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TRANSIENT-PHASE KINETICS OF α-CHYMOTRYPSIN AND OTHER ENZYME SYSTEMS

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SUMMARY

A transient-phase method is described for obtaining values for the individual rate constants for a single-substrate two-intermediate enzyme mechanism. In principle, values of k_1, k_{-1}, k_2 and k_3 can be obtained from such studies, as can values of inhibition constants k_q and k_{-q} from studies of a competitively-inhibited reaction. An experimental stopped-flow study is made for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate, in the absence and presence of the inhibitor indole, and the results are consistent with the theoretical predictions. Values of k_1, k_{-1}, k_2 and k_3 were obtained.

The transient-phase equations for many enzyme systems, at limiting enzyme concentration, are in all cases of the form

$$x = vt + \beta + \sum_{i=1}^{n} \beta_i e^{-\lambda_i t}$$

where n, the number of exponential terms, is equal to the number of enzyme-containing intermediates in the mechanism (not counting the free enzyme). The sum of the exponents λ, i.e. $\sum_{i=1}^{n} \lambda_i$, is equal to the sum of all of the first-order rate constants, plus the sum of all the second-order rate constants each multiplied by the corresponding substrate or modifier concentration:

$$\sum_{i=1}^{n} \lambda_i = \sum_i k_i c_i + \sum_j k_j$$

where k_i is a second-order rate constant and k_j a first-order constant. This suggests general procedures for obtaining rate constants. Published results on α-chymotrypsin and alkaline phosphatase are reinterpreted in the light of the theory.

Abbreviations: α-CT, α-chymotrypsin; NPA, *p*-nitrophenyl acetate.

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INTRODUCTION

A number of reactions catalyzed by enzymes are known to occur by a mechanism involving a second intermediate, the general scheme being

$$\begin{array}{l} \mathrm{E} + \mathrm{A} \rightleftharpoons \mathrm{EA} \rightarrow \mathrm{EA}' \rightarrow \mathrm{E} + \mathrm{Y} \\ \qquad\qquad\quad \downarrow \\ \qquad\qquad\quad \mathrm{X} \end{array}$$

Here E is the enzyme, A the substrate, and EA is an addition complex which splits off one product X to form a second intermediate EA′, which finally forms enzyme and a second product Y. The reactions of proteolytic and other hydrolytic enzymes have frequently been found to occur with two identifiable intermediates, EA′ being an acylated enzyme.

In the present paper we first give a treatment of the transient phase for such a system, and apply it to results we have obtained for the action of α-chymotrypsin on *p*-nitrophenyl acetate, in the absence and presence of the competitive inhibitor indole. We later consider the transient-phase equations that apply to a variety of other reaction schemes, and suggest some general procedures for the analysis of experimental results.

THEORETICAL

This analysis is confined to the case in which substrate and inhibitor concentrations are greatly in excess of the concentration of the enzyme. The mechanism is written as

$$\begin{array}{ccccccccc} & \mathrm{E} & & & \overset{k_1}{\rightleftharpoons} & & \overset{k_2}{\longrightarrow} & & \overset{k_3}{\longrightarrow} \\ e_0 - m - n & + & \mathrm{A} & \underset{k_{-1}}{} & \mathrm{EA} & m & \downarrow & \mathrm{EA}' \; n & \longrightarrow \mathrm{E} + \mathrm{Y} \; y \\ & & a_0 & & & & \mathrm{X} & & \\ & & & & & & x & & \end{array}$$

where the k's are the rate constants, and the other lower-case letters refer to the concentration of the appropriate species at any time t. Previous treatments of this case have been given by Ouellet and Laidler [1] and by Ouellet and Stewart [2]: an alternative and more satisfactory treatment, involving the use of operators, is now given. The differential rate equations for the system are

$$\dot{m} = k_1 a_0 (e_0 - m - n) - \bar{k} m \tag{1}$$

$$\dot{n} = k_2 m - k_3 n \tag{2}$$

$$\dot{x} = k_2 m \tag{3}$$

$$\dot{y} = k_3 n \tag{4}$$

where $\bar{k} = k_{-1} + k_2$. Replacement of the differentials by operators P, according to the Laplace–Carson transform method [3], leads to

$$m = \frac{k_1 a_0 e_0 (\mathrm{P} + k_3)}{(\mathrm{P} + \lambda_1)(\mathrm{P} + \lambda_2)} \tag{5}$$

$$n = \frac{k_1 k_2 a_0 e_0}{(\mathrm{P} + \lambda_1)(\mathrm{P} + \lambda_2)} \tag{6}$$

where λ_1 and λ_2 are the negative roots of the quadratic

$$P^2 + P(k_1a_0 + \bar{k} + k_3) + k_3\bar{k} + k_1a_0(k_2 + k_3) = 0 \tag{7}$$

and the following relations are obeyed by λ_1 and λ_2:

$$\lambda_1 + \lambda_2 = k_1a_0 + \bar{k} + k_3 \tag{8}$$

$$\lambda_1\lambda_2 = k_1a_0(k_2 + k_3) + \bar{k}k_3 \tag{9}$$

The originals for m and n are

$$m = \frac{k_1k_3a_0e_0}{\lambda_1\lambda_2} - \frac{k_1a_0e_0(k_3 - \lambda_1)}{\lambda_1(\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1a_0e_0(k_3 - \lambda_2)}{\lambda_2(\lambda_1 - \lambda_2)} e^{-\lambda_2 t} \tag{10}$$

$$n = \frac{k_1k_2a_0e_0}{\lambda_1\lambda_2} - \frac{k_1k_2a_0e_0}{\lambda_1(\lambda_2 - \lambda_1)} e^{-\lambda_1 t} - \frac{k_1k_2a_0e_0}{\lambda_2(\lambda_1 - \lambda_2)} e^{-\lambda_2 t} \tag{11}$$

Substitution of Eqns 10 and 11 into Eqns 3 and 4, respectively, and integration with the boundary conditions $t = 0$, $x = y = 0$, leads to

$$x = \frac{k_1k_2k_3a_0e_0}{\lambda_1\lambda_2} t + \frac{k_1k_2a_0e_0(k_3 - \lambda_1)}{\lambda_1^2(\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1k_2a_0e_0(k_3 - \lambda_2)}{\lambda_2^2(\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1) \tag{12}$$

$$y = \frac{k_1k_2k_3a_0e_0}{\lambda_1\lambda_2} t + \frac{k_1k_2k_3a_0e_0}{\lambda_1^2(\lambda_2 - \lambda_1)} (e^{-\lambda_1 t} - 1) + \frac{k_1k_2k_3a_0e_0}{\lambda_2^2(\lambda_1 - \lambda_2)} (e^{-\lambda_2 t} - 1) \tag{13}$$

In both Eqns 12 and 13 there is a biphasic exponential approach to the initial steady state, which corresponds to the linear term in t.

In the present study we are interested in the variation of x as a function of time. Eqn 12 may be written as

$$x = vt + \beta_1 + \beta_2 - \beta_1 e^{-\lambda_1 t} - \beta_2 e^{-\lambda_2 t} \tag{14}$$

where

$$\beta_1 = -\frac{k_1k_2a_0e_0(k_3 - \lambda_1)}{\lambda_1^2(\lambda_2 - \lambda_1)} \tag{15}$$

$$\beta_2 = -\frac{k_1k_2a_0e_0(k_3 - \lambda_2)}{\lambda_2^2(\lambda_1 - \lambda_2)} \tag{16}$$

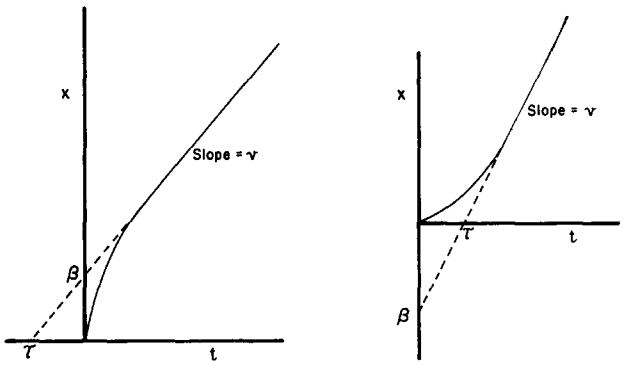
$$v = \frac{k_1k_2k_3a_0e_0}{\lambda_1\lambda_2} \tag{17}$$

We can arrange the equation into

$$-x + vt + \beta = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} \tag{18}$$

where $\beta = \beta_1 + \beta_2$. The variation of x with t is illustrated schematically in Fig. 1; y shows a similar variation.

Analysis of experimental results can conveniently be done with reference to Eqn 18. The quantities β and v can be determined from the variation of x with t (cf. Fig. 1). A plot of ln $(-x + vt + \beta)$ vs t will consist to a good approximation



(a) EARLY BURST (b) LAG

Fig. 1. Variation with time of the concentration, x, of a reaction product.

(if λ_1 and λ_2 are not too close together) of two straight regions with slopes of $-\lambda_1$ and $-\lambda_2$. A plot of $\lambda_1 + \lambda_2$ against a_0 (cf. Eqn 8) is linear with a slope of k_1 and an intercept of $k_{-1} + k_2 + k_3$. A plot of $\lambda_1\lambda_2$ against a_0 is also linear (cf Eqn 9) with a slope of $k_1(k_2 + k_3)$ and an intercept of $(k_{-1} + k_2)k_3$. It follows that the individual constants k_1, k_{-1}, k_2, and k_3 can be separated.

Hijazi and Laidler [4] have used this method of integration and obtained the equations for the transient phase in the presence of a competitive inhibitor where the mechanism is

$$\begin{array}{c} \mathrm{Q} \\ + \\ \mathrm{E} \\ k_q \Uparrow k_{-q} \\ \mathrm{EQ} \end{array} + \mathrm{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA} \xrightarrow[\downarrow]{k_2} \mathrm{EA'} \xrightarrow{k_3} \mathrm{E} + \mathrm{Y}$$
$$\mathrm{X}$$

The variation of x with t was found to be

$$-x + vt + \beta = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} + \beta_3 e^{-\lambda_3 t} \tag{19}$$

where v, β, and the β_i's are obviously different from those for the uninhibited reaction, and

$$\lambda_1 + \lambda_2 + \lambda_3 = k_1 a_0 + k_{-q} + \bar{k} + k_3 + k_q q_0 \tag{20}$$

$$\lambda_1\lambda_2\lambda_3 = \bar{k}k_3(k_q q_0 + k_{-q}) + k_1 k_3 k_{-q} q_0 + k_1 k_2 k_{-q} a_0 \tag{21}$$

Analysis of data with reference to Eqn 19 can be carried out as described above

for the uninhibited case. Plots of ln $(-x + vt + \beta)$ against t will consist of three straight regions with slopes of $-\lambda_1$, $-\lambda_2$ and $-\lambda_3$. The λ_i's follow the relations in Eqns 20 and 21, and plots of $\lambda_1 + \lambda_2 + \lambda_3$ against q_0 can lead directly to values of k_q. The ratio $k_{-q}/k_q = K_q$ is known from steady-state analysis, so that k_{-q} can be calculated. On the other hand, plots of $\lambda_1\lambda_2\lambda_3$ against a_0 are linear with a slope of $k_1k_2k_{-q}$, and since k_1 and k_2 are known k_{-q} can be calculated, and hence k_q.

In the present work, the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin was studied because the kinetics and mechanism of this reaction are well documented, and the reaction is slow enough that the transient phase can be observed conveniently with a stopped-flow apparatus. We observed the rate of release of *p*-nitrophenol, which is X in the reaction scheme shown above. Indole was used as the competitive inhibitor.

EXPERIMENTAL PROCEDURE

Thrice crystallized α-chymotrypsin (lot CD1 6JF) was obtained from Worthington Biochemical Corp., and concentrations were determined using $\varepsilon_{280\ nm} = 5.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. Purified *p*-nitrophenyl acetate (lot 107B-5180) was obtained from Sigma Chemical Co. All other chemicals were reagent grade and were used without further purification. All experiments were carried out at 25 °C in solutions of pH 7.90 Tris buffer ($I = 0.055$) containing 5% v/v isopropanol. The pK_a of *p*-nitrophenol under these conditions was determined spectrophotometrically to be 6.83.

The kinetics of the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin were monitored at 400 nm with a Durrum–Gibson stopped-flow apparatus; photographs of % transmittance versus time were taken of the oscilloscope reaction traces and subsequently analyzed. The difference extinction coefficient was determined to be $\Delta\varepsilon_{400\ nm} = 1.80 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. Fig. 2 shows the results of a typical experiment,

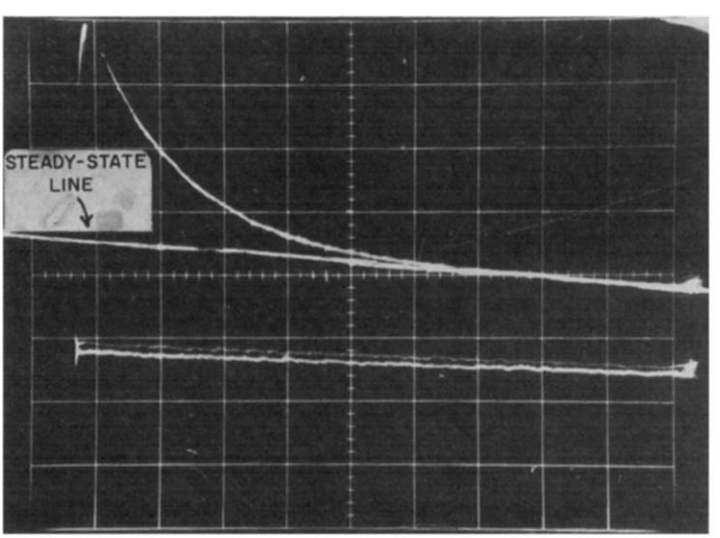


Fig. 2. A representative oscilloscope trace. Vertical scale 50–100% T. Horizontal scale 0.2 s/box $[\alpha\text{-CT}]_0^1 = 6.95 \cdot 10^{-6}$ M, $[NPA]_0 = 4.65 \cdot 10^{-3}$ M.

and illustrates the transient phase approaching the steady-state rate of production of *p*-nitrophenol.

Steady-state parameters in the presence and absence of the inhibitor indole were determined by analyzing stopped-flow oscilloscope traces at slow sweep speeds. That indole is a competitive inhibitor was shown by a plot of v_0/v vs the concentration of indole, in which the slope varied with *p*-nitrophenyl acetate concentration (Laidler [5]) Values of K_m and K_q were found to be $1.00 \cdot 10^{-3}$ M and $5.48 \cdot 10^{-4}$ M, respectively.

RESULTS

Figs 3 and 4 are plots of log $(-x + vt + \beta)$ vs time for the uninhibited reaction and the reaction competitively inhibited by indole, respectively. All plots of the uninhibited reaction showed two slopes, indicating a biphasic exponential approach to the steady state. Fig. 4 shows the triphasic behaviour observed for the competitively

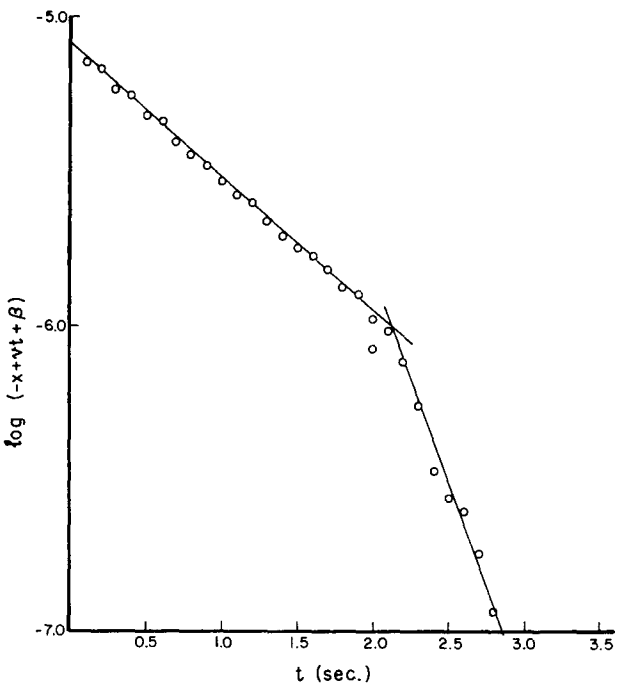


Fig. 3. A plot of log $(-x + vt + \beta)$ vs time. $[\alpha\text{-CT}]_0 = 6.75 \cdot 10^{-6}$ M, $[\text{NPA}]_0 = 3.09 \cdot 10^{-4}$ M, $[\text{indole}]_0 = 0$.

inhibited reaction in the range of indole concentration $4 \cdot 10^{-3}$–$9 \cdot 10^{-3}$ M; below this range only two slopes were seen, and the upper limit was set by solubility limitations.

The slopes of plots such as Fig. 3 were analyzed as a function of $[\text{NPA}]_0$, and the values of λ_1 and λ_2 obtained. Values of λ_1 and λ_2 obtained under identical experimental conditions were reproducible within 10%. Figs 5 and 6 are plots of $\lambda_1 + \lambda_2$

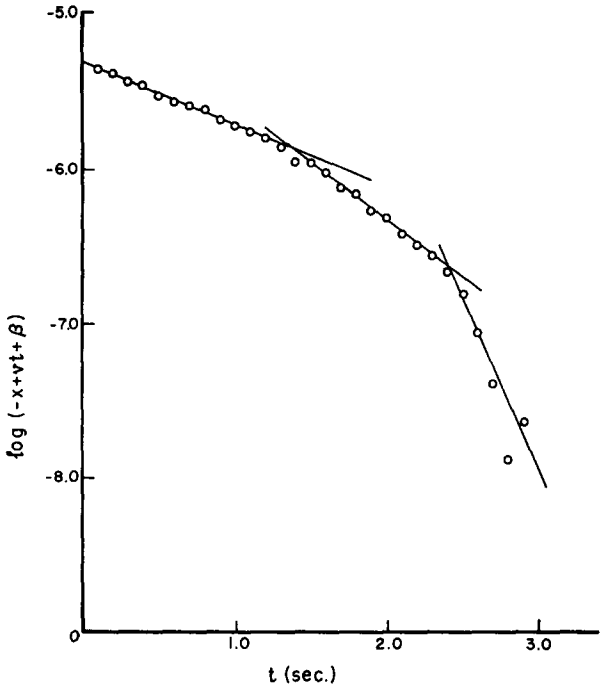


Fig. 4. A plot of log $(-x + vt + \beta)$ vs time. $[\alpha\text{-CT}]_0 = 6.45 \cdot 10^{-6}$ M, $[\text{NPA}]_0 = 2.17 \cdot 10^{-3}$ M, $[\text{indole}]_0 = 8.50 \cdot 10^{-3}$ M.

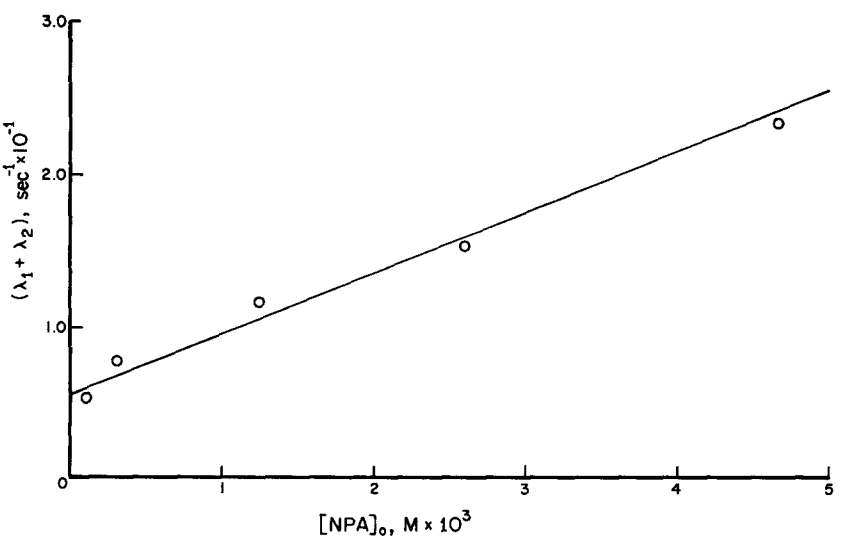


Fig. 5. A plot of $\lambda_1 + \lambda_2$ vs $[\text{NPA}]_0$. $[\alpha\text{-CT}]_0 = 6.97 \cdot 10^{-6}$ M, $[\text{indole}]_0 = 0$.

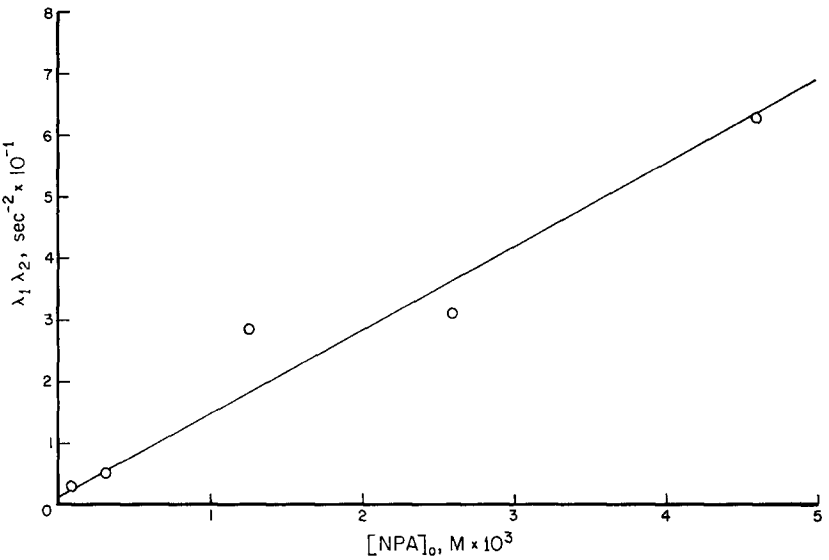


Fig. 6. A plot of $\lambda_1\lambda_2$ vs $[NPA]_0$. Same experimental conditions as for Fig. 5.

and $\lambda_1\lambda_2$ vs $[NPA]_0$, respectively. From these plots, the following values for the rate constants were obtained by least-squares analysis:

$k_1 = (4.0 \pm 0.5) \cdot 10^3\ M^{-1} \cdot s^{-1}$

$k_{-1} = 1.15 \pm 0.2\ s^{-1}$

$k_2 + k_3 = 3.3 \pm 0.3\ s^{-1}$

It would have been possible to separate k_2 and k_3 from the transient-phase data, employing the methods previously described, but it was considered more reliable to make use of the steady-state value

$k_3 = 5.0 \cdot 10^{-4}\ s^{-1}$

which leads to

$k_2 = 3.3 \pm 0.3\ s^{-1}$

Values of k_q and k_{-q} were not determined because of experimental error.

DISCUSSION

To our knowledge, this is the first time that k_1 and k_{-1} have been determined for the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin. The value of $k_2 + k_3$ is in agreement with values summarized by Frankfater and Kézdy [6]. It should be pointed out that poor definition of slopes in plots of $\ln(-x + vt + \beta)$ vs time can be a source of considerable error; nevertheless, we have demonstrated that the uninhibited and competitively inhibited reactions exhibit biphasic and triphasic exponential approaches to the steady state, respectively, in agreement with theory, and that values of individual rate constants in two-intermediate reaction mechanisms may, at least in principle, be determined by the technique described.

GENERAL DISCUSSION

A number of publications from this laboratory [1, 2, 4, 7–13] have been concerned with pre-steady-state kinetics for a variety of enzyme systems. Use of the Laplace–Carson transform method has permitted solutions to be obtained for systems that are too complicated to be analyzed by ordinary methods. During the course of these more recent studies we have become aware of a general pattern that underlies all of the mathematical solutions for the transient phase, and of general methods that may be used to obtain rate constants from the experimental data.

The systems that have been treated up to now are listed in Table I. In all of the cases investigated, the variation in the concentration x of any product with time has the general form

$$x = vt + \beta + \sum_{i=1}^{n} \beta_i e^{-\lambda_i t} \tag{22}$$

The variation was shown schematically in Fig. 1. The rate v is the steady-state rate; β which is equal to $-\sum_{i=1}^{n} \beta_i$, is the intercept on the x axis, and in general can be positive or negative; both cases are shown in the figure. In the former case one may speak of an "initial burst" or "early burst", and the apparent induction period is negative; in the latter case there is a "lag" and the induction period is positive. For the simple Michaelis–Menten scheme (one intermediate, EA) there must be a lag; an initial burst is therefore evidence for a second intermediate. This early burst is only found for the first product X; the second product Y always shows a positive induction period.

The steady state is approached by a curve that is the sum of a number, n, of exponentials. In all of the cases investigated n has turned out to be equal to the number of enzyme-containing intermediates (not counting the free enzyme). We have no general proof of this, but it seems a reasonable presumption that the result is universal. In any case, it provides a useful guideline in the analysis of experimental data.

Sometimes, visual inspection of the x vs t curves reveals a number of exponential terms; a good example is provided by the traces obtained by McConn et al. [14] for an inhibited chymotrypsin system; these traces clearly reveal four exponential terms. Analysis of the traces can be made by numerical fitting, or by plotting ln $(x - vt - \beta)$ against t. Such plots will have n linear regions, the slopes being the λ_i's. We thus have a method of determining the number of experimentally detectable intermediates. Sometimes the different λ_i values are not easily distinguished, but variation of the concentrations of substrates (and of modifiers, if present) frequently helps in their separation.

Table I shows that the sum of the λ_i values is in all cases given by

$$\sum_{i=1}^{n} \lambda_i = \Sigma k_i c_i + \Sigma k_j \tag{23}$$

where k_i is a second-order rate constant and c_i the corresponding substrate or modifier concentration; k_j is a first-order rate constant. The summations are over all terms of the particular type. We have no proof that this relationship always holds, but it is certainly true for a wide variety of mechanisms and we know of no exceptions.

TABLE I

SUMMARY OF TRANSIENT-PHASE EQUATIONS

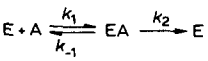
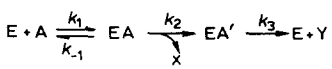
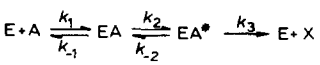
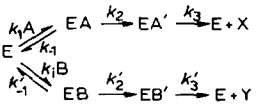
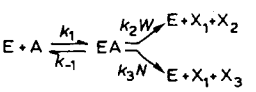
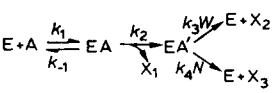
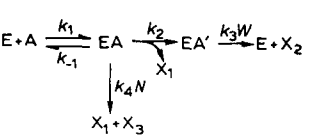
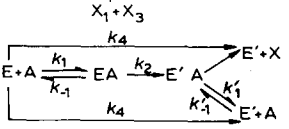
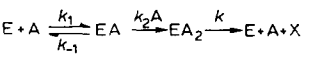
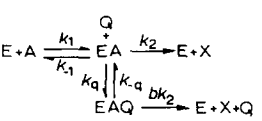
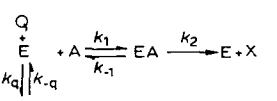
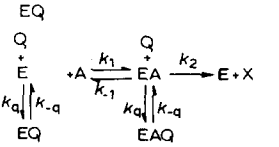
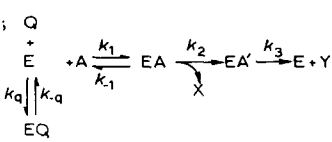
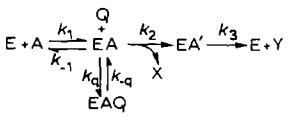
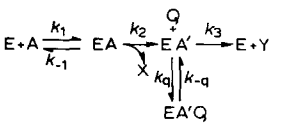
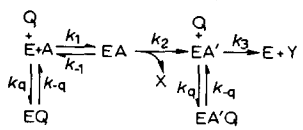
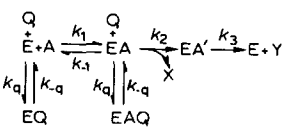
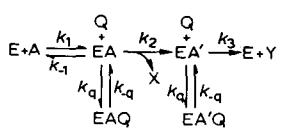
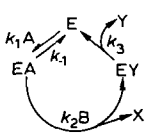
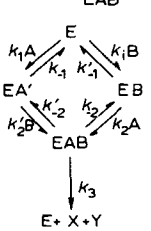
Type of mechanism	mechanism	No. of exponentiials, n	$\sum_{i=1}^{n}\lambda_i$	Ref
Simple Michaelis-Menten		1	$k_1 a_0 + k_{-1} + k_2$	7
Michaelis-Menten with second intermediate		2	$k_1 a_0 + k_{-1} + k_2 + k_3$	1
Reversible conformation change in EA		2	$k_1 a_0 + k_{-1} + k_2 + k_{-2} + k_3$	13
Two competing substrates		4	$k_1 a_0 + k_i' b_0 + k_{-1} + k_{-1}' + k_2 + k_2' + k_3 + k_3'$	*
Simple Michaelis-Menten with competing nucleophile N		1	$k_1 a_0 + k_{-1} + k_2 W + k_3 N$	*
Second intermediate with competing nucleophile		2	$k_1 a_0 + k_3 W + k_4 N + k_{-1} + k_2$	*
Second intermediate with competing nucleophile		2	$k_1 a_0 + k_3 W + k_4 N + k_{-1} + k_2$	*
A mechanism for sigmoid kinetics		3	$(k_1 + k_{-1}')a_0 + k_{-1} + k_2 + k_3 + k_1' + k_4$	*
A mechanism for substrate activation		2	$(k_{-1} + k_2)a_0 + k_{-1} + k_3$	*
Classical anticompetitive inhibition		2	$k_1 a_0 + k_q q_0 + k_{-1} + k_2 + k_{-q} + bk_2$	11
Classical competitive inhibition		2	$k_1 a_0 + k_{-1} + k_2 + k_q q_0 + k_{-q}$	11
Classical non-competitive inhibition		3	$k_1 a_0 + 2k_q q_0 + 2k_{-q} + k_{-1} + k_2$	11
Competitive inhibition; two intermediates		3	$k_1 a_0 + k_q q_0 + k_{-q} + k_{-1} + k_2 + k_3$	4

TABLE I (continued)

Type of mechanism	mechanism	No. of exponentials, n	$\sum_{i=1}^{n}\lambda_i$	Ref.
Inhibition; two intermediates		3	$k_1a_0+k_qq_0+k_{-q}+k_{-1}+k_2+k_3$	4
Inhibition; two intermediates		3	$k_1a_0+k_qq_0+k_{-q}+k_{-1}+k_2+k_3$	4
Inhibition; two intermediates		4	$k_1a_0+2k_qq_0+k_{-1}+k_2+k_3+2k_{-q}$	4
Inhibition; two intermediates		4	$k_1a_0+2k_q\,q_0+k_{-1}+k_2+k_3+2k_{-q}$	4
Inhibition; two intermediates		4	$k_1a_0+2k_qq_0+k_{-1}+k_2+k_3+2k_{-q}$	4
Theorell-Chance		2	$k_1a_0+k_{-1}+k_2b_0+k_3$	12
Ping Pong bi bi		3	$k_1a_0+k_3b_0+k_{-1}+k_2+k_4$	12
Ordered ternary complex		3	$k_1a_0+k_2b_0+k_{-1}+k_{-2}+k_3+k_4$	12
Random ternary complex		4	$(k_1+k_2)a_0+(k_1'+k_2')b_0+k_{-1}+k_{-1}'+k_{-2}'+k_3$	12

* To be published.

a_0, initial concentration of substrate A; b_0, initial concentration of substrate B; W, concentration of water; N, concentration of alternative nucleophile; q_0, initial concentration of modifier Q.

Eqn 23 leads to a procedure for obtaining the second-order constants from the experimental results, by observing the dependence of $\Sigma\lambda_i$ on the concentrations c_i. It also gives rise to Σk_j. Other procedures have to be used for separating the individual k_j values. These methods are different for the different mechanisms; some of them involve studying the dependence of $\prod_{i=1}^{n} \lambda_i$ or of $v \prod_{i=1}^{n} \lambda_i$ on the c_i values. Details have been given in some of the earlier publications [4, 10–12].

These principles will now be applied to some results for α-chymotrypsin and alkaline phosphatase.

α-Chymotrypsin

The transient-phase kinetics of α-chymotrypsin action have been studied in two different ways:

(1) Measurement of reaction products as a function of time.

(2) Displacement of an inhibitor by the substrate, the concentration of enzyme–inhibitor complex being followed as a function of time.

Method 1 has been used in the classical studies of Hartley and Kilby [15], and another investigation using this method has been described earlier in this paper. The results provide strong evidence that the second intermediate EA′ is involved. This method can only be used if one of the products can be monitored by spectroscopic means; in the investigations just referred to the substrate was *p*-nitrophenyl acetate, which yields the easily detected coloured product *p*-nitrophenol.

Specific substrates of α-chymotrypsin cannot easily be investigated by Method 1, since the products cannot be monitored spectroscopically. Some recent investigations have therefore been carried out by Method 2, using the competitive inhibitor proflavin, the absorption spectrum of which changes upon binding to the enzyme. The reaction mechanism is

$$\begin{array}{ccccccccc} & & \mathrm{F} & & & & & & \\ & & + & & & & & & \\ & & \mathrm{E} & \overset{k_1}{\rightleftharpoons} & \mathrm{EA} & \xrightarrow{k_2} & \mathrm{EA'} & \xrightarrow{k_3} & \mathrm{E} + \mathrm{Y} \\ k_q & \Updownarrow & k_{-q} & & k_{-1} & & \downarrow & & \\ & & \mathrm{EF} & & & & \mathrm{X} & & \end{array} + \mathrm{A}$$

Enzyme and proflavin (F) are premixed in one syringe, and then mixed with substrate solution at $t = 0$. The transient-phase equations for the above mechanism, for no premixing, have been presented in a previous publication [4], which gave solutions for the rates of formation of product. The equation for the change in concentration of EF with time is easily worked out on the basis of the preceding treatment, and is

$$[\mathrm{EF}] = \frac{k_q f_0 \mathrm{Q}}{\lambda_1\lambda_2\lambda_3\lambda_4} - \sum_{i=1}^{4} \frac{k_q f_0 e_0(-\lambda_i^3 + \mathrm{M}\lambda_i^2 - \mathrm{L}\lambda_i + \mathrm{Q})}{\lambda_i(\mathrm{P} - \lambda_i)} \mathrm{e}^{-\lambda_i t} \tag{24}$$

The λ_i's are the roots of a complicated polynomial of the fourth degree, P is the differential operator, and f_0 is the initial proflavin concentration; also

$$\sum_{i=1}^{4} \lambda_i = k_1 a_0 + k_q f_0 + k_{-q} + k_2 + k_{-1} + k_3 \tag{25}$$

$$\prod_{i=1}^{4} \lambda_i = (k_q f_0 + k_{-q})Q + k_1 k_{-q} e_0 a_0 (k_2 + k_3) \tag{26}$$

$$Q = (k_{-1} + k_2)(k_q f_0 + k_{-q}) + k_1 k_{-q} a_0 (k_2 + k_3) \tag{27}$$

It has been shown in an earlier paper [13] that premixing, e.g. of E and F, only affects the pre-exponential terms; the equation for [EF] will therefore be of the same form as Eqn 24, and Eqns 25, 26 and 27 will still apply.

The experiments done so far on this system were carried out in the absence of a complete theory. Hess et al. [14, 16–21], however, intuitively envisioned the reaction as involving the following four steps:

(1) An initial rapid decrease in concentration of EF, which was considered to reflect the formation of EA.

(2) A second decrease in the concentration of EF, considered to occur as a result of the formation of EA′.

(3) A period where the concentration of EF hardly changes, considered to reflect the period during which there is a steady concentration of EA′.

(4) A final period during which there is an increase in the concentration of EF, considered to be a result of the decomposition of EA′ and regeneration of the enzyme.

This intuitive breakdown is certainly along the right lines, but oversimplifies the situation. The oscilloscope traces of Hess et al. do clearly reveal four exponential terms, which is consistent with the above explanation and with Eqn. 24. We are planning further experimental work along these lines.

Alkaline phosphatase

A considerable number of transient-phase studies have been carried out with alkaline phosphatase [22–36], and a variety of interpretations have been given. Fernley and Walker [32] worked with 4-methylumbelliferyl phosphate as substrate, and monitored the first product released (4-methylumbelliferone). At substrate concentrations of approx. 5 μM and in the pH range of 3.8–6.3 there was an early burst (negative induction period). At lower substrate concentrations (0.1 μM) in the pH range of 4.9 to 5.9 there was an initial lag (positive induction period). The results also indicated two exponential terms. These findings are all consistent with a simple two-intermediate mechanism, for which

$$x = vt + \beta + \sum_{i=1}^{2} \beta_i e^{-\lambda_i t} \tag{28}$$

with an induction period of

$$\tau = \frac{v}{\beta} = \frac{k_3^2 - k_1 k_2 a_0}{k_3^2(k_{-1} + k_2) + k_1 k_3 a_0 (k_2 + k_3)} \tag{29}$$

τ can thus be positive or negative according to the relative magnitudes of k_3^2 and $k_1 k_2 a_0$.

Other workers have proposed mechanisms that are more complicated than required to explain the results. Thus Gutfreund et al. [22, 23, 30, 31] observed only one exponential approach to the steady state, and discussed their results with respect to the mechanism

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*A \xrightarrow{k_3} E^*A' \xrightarrow{k_4} E + Y$$

$$\downarrow X$$

E^*A is a conformationally-different form of EA. Studies with added nucleophiles do, in fact, provide support for such a mechanism [37]. However, theory indicates that there should be a triphasic rise to the steady state; it may be that two of the exponential terms were too large to be observed under the experimental conditions. The results of Gutfreund et al. do not provide evidence for the three-intermediate mechanism they proposed. An alternative mechanism involving conformational changes was proposed by Reid and Wilson [36], but again the transient-phase work provides no support.

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