Biochimica et Biophysica Acta, 480 (1977) 1—13

BBA 68000

A SIMPLE METHOD FOR CALCULATING K_m AND V FROM A SINGLE ENZYME REACTION PROGRESS CURVE

SHYUN-LONG YUN and CLARENCE H. SUELTER

Department of Biochemistry, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)

(Received February 27th, 1976)
(Revised manuscript received July 14th, 1976)

Summary

A method of calculating K_m and V from a single reaction progress curve is presented. The integrated Michaelis-Menten equation $Vt = S_0 + K_m \ln(S_0/S)$, may be rearranged to the form $1/\bar{v} = 1/V + K_m/VS$, where $\bar{v} = (S_i - S_j)/\Delta t = \Delta S/\Delta t$ and $S = (S_i - S_j)/\ln(S_i/S_j)$ or S is approximated by $(S_i + S_j)/2$. S_i and S_j represent substrate concentrations at two points along a reaction progress curve separated by the time interval, Δt. The error resulting from the approximation depends on the magnitude of $\Delta S_i/S_i$; when $\Delta S_i/S_i < 0.3$, the error is insignificant; when $\Delta S_i/S_i > 0.3$, the error becomes significant. Procedures are presented to correct this error. Simulated data and application to the direct spectrophotometric assay of AMP aminohydrolase and the lactate dehydrogenase coupled assay of pyruvate kinase are provided.

The method is recommended when routine K_m and V values are desired. Compared to the initial rate method, it is faster, requires less substrate, and eliminates pipetting errors.

Introduction

Kinetic parameters (K_m and V) for an enzyme-catalyzed reaction are normally obtained from initial velocities at a series of substrate concentrations (differential method) or by analysis of a total reaction progress curve (integration method). The former method, since introduced by Michaelis and Menten [1] in 1913, is simple and thus widely used. The latter integrated method, which has not been popular with enzymologists, is usually employed by plotting $(S_0 - S)/t$ versus $1/t \ln (S_0/S)$ [2—6] where S_0 and S are the concentrations of substrate at $t = 0$ and t, respectively. The parameters, K_m and V, are calculated from the slope and intercept. The analysis suffers primarily from the fact that S appears in both variables, thus negating the use of a simple regression analysis [7]. The treatment of data according to the procedure of Klesov

and Berezin [8], which is similar to the approach used by Guggenheim [9] for the analysis of a first-order reaction, also suffers from the disadvantage of having S appear in both variables. The application of various computer techniques has provided avenues to circumvent some of these problems [10—14]; however, since the computer is not as yet a normal laboratory instrument, another approach for examing total progress curves was examined.

In the present paper, we outline a method to estimate K_m and V from a single reaction progress curve. In principle, one estimates the velocities at several substrate concentrations throughout the course of a complete reaction. These velocities can then be treated by standard accepted methods for treating normal Michaelis-Menten kinetic data e.g. Lineweaver-Burk [15] and Hofstee plots [16], or others as reviewed by Atkins and Nimmo [17]. The advantages over the initial velocity method are: (1) much less time is required to obtain the kinetic parameters; (2) more information is obtained from a complete reaction curve; (3) the method requires less substrate; (4) pipetting errors are eliminated since the substrate concentration is obtained from the reaction progress curve. Simulated data and application to the direct spectrophotometric analysis of AMP aminohydrolase and lactate dehydrogenase coupled pyruvate kinase reactions are provided. The results are in excellent agreement with those obtained by the conventional initial rate method.

Theory

(I) Irreversible reactions

For the integrated Michaelis-Menten equation:

$$Vt = (S_0 - S_t) + K_m \ln (S_0/S_t) \tag{1}$$

there exist pairs of data (t_i, S_i) such that, $Vt_i = S_0 - S_i + K_m \ln (S_0/S_i)$; $Vt_j = S_0 - S_j + K_m \ln (S_0/S_j)$, from which one obtains by subtraction:

$$-V(t_i - t_j) = S_i - S_j + K_m \ln (S_i/S_j) \tag{2}$$

this is followed by

$$-\frac{t_i - t_j}{s_i - s_j} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{\ln(S_i/S_j)}{S_i - S_j}$$

i.e.

$$\frac{1}{v_i} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{\ln(S_i/S_j)}{S_i - S_j} \tag{3}$$

where

$$v_i = -\frac{S_i - S_j}{t_i - t_j} = \frac{\Delta S_i}{\Delta t_i}$$

If we let $S = \dfrac{S_i - S_j}{\ln(S_i/S_j)}$, Eqn. 3 becomes the familiar Lineweaver-Burk equation

$$\frac{1}{v_i} = \frac{1}{V} + \frac{K_m}{VS} \tag{4}$$

Alternatively, we may, make the approximation given in Eqn. 5

$$\frac{S_i - S_j}{\ln(S_i/S_j)} \approx \frac{S_i + S_j}{2} = \bar{S} \tag{5}$$

The error of this approximation * initially suggested by Lee and Wilson [18], depends on $(S_i - S_j)/S_i$. It may be overcome by introducing a correction factor as outlined later such that $S = \bar{S}/(1 + \alpha_i)$. In any case whatever method is chosen to estimate S, K_m and V can be determined from v and S or $\bar{S}$ measured throughout a complete reaction progress curve.

(II) Equilibrium reaction

The integrated equation for a single substrate reaction at equilibrium:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} X \underset{k_4}{\overset{k_3}{\rightleftarrows}} E + P$$

has been given by Alberty [20] as:

$$\frac{V_s}{K_s}\left(1 + \frac{1}{K_{eq}}\right) \cdot t = \left(\frac{1}{K_s} - \frac{1}{K_p}\right) \cdot P - \left[1 + \frac{(S_0 + P_0)}{K_p} + \frac{\frac{1}{K_s} - \frac{1}{K_p}}{1 + K_{eq}}(S_0 + P_0)\right] \ln\left(1 - \frac{P}{P_{eq}}\right) \tag{6}$$

where K_s and K_p are the Michaelis-Menten constants for substrate, S, and product, P; S_0 and P_0 are the concentration of substrate and product at $t = 0$; V_s is the maximal velocity of the forward reaction; K_{eq}, the equilibrium constant. After substituting $S_0 + P_0 = S + P = S_{eq} + P_{eq}$, Eqn. 6 becomes:

$$t = a(S_0 - S) + b \ln \frac{S_0 - S_{eq}}{S - S_{eq}} \tag{7}$$

where

$$a = \frac{1/K_s - 1/K_p}{(1 + 1/K_{eq}) \cdot V_s/K_s} \tag{8a}$$

$$b = \frac{1}{(1 + 1/K_{eq}) \cdot V_s/K_s}\left\{1 + \frac{S_0 + P_0}{K_p} + \frac{(1/K_s - 1/K_p)(S_0 + P_0)}{1 + K_{eq}}\right\} \tag{8b}$$

Following the same approach as outlined in Eqns. 1—5, one obtains:

$$1/\bar{v} = a + b/(\bar{S} - S_{eq}) \tag{9}$$

or

$$\frac{1}{\bar{v}_i} = a + b \cdot \frac{1 + \alpha_i}{\bar{S} - S_{eq}} \tag{10}$$

An examination of Eqns. 8a and 8b indicates that the slope, b, is dependent

---

* This approximation is identical to that suggested by Cornish-Bowden [19] to obtain initial velocities for use in the direct linear plot. If we let $1 + x = S_i/S_j$, then by taking the first term of an infinite series $\ln(1 + x) = 2\left[\left(\frac{x}{2+x}\right) + 1/3\left(\frac{x}{2+x}\right)^3 + \ldots\right]$, $\ln(S_i/S_j) = 2\left[\frac{\frac{S_i}{S_j} - 1}{2 + \frac{S_i}{S_j} - 1}\right] = \frac{2(S_i - S_j)}{(S_i + S_j)}$

on S_0. The secondary plot of b (Eqn. 8b) vs. S_0 gives for the new intercept, A (Eqn. 11a) and slope, B (Eqn. 11b):

$$A = \frac{1 + \frac{P_0}{K_p} + \frac{(1/K_s - 1/K_p)P_0}{1 + K_{eq}}}{(1 + 1/K_{eq}) \cdot \bar{V}/K_s} \tag{11a}$$

$$B = \left[1/K_p + \frac{1/K_s - 1/K_p}{1 + K_{eq}}\right] \Big/ (1 + 1/K_{eq}) \cdot V_s/K_s \tag{11b}$$

From Eqns. 8a, 11a, and 11b, one obtains:

$$1/K_s = B/(A - P_0B) + \frac{a}{(A - P_0B)(1 + 1/K_{eq})} \tag{12a}$$

$$1/K_p = B/(A - P_0B) - \frac{a}{(A - P_0B)(1 + K_{eq})} \tag{12b}$$

$$1/V_s = a + B(1 + 1/K_{eq}) \tag{12c}$$

$$1/V_p = (1 + K_{eq})B - a \tag{12d}$$

Thus, using several different concentrations of substrate, S_0, one is able to calculate kinetic parameters (K_s, K_p, V_s and V_p) providing that K_{eq} is available.

(III) Competitive, uncompetitive, and non-competitive inhibition

The normal diagnostic procedures and equations used to analyze the inhibition of an enzyme reaction are applicable in this analysis. The modifier $(1 + I/K_i)$ where I is the concentration of inhibitor and K_i, the inhibition constant, can modify: (1) the intercept term of Eqn. 5 to give uncompetitive inhibition; (2) the slope term to give competitive inhibition; (3) both the intercept and slope terms to give non-competitive inhibition. The reactions are completed at several concentrations of inhibitor and plotted to assess the inhibition.

(IV) Product inhibition

One of the criticisms sometimes made of this approach is the unsuspected inhibition by product accumulating during reaction which, in turn, affects the kinetic parameters. However, as indicated below, it is possible to detect and use this inhibition to obtain valuable information regarding the reaction. For example, a reaction inhibited by a product can be expressed by the following equation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} X \xrightarrow{k_3} E + P$$

$$E + P \underset{k_5}{\overset{k_4}{\rightleftarrows}} EP \qquad K_p = k_4/k_5$$

Using the same approach as outlined in Eqns. 1—5, one obtains the following equation for the approximation to the integrated rate equation [21,22]:

$$1/v = 1/V[1 - (K_s/K_p)] + 1/V(K_s + S_0K_s/K_p)1/\bar{S}$$

The plot of $1/v$ vs. $1/S$ gives intercept, $a = [1 - K_s/K_p)]/V$, independent of

substrate concentration and slope, $b = [(K_s + S_0 (K_s/K_p)]/V$, which is a function of the initial concentration of substrate. From a series of values of b obtained at different initial concentrations of substrate, S_0, a second plot of b vs. S_0 will give, for simple inhibition, a straight line with an intercept on b-axis equal to K_s/V and intercept on S_0-axis equal to K_p. The K_s and V may be calculated from the equation, $a = [(1 - (K_s/K_p)]/V$. Other, more complicated, types of inhibition may affect the intercept as well as the slope term. In any case, by examining reactions at more than one substrate concentration, product inhibition can be detected, accounted for, and utilized.

(V) Two substrate reactions

For a reaction involving two or more substrates, the first approximation of K_m and V is best made by analysis of K_m^A and V with saturating levels of substrate B and setting the concentration of substrate A at levels near the K_m. Alternatively, we can determine K_m^B by reversing the procedure. The data for yeast pyruvate kinase is presented later to illustrate the application. The analysis of all kinetic constants and mechanisms of a two or more substrate reaction is too complicated to present here. Frieden [23] has discussed an approach which is applicable.

Methods

The recommended procedure for obtaining data pairs, v and S, is outlined in Fig. 1. We will use $\bar{S}$ throughout the remainder of this paper since its determination is simpler and more rapid. Several sets of data pairs, $\bar{v}$ and $\bar{S}$ are determined from points taken along a reaction progress curve * using the approximation given by Eqn. 5, as follows:

$$\bar{v}_i = -(S_i - S_j/(t_i - t_j), \quad \bar{S}_i = (S_i + S_j)/2$$

$$\bar{v}_{i+1} = -(S_{i+1} - S_{j+1})/(t_i - t_{j+1}), \quad \bar{S}_{i+1} = (S_{i+1} + S_{j+1})/2$$

$$\bar{v}_{i+n} = -(S_{i+n} - S_{j+n})/(t_{i+n} - t_{j+n}), \quad \bar{S}_{i+n} = (S_{i+n} + S_{j+n})/2$$

The values for S and t are derived as indicated in Fig. 1 to overcome the objections noted by Roseveare [24]. It is not necessary to use fixed intervals of ΔS and Δt as implied in Fig. 1. However, it should be emphasized that the error arising from the approximation made in Eqn. 5 is inversely related to ΔS, i.e. the accuracy of the approximation will be greater as $\Delta S_i/S_i$ becomes smaller. On the other hand, as ΔS becomes smaller its relative error becomes larger since the absolute error in reading absorbances from a recorded reaction progress

* To use this method, one must be able to determine the substrate concentration at each point in the reaction profile. If one records continuously with a recorder, the simplest approach for an irreversible reaction is to let the reaction proceed to completion and calculate, from the change in absorbance and the extinction coefficient for that change, the concentration of substrate remaining at each point. If the reaction proceeds to an equilibrium point, then one must know the concentrattion of substrate at time zero and from the change in absorbance observed, calculate the concentration of S remaining at each point of the assay. The application of this method can be more convenient if data are collected as a function of time with a digital computer. Alternatively, data listers which print absorbances at preselected time intervals are less tedious than taking data from a recorded reaction progress curve.

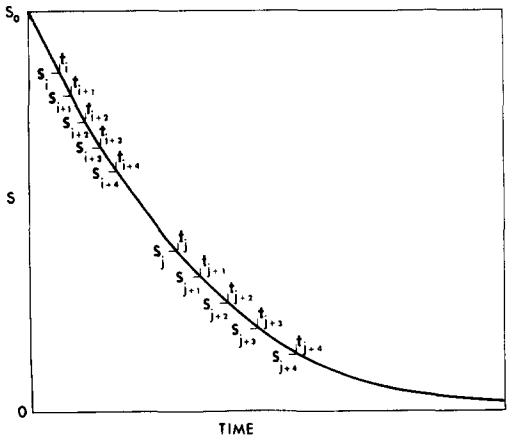

Fig. 1. Determination of V and S from a progress curve of an enzyme-catalyzed reaction. S_0 represents the initial substrate concentration and S_i is the concentration at time t_i. To determine the average velocity, v_i, and average substrate concentration, S_i, S_i at various time t_i is measured. $\Delta S_i = S_i - S_j$, $\Delta t_i = t_i - t_j$, and $v_i = \Delta S_i/\Delta t_i$, $S_i = (S_i + S_j)/2$ are then calculated for each pair of data. $\Delta S_i/S_i$ is also calculated for the determination of relative error, α_i.

curve is rather constant. For instance, if the reaction progress curve is recorded over the full scale of recording chart paper, the absolute error in reading an absorbance is usually less than 0.2% of the full scale value. As outlined later, this error is significantly reduced by increasing the ratio of $\Delta S_i/S_i$ in the calculation of v and S.

The initial substrate concentration chosen for a reaction may range from 0.2 K_m to 5 K_m depending on the assay. We recommend because of the reasoning outlined previously and later that three substrate concentrations (0.2 K_m, K_m, and 5 K_m) be used to determine the kinetic parameters. After being assured that accumulating products do not inhibit, it is convenient for routine work to use substrate concentrations equivalent to 2—3 times K_m.

Error

The errors caused by the approximation made in Eqn. 5 have been analyzed previously by Lee and Wilson * [18].

$$\frac{\ln S_i/S_j}{\frac{2(S_i - S_j)}{(S_i + S_j)}} = (1 + \alpha_i), \tag{13}$$

then the relative error, α_i, will depend on the ratio of $\Delta S_i/S_i$.

Eqn. 13, after substituting ΔS_i for $(S_i - S_j)$ and $R_i = \Delta S_i/S_i$, yields

$$(1 + \alpha_i) = \left(\frac{1}{R_i} - 1/2\right) \ln \frac{1}{(1 - R_i)} \tag{14}$$

* The approximation suggested by Lee and Wilson [18] to estimate initial velocities for an enzyme reaction must also be corrected if products of the reaction inhibit.

which shows that (1 + α_i) is constant when R_i is constant. To determine the effect of the approximation error on K_m and V, the above is substituted into Eqn. 3 and rearranged to give Eqn. 15 which is formally equal to Eqn. 3.

$$\frac{1}{v_i} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{(1 + \alpha_i)}{\bar{S}_i} \tag{15}$$

α_i is not significant when $\Delta S_i/S_i$ or $R_i < 0.3$. As indicated above when R_i is kept constant for all pairs of data ($\bar{S}_i$ and $\bar{v}_i$), the apparent K_m obtained from a plot of Eqn. 5 can be divided by (1 + α_i) to give the actual K_m. Alternatively, one may correct the error when R_i is greater than 0.3 by dividing $\bar{S}_i$ by (1 + α_i) as indicated in Eqn. 15.

Results

(I) Demonstration of method

Data from a complete reaction progress curve for the hydrolysis of phenylphosphate catalyzed by prostate acid phosphatase previously presented by

TABLE I

SUMMARY OF CALCULATION OF $\bar{v}$ AND $\bar{S}$

Data in columns 1 and 2 are taken from Schonheyder [22] showing the formation of product at various times, t. The reaction was 98.7% complete at the final determination. The substrate S_i, remaining at time, t, was calculated from column 2. S_j was the values of column 3 after t = 4.57 min. The $\Delta S_i = S_i - S_j$ and time elapsed, Δt, are listed in columns 5 and 6. The average velocity and substrate concentrations are then calculated from $\bar{v} = \Delta S/\Delta t$ and $\bar{S} = 1/2\ (S_i + S_j)$. The correction (1 + α) at various values of $\Delta S_i/S_i$ was calculated according to Eq. 14.

1	2	3	4	5	6	7	8	91	10
t (min)	P (mM)	S_i (mM)	S_j (mM)	ΔS_i (mM)	Δt (min)	$\bar{v} = \Delta\bar{S}/\Delta t$	$\bar{S} = (S_i + S_j)/2$	$\Delta S_i/S_i$	$\bar{S}/(1 + \alpha)$
0.47	0.0252	0.9586	0.7694	0.189	4.1	0.0461	0.864	0.197	0.860
1.20	0.0631	0.9207	0.7190	0.202	4.5	0.0448	0.820	0.219	0.816
2.35	0.1135	0.8703	0.6685	0.202	4.55	0.0444	0.769	0.232	0.765
3.33	0.1639	0.8199	0.6181	0.202	4.93	0.0409	0.719	0.246	0.714
4.57	0.2144	0.7694	0.5677	0.189	5.13	0.0393	0.669	0.262	0.663
5.70	0.2648	0.7190	0.5298	0.176	5.10	0.0371	0.624	0.263	0.620
6.90	0.3153	0.6685	0.4920	0.164	5.13	0.0344	0.580	0.264	0.576
8.26	0.3657	0.6181	0.4542	0.151	5.07	0.0323	0.536	0.265	0.532
9.70	0.4161	0.5977	0.4163	0.151	5.18	0.0292	0.492	0.267	0.489
10.80	0.4540	0.5298	0.3785	0.151	5.40	0.0280	0.454	0.286	0.450
12.03	0.4918	0.4920	0.3407	0.151	5.82	0.0260	0.416	0.308	0.412
13.33	0.5296	0.4542	0.3029	0.151	6.46	0.0234	0.379	0.333	0.374
14.88	0.5675	0.4163	0.2650	0.151	6.63	0.0228	0.341	0.363	0.335
16.20	0.6053	0.3785	0.2272	0.151	7.85	0.0193	0.300	0.400	0.296
17.85	0.6431	0.3407	0.1894	0.151	8.93	0.0169	0.265	0.444	0.258
19.79	0.6809	0.3029	0.1515	0.151	9.71	0.0156	0.277	0.500	0.218
21.51	0.7188	0.2650	0.1263	0.139	10.80	0.0128	0.196	0.521	0.187
24.05	0.7566	0.2272	0.1011	0.126	11.30	0.0112	0.164	0.555	0.156
26.78	0.7944	0.1894	0.0759	0.114	12.60	0.0090	0.133	0.598	0.124
29.50	0.8323	0.1515	0.0507	0.101	15.50	0.0065	0.101	0.665	0.092
32.33	0.8575	0.1263	0.0254	0.101	22.50	0.0045	0.076	0.799	0.063
35.35	0.8827	0.1011	0.0128	0.088	33.60	0.0026	0.057	0.873	0.032
39.40	0.9079	0.0759							
45.00	0.9331	0.0507							
54.90	0.9584	0.0254							
69.00	0.9710	0.0128							

Schonheyder [22] were analyzed with this method to demonstrate its application. Columns 1 and 2 of Table I were taken from Table I of ref. 22; column 3 lists the concentration of S remaining at times t; values in column 4 were taken from column 3 starting at time t = 4.57 min. This value was chosen to provide a significant ΔS. A larger or smaller value could have been selected. Columns 5, 6, 7, 8, and 9 were calculated as indicated. The values given in column 10 were calculated with the $(1 + \alpha)$ values determined with Eqn. 14 and are equal to $(S_i - S_j)/\ln(S_i/S_j)$. The reciprocal values of $\bar{v}$ and $\bar{S}$ or $\bar{v}$ and $\bar{S}/(1 + \alpha_i)$ are plotted in Fig. 2. You will note that when $\Delta S_i/S_i$ exceeds 0.5, the reciprocal plot for the uncorrected data deviates significantly from a straight line. The values of K_m and V for the note that when $\Delta S_i/S_i$ exceeds 0.5, the reciprocal plot for the uncorrected data corrected S when calculated according to Wilkinson [25] were 2.35 ± 0.186 and 0.175 ± 0.011, respectively. These values are in excellent agreement with 2.42 and 0.179 calculated by Schonheyder [22] using a graphical solution of simultaneous equations to obtain K_m and V from $t = K_m/S \ln S_0/S + 1/V (S_0 - S)$.

(II) Simulated data

To demonstrate the accuracy of the present method, a theoretical progress curve for a reaction following Eqn. 1 was simulated with $V = K_m = 10$ using $S_0 = 0.2\ K_m$, K_m and $5\ K_m$. Data were simulated with a fixed random error of 0.2 and 0.5% of the initial substrate concentration. As indicated earlier, we estimate that the error in reading absorbances from a recorded reaction progress curve is less than 0.2% of the full scale value. These simulated progress curves were then analyzed according to Eqn. 15 and the results are presented in Table II. As expected K_m and V determined with data containing 0.5% random error had larger standard deviations than those from data with 0.2% error. Further the larger the value of $\Delta S_i/S_i$ used or the larger the number of v_i and

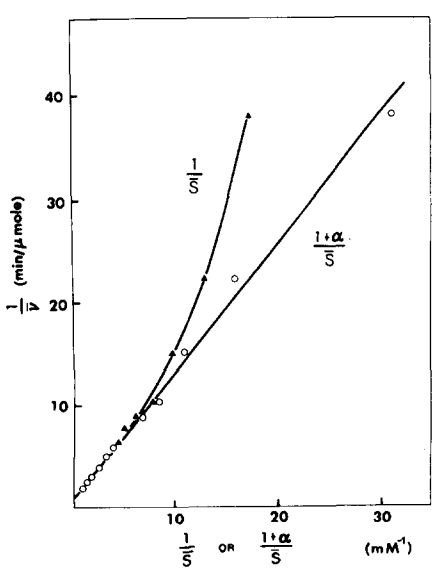

Fig. 2. Lineweaver-Burk plot of $1/v$ vs. $1/S$ or $1/v$ vs. $(1 + \alpha)/S$ taken from data of Table I.

TABLE II

SUMMARY OF SIMULATED DATA

The time couse of substrate concentration for an enzyme-catalyzed reaction following simple Michaelis-Menten kinetics was simulated by a computer with a random error of 0.2 and 0.5% of three initial substrate concentrations, 0.2 K_m, K_m and 5 K_m and assuming $V = K_m = 10$. The K_m and V were then calculated according to Eq. 15.

S_0	Error	$\Delta S_i/S_i$	Points *	K_m	V	Slope
$0.2K_m$	0.2%	<0.1	25	10.74 ± 3.26	10.81 ± 3.44	0.99
		~0.15	24	8.31 ± 1.10	8.44 ± 1.00	0.99
		0.22—0.25	23	8.85 ± 0.95	8.94 ± 0.86	0.99
		0.35—0.4	21	9.12 ± 0.70	9.18 ± 0.64	0.99
	0.5%	0.15—0.18	22	8.13 ± 1.38	9.22 ± 1.77	0.88
		0.35—0.4	21	13.72 ± 2.9	13.82 ± 3.19	0.99
		0.58—0.62	16	13.384 ± 3.76	13.26 ± 0.38	1.01
K_m	0.2%	0.1—0.16	30	9.73 ± 1.00	9.86 ± 0.68	0.99
		0.36—0.5	24	9.99 ± 0.45	9.99 ± 0.45	1.00
	0.5%	0.14—0.24	11	8.48 ± 1.84	9.22 ± 1.4	0.92
		0.14—0.24	31	8.81 ± 1.01	9.28 ± 0.74	0.95
		0.23—0.33	21	8.68 ± 1.1	9.15 ± 0.69	0.95
		0.36—0.51	27	10.08 ± 0.68	10.07 ± 0.47	1.00
$5K_m$	0.2%	0.083—0.28	66	10.03 ± 0.43	10.01 ± 0.13	1.00
		<0.1	21	8.16 ± 1.34	9.62 ± 0.31	0.85
		0.24—0.59	21	9.7 ± 0.30	9.89 ± 0.10	0.98
		0.32—0.68	52	9.98 ± 0.15	10.00 ± 0.05	1.00
	0.5%	0.24—0.59	21	11.68 ± 1.11	10.33 ± 0.35	1.13
		0.32—0.67	51	9.51 ± 0.44	9.86 ± 0.14	0.97

* This value represents the number of data pairs, v and S, used for the calculations of K_m and V. K_m and V were calculated using Wilkinsons [25] weighting to the Lineweaver-Burk equation.

S_i pairs used to calculate the data, the smaller the standard deviation. The smallest deviations were noted when the initial substrate concentrations were $5 \times K_m$.

(III) Experimental data

Fig. 3 illustrates a progress curve for the reaction catalyzed by muscle AMP aminohydrolase with $S_0 = 1.8$ mM. The plot of the data according to Eqn. 15 is shown in the insert. The K_m was determined to be 0.46 mM in agreement with

TABLE III

SUMMARY OF K_m AND V OF RABBIT MUSCLE AMP AMINOHYDROLASE DETERMINED FROM PROGRESS CURVE AT THREE DIFFERENT INITIAL CONCENTRATION OF SUBSTRATE

The reaction mixture contained 0.1 M Tris/2-(*N*-morpholino)-ethane sulfonic acid, pH 6.4; 0.1M KCL; and AMP as indicated. Reaction was run at 30°C; 3 μg of enzyme was used.

AMP (μM)	K_m (mM)	V (units/min)
90 [a]	0.476	4.0
900 [b]	0.37	3.6
1800 [b]	0.475	3.8

[a] Reaction recorded at 265 nm.
[b] Reaction recorded at 285 nm.

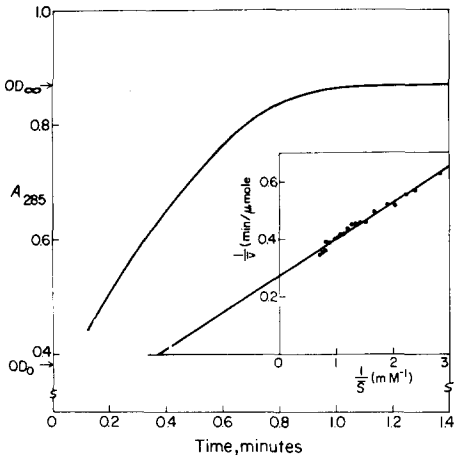

Fig. 3. A progress curve of the reaction catalyzed by rabbit muscle AMP aminohydrolase. Reaction was carried in 1 ml of 0.1 M Tris 2-(*N*-morpholino)-ethane sulfonic acid buffer, pH 6.4, containing 0.1 M KCl and 1.8 mM AMP at 30°C. The reaction was started by adding 5 μl of enzyme (3 μg) and monitored at 285 nm with recording speed of 5 inches/min. Insert shows the Lineweaver-Burk plot of the data with $\Delta S_i/S_i = 0.08$—0.15.

the reported value of 0.4 mM by the initial velocity method [26]. Table III summarizes the values of K_m and V from three different initial substrate concentrations.

Fig. 4 shows the progress curve for the reaction catalyzed by yeast pyruvate

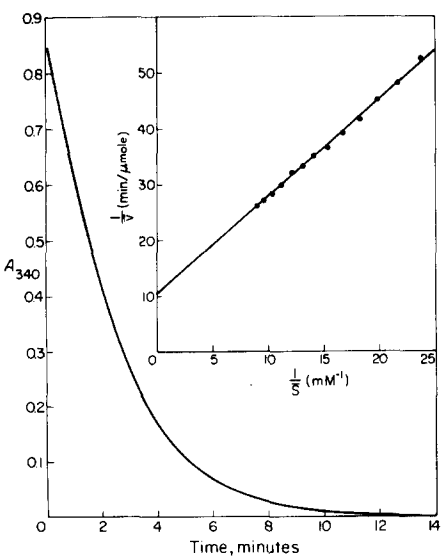

Fig. 4. The progress curve of the reaction catalyzed by yeast pyruvate kinase, coupled to lactate dehydrogenase. Reaction was started by adding 20 μl of enzyme (0.28 μg) $[ADP]_0 = 0.141$ mM. Insert shows the Lineweaver-Burk plot of the data with $\Delta S/S \approx 0.3$. The other conditions are given in Table IV and Methods.

TABLE IV

COMPARISON OF K_m VALUES OF YEAST PYRUVATE KINASE BY THE PRESENT METHOD AND THE INITIAL RATE METHOD

Pyruvate kinase activity was determined by the spectrophotometric method after coupling to lactate dehydrogenase [27]. The reaction mixture contained per ml: tetramethylamine/2-(*N*-morpholino)-ethane sulphonic acid [3] buffer, 100 μmol, pH 6.2; KCl, 100 μmol; $MgCl_2$, 24 μmol; rabbit muscle lactate dehydrogenase, 33 μg; NADH, 0.2 μmol; enzyme 0.2—0.3 μg; phospho*enol*pyruvate and ADP. The concentration of the testing substrate was 0.12—0.14 mM, while the other was at saturating concentration (10 mM). Reactions were carried out at 30°C. Number in parentheses represent the number of experiments.

Substrate	Present method		Initial rate method K_m (mM)
	V (μmol/min per mg)	K_m (mM)	
Phospho*enol*-pyruvate	368 ± 5.6 [b] (6)	0.089 ± 0.019 [b] (6)	0.08—0.125
ADP [a]	344 ± 2.5 (3)	0.167 ± 0.005 (3)	0.16
ADP	334 ± 3.0 (3)	0.427 ± 0.026 (3)	0.34

[a] Fru-1,6 P_2, 1 mM final concentration, was added.
[b] Standard deviation.

kinase coupled to lactate dehydrogenase, and the insert shows the plot of Eqn. 15. Table IV summarizes the results of K_m values and V of yeast pyruvate kinase for its substrates, phospho*enol*pyruvate and ADP. The K_m values determined from the progress curve are in good agreement with the values reported earlier [28], as determined by the initial rate method. V obtained from different determinations were close to the rate obtained at saturating substrate concentration.

Discussion

We have suggested a procedure to allow use of a complete reaction progress curve and the integrated Michaelis-Menten equation to determine the kinetic parameters for an enzyme reaction. After being assured that the enzyme is not inactivated during assay and that accumulating products do not inactivate or inhibit, a single substrate concentration suffices for an experiment to determine K_m and V. Inhibition constants and types of inhibition may also be determined.

To use the integrated equation, one obtains a complete reaction progress curve either by continuous recording or by taking points at various time intervals throughout the time course of a reaction. The estimated velocities and substrate concentrations at user-selected points of a reaction profile are treated in the same manner as that used for the initial rate method to calculate the kinetic constants. We suggest for the initial examination of an enzyme reaction that complete reaction progress curves be obtained at three different initial substrate concentrations equivalent to 5 K_m, K_m, and 0.2 K_m to ascertain reversibility, product, or substrate inhibition. To be sure that the enzyme is not inactivated during the assay, the reaction should be run at a minimum of two enzyme concentrations as suggested by Selwyn [29]. If complete reaction progress curves are obtained under identical conditions but at two different enzyme concentra-

tions, then the $1/v$ versus $1/S$ plots after correction for enzyme concentration should be identical. Non-identical behavior suggests inactivation during assay.

The method suggested here requires less time and material than the initial velocity method to calculate K_m and V. Furthermore, it is more precise and less tedious than determining the tangents to a curve at various points throughout a reaction progress curve. For reactions going to equilibrium or for product-inhibited reactions, the measurement of reaction in one direction should provide the necessary data to calculate all kinetic constants. This may be important if substrates or products are not readily available. Another advantage of this method arises from the higher internal consistency since errors of pipetting are eliminated. If enzyme becomes inactivated during assay, it may be possible to obtain data under stopped flow conditions; that is, under high enzyme concentrations where the reaction may be complete at much shorter time intervals.

Cornish-Bowden [19], after summarizing several attempts to use the integrated equation to determine K_m and V, argued that it is a highly dubious approach. The argument is based on the fact that product inhibition, imprecise knowledge of product concentrations at infinite time, and possible enzyme inactivation during assay, lead to values of K_m and V which are difficult to interpret with confidence. On the other hand, as discussed earlier, it is possible to assess the extent of each of these problems. Our experience to date suggests that many enzymes will be stable under conditions of a complete reaction. It is realized, of course, that under extreme conditions of pH or temperature, enzymes will become inactivated. This is also a problem when the initial rate method is used. In our opinion, then, it would be unwise to discard a procedure which is theoretically sound but, for practical reasons, cannot be applied to all enzyme reactions.

Acknowledgements

This work was supported in part by Grant GM 20716-02, National Institutes of Health, Grant BMS74-20107, National Science Foundation, and Michigan State Agricultural Experiment Station, Michigan State Journal Article No. 7393. We thank Mr. John C.W. Campbell for providing rabbit muscle AMP aminohydrolase. C.H.S. is the recipient of Research Career Development Award 1K3 9725 of the N.I.H.

References

1 Michaelis, L. a nd Menten, M.L. (1913) Biochem. Z. 49, 333—369
2 Foster, R.A. and Niemann, C. (1953) Proc. Natl. Acad. Sci. U.S. 39, 999—1003
3 Booman, K.A. and Niemann, C. (1955) J. Am. Chem. Soc. 77, 5733—5735
4 Jennings, R.R. and Niemann, C. (1955) J. Am. Chem. Soc. 77, 5432—5433
5 Alberty, R.A. and Koerber, B.B. (1957) J. Am. Chem. Soc. 79, 6379—6382
6 Schwert, G.W. (1969) J. Biol. Chem. 244, 1285—1290
7 Fleisher, C.A. (1953) J. Am. Chem. Soc. 75, 4487—4490
8 Klesov, A.A. and Berezin, I.V. (1972) Biokhimiya (English edn.) 37, 141—151
9 Guggenheim, E.A. (1926) Phil. Mag. 2, 538—543
10 Darvey, I.G., Shrager, R. and Kohn, L.D. (1975) J. Biol. Chem. 250, 4696—4701
11 Bizzozero, S.A., Kaiser, A.W. and Dutler, H. (1973) Eur. J. Biochem. 33, 292—300
12 Atkins, G.L. and Nimmo, I.A. (1973) Biochem. J. 135, 779—784
13 Fernley, H.N. (1974) Eur. J. Biochem. 43, 377—378
14 Nimmo, I.A. and Atkins, G.L. (1974) Biochem. J. 141, 913—914

15 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
16 Hofstee, B.H.J. (1952) Science 116, 329—331
17 Atkins, G.L. and Nimmo, I.A. (1975) Biochem. J. 149, 775—777
18 Lee, H.J. and Wilson, I.B. (1971) Biochim. Biophys. Acta 242, 519—522
19 Cornish-Bowden, A. (1975) Biochem. J. 149, 305—312
20 Alberty, R.A. (1959) The Enzymes 2nd edn., Vol. 1, pp. 143—155, Academic Press, New York
21 Huang, H.T. and Niemann, C. (1951) J. Am. Chem. Soc. 73, 1541—1548
22 Schonheyder, F. (1952) Biochem. J. 50, 378—384
23 Frieden, C. (1975) J. Biol. Chem. 250, 2111—2113
24 Roseveare, W.E. (1931) J. Am. Chem. Soc. 1651—1661
25 Wilkinson, G.N. (1961) Biochem. J. 80, 324—332
26 Smiley, Jr., K.L. and Suelter, C.H. (1967) J. Biol. Chem. 242, 1980—1981
27 Bucher, T. and Pfleiderer, G. (1955) Methods Enzymol. 1, 435—440
28 Aust, A., Yun, S.L. and Suelter, C.H. (1975) Methods Enzymol. 42, 176—182
29 Selwyn, M.J. (1965) Biochim. Biophys. Acta 105, 193—195