A Genetic Approach to Variation in Influenza Viruses

1. The Characters of Three Substrains of Influenza Virus A (WS)

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SUMMARY: The three virus types derived from the classical WS strain of influenza virus can be distinguished by a relatively wide range of 'marker' characters, which are available for the recognition of virus characteristics in studies of variation and recombination. Despite their common origin the three WS strains have diverged very markedly one from another. Each has unique characteristics: NWS its absence of enzymic and indicator activity, its neurotropism; WSE its firm non-eluting adsorption to red cells; WSM its extremely heat-resistant haemagglutinin. From the point of view of the general pathology of virus infection the neurotropic character imposed on an influenza virus is the most important of the variant qualities.

A long experience of work with influenza viruses impresses one very strongly with the importance of variation both in epidemiological phenomena and in the laboratory manipulation of the virus. In this laboratory much attention has been directed to the initial variation by which influenza virus A, isolated directly from the human patient, is converted to forms that can multiply in the allantoic cavity of the chick embryo—the O-D change of Burnet & Bull (1943). This change appears to be due to mutation and selective overgrowth of the variant rather than to any direct influence of the environment on the multiplying virus population.

In recent years rapid progress has been made in developing a genetic approach to variation in bacteria (Avery, McLeod & McCarty, 1944; Tatum & Lederberg, 1947) and in bacterial viruses (Delbrück & Bailey, 1946; Hershey & Rotman, 1948). In both groups it was possible to demonstrate recombination of characters in the sense that when two strains, each with two or more 'marker' characteristics are mixed under conditions where multiplication of both can take place, a certain proportion of 'recombinants' can be subsequently isolated. If two strains designated by their marker characters AB and CD respectively, give rise to a proportion showing AD or BC characters and if under similar conditions AB or CD alone never give rise to such types, then it is legitimate to consider that recombination has occurred. Amongst the bacterial viruses studied by Delbrück & Bailey (1946) recombination is only demonstrable between strains of the same general type, e.g. amongst the morphologically similar even-numbered members of the T series of coliphages. Only under such conditions does it appear to be possible for two or more virus particles to infect a single host bacterium.

Interest in the possibility of a similar genetic approach to the influenza viruses was stimulated in the course of work with Stuart-Harris's neurotropic variant of the strain WS (influenza virus A). These experiments were concerned with the possible effect of infection on the haemagglutinin-inhibitor content of mouse brain. It was then realized that there were available in the laboratory

three strains, all derivatives from the original WS of Andrewes, Laidlaw & Smith (1934) which had well-defined easily demonstrable differences and which, by analogy with bacterial viruses, should offer an opportunity to test whether recombination of characters could occur in influenza viruses.

In designing experiments of this type it became clear that a primary requirement was the use of pure clones of virus. Most of the marker characters that might be used were *in vitro* reactions shown by fluids containing high concentrations of virus particles. Typical examples are the capacity to agglutinate red cells of different species and the temperature at which this power to agglutinate is destroyed. A number of other markers are related to the enzymic function of the viruses in regard to soluble or cellular mucoids. All such tests reflect the activity of a large population of virus particles, and it is obviously desirable to ensure, as far as possible, the uniformity of the individuals making up that population.

By analogy with all other work on variation in micro-organisms we can feel confident that whenever a large population of virus particles develops from a small uniform inoculum, this population will contain a certain proportion of mutant forms. For *in vitro* tests of the type used in influenza virus work any proportion of mutants in the population below 1 % can probably be neglected. The presence of the mutants becomes important when successive transfer provides an opportunity for one or more of them to outgrow and replace the original form. In genetic work the necessity is to retain each significant type in a constant condition so that whenever required a clone of virus (in the form of an infected egg fluid) with consistent properties can be prepared.

This can readily be accomplished provided we are content to obtain only the predominant form, the type present in greatest numbers, in the material under study. All the evidence suggests that when a virus suspension is serially diluted until infection results in only a small proportion of highly susceptible hosts, those infections are initiated by a single virus unit. This holds particularly where an all or nothing response of the host occurs, as in chick embryo infections with well-adapted influenza virus strains. Pure clone technique with influenza viruses depends, then, on diluting the starting material till it is slightly below the 50 % infective level and on examining several of the fluids obtained from embryos infected by such a dilution. If these fluids are uniform in their relevant characteristics any one of them may be taken as representing a pure clone of virus, subject of course to the qualification that some production of mutants may have occurred by the time the virus is harvested.

Minor components of a virus population cannot be isolated at will. It may be possible to provide an alternative type of host cell which will favour the multiplication of a minority variant sufficiently so that it will overgrow the original predominant type and be isolated by the limit-dilution method. Exposure of a population to graded doses of destructive agents, heat, ultra-violet light, antiserum, etc., may also allow survival of variant forms initially in a minority. Both methods, however, allow the isolation only of mutants of types appropriate to the different techniques. Transfer of a mixture of clones to a new host may not necessarily allow the immediate demonstration of a mutant

pathogenic for the new host even when such a mutant is present. The presence of an excess of the non-pathogenic form may 'interfere' with the multiplication of the pathogenic minority so that no lesions result and no isolation is possible.

The present work represents essentially an application of the principle of pure clone technique to a study of the influenza strains that have been derived from the classical WS strain and particularly the neurotropic variant developed by Stuart-Harris (1939). This strain is unique amongst existing influenza viruses in being able to multiply and produce fatal encephalitis in the mouse brain. This provides a specially valuable marker for recombination studies. This first paper deals with a preliminary study of the characteristics of pure clones of the three established variants of WS.

MATERIALS AND METHODS

It will be convenient to include here a general account of the methods used throughout the experiments reported in this paper and the three subsequent papers, leaving only special aspects of technique to be included in the latter.

The virus strains used were the standard strains maintained in this laboratory as infected allantoic fluids stored in a dry-ice refrigerator. Seed virus was prepared from embryos infected with such material and stored with the addition of glycerol to 33 % at -10° . Experimental virus as used in all tests was in the form of allantoic fluid harvested after 2 days at 35° from eggs inoculated at 11 days' incubation with 0.05 ml. of 10^{-4} or 10^{-6} dilution of seed virus. Throughout the whole series of experiments one or more of the three sub-strains of WS have been of central importance and a fairly full account of their origin is, therefore, desirable.

The strain WS was isolated at Hampstead in the spring of 1933 and given eighty-eight ferret passages before being transferred to mice early in 1934. Dried mouse-passage material was received from Dr C. H. Andrewes in 1937 and from this, strains WSM and WSE are derived.

WSM. A mouse-passage series was initiated with the mouse-lung material received from Hampstead in 1937. In 1940 the mouse virus underwent 100 passages by the amniotic route without showing more than trivial changes in character. Amniotic fluid from the 102nd passage was maintained as seed virus in a dry-ice refrigerator. It was revived by inoculating a dilution of 1/100 into embryos by the amniotic route and infected amniotic fluid from these, mixed with an equal volume of pure glycerol, was used as seed virus for the preparation of allantoic fluids.

WSE. The same dried mouse-lung WS received in 1937 was passaged on the chorioallantois as described by Burnet & Lush (1938); 56th passage virus was passed to the amniotic cavity and the strain kept in the dry-ice refrigerator with only one intervening passage between 1942 and 1949. This was revived and handled in the same fashion as WSM.

NWS. This was derived by Stuart-Harris (1939) from mouse passage WS, first by adaptation to the chorioallantois with the development of haemorrhagic brain lesions, followed by inoculation of embryo brain material to very young mice and serial passage. After twelve passages deaths occurred amongst mice

and continued in subsequent passages. We received the strain from Prof. Stuart-Harris through Dr Alick Isaacs in the form of dried mouse-brain 'M103 dried 27/7/48'. When redissolved this was infective for chick embryos and mice. After one further allantoic passage the resulting fluid was titrated and showed two of four eggs infected at dilution 10^{-6} . One of these fluids was glycerolated and used as seed virus either directly or at one remove in all subsequent work.

Haemagglutinin titrations. Haemagglutinin tests were made by adding 1 % fowl cells to equal (0.25 ml.) volumes of virus dilutions in saline and allowing them to sediment at room temperature. The reading is made from the pattern of deposited cells, the end-point being a standard degree of partial agglutination. All the other *in vitro* tests based on haemagglutination are derived in the simplest possible way from this basic technique.

Conversion of strains to indicator state. When a typical influenza B virus is heated to 55° for 30 min. it retains its power to agglutinate red cells apparently unaltered, but differs from the original active virus in two important respects. It no longer possesses the capacity of eluting from cells or showing other evidence of enzyme action, and its haemagglutinating action is readily inhibited by a variety of soluble mucoid materials. These two characters define what we have called the indicator state of these viruses. With many influenza A strains simple heating will not produce such a conversion, and as a routine Stone's (1949) method was used. Three volumes of allantoic fluid virus were mixed with one volume of 2 % sodium citrate saline and one volume of borate buffer pH 8.5. This mixture was heated at 50° for 30 min. Occasional modification of this temperature for special reasons is indicated in the text.

In many instances it was desirable to determine whether simple heating converted a virus to the indicator state. This was done by preparing, on the results of preliminary titration, a dilution in saline containing five haemagglutinating doses (5AD) of virus. An ovomucin preparation made by Gottschalk & Lind's technique (1949) was diluted 1/5 in 0.85 % saline, spun, and the supernatant used as starting-point for tenfold dilutions in saline. To 0.25 ml. of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilutions of ovomucin an equal volume of indicator virus (5AD) was added, and after 30 min. at room temperature a further volume of 1 % fowl cells. Full development of the indicator state would be shown by failure of agglutination with 10^{-3} and sometimes with 10^{-4} mucin dilution.

The position of a strain in the receptor gradient. It was shown by Burnet, McCrea & Stone (1946) that viruses could be placed in a linear order corresponding to the sequence with which red cells, treated with viruses or with the receptor-destroying enzyme (RDE) of the cholera vibrio, lost ability to be agglutinated by the viruses. The most convenient test for the position of a virus in this 'receptor gradient' is to test it with the standard viruses or red cells treated with graded amounts of RDE. Twofold dilutions of a crude stock filtrate containing 1200 units of enzyme/0.25 ml. were prepared in 4 ml.-volumes of calcium acetate saline pH 6.2 from 1/25 to 1/1600. To these 0.1 ml. of packed fowl cells was added and the tubes held at 37° for 30 min. Sodium citrate solution (0.4 ml. of 20 %) was then added to each tube to stop all RDE

action. The cells were washed and made up to 1 % suspension in 2 % citrate saline. Test and standard viruses diluted to 5AD were then tested with each set of treated cells.

The characters of the three strains WSM, WSE and NWS

All these strains have a number of characters in common. They multiply freely in the allantoic and amniotic cavities of chick embryos, producing actively haemagglutinating virus. They give typical influenzal lesions on intranasal inoculation in mice. Serologically they are very closely related though, as will be shown later (Burnet & Lind, 1951) NWS seems to lack a minor antigenic component present in the others. They give rise to soluble complement-fixing antigen of A type. None of the strains shows visible filaments on dark-ground microscopy. They are presumably composed of spherical particles but they have not yet been compared by electron microscopy. They differ as follows.

Pathogenicity. The neurotropic strain NWS alone produces symptoms and fatal infection on intracerebral inoculation in mice. A typical allantoic-fluid virus gives fatal infections when diluted 10^{-5} but only a proportion at 10^{-6} . Deaths occur as a rule within 4-7 days and are usually preceded for 24 hr. by some signs of encephalitis. When the brain is removed from a mouse dying or just dead with typical signs, relatively large amounts of virus are present. As a routine the brain of each mouse tested was ground and suspended in 2 ml. saline. After centrifugation the supernatant fluid was infective to chick embryos to dilutions of 10^{-4} or 10^{-5} . Neither of the other strains produced any symptoms in mice after intracerebral inoculation with infected fluids either undiluted or diluted 10^{-2} . On the chorioallantois of 12-day embryos WSE and NWS produce similar well-defined foci at high dilution, but WSM produces lesions of weaker and more irregular character.

When inoculated intravenously in 12-day chick embryos the strains WSE and NWS produce fatal infections with characteristic massive haemorrhages in the brain and less regularly in other tissues, especially skin and lung. Even with very small inocula (dilution 10^{-5}) embryos after 3 days show brain and lung lesions, and both organs contain sufficient virus to allow 10 % extracts in saline to agglutinate fowl cells. When these extracts are heated to 65° and appropriately tested no inhibitor for haemagglutination by heated LEE virus is demonstrable. Extracts similarly prepared from uninfected organs have an inhibitor titre *c.* 60.

The strain WSM is not lethal within 3 days and only at relatively high concentrations (dilution 10^{-2}) is the lung infected sufficiently to show the presence of haemagglutinating virus and disappearance of virus inhibitor. No gross lesions are produced.

Haemagglutination. All these strains agglutinate human, guinea-pig, fowl and pigeon red cells readily and to comparable titres. Mouse cells are agglutinated less readily, WSE being the only one to produce complete agglutination; the other two give partial agglutination to a titre about one-fourth that shown with fowl cells. Rabbit cells are slightly agglutinated by WSE, not by the others.

In the cold (0°) fowl cells adsorb any of the strains but NWS somewhat less readily than the others. On prolonged treatment at 37° fowl cells are stabilized by WSM and less completely by NWS, with elution of virus. With WSE, however, virtually no liberation of virus occurs at 37° and the cells remain persistently unstable. As was first shown by Briody (1948) the enzymic action of WS strains is potentiated by Ca ions and inhibited by calcium deionizers such as citrate. Even in the presence of Ca ions, however, no elution of WSE is observed.

Table 1. *Position of WS virus strains in receptor gradient*

(The action is shown of five agglutinating doses (AD) of each virus on fowl red cells treated with excess of virus and in the case of WSE stabilized with immune serum, or treated with graded amounts of receptor destroying enzyme RDE corresponding to the dilutions shown.)

Virus	Cells treated with excess of			Cells treated with receptor-destroying enzyme RDE					
	NWS	WSM	WSE	Dilutions of RDE					
				1/50	1/100	1/200	1/400	1/800	1/1600
NWS	—	—	+	—	—	—	—	± ±	++
WSM	+	—	+	—	—	—	±	++	++
WSE	++	+	++	—	—	±	++	++	++

++ = complete agglutination; ± ±, +, ± = degrees of partial agglutination;
— = no agglutination.

The order of the viruses in the receptor gradient is NWS, WSM and WSE. When fowl cells are treated with virus in the presence of excess Ca ions and in the case of WSE stabilized with immune serum, the reactions with different viruses are as shown in Table 1. In the same table are shown the reactions with cells treated as described under methods with graded amounts of RDE. The discrepancy with cells treated with WSE is due simply to the failure of receptor destruction by the virus. In relation to the other viruses of the receptor gradient all the WS strains come early in the series. There are the usual minor differences according to the agents used in treating the cells but with fowl cells the order observed is MU, NWS, NDV, WSM, MEL, WSE and LEE.

There are marked differences in the thermostability of the three haemagglutinins. In the experiment shown in Table 2 freshly harvested allantoic fluids were buffered by the addition of M/3 phosphate to give a final M/30 concentration with pH 7.35. These were heated for 30 min. at each of the temperatures shown. Haemagglutinin titrations were made and each fluid, diluted 1/10, inoculated into chick embryos. The striking thermostability of WSM haemagglutinin is to be particularly noted. The addition of phosphate buffer was probably unnecessary as in many other tests untreated fluids were heated for 30 min. at 54° and 62°. The lower temperature always decreased NWS haemagglutinin concentration to a very low level while 62° had practically no action on any WSM fluid. The heat resistance of infectivity for chick embryos is in the same order but as would be expected the differences cover a much smaller temperature range.

Enzymic activity and indicator state. The WS strains are all relatively weak in their enzymic action on ovomucin. When tested under the usual conditions

Table 2. *Thermostability of haemagglutinin and infectivity of WS strains heated for 30 min. at various temperatures*

Temp. (° C.)	NWS		WSE		WSM	
	HA	Inf.	HA	Inf.	HA	Inf.
Unheated control	400	.	1000	.	600	.
52	270	++	500	++	1000	++
54	4	--	350	++	800	++
56	<2	.	170	---	650	+++
58	.	.	<10	---	480	---
60	.	.	.	.	640	.
62	.	.	.	.	400	.
64	.	.	.	.	360	.
65.5	.	.	.	.	80	.

HA = Haemagglutinin titre; Inf. = Infectivity for chick embryos; each + represents an egg giving haemagglutination; -- = no haemagglutination.

none of the three show any power to diminish its inhibitory titre against any of the indicator viruses. When, however, the ovomucin is diluted 1/50 instead of the usual 1/5 in calcium acetate saline, WSM and WSE both destroy the inhibitory action against H-WSE but not against the other indicators H-LEE and T-MEL (see Stone, 1949). NWS under these conditions has no demonstrable action.

A very sensitive test for enzymic activity has been described by Edney & Isaacs (1951). This depends on the well-known fact that normal allantoic fluid contains virus inhibitor, the behaviour of which is essentially the same as that of dilute ovomucin. Infective fluids of strain WSE harvested at 48 hr. and heated at 65° to destroy haemagglutinin are found to lack all inhibitors. This is also the finding with all other strains tested by Edney & Isaacs, but the influence of WSE is particularly striking because *in vitro* it does *not* destroy inhibitory action against strains H-LEE or T-MEL. The only exception to the rule that H-LEE inhibitors are always absent from an infective fluid of reasonable haemagglutinin titre is NWS. Fluids infected with NWS show practically normal content of H-LEE and T-MEL inhibitors but the H-WSE inhibitor is reduced or eliminated. One of the most delicate tests as to whether only NWS is present in an infected allantoic fluid is the estimation of H-LEE inhibitor after the fluid has been heated to destroy haemagglutinin. If it shows the normal value of 50–100 no other variant than NWS is present.

Alone amongst influenza viruses it has not been found possible to convert NWS into the indicator state. With all other WS variants heating in citrate at pH 8.5 for 30 min. at 50° converts them to a form, haemagglutination by which is inhibited by high dilutions of ovomucin and similar substances. A second test for identifying NWS is therefore to heat the fluid under these conditions and prepare a dilution to contain 5AD of the modified virus. This is tested against tenfold dilutions of stock ovomucin solution. With the three viruses the usual findings are as shown in Table 3.

Table 3. Conversion of WS viruses to the indicator state

(Each virus was treated as described in the text by heating to 50° at pH 8.5 in the presence of citrate and 5 AD then mixed with an equal volume of the ovomucin dilutions shown. After 30 min. a third volume of red cells was added giving the degrees of agglutination shown.)

Treated virus	Ovomucin dilutions			
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
WSM	—	—	—	+
WSE	—	—	—	++
NWS	±	+	++	++

++ = Complete agglutination; +, ± incomplete agglutination;
— = no agglutination.

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