The Fixation of Complement by Virus-Antibody Complexes: Equivalence and Inhibition in the Reactions of the Viruses of Tomato Bushy Stunt and Foot-and-Mouth Disease with Rabbit and Guinea-Pig Antisera

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SUMMARY

A sensitive and simply calculated complement-fixation test is described in which viral antigen and antibody react to completion for up to 90 min. at 37°C before the addition of one 70% haemolysis unit of complement. The test has been applied to reactions between the components of the virus system of foot-and-mouth disease, tomato bushy stunt virus, bovine plasma albumin and their rabbit or guinea-pig hyperimmune sera. In the restricted region of proportional fixation each µl of complement represents about $3 \times 10^8$ virus particles and $6 \times 10^{11}$ molecules of albumin. In suspensions of foot-and-mouth disease virus of highest infectivity ($10^{10}$ mouse ID$_{50}$/ml.), probably less than 1 in 30 of the characteristic 25 mp particles are infective.

Quantitative data define the regions of antibody excess, equivalence and antigen excess, and show that complement is bound as a secondary process to that of antigen/antibody complex formation, that complement is bound only by relatively massive complexes and that the independent formation of unrelated complexes in the same system may be sensitively detected. The location of the optimum reaction and the confirmation of equivalence allow fixation data to be related closely to parallel data on the neutralization of infectivity. Since the concentrations of antigen and of antibody for optimal fixation are almost independent, it is concluded that the maintenance of equivalence in terms of a constant antigen/antibody ratio is not a valid principle for the interpretation of such data.

INTRODUCTION

Recent comparative studies by neutralization of infectivity and by fixation of complement of the foot-and-mouth disease virus system (Bradish & Farley, 1960; Bradish, Farley & Ferrier, 1962) have shown a close quantitative correspondence between these distinct manifestations of a common antigen/antibody interaction. The definition of the optimum reaction is of particular significance in this correlation. Most studies of neutralization and complement fixation in animal virus systems have been restricted to reactions in the region of antibody excess; few data have been related to block-assays covering the critical regions of optimum reaction in which certain features are most apparent. The present work parallels that on the neutralization of infectivity and investigates, through complement fixation, the antibody-combining properties of the viruses of foot-and-mouth disease and tomato bushy stunt, particularly in and beyond the regions of antigen/antibody equiva-
lence. Concurrent experiments with bovine serum albumin as antigen illustrate the
generality of the pattern of reactions described. It is shown that particular condi-
tions with respect to the relative concentrations of reactants must be satisfied before
a meaningful titration of antigen or of antibody can be completed, and before the
properties of the antigen/antibody system can be resolved from those of the comple-
ment assay system. Reference may be made to Kabat & Mayer (1961) and Rice
(1959b, c, 1960) for discussions of earlier studies.

METHODS

Preparation of antigens. The 25 mp infective component and 7 mp non-infective
component of the virus system of foot-and-mouth disease were separated from
guinea-pig pad vesicular fluid or epithelium suspension by procedures described
elsewhere (Bradish, Brooksby, Dillon & Norambuena, 1952; Bradish, Henderson &
Kirkham, 1960). The concentrates of the 25 mp infective component contained up
to $10^{10}$ mouse ID50/ml.

Tomato bushy stunt virus was available as a purified concentrate through the
courtesy of Drs C. A. Knight and G. Rushizky (Virus Laboratory, Berkeley,
California, U.S.A.). This preparation was standardized by electron microscopy and
by observation of ultraviolet (u.v.) absorption at 260 mp and it was diluted to the con-
centrations required in the appropriate buffer solutions. Bovine plasma albumin frac-
tion V as a dry powder was obtained from the Armour Pharmaceutical Company Ltd.

Estimation of virus infectivity and concentration. The infectivity of suspensions of
foot-and-mouth disease virus was determined by the intraperitoneal inoculation of
serial 10-fold dilutions into unweaned white mice of the Pirbright strain (Skinner,
1951). The volume of inoculum was 0.03 ml. and five or six mice from randomized
litters were inoculated at each dilution; the scores of dead and paralysed mice were
counted up to the third day. The concentration of 50 % infective units (ID50/ml. of
undiluted sample) was calculated by the method of Reed & Muench (1938).

The concentration of characteristic particles in suspensions of each virus was
determined by direct counting, by means of electron microscopy (RCA type EMU-
2B), of the number of such particles in at least 10 microdrops. The mixture of virus
and the 88 mp polystyrene marker was sprayed on to the mounting films by means
of a special low-pressure totally enclosed glass gun. The procedures generally fol-
lowed those already described (Backus & Williams, 1950; Breese & Trautman, 1960).

Preparation of antisera in guinea-pigs and rabbits. Guinea-pigs were hyperim-
munized against foot-and-mouth disease virus and their antisera collected and
stored in the manner described by Brooksby (1952). Rabbits (1.7–2.3 kg. initial
weight) were immunized by the inoculation of the fractions described below into the
marginal vein of the left ear. Daily inoculations of 1 ml. virus suspension in phos-
phate buffer (0.04 M, pH 7.6) continued for 10 days. After a rest for 11 days and
starvation for 24 hr, about 30 ml. blood were collected from the right ear of each
animal. Following separation of clot, the antisera were inactivated for 30 min. at 56°
and then stored at $-20^\circ$ in 1 ml. samples. The courses of immunization were repeated
on the same basis after a rest period of at least 6 weeks. These procedures in rabbits
follow those described by Matthews (1957).

The inocula for immunization of rabbits were as follows. Foot-and-mouth disease
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Virus: preparations of the 25 μm infective component of appropriate virus type isolated as defined in the previous section; each ml. of inoculum contained about $10^7$ mouse ID 50 and $10^{10}$ characteristic particles. Thus, in 10 days, each rabbit received about 1 μg. of virus.

Tomato bushy stunt virus: rabbits received daily 4 mg. for 10 days.

Bovine plasma albumin: 4 mg. in 1 ml. daily for 10 days.

Complement-fixation test. The reagents used in this test were generally those described by Brooksby (1952). Pooled normal guinea-pig serum was the source of complement (C') and this was freeze-dried or stored frozen at $-20^\circ$C. All dilutions and controls were made in veronal buffer (0.005M, pH 7.6) with added calcium and magnesium (Mayer, Osler, Bier & Heidelberger, 1946). The haemolytic indicator was a suspension of sheep erythrocytes maximally sensitized by 4 minimal haemolytic units of haemolysin. Haemolysin and cell suspension were thoroughly mixed and incubated for 80 min. at 37°C immediately before addition to the antigen + antibody + complement mixture, also at 37°C. The haemolytic indicator was adjusted to contain $45 \times 10^6$ cells/ml. by preliminary dilution of the red cell suspension. The final mixture of 1 ml. indicator + 1.8 ml. reaction (antigen + antibody + complement) mixture then gave 50% haemolysis for 50–60% light transmission at 520 μm in the matched 1 cm. diameter tubes of the Coleman Junior Spectrophotometer. A maximum sensitivity of observation of percentage haemolysis was thus obtained in the subsequent test.

The complement-fixation test used was initially the '50% haemolysis procedure' of Wadsworth, Maltaner & Maltaner (1938) as described by Brooksby (1952) and used in previous studies of components of the foot-and-mouth disease virus system (Bradish, Brooksby & Tsubahara, 1960; Bradish & Brooksby, 1960). However, the need to analyse many antigen/antibody mixtures covering wide ranges of reactant concentrations demanded a diminution in the quantities of reactants required per tube and in the number of tubes to be set up with each reaction mixture. Studies of reactions in antigen excess were particularly restricted by the available quantities of virus fractions since only about 10 μg./week of the separated 25 μm component of the virus system of foot-and-mouth disease was available.

The following 'haemolysis-depression' procedure was therefore developed; this is an extension of the procedure proposed by Stein & van Ngu (1950). Each reaction mixture was prepared with a constant volume of complement rather than with the 5 or more graded volumes of complement used in the earlier 50% haemolysis procedures. The constant volume of complement used in these studies was that required to produce 70% haemolysis (65–75%) of the red cells in 1 ml. of standardized indicator system defined above. The 70% haemolysis point was selected as being conveniently high on the central linear region of the haemolysis sigmoid curve, yet below the region of sharp curvature above 80% haemolysis. The 70% haemolysis dose of complement was determined for each experiment by a preliminary standardization (made as in Fig. 1) of a 1/50 or 1/100 dilution of pooled normal guinea-pig serum. An appropriate further dilution containing one 70% haemolysis dose/ml. was then prepared for use in the main experiment. The main experiment always included a re-standardization of the final dilution of complement. Since, in the range from 20% to 80% haemolysis, the percentage haemolysis increases essentially linearly with the volume of available complement, it follows that any 'fixation' of
Table 1. Distribution of reactants in titration of antigen in presence of optimal* concentration of homologous antibody

<table>
<thead>
<tr>
<th>Tube number and contents</th>
<th>Antigen titration and controls</th>
<th>Antiserum Complement control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Compensating volume of veronal buffer (ml.)</td>
<td>0</td>
<td>0-4</td>
</tr>
<tr>
<td>Hyperimmune serum at optimal* dilution (ml.)</td>
<td>0-4</td>
<td>0</td>
</tr>
<tr>
<td>Complement standardized† for 70 % haemolysis in control tubes (ml.)</td>
<td>1-0</td>
<td>1-0</td>
</tr>
<tr>
<td>Volume (ml.) and dilution* of antigen taken</td>
<td>0-4 at 1/50</td>
<td>0-4 at 1/100</td>
</tr>
<tr>
<td>Equivalent volume of undiluted antigen taken (µl.)</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Standardized haemolytic indicator (ml.)</td>
<td>1-0</td>
<td>1-0</td>
</tr>
<tr>
<td>Optical density† of clarified supernatant fluid (o.d.)</td>
<td>(A)</td>
<td>(A,70)</td>
</tr>
<tr>
<td>Depression of optical density (Δ o.d.)</td>
<td>((A,70 - A))</td>
<td>((B,70 - B))</td>
</tr>
</tbody>
</table>

* (1) The appropriate dilution of antiserum for optimal fixation of complement is determined by preliminary tests in which tables of this type are extended to include a range of antiserum dilutions from 1/50 to about 1/500 or beyond. The appropriate dilutions of viral antigens usually lie between 1/10 and 1/500.

† (2) The standardized complement produces 70 % haemolysis in the 2-8 ml. total volume if no fixation occurs. This corresponds with the optimal density \(D\,70\) for the complement control tube no. 8. If the antiserum is anti-complementary the optical density for tube no. 7 is less than \(D\,70\) although at the dilutions of antiserum usually required no significant difference is detected. Similarly, if the antigen is anti-complementary at the dilutions tested, the optical densities \(A\,70\), \(B\,70\), \(C\,70\) for the control tubes no. 2, 4, 6 are less than \(D\,70\). Thus, the optical density depressions (Δ o.d.) shown in the table include corrections for any anti-complementariness of the corresponding antigen dilutions.

(3) The mean optical density depression due to fixation of complement in the proportional region is obtained from these data as

\[
\Delta \text{ o.d. per } \mu\text{l. of undiluted antigen} = \frac{(A\,70 - A) + (B\,70 - B) + (C\,70 - C)}{8 + 4 + 2}.
\]

(4) In pre-incubation procedures the complement is added to the systems after completion of the primary reaction between antigen and antibody. The titration and calculations are otherwise unchanged.
complement by antigen/antibody complexes will cause the percentage haemolysis now observed (x on line B in Fig. 1) to be below 70 % (s on line B) by an amount proportional to the volume of complement fixed (ΔC'). The operational basis of this procedure is developed in the next section.

Table 1 illustrates the scheme of mixtures and controls required for an antigen titration by the haemolysis-depression procedure. In this case a constant and ‘optimal’ concentration of serum antibody is shown. All reactants were kept in iced water during the preparation of the mixtures and were added in the order shown. The reaction mixtures and controls of 1.8 ml. total volume (1.0 ml. complement + 0.4 ml. each of antibody and antigen dilutions) were prepared in duplicate in the matched 1 cm. diam. tubes of the spectrophotometer. In the ‘30 min. test’ the reaction mixture was incubated at 37° for 30 min. before addition of 1 ml. haemolytic indicator system, also at 37°. After further incubation for 30 min. at 37° the samples were centrifuged to remove intact red cells and the optical densities of the clear supernatant fluids observed at 520 μ. The final observation of the optical density of the supernatant fluid was reproducible to better than ± 5 % at the central point. The ‘pre-incubation’ procedure is defined in a later section.

Since the antigens, and occasionally the antisera, were slightly anticomplementary, the corrected depression of optical density for a given reaction tube was obtained by subtracting its optical density value from that of the appropriate ‘antigen-only’, or ‘antibody-only’, control. The method for calculation of results is shown in Table 1; it is equivalent to that followed in previous investigations (Bradish, Brooksby & Tsubahara, 1960) using the earlier procedure of ‘50 % haemolysis’.

**Interpretation of complement-fixation data in terms of haemolysis depression.** Consider a series of reaction mixtures of constant volume V (2.8 ml.) containing virus + antisera + complement with 1 ml. standardized haemolytic indicator. The haemolytic indicator contains N red cells/ml. After incubation and centrifugation the supernatant fluids show the optical densities D₁₀₀, D₇₀, D₀, D₀, corresponding with the percentage haemolysis, 100, 70, H, 0.

We may now write

\[ N = K(D_{100} - D_0) \cdot V, \]

(1)

where the constant K defines the number of red cells in 1 ml. which gives an optical density of 1.0 at 100 % haemolysis. The value \( K = 225 \times 10^6 \) red cells/ml. was obtained when dilutions of suspensions of washed sheep red cells were counted on a Fuchs–Rosenthal slide before haemolysis in distilled water and observation of optical density.

Control tubes without virus or antiserum show 70, 50, H % haemolysis when the volumes of complement C'70, C'50, C' ml. are included (Fig. 1). The empirical equation of von Krogh (Kabat & Mayer, 1961) now relates the volume of complement to the percentage haemolysis through the expression

\[ C' = C'50 \left( \frac{H}{100-H} \right)^{\frac{1}{n}}, \]

where \( n \) is a constant of value near 4. Over the experimentally significant central region from 20 to 80 % haemolysis, this relationship differs by less than ± 2 % from the linear form

\[ C' = C'50 \left( 1 + \frac{H-50}{25 \cdot n} \right). \]
This equation is illustrated by the data of the typical complement standardizations shown in Fig. 1. All such complement standardization lines cut the axis of haemolysis at the value \((50 - 25n)\%\). In 40 standardizations of the type shown in Fig. 1, the mean value of \(n\) was 3.60 ± 0.5%. The volume of complement for 50% haemolysis \((C'50)\) in this standardized system varied in different batches of guinea-pig serum over the range 2 to 10 μl.; the median value was about 6 μl. If now \(C'70\) indicates the initial complement volume contained by every tube (point \(s\) on line \(B\) in Fig. 1) and \(C'\) the residual complement volume (point \(x\)) observed after fixation of the volume \(\Delta C'\) by antigen/antibody complexes, then we may write, using equation (2),

\[
\Delta C' = C'70 - C' = \frac{C'50}{25\cdot n}(70 - H).
\]

or, in terms of the actually observed optical densities,

\[
\Delta C' = \frac{4C'50}{n} \frac{D70 - D}{D100 - D0}.
\]

The \(N\) red cells in the reaction mixture are 50% haemolysed by the complement volume \(C'50\). It follows that, under these reaction conditions, the volume of complement fixed by antigen/antibody complexes is equivalent to that required for the 50% haemolysis of \(F\) red cells, according to the equation

\[
F = \frac{\Delta C'}{C'50} N.
\]

Combination of expressions (1), (4) and (5) now gives

\[
F = \frac{4K \cdot V}{n} (D70 - D).
\]

This number of red cells which, at 50% haemolysis, bind as much complement as that bound by the specific complexes, is thus obtained directly from the depression of optical density \((D70 - D)\) by multiplication by the method constant \(4K \cdot V/n\). Using the numerical data quoted above, this constant has the value \(7 \times 10^8\), and is essentially independent of the concentration of complement and red cells in the reaction mixture.

In the present report, later fixation data are given in terms of the directly observed depression of optical density \(\Delta \text{o.o.d.} = (D70 - D)\). Previous reports from this laboratory have quoted experimental data in terms of units of complement fixed but this is less absolute than the present statement of \(F\) or of optical density depression. The simple numerical equivalence between these methods of presentation is defined by equation (4). The simplicity of this treatment of the sensitive linear region of the sigmoid response curve will be noted.

RESULTS

Quantitative aspects of antigen titration

*Foot-and-mouth disease virus.* The titration of antigen in the presence of excess antibody is a major application of the complement-fixation reaction. The proportionality between the volume or mass of reacting antigen and the volume of complement fixed has been shown for many systems and provides the basis for the
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quantitative assessment of such titration data (Rice, 1946, 1947, 1959a, b, c, 1960). This proportionality appears as an initial linearity in the plot of optical density depression against volume or mass of antigen (Fig. 10). For the systems investigated here, however, this simple linear relationship is valid only over a relatively narrow range of antigen/antibody ratio. Outside this range, as discussed in following sections, the extent of fixation is limited or inhibited by either antigen or antibody.

When attention was confined to the titration of antigen in the presence of a nearly optimal content of antibody, data of the type shown in Fig. 2 were obtained. Such data define the maximum fixation of complement for a given mass of antigen.

Fig. 1. Standardization of two normal guinea-pig sera (A and B) as sources of complement. The indicated volumes of complement (1 ml. of normal serum diluted 1/50 = 20 μl.) were made up to 1.8 ml. with veronal buffer and incubated 30 min. at 37° with 1 ml. standardized haemolytic indicator. The supernatant fluids were then clarified and their optical densities observed. Percentages of haemolysis were calculated from the mean optical density for two tubes. Points on line $B$ indicates that one 70% haemolysis unit of complement is contained by 1 ml. of the normal serum at a dilution of about 1/100. At a dilution of 1/250 the serum A contains one 70% haemolysis unit of complement in 1 ml. If 1 ml. of a standardized complement yields 70% haemolysis in control tubes but only $H$% haemolysis when incubated with an antigen/antibody mixture (Table 1), then the corresponding points $s$ and $x$ on line $B$ indicate that the volume of complement $C' s - C' x = \Delta C'$ has been fixed.

Fig. 2. Relationship between infectivity, complement-fixing activity and absolute number of 25 mμ virus particles in 24 separations from guinea-pig pad vesicular fluid and epithelium.

The antigen used in the experiments was the infective 25 mμ component of the foot-and-mouth disease virus system (type O/VI) isolated from guinea-pig pad vesicular fluid and epithelium. Each 1 ml. of antigen suspension represented the virus yield from the lesions on the ten plantar pads of five guinea pigs. The homologous guinea-pig antisera, at dilutions from 1/30 to 1/300, were mixed with complement and antigen and incubated for 30 min. at 37°. The data for 24 virus suspensions of extreme infectivity are shown in Fig. 2 in terms of the number of 25 mμ particles required for each ml. of complement fixed (upper line) and for each mouse infective unit (lower line). The number of 25 mμ particles for each ml. of complement fixed was essentially constant at $10^{9.3 \pm 0.1}$, regardless of the wide range of initial infectivity from $10^6$ to $10^{10}$ ID50/ml. A linear histogram of the same data is given in Fig. 3. The
complement-fixation procedure may evidently be used to provide an estimate of the absolute count of $25 \mu$ particles on the basis of $2 \times 10^9$ particles/µl. complement fixed.

In contrast to the complement-fixation data, the number of $25 \mu$ particles for each mouse ID$_{50}$ (Fig. 2) decreased steadily as the infectivity of the virus suspension increased. Preparations of highest infectivity showed about 300 $25 \mu$ particles/mouse ID$_{50}$. These data relate only to the intraperitoneal route of inoculation in unweaned mice (Pirbright strain), which was shown to be up to 1 log. unit less sensitive than the intramuscular route (Heatley, Skinner & Subak-Sharpe, 1960).

It follows that, in the most infective suspensions, one mouse ID$_{50}$ by the intramuscular route might represent as few as 30 virus particles or even less, for infectivity assays of even higher efficiency. Bachrach & Breese (1958) observed particle/plaque ratios of 80–1000 in their studies of virus concentrates derived from cultures of bovine kidney cells. Such data are unrelated to the ‘purity’ of the preparations.

The slope of the lower line of Fig. 2 indicates that the virus suspensions contained about $10^{12}$ particles/ml. regardless of the initial infectivity. This implies that the epithelium from each guinea-pig pad yielded $10^{12}$ characteristic $25 \mu$ particles, of which, if every infective particle initiated infection, any proportion up to 1 in 30 might be infective. Thus, even in the most infective preparations of $10^{10} \sim 10^{11}$ID$_{50}$/ml., at least 97% of the specific antigenic mass due to $25 \mu$ particles was non-infective or failed to initiate infection, and could be detected only by observation of complement fixation or other in vitro activity. Similar observations have been made for poliomyelitis virus (Mayer et al. 1957). Although the accountable infective component cannot contribute significantly to the observed fixation of complement, a coincidental correlation between infectivity and degree of complement fixation might be observed if, in the range of samples investigated, the concentrations of the infective component happened to be about proportional to the concentrations of the non-infective antibody-combining components.

*Tomato bushy stunt virus and bovine plasma albumin.* Tittrations by complement fixation of these antigens were made under the same conditions as those defined in the previous section for the $25 \mu$ component of foot-and-mouth disease virus. The specific rabbit antisera were present at nearly optimal concentrations and incubation of the reaction mixture with complement continued for 30 min. at $37^\circ$ (short test).

The complement-fixation data obtained are summarized in Table 2. Each entry relates to maximal fixation of complement by a given mass of antigen in the presence of nearly optimal antibody concentration. The mean maximal fixation extended only from 14 to 50 µl. complement/µg. antigen despite the use of many preparations of different antigens and antisera. Thus, a single conversion factor of 30 µl. complement/µg. antigen or 3 A o.d./µg. antigen summarizes to within a factor of 2 the data for the three systems entered in Table 2.

For the small ‘spherical’ particles of foot-and-mouth disease and bushy stunt viruses, each µl. normal guinea-pig serum as complement (Δ o.d. = 0.10) was equivalent to about $8 \times 10^9$ particles. At 50% haemolysis each red cell in the haemolytic indicator system presents a complement-binding capacity equivalent to that of about 40 virus particles or 9000 molecules of bovine plasma albumin. Since the present procedure is sensitive to the fixation of less than 0.3 µl. complement
Table 2. *Maximal fixation of complement by three antigens in presence of optimal concentration of homologous antibody*

<table>
<thead>
<tr>
<th>Antigen and molecular weight</th>
<th>Antiserum and dilution</th>
<th>Maximal fixation</th>
<th>Mean maximal fixation activity</th>
<th>Molecules of antigen equivalent to one red cell at 50% haemolysis (equation 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 Kd component of foot-and-mouth disease; ~7 x 10⁶</td>
<td>Guinea pig at 1/30 to 1/300</td>
<td>See data of Figs. 2 and 3</td>
<td>50</td>
<td>1-5 x 10⁹</td>
</tr>
<tr>
<td>Tomato bushy stunt virus: 9 x 10⁶</td>
<td>Rabbit at 1/100 to 1/3000</td>
<td>1.00 1.32 1.46</td>
<td>1.58 1.58 1.62</td>
<td>1.66 1.97 2.27</td>
</tr>
<tr>
<td>Bovine plasma albumin: 7 x 10⁴</td>
<td>Rabbit at 1/300</td>
<td>0.86 1.01 1.37</td>
<td>1.55 2.12</td>
<td></td>
</tr>
</tbody>
</table>
(± 0·03 C'50 unit or ± 0·03 in optical density), it follows that 0·01 µg. antigen may be detected under appropriate conditions of maximal fixation. The fixation data published by Mayer and colleagues (1957) for purified preparations of poliomyelitis virus showed that about 10^{10} physical particles corresponded to one C'50 unit, or about 2 × 10^9 particles/µl. complement. This conforms closely to the data of Table 2, despite great differences in procedure.

**Time of incubation of reaction mixtures**

In previous studies from this laboratory, the mixtures of complement + antibody + antigen (Table 1) were placed together in the reaction tube and incubated for 30 min. at 37° before addition of the haemolytic indicator and further incubation for 30 min. at 37°. However, preliminary tests on the rate of complement uptake by preformed complexes showed that, although complement was absorbed completely within 20–30 min. at 37° (Barbaro & Becker, 1962), the actual formation of the antigen/antibody complexes responsible for fixation was not always complete at this time. Figure 4 illustrates experiments in which antigen and antibody were allowed to react for periods of up to 3·5 hr at 37° before addition of standardized complement and further incubation for 30 min. at 37°. The addition of the stan-
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Standardized haemolytic indicator and observation of the optical density of the supernatant fluid then followed, as already described. The data of Fig. 4 show that the fixation of complement, or depression of optical density (Δ o.d.), was not complete in the then standard 30 min. incubation period, but that a pre-incubation of antigen and antibody for at least 1·5 hr at 37° was often necessary if a maximal reaction in terms of complement fixation was to be assured. Under conditions of lower antigen content, the terminal fixation was then 3 to 4 times greater than that observed in the '30 min. test'.

These experiments extend the previous observations on this system (Bradish et al. 1960) that the fixation of complement is secondary to that of complex formation, and that essentially as much complement is fixed by pre-incubated complexes to which complement is added later as is fixed by the same reactants when these are incubated with complement from the start. In equilibrium experiments like those of Fig. 4 it is, in any case, undesirable to incubate complement for the whole period with the other reactants since haemolytic activity will decay appreciably during the necessarily longer periods of incubation. For this reason, the standardized complement at 37° was added to all reaction systems only for the last 30 min. of incubation. This also served to minimize further the anti-complementariness of the reactants, which increased with time of incubation with complement.

A 'pre-incubation' test in which mixtures of antigen + antibody were incubated for up to 1·5 hr at 37° before addition of the '70 % haemolysis' unit of complement for a further 30 min. at 37° was adopted in the following experiments; the preparation and composition of reaction mixtures otherwise followed the scheme of Table 1. The use of a 'pre-incubation' procedure in place of the '30 min. test' ensured that maximal fixation of complement by equilibrated complexes was observed under conditions of extreme antigen or antibody excess.

The observation through complement fixation that under many conditions the formation of antigen/antibody complexes only reached completion in about 1·5 hr at 37° contrasted sharply with parallel observations through neutralization of infectivity (Bradish et al. 1962), which indicated that complex formation was essentially complete within 10 min. at 37°. This disparity suggested that the neutralization of infectivity was complete when only small early complexes of antigen + antibody had been formed. At this stage, the specific complex was still too small to show significant fixation of complement; the larger complexes required for maximal fixation of complement only appeared much later.

Inhibition of fixation by antigen excess

It was shown in a recent report concerning complement fixation and neutralization of infectivity (Bradish & Farley, 1960) that the fixation of complement was inhibited completely when a constant dilution of guinea-pig or rabbit hyperimmune serum reacted with the 25 mμ component of foot-and-mouth disease virus at a sufficiently high concentration. Similar inhibitions of complement fixation occurred when the antigen was the 7 mμ component of the virus system. The complexes formed in the virus/antibody reactions thus showed fixation properties which were similar to those observed for many non-viral systems. The present comparative data for the viruses of foot-and-mouth disease (Fig. 5) and tomato bushy stunt (Fig. 6) in combination with constant antibody show that, in the initial region of low antigen
content, the fixation of complement increased with mass of available antigen. This is the titration region of proportional fixation discussed in an earlier section. Reaction mixtures of higher antigen content showed a fixation of complement which was below that required by the initial linear relationship, through partial inhibition by slight antigen excess. With yet higher concentrations of antigen (Figs. 5, 6), the extent of fixation passed through a maximum value and was then depressed to a negligible value. The value of complement fixation at the maximum is defined by the antibody content of the system and occurs at a definite equivalent concentration of antigen. In general, the degree of fixation is 50% of the maximum value at an antigen concentration of 2 to 3 equivalents, and is negligible at an antigen concentration of 5 to 10 equivalents.

The maximal fixation of complement/unit mass antigen occurred only in the initial proportional region where antibody was in adequate excess with respect to antigen. The maximal fixation of complement for unit mass of antibody occurred at the peak of fixation (Figs. 5, 6) where antigen was in slight critical excess with respect to antibody. Thus, antigen and antibody cannot be present simultaneously under conditions appropriate to their individual maximal fixation activities. This is illustrated by the second curves (filled circles) of Figs. 5 and 6, which show that the maximal fixation of complement with respect to antigen occurred for significantly lower concentration of antigen than that for the maximum of total fixation (open circles).

The confirmation of antigen excess

Direct tests of the actual availability of free antigen or of antibody-combining sites in reaction mixtures of higher antigen content than that which gave maximal fixation of complement were set up as shown in Fig. 7. Dilutions of the separated
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7 mp (curves A and B) and 25 mp (curves C and D) components of foot-and-mouth disease virus (type A/GB) were mixed with homologous guinea-pig antiserum at dilutions 1/150 or 1/300. After incubation for 30 min. at 37°, the 70 % haemolysis unit of complement was added with and without a further portion of antiserum. The fixation test continued in the standard manner after a second incubation for 30 min. at 37°. The data of Fig. 7 for the control mixtures without the second addition of antiserum (curves B and D) showed the usual maxima of fixation with later inhibition by excess antigen. When second additions of antiserum were made (curves A and C) the degrees of fixation were unchanged at antigen concentrations below the optimum, but were significantly increased in the regions to the right of the fixation maxima. It therefore appeared that only in such regions was antigen in excess and free to combine with later additions of antibody and to fix more complement. In regions to the left of the fixation optima (Fig. 7) the addition of complement and more antiserum did not increase the degree of fixation. Thus antibody was already in effective excess in this region. It seems probable that, in the region of 'antigen excess' to the right of the fixation maximum, the antigen was present as small complexes with antibody which were able to bind more antibody and thereby fix more complement. An increasing proportion of free uncombined antigen became available only at higher excess of antigen. Identical conclusions follow from parallel studies of the reactions of other foot-and-mouth disease viruses, tomato bushy stunt virus and bovine plasma albumin with their specific rabbit antisera.

Cross-reactions in regions of antigen excess

Since the present studies are concerned with complement fixation by specific antigen/antibody complexes, it was necessary to confirm that fixation by one antigen/antibody system was uninfluenced by reactions in a second system present in the same medium (Hoyle, 1945–46; Fulton, 1958; Rice, 1959a, b, c). If specific cross-reactions between two systems are to be detected by complement fixation, these reactions must occur because the antigens and antibodies involved are related and not because hybrid complexes are formed through the non-specific association or entanglement of unrelated complexes.

The independent formation of unrelated complexes was tested through the reactions of a mixture of two rabbit hyperimmune sera with a mixture of homologous antigens. The mixture of rabbit anti-sera contained anti-bushy stunt serum at 1/64,000 and anti-foot-and-mouth disease virus serum at 1/32. These dilutions were established by preliminary titrations as being suitable for the separation of the anticipated fixation peaks. Samples of the mixture of antisera were incubated with dilutions of a suspension of bushy stunt virus, dilutions of a suspension of the 25 mp component of foot-and-mouth disease virus (type O/VI) and dilutions of a mixed suspension containing these two antigens at the same concentrations. After 30 min. at 37°, the 70 % haemolysis dose of complement was added and the test continued in the standard manner.

The results of this experiment are shown in Fig. 8. The reaction with foot-and-mouth disease virus (F) or tomato bushy stunt virus (B) resulted in a typical fixation curve with maximal fixation of complement for 50 and 3 μl. of antigen, respectively. The reaction with the mixed antigens (M) indicated a composite fixation which approximated to the total of the fixations due to each antigen when
used separately. Thus each antigen/antibody system was effectively responsible for a definite fixation of complement; and neither the formation of specific complexes nor the subsequent fixation of complement by such complexes was modified by the simultaneous reactions of other antigens. The curve of composite fixation (M) shows the maximal fixations due to each component of the mixture of antisera almost as well as these are shown by the individual reaction curves (F and B). Similar data are shown in Fig. 9 for the mixed system of foot-and-mouth disease virus + bovine plasma albumin, reacting with their homologous rabbit antisera.

The consequence of reactions between related antigen/antibody systems is shown in Fig. 10. The antigens in this experiment were the separated 7 and 25 mp components of the foot-and-mouth disease virus system (type O/VI). These antigens were mixed in the proportions shown by the volume scales in Fig. 10. Homologous guinea-pig antiserum at an optimum dilution of 1/40 replaced the mixture of antisera of the previous experiments. After incubation for 30 min. at 37° the fixation by the system containing the 7 mp component as the only antigen followed the almost proportional relationship previously discussed. The incorporation of 0.25-1 pl. of the 25 mp component in mixtures with the 7 mp component did not disturb this proportionality. The fixation of complement was greatly decreased, however, by
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the incorporation of 2 μl. of the 25 mμ component and wholly suppressed by the incorporation of 4 μl. Similar data were obtained when increasing proportions of the 7 mμ component were mixed with the 25 mμ component before reaction with antiserum.

Such data indicate that the 7 and 25 mμ components of the foot-and-mouth disease virus system were competitive antigens which reacted with common antibodies in guinea-pig and rabbit antisera in accordance with their relative concentrations and activities. Such hyperimmune sera apparently contained no significant proportion of antibody which reacted with only the 25 mμ or the 7 mμ component of the virus system. These data extend those of earlier reports (Bradish et al. 1960; Bradish et al. 1962).

Fig. 9. Demonstration of independent fixation of complement by two systems reacting simultaneously. Mixed rabbit antiserum used throughout contained anti-bovine plasma albumin serum diluted 1/300 and foot-and-mouth disease antiserum diluted 1/15. Pre-incubation of reactants for 1-5 hr at 37°C before addition of complement.

\( A \): Reaction with dilutions of solution of bovine plasma albumin, optimum at 0.0063 pl. or 0.18 μg.

\( M \): Reaction with mixture of antigens \( P \) and \( A \) above adjusted to give same concentrations as in reactions with single antigens.

Fig. 10. Demonstration of competition for available antibody by 25 mμ and 7 mμ components of foot-and-mouth disease virus system (type O/VI). A constant 0.4 ml. hyperimmune guinea-pig serum at dilution 1/40 reacts for 30 min. at 37°C with 0.8 ml. of a series of mixed antigens prepared from fractions of the virus components. The scales show the final content of each antigen in the reaction mixtures. The 70% haemolysis dose of complement was added for a final 30 min. at 37°C.

Proportions of antigen and antibody in relation to extent of fixation

The discussion of antigen/antibody reactions in terms of the combination of a constant concentration of one reactant with a series of concentrations of the other requires extension to the general case in which both reactants are used in a single experiment over a range of concentrations. Figures 11 and 12 show fixation data of this kind for a series of reaction mixtures containing up to 9 μg. tomato bushy stunt virus and up to 1.6 pl. rabbit antisera. Such data are representative of other experiments with bovine plasma albumin or the components of the foot-and-mouth
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disease virus system. In Fig. 11 each curve represents a single antibody content and in Fig. 12 a single antigen content. The constant initial slope of the family of curves in Fig. 11 characterizes the fixation due to antigen as limiting reactant in presence of a slight excess of antibody; such data have been discussed in an earlier section. The subsequent inhibition of fixation by excess of antigen is shown clearly by the curves for the lowest contents of antibody. At higher concentrations of antibody, with antigen still in excess, the fixation of complement was limited by the content of antibody, but was not proportional to the concentration. An overall maximum of fixation occurred for a reaction mixture containing 1 μg. virus and about 0·4 μl. rabbit antiserum.

![Antiserum vs. Mass of virus](image1)

**Fig. 11**

Figs. 11 and 12. Fixation characteristics for reaction of tomato bushy stunt virus with hyperimmune rabbit serum. Incubation of 0·4 ml. of each reactant for 30 min. at 37° before and after addition of 70 % haemolysis dose of complement in 1 ml. In Fig. 11 the data are shown as curves of constant antiserum content and in Fig. 12 as curves of constant virus content. The scales show the actual content of virus in μg. and of undiluted antiserum in μl.

In Fig. 12 the curves of constant antigen content do not show a common initial slope, indicative of the antiserum activity, since fixation was inhibited by the initial excess of antigen. At the highest values of antigen content the initial fixation was completely depressed and the activity of antiserum was indicated only by the steepest initial slope for the curves of lowest antigen content. Later fixation was limited by the antigen content of the reaction mixtures. The fixation of complement in this reaction series was proportional to the antigen content only below 0·14 μg.; the overall maximum of fixation is shown as in Fig. 11.

Figures 11 and 12 show that a given degree of fixation may be shown by many different values of antigen and antibody concentration. A quantitative titration of the concentration of either reactant required that attention be confined to the initial linear region in which the extent of fixation was limited proportionally by the concentration of one reactant and was not limited or inhibited by the concentration of the other. Similar data for the 25 mPas infective component of the foot-and-mouth disease virus system (type O/VI) are shown in Table 3. The significant inhibition of complement fixation by excess of rabbit antiserum or guinea-pig antiserum has been regularly observed, but the effect seems to be more frequent with rabbit antiserum.
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It is apparent from these data (Table 3; Figs. 11, 12) that, although the maximal fixation observed in the presence of a given concentration of antiserum occurred at a definite concentration of antigen, an increased concentration of antiserum did not correspond at the new optimum with a proportionally increased concentration of antigen. If a law of simple proportionality was applicable, then the maximal fixations in the rows and columns of Table 3 would lie on a single diagonal, and there would be no overall maximum of fixation. All of these data indicate that the concentrations of antigen and antibody giving maximal fixation of complement cannot be related by a law of simple proportions. The optimal concentrations of antigen and antibody are, in fact, almost independent since the maximal fixation of complement is given by an almost constant concentration of antigen regardless of the antiserum dilution employed. The concentration of antiserum for maximal fixation is similarly very little influenced by variation of antigen concentration.

The formation of complexes in the presence and absence of complement

In these experiments the 25 μ infective component of foot-and-mouth disease virus used as antigen was separated from the supernatant fluids of cultures of baby hamster kidney cells. Complexes with homologous guinea-pig antiserum were formed in the presence and absence of the 70 % haemolysis unit of complement. After incubation for 1.5 hr at 37° these reaction mixtures were centrifuged in Spinco L 40 tubes for 1 hr at 30,000 rev./min. Supernatant fluids of 5 ml. volumes were then carefully withdrawn from the 8 ml. volumes loaded, and taken forward for fixation assay together with non-centrifuged controls. Samples containing no complement received the 70 % haemolysis unit after ultracentrifugation and all samples were then incubated for a further 30 min. at 37°. The 'virus-only' controls received antiserum and complement after ultracentrifugation.

The data of Fig. 13 show that, when complexes were formed in the presence of complement, the supernatant fluids (F) and non-centrifuged controls (E) retained the same high degree of fixation activity. Thus the presence of complexes with bound complement (23) did not contribute to the haemolysis by free complement. When complexes were formed in absence of complement, the activity of the supernatant fluids (D) was eliminated although the non-centrifuged control (C) showed a fixation activity like that of the complexes formed in the presence of complement (E, F). Thus, in F the complexes responsible for fixation were removed together with their bound complement, whereas in D the complexes were removed before complement was added. When virus only was centrifuged (B) the activities of the supernatant fluids were almost eliminated, whereas those of the non-centrifuged controls (A) were almost the same as those of the complexes formed in presence or absence of complement (E, F, C). Hence, as in experiments with bushy stunt virus as antigen, complement was effectively bound by preformed 'soluble' complexes.

The irreversibility of complex formation and complement binding

The irreversibility of complex formation and complement binding was tested by forming antigen/antibody complexes under optimal conditions and adding to these equilibrated mixtures more antigen in excess. Incubation and addition of complement then continued as defined previously. For the foot-and-mouth disease virus and bovine plasma albumin systems quoted in Table 4, the reactions under optimal
Table 3. Complement-fixation data for infective 25 μm component of foot-and-mouth disease virus separated from suspension of guinea-pig pad vesicular epithelium (type O/VI) in reaction with homologous rabbit and guinea-pig antisera

<table>
<thead>
<tr>
<th>Volume of antigen in reaction mixture (μl.)</th>
<th>Rabbit antiserum (μl.)</th>
<th>Guinea-pig antiserum (μl.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Depression of optical density Δ o.d.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>0.12</td>
<td>0.17</td>
</tr>
<tr>
<td>8</td>
<td>0.13</td>
<td>0.21</td>
</tr>
<tr>
<td>16</td>
<td>0.18</td>
<td>0.27</td>
</tr>
</tbody>
</table>

The lines in the table show the positions of the α-optimal (horizontal) and β-optimal (near vertical) reactions.

Table 4. Demonstration of irreversibility of complex formation following addition of antigen in excess

<table>
<thead>
<tr>
<th>Antigen (G)</th>
<th>Homologous Antiserum (S)</th>
<th>Reaction conditions</th>
<th>Reaction mixture (first incubation mixture shown in brackets)</th>
<th>Incubation time at 37° after each addition</th>
<th>Complement fixation as depression of optical density (Δ o.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension of 25 μm component of foot-and-mouth disease (type C/GC) from guinea-pig pads</td>
<td>Guinea-pig at dilution 1/150</td>
<td>Optimal proportions Initially optimal</td>
<td>(400 μl. G/16 + 400 μl. S) + 400 μl. buffer (400 μl. G/16 + 400 μl. S) + 400 μl. G (400 μl. G/16 + 400 μl. G) + 400 μl. S</td>
<td>30 min.</td>
<td>0.32</td>
</tr>
<tr>
<td>Solution of bovine plasma albumin, 100 μg/ml.</td>
<td>Rabbit at dilution 1/3000</td>
<td>Optimal proportions Initially optimal</td>
<td>(400 μl. G/30 + 400 μl. S) + 80 μl. buffer (400 μl. G/30 + 400 μl. S) + 80 μl. G (400 μl. G/30 + 80 μl. G) + 400 μl. S</td>
<td>90 min.</td>
<td>0.36</td>
</tr>
</tbody>
</table>

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conditions gave maximal fixation of complement. This degree of fixation was not disturbed by further incubation with excess of antigen. However, when the antiserum and the total excess of antigen were incubated together from the start, fixation of complement was decreased. Thus, once formed, the complexes responsible for the later optimal fixation of complement were not modified by continued incubation with antigen in excess. This effective irreversibility of the reaction between antigen and antibody paralleled that already shown as a feature of the neutralization of the infectivity of the foot-and-mouth disease virus. The dissociation of antigen/antibody complexes by modifications of medium introduces additional features not involved in the present considerations of irreversibility with respect to changes of reactant concentrations.

DISCUSSION

The most obvious conditions which define the reaction between antigen and antibody are the concentrations of the reactants; the reactant concentrations control the rate at which antigen/antibody complexes are formed and the nature of the reaction product at equilibrium. However, the rate effect cannot properly be investigated by complement fixation since the initial combination of antigen and antibody is not only more rapid than the binding of complement, but produces many small early complexes which fix little or no complement. Thus complement fixation procedures are restricted to studies of antigen/antibody combination at or near equilibrium, where the rate factors which arise from the distinct primary and secondary reactions are not involved.

In many of the experiments described here the complement system was added to the antigen/antibody mixture only after the completion of the primary reaction. It has been shown, subject to the stability of complement and the completion of the primary and secondary reactions, that the time at which complement is added to the primary reaction mixtures has little effect on the degrees of fixation subsequently observed. It thus became possible to impose experimental modifications upon the fundamental primary reaction without alteration to the standardized secondary reaction involving complement and haemolytic indicator.

The distinction between the complement-binding capacity of antigen/antibody complexes formed in the presence or absence of complement was summarized by Barbaro & Becker (1962). These authors studied the albumin/anti-albumin reaction and suggested that the greater fixation shown by complexes formed in the presence of complement was a unique property of rabbit antisera, since the reverse situation was observed with certain horse and sheep antisera. Although the present studies are not consistent with this conclusion, it may be pointed out that the fixation tests involved are very different. Barbaro & Becker (1962) used the procedure of Mayer, Osler, Bier & Heidelberger (1948) in which the reactants combine in the presence or absence of 50–100 C’ 50 units and are subsequently chilled and diluted before addition of the haemolytic indicator. Complement-fixation tests in virology use reactants at much lower concentrations; with a total complement load of usually not more than 5 C’ 50 units there is no dilution before addition of the haemolytic indicator. In the present work only one C’ 70 unit or about 1.2 C’ 50 units of complement was used.
Preliminary experiments with the bovine plasma albumin system have shown that the degree of fixation at the optimum is very similar by the present and by the high-complement-load procedures but that the sharpness of the fixation optimum increases markedly as the complement load is decreased to the present low value. Clearly it cannot be assumed that the complement-binding properties of complexes formed by reactants at low concentrations will correspond with those formed at high reactant concentrations.

The relative activities of antigen and antibody are reflected in the present complement-fixation data by sharp maxima of fixation. Such maxima occur for definite concentrations of antigen and antibody, which are thereby defined as optimal. Although limitations of antigen concentration have caused this feature of the reaction to be exa-

Fig. 13. Fixation of complement by preformed ‘soluble’ complexes in reactions of 25 μ component of foot-and-mouth disease virus (type O/M11, 10th passage on BHK cells) with an equal volume of hyperimmune guinea-pig serum at dilution 1/300. Sequence in reactions: A. (Virus + antiserum) + complement. B. (Virus supernatant + antiserum) + complement. C. (Virus + antiserum) + complement. D. (Supernatant fluid of (virus + antiserum) + complement). E. (Virus + antiserum + complement). F. Supernatant fluid of (virus + antiserum + complement). The round bracket indicates incubation for 1-5 hr at 37° and the square bracket for 30 min. at 37°. Virus and antiserum were present as 0.4 ml. each and complement as the 70% haemolysis dose in 1 ml.

Fig. 14. Pattern of reactions shown in complement fixation by virus/antibody complexes. ‘g’ and ‘G’ signify antigen concentrations below and above the α-optimum value. ‘b’ and ‘B’ signify antibody concentrations below and above the β-optimum value. The α-optimum is that antigen concentration giving maximal reaction against a constant antibody content. The β-optimum is that antibody concentration giving maximal reaction against a constant antigen content.

ried only rarely for animal virus systems (Mayer et al., 1957; Fulton, 1958; Bradish & Farley, 1960), it has long been recognized as a feature of the reactions of the small protein and bacterial antigens (Dean, 1912; Opie, 1923; Brown, 1934) and may be regarded as the demonstration by complement fixation of the classical precipitation curve (Heidelberger, 1956). Such parallel fixation and precipitation studies indicate the reactions of common antigen/antibody systems; distinct antibody species for each experimental procedure were not postulated. In appropriate tests the observations of complement-fixation reactions relate only to ‘soluble’ complexes, and differ
from those in precipitation or flocculation studies by responding to much lower concentrations of reactants and by not requiring the formation of gross insoluble complexes and the interpretation of a variety of non-specific physico-chemical factors. The distribution of antigen/antibody complexes formed in the system at equilibrium is interpreted as being determined by concentrations of reactants and by their valencies (Goldberg, 1952, 1953). This model has received some confirmation in virology through studies on tomato bushy stunt virus and its rabbit antiserum (Bradish & Crawford, 1960). In terms of this model, the region of limited antibody excess or near-equivalence is such that all reactive sites on the antigen are saturated by specific antibody, and the number of antigen/antibody bonds or of complexes formed will be approximately proportional to the concentration of antigen in the system. Since it has been shown that in this restricted zone the fixation of complement is proportional to the concentration of antigen, it thus appears that each virus particle or virus + antibody bond is associated with the fixation of a definite 'unit' of complement. Subject to restrictions related to the quality of the antiserum used, the viruses of foot-and-mouth disease, tomato bushy stunt and poliomyelitis all require about $3 \times 10^9$ particles for the maximal fixation of the complement content of 1 µl normal guinea-pig serum. About $6 \times 10^{11}$ molecules of bovine plasma albumin are required for this degree of fixation.

The region of antigen excess is characterized by a lower degree of complement fixation for mixtures of higher antigen concentration. Most reactive sites on the antibody are now saturated by specific antigen and the degree of fixation is controlled by the sizes of the complexes formed in relation to the degree of antigen excess. Since increasing antigen excess is associated with the elimination of the larger complexes, it appears that complement is bound effectively only by such complexes and not significantly by greater masses of antigen and antibody distributed as smaller complexes.

The interpretation of complement-fixation data in the region of the optimum presents some difficulties, since the results for different systems do not support the assumption that equivalent concentrations of antigen and antibody are proportional. The optimal fixation of complement occurs at a definite concentration of antigen which varies little with the constant concentration of antibody used in the test. Similarly, in tests with constant antigen content, the optimal fixation occurs at a definite concentration of antibody which is almost independent of the homologous antigen. In the nomenclature of plant virus serology (Matthews, 1957) this is equivalent to the statement that the $\alpha$-optimum line (value of antigen concentration giving maximal fixation in 'constant-antiserum' tests) and the $\beta$-optimum line (values of antibody concentration giving maximal fixation in 'constant-antigen' tests) are almost at right angles and intersect at that unique antigen/antibody mixture which gives the overall maximum of fixation; this situation is summarized in Fig. 14.

The dilution of a virus sample to its $\alpha$-optimum concentration allows maximal fixation against a range of dilutions of homologous antisera. Similarly, the dilution of an antiserum to its $\beta$-optimum concentration allows maximal fixation against a range of dilutions of homologous antigens. Such $\alpha$- and $\beta$-optimum dilutions constitute a sensitive indication of the combining activities of the antigen and the antiserum used, and indicate an alternative procedure for titration of these reactants and...
for standardization of the fixation test. The near-constancy of the optimal concentrations of antigen and antibody distinguishes the present complement-fixation data from the more familiar pattern of simple proportions which appears to characterize most gross flocculation data (Matthews, 1957; Belyavin, 1957; Smith, 1958) in which the $\alpha$- and $\beta$-optimum lines pass diagonally across the reaction area from the origin.

It is apparent that the intersection of the $\alpha$- and $\beta$-optimum lines (Fig. 14) divides the area of the antigen/antibody reaction into four zones of distinct fixation character. These zones may be considered as follows:

- **gB**: the zone of antibody excess in which antigen is the limiting reactant.
- **Gb**: the zone of antigen excess in which antibody is the limiting reactant.
- **$gb$**: the zone in which both antigen and antibody are present at sub-optimal concentration.
- **GB**: the zone in which both antigen and antibody are present at supra-optimal concentration.

Thus, for example, a fixation experiment with variable antigen and constant antibody represents a horizontal section of the reaction area with maximal fixation at the $\alpha$-optimum. The detailed character of such an experiment depends upon the 'quality' of the antibody and the choice of antibody concentration as low, optimal or high. At constant low antibody content the reaction zones $gb$-Gb indicate an initial fixation proportional to the antigen content (zone $gb$) which passes rapidly, after maximal fixation at the $\alpha$-optimum, into the region of antigen excess inhibition (zone Gb). These reaction zones have been discussed previously in relation to the neutralization of infectivity (Bradish et al. 1962). At constant high-antibody content in the zones gB-GB the initial fixation (zone gB) may be inhibited by antibody excess. The later fixation increases non-proportionally with the antigen content and, after maximal fixation at the $\alpha$-optimum, passes into the supra-optimal zone GB in which, again according to the antiserum used, the degree of fixation may remain near-maximal or decrease only slowly with increasing antigen content. These regions (gB-GB) typify the data previously published from this Institute in which relatively high concentrations of antiserum were used (Bradish et al. 1960). The marked inhibition of fixation by antigen excess is a feature of the zone Gb only. The data obtained by Mayer et al. (1957) for the poliomyelitis virus/rabbit antiserum system show many of these features.

Similar considerations apply to the vertical sections of the reaction area which represent fixation experiments with constant antigen and a range of antibody concentrations. However, the complexity of the reaction area shown in Fig. 14 indicates that the quantitative assay of antibody by complement fixation requires particular attention (Fulton, 1958; Rice, 1947, 1959b). Wallace, Osler & Mayer (1950) have emphasized that in the bovine serum albumin/rabbit antiserum system the degree of complement fixation is greatly influenced by the quality of the antiserum and does not relate simply to the quantity or content of antibody. These difficulties in the present systems are largely due to the limited range of antibody concentration over which fixation is proportional to the antibody content without being inhibited by antigen excess or limited by approach to antibody excess. The observed antibody activity (depression of optical density/pl. undiluted antiserum) is acceptable as an assay conclusion only if independent of the concentration of both antigen
and antibody over a significant range of these variables. Subject to adequate controls, the analysis of the data for this region of confirmed proportional fixation then follows as described for the titration of antigen. The most sensitive titration of antibody is thus available in the region of the $\alpha$-optimum line (shaded in Fig. 14), between the reaction zones gb and Gb. Similarly, and as previously discussed, the most sensitive titration of viral antigen is obtained in the region of the $\beta$-optimum line between the reaction zones gb and Gb.

Fulton & Almeida (1962) have discussed a procedure for titration of antiserum in antigen excess which depends upon a linear relationship between the amount of antiserum and the degree of complement fixation in this region (GB and Gb in Fig. 14). Fixation data for the systems studied here do not confirm this relationship as a basis for the titration of antiserum. These authors also discuss the selection of linear or logarithmic scales for the presentation of fixation data. In the present paper both scales have been used as a matter of convenience according to the experiment and the range of variables to be presented in the figure. The generally non-monotonic character of the fixation data is such that neither linear nor logarithmic scales have any special significance in relation to the description of the overall reaction. Over the limited regions of proportional fixation already discussed, the use of linear relationships and scales appears to be well justified.

The failure of the law of simple proportions in the correlation of fixation data blocks any statement about an absolute molecular equivalence of virus and antibody. Thus, the direct enumeration of virus particles by electron microscopy cannot be related through complement fixation to an indirect enumeration of antibody molecules. It is clear that fixation data may not be extended in terms of simple proportions or of constancy of antigen/antibody ratio to other conditions of reactant concentrations.

Notwithstanding the complexity of the fixation of complement by the antigen/antibody complex it has been shown that many features of the reaction may be indicated very sensitively by such methods. Thus, the state of irreversibility of the reaction, the recognition of independent or cross-combining systems, the indirect assay of virus particle concentration, the correlation of fixation with neutralization and adsorption data, the location of the optimum reaction and the confirmation of equivalence, all illustrate the information available beyond that of the routine assay or typing experiment.

Parallel studies on the components of the complex virus system of vesicular stomatitis (major infective component $175 \times 70$ m$\mu$) have confirmed that the present methods and general conclusions are not confined to the smaller solvent-insensitive viruses.

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REFERENCES


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