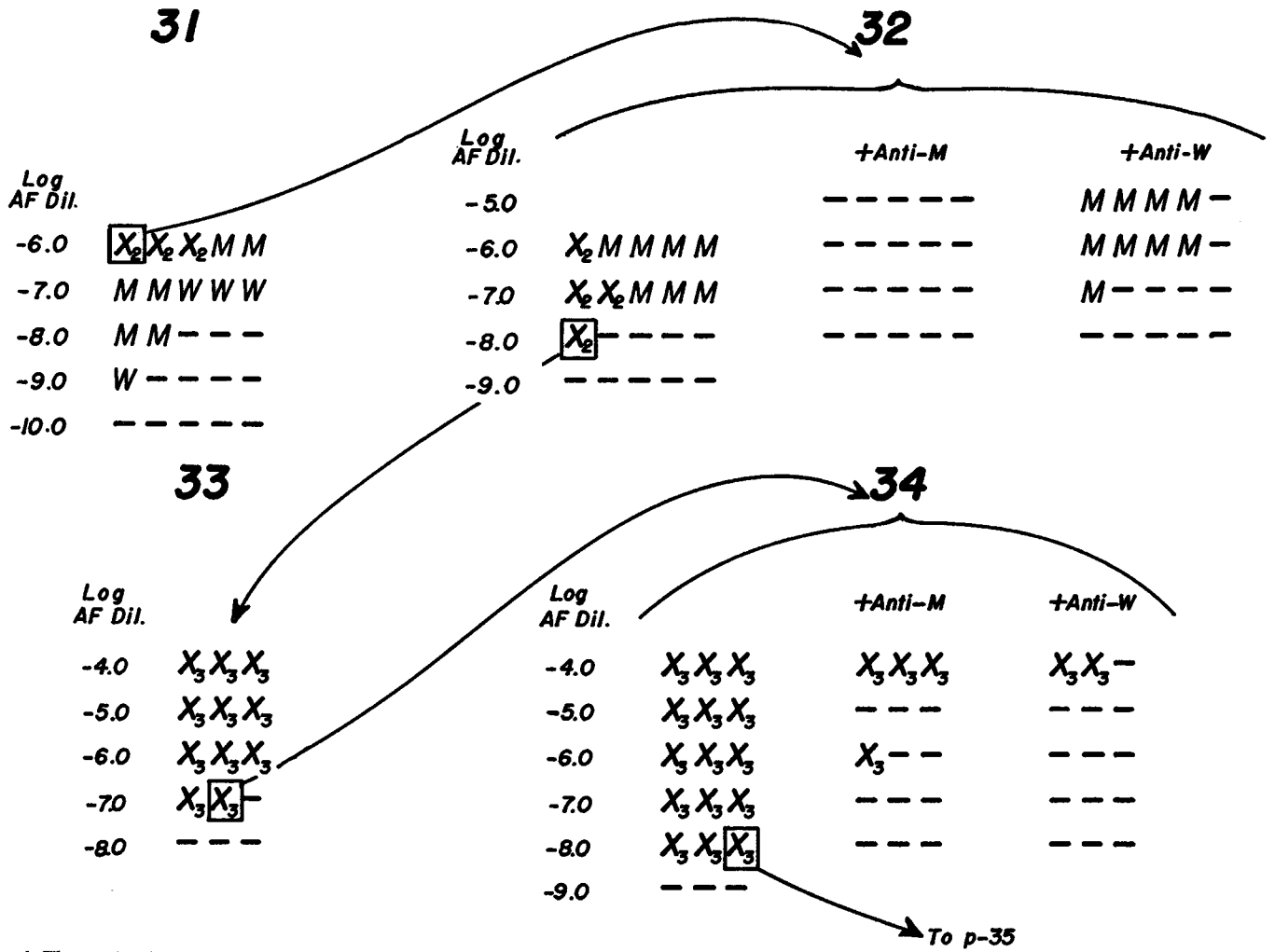

FIG. 6. The mode of transfer of virus in those passages in which X_3 was first noted. The X_2 fluid (boxed) in the 10^{-6} dilution of p-31 was studied by *in vitro* serological methods (Tables II and III). Titrations of p-32 and p-34 were carried out in normal and immune sera (see Fig. 5). The point of shift from X_2 to X_3 as shown is somewhat arbitrary and was based on the first evidence that there was no contamination with X_2.

This same fluid from p-31 was passed in dilutions to p-32 and titrations of the virus in specific M and W sera were carried out as well (Fig. 6). The suspension from p-31 titered $10^{-7.6}$ in normal serum and the yield of types in the various fluids was not unusual. The W titer of the fluid was low, being less than 0.1 per cent of the total viable virus. This sort of result had been found in previous passages. However, the M titer of the p-31 fluid as determined in the presence of W serum was only $10^{-6.6}$, indicating that only about 10 per cent of the virus present was M. Therefore, approximately 90 per cent of the viable virus in this fluid was neutralized by both sera, and hence was by definition X virus.

Passage 33 was made from the limiting dilution X fluid of p-32. It was passed in dilutions 10^{0} to $10^{-9.0}$. Only the higher dilutions are shown in Fig. 6. Every egg in this titration yielded an allantoic fluid in which X virus was predominant. This was the first time in 33 passages that an unmixed yield of X was obtained. *In vitro* serological tests on many of these fluids showed the same characteristics as the X fluid from p-31; *i.e.*, the hemagglutinin was inhibited by small amounts of W serum but only by large amounts of M serum.

Virus was passed from a limiting dilution of p-33 to p-34 and again titrations were made in normal, anti-M, and anti-W sera (Fig. 6). Again, only X virus was found. The titer in normal serum was $10^{-8.5}$, while in M and W sera it was $10^{-4.7}$ and $10^{-4.2}$, respectively. Whether these latter figures represent the W and M *in ovo* titers is questionable since only X virus was found in the limiting dilutions of these titrations. It is more likely that these two sera were able to neutralize only about 10,000 ID_{50} of what appeared to be a new variety of X virus which we shall call X_3.

Although X_3 gave every appearance of being pure at this stage, further efforts were made to insure this point. From the limiting dilution of p-34 virus was passed to p-35 by making dilutions in twofold steps. In the limiting dilution of p-35, one egg out of ten was infected and virus was passed from this to p-36, when again dilutions were made in twofold steps and again the limiting dilution contained only one infected egg of ten inoculated. At this point the virus had passed through five consecutive limiting dilution passages and in p-37 titrations were again made in normal and immune sera. The titer in normal serum was $10^{-7.5}$, in anti-M serum it was $10^{-2.7}$, and in anti-W serum was less than $10^{-2.0}$. Further limiting dilution passages were carried on from here and titration in normal and immune sera was repeated with results similar to those above but the details will not be given at this time.

Throughout all these passages, X_3 virus retained its distinctive serological characteristics, first seen in p-31, p-32, and p-33. The most striking of these was the complete absence of any tendency to reversion to parent types. No M or W fluids were encountered from p-33 on. The distinctive pattern of

in vitro inhibition was retained; *i.e.*, efficient inhibition by W sera, and weak but significant inhibition with M sera. From p-32 through p-37 increasingly significant neutralization of the virus by specific M or W antisera was noted. The ability to demonstrate the double neutralization was made possible by the lack of a tendency toward reversion in X_3.

The passage of X_3 through more than five limiting dilution passages, with retention of its essential serological characteristics, and the high degree of neutralization of the virus by both antisera in several passages is believed to be sufficient evidence that we have isolated a virus which is free from parent types. Both the *in vitro* and *in vivo* serological tests indicate that this virus contains antigens from both of two parents. The strain appears to be a stable one in that it has given no evidence of reversion to parent types. Whether X_3 is in itself "pure" or is a mixture of combinants and whether it will retain its antigenic character through prolonged passage must be decided in the future.

DISCUSSION

The experiment just described proved large and unwieldy and since this outcome was not clearly foreseen at the beginning there are many loose ends which remain to be gathered up. Some of the results, such as the shift from X_1 to X_2 virus and the emergence of X_3, were apparently chance events and we know nothing of the probability of their recurrence under similar circumstances. It would, of course, add greatly to the weight of evidence if this same passage series were repeated in its entirety, and this may be done in due time.

It is assumed throughout that X virus was a type of agent produced by interaction between two serologically distinct viruses, in which part or possibly all of the specific antigenic components of both parent types were included in a single particle. The reasons for this assumption, based on double inhibition and double neutralization of X virus, have already been thoroughly discussed. A second assumption, which seems equally clear, is that the X_2 type of particle reverted to either parent type in the course of infection of a single egg. Virus which was completely neutralized by both of two highly specific (M and W) sera yielded virus which was neutralized by only one or the other serum (Fig. 5). From circumstantial and evidence as yet unpublished, it seems almost equally conclusive that the X_1 virus also reverted to parent types, probably at a rate exceeding that of X_2.

The maintenance of the X_1 form on passage was accomplished by the transfer of very large amounts of virus, and it is conceivable that during the first seven passages there was no multiplication of X_1 as such. It is possible that X_1 particles reverted to parent types on infection of an egg and the latter after multiplication were reformed into X_1 virus by the same mechanism

which operated in p-0. Since the reversion rate of X_1 is not known, this interpretation must remain in doubt and we prefer the view that X_1 did multiply as such but that reversion and reformation of X virus probably also occurred in each passage. These latter events may have led to the chance union between an M and a W particle in which the tendency to reversion was less than with X_1. This lower rate permitted the new X form to occasionally predominate in an infection initiated by one particle, and thus permitted us to separate X_2 from X_1.

Serial passage of X_2 virus gave very complicated results, at least as viewed from the varied predominance of the three virus forms in titrations. Some X_2 allantoic fluids induced combination forms in the majority of eggs in all dilutions, while a few yielded only M or W fluids on passage. The majority of the results were between these two extremes but wide variations followed each other in series for no apparent reason. The extent to which these inconsistencies can be explained is very limited in our present state of knowledge. A few of the factors involved will be pointed out.

The two-tube serological test showed that X_2 was the major component in fluids used for passage but this test could not differentiate between active and inactive virus. Hence, in some of these fluids it is possible that the titer of viable M or W exceeded that of viable X_2. Such a fluid could be expected to give only M or W fluids on passage, as was occasionally the case. It has already been noted that X_2 sometimes reverted mainly to W, at other times to M, and it is possible, though unproven, that the reversion rate itself may have varied. Such factors as these would obviously influence the yield of types in a quite unpredictable manner.

W fluids appeared mainly at or near the limiting infective dilution in X_2 titrations. When X_2 fluids were titrated in the presence of M serum it was found that the W content was usually 1 per cent or less of the total viable virus. Therefore, it would be expected that the W fluids which occurred in limiting dilutions did not come from infection with W virus in the inoculum but arose mainly from X_2 infections in which there was reversion toward the the W parent. W fluids did not occur at lower dilutions where M virus was always present in the inoculum, in which case the faster growing M virus was usually able to predominate in the final yield.

The most constant feature in over 100 titrations of X_2 was the consistent predominance of the X form in the lowest dilutions. If we eliminate from consideration those instances in which the fluids of a titration were nearly all M or all W (*i.e.* in which *viable* X_2 may not have predominated in the inoculum) and other cases in which the total virus titer of the inoculum was low (as in p-12 after filtration) then the results of inoculating X_2 at a 10^1 dilution resulted in very close to a 100 per cent yield of X fluids. Not only did 10^1 inocula give X fluids more frequently than higher dilutions but they gave fluids which contained a much higher percentage of viable X_2. X fluids

from high dilutions were found to contain roughly 50 to 75 per cent X_2 by *in ovo* test while an X fluid from a 10^1 dilution (shown in Fig. 5) contained nearly 99 per cent of its viable virus in the combination form. All this may have been due (as was postulated with X_1) to the very high multiplicity of allantoic cell infection at 10^1, which thus furnished the most ideal conditions for reformation of X virus, not only from M and W in the inoculum but also from M and W which arose by reversion of X_2.

The reasons for believing that X_3 is a combinant form from two parents has already been sufficiently discussed in the experimental section. It may not be amiss, however, to review the evidence which leads us to believe that X_3 is not a simple mixture of two viruses. The most important is the demonstration that the immune sera used were both potent and completely specific. Lack of specificity would render the results most difficult to interpret. The sera employed each gave a neutralization index of approximately one million with the homologous strain, *e.g.* when W virus was titrated *in ovo* in the presence of 1:16 absorbed W serum the titer was a million times less than when the virus was tested in the presence of normal serum. However, when W virus was tested in the presence of 1:16 M serum the titer was the same as that obtained in the presence of normal serum. The latter type of test was repeated several times.

When artificial mixtures of two viruses are prepared it is a simple matter to determine the titer of each by inoculating dilutions of the mixture into eggs with M or W serum. If exactly equal proportions of the two agents are mixed in terms of ID_{50}), the *in ovo* titer in the presence of each serum will be 0.3 log less than the total titer, a difference which is within the limits of error of the test as usually performed. If the M/W ID_{50} ratio in the mixture is, say 5:1, then titration of this suspension in the presence of M serum will give a significant reduction in titer compared to a titration in normal serum, but titration in W serum will not. In other words, this test, like the inhibition test, gives significant neutralization of an artificial virus mixture with one serum or with neither serum but never with both. Thus, the neutralization of the inoculum for p-34 and p-37 by both M and W sera is good evidence that they contain a third or combination form.

Since the inoculum for p-34 was reduced in titer by 3.5 logs or more by both M and W sera, it may be inferred that it contained one part in 3000 or less of either M and W. The chance of transferring M or W from the limiting dilution of p-34 was then fairly remote and with a second similar passage the possibility becomes negligible. In p-37, the degree of neutralization by both sera was such that contamination could be said to be less than one part in 100,000 and was probably much less. Such a small degree of possible contamination could hardly be invoked to explain the serological behavior of X_3.

Further speculation on the nature of any of the X forms described will be deferred until more complete data become available.

SUMMARY

Double infection of the allantoic sac with Melbourne and WSN viruses induced the formation of a combination virus, which had some of the antigenic properties of both parents and which maintained itself through serial passage in the chorioallantoic sac. In the course of prolonged passage in the egg, three varieties of combination virus were found. The first (X_1) occurred in ten passages and was characterized as follows: X_1 was produced in and could be successfully passed from those chick embryos that had received very large inocula of virus. The X_1 hemagglutinin was efficiently inhibited by both M and W antisera. Each passage fluid, containing X_1 as the predominant strain, also contained large amounts of the parent forms M and W. It is very likely that X_1 reverted to parent types of virus at a high rate.

The second variety (X_2) arose from the passage series of X_1 and was carried for approximately 20 passages without definite alteration in its character. It differed from X_1 in that it had the ability to appear as the predominant form occasionally in embryos that had received a limiting infective dilution of virus. Because of this, it seems probable that X_2 reverted to parent types at a slower rate than X_1. Like X_1, this virus was never obtained in pure form and suspensions in which it predominated contained large amounts of M and W, especially the former. Good evidence was obtained that X_2 reverted to M and W virus while multiplying in the chorioallantoic sac.

X_3 was derived from passage of X_2 virus after 32 transfers and can be characterized in several ways: (*a*) X_3 yielded the only X_3 fluids on passage in eggs. This was in striking contrast to X_2 which could not be passed without giving rise to some fluids in which M and W predominated. (*b*) The X_3 hemagglutinin was weakly inhibited by all but the highest concentrations of M serum, but was strongly inhibited by W serum. X_2 was readily inhibited by relatively small amounts of both sera. (*c*) X_3 virus, after several passages at limiting dilutions, was neutralized to a highly significant degree by specific M and W sera. This is not necessarily an essential difference from the behavior of X_2 since technical difficulties (large amounts of M and W in X_2 fluids) may have prevented the demonstration of equally striking double neutralization of this virus. (*d*) After five limiting dilution passages, the X_3 virus retained the characteristics listed under (*a*) to (*c*). It is felt that the foregoing facts justify the conclusion that X_3 is a stable virus which combines some of the specific antigens of two parent types.

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