AN ANALYSIS OF THE COMPLEMENT-FIXATION REACTION IN INFLUENZA*

By L. HOYLE

(With 11 Figures in the Text)

This paper describes a study of the mechanism of the complement-fixation reaction in influenza. Its purpose was to determine the relative parts played in the reaction by the virus elementary body and the soluble antigen, and to find out whether any specific differences could be demonstrated between different strains of influenza virus A. Almost all the work has been done by means of 'chess-board' experiments of the type first used by Dean & Webb (1926) in their classical work on the complement-fixation reaction. These experiments, in which a series of antigen dilutions is tested against a series of serum dilutions with a constant dose of complement, convey very much more information about the nature of a complement-fixation reaction than is obtainable by a simple titration of antigen or serum.

Table 1. *Complement-fixation reaction between a concentrated antigen derived from mouse lungs infected with W.S. virus, and a human convalescent serum M.J.H.*

Short fixation, 2½ M.H.D. of complement.

++++ = 100% haemolysis; +++, ++, + = 75, 50 and 25% haemolysis. 0 = no haemolysis.

Serum dilution	Antigen dilution 1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
1 : 4	0	0	0	0	0	++++	++++
1 : 8	0	0	0	0	0	++++	++++
1 : 16	0	0	0	0	0	++++	++++
1 : 32	0	0	0	0	0	++++	++++
1 : 64	0	0	0	0	0	++++	++++
1 : 128	+++	0	0	0	+	++++	++++
1 : 256	++++	++++	+++	+	+++	++++	++++
1 : 512	++++	++++	++++	++++	++++	++++	++++

TECHNICAL METHODS

In order to conserve serum and antigen a very small unit volume (0·05 c.c.) has been used in the complement-fixation tests. Special constant volume delivering pipettes were used to ensure accuracy in measuring such small volumes, and the tests were done in small tubes 1¼ × ¼ in. Each tube contained 0·05 c.c. serum dilution, 0·05 c.c. complement, and 0·05 c.c. antigen dilution. Tubes were incubated 1 hr. at 37° C. and then 0·1 c.c. of 3% sensitized sheep cells (5 doses of I.B.) was added, the tubes incubated 45 min., the cells allowed to sediment and readings made. To facilitate reading intermediate degrees of haemolysis a series of artificial standards of 0, 25, 50, 75 and 100% haemolysis was prepared and the tubes matched against these.

A constant dose of complement (2½ M.H.D.) has been used in most of the experiments. The complement was the pooled serum of several guinea-pigs preserved by the boric acid-sorbite-azide method of Richardson (1941). This preserved liquid complement has been found very satisfactory as its titre remains unchanged for weeks and enables a large number of experiments to be done with the same batch of complement.

The sera used in this work were mainly human convalescent sera from the epidemic of 1943–4. They were inactivated at 56° C. for 30 min. and preserved by addition of 0·08% sodium azide. Some of the antigens used in the work have also been preserved by sodium azide.

GENERAL CONSIDERATIONS AND QUANTITATIVE MEASUREMENTS

A typical 'chess-board' experiment is shown in Table 1. Falling dilutions of the serum of a human influenza convalescent were tested against falling dilutions of a concentrated complement-fixing antigen prepared from mouse lungs infected with the W.S. strain of influenza virus A. Hoyle & Fairbrother (1937) showed that the complement-fixation reaction given by infected mouse lungs was due to a soluble substance distinct from the infective virus elementary body. This soluble antigen could be purified and concentrated by precipitation with acetic acid at *p*H 5·0 followed by resolution in

* A report to the Medical Research Council from the Emergency Public Health Laboratory, Northampton.

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phosphate buffer at *p*H 7·4, and the antigen used in this experiment was prepared in this way.

The results of chess-board experiments of this type can be most conveniently represented graphically as in Fig. 1. This curve is derived by drawing a line through all mixtures giving 50 % haemolysis. Where in a given series of tubes no single one gives exactly 50 % haemolysis, then the 50 % point is obtained by interpolation. Thus in the first horizontal row of Table 1 the 1 : 32 antigen dilution gives no haemolysis while the 1 : 64 dilution gives complete haemolysis. The 50 % haemolysis point therefore lies midway between 1 : 32 and 1 : 64.

Fig. 1 is derived from Table 1, the circles being derived from the horizontal rows of the table and

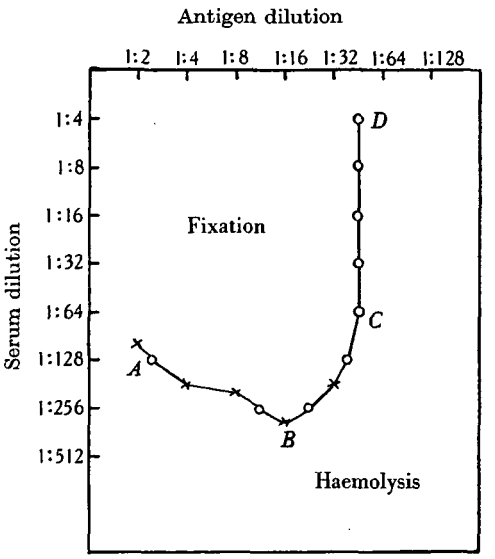

Fig. 1. 50% haemolysis curve from Table 1. Antigen: concentrated mouse-lung preparation of W.S. virus. Serum: human convalescent M.J.H.

the crosses from the vertical rows. A line *ABCD* joining these points is the 50 % haemolysis curve. All mixtures within the area bounded by this curve show complete fixation, while all mixtures outside it give complete haemolysis. The curve shows a single sharp optimal point *B* which gives a reading of the *maximal serum titre* and the *optimal antigen titre*. Excess of antigen over the optimal amount results in reduced fixation—the zone phenomenon. The line *CD* gives a measure of the *maximal antigen titre*, but there is no optimal serum titre since no zone phenomenon occurs with excess of serum.

This type of curve is the commonest seen in complement-fixation reactions, but it is not the only type which may occur. Reactions may be encountered in which fixation fails with both antigen and serum excess so that the 50 % haemolysis curve shows two optimal points. This type of curve was encountered by Dean & Webb in their original analysis of the complement-fixation reaction. Also complement-fixation reactions may occur in which no zone phenomena are seen and there are no optimal points in the 50 % haemolysis curve. This type commonly occurs where the particle size of the antigen is very large and is often seen in the Wassermann reaction.

Where two or more antigen-antibody reactions occur simultaneously a variety of irregular curves may be encountered, and even double zoning may be seen.

With curves of the type shown in Fig. 1 three quantitative measurements can be made, the *maximal serum titre*, *maximal antigen titre* and *optimal antigen titre*. In addition, the ratio of maximal to optimal antigen titre is of importance, since when this ratio (3 : 1 in Fig. 1) is known it is possible to derive the optimal antigen titre from the maximal titre. This is of great practical value since the maximal antigen titre is easily found by a simple antigen titration with serum in excess, while a full chess-board experiment is necessary to determine the optimal titre.

Effect of time period of fixation

It is well known that the sensitivity of complement-fixation reactions can be increased by prolonging the time of fixation. This is shown in Fig. 2. Parallel chess-board experiments were done, one with short fixation (1 hr. at 37° C.) and one with long fixation (overnight at 2° C. followed by 1 hr. at 37° C.). The effect of prolonging the fixation period was to increase the maximal serum titre, the optimal antigen titre, the maximal antigen titre, and the ratio of maximal to optimal antigen titres. The reaction was therefore rendered more sensitive and it might be thought that prolonged fixation was the better technique. This, however, is not the case. A price has to be paid for the increased sensitivity. This is partly shown in Fig. 2 where it will be seen that prolonged fixation had the effect of making the lower dilutions of antigen anticomplementary. This is a very usual effect and may sometimes more than annul the advantages of increased sensitivity. Low dilutions of serum may also become anticomplementary, and, in addition, irregularities may appear in the 50 % haemolysis curve which render it difficult to make accurate quantitative measurements. Thus the increased sensitivity given by prolonged fixation may be balanced by a loss of accuracy. As the complement-fixation reaction in influenza is one of the most powerful known it is only rarely that great sensitiveness is necessary, and for most purposes the more accurate short-fixation method is preferable.

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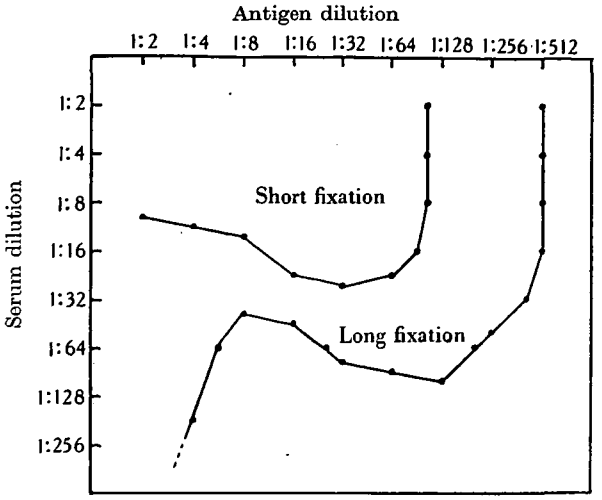

Fig. 2. Effect of time of fixation. Short fixation: 1 hr. at 37° C. Long fixation: overnight at 2° C. followed by 1 hr. at 37° C. Antigen: concentrated mouse-lung preparation of W.S. virus. Serum: human convalescent 2771.

Effect of variation of the dose of complement

Parallel chess-board experiments were set up with 2, 3, 4, 6 and 8 M.H.D. of complement (Fig. 3). The experiment shows that the maximal serum titre, maximal antigen titre, and optimal antigen titre are all dependent on the dose of complement used, but the ratio of maximal antigen titre to optimal antigen titre remains the same.

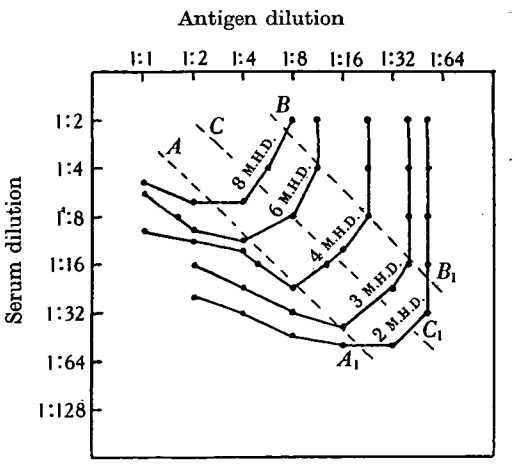

Fig. 3. Effect of variation of dose of complement. Antigen: concentrated mouse-lung preparation of W.S. virus. Serum: human convalescent Miss T.

Nature of the antigen-antibody union. A linear relationship exists between the maximal serum titre and the maximal and optimal antigen titres (Fig. 4 A) showing that the antigen-antibody reaction obeys the law of constant proportions.

Linear relations also exist between the dose of complement fixed and the serum and antigen titres (Fig. 4 B, C), but the relationship is not a simple quantitative one. Thus in order to double the amount of complement fixed it is necessary to increase serum and antigen by approximately three times.

The experiment of Fig. 3 also enables us to determine at what point the antigen-antibody mixture is neutral. If we consider the optimal points on the line AA_1 it will be seen that at any point on this line the amount of complement fixed cannot be increased by adding more antigen, but is increased by addition of more serum. At these points therefore the antigen-antibody complex is saturated with antigen but is capable of uniting with more antibody. A similar argument applied to the line BB_1 shows that at points on this line the antigen-antibody complex is saturated with antibody but is capable of uniting with more antigen and in so doing fixing more complement. There is therefore no sharply defined neutral point but rather a zone of neutrality lying between the lines AA_1 and BB_1. Mixtures within this zone can unite with both antigen and antibody. As a practical point a neutral mixture will be produced by mixing antigen and antibody in the proportions of their maximal titres. Such mixtures fall along the line CC_1 which is almost central in the neutral zone.

COMPLEMENT FIXATION WITH THE SOLUBLE ANTIGEN

Hoyle & Fairbrother (1937) showed that the complement-fixing antigen present in extracts of infected mouse lungs was a soluble substance distinct from the infective elementary body, the two being separable by differential centrifugation. This observation was confirmed by Lennette & Horsfall (1940), Friedewald (1943), and Henle, Henle, Groupé & Chambers (1944). It was, however, shown that the infective elementary body could play a part in complement fixation; Hoyle & Fairbrother could demonstrate this only by the use of prolonged fixation, but Friedewald showed that in the case of allantoic fluid antigens from infected developing eggs the complement fixation due to the infective virus particles might be greater than that due to soluble antigen.

Preparations of soluble antigen almost entirely free from infective virus can be readily obtained by a variety of methods of which the following are among the simplest:

(1) Dried infected mouse lungs are ground with sand and saline to make a 0·5 % suspension. This is

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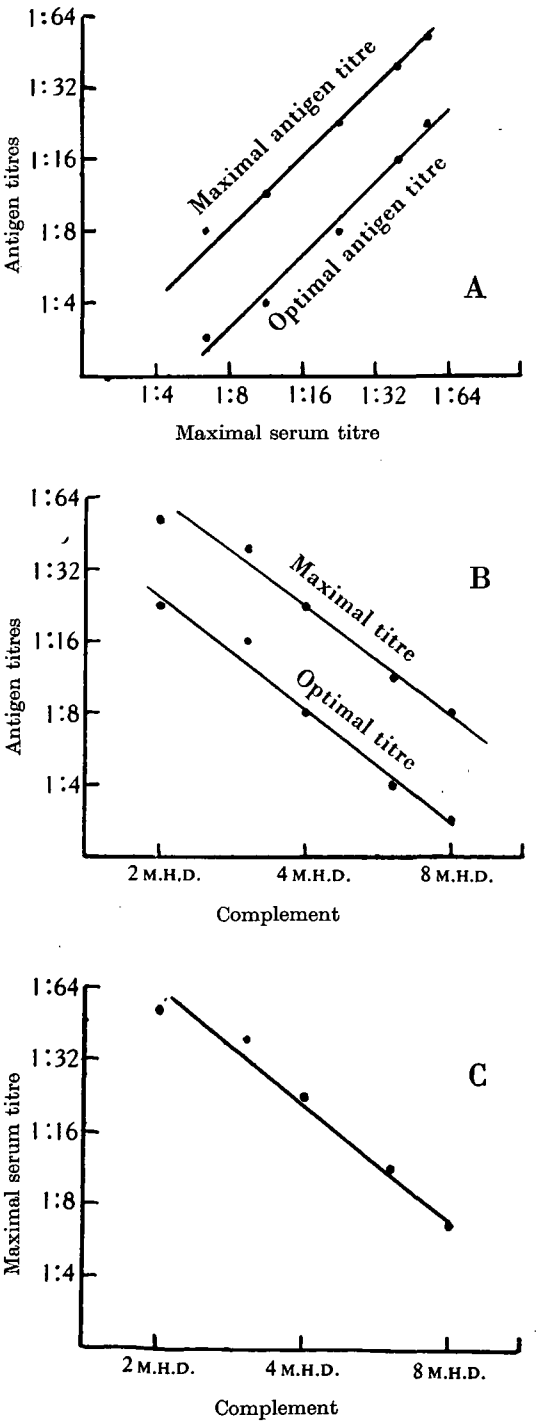

Fig. 4. Relations derived from experiment of Fig. 3. A, relation between antigen and serum titres; B, relation between complement dose and antigen titres; C, relation between complement dose and serum titre.

centrifuged and the supernatant removed and adjusted to *p*H 5·0 by addition of 0·03 % acetic acid. The precipitate formed is centrifuged off and redissolved in a small volume of phosphate buffered saline *p*H 7·4, insoluble matter being removed by centrifugation. A concentrated almost non-infective soluble antigen is obtained.

(2) A 2 % suspension of dried infected mouse lungs is prepared, centrifuged, the supernatant removed, and a few drops of chloroform added to it. The liquid is then allowed to stand for 3–4 days in the refrigerator. The addition of chloroform causes further sedimentation of particulate matter and much of the infective virus is also carried down. On again centrifuging a clear preparation of soluble antigen is obtained.

(3) Soluble antigen can be prepared from the allantoic fluid of infected developing eggs if the infective virus is removed by absorption with guinea-pig red cells. Three successive absorptions with 2 % cells is usually adequate. The final fluid should no longer agglutinate red cells.

Identity of the soluble antigens of all strains of influenza virus A

A preparation of soluble antigen from mouse lungs infected with W.S. virus was titrated with four different human convalescent sera of different titres (Fig. 5). The experiment shows that the optimal antigen titre is the same (1 : 16) whatever the serum used to titrate it, as also is the maximal antigen titre.

The converse experiment is shown in Fig. 6. Here a single convalescent serum was titrated with five different preparations of soluble antigen:

(1) A mouse-lung preparation of W.S. (1933) virus.

(2) A mouse-lung preparation of R.W.F. (1937) virus.

(3) A mouse-lung preparation of E.L. (1937) virus.

(4) A mouse-lung preparation of swine virus.

(5) An allantoic fluid preparation of D.S.P. (1943) virus.

The maximal serum titre is seen to be the same no matter what preparation of soluble antigen is used.

These two experiments demonstrate clearly that the soluble antigens of all strains of influenza A virus are identical.

This identity was also demonstrated in a conclusive manner by the mixing experiment described below. It can be shown that if chess-board experiments are done with a mixture of two antigens and a single serum the result will differ according to whether the antigens are identical or different. A human convalescent serum D.S.P. was titrated with three soluble antigens W.S., Swine, and the Lee strain of influenza virus B (Francis, 1940). The

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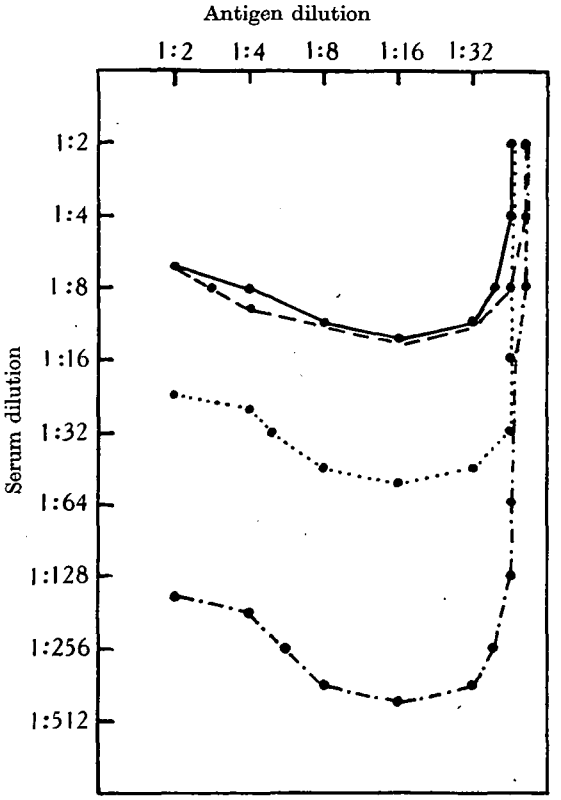

Fig. 5. Titration of a single soluble antigen (from mouse lung infected with W.S. virus) with four different convalescent sera.

Serum: McC. ———— 2771 – – – – –
Miss T. ····· M.J.H. –·–·–·

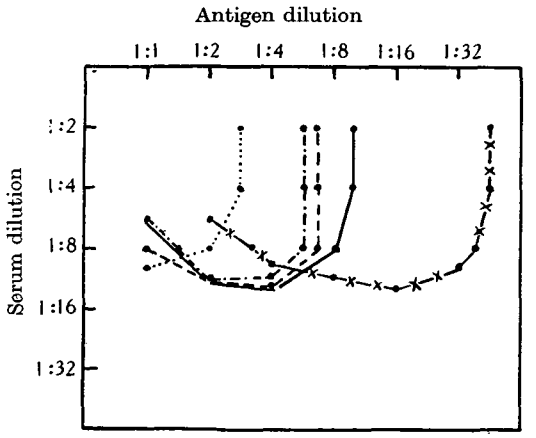

Fig. 6. Titration of serum 2771 with five different soluble antigens.

Antigens: W.S. –×–×– D.S.P. ·····
R.W.F. –·–·–· E.L. ———— Swine – – – – –

results are shown in Fig. 7A. Now consider the probable result of using a mixture of equal parts of W.S. and swine antigens. If these are identical then the curve 1 in Fig. 7B will be obtained, the curve being identical in form with each of its components and being simply the average of the two separate parts. The curve will have a sharp optimal point and there will be a clear-cut zone phenomenon. If, however, the antigens are different then the optimal point due to the swine portion will fall in the zoning region of the W.S. portion with the result that the zone phenomenon will be obliterated and a curve 2 obtained which is the sum of the two component curves and is quite different in form from them. The experiment was carried out and the observed curve is seen to agree closely with curve 1, indicating the identity of W.S. and swine antigens. A similar mixing experiment done with W.S. and Lee strain of B virus (Fig. 7C) shows these antigens to be different.

The soluble antigens of all strains of influenza virus A are therefore identical, and are different from the soluble antigen of virus B.

COMPLEMENT FIXATION WITH THE VIRUS ELEMENTARY BODY

Fairbrother & Hoyle (1937) had been unable to detect any antigenic difference between the human and swine viruses by complement-fixation methods, but subsequent workers (Lush & Burnet, 1937; Eaton, 1941; Lennette & Horsfall, 1941) found that slight differences could be demonstrated, especially if antigens from developing eggs were used. The differences were more pronounced in the sera of experimental animals than with human sera, but even with human sera antigens usually reacted more powerfully with homologous than heterologous sera. As these effects were best demonstrated with egg antigens which contain much more infective virus than antigens from mouse lung (Friedewald, 1943), it seemed probable that the specific phenomena were due to the infective elementary body, and that the fixation due to the elementary body differed in nature from that due to soluble antigen. This has been found to be the case.

Allantoic fluid from developing eggs infected with the D.S.P. strain of virus A was fractionated by means of red cell absorption. The fluid was absorbed successively three times with guinea-pig red cells added to a concentration of 2%. The residual fluid no longer agglutinated red cells and constituted the *soluble antigen fraction*. The red cells used in the absorption were then pooled, washed twice with saline, and the virus eluted from them into a small volume (one-quarter of the original) of phosphate buffered saline *p*H 7·4 at 37°C. for 4 hr. The cells were then centrifuged out and the supernatant,

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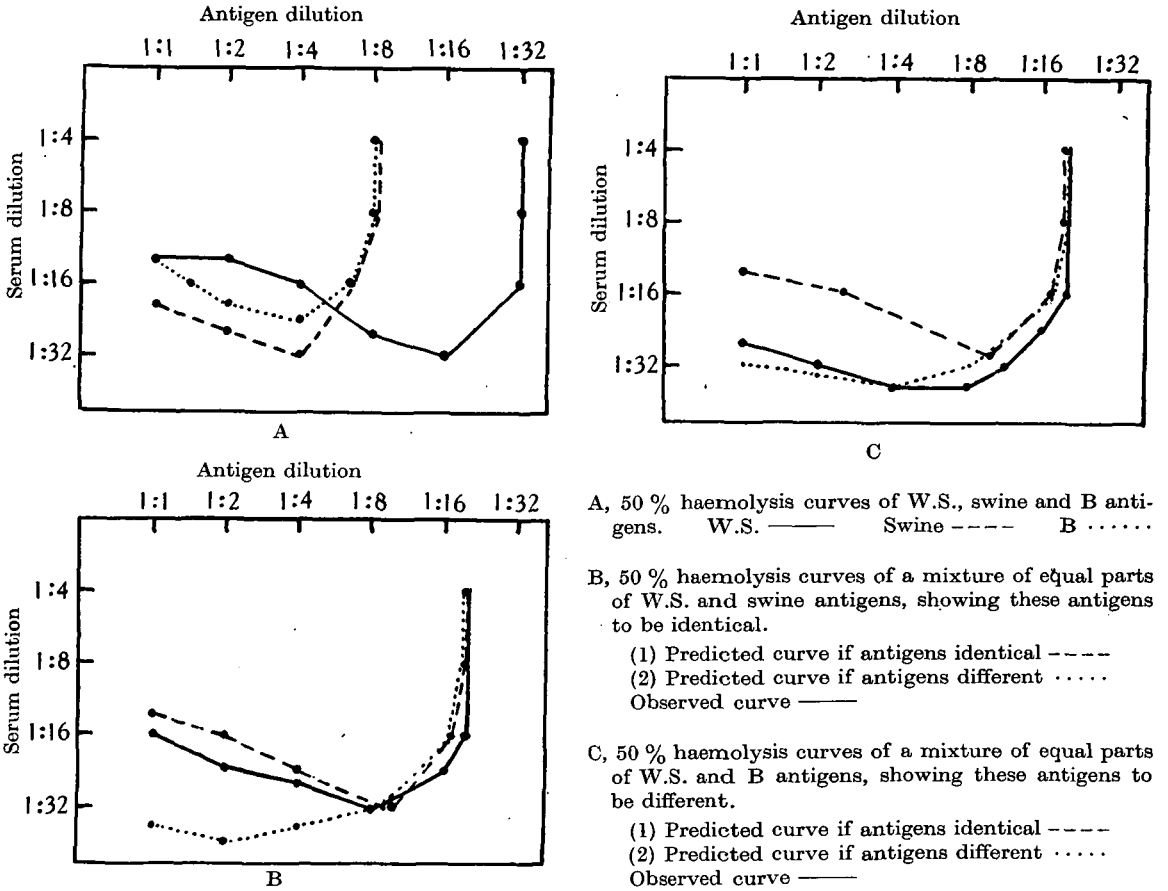
which powerfully agglutinated red cells, constituted the *virus elementary body fraction*.

Parallel chess-board experiments were done with the two fractions against a human convalescent serum (Fig. 8). Not only did the curves of the two fractions differ considerably in form, but the maximal serum titre was higher against the elementary body fraction than against the soluble antigen. The complement fixation due to the two fractions is therefore different in nature.

These results could only mean that the elementary body contained several different antigens and that the observed irregularities of the curves were due to multiple antigen-antibody reactions occurring simultaneously. A careful study of these and other similar curves suggested that at least three and possibly four different antigens could be detected in the elementary body.

The converse experiment is shown in Figs. 10 and 11. Here three different elementary body anti-



A, 50 % haemolysis curves of W.S., swine and B antigens. W.S. ——— Swine – – – – B ·····

B, 50 % haemolysis curves of a mixture of equal parts of W.S. and swine antigens, showing these antigens to be identical.

(1) Predicted curve if antigens identical – – – –
(2) Predicted curve if antigens different ·····
Observed curve ———

C, 50 % haemolysis curves of a mixture of equal parts of W.S. and B antigens, showing these antigens to be different.

(1) Predicted curve if antigens identical – – – –
(2) Predicted curve if antigens different ·····
Observed curve ———

Fig. 7. Mixing experiment with soluble antigens of W.S., swine and B viruses. Serum D.S.P.

The infective elementary body fraction was then titrated with several different convalescent sera (Fig. 9). The curves obtained showed marked differences in form, the maximal antigen titre was not the same for all the sera, and in those curves showing an optimal point the optimal antigen titre also varied with the serum used. With some of the sera the zone phenomenon was observed, but with others it was almost absent. One serum (2699) showed double zoning.

gens were tested against single sera. Again the curves obtained differed markedly in form, and there were small but definite differences between the maximal serum titres obtained with different antigens. Double zoning was seen with the swine antigen in Fig. 10 and with W.S. antigen in Fig. 11. In Fig. 10 the maximal serum titre was slightly higher with the homologous antigen D.S.P. than with W.S. and swine antigens.

These experiments can only be explained by

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supposing that the elementary body contains several different antigens, and it is clear that we have here an explanation of the specific phenomena observed in complement-fixation reactions with antigens containing much infective virus. The differences between various strains of virus A are probably more

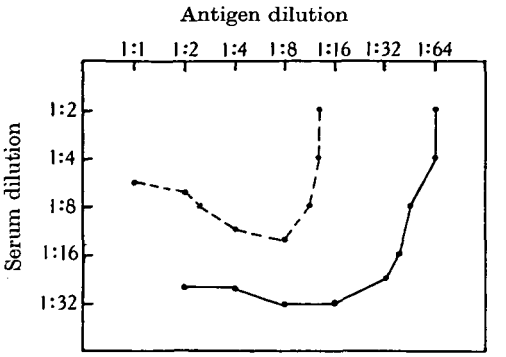

Fig. 8. Titration of serum 2771 with allantoic fluid fractions. D.S.P. virus.

Virus elementary body fraction ———
Soluble antigen fraction – – – –

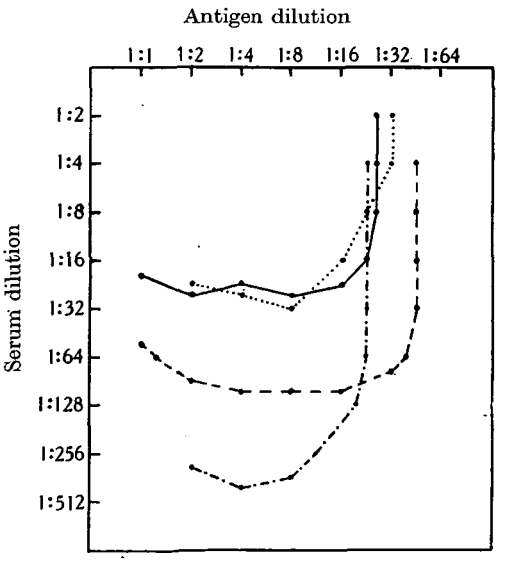

Fig. 9. Titration of an elementary body preparation of D.S.P. virus with four different sera.

Serum:
2699 ——— L.H. – – – – M.J.H. –·–·– A.C. · · · · ·

of a quantitative than a qualitative nature, and there are certainly some antigens common to all strains. Thus it is not possible by the methods so far used to free the elementary body entirely from soluble antigen. This is shown by the fact that in experiments of the type shown in Fig. 8 where a serum is titrated with elementary body and soluble antigen fractions the maximal serum titre is never greater with the soluble antigen than with the elementary body. Usually the titre is slightly higher with the elementary body fraction; occasional sera have been seen where the two fractions gave almost identical maximal serum titres, but in no case has

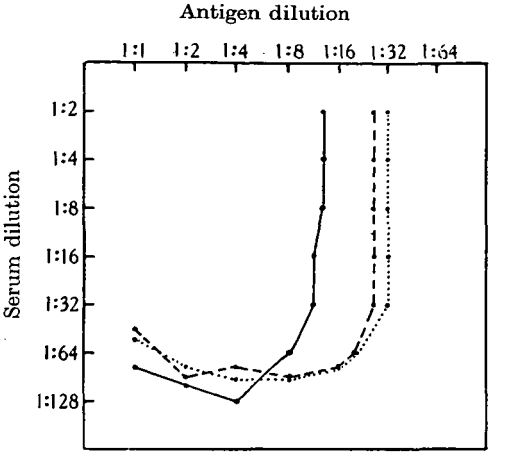

Fig. 10. Titration of D.S.P. serum with three different elementary body antigens.

Antigen: D.S.P. ——— W.S. · · · · · Swine – – – –

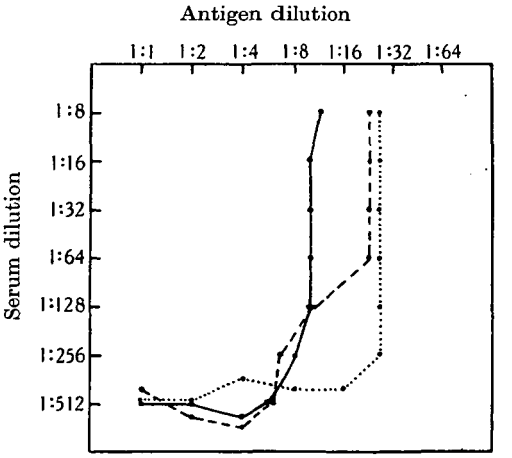

Fig. 11. Titration of M.J.H. serum with three different elementary body antigens.

Antigen: D.S.P. ——— W.S. · · · · · Swine – – – –

the soluble antigen given the higher titre. This phenomenon which has also been observed by Henle *et al.* (1944) must mean that the elementary body preparations contain soluble antigen. It is impossible to tell whether this soluble antigen is an intrinsic part of the elementary body or is merely adsorbed.

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DISCUSSION

The experiments described in this paper show clearly that the complement-fixation reaction in influenza is a complex one, and that two distinct antigens, the soluble antigen and the elementary body, are involved.

The soluble antigen appears to be a single substance and the soluble antigens of all strains of virus A are identical. The B virus has a different soluble antigen.

The elementary body is shown to be a complex of several different antigens, and the specific phenomena, sometimes seen in complement-fixation reactions, are due to the differences in antigenic structure of the elementary bodies of different strains of virus A.

The suggestion has often been made that the soluble antigen is merely disintegrated elementary bodies. This work does not support this view, as if it were true several different antigens should be detected in the soluble fraction, since the elementary body is a complex of a number of antigens.

The chief practical value of the complement-fixation reaction in influenza has been its use in studies of the epidemiology of influenza. For this purpose the ideal antigen is clearly one containing mainly the soluble antigen, since such an antigen will react with the serum of anyone previously infected by virus A, and the titre of the serum will not be affected by any chance relationship or absence of relationship between the infecting strain and that used as antigen. Studies of antigenic differences between strains of virus A are much better carried out by the neutralization test or by inhibition of red cell agglutination.

Allantoic fluid antigens were strongly recommended by Nigg, Crowley & Wilson (1941) and have been much used, but their high content of infective virus in relation to soluble antigen renders them unsuitable for epidemiological studies.

Experience of all types of antigen extending over many years has convinced the author that the original dried mouse-lung antigen of Fairbrother & Hoyle is still the antigen of choice. It is very stable; samples dried in 1937 were tested recently and found apparently unchanged in potency after 7 years' storage. The following description of the method of preparation and use of such an antigen may therefore be of value.

Preparation and use of mouse-lung antigen

A strain of virus of high mouse virulence such as W.S. or P.R. 8 should be used. Mice are infected by intranasal inoculation, and the lungs of mice dying on the 3rd or 4th days are cut into small pieces and desiccated *in vacuo* over calcium chloride. The dried lung is then stored in sealed tubes containing calcium chloride, preferably in accurately weighed quantities of 0·2 g. The tubes may with advantage be evacuated but this is not necessary.

When antigen is required for use the contents of one tube are ground with sand and a few drops of saline, diluted to 10 c.c. with saline thus making a 2 % suspension, and centrifuged at 4000 r.p.m. for 20 min. The supernatant is removed, 0·08 % sodium azide added as preservative, and a few drops of chloroform. Allow to stand for 2–3 days in the refrigerator when much particulate matter will sediment. Centrifuge again and remove from the chloroform layer. The fluid azide-preserved antigen will keep for many weeks. It is almost devoid of anti-complementary properties and the treatment with chloroform also has the advantage of removing Forssman antigen.

The fluid antigen should be titrated against an excess of known positive serum so as to obtain a reading of the maximal antigen titre. If short fixation (1 hr. at 37° C.) is to be used, then the optimal concentration of antigen for use in serum titrations will be found by dividing the maximal titre by 3. Thus in practice a 2 % suspension usually has a maximal titre of the order of 1 : 12, so that the optimal titre is 1 : 4, i.e. a 0·5 % suspension. Alternatively, a full chess-board experiment may be done so as to obtain a direct reading of the optimal titre.

An antigen made up in this way and accurately titrated is almost foolproof in use. The only false positives the author has encountered with it were given by the sera of certain soldiers who had been immunized against yellow fever by mouse-brain vaccine, and these were of a very feeble nature.

SUMMARY

1. An analysis of the complement-fixation reaction in influenza has been made by the use of chess-board experiments in which serial dilutions of antigen are tested against serial dilutions of serum with a constant dose of complement.

2. The reaction has been shown to be a complex one, involving two different antigens, the virus elementary body and the soluble antigen.

3. The soluble antigens of all strains of influenza virus A are identical, and different from the antigen of virus B.

4. The elementary body is a complex of several different antigens, and differences in antigenic structure can be detected between different strains of virus A. All elementary body preparations, however, contain soluble antigen which may be an intrinsic part of the elementary body or may be adsorbed.

5. The most suitable antigen for use in epidemiological studies is one containing chiefly soluble

antigen, and a description is given of the method of preparation and use of such an antigen derived from infected mouse-lung tissue.

The author wishes to express his indebtedness to Dr C. H. Andrewes for some of the strains of influenza virus used in this work.

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(*MS. received for publication* 6. IX. 44.—Ed.)