Computer Technology for the Realistic Calculation of Properties of Enzyme Systems

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Traditional pencil-and-paper analyses of enzyme kinetic experiments assume so much simplification that the results have limited biological significance. Although performing initial velocity experiments with negligibly low enzyme concentrations in the presence of single inhibitors facilitates interpretation, there are a number of enzymes for which this method fails completely. Software has been developed for the economical simulation of enzyme behavior under realistic conditions. Enzyme activity is computed as a function of either time or concentration by solution of either differential or algebraic equations with any desired ratio of enzyme/substrate concentrations. Simulation of experiments with hexokinase from mouse ascites cells has permitted resolution of some apparently contradictory results and indicated guidelines for assuring reliability of data. Applications of lab. analyzers to improve interpretation of experiments and perhaps actually perform them are discussed.

KEY WORDS AND PHRASES: enzyme kinetics, chemical reaction kinetics, data fitting, rate laws, statistical weighting programs, matrix inversion, differential equations, simulation, mathematical models, enzymes (hexokinase, triosephosphateisomerase, glyceraldehyde phosphate dehydrogenase).
CR CATEGORIES: 3.13,3.12,3.22,4.12,4.22, 5.16,5.17,5.5

INTRODUCTION

Enzymes have been studied kinetically for a long time. The first theories of enzyme reactions were developed during the early part of the twentieth century, well before the appearance of electronic computers. Except for one extremely simple case (1), the inhomogeneous non-linear differential equations of enzyme kinetics cannot be solved analytically. As a result, various simplifying assumptions are made in order to interpret experimental results. Linear transformations are applied to the normally hyperbolic concentration vs. velocity curves, and constants characteristic of the enzyme under investigation are derived. Although these methods are easy to apply, the results may have limited validity. The commonly used graphical method based on taking reciprocals is inherently inaccurate. Under physiological conditions, the results obtained with the idealized conditions may be invalid.

This paper is concerned with the application of computers to the analysis and simulation of enzyme kinetics. It includes both a review of the current state-of-the-art, descriptions of the current contributions of the authors to this art, and some possibilities for the near future.

The kinetics of enzyme reactions are usually studied by means of initial velocities under classical steady state conditions, where the following conditions are

assumed to apply:

Enzyme concentrations are much smaller than substrate concentrations

There is no product present initially; and what is measured is its rate of formation

The effects of inhibitors and other modifiers are considered only one at a time

The concentration of any enzyme-containing forms is a constant or changes only slowly with time (steady-state assumption).

This last assumption changes the problem of simulating enzyme systems from the solution of nonlinear differential equations to the much simpler solution of linear homogeneous equations. These may be solved by standard methods such as matrix inversion or the graphical method due to King and Altman (2). Cleland (3) has developed a system for classifying enzymes involved with more than one substrate, and has derived a large number of rate laws for such situations. Since these manual derivations become unwieldly , a number of workers have developed computer programs for deriving rate laws. Unfortunately the mechanism of any enzyme reaction involving more than one substrate is not known with certainty.

In practice, a series of initial velocities is observed, and the results graphed as double reciprocal (Lineweaver-Burk) plots. The maximum velocity and Michaelis constant, which are characteristic of the enzyme studied are then evaluated from the horizontal and vertical intercepts of these plots. Here we find a simple, widely used method which is inherently inaccurate since it gives the greatest weight to points having the smallest magnitude (4).

The effect of inhibitors or activators is studied by similar means. One of the clearest descriptions of this method is due to Cleland (3,5). It is illustrated in Fig. 1. Various enzyme mechanisms are distinguished on the basis of how these modifiers affect the double reciprocal plots. Although characteristic changes can be ascribed to specific reactions for simple mechanisms, these need not apply when the reactions are more complex. There are a number of systems for classifying these inhibitions, with no general agreement regarding definition of terms. The use of the term "allosteric" is particularly confusing. As a result controversies arise from poor communication as well as genuine disagreement.

Thus far attempts to improve the accuracy of double reciprocal plots have involved the application of statistical weighting factors (6,7,8). However, such methods appear to be more applicable to cases where perhaps 100 experiments rather than the customary 3 to 6 are performed.

Although these methods probably yield useful information in a majority of cases, there are a number of important exceptions. Those which are at least partly due to the presence of high enzyme concentrations will be considered in detail. The glycolytic enzyme glyceraldehyde phosphate dehydrogenase illustrates almost all the ways in which an enzyme can deviate from this simplified scheme. It catalyzes the reaction:

3-Glyceraldehyde Phosphate + NAD + inorganic phosphate ⇄ 1,3-diphosphoglyceric acid + NADH

All five of its substrates and products are either activators or inhibitors. Under physiological conditions, three of these are present in concentrations 2 orders of magnitude smaller than the enzyme concentration. The enzyme is present within the cell in high concentration; in yeast cells it constitutes a significant part of all the cellular protein. The enzyme itself associates and dissociates into various oligomers, and shows other complex allosteric properties.

Triose phosphate isomerase, which catalyzes the interconversion of the triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is another interesting example of anomalous behavior (9, 10). In a variety of tissues, it is almost always out of equilibrium, sometimes even moving material in the wrong direction, although activity profiles of glycolytic enzymes usually show it to be the most active enzyme (11). To some extent this may be due to differences between assay and physiological conditions. The enzyme is assayed at much higher substrate and product concentrations than actually exist in the cell.

One of the classical assumptions clearly does not apply when an enzyme is present in concentrations equal to or greater than its reactants. The existing theory to cover this situation has been developed for simple cases where only the reaction of the enzyme with a substrate or modifier was considered. One of the first theoretical studies was by Strauss and Goldstein (12) who were concerned with the enzymatic effects of drugs *in vivo* as well as *in vitro*. They derived an equation for the rate of a one-substrate enzyme reaction in terms of total substrate concentration as well as the more usual parameters. They then considered in detail the case where the substrate was an inhibitor (intermediate complex did not dissociate to form products) and derived properties for the inhibitor when it was mostly in the free form (classical case), mostly combined, or present in both forms. They found that in the presence

of high enzyme concentrations the effects of all inhibitors were similar, and that no constants characteristic for a specific inhibitor could be derived. Although they did verify their theory with one enzyme system, the derivation was so simplified that reaction between normal substrate and enzyme was neglected.

Cha (13) has studied the effect of high enzyme concentration on the conventionally derived kinetic constants, and finds intolerably high errors (900%) when the classical (Michaelis-Menten) scheme is used for high enzyme concentrations. He compares the equation of Strauss and Goldstein and a number of other approximations, and gives conditions where each approximation is likely to be most accurate. The extension of these methods to more complex reactions is discussed in a later section.

Although the classical methods of studying enzymes have produced valuable insights into how enzymes operate, it is desirable to extend this methodology. Computers can be used in a variety of ways to make enzyme experiments more meaningful, especially if computations are sufficiently rapid to enable experimenters to consider computed results when planning subsequent experiments.

APPLICATIONS OF COMPUTERS TO PROBLEMS IN ENZYME KINETICS

The computer methodology which now exists falls into two classes: data fitting and simulation. While statistical methods can be used to determine some features of enzyme mechanisms, simulation is needed to test for overall consistency, especially when a variety of experiments have been performed with a complicated enzyme.

Cleland (14,15) has developed a number of programs which apply weighting factors to points of a double reciprocal plot according to the method of Wilkinson (6) and Johanson and Lumry (7). Hanson, Ling, and Havir (16) have programmed the second-order method due to Bliss and James (8) for computer use. It is probably preferable to match experimental data directly. Arihood and Trowbridge (17) report differences of 50% in model parameters when using different linearizations of the same equation to fit a single set of data. Grzybowski has written a number of programs for performing computations needed during enzyme kinetics studies (personal communication).

We have been concerned with simulating the kinetic behavior of biochemical systems and developed a generalized problem-oriented language for doing this by solving differential equations (9,10). Various pathways as well as individual enzymes have been studied. The generalized simulation program has been used to develop models of phosphofructokinase (18) and pyruvate kinase (19). However, the "stiffness" of the resulting equations makes this procedure very inefficient. We are currently attempting to adapt a more rapid method of solving differential equations, originally due to Gear (20a).

We have also developed a variety of programs using rate laws or matrix-inversion techniques for studying individual enzymes. For fairly simple enzymes, the methods of Cleland (3) permit development of models by pencil-and-paper methods (20). Application of computer optimization permits the development of more accurate models with any desired kinetic constants for given reaction mechanisms (21).

Green and Garfinkel (22) have written an interactive-graphics program that uses a run-time generator and matrix inversion to simulate steady state experiments. Input is in the form of chemical reactions, rate constants, and concentrations. Output is primarily via scope display as double reciprocal plots, with experimental points also displayed to facilitate matching.

The enzyme phosphoglyceromutase was studied with this program (23) and found to have allosteric properties not evident to the experimentalists. This program could be used to help design experiments since results are obtained very rapidly.

The Chemist program of the Rand Corporation (24) which also utilizes matrix inversion should be applicable here. Its goal-seeking routines might facilitate matching of data. However, this program requires as input free energies of all components; these are presently not known for enzyme intermediates. It may be possible to estimate these from the kinetic parameters, but more investigation will be needed.

Computer programs for deriving rate laws have been prepared, since for random-order mechanisms, even the simplified method of King and Altman (2) becomes unwieldly. Such programs have been prepared by Rhoads and Pring (25), Hurst, (26), Silverstri and Zahner (27), and Fisher and Schulz (28). The program of Rhoads and Pring has been incorporated into our generalized simulation language where the use of rate laws of enzymes in a steady state speeds up computations. However, none of these programs are applicable to the physiologically important situation where large amounts of enzyme are present; here ordinary methods for solving differential equations may become inefficient as low substrate concentrations give rise to "stiffness".

Rhoads has written programs based

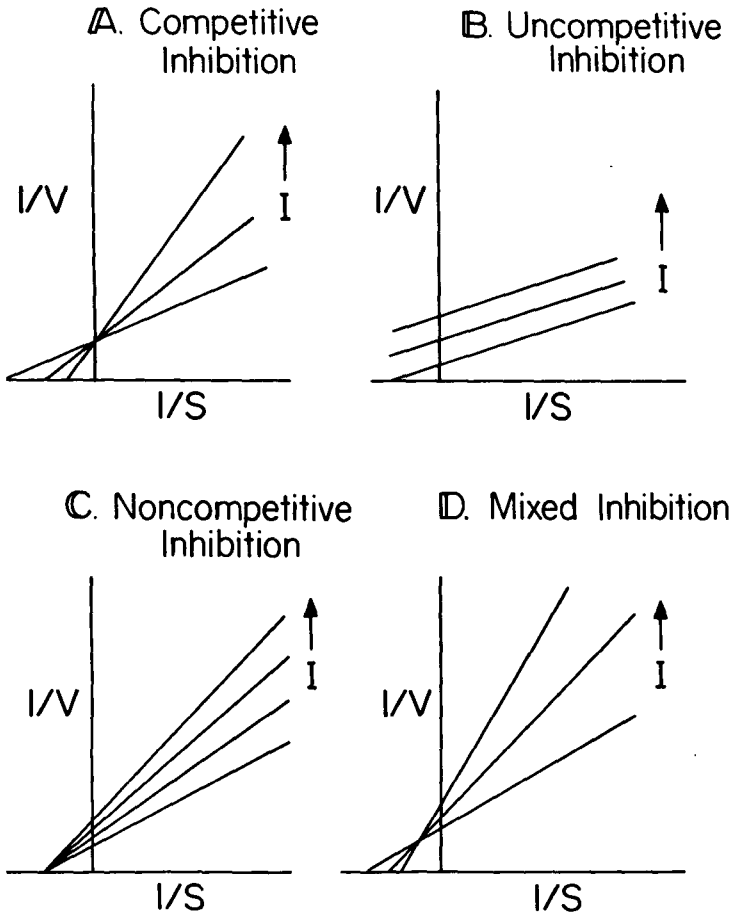

FIGURE 1— Effect of Inhibitor on Activity of an Enzyme.

Inhibitors can change either the slope or the intercept. Arguments regarding the nature of an enzyme mechanism are often based on how modifiers affect these lines.

TABLE I

The mechanisms studied are in the nomenclature of Cleland (3):

1. Uni-uni, $E + S \rightleftarrows ES \rightleftarrows E + P$

2. Ping pong bi bi
$E + AX \rightleftarrows EA \rightleftarrows EX + A$
$EX + P \rightleftarrows EPX \rightleftarrows PX + E$

3. Ordered bi bi
$E + A \rightleftarrows EA$
$EA + B \rightleftarrows EAB \rightleftarrows EPQ \rightleftarrows Q + EP$
$EP \rightleftarrows E + P$

4. Random bi bi, similar to ordered bi bi, except that either A or B may add first, and either P or Q may leave first.

primarily on rate laws for simulating enzymes systems (ROMP) which will simulate enzymes present in high concentrations. One program follows the time course of a system while the other computes the initial velocity for a specific reactant with concentrations of two reactants being used as variables. In the case of time-course program, the initial concentrations are the independent variable. Rates are computed, an appropriate time interval is selected, and new concentrations are computed. Then this process is repeated for each subsequent iteration. For the concentration program, new concentrations must be specified for every iteration.

The user can simulate chemical or enzymatic systems using several methods: two types of rate laws, differential equations, or specially programmed subroutines. The choice of rate law depends on whether or not binding of enzymes and reactants is significant. At high enzyme concentrations, free reactant concentration is corrected for the amount of bound reactants; this is equated with the concentration of the enzyme form to which the reactant moiety is bound (e.g., in the mechanism $E + S \rightleftarrows ES \rightleftarrows E + P$ the concentration of bound S is the concentration of (ES)). It is difficult in some cases to make a clear unambiguous assignment of enzyme forms to various reactants. Output for the concentration program consists of scope, plotter or line printer graphs or the actual data expressed in any of 8 modes, such as v vs S plots, I/v vs I/S plots, log (v/V-v) vs. log S (Hill plots), v/S vs S, I/v vs I, and a user-supplied relationship.

The program allows the user to change almost any parameter short of changing the chemical reactions themselves, either between runs or during a run. The program, which is written mostly in FORTRAN IV, assumes 36 bit words, 7 bit ASCII, and several specific subroutines written for the PDP-6.

SPECIFIC APPLICATIONS

Ascites Hexokinase

A detailed simulation of hexokinase from mouse ascites tumor cells has been carried out with the MATRX program of Green and Garfinkel (22). This enzyme catalyzes the reaction.

$$\text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP}$$

There is at least some evidence that its rate law is too complex for hand calculations. Hexokinase is the first, and one of the most important, enzymatic steps in the glycolytic pathway, where glucose is degraded to lactate and two molecules of the high-energy compound ATP are formed. There is a considerable body of data regarding the behavior of glycolysis in ascites cells (29,30). Hexokinase can bind to mitochondria, with the bound form being more reactive and less sensitive to inhibition by glucose-6-phosphate. The existence of the enzyme in the bound or particulate form may explain why the enzyme is sometimes active in the presence of normally inhibitory concentrations of glucose-6-phosphate.

From a biochemical point of view, the study of hexokinase presents several problems. In mammalian tissues it is irreversible so that neither the reverse reaction nor radioactive exchange at equilibrium can be observed. It is present in different forms (isozymes) which are separable by physical means, have different kinetic properties, and are probably distributed differently in different organs. Experiments where a mixture of isozymes are used may give rise to anomalous results. Hexokinase preparations undergo aging and are known to change properties during storage.

The importance of hexokinase has made it the subject of many studies. There is therefore a large mass of data from a variety of sources for mammalian hexokinase, which must be explained. Unfortunately much of it appears to be inconsistent, even when taken from a single publication. In part this is due to the instability of the enzyme. Experimental conditions which need to be carefully controlled are pH, ionic strength, and magnesium ion concentration. Experimenters have reported that their initial velocity data indicate a number of different reaction mechanisms.

The data chosen for simulation were reported by Kossow and Rose (31,32). A random mechanism with a rate limiting step is indicated. It has thus far been possible to simulate all of the experimental results, but not always with the same model. Similar discrepancies have been reported for other enzymes (33). Fitting data for the stronger inhibitors where small concentrations yield high fluxes tends to magnify the apparent discrepancies. Since glucose stabilizes the enzyme, it has generally been easier to simulate experiments where glucose was present in excess (ATP was the varied substrate). Loss of enzyme activity is probably involved here since unique models can be used for the soluble enzyme if its activity is increased by about 15%. Further support for the supposed loss of soluble enzyme activity is lent by the applicability of these same inhibition reactions to the mitochondrially bound enzymes. In fact there is greater incon-

sistency in the unihibited reactions of the bound enzyme than in the inhibition reactions for both the bound and the soluble forms. The latest models indicate that inhibition by ANG6P depends on both binding to mitochondria and the ATP concentration. This may help to explain why hexokinase in glycolyzing ascites cells is active in the presence of normally inhibitory glucose-6-phosphate concentrations.

It has been generally possible to match the experimental data quite closely, but the qualitative match of inhibition types has not been satisfactory. The data for ADP require a different rate limiting step in the uninhibited mechanism than those for AMP and ANG6P. Perhaps some of the inhibitors actually alter the rate-limiting step, since changes of less than an order of magnitude can produce the desired inhibition patterns. Thus far computer difficulties have prevented the testing of this hypotheses.

If one considers all of the data that have been published on hexokinase, it would appear to have a random mechanism. The ordered mechanisms proposed can usually be shown to be due to the fact that one of the substrates was present in excess; however these excesses may correspond to physiological conditions for certain organs.

Computations with ROMP

The ROMP programs have been used to determine the magnitude of the errors associated with the classical assumptions used in the derivation of rate laws. The four mechanisms which were studied are given in Table I. Parameter values representative of those encountered during experimental conditions were used.

Reaction rates were determined by three methods. In method 1, initial velocities were computed using rate laws which did not correct for the reactant concentrations that were bound to the enzyme. Method 2 is similar to method 1, except that corrections were made for such binding. In method 3, the maximum rates of production of each product were determined by solving differential equations for the early time course of the reaction. The purpose of method 3 is to simulate the enzyme system as it actually might be encountered experimentally. In fact, one of the major purposes of this work is to determine the sort of errors that might be expected if the experimentalist analyzed his data using enzymatic rate laws.

All three methods used gave the same results only when substrate concentrations were much higher than enzyme concentrations.

When the enzyme concentration was relatively high, rate law method 1 gave consistently high values, sometimes over 1000 times greater than those obtained by rate law method 2, the errors being largest when the highest fractions of substrate were bound to the enzyme. The rates computed by these two methods approached each other only at saturating substrate concentrations. Where directly comparable, these results agree with those of Cha (13).

Rate law method 2, which corrects for bound reactant, yielded velocities which were, in all cases studied, within a factor of 2.6 of these obtained by method 3 for the irreversible system. In most cases, the difference was less than a factor of 1.3. In almost all cases, the rate from method 2 was greater than that from method 3. This difference is probably a function of how far the system is from a steady state condition.

Very different rates were obtained when the differential equations (Method 3) were made to represent a reversible enzyme system. In many cases, especially at relatively high enzyme concentrations, the rate of the reversible system was much less than that of the irreversible system. These differences are probably related to the relative rate at which products accumulate and hence inhibit or reverse the forward reaction. These studies quantitate the errors that occur in rate law computations when the assumptions used in their derivation are violated. In irreversible systems, the errors due to failure to correct for bound reactants are much the larger.

POSSIBLE FUTURE DEVELOPMENTS

In addition to continuing the projects discussed previously, further work in this area should include investigation into the best methods for fitting data and achieving better quality control. Standards for what constitutes sufficient data need to be established and made available to biochemists. Analysis of data by means of statistics and simulation should be useful in showing in what concentration ranges further investigation is needed.

More attention should be given to quality control in experiments. Enzyme activities are not always assayed frequently enough to yield internally consistent data when unstable enzymes are studied. The simulation of recently performed experiments should encourage attention to such details of experimental proceedures that tend to be neglected without computer assistance.

The use of the double-reciprocal plots to analyze initial velocity data is such a popular procedure that it may be necessary to accept some of its weaknesses. Most recent reviews of enzyme mechanism determ-

inations are based on analyzing the properties of these plots. Their accuracy could be improved by developing better procedures for analyzing errors than the univariant analyses now used in the available programs. The usual assumptions that concentrations are free from error, and all the error is due to velocity measurements, is an obvious oversimplification.

It may be desirable to prepare diagnostic programs based on the logic by which enzyme mechanisms could be determined. Such programs could indicate which are the critical experiments for distinguishing among what appear to be equally probable mechanisms.

Perhaps such programs should be operated as a service to experimenters. Many enzymologists are not skillful computer programmers and in particular will not use programs which do not run on their computers, regardless of the reason. These developments are being undertaken because, in its present form, the available methodology is difficult to use, and therefore is not always applied.

A more radical application would be the use of computers to actually perform the experiments, by having a mini-computer (perhaps with communication to a larger computer) controlling one of the generalized clinical chemistry testing devices which are now becoming commercially available. These devices can perform initial velocity determinations and are fast enough to eliminate problems due to loss of enzyme activity. Human errors should also be eliminated by use of these devices.

The converse interaction is also desirable: to apply the available knowledge regarding enzyme kinetics to the more accurate determination of enzyme activities, which is often required for clinical purposes.

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GLOSSARY

ADP	-adenosinediphosphate (a coenzyme)
AMP	-adenosinemonophosphate (a coenzyme)
ANG6P	-anhydroglucitol-6-phosphate
ATP	-adenosinetriphosphate (a "high-energy" coenzyme)
bi bi	-The class of enzyme reactions with two substrates and two products
E	-enzyme
ES	-Enzyme-substrate complex, an intermediate in an enzyme reaction
Michaelis constant	-The substrate concentration at which 2v = V (see below)
NAD	-nicotine adenine dinucleotide (a coenzyme)
NADH	-reduced form of nicotine adenine dinucleotide
P	-product
ping-pong	-A class of enzyme reaction where a partial reaction may occur and be reversed (bounce back and forth) many times before the next one starts
Random mechanism	-Enzyme mechanism where any one of more than one required substrate can add to the enzyme first.
Rapid equilibrium random mechanism	-A special case of random mechanism where the mathematics is greatly simplified. For a two substrate, two product reaction, all steps but the limiting one (interconversion of ternary complexes) are in equilibrium and all are significantly more rapid than this step.
S	-substrate
v	-reaction velocity
V	-maximal reaction velocity
uni-uni	-The class of enzyme reactions with one substrate and one product