[CONTRIBUTION FROM THE SECTION OF BIOCHEMISTRY, MAYO CLINIC]

Curve Fitting of Enzymatic Reactions Based on the Michaelis–Menten Equation[1]

BY GERARD A. FLEISHER

RECEIVED JANUARY 2, 1953

The integrated Michaelis–Menten equation can be converted to a form which is suitable for curve fitting of enzyme reactions. The two parameters of this equation are the initial rate m and the quotient r of substrate concentration and Michaelis constant. For reactions in which competitive inhibitors are initially present or formed as reaction products the same type of equation can be derived. In this case r is replaced by r' which contains also the concentrations and dissociation constants of all inhibitors. The parameters can be calculated by an application of the method of least squares. This method of curve fitting is illustrated for the hydrolytic splitting of glycylglycylglycine by serum and red blood cells, reactions which cannot be described by either first-order or zero-order kinetics. In some cases the method may allow the calculation of Michaelis constants, dissociation constants of the enzyme-inhibitor complexes and maximal velocities from the parameters of the fitted straight lines. This is shown for cholinesterase in human serum.

In the course of their studies on the kinetics of histidase, Walker and Schmidt[2] devised a novel way of determining the Michaelis constant K_S[3] and the maximal velocity V_m. In its integrated form the Michaelis–Menten equation was treated as a regression of $\frac{[P]}{t}$ on $\frac{1}{t} \ln \frac{[S]_0}{[S]_0 - [P]}$, in which $[S]_0$ and $[P]$ represent the concentrations of substrate initially present and of product formed during the time t, respectively. The slope of the straight line was $-1/K_S$ and the intercept on the $[P]/t$ axis, V_m. Other investigators, among them Elkins-Kaufman and Neurath[4] and Huang and Niemann,[5] have tested the integrated form of the Michaelis–Menten equation, but with the constants determined by the customary Lineweaver–Burk plot rather than by the method of Walker and Schmidt.

In our studies on various peptidases in blood, my colleagues and I were interested in finding a more accurate way of expressing enzyme activities than has conventionally been done by assuming a reaction to follow either first-order or zero-order kinetics. The analytic methods available at present for determining the degree of peptide hydrolysis preclude the evaluation of initial reaction rates for use in a Lineweaver–Burk plot. The possibility of using the integrated form of the Michaelis–Menten equation to fit our reactions was, therefore, investigated. When the method of Walker and Schmidt was applied to a number of peptidase reactions, it was frequently impossible to fit a straight line through the points of the plot. We had good reason to suspect that our difficulties were due to the greater error inherent in our method of analysis as compared with the ammonia titration in the determination of histidase. For it can easily be seen that in a plot of $\frac{[P]}{t}$ *vs.* $\frac{1}{t} \ln \frac{[S]_0}{[S]_0 - [P]}$ any error in [P] will enter into both coördinates, displacing the points in a peculiar fashion along curved lines. This is illustrated by a hypothetical experiment in Fig. 1, in which the upper and lower curves represent the displacements from the straight line for a constant error of plus and minus 2%, respectively. An additional difficulty was often encountered because of an error in the initial determination, the base line for all subsequent analyses. Such an error has the greatest effect on the smallest [P]-values, resulting in a curve rather than a straight line.

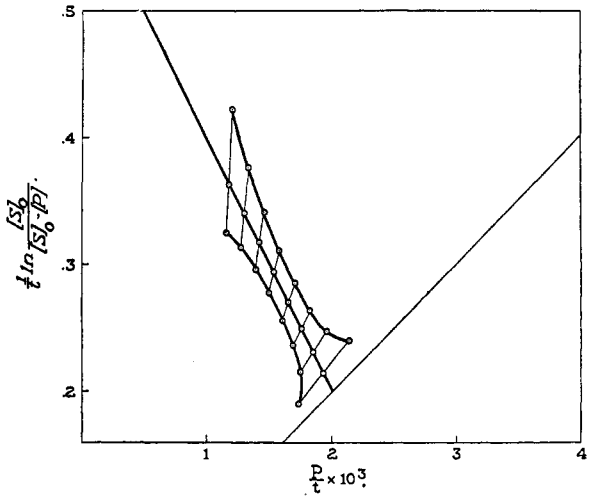

Fig. 1.—Effect of errors in P on the Walker–Schmidt plot of a hypothetical reaction, with $K_S = 5 \times 10^{-3}$ molar and $V_m = 3 \times 10^{-3}$ mole per hour. The points on the straight line would represent hourly determinations: upper curve, +2% error; lower curve, −2% error.

In the present investigation an attempt has been made to develop a new method of curve fitting in which the difficulties of the procedure of Walker and Schmidt are largely eliminated. Derivation of suitable equations has been made so as to include the effect of competitive inhibitors, both initially present and formed during the reaction. From such equations a calculation of Michaelis constants, dissociation constants of the enzyme–inhibitor complexes and maximal velocities is possible, if the accuracy of the analytic method is sufficiently great. This has been examined for the case of serum cholinesterase.

As will be seen, these equations may also serve to extend the method of Walker and Schmidt to enzyme reactions which proceed in the presence of inhibitors.

(1) Read at the Meeting of the American Chemical Society, Los Angeles, Calif., March 15 to 19, 1953. The following changes in the nomenclature have now been adopted: m instead of k, and f instead of l.

(2) A. C. Walker and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 445 (1944).

(3) Some notations in formulas taken from reference papers have been changed so as to conform with my own nomenclature.

(4) Elaine Elkins-Kaufman and Hans Neurath, *J. Biol. Chem.*, **175**, 893 (1948).

(5) H. T. Huang and Carl Niemann, THIS JOURNAL, **73**, 1541 (1951).

Derivation of Equations for Curve Fitting

In order to change the integrated Michaelis–Menten equation[2,5]

$$k_3[E]t = K_S \ln \frac{[S]_0}{[S]_0 - [P]} + [P] \quad (1)$$

where $k_3[E] = V_m$, to a form which is suitable for treatment as a regression of t on a function of [P], it is necessary to refer the concentration [P] to unity substrate. If this quantity is designated as p, then $p = [P]/[S]_0$. Introducing a new term, r, defined as $r = [S]_0/K_S$, we obtain the useful form of equation (1)

$$t = \frac{K_S}{k_3[E]}\left(\ln \frac{1}{1-p} + rp\right) \quad (2)$$

While this equation may be subjected to the method of least squares, I have preferred to introduce the following additional change. In a plot of p *vs.* t the initial rate is given by the slope m of the tangent through the point of origin, which is, according to the differential Michaelis–Menten equation

$$m = \frac{k_3[E]}{K_S + [S]_0} = \frac{k_3[E]}{K_S(r+1)} \quad (3)$$

Combining equations 2 and 3 one obtains

$$t = \frac{1}{m(r+1)}\left(\ln \frac{1}{1-p} + rp\right) \quad (4)$$

In words, a plot of $\frac{1}{r+1}\left(\ln \frac{1}{1-p} + rp\right)$ *vs.* t is identical with the tangent of the reaction curve, plotted as p *vs.* t, whose slope is the initial rate.

For reactions which proceed in the presence of competitive inhibitors, either present initially or formed during the course of the reaction, an equation formally identical with (4) may be derived. If an inhibitor of the first type is designated by I and an inhibitory reaction product by P, the integrated form of the Michaelis–Menten equation for such a case is, according to Huang and Niemann[5]

$$k_3[E]t = K_S\left(1 + \frac{[S]_0}{K_P} + \frac{[I]}{K_I}\right)\ln \frac{[S]_0}{[S]_0 - [P]} + \left(1 - \frac{K_S}{K_P}\right)[P] \quad (5)$$

where K_I and K_P are the enzyme–inhibitor dissociation constants. Let

$$\frac{1 - K_S/K_P}{1 + [S]_0/K_P + [I]/K_I} \times \frac{[S]_0}{K_S} = r' \quad (6)$$

and

$$\frac{k_3[E]}{[S]_0 + K_S(1 + [I]/K_I)} = m' \quad (7)$$

where m' is again the initial rate in a plot of p *vs.* t, according to the differential equation of Michaelis and Menten. With these definitions of r' and m' equation 5 becomes

$$t = \frac{1}{m'(r'+1)}\left(\ln \frac{1}{1-p} + r'p\right) \quad (8)$$

If we define $\ln \frac{1}{1-p} - p = f$, equation 8 reduces to the simpler form

$$t = \frac{1}{m'}\left(p + \frac{f}{r'+1}\right) \quad (9)$$

The value of f may be obtained readily from a table[6] for any experimental value of p.

To determine the parameter r' the method of least squares is applied with the condition that $d\Sigma d_i^2/dr' = 0$, where d_i are the deviations of the function $p + f/(r'+1)$ of the experimental data from the points of the straight line. The final result of the calculation is

$$r' = \frac{\Sigma(bt_i + d - f_i)^2}{\Sigma(p_i - at_i - c)(bt_i + d - f_i)} - 1 \quad (10)$$

where

$$a = \frac{(n+1)\Sigma t_i p_i - \Sigma t_i \Sigma p_i}{(n+1)\Sigma t_i^2 - (\Sigma t_i)^2} \quad b = \frac{(n+1)\Sigma t_i f_i - \Sigma t_i \Sigma f_i}{(n+1)\Sigma t_i^2 - (\Sigma t_i)^2}$$

$$c = \frac{\Sigma t_i^2 \Sigma p_i - \Sigma t_i \Sigma t_i p_i}{(n+1)\Sigma t_i^2 - (\Sigma t_i)^2} \quad d = \frac{\Sigma t_i^2 \Sigma f_i - \Sigma t_i \Sigma t_i f_i}{(n+1)\Sigma t_i^2 - (\Sigma t_i)^2}$$

With r' thus determined, the slope and intercept are calculated according to the equations

$$m' = a + \frac{b}{r'+1}; \quad -d_0 = c + \frac{d}{r'+1} \quad (11)$$

The intercept is taken as the negative deviation of the determination at $t = 0$, which increases the number of terms to $n + 1$. As a measure of the "goodness" of the fit the standard deviation s is calculated with the formula

$$s = \pm\sqrt{\frac{\Sigma[(p_i - at_i - c) - (bt_i + d - f_i)/(r'+1)]^2}{n-1}}$$

Experimental Results

This method of curve fitting has been applied to a number of different enzymatic reactions with satisfactory results. Some examples are given in Figs. 2, 3 and 4 and in Table I, concerning the tripeptidase activity in human red blood cells and serum. The substrate used was glycylglycylglycine (GGG) in a concentration of 5×10^{-3} molar. The reactions were allowed to proceed at 38° in the presence of $M/30$ phosphate buffer of pH 7.0 and 6.8, respectively, for the hemolyzed cells and serum. No activating ion was added. Other conditions were those indicated in the figures. The progress of the reaction was followed by means of a colorimetric ninhydrin method,[7] essentially the same as that of Schwartz and Engel.[8] It may be seen that in every instance the straight line fits the experimental values rather well. As may be expected, the fit is even better (see comment below) in the rate curves ($F = p$), which were reconstructed from the fitted straight lines.

TABLE I

TRIPEPTIDASE ACTIVITY IN 5 NORMAL SERUMS

Number	r'	$m' \times 10^3$ per minute[a]	$s \times 10^3$[b]
1	1.5	2.9	±15
2	1.6	2.1	±20
3	1.7	2.1	±26
4	1.6	2.2	±17
5	2.3	1.9	±28
Mean	1.7		

[a] For serum dilution 1:5. [b] Standard deviation of the function $p + f/(r'+1)$.

(6) Copies of this table may be obtained on request from the author

(7) G. A. Fleisher, unpublished data.

(8) T. B. Schwartz and F. L. Engel, *J. Biol. Chem.*, **184**, 197 (1950).

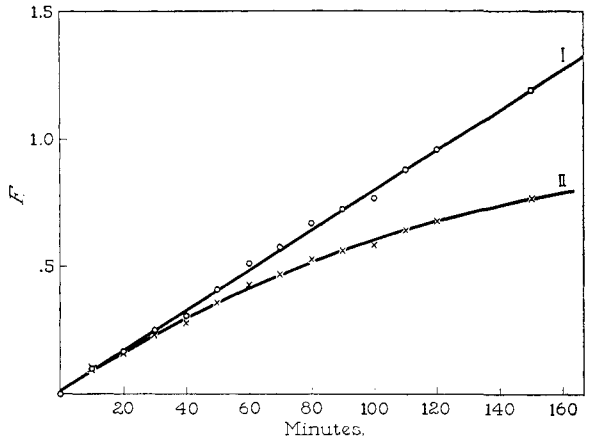

Fig. 2.—Reaction rate of tripeptidase in human red cells (8.8 × 10^7 cells per ml.) with GGG (5 × 10^{-3} molar): I, $F = p + f/1.59$; II, $F = p$.

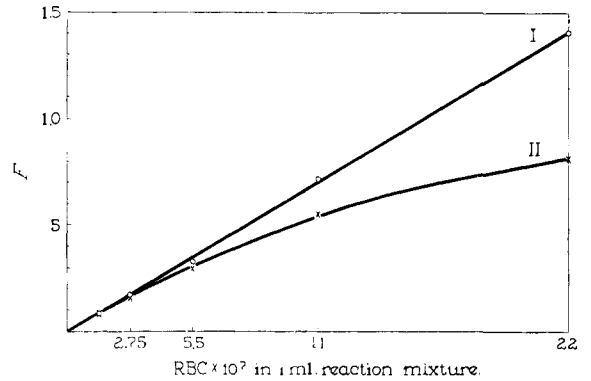

Fig. 3.—Reaction rate of tripeptidase in human red cells (varied) with GGG (5 × 10^{-3} molar); time 60 minutes: I, $F = p + f/1.49$; II, $F = p$.

The reactions shown in Figs. 2 and 3 were performed on hemolyzed cells obtained from the same subject; in the first experiment the time was chosen as the variable, in the second, the enzyme concentration, which, according to equation (5), is inversely proportional to the time. The parameters r' were calculated to be 0.59 and 0.49, a satisfactory agreement, since interchanging these values will alter the rates m' by less than 3%.

Table I is a summary of five experiments performed on the serum from different subjects. With one exception the values of r' agree quite well. This difference may be due to the inaccuracy of the analytic method, as indicated in the standard deviations. It would be fallacious, however, to assume constancy of r' in enzyme preparations as crude as serum, because it may well contain inhibitory substances in varying amounts. If, nevertheless, the individual values of m' were recalculated using the mean $r' = 1.7$, the difference from the corresponding figures in the table would be less than 5%.

Comment

It is fully realized that the calculation of parameters of the integrated Michaelis–Menten equation by the method outlined may have several sources of error. One, inherent in any curve fitting, is that the best fit of the variable $F = p + f/(r' + 1)$ may not necessarily be best for the variable p. If the errors in p and F are called dp_i and dF_i, then $dF_i = (dF/dp)dp_i$. The solution of this equation is $dF_i = [1/(r' + 1)][1/(1 - p) + r']dp_i$. This shows that the quotient dF_i/dp_i increases continuously during the reaction and that this increase is greatest for the smallest r'.

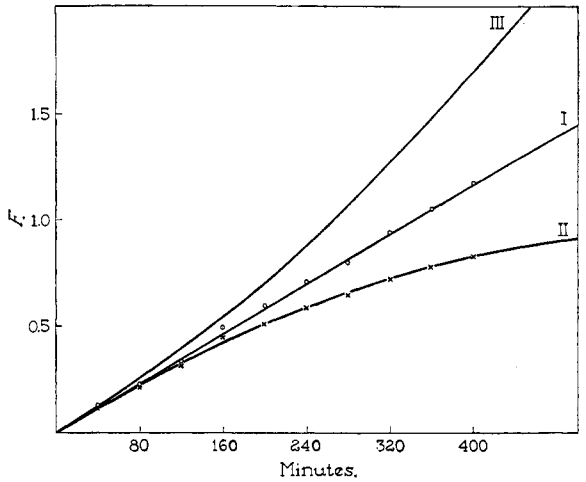

Fig. 4.—Reaction rate of tripeptidase in human serum (dilution 1:5) with GGG (5 × 10^{-3} molar): I, $F = p + f/2.5$; II, $F = p$; III, $F = \ln 1/(1 - p)$.

An additional source of error is introduced by the intercept d_0, since all values for p are actually based on the assumption that $p = 0$ for $t = 0$. This discrepancy must be expected to result in some error in r' and m', depending on the magnitude of d_0. A third error may stem from the assumption that only for the "true" value of r' does the condition $d\Sigma d_i^2/dr' = 0$ hold. This condition is absolutely valid only for the ideal case in which $dp_i = 0$, but in practice it may not hold nearly as well if the experimental points are few, the errors excessively large and their distribution quite irregular.

Determination of Dissociation Constants from the Parameters

For enzyme reactions which can be determined with sufficient accuracy the parameters of the fitted straight lines may be used to calculate dissociation constants and the maximal velocity defined as $V = k_3[E]/\epsilon$. (ϵ is used here for the enzyme concentration based on an arbitrary standard, such as unit volume, unit weight or others, while [E] represents the molar concentration.) If inhibitors are absent, a single reaction will yield K_S and V. With one of the products a competitive inhibitor, two reactions at different initial substrate concentrations are required to solve for K_S, K_P and V. If subscripts 1 and 2 are used to indicate the initial substrate concentrations and the parameters of the two reactions, then according to equation (6)

$$K_S = \frac{r_2' - r_1'}{(r_1'/[S_1])(r_2' + 1) - (r_2'/[S_2])(r_1' + 1)}, \quad K_P = \frac{r_2' - r_1'}{(r_1'/[S_1]) - (r_2'/[S_2])} \qquad (12)$$

while equation (7) results in

$$K_S = \frac{m_2[S_2] - m_1[S_1]}{m_1 - m_2}, \quad V = \frac{[S_2] - [S_1]}{1/m_2 - 1/m_1} \times \frac{1}{\epsilon} \qquad (13)$$

These equations were put to the test for the case of serum cholinesterase (human) and its substrate acetylcholine. This enzyme is known to be competitively inhibited by choline, one of the products of the reaction, and to be completely stable during the reaction. It was also established that this reaction is not reversible to any measurable degree. Two different concentrations of acetylcholine chloride, 6×10^{-3} and 12×10^{-3} molar, were chosen. The buffer was bicarbonate–carbon dioxide at *p*H 7.8 (R_{30}),[9] the serum dilution 1:40, and the temperature 38°. The course of the hydrolysis was followed by the manometric technique. At the higher concentration of acetylcholine measurements were taken at fourteen time intervals before the reaction was about 50% complete. Because of the necessity to maintain the *p*H as constant as possible, higher values of p were not included in the calculation. The lower concentration, however, was followed to completion. Corrections were applied for the non-enzymatic hydrolysis for which first-order kinetics had been established ($k_n = 0.25 \times 10^{-3}$ per minute). Because its contribution to the over-all reactions is small, a correction factor of $m'/(m' - k_n)$ for the values of r' appears to be a reasonable approximation. (If the non-enzymatic reaction becomes more prominent, the Michaelis–Menten kinetics may not be expected to hold.) The values for r' and m' are listed in Table II; while these were determined by calculation, the graphic method gave almost identical results. Table II shows also the dissociation constants and V obtained from the parameters with equations 12 and 13 and by the conventional Lineweaver–Burk plot on the same serum. It may be seen that the agreement of the results obtained by the different methods is satisfactory.

(9) K. B. Augustinsson, *Acta physiol. Scand.*, suppl. 52, **15**, 1 (1948).

TABLE II

DETERMINATION OF K_S, K_P AND V FOR CHOLINESTERASE

a. Parameters of integrated Michaelis–Menten equation (corrected for blanks)

$S_0 \times 10^3$, moles/liter	6.0	12.0
r'	2.42	3.56
$m' \times 10^3$, per minute	11.6	6.4
$s \times 10^{3a}$	±3.3	±2.3

b. Enzyme constants

$K_S \times 10^3$, moles/liter	1.5	Eq. 12
	1.4	Eq. 13
	1.4	Lineweaver–Burk
$K_P \times 10^3$	10.7	Eq. 12
	9.1	Lineweaver–Burk
$V \times 10^3$, moles/liter serum/minute	3.4	Eq. 13
	3.3	Lineweaver–Burk

[a] Standard deviation of the function $p + f/(r' + 1)$.

ROCHESTER, MINN.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Primer Specificity of Potato Phosphorylase[1,2]

BY DEXTER FRENCH AND GENE M. WILD

RECEIVED JUNE 4, 1953

Variation in the ability of various saccharides to "prime" potato phosphorylase is shown by spraying the resolved compounds on paper chromatograms with a mixture of glucose-1-phosphate and phosphorylase, incubating in a moist atmosphere, and locating the regions in which starch synthesis has occurred by spraying with iodine solution. Primer areas appear as spots ranging from nearly colorless or yellow to deep blue, depending on the amount and nature of the saccharide. Inhibiting saccharides appear as characteristic white areas. In order to function as a primer for potato phosphorylase, an amylose chain must contain three glucose units (poor primers) or four or more glucose units (good primers). Substitution at the reducing end-group of amylose chain fragments does not qualitatively change the priming ability.

It is generally recognized that synthesis of polysaccharide from glucose-1-phosphate (G-1-P) by phosphorylase action requires the presence of co-substrate ("primer," "activator" or "catalyst") which may be starch, glycogen or oligosaccharides produced by acid or enzymatic hydrolysis of amylaceous materials.[3-8] However, glucose, maltose and other end-products of diastatic action on starch are non-priming and the cyclic Schardinger dextrins are strong inhibitors of potato phosphorylase.

Swanson and Cori[8] estimated the minimum primer size as 4–5 glucose units, while Weibull and Tiselius[6] claimed that amylotriose (maltotriose) is as effective a primer as higher saccharides. Bailey, Whelan and Peat[9] state that amylotriose is a poor primer and that amylotetraose and higher oligosaccharides are effective primers of essentially equivalent priming ability.

In the initial experiments we explored the problem of the minimum primer requirements in potato phosphorylase synthesis by examining the reverse reaction. Amyloheptaose[10] was subjected to phosphorylase action in the presence of phosphate or

(1) Journal Paper No. J-2336 of the Iowa Agricultural Experiment Station, Ames, Iowa Project 1116. Supported by a grant from the Corn Industries Research Foundation.

(2) Presented before the Division of Biological Chemistry, American Chemical Society, Sept., 1950. Abstracts, 51 C. Taken in part from the M.S. thesis of Gene M. Wild, Iowa State College, 1949.

(3) G. T. Cori and C. F. Cori, *J. Biol. Chem.*, **131**, 397 (1939); **135**, 733 (1940).

(4) C. S. Hanes, *Nature*, **145**, 348 (1940); *Proc. Roy. Soc. (London)*, **B129**, 174 (1940).

(5) D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, **142**, 355 (1942).

(6) C. Weibull and A. Tiselius, *Arkiv Kemi, Mineral. Geol.*, **A19**, No. 19 (1945).

(7) P. H. Hidy and H. G. Day, *J. Biol. Chem.*, **152**, 477 (1944); **160**, 273 (1945); E. C. Proehl and H. G. Day, *ibid.*, **163**, 667 (1946).

(8) G. T. Cori, M. A. Swanson and C. F. Cori, *Federation Proc.*, **4**, 234 (1945); M. A. Swanson and C. F. Cori, *J. Biol. Chem.*, **172**, 815 (1948).

(9) J. M. Bailey, W. J. Whelan and S. Peat, *J. Chem. Soc.*, 3692 (1950).

(10) D. French, M. L. Levine and J. H. Pazur, THIS JOURNAL, **71** 356 (1949).