The chemical reactions
of the haemagglutinins and neuraminidases of
different strains of influenza viruses

II. Effects of reagents modifying the higher order
structure of the protein molecule

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In previous work (Hoyle & Hana, 1966) it was found that the enzymic activity of the DSP and LEE strains of influenza virus was reduced by agents acting on disulphide bonds. The results, however, tended to be irregular, except in the case of mercuric chloride, these irregularities being attributed to reversibility of the reaction with many of the chemical reagents used. More recent work using dithiothreitol (Cleland, 1964) which reacts completely and irreversibly with the disulphide bond have shown that the disulphide bonds in the virus proteins are inaccessible to attack except under conditions permitting some disruption of hydrogen bonds. A study has therefore been made of the effects on the virus properties produced by reagents acting on non-covalent bonds alone and in combination with dithiothreitol.

METHODS

In this work purified 'virus concentrate' preparations have been used, prepared as described in the previous paper (Hoyle, 1969). Two mixtures were first made, one consisting of equal volumes of virus concentrate and buffer pH 8·0, and the second of virus concentrate and 1/100 dithiothreitol in buffer pH 8·0. The two mixtures in 0·05 ml. amounts were then mixed with 0·15 ml. of buffer pH 8·0 containing amounts of urea or guanidine sufficient to give final concentrations of 0, 4 M and 6 M urea or 2 M, 4 M and 6 M guanidine. Mixtures were held at 20° C. for 30 min. and were then diluted to 2 ml. with buffer pH 6·0. Haemagglutinin titres were then measured and neuraminidase activity assessed by the elution test as described in the previous paper (Hoyle, 1969). Each virus was therefore exposed to concentrations of urea of 0, 4 M and 6 M, and of 2 M, 4 M and 6 M guanidine, alone and in combination with 1/800 dithiothreitol.

In addition the action of mercuric chloride was tested by exposing virus dilution preparations to 1/10,000 $HgCl_2$ at pH 6·0 for 2 hr. at 37° C.

RESULTS

In contrast to the results obtained with reagents reacting directly with amino acids in the active centres of the virus haemagglutinin and neuraminidase, very

striking differences were observed between individual strains of virus and also between haemagglutinin and neuraminidase as a result of treatment with reagents acting on the higher-order structure of the protein molecule.

Exposure to high concentrations of urea disrupts hydrogen bonds. With concentrations of less than 4 M there was no visible disruption of the virus particle and no effects were produced on the virus properties, but with 4 M urea the virus preparations became less opalescent indicating some disruption of the particles and the opalescence completely cleared with 6 M urea. Guanidine is a more powerful reagent disrupting non-covalent bonds and significant effects were produced by concentrations as low as 2 M. The opalescence of concentrated virus preparations was completely cleared by 4 M guanidine.

Table 1. *Effect of reagents disrupting the higher-order structure
of the virus protein on the virus haemagglutinin*

(4 = complete destruction of haemagglutinin; 3, 2, 1 = intermediate degrees
of haemagglutinin destruction; 0 = no action.)

	A			A_1		A_2		B
Reagent	SWINE	PR 8	DSP	BURCH	BRATI-SLAVA	TAIWAN	ENG/67	LEE
4 M urea	0	0	0	0	0	0	0	0
6 M urea	2	4	0	0	0	0	2	0
4 M urea + 1/800 DTT	1	0	1	0	1	0	0	0
6 M urea + 1/800 DTT	4	4	4	4	4	0	1	4
2 M guanidine	4	4	0	0	0	4	4	0
4 M guanidine	4	4	0	0	0	4	4	0
6 M guanidine	4	4	0	0	0	4	4	0
2 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
4 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
6 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
Dithiothreitol (DTT) 1/800	0	0	0	0	0	0	0	0
Mercuric chloride 1/10,000	0	0	0	0	0	0	0	0

The results of treatment of eight strains of virus with these reagents is shown in Tables 1 and 2. The haemagglutinin titre and neuraminidase activity of all eight strains was completely unaffected by dithiothreitol alone, suggesting that the disulphide bonds are inaccessible to attack by this reagent. Haemagglutinin titres were not significantly reduced by mercuric choloride but with 6 of the 8 strains the neuraminidase activity was completely destroyed. The A_2 strains of virus were remarkable in the possession of a neuraminidase that was completely resistant to mercuric chloride.

With three strains of virus—the DSP strain of virus A and the two A_1 strains— there was a very considerable difference in sensitivity between haemagglutinin and neuraminidase. The neuraminidase activity of these strains was completely destroyed by mercuric chloride and by all the concentrations of urea and guanidine used. The haemagglutinins of these three strains were completely resistant to mercuric chloride and to urea and guanidine, but were destroyed by combinations

of guanidine and dithiothreitol and also by 6 M urea + dithiothreitol. It appears that with these strains neuraminidase activity is destroyed by any interference with the higher-order structure of the virus protein, but that the haemagglutinin is unaffected by reagents acting on non-covalent bonds and is also unaffected by mercuric chloride which introduces a mercury atom into the disulphide bridges without disrupting the bond. Haemagglutinin is only destroyed as a result of the complete disruption of the disulphide bonds by dithiothreitol in combination with urea or guanidine.

Table 2. *Effect of reagents disrupting the higher-order structure*
of the virus protein on neuraminidase activity

(4 = complete destruction of neuraminidase activity; 3, 2, 1 = intermediate degrees of neuraminidase destruction; 0 = no action.)

| | A | | | A_1 | | A_2 | | B |
Reagent	SWINE	PR 8	DSP	BURCH	BRATI-SLAVA	TAIWAN	ENG/67	LEE
4 M urea	4	4	4	4	3	0	0	0
6 M urea	4	4	4	4	4	2	2	0
4 M urea + 1/800 DTT	4	4	4	4	4	1	0	2
6 M urea + 1/800 DTT	4	4	4	4	4	4	4	4
2 M guanidine	4	4	4	3	3	4	4	2*
4 M guanidine	4	4	4	4	4	4	4	2*
6 M guanidine	4	4	4	4	4	4	4	4
2 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
4 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
6 M guanidine + 1/800 DTT	4	4	4	4	4	4	4	4
Dithiothreitol (DTT) 1/800	0	0	0	0	0	0	0	0
Mercuric chloride 1/10,000	4	4	4	4	4	0	0	4

* Two types of sub-unit present; one sensitive to guanidine and one resistant

The neuraminidases of the SWINE and PR 8 strains of virus A were also completely destroyed by all reagents acting on the higher-order structure of the virus protein. The haemagglutinin of these strains was highly sensitive to guanidine, but showed some resistance to urea, being unaffected by 4 M urea but reduced or destroyed by 6 M urea. Combinations of urea and dithiothreitol were only slightly more destructive than urea alone and mercuric chloride had no effect.

The remarkable sensitivity of the neuraminidase of the A and A_1 strains to urea and guanidine suggested the possibility that urea and guanidine did not actually destroy neuraminidase but split it off the virus particle in a form which was not adsorbed by red cells and therefore was not detectable in the elution test. Fortunately urea and guanidine do not significantly interfere with the estimation of neuraminic acid by the Aminoff (1961) technique so that it was possible to test for the presence of a non-haemagglutinating neuraminidase by the use of the Aminoff method.

Preparations of DSP virus and the A_1 BRATISLAVA virus in volumes of 0·05 ml. were exposed to 4 M urea and to 4 M guanidine for 30 min. at 20° C. They were then diluted to 1·0 ml. with buffer pH 6·0. Control preparations of untreated virus and

virus treated with diluted urea or guanidine were also set up. To all preparations 0·4 ml. of packed guinea-pig red cells was added as a substrate for neuraminidase action and the mixtures incubated at 37° C. for 30 min. The cells were then centrifuged out and the concentration of N-acetylneuraminic acid in the supernatant fluids measured. Results are shown in Table 3. With the DSP virus neuraminidase activity was unaffected by treatment with diluted urea but was greatly reduced by 4 M urea. The activity was completely destroyed by 4 M guanidine and was even slightly reduced by diluted guanidine. The neuraminidase activity of the A₁ BRATISLAVA strain was destroyed by 4 M urea or guanidine and was reduced by diluted guanidine and very slightly affected by diluted urea. These results show that urea and guanidine actually destroy neuraminidase and do not merely split it off the virus particle.

Table 3. *Action of urea and guanidine on the neuraminidase activity of A DSP and A₁ BRATISLAVA viruses*

(Effects tested by determining the amount of N-acetylneuraminic acid released from 0·4 ml. of guinea-pig red cells in 30 min. at 37° C. The figures given are absorptiometer readings at 549 μ.)

	Virus	
	A DSP	A₁ BRATISLAVA
Untreated virus control	0·205	0·240
Virus + diluted urea	0·200	0·200
Virus + 4 M urea diluted after 30 min. at 20° C.	0·035	0·000
Virus + diluted guanidine	0·160	0·135
Virus + 4 M guanidine diluted after 30 min. at 20° C.	0·000	0·005

With the A₂ strains there was much less difference in sensitivity between haemagglutinin and neuraminidase. Both activities were resistant to mercuric chloride and sensitive to guanidine. The neuraminidase of the A₂ TAIWAN strain was partially sensitive to urea while the haemagglutinin was resistant, and the neuraminidase of the A₂ ENG/67 strain was slightly more sensitive to urea + dithiothreitol than the haemagglutinin.

With the LEE strain of virus B also the reactions of haemagglutinin and neuraminidase were very similar. The haemagglutinin was resistant to urea and guanidine and to mercuric chloride but was highly sensitive to dithiothreitol in combination with urea or guanidine. The neuraminidase was also resistant to urea and highly sensitive to dithiothreitol in combination with urea or guanidine and was also sensitive to mercuric chloride. Treatment with guanidine alone led to an unusual type of result. With the LEE strain 2 M guanidine produced a considerable increase in haemagglutinin titre indicating disruption of the virus particle. When preparations treated with 2 M and 4 M guanidine were tested for neuraminidase activity in the elution test it was found that some of the adsorbed haemagglutinin eluted very rapidly, but the rest remained attached to the cells and at the end of the elution period, when the control preparation was completely eluted, the cells in

the guanidine-treated preparations still showed a fine agglutination and this agglutination was increased by addition of fresh virus. It appeared that there were two types of enzymic sub-unit present in the virus particle, one sensitive to guanidine and one resistant to it, and it seemed possible that these were reacting with different substrate molecules in the red cell since the guanidine-resistant enzyme was not able to release the guanidine-sensitive haemagglutinating particles from combination with red cells. This type of result has only been seen with the LEE virus.

DISCUSSION

The results described above suggest that the neuraminidase activity of A and A_1 strains of influenza virus is a function of a higher-order of structure of the protein molecule than is the haemagglutinating activity. There is much evidence that the protein of the outer component of the influenza virus particle can exist in a number of different structural states. Jagger & Pollard (1956), from studies of the resistance of virus properties to irradiation by deuterons, α-particles and electrons, calculated the haemagglutinin in the intact virus to have a molecular weight of 190,000. Mayron, Robert, Winzler & Rafaelson (1961) first showed that neuraminidase could be separated from the virus particle by trypsin treatment and Noll, Aoyagi & Orlando (1962) showed that the neuraminidase separable from LEE virus had a sedimentation constant of 9S corresponding to a molecular weight of 190,000. Laver (1963) separated a neuraminidase of sedimentation constant 9S from LEE virus by treatment with dodecyl sulphate, but similar treatment of A viruses destroyed the neuraminidase. This result agrees with the finding in the present paper that the neuraminidase of A viruses is destroyed by agents reacting on non-covalent bonds while the LEE virus possesses a neuraminidase resistant to these agents. Reginster (1966a, b) separated neuraminidase from PR 8 virus by treatment with caseinase C or pronase and found it to have a sedimentation constant of 6S, but in later work (Reginster, 1968) a value of 8S was found. Seto, Drzeniek & Rott, (1966) separated a neuraminidase of 8·8S from A_2 virus by pronase treatment, and Drzeniek, Seto & Rott (1966) obtained neuraminidases of S value 9–10 from A_2 and fowl-plague viruses. Reginster (1968) showed that treatment of PR 8 virus with caseinase C resulted in loss of the surface projections and the residual particles no longer agglutinated red cells and were not adsorbed by them.

The released neuraminidases do not agglutinate red cells and are either not adsorbed or adsorbed by them only with great difficulty. As it is difficult to believe in enzymes which do not unite with their substrate the probable explanation of the failure of the isolated neuraminidases to agglutinate red cells is that they carry only one active centre and are therefore unable to bridge red cells. Such a monovalent neuraminidase will be very difficult to adsorb on red cells. An intact virus particle carrying some 400–500 projections may be adsorbed by a single red cell receptor, but 400–500 receptors will be needed to adsorb the released separate projections.

Although the released neuraminidases do not agglutinate red cells they may act

as a blocking antigen in haemagglutination-inhibition tests (Reginster 1968) and antisera to them may inhibit haemagglutination (Rafaelson, Wilson, & Schneir, 1962; Reginster, 1968) though not apparently in all cases (Drzeniek *et al.* 1966).

It is very probable that the molecular weight of 190,000 represents the molecular weight of one of the surface projections on the virus particle and that the released neuraminidases of sedimentation constant 8–10S are single projections. The virus protein can, however, be disintegrated into smaller units.

Disruption of viruses by dodecyl sulphate results in the release of protein sub-units of sedimentation constant 3·4S which have no enzymic activity and do not agglutinate red cells (Laver, 1963). By treatment of WS virus with deoxycholate and ether Hobson (1966) produced a sub-unit which did not agglutinate red cells but which was active as a serum-blocking agent in haemagglutination-inhibition tests and also induced haemagglutinin-inhibiting antibody in guinea-pigs. Eckert (1966*a*, *b*, 1967) extracted the lipid from PR 8 virus and then treated the protein either with 8 M urea + dithiothreitol or with 67 % acetic acid. In each case the protein was disrupted to sub-units with a sedimentation constant of 2S which reassociated to a 4S unit. The 4S units produced by urea-DTT treatment had no enzymic activity and did not agglutinate red cells, but reacted as a blocking antigen in complement fixation tests. Treatment with dithiothreitol produces irreversible disruption of disulphide bonds and prevents restoration of the tertiary structure of the protein molecule. The sub-units produced by acetic acid treatment reassociated to give a product which agglutinated red cells in the cold and could be adsorbed by red cells if a sufficiently large amount of cells was used. It had no neuraminidase activity.

Relation between haemagglutinin and neuraminidase

Many workers have suggested that haemagglutinin and neuraminidase are separate protein molecules, and Laver & Kilbourne (1966) produced a recombinant strain X 7 from the NWS strain of virus A and the A_2 (R 1) 5 strain which apparently contained the haemagglutinin of the A parent and the neuraminidase of the A_2 strain.

There is, however, reason to believe that while haemagglutinin and neuraminidase are certainly not precisely identical they are more closely related than would be expected if they were entirely distinct proteins. Both are associated with the surface projections, both unite with the same substrate molecule and both appear to depend for their activity on the presence of active centres containing the same amino acids. All chemical reagents which destroy haemagglutinin also destroy neuraminidase. Neuraminidase activity is, however, more sensitive to destruction by heat and chemical reagents than haemagglutinin

The following hypothesis is advanced to explain the relation between haemagglutinin, neuraminidase, and specific antigen. The surface projections on the virus particle are protein polymers of sedimentation constant 8–10S and a molecular weight of about 190,000. Each polymer is made up of three or four monomers of sedimentation constant 4S and a molecular weight of 50,000–60,000. Each of the monomers carries an active centre capable of uniting with red cell receptors, but

neuraminidase activity is only developed when the active centres of the monomers become closely associated in the polymer. Neuraminidase activity is therefore a function of the quaternary structure of the projections while the haemagglutinating activity is a function of the tertiary structure of the monomers. Antigenic activity resides in the primary or secondary structure of the monomers which represent the various components of the V antigen complex. On this basis the results of treatment of virus strains with agents acting on the higher-order structure is interpreted as follows.

(1) With the A and A_1 strains the quaternary structure of the polymer is maintained by hydrogen bonds between the monomers, and neuraminidase activity is destroyed by 4 M urea and by guanidine. The haemagglutinin of the two A_1 strains and the DSP strain of virus A is resistant to both urea and guanidine and is only destroyed by combinations of these agents with dithiothreitol. The tertiary structure of the monomers in these strains is therefore maintained primarily by disulphide bonds.

The haemagglutinin of the SWINE and PR 8 strains is destroyed by urea + dithiothreitol but is also very sensitive to guanidine, so that in these strains both disulphide bonds and guanidine-sensitive urea-resistant bonds are needed to maintain the tertiary structure. This guanidine-sensitive bond may possibly be an ionic bond between amino acids with basic and acidic side-chains.

Neuraminidase activity is not only sensitive to disruption of the quaternary structure but is also highly sensitive to modifications of the tertiary structure of the monomers. Thus even the introduction of a mercury atom into the disulphide bonds produces enough distortion of the active centre to destroy neuraminidase activity.

(2) In the A_2 and LEE viruses the quaternary structure of the polymers is maintained by stronger bonds than with the A and A_1 strains. Neuraminidase activity is not destroyed by 4 M urea and Laver (1963) found that while treatment of A viruses with dodecyl sulphate produced sub-units of sedimentation constant 3·4S, with the LEE virus it only produced 9S sub-units.

The haemagglutinin of the A_2 strains is resistant to urea + dithiothreitol but is highly sensitive to guanidine, suggesting that the tertiary structure is maintained by guanidine-sensitive bonds. The neuraminidase of A_2 strains is guanidine sensitive and is resistant to 4 M urea + dithiothreitol, but is destroyed by 6 M urea + dithiothreitol and is slightly sensitive to 6 M urea alone. The quaternary structure may be maintained by a combination of hydrogen and disulphide bonds. The A_2 strains, however, are unique in the possession of a neuraminidase which is resistant to mercuric chloride. This would suggest that disulphide bonds are not involved in the tertiary structure and that introduction of a mercury atom into the disulphide bond does not disrupt the quaternary structure.

The haemagglutinin of the LEE virus is completely resistant to urea and guanidine but destroyed by combination of these agents with dithiothreitol, indicating that the tertiary structure is maintained by disulphide bonds. Neuraminidase activity is also sensitive to urea + dithiothreitol and to mercuric chloride but is resistant to urea. It is destroyed by high concentrations of guanidine and is

partially sensitive to low concentrations. The quaternary structure may be maintained by guanidine-sensitive bonds, but there is some evidence that there are two types of neuraminidase, one sensitive and one partially resistant to guanidine.

Table 4. *Classification of influenza viruses by the chemical reactions of their haemagglutinins*

($+$ = haemagglutinin sensitive to reagent; $-$ = heamagglutinin not sensitive to reagent.)

Group	Strain	Fluoro-dinitro-benzene, pH 8·0	Iodine, pH 6·0, 0° C.	Urea	Guanidine	Urea + dithio-threitol	Mercuric chloride
1	A SWINE	--	$-$	$-$	$+$	$+$	$-$
	A PR 8	$-$	$-$	$-$	$+$	$+$	$-$
2	A DSP	$-$	$-$	$-$	$-$	$+$	$-$
3	A_1 BURCH	$+$	$-$	$-$	$-$	$+$	$-$
	A_1 BRATISLAVA	$+$	$-$	$-$	$-$	$+$	$-$
4	A_2 TAIWAN/64	$+$	$-$	$-$	$+$	$-$	$-$
	A_2 ENG/67	$+$	$-$	$-$	$+$	$-$	$-$
	B LEE	$+$	$-$	$-$	$-$	$+$	$-$

Table 5. *Classification of influenza viruses by the chemical reactions of their neuraminidases*

($+$ = neuraminidase sensitive to reagent; $-$ = neuraminidase not sensitive to reagent.)

Group	Strain	Fluoro-dinitro-benzene, pH 8·0	Iodine pH 6·0, 0° C.	Urea	Guanidine	Urea + dithio-threitol	Mercuric chloride
1	A SWINE	$+$	$+$	$+$	$+$	$+$	$+$
	A PR 8	$+$	$+$	$+$	$+$	$+$	$+$
2	A DSP	$+$	$-$	$+$	$+$	$+$	$+$
	A_1 BURCH	$+$	$-$	$+$	$+$	$+$	$+$
	A_1 BRATISLAVA	$+$	$-$	$+$	$+$	$+$	$+$
3	A_2 TAIWAN/64	$+$	$-$	$-$	$+$	$+$	$-$
	A_2 ENG/67	$+$	$-$	$-$	$+$	$+$	$-$
	B LEE	$+$	$+$	$-$	$\pm$	$+$	$+$

Chemical classification of influenza virus strains

The virus strains used in the present work can be classified into groups by means of the chemical reactions shown by their haemagglutinins and neuraminidases (Tables 4, 5). The A virus haemagglutinins fall into four groups; SWINE + PR 8, A (DSP), A_1, and A_2, while the neuraminidases fall into three groups: SWINE + PR 8, A (DSP) + A_1, and A_2. Paniker (1968), studying the serological relationships of the haemagglutinins and neuraminidases of strains of influenza viruses, also found that the haemagglutinins fall into four groups: SWINE, A_0, A_1, and A_2, while the neuraminidases fall into three groups; SWINE, $A_0 + A_1$, and A_2. The results of chemical and serological classifications are therefore very similar, the only difference being that the PR 8 strain was chemically indistinguishable from SWINE.

SUMMARY

The results of treatment of influenza virus strains with chemical reagents acting on the higher-order structure of protein molecules shows that both the haemagglutinating and enzymic activities are susceptible to these agents but there are considerable differences between the different strains and the neuraminidase activity is more sensitive than the haemagglutinating activity.

The neuraminidase activity of A and A_1 strains is destroyed by urea, guanidine, urea + dithiothreitol and mercuric chloride. The haemagglutinin of the PR 8 and SWINE strains is resistant to urea and mercuric chloride but destroyed by guanidine and by urea + dithiothreitol. The haemagglutinin of the DSP strain of virus A and the A_1 strains is resistant to urea, guanidine and mercuric chloride but is destroyed by urea + dithiothreitol.

The neuraminidase activity of the A_2 strains is more resistant than that of the A and A_1 strains. It is resistant to mercuric chloride and partially resistant to urea but is destroyed by guanidine and by urea + dithiothreitol. The A_2 haemagglutinin is resistant to urea, urea + dithiothreitol, and mercuric chloride but is destroyed by guanidine.

The LEE virus neuraminidase is resistant to urea and partially resistant to guanidine but is destroyed by urea + dithiothreitol and mercuric chloride. The LEE haemagglutinin is resistant to urea, guanidine and mercuric chloride but is destroyed by urea + dithiothreitol.

It is suggested that the surface projections of the virus particle are protein polymers each made up of three or four monomers which are the components of the V antigen complex. Antigenic activity is a function of the primary or secondary structure of the monomers, haemagglutinin activity is a function of the tertiary structure of the monomers, while neuraminidase activity is a function of the quaternary structure of the polymer.

From studies of the chemical reactions of their haemagglutinins and neuraminidases strains of influenza virus A can be classified into groups. These groups are very similar to but not precisely identical with groupings made by serological methods.

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