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DETERMINATION OF RATE CONSTANTS OF FIRST ORDER ENZYME REACTION WITH DISSOCIABLE MODIFIER

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The Laplace transformation has been applied to the study of the analysis of the rate equations for some first order enzyme reaction systems with some dissociable activator and inhibitor. According to the analytical results the method of determination of rate constants for Michaelis scheme by measuring the lag time has been expanded to the first order enzyme reaction system modified by some dissociable modifier and the new method has been applied to the determination of the rate constants of activation of papain by cysteine using α-tosyl-L-arginine methyl ester as substrate.

There are many ways of studying the mechanism of the reaction catalyzed by a given enzyme. Among them the determination of rate constants of the reaction, assuming that it proceeds according to an appropriate mechanism, is the most typical one. The rate constant of the formation of some enzyme-substrate complex was determined by Gutfreund from the lag time of presteady-state part of the reaction[1] and also by Slater from the relationship between Michaelis constant and velocity at infinite substrate concentration[2], respectively. The methods which were employed in their works were to determine the rate constant of simple first order enzyme reaction (Scheme I).

$$\overset{e}{\mathrm{E}} + \overset{s}{\mathrm{S}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \overset{x}{\mathrm{ES}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E} + \overset{p}{\mathrm{P}}$$

Scheme I

It is excellent to use the lag time for the determination of rate constants, because it gives us the most precise information from the transient state experiments. Some modification is necessary to apply this method to the reactions of other schemes.

The author has studied the analysis of first order enzyme reaction with some dissociable activator and inhibitor. This study has been carried out with the system in which the substrate concentration was very high compared with the enzyme concentration and did not fall so rapidly in the beginning of the reaction. It has been found that three kinds of experimental methods are available for the study of enzyme reactions. Any two of the following constituents, enzyme, substrate and activator or inhibitor, were first mixed and after a certain lapse of time enough

1) H. Gutfreund, *Discuss. Faraday Soc.*, 20, 167 (1956)
2) E. C. Slater, *ibid.*, 20, 231 (1956)

to reach an equilibrium the rest was added and the reaction was initiated. During the course of the reaction initiated in various manners, distinct difference could be seen only in the beginning of reaction.

The author applied the Laplace transformation to this study and introduced r as the variable for response transform, $F(r)$, of a function, $f(t)$, of time, t.

An example of the application of the Laplace transformation will be shown in the first paragraph, (A). The enzyme reaction which yields two resultants will be discussed in the second, (B). The enzyme reaction modified by an activator is classified into several typical cases and discussed in the third, (C). The enzyme reaction modified by an inhibitor is also classified into several cases and discussed in the fourth, (D). The results of the determination of rate constants of activation of papain by cysteine will be presented in the last paragraph, (E).

(A) Scheme I

Gutfreund discussed the rate equations of Scheme I and stated that the lag-time, τ, is an important quantity for studying the reaction mechanism[1]. We will take it up again as an illustration example of applying the method of the Laplace transformation.

The rate equations are given by

$$\left.\begin{aligned} \frac{dx}{dt} &= k_1 se - x(k_{-1} + k_2) + k_{-2}ep\,, \\ \frac{dp}{dt} &= k_2 x - k_{-2}ep\,, \\ e_0 &= e + x\,, \end{aligned}\right\} \qquad (1)$$

where x, e, s and p are the concentrations of enzyme-substrate complex, free enzyme, substrate and product respectively, and k_1, k_{-1}, k_2 and k_{-2} are the rate constants, whosem eanings are shown in Scheme I. We have neglected the terms of the second order, *i.e.* terms involving ep.

The Laplace transform of a function of t, say $f(t)$, will be denoted by $F(r)$:

$$F(r) = \int_0^\infty f(t) e^{-rt} dt\,. \qquad (2)$$

We shall have frequent occasions to apply the following relation:

$$\int_0^\infty \frac{df}{dt} e^{-rt} dt = rF(r) - f(0+)\,, \qquad (3)$$

where $0+$ means to take the limit at $t=0$ from the right on the time axis. Now (1) can be transformed into the following equations:

$$\begin{aligned} rX(r) - x(0+) &= \frac{k_1 s e_0}{r} - X(r)(k_1 s + k_{-1} + k_2)\,, \\ rP(r) - p(0+) &= k_2 X(r)\,. \end{aligned} \qquad (4)$$

Substituting the initial conditions: $x(0+)=0$ and $p(0+)=0$,

$$P(r)=\frac{k_1k_2e_0s}{r^2(r+k_1s+k_{-1}+k_2)}. \quad (5)$$

If we develop (5) into a partial fractional formula, we have

$$P(r)=\frac{-k_1k_2e_0s}{r(k_1s+k_{-1}+k_2)^2}+\frac{k_1k_2e_0s}{r^2(k_1s+k_{-1}+k_2)}+\frac{k_1k_2e_0s}{(k_1s+k_{-1}+k_2)^2(r+k_1s+k_{-1}+k_2)}. \quad (6)$$

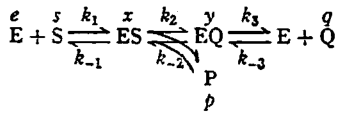
To get an explicit expression for $p(t)$, we may take the inverse transform of (6). Thus, the first term of the right-hand side gives a constant, the second a term proportional to t which corresponds to the steady state, and the last an exponential function of t corresponding to the transient state. In order to obtain the expression for the lag time, we only have to find the intercept of the steady state part of $p(t)$ extrapolated back to the time axis. It is given by the ratio of the coefficients of $1/r$ and $1/r^2$ with reversed sign in (6).

$$\tau=\frac{1}{k_1s+k_{-1}+k_2}=\frac{1}{k_1(s+K_M)}. \quad (7)$$

This result is just the same as Gutfreund's, as expected.

(B) Scheme II

In Scheme II another reaction which yields two different substances, P and Q, as products is considered, such as the case of enzymatic hydrolysis. Two lag times, τ_p and τ_q, will be shown.



Scheme II

The rate equations are given by

$$\left.\begin{aligned}
\frac{dx}{dt}&=k_1se+k_{-2}yp-x(k_{-1}+k_2),\\
\frac{dy}{dt}&=xk_2-y(k_3+k_{-2}p)k_{-3}eq,\\
\frac{dq}{dt}&=k_3y-k_{-3}eq,\\
\frac{dp}{dt}&=k_2x-k_{-2}yp,\\
e_0&=e+x+y.
\end{aligned}\right\} \quad (8)$$

Our interest is concerned mainly with the early stage of the reaction, where e, p, q and y may be assumed to be very small compared with s. Thus we can omit the terms of the second order, *i.e.* terms involving yp or eq. The transforms of $p(t)$ and $q(t)$ are now written down as follows:

$$P(r)=\frac{k_1 s k_2 e_0(r+k_3)}{r^2[(r+k_1 s+k_{-1}+k_2)(r+k_3)+k_1 s k_2]}, \tag{9}$$

$$Q(r)=\frac{k_1 s k_2 e_0 k_3}{r^2[(r+k_1 s+k_{-1}+k_2)(r+k_3)+k_1 s k_2]}. \tag{10}$$

The coefficients of $1/r^2$ are common for two response transforms:

$$\left[\frac{dp}{dt}\right]_{t\to\infty}=\left[\frac{dq}{dt}\right]_{t\to\infty}=\frac{k_1 s k_2 e_0 k_3}{(k_1 s+k_{-1}+k_2)k_3+k_1 s k_2}=\frac{s\dfrac{k_1 e_0 k_2}{k_2+k_3}}{s+\dfrac{k_3(k_{-1}+k_2)}{k_1(k_2+k_3)}}. \tag{11}$$

Comparing the last expression of (11) with the well-known formula of the steady state:

$$V=\frac{sV_m}{s+K_M}, \tag{12}$$

we can identify $\frac{k_3(k_{-1}+k_2)}{k_1(k_2+k_3)}$ with the apparent Michaelis constant, K_M, and $\frac{k_1 e_0 k_2}{k_2+k_3}$ with the maximnm velocity, V_m, respectively.

Dividing each coefficient of $1/r$ in Eqs. (9) and (10) by (11), we obtain two lag times τ_p and τ_q for the production of P and Q, respectively:

$$\tau_p=\frac{k_1 s+k_{-1}+k_2+k_3}{(k_1 s+k_{-1}+k_2)k_3+k_1 s k_2}-\frac{1}{k_3}, \tag{13}$$

$$\tau_q=\frac{k_1 s+k_{-1}+k_2+k_3}{(k_1 s+k_{-1}+k_2)k_3+k_1 s k_2} \tag{14}$$

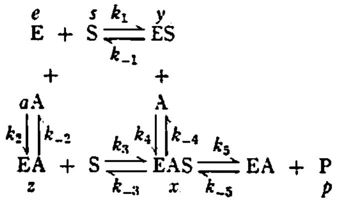
and thus

$$\tau_q-\tau_p=\frac{1}{k_3}. \tag{15}$$

The values of K_M and V_m can be determined from usual steady state experimenis, and those of τ_q and $\tau_q-\tau_p$ are also determined by transient state experiments. Four rate constants in Scheme II would be calculated from those four quantities, K_M, V_m, τ_q and $\tau_q-\tau_p$.

(C) Scheme III, Enzyme Reactions with Dissociable Activator

Let us consider Scheme III.



Scheme III

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The rate equations are given by

$$\left.\begin{aligned}
\frac{dx}{dt} &= k_3sz + k_4ay - x(k_{-3} + k_{-4}) + k_{-5}zp - k_5x\,,\\
\frac{dy}{dt} &= k_1se + k_{-4}x - y(k_{-1} + k_4a)\,,\\
\frac{dz}{dt} &= k_2ae + k_{-3}x - z(k_{-2} + k_3s) + k_5x - k_{-5}zp\,,\\
\frac{dp}{dt} &= k_5x - k_{-5}zp\,,\\
e_0 &= e + x + y + z\,.
\end{aligned}\right\} \tag{16}$$

(16) are transformed into,

$$\left.\begin{aligned}
&(r + k_{-3} + k_{-4} + k_5)X(r) - k_4aY(r) - k_3sZ(r) = x(0+)\,,\\
&(k_1s - k_{-4})X(r) + (r + k_1s + k_{-1} + k_4a)Y(r) + k_1sZ(r) = \frac{k_1se_0}{r} + y(0+)\,,\\
&(k_2a - k_{-3} - k_5)X(r) + k_2aY(r) + (r + k_2a + k_{-2} + k_3s)Z(r) = \frac{k_2ae_0}{r} + z(0+)\,,\\
&rP(r) = k_5X(r)\,,
\end{aligned}\right\} \tag{17}$$

where the terms involving k_{-5} are omitted because they are second order. From the first three of the Eq. (17) we obtain

$$X(r) = N_X/M\,, \tag{18}$$

where

$$N_X = \begin{vmatrix} x(0+) & -k_4a & -k_3s \\ \dfrac{k_1se_0}{r} + y(0+) & x + k_1s + k_{-1} + k_4a & k_1s \\ \dfrac{k_2ae_0}{r} + z(0+) & k_2a & r + k_2a + k_{-2} + k_3s \end{vmatrix}\,, \tag{19}$$

and

$$M = \begin{vmatrix} r + k_{-3} + k_{-4} + k_5 & -k_4a & -k_3s \\ k_1s - k_{-4} & r + k_1s + k_{-1} + k_4a & k_1s \\ k_2a - k_{-3} - k_5 & k_2a & r + k_2a + k_{-2} + k_3s \end{vmatrix}\,. \tag{20}$$

Substituting (18) into the last equation of (17) we get the expression for $P(r)$:

$$P(r) = \frac{k_5g}{r^2(r^3 + ar^2 + br + c)}\,, \tag{21}$$

where a, b and c are constants and g may be either a constant or a function of r.

From the above equations we can derive the expressions for various quantities which can be determined directly or indirectly from experiments.

Three different programs of the initialization of reaction are introduced here. They are tentatively called enzyme-start, substrate-start and activator-start, which were already explained in the introduction. Corresponding with each program, expressions for $P(r)$, N_X and τ for each case, are given by the notations, such as $P_K(r)$, ${}_KN_X$ and τ_K, for the enzyme-start.

$$\tau_E = -\frac{\left[\frac{d}{dr}\left(\frac{r\cdot {}_EN_X}{M}\right)\right]_{r=0}}{\left[\frac{r\cdot {}_EN_X}{M}\right]_{r=0}} = -\left[\frac{M\frac{d}{dr}(r\cdot {}_EN_X) - r\cdot {}_EN_X\cdot\frac{dM}{dr}}{M\cdot r\cdot {}_EN_X}\right]_{r=0}. \tag{22}$$

Although τ_S and τ_A for substrate-start and activator-start respectively can be given by similar equations, it is better to use the differences $\tau_E-\tau_S$ and $\tau_E-\tau_A$ in the place of τ_S and τ_A, because it is noticed to become much simpler.

The differences between the lag-times mentioned above, are obtained in the following way. Since

$$P_E - P_S = \frac{k_5({}_EN_X - {}_SN_X)}{r\cdot M}, \tag{23}$$

and this equation contains no term of $1/r^2$, we can develop it as follows:

$$P_E - P_S = \frac{A_0}{r} + \frac{A_M'}{M'}. \tag{24}$$

From this we obtain,

$$A_0 = [r(P_E - P_S)]_{r=0} = k_5\left[\frac{{}_EN_X - {}_SN_X}{M}\right]_{r=0}. \tag{25}$$

On the other hand we get,

$$P_E = \frac{A_1}{r^2} + \frac{A_M''}{M''}. \tag{26}$$

Then

$$A_1 = [r^2P_E]_{r=0} = k_5\left[\frac{r\cdot {}_EN_X}{M}\right]_{r=0}. \tag{27}$$

Hence

$$\tau_E - \tau_S = -\left[\frac{{}_EN_X - {}_SN_X}{r\cdot {}_EN_X}\right]_{r=0}, \tag{28}$$

$$\tau_E - \tau_A = -\left[\frac{{}_EN_X - {}_AN_X}{r\cdot {}_EN_X}\right]_{r=0}, \tag{29}$$

Many distinctive cases are obtained from the results of steady-state and transient-state experiments, as follows:

Case i) $\alpha = 1$,

Case ii) $\alpha = 0$, $k_{-1}/k_1 = \infty$, k_{-2}/k_2 is finite, and k_{-3}/k_3 is finite, $k_{-4}/k_4 = 0$,

Case iii) $\alpha = 0$, k_{-1}/k_1 is finite, $k_{-2}/k_2 = \infty$, and $k_{-3}/k_3 = 0$, k_{-4}/k_4 is finite,

Case iv) $0 < \alpha < \infty$ and $\alpha \neq 1$,

a) k_1s, k_{-1}, k_3s and k_{-3} are much larger than k_2a, k_{-2}, k_4a and k_{-4} respectively,

b) k_2a, k_{-2}, k_4a and k_{-4} are much larger than k_1s, k_{-1}, k_3s and k_{-3} respectively,

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c) all rate constants take comparable values with each other,

where

$$\alpha=\frac{k_{-3}}{k_3}\Big/\frac{k_{-1}}{k_1}=\frac{k_{-4}}{k_4}\Big/\frac{k_{-2}}{k_2}.$$

The three initial conditions are expressed as follows:

Enzyme-start; $x(0+)=0$, $y(0+)=0$, $z(0+)=0$,

Substrate-start; $x(0+)=0$, $y(0+)=0$, $z(0+)=k_2ae_0/(k_2a+k_{-2})$,

Activator-start; $x(0+)=0$, $y(0+)=k_1se_0/(k_1s+k_{-1})$, $z(0+)=0$,

except the cases iv) a) and b).

Case i)

If $\gamma k_{\pm1}=k_{\pm3}$ and $\delta k_{\pm2}=k_{\pm4}$.

$$N_X=\begin{vmatrix} x(0+) & -\delta k_2a & -\gamma k_1s \\ \dfrac{k_1se_0}{r}+y(0+) & r+k_1s+k_{-1}+\delta k_2a & k_1s \\ \dfrac{k_1se_0}{r}+z(0+) & k_2a & r+k_2a+k_{-1}+\gamma k_1s \end{vmatrix}, \tag{30}$$

and

$$M=\begin{vmatrix} r+\gamma k_{-1}+\delta k_{-2}+k_5 & -\delta k_2a & -\gamma k_1s \\ k_1s-\delta k_{-2} & r+k_1s+k_{-1}+\delta k_2a & k_1s \\ k_2a-\gamma k_{-1}-k_5 & k_2a & r+k_2a+k_{-2}+\gamma k_1s \end{vmatrix}. \tag{31}$$

Hence

$$\tau_E-\tau_A=\frac{\gamma(k_1s+k_{-1})+(\gamma-1)\delta k_2a}{(k_2a+k_{-2})\{\delta(\gamma k_1s+k_{-2})+\gamma(k_{-1}+\delta k_2a)\}}. \tag{32}$$

As a special case if $\gamma=\delta=1$, Eq. (32) becomes

$$\tau_E-\tau_A=\frac{k_1s+k_{-1}}{(k_2a+k_{-2})(k_1s+k_{-1}+k_2a+k_{-2})}. \tag{33}$$

Similarly to (32) we obtain

$$\tau_E-\tau_S=\frac{\delta(k_2a+k_{-2})+(\delta-1)\gamma k_1s}{(k_1s+k_{-1})\{\delta(\gamma k_1s+k_{-1})+\gamma(k_{-2}+\delta k_2a)\}}. \tag{34}$$

And if $\gamma=\delta=1$,

$$\tau_E-\tau_S=\frac{k_2a+k_{-2}}{(k_1s+k_{-1})(k_1s+k_{-1}+k_2a+k_{-2})}. \tag{35}$$

In this case τ_E is considerably complicated. Even if $\gamma=\delta=1$, and if $k_5\ll k_{-1}$,

$$\tau_E=\frac{B_1^2+B_2^2+B_1B_2+2B_1+2B_2}{B_1B_2(B_1+B_2)}, \tag{36}$$

where $B_1=k_1s+k_{-1}$ and $B_2=k_2a+k_{-2}$.

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Case ii)

Since $y=0$,

$$N_X=\begin{vmatrix} x(0+) & -k_3s \\ \dfrac{k_2ae_0}{r}+z(0+) & r+k_2a+k_{-2}+k_3s \end{vmatrix}, \tag{37}$$

and

$$M=\begin{vmatrix} r+k_{-3}+k_5 & -k_3s \\ k_2a-k_{-3}-k_5 & r+k_2a+k_{-2}+k_3s \end{vmatrix}. \tag{38}$$

Hence

$$\tau_E=\frac{k_2a+k_{-2}+k_3s+k_{-3}+k_5}{k_2a(k_3s+k_{-3}+k_5)+k_{-2}(k_{-3}+k_5)}, \tag{39}$$

and

$$\tau_E-\tau_S=\frac{1}{k_2a+k_{-2}}. \tag{40}$$

Case iii)

Since $z=0$,

$$N_X=\begin{vmatrix} x(0+) & -k_4a \\ \dfrac{k_1se_0}{r}+y(0+) & r+k_1s+k_{-1}+k_4a \end{vmatrix}, \tag{41}$$

and

$$M=\begin{vmatrix} r+k_{-4}+k_5 & -k_4a \\ k_1s-k_{-4} & r+k_1s+k_{-1}+k_4a \end{vmatrix}. \tag{42}$$

Hence

$$\tau_E=\frac{k_1s+k_{-1}+k_4a+k_{-4}+k_5}{k_1s(k_4a+k_{-4}+k_5)+k_{-1}(k_{-4}+k_5)}, \tag{43}$$

$$\tau_E-\tau_A=\frac{1}{k_1s+k_{-1}}. \tag{44}$$

Case iv) a)

For the slower reaction, we obtain

$$\begin{aligned}\frac{d(x+z)}{dt}&=\frac{dx}{dt}\left(1+\alpha\frac{K_S}{s}\right)=ya\left(\frac{K_S}{s}k_2+k_4\right)-x\left(\alpha\frac{K_S}{s}k_{-2}+k_{-4}\right)\\&=(ya-x\alpha K_A)\left(\frac{K_S}{s}k_2+k_4\right)\\&=\left\{a\frac{e_0-x\left(1+\alpha\dfrac{K_S}{s}\right)}{1+\dfrac{K_S}{s}}-x\alpha K_A\right\}\left(\frac{K_S}{s}k_2+k_4\right),\end{aligned} \tag{45}$$

where $K_S=k_{-1}/k_1$ and $K_A=k_{-2}/k_2$. It is assumed that k_5 is negligible compared with k_{-3}. Then

$$\frac{dx}{dt}=\left\{\frac{ae_0}{\left(1+\dfrac{K_S}{s}\right)\left(1+\alpha\dfrac{K_S}{s}\right)}-x\left(\frac{a}{1+\dfrac{K_S}{s}}+\frac{\alpha K_A}{1+\alpha\dfrac{K_S}{s}}\right)\right\}\left(\frac{K_S}{s}k_2+k_4\right). \tag{46}$$

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From the Laplace transform of this equation,

$$X(r)=\frac{\dfrac{ae_0/r}{\left(1+\dfrac{K_S}{s}\right)\left(1+\alpha\dfrac{K_S}{s}\right)}\left(\dfrac{K_S}{s}k_2+k_4\right)+x(0+)}{r+\left(\dfrac{a}{1+\dfrac{K_S}{s}}+\dfrac{K_A}{1+\alpha\dfrac{K_S}{s}}\right)\left(\dfrac{K_S}{s}k_2+k_4\right)}. \tag{47}$$

The initial conditions are as follows:

Enzyme-start: $x(0+)=0$,

Substrate-start: $X(0+)=\dfrac{k_2ae_0}{(k_2a+k_{-2})\left(1+\alpha\dfrac{K_S}{s}\right)}$.

Hence

$$\tau_K=\frac{1}{\left(\dfrac{a}{1+\dfrac{K_S}{s}}+\dfrac{\alpha K_A}{1+\alpha\dfrac{K_S}{s}}\right)\left(\dfrac{K_S}{s}k_2+k_4\right)}, \tag{48}$$

and

$$\tau_K-\tau_S=\frac{1+\dfrac{K_S}{s}}{(a+K_A)\left(\dfrac{K_S}{s}k_2+k_4\right)}. \tag{49}$$

In the case of the faster reactions, only the experiment for the substrate-start reaction is available. This is equal to the case of Scheme I expect enzyme concentration. In this case the enzyme concentration is expressed as $k_2ae_0/(k_2a+k_{-2})$ in place of e_0 in the case of Scheme I.

Hence

$$\tau_S=\frac{1}{k_3(s+\alpha K_S)}. \tag{50}$$

Since $k_{\pm1}$ are masked by the slower reactions, it is difficult to dertermine them, as long as the objects of the determination are set on the velocity of formation of the last product or the concentration of active complex.

Case iv) b)

From the style of the Scheme III, this case may be treated as the case a).

For the slower reaction τ_K and $\tau_K-\tau_A$ becomes

$$\tau_K=\frac{1}{\left(\dfrac{s}{1+\dfrac{K_A}{a}}+\dfrac{\alpha K_S}{1+\alpha\dfrac{K_A}{a}}\right)\left(\dfrac{K_A}{a}k_1+k_3\right)}, \tag{51}$$

and

$$\tau_K-\tau_A=\frac{1+\dfrac{K_A}{a}}{(s+K_S)\left(\dfrac{K_A}{a}k_1+k_3\right)}. \tag{52}$$

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For the faster reaction

$$\tau_A = \frac{1}{k_4(a+\alpha K_A)}. \tag{53}$$

Case iv) c)

τ_E is given by Eq. (22), and $\tau_E - \tau_S$ and $\tau_E - \tau_A$ are given by

$$\tau_E - \tau_S = \frac{-\dfrac{k_2 a}{k_2 a + k_{-2}}\begin{vmatrix} -k_4 a & -k_3 s \\ k_1 s + k_{-1} + k_4 a & k_1 s \end{vmatrix}}{{}_E N'_X}, \tag{54}$$

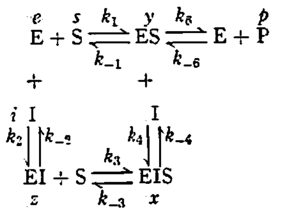
$$\tau_E - \tau_A = \frac{\dfrac{k_1 s}{k_1 s + k_{-1}}\begin{vmatrix} -k_4 a & -k_3 s \\ k_2 a & k_2 a + k_{-2} + k_3 s \end{vmatrix}}{{}_E N'_X}, \tag{55}$$

where

$${}_E N'_X = \begin{vmatrix} 0 & -k_4 a & -k_3 s \\ k_1 s & k_1 s + k_{-1} + k_4 a & k_1 s \\ k_2 a & k_2 a & k_2 a + k_{-2} + k_3 s \end{vmatrix}. \tag{56}$$

(D) Scheme IV, Enzyme Reaction with Dissociable Inhibitor

Let us consider Scheme IV.



Scheme IV

The rate equations are given by

$$\left.\begin{aligned}
\frac{dx}{dt} &= y k_4 i + z k_3 s - x(k_{-3} + k_{-4}), \\
\frac{dy}{dt} &= e k_1 s + x k_{-4} - y(k_{-1} + k_4 i) + k_{-6} e p - k_6 y, \\
\frac{dz}{dt} &= e k_2 i + x k_{-3} - z(k_{-2} + k_3 s), \\
\frac{dp}{dt} &= k_6 y - k_{-6} e p, \\
e_0 &= e + x + y + z.
\end{aligned}\right\} \tag{57}$$

(57) is transformed into

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$$\left.\begin{aligned}&(r+k_{-3}+k_{-4})X(r)-k_4iY(r)-k_3sZ(r)=x(0+)\,,\\&(k_1s-k_{-4})X(r)+(r+r_1s+k_{-1}+k_4i+k_6)Y(r)+k_1sZ(r)=\frac{k_1se_0}{r}+y(0+)\,,\\&(k_2i-k_{-3})X(r)+k_2iY(r)+(r+k_2i+k_{-2}+k_3s)Z(r)=\frac{k_2ie_0}{r}+z(0+)\,,\\&rP(r)=k_6Y(r)\,.\end{aligned}\right\}\quad(58)$$

From (58) we obtain

$$Y(r)=N_Y/M\,,\qquad(59)$$

where

$$N_Y=\begin{vmatrix}r+k_{-3}+k_{-4} & x(0+) & -k_3s\\ k_1s-k_{-4} & \frac{k_1se_0}{r}+y(0+) & k_1s\\ k_2i-k_{-3} & \frac{k_2ie_0}{r}+z(0+) & r+k_2i+k_{-2}+k_3s\end{vmatrix},\qquad(60)$$

and

$$M=\begin{vmatrix}r+k_{-3}+k_{-4} & -k_4i & -k_3s\\ k_1s+k_{-4} & r+k_1s+k_{-1}+k_4i+k_6 & k_1s\\ k_2i+k_{-3} & k_2i & r+k_2i+k_{-2}+k_3s\end{vmatrix}.\qquad(61)$$

Since

$$rP(r)=k_6Y(r)\,,\qquad(62)$$

we get an expression of the same form as in the paragraph (C). Therefore, the lag times and those differences for this case are obtained in the way similar to (C). The following typical cases are possibly distinguishable.

Case v) $\alpha=1$,

Case vi) $\alpha=0$, k_{-1}/k_1 is finite, $k_{-2}/k_2=\infty$, $k_{-3}/k_3=0$, k_{-4}/k_4 is fiite,

Case vii) $\alpha=\infty$, k_{-1}/k_1 and k_{-2}/k_2 are finite, k_{-3}/k_3 and $k_{-4}/k_4=\infty$,

Case viii) $0<\alpha<\infty$ and $\alpha\neq1$,

a) k_1s, k_{-1}, k_3s and k_{-3} are much larger than k_2i, k_{-2}, k_4i and k_{-4} respectively,

b) k_2i, k_{-2}, k_4i and k_{-4} are much larger than k_1s, k_{-1}, k_3s and k_{-3} respectively,

c) all rate constants take comparable values to each other.

where

$$\alpha=\frac{k_{-3}}{k_3}\Big/\frac{k_{-1}}{k_1}=\frac{k_{-4}}{k_4}\Big/\frac{k_{-2}}{k_2}\,.$$

The three starting conditions are expressed as follows:

Enzyme-start; $x(0+)=0$, $y(0+)=0$, $z(0+)=0$,

Substrate-start; $x(0+)=0$, $y(0+)=0$, $z(0+)=k_2ie_0/(k_2i+k_{-2})$,

Inhibitor-start; $x(0+)=0$, $y(0+)=k_1se_0/(k_1s+k_{-1})$, $z(0+)=0$,

except the cases viii) a) and b).

Case v)

If $\gamma k_{\pm1}=k_{\pm3}$ and $\delta k_{\pm2}=k_{\pm4}$,

$$N=r\begin{vmatrix} r+\gamma k_{-1}+\delta k_{-2} & x(0+) & -\gamma k_1 s \\ k_1 s-\delta k_{-2} & \frac{k_1 s e_0}{r}+y(0+) & k_1 s \\ k_2 i-\gamma k_{-1} & \frac{k_2 i e_0}{r}+z(0+) & r+k_2 i+k_{-2}+\gamma k_1 s \end{vmatrix}, \tag{63}$$

and

$$M=\begin{vmatrix} r+\gamma k_{-1}+\delta k_{-2} & -\delta k_2 i & -\gamma k_1 s \\ k_1 s-\delta k_{-2} & r+k_1 s+k_{-1}+\delta k_2 i+k_6 & k_1 s \\ k_2 i-\gamma k_{-1} & k_2 i & r+k_2 i+k_{-2}+\gamma k_1 s \end{vmatrix}, \tag{64}$$

Hence

$$\tau_K-\tau_S=-\frac{k_2 i\{\gamma(k_1 s+k_{-1})+(1-\gamma)\delta k_{-2}\}}{k_{-2}(k_2 i+k_{-2})\{\gamma k_{-1}+\delta k_{-2}+\gamma\delta(k_1 s+k_2 i)\}}. \tag{65}$$

As a special case, if $\gamma=\delta=1$, Eq. (65) becomes

$$\tau_K-\tau_S=-\frac{k_2 i(k_1 s+k_{-1})}{k_{-2}(k_2 i+k_{-2})(k_1 s+k_{-1}+k_2 i+k_{-2})}. \tag{66}$$

Similatly we obtain

$$\tau_K-\tau_I=\frac{\gamma k_1 s(k_2 i+\delta k_{-2})+(\gamma k_{-1}+\delta k_{-2})(k_2 i+k_{-2})}{(k_1 s+k_{-1})k_{-2}\{\gamma k_{-1}+\delta k_{-2}+\gamma\delta(k_1 s+k_2 i)\}}. \tag{67}$$

And if $\gamma=\delta=1$,

$$\tau_K-\tau_I=\frac{(k_2 i+k_{-2})(k_1 s+k_{-1}+k_{-2})}{k_{-2}(k_1 s+k_{-1})(k_1 s+k_{-1}+k_2 i+k_{-2})}. \tag{68}$$

In this case τ_K is considerably complicated, even if $\gamma=\delta=1$, and if $k_6 \ll k_{-1}$,

$$\tau_K=\frac{B_1^2+B_2^2+B_1B_2+2B_1+2B_2-B_1^2B_2}{k_{-2}B_1B_2(B_1+B_2)}, \tag{69}$$

where $B_1=k_1s+k_{-1}$ and $B_2=k_2i+k_{-2}$.

Case vi)

Since $z=0$,

$$N_Y=\begin{vmatrix} r+k_{-4} & x(0+) \\ k_1 s-k_{-4} & \frac{k_1 s e_0}{r}+y(0+) \end{vmatrix}, \tag{70}$$

and

$$M=\begin{vmatrix} r+k_{-4} & -k_4 i \\ k_1 s-k_{-4} & r+k_1 s+k_{-1}+k_4 i+k_6 \end{vmatrix}. \tag{71}$$

Hence

$$\tau_K=\frac{k_{-4}(k_4 i+k_{-4})-k_4 i k_1 s}{k_{-4}(k_1 s+k_{-1}+k_6)+k_4 i k_1 s}, \tag{72}$$

and

$$\tau_K-\tau_I=\frac{1}{k_1 s+k_{-1}}. \tag{73}$$

Case vii)

Since $x=0$,

$$N_Y=\begin{vmatrix}\frac{k_1se_0}{r}+r(0+) & k_1s\\ \frac{k_2ie_0}{r}+z(0+) & r+k_2i+k_{-2}\end{vmatrix}. \tag{74}$$

and

$$M=\begin{vmatrix}x+k_1s+k_{-1}+k_6 & k_1s\\ k_2i & r+k_2i+k_{-2}\end{vmatrix}. \tag{75}$$

Hence

$$\tau_E=\frac{k_{-2}(k_2i+k_{-2})-k_2i(k_{-1}+k_6)}{k_{-2}(k_{-1}+k_6)(k_2i+k_{-2})+k_1sk_{-2}}, \tag{76}$$

$$\tau_E-\tau_S=\frac{k_2i}{k_{-2}(k_2i+k_{-2})}, \tag{77}$$

and

$$\tau_E-\tau_I=\frac{-(k_2i+k_{-2})}{k_{-2}(k_1s+k_{-1})}. \tag{78}$$

Case viii) a)

Considering the slower reaction, the following differential equation is obtained.

$$\frac{d(e+y)}{dt}=\frac{dy}{dt}\left(1+\frac{K_S}{s}\right)=x\left(\alpha\frac{K_S}{s}k_{-2}+k_{-4}\right)-yi\left(\frac{K_S}{s}k_2+k_4\right)$$

$$=(x\alpha K_I-yi)\left(\frac{K_S}{s}k_2+k_4\right)=\left\{\alpha K_I\frac{e_0-y\left(1+\frac{K_S}{s}\right)}{1+\alpha\frac{K_S}{s}}-yi\right\}\left(\frac{K_S}{s}k_2+k_4\right), \tag{79}$$

where $K_S=k_{-1}/k_1$ and $K_I=k_{-2}/k_2$. It is assumed that k_6 is negligible compared with k_{-1}. Then

$$\frac{dy}{dt}=\left\{\alpha K_Ie_0\frac{1}{\left(1+\frac{K_S}{s}\right)\left(1+\alpha\frac{K_S}{s}\right)}-y\left(\frac{1}{1+\alpha\frac{K_S}{s}}+\frac{i}{1+\frac{K_S}{s}}\right)\right\}\left(\frac{K_S}{s}k_2+k_4\right). \tag{80}$$

From the Laplace transform of Eq. (80), we obtain

$$Y(r)=\frac{\frac{\alpha K_Ie_0/r}{\left(1+\frac{K_S}{s}\right)\left(1+\alpha\frac{K_S}{s}\right)}\left(\frac{K_S}{s}k_2+k_4\right)+y(0+)}{r+\left(\frac{1}{1+\alpha\frac{K_S}{s}}+\frac{i}{1+\frac{K_S}{s}}\right)\left(\frac{K_S}{s}k_2+k_4\right)}. \tag{81}$$

The initial conditions are as follows;

Enzyme-start; $y(0+)=e_0/(1+K_S/s)$,

Substrate-start ; $y(0+)=\frac{k_2 i}{k_2 i+k_{-2}}\frac{e_0}{1+\frac{K_S}{s}}$.

Hence

$$\tau_K=\frac{1}{\left(\frac{1}{1+\alpha\frac{K_S}{s}}+\frac{i}{1+\frac{K_S}{s}}\right)\left(\frac{K_S}{s}k_2+k_4\right)}, \tag{82}$$

and

$$\tau_K-\tau_S=-\frac{k_2 i}{\alpha K_I}\frac{1+\alpha\frac{K_S}{s}}{(k_2 i+k_{-2})\left(\frac{K_S}{s}k_2+k_4\right)}. \tag{83}$$

Regarding the faster reaction of Scheme IV, it is easily understood that the experiment without any inhibitor conforms to this case.

Case viii) b)

Taking account of the slower reaction, we obtain

$$\frac{d(y+x)}{dt}=\frac{dy}{dt}\left(1+\alpha\frac{i}{K_I}\right)=es\left(\frac{i}{K_I}k_3+k_1\right)-y\left(\alpha\frac{i}{K_I}k_{-3}+k_{-1}\right)$$
$$=(es-yK_S)\left(\frac{i}{K_I}k_3+k_1\right)=\left\{\frac{e_0-y\left(1+\alpha\frac{i}{K_I}\right)}{1+\frac{i}{K_I}}s-yK_S\right\}\left(\frac{i}{K_I}k_3+k_1\right). \tag{84}$$

Then

$$\frac{dy}{dt}=\left\{\frac{e_0 s}{\left(1+\frac{i}{K_I}\right)\left(1+\alpha\frac{i}{K_I}\right)}-\left(y\frac{s}{1+\frac{i}{K_I}}+\frac{K_S}{1+\alpha\frac{i}{K_I}}\right)\right\}\left(\frac{i}{K_I}k_3+k_1\right). \tag{85}$$

From the Laplace transform of Eq. (85), we obtain,

$$Y(r)=\frac{\frac{e_0 s\left(\frac{i}{K_I}k_3+k_1\right)}{\left(1+\frac{i}{K_I}\right)\left(1+\alpha\frac{i}{K_I}\right)}+y(0+)}{r+\left(\frac{s}{1+\frac{i}{K_I}}+\frac{K_S}{1+\alpha\frac{i}{K_I}}\right)\left(\frac{i}{K_I}k_3+k_1\right)}. \tag{86}$$

The initial conditions are as follows ;

Enzyme-start ; $y(0+)=0$,

Inhibitor-start ; $y(0+)=\frac{k_1 s e_0}{(k_1 s+k_{-1})\left(1+\alpha\frac{i}{K_I}\right)}$.

Hence

$$\tau_E = \frac{1}{\left(\frac{s}{1+\frac{i}{K_I}} + \frac{K_S}{1+\alpha\frac{i}{K_I}}\right)\left(\frac{i}{K_I}k_3 + k_1\right)}, \tag{87}$$

and

$$\tau_E - \tau_S = \frac{k_1\left(1+\frac{i}{K_I}\right)}{\left(\frac{i}{K_I}k_3 + k_1\right)(k_1 s + k_{-1})}. \tag{88}$$

For the consideration of the faster reaction an experiment for inhibitor-start should be tried. In this condition the following differential equation is deduced,

$$\frac{dy}{dt} = xk_{-4} - yk_4 i = \left(\frac{e_0}{1+\frac{K_S}{s}} - yk_{-4}\right) - yk_4 i = \frac{e_0 k_{-4}}{1+\frac{K_S}{s}} - y(k_4 i + k_{-4}). \tag{89}$$

From the Laplace transform of Eq. (89), we obtain

$$Y(r) = \frac{\frac{e_0 k_{-4}/r}{1+\frac{K_S}{s}} + y(0+)}{r + k_4 i + k_{-4}}, \tag{90}$$

where $y(0+) = e_0/(1+K_S/s)$.
Hence

$$\tau_I = \frac{1}{k_4 i + k_{-4}}. \tag{91}$$

Case viii) c)

τ_E is given by the same equation as (22), and $\tau_E - \tau_S$ and $\tau_E - \tau_I$ are given by

$$\tau_E - \tau_S = \frac{\frac{k_2 i}{k_2 i + k_{-2}}\begin{vmatrix} k_{-3}+k_{-4} & -k_3 s \\ k_1 s - k_{-4} & k_1 s \end{vmatrix}}{{}_E N'_Y}, \tag{92}$$

$$\tau_E - \tau_I = \frac{\frac{-k_1 s}{k_1 s + k_{-1}}\begin{vmatrix} k_{-3}+k_{-4} & -k_3 s \\ k_2 i - k_{-3} & k_2 i + k_{-2} + k_3 s \end{vmatrix}}{{}_E N'_Y}, \tag{93}$$

where

$$ {}_E N'_Y = \begin{vmatrix} k_{-3}+k_{-4} & 0 & -k_3 s \\ k_1 s - k_{-4} & k_1 s & k_1 s \\ k_2 i - k_{-3} & k_2 i & k_2 i + k_{-3} + k_3 s \end{vmatrix}. \tag{94}$$

(E) Experiment

Papain is a well-known phytoproteinase. The activity of the enzyme is proved to have originated from thiol groups in the enzyme molecule. As SH group is the reduced from of

–S–S–, it has been observed by many investigators that this enzyme is activated by some reductants, for instance, H_2S, cysteine, CN^-. As hydrolysis, the specific reaction catalyzed by papain, is known to be caused by the combination of the substrate with SH groups of the enzyme produced by reduction, the mechanism of reaction by papain may be classified into Friedenwald's coupling activation[3]. The author et al. studied about the mechanism of hydrolysis of α-benzoyl-L-arginine amide catalyzed by papain and confirmed that the above was held well[4]. In this article, the experimental results obtained about the reaction mechanism of papain upon α-tosyl-L-arginine methyl ester (TsAME) using cysteine as the activator, are shown. It was found, however, that, when TsAME was used as the substrate, papain combined with substrate molecule even before occuring of the reduction by the activator and that the factor α was larger than 1. In this paragraph the EA does not mean papain-cysteine complex but papain activated by cysteine.

Materials

Papain was prepared from a commercial dried papaya latex by the purification method of Emil L. Smith, with the modification of EDTA 2Na salt used in place of cysteine. The preparation was purified several times by the salting-out method with NaCl. and was stored in a state of packed solution or as about 1 per cent clear solution.

TsAME was prepared by the method of Bergman, Fruton and Pollok[5].

Determination of proceeding of reaction

The method of Schwert, Neurath, Kaufman and Snoke[6] was employed. The reaction vessel

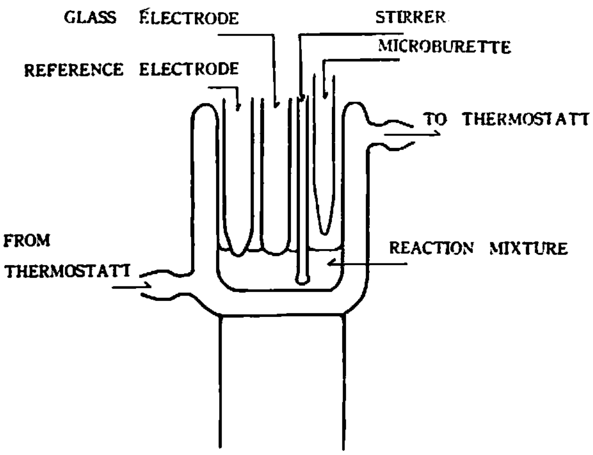

Fig. 1 Reaction vessel

3) J. S. Friedenwald, G. D. Maengwyn-Davies, *A Simposium on the Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, p. 154 (1954)

4) K. Ozawa, T. Ohnishi and S. Tanaka, *J. Biochem.* (Tokyo), **51**, (1962) in press

5) M. Bergman, J. S. Fruton and H. Pollok, *J. Biol. Chem.*, **127**, 643 (1939)

6) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *ibid.*, **72**, 221 (1948)

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used for this experiment is shown in Fig. 1. Water kept at constant temperature from a bath with a thermostatt was circulated throughout the experiments. The reactions were started by the following three programs, enzyme-start, substrate-start, activator-start. About 1 μl of N NaOH solution was added to a reaction mixture at every step of determination. The materials and instruments used in these experiments are shown in Table 1.

Table 1 Materials and instruments

	Material		ml	Final concentration
i)	Substrate	TsAME	2	
ii)	Buffer	McIlvaine $\times \frac{1}{2}$	1	With 1/1000 M EDTA 2Na
iii)	Activator	Cysteine	1	1/20~1/1000 M
iv)	Enzyme	Papain	1	$1.23 \times 10^{-5} M$
v)		1 N NaOH		
vi)	pH Meter	Beckman model GS. using sensitivity switch turned on B		
vii)	Microburette	Mitamura Riken Industry		
viii)	Stirrer	A small glass rod stirrer driven by an AC toy motor with a flexible shaft derived from camera relese		

a) Enzyme-start :——In this case, the substrate, the buffer solution and the activator were first mixed and pH was adjusted to 6.0. The zero-time was chosen to be the time of the addition of the enzyme solution.

b) Substrate-start :——Buffer solution, activator and enzyme were mixed first. pH was adjusted also to 6.0 and the mixture was preincubated for 30 minutes or more. The zero-time is the time of the addition of the substrate. pH of substrate solution was also adjusted to 6.0 before addition.

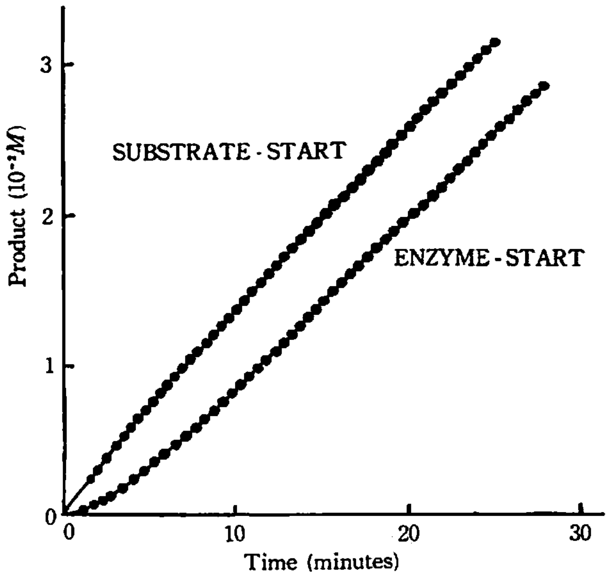

Fig. 2 Hydrolysis of TsAME, catalyzed by cysteine activated papain at 40°C and pH 6.0

Each reaction mixture contained M/10 TsAME, M/20 cysteine and $1.23 \times 10^{-5} M$ papain.

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c) Activator-start:——Substrate, buffer and enzyme were mixed first. The same pH was adopted. The mixture was preincubated for 30 minutes or more. The zero-time is the time of the addition of the activator. pH of the activator solution was adjusted to 6.0.

Lineweaver-Burk plot

Fig. 2 shows some of the experimental results obtained. A linear relationship should be noticed to be held between the concentration of the product and the lapse of time when the substrate concentration is sufficiently high except in the beginning of the reaction. Abnormality in the relationship is observed probably owing to the inadequate concentration of the substrate. The initial velocity, therefore, is difficult to be determined from these results. To make the Lineweaver-Burk plot from them, the following method is applied.

A relationship between $1/V$ and $1/s$ was calculated from the experimental data, p versus $1/V$, and it is plotted. Fig. 3 shows a model for several experimental data of one fixed con-

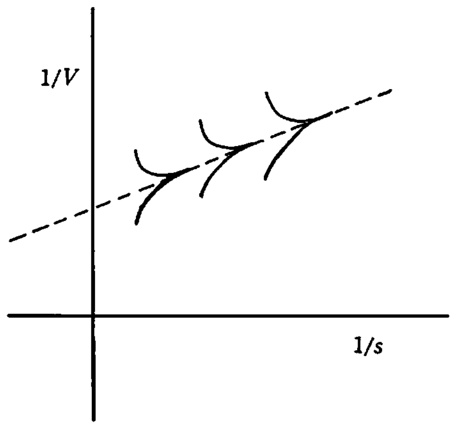

Fig. 3 Method of Lineweaver-Burk plot employed

centration of the activator. The two groups of similar curves above and below the oblique broken line correspond to the experimental results of enzyme-start and substrate-start respectively. Each curve has a sharply bended part and a straight part which has a slope similar to each other. The former is the initial part of the reaction and the latter is the steady state part.

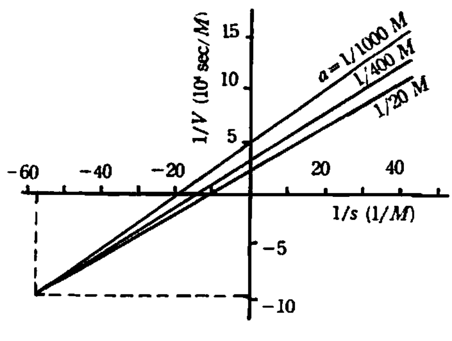

Fig. 4 A plot of $1/V$ against $1/s$ for hydrolysis of TsAME, catalyzed by cysteine activated papain

And these latter parts are the parts of the same straight line, by the extrapolation of which Lineweaver-Burk plot is obtained. This treatment is based on an assumption that the concentration of the product has no effect on the reaction. Fig. 4 was obtained by this method, from

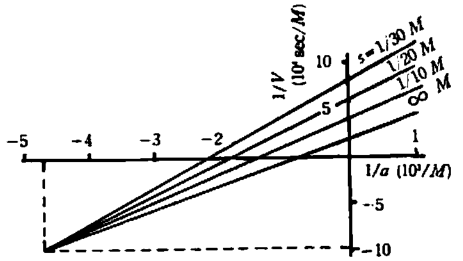

Fig. 5 A plot of $1/V$ against $1/a$ for hydrolysis of TsAME, catalyzed by cysteine activated papain

which Fig. 5 was derived. The intersection of plot is in the third quadrant and indicates that $1<\alpha<\infty$. The experimental results of the activator-start corresponded completely to those of the enzyme-start and it is presumed that the equilibriums of $E+S \rightleftharpoons ES$ and $EA+S \rightleftharpoons EAS$ are accomplished in a very short time compared with the other equilibriums and the transient state part, in which these equilibriums take part, is unable to be traced by Schwert's method.

The Case vi) a) is conformable to determine the rate constants of this case.

Rate constants

As has been stated it is unable to know directly the initial velocity from the experimental data and also from the other quantities concerned with the various properties of the reaction system, for example, the lag time. Hence the data were corrected by,

$$\frac{\dfrac{1}{s_t}+\dfrac{1}{K_S}}{\dfrac{1}{s_0}+\dfrac{1}{K_S}}\left\{\frac{1}{V_t}+\frac{1}{V_m}(\alpha-1)\right\}-\frac{1}{V_m}(\alpha-1)=\frac{1}{V_{t0}}, \qquad (95)$$

where s_0 and s_t are the substrate concentrations at zero time and time t respectively, and V_t is the hydrolytic velocity at time t and V_{t0} is the corrected velocity of V_t by the initial substrate concentration.

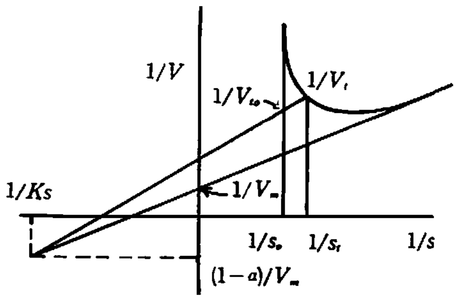

Fig. 6 Correction of experimental data obtained at inadequate substrate concentration

The relationship among these values is shown in Fig. 6. The corrected curve of the time

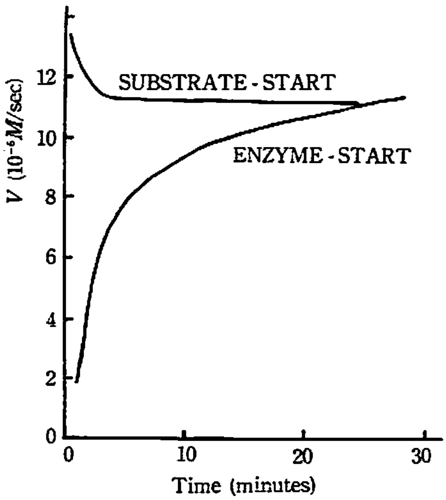

Fig. 7 A corrected plot of V against time for hydrolysis of TsAME, catalyzed by cysteine activated papain, derived from Fig. 2

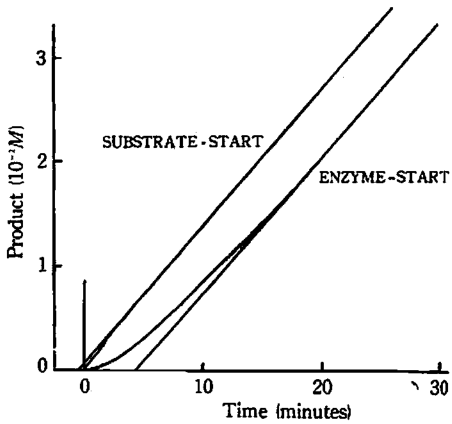

Fig. 8 Hydrolysis of TsAME, catalyzed by cysteine activated papain (corrected)

versus the reaction velocity is available from plotting V_{t_0} versus t.

Fig. 7 was obtained from Fig. 2 by this correction, which shows sharp changes in the initial parts and then constant values for steady state parts. By integrating these figures and plotting the product concentration versus time, Fig. 8 was obtained. From these treatments, the lag time is derived.

The rate constants of activation of papain by cysteine and other constants, shown in Table 2, were calculated from a pair of simultaneous for the lag times which were obtained by the experiments of different substrate concentrations.

Table 2 Various constants of hydrolysis of TsAME, catalyzed by cysteine activated papain at 40°C and pH 6.0

$K_S = 1.74 \times 10^{-2}\,M$	$K_A = 2.1 \times 10^{-4}\,M$
$x = 5.9$	$k_5 = 4.1$/mol/mol/sec
$k_2 = 2.15 \times 10^{-1}$/mol/sec	$k_{-2} = 4.51 \times 10^{-5}$/sec
$k_4 = 4.32 \times 10^{-2}$/mol/sec	$k_{-4} = 5.35 \times 10^{-5}$/sec

Summary

1. The Laplace transformation has been applied to analyze the properties of the rate equations for some first order enzyme reaction systems.

2. The method of determination of rate constants for Michaelis scheme by the lag time has been expanded to the first order enzyme reaction system modified by some dissociable modifier, which is classified in several groups from its properties of steady state and transient state.

3. The rate constants of activation of papain by cysteine using α-tosyl-L-arginine methyl ester as substrate has been determined.

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