BIOASSAY: THE QUANTAL RESPONSE ASSAY

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I. INTRODUCTION. In many instances of interest in medical and biological research, the properties, activity or potency of certain substances cannot be measured directly by common in vitro chemical or physical methods, but can be measured (quantitated) only in terms of some effect they evoke in a living test subject, - animal, plant or microorganism.

Substances in this category include many hormones, vitamins, pharmacologically and toxicologically active substances, antibiotics, and immunologically active substances, - vaccines, toxins, toxoids, antisera, allergens, etc. Measurement or quantitative assessment of the activity of such substances constitutes the subject matter of biological assay.

Design of bioassay experiments and statistical analysis of the resultant data involve mainly an extension of principles and procedures readily available in standard references on experimental design and statistical analysis with major emphasis on regression analysis and analysis of variance with or without transformation of the data originally recorded in conventional units.

II. TYPES OF BIOASSAYS

1. On a basis of intent: On the basis of intent, bioassays can be classified in one of two main groups, - absolute or comparative.

Absolute assays: Absolute assays involve an attempt to obtain some quantitative measurement that can be expressed in absolute terms, such as a Minimal Lethal Dose (MLD) or Median Effective Dose (ED_{50}, LD_{50}, etc.). Such attempts are based on the assumption or belief that some such absolute value exists and that universally it can be determined with adequate precision. However, the absolute potency of substance X for "the cat" typically depends on just which cat is used and, unfortunately, cats invariably do differ. Laudable though the goals and objectives may be, absolute assays of biologically active substances, with

few (if any) exceptions, have little useful quantitative meaning.

Comparative assays: Although absolute assays seldom if ever yield adequately reproducable results, it generally is possible to achieve experimental quantitation of many biologically active substances through assessment of the substance of interest (unknown) in direct comparison with a reference substance (standard) qualitatively identical or, at least, similar in terms of the response evoked in the test subject of choice. While the absolute potency of either may never be known, the comparative or relative activity of the two may be assessed and the biological activity of the unknown expressed in relation to that of the standard in terms of relative potency, - whether expressed in proportions, percentages or in arbitrarily defined units. By using a common reference or standard substance, various investigators may obtain quantitative results with a degree of comparability adequate for their needs. Such relative potency estimates are subject to uncertainty (experimental error), of course, but ideally this may be kept within manageable proportions. It is this innate element of uncertainty that makes bioassay a candidate for statistical consideration.

2. On the basis of response: On the basis of the response evoked in the test subjects of choice, most bioassays may be categorized into one of the following types:

Direct assays: In these the response in the individual test subject is absolute (live, die; response, non-response; etc.) and critical (thresh-hold) levels of the assayed material are determinate, at least within reasonable limits. Computations mainly involve calculation of means and ratios, and estimation of standard errors or confidence limits of such statistics. Example: the cat assay of digitalis.

Graded response-parallel line assays: In these, the response in the individual is proportional to the dose of test substance administered and the degree of response is experimentally determinable. Typically, the degree of response is a linear function of log-dose and the dosage-response regression lines of "Unknown" and "Standard" will be parallel denoting identity or similarity of action. Statistical analysis involves mainly regres-

sion analysis and analysis of variance. With proper design (balanced or partially balanced factorial assays), analysis can be simplified greatly through the use of coefficients. Example: assay of insulin in the rabbit.

Slope-ratio assays: These include mainly the microbiological assays, a group of rather limited general interest in which the degree of measurable response in the individual probably is absolute, but since masses of test subjects (microorganisms) are dealt with, the total response measured, as density, acid formation, etc., approaches a continuous function. Statistical analysis involves multiple regression and relative potency is estimated from the ratio of the partial regression coefficients. Example: microbiological assay of riboflavin.

Quantal response assays: In these, response in the individual test subject is absolute (frequently, live or die) but the critical dose of test material necessary to evoke the response is not directly determinable. Quantitation is achieved through the use of groups of test subjects and determination of the proportion responding to various dosage levels of "Unknown" and "Standard" test products. Following suitable transformation of the data (probits, angles, etc.,) response typically is a linear function of log dose and statistical analysis is essentially similar to that employed with the graded response-parallel line bioassays. Examples: mouse-protective potency assays of typhoid, pertussis and rabies vaccines.

III. REQUIREMENTS OF A VALID BIOASSAY. The following requirements of a "valid" bioassay have evolved from recommendations originally made by Gaddum (1) with modifications made by Bliss, Finney, and others, and are practically universally accepted by students of bioassay. Perhaps the word "valid" should be replaced by "good" or "acceptable."

1. The assay should involve a direct comparison of an unknown with a standard in identical, concomitant tests.

   a. Ideally, the two products should be of essentially equal potency.

2. There should be a significant progressive relationship between dosage and response.

a. Linear following transformation as required.

b. Highly significant slope.

c. No significant curvature; combined or opposed.

3. Dosage-response regression lines for the two products should be parallel, denoting identity or similarity of action.

4. There should be internal evidence of homogeneity (of the data) establishing validity of statistical analysis and adequacy of the testing situation.

5. Analysis should include an estimate of assay error (uncertainty) calculated directly from the data.

Obviously, not all requirements can be applied to each type of assay. Requirements pertaining to slope do not apply to direct assays; those pertaining to parallelism do not apply to slope-ratio assays, etc. However, all do apply to parallel-line graded response assays and most quantal response assays are of similar design.

IV. REDUCTION OF UNCERTAINTY (ERROR) OF BIOASSAYS.

All experienced bioassayists are aware of the innate uncertainty and poor reproducibility of such assays as a whole. The degree of variability differs markedly with various assays, perhaps being least with slope-ratio assays and greatest with quantal response assays. This variability can be reduced to some extent in a variety of ways including:

1. Perfection of technique: equipment, reagents, etc.

2. Control of environment: constant temperature, humidity, etc.

3. Increased homogeneity of test subjects: selection of strains, sex and size of test animals; use of litter mates, etc.

4. Use of restricted designs: randomized blocks (complete or incomplete), Latin squares, cross-over designs, confounding, etc.

5. Statistical adjustment of data: covariance analysis, adjusting response data on the basis of a pertinent associated measurement.

6. Increasing the number of observations (test subjects), either by using more subjects per assay or, preferably, by independent replication of the assay as a whole.

In many quantal response assays, particularly assays of vaccines, antisera, etc., most of the above conventional approaches accomplish only modest reduction in assay error. Slopes of the dosage-response regression lines characteristically are low, constituting a major source of assay error, and the main direct compensating approach is to increase the number of test subjects.* A major reduction in assay error, however, would require impractically large numbers of subjects. Practical solution to many of these problems probably lies in the development of assay procedures involving new experimental approaches. If some meaningful response or attribute of the individual test subject can be measured as a continuous variable, a graded response-parallel line assay procedure should be possible. Typically, errors of these assays are much less than of quantal response assays. In some situations, "time to death" has shown promise as a meaningful quantitative response metameter.

V. REQUIREMENTS OF AN ADEQUATE STATISTICAL ANALYSIS.

1. The analysis should provide for the acceptance or rejection of the assay results as a whole; - such acceptability based

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*In simplified probit analysis, a crude approximation of the standard error of M (log-ratio of potency) is given by

$$s_M = \frac{1}{b_c}\sqrt{\frac{2}{N_U} + \frac{2}{N_S}}$$

where b_c = combined (average) slope, and N_U and N_S = the number of test subjects assigned to the unknown and standard, respectively

upon the requirements outlined in part III.

2. The analysis should provide for a reliable, unbiased estimate of relative potency that is independent of dosage throughout the maximum possible range.

3. The analysis should provide for an estimate of assay uncertainty, - preferably expressed as confidence limits of the relative potency, - provided meaningful alternatives for action based upon such resultant estimates can be established.

Of the above requirements, the first is considered by this writer to be the most essential and the one most commonly unrecognized or neglected in routine analysis of bioassay data. Specific computational procedures and illustrative examples for all the main types of bioassays are given in standard reference books such as Burn (2), Bliss (3), and Finney (4, 5).

VI. STATISTICAL ANALYSIS OF QUANTAL RESPONSE BIOASSAY DATA. A surprising number and variety of computational procedures for analysis of quantal response bioassay data have been proposed. In terms of statistical rigor and sophistication, they range from simple "quick-and-dirty" graphic approximations to formal iterative procedures involving a degree of complexity and tedious computational detail which is difficult to justify except, possibly, in the most critically extenuating circumstance.

Most, or perhaps all, of these methods have some advantages or disadvantages dependent upon their contemplated use but any critical comparison is far beyond the scope of this presentation. It is consoling to find, however, that they all lead to closely similar estimates of relative potency (or end-points) when applied to truly good data as defined in Part III. Unfortunately, the simpler approximate methods generally do not provide a basis for discrimination between acceptable and non-acceptable data and when applied unwittingly to truly unreliable data may yield estimates which are seriously misleading.

The more commonly used computational procedures can be classified into four general categories. These general categories, examples of methods included in each, and minimal comments regarding each, are given below:

Class	Examples	Comments
Graphic approximations	Miller-Tainter (6).	Minimal calculations; adequate reliability provided good data; some discriminatory power by inspection.
Calculated approximations	Reed-Muench Behrens (7).	Most widely used and probably least reliable of all methods; limited to estimating 50% endpoint.
Formal procedures	Probit analysis; Bliss (8), Finney (5). Knudsen-Curtis (9).	Laborious calculations; maximum reliability and discriminatory power.
Compromise methods	Litchfield-Wilcoxon (10).	Generally adequate reliability and discriminatory power; appreciably less calculations than formal methods.

Another method, involving a factorial χ^2 approximation, is proposed by this writer. This should be considered a compromise method and is presented in some detail in part VII of this presentation.

The factorial χ^2 approximation is based essentially on analysis of variance of quantal response data espressed in terms of per cent response and log dose. When used with data from balanced factorial bioassays involving a constant number of test subjects per experimental unit, adequate tests for acceptability of the data, the relative potency estimate and an approximation to confidence limits of the relative potency estimate can be obtained with only moderately extensive calculations. Analysis of the data from numerous factorial quantal response bioassays by this method has yielded results in close agreement with those obtained by formal probit analysis (5) and the Knudsen-Curtis method (9).

VII. FACTORIAL χ^2 ANALYSIS OF QUANTAL RESPONSE BIOASSAY DATA. In a previous report (11) the essential computational details of factorial analysis of attribute (enumeration) data, as developed by Brandt, were presented together with illustrations of applications of the method to selected experiments in industrial chemistry. Two forms of the basic formula were presented. The first "(Formula 1)" being the form for

calculating values of χ^2 for individual degrees of freedom from complete factorial experiments in which the experimental units are of equal size, was given as

$$\chi^2_{[1]} = \frac{N^2}{SxF} \times \frac{T^2}{D}$$

where N = total individuals or observations; S = total successes; F = total failures; T = the total of the sums of products of factorial coefficients and the number of successes in the corresponding experimental units; D = the product of the sums of the squares of the factorial coefficients and the number of individuals per experimental unit; and, the subscript in brackets indicates the degrees of freedom. Either of the outcomes (yes or no, response or non-response, survival or death, etc.) can be designated as success; the other outcome as failure.

In many instances, quantal response bioassay data can be subjected to factorial χ^2 analysis;, the major restrictions being that the experimental units are of equal size and that successive doses of the independent variable (i.e., the toxic or protective substance being assayed) differ by a constant interval when expressed in appropriate units of measurement. In most (perhaps all) assays of immunologically active substances, the successive doses (levels of X) should be increased or decreased in a geometric series such as 1, 2, 4, 8, 16; 1, 3, 9, 27;, etc., as the differences between the logarithms of successive doses are constant in value. When these restrictions are complied with, factorial coefficients (3) can be used directly in analysis of the data and χ^2 values can be computed by the formula given above. In this manner it is possible to obtain statistical information regarding the validity or adequacy of the data (Part III) and, as shown below, to obtain a direct estimate of relative potency and its approximate confidence limits.

The procedures are illustrated with actual examples of both 2-dose (4-point) and 3-dose (6-point) assays of the mouse protective potency of typhoid vaccine performed by the author at the Army Medical Service Graduate School.* Details of the assay procedure employed have been published previously (12); attention here will be limited primarily to statistical treatment of the data.

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* Now known as Walter Reed Army Institute of Research.

1. Factorial χ^2 analysis of a 2-dose quantal response bioassay

As a part of a study to determine the reproducability of mouse protection potency assays of typhoid vaccine (13), a series of 6 assays were run on identical aliquots of a reference vaccine. The aliquotes were identified only as A and B and prior to the assay it was decided to calculate their relative potency, B as per cent of A. Data from the sixth trial are reproduced in Table I.

Table I
Two-dose assay of the mouse protective potency of typhoid vaccines
(Survivals/totals)

Vaccine	Vaccine dose (ml)	
	0.015	0.15
A	5/20	13/20
B	2/20	15/20

For factorial χ^2 analysis, these data are rearranged to the form given in Table IA. For purposes of obtaining tests of significance (χ^2) it is of no consequence in which order the vaccines are entered in the table or which comparison groups are assigned + and - coefficients. However, in the estimation of relative potency, slope, etc., computations are more convenient if certain orders are followed. For the comparison between products (designated as comparison a), positive coefficients should be assigned to the "unknown" (vaccine B in this case). Likewise, for the estimation of slope (comparison a), positive coefficients should be assigned to the higher dose level. Assignment of coefficients to the interaction comparison (ab) is uniquely determined as the cross products of coefficients for the first 2 comparisons, of course. This assignment of coefficients is consistent with that employed by Bliss (3) and others.

Table IA
Factorial χ^2 analysis of the data on Table I

Vaccine	B (unknown)		A (standard)								
Dose	Low	High	Low	High							
Success (survivors) / 20	2	15	5	13	$\Sigma+$	$\Sigma-$	T	T^2	D	T^2/D	χ^{2*}
Comparisons											
a Unknown vs standard	+	+	-	-	17	18	-1	1	80	0.0125	0.05
b Slope (high vs low dose)	-	+	-	+	28	7	21	441	80	5.5125	22.38
ab Departure from parallelism (products x doses)	-	+	+	-	20	15	5	25	80	0.3125	1.27

* $\chi^2 = \frac{N^2}{SxF} \times \frac{T^2}{D} = \frac{80^2}{35 \times 45} \times \frac{T^2}{D} = 4.06 \times \frac{T^2}{D}$.

Evidence of assay validity: All calculations are performed in the manner previously described (11). From comparison a, it is found that the 2 vaccines do not differ appreciably in total effect ($\chi^2[1] = 0.05$). From comparison b it can be seen that there is a highly significant relationship between dosage and response ($\chi^2[1] = 22.38$), and by comparison ab it is determined that there is no significant departure from parallelism exhibited by the dosage response lines for the unknown and standard. No information is available concerning curvature of the dosage response curves. Such can be obtained only when 3 or more dosage levels are employed.

As the assay actually was conducted, the 20 mice in each experimental unit were not handled as a single group but as 4 independent groups of 5 each. These groups were selected, assigned spaces in the test room, immunized and challenged in random order and the number of survivors originally were recorded per group of 5. Thus it is possible to calculate a "within groups" χ^2 with 12 degrees of freedom which can be used as a measure of internal homogeneity (requirement 4). The procedure will be illustrated with data from the next example (Table II).

Estimation of relative potency: It is possible to obtain an estimate of relative potency (RP) from the data and calculations of Table IA by use of the formula for estimating relative potency from

a 2-dose factorial assay as given by Bliss (3).

$$M = \frac{i \times T_a}{T_b}$$

where M = the log ratio of potency; i = the log-dose increment*; and, T_a and T_b are the values in the column headed T for comparisons a and b, respectively. In this assay, the dosage increment was 10-fold, so i = log 10 = 1. T_a = -1 and T_b = 21. Substituting these values in the formula, M is calculated as

$$M = \frac{1 \times -1}{21} = -0.0476.$$

This value is a logarithm and must be converted to the usual form $\bar{1}.9524$. The antilogarithm of $\bar{1}.9524$ is the relative potency which is found to be 0.896; or, in terms of percentage, vaccine B is 89.6 per cent as potent as vaccine A. This estimate is in reasonably close agreement with that obtained by probit analysis, 85.4 per cent.

Approximate confidence limits of relative potency: It also is possible to obtain an approximation of the confidence limits of the relative potency estimate from the data and calculations presented in Table IA. This is most easily done by first determining the approximate confidence interval for $M(CI'_M)$ which for a 2-dose assay is calculated as

$$CI'_M = \frac{1.96 \times 2n\sqrt{N} \times i}{T_b} \quad **$$

where n = individuals per experimental unit; N = 4n or grand total individuals, and i and T_b have the same meaning as before. The

*Logarithms of dosage increments from 2-fold to 10-fold are tabulated in Table 1, Appendix 1 and designated as constants $c_{M.2}$.

**The term "confidence interval" typically is used to denote the entire range included between lower and upper confidence limits. The quantity approximated by CI'_M, as used here, is one-half the entire range expressed in logarithmic units. Derivation of this approximation is given in Appendix II to this paper.

95 per cent confidence limits of M then are determined as

$$M \pm CI'_M$$

and the 95 per cent confidence limits of the relative potency ($95\%\ CL_{RP}$) are found as the antilogarithms of these 2 values.

$$95\%\ CL_{RP} = \text{antilogarithms of } M\text{-}CI'_M \text{ and } M\text{+}CI'_M.$$

These limits will be in the form of ratios which can be converted to percentage by multiplying by 100. For the illustrative problem dealt with here (Tables I and IA)

$$CI'_M = \frac{1.96 \times 40/\sqrt{80} \times \log 10}{21} = 0.4174$$

Then

$$95\%\ CL_M = -0.0476 + 0.4174 = -0.4650 \text{ and } 0.3698$$

$$= 1.5350 \text{ and } 0.3698$$

Taking antilogarithms

$$95\%\ CL_{RP} = 0.34 \text{ and } 2.34$$

or 34% and 234%.

Thus, the best estimate of relative potency (B as per cent of A) is 89.6 per cent and the odds are approximately 19 out of 20 that the true potency is between 34 and 234 per cent.

For a factorial assay of set design, where i and n are constant, assay to assay, the foregoing calculations can be simplified as all elements in the formula for CI'_M will be the same

except for T_b. Thus constants for 2-dose assays ($C_{I.2}$) and 3-dose assays ($C_{I.3}$) for fold-increments of dosage from 2 to 10, and for values of n from 10 to 20, have been calculated and are presented in Appendix I, Tables 2 and 3.

It must be emphasized that this estimate of the confidence limits of the relative potency is only an approximation. Yet the results obtained were in reasonably close agreement with those obtained by probit analysis, 31.6 and 230.4 per cent.

2. Factorial χ^2 analysis of a 3-dose quantal response bioassay.

Factorial χ^2 analysis of a 3-dose quantal response assay for determining the validity of the assay and the estimation of relative potency and approximate 95% confidence limits of the potency estimate, are illustrated with data from another typhoid vaccine mouse protection potency test performed at the Army Medical Service Graduate School. The vaccines tested were a routine production lot (unknown) and a reference standard. Results of the assay are summarized in Table II, and are arranged in the form suitable for factorial χ^2 analysis in Table IIA.

Table II

Three-dose assay of the mouse protective potency of an unknown typhoid vaccine in respect to a standard

(Survivors/totals)

Vaccine	Vaccine dose (ml)		
	0.02	0.08	0.32
Unknown	1/10	5/10	8/10
	1/10	7/10	9/10
Standard	2/10	4/10	8/10
	1/10	5/10	7/10

Table IIA
Factorial χ^2 analysis of the data of Table II

Vaccine	Unknown			Standard									
Dose	D_1	D_2	D_3	D_1	D_2	D_3							
Success (survivors) / 10	1 1	5 7	8 9	2 1	4 5	8 7							
Successes/20	2	12	17	3	9	15	E+	Σ-	T	T^2	D	T^2/D	χ^{2*}
Comparisons													
a Unknown vs standard	+	+	+	-	-	-	31	27	4	16	120	0.13	0.52
b Slope	-	0	+	-	0	+	32	5	27	729	80	9.11	36.44
ab Parallelism	-	0	+	+	0	-	20	17	3	9	80	0.11	0.44
c Combined curvature	+	-2	+	+	-2	+	37	42	5	25	240	0.10	0.40
ac Opposed curvature	+	-2	+	-	+2	-	37	42	5	25	240	0.10	0.40

$$^{*}\chi^2 = \frac{N^2}{S \times F} \times \frac{T^2}{D} = \frac{120^2}{58 \times 62} \times \frac{T^2}{D} = 4.00 \times \frac{T^2}{D}$$

Between groups within experimental units:

$$\chi^2[6] = 4.00 \times \frac{(1-1)^2 + (7-5)^2 + (9-8)^2 + (2-1)^2 + (5-4)^2 + (8-7)^2}{20} = 4.00 \times \frac{8}{20}$$

$$= 1.60.$$

There is little need for comment regarding the computational procedure employed. Factorial coefficients were assigned in conventional order (3) and χ^2 values for each comparison were computed in the manner previously described. Calculation of χ^2 "between groups within experimental units" was accomplished by summation of all T^2/D values between pairs of groups of 10 each and multiplying the total by the constant $\frac{N^2}{S \times F}$.

Evidence of validity

There was no evidence of significant differences between the pairs of groups within experimental units ($\chi^2[6] = 1.60$). This yields assurance that the randomization procedures employed during the assay were adequate to prevent appreciable bias due to technical and environmental factors. Since 3 dosage levels of vaccine were employed, it was possible to gain information regarding curva-

ture of the dosage response lines, both combined and in opposition. There was no evidence of systematic departure from linearity. Thus, all requirements for assay validity (Part III) were satisfied.

Estimation of relative potency

The relative potency of the unknown in respect to the standard was estimated by the formula given by Bliss (3) for calculating M in 3-dose factorial assays

$$M = \frac{4 \times i \times T_a}{3 \times T_b} \quad *$$

The dosage increment employed in this assay was 4-fold, so i= log 4 = O. 6O21. Substituting calculated values of T_a and T_b into the formula, M was calculated as

$$M = \frac{4 \times O.6021 \times 4}{3 \times 27} = O.1189$$

and the relative potency = 1OO x antilog O.1189 = 131. 5 per cent.

Approximate confidence limits of relative potency

The formula for estimating the approximate confidence interval of M in a 3-dose assay differs from that for 2-dose assay only in that 4n must be substituted for 2n. Thus, for a 3-dose factorial assay.

$$CI'_M = \frac{1,96 \times 4n/\sqrt{N} \times i}{T_b}$$

* Values of $\frac{4 \times i}{3}$ dosage increments of 2-fold through 1O-fold have been calculated and are given as constants $c_{M.3}$ in Table 1, Appendix I. M is determined by multiplying the ratio T_a/T_b by the appropriate value of $c_{M.3}$ (O. 8O2O in this example).

For the data dealt with here (Tables II and IIA), n = 20, N = 120, and i = log 4 = 0.6021. Then

$$CI'_M = \frac{1.96 \times 80/\sqrt{120 \times 0.6021}}{27}$$

$$= 0.3192.$$

The confidence limits of M are found as

$$95\% \, CL_M = M \pm CI'_M$$

$$= 0.1189 \pm 0.3192 = -0.2003 \text{ and } 0.4381$$

$$= \overline{1}.7997 \text{ and } 0.4381$$

Then the 95 per cent confidence limits of the relative potency are obtained as the antilogarithms of these values.

$$95\% \, CL_{RP} = 0.63 \text{ and } 2.74$$

or $$= 63 \text{ and } 274 \text{ per cent}$$

These data also were analyzed by the probit analysis. The relative potency estimate was 132.2 per cent and the 95 per cent confidence limits were 64.2 per cent and 272.2 per cent.

3. Resumé of computational procedure: Chi square analysis of quantal response factorial assays yielding (1) statistical evidence regarding reliability of the data, (2) an estimate of relative potency, and (3) approximate confidence limits of the relative potency, involves a series of 7 main steps.

1. Arrange the data on a work sheet of the form used in Tables IA and IIA.

2. Assign the factorial coefficients in accordance with the actual design of the experiment. Compute N^2/SxF from the grand

*Constants $c_{I.3}$ for estimating values of CI'_M in 3-dose factorial assays for dosage increments of 2-fold through 10- fold and for values of n from 10 through 20, have been calculated and are given in Table 3 of the appendix. For this problem, $c_{I.3} = 8.6183$. This divided by 27 $(T_b) = 0.3192$, the same as calculated above.

totals and then $\Sigma +$, $\Sigma -$, T, T^2, D, T^2/D and χ^2 for each comparison (row). Also, if data on subgroups within experimental units are available, calculate the "between groups" χ^2 (cf. Table IIA). From the various values of χ^2 determine if there is sufficient evidence of validity to justify estimation of potency.

3. If justified, compute the ratio T_a/T_b and calculate M as:

   a. Two dose assay: $M = i \times T_a/T_b$. Values of i are given as the constants $c_{M.2}$ in Table 1, Appendix I.

   b. Three-dose assay: $M = \frac{4 \times i}{3} \times T_a/T_b$. Values of $\frac{4 \times i}{3}$ are given as the constants $c_{M.3}$ in Table 1, Appendix I.

4. Determine the relative potency (RP) as a ratio or percentage as antilog M, or as 100 x antilog M, respectively.

5. Compute CI'_M as:

   a. Two-dose assay:

$$CI'_M = \frac{1.96 \times 2n\sqrt{N} \times i}{T_b}$$

   or, using constants $c_{I.2}$ from Table 2, Appendix I:

$$CI'_M = \frac{c_{I.2}}{T_b}.$$

   b. Three-dose assay:

$$CI'_M = \frac{1.96 \times 4n/\sqrt{N} \times i}{T_b}$$

   or, using constants $c_{I.3}$ from Table 3, Appendix I:

$$CI'_M = \frac{c_{I.3}}{T_b}.$$

6. Calculate the 95 per cent conficence limits of M as

$$95\%\ CL_M = M \pm CI'_M.$$

7. Determine the 95 per cent confidence limits of the relative potency as

$$95\%\ CL_{RP} = \text{antilog } M\text{-}CI'_M \text{ and antilog } M + CI'_M.$$

If it is desired to express the limits as percentages, multiply each value by 100.

APPENDIX I

Table 1

Values of $c_{M.2}$ and $c_{M.3}$ for obtaining estimates of M, the log ratio of potency, from 2-dose and 3-dose factorial assays

$$(M = c_{M.i} \times T_a/T_b)\ *$$

Fold-increment in dosage	$c_{M.2}$ (2-dose assays)	$c_{M.3}$ (3-dose assays)
2	0.3010	0.4013
3	0.4771	0.6361
4	0.6021	0.8028
5	0.6990	0.9320
6	0.7782	1.0376
7	0.8451	1.1268
8	0.9031	1.2041
9	0.9542	1.2722
10	1.0000	1.3333

*Relative potency = antilog M.

Relative potency in % = 100 x antilog M.

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APPENDIX I
Table 2
Values of $c_{I.2}$ *

Constants for calculating CI'_M, the confidence interval of M, for 2-dose factorial assays. Select the values of $c_{I.2}$ determined by the dosage increment (row) and group size (column).

$$\left(CI'_M = \frac{c_{I.2}}{T_b}\right)$$

Fold-increment in dosage	n (individuals per group)										
	10	11	12	13	14	15	16	17	18	19	20
2	1.8656	1.9567	2.0437	2.1271	2.2074	2.2849	2.3598	2.4325	2.5030	2.5716	2.6384
3	2.9571	3.1014	3.2393	3.3716	3.4989	3.6217	3.7405	3.8556	3.9674	4.0761	4.1820
4	3.7318	3.9104	4.0880	4.2550	4.4156	4.5705	4.8658	4.7205	5.0068	5.1440	5.2776
5	4.3324	4.5439	4.7459	4.9398	5.1263	5.3061	5.4802	5.6488	5.8126	5.9718	6.1270
6	4.8233	5.0588	5.2837	5.4995	5.7071	5.9073	6.1011	6.2889	6.4712	6.6485	6.8212
7	5.2379	5.4937	5.7379	5.9722	6.1977	6.4152	6.6256	6.8295	7.0275	7.2200	7.4076
8	5.5974	5.8707	6.1317	6.3821	6.6231	6.8554	7.0803	7.2982	7.5098	7.7155	7.9160
9	5.9141	6.2029	6.4786	6.7432	6.9978	7.2433	7.4809	7.7112	7.9347	8.1521	8.3639
10	6.1980	6.5006	6.7896	7.0669	7.3337	7.5910	7.8400	8.0813	8.3156	8.5434	8.7654

*Calculated as $\frac{1.96 \times 2n \times i}{N}$

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APPENDIX I

Table 3

Values of $c_{I.3}$ *

Constants for calculating CI'_M, the confidence interval of M, for 3-dose factorial assays.

Select the values of $c_{I.3}$ determined by the dosage increment (row) and group size (column).

$$(CI'_M = \frac{c_{I.3}}{T_b})$$

Fold-increment in dosage	n (individuals per group)										
	10	11	12	13	14	15	16	17	18	19	20
2	3.0465	3.1952	3.3373	3.4736	3.6047	3.7313	3.8536	3.9722	4.0874	4.2013	4.3085
3	4.8289	5.0646	5.2898	5.5058	5.7136	5.9142	6.1081	6.2961	6.4787	6.6593	6.8291
4	6.0941	6.3915	6.6757	6.9483	7.2106	7.4638	7.7084	7.9457	8.1761	8.4041	8.6183
5	7.0749	7.4202	7.7501	8.0665	8.3710	8.6649	8.9490	9.2245	9.4919	9.7566	10.0053
6	7.8765	8.2609	8.6282	8.9805	9.3195	9.6467	9.9630	10.2697	10.5674	10.8620	11.1390
7	8.5536	8.9711	9.3670	9.7525	10.1207	10.4760	10.8195	11.1525	11.4759	11.7958	12.0966
8	9.1406	9.5868	10.0130	10.4219	10.8153	11.1950	11.5620	11.9179	12.2635	12.6054	12.9268
9	9.6578	10.1292	10.5796	11.0116	11.4272	11.8285	12.2162	12.5923	12.9574	13.3186	13.6582
10	10.1214	10.6154	11.0874	11.5401	11.9757	12.3962	12.8026	13.1967	13.5793	13.9579	14.3138

* Calculated as $\frac{1.96 \times 4n \times i}{N}$

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APPENDIX II

Approximation of the Confidence Interval of M

The standard error of M (s_M) from a balanced factorial bioassay is given by Bliss (3) as:

$$s_M = \frac{s}{b_c}\sqrt{\frac{4}{N}\left[1 + \frac{D^2}{B^2 - s^2 t^2}\right]}$$

where D^2 = mean square between products; B^2 = mean square for combined slope; s^2 = mean square; t = Student's statistic; N = total number of test subjects (possible responses); and b_c is the combined or average slope of the dose-response regression line.

For a 2-dose (4-point) assay, b_c is estimated as $\frac{Tb}{2 \times i \times n}$; for a 3-dose (6-point) assay, as $\frac{Tb}{4 \times i \times n}$. In these, Tb is found as shown in Tables IA and IIA, i is the log ratio of dosage increment, and n is the number of test subjects per experimental group.

In a good bioassay (statistically acceptable), D^2 will be small and B^2 will be large. Thus, the quantity enclosed in brackets approaches unity and can be ignored. In the binomial, the variance (s^2) has a maximum value of O. 25 and s has a maximum value of O. 5. In a balanced assay of fixed design, N will be 4n or 6n for a 2-dose and 3-dose assay, respectively. Substituting the appropriate formula for b_c as given above, and introducing $t_\infty = 1.96$, the confidence intervals of M can be reduced to the following approximations:

$$\text{2-dose assay: } CI_M = \frac{(1.96 \times 2n \times i)/\sqrt{N}}{T_b}$$

$$\text{3-dose assay: } CI_M = \frac{(1.96 \times 4n \times i)/\sqrt{N}}{T_b}$$

These approximations were used for calculating the constants presented in Tables 2 and 3 of Appendix I.

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