2. Permanganate oxidation of the metabolite of MTX yields a simple pteridine with the properties of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid.

3. Heating of the latter compound in 2 *N* sodium hydroxide solution at 70° leads to slow hydrolysis of the 4-amino group.

4. The nonpteridine moiety of the metabolite of MTX is identical with the nonpteridine moiety of MTX.

5. The structure 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid (7-hydroxy-MTX) is proposed for the MTX metabolite.

REFERENCES

(1) Johns, D. G., *et al.*, *Biochim. Biophys. Acta*, **105**, 380(1965).
(2) Redetzki, H. M., Redetzki, J. E., and Elias, A. L., *Biochem. Pharmacol.*, **15**, 425(1966).
(3) Johns, D. G., *et al.*, *ibid.*, **15**, 555(1966).
(4) Loo, T. L., and Adamson, R. H., *ibid.*, **11**, 170(1962).
(5) Loo, T. L., and Adamson, R. H., *J. Med. Chem.*, **8**, 513(1965).
(6) Albert, A., *Quart. Rev.*, **6**, 197(1952).
(7) Rajagopalan, K. V., Fridovich, I., and Handler, P., *J. Biol. Chem.*, **237**, 922(1962).
(8) Waddell, W. J., *J. Lab. Clin. Med.*, **48**, 311(1956).
(9) Johns, D. G., *et al.*, *Life Sci.*, **3**, 1383(1964).
(10) Bertino, J. R., *et al.*, *J. Biol. Chem.*, **239**, 479(1964).
(11) Zakrzewski, S. F., *ibid.*, **238**, 1485(1963).
(12) Werkheiser, W. C., *ibid.*, **236**, 888(1961).
(13) Elion, G. B., Hitchings, G. H., and Russell, P. B., *J. Am. Chem. Soc.*, **72**, 78(1950).
(14) Fu, S.-C. J., Reiner, M., and Loo, T. L., *J. Org. Chem.*, **30**, 1278(1965).
(15) Freireich, E. J., *et al.*, *Cancer Chemotherapy Rept.*, **50**, 219(1966).
(16) Freeman, M. V., *J. Pharmacol. Exptl. Therap.*, **122**, 154(1958).
(17) Johns, D. G., *et al.*, *J. Clin. Invest.*, **43**, 621(1964).
(18) Henderson, E. S., Adamson, R. H., and Oliverio, V. T., *Cancer Res.*, **25**, 1018(1965).

Statistical Analysis of Log-Dose Response Bioassay Experiments with Unequal Dose Ratios for the Standard and Unknown Preparations

By C. PHILIP COX

Well-known statistical analyses are available for the analysis of log-dose response assays when successive doses are in the same ratio for both the standard and unknown preparations. It is, however, sometimes convenient and advantageous in practice to use unequal dose ratios. Appropriate analyses are offered for such cases, analyses which reduce to the usual ones when the ratios are equal. It is seen that the operational flexibility thus permitted is obtained in return for only slightly increased computation. Four and six-point assays are discussed in detail together with a numerical example of the former. An improved method for calculating confidence interval estimates in log-dose response assays is also given.

STATISTICAL ANALYSES for log-dose response parallel line assays in which the ratios of the successively higher doses are the same for both the standard and unknown preparations are well known. (For example, see *Reference 1.*) The case to be considered here is that in which the ratios of successive doses are constant within each preparation but the constant ratio differs from one preparation to the other. Such a relaxation of the usual single constant ratio condition is occasionally desirable in practice (2) and, for example, may permit the linear response log-dose range of the standard preparation to be exploited in the presence of more uncertainty about the linear range for the unknown preparation.

It will be shown that such flexibility can be achieved with only small changes from the single ratio analyses and, in fact, the latter can be regarded as special cases of the more general analysis proposed here. This analysis has some computational advantages over the alternative based on a single log-dose transformation which gives integral dose-metameter values for one preparation but nonintegral values for the other.

SPECIFICATION AND DOSE TRANSFORMATIONS

It will be supposed that, in an $(h + k)$-point assay, the h and k doses (concentrations) of the standard and unknown preparations, respectively, are chosen so that,

$$\left.\begin{aligned} \frac{z_{Si}}{z_{Si-1}} &= D_S,\ i = 1, 2, \ldots, h \\ \text{and} \\ \frac{z_{Uj}}{z_{Uj-1}} &= D_U,\ j = 1, 2, \ldots, k \end{aligned}\right\} \quad \text{(Eq. 1)}$$

where z_{Si} and z_{Uj} are, respectively, the ith dose of the standard and the jth dose of the unknown preparation.

As usual, it will be assumed that the observed

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responses are subject to residual variability components which are normally and independently distributed about population responses and that these are linearly related to logarithms of the doses over, at least, the dose ranges used. Then, writing

$$d_S = \log D_S \text{ and } d_U = \log D_U$$

dose transformations are defined as,

$$\left.\begin{aligned} x_S &= \frac{1}{d_S}\log(z_S/z_{S1}) \\ \text{and} \\ x_U &= \frac{1}{d_U}\log(z_U/z_{U1}) \end{aligned}\right\} \quad \text{(Eq. 2)}$$

where z_{S1} and z_{U1} are the two lowest doses. Hence, because for the ith dose, from Eq. 1,

$$z_{Si} = D_S z_{Si-1} = \ldots = D_S^{i-1} z_{S1}$$

so that,

$$x_{Si} = \frac{1}{d_S}\log(D_S^{i-1} z_{S1}/z_{S1}) = i - 1$$

it is apparent that the integral x_S values 0, 1, 2, . . . , $(h - 1)$, correspond to the z_S values z_{S1}, z_{S2}, . . . z_{Sh}, and similarly, for the unknown preparation, the second of Eq. 2 transforms the z_U values into the integral x_U values 0, 1, 2, . . ., $k - 1$.

EQUIVALENT DILUTION CONDITION AND RELATIVE POTENCY FORMULA

A dilution assay is one in which the unknown preparation acts simply as a dilution (concentration) of the standard preparation in an inert diluent. In this case if z_{ES} is a dose of the standard preparation which is equipotent to, that is, produces the same response value as a dose z_{EU} of the unknown preparation, any two such equipotent doses are connected by the equivalent dilution relationship

$$z_{ES} = \rho_p z_{EU} \quad \text{(Eq. 3)}$$

where ρ_p is the relative potency.

It is well known that, in the single dose-ratio case, this expression leads to a pair of parallel lines for the relationship between responses and log-dose values. The corresponding implication from the equivalent dilution condition based on the transformations in Eq. 2 is readily obtained as follows.

Let x_{ES} and x_{EU} denote equipotent log-dose-values so that, from Eqs. 2 and 3,

$$d_S x_{ES} - d_U x_{EU} = \log\left(\frac{z_{ES}}{z_{EU}}\right)\left(\frac{z_{U1}}{z_{S1}}\right) = \log(\rho_p z_{U1}/z_{S1}) \quad \text{(Eq. 4)}$$

and it may be noted that, if $d_S = d_U$, Eq. 4 reduces to $x_{ES} - x_{EU}$ = a constant, that is to the usual parallelism condition.

The next step is to find the relations between the parameters of the two response lines when these are such that the condition in Eq. 4 obtains. These relations will further lead to the expression from which the relative potency may be estimated.

For these suppose that population values η_S and η_U of standard and unknown preparation responses are linearly related to their corresponding x_S and x_U values by the equations:

$$\left.\begin{aligned} \eta_S &= \alpha_S + \beta_S x_S \\ \eta_U &= \alpha_U + \beta_U x_U \end{aligned}\right\} \quad \text{(Eq. 5)}$$

α_S and α_U being the intercepts, while β_S and β_U are the slopes of the lines. Then, since $\eta_S = \eta_U$ for equipotent doses,

$$\alpha_S + \beta_S x_{ES} = \alpha_U + \beta_U x_{EU}$$

and, multiplying both sides by d_S,

$$d_S\alpha_S + \beta_S d_S x_{ES} = d_S\alpha_U + \beta_U d_S x_{EU}$$

Hence, substituting for $d_S x_{ES}$ from Eq. 4 into the lefthand side,

$$d_S\alpha_S + \beta_S\{d_U x_{EU} + \log(\rho_p z_{U1}/z_{S1})\} = d_S\alpha_U + \beta_U d_S x_{EU}$$

that is,

$$d_S(\alpha_S - \alpha_U) + \beta_S\log(\rho_p z_{U1}/z_{S1}) + (\beta_S d_U - \beta_U d_S)x_{EU} = 0$$

But, for this relation to hold for all x_{EU} in the linear range, it must be an identity and hence,

$$d_S(\alpha_S - \alpha_U) + \beta_S\log(\rho_P z_{U1}/z_{S1}) = 0 \quad \text{(Eq. 6)}$$

and

$$\beta_S d_U - \beta_U d_S = 0 \quad \text{(Eq. 7)}$$

The relation in Eq. 7 is the new statement of the equivalent dilution condition and which reduces to the parallelism condition, $\beta_S = \beta_U$, when $d_S = d_U$. For the relative potency itself it is first convenient to write the condition in Eq. 7 as:

$$\frac{\beta_S}{d_S} = \frac{\beta_U}{d_U} = \beta \quad \text{(Eq. 8)}$$

Then, from Eq. 6,

$$\log(\rho_p z_{U1}/z_{S1}) = -(\alpha_S - \alpha_U)/\beta \quad \text{(Eq. 9)}$$

so that the population value of the relative potency is given by

$$\rho_p = \frac{z_{S1}}{z_{U1}}\,\text{antilog}\left\{-(\alpha_S - \alpha_U)/\beta\right\}$$

and the related estimation procedure is next considered.

RELATIVE POTENCY ESTIMATE

Writing Eq. 5 in the equivalent form,

$$\eta_S = \mu_S + \beta_S(x_S - \bar{x}_S) = \mu_S + \beta d_S(x_S - \bar{x}_S)$$

$$\eta_U = \mu_U + \beta_U(x_U - \bar{x}_U) = \mu_U + \beta d_U(x_U - \bar{x}_U)$$

it follows that

$$(\alpha_S - \alpha_U) = (\mu_S - \mu_U) - \beta(d_S\bar{x}_S - d_U\bar{x}_U)$$

and hence, from Eq. 9, that

$$\log(\rho_p z_{U1}/z_{S1}) = d_S\bar{x}_S - d_U\bar{x}_U - \frac{(\mu_S - \mu_U)}{\beta} \quad \text{(Eq. 10)}$$

Conventional statistical methodology then leads to estimates of μ_S, μ_U, and β as $\bar{y}_S$, $\bar{y}_U$, and b, respectively, where

$$b = \frac{d_S\Sigma_S'xy + d_U\Sigma_U'xy}{d_S^2\Sigma_S'xx + d_U^2\Sigma_U'xx} \quad \text{(Eq. 11)}$$

in which quantities such as,

$$\Sigma_S'xy = \Sigma_S(x_S - \bar{x}_S)(y_S - \bar{y}_S) = \Sigma_S(x_S - \bar{x}_S)y_S$$

can be calculated by the usual computation formula, suffixes S and U denoting summations over the standard and test preparation values, respectively.

From Eq. 10 the relative potency estimate R is then given by

$$R = \frac{z_{S1}}{z_{U1}} \text{antilog } [d_S\bar{x}_S - d_U\bar{x}_U - (\bar{y}_S - \bar{y}_U)/b] \quad \text{(Eq. 12)}$$

Pursuance of the general case beyond this point is straightforward but likely to be of limited practical appeal; more usefully the commonly occurring cases of four and six-point assays will now be examined in detail.

FOUR-POINT LOG-DOSE RESPONSE ASSAY

Suppose that a four-point assay is carried out in a completely randomized design and that n responses are observed at each of the four preparation-dose combinations. Let S_1 and S_2 be the response totals for the lower and higher doses of the standard preparation, U_1 and U_2 being the corresponding totals for the unknown preparation. Then, as in the usual case when $d_S = d_U$, it is convenient for the analysis of variance (ANOV) and the estimation of relative potency to obtain three orthogonal contrasts between the four treatment totals.

The difference between the means for the two preparations $(\bar{y}_S - \bar{y}_U)$ is

$$\bar{y}_S - \bar{y}_U = \frac{1}{2n}(S_1 + S_2 - U_1 - U_2) = -L_p/2n \quad \text{(Eq. 13)}$$

where

$$L_p = -S_1 - S_2 + U_1 + U_2 \quad \text{(Eq. 14)}$$

is the first of the contrasts required.

The second contrast is one proportional to the regression coefficient b. In the four-point assay case the transformations in Eq. 2 lead to x-values of 0 and 1 for the upper and lower doses, respectively. Then:

$$\Sigma_S'xy = \Sigma_S(x_S - \bar{x}_S)y_S = \frac{1}{2}(-S_1 + S_2)$$

and

$$\Sigma_S'xx = \Sigma_S(x_S - \bar{x}_S)^2 = n/2$$

and similarly for unknown preparation summations. Hence, from Eq. 11,

$$b = \frac{d_S(-S_1 + S_2) + d_U(-U_1 + U_2)}{n(d_S^2 + d_U^2)} = \frac{L_r}{n(d_S^2 + d_U^2)} \quad \text{(Eq. 15)}$$

so that

$$L_r = -d_SS_1 + d_SS_2 - d_UU_1 + d_UU_2 \quad \text{(Eq. 16)}$$

will be taken as the second contrast.

The third contrast is one for testing the relevance of the model, in particular, being sensitive to departure from the equivalent dilution condition as now expressed by Eq. 8. This contrast can therefore be taken as proportional to the quantity $(d_Ub_S - d_Sb_U)$ where b_S and b_U are regression coefficients for the standard and unknown preparations taken separately. With

$$b_S = \Sigma_S'xy/\Sigma_S'xx = -(S_1 - S_2)/n$$

and

$$b_U = -(U_1 - U_2)/n$$

it follows that

$$d_Ub_S - d_Sb_U = -\frac{1}{n}(d_US_1 - d_US_2 - d_SU_1 + d_SU_2)$$

The third contrast will accordingly be taken as

$$L_d = d_US_1 - d_US_2 - d_SU_1 + d_SU_2 \quad \text{(Eq. 17)}$$

where the suffix d here denotes divergence from the condition in Eq. 8.

The statistical analysis is based on the usual assumptions that the responses are independent and normally distributed with constant variance. Accordingly, because each contrast is a linear combination of independent observations, the appropriate divisors can be found by conventional statistical procedures and it is also readily checked that the three contrasts are orthogonal. From Eqs. 14, 16, and 17 the complete scheme of contrasts together with the ANOV entries, may then be set out as in Table IA.

Defining the correction term as

$$C = (S_1 + S_2 + U_1 + U_2)^2/4n$$

the ANOV is therefore as shown in Table IB.

Model relevance may be tested by comparing the ratio of the mean squares for divergence and for within preparations and levels against the tabulated critical value, $F_c = F(1, 4n - 4, 0.05)$ say. Assuming satisfactory relevance, the point estimate of relative potency is next obtained. For this, values of $(\bar{y}_S - \bar{y}_U)$ and b can be substituted, from Eqs. 13 and 15 into Eq. 12, to give with $\bar{x}_S = \bar{x}_U = \frac{1}{2}$,

$$R = \frac{z_{S1}}{z_{U1}} \text{antilog } \frac{1}{2}\left[d_S - d_U + (d_S^2 + d_U^2)\frac{L_p}{L_r}\right] \quad \text{(Eq. 18)}$$

With the usual assumptions the only quantities subject to statistical variability are the contrasts L_p and L_r. Accordingly, an interval estimate of the relative potency can be obtained from an interval estimate for the population mean of

$$M = (d_S^2 + d_U^2)L_p/L_r \quad \text{(Eq. 19)}$$

The result due to Fieller (3, 4) for interval estimation of a ratio can be applied in this case using the method of Cox and Ruhl (5). The authors showed that r_L and r_H, the lower and upper limits, respectively, of the 95% confidence interval (for example) for the population mean of a ratio $r = L_p/L_r$ can be expressed as

$$r_L, r_H = \frac{1}{(F_r - F_c)} \times \left[rF_r \mp \sqrt{\frac{a_pF_c}{a_r}(F_p + F_r - F_c)}\right] \quad \text{(Eq. 20)}$$

where

TABLE IA—ORTHOGONAL CONTRASTS FOR AN UNEQUAL DOSE RATIO FOUR-POINT ASSAY (n RESPONSES PER DOSE)

Contrast	Response Total S_1	S_2	U_1	U_2	ANOV Sum of Sq.
Prepn.: L_p	-1	-1	1	1	$L_p^2/4n$
Regression: L_r	$-d_S$	d_S	$-d_U$	d_U	$L_r^2/2n(d_S^2 + d_U^2)$
Divergence: L_d	d_U	$-d_U$	$-d_S$	d_S	$L_d^2/2n(d_S^2 + d_U^2)$

TABLE IB—ANOV

Variation Source	d.f.	s.s.	m.s.
Prepn.	1	$L_p^2/4n$	
Regression	1	$L_r^2/2n(d_S^2 + d_U^2)$	
Divergence	1	$L_d^2/2n(d_S^2 + d_U^2)$	
Within prepn. and levels	$4n - 4$	By subtraction	$s^2 = \text{s.s.}/4n - 4$
Total	$4n - 1$	$\Sigma\,(\text{response})^2 - C$ all	

$$F_c = F(1, 4n - 4, 0.05)$$

is, as previously noted, the critical F-value read from standard statistical tables, and

$$\left.\begin{aligned} F_p &= \text{the } F\text{-ratio for preparations in the ANOV} = L_p^2/a_p s^2 \\ F_r &= \text{the } F\text{-ratio for regression in the ANOV} = L_r^2/a_r s^2 \end{aligned}\right\} \quad \text{(Eq. 21)}$$

An improved method can, however, be derived as follows. From Eq. 21,

$$\frac{a_p}{a_r} = \frac{r^2 F_r}{F_p}$$

and substituting this in Eq. 20 gives

$$\begin{aligned} r_L, r_H &= \frac{1}{(F_r - F_c)} \\ &\times \left[rF_r \mp \sqrt{\frac{r^2 F_r F_c}{F_p}(F_p + F_r - F_c)} \right] \\ &= \frac{rF_r}{F_r - F_c} \\ &\times \left[1 \mp \sqrt{\frac{F_c}{F_p F_r}(F_p + F_r - F_c)} \right] \\ &= \frac{r}{\left(1 - \frac{F_c}{F_r}\right)} \\ &\times \left[1 \mp \sqrt{\frac{F_c}{F_r} + \frac{F_c}{F_p}\left(1 - \frac{F_c}{F_r}\right)} \right] \end{aligned} \quad \text{(Eq. 22)}$$

Applied in the present case the lower and upper limits, M_L and M_H, with M as defined in Eq. 19 are then, from Eq. 22,

$$\begin{aligned} M_L, M_H &= \frac{M}{\left(1 - \frac{F_c}{F_r}\right)} \\ &\times \left[1 \mp \sqrt{\frac{F_c}{F_r} + \frac{F_c}{F_p}\left(1 - \frac{F_c}{F_r}\right)} \right] \end{aligned} \quad \text{(Eq. 23)}$$

The final assay results may therefore be cited as:

$$R = \frac{z_{S1}}{z_{U1}} \text{ antilog } \frac{1}{2}(d_S - d_U + M) \quad \text{(Eq. 24)}$$

where the 95% confidence interval is defined by

$$\begin{aligned} R_L &= \frac{z_{S1}}{z_{U1}} \text{ antilog } \frac{1}{2}(d_S - d_U + M_L), \\ R_H &= \frac{z_{S1}}{z_{U1}} \text{ antilog } \frac{1}{2}(d_S - d_U + M_H) \end{aligned} \quad \text{(Eq. 25)}$$

Numerical Example—To illustrate the above method, responses from an assay (6) are used, unequal dose ratios being obtained by assuming that the upper dose of the unknown preparation was 0.9 mg. instead of 1 mg. as used in the actual experiment. The totals, each of 5 individual responses, and the doses taken for present purposes are shown in Table II.

Here then,

$$D_S = 4,\ D_U = 3.6$$

so that,

$$d_S = \log 4 = 0.6021,\ d_U = 0.5563$$

giving,

$$d_S - d_U = 0.0458,\ d_S^2 + d_U^2 = 0.6720$$

The contracts L_p, L_r, and L_d are then calculated in accordance with the scheme in Table IA as,

$$L_p = -350.1 - 213.9 + 335.8 + 240.7 = 12.5$$

$$\begin{aligned} L_r &= -d_S(S_1 - S_2) - d_U(U_1 - U_2) \\ &= (-0.6021)(136.2) - (0.5563)(95.1) \\ &= -134.9102 \end{aligned}$$

$$\begin{aligned} L_d &= d_U(S_1 - S_2) - d_S(U_1 - U_2) \\ &= (0.5563)(136.2) - (0.6021)(95.1) = 18.5083 \end{aligned}$$

With $n = 5$ the corresponding ANOV entries are then,

$$L_p^2/20 = 7.8125$$

$$L_r^2/10(d_S^2 + d_U^2) = (134.9102)^2/6.72 = 2708.4467$$

$$L_d^2/6.72 = (18.5083)^2/6.72 = 50.9758$$

TABLE II—DOSES AND RESPONSE TOTALS

	Std. Prepn., mcg./LH		Unknown Prepn., mg. Tissue	
Dose	0.6	2.4	0.25	0.9
Response total	350.1	213.9	335.8	240.7

From the original ANOV, the total corrected sum of squares was 3692.1775 so that for the present case the ANOV (in which the ratios F_p and F_r are also indicated) is as shown in Table III.

TABLE III—ANOV

Variation Source	d.f.	s.s.	m.s.	
Prepn.	1	7.8125		$F_p = 0.1351$
Regression	1	2708.4467		$F_r = 46.8517$
Divergence	1	50.9758		
Residual	16	924.9425	57.8089	
Total	19	3692.1775		

Since the mean square for divergence is less than the residual mean square relevance may be considered satisfactory. Proceeding to the estimation of relative potency, from Eq. 19,

$$\begin{aligned} M &= (d_S^2 + d_U^2)L_p/L_r \\ &= (0.6720)(12.5)/-134.9102 \\ &= -0.0623 \end{aligned}$$

so that, from Eq. 24,

$$R = \frac{0.6}{0.25} \text{ antilog } \frac{1}{2}(0.0458 - 0.0623)$$

$$= 2.36 \text{ mcg./mg.}$$

For the interval estimate F_c, from statistical tables, is $F_c = F(1, 16, 0.05) = 4.49$ or, if desired, F_c can be obtained from the t-table for 16 degrees of freedom as

$$F_c = t^2(16, 0.05) = 2.12^2 = 4.49$$

Then, with F_p and F_r from the ANOV,

$$\frac{F_c}{F_p} = \frac{4.49}{0.1351} = 33.2346$$

$$\frac{F_c}{F_r} = \frac{4.49}{46.8517} = 0.0958$$

so that, from Eq. 23,

$$\begin{aligned} M_L, M_H &= \frac{-0.0623}{0.9042} \\ &\quad \times [1 \pm \sqrt{0.0958 + (33.2346)(0.9042)}] \\ &= -0.4472, 0.3094 \end{aligned}$$

and, finally, from Eq. 25,

$$R_L, R_H = 2.4 \text{ antilog } \frac{1}{2}(0.0458 - 0.4472),$$

$$2.4 \text{ antilog } \frac{1}{2}(0.0458 + 0.3094)$$

$$= 1.51, 3.61 \text{ mcg./mg.}$$

SIX-POINT LOG-DOSE RESPONSE ASSAY

Suppose that a six-point assay is carried out using a completely randomized design and that n responses are observed at each of doses z_{S1}, $D_S z_{S1}$, $D_S^2 z_{S1}$ for the standard preparation and at doses z_{U1}, $D_U z_{U1}$, $D_U^2 z_{U1}$ for the unknown preparation. Let the respective response totals be denoted in the usual way as S_1, S_2, S_3, and U_1, U_2, U_3. Five orthogonal contrasts together with their ANOV entries can then be defined as shown in Table IV, where $d_S = \log D_S$ and $d_U = \log D_U$.

The first three contrasts are analogous to the three defined for the four-point assay while the contrasts Q_S and Q_U are sensitive to quadratic curvature in the log-dose response lines for the standard and the unknown preparations, respectively. An alternative formulation for the latter two contrasts would be to replace them by contrasts for combined and opposed curvatures Q_1 and Q_2 say, where,

$$Q_1 = Q_S + Q_U = S_1 - 2S_2 + S_3 + U_1 - 2U_2 + U_3$$

$$Q_2 = Q_S - Q_U = S_1 - 2S_2 + S_3 - U_1 + 2U_2 - U_3$$

for which the ANOV entries would be $Q_1^2/12n$ and $Q_2^2/12n$, respectively. The formulation in Table IV, however, is slightly easier to compute and provides for the relevance tests required because appreciable curvature in either response line will render the present analysis inapplicable. The ANOV is then as shown in Table V.

On the assumption that the relevance tests for divergence, curvature(S) and curvature(U) are satisfactory, the relative potency estimate is now obtained by finding appropriate quantities to substitute into Eq. 12. For the 6-point assay the transformations in Eq. 2 lead to x-values of 0, 1, and 2 for both preparations, so that $\bar{x}_S = \bar{x}_U = 1$ and

$$d_S\bar{x}_S - d_U\bar{x}_U = d_S - d_U$$

Next

$$\bar{y}_S - \bar{y}_U = \frac{1}{3n}(S_1 + S_2 + S_3 - U_1 - U_2 - U_3)$$

$$= -L_p/3n \quad \text{(Eq. 26)}$$

with L_p as defined in Table IV.

For the regression coefficient the quantities required are

$$\Sigma_S' xy = -S_1 + 0S_2 + S_3 = -S_1 + S_3$$

$$\Sigma_S' xx = 2n$$

and similarly for the unknown preparation. Hence, from Eq. 11

$$b = \frac{d_S(-S_1 + S_3) + d_U(-U_1 + U_3)}{2n(d_S^2 + d_U^2)}$$

$$= L_r/2n(d_S^2 + d_U^2) \quad \text{(Eq. 27)}$$

From Eqs. 26, 27, and 12 the relative potency estimate is then

$$R = \frac{z_{S1}}{z_{U1}} \text{ antilog } \left[d_S - d_U + \frac{2}{3}(d_S^2 + d_U^2)\frac{L_p}{L_r}\right]$$

$$= \frac{z_{S1}}{z_{U1}} \text{ antilog } [d_S - d_U + M] \quad \text{(Eq. 28)}$$

where

$$M = \frac{2}{3}(d_S^2 + d_U^2)\frac{L_p}{L_r} \quad \text{(Eq. 29)}$$

For the interval estimate M_L and M_H are calculated, using the now appropriate values, from Eq. 23 so that, for the relative potency itself the interval estimate is defined by

TABLE IV—ORTHOGONAL CONTRASTS FOR AN UNEQUAL DOSE RATIO SIX-POINT ASSAY (*n* RESPONSES PER DOSE)

Contrast		Response Total S_1	S_2	S_3	U_1	U_2	U_3	ANOV Sum of Sq.
Prepn.:	L_p	−1	−1	−1	1	1	1	$L_p^2/6n$
Regression:	L_r	$-d_S$	0	d_S	$-d_U$	0	d_U	$L_r^2/2n(d_S^2 + d_U^2)$
Divergence:	L_d	d_U	0	$-d_U$	$-d_S$	0	d_S	$L_d^2/2n(d_S^2 + d_U^2)$
Curvature (S):	Q_S	1	−2	1	0	0	0	$Q_S^2/6n$
Curvature (U):	Q_U	0	0	0	1	−2	1	$Q_U^2/6n$

$$R_L, R_H = \frac{z_{S1}}{z_{U1}} \text{ antilog } [d_S - d_U + M_L], \quad \frac{z_{S1}}{z_{U1}} \text{ antilog } [d_S - d_U + M_H] \quad \text{(Eq. 30)}$$

CONCLUSION

For a given assay response it has been established in bioassay literature that two highly important requirements for the precision of relative potency estimations are: (*a*) the range, from the lowest to the highest dose investigated, should be as large as possible subject to linearity of the relationship between the response and the log-dose and to the requirement that responses should be normally distributed with constant variance and (*b*) the difference between the mean response of the standard and unknown preparations should be as small as possible.

TABLE V—ANOV

Variation Source	d.f.	s.s.	m.s.
Prepn.	1	$L_p^2/6n$	
Regression	1	$L_r^2/2n(d_S^2 + d_U^2)$	
Divergence	1	$L_d^2/2n(d_S^2 + d_U^2)$	
Curvature (S)	1	$Q_S^2/6n$	
Curvature (U)	1	$Q_U^2/6n$	
Residual	$6n - 6$	By subtraction	s.s./$6n-6$
Total	$6n - 1$		

In planning an assay, therefore, a basic problem is just that of using what is known about the unknown preparation to best meet these requirements. And in early stages, following perhaps some pilot investigations of log-dose response relationships, it may not be desirable to use equal spacing of log-doses for either the standard or the unknown preparations. The analyses of such general assays can be carried through quite straightforwardly using the actual log-doses instead of being based on log-dose transformations giving integral values for at least one of the two sets of log-doses. The advantages of such transformations are primarily computational so that such transformations are commonly used in relatively well-established assays. In this context the present proposals may be regarded as providing a convenient procedure for intermediate cases. It gives simple computations and allows greater flexibility in the choice of doses than is permitted by the completely canonical form which requires the same dose ratio to be used for both preparations.

REFERENCES

(1) Bliss, C. I., "The Statistics of Bioassay," Academic Press Inc., New York, N. Y., 1952.
(2) Folley, S. J., personal communication, 1956.
(3) Fieller, E. C., *Quart. J. Pharm. Pharmacol.*, **17**, 117 (1944).
(4) Fieller, E. C., *J. R. Statist. Soc.*, **B16**, 175(1954).
(5) Cox, C. P., and Ruhl, D. J., *J. Pharm. Sci.*, **55**, 368 (1966).
(6) Schultz, J., personal communication, 1965.