STRUCTURE OF THE INFLUENZA VIRUS

THE RELATION BETWEEN BIOLOGICAL ACTIVITY AND CHEMICAL STRUCTURE OF VIRUS FRACTIONS

By L. HOYLE

Public Health Laboratory (Medical Research Council), Northampton

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INTRODUCTION

From studies of the growth cycle of influenza virus A in the fertile egg (Hoyle, 1948, 1950) it was concluded that on entry into a susceptible cell the virus elementary body becomes disintegrated into smaller units which multiply in the cell. The elementary bodies which are ultimately released from the infected cell consist of aggregates of these smaller units enclosed in a lipid membrane derived from the wall of the host cell. By treatment of elementary bodies with ether the lipid envelope can be removed and the particle disintegrates into small units of two different types carrying different biological activities. This paper describes the chemical properties of the units into which the elementary body can be disintegrated by ether treatment.

PREPARATION OF ELEMENTARY BODY SUSPENSIONS AND THEIR DISINTEGRATION BY ETHER

Most of the work has been done with the highly egg-adapted D.S.P. strain of influenza virus A. The D.S.P. strain was isolated in 1943 and is serologically distinct from the A prime virus of the 1949 epidemic. Elementary body suspensions were prepared in the following way.

Twelve-day-old fertile eggs were inoculated into the allantoic sac with 0·1 ml.

of infected allantoic fluid diluted 1:100 and incubated at 35° C. for 20 hr. The eggs were then opened and the chorio-allantoic membranes torn through and allowed to bleed into the allantoic fluid. The blood-stained allantoic fluid was collected, chilled on ice and centrifuged. The sedimented red cells carrying the absorbed virus were washed with ice-cold saline and then suspended in saline, 1 ml. per egg, and incubated at 37° C. for 4 hr. to allow elution of the virus. The fluid was then centrifuged and 0·08 % of sodium azide added to the supernatant. This constituted the *original elementary body suspension.*

Ether disintegration of the elementary body was carried out as follows. The suspension was mixed with half its volume of pure ethyl ether, shaken vigorously, and incubated at 37° C. for 2 hr., and fresh ether added at intervals to counter loss by evaporation. The fluid was then centrifuged, removed from the ether layer, and incubated in an open tube overnight to ensure complete removal of ether. A slight precipitate of denatured protein was removed by centrifugation, and the supernatant fluid constituted the *ether-treated elementary body suspension.*

The fluid was then fractionated by adsorption with red cells. Fowl or guinea-pig red cells were added in a concentration of 10 %, allowed to stand 5 min., and centrifuged. The sedimented cells were washed with saline, resuspended in saline and incubated for 6 hr. at 37° C. to allow elution of the adsorbed agglutinin. The cells were then centrifuged out and 0·08 % sodium azide added to the supernatant. This constituted the *agglutinin fraction.*

The supernatant fluid after adsorption of the ether-treated elementary body suspension with red cells was again adsorbed with 10 % red cells to ensure complete removal of agglutinin, and centrifuged. The supernatant fluid constituted the *soluble antigen fraction.*

BIOLOGICAL PROPERTIES OF THE SUSPENSIONS

The original elementary body suspension was infective, agglutinated red cells, contained both specific and group complement-fixing antigens, and showed enzymic activity against fowl and guinea-pig red cells, modifying the cells so that they were no longer agglutinated by fresh virus. All these properties were completely removed from the fluid by adsorption with red cells. Electron microscope studies showed that the suspension contained large numbers of elementary bodies of particle size 1250 Å., but smaller particles were also present (Hoyle, Reed & Astbury, 1952).

Treatment with ether resulted in complete or almost complete loss of infectivity, associated with disintegration of the elementary bodies. The ether-treated suspension showed in the electron microscope large numbers of particles 120 and 250 Å. and a small number 500 Å. in diameter. The agglutinin titre of the ether-treated suspension was higher than that of the original suspension. When titrated by the short complement-fixation technique the ether-treated suspension reacted to the same titre as the original suspension with human convalescent serum containing mainly antibody to the group or soluble antigen, but no longer reacted with a specific ferret serum containing only antibody to the specific antigen.

Adsorption of the ether-treated suspension with red cells resulted in almost complete separation of the agglutinating and complement-fixing properties. The agglutinin was adsorbed by the cells and could be recovered from them by elution, while the complement-fixing antigen was not adsorbed.

The results of a typical fractionation of the elementary body are shown in Table 1.

Table 1. *Fractionation of elementary body suspension*

Material	Infectivity	Agglutinin titre	Complement-fixation titre (short fixation)	
			Specific serum	Group serum
Original elementary body suspension	+ + +	12,800	32	48
Ether-treated elementary body suspension	–	51,200	Nil	48
Agglutinin fraction	–	51,200	Nil	Trace (less than 1·0)
Soluble antigen fraction	–	Nil	Nil	40

Note. The specific complement-fixing serum was the serum of a ferret convalescent from infection with D.S.P. virus. It reacted to a titre of 1:128 with D.S.P. elementary body suspension but reacted to a titre of less than 1:2 with soluble antigen preparation (extract of infected chorio-allantoic membrane from which infective virus and agglutinin had been removed by red cell adsorption).

The group serum was pooled human convalescent serum from the A prime epidemic of 1949. It reacted to a titre of 1:128 with soluble antigen preparations but had little ability to neutralize D.S.P. virus or to inhibit red cell agglutination by it. The D.S.P. strain was isolated in 1943 and is serologically distinct from the A prime virus of the 1949 epidemic.

NATURE AND PROPERTIES OF THE SOLUBLE ANTIGEN

The soluble antigen was first detected in extracts of infected mouse lung by Hoyle & Fairbrother (1937). The antigen is also present in extracts of infected chorio-allantoic membrane, in the allantoic fluid of infected eggs, and in the embryo lungs, amnion and amniotic fluid of eggs inoculated by the amniotic route. Soluble antigen is released when the elementary body is disintegrated by ether. Soluble antigen from all these sources appears to be identical in chemical and serological behaviour and in particle size. The soluble antigens of all strains of influenza virus A appear to be serologically identical (Hoyle, 1945). The antigen is not adsorbed by red cells or by the cells of the chorio-allantoic membrane, and is not infective. Hoyle & Fairbrother (1937) showed that the soluble antigen in infected mouse lung had a particle size smaller than that of the elementary body. Henle & Wiener (1944) estimated the particle size of soluble antigen in infected allantoic fluid at 100–150 Å. by centrifugation methods, and Hoyle *et al.* (1952) showed that soluble antigen derived from the elementary body by disintegration with ether had a particle size of 120 Å. by electron microscopy.

The antigen is heat labile. Preparations of D.S.P. elementary bodies before and after treatment with ether were heated for 20 min. at various temperatures, and then tested for complement-fixing power with human convalescent serum. The

antigen was unaffected at 60° C., was greatly reduced at 65° C. and destroyed at 70° C.

The antigen is stable in the pH range 4·0–10·0, but is destroyed by acid or alkali beyond these extremes. The antigen is strongly adsorbed by filter-paper and by Seitz disks and this adsorption is maximal at pH 5·0.

Soluble antigen from all sources is precipitated by half saturation with ammonium sulphate and can be recovered by dissolving the precipitate in water. The author has made many attempts to purify and concentrate the antigen by serial precipitation with ammonium sulphate. Such procedures have always been associated with a steadily increasing loss of complement-fixing power which is probably not due to destruction of the antigen but to an increasing tendency to aggregation of the particles as the preparations become more pure.

The properties of the soluble antigen are clearly those of a protein. The following experiments indicate that it is a ribonucleoprotein.

(1) *Precipitation with lanthanum acetate*

Nucleoproteins form insoluble complexes with salts of metals of the alkaline earth group.

Soluble antigen preparations were made (*a*) from a saline extract of infected chorio-allantoic membrane, agglutinin having been removed by red cell adsorption, and (*b*) from the elementary body by ether disintegration.

From both preparations the antigen was completely precipitated by addition of an equal volume of 1 % lanthanum acetate, the supernatant fluid after removal of excess lanthanum by precipitation with disodium phosphate having no complement-fixing power.

This precipitation of the antigen was not a simple salt precipitation since the antigen could not be recovered from the precipitate by repeated washing with water. About 60 % of the antigen could be recovered by two successive extractions of the precipitate with 2·5 % disodium phosphate solution. It appears that the antigen forms a specific insoluble complex with lanthanum.

(2) *Digestion with proteolytic enzymes and with nucleases*

The soluble antigen is destroyed by digestion with trypsin or chymotrypsin. A preparation of soluble antigen derived from the elementary body was digested overnight at 37° C. with crystalline trypsin and crystalline chymotrypsin in concentrations of 1:2000 and 1:20,000 at pH 8·5 with the following results:

Control undigested antigen:	Complement-fixation titre, 56
Digested with 1:2000 trypsin:	Complement-fixation titre, less than 1·0
Digested with 1:20,000 trypsin:	Complement-fixation titre, 4
Digested with 1:2000 chymotrypsin:	Complement-fixation titre, 1·5
Digested with 1:20,000 chymotrypsin:	Complement-fixation titre, 6

It will be noted that the amount of enzyme required is large, and total destruction of the antigen is not easily achieved.

The antigen is unaffected by digestion with deoxyribonuclease, but its titre is reduced to 50 % or less by digestion with ribonuclease. The following result is typical. A preparation of soluble antigen derived by ether disintegration of the elementary body was digested for 48 hr. at 37° C. with crystalline deoxyribonuclease and crystalline ribonuclease in concentrations of 1:1000. Digestion at pH 8·5 in the presence of 0·1 % $MgCl_2$ $6H_2O$:

Control undigested antigen:	Complement-fixation titre, 32
Digested with 1:1000 deoxyribonuclease:	Complement-fixation titre, 32
Digested with 1:1000 ribonuclease:	Complement-fixation titre, 14

Samples of soluble antigen from mouse lung and chorio-allantoic membrane were similarly reduced in titre by ribonuclease. More prolonged digestion produced little increase in effect. Two different samples of crystalline ribonuclease from different sources each reduced the titre of soluble antigen preparations to about 40 %. Neither enzyme preparation had any demonstrable proteolytic action on gelatin.

(3) *Effect of ethanol on soluble antigen*

The soluble antigen is completely precipitated by treatment with an equal volume of absolute ethanol at room temperature. The precipitate was denatured and largely insoluble, but some antigenically active material was recoverable from the precipitate by extraction with saline.

Original soluble antigen:	Complement-fixation titre, 80
Treated with 50 % ethanol, Supernatant:	Complement-fixation titre, nil
Treated with 50 % ethanol, Saline extract of deposit:	Complement-fixation titre, 6

Even when the antigen was treated with 90 % ethanol at room temperature some antigen could be recovered from the deposit by extraction with saline.

These results suggest that the soluble antigen is a ribonucleoprotein, and that both the protein and the nucleic acid parts determine its serological behaviour with the protein playing the predominant role. Ethanol denatures the protein but leaves serologically active nucleic acid. Trypsin and chymotrypsin destroy the protein, but some antigenic activity may persist even on prolonged digestion, suggesting that the nucleic acid is serologically active. Lanthanum salts precipitate the antigen by combination with the nucleic acid, but serologically active material can be extracted from the precipitate with disodium phosphate solution. Ribonuclease attacks the nucleic acid but has no effect on the protein part of the antigen.

NATURE AND PROPERTIES OF THE AGGLUTININ

When an elementary body suspension is disintegrated by ether the agglutinin fraction can be adsorbed on red cells and recovered by elution. On examination in the electron microscope the preparation shows a large number of particles of 120 Å. in diameter and also particles of larger size of which one of 500 Å. is common (Hoyle *et al.* 1952). Centrifugation studies indicate that the agglutinating power of the preparation is carried by the particles.

The agglutination of red cells is inhibited by convalescent serum, and this inhibition shows considerable strain specificity. Thus the serum of a ferret convalescent from infection with the D.S.P. strain of virus A powerfully inhibited the agglutination of red cells by D.S.P. virus but had little inhibiting activity against other strains of virus A.

The agglutinin is heat labile, being slightly more sensitive to heat than the soluble antigen. A preparation of D.S.P. agglutinin was unaffected by heating for 20 min. at 55° C., was largely destroyed at 60° C. and completely inactivated at 65° C. The agglutinin was completely destroyed by treatment with an equal volume of absolute ethanol at room temperature. No agglutinin was detectable in the supernatant, and none could be recovered from the precipitate by extraction with saline.

Half saturation with ammonium sulphate precipitates the agglutinin almost completely, and it can be recovered by dissolving the precipitate in water.

These properties suggest that the agglutinin is a protein.

The agglutinin has two major biological properties; it agglutinates red cells and possesses enzymic activity against the red cell receptors, destroying the receptors and rendering the red cells insusceptible to agglutination by fresh virus. By certain methods the enzymic property can be destroyed without affecting the agglutinating power.

Hirst (1948) showed that by heating at 56° C. for 30 min. the ability of virus to elute from red cells could be destroyed while the agglutinating power was retained. A similar effect is produced by partial digestion with trypsin (Stone, 1949).

(1) *Effect of trypsin on the agglutinin*

A preparation of D.S.P. agglutinin was digested at 37° C. for 48 hr. at pH 8·5 with 5 % *Liq. trypsin. co.* A control preparation was incubated without enzyme. The preparations were then adsorbed with 10 % guinea-pig red cells, centrifuged and the agglutinin titre of the supernatant measured. The tubes were then incubated for 1 hr. at 37° C., and the agglutinin titres of the supernatants again measured.

Untreated agglutinin	Original agglutinin titre	102,400
	Titre after adsorption with red cells	256
	Amount of virus adsorbed	99·75 %
	Titre after elution for 1 hr.	102,400
	Amount of virus eluted	100 %
Trypsinized agglutinin	Agglutinin titre	102,400
	Titre after adsorption with red cells	64
	Amount of virus adsorbed	99·94 %
	Titre after elution for 1 hr.	8192
	Amount of virus eluted	12·0 %

Trypsin had no effect on the agglutinin titre of the preparation and the tryptic digested agglutinin was adsorbed by red cells in the same way as untreated agglutinin, but its ability to elute from the red cell was very greatly reduced. The

agglutinin was also modified in other ways by tryptic digestion. The untreated agglutinin was precipitated by half saturation with ammonium sulphate; after tryptic digestion it was no longer precipitated. The trypsinized agglutinin, however, was as susceptible to heat and to destruction by ethanol as the untreated agglutinin.

When the above experiments were repeated with crystalline trypsin and chymotrypsin similar results were obtained if the enzyme concentration was 1:20,000, but with larger amounts of enzyme (1:2000) the agglutinin titre was greatly reduced, to 25 % by chymotrypsin and to 10 % by trypsin.

It appears that the agglutinin is susceptible to tryptic digestion but that the enzymic property is destroyed before the ability to combine with the red cell. The agglutinating power may be retained even when the digestion has proceeded to such an extent as to render the preparation no longer precipitable by ammonium sulphate.

(2) *Effect of nucleases*

A preparation of D.S.P. agglutinin was digested for 48 hr. with crystalline ribonuclease and crystalline deoxyribonuclease in concentrations of 1:1000. Neither enzyme produced any demonstrable effect on the agglutinin titre.

These experiments indicate that the agglutinin is a protein with enzymic properties, which can combine with the receptors of red cells and destroy them. The combining group is relatively resistant to proteolytic enzymes. No evidence of the presence of nucleic acid in the agglutinin has been found, but this possibility cannot be excluded.

RELATION BETWEEN AGGLUTININ AND SPECIFIC ANTIGEN

In previous work it has been considered that the specific complement-fixing antigen was identical with the agglutinin, for the following reasons:

(1) Sera containing specific complement-fixing antibody always inhibit the agglutination of red cells by virus.

(2) In experiments on the growth cycle of influenza viruses (Hoyle, 1950) the appearances of agglutinin and specific antigen coincide in time and they increase in parallel.

(3) If the agglutinin is removed from an extract of infected tissue by adsorption with red cells the specific antigen is also adsorbed and both are recovered from the cells by elution.

However, as described above, a striking difference in behaviour is observed when elementary body preparations are treated with ether. After ether treatment the fluid shows an increased agglutinin titre, but no longer gives complement fixation with sera reacting with the specific antigen by the short fixation method.

It was at first thought that the specific antigen might be ether soluble, but this proved to be incorrect. An elementary body preparation was extracted with ether, the ethereal extract evaporated at 37° C. to very small bulk and then dispersed by squirting it into saline at 37° C. from a fine pipette. The result was an opalescent suspension resembling Wassermann antigen. This 'lipid antigen'

gave no complement-fixation reaction with a human serum containing both specific and group complement-fixing antibody. When tested with a strain-specific ferret serum complement fixation occurred to a low titre, but on further investigation this proved to be a non-specific reaction since complement fixation also occurred with normal ferret serum. It was further found that normal ferret sera would react with alcoholic or ethereal extracts of normal chorio-allantoic membrane and with extracts of chick red cells. Ferret sera do not react with saline extracts of chorio-allantoic membrane since such extracts do not contain enough lipid.

Ether can extract from the elementary body preparation a lipid which has similar serological properties to the lipids of the host cell from which the virus was derived. Hoyle (1950) suggested that the elementary body was enclosed in a lipid envelope derived from the wall of the host cell, and the results described above are in agreement with this view.

Further experiments have shown that although after ether treatment no specific complement-fixing antigen can be demonstrated in elementary body suspensions by the short fixation technique, the antigen is in fact still present, but in an altered form. This is shown by the finding that an ether-treated elementary body suspension is capable of combining with specific antibody and preventing it from giving complement fixation with an untreated elementary body suspension added later. The antigen can thus be detected by an indirect complement-fixation test.

Demonstration of specific antigen in ether-treated elementary body suspensions by indirect complement fixation

A D.S.P. elementary body suspension was treated with ether in the usual way and the original and treated suspensions were then titrated against a suitable dose of strain-specific ferret serum by the short fixation method (Table 2). The original suspension gave an antigen titre of 24, while the ether-treated suspension gave no complement fixation.

A range of dilutions of the ether-treated suspension was then mixed with specific serum, and the tubes incubated for 1 hr. at 37° C. At the end of this time complement and an amount of untreated elementary body suspension sufficient to give complete fixation with the serum were added. Tubes were incubated for 1 hr.; sensitized cells were then added, tubes incubated for a further 30 min. and readings made (Table 3). The ether-treated elementary body suspension inhibited complement fixation to a dilution of 1:7. There is therefore an agent present in the ether-treated suspension which, though unable to fix complement with specific serum, nevertheless unites with antibody and thus prevents the occurrence of complement fixation when an untreated elementary body suspension is added later. This must be specific antigen in an altered form. The properties of this antigen were studied using the indirect complement-fixation test. It was found that the antigen was adsorbed by red cells and could be eluted. By centrifugation methods it was shown that the particle size of the altered specific antigen was the same as the particle size of the agglutinin. An ether-treated elementary body suspension was centrifuged at 152,000 g for $2\frac{1}{2}$ hr. and the agglutinin and specific

Table 2. *Demonstration of specific antigen in ether-treated elementary body suspensions by direct complement fixation*

(Direct complement-fixation test with D.S.P. elementary body suspension and with ether-treated suspension. Serum: specific ferret serum titre 1:128 used 1:20. Control: + + + +.)

Antigen	Antigen control	Antigen dilution 1:1	1:2	1:4	1:8	1:16	1:32	1:64	Antigen titre
D.S.P. elementary body suspension	+ + + +	0	0	0	0	0	+ + + +	+ + + +	24
Ether-treated suspension	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	Nil

Table 3. *Demonstration of specific antigen in ether-treated elementary body suspensions by indirect complement fixation*

(Dilutions of ether-treated elementary body suspension mixed with ferret serum (1:20) and incubated 1 hr. at 37° C. Complement then added followed by untreated D.S.P. elementary body suspension in dilution 1:6. After incubating 1 hr. sensitized cells added, tubes incubated 30 min. and readings made.)

Control (No D.S.P. ether elementary body suspension	Dilution of D.S.P. ether-treated elementary body suspension 1:1	1:2	1:4	1:8	1:16	1:32	1:64	Titre (indirect)
0	+ + + +	+ + + +	+ + + +	+	0	0	0	1:7

+ + + + = complete haemolysis; + + +, + +, + = intermediate degrees of haemolysis; 0 = no haemolysis: complete fixation of complement. The titre is measured as the dilution giving 50 % haemolysis.

antigen contents of various fractions of the supernatant and the deposit measured (Table 4). Both specific antigen and agglutinin were sedimented together.

Table 4. *Centrifugation of agglutinin and specific antigen*

(Material spun at 152,000 *g* for 2½ hr.)

Material	Agglutinin titre	Specific antigen titre (indirect C.F. test)
Original ether-treated elementary body suspension (12·0 ml.)	51,200	8
Top supernatant (3·0 ml.)	128	Nil
Middle supernatant (6·0 ml.)	256	Nil
Bottom supernatant (3·0 ml.)	6,400	1·75
Deposit suspended in 1·0 ml. saline	204,800	32

Note. Much of the deposit was aggregated so that the titres of the resuspended deposit did not accurately represent the content of agglutinin and specific antigen.

It is evident that the altered specific antigen is identical with the agglutinin. In the intact elementary body the agglutinin will give complement fixation with specific serum, but when the soluble antigen and agglutinin are dissociated by ether treatment the agglutinin no longer gives complement fixation by the short fixation method. Complement fixation may, however, occur if the time of fixation is greatly prolonged. An ether-treated elementary body suspension was titrated with specific serum using both short fixation (1 hr. at 37° C.) and prolonged fixation (overnight at 4° C. followed by 2 hr. at 37° C.). No reaction occurred by the short fixation method, but with prolonged fixation the suspension reacted with specific serum to a dilution of 1:14.

POSSIBLE PRESENCE OF SOLUBLE ANTIGEN IN THE AGGLUTININ

Agglutinin preparations do not usually react at all with antisera to the soluble antigen by the short fixation method. If, however, the preparations are concentrated by centrifugation the resuspended deposit does react. This may merely mean that the separation of agglutinin and soluble antigen resulting from ether treatment of the elementary body is not entirely complete. However, since agglutinin preparations do not react with strain-specific ferret serum by the short fixation method it was thought that the failure to react with human convalescent serum containing mainly antibody against soluble antigen might not be satisfactory evidence of the absence of soluble antigen in the agglutinin. Experiments were therefore set up using prolonged fixation, and it was found that under these conditions agglutinin preparations reacted with antisera to soluble antigen to much the same titre (1:16) as with specific serum (1:14). It appeared that the agglutinin did contain soluble antigen. If this was so it was thought that if the agglutinin were denatured with ethanol it might be possible to recover a serologically active fraction (?nucleic acid) by extraction of the precipitate in the same way as with ethanol-treated soluble antigen. An agglutinin preparation was

treated with an equal volume of ethanol, and the precipitate extracted with a small volume of saline and the extract titrated with antiserum to soluble antigen by the short fixation method. No fixation occurred. If therefore the agglutinin does contain soluble antigen it is probably the protein part of the antigen and not the nucleic acid which is present.

A very close relationship between soluble antigen and agglutinin is also suggested by studies of the enzymic properties of virus and virus fractions. Soluble antigen preparations are not adsorbed by red cells, and red cells exposed to powerful soluble antigen preparations are quite unaffected in agglutinability. Soluble antigen alone has no demonstrable enzymic activity. However, the enzymic activity of agglutinin-containing virus and virus fractions seems to be conditioned to some extent by the presence of soluble antigen.

Enzymic activity of virus and virus fractions

The agglutinating and enzymic activities of a virus preparation can be measured at the same time by the following method. An ordinary agglutination test is set up by the Salk method and the agglutinin titre read after 2 hr. at room temperature. The cells are then resuspended by shaking, the tubes incubated at 37° C. for 4 hr., again shaken, and the cells allowed to settle at room temperature. When this is done it is found that with large amounts of virus the agglutination pattern does not reappear, since the red cell receptors have been destroyed during the incubation. It is therefore possible to read an 'enzyme titre' to which this destruction of agglutination pattern occurs. The enzyme titre is always much less than the agglutinin titre.

Experiments of this type were set up with untreated elementary body suspension, ether-treated suspension, agglutinin fraction, and agglutinin fraction partially digested with trypsin with the result shown in Table 5.

Table 5. *Agglutinin titre and enzymic activity of virus fractions*

(Agglutinin titres measured by Salk test, the tubes then shaken and incubated for 4 hr. at 37° C. to allow enzymic destruction of red cell receptors. Cells then allowed to settle and the dilution to which the normal agglutinin pattern was destroyed was read as the 'enzyme titre'.)

Material	Agglutinin titre	Enzyme titre
D.S.P. elementary body suspension	65,000	4,000
Ether-treated elementary body suspension	130,000	256
Agglutinin fraction	130,000	8
Tryptic digest of agglutinin fraction	130,000	Nil

The enzymic activity of the original elementary body suspension is greatly reduced by treatment with ether, the agglutinin fraction is still less active, while a tryptic digested agglutinin shows no enzymic activity at all. These results might be explained by supposing that the enzymic activity of the virus is a function of the protein part of the soluble antigen, and that the agglutinin consists of soluble

antigen protein plus a specific antigen which is responsible for union with the red cell and is therefore essential to activity. The intact elementary body contains the maximum amount of soluble antigen protein in combination with specific antigen, and therefore shows maximal enzymic activity. After ether treatment much of the soluble antigen is dissociated from the specific antigen so that enzymic activity is reduced. When agglutinin and soluble antigen fractions are separated the agglutinin fraction is less active than when soluble antigen is also present (possibly slight recombination may occur if the two are simultaneously present) while treatment with trypsin, to which soluble antigen is very susceptible, destroys all enzymic activity.

From studies of the growth cycle of the virus the author (Hoyle, 1950) had suggested the possibility that the agglutinin was a complex of soluble antigen and specific antigen, and that the specific antigen might be a polysaccharide. The virus was known to contain a large amount of carbohydrate (Knight, 1947); the high degree of serological specificity of the agglutinin suggested that polysaccharide groups might be present, and it was known that the agglutinin could be at first modified and later destroyed by sodium periodate, an agent known to attack carbohydrates (Hirst, 1949; Fazekas de St Groth & Graham, 1949).

However, the properties of the agglutinin described above, especially the sensitiveness to heat, ethanol and trypsin, do not suggest that its combining group is a polysaccharide. It was therefore decided to examine more closely the effect of sodium periodate.

ACTION OF SODIUM PERIODATE ON THE AGGLUTININ

Hirst (1949) showed that virus treated with sodium periodate was modified, so that while still able to agglutinate red cells it no longer eluted from them. Fazekas de St Groth & Graham (1949) obtained the same result and also showed that very large amounts of periodate destroyed the agglutinin. The author has confirmed these findings. The amount of periodate required to produce these effects is very great, and is much greater than the amount of periodate which will modify the receptors of red blood cells so that virus is adsorbed but does not elute (Anderson, 1947). Thus in the agglutination reaction a 1 % suspension of red cells is equivalent to virus with an agglutinin titre of 1:500 in the sense that this quantity of cells will almost completely adsorb the virus and recovery of virus by elution is complete. If a 1 % suspension of red cells is treated with periodate it is found that a 1:30,000 dilution of periodate will modify the red cell receptors in such a way that virus is no longer eluted from them. If, however, a virus suspension with an agglutinin titre of 1:500 is treated with periodate it requires a 1:1000 dilution of periodate to modify the virus so that it no longer elutes from red cells. Still larger amounts are required to destroy the agglutinin. Thus a preparation of D.S.P. agglutinin with a titre of 1:500 was only reduced to 1:100 by 1:500 periodate. This amount of periodate applied to a 1 % red cell suspension causes complete haemolysis.

It is probable that periodate modifies the red cell receptors by virtue of its action on carbohydrate, but it seems unlikely that the action on the virus can be of the

same nature in view of the very much larger amount required. The red cell receptors can be completely destroyed by the receptor-destroying enzyme of *Vibrio cholerae* without the occurrence of haemolysis, but amounts of periodate which have demonstrable action on the virus produce haemolysis when applied to red cells. It is probable that large amounts of periodate have some action on protein. Periodate destroys the enzymic properties of the agglutinin before the combining power with red cells is affected. There is therefore very little reason to suppose that the combining group of the agglutinin is a carbohydrate, and the results with periodate do not support the view that the specific complement-fixing antigen is a polysaccharide.

DISCUSSION

The experiments described above show that when influenza virus elementary bodies are treated with ether they disintegrate into two different types of particle, soluble antigen and agglutinin.

The soluble antigen is not infective and does not agglutinate red cells. It gives complement fixation with human convalescent sera by the short fixation method, but does not react with strain specific ferret serum. Its chemical properties are those of a protein, and there is strong evidence that it is a ribonucleoprotein since it contains some fraction which resists denaturation with ethanol, it forms an insoluble complex with lanthanum, and it is partially destroyed by crystalline ribonuclease.

The agglutinin appears to be an enzyme. It combines with red cells and destroys the red cell receptor without being itself modified. Its chemical properties are those of a protein. By heating at 56° C. or by treatment with suitable amounts of trypsin or sodium periodate the enzymic properties can be destroyed while still leaving the ability to combine with red cells unaffected. The red cell combining group of the agglutinin must have a comparatively low molecular weight, since the agglutinin can be digested with trypsin to a point when it no longer precipitates with ammonium sulphate without the agglutinating power being reduced. The combining group is, however, totally destroyed by ethanol denaturation.

The agglutinin is a very unsatisfactory antigen in complement-fixation tests. It gives no complement fixation by the short fixation technique, but if prolonged fixation is used it reacts with both human convalescent serum containing antibody to soluble antigen and also with strain-specific ferret serum containing only antibody to specific antigen. The intact elementary body reacts with strain-specific ferret serum by the short fixation method. After ether treatment of the elementary body this reaction disappears, but the specific antigen is still present, since it can be detected by its ability to unite with antibody and by so doing to prevent complement fixation with intact elementary bodies added later. The specific antigen detectable by this indirect complement-fixation test appears to be identical with the agglutinin since it has the same particle size and is adsorbed and eluted from red cells in the same way. No satisfactory explanation can be given for the peculiar behaviour of the agglutinin in complement-fixation tests. It may be that the strain specific antibody is unable to build up rapidly aggregates with specific

antigen although uniting with it, so that complement fixation only occurs by the short fixation method if the antigen particle is already of large size. The elementary body would thus give fixation but the agglutinin of much smaller size might be unable to do so. But it is also possible that the lipid present in the elementary body is linked to the specific antigen and that removal of the lipid reduces the ability to give complement fixation by the short fixation method.

It seems reasonable to suppose that the agglutinin is an enzyme with a combining group of small molecular weight which is identical with the specific complement-fixing antigen and which unites with red cell receptors, while there is also a protein of larger molecular weight which carries the enzymic power. The enzymic activity of various types of agglutinating particle appears to be in some way related to the presence of soluble antigen; thus the intact elementary body destroys red cell receptors more powerfully than the agglutinin after ether treatment. There is some serological evidence for the presence of soluble antigen in the agglutinin, and it may be that the protein of the agglutinin is identical with the protein of the soluble antigen, and that the agglutinin represents soluble antigen protein modified by the presence of a combining group with affinity for red cells. There is, however, no evidence of the presence of nucleic acid in the agglutinin, though this possibility cannot be entirely discounted.

All the biological properties of the intact elementary body except the infectivity can be accounted for in the fractions obtained by ether treatment. The soluble antigen is responsible for the complement-fixing power with heterologous human convalescent serum. The agglutinin carries the red cell combining power and the enzymic properties, and appears also to contain the specific complement-fixing antigen.

Chemical analysis of the elementary body by Knight (1947) showed that it contained both ribonucleic and deoxyribonucleic acid, a large amount of lipid and also a carbohydrate built up of glucosamine, galactose and mannose. The ribonucleic acid is almost certainly present in the soluble antigen fraction, but none of the known biological properties of the virus can be related to the presence of deoxyribonucleic acid, lipid or carbohydrate. Hoyle (1950) brought evidence to show that the elementary body was excreted from the cell in the form of an aggregate of agglutinating and complement-fixing particles enclosed in a membrane derived from the wall of the host cell. The elementary body therefore contains some material derived from the host cell. Knight (1946), himself, noted the presence of material with serological characters of host origin. When the elementary body is disintegrated with ether a serologically active lipid can be recovered from the ether, but this material has similar characters to lipid from the host cell. The carbohydrate is probably also of host origin. The cell wall contains mucoprotein and the fact that the virus carbohydrate is built up of glucosamine, galactose and mannose suggests that it may be mucin. The virus appears to escape from the host cell by means of its production of an enzyme which attacks mucoprotein, but the work of Burnet and his colleagues on the receptor gradient has shown that most strains of influenza virus do not destroy the whole of the mucoprotein receptors of the cell, so that undamaged mucoprotein of host cell origin may well be present

in the membrane surrounding the virus. None of the biological properties of the virus or fractions obtained by ether treatment is affected by digestion with deoxyribonuclease. It is of course possible that the virus contains deoxyribonucleic acid of a type insusceptible to the action of the particular enzyme used, but it is also possible that the deoxyribonucleic acid found by Knight is of host cell origin, since deoxyribonucleic acid appears to be concerned with polysaccharide synthesis and may therefore be present in the cell wall. Recent work by Graham (1950) indicates that the amount of deoxyribonucleic acid present in the virus is very small in relation to the amount of ribonucleic acid.

The results reported in this paper support the conception of the intracellular growth of the virus advanced by Hoyle (1948, 1950). It was suggested that the elementary body on entry into a susceptible cell became disintegrated, liberating soluble antigen. This soluble antigen was believed to be a self-replicating unit which multiplied in the cell, and later recombined with specific antigen derived from the original inoculum. More specific antigen was then synthesized and the complex of soluble and specific antigen was believed to be identical with the agglutinin and to have enzymic properties which enabled it to attack the cell wall. The virus escaped from the cell as a result of this attack on the cell wall, and the elementary body consisted of an aggregate of soluble antigen and agglutinin enclosed in a lipid membrane derived from the wall of the host cell.

The identification of the soluble antigen as a ribonucleoprotein supports the idea that it is a self-replicating unit. The agglutinin contains specific antigen and carries the enzymic activity. Serological evidence suggests that the agglutinin contains some protein with serological characters similar to the soluble antigen, but possibly does not contain nucleic acid. The results are consistent with the view that the soluble antigen is a replicating nucleoprotein and that it is responsible for the production of the agglutinin. The soluble antigen would thus be an enzyme precursor.

As a result of studies of variation in influenza viruses Burnet & Lind (1951) suggested that on entry into a susceptible cell the virus elementary body became disintegrated into a number of independent genetic units which then replicated in the cell, infective virus being produced later by recombination of individual genetic units. Each of the several biological characters of the virus were supposed to be carried by separate genetic units. On this view the soluble antigen would represent one type of genetic unit while the agglutinin represented another. If this were so the various genetic units would probably all be nucleoproteins, since there is no evidence that any protein other than nucleoprotein possesses replicating powers. There is, however, no evidence that the agglutinin contains nucleic acid. The two concepts might be fused if it could be shown that the independent genetic units of Burnet & Lind each contained soluble antigen which would be responsible for the replicating power, together with some enzyme or other group carrying the biological properties. The problem really resolves itself into a choice between two possibilities. According to Burnet & Lind there is a specific replicating unit for each enzyme or other biologically active group demonstrable in the virus—the 'one gene one enzyme theory'. The author considers that there is one replicating

unit, the soluble antigen, which may produce a number of different enzymes or other active groups, the nature of those which appear being conditioned by the presence of material from the original inoculum acting as a pattern molecule, or in the case of an enzyme, being developed as a result of the presence of its substrate or the products of its action. It is impossible in the present state of knowledge to decide in favour of either of these two alternatives. Northrop, Kunitz & Herriot (1948) have suggested a mechanism of protein and enzyme synthesis in cells which is very similar to the author's concept. They suggest that the first step is the synthesis of a species—specific proteinogen, which is self replicating in that it determines its own structure, probably contains nucleic acid and has some of the characters of a denatured protein. The second step is the formation of individual native proteins from the denatured proteinogen by a catalytic reaction, an example being the formation of an enzyme from its precursor. This second step would not require energy. The soluble antigen appears to have the characters of such a proteinogen, and the agglutinin might well be derived from the soluble antigen in the manner visualized by Northrop *et al.* (1948).

SUMMARY

Influenza virus elementary bodies can be disintegrated by treatment with ether with the liberation of two types of smaller particle, soluble antigen and red cell agglutinin.

Soluble antigen derived from the elementary body is identical in serological and chemical behaviour with soluble antigen recovered from infected tissues. Its chemical properties are those of a ribonucleoprotein.

The agglutinin is an enzyme, with a protein part carrying the enzymic activity and a combining group with affinity for red cells. The agglutinin does not react in complement-fixation tests by the short fixation technique, but by the use of prolonged fixation or by indirect complement fixation it can be shown to contain a strain specific antigen and also a non-specific antigen. The specific antigen is identical with the specific complement-fixing antigen demonstrable in the intact elementary body, and has combining affinity for red blood cells. The non-specific antigen is probably similar to the protein part of the soluble antigen. No evidence has been found that the agglutinin contains either carbohydrate or nucleic acid.

From the ether used to disintegrate the elementary body a serologically active lipid can be recovered which has properties suggesting that it is derived from the host cell.

The author is greatly indebted to Dr L. Dmochowski for assistance in experiments involving high-speed centrifugation, to Sir Macfarlane Burnet and Dr G. L. Ada for supplies of receptor-destroying enzyme, to Dr E. S. Duthie for crystalline trypsin and chymotrypsin, and to Dr R. R. Porter for a sample of crystalline ribonuclease.

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(*MS. received for publication* 8. XI. 51.)