METHOD FOR CALCULATION OF INITIAL AND MAXIMUM REACTION RATES, MICHAELIS CONSTANT, AND DETERMINATION OF THE KIND OF INHIBITION

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The title method was proposed for the use in enzyme reaction kinetics to replace by its simplicity, rapidity and much higher accuracy the tedious and little accurate graphical methods used in biochemical laboratories. The new method can be used in connexion with a common electronic calculator; it is suitable for such enzyme reactions where the measured quantity changes nonlinearly. The values of initial and maximum reaction rates, Michaelis constant, dissociation constant of the enzyme-inhibitor complex, and the kind of inhibition can be directly determined.

The initial and maximum reaction rates, K_m, K_i and the kind of inhibition in enzyme reactions are commonly determined by graphical methods[1]. To determine the initial reaction rates, which serve as a base, the dependences of the reaction rate on time at various substrate concentrations are plotted and the tangents at $t = 0$ are drawn. This method is subject to a large error which can be diminished by graphical differentiation[2]. This, however, in certain cases is also not too accurate and the resulting value of K_m is accordingly influenced. Some authors therefore proposed numerical methods which — with a sufficient accuracy — led as a rule to the use of complicated computation techniques[3-6]. For routine laboratory work, the method of Algranati[7] is preferable, where the calculation of the initial reaction rates is based on the Gregory–Newton interpolation method; however the calculation is rather tedious.

We therefore propose another method that enables to determine besides the initial reaction rates also the maximum reaction rate together with K_m, K_i and the kind of inhibition. This method can replace by its simplicity, rapidity and higher accuracy the tedious and inaccurate graphical methods used in biochemical laboratory work and it requires only a small, common electronic calculator.

THEORETICAL

The dependence of the formation of the reaction product on time has in the case of enzyme reactions the form similar as shown in Fig. 1; in the case of consumption of the determined product they are similar to those in Fig. 2. In this work we replace

these experimental curves by a suitable function, namely for Fig. 1

$$k_i = at_i + bt_i^2 \qquad (1)$$

and for Fig. 2

$$k_i = A + Bt_i + Ct_i^2 , \qquad (2)$$

where k_i denotes concentration of the formed product, t_i time, and a, b, A, B, C coefficients. The replacement of the experimental curve by the function (*1*) with the use of different time intervals of the determination of the enzyme activity is shown in Fig. 3. It is apparent that shortening of the measured time intervals leads to a better approaching of the experimental curve; in our case a five-second interval is sufficient to express the mentioned function with a satisfactory accuracy.

The coefficients a, b, A, B, and C were found for various substrate concentrations by the least squares method. It is necessary to observe the condition that in the case of nonlinear expressions the number of pairs of results must be higher than the number of the coefficients[8]. The coefficients are calculated from equations that express the minimum sum of the squares of the deviations. We proceeded so that

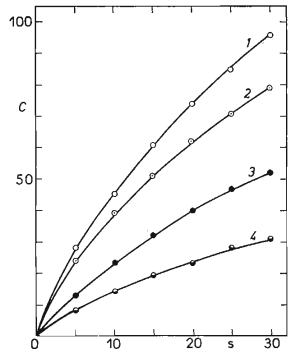

FIG. 1

Activity of Alcoholdehydrogenase C (μmol/dm³ of Formed NADH) at Different Ethyl Alcohol Concentrations

1 0·10; *2* 0·05; *3* 0·02; *4* 0·01 mol/dm³.

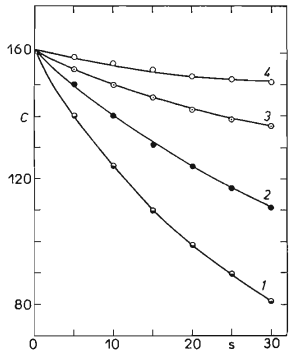

FIG. 2

Activity of Alcoholdehydrogenase C (μmol/dm³ of Oxidised NADH) at Different Acetaldehyde Concentrations

1 100; *2* 50; *3* 20; *4* 10 μmol/dm³.

the chosen expression was rewritten in the form of the initial equation, namely for the expression (*1*):

$$at_i + bt_i^2 - k_i = \Delta_i \,, \tag{3}$$

from which we further obtain

$$\sum \Delta_i^2 = \sum (at_i + bt_i^2 - k_i)^2 \equiv \varphi \,. \tag{4}$$

The coefficients a, b follow from the conditions $(\partial\varphi/\partial a)_b = 0$ and $(\partial\varphi/\partial b)_a = 0$. The resulting equations are

$$a = (\sum k_i - b \sum t_i^2)/\sum t_i \tag{5}$$

$$b = \frac{\sum t_i \sum t_i k_i - \sum t_i^2 \sum k_i}{\sum t_i \sum t_i^3 - (\sum t_i^2)^2} \,, \tag{6}$$

where all summations proceed from $i = 1$ to $i = n$.

Starting from Eq. (*2*) we proceeded by the same method with the only difference that the coefficients A, B, and C were calculated by means of determinants of the third order which were rearranged according to the Sarrus rule[9]:

$$A = D_1/D \,, \quad B = D_2/D \,, \quad C = D_3/D \,, \tag{7}$$

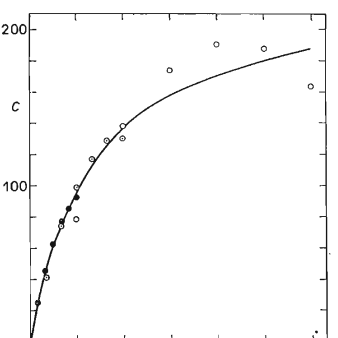

FIG. 3

Experimental Course of Alcoholdehydrogenase Activity (EtOH Concentration 0·1M) and Course of Function *I* Calculated for Duration of Measurement

○ 0—3 min, measuring interval 30 s; ⊙ 0—60 s, interval 10 s; ● 0—30 s, interval 5 s.

$$D = n\sum t_i^2\sum t_i^4 + 2\sum t_i\sum t_i^2\sum t_i^3 - (\sum t_i^2)^3 - n(\sum t_i^3)^2 - (\sum t_i)^2(\sum t)_i^4$$

$$D_1 = \sum t_i^2\sum t_i^4\sum k_i + \sum t_i\sum t_i^3\sum t_i^2k_i + \sum t_i^2\sum t_i^3\sum t_ik_i - \\ -(\sum t_i^2)^2\sum t_i^2k_i - (\sum t_i^3)^2\sum k_i - \sum t_i\sum t_i^4\sum t_ik_i$$

$$D_2 = n\sum t_i^4\sum t_ik_i + \sum t_i^2\sum t_i^3\sum k_i + \sum t_i\sum t_i^2\sum t_i^2k_i - \\ -(\sum t_i^2)^2\sum t_ik_i - n\sum t_i^3\sum t_i^2k_i - \sum t_i\sum t_i^4\sum k_i$$

$$D_3 = n\sum t_i^2\sum t_i^2k_i + \sum t_i\sum t_i^2\sum t_ik_i + \sum t_i\sum t_i^3\sum k_i - \\ -(\sum t_i^2)^2\sum k_i - n\sum t_i^3\sum t_ik_i - (\sum t_i)^2\sum t_i^2k_i .$$

All summations proceed from $i = 1$ to n.

Further we must find the initial reaction rate, v_i, at time $t = 0$. This is defined as the tangent to the reaction curve and can be obtained by differentiation of the functions used. The first derivative of the function (*1*) at $t = 0$ is $k_i' = a$ and of (*2*) is $k_i' = B$. Thus, the coefficients a, B give the slopes of the tangents of the mentioned functions in the origin of coordinates. The value of A gives also the intersection of the curve with the concentration axis of the formed product, hence it should be in cases similar to that mentioned at the end of this paper approximately constant at different substrate concentrations.

To calculate K_m, we use the linear transformation according to Lineweaver and Burk[1] because of its illustrativeness in determining the type of inhibition. Dowd and Riggs[10] do not consider it as the most suitable one of three common linear transformations used, however in this case where the results are doubly corrected by the least squares method it is sufficiently accurate. According to this method during the calculation of K_m we first find the reciprocal values of the initial rates obtained by preceding calculations, the corresponding reciprocal concentrations of the substrate, and we calculate the regression line leading through these points. The value of K_m is then determined, *e.g.*, from the intersection of this line with the axis of reciprocal concentrations, which is equal to $-1/K_m$.

In practice we proceed so that we calculate the reciprocal values of the initial reaction rates v_i and substrate concentrations $[S]$, and introduce them into the linear equation

$$v_i^{-1} = p + r[S]^{-1} . \qquad (8)$$

The coefficients p and r are determined by the least squares method analogously as the coefficient a and b. They are calculated from the following equations:

$$p = \frac{1}{n}(\sum v_i^{-1} - r\sum[S]^{-1}) , \qquad (9)$$

$$r = \frac{\sum[S]^{-1} \sum v_i^{-1} - n \sum[S]^{-1} v_i^{-1}}{(\sum[S]^{-1})^2 - n \sum[S]^{-2}}, \qquad (10)$$

where again all summations proceed from $i = 1$ to n.

By solving Eq. (8) with respect to $[S]^{-1}$ with the equation $v_i^{-1} = 0$ we obtain the intersection of this line with the axis of $[S]^{-1}$. Then from $[S]^{-1} = -K_m^{-1}$ we obtain the value of K_m. By this procedure we obtain the final relation

$$K_m = r/p . \qquad (11)$$

In determining the type of inhibition, we started from the linear relation of Lineweaver and Burk[1]. In the case where the concentration of the substrate changes in the presence of a constant concentration of the inhibitor, in contrast to noninhibited substrate reactions there are, as a rule, two alternatives: the straight lines corresponding to the linear relation between v_i^{-1} and $[S]^{-1}$ intersect either on the axis of $[S]^{-1}$, then the inhibition is noncompetitive, or on the axis of v_i^{-1}, then the inhibition is competitive.

Information about the intersection of the straight lines with the $[S]^{-1}$ axis can be obtained from the value of K_m corresponding to the pure substrate and the apparent values of K'_m corresponding to the substrate with the inhibitor. The intersection of the straight line with the v_i^{-1} axis gives the coefficient p. Hence, if we calculate K_m and K'_m as described above (and so obtain the coefficients p and p'), then we have the following criterions for the type of inhibition:

1) competitive inhibition: $K_m \neq K'_m$, $p \approx p'$,

2) noncompetitive inhibition: $K_m \approx K'_m$, $p \neq p'$. (12)

In cases where the mutual agreement of the K_m or p values is not so apparent as in the example given below, it is necessary to evaluate statistically the significance of the agreement or difference between the parameters K_m and p obtained from repeated measurements and the parameters K'_m and p'. Thus, it is possible to distinguish the competitive, noncompetitive, and mixed inhibition types.

With aid of the coefficient p we can further determine also the maximum reaction rate V, since the section on the v_i^{-1} axis (*i.e.* the parameter p) is equal to V^{-1}. Hence,

$$V = 1/p . \qquad (13)$$

The value of V can be further derived also from the slope of the straight line, *i.e.* from the equation $r = K_m/V$. Hence,

$$V = K_m/r . \qquad (14)$$

TABLE I

Activities of Alcoholdehydrogenase (μmol/dm³ of Formed NADH) for Different Concentrations of Substrate and Inhibitors, Calculation of Coefficients a, b, p, r and Values of V, K_m, K_i, and Determination of Type of Inhibition

Inhibitor	Without				Hydroxylamine, 10^{-2} mol/dm³				*p*-Chloromercuribenzoate $4 . 10^{-6}$ mol/dm³			
Substrate	ethyl alcohol, mmol/dm³				ethyl alcohol, mmol/dm³				ethyl alcohol, mmol/dm³			
	100	50	20	10	100	50	20	10	100	50	20	10
5	28	24	13	8	28	19	12·5	7	18	12	6	3·5
10	45	45	23	14	52	32	24·5	13	34·5	22·5	11	9
t_i, s 15	61	51	32	19	68	44	33·5	18	50	31	16	12
20	74	62	40	23	82	54	40·5	22	62·5	38·5	21·5	15·5
25	85	71	47	28	95	63	47	26	73	44	24	17
30	96	79	52	31	104	72	53	30	85	51	28	20
	$\sum t = 105$, $\sum t^2 = 2275$, $\sum t^3 = 55125$											
$\sum k$	389	326	207	123	329	284	211	116	323	199	107	77
$\sum tk$	7 990	6 660	4 310	2 555	8 815	5 890	4 385	2 425	6 810	4 150	2 250	1 623
a	5·33	4·60	2·62	1·58	5·87	3·68	2·70	1·43	3·78	2·50	1·22	0·90
$-b . 10^2$	7·51	6·91	3·00	1·89	8·23	4·51	3·20	1·51	3.23	2·77	0·99	0·79

$n = 4;\ \sum[S]^{-1} = 0{\cdot}18;\ \sum[S]^{-2} = 0{\cdot}013$			
$\sum v^{-1}$	1 419	1 510	2 585
$\sum[S]^{-1} v_i^{-1}$	88·55	95·47	161·91
p	127·9	125·0	227·6
r	5 041	5 613	9 304
K_m, mol/dm^3	$3{\cdot}94 \cdot 10^{-2}$	$4{\cdot}49 \cdot 10^{-2}$	$4{\cdot}09 \cdot 10^{-2}$
V, mol/dm^3 s	$7{\cdot}8 \cdot 10^{-3}$	$8{\cdot}0 \cdot 10^{-3}$	$4{\cdot}4 \cdot 10^{-3}$
Inhibition	—	competitive	noncompetitive
K_i, mol/dm^3	—	$8{\cdot}81 \cdot 10^{-2}$	$4{\cdot}73 \cdot 10^{-4}$

Assuming the validity of the reaction mechanism described by Lineweaver and Burk[1], we can use the coefficient r, which gives the slope of the straight line, to calculate the dissociation constant of the enzyme–inhibitor complex, K_i. These authors found that the slope of the straight line with both inhibition types is larger by the value of $1 + [I]/K_i$ as compared with the straight line of the pure substrate without the inhibitor. If r' denotes the new slope of the line then

$$r' = r(1 + [I]/K_i), \tag{15}$$

where $[I]$ denotes inhibitor concentration. Hence,

$$K_i = r[I]/(r' - r). \tag{16}$$

TABLE II

Activities of Alcoholdehydrogenase (μmol/dm^3 of Oxidized NADH) for Different Concentrations of Acetaldehyde, Calculation of Coefficients A, B, C, p, r and Values of K_m and V

Substrate	Acetaldehyde, μmol/dm^3			
	100	50	20	10
5	140	150	155	159
10	134	140	150	157
15	110	131	146	155
t_i, s 20	99	124	142	153
25	90	117	139	152
30	81	111	137	151
$n = 6$, $\sum t = 105$, $\sum t^2 = 2\,275$, $\sum t^3 = 55\,125$, $\sum t^4 = 1\,421\,875$				
$\sum k$	654	773	869	927
$\sum tk$	10 350	12 850	14 890	16 080
$\sum t^2 k$	210 400	269 850	318 700	346 650
A	160·3	160·6	160·7	161·7
$-B$	3·63	2·25	1·20	0·55
$C \cdot 10^2$	3·21	2·00	1·36	0·64

$n = 4$; $\sum [S]^{-1} = 0{\cdot}18$; $\sum [S]^{-2} = 0{\cdot}013$; $\sum v_i^{-1} = -3{\cdot}369$; $\sum [S]^{-1} v_i^{-1} = -0{\cdot}2349$

$p = -7{\cdot}736 \cdot 10^{-2}$; $r = -16{\cdot}998$; $K_m = 2{\cdot}19 \cdot 10^{-4}$ mol/dm^3; $V = 1{\cdot}29 \cdot 10^{-5}$ mol/dm^3 s

As an illustration, we shall discuss the following example. We chose alcoholdehydrogenase to show that our method can be used not only for simple one-substrate reactions but also here for a two-substrate reaction, where the other substrate from the kinetic point of view is NAD, subject to an ordered mechanism, and where the modified Michaelis equation applies. At the same time we can show the solution for both types of enzyme reactions, where the measured quantity can either increase or decrease.

Example

Alcohol dehydrogenase (EC 1.1.1.1 and 1.1.1.2) catalyses the general reaction $R{-}CH_2{-}OH + NAD^+ \rightleftarrows R{-}CHO + NADH + H^+$. We used the enzyme isolated from yeast and worked at 25°C and pH 7·9. The activities for different concentrations of substrates (ethyl alcohol, acetaldehyde) and inhibitors (hydroxylamine, *p*-chloromercuribenzoate) are given in Tables I and II.

First we shall discuss the case of splitting of ethyl alcohol (values from Table I are illustrated in Fig. 1) and inhibition of this reaction by both inhibitors. From the text follows the use of Eq. (*1*). To calculate the coefficients *a* and *b* from (*5*) and (*6*), we shall need the values of $\sum t$, $\sum t^2$, $\sum t^3$, $\sum k$, and $\sum tk$. As already mentioned, the coefficient *a* gives the initial reaction rate v_i, in our case in μmol/dm^3 s. This value, expressed in mmol/dm^3 s, is introduced in Eq. (*8*) (also the alcohol concentration is expressed in mmol/dm^3), then the coefficients *p* and *r* are calculated with the aid of Eqs (*9*) and (*10*), further the values of K_m, *V*, and K_i according to Eqs (*11*)—(*13*) and (*16*), and the type of inhibition is determined. The results are given in Table I. It is seen that hydroxylamine is a competitive inhibitor since $K_m \neq K'_m$ and $p \approx p'$. The other inhibitor, *p*-chloromercuribenzoate, is noncompetitive since $K_m \approx K'_m$ and $p \neq p'$.

If we solve this example for an opposite reaction course, *i.e.*, the formation of ethyl alcohol from acetaldehyde (Fig. 2), we must use Eq. (*2*) for the calculation of the mentioned values. The method of calculations with the aid of Eq. (*7*) will be the same. The initial reaction rate v_i is here given by the coefficient *B* (in μmol/dm^3 s), which is together with the corresponding acetaldehyde concentration introduced into Eq. (*8*) (the μmol/dm^3 units are used), then with the aid of Eqs (*9*) and (*10*) the coefficients *p* and *r* are found, and finally from (*11*) and (*13*) the quantities K_m and *V*. The calculation procedure is illustrated by Table II.

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