7

Slope ratio assays

7.1 The power dose metameter

The assays in Chapters 4 to 6 used a logarithmic dose metameter, corresponding to $\lambda = 0$ in §3.7. The alternative form of regression with $\lambda \neq 0$ must now be considered. As in §3.7, this requires the dose metameter

$$x = z^{\lambda}, \tag{7.1.1}$$

in terms of which the regression lines for the two preparations are

$$\left.\begin{aligned} Y_S &= \alpha + \beta x, \\ Y_T &= \alpha + \beta\rho^{\lambda} x. \end{aligned}\right\} \tag{7.1.2}$$

These equations represent two lines, of slopes β and $\beta\rho^{\lambda}$, intersecting at $x = 0$: obviously the expected response to zero dose must be the same for both preparations. If b_S, b_T are estimates of the regression coefficients,

$$R = \left(\frac{b_T}{b_S}\right)^{1/\lambda} \tag{7.1.3}$$

is an estimate of the relative potency, whence comes the name *slope ratio assay*. Throughout this book, $\lambda = 1$ will be assumed, because in all current applications, it appears to be an adequate approximation to the truth. The restriction is not very serious. Results appropriate to any other λ can be derived by applying the same methods to give estimates of R^{λ} and its fiducial limits, and then raising each to the power $1/\lambda$. If λ itself had to be estimated for each assay, instead of being regarded as a part of the definition of the dose metameter with a value determined from preliminary investigations, the analysis would be more complicated. The method of calculation for that problem will not be described here; rarely would data from one assay suffice to estimate λ with reasonable precision.

Though their discussion did not explicitly recognize the nature of the analysis, Birch and Harris (1934) appear to have been the first to publish a slope ratio assay.(*) They found the duration of cure of bradycardia in vitamin B_1 deficient rats to be directly proportional to dose of vitamin. They therefore estimated the potency of a test preparation by adjusting dose scales until its response curve coincided with that for the standard, essentially the procedure now used but performed graphically rather than arithmetically.

Equations (7.1.2) were first systematically discussed for vitamins (Burn *et al.*,

(*)Mr L.E. Hudson has told me that, as early as 1908, W.S. Gosset (better known as 'Student') was using analogous methods with $\lambda = 2$ for estimating the effect of hops on the life of beer.

1950, Chapter III; Emmens, 1948, Chapter 20; Finney, 1945a, 1947d; Wood, 1945, 1946a). Because microbiological assays have always formed the major application of slope ratio methods, microbiological terminology is adopted here. The test subject is not a single animal but an inoculum (of specified size) of a bacterial culture, which is added to a dose of S or T and incubated under standard conditions. The response is some measure of bacterial growth in a fixed time, perhaps a measurement of turbidity or of the amount of alkali required to neutralize the acid formed during growth. Moreover, the response actually measured seems usually to show a homoscedastic linear regression. Consequently, the response metameter is defined by

$$Y = U. \tag{7.1.4}$$

The statistical theory is equally applicable to macrobiological techniques such as that of Birch and Harris; more recently, Carpenter, McDonald and Miller (1972) have used slope ratio estimation for assaying methionine in feeding stuffs with either weight gain or food conversion efficiency in chicks as the response.

7.2 The multiple regression equation

Write x_S, x_T for the doses of the two preparations. If equations (7.1.2) remain valid down to $x_S = x_T = 0$, it will be natural to run some tests on 'blank' or control subjects with zero dose; Chapter 8 shows this also to be expedient in respect of efficiency. To fit regression equations independently for S and T would be improper, even if blank tests have not been included. The constraint that equations (7.1.2) have a common α, which corresponds to the constraint of parallelism with a logarithmic dose metameter, must be introduced.

Equations (7.1.2) can alternatively be written

$$Y = \alpha + \beta_S x_S + \beta_T x_T, \tag{7.2.1}$$

with the understanding that, for assay purposes at least, interest is restricted to situations in which one (or both) of x_S, x_T is zero. Whether or not responses at $x_S = x_T = 0$ have been measured, the ordinary procedure of multiple linear regression (cf. §§2.8, 4.23) can be used to give the estimating equation

$$Y = a + b_S x_S + b_T x_T. \tag{7.2.2}$$

The regression equations are obtained from

$$\left.\begin{aligned} b_S S_{x_S x_S} + b_T S_{x_S x_T} &= S_{x_S y}, \\ b_S S_{x_S x_T} + b_T S_{x_T x_T} &= S_{x_T y}. \end{aligned}\right\} \tag{7.2.3}$$

The summations are to be taken over all subjects, as illustrated in §7.4. The estimate of ρ is

$$R = b_T/b_S, \tag{7.2.4}$$

and limits of error can be obtained by Fieller's theorem.

7.3 An unsymmetric slope ratio assay

Kent-Jones and Meiklejohn (1944) described an assay of nicotinic acid in a meat extract, the test preparation being a solution containing 0·2 mg extract per ml. Duplicate assay tubes were prepared for each of five doses of standard nicotinic acid and three doses of T, as well as for zero dose. The eighteen tubes were inoculated from a culture of *Lactobacillus arabinosus*, and incubated at 37°C for 72 hours. The acidity of each tube was measured by titration with N/14 sodium hydroxide, with bromothymol blue as an indicator in a colour comparator. Table 7.3.1 records the responses. The design was scarcely ideal (Chapter 8), but the lack of symmetry may help in emphasizing the main features of the full statistical analysis. The authors intended to use a form of standard curve estimation, inadequate by modern criteria but in this instance giving about the right answer.

TABLE 7.3.1 Responses in an assay of nicotinic acid in a meat extract (ml N/14 NaOH)

Dose of standard preparation (μg per tube)				
0·05	0·10	0·15	0·20	0·25
3·5	5·0	6·2	8·0	9·4
3·2	4·7	6·1	7·7	9·5

Dose of test preparation (ml per tube)			Blanks
1·0	1·5	2·0	
4·9	6·3	7·7	1·5
4·8	6·5	7·7	1·4

Fig. 7.3.1 shows each response plotted against dose. In a slope ratio assay, numerical values of x_S and x_T may be very different. For convenience of representation, separate dose scales can be used but should be drawn with a common zero; the scales should be chosen to keep the two sets of points quite distinct, so that two straight lines intersecting near the point for blanks $x_S = x_T = 0$ can easily be drawn. Of course, the mean response for blanks has no more reason to lie exactly on the lines than has any other point. Lines drawn by eye in Fig. 7.3.1 show for S an increase in response of about 3·1 units (ml NaOH) per 0·1 μg, and for T an increase in response of about 3·1 units per ml. Hence the potency of the test preparation is about 0·1 μg per ml: 1 g of the undiluted meat extract contains about 500 μg nicotinic acid.

7.4 Analysis of variance

As usual, an analysis of variance aids examination of the validity of the assay. The regression coefficients must first be calculated. With S symbolizing summation over the 18 tubes,

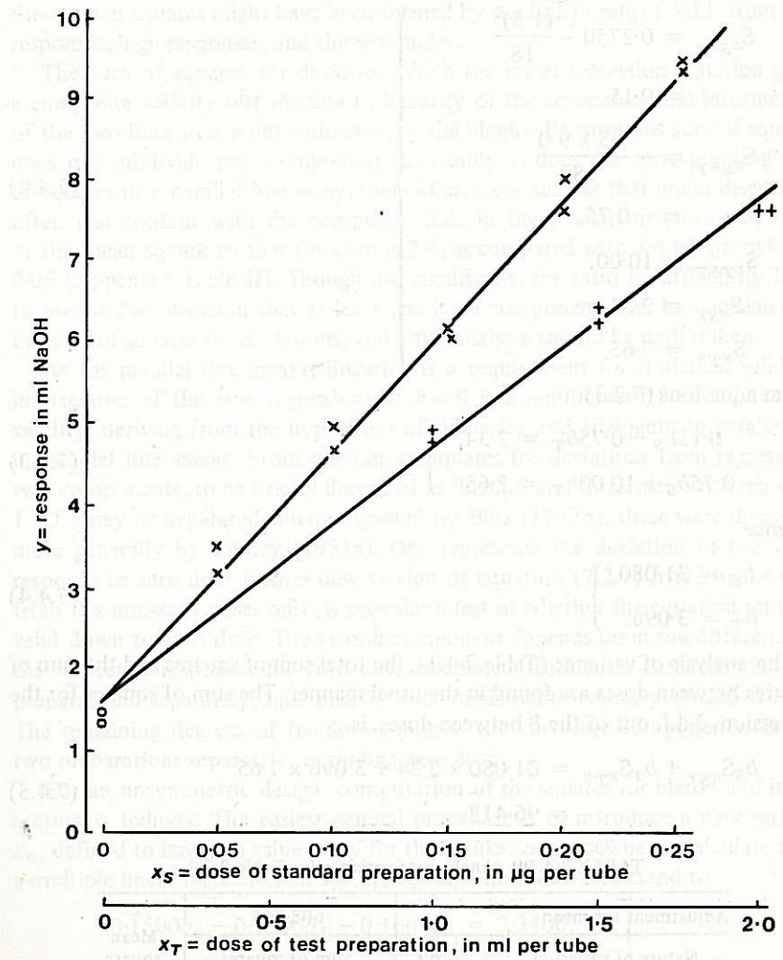

Fig. 7.3.1 Linear dose–response regressions for the assay of nicotinic acid, Table 7.3.1

X: Responses to standard preparation

+: Responses to test preparation

o: 'Blanks'

The straight lines are those mentioned in §7.3 as drawn by eye, but the calculated lines on which equation (7.6.1) is based are almost identical with them.

$$\left.\begin{aligned} S x_S &= 1{\cdot}5, \\ S x_T &= 9{\cdot}0, \\ S y &= 104{\cdot}1, \end{aligned}\right\} \qquad (7.4.1)$$

since $x_S = 0$ except for tubes of the standard and $x_T = 0$ except for tubes of the test preparation. The sums of squares and products of deviations are

$$\left.\begin{aligned} S_{x_S x_S} &= 0{\cdot}2750 - \frac{(1{\cdot}5)^2}{18} \\ &= 0{\cdot}15, \\ S_{x_S x_T} &= -\frac{1{\cdot}5 \times 9{\cdot}0}{18} \\ &= -0{\cdot}75, \\ S_{x_T x_T} &= 10{\cdot}00, \\ S_{x_S y} &= 2{\cdot}34, \\ S_{x_T y} &= 7{\cdot}65. \end{aligned}\right\} \qquad (7.4.2)$$

From equations (7.2.3),

$$\left.\begin{aligned} 0{\cdot}15b_S - 0{\cdot}75b_T &= 2{\cdot}34, \\ -0{\cdot}75b_S + 10{\cdot}00b_T &= 7{\cdot}65, \end{aligned}\right\} \qquad (7.4.3)$$

whence

$$\left.\begin{aligned} b_S &= 31{\cdot}080, \\ b_T &= 3{\cdot}096. \end{aligned}\right\} \qquad (7.4.4)$$

In the analysis of variance (Table 7.4.1), the total sum of squares and the sum of squares between doses are found in the usual manner. The sum of squares for the regression, 2 d.f. out of the 8 between doses, is

$$\begin{aligned} b_S S_{x_S y} + b_T S_{x_T y} &= 31{\cdot}080 \times 2{\cdot}34 + 3{\cdot}096 \times 7{\cdot}65 \\ &= 96{\cdot}412. \end{aligned} \qquad (7.4.5)$$

TABLE 7.4.1 Analysis of variance for Table 7.3.1

Adjustment for mean		602·045	
Nature of variation	d.f.	Sum of squares	Mean square
Regression	2	96·412	
Deviations from regression	6	0·278	0·0463
Between doses	8	96·690	
Error	9	0·175	0·0194
Total	17	96·865	

7.5 Validity tests

Table 7.3.1 gives no hint of heteroscedasticity. Bartlett's test (§3.11) would scarcely be a reliable indicator when each variance estimate is based on 1 d.f., especially when the accuracy of measurement (to the nearest 0·1 ml) is low in relation to the magnitude of the standard deviation. Had a test been needed,

three mean squares might have been formed by pooling in sets of 3 d.f. from low responses, high responses, and the remainder.

The sum of squares for deviations from the linear regression equation gives a composite validity test relating to linearity of the regressions and intersection of the two lines in a point estimated by the blanks. Because this sum of squares does not subdivide into components as readily as does the corresponding sum of squares in a parallel line assay, users of an assay such as that under discussion often rest content with the composite test. In the present instance, the ratio of the mean square to that for error is 2·4, as compared with 3·4 for probability 0·05 (Appendix Table II). Though not significant, the ratio is sufficiently large to arouse the suspicion that at least one large component may be concealed in the sum of squares for deviations, and a full analysis should be undertaken.

As for parallel line assays, linearity is a requirement for statistical validity; intersection of the true regressions at $x = 0$ is a requirement for fundamental validity, deriving from the hypothesis of similarity and analogous to parallelism in parallel line assays. From the sum of squares for deviations from regression, two components, to be briefly described as 'Blanks' and 'Intersection', each with 1 d.f., may be separated. First suggested by Bliss (1947b), these were discussed more generally by Finney (1951a). One represents the deviation of the mean response to zero dose from a new version of equation (7.2.1) fitted to the data from the non-zero doses only; it provides a test of whether the equation remains valid down to zero dose. The second component depends upon the difference in the expected responses for zero dose calculated from lines fitted to the two preparations separately, and thus it tests fundamental validity (Wood, 1945). The remaining degrees of freedom comprise the curvature components for the two preparations separately, excluding zero dose.

For an unsymmetric design, computation of the squares for blanks and intersection is tedious. The easiest general procedure is to introduce a new variate, x_0, defined to have the value unity for the blanks, zero elsewhere. Calculate then a multiple linear regression on x_S, x_T, x_0: equations (7.4.3) extend to

$$\left.\begin{aligned} 0{\cdot}1500b_S - 0{\cdot}7500b_T - 0{\cdot}1667b_0 &= 2{\cdot}3400, \\ -0{\cdot}7500b_S + 10{\cdot}0000b_T - 1{\cdot}0000b_0 &= 7{\cdot}6500, \\ -0{\cdot}1667b_S - 1{\cdot}0000b_T + 1{\cdot}7778b_0 &= -8{\cdot}6667. \end{aligned}\right\} \qquad (7.5.1)$$

The values of b_S, b_T are no longer those of equations (7.4.4), but the same notation is retained. The solutions of equations (7.5.1) are

$$\left.\begin{aligned} b_S &= 30{\cdot}1253, \\ b_T &= 2{\cdot}9874, \\ b_0 &= -0{\cdot}3704; \end{aligned}\right\} \qquad (7.5.2)$$

note the necessity for a large number of decimal places in order to give sufficient accuracy in the next calculation. The sum of squares for the new regression is

$$2{\cdot}3400b_S + 7{\cdot}6500b_T - 8{\cdot}6667b_0 = 96{\cdot}557.$$

The difference between this and the sum of squares with 2 d.f. in equation (7.4.5) is the required component for blanks. That for intersection is obtained indirectly by finding the residual sum of squares after fitting two separate linear regressions to the non-zero doses. By calculation from the dose totals for the five doses of S and three of T, the sums of squares between doses are 46·296 and 8·143, with 4 d.f. and 2 d.f. respectively. Linear regressions account for

$$(-2 \times 6{\cdot}7 - 9{\cdot}7 + 15{\cdot}7 + 2 \times 18{\cdot}9)^2/20 = 46{\cdot}208$$

and

$$(-9{\cdot}7 + 15{\cdot}4)^2/4 = 8{\cdot}122$$

respectively. The residual for all types of curvature is therefore

$$(46{\cdot}296 - 46{\cdot}208) + (8{\cdot}143 - 8{\cdot}122) = 0{\cdot}109$$

with 4 d.f., which could be further split into quadratic, cubic, and other components. The square for intersection is now obtained by subtraction (0·278 − 0·144 − 0·109) and Table 7.5.1 is completed.

TABLE 7.5.1 Complete analysis of variance for Table 7.3.1

Adjustment for mean		602·045	Mean square
Nature of variation	d.f.	Sum of squares	
Regression	2	96·412	
Blanks	1	0·145	0·145
Intersection	1	0·025	0·025
Curvature	4	0·108	0·027
Between doses	8	96·690	
Error	9	0·175	0·0194
Total	17	96·865	

The mean square for blanks is significantly greater than error – clear evidence of invalidity. Fortunately, this is only statistical invalidity, the mean response for the blanks being appreciably lower than is predicted by fitting equation (7.2.1) to the other data. Because a slight curvature at very low doses is not uncommon, Wood (1946a) suggested that, for riboflavin assays using *Lactobacillus helveticus* as test subject, a small amount of the standard preparation (say 0·03 μg per tube) might be added to the basal medium, and only quantities in excess of this regarded as experimental doses. The conventional zero dose should then be brought on to the linear portions of both response curves. For a true analytic dilution assay, this procedure seems unexceptionable and adaptable to assays of other materials. For a comparative assay, the risk of chemical or biological complications through mixing the standard and test preparations must be considered.

The practice of placing replicate tubes of a dose adjacent to one another in the incubator has been criticized in §6.7 (cf. §§4.19, 4.20): it can be one reason for

variance heterogeneity or for the mean square for deviation from regression being larger than the error mean square. Such a flaw casts doubt on any assessment of fiducial limits of the relative potency. Similar considerations apply to titrating the acidity after incubation. To match duplicate tubes with one another for colour, instead of independently with a standard, may produce a correlation of response measurements and underestimation of the error variance. Experimenters are tempted to believe that consecutive treatments of, or consecutive measurements on, duplicate tubes give independent observations, and to ignore the possible correlation of subjective errors; the risk of biases that may pass unsuspected is not negligible. In the present assay, curvature at very low doses seems a more plausible explanation of the evidence for invalidity, since only the square for blanks is much greater than the error mean square.

7.6 Potency estimation

In view of the anomalous behaviour of the responses at zero dose (Table 7.5.1), estimation of potency from the non-zero dose levels seems desirable. Omission of the blanks makes a trivial difference to R itself, but it affects the precision; use of the blanks would give an apparently more precise but possibly misleading estimate. The natural method of computation is to proceed exactly as in §7.4 but to use only the data from the 16 tubes. As is obvious from theoretical considerations, the values of b_S, b_T are those in equations (7.5.2). Therefore

$$R = 2{\cdot}987/30{\cdot}125 = 0{\cdot}0992; \tag{7.6.1}$$

adjustment for the dilution then gives 496 μg per g as the estimated potency of the meat extract.

Evaluation of variances requires the inverse matrix of the coefficients in equations (7.5.1); as in §4.23, this is found to be

$$\mathbf{V} = \begin{pmatrix} 16{\cdot}9663 & 1{\cdot}51685 & 2{\cdot}44382 \\ 1{\cdot}51685 & 0{\cdot}241573 & 0{\cdot}278090 \\ 2{\cdot}44382 & 0{\cdot}278090 & 0{\cdot}948034 \end{pmatrix}. \tag{7.6.2}$$

In routine computation, $\mathbf{V}$ would be found first, and the regression coefficients obtained from it, as

$$b_S = 2{\cdot}3400v_{11} + 7{\cdot}6500v_{12} - 8{\cdot}6667v_{13}, \text{ etc.} \tag{7.6.3}$$

Multiplication of s^2 by v_{11}, v_{22}, and v_{12} in turn gives the variances of b_S, b_T and their covariance. For s^2, there appears to be no objection to using the error mean square in Table 7.5.1, in spite of its inclusion of 1 d.f. from the blanks:

$$s^2 = 0{\cdot}0194. \tag{7.6.4}$$

Fieller's theorem (§4.12) then gives the fiducial limits of R as

$$R_L, R_U = \left[R - \frac{gv_{12}}{v_{11}} \pm \frac{ts}{b_S}\left\{v_{22} - 2Rv_{12} + R^2v_{11} - g\left(v_{22} - \frac{v_{12}^2}{v_{11}}\right)\right\}^{1/2}\right] \Big/ (1-g), \tag{7.6.5}$$

where

$$g = \frac{t^2 s^2 v_{11}}{b_S^2}. \tag{7.6.6}$$

In a good microbiological assay, the variation between replicate tubes should be relatively much less than that between animals in a macrobiological assay. If g is negligible, fiducial limits may be based upon the variance formula

$$\mathrm{Var}(R) = \frac{s^2}{b_S^2}[v_{22} - 2Rv_{12} + R^2 v_{11}]. \tag{7.6.7}$$

In the example under discussion,

$$g = \frac{(2{\cdot}262)^2 \times 0{\cdot}0194 \times 16{\cdot}966}{(30{\cdot}125)^2}$$
$$= 0{\cdot}0019,$$

which is sufficiently small to be neglected. From (7.6.5), the fiducial limits are

$$R_L, R_U = \left[0{\cdot}099\,15 - 0{\cdot}000\,17 \pm \frac{2{\cdot}262}{30{\cdot}125}\{0{\cdot}0194 \times (0{\cdot}241\,57 - 0{\cdot}300\,79 + 0{\cdot}166\,79 - 0{\cdot}000\,20)\}^{1/2}\right] \Big/ 0{\cdot}9981$$
$$= 0{\cdot}0957, 0{\cdot}1026.$$

Since the meat extract was diluted 5000-fold for use as a test preparation, it is estimated to contain 496 μg per g, with fiducial limits at 478 μg and 513 μg per g. The same result may be obtained by using equation (7.6.7) to give the SE of 7·6 μg per g to the potency estimate. Had the indications of invalidity been ignored and the data from the blanks used, R would have been obtained from equations (7.4.4); the estimate of potency would then have been 498 ± 7·3 μg per g, with limits at 482 μg and 514 μg per g. In spite of the significance of Blanks in Table 7.5.1, the difference in conclusion is clearly unimportant here.

7.7 General formulae

The general variance and covariance matrix may be written

$$\mathbf{V} = \begin{pmatrix} \frac{S_{x_T x_T}}{\Delta} & -\frac{S_{x_S x_T}}{\Delta} \\ -\frac{S_{x_S x_T}}{\Delta} & \frac{S_{x_S x_S}}{\Delta} \end{pmatrix}, \tag{7.7.1}$$

where

$$\Delta = S_{x_S x_S} S_{x_T x_T} - (S_{x_S x_T})^2. \tag{7.7.2}$$

The regression coefficients, solutions of equations (7.2.3), are

$$\left.\begin{aligned} b_S &= v_{11}S_{x_S y} + v_{12}S_{x_T y}, \\ b_T &= v_{12}S_{x_S y} + v_{22}S_{x_T y}. \end{aligned}\right\} \tag{7.7.3}$$

Equation (7.2.4) gives R. If g, equation (7.6.6), is small, the variance formula

$$\mathrm{Var}(R) = \frac{s^2}{b_S^2 \Delta}(S_{x_S x_S} + 2RS_{x_S x_T} + R^2 S_{x_T x_T}) \tag{7.7.4}$$

may be used; if g is not small, equation (7.6.5) is needed. An analysis of variance like that in Table 7.4.1 can be constructed, with the aid of formula (7.4.5) for the sum of squares that is accounted for by the regression. If the replicates are not classified into blocks, the error sum of squares can be calculated directly from the pooled variation within doses. The usual procedure of analysis of variance for the elimination of block effects would be necessary, for example, if the tubes in the assay just discussed had been arranged in the incubator in two randomized blocks of nine.

These formulae apply to any spacing of doses, to any numbers of tubes at each dose, and whether or not tests are made at zero dose. If the sum of squares for deviations from regression is to be subdivided as in Table 7.5.1, the formulae may be applied twice, once including and once excluding the blanks, so as to give the 1 d.f. for blanks as the difference between two residual sums of squares, but the procedure in §7.5 is preferable.

Important though the general formulae are, symmetric designs should be adopted whenever possible, because of their efficiency and their relative simplicity of execution and analysis.

7.8 The symmetric $(1, k, k)$-point design

A slope ratio design analogous to the (k, k) for parallel lines is the $(1, k, k)$; in its symmetric form, this has equal numbers of subjects at zero dose and at k equally spaced doses of each preparation. Without loss of generality, the scales may be so chosen that the highest dose of each preparation is unity; if on the original scales these doses are X_S, X_T, the relative potency calculated on the conventional scales must finally be multiplied by X_S/X_T. The total number of subjects,

$$N = n(2k+1), \tag{7.8.1}$$

includes n at zero dose and n at doses $\frac{1}{k}, \frac{2}{k}, \ldots, \frac{k-1}{k}, 1$ of each preparation.

The algebra may be developed as in §5.3. If $C, S_1, S_2, \ldots, S_k, T_1, \ldots, T_k$ represent dose totals for the blanks and for the two preparations,

$$\left.\begin{aligned} S_{x_S y} &= \frac{1}{k}(S_1 + 2S_2 + \ldots + kS_k) - (k+1)Sy/(4k+2), \\ S_{x_T y} &= \frac{1}{k}(T_1 + 2T_2 + \ldots + kT_k) - (k+1)Sy/(4k+2). \end{aligned}\right\} \tag{7.8.2}$$

Equation (7.7.1) then becomes

$$\mathbf{V} = \frac{3k}{N(k+1)(k^2+k+1)} \begin{pmatrix} 5k^2+5k+2 & 3k(k+1) \\ 3k(k+1) & 5k^2+5k+2 \end{pmatrix}, \quad (7.8.3)$$

the factor outside the matrix being understood as multiplying each of the elements. Equations (7.7.3) give the regression coefficients. The components of the analysis of variance for blanks and intersection are given by two orthogonal contrasts, L_B and L_I, defined by

$$L_B = k(k-1)C-(2k-2)(S_1+T_1)-(2k-5)(S_2+T_2) - (2k-8)(S_3+T_3)+\ldots+(k-1)(S_k+T_k) \quad (7.8.4)$$

for which the divisor is

$$Nk(k-1)(k^2+k+1)/(2k+1), \quad (7.8.5)$$

and

$$L_I = (2k-2)(S_1-T_1)+(2k-5)(S_2-T_2)+(2k-8)(S_3-T_3)+\ldots - (k-1)(S_k-T_k) \quad (7.8.6)$$

for which the divisor is

$$Nk(k-1). \quad (7.8.7)$$

The sum of squares for the remaining $(2k-4)$ d.f. between doses is then found by subtraction of

$$b_S S_{xS y} + b_T S_{xT y}, \quad (2 \text{ d.f.}), \quad (7.4.5)$$

$$\frac{(2k+1)L_B^2}{Nk(k-1)(k^2+k+1)} \quad (1 \text{ d.f.}), \quad (7.8.8)$$

and

$$\frac{L_I^2}{Nk(k-1)} \quad (1 \text{ d.f.}) \quad (7.8.9)$$

from the complete sum of squares between doses with $2k$ d.f.; it may be further partitioned into quadratic, cubic and higher-order components for each preparation, by applying to the S totals and the T totals separately the same orthogonal coefficients as were discussed in Chapter 5.

In the absence of evidence of invalidity, the estimate and its fiducial limits are assessed by equations (7.2.4) and (7.6.5). The variances and covariance of the regression coefficients are obtained from $\mathbf{V}$ as

$$\left.\begin{aligned} \mathrm{Var}(b_S) &= s^2 v_{11}, \\ \mathrm{Var}(b_T) &= s^2 v_{22}, \\ \mathrm{Cov}(b_S, b_T) &= s^2 v_{12}, \end{aligned}\right\} \quad (7.8.10)$$

whence

$$g = \frac{3t^2 s^2 k(5k^2+5k+2)}{Nb_S^2(k+1)(k^2+k+1)}. \quad (7.8.11)$$

The formula for $\mathrm{Var}(R)$ and the fiducial limits can be written in terms of k, but they do not take any particularly simple form and the general expressions are more easily remembered.

If the assay is statistically invalid because of the significance of L_B, the data for the blanks may be rejected; the remainder may be treated as a $(0, k, k)$ design, with a new analysis as described in § 7.12, remembering that the total number of subjects is now only $2nk$ or $2Nk/(2k+1)$.

7.9 The (1, 1, 1) assay

The simplest special case, the (1, 1, 1) design, is even less satisfactory than is the (2, 2) parallel line assay. Although it provides no validity tests of any kind, it is of interest as a standard of comparison, since, if the experimenter were certain of validity *a priori*, it would lead to the most reliable estimate of potency. In any (1, 1, 1) assay, the regression lines are obtained by joining the points representing the mean S and T responses to the point for the blanks, so giving a perfect fit of the data to the regression equations; no degrees of freedom remain for tests of statistical or fundamental validity.

In the symmetric design, $N/3$ subjects are tested at zero dose and at unit dose of S, T. If the totals of the responses at the three doses are C, S, T, the general equations of § 7.8 agree that

$$\left.\begin{aligned} b_S &= 3(S_1-C)/N, \\ b_T &= 3(T_1-C)/N, \end{aligned}\right\} \quad (7.9.1)$$

and

$$R = (T_1-C)/(S_1-C). \quad (7.9.2)$$

Moreover,

$$g = \frac{6t^2 s^2}{Nb_S^2} = \frac{2Nt^2 s^2}{3(S_1-C)^2}. \quad (7.9.3)$$

The fiducial limits might be expressed directly in terms of the totals C, S_1, T_1, but they are more conveniently written

$$R_L, R_U = \left[R - \tfrac{1}{2}g \pm \frac{t}{b_S}\left\{\frac{3s^2}{2N}(4-4R+4R^2-3g)\right\}^{1/2}\right] \Big/ (1-g). \quad (7.9.4)$$

When g is small, a satisfactory approximation is

$$\mathrm{Var}(R) = \frac{6s^2(1-R+R^2)}{Nb_S^2}. \quad (7.9.5)$$

7.10 The (1, 2, 2) assay

The (1, 2, 2) design is usually preferable to the (1, 1, 1), though its advantages are achieved at the cost of a reduction in relative reliability (Chapter 8). The symmetric form is perhaps the most useful of all slope ratio designs. To each of

the five doses (zero and $\frac{1}{2}$, 1 unit of each preparation) $N/5$ subjects are assigned. Equation (7.8.3) becomes

$$\mathbf{V} = \frac{4}{7N}\begin{pmatrix} 16 & 9 \\ 9 & 16 \end{pmatrix}. \qquad (7.10.1)$$

The regression coefficients can be calculated from equations (7.7.3) and the corresponding sum of squares from (7.4.5).

Alternatively, the calculations may be made directly from contrasts between responses. This is analogous to the procedure in Chapter 5, but the contrasts for b_S and b_T are not mutually orthogonal (§ 4.16); consequently, the two regression coefficients cannot be made to give independent squares for the analysis of variance, and use of formula (7.4.5) is unavoidable. The remaining dose contrasts can be subdivided into components orthogonal with one another and with b_S and b_T as in § 7.8. In Table 7.10.1, the first two divisors are to be used only for forming b_S and b_T, and the others only for forming the appropriate squares. The reader should verify the non-orthogonality of b_S and b_T, and the orthogonality of every other pair. The contrasts L_B and L_I, together with the regression, account for the whole of the sum of squares between doses, as will be numerically verified in Tables 7.10.2 and 7.10.3.

TABLE 7.10.1 Coefficients of regression and orthogonal contrasts for the (1, 2, 2) design

Contrast	C	S_1	S_2	T_1	T_2	Divisor
b_S	−15	1	17	−6	3	$35n/2$
b_T	−15	−6	3	1	17	$35n/2$
L_B	2	−2	1	−2	1	$14n$
L_I	0	2	−1	−2	1	$10n$

The analysis of variance analogous to Table 7.5.1 may now be completed. If examination of L_B, L_I and any other relevant tests does not indicate invalidity, the fiducial limits to R are

$$R_L, R_U = \left[R - \frac{9g}{16} \pm \frac{t}{b_S}\left\{\frac{8s^2}{7N}\left(8 - 9R + 8R^2 - \frac{175g}{32}\right)\right\}^{1/2}\right] \Big/ (1-g), \qquad (7.10.2)$$

where

$$g = \frac{64t^2s^2}{7Nb_S^2}. \qquad (7.10.3)$$

When g is small enough to be ignored,

$$\mathrm{Var}(R) = \frac{8s^2(8 - 9R + 8R^2)}{7Nb_S^2}. \qquad (7.10.4)$$

Formulae for unsymmetric (1, 2, 2) designs (Wood and Finney, 1946) are rarely needed and will not be reproduced here.

The computational simplicity of the symmetric (1, 2, 2) design may be illustrated by an assay of a sample of malt for its riboflavin content, using *Lactobacillus helveticus* as the test organism and titrating for acidity with sodium hydroxide. The data in Table 7.10.2 were reported by Wood (1946a), who used 20 tubes, 4 each for blanks, 0·1, 0·2 μg of standard riboflavin and 0·025, 0·05 g malt per tube. Responses were measured to the nearest 0·05 ml: for arithmetical convenience, this may be taken as the unit of response, so that all values of y are integers.

TABLE 7.10.2 Responses in an assay of riboflavin in malt (measured in units of 0·05 ml N/10 NaOH)
Standard preparation: 1 unit = 0·2 μg riboflavin
Test preparation: 1 unit = 0·05 g malt

Blanks	Standard		Test	
$x_S = 0$	$x_S = \frac{1}{2}$	$x_S = 1$	$x_S = 0$	$x_S = 0$
$x_T = 0$	$x_T = 0$	$x_T = 0$	$x_T = \frac{1}{2}$	$x_T = 1$
38	97	167	80	121
45	100	164	88	124
40	105	159	90	122
44	98	156	82	122
167	400	646	340	489

Equations (7.2.3) become

$$3{\cdot}2b_S - 1{\cdot}8b_T = 233{\cdot}4,$$
$$-1{\cdot}8b_S + 3{\cdot}2b_T = 46{\cdot}4.$$

Alternatively, for the dose totals in Table 7.10.2, the contrasts defined in Table 7.10.1 give

$$b_S = 8304/70 = 118{\cdot}629,$$
$$b_T = 5686/70 = 81{\cdot}229.$$

The sum of squares for regression is therefore

$$118{\cdot}629 \times 233{\cdot}4 + 81{\cdot}229 \times 46{\cdot}4 = 31\,456{\cdot}9.$$

Again using Table 7.10.1,

$$L_B = -11,$$
$$L_I = -37,$$

which make contributions $11^2/56$, $37^2/40$ to the analysis of variance in Table 7.10.3. Direct computation of the sum of squares between doses (4 d.f.) checks the total of these three items. The error sum of squares may be obtained by subtraction, or by pooling contributions, each with 3 d.f., from the columns of Table 7.10.2. The mean squares from these five contributions, 10·9, 12·7, 24·3,

22·7 and 1·6, show no association with the magnitude of the mean response; randomization had been strictly conducted, so that the small component from the higher dose of malt has no obvious explanation, and Bartlett's test discloses no heterogeneity. Table 7.10.3 gives no indication of invalidity in respect of blanks or intersection.

TABLE 7.10.3 Analysis of variance for Table 7.10.2

Adjustment for mean		208 488·2	Mean square
Nature of variation	d.f.	Sum of squares	
Regression	2	31 456·9	
Blanks	1	2·2	2·2
Intersection	1	34·2	34·2
Between doses	4	31 493·3	
Error	15	216·5	14·43
Total	19	31 709·8	

In the units for analysis, the estimate of potency is, by equation (7.2.4),

$$R = 81{\cdot}229/118{\cdot}629$$
$$= 0{\cdot}6847.$$

Equation (7.10.3) gives

$$g = \frac{16 \times (2{\cdot}131)^2 \times 14{\cdot}43}{35 \times (118{\cdot}63)^2} = 0{\cdot}0021,$$

so small that the standard error of R, 0·0181, could safely be used. Equation (7.10.2) gives

$$R_L, R_U = 0{\cdot}6464, 0{\cdot}7235$$

as the fiducial limits. In order to express the results as μg riboflavin per g malt, they must be multiplied by the ratio of units, 0·2/0·05. The conclusion is that the malt contains 2·74 μg riboflavin per g with fiducial limits at 2·59 and 2·89 μg per g.

7.11 Other $(1, k, k)$ assays

Designs with higher values of k are seldom chosen. As shown in Chapter 8, they are appreciably less efficient, and their additional validity tests are not often needed. Uncertainty about the upper limit of the range of linearity occasionally makes desirable an assay with one or two high dose levels that can be rejected from the analysis if they are clearly beyond the linear region.

For the (1, 3, 3) design,

$$\mathbf{V} = \frac{9}{26N}\begin{pmatrix} 31 & 18 \\ 18 & 31 \end{pmatrix}. \qquad (7.11.1)$$

Table 7.11.1 shows the non-orthogonal contrasts for the regression coefficients. The remaining 4 d.f. between doses may be divided orthogonally into blanks, intersection, and a quadratic component for each preparation. The contrasts are shown in Table 7.11.1; since the regression curves are not parallel, no meaning attaches to average measures of curvature, and the use of separate components for the two preparations seems preferable. The divisors are used to give the magnitudes of b_S and b_T, and to give the squares for the other contrasts; the sum of squares for the regression must as usual be calculated from formula (7.4.5).

TABLE 7.11.1 Coefficients of regression and orthogonal contrasts for the (1, 3, 3) design

Contrast	C	S_1	S_2	S_3	T_1	T_2	T_3	Divisor
b_S	−42	−11	20	51	−24	−6	12	$182n/3$
b_T	−42	−24	−6	12	−11	20	51	$182n/3$
L_B	6	−4	−1	2	−4	−1	2	$78n$
L_I	0	4	1	−2	−4	−1	2	$42n$
L_{2S}	0	1	−2	1	0	0	0	$6n$
L_{2T}	0	0	0	0	1	−2	1	$6n$

The (1, 4, 4) design is easily incorporated into a programme that ordinarily uses (1, 2, 2), as it requires only that additional tests be made at doses one-quarter and three-quarters of the highest for each preparation. For this design,

$$\mathbf{V} = \frac{24}{35N}\begin{pmatrix} 17 & 10 \\ 10 & 17 \end{pmatrix}. \qquad (7.11.2)$$

The regression and other contrasts are in Table 7.11.2; a factor 3 has been removed from L_B and L_I. The additional degrees of freedom are associated with cubic components for the two preparations, L_{3S} and L_{3T}. The relation of the quadratic and cubic components to L_2 and L_3 in Table 5.6.1 should be clear. For most purposes, a composite test of residual curvature with 4 d.f. will suffice; the sum of squares is obtained by subtracting the regression, blanks, and intersection components from the total between doses.

TABLE 7.11.2 Coefficients of regression and orthogonal contrasts for the (1, 4, 4) design

Contrast	C	S_1	S_2	S_3	S_4	T_1	T_2	T_3	T_4	Divisor
b_S	−30	−13	4	21	38	−20	−10	0	10	$105n/2$
b_T	−30	−20	−10	0	10	−13	4	21	38	$105n/2$
L_B	4	−2	−1	0	1	−2	−1	0	1	$28n$
L_I	0	2	1	0	−1	−2	−1	0	1	$12n$
L_{2S}	0	1	−1	−1	1	0	0	0	0	$4n$
L_{2T}	0	0	0	0	0	1	−1	−1	1	$4n$
L_{3S}	0	−1	3	−3	1	0	0	0	0	$20n$
L_{3T}	0	0	0	0	0	−1	3	−3	1	$20n$

7.12 The symmetric $(0, k, k)$-point design

A second type of symmetric slope ratio assay has no blanks, but instead has k doses equally spaced between c and 1 for each preparation, c being taken as small yet sufficiently large to avoid curvature. Thus the doses are

$$c, c+\frac{1-c}{k-1}, c+\frac{2(1-c)}{k-1}, \ldots, c+\frac{(k-2)(1-c)}{k-1}, 1,$$

with $N/2k$ subjects at each. This design would not be chosen if the linear regression of response on dose were believed to hold down to zero dose, for it would then fail to use the whole range of linearity and so would give results less precise than the best obtainable. For an assay based upon a response that is known to depart from a linear regression at low doses, a $(0, k, k)$ design can be a good choice; the value of c might perhaps be about 0·1.

General formulae involve both c and k, and offer no particular advantages over the complete regression calculations. One special case is that of a $(1, k, k)$ converted into a $(0, k, k)$ by the necessity of rejecting the tests on the blanks, because of significant deviation from the linear regression equation (cf. § 7.8). For this design, $c = 1/k$, and N is now the original N multiplied by $2k/(2k+1)$. Hence

$$\mathbf{V} = \frac{6k^2}{N(k^2-1)(2k+1)} \begin{pmatrix} 5k+1 & 3(k+1) \\ 3(k+1) & 5k+1 \end{pmatrix}. \qquad (7.12.1)$$

The blanks contrast no longer occurs; that for intersection is still L_I as defined by (7.8.6), with divisor $\frac{1}{2}N(k-1)(2k+1)$ in terms of the new N.

TABLE 7.12.1 Coefficients of regression and orthogonal contrasts for the (0, 2, 2) design with $c = \frac{1}{2}$

Contrast	S_1	S_2	T_1	T_2	Divisor
b_S	−4	7	−6	3	$5n$
b_T	−6	3	−4	7	$5n$
L_I	2	−1	−2	1	$10n$

TABLE 7.12.2 Coefficients of regression and orthogonal contrasts for the (0, 3, 3) design with $c = \frac{1}{3}$

Contrast	S_1	S_2	S_3	T_1	T_2	T_3	Divisor
b_S	−3	1	5	−4	−1	2	$14n/3$
b_T	−4	−1	2	−3	1	5	$14n/3$
L_I	4	1	−2	−4	−1	2	$42n$
L_2S	1	−2	1	0	0	0	$6n$
L_2T	0	0	0	1	−2	1	$6n$

For ease of reference, the contrasts required for $k = 2, 3, 4$ in designs resulting from rejection of the blanks in a $(1, k, k)$ assay are summarized in Tables 7.12.1–7.12.3; these are arranged to correspond to Tables 7.10.1, 7.11.1, and 7.11.2. The variance matrices are

TABLE 7.12.3 Coefficients of regression and orthogonal contrasts for the (0, 4, 4) design with $c = \frac{1}{4}$

Contrast	S_1	S_2	S_3	S_4	T_1	T_2	T_3	T_4	Divisor
b_S	−8	−1	6	13	−10	−5	0	5	$15n$
b_T	−10	−5	0	5	−8	−1	6	13	$15n$
L_I	2	1	0	−1	−2	−1	0	1	$12n$
L_2S	1	−1	−1	1	0	0	0	0	$4n$
L_2T	0	0	0	0	1	−1	−1	1	$4n$
L_3S	−1	3	−3	1	0	0	0	0	$20n$
L_3T	0	0	0	0	−1	3	−3	1	$20n$

$$\mathbf{V} = \frac{8}{5N} \begin{pmatrix} 11 & 9 \\ 9 & 11 \end{pmatrix}, \qquad (7.12.2)$$

$$\mathbf{V} = \frac{27}{7N} \begin{pmatrix} 4 & 3 \\ 3 & 4 \end{pmatrix}, \qquad (7.12.3)$$

and

$$\mathbf{V} = \frac{32}{15N} \begin{pmatrix} 7 & 5 \\ 5 & 7 \end{pmatrix} \qquad (7.12.4)$$

respectively. In the formulae, N always represents the total number of subjects in the assay as analyzed, and n the number of subjects per dose, so that equation (7.8.1) must be replaced by

$$N = 2nk. \qquad (7.12.5)$$

7.13 Routine assays

With the obvious modifications, much of §§ 5.7–5.10 applies to slope ratio assays. If a particular design is used frequently as a routine, the calculations should be standardized and reduced to a minimal labour consistent with extracting adequate information. For desk calculation of any of the symmetric designs, the formulae in earlier sections are easily applied systematically; a computer program that both draws attention to any evidence of invalidity and completes the potency estimation is simple to write. Nomographs could be developed. Approximations such as range estimation of standard deviations (Wood, 1947b) should have the same status as for parallel line assays.

For routine assays, control charts should prove a valuable guard against unsuspected changes in experimental conditions. Control charts might be set up for s^2 and b_S, and also for L_B and L_I or for the ratios of these two quantities to their standard errors. In order to give better values for s^2 and b_S, some pooling of estimates from previous assays might be permitted in a series of assays showing satisfactory control. The suggestions in § 5.10 are readily adapted.

7.14 Other slope ratio problems

Whereas the study of parallel regression lines preceded their special use in biological assays, development of statistical methods for concurrent pencils of regression lines seems to have begun with slope ratio assays. Neither the designs nor the arrangement of the analysis of variance suited to assays are necessarily the best in other circumstances. Claringbold (1959) pointed out that, when chief interest lies in tests of significance of slope differences, sets of mutually orthogonal contrasts may be preferable and also that the blanks may give little relevant information. His valuable paper should be seen by any who have problems allied to the slope ratio situation but not strictly of an assay type. Other special methods of analysis appropriate to pencils of lines have been well presented by Williams (1959).

8
Efficiency in slope ratio assays

8.1 General principles

The principles of assay design described in §§ 6.1–6.7 are as relevant and as important in slope ratio as in parallel line assays. Their application leads to different advice, because the formulae expressing precision and reliability are different. The requirements of good design for slope ratio assays are here discussed under the assumption that conditions (a), (b), and (c) of § 6.8 again apply, except that the regression is now known to be linear on the absolute measure of dose. The problem for the assayist is still that of making the best use of a total of N subjects.

Suppose that the highest doses of S, T used in an assay are X_S, X_T. As in § 7.7, define v_{11}, v_{12}, v_{22} to be the elements of the variance matrix after rescaling all doses so that the highest dose of each preparation is unity; that is to say, these quantities relate to an assay in which all doses of S have been divided by X_S, all doses of T by X_T. Then, for the assay as actually performed,

$$\mathbf{V} = \begin{pmatrix} \dfrac{v_{11}}{X_S^2} & \dfrac{v_{12}}{X_S X_T} \\ \dfrac{v_{12}}{X_S X_T} & \dfrac{v_{22}}{X_T^2} \end{pmatrix}. \tag{8.1.1}$$

This definition enables the effect of the range of doses to be kept distinct from that of changing the distribution of doses over their range. From equation (7.6.5) the quarter-square of the fiducial interval for R is

$$I = \frac{t^2 s^2}{b_S^2 (1-g)^2}\left[\frac{v_{22}}{X_T^2} - \frac{2Rv_{12}}{X_S X_T} + \frac{R^2 v_{11}}{X_S^2} - \frac{g}{X_T^2}\left(v_{22} - \frac{v_{12}^2}{v_{11}}\right)\right], \tag{8.1.2}$$

where

$$g = \frac{t^2 s^2 v_{11}}{b_S^2 X_S^2}. \tag{8.1.3}$$

Equation (8.1.2) may be written

$$I = \frac{R^2 t^2 s^2}{b_S^2 X_S^2 h^2 (1-g)^2}\left[v_{22} - 2hv_{12} + h^2 v_{11} - g\left(v_{22} - \frac{v_{12}^2}{v_{11}}\right)\right]; \tag{8.1.4}$$

the quantity

$$h = RX_T/X_S, \tag{8.1.5}$$

the ratio of estimated relative potency to relative magnitudes of the highest