Selected Methods in Enzymology Series

Series Editors

Sidney P. Colowick and Nathan O. Kaplan

CONTEMPORARY ENZYME KINETICS AND MECHANISM

Edited by

DANIEL L. PURICH

Contemporary Enzyme Kinetics and Mechanism

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Foreword

The *Methods in Enzymology* series, which was originally published as a four-volume treatise over twenty-five years ago, has now grown to over 100 volumes. It has become more and more difficult for an individual investigator to locate particular methods of interest, especially in rapidly developing fields in which pertinent information now appears in many volumes of the series. Although individual and cumulative indexes are provided, the task of information retrieval is still formidable. We have, therefore, undertaken to provide such investigators and their students with a single volume work in a given area of interest, compiled by selection of the most essential and widely used procedures published in volumes of *Methods in Enzymology* in that particular area. The aim is to permit the individual investigator or student to have conveniently at hand all of the basic methodology in that field at relatively low cost. The articles, which are selected by the editors in that area, will be unabridged. A new Subject Index will be prepared for each volume of "Selected Methods in Enzymology."

It is our intention that one volume of "Selected Methods" will be derived from several related volumes of equivalent size in the parent series. This volume of the "Selected Methods" series deals with Contemporary Enzyme Kinetics and Mechanism and is comprised of articles selected by Dr. Purich from volumes of the *Methods in Enzymology* series for which he served as editor. It also includes a supplementary chapter [19] described in the Preface. We hope that this experiment in publication proves useful to the broad audience for this new series.

SIDNEY P. COLOWICK
NATHAN O. KAPLAN

Preface

While the pace of research on enyzmic catalysis has increased as a result of many experimental and theoretical approaches, certain methods and perspectives in the area of enzyme kinetics and mechanism have constituted a nucleus about which the field continually grows. Thus, for even the very latest emerging concepts concerning enzyme action, one may trace their roots back to this nucleus of theory and practice.

This particular volume of *Selected Methods in Enzymology* was organized to provide researchers, students, and other interested readers with a reasonably representative view of "Contemporary Enzyme Kinetics and Mechanism" by covering these central areas. The basic idea was to include a limited number of chapters from Volumes 63, 64, and 87 of *Methods in Enzymology,* "Enzyme Kinetics and Mechanism," Parts A, B, and C, respectively. I believe that this selection will provide the interested reader with an excellent view of contemporary methods and perspectives as well.

A new chapter [19], entitled "Selected Exercises and Problems," has been added. This addition should serve to make the volume more useful to students and other interested readers. It includes a series of exercises and problems which build on each other in a progressive manner. This chapter should convey to the reader the importance of achieving proficiency in formulating quantitative relationships describing enzyme behavior. Only when one has the ability to derive, manipulate, and understand such relationships can one begin to explore and to unravel the subtleties of enzymic catalysis. The problem set is, nonetheless, only a starting point, and I have added many references to the literature to encourage further awareness of the field. The exercises and problems were not meant to cover all of the chapters in this volume, so to estimate the importance of any single chapter by the number of questions associated with it would be an error. It was also apparent that step-by-step solutions would be difficult to provide because of space limitations. Yet, it is also true that the presentation of one approach to the exclusion of another might inhibit the reader's development or mastery of other means for achieving an equally adequate solution. A list of answers or hints for solution appears immediately after the exercises and problems section and should be helpful in checking one's progress. I am grateful to Drs. Charles Y. Huang, Bryce V. Plapp, and R. Donald Allison for suggesting or providing several of the problems associated with their chapters.

This has been an interesting effort to organize a volume which will introduce the reader to enzyme kinetics and mechanism at an intermediate level. As a reference book for individuals or as an additional textbook

for specialty courses on enzyme action, "Contemporary Enzyme Kinetics and Mechanism" should prove useful.

Please note that where a cross reference is given to a volume or paper in this series, it refers to the *Methods in Enzymology* series. Where only volumes and paper numbers are referred to, the volumes too are those in the *Methods in Enzymology* series.

DANIEL L. PURICH

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Volume 64

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Section I. Enzyme Intermediates

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[1] Derivation of Initial Velocity and Isotope Exchange Rate Equations

By CHARLES Y. HUANG

A rate equation for an enzymic reaction is a mathematical expression that depicts the process in terms of rate constants and reactant concentrations. It serves as a link between the experimentally observed kinetic behavior and a plausible model or mechanism. The characteristics of the rate equation permit tests to be designed to verify the mechanism. Conversely, the experimental observations provide clues to what the mechanism may be, hence, what form the rate expression shall take.

Derivation of rate equations is an integral part of the effective usage of kinetics as a tool. Novel mechanisms must be described by new equations, and familiar ones often need to be modified to account for minor deviations from the expected pattern. The mathematical manipulations involved in deriving initial velocity or isotope exchange-rate laws are in general quite straightforward, but can be tedious. It is the purpose of this chapter, therefore, to present the currently available methods with emphasis on the more convenient ones.

Derivation of Initial Velocity Equations

The derivation of initial velocity equations invariably entails certain assumptions. In fact, these assumptions are often conditions that must be fulfilled for the equations to be valid. Initial velocity is defined as the reaction rate at the early phase of enzymic catalysis during which the formation of product is linear with respect to time. This linear phase is achieved when the enzyme and substrate intermediates reach a steady state or quasi-equilibrium. Other assumptions basic to the derivation of initial rate equations are as follows:

1. The enzyme and the substrate form a complex.
2. The substrate concentration is much greater than the enzyme concentration, so that the free substrate concentration is equivalent to the total concentration. This condition further requires that the amount of product formed is small, such that the reverse reaction or product inhibition is negligible.
3. During the reaction, constant pH, temperature, and ionic strength are maintained.

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Steady-State Treatment

During the steady state, the concentrations of various enzyme intermediates are essentially unchanged; that is, the rate of formation of a given intermediate is equal to its rate of disappearance. This assumption was first introduced to the derivation of enzyme kinetic equation by Briggs and Haldane.[1]

To derive a rate equation, the first step is to write a reaction mechanism. The nomenclature used by Fromm in Volume 63 [3] will be adopted here with the exception that rate constants in the forward and reverse directions will be denoted by positive and negative subscripts. For example, the simplest one substrate–one product reaction can be written as:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P \quad \text{or} \quad E \underset{k_{-1}}{\overset{k_1 A}{\rightleftharpoons}} EA \xrightarrow{k_2} \overset{P}{E} \tag{1}$$

Since both the k_{-1} and k_2 steps (or branches) lead from EA to E, the two branches, as has been shown by Volkenstein and Goldstein,[2] can be combined into a single branch. This simplification procedure will be used whenever feasible.

$$E \underset{k_{-1}+k_2}{\overset{k_1 A}{\rightleftharpoons}} EA$$

The intial rate is given by

$$v = dP/dt = k_2\,(EA)$$

Applying the steady-state assumption, we have

$$d(EA)/dt = k_1 A(E) - (k_{-1} + k_2)(EA) = 0 \tag{2}$$

To obtain an expression for (EA), the enzyme conservation equation

$$\text{Total enzyme} = E_0 = E + EA \tag{3}$$

is required. Substitution of $(E) = (E_0 - EA)$ into Eq. (2) yields

$$(EA) = \frac{E_0 A}{[(k_{-1} + k_2)/k_1] + A}$$

[1] G. E. Briggs and J. B. S. Haldane, *Biochem. J.* **19**, 338 (1925).

[2] M. V. Volkenstein and B. N. Goldstein, *Biochim. Biophys. Acta* **115**, 471 (1966).

and

$$v = k_2(\mathrm{EA}) = \frac{k_2\mathrm{E}_0\mathrm{A}}{[(k_{-1} + k_2)/k_1] + \mathrm{A}} = \frac{V_1\mathrm{A}}{K_\mathrm{m} + \mathrm{A}} \tag{4}$$

where V_1 is the maximum velocity in the forward direction and K_m is the Michaelis constant.

It should be noted that the validity of the steady-state method does not depend on the assumption $d(\mathrm{EA})/dt = 0$. Without setting Eq. (2) equal to zero, one can obtain the following expression from Eqs. (2) and (3):

$$(\mathrm{EA}) = \frac{k_1\mathrm{AE}_0 - d(\mathrm{EA})/dt}{k_1\mathrm{A} + k_{-1} + k_2}$$

Wong[3] has pointed out that the steady-state approximation only requires that $d(\mathrm{EA})/dt$ be small compared with $k_1\mathrm{AE}_0$. In the early phase of the reaction, if $\mathrm{A} \gg \mathrm{E}_0$, the rate of change of EA due to diminishing A will be relatively slow. It is clear that the validity of steady state is intimately tied to the condition of high substrate to enzyme ratio.

The Determinant Method

For a mechanism involving several enzyme-containing species, derivation of the rate equation can be done by solving the simultaneous algebraic equations by the determinant method. Consider the mechanism described by Eq. (1) with the addition of an EP intermediate.

$$\mathrm{E} \underset{k_{-1}}{\overset{k_1\mathrm{A}}{\rightleftharpoons}} \mathrm{EA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EP} \xrightarrow{k_3} \mathrm{E} + \mathrm{P} \tag{5}$$

The three simultaneous equations are given in the following form:

$$\begin{array}{r|ccc|c} & \mathrm{E} & \mathrm{EA} & \mathrm{EP} & \\ d\mathrm{E}/dt = & -k_1\mathrm{A} & k_{-1} & k_3 & = 0 \\ d\mathrm{EA}/dt = & k_1\mathrm{A} & -(k_{-1} + k_2) & k_{-2} & = 0 \\ d\mathrm{EP}/dt = & 0 & k_2 & -(k_{-2} + k_3) & = 0 \end{array}$$

The determinant, or distribution term, for E, for example, can be calculated from the coefficients listed above, after deleting the E column. For a mechanism of n intermediates, only $n - 1$ equations are needed. Thus, by leaving out the $d\mathrm{EP}/dt$ row, we can write

[3] J. T. Wong, "Kinetics of Enzyme Mechanisms." Academic Press, New York, 1975.

$$(\mathrm{E}) = \begin{vmatrix} k_{-1} & k_3 \\ -(k_{-1} + k_2) & k_{-2} \end{vmatrix} = k_{-1}k_{-2} + k_3(k_{-1} + k_2)$$

If the $d\mathrm{E}/dt$ row is omitted instead, we have

$$\begin{aligned}(\mathrm{E}) &= \begin{vmatrix} -(k_{-1} + k_2) & k_{-2} \\ k_2 & -(k_{-2} + k_3) \end{vmatrix} \\ &= k_{-1}(k_{-2} + k_3) + k_2(k_{-2} + k_3) - k_2k_{-2} \\ &= k_{-1}k_{-2} + k_3(k_{-1} + k_2)\end{aligned}$$

Note that deletion of different equations often leads to different amounts of algebraic manipulations. Application of the same operations to EA and EP yields

$$\begin{aligned}(\mathrm{EA}) &= k_1(k_{-2} + k_3)\mathrm{A} \\ (\mathrm{EP}) &= k_1k_2\mathrm{A}\end{aligned}$$

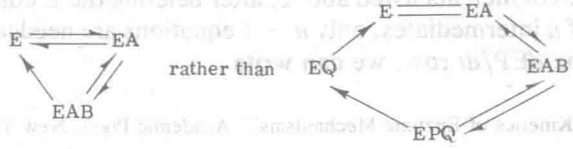
The rate equation is readily obtained as

$$\begin{aligned}\frac{v}{\mathrm{E}_0} &= \frac{k_3(\mathrm{EP})}{(\mathrm{E}) + (\mathrm{EA}) + (\mathrm{EP})} \\ &= \frac{k_1k_2k_3\mathrm{A}}{k_{-1}k_{-2} + k_3(k_{-1} + k_2) + k_1(k_{-2} + k_3)\mathrm{A} + k_1k_2\mathrm{A}}\end{aligned}$$

or

$$\begin{aligned}v &= \frac{k_2k_3\mathrm{E}_0\mathrm{A}/(k_2 + k_{-2} + k_3)}{\{[k_{-1}k_{-2} + k_3(k_{-1} + k_2)]/[k_1(k_2 + k_{-2} + k_3)]\} + \mathrm{A}} \\ &= \frac{V_1\mathrm{A}}{K_\mathrm{m} + \mathrm{A}}\end{aligned} \tag{6}$$

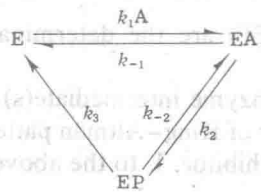
Equation (6) is identical in form with Eq. (4). In fact, if $k_3 \gg k_2, k_{-2}$, Eq. (6) reduces to Eq. (4). Although Eq. (5) is a more realistic mechanism compared with Eq. (1), especially when the rapid-equilibrium treatment is applied to the reversible reaction, the information obtainable from initial-rate studies of such unireactant system remains nevertheless the same: V_1 and K_m. This serves to justify the simplification used by the kineticist; that is, the elimination of certain intermediates to maintain brevity of the rate equation (provided the mathematical form is unaltered). Thus, the *forward* reaction of an ordered Bi Bi mechanism is generally written as diagrammed below.



The use of the determinant method for complex enzyme mechanisms is time-consuming because of the stepwise expansion and the large number of positive and negative terms that must be canceled. It is quite useful, however, in computer-assisted derivation of rate equations (cf. Chapter [5] by Fromm, in Volume 63).

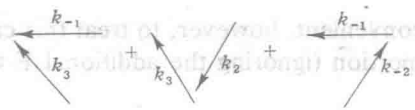
The King and Altman Method

King and Altman[4] developed a schematic approach for deriving steady-state rate equations, which has contributed to the advance of enzyme kinetics. The first step of this method is to draw an *enclosed* geometric figure with each enzyme form as one of the corners. Equation (5), for instance, can be rewritten as:



The second step is to draw all the possible patterns that connect all the enzyme species without forming a loop. For a mechanism with n enzyme species, or a figure with n corners, each pattern should contain $n - 1$ lines. The number of valid patterns for any single-loop mechanism is equal to the number of enzyme forms. Thus, there are three patterns for the triangle shown above:

The determinant for a given enzyme species is obtained as the summation of the product of the rate constants and concentration factors associated with all the branches in the patterns *leading toward* this particular enzyme species. The same patterns are used for each species, albeit the direction in which they are read will vary. However, when an irreversible step is present, e.g., the EP → E step, some patterns become invalid for certain enzyme forms.



$$= k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2}$$

[4] E. L. King and C. Altman, *J. Phys. Chem.* **60**, 1375 (1956).