

## ANALYSIS OF RELAXATION KINETICS DATA BY A NONLINEAR LEAST SQUARES METHOD

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Received 15 October 1973

Revised manuscript received 25 February 1974

Recent improvements in measuring and data acquisition techniques have increased greatly the precision of chemical relaxation data. This has necessitated more accurate methods for data analysis in cases of complex relaxation spectra, as is often observed in biochemical systems. We have developed and applied a method capable of decomposing a wave form containing up to three overlapping exponentials. The method is based upon a nonlinear least squares algorithm. Analysis of the method shows that when it is applied to a two exponential function where the signal-to-noise ratio,  $\Delta S/N$ , is fifty, using 100 data points, the resulting four coefficients (two amplitudes and two relaxation times) are each accurate to within 5–10 % over a wide range of conditions, i.e., relative amplitudes from 10–90 % and ratios of relaxation times of 2.5 or greater. The influence of the number of data points and of random noise is as expected from statistical theory. Applications of the curve fitting methods to experimental temperature-jump data from oxygen binding to hemoglobin and hemocyanin yields internally consistent results. The values of the root-mean-square of the residuals of the fit approach those expected on the basis of the experimental signal-to-noise ratios.

### 1. Introduction

Curve fitting to nonlinear equations is a common problem in data analysis. Several methods are available in principle; the success of a particular method in practice depends upon the magnitude and kinds of noise in the data. We have applied a well-known nonlinear least squares method ([9], p. 451; [13], p. 149; [2]; [6]) specifically to simulated and real temperature-jump relaxation kinetics data. The method has been tested and used for more than one year in the form of a user oriented Fortran IV program and has proved to be practical and reliable in a variety of applications to the analysis of temperature-jump kinetics data from the binding of oxygen to hemocyanin and hemoglobin.

It has been shown theoretically that chemical relaxa-

tion (the time course of the change in concentration of some dependent variable) is described exactly as the sum of exponential curves [5]. The following analytical expressions apply to any problem involving fitting to multiple exponential curves, such as fluorescent decay kinetics [4, 16], isotope exchange kinetics [12], equilibrium sedimentation [7, 21] and the correlation spectrum of concentration fluctuations from equilibrium [3].

In a relaxation kinetics experiment the time course of the approach to an equilibrium state is given by

$$y(t) = \sum_i A_i \exp(-t/\tau_i). \quad (1)$$

$A_i$  is some dependent variable which is related to the extent of the reaction, such as optical absorbance or conductivity;  $t$ , the time is the independent variable; and  $\tau_i$  the relaxation times. The quantities  $A_i$  and  $\tau_i$  are the direct observables which can be related to the "normal reaction variables" of the reaction matrix for a given kinetic mechanism [5]. The accuracy with which  $A_i$  and  $\tau_i$  can be evaluated from the experimen-

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‡ A preliminary account of these results was presented at the IUPAB Symposium on "Relaxation Methods in Molecular Biology", Copenhagen, August, 1972.

tal kinetics curve determines the accuracy with which the rate constants, enthalpies, and activation parameters of the corresponding reaction mechanism can be determined. In a more general sense it determines the number of elementary chemical reactions which can be characterized and thus defines the resolution of the entire kinetic analysis [5, 8, 20].

Recent developments in photometric detection [15] and on-line computer assisted data acquisition systems [17] have made it possible to obtain sufficiently accurate data to warrant the evaluation and use of improved curve fitting methods for obtaining  $A_i$  and  $\tau_i$ .

## 2. Methods

The Gauss–Newton method (and any other method of successive approximation) is an algorithm for refining guesses of the values of the coefficients of a function. The algorithm can thus be applied repeatedly until the guesses have been refined to any specified precision. For example, for a 2 exponential fit, given a set of data points  $t_i$  (independent variable, in this case time) and  $y_i$  (dependent variable, in this case concentration) and initial guesses  $\alpha_1^{(0)}$ ,  $\alpha_2^{(0)}$ ,  $\alpha_3^{(0)}$ , and  $\alpha_4^{(0)}$  (coefficients, i.e., amplitudes and reciprocal time constants) the algorithm generates a new set of improved guesses  $\alpha_1^{(1)}$ ,  $\alpha_2^{(1)}$ ,  $\alpha_3^{(1)}$ , and  $\alpha_4^{(1)}$  (using Magar's notation [13]). The Gauss–Newton iteration generates a series of approximations for each variable (such as  $\alpha_j^{(0)}$ ,  $\alpha_j^{(1)}$ ,  $\alpha_j^{(2)}$ , ...,  $\alpha_j$ ) which approach the convergence values of the coefficients ( $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_4$ ). These convergence values yield a minimum root-mean-square (RMS) of the residuals for fitting to the function,  $F$ , for  $m$  data points.

$$F(\alpha_1, \alpha_2, \alpha_3, \alpha_4, t) = \alpha_1 e^{-t\alpha_2} + \alpha_3 e^{-t\alpha_4}, \quad (2)$$

$$(\text{RMS})^2 = (1/m) \sum_{i=1}^m [F(\alpha_1, \alpha_2, \alpha_3, \alpha_4, t_i) - y_i]^2. \quad (3)$$

The Gauss–Newton method calls for approximation of the function  $F(\alpha, t_i)$  by the Taylor expansion

$$y_i \approx F(\alpha^{(q)}, t_i) \\ \approx F(\alpha^{(q-1)}, t_i) + \sum_{j=1}^n [\delta F(\alpha^{(q-1)}, t_i) / \delta \alpha_j] \epsilon_j \quad (4)$$

at each of the  $i$  data points ([13], p. 150). In this equa-

tion  $\alpha$  is a vector whose elements are the parameters  $\alpha_1, \alpha_2, \alpha_3, \alpha_4$  and  $\epsilon_j$  is the difference between the values of the  $j$ th parameter for successive iterations ( $q$  and  $q-1$ ). This set of equations can then be rewritten in matrix notation as

$$y^* = P\epsilon, \quad (5)$$

where  $y^*$  is the vector whose elements are  $y_i - F(\alpha^{(q-1)}, t_i)$  and  $\epsilon$  is the vector whose elements are  $\epsilon_1, \epsilon_2, \epsilon_3, \epsilon_4$ , and  $P$  is the matrix of the partial derivatives in eq. (4). The vector  $\epsilon$  can be evaluated from its matrix equivalent

$$\epsilon = (P'P)^{-1} P'y^*, \quad (6)$$

where  $P'$  is the transpose of  $P$ .

The correction vector,  $\epsilon$ , can then be added to  $\alpha$  to get a better approximation of  $\alpha$ ,

$$\alpha^{(q)} = \alpha^{(q-1)} + \Pi\epsilon \quad (7)$$

where  $\Pi \leq 1$ , which is used to speed convergence ([13], p. 146). It should be noted that this constant is unity at convergence and is varied during the iterative process if required to prevent divergence.

The computer program we have developed [11] chooses the initial values of  $\alpha$  by first approximating the reciprocal time constants and then using a linear least squares fit to obtain the best amplitudes corresponding to these time constants. For a two exponential fit the initial values of the reciprocal time constants are found by fitting a straight line to a log plot of the first and last half of the data points. For a three exponential fit the starting values for the reciprocal time constants are obtained using the values of the reciprocal time constants ( $\alpha_2, \alpha_4$ ) determined in a prior two exponential fit. The starting values of the reciprocal time constants for a three exponential case are taken to be (1) the average of  $\alpha_2$  and  $\alpha_4$ , (2) a number 20% greater than either  $\alpha_2$  or  $\alpha_4$ , and (3) a number 20% less than the other.

For a linear least squares fit the standard errors (SE) of the coefficients,  $\alpha_j$ , would be given by ([9], p. 258)

$$SE_j = [(P'P)_{jj}^{-1} (\text{RMS})^2]^{1/2}, \quad (8)$$

where  $(P'P)_{jj}^{-1}$  is the  $j$ th diagonal element of the ma-

trix  $(P'P)^{-1}$ .

### 3. Results and discussion

The use of eq. (8) for nonlinear fitting is only an approximation which in this case involves three assumptions, (1) that the nonlinear problem is linear at the solution [see eq. (4)], (2) that the error in each parameter is independent and normally distributed, and (3) that the variance of the noise is given by the RMS of the fit. For convenience of notation the parameters obtained in a two exponential fit [see eq. (2)] are denoted by  $a, b, c, d$  for  $\alpha_1, \alpha_2, \alpha_3, \alpha_4$ , respectively, and  $a_0, b_0, c_0, d_0$  are the values used in generating the test data.

We have used this approximation as a measure of the standard error of the parameters of a fit and refer to it as the "confidence limit" (CL). A more complete statistical analysis of standard errors can be obtained by an analysis of the confidence contours of the parameters as shown by Magar ([13], p. 252). However, the interpretation of such analysis is complex for more than three parameters.

One method of defining the meaning of the CL of a parameter is to compare it with the SE of the mean (correct) value obtained by analysis of a single set of test data perturbed by several different sets of random noise. This procedure can be used to calibrate the CL.

Table 1

Test of the algorithm for finding the "confidence limit" (CL) of a variable using a linear approximation [eq. (8)]. For the function,

$$y = a_0 e^{-b_0 t} + c_0 e^{-d_0 t}$$

one hundred data points equally spaced in time were calculated and perturbed by six different sets of pseudo-random noise (RMS = 0.02;  $\Delta S/N = 50$ ). CL, the "confidence limit", is seen to be about three times the standard error (SE)

True value	Mean value	SE of mean	Mean CL
$a_0 = 0.50$	$a = 0.4956$	0.008	0.033
$b_0 = 1.00$	$b = 0.998$	0.013	0.051
$c_0 = 0.50$	$c = 0.5009$	0.010	0.034
$d_0 = 5.00$	$d = 5.054$	0.15	0.511

Table 1 shows the results of a typical test of this definition of the "confidence limit". In this case the CL was about three times the standard error of the mean.

Extensive testing of the method was performed by analyzing generated data and comparing the calculated and the true time constants and amplitudes under a variety of conditions. These conditions, although realistic, do not always represent the optimum ones available with present instrumentation. For example, the  $\Delta$  signal-to-noise ratio ( $\Delta S/N$ ) used for most of these evaluations is 50 but in practice values of 200 can be obtained, depending on the system under study. Also, although the use of one hundred data points represents a reasonable number for analog recording, more recent digital recording methods provide for as many as one thousand data points with time spacing varying according to the particular transient [17]. The present method does not require equally spaced data points.

The examples shown in figs. 1–5 are all for a two exponential fit to generated data. In each case all four parameters, two time constants and two amplitudes, were varied simultaneously. In all cases the time range of the data extended to three times the slowest time constant and, except for fig. 4, the baseline was assumed.

Fig. 1 shows that as  $\Delta S/N$  decreases the uncertainty of each of the four parameters varies in about the same way. At  $\Delta S/N = 100$  the CLs of the time constants are within 5%, and of the amplitudes within 2%, of the correct values. It should be recalled that the corresponding standard error is about one-third of these CLs (see table 1).

The detection and measurement of a second relaxation having a small amplitude can be accomplished over a rather wide range of relative amplitudes, as shown in fig. 2. Between the relative amplitudes of 0.10 and 0.90 the time constants were determined to within 8% in both cases.

Fig. 3 shows the influence of the number of data points on the CL of each coefficient. An accuracy of about 5% is obtained with 100 data points in all but one case, the faster time constant, for which it is about 10%. If, however, the data points had been taken unequally spaced in time so as to increase the fraction of points within the fast phase, the CL of the faster time constant can be brought to within 5%. Such a procedure is possible in practice by optimizing the data sampling profile.

In practice, determining the baseline can cause er-

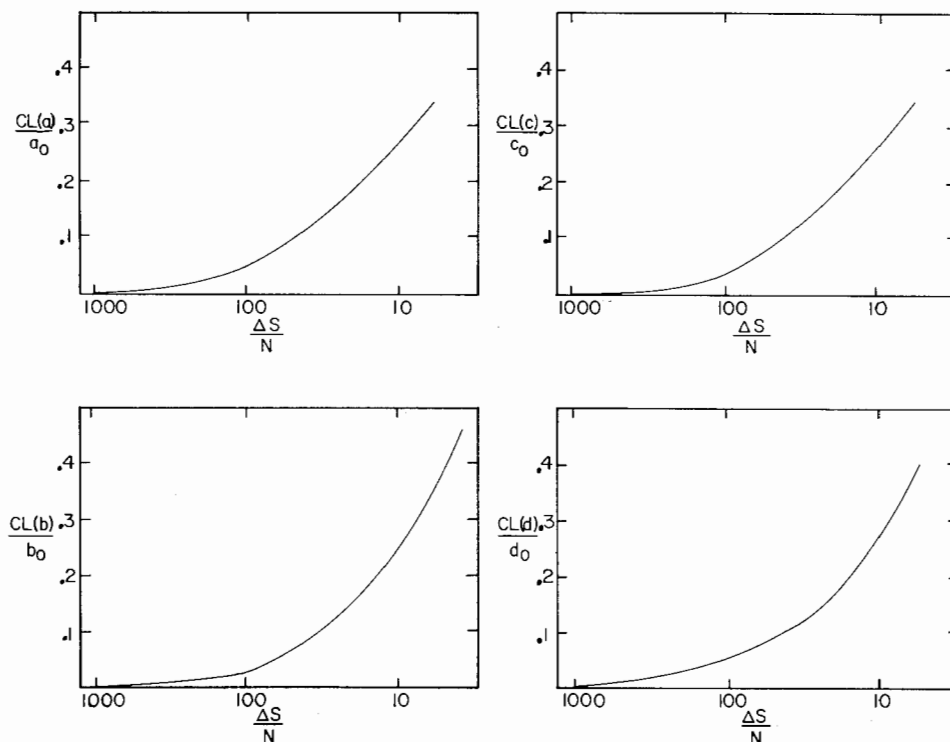


Fig. 1. Error in amplitudes and time constants as a function of  $\Delta S/N$ . Ordinate: Confidence limit (CL, approximately  $3 \times \text{SE}$ ) of the calculated variable ( $a$ ,  $b$ ,  $c$ ,  $d$ ) divided by the true value of that variable;  $\text{CL}(a)/a_0$ ,  $\text{CL}(b)/b_0$ ,  $\text{CL}(c)/c_0$ , or  $\text{CL}(d)/d_0$ . Abscissa: Absolute difference in signal level before and after reaction divided by RMS noise,  $\Delta S/N$ . Calculated using the function  $y = a_0 e^{-b_0 t} + c_0 e^{-d_0 t}$ , where  $a_0 = 0.5$ ,  $b_0 = 1.0$ ,  $c_0 = 0.5$ ,  $d_0 = 5.0$ . All four parameters were varied simultaneously using 100 data points equally spaced in time.

rors and the influence of such errors is shown in fig. 4. A 1% error in the baseline causes about 5% uncertainty in each of the four coefficients, emphasizing the importance of the accuracy of this measurement.

Baseline error is basically an instrumental problem which results from cooling after the temperature perturbation and/or very slow decay processes which are not complete by the time cooling occurs. These sources of baseline error can be minimized significantly by precise calibration procedures and automated data acquisition methods with varying sampling profiles. This has been found to essentially solve this problem for baseline corrections of a few percent [11].

The present method yields reliable values when the ratio of the time constants is as small as about 2.5, as shown in fig. 5. For more closely overlapping time constants, which display essentially a spectrum of coefficients, it is possible to use average techniques [18].

We have avoided data smoothing procedures since, without a priori knowledge of the form of the noise,

such methods are expected to bias the data. If all data points have the same absolute accuracy and are equally spaced in time, no weighting factors should be used. This is because the present method does not introduce any weighting factors. However, weighting factors should be used if the data are recorded using a variable bandwidth or if there are any unusual or time dependent noise distributions [9].

The above analysis has demonstrated the sensitivity and limitations of the method emphasizing a two exponential function. Selected analyses for a three exponential function have revealed similar trends. A presentation of the corresponding complete analysis for a three exponential function would be too extensive for this report. In principle, the method can be applied to a function having a larger number of exponential terms, provided the data justify such an analysis. Furthermore, the method can be extended to transcendental, non-homogeneous and periodic functions making it useful for the analysis of a variety of data.

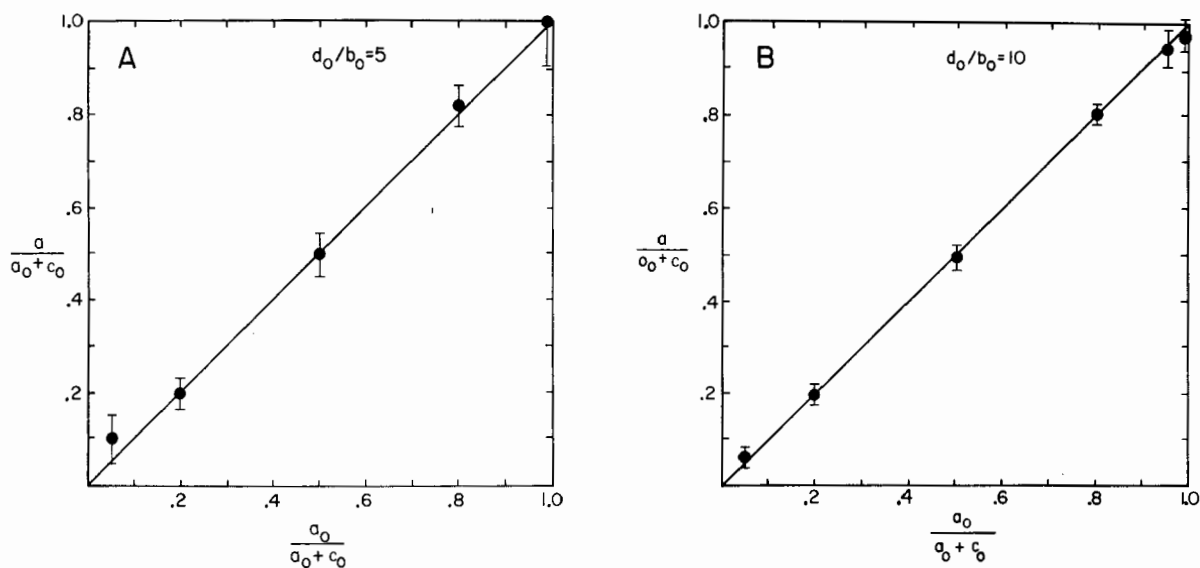


Fig. 2. Influence of relative amplitudes on determination of true amplitudes. Ordinate: Calculated relative amplitude. Abscissa: True relative amplitude. Definitions as in fig. 1,  $\Delta S/N = 50$ , 100 data points equally spaced in time, all four parameters were varied simultaneously for two different ratios of relaxation times,  $d_0/b_0 = 5$  (A), 10 (B). Straight lines indicate unity slope.

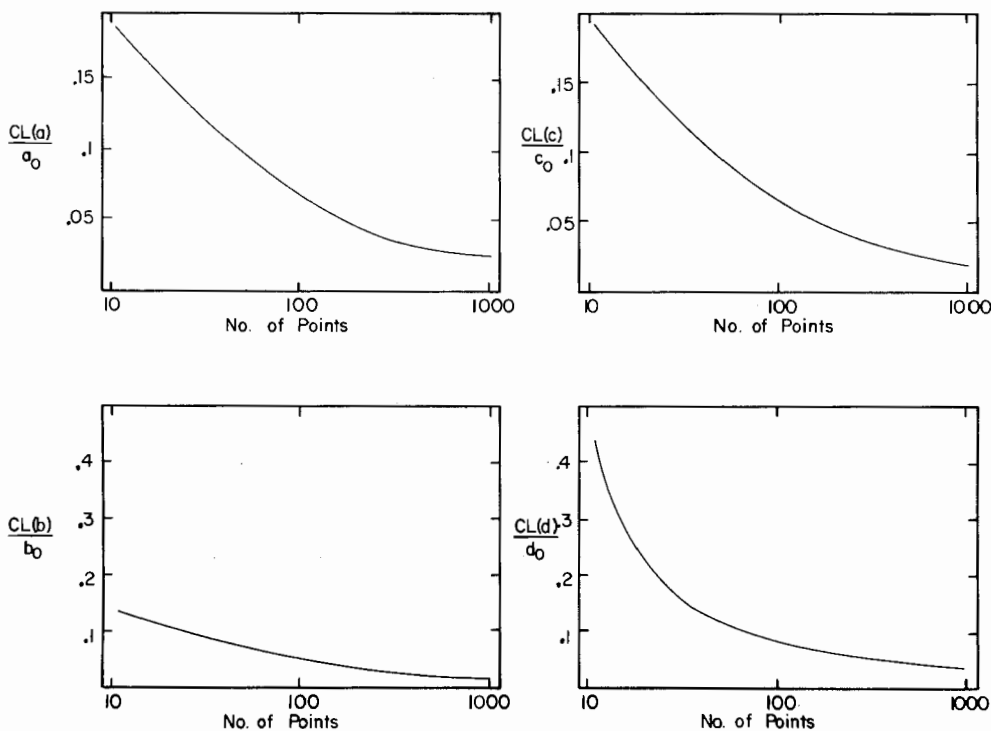


Fig. 3. Influence of the number of data points on the determination of amplitudes and time constants. Ordinate: As in fig. 1. Abscissa: Total number of data points spaced equally in time over identical time ranges.  $\Delta S/N = 50$  and  $a_0, b_0, c_0, d_0$  as in fig. 1.

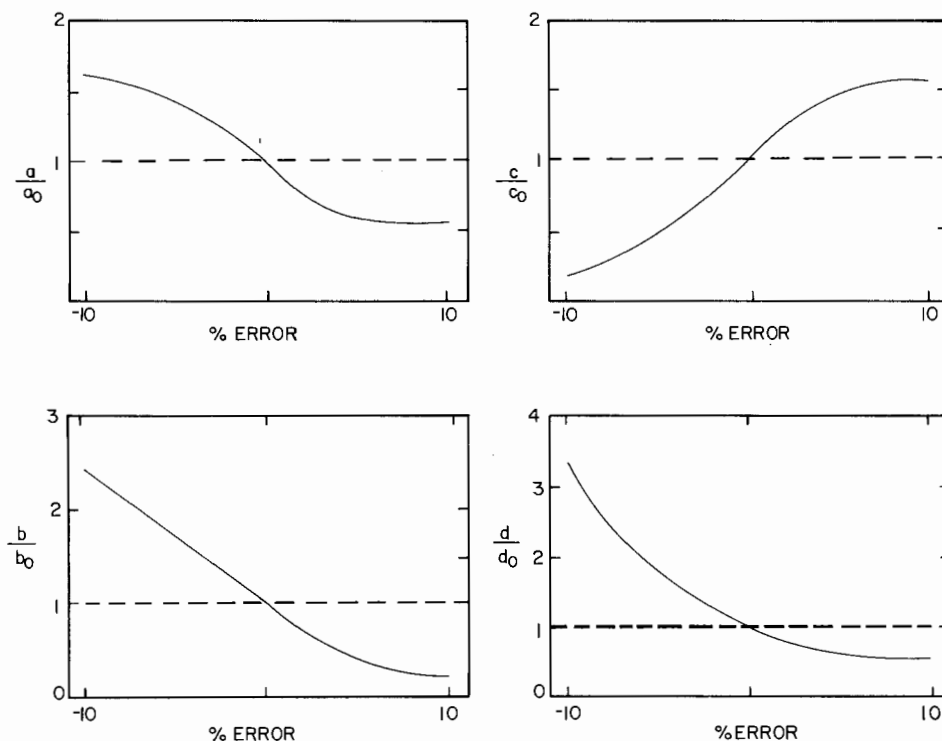


Fig. 4. Influence of baseline error on the determination of amplitudes and time constants. Ordinate: Parameters have same meaning as in fig. 1. Abscissa: Baseline error, as percent of total amplitude. All four parameters were varied simultaneously, using 100 equally spaced points. Other parameters as in fig. 1.

The computer program in which the present method has been used for analyzing relaxation data containing up to three exponentials includes options which allow for (1) determining baselines by fitting to polynomials, (2) making cooling corrections, (3) signal averaging from repetitive experiments, (4) amplitude normalization, (5) conversion of data from transmittance to absorbance, (6) data sorting and editing according to the RMS noise, (7) a routine for simulating and refitting relaxation spectra based upon measured relaxation times, amplitudes and RMS noise, and (8) evaluation of average relaxation times and amplitudes [18].

#### 4. Experimental applications

We have applied this method of analysis to data from equilibrium centrifugation [11] and temperature-jump relaxation kinetics [14, 17] and cite briefly examples of the latter. Two proteins whose mechanism

of oxygen binding are presently under investigation are hemocyanin and hemoglobin, examples of which are summarized in tables 3, 4, and fig. 6.

Numerically, there is "no satisfactory way" ([13], p. 255) of determining the number of exponential terms required to describe a set of experimental data. However, knowledge of the chemical properties, composition or structure of the system under study often provides information about the minimum number of parameters required to describe the time course of the reaction. We have used two numerical methods to attempt to determine the minimum number of exponential terms: (1) comparison of the RMS of each fit with the expected RMS based on a determination of the magnitude of noise in the data, and (2) an analysis of the randomness of the residuals of the fit. The determination of the number of exponentials is made using data from replicate experiments since both methods are sensitive to nonrandom noise.

We estimate the randomness of the residuals by fit-

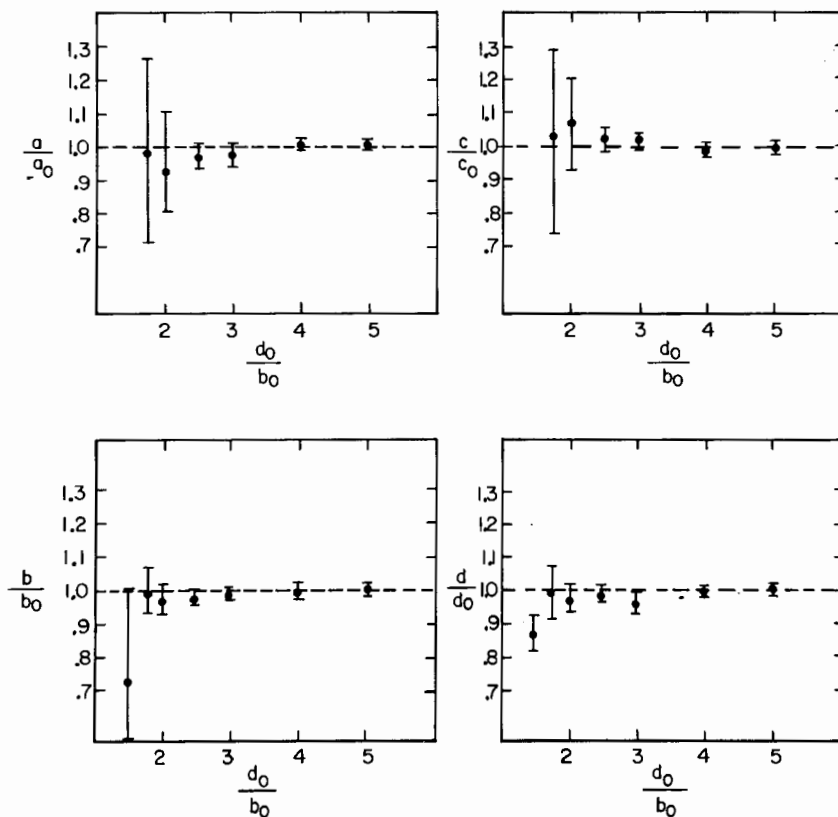


Fig. 5. Influence of the ratio of time constants on the determination of time constants and amplitudes. Ordinate: As in fig. 1,  $\pm$  CL. Abscissa: Ratio of true time constants. All four parameters were varied simultaneously using 1000 data points equally spaced in time, equal amplitudes, and  $\Delta S/N = 100$ .

ting the residuals as a function of the independent variable (time) to four polynomials, of degree zero through three (yielding ten coefficients), and comparing the value of each coefficient with twice its standard error ([9], p. 258). If the residuals were truly random all of the coefficients would be approximately zero and statistically only one in ten would be larger than twice its standard error. This simple approach is utilized by counting the number of coefficients which are greater than twice the respective standard error thus giving an estimate of the nonrandomness which will vary between zero and ten, where the larger numbers indicate less randomness.

Table 2 demonstrates this measure of randomness for a test case where the data were calculated for two exponential terms. The RMS and nonrandomness are seen to decrease when the correct minimum number

of exponential terms is used. (Observe that the confidence limits increase when analyzing this two exponential data as three exponentials.) Fitting to three exponentials does not improve the fit as judged from the RMS or the nonrandomness. In addition, the uncertainty of each coefficient is increased in the three exponential fit as seen in the values of CL, as expected from eq. (8).

Table 3 and fig. 6 show that under some conditions at least three exponentials are required to describe the kinetics of  $O_2$  binding to hemoglobin. This can be seen from the decrease in the RMS of the fits and nonrandomness of the residuals. However, since even the three exponential fit yields a high value for the estimate of the nonrandomness and the value of  $\Delta S/\text{RMS}$  is only about 75 % of the value expected on the basis of the RMS noise monitored prior to the temperature-jump,

Table 2

Analysis of the behavior of the nonrandomness of the residuals as a function of the number of exponential terms included in the fit. One thousand data points were calculated according to the function

$$y_i = 0.5 \exp^{10.0t_i} + 0.5 \exp^{3.0t_i},$$

where the time values were equally spaced from zero to one. These data were then perturbed with pseudo-random noise, normally distributed, such that the  $\Delta$  signal-to-noise ratio,  $\Delta S/\text{RMS}$ , was 50.0

Fit	RMS (volt)	$\Delta S/\text{RMS}$	NR <sup>1)</sup>	$A_i/\Delta S \pm \text{CL}^1)$	$1/\tau(\text{sec}^{-1}) \pm \text{CL}^1)$
One exp.	0.02994	33.2	9	100.0 $\pm$ 0.45	4.48 $\pm$ 0.028
Two exp.	0.01995	50.0	0	46.2 $\pm$ 2.5 53.8 $\pm$ 2.8	10.77 $\pm$ 0.49 3.11 $\pm$ 0.07
Three exp.	0.01993	50.0	0	27.7 $\pm$ 52.4 32.6 $\pm$ 28.6 39.7 $\pm$ 58.6	13.6 $\pm$ 7.8 6.1 $\pm$ 9.0 2.8 $\pm$ 1.0

1) See text for meaning of NR, "nonrandomness", and CL, "confidence limit".

Table 3

Analysis of temperature-jump relaxation data from oxygen binding to human hemoglobin-A. Protein concentration  $10^{-4}$  M (heme); 22 percent saturated with  $\text{O}_2$ ; pH 7.0, 0.1 M phosphate plus  $1.25 \times 10^{-3}$  M inositol hexaphosphate; initial temperature =  $16^\circ\text{C}$ ,  $\Delta T = 4^\circ\text{C}$ ; wavelength of observation 365 nm; heating time about 3 microseconds. Total change in optical absorbance  $\Delta S = 4.42$  volt (0.0230 A). Data were recorded using an on-line computer for data acquisition [17]. One thousand ten-bit data points were recorded digitally at 100 kilohertz band width with a sampling frequency that decreased with the slope of the transient

Fit	RMS (volt)	$\Delta S/\text{RMS}$	NR <sup>1)</sup>	$A_i/\Delta S \pm \text{CL}^1)$	$1/\tau(\text{sec}^{-1}) \pm \text{CL}^1)$
One exp.	0.281	15.6	10	100.0 $\pm$ 0.89	201.0 $\pm$ 4.2
Two exp.	0.0617	71.2	8	67.4 $\pm$ 0.49 32.6 $\pm$ 0.32	977.0 $\pm$ 10.7 62.0 $\pm$ 0.8
Three exp.	0.0259	169.0	7	57.0 $\pm$ 0.33 33.7 $\pm$ 0.28 9.3 $\pm$ 0.25	1386.0 $\pm$ 12.2 150.0 $\pm$ 2.2 19.5 $\pm$ 0.6

1) See text for the meaning of NR, "nonrandomness", and CL, "confidence limit".

$\Delta S/\text{RMS} = 200$ , it is possible that a fourth term is required to adequately characterize these data.

In contrast, the oxygen binding reaction with *Limulus* (horseshoe crab) hemocyanin monomer with a single binding site (table 4) is characterized by a minimum of two exponentials under some conditions. This

conclusion is arrived at by noting that the RMS of the two exponential fit is approximately what would be expected from the magnitude of the noise before the temperature jump,  $\Delta S/\text{RMS} \approx 120$ , and the marked decrease in the nonrandomness which results from including the second exponential.

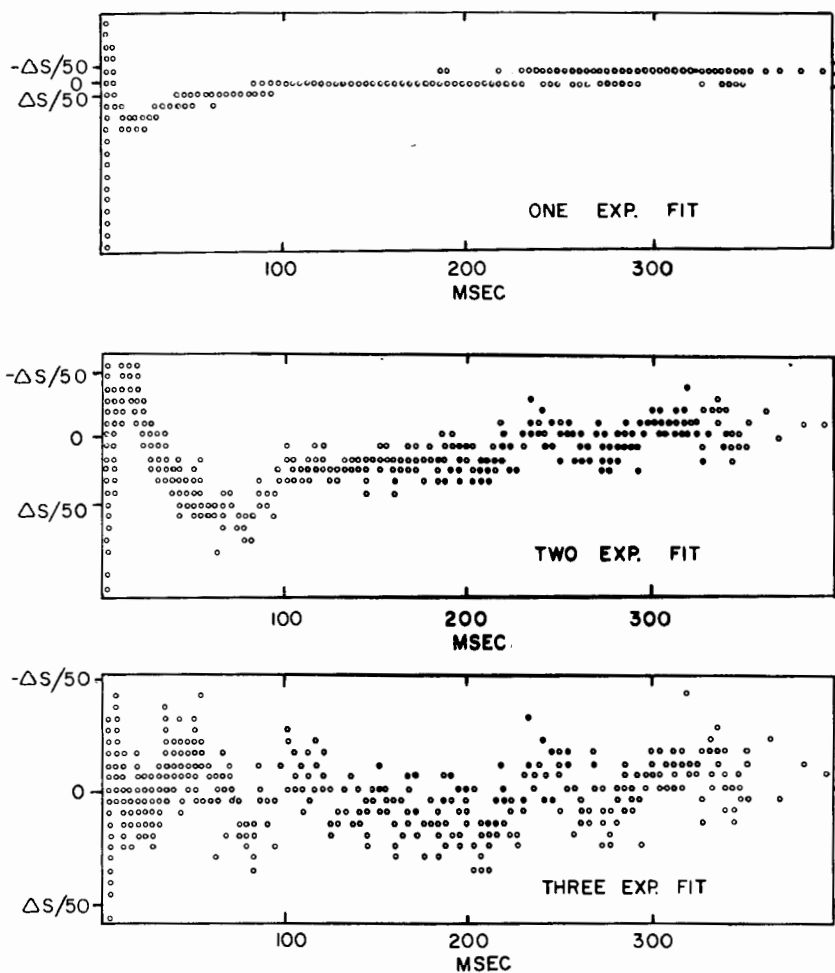


Fig. 6. Plots of residuals for the three fits of hemoglobin data as described in table 3. Ordinate: Deviation of residuals defined as the difference between the data point and the corresponding fitted function at that time. *Note scale differences.* Abscissa: Time in milliseconds. In each case all parameters were varied simultaneously. Baseline was determined independently and is the same for all fits. Experimental conditions and data recording are as described in table 3. Note the increasing randomness in the residuals with increasing number of time constants, this is an additional criterion for the significance of the minimum number of coefficients.

#### Acknowledgement

The advice of Dr. D.A. Yphantis and the stimulation of Dr. R. Dyson are gratefully acknowledged. This research was supported by the following grants: National Science Foundation, GJ-9 to the University of Connecticut Computer Center, GB-30825X (to D.A.Y.), GB-31097X (to T.M.S. and D.A.Y.). USPHS-NIH: GM-18472 (to T.M.S.) and Training Grant GM-00317 (M.L.J.).

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