Published November 1, 1960

STUDIES OF TWO KINDS OF VIRUS PARTICLES WHICH COMPRISE INFLUENZA A2 VIRUS STRAINS

I. Characterization of Stable Homogeneous Substrains in Reactions with Specific Antibody, Mucoprotein Inhibitors, and Erythrocytes*

By PURNELL W. CHOPPIN, M.D., and IGOR TAMM, M.D.

(*From The Rockefeller Institute*)

(Received for publication, July 1, 1960)

Influenza A2 (Asian) viruses, which caused the 1957 pandemic, are antigenically closely related, but they differ markedly in sensitivity to specific antibodies and also in reactivity with serum inhibitors, urinary mucoprotein, and erythrocytes (1–11). The characteristics of variation in antibody sensitivity and in reactivity with mucoprotein receptors suggested that it might be due to the presence in the strains of two kinds of virus particles in varying proportions (10). This possibility was explored in studies with New York strains which were isolated and passed in the chicken embryo, and isolation of substrains with contrasting properties from four influenza A2 virus strains has been reported (10). Antibody- and inhibitor-sensitive substrains were designated "+" and insensitive substrains "−."

In the present communication the genetic stability of the pure populations of "+" or "−" particles is reported. The relative proportions of the two kinds of virus particles in parent strains and changes which occur in the proportions on passage of strains in the chicken embryo are described. Quantitative information is presented concerning the following properties of parent and substrains: sensitivity to serum inhibitors and to urinary mucoprotein; receptor-destroying enzymatic activity using urinary mucoprotein as a substrate; rate of elution from erythrocytes; and ability to adsorb to and agglutinate erythrocytes which have been treated with receptor-destroying enzyme of *Vibrio cholerae*. The relationship between varying proportions of "+" and "−" particles and varying properties of parent strains is demonstrated. Antigenic similarity of "+" and "−" particles is reported.

In a second communication (12) evidence is presented concerning the chemical nature and biological properties of virus inhibitory components in normal animal sera, and the basis of the profound difference in reactivity of "+" and "−" particles with serum inhibitors is explored.

In a third communication (13) changes in morphological characteristics

* Aided by a grant from The National Foundation, Inc.

exhibited by influenza A2 viruses on passage in the chicken embryo are described. Lack of correlation of biological properties of "+" and "−" particles with morphological characteristics is reported.

Materials and Methods

Viruses.—The influenza A2 (Asian) strains A/RI/1/57 through A/RI/6/57 were isolated in this laboratory in 1957 (4). The A/Japan/305, 57 strain (1) was kindly provided by Miss M. L. Miesse of the Walter Reed Army Institute of Research. These strains are referred to in this communication by the abbreviated strain designations; *i.e.*, RI/1, Japan/305, etc. The above influenza A2 strains were passed only in the chick embryo. In addition, influenza A prototype strain PR8 and influenza B prototype strain Lee were also employed.

Phosphate-Buffered Saline.—The solution designated phosphate-buffered saline (PBS) consisted of 0.85 per cent NaCl buffered at pH 7.2 with 0.01 M phosphate.

Calcium Borate Buffered Saline.—The solution designated calcium borate–buffered saline (CaBBS) consisted of 8.50 gm. NaCl, 1.0 gm. $CaCl_2$, 1.203 gm. H_3BO_3, 0.052 gm. $Na_2B_4O_7 \cdot 10$ H_2O and 1000 gm. deionized distilled water. The pH was approximately 7.0.

Erythrocytes.—Chicken and human type O blood was obtained by venipuncture. Erythrocytes were washed in the centrifuge 3 times with PBS, and suspended in the desired concentration in the appropriate buffer. All erythrocyte suspensions were used within 5 days of preparation.

Sera.—Normal horse serum was obtained through the Department of Health of the City of New York. Normal rabbit serum was obtained from male rabbits weighing 2.5 to 3.0 kg. Rabbit antisera were prepared by a single intravenous injection of 10 ml. of virus-infected allantoic fluid; rabbits were bled 2 weeks after injection. Human convalescent serum was obtained from patients 2 to 3 weeks after the onset of influenza. Clotted blood was allowed to stand at 4°C. overnight, and serum then separated from the clot by centrifugation. Sera were stored at −25°C.

Urinary Mucoprotein.—Mucoprotein was isolated from human urine by the method of Tamm and Horsfall (14). Stock suspensions of urinary mucoprotein were dissolved in deionized distilled water and stored at 4°C.

V. cholerae Filtrate.—The Inaba strain of *V. cholerae* was grown in neopeptone broth. The preparation of culture filtrate and the treatment of sera with filtrate were carried out by the procedure of Tyrrell and Horsfall (15).

Purified Neuraminidase.—Purified neuraminidase (16) was obtained from Dr. H. E. Schultz, Behringwerke, Marburg/Lahn, Germany.

Hemagglutination Titrations.—Titrations were carried out by the procedure previously described (4). The amount of virus present at the titration end point is defined as 1 hemagglutinating (HA) unit.

Hemagglutination-Inhibition Titrations.—Determinations of hemagglutination-inhibiting activity of normal or immune sera were carried out by the procedure previously described (4). The amount of virus used was 4 HA units except when stated otherwise. Hemagglutination-inhibition titrations with urinary mucoprotein were done by the same procedure except that after the addition of virus to serial dilutions of mucoprotein, the mixtures were held at 24°C. for 1 hour before the addition of erythrocytes.

Neutralization Titrations.—Two types of neutralization titration were employed: (*a*) the constant virus-serum dilution method, and (*b*) the constant serum-virus dilution method. The constant virus-serum dilution titrations were done by the method previously described (4), with a virus inoculum of 1000 fifty per cent egg infective doses (EID_{50}) and serial twofold dilutions of serum. Titrations in which urinary mucoprotein was employed instead of serum were done by the same procedure.

The constant serum-virus dilution titrations were done by the following method. Serial 0.5 log dilutions of infected allantoic fluid were made in Pfanstiehl's peptone broth, and 0.4 ml. volumes of each dilution were added to 0.4 ml. volumes of serum, or of PBS as a control. Mixtures were held at 24°C. for 15 minutes, then chilled and inoculated into 10-day chick embryos. Four embryos were inoculated with each mixture. After 48 hours of incubation the allantoic fluids were harvested and the presence of virus determined by hemagglutination of chicken erythrocytes. Fifty per cent egg infective end points were calculated (17). In neutralization titrations by the constant serum-virus dilution procedure in which urinary mucoprotein was employed, the presence of virus in allantoic fluids was determined by hemagglutination at a final dilution of allantoic fluid of 1:20. At lower dilutions agglutination of erythrocytes occurred occasionally due to the urinary mucoprotein in the inoculum.

EXPERIMENTAL

I. Composition and Stability on Passage of Parent Strains; Separation and Stability of Substrains

Isolation of Substrains.—As reported previously (7, 9), and described in detail in the accompanying communication (12), normal horse serum possesses extremely high neutralizing activity for certain influenza A2 virus strains. This property made possible the isolation of pure substrains of antibody- and inhibitor-sensitive or "+," and insensitive or "−" particles (10).

Pure substrains of the two kinds of particles were separated by passage of strains at limiting dilutions in the presence or absence of horse serum. The sensitive or "+" substrains were isolated by passage in the absence of serum. Single allantoic fluids containing inhibitor-sensitive virus were selected, and passage at high dilution was repeated. The absence of infective "−" particles in a substrain obtained by this procedure was indicated by complete neutralization of the substrain *in ovo* by 0.05 ml. of normal horse serum which had been heated at 56°C. for 30 minutes. Insensitive or "−" substrains were selected by passage in a similar manner in the presence of 0.05 ml. of heated horse serum per egg. Substrains obtained by this procedure were not neutralized by horse serum.

It is apparent that neutralization of all "+" particles by horse serum provides a means of isolating a pure substrain of "−" particles regardless of the number of "+" particles present in a strain. However, the isolation of "+" particles can be done only by the limiting dilution technique, and if a strain contains a large majority of "−" particles, isolation of "+" particles from that strain is extremely difficult and may be practically impossible.

Isolation of "+" and "−" particles was attempted from six New York strains and the Japan/305 strain, and was successful with four; *i.e.*, RI/1, RI/3, RI/4, and RI/5. The three remaining strains behaved as inhibitor-insensitive strains, and a sensitive, "+" substrain could not be separated by the limiting dilution technique. However, as emphasized above, this does not exclude the possibility of the presence of a small number of "+" particles in the insensitive parent strain.

Isolation of substrains has been possible from first passage material as well as from later passages of the four parent strains.

Proportions of the Two Kinds of Particles in Early Passages of Parent Strains.—The neutralization of "+" particles and the failure of neutralization of "−"

particles by normal horse serum provide a means of determining the numbers of "+" and "−" particles present in a strain. Thus, in neutralization test done by the constant serum-virus dilution technique, the virus which is not neutralized by normal horse serum indicates the amount of "−" virus, and the virus which is neutralized (determined by subtracting the amount of virus not neutralized from the total amount in the control) indicates the amount of "+" virus. The ratios of "+" to "−" particles can then be calculated. Since the amounts of "+" and "−" virus are determined on the basis of measurements of infective virus, these ratios refer to ratios of infective particles.

TABLE I

Proportions of "+" and "−" Particles in Influenza A2 Virus Strains

Strain	Infective units			
	Total	"−"	"+"	Ratio "+"/"−"
Jap/305	7.7×10^6	5.6×10^6	2.1×10^6	0.37/1
RI/1	5.6×10^6	5.6×10^5	5.0×10^6	9/1
RI/2	1.5×10^8	1.2×10^8	0.3×10^8	1/4
RI/3	1.8×10^7	0.8×10^7	1.0×10^7	1.3/1
RI/4	1.7×10^6	2.4×10^4	1.7×10^6	71/1
RI/5	7.6×10^7	7.7×10^3	7.6×10^7	9900/1
RI/6	8.3×10^5	1.8×10^6	0	0/1

Table I shows the results obtained with the RI/1 through RI/6 and the Japan/305 strains of influenza A2 virus. Significant neutralization by horse serum was obtained with RI/1, RI/4, and RI/5 strains, in ascending order of neutralization. Questionable neutralization was obtained with RI/3; no significant neutralization was obtained with the other strains, indicating that few if any "+" particles were present. The ratio of "+" to "−" particles in the RI/1 strain was approximately 9:1; RI/4, 71:1; and in RI/5, 9,900:1. Thus the proportions of the two kinds of particles varied widely in different strains. The PR8 strain of influenza A and the Lee strain of influenza B virus were also tested in similar titrations, and there was no neutralization of either virus by normal horse serum.

Effect of Serial Passage in the Chick Embryo on the Relative Proportions of "+" and "−" Particles.—The marked variation in the proportions of the two kinds of virus particles in different strains prompted an investigation of the effect of serial passages on the relative proportions of the two particles.

Eleven serial passages of the RI/5 parent strain were made in the allantoic cavity of 10-day chick embryos. All passages were made in low (10^{-3}) dilutions. The number of "+" and "−" particles present in the infected allantoic fluids was determined by neutralization titrations with horse serum done by the constant serum-virus dilution technique.

In every passage assayed there was a marked preponderance of "+" particles, and there was, in general, a gradual increase in the proportion of "+" particles until by the 11th passage the population consisted wholly of "+" particles. These results suggest a reproductive advantage of "+" particles over "−" particles. However, it should be mentioned that in occasional passages the relative proportion of "+" particles decreased or remained unchanged, rather than increased. Also, the fact that there was a great majority of "+" particles initially may have influenced the growth of the two particles in a mixed infection.

To investigate further the effect of serial passage on the relative numbers of the two kinds of particles, known mixtures of "+" and "−" particles were prepared and passed serially.

Mixtures were prepared by combining suspensions of "+" and "−" particles previously diluted to the desired concentration in Pfanstiehl's peptone broth. After mixing, 0.1 ml. was inoculated into the allantoic cavity of 10-day-old chick embryos. Embryos were incubated for 48 hours at 35°C. Subsequent passages were made at 10^{-3} dilution of infected allantoic fluid. The number of "+" and "−" virus particles present in each passage was determined by infectivity titrations in the presence and absence of normal horse serum as described above.

A mixture of RI/4^{+} and RI/4^{-} virus particles was prepared in which the ratio of the "+" to "−" particles was approximately 3.3:1. The ratio of "+" to "−" particles in the infected allantoic fluid after one passage of this mixture was 22:1. After seven subsequent passages the ratio was 15:1 with variations in the intervening passages from 6.5:1 to 157:1.

A mixture of RI/5^{+} and RI/5^{-} virus particles was prepared in which the ratio of "+" to "−" particles was 1:5.6. The ratio of "+" to "−" particles could not be determined in the first six passages because there was insufficient neutralization by horse serum to calculate a ratio. By the eighth passage the ratio was 1:1 and by the fourteenth passage, 2.5:1.

These results indicate that changes in the relative proportions of "+" and "−" particles occur with serial passage in the chick embryo, but marked changes in proportions usually did not occur within a few passages.

Stability of Substrains.—To determine the stability of pure substrains on passage, "+" and "−" substrains were passed serially at low (10^{-3}) dilution, and the composition of the virus population determined at each passage with "+" substrains and frequently with "−" substrains.

The purity of "+" substrain passages was determined by inoculating 10-day-old chick embryos with 0.1 ml. of a mixture of equal volumes of undiluted infected allantoic fluid and heated normal horse serum. Nine to 12 eggs were used with material from each passage, and after 48 hours' incubation the allantoic fluids were tested for the presence of virus by the hemagglutination technique. The presence of a single "−" infective dose in the inoculum would be revealed by infection of the egg, and absence of virus in the allantoic fluids from the test eggs would indicate a pure population of "+" particles.

The purity of "−" substrains could only be determined by the somewhat less sensitive

method of infectivity titration in the presence and absence of horse serum; any neutralization of infectivity by the serum would indicate the presence of "+" particles.

The pure RI/4$^+$ and RI/5$^+$ substrains were passed nine and six times respectively and remained homogeneous; no "−" particles were detected in any of the passages. Table II shows the complete neutralization by horse serum of the first passages, and the ninth and sixth passages respectively, of RI/4$^+$ and RI/5$^+$ substrains. Similar results were obtained with all intermediate passages. In further passages of these substrains "−" particles have occasionally been found in very small numbers.

TABLE II

Stability of "+" and "−" Substrains on Serial Passage

Substrain	Passage	Infective units	
		Control	In the presence of horse serum
RI/4$^+$	1	4.7×10^7	<14
RI/4$^+$	9	8.3×10^7	<14
RI/5$^+$	1	5.6×10^7	<14
RI/5$^+$	6	4.7×10^8	<14
RI/4$^-$	1	4.3×10^7	3.7×10^7
RI/4$^-$	15	0.9×10^9	1.1×10^9
RI/5$^-$	1	2.6×10^8	1.8×10^8
RI/5$^-$	15	0.9×10^9	1.4×10^9

The pure RI/3$^-$, RI/4$^-$ and RI/5$^-$ substrains were each passed 15 times, and at intervals passages were examined for "+" and "−" particles. As shown in Table II there was no neutralization by horse serum of the first or fifteenth passages of RI/4$^-$ and RI/5$^-$ strains. There was also no neutralization of the intermediate passages of these substrains or of the passages of RI/3$^-$ substrain. Thus, no "+" particles could be detected, and the "−" substrains appeared to remain homogeneous.

These results indicate that the "+" and "−" substrains remained stable during numerous serial passages at low dilution, and suggest that if mutations of one kind of particle to the other occur, the frequency of mutation is low.

Neutralization of Infectivity by Normal Rabbit Serum.—The sensitivity of six strains of influenza A2 virus to human and rabbit serum inhibitors in hemagglutination-inhibition tests has been previously reported (4). Two strains were found to be highly sensitive, RI/4 and RI/5; one strain moderately

sensitive, RI/1; and three strains apparently completely insensitive, RI/2, RI/3, and RI/6. Neutralization of some influenza A2 strains by normal rabbit serum has been reported from several laboratories (7, 9, 18, 19). The RI/1 to RI/6 and the Japan/305 strains were examined in neutralization reactions.

Neutralization titrations were done using a virus inoculum of 1000 EID_{50} and varying dilutions of serum which had been heated at 56°C. for 30 minutes. Five to 8 pairs of normal rabbit sera were tested with each virus and the geometric mean neutralization titer was calculated.

As can be seen in Table III, only the RI/5 strain was neutralized in this type of test. The failure of RI/4, which was highly sensitive in hemagglutination-inhibition experiments (4), to be neutralized might appear to indicate that neutralization of infectivity and inhibition of hemagglutination are brought about by different mechanisms. However, the explanation of this finding lies in the fact that the RI/4 strain is a mixture of inhibitor-sensitive ("+") and insensitive ("−") particles in a ratio of 71:1. Since an inoculum of 1000 EID_{50} of virus was used in the neutralization test, only strains in which the ratio of "+" to "−" particles is greater than 1000:1 can be expected to be neutralized in this type of test, as only one infective dose per egg is necessary to produce infection. The inoculum of RI/4 virus contained 14 infective doses of insensitive ("−") particles along with 986 infective doses of sensitive ("+") particles, and therefore neutralization was not observed. The situation is different in hemagglutination-inhibition reactions because a very large number of virus particles is required to cause hemagglutination in the pattern test. The presence of a small minority of insensitive particles does not obscure the inhibitory effect of serum on sensitive particles present in great majority, as in RI/4.

TABLE III

Neutralization of Influenza A2 Virus Strains by Normal Rabbit Serum

Virus*		Serum neutralization titer
Strain	Passage	
Jap/305	8	< 8
RI/1	4	< 8
RI/2	4	< 8
RI/3	4	< 8
RI/4	4	< 8
RI/5	3	24
RI/6	3	< 8

* Inoculum: 1000 EID_{50} per egg.

Neutralization of sensitive particles in mixtures in which the ratio of "+" to "−" particles is less than 1000:1 was detected by the procedure employing a constant amount of serum and dilutions of virus as shown in Table I.

II. Interactions of Substrains and Strains with Antibody and Soluble Inhibitors

Sensitivity of Substrains to Antibodies and to Serum Inhibitors.—The "+" and "−" substrains of RI/4 and RI/5 strains were examined in hemagglutination-inhibition and neutralization reactions with antibodies in human convalescent sera and with normal horse and rabbit sera.

TABLE IV

Sensitivity of "+" and "−" Particles to Antibody and Serum Inhibitors in Hemagglutination-Inhibition and Neutralization Reactions

Virus	Serum titer				
	Hemagglutination-inhibition*			Neutralization‡	
	Antibody	Inhibitors		Antibody	Inhibitors
	Human	Horse	Rabbit	Human	Horse
RI/4+	1,024	131,000	2,890	40	8,200
RI/4−	256	<16	<16	< 8	<4
RI/5+	810	131,000	2,048	35	5,900
RI/5−	128	<16	<16	5	<4

* Amount of virus: 4 HA units per tube.

‡ Inoculum: 1000 EID_{50} per egg.

In hemagglutination-inhibition reactions 4 HA units of virus was present in the final mixtures of virus and serially diluted serum. In neutralization titrations the procedure employing constant virus and dilutions of serum was used. The virus inoculum consisted of 1000 EID_{50} per egg. Normal rabbit serum was heated at 65°C. for 30 minutes, and normal horse serum was heated at 56°C. for 30 minutes. The human convalescent serum used to measure the sensitivity of "+" and "−" particles to antibody was heated at 56°C. for 30 minutes and treated with *V. cholerae* filtrate to remove serum inhibitors. The effectiveness of *V. cholerae* filtrate treatment was verified by simultaneous treatment of normal human serum containing no antibodies against the viruses employed.

As can be seen in Table IV the "+" substrains gave higher titers with antibody in human convalescent serum than did the "−" substrains in both hemagglutination-inhibition and neutralization reactions. The "+" substrains were highly sensitive to inhibitors in normal horse serum in both hemagglutination-inhibition and neutralization reactions, and they were also sensitive to inhibitors in rabbit serum. The "−" substrains were completely insensitive to inhibitors in the sera of both animals. Thus with the pure substrains there is a

positive correlation between sensitivity to antibodies and sensitivity to normal serum inhibitors. A similar correlation between sensitivity to serum inhibitors and sensitivity to specific antibodies has been found by many workers with strains of influenza A2 virus (4–8, 20).

The Effect of Varying Proportions of "+" and "−" Particles on Hemagglutination-Inhibition Titers with Antibodies and Normal Serum Inhibitors.—Differences in sensitivity to antibodies and to serum inhibitors of pure substrains prompted an investigation of the relationship between varying proportions of "+" and "−" particles in prepared mixtures and sensitivity of the mixtures

TABLE V

Effect of Varying Proportions of "+" and "−" Particles on Hemagglutination-Inhibition Titers with Antibody and Serum Inhibitors

Artificial mixtures of RI/5$^+$ and RI/5$^-$ particles, *ratio* "+"/"−"	Serum hemagglutination-inhibition titer*		
	Antibody	Inhibitors	
	Human	Horse	Rabbit
Only "−"	320	<8	<16
1:10	405	<8	<16
1:4	405	<8	<16
1:2	455	<8	<16
1:1	640	<8	<16
2:1	1,280	1,024‡	—
4:1	2,040	22,340‡	128‡
10:1	2,560	131,100	512
Only "+"	2,560	131,100	512

* Amount of virus: 4 HA units per tube.

‡ ± agglutination in all tubes before end point.

to inhibition by antibody or serum inhibitors. Such a study provides a means of confirming the conclusion that variable sensitivity of parent strains is due to the presence of "+" and "−" particles in varying proportions.

Diluted suspensions of "+" and "−" particles were prepared in PBS and mixed in various proportions. Hemagglutination-inhibition reactions with human convalescent and normal horse and rabbit sera were carried out using 4 hemagglutinating units of "+" particles, "−" particles, or mixtures of "+" and "−" particles.

The results shown in Table V indicate that in the reactions with human antibody, inhibition titers increased as the relative proportion of "+" particles was increased. In the reactions with normal animal sera some inhibition became evident when the ratio of "+" to "−" particles reached 2:1, and the inhibition titers increased as the proportion of "+" particles was increased.

In hemagglutination-inhibition reactions the end point is usually taken as the highest dilution at which there is complete inhibition of agglutination. However, in these experiments, with certain mixtures there was ± to 1+ agglutination at every serum dilution up to a point at which there was strong agglutination. This phenomenon was apparently caused by the "−" particles, present in relatively small but significant amounts in mixtures which mainly consisted of the sensitive, "+" particles. Being unaffected by the horse serum the "−" particles caused some agglutination. In these instances, indicated in Table V, the end point was taken to be in the last tube showing no more than 1+ agglutination. This phenomenon has also been found in some passages of parent strains in which the proportions of particles were similar. The apparent absence of inhibition of virus mixtures containing "+" particles in proportions as great as 1:1 is also expected, since in such mixtures there are enough "−" particles present in the final 4 HA units in each tube to produce complete agglutination of all cells.

TABLE VI

Sensitivity of "+" and "−" Particles to Urinary Mucoprotein

Virus	Urinary mucoprotein, μg.	
	Hemagglutination-inhibition*	Neutralization‡
RI/4+	0.008	19
RI/4−	0.085	>200
RI/5+	0.008	23
RI/5−	0.085	>200

* Micrograms of mucoprotein required to inhibit 1 HA unit of virus.

‡ Micrograms of mucoprotein required to neutralize 1000 EID_{50} of virus.

These results provide a definitive demonstration that antibody or inhibitor titers obtained with viral populations which are mixtures depend on the relative proportions of the two kinds of virus particle.

Sensitivity of Substrains to Urinary Mucoprotein.—Urinary mucoprotein (14, 21) is a well characterized inhibitor of influenza virus which is readily obtainable in pure state. The sensitivities of "+" and "−" substrains to urinary mucoprotein were examined in hemagglutination-inhibition and neutralization reactions.

In hemagglutination-inhibition reactions serial dilutions of mucoprotein solution were made in PBS, and 8 HA units of virus was added per tube. The mixtures were held at 24°C. for 1 hour, after which chicken erythrocytes were added. Hemagglutination patterns were recorded 1 hour after addition of erythrocytes. The end point was taken to be at the highest dilution of mucoprotein which completely prevented hemagglutination. In neutralization tests serial dilutions of urinary mucoprotein were made in PBS and aliquots were mixed with virus suspensions diluted to give a virus inoculum of 1000 EID_{50} per egg. The virus-mucoprotein mixtures were held at 24°C. for 15 minutes before inoculation.

As can be seen in Table VI, marked differences in sensitivity of "+" and "−" substrains to inhibition by mucoprotein were found. Hemagglutination

by the RI/4$^+$ and RI/5$^+$ substrains was inhibited by 0.008 μg. of mucoprotein per 1 HA unit of virus. This compares closely with the results obtained earlier with the Lee strain of influenza B virus which had been heated at 56°C. for 30 minutes (14). However, 0.085 μg. of mucoprotein was required to prevent hemagglutination by RI/4$^-$ and RI/5$^-$ substrains.

In neutralization tests the two "+" substrains were neutralized by 19 and 23 μg. of mucoprotein respectively, whereas the "−" substrains were unaffected by 200 μg. In neutralization tests reported previously (10) in which serial dilutions of pure "+" and "−" substrains and a constant amount, 100 μg., of mucoprotein were used, there was a decrease in infectivity titer of 5.84 log units with RI/4$^+$ and 4.32 log units with RI/5$^+$, whereas with RI/4$^-$ and RI/5$^-$ there was a decrease of only 0.75 and 0.22 log units, respectively.

These results show that the difference in sensitivity of "+" and "−" particles to urinary mucoprotein qualitatively parallels those in sensitivity to specific antibody and serum inhibitors.

Sensitivity of Parent Strains to Urinary Mucoprotein.—The sensitivities of the parent strains RI/2 and RI/5 to urinary mucoprotein were determined in hemagglutination-inhibition and neutralization reactions using the same procedures as in experiments with substrains.

Hemagglutination by the RI/5 strain was inhibited by 0.008 μg. of mucoprotein per hemagglutinating unit, and 33 μg. neutralized 1000 EID_{50} of this strain. With the RI/2 strain 0.03 μg. of mucoprotein was required to inhibit 1 hemagglutinating unit, and there was no neutralization by 200 μg. Since the RI/5 strain consists predominantly of "+" particles, and the RI/2 strain predominantly of "−" particles, these results correlate well with those obtained above in reactions with pure substrains and urinary mucoprotein.

In neutralization tests reported previously (10) in which serial dilutions of parent strains and a constant amount, 100 μg., of mucoprotein were used, there was a decrease in infectivity of 3.80 log units with RI/5 and 3.49 log units with RI/4. In a similar experiment with RI/2, there was a decrease of 1.56 log units.

If reference is made to Table I which gives the proportions of "+" and "−" particles in the parent strains, it is evident that the variable sensitivity of strains to urinary mucoprotein reflects differences in the ratio of "+" to "−" particles in strains.

As the proportions of "+" and "−" particles vary on serial passage, the passage materials of RI/5 strain which had been used to study fluctuations in the proportions of the two kinds of particles were examined in neutralization tests with urinary mucoprotein. The degree of neutralization by 100 μg. of mucoprotein was again proportional to the number of "+" particles present.

Enzymatic Activity of Substrains.—The ability of the viral enzyme of "+" and "−" substrains to destroy influenza virus receptors on the urinary mucoprotein molecule was investigated.

The procedure of Tamm and Horsfall (14) was employed. Infected allantoic fluids diluted in 0.025 M phosphate buffer to contain 32 or 64 HA units of virus per ml. was used as the enzyme preparation. One ml. aliquots of such diluted virus suspensions were mixed with 1 ml. volumes of a solution of urinary mucoprotein containing 50 μg of mucoprotein per ml. As controls, 1 ml. aliquots of the mucoprotein solution were mixed with equal volumes of 0.025 M phosphate buffer. All mixtures were incubated at 37°C. in a waterbath. After 20, 40, 60, and 80 minutes of incubation groups of tubes were removed and heated at 65°C. for 30 minutes to destroy the hemagglutinating ability of the virus. The tubes were then stored at 4°C. until the completion of the experiment, at which time all reaction mixtures were assayed for hemag-

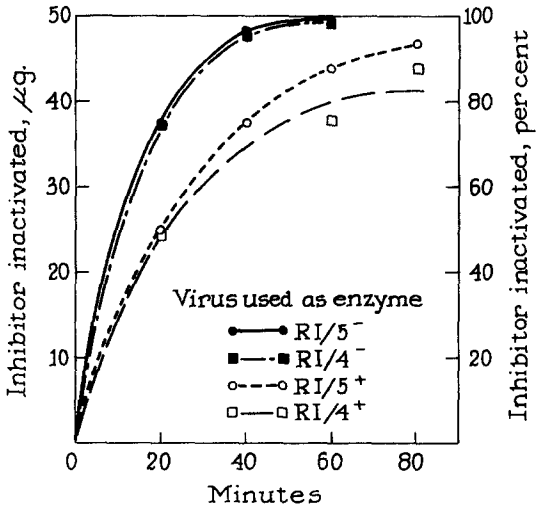

FIG. 1. Rate of inactivation of inhibitory activity of urinary mucoprotein by "+" and "−" substrains of influenza A2 viruses. The temperature of the reaction mixture was 37°C., and 64 units of each virus was used. Residual inhibitory activity was determined with heated Lee virus.

glutination-inhibiting activity with 8 HA units of Lee virus which had been heated at 56°C. for 30 minutes. The inhibitory activity of the urinary mucoprotein in control solutions remained equivalent to 0.008 μg. per unit of heated Lee virus throughout the duration of the experiments.

The results obtained in four such experiments with the "+" and "−" substrains of RI/4 and RI/5 virus strains were similar. Fig. 1 shows the results of one, in which 64 HA units of each substrain was used as the viral enzyme preparation. As can be seen, both "+" and "−" particles were able to destroy rapidly the inhibitory activity of urinary mucoprotein. In earlier experiments with Lee virus a similar rate of inactivation of mucoprotein was obtained (14). Both RI/4$^+$ and RI/5$^+$ substrains inactivated the mucoprotein somewhat more rapidly than did RI/4$^+$ and RI/5$^+$ substrains. Similar results were obtained when the RI/4$^+$ strain was used to assay the remaining inhibitory activity instead of heated Lee virus.

These results indicate that the "+" particles, which are highly sensitive to inhibition by urinary mucoprotein, are capable of destroying virus receptors on the mucoprotein molecule, though at a somewhat slower rate than the "—" particles.

Enzymatic Activity of Parent Strains.—The ability of viral enzyme of parent strains, RI/2 and RI/5, and the Lee strain to destroy virus receptors on the urinary mucoprotein molecules was examined.

The procedure employed was identical with that described above. The amount of viru used as enzyme was 128 HA units. Heated Lee virus, 8 HA units per tube, was employed a

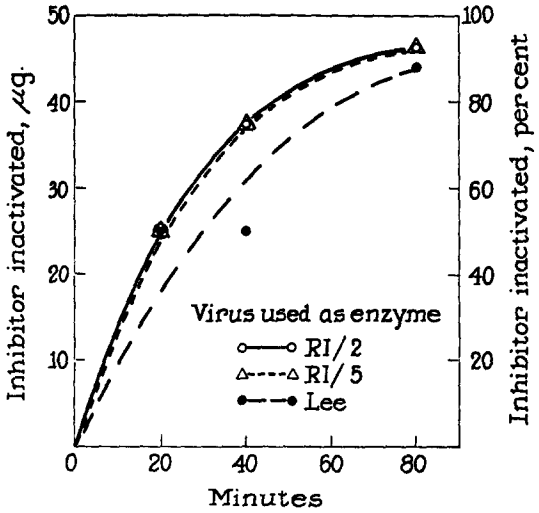

FIG. 2. Rate of inactivation of inhibitory activity of urinary mucoprotein by influenza A2 and B virus strains. The temperature of the reaction mixture was 37°C., and 64 units of each virus was used. Residual inhibitory activity was determined with heated Lee virus.

the indicator of remaining inhibitory activity. The inhibitory activity of the mucoprotein in the controls remained equivalent to 0.004 μg. per unit of heated Lee virus throughout the experiments.

As can be seen in Fig. 2, there was little difference in the rates of destruction of inhibitory activity of mucoprotein by the three strains. These results indicate that the differences in enzymatic activity among these strains are not large. In contrast, as will be shown below, they differ markedly in elution rates from erythrocytes. Seto and coworkers (22) have also studied the enzymatic activity of Asian strains and have found discrepancies between elution and enzymatic activity of these strains.

The possibility that the viral enzyme of the different strains was destroying receptors on the mucoprotein for one strain, *i.e.* heated Lee, and not the others was investigated. Experiments were carried out by the method described above,

with RI/2, RI/5, and Lee strains and *V. cholerae* filtrate as enzyme preparations. The amount of inhibitory activity destroyed was determined using RI/2, RI/5, and the heated Lee virus as indicators of the remaining activity. There was no significant difference in the reduction in inhibitory activity after 20, 40, or 80 minutes of treatment, regardless of which virus was used to measure inhibitory mucoprotein. Thus, the different virus strains appear to be capable of destroying receptors for each of the three viruses at similar rates.

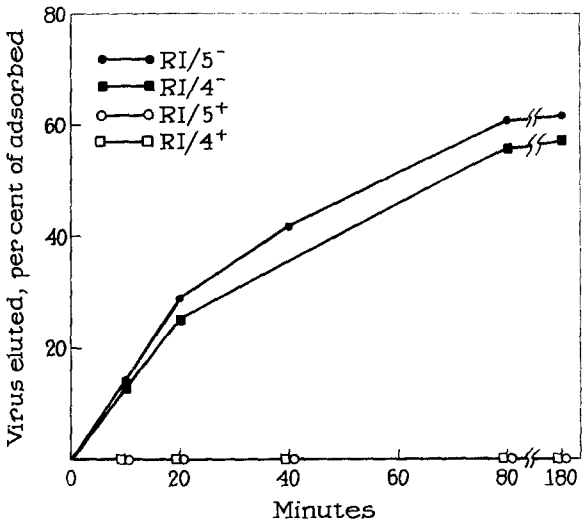

FIG. 3. Rates of elution of "+" and "−" substrains of influenza A2 viruses from human O erythrocytes. Experiments were carried out at 37°C. Eluted virus was measured by hemagglutination titrations with chicken erythrocytes.

III. Interactions of Substrains and Strains with Receptors on Erythrocytes

Elution of "+" and "−" Virus Particles from Human Erythrocytes.—The elution from human erythrocytes of the "+" and "−" substrains separated from the RI/4 and RI/5 strains was studied.

A modification of the procedure of Smith and Cohen (23) was employed. Virus concentrations were adjusted to approximately equal levels by dilution of infected allantoic fluid in PBS. Two ml. volumes of virus suspension containing approximately 350 HA units were mixed with equal volumes of a 0.5 per cent suspension of human erythrocytes in PBS. The mixtures were held at 4°C. for 30 minutes to allow adsorption of virus to erythrocytes to occur, and they were then centrifuged at 2000 R.P.M. for 3 minutes in the International PR-2 centrifuge at 4°C. The supernate was removed and stored at 4°C. The cells were resuspended in 4.0 ml. of PBS which had been previously warmed to 37°C., and the suspension was then incubated at 37°C. At intervals groups of tubes were removed, centrifuged at 4°C. as above, and the supernate containing the eluted virus removed and stored at 4°C. At the completion

of the experiment the original virus suspension, the supernate collected after the adsorption period, and the eluates were titrated by the hemagglutination procedure with chicken erythrocytes. The amount of virus adsorbed and eluted was calculated.

Fig. 3 illustrates the elution of substrains as observed during a 3 hour period. The amount of virus eluted is expressed as a percentage of the amount of virus which adsorbed initially to erythrocytes. As shown, within 80 minutes over 50 per cent of the two "−" substrains had eluted, but the "+" substrains showed no elution in 3 hours. These experiments were extended to 6 hours without demonstrable elution of the "+" particles.

Padgett and Walker (24, 25) have described variants of Lee strain of influenza B virus which eluted more rapidly at 24°C. than at 37°C. and more rapidly in the presence of calcium than in PBS. Elution experiments were therefore carried out with the RI/4$^+$ substrain at 25°C. in PBS and at 25°C. and 37°C. in CaBBS, but no elution was demonstrable in 80 minutes.

The "+" virus particles thus apparently form a more stable combination with the receptors on erythrocytes than do the "−" virus particles. This correlates well with the findings that "+" particles formed more stable combinations with serum inhibitors and urinary mucoprotein.

Elution of Parent Strains from Human Erythrocytes.—The elution rates from human erythrocytes of RI/1 to RI/6, Japan/305, and Lee viruses were studied.

The procedure employed was described above. The seventh egg passage virus of each of the 1957 influenza A strains was used. The amount of virus used was such that the amounts of virus adsorbed to the cells were similar with all viruses.

Fig. 4 shows the results obtained in elution experiments with these strains. Most of the strains eluted rapidly with comparable rates. The RI/4 and RI/5 strains, however, showed no elution in 80 minutes. The RI/3 strain showed an intermediate elution rate. In some experiments the elution time was extended to 18 hours without any demonstrable elution of RI/4 or RI/5, but in a few experiments a small amount of virus eventually eluted. In these instances the largest amount of virus which eluted in 18 hours was 15 per cent of that adsorbed to erythrocytes.

The effect of the proportion of the two kinds of virus particles on the rate of elution is illustrated by the elution experiment summarized in Fig. 4. The ratio of "+" to "−" particles in the passages of virus used in the experiment was determined by the procedure of infectivity titrations in the presence and absence of horse serum. It was found that in the RI/5 strain which failed to elute the ratio of "+" to "−" particles was 4.6×10^6:1. In the RI/1 strain which eluted rapidly, no "+" particles could be demonstrated. In the RI/3 strain, which eluted at an intermediate rate, the ratio was 2.6 "+" particles to 1 "−" particle. It is therefore apparent that the elution rates obtained with parent strains composed of "+" and "−" particles depend on the proportion

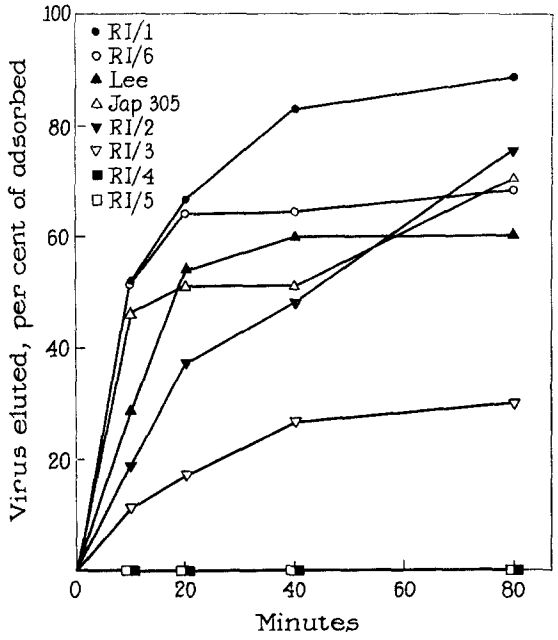

FIG. 4. Rate of elution of influenza A2 and B virus strains from human O erythrocytes. Experiments were carried out at 37°C. Eluted virus measured by hemagglutination titrations with chicken erythrocytes.

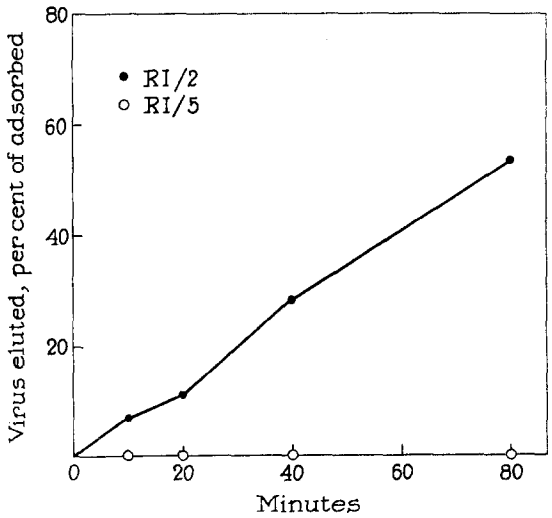

FIG. 5. Rates of elution of RI/2 and RI/5 strains of influenza A2 virus from chicken erythrocytes. Experiments were carried out at 37°C. Eluted virus was measured by hemagglutination titrations with chicken erythrocytes.

of the two kinds of particles present, and that the results obtained in the above experiments with parent strains are consistent with what would be expected from the experiments with pure substrains.

Elution of Parent Strains from Chicken Erythrocytes.—The elution rates of RI/2 and RI/5 strains from chicken erythrocytes were determined by the same procedure used with human erythrocytes. Fig. 5 shows that the results obtained with chicken erythrocytes were similar to those obtained with human erythrocytes. The RI/5 strain failed to elute, whereas the RI/2 strain eluted relatively rapidly, although not as rapidly as from human erythrocytes.

Takátsy *et al.* (26) have also shown that influenza A2 virus strains which are sensitive to serum inhibitors and antibody elute slowly from fowl erythrocytes.

TABLE VII

Prevention of Viral Hemagglutination by V. cholerae Filtrate Treatment of Chicken Erythrocytes

Virus*	Reciprocal of highest dilution of *V. cholerae* filtrate which prevented agglutination
Lee	512
RI/4$^+$	< 8
RI/4$^-$	32
RI/5$^+$	< 8
RI/5$^-$	16

* 8 HA units per tube.

Effect of V. cholerae Filtrate Treatment of Erythrocytes on Agglutination by "+" and "−" Virus Particles.—Destruction of receptors on erythrocytes for influenza, mumps, and Newcastle disease viruses by an enzyme produced by the bacterium *V. cholerae* is well known. The ability of substrains of 1957 isolates to agglutinate *V. cholerae*-treated erythrocytes was investigated in experiments with chicken erythrocytes.

Serial dilutions of *V. cholerae* culture filtrate were made in 0.3 ml. volumes of CaBBS. To each tube was added 0.6 ml. of 0.36 per cent chicken erythrocytes suspended in 0.85 per cent NaCl. Mixtures were incubated at 37°C. for periods of 30 minutes to 2 hours. The tubes were then brought to 24°C., the erythrocytes resuspended, and 8 HA units of virus contained in 0.3 ml. was added to each tube. The patterns formed by settled erythrocytes were examined after 70 minutes at 24°C., and the end point was taken to be the highest dilution of filtrate which completely prevented hemagglutination.

Table VII shows the results obtained in one such experiment with the four substrains, and the Lee strain of influenza B virus. Hemagglutination by the Lee strain was prevented by a high dilution of *V. cholerae* filtrate; hemagglutination by the RI/4$^-$ and RI/5$^-$ substrains was prevented only by much lower

dilutions; and hemagglutination by the RI/4$^+$ and RI/5$^+$ substrains was not prevented by the lowest dilution of filtrate employed. This indicates that destruction of all receptors for the "+" virus particles was not accomplished by treatment with *V. cholerae* filtrate.

Thus, the "+" substrains, in addition to forming stable combinations with receptors on soluble inhibitors and erythrocytes, are also capable of agglutinating erythrocytes which have been extensively treated with *V. cholerae* filtrate. Treatment with the filtrate did prevent agglutination of erythrocytes by the "−" strains, but higher concentrations of filtrate were required than was necessary to render the erythrocytes inagglutinable by the Lee virus.

The ability of two of the parent strains, RI/2 and RI/5, to agglutinate *V. cholerae* filtrate-treated erythrocytes was also studied. In an experiment in which *V. cholerae* filtrate at a dilution of 1:1024 prevented agglutination by the Lee strain, a dilution of 1:128 was required to prevent agglutination by the RI/2 strain and a dilution of 1:2 did not prevent agglutination by the RI/5 strain. Treatment of erythrocytes for periods extending to 24 hours did not prevent agglutination by the RI/5 strain. These results are in agreement with those of Takátsy and coworkers (26) who also found that antibody- and inhibitor-sensitive influenza A2 virus strains were able to agglutinate *V. cholerae* filtrate-treated erythrocytes which no longer were agglutinable by other strains of influenza virus.

Effect of Purified Neuraminidase Treatment of Erythrocytes on Adsorption of "+" and "−" Virus Particles.—The agglutination of *V. cholerae* filtrate-treated erythrocytes by "+" virus particles, described above, indicates that such treatment did not completely destroy virus receptors on erythrocytes. Since destruction of virus receptors on the erythrocyte surface prevents adsorption of virus to erythrocytes, the diminished capacity of treated erythrocytes to adsorb virus provides a measure of receptor destruction. The ability of RI/5$^+$, RI/5$^-$ substrains, and the Lee strain of influenza virus to adsorb to erythrocytes treated with purified neuraminidase from *V. cholerae* was, therefore, determined.

Infected allantoic fluids were diluted with CaBBS to contain approximately the same concentration of virus. One ml. volumes of 1.0 per cent chicken erythrocytes in CaBBS were mixed with 1.0 ml. aliquots of purified neuraminidase diluted in CaBBS to contain 25 units of enzyme per ml. (16). Controls consisted of erythrocytes in CaBBS. Mixtures were incubated at 37°C. for 2 hours and then chilled in an ice bath. The cells were sedimented at 2000 R.P.M. for 3 minutes at 4°C. and resuspended in 2.0 ml. of virus suspension. The tubes were rocked at 8 oscillations per minute for 30 minutes at 4°C. to allow adsorption and the cells again sedimented as above and the supernate collected. The original virus suspension, the supernate from the neuraminidase-treated cells, and the supernates from the control cells were titered by a fractional hemagglutination titration procedure (27). The amount of virus absorbed to treated erythrocytes was expressed as a percentage of the amount adsorbed to untreated cells.

Table VIII shows the results obtained in such an experiment. With the RI/5$^+$ substrain, the amount of virus which adsorbed to treated cells represented

54 per cent of the amount adsorbed to untreated cells; the percentage adsorption of RI/5⁻ substrain and Lee strain were 5 per cent and 2 per cent respectively. Thus a large amount of RI/5⁺ particles were still able to adsorb to cells although almost all receptors for the RI/5⁻ and Lee virus particles had been destroyed.

Experiments to determine whether purified neuraminidase would fail to prevent agglutination of erythrocytes by RI/5⁺, as described above with *V. cholerae* filtrate, could not be done, because neuraminidase caused spontaneous agglutination of the chicken erythrocytes.

IV. Antigenic Comparison of Substrains

The strains of influenza A virus isolated in New York City in 1957 which were used in the present studies have been shown to be antigenically closely

TABLE VIII

Adsorption of Virus to Neuraminidase-Treated Chicken Erythrocytes

Virus	Hemagglutinating units of virus			Virus adsorbed to treated RBC, expressed as per cent of amount adsorbed to untreated RBC
	Initial suspension	Adsorbed to		
		Untreated RBC	Neuraminidase-treated RBC	
Lee	1260	1220	30	2
RI/5⁺	1260	1180	630	54
RI/5⁻	1316	1060	56	5

similar (4). Because of the marked differences in biological properties of the "+" and "−" particles isolated from these strains, two "+" and two "−" substrains were compared in cross hemagglutination-inhibition reactions with rabbit antisera.

The antisera employed in these reactions were prepared by single intravenous injections of pairs of rabbits with 10 ml. of infected allantoic fluid. The rabbits were bled 2 weeks later. Serum from two rabbits immunized with the same virus was pooled, and all serum pools were treated with *V. cholerae* filtrate to remove inhibitors. The antibody titers of each serum pool were then determined with "+" and "−" substrains by the hemagglutination-inhibition procedure. The pre-immunization sera pooled in the same manner were also treated with *V. cholerae* filtrate and tested for hemagglutination-inhibiting activity. Such sera either showed no hemagglutination-inhibiting activity or the hemagglutination-inhibition titers were insignificant compared to antibody titers.

Table IX shows the results obtained in cross-reactions with the "+" and "−" substrains isolated from the RI/4 and RI/5 strains. The greater sensitivity to antibody of the "+" substrains is apparent. In every case the "+" sub-

strains gave a higher titer than the "−" substrains, regardless of whether the antiserum had been prepared to a "+" or a "−" substrain.

However, the results also show that there were no significant antigenic differences between "+" and "−" particles. The antigenic relationship between any two viruses may be expressed as the geometric mean of $\frac{\text{heterologous titer}}{\text{homologous titer}}$

TABLE IX

Cross Hemagglutination-Inhibition Reactions with "+" and "−" Particles and Corresponding Rabbit Antisera

Rabbit antiserum	Virus			
	RI/4+	RI/4−	RI/5+	RI/5−
RI/4+	**2048**	512	4096	256
RI/4−	1024	**512**	2048	256
RI/5+	2048	512	**2048**	256
RI/5−	512	256	512	**128**

TABLE X

Antigenic Similarity of "+" and "−" Particles

Virus	Virus			
	RI/4+	RI/4−	RI/5+	RI/5−
RI/4+	1.0*	0.7	1.4	0.7
RI/4−		1.0	1.0	1.0
RI/5+			1.0	0.7
RI/5−				1.0

* Antigenic relationship expressed as $R = \sqrt{r_1 \times r_2}$

ratios, or R value (28, 29). This is a convenient procedure for evaluation of antigenic relatedness uninfluenced by the sensitivity of viruses to antibody. An R value of 1 indicates that there is no demonstrable difference between two strains. The antigenic relationships among "+" and "−" substrains of RI/4 and RI/5 viruses expressed as R values are shown in Table X. The small deviations from unity observed in some of the comparisons are not significant.

In conclusion, "+" and "−" particles are antigenically closely related, yet they differ markedly in sensitivity to specific antibody and in reactivity with mucoprotein receptors.

Summary of Biological Properties of "+" and "−" Particles

The properties of the two kinds of virus particles described in this communication can be briefly summarized as follows: The "+" particles are highly sen-

sitive to serum inhibitors, specific antibody and urinary mucoprotein. They elute slowly or fail to elute from erythrocytes and agglutinate erythrocytes extensively treated with *V. cholerae* filtrate. In contrast, the "−" particles are completely insensitive to serum inhibitors and relatively insensitive to antibody and urinary mucoprotein. They elute rapidly from erythrocytes, and are unable to agglutinate erythrocytes which have been extensively treated with *V. cholerae* filtrate.

Both "+" and "−" particles, acting enzymatically, are able to destroy rapidly virus receptors on urinary mucoprotein. The "−" particles appear to act somewhat faster than the "+" particles. Antigenically the "+" and "−" particles are closely related.

The properties of "+" and "−" particles indicate that there are important differences in the reactions of the two kinds of particles with mucoprotein receptors and specific antibodies. The nature of these differences is discussed in the accompanying communication (12).

DISCUSSION

The discovery (10) of two kinds of virus particles with contrasting properties in influenza A2 virus strains has provided the basis for an adequate explanation of strain variation in sensitivity to specific antibody and in reactivity with mucoprotein receptors on soluble inhibitors and on erythrocytes. Variation among influenza A2 virus strains can be readily understood when it is considered that most A2 virus strains are mixtures of sensitive ("+") and insensitive ("−") particles in varying proportions. It should be emphasized that the ratio of "+" to "−" particles varied widely from strain to strain as did the strain properties.

The demonstration of the two kinds of virus particles in the first egg passage material of strains, and the finding of "+" and "−" particles in varying proportions in different strains suggest that the two types of particles were present in varying proportions in the throat washings of different patients. Additional support for this view derives from the following findings: (*a*) the strains from which substrains of the two kinds of particles were separated were all isolated within a 2 month period in 1957; (*b*) the early egg passage materials of these strains contained the two kinds of particles in widely different numbers, and, in addition, other strains from which only "−" particles could be obtained were isolated during this period; (*c*) finally, although the "+" and "−" substrains are genetically stable in passage in the chicken embryo, the ratio of "+" to "−" particles in mixtures undergoes changes; it seems possible that such changes could occur as virus was transmitted from person to person under natural conditions.

These findings do not exclude the possibility that one or the other of the two kinds of particles appeared as a result of the first egg passage. However, such a possibility appears highly unlikely.

No other laboratories have reported quantitative determinations of two kinds of particles present in varying proportions in influenza A2 virus isolates from the 1957 pandemic. However, the available information concerning variation of strains in antibody and inhibitor sensitivity suggests that the two kinds of virus particles were present in many strains and that variation in the proportions of "+" and "−" particles in influenza virus strains was occurring on a world-wide basis during the influenza pandemic of 1957.

It should be emphasized that variation of influenza viruses in sensitivity to specific antibody occurred frequently among A and A1 viruses which were prevalent before 1957 (30–33), although quantitatively the degree of variation appears not to have been as marked as with influenza A2 viruses prevalent since 1957.

Based on antibody sensitivity of what were thought to be antigenically closely similar strains, Mulder and coworkers (30, 34) proposed a classification of strains in P, Q, and R phases. In a modified form (32), with R omitted, this terminology has been widely used. The term P phase, as now used, refers to strains which are highly sensitive both to homologous and heterologous antisera. Q phase refers to virus strains which are relatively insensitive both to homologous and heterologous antisera. Because the P-Q terminology does not seem appropriate for variation dependent on proportions of different kinds of particles, and because the "+" and "−" particles differ in many other respects in addition to antibody sensitivity, this terminology has not been used in the present studies.

There is some evidence (33, 35, 36) that influenza A and A1 virus strains contained particles which differed in sensitivity to specific antibody and to serum inhibitors. Thus, the heterogeneity of A and A1 virus strains may have been similar to that of A2 strains in which two kinds of virus particles with contrasting properties were found in varying proportions which markedly affected the biological characteristics of strains. However, separation and characterization of pure, genetically stable substrains from influenza A or A1 virus strains have not been reported.

In the absence of such information comparisons are not possible between "+" and "−" particles on the one hand, and earlier influenza virus species on the other, and no precise inquiry into the mutational steps through which some earlier influenza virus species may have given rise to "+" and "−" particles can be carried out.

When "+" and "−" particles are compared with influenza A and A1 virus strains, it does appear that "+" particles are more reactive and "−" particles less reactive with mucoprotein receptors than most of the influenza A and A1 virus strains about which information is available. This qualified comparison suggests that the change from the ancestor of influenza A2 viruses to A2 "+" and "−" particles involved a radical change not only in the antigenic composition of particles but in the viral components which react with mucoprotein

receptors. The simplest hypothesis would be that the precursor of A2 viruses gave rise to either "+" or "−" particles and of the latter two, one gave rise to the other. Alternatively the possibilities must be considered that "+" and "−" particles each had a precursor among pre-A2 virus species, or that one and the same precursor gave rise to both "+" and "−" particles. Whichever mechanism is correct, it appears that two or more mutational changes were necessary to give A2 "+" and "−" particles. Whether the precursors of A2 "+" and "−" particles were human or animal influenza virus species cannot be decided at this time.

Although the origin of "+" and "−" particles is not clear, the opportunities for genetic studies with "+" and "−" substrains appear to be extensive. That the pure substrains provide highly useful material for viral genetic studies has already been demonstrated (37).

The successful isolation from influenza A2 virus strains of two kinds of particles which account for the properties of parent strains, and the demonstration that "+" and "−" substrains are genetically homogeneous and stable emphasize the need for vigorous efforts to separate other heterogeneous influenza virus strains into populations of different species of particles which may make up such strains. Studies on influenza virus variation would be much advanced by more frequent application of the principle that homogeneous populations of particles are required for meaningful analysis of strain properties.

One of the interesting aspects of the biological characteristics of "+" and "−" particles is that high sensitivity of "+" particles to specific antibodies is paralleled by high reactivity with mucoprotein receptors, and low sensitivity of "−" particles to antibody, by low reactivity with receptors. Findings which are similar in certain respects have been obtained with variant populations of pre-1957 influenza viruses (23, 36, 38).

Whatever the precise mechanism of parallelism in antibody sensitivity and reactivity with mucoprotein receptors may be, the phenomenon suggests that the features of the surface structure of virus particles which determine antibody sensitivity may also affect reactivity with mucoprotein receptors. This may be so in spite of the fact that the specific chemical groups on virus surface involved in reactions with antibody are probably different from those which react with mucoprotein receptors. If it be true that the same factors determine antibody sensitivity and reactivity with mucoproteins, it follows that the same genetic mechanism may be responsible for viral behavior in different reactions; *i.e.*, with antibody and mucoprotein receptors.

It should be stressed that variation in antibody sensitivity and reactivity with mucoprotein receptors is independent of variation in antigenic specificity of virus particles.

In the communication which follows additional evidence is presented which bears on the nature of reactive groupings of "+" and "−" particles and mucoprotein receptors, and the mechanism of differences between "+" and

"−" particles in sensitivity to specific antibody and mucoprotein inhibitors and in stability of virus-erythrocyte complexes is further discussed. Some of the general considerations and some of the historical background are presented elsewhere (39).

SUMMARY

Two kinds of virus particles have been found in varying proportions in influenza A2 strains isolated during the 1957 pandemic. Pure populations of the different particles were obtained, and these substrains were genetically stable on serial passage in the chick embryo.

The two virus particles differ markedly in several biological properties though they are antigenically similar. One kind of particle, designated "+," is relatively sensitive to specific antibody, is highly sensitive to inhibition by serum inhibitors and urinary mucoprotein, fails to elute or elutes very slowly from human erythrocytes, and is capable of agglutinating erythrocytes treated extensively with *V. cholerae* filtrate. The other particle, designated "−," is relatively insensitive to antibodies and urinary mucoprotein, completely insensitive to serum inhibitors, elutes rapidly from erythrocytes, and can agglutinate erythrocytes treated extensively with *V. cholerae* filtrate. Both "+" and "−" particles destroy virus receptors on urinary mucoprotein.

The relative proportions of these two particles determine the characteristics of parent strains in reactions with specific antibody, mucoprotein inhibitors, and erythrocytes. The "+" and "−" particles with several easily identifiable markers are well suited for genetic studies.

BIBLIOGRAPHY

1. Meyer, H. M., Jr., Hilleman, M. R., Miesse, M. L., Crawford, I. P., and Bankhead, A. S., New antigenic variant in Far East influenza epidemic, 1957, *Proc. Soc. Exp. Biol. and Med.*, 1957, **95,** 609.
2. Jensen, K. E., New set of type A influenza viruses, *J. Am. Med. Assn.*, 1957, **164,** 2025.
3. Levy, A. H., and Wagner, R. R., Increased avidity of antibody for Baltimore 1957 strains of Asian influenza virus (P-Q-R variation), *Proc. Soc. Exp. Biol. and Med.*, 1958, **98,** 357.
4. Choppin, P. W., Osterhout, S., and Tamm, I., Immunological characteristics of N. Y. strains of influenza A virus from the 1957 pandemic, *Proc. Soc. Exp. Biol. and Med.*, 1958, **98,** 513.
5. Zhdanov, V. M., Zakstelskaya, L. Y., Yefimova, V. E., Yachno, M. A., and Khait, S. L., Antigenic peculiarities of 1957 influenza viruses and serologic indexes of immunity among the population, *J. Am. Med. Assn.*, 1958, **167,** 1469.
6. Tokuda, M., Studies on the antigenicity of influenza virus isolated in the epidemic of early summer, 1957, *J. Immunol.*, 1958, **81,** 107.
7. Cohen, A., and Belyavin, G.. Hemagglutination inhibition of Asian influenza viruses: a new pattern of response, *Virology*, 1959, **7,** 59.

8. Takátsy, G., and Barb, K., New inhibitor and erythrocyte receptor substances for certain Asian strains of influenza virus, *Nature*, 1959, **183,** 52.
9. Choppin, P. W. and Tamm, I., Interaction of influenza A viruses, New York 1957, and receptors, *Bact. Proc.*, 1959, 65.
10. Choppin, P. W., and Tamm, I., Two kinds of particles with contrasting properties in influenza A virus strains from the 1957 pandemic, *Virology*, 1959, **8,** 539.
11. Influenza, *Bull. World Health Org.*, 1959, **20,** 183.
12. Choppin, P. W. and Tamm, I., Studies of two kinds of virus particles which comprise influenza A2 virus strains. II. Reactivity with virus inhibitors in normal sera, *J. Exp. Med.*, 1960, **112,** 921.
13. Choppin, P. W., Murphy, J. S., and Tamm, I., Studies of two kinds of virus particle which comprise influenza A2 virus strains. III. Morphological characteristics; independence of morphological and functional traits, *J. Exp. Med.*, 1960, **112,** 945.
14. Tamm, I., and Horsfall, F. L., Jr., A mucoprotein derived from human urine which reacts with influenza, mumps, and Newcastle disease viruses, *J. Exp. Med.*, 1952, **95,** 71.
15. Tyrrell, D. A. J., and Horsfall, F. L., Jr., A procedure which eliminates non-specific inhibitor from human serum but does not affect specific antibodies against influenza viruses, *J. Immunol.*, 1952, **69,** 563.
16. Schramm, G., and Mohr, E., Purification of neuraminidase from *Vibrio cholerae*, *Nature*, 1959, **183,** 1677.
17. Reed, L. J., and Muench, H., A simple method of estimating fifty per cent endpoints, *Am. J. Hyg.*, 1938, **27,** 493.
18. Levy, A. H., Norman, P. S., and Wagner, R. R., Electrophoresis of serum inhibitors of influenza viruses, *Fed. Proc.*, 1959, **18,** 580.
19. Zhdanov, V. M., Hamburg, V. P., and Svet-Moldavsky, G. J., Antigenicity of the inhibitor of influenza virus strain A/Asia/57, *J. Immunol.*, 1959, **82,** 9.
20. Jensen, K. E. and Hogan, R. B., Laboratory diagnosis of Asian influenza, *Public Health Rep.*, 1958, **73,** 140.
21. Tamm, I., and Horsfall, F. L., Jr., Characterization and separation of an inhibitor of viral hemagglutination present in urine, *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 108.
22. Seto, J. T., Hickey, B. J., and Rasmussen, A. F., Jr., Sialidase activity and related properties of influenza A2 viruses, *Virology*, 1959, **9,** 598.
23. Smith, W., and Cohen, A., The enzymatic activity of influenza viruses, *Brit. J. Exp. Path.*, 1956, **37,** 612.
24. Padgett, B. L., and Walker, D. L., Enzymatic variants of influenza virus. I. Isolation and characterization of slowly reacting enzymatic variants of influenza B virus, *J. Exp. Med.*, 1957, **106,** 53.
25. Padgett, B. L., and Walker, D. L., Enzymatic variants of influenza virus. II. Effect of environmental factors on enzymatic characteristics of a variant of influenza B virus, *J. Exp. Med.*, 1958, **108,** 651.
26. Takátsy, G., Barb, K., and Farkas, E., A new erythrocyte receptor with exclusive affinity to avid Asian strains of influenza A virus, *Acta Virol.*, 1959, **3,** 79.
27. Tamm, I., Enhancement of influenza virus multiplication by 5-methyl-2-D-ribobenzimidazole, *Virology*, 1956, **2,** 517.

28. Archetti, I., and Horsfall, F. L., Jr., Persistent antigenic variation of influenza A viruses after incomplete neutralization *in ovo* with heterologous immune serum, *J. Exp. Med.*, 1950, **92,** 441.
29. Chu, C. M., Andrewes, C. H., and Gledhill, A. W., Influenza in 1948–1949, *Bull. World Health Org.*, 1950, **3,** 187.
30. van der Veen, J., and Mulder, J., Studies on the antigenic composition of human influenza virus A strains with the aid of the hemagglutination inhibition technique, Onderzoekingen en mededelingen uit het Institut voor Praeventieve Geneeskunde, Leiden, Stenfert Kroese, 1950, No. 6.
31. Isaacs, A., Gledhill, A. W., and Andrewes, C. H., Influenza A viruses; laboratory studies, with special reference to European outbreak of 1950–51, *Bull. World Health Org.*, 1952, **6,** 287.
32. Mulder, J., Brans, L. M., and Masurel, N., Studies on the antigenic composition of the influenza virus-A strains isolated in the Netherlands in the period 1947–1953, Onderzoekingen en mededelingen uit het Institut voor Praeventieve Geneeskunde, Leiden, Stenfert Kroese, 1956, No. 15.
33. JENSEN, K. E., The nature of serological relationships among influenza viruses, *Advances Virus Research*, 1957, **4,** 279.
34. Mulder, J., van der Veen, J., Brans, J. J., and Enserink, S. W., Rapid diagnosis of the sub-group of influenza-A virus strains isolated during the epidemic of 1949 in the Netherlands, *Antonie van Leeuwenhoek*, 1949, **15,** 125.
35. Isaacs, A., P-Q variation in influenza viruses, *Lancet*, 1953, **1,** 676.
36. Cohen, A., and Smith, W., The heterogeneity of infective virus particles within a single influenza virus strain, *Brit. J. Exp. Path.*, 1957, **38,** 385.
37. Kilbourne, E. D., and Murphy, J. S., Genetic studies of influenza viruses. I. Viral morphology and growth capacity as exchangeable genetic traits. Rapid *in ovo* adaptation of early passage Asian strain isolates by combination with PR8, *J. Exp. Med.*, 1960, **111,** 387.
38. Smith, W., Westwood, M. A., Westwood, J. C. N. and Belyavin, G., Spontaneous mutation of influenza virus A during routine egg passage, *Brit. J. Exp. Path.*, 1951, **32,** 422.
39. Tamm, I., and Choppin, P. W., Influenza viruses: a study in variation, *in* Immunochemical Approaches to Problems in Microbiology, New Brunswick, New Jersey, Rutgers University Press, in press.