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Estimating titres and their approximate standard errors in complement fixation tests

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INTRODUCTION

In the complement fixation test indirect measures are obtained of an antigen-antibody reaction. Each test comprises a three-dimensional array of reaction mixtures, the three variables being antigen, antiserum and complement. The different levels of each variable are equally spaced on a logarithmic scale, except that one of the levels is the zero level, with none of the variable present. For a full account of the test the reader is referred to Fulton (1958), who describes the calculation of antigen and antiserum maxima titres but notes that, to judge the significance of a difference between two titres, it is usual to set arbitrary conservative limits to the estimates in view of the difficulty of obtaining their variances. The main purpose of this paper is to give formulae whereby these variances may be approximately calculated in a relatively simple fashion, and to give an appropriate method for the comparison of the titres of two antisera (or antigens) when they react homologously.

METHOD AND RESULTS

(1) *The amount of complement fixed*

The amount of complement fixed at any particular level of antigen and antiserum is measured indirectly by the amount required so that enough is left over, after fixation, to cause 50 % haemolysis in a standard indicator system of sensitized red blood cells. Let the true amount required be ζ. Then Fulton (1958) suggests the following method to obtain an estimate z of ζ. If we look at the reaction mixtures corresponding to the different levels of complement, we find, for an appropriate indicator system, that the transition from no lysis to complete lysis is so rapid that only one of two effects can be observed. Either the reaction mixtures can be divided into two sets, one set appearing to have all the indicator cells lysed and the other no indicator cells lysed; or else just one reaction mixture shows partial lysis of the indicator cells. If the former, we take z to be the geometric mean of the levels of complement at the transition point; if the latter, we take z to be the level of complement that shows partial lysis.

Let the spacing between successive levels of complement be h when logarithms to base 10 are taken, i.e. if c is the constant dilution factor $h = \log_{10} c$. Let $y = \log_{10} z$ and $\eta = \log_{10} \zeta$. Thus any estimate y of η is either one of the log levels of

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complement used or an average of two consecutive log levels of complement used. Suppose that of a large number of estimates obtained in this way θ_1 is the proportion that are log levels of complement used, i.e. that are determined by a partial lysis. Then, in any interval h that can give rise to an estimate, a range $\theta_1 h$ would give rise to an estimate of this type. Thus it is reasonable to assume that if a log level of complement used falls in the interval $(\eta-\theta_1 h/2,\ \eta+\theta_1 h/2)$ then that log level will be taken as the estimate y. We can therefore consider such an estimate y to be a random variable uniformly distributed on the interval $(\eta-\theta_1 h/2,\ \eta+\theta_1 h/2)$, with the result that it is an unbiased estimate of η and has a variance of $\theta_1^2 h^2/12$. Similarly any estimate y that is the average of two log complement levels used can be considered to be a random variable uniformly distributed on the interval $(\eta-(1-\theta_1)\frac{1}{2}h,\ \eta+(1-\theta_1)\frac{1}{2}h)$, with the result that it is again an unbiased estimate of η but in this case the variance of the estimate is $(1-\theta_1)^2 h^2/12$. Expressing the variance of z by var (z) we have

$$\text{var}\ (z) \doteqdot z^2\ \text{var}\ (\log_e z) = (z \log_e 10)^2\ \text{var}\ (\log_{10} z) = (z \log_e 10)^2\ \text{var}\ (y).$$

Thus

$$\text{var}\ (z) \doteqdot (zth \log_e 10)^2/12, \tag{1}$$

where
$$t = \left\{ \begin{array}{c} \theta_1 \text{ if } z \text{ is a level of complement used,} \\ 1-\theta_1 \text{ if } z \text{ is a geometric mean of two} \\ \text{levels of complement used.} \end{array} \right\} \tag{2}$$

Now with some indicator systems the transition from no lysis to complete lysis is not so rapid, and the reaction mixtures can be classified into more states than the three simple ones of 'no lysis,' 'partial lysis' and 'complete lysis'. In such cases the variances of the estimates z, and so also the variance of the final estimate of the titre, can be materially reduced by making use of the extra information available. To illustrate this we shall consider in some detail the situation in which there are altogether five recognizable states, i.e. those that are judged as being '0 % lysis', '25 % lysis', '50 % lysis', '75 % lysis' and '100 % lysis'.

Let the five states, in sequence of increasing lysis, be scored 0, 1, 2, 3 and 4. Then for a given indicator system and a given value of h there are three possible cases.

Case 1. h is so large that the two levels of complement on either side of η can never be scored 1 and 3. Then one of the four following effects may be observed:

(i) Just one reaction mixture is scored 2; y is taken to be the level of complement at which this occurs.

(ii) Two consecutive reaction mixtures are scored 0 and 3; y is taken to be a weighted average of the two corresponding complement levels.

(iii) Two consecutive reaction mixtures are scored 1 and 4; y is taken to be a weighted average of the two corresponding complement levels.

(iv) Two consecutive reaction mixtures are scored 0 and 4; y is taken to be the simple average of the corresponding complement levels.

Case 2. h is so small that the two levels of complement on either side of η can never be scored 0 and 3 or 1 and 4 (nor, *a fortiori*, 0 and 4). Then only two effects are possible, and y will be taken to be either a level of complement scored 2 or the

average of two levels scored 1 and 3. This case is thus equivalent to the situation in which only three states are recognizable, and this has been dealt with above.

Case 3. h is intermediate in size between the two extremes of cases 1 and 2. In this case the two levels of complement on either side of η can never be scored 0 and 4. There are four possible effects, given by (i), (ii) and (iii) under case 1 above and (iv) Two consecutive reaction mixtures are scored 1 and 3; y is taken to be the simple average of the corresponding complement levels.

The weights used to obtain the weighted averages in cases 1 and 3 are so chosen that the estimates y are unbiased, and for this purpose it is necessary to know the expected proportion θ_1 of estimates of type (i) and the expected proportion θ_2 of estimates of type (ii). It is reasonable to assume that θ_2 is also the proportion of type (iii), and so θ_2 should be taken to be half the proportion of a large number of estimates of either types (ii) or (iii). Then the proportion of type (iv) is $1-\theta_1-2\theta_2$. Let y_0, y_1, y_3 and y_4 be log complement levels scored 0, 1, 3 and 4, respectively. An estimate of type (ii) is then $wy_0+(1-w)y_3$, for a suitably chosen w, and, since $y_0 = y_3-h$, this estimate is equal to y_3-wh. Now by an argument analogous to that used previously y_3 can be considered to be a random variable uniformly distributed on the interval $(\eta+\theta_1 h/2, \eta+\theta_1 h/2+\theta_2 h)$, and so for the estimate to be unbiased w must be so chosen that

$$\int_{\eta+\theta_1 h/2}^{\eta+\theta_1 h/2+\theta_2 h} [(y_3-wh)/\theta_2 h]\,dy_3 = \eta,$$

the solution to which is $w = (\theta_1+\theta_2)/2$. The variance of this estimate is the same as that of y_3, i.e. $\theta_2^2 h^2/12$. In a similar manner it is found that an estimate of type (iii) is $(1-w)y_1+wy_4$, with the same value of w as before, and the variance of this estimate is $\theta_2^2 h^2/12$.

By the same argument estimates of type (i) and (iv) are also unbiased, and their variances are respectively $\theta_1^2 h^2/12$ and $(1-\theta_1-2\theta_2)^2 h^2/12$. In each case z is taken to be the antilog of y, and so the variances of these estimates z are again given by (1), but where now

$$t = \left\{ \begin{array}{l} \theta_1 \text{ for estimates of type (i),} \\ \theta_2 \text{ for estimates of types (ii) or (iii),} \\ 1-\theta_1-2\theta_2 \text{ for estimates of type (iv).} \end{array} \right\} \qquad (3)$$

A value of z can be found for each combination of levels of antigen and antiserum used in the test. In particular, let k be the value found when both antigen and antiserum are at the zero level. Then, provided the effect is additive, $z-k$ estimates the amount of complement fixed at any given level of antigen and antiserum; it also estimates the amount of complement fixed by the antigen-antibody complex formed at that level provided there are no anticomplementary or procomplementary effects.

These effects, however, may be present. Now for any particular estimate z let u be the corresponding estimate at the same level of antiserum, but at the zero level of antigen, and let v be the corresponding estimate at the same level of antigen but

at the zero level of antiserum. Then, provided the various effects are additive, the amount of complement fixed by the antigen-antibody complex is given by

$$f = z-u-v+k. \tag{4}$$

This reduces to $z-v$ if the antiserum shows no anticomplementary or procomplementary effects ($u = k$), to $z-u$ if the antigen shows no such effects ($v = k$), and to $z-k$ if neither antiserum nor antigen show such effects ($u = k = v$). The variances of u, v and k are all given approximately by (1), with u, v and k respectively replacing z, and so the variance of (4) is given approximately by a sum of such expressions.

(2) *Antiserum (antigen) maxima titre*

In many systems the antiserum (or antigen) maxima line can be determined by only a few points. We shall here consider in detail the case where only two points are used to define the maxima line, and obtain the appropriate variance for the estimate of the antiserum (or antigen) titre. Only the antiserum maxima line need be discussed, as analogous results are obtained for the antigen maxima line by interchanging the words 'antigen' and 'antiserum', and the definitions of u and v, in what follows.

Fulton & Almeida (1962) have shown that the antiserum maxima line can be defined either as the linear relation between the maximum value of f at any one level of antiserum and the concentration of antiserum at that level, or as the linear relation between the logarithm of the maximum value of f and the logarithm of the antiserum concentration. The latter definition is preferable, but in many practical situations the two definitions are equivalent. In view of this, and since the assumption of a linear relation between the logarithms leads to a computationally much more cumbersome expression for the variance of the antiserum maxima titre, the former definition will be used here. The antiserum maxima titre is then taken from this line to be that antiserum concentration at which f is equal to k (i.e. k is taken to be the unit of measurement).

Suppose we have two points (f_1, x_1) and (f_2, x_2) on the antiserum maxima line, where corresponding to (4) we have

$$f_1 = z_1-u_1-v_1+k \quad \text{and} \quad f_2 = z_2-u_2-v_2+k.$$

Then the antiserum maxima titre is estimated by

$$x_t = G/F, \tag{5}$$

where

$$F = f_1-f_2 \tag{6}$$

and

$$G = (f_1-k)\,x_2-(f_2-k)\,x_1 \tag{7}$$

The variance of (5) can be approximately determined as follows. We have (provided, as would always occur in practice, F^2 is large relative to its variance)

$$s_x^2 \doteq s_G^2/F^2-2Gs_{FG}/F^3+G^2s_F^2/F^4, \tag{8}$$

where

s_x^2 = variance of x_t,
s_F^2 = variance of F,
s_G^2 = variance of G,
s_{FG} = covariance of F and G.

These variances and covariance depend upon whether anticomplementary or

procomplementary effects are shown by the antiserum alone ($u \neq k = v$), by the antigen alone ($u = k \neq v$), by both ($u \neq k \neq v$), or by neither ($u = k = v$). For this reason it is convenient to define three quantities, S_1, S_2 and S_3, in four different ways according to which of these conditions holds, and then it will be possible to express s_x^2 in terms of S_1, S_2 and S_3. Let

$$\text{if } u \neq k = v\text{: } \left.\begin{aligned} S_1 &= (z_1 t)^2 + (u_1 t)^2, \\ S_2 &= (z_2 t)^2 + (u_2 t)^2, \\ S_3 &= (kt)^2; \end{aligned}\right\} \tag{9}$$

$$\text{if } u = k \neq v\text{: } \left.\begin{aligned} S_1 &= (z_1 t)^2 + (v_1 t)^2, \\ S_2 &= (z_2 t)^2 + (v_2 t)^2, \\ S_3 &= (kt)^2; \end{aligned}\right\} \tag{10}$$

$$\text{if } u \neq k \neq v\text{: } \left.\begin{aligned} S_1 &= (z_1 t)^2 + (u_1 t)^2 + (v_1 t)^2, \\ S_2 &= (z_2 t)^2 + (u_2 t)^2 + (v_2 t)^2, \\ S_3 &= 0; \end{aligned}\right\} \tag{11}$$

$$\text{if } u = k = v\text{: } \left.\begin{aligned} S_1 &= (z_1 t)^2, \\ S_2 &= (z_2 t)^2, \\ S_3 &= 4(kt)^2; \end{aligned}\right\} \tag{12}$$

where in each product t is given by (2) or (3) and is determined by the type of estimate with which it is multiplied. Then using (1) it follows that

$$\begin{aligned} s_F^2 &= (h \log_e 10)^2 (S_1 + S_2)/12, \\ s_G^2 &= (h \log_e 10)^2 (S_1 x_2^2 + S_2 x_1^2 + S_3 [x_2 - x_1]^2)/12, \\ s_{FG} &= (h \log_e 10)^2 (S_1 x_2 + S_2 x_1)/12. \end{aligned}$$

Finally, substituting into (8) these expressions, (5) and $(\log_e 10)^2/12 = 0{\cdot}442$, we obtain for the variance of x_t

$$s_x^2 \doteqdot 0{\cdot}442 h^2 [(x_2 - x_t)^2 S_1 + (x_1 - x_t)^2 S_2 + (x_2 - x_1)^2 S_3]/F^2.$$

The standard error of the antiserum maxima titre is therefore taken to be approximately,

$$0{\cdot}665 h [(x_2 - x_t)^2 S_1 + (x_1 - x_t)^2 S_2 + (x_2 - x_1)^2 S_3]^{\frac{1}{2}}/F. \tag{13}$$

(3) *Comparing two antiserum (antigen) maxima titres*

One of the main purposes of estimating an antiserum maxima titre and its standard error is to compare it with another such estimate. If the two antiserum preparations that are being compared contain entirely different antibodies, or at any rate antibodies that do not react in a homologous manner, then the interpretation of such a comparison is hazardous. If, on the other hand, the one preparation is a simple concentration of the other, or else the two antisera react homologously (so that one can be considered to be a concentration of the other), then a comparison of their maxima titres is the same as a comparison of their average titres, and this is essentially the estimation of the concentration of the one relative to that of the other. On a logarithmic scale the relative concentration (i.e. multiplicative factor) is given by a log difference (i.e. additive quantity), and we shall proceed to give a method of estimating this and its standard error. As before, it is not necessary to go into the comparison of two antigens, since this is completely analogous.

There are standard procedures for estimating a relative concentration when some known single-valued function of the response involved bears a linear relationship to some known single-valued function of the concentration (Finney, 1952). But in a complement fixation test, in which procomplementary or anti-complementary effects may be present, the functional form of the relation between the response (i.e. amount of complement fixed) and the concentration of antiserum is in general unknown; and furthermore the response may not be strictly monotonic, in which case no transformation can lead to a linear relationship. It is reasonable, however, to assume that over a limited range the response curve can be well represented by a parabola, and a full account of a method for estimating relative concentration in such a situation is presented elsewhere (Elston, 1965). An outline of the computational details, adapted to the special case of estimating the relative concentration of two antisera by the complement fixation test, will be given here.

For computational simplicity we wish all the responses that are measured in a given test to have approximately the same variance; this will be so for the estimates y on a logarithmic scale. It will not be possible to use the estimates obtained at the zero level of antiserum, and it is preferable not to use the estimates obtained at the zero level of antigen. Suppose that for each of the antiserum preparations a test has been carried out at d non-zero levels of antiserum and r non-zero levels of antigen, so that for each preparation we have dr estimates y. If, for one of these preparations, p_j is the proportion of these dr estimates of the jth type, the weighted average variance of y for that preparation is

cases 1 and 3: $$[p_1\theta_1^2+(p_2+p_3)\,\theta_2^2+p_4(1-\theta_1-2\theta_2)^2]\,h^2/12. \tag{14}$$

case 2 (and only three states recognizable):

$$[p_1\theta_1^2+(1-p_1)\,(1-\theta_1)^2]\,h^2/12. \tag{15}$$

In (15) p_1 is the proportion of estimates y scored 2 (or determined by a partial lysis if only three states are recognizable).

Denote the two antiserum preparations A and B, and suppose we wish to determine the concentration of B relative to A. Define the orthogonal polynomial coefficients

$$x_{1i} = \begin{cases} i-\frac{1}{2}(d+1) & \text{if } d \text{ is odd,} \\ 2i-(d+1) & \text{if } d \text{ is even} \end{cases} \tag{16}$$

and $$x_{2i} = x_{1i}^2-\sum_i x_{1i}^2/d \quad (i = 1, 2, \ldots, d). \tag{17}$$

Let $$C = r\sum_i x_{1i}^2 \quad \text{and} \quad D = r\sum_i x_{2i}^2. \tag{18}$$

Let the sum of the r estimates y at the ith level of antiserum be $y_{i.}$ for preparation A and $y'_{i.}$ for preparation B (the first level is the most dilute and the dth level the most concentrated). The following sums of squares are calculated to check the validity of the method of estimation:

$$L = (\sum_i x_{1i}y_{i.})^2/\mathrm{C}, \quad \text{with 1 degree of freedom,}$$

$$Q = (\sum_i x_{2i}y_{i.})^2/\mathrm{D}, \quad \text{with 1 degree of freedom,}$$

and $$R = \sum_i y_{i.}{}^2/r-(\sum_i y_{i.})^2/dr-L-Q, \quad \text{with } d-3 \text{ degrees of freedom.}$$

L, Q and R are divided by the weighted average variance of y for A and compared with the tabulated values of the χ^2-distribution with the degrees of freedom indicated. L and/or Q should be significantly large, but R, which is a measure of the deviation from a parabolic response curve, should not be significantly large. Similarly, three quantities L, Q and R are calculated for preparation B, using $y'_{i.}$ instead of $y_{i.}$, and these are divided by the weighted average variance of y for B; as before L and/or Q should be significant, but R should be not significant. Let the sum of the two weighted average variances of y for A and B be s^2. L, Q and R are now calculated using $y'_{i.}+y_{i.}$ instead of $y_{i.}$, divided by s^2 and referred to the same χ^2-distributions; as before L and/or Q should be significant and R should not be significant. Finally, these three sums of squares are computed using $y'_{i.}-y_{i.}$ instead of $y_{i.}$, divided by s^2, and again referred to the same χ^2-distributions; in this case neither Q nor R should be significantly large—if Q is significant this may indicate that the two preparations are not reacting homologously.

Provided the above checks do not indicate any invalidity of the method, the estimate of log relative concentration is obtained by calculating, in sequence:

$$\begin{aligned} a &= \sum_i (y'_{i.}-y_{i.})/dr, & b &= \sum_i x_{1i}\,(y'_{i.}-y_{i.})/C, \\ b_1 &= \sum_i x_{1i}\,(y'_{i.}+y_{i.})/C, & b_2 &= \sum_i x_{2i}\,(y'_{i.}+y_{i.})/D, \\ m_1 &= 2a/b_1, & m_2 &= b/b_2, \\ W_1 &= (m_1^2/C+4/dr)/b_1^2, & W_2 &= (m_2^2/D+1/C)/b_2^2 \end{aligned} \tag{19}$$

$$m = (W_1 m_2 + W_2 m_1)/(W_1+W_2). \tag{20}$$

At this point m is substituted for m_1 and m_2 in (19) to obtain new values of W_1 and W_2, which are then used in (20) to recalculate m; this process is repeated until m becomes stable. Then we calculate

$$s_m^2 = s^2\, W_1 W_2/(W_1+W_2), \tag{21}$$

using the last values of W_1 and W_2.

Now the r levels of antigen used for A must be the same as the r levels used for B, but the d levels of antiserum used need not be the same for both preparations; it is essential, however, that the d levels of antiserum used for A should be a constant multiple of the d levels used for B. Let the logarithm of this constant multiple be k, so that if the concentration of A at the ith level is z_i, and that of B is z'_i, $k = \log_{10}(z_i/z'_i)$ and is the same for all i. Then the concentration of B relative to A, in $\log_{10}$ units, is estimated by

$$\begin{cases} mh+k, \text{ with standard error } s_m h, \text{ if } d \text{ is odd}, & (22) \\ \frac{1}{2}mh+k, \text{ with standard error } \frac{1}{2}s_m h, \text{ if } d \text{ is even}. & (23) \end{cases}$$

(4) *Examples*

Table 1 (sample A) summarizes the results of a complement fixation test in which the antigen is human albumin and the antibody is made in rabbits. The successive concentrations used for all three variables were arranged to be 0·2 $\log_{10}$ units apart. The reaction mixtures were classified into five states, and so there are four different types of estimates. From about 200 such estimates obtained in

different tests, all using the same indicator system as in this example and with $h = 0{\cdot}2$, the proportions of the different types were found to be $\theta_1 = 0{\cdot}23$ and $\theta_2 = 0{\cdot}24$. The estimates in the table were therefore obtained using $w = 0{\cdot}47/2$.

Table 1. *Estimates, z, of amount of complement required for 50 % haemolysis in indicator system—sample A*

Antiserum concentration	Antigen concentration					
	0·0251	0·0158	0·0100	0·0063	0·0040	0
0·0251	0·178	0·158	0·112	0·100	0·079	0·020
0·0158	0·141	0·141(iii)	0·141	0·100	0·079	0·025(i)
0·0100	0·112	0·112	0·112	0·089	0·079	0·032
0·0063	0·089	0·089(iii)	0·089	0·071	0·063	0·040(i)
0	0·032	0·032(iv)	0·032	0·040	0·045	0·050

(i), (iii), (iv), type of estimate.

The last column of Table 1 gives the values of u, the last row the values of v; k is given in the lower right corner, i.e. $k = 0{\cdot}050$. From this table it is found that the largest values of f at each of the four antisera concentrations are:

$$f = 0{\cdot}176 \quad \text{at} \quad x = 0{\cdot}0251,$$
$$f = 0{\cdot}134 \quad \text{at} \quad x = 0{\cdot}0158,$$
$$f = 0{\cdot}098 \quad \text{at} \quad x = 0{\cdot}0100,$$
$$f = 0{\cdot}067 \quad \text{at} \quad x = 0{\cdot}0063.$$

However, inspection of the first line of Table 1 shows that at antiserum concentration 0·0251 a maximum has not necessarily been reached, and so it is best to ignore the point ($f = 0{\cdot}176$, $x = 0{\cdot}0251$). The other three points lie approximately on a straight line, and the two points out of these three that best determine the antiserum maxima line are the two extreme points ($f_1 = 0{\cdot}134$, $x_1 = 0{\cdot}0158$) and ($f_2 = 0{\cdot}067$, $x_2 = 0{\cdot}0063$). We shall use these two points to determine an estimate of the antiserum maxima titre and its standard error.

Using (6), (7) and (5) we obtain

$$F = 0{\cdot}134 - 0{\cdot}067 = 0{\cdot}067,$$
$$G = (0{\cdot}134 - 0{\cdot}050)\,(0{\cdot}0063) - (0{\cdot}067 - 0{\cdot}050)\,(0{\cdot}0158) = 0{\cdot}0002606$$

and $x_t = 0{\cdot}0002606/0{\cdot}067 = 0{\cdot}00389$ or 1:257.

It is evident that both antiserum and antigen show procomplementary effects ($u \neq k \neq v$), and so to obtain the standard error of this estimate we use (11) to define S_1, S_2 and S_3. Thus

$$S_1 = (0{\cdot}141 \times 0{\cdot}24)^2 + (0{\cdot}025 \times 0{\cdot}23)^2 + (0{\cdot}032 \times 0{\cdot}29)^2 = 0{\cdot}001264,$$
$$S_2 = (0{\cdot}089 \times 0{\cdot}24)^2 + (0{\cdot}040 \times 0{\cdot}23)^2 + (0{\cdot}032 \times 0{\cdot}29)^2 = 0{\cdot}000627,$$
$$S_3 = 0.$$

Therefore, using (13), the approximate standard error is

$$0{\cdot}665 \times 0{\cdot}2 \times [(0{\cdot}0024)^2 \times 0{\cdot}001264 + (0{\cdot}0119)^2 \times 0{\cdot}000627]^{\frac{1}{2}}/0{\cdot}067 = 0{\cdot}00062.$$

From this we obtain an approximate 95 % confidence interval for x_t as $0{\cdot}00389 \pm 2 \times 0{\cdot}0062$, i.e. 0·00265 to 0·00513.

Table 2 (sample B) summarizes the results of a similar complement fixation test, the only differences from sample A being that the antiserum is different, but believed to act as a concentration of the antiserum in sample A, and that different levels of antiserum are used. If we estimate the antiserum maxima titre in the same way as for sample A we find $x_t = -\ 0{\cdot}00009$ with a standard error of 0·00036. Thus this estimate of the titre is not significantly different from zero, in accordance with the fact that the true titre cannot be negative. This is a case in which it would be distinctly preferable to define the antiserum maxima line as the linear relation

Table 2. *Estimates, z, of amount of complement required for* 50 % *haemolysis in indicator system—sample B*

Antiserum concentration	Antigen concentration					
	0·0251	0·0158	0·0100	0·0063	0·0040	0
0·0100	0·251	0·224	0·141	0·100	0·063	0·020
0·0063	0·200	0·200(iv)	0·141	0·112	0·071	0·020(iv)
0·0040	0·141	0·141	0·141	0·112	0·079	0·020
0·0025	0·112	0·112(ii)	0·112	0·089	0·079	0·020(iv)
0	0·032	0·032(iv)	0·032	0·040	0·045	0·050

(ii), (iv), type of estimate.

between the logarithm of the maximum value of f and the logarithm of the antiserum concentration, for then estimates of the titre cannot be negative. However, if our main purpose is to estimate the antiserum concentration of sample B relative to that of sample A, rather than to estimate the individual titres, the method given in §3 should be used; this method will now be illustrated.

Logarithms of the estimates z in Tables 1 and 2 are given, multiplied by minus one, in Table 3; multiplication of all the y-values by minus one has no effect on the results, and so is done to avoid unnecessary minus signs. The type of each estimate is indicated, and the proportions of the types in the two samples are:

$$\begin{aligned} \text{sample A:} \quad p_1 &= 3/20 = 0{\cdot}15 \\ p_2+p_3 &= 14/20 = 0{\cdot}70 \\ p_4 &= 3/20 = 0{\cdot}15 \\ \text{sample B:} \quad p_1 &= 3/20 = 0{\cdot}15 \\ p_2+p_3 &= 13/20 = 0{\cdot}65 \\ p_4 &= 4/20 = 0{\cdot}20 \end{aligned}$$

Taking $\theta_1 = 0{\cdot}23$, $\theta_2 = 0{\cdot}24$ and $h = 0{\cdot}2$ as before, (14) gives the weighted average variance to be 0·000203 for sample A and 0·000207 for sample B. These two values and their sum are entered in the last row of Table 4.

For these data $d = 4$ and $r = 5$, and so from (16), (17) and (18):

$$\begin{aligned} &x_{11} = -3, \quad x_{12} = -1, \quad x_{13} = 1, \quad x_{14} = 3, \\ &x_{21} = 4, \quad x_{22} = -4, \quad x_{23} = -4, \quad x_{24} = 4, \\ &C = 100 \quad \text{and} \quad D = 320. \end{aligned}$$

Each of the four sets of sums of squares L, Q and R in Table 4 is then computed from these values and the appropriate set of sums or differences given in the last

two columns of Table 3. For example, the last column of Table 4 is obtained as follows:

$$L = [(-3)(-0{\cdot}50)+(-1)(-0{\cdot}40)+(1)(-0{\cdot}30)+(3)(-0{\cdot}15)]^2/100 = 0{\cdot}0132,$$
$$Q = [(4)(-0{\cdot}50)+(-4)(-0{\cdot}40)+(-4)(-0{\cdot}30)+(4)(-0{\cdot}15)]^2/320 = 0{\cdot}0001,$$
$$R = [(-0{\cdot}50)^2+(-0{\cdot}40)^2+(-0{\cdot}30)^2+(-0{\cdot}15)^2]/5-[-0{\cdot}50-0{\cdot}40-0{\cdot}30-0{\cdot}15]^2/20-L-Q = 0{\cdot}0001.$$

Table 3. *Negative logarithms of estimates, z, in Tables* 1 *and* 2 *(non-zero levels of antigen and antiserum), together with required sums and differences*

Anti-serum level, i.	Sample A					Total $y_{i\cdot}$	$y'_{i\cdot}+y_{i\cdot}$
4	$0{\cdot}75^{(II)}$	$0{\cdot}80^{(I)}$	$0{\cdot}85^{(III)}$	$0{\cdot}95^{(II)}$	$1{\cdot}10^{(IV)}$	4·45	8·75
3	$0{\cdot}85^{(III)}$	$0{\cdot}85^{(III)}$	$0{\cdot}85^{(III)}$	$1{\cdot}00^{(I)}$	$1{\cdot}10^{(IV)}$	4·65	9·00
2	$0{\cdot}95^{(II)}$	$0{\cdot}95^{(II)}$	$0{\cdot}95^{(II)}$	$1{\cdot}05^{(III)}$	$1{\cdot}10^{(IV)}$	5·00	9·60
1	$1{\cdot}05^{(III)}$	$1{\cdot}05^{(III)}$	$1{\cdot}05^{(III)}$	$1{\cdot}15^{(II)}$	$1{\cdot}20^{(I)}$	5·50	10·50
	Sample B					Total $y'_{i\cdot}$	$y'_{i\cdot}-y_{i\cdot}$
4	$0{\cdot}60^{(I)}$	$0{\cdot}65^{(III)}$	$0{\cdot}85^{(III)}$	$1{\cdot}00^{(I)}$	$1{\cdot}20^{(I)}$	4·30	−0·15
3	$0{\cdot}70^{(IV)}$	$0{\cdot}70^{(IV)}$	$0{\cdot}85^{(III)}$	$0{\cdot}95^{(II)}$	$1{\cdot}15^{(II)}$	4·35	−0·30
2	$0{\cdot}85^{(III)}$	$0{\cdot}85^{(III)}$	$0{\cdot}85^{(III)}$	$0{\cdot}95^{(II)}$	$1{\cdot}10^{(IV)}$	4·60	−0·40
1	$0{\cdot}95^{(II)}$	$0{\cdot}95^{(II)}$	$0{\cdot}95^{(II)}$	$1{\cdot}05^{(III)}$	$1{\cdot}10^{(IV)}$	5·00	−0·50

$^{(I)}$, $^{(II)}$, $^{(III)}$, $^{(IV)}$, type of estimate.

Table 4. *Sums of squares and χ^2-values for checking the validity of the method*

Com-ponent	D.F.	$y_{i\cdot}$ S.S.	$y_{i\cdot}$ χ^2	$y'_{i\cdot}$ S.S.	$y'_{i\cdot}$ χ^2	$y'_{i\cdot}+y_{i\cdot}$ S.S.	$y'_{i\cdot}+y_{i\cdot}$ χ^2	$y'_{i\cdot}-y'_{i\cdot}$ S.S.	$y'_{i\cdot}-y'_{i\cdot}$ χ^2
L	1	0·1221	601	0·0552	267	0·3422	835	0·0132	32
Q	1	0·0045	22	0·0061	29	0·0211	51	0·0001	< 1
R	1	0·0000	< 1	0·0001	< 1	0·0001	< 1	0·0001	< 1
Weighted average variance		0·000203		0·000207		0·000410			

(A computational check is afforded by noting that for each component the sum of the sums of squares in the first two columns should be half the sum in the last two columns.) The χ^2-values in Table 4 are obtained by dividing each corresponding sum of squares by the variance at the bottom of its column. We see that L is significantly large in all four columns, Q is significant in all columns but the last and R is never significant. There is thus no reason to doubt the validity of the method, and so we can proceed to estimate the relative concentration.

From the last column of Table 3 we find

$$a = -0{\cdot}0675, \quad b = 0{\cdot}0115,$$
$$b_1 = -0{\cdot}0585, \quad b_2 = 0{\cdot}008125,$$

and so $m_1 = 2{\cdot}308$ and $m_2 = 1{\cdot}415$.

Substituting these values into (19) we obtain

$$W_1 = 74{\cdot}0 \quad \text{and} \quad W_2 = 246{\cdot}3,$$

so that from (20) $m = 2{\cdot}10$. Substituting this value for m_1 and m_2 in (19) we obtain

$$W_1 = 71{\cdot}3 \quad \text{and} \quad W_2 = 360{\cdot}2,$$

so that using these new values in (20) $m = 2{\cdot}16$. Finally, if this value is substituted for m_1 and m_2 in (19) we obtain

$$W_1 = 72{\cdot}1 \quad \text{and} \quad W_2 = 372{\cdot}3$$

and m remains 2·16. Then using (21) we calculate

$$s_m^2 = (0{\cdot}000410)\ (72{\cdot}1)\ (372{\cdot}3)/444{\cdot}4 = 0{\cdot}0248.$$

Inspecting Tables 1 and 2 we see that each concentration of antiserum for sample A is 2·51 times the corresponding concentration for sample B. Thus $k = \log_{10} 2{\cdot}51 = 0{\cdot}4$ (i.e. two levels that were arranged to be 0·2 $\log_{10}$ units apart), and from (23) the concentration of B relative to A, in $\log_{10}$ units, is estimated by

$$(2{\cdot}16)\ (0{\cdot}2)/2 + 0{\cdot}4 = 0{\cdot}616$$

with standard error $[\sqrt{(0{\cdot}0248)}]\ (0{\cdot}2)/2 = 0{\cdot}0157$. It follows that an approximate 95 % confidence interval for the log concentration of B relative to A is given by $0{\cdot}616 \pm 0{\cdot}031$, and taking antilogs the 95 % confidence interval for the concentration of B relative to A is 3·85–4·44.

DISCUSSION

In the examples only two points were used to estimate the absolute antiserum maxima titre and its standard error. It is not suggested that the other points on the maxima line should be ignored. All the points should be used to determine the titre, fitting a line either by eye or by least squares (in this latter case it would be possible to take account of the different variances and covariances among the points, but this would hardly be worth while). It is, however, suggested that for computational simplicity only the two most extreme points on the line be used to obtain an approximate standard error for the estimate. If all the points were uncorrelated and supplied an equal amount of information about the titre, then using just two out of n points would result in estimating $\sqrt{\frac{1}{2}n}$ times the required standard error. But since the most extreme points contain most of the information, and since the points are all correlated, the estimate that is obtained is less than $\sqrt{\frac{1}{2}n}$ times too big; it can therefore be regarded as a conservative estimate of the true standard error. Thus if, for sample A, a regression line is fitted to the three points by unweighted least squares, the antiserum maxima titre is found to be 0·00358. This estimate is probably better than the estimate 0·00389 previously obtained, and it is reasonable to assume that 0·00062 is a conservative estimate of its standard error; since three points are available, we may expect this standard error to be less than $\sqrt{\frac{3}{2}} = 1{\cdot}2$ times too big. Thus a conservative 95 % confidence interval is given by $0{\cdot}00358 \pm 2 \times 0{\cdot}00062$, i.e. 0·00234 (about 1:430) to 0·00482 (about 1:210).

Provided it is valid the method given in §3 should always be used to compare two titres, in preference to the simpler method of estimating the absolute titres separately and comparing these estimates, as it uses much more of the information available. Checking the validity of the method is an essential part, requiring little extra computation (it should be noted that b, b_1 and b_2 can be calculated at the same time as some of the sums of squares are calculated). Occasionally both components L and Q may be non-significant for $y_{i.}$ and/or $y'_{i.}$. In such a case the antigen contours should be plotted and a set of antigen concentrations that give similarly shaped contours (the same set for both A and B) used to estimate m and s_m^2. It may be possible to pick out another set of antigen concentrations and so obtain a second pair of values, say m' and s'^2_m. (For each set of antigen concentrations L and/or Q must be significant for both $y_{i.}$ and $y'_{i.}$.) Then instead of m take the weighted average $(ms'^2_m + m's_m^2)/(s_m^2 + s'^2_m)$ with standard error $s_m s'_m/\sqrt{(s_m^2 + s'^2_m)}$.

In certain cases the calculation of m can be simplified. If W_1 is very large compared with W_2, m is virtually equal to m_2 and $s_m^2 = s^2 W_2$; while if W_2 is very large compared with W_1, m is virtually equal to m_1 and $s_m^2 = s^2 W_1$. However if, as in the example, W_1 and W_2 are of the same order of magnitude, m must be obtained by an iterative process.

Finally, it should be noted that the method given for comparing two titres gives the most precise result when all d quantities $y'_{i.} - y_{i.}$ are near zero. This can to a large extent be arranged, as in the example, by having different antiserum concentrations of the two preparations, i.e. by appropriately choosing k.

SUMMARY

A method is given for obtaining the approximate standard error of the antiserum (or antigen) maxima titre in the complement fixation test when the maxima line is determined by only two points; the same formulae may be used to obtain a conservative estimate of the standard error when more than two points are available. If the degree of lysis in each reaction mixture can be scored more finely than into the three simple states of 'no lysis', 'partial lysis' and 'complete lysis', then more accurate estimates can be obtained. A method is also given for estimating the concentration of one antiserum (or antigen) relative to that of another when they react homologously.

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