

Antigenic Composition of Recombinant Virus Strains Produced from Human and Avian Influenza A Viruses

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(Accepted 11 February 1969)

SUMMARY

The antigenic structure of a recombinant virus, FPV-A₂ (R₄), obtained by the interaction of an avian and a human influenza A virus was investigated by means of haemagglutination-inhibition, neuraminidase-inhibition and immunoprecipitation tests. The neuraminidase of the recombinant was found to be antigenically identical to that of its A₂/SINGAPORE/1/57 parent whilst its haemagglutinin was identical to that of fowl plague virus, the other parent. Similar studies on a recombinant obtained by the interaction of two avian influenza A virus strains, fowl plague virus and A/turkey/MASSACHUSETTS/65 virus, indicated that it contained neuraminidase of the antigenic type found in the turkey virus and haemagglutinin like that of fowl plague virus. A/turkey/MASSACHUSETTS/65 was selected for study since its neuraminidase is antigenically closely related to that of human A₂ virus strains.

Studies on the electrophoretic mobilities of the structural proteins of FPV-A₂ (R₄) indicated that it contained structural components derived from each parent and thus supported the immunological findings.

The significance of these findings in relation to possible genetic interactions between human and avian influenza viruses in nature is discussed.

INTRODUCTION

Tumova & Pereira (1965) reported the reactivation of the plaque forming capacity of u.v.-inactivated fowl plague virus in monolayers of chick embryo cells doubly infected with the inactive fowl plague virus and live influenza A₂/SINGAPORE/1/57. The influenza A₂ virus did not produce plaques under the conditions of the experiment. Antigenic analysis of the recombinant virus by haemagglutination-inhibition and strain-specific complement fixation tests indicated that it contained subtype-specific antigens which were immunologically related to those of both parent strains. This finding suggested that during reactivation an antigen was transferred from A₂/SINGAPORE/1/57 to the recombinant virus. Although the nature of the transferred antigen was not firmly established it did not appear to be related to the haemagglutinin of the A₂ parent. Persistence of the A₂ antigen in recombinant virus populations after several cycles of plaque-to-plaque transfer, a process which exerts selective pressure against the A₂ virus, precluded phenotypic or simple mixing as an explana-

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tion for the results. The authors thus concluded that genetic exchange had taken place during reactivation.

Similar findings of the presence in recombinant influenza viruses of subtype-specific antigens derived from both parent strains have been described for a number of reactivant viruses by Kilbourne and his co-workers (Kilbourne & Schulman, 1965; Sugiura & Kilbourne, 1966; Kilbourne *et al.* 1967). In these studies heat inactivated A0/NWS virus and live A2/JAPAN/107/57 were used as the parent viruses. Detailed immunological and biochemical study (Laver & Kilbourne, 1966) of one of the recombinant viruses, X7, indicated that it contained neuraminidase identical to that of the live A2 parent and haemagglutinin identical to that of the inactivated A0 parent.

In the present report we describe studies on the nature of the antigens present in two recombinant viruses. One of the virus strains studied was FPV-A2 (R4), a recombinant obtained from fowl plague virus and A2/SINGAPORE/1/57 (Tumova & Pereira, 1965); the other was FPV-T/M/65 (R4), a virus strain obtained by the reactivation of fowl plague virus by an avian influenza A virus, A/turkey/MASSACHUSETTS/65. The influenza virus of turkey origin was selected for use as a parent virus in this study because it is known to contain neuraminidase immunologically identical to that present in A2/SINGAPORE/1/57 (Pereira, Tumova & Webster, 1967; Webster & Pereira, 1968).

METHODS

Virus strains. The origin of the DUTCH strain of fowl plague virus and of A/turkey/MASSACHUSETTS/3740/65 used in the studies have been described (Pereira *et al.* 1966; Pereira *et al.* 1967). Recombinant virus FPV-A2 (R4) was obtained after four serial reactivation cycles of u.v.-inactivated fowl plague virus using live influenza A2/SINGAPORE/1/57 in each reactivation cycle. The origin of this virus was described in detail by Tumova & Pereira (1965).

FPV-T/M/65 (R4) was obtained by four serial cycles of inactivation and reactivation using A/turkey/MASSACHUSETTS/3740/65 as the live parent strain. The methods used were identical with those used by Tumova & Pereira (1965) in obtaining the recombinant virus FPV-A2 (R4) except that the turkey influenza virus was used instead of influenza A2/SINGAPORE/1/57.

Virus purification and concentration. Virus used for the preparation of rabbit antisera and in electrophoresis and immunodiffusion studies was grown in the allantoic sac of 11-day-old chick embryos and purified by adsorption to and elution from chicken erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10 to 40% sucrose in 0.15 M-NaCl) (Laver & Webster, 1966; Schild & Pereira, 1969).

Antisera. Antiserum against purified influenza A2 neuraminidase from a hyper-immunized rabbit was prepared as described by Laver & Webster (1966) using the recombinant virus X7 (F1) (Kilbourne *et al.* 1967) as the source of enzyme. Rabbit antisera were prepared against purified inactivated influenza viruses (Laver & Webster, 1966). Guinea pig anti-V antisera were obtained by methods of Lief & Henle (1959).

Electrophoresis of disrupted virus and the location of neuraminidase activity. Viruses were disrupted with sodium dodecyl sulphate; their protein components were separated by electrophoresis on cellulose acetate strips and the virus antigen bands stained with

Procion brilliant blue as described by Laver (1964). To identify the protein bands containing neuraminidase, side pieces were cut from the cellulose acetate strips and stained for protein. The unstained centre piece was then cut into segments and each segment was eluted with 0.25 ml. of 0.1 M-phosphate buffer, pH 5.9, and the eluates were assayed for neuraminidase activity using fetuin as substrate by a modification of Warren's method as previously described (Laver & Webster, 1966).

Immunodiffusion studies. A micro-double-immunodiffusion method (Crowle, 1958) was used. The tests were made with virus disrupted by sodium dodecyl sulphate as described by Schild & Pereira (1969).

Neuraminidase inhibition tests. Neuraminidase-inhibiting antibody was assayed as described by Webster & Pereira (1968) except that the antiserum and neuraminidase preparations were incubated for 16 to 18 hr at 4° before the addition of substrate. Neuraminidase-inhibiting antibody titres were expressed as that dilution of serum which neutralized 50% of the neuraminidase activity of the virus preparation. In some neuraminidase-inhibition tests the enzyme preparations used were obtained by incubating purified virus for 1 hr at 37° with 0.05% pronase (British Drug Houses Ltd) in 0.01 M-phosphate at pH 7.2, to destroy virus haemagglutinin (Seto, Drzeniek & Rott, 1966; Biddle, 1968).

Haemagglutination assays and haemagglutination-inhibition tests were done by the microtitration technique of Takatsy (1955). For haemagglutination-inhibition tests, the sera were treated with receptor destroying enzyme before testing. Four haemagglutinating units of virus were used and the serum + virus mixtures were kept at room temperature for 1 hr before adding fowl erythrocytes (0.5% suspension).

Complement fixation tests were made in plastic trays as described by Pereira, Pereira & Law (1964).

Table 1. *Cross-reactions of parent strains and reactivant viruses in haemagglutination-inhibition and strain-specific complement fixation tests*

Virus strain	Antiserum							
	Anti-fowl plague virus		Anti-A2/SINGAPORE/1/57		Anti-A/turkey/MASSACHUSETTS/65		Anti-purified A2/57 neuraminidase	
	HI	CF	HI	CF	HI	CF	HI	CF
Fowl plague virus	120	640	<	<	<	<	<	<
A2/SINGAPORE/1/57	<	<	1280	640	<	80	<	640
A/turkey/MASSACHUSETTS/65	<	<	<	320	480	640	<	640
FPV-A2 (R4)	480	640	<	320	<	80	<	640
FPV-A/turkey/MASS/65	240	640	<	320	<	480	<	640

HI, Reciprocal haemagglutination-inhibiting antibody titres in tests with antisera from hyperimmunised rabbits.

CF, Reciprocal complement fixing antibody titres of guinea-pig (anti-V) antisera.

<, No reaction with serum dilutions of 1/20 or greater.

RESULTS

Immunological studies

Haemagglutination-inhibition tests were made with rabbit antisera prepared against the purified parent virus strains and against a purified influenza A2 neuraminidase preparation (Table 1). The antiserum against fowl plague virus reacted with the

recombinant strains FPV-A2 (R4) and FPV-T/M/65 (R4) at titres similar to that obtained with the homologous virus. However, there was no reaction with A2/SINGAPORE/1/57 and A/turkey/MASSACHUSETTS/65. Antisera prepared against A2/SINGAPORE/1/57 and A/turkey/MASSACHUSETTS/65 reacted with only the homologous viruses in the haemagglutination-inhibition test. These results indicated that the haemagglutinins present in the two recombinant virus strains were immunologically related to that of fowl plague virus but distinct from that present in the parent strains, A2/SINGAPORE/1/57 and A/turkey/MASSACHUSETTS/65. The antiserum against purified A2 neuraminidase had no haemagglutination-inhibiting activity.

Table 2. Serum dilutions producing 50% inhibition of neuraminidase activity

Virus*	Antiserum				Normal rabbit serum
	Anti-fowl plague virus	Anti-A2/SINGAPORE/1/57	Anti-A/turkey/MASSACHUSETTS/65	Anti-purified A2/57 neuraminidase	
Fowl plague virus	126*	<	<	<	<
A2/SINGAPORE/1/57	<	501	631	7943	<
A/turkey/MASSACHUSETTS/65	<	794	3162	3162	<
FPV-A2 (R4)	79	630	501	3981	<
FPV-T/M/65 (R4)	50	398	316	2512	<

* Reciprocal of serum dilution producing 50% inhibition of neuraminidase activity.

<, Less than 50% inhibition of neuraminidase with 1/10 serum.

The immunological relationships between the recombinant viruses and the parent strains in strain-specific complement fixation tests were studied with guinea pig (anti-V) antisera (Table 1). Anti-V antiserum for fowl plague virus reacted to high titres with the homologous virus and also with the two recombinant virus strains but did not react with A2/SINGAPORE/1/57 or A/turkey/MASSACHUSETTS/65. This pattern of cross-reactions was the same as that detected in haemagglutination-inhibition tests and the results thus suggested similarities in the haemagglutinins of fowl plague virus and the recombinant virus strains. In contrast, anti-V antisera for A2/SINGAPORE/1/57 and A/turkey/MASSACHUSETTS/65 did not react with fowl plague virus although each reacted with both of the recombinant virus strains. A similar pattern of reactions was given by the rabbit antiserum prepared against purified A2 neuraminidase. This antiserum, which contained no antibody against haemagglutinin, reacted to similar titres with A2/SINGAPORE/1/57, A/turkey/MASSACHUSETTS/65 and the two recombinant viruses but failed to react with fowl plague virus. This finding indicated that a common antigenic component, probably neuraminidase, was present in A2/SINGAPORE/1/57 and A/turkey/MASSACHUSETTS/65 and the two recombinant viruses.

The immunological relationships between the parent and recombinant virus strains was further studied in neuraminidase-inhibition tests with sera from hyperimmunized rabbits (Table 2). Suspensions of intact, purified influenza viruses were used as sources of the enzyme. Antisera against purified A2 neuraminidase and the parent virus strains A2/SINGAPORE/1/57 and A2/turkey/MASSACHUSETTS/65 inhibited the neuraminidase activity of all the test viruses except fowl plague virus. Antiserum against fowl plague virus inhibited the neuraminidase of the homologous virus and also inhibited the enzymic activity of both recombinant viruses but at lower titres than

those obtained with the homologous virus. This may indicate that the neuraminidase of these viruses was immunologically related to that of fowl plague as well as to that of A2/SINGAPORE/1/57. An alternative possibility is that the neuraminidase present in the recombinant viruses was identical to that of the A2 influenza virus but partial inhibition of the enzyme was brought about by antibody to haemagglutinin of fowl plague virus. It has been suggested that steric hindrance of the access of substrate to influenza virus neuraminidase may be brought about by antibody to haemagglutinin coating the surface of the intact virus particle (Paniker, 1968; Webster & Pereira, 1968). This apparent neutralization of enzyme would be expected to occur only when intact virus particles were used as a neuraminidase source. Thus, some of the neuraminidase-inhibition tests were repeated using preparations of neuraminidase isolated from FPV-A2 (R4) virus by sodium dodecyl sulphate treatment followed by cellulose acetate electrophoresis (Plate 1a). In addition, tests were made with neuraminidase from pronase-treated virus. Treatment of influenza viruses by proteases has been shown to destroy virus haemagglutinin and to release biologically active neuraminidase from the virus particle (Seto *et al.* 1966; Biddle, 1968). Neuraminidase from FPV-A2 (R4) was not neutralized by the antiserum for fowl plague virus when separated from the virus by electrophoresis or by treatment of virus by pronase. The activity of the same neuraminidase preparations was inhibited by antiserum against A2/SINGAPORE/1/57 and by antiserum against purified A2/57 neuraminidase. As a control, A2/SINGAPORE/1/57 virus was treated in the same way and neuraminidase which had been separated from the virus by electrophoresis and the neuraminidase of pronase-treated virus were neutralized to the same extent as the neuraminidase of the intact virus (Table 3).

Table 3. Comparison of neuraminidase-inhibition antibody titres using intact virus, pronase-treated virus and isolated neuraminidase

Source of neuraminidase	Antisera		
	Anti-fowl plague virus	Anti-A2/ SINGAPORE/1/57	Anti-purified A2 neuraminidase
Intact FPV-A2 (R4) virus	80*	550	3160
Neuraminidase isolated from FPV-A2 (R4) by electrophoresis	<	750	3980
Pronase-treated FPV-A2 (R4) virus	<	630	6300
Intact A2/SINGAPORE/1/57 virus	<	500	6300
Neuraminidase isolated from A2/SINGAPORE/1/57 by electrophoresis	<	750	3980
Pronase-treated A2/SINGAPORE/1/57 virus	<	750	2510

* Reciprocal of serum dilution producing 50% inhibition of neuraminidase activity.

<, Less than 50% inhibition of neuraminidase by a 1/10 serum dilution.

These findings suggested two possibilities: that the FPV-A2 (R4) recombinant contains two neuraminidases, one derived from fowl plague virus and one from the A2 parent strain, or that the effect of fowl plague antiserum on the recombinant was due to steric hindrance. Since fowl plague antiserum did not inhibit the neuraminidase released from the particles of the recombinant virus by pronase treatment, a process which did not destroy the neuraminidase activity of either parent strain, it was concluded that the second possibility is the correct one. This conclusion was supported by immunodiffusion studies described below.

Immunodiffusion was used as a method to detect A2 neuraminidase in influenza virus strains by Schild & Pereira (1969), who showed that a rabbit antiserum against purified A2/57 neuraminidase gave a single precipitin line with virus strains which contained A2 neuraminidase but not with other influenza A viruses. Antiserum against purified A2 neuraminidase was tested by immunoprecipitation against fowl plague virus, A2/SINGAPORE/1/57 and the two recombinant viruses (Fig. 1). No precipitin line was seen for fowl plague virus. However, A2/SINGAPORE/1/57 and the two recombinant virus strains produced precipitin lines which were continuous for the various viruses and also showed continuity with the precipitin line given by a preparation of isolated neuraminidase from A2/SINGAPORE/1/57 virus. These immunodiffusion techniques could not be used to test for the presence of A2 neuraminidase in A/turkey/MASSACHUSETTS/65 virus; concentrates suitable for immunodiffusion studies could not

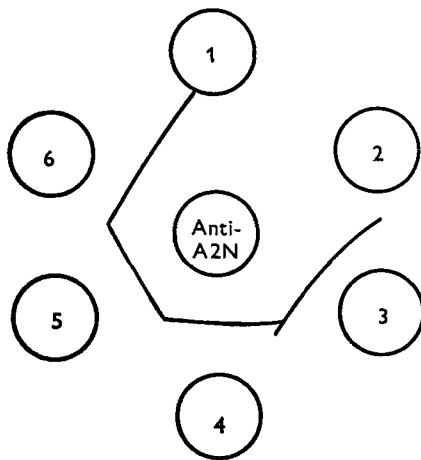


Fig. 1

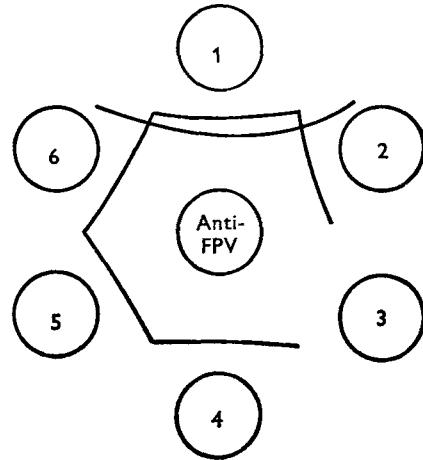


Fig. 2

Fig. 1 and 2. Precipitin reactions of parent and recombinant influenza virus strains. The virus concentrates were treated with sodium dodecyl sulphate (1%) before being added to the wells.

Fig. 1. Centre well contains antiserum against purified A2 neuraminidase. Peripheral wells: 1, fowl plague virus; 2, B/ENGLAND/5/66; 3, Purified A2 neuraminidase; 4, A2/SINGAPORE/1/57; 5, FPV-T/M/65 recombinant; 6, FPV-A2 (R4) recombinant.

Fig. 2. Centre well contains antiserum against pronase treated fowl plague virus. Peripheral wells: 1, fowl plague virus; 2, AO/BEL; 3, B/ENGLAND/5/66; 4, A2/SINGAPORE/1/57; 5, FPV-T/M/65 recombinant; 6, FPV-A2 (R4) recombinant.

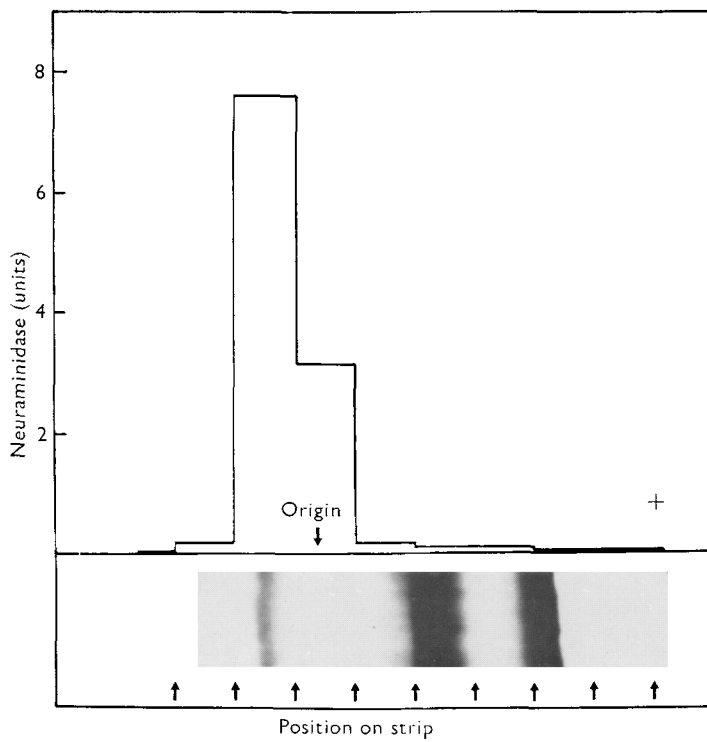
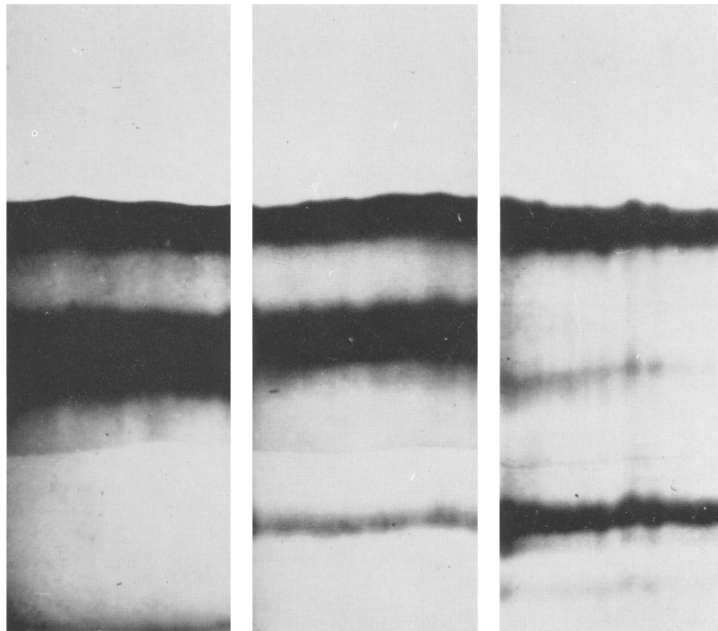
EXPLANATION OF PLATE

PLATE 1a

Cellulose acetate strips stained with Procion brilliant blue, showing the electrophoretic separation of the protein components of the two parent viruses (FPV and A2/SINGAPORE) and of the recombinant virus (FPV-A2/SINGAPORE R4) after disruption of the virus particles with sodium dodecyl sulphate.

PLATE 1b

Location of neuraminidase activity on a cellulose acetate strip after electrophoresis of the disrupted sodium dodecyl sulphate recombinant virus (FPV-A2/SINGAPORE R4). Arrows indicate where the unstained portion of the strip was cut. Segments were eluted with 0.1 M-phosphate buffer, pH 5.9, and the eluates were tested for neuraminidase activity.



(b)

be obtained because of the relatively poor growth of this virus. Further immunodiffusion studies were made using an antiserum prepared against purified, concentrated fowl plague virus treated with pronase (0.05 % for 60 min. at 37°) to destroy haemagglutinin. This antiserum had a high titre of neuraminidase-inhibiting antibody (titre 1/5000) and no haemagglutination-inhibiting antibody (titre < 1/40) for fowl plague virus. It also contained antibody for the type-specific ribonucleoprotein of influenza A. A common precipitin line, apparently corresponding to type-specific antigen (Schild & Pereira, 1969), was seen for all the influenza A viruses but not with an influenza B virus. (Fig. 2). In addition, fowl plague virus, but not A2/SINGAPORE/1/57 or the two recombinant virus strains, gave a further precipitin line. This finding indicated that an antigen was present in fowl plague virus which was absent from the recombinant viruses. The presence in the rabbit antiserum of high titres of antibody for neuraminidase but not for haemagglutinin strongly suggested that the precipitin line found with fowl plague virus corresponded with fowl plague neuraminidase and that the enzyme of fowl plague virus was not present in the recombinant virus strains.

Electrophoretic studies of virus proteins

Samples of purified parent viruses, fowl plague and A2/SINGAPORE/1/57 and the recombinant virus FPV-A2 (R4) were disrupted by treatment with sodium dodecyl sulphate and their component proteins separated by electrophoresis on cellulose acetate. The electrophoretic pattern of the two parent viruses differed considerably (Plate 1*a*). For fowl plague virus, two protein bands were seen on the stained strips. Both the protein bands moved towards the anode. For A2/SINGAPORE/1/57, three principal protein bands were detected. Two bands moved towards the anode and one moved towards the cathode. The electrophoresis of FPV-A2 (R4) also revealed three protein bands. One band, like one of the bands produced by A2/SINGAPORE/1/57, moved towards the cathode. The remaining two bands produced by the recombinant virus were of approximately the same mobility and stained with about the same intensity as the bands given by fowl plague virus. The position of the neuraminidase activity was located on duplicate unstained electrophoresis strips by testing eluates from sections of strip for enzymic activity. In A2/SINGAPORE/1/57 (Plate 1*b*) the band which moved towards the cathode contained neuraminidase activity. The neuraminidase activity of the recombinant, FPV-A2 (R4), was associated with a band of similar mobility to that which contained the neuraminidase of the A2 parent. Neuraminidase activity could not be detected in the eluate of strips after the electrophoretic separation of fowl plague virus and the enzyme of this virus was found to be inactivated by the addition of sodium dodecyl sulphate.

DISCUSSION

The immunological reactions of the recombinant virus FPV-A2 (R4) and the electrophoretic mobilities of its structural proteins strongly suggested that this virus contained subtype-specific antigens derived from each of its parent viruses. The neuraminidase of the recombinant appeared to be identical to that of the human influenza virus, A2/SINGAPORE/1/57, which was used as the live parent, whilst the haemagglutinin of the recombinant was identical to that of fowl plague virus, the inactivated parent virus. This finding confirms the earlier observations of Tumova & Pereira (1965), based on strain-specific complement fixation tests, that the genetic interaction of

inactivated fowl plague virus and live influenza A2 virus was accompanied by the transfer of an antigen from the live to the inactivated virus. Although the electrophoretic mobilities of its component proteins were not studied there was strong evidence from immunological tests that the recombinant virus FPV-T/M/65 also contained haemagglutinin derived from fowl plague virus and neuraminidase derived from its live avian influenza A virus parent, A/turkey/MASSACHUSETTS/65. Our findings are thus strikingly similar to those obtained for a recombinant virus, X7, by Laver & Kilbourne (1966). X7, which was obtained by the reactivation of heat-inactivated A0/NWS virus by a live A2 virus, was found to contain neuraminidase identical to that of its influenza A2 virus parent and haemagglutinin derived from A0/NWS.

The study of the genetic interactions between influenza A viruses is of particular importance in regard to the origin of the new subtypes of virus which arise from time to time in the human population. The discovery that an avian virus, A/turkey/MASSACHUSETTS/65, contained a type of neuraminidase identical to that of human influenza A2 virus although its haemagglutinin was distinct from that of human influenza A virus (Pereira *et al.* 1967; Webster & Pereira, 1968) suggested the possibility that genetic interaction might take place under natural conditions between human and animal influenza A viruses. The present study indicates that under controlled laboratory conditions two avian viruses or an avian and a human influenza A virus interact in such a way that antigenic exchange takes place resulting in 'hybrid' virus strains with antigenic specificities derived from each of the parent virus strains.

An attractive prospect arising from the present study and from that of Laver & Kilbourne (1966) is the possibility of 'tailor making' influenza viruses in the laboratory which contain neuraminidase and haemagglutinin of types which are predetermined by the selection of appropriate parent virus strains. Such procedures would enhance the detailed antigenic analysis of influenza virus strains and might also be useful in providing a source of viruses from which strains may be selected with immunological and biological characteristics which are appropriate to particular types of experimental work.

We wish to thank Mrs S. Piddington, Mr R. W. Newman and Miss C. A. Potts for capable technical assistance.

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(Received 23 December 1968)