Integrated Steady State Rate Equations and the Determination of Individual Rate Constants

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Integrated steady state rate equations have been used to determine the kinetic constants (\(V_a, K_a, V_p,\) and \(K_p\)) and rate constants (\(k_1, k_2, k_3,\) and \(k_4\)) of the reversible enzyme mechanism:

\[
E + S \xrightarrow{k_1} \frac{k_1}{k_2} \times \frac{k_1}{k_4} E + P
\]

The fumarase reaction has been used as a model to illustrate the procedures for determining these constants.

In contrast to initial velocity studies, the values of the constants have been obtained by examining the enzyme reaction in only one direction rather than in both forward and reverse directions. To accomplish this, a new procedure is described for fitting data to integrated rate equations which eliminates problems encountered when data are analyzed graphically. The advantages of examining an enzyme reaction in one direction with these new procedures allow this method to be extended to the examination of enzymes with simple mechanisms where initial velocities are difficult to measure because either the substrate or product is not readily available, or because the reaction is not readily reversible.

Steady state kinetic studies of enzyme reactions which are based on measurements of initial reaction rates in only one direction usually provide limited information about kinetic constants. To estimate all the kinetic constants (e.g. \(V_a, K_a, V_p,\) and \(K_p\), defined below) appearing in a steady state rate equation thus requires measurements of initial reaction rates in both the forward and reverse directions of a reaction. Unfortunately, these studies which restrict experimental measurements to the initial stages of a reaction suffer from a number of disadvantages (1-10), i.e. difficulties are sometimes encountered in accurately measuring the slope of a reaction curve extrapolated to zero time (1-3, 6, 9, 11, 12); not all enzyme reactions are easily measured in the forward and reverse directions, and most of the available information contained in the whole progress curve of a reaction is wasted when only initial rates are measured (1, 3-6, 8-10).

These considerations have resulted in several attempts to make use of the whole experimental progress curve of a reaction and to fit such data to equations describing the entire steady state course of a reaction. These attempts used either the differential (6, 9) or the integrated (1-3, 5, 7, 11, 13-15) forms of steady state rate equations. In fitting data to the differential form of a steady state rate equation, it was necessary to determine the velocity at various times during the course of a reaction. The process of computing the slope of a curve is a hazardous operation, especially when dealing with low accuracy empirical data (16). In fitting data to the integrated forms of rate equations, applications were restricted to graphical analyses of experimental data (1-3, 5, 7, 13, 14). These graphical analyses suffered from the same disadvantages as the graphical analyses which were used in evaluating initial velocity data (17-19); in particular, too much weight was often given to data points which could be grossly in error, and systematic errors were often introduced by assuming that certain experimentally determined parameters were without error.

A third approach to analyzing the whole progress curve of a reaction, including the transient phase, has been suggested by
Bates and Frieden (10, 20). They obtain a numerical solution of
the differential equations describing a particular mechanism
and compare this solution with experimental data. The param-
eters of the computed curve are adjusted until the best visual
fit with the experimental data is obtained. This approach is
basically a trial and error procedure or "analogous to fitting
data by eye" and does not provide any estimate of the
reliability of the fitted constants or any systematic strategy on
how to adjust the parameters once a computed curve is
compared with experimental data.

Some of the above problems are readily illustrated by
considering the integrated steady state equation for the
simplest one substrate-one product enzymic mechanism.

\[
E + S \times \frac{k_1}{k_2} \times \frac{k_3}{k_4}E + P
\]

**MECHANISM I**

Alberty (11, 15) expressed the integrated equation for
Mechanism I in the form of Equation 1. In this equation \(E_0\) and \(S_0\) are
the initial concentrations of enzyme and substrate, respec-
tively; \(P\) is the steady state concentration of product at time \(t\);
\(P_{eq}\) is the concentration of product at equilibrium; and the
following relations exist:

\[
V_s = k_3S_0; \quad V_p = k_2S_0; \quad V_s = k_1; \quad V_p = k_4
\]

Alberty (15) pointed out that, according to this equation, plots
of \(P/t\) against \([\ln(1-P/P_{eq})]/t\) should be linear, and that by
measuring the slope and intercepts of such plots at two values of
\(S_0\), the values for the four kinetic constants, \(V_s\), \(K_s\), \(V_p\), and
\(K_p\) could be calculated. In other words, one could obtain
estimates of all the steady state kinetic parameters for Mecha-
nism I by studying the reaction in only one direction.

Taraszka and Alberty (21) attempted to use this graphical
procedure to analyze data collected over the entire time course
of the fumarase reaction. They found, however, that plots of
\(P/t\) against \([\ln(1-P/P_{eq})]/t\) were not linear, especially at high
substrate concentrations. They did not pursue their studies on
the application of integrated rate equations to fumarase; instead,
they accounted for their nonlinear plots of \(P/t\) against
\([\ln(1-P/P_{eq})]/t\) by examining the effects of substrate activa-
tion (which occur at high substrate concentrations (22, 23))
using initial rate measurements.

Several types of errors could have accounted for nonlinear
plots of \(P/t\) against \([\ln(1-P/P_{eq})]/t\) obtained by Taraszka and
Alberty (21) even at low substrate concentrations where
substrate activation effects could be ignored.

1. \(P_{eq}\) was estimated from experimental data and any error
in its estimated value could have produced a systematic error in the
value of \([\ln(1-P/P_{eq})]/t\).

2. Errors in \(P/t\) and in \([\ln(1-P/P_{eq})]/t\) could have been
magnified at the start of the reaction because both \(P\) and \(t\) were
small, and inaccurate values are obtained at early time points.

3. Errors in \([\ln(1-P/P_{eq})]/t\) could have been magnified as
the reaction approached equilibrium, since \((1 - P/P_{eq})\) ap-
proaches zero and \([\ln(1-P/P_{eq})]/t\) approaches minus infinity as \(P\)
approaches equilibrium.

\[
\left(\frac{V_s}{K_s} + \frac{V_p}{K_p}\right) t = \left(\frac{1}{1 + \frac{1}{K_s}} - \frac{1}{1 + \frac{1}{K_p}}\right) P - \left(\frac{1}{1 + \frac{1}{K_s}} - \frac{1}{1 + \frac{1}{K_p}}\right) P_{eq} S_0 \ln(1-P/P_{eq})
\]

(1)

Taken together these errors indicate that one of the major
obstacles in using either Equation 1 or integrated rate equa-
tions in general is the lack of adequate procedures for fitting
data by least squares methods which give proper weighting to
the \((t, P)\) data points. As a consequence, and as far as the
authors are aware, there has been no attempt to fit data for a
reversible enzyme reaction to Equation 1 in order to obtain
estimates of the values of all four parameters appearing in the
equation.

In the present report, we have reinvestigated the fumarase
alyzed conversion of malate to fumarate using integrated
steady state rate equations. We have avoided the problem of
substrate activation (22, 23) by working with low malate
concentrations and we describe a new procedure for fitting data
to integrated rate equations which is based on the method of
least squares. This procedure eliminates the problems which
are encountered when data are analyzed graphically, by giving
each \((t, P)\) data point (for a fixed \(E_0\) and \(S_0\)) the same weight.

Further, instead of setting \(P_{eq}\) in Equation 1 to a fixed value, it
is treated as a parameter which is estimated from the data at
the same time as estimates are found for the other parameters.
We show that this procedure yields estimates of all the kinetic
constants \(V_s, K_s, V_p,\) and \(K_p\) of the fumarase reaction from
studies in only one direction of the enzyme reaction. These
constants are in good agreement with values reported in the
literature and with constants determined from initial velocity
studies in both directions of the reaction.

**MATERIALS AND METHODS**

**Enzyme**—Heart muscle fumarase was obtained from the Boehringer
Mannheim Corporation as a crystalline suspension in an ammonium
sulfate solution. Enzyme preparations were recrystallized and stored as
described (24). Enzyme preparations exhibited a single band on
analytical disc gel electrophoresis at pH 8.9, sedimented as a single,
symmetrical peak throughout the course (3 hours) of sedimentation-
velocity analysis in the ultracentrifuge, and behaved as a single protein
species of molecular weight 194,000 ± 8,000 by sedimentation equilib-
rium analyses. Techniques and conditions used for these evaluations
were those described (24-26).

Prior to use, crystals were washed with water, centrifuged, dissolved
in 10 mM Tris-acetate at pH 7.4 containing 15 mM EDTA, and stored in
polyethylene tubes at -20°C at a concentration of 0.1 mg per ml.
Enzyme concentrations were determined spectrophotometrically
(23-25). Since the enzyme was unstable in 10 mM Tris at 0°C after
thawing (27), the enzyme was diluted 10-fold with 0.1 M Tris-acetate,
pH 7.4, containing 1 mg/ml of bovine serum albumin, and was kept at
25°C during the course of a set of experiments. The enzyme was stable
for about 8 hours under these conditions and retained its maximum
activity for 45 min after addition to the reaction mixture with or
without substrate. This last point is important since the previous
studies of integrated rate equations had noted significant losses of
fumarase activity when diluted enzyme solutions were placed in
cuvettes (11). To evaluate any losses in enzyme activity that might
have occurred, a standard assay was used. Activity was measured as
the increase in absorbance at 240 nm in cuvettes with a 1-cm light
path. The assay mixture contained 0.15 M l-malic acid as substrate in 0.1
mM sodium phosphate buffer, pH 7.3. Measurements were made at 25°C
in a Gilford recording spectrophotometer.

**Substrates**—Fumaric acid and L-malic acid were recrystallized and
characterized as described (28). Fumarate concentrations were mea-
sured spectrophotometrically at 240 nm using an \(E_{240}^{fumarate} = 2.44 \times
10^4\) cm²/mole, t-malate concentrations were checked enzymatically
using malic dehydrogenase and NAD in the presence of hydrazine. The
amount of NADH formed under these conditions is equivalent to the amount of malate in the cuvette.

**Kinetic Assays**—Kinetic experiments were performed at 240 nm (23, 29) with a Cary 14 or Cary 15 recording spectrophotometer; cuvettes with a 3-mm volume and a 10-mm light path were used. Temperature was maintained at 25 °C with a constant temperature circulating water system and a jacketed cell housing. Assays were performed in 0.1 M Tris acetate, pH 7.4, using substrate concentrations between 0.01 and 0.7 μmol/ml; enzyme concentrations were between 0.03 and 0.3 μg per ml. Assays were initiated either with enzyme or substrate, and conditions were chosen to ensure that the equilibrium state was reached within 45 min.

**THEORETICAL CONSIDERATIONS AND DATA MANIPULATION**

**Choice of Equation for Evaluation of P**

We have rewritten Equation 1 in the following form:

\[ E_o t = -a_2 - (a_3 + a_4) S_o \ln (1 - P / P_{eq}) \]  
(2)

Expressions for the parameters \( a_2, a_3, \) and \( a_4 \) in terms of the kinetic constants \( V_p, K_p, V_s, \) and \( K_s \) and in terms of the velocity constants \( k_1, k_2, k_3, \) and \( k_4 \) are given in Table I. We use the term experiment to denote a set of measured values of \( P (P_{obs}) \) obtained at various times \( (t_{obs}) \) for a fixed \( E_o \) and \( S_o \).

In fitting data to this equation, we have assumed that the values measured for \( E_o, S_o, \) and \( t \) are without significant error given reasonable experimental technique and attention. In addition, we have assumed that for each value of \( t, P \) is a random variable which is normally distributed, that for each experiment the variance of \( P \) [denoted by \( \text{var} (P_{obs}) \)] is the same for all values of \( t \), and that errors in \( P_{obs} \) are independent of each other.

To ensure that the above assumptions are not far from reality, we have measured \( E_o, S_o, \) and \( t \) in every experiment and have not relied on a master batch of enzyme or substrate. To ensure that a series of measurements of \( P_{obs} \) from one experiment did not systematically distort our data, we have performed several (about 8) independent experiments using values of \( S_o \) that cover the Michaelis-Menten curve.

1 Many more than 8 curves were evaluated in the studies leading to the present report, all of which satisfied the results to be presented. The choice of 8 for presentation in this report serves to illustrate the results that can be obtained from a reasonable and relatively small number of curves. Analysis of as few as 4 curves could yield the data to be presented; analysis of 8 curves improved the error values considerably; and analysis of more than 8 curves improved the results only minimally. Eight curves seemed, therefore, to achieve a reasonable compromise between experimental error and experimental effort.

To compute a theoretical \( P \) versus \( t \) curve to match the \( P_{obs} \) values and to easily judge the behavior of \( P \) by inspection of a formula, we rearranged Equation 2 to yield another implicit equation in \( P \):

\[ P(t) = a_1 S_o (1 - e^{-t}) \]  
(3)

In this equation \( a_1 = P_{eq} / S_o \) and \( \tau = (E_o t + a_3 S_o) / (a_1 + a_4 S_o) \). The parameter \( a_1 \) was introduced into Equation 3 since \( P_{eq} \) is estimated experimentally.

The advantage of using this equation was that we had a better idea about the appropriate weights to apply to each data point. Since the parameters \( a_2, a_3, a_4, \) and \( a_1 \) did not enter Equation 3 linearly, linear least squares methods (as described in the standard texts) could not be used to compute, in one step, a unique set of parameters from any first estimate. We had, instead, to provide good first estimates of \( a_1, a_2, a_3, \) and \( a_4 \), to refine them by some iterative technique (30-32), and to assure that our computed values were unique. Because \( P \) appears on both sides of Equation 3, the evaluation of \( P \) was accomplished by finding the single root of the following equation using the Newton-Raphson technique (32).

\[ \frac{dP}{dt} - 1 = 0 \]  
(4)

**Procedure for Generating First Estimates of Parameters**

Since Equation 2 is linear in \( a_2, a_3, \) and \( a_4, \) it can be solved easily and uniquely for \( a_2, a_3, \) and \( a_4 \) by using linear least squares methods and the following a priori weights (see below).

\[ \frac{a - (P_{eq} - P_{obs})^2}{\text{var} (P_{obs})} = \frac{(P_{eq} - P_{obs})^2}{S_o^2} \]  
(5)

A first estimate of the parameter \( a_1 \) is found from a weighted least squares fit of \( P_{eq} \) values obtained at various \( S_o \) values using the equation \( P_{eq} = a_1 S_o \).

**Statistical Weights Applied to Data**

In Equation 2, \( t \) is a function of \( P \). If we know the error in \( P_{obs} \) (\( P_{obs} \)), we may attribute it to \( t \) by differentiating Equation 2 and substituting the result in Equation 6 to obtain Equation 7.

\[ \frac{dP_{obs}}{dt} = \frac{P_{obs} - P_{obs}}{\text{var} (P_{obs})} \left( \frac{a - (a_1 + a_4 S_o) / (P_{eq} - P_{obs})}{S_o} \right) \]  
(7)

**Relations between kinetic constants \((K_p, K_s, V_p, V_s, a)\) and velocity constants \(k_j (j = 1, 2, 3, \) and 4)**

Let \( b = a_4 - a_3 a_2; c = b + a_4; \) and \( d = k_1 k_3 + k_4 k_2 \).

<table>
<thead>
<tr>
<th>( j )</th>
<th>( a_j )</th>
<th>( b_j )</th>
<th>( c_j )</th>
<th>( d_j )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( V_p K_p / S_o )</td>
<td>( V_s K_p / S_o )</td>
<td>( (V_s + V_p) K_p / S_o )</td>
<td>( (V_s + V_p) K_p / S_o )</td>
</tr>
<tr>
<td>2</td>
<td>( k_1 k_3 / d )</td>
<td>( (k_4 - k_1) / d )</td>
<td>( (k_2 + k_3) / d )</td>
<td>( k_1 k_4 (k_2 + k_3) / d^2 )</td>
</tr>
<tr>
<td>3</td>
<td>( a_4 / (a_3 a_2) )</td>
<td>( (1 - a_1) / c )</td>
<td>( a_1 / b )</td>
<td>( a_4 / (a_3 b) )</td>
</tr>
</tbody>
</table>
When fitting data to Equation 2, the a's are unknown, so that we further simplify Equation 1 by assuming that all effects except those due to the \( (P_{eq} - P_{obs}) \) term are relatively unimportant, i.e.,

\[
\frac{e_{\text{obs}}}{P_{eq}} = \frac{e_{\text{obs}}}{P_{obs}}
\]

Therefore, to partially compensate for the effect of weighting which is introduced by fitting data to Equation 2, we apply a weight \( (P_{eq} - P_{obs})^2/\text{var} \) to each \((t_{obs}, P_{obs})\) data point which is introduced by fitting data to Equation 2, we apply a weight \( (P_{eq} - P_{obs})^2/\text{var} \) to each \((t_{obs}, P_{obs})\) data point. This is accomplished by the Newton-Raphson procedure (32).

\[\text{Experimental results} \]

Application to Fumarase-A computer program\(^1\) based on the above principles was prepared and used to analyze steady state data obtained over the whole time course of the fumarase reaction. Experimental data which measured the amount of fumarate formed at various times and starting with different initial concentrations of malate were then compared with curves generated by the computer on the basis of the rate constants it calculated using the program and the experimental measurements (Fig. 1). Good agreement was found between the experimental and theoretical curves during the entire time course of the reaction.

The values of the rate constants used to generate the theoretical curves in Fig. 1 are listed in Table II. Values for these rate constants and the kinetic constants which can be computed from them are compared to values for the same constants derived from initial velocity measurements in both the forward and reverse directions of the reaction (Table III). Reasonable agreement again exists. The initial velocity measurements used to compute the rate constants and kinetic constants are plotted in Fig. 2 according to the Lineweaver-Burk procedure (35).

Kinetic constants obtained under identical conditions have not been reported previously, however, constants have been obtained under analogous conditions, i.e. 25° in 0.01 \(M\) Tris-acetate, pH 7.0, and varying amounts of sodium chloride (28). Comparisons of these values reported in the literature and our data are made in Table III.

\[\text{Discussion} \]

In this report we have shown that integrated steady state rate equations can be used to fit steady state kinetic data for reversible enzymic reactions provided proper attention is given to the weighting of the data. By properly weighting the data, one avoids the problems encountered by other workers (1-3, 5, 7, 13, 14, 21) who used graphical procedures for analyzing their data. Such graphical procedures usually bias the data and therefore provide biased values for the parameters. The procedure outlined here is, in principle, similar to that suggested by Wilkinson (18) for fitting initial steady state velocity data to the Lineweaver-Burk form (35) of the Henri-Michaelis-Menten equation.

By using integrated steady state rate equations, all four steady state kinetic parameters for the fumarase reaction have been obtained by studying the reaction in only one direction, i.e. in the direction malate to fumarate. This procedure has obvious advantages over methods which use initial steady state velocity measurements where one has to follow the reaction in both directions in order to obtain all four steady state kinetic parameters.

We chose fumarase as a model to illustrate the application of

\[\text{Note:} \]

\[\text{Copies of the computer program used by these investigators have been supplied to the Journal and can be obtained from the authors by writing to them. If ordering from the Journal, please specify the document number 74M-1623 and remit $1.00 for the three-page photocopy.}\]
FIG. 1. A typical plot of experimental (----) and computed (-----) curves for the entire time course of the malate to fumarate reaction catalyzed by fumarase (4.3 × 10⁻¹⁰ M). S₀ concentrations ranged from 0.025 to 0.295 mM.

**TABLE II**
Values for parameters aᵢ and velocity constants kᵢ obtained from the data of Fig. 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>a₁</td>
<td>2.3 × 10⁻¹³</td>
<td>1 × 10⁻²</td>
</tr>
<tr>
<td>a₂</td>
<td>10.0 × 10⁻⁸ min</td>
<td>3 × 10⁻⁶</td>
</tr>
<tr>
<td>a₃</td>
<td>3.8 × 10⁻⁷ mM min</td>
<td>1 × 10⁻⁴</td>
</tr>
<tr>
<td>a₄</td>
<td>3.8 × 10⁻⁶ min</td>
<td>4 × 10⁻⁴</td>
</tr>
<tr>
<td>k₁</td>
<td>8.7 × 10⁶ mM⁻¹ min⁻¹</td>
<td>0.9 × 10⁴</td>
</tr>
<tr>
<td>k₂</td>
<td>6.7 × 10⁵ min⁻¹</td>
<td>1 × 10⁴</td>
</tr>
<tr>
<td>k₃</td>
<td>1.6 × 10⁵ min⁻¹</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>k₄</td>
<td>7.0 × 10⁴ mM⁻¹ min⁻¹</td>
<td>1 × 10⁴</td>
</tr>
</tbody>
</table>

**TABLE III**
Constants determined from integrated rate equation by analysis of forward reaction compared to constants calculated from initial velocity measurements in both forward and reverse reactions

| Constant | Computer constants | Constants from initial velocity studies | Constants from Frieden, Wolff, and Alberty
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>k₁ (mM⁻¹ min⁻¹)</td>
<td>8.7 × 10⁴</td>
<td>5.2 × 10⁴</td>
<td>6.7</td>
</tr>
<tr>
<td>k₂ (min⁻¹)</td>
<td>6.7 × 10⁴</td>
<td>4.0 × 10⁴</td>
<td>4.0</td>
</tr>
<tr>
<td>k₃ (min⁻¹)</td>
<td>1.6 × 10⁴</td>
<td>1.1 × 10⁴</td>
<td>11</td>
</tr>
<tr>
<td>k₄ (mM⁻¹ min⁻¹)</td>
<td>7.0 × 10⁴</td>
<td>3.7 × 10⁴</td>
<td>41</td>
</tr>
<tr>
<td>Vₑ/Eₑ × 10⁻⁴ (min⁻¹)</td>
<td>6.7</td>
<td>4.0</td>
<td>41</td>
</tr>
<tr>
<td>Vₑ/Eₑ × 10⁻⁴ (min⁻¹)</td>
<td>16</td>
<td>11</td>
<td>70</td>
</tr>
<tr>
<td>Kₛ × 10⁶ (M)</td>
<td>33</td>
<td>41</td>
<td>144</td>
</tr>
<tr>
<td>Kₚ × 10⁶ (M)</td>
<td>264</td>
<td>259</td>
<td></td>
</tr>
</tbody>
</table>

where S and P are the steady state concentrations of substrate and product at time t, and Vₑ, Kₛ, Vₑ, Kₚ, θₑ, θₑ, θₚ, Kₑₛ, and θₑₚ are steady state kinetic parameters for the reaction (21). Equation 11 is consistent with the mechanism proposed by Taraszka and Alberty (21) (based on initial velocity studies) and with the mechanism proposed by Hansen et al. (36) (based on isotope exchange studies). By working with low S₀ values, the terms in S², P², and SP in the numerator and denominator of Equation 11 can be neglected and Equation 11 reduces to:

\[
\begin{align*}
\frac{dS}{dt} = & \frac{(Vₚ/Kₚ)S - (Vₑ/Kₑ)P}{1 + S/Kₛ + P/Kₚ + S/Kₑₚ + P/Kₑₚ + S²/Kₛₚ + P²/Kₑₚ + S/Kₑₚ + P/Kₑₛ} \\
\text{where } S & = \text{substrate concentration, } P = \text{product concentration, } Vₑ = \text{forward velocity, } \text{and } Kₛ = \text{forward equilibrium constant.}
\end{align*}
\]

Integration of Equation 12 yields Equation 1.

While future work on the application of integrated steady state rate equations may involve attempts to fit data to complex equations for which no straightforward procedures are available for obtaining first estimates of all the parameters in the equation, the procedures we have developed here should be applicable to a large class of mechanisms involving two substrates and two products. Some examples of the applicable mechanisms which yield integrated rate equations which can be handled using the techniques presented in this paper can be found in a paper by Darvey and Williams (37).

**REFERENCES**