BRADISH, C. J., BROOKSBY, J. B. & TSUBAHARA, H. (1960). *J. gen. Microbiol.* **22**, 392-404

The Complement-Fixation Test in Studies of Components of the Virus System of Foot-and-Mouth Disease and its Antibodies

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SUMMARY: The antibody combining properties of the U- and D-fractions of the virus system of foot-and-mouth disease were investigated by complement-fixation methods. The virus fractions were titrated in antibody excess and used as excess antigens in titrations of antisera. The results demonstrate that the complement-fixing activity in antibody excess increased with time of incubation by a threefold or higher factor when the U-fraction was used as antigen than when the D-fraction was used. Titrations of antisera showed that distinct combining activities were involved according to the virus fraction used as antigen in excess. Data in support of this were obtained in adsorption experiments in the absence of complement by titration of the residual antibody activity following sedimentation of the complexes formed with the U- or D-fractions. The reaction in the region of equivalence, in which neither antigen nor antibody is in effective excess, was investigated. A rapid and objective procedure for the treatment of complement-fixation data is presented.

The complement-fixation test is of particular importance in foot-and-mouth disease research since it offers the only quantitative *in vitro* procedure of sufficient sensitivity for the investigation of the majority of field and experimental samples. A review of the complement-fixation test in the study of animal virus diseases was published by Fulton (1958). In the present paper and the following one (Bradish & Brooksby, 1960) the separated U- and D-fractions of the virus system (Bradish, Brooksby, Dillon & Norambuena, 1952; Bradish, Henderson & Kirkham, 1960) were used as distinct antigens in investigations of antigen-antibody complex formation in the presence and absence of complement. The activities of the virus system are shown to be determined by those of its components, and, in the complement-fixation test, by the nature of the antiserum used and the test itself.

METHODS

Antigens and antibodies. The foot-and-mouth disease 'antigens' used in this work were prepared from suspensions in phosphate buffer (M/25; pH 7.6) of the vesicular epithelium from the plantar pads of infected guinea pigs or from the tongues of infected cattle. The highly infective suspensions were clarified by centrifugation in the Spinco L40 or C rotors to retain about 90 % of initial infectivity in the supernatant fluids and thus provide starting materials free from gross cellular debris. Such clarified starting materials were subsequently fractionated by ultracentrifugation to provide purified D-fraction

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tions of the 25 m μ infective component, and supernatant U-fractions of low infectivity of the 7 m μ complement-fixing component. These components and their methods of separation have been described previously (Bradish *et al.* 1952, 1960).

The complement-fixation test. This test was made as described by Brooksby (1952) except where departures are indicated in the text.

The complement used was normal guinea-pig serum freeze-dried on the day of collection. This was reconstituted to a 1/25 dilution in veronal buffer with added calcium and magnesium. Subdilutions were prepared to give a series containing 5.2, 8, 12, 18, 27 μ l. of normal guinea-pig serum per ml. One ml. of each was then added to a series of five reaction tubes for each antigen dilution to be tested.

The antisera were obtained from guinea-pigs convalescent from infection with the appropriate type of virus and hyperimmunized 3 months later by the intramuscular inoculation of a suspension of infective guinea-pig pad vesicular epithelium. The antisera were collected 10 days after hyperimmunization, inactivated at 56° for 30 min., and then either stored at -20° or freeze-dried and stored at room temperature. For use in the test 1/16 dilutions of the antisera were prepared in veronal buffer solution and 0.4 ml. added to the appropriate reaction tubes.

Each virus fraction to be titrated as antigen was diluted in phosphate buffer (M/25; pH 7.6) to provide three two-fold dilutions within the range from $\frac{1}{2}$ to 1/64. At each dilution 0.2 ml. was added to five reaction tubes containing graded doses of complement with constant excess antiserum.

The total volume of reaction mixture was 1.6 ml. throughout. The M/200 concentration of phosphate in the final reaction mixture was not anti-complementary. Phosphate buffer was used routinely in the purification of foot-and-mouth disease virus in view of the stability of infectivity in this medium and its lack of nitrogen.

In the 'short' or 'routine' test the mixtures were shaken and incubated at 37° for 30 min. before addition of the haemolytic indicator system. In the 'long' or 'overnight' test the mixtures were held at 4° for 16-20 hr. followed by 30 min. at 37° before adding the indicator. Mixtures with indicator were held for a further 30 min. at 37° before clarification and observation of optical density at 520 m μ in a photoelectric absorptiometer. Control mixtures in which the antigen, the antibody or both were replaced by buffer solution were included in every test.

The calculation sequence described by Brooksby (1952; optical density, percentage haemolysis, probit value, log. dose complement for 50% haemolysis, log. dose complement fixed) was replaced by that outlined in the next section.

The introduction of probits in complement-fixation work of the present type is an unprofitable complication since in the 15-85% range of haemolysis values used the conversion to probits may be regarded as a linear transformation; probit value = 0.028 (percentage haemolysis) + 3.6. The simplification of replacing the probit conversion by a direct linear interpolation of percentage

haemolysis introduces a maximum error of $\pm 1.3\%$ haemolysis which is negligible in comparison with other experimental errors.

A special slide-rule calculator or interpolation grid was employed for the rapid conversion of absorptiometer readings of optical density to complement dose for 50% haemolysis.

In practice the complement dose for 50% haemolysis was derived from the readings on the two or three adjacent tubes showing between 20 and 80% haemolysis. The final complement-fixing activity was derived from the paired observations of antigen dilution and complement dose for 50% haemolysis by the procedure shown below.

Assessment of complement-fixation data

It has been shown for the present system (Fig. 2) that within the limits of experimental error the quantity of complement fixed by antigen in antibody excess or by antibody in antigen excess is linearly related to the quantity of the limiting reactant. This is also apparent from the unit slope of much logarithmically plotted data (Brooksby, 1952; Le Bouvier, 1953; Fulton, 1958). It is an implicit assumption in this type of work that complement may be regarded as a stable indicator.

Consider a series of mixtures containing graded volumes of complement, antibody in excess and v_i ml. of an antigen sample of concentration G g./ml. We may now write the linear relationship

$$\alpha G = (V_i - V_0)/v_i, \quad (1)$$

where V_i is the volume of complement indicated for 50% haemolysis and V_0 the 50% haemolytic unit of complement indicated by control tests in which antigen is replaced by the diluent. In the present study volumes of complement (C') are expressed as microlitres (μ l.) of guinea-pig serum. The constant, α , defines the intrinsic complement-fixing activity of the antigen as μ l. C' /g. antigen. Thus αG is the complement-fixing activity of the antigen sample expressed in units of μ l. C' /ml.

In practice several volumes of antigen in a twofold dilution series ($v_i = \frac{v}{1}, \frac{v}{2}, \frac{v}{4}$) are set up with graded doses of complement and excess antibody. The corresponding volumes of complement (V_1, V_2, V_4) for 50% haemolysis are determined and provide paired values of v_i and V_i for entry into equation (1). The complement-fixing activity of the antigen samples (αG) may now be evaluated as the best fitting slope of the line relating complement volume for 50% haemolysis (V_i) to antigen volume (v_i). This line passes through V_0 at $v_i = 0$.

The best fitting value for the complement-fixing activity is calculated by dividing the sum of all ($V_i - V_0$) values by the sum of all corresponding v_i values. Thus

$$\alpha G = \frac{\Sigma(V_i - V_0)}{\Sigma v_i}. \quad (2)$$

Estimates of activity by this procedure are reproducible to better than

$\pm 10\%$. The failure of antibody to remain in adequate excess is shown by the approach of V_i to a limiting value as v_i increases. Only data for the initial linear portion of the V_i , v_i relationship should be included in equation (2).

The treatment of complement-fixing activity (αG) as the product of the intrinsic complement-fixing activity (α) by the concentration of the antigen (G) is of particular importance in specificity studies. This is shown in the following paper (Bradish & Brooksby, 1960).

These equations provide a rapid, non-graphical and objective procedure for the assessment of the available data. Through a series of antigen titrations employing the same antibody and complement the 'blank fixation' or haemolytic unit, V_0 , is constant and should be small (about $\times \frac{1}{10}$) compared with V_1 . A correction for the anti-complementariness of the antigen may be applied by subtracting from G the corresponding value (also calculated from equation (2)) obtained when antibody in the reaction mixtures is replaced by buffer solution. This correction is negligible in the present titrations of fractions of the virus system of foot-and-mouth disease.

With appropriate reinterpretation of symbols these equations may be applied to the data of antibody titrations in antigen excess.

RESULTS

Influence of incubation conditions

To investigate the effect of incubation conditions fractions of the virus system were mixed with excess antibody and complement and held at 4° for 0, 1, 4 and 20 hr. before incubation at 37° as indicated in Methods. The complement-fixing activities observed in a typical experiment are shown in

Table 1. *Influence of conditions of incubation on observed complement-fixing activity of virus fractions*

Fraction of virus system	Virus type	Type O-antiserum in excess			Type A-antiserum in excess		
		Short fixation test	1-4 hr. at 4°	Long fixation test	Short fixation test	1-4 hr. at 4°	Long fixation test
			followed by 30 min. at 37°			followed by 30 min. at 37°	
			Complement-fixing activity as μ l. C'/ml. undiluted fraction (cross-fixation ratio shown in parentheses)				
U-	O	8	25	150	5 (0.62)	14 (0.56)	120 (0.80)
U-	A	4 (0.31)	9 (0.21)	100 (0.56)	13	43	180
D-	O	3	5	10	0.2 (0.07)	0.3 (0.06)	0.4 (0.04)
D-	A	0.2 (0.10)	0.1 (0.03)	0.1 (0.02)	2	3	6

Table 1. The complement-fixing activities of the D-fractions increased by a factor of about 3 in 20 hr., whereas those of the U-fractions increased 25-fold in the presence of heterotypic antibody, and 16-fold in the presence of homotypic antibody. Thus the increase of activity after 20 hr. at 4° was 5 to 8 times greater for the 7 $m\mu$ component (U-fraction) than for the 25 $m\mu$ component (D-fraction).

Table 1 also demonstrates the specificity of these fractions. The cross-fixation ratio (the ratio of the complement-fixing activity in excess of the type heterologous serum to that in excess of type homologous serum) for the D-fractions in the short test was only 0.01–0.10, whereas that for the U-fractions was 0.20–0.80. Thus the activities of these fractions of the virus system differ both in specificity and in response to incubation. The problem of specificity is discussed more fully in the following paper (Bradish & Brooksby, 1960).

In further experiments a comparison was made between the short and long incubation procedures. Data for twelve experiments are shown in Fig. 1 as the ratio of the activity observed in the long incubation test to that observed in the short incubation test. The activity of the D-fraction increased during incubation by a mean factor of 2.0, whereas that of the U-fractions increased by a mean factor of 6.8. Thus the increase in the activity of the U-fractions was

Fig. 1. Relative activities of U- and D-fractions of virus system in long and short complement-fixation tests

at least 3 times greater than that of the D-fraction. This significant difference between the behaviour of the two fractions indicates that the behaviour of an unfractionated sample must be interpreted in terms of its content of the 7 and 25 $m\mu$ components. Despite an increase of 6-fold or more in total fixation, due largely to the heterotypic U-fraction, the sensitivity of the long incubation test as a means of typing is inferior to that of the short incubation test. This is demonstrated further in the following paper (Bradish & Brooksby, 1960). It is clear that the results of a complement-fixation test are greatly influenced by the manner in which the test is conducted, especially with a virus system of at least two distinct components.

Titration of antibody with antigen in excess

In the previous section fractions of the virus system were titrated in the presence of excess antibody provided by 1/16 or 1/32 dilutions of hyper-immune guinea-pig serum. The complementary data for antibody titrations in a 3- to 6-fold excess of antigen are shown in Fig. 2*b* and Table 2. The proportionality between quantity of complement fixed and quantity of antibody present is similar to that for antigen titrations (Fig. 2*a*).

Fig. 2. Typical complement-fixation data obtained in reactions of a single guinea-pig antiserum (O-type) with various virus fractions as antigen: (a) titration of antibody in excess; (b) titration of antibody with antigen in excess.

Table 2. *Antibody titrations of clarified antisera in presence of excess of separated fractions of the homotypic virus system*

Type, strain and batch of antiserum tested	Excess antigen			Antibody activity ratio: <u>U-detectable activity</u> <u>D-detectable activity</u>
	U-fraction	D-fraction	(U + D) or clarified starting material*	
Antibody activity (ml. C'/ml.)				
O/V1/166	13.5	3.7	17.6	3.6
O/V1/164	17.2, 20.3, 18.2 19.8	5.4, 7.6, 5.8, 7.0	—	3.2, 2.7, 3.1, 2.8
C/GC/163	8.6, 7.0	7.8, 5.6	—	1.1, 1.3
O/V1/155	3.0, 2.8	3.1, 3.0	—	1.0, 0.9
O/V1/138	14.7, 20.3	40.3, 53.9	60.0*, 77.2*, 97.2	0.36, 0.38
O/V1/77	12.0	11.0	30.0*	1.1
C/GC/76	25.0	18.0	44.0*	1.4

Antibody activity in the presence of unfractionated starting material as excess homologous antigen was greater than that in the presence of either U- or D-fraction separately. Antibody activity in the presence of a mixture of the homologous U- and D-fractions as excess antigen approximated to that in the presence of unfractionated starting material and to the sum of the

activities separately observed in the presence of the U- and D-fractions. In these experiments the concentrations of the U- and D-fractions in mixtures were equal to their concentrations when used singly.

The ratio of the antibody activity detectable in the presence of excess U-fraction to that detectable in the presence of excess D-fraction was essentially constant for each batch of antiserum but varied from batch to batch. Table 2 shows ratios of U-detectable activity to D-detectable activity ranging from $\frac{1}{4}$ to 4.

These data indicate that immune sera contain either separate antibodies or separate antibody sites corresponding to the 7 and 25 $m\mu$ components of the virus system. The 25 $m\mu$ component (D-fraction) in excess combines type specifically with antibody, whereas the 7 $m\mu$ component (U-fraction) in excess shows only 4 to 5 times greater fixation with homotypic than with heterotypic antibody. It appears from these data and those of the following paper (Bradish & Brooksby, 1960) that a typing test will be more sensitive when the unknown antigen is used in excess for titration of known antisera than when the unknown antigen is titrated in the presence of excess known antisera. A further refinement is the titration of known antisera in the presence of excess of the separated D-fraction of the unknown antigen. This is illustrated in Fig. 2.

Titration of complement-fixing activity in regions of antigen-antibody equivalence

Consideration of the intermediate region of antigen-antibody equivalence, in which neither reactant is in effective excess, is necessary in order to establish the limits of antibody-antigen ratio within which simple proportional relationships are valid interpretations of complement-fixation data. The results of complement-fixation titrations by the short incubation test of constant volumes of the U- or D-fractions are shown in Fig. 3. The volumes of undiluted antibody (u) and antigen (v) in the reaction mixture are expressed by the antibody-antigen ratio u/v which increases from zero, at antigen excess, to 0.2 at antibody excess. The standard antibody excess in routine antigen titrations corresponds to $u/v = 0.13$. The volume of complement fixed is shown as a fraction of that fixed in the presence of excess antibody, $C'_{\max}$. The ratio $C'/C'_{\max}$ rises steeply (Fig. 3) at low antibody-antigen ratios and turns sharply to assume near maximum fixation for antibody-antigen volume ratios above 0.04. Within the limits of experimental error this reaction between u ml. of antibody (of intrinsic complement-fixing activity β and concentration B) and v ml. of antigen (of intrinsic complement-fixing activity α and concentration G) may be expressed by the equation

$$C' = V - V_0 = \alpha v G \left\{ 1 - \exp \left(- \frac{\beta u B}{\alpha v G} \right) \right\}.$$

This reduces to the linear forms quoted earlier when either antigen or antibody passes into limited excess. No arbitrary constants are introduced. The solid line in Fig. 3 shows this exponential relationship for the value $\beta B/\alpha G = 88$,

which indicates that in this experiment 1 ml. of antibody titrated in antigen excess fixed about 83 times more complement than 1 ml. of antigen titrated in antibody excess.

These data imply that when samples of antigen and antibody are titrated separately, each in excess of the other, and then adjusted by dilution so that their complement-fixing activities are the same, the direct mixture of such nominally equivalent dilutions will show only about 60% of the complement-fixing activity observed when the same dilutions of antigen or antibody are titrated each in excess of the other. The results of a direct test of this condition

Fig. 3

Fig. 4

Fig. 3. Influence of composition of reaction mixture on level of complement fixation.

Fig. 4. Levels of complement fixation observed at nominal equivalence: ○○, D-fractions of virus system as antigen; ●●, U-fractions of virus system as antigen.

are shown in Fig. 4. Antigen and antibody samples were diluted to equal complement-fixing activity (nominal equivalence) following preliminary titrations of each in excess of the other. These dilutions of antigen and antibody were then mixed and the complement-fixing activity observed. The observed fixation was only $58 \pm 14\%$ of that observed in the preliminary 'excess' titrations and corresponds well with the result of 64% anticipated by the above relationship. It is of interest to note that when Heidelberger's (1956) treatment of the precipitation reaction is applied to the present experiment in complement fixation, the anticipated fixation at 'nominal equivalence' is 75% of the maximum fixation observed in titrations with one reactant in excess.

It appears that the condition of nominal equivalence, indicated by equal complement-fixing activity in preliminary 'excess' titrations, differs from the condition of actual equivalence when neither reactant is in excess. Actual equivalence in a mixture, for example, of constant antibody with variable antigen, may be defined as occurring at that minimum antigen concentration which gives effectively maximum complement fixation. A decrease in the concentration of either antigen or antibody then decreases the degree of complement fixation. Actual equivalence requires about 3 to 5 nominal equivalents of the limiting reactant.

Since antigen or antibody cannot be in infinite excess in practical experiments, the use of proportional relationships for the calculation of antigen or antibody activity in terms of complement-fixing activity introduces a systematic underestimation of activity which increases as the degree of excess of, say, antibody in an antigen titration, is diminished. This error of underestimation amounts to about 17 N %, where N is the maximum concentration in nominal equivalents of the limiting reactant in the titration. Thus, if this error must not exceed 10 %, the concentration of the limiting reactant must not exceed 0.6 nominal equivalents at the lowest dilution used in the test. This corresponds with a maximum antibody-antigen volume ratio (u/v) of about 0.007 in the experiment of Fig. 3 and confines attention to the closely linear initial part of the curve.

Antigen-antibody combination in the absence of complement: serum absorption

An alternative approach to the study of equivalence and antibody complexity was made by estimating the antibody activity of immune sera by titration in antigen excess before and after a part of the antibody had combined with known amounts of added antigen. Serial dilutions of clarified homotypic antisera were mixed with equal volumes of the U- or D-fractions of the virus system. These mixtures, of antigen-antibody volume ratio from 8 to 450, were held at 25° for 1 hr. before deposition of antigen-antibody complexes by ultracentrifugation in the 8 ml. tubes of the Spinco C rotor at 12 G (35,000 rev./min. for 50 min.). The upper two-thirds of the supernatant fluids were sampled with a fine J-shaped capillary pipette and the free antibody titrated in the presence of added excess antigen. Deposition of complexes was complete without significant decrease of the free antibody activity of the supernatant fluids (see next section). Typical experimental data are shown in Fig. 5. The line shows the initial absorption anticipated from the equivalence of antibody activity in antigen excess ($\beta B = 2.5$ ml. $C'/\text{ml.}$) and antigen activity in antibody excess ($\alpha G = 35$ $\mu\text{l.}$ $C'/\text{ml.}$). In this case 1 ml. of antiserum was nominally equivalent to 71 ml. of antigen and $f = \frac{1}{71} (v/u)$ defined the anticipated fraction of antibody absorbed. This was in satisfactory agreement with that observed. Further typical data are shown in Fig. 6. Thus the results of antigen and antibody titrations may be related directly to those of adsorption experiments.

In experiments in which the complexing and testing antigens were the same, all detectable antibody was ultimately absorbed by a sufficient excess of antigen. Figure 6 also illustrates other experiments in which homologous antisera were complexed with the U- or D-fractions of the virus system before centrifugation, and the titration of residual antibody activity by complement-fixation tests in the presence of excess of both the U- and D-fractions. The levels of U-detectable and D-detectable antibody were thus titrated before and after absorption by the U- and D-fractions. The results indicate that effectively all of the U-detectable antibody activity was removed by absorption by the D-fraction. Similarly, the D-detectable antibody activity was

almost completely absorbed by the U-fraction. These properties, taken with those discussed in relation to Table 2, suggest a system in which all antibodies are capable of combining, more or less rapidly, with all antigens. One group of antibodies, or antibody sites, reacts more avidly with the 25 $m\mu$ component of the D-fraction, while another reacts more avidly with the 7 $m\mu$ component of the U-fraction.

Not more than about 10% adsorption of initial antibody activity was observed following complexing with the U- and D-fractions of the heterologous virus system. This result conforms with the type specificity of combination observed for the D-fractions in direct titrations by complement fixation with antibody in excess. The almost type-specific adsorption of antibody by the U-fractions in excess is distinct, however, from the heterotypic combination observed in direct complement-fixation tests with antibody in excess. This is the subject of further investigations.

Fig. 5

Fig. 6

Fig. 5. Adsorption of antibody by homotypic U-fraction of virus system. The dotted line shows the initial adsorption anticipated from preliminary titrations of antibody in antigen excess and of antigen in antibody excess.

Fig. 6. Adsorption of antibody under various conditions. The dotted lines show the initial adsorptions anticipated from preliminary titrations of antibody in antigen excess and of antigen in antibody excess: 1, antibody complexed with D-fraction, tested with D-fraction; 2, antibody complexed with D-fraction, tested with U-fraction; 3, antibody complexed with U-fraction, tested with D-fraction; 4, antibody complexed with U-fraction, tested with U-fraction.

Sedimentation properties of the antigen-antibody complex

In the previous section ultracentrifugation at 12 G was employed to deposit the antigen-antibody complexes and thereby provide supernatant samples containing free antibody activity. Particular significance attaches to the rate of complex sedimentation since this is an indication of the nature of the complex formed.

Samples of the U-fraction derived as before from guinea-pig or tissue culture sources, were mixed with equal volumes of 1/4 dilutions of normal serum or homotypic or heterotypic antiserum. Control mixtures contained phosphate

buffer in place of serum. Following a combination period of 1 hr. at room temperature the mixtures were distributed in the 8 ml. tubes of the Spinco C rotor and centrifuged for up to 50 min. at 35,000 rev./min. (G 12; see Bradish *et al.* 1952, 1960). Standard 6 ml. samples of supernatant fluid were then withdrawn carefully with J-shaped capillary pipettes and titrated for complement-fixing activity in the standard manner. Serum antibody was already present to excess in all except the normal serum and phosphate buffer control mixtures. Fig. 7 shows the results obtained in a number of experiments. The complement-fixing activities of the supernatant fluids, expressed as fractions of those of the uncentrifuged control mixtures, fell below $\frac{1}{2}$ following centrifugation at 2.5 G and below $\frac{1}{4}$ at 6 G and 1/10 at 12 G. Complexes formed by the

Fig. 7. Sedimentation properties of 7 m μ component of U-fraction before and after complexing with antibody. U-fraction of O-type virus system held for 1 hr. at room temperature with an equal volume of: 1, phosphate buffer; 2, normal serum at $\frac{1}{4}$; 3, 5, homotypic antiserum, O-type, at $\frac{1}{4}$; 4, heterotypic antiserum, C-type, at $\frac{1}{4}$.

U-fractions with either homotypic or heterotypic antibody appeared to sediment at essentially the same rate. Mixtures of the antigen with normal serum or phosphate buffer showed no significant diminution of complement-fixing activity in supernatant fluids obtained below 8 G and retained at least $\frac{3}{4}$ of the initial activity at 12 G. Thus the antigen-antibody complex sedimented 8 or more times more rapidly than the initial free 7 m μ component of the U-fraction, and almost as rapidly as the free 25 m μ infective component of the D-fraction.

These data for the small complement-fixing component indicate that the mass of the average complex with antibody may be as much as 30 times greater than that of the single free antigen. This corresponds with an increase of the equivalent free particle diameter from about 7 m μ to at least 15 or 20 m μ . If it may be assumed that the antibody is a bivalent immune globulin and that the antigen has a valency of 10 then the average massive complex contains about 13 antigen molecules combined with about 40 antibody molecules.

These tentative calculations depend upon many obvious assumptions but illustrate the magnitude involved in considerations of the nature of this antigen-antibody complex.

Similar preliminary experiments with the D-fractions as antigens showed no significant difference between the sedimentation properties of free and complexed antigen, but these experiments must be extended in view of the ready disintegration of the 25 m μ component. If such disintegration occurs during complexing then the 7 m μ component produced will form a complex with antibody different from that formed with the intact 25 m μ component.

DISCUSSION

Most conclusions in this work are based upon complement-fixation data and the attendant assumption that complement itself may be regarded as a stable system which does not affect the reaction fundamentally. This is equivalent to the assumption that the same antigen-antibody complexes are formed in the presence or absence of complement. In support of this assumption it has been shown that the combining proportions of antigen and antibody in homotypic mixtures are similar in the presence and absence of complement.

Under the conditions of the present experiments no dissolution of the antigen-antibody complex in regions of antigen or antibody excess was observed. More recent experiments with Mrs J. O. Farley have shown that inhibition of fixation may be significant if one equivalent of antibody combines with more than 6 equivalents of antigen. The degree of fixation is halved at an antigen excess of about 15 equivalents. These data will be presented in a later paper. It is evident that in the present system of low antigen concentration the antigen-antibody complexes formed, with or without complement, must be classified as 'soluble' in the sense that all studies relate to a suspended phase and that precipitation only occurs as sedimentation during ultracentrifugation. In this respect the present data are more immediately related to those obtained by light-scattering methods (Bradish & Crawford, 1960) than to those obtained in precipitation or gel-diffusion studies.

The data obtained by titration and absorption studies demonstrate a complexity of the antibody system which may be interpreted in terms of two distinct antibodies or antibody sites. It is probable that the 'antibody' which reacts more avidly with the infective 25 m μ component (D-fraction) may be identified with the neutralizing antibody investigated by *in vivo* methods. The 'antibody' which reacts more avidly with the 7 m μ component (U-fraction) may be detectable only by *in vitro* methods. These diverse *in vitro* and *in vivo* reactions justify further investigation in view of their interpretation in terms of the structure and properties of the virus system.

The authors wish to thank Miss A. Cumming, Miss J. Rogers, Mr E. Scoates, and Mr W. Painter for their help in much of the complement-fixation work described, and Miss H. Wigman and Mr T. Burrows for assistance with the ultracentrifugation procedures.

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(Received 13 August 1959)