EVIDENCE FOR GENETIC INTERACTION BETWEEN NON-INFECTIOUS AND INFECTIOUS INFLUENZA A VIRUSES*

By SAMUEL BARON, M.D.,‡ and KEITH E. JENSEN, Ph.D.

(From the Department of Epidemiology and Virus Laboratory School of Public Health, University of Michigan, Ann Arbor)

(Received for publication, August 3, 1955)

Genetic interaction has been observed among several kinds of bacteria (1) and among bacteriophage (2). Evidence also has been accumulated recently which indicates genetic traits of animal viruses can be interchanged (3–5). These results were obtained employing fully infectious microorganisms. Early demonstrations with bacteria indicated genetic transfer could take place between heat-killed pneumococci and fully infectious pneumococci (6). Desoxyribose nucleic acid from disrupted diplococci was shown to be the genetic carrier material (7). Thus, it became evident that intact microorganisms are not essential for genetic activity. Other demonstrations of bacterial transformation have subsequently confirmed this concept (8).

Mechanisms of genetic interaction among bacteriophage and animal viruses are currently being investigated. To date, it is known that ultraviolet-treated coliphages or influenza viruses may be revived by multiple infection of a host cell (9, 10). It has also been shown that when a mixture of heat-killed myxoma virus and infectious fibroma virus is inoculated into rabbits, fully infectious myxoma virus can be recovered (11).

It appeared that information concerning mechanisms involved in these phenomena might be derived by studying residual genetic activity of non-infectious influenza virus. The approach has been to degrade virus to various degrees by several means and to follow resultant genetic combining activity with a related infective virus. Data obtained clearly indicate that non-infectious virus may genetically combine with infectious virus within the host cell.

Results of exploratory experiments designed to elucidate certain aspects of these phenomena are reported, and possible mechanisms involved will be discussed.

* These studies were conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and were supported by the Office of the Surgeon General, Department of the Army, Washington, D. C.

‡ Fellow of the National Foundation for Infantile Paralysis, Inc.

Present address: National Institutes of Health, Division of Biological Standards, Bethesda 14.

Materials and Methods

Virus.—Two strains of Type A influenza virus were chosen for this study. The first was neurotropic WS, isolated in ferrets in 1933, and passed in mice. It has since undergone an unknown number of passages in minced chick embryo tissue culture, 7 passages in minced chick embryo brain tissue culture, and 101 passages in mouse brain (12). Intranasal inoculation of mice with NWS produces lung lesions and death. Growth in 9 to 11 day embryonate eggs following allantoic inovulation yields fluids with hemagglutinin titers of 1:160 per 0.5 ml. and approximately $10^{7.3}$ EID_{50} per 0.1 ml.

Wright (Wr) is an A prime strain isolated in 1953 in the amniotic cavity of the embryonate egg, and then passaged 22 times in the allantoic cavity. It lacks mouse neurotropism, will not produce lesions in the mouse lung, and is antigenically quite distinct from NWS. When grown in eggs hemagglutinin titers of 1:480 per 0.5 ml. and about $10^{7.5}$ EID_{50} per 0.1 ml, were obtained as a routine.

Since it is desirable to work with relatively homogeneous virus populations in order to minimize unexpected selection of variants (3), both strains were passaged 3 times at limiting infectious dilutions in eggs. The final infected allantoic fluid was quick-frozen, stored in small aliquots, and used to make pools of seed virus and reagent virus which were similarly frozen and stored at −60°C. in a mechanical freezer.

Antisera.—Immune sera were produced in rabbits by intravenous injection of 2 ml. infected allantoic fluid for 3 consecutive days every 2 weeks for three immunization courses. Animals were bled 11 to 14 days after the last injection (4). Sera were heated at 56°C. for 30 minutes before use. In instances requiring the removal of non-specific inhibitor from serum, 1 ml. of serum was mixed with 4 mg. of Difco trypsin dispersed in 0.5 ml. of phosphate buffered saline at pH 8.2. This was immediately immersed in a 56°C. water bath for 30 minutes (13, 14).

Diluents.—Preparations intended for egg inoculation were diluted in broth containing 250 units of penicillin and 1 mg. of streptomycin per ml. Those destined for mouse brain inoculation and for hemagglutinin testing were diluted in 0.01 M phosphate buffered saline at pH 7.2. Since virus is less stable to the effect of visible light in protein free diluents (15), tubes containing this fluid were guarded from light by being placed in wooden racks.

Virus Titrations.—Infectivity was determined by inoculating 0.1 ml. each of serial tenfold broth dilutions into 4 eggs. After 2 to 3 days' incubation at 37°C., 0.5 ml. of allantoic fluid from each egg was tested for hemagglutinin by addition of 0.5 ml. of 0.5 per cent chicken red blood cells.

Hemagglutinin titer was determined by mixing 0.5 ml. serial twofold buffered saline dilution of virus preparations with 0.5 ml. of 0.5 per cent chicken erythrocytes (16). Patterns were read after 45 minutes at room temperature, the end-point being the last tube exhibiting hemagglutinin agglutination.

Hemagglutinin Inhibition.—A modification of the standard pattern test (17) was employed as a rapid method for antigenic identification of virus (4). Four hemagglutinating units of an unknown virus preparation were mixed with antisera for NWS or Wr at dilutions known to render them specific. Thus hemagglutination of homologous antigen was prevented in one tube but allowed to proceed in a second tube containing a heterologous antigen.

Virus Neutralization.—This titration was done *in ovo* by mixing 1000 EID_{50} of virus with equal volumes of serial fourfold serum dilutions. Mixtures were incubated at room temperature for 30 minutes, and 0.2 ml. inoculated into each of 2 eggs per level. After 3 days at 37°C., 0.5 ml. of allantoic fluid was tested for hemagglutinin by addition of 0.5 ml. of 0.5 per cent chicken erythrocytes.

Procedure for Studying Genetic Interaction.—The routine procedure developed from preliminary tests, which are described in the experimental section, is outlined in Fig. 1. One

of the virus strains (NWS) was subjected to ultraviolet light, as described below, to destroy infectivity. Allantoic fluid containing NWS was dialyzed against 20 volumes of phosphate buffered saline (pH 7.2) for 24 hours at 4°C. It was then quick-frozen in 12 ml. aliquots and stored at −60°C. in a mechanical freezer. Samples of 12 ml. each were thawed and placed in a 9.5 cm. diameter open Petri dish suspended 7 inches from a 15 watt G. E. germicidal lamp. During irradiation the Petri dish was agitated alternately in a vertical and then in a horizon-

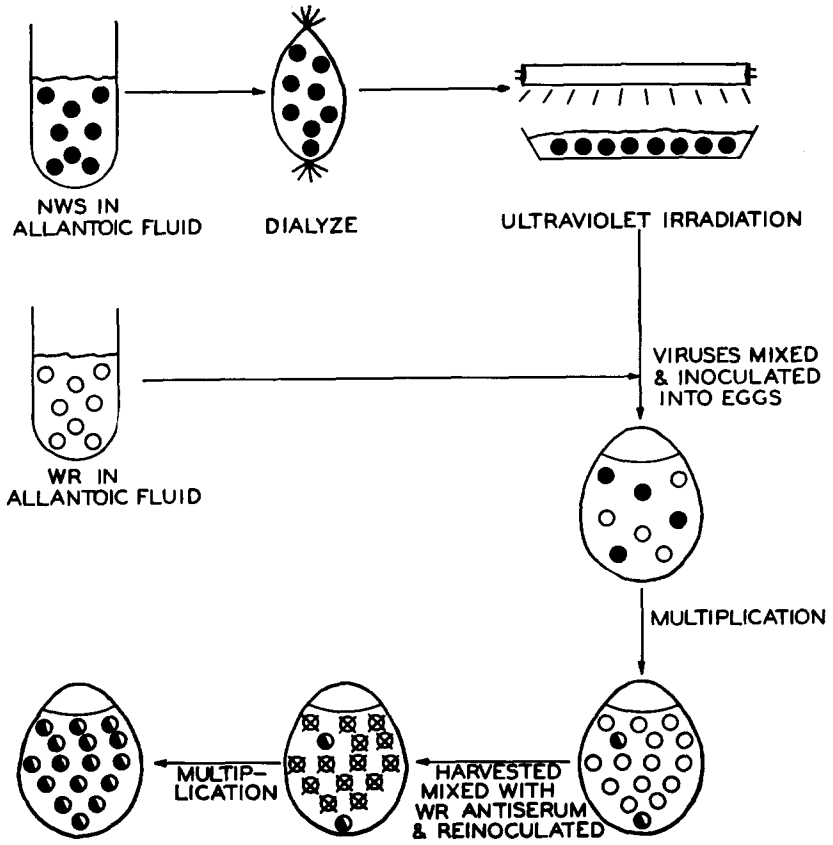

FIG. 1. Experimental procedure for studying genetic interaction.

tal direction (18). Complete loss of infectivity occurred between 60 and 120 seconds, but the optimal time of irradiation within this interval was determined individually for each lot. Aliquots of this non-infectious material were quick-frozen and stored at −60°C. in a mechanical freezer. The preparation remained stable for at least 2 months at this temperature and also at 4°C.

The irradiated non-infectious preparation was diluted 10^{-1}, 10^{-2}, and 10^{-3} in broth, mixed with equal volumes of infectious Wr at dilutions of 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} and 0.2 ml. of each mixture was inoculated into each of 2 eggs. After 2 days at 37°C., 0.15 ml. of allantoic fluid was aspirated by syringe from each egg. This material was mixed with an equal volume

of Wr antiserum containing 3 to 4 times the minimal quantity necessary to neutralize undiluted allantoic fluid containing $10^{7.5}$ EID_{50} of Wr per 0.1 ml. The final dilution of different sera used varied from 1:4 to 1:10. After standing at room temperature for 30 minutes the contents of each tube were inoculated into one egg. After incubation at 37°C. for 2 days allantoic fluids were tested for hemagglutinin. Positive fluids were designated as containing derived virus and were subjected to further analysis.

Wr antisera at dilutions used in these experiments, were pretested for specificity by neutralization tests with 100 EID_{50} of NWS virus. Only those sera which specifically neutralized Wr were employed.

Autointerference.—It was necessary to determine whether irradiated non-infectious preparations were capable of masking any residual infectious virus (autointerference). The ability to interfere with growth of added homologous virus was used as an indicator of autointerference. For this procedure, 0.2 ml. containing a mixture of 10 or 100 EID_{50} of homologous infec-

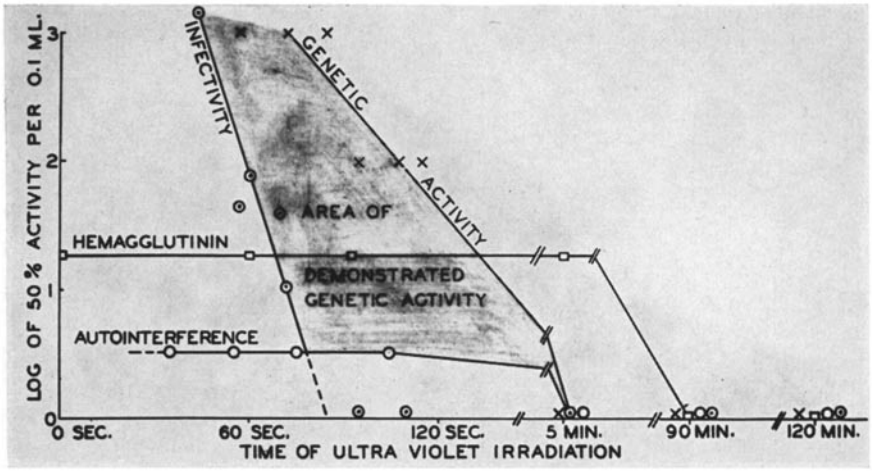

FIG. 2. Effect of ultraviolet irradiation on 4 properties of NWS.

tive virus plus an equal volume of serial tenfold dilutions of irradiated material was inoculated into each of 4 eggs per level. Fluids which after 2 days lacked hemagglutinin indicated homologous interference by that dilution of irradiated virus. Thus at these dilutions autointerference could be responsible for masking residual infective virus (Fig. 2).

EXPERIMENTAL AND RESULTS

Genetic Activity of Non-Infectious NWS.—The initial objective was to prepare a non-infectious influenza virus which could be tested for genetic activity. Accordingly, ultraviolet irradiation of NWS strain of influenza was employed as described in Materials and Methods. Infectivity decreased rapidly with increasing time of irradiation, so that complete loss of demonstrable infectivity occurred between 60 and 90 seconds (Fig. 2), as evidenced by lack of viral growth in two consecutive egg passages (19). Chorioallantoic membranes from these eggs were also shown not to contain infective virus. In

some instances preparations which showed no infectivity on first inoculation were positive on second passage, indicating that two transfers provide greater confidence in the results. This determination was also complicated by the fact that high concentrations of non-infectious virus retain the property of auto-interference, which may mask residual infectivity (Fig. 2 and Materials and Methods). Thus, lack of infectivity could be determined with assurance only when autointerference was eliminated by dilution of irradiated virus. Preparations containing non-infectious NWS were shown to retain genetic activity in the following manner. Dialyzed NWS was irradiated for varying periods of time beyond the point of complete loss of infectivity, and tested for genetic activity as described in Materials and Methods. In essence the technique involved inoculating a mixture of irradiated NWS and fully infectious Wr

TABLE 1

Virus Isolations at Completion of Test for Genetic Activity with Inactive NWS

Irradiated NWS Dilution	Ultraviolet irradiation time															
	1 min.				2 min.				5 min.				90 min.			
10^{-3}	⊕*	—	⊕	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	⊕	—	—	—	—	—	—	—	—	—	—	—	—
10^{-2}	—	⊕	⊕	⊕	—	—	—	⊕	—	—	—	—	—	—	—	—
	—	⊕	—	⊕	—	—	—	⊕	—	—	—	—	—	—	—	—
10^{-1}	—	—	—	⊕	—	⊕	—	⊕	—	—	—	—	—	—	—	—
	—	⊕	⊕	⊕	⊕	—	—	—	—	—	—	—	—	—	—	—
Wr Dilution..	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{0}	10^{-1}	10^{-2}	10^{-3}

* ⊕ indicates eggs containing viral hemagglutinin.

viruses into eggs. After incubation for 2 days infected allantoic fluids were mixed with Wr antiserum and reinoculated into eggs (Fig. 1).

Table I illustrates the results of a typical experiment in which many positive fluids were obtained. Resultant virus was thought to be neither the original Wr nor NWS strain because it had multiplied despite incubation with Wr antisera, and the NWS had been proven non-infectious. Subsequent characterizations confirmed this belief and indicated genetic combination. Further passage of negative fluids with or without Wr antiserum, did not result in additional isolations.

The reaction did not occur if more than 2 minutes of ultraviolet irradiation were used, nor did it proceed if inactive NWS was diluted beyond 10^{-3}. These observations indicate that recovered virus is dependent upon the non-infectious NWS component of the inoculum.

It was noted that fewer viruses were derived when a large inoculum of Wr

was employed. This was confirmed with additional experiments, but elucidation of the mechanism awaits further study.

In addition to previously mentioned precautions against infectivity in irradiated NWS preparations, the following controls were included. Parallel genetic activity experiments were carried out in which broth was substituted for Wr virus, and another in which non-infectious NWS was replaced by broth. No virus was isolated in either instance, indicating that recovery of virus required the presence of Wr and NWS in the original inoculum. Anti-

TABLE II

Antigenic Identification by 2 Tube Hemagglutination-Inhibition Test

Virus	NWS antiserum	Wr antiserum
NWS	−	+
Wr	+	−
Derived Virus 1	−	+
2	−	+
3	−	+
4	−	+
5	−	+
6	−	+
7	−	+
8	−	+
9	−	+
10	−	+
11	−	+
12	−	+
13	−	+
14	−	+

+ indicates hemagglutination, − indicates inhibition of 4 hemagglutinin units (identification).

sera were also shown to be free of contaminating virus by 2 consecutive egg passages.

Characterization of Resultant Virus.—Influenza virus possesses inheritable traits which are often distinct for various strains and which have been applied to characterize virus populations (20). The following 4 traits were used to delineate the original NWS and Wr strains as well as derived viruses—antigenicity, heat stability of hemagglutinin, mouse lung pathogenicity, and neurotropism in mice.

Antigenic identifications using the 2 tube hemagglutination inhibition test are outlined in Table II. It should be noted that the original NWS and Wr strains are completely distinct (with serum dilutions of 1:300) so that only homologous antiserum prevents hemagglutina-

tion. Virus resulting from interaction of infectious Wr and irradiated NWS is seen to be antigenically similar to NWS, as was anticipated because of its ability to multiply in the presence of Wr antiserum. A total of 50 isolations tested in this way were antigenically similar. Con-

TABLE III

Comparison of Titers Obtained by Hemagglutination-Inhibition

Virus	Hemagglutination-inhibition titer	
	NWS antiserum	Wr antiserum
NWS	960	<10
Wr	<10	960
Derived virus E	480	<10
H	480	<10
K	480	<10
W	960	<10
14	480	<10

TABLE IV

Comparison of Titers Obtained by in Ovo Neutralization

Virus	Neutralization titer	
	NWS antiserum	Wr antiserum
NWS	1600	<100
Wr	<100	1600
Derived virus A	400	<100
H	400	<100
Q	1600	<100
R	1600	<100
14	400	<100

firmation of identity with standard hemagglutination-inhibition tests (Table III) and *in ovo* neutralization tests (Table IV) were also carried out.

Since these tests allow some quantitation it is possible to compare relative similarity of virus strains. The above results indicate that viruses arising from this interaction are antigenically quite similar to NWS and distinct from Wr.

Mouse lung pathogenicity was evaluated by intranasal inoculation of 1 hemagglutinating unit of virus in 0.05 ml. of buffered saline, and observation for 10 days. Surviving mice sacrificed after this time were examined for lung lesions (Table V).

The original NWS strain was shown to be highly pathogenic for mice, producing death with complete lung consolidation in 5 to 8 days. In contrast, Wr strain did not produce lesions at this dosage level. The derived viruses

TABLE V

Comparative Mouse Lung Pathogenicity

Virus	Mouse No.			
	1	2	3	4
NWS	5*	5	5	5
Wr	0	0	0	0
Derived Virus A	1	0	0	0
H	0	0	3	0
Q	0	0	0	2
R	0	0	0	0
14	0	0	0	0

* Lesions were scored 0 to 4: 0, normal lung; 4, complete consolidation; 5, death in 5 to 8 days.

TABLE VI

Comparative Neurotropism for Mice

Virus*	Effect with dilution tested					
	10^{-1}		10^{-2}		10^{-3}	
NWS	D	D	D	D	D	D
Wr	S	S	S	S	S	S
Derived Virus A	S	S	S	S	S	S
H	S	S	S	S	S	S
Q	S	S	S	S	S	S
R	S	S	S	S	S	S
14	S	S	S	S	S	S

D, indicates death in 5 to 8 days, S, indicates survival.

* EID_{50} varied from 10^7 to 10^8 per 0.1 ml.

rarely caused lung lesions and were therefore unlike NWS but similar to Wr strain in this respect.

Neutrotropism of viruses was determined by intracerebral inoculation of mice with 0.03 ml. of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of virus in buffered saline. Animals were observed for fatalities during a period of 10 days (Table VI).

Again the resultant viruses were seen to be unlike NWS and similar to Wr since all mice inoculated with test viruses survived.

Relative heat stability of viral hemagglutinin was determined by heating 0.2 ml. of undiluted virus in allantoic fluid at 60°C. for 7 minutes. NWS hemagglutinin was destroyed by this stress while significantly less effect was noted on Wr strain. Strains derived from genetic activity experiments manifested a characteristic similar to NWS in that hemagglutinins were destroyed under these conditions (Table VII).

Genetic characteristics determined for reagent and derived virus strains are summarized in Fig. 3. Heat lability of hemagglutinin and antigenicity of derived strains indicate similarity to NWS, while lack of virulence in either mouse lung or brain suggests Wr strain. In no instance was a derived virus obtained which manifested all 4 properties of either NWS or Wr.

It remained to be established whether the characteristics observed were those of a homogeneous virus population, or whether results were due to mixture of 2 or more strains with different traits. Therefore, each of 5 derived

TABLE VII

Relative Heat Stability of Hemagglutinins

Virus	Original hemagglutinin titer	Hemagglutinin titer after 7 min. at 60°C.
NWS	320	<10
Wr	640	240
Derived Virus A	160	<10
H	480	<10
Q	240	<10
R	480	<10
14	640	<10

strains were passaged at limiting infectious dilutions, for 3 or 4 transfers in order to isolate single clones (21). These were examined for the 4 inheritable characteristics and found to combine the same traits as did the initially isolated strains. Thus it seemed clear that results were not due to a mixture of viruses, and that the traits were genetically transferable.

It is probable that new strains resulted from genetic interaction between non-infectious NWS and infectious Wr virus.

Recombination with Dialyzed Irradiated Wr.—Genetic crossing experiments were attempted using the reverse arrangement of viruses, *i.e.*, irradiated Wr and infectious NWS.

Wr was dialyzed, quick-frozen, and stored at −60°C. as had been done with NWS in previous experiments. Thawed aliquots were similarly irradiated for varying periods of times and it was found that infectivity approached zero between 70 and 100 seconds. Controls were included to confirm loss of infectivity and to avoid autointerference.

The non-infectious Wr preparation was diluted and mixed with NWS in the previously described manner. In this case NWS antiserum was substituted for Wr antiserum as the selective agent.

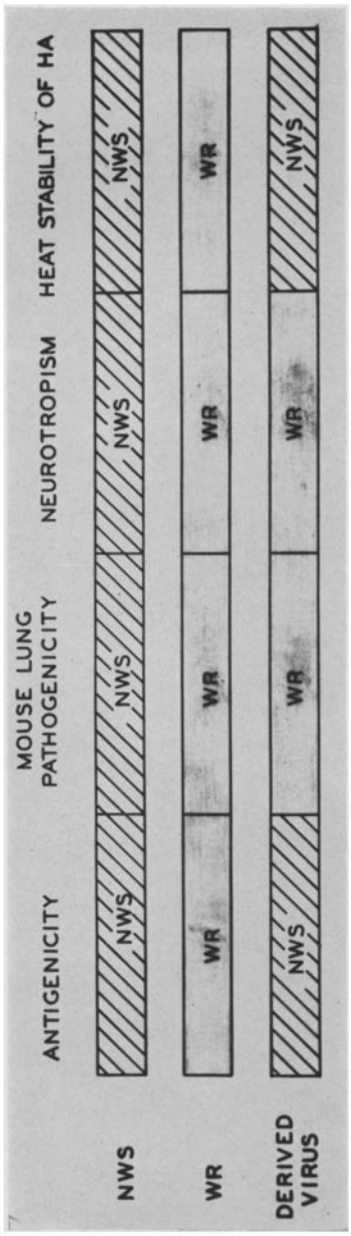

FIG. 3. Representation of genetic traits of original and derived viruses.

Results of such an experiment are summarized in Table VIII, in which two irradiation time intervals were used.

Tenfold dilutions of non-infectious Wr were mixed with each of undiluted and diluted NWS and the procedure described in Materials and Methods followed. As a control this same procedure was carried out in parallel with the omission of NWS. All eggs tested negative for virus in control experiments.

Virus isolations indicated in Table VIII were analyzed for the previously described genetic markers. This was carried out with first isolation materials as well as with those obtained after 2 passages at limiting infectious dilution. The viruses derived from this arrangement were antigenically similar to Wr strain, but manifested varying degrees of mouse lung pathogenicity. Only one isolate seemed to be as virulent as NWS, while others exhibited diminished capacity to produce lesions. Observations of other workers (22) with influenza virus recombinants resulting from mixture of 2 infectious strains,

TABLE VIII

Virus Isolations from Tests for Genetic Activity of Irradiated Wr Virus Preparations

70 sec. irradiation					100 sec. irradiation				
Irradiated Wr dilution 10^{-5}	—	—	—	—	Irradiated Wr dilution 10^{-2}	—	—	—	—
10^{-4}	—	—	—	—	10^{-1}	⊕	—	⊕	—
10^{-3}	⊕*	⊕	⊕	—	NWS dilution	10^{0}		10^{-3}	
10^{-2}	—	—	⊕	⊕					
NWS dilution	10^{0}		10^{-3}						

* ⊕ indicates eggs containing virus.

have similarly indicated that a virulent parent virus may transmit only part of this characteristic to combined forms. None of the viruses isolated was neurotropic for mice. Finally, the hemagglutinin was heat-stable, indicating similarity to Wr in this respect.

It was, therefore, concluded that new strains had been obtained, which like those isolated using irradiated NWS, possess a set of inheritable characteristics derived from both viruses in the original inoculum. However, as will be noted by comparison in Fig. 4, combination of traits is quite different from that found for virus derived by using non-infectious NWS.

It is interesting to note that antigenicity and heat stability were transmitted in association, but no other correlations were apparent from the data.

Effect of Ultraviolet Irradiation on Genetic Traits of NWS.—The question arose whether the experimental procedure, which included ultraviolet irradiation and 2 egg passages, resulted in loss of mouse lung pathogenicity and neurotropism by undetected infectious NWS, thus simulating combined virus.

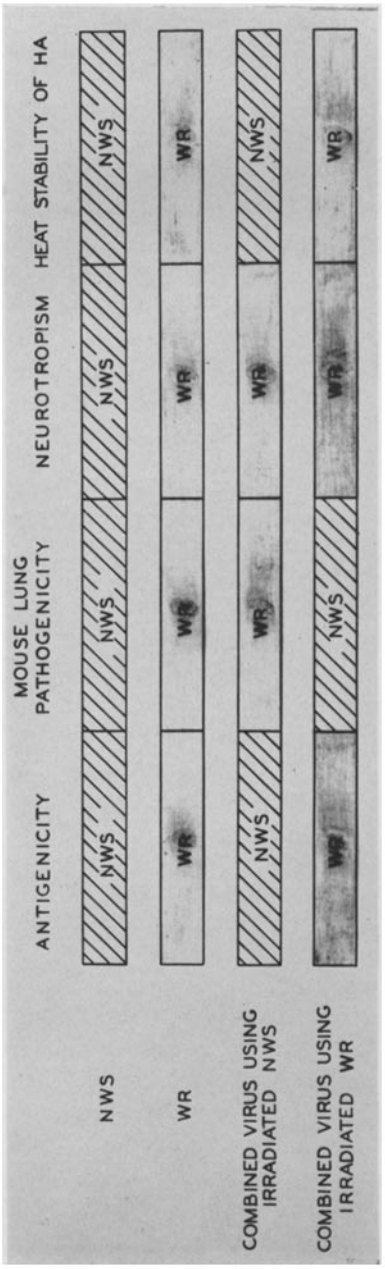

FIG. 4. Comparison of genetic traits of original and combined viruses.

Therefore, dialyzed NWS was irradiated in the previously described manner except that the time of exposure was reduced to 45 seconds in order to retain some infectivity. This material was passaged at dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} into 4 eggs per level. There were 3 positive eggs: 2 at 10^{-2} dilution and 1 at 10^{-4} dilution. Each fluid was then mixed with Wr antiserum and reinoculated into eggs. In essence NWS virus which had received a subminimal dose of ultraviolet light was manipulated as in a test for genetic activity except no active Wr was added.

After completion of the last egg passage, the 3 viruses were examined for genetic traits and found to retain all 4 properties of NWS. It was concluded that experimental procedures did not result in production of modified virus which resembled the genetic combination obtained by interaction of two virus strains.

Sedimentation of Genetically Active Material in Irradiated NWS Preparations.—Following observations that non-infectious NWS preparations were

TABLE IX

Sedimentation of Genetically Active Material in Irradiated NWS Preparations

Dilution of inactive NWS	Genetic activity of supernatant						Genetic activity of sediment resuspended in supernatant					
10^{-4}	—	—	—	—	—	—	—	—	—	—	—	—
10^{-3}	—	—	—	—	—	—	⊕	—	—	—	—	—
10^{-2}	—	—	—	—	—	—	⊕	⊕	⊕	—	—	—
10^{-1}	—	—	—	—	—	—	⊕	⊕	⊕	⊕	—	—

⊕ indicates eggs containing genetically crossed virus.

genetically active, it became of great interest to determine whether the active material resides in morphologically intact virus or consists of virus fragments.

To this point 8 ml. of irradiated virus preparation contained in plastic tubes was centrifuged at 13,000 R.P.M. for 3 hours at 6°C. in an angle-head centrifuge with a mean radius of approximately 3 inches. The force developed by this procedure was known to be sufficient to sediment intact influenza virus but not a significantly smaller particle. After centrifugation the plastic tube was pierced 3/4 inch below the fluid surface with a 25 gauge needle on a 0.25 ml. tuberculin syringe and 0.2 ml. of supernatant fluid carefully removed. The sediment was resuspended in the remaining fluid and another 0.2 ml. portion removed. Each sample was tested for genetic activity as shown in Table IX.

It can be seen that genetically active material was sedimented under these conditions and was not contained in relatively small molecules which were dissociated from the virus particle.

Attempts to Extract Genetically Active Materials from NWS Virus.—It now became of interest to determine whether genetically active material could be extracted from disrupted influenza virus following the lead of bacterial transformation studies.

Various methods were employed in attempts to fractionate the NWS strain. These included: (*a*) prolonged ultraviolet light irradiation; (*b*) enzymatic digestion; (*c*) treatment with sodium desoxycholate; and (*d*) osmotic shock. In addition, attempts were made to extract active material from infected chorioallantoic membranes. The procedures utilized and results obtained will be presented in a brief manner since no genetic activity was demonstrated.

1. Prolonged Ultraviolet Light Irradiation.—In previously described experiments ultraviolet irradiation was applied to NWS for long periods of time (Table I). In some instances treatment was applied until the viral hemagglutinating property was lost. This was considered an example of strenuous application of disruptive force to NWS since the relatively stable property of

TABLE X

Digestion of NWS with Trypsin

0.5 ml. of trypsin* diluted to:	Undiluted NWS, 37°C. for 1 hour	Hemagglutinin‡	Infectivity for eggs§	
1:500	0.5 ml.	−	−	+
1:1000	"	−	−	−
1:2000	"	−	+	+
1:4000	"	−	+	+
1:8000	"	±	+	+
1:16000	"	+	+	+
1:32000	"	+	+	+
1:64000	"	+	+	+
Buffered Saline	"	+	+	+

All eggs remained viable.

* Armour crystalline trypsin diluted in citrate-saline.

‡ 0.5 ml. of undiluted material plus 0.5 ml. of 0.5 per cent chick red blood cells.

§ 0.1 ml. of undiluted material per egg incubated at 10°C. for 2 days.

hemagglutination (18) was lost. No genetic activities could be demonstrated with such preparations.

2. Enzymatic Digestion of NWS-Trypsin.—It was believed this enzyme would break down protein while sparing nucleic acids and thus free genetic materials. Results of preliminary experiments established that infectivity and hemagglutinin of NWS could be destroyed with trypsin (Table X). Preparations treated with 1:125 final dilution of crystalline trypsin retained $10^{2.0}$ EID_{50} per 0.1 ml. Further reduction of infectivity to $10^{1.0}$ EID_{50} per 0.1 ml. was accomplished by incubating digests with 1:5 final dilution of NWS chicken antiserum at 6°C. for 30 minutes followed by removal of any residual NWS antibodies by adsorption with WS-coated formalinized human red blood cells (23).

These extracts were each tested for genetic activity in the standard manner at tenfold dilutions beyond the last level of infectivity. No virus was derived by any of these procedures.

Lipase.—Since it has been postulated that influenza virus is enclosed in a lipide membrane, it was considered possible that lipase (Nutritional Biochemical Corporation, Cleveland) might specifically digest the membrane and leave genetically active fragments. Table XI illustrates the effect of varying concentrations of lipase on NWS. It was found that hemagglutinin and infectivity decreased in parallel indicating equal susceptibility to the action of lipase. This determination was repeated using twofold dilutions of enzyme and results indicated that 1:10 initial dilution of lipase would be sufficient to

TABLE XI

Digestion of NWS with Lipase

0.5 ml. of lipase diluted to:	Undiluted NWS, 37°C. for 1 hour	Hemagglutinin titer	Infectivity for eggs of 20 EID_{50}			
1:20	0.5 ml.	80	−	−	−	−
1:200	"	80	+	−	−	−
1:2000	"	240	+	+	+	+
1:20,000	"	240	+	+	+	+
1:200,000	"	240	+	+	+	+
Buffered Saline	"	240	+	+	+	+

TABLE XII

Treatment of NWS with Sodium Desoxycholate

0.05 ml. of sodium desoxycholate diluted to:	Undiluted NWS	Hemagglutinin titer, 22°C. for 30 min.	Infectivity for eggs of 10 EID_{50}		
1:50	0.5 ml.	10	−	−	−
1:100	"	40	+	−	−
1:200	"	320	+	+	−
1:400	"	320	+	+	−
1:800	"	480	+	+	+
Buffered Saline	"	480	+	+	−

destroy viral infectivity and hemagglutinin. However, at such concentrations it is quite likely that contaminating enzymes were present in significant amounts. Infectivity of virus suspensions, treated with the enzyme preparation, dropped to less than $10^{1.0}$ EID_{50} per 0.1 ml. Tests for genetic activity of the digest yielded negative results.

3. Sodium Desoxycholate Treatment of NWS.—Treatment of bacteria with sodium desoxycholate is a classic method for bacterial disruption which releases genetically active desoxyribose nucleic acid (7). With this in mind the effect on influenza virus was studied and results obtained are presented in Table XII. As with lipase the loss of hemagglutinin and infectivity oc-

curred at the same treatment level. Results of similar experiments indicated that 1:8 initial dilution of sodium desoxycholate would decrease infectivity to $10^{3.7}$ EID_{50}. Tests for genetic activity with materials prepared in this manner did not result in any virus recoveries.

4. Attempts to Disrupt NWS by Osmotic Shock.—Osmotic shock will cause viral desoxyribose nucleic acid to be separated from the protein portion of bacteriophage resulting in 100-fold decrease of infectivity (24). Osmotic shocking is accomplished by mixing equal volumes of virus and 3 M salt solution, with subsequent rapid dilution with 50 volumes of distilled water. Various salts (NaCl, KNO_3, $NaHCI_3$, Na_2SO_4, and K_2HPO_4) tested in this manner did not result in detectable loss of infectivity with suspensions of influenza virus.

5. Extraction of Infected Chorioallantoic Membranes.—Since viral genetic determinants must be present in host cells after infection but before mature virus is produced, attempts have been carried out to extract such materials from infected chorioallantoic membranes.

To this end, 11 to 15 day old embryonate eggs were inoculated with 0.5 ml. undiluted allantoic fluid containing $10^{7.8}$ EID_{50} and incubated at 37°C. for 15, 30, 60, 90, and 120 minutes. Time intervals were designed to include various stages of virus development prior to the formation of complete progeny. Infected chorioallantoic membranes were removed from the eggs, washed in cold buffered saline, ground with alundum at 4°C., and suspended in 2.0 ml. of 0.1 M sodium citrate and 0.1 M sodium chloride (citrate-saline) per membrane. Citrate was employed to stop the action of any desoxyribonuclease in the preparation (7). Extracts were centrifuged at 2500 R.P.M. for 30 minutes at 4°C. Supernatant fluids contained as much as $10^{8.3}$ EID_{50} per 0.1 ml. Infectivity was reduced to $10^{2.5}$ EID_{50} by high speed centrifugation or treatment with antiserum as described previously. Digestion with trypsin dropped infectivity levels to approximately $10^{0.5}$ EID_{50} per 0.1 ml.

The freshly prepared extract, centrifuged extract, antiserum-treated extract, and trypsin-digested extract were each tested for genetic activity by the standard method at tenfold dilutions beyond the level of infectivity.

No virus recoveries were made indicating that genetic materials were not demonstrable in the extracts.

Since the possibility existed that genetic material was present but not in a form necessary for host cell penetration an attempt was made to increase cellular permeability by injection of 1.0 ml. of water into the allantoic sac prior to testing. Again no genetic effects were demonstrated.

Negative results obtained with this procedure do not, however, exclude the possibility that genetically active materials were present in the extracts, and in fact, the critical factor may be to determine more suitable methods for permitting host cell penetration.

Effect of Time Interval between Virus Inoculations on Genetic Combination.—Evidence has been presented (25) that in order for recombination to occur with infectious influenza viruses it is necessary that the second virus be in-

jected not more than 1 hour after the first. However, it was believed that the effective time interval was considerably longer with the system under study because experiments employing small amounts of reagent viruses resulted in undiminished genetic crossing despite the fact that double "infection" of a single cell in the first infectious cycle would be a theoretical rarity. It was reasoned that inactive virus within the egg remains susceptible to genetic crossing for the many hours it takes the infectious virus to multiply sufficiently so that double "infection" could occur.

To test the hypothesis that irradiated NWS determinates remain genetically active in host cells for long periods, 0.1 ml. of a 10^{-2} dilution of irradiated NWS was first inoculated into the allantoic cavity of 10 day eggs and at increasing time intervals up to 7 hours 0.1 ml. of undiluted or diluted allantoic fluid containing Wr was inoculated.

With the use of the previously described standard procedure, combined virus was isolated at each of the time intervals. Succeeding experiments have shown that the combining property is retained within the egg for at least 4 days after inoculation with irradiated virus. Aging of eggs appears to be the limiting factor in determining the duration of this interval.

DISCUSSION

It is now clear that non-infectious influenza virus may retain functioning genetic materials. When virus irradiated with ultraviolet light under specific conditions lost the ability to propagate, as shown by elaborate controls, it was not genetically inactivated. Infection of embryonate eggs with a mixture of such a preparation and infectious related influenza virus resulted in formation of viral forms which possessed inheritable characteristics of both the infectious virus and non-infectious virus strains. Appropriate controls ruled out the probability that these forms could be derived from infection with only one virus strain.

The nature of mechanisms involved in this reaction is an intriguing problem. Genetic activity of irradiated virus suspensions was sedimented by gravitational forces sufficient to remove intact influenza virus from suspension but which were unable to sediment significantly smaller particles. This leads to the assumption that the activity does not reside in small portions of virus material split off by irradiation, but rather is a part of the intact virus particle. However, it is possible that fragmented virus would adsorb to viral elementary bodies and be sedimented with them. The first alternative appears more likely at this time.

The series of experiments designed to fragment influenza virus and extract genetic carrier materials from infected tissues led only to negative results. Several explanations appear possible: (*a*) the entire virus particle may be necessary for genetic transfer so that demolition leads to loss of this func-

tion; (*b*) freed genetic materials perhaps could not be demonstrated because of inefficient extraction methods, or because inhibitory substances masked their presence; (*c*) although successful extraction may have been accomplished, the material could not gain entrance into the host cell in the necessary form and quantity. Circumvention of this potential difficulty might be achieved by methods permitting cellular penetration. It is of interest to note that reactivation and recombination of ultraviolet irradiated bacteriophage have, to date, been associated only with phage particles (9).

When genetically active irradiated influenza virus is introduced into a cell the genetic influence appears to remain for an undetermined length of time. Thus eggs which have been injected with altered virus preparations could be inoculated as long as 4 days later with the infective strain to produce combined forms of influenza virus. Although it is not clear when the irradiated virus enters host cells, it must do so in order to engage in recombination. Perhaps the virus remains on the cell surface or enters the cell prior to penetration by the infectious strain. In these sites it could remain latent or initiate cellular orientation for virus production. The role of infectious virus may be either to contribute genetic material to replace ultraviolet damaged portions, or to set in motion "stalled" virus producing mechanisms which were only partially activated by irradiated virus. Another possibility is that the cell has produced viral genetic material which is synchronized with subsequently introduced genetic materials contributed by the infective virus.

Implications of these concepts suggest application in several problems. One is virus evolution. The general theory of evolution proposes that natural selection favors variants possessing survival advantage. Variants are thought to begin as mutations which may be injected into the population by recombination. Resultant new genetic associations allow many different phenotypic expressions, which substantially increase the probability that an individual with survival advantage will appear. "Segregation and recombination . . . are the main genetic processes involved in population variation." (26) Any mechanism increasing opportunity for recombination is a potential asset. Irradiated non-infectious virus appears to be such an asset to the species since it remained in genetic recombining form at least 100 times longer than infectious virus.

It is not difficult to imagine virus in expired droplets from infected individuals undergoing various degrees of inactivation due to disruptive forces such as ultraviolet light, visible light, desiccation, and thermal effects (15, 27). Upon contacting a susceptible host cell, virus may enter the latent genetic stage for an undetermined period. Subsequent superinfection by fully infectious related virus might result in genetic combinations leading to variation. Variants with survival value could replace the existing viral population. Antigenic variants among strains of human influenza may arise through

this process of delayed recombination. The existing antibody pattern in the human population would be a selective factor favoring virus forms with antigenic structure not readily neutralized. Experimental support for this hypothesis comes from studies demonstrating viral antigenic change resulting from exposure to antibody (28, 29) and from antigenic combination arising during recombination experiments (4).

Delayed recombination like bacterial transformation may also provide a mechanism whereby individual, desirable genetic traits may be preserved for the species. It has been shown experimentally that penicillin-killed, streptomycin-resistant, encapsulated pneumococci when mixed with living unencapsulated pneumococci resulted in forms which were streptomycin-resistant or encapsulated (30). The result was to salvage the potentially valuable "genes" of drug resistance or encapsulation from non-infectious organisms. Similarly it has been shown in studies reported here that influenza virus is able to recover antigenic traits and virulence from non-infectious influenza virus.

These concepts are, of course, highly speculative and await confirmation with more direct experiments using higher animal forms.

Other reports have appeared in which virus strains studied in genetic recombination or reactivation experiments have been heated (11, 31) or subjected to ultraviolet irradiation (10, 32). None of these experiments was completely analogous to those reported here in that either the virus forms were not proven non-infectious or that a combination of genetic characteristics was not described for resultant virus.

Of these rabbit fibroma-myxoma transformation (11) and influenza multiplicity reactivation (10) are strikingly similar to the non-infectious recombination phenomenon studied here. The mechanism of occurrence, which may also be similar, might be definitely determined by employing genetic markers and sedimentation studies. If the fibroma-myxoma reaction occurs in this way, it can be anticipated that ultraviolet irradiation should eliminate the inconsistencies resulting from the less selective method of heat inactivation which has been utilized (33).

Use of heat-killed influenza virus in recombination studies was reported (31) while experiments described here were in progress. Controls for live virus, including autointerference, were not rigorous so that reactions may have occurred between infective virus of each strain. Nevertheless, good statistical evidence was provided for genetic combination involving non-infectious virus. It is interesting to note that these investigators had difficulty in obtaining heat-killed virus preparations that retained genetic activity. In contrast ultraviolet light has readily rendered virus non-infectious while retaining recombining activity. Ultraviolet light inactivation has also been found more efficient than heat in multiplicity reactivation experiments (10). Perhaps these observations indicate greater selectivity of ultraviolet irradiation. There

is ample evidence that this force may inactivate properties of influenza virus in ordered sequence (18).

Placement of the recombining property within this sequence seems desirable. It was noted that demonstrable genetic activity titer was about 10^8 when interference titer was 10^7 (assuming that interference within one egg requires at least 10^7 particles (34)). This suggests that the genetic property is inactivated more rapidly than interference and thus occurs sometime before interference and after infectivity in the ultraviolet inactivation gradient. However, recombination may occur with much greater frequency than is observable with these procedures because the method selects only a particular kind of recombinant, *i.e.*, those resistant to the antiserum. Also multiple cellular infection, which is required for recombination, may result in interference phenomena, and therefore lessen the number of derived viruses appearing.

It is apparent that phenomena described here merit additional study. Investigations utilizing a delayed recombination system may result in better understanding of mechanisms involved in virus propagation.

SUMMARY

Influenza virus rendered non-infectious by ultraviolet irradiation retained ability to "exchange" genetic traits with related virus, resulting in recombined forms. Sedimentation studies indicated association of recombining activity with particles approximately the size of influenza virus. Genetic activity was not demonstrated when virus was more severely disrupted in attempts to observe phenomena analogous to bacterial transformation. Irradiated virus was also shown to remain capable of genetic exchange for at least 4 days after inoculation into embryonate eggs. In contrast infectious virus becomes insusceptible to genetic exchange after 1 hour incubation in eggs. The importance of this delayed recombination phenomenon to processes of virus evolution and influenza strain variation was discussed.

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