酵素觸媒反應의 速度變數決定의 새로운 方法

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(1978. 11. 17 접수)

A New Method of Estimation of Kinetic Parameters
for Enzyme-Catalyzed Reactions

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(Received Nov. 17, 1978)

요 약. 효소촉매반응의 전체 시간과정을 최소제곱법에 의해 분석하는 새로운 방법을 제시하였다. 이 방법은 프로그램이 가능한 소형능력의 계산기만으로도 가능하며 저해받지 않는 효소반응에도 적용할 수 있고 생성물이나 외부에서 가해준 화합물에 의해 저해받는 효소반응에도 적용할 수 있다. 이 방법은 데이타를 비선형 관계의 반응물 농도와 시간 곡선에 맞추기 때문에 직선의 식으로 변환시켜 최소제곱법을 적용시키는 다른 방법보다 속도변수의 값을 더 정확하게 평가할 수 있다.

ABSTRACT. A new least square method for analysis of the whole time course of enzyme-catalyzed reactions is presented. This method requires only a programmable calculator with small capacity and is applicable to both uninhibited reactions and reactions inhibited by products or added compounds. This method fits the data to the nonlinear plot of substrate concentration *vs.* time, and, consequently, estimates the kinetic parameters better than the least square method based on linearly transformed equations.

INTRODUCTION

The importance of kinetic measurements in elucidation of enzyme mechanisms cannot be overemphasized. [1~3] Kinetic parameters such as k_{cat}, K_m, and K_i are estimated from the kinetic results and these give plenty of information about the nature of catalytic residues on the active site and binding mode of substrates and inhibitors to the active site. Conventional methods for estimation of kinetic parameters consist of measurement of initial velocity (v_0) and analysis of the dependency of v_0 on the initially-added substrate concentration (S_0) by Lineweaver-Burk plot or its analogs[3]. Thus, for a given run emphasis is given to only the initial part of the reaction in order to obtain the initial velocity. Furthermore, runs at several different S_0 values are needed to obtain values of kinetic parameters with Lineweaver-Burk plot or its analogs.

If the whole time course of an enzymatic reaction is analyzed according to the integrated rate expression, the kinetic parameters for reactions following the simple Michaelis-Menten scheme can be estimated from a single run. In addition, this analysis is the best way to demonstrate that the kinetic scheme under con-

sideration is consistent with the data once the enzyme activity, temperature, pH, the ionic strength, and other conditions are known to be maintained constant during the reaction.

Recent advent of high-speed computers allowed more efficient and accurate data analysis of the kinetic runs. However, computer analysis is limited to those who have the pertinent programs. Besides, computer analysis is still uneconomical in terms of the time needed for preparation of input cards as well as the cost.

In this article, a new method for analyzing the whole time course of an enzymatic reaction by the least square method is presented. A commercially available programmable-calculator with small capacity is enough for data treatment with this method.

METHODS

Data analysis by linear and nonlinear least square method was performed with a Texas Instruments Programmable Slide-Rule Calculator Model SR-56.

RESULTS AND DISCUSSION

The schemes for reactions catalyzed by unisubstrate enzymes[2] are listed in *Table* 1. Also included in this table are reactions inhibited in various modes. These schemes lead to rate expressions generally represented by equation 1[2] when steady-state approximation is applied to ES and ES' under the condition of E_0 (total enzyme concentration) $\ll S_0$.

$$v = \frac{-dS}{dt} = \frac{aE_0(S)}{b+(S)} \tag{1}$$

The integrated form of equation 1 is

$$t = A\ln\frac{S_0}{(S)} + B[S_0 - (S)] \tag{2}$$

The expressions of A and B for various

Table 1. Schemes for the enzymatic reactions considered.

No.	Scheme	Remark
I	$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E+P_i$	Simple Michaelis-Menten Scheme
II	$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} ES' \overset{k_3}{\longrightarrow} E+P_2$ $+$ P_1	ES' is a covalent intermediate.
III	$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E+P_i$ $E+P_1 \underset{K_i}{\rightleftharpoons} EP_1$	Competitive product inhibition
IV	$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E+P_i$ $E+I \underset{K_i}{\rightleftharpoons} EI$	Competitive inhibition
V	$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E+P_i$ $K_i \| I \qquad I \| K_i$ $EI+S \rightleftharpoons ESI$	Noncompetitive inhibition
VI	$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E+P_i$ $I \| K_i$ ESI	Uncompetitive inhibition

徐 正 憲

Table 2. Expression for parameters of equation 2 and methods of estimation of enzymatic kinetic parameters. [a]

Scheme	A	B	Methods of estimation of kinetic parameters
I	$\dfrac{K_m}{k_{cat}E_0}$	$\dfrac{1}{k_{cat}E_0}$	$k_{cat}=\dfrac{1}{E_0B}$, $\quad K_m=\dfrac{A}{B}$, $\quad \dfrac{k_{cat}}{K_m}=\dfrac{1}{E_0A}$
II	$\dfrac{K_m\text{app}}{k_{cat}E_0}$	$\dfrac{1}{k_{cat}E_0}$	$k_{cat}=\dfrac{1}{E_0B}$, $\quad K_m\text{app}=\dfrac{A}{B}$, $\quad \dfrac{k_{cat}}{K_m\text{app}}=\dfrac{1}{E_0A}$
III	$\dfrac{\dfrac{K_m}{K_i}(S_0+K_i)}{k_{cat}E_0}$	$\dfrac{1-\dfrac{K_m}{K_i}}{k_{cat}E_0}$	Plot of E_0A vs. S_0 : slope $=\alpha=(K_m/K_i)/k_{cat}$ and intercept $=\beta=K_m/k_{cat}$ $\quad \therefore k_{cat}=\dfrac{1}{E_0B+\alpha}$, $\quad K_m=\dfrac{\beta}{E_0B+\alpha}$, $\quad \dfrac{k_{cat}}{K_m}=\dfrac{1}{\beta}$, $\quad K_i=\dfrac{\beta}{\alpha}$
IV	$\dfrac{K_m\left(1+\dfrac{I_0}{K_i}\right)}{k_{cat}E_0}$	$\dfrac{1}{k_{cat}E_0}$	Plot of E_0A vs. I_0: slope $=\alpha=K_m/k_{cat}K_i$ and intercept $=\beta=K_m/k_{cat}$ $\quad \therefore k_{cat}=\dfrac{1}{E_0B}$, $\quad K_m=\dfrac{\beta}{E_0B}$, $\quad \dfrac{k_{cat}}{K_m}=\dfrac{1}{\beta}$, $\quad K_i=\dfrac{\beta}{\alpha}$
V	$\dfrac{K_m\left(1+\dfrac{I_0}{K_i}\right)}{k_{cat}E_0}$	$\dfrac{1+\dfrac{I_0}{K_i}}{k_{cat}E_0}$	Plot of E_0A vs. I_0 : slope $=\alpha_1=(K_m/K_i)/k_{cat}$ and intercept $=\beta_1=K_m/k_{cat}$ Plot of E_0B vs. I_0 : slope $=\alpha_2=1/k_{cat}K_i$ and intercept $=\beta_2=1/k_{cat}$ $\quad \therefore k_{cat}=\dfrac{1}{\beta_2}$, $\quad K_m=\dfrac{\beta_1}{\beta_2}$, $\quad \dfrac{k_{cat}}{K_m}=\dfrac{1}{\beta_1}$, $\quad K_i=\dfrac{\beta_1}{\alpha_1}$ or $\dfrac{\beta_2}{\alpha_2}$
VI	$\dfrac{K_m}{k_{cat}E_0}$	$\dfrac{1+\dfrac{I_0}{K_i}}{k_{cat}E_0}$	Plot of E_0B vs. I_0 : slope $=\alpha=1/k_{cat}K_i$ and intercept $=\beta=1/k_{cat}$ $\quad \therefore k_{cat}=\dfrac{1}{\beta}$, $\quad K_m=\dfrac{E_0A}{\beta}$, $\quad \dfrac{k_{cat}}{K_m}=\dfrac{1}{E_0A}$, $\quad K_i=\dfrac{\beta}{\alpha}$

[a] $k_{cat}=k_2$ and $K_m=(k_{-1}+k_2)/k_1$ for schemes except Scheme II. For Scheme II, $k_{cat}=k_2k_3/(k_2+k_3)$ and $K_m\text{app}=k_3(k_{-1}+k_2)/(k_2+k_3)k_1$. k_i's are as defined in *Table* 1.

Schemes are summarized in *Table* 2. Thus, equation 2 represents the general equation of the whole time course of enzyme-catalyzed reactions.

The least square treatment on equation 2 requires minimization of χ^2 defined by equation 3[4].

$$\chi^2=\sum_i \frac{\{t_i-A\ln\dfrac{S_0}{(S)_i}-B[S_0-(S)_i]\}^2}{\sigma_{t_i}{}^2} \qquad (3)$$

Here, σ_{t_i} is the standard deviation of t_i. Because t_i is the time reading for each data point, σ_{t_i} can be regarded as being constant under normal experimental conditions. Thus, one obtains

$$\chi^2=\frac{1}{\sigma_t^2}\sum_i \{t_i-A\ln\frac{S_0}{(S)_i}-B[S_0-(S)_i]\}^2 \qquad (4)$$

By setting $\dfrac{\partial\chi^2}{\partial A}=0$ and $\dfrac{\partial\chi^2}{\partial B}=0$ in order to minimize χ^2, one obtains

$$A=\frac{1}{D}\begin{vmatrix} \sum_i t_i\ln\dfrac{S_0}{(S)_i} & \sum_i[S_0-(S)_i]\ln\dfrac{S_0}{(S)_i} \\ \sum_i t_i[S_0-(S)_i] & \sum_i[S_0-(S)_i]^2 \end{vmatrix} \qquad (5)$$

$$B=\frac{1}{D}\begin{vmatrix} \sum_i[\ln\dfrac{S_0}{(S)_i}]^2 & \sum_i t_i\ln\dfrac{S_0}{(S)_i} \\ \sum_i[S_0-(S)_i]\ln\dfrac{S_0}{(S)_i} & \sum_i t_i[S_0-(S)_i] \end{vmatrix} \qquad (6)$$

$$D=\begin{vmatrix} \sum_i[[\ln\dfrac{S_0}{(S)_i}]]^2 & \sum_i[S_0-(S)_i]\ln\dfrac{S_0}{(S)_i} \\ \sum_i[S_0-(S)_i]\ln\dfrac{S_0}{(S)_i} & \sum_i[S_0-(S)_i]^2 \end{vmatrix} \qquad (7)$$

Standard deviations of A and B can be derived by equations 8 and 9[4].

$$\sigma_A^2=\sum_j\sigma_{t_j}^2\left(\frac{\partial A}{\partial t_j}\right)^2=\sigma_t^2\sum\left(\frac{\partial A}{\partial t_j}\right)^2 \qquad (8)$$

$$\sigma_{\dot{B}}^2 = \sum_j \sigma_{t_j}^2 \left(\frac{\partial B}{\partial t_j}\right)^2 = \sigma_t^2 \sum_j \left(\frac{\partial B}{\partial t_j}\right)^2 \qquad (9)$$

Thus,

$$\sigma_A = \sigma_t \sqrt{\frac{\sum_i [S_0 - (S)_i]^2}{D}} \qquad (10)$$

$$\sigma_B = \sigma_t \sqrt{\frac{\sum_i [\ln \frac{S_0}{(S)_i}]^2}{D}} \qquad (11)$$

where σ_t can be given by equation 12[4].

$$\sigma_t^2 = \frac{1}{N-2} \sum_i \{t_i - A \ln \frac{S_0}{(S)_i} - B(S_0 - (S)_i)\}^2 \qquad (12)$$

Here, N is the number of data points.

Analysis of each kinetic run according to equations 5, 6, 10, and 11 leads to the values of A, B, and their standard deviations.

When the enzyme reaction follows Scheme I or II, values of the kinetic parameters, k_{cat}, K_m, and k_{cat}/K_m, can be calculated directly from the values of A and B of a single run as indicated in *Table* 2. When several runs are performed, weighted average of the values of the kinetic parameters determined from each run can be obtained according to equations 13 and 14[4].

$$\bar{P} = \frac{\sum_i (P_i/\sigma_{P_i})^2}{\sum_i (1/\sigma_{P_i})^2} \qquad (13)$$

$$\bar{\sigma}_P = \{\sum_i (1/\sigma_{P_i}^2)\}^{-\frac{1}{2}} \qquad (14)$$

Here, $\bar{P}$ and $\bar{\sigma}_P$ are the average value and the average standard deviation, respectively, of the parameter P (*i.e.* A or B) and P_i and σ_{P_i} are the values of P and its standard deviation, respectively, determined from the ith kinetic run. In order to obtain good estimation of the parameter values from these equations, runs with S_0 values near K_m must be performed.

For Schemes III~VI, linear plots of E_0A or E_0B obtained at several S_0 or I_0 values *vs.*

respective S_0 or I_0 are needed to obtain the values of the kinetic parameters as shown in *Table* 2. Weighted regression of lines represented by $y = \alpha x + \beta$ can be carried out with equations 15~19[4].

$$\alpha = \frac{1}{\varDelta} \left\{ \sum_i \frac{1}{\sigma_i^2} \sum_i \frac{x_i y_i}{\sigma_i^2} - \sum_i \frac{x_i}{\sigma_i^2} \sum_i \frac{y_i}{\sigma_i^2} \right\} \qquad (15)$$

$$\beta = \frac{1}{\varDelta} \left\{ \sum_i \frac{x_i^2}{\sigma_i^2} \sum_i \frac{y_i}{\sigma_i^2} - \sum_i \frac{x_i}{\sigma_i^2} \sum_i \frac{x_i y_i}{\sigma_i^2} \right\} \qquad (16)$$

$$\sigma_\alpha = \frac{1}{\varDelta} \sum_i \frac{1}{\sigma_i^2} \qquad (17)$$

$$\sigma_\beta = \frac{1}{\varDelta} \sum_i \frac{x_i^2}{\sigma_i^2} \qquad (18)$$

$$\varDelta = \sum_i \frac{1}{\sigma_i^2} \sum_i \frac{x_i^2}{\sigma_i^2} - \left\{\sum_i \frac{x_i}{\sigma_i^2}\right\}^2 \qquad (19)$$

Here, σ_α, σ_β, and σ_i are the standard deviations of α, β, and y_i (the value of y determined from the ith run), respectively. It should be kept in mind that several y_i's must be measured at low x_i's to obtain good estimation of the intercept and a relatively wide range of x_i must be tried to obtain good estimation of the slope.

From the weighted average of E_0A or E_0B whichever is independent of S_0 or I_0 and from the values of α and β, the values of the kinetic parameters are calculated as summarized in *Table* 2. For example, when Scheme III is operative, values of E_0A and E_0B are obtained at several S_0 values. Because E_0B is a constant value, its weighted average and weighted standard deviation ($\overline{E_0B}$ and $\bar{\sigma}_{E_0B}$) are calculated with equations 13 and 14. Weighted linear regression of the plot of $(E_0A)_i$ *vs.* $(S_0)_i$ produces the values of the intercept, the slope, and their standard deviations (β, α, σ_β, and σ_α). Then, the values of k_{cat}, K_m, k_{cat}/K_m, and K_i are taken as $1/(\overline{E_0B}+\alpha)$, $\beta/(\overline{E_0B}+\alpha)$, $1/\beta$, and β/α. The values of standard deviations of these kinetic parameters can be obtained by the rule of propagation of error[4] as indicated by equation 20.

$$\sigma_R^2=\sigma_U^2\left(\frac{\partial R}{\partial U}\right)^2+\sigma_V^2\left(\frac{\partial R}{\partial V}\right)^2 + \cdots\cdots \quad (20)$$

when R is a function of U, V, $\cdots\cdots$.

Linear equations 21~24 which are obtained by rearranging equation 2 have been used in analyzing the rate data[3].

$$\frac{\ln\frac{S_0}{(S)}}{t}=A_1\frac{S_0-(S)}{t}+B_1 \quad (21)$$

$$\frac{t}{S_0-(S)}=A_2\frac{\ln\frac{S_0}{(S)}}{S_0-(S)}+B_2 \quad (22)$$

$$\frac{t}{\ln\frac{S_0}{(S)}}=A_3\frac{S_0-(S)}{\ln\frac{S_0}{(S)}}+B_3 \quad (23)$$

$$\frac{S_0-(S)}{t}=A_4\frac{\ln\frac{S_0}{(S)}}{t}+B_4 \quad (24)$$

In principle, linear regression on these equations can produce the parameter values. Thus, plot of $\dfrac{\ln\frac{S_0}{(S)}}{t}$ vs. $\dfrac{S_0-(S)}{t}$ gives a slope of $A_1(=-B/A)$ and an intercept of $B_1(=1/A)$. For a linear line represented by equation 25, the least square method minimizes $\chi^{2\prime}$ defined by equation 26.

$$y=ax+b \quad (25)$$

$$\chi^{2\prime}=\sum_i\frac{\{y_i-ax_i-b\}^2}{\sigma_{y_i}^2} \quad (26)$$

where σ_{y_i} is the standard deviation of the individual measurement y_i. Assuming that $\sigma_{y_i}^2$ is constant, equation 26 becomes

$$\chi^{2\prime} = 1/\sigma_y^2\sum\{y_i-ax_i-b\}^2 \quad (27)$$

By putting $\dfrac{\partial\chi^{2\prime}}{\partial a}=0$ and $\dfrac{\partial\chi^{2\prime}}{\partial b}=0$, the widely used epuations for the linear least square method are obtained[4]. Thus, the validity of the assumption that the standard deviations of y_i's are equal, is essential for this regression method.

This assumption is hardly met for equations 21~24. For example, when equation 21 is considered, equation 20 gives $\sigma_{y_i}^2$ as

$$\sigma_{y_i}^2=\sigma^2\left\{\frac{\ln\frac{S_0}{S}}{t_i}\right\}=\frac{1}{t_i^2(S)_i^2}\sigma_{(S)_i}^2$$

$$+\left\{\frac{\ln\frac{S_0}{(S)_i}}{t_i^2}\right\}^2\sigma_{t_i}^2 \quad (28)$$

Under normal instrumental conditions, $\sigma_{(S)_i}^2$ and $\sigma_{t_i}^2$ can be regarded as being constant. Thus, equation 28 shows that $\sigma_{y_i}^2$ cannot be constant.

Besides, one of the basic assumptions in least square method is that the independent variables are correctly measurable, their standard deviations being zero. For example, when equation 21 is considered.

$$\sigma^2\left\{\frac{S_0-(S)_i}{t_i}\right\}=\frac{1}{t_i^2}\sigma_{(S)_i}^2+\left\{\frac{S_0-(S)_i}{t_i^2}\right\}^2\sigma_{t_i}^2=0 \quad (29)$$

Thus, $\sigma_{(S)_i}^2$ and $\sigma_{t_i}^2$ are assumed to be zero when the least square treatment, on equation 21 is performed. This means that $\sigma_{y_i}^2$ given by equation 28 is also zero and, consequently, the dependent variables as well as the independent ones are correctly measurable.

This consideration indicates that linear regression on rate data according to the linearly transformed equations 21~24 is less logical.

Sample data were collected from carboxypeptidase A-catalyzed hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate which was carried out in 0.5 M NaCl, 0.05 M Tris, pH7.50 buffer solutions at 25°C. The data are illustrated in *Fig.* 1. Also illustrated in *Fig.* 1 is the theoretical curve obtained by nonlinear regression based on equations 5~12. The same data have been analyzed according to equation 21, one of the linearly transformed rate expressions, and the linear plot and the calculated line are illustrated in *Fig.* 2. As shown by *Fig.* 2, data points obtained in the early stage of the reaction are scattered more than those obtained in the later stage, as expected by the propagation of error expressed by equation 28.

The new method developed in this article permits estimation of enzymatic kinetic parameters using commercially available calculators. The calculator used for analysis of the data illustrated in *Fig.* 1 had a programming capacity of only 100 steps. Besides, this method is based on the integrated rate expression instead of the initial velocities, and, thus, much smaller number of enzymatic runs are needed for estimation of kinetic parameters.
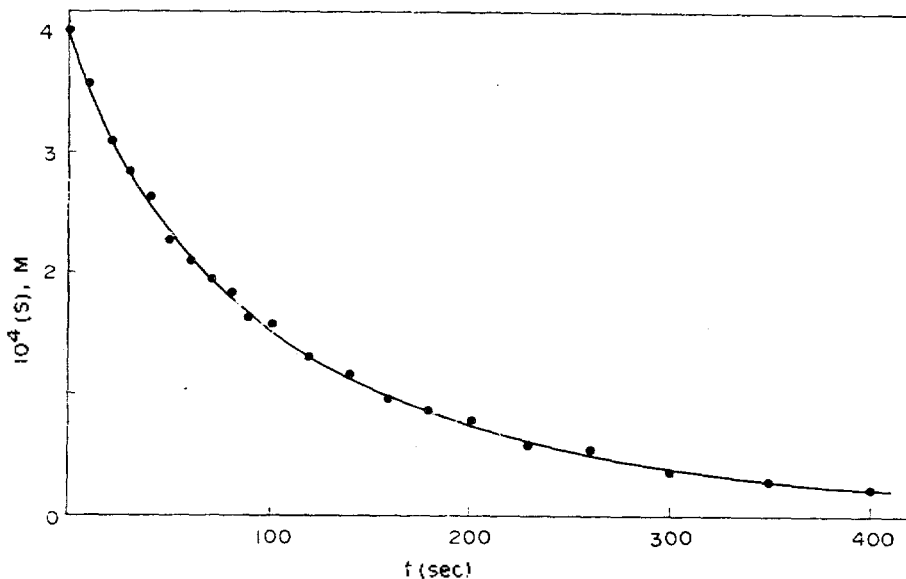

Fig. 1. Sample data obtained from carboxypeptidase A $(1.35 \times 10^{-7} M)$ catalyzed hydrolysis of O-(*trans*-cinnamoyl)-L-β-phenyllactate $(0.4 \, mM)$ at pH 7.5 and 25°C. The line drawn is constructed according to equation 2. The estimated parameter values are: $A = (1.67 \pm 0.03) \times 10^2$ sec and $B = (-2.43 \pm 0.15) \times 10^5$ sec·M^{-1}. The value of B is negative because the reaction is competitively inhibited by the product (Scheme III).
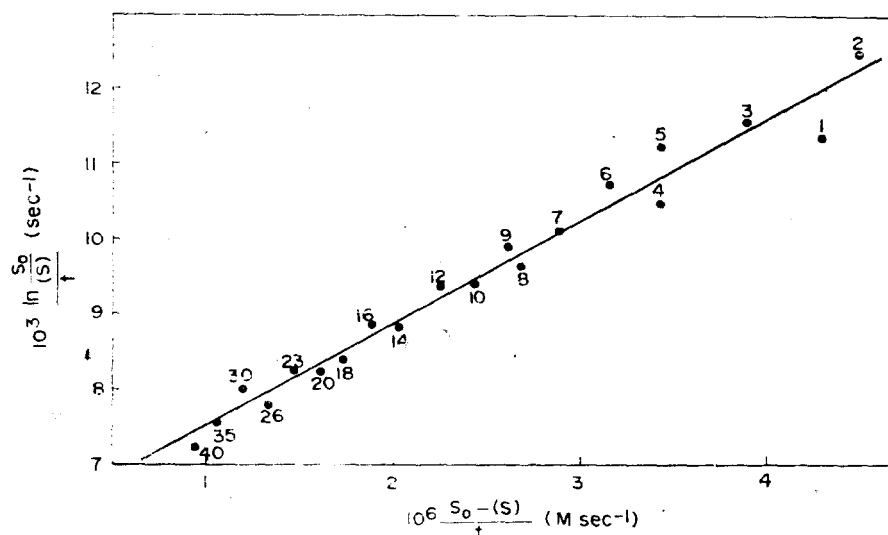

Fig. 2. Plot of the sample data according to equation 21. The estimated parameter values are $A_1 = (1.36 \pm 0.06) \times 10^3 \, M^{-1}$ and $B_1 = (6.17 \pm 0.16) \times 10^{-3}$ sec^{-1}. The number near each data point indicates the time (sec) for each measurement divided by 10.

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