THE EXPERIMENTAL PRODUCTION OF COMBINATION FORMS OF VIRUS

III. The Formation of Doubly Antigenic Particles from Influenza A and B Virus and a Study of the Ability of Individual Particles of X Virus to Yield Two Separate Strains*

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It was shown in previous work that infection of the allantoic sac with two influenza strains could give rise to a new kind of virus which contained antigens characteristic of both parent types (1). This doubly antigenic virus was called X virus and in the course of studying it three varieties of the new agent were found (2). These may be contrasted briefly:—

X_1 virus did not reproduce itself when introduced into an egg but, instead, gave rise to parent-like strains of a single serotype.

X_2 virus was successfully propagated by serial passage in eggs, using limiting infective dilutions. It appeared to multiply in the chick embryo, but, in addition, it gave rise to viruses of single antigenic types.

X_3 virus contained very unequal quantities of the two parent antigens (in contrast to X_1 and X_2) and was stable during egg passage. It is not considered in this report.

When X_1 and X_2 viruses were first isolated, their very striking double serotype led at once to the suspicion that single particles might contain the total genetic as well as antigenic specificity of both parents. This view seemed less justified after it was discovered that infections with influenza A and B viruses gave rise to X forms, for this would have meant that two antigenically unrelated viruses could interact genetically. Partly as a consequence of this, we suggested that the X_1 form might be caused by the phenomenon known as phenotypic mixing (3), a reaction already well known for bacteriophage, which is not due to genetic interaction between particles in the usual sense (4, 5). However, phenotypic mixing (as will be made plain in the discussion) was inadequate to explain the behavior of a doubly antigenic particle which could multiply, such as X_2. Furthermore, the evidence strongly suggested that a single particle of X_2 virus was capable of initiating infections which yielded *both* parent types, since it

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could be propagated in series, utilizing limiting infectious dilutions. It was therefore assumed that the X_2 particle had that part of the genetic complex of each parent which transmits serological specificity (heterozygous for antigenic type) or else that X_2 was diploid. In summary, it was suggested that X_1 viruses arose as the result of phenotypic mixing and that the X_2 viruses were either heterozygous or diploid.

We were dissatisfied with the evidence that a single X_2 particle could give rise to both parent serotypes and the work reported here is an attempt to get more information on this point. This was achieved in part by a detailed study of limiting infectious dilutions of virus in the egg, first of normal virus and then of various X forms. These new experiments lead, we believe, to a more satisfactory hypothesis regarding X virus. In the experimental section below, the demonstration of X forms from mixed A-B infections is given first and this is followed by the analysis of virus yields of individual eggs from limiting infectious dilutions.

Methods

The following strains of influenza virus were employed in the present work: PR8 (P), Lee (L), Melbourne (M), and WSN (W). All are influenza A strains, except Lee which is influenza B.

In the first part of the experimental procedure reported below, a pattern was followed which is essentially the same as that which was previously employed with other strains (1). For this reason, a detailed description of the methods of rabbit serum production, antibody absorption, hemagglutinin titration, etc., will not be repeated here.

Some difficulty was experienced with Lee antisera which sometimes contained non-specific inhibitor for A strains. This inhibitor was not much affected by RDE treatment but could be removed by absorption with heterologous virus. The Lee and PR8 sera were absolutely specific in neutralization tests.

The methods used in the study of limiting infective dilutions are given in the text.

EXPERIMENTAL

The Occurrence of X Forms Following the Simultaneous Inoculation of Influenza A and B Virus into the Allantoic Sac

The formation of X_1 virus was first described with two pairs of influenza A strains, Melbourne-WSN and WSN-FM-1 (1). Several combinations of these viruses with the PR8 strain have also been tested and no pair failed to give the X_1 form when the infecting strains were inoculated in a ratio of about 1/1 (in terms of ID_{50}). We tested influenza B virus (Lee strain) with each of the A strains mentioned above and again obtained X_1 forms with regularity from every combination. The description below concerns more detailed studies on a single pair.

PR8 (P) and Lee (L) virus were freshly prepared by inoculating eggs with dilute (10^{-6}) seed and the yield was concentrated 100 times by centrifugation. Twofold dilutions were

made of each concentrate and those of strain PR8, for example, were added in equal volume to concentrated Lee virus and *vice versa*. These mixtures, which covered a wide range of P/L ratios (in terms of ID_{50}) were inoculated into 6 eggs each. After 18 hours' incubation, the eggs were chilled and the allantoic fluids removed and tested for HA titer. 4 HA units of each sample were tested for inhibition by PR8 and Lee antisera (1/32 dilution final). This gave information concerning the type of the predominant virus and the results are shown in Table I.

From this experiment it will be seen that the prevailing virus shifted from L to P with a very small change in the proportions in the inoculum and that X virus occurred within this narrow compass. A PR8/Lee ratio of 16/1 in the inoculum gave the best yield of X virus, a fact confirmed in other tests. With other virus pairs, such as M and W the optimum ratio was closer to 1/1. The very large amounts of virus used in the inoculation of these eggs were not a

TABLE I

Simultaneous Inoculation of the Allantoic Sac with Influenza Strains PR8 and Lee (A and B)

Total ID_{50} inoculated Log		Ratio of ID_{50} inoculated PR8/Lee	Predominant virus type in allantoic fluids after incubation					
PR8	Lee							
9.8	9.5	2/1	B	B	B	B	B	B
10.1	9.5	4/1	B	B	B	B	B	B
10.1	9.2	8/1	X	X	B_x	B	B	B
10.1	8.9	16/1	X	X	X	X	X	B_x
10.1	8.6	32/1	X	A_x	A_x	A	A	A
10.1	8.3	64/1	A	A	A	A	A	A

The inoculum was given in a volume of 0.2 ml. and the eggs were incubated at 37°C. for 18 hours before chilling and harvesting the allantoic fluid. The fluid was tested for HA titer and 4 HA units was used against standard specific antisera used at a final dilution of 1/128 in HAI tests. B_x or A_x indicates that one of the serological tests showed complete inhibition while the other gave a partial reaction. B, A, or X indicates that Lee, PR8, or X virus predominated in the fluid.

necessary condition for production of X virus, which was also found when the inoculum was less than 100 ID_{50}.

A number of X fluids were studied in more detail by other serological methods and the results are summarized in Tables II and III. In the first of these tables are given the data showing the amount of X virus in various allantoic fluids as determined by the hemagglutination (HA) and the hemagglutination inhibition (HAI) tests. This approach is based on the previously established fact (1) that the HA titer of a mixture of virus strains is the sum of the titers of individual components. When the sum of the HA titers of the two parent strains fails to equal the total HA titer the difference (titer deficiency) is the X titer or that portion of the total virus HA which was inhibited by both sera. X fluids were taken from an experiment like that shown in Table I and were tested for

the content of X virus by this method. Table II shows a representative cross-section of the results. Occasional fluids (No. 1) showed a 75 per cent discrepancy

TABLE II

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

Fluid No.	HA titer			HA titer discrepancy (X virus)	X virus per cent of total virus
	Saline	(P Virus)	(L virus)		
1	1000	125	125	750	75
2	3000	500	750	1750	59
3	1000	160	250	590	59
4	2000	500	700	800	40
5	1500	750	500	250	17
Control P	1000	1000	<16	None	—
Control L	1000	<16	1000	None	—

The fluids tested were selected for the fact that they showed inhibition with both L and P sera in HAI tests. The HA was titered in saline and both immune sera. The failure of the latter values to add up to the former gives the discrepancy entered in the fourth column which is believed to be indicative of the X titer.

TABLE III

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

Fluid No.	Log 50 per cent infectivity titer, dilutions made in			Per cent of infective virus neutralized by both sera X virus
	Normal serum Total virus	P serum L virus	L serum P virus	
1*	5.5	4.2	4.5	85
2*	5.2	4.5	3.8	76
3	6.6	5.6	4.6	89
4*	5.8	5.2	4.5	70
5	6.7	5.4	6.0	75

Fluids were taken from an experiment like that shown in Fig. 1. All of those which were predominantly X virus by *in vitro* methods were tested for doubly neutralizable virus. Each fluid was titered in normal, P, and L serum to get the infective titer. The P and L titer was subtracted from the total titer and what remained (in per cent of total titer) had been neutralized by both sera.

* These fluids were tested in titrations with virus dilutions done in threefold steps and 8 eggs per dilution.

but the majority (Nos. 2 and 3) contained about 60 per cent X virus and sometimes, as with No. 5, the discrepancy was not significant. The values found were on the whole less than were obtained by the same technique with X virus made from strains M and W, which occasionally reached 95 per cent (Table VI).

A few experiments were carried out to test the efficiency of our immune sera

in inhibiting this variety of X virus. This was done as before (1) by comparing the amount of serum necessary to inhibit 4 HA units of X virus with that required to inhibit 4 HA units of the homologous strain. X virus required two to four times as much serum as the parent strains to bring about complete inhibition. This difference was surprisingly small when one considers that the two antigenic complexes (P and L) were totally different.

In ovo neutralization was also employed for determining the X titer and is the same in principle as the use of HAI tests for measuring X virus concentrations. The total ID_{50} of a virus suspension was determined with a normal serum diluent. The ID_{50}'s of L and P virus were then measured by titrations done in immune sera. The difference between the sum of L and P titers and the total titer was a measure of the viable X titer and this was expressed as per cent of total titer. This is less sensitive than the *in vitro* method for obtaining X titers because of the large inherent error in the neutralization test.

About one-third of the fluids which showed X virus by *in vitro* tests also had significant titer deficiencies by the *in ovo* method. The results of a few of these titrations are shown in Table III. Since the P and L sera employed were shown to be absolutely specific and since a number of the titrations were carried out with closely spaced dilutions, it is felt that the existence of doubly antigenic virus was demonstrated beyond a reasonable doubt.

In an occasional fluid, the X titer was high enough so that a single dilution of it, which could infect a fair proportion of eggs inoculated, was rendered non-infective by both P and L antisera and, hence, this dilution contained mostly X virus. Such fluids were found which yielded one or both original serotypes, indicating that X virus could give rise to either parent form.

X virus was carried through 12 egg passages when undiluted allantoic fluid was used for transfer. Inoculation of smaller amounts of virus usually resulted in yields which were predominantly P or L, except for a rare egg which contained mostly X virus following an inoculum of 10^{-3} or 10^{-4}. All attempts failed to produce convincing evidence that this X form multiplied as such in the egg and hence, by definition, it was X_1.

In summary, it was found that A-B, as well as A-A combinations of influenza virus, gave X_1 forms in the egg. The A-B X virus was qualitatively like A-A X_1 virus in that single particles could react with both parent antisera as shown by means of *in vitro* and *in vivo* tests. These X forms yielded either parent type on passage.

The Frequency of Mixed Infections in Eggs Following Inoculation of a Mixture of Two Viruses at Limiting Infective Dilutions

The fact that a virus particle had antigens from two different parents suggested that it might have the genetic apparatus of each parent as well. If such were the case, then such an X particle might conceivably give rise to both

parent types. Neutralization tests on X fluids had shown that some X particles yielded one parent while other X particles gave rise to the alternate serotype. There was nothing in such evidence which confirmed or ruled out the possibility that a single X particle could give rise to both serotypes. The only suggestion in favor of this event came from a consideration of the facts about X_2 virus.

X_2 virus was propagated by serial passage through more than 20 egg to egg transfers (2). About half of these passages were made from eggs that had received one ID_{50} or less of virus and in several instances the inoculum was as small as one-eighth ID_{50}. If infection is ever initiated in the egg by a single particle (a possibility discussed below), then the X_2 virus from this passage series may have gone through a single particle stage, not once but several times, and yet both parent types were always present. The evidence bearing on this point is unsatisfactory because so little is known about the potentialities of limiting infectious dilutions for isolating pure virus lines.

To gain an answer to the difficult question of whether a single particle can give rise to two types of virus it was first necessary to get an idea of the frequency with which infections are initiated by one and by two or more particles when the inoculum is very small (one ID_{50} or less). To obtain this information it was decided to infect eggs with artificial mixtures of two strains, each of which could be readily detected in the presence of the other by serological test. By examining individual eggs to determine whether they had been infected with one or two strains, we hoped to establish frequencies for the occurrence of singly and multiply induced infections with well defined dosages of virus. Only those multiple infections initiated by two or more serologically different particles could be detected.

Artificial mixtures of strains M and W (A-A) and P and L (A-B) were used for these tests. Fresh suspensions of each of the strains to be used were prepared and the two viruses were carefully titered in eggs using threefold virus dilutions. On the basis of these preliminary titers, the two viruses were combined in such a way that the final mixture contained equal amounts of each. The mixture was diluted in twofold steps over the limiting infectious range and each dilution was inoculated into 30 to 40 eggs. An attempt was made to test those dilutions which contained one ID_{50} or less and to include a dilution which failed to infect any eggs. After 40 hours, the eggs were chilled and the allantoic fluids tested for HA. Those which were positive were tested for the predominant serological type by the HAI test. Each positive fluid was then tested for the presence of the alternate serological type by inoculating 2 or 3 eggs with allantoic fluid at 10^{-1} (A-B) or 10^{-2} (A-A) plus a specific absorbed serum against the type of agent known to be present. All hemagglutinins found in these eggs were checked for serological type by the HAI test. It seems virtually certain that virus was recovered from eggs only after an infection had taken place. Because such small inocula were used to infect, the chance of obtaining growth from unabsorbed virus particles was very remote.

Three experiments were done to determine the number of mixed infections after inoculation of artificial mixtures. Two were performed with strains M and

W and one with P and L. Liu and Henle did essentially the same experiment with strains P and L (6) as part of a larger program. Our results are very similar

TABLE IV

Data on the Yield of Single and Mixed Infections in Individual Eggs Inoculated with Limiting Infectious Dilutions of Two Virus Strains Mixed in Vitro

Experiment No.	Strains inoculated	Ratio of total eggs infected with each strain	Dilution of allantoic fluid (log)	No. of eggs inoculated	No. of eggs infected	No. of eggs infected with two strains	Per cent of total eggs infected	Per cent of infected eggs that showed mixed infection
1	M and W	49 M / 70 W	7.0	40	34	15	85	44
			7.3	40	24	4	60	17
			7.6	40	13	4	33	31
			7.9	40	12	2	30	17
			8.2	40	10	0	25	0
			8.5	25	1	0	4	0
2	M and W	52 M / 50 W	7.5	30	24	17	80	71
			7.8	29	19	10	65	53
			8.1	29	11	2	38	18
			8.4	30	10	1	33	10
			8.7	30	3	0	10	0
			9.0	30	5	0	17	0
3	P and L	23 P / 24 L	8.3	40	20	8	50	40
			8.6	40	10	0	25	0
			8.9	38	5	0	13	0
			9.2	39	1	0	2	0
			9.5	39	3	0	7	0
			9.8	40	0	0	0	0
4*	P and L	46 P / 39 L		69	36	13	56	36
				68	19	6	28	32
				71	7	0	10	0
				30	4	0	13	0

In each test all the eggs infected with each virus were added together to give the figures in the second column. These ratios give the most accurate information available on the relative amounts of the two agents present in the inoculum.

* Data on No. 4 were taken from the experiment of Liu and Henle (6), and the figures given were obtained by a technique similar to that used for the other tests. Dilutions were in twofold steps.

to theirs. All four titrations are given in detail in Table IV and much of the same data are expressed graphically in Fig. 1. From the figures on the left side of Table IV, relating to number of eggs infected with each virus, it will be seen that the proportion of two kinds of particles in the same mixture was nearly equal and, hence, was satisfactory for obtaining the maximum number of mixed infections by chance.

In Fig. 1, the size of the infecting dose of virus was plotted against the per cent of infections that were mixed infections. The curves obtained with two different pairs of agents, as well as that from the data of Liu and Henle, all agree reasonably well with a spread no larger than might be expected from the small number of eggs involved. If we assume that there was an average of one infective particle per inoculum at one ID_{50}, then on the basis of Poisson distribution we could expect 33 per cent of the infections to be mixed and actually when one ID_{50} was inoculated an average of 32 per cent of the infected eggs yielded mixed infections. This strongly suggests that most mixed infections were due to the chance inclusion of two types of virus in the inoculum,

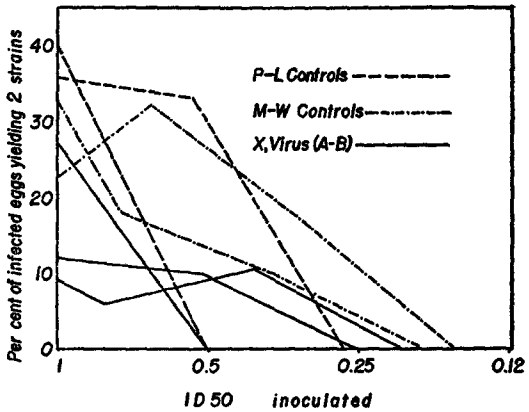

FIG. 1. The effect of the number of ID_{50} of virus inoculated on the per cent of infected eggs that yield two strains. The broken lines show control titrations in which two normal strains were mixed before inoculation into eggs. The solid lines indicate the results with X_1 virus made from A and B strains. All data are taken from Tables IV and V.

and the curves give a rough but sufficiently accurate estimate of the probability of this chance.

The Frequency of Mixed Infections in Eggs Inoculated with Limiting Infectious Dilutions of X Virus (A-B Type)

Our first experiments on the frequency of mixed infections after inoculation of X virus were done with A-B combinations and were carried out as follows:

X virus was produced from influenza A and B by the same method used in the experiment shown in Table I. Strains P and L and W and L were employed. Virus from allantoic fluid was concentrated 100 times by centrifugation and different proportions of the two strains were inoculated into eggs. Some of the fluids which showed a predominance of X virus by *in vitro* test were examined for their content of X virus as determined by double neutralization tests (see Table III). X virus was found to be present to the extent of 63, 75, and 89 per cent in three fluids and the fourth was not tested. These four preparations were inoculated

into eggs (30 to 40 per dilution) over the range of one to one-sixteenth ID_{50} and after 40 hours' incubation each infected egg was examined for the presence of the parent serotypes. Data from these tests are shown in Table V and Fig. 1.

TABLE V

Data on the Yield of Single and Mixed Infections in Individual Eggs Inoculated with Limiting Infectious Dilutions of X Viruses (A-B Types)

Experiment No.	Parent strains	X virus per cent of total virus	Dilution of allantoic fluid (log)	No. of eggs inoculated	No. of eggs infected	No. of eggs infected with two strains	Per cent of total eggs infected	Per cent of infected eggs that showed mixed infection
1	P and L	—	6.3	30	17	2	57	12
			6.6	30	9	1	30	10
			6.9	30	5	0	17	0
			7.2	30	0	0	0	0
			7.5	30	3	0	10	0
2	P and L	75	7.0	40	29	5	63	17
			7.3	40	18	1	45	5
			7.6	40	8	1	20	12
			7.9	40	4	0	10	0
			8.2	40	5	0	12	0
			8.5	40	2	0	5	0
			8.8	30	1	0	2	0
3	L and W	89	7.0	30	22	6	73	27
			7.3	29	14	0	48	0
			7.6	29	9	0	31	0
			7.9	40	8	0	20	0
			8.2	40	1	0	2	0
			8.5	40	3	0	7	0
4	P and L	63	6.8	40	12	3	30	25
			7.1	40	8	3	20	37
			7.4	40	2	0	5	0
			7.7	40	4	1	10	25
			8.0	40	0	0	0	0
			8.3	40	1	0	2	0

The percentage of X virus was determined by *in ovo* titrations of virus in the presence of normal and immune sera.

Three of the fluids yielded fewer mixed infections than the controls and yet in two of these specimens there was a very high content of X virus. Results with fluid No. 4 were not entered in Fig. 1 because the ID_{50} was not determined and the small number of eggs infected at higher dilutions makes it difficult to say whether the small increase in mixed infections was significant. The intrinsic

nature of this test makes it obvious that only large changes in the yield of mixed infections would be important.

TABLE VI

Data on the Yield of Single and Mixed Infections in Individual Eggs Inoculated with Limiting Infectious Dilutions of X Viruses (M-W Types)

Experiment No.	X type and passage No.	X virus per cent of total virus	Dilution of allantoic fluid (log)	No. of eggs inoculated	No. of eggs infected	No. of eggs infected with two strains	Per cent of total eggs infected	Per cent of infected eggs that showed mixed infection
1	X_1 p-0	74	5.9	40	34	29	85	85
			6.2	40	26	21	65	81
			6.5	30	11	6	38	55
			6.8	30	11	7	38	64
			7.1	20	1	0	5	0
2	X_1 p-0	30*	5.0	40	17	13	42	77
			5.3	40	6	3	15	50
			5.6	40	2	0	5	0
			5.9	40	3	1	7	33
			6.2	40	0	0	0	0
3	X_2 p-0	11*	4.5	30	20	16	67	80
			4.8	30	11	10	37	91
			5.1	30	8	6	27	75
			5.4	30	1	0	3	0
			5.7	30	3	1	10	33
4	X_2 p-15	95	6.0	10	9	8	90	89
			6.3	9	5	4	55	80
			6.6	10	6	4	60	67
			6.9	20	8	4	40	50
			7.2	39	7	6	18	86
			7.5	40	4	1	10	25
			7.8	40	3	1	7	33
			8.1	37	1	0	3	0

* These fluids showed no significant degree of titer deficiency by the usual method of double neutralization. The figures are an estimate based on the testing of a high dilution of virus in a large number of eggs, both with and without immune serum.

The Frequency of Mixed Infections in Eggs Inoculated with Limiting Infectious Dilutions of X Virus (A-A Type)

Four X fluids made from M and W viruses were tested for their ability to produce mixed infections with inocula of one or less ID_{50}. The results are shown in Table VI. Specimens 1 and 2 were X_1 fluids from eggs that had been inoculated with concentrated M and W viruses. Fluids 3 and 4 were X_2 fluids, one

from p-0 and the other from p-15. All fluids were tested for X virus content by the neutralization test.

The data from Table VI are summarized in Fig. 2 which also includes a line showing the average of four control tests (from Table IV). As will be readily

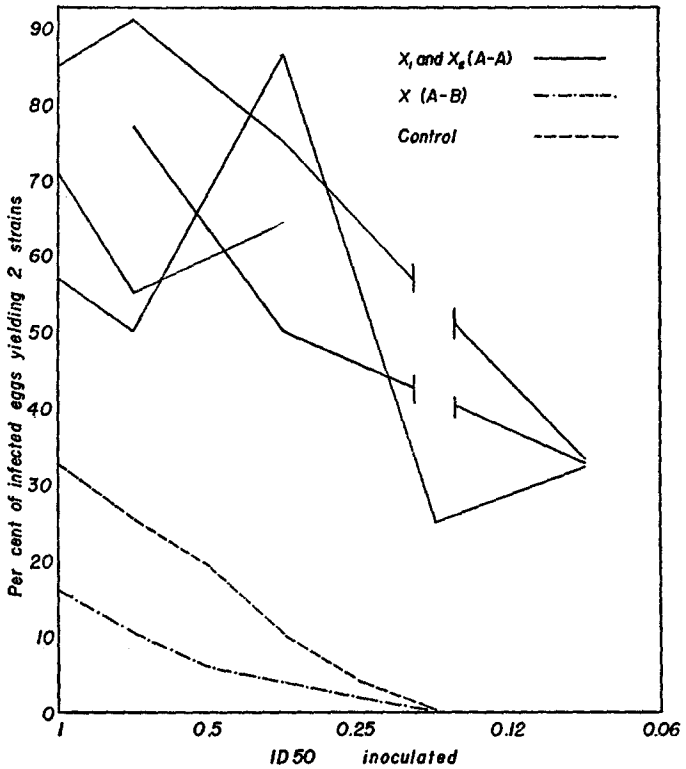

FIG. 2. The effect of the number of ID_{50} of virus inoculated on the per cent of infected eggs that yield two strains. The broken lines show (1) the average of four control titrations and (2) the average of three X virus titrations (A-B type). The solid lines indicate the yield of double infections from both X_1 and X_2 virus made from strains M and W. Points where less than three fluids were involved were not plotted on this graph. All data are taken from Tables IV, V, and VI.

seen, all four X viruses yielded doubly infected eggs in much higher percentage than the control virus, over the range of one to one-sixteenth ID_{50}. Although the number of eggs involved at some points was small, there could be no doubt that the difference was highly significant. The yields from X_1 and X_2 were almost equally good. With a variation in X titer content from 11 to 95 per cent there was no consistent variation in ability of a preparation to induce mixed infections. This result contrasts sharply with the results obtained from A-B

combinations in which a high X content was accompanied by an ability to yield mixed infections which was less than that of artificial mixtures.

DISCUSSION

Simultaneous infection of the egg with influenza A and B gave results which were quite similar to those found when two A strains were employed. Individual virus particles were formed which had antigenic components of both parent types and which were like the X_1 variety of other combinations (1). These new A-B forms had *no* unusual capacity to induce mixed infections at limiting dilutions, a fact which is of fundamental importance in understanding the formation of X virus.

The studies on limiting infectious dilutions in eggs may be recapitulated briefly. An examination of artificial mixtures of virus strains, inoculated into eggs at high dilutions, showed that the number of eggs yielding mixed infections was a function of the dose of virus employed. The chance inclusion of two particles of different serotype in a single inoculum seems adequate to explain the frequency of mixed infections. X_1 virus from A-B combinations yielded fewer mixed infections than the control but the difference was small and may have been due to the existence of unequal amounts of the two parent genotypes in the inoculum. X viruses (M-W type) gave much higher yields of mixed infections than the control. With an inoculum of one-fourth ID_{50} or less, there were *no* mixed infections among 39 control eggs but there were four among 18 eggs inoculated with X virus. Though the number of eggs is small the latter figure gives the best available estimate of the proportion of particles in these X suspensions which were able to give rise to two infections (22 per cent). Where the inoculum was one-quarter to one ID_{50}, the per cent of mixed infections with control virus varied from 5 to 30 per cent and with X virus from 50 to 90 per cent.

One way of explaining the high rate of mixed infection induced by M-W X particles would be to assume that aggregation had occurred. This factor is difficult to rule out with certainty, but a number of considerations make it seem unlikely. It would be necessary to assume, in addition, that M-W X forms tend to aggregate while all types of A-B X forms do not. More striking is the observation,—which will be reported at a later time,—that strains isolated from double infections show basic differences from the parent type, such as the acquisition of new antigens and the loss of mouse virulence, which could not be explained if the particle which gave rise to two strains were merely an aggregate. Studies on the sensitivity of X forms to ultraviolet light and on sedimentation of X virus in the centrifuge also suggest that we were not dealing with aggregates; but these and other avenues of approach to this problem need to be more fully explored.

A second way of explaining the high rate of double infection involves the assumption that a certain proportion of the particles found in X suspensions

are capable of giving rise to mixed infections. Since the two strains which arose from a single particle of this sort differed in antigenic structure, we may say that the particle was heterozygous for antigenic type. Since the two strains recovered were like their parents and different from each other in a number of other respects (rate of growth, formation of indicator state with heat, etc.), it seems likely that the single particles may have been diploid. This, in turn, suggests that other "diploids" may have been present also, but being of formula W-W or M-M were unrecognizable; and it is not impossible that such forms may constitute an important fraction of so called normal virus as produced with small inocula.

One of the facts established by examining eggs from limiting infectious dilutions was that X virus of the A-B variety had no intrinsic ability to initiate mixed infections and it was earlier established that such X particles gave rise to either A or B progeny. These facts may be restated as follows: a virus of phenotype A-B may be genotypically A or B but not A-B. This fulfills the requirements for one kind of phenotypic mixing.

Phenotypic mixing is a term which has been employed in studies on bacteriophage to indicate a particle which has a genotype from one kind of parent and a phenotype from another (5). This phenomenon was first described in 1946 (4) but recently Streisinger (7) has discovered further facts about the original example which give a much clearer picture of the reaction.

It is well known that bacteria infected with bacteriophage types T2 and T4 yield both T2 and T4 genotypes. Streisinger found, however, that the bacteriophage tails in this yield consisted of some which were antigenically T2, some T4, while some were of mixed type (T2-T4). These three kinds of tails were distributed *at random* among the two kinds of genotypes. When a phenotypically mixed particle infected a new cell the newly produced particles all had tails and genotypes of matching type; *i.e.*, the phenotypic mixing disappeared. These facts indicate rather clearly that the formation of tails is not a function of that part of the particles which transmits specificity but that more probably tails come from a common pool of material in the infected cell.

There is a very close analogy between some aspects of this example and what was found with X virus (A-B type). The latter has already been described as phenotypically A-B and genotypically A or B, while one of the products of mixed bacteriophage infection could be described as T2-T4 phenotype and a T2 or T4 genotype. In both cases infection with phenotypically mixed virus results in a yield of normal particles. In both cases parent type sera react efficiently with the mixed antigenic form. The antigens involved in both instances are affected by neutralizing antibody and both antigens are part of mechanisms for attaching their respective viruses to the host cell. A few small elements in the analogy are missing; *e.g.*, evidence for an influenza particle which is phenotypically A and genotypically B or evidence for phenotypic mixing between antigenically unrelated bacteriophage particles. However, the

resemblance is so striking that there is little doubt that the double antigenicity of the X particle is due to phenotypic mixing.

In a previous attempt to explain the various X forms, it was suggested that X_1 was due to phenotypic mixing and X_2 to heterozygosis or diploidy (3). At the present time, it seems more likely that phenotypic mixing accounts for the double antigenicity of both X_1 and X_2 and, in addition, the evidence for diploidy or heterozygosis is equally good with both forms. Thus, the behavioral difference between X_1 and X_2 is not accounted for unless we assume, as seems most likely that the latter has a more stable diploid form than the former.

SUMMARY

The simultaneous inoculation of influenza A and B strains into eggs was found to result in the formation of virus which had antigenic properties of both parent strains. The hemagglutinin of the new virus could be inhibited by both A and B antisera and the agent was also neutralized by both sera. This X particle displayed the characteristics of what we have previously called X_1. It did not reproduce itself on multiplying in the egg, but instead yielded parent types.

Limiting infectious dilutions of influenza virus were studied in eggs by inoculating two different strains at once to act as markers for singly or doubly initiated infections. A base line was thus established for the frequency of single and mixed infections at virus dosages which were fractions of one ID_{50}. These frequencies were compared with those obtained with X viruses. X_1 virus from A-B infections did not initiate an unusual number of mixed infections at the end point. By contrast, X_1 and X_2 (M-W type) gave much higher numbers of mixed infections than did the control virus.

In the discussion it is proposed (*a*) that doubly antigenic viruses are due to an effect known as phenotypic mixing and (*b*) that the ability of individual virus particles to give rise to two separate strains is due to heterozygosis or diploidy.

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