Determination of initial velocities of enzymic reactions from progress curves

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The present communication describes a novel method for estimating initial velocities (v) of enzyme-catalysed reactions. It is based on an approximation of experimental data obtained by the cubic spline function. The initial velocity of a reaction is calculated as a derivative of the approximating function at a time value equal to zero. The proposed method is usable on a computer with a FORTRAN IV program. The method can be successfully used in such cases as substantial extents of substrate conversion, the inactivation of an enzyme in the course of a reaction, the existence of large experimental error or when the reaction mechanism is unknown.

INTRODUCTION

There are two principal ways for determining the kinetic parameters of enzyme-catalysed reactions: (i) the method of initial velocities and (ii) the method of full kinetic curves. Of these the method of initial velocities, most widely used when the parameters of the Michaelis–Menten equation are to be calculated, is generally employed to ensure more accurate results (Atkins & Nimmo, 1980). It is therefore essential to obtain precise values of initial velocities of enzyme-catalysed reactions. The accurate determination of v is especially important in detailed investigations of kinetic mechanisms and kinetic enzyme variants. Furthermore, difficulties are bound to arise during determination of the initial velocity of a reaction at low substrate concentration (K_m approx. $10\ \mu\text{M}$) or solubility, high degree of product inhibition or in investigations of unstable enzymes. In these cases the determination of the initial velocity on the basis of product concentration at a fixed time of a reaction ($\Delta p/\Delta t$, where p is the product concentration and t is time) is most likely to generate errors, and therefore the use of full kinetic curves (progress curves) is more reliable (Atkins & Nimmo, 1980). Initial velocities may be determined from progress curves by following three approaches (Cornish-Bowden, 1975; Nimmo & Atkins, 1978; Waley, 1981; Boeker, 1982): (i) graphically, by drawing a tangent line to the initial part of kinetic curves, (ii) by employing an integral form of the kinetic equation and (iii) by utilizing a polynomial, produced by statistical methods. The advantages and disadvantages of these methods received a detailed discussion by Cornish-Bowden (1975).

The present paper discusses a new method for determining initial velocities. It is based on the approximation of experimental data obtained by the cubic spline function. It should be mentioned that reference is made to the application of the spline function in the analysis of progress curves by Markus & Plesser (1981). However, their paper does not present either a description of a method or an analysis of the possibilities of its application.

METHOD

The functions of cubic smoothing splines are used for the approximation of ‘noised’ experimental data (Reinsch, 1967). It is possible to show with mathematical precision that the smoothing spline is a group of coupled cubic polynomials that goes near the experimental points, and their first and second derivatives are continuous in the whole range of data under consideration. Further, the second derivative should have a minimal value. By means of physical interpretation, the smoothing spline function may be presented as a flexible ruler, deformable to such an extent that would bring it close to the experimental points (in accordance with the precision of measurements) with a minimal bending effort.

It is convenient to use the cubic smoothing spline function in the analysis of data on enzyme kinetics. The mathematical principles of this method are discussed below. Let us assume that, in the time interval $[0, t_n]$, some measurements of the variable f_i , $i = 0, 1, \dots, n$, at points t_i are made. Smoothing cubic splines are formed by minimizing the following functional:

$$1 = \int_0^t [m(t)]^2 dt + C \sum_{i=0}^n r_i \frac{(f_i - \tilde{f}_i)^2}{f_i} \rightarrow \text{minimum} \quad (1)$$

where $m(t)$ is the second derivative of the approximating function, C is the coefficient (multiplier), the value of which is selected in such a way that the discrepancy between the values of the approximating function and the experimental data correspond to the known precision of the performed measurements, r_i values are weighting factors of experimental points, which are inversely proportional to reaction time (in the case when the relative error is not identical for all experimental points, then r_i is also inversely proportional to the experimental error), f_i is the value of approximating function, and $\tilde{f}_i$ values are experimental data of product concentration.

If we are to presuppose that the second derivatives of the approximating function at boundary points $t = 0$ and $t = t_n$ equal zero (i.e. $m_0 = m_n = 0$), which is necessary as a consequence of the mathematical nature of the spline

method, the values of second derivatives m_i of the approximating function can be obtained from the eqn. (2) by employing the variation calculation principle:

$$m = H \cdot \tilde{F} \cdot (A + H \cdot R^{-1} \cdot H^T)^{-1}$$

in which

$$m = |m_1 \ m_2 \ \dots \ m_{n-1}|^T,$$

$$\tilde{F} = |\tilde{f}_0 \ \tilde{f}_1 \ \dots \ \tilde{f}_n|^T,$$

T indicates matrix transposition,

A is a square matrix, the elements of which are assigned as follows:

$$A = \begin{vmatrix} \frac{h_1+h_2}{3} & \frac{h_2}{6} & \dots & 0 & 0 \\ \frac{h_2}{6} & \frac{h_1+h_3}{3} & \frac{h_3}{6} & \dots & 0 & 0 \\ 0 & \frac{h_3}{6} & \frac{h_3+h_4}{3} & \dots & 0 & 0 \\ \dots & \dots & \dots & \dots & \dots & \dots \\ 0 & 0 & 0 & \dots & \frac{h_{n-1}}{6} & \frac{h_n+h_{n-1}}{3} \end{vmatrix},$$

$$h_i = t_i - t_{i-1},$$

H is a rectangle matrix, the elements of which are thus assigned:

$$H = \begin{vmatrix} \frac{1}{h_1} \left(-\frac{1}{h_1} - \frac{1}{h_2} \right) & \frac{1}{h_2} & \dots & 0 & 0 \\ 0 & \frac{1}{h_2} & \left(-\frac{1}{h_2} - \frac{1}{h_3} \right) & \dots & 0 & 0 \\ \dots & \dots & \dots & \dots & \dots & \dots \\ 0 & 0 & 0 & \dots & \left(-\frac{1}{h_{n-1}} - \frac{1}{h_n} \right) & \frac{1}{h_n} \end{vmatrix},$$

R is a diagonal matrix of the weighting factors of the measurements:

$$R = C \begin{vmatrix} r & 0 & \dots & 0 \\ 0 & r_1 & \dots & 0 \\ \dots & \dots & \dots & \dots \\ 0 & 0 & \dots & r_n \end{vmatrix}.$$

All the elements of matrix R are respectively multiplied by C if the value of C is not equal to one.

When the value of m is determined by the use of eqn. (2) the value of the approximating function F at measurement points t_i is estimated from the expression:

$$F = \tilde{F} - R^{-1} \cdot H^T \cdot m \quad (3)$$

and

$$F = |f_0 \ f_1 \ \dots \ f_n|^T$$

The value of the derivative of approximating function $\dot{f}_i$ at measurement points t_i is established from eqn. (4):

$$\dot{f}_i = \frac{h_{i+1}}{3} \cdot m_i + \frac{h_{i+1}}{6} \cdot m_{i+1} + \frac{f_{i-1} - f_i}{h_{i+1}} \quad (4)$$

The approximating function values between two points, i.e. at a time moment t within $[t_i, t_{i+1}]$, is determined from the expression:

$$f(t) = \frac{m_{i-1}(t_i - t)^3}{6h_i} + \frac{m_i(t - t_{i-1})^3}{6h_i} + \left(f_{i-1} - \frac{m_{i-1}h_i^2}{6} \right) \cdot \left(\frac{t_i - t}{h_i} \right) + \left(f_i - \frac{m_ih_i^2}{6} \right) \cdot \left(\frac{t - t_{i-1}}{h_i} \right) \quad (5)$$

whereas the values of the approximating function derivative at a time moment t within $[t_i, t_{i+1}]$ are calculated from the expression:

$$\dot{f}(t) = \frac{-m_{i-1}(t_i - t)^2}{2h_i} + \frac{m_i(t - t_{i-1})^2}{2h_i} + \frac{f_i - f_{i-1}}{h_i} - \frac{m_i - m_{i-1}}{6} \cdot h_i \quad (6)$$

The initial velocity of an enzyme-catalysed reaction is defined by an equation of the following form:

$$v = \dot{f}(0)$$

Eqns. (2)–(6) fully determine the algorithm for the formation of smoothing splines and can be put to direct use when writing a computer program. We have formed smoothing spline programs in FORTRAN IV with an automatic choice of the coefficient C , which is dependent upon the precision of measurements. The programs are utilized through type PDP-11 computers and are available on request from the Department of Automation, Kaunas Polytechnic Institute.

For the determination of the coefficient C the method of pseudo-position (Shoup, 1979) was used. At the first iteration C was taken as a small number, for example $C_1 = 0.01$, and, consequently, the error of the smoothing spline was larger than the error of measurement known *a priori*. At the second iteration coefficient C was taken as a large number, for example $C_2 = 100$, and, in accordance with that, the error of smoothing spline was less than the error of measurement. At the third iteration C_3 was taken to be equal to $(C_1 + C_2)/2$. The error of smoothing spline was calculated. If this error was larger than the known error of measurement the next iteration of C was from the interval $[C_2, C_3]$, otherwise it was from the interval $[C_1, C_2]$. Iterative calculations of C continued until the error of the smoothing spline and error of measurement were equal with a 10% precision.

RESULTS AND DISCUSSION

Values of the elements of the weight matrix R were estimated as follows. A high value of r_0 (exceeding r_1 by 10000-fold) was chosen on the basis of knowledge *a priori* to the effect that the spline function should commence from the origin in the co-ordinate system (t, p) . It was also assumed that the significance of the experimental points for the determination of the initial velocity is inversely proportional to duration of measurement, e.g. $r_i = 1/t^k$, $i = 1 \dots n$. Experimental computer simulation revealed that the greatest accuracy and precision of determination of initial velocity was achieved at $k = 2$. Table 1 demonstrates the dependence of the accuracy and precision of v on the value of k (power). Consequently, the elements of matrix R were determined as follows: $r_0 = 10000 \ r_1$, $r_1 = 1/t_i^2$, $i = 1, \dots, n$.

To evaluate the accuracy and precision of the method, we performed a computer simulation of some selected cases of enzyme catalysed reactions, which include Michaelis kinetics at different extents of substrate conversion, enzyme deactivation in the course of the reaction, substrate inhibition, product inhibition or

Table 1. Precision of calculations of initial velocity as a function of power k in the weighting-factor equation

The enzymic reaction was examined by computer simulation in accordance with a simple Michaelis–Menten mechanism. Calculations for the deactivating enzyme are also included. Theoretical values: $V = 1$, $K_m = 2$, substrate conversion $X = 0.5$ and variation coefficient of p measurement $C.V.(p) = 5\%$. Four points equidistant in relation to p were taken from the progress curve ($N = 4$). Number of progress curves used for the calculation of v was $n = 100$. Table values of the initial velocity were calculated from the equation:

$$v = \frac{1}{n} \sum \left(\frac{v_{\text{calc.}}}{v_{\text{theor.}}} \cdot 100 \right)$$

where $v_{\text{calc.}}$ is the computer result from one progress curve and $v_{\text{theor.}}$ is the theoretical value. C.V. is the variation coefficient of v determination, calculated with respect to the theoretical value of v ; A_X is the residual activity of deactivating enzyme at extent of substrate conversion X .

k	$s = 1, A_X = 100$		$s = 20, A_X = 10$	
	v (%)	C.V. (%)	v (%)	C.V. (%)
0	92.2	3.7	85.3	4.2
1	92.9	3.9	86.9	4.0
2	94.7	4.3	90.4	4.5
3	96.0	5.1	92.0	5.4
4	96.5	5.7	92.5	6.0
5	96.6	6.0	92.6	6.2

activation. The following kinetic schema illustrates these cases:

The theoretical dependence of product concentration (p) versus time (t) were calculated by using assigned kinetic constants from the following equations:

(i) In the case of the common mechanism by Michaelis–Menten equation:

$$p = V \cdot t - K_m \cdot \ln \left(\frac{s}{s-p} \right)$$

where V is maximal velocity, K_m is the Michaelis constant and s is substrate concentration.

(ii) In the case of substrate inhibition:

$$p = V \cdot t - K_m \cdot \ln \left(\frac{s}{s-p} \right) - \frac{s^2 - (s-p)^2}{2K_s}$$

where K_s is the substrate inhibition constant.

(iii) In the case of product inhibition or activation:

$$t = \left(\frac{c}{a} - \frac{2d}{b} - \frac{b \cdot g}{a^2} \right) \cdot \ln \left(\frac{a \cdot s + b}{a(s-p) + b} \right) + \frac{d}{b} \cdot \ln \left(\frac{s}{s-p} \right) + \frac{g}{a} \cdot p$$

where $a = -\beta V / \alpha K_p$, $b = [1 + (\beta s / \alpha K_p) V]$, $c = 1 + s / \alpha K_p - K_m / K_p$, $d = K_m(1 + s / K_p)$, $g = 1 / \alpha K_p$ and K_p is the product inhibition constant.

Table 2. Influence of point number on the precision of calculations of initial velocity

Computer simulation was accomplished under conditions presented in the legend to Table 1. $s = 1$, $k = 2$.

N	v (%)	C.V. (%)
2	91.8	4.9
3	94.1	4.4
4	94.7	4.3
5	96.4	4.5
6	96.8	4.1

(iv) In the case of enzyme deactivation during the biocatalytical process:

$$p = \frac{V[1 - \exp(-k_d t)]}{k_d} - K_m \cdot \ln \left(\frac{s}{s-p} \right)$$

where k_d is the first-order enzyme deactivation constant.

The theoretical values of p_t were 'noised' corresponding to variation coefficients of measurements at levels equal to 1, 5, 10 or 33%. In order to get measurement 'noises' with normal distribution we have used the standard SSP GAUSS subroutine with corresponding dispersion and mathematical means equalling zero.

For the determination of v , several points approximately equidistant in relation to product concentration were taken from the progress curve. The number of necessary points was estimated by means of computer experimentation. Table 2 demonstrates how the number of points influences the accuracy and precision of v determination. It is obvious that four points (regardless of the extra point at co-ordinate origin) are sufficient for the determination of v by means of the cubic spline function.

Table 3 presents simulation data reflecting the influence of such factors as extent of substrate conversion, enzyme deactivation, substrate inhibition, product inhibition and activation and possible experimental errors on the determination of v . It is evident that the extent of substrate conversion has little influence upon the accuracy of v determination. However, the increase of conversion raises the systematic error of v determination, which reaches the 8% level in the case of combination of substantial extent of conversion (90%) and a considerable error of measurement (variation coefficient 10%). Usually the coefficient of variation of biochemical analytical methods does not exceed 5%. In such cases v values should be regarded as more or less correct. Such factors as product inhibition or activation, substrate inhibition or enzyme deactivation during the reaction have little effect on the results. As can be seen in Table 3, even a 90% loss in the initial activity of an enzyme without any further complicating circumstances results in satisfactory results for the determination of v .

In order to compare the proposed method with other methods known from the literature, we calculated the initial velocities of a number of enzymic reactions using the same theoretical constants for calculations.

The following methods were examined: (i) linearization of experimental data in the co-ordinate system p/t versus p (Boeker, 1982), (ii) approximation by cubic polynomial $t = b_0 + b_1 p + b_2 p^2 + b_3 p^3$ (Nimmo & Atkins,

Table 3. Computer simulation data on the calculations of initial velocity

Constants and K_s , K_p , α and β are explained in the text. Theoretical values: $V = 1$, $K_m = 2$, $N = 4$, $n = 100$ and $k = 2$. See also the legend to Table 1.

Linearization p/t versus p										Cubic in p				Cubic in t				Direct linear plot		Spline	
K_s	K_p	β	α	C.V.(p) (%)	X	s	A_x	(i)		(ii)		(iii)		(iv)		(v)					
								v (%)	C.V. (%)	v (%)	C.V. (%)	v (%)	C.V. (%)	v (%)	C.V. (%)	v (%)	C.V. (%)	v (%)	C.V. (%)		
∞	∞	0	∞	1	0.5	20	100	100.2	1.1	99.9	3.6	99.9	99.9	3.9	99.9	0.9	99.2	0.9	99.2		
∞	∞	0	∞	5	0.5	20	100	99.2	5.5	99.8	18.0	99.8	101.4	20.4	99.4	22.6	98.6	2.9	98.6		
∞	∞	0	∞	10	0.5	20	100	97.5	10.8	99.6	36.0	99.6	115.2	79.3	116.9	169.2	97.9	5.7	97.9		
∞	∞	0	∞	33	0.5	20	100	82.3	29.1	99.1	118.7	99.1	48.0	979.7	321.2	2010.8	91.5	24.0	91.5		
∞	∞	0	∞	1	0.1	1	100	99.9	1.1	100.0	3.6	100.0	100.0	3.9	99.9	1.1	98.9	0.9	98.9		
∞	∞	0	∞	5	0.1	1	100	98.9	5.4	99.9	18.0	99.9	101.5	20.4	102.3	9.1	98.4	2.8	98.4		
∞	∞	0	∞	10	0.1	1	100	97.1	10.8	99.8	36.0	99.8	115.3	78.2	160.5	399.3	97.6	5.7	97.6		
∞	∞	0	∞	33	0.1	1	100	82.0	29.1	99.3	118.7	99.3	-261.0	3942.0	38.1	371.4	91.0	24.1	91.0		
∞	∞	0	∞	1	0.5	1	100	101.1	1.1	100.0	2.9	100.0	98.9	5.0	99.9	1.2	97.1	1.1	97.1		
∞	∞	0	∞	5	0.5	1	100	100.1	5.5	99.7	14.6	99.7	102.5	28.0	102.5	9.4	94.7	4.3	94.7		
∞	∞	0	∞	10	0.5	1	100	98.7	10.9	99.3	29.2	99.3	127.8	175.6	174.8	533.1	92.8	6.4	92.8		
∞	∞	0	∞	33	0.5	1	100	83.0	29.3	97.4	96.3	97.4	47.5	224.1	67.0	100.3	85.6	21.3	85.6		
∞	∞	0	∞	1	0.9	1	100	108.2	1.2	100.8	1.9	100.8	70.0	5.2	99.9	1.4	94.8	1.2	94.8		
∞	∞	0	∞	5	0.9	1	100	107.1	5.7	100.2	9.7	100.2	78.2	36.1	105.4	17.8	93.2	4.6	93.2		
∞	∞	0	∞	10	0.9	1	100	105.5	11.4	99.5	19.4	99.5	194.8	540.9	121.1	210.4	91.0	8.0	91.0		
∞	∞	0	∞	33	0.9	1	100	88.4	30.5	96.2	64.0	96.2	11.2	202.8	72.0	191.2	80.7	20.2	80.7		
∞	∞	0	∞	5	0.5	20	90	99.3	5.5	99.7	17.0	99.7	101.7	22.2	100.2	12.9	97.0	3.2	97.0		
∞	∞	0	∞	5	0.5	20	50	100.2	5.5	99.3	12.8	99.3	103.3	35.3	105.7	13.3	93.1	4.5	93.1		
∞	∞	0	∞	5	0.5	20	10	103.9	5.6	97.4	8.8	97.4	85.4	58.0	55.9	935.2	90.4	4.5	90.4		
∞	∞	0	∞	5	0.5	100	100	98.4	5.4	100.5	21.8	100.5	100.5	15.8	105.7	10.2	103.1	4.3	103.1		
1	∞	0	∞	5	0.5	100	100	96.1	5.3	105.4	26.6	105.4	100.0	12.4	104.4	5.1	104.4	6.1	104.4		
∞	200	0.1	100	5	0.5	20	100	91.0	5.0	92.0	15.9	92.0	94.0	20.1	91.4	15.4	89.6	2.7	89.6		
∞	20	0.1	100	5	0.5	20	100	85.0	4.7	85.2	14.0	85.2	87.1	20.1	86.0	9.0	82.2	3.0	82.2		
∞	1000	1.1	0.001	5	0.1	20	100	101.4	5.5	99.4	21.2	99.4	100.9	16.6	109.2	36.2	104.6	4.1	104.6		
∞	1000	1.5	0.001	5	0.1	20	100	117.3	6.3	109.1	27.9	109.1	114.1	16.0	126.4	17.8	122.5	6.4	122.5		

1978), (iii) approximation by cubic polynomial $p = a_0 + a_1t + a_2t^2 + a_3t^3$ (Nimmo & Atkins, 1978), and (iv) direct-linear-plot method (Cornish-Bowden, 1975).

From the data presented in Table 3 it follows that method (i) is sufficiently accurate, although it produces a slightly greater systematic error in comparison with the spline method. The complexity of the kinetic schema has a comparatively little effect on v determination by this method. Method (ii) is notable for its great accuracy, yet the rate of precision is very low. Method (iii) is inaccurate and extremely sensitive to errors of measurement. Method (iv) gives excellent results in the case of precise measurements irrespective of the reaction mechanism. However, method (iv) is bound to lead to erroneous results when experimental data contain significant errors.

From the above analysis it is obvious that in some cases, particularly when the progress curve roughly coincides with the form of the approximating function, the known methods for the determination of initial velocity may be regarded as superior to the method of cubic smoothing splines. However, the advantage of the cubic smoothing spline function lies in the accuracy and precision of v determination for different kinds of progress curves, with the type of kinetic mechanism playing insignificant role in the determination of v .

Because of its wide application, the method of cubic smoothing splines may be successfully used during the initial stages of analysis of kinetic data, or in the case when the kinetic schema, and subsequently the shape of the progress curve, is unknown. Moreover, this method is especially efficient under the following circumstances: great inaccuracies of measurements, unstable enzyme, non-saturation of enzymes with their substrates etc. Although experimentally more laborious and time-demanding for computer calculation, the proposed method is particularly appropriate for use in complicated circumstances in order to obtain accurate and precise values of the initial velocity.

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