Behavior of ENZYME SYSTEMS

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AN ANALYSIS OF KINETICS AND MECHANISM

by

JOHN M. REINER
Dept. of Bacteriology and Immunology
Emory University
Atlanta, Georgia

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A FOREWORD
FOR TIMID SOULS

Enzymes and enzyme systems have increasingly taken a deserved place in the forefront of biological and medical research. Those who do not study enzymes as such are studying metabolism - that is to say, the behavior of complex systems of enzymes. The embryologist and cytologist are trying to analyze development and differentiation in terms of enzymes, their changes with time, their redistribution, their characteristic products. The pharmacologist is looking to enzymes as the specific targets of drugs. The physiologist is seeking in enzymatic mechanisms the key to such different processes as renal function, muscle contraction, and nerve activity. The research clinician is studying enzyme systems in search of fundamental understanding of disease processes, not only in such settings as cancer and muscular dystrophy but also in mental disease and mental deficiency.

To analyze and interpret metabolic data in a rough qualitative way, it is often possible to view them in a framework of existing knowledge about metabolic paths and cycles. To analyze and interpret such data quantitatively and with confidence, a mathematical formulation of enzyme behavior is necessary.

This book is written for those research workers who feel the need of tools for quantitative interpretation of their work. It is addressed very particularly to those who not only feel the need, but also are in some doubt as to whether their mathematical training and facility are good enough for the mastery and use of a mathematical analysis of enzyme activity. Looking over the field for help, such workers will find that standard texts of biochemistry dismiss this important technique with a lick and a promise.* They will find a review or two; and these would be very convenient compendia of formulas, provided one already knew what this field is all about, or providing one were willing to use the formulas without understanding them too well. Finally, resorting to the "literature", they will find a scattering of papers, of which the most impressive - and indeed some of the best - ones appear to have been written by experts for experts alone. For themselves and others like them, they will find nothing

The foremost purpose of this book, accordingly, is: To make it possible for anyone to begin the book knowing substantially nothing, and to finish it an expert for all practical purposes. The manner of presentation is based on the explicit assumption that most of those who should know the contents of the book will have had a relatively meagre preparation in mathematics, and that even this little has probably gotten rusty from lack of use. The only prerequisite is the desire to know what there is to be known, and the willingness to work at it just a little.

The operative principle behind this is a simple one: There is no such thing as a "mathematical mind" - or the lack of one (I speak now of learning and use, not of creation). Anyone with sense enough to design an experiment properly has all the mind he needs for mathematics. If the presentation is clear and simple, he will master the material at any level whatever. Of course, it is possible to treat this book like any other book which deals with an application of mathematics: namely, "lift" the formulas, and ignore the discussion and derivations. But we would warn the prospective reader that to do so will be to cheat himself out of a large part of his money's worth.

* Note added in press: A recent and honorable exception to this is Dixon, M., and Webb, E. C., "Enzymes", Academic Press, 1958.

If mathematical analysis of enzyme behavior is to be conducted intelligently, it must be completely understood. We have therefore stated explicitly and often examined rather critically the assumptions and approximations underlying each piece of theory. The range of applicability of each formula is considered. The pitfalls of a superficial use of the formulas are frequently indicated.

We have tried to make this book very much of a practical handbook for the analysis and diagnosis of metabolic behavior. The analytical and above all the graphical methods for seeing what a theory means are presented at great length. Necessary bits of mathematical background are in virtually every case derived or reviewed - nothing has been taken for granted. Here again the aim held perpetually in view has been: To give the reader every conceivable technique for applying theoretical equations instead of simply admiring them - to furnish the tools for finding out what his experiments mean and for designing better experiments.

While this book was not written primarily for the experts, they will find it as useful as will the novice. Besides covering the principal results of published work in the field, a considerable portion of the material covers hitherto unpublished work which for the past 18 years has represented something of a hobby for the author. The book does not pretend to be exhaustive, however; and we hope that no one will feel seriously hurt if he fails to find one of his papers in the bibliography.

We will welcome correspondence from readers who have suggestions, criticisms, or complaints. We have tried our utmost within the limitations of time and space to be completely clear. But if someone will show us where we have failed, we will be grateful, and the next edition will be that much better.

John M. Reiner

Emory University,
Atlanta, Ga.

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Chapter I
FUNDAMENTALS OF ENZYME ACTION

PROPERTIES OF ENZYMES

We begin by stating those properties of enzymes which are relevant for this book in a simple, dogmatic fashion. In doing so, we ignore historically controversial ideas which are now defunct, as well as current unconfirmed speculations. We shall omit the evidence for our statements, and refer the reader to the general texts and handbooks on enzymes (cited in the Bibliography) for the details of such evidence.

1. Enzymes are compounds which cause chemical reactions to proceed at higher rates than would be the case in the absence of the enzyme. In many important cases the rate in the absence of enzyme, the "spontaneous" rate, is to all intents and purposes equal to zero. With appropriate experimental precautions, it may be shown that the enzyme is unchanged in amount and properties after the chemical reaction has occurred. This places enzymes in the class of those substances known as "catalysts", which are in fact defined in just this way. Enzymes are distinguished from other catalysts by the fact that they occur only within or as secretions of living organisms.

2. All enzymes about which sufficient information is available are known to be proteins (simple or conjugated). They consequently share all the special properties of proteins. Thus: (a) they are antigenic; (b) they are denatured by such agents as elevated temperature and extreme pH values; (c) their physical state and their catalytic function depend markedly upon a number of physical factors such as pH, temperature, and ionic strength.

3. When enzyme activity (the rate of the catalyzed reaction) is plotted against either pH or temperature, the curve usually has a peak or optimum. The same is true when enzyme stability is plotted against pH. The region of optimum pH (or temperature) for activity is not necessarily at or near the pH (or temperature) values normal for the living cell from which the enzyme was taken. Nor is the region of optimum pH for activity necessarily at or near the region of optimum pH for stability (and similarly for temperature).

4. All enzymes are functionally specific to varying degrees. This is not the place to discuss all the aspects of this specificity, which enter into various schemes for classifying enzymes. Some examples of various kinds of specificity will suffice:

   a. A given enzyme catalyzes only reactions of a certain class, for example hydrolyses: $AB + H_2O \rightarrow AH + BOH$. Usually the range is still narrower, as the hydrolysis of oxygen esters of orthophosphoric acid: $ROPO_3H_2 + H_2O \rightarrow ROH + H_3PO_4$. Enzymes of the broader class are known as hydrolases, those of the narrower class as phosphatases.

   b. Within classes such as those described in (a), the rate of the catalyzed reaction differs with substrate (in the case of the phosphatases, with the structure of the group R). Thus there will be a phosphatase for which the rates of hydrolysis of phenyl phosphate and adenosine-3'-phosphate are quite different under otherwise identical experimental conditions.

c. As a consequence of the specificity described in (b), we can distinguish enzymes which are otherwise very much alike but which differ in their "substrate-activity profile". That is, if we take two such enzymes, and let them act on each of a list of five compounds (e.g., five different phosphate esters in the case of phosphatases), the list of rates for enzyme I acting on substrates A, B, C, D, and E differs from the corresponding list for enzyme II (other conditions being of course the same in all cases).

d. The specificity may be still narrower - the enzyme may act on one and only one substrate. This situation is less common than is usually supposed. At a given date it depends in part on how many compounds have been tested. Thus, for example, there is a class of enzymes which hydrolyze only the terminal phosphate anhydride linkage of adenosine triphosphate (abbreviated ATP), which do not affect any ordinary phosphate esters, which on the other hand do not split such phosphate anhydride links as those of pyrophosphate or adenosine diphosphate. However, such very specific adenosine triphosphatases do act, though at a much reduced rate, upon inosine triphosphate (ITP), a compound which differs from ATP only in the substitution of -OH for $-NH_2$ on the number 6 carbon atom of the adenine ring. Some enzymes are more discriminating than others, but it seems fair to say that any enzyme can be fooled if one goes to enough trouble.

e. In addition to specificity with respect to type of bond attacked and chemical structure of compound, most enzymes are highly specific with respect to stereoisomers, and especially with respect to optical isomers. Thus they discriminate, usually quite strictly, between the D- and the L- forms of amino acids, of simple sugars, between the α and β forms of glycosides, and between pyranosides and furanosides.

f. If the same enzyme ("same" in the sense of having exactly the same kind of enzymatic activity) is obtained from two different organisms, the two samples of enzyme always differ antigenically. But, in addition to this, other striking and unexpected differences may be found. Consider, for instance, aldolase, which is defined as the enzyme that (non-hydrolytically) splits 1, 6-diphosphofructose into two molecules of triose phosphate (performing essentially the reverse of an aldol condensation). Aldolase obtained from muscle is a simple protein, which is not known to require any added substance other than the substrate for full activity. Aldolase obtained from yeast, on the other hand, is activated by ferrous ion in the natural state, may be activated by zinc, and in the natural state is readily inhibited by iron-complexing substances such as pyrophosphate.

THE PROBLEMS OF A THEORY OF ENZYME BEHAVIOR

The task of theoretical enzymology is to take account of these basic properties of enzymes, to correlate them, and to explain them in terms of fundamental chemical and physical laws. This task may be subdivided into the following problems:

1. To show how the rates of enzyme-catalyzed reactions are related to various environmental factors, such as: (a) the concentration of the enzyme; (b) the concentration of the reacting substances (generally known as the "substrates" of the enzyme); (c) the concentrations of various substances which specifically activate or inhibit the catalysis; and (d) physical factors such as the pH, temperature, and ionic strength of the reaction mixture.

2. To account for the specificities of enzymes in terms of their chemical structure and the chemical structures of their substrates.

3. To explain the occurrence of enzymatic catalysis in physicochemical terms - that is, to show, in terms of the atomic and electronic structures of enzyme and substrate, precisely how the presence of the enzyme raises the rate of chemical reaction above the "spontaneous" rate.

The answer to the first of these three problems will occupy most of this book, for the simple reason that this problem (that of "enzyme kinetics") has in large measure been satisfactorily solved. The other two problems, for a variety of reasons which include limitations of experimental technique, are only now beginning to be attacked effectively. Thus only the last chapter of the book deals explicitly with the mechanism of enzyme action, although it will be seen that certain kinds of kinetic studies contribute to the analysis of mechanism.

THE ASSUMPTIONS OF ENZYME KINETICS

In order to develop a theory of the rates of enzyme-catalyzed reactions, it is necessary to make some concrete assumption about the way in which the enzyme and the substrate molecules interact.

For example, one might assume that enzyme molecules increase the energy of substrate molecules by some sort of action at a distance (e.g., electrostatic attractions or repulsions, or electromagnetic radiation). One would then have to add a second assumption: that substrate molecules whose energy distribution in certain bonds exceeds a certain critical or threshold value will react, and that the rate of reaction is proportional to the number of such "hot" or activated substrate molecules.

Alternatively, one might assume that energy is transmitted from enzyme to substrate by way of inelastic collisions, again with the added proviso that only sufficiently energized molecules finally react.

Again, one might assume that enzyme and substrate form a compound, and that this compound then undergoes internal rearrangement, after which the reaction product and the original enzyme molecule are formed from the rearranged compound.

The notion of action at a distance was not entertained until recently (1). It was supported by evidence which has been severely criticized on technical grounds (2), and which has not been confirmed. A quantitative theory based on the idea has not been developed; but it is not difficult to show that it would lead to relations rather different from those found experimentally to date (3).

The idea of energy transmission by inelastic collision was proposed and worked out by Medwedew, and has been elaborated by Hearon and Katzman (4, 5). It leads to a relation between reaction rate and substrate concentration which superficially resembles the relation derived by assuming compound formation between substrate and enzyme; and in a few cases it fits experimental data rather well. However, it will not stand a more critical test, to be described later in this chapter.

The hypothesis of complex or compound formation by enzyme and substrate meets the test of experimental data on reaction rates (with such additional assumptions as may be needed, such as will be described in the course of this book). In recent years, it has received the further support of direct experimental confirmation (6, 7). The case for this model of enzyme action is therefore so strong that it alone will be used as the basis for all the further theoretical developments to be presented.

Before developing the idea in quantitative form, however, it will be worth while to consider in detail some of the fundamental experiments which led up to it.

THE CONCEPT OF THE ENZYME-SUBSTRATE COMPLEX

Just when the notion of a direct stoichiometric combination of enzyme and substrate was first stated is not certain. There is no doubt that it pervades the literature of the late nineteenth and very early twentieth centuries, usually in a fairly mature and clear form. For example, H. E. Armstrong, in a Presidential Address to the Chemical Society entitled "The Nature of Chemical Change", discussed enzyme action as merely one case among many, and asserted that "complication, not simplification, precedes most, if not all, chemical change, that complex molecular systems are first formed from the interacting substances, and that these, on breakdown, suffer rearrangement of the parts - such rearrangements taking place in consequence of elements which were previously separated being brought into one common 'sphere of activity' within which it is possible for them to interact. ... The function of enzymes in promoting hydrolysis (of saccharides), on this hypothesis, consists in bringing water into conjunction with the carbohydrate by combining with both" (8). The idea that the sole function of the enzyme is a passive geometrical one is, of course, no longer entertained; but the picture of the stoichiometric relations of enzyme, enzyme-substrate combination, and products is precisely the one which prevails at present.

The consequences of such a point of view were recognized fairly early, but were not at first fully expressed in algebraic form. Perhaps the earliest extensive quantitative study of enzyme kinetics was performed by O'Sullivan and Tomson (9). Using yeast invertase, these investigators studied methods of extraction and purification, and studied the kinetics of invertase as a function of temperature, pH, substrate concentration, and similar variables. They concluded that the hydrolysis of sucrose by the enzyme followed a "mass action" law, by which they meant that the reaction was first order, a constant proportion, or per cent, of the substrate being split per unit time.

These conclusions were criticized by Brown (10). Working with the invertase of live yeast, Brown found that a constant weight (rather than a constant proportion) of the sugar present was split in unit time. Brown then repeated and varied the experiments of O'Sullivan and Tomson, and confirmed his previous results.

In spite of the temptation to be satisfied with this, Brown analyzed the data of both laboratories in a remarkably lucid fashion, employing essentially those concepts of enzyme action which we now hold. He cited evidence from O'Sullivan and Tomson on protection of enzyme against heat by substrate, experiments on compound formation between papain and fibrin (11), and Fischer's concept of configurational specificity of enzymes, to support the view that the invertase must combine with sucrose in the course of its action.

Assuming a mean lifetime of 0.01 seconds for this complex before it reacts, he pointed out that in consequence the maximum number of elementary reactions which one molecule of enzyme could promote per second (what is now called the turnover number of the enzyme) would in such a case be 100. At low sugar concentrations, the number of complexes formed and able to react per unit time would be proportional to the number of collisions of enzyme and substrate, which would also have to be proportional to the amount of substrate present. If the concentration of sugar is increased, however, till the number of collisions per unit time is greater than the number of conversions the enzyme could perform in that time, the rate could still not exceed 100 conversions per enzyme molecule per second. Under these conditions a constant weight of sugar would be split per unit time, this being the maximal possible amount. Brown reasoned that the results of O'Sullivan and Tomson should be valid at low substrate concentrations, performed the experiment, and did indeed get the expected first-order reaction rate.

In the same year E. F. Armstrong investigated a number of disaccharide-hydrolyzing enzymes such as maltases and lactases of various origins (12). The amount of

hydrolysis was measured at various times, in the presence of different amounts of enzyme and sugar, and with different enzyme-sugar ratios. In the presence of a large initial excess of sugar, the actual weight of sugar hydrolyzed after a given time was independent of substrate concentrations (provided it remained in large excess), and the amount split was proportional to the time during which the enzyme was allowed to act. Where a large excess of enzyme was used, on the other hand, the amount of hydrolysis was directly proportional to the amount of substrate present. Under all conditions, the hydrolysis during a given period of time was proportional to the amount of enzyme present.

From these results Armstrong inferred that the outcome of such experiments would be principally determined by the relation between amount of enzyme and amount of substrate. He proposed that the "active mass" of the substrate (the amount which is ready to react at any time) is equal to the amount combined with enzyme.

In a remarkable anticipation of the "steady state" idea which will be presented later, Armstrong described how the amount of this active mass is determined. Enzyme and substrate combine, but then water, the remaining reactant, "competes" with the enzyme for the substrate - that is, the water attacks the active complex and releases the products of hydrolysis. An "equilibrium" is soon reached between the process which forms the active mass and the process which consumes it (the word "equilibrium" is not strictly correct, but it is clear that Armstrong really meant a steady state, in which the reactions going to form active complex are just balanced by the reactions which remove it). The level of this "equilibrium" depends on the relation between amount of enzyme and amount of substrate.

If one sets up the experiment with an excess of sugar, the enzyme is, to use current terminology, saturated with substrate ("the maximum possible number of effective combinations", in Armstrong's words, is formed). The "active mass" is therefore constant in time, and the amount split is simply proportional to elapsed time. As the amount of unhydrolyzed sugar decreases, the enzyme eventually is no longer saturated, and the active mass becomes a function of the amount of sugar present. If one begins the experiment with an excess of enzyme, the phase of hydrolysis proportional to time is no longer observed, and only the later phase is found.

ASSUMPTIONS FOR THE SIMPLEST KINETIC EQUATIONS

At the turn of the century, then, the major notions of enzyme kinetics existed in complete form: the idea of the enzyme-substrate complex, of the turnover number, and of saturation, as well as the concept of specific inhibition of enzymes (e.g., by their reaction products). The mathematical formulation of these ideas is quite straightforward. Let us consider the simple case with which most books begin (and end): that of a reaction involving only one substrate.

The assumptions that must be made for such a simple case are:

1. A molecule of enzyme and a molecule of substrate combine reversibly to form a complex or compound. (We will consider in Chapter VI the situation where more than one substrate molecule unites with one enzyme molecule.)

2. The complex breaks up irreversibly, giving a molecule of product and the original enzyme molecule. (The consequences of reversible action at this stage will be studied in Chapter II.)

3. The only forms of the enzyme are: free enzyme and enzyme incorporated into complex. The total amount of enzyme is constant throughout the experimental period.

4. The amount of substrate which is bound by enzyme is very small compared with the total amount of substrate present in the mixture. (The result of abandoning this assumption will be explored in Chapter III.) Consequently, the concentration of free substrate is equal to the concentration of total substrate at any time.

We shall represent the substrate by S, the free enzyme by E, and the E-S complex by C (the reaction product, which will not enter the formulae, being denoted by P). To simplify notation, we use the same symbols, both in the stoichiometric chemical equations, where they denote the substances, and in all other equations, where they represent concentrations (e.g., in moles per liter); the usual brackets to denote concentrations will be omitted, there being no cases where confusion is likely to occur. The total concentration of enzyme is denoted by E_t. Forward reactions will be numbered, and the corresponding back reactions, if any, will be denoted by the same number preceded by a minus (-) sign.

If assumptions 1 and 2 are translated into the symbolism of chemical equations, we get the stoichiometric scheme or set of stoichiometric equations*:

I.A. $$E + S \underset{-1}{\overset{1}{\rightleftharpoons}} C; \qquad C \xrightarrow{2} E + P$$

* It is possible to condense the way in which two or more equations like I.A. are written. Thus, we can write:

$$E + S \underset{-1}{\overset{1}{\rightleftharpoons}} C \xrightarrow{2} E + P$$

This can be done only when the equations have a side completely in common. In the case of the example given, the expression C is the common side. To see what cannot be done under this rule, compare the following:

Complete: $A + B \rightarrow C$; $C \rightarrow X$ Condensed: $A + B \rightarrow C \rightarrow X$.

Complete: $A + B \rightarrow C + D$; $C \rightarrow X$ Condensed: $A + B \rightarrow C + D \rightarrow X$.

The first condensation is correct; the second is wrong - because it implies that X is formed from C and D together, where in fact X is formed from C only. If the condition of a complete side of an equation in common is not fulfilled, no condensed version is possible.

The direction of arrows must also be observed. Thus, if we have

$$A \rightarrow X; \qquad B \rightarrow X;$$

it obviously is not sensible to write either

$$A \rightarrow X \rightarrow B \quad \text{or} \quad B \rightarrow X \rightarrow A.$$

According to the two original equations, X is a dead end, and therefore cannot be a link between A and B in a condensed version.

In the same way, any condensed chemical equation can be broken down into a set of simpler equations, each involving only one reaction.

We refer to a set of stoichiometric equations as a stoichiometric scheme (or simply a scheme for brevity) when they describe completely all of the chemical reactions which are supposed to be going on under our particular assumptions.

The stoichiometric equations specify what reactions occur, what the reactants and products are, and in what molecular proportions they react or are formed. Thus $A + B \rightarrow C$ specifies that each elementary reaction consists of one molecule of A and one of B giving one of C. If the equation were to read $2A + B \rightarrow 3C$, it would tell us that in

each elementary reaction two molecules of A react with one of B to form three of C. The equations thus give an overall bookkeeping picture of the reaction. However, they say nothing about the rate at which the reaction occurs.

HOW TO DERIVE KINETIC EQUATIONS:

To express rates, we make use of kinetic equations. There is a separate kinetic equation for each compound, reactant or product, pictured in a stoichiometric scheme. Thus, for the scheme I.A, there will be four kinetic equations, one for each of the four substances E, C, S, and P.

A kinetic equation for a given substance expresses the rate of change of that substance with time. In other words, if we are calling the substance X, the kinetic equation for X expresses the quantity dX/dt.

The rate of change of a substance is made up of the rates of the individual chemical reactions in which that substance is used or formed (and only those reactions). Each reaction step, forward or backward, contributes one term to the net rate of change. Thus an irreversible reaction will contribute just one term; a reversible reaction will contribute two, one for the forward step and one for the backward step.

Thus, if we were confronted with the simple equation $S + E \underset{-1}{\overset{1}{\rightleftharpoons}} C$, we could write three kinetic equations, one each for S, E, and C. Suppose we pick C to begin with: its rate of change is dC/dt, and it is made up of a term from the forward reaction step (labelled 1) and a term from the reverse reaction step (labelled -1). The first term is positive, because the forward step makes C, and so would tend to make C increase with time; the second term will be negative, because the reverse reaction breaks C down again to S and E, and so would make C decrease with time. Thus we could write the kinetic equation for C in a sort of pseudomathematical way as dC/dt = (Rate 1 forward) - (Rate -1 back). The equation for dE/dt would involve the same terms, but the signs would be opposite; the forward step removes E, and so makes E decrease with time, while the back reaction forms E again, and so makes E increase with time. The pseudomathematical form for dE/dt would be dE/dt = -(Rate 1 forward) + (Rate -1 back). The same will be true for dS/dt.

To go from the pseudomathematical equations just indicated to actual usable equations, we must have a way of expressing each of the individual rates mathematically. This is given by the Law of Mass Action (13): The rate of a reaction is proportional to the product of appropriate powers of the concentrations of the reactants; the "appropriate power" for each reactant is the number of molecules reacting according to the stoichiometric equation.

Thus, reverting to our illustrative equation, $S + E \underset{-1}{\overset{1}{\rightleftharpoons}} C$, the term which we have referred to as Rate 1 forward is proportional to S^1xE^1, since one molecule each of S and E reacts. This can, of course, simply be written SE, by the mathematical convention which leaves out the index of a power when it is equal to one. In the same way, Rate -1 back is proportional to C^1 or C. If we were working with an equation like $A + 2B \rightarrow X$, the forward rate would be proportional to AB^2; since two B molecules react at each step, the appropriate power of B is the second power.

The phrase "proportional to", of course, means "multiplied by a constant". Thus the concentration factors in every rate term will be multiplied by a constant - that is, a factor that does not depend on any of the concentrations. Each rate has its own individual constant. To express such constants, we propose to use the letter "k", labelled with a subscript which is the number of the reaction step involved. Thus the constant for Rate 1 forward would be "k_1", and that for Rate -1 back would be "k_{-1}". Such constants are termed "rate constants" or "velocity constants".