Linear relationships in complement fixation

By F. FULTON and J. O. ALMEIDA*

London School of Hygiene and Tropical Medicine

INTRODUCTION

Wadsworth, Maltaner & Maltaner (1931) have shown that, over a certain range, there is a linear relationship between the amount of complement required for 50 % lysis of the indicator system in a complement-fixation test and the amount of antiserum used, provided that the concentrations of antigen are adjusted so that maximal fixation occurs.

The straight line defined in this way may be called the antiserum maxima line. Similarly an antigen maxima line may be drawn.

Hoyle (1945–46) using influenza virus antigens, found a linear relationship between the logarithm of the amounts of complement fixed and the logarithm of the maximal antiserum or maximal antigen titres. Logarithmic co-ordinates have been used by many other workers to define antiserum and antigen titres in complement fixation tests (Fulton, 1958).

We shall show that the linear relationship with arithmetic co-ordinates and the linear relationship with logarithmic co-ordinates provide the same information in different ways.

LINEAR RELATIONSHIPS WITH ARITHMETIC CO-ORDINATES

The equation of a straight line may be written in the slope intercept form,

$$y = a + bx.$$

The parameter b is the slope of the line and the parameter a is the point where the straight line intercepts the y co-ordinate axis where $x = 0$.

I. $a = 1$

If x is the amount of antiserum in the primary mixture of a complement-fixation test and y is the number of units of complement required for 50 % lysis of the indicator system at the conclusion of the test, the antiserum maxima line can, in many cases, be defined by the equation,

$$y = 1 + bx.$$

Similarly if x is the amount of antigen, the antigen maxima line can often be defined by the same equation. When $x = 0$, one unit of complement is required for the lysis of half the erythrocytes in the indicator system.

* Present address: Departamento de Microbiologia e Imunologia, Faculdade de Medicina de Ribeirao Preto, Universidade de Sao Paulo, Brazil.

Since one of the parameters of the straight line is determined by the fixed intercept point, $y = 1$, the slope b is the unique characteristic of the reaction.

The numerical value of b can be used to define the titre of the antigen or antiserum.

In many cases, the linear relationship between x and y can only be demonstrated over a certain range. For example, if x is the amount of antiserum, the potency of the undiluted antigen may be insufficient to give the maximal fixation of complement of which the larger amounts of antiserum are capable. However, the maxima line may be extrapolated beyond the defined range to some arbitrary, fixed value of x, and the titre may be defined as the number of units of complement required for 50 % lysis of the indicator system with this fixed amount.

If an antigen or antiserum maxima line can be defined by the equation,

$$y = 1 + bx$$

there is also a linear relationship between the logarithm of the number of units of complement fixed maximally and the logarithm of the amount of antigen or antiserum.

For, $$\log(y-1) = \log b + \log x.$$

After the logarithmic transformation, the slope of the straight line is no longer a characteristic of the reaction, for the line always has unit slope. The line cuts the ordinate axis at the point,

$$\log(y-1) = \log b$$

and this intercept point is the unique characteristic of the reaction.

Thus, with these types of maxima lines, differences in slopes with arithmetic co-ordinates are transformed into differences in the position of the lines with logarithmic co-ordinates.

II. $a \neq 1$

(i) *Maxima lines: $a < 1$*. In many cases when an antigen or antiserum maxima line is defined with arithmetic co-ordinates, although there is a linear relationship between x and y when x is confined to a range of small positive values, if the line is extrapolated to the ordinate axis, the point of intercept is not $y = 1$.

The maxima line can be defined by the equation,

$$y = a + bx, \quad (a \neq 1)$$

Most commonly the intercept parameter is less than 1. An example, using influenza virus antigens and homologous guinea-pig antiserum is shown in Fig. 1. This antiserum maxima line is defined by the equation,

$$y = -1{\cdot}79 + 14{\cdot}25x$$

for values of x in the range,

$$0{\cdot}3125 \,\mu\text{l.} \leqslant x \leqslant 1{\cdot}25 \,\mu\text{l.}$$

Since when $x = 0$, 1 unit of complement is required for 50 % lysis of the indicator system, it follows that there is not a linear relationship between x and y over the range

$$0 \leqslant x < 0{\cdot}3125 \,\mu\text{l.}$$

In a reaction of this type using human serum and a cardiolipin antigen, Almeida (1958) has demonstrated the non-linearity of the relationship between x and y as the values of x approach zero (see Fig. 12 in the reference quoted).

The non-linear zone implies that with low concentrations of the reagents the complement fixation is inhibited. The numerical value of the parameter a is a measure of the extent of the zone of inhibition. If the titre of the antigen or antiserum is defined solely by the numerical value of the slope b of the linear part of the maxima line, the information implicit in the parameter a is lost.
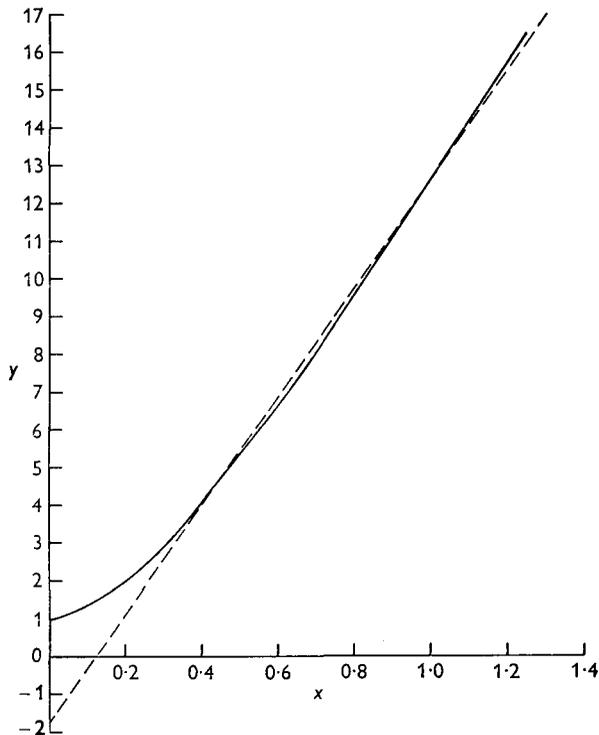

Fig. 1. Titration of an antiserum: arithmetic co-ordinates. y = units of complement required for 50 % lysis. x = μl. of antiserum.
Equation defining the maxima line:

$$y = a + bx$$
$$= -1 \cdot 79 + 14 \cdot 25x.$$

Equation defining the curve:

$$y = 1 + \alpha x^{\beta}$$
$$= 1 + 10 \cdot 96 x^{1 \cdot 5}.$$

If the antiserum maxima line illustrated in Fig. 1 is transformed to logarithmic co-ordinates, a linear relationship between x and y is defined by the equation,

$$\log(y - a) = \log b + \log x.$$

This line has unit slope and therefore the reaction is characterized by the intercept point, $\log b$. But, again, some information is lost by including the parameter a in the ordinate scale.

7   Hyg. 60, 1

(ii) *Maxima lines: a > 1*. Occasionally the intercept parameter of a maxima line is greater than 1. Since when $x = 0$, 1 unit of complement is required for 50 % lysis of the indicator system, there is, in this case also, a non-linear zone as the values of x approach zero.

Almeida (1958) gives an example of this type of reaction which occurs with highly avid systems that rapidly fix complement as soon as a very small amount of the antigen-antibody complex begins to appear in the primary mixture (see fig. 19 in the reference quoted).

(iii) *Complement fixation with antigen or antiserum in excess.* Almeida (1956) has shown that if an antiserum is titrated by complement fixation with a fixed concentration of antigen which is in excess for the whole range of antiserum dilutions, there is a linear relationship between the amount x of antiserum and the number, y of units of complement required for 50 % lysis of the indicator system.
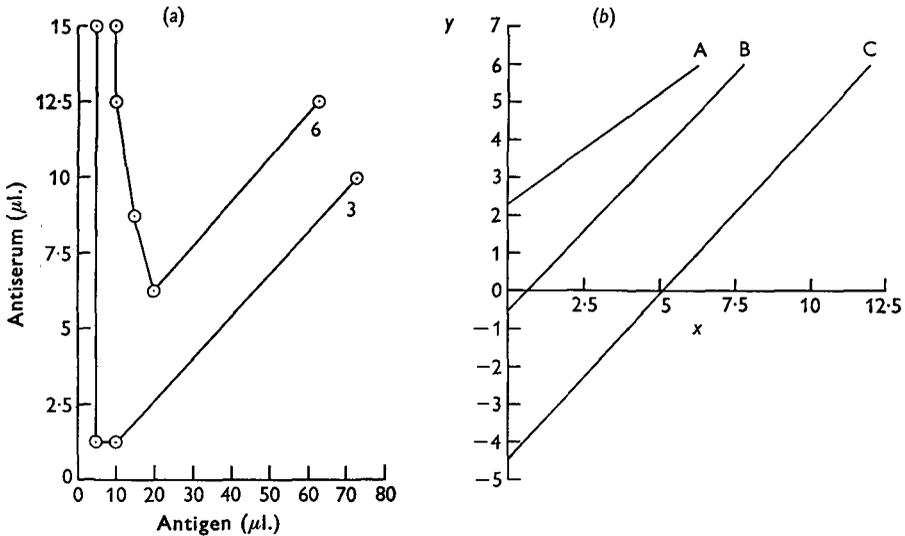

Fig. 2. (*a*) Isohaemolytic curves for 3 and 6 units of complement: arithmetic co-ordinates. (*b*) Titration of an antiserum: arithmetic co-ordinates. y = units of complement required for 50 % lysis. $x = \mu$l. of antiserum. A. Maxima line. B. With 30 μl. of antigen. C. With 60 μl. of antigen.

The use of a fixed concentration of antigen in excess simplifies the test because a much larger number of reaction mixtures is required to locate the maxima points.

The antigen can only be used in excess within the range defined by a family of isohaemolytic curves where all the curves are parallel to one another; if they are not parallel, there will not be a linear relationship between x and y. If the iso-haemolytic curves are not only parallel to each other but also parallel to the corresponding co-ordinate axis (type I), the linear relationship defined in the excess zone will be identical with the maxima line. If, however, the parallel isohaemolytic curves are not parallel to the corresponding co-ordinate axis (type II), the numerical value of the intercept point, a, will depend on the concentration of the antigen. With a relatively large excess, the parameter a will take a large

negative value, but as the selected concentration of antigen is reduced, the value of the parameter a converges to the value of a for the maxima line. For any of the lines in the zone of antigen excess, the numerical value of the slope b' is the same, but is not necessarily equal to the slope of the maxima line. An example is shown in Fig. 2.

As in the case of maxima lines, the complement-fixation reaction is characterized uniquely by the slope, b' in the range of values of x where the linear equation is valid. But the numerical value of the parameter, a, is no longer necessarily a measure of the zone of inhibition.

Similar linear relationships can be demonstrated when antigens are titrated with excess antiserum.

Although lines in excess zones are more easily determined than maxima lines, the simplification is at the cost of the information about the extent of the non-linear zone.

LINEAR RELATIONSHIPS WITH LOGARITHMIC CO-ORDINATES

If an antigen or antiserum maxima line is defined by the equation

$$y = a + bx$$

there is also a linear relationship between $\log(y - a)$ and $\log x$, for

$$\log(y - a) = \log b + \log x.$$

This line has unit slope.

In the special case when $a = 1$, there is a linear relationship between the logarithm of the number, $(y - 1)$ of units of complement fixed and $\log x$, for

$$\log(y - 1) = \log b + \log x.$$

This line also has unit slope.

If the parameter a is not equal to 1, there is an excellent linear relationship between the logarithm of the number of units of complement fixed maximally and $\log x$.

This logarithmic maxima line is defined by the equation,

$$\log(y - 1) = \log \alpha + \beta \log x.$$

The slope of this line is β.

In Fig. 1, the antiserum maxima line with arithmetic co-ordinates is defined by the equation,

$$y = -1 \cdot 79 + 14 \cdot 25x.$$

But another antiserum maxima line with logarithmic co-ordinates is defined by the equation,

$$\log(y - 1) = 1 \cdot 04 + 1 \cdot 5 \log x.$$

This line is shown in Fig. 3.

A logarithmic maxima line is characterized by two parameters, $\log \alpha$ and β. To use all of this information, Fulton (1958) defined the titre of the antigen or antiserum as the dilution which will fix maximally one unit of complement. This dilution is located by extrapolating the logarithmic maxima line to the $\log x$ co-ordinate axis when $\log(y - 1) = 0$.

7-2

If $\beta = 1$, the intercept point is

$$\log x = -\log \alpha.$$

In this special case, $\alpha = b$, where b is the slope of the corresponding maxima line with arithmetic co-ordinates.

If $\beta \neq 1$, the intercept point is

$$\log x = \frac{-\log \alpha}{\beta}.$$

Here $\alpha \neq b$.

In the example in Fig. 3, the titre of the antiserum is

$$\log x = \frac{-1\cdot04}{1\cdot5} = \overline{1}\cdot306$$
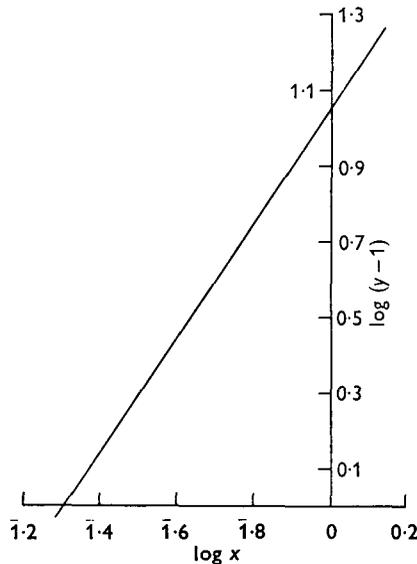
and so, $x = 0\cdot2023\,\mu\mathrm{l}.$

Fig. 3. Titration of an antiserum: logarithmic co-ordinates. $(y-1)$ = units of complement fixed, $x = \mu\mathrm{l}.$ of antiserum. Equation defining the logarithmic maxima line:

$$\log(y-1) = \log\alpha + \beta\log x$$
$$= 1\cdot04 + 1\cdot5\log x.$$

The component volumes of this complement-fixation test were $20\,\mu\mathrm{l}.$, therefore, the titre of the antiserum is $1/99$.

The introduction of the parameter $\beta \neq 1$ is a consequence of the non-linear zone in the reaction for small values of x, when arithmetic co-ordinates are used. For if the equation with logarithmic co-ordinates is transformed to arithmetic co-ordinates, a curve is defined for which $y = 1$ when $x = 0$. The equation of the curve is $y = 1 + \alpha x^{\beta}$. The logarithmic maxima line shown in Fig. 3 is reproduced in Fig. 1 after transformation to arithmetic co-ordinates. The steep part of the curve is congruous with the arithmetic maxima line. The titre of the antiserum derived from the arithmetic maxima line is $1/77$ ($0\cdot26\,\mu\mathrm{l}.$). The corrected titre, $1/99$, derived from the logarithmic maxima line, allows for the non-linear zone.

If the complement-fixation test is made by titrating antigen or antibody with an excess of the other reagent, the titre cannot be determined from the straight line defined by the equation

$$\log(y-1) = \log\alpha + \beta\log x.$$

For example, if an antiserum is titrated with an excess of antigen and a straight line is defined with arithmetic co-ordinates

$$y = a + b'x$$

the numerical value of the parameter, a, depends on the concentration of antigen, that is, the position of the line but not the slope of the line depends on the antigen concentration.

If logarithmic lines analogous to logarithmic maxima lines are drawn, both their positions and their slopes will depend upon the concentration of the antigen, and they do not define a unique titre.

However, it is easy to obtain a linear relation in excess zones using logarithmic co-ordinates, for

$$\log(y-a) = \log b' + \log x.$$

As before, the characteristic of the reaction is the intercept point, $\log b'$, but some information is lost by including the parameter a in the ordinate scale.

COMPARISON OF THE PRINCIPAL METHODS OF TITRATION

The titre of an antigen or antiserum derived from a logarithmic maxima line reflects both the characteristic slope of the complement-fixation reaction and the extent of the non-linear zone with small amounts of antigen or antiserum. However, maxima lines are difficult to determine and in many practical applications the characteristic slope of the reaction provides sufficient information; for these purposes, titration in excess zones is more convenient. For example, if an antiserum is titrated with a constant amount of antigen in excess, the characteristic slope of the reaction when arithmetic co-ordinates are used is unaffected by choosing different antigen concentrations over a wide range. In order to determine the corresponding maxima line, a precise amount of antigen must be selected for each antiserum dilution in the titration.

It is often necessary to titrate a number of antisera with one antigen. If maxima lines are defined, the antigen concentrations must be adjusted independently for each antiserum. If only the characteristic slope is determined a single antigen concentration may usually be chosen for the titration of all the antisera.

OUTLINE OF A COMPLEMENT-FIXATION TEST

A new design for a complement-fixation test defining the characteristic slope of an antigen-antiserum reaction is proposed. The antigen and the antiserum must not be anticomplementary over the range of dilutions used, and the specificity of the reaction must have been established.

The plate method of complement fixation is used (Fulton & Dumbell, 1949; Fulton, 1958).

Preliminary titration of complement

A geometric series of complement dilutions is prepared (log factor 0·05), using borate-saline as diluent (Wadsworth, 1947). For Maltaner & Almeida (1949) have shown that if either the antiserum or the antigen in the main test contains phosphate ions, anticomplementary effects may be observed if a diluent containing magnesium salts is used. 0·4 ml. of each complement dilution is transferred to a set of glass tubes and to each is added 0·8 ml. of diluent and 0·8 ml. of a 0·1 % suspension of sensitized sheep cells.

The total volume in the tubes is 2·0 ml. but since the concentrations of the reagents is the same as those used in the plate method when the total volume is only 0·1 ml., the unit of complement defined in the tubes is also the unit of complement required for the plates.

The set of tubes is placed in a water-bath at 37° C. for 45 min. and, with the unlysed cells in suspension, the degree of haemolysis is measured with a nephelometer.

The initial dilution of complement which lyses half the cells defines the unit.

Isohaemolytic curves

A geometric series of nine dilutions of antiserum and of nine dilutions of antigen is prepared in borate-saline. The series should have a log factor of about 0·2, but a larger factor may be chosen if a greater range must be covered.

Three plates are set out with the antiserum as the row variable and the antigen as the column variable. On each plate the tenth row and the tenth column are controls which contain no antiserum and no antigen, respectively.

For the first plate, the plate constant is 3 units of complement, for the second 6 units, and for the third 9 units of complement. The plates are kept overnight at 4° C. for primary fixation. Complement suffers very little deterioration when the dilution contains at least 3 units. When the plates are set out, the residue of the dilution of complement containing 3 units is preserved in a glass tube at 4° C. Next day, when the plates are removed from the refrigerator, this stored complement is titrated again on a fourth plate by setting out in triplicate, one, two and three drops of the dilution originally containing 3 units. Diluent is added so that each of the nine reaction mixtures is composed of three drops and contains 1, 2 or 3 units of complement.

The indicator system is added to all the plates which are then kept at 37° C. for 2 hr. Reactions are evaluated in terms of antigen or antiserum required for 50 % lysis, and a set of three isohaemolytic curves is drawn using logarithmic co-ordinates. In reading the plates the mixtures showing 50 % lysis should be recorded first by rows and then by columns.

The titration of antiserum

Choose a dilution of antigen in about the middle of the range where the three isohaemolytic curves are parallel to one another; this dilution is the plate constant for the titration of the antiserum. Prepare an arithmetic series of nine dilutions

of the antiserum covering the range indicated by the isohaemolytic curves for the chosen dilution of antigen.

Titrate the complement in glass tubes as before, and prepare dilutions containing 3, 6 and 9 units; keep these dilutions in an ice bath. On a plate, set out the antiserum dilutions in rows, and the complement in triplicate as the column variable; the tenth column is a serum control with 3 units of complement. Finally add the chosen dilution of antigen.

As before the complement dilution containing 3 units is preserved for titration next day. At the conclusion of the test, the reactions are evaluated in terms of the antiserum required for 50 % lysis with 3, 6 and 9 units of complement added initially.

Using as co-ordinates the number of units required for 50 % lysis and the corresponding amounts of antiserum, fit a straight line to the nine observed points using the method of least squares. The titre of the antiserum is defined as the slope of this line. Confidence limits can be derived by an analysis of variance. When a number of antisera are to be titrated with the same antigen, it will usually be possible to choose the antigen concentration to be used for all the antisera by reference to isohaemolytic curves of the antigen with one of the sera.

The titration of antigen

From a set of isohaemolytic curves, an antiserum dilution is chosen in about the middle of the range when they are parallel to one another. With this antiserum dilution as a plate constant, the row variable is an arithmetic series of antigen dilutions, and the column variable, as before, 3, 6 and 9 units of complement in triplicate.

SUMMARY

Over a certain range there is a linear relationship between the amount of complement required for 50 % lysis and the amounts of one of the variables in the primary reaction mixture of a complement-fixation test when the third variable is present in a quantity sufficient to allow maximum fixation.

If this linear relationship were maintained as the concentration of the selected variable is progressively reduced, there would also be a linear relationship between the logarithms of the amounts of complement fixed and the logarithms of the amounts of the selected variable; in this case, the line with logarithmic co-ordinates will necessarily have unit slope.

When the fixation of small amounts of complement by several virus systems is measured, an approximately linear relationship using logarithmic co-ordinates has been demonstrated, but in many cases the line does not have unit slope. In this range, therefore, there is not a linear relationship when arithmetic co-ordinates are used.

A new design for a complement-fixation test is proposed, using isohaemolytic curves.

REFERENCES

ALMEIDA, J. O. (1956). *J. Immunol.* **76**, 259.
ALMEIDA, J. O. (1958). *Rev. bras. Leprol.* **26**, 181.
FULTON, F. (1958). *Advanc. Virus Res.* **5**, 247.
FULTON, F. & DUMBELL, K. R. (1949). *J. gen. Microbiol.* **3**, 97.
HOYLE, L. (1945–46). *J. Hyg., Camb.,* **44**, 170.
MALTANER, F. & ALMEIDA, J. O. (1949). *Blood,* **4**, 728.
WADSWORTH, A. (1947). *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health,* 3rd ed., p. 369. Baltimore: The Williams and Wilkins Company.
WADSWORTH, A., MALTANER, F. & MALTANER, E. (1931). *J. Immunol.* **21**, 313.