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MULTIPOINT REGRESSION DATA-PROCESSING METHODS APPLIED TO NONLINEAR KINETIC DATA

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Abstract - This paper describes the application of multipoint regression methods to a variety of situations that involve nonlinear kinetic data, and illustrates advantages that can be achieved with these data-processing methods. Examples include kinetic procedures that are virtually independent of variations in experimental parameters such as temperature, pH, reagent concentration, etc., kinetic procedures that have substantially increased linear ranges, procedures that can resolve two-component mixtures such as unconjugated and conjugated bilirubins or the H and M subunits of lactate dehydrogenase, procedures that can compensate for substrate inhibition in the quantitation of enzyme activity, and procedures that have the potential to yield linear (not "linearized") calibration curves in competitive binding immunoassays. Data are presented to illustrate most of these points.

INTRODUCTION

Although it has become common practice in recent years to use the method of least squares (1,2) to process data that vary linearly with time, there have been relatively few applications to clinical problems of these regression methods to process data that vary nonlinearly with time. Some of the more common situations that involve nonlinear time-dependent data include reactions that follow first-order or Michaelis-Menten kinetics as well as the so-called lag phases in enzyme catalyzed reactions. These situations occur with sufficient frequency, or experimental procedures are designed to avoid them sufficiently often, that they merit explicit attention. This paper describes applications of nonlinear regression methods to selected situations, and discusses some of the potential advantages of the resulting procedures.

APPLICATIONS OF NONLINEAR REGRESSION METHODS

Several applications of nonlinear regression methods to nonlinear kinetic data are presented here as illustrative examples.

First-order processes

The application of nonlinear regression methods to process single-component, first-order, kinetic data has resulted in a procedure that is virtually independent of variations in parameters such a pH, temperature, activators, inhibitors, and enzyme denaturation that can influence rate constants; applications to two-component systems have resulted in procedures that permit the quantitative resolution of individual components without a separation step.

Single-component samples. Figure 1 illustrates an approach used to process first-order, single-component data (3,4). Absorbance vs. time data collected early in the process are fit to a first-order model in a manner such that predicted values of the initial absorbance, $\hat{A}_o$, the final absorbance, $\hat{A}_\infty$, and the pseudo-first-order rate constant, $\hat{k}$, are obtained for each individual sample. Thus, the absorbance change, $\Delta\hat{A} = \hat{A}_\infty - \hat{A}_o$, that would be obtained if the reaction were to proceed to equilibrium is obtained without the need to wait until equilibrium is established. In addition, the $\Delta\hat{A}$ value obtained for each individual sample is predicted on the basis of the first-order rate constant that applies for the measurement conditions associated with each sample. There are advantages associated with each of these features, and both are illustrated below.

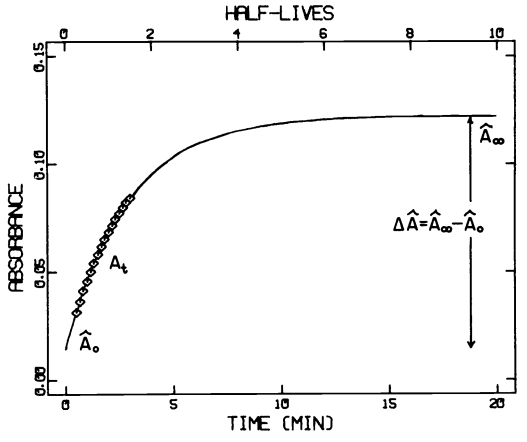

Fig. 1

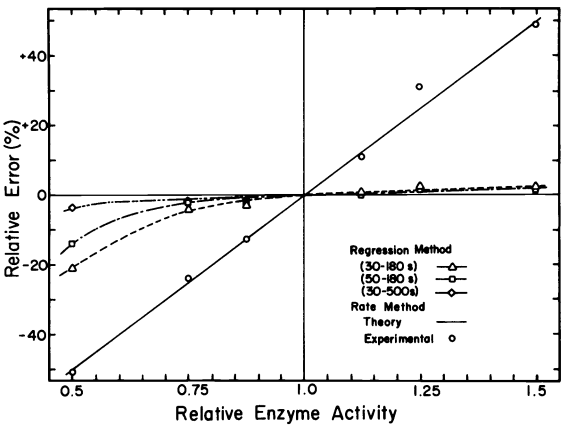

Fig. 2

Fig. 1. Conceptual representation of multiple-linear regression method applied to selected glucose data. ◇ - experimental points, —— fit to first-order model. Reprinted with permission from Clin. Chem., 25, 1581 (1979).

Fig. 2. Effects of relative enzyme activity on the relative error for initial rate and regression kinetic methods. Reprinted with permission from Clin. Chem., 25, 1598 (1979).

In experiments with the hexokinase/glucose-6-phosphate dehydrogenase coupled reaction system for quantitation of glucose (4), activities and concentrations of hexokinase, glucose-6-phosphate dehydrogenase, NAD^+, and ATP were all varied from 50% of a predetermined set of suitable concentrations to 150% of the reference values, and the effects of these variations on errors observed for a conventional initial-rate and the proposed regression method were evaluated. Results are shown in Fig. 2. The linear plot ranging from errors of -50% to +50% corresponds to the initial-rate approach; the flatter curves with most values less than 5% error correspond to the regression method (4). Clearly, the regression method is very insensitive to these rather drastic changes in enzyme activity and reactant concentration while the more traditional initial rate approach is very intolerant of the changes.

The reason for this insensitivity to experimental conditions is illustrated in Fig. 3 which shows experimental data and fitted curves for two sets of conditions. Although initial rates are very different, extrapolated values of final absorbance values are about the same.

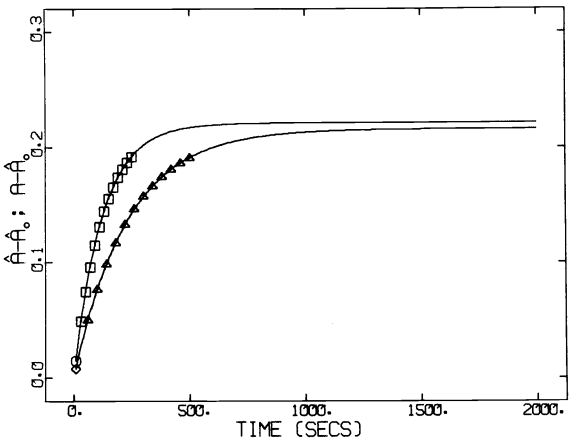

Fig. 3. Blank corrected absorbance vs. time data for glucose at two values of relative enzyme activity. Δ - experimental, relative activity of 0.75. □ - experimental, relative activity of 1.50. —— fit of data to first-order model. Reprinted with permission from Clin. Chem., 25, 1581 (1979).

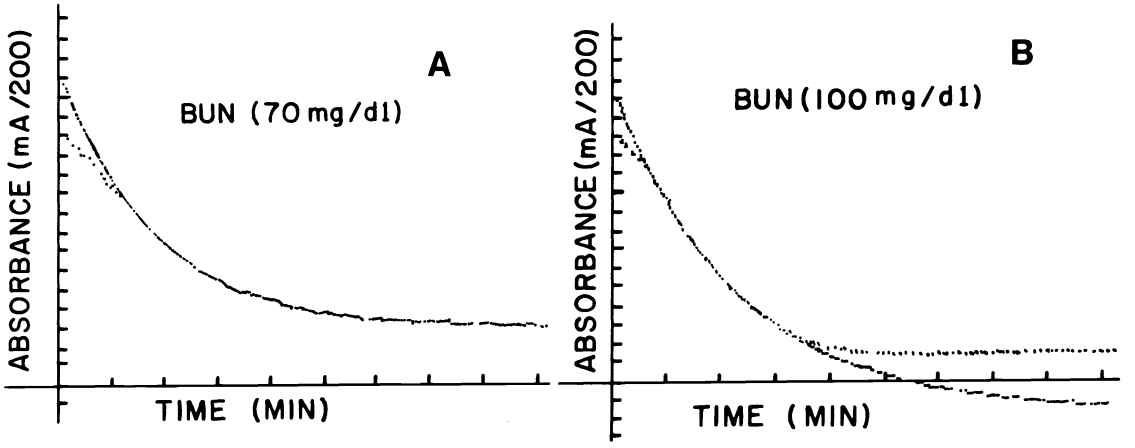

Fig. 4A. Response curve for the enzymatic quantitation of 70 mg/dl urea. ···· experimental data; —— fitted data. With permission (5).

Fig. 4B. Response curve for the enzymatic quantitation of 100 mg/dl of urea. ···· experimental data; —— fitted data. With permission (5).

An advantage of not having to wait for reactions to reach equilibrium is illustrated with data for the enzymatic quantitation of urea with urease. Figures 4A and 4B show data obtained (5) for a low and a high concentration of urea with a prototype of a commercial instrument (DACOS®, Coulter Electronics, Inc.). The dotted curve represents experimental data and solid curve represents results predicted with a program similiar to that described earlier (4). At the higher urea concentrations, there is inadequate NADH to react with all urea present, and the A vs. t plots cease changing before all urea has reacted. The lower curve in Fig. 5 shows the effect on the calibration plot; it bends toward zero slope at the higher concentrations. However, the regression fit of A vs. t data (Fig. 4) data during the early part of the reaction extrapolates the absorbance change to the full value that would be achieved if sufficient NADH were added to react with all urea present. The effect of the extrapolation procedure on the calibration curve is illustrated in Fig. 5; the linear range is extended to an absorbance change of almost four absorbance units. Because few if any photometers are capable of measuring absorbances accurately over such a wide range, it would not be practical to use sufficient NADH to react with this wide range of urea concentrations and accordingly, the regression method extends the useful range of the method. The only requirement is that the reaction obey pseudo-first-order kinetics.

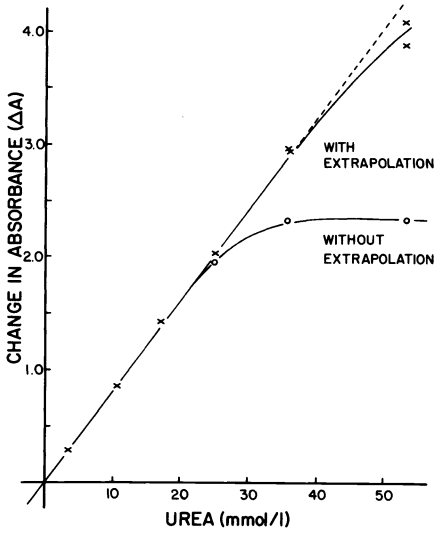

Fig. 5. Calibration curves for urea without and with extrapolotion procedure. With permission (5).

Two-component samples. For samples that include two (or more) components that undergo similar first-order reactions, nonlinear regression methods can be used to quantify the individual components provided the rate constants for the components differ by a factor of two or more. For example, it has been shown (6) that for appropriate conditions, unconjugated and conjugated bilirubin react with p-diazobenzenesulfonic acid at different rates; at pH 7.54, and other controlled conditions (7), rate constants for unconjugated and conjugated bilibrubins have values of k_u - 11.3 s^{-1} and k_c = 3.3 s^{-1}, respectively. Thus, a single response curve that is characteristic of these two simultaneous first-order processes can be resolved in terms of each individual process. Experiments with prepared mixtures of the two forms of bilirubin gave the following regression data for unconjugated, conjugated, and

$$y_u = 0.99\ x_T - 4.3\ \mu mol\ L^{-1} \text{ with } S_{yx} = 13\ \mu mol\ L^{-1}$$

$$y_c = 1.05\ x_T + 7.8\ \mu mol\ L^{-1} \text{ with } S_{yx} = 14\ \mu mol\ L^{-1}$$

$$y_T = 1.01\ x_T + 7.2\ \mu mol\ L^{-1} \text{ with } S_{yx} = 12\ \mu mol\ L^{-1}$$

total bilirubin where y and x are the concentrations (μmol/L) found and added, respectively, and S_{yx} is the standard error. All plots are linear with reasonalbe scatter (S_{yx}) about the least-squares line. When perfected, such a procedure could help avoid the need for two-step or separation procedures.

First-order/zero-order

It is judged that several situations such as progressively inhibited enzyme reactions, lag phases in coupled enzyme reactions, and antibody/antigen (immunoassay) reactions involving enzymes as the label may result in simultaneous first-order/zero-order processes. One example involving lactate dehydrogenase is used to illustrate how such systems can be resolved and some advantages that might result.

Lactate dehydrogenase subunits. It has been shown (8,9) that under controlled conditions, the inhibition by pyruvate of the activity of the heart (H) and muscle (M) subunits of LDH follows pseudo-first-order kinetics with different rate constants for the two subunits (eg. k_H = 0.7 s^{-1} and k_M = 1.4 s^{-1} (9)). Thus, it is expected that if one of the subunits were mixed rapidly with pyruvate and NADH, the response should be that of combined first-order (inhibition) and zero-order (catalytic reaction) processes, and a fit of the data to a model for parallel zero- and first-order processes should permit computation of the initial (uninhibited) rate. Furthermore, if a mixture of H and M subunits were mixed rapidly with pyruvate and NADH, the response should be characteristic of the mixture, and it should be possible to use an appropriate model to resolve the mixture quantitatively.

Figure 6A is an A vs. t response curve for the H_4 subunit mixed rapidly with pyruvate and NADH and Fig. 6B is a similar data set with the fit to a combined first-order/zero-order model superimposed. The horizontal (scatter) plot represents differences between observed and predicted A vs. t data scalled to standard deviation units (scale at right). There is

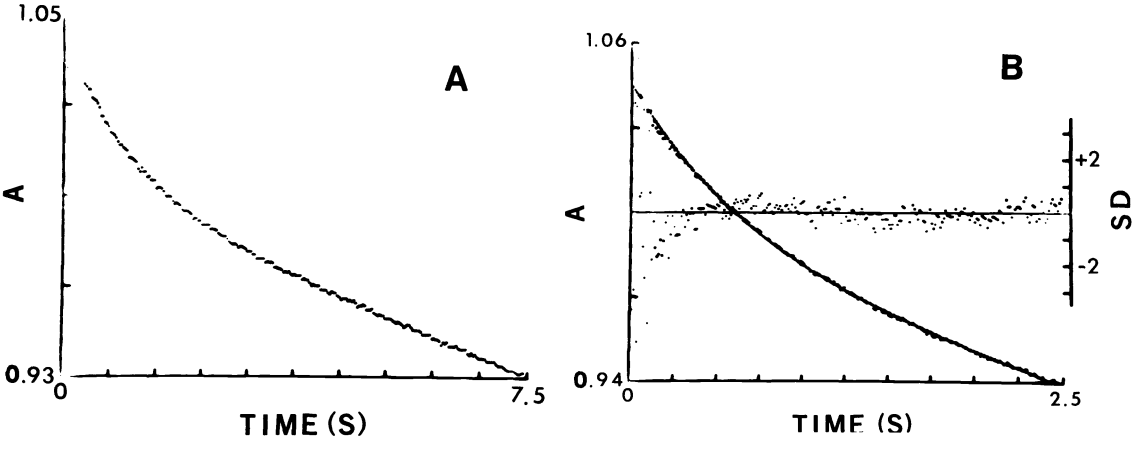

Fig. 6A. Response curve for H_4 lactate dehydrogenase subunit. Reprinted with permission from Anal. Chim. Acta, 127, 23 (1981).

Fig. 6B. Rsponse curve for H_4 subunit with fit to first-order/zero-order model superimposed. Horizontal plot represents residuals scaled to standard deviation units. Reprinted with permission from Anal. Chim. Acta, 127, 23 (1981).

excellent agreement between observed and fitted data; most of the residuals are scattered about zero and most are within the ± 2 sd. units (95% confidence level). Similarly good fits were obtained for the M_4 subunit and mixtures of the two subunits.

It is apparent from Figs. 6A and 6B that the initial velocity, V_i, is much higher than the steady-state velocity, V_{ss}, after the inhibition process has gone to completion. Figure 7 is a plot of initial and steady-state velocities vs. concentration for the M_4 subunit. The steady-state velocity is only about 40% of the true initial velocity, indicating that many so-called "optimized procedures" that quantify steady-state rates and do not take inhibition into account have very large errors associated with them. For the H_4 subunit, the steady-state velocity is only about 15% of the initial velocity ($V_{ss} \cong 0.15\ V_i$).

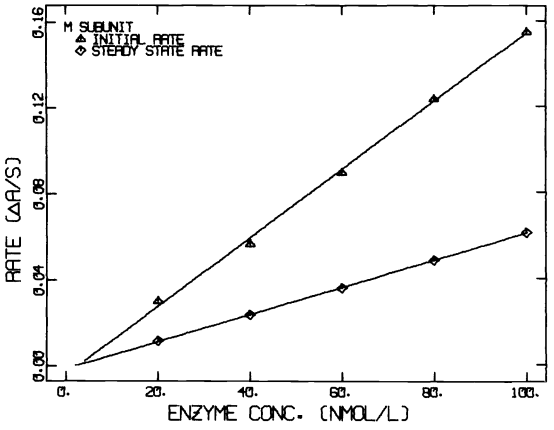

Fig. 7. Comparison of initial and steady-state velocities for H_4 lactate dehydrogenase subunit. △ - initial velocity; ◇ - steady-state velocity.

Several mixtures of the H and M subunits were evaluated (9) by the regression method. The least-squares data are

$$y_H = 0.97\ x_H + 0.21 \text{ nmol } L^{-1} \text{ with } S_{yx} = 0.74 \text{ nmol } L^{-1} \text{ and } r = 0.994$$

$$y_M = 0.98\ x_M + 0.01 \text{ nmol } L^{-1} \text{ with } S_{yx} = 0.46 \text{ nmol } L^{-1} \text{ and } r = 0.997$$

and

$$y_T = 1.01\ x_T - 0.66 \text{ nmol } L^{-1} \text{ with } S_{yx} = 0.44 \text{ nmol } L^{-1} \text{ and } r = 0.998$$

where y_H, y_M, and y_T represent amounts of heart, muschle and total (H + M) found in the mixtures and x_H, x_M, and x_T represent amounts added.

Other applications. It is highly probable that the lag phase in many coupled enzyme reactions follow combined first-order/zero-order kinetics, and that procedures such as this could be used to better study these lag phases and perhaps exploit information contained therein. Thus it could be possible to understand and utilize a feature of enzyme reactions that at the present time creates more problems than useful information.

Another more general and potentially more useful possibility involves homogeneous immunoassays. The traditional approach to immunoassays is to permit antibody-antigen reactions to proceed to equilibrium, and to base the final result on the equilibrium conditions. Because equilibrium concentration are frequently nonlinear functions of initial concentrations, calibration plots from such procedures are nonlinear and complex functions such as the logit-log transformation are used to "linearize" the data. However, because initial rates frequently vary linearly with initial concentration, procedures bases on kinetic aspects of antibody-antigen reactions rather than equilibrium conditions offer better opportunities for linear responses. The regression methods described above should be applicable to such systems with the first-order model being applicable to systems that are monitored directly by some suitable physical property (absorbance or fluorescence) and with the first-order/zero-order model being applicable to systems in which the antibody-antigen reaction is monitored by the activity of an enzyme label. Preliminary data in our laboratory for one system (10) confirm that the nonlinear regression method yields linear calibration plots whereas a more conventional two-point, fixed-time method yielded nonlinear calibration data (10).

Michaelis-Menton kinetics

A traditional problem associated with the use of kinetic data to quantitate substrates in enzyme catalzyed reactions is the need for the Michaelis constant to be much larger than substrate concentration ($K_M >> C_S^o$) so that the reaction will be pseudo-first-order in substrate concentration. Using nonlinear regression methods similar to those discussed above, it is possible to alleviate this problem to a substantial extent. The procedure is to fit rate ($\Delta A/\Delta t$) and absorbance using V_{max}, K_M, and C_S^o as fitting parameters. Preliminary results in our laboratory with uricase used to quantify uric acid via the change in absorbance at 280 nm give linear results between 6×10^{-6} and 8×10^{-5} mol/L. Considering a value of the Michaelis constant of about 3×10^{-5} mol/L for uricase, it is apparent that more traditional kinetic methods would yield a nonlinear relationship for concentrations in this range.

SUMMARY

In the traditional approach to the quantitation step in clinical chemistry, much effort is usually devoted to adjusting procedures to yield linear response curves. Although this was a desirable and perhaps even necessary aim when data were collected and processes primarily by manual methods, it is not so desirable or necessary with the current trend toward the use of computers to mechanize data-acquisition and data-processing steps in quantitative procedures. It is shown above that not only can nonlinear data be processed as readily as linear data, but that substantial advantages can be appreciated via the use of nonlinear data. As examples, the insensitivity to experimental parameters demonstrated by data in Fig. 2, the extended linear range illustrated by data in Fig. 5, the ability to resolve two-component mixtures such as unconjugated and conjugated bilirubins or the H and M subunits of lactate dehydragenase, the ability to quantify true initial rates for LDH (Fig. 7), and the ability to obtain linear calibration data for uric acid concentrations that approach and exceed the Michaelis constant for uricase all depend upon the exploitation of the nonlinear characteristics of response curves. We have prliminary data to suggest that the use of nonlinear data to obtain initial velocities of antibody-antigen reactions may permit one of the major problems associated with these procedures, namely the insensitivity to antigen concentration at high concentrations, to be alleviated. Thus, a feature of data sets that was viewed as a nemisis with manual data-processing methods can be used to substantial advantage with mechanized data-processing methods, and it is believed that these methods merit more attention from clinical chemists and clinical instrument manufacturers.

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