A Genetic Approach to Variation in Influenza Viruses

4. Recombination of Characters Between the Influenza Virus A Strain NWS and Strains of Different Serological Subtypes

BY F. M. BURNET AND P. E. LIND

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

SUMMARY: Mice were inoculated intracerebrally with mixtures of the influenza virus strain NWS and the serologically distinct non-neurotropic strains MEL, OcI and SW. From mouse brains removed about the 5th or 6th day virus strains were obtained which, after at least two passages at limiting infective dilution, showed active encephalitogenic power with the serological character of the second strain of the original mixture. These are regarded as recombinants. The strains in general show *in vitro* characters close to those of the serologically similar 'parent'. They have, however, diminished enzymic action on ovomucin and do not readily elute from red cells; in this they resemble the strain NWS. They are more readily converted to the indicator state than either of the original strains.

The process by which these unusual types arise is discussed in the light of recent views that intracellular multiplication of bacterial viruses and of some animal viruses takes place not by binary fission but by the breaking down of the infective unit into smaller particles which are the replicating units. It is concluded that the recombinants arise by reconstitution of infective particles from the pool of replicating units produced by double infection of a single cell by virus particles of both parent types.

In a previous paper (Burnet & Lind, 1951) we described the isolation from the brains of mice infected intracerebrally with mixtures of the strains NWS and WSM, of four strains which had characters apparently derived from both 'parents'. It was of obvious interest to see whether similar recombination and characters could be obtained by this method, using strains which, as well as having no encephalitic action, were serologically distinct from NWS.

In this paper we report the production of several strains in which serological characters corresponding to MEL, SW and OcI were combined with encephalitogenic action. In this way we have now available recombinant strains of each of the major serological subtypes of influenza A virus resulting from their interaction with the strain NWS.

MATERIALS AND METHODS

The methods used were described by Burnet (1951*a, b*), Burnet & Lind (1951) in earlier papers of this series. The only important modification used in our later experiments was to emulsify brains from mice infected with mixtures of NWS and another virus in a 10% solution in saline of serum from a fowl immunized against NWS. In this way it was possible to inhibit, not always completely, growth of any NWS that might be present when the brain emulsions were tested in chick embryos.

Virus strains

The NWS strain used was described by Burnet (1951*a*).

MEL. The Melbourne strain adapted to mouse passage during 1935-6, but since 1940 maintained by storage of amniotic or allantoic fluid at the temperature of solid CO₂. For the experiments from which Nr-MEL II, III and IV were derived a 'pure clone' stock of MEL was used.

SW. Shope's strain swine 15 received through Dr C. H. Andrewes in 1936, and maintained as was MEL.

OcI. The strain isolated from the Ocean Island epidemic of 1948 by Isaacs, Edney, Donnelley & Ingram (1949). This is antigenically of the CAM-FMI (A¹) group and shows the characteristic filamentous morphology under dark-ground examination. After isolation by amniotic inoculation in chick embryos it was adapted to allantoic passage, re-isolated at limit infective dilution and maintained as glycerolated seed virus.

EXPERIMENTAL

There are two main sets of experiments. The first set deals with the origin and characters of the first two strains which were regarded as recombinants of NWS with MEL and SW respectively; the second set with experiments carried out in 1950, essentially to establish the reproducibility of the phenomena. In these anti-NWS serum was used to assist the recovery of recombinants in the presence of NWS and the procedure thereby greatly simplified. Both the strains to be described in the first section of the paper were derived in a single experiment designed primarily to observe the range of viruses which on simultaneous injection would interfere with the encephalitogenic action of NWS.

NWS fluid 54,026 was diluted 10⁻³ and 0.25 ml. volumes were mixed with equal amounts of undiluted allantoic fluid viruses as shown in Table 1. In the case of MEL, dilutions of 10⁻¹, 10⁻², 10⁻³ were also included, with a control mixture in which an extra volume of the diluent, normal horse serum saline (10 % serum), was used. The viruses in this experiment were stock fluids harvested from 0 to 6 days previously.

Each mixture was inoculated intracerebrally into four mice (4-5 weeks old) in 0.03 ml. amounts. The mice were observed for 12 days. A large proportion of those showing signs of encephalitis or dying later than the control mice were examined for amount and type of virus present in the brain. The whole brain was removed aseptically and ground with coarse alundum powder before being emulsified in 2 ml. physiological saline. The suspension was spun lightly and the supernatant fluid inoculated at dilutions from undiluted to 10⁻³ in 11-day chick embryos. All positive fluids at or near the limiting infective dilution were tested for serological type with immune ferret sera. Those which showed evidence of serological character other than WS were then studied in detail.

The results are shown in Table 1 from which it will be seen that interference was complete with the two WS strains and PR8, and virtually complete with the other A strains, MEL and BEL. With SW there was some evidence of interference, none with LEE, Mumps or NDV. Most of the strains isolated

Table 1. *Interference with encephalitogenic action in mice of NWS by other viruses*

Added virus	Deaths	Virus isolations in eggs from mouse brains indicated by A, B or C
MEL undiluted	(8)A, — — —	A — — —
MEL diluted 10 ⁻¹	5A, 5B, 6C, 8	A: N, N, N; B: N, N, MEL; C: N, + +
Mumps	5, 6, 6, —	.
NDV	5, 5, 5, 6	.
WSM	— — — —	.
WSE	— — — —	.
BEL	(8)A, — — —	A: N, N
PR8	+ — — —	.
SW	5A, (7)B, 7C, —	A: N, SW; B: N, N, N; C: N, N
LEE	5A, 5B, 7C, 7	A: N, N; B: N, N; C: N, N, N
Control	5, 6, 6, 6	.

5, etc.=death on 5th day after injection; (8)=mouse sick and killed on 7th day, which would probably have died on 8th day; —=survival for 12 days.

N=virus resembling NWS serologically.

MEL, SW=virus showing this serological character present in the allantoic fluid.

+ =virus present but not further characterized, usually because of low titre of fluid.

from brain emulsions were of the NWS type. Some may have been of N' type (Burnet & Lind, 1951) but this was not studied. The two strains indicated as MEL and SW had the serological character of the corresponding non-neurotropic strain in the mixture used. Details of the titration of the mouse brains are shown in Table 2.

Table 2. *Titration of mouse brains from which recombinants were obtained*

Mouse no.	Virus added	Virus isolated in eggs			
		Undiluted	Dilution		
			10 ⁻¹	10 ⁻²	10 ⁻³
70,555	MEL	+ + —	N, N, +	MEL, — —	— — —
70,594	SW	+ + +	SW, N, —	— — —	.

Symbols as in Table 1.

Purification and characterization of the strain Neuro-Swine Nr-SW I

The allantoic fluid shown in Tables 1 and 2 as of SW serological character (fluid from egg 54,598 inoculated with mouse-brain 70,594) was inoculated undiluted into the brains of four mice. Five days later one was dead and two were very sick. From the brains of these three animals virus of the same SW serological type was isolated. One fluid of this group, (54,914) whose characters are shown in Table 3, was titrated in eggs, and the fluids from the limiting infective dilution of 10⁻⁸ tested. All positive fluids showed the typical serological reactions of a swine strain and one of these (55,198) tested in mice at a dilution of 10⁻³ produced typical symptoms in all, with death in the two not killed for culture. Emulsions of these two brains were infective for chick embryos up to a dilution of 10⁻³, and allantoic fluids from this dilution were of Swine serological type. All the allantoic fluids that have been mentioned had

similar characteristics, and we refer to the type of virus involved as Neuro-Swine (Nr-SW) this particular strain being Nr-SW I.

The sequence of passages from which the stock glycerolated seed virus of Nr-SW I was derived can be represented as follows:

$$\text{Mixture NWS} + \text{SW} \rightarrow \text{M} \rightarrow \text{E} \rightarrow \text{M} \rightarrow \text{E} \rightarrow \text{E} \quad \text{L} \quad (55,198),$$

where M is mouse inoculation, E is allantoic inoculation and L is passage at the limiting dilution.

The characters of pathogenicity for mice by intracerebral injection and neutralization in anti-haemagglutinin tests by Swine antisera are shown in Table 3.

Table 3. *Serological character and pathogenicity of Nr-SW strain*

(Titres shown represent dilution of ferret serum neutralizing five agglutinating doses of virus in anti-haemagglutinin tests.)

	Passage	'Antisera'				Intracerebral pathogenicity for mice
		NWS 304	SW 303	MEL 310	LEE 309	
E 54,598	0	10	100	15	< 10	10 ⁰ 5, (6), 6
E 57,914	2	15	120	.	.	10 ⁴ (7), 7, 7, 8
E 55,198	3	< 50	240	.	.	10 ^{-2.2} (7), (8), 8, 8
SW	.	20	320	< 20	< 20	10 ⁰ — — —

The capacity to produce encephalitis is well marked, mice dying when inoculated with fluids diluted 10⁻⁴ and their brains yielding relatively large amounts of virus. Brain emulsions were usually infective for eggs up to a dilution of 10⁻³. The stock SW strain from which the recombinant was derived had no influence on mice inoculated intracerebrally either with undiluted or 10⁻² allantoic fluid virus. In the only experiment so far completed interference with the encephalitogenic action of Nr-SW I is strongly shown by MEL and WSM and partially by LEE. Mixtures of Nr-SW I diluted 10⁻³ with equal volumes of undiluted virus gave no deaths with MEL and WSM, three delayed deaths and one survival with LEE.

Purification of the strain Nr-MEL I

The original fluid from eggs inoculated with mouse-brain emulsion was heavily contaminated with bacteria but had a high enough haemagglutinin titre to allow its characterization as a MEL-type strain. This was treated with penicillin and diluted 10⁻⁸ for passage. Two positive and two negative fluids were obtained, but both positive fluids were still contaminated. We then applied the simple procedure of shaking the fluid with toluene and after leaving for 1.5 hr. at 37° inoculating eggs with a 10⁻² dilution. These gave virus-containing fluids free from bacteria. One of these fluids, 55,014, was inoculated into mice undiluted and also diluted 10⁻². One of four mice inoculated with the undiluted material (M70,738) died on the 6th day, the other three survived. In the group inoculated with 10⁻² dilution of virus there was also only one death (M 70,734), in this case delayed until the 12th day. From mouse 70,738 two

eggs inoculated with brain emulsion diluted 10^{-1} gave positive fluids, both with MEL serological character. A further allantoic passage at limiting infective dilution 10^{-9} gave fluid 55,841. On testing this in mice intracerebrally the virus was much more pathogenic than in the first passage, giving deaths on the days indicated as follows: undiluted, 3, (5), 7; diluted 10^{-2} , (6), 7, 7. Brains from one mouse in each group showed virus to a dilution of 10^{-3} of MEL serological type. Stock virus Nr-MEL I is derived from a further limit-dilution passage of virus derived from this series, its history being expressible as follows:

L   L   L
mixture MEL + NWS → M → E → E → M → E → E → M → E → E stock 56,608.

The *in vitro* characters of Nr-MEL described in the next section were assessed on fluids from eggs inoculated with the glycerolated stock fluid 56,608.

In vitro characters of Nr-SW and Nr-MEL strains

It is obviously of the greatest interest to find whether the development of neurotropism is regularly associated with any other demonstrable change in the virus. The strains Nr-SW I and Nr-MEL I were therefore examined in parallel with the normal strains and NWS for most of the measurable *in vitro* characters of influenza viruses. All tests were made with allantoic fluid virus not more than 5 days old, obtained by inoculating 11-day embryos with 10^{-6} dilution of glycerolated seed virus and incubating at 35° for 2 days.

The thermostability of the haemagglutinin of the neuro-strains was almost identical with that of the normal types. The result of heating virus buffered with phosphate to pH 7.35 for 30 min. at temperatures between 52 and 60° is shown in Table 4. There is no significant change in position in the receptor

Table 4. *Thermostability of haemagglutinin from original and recombinant strains*

(Figures show haemagglutinin titre given by allantoic fluid virus heated for 30 min. at the temperature shown.)

Virus strain	Titre of unheated strain	Temperature				
		52°	54°	56°	58°	60°
		Haemagglutinin titre				
MEL	480	.	420	480	360	25
Nr-MEL I	300	.	240	280	110	30
SW	200	160	70	< 2	.	.
Nr-SW I	250	80	50	< 2	.	.
NWS	320	.	< 2	.	.	.

gradient. Using the standard method with fowl cells treated with graded amounts of RDE the results shown in Table 5 were obtained. When attempts were made to compare the reactions of cells which had been treated directly by the two viruses a clear distinction between Nr-SW and SW was found. After 5 hr. action of excess virus at 37° cells treated with SW were found to be stable and to have lost their agglutinability by all the available viruses. Cells treated with Nr-SW, on the other hand, showed no sign of stabilization and they could

be stabilized only by the use of an appropriate dilution of anti-SW ferret serum. When tested the only virus failing to agglutinate these cells was NDV. Both SW and Nr-SW were fully active. The deduction that Nr-SW was adsorbed to, but had little or no enzymic action on, the receptor substance of the cell surface was confirmed by measuring directly the adsorption and elution of the viruses

Table 5. *Place in receptor gradient with fowl cells treated with graded dilutions of receptor-destroying enzyme (RDE)*

(Symbols show the relative degrees of agglutination by five agglutinating doses of virus, of cells treated for 30 min. with dilutions of stock enzyme (titre 1/1500.))

Virus strain	RDE dilutions					
	1/25	1/50	1/100	1/200	1/400	1/800
	Degree of agglutination					
MEL	—	—	—	±	++	++
Nr-MEL I	—	—	—	+	++	++
SW	—	±	++	++	++	++
Nr-SW I	—	+	++	++	++	++
NWS	—	—	—	—	—	++

to fowl cells. Both were adsorbed well but after 5 hr. at 37° the supernatant fluids showed titres of SW 160, Nr-SW 2, compared with original haemagglutinin titres of 500 in both instances.

An almost identical relationship exists between Nr-MEL and MEL. In one experiment the two stock viruses were diluted so that each had a haemagglutinin titre of 250 and 0.125 ml. of packed fowl cells added in the cold to 4 ml. of virus. After 20 min. at 0° the tubes were spun and the supernatant fluid removed. This was replaced by 4 ml. of saline and the resuspended cells placed in a water-bath at 37°. At the end of 2 hr. it was obvious that the cells treated with MEL were relatively stable while those treated with Nr-MEL were grossly unstable; this persisted when the tubes were removed after 4 hr. at 37°. After separation of the supernatant fluid the cells were washed (after stabilization with immune serum in the case of Nr-MEL cells) and tested for their susceptibility to agglutination by different viruses; the results are recorded in Table 6. The cell reactions are unusual in that Nr-MEL leaves NDV and MEL receptors apparently intact while greatly diminishing its own reactivity; MEL, on the other hand, removes NDV and MEL receptors but has no greater action on Nr-MEL receptors than the homologous virus.

Both new strains are more readily converted to the indicator state than the originals. Nr-MEL is of special interest in being convertible to indicator by simple heating for 30 min. at 56° which, as Stone (1949) has shown, is without effect on the standard virus. In the presence of citrate at pH 8.5 heating to 50° converts Nr-MEL to a sensitive indicator while MEL requires a temperature between 52 and 54°.

The SW and Nr-SW strains are much more readily converted to the indicator state. Simple heating at 50° brings out the full susceptibility of Nr-SW I but has only a minimal effect on most samples of SW. Occasionally older fluids of

Table 6. *Comparison of action of MEL and Nr-MEL on fowl cells*

(The upper half of the table shows the differences between MEL and Nr-MEL when absorbed to fowl cells in the cold and then allowed to elute at 37°. The figures show the haemagglutinin titres of supernatant fluids. Nr-MEL is adsorbed but not eluted. The second half of the table shows the reactions of cells treated with MEL or Nr-MEL and stabilized when tested with five agglutinating doses of the six viruses shown.)

Fluids	Virus	
	MEL	Nr-MEL
	Haemagglutinin titre	
First supernatant 0°	2	22
Supernatant 2 hr. at 37°	280	16
Supernatant 4 hr. at 37°	300	10

Cells treated with virus	Testing virus					
	NDV	NWS	WSM	Nr-MEL	MEL	LEE
	Reaction of cells					
Nr-MEL	++	—	—	+	++	++
MEL	—	—	—	+	—	++

Table 7. *Conversion of normal and 'neuro' types of MEL and SW to the indicator state*

(Figures shown are dilutions of ovomucin solution giving end-point inhibition of haemagglutination by five agglutinating doses of virus. For all instances not otherwise shown, heating was for 30 min.)

Treatment	MEL	Nr-MEL
	Dilution of ovomucin	
Heated 56°	< 10	3,000
Heated with citrate at pH 8.5		
50.5°	20	30,000
52°	2,000	.
54°	30,000	30,000
56°	30,000	.
	SW	Nr-SW
Heated 50°	100	< 10,000
Heated with citrate at pH 8.5		
50°	10,000	20,000
37° (2 hr.)	300	20,000

Nr-SW have been found which showed indicator activity without being heated (see Table 7).

Enzymic action on ovomucin. Both neuro-variants are much less active than the standard strains in destroying the inhibitor quality of ovomucin. As pointed out in previous papers the extent to which inhibitor, especially inhibitor for the indicator-treated MEL (T-MEL) (Stone, 1949), is destroyed, often depends greatly on the concentration at which the ovomucin is present in the reacting mixture. With many samples of ovomucin, MEL fails to act unless the ovomucin is diluted to a level equivalent to about 2000–3000 inhibitor units.

With the sample used in the present experiments both MEL and SW completely destroyed inhibitors at the highest concentration of ovomucin used. The method described by Burnet (1949) was used, a final dilution of twenty agglutinating units of virus being present in a final volume of 1.25 ml. Ovomucin prepared according to the method of Gottschalk & Lind (1949) was diluted 1/5 in Ca saline lightly buffered with acetate at pH 6.2, then centrifuged. The supernatant fluid was used in 1 ml. vol. and 0.25 ml. of each virus, adjusted to a strength of 100 agglutinating units was added. A drop of toluene served as a bacteriostatic substance and the corked tubes were held for 18 hr. at 37°. Residual haemagglutinin was eliminated by heating to 62.5° for 30 min. The mixtures were then titrated for inhibitor using six indicator viruses WSE, LEE, MEL, Nr-MEL, SW and Nr-SW, each treated in the fashion appropriate to the strain to give a fully sensitive indicator. The results are recorded in Table 8.

Table 8. *Action of viruses on the inhibitor qualities of ovomucin*

(Figures show dilution to which five agglutinating doses of indicator virus are inhibited.)

Ovomucin acted on by	Indicator strain					
	H-WSE	H-LEE	T-SW	T-Nr-SW	T-MEL	T-Nr-MEL
Nil	3,000	3,000	8,000	8,000	30,000	20,000
MEL	< 10	< 10	< 10	< 10	< 10	< 10
Nr-MEL	100	80	250	250	8,000	6,000
SW	< 10	< 10	< 10	< 10	< 10	< 10
Nr-SW 1	80	80	250	80	30,000	12,000

With ovomucin initially diluted 10^{-1} there is a much less evident difference. Both Nr-MEL and Nr-SW destroy all inhibitor for T-MEL or T-Nr-MEL under these conditions, but Nr-MEL leaves a small residual inhibition against heated WSE (H-WSE) and T-SW.

Isolation of further strains of Nr-MEL and Nr-SW

It seemed desirable to make a fairly elaborate study of the first two strains before attempting to obtain further recombinants of the same type. In two more experiments on similar lines we had no difficulty in obtaining several more strains. It will have been noted that the neuro-strains studied can be distinguished from normal strains by a simple *in vitro* procedure. Nr-MEL strains heated to 56° for 30 min. take on indicator status, whereas MEL strains do not. The same difference between Nr-SW and SW strains is found when the temperature is 50°. If these differences are typical then any infected fluid obtained by subculture from mouse brain can be rapidly characterized by testing it serologically against the two appropriate ferret sera and, after heating, for indicator status against ovomucin dilutions. With one exception discussed below there has been general concordance of mouse pathogenicity by intracerebral route with these *in vitro* characters. Another refinement of technique has been to add anti-NWS serum (made by immunization of fowls) in a final concentration of 1/10 to all dilutions of mouse-brain emulsions, and to hold the dilutions thus prepared at 4° for 1 hr. before inoculating into eggs. Thus we can

prevent the appearance of the original non-neurotropic form, since it cannot survive for 5–6 days in the brain and by means of the anti-NWS serum we can prevent growth of the original NWS unless there is very active multiplication.

The procedure was standardized along the following lines. Mice were inoculated with mixtures containing NWS diluted 10^{-3} and equal volumes of the non-neurotropic virus fluid, undiluted or diluted 10^{-1} and 10^{-2} . In all these experiments the seed viruses used had been prepared from 'pure clone' fluids. At the 5th or 6th day mice with the smallest proportion of the second virus were usually dead or dying and at this stage mice with minimal symptoms in the other groups were killed for culture. The brains were removed, ground with saline containing 10 % of serum from fowls 285 or 286 (anti-NWS) and 100 units penicillin/ml. After centrifugation and a variable period in the refrigerator the supernatant fluid was inoculated undiluted or diluted 10^{-1} , 10^{-2} , 10^{-3} in horse-serum saline containing anti-NWS serum 1/10 into 11-day embryos by the allantoic route. After 3 days all positive fluids were tested for neutralization of haemagglutinin by ferret antisera corresponding to the two strains in the mixture. All strains serologically NWS were discarded. Those neutralized by the other serum were then tested for conversion to the indicator state by simple heating, and a representative example from the highest infective dilution of brain substance chosen for passage at limiting dilution. Dilutions of 10^{-7} , 10^{-8} , 10^{-9} were subinoculated to 11-day embryos and the limit positive fluid tested similarly *in vitro* and then inoculated diluted 10^{-1} into mice intracerebrally. From a sick or dead mouse at 5 or 6 days virus was re-isolated by the same procedure and a further limit-dilution passage made. This final fluid was then tested for its power to produce encephalitis when injected intracerebrally at 10^{-1} dilution and its *in vitro* characteristics checked.

The history of the strain Nr-SW III was as follows:

$$\text{NWS } 10^{-3} + \text{SW } 10^0 \rightarrow \overset{\text{L}}{\text{M}_1} \rightarrow \overset{\text{L}}{\text{E}_1} \rightarrow \overset{\text{L}}{\text{E}_2} \rightarrow \overset{\text{L}}{\text{M}_2} \rightarrow \overset{\text{L}}{\text{E}_3} \rightarrow \text{E}_4 \rightarrow \text{M}_3$$

In Tables 9 and 10 is shown the character of the mixture inoculated and the primary egg fluids obtained. The first-limit egg passage corresponds to fluid E_2 in the schema and the second to E_4 . For each is shown the number of the fluid used, the dilution of the previous fluid that infected the embryo in question, and the death-times of mice inoculated intracerebrally with fluid diluted 10^{-1} . The Nr-SW strains were uniform in character, indistinguishable from Nr-SW I and approximately equally pathogenic for mice. Similarly, Nr-MEL III and IV closely resemble Nr-MEL I and no further discussion of these is required.

The results with one mouse-brain emulsion from an animal, M 70,903, inoculated with MEL + NWS were however extremely complex and a variety of types was obtained. These results may be highly relevant for an understanding of the process of recombination and we therefore give them in some detail. In the first egg culture of the brain emulsion the 10^{-1} dilution gave four positive fluids all serologically MEL; but two showed the Nr-MEL character of conversion to indicator by simple heating at 56° (57,210, 57,212) and two failed to do so (57,209, 57,211). Application of the usual procedure to 57,212 gave the results shown in Table 11. The first limit-passage fluid produced sickness but

Table 9. *Isolation of recombinants from mixed infections with NWS and SW*

Strain	From mouse no.	Inoculum	Primary fluids	First-limit passage	Second-limit passage
Nr-SW II	70,914	NWS dil. 10^{-3} + SW dil. 10^0	10^0 : SW, SW, SW 10^{-1} : SW*, SW, SW	10^{-8} 57,505, 5, 6, 6	
Nr-SW III	70,915	NWS dil. 10^{-3} + SW dil. 10^0	10^0 : N, N, N, SW* 10^{-1} : N, N, — —	10^{-8} 58,437, 5, 5, 5	10^{-8} 59,186, 5, 5, 5, 6
Nr-SW IV	71,026	NWS dil. 10^{-3} + SW dil. 10^0	10^0 : N, SW, SW, + 10^{-1} : N, N, SW*, +	10^{-8} 58,091, 5, 5, 6, (6)	10^{-8} 58,915, 3, 4, 8, 9
Nr-SW V	71,028		10^0 : N, — — — 10^{-1} : SW*, N, — —	10^{-7} 58,339, 5, 5, 5, 5	10^{-8} 59,173, 4, 6, 6

N = strain with serological character of NWS; SW = strain with serological character of SW.

* Fluids used for further passage. All fluids shown had serological character of SW.

Days of death of mice inoculated with 10^{-2} fluid are shown after each.

Table 10. *Isolation of recombinants from mixed infections with NWS and MEL*

Strain	From mouse no.	Inoculum	Primary fluids	First-limit passage	Second-limit passage
Nr-MEL II	70,903	NWS dil. 10^{-3} + MEL dil. 10^0	10^0 : M, M, M, M, M 10^{-1} : M, M, M, M*	A 10^{-9} 58,586, (6), (6), S, S B 10^{-9} 58,450, 3, 5, 5, (6)	10^{-8} 59,152, S, S, S, S 10^{-7} 59,360
Nr-MEL III	71,016	NWS dil. 10^{-3} + MEL dil. 10^0	10^0 : M, M, M, M 10^{-1} : M, M, M, M*	10^{-9} 58,384, 3, 3, 4, 4	10^{-9} 59,062, 5, 5, 5, 6
Nr-MEL IV	71,017	NWS dil. 10^{-3} + MEL dil. 10^0	10^0 : M, M, M, — 10^{-1} : M, M, M*, —	10^{-9} 58,397, 5, (6, 6, 6)	10^{-9} 59,217, 5, 6

M = fluid with serological character of MEL; S = survived for 12 days.

Other symbols as Table 9.

did not kill mice intracerebrally. There was, however, virus to a dilution of 10^{-2} in the brain of one of the sick mice. A further limit passage from egg to egg gave fluid 59,152 which failed to kill any of four mice, although all showed minor symptoms around the 6th day. All fluids in this series were of Nr-MEL type *in vitro*.

Fluid 57,209, of normal MEL character, was injected intracerebrally into three mice. One, killed when sick at 5 days, gave fluids all of Nr-MEL character by *in vitro* test. The second, killed when moribund at the 6th day, gave two types of fluid in approximately equal numbers, one typified by 58,255 had the *in vitro* qualities of Nr-MEL. The other typified by 58,260 was not significantly neutralized by either MEL, NWS or SW antisera and was convertible into indicator by simple heating. This strain has maintained its character on limit-dilution passage and is reserved for future study. Fluid 58,255 of Nr-MEL character was passed at limit dilution and killed four mice at 3, 5, 5 and (6) days. Nr-MEL type virus was isolated from the brains and after another limit-dilution transfer was inoculated to mice intracerebrally. These died at 5, 6, 6, (7) days.

Table 11. *Character of different substrains obtained from 'mouse' 70,903*

(All strains were of the serological type MEL.)

Representative fluid	Indicator heated at 56°	Intracerebral pathogenicity for mice
58,255	+	3, 5, 5, (6)
57,508	+	3, 6, (6), (7), S, S
57,579	+	S, S, S, S Mice appeared sick around
58,586	+	S, S, S, S 6th day
58,574	±	S, S, S, Non-pathogenic
57,582	—	S, S, S, Non-pathogenic

+ = fluid converted to indicator for inhibition by ovomucin and giving full titre; ± = indicator but titre low. 5, etc. = death on 5th day after injection; (6), etc. = mouse sick and killed 7th day, would probably have died on 8th day; S = survived for 12 days.

Several other lines of descent were followed from mouse-brain 70,903 and a summary of those series in which at least one limit-dilution fluid was tested is given in Table 11. The interest of this series lies in the strong indication of a graded series of strains of increasing intracerebral pathogenicity. If we are concerned with recombination of characters there is here a possible opportunity of analysing what appears to be a 'dosage effect'. As soon as circumstances allow it is hoped to return to this aspect of the problem.

Isolation of a recombinant of NWS with a filamentous influenza A¹ strain

In the hope that an additional genetic 'marker' could be obtained in the filamentous character of recent influenza A¹ strains, limited experiments were made with the strain, Ocean Island (Ocl.) (Isaacs *et al.* 1950). The procedure was exactly as with MEL and SW and three lines were followed. The results resembled in many respects those obtained with MEL in the appearance of three more or less distinct subtypes.

The most encephalitogenic substrain Nr-OcI. II was obtained from fluid 58,215 derived from 10^{-1} dilution of mouse brain 71,119 inoculated with (NWS diluted 10^{-3} + OcI. diluted 10^{-1}). Fluid 58,491 obtained by limit-dilution passage (dilution 10^{-7}) of 58,215 was diluted 10^{-1} and inoculated intracerebrally into mice one of which died on the 7th day; the others were killed and cultured at 5, 5 and 7 days respectively, all appearing sick at the time. The brain emulsion from one was infective for eggs to dilution 10^{-3} , the fluids having OcI. serological character and showing no filamentous forms on dark-ground microscopy. Fluid 59,211 representing a second limit passage (dilution 10^{-9}) of 58,215 gave, on intracerebral inoculation in mice, one death at 2 days, while the three remaining mice were all very sick on the 7th day but recovered subsequently.

Another strain Nr-OcI. I, similarly derived from mouse 71,118, gave throughout OcI. serological reactions and showed definite but non-fatal illness in mice inoculated with first limit-dilution egg-passage fluid. The sick mouse killed on the 5th day gave a brain emulsion infecting two of four chick embryos at dilution 10^{-3} . A further limit-dilution passage gave at dilution 10^{-7} the fluid 59,189. In mice this fluid produced sickness at 6–8 days but all survived. Our impression was that the symptoms of excitability and undue disorientation after being spun by the tail and dropped back into the tin, were less marked than in mice inoculated with the first limit-passage egg fluid.

The third series was started with fluid 58,189 from a 10^{-2} dilution of brain emulsion of mouse M 71,083 killed when sick at 5 days. This fluid was serologically OcI. and, unlike the other fluids mentioned above, contained an almost normal proportion of filamentous forms. None of the mice inoculated became sick, and eggs inoculated from the brain of one killed at 5 days were not infected. Apart from the fact of its survival for 5 days in the first mouse of the series there is nothing to indicate any difference from normal OcI. The association of long filaments with non-neurotropic forms and their almost complete disappearance in the encephalitogenic Nr-OcI. II is a point of considerable interest which it is hoped to investigate more fully.

DISCUSSION

We have taken the obvious viewpoint that when two viruses are inoculated into a mouse brain, one neurotropic of serological character A, the other non-neurotropic of serological character B, and a neurotropic strain serologically B is obtained, then a recombination of characters has occurred. The fact that a similar phenomenon has been described for bacterial viruses (Hershey & Rotman, 1948; Delbrück & Bailey, 1946) makes it likely that an important general character of viruses is concerned and justifies a certain amount of speculation as to the mechanism by which recombination of characters occurs.

It must be admitted that no direct information is available on the morphological changes undergone by viruses in the course of multiplication in the cell. Electron micrographs have not shown any unequivocal evidence of partially completed binary fission in the purified suspensions needed for electron micro-

scope work. There is, however, no decisive evidence against the view that the standard method of reproduction by animal viruses is by simple binary fission although there is undoubtedly a strong trend of present opinion away from this view. Hypotheses as to the basis of the recombination phenomena described in these papers must therefore be shaped according to whether reproduction of influenza viruses is assumed to be by binary fission or by some alternative method.

A. The available hypotheses on the assumption of binary fission appear to be three in number:

(1) Under certain circumstances two virus particles in the same cell may undergo a primitive type of sexual fusion allowing some redistribution of characters when the 'haploid' form is regained. This would be analogous to the situation which appears to obtain in regard to the formation of bacterial recombinants with the *E. coli* strain K 12 (Tatum & Lederberg, 1947).

(2) Mutation is relatively frequent in viruses, and it is possible that in the course of intracellular multiplication non-viable mutants are abundantly produced. By analogy with the work of Avery *et al.* (1944) on pneumococcal type transformation, one could conceive that a unit analogous to the deoxyribosenucleic acid of the pneumococcus could be transferred from one type of virus to a mutant deficient in the corresponding allele, thereby rendering it viable and giving opportunity for recombination of characters.

(3) It is possible that a strain adapted to growth in a special intracellular environment such as brain, may in some way facilitate the rapid transformation of another virus into a form capable of growth in the unusual environment. There is perhaps a remote analogy in the phenomenon of syntrophism in bacteria when two variants lacking capacity to synthesize two different essential metabolites may be able to grow in mixed culture in a medium unable to support growth of either alone. We know of no closer analogy.

B. If we follow the current trend of opinion amongst workers with bacterial viruses that the coliphages T₂, T₄ and T₆ at least multiply by a method totally distinct from binary fission, a second general hypothesis can be formulated.

Workers with bacterial viruses of this group consider that after the virus particle penetrates the bacterial surface it breaks up into 30–50 sub-units (genes). These replicate independently and towards the end of the phase of intracellular development, infective virus particles are reconstituted ready for liberation with lysis of the host cell. The evidence is based first on Luria's (1947) work on the infectivity of virus treated with ultra-violet light which indicates that while one virus particle is non-infective a high proportion of multiple infections give rise to multiplication and lysis. In addition, there is the extensive evidence of recombination provided by Hershey & Rotman (1948) and by Delbrück & Bailey (1946).

Hoyle (1948), from his work on the disappearance of influenza virus for a few hours after infection of the allantoic lining, and Bauer (1949), from a general consideration of the problem of virus multiplication, have already suggested that similar processes are concerned in the intracellular growth of animal viruses. Hoyle considers that the disappearance of demonstrable virus in the

allantoic membrane is due to a disintegration of the infective particles into a similar non-infective form. The existence of this form can be demonstrated by the presence of the 'soluble' complement-fixing antigen in the tissues before the reappearance of infective and haemagglutinating virus. Whether this overall identification of the 'non-infective phase' of the virus with the complement-fixing antigen is justifiable may be doubtful but the temporary disappearance of demonstrable virus has been confirmed by Henle & Henle (1949) and for inactive (indicator) virus by Isaacs & Edney (1951) and must in some way be accounted for.

Obviously our results could readily be covered by a slight elaboration of Hoyle's hypothesis along the lines that have been adopted by Delbrück (1949) to interpret the genetics of bacterial viruses. One would have to assume not that the virus disintegrated into a soluble phase composed of uniform particles, but that the units produced were of the nature of individual genetic units replicating true to type and that the equivalent units in related but distinct virus strains had allelomorphous relations to one another.

In the Nr-OcI strain derived from recombination of NWS and OcI, it has been shown that *two* characters of NWS have been transferred to the recombinant, neurotropism and spherical morphology. We have taken the serological type of the recombinant as the most fundamental character and have usually thought of the recombinants as representing the non-neurotropic form on to which the neurotropic character has been in some way grafted. However, considering the relatively small number of markers that are available, our results are quite compatible with the view that the process envisaged by Hoyle does take place and allows wide possibilities of recombination of characters. The strains isolated are of course only those combinations capable of survival and growth in the conditions provided by brain tissue and the manipulations of isolation in the allantoic cavity.

It should be stressed that any genetic work with influenza viruses must be done within the strict technical limitation that there is no equivalent to a plating method by which all the offspring of a given 'cross' can be determined and no possibility of isolating single cells in which double infections have been initiated. The technique must always be to introduce the two parent strains in relatively high concentration into the susceptible tissue and then so to arrange circumstances that the multiplication of both original types is inhibited relative to that of the recombinant one is seeking. The obvious procedure is to take strains which differ (*a*) serologically, (*b*) in ability to multiply in a given tissue. The strains are now placed in an environment in which one only can multiply and at an appropriate stage anti-serum to inhibit the activity of this strain is used; only recombinants should then appear, as in the experiments we have detailed. But any such method can obviously only allow the isolation of strains with the capacity to overcome both the obstacles provided. If recombination were a straightforward sexual interchange of qualities $AB \times A'B'$, giving the possible recombinants AB' and $A'B$, only one of these could be isolated. With our mouse-brain technique and NWS as a constant component, for example, it is impossible to obtain as a recombinant non-neurotropic WS.

There are already hints from the experiments with MEL that more than one type of recombinant can be obtained and further work in this direction should be illuminating. We feel that our results require the acceptance of some process allowing free recombination of characters either by sexual fusion of two individuals or by the diffuse replication of individual 'genes' according to Hoyle's hypothesis. Without by any means feeling that the last word has been said we rather strongly favour the latter alternative.

Many workers have been impressed by the general resemblance between the larger bacterial viruses and influenza viruses, and the evidence in regard to bacteriophage multiplication and recombination is extremely cogent. When all the demonstrable characters of influenza virus multiplication and recombination agree with such an hypothesis it is natural to lay much stress on the analogy, although technical considerations make it impossible to devise with influenza virus the fully quantitative studies possible with bacteriophages. The well-established temporary disappearance of demonstrable influenza virus in the early stages of infection is important evidence against binary fission as the standard process of multiplication. The existence of filamentous forms of virus (Chu, Dawson & Elford, 1949) in recent influenza A strains, in itself seems to favour strongly a method of growth in which the final infective form is built up from a pool of self-replicated sub-units. With such a method it is easy to conceive of the production of a variety of infective units from spheres to long filaments. It is not easy to see how filaments could be produced by any process resembling binary fission.

We feel that our findings add strong circumstantial evidence in favour of the hypothesis that influenza virus particles are composite, and on entry into the host cell break down into sub-units which replicate independently, giving rise to a pool of virus material from which infective units can be reconstituted. This hypothesis has not been established, but it has implications which could be tested. (1) The initial stage of virus disintegration soon after entering the cell is presumably due to a relatively simple enzyme action and should be capable of imitation *in vitro*. Disintegration should be demonstrable by the disappearance of infectivity and haemagglutination and by electron microscope studies. (2) A simple accumulation of data on the types of recombinant obtained by the experimental technique we have been using should provide indication of how far the recombination process is a symmetrical one. (3) There seems to be no intrinsic reason why classical genetic procedures such as backcrossing should not be modified to the circumstances and provide valuable information at the genetic level. (4) With advanced optical and electron microscope techniques the fate of filamentous virus in tissue culture might be followed and provide direct evidence of breakdown and reconstitution.

This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

REFERENCES

- AVERY, O. T., McLEOD, C. M. & McCARTY, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. exp. Med.* **79**, 137.
- BAUER, D. J. (1949). Multiplication of the animal viruses. *Nature, Lond.*, **164**, 767.
- BURNET, F. M. (1949). Ovomucin as a substrate for the mucinolytic enzymes of *V. cholerae* filtrates. *Aust. J. exp. Biol. med. Sci.* **27**, 245.
- BURNET, F. M. (1951*a*). A genetic approach to variation in influenza viruses. 1. The characters of three substrains of influenza virus A (WS). *J. gen. Microbiol.* **5**, 46.
- BURNET, F. M. (1951*b*). A genetic approach to variation in influenza viruses. 2. Variation in the strain NWS on allantoic passage. *J. gen. Microbiol.* **5**, 54.
- BURNET, F. M. & LIND, P. E. (1951). A genetic approach to variation in influenza viruses. 3. Recombination of characters in influenza virus strains used in mixed infections. *J. gen. Microbiol.* **5**, 59.
- CHU, C. M., DAWSON, I. M. & ELFORD, W. J. (1949). Filamentous forms associated with newly-isolated influenza virus. *Lancet*, **i**, 602.
- DELBRÜCK, M. (1949). Genetique du Bacteriophage. *Colloques Internationaux du Centre National de la Recherche Scientifique*, **8**, 91.
- DELBRÜCK, M. & BAILEY, W. T. (1946). Induced mutations in bacterial viruses. *Cold Spr. Harb. Symp. quant. Biol.* **11**, 55.
- GOTTSCHALK, A. & LIND, P. E. (1949). Ovomucin, a substrate for the enzyme of influenza virus. 1. Ovomucin as an inhibitor of haemagglutination by heated LEE virus. *Brit. J. exp. Path.* **30**, 85.
- HENLE, W. & HENLE, G. (1949). Studies on host-virus interactions in the chick embryo-influenza virus system. III. Development of infectivity, haemagglutination and complement fixation activities during the first infectious cycle. *J. exp. Med.* **90**, 23.
- HERSHEY, A. D. & ROTMAN, R. (1948). Linkage among genes controlling inhibition of lysis in a bacterial virus. *Proc. nat. Acad. Sci., Wash.*, **34**, 89.
- HOYLE, L. (1948). The growth cycle of influenza virus A. A study of the relations between virus, soluble antigen and host cell in fertile eggs inoculated with influenza virus. *Brit. J. exp. Path.* **29**, 390.
- ISAACS, A. & EDNEY, M. E. (1951). Interference between inactive and active influenza viruses in the chick embryo. IV. The early stages of virus multiplication and interference. *Aust. J. exp. Biol. med. Sci.* (In the Press.)
- ISAACS, A., EDNEY, M. E., DONNELLEY, M. & INGRAM, M. W. (1950). Influenza in an isolated community. An epidemic on Ocean Island. *Lancet*, **i**, 64.
- LURIA, S. E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. *Proc. nat. Acad. Sci., Wash.*, **33**, 253.
- STONE, J. D. (1949). Inhibition of influenza virus haemagglutination by mucoids. I. Conversion of virus to indicator for inhibition. *Aust. J. exp. Biol. med. Sci.* **27**, 337.
- TATUM, E. L. & LEDERBERG, J. (1947). Gene recombination in the bacterium *Escherichia coli*. *J. Bact.* **53**, 673.

(Received 20 March 1950)