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Intermediate Compounds in Enzymic Catalysis and Their Kinetic Investigation

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Reactions catalysed by enzymes usually involve the successive formation of several intermediate compounds (enzyme-substrate complexes, covalent intermediates, conformers of the enzyme or the substrate). Kinetic methods have provided a fertile approach to the investigation of such labile intermediates. The Review summarises the kinetic laws of catalytic reactions involving labile intermediates in prestationary and stationary states. The relaxation kinetics of a multistage catalytic reaction is also examined.
The investigation of labile intermediate compounds is inseparably connected with the development of methods of "fast" kinetics. The Review therefore discusses "flow", "flash", and "temperature jump" methods and the possibility of applying them to enzymic reactions. Rather more detailed consideration is given to the formal kinetics of the appearance of intermediate compounds in reactions of the most thoroughly studied enzymes-catalase, α-chymotrypsin, peroxidase, ribonuclease, and aspartate aminotransferase. A list of 236 references is included.

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I. INTRODUCTION

Physical organic chemistry is paying increasing attention to research on reactions involving biopolymer catalysts[1,2], because knowledge of the specific mechanisms through which enzymes accelerate chemical reactions should help to elucidate the driving forces of enzymic catalysis. As a result, application of the principles of the action of the active centres functioning in enzymes might facilitate solving the problem of producing selective chemical catalysts with controllable properties[3-5].

The efforts of several scientific biochemical schools during the past decade have produced considerable progress in research on catalysis by enzymes[1,2,6]. Investigations in this field are developing in two main directions: (i) the structures both of enzymes and of the active centres are being studied, the most valuable information being given by X-ray diffraction[7-9]; and (ii) a study is being made of the dynamics of enzymic action, i.e. the sequence of changes undergone by a substrate molecule at an active centre of the enzyme, leading to formation of a product molecule. The present Review is devoted mainly to discussing the achievements of the latter group of investigations.

A fundamental idea in enzymic catalysis is the multistage transformation of substances under the influence of a protein catalyst. This was first pointed out by Michaelis and Menten[10], who concluded that the mechanism of the action of invertase involves formation of an intermediate enzyme-substrate complex (a Michaelis complex)†.

Subsequently a detailed investigation of other enzymic systems confirmed that reactions involving biological catalysts actually comprise the successive formation of several intermediate compounds (non-covalent enzyme-substrate complexes, covalently formed intermediate compounds, conformers of enzyme or substrate). Thus study of the mechanism of enzyme action encounters the specific problem of determining the number and nature of intermediate compounds involved. These intermediates are usually extremely labile, and in rare cases can be isolated in individual form[1]. Chemical kinetic methods have proved very fruitful for investigating such labile intermediates.

The present Review summarises the kinetic laws observed in enzymic reactions, paying attention primarily to the kinetic evidence for intermediate compounds in both transitional and stationary stages of the reaction. More detailed acquaintance with procedural features of the kinetic methods used can be obtained by referring to reviews[1,11-13]. Certain formal kinetic aspects of the investigation of intermediate compounds in enzymic catalysis may prove useful also for analysing other classes of reactions likewise involving labile intermediates.

The simplest general mechanism of a multistage consecutive catalytic reaction occurring on an active centre of an enzyme E and involving a substance S (the substrate) can be written

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \dots \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} X_i \rightleftharpoons \dots X_n \underset{k_{-(n+1)}}{\overset{k_{(n+1)}}{\rightleftharpoons}} E + P \qquad (1)$$

where the X_i ($i = 1,2,3,\dots,n$) are intermediate compounds, P is the reaction product, and k_i and k_{-i} are the rate constants of forward and reverse elementary steps. This scheme covers both unimolecular [k_i, $i \neq 1$, $-(n+1)$] and bimolecular [k_1, $k_{-(n+1)}$] elementary reactions. According to the laws of chemical kinetics a differential equation describing the variation of concentration with time can be written for each of the $n + 3$ participants in the reaction. Only $n + 1$ of the resulting $n + 3$ differential equations are linearly independent, since the variable

† The term "Michaelis complex" often implies the primary non-covalent enzyme-substrate complex. This is not quite exact, since the "saturation" observed in the dependence of reaction velocity on substrate concentration, which indicates the existence of a "Michaelis complex", may represent the presence of any other intermediate compound in the reaction, including one formed covalently. In its general meaning the term "Michaelis complex" represents the totality of intermediate compounds in enzymic catalysis.

component concentrations are related by the two linear algebraic equations of material balance

$$[E]_0 = [E] + \sum_{i=1}^{n} [X_i]$$
$$[S]_0 = [S] + [P] + \sum_{i=1}^{n} [X_i] \quad (2)$$

Thus the progress of all the elementary reactions in scheme (1) with time is described by the set of $n + 1$ differential equations

$$\begin{aligned}
&\frac{d[X_1]}{dt} + (k_{-1} + k_2)[X_1] = k_1[E][S] + k_{-2}[X_2] \\
&\vdots \quad \cdots \\
&\frac{d[X_i]}{dt} + (k_{-i} + k_{i+1})[X_i] = k_i[X_{i-1}] + k_{-(i+1)}[X_{i+1}] \\
&\vdots \quad \cdots \\
&\frac{d[X_n]}{dt} + (k_{-n} + k_{(n+1)})[X_n] = k_n[X_{n-1}] + k_{-(n+1)}[E][P] \\
&\frac{d[P]}{dt} + k_{-(n+1)}[P][E] = k_{(n+1)}[X_n]
\end{aligned} \quad (3)$$

together with the two algebraic equations (2). Simultaneous solution of Eqns. (2) and (3) yields expressions for all the reaction components as functions of time. The above differential and algebraic equations constitute a non-linear set with constant coefficients (non-linear because of the presence of second-order terms containing the products of variable concentrations—$k_1[E][S]$ and $k_{-(n+1)}[E][P]$). A solution can be found by numerical integration on a digital computer[14] or by simulation on an analogue computer[15]. The character of the transitional processes in enzymic reactions involving intermediate compounds has been investigated qualitatively for various initial conditions[16]. However, the possibility of an analytical solution of the set of equations (2) and (3) is of the greatest interest for comparing the reaction mechanism with experimental kinetic results. This task can be simplified considerably by using certain conditions permitting transformation to a linear set of equations. In the kinetic analysis of the mechanism of the action of enzymes three approaches based on the premises simplifying the formal kinetic treatment—(*a*) kinetics of reactions in the transitional stage or "prestationary" kinetics, (*o*) kinetics of steady-state enzymic conversion, and (*c*) relaxation kinetics—have at present found the greatest development and application. These approaches and their application to the investigation of reaction mechanisms will be examined in detail below with reaction (1) as example.

II. TRANSITIONAL STAGE OF REACTION

1. "Prestationary" Kinetics of an Enzymic Reaction

The non-linear set of equations (2) and (3) can be simplified by conducting the bimolecular stages of reaction (1) under pseudounimolecular conditions, i.e. in the presence of a large excess of one of the reactants. Changes in the concentration of this reactant with time can then be neglected, so that the bimolecular terms in the differential equations (3) become linearly dependent only on the variable concentration of one of the reactants. This approach is usually made for the analytical treatment of so called "prestationary" kinetics.

With $[S]_0 \gg [E]_0$ and only slight occurrence of reaction (1) ($[P] \simeq 0$ is formally equivalent to formation of the product P at an irreversible stage of the reaction, $k_{-(n+1)} = 0$) under conditions such that consumption of the substrate can be neglected, $[S] = [S]_0$, the set of equations (2) and (3) becomes

$$\left\{\begin{aligned}
&\frac{d[X_1]}{dt} + (k_{-1} + k_2)[X_1] = k_1[S_0][E] + k_{-2}[X_2] \\
&\vdots \quad \cdots \\
&\frac{d[X_i]}{dt} + (k_{-i} + k_{i+1})[X_i] = k_1[S]_0[E] + k_{-2}[X_2] \\
&\vdots \\
&\frac{d[X_n]}{dt} + (k_{-n} + k_{n+1})[X_n] = k_n[X_{n-1}] \\
&[E]_0 = [E] + \sum_{i=1}^{n} [X_i]
\end{aligned}\right. \quad (4)$$

$$\frac{d[P]}{dt} = k_{(n+1)}[X_n] \quad (5)$$

Several standard procedures—reduction to a single linear differential equation of higher order, application of Laplace transforms, etc.—are available for solving sets of linear differential equations[17]. The method of solution in the kinetic analysis of complicated chemical reactions has been described[18-22], and several approaches to a solution have been discussed for the progress of enzymic reactions[15,23-26]. The use of graph theory for these purposes has been described[27,28].

The general solution of Eqns. (4) is

$$[X_n] = \sum_{j=1}^{n} A_j e^{\lambda_j t} + B \quad (6)$$

where the λ_j are the n eigen values or real roots of the corresponding characteristic equation of order n. The quantity $-1/\lambda_j$ has the dimension of time and is a complicated function of the elementary rate constants of reaction (1). Particular solutions describing the time variation of the concentrations of all intermediate compounds X_i can be obtained from the general solution (6) by introducing initial conditions. The dependence of the concentration of the reaction product on time follows from the differential equation (5) when (6) is substituted and the resulting expression is integrated:

$$[P] = k_{n+1} \sum_{j=1}^{n} \frac{A_j}{\lambda_j} (e^{\lambda_j t} - 1) + k_{n+1} B t \quad (7)$$

Equations (6) and (7) generalise for n intermediate compounds earlier equations obtained for the kinetics of reactions involving one intermediate[29-33]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X \xrightarrow{k_2} E + P \quad (8)$$

or two intermediates[15,34]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} X_2 \xrightarrow{k_3} E + P \quad (9)$$

Considering scheme (8) in greater detail, we find that the time variation of the concentration of the intermediate X is given by

$$[X] = \frac{k_1[S]_0[E]_0}{k_{-1} + k_2 + k_1[S]_0} (1 - e^{-t/\tau}) \quad (10)$$

Analysis of the curve corresponding to function (10) (Fig. 1*A*) enables the characteristic time τ to be determined. The dependence of τ^{-1} on the substrate concentration

$$\tau^{-1} = k_1[S]_0 + k_{-1} + k_2 \qquad (11)$$

will then yield the formation and decomposition rate constants k_1 and $k_{-1} + k_2$ of the intermediate compound.

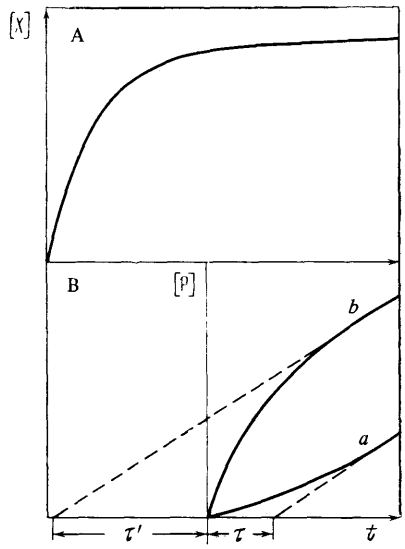


Figure 1. Kinetics of an enzymic reaction involving one intermediate under prestationary conditions.
(A) Dependence of concentration of intermediate compound X on time for the prestationary occurrence of the reaction by mechanism (8). (B) Rate curve for the prestationary formation of the product by different mechanisms:
a) Michaelis–Menten (scheme 8); *b*) Henry (scheme 14).

If direct experimental observation of X is difficult, information on the presence of such intermediate and on the reaction mechanism can be obtained by examining the kinetics of formation of the final product. The concentration of P as a function of time is given [32] by

$$[P] = \frac{k_2[E]_0[S]_0}{[S]_0 + K_m}t + \frac{k_1k_2[E]_0[S]_0}{k_1[S]_0 + k_{-1} + k_2}\left(e^{-(k_1[S]_0 + k_{-1} + k_2)t} - 1\right) \qquad (12)$$

where $K_m = (k_{-1} + k_2)/k_1$. The [P] curve approaches asymptotically a straight line whose slope is the steady-state rate of the process (Fig. 1*B*, curve *a*):

$$v_{stat} = \left(\frac{d[P]}{dt}\right)_{max} = \frac{k_2[E]_0[S]_0}{[S]_0 + K_m} \qquad (13)$$

Formula (11) holds for the observed induction period τ. The presence of an induction period in the product–time curve (in the absence of a chain or autocatalytic reaction) is a reliable indication of the presence of an intermediate (or at least one intermediate—see below). Analysis of the dependence of $(d[P]/dt)_{max}$ and τ on the enzyme and substrate concentrations will enable rate constants to be determined for all the elementary reactions.

Consecutive formation of an enzyme–substrate complex (Michaelis–Menten, scheme 8) and its concurrent formation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad (14)$$
$$E + S \xrightarrow{k_2} P + E$$

(Henry [35]) can be distinguished by the formal kinetic analysis of enzymic reactions in the prestationary state. Scheme (14), which assumes the formation of an unreactive complex ES and a bimolecular reaction of the substrate with the free enzyme, leads to the steady-rate equation (13) for scheme (8). However, significant differences have been found [36] between the kinetics of reactions (8) and (14) in the prestationary stage. No induction period is present in the product–time curve for reaction (14) (Fig. 1*B*, curve *b*), and the asymptote corresponding to the steady state makes the negative intercept

$$\tau' = -\frac{[S]_0}{k_1K_m(K_m + [S]_0)} \qquad (15)$$

on the abscissa axis. Mechanisms (8) and (14) can be distinguished also by the methods of relaxation kinetics [37].

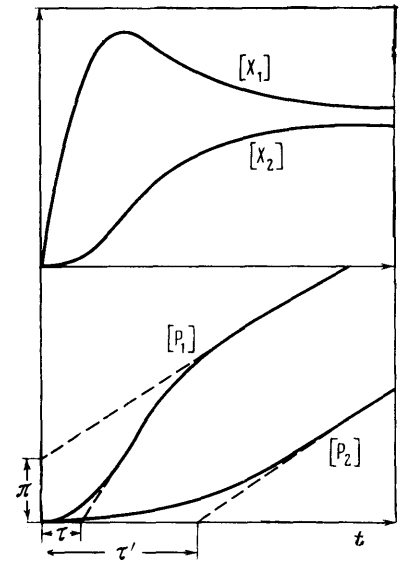


Figure 2. Prestationary kinetics of the formation of intermediates and products for a three-stage mechanism (16).

The mechanism of the action of proteolytic enzymes has been discussed in detail in terms of the formation of two intermediates and two products

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \rightarrow X_2 \overset{k_3}{\rightarrow} E + P_2 \qquad (16)$$
$$\searrow P_1$$

Fig. 2 illustrates the variation of $[X_1]$ and $[X_2]$. The analytical time dependence of the concentration of product P_1 (Fig. 2) is [38]

$$[P_1] = \frac{k_2R}{Q}t + \frac{k_2N}{G}(e^{Gt} - 1) + \frac{k_2M}{F}(e^{Ft} - 1) \qquad (17)$$

where R, Q, F, G, M, and N are functions of the rate constants k_1, k_{-1}, k_2, and k_3. All the elementary rate constants of mechanism (16) can be determined[38] by analysing the steady-state reaction velocity and also τ, τ', and π

$$\tau = \frac{1}{k_1[S]_0 + K_m}; \quad K_m = \frac{k_2 + k_{-1}}{k_1} \tag{18}$$

$$\tau' = \frac{k_2^2 - k_1 k_2 [S]_0}{k_1 k_3 (k_2 + k_3)([S]_0 + K_m')}; \quad K_m' = K_m \frac{k_3}{k_2 + k_3} \tag{19}$$

$$\pi = [E]_0 \left\{ \frac{k_2^2 [S]_0^2}{(k_2 + k_3)^2 ([S]_0 + K_m')^2} - \frac{k_2 k_3^2 [S]_0}{k_1 (k_2 + k_3)^2 ([S]_0 + K_m')^2} \right\} \tag{20}$$

as functions of enzyme and substrate concentrations (see also Fig. 2).

We must emphasise an important general kinetic peculiarity of multistage catalytic reactions of type (1) in the prestationary state. Equations (6) and (7) show that the functional time dependence of the concentration of intermediate compounds is given by the sum of n exponential terms and a constant, whereas that of the concentration of the final product is represented by the sum of n exponential terms $(e^{\lambda_j t} - 1)$ together with a linear function of time. Hence the number of exponential terms in Eqns. (6) and (7) corresponds to the number of intermediate compounds in reaction (1) (cf. in particular mechanisms (8) and (16) with Eqns. (10) and (17)). Thus a reaction involving n intermediate compounds will ideally yield an experimental set of n characteristic times. In an actual experiment, however, a smaller number of exponential terms may be observed, primarily because of the method of recording the kinetic curve. The most widely used spectrophotometric method requires appreciable spectral differences between the components of reaction (1). Furthermore, the time resolution of the apparatus imposes significant limitations. A characteristic time can be determined experimentally only provided that it exceeds the "dead" time of the apparatus. As a consequence of these limitations, the number of exponential terms observed experimentally will give only an estimate of the minimum number of intermediate compounds involved in the reaction.

2. Experimental Methods

The kinetics of the prestationary stage of enzymic reactions (approximated by the above restriction to excess of one of the reactants and a slight extent of conversion of the substrate) has been studied mainly by flow methods. Such methods for measuring the kinetics of fast reactions in solution were first used by Hartridge and Roughton[39-41] and have since been developed by Chance[42-47]. At the present time the stopped-flow method is most widely used[48-53,181]. The main feature of flow methods is the rapid mixing of solutions of the reactants in a specially designed mixing chamber. Much attention has been paid to the problem of efficient mixing[11,54]. The main conclusion was that rate constants determined by means of flow apparatus can be regarded as reliable only for reactions with half-lives exceeding 0.2–1 ms. The "dead" time of current flow equipment is about 0.1–0.3 ms[50,51,55]. Nevertheless, the development of sufficiently simple and convenient apparatus has led to the widespread use of flow methods (especially the stopped-flow method) for studying intermediate compounds in enzymic reactions. Table 1 lists the enzymic systems that have been studied by flow methods.

The use of flash photolysis in enzymology will further increase the resolving power of apparatus for investigating non-stationary kinetics[109-112]. In contrast to flow methods, the flash method does not require rapid mixing of the reactants. With use of a photosensitive substrate precursor, capable of conversion into the substrate by the action of light, two parameters—(i) duration of the flash and (ii) time of photochemical formation of the substrate—govern the "dead" time of the method[110]. The xenon pulse technique yields powerful flashes of light lasting 10–100 ms.[113] The duration of a flash can be shortened without diminishing the power by use of a laser technique[111]. The duration of the photochemical reaction may be quite short.

Table 1.

Enzyme	Substrate or activator	Ref.
Alcohol dehydrogenase	aromatic aldehydes	56
	p-nitrosodimethylaniline	57
	benzaldehyde, acetaldehyde	58-60
α-Amylase	*o*-nitrophenol-α-maltose	61
Hydroxylase	*p*-hydroxybenzoic acid	62
Glyceraldehyde phosphate dehydrogenase	NAD*	63
Glutamate dehydrogenase	glutamic acid	64,65
Enolase	interaction of subunits	66
Carbamate kinase	carbamoyl phosphate, ADP**	67
Carbonic anhydrase	sulphonamides	68,69
	Zn^{2+}	70
Carboxypeptidase A	*N*-dansylated peptides	71
Catalase	hydrogen peroxide	72
Catalase-hydrogen peroxide	hydrogen peroxide, ethanol	72,73
Xanthin oxidase	xanthin oxygen	74
Lactate dehydrogenase	NAD*, lactic acid	75-77
D-Amino-acid oxidase	β-chloroalanine	78
Papain	nitrophenyl esters of benzyloxycarbonyl-glycine	79
	p-nitrophenyl ester of benzyloxycarbonyl-L-lysine	80
Peroxidase	hydrogen peroxide, ethyl and methyl hydroperoxides	81
Peroxidase-hydrogen peroxide	ascorbic acid, quinol	73
	cytochrome *C*	82
	hexacyanoferrate(II)	83
Staphylococcal nuclease	isomerisation (pH jump)	84
Trypsin	ethyl ester of *N*-benzoyl-L-arginine	31,86
	p-nitroanilide of *N*-benzoyl-L-arginine	87
	p-nitrophenyl acetate	108
	structural changes	89
Tryptophan synthetase	L-serine, indole, tryptophan	90
Ficin	ethyl ester of *N*-acetyl-L-arginine	31,86
	p-nitrophenyl hippurate	88
	p-nitrophenyl ester of benzyloxycarbonyl-L-lysine	80
Chymotrypsin	*p*-nitrophenyl acetate	85,91-94
	methyl and ethyl esters of *N*-acetylated L-amino-acids	95-100
	amide of *N*-furovlacryloyl-L-tryptophan	98
	methyl ester of *N*-furoylacryloyl-L-tryptophan	99
	p-nitrophenyl esters of *N*-benzyloxycarbonyl-L-amino-acids	101,102
	N-furoylacryloyl-L-tryptophan (pH jump)	103
	cyclic sulphonates	104
	Proflavin	105
Cytochrome C	Cr^{2+}	106
Alkaline phosphatase	*O*-*p*-phenylazophenyl phosphorothioate	107

[* NAD = nicotinamide–adenine dinucleotide.]
[** ADP = adenosine diphosphate.]

Catalase, peroxidase, and α-chymotrypsin have been studied in greatest detail by the above methods, and therefore the mechanism by which these enzymes function will be more fully discussed below.

3. α-Chymotrypsin

The proteolytic enzyme α-chymotrypsin catalyses the hydrolysis of peptide linkages, as well as that of ester and amide bonds in synthetic model substrates. The first hint that an intermediate compound of the enzyme with the acidic portion of the substrate is formed at the stage of

formation of the main product P_1 (scheme 16) was Hartley and Kilby's observation[114,115] that the separation of *p*-nitrophenol in the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin takes place in two stages: an initial "burst" of *p*-nitrophenol in approximately stoichiometric concentration with respect to the added enzyme is followed by a slower, steady-state further hydrolysis of the substrate (a critique of the conclusions of this research will be found in Ref. 1). Careful investigation of the kinetics of both stages by later workers[85,91-94], using the stopped-flow method, showed that the rate curve for the separation of *p*-nitrophenol is described by Eqn. (17) provided that $k_1[S]_0 \gg k_2$, k_3. The time variation of $[P_1]$ then assumes the simpler form[91]

$$[P_1] = At + B(1 - e^{-kt}) \tag{21}$$

$$A = \frac{\frac{k_2 k_3}{k_2 + k_3}[E]_0[S]_0}{[S]_0 + K_m \frac{k_3}{k_2 + k_3}}; \quad K_m = \frac{k_{-1} + k_3}{k_1} \tag{22}$$

$$B = \frac{\left(\frac{k_2[S]_0}{K_m + [S]_0}\right)^2 [E]_0}{\left(\frac{k_2[S]_0}{K_m + [S]_0} + k_3\right)^2} \tag{23}$$

$$k = \frac{k_2[S_0]}{K_m + [S]_0} + k_3 \tag{24}$$

In fact, when $[S]_0 \gg K_m$ and $k_2 \gg k_3$, we have $B = [E]_0$, so that the initial "burst" of *p*-nitrophenol corresponds to the initial concentration of the enzyme (Fig. 3)‡. These results established the presence of the intermediate compound X_2 (the acylated enzyme), which was subsequently shown to be the ester of the hydroxyl group of serine-195, present in the active centre of the enzyme, and the acyl part of the substrate[117,118]§.

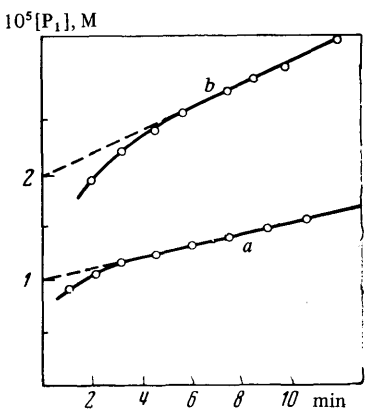


Figure 3. Kinetics of the formation of *p*-nitrophenol in the hydrolysis of *p*-nitrophenyl acetate catalysed by α-chymotrypsin[115] (enzyme concentration for curve *b* double that for *a*).

‡ Current methods for determining the concentration of active centres in many enzymes are based on this principle[116].

§ Several acylated enzymes, which are stable at low pH, have been isolated in pure form (acetylchymotrypsin[117], the homologous series of acylated enzymes formed by aliphatic carboxylic acids from C_1 to C_7[119], *cis*- and *trans*-isomers of cinnamoylchymotrypsin[120-122], etc.).

The kinetics of acylation of the active centre in α-chymotrypsin by *p'*-nitrophenyl *trans*-*p*-nitrocinnamate has been studied[110] by flash photolysis using the *cis*-ester as photosensitive substrate precursor. Steric features of the active centre are such that the reactivity of the *trans*-isomers of substrate derivatives of β-arylacrylic acids exceeds that of the corresponding *cis*-isomers by factors of 10^3–10^4 (*trans*-specificity)[123]. The action of a flash of light on a solution of α-chymotrypsin with *p'*-nitrophenyl *cis*-*p*-nitrocinnamate forms the highly reactive *trans*-isomer, which acylates the active centre of the enzyme. Spectrophotometric monitoring of the separation of *p*-nitrophenol was used to study the kinetics of the reaction.

p-Nitrophenyl acetate and the other substrates used in the above researches are relatively unreactive ("non-specific") substrates of α-chymotrypsin. Gutfreund and Sturtevant[91,92] suggested that the catalysis by chymotrypsin of specific ester substrates derived from α-*N*-acylated L-amino-acids takes place by the same mechanism (16) as does the hydrolysis of *p*-nitrophenyl acetate. Special attention was paid to proving this hypothesis[96-100].

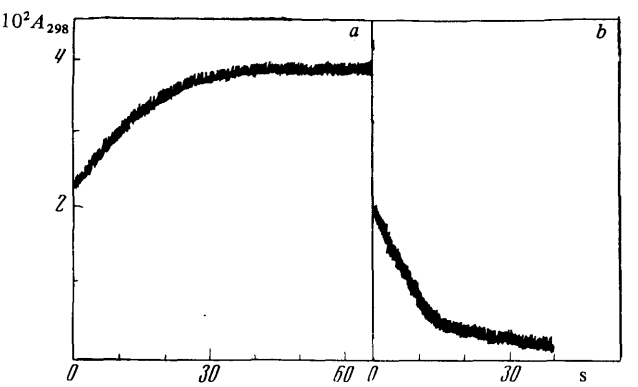


Figure 4. Prestationary kinetics of the hydrolysis of the methyl ester of *N*-acetyl-L-phenylalanine investigated by stopped-flow method[98]: *a*) variation of optical density when 3.7×10^{-5} M α-chymotrypsin and 0.017 M substrate solutions are mixed at pH 3.2; *b*) pH of solution obtained in expt. (*a*) raised from 3.2 to 5.7.

Formation of an acylated enzyme in the hydrolysis of the ethyl ester of *N*-furoylacryloyl-L-tyrosine is indicated by the effect that separation of the alcohol anticipates establishment of a steady-state concentration of the intermediate compound (as with *p*-nitrophenyl acetate).[95] Direct kinetic confirmation of the formation of intermediate compounds X_1 and X_2 in the catalysed hydrolysis of esters of *N*-acylated L-amino-acids has been obtained[98,124]. In the latter case analysis of the reaction kinetics at the wavelengths of absorption by the intermediate compounds (~ 290 nm) revealed that the rate curve was represented by the sum of at least two exponential terms, which according to Eqn. (6) indicates the formation of at least two intermediate compounds. Thus mixing a solution of α-chymotrypsin with the methyl ester of *N*-acetyl-L-phenylalanine produces a rapid, kinetically unresolved variation of the spectrum (with a half-time < 3 ms), which evidently indicates formation of the primary enzyme–substrate complex X_1; this is followed by slow formation of the acylated enzyme (Fig. 4, curve *a*). In the steady

state, under conditions such that consumption of the substrate can be neglected, the concentration of the acylated enzyme remains constant; consumption of the substrate ultimately leads to disappearance of the intermediate compounds from the solution (curve *b*). A similar pattern is found in the chymotrypsin hydrolysis of other specific ester substrates—the methyl esters of the *N*-acetylated amino-acids L-tyrosine, L-tryptophan, and L-leucine, and also the ethyl ester of *N*-acetyl-L-tyrosine—the acylation rate curves for all these substrates being used to determine the formation and hydrolysis rate constants k_2 and k_3 of the acylated enzymes by means of Eqn. (24).[124]

The rate of hydrolysis of amides is limited at the stage of formation of the acylated enzymes ($k_2 \ll k_3$)[97,98]. A more detailed picture of formation of the enzyme–substrate complex X_1 could be obtained for this class of substrates. Use of a combined "stopped flow–temperature jump" apparatus (see below) showed[98] that formation of a reactive enzyme–substrate complex in the hydrolysis of *N*-furoylacryloyl-L-tryptophanamide involves formation of a metastable intermediate compound:

$$E + S \underset{10^4\ s^{-1}}{\overset{6\cdot10^7\ M^{-1}\ s^{-1}}{\rightleftharpoons}} ES \underset{30\ s^{-1}}{\overset{1.5\ s^{-1}}{\rightleftharpoons}} ES' \rightarrow E + P \qquad (25)$$

The rate constants of formation of the primary complex ES and of the conformational change ES $\rightleftharpoons$ ES′ (scheme 25) were determined from an analysis of the rate curve described by the sum of two exponential terms.

The conformational mobility of the active centre of α-chymotrypsin was discovered kinetically from an analysis of the interaction of the acridine colorant Proflavin with the active centre. Analysis of the kinetics of complex formation shows[105] that the active centre exists in solution as two conformations, which are able to bind the colorant at significantly different rates. The concentration ratio of the conformers depends on the pH.

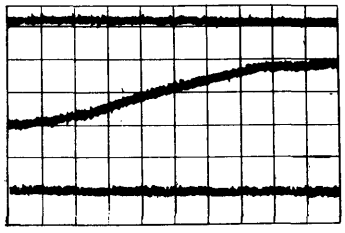


Figure 5. Oscillogram of variations of transmittance after the flash exposure of *cis*-*p*-nitrocinnamoylchymotrypsin (0.1 M KCl + 0.01 M KH_2PO_4 (pH 8.8) at 25°C). The horizontal lines mark 0 and 100% transmittance, and the time base is 0.25 s per large division[109].

A conformational change in the active centre has been recorded at the hydrolysis stage of the acylated enzyme in the hydrolysis of *trans*-*p*-nitrocinnamoylchymotrypsin induced by flash exposure of the *cis*-isomer[109]. Such exposure at the absorption wavelength of the cinnamoyl chromophore (320–360 nm) produces a variation in optical density comprising three kinetically resolved processes (Fig. 5). The reaction mechanism involves three stages:

$$cis\text{-EA} \overset{h\nu}{\rightleftarrows} trans\text{-EA}' \overset{k}{\rightarrow} trans\text{-EA} \overset{k_3}{\rightarrow} E + P \qquad (26)$$

The spectrophotometric analysis and the pH dependence show that the first, rapid stage involves photochemical *cis*–*trans* isomerisation of the cinnamoyl group in the active centre of the enzyme, whereas the slow concluding stage entails hydrolysis of the resulting *trans*-isomer. The induction period observed on the rate curve for separation of *trans*-nitrocinnamic acid (the stage *trans*-EA′ → *trans*-EA) is probably associated with specific conformational changes in the active centre, which will thus acquire a catalytically active structure.

Thus the detailed kinetic analysis which has hitherto been made of reactions catalysed by α-chymotrypsin shows that the mechanism by which the enzyme acts involves labile intermediate compounds comprising the covalently formed acetylated enzyme, non-covalent complexes, and conformers of the enzyme.

4. Catalase and Peroxidase

The visible absorption spectra of the complexes formed by the haem-containing enzymes—catalase and peroxidase—with hydrogen peroxide and organic peroxides differ substantially from those of the original enzymes. This enabled Chance to monitor directly the formation and consumption of these labile intermediates by the accelerated- and stopped-flow methods using highly sensitive spectrophotometric apparatus[72,73,81,82].

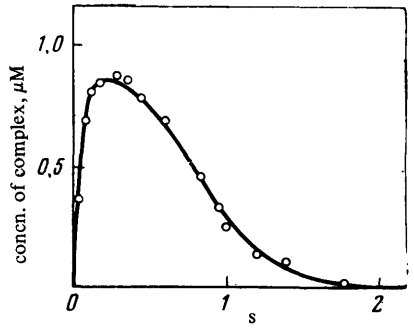


Figure 6. Variation of concentration of peroxidase–hydrogen peroxide complex when 1.0 μM peroxidase and 4.0 μM H_2O_2 solutions are mixed (pH 4.0).[126]

Rapid mixing of solutions of catalase with hydrogen peroxide enabled the rates of formation of the complexes formed by the enzyme with hydrogen peroxide and with organic peroxides to be recorded, as well as the slower rate of disappearance resulting from consumption of the peroxy-compound (Fig. 6). The rate of disappearance of the complex is increased by the presence of electron-donating substrates (aliphatic alcohols and glycols were used for this purpose)[125,128]. These results suggested that the mechanism of the peroxidase action of catalase involved a stage of formation of an intermediate enzyme–substrate compound and a stage of bimolecular reaction of this compound with a donor molecule AH_2:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[(AH_2)]{k_2} E + P \qquad (27)$$

The formal kinetic description of a two-substrate reaction with one intermediate compound has been discussed [32,125-127]. Provided that the concentration of the second substrate remains almost unchanged during the reaction ($[AH_2] \gg [E]_0$, $[S]_0$) the kinetics of reaction (27) is described by the equations

$$\frac{d[S]}{dt} = k_{-1}[ES] - k_1([E]_0 - [ES])[S] \quad (28)$$

$$\frac{d[ES]}{dt} = k_1([E]_0 - [ES])[S] - (k_{-1} + k_2[AH_2]_0)[ES] \quad (29)$$

The rate constant k_1 was determined [125] in conformity with Eqn. (29) from the initial rate of formation of the intermediate compound when formation of the complex has not proceeded far:

$$k_1 \cong \frac{1}{([E]_0 - [ES])[S]_0} \cdot \frac{d[ES]}{dt} \quad (30)$$

Adding Eqns. (28) and (29) yields the relation

$$\frac{d[S]}{dt} + \frac{d[ES]}{dt} = -k_4[AH_2]_0[ES] \quad (31)$$

Integration of this formula for complete consumption of intermediate compound and substrate ($t = 0$, $[S] = [S]_0$, $[ES] = 0$; $t \to \infty$, $[S] = 0$, $[ES] = 0$) gives

$$k_2 = \frac{[S]_0 \int_0^\infty}{[AH_2]_0 \cdot \int_0^\infty [ES]\,dt} \quad (32)$$

which enables us to find the rate constant k_2 (scheme 27). The integral $\int_0^\infty [ES]dt$ was evaluated numerically from the area bounded by the kinetic curve representing formation and consumption of the enzyme–substrate complex (Fig. 4). Reaction of the complex ES with the donor molecule is bimolecular, but does not involve formation of an enzyme–peroxide–donor ternary complex [129]. Thus the value of k_2 determined for the catalase–methyl hydroperoxide–methanol system was found to be independent of the methanol concentration (0.2–0.08 M).

According to Chance [125] the mechanism of the catalase action of catalase does not differ significantly from that of its peroxidase action. A second hydrogen peroxide molecule acts as donor in this case:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow[[S]]{k_2} E + P \quad (33)$$

The mechanism of catalysis by peroxidase is more complicated than the reaction mechanisms involving catalase. Peroxidase forms at comparable rates two spectroscopically distinct complexes with hydrogen peroxide. Complex (I) is formed immediately on the addition of hydrogen peroxide, and is an unstable green compound rapidly converted into the red complex (II). This conversion takes place more rapidly in the presence of a hydrogen-donor. The general scheme of the process based on fast kinetic measurements can be written [125,130]

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES^I \xrightarrow[(AH_2)]{k_2} ES^{II} \xrightarrow[(AH_2)]{k_3} E \quad (34)$$

$$\searrow AH^\cdot \qquad \searrow AH^\cdot$$

$$2AH^\cdot \to A + AH_2$$

III. STATIONARY STAGE OF REACTION

1. Multistage Catalytic Reactions

Steady-state occurrence of reaction (1) permits considerable simplification of its kinetic description. Experience shows that a steady state is usually established some time after the start of the reaction. This follows from Eqns. (6) and (7), since, at sufficiently large values of t, the concentrations of the intermediate compounds reach constant (stationary) values

$$[X_n]_{t\to\infty} = B \quad (35)$$

while the concentration of the product increases linearly with time

$$[P]_{t\to\infty} = k_{n+1}Bt - k_{n+1}\sum_{j=1}^{n}\frac{A_j}{\lambda_j} \quad (36)$$

provided that consumption of the substrate during the initial period of reaction can in effect be neglected.

The conditions limiting the validity of using the equations of stationary kinetics to investigate the mechanisms of enzymic reactions have been analysed [15,131,132]. If the rate of change of the concentration of the intermediate compounds X_i is much smaller than the rates of change of the concentrations of reaction products and starting materials, each of the derivatives of the intermediate concentrations can be equated to zero: $d[X_i]/dt = 0$ (see Eqns. 3). The problem reduces to the simultaneous solution of $n + 2$ algebraic equations and one differential equation [133-136]. The steady-state reaction velocity in this case is given by the usual Michaelis equation for reversible reactions

$$v = \frac{\frac{V_S}{K_S}[S]_0 - \frac{V_P}{K_P}[P]_0}{1 + [S]_0/K_S + [P]_0/K_P} \quad (37)$$

in which, however, the parameters—the maximum rates V_S, V_P and the Michaelis constants K_S, K_P—are functions of the rate constants of the elementary stages. These quantities have been expressed in terms of the rate constants of the kinetic scheme (1). [133]

Many papers have appeared on the theory of stationary kinetics and its practical application to investigating the specific mechanisms of enzymic reactions [15,131-145]. It must be noted, however, that study of the steady-state kinetics of an enzymic reaction is of very limited value for an understanding of the multistage mechanism of enzyme action. The primary reason is that in general, without the use of additional data, experimental rate constants cannot be assigned unambiguously to elementary chemical stages, since the dependence of the rate of the process on the initial concentrations of S and P will yield only the four kinetic parameters—K_S, K_P and V_S, V_P—whereas the number of rate constants of elementary reactions in process (1) is $2(n + 1)$. Determination of all the elementary constants of reaction (1) obviously becomes impossible when $n > 1$.

Nevertheless, lower limits to rate constants of elementary stages can be determined from steady-state kinetics. The reason is that the parameters of the stationary kinetics are determined by the limiting stage. The lower limit to the rate constant of any unimolecular stage in the forward direction of reaction (1) is given by [133]

$$k_{i+1} \geqslant \frac{V_S}{[E_0]}; \quad i \neq 0 \quad (38)$$

Similarly, for the reverse reaction we have

$$k_{-i} \geqslant \frac{V_P}{[E]_0}; \quad i \neq n+1 \quad (39)$$

These formulae enable data on the stationary kinetics of enzymic reactions to be analysed, and the rates of the slowest stages to be estimated. For this purpose Table 2 lists values of $V_{max}/[E]_0$ for several enzyme systems [12].

These results represent the efficiency of enzymic catalysis. Table 2 shows that rate constants of the limiting processes of unimolecular conversion of intermediate compounds lie in the range 10^2–10^3 s^{-1} for the majority of enzymes.

Table 2.

Enzyme	$V_{max}/[E]_0$, s^{-1}	Enzyme	$V_{max}/[E]_0$, s^{-1}
Acetylcholinesterase	10^3	Kinase	10^3
Chymotrypsin	10^2–10^3	Urease	10^4
Trypsin	10^2–10^3	Fumarase	10^3
Ribonuclease	10^2	Aldolase	10^2
Papain	10	Dehydrogenase	10^3
Carboxypeptidase	10^2	Catalase	10^7
Myosin-ATPase*	10^2	Peroxidase	10

[* ATP = adenosine triphosphate.]

Lower limits to the rate constants can be determined[133] also for the bimolecular stages of reaction (1). They are respectively

$$k_1 \geqslant \frac{V_S + V_P}{[E]_0 K_S} \tag{40}$$

$$k_{-(n+1)} \geqslant \frac{V_S + V_P}{[E]_0 K_P} \tag{41}$$

Table 3 gives the results of analyses[12,133] of the limiting values of rate constants for several specific enzymic reactions. The minimum value of the bimolecular rate constant k_1 for certain reactions (of fumarase) approaches the limit predicted by the theory of reactions controlled by diffusion of the reactants.

The study of stationary kinetics may be useful, not only for estimating lower limits to the reactivity of intermediate compounds, but also for obtaining more detailed information on their properties. However, this requires that the reaction mechanism should be sufficiently well known. As example we consider some results of the investigation of catalysis by α-chymotrypsin.

2. Steady-state Kinetic Study of Intermediate Acylated Enzyme in Catalysis by α-Chymotrypsin

Investigation of the prestationary kinetics of reactions catalysed by α-chymotrypsin showed that the mechanism of catalysis involved a stage of formation and hydrolysis of an intermediate acylated enzyme (scheme 16). The steady-state rate of such a reaction is described by the equation[146]

$$v = \frac{k_{cat}\,[E]_0\,[S]_0}{K_{m(app)} + [S]_0} \tag{42}$$

where

$$k_{cat.} = k_2 k_3/k_2 + k_3 \tag{43}$$

$$K_{m(app)} = K_S k_3/k_3 + k_2; \quad K_S = \frac{k_{-1} + k_2}{k_1} \tag{44}$$

Equations (42)–(44) indicate that the experimental parameters k_{cat} and $K_{m(app)}$ are complicated functions of the rate constants of both chemical stages of the enzymic process. Hence it follows that, with comparable values of k_2 and k_3, such parameters do not characterise the reactivity of any individual intermediate. Nevertheless, abundant experimental information, describing the reactivity both of the enzyme–substrate complex and of the intermediate acylated enzyme, has now been obtained from stationary kinetic data. Apart from certain indirect methods[146] two main approaches have been used for analysing reactivity in catalysis by α-chymotrypsin.

Table 3.

Enzyme	Substrate	$k_{1,min}$, $M^{-1}\,s^{-1}$
β-Amylase	amylose	5.8×10^7
Urease	urea	5.0×10^6
ATPase	ATP	8×10^6
Cytochrome C reductase	cytochrome C	9×10^7
Acetylcholinesterase	acetylcholine	10^9
Fumarase	fumaric acid	0.5×10^8
	L-malic acid	1.6×10^8
	fumaric acid at zero ionic strength	3×10^{10}

(a) Investigation of substrates with a known limiting stage

The rate constant of the limiting stage can be determined from steady-state kinetic data k_{cat} and $K_{m(app)}$ only if the difference in reactivity between the intermediate species ES and EA is sufficiently large. This is evident from Eqns. (42)–(44), whence it follows that, when $k_2 \gg k_3$,

$$k_{cat.} = k_3; \quad K_{m(app)} = K_S k_3/k_2 \tag{45}$$

Different relations hold when $k_3 \gg k_2$:

$$k_{cat.} = k_2; \quad K_{m(app)} = K_S \tag{46}$$

This approach has been used[147–150] to analyse the relation between structure and reactivity k_3 of the intermediate acylated enzymes formed in the hydrolysis of p-nitrophenyl esters of aliphatic carboxylic acids (C_1–C_7).

(b) Determination of elementary constants by selective action on rates of individual stages of enzymic reaction

Use of this method has been considered for a reaction with a single intermediate[151–153]. The approach has been analysed[154–161] for the three-stage catalytic reaction mechanism (16). Thus the reversible inhibiting effect of heavy-metal cations on the acylation stage k_2 of α-chymotrypsin[162–165] or even the effect of the ionic strength of the solution[155] on the same stage has been used for this purpose. Fig. 7 illustrates the effect of ionic strength on the steady-state hydrolysis of the ethyl ester of *N*-benzoyl-L-tyrosine in the presence of α-chymotrypsin. The abscissa of the point of intersection gives[155] the value of $-1/K_S$, and the ordinate $1/k_3$.

However, the method which has been most greatly developed and systematically applied to reactions involving migration of an acyl group is that of competing reactions,

based on competition between water and an added nucleophilic reagent for reaction with the acylated enzyme[156-159, 166]. According to the scheme

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \begin{cases} \xrightarrow{k_3[H_2O]} E + P_2 \\ \xrightarrow{k_4[N]} E + P_3 \end{cases} \quad (47)$$

the experimental parameters are related to the concentration of the nucleophile by the formulae

$$k_{cat(1)} = \frac{k_2(k_3 + k_4[N])}{k_2 + k_3 + k_4[N]} \quad (48)$$

$$k_{cat(2)} = \frac{k_2 k_3}{k_2 + k_3 + k_4[N]} \quad (49)$$

$$K_{m(app)} = K_S \frac{k_3 + k_4[N]}{k_2 + k_3 + k_4[N]} \quad (50)$$

where $k_{cat(1)}$ and $k_{cat(2)}$ are the kinetic parameters determined by means of Eqn. (42) for the steady-state rates of formation of the products P_1 and P_2 respectively. From the dependence of k_{cat} and $K_{m(app)}$ on the concentration of the added nucleophile, using Eqns. (48)–(50), we can determine separately the rate constants at the stages of acylation and hydrolysis of the acylated enzymes, as well as the equilibrium constant K_S.

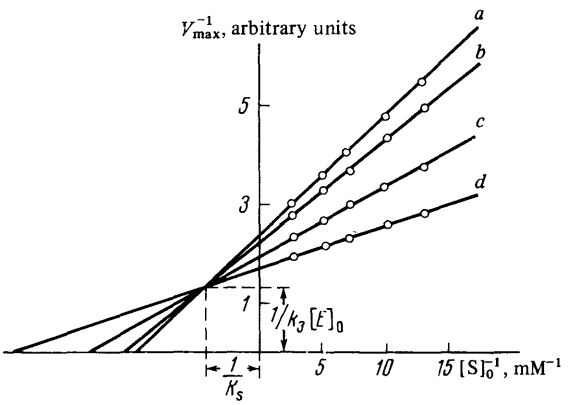


Figure 7. Dependence of initial steady-state rate of α-chymotrypsin hydrolysis of ethyl ester of *N*-benzoyl-L-tyrosine (6 vol.% of acetonitrile with pH 7.8 (pH-stat) at 25°C) on ionic strength (KCl, M): *a*) 0.1; *b*) 0.2; *c*) 0.5.[155]

Among the disadvantages of this technique are uncertainty on the mechanism of the effect of the nucleophile on the acylated enzyme and also the need to allow for the inhibiting effect of the nucleophile[158].

This approach has found systematic application in an investigation[158,159] of the reactivity of intermediate acylated enzymes using butane-1,4-diol as added nucleophile. Rate constants of formation and hydrolysis have been determined[159] for the homologous series of acylated enzymes formed by *N*-acylated derivatives of L-amino-acids. The results enabled certain conclusions to be drawn on the structure of the active centre and the mechanism of the specificity of α-chymotrypsin[147,160].

IV. METHODS OF RELAXATION KINETICS

1. Multistage Catalytic Reactions

Relaxation methods of investigating the kinetics of chemical reactions are based on the principle that, when a system is subjected to a rapid external constraint (change of temperature, pressure, or electric field), the time required for the system to reach a new equilibrium (or stationary) state will depend on the rate of chemical reaction (or sometimes on the rate of diffusion of the reactants). Transition of the system to the new equilibrium reactant concentrations is termed "chemical relaxation"[167]. The external constraint perturbing the system may be either discontinuous (temperature jump, pressure, or electric field pulse) or periodic (ultrasonic absorption). The resolving power of relaxation methods lies between 10^{-5} s (temperature jump) and 10^{-10} s (absorption of ultrasound)[11-13,26]. Formal aspects of the relaxation kinetics of complicated chemical reactions have been discussed[12,26,168,182].

The relaxation kinetics of an enzymic reaction involving *n* intermediate compounds (reaction 1) was analysed by Hammes and Alberty[169] and also by Eigen and Hammes[12]. With the system at equilibrium the concentrations

$$[\bar{E}],\ [\bar{S}],\ [\bar{X}_1] \ldots [\bar{X}_n],\ [\bar{P}]$$

of all the species are constant. When a rapid external constraint is applied, the reactant concentrations assume new values corresponding to the new equilibrium. The instantaneous concentrations of the components can be written

$$([\bar{E}]+\Delta[E]),\ ([\bar{S}]+\Delta[S]),\ ([\bar{X}_1]+\Delta[X_1]) \ldots ([\bar{X}_n+\Delta[X_n]),\ ([\bar{P}]+\Delta[P]),$$

where the symbol Δ denotes the changes in the reactant concentrations. In order to find the functional dependence of each component concentration on time, we must solve Eqns. (2) and (3). This set of equations will be linear if the displacement of the equilibrium is small. The bimolecular terms $k_1[E][S]$ and $k_{n+1}[E][P]$, which are responsible for the non-linearity of the system, can then be written

$$k_1([\bar{S}]+\Delta[S])\,([\bar{E}]+\Delta[E]) = k_1([\bar{E}][\bar{S}]+[\bar{E}]\Delta[S]+ +[\bar{S}]\Delta[E]+\Delta[S]\Delta[E])$$

and hence

$$k_{(n+1)}([\bar{E}][\bar{P}] + [\bar{E}]\Delta[P]+[\bar{P}]\Delta[E]+\Delta[P]\Delta[E]).$$

For a small shift of the equilibrium, terms containing Δ^2 can be neglected. Hence Eqns. (2) and (3) can be simplified to

$$\frac{d\Delta[S]}{dt} = a_{1,1}\Delta[S] + \sum_{j=1}^{n} a_{i,j+1}\Delta[X_j]$$
$$\frac{d\Delta[X_j]}{dt} = a_{j,1}\Delta[S] + \sum_{j=1}^{n} a_{i,j}\Delta[X_j] \quad (51)$$

where the constants $a_{i,j}$ are functions of the rate constants of the elementary chemical reactions and the initial equilibrium concentrations of the components of the reaction

$$E + S \underset{k_{2,1}}{\overset{k_{1,2}}{\rightleftarrows}} X_1 \underset{k_{3,2}}{\overset{k_{2,3}}{\rightleftarrows}} \ldots \rightleftarrows X \rightleftarrows \ldots \underset{k_{n+1,n}}{\overset{k_{n,n+1}}{\rightleftarrows}} X_n \underset{k_{n+2,n+1}}{\overset{k_{n+1,n+2}}{\rightleftarrows}} E + P \quad (52)$$

Somewhat as for the set of equations (4), a general solution can be sought (accurate to a constant) in the form

$$\Delta[S] = \sum_{j=1}^{n+1} A_j e^{\lambda_j t}$$
$$\Delta[X_j] = \sum_{j=1}^{n+1} B_{i,j} e^{\lambda_j t} \quad (53)$$

where the λ_j are the roots of the characteristic equation of degree $n + 1$.

An important conclusion which follows from the above survey is that, when n intermediate compounds are involved, the number of exponential terms is $n + 1$. Thus the number of exponentials observed experimentally can be regarded as a criterion of the minimum number of intermediate compounds taking part in the reaction. The measured relaxation times $-1/\lambda_j$ are functions of all the rate constants of elementary reactions and the equilibrium concentrations of the reactants.

Table 4.

Enzyme	Substrate or activator	Ref.
Alcohol dehydrogenase	NAD-imidazole	176
Aspartate aminotransferase	aspartic, glutamic, oxosuccinic, oxoglutaric acids	177
	α-methylaspartic acid	178
	erythro-β-hydroxyaspartic acid	179
Aspartate transcarbamoylase	5-bromocytidine phosphate	180
	carbamoyl phosphate, *L*-malic and succinic acids	183-185
Glyceraldehyde 3-phosphate dehydrogenase	NAD	186-192
Acid phosphatase	*p*-nitrophenyl phosphate	193
Creatine kinase	creatine phosphate	194
Lactate dehydrogenase	NADH	195,196
Lysozyme	saccharides	197,198
	Biebrich Scarlet	199
Malate dehydrogenase	NADH, malic acid	200,201
Pyruvate kinase	bivalent metal ions, ADP	202
Ribonuclease	nucleotides	202-209
	isomerisation	210-213
Serine dehydrogenase	isomerisation	214
Serine transhydroxymethylase	glycine	215
Trypsin	isomerisation	210,211
	proflavin-benzamidine	174,216
Tryptophan synthetase	L-serine, indole, tryptophan	217,218
Urease	urea	219
Flavodoxin	interaction of coenzyme with apoprotein	220-222
Chymotrypsin	Proflavin	175,223,224
	Proflavin and methyl ester of *N*-toluene-*p*-sulphonyl-L-arginine	225
	Biebrich Scarlet	226
	isomerisation	211
Cytochrome C	hexacyanoferrate(II)	175

Particular cases of scheme (52) (one and two intermediate compounds) and specific approaches to determining the rate constants of elementary stages have been described[13,167-173]. It has been shown[13,26,174] that data on relaxation amplitudes may prove useful, besides kinetic data (relaxation times), in the analysis of reaction mechanisms.

Many enzyme systems have now been studied by relaxation methods. Those listed in Table 4 have been examined mainly by the temperature-jump method. A rapid change of temperature is usually effected by discharging a high-voltage capacitor through the reaction cell containing, in addition to the reactants, a certain quantity of an inert electrolyte to lower the electrical resistance. A temperature change of 3–10 deg occurs within 10^{-6} s.[11-13] Equipment combining stopped-flow and temperature-jump apparatus was proposed for the kinetic investigation of fast reversible stages preceding the *irreversiole* limiting stage[175]. The temperature jump can be effected in an interval from milliseconds to a few seconds after cessation of flow. Further shortening of the "dead" time of the "temperature jump" method entails the use of laser illumination to heat up the reaction cell[227,228].

The possibilities of using flash photolysis to study the relaxation kinetics of enzyme reactions have been discussed[229-231]. In this case the equilibrium is changed as a consequence of a concerted fast photochemical reaction. Light-induced rapid changes in pH (flash-induced pH jump) merit special attention[229].

2. Aspartate Transaminase and Ribonuclease

A review[232] of the study of enzyme reactions by relaxation methods discusses the mechanisms of the following—(i) reactions of aspartate transaminase with amino- and keto-acids, (ii) reaction of cytochrome C with ferrocyanide, (iii) reaction of alcohol dehydrogenase with NAD–imidazole and the combination of NADH with malate and lactate dehydrogenases, (iv) hydrolysis of *p*-nitrophenyl phosphate by acid phosphatase, and (v) reactions of pancreatic ribonuclease A. Table 4 shows that the reaction mechanisms of aspartate transaminase and ribonuclease have been studied in greatest detail. These examples give an idea both of the possibilities of relaxation kinetics and of the achievements of enzymology in investigating the mechanisms of enzymic catalysis.

Aspartate aminotransferase (AAT) catalyses the reversible transfer of an amino-group between aspartic and oxoglutaric acids with the formation of oxosuccinic and glutamic acids:

$$\overset{\ominus}{\mathrm{COO}}\mathrm{CH_2CH}(\overset{+}{\mathrm{NH_3}})\overset{\ominus}{\mathrm{COO}} + \overset{\ominus}{\mathrm{COO}}(\mathrm{CH_2})_2\,\mathrm{COCO}\overset{\ominus}{\mathrm{O}} \xrightleftharpoons{\mathrm{AAT}}$$
$$\rightleftarrows \overset{\ominus}{\mathrm{COO}}\mathrm{CH_2COCO}\overset{\ominus}{\mathrm{O}} + \overset{\ominus}{\mathrm{COO}}(\mathrm{CH_2})_2\mathrm{CH}(\overset{+}{\mathrm{NH_3}})\overset{\ominus}{\mathrm{COO}} \quad (54)$$

Pyridoxal phosphate acts as coenzyme in this reaction, undergoing substantial spectral changes, which provides a convenient method for monitoring the kinetics. The reaction mechanism consists roughly in transfer of the amino-group from the amino-acid to the pyridoxal phosphate to form pyridoxamine phosphate and a keto-acid, followed by its transfer to the other keto-acid with formation of a new amino-acid[233]:

$$E_l + \mathrm{Asp.} \rightleftarrows X_1 \ldots X_n \rightleftarrows E_m + \mathrm{Osu}$$
$$E_m + \mathrm{Ogl} \rightleftarrows Y_n \ldots Y_1 \rightleftarrows E_l + \mathrm{Glu} \quad (55)$$

in which E_l and E_m represent the enzyme in the pyridoxal and pyridoxamine forms respectively, Asp is aspartic acid, Osu oxosuccinic acid, Ogl oxoglutaric acid, and Glu glutamic acid, while the X_i and Y_i are the intermediate compounds formed.

Study of this system by the temperature-jump method[177] revealed at least two intermediate compounds in each reaction (conversion of aspartic into oxosuccinic acid and of oxoglutaric into glutamic acid); three relaxation times in the range 50–20 μs were detected in the examination of each reaction. The dependence of the relaxation times on the concentrations of the substrates and the enzyme was

studied, and rate constants characterising the individual stages were determined (or estimated). The spectral characteristics of the intermediates showed them to be probably covalent compounds of the substrate with pyridoxal phosphate, including Schiff bases.

Besides the stages that were studied, more rapid spectral changes were observed in the reaction of aspartate aminotransferase, whose kinetics could not be analysed by the temperature-jump technique. In order to slow down these stages, the reaction of the enzyme with certain modified substrates was investigated. The analogous L-α-methylaspartic acid is a competitive inhibitor of amino-acid substrates and able to form an aldimine with pyridoxal phosphate on the enzyme. Because of the presence of the α-methyl group, however, transamination cannot take place. Kinetic study of this system by a combined "stopped-flow–temperature jump" method revealed that the reaction occurred in three stages[178]:

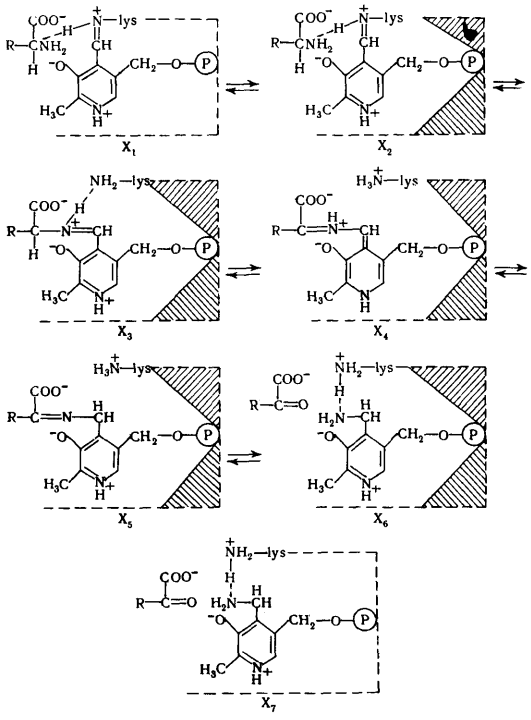
$$E + S \rightleftarrows X_1 \rightleftarrows X_2 \rightleftarrows X_3 \qquad (56)$$

All the rate constants in this mechanism were determined, and the spectral properties of the intermediate compounds were studied. The reaction was regarded[178] as involving formation of a primary complex X_1, followed by a conformational change ($X_1 \rightleftharpoons X_2$) and formation of an aldimine X_3.

The *erythro*-isomer of L-β-hydroxyaspartic acid is a substrate of the enzyme, but transamination occurs very slowly. Eight relaxation times were detected[179] in the reaction of this substrate with aspartate transaminase. The simplest mechanism consistent with this finding is

$$E_l + S \rightleftarrows X_1 \rightleftarrows X_2 \rightleftarrows X_3 \rightleftarrows X_4 \rightleftarrows X_5 \rightleftarrows X_6 \rightleftarrows X_7 \rightleftarrows E_m + S' \qquad (57)$$

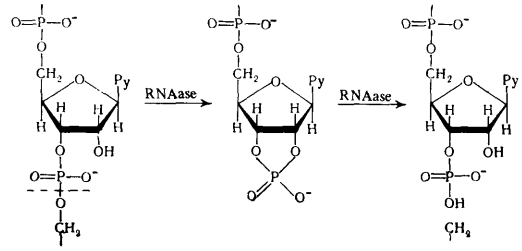
where S' represents the keto-form of the substrate. Detailed analysis of the concentration dependence of relaxation times and equilibrium constants enabled 14 and 16 rate constants to be calculated representing mechanism (57). Furthermore, the spectral characteristics of the intermediates were determined. A suggested reaction mechanism consistent with the results can be represented by the scheme[234]



(58)

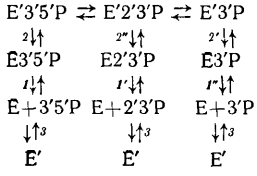
The first three intermediates have spectral characteristics similar to those of compounds X_1, X_2, and X_3 in the reaction of L-α-methylaspartic acid, and at these stages the reaction probably also terminates in formation of an aldimine (X_3). The intermediate X_4 has absorption maxima at 460 and 490 nm, the molar absorption at the latter wavelength exceeding 10^5 M^{-1} cm^{-1}. According to the authors[179] this compound has a quinoid structure; the other three compounds have absorption maxima in the region of 330 nm and are ketimines (scheme 58).

Hammes studied the mechanism of the action of ribonuclease A in as great detail as that of catalysis by aspartate aminotransferase[203–209]. The reaction catalysed by the former enzyme involves transesterification of a 3',5'-dinucleotide with formation of a 2',3'-cyclophosphate, followed by hydrolysis of the cyclic phosphate with formation of a 3'-nucleotide:



(59)

Synthetic substrates were used to investigate the relaxation kinetics, which considerably simplified the kinetic analysis. The interaction of the enzyme was studied with all three classes of substrates. On the basis of these investigations the "minimal" mechanism



(60)

was suggested. With each class of substrates (3',5'-dinucleotides, 2',3'-cyclophosphates, and 3'-nucleotides) the reaction involves a bimolecular stage of formation of the primary complex (stages *1*, *1'*, *1''*), followed by its isomerisation (stages *2*, *2'*, *2''*). Furthermore, examination of the free enzyme showed that it existed in two conformations (stage *3*). Comparison of these kinetic results with X-ray diffraction data[235,236] enabled a detailed mechanism to be postulated for the action of the enzyme[209,232].

The investigations on the mechanisms of the action of aspartate aminotransferase and ribonuclease A provide an excellent illustration of the possibilities and achievements of "fast" kinetic methods for studying the mechanisms of enzymic catalysis. The results of these investigations may be instructive for the choice of approaches to the study of other catalytic systems.

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