EVALUATION AND PROPAGATION OF CONFIDENCE INTERVALS IN NONLINEAR, ASYMMETRICAL VARIANCE SPACES

Analysis of Ligand-Binding Data

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ABSTRACT Two problems that are often overlooked in studies employing nonlinear least-squares techniques for parameter estimation are confidence-interval estimation and propagation. When the parameters are correlated, the variance space and consequently the confidence intervals are nonlinear and asymmetrical. The presented mathematical method for the evaluation of confidence intervals and error propagation addresses these problems. The examples employed to demonstrate these methods include linear least-squares and the nonlinear least-squares analysis of ligand-binding problems, such as hormone receptor interactions and oxygen binding to human hemoglobin. The mathematical procedures have proven very useful for analyzing the molecular mechanism of cooperativity in human hemoglobin (Johnson, M. L., and G. K. Ackers, 1982. *Biochemistry* 21:201–211).

INTRODUCTION

Our investigations into the molecular mechanism of cooperativity in human hemoglobin have relied heavily on nonlinear least-squares parameter estimation (Ackers et al., 1975, 1976; Ip et al., 1976; Mills et al., 1976; Johnson et al., 1976; Johnson and Ackers, 1977; Atha et al., 1979; Ackers and Johnson, 1981; Johnson and Ackers, 1982). These investigations of the oxygenation-linked dimer-tetramer assembly involve formulating a variety of interesting thermodynamic (Ackers and Halvorson, 1974) and mechanistic models (Ackers and Johnson, 1981; Johnson and Ackers, 1982), and then testing these by least-squares techniques. This multiplicity of possible formulations forced the solution of the two major numerical analysis questions to be addressed in this report.

The first problem is how accurately the desired parameters can be determined by least-squares fitting of a specific set of experimental data to a specific functional form. We found that the standard methods of approaching this question were inadequate for the human hemoglobin problem, and were thus forced to adapt and modify a rarely used method to our particular problem.

An example of the inadequacy of the standard methods is shown in Fig. 1. A set of synthetic data was generated corresponding to a straight line with slope and intercept equal to 1.0 and with normally distributed pseudorandom noise of standard deviation 0.1 added (data not shown). The best least-squares line through these points corresponds to a slope of 0.82 ± 0.11 (±SEM) and intercept of 1.29 ± 0.17. The standard errors as predicted by the standard statistical methods do not include the correct answers. However, if assumptions are made that the parameters are correlated and that the variance space is nonlinear and asymmetrical, then a confidence region that is approximately elliptically shaped can be predicted by the methods presented. The solid curve in Fig. 1 is the true one standard-deviation confidence region corresponding to the described data. In this figure the single filled square corresponds to the correct answers, the solid lines intersect at the least-squares determined values, and the dashed lines correspond to the confidence intervals as determined by standard statistical methods. This example is typical of a large set of tests of this type in which the standard statistical methods are significantly worse at predicting realistic confidence intervals than the methods presented here.

The second problem arose from the multiplicity of possible mathematical formulations of the linkage between subunit assembly and ligand binding in human hemoglobin (Ackers and Halvorson, 1974; Ackers and Johnson, 1981; Johnson and Ackers, 1982). Once the values and confidence intervals of a set of parameters have been determined by nonlinear least-squares techniques, how can these be propagated to find the values and confidence intervals of the parameters for some other formulation? This report presents the numerical procedures employed to solve these problems, and provides examples of the use of

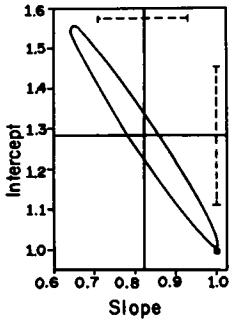

FIGURE 1 Confidence-interval ellipse for a linear least-squares analysis of simulated data.

these procedures as applied to ligand-binding problems, and makes a general purpose FORTRAN IV program available.

NUMERICAL METHODS AND EXAMPLES

To describe the methods of confidence-interval evaluation and propagation, the curve fitting method to be employed must also be briefly described since they are interrelated. Consequently, this section will consist of a description of (*a*) the curve-fitting method, (*b*) the determination of the confidence intervals (with examples), and (*c*) the method of error propagation (with examples).

Curve-Fitting Method

The basic algorithm takes some function, G, and a series of data points, X_i and Y_i, and determines a vector, α, of fitted parameters such that the sum of the squares of the residuals, the differences between the function and the data points, is a minimum. The numerical procedure used for this least-squares curve fitting is a modification of the basic Gauss-Newton procedure (Box, 1960; Hartley, 1961; Magar, 1972; Johnson et al., 1976, 1981; Ackers and Johnson, 1981; Johnson and Ackers, 1982). This procedure is simply an algorithm that, when given an initial guess for the vector α, will find a better guess for α. The procedure is then applied in an iterative fashion until the vector α does not change within some specified tolerance.

During this iterative process a matrix, P, is employed as part of the algorithm to predict the next set of parameters. This matrix P is defined as

$$P_{i,j} = \frac{\partial G(X_i, \alpha)}{\partial \alpha_j}, \qquad (1)$$

where the i subscripts represent a particular data point, and the j subscripts represent a particular element of α. This same matrix and its transpose P', evaluated at the minimum of the variance space is also used for the prediction of the confidence intervals.

Evaluation of Confidence Intervals

The true confidence intervals of the fitted parameters are defined by the F statistic calculated by the ratio of the variance at any point in space to the variance at the minimum of that space (Box, 1960; Endrenyi and Kwong, 1973). This is true for any functional form, irrespective of the nonlinearity and asymmetricalitry of the variance space. An example of the use of this procedure to find the confidence interval for a one parameter least-squares estimation is given in Fig. 2. This example is a simulation of a simple ligand-binding problem, where the number of binding sites is known independently and there is no cooperative interaction of any type between the binding sites. With these assumptions, the fractional occupancy can be expressed as $K[X]/(1 + K[X])$, where K is the equilibrium binding constant of the ligand and $[X]$ is the free concentration of the ligand. This is the commonly used functional form for the evaluation of hormone receptor interactions.

Each curve of Fig. 2 was calculated by generating 50 data points with equal logarithmic spacing in $[X]$. The data were then perturbed with Gaussian distributed pseudorandom error of standard deviation of 0.05. The variance that corresponds to any value of K can be calculated from the residuals, that is the difference between the data points and the function evaluated at the corresponding free-ligand concentration. The variance ratio, F statistic, corresponding to a given value of K can then be calculated as the ratio of the variance for that particular value of K and the best estimated value for the data, $K = 1$. The solid line in Fig. 2 was generated with data, where the free-ligand concentration varied over four orders of magnitude centered on the simulated dissociation constant, $0.01 < [X] < 100$. The critical value of the F statistic for a 67% confidence interval here is 1.14. This predicts that the equilibrium constant from these data is 1.03 with a one standard-deviation confidence interval of 0.89 to 1.21. However, note that in real experiments, data are rarely obtainable over a perfectly selected four orders of magnitude in concentration. Consequently, the dashed line in Fig. 2 was calculated by the same procedure with the range of $[X]$ being only one order of magnitude in concentration

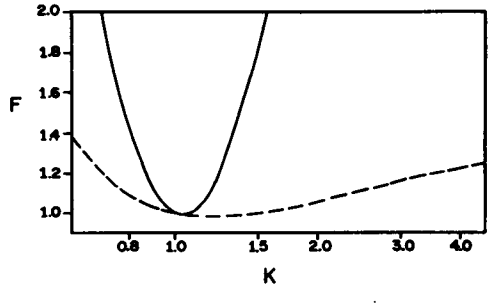

FIGURE 2 Confidence intervals for a simple single parameter least-squares analysis of a rectangular hyperbola. See text for details.

and not centered around the dissociation constant, $10 < [X] < 100$. The same critical value of the F statistic predicts that the equilibrium constant is 1.20 with a one standard-deviation confidence interval of 0.75 to 2.79. Note that this pseudorealistic one parameter curve-fitting problem can yield very asymmetrical confidence intervals. Such asymmetrical confidence intervals can only be evaluated by searching the variance space for the critical value of the F statistic. The classical methods of evaluating confidence intervals assume that the dashed line in Fig. 2 can be approximated as a symmetrical parabola centered about the best approximation of the answer. Consequently, they will predict that the confidence interval, corresponding to the dashed line in Fig. 2, is symmetrical.

This search method can easily be expanded to multiple dimensions to evaluate the confidence intervals when multiple parameters are simultaneously evaluated. Note that this mapping of the F statistic must include both mapping along each of the parameters independently, as in Fig. 2, as well as mappings where multiple parameters can vary simultaneously. Consequently, the amount of computer time required to perform the mapping increases as the power of the number of parameters. Clearly, for a complex multiple parameter evaluation a faster approximation is required.

Previous authors (Box, 1960; Endrenyi and Kwong, 1973) assumed that the variance space could be approximated by a multidimensional ellipse. This assumption allows the size and shape of the ellipse, and consequently the variance space, to be predicted by the curvature of the space at the minimum of the variance space, i.e., from the $P'P$ matrix, (see Eq. 1). This method, however, assumes that the confidence region in the multidimensional space is symmetrical. The example shown in Fig. 2 has clearly shown that this is not always the case. A modified version of this procedure is the basis for the method of evaluating the confidence intervals that we employed.

Our adaptation of this method for the determination of confidence limits of the fitted parameters is to evaluate them by searching the variance space for an F statistic corresponding to approximately a 67% confidence probability (Ackers et al., 1976; Johnson et al., 1976; 1981). The 67% confidence region for a Gaussian distribution is the mean plus or minus approximately one standard deviation. Rather than search all of the space, we limit the search to the parameters independently and to directions predicted by the axis of the multidimensional ellipse predicted by the method of Box (1960).

Fig. 3 provides an example of four methods for finding the confidence limits of fitted parameters in a multidimensional space. The experimental data points in this example describe the binding of oxygen to hemoglobin. The oxygenation-linked subunit assembly of human hemoglobin can be described by seven independent parameters: four for oxygen binding to tetramers, two for oxygen binding to dimers, and the dimer-tetramer association constant (Ackers and Halvorson, 1974). However, if the experimental data are measured at very high concentrations, then only the oxygen binding to tetramers need be considered. These experiments were performed at such a high hemoglobin concentration (Johnson and Ackers, 1982). Consequently, these data can be used as a simple, but real, example of the nonlinear least-squares technique.

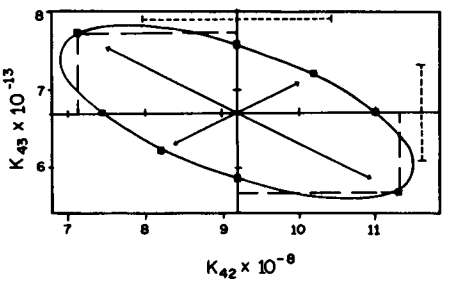

FIGURE 3 Confidence intervals for the analysis of the second and third product Adair binding constants (K_{42} and K_{43}) from the data of Roughton and Lyster (1965). Data were at pH 7 in 0.6 M phosphate and ~2% hemoglobin.

At high concentrations, the oxygen binding to tetrameric human hemoglobin can be described by four product Adair constants: K_{41}, for the average affinity of the first oxygen; K_{42}, for the average affinity for binding the first two oxygens; K_{43}, for the average affinity for binding the first three oxygens; and K_{44}, for the average affinity for binding all four oxygens (Johnson and Ackers, 1977). The fractional saturation can then be expressed as

$$\bar{Y} = \frac{\sum_{i=1}^{4} iK_{4i}[X]^i}{4\sum_{i=0}^{4} K_{4i}[X]^i}, \qquad (2)$$

where $K_{40} = 1$.

Fig. 3 was generated by first performing a four parameter least-squares fit of the data of Roughton and Lyster (1965) to Eq. 2. The parameter values determined by the least-squares fit are given in Table I. Because it is difficult to visualize a four dimensional space, a second least-squares fit was performed to generate the example in Fig. 3. In this second fit, an assumption was made that the values of K_{41} and K_{44} were known to be the values given in Table I. Consequently, this second least-squares fit is a two parameter least-squares fit to determine only K_{42} and K_{43}. The fitted parameters for this second fit are presented in Table II.

The solid elliptically shaped curve in Fig. 3 was calculated by exhaustively searching the two parameter space (K_{42} vs. K_{43}) for the dividing line between acceptable and unacceptable parameter pairs that yield an F statistic as determined by the desired confidence probability and number of degrees of freedom ($F = 1.22$ in this case). The best method of approximating the true confidence interval is to take the extreme values determined by such a search. However, this search takes an excessively large amount of

TABLE I
PARAMETER VALUES AS DETERMINED BY LEAST-SQUARE FIT*

Constant	Value	67% Confidence interval	
K_{41}	2.96×10^4	1.98×10^4	4.00×10^4
K_{42}	9.21×10^8	0.16×10^8	17.63×10^8
K_{43}	6.69×10^{13}	0.35×10^{13}	10.18×10^{13}
K_{44}	1.51×10^{19}	1.44×10^{19}	1.59×10^{19}
k_{41}	2.96×10^4	1.98×10^4	4.00×10^4
k_{42}	3.11×10^4	0.04×10^4	8.89×10^4
k_{43}	7.27×10^4	1.96×10^4	627.66×10^4
k_{44}	2.26×10^5	1.50×10^5	4.35×10^5

*Macroscopic product Adair constants as determined by a least-squares curve fit of the data of Roughton and Lyster (1965) to Eq. 2. Also given are the stepwise Adair binding constants as determined by an error propagation of the product Adair constants.

computer time when least-squares fitting to multiple parameters and consequently some approximation is needed. Fig. 3 also depicts the results of several approximations and these will be discussed in detail.

Most of the available canned computer programs employ a method for the evaluation of confidence intervals that is based on the method used for linear least-squares fitting. This linear approximation assumes that the variance of a fitted parameter is equal to the corresponding diagonal element of the inverse of the $P'P$ matrix times the variance of the experimental noise (Hildebrand, 1956). This method uses less computer time and assumes that the parameters are not correlated with each other, that the variance space can be predicted from the curvature at the minimum, and that the solution can be approximated by a linear least-squares fit. An example of the use of this method is also given as the short dashed lines at the top and right-hand side in Fig. 3. Note that even for this relatively simple example the classical method does not perform well.

A second method of approximating the confidence intervals of the fitted parameters assumes that the variance space can be approximated by a multidimensional hyperellipsoid defined by the solutions, Ω, of the following matrix equation (Box, 1960; Magar, 1972; Johnson et al., 1981).

$$(\alpha - \Omega)PP'(\alpha - \Omega) \leq n\sigma^2 F, \quad (3)$$

where n is the number of parameters, σ^2 is the variance, and F is the value of the F statistic for the desired probability and degrees of freedom. Confidence intervals evaluated by this procedure are by definition symmetrical about the optimal values determined as the minimum by the least-squares technique. This analysis assumes that the shape of the variance space can be predicted from the curvature of the minimum. These last two assumptions are at best tenuous in some cases. One such case will be described in the section on error propagation. The arrows at the center of Fig. 3 show the results of this method of evaluation of the confidence intervals. This method is still not a good approximation for this example, although it is better than the previous one.

The method of evaluation of the confidence intervals that we prefer is to search the variance space for the desired F statistic. This search method does not assume that the confidence region can be described as an ellipse. Furthermore, the method does not assume that the confidence region is symmetrical or that it can be accurately predicted by the curvature of the space at the minimum. We have only searched for the confidence-region contour along particular sets of directions. First, each of the parameters is searched independently in both directions until the desired F statistic is found. Second, each of the axes of the hyperellipse defined by Eq. 3 is searched in both directions for the desired F statistic. The solid squares in Fig. 3 show the results of this search. The confidence region for a given parameter is then determined by projecting each of the mapped points onto the axis for the given parameter and taking the maximum deflection in each direction. The long dashed lines in Fig. 3 show an example of this procedure. The 67% confidence intervals presented in Tables I and II, corresponding to plus or minus one standard deviation, were determined by this method. The amount of computer time required is proportional to the number of parameters being determined, not a power of the number of parameters.

TABLE II
LEAST-SQUARE CURVE FIT WITH TWO KNOWN PARAMETERS*

Constant	Value	67% Confidence interval	
K_{41}	2.96×10^4	—	—
K_{42}	9.21×10^8	7.07×10^{13}	11.34×10^8
K_{43}	6.69×10^{13}	5.65×10^{13}	7.74×10^{13}
K_{44}	1.51×10^{19}	—	—
k_{41}	2.96×10^4	—	—
k_{42}	3.11×10^4	2.39×10^4	3.84×10^4
k_{43}	7.27×10^4	4.98×10^4	10.95×10^4
k_{44}	2.26×10^5	1.95×10^5	2.67×10^5

*Macroscopic product Adair constants as determined by a least-squares parameter estimation of the data of Roughton and Lyster (1965) to Eq. 2, with the assumption that K_{41} and K_{44} are known. Consequently, the least-squares fit presented here is to two parameters only, i.e., K_{42} and K_{43}. Also given are the stepwise Adair binding constants as determined by an error propagation of the product Adair constants.

Error Propagation of Confidence Intervals

Once a set of parameters has been determined for a given set of data and a particular functional form, G, it is sometimes desirable to propagate these parameter values into the values of a different set of parameters that are of interest. It would be most desirable to perform this propagation without simply repeating the least-square fit for a different function and set of parameters. The first least-squares fit to function G has imposed constraints on the allowable values of its parameters, and it is of interest to

see how these implied constraints will propagate into a different set of parameters. Furthermore, a simple method of propagating these parameters and confidence intervals will save considerable amounts of computer time.

When the fitted parameters have been evaluated, they can be used to evaluate other parameters of interest without repeating the least-squares process. Eq. 3 can also be expressed in terms of the stepwise Adair constants: k_{41} for the affinity of binding the first oxygen; k_{42} for the affinity for adding the second oxygen to singly oxygenated hemoglobin; k_{43} for the affinity for adding the third oxygen to doubly oxygenated hemoglobin; k_{44}, etc. These stepwise and product Adair constants are, of course, related by Eqs. 4–7.

$$K_{41} = k_{41} \tag{4}$$

$$K_{42} = k_{41}k_{42} \tag{5}$$

$$K_{43} = k_{41}k_{42}k_{43} \tag{6}$$

$$K_{44} = k_{41}k_{42}k_{43}k_{44} \tag{7}$$

For a more complete description of the linkage between subunit assembly and oxygen binding in human hemoglobin, the reader is referred to Ackers and Halvorson (1974), Ackers et al. (1975), and Johnson et al. (1976). It is these relationships that will be used as the example of the propagation of confidence intervals determined by least-squares curve fitting in one space to parameters of another space. The actual example will be to determine the product Adair constants and their associated confidence intervals, and then determine from them the stepwise Adair constants and corresponding confidence intervals without repeating the least-squares fit.

Serious problems arise in propagating the confidence intervals to determine the confidence intervals of the stepwise Adair constants. Note that standard methods of error propagation assume that the confidence interval is symmetrical about the estimated best value, and most assume that the parameters are not correlated. However, the cross-correlation coefficient between the parameters used to generate Fig. 3 was -0.65 and the suboptimal case in Fig. 4 is obviously asymmetrical.

The basic method of confidence-interval propagation is actually a simple problem of mapping one parameter space into another parameter space. The optimal method is to map the entire critical F statistic contour from one space to another. This requires a large amount of computer time, as previously noted, since it again requires that the entire contour be evaluated. We therefore developed a method to approximately map the entire space.

An example of this mapping from parameter space to another is given in Fig. 4. The elliptically shaped confidence contour in Fig. 3 is a two-dimensional contour, i.e., K_{43} vs. K_{42}. When this contour in product Adair constant space is mapped to the stepwise Adair constant space, it becomes a three-dimensional contour in k_{42}, k_{43}, and k_{44}. This added dimension arises from the assumption that K_{41} and K_{44} are previously known. For ease of visualization, a two-dimensional projection, k_{44} vs. k_{43}, is presented in Fig. 4. It is obvious that this contour is neither symmetrical nor elliptical. Also shown in Fig. 4 are the eight points presented in Fig. 3 that were mapped into the new parameter space. These points are sufficient to define the contour, and thus the projections of these points onto the individual stepwise Adair constant axes are close approximations of the true confidence intervals of the propagated parameters.

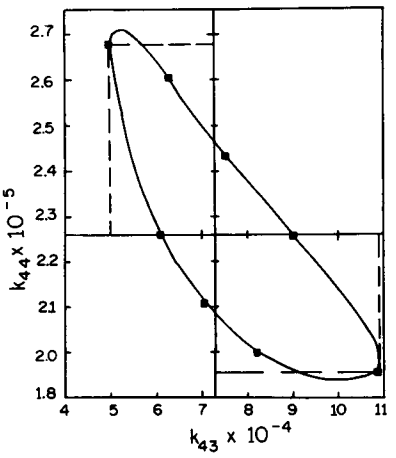

FIGURE 4 Propagation of the confidence intervals shown in Fig. 2 to derive the third and fourth stepwise Adair constants (k_{43} and k_{44}). See text for details.

The effects of cross correlation between parameters can also be seen by a comparison between Tables I and II. If, as in Table II, the assumption is made that K_{41} and K_{44} are known, then the value of K_{42} is 9.21×10^8 with a confidence interval of 7.07 to 11.34×10^8. If the effects of the possible variation of all four parameters are included, as in Table I, then the confidence intervals are substantially larger. Also shown in Table I are the values of the stepwise Adair constants propagated in the four-dimensional parameter space.

CONCLUSIONS

The numerical procedures presented here have proven very useful in modeling the molecular mechanisms of hemoglobin. These procedures are also generally applicable to a large range of nonlinear curve-fitting problems. To this end, the author is willing to supply a general purpose nonlinear curve-fitting program, written in FORTRAN IV for PDP-11 computers, which performs a least-squares fit and confidence-interval evaluation as described here.

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