

Quantitative Analysis of the Time Courses of Enzyme-Catalyzed Reactions

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The catalytic properties of enzymes are usually evaluated by measuring and analyzing reaction rates. However, analyzing the complete time course can be advantageous because it contains additional information about the properties of the enzyme. Moreover, for systems that are not at steady state, the analysis of time courses is the preferred method. One of the major barriers to the wide application of time courses is that it may be computationally more difficult to extract information from these experiments. Here the basic approach to analyzing time courses is described, together with some examples of the essential computer code to implement these analyses. A general method that can be applied to both steady state and non-steady-state systems is recommended. © 2001 Academic Press

Enzymes are both measured and defined by their effect on the rates of reactions. Similarly, mathematical descriptions of the overall reaction, or individual steps in the catalytic cycle, are formulated in terms of rates. In contrast, experimental measurements of these reactions rarely, if ever, determine rates directly. Rather, substrate or product concentrations are determined at various times, and rates are calculated from the change in concentration with time.

This process of differentiating the data is necessarily inexact. The errors may be small when the assay method allows continuous monitoring of the concentration and when the change in concentration is approximately linear with time. However, if the assay method is discontinuous and the change in concentration is not linear with time, the rates that are determined may be of little reliability. This, in turn, limits the amount of

quantitative and mechanistic information that can be deduced about the enzyme.

There is an alternative to differentiating the data and that is to integrate the model that describes the reaction. This creates a mathematical description that is formulated in the same terms as the experimental measurements, that is, as concentration as a function of time. Integration can be performed with no, or at worst infinitesimal, error.

Apart from resolving the basic incompatibility between the enzyme model and the experimental data, measurements of time courses (or progress curves as they are often known) have other advantages.

1. A single reaction mixture can yield multiple experimental points, allowing more data to be collected with little additional experimentation. In contrast, when rates are measured it is usual to determine one rate only from each assay.

2. All data collected from a single assay are obtained at exactly the same concentration of enzyme, activators, inhibitors, buffer, and most other components that are not consumed or formed by the reaction itself.

3. The concentrations of components that are consumed or formed by the reaction itself are varied automatically. As a result, a full description of the effects of these components on the reaction is contained within the data. Moreover, products are formed in an enantiomerically pure form, allowing the effects of such products, or of products that are difficult to obtain, to be studied readily.

4. Enzymes for which the assay is relatively insensitive are easily studied. This is because it is possible, and usually desirable, to measure the reaction over a period during which a substantial fraction of the substrate is used. In contrast, rate measurements must be

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restricted to the first few percent of substrate utilization so that its concentration remains nearly constant.

In addition to these advantages, some properties of enzymes are necessarily not amenable to rate measurements because they change over time. Probably the most important of these are the interactions with slow-binding inhibitors, but any process that is not in the steady state is best studied through time courses.

Given these advantages, it is useful to inquire why such limited use has been made of time courses, with the vast majority of enzyme studies continuing to focus on rates, and usually initial rates. There are several disadvantages and, paradoxically, the first two of these are the result of the same features of time courses that were listed as advantages 2 and 3 above.

1. All data collected from a single assay contain systematic errors if there are any departures from the desired concentration of enzyme, activators, inhibitors, buffer, and any other component, including those that are consumed or formed by the reaction itself.

2. The concentrations of components that are consumed or formed by the reaction itself vary simultaneously. As a result, it is difficult to isolate the effect of any single component without also considering the effects of all components and the interactions between them.

3. Side reactions, instability, or volatility of substrates, products, activators, and inhibitors, as well as time-dependent changes in enzyme activity, all affect time courses.

4. Partly as a result of the previous two points, the equations that describe time courses are very much more complex than rate equations and require sophisticated methods and computer programs for their analysis.

Provided that these disadvantages are not overwhelming, then studying enzymes by means of time courses is a useful weapon in the enzymologist's armory. For a "one-off" study, it would not usually be worthwhile to expend the time and effort needed to exploit the potential of time courses. Rate measurements have stood the test of time and this author would not advocate discarding them. Nevertheless, for systems that are amenable to analysis by time courses, the extra effort can pay great dividends.

My intention here is to provide some advice and suggestions to the novice who might wish to explore the potential of time courses. It is not the intention here to provide a historical account of the analysis of enzyme time courses, which extends back more than a century. Neither shall I provide a comprehensive review of the

contributions of many enzymologists to the field over this period.

DESCRIPTION OF METHOD

Assay

The study of any enzyme requires a suitable assay method that can measure the changing concentration of a substrate or a product. The requirements of the assay in time course analysis are somewhat different, and in some ways far less stringent, than those used for rate measurements. Generally, rate measurements require that the assay is sufficiently sensitive that the concentrations of substrates and products can be assumed to be unchanged during the measurement period. In contrast, time course measurements generally rely on the fact that the concentrations of substrates and products change substantially and usually the reaction is followed until substrate exhaustion (or thermodynamic equilibrium) is approached. For this reason, very insensitive assays can be used. Indeed, conventional assays may be far too sensitive and special adaptations may be required. For example, if the assay involves spectrophotometric monitoring of a substrate or product, it may be necessary to move well away from the absorbance peak of the chromophore to enable data from the entire reaction time course to be collected.

Selwyn's Test

The steady-state rate equations for most enzyme-catalyzed reactions may be written as

$$v = \frac{k_{\text{cat}} [E]_0}{f([A_1], [A_2], \text{etc.}; [P_1], [P_2], \text{etc.}; [M_1], [M_2], \text{etc.})} \quad [1]$$

In this equations, $[E]_0$ is the total enzyme concentration, $[A_i]$ represents the concentrations of various substrates, $[P_i]$ represents the concentrations of various products, and $[M_i]$ represents the concentrations of various modulators (e.g., activators and inhibitors). If we define the rate (v) in terms of the formation of product P_1 with time, Eq. [1] may be rewritten as

$$\frac{d[P_1]_t}{dt} = \frac{k_{\text{cat}} [E]_0}{f([A_1]_0 - [P_1]_t, [A_2]_0 - [P_1]_t, \text{etc.}; [P_1]_0 + [P_1]_t, [P_2]_0 + [P_1]_t, \text{etc.}; [M_1], [M_2], \text{etc.})} \quad [2]$$

For any given amount of product P_1 formed, the denominator of Eq. [2] is a constant; therefore, integrating Eq. [2] yields the simple result

$$k_{\text{cat}} [E]_0 t = \text{constant.} \quad [3]$$

What this means is that several time courses measured under identical conditions except for the amount of enzyme added should be superimposable when the time axis is multiplied by the enzyme concentration. This important check is known as Selwyn's test (1).

Failure to pass Selwyn's test means that the shape of the time course curve is not entirely governed by the change in rate due to substrate consumption and product formation by the catalyzed reaction. Most commonly, instability of the enzyme would be responsible, although instability of substrates, products, or modulators might also be involved in some instances. Systems that are not in the steady state will also fail Selwyn's test.

There are two approaches to rectify such difficulties. The first is to alter the reaction conditions to overcome the instability. However, this might not always be possible or desirable. The second approach is to include allowance for the instability in the data analysis, as described in a later section. This approach can also be applied to non-steady-state systems, such as occurs with slow-binding inhibitors. However, for the time being, it is assumed that the system passes Selwyn's test.

Simple Systems

To understand the basic principle of analyzing time courses, the simplest system is described. This is an enzyme that obeys Michaelis–Menten kinetics, is not inhibited by its product(s), and catalyzes an irreversible reaction with a single substrate, A. For such a reaction, Eq. [2] reduces to

$$\frac{d[P]_t}{dt} = \frac{V_m}{1 + K_a/([A]_0 - [P]_t)}, \quad [4]$$

where the maximum velocity (V_m) has its usual definition ($k_{\text{cat}} [E]_0$) and K_a is the Michaelis constant for substrate A. The corresponding integrated form is

$$[P]_t - K_a \ln(1 - [P]_t/[A]_0) - V_m t = 0. \quad [5]$$

Various approaches have been suggested to fit this equation to experimental data. For example, rearrangement to the linear form

$$[P]_t/t = V_m + K_a \ln(1 - [P]_t/[A]_0)/t \quad [6]$$

allows the parameters V_m and K_a to be calculated from the intercept and slope, respectively, of a plot of $[P]_t/t$ versus $\ln(1 - [P]_t/[A]_0)/t$. This demonstrates that, at least in principle, it is possible to determine both V_m and K_a from a single time course. However, it should be recognized that a function of the experimentally measured variable ($[P]_t$) appears on both axes and simple linear regression (which assumes that experimental errors are associated with the ordinate only) cannot yield best-fit values for the parameters. A variation of the direct linear plot (2) overcomes this statistical objection but is not sufficiently general that it can be used in anything other than simple Michaelis–Menten systems.

To obtain best-fit values it is necessary to calculate $[P]_t$ for any given values of V_m , K_a , $[A]_0$, and t . There is no algebraic rearrangement of Eq. [5] that allows this to be done so various numerical approximation methods have been applied (see 3, 4). Probably the easiest of these to understand is the procedure known as bracketing. It is obvious that $[P]_t$ must be between a lower limit of zero and an upper limit of $[A]_0$ at any finite time. If a value of $[P]_t$ is chosen at halfway between these limits and this value is substituted into the left-hand side of Eq. [5], then the result will be less than, greater than, or equal to zero. If it is less than zero then this guess must be too low and the chosen value of $[P]_t$ can be used as a new lower limit. Similarly, if the calculation yields a left-hand side of Eq. [5] that is greater than zero, then the value of $[P]_t$ can be used as a new upper limit. Using these new limits, another guess is made of $[P]_t$ and the process is repeated. Eventually, an exact solution is found (the left-hand side of Eq. [5] is equal to zero) or the upper and lower limits are so close together that further refinement is unnecessary.

The bracketing algorithm can be incorporated into any of several published or commercial nonlinear regression programs, allowing best-fit values (and standard errors) of the parameters V_m and K_a to be calculated. The program code for use in conjunction with the GraFit program (Erithacus Software) is illustrated in Fig. 1.

It is worth mentioning that bracketing is a general procedure that can be used to solve a variety of equations. It is only necessary to write the equation in the same form as Eq. [5] (i.e., with zero on the right-hand side) and to provide reliable starting values that encompass the solution.

Experimental Design

For reliable determination of both V_m and K_a some thought must be given to the appropriate starting substrate concentration to use. If it is too high, the time course will approximate a straight line because the enzyme will be saturated and the curve dominated by V_m alone. Conversely, if the substrate concentration is too low, the time course will approximate a first-order curve with a shape that reflects V_m/K_a only. An appropriate choice has been suggested to be two to three times K_a (5).

In practice, using a single time course is not very reliable for determining V_m and K_a . Even small deviations between the intended and actual substrate concentrations can substantially alter the estimates (6). It is preferable to use several starting substrate concentrations and combine the data for analysis. This has a second advantage that is discussed in the next section.

Using several progress curves over a wide range of starting substrate concentrations results in time courses that may be inconvenient to follow over a single time span. A starting concentration that is well above K_a will reach any given fractional completion more slowly than one that starts well below K_a . However, because the system passes Selwyn's test, there is no requirement that all time courses are collected at the same enzyme concentration. For higher starting substrate concentrations, the amount of enzyme can be increased and the time axis of the data later normalized to a common enzyme concentration.

```
Pt_lo := 0
Pt_hi := Ao
:Bisect
if (Pt_hi - Pt_lo)/(Pt_hi + Pt_lo) < 1E-5 then
goto Done
endif
Pt := (Pt_hi + Pt_lo)/2
Diff := Pt - Ka * ln(1 - Pt/Ao) - Vm * t
If Diff > 0 then
Pt_hi := Pt
goto Bisect
endif
If Diff < 0 then
Pt_lo := Pt
goto Bisect
endif
:Done
return (Pt)
```

FIG. 1. Algorithm to solve the integrated Michaelis–Menten equation (Eq. [5]) by the bracketing method. This particular example is designed for use with the GraFit program (Erithacus Software) but a similar code could be used with other programs and computer languages.

Competitive Product Inhibition

The simple Michaelis–Menten system described above does not allow for inhibition by the product(s) of the reaction. If an accumulating product inhibits the enzyme competitively, this will not be immediately obvious from the shape of the time course and any given curve will be described exactly by Eq. [5], except that V_m and K_a will now be apparent values defined by Eqs. [7] and [8] in which K_i is the inhibition constant for the product:

$$V_m' = V_m K_i / (K_i - K_a), \quad [7]$$

$$K_a' = K_a (K_i + [A]_0 + [P]_0) / (K_i - K_a). \quad [8]$$

It is evident that no single time course can be used to determine all of V_m , K_a , and K_i but a series of reactions, at different starting values of $[A]_0$ and/or $[P]_0$, will yield sufficient information to define all three parameters. It is of interest that the apparent V_m and apparent K_a calculated from a single curve have negative values if the product is a strong inhibitor ($K_i < K_a$). The value of V_m' should be independent of the starting substrate concentration and should be unaffected by added product (Eq. [7]). In contrast, K_a' should be a linear function of $[A]_0 + [P]_0$ (Eq. [8]), and the observation of a trend between K_a' and $[A]_0$ suggests that the system is affected by product inhibition.

Reversible Reactions

Single-substrate/single-product reversible reactions also obey Eq. [5] but again the values of V_m and K_a are apparent values that depend on the maximum velocities and Michaelis constants in each direction, as well as the initial reactant concentrations (7). Determining all four kinetic parameters requires a series of reactions at different starting substrate concentrations, preferably with the reaction studied in both directions (8).

Complex Systems

Reactions with more than one substrate, with two or more inhibitory products, or with a single product that is a noncompetitive or uncompetitive inhibitor do not obey Eq. [5]. There may be additional terms involving $[P]_t^2$ and higher powers, more than one logarithmic term, or both. Boeker (9–11) has developed a general framework for the integrated rate equations for such systems, although there has been little work where this has been applied to experimental systems. In this author's opinion, a full kinetic analysis of such a system

is unlikely to yield reliable results due to the large number of kinetic parameters that must be extracted from the data. However, the complexity of the system may sometimes be manipulated experimentally to reduce the number of parameters. For example, aspartate aminotransferase catalyzes a reversible two-substrate/two-product ping-pong reaction, but by using glutamate and oxaloacetate as substrates and coupling the reaction to glutamate dehydrogenase, it can be greatly simplified. Using this coupling system, 2-ketoglutarate does not accumulate (and therefore cannot inhibit), the reaction becomes irreversible, and the concentration of glutamate remains fixed due to recycling (12). In this way, this eight-parameter system is simplified to a more manageable five parameters.

The second problem with these complex systems is the difficulty of writing the integrated rate equations in a form suitable for incorporation into a nonlinear regression analysis program. A computer program that writes these equations has been described (8) but has not been widely used. In the next section I describe an alternative that is substantially simpler and has the additional and significant advantage that it can be applied to both steady-state and non-steady-state systems.

Numerical Integration

The alternative to using algebraic methods to integrate rate equations is to integrate them numerically. In its simplest form, this involves using Eq. [4] to calculate the rate at any given starting point, then estimating the new concentration of P after a small increment in time (Δt)

$$[P]_{t+\Delta t} = [P]_t + \Delta t (d[P]_t/dt). \quad [9]$$

This “first-order” method is not sufficiently accurate to be generally useful but more sophisticated procedures exist that are highly reliable. Descriptions of these methods may be found in standard texts (e.g., 13).

In the past, the main difficulty with using numerical integration for analyzing time courses was that it requires a substantial amount of computation, making the method rather slow. The enormous advances that have occurred in desktop computers over the past 20 years have largely overcome this problem.

There are a number of published and commercial computer programs available that combine numerical integration with nonlinear regression. It is not the intention to survey these programs here or to argue that any one them is superior to all others. However, the DNRP-RKF program, written in BASIC and developed

in this laboratory, is available over the World Wide Web (<http://www.biosci.uq.edu.au/~duggleby/rgd3a.htm>) and has been shown to be quite versatile (14). The main drawback is that it has no graphics capability. An inexpensive Windows-based program with good graphics is Berkeley Madonna, available from the web site <http://www.berkeleymadonna.com/>. Unfortunately, in its present form, it has a very awkward way of dealing with multiple time courses, and does not provide standard errors of the estimated parameters. One hopes that these shortcomings of Berkeley Madonna will be overcome in future versions. Alternatively, this author invites any collaborator who is interested in constructing a graphical interface for DNRP-RKF.

DNRP-RKF

The use of DNRP-RKF is illustrated by example, using first a simple Michaelis–Menten system. The data (Fig. 2A) are from Schönheyder (15) and represent the hydrolysis of phenyl phosphate by human prostate acid phosphatase. This reaction is adequately described by Eq. [4] and the representation of this equation for use with DNRP-RKF is shown in Fig. 2B. The best fit to the data (line in Fig. 2A) obtained using DNRP-RKF

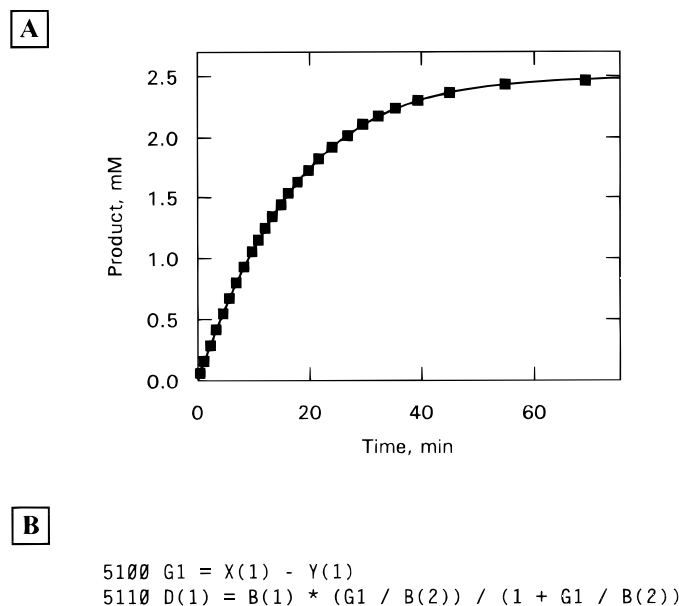


FIG. 2. Analysis of the hydrolysis of phenyl phosphate catalyzed by human prostate acid phosphatase. (A) The data are calculated from Table 1 of Schönheyder (15) and the line represents the best fit to the data of Eq. [4], with $V_m = 0.4569$ mM/min and $K_a = 6.155$ mM. (B) The computer code for analyzing this system using DNRP-RKF. X(1) represents the initial substrate concentration while the fitted parameters (V_m and K_a) are represented by B(1) and B(2), respectively. Y(1) and D(1) correspond to $[P]_t$ and $d[P]_t/dt$, respectively, while G1 is an intermediate variable that is equivalent to $[A]_t$.

yielded values of $V_m = 0.4569 \pm 0.0140$ mM/min and $K_a = 6.155 \pm 0.236$ mM. These values are in quite good agreement with those of Schönheyder (0.453 mμ/min and 6.11 mM, respectively), which were obtained using a direct linear plot method that was later rediscovered by Cornish-Bowden (2).

The second example is the hydrolysis of *p*-nitrophenyl phosphate catalyzed by bovine intestinal alkaline phosphatase (16). The reaction is irreversible and converts a single substrate (ignoring the water) to two products. Of these, only phosphate causes any significant inhibition so the enzyme behaves as a single-substrate/single-product system. However, as revealed by Selwyn's test, the enzyme is unstable under the buffer conditions used (which included EGTA, which extracts the essential zinc ion from the enzyme). Due to this instability, the integrated rate equation defined by Eqs. [5], [7], and [8] is not appropriate.

A general model for this system is illustrated in Fig. 3A. However, it was shown (16) that it is only the free enzyme that is susceptible to inactivation, and, in the enzyme-substrate and enzyme-phosphate complexes, the zinc is locked in. This simplifies the model by eliminating the inactivation routes governed by j_2 and j_3 . The rate equations for product formation and enzyme inactivation are

$$\frac{d[P]_t}{dt} = \frac{k_{\text{cat}}[E]_t([A]_0 - [P])_t/K_a}{1 + ([A]_0 - [P])_t/K_a + [P]_t/K_p} \quad [10]$$

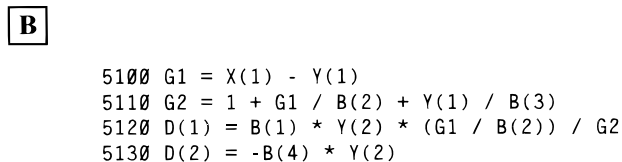
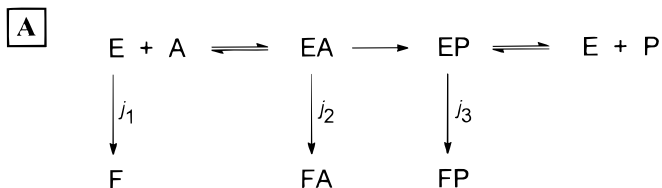


FIG. 3. Analysis of the time course for an unstable enzyme. (A) The model is for an enzyme that catalyzes a single-substrate/single-product irreversible reaction that is competitively inhibited by the product, and where each enzyme form is unstable. (B) The computer code for analyzing this system using DNRP-RKF when only the free enzyme is unstable (Eqs. [10] and [11]). Definitions of the various symbols are as in Fig. 2, except that: B(1) is k_{cat} ; there are two additional fitted parameters (K_p and j_i) represented by B(3) and B(4), respectively; Y(2) and D(2) correspond to $[E]_t$ and $d[E]_t/dt$, respectively; and there is an additional intermediate variable (G2) that is equivalent to the denominator Eqs. [10] and [11].

and

$$\frac{d[E]_t}{dt} = \frac{-j_1[E]_t}{1 + ([A]_0 - [P])_t/K_a + [P]_t/K_p}, \quad [11]$$

respectively, and the appropriate computer code for analyzing this system using DNRP-RKF is shown in Fig. 3B.

The third example illustrates the analysis of the effect of a slow-, tight-binding inhibitor. The model (Fig. 4A) is taken from Williams *et al.* (17) for the inhibition of dihydrofolate reductase by methotrexate. There is rapid formation of the complex EI, and this slowly isomerizes to a second complex EI*. Some representative data are illustrated in Fig. 4B. The concentration of the limiting substrate (dihydrofolate) was 76 μM and can be considered to remain constant during the course of the experiment. The enzyme concentration that was used in this experiment was 6 nM, which is comparable to that of the inhibitor, methotrexate. Consequently, reduction of the inhibitor concentration as a result of binding to the enzyme must be included in the analysis. Given a value for the concentration of EI* at any time, the concentration of EI is calculated by solving the quadratic equation

$$K'_i = K_i(1 + [A]/K_a), \quad [12]$$

$$[I']_t = [I]_0 - [EI^*]_t, \quad [13]$$

$$[E']_t = [E]_0 - [EI^*]_t, \quad [14]$$

$$[EI]_t^2 - [EI]_t([E']_t + [I']_t + K'_i) + [E']_t[I']_t = 0. \quad [15]$$

Using this value, the concentration of EA can be calculated,

$$[EA]_t = ([E']_t - [EI])_t / (1 + K_a/[A]), \quad [16]$$

and all quantities can now be supplied to the coupled differential equations

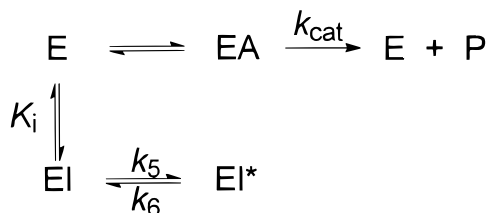
$$d[EI^*]_t/dt = k_5[EI]_t - k_6[EI^*]_t, \quad [17]$$

$$d[P]_t/dt = k_{\text{cat}}[EA]_t, \quad [18]$$

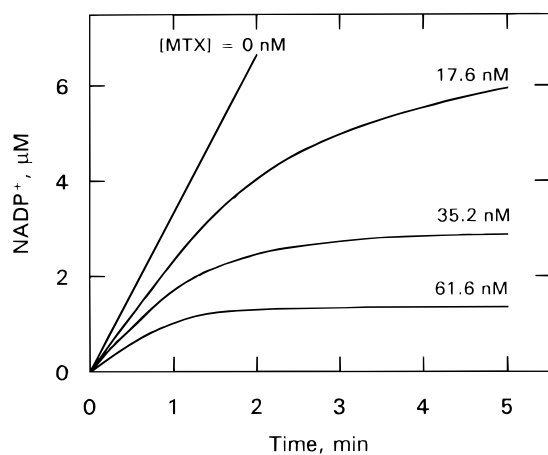
The computer code for using DNRP-RKF to analyze such data is shown in Fig. 4C.

These examples illustrate how DNRP-RKF can be

A



B



C

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5100 Eo = X(1)
5101 G1 = X(2)
5102 Io = X(3)
5110 Kiapp = B(2) * (1 + G1)
5111 Iapp = Io - Y(2)
5112 Eapp = Eo - Y(2)
5120 G2 = -(Eapp + Iapp + Kiapp)
5121 G3 = Eapp * Iapp
5122 EI = (-G2 - SQR(G2 * G2 - 4 * G3)) / 2
5125 EA = (Eapp - EI) / (1 + 1 / G1)
5130 D(1) = B(1) * EA
5131 D(2) = B(3) * EI - B(4) * Y(2)

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FIG. 4. Analysis of the time course for a slow-, tight-binding inhibitor. (A) The model is for the inhibition of dihydrofolate reductase by methotrexate (MTX) and is taken from Williams *et al.* (17). (B) Representative data for this system, redrawn from Fig. 1 of Williams *et al.* (17). (C) The computer code representing Eqs. [12]–[18] for analyzing this system using DNRP-RKF. Lines 5100–5102 definesome constants, where X(2) represents the known ratio between the substrate concentration (dihydrofolate = 76 μM) and its Michaelis constant (15 μM). Lines 5110–5112 correspond to Eqs. [12]–[14], while lines 5120–5122 solve the quadratic Eq. [15]. Line 5125 represents Eq. [16] and the differential equations (Eqs. [17] and [18]) are defined by lines 5130 and 5131.

used for a steady-state system that passes Selwyn's test, a system that fails Selwyn's test due to enzyme instability, and a non-steady-state system resulting from slow-binding inhibition. It has also been applied (14) to the analysis of a pre-steady-state system and simplified metabolic pathway consisting of a pair of a coupled reactions.

CONCLUDING REMARKS

The time course of an enzyme-catalyzed reaction contains a wealth of information but this was, in the past, difficult to extract due to the complexity of the calculations involved. The increased speed and capacity of desktop computers have now removed this limitation and should allow the better use of reaction time courses. The approach recommended here is to use the familiar descriptions of enzyme-catalyzed reactions that are couched in terms of rates. Numerical integration coupled with nonlinear regression is then used to fit the model to the time courses and thereby estimate kinetic parameters. One of the advantages of this approach is that it can be applied to both steady-state and non-steady-state systems.

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