0022-1767/87/1382-0333$02.00/0
THE JOURNAL OF IMMUNOLOGY
Vol. 138, 333-341, No. 2, January 15, 1987
Printed in U.S.A.

A SOLUTION TO THE PROBLEMS OF CYTOLYSIS ASSAYS WITH ADDITIONAL APPLICATIONS TO OTHER IMMUNOLOGICAL AND BIOCHEMICAL ASSAYS[1]

CARL TASWELL

From the Department of Biostatistics, Harvard University School of Public Health, Boston, MA 02115

After cellular immunoassays are compared with classical bioassays, conventional methods and consequent problems of data analysis for cytolysis assays are reviewed and a new solution is proposed. This solution incorporates new methods, called dose-response surface assays and analysis (DRSA), which estimate cytolytic activity coefficients on a surface in a three-dimensional space with two dose variables (killers and targets) and one response variable (counts). These new methods based on dose-response surfaces are demonstrated to be more informative and reliable than classical methods based on dose-response curves. In a test of the methods' robustness (sensitivity of parameter estimates to changes in the dose levels of the assay design), cytolytic activity coefficients estimated by DRSA varied by ≤30% over a reduction of three to four orders of magnitude in the dose levels. This remarkable robustness should be compared with the corresponding figures of as much as 500% over <1 order of magnitude for previously published results of coefficients estimated by conventional methods. DRSA is distinguished from replot-of-plots methods such as those used for enzyme inhibition assays in biochemistry, and is recommended as a more efficient method that should replace replot-of-plot methods now antiquated by the advent of microcomputers. DRSA can be applied to any experimental system that requires an activity coefficient to be estimated on a dose-response surface in a space of ≥3 dimensions (≥2 dose variables and one response variable), regardless of the mathematical model and statistical estimators used to analyze the dose-response interaction. Finally, DRSA is compared with the methods known as response surface methodology (RSM), and is described as a new class of methods to be added to those that constitute RSM.

SECTION 1: INTRODUCTION AND COMPARISON OF IMMUNOASSAYS WITH OTHER BIOASSAYS

Classical biochemical and biological assays, such as enzyme kinetics and animal toxicity assays, analyze homogeneous molecular solutions by observing, respectively, the formation of products in chemical reactions and the responses[2] of individual living organisms. Cellular immunological assays, such as cytolysis assays, analyze heterogeneous cellular suspensions[3] by observing the responses of other heterogeneous cellular suspensions. All of these assays evaluate the response of a tested subject to a tested object as observed within a test system. For enzyme kinetics assays, the response is product formation in a test system that is a reaction solution composed of the enzyme as tested object and the substrate as tested subject, both of which are homogeneous molecular solutions. For animal toxicity assays, the response is death of an individual animal that is both the test system as well as the tested subject into or to which the tested object is injected or applied. For cytolysis assays, the response is label release from target cells into the fluid supernatant of a test system that is a culture mixture composed of the killer cells as tested object and the target cells as tested subject, both of which are heterogeneous cellular suspensions.

Given the dramatic differences in order of complexity revealed by these simple comparisons, is it appropriate to apply classical methods of data analysis to cytolysis, proliferation, and other cellular immunoassays? Certainly, traditional approaches have not led to the development of standard methods for the reporting of reliable and reproducible results in a manner that would permit the valid comparison of activities for different immunological systems from laboratories around the world. More important, immunoassays already influence clinical decisions and thus affect therapy and prognoses of patients: for example, consider the use of proliferation assays for decisions about organ transplantation. It is not possible, therefore, to ignore the lack of standards and the consequent need to develop new methods that will enable appropriate standards to be established. In this article, I describe several principles of data analysis that were developed for and applied to cytolysis assays and that have provided the basis for a new paradigm. Because the principles can be applied regardless of the mathematical model and statistical estimators used to analyze the dose-response interaction between tested object and subject, they can also be applied to other assays that do not fit into the framework of analysis of the classical paradigms.

Received for publication July 19, 1986.
Accepted for publication September 29, 1986.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was funded by a National Institutes of Health Training Grant in Biostatistics, 5 T32 CA09337-06, from the National Cancer Institute, and by a cooperative agreement with the SIAM Institute for Mathematics and Society and the Environmental Protection Agency.

[2] The word "response" is used as a statistical and not immunological term. Thus, in a dose-response bioassay, a response is the value of a measurable characteristic of a subject after the application of a measured dose of a stimulus to the subject (1).

[3] In the phrase "heterogeneous cellular suspension," "heterogeneous" applies to both "cellular" (the types and specificities of the cells in the population) and "suspension" (the spatial distribution of the cells in the culture).

SECTION 2: CONVENTIONAL METHODS AND CONSEQUENT PROBLEMS OF DATA ANALYSIS FOR CYTOLYSIS ASSAYS

Since their development by Brunner et al. (2) in 1968, cytolysis assays have been performed by observing a measured response C[4] of radiolabel counts released from target cells as a function of a varying dose K of killer cells at an arbitrarily chosen fixed dose T of target cells. Dose-response curves have been plotted as the percent specific release %SR vs the ratio K:T of killers to targets with the assumption that the results are independent of the dose T. %SR is defined as

$$\%SR = 100(C_{exp} - C_{spo})/(C_{max} - C_{spo}) \quad [1]$$

where C_{exp}, C_{spo}, and C_{max} are the experimental-, spontaneous-, and maximum-release counts. Cytolytic activities for different preparations have been compared by choosing a %SR level and comparing the corresponding K:T ratios. Alternatively, the K:T ratio has been chosen and %SR levels compared.

Cytolysis assays performed and analyzed in this manner have been the subject of numerous investigations in an effort to resolve the controversial problem of how to compare dose-response curves that are different in shape or that do not reach the level at which comparisons are made. A diverse variety of biomathematical models based on theoretical equation derivation and biostatistical models based on empirical curve fitting have been proposed as solutions to this problem, but none have gained widespread acceptance. The biomathematical models can be classified into two groups: saturation models based on reaction rates and enzyme kinetics theory (3–9), and collision models based on interaction probabilities and Poisson statistics theory (10–12). The biostatistical models have so far included two-parameter exponential and three-parameter sigmoidal curves (13–15). The biomathematical models have been criticized for their dependence on a complicated set of biological, chemical, and physical assumptions. Both the biomathematical and biostatistical models have been criticized for their lack of robustness, with parameter estimates varying several-fold and depending upon the region of the true curve that is estimated by the observed curve as viewed through the window of the experimental design (15, 16).

All of these proposals have employed a dose-response curve analysis and assumed an analogy to animal toxicity assays: the percentage of target cells lysed corresponds to the percentage of animals killed. But individual targets are not examined for lysis as are individual animals for death. This difference should invalidate the analogy (see below). Some have also assumed an analogy to Michaelis-Menten enzyme kinetics assays: killer cells and target cells correspond to enzyme and substrate. But the Michaelis-Menten constant for lysis of targets by killers has been found to be dependent upon the dose K, which plays the role of the "enzyme" concentration (3–9). This observation should invalidate the analogy. Indeed, Callewaert and Mahle (9) have recently concluded that "it is not possible to assign a simple physical or biological significance to values obtained for K_M^{app}" where K_M^{app} is the apparent Michaelis-Menten constant. Thus, all have applied traditional methods based on dose-response curve analyses analogous to classical assays but have not yet considered alternative approaches that may be more informative.

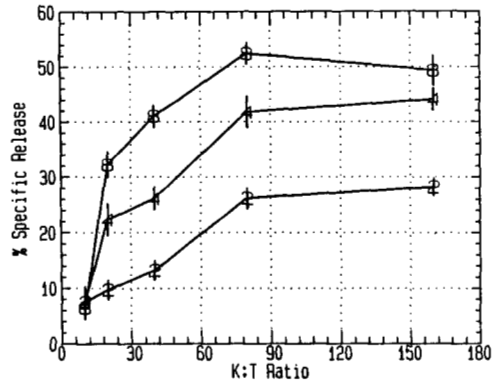

Figure 1. Plot of %SR levels vs K:T ratios for the cytolysis assay coded ECL041584 in Table II. Points on the curve are labeled with the values × 10^{-3} of the dose T, which is fixed at a different level for each curve. Vertical bars represent ± 1 SE calculated by a bootstrap procedure (17) with 24 experimental replicates per data point.

SECTION 3: UNDERLYING PROBLEMS, PRINCIPLES OF SOLUTION, AND APPROACHES TO IMPLEMENTATION OF A SOLUTION FOR CYTOLYSIS ASSAYS

There are two underlying problems and corresponding principles of solution. 1) Contrary to convention, the response, whether expressed as C or %SR, is not independent of the arbitrarily chosen fixed dose T when the dose K is varied or of fixed K when T is varied (3–9) (Fig. 1 and Table I). Therefore, the response C or %SR should be observed as a function of both the dose K and the dose T. 2) Contrary to convention, transformation of response data from the C scale to the %SR scale does not permit adequate data analysis. In fact, it amplifies error and results in values <0 and >100 (Table I). It prevents proper data analysis because the experimental values of the fraction specific release are not calculated independently of each other and each value is not calculated as the ratio of a binomial response variable to its parameter n for the number of trials.[5] Thus, the fraction specific release is not restricted to the interval [0, 1], and the usual error assumptions for binomial response variables distributed identically and independently with parameter p in [0, 1] cannot be made. Therefore, dose-response data should be analyzed on the C scale and not the %SR scale.

Given analysis of C as a function of both K and T as the principles for a solution, there are then two approaches to implementation of a solution. 1) A replot of plots: a two-stage estimation method based on a family of dose-response curves where each curve in the family is fit separately to the appropriate data points and then the curves' slopes are replotted as a function of the dose variable that was fixed at a different level for each curve. This method would be analogous to Dixon plots as used in enzyme inhibition assays (18). With the simplest possible model (two lines), this method would require at least

[4] Abbreviations used in this paper: DRSA, dose-response surface assays and/or analysis; RSM, response surface methodology. Abbreviations used as notation for mathematical variables, parameters, and statistics: K, killers; T, targets; C, counts; %SR, percent specific release; TK, target-to-killer ratio; TI, target index; KI, killer index; CA, cytolytic activity; RP, relative potency; SE, standard error; CL, confidence limit.

[5] The fraction specific release is not calculated as the ratio of the number of individual cells lysed to the total number of individual cells examined for lysis because these numbers are not observed experimentally.

TABLE I

Data from the cytolysis assay coded CSR031186-1 in Table II[a]

K	X	T						
		412	825	1650	3300	4950	6600	8250
		C						
0	0	74	110	143	222	300	443	662
2,500	0	130	173	212	278	332	487	512
5,000	0	155	230	256	370	410	598	603
10,000	0	165	300	442	586	689	752	1040
20,000	0	171	284	544	777	1105	1021	1222
30,000	0	158	298	576	1034	1182	1203	1553
40,000	0	175	306	609	1139	1356	1571	2171
50,000	0	221	285	587	1150	1339	1714	1995
0	1	201	402	1150	1413	2134	3166	3524
		%SR						
0	0	0.	0.	0.	0.	0.	0.	0.
2,500	0	44.1	21.4	6.9	4.7	1.7	1.6	−5.2
5,000	0	63.8	40.9	11.3	12.4	6.0	5.7	−2.1
10,000	0	71.7	64.9	29.7	30.5	21.2	11.3	13.2
20,000	0	76.4	59.6	39.8	46.6	43.9	21.2	19.5
30,000	0	66.1	64.2	43.0	68.2	48.1	27.9	31.1
40,000	0	79.1	67.1	46.3	77.0	57.6	41.4	52.7
50,000	0	115.0	59.9	44.1	77.9	56.6	46.7	46.6
0	1	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a] The response in counts C or percent specific release %SR is a function of the doses in numbers K and T of killer and target cells. The first and last rows of each matrix are, respectively, the spontaneous- and maximum-release data points, with X an indicator variable for the latter. In referring to the size of the matrices, only the experimental-release data points are considered. Thus, these matrices are 7 x 7 in size.

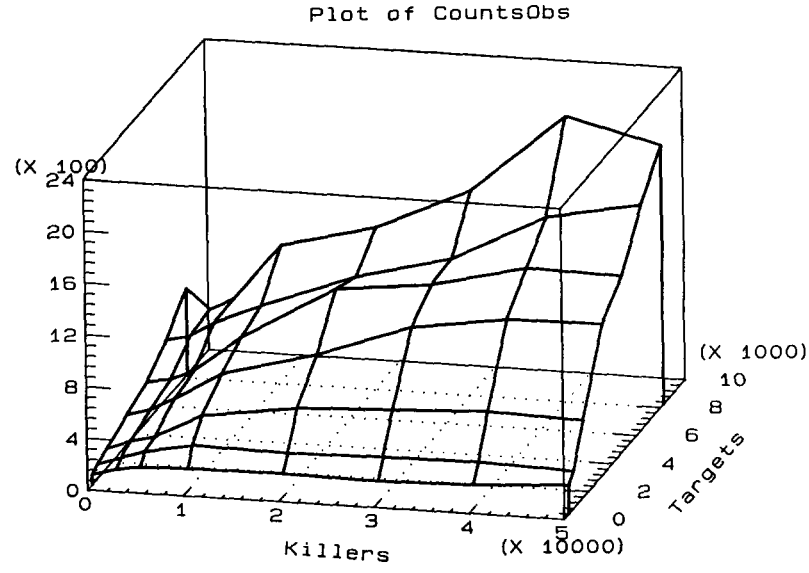

Figure 2. Plot of the observed dose-response surface for the data in Table I from the cytolysis assay coded CSR031186-1 in Table II. Maximum-release data points are not plotted.

four data points. 2) A dose-response surface analysis (DRSA): a one-stage estimation method based on a dose-response surface where the entire surface is fit simultaneously to all data points (Figs. 2 and 3). With the simplest possible model (a plane), this method would require at least three data points.

DRSA provides the better approach for several reasons. 1) It requires fewer data points and evaluates all of them as random variables from the same probability distribution. 2) It permits the reduction of multiple estimated parameters from the model to a single summary statistic by using simple geometric principles that can be applied regardless of the particular model and estimators used. 3) It provides a convenient solution to an additional problem not accounted for by conventional methods nor considered in previous proposals: the comparison of results from assays with different ratios of spontaneous- to maximum-release levels (which typically can range from 0.03 to 0.3).

SECTION 4: MODEL-INDEPENDENT PRINCIPLES OF DRSA APPLIED TO CYTOLYSIS ASSAYS

Assuming that the response C is a sufficiently smooth function of both doses K and T, summary statistics calculated at standardized comparison points (K*, T*, C*) on the dose-response surface should provide an approximate description of the behavior of the system. Points (K*, T*, C*) should be located at prescribed distances from the origin along prescribed paths.[6] Because different paths lead to different points (K*, T*, C*), they should be standardized in order to enable valid comparisons. These paths can be standardized to be the path of steepest ascent from the origin, or the path such that its projection onto the (K, T) plane is either the K or T axis or the line with T/K = 1. The distances can be standardized to be

[6] The term "path" refers to a curve traced by a point as it moves through space changing its direction freely. In general, this path is a nonlinear curve but may also be a straight line. The phrase "direction at a point of the path" refers to the unit vector tangent to that point of the curve.

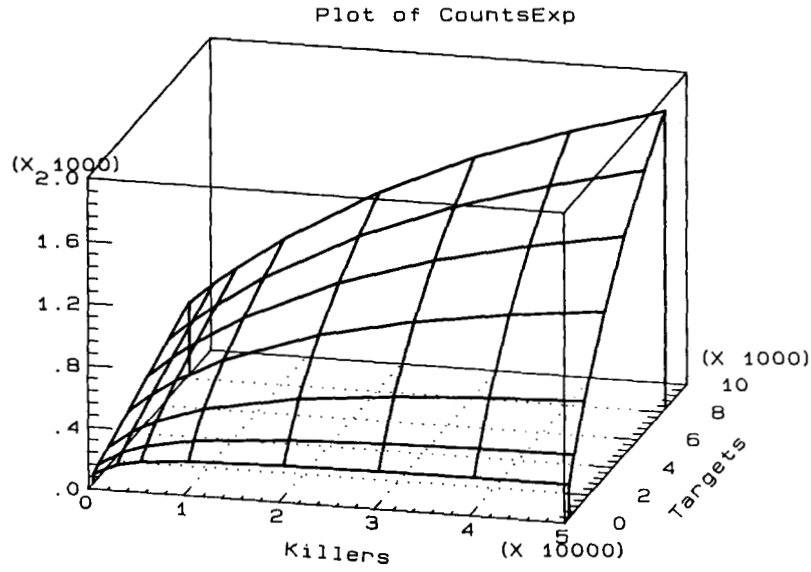

Figure 3. Plot of the expected dose-response surface for the data in Table I from the cytolysis assay coded CSR031186-1 in Table II. The surface was generated by using the model-parameter estimates in Table III. Maximum-release data points are not plotted.

one unit, or the mean distance from the origin of the design points (K and T dose levels on the K and T axes), or the distance such that $C^*/C_\infty = 0.5$, where C_∞ is the asymptotic maximum level of C attainable in the assay. Summary statistics calculated at (K^*, T^*, C^*) should be defined to possess interpretations with real-world units of response per dose. Thus, if the response C is assumed to be a function of the number T_{lys} of targets lysed, then the response per dose can be expressed as the target index TI in units of T_{lys}/T, the killer index KI in units of T_{lys}/K, and the cytolytic activity CA in units of $T_{lys}/T/K$.

These statistics are calculated by the following algorithm: Fit the data to the model and estimate the parameters of the model. Using the estimated parameters, find (K^*, T^*, C^*). Calculate the target-to-killer ratio TK at (K^*, T^*, C^*) defined as

$$TK = T^*/K^* \quad [2]$$

Calculate the predicted values $\hat{C}^*_{exp}$, $\hat{C}^*_{spo}$, and $\hat{C}^*_{max}$ of C_{exp}, C_{spo}, and C_{max} corresponding to (K^*, T^*, C^*). Assuming that the fraction specific release approximates the fraction targets lysed

$$(\hat{C}_{exp} - \hat{C}_{spo})/(\hat{C}_{max} - \hat{C}_{spo}) \simeq T_{lys}/T \quad [3]$$

calculate the target index TI defined as

$$TI = (\hat{C}^*_{exp} - \hat{C}^*_{spo})/(\hat{C}^*_{max} - \hat{C}^*_{spo}) = T^*_{lys}/T^* \quad [4]$$

Note that TI is analogous to the conventional fraction specific release, with the crucial difference being, however, that TI can be calculated at any point on the response surface by using predicted values inferred from the model-parameter estimates. Multiply TI by TK to obtain the killer index KI defined as

$$KI = (TI)(TK) = (T^*_{lys}/T^*)(T^*/K^*) = T^*_{lys}/K^* \quad [5]$$

Divide TI by K^* to obtain the cytolytic activity CA defined as

$$CA = (TI)/K^* = T^*_{lys}/T^*/K^* \quad [6]$$

All of these statistics increase as cytolysis increases. It is also possible to construct many alternative statistics by using the geometry of the dose-response surface.[7]

SECTION 5: APPLICATION OF DRSA TO A PARTICULAR MODEL FOR CYTOLYSIS ASSAYS

DRSA has been successfully applied to data from a total of 19 assays of immune and natural cytolysis (Table II). Data were provided by E. C. Lattime of the Sloan-Kettering Memorial Cancer Center, R. A. Miller of the Boston University School of Medicine, and C. S. Reiss of Harvard Medical School. Data sets were examined to determine the simplest functional relationships between dose and response variables applicable to all data sets. A model was then constructed based only on these functional relationships: the responses C_{spo} and C_{max} increase as linear functions of the dose T,

$$C_{spo} = sT + f \quad [7]$$

$$C_{max} = mT + g \quad [8]$$

and the response C_{cor} increases as a hyperbolic function of both the doses T and K (a hyperbolic function of T when K is fixed and of K when T is fixed),

$$C_{cor} = C_{exp} - C_{spo} = aKbT/(aK + bT) \quad [9]$$

where C_{cor} is the spontaneous-release-corrected experimental-release counts. The relevant subsets of each data set were fit separately to the appropriate equations in this system to estimate the parameters a, b, s, m, f, and g. The entire data set was also fit simultaneously to the single combined equation

$$C = [aKbT/(aK + bT) + sT](1 - X) + mTX + e \quad [10]$$

to estimate the parameters a, b, s, m, and e where X is an indicator variable such that X = 0 for C_{spo} and C_{exp} and X = 1 for C_{max}. Estimates for the parameters a, b, s,

[7] For example, the directional and magnitudinal components of the gradient vector at the standardized comparison point can be related to components of gradient vectors on the spontaneous- and maximum-release planes. These planes are constructed by projecting the spontaneous- and maximum-release lines, which are located in the plane defined by the T and C axes, throughout the third dimension defined by the K axis. The experimental-release surface is then sandwiched between the spontaneous- and maximum-release planes.

TABLE II

Identification of the eleven murine cytolysis assays for which results are presented in Tables I, III, IV, and V, and Figures 1, 2, and 3[a]

Assay	Killer	Target	Reference
ECL120882-1	C57BL/6 NK	YAC-1	(19)
ECL120882-2	C57BL/6 NK + poly-IC	YAC-1	(19)
ECL121082-1	C57BL/6 NK	YAC-1	(19)
ECL121082-2	C57BL/6 NK	YAC-1A	(19)
ECL041584	C57BL/6 NK	YAC-1	(19)
RAM011586-1	CBA anti-(CBA × DBA/2)F_1	P815	(20)
RAM011586-2	CBA anti-(CBA × C57BL/6)F_1 + PHA	P815	(20)
CSR020686	BALB/c dm2 anti-BALB/c	p815	(21)
CSR031186-1	C57BL/6 anti-B10.D2 dm1	P815	(22)
CSR031186-2	BALB/c dm2 anti-BALB/c	P815	(21)
CSR031186-3	BALB/c dm2 anti-BALB/c	T1.1.1	(21)

[a] Eight additional assays for similar strain combinations were also analyzed with similar results.

and m used to calculate the summary statistics TK, TI, KI, and CA were similar for both separate and simultaneous fits.

The simultaneous fit was chosen as the preferable method because it constrains all of the residuals to the same probability distribution and does so with one less parameter. Final estimates were calculated by using iteratively reweighted nonlinear least squares with a modified Gauss-Newton algorithm (23) and bisquare weights (24) on iterative weights equal to the reciprocal of the fitted values (23) assuming an error structure with variance proportional to the expected mean (23, 25). R^2 coefficients were greater than 0.95 with no departure from normality of the residuals revealed by skewness and kurtosis statistics or by plots of observed vs predicted values, residuals vs predicted values, and residuals vs each of the dose variables.

For this particular model, results for summary statistics TK, TI, and KI depend only on the direction[6] at the origin of the chosen path to the comparison point (K*, T*, C*) and not on the point's distance from the origin. Furthermore, the only component of the direction that is relevant is the angle in the (K, T) plane. Therefore, transforming from Cartesian coordinates in the (K, T) plane to polar coordinates in the (r, θ) plane (with $\theta = 0$ on the K axis and $\theta = \pi/2$ on the T axis) enables equations to be expressed as a function of one variable instead of two. Thus, expressed in polar coordinates, results for the statistics TK, TI, and KI depend only on θ and not on r with

$$TK(\theta) = \tan\theta \quad [11]$$

$$TI(\theta) = [(a^{-1}\tan\theta + b^{-1})(m - s)]^{-1} \quad [12]$$

$$KI(\theta) = \{[a^{-1} + (b\tan\theta)^{-1}](m - s)\}^{-1} \quad [13]$$

Results for the summary statistic CA depend on both r and θ with

$$CA(r, \theta) = [r(a^{-1}\sin\theta + b^{-1}\cos\theta)(m - s)]^{-1} \quad [14]$$

However, the statistic CA can be standardized by fixing the variable r at the value r = 1 with the compelling rational that $CA(r = 1, \theta)$ equals $TI(\theta)$ on the K axis and $KI(\theta)$ on the T axis. Refer to *Appendix* for a proof of this statement as well as all derivations and proofs relevant to this section.

By using the model-parameter estimates $\hat{a}$, $\hat{b}$, $\hat{s}$, and $\hat{m}$, the summary statistics TK and CA were calculated at four different points (K*, T*, C*) located on: 1) the K axis with

$$TK_{kax} = 0 \quad [15]$$

$$CA_{kax} = CA(r = 1; \theta = 0) = TI(\theta = 0) = b/(m - s) \quad [16]$$

interpreted as the maximum value of TI as TK → 0 (i.e., of T_{lys}/T as T/K → 0); 2) the path of steepest ascent on the spontaneous-release-corrected response surface C_{cor} with

$$TK_{csa} = (a/b)^{1/3} \quad [17]$$

$$CA_{csa} = CA(r = 1; \theta = \arctan(TK_{csa})) \quad [18]$$

3) the path of steepest ascent on the experimental response surface C_{exp} with

$$\theta^* = \{\theta \mid 0 = (a^2b\cos^3\theta - ab^2\sin^3\theta)/(a\cos\theta + b\sin\theta)^2 + s\cos\theta\} \quad [19]$$

$$TK_{esa} = \tan\theta^* \quad [20]$$

$$CA_{esa} = CA(r = 1; \theta = \theta^*) \quad [21]$$

and 4) the T axis with

$$TK_{tax} = \infty \quad [22]$$

$$CA_{tax} = CA(r = 1; \theta = \pi/2) = KI(\theta = \pi/2) = a/(m - s) \quad [23]$$

interpreted as the maximum value of KI as TK → ∞ (i.e., of T_{lys}/K as T/K → ∞).

Estimates of standard errors of model parameters (Table III) and summary statistics (Table IV) were calculated by using a jackknife procedure (17) in which the data set was divided into groups by successively deleting from the data matrix one T column at a time (thus maintaining a balanced design structure with equal numbers of C_{spo}, C_{exp}, and C_{max} data points in each of the groups). Relative potencies were calculated for test and standard killer-target pairs based on their cytolytic activities. The relative potency is defined as the ratio of the test preparation's statistic to the standard preparation's statistic (1). Thus,

$$RP_i = CA_i(\text{test pair})/CA_i(\text{standard pair}) \quad [24]$$

for each of the above four comparison points (kax, csa, esa, and tax) indexed here by i. Confidence limits for relative potencies were calculated by using Fieller's theorem with the normal approximation (1).

Results for relative potencies were demonstrated to be consistent with general previous knowledge of relative cytolytic activities for a variety of test and standard killer-target pairs (Table IV). CA_{esa} appears to be the most sensitive of the four CA_i statistics, resulting in values for

TABLE III

Estimates with standard errors for the model parameters in equation [10] for the cytolysis assays identified in Table II[a]

Assay	$\hat{a}$	$\hat{b}$	$\hat{s}$	$\hat{m}$
ECL120882-1	0.0011 ± 0.0001	0.0566 ± 0.0079	0.0068 ± 0.0008	0.163 ± 0.001
ECL120882-2	0.0032 ± 0.0003	0.0865 ± 0.0165	0.0070 ± 0.0009	0.163 ± 0.001
ECL121082-1	0.0013 ± 0.0001	0.0420 ± 0.0034	0.0076 ± 0.0004	0.072 ± 0.010
ECL121082-2	0.0018 ± 0.0001	0.0652 ± 0.0054	0.0151 ± 0.0010	0.318 ± 0.005
ECL041584	0.0018 ± 0.0004	0.0639 ± 0.0027	0.0598 ± 0.0017	0.171 ± 0.003
RAM011586-1	0.0030 ± 0.0005	0.109 ± 0.037	0.0196 ± 0.0012	0.111 ± 0.001
RAM011586-2	0.0080 ± 0.0013	0.0960 ± 0.0044	0.0191 ± 0.0010	0.111 ± 0.001
CSR02086	0.140 ± 0.019	0.248 ± 0.024	0.0321 ± 0.0029	0.269 ± 0.030
CSR031186-1	0.0527 ± 0.0070	0.389 ± 0.041	0.0520 ± 0.0054	0.437 ± 0.017
CSR031186-2	0.0682 ± 0.0064	0.351 ± 0.059	0.0460 ± 0.0046	0.418 ± 0.031
CSR031186-3	0.0336 ± 0.0079	0.0925 ± 0.0229	0.112 ± 0.012	0.875 ± 0.078

[a] Calculation methods are explained in *Section 5*.

TABLE IV

Summary statistics with standard errors or confidence limits calculated at four different response-surface comparison points indexed by i for the cytolysis assays identified in Table II[a]

Assay	i	$TK_i \pm SE(TK_i)$[b]	$CA_i \pm SE(CA_i)$[c]	RP_i[d]	[95% CL (RP_i)]
ECL120882-1	kax	0	0.362 ± 0.050	1	
	csa	0.269 ± 0.017	0.0252 ± 0.0011	1	
	esa	6.20 ± 1.30	0.00708 ± 0.00061	1	
	tax	∞	0.00702 ± 0.00059	1	
ECL120882-2	kax	0	0.553 ± 0.105	1.53	[0.91–2.39]
	csa	0.333 ± 0.030	0.0581 ± 0.0024	2.31	[2.05–2.60]
	esa	2.26 ± 0.46	0.0219 ± 0.0026	3.09	[2.28–4.09]
	tax	∞	0.0204 ± 0.0020	2.91	[2.24–3.74]
ECL121082-1	kax	0	0.650 ± 0.057	1	
	csa	0.315 ± 0.017	0.0614 ± 0.0094	1	
	esa	5.86 ± 0.88	0.0205 ± 0.0042	1	
	tax	∞	0.0203 ± 0.0041	1	
ECL121082-2	kax	0	0.216 ± 0.018	.332	[0.262–0.423]
	csa	0.303 ± 0.015	0.0190 ± 0.0005	.309	[0.237–0.443]
	esa	8.34 ± 1.19	0.00605 ± 0.00043	.295	[0.204–0.500]
	tax	∞	0.00602 ± 0.00042	.297	[0.206–0.498]
ECL041584	kax	0	0.576 ± 0.025		
	csa	0.301 ± 0.022	0.0501 ± 0.0078		
	esa	33.4 ± 8.5	0.0158 ± 0.0036		
	tax	∞	0.0158 ± 0.0036		
RAM011586-1	kax	0	1.20 ± 0.42	1	
	csa	0.301 ± 0.034	0.104 ± 0.023	1	
	esa	6.69 ± 1.51	0.0328 ± 0.0056	1	
	tax	∞	0.0326 ± 0.0054	1	
RAM011586-2	kax	0	1.04 ± 0.06	0.87	[0.51–2.77]
	csa	0.436 ± 0.018	0.181 ± 0.022	1.74	[1.11–3.18]
	esa	2.57 ± 0.43	0.0896 ± 0.0161	2.73	[1.63–4.53]
	tax	∞	0.0862 ± 0.0145	2.64	[1.63–4.29]
CSR020686	kax	0	1.05 ± 0.20		
	csa	0.827 ± 0.045	0.552 ± 0.088		
	esa	1.05 ± 0.04	0.531 ± 0.084		
	tax	∞	0.592 ± 0.094		
CSR031186-1	kax	0	1.01 ± 0.11	1.07	[0.72–1.75]
	csa	0.514 ± 0.036	0.237 ± 0.023	0.87	[0.67–1.11]
	esa	1.29 ± 0.15	0.157 ± 0.028	0.74	[0.47–1.06]
	tax	∞	0.137 ± 0.022	0.75	[0.50–1.05]
CSR031186-2	kax	0	0.943 ± 0.175	1	
	csa	0.579 ± 0.043	0.274 ± 0.023	1	
	esa	1.10 ± 0.07	0.211 ± 0.018	1	
	tax	∞	0.183 ± 0.017	1	
CSR031186-3	kax	0	0.121 ± 0.027	0.128	[0.067–0.229]
	csa	0.713 ± 0.108	0.0502 ± 0.0058	0.183	[0.136–0.241]
	esa	3.99 ± 1.18	0.0416 ± 0.0104	0.197	[0.099–0.307]
	tax	∞	0.0440 ± 0.0123	0.240	[0.107–0.390]

[a] Comparison points are located on paths with the subscript codes: kax, K axis; csa, corrected steepest ascent; esa, experimental steepest ascent; and tax, T axis. Calculation methods are explained in *Section 5*.

[b] TK_i are standardized T:K ratios defined by equations [15], [17], [20], and [22].

[c] CA_i are standardized cytolytic activities defined by equations [16], [18], [21], and [23].

[d] RP_i are relative potencies defined by equation [24]. Relative potencies are calculated only for different assays in the same experiment.

RP_{esa} either greater or lower than the values for the other three RP_i (in four out of five examples in Table IV). More important, however, the methods were demonstrated to be free of the problems that have characterized cytolysis assays in the past. In particular, cytolytic activities varied by ≤30% (with overlapping confidence intervals) over a reduction of three to four orders of magnitude in the dose levels for data point deletion experiments on the dose-response surface (Table V). This remarkable robustness should be compared with the corresponding figures from previously published results of as much as 500% (with no confidence intervals reported) over less than one order of magnitude for data point deletion experiments on dose-response curves (15).

The statistics CA_{kax} and CA_{tax} can be interpreted, respectively, as the maximum number of targets lysed per

TABLE V

The effect of matrix size reduction (data point deletion) on the summary statistic CA_{esa} for the cytolysis assay coded CSR020686 in Table II[a]

Matrix Size	Dose Range		Lower 95% CL (CA_{esa})	CA_{esa}	Upper 95% CL (CA_{esa})
	781 ≤ K ≤	391 ≤ T ≤			
9 x 9	200,000	100,000	0.366	0.531	0.696
8 x 8	100,000	50,000	0.321	0.587	0.854
7 x 7	50,000	25,000	0.327	0.514	0.701
6 x 6	25,000	12,500	0.304	0.475	0.647
5 x 5	12,500	6,250	0.423	0.564	0.704
4 x 4	6,250	3,125	0.427	0.618	0.809
3 x 3	3,125	1,562	0.501	0.594	0.687

[a] Similar results were obtained for the other summary statistics and assays. Matrix size is explained in Table I. Confidence limits were calculated by using the normal approximation.

target and the maximum number of targets lysed per killer. Note that the term "killer" refers here to all cells in the killer cell population, some of which in fact are not killer cells. Thus, depending upon the assumptions made, various inferences can be drawn about the frequency of killer cells in the killer cell population. The statistics CA_{esa} and CA_{csa} can both be interpreted as the number of targets lysed per target per killer at doses where the increase in response (C_{exp} and C_{cor}, respectively) is greatest. CA_{esa} properly accounts for varying levels of spontaneous release and spontaneous-to-maximum release ratios, whereas CA_{csa} does not. Because CA_{esa} is derived from the slope of the steepest path from the origin on the true (not spontaneous-release-corrected) experimental response surface, it should be the most sensitive statistic, and appears to be so. If a single statistic is desired to calculate relative potencies, CA_{esa} seems to be the most appropriate. CA_{kax} and CA_{tax} provide ancillary information with the interpretations discussed above.

SECTION 6: DISCUSSION AND COMPARISON OF DRSA WITH RSM

I have chosen the specific methods (*Section 5*) used here as simple initial implementations of the general principles (*Section 4*) of a new approach (*Section 3*) for analysis of data from cytolysis assays. As such, they are not necessarily the best methods, but rather only the first methods, to implement the general principles of this new approach. Certainly, future work should investigate more extensively the particular model and estimators used here and compare them with alternative models and estimators. Most important, however, the data analysis methods for cytolysis assays derived and presented here provide the first working example of a new paradigm for analysis of data from a newly defined class of biological assays: those that require an activity coefficient to be defined and estimated on a dose-response surface in a space of three or more dimensions. This space is composed of one dimension for the response variable and two or more dimensions for the dose variables, which can include time and other factors.

In this investigation, I have assumed that a single activity coefficient can characterize a single dose-response surface. For cytolysis assays, the most likely candidate is CA_{esa} because of its apparently greater sensitivity than that of the other CA_i coefficients. This assumption may not be valid for cytolysis assays, however, and is probably not valid for at least some biological systems. In the case of cytolysis assays, it necessitates a discussion of the validity and meaning of the comparison of different killers for the same target vs different targets for the same killer. Even if the assumption is valid, it may nevertheless be more informative to estimate the potency of a test surface relative to a standard surface with the data fit to both surfaces simultaneously in a manner analogous to that of traditional bioassays, such as slope-ratio and parallel-line assays (1). Thus, the relative potency could be defined as the distance in some metric between test and standard dose-response surfaces or some combination of the ratios of components of their gradient vectors.

The principles of what I have called dose-response surface assays and analysis (DRSA) can be contrasted with those of response surface methodology (RSM) as it has been used until now (26). DRSA seeks a single value representing the activity characteristic of a dose-response surface or the relative potency characteristic of a pair of surfaces. RSM seeks the values of the dose variables corresponding to the maximum or some other defined level of the response variable. The goal of DRSA is characterization of the tested object at the lowest possible levels of the dose variables in order to minimize the cost of the characterization analysis. The goal of RSM is production of an optimal response in tested subjects at whatever levels of the dose variables are required in order to maximize the efficiency of the production yield. Finally, the number of elements that constitute each tested subject (the number of cells in a suspension or molecules in a solution) is considered a dose variable in DRSA but not in RSM.

These comparisons are intended not to define but only to contrast DRSA as used in this article and RSM as used in previous publications by other authors. Despite these differences, DRSA and RSM share many similarities. Indeed, DRSA can be described as a new class of methods to be added to those that constitute RSM. Usage of the terms DRSA and RSM will undoubtedly continue to evolve. Some aspects of the above description of DRSA may be too restrictive. For example, the first criterion listed above would exclude enzyme inhibition assays where the individual model parameters have real meanings and there is no reason to reduce them to a single summary statistic representing an activity. However, the other criteria would certainly include enzyme inhibition assays in the specific domain of DRSA rather than the more general domain of RSM. Regardless of debates about nomenclature, a dose-response-surface rather than replot-of-plots estimation procedure should provide a more efficient method for analyzing data from these biochemical assays as well as any other systems where replots of plots have been used in the past.

Biological research on DRSA should explore applications to a wide variety of assay systems in immunology, biochemistry, and other sciences. Preliminary results from proliferation assays suggest that DRSA may soon be successfully applied to these immunoassays in addition to the cytolysis assays reported in this article. Statistical research on DRSA should investigate issues of experimental design and all aspects of data analysis including the theoretical and experimental performance of alternative models, estimation procedures, summary statistics, and validity (goodness-of-fit) tests. However, even at their present stage of development as described here, DRSA should provide a significant methodological ad-

vance promoting progress toward the standardization of results from cytolysis and other immunoassays.

COMPUTER SOFTWARE

Computer programs for all computational methods described in this article have been developed for the Commodore Amiga, Apple Macintosh, IBM PC, and compatible microcomputers. Contact the author for information about the availability of this software.

Acknowledgments. I thank E. C. Lattime (Sloan-Kettering Memorial Cancer Center), R. A. Miller (Boston University School of Medicine), and C. S. Reiss (Harvard Medical School) for their participation in this investigation; D. J. Finney (University of Edinburgh) for helpful discussions; H. A. Feldman, J. H. Ware, and other members of the Department of Biostatistics (Harvard School of Public Health) for reviewing the manuscript; and especially C. S. Reiss for her enthusiastic support.

APPENDIX

In each of the following derivations, the "*" and "^" are dropped for simplicity, and r cos θ and r sin θ are substituted for K and T in the transformation to polar coordinates.

Target-to-killer ratio TK:

$$\begin{aligned} TK &= T/K && [2]\\ &= r\sin\theta/r\cos\theta \\ &= \tan\theta && [11]\end{aligned}$$

Target index TI:

$$\begin{aligned} TI &= (C_{exp} - C_{spo})/(C_{max} - C_{spo}) && [4]\\ &= [aKbT/(aK + bT) + sT + e - (sT + e)]/ \\ &\quad [mT + e - (sT + e)] \\ &= [(a^{-1}T/K + b^{-1})(m - s)]^{-1} && [25]\\ &= [(a^{-1}\tan\theta + b^{-1})(m - s)]^{-1} && [12]\end{aligned}$$

Taking the limit as $K \to \infty$ and $T \to 0$ in equation [25] or as $\theta \to 0$ in equation [12] both give

$$TI = b/(m - s) \quad [16]$$

interpreted as the maximum value of TI as $TK \to 0$.

Killer index KI:

$$\begin{aligned} KI &= (TI)(TK) && [5]\\ &= [(a^{-1}T/K + b^{-1})(m - s)]^{-1}(T/K) \\ &= \{[a^{-1} + (bT/K)^{-1}](m - s)\}^{-1} && [26]\\ &= \{[a^{-1} + (b\tan\theta)^{-1}](m - s)\}^{-1} && [13]\end{aligned}$$

Taking the limit as $T \to \infty$ and $K \to 0$ in equation [26] or as $\theta \to \pi/2$ in equation [13] both give

$$KI = a/(m - s) \quad [23]$$

interpreted as the maximum value of KI as $TK \to \infty$.

Cytolytic activity CA on the K and T axes:

$$\begin{aligned} CA &= (TI)/K && [6]\\ &= [(a^{-1}T/K + b^{-1})(m - s)]^{-1}/K \\ &= [(a^{-1}T + b^{-1}K)(m - s)]^{-1} \\ &= [r(a^{-1}\sin\theta + b^{-1}\cos\theta)(m - s)]^{-1} && [14]\end{aligned}$$

Evaluating at r = 1 and $\theta = 0$ on the K axis gives

$$CA_{kax} = CA(r = 1;\ \theta = 0) = b/(m - s) = TI(\theta = 0) \quad [16]$$

and evaluating at r = 1 and $\theta = \pi/2$ on the T axis gives

$$\begin{aligned} CA_{tax} &= CA(r = 1;\ \theta = \pi/2) \\ &= a/(m - s) = KI(\theta = \pi/2) && [23]\end{aligned}$$

Thus, CA(r = 1) provides a convenient statistic that incorporates the limit of TI on the K axis and the limit of KI on the T axis.

Cytolytic activity CA on the paths of steepest ascent: Using the general equation

$$C = aKbT/(aK + bT) + cK + dT + e \quad [27]$$

for the convenience of its symmetry, transforming Cartesian coordinates in the (K, T) plane to polar coordinates in the (r, θ) plane, and taking partial derivatives with respect to r and θ gives the gradient vector ($\delta C/\delta r$, $\delta C/\delta\theta$) for the response surface C where

$$\begin{aligned} \delta C/\delta r &= ab\cos\theta\sin\theta/(a\cos\theta + b\sin\theta) \\ &\quad + c\cos\theta + d\sin\theta && [28]\end{aligned}$$

$$\begin{aligned} \delta C/\delta\theta &= r[(a^2b\cos^3\theta - ab^2\sin^3\theta)/(a\cos\theta + b\sin\theta)^2 \\ &\quad - c\sin\theta + d\cos\theta] && [29]\end{aligned}$$

Thus, the gradient is constant in the radial direction for a given angular direction, and can be maximized as a function of the angular direction in order to find the path of steepest ascent from the origin. Solving

$$\begin{aligned} 0 &= (a^2b\cos^3\theta - ab^2\sin^3\theta)/(a\cos\theta + b\sin\theta)^2 \\ &\quad - c\sin\theta + d\cos\theta && [30]\end{aligned}$$

for θ when c = d = 0 gives

$$\theta^* = \arctan[(a/b)^{1/3}] \quad [31]$$

for the path of steepest ascent on the spontaneous-release-corrected response surface C_{cor}, and equations [17] and [18] for TK_{csa} and CA_{csa} follow as a consequence. Solving equation [30] for θ when c = 0 and d = s gives

$$\begin{aligned} \theta^* &= \{\theta \mid 0 = (a^2b\cos^3\theta - ab^2\sin^3\theta)/(a\cos\theta \\ &\quad + b\sin\theta)^2 + s\cos\theta\} && [19]\end{aligned}$$

(which must be solved numerically rather than analytically) for the path of steepest ascent on the experimental response surface C_{exp}, and equations [20] and [21] for TK_{esa} and CA_{esa} follow as a consequence.

REFERENCES

1. **Finney, D. J.** 1978. *Statistical Method in Biological Assay.* 3rd ed. Charles Griffin & Co., London.
2. **Brunner, K. T., J. Mauel, J.-C. Cerottini, and B. Chapuis.** 1968. Quantitative assay for the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology 14:181.*
3. **Thorn, R. M., and C. S. Henney.** 1976. Kinetics analysis of target cell destruction by effector T cells. I. Delineation of parameters

related to the frequency and lytic efficiency of killer cells. *J. Immunol. 117:2213.*

4. **Zeijlemaker, W. P., R. H. J. van Oers, R. E. Y. de Goede, and P. T. A. Schellekens.** 1977. Cytotoxic activity of human lymphocytes: quantitative analysis of T cell and K cell cytotoxicity, revealing enzyme-like kinetics. *J. Immunol. 119:1507.*
5. **Thoma, J. A., M. H. Touton, and W. R. Clark.** 1978. Interpretation of ^{51}Cr-release data: a kinetic analysis. *J. Immunol. 120:991.*
6. **Callewaert, D. M., D. F. Johnson, and J. Kearney.** 1978. Spontaneous cytotoxicity of cultured human cell lines mediated by normal peripheral blood lymphocytes. III. Kinetic parameters. *J. Immunol. 121:710.*
7. **Herrick, M. V., and S. B. Pollack.** 1978. Kinetic analysis of antibody-dependent cellular cytotoxicity: evidence for noncompetitive inhibition by autologous lymphoid cells. *J. Immunol. 121:1348.*
8. **Merrill, S. J.** 1982. Foundations of the use of an enzyme-kinetic analogy in cell-mediated cytotoxicity. *Math. Biosciences 62:219.*
9. **Callewaert, D. M., and N. H. Mahle.** 1985. Kinetic models for natural cytotoxicity and their use for studying activated NK cells. In *Mechanisms of Cytotoxicity by NK Cells.* Edited by R. Herberman and D. M. Callewaert. Academic Press, New York. P. 381.
10. **Henney, C. S.** 1971. Quantitation of the cell-mediated immune response. I. The number of cytolytically active mouse lymphoid cells induced by immunization with allogeneic mastocytoma cells. *J. Immunol. 107:1558.*
11. **Miller, R. G., and M. Dunkley.** 1974. Quantitative analysis of the ^{51}Cr release cytotoxicity assay for cytotoxic lymphocytes. *Cell. Immunol. 14:284.*
12. **Chu, G.** 1978. The kinetics of target lysis by cytotoxic T lymphocytes: a description by Poisson statistics. *J. Immunol. 120:1261.*
13. **Pross, H. F., M. G. Baines, P. Rubin, P. Shragge, and M. S. Patterson.** 1981. Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. *J. Clin. Immunol. 1:51.*
14. **Bloom, E. T., and E. L. Korn.** 1983. Quantification of natural cytotoxicity by human lymphocyte subpopulations isolated by density: heterogeneity of the effector cells. *J. Immunol. Methods 58:323.*
15. **Yron, I., E. Sahar, L. Shohat, and M. Efrati.** 1985. The analysis of measurements of murine natural killer cell activity. *J. Immunol. Methods 79:109.*
16. **Holmberg, A.** 1982. On the practical identifiability of microbial growth models incorporating Michaelis-Menten type nonlinearities. *Math. Biosciences 62:23.*
17. **Efron, B., and G. Gong.** 1983. A leisurely look at the bootstrap, the jackknife, and cross-validation. *Am. Statist. 37:36.*
18. **Segel, I. H.** 1976. *Biochemical Calculations.* 2nd ed. John Wiley & Sons, New York.
19. **Lattime, E. C., G. Pecoraro, and O. Stutman.** 1982. Natural cytotoxic cells against solid tumors in mice. IV. Natural cytotoxic (NC) cells are not activated natural killer (NK) cells. *Int. J. Cancer 30:471.*
20. **Bevan, M. J., and M. Cohn.** 1975. Cytotoxic effects of antigen- and mitogen-induced T cells on various targets. *J. Immunol. 114:559.*
21. **Reiss, C. S., G. A. Evans, D. H. Margulies, J. G. Seidman, and S. J. Burakoff.** 1983. Allospecific and virus-specific cytolytic T lymphocytes are restricted to the N or C1 domain of H-2 antigens expressed on L cells after DNA-mediated gene transfer. *Proc. Natl. Acad. Sci. USA 80:2709.*
22. **Reiss, C. S., J. L. Greenstein, M. A. V. Crimmins, L. L. M. Liu, A. Rao, R. T. Maziarz, C. Murre, and S. J. Burakoff.** 1986. Recognition of the α-1 and α-2 domains of H-2 molecules by allospecific cloned T cells. *J. Immunol. 136:2191.*
23. **Jennrich, R. I., and M. L. Ralston.** 1979. Fitting nonlinear models to data. *Annu. Rev. Biophys. Bioeng. 8:195.*
24. **Mosteller, F., and J. W. Tukey.** 1977. *Data Analysis and Regression.* Addison-Wesley Publ. Co., Reading, MA.
25. **McCullagh, P., and J. A. Nelder.** 1983. *Generalized Linear Models.* Chaman and Hall Ltd., London.
26. **Morton, R. H.** 1983. Response surface methodology. *Math. Scientist 8:31.*