J. Hyg., Camb. (1969), **67**, 289
Printed in Great Britain

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

I. Effect of reagents reacting with amino acids in the active centres

By L. HOYLE
Public Health Laboratory, Northampton

(*Received* 18 *September* 1968)

The results of disintegration of influenza virus particles by ether treatment, and studies of the effects of proteolytic agents on the particles, show that the haemagglutinating, enzymic, and specific complement-fixing activities are closely related properties of the outer component of the virus particle, being associated with the surface projections. Some activity is probably also present in the interior of the particle. There are serological differences between the specific complement fixing antigens of different strains of virus, differences in the ability to agglutinate different types of red cell and in susceptibility to different mucoprotein inhibitors of haemagglutination, and differences in the enzymic activity against different substrates. By treatment of virus with chemical reagents acting on the reactive groups of protein molecules it was shown by Hoyle & Hana (1966) that there were differences in the chemical structure of the active centres of the haemagglutinins and neuraminidases of the DSP strain of virus A and the LEE strain of virus B. This work has now been extended to a range of strains of virus A.

Chemical reagents used in the work fall into two groups. The first consists of reagents which probably owe their activity to direct reaction with amino acids present in the active centres of the virus haemagglutinin or neuraminidase. The second group consists of reagents which do not act directly on the active centres but which alter the higher order structure of the protein molecule by reaction with hydrogen or other non-covalent bonds or with disulphide bridges. The results with the two groups of reagents were very different and they will be considered separately. This paper deals with reagents acting on the active centres, while the following paper (Hoyle, 1969) describes the results obtained with reagents of the second group.

METHODS

Virus preparations

Highly purified virus preparations were used in the work. Infected egg allantoic fluids were subjected to two cycles of adsorption–elution from guinea-pig red cells, concentrating $\times 15$, followed by a cycle of differential centrifugation. The final deposited virus was suspended in phosphate-buffered saline of pH 7·0 to a concentration of about 1 % by volume. This material is referred to as 'virus concentrate'.

For use in most of the tests the concentrate was diluted 1/10 with saline—'virus dilution'. In addition virus dilution preparations were disintegrated by shaking with ether at room temperature. After removal of ether and centrifugation to remove precipitated lipoprotein the supernatant 'ether-treated virus dilution' was used in the tests. The haemagglutinin titre of virus dilution preparations ranged from 8000 to 16,000 with predominantly spherical strains such as DSP or PR 8 to from 2000 to 8000 with filamentous strains. Ether-treated preparations were less variable, most giving titres of 16,000–32,000.

Eight strains of virus have been used in the work, the SWINE, PR 8, and DSP strains of virus A; two strains of A_1 virus, A_1 BURCH isolated in Northampton in 1947 and a Czechoslovak strain A_1 BRATISLAVA; two strains of A_2 virus, A_2/TAIWAN/64 and A_2/ENGLAND/67; and the LEE strain of virus B.

Conditions for chemical reaction

Virus dilution and ether-treated virus dilution preparations in volumes of 0·4 ml. were mixed with an equal volume of chemical reagent dissolved in buffered saline of suitable pH, 0·5% phosphate buffered saline being used for pH 5–8·0, and 0·5% borate saline for pH 9·0. Reagent-free controls were set up and exposed to the same pH and temperature for the same duration as the test samples.

After interaction with reagent the preparations were diluted to 2·0 ml. with buffered saline, pH 6·0, and immediately tested for haemagglutinin titre and neuraminidase activity.

Haemagglutinin titrations

Haemagglutinin was titrated by the Salk method with 0·5% guinea-pig red cells at pH 6·0, preparations being tested in dilutions from 1/32 to 1/65, 536.

Test for neuraminidase activity

Many of the chemical reagents used in the work interfere with the estimation of neuraminic acid by the Aminoff (1961) method and for this reason neuraminidase activity was tested by determining the ability of the treated virus to elute from red blood cells and to destroy the cell receptors.

After estimation of haemagglutinin titre the diluted virus-reagent mixtures were chilled to 4° C. and adsorbed with 0·05 ml. of a 1/3 dilution of packed guinea-pig red cells. The cells were then centrifuged out and resuspended in 1·0 ml. of buffered saline, pH 6·0, and incubated at 37° C., shaking at intervals of 10 minutes. At two intervals the tubes were centrifuged and the haemagglutinin titre of the supernatants measured. The first test was made as soon as the control preparation showed any sign of elution as indicated by a reduced rate of sedimentation of the red cells, and the second test was made after a four times longer period of elution. Thus with strains of virus which eluted rapidly tests were made at 20 and 80 min., with more slowly eluting strains at 30 and 120 min. while with very slowly eluting strains tests were made at 45 and 180 min.

The amount of virus used in the tests was much more than sufficient to saturate completely the red cells used in the elution test, so that neuraminidase activity

could be assessed provided the chemical reagent used did not reduce the haemagglutinin titre to less than 25% of the original. But if the haemagglutinin was destroyed then none could be adsorbed or eluted. In such cases the elution test was carried out as if haemagglutinin was present and at the end of the elution period the red cells were centrifuged out and tested for agglutinability by fresh virus in a simple slide test. In all such cases it was found that the cells remained agglutinable, indicating that destruction of haemagglutinin also destroyed enzymic activity.

Design of experiments and assessment of results

Reaction of chemical reagents with the virus strains was assessed in two types of experiment. In the first a single strain of virus was tested simultaneously against a range of different reagents, and in the second several strains of virus were tested simultaneously against the same reagent. In each case both virus dilution and ether-treated virus dilution preparations were used. The activity of each reagent against each virus was thus tested in at least four different experiments. The results with intact and ether-disrupted virus were the same with reagents acting at pH 9·0 as at this pH the virus particles tend to be slowly disrupted, but at more acid reactions the two types of preparation showed slight differences in sensitivity, haemagglutinin of intact virus being more resistant than that of ether-treated virus, while neuraminidase activity was more resistant to attack in the ether-treated than in the intact virus preparations. These differences are explained in the following way. Intact virus particles carry several hundred surface projections and to prevent such a particle agglutinating red cells it might be necessary to destroy over 99% of the haemagglutinin. The haemagglutinin particles in the ether-treated preparations carry a much smaller number of haemagglutinating units per particle and a smaller percentage reduction will be needed to prevent bridge formation between red cells. Neuraminidase activity of intact virus will be destroyed if the surface projections alone are inactivated, but on ether treatment internal neuraminidase is released and the enzyme is distributed over a much larger number of separate particles and may be more efficient. Ether-treated preparations frequently elute more rapidly in the elution test than intact virus.

In addition to these differences it is important to realize that chemical reaction with the active centre is more easily demonstrated in the case of the neuraminidase than with the haemagglutinin. A 50% reduction in neuraminidase activity is detectable in the elution test, but a much greater destruction of haemagglutinin is necessary to prevent bridge formation between red cells.

From the mean results of four or more experiments the activity of each reagent against each virus was assessed and given a value from 0–4. In the case of haemagglutinin titrations the significance of the values is as follows:

0 = no reduction in haemagglutinin titre;
1 = reduction of titre to $\frac{1}{4}$ of the control;
2 = reduction of titre to $\frac{1}{16}$ of the control;
3 = reduction of titre to $\frac{1}{64}$ of the control;
4 = total destruction of haemagglutinin.

With the test for neuraminidase activity:

0 = haemagglutinin titres in the elution tests the same as in the control;
1 = reduced titre at the first elution test with complete elution at the second test;
2 = greatly reduced titre at the first test and some reduction at the second test;
3 = no elution at the first test and reduced titre at the second test;
4 = total failure to elute and complete sensitivity of the red cells used to agglutination by fresh virus.

Chemical reagents

Previous studies (Hoyle & Hana, 1966) did not suggest that reagents reacting with carboxyl or alcoholic hydroxyl groups could be usefully employed in the present work. Reagents reacting with —COOH groups precipitate the virus and destroy all virus properties. Reagents reacting with alcoholic —OH groups are almost all non-specific and results obtained with them were attributed to action on other groups. The following reagents have been used under the conditions described.

Sodium dichromate. Preparations were exposed to dichromate in a concentration of 1/10,000 at pH 6·0 for 4 hr. at 37° C. The reagent reacts specifically with the sulphydryl group —SH of cysteine.

Iodacetamide. Reacts specifically with the SH group when used in a concentration of 1/1000 at pH 6·0 for 4 hr. at 37° C.

Acrylonitrile. Used in a concentration of 1/200 at pH 7·0 for 2 hr. at 37° C. Reacts with —SH and amino groups (McKinney, Uhing, Setzkorn & Cowen, 1950).

Phenylisothiocyanate. Reacts primarily with amino groups. Virus preparations were treated at pH 9·0 for 18 hr. at 4° C. The reagent was added to virus–buffer mixtures in solution in 0·05 ml. of ether and shaken to give an emulsion with a final concentration of 1/1000. Control preparations were treated with reagent-free ether. This reagent could only be used in studies of the action on haemagglutinin as it rapidly lysed the red cells used in the elution test and this interfered with assessment of neuraminidase activity.

β propiolactone. Used in a concentration of 1/800 at pH 8·0 for 2 hr. at 37° C.; 2 % sodium acetate was added to give extra buffering. Reacts with amino groups and slightly with the —NH group of histidine.

Formaldehyde. Reacts rapidly with amino groups. Prolonged exposure to formaldehyde results in the formation of methylene bridges between amino and amide or guanidyl groups (Fraenkel-Conrat & Olcott, 1948). Preparations were tested under three different conditions. (*a*) Treatment with 1/1000 formaldehyde at pH 7·0 for 1 hr. at 37° C. Only amino groups react. (*b*) Treatment with 1/1000, and (*c*) with 1/100 formaldehyde at pH 7·0 for 24 hr. at 37° C. (under these conditions it was expected that amide and guanidyl groups would react in addition to amino groups).

Glyoxal. Used in a concentration of 1/200 at pH 8·5 for 4 hr. at 37° C. Reacts powerfully with amino groups and less actively with the guanidyl group of arginine (Nakaya, Norinishi & Shibata, 1967).

Xanthydrol. Reacts with amide and guanidyl groups at acid reactions. Reagent was added to virus–buffer mixtures in solution in 0·05 ml. of methanol and the mixture shaken to produce an emulsion with a final concentration of 1/1000. Controls were treated with reagent-free methanol. Preparations were treated at pH 5·5 for 4 hr. at 37° C. The reagent was more active at pH 5·0 but some of the virus strains were susceptible to acid at this reaction. Only ether-treated virus preparations gave satisfactory results; variable results were obtained with intact virus apparently because the reagent produced a slow disruption of the virus particle with release of internal haemagglutinin.

Iodine. Iodine at pH 6·0 reacts with cysteine, methionine, tryptophan, tyrosine, histidine, cystine and proline, but there are very great differences in the rate of reaction. In a concentration of 1/10,000 at 0° C. iodine reacts instantly with cysteine and methionine and in a few seconds with tryptophan. Tyrosine reacts more slowly with a half-life of some 20 min. Histidine reacts very slowly with a half-life of over 24 hr. Cystine and proline do not react at 0° C. At 37° C. cysteine, methionine, tryptophan and tyrosine react rapidly and histidine within 1 hr. Cystine and proline react much more slowly. Iodine was used at pH 6·0 in a concentration of 1/10,000 (dilutions prepared from a stock solution of 1/1000 iodine in 1 % KI) under three conditions:

(*a*) At 0° C. for 2 min.—Reaction with the SH group of cysteine, the CH_3S group of methionine and the indole ring of tryptophan.

(*b*) At 0° C. for 1 hr.—reaction also with the phenol ring of tyrosine.

(*c*) At 37° C. for 1 hr.—reacts also with the imidazole ring of histidine.

After interaction the iodine was neutralized with thiosulphate. The controls were treated with iodine previously neutralized with thiosulphate.

2,4-*dinitro*-1-*fluorobenzene* (*FDNB*). The activity of this reagent increases with increasing pH. Sulphydryl and amino groups react rapidly and the —NH group of histidine and the phenolic hydroxyl group of tyrosine more slowly. Because of the reaction with amino groups it was not possible to determine directly the rates of reaction with histidine and tyrosine but studies were made using iminazole and phenol and these indicated that at pH 8·0 the —NH group reacted more rapidly than the phenolic hydroxyl group. At pH 9·0 both groups reacted rapidly. In an attempt to differentiate between action on histidine and tyrosine the reagent was used at both pH 8·0 and 9·0. In each case the reagent was added to the virus–buffer mixture in solution in ether and the mixture was shaken to give an emulsion with a final concentration of 1/800. The mixtures were held at 20° C. for 30 min. followed by 18 hr. at 4° C. Controls were treated with reagent-free ether.

At pH 8·0 it was considered that effects produced would be due mainly to action on the —NH group of histidine (apart from reaction with —SH and amino groups). No significant hydrolysis of the reagent occurred at pH 8·0. At pH 9·0 the mixtures developed a yellow colour indicating hydrolysis of the reagent and it was considered that at pH 9·0 both the histidine NH group and the tyrosine OH group would react.

Diazotized sulphanilic acid. Reacts with histidine, tyrosine and tryptophan. It was used in a concentration of 1/5600 at pH 9·0 for 18 hr. at 0° C.

RESULTS

The haemagglutinin titre and enzymic activity of all the virus strains tested were completely unaffected by treatment with dichromate, iodacetamide or acrylonitrile. This shows that the SH group is not involved in the interaction of virus with red cells, and that the active centres of the haemagglutinins and neuraminidases do not contain cysteine.

The amino group also is apparently not involved. The haemagglutinin titre of all the virus preparations was unaffected by treatment with acrylonitrile, phenyl isothiocyanate, β-propiolactone, glyoxal, or short periods of treatment with

Table 1. *Action of reagents reacting with aromatic amino acids on the haemagglutinin of influenza virus strains*

(4 = complete destruction of haemagglutinin; 3, 2, 1 = intermediate degrees of reduction of haemagglutinin titre; 0 = no action. Trypt. = tryptophan; tyr. = tyrosine; hist. = histidine; int. = intact virus; E.T. = ether-treated virus.)

	Iodine 1/10,000, pH 6·0						Fluorodinitrobenzene, 1/800, 30 min. at 20° C + 18 hr. at 4° C.			Diazotized sulphanilic acid, 1/5600 pH 9·0, 18 hr. at 0° C.
	0° C.				37° C. 1 hr.					
Probable reacting groups ...	2 min. SH, CH_3S, trypt.		1 hr. SH, CH_3S, trypt., tyr.		SH, CH_3S, trypt., tyr., hist.		pH 8·0 SH, NH_2, NH (hist.)		pH 9·0 SH, NH_2, NH (hist.), OH (tyr.)	Hist., tyr., trypt.
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int. + E.T.
A SWINE	0	0	0	0	1	2	0	0	4	4
A PR 8	0	0	0	0	1	4	0	0	4	4
A DSP	0	0	0	0	2	4	0	0	4	4
A_1 BURCH	0	0	0	0	3	4	2	2	3	4
A_1 BRATISLAVA	0	0	0	0	2	4	2	3	4	4
A_2 TAIWAN	0	0	0	0	1	4	3	2	4	4
A_2 ENG/67	0	0	0	0	2	2	2	1	3	4
B LEE	0	0	0	0	0	2	2	2	4	4

formaldehyde. In studies of neuraminidase activity phenyl isothiocyanate could not be used because of its lytic effect on red cells, but the neuraminidase activity of all the virus strains was unaffected by acrylonitrile, glyoxal or by short periods of formaldehyde treatment. In some experiments a very slight reduction in neuraminidase activity was found on treatment with β-propiolactone, but these effects were probably due to the slight reaction of the reagent with histidine.

Action of reagents acting on the aromatic amino acids

Significant effects were produced by agents reacting with the imino group of histidine, the phenolic hydroxyl group of tyrosine, or with the aromatic rings. Effects of these reagents on the haemagglutinins of the various virus strains are shown in Table 1. None of the haemagglutinins were affected by iodine at 0° C., showing that the —SH group of cysteine, the —CH_3S group of methionine, and tryptophan play no part in haemagglutination. All the haemagglutinins were completely destroyed by diazotized sulphanilic acid, completely or nearly completely destroyed by FDNB at pH 9·0, and were reduced in titre by iodine at 37° C., ether-treated preparations being more sensitive than intact virus. All these reagents

react with both tyrosine and histidine. The results of treatment with FDNB at pH 8·0 show that the virus strains fall into two groups. The haemagglutinin titres of the SWINE, PR 8, and DSP strains of virus A were unaffected by FDNB at pH 8·0, while significant reductions in titre occurred with the A_1 and A_2 strains and with the LEE strain of virus B. The haemagglutinin titres of the A_1 and A_2 strains and the LEE virus were unaffected by 1 hr. exposure to iodine at 0° C., so that it appears that the haemagglutinating centres of these viruses contain histidine but not tyrosine. The haemagglutinin titres of the A strains were unaffected by 1 hr. exposure to iodine at 0° C., which reacts with tyrosine, and were also unaffected by FDNB at pH 8·0 which reacts with histidine, and with these viruses haemagglutinating activity can only be destroyed by agents acting on both tyrosine and histidine.

Table 2. *Action of reagents reacting with aromatic amino acids on the neuraminidase activity of influenza viruses*

(4 = complete destruction of neuraminidase; 3, 2, 1 = intermediate degrees of reduction in activity; 0 = no action. Trypt. = tryptophan; tyr. = tyrosine; hist. = histidine; Int. = intact virus; E.T. = ether-treated virus.)

	Iodine 1/10,000, ph 6·0						Fluorodinitrobenzene, 1/800, 30 min. at 20° C. + 18 hr. at 4° C.			Diazotized sulphanilic acid, 1/5600, pH 9·0, 18 hr. at 0° C.
	0° C.				37° C					
Probable reacting groups ...	2 min. SH, CH_3S, trypt.		1 hr. SH, CH_3S, trypt., tyr.		1 hr. SH, CH_3S, trypt., tyr.		pH 8·0 SH, NH_2, NH (hist.)		pH 9·0 SH, NH_2, NH (hist.), OH (tyr.)	Hist., tyr., trypt.
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int. + E.T.
A SWINE	0	0	1	0	4	4	2	2	4	4
A PR 8	0	0	3	1	4	4	2	3	4	4
A DSP	0	0	0	0	4	4	2	2	4	4
A_1 BURCH	0	0	0	0	4	4	4	4	4	4
A_1 BRATISLAVA	0	0	0	0	4	4	4	4	4	4
A_2 TAIWAN	0	0	0	0	4	4	4	3	4	4
A_2 ENG/67	0	0	0	0	4	3	2	1	4	4
B LEE	0	0	1	1	4	4	2	2	4	4

The results suggest the existence of two types of haemagglutinating centre, one containing histidine and one tyrosine. The SWINE, PR 8 and DSP strains appear to contain both types of centre, but only the histidine-containing centre can be detected in the A_1, A_2 and LEE strains.

The action of these reagents on the neuraminidase activity is shown in Table 2. No reduction in activity of any of the strains was produced by 2 min. exposure to iodine at 0° C., indicating that cysteine, methionine and tryptophan are not present in the active centres. All the neuraminidases were completely destroyed by agents reacting with both tyrosine and histidine. But the results obtained with 1 hr. exposure to iodine at 0° C. and with FDNB at pH 8·0 show that two types of neuraminidase can be detected. The neuraminidase activity of the DSP, A_1 and A_2 strains is reduced or destroyed by FDNB at pH 8·0 but is unaffected by iodine at 0° C., so that the active centres of these strains appear to contain histidine but not tyrosine. With the SWINE, PR 8 and LEE strains neuraminidase activity is reduced both by FDNB at pH 8·0 and by iodine at 0° C. but is only completely

destroyed by reagents acting on both tyrosine and histidine. These strains appear to contain two types of neuraminidase: one containing histidine and one tyrosine.

Action of reagents acting on amide and guanidyl groups

Results obtained with these reagents are shown in Tables 3 and 4. The haemagglutinating and neuraminidase activities of all the strains were unaffected by glyoxal or by 1 hr. exposure to 1/1000 formaldehyde, showing that amino groups

Table 3. *Action of reagents reacting with amino, amide and guanidyl groups on the haemagglutinin of influenza virus strains*

(4 = complete destruction of haemagglutinin; 3, 2, 1 = intermediate degrees of destruction of haemagglutinin; 0 = no action. Int. = intact virus; E.T. = ether-treated virus.)

	Formaldehyde, pH 7, 37° C.								
Probable reacting groups ...	1:1000, 1 hr. Amino		1:1000, 24 hr. Amino, amide guanidyl		1:100, 24 hr. Amino, amide, guanidyl		Glyoxal, 1:100, pH 8·5, 4 hr., 37° C. Amino, guanidyl	Xanthydrol, 1:1000, pH 5·5, 4 hr., 37° C. Amide guanidyl	
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int.	E.T.
A SWINE	0	0	0	1	4	4	0	1	3
A PR 8	0	0	0	0	4	4	0	0	1
A DSP	0	0	0	0	0	4	0	2	3
A_1 BURCH	0	0	0	0	4	4	0	1	3
A_1 BRATISLAVA	0	0	0	2	4	4	0	0	3
A_2 TAIWAN	0	0	0	0	4	4	0	0	3
A_2 ENG/67	0	0	0	1	4	4	0	1	2
B LEE	0	0	0	1	4	4	0	0	2

Table 4. *Action of reagents reacting with amino, amide and guanidyl groups on the neuraminidase activity of influenza virus strains*

(4 = complete destruction of neuraminidase; 3, 2, 1 = intermediate degrees of neuraminidase destruction; 0 = no action. Int. = intact virus; E.T. = ether-treated virus.)

	Formaldehyde, pH 7, 37° C.								
Probable reacting groups ...	1:1000, 1 hr. Amino		1:1000, 24 hr. Amino, amide, guanidyl		1:100, 24 hr. Amino, amide, guanidyl		Glyoxal, 1:100, pH 8·5, 4 hr., 37° C. Amino, guanidyl	Xanthydrol, 1:1000, pH 5·5, 4 hr., 37° C. Amide, guanidyl	
	Int.	E.T.	Int.	E.T.	Int.	E.T.	Int. + E.T.	Int.	E.T.
A SWINE	0	0	2	2	4	4	0	2	3
A PR 8	0	0	3	1	4	4	0	1	3
A DSP	0	0	3	2	3	4	0	3	3
A_1 BURCH	0	0	4	2	4	4	0	1	4
A_1 BRATISLAVA	0	0	4	2	4	4	0	1	4
A_2 TAIWAN	0	0	1	4	4	4	0	2	4
A_2 ENG/67	0	0	0	2	4	4	0	1	2
B LEE	0	0	1	2	4	4	0	2	2

are not involved in the activities. All the strains showed a reduction in both haemagglutinating power and neuraminidase activity as a result of treatment with xanthydrol. Prolonged exposure to 1/1000 formaldehyde reduced the enzymic activity of all the strains but had little effect on haemagglutinin titres, but with 1/100 formaldehyde both haemagglutinins and neuraminidases were destroyed,

except with intact DSP virus which showed some resistance even to 1/100 formaldehyde. These results suggest that an amide group is present in the active centres of both haemagglutinin and neuraminidase in all the virus strains.

It is, however, possible that the effects produced by these reagents are non-specific. The methylene bridges produced by prolonged treatment with formaldehyde may produce steric effects and distort the structure of the protein molecule with a resulting interference with the virus activities even if amide groups are not present in the active centres. Xanthydrol is specific for the amide group but is effective only at reactions close to the iso-electric point of the viruses and also produces a precipitate, so that loss of activity might be due to aggregation of virus or adsorption on the precipitate. However, the most probable explanation of the results is that an amide group is present in all the active centres of haemagglutinin and neuraminidase in all the virus strains. If this is so then the amino acid involved is probably not arginine, as the activities are unaffected by glyoxal, but may be either asparagine or glutamine.

DISCUSSION

The results described above indicate a very considerable similarity in the chemical constitution of the active centres of haemagglutinin and neuraminidase in all the strains of virus tested. The active centres do not appear to contain cysteine, methionine, tryptophan, lysine or arginine, while all the active centres are partially or completely inactivated by action on amide groups and are completely inactivated by agents acting on both tyrosine and histidine. The only differences observed were in the results of treatment with iodine for 1 hr. at 0° C. which reacts with tyrosine but not with histidine, and with FDNB at pH 8·0 which probably reacts mainly with histidine and only weakly with tyrosine.

It would, of course, be expected that the chemical structure of haemagglutinating or enzymically active centres acting on the same substrate molecule would be very similar, and the possibility has to be considered that the centres may in fact all be chemically identical and that the differences observed in experiments with iodine and FDNB were due to non-specific factors such as variations in morphology of different virus strains or differences in the degree of purification attained.

However, the great differences in the rate of elution of the strains from red cells suggests that the enzymes are not identical, as also does the well-known differences in susceptibility to different haemagglutinin inhibitors. Also, in experiments in which strains were tested simultaneously against the same reagent consistent differences in sensitivity were seen; thus in numerous experiments with FDNB the A_1 and A_2 haemagglutinins were always more sensitive than the haemagglutinin of the A strains.

If, therefore, we accept the observed results at their face value we can detect a number of different types of centre. The results with the A_1 and A_2 strains can be explained by supposing that these strains contain a single active centre containing histidine and an amide group but not containing tyrosine, the centre possessing both haemagglutinating and enzymic activity.

With the A and B strains the results indicate the presence of two types of active centre, one containing histidine and one tyrosine. With the SWINE and PR 8 strains both centres appear to possess both haemagglutinating and enzymic activity as haemagglutination can only be prevented by action on both histidine and tyrosine, while neuraminidase activity is reduced by action on either tyrosine or histidine but is only completely destroyed by action on both. With the DSP strain, however, the tyrosine-containing centre has no demonstrable neuraminidase activity. It is possible that a similar centre without neuraminidase activity may also be present in the SWINE and PR 8 strains as these strains elute very slowly from red cells, a finding which is difficult to explain if all the haemagglutinating centres present also had neuraminidase activity.

The LEE strain of virus B also has two centres: one containing histidine and one tyrosine. Both centres have neuraminidase activity and the strain elutes rapidly from red cells. The tyrosine-containing centre of the LEE strain does not appear to play any part in haemagglutination. As it is difficult to believe in the existence of enzymes which do not unite chemically with their substrates one would expect that all neuraminidases present in the intact virus would be potential haemagglutinins, and it may be that the tyrosine-containing centre of the LEE strain also contains histidine so that with this strain haemagglutination can be prevented by action on histidine alone. The neuraminidase activity of all the strains is reduced by FDNB at pH 8·0 and it is possible that histidine is an essential constituent of all neuraminidases, but that tyrosine can substitute for histidine in haemagglutination, so that there exist haemagglutinating centres without neuraminidase activity.

Finally, it must be recognized that the chemical procedures used represent a very crude method of investigating the active centres and that only active centres which are dominant in the virus particle can be demonstrated; minor components would probably be undetectable.

SUMMARY

Studies of the chemical reactions of the haemagglutinins and neuraminidases of eight strains of influenza viruses have been made by the use of chemical reagents reacting with chemically active groups in the protein molecule. The results indicate a close resemblance between the active centres of the haemagglutinins and neuraminidases in all the strains tested. In all cases the activities were unaffected by reagents reacting with the —SH group of cysteine, the —CH_3S group of methionine, the amino group of lysine, the guanidyl group of arginine, or the indole ring of tryptophan. In all cases both the haemagglutinating and enzymic activities were reduced or destroyed by agents reacting with amide groups or reacting with both tyrosine and histidine.

By the use of iodine under conditions in which tyrosine reacts but not histidine, and fluorodinitrobenzene under conditions in which histidine reacts more strongly than tyrosine, it was possible to detect a number of different active centres.

(1) An active centre containing histidine and an amide group but not containing tyrosine was present in all the virus strains and was the only centre detectable in

A_1 and A_2 strains. This type of centre appeared to possess both haemagglutinating and neuraminidase activity.

(2) Active centres containing tyrosine and an amide group were detected in strains of A and B viruses. There was some evidence suggesting that tyrosine-containing centres were of two types: one possessing both haemagglutinating and enzymic activity while the other was a haemagglutinin without neuraminidase activity.

The results could be explained by supposing that the presence of histidine in the active centre was essential for neuraminidase activity and that enzymically active tyrosine-containing centres also contained histidine, but that tyrosine could substitute for histidine in haemagglutinating centres.

REFERENCES

AMINOFF, D. (1961). Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**, 384–92.

FRAENKEL-CONRAT, H. & OLCOTT, H. S. (1948). The reaction of formaldehyde with proteins. Cross linking between amino and primary amide or guanidyl groups. *J. Am. chem. Soc.* **70**, 2673.

HOYLE, L. (1969). 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. *J. Hyg., Camb.* **67**, 301.

HOYLE, L. & HANA, L. (1966). The chemical reactions of influenza virus proteins. *J. Path. Bact.* **92**, 447–60.

MCKINNEY, L. L., UHING, E. H., SETZKORN, E. A. & COWAN, J. C. (1950). Cyanoethylation of α-amino acids. I. Monocyanoethyl derivatives. *J. Am. chem. Soc.* **72**, 2599–603.

NAKAYA, K., HORINISHI, H. & SHIBATA, K. (1967). States of amino acid residues in protein. XIV. Glyoxal as a reagent for discrimination of arginine residues. *J. Biochem., Tokyo* **61**, 345–51.