Flocculation of Influenza Virus by Specific Anti-neuraminidase Antibody

By

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With 2 Figures

Summary

1. Flocculation of purified preparations of influenza virus has been demonstrated to occur in the presence of low dilutions of specific anti-neuraminidase sera. High dilutions of the sera caused microscopic aggregation of virions. It is suggested that the effects of anti-neuraminidase antibody on the replication of influenza virus observed *in vitro* could be caused by antibody binding virions to infected cells, rather than by inhibition of neuraminidase enzymic activity.

2. On examination in the electron microscope of virus floccules obtained with specific anti-neuraminidase sera, surface spikes were clearly seen on the virions. However, when influenza virus was similarly flocculated by serum containing antibodies that inhibited both haemagglutination and neuraminidase activities, the ends of the spikes were almost all completely obscured.

3. Measurement of the Michaelis constant (Km) of X-7 (F1) neuraminidase shows that the enzyme active site is related to that of A_2/Singapore/57 neuraminidase.

1. Introduction

The presence of an enzymic component in the influenza viruses has been recognised for many years, but it is only more recently that the antigenic nature of this enzyme, neuraminidase (E.C.3.2.1.18), has become apparent (Rafelson, Schneir and Wilson, 1963; Ada, Lind and Laver, 1963). This property of neuraminidase has since been studied to great advantage, and evidence has been obtained by use of recombinant virus strains to show that the antigenic sites on the viral surface which are associated with haemagglutinating and neuraminidase activities are coded by separate parts of the viral genome (Kilbourne and Schulman, 1965; Laver and Kilbourne, 1966; Kilbourne *et al.*, 1967; Webster and Laver, 1967; Kilbourne, 1968; Webster, Laver and Kilbourne, 1968). Kinetic studies of the chemical inactivation of influenza viruses lead to the

same conclusion (SCHOLTISSEK and ROTT, 1964; SCHOLTISSEK, BECHT and DRZE-
NIEK, 1967), and the current view is generally held that neuraminidase and
haemagglutinating activities are the properties of different viral-specified proteins.

Thus, investigations of the function of neuraminidase in influenza virus
infections have often adopted the use of specific anti-neuraminidase antibody to
selectively inhibit the activity of this viral component (SETO and ROTT, 1966;
JAHIEL and KILBOURNE, 1966; SETO, OKUDA and HOKAMA, 1967; WEBSTER and
LAVER, 1967; SETO and CHANG, 1969). In the present report, we have examined
and commented on a complementary property of anti-neuraminidase antibody,
the ability to aggregate influenza virions.

2. Methods
2.1. Propagation of Virus

An inhibitor-sensitive variant of the virus strain A_2/Singapore/1/57 was grown in
the allantoic cavity of $10-11$ day old embryonated hens' eggs, as previously described
(KENDAL et al., 1968). Seed of the recombinant virus strain X-7 (F1) was kindly
provided by Dr. G. Schild, of the Medical Research Council Laboratories, Mill Hill,
England, and then grown as for A_2/Singapore/1/57. Purified virus was obtained from
infected allantoic fluid by one cycle of differential centrifugation followed by rate
zonal density gradient centrifugation (KENDAL et al., 1968).

2.2. Determination of Haemagglutination and Haemagglutination
Inhibition

This was carried out as described by BIDDLE and SHORTRIDGE (1967), except that
all volumes were adjusted for use in microtrays. Virus samples for the haemaggluti-
nation inhibition test consisted of infected allantoic fluids diluted in 0.15 M saline to
contain 16 haemagglutinating units (HAU)/ml. 0.025 ml aliquots were added to equal
volumes of serum dilutions and stood at room temperature for 15 minutes before
adding fowl red cells. Rabbit sera were treated with periodate before use (LENNETTE
and SCHMIDT, 1969), and inhibition titres (HIT) were expressed as the serum dilution
causing 50% inhibition of haemagglutination.

2.3. Measurement of Neuraminidase Inhibition

This was performed using the automated procedure described previously for
human influenza virus strains, using serum glycoprotein as the enzyme substrate
(KENDAL and MADELEY, 1969). Titres (NIT) are expressed as the reciprocal of the
serum dilution causing 50% inhibition of observed neuraminidase activity.

2.4. Flocculation of Virus

This was performed essentially as described by BELYAVIN (1955). To serial two-fold
dilutions of serum ($0.2-0.3$ ml) in Dreyer agglutination tubes were added equal
volumes of purified virus suspensions, containing $500-1,000$ HAU/ml. The tubes
were half-immersed in a 37°C water-bath for about 17 hours and the flocculation
titre (FT) of each serum was determined as the highest serum dilution giving visible
virus floccules at the base of the tubes, as observed with the naked eye using indirect
illumination and a dark background.

2.5. Isolation of Neuraminidase

Neuraminidase was isolated from purified concentrates of influenza virus by
incubation with the bacterial protease nagarse (subtilisin BPN[1]). This treatment
digests more than 99% of the viral haemagglutinin to peptides, releases active neura-
minidase, and produces degraded virus particles of reduced buoyant density and
sedimentation coefficient, but still containing the bulk of viral nucleic acid (BIDDLE,
1968; KENDAL et al., 1968; KENDAL, APOSTOLOV and BELYAVIN, 1969). The degraded

virus particles were removed by centrifugation through 12—60% (w/v) sucrose gradients (100,000 $\times g$ for 90 minutes) and the neuraminidase in solution was recovered in the upper fractions of these gradients. Neuraminidase was then separated from protease and slowly sedimenting viral degradation products by rate zonal centrifugation (ca. 70,000 $\times g$ for 17 hours) through further 12—60% (w/v) sucrose density gradients. The fractions from these gradients containing the peak of neuraminidase activity were pooled and dialysed against 0.15 M NaCl. This procedure has been described in full previously (KENDAL et al., 1968).

2.6. Production of Antisera

Anti-neuraminidase. Two preparations of neuraminidase were isolated from influenza virus A_2/Singapore/57, as described above. These preparations N.1. and N.2., obtained from a nominal 400,000 and 10,000 HAU of virus, respectively, were inoculated into an adult rabbit, and sera were obtained, according to the schedule shown in Table 1.

Anti-whole virus. A rabbit was given two intravenous inoculations with preparations of purified influenza virus at an interval of 3 weeks, and bled 5 days after the second inoculation.

2.7. Determination of Michaelis Constant of Neuraminidase

This was performed by the automated procedure previously described, using sialo-lactose substrate (KENDAL and MADELEY, 1969).

2.8. Electron Microscopy of Virus Flocculation

All floccules were recovered for electron microscopy directly from the agglutination tubes, without centrifugation. For examination by negative staining, the samples were diluted 2—4 fold in distilled water, mixed with an equal volume of 3% phosphotungstic acid, pH 6.0, and a drop was applied to carbon-formwar coated grids. Micrographs were taken in a Philips EM 200, using double condenser illumination and an instrumental magnification of 62,000.

3. Results

3.1. Inhibition of Viral Haemagglutinating and Neuraminidase Activities by Antisera

These tests, performed as described in methods, were carried out against both A_2/Singapore/57 virus, and the recombinant virus strain X-7 (F1). As shown in Table 2, sera prepared against antigens of the Asian parent produced no detectable inhibition of haemagglutination by X-7 (F1) virus, although high neuraminidase inhibition could be demonstrated. Serum prepared against isolated neuraminidase from A_2/Singapore/57 virus had high neuraminidase inhibitory activity, and a significant level of haemagglutination inhibition was also produced against the homologous virus but not against X-7 (F1). Normal rabbit serum produced very low levels of inhibition in all cases. Horse serum had no significant anti-neuraminidase activity against the Asian virus enzyme (NIT <15), although the haemagglutinin of this sensitive variant of A_2/Singapore/57 was strongly inhibited (HIT=1,000).

3.2. Kinetic Characteristics of X-7 (F1) Virus Neuraminidase

The immunological results described above confirmed other similar evidence that the neuraminidase, but not the 'haemagglutinin', of the recombinant virus X-7 (F1) is derived from the parent virus A_2/Singapore/57 (KILBOURNE et al., 1967; WEBSTER et al., 1968). However, we have previously suggested that the

antigenic sites and active sites of influenza virus neuraminidases might segregate independently in genetic recombination experiments (KENDAL and MADELEY, 1969). Accordingly, the Michaelis constants of X-7 (F1) and A_2/Singapore/57 virus neuraminidases were determined, using sialolactose substrate. A_0/BEL/35 virus was also tested, as a representative of the A_0/antigenic-type neuraminidases (PANIKER, 1968). As shown in Table 3, the kinetic evidence provides further support for the proposed derivation of X-7 (F1) neuraminidase. The Km of this viral enzyme was very similar to that of A_2/Singapore/57, and quite different to the present value determined for A_0/BEL/35, and the previously reported values for A_0/PR8/34, A_0/MEL/35, and A_0/NWS/39 (KENDAL and MADELEY, 1969).

Table 1. *Schedule Used for Production of Rabbit Anti-serum against Purified Neuraminidase from Influenza Virus A_2/Singapore/57*

Day	Remarks
1	Pre-immunisation bleed (normal serum)
1	Intramuscular inoculation of 0.25 ml Freund's complete adjuvant + 0.25 ml neuraminidase preparation N.1. ($\equiv$ enzyme from 200,000 HAU of virus)
14	Inoculation as above ($\equiv$ enzyme from 200,000 HAU of virus)
69	Inoculation as above, with neuraminidase preparation N.2. ($\equiv$ enzyme from 10,000 HAU of virus)
158	Sample bleed used in neuraminidase inhibition and flocculation experiments

(days 8, 14, 17, 21, 29 and 78 other sample bleeds)

3.3. Flocculation of Influenza Virus by Antibodies Specific for Neuraminidase

Initially, A_2/Singapore/57 virus was used in these tests, and it was found that this virus could be flocculated by serum prepared against whole virus, and by serum prepared against isolated neuraminidase (Table 2). In repeated experiments using different preparations of A_2/Singapore/57 virus, the flocculating titre of the 'anti-neuraminidase' serum was consistently about 2-fold less than that of the anti-whole virus serum. Although the anti-neuraminidase serum was obtained in response to purified neuraminidase antigen, the HI activity of this serum indicated that some antibodies directed against the viral haemagglutinin might also have been present. As varying degrees of inhibition of haemagglutination by different virus recombinants can apparently be produced by anti-neuraminidase antibodies (KILBOURNE, 1968), it is quite possible that the HI activity observed in the present experiment was the result only of steric hindrance by anti-neuraminidase antibodies. However, to confirm that flocculation of influenza virus particles could be induced by antibodies specific for neuraminidase, the X-7 (F1)

virus was used, since none of the immune sera had any detectable haemagglutinating activity against this recombinant virus. As seen from Table 2, macroscopic aggregates of X-7 (F1) virus formed on mixing with these immune sera.

3.4. Electron Microscopic Examination of Virus Floccules

To verify that the aggregated material seen in the flocculation tests contained virus particles, and not just serum deposits, floccules were examined by negative staining technique in the electron microscope. Shown in Fig. 1 is the appearance

Table 2. *Immunologic Reactions of X-7 (F1) and A$_2$/Singapore/57 Viruses with Rabbit Anti-sera*

Serum prepared against	Serum titres[1]					
	Anti-neuraminidase		Anti-haemagglutinin		Flocculation	
No antigen (normal serum)	75[2]	(65)	<5	(<5)	8	(8)
A$_2$/Singapore/57 isolated neuraminidase	4,500	(9,000)	<5	(200)	60	(80)
Whole virus A$_2$/Singapore/57	4,500	(6,750)	<5	(1,250)	30	(120)

[1] For details of expression of serum titres, see "Methods".
[2] 1st Figure — reaction with X-7 (F1) virus.
  2nd Figure, in parenthesis — reaction with A$_2$/Singapore/57 virus.

Table 3. *Michaelis Constant (Km) of Influenza Virus Neuraminidases for Sialo-lactose Substrate*

Virus strain	Km M/L × 10^4	
	Mean[1]	Range
A$_2$/Singapore/57	9.8	9.0—11
A$_2$/Singapore/57[2]	13.0	9.0—18
X-7 (F1)	9.1	7.7—10
A$_0$/BEL/35	3.1	2.8—3.4
A$_0$/NWS/39[2]	3.5	2.9—4.7

[1] Mean Km values obtained from a minimum of 3 determinations.
[2] Previously determined values (KENDAL and MADELEY, 1969).

of the precipitate obtained when A$_2$/Singapore/57 virus was reacted with fully homologous anti-serum at a dilution of 1/20. Many aggregated virus particles were seen, no longer clearly showing the characteristic spikes seen in negatively stained control preparations. A number of the virions, particularly at the edge of the floccules, appeared to be surrounded by a 'halo' of material. This suggests that antibody molecules were binding around the tips of the spikes, and possibly not along their whole length.

When virus precipitated by specific anti-neuraminidase serum was examined, the spiky appearance of control virions was still seen quite clearly. Little dif-

ference was observed between particles of A_2/Singapore/57 or X-7 (F1) virus reacted with this serum (Fig. 2). In some areas around the particles material apparently binding to the spikes was present. Such areas were randomly distributed, and a diffuse 'halo' was not seen. Even when immune sera were reacted
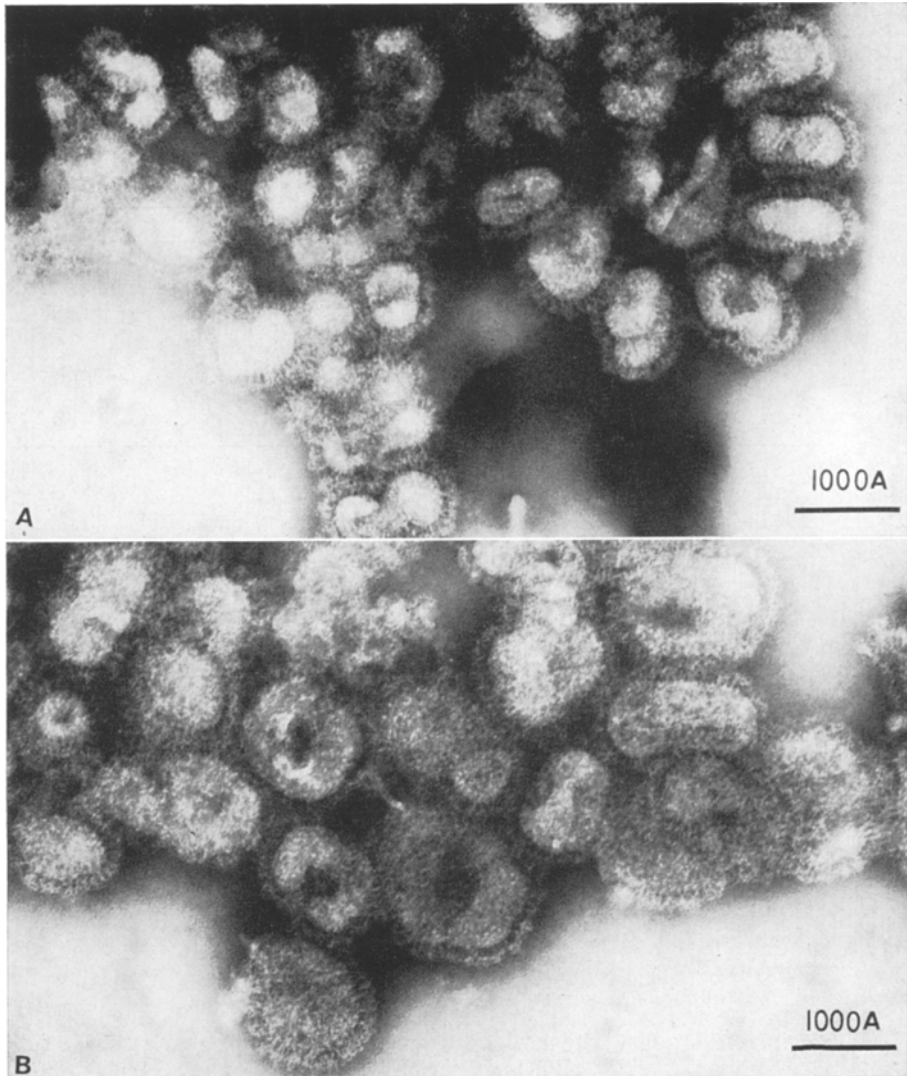

Fig. 1. A and B. Floccules of A_2/Singapore/57 virus obtained with anti-A_2/Singapore/57 serum (serum dilution, 1:20). Preparation negatively stained with P.T.A. The outermost ends of all the surface spikes are obscured by antibody

at a high dilution (1/400) so that no macroscopic virus precipitate formed, few single virus particles could be seen in the mixtures, most virions occurring in aggregates. Control virus preparations mixed with normal rabbit serum (1/20 dilution) contained predominantly individual virions, with occasional small groups.

4. Discussion

The structural relationship of influenza virus haemagglutinin and neuraminidase components on the surface of the virions is still the subject for some speculation, and information on this subject is particularly required in connec-
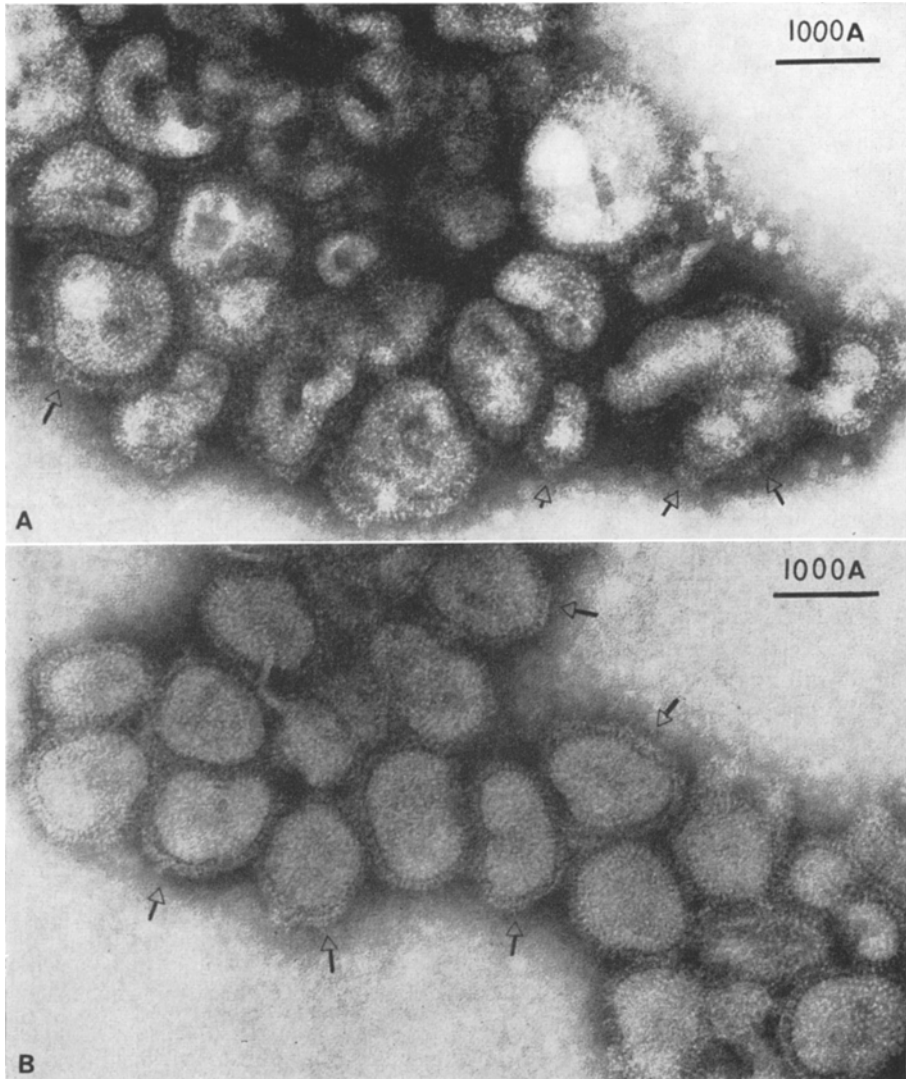

Fig. 2. Influenza virus floccules obtained with anti-A₂/Singapore/57 neuraminidase serum (serum dilution, 1:20). A. A₂/Singapore/57 virus floccules. B. X-7 (F 1) virus floccules
Preparations negatively stained with P.T.A.
Large areas of the surface spikes appear unobscured, but the possible presence of antibody around the tips of the spikes may be seen localised in some places (arrowed)

tion with the possible interference by anti-neuraminidase antibody in haemagglutination inhibition tests used for typing viruses (see SCHULMAN and KILBOURNE, 1969).

Although it has been known for many years that influenza virus particles could be flocculated by specific immune sera (BELYAVIN, 1955), in retrospect the participation of viral haemagglutinin and neuraminidase in the aggregation reactions described could not be differentiated at that time. Subsequent experiments have shown that influenza viruses may be flocculated by non-antibody serum haemagglutination inhibitors (BIDDLE and STEVENSON, 1966), and the present experiments were designed to determine whether virus particles could be flocculated through their neuraminidase components.

For this conclusion to be drawn, it was important to demonstrate viral aggregation in a system where the effect of anti-haemagglutinin antibodies could be excluded. Therefore, X-7 (F1) virus was tested with sera obtained against neuraminidase from A$_2$/Singapore/57 virus, since neuraminidase is the only surface antigen so far demonstrated to be common between these viruses (WEBSTER et al., 1968). Our finding of a strong similarity between the kinetic behaviour of the enzymes from these two viruses indicates that the resemblance between their neuraminidases extends to the molecular configuration at their active sites, and this is additional evidence for the proposed derivation of the X-7 (F1) virus used in the aggregation reaction described here.

The present results therefore show conclusively that, under controlled experimental conditions, flocculation of influenza virus particles by specific anti-neuraminidase antibody does occur. We also found that even quite low anti-neuraminidase antibody concentrations produced microscopic aggregates of influenza virus, in agreement with the observations of SETO and CHANG (1969).

Aggregation of virions can only occur if antibody can form bridges between one particle and another. Since the 'spikes' on each influenza virion are 100 Å long, it will not be possible for flocculation to occur if viral antigen is located near the base of the spikes, and the rabbit antibody molecule Fab sites cannot stretch more than 120 Å apart (VALENTINE and GREEN, 1967). Thus it would seem that neuraminidase antigens are not confined to the base of the spikes. This is further supported by the evidence in Plate 2, where antibody appears to be binding to the tips of the spikes, leaving the base visible. In addition, attachment of the anti-neuraminidase antibody seems to be localised to groups of spikes, rather than uniformly distributed over the surface. Such a possibility has been considered previously, in a discussion of how neuraminidase might be arranged on the surface of influenza virus particles so that both detergents and protease released enzymic structures of similar sedimentation coefficients (APOSTOLOV, FLEWETT and KENDAL, 1969).

The ability of anti-neuraminidase antibody to aggregate virions may be highly significant in interpreting the results of experiments designed to investigate the influence of such antibody on influenza virus replication. From the evidence that anti-neuraminidase antibody reduces the plaque size or yield of virus in influenza-infected cells (SETO and ROTT, 1966; JAHIEL and KILBOURNE, 1966; WEBSTER and LAVER, 1967; KILBOURNE et al., 1968; SETO and CHANG, 1969), it has previously been concluded that the enzymic function of neuraminidase in influenza replication is the release of matured virus from the cells (KILBOURNE et al., 1968).

However, we would suggest that there is equally good reason to believe that the release of virus particles may be inhibited simply by anti-neuraminidase antibody aggregating virions to infected cells. Neuraminidase activity may be detected on the plasma membrane of influenza-infected cells, and is available for reaction with antibody (KENDAL and APOSTOLOV, 1969). Therefore, as the present experiments have shown that the neuraminidase present on influenza virions is available for aggregation reactions with antibody, it seems probable that antibody could bind virions to infected cells by attaching to neuraminidase molecules on each.

This hypothesis is in agreement with the electron microscopic observations of SETO and CHANG (1969), and in addition explains the otherwise paradoxical behaviour of the recombinant influenza viruses X-7 (F1) and X-7 in plaque inhibition tests. For, if the release of virus particles from infected cells was dependant on the enzymic activity of neuraminidase, it would be anticipated that the release of X-7 (F1) virus would be less affected than X-7 virus by anti-neuraminidase antibody, since X-7 (F1) virus contains twice the amount of neuraminidase present in X-7 (WEBSTER et al., 1968). In practice, the reverse has been found to be the case, for complete abolition of X-7 (FI) plaques is produced by anti-neuraminidase sera, whereas only the size, but not the number, of X-7 plaques is reduced under the same conditions (JAHIEL and KILBOURNE, 1966; KILBOURNE et al., 1968).

This previously reported result is consistent with our present suggestion that the observed effects of anti-neuraminidase antibody in tissue culture and in ovo could be due to aggregation of virions to infected cells, since an increase in the number of neuraminidase sites on the virions (and by inference on the membrane of infected cells) would result in the formation of more antibody links, and correspondingly increase the stability of any virus-cell aggregates.

Experiments with mice have shown that the presence of circulating anti-neuraminidase antibodies correlates with a degree of protection against pulmonary virus infection (SCHULMAN, KHAKPOUR and KILBOURNE, 1968). We have found a significant degree of protection associated with anti-neuraminidase antibodies in lethal neurotropic infection of chickens by avian influenza viruses (ALLAN, MADELEY and KENDAL, in preparation). These results suggest that anti-neuraminidase antibodies can limit the extent of influenzal infections in natural hosts. However, there is no indication whether the antibody effect observed was due to inhibition of neuraminidase enzymic activity or an aggregation phenomenon. Further experiments are now being planned to investigate the influence of anti-neuraminidase antibody on the replication of influenza viruses, in tissue culture and in natural hosts, under conditions where the possibility of virus-cell aggregation is prevented. In this way, it is hoped to obtain a better understanding of the enzymic function of neuraminidase.

A further observation from the present work was the indistinguishable appearance of floccules of A_2/Singapore/57 virus and X-7 (F1) virus obtained with anti-neuraminidase serum (Fig. 2 A and B), and it seems probable, therefore, that the haemagglutination inhibition activity of this serum against A_2/Singapore/57 virus was due to anti-neuraminidase antibodies. A similar effect has been observed by KILBOURNE (1968) with an inhibitor-sensitive variant of A_2/Singapore/57. That the haemagglutinin of X-7 (F1) virus was not inhibited by this

anti-neuraminidase serum confirms that where the possibility exists of anti-neuraminidase antibodies interfering with viral haemagglutinin (and *vice versa*), each strain of influenza virus must be considered independently. It may also be concluded from the serological studies with X-7 (F1) virus that the virus concentration used in the standard haemagglutination tests was too low to be flocculated by anti-neuraminidase sera. This question has also recently been discussed by WEBSTER (1969).

Acknowledgements

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