ENZYME KINETICS

Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems

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CHAPTER ONE

INTRODUCTION—ENZYMES AS BIOLOGICAL CATALYSTS

A. THE DISCOVERY OF ENZYMES AND THE DEVELOPMENT OF ENZYMOLOGY

It is hard to pinpoint the exact discovery of enzymes. Cell-free activity was observed as early as 1783 when Spallanzani noted that meat was liquefied by gastric juice of hawks. In the following years, numerous similar observations were made. For example, in 1814 Kirchhoff observed that a "glutinous" (i.e., proteinaceous) component of wheat was capable of converting starch to sugar. Robiquet and Boutron and also Chaland discovered the hydrolysis of amygdalin by bitter almonds in 1830. Leuchs, in 1831, described the diastatic action of salivary ptyalin. The first discovery of an enzyme is usually credited to Payen and Persoz, who, in 1833, treated an aqueous extract of malt with ethanol and precipitated a heat-labile substance which promoted the hydrolysis of starch. They called their fraction "diastase," from the Greek word for *separation*, since their material separated soluble sugar from starch. Today we recognize that the diastase of Payen and Persoz was an impure preparation of amylase. The next enzyme to be partially purified was from an animal source. In 1834 Schwann described pepsin, and in 1836 he extracted the active agent with acid from the stomach wall. It is noteworthy that the first observations of enzyme activity preceded a clear notion of catalysis. Berzelius, in 1835 to 1837, described this unknown force, which by its mere presence could "exert its influence and arouse affinities and reactivities in the other complex bodies thereby causing a rearrangement of the constituents of the complex body." This concept of catalysis evolved from observations of the action of diastase and pepsin, and from the seemingly similar action produced by yeast during fermentation. In all cases, one substance was changed to another under the influence of an active agent—the catalyst. It was not yet recognized that yeast were living

cells. In 1838, Cagniard de Latour showed that fermentation was caused by living organisms, an idea confirmed and extended by Pasteur between 1858 and 1871. Pasteur regarded the chemical changes occurring in fermentation as an essential part of the life processes of the microorganisms involved. The chemists of the day, most notably Stahl and Liebig, favored a purely chemical theory of fermentation. A distinction was made between the "organized ferments," such as those catalysts presumed to be present in or on the surface of yeast and lactic acid bacteria, and the "unorganized ferments," such as diastase and pepsin whose activities were clearly not associated with microorganisms. Liebig's theory visualized a ferment as a chemical substance produced by a decomposing organism. The atoms of the ferment were supposed to be in "ceaseless movement, constantly changing their position." This highly agitated state was somehow transmitted to the atoms of a sugar molecule "whose elements are held together by weak forces." As a result, the sugar breaks down to compounds (CO_2 and ethanol) whose atoms are held together more tightly. In 1860, Berthelot macerated yeast and obtained an alcohol-precipitable fraction which converted sucrose to glucose plus fructose. He concluded that this invertase (as the active agent was called) was one of many ferments present *in* yeast. In 1878, Kühne suggested the name *enzyme* (meaning "in yeast") for both organized and unorganized ferments. The suffix "*ase*" was proposed by Duclaux in 1883. The end of the Pasteur-Liebig controversy came in 1897 when Hans and Edouard Buchner were able to extract from yeast a cell-free juice which carried out the complete fermentation of sugar. The Buchner brothers were primarily interested in obtaining a yeast juice for therapeutic purposes. Since their preparation was intended for human consumption, it could not be preserved with the usual bacteriocidal agents. An assistant suggested that they add a large quantity of sucrose, since it was known that the growth of microorganisms was inhibited by high sugar concentration. Upon adding the sugar, the yeast juice bubbled vigorously as ethanol and CO_2 were produced. In the same year, Bertrand observed that some enzymes required dialyzable factors for catalytic activity. He named these substances *coenzymes*. By 1900, the catalysts of cellular oxidation were recognized as enzymes.

During the early part of the twentieth century, serious attempts were made to purify enzymes and describe their catalytic activity in precise mathematical terms. In 1902, Henri and Brown independently suggested that an enzyme-substrate complex was an obligate intermediate in the catalytic reaction. Their suggestion was based on the type of curve obtained when the initial velocity of the reaction was plotted against the substrate concentration and was in agreement with the lock-and-key concept proposed by Emil Fischer in 1894 to account for the high degree of specificity exhibited by enzymes. Henri derived a mathematical equation to account for the effect of substrate concentration on the velocity. The effect of pH on enzyme activity was pointed out by Sörensen in 1909. In 1913, Michaelis and Menten rediscovered the equation derived by Henri 11 years earlier. The Henri-Michaelis-Menten equation was based on simple chemical equilibrium principles. In 1925, Briggs and Haldane introduced the steady-state concept to enzyme kinetics. Today, both approaches are used to explain the kinetic properties of enzymes.

The fact that enzymes are proteins was not accepted until the late 1920's (although as early as 1877 Traube suggested that all cellular activities including fermentation, respiration, and putrefaction were catalyzed by substances allied to proteins). In 1926, Sumner crystallized the enzyme urease, but many argued that the enzyme was simply an impurity adsorbed onto or occluded within the protein crystals. However, during the 1930's, Northrop and co-workers crystallized pepsin, trypsin, and chymotrypsin, and demonstrated conclusively that the protein crystals were pure enzymes. By 1943, about 25 enzymes had been crystallized.

In the 1940's and 1950's hundreds of new enzymes were discovered, and many of them purified to homogeneity and crystallized. Dozens of key metabolic pathways were elucidated, and biochemists started focusing on the mechanisms of enzyme activity and regulation. Genetics and biochemistry joined to produce the field of molecular biology. New chemical and physical techniques were used to purify proteins and probe their structures. In 1955, Sanger reported the complete amino acid sequence of insulin, a small protein of molecular weight 6000. Five years later, the first enzyme (ribonuclease, molecular weight 13,700) was sequenced, and finally, in 1969, the first chemical synthesis of an enzyme (ribonuclease) was achieved. In 1957, Kendrew deduced the three-dimensional structure of myoglobin from X-ray diffraction studies.

Up until the 1950's most studies on the kinetics of enzyme activity were based on the Henri-Michaelis-Menten or Briggs-Haldane equations for unireactant enzymes. From the mid-1950's to the early 1960's attempts were made to analyze the kinetics of bireactant and terreactant enzymes. Equations based on the rapid equilibrium assumptions of Henri, Michaelis, and Menten could be derived quite easily, but many enzymes did not follow rapid equilibrium kinetics. Equations based on steady-state concepts were derived by Dalziel, Alberty, Hearon, and others, but, in general, these were rather complex and were not expressed in the familiar terms of K_m, K_i, and V_{max}. In 1963, Cleland presented a clear, uniform procedure for writing kinetic equations for multireactant steady-state enzyme systems together with a convenient shorthand nomenclature for describing the kinetic mechanisms (Chapter Nine). In 1965, Monod, Wyman, and Changeux presented a kinetic model for *allosteric enzymes* (regulatory enzymes which

displayed sigmoidal rather than hyperbolic velocity curves). A year later, Koshland, Nemethy, and Filmer presented an alternate model based on the flexible enzyme-induced fit model of Koshland (1959). (These and other models are described in detail in Chapter Seven.)

Before embarking on our survey of enzyme kinetics, let us first examine exactly what an enzyme does.

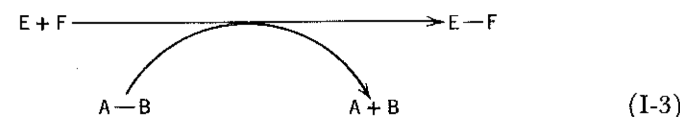
B. LIFE, ENERGY, AND COUPLED REACTIONS

Chemical reactions can be classified as exergonic (energy-yielding) or endergonic (energy-requiring). A unique property of a living cell is its ability to couple exergonic and endergonic reactions and, thereby, grow and reproduce at the expense of its environment. It does not take a knowledge of thermodynamics to recognize that growth is an endergonic process. Living cells are composed of an organized assemblage of fragile macromolecules, each with a highly specific structure. It takes energy to build large molecules from small molecules, that is, work must be done to build the complex structures of proteins, nucleic acids, cell membranes, and such, from the basic building blocks. Indeed, the resulting structures are so fragile that work must be done continually just to maintain the integrity of the cell. The energy for this work is derived from exergonic reactions. Thus if we had to summarize "life" in a series of simple equations, we could write:

$$\text{A–B} \longrightarrow \text{A} + \text{B} + \text{energy} \qquad \Delta G' \text{ is negative} \qquad \text{(I-1)}$$

$$\text{E} + \text{F} + \text{energy} \longrightarrow \text{E–F} \qquad \Delta G' \text{ is positive} \qquad \text{(I-2)}$$

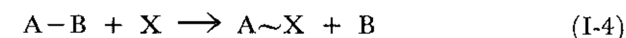
Reaction I-1 represents the catabolic reactions that occur in a living cell while reaction I-2 represents the anabolic (biosynthetic) reactions. The energy released by reaction I-1 must be made available for the endergonic reaction I-2; that is, the two reactions cannot take place randomly in different parts of the cell, but rather, they must somehow be coupled as in reaction I-3.



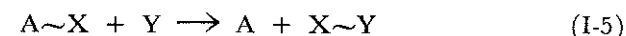
(I-3)

The overall biosynthesis of E–F at the expense of A–B is more likely to

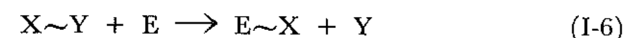
proceed via a series of steps:



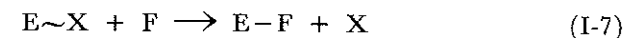
$$\text{A}-\text{B} + \text{X} \longrightarrow \text{A}{\sim}\text{X} + \text{B} \qquad \text{(I-4)}$$



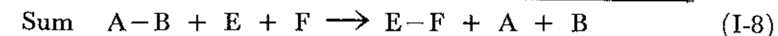
$$\text{A}{\sim}\text{X} + \text{Y} \longrightarrow \text{A} + \text{X}{\sim}\text{Y} \qquad \text{(I-5)}$$



$$\text{X}{\sim}\text{Y} + \text{E} \longrightarrow \text{E}{\sim}\text{X} + \text{Y} \qquad \text{(I-6)}$$



$$\text{E}{\sim}\text{X} + \text{F} \longrightarrow \text{E}-\text{F} + \text{X} \qquad \text{(I-7)}$$



$$\text{Sum} \quad \text{A}-\text{B} + \text{E} + \text{F} \longrightarrow \text{E}-\text{F} + \text{A} + \text{B} \qquad \text{(I-8)}$$

In reaction I-4, A–B is cleaved and a portion of the energy made available is used to condense A with X to yield a transient activated A. The potential energy of A∼X is conserved when X is transferred to Y, producing a mobile, energy-rich X∼Y (e.g., ATP). The condensation of E and F occurs in two steps: first E is activated to yield E∼X, and then the potential energy of E∼X is discharged by the formation of E–F.

C. ENZYMES AS CATALYSTS

The fact that a reaction has a negative ΔG does not mean that it will proceed at a detectable rate. A negative ΔG means that the existing [P]/[S] ratio is less than that at equilibrium. The rate at which the reaction approaches equilibrium cannot be deduced from the magnitude or sign of ΔG. For example, the oxidation of glucose to $CO_2 + H_2O$ has a $\Delta G°$ of $-686{,}000$ cal/mole; that is, glucose in the presence of oxygen is unstable in a *thermodynamic* sense. Yet, glucose does not immediately oxidize to $CO_2 +$ H_2O in the presence of oxygen. Thus glucose is quite stable in a *kinetic* sense. It is obvious that some barrier exists even for so-called spontaneous reactions. The barrier is the *activation energy* that is required. This is illustrated in Figure I-1 where we see that the reaction S→P has a negative ΔG, but before a molecule of S can become a molecule of P, it must possess a certain minimum energy to pass into an activated transition state, $S \cdot \cdot P^{\ddagger}$. The activated state represents a sort of halfway point where the bonds of S are distorted sufficiently so that conversion to P becomes possible. Molecules of S that attain less than the minimum energy simply fall back to the ground state. The rate at which S is converted to P depends on the number of molecules that make it to the transition state per unit time. Glucose is stable in air at room temperature because virtually none of the molecules are sufficiently activated. There are two ways of accelerating the reaction S→P. One is to raise the temperature until a significant number of S molecules attain the transition state. Another way is to lower the activation energy.

Living cells exist at relatively low temperatures—between 0°C and 100°C. At life temperatures few, if any, of the exergonic and endergonic reactions of intermediary metabolism would occur at a rate sufficient to permit cell maintenance and growth. Furthermore, even if a living cell could increase its temperature sufficiently, it would have no way of specifically increasing the temperature of one reaction relative to another. Living cells can operate under relatively mild environmental conditions because they possess *enzymes, which selectively lower the energies of activation* of the vital chemical reactions. In the presence of the appropriate enzyme, the ambient temperature provides a substantial fraction of the reactant molecules with the required activation energy. Enzymes, then, are *catalysts* which speed up the rate of a chemical reaction without themselves being consumed. In the process, enzymes act as mediators of the coupled reactions that constitute metabolism (e.g., reactions I-4 to I-7 would each be catalyzed by a specific enzyme). The equilibrium constant for a reaction is unaltered. Only the rate at which the reaction proceeds toward equilibrium is affected by an enzyme. For example, in the reaction $S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P$, k_1 might be 10^{-3} min^{-1} while k_{-1} might be 10^{-5} min^{-1}. At equilibrium the forward and reverse velocities are equal. Therefore:

$$v_f = k_1[S]_{eq} = v_r = k_{-1}[P]_{eq}$$

The equilibrium constant for the reaction (defined as $[P]_{eq}/[S]_{eq}$) is:

$$K_{eq} = \frac{k_1}{k_{-1}} = \frac{10^{-3}}{10^{-5}} = 100$$

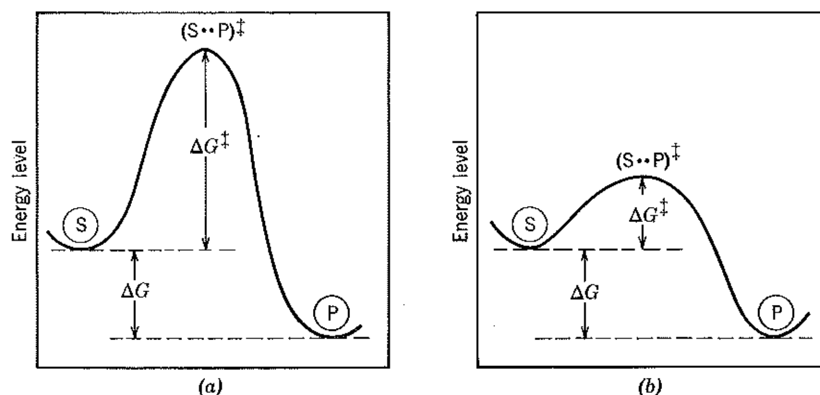


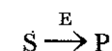
Fig. I-1. ΔG and $\Delta G^\ddagger$ of (*a*) nonenzymatic and (*b*) enzymatic reactions.

In the presence of an appropriate enzyme, both k_1 and k_{-1} are enhanced to the same degree. Thus k_1 might increase 10,000-fold to 10 min^{-1}; k_{-1} must also increase 10,000-fold to 10^{-1} min^{-1}; and K_{eq} is unchanged.

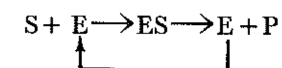
$$K_{eq} = \frac{10}{10^{-1}} = 100$$

D. THE ACTIVE SITE

The enzyme-catalyzed production of P from S can be written:



but it was recognized early that the enzyme and the reactant (hereafter called the substrate) must combine in some way during the course of the reaction. The overall catalytic sequence can be written:



The existence of an enzyme-substrate complex, ES, was inferred from (*a*) the high degree of specificity exhibited by enzymes (Fischer, 1894), (*b*) the shape of the velocity versus substrate concentration curve (Brown, 1902; Henri, 1902), and (*c*) the fact that substrates frequently protected enzymes from inactivation (O'Sullivan and Tompson, 1890). The high degree of specificity of enzymes prompted Emil Fischer in 1894 to suggest the *template* or *lock-and-key* analogy of enzyme-substrate interaction. This relationship, shown schematically in Figure I-2, assumes that the enzyme possesses a region (called the substrate binding site, the active site, or the catalytic site) that is complimentary in size, shape, and chemical nature to the substrate molecule. Thus only a single substance, or, at most, a limited range of substances can bind to the enzyme and act as substrates. Only when the substrate is anchored in the site can it undergo the chemical change that converts it to the product. Today, we recognize that the active site need not be a rigid geometrical cavity or pocket, but rather a very specific and precise spatial arrangement of amino acid residue R-groups that can interact with complimentary groups on the substrate.

All enzymes are proteins with molecular weights in the tens of thousands or greater. Most substrates are low molecular weight substances. (The latter statement is true even for enzymes that accept high molecular weight polymers as substrates. The polymer itself is not recognized as the substrate, but rather, a specific region or bond of the polymer.) Thus only a small

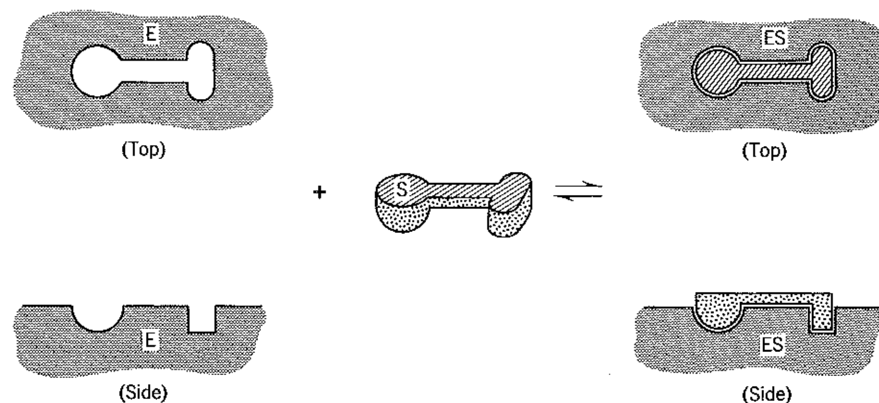


Fig. I-2. Lock and key (template) hypothesis of enzyme specificity.

fraction of the enzyme is actually involved in catalysis. To put it another way, the active site occupies only a very small fraction of the enzyme. In fact, there may be less than a dozen amino acid residues surrounding the absorption pocket of the active site, and, of these, only two or three may actually participate in substrate binding and/or catalysis. Why, then, are enzymes large proteins as opposed to small tripeptides or dodecapeptides? The answer is obvious when we consider that the two or three essential R-groups must be perfectly juxtaposed in three-dimensional space. A linear tripeptide might contain all the essential binding and catalytic groups, but the fixed bond distances and relatively fixed bond angles would not allow the essential R-groups to assume the required spatial relationship. With a large protein composed of a hundred or more amino acids the polypeptide chain could bend, twist, and fold back upon itself and in this way the positions of the three essential R-groups could be fixed exactly in space. Figure I-3 shows the tertiary structure of a hypothetical enzyme. The shaded area represents the absorption pocket of the active site while A, B, and C represent three essential R-groups that contribute to substrate binding and catalytic activity. The A and B might be only two or three residues apart but amino acid C might be 50 residues away from B. Even if the absorption pocket is lined with 12 amino acid residues, a dodecapeptide could never bend into the proper shape. Although only three amino acid residues are involved in the activity of our hypothetical enzyme, it is obvious that a great many of the other residues play an equally important role: that of maintaining the protein in its tertiary structure (via electrostatic interactions, hydrogen bonds, disulfide bonds, hydrophobic interactions, and dipole-dipole interactions).

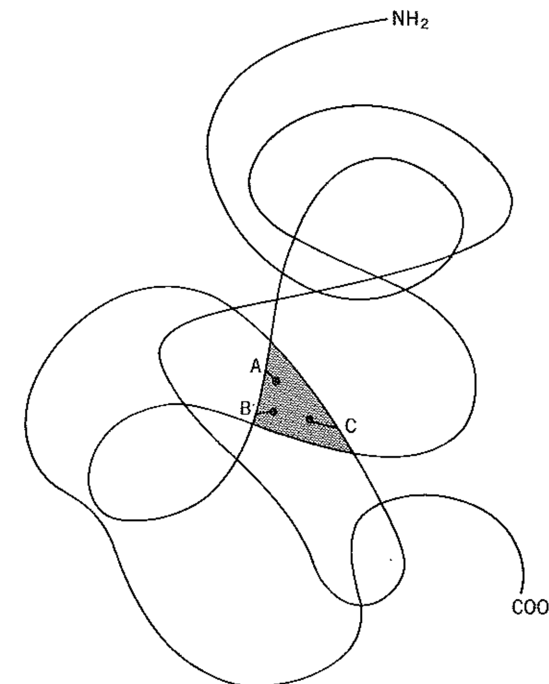
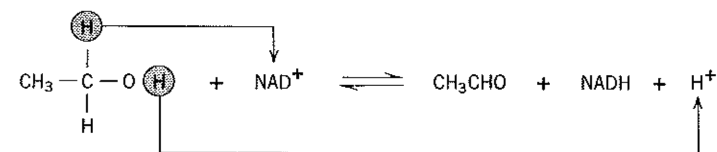


Fig. I-3. The active site (shaded area) occupies only a small region of the enzyme. A, B, and C are the amino acid R-groups responsible for substrate binding and catalytic activity.

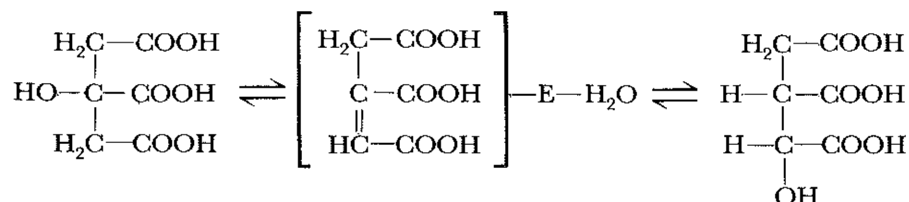
E. THREE-POINT ATTACHMENT

The combination of enzyme and substrate can be even more specific than we might deduce from the lock-and-key concept. For example, alcohol dehydrogenase catalyzes the reaction:



A given alcohol dehydrogenase always transfers the same methylene hydrogen to NAD^+ and vice versa. This high degree of stereospecificity can be explained if it is assumed that ethanol binds to the enzyme by a three-point attachment through the methyl group, the OH group, and one hydrogen of

the methylene group (Fig. I-4*a*). Actually, only the methyl group and the OH group need be bound. When these two groups are bound by the complimentary sites on the enzyme only one of the two methylene hydrogens (always the same one) will be situated next to the NAD^+ site. Aconitase exhibits a similar specificity. In this case the substrate, citric acid, is a symmetrical molecule, yet the OH group is always transferred in the same direction.



The specificity can be explained if citric acid binds to the enzyme via a three-point attachment as shown in Figure I-4*b* and the catalytic group lies in the region of the A–B sites.

F. THE FLEXIBLE ENZYME-INDUCED FIT HYPOTHESIS

Although the lock-and-key hypothesis successfully explained the great majority of specificity patterns exhibited by enzymes, there were several phenomena that the hypothesis could not explain. For example, compounds that resembled the normal substrate chemically but possessed less bulky groups often failed to react, yet they certainly should have fit the template. Compounds with more bulky groups often failed to react (as expected), yet they were found to bind tightly to the enzyme. Many bireactant enzymes would not bind substrate B before substrate A, yet according to the lock-and-key hypothesis, the binding site for substrate B on the free enzyme should be accessible to B. These and other considerations led Koshland to propose the *flexible enzyme* or *induced fit* hypothesis. The hypothesis states that the substrate induces a conformational change in the enzyme that results in a precise alignment of the catalytic groups with the susceptible bonds on the substrate (Fig. I-5*a*, *b*). Substrate analogs with larger (Fig. I-5*c*) or smaller (Fig. I-5*d*) groups may bind to the enzyme, but may not induce the proper alignment of the catalytic groups. In ordered bireactant systems, substrate A is assumed to induce a conformational change that exposes the binding site for substrate B. There is considerable physical evidence supporting the induced fit hypothesis including (*a*) substrate-induced changes in the chemical reactivity of certain R-groups of the enzyme and (*b*) substrate-induced changes in the fluorescence and absorbance properties of certain R-groups. The concept of

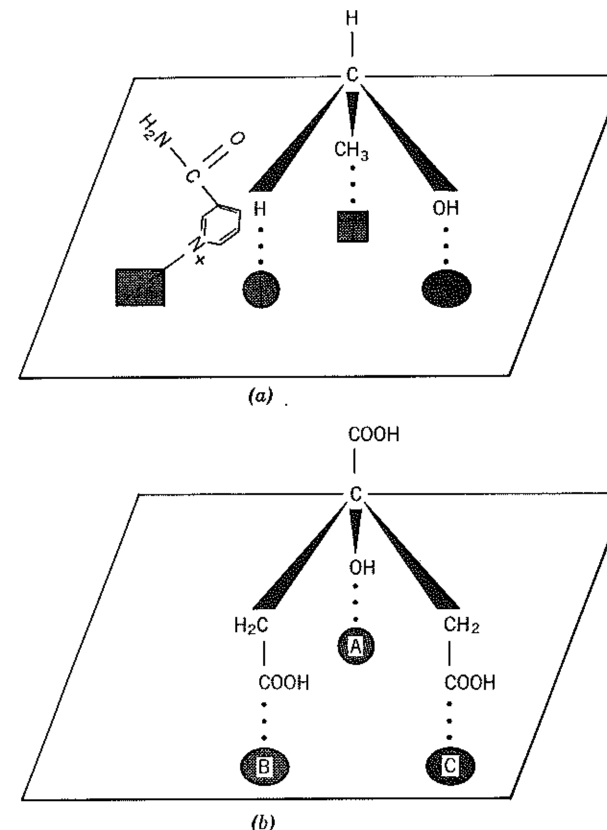


Fig. I-4 Three point attachment hypothesis to account for stereospecific catalysis. (*a*) Alcohol dehydrogenase. (*b*) Aconitase.

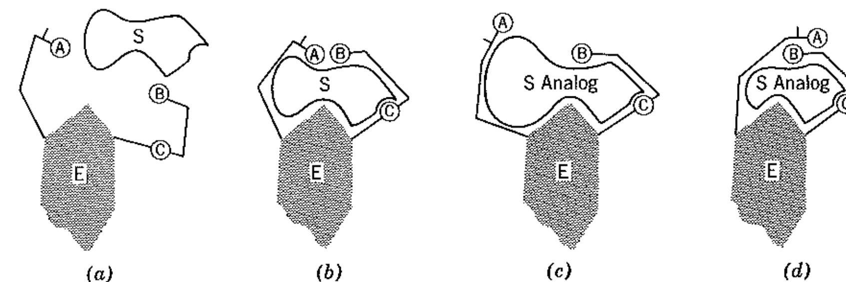


Fig. I-5. Induced-fit hypothesis of Koshland. (*a*) The substrate approaches the active site. (*b*) Substrate binding induces the proper alignment of the catalytic groups, A and B. (*c*) and (*d*) Substrate analogs (competitive inhibitors) bind to the enzyme (aided by group C) but the catalytic groups are not aligned properly. [Redrawn from Koshland, D. E. Jr., *Cold Spring Harbor Symposium on Quantatative Biology*, **28**, 473, (1963).]

a flexible enzyme is the basis for several theories of allosterism (Chapter Seven).

G. FACTORS RESPONSIBLE FOR THE CATALYTIC EFFICIENCY OF ENZYMES

Enzymes have a phenomenal ability to accelerate the rates of reactions. When it is possible to compare the nonenzymatic and enzymatic rates, we find that enzymes enhance the reaction rate by a factor of up to 10^{15}. By stating that "enzymes enhance reaction rates by lowering the activation energy required," we can discuss enhancement factors in a quantitative way, but the real question remains: How do enzymes lower the activation energy? A number of factors have been suggested which we can examine in a very qualitative way. First of all, it is generally agreed that most enzyme-catalyzed reactions proceed via recognized organic reaction mechanisms (e.g., general acid-base catalysis, nucleophilic and electrophilic displacements) in which the enzyme provides the catalytic groups. Certainly, some of the rate enhancement by an enzyme stems from *proximity and orientation* factors. In order for two substrate molecules (or a substrate molecule and a catalytic group) to react they must get close enough to each other and the approach must occur at the proper angle. In solution, the random motion of the two molecules would yield a low probability of an effective collision. When the two molecules are adsorbed onto the active site of the enzyme (or when one of the reactants is the substrate and the other reactant is an R-group of the active site), then both the intermolecular distance and orientation may be optimized. (The effective concentration of substrates in the volume of the active site is considerably greater than that in the solution from which they were adsorbed). A second-order intermolecular reaction between A and B in solution becomes a first-order intramolecular reaction when both A and B are enzyme-bound. Koshland and co-workers have proposed that the active sites of enzymes are so constructed that they align the orbitals of the substrate and catalytic groups optimally to enter the transition state. This concept of *orbital steering* is very similar to one proposed in 1960 by Bruice and Pandit. A qualitatively similar concept of *stereopopulation control* has been discussed by Milstien and Cohen. These workers point out that the combined effect of multipoint attachment and the precise fit of the substrate into the active site would tend to restrict the rotational freedom of the substrate and "freeze" it into a unique conformation. Also, substrates confined to the active site of an enzyme may have a relatively *long residence time* (compared to the time interval that the same substrates would be within striking distance of each other if they were in random motion in solution).

As a consequence of this *substrate anchoring* (as termed by Reuben), the number of substrate molecules attaining the activated transition state per unit time may be increased tremendously.

The idea that certain bonds of the substrate are distorted upon binding to the enzyme has been suggested by several workers. This so-called *rack mechanism* assumes that the substrate fits loosely into the active site, but the bonds that are formed between the enzyme and the substrate are so strong that a susceptible bond within the substrate is distorted producing an activated transition state (Fig. I-6). In this mechanism, a portion of the activation energy is provided directly by the binding forces between E and S. Although the mechanism is illustrated for a cleavage reaction, similar distortion models can easily be imagined for condensations and group transfer reactions in which the transition states of the substrates are more tightly bound to the enzyme than the unactivated substrates.

In the rack mechanism, the substrate molecule distorts to accommodate itself to a static enzyme. In 1947, Fano proposed that the substrate may be distorted by conformational changes in the enzyme. He visualized substrate binding as occurring in two steps (Fig. I-7). First, one part of the substrate binds to one site, then normal thermal motion brings the second site into a position that permits another part of the substrate to bind. As a result of the two-point binding of substrate, the enzyme is locked momentarily into a low entropy conformation. If the enzyme-substrate bonds are quite strong, then the substrate will be distorted to a transition state when the enzyme molecule swings back to its "open" conformation.

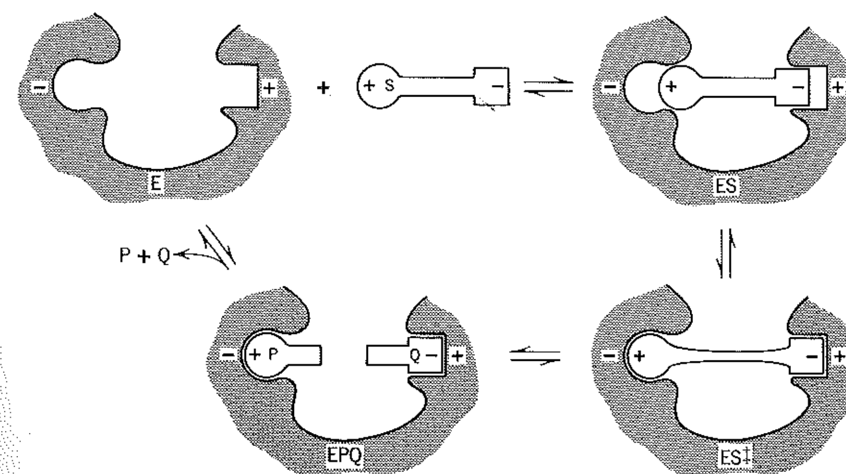


Fig. I-6. Distortion or rack mechanism.

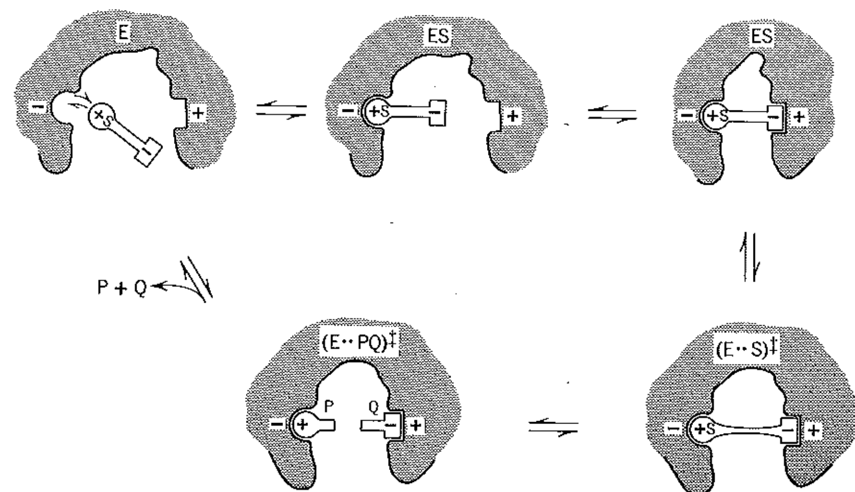


Fig. I-7. Two-step binding sequence leading to substrate distortion.

In spite of the many proposed mechanisms of rate enhancement and their success in accounting for the activity of some enzymes, we still understand very little about the factors responsible for the tremendous catalytic efficiency of the vast majority of enzymes.

H. ENZYME KINETICS

The sole function of an enzyme is to catalyze a reaction. *Enzyme kinetics* is that branch of enzymology that deals with the factors affecting the rates of enzyme-catalyzed reactions. The most important factors are: enzyme concentration, ligand concentrations (substrates, products, inhibitors, and activators), pH, ionic strength, and temperature. When all these factors are analyzed properly, it is possible to learn a great deal about the nature of the enzyme. For example, by varying the substrate and product concentrations, it is possible to deduce the *kinetic mechanism* of the reaction, that is, the order in which substrates add and products leave the enzyme. Such studies establish the kinds of enzyme-substrate and enzyme-product complexes that can form and thereby tell us something about the architecture of the active site. In some cases the kinetics of a reaction provide evidence for stable, covalently-bound intermediates that are undetectable by ordinary chemical analyses. Certain kinetic constants can be determined and from these we can make an educated guess concerning the usual intracellular concentrations of substrates and products and the physiological direction of the reaction. The kinetics of a reaction may indicate the way in which the activity of the enzyme is regulated *in vivo*. A study of the effect of varying pH and temperature on the kinetic constants can provide information concerning the identities of the amino acid R-groups of the active site. A kinetic analysis can lead to a model for an enzyme-catalyzed reaction and, conversely, the principles of enzyme kinetics can be used to write the kinetic equation for an attractive model. The kinetic equation tells us exactly how all the ligands of a system interact to affect the velocity of the reaction. Consequently, once we have a possible equation, the model can be tested experimentally. For many biologists, a thorough understanding of enzyme kinetics is indispensable to their research. A knowledge of the basic principles of enzyme kinetics is useful even to the average biologist who only occasionally assays an enzyme. With these principles, he will be able to design experiments and tell, for example, whether he is dealing with a single enzyme, or whether his preparation contains multiple enzymes that catalyze the same reaction. He will be able to tell whether his enzyme preparation contains inhibitors or activators. By comparing the kinetic constants of two seemingly-identical enzymes from different tissues, or from the same tissue at different stages of development, it is possible to decide whether the two enzymes are indeed the same gene product, or whether they are distinct proteins that catalyze the same reaction. For many biologists, the subject of enzyme kinetics provides no more than a satisfying intellectual experience while others are convinced that the maze of algebra is not worth going through. The following chapters are designed to act as a step-by-step guide through the maze. The basic principles and their application to specific types of enzyme systems are described in detail. An attempt is made to prove that the kinetic equations and graphical analyses are not as formidable as they seem at first glance. It is worth mentioning here that many of the velocity (kinetic) equations presented in Chapters Two through Eight and in Chapter Eleven are, in fact, equilibrium ligand binding equations. These equations, with only slight modification, are directly applicable to studies of drug and hormone binding to receptor sites, the interaction of inducers with repressor proteins, and the binding of substrates to membrane transport proteins.

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CHAPTER TWO

KINETICS OF UNIREACTANT ENZYMES

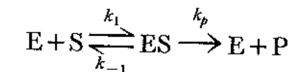
A. THE HENRI EQUATION AND THE MICHAELIS-MENTEN EQUATION

During the late nineteenth century, many workers sought to explain the progress of reactions involving enzymes in terms of the known principles of equilibrium and mass action. Most of the work at that time was concerned with measurements of substrate and product concentrations during the course of sucrose and starch hydrolyses, from which apparent rate constants were calculated. The first general rate equation for reactions involving enzymes was derived in 1903 by Henri. Henri's equation accounted for the observation that the initial rate of a reaction was directly proportional to the concentration of enzyme preparation, but increased in a nonlinear manner with increasing substrate concentration up to a limiting maximum rate. The derivation of Henri's equation was based on the assumptions that:

1. The enzyme is a catalyst (proposed in 1835 to 1837 by Berzelius).
2. The enzyme and substrate react rapidly to form an enzyme-substrate complex (proposed in 1902 by Brown).
3. Only a single substrate and a single enzyme-substrate complex are involved and the enzyme-substrate complex breaks down directly to form free enzyme and product.
4. Enzyme, substrate, and the enzyme-substrate complex are at equilibrium; that is, the rate at which ES dissociates to E + S is much faster than the rate at which ES breaks down to form E + P.
5. The substrate concentration is very much larger than the enzyme concentration so that the formation of an ES complex does not alter the substrate concentration.
6. The overall rate of the reaction is limited by the breakdown of the ES complex to form free enzyme and product.

7. The velocity is measured during the very early stages of the reaction so that the reverse reaction is insignificant.

The assumption that only the early components of the reaction are at equilibrium is called the quasi-equilibrium or rapid equilibrium assumption. The overall reaction was visualized as:



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$

The Henri equation is shown below.

$$\boxed{v = \frac{K[S]}{1 + \frac{[S]}{K_S}}} \qquad \text{(II-1)}$$

where $[S]$ = a fixed substrate concentration
- v = initial velocity (the instantaneous velocity, $d[P]/dt$ or $-d[S]/dt$) at the given substrate concentration. In practice, v can be taken as $\Delta[P]/\Delta t$ or $-\Delta[S]/\Delta t$ provided the appearance of P is linear with time for the duration of the assay and no more than 5% of the original [S] is utilized.
- k_p = rate constant for the breakdown of ES to E + P
- K_S = the dissociation constant of the ES complex $= k_{-1}/k_1 = [E][S]/[ES]$
- K = a constant, characteristic of the particular enzyme preparation $= k_p[E]_t/K_S$, where $[E]_t$ is the total concentration of enzyme, $[E] + [ES]$

Ten years later Michaelis and Menten confirmed Henri's experimental work and presented a slightly modified version of the rate equation.

$$v = \frac{k_p[E]_t[S]}{K_S + [S]} \qquad \text{(II-2)}$$

If $v = k_p[ES]$, then $k_p[E]_t$ can be taken as V_{max}, the limiting maximal velocity that would be observed when all the enzyme is present as ES. This gives the familiar "Michaelis-Menten" equation (II-3). As pointed out by a number of people, it would be appropriate to refer to the equation as the Henri-Michaelis-Menten equation.

$$\boxed{\frac{v}{V_{max}} = \frac{[S]}{K_S + [S]}} \qquad \text{(II-3)}$$

The derivation of the Henri-Michaelis-Menten equation from rapid equilibrium considerations is given below. The general procedure can be used to obtain velocity equations for all rapid equilibrium systems, including those involving multiple ligands.

1. Write the reactions involved in the overall conversion of S to P. For the simple reaction involving one ligand, one catalytic site, and one enzyme-substrate complex, the reactions are:

$$\mathrm{E} + \mathrm{S} \overset{K_S}{\rightleftharpoons} \mathrm{ES} \overset{k_p}{\longrightarrow} \mathrm{E} + \mathrm{P}$$

2. Write the mass balance (conservation) equation expressing the distribution of the total enzyme, $[E]_t$, among the various species. For the simple reaction the equation is:

$$\boxed{[\mathrm{E}]_t = [\mathrm{E}] + [\mathrm{ES}]} \qquad \text{(II-4)}$$

3. Write the velocity-dependence equation. This equation states that v is equal to the concentrations of all product-forming species, each multiplied by its catalytic rate constant. When there is only one product-forming species, ES, the equation is:

$$\boxed{v = k_p[\mathrm{ES}]} \qquad \text{(II-5)}$$

4. Divide the left-hand term of the velocity-dependence equation by $[E]_t$, and the right-hand term by $[E]_t$ expressed as the sum of all the enzyme species:

$$\boxed{\frac{v}{[\mathrm{E}]_t} = \frac{k_p[\mathrm{ES}]}{[\mathrm{E}] + [\mathrm{ES}]}} \qquad \text{(II-6)}$$

5. Express the concentration of each enzyme species in terms of free E. This is accomplished by rearranging the expressions for the various equilibria. For the simple reaction, there is only one equilibrium.

$$K_S = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]}, \qquad \therefore \quad [\mathrm{ES}] = \frac{[\mathrm{S}]}{K_S}[\mathrm{E}]$$

6. Substitute the expressions for each complex, in terms of [E], into equation II-6.

$$\boxed{\frac{v}{[\mathrm{E}]_t} = \frac{k_p \dfrac{[\mathrm{S}]}{K_S}[\mathrm{E}]}{[\mathrm{E}] + \dfrac{[\mathrm{S}]}{K_S}[\mathrm{E}]}} \qquad \text{(II-7)}$$

Or, canceling [E] and designating $k_p[E]_t$ as V_{max}:

$$\boxed{\frac{v}{V_{max}} = \frac{\dfrac{[\mathrm{S}]}{K_S}}{1 + \dfrac{[\mathrm{S}]}{K_S}}} \qquad \text{(II-8)}$$

The proportion of the total enzyme present as any one species is:

$$\frac{[\mathrm{E}]}{[\mathrm{E}]_t} = \frac{[\mathrm{E}]}{[\mathrm{E}] + [\mathrm{ES}]} = \frac{[\mathrm{E}]}{[\mathrm{E}] + \dfrac{[\mathrm{S}]}{K_S}[\mathrm{E}]} = \frac{1}{1 + \dfrac{[\mathrm{S}]}{K_S}}$$

$$\frac{[\mathrm{ES}]}{[\mathrm{E}]_t} = \frac{[\mathrm{ES}]}{[\mathrm{E}] + [\mathrm{ES}]} = \frac{\dfrac{[\mathrm{S}]}{K_S}[\mathrm{E}]}{[\mathrm{E}] + \dfrac{[\mathrm{S}]}{K_S}[\mathrm{E}]} = \frac{\dfrac{[\mathrm{S}]}{K_S}}{1 + \dfrac{[\mathrm{S}]}{K_S}} = Y_S = \frac{v}{V_{max}}$$

The ratio of occupied to total sites, Y_S, is equivalent to v/V_{max}.

Equation II-8 is a useful form of the velocity equation. The numerator contains one term expressing the fact that there is only one product-forming species. The denominator contains two terms expressing the fact that there are a total of two species (free E and one complex). Equation II-8 can be converted to the usual form of the Henri-Michaelis-Menten equation by multiplying the numerator and denominator of the right-hand part by K_S:

$$\boxed{\frac{v}{V_{max}} = \frac{[\mathrm{S}]}{K_S + [\mathrm{S}]}} \qquad \text{(II-9)}$$

The rapid equilibrium treatment described above permits us to express [ES] in terms of [E], [S], and K_S. The derivation gives nothing more than an *equilibrium* expression for the binding of the substrate to the enzyme. We obtain a *kinetic* expression (i.e., a velocity equation) only when we insert the expression for [ES] into the velocity dependence equation, or when we equate $[ES]/[E]_t$ to v/V_{max}. When k_p is of the same order of magnitude as k_{-1} (as is true for many, and very likely most, enzymes), the concentration of ES is no longer fixed solely by the concentrations of E and S and the equilibrium constant, K_S. In this case, the velocity equation must be derived by the more exact steady-state treatment (described below and in more detail in Chapter Nine). Nevertheless, we will make extensive use of the rapid equilibrium treatment for the following reasons. (*a*) The rapid equilibrium treatment is the simplest and most direct technique for deriving velocity equations in the absence of any prior knowledge of the relative magnitudes of the rate constants. The rapid equilibrium treatment permits us to write velocity equations for complex multiligand systems after simple inspection of the equilibria between the various enzyme species. If the experimental data fit the velocity equation, then we have the simplest kinetic mechanism for the system. If the data do not fit, then we can proceed to more complex models and velocity equations. (*b*) For many situations, the rapid equilibrium and steady-state treatments yield the same final velocity equation. That is, the *form* of the velocity equation is the same but the definitions of the constants are not the same. (*c*) The major theories of allosteric enzymes are based on rapid equilibrium assumptions. A steady-state treatment yields velocity equations that are, at present, too complex for practical consideration.

B. GENERAL RULES FOR WRITING VELOCITY EQUATIONS FOR RAPID EQUILIBRIUM SYSTEMS

Before proceeding further, it would be useful to point out the ease with which velocity equations for seemingly complex systems can be obtained if rapid equilibrium conditions prevail. No derivation is really necessary. In fact the velocity equation for any rapid equilibrium system can be written directly from an inspection of the equilibria between enzyme species. We start by writing the velocity dependence equation:

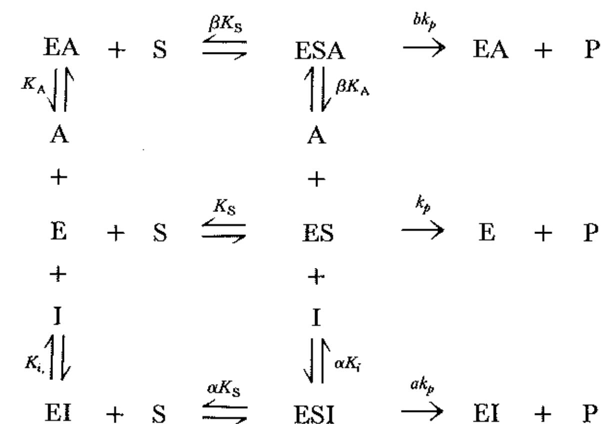
$$v = k_p[ES] + bk_p[ESA] + ak_p[ESI] \qquad \text{and so on}$$

where [ES], [ESA], [ESI], and so on are the concentrations of those species which yield product while k_p, bk_p, ak_p, and such are the respective catalytic

rate constants. Next, both sides of the velocity dependence equation are divided by $[E]_t$, where $[E]_t$ on the right-hand side is expressed as the sum of the concentrations of all species: [E]+[ES]+[EA]+[EI]+[ESA]+[ESI], and so on.

$$\frac{v}{[E]_t} = \frac{k_p[ES] + bk_p[ESA] + ak_p[ESI] + \cdots}{[E]+[ES]+[EA]+[EI]+[ESA]+[ESI]+\cdots} \qquad \text{(II-10)}$$

Now all we need do is express the concentration of each species in terms of [E]. The term for any given complex is composed of a numerator and a denominator. The numerator is the product of the concentrations of all ligands in the complex. The denominator is the product of all dissociation constants between the complex and free E. Suppose I is a mixed-type inhibitor and A is a nonessential activator. (Mixed-type inhibition and nonessential activation are described in Chapters Four and Five, respectively. For the moment, all we need to know is that the equilibria between enzyme species can be represented as shown below.)



The term for [ESA] is $[S][A]/\beta K_S K_A$. The term for [ESI] is $[S][I]/\alpha K_S K_i$, and so on. Substituting into equation II-10, we obtain:

$$\frac{v}{[E]_t} = \frac{k_p\frac{[S]}{K_S} + bk_p\frac{[S][A]}{\beta K_S K_A} + ak_p\frac{[S][I]}{\alpha K_S K_i}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[I]}{K_i} + \frac{[S][A]}{\beta K_S K_A} + \frac{[S][I]}{\alpha K_S K_i}}$$

where the 1 represents free [E]. If $k_p[\mathrm{E}]_t$ is taken as $V_{\max}$, the equation becomes:

$$\frac{v}{V_{\max}} = \frac{\dfrac{[\mathrm{S}]}{K_\mathrm{S}}\left(1+\dfrac{b[\mathrm{A}]}{\beta K_\mathrm{A}}+\dfrac{a[\mathrm{I}]}{\alpha K_i}\right)}{\text{same denominator}}$$

Multiplying numerator and denominator by K_S and factoring:

$$\frac{v}{V_{\max}} = \frac{[\mathrm{S}]\left(1+\dfrac{b[\mathrm{A}]}{\beta K_\mathrm{A}}+\dfrac{a[\mathrm{I}]}{\alpha K_i}\right)}{K_\mathrm{S}\left(1+\dfrac{[\mathrm{A}]}{K_\mathrm{A}}+\dfrac{[\mathrm{I}]}{K_i}\right)+[\mathrm{S}]\left(1+\dfrac{[\mathrm{A}]}{\beta K_\mathrm{A}}+\dfrac{[\mathrm{I}]}{\alpha K_i}\right)}$$

Or, in the usual Henri-Michaelis-Menten form:

$$\boxed{\frac{v}{V_{\max}} = \frac{[\mathrm{S}]}{K_\mathrm{S}\dfrac{\left(1+\dfrac{[\mathrm{A}]}{K_\mathrm{A}}+\dfrac{[\mathrm{I}]}{K_i}\right)}{\left(1+\dfrac{b[\mathrm{A}]}{\beta K_\mathrm{A}}+\dfrac{a[\mathrm{I}]}{\alpha K_i}\right)}+[\mathrm{S}]\dfrac{\left(1+\dfrac{[\mathrm{A}]}{\beta K_\mathrm{A}}+\dfrac{[\mathrm{I}]}{\alpha K_i}\right)}{\left(1+\dfrac{b[\mathrm{A}]}{\beta K_\mathrm{A}}+\dfrac{a[\mathrm{I}]}{\alpha K_i}\right)}}} \qquad \text{(II-11)}$$

Or, in general terms:

$$\boxed{\frac{v}{V_{\max}} = \frac{[\mathrm{S}]}{K_\mathrm{S}(\text{slope factor})+[\mathrm{S}](\text{intercept factor})}} \qquad \text{(II-12)}$$

As shown later, the slope and $1/v$-axis intercept of the reciprocal plot are given by:

$$slope_{1/\mathrm{S}} = \frac{K_\mathrm{S}}{V_{\max}}(\text{slope factor}), \qquad \frac{1}{V_{\max_{\mathrm{app}}}} = \frac{(\text{intercept factor})}{V_{\max}}$$

The $K_{\mathrm{S}_{\mathrm{app}}}$ (apparent Michaelis constant) is given by:

$$K_{\mathrm{S}_{\mathrm{app}}} = K_\mathrm{S}\frac{(\text{slope factor})}{(\text{intercept factor})}$$

C. THE VAN SLYKE EQUATION

In 1914, Van Slyke and co-workers independently derived a general rate equation based on their studies of the urease reaction. These workers assumed that the overall reaction occurred in two irreversible steps.

$$\mathrm{E}+\mathrm{S} \xrightarrow{k_1} \mathrm{ES} \xrightarrow{k_p} \mathrm{E}+\mathrm{P}$$

The rate equation was obtained by assuming that the time required for the overall reaction was the sum of the times for each step. This may be expressed as:

$$t = \frac{1}{k_1[\mathrm{S}]} + \frac{1}{k_p} = \frac{k_p + k_1[\mathrm{S}]}{k_1 k_p[\mathrm{S}]}$$

The units of t might be "minutes per mole of S converted to P per mole of enzyme." The reciprocal is:

$$\frac{1}{t} = \frac{k_1 k_p[\mathrm{S}]}{k_p + k_1[\mathrm{S}]} \qquad \text{moles S} \longrightarrow \text{P/min/mole of enzyme}$$

The overall velocity for a given amount of enzyme is $[\mathrm{E}]_t(1/t)$:

$$v = \frac{[\mathrm{E}]_t k_1 k_p[\mathrm{S}]}{k_p + k_1[\mathrm{S}]}$$

Dividing numerator and denominator by k_1:

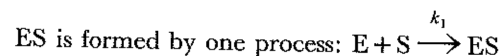
$$\boxed{v = \frac{k_p[\mathrm{E}]_t[\mathrm{S}]}{\dfrac{k_p}{k_1}+[\mathrm{S}]} = \frac{k_p[\mathrm{E}]_t[\mathrm{S}]}{K+[\mathrm{S}]}} \qquad \text{(II-13)}$$

The equation is essentially identical to that derived by Henri and by Michaelis and Menten. Now, however, the constant K in the denominator is the ratio of forward rate constants instead of an equilibrium constant.

D. THE BRIGGS-HALDANE STEADY-STATE APPROACH

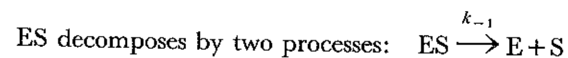
In 1925, Briggs and Haldane derived a general rate equation that did not require the restriction of equilibrium required by the methods of Henri, Michaelis, and Menten, nor the restriction of irreversible reactions required

by the method of Van Slyke. Briggs and Haldane pointed out that the enzyme-substrate complex need not be in equilibrium with free enzyme and substrate, but within a very short time after starting the reaction ES would build up to a near-constant, or "steady-state," level (a concept introduced in 1913 by Bodenstein) (Fig. II-1). After the initial pre-steady-state period, ES would be formed at the same rate at which it decomposed. The steady-state level would be very close to the equilibrium level if the rate of equilibration is very rapid compared to the rate at which ES decomposes to E + P (i.e., if k_p is very small compared to k_{-1}). On the other hand, if k_p is comparable to k_{-1} or larger, then the steady-state level would be lower than the equilibrium level (i.e., ES decomposes to E + P so fast that it never can attain a level that would be in equilibrium with E and S). The rate at which P is formed will be proportional to the steady-state concentration of ES. The steps involved in deriving the general rate equation from steady-state considerations are similar to those described earlier for the derivation from equilibrium considerations. This time, however, the concentration of the product-forming species (ES) is obtained from steady-state equations instead of from equilibrium expressions. The derivation is outlined below.

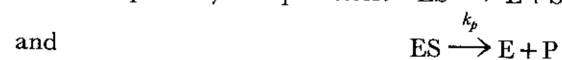


ES is formed by one process: $E + S \xrightarrow{k_1} ES$

(The reverse reaction $E + P \rightarrow ES$ is neglected because during the early stage of the reaction the concentration of product is essentially zero.)



ES decomposes by two processes: $ES \xrightarrow{k_{-1}} E + S$



and $ES \xrightarrow{k_p} E + P$

$$\therefore \quad \text{rate of ES formation} = \left(+\frac{d[\mathrm{ES}]}{dt}\right) = k_1[\mathrm{E}][\mathrm{S}]$$

$$\text{rate of ES decomposition} = \left(-\frac{d[\mathrm{ES}]}{dt}\right) = k_{-1}[\mathrm{ES}] + k_p[\mathrm{ES}] = (k_{-1} + k_p)[\mathrm{ES}]$$

$$\text{at steady-state:} \quad \left(+\frac{d[\mathrm{ES}]}{dt}\right) = \left(-\frac{d[\mathrm{ES}]}{dt}\right) \quad \text{or} \quad \frac{d[\mathrm{ES}]}{dt} = 0$$

$$k_1[\mathrm{E}][\mathrm{S}] = (k_{-1} + k_p)[\mathrm{ES}]$$

Rearranging:

$$[\mathrm{ES}] = \frac{k_1[\mathrm{S}]}{(k_{-1} + k_p)}[\mathrm{E}] \qquad \text{(II-14)}$$

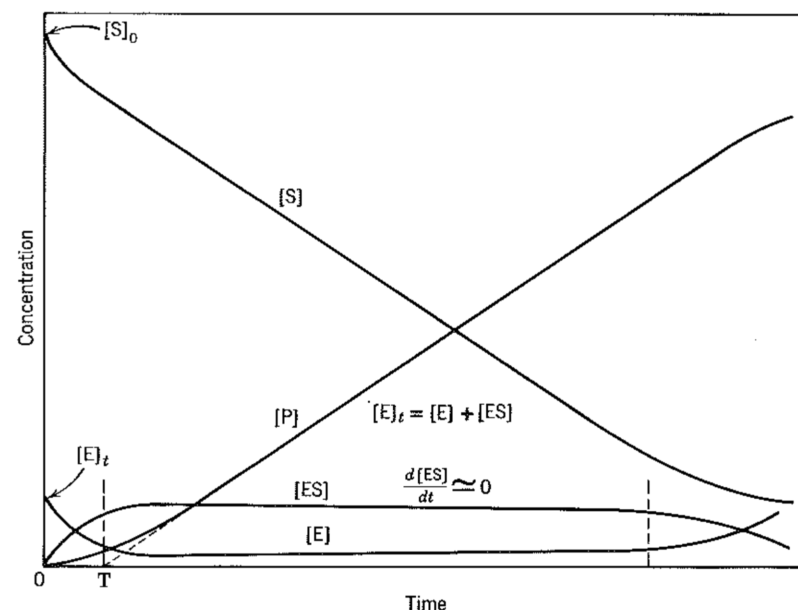


Fig. II-1. Progress curve for a catalyzed reaction where the initial substrate concentration, $[\mathrm{S}]_0$, is significantly greater than the initial enzyme concentration, $[\mathrm{E}]_t$. As the ratio of $[\mathrm{S}]_0/[\mathrm{E}]_t$ increases, the steady-state region accounts for an increasing fraction of the total reaction time.

Dividing both sides of the velocity-dependence equation by $[\mathrm{E}]_t$:

$$\frac{v}{[\mathrm{E}]_t} = \frac{k_p[\mathrm{ES}]}{[\mathrm{E}] + [\mathrm{ES}]}$$

Rearranging and substituting for [ES]:

$$\frac{v}{k_p[\mathrm{E}]_t} = \frac{\dfrac{k_1[\mathrm{S}]}{(k_{-1} + k_p)}[\mathrm{E}]}{[\mathrm{E}] + \dfrac{k_1[\mathrm{S}]}{(k_{-1} + k_p)}[\mathrm{E}]}$$

Canceling [E] and substituting $V_{\max}$ for $k_p[\mathrm{E}]_t$:

$$\frac{v}{V_{\max}} = \frac{\dfrac{k_1[\mathrm{S}]}{(k_{-1} + k_p)}}{1 + \dfrac{k_1[\mathrm{S}]}{(k_{-1} + k_p)}}$$

Grouping the rate constants as K_m, the Michaelis constant:

$$\boxed{\frac{v}{V_{\max}} = \frac{\dfrac{[S]}{K_m}}{1+\dfrac{[S]}{K_m}}} \quad \text{or} \quad \boxed{\frac{v}{V_{\max}} = \frac{[S]}{K_m+[S]}} \qquad \text{(II-15)}$$

where

$$K_m = \frac{k_{-1}+k_p}{k_1}$$

Equation II-14 can be rearranged to:

$$\frac{[E][S]}{[ES]} = \frac{k_{-1}+k_p}{k_1} = K_m \qquad \text{(II-15a)}$$

Thus K_m is a dynamic or pseudoequilibrium constant expressing the relationship between the actual steady-state concentrations rather than equilibrium concentrations.

Note that it is possible to write four differential equations describing the overall reaction sequence. Each equation states that the rate at which the concentration of a given component changes equals the difference between the rate at which it is formed and the rate at which it is utilized.

$$\frac{d[E]}{dt} = (k_{-1}+k_p)[ES] - k_1[E][S]$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1}+k_p)[ES]$$

$$\frac{d[S]}{dt} = k_{-1}[ES] - k_1[E][S]$$

$$\frac{d[P]}{dt} = k_p[ES]$$

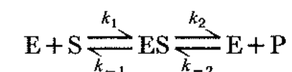
Together with the mass balance equation $[E]_t = [E]+[ES]$ we have five equations and four unknowns. Yet it is impossible to derive a general equation expressing v (i.e., $-d[S]/dt$ or $d[P]/dt$) as a function of [S] or an

integrated equation expressing [S] or [P] as a function of time *unless* we assume steady-state conditions (i.e., $d[ES]/dt = 0$). The five equations can be solved numerically to yield [E], [S], [ES], and [P] at any time if $[S]_0$, $[E]_t$, k_1, k_{-1}, and k_p are known. (A computer would greatly facilitate the calculations.) The results would resemble those shown in Figure II-1. As the ratio of $[S]_0/[E]_t$ increases, the steady state assumption becomes more valid. That is, as $[S]_0/[E]_t$ increases, the time interval *before* $d[ES]/dt \cong 0$ decreases and the extent of the reaction during which $d[ES]/dt \cong 0$ increases. Because (a) most *in vitro* enzyme studies are conducted with "catalytic" concentrations of enzyme, and (b) v is taken as the velocity observed when only a small fraction of $[S]_0$ is utilized, the steady-state assumption is quite valid.

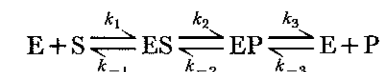
Equation II-15 is identical to that derived by Henri, Michaelis and Menten, and Van Slyke, but now the constant has a different meaning. We see that the restrictive assumptions of Henri, Michaelis and Menten, and Van Slyke are special cases of the Briggs-Haldane steady-state treatment. When k_p is very small compared to k_{-1}, $K_m \cong k_{-1}/k_1 \cong K_S$; that is, K_m is essentially the dissociation constant of the enzyme-substrate complex. When k_{-1} is $\ll k_p$, $K_m \cong k_p/k_1$; that is, K_m is a kinetic constant. The rate constants comprising K_m are not restricted to k_1, k_{-1}, and k_p. It may appear so because in deriving the velocity equation it was assumed that there was only a single central complex, ES, that decomposed directly to free E+P.

E. REVERSIBLE REACTIONS—EFFECT OF PRODUCT ON FORWARD VELOCITY

Strictly speaking, all enzyme-catalyzed reactions are reversible. The simplest representation of the overall reaction is:



A more realistic sequence involves two central complexes:



Under the usual assay conditions, velocities are measured very early in the reaction before the product concentration has increased to a significant level. We can calculate the initial velocity for the reaction in each direction from

the appropriate Henri-Michaelis-Menten equations:

$$v_f = \frac{V_{\max_f}[\mathrm{S}]}{K_{m_\mathrm{S}} + [\mathrm{S}]} \qquad \text{in the absence of P}$$

$$v_r = \frac{V_{\max_r}[\mathrm{P}]}{K_{m_\mathrm{P}} + [\mathrm{P}]} \qquad \text{in the absence of S}$$

It would be instructive to examine the effect of product on the initial forward velocity. For example, suppose we have a solution containing a certain concentration of S and a certain concentration of P. In the absence of an appropriate enzyme, the reaction does not occur at a measurable rate. Now we add an enzyme catalyzing the reversible reaction $\mathrm{S} \rightleftharpoons \mathrm{P}$. In which direction and at what rate will the reaction progress? The direction of the reaction will depend on the ratio of [P]/[S] relative to the equilibrium ratio. If $K_{\mathrm{eq}} = 1$, [P] = 5 mM, and [S] = 1 mM, the reaction will proceed in the direction P→S and approach an equilibrium where [P] = 3 mM and [S] = 3 mM. The initial rate at which the reaction starts toward equilibrium *cannot* be calculated simply by taking the difference between v_f and v_r as given by their respective Henri-Michaelis-Menten expressions. These expressions were derived assuming [P] = 0 or [S] = 0. To calculate the initial rate when either [P] or [S] are not zero, we must derive a different general rate equation taking into account the reverse reactions. If we assume only one central complex the *net* velocity in the forward direction is given by:

$$v_{\mathrm{net}} = k_2[\mathrm{ES}] - k_{-2}[\mathrm{E}][\mathrm{P}]$$

The steady-state relationships are:

$$\left(+\frac{d[\mathrm{ES}]}{dt}\right) = \left(-\frac{d[\mathrm{ES}]}{dt}\right) \qquad \text{or} \qquad \frac{d[\mathrm{ES}]}{dt} = 0$$

$$\left(+\frac{d[\mathrm{ES}]}{dt}\right) = k_1[\mathrm{E}][\mathrm{S}] + k_{-2}[\mathrm{E}][\mathrm{P}]$$

$$\left(-\frac{d[\mathrm{ES}]}{dt}\right) = k_2[\mathrm{ES}] + k_{-1}[\mathrm{ES}]$$

$$k_1[\mathrm{E}][\mathrm{S}] + k_{-2}[\mathrm{E}][\mathrm{P}] = (k_2 + k_{-1})[\mathrm{ES}]$$

$$[\mathrm{ES}] = \frac{k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]}{k_2 + k_{-1}}[\mathrm{E}]$$

Dividing the velocity-dependence equation by $[\mathrm{E}]_t$, where $[\mathrm{E}]_t = [\mathrm{E}] + [\mathrm{ES}]$:

$$\frac{v_{\mathrm{net}}}{[\mathrm{E}]_t} = \frac{k_2[\mathrm{ES}] - k_{-2}[\mathrm{E}][\mathrm{P}]}{[\mathrm{E}] + [\mathrm{ES}]}$$

Substituting for [ES]:

$$\frac{v_{\mathrm{net}}}{[\mathrm{E}]_t} = \frac{k_2\left(\dfrac{k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]}{k_2 + k_{-1}}\right)[\mathrm{E}] - k_{-2}[\mathrm{E}][\mathrm{P}]}{[\mathrm{E}] + \left(\dfrac{k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]}{k_2 + k_{-1}}\right)[\mathrm{E}]}$$

$$= \frac{\dfrac{k_2k_1[\mathrm{S}]}{k_2 + k_{-1}} + \dfrac{k_2k_{-2}[\mathrm{P}]}{k_2 + k_{-1}} - \dfrac{k_{-2}(k_2 + k_{-1})[\mathrm{P}]}{k_2 + k_{-1}}}{1 + \dfrac{k_1[\mathrm{S}]}{k_2 + k_{-1}} + \dfrac{k_{-2}[\mathrm{P}]}{k_2 + k_{-1}}}$$

$$v_{\mathrm{net}} = \frac{\dfrac{k_2k_1[\mathrm{S}][\mathrm{E}]_t}{k_2 + k_{-1}} - \dfrac{k_{-2}k_{-1}[\mathrm{P}][\mathrm{E}]_t}{k_2 + k_{-1}}}{1 + \dfrac{k_1[\mathrm{S}]}{k_2 + k_{-1}} + \dfrac{k_{-2}[\mathrm{P}]}{k_2 + k_{-1}}}$$

$$\boxed{v_{\mathrm{net}} = \frac{V_{\max_f}\dfrac{[\mathrm{S}]}{K_{m_\mathrm{S}}} - V_{\max_r}\dfrac{[\mathrm{P}]}{K_{m_\mathrm{P}}}}{1 + \dfrac{[\mathrm{S}]}{K_{m_\mathrm{S}}} + \dfrac{[\mathrm{P}]}{K_{m_\mathrm{P}}}}} \qquad \text{(II-16)}$$

where:

$$k_2[\mathrm{E}]_t = V_{\max_f}, \qquad k_{-1}[\mathrm{E}]_t = V_{\max_r}$$

$$\frac{k_2 + k_{-1}}{k_1} = K_{m_\mathrm{S}}, \qquad \frac{k_2 + k_{-1}}{k_{-2}} = K_{m_\mathrm{P}}$$

Solving for the individual rate constants:

$$k_1 = \frac{V_{\max_f} + V_{\max_r}}{K_{m_\mathrm{S}}[\mathrm{E}]_t}, \qquad k_{-1} = \frac{V_{\max_r}}{[\mathrm{E}]_t}$$

$$k_2 = \frac{V_{\max_f}}{[\mathrm{E}]_t}, \qquad k_{-2} = \frac{V_{\max_f} + V_{\max_r}}{K_{m_\mathrm{P}}[\mathrm{E}]_t}$$

The $[E]_t$ in the equations above represents the concentration of catalytic sites. If the enzyme contains one catalytic site per molecule, $[E]_t$ is the molar concentration of enzyme. As shown later, an enzyme with multiple identical and independent catalytic sites is indistinguishable kinetically from an enzyme with only one site. The constants k_1 and k_{-2} are second-order rate constants:

$$k_1 = \frac{V_{\max_f} + V_{\max_r}}{K_{m_S}[E]_t} = \frac{\dfrac{\text{moles}}{1 \times \text{min}} + \dfrac{\text{moles}}{1 \times \text{min}}}{\left(\dfrac{\text{moles}}{1}\right)\left(\dfrac{\text{moles}}{1}\right)} = M^{-1} \times \text{min}^{-1}$$

At a fixed enzyme concentration, the reaction $E + S \rightleftharpoons ES$ is pseudo-first-order with an observed first-order rate constant equal to $k_1[E]$. The constants k_{-1} and k_2 are true first-order rate constants:

$$k_2 = \frac{V_{\max_f}}{[E]_t} = \frac{M \times \text{min}^{-1}}{M} = \text{min}^{-1}$$

At a fixed enzyme concentration, $k_2[E]_t$ is a pseudo-zero-order constant:

$$k_2[E]_t = V_{\max_f} = M \times \text{min}^{-1}$$

The constant, k_2 (or k_p or k_{cat}), is called the *turnover number* (or *molecular activity* or *catalytic rate constant*) and represents the maximum velocity per mole of enzyme (or per mole of catalytic site if $[E]_t$ is expressed in concentration of catalytic sites).

$$k_2 = \frac{V_{\max_f}}{[E]_t} = \text{moles of product formed per minute per mole of enzyme}$$

The reciprocal of k_2 represents the time required to complete one catalytic cycle. The k_1 values are usually in the range 10^7 to 10^{10} $M^{-1} \times \text{min}^{-1}$. The maximum value is about $10^{11} M^{-1} \times \text{min}^{-1}$, limited by the rate of diffusion of a small molecule in aqueous solution to the active site of the enzyme. The k_{-1} values are usually 10^2 to 10^6 min^{-1}, while k_2 values vary from 50 to 10^7 min^{-1}. The K_m values are usually in the range 10^{-6} to 10^{-2} M.

The complete velocity equation for the more realistic reaction sequence involving two central complexes is easily derived for rapid equilibrium conditions, as shown below.

$$\frac{v_{net}}{[E]_t} = \frac{k_2[ES] - k_{-2}[EP]}{[E] + [ES] + [EP]}$$

$$v_{net} = \frac{k_2[E]_t\dfrac{[S]}{K_S} - k_{-2}[E]_t\dfrac{[P]}{K_P}}{1 + \dfrac{[S]}{K_S} + \dfrac{[P]}{K_P}}$$

$$\boxed{v_{net} = \frac{V_{\max_f}\dfrac{[S]}{K_S} - V_{\max_r}\dfrac{[P]}{K_P}}{1 + \dfrac{[S]}{K_S} + \dfrac{[P]}{K_P}}} \qquad \text{(II-17)}$$

A steady-state treatment (Chapter Nine) yields:

$$v_{net} = \frac{[E]_t(k_1k_2k_3[S] - k_{-1}k_{-2}k_{-3}[P])}{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3 + k_1(k_2 + k_{-2} + k_3)[S] + k_{-3}(k_{-1} + k_2 + k_{-2})[P]}$$

The equation above may be further modified by grouping rate constants into K_m and $V_{\max}$ values. The final equation has the same form as that derived for rapid equilibrium conditions and for steady-state conditions assuming only one central complex. Only the definitions of K_m and $V_{\max}$ in terms of rate constants change.

$$K_{m_S} = \frac{k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3}{k_1(k_2 + k_{-2} + k_3)}, \qquad K_{m_P} = \frac{k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3}{k_{-3}(k_{-1} + k_2 + k_{-2})}$$

$$V_{\max_f} = \frac{k_2k_3[E]_t}{k_2 + k_{-2} + k_3}, \qquad V_{\max_r} = \frac{k_{-1}k_{-2}[E]_t}{k_{-1} + k_2 + k_{-2}}$$

It is obvious that the physical significance of K_m cannot be stated with any certainty in the absence of other data concerning the relative magnitudes of the various rate constants. Nevertheless, K_m represents a valuable constant that relates the velocity of an enzyme-catalyzed reaction to the substrate concentration. Inspection of the Henri-Michaelis-Menten equation shows that K_m is numerically equivalent to the substrate concentration that yields

half-maximal velocity:

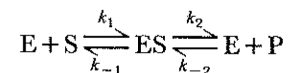
$$v=\frac{[S]}{K_m+[S]}V_{max} \quad \therefore \text{ when } \quad [S]=K_m:$$

$$v=\frac{K_m}{K_m+K_m}V_{max}=\tfrac{1}{2}V_{max}$$

The numerical value of K_m is of interest for several reasons. (*a*) The K_m establishes an approximate value for the intracellular level of the substrate. It is unlikely that this level would be significantly greater or significantly lower than K_m. If $[S]_{intracell} \ll K_m$, v would be very sensitive to changes in [S], but most of the catalytic potential of the enzyme would be wasted, since v would be $\ll V_{max}$. There is also no physiological sense in maintaining $[S] \gg K_m$, since v cannot exceed V_{max}, and the difference between v at $[S]=K_m$ and $[S]=1000K_m$ is only twofold. Also at $[S] \gg K_m$, v becomes insensitive to small changes in [S]. (*b*) Since K_m is a constant for a given enzyme, its numerical value provides a means of comparing enzymes from different organisms or from different tissues of the same organism, or from the same tissue at different stages of development. In this way, we might determine whether enzyme A is identical to enzyme B, or whether they are different proteins that catalyze the same reaction. (*c*) A ligand-induced change in the effective value of K_m is one mode of regulating the activity of an enzyme. If K_m determined *in vitro* seems "unphysiologically" high then we might search for activators that function *in vivo* to lower the effective K_m. By measuring the effects of different compounds on K_m we might identify physiologically important inhibitors as well. (*d*) If we know K_m, we can adjust the assay conditions so that $[S] \gg K_m$, and thereby determine V_{max}, which is a measure of $[E]_t$.

F. HALDANE RELATIONSHIP BETWEEN KINETIC CONSTANTS AND EQUILIBRIUM CONSTANT

The constants K_m and V_{max} were derived in terms of the various rate constants of the overall reaction. The equilibrium constant for the overall reaction is composed of the same rate constants. Consequently, it should be possible to express K_{eq} in terms of K_m and V_{max}. For example, consider the simple two-step reaction shown below.



$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E+P$$

The overall equilibrium constant for the reaction reading left to right is the product of the equilibrium constants for the individual steps, which may be expressed in terms of the rate constants:

$$K_{eq}=\frac{[P]_{eq}}{[S]_{eq}}=K_1K_2=\frac{k_1k_2}{k_{-1}k_{-2}}$$

We can express this grouping of the rate constants in terms of K_m and V_{max} values as shown below.

$$\frac{V_{max_f}}{K_{m_S}}=\frac{k_1k_2[E]_t}{k_2+k_{-1}}, \qquad \frac{V_{max_r}}{K_{m_P}}=\frac{k_{-2}k_{-1}[E]_t}{k_2+k_{-1}}$$

Now dividing one ratio by the other:

$$\frac{V_{max_f}/K_{m_S}}{V_{max_r}/K_{m_P}}=\frac{(k_1k_2[E]_t)(k_2+k_{-1})}{(k_2+k_{-1})(k_{-2}k_{-1}[E]_t)}$$

$$\boxed{\frac{V_{max_f}K_{m_P}}{V_{max_r}K_{m_S}}=\frac{k_1k_2}{k_{-1}k_{-2}}=\frac{[P]_{eq}}{[S]_{eq}}=K_{eq}} \tag{II-18}$$

The relationship between K_{eq} and the K_m and V_{max} values is known as the Haldane equation. The Haldane equation can be obtained directly from the equation for v_{net}.

$$v_{net}=\frac{V_{max_f}\dfrac{[S]}{K_{m_S}}-V_{max_r}\dfrac{[P]}{K_{m_P}}}{1+\dfrac{[S]}{K_{m_S}}+\dfrac{[P]}{K_{m_P}}} \tag{II-19}$$

At equilibrium, $v_{net}=0$. Thus the numerator of equation II-19 must equal zero, or:

$$\frac{V_{max_f}[S]_{eq}}{K_{m_S}}=\frac{V_{max_r}[P]_{eq}}{K_{m_P}} \quad \text{or} \quad \frac{V_{max_f}K_{m_P}}{V_{max_r}K_{m_S}}=\frac{[P]_{eq}}{[S]_{eq}}=K_{eq}$$

Substituting $V_{\max_f}/K_{m_S}K_{eq}$ for $V_{\max_r}/K_{m_P}$ in equation II-19 yields equation II-20 for v_{net}:

$$\boxed{v_{net}=\frac{V_{\max_f}\left([S]-\dfrac{[P]}{K_{eq}}\right)}{K_{m_S}\left(1+\dfrac{[P]}{K_{m_P}}\right)+[S]}} \qquad \text{where} \quad \frac{[P]}{K_{eq}}=[S]_{eq} \tag{II-20}$$

Equation II-20 can be written as:

$$v_{net}=\frac{V_{\max_f}\Delta S}{K_{m_S}\left(1+\dfrac{[P]}{K_{m_P}}\right)+\dfrac{[P]}{K_{eq}}+\Delta S} \qquad \text{where} \quad \Delta S=[S]-\frac{[P]}{K_{eq}} \tag{II-21}$$

In place of the usual [S] in the numerator, we have the *difference* between [S] and the equilibrium value of [S]. The K_{m_S} term in the denominator is modified in a manner consistent with the product acting as a competitive inhibitor with respect to the substrate. In other words, the initial net velocity depends on the displacement of the system from equilibrium, (that is, the thermodynamic driving force) and the amount of enzyme tied up with product. A more detailed account of competitive inhibition is given in a later section. The effect of a fixed [P] on the [S]-dependence of initial net velocity is shown in Figure II-2. We see that at any substrate concentration below the equilibrium value of 5 mM the net velocity is negative, that is, in the direction of P→S. When [S] = 5 mM the reaction is at equilibrium and $v=0$.

The net velocity of S→P in the presence of preexisting P is always less than the initial velocity at the same [S] in the absence of preexisting P. There are two reasons for the decreased rate: (*a*) at any time, some of the P is being converted back to S, and (*b*) at any time, some of the enzyme is combined with P so that less enzyme is available for combination with S. Reason *a* is expressed by the $([S]-[P]/K_{eq})$ factor in the numerator of the velocity equation, which makes it seem as if less substrate is available. Reason *b* is expressed by the $(1+[P]/K_{m_P})$ factor in the denominator, which increases the apparent K_m value for S. The net rate (equation II-19) can be expressed as:

$$v_{net}=\frac{V_{\max_f}[S]}{K_{m_S}\left(1+\dfrac{[P]}{K_{m_P}}\right)+[S]}-\frac{V_{\max_r}[P]}{K_{m_P}\left(1+\dfrac{[S]}{K_{m_S}}\right)+[P]}$$
$$=v_f-v_r \tag{II-22}$$

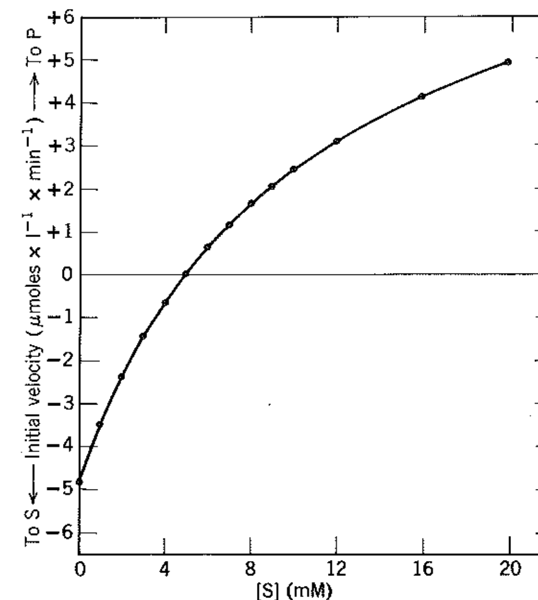


Fig. II-2. Velocity curve in the presence of product. [P] = 5 mM, $K_{eq}=1.0$, $K_{m_S}=0.4$ mM, $K_{m_P}=0.2$ mM, $V_{\max_f}=10$ μmoles × l^{-1} × $\min^{-1}$, $V_{\max_r}=5$ μmoles × l^{-1} × $\min^{-1}$.

The absolute rates in either direction (v_f or v_r) can be measured by using radioactively labeled S or P. For example, suppose we have a solution containing 3 mM S, 3 mM P, and an enzyme that catalyzes the reversible reaction S⇌P. Assume that $K_{eq}=1$ so that the reaction is at equilibrium. If the concentrations of S and P are determined by chemical means, no change in either will be detected with time (i.e., $v_{net}=0$). However, the equilibrium is a dynamic one. The v_{net} equals zero because $v_f=v_r$. If S is made radioactive by introducing a small amount of high specific activity S (such that the concentration of S is unchanged) then v_f can be measured as the initial rate at which radioactivity appears in P (see Chapter Ten).

G. SPECIFIC (OR RELATIVE OR REDUCED) SUBSTRATE CONCENTRATION AND VELOCITY

Strictly speaking, the initial velocity of an enzyme-catalyzed reaction depends not on the substrate concentration, but rather on the ratio of [S] to K_m. This ratio, $[S]/K_m$, has been called "specific substrate concentration," "reduced substrate concentration," "relative substrate concentration," or "normalized substrate concentration" and denoted [S′], α, or σ. The Henri-

Michaelis-Menten equation may be expressed in terms of [S′].

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_m}}{1+\frac{[S]}{K_m}} = \frac{[S']}{1+[S']}$$

When $[S']=1$, $v=\frac{1}{2}V_{max}$. The initial velocity at any given [S] may also be expressed as a fraction of V_{max}, that is, in terms of "specific velocity" or "relative velocity," v' or ϕ, giving:

$$\boxed{v' = \frac{[S']}{1+[S']}} \quad \text{(II-23)}$$

The use of "specific" or "relative" values provides a way of normalizing data for a variety of enzymes. For example, when $[S']=6$, $v'=0.857$, or in other words, when the substrate concentration is six times the K_m value, the initial velocity is always $\frac{6}{7}$ or 85.7% of V_{max}, regardless of the actual values of K_m or V_{max}. Equation II-23 may be rearranged to solve for the specific substrate concentration required for any fraction of V_{max}:

$$\boxed{[S'] = \frac{v'}{1-v'}} \quad \text{(II-24)}$$

H. VELOCITY VERSUS SUBSTRATE CONCENTRATION CURVE

The velocity equation can be written as $(V_{max}-v)(K_m+[S])=K_mV_{max}$ or $(a-y)(b+x)=$ a constant. This equation describes a right rectangular hyperbola with limits of V_{max} and $-K_m$. The curvature is fixed regardless of the values of K_m and V_{max}. Consequently, the ratio of substrate concentrations for any two fractions of V_{max} is constant for all enzymes that obey Henri-Michaelis-Menten kinetics. For example, the ratio of substrate required for 90% of V_{max}, $[S]_{0.9}$, to the substrate required for 10% of V_{max}, $[S]_{0.1}$, is always 81 as shown below and illustrated in Figure II-3.

When $v=0.9V_{max}$:

$$[S']_{0.9} = \frac{v'}{1-v'} = \frac{0.9}{1-0.9} = \frac{0.9}{0.1} = 9$$

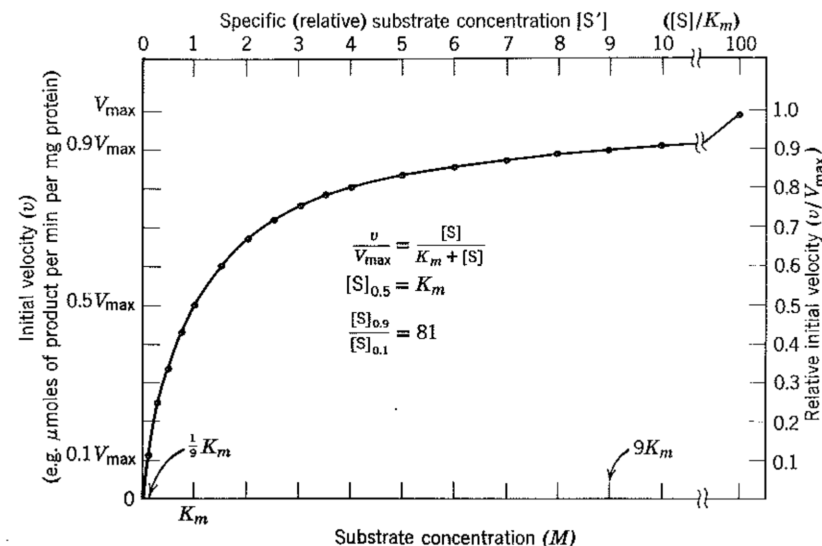


Fig. II-3. The curvature of the v versus [S] plot is constant; $[S]_{0.9}/[S]_{0.1}$ always equals 81 regardless of the absolute values of K_m and V_{max}.

When $v=0.1V_{max}$:

$$[S']_{0.1} = \frac{v'}{1-v'} = \frac{0.1}{1-0.1} = \frac{0.1}{0.9} = 0.111$$

$$\therefore \quad \frac{[S]_{0.9}}{[S]_{0.1}} = \frac{9}{0.111} \quad \text{or} \quad \boxed{\frac{[S]_{0.9}}{[S]_{0.1}} = 81} \quad \text{(II-25)}$$

I. REACTION ORDER

If we examine the v versus [S] curve, we find three distinct regions where the velocity responds in a characteristic way to increasing [S] (Fig. II-4*a*). At very low substrate concentrations (e.g., $[S]<0.01K_m$), the v versus [S] curve is essentially linear; that is, the velocity (for all practical purposes) is directly proportional to the substrate concentration (Fig. II-4*b*). This is the region of *first-order kinetics*. At very high substrate concentrations (e.g., $[S]>100K_m$), the velocity is essentially independent of the substrate concentration. This is the region of *zero-order kinetics* (Fig. II-4*c*). At intermediate substrate concentrations, the relationship between v and [S] follows neither first-order nor

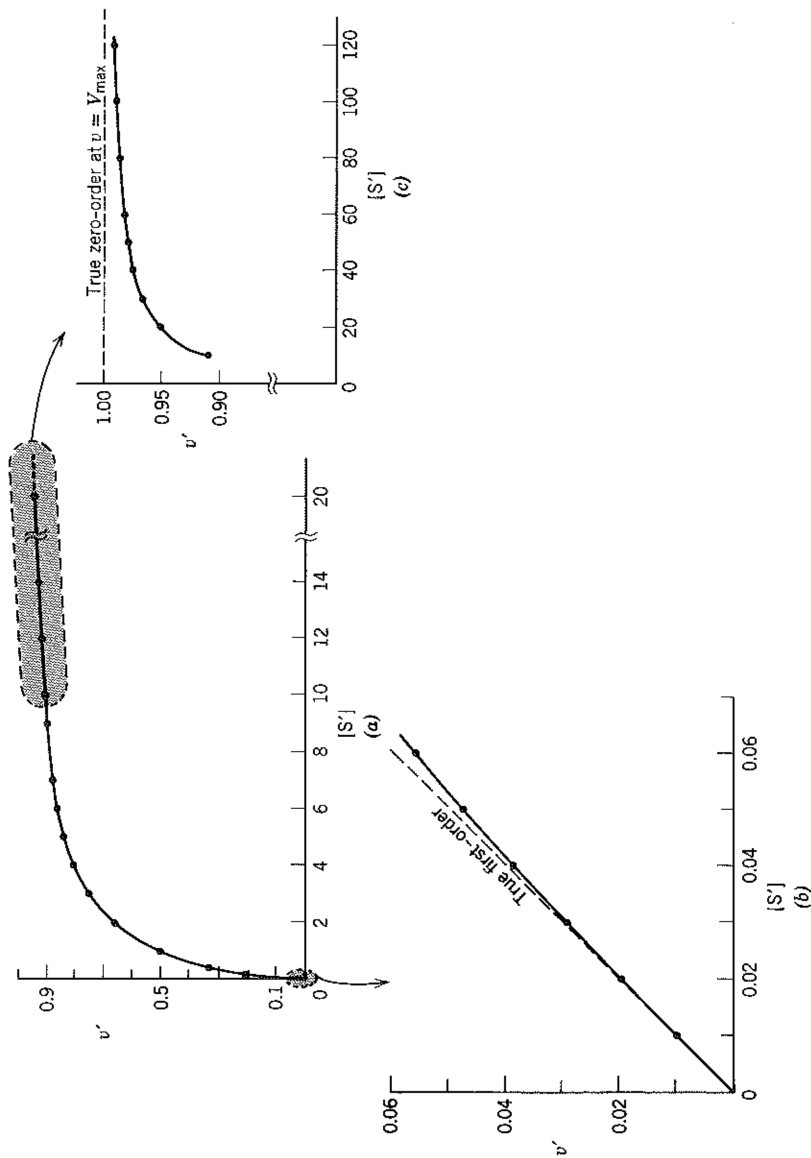


Fig. II-4. (*a*) v versus [S] plot over a wide range of [S]. (*b*) v versus [S] plot in a narrow range where $[S] \ll K_m$. (*c*) v versus [S] plot in a range where $[S] > K_m$.

zero-order kinetics. The characteristics of the first-order and zero-order regions are described below.

First-Order Kinetics

The linear relationship between v and [S] when $[S] \ll K_m$ can be derived from the Henri-Michaelis-Menten equation.

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

When $[S] \ll K_m$, the [S] in the denominator may be ignored and the equation reduces to:

$$v = \frac{V_{max}}{K_m}[S] \qquad \text{or} \qquad v = k[S] \qquad \text{(II-26)}$$

where k is a first-order rate constant equivalent to V_{max}/K_m. The units of k are min^{-1} if v is expressed as $\text{moles} \times \text{l}^{-1} \times \text{min}^{-1}$ and K_m is expressed as $\text{moles} \times \text{l}^{-1}$:

$$k = \frac{V_{max}}{K_m} = \frac{\text{moles} \times \text{l}^{-1} \times \text{min}^{-1}}{\text{moles} \times \text{l}^{-1}} = \text{min}^{-1}$$

In terms of the rate constants of the individual steps, $k = k_1 k_p [E]_t/(k_p + k_{-1})$. Equation II-26 expresses the fact that when [S] is very small, the absolute velocity decreases from moment to moment as [S] decreases (Fig. II-5*a*). However, at any given moment, a constant *fraction* of the substrate present undergoes conversion to product:

$-\frac{d[S]}{dt} =$	$v =$	k	[S]
The amount of S used up per small increment of time...	that is, the velocity...	is some constant fraction...	of the substrate present at that time

Thus the physical significance of the first-order rate constant is that it approximates the fraction of the substrate present that is converted to product per unit of time. For example, if $k = 0.02\ \text{min}^{-1}$, then approximately 2% of the substrate present at any time is converted to product in a minute. A $k > 1\ \text{min}^{-1}$ means that more than 100% of the substrate present at

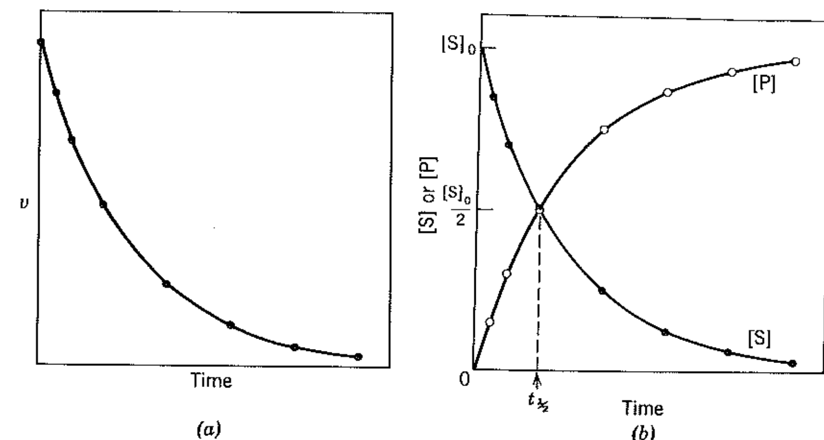


Fig. II-5. First-order region of the velocity curve. (*a*) v decreases continually with time. (*b*) The appearance of P and the disappearance of S are not linear with time.

zero-time could be utilized in a minute *if* v remained constant for a minute. It may be more meaningful to express k in units that yield numerical values less than unity. For example, if $k = 2.3\ \text{min}^{-1}$:

$$k = \frac{2.3\ \text{min}^{-1}}{60\ \text{sec} \times \text{min}^{-1}} = 0.0383\ \text{sec}^{-1}$$

Thus $k = 2.3\ \text{min}^{-1}$ means that approximately 3.83% of [S] is utilized per second.

Because v decreases with time in the first-order region, the plots of [S] versus time and [P] versus time are curved (Fig. II-5*b*). We can determine the amount of substrate utilized or product formed during any given time interval by using the integrated first-order rate equation:

$$v = -\frac{d[\text{S}]}{dt} = k[\text{S}] \quad \text{or} \quad -\frac{d[\text{S}]}{[\text{S}]} = k\,dt$$

Integrating between $[\text{S}] = 0$ at $t = 0$ and [S] at any other time, t, we obtain:

$$-\int_{[\text{S}]_0}^{[\text{S}]} \frac{d[\text{S}]}{[\text{S}]} = k\int_0^t dt$$

$$\boxed{2.3\log\frac{[\text{S}]_0}{[\text{S}]} = kt} \quad \text{or} \quad \boxed{[\text{S}] = [\text{S}]_0 e^{-kt}} \qquad \text{(II-27)}$$

Equation II-27 may be rearranged to:

$$\boxed{\log[\text{S}] = -\frac{k}{2.3}t + \log[\text{S}]_0} \qquad \text{(II-28)}$$

Thus a plot of log [S] versus t is linear with a slope of $-k/2.3$ and an intercept of log $[\text{S}]_0$ on the log [S]-axis (Fig. II-6). When $[\text{S}] = \frac{1}{2}[\text{S}]_0$, $t =$ the "halflife," $t_{1/2}$, the time required to convert half the substrate originally present to product. The $t_{1/2}$ is constant for first-order reactions and is related to k as shown below.

$$2.3\log\frac{1}{0.5} = kt_{1/2} \qquad \therefore \frac{0.693}{k} = t_{1/2} \qquad \text{(II-29)}$$

Zero-Order Kinetics

When $[\text{S}] \gg K_m$, the K_m in the denominator of the Henri-Michaelis-Menten equation may be ignored and the equation simplifies as shown below.

$$v = \frac{V_{\max}[\text{S}]}{K_m + [\text{S}]} \xrightarrow{[\text{S}] \gg K_m} \frac{V_{\max}[\text{S}]}{[\text{S}]} \quad \text{or} \quad v = V_{\max}$$

$$\therefore \boxed{[\text{P}] = [\text{S}]_0 - [\text{S}] = V_{\max}t} \qquad \text{(II-30)}$$

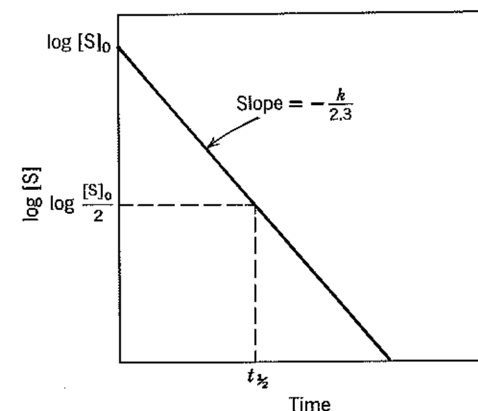


Fig. II-6. Semilog plot of the integrated first-order velocity equation.

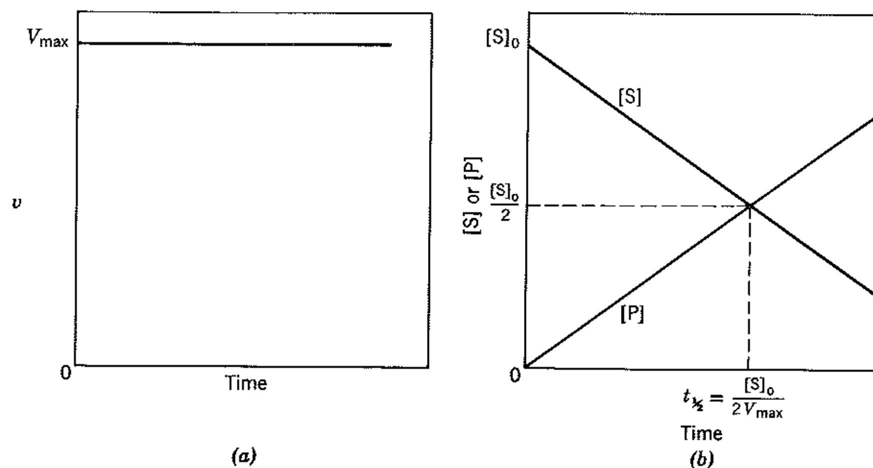


Fig. II-7. Zero-order region of the velocity curve. (*a*) The velocity is constant over time. (*b*) P appears and S disappears linearly with time.

For all practical purposes, the velocity is constant and independent of [S] (Fig. II-7*a*). Plots of [S] versus time and [P] versus time are linear (Fig. II-7*b*).

J. GRAPHICAL DETERMINATION OF K_m AND V_{max}

Because the v versus [S] curve is a hyperbola, it is extremely difficult to determine V_{max} and K_m. An early attempt to remedy the situation involved plotting v versus log [S] as shown in Figure II-8. This plot is based on the rearrangement of the Henri-Michaelis-Menten equation as shown below:

$$\frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}$$

Inverting:

$$\frac{V_{max}}{v} = \frac{K_m + [S]}{[S]} = \frac{K_m}{[S]} + 1$$

$$\frac{V_{max}}{v} - 1 = \frac{K_m}{[S]}$$

$$\boxed{\log\left(\frac{V_{max}}{v} - 1\right) = \log K_m - \log [S]} \qquad \text{(II-31)}$$

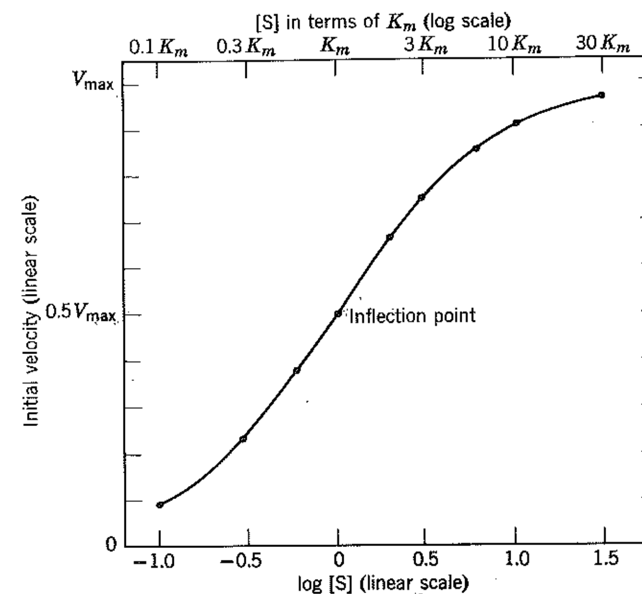


Fig. II-8. Plot of v versus log [S]; [S] at the inflection point equals K_m.

When $v = 0.5\,V_{max}$, $(V_{max}/v - 1)$ equals 1 and log $(V_{max}/v - 1)$ equals zero. At this point log [S] = log K_m, or [S] = K_m. To determine K_m, it is only necessary to identify the midpoint (inflection point) of the curve.

A slightly different rearrangement will yield a familiar equation:

$$\boxed{p[S] = pK_m + \log \frac{V_{max} - v}{v}} \qquad \text{(II-32)}$$

The equation is similar to the Henderson-Hasselbalch equation relating pH to pK_a and the ratio of conjugate base to conjugate acid; $(V_{max} - v)/v$ is analogous to [original HA − amount titrated to base]/[base] or $[HA]/[A^-]$.

In 1934, the more useful Lineweaver-Burk double reciprocal plot was introduced. (The double reciprocal plot was first proposed in 1932 by Haldane and Stern, as a result of a suggestion by Woolf.) This plot is based on the rearrangement of the Henri-Michaelis-Menten equation into a linear $(y = mx + b)$ form.

Lineweaver-Burk Reciprocal Plot: $1/v$ versus $1/[S]$

$$\frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}$$

Inverting:

$$\frac{V_{max}}{v} = \frac{K_m + [S]}{[S]} = \frac{K_m}{[S]} + 1$$

Cross multiplying V_{max}:

$$\boxed{\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}} \qquad \text{(II-33)}$$

Thus if we plot $1/v$ versus $1/[S]$, the slope $= K_m/V_{max}$ and the intercept on the $1/v$ axis $= 1/V_{max}$. We can also see that when $1/v = 0$, $1/[S] = -1/K_m$. As we see later, any factor that multiplies the K_m term of the original Henri-Michaelis-Menten equation will turn out to be a factor of the slope (i.e., of K_m/V_{max}) in the reciprocal equation. Any factor that multiplies the denominator [S] term of the original equation will turn out to be a factor of the $1/v$-axis intercept (i.e., of $1/V_{max}$) in the reciprocal equation.

Substrate Concentration Range

The concentrations of substrate chosen to generate the reciprocal plot should be in the neighborhood of K_m (Fig. II-9). If the concentrations chosen are very high relative to K_m, the curve will be essentially horizontal (Fig. II-10). This will allow V_{max} to be determined, but the slope of the line will be near zero. Consequently, it will be difficult to determine K_m accurately. If the substrate concentrations chosen are very low relative to K_m, the curve will intercept both axes too close to the origin to allow either V_{max} or K_m to be determined accurately (Fig. II-11). (At very low substrate concentrations, the reaction is essentially first-order. There is no hint of saturation. V_{max} and K_m appear to be infinite.)

Generally, substrate concentrations are chosen that give evenly spaced reciprocals (e.g., 1.0, 1.11, 1.25, 1.43, 1.67, 2.0, 2.5, 3.33, 5.0, 10). If a constant increment of substrate concentration is used (e.g., 1.0, 2.0, 3.0, 4.0, etc.) the points will cluster close to the $1/v$-axis.

Labeling the Axes of Reciprocal Plots

The beginning student sometimes is uncertain about the units used in labeling axes or columns of data. The uncertainty arises because there are two ways of interpreting units containing factors (see Table II-1). For

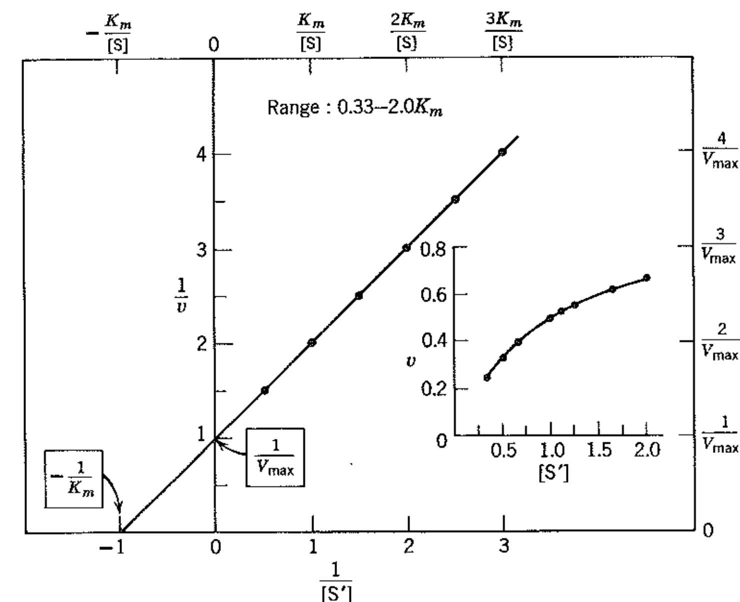


Fig. II-9. Double reciprocal ($1/v$ versus $1/[S]$) Lineweaver-Burk plot. The [S] range chosen is optimal for the determination of K_m and V_{max}.

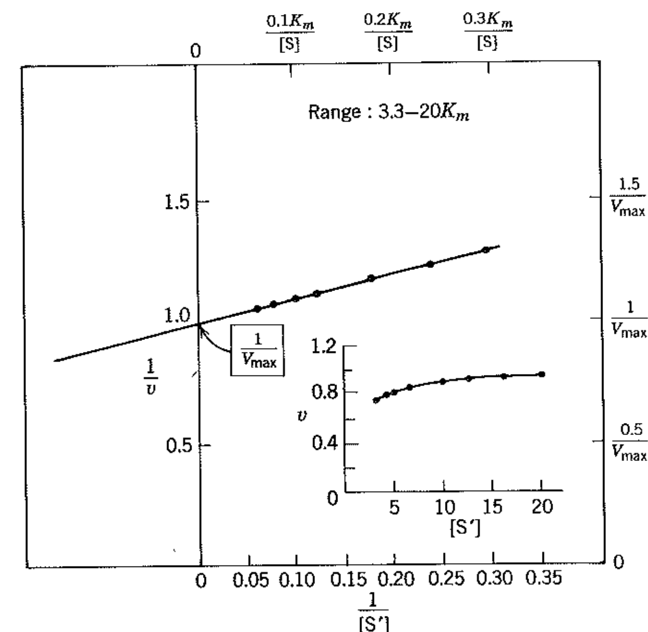


Fig. II-10. $1/v$ versus $1/[S]$ plot. The [S] range chosen is higher than optimal; v is relatively insensitive to changes in [S].

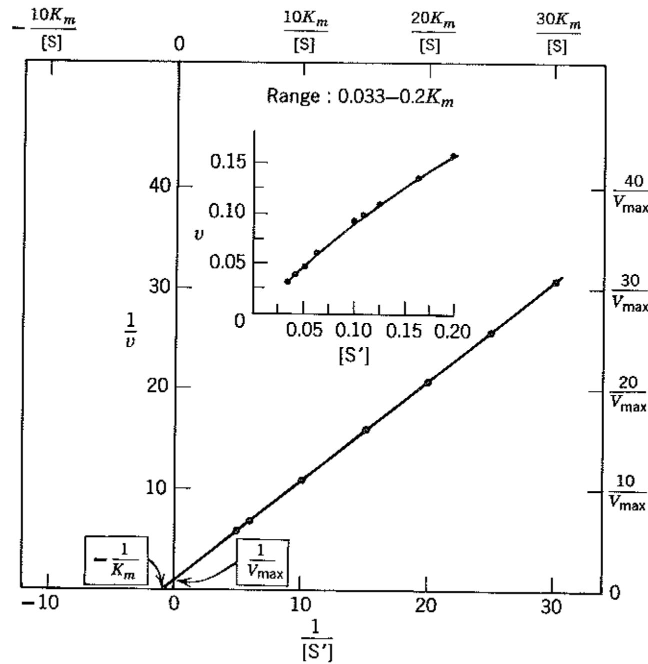


Fig. II-11. $1/v$ versus $1/[S]$ plot. The [S] range chosen is lower than optimal. The reaction is almost first-order with respect to [S].

example, we might find a column headed "substrate concentration" with units of mM$\times 10^2$. Below the heading we might find the figure 0.1. Some people interpret the heading as "the units of the data;" hence the 0.1 really represents 0.1×10^2 mM or 10 mM. Others interpret the heading as "the numbers shown below are 100 times the mM concentrations." The figure 0.1 then represents 0.1×10^{-2} mM or 0.001 mM. Most biochemists use the latter convention. To avoid confusion, it is desirable, whenever possible, to reduce the data to units that do not include factors. In the example above, the column may have been headed "substrate concentration, μM." Then 0.001 mM could have been entered as 1.0.

In the reciprocal plot shown in Figure II-9, the substrate concentration range was $0.33K_m$ to $2.0K_m$. If $K_m = 1 \times 10^{-5}$ M, the range becomes 0.33×10^{-5} M to 2.0×10^{-5} M. The various ways of expressing the $1/[S]$-axis are shown below. Obviously, the most convenient way would be to use units of μM^{-1} and label the axis at 0.1, 0.2, and 0.3.

Table II-1 Different Ways of Labeling the 1/[S]-Axis of Reciprocal Plots

Values	Low Substrate			High Substrate		
	(a)	(b)	(c)	(a)	(b)	(c)
[S]	0.33×10^{-5} M	3.3×10^{-6} M	3.3 μM	2×10^{-5} M	20×10^{-6} M	20 μM
$\frac{1}{[S]}$	3×10^5 M^{-1}	0.3×10^6 M^{-1}	0.3 μM^{-1}	0.5×10^5 M^{-1}	0.05×10^6 M^{-1}	0.05 μM^{-1}
Number on axis	3	0.3	0.3	0.5	0.05	0.05
Units	$M^{-1} \times 10^{-5}$ or 10^{-5} M^{-1} or $10^{-5}/M$ or $(M \times 10^5)^{-1}$ or $(10^5$ $M)^{-1}$	$M^{-1} \times 10^{-6}$ or 10^{-6} M^{-1} or $10^{-6}/M$ or $(M \times 10^6)^{-1}$ or $(10^6$ $M)^{-1}$	μM^{-1}	(same as low [S])		

Graphical Analysis as a Method of Solving Simultaneous Equations

If we know the velocity, v_1, at one substrate concentration, $[S]_1$, and we also know the velocity, v_2, at a different higher substrate concentration, $[S]_2$, it is a simple matter to solve the two simultaneous equations for the two unknowns, K_m and V_{max}. All we need do is take the ratio of v_2/v_1:

$$\frac{v_2}{v_1}=\frac{\dfrac{[S]_2 V_{max}}{K_m+[S]_2}}{\dfrac{[S]_1 V_{max}}{K_m+[S]_1}}=\frac{[S]_2(K_m+[S]_1)}{[S]_1(K_m+[S]_2)}$$

Solving for K_m:

$$\boxed{K_m=\frac{[S]_2[S]_1(v_1-v_2)}{v_2[S]_1-v_1[S]_2}} \qquad \text{(II-34)}$$

The V_{max} can be obtained by substituting the value of K_m into the original Henri-Michaelis-Menten expression for v_2 or v_1. In effect, the Lineweaver-Burk reciprocal plot has solved two simultaneous equations. We need at least two $1/v-1/[S]$ points to draw a straight line. Once the line is drawn, we automatically obtain the two unknowns as intercepts. In Chapter Three we see that in the presence of an inhibitor, the velocity equation contains an additional constant, K_i. Again, we could determine all the constants by solving three simultaneous equations (or four equations if we do not know the type of inhibition), but it is far simpler to plot the data as two (or more) straight lines and extract the constants from intercepts and slopes (or replots thereof). Other methods of plotting enzyme kinetics data are described in Chapter Four.

Effect of Impure Substrate on K_m and V_{max}

Suppose that the substrate is quite impure but the contaminant is not inhibitory. The velocity is given by:

$$\boxed{\frac{v}{V_{max}}=\frac{y[S]_{add}}{K_m+y[S]_{add}}=\frac{[S]_{add}}{\dfrac{K_m}{y}+[S]_{add}}} \qquad \text{(II-35)}$$

where $[S]_{add}$ = the concentration of added substrate (i.e., the assumed concentration of S which will be plotted)

y = the fractional purity of added S (as a decimal)

$\therefore\ y[S]_{add}$ = the true concentration of S.

The intercept on the $1/v$-axis gives the true $1/V_{max}$ but the observed K_m will be higher than the true K_m because the assumed (plotted) values of $1/[S]$ will be lower than the true values. If, for example, $y=0.50$ (S is only 50% pure), the K_m determined from the reciprocal plot will be high by a factor of 2. If the impurity is inhibitory, then both the K_m and V_{max} will change, as described in Chapter Three.

Eisenthal, Cornish-Bowden Plot and New Dixon Plot

While we might assume that all the properties of the hyperbolic velocity curve have been discovered by now, every few years something new turns up. For example, in 1974, Eisenthal and Cornish-Bowden showed that if the experimental [S] values are plotted on a negative horizontal axis, and the observed v values are plotted on a vertical axis, then straight lines drawn through the corresponding $-[S]$ and v points intersect at $[S]=K_m$ and $v=V_{max}$ (Fig. II-12*a*) (lines drawn through positive [S] and v points intersect at $-K_m$).

Another property of the hyperbolic velocity curve described by Dixon in 1972 is illustrated in Figure II-12*b*. If V_{max} can be determined easily (as v observed with a large excess of substrate), then straight lines can be drawn from the origin through various $[(n-1)/n]\ V_{max}$ points on the velocity curve to intersect the horizontal V_{max} line at some value of [S], called $[S]_n$. The n represents various whole numbers such that the lines are drawn through $\frac{1}{2}V_{max}$ ($n=2$), $\frac{2}{3}V_{max}$ ($n=3$), $\frac{3}{4}V_{max}$ ($n=4$), and so on. The intercept, $[S]_n$, is related to [S], the actual substrate concentration needed for $[(n-1)/n]\ V_{max}$, as shown in the insert to Figure II-12*b* and equation II-36.

$$\boxed{[S]_n=\left(\frac{n}{n-1}\right)[S]} \qquad \text{(II-36)}$$

The distance between one intercept and the next is always equal to K_m. For example, when $n=4$, $v=\frac{3}{4}V_{max}$. Therefore, $[S]_1=3K_m$ and $[S]_{n_1}=\frac{4}{3}[S]_1$; when $n=5$, $v=\frac{4}{5}V_{max}$. Therefore, $[S]_2=4K_m$ and $[S]_{n_2}=\frac{5}{4}[S]_2$. Then $\Delta=[S]_{n_2}-[S]_{n_1}=(1.25)(4K_m)-(1.33)(3K_m)=5K_m-4K_m=K_m$. If the V_{max} line is drawn too low, the increments will decrease in size as n increases. If the

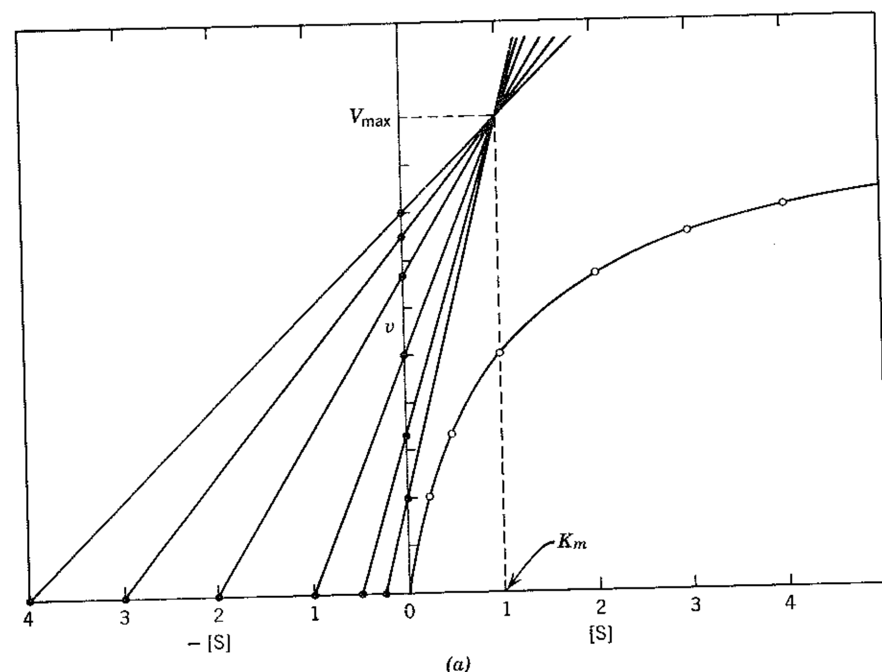


Fig. II-12. (*a*) Direct linear plot of v–[S] data described by Eisenthal and Cornish-Bowden [*Biochem. J.* **139**, 715 (1974)] and by Merino [*Biochem. J.* **143**, 93 (1974)].

V_{max} line is drawn too high, the increments will increase in size as n increases. Thus the method provides a check on the assumed value of V_{max}. The first line is drawn through $\frac{1}{2} V_{max}$. The distance between the v-axis and the intercept of this line on the V_{max} line equal $2K_m$. The hypothetical line for $n=1$ divides this distance into two equal lengths of K_m each. The $n=1$ line is tangent to the velocity curve at the origin. If, after setting off an increment of K_m to the left of the $n=2$ line (i.e., after drawing the $n=1$ line) the remaining increment (between the v-axis and the $n=1$ line) is greater than K_m, then we may conclude that a substantial portion of the total substrate added is bound by the enzyme. In fact, the major advantage of this new Dixon plot is that it provides a way of determining K_m and $[E]_t$ for an enzyme that has a very high affinity for its substrate such that under assay conditions $[S]_{free} \neq [S]_{total}$. A similar plot can be used for inhibition systems where I is very strongly bound by the enzyme such that $[I]_{free} \neq [I]_{total}$. The equations and the plots are described in more detail in a following section (p. 74) and Chapter Three.

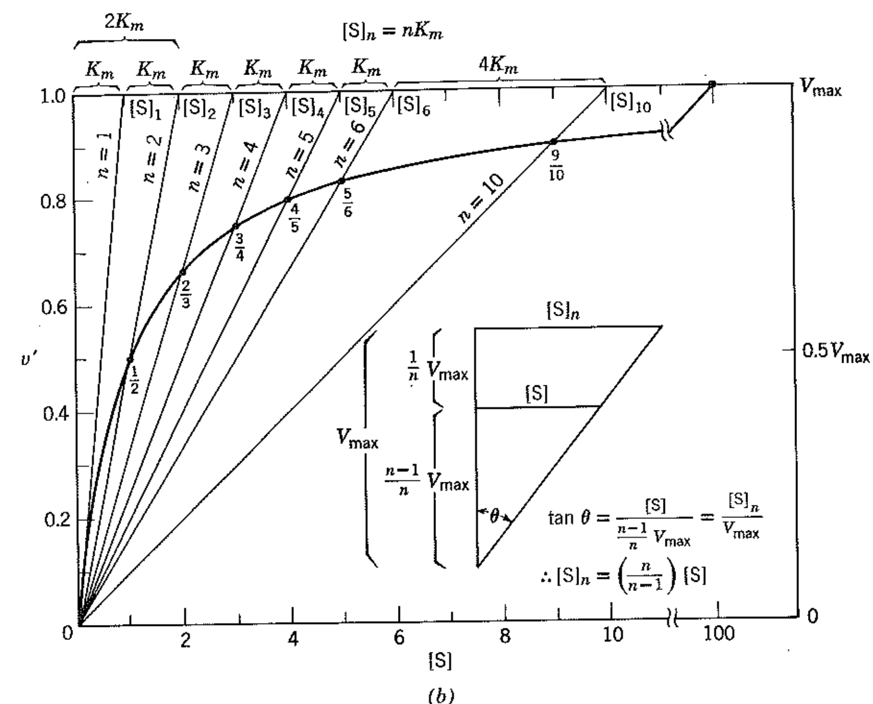


Fig. II-12. (*b*) An interesting geometric property of the velocity curve described by Dixon [*Biochem. J.* **129**, 197 (1972)]. Lines drawn from the origin through various $V_{max}(n-1)/n$ points intersect a horizontal line at V_{max} at increments of K_m.

Log v Versus Log [S] Plot

The log v versus log [S] plot is the most convenient way of expressing initial velocity data obtained over a very wide range of substrate concentrations. The plot provides a preliminary estimate of K_m and V_{max} and in some cases will disclose the presence of multiple enzymes catalyzing the same reaction (as described in a following section). Figure II-13 shows the log-log plot obtained in the author's laboratory for methylamine transport by *Pencillium chrysogenum*. The plot shows that methylamine uptake by nitrogen-sufficient cells is apparently first-order throughout the entire concentration range studied. Nitrogen deficiency derepresses (or deinhibits) a saturable transport system with an apparent K_m in the region of 10^{-5} M. At high methylamine concentrations (ca. 10^{-3} M), there is little difference between the two types of cells in their ability to transport methylamine. However, at low substrate concentrations ($<10^{-5}$ M), the nitrogen deficient cells can transport methylamine about 1000-times faster than the nitrogen sufficient cells.

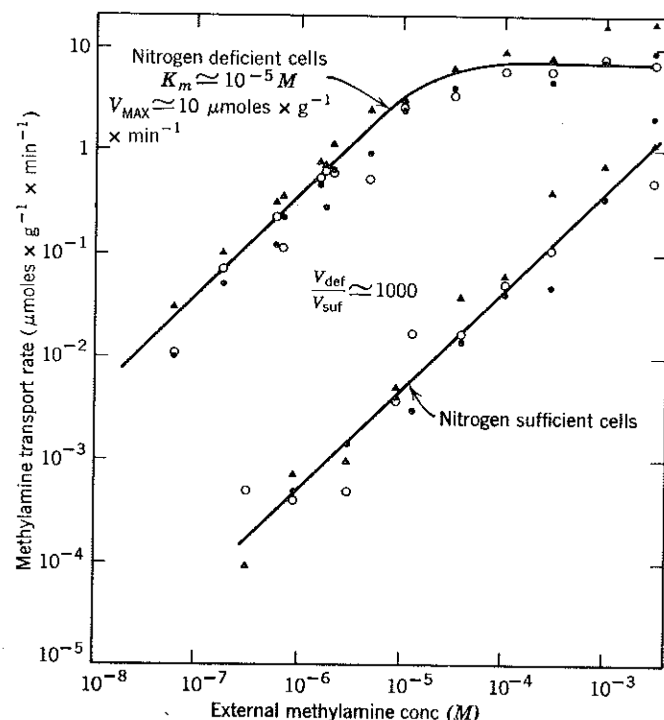


Fig. II-13. Log v versus log [S] plot for methylamine transport by *Penicillum chrysogenum*. [Redrawn from Hackette, S. L., Skye, G. E., Burton, C. and Segel, I. H., *J. Biol. Chem.* **245**, 4241, (1970).]

K. INTEGRATED FORM OF THE HENRI-MICHAELIS-MENTEN EQUATION

Integrated Rate Equation Assuming No Product Inhibition ($K_{m_S} \ll K_{m_P}$) and that K_{eq} Is Very Large

The Henri-Michaelis-Menten equation is a differential velocity equation where v represents $d[P]/dt$ or $-d[S]/dt$. Thus, to determine K_m and V_{max} by one of the usual linear plots based on the Henri-Michaelis-Menten equation (e.g., $1/v$ versus $1/[S]$), the reaction must proceed to a negligible extent during the course of the assay (e.g., less than 5% of $[S]_0$ converted to P). Under some experimental conditions it may not be feasible to restrict the reaction extent to 5% or less. For example, it may be difficult to determine very low product concentrations and, consequently, it becomes necessary to allow the reaction to proceed until a substantial fraction of the initial $[S]_0$ is converted to P. In some assays, P is not measured directly, but rather is determined from the decrease in substrate concentration: $[P]=[S]_0-[S]$. Again, a reliable estimate of [P] may require that [S] be significantly less than $[S]_0$. These situations can be handled by using the integrated rate equation and incorporating as many points as desired between, for example, 10 and 90% conversion of S to P. The integrated equation is valid over the entire course of the reaction. The simplest integrated rate equation is derived below. The derivation assumes that the decrease in velocity with time results only from decreasing saturation of the enzyme, and not from product inhibition or approach to equilibrium.

Let

$$v = -\frac{d[S]}{dt} = \frac{V_{max}[S]}{K_m+[S]}$$

Rearranging and inverting:

$$V_{max}\,dt = -\frac{K_m+[S]}{[S]}\,d[S]$$

Integrating between any two times (e.g., zero-time, t_0, and any other time, t) and the corresponding two substrate concentrations ($[S]_0$ and [S]):

$$V_{max}\int_{t_0}^{t} dt = -\int_{[S]_0}^{[S]} \frac{K_m+[S]}{[S]}\,d[S]$$

Separating the terms in the right-hand expression:

$$V_{max}\int_{t_0}^{t} dt = -K_m\int_{[S]_0}^{[S]} \frac{d[S]}{[S]} - \int_{[S]_0}^{[S]} d[S]$$

$$V_{max}t = -K_m \ln\frac{[S]}{[S]_0} - ([S]-[S]_0)$$

or

$$\boxed{V_{max}t = 2.3K_m \log\frac{[S]_0}{[S]} + ([S]_0-[S])} \qquad \text{(II-37)}$$

where

$([S]_0 - [S])$ = concentration of substrate utilized by time t

= [P], the concentration of product produced by time t

Unlike the integrated first-order rate equation (II-27) or integrated zero-order rate equatiion (II-30), equation II-37 can not be solved for [S] or [P] at any time t even if K_m and V_{max} are known. On the other hand, if $[S]_0$ and [S] at two or more times are known, K_m and V_{max} can be calculated. Equation II-37 can be rearranged to a linear form:

$$\frac{2.3}{t}\log\frac{[S]_0}{[S]} = -\frac{1}{K_m}\frac{([S]_0-[S])}{t} + \frac{V_{max}}{K_m} \qquad \text{(II-38)}$$

Thus K_m and V_{max} may be determined by measuring the concentration of substrate utilized (or product produced) several times during the reaction and then plotting the appropriate values as shown in Figure II-14.

There are several other linear forms of the integrated rate equation:

$$\boxed{\frac{t}{[P]} = \frac{K_m}{V_{max}}\left(\frac{2.3\log\frac{[S]_0}{[S]}}{[P]}\right) + \frac{1}{V_{max}}} \qquad \text{(II-39)}$$

$$\boxed{\frac{t}{2.3\log\frac{[S]_0}{[S]}} = \frac{1}{V_{max}}\left(\frac{[P]}{2.3\log\frac{[S]_0}{[S]}}\right) + \frac{K_m}{V_{max}}} \qquad \text{(II-40)}$$

$$\boxed{\frac{[P]}{t} = -K_m\left(\frac{2.3}{t}\log\frac{[S]_0}{[S]}\right) + V_{max}} \qquad \text{(II-41)}$$

where $[P] = [S]_0 - [S]$. Equation II-39 has the same slope and vertical axis intercept as the plot of $1/v$ versus $1/[S]$. Equation II-40 is analogous to the plot of $[S]/v$ versus [S], while equation II-41 is analogous to the plot of v versus $v/[S]$. These alternate linear plots for initial velocity studies are described in Chapter Four. When plotting data according to one of the

integrated rate equations a good spread of points can be obtained by starting with an $[S]_0$ that is several-fold greater than K_m (e.g., $10K_m$) and allowing the reaction to proceed until [S] is significantly below K_m (e.g., $0.1K_m$).

Determination of K_m and V_{max} from $[\bar{S}]$ and $\bar{v}$

If a substantial fraction of the substrate is utilized during the assay, the K_m and V_{max} values determined from the reciprocal plot (or any other linear plot described in Chapter Four) will be in error. First of all, if $[S]_0 - [S] = \Delta[S]$ is an appreciable fraction of $[S]_0$, then neither $[S]_0$ nor [S] can be taken as the [S] variable. Secondly, $[P]/t$ represents an overall velocity which is neither the initial velocity $(-d[S]/dt)$ at $[S]=[S]_0$ nor the final velocity at [S]. The Lineweaver-Burk plot could be used if we knew exactly which substrate concentration between $[S]_0$ and [S] yields an instantaneous velocity the same as the observed overall velocity. Certainly, there is *some* value of [S] that gives a $-d[S]/dt = \Delta S/\Delta t$. Lee and Wilson (1971) have shown that the arithmetic mean substrate concentration, $\frac{1}{2}([S]_0+[S])$ is an excellent approximation and with this value the modified Lineweaver-Burk equation can yield reliable estimates of the kinetic constants even though the extent of the reaction is significant. The modified plot is based on equation II-42:

$$\boxed{\frac{1}{\bar{v}} = \frac{K_m}{V_{max}}\frac{1}{[\bar{S}]} + \frac{1}{V_{max}}} \qquad \text{(II-42)}$$

where

$$\bar{v} = \frac{[P]}{t} = \frac{[S]_0-[S]}{t} = \text{the overall velocity}$$

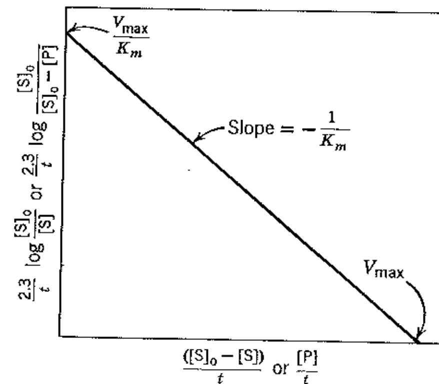


Fig. II-14. Plot of the integrated Henri-Michaelis-Menten equation.

and

$$[\bar{S}] = \frac{[S]_0 + [S]}{2}$$ = the arithmetical average substrate concentration over the course of the assay

The modified equation can be compared to the integrated rate equation II-39, which can be written as:

$$\boxed{\frac{1}{\bar{v}} = \frac{K_m}{V_{max}}\left(\frac{2.3 \log \frac{[S]_0}{[S]}}{[S]_0 - [S]}\right) + \frac{1}{V_{max}}} \qquad \text{(II-43)}$$

The validity of equation II-42 depends on how closely $1/[\bar{S}]$ approximates the parenthetical term in equation II-43. (Remember that the integrated rate equation is valid for any extent of the reaction.) We can easily check the approximation. For example, suppose that the reaction proceeds until 30% of $[S]_0$ is utilized. Let $[S]_0 = 1.0$:

$$\frac{[S]_0 + [S]}{2} = [\bar{S}] = \frac{1.7}{2} = 0.85 \qquad \frac{1}{[\bar{S}]} = 1.18$$

$$\frac{2.3 \log \frac{[S]_0}{[S]}}{[S]_0 - [S]} = \frac{2.3 \log 1.43}{0.3} = 1.19$$

Thus the modified Lineweaver-Burk plot introduces only about a 1% error in the determination of K_m even when the reaction has proceeded to the extent of 30%. A similar calculation will show an error of only about 4% when the reaction has proceeded to 50% utilization of initial substrate. Figure II-15 shows the relative error in K_m as a function of the extent of the reaction when the modified equation is used. For comparison, the error obtained by using $[S]_0$ instead of $[\bar{S}]$ is also shown. It is apparent that the Lineweaver-Burk plot (and the other linear plots described in Chapter Four) can be used to determine K_m and V_{max} even when the reaction extent is large, *provided* that (*a*) $[\bar{S}]$ rather than $[S]_0$ is used as the horizontal axis variable, (*b*) all cosubstrates are saturating, (*c*) the reaction is not significantly reversible, and (*d*) there is no product inhibition. Factors *c* and *d* can be checked easily. If necessary, the reaction can be made irreversible and product inhibition minimized by continually removing the products with trapping agents or coupling enzymes.

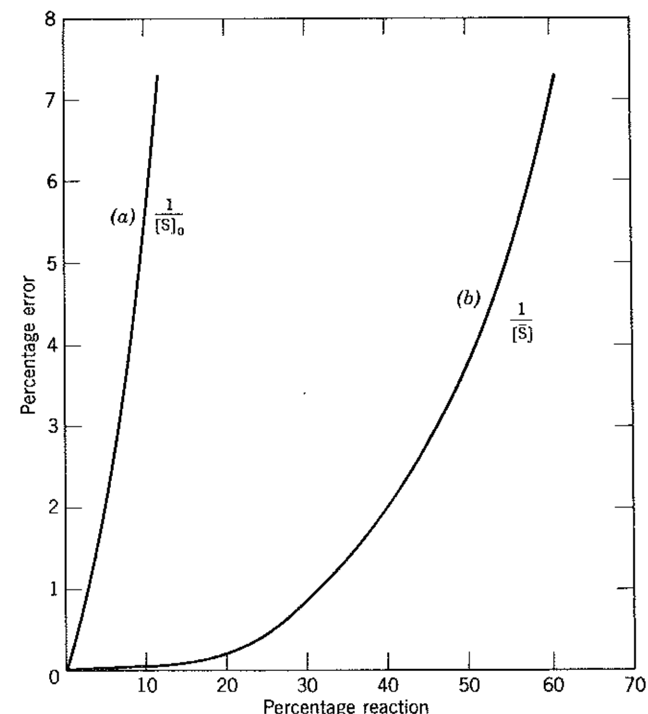


Fig. II-15. Percentage error in the value of K_m as a function of the extent of the reaction. Curve *a* plotting $1/[S]_0$. Curve *b* plotting $1/[\bar{S}]$. [Redrawn from Lee, H-J. and Wilson I. B., *Biochim. Biophys. Acta* **242**, 519, (1971).]

Integrated Rate Equation Where $K_{m_P} \cong K_{m_S}$ and K_{eq} Is Very Large

If the enzyme has an appreciable affinity for the product, but K_{eq} is still very large, then the decrease in velocity with time will result from a combination of decreasing saturation of the enzyme with substrate and increasing product inhibition. The velocity at any time t is given by:

$$v = -\frac{d[S]}{dt} = \frac{V_{max}[S]}{K_{m_S}\left(1 + \frac{[S]_0 - [S]}{K_{m_P}}\right) + [S]}$$

where

$[S]_0 - [S] = [P]$ = the product concentration at time t.

Rearranging and inverting:

$$V_{\max}\,dt = \left(-\frac{K_{m_S}}{[S]} - \frac{K_{m_S}[S]_0}{K_{m_P}[S]} + \frac{K_{m_S}[S]}{K_{m_P}[S]} - \frac{[S]}{[S]} \right) d[S]$$

Grouping the constants K_{m_S}, K_{m_P}, and $[S]_0$ and integrating between $[S]_0$ and $[S]$ and time zero and time t:

$$V_{\max}\int_{t_0}^{t} dt = -K_{m_S}\left(1+\frac{[S]_0}{K_{m_P}}\right)\int_{[S]_0}^{[S]}\frac{d[S]}{[S]} + \left(\frac{K_{m_S}}{K_{m_P}}-1\right)\int_{[S]_0}^{[S]} d[S]$$

$$V_{\max}\,t = 2.3K_{m_S}\left(1+\frac{[S]_0}{K_{m_P}}\right)\log\frac{[S]_0}{[S]} + \left(1-\frac{K_{m_S}}{K_{m_P}}\right)([S]_0-[S]) \qquad \text{(II-44)}$$

The equation can be rearranged to several linear forms analogous to those shown above for $K_{m_P} \gg K_{m_S}$:

$$\frac{2.3}{t}\log\frac{[S]_0}{[S]} = -\frac{1}{K_{m_S}}\left(\frac{K_{m_P}-K_{m_S}}{K_{m_P}+[S]_0}\right)\frac{[P]}{t} + \frac{V_{\max}}{K_{m_S}\left(1+\dfrac{[S]_0}{K_{m_P}}\right)} \qquad \text{(II-45)}$$

$$\frac{t}{[P]} = \frac{K_{m_S}}{V_{\max}}\left(1+\frac{[S]_0}{K_{m_P}}\right)\left(\frac{2.3\log\dfrac{[S]_0}{[S]}}{[P]}\right) + \frac{1}{V_{\max}}\left(1-\frac{K_{m_S}}{K_{m_P}}\right) \qquad \text{(II-46)}$$

The series of plots based on equation II-46 will have a common intersection point on the vertical axis for all values of $[S]_0$.

$$\frac{t}{2.3\log\dfrac{[S]_0}{[S]}} = \frac{1}{V_{\max}}\left(1-\frac{K_{m_S}}{K_{m_P}}\right)\left(\frac{[P]}{2.3\log\dfrac{[S]_0}{[S]}}\right) + \frac{K_{m_S}}{V_{\max}}\left(1+\frac{[S]_0}{K_{m_P}}\right) \qquad \text{(II-47)}$$

The family of curves for different values of $[S]_0$ will be parallel, since the slope of equation II-47 is independent of $[S]_0$.

$$\frac{[P]}{t} = -K_{m_S}\left(\frac{K_{m_P}+[S]_0}{K_{m_P}-K_{m_S}}\right)\left(\frac{2.3}{t}\log\frac{[S]_0}{[S]}\right) + \frac{V_{\max}}{\left(1-\dfrac{K_{m_S}}{K_{m_P}}\right)} \qquad \text{(II-48)}$$

Plots of equation II-48 will intersect on the vertical axis for all values of $[S]_0$. Plots based on equations II-45, II-47, and II-48 can have positive or negative slopes, depending on the relative values of K_{m_S} and K_{m_P}. Similarly, vertical axis intercepts of plots based on equations II-46 and II-48 can be positive or negative. When $K_{m_S} = K_{m_P}$, equations II-45 and II-47 yield plots with zero slope; equation II-48 will yield vertical lines (infinite slope and vertical axis intercept). Equation II-46 will yield plots that intersect at the origin. All three unknowns, K_{m_S}, K_{m_P}, and $V_{\max}$ can be determined from a series of plots obtained for different initial substrate concentrations, $[S]_0$. For example, the slope of the plot described by equation II-48 is given by:

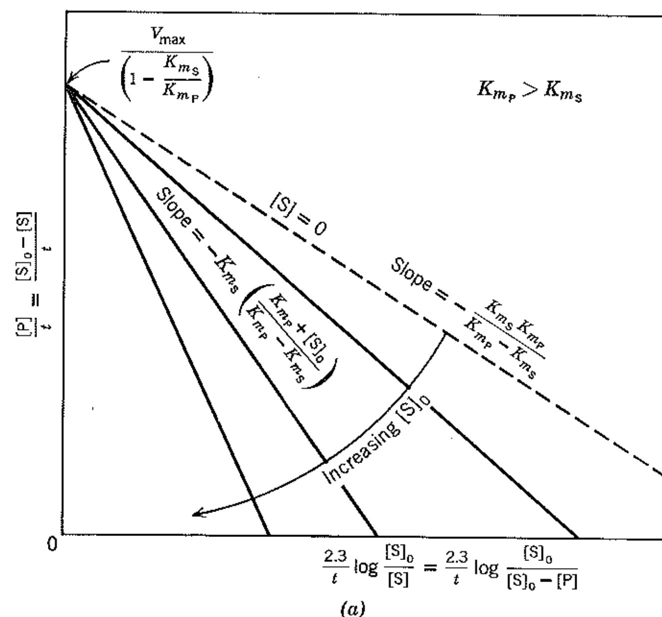
$$slope = -K_{m_S}\left(\frac{K_{m_P}+[S]_0}{K_{m_P}-K_{m_S}}\right) = -\frac{K_{m_S}}{(K_{m_P}-K_{m_S})}[S]_0 - \frac{K_{m_S}K_{m_P}}{(K_{m_P}-K_{m_S})}$$

Thus the replot of *slope* versus $[S]_0$ is linear. When *slope* = 0, the intercept on the horizontal axis is $-K_{m_P}$. With K_{m_P} known, K_{m_S} can be calculated from the slope or vertical axis intercept of the replot. Then $V_{\max}$ can be calculated from the vertical axis intercept of the original plot. The plot of equation II-48 and the *slope* replot are shown in Figure II-16 for a system where $K_{m_P} > K_{m_S}$. For convenience, $-$*slope* is plotted versus $[S]_0$ in the replot.

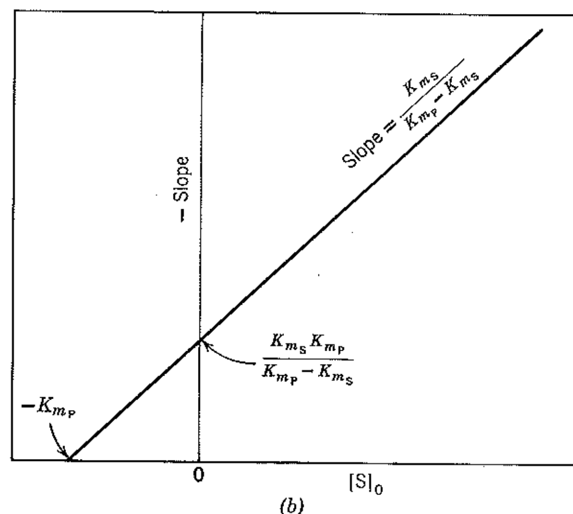
Integrated Rate Equation Where $K_{m_P} \cong K_{m_S}$ and K_{eq} Is Not Very Large

The complete integrated rate equation, taking into account both product inhibition and the reverse reaction is:

$$\left(\frac{V_{\max_f}}{K_{m_S}} + \frac{V_{\max_r}}{K_{m_P}}\right)t = \left(\frac{1}{K_{m_S}} - \frac{1}{K_{m_P}}\right)[P] - 2.3\log\left(1-\frac{[P]}{[P]_{eq}}\right)\left(1+\frac{[S]_0}{K_{m_P}} + \frac{\left(\dfrac{1}{K_{m_S}}-\dfrac{1}{K_{m_P}}\right)}{\left(\dfrac{V_{\max_f}}{K_{m_S}}+\dfrac{V_{\max_r}}{K_{m_P}}\right)}\frac{V_{\max_r}}{K_{m_P}}[S]_0\right) \qquad \text{(II-49)}$$



(a)



(b)

Fig. II-16. *(a)* Plot of the integrated velocity equation II-48 where $K_{m_P} > K_{m_S}$ and $K_{eq} \gg 1$. *(b)* Slope replot.

The four kinetic constants are not independent, but rather are related through the Haldane equation:

$$K_{eq} = \frac{[P]_{eq}}{[S]_{eq}} = \frac{V_{max_f} K_{m_P}}{V_{max_r} K_{m_S}}$$

Substituting for V_{max_r} from the Haldane equation:

$$\frac{V_{max_f}}{K_{m_S}}\left(1+\frac{1}{K_{eq}}\right)t = \left(\frac{1}{K_{m_S}} - \frac{1}{K_{m_P}}\right)[P] - 2.3\log\left(1-\frac{[P]}{[P]_{eq}}\right)\left(1+\frac{[S]_0}{K_{m_P}} + \frac{\left(\frac{1}{K_{m_S}}-\frac{1}{K_{m_P}}\right)}{1+K_{eq}}[S]_0\right) \tag{II-50}$$

The equation can be rearranged to a linear form:

$$\frac{2.3}{t}\log\left(1-\frac{[P]}{[P]_{eq}}\right) = \left(\frac{(K_{m_P}-K_{m_S})(1+K_{eq})}{K_{m_P}K_{m_S}(1+K_{eq}) + (K_{m_P}+K_{m_S}K_{eq})[S]_0}\right)\frac{[P]}{t} - \frac{V_{max}\left(1+\frac{1}{K_{eq}}\right)}{K_{m_S} + \left(\frac{K_{m_S}K_{eq}+K_{m_P}}{K_{m_P}(1+K_{eq})}\right)[S]_0} \tag{II-51}$$

The equations are complex, yet all that is required is a knowledge of K_{eq} and the product concentration during the course of the reaction at two or more initial substrate concentrations, $[S]_0$, and all four kinetic constants can be determined.

The integrated rate equations were derived for a one substrate-one product (Uni Uni) system. The corresponding equations for multisubstrate-multiproduct systems are exceedingly complex and are rarely employed, although sometimes it may be possible to treat a complex reaction as a Uni Uni reaction. For example, consider the one substrate-two product (Uni Bi) reaction: $E + S \rightleftharpoons ES \rightleftharpoons E + P + Q$. Fructose diphosphate aldolase catalyzes a

Uni Bi reaction, while many hydrolytic reactions yield apparent Uni Bi kinetics because the concentration of the second substrate (water) is constant (Chapter Nine). One or both of the products, P and Q, may compete with the substrate for free enzyme, depending on whether the release of P and Q is random or ordered. In a random release, P and Q both can combine with free enzyme, and thus act as competitive inhibitors. In an ordered reaction, only the last product released (Q) is a competitive inhibitor with respect to S, but P may act as a noncompetitive inhibitor if it binds appreciably to the EQ complex. If P is trapped chemically, or removed as it is formed by a coupled enzyme reaction, then the reaction can be treated as a Uni Uni reaction with Q as the sole accumulating competitive product inhibitor. The removal of P will also eliminate the reverse reaction. (In the ordered reaction, it is necessary to know beforehand which product is P and which is Q.) The calculated K_Q constant is an inhibition constant and may not equal the K_m value for Q in the reverse direction. In general, however, integrated rate studies for multireactant enzymes are far less profitable than initial velocity studies (Chapter Nine).

L. MULTIPLE ENZYMES CATALYZING THE SAME REACTION

Occasionally, a preparation may contain two or more enzymes (or multiple forms of the same enzyme) that catalyze the same reaction. The velocity at any substrate concentration is the sum of the velocities contributed by each enzyme. For two enzymes the velocity is given by:

$$v = \frac{[S]\,V_{max_1}}{K_{m_1} + [S]} + \frac{[S]\,V_{max_2}}{K_{m_2} + [S]} \qquad \text{(II-52)}$$

The multiplicity may go undetected even if the K_m and V_{max} values of the enzymes are quite different. Figure II-17 shows the reciprocal plots for five different combinations of two enzymes with K_m values differing by a factor of 10. All the plots for two enzymes are curved although the points over any limited range of $1/[S]$ may appear to fall on a straight line. In all cases the greatest curvature is in the region close to the $1/v$-axis, that is, at very high substrate concentrations. Unfortunately, this is also the region where the limitations of the assay frequently make it difficult to detect changes in initial velocity as the substrate concentration is increased. The relative contributions of two enzymes to the observed total initial velocity are shown in Figures II-18 to II-22. In the system where the enzyme with the higher

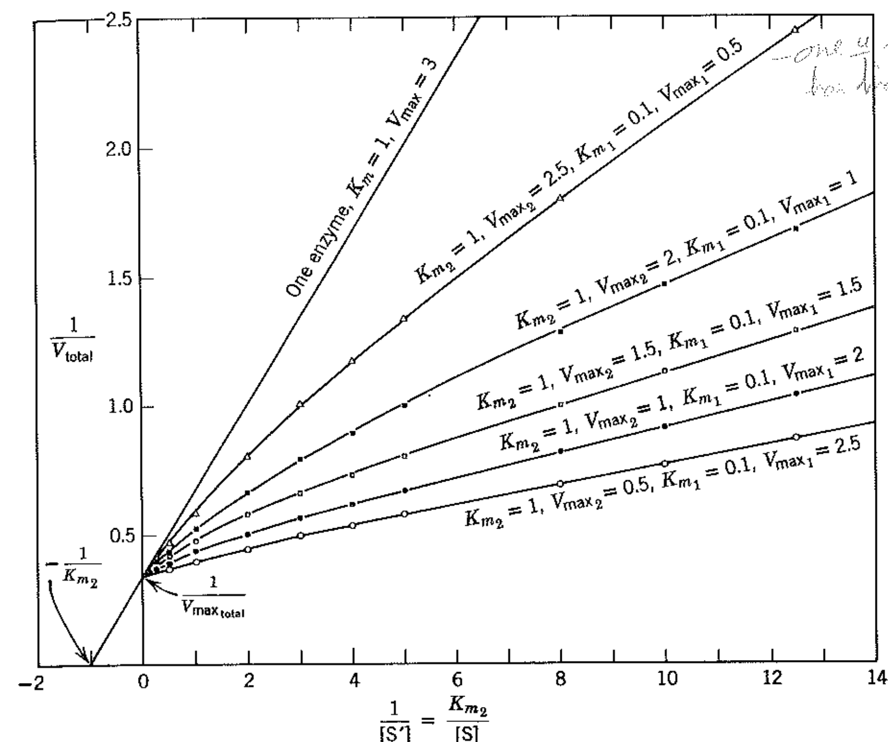


Fig. II-17. The $1/v$ versus $1/[S]$ plots when two enzymes are present: $V_{max_1} + V_{max_2} = 3$, and $K_{m_1} = 0.1 K_{m_2}$.

V_{max} has the lower K_m, the contribution of the lower V_{max}–higher K_m enzyme will be completely obscured (Fig. II-18). A reciprocal plot will appear linear over a wide range of $1/[S]$ and extrapolate close to the $1/V_{max}$ and $-1/K_m$ values for the lower K_m–higher V_{max} enzyme. If the two enzymes have the same K_m but different V_{max} values (Fig. II-19), the mixture behaves as a single enzyme with $V_{max} = V_{max_1} + V_{max_2}$. When the enzymes have the same V_{max} but different K_m values (Fig. II-20), the reciprocal plot bends downward close to the vertical axis as the contribution of the higher K_m enzyme to the total velocity increases at high substrate concentrations. The greatest curvature of the reciprocal plot occurs when the V_{max} and K_m of one enzyme are significantly higher than that of the other (Fig. II-22 and Fig. II-23). At low substrate concentrations, the relative contributions of the two enzymes will depend on the V_{max}/K_m ratios (i.e., the first-order rate constants) of the two enzymes. For example, in the system described in Figure II-21, both enzymes have V_{max}/K_m ratios of 100, and both contribute about equally to

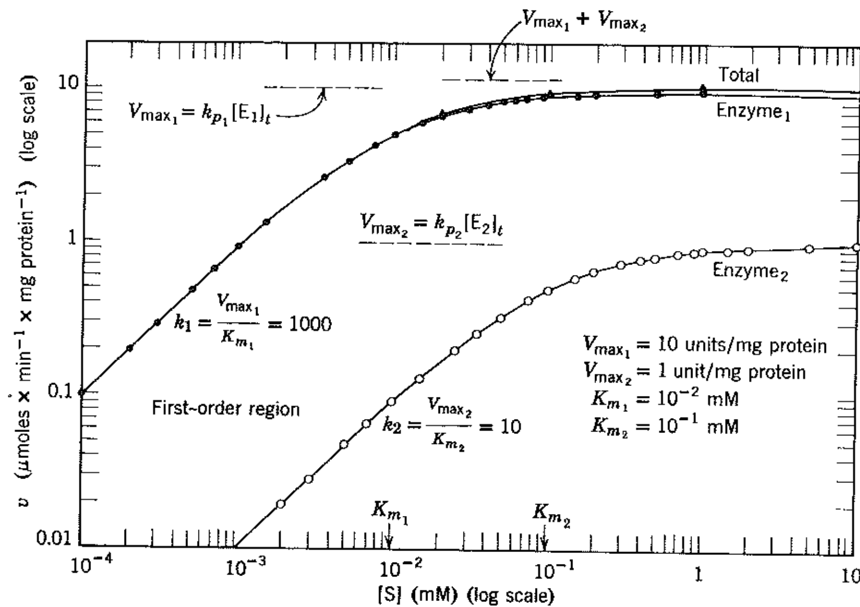


Fig. II-18. Log v versus log [S] plot in the presence of two enzymes: $V_{max_1} > V_{max_2}$, $K_{m_1} < K_{m_2}$.

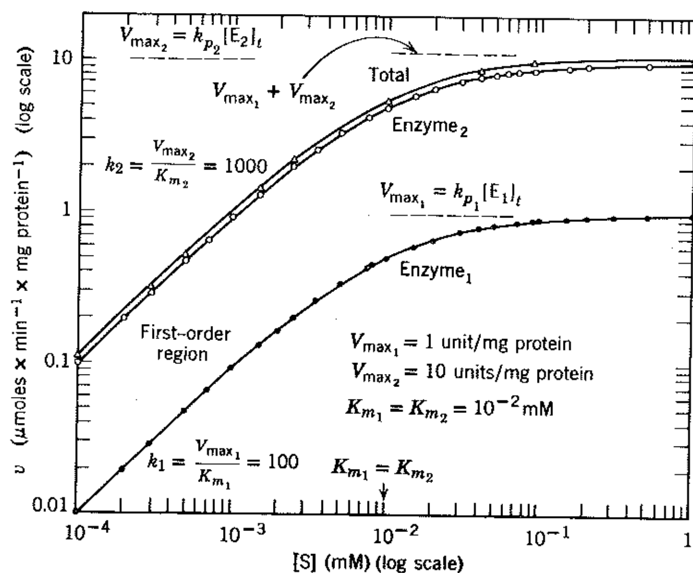


Fig. II-19. Log v versus log [S] plot in the presence of two enzymes: $V_{max_1} < V_{max_2}$, $K_{m_1} = K_{m_2}$.

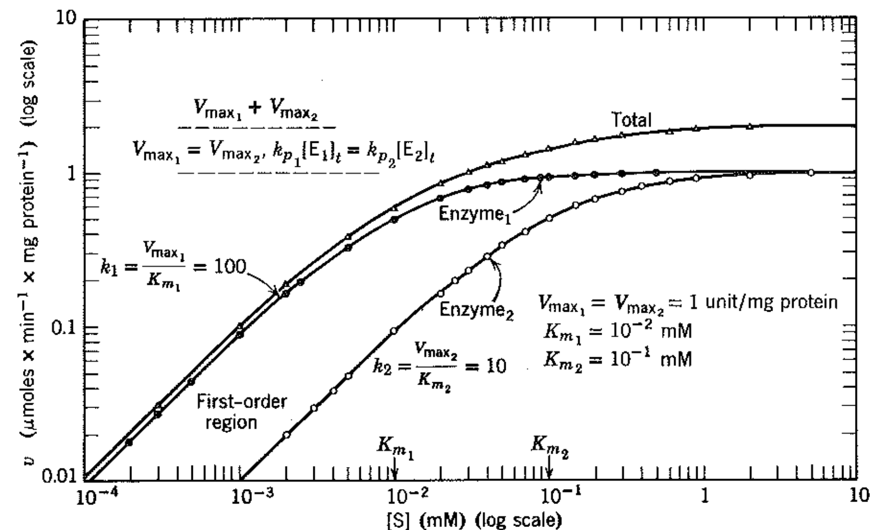


Fig. II-20. Log v versus log [S] plot in the presence of two enzymes: $V_{max_1} = V_{max_2}$, $K_{m_1} < K_{m_2}$.

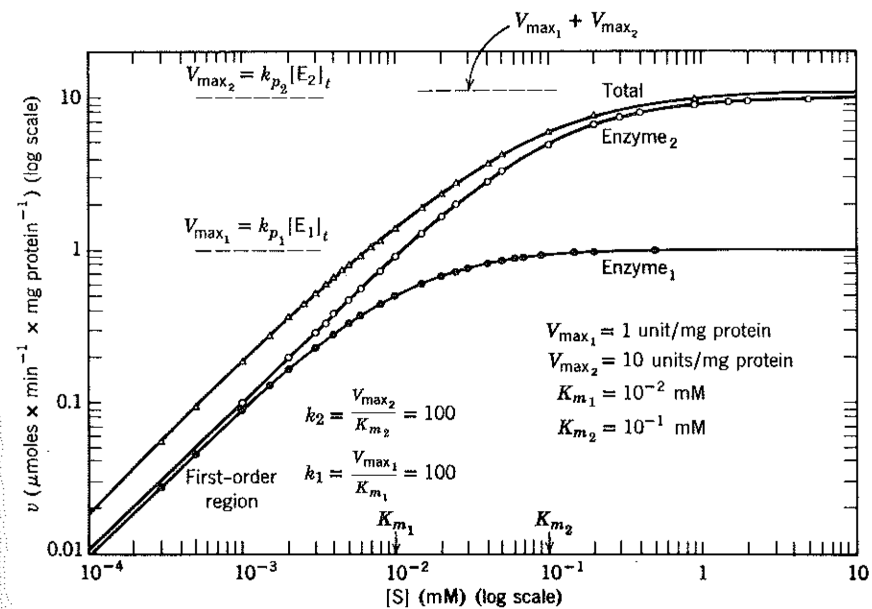


Fig. II-21. Log v versus log [S] plot in the presence of two enzymes: $V_{max_1} < V_{max_2}$,

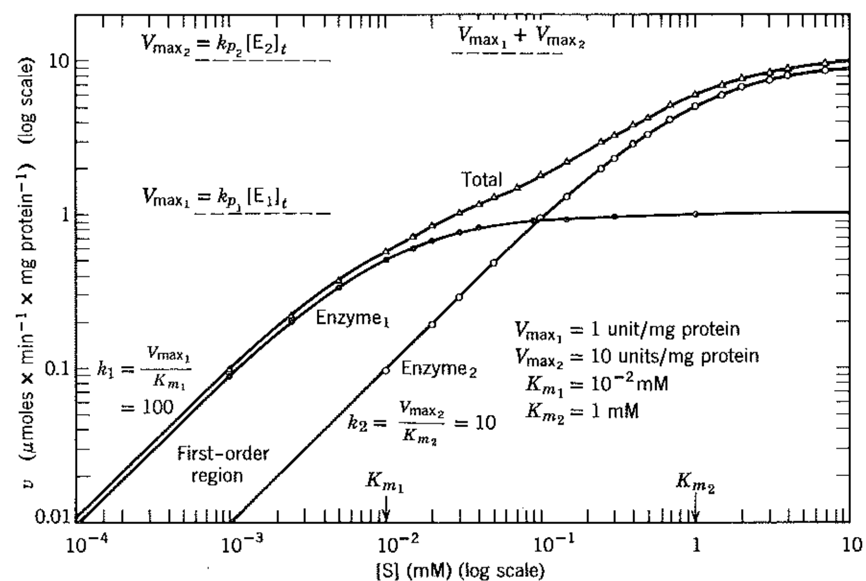


Fig. II-22. Log v versus log [S] plot in the presence of two enzymes: $V_{max_1} < V_{max_2}$, $K_{m_1} < K_{m_2}$.

the observed initial velocity in the first-order region. In the system described in Figure II-22, the V_{max}/K_m ratio of the low K_m–low V_{max} enzyme is 100, while the ratio for the high K_m–high V_{max} enzyme is 10. Accordingly, the low K_m–low V_{max} enzyme is the major contributor to the observed initial velocity at low [S]. In this system, the presence of two different enzymes is clearly indicated by the obvious inflection in the (total) velocity curve. Estimates of the K_m and V_{max} values can be obtained from reciprocal plots taking points from the region below the first inflection for the estimation of K_{m_1} and V_{max_1}, and points well within the second phase of the velocity curve for the estimation of K_{m_2} and $V_{max_1} + V_{max_2}$. At very low substrate concentrations, only the lower K_m enzyme contributes significantly to the observed velocity. Consequently, the reciprocal plot for the low substrate region approximates a straight line as 1/[S] increases (Fig. II-23). Extrapolation of the highest 1/[S]–$1/v$ points to the 1/[S]-axis gives $-1/K_{m_1}$. Under experimental conditions, it may be difficult to obtain accurate measurements of the initial velocity at sufficiently low substrate concentrations, and the highest 1/[S] points may not be high enough to approximate a good straight line.

Occasionally, linear plots are obtained which seem to have two distinct regions that can be fitted with different straight lines. Barring experimental error, the plot suggests the presence of two different enzymes. This is illustrated in Figures II-24, II-25, and II-26. The curves were calculated

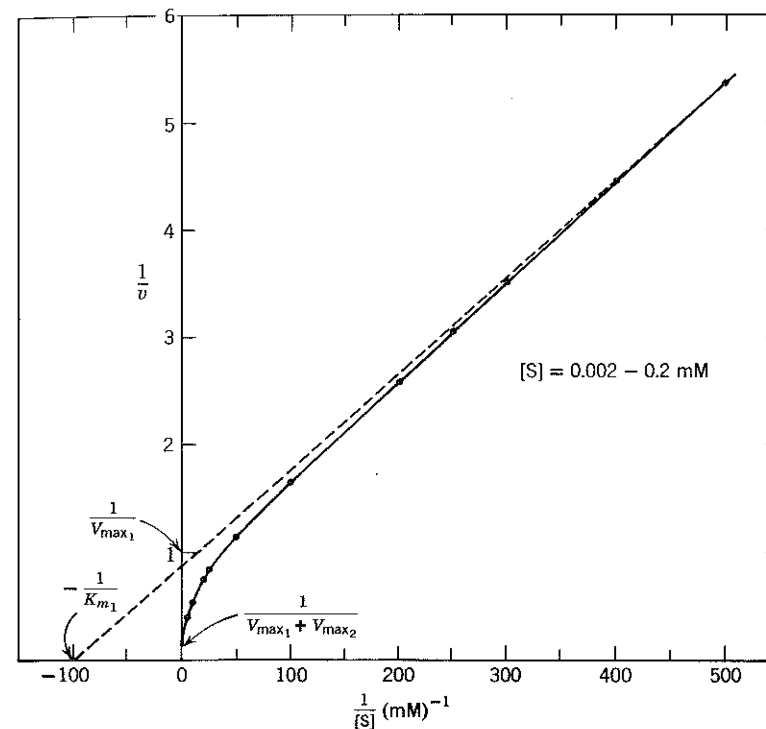


Fig. II-23. The $1/v$ versus 1/[S] plot in the presence of two enzymes: $V_{max_1} < V_{max_2}$, $K_{m_1} < K_{m_2}$ as in Fig. II-22. The [S] plotted is in the region of K_{m_1}.

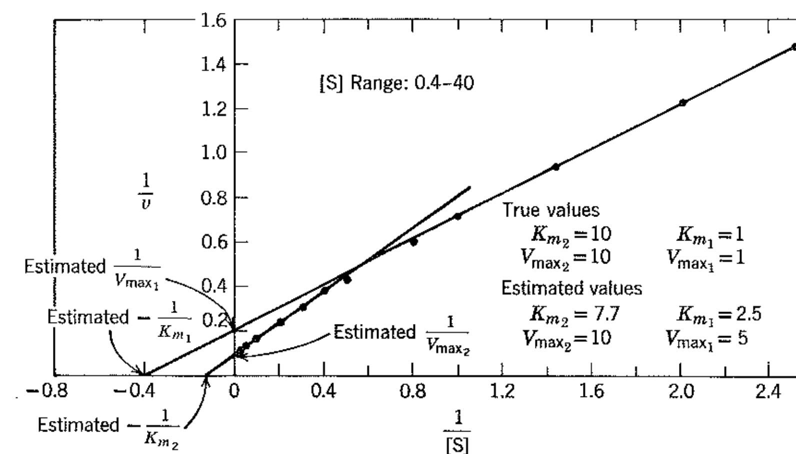


Fig. II-24. The $1/v$ versus 1/[S] plot in the presence of two enzymes.

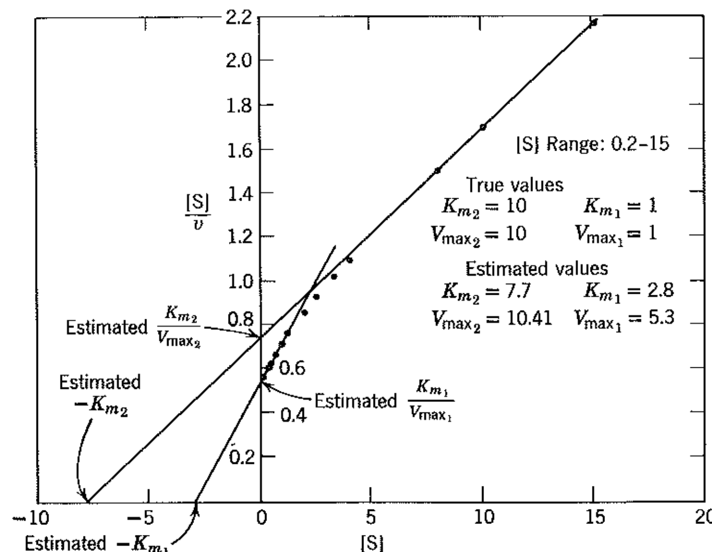


Fig. II-25. The [S]/v versus [S] plot in the presence of two enzymes.

assuming the additive velocities of two enzymes where $K_{m_1} = 1$, $V_{max_1} = 1$, and $K_{m_2} = 10$, $V_{max_2} = 10$. (The log-log plot would resemble that shown in Figure II-21.) The [S]/v versus [S] and v/[S] versus v plots are described in detail in Chapter Four. For the moment, however, we can see that these plots weight points at high and low v or high and low [S] more equally than the

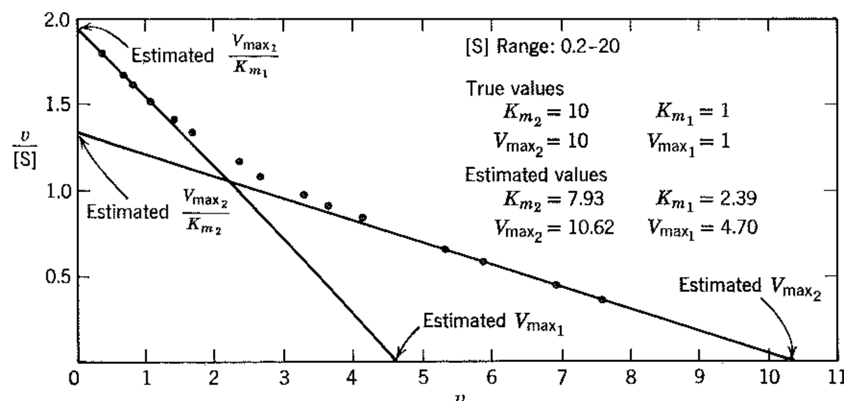


Fig. II-26. v/[S] versus v plot in the presence of two enzymes. Of the three linear plots, this one gives the best indication that more than one enzyme is present.

$1/v$ versus $1/[S]$ plot, and consequently, are more useful in detecting the presence of multiple enzymes. In spite of the apparent fit of the two straight lines on all three plots, the extrapolated intercepts do not yield the correct values for the constants. Spears, Sneyd, and Loten (1971) have described a procedure whereby the constants for two enzymes can be obtained by successive approximations. First, a linear plot is constructed from velocity measurements obtained at substrate concentrations above the approximate K_m of the high K_m enzyme (enzyme 2) (e.g., [S] = 5 to 100 for the example shown in Figures II-24 to II-26). The plot will give a first estimate of V_{max_2} and K_{m_2} (of about 10.8 and 8.3, respectively). The estimated constants are then used to calculate the velocity of enzyme 2 at substrate concentrations below the approximate K_m of the low K_m enzyme (enzyme 1) (e.g., [S] = 0.1 to 2 for the system shown in Figures II-24 to II-26). The contribution of enzyme 2 in this [S] region is subtracted from the observed velocity, and a linear plot is constructed from the corrected v values. This plot gives a first estimate of V_{max_1} and K_{m_1} (of about 0.46 and 0.67, respectively). These values in turn are then used to calculate the contribution of enzyme 1 in the high [S] region that is used to determine V_{max_2} and K_{m_2}. The contribution of enzyme 1 is subtracted from the observed velocities, and the corrected values plotted to obtain a second estimate of V_{max_2} and K_{m_2} (of about 10.4 and 9.1, respectively). The better estimates of V_{max_2} and K_{m_2} are used to correct the observed velocity at low [S] and the corrected values are plotted to obtain a second (and closer) estimate of V_{max_1} and K_{m_1} (0.71 and 0.80, respectively). The entire procedure can be repeated if closer estimates are desired. In general, the presence of two enzymes will be obvious, and reliable estimates of the four constants can be obtained only if $K_{m_2} > K_{m_1}$ and $V_{max_2} \geqslant V_{max_1}$. In addition, V_{max_2}/K_{m_2} (i.e., the first order rate constant, k_2) must be about equal to or less than V_{max_1}/K_{m_1} (i.e., k_1) so that a substrate concentration region exists where the low K_m enzyme contributes significantly to the observed velocity. If k_2 is much greater than k_1, enzyme 2 will obscure enzyme 1 even at low [S] concentrations. (If $k_2 > k_1$, the *estimated* contribution of enzyme 2 to the velocity at low [S] may be equal to or greater than the actual observed velocity because of experimental error and the fact that the first estimate of K_{m_2} will be slightly low.) In this case, it will be necessary to fractionate the preparation to obtain separate fractions significantly enriched with only one of the enzymes. Alternatively, it may be possible to increase the apparent K_{m_2}, or decrease the apparent V_{max_2}, or both, with an inhibitor that is specific for enzyme 2. Finally, keep in mind that curved reciprocal plots and multiphasic velocity curves do not necessarily indicate the presence of multiple enzymes. For example, multisite enzymes that have substrate binding sites of different affinities or display negative cooperativity also yield curved reciprocal plots.

M. KINETIC BEHAVIOR AT HIGH ENZYME CONCENTRATIONS

The [S] term in the Henri-Michaelis-Menten equation refers to *free* substrate, $[S]_f$. Generally, we do not bother to discriminate between $[S]_f$ and total substrate, $[S]_t$. That is, the derivation of the Henri-Michaelis-Menten equation assumes that $[S]_t$ is much greater than the total enzyme concentration, $[E]_t$, so that any decrease in the concentration of S by formation of ES is negligible. Thus we include the mass balance $[E]_t = [E] + [ES]$ in the derivation, but we neglect the mass balance $[S]_t = [S]_f + [ES]$. Under most *in vitro* assay conditions the enzyme concentration ranges from about 10^{-12} to $10^{-7} M$, while the substrate concentration ranges from about 10^{-6} to $10^{-2} M$. Under these conditions, the assumption that $[S]_f = [S]_t$ is quite valid.

Intracellular Concentrations of Enzymes

We might ask ourselves how valid is the assumption that enzymes are present at "catalytic" concentrations *in vivo*? We can estimate the intracellular enzyme concentration in several ways. For example, the protein content of most cells represents about 15 to 20% of the fresh weight. The average molecular weight of soluble proteins is about 100,000 to 150,000. If we assume that the entire fresh weight of cells is water and that all the soluble proteins are enzymes, then the total molarity of enzymes is:

$$\frac{150\text{ g/l}}{150{,}000\text{ g/mole}} = 10^{-3} M$$

If we assume that there are about 1000 different enzymes in the average cell, then the average concentration of an enzyme is about $10^{-6} M$ and we might expect a range between 10^{-8} and $10^{-4} M$ for individual enzymes.

Another estimate can be obtained from the enzymic activity of a given tissue and the known molecular weight and turnover number of the enzyme. For example, suppose we observe 40 units of enzyme activity per gram of fresh tissue for an enzyme with a specific activity of 3×10^9 units/mole. The molarity of the enzyme in the tissue is:

$$\frac{40 \times 10^3 \text{ units/l tissue water}}{3 \times 10^9 \text{ units/mole enzyme}} = 1.33 \times 10^{-5} M$$

The estimates above assume that the entire fresh weight of tissue is water and that there is no compartmentation. For mitochondrial enzymes, the

apparent concentrations would be about five times higher because in most cells of higher organisms mitochondria occupy 20% of the cell volume.

Most enzymes are composed of subunits which range in molecular weight from about 15,000 to 60,000. The average subunit molecular weight is about 40,000. If each subunit carries a substrate binding site, then the actual molar concentration of the catalytic unit is increased by another factor of 2 to 4. The K_m values for different enzymes range from about 10^{-6} to $10^{-2} M$. It seems reasonable to expect that the intracellular concentrations of substrates are in the neighborhood of their K_m values (otherwise the full potential of the enzymes would not be realized). Thus it seems likely that enzyme concentrations within cells are of the same order of magnitude as their substrates and, consequently, a significant fraction of $[S]_t$ may be bound as ES complexes. Substrate depletion by binding will also occur when $[E]_t$ is very low if the enzyme has a very high affinity for S. For example, if $[E]_t = 2 \times 10^{-8} M$ and $K_S = 10^{-8} M$, then 50% of the total substrate will be bound as ES when $[S]_t = 2 \times 10^{-8} M$. A general velocity equation, taking substrate depletion into account, is derived below. The equation expresses v/V_{max} in terms of $[E]_t$ and $[S]_t$.

$$\frac{[E][S]}{[ES]} = K_S$$

$$[E] = [E]_t - [ES]$$

$$[S] = [S]_f = [S]_t - [ES]$$

$$\frac{([E]_t - [ES])([S]_t - [ES])}{[ES]} = K_S \qquad \text{(II-53)}$$

$$[E]_t[S]_t - [E]_t[ES] - [S]_t[ES] + [ES]^2 = K_S[ES] \qquad \text{(II-53a)}$$

$$[ES]^2 - ([E]_t + [S]_t + K_S)[ES] + [E]_t[S]_t = 0$$

$$[ES] = \frac{([E]_t + [S]_t + K_S) - \sqrt{([E]_t + [S]_t + K_S)^2 - 4[E]_t[S]_t}}{2} \qquad \text{(II-53b)}$$

The positive root solution of the quadratic equation was neglected because the situation requires that $[ES] = 0$ when either $[S_t] = 0$ or $[E]_t = 0$. This can only be true when the negative root solution is taken. A binding or velocity

equation can be obtained by dividing by $[E]_t$:

$$\frac{[ES]}{[E]_t} = Y_S = \text{fraction of total sites occupied} = \frac{v}{V_{max}}$$

$$\therefore \quad \boxed{\frac{v}{V_{max}} = \frac{([E]_t + [S]_t + K_S) - \sqrt{([E]_t + [S]_t + K_S)^2 - 4[E]_t[S]_t}}{2[E]_t}} \qquad \text{(II-54)}$$

Equation II-54 is valid for all values of $[S]_t$ and $[E]_t$. When $[E]_t \ll K_S$, equation II-54 yields the same value for v/V_{max} or $[ES]/[E]_t$ as the Henri-Michaelis-Menten equation. As $[E]_t$ increases, the ratio at any fixed $[S]_t$ decreases because $[S]_f$ decreases.

The quadratic equation II-53a can be rearranged to a linear form which can be plotted to obtain K_S and $[E]_t$ if V_{max} is known (Henderson, 1973):

$$\boxed{\frac{[S]_t}{v} = K_S \frac{1}{V_{max} - v} + \frac{[E]_t}{V_{max}}} \qquad \text{(II-54a)}$$

For binding studies, v and V_{max} can be replaced with $k_p[ES]$ and $k_p[E]_t$, respectively. The equation becomes:

$$\boxed{\frac{[S]_t}{[ES]} = K_S \frac{1}{[E]_t - [ES]} + 1} \qquad \text{(II-54b)}$$

(See Chapter Four for a further discussion of equilibrium binding studies.)

Dixon Plot for Determining K_m and $[E]_t$

Dixon (1972) has described an elegant and simple direct plot for determining K_m and $[E]_t$ when a substantial fraction of the added $[S]_t$ is bound. Consider some point on the velocity curve where:

$$v = V_{max}\left(\frac{n-1}{n}\right) \qquad \text{(II-55)}$$

where n is a whole number. The velocity at any [S] is given by:

$$v = k_p[ES] \qquad \text{(II-56)}$$

Therefore, at the given point on the velocity curve:

$$k_p[ES] = V_{max}\left(\frac{n-1}{n}\right) \qquad \text{(II-57)}$$

As usual:

$$V_{max} = k_p[E]_t \qquad \text{(II-58)}$$

$$\therefore \quad k_p[ES] = k_p[E]_t\left(\frac{n-1}{n}\right) \quad \text{or} \quad [ES] = [E]_t\left(\frac{n-1}{n}\right) \qquad \text{(II-59)}$$

From equations II-15a and II-53, we can write:

$$K_m = \frac{([E]_t - [ES])([S]_t - [ES])}{[ES]} = ([E]_t - [ES])\left(\frac{[S]_t}{[ES]} - 1\right) \qquad \text{(II-60)}$$

Substituting for [ES] from equation II-59 and simplifying:

$$K_m = \frac{[S]_t}{n-1} - \frac{[E]_t}{n} \qquad \text{(II-61)}$$

A series of lines are drawn from the origin through points on the velocity curve where $v = ((n-1)/n)V_{max}$ (Fig. II-27*a*). Since n is a whole number (2,3,4, etc.), the points correspond to $\frac{1}{2}V_{max}$, $\frac{2}{3}V_{max}$, $\frac{3}{4}V_{max}$, and so on. Each line intersects a horizontal line of height V_{max} at different $[S]_n$ values, called $[S]_2$ (for the line drawn through $\frac{1}{2}V_{max}$), $[S]_3$ (for the line drawn through $\frac{2}{3}V_{max}$), and so on. The value of each $[S]_n$ (as shown earlier in Fig. II-12*b*) is given by:

$$[S]_n = \left(\frac{n}{n-1}\right)[S]_t \qquad \text{(II-62)}$$

where $[S]_t$ is the total substrate concentration required for a given n. Consequently,

$$[S]_t = \left(\frac{n-1}{n}\right)[S]_n \qquad \text{(II-63)}$$

and from equation II-61:

$$K_m = \left(\frac{n-1}{n}\right)\frac{[S]_n}{n-1} - \frac{[E]_t}{n} = \frac{[S]_n}{n} - \frac{[E]_t}{n}$$

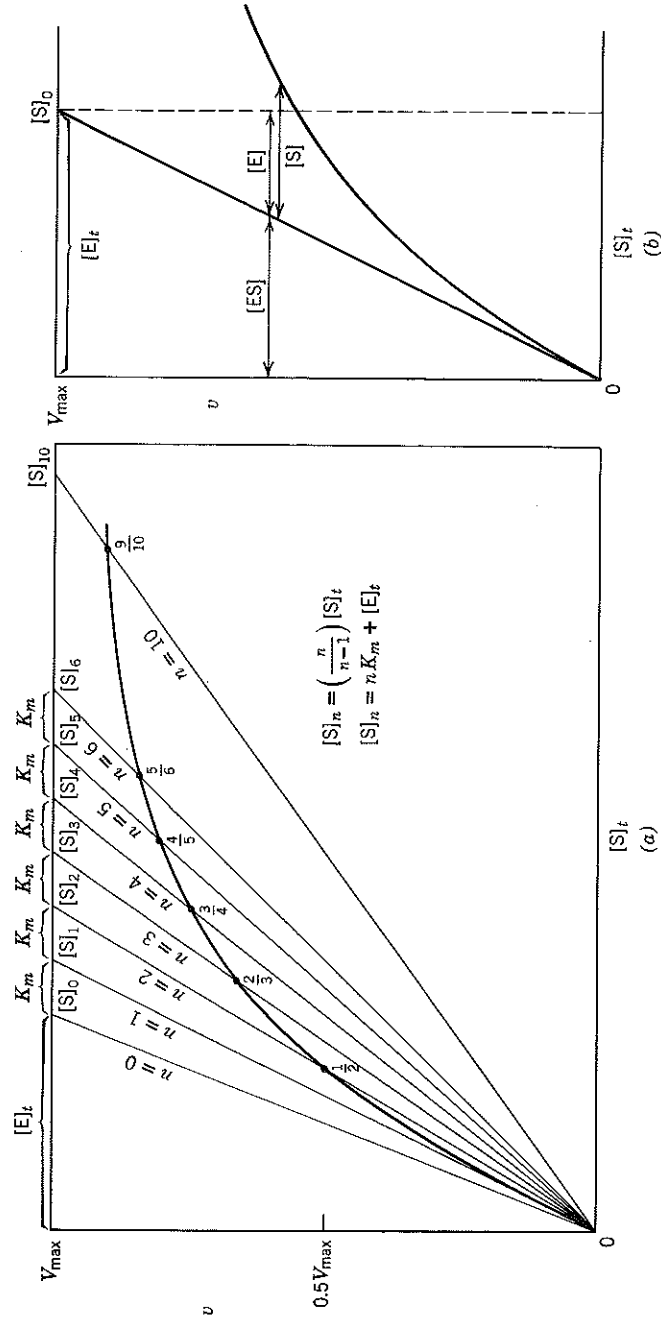


Fig. II-27. (*a*) Plot suggested by Dixon for determining $[E]_t$ when a significant fraction of the added substrate is bound to the enzyme. (*b*) Left-hand portion of (*a*).

or

$$[S]_n = nK_m + [E]_t \tag{II-64}$$

The intercepts on the V_{max} line occur at increments of K_m; that is,

$$[S]_n - [S]_{n-1} = K_m \tag{II-65}$$

A line drawn from the origin to a point on the horizontal V_{max} line one K_m distance to the left of the $n=2$ intercept represents the $n=1$ line. This line is tangent to the velocity curve at the origin. A line drawn similarly for $n=0$ intercepts the horizontal V_{max} line at $[E]_t$. Thus if $[E]_t \ll [S]_t$, this line is vertical and corresponds to the v-axis (as shown earlier in Fig. II-12*b*). The procedure requires a knowledge of V_{max}. As noted earlier, if the V_{max} value chosen is too low, the distances between intercepts (i.e., K_m) will decrease toward the right. If the V_{max} chosen is too high, the intervals will increase toward the right. Thus the method gives a check on the assumed value of V_{max}. The method also allows the concentration of all components of the system to be determined, as shown in Figure II-27*b*, which is just the left hand part of Figure II-27*a*. All that is required is a vertical line from $[S]_0$ to the $[S]_t$-axis and a diagonal line from $[S]_0$ to the origin. The components [ES], [E], and [S] (i.e., free S) as well as $[E]_t$ can be read off directly as appropriate distances between lines.

N. ENZYME ASSAYS

Initial Velocity as a Function of $[E]_t$

Under the usual *in vitro* assay conditions, the enzyme is present in limiting or "catalytic" amounts. The $[E]_t$ is generally 10^{-12} to $10^{-7}M$ while $[S]_t$ is generally 10^{-6} to $10^{-2}M$. At any substrate concentration the instantaneous or initial velocity is given by:

$$v = \frac{[S]\,V_{max}}{K_m + [S]} = \frac{[S]k_p[E]_t}{K_m + [S]} = \frac{k_p}{\left(1 + \frac{K_m}{[S]}\right)}[E]_t \tag{II-66}$$

Thus the initial velocity is directly proportional to $[E]_t$ at all substrate concentrations, and this fact can be used to quantitate the concentration of enzyme in any preparation, at any stage of purification. It should be stressed that the relationship between v and $[E]_t$ is linear only if true initial velocities are measured; that is, the rate of product formation must be constant over

the entire time interval of the assay. Since v varies with [S], the assay period must be short enough to ensure that only a small fraction of the substrate is utilized (about 5% or less). Figure II-28a shows the appearance of product at different concentrations of enzyme and a fixed substrate concentration. The rate of product formation, $d[P]/dt$, is constant for $[E]_{t_1}$ to $[E]_{t_4}$ up until time $= t_1$ (Fig. II-28b). If a longer assay time is chosen (e.g., t_2), the response would not be linear over the entire range of $[E]_t$. Similarly, if an enzyme concentration greater than $[E]_{t_4}$ is used, the response would not be linear for an assay time of t_1. Thus the first thing to do in any kinetic study of an enzyme is to establish the limits of linearity, that is, establish the maximum concentration of product that can accumulate before the [P] versus time and v versus $[E]_t$ responses become nonlinear. This applies to all kinetic studies where v means initial velocity. In initial velocity studies with multireactant enzymes, a preliminary check for linearity should be made at several combinations of [A], [B], [C], and so on, especially at (a) the lowest concentrations of all substrates that will be used together and (b) the lowest concentration of each substrate that will be used in the presence of the highest concentrations of the others. If a linear response of [P] versus time cannot be obtained with any $[E]_t$, because the concentration of one of the substrates changes markedly during the assay, then the equation of Lee and Wilson (equation II-42) can be used when that substrate is varied provided the concentrations of the other substrates do not change significantly.

Enzyme Units and Specific Activities—Quantitating $[E]_t$

In most preparations the actual molar concentration of enzyme is unknown. Consequently, the amount of enzyme present can be expressed only in terms of its activity. To standardize the reporting of enzyme activities, the Commission on Enzymes of the International Union of Biochemistry has defined a standard unit:

> *One International Unit (1 U) of enzyme is that amount which catalyzes the formation of 1 μmole of product per minute under defined conditions.*

The concentration of enzyme in an impure preparation can be expressed in terms of units/ml (i.e., mM/min), and the specific activity of the preparation as units/mg protein. As the enzyme is purified, the specific activity will increase to a limit (that of the pure enzyme). Since v varies with [S], pH, ionic strength, temperature, and so on, a given preparation can have an infinite number of specific activities. Consequently, specific activities are usually reported for optimal assay conditions at a fixed temperature (usually 25°C, 30°C, or 37°C), with all substrates present at saturating concentrations. Thus the specific activity of a preparation represents V_{max}/mg protein.

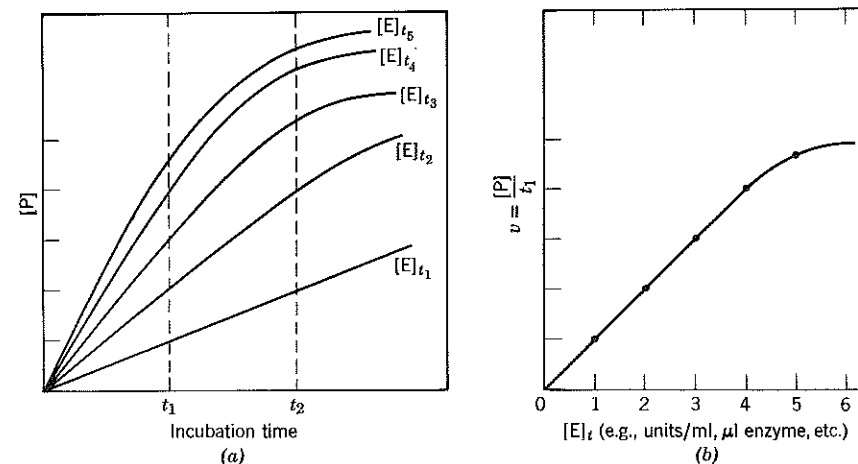


Fig. II-28. Enzyme assays: (a) Product formation as a function of time at different concentrations of enzyme. (b) Initial velocity (calculated as $[P]/t_1$) as a function of enzyme concentration.

Turnover Number

The term "turnover number" can be used in two ways. One way, which has been recently redefined as "molecular activity," is *the number of moles of substrate transformed per minute per mole of enzyme (units per micromole of enzyme) under optimum conditions*. Since many enzymes are oligomers containing n subunits, another possible "turnover number" is *the number of moles of substrate transformed per minute per mole of active subunit or catalytic center under optimum conditions*. This latter definition of catalytic power is called "catalytic center

activity." Both values are sometimes given simply as a number with dimensions of min^{-1}.

$$k_p = \frac{V_{max}}{[E]_t} = \frac{\mu\text{moles (of S}\longrightarrow\text{P)}\times\text{min}^{-1}\times\text{ml}^{-1}}{\mu\text{moles (of E)}\times\text{ml}^{-1}} = \text{min}^{-1}$$

Values of k_p range from about 50 to about $10^7\,\text{min}^{-1}$. Carbonic anhydrase has one of the highest turnover numbers known ($36\times10^6\,\text{min}^{-1}$). The reciprocal of k_p gives the time required for a single catalytic cycle. Thus, for carbonic anhydrase, $1/k_p = 1/(36\times10^6) = 0.028\times10^{-6}\,\text{min} = 1.7\ \mu\text{sec}$.

Quantitation of $[E]_t$ Using the Integrated Velocity Equation

One form of the integrated velocity equation is:

$$\boxed{V_{max}t = 2.3K_m \log\frac{[S]_0}{[S]_0-[P]} + [P]} \qquad \text{(II-67)}$$

Thus for any fixed $[S]_0$, the time required for the formation of a given [P] is inversely proportional to V_{max}; that is, $V_{max}t$ is constant for a given $[S]_0$ and [P]. Keep in mind that V_{max} is a measure of $[E]_t$. Thus the rule is usually stated as:

$$\boxed{[E]_t t = \text{constant}} \qquad \text{(II-68)}$$

If n units of enzyme produce 1 mM product in 5 min, then $2n$ units will yield 1 mM product in 2.5 min while $0.5n$ units will require 10 min. This relationship holds for all regions of $[S]_0$. Although $2n$ units yields 1 mM product in 2.5 min, it is not necessarily true that $2n$ units will yield 2 mM product in 5 min (unless $[S]_0 \gg K_m$ so that the reaction is zero order).

Reporting Data

Published data should always be reported in meaningful terms. Velocities and specific activities should be given in terms of units rather than as "cpm incorporated" or "ΔOD/min," although it is a good idea to include an extra vertical axis on at least one of the published figures where the actual raw data are shown. This serves to inform readers of the magnitudes of the actual observations. Raw data should not be reported in misleading terms. For example, do not report "cpm/mg protein," if only 5 μg of protein are used. Also, nonstandard "units" should be clearly defined (e.g., μmoles/hr or $n$moles/min).

Enzyme Purification

Enzymes are purified by employing successive chemical or physical fractionation procedures. The object of each step is to retain as much of the desired enzyme as possible while getting rid of as much of the other proteins, nucleic acids, and such, as possible. The efficiency of each step is given by the "yield" or "recovery" (the percentage of the total enzyme activity originally present that is retained) and the "purification" or "purification factor" (the factor by which the specific activity of the preparation has increased). The object is to optimize both factors. Sometimes a good yield is sacrificed for the sake of an excellent purification step; sometimes a good purification step is not used because the yield is too low. If the crude cell-free extract contains inhibitors, yields greater than 100% may be observed in the early stages of purification. Table II-2 shows a hypothetical purification scheme. The crude cell-free extract may be prepared by a number of means depending on the nature of the starting tissue or cells and the size of the preparation. Some common cell breakage methods include autolysis, freeze-thaw, sonic oscillation, mechanical grinding (with or without an abrasive), ballistic homogenization, or disruption in any one of a number of pressure cells (X-press, French press). The resulting homogenate is usually centrifuged to remove unbroken cells and large debris. There are no general rules concerning the order of the purification steps, although heat treatment (where possible) and ammonium sulfate precipitations are usually done early in the purification sequence. Gel filtration can follow ammonium sulfate precipitation and, thereby, desalt the preparation as well as fractionate the proteins according to size. If ion-exchange chromatography is to follow the ammonium sulfate step, then it is a good idea to dialyze the preparation first, or pass the preparation through a rapid gel filtration column (e.g., Sephadex G-25). The removal of the ammonium sulfate will facilitate the binding of the proteins to the ion-exchange column. Other steps not shown in Table II-2 that may be highly effective for certain enzymes include differential centrifugation (for mitochondria, chloroplasts, nuclei, microsomes, ribosomes), pH precipitation, organic solvent precipitation (e.g., ethanol, acetone), protamine sulfate or streptomycin sulfate pre-

Table II-2 Hypothetical Enzyme Purification Scheme

Step	Volume of Fraction (ml)	Protein: Concentration (mg/ml)	Protein: Total Amount (mg)	Enzyme: Concentration (units/ml)	Enzyme: Specific Activity (units/mg protein)	Enzyme: Total Amount (units)	Yield (%)	Purification Factor Fold
Crude cell-free extract	1000	12	12,000	5	0.416	5000	"100"	"1.00"
Heat step: 50°C for 5 min, then remove denatured protein	1000	8	8,000	4.8	0.60	4800	96	1.44
Ammonium sulfate precipitation: 30 to 50% saturation fraction	250	3	750	11.0	3.67	2730	55	8.83
Ion-exchange chromatography: DEAE-sephadex: elution via pH gradient. Fractions 50 to 60, 5 ml each, pooled, dialyzed and concentrated	25	9	225	88	9.8	2200	44	23.6
Ion exchange chromatography: DEAE-sephadex: elution via KCl gradient. Fractions 21 to 31, 2 ml each, pooled and concentrated	5	7	35	364	52	1820	36.4	125
Gel filtration: BioGel P-100. Fractions 30 to 40, 1 ml each, pooled.	10	0.92	9.2	170	185	1700	34	444
Hydroxyl apatite chromatography: Elution via phosphate buffer gradient. Fractions 15 to 18, 1 ml each, pooled.	4	0.75	3	375	500	1500	30	1200

cipitation (to precipitate nucleic acids and acidic proteins), affinity chromatography, and preparative gel electrophoresis. The purity of the final preparation should be checked by several methods before concluding that the preparation is homogeneous. Suitable methods include analytical disc gel electrophoresis at several pH values and gel concentrations, and ultracentrifugation. A homogeneous enzyme preparation should elute from an ion-exchange or gel filtration column as a single symmetrical activity and protein peak with a constant specific activity throughout. A homogeneous preparation is by no means necessary for kinetic analyses, but the purer the enzyme, the less the complications from competing reactions that may use up the substrate or the product.

Determination of v

An assay requires some method of measuring the rate of appearance of a product, $+d[\mathrm{P}]/dt$, or the rate of disappearance of a substrate, $-d[\mathrm{S}]/dt$. Theoretically, either measurement is sufficient but in practice the measurement of product appearance is usually more precise because the value is based on the difference between zero and a finite concentration. To measure v as $-d[\mathrm{S}]/dt$, the difference between $[\mathrm{S}]_0$ and $[\mathrm{S}]$ at time t must be large enough to determine accurately, but, at the same time, the difference must not be so large as to cause a significant change in the substrate concentration. The same problem arises in product inhibition studies. In this case, it is desirable to arrange conditions so that the product formed during the reaction can be distinguished from the product added to the reaction mixture. This can be accomplished by using radioactively labeled S and measuring the rate of labeled P appearance (assuming P* can be separated from S*). When two different products are formed, P and Q, it would be desirable to measure v as $d[\mathrm{Q}]/dt$ when P is the product inhibitor, and vice versa.

Assays with Auxiliary Enzymes

Frequently, the product of a reaction cannot be detected and quantitated directly, but it is possible to add an auxiliary enzyme which converts the product quantitatively to another substance which can be measured. The overall reaction sequence is:

$$\mathrm{A} \xrightarrow{\mathrm{E}_1} \mathrm{S} \xrightarrow{\mathrm{E}_2} \mathrm{P}$$

where E_1 is the enzyme being assayed and E_2 is the auxiliary enzyme. The conditions for the conversion of S to P by E_2 may not be compatible with those for the conversion of A to S. (One of the cosubstrates for the E_2

reaction may be an inhibitor of E_1; the pH optimum for the E_2 reaction may be quite different from that of the E_1 reaction; etc.) In this case, the assay is run in two stages. First A is incubated with E_1 (plus any cosubstrates) for a time sufficient to accumulate a detectable concentration of S. The reaction is then stopped (by boiling, or changing the pH, etc.). Then E_2 and all necessary cosubstrates are added, and the reaction is allowed to proceed until all the S accumulated in stage 1 is converted to P, which is then measured. The question is: how long should the second stage incubation time be to convert the maximum level of S to P for a fixed amount of E_2 and saturating cosubstrates of E_2? The incubation time for any level of S can be calculated from the integrated velocity equation:

$$t = \frac{2.3K_{m_S}}{V_{\max_{E2}}}\log\frac{[S]_0}{[S]_0-[P]} + \frac{[P]}{V_{\max_{E2}}} \qquad \text{(II-69)}$$

Since part of the reaction will be first-order, a 100% conversion of S to P will take an infinite time. Suppose we settle for 98% conversion, which would not introduce any significant error. If $K_{m_S} = 2\times10^{-4}M$, $V_{\max_{E2}} = 5\times10^{-5}M/\text{min}$ for the amount of auxillary enzyme used (i.e., 0.05 mM/min = 0.05 units/ml) and the maximum concentration of S that is allowed to accumulate is $1\times10^{-4}M$, the incubation time required to yield $0.98\times10^{-4}M$ P is:

$$t_{98\%} = \frac{(2.3)(2\times10^{-4})}{(5\times10^{-5})}\log\frac{10^{-4}}{2\times10^{-6}} + \frac{0.98\times10^{-4}}{5\times10^{-5}}$$

$$= (9.2)(1.7) + 1.96 = 18\,\text{min}$$

If the auxillary enzyme is very expensive, then we may wish to calculate the minimum amount needed to "complete" the reaction in a reasonable time. For example, to obtain 98% conversion of $10^{-4}M$ S in 30 min, we require:

$$V_{\max_{E2}} = \frac{(2.3)(2\times10^{-4})}{30}\log\frac{10^{-4}}{2\times10^{-6}} + \frac{0.98\times10^{-4}}{30}$$

$$= 2.94\times10^{-5}M/\text{min} = 0.0294\,\text{mM/min} \cong 0.03 \text{ units of } E_2/\text{ml}$$

If the K_{m_S} is 100 times or more greater than the maximum concentration of S that is allowed to accumulate, then the reaction S→P will always be first-order with respect to [S]. In this case, the concentration of S can be determined by measuring the *initial velocity* of the second stage reaction.

$$v = k[S] = \frac{V_{\max_{E2}}}{K_{m_S}}[S] \qquad \text{or} \qquad [S] = \frac{vK_{m_S}}{V_{\max_{E2}}}$$

Kinetics of Coupled Assays

If none of the conditions required for the $S \xrightarrow{E_2} P$ reaction are detrimental to the reaction $A \xrightarrow{E_1} S$ (and vice versa), then both stages of the assay can be carried out simultaneously. The amount of E_1 present can be determined by measuring the velocity of P formation. The following conditions are necessary for a valid coupled assay: (*a*) the primary reaction must be zero-order with respect to [A] over the assay time and irreversible, and (*b*) the second-stage reaction must be first order with respect to [S] and irreversible. Condition *a* is easily met if only a small fraction of $[A]_0$ is utilized during the assay period or if $[A]_0 \gg K_{m_A}$ for E_1 and all cosubstrates are saturating. Irreversibility is assumed by the removal of S in the second-stage reaction. Condition *b* is met if $[S]_{ss}$, the steady-state concentration of S, is $\ll K_{m_S}$ for E_2. The $[S]_{ss}$ can be maintained $\ll K_{m_S}$ by using a sufficient excess of E_2. Irreversibility can be assumed if the equilibrium of the E_2 reaction lies far to the right, or one of the coproducts of the reaction is continuously removed, or if the reaction proceeds to only a small extent. Under these conditions, A will yield a certain $[S]_{ss}$ *after a short lag*; thereafter, the rate of P formation will be constant and proportional to $[E_1]$ (Fig. II-29). If $[E]_1$ is doubled, $[S]_{ss}$ will double, and the rate of P formation will double. On the other hand, doubling $[E_2]$ halves $[S]_{ss}$ but because the velocity of the reaction S→P is given by $v_2 = k_2[S]_{ss}[E_2]$, v_2 is unchanged. Thus once a sufficient excess of E_2 is present, the rate of P formation will be independent of $[E_2]$. The amount of E_2 required to make the overall velocity dependent only on $[E_1]$ can be determined by trial and error, or calculated as shown by McClure (1969) and outlined below.

The differential rate equation for the conditions of the coupled assay is:

$$\frac{d[S]}{dt} = k_1 - k_2[S] \qquad \text{(II-70)}$$

where $k_1 = v_1$ = the pseudo-zero-order velocity of the A→S reaction
k_2 = the first-order rate constant of the S→P reaction

Equation II-70 integrates to:

$$[S] = \frac{k_1}{k_2}(1 - e^{-k_2 t}) \qquad \text{or} \qquad [S] = \frac{k_1}{k_2}(1 - 0.5^{t/t_{1/2}}) \qquad \text{(II-71)}$$

where [S] = the concentration of S at any time, t
$t_{1/2}$ = the half-life of the second stage reaction = $0.693/k_2$

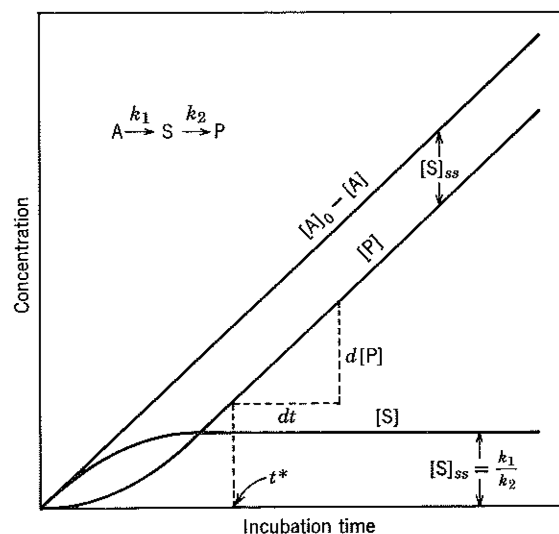


Fig. II-29. Kinetics of coupled assays: Substrate A disappears linearly from time zero, but the appearance of P becomes linear with time only after a lag period.

As $t \rightarrow \infty$, $[S] \rightarrow [S]_{ss}$, which is given by:

$$[S]_{ss} = \frac{k_1}{k_2} = 1.44 t_{1/2} k_1 \quad \text{(II-72)}$$

Thus the steady-state level of S represents the amount of A converted to S in 1.44 half-lives of the second stage reaction. Equation II-72 can be obtained directly by recalling that $v_1 = v_2$ when the steady-state is attained (i.e., $d[S]/dt = 0$).

$$v_1 = k_1, \qquad v_2 = k_2[S]_{ss} \qquad \therefore \quad [S]_{ss} = \frac{k_1}{k_2} \quad \text{(II-73)}$$

To obtain an expression for the time required to attain a practical fraction of $[S]_{ss}$, equation II-71 is rearranged and put into logarithmic form:

$$1 - \frac{k_2}{k_1}[S] = e^{-k_2 t}$$

$$2.3 \log\left(1 - \frac{k_2}{k_1}[S]\right) = -k_2 t \qquad \text{or} \qquad 2.3 \log\left(1 - \frac{[S]}{[S]_{ss}}\right) = -k_2 t \quad \text{(II-74)}$$

Letting F_S = the fraction of $[S]_{ss}$ desired at time $= t^*$, we obtain:

$$\boxed{t^* = -\frac{2.3 \log(1 - F_S)}{k_2}} \quad \text{(II-75)}$$

Substituting $V_{\max_{E2}}/K_{m_S}$ for k_2 and rearranging:

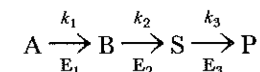
$$\boxed{V_{\max_{E2}} = -\frac{K_{m_S} 2.3 \log(1 - F_S)}{t^*}} \quad \text{(II-76)}$$

Equation II-76 gives the amount of auxillary E_2 required to obtain some practical fraction of $[S]_{ss}$ in time $= t^*$. Note that the time required to achieve a practical fraction of $[S]_{ss}$ is independent of k_1. As an example, suppose K_{m_S} for $E_2 = 2 \times 10^{-4} M$ at the pH and temperature used to assay E_1 and we accept $0.99[S]_{ss}$ as being experimentally equivalent to $[S]_{ss}$. How much E_2 must be added so that $[S]_t = 0.99[S]_{ss}$ in 6 sec?

$$V_{\max_{E2}} = \frac{-2 \times 10^{-4} M(2.3 \log 0.01)}{0.10\,\text{min}} = \frac{-(-9.2) \times 10^{-4}}{10^{-1}} = 9.2\,\text{mM/min} = 9.2\,\text{units/ml}$$

The 9.2 units/ml is the amount of auxillary enzyme required under the specific conditions used to assay E_1. If the stock solution of E_2 is given as 4600 units/ml at pH 8.5 and 37°C (optimum for E_2), but E_1 is being assayed at pH 6.8 and 25°C, then 2 μl of stock E_2 (i.e., "9.2 units") will not provide 9.2 units of activity. Obviously, the K_m and $V_{\max}$ of the auxillary enzyme should be determined under the conditions of use.

When two auxillary enzymes are used, the overall sequence is:



The object is to arrange conditions so that k_1 is a pseudo-zero-order rate constant while k_2 and k_3 are first-order rate constants; that is, $[B]_{ss} \ll K_{m_B}$ of E_2 and $[S]_{ss} \ll K_{m_S}$ of E_3, and only a small fraction of $[A]_0$ is utilized over the assay period. Under these conditions, the rate of P appearance will be constant (after a short lag) and proportional to $[E_1]$. The differential rate equation is:

$$\frac{d[S]}{dt} = k_2[B] - k_3[S]$$

Substituting for [B] from equation II-71:

$$\frac{d[\mathrm{S}]}{dt}=k_1(1-e^{-k_2t})-k_3[\mathrm{S}]$$

or

$$\frac{d[\mathrm{S}]}{dt}=k_1-k_1e^{-k_2t}-k_3[\mathrm{S}] \qquad \text{(II-77)}$$

As $t\rightarrow\infty$, $d[\mathrm{S}]/dt\rightarrow 0$ and $[\mathrm{S}]\rightarrow[\mathrm{S}]_{ss}$ given by:

$$[\mathrm{S}]_{ss}=\frac{k_1}{k_3} \qquad \text{(II-78)}$$

At any time, t, the concentration of S is given by:

$$[\mathrm{S}]=\frac{k_1}{k_3}-\frac{k_1}{k_3-k_2}\left(e^{-k_2t}-\frac{k_2}{k_3}e^{-k_3t}\right) \qquad \text{(II-79)}$$

When $t=\infty$, the parenthetical term reduces to zero and $[\mathrm{S}]=k_1/k_3=[\mathrm{S}]_{ss}$. Equation II-79 can be rearranged to:

$$(k_3-k_2)(1-F_\mathrm{S})=k_3e^{-k_2t^*}-k_2e^{-k_3t^*} \qquad \text{(II-80)}$$

where F_S = some fraction of $[\mathrm{S}]_{ss}$ attained at time $=t^*$. Equations II-79 and II-80 are symmetrical with respect to k_2 and k_3; that is, a pair of values for k_2 and k_3 can be interchanged without affecting the time required to attain F_S. When $k_3 \gg k_2$, equation II-80 becomes:

$$\boxed{(1-F_\mathrm{S})=e^{-k_2t^*}} \quad \text{or} \quad \boxed{t^*=-\frac{2.3\log(1-F_\mathrm{S})}{k_2}} \qquad \text{(II-81)}$$

In other words, as k_3 becomes very large compared to k_2, [S] approaches $[\mathrm{S}]_{ss}$ as fast as [B] approaches $[\mathrm{B}]_{ss}$. When $k_2 \gg k_3$ the equations are:

$$\boxed{(1-F_\mathrm{S})=e^{-k_3t^*}} \quad \text{or} \quad \boxed{t^*=-\frac{2.3\log(1-F_\mathrm{S})}{k_3}} \qquad \text{(II-82)}$$

Thus if a large excess of one of the auxillary enzymes is used, the amount of the other auxillary enzyme required for a given lag time, t^*, can be

calculated quite easily. In fact, if one constant is only 4 to 5 times greater than the other, equation II-81 or II-82 will predict t^* satisfactorily (a small difference between k_2 and k_3 causes a large difference in the exponential terms of equation II-80). When neither auxillary enzyme is in significant excess (i.e., $k_2 \cong k_3$), then there is no single solution for t^* (any number of combinations of $[\mathrm{E}_1]$ and $[\mathrm{E}_2]$ can produce a given lag). McClure (1969) has solved equation II-80 numerically and presented the solutions as a nomogram. Figures II-30*a* and *b* show the times required to reach 99% of $[\mathrm{S}]_{ss}$ for different values of k_2 and k_3.

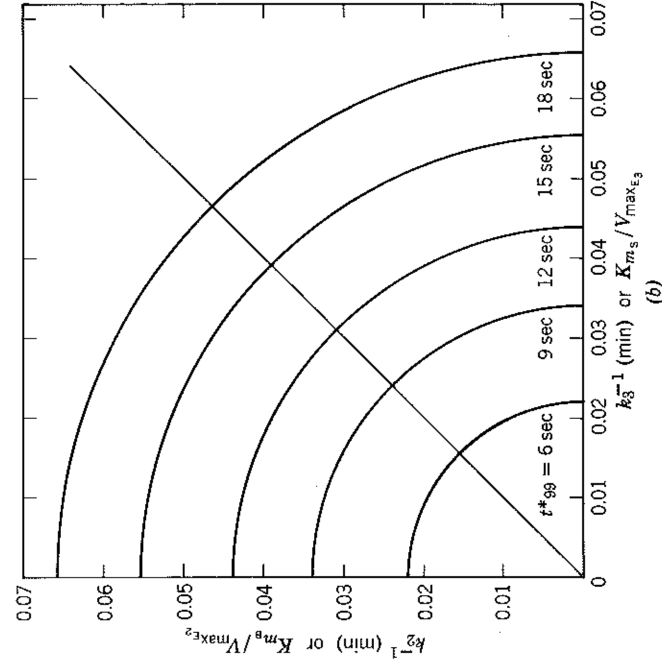
Although we have been concerned with using coupled assays to quantitate E_1, the same procedures can be used for initial velocity and product inhibition studies. For the latter, the inhibitory product of the E_1 reaction must be one other than that utilized by E_2 and must not be inhibitory to E_2 or E_3.

O. EFFECTS OF ENDOGENOUS SUBSTRATES

A crude cell-free extract may contain endogenous substrates of the enzyme under investigation. This extra substrate will be introduced into the assay mixture together with the enzyme and may contribute to the observed reaction velocity. A plot of v versus $[\mathrm{E}]_t$ at some subsaturating substrate concentration will not be linear since increasing $[\mathrm{E}]_t$ also increases the total [S]. For example, suppose the substrate concentration added to the assay mixture is equivalent to $0.1K_m$. Suppose further that the enzyme preparation contains 100 units of activity per 10 μl and is contaminated with endogenous substrate such that each 10 μl of extract contributes additional substrate equivalent to $0.1K_m$. The observed velocity for any amount of preparation is given by:

$$v=\frac{k_p[\mathrm{E}]_t\dfrac{[\mathrm{S}]_t}{K_m}}{1+\dfrac{[\mathrm{S}]_t}{K_m}}=\frac{k_p[\mathrm{E}]_t\dfrac{[\mathrm{S}]_{\mathrm{add}}+[\mathrm{S}]_{\mathrm{endog}}}{K_m}}{1+\dfrac{[\mathrm{S}]_{\mathrm{add}}+[\mathrm{S}]_{\mathrm{endog}}}{K_m}} \qquad \text{(II-83)}$$

where $k_p[\mathrm{E}]_t$ = the units of enzyme activity
= $V_{\max}$ for the given enzyme concentration at saturating [S]
$[\mathrm{S}]_{\mathrm{add}}$ = the substrate concentration added to the assay mixture
$[\mathrm{S}]_{\mathrm{endog}}$ = the endogenous substrate concentration introduced along with the enzyme preparation
$[\mathrm{S}]_t$ = the total substrate concentration in the assay mixture



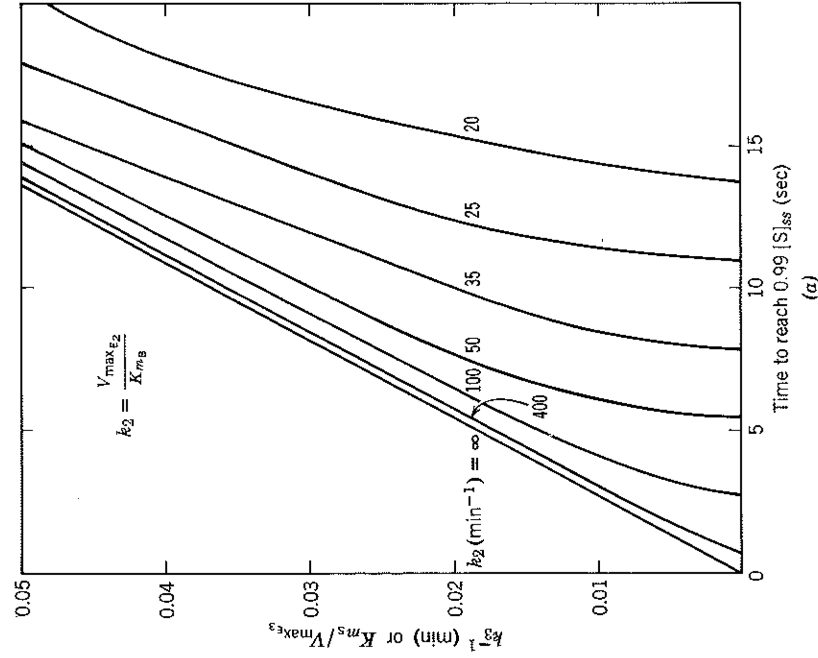


Fig. II-30. Nomograms of McClure showing the time required for [S] to attain 99% of its steady-state level in a coupled assay; k_2 and k_3 represent the effective first-order rate constants ($V_{\max}/K_m$) of the two coupling enzymes. [Redrawn with permission from McClure, W. R., *Biochemistry* **8**, 2782 (1969) Copyright by the American Chemical Society.]

At 10 μl of preparation:

$$v = \frac{(100)(0.1+0.1)}{1+(0.1+0.1)} = 16.65 \text{ units}$$

At 20 μl of preparation:

$$v = \frac{(200)(0.1+0.2)}{1+(0.1+0.2)} = 46.2 \text{ units}$$

Thus doubling the enzyme concentration more than doubles the observed velocity; halving the enzyme concentration yields a velocity less than half the original velocity (Fig. II-31). As the amount of enzyme preparation increases, $[S]_t$ increases. Eventually, the enzyme becomes saturated, and the v versus $[E]_t$ curve approaches a straight line with a limiting positive slope. If the assays were conducted with $[S]_{add} \gg K_m$, then the enzyme would always be saturated with S and the endogenous substrate would have no significant effect on the observed velocity. The observed v would always equal $V_{\max}$ for the amount of enzyme added, and the $V_{\max}$ versus $[E]_t$ curve would be linear. Nonlinear v versus $[E]_t$ curves may also be observed if the enzyme preparation contains endogenous activators. If the activator functions by decreasing the apparent K_m, then the nonlinearity will disappear when the assay is run at saturating [S]. However, if the activator increases the apparent $V_{\max}$, then nonlinear v versus $[E]_t$ plots will be seen even when $[S] \gg K_m$.

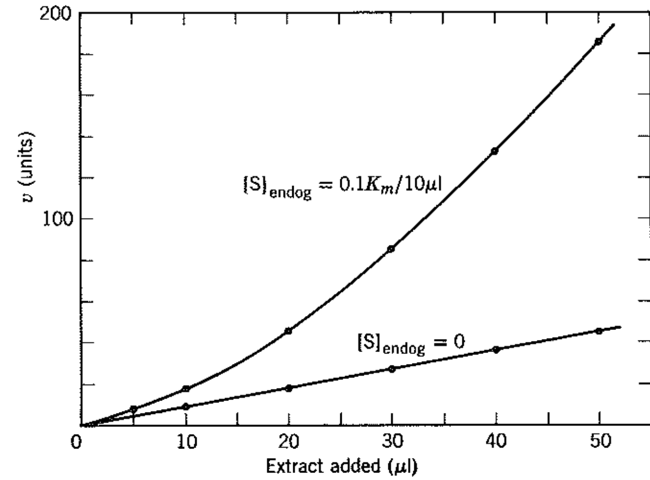


Fig. II-31. Effect of endogenous substrate on the plot of v versus amount of enzyme preparation added. $[E]_t = 100$ Units/10 μl of extract; $[S]_{endog} = 0.1\ K_m/10\ \mu$l of extract. The assays are run at $[S]_{add} = 0.1\ K_m$.

We can also consider the effect of endogenous substrates on the determination of K_m and V_{max}. The raw data would consist of a series of initial velocities observed at various concentrations of substrate and a fixed concentration of enzyme. If the enzyme preparation contains endogenous substrates, then we would observe a reaction velocity at zero added substrate. (The endogenous substrate would not have been used up prior to the assay if the cells were broken and centrifuged at 4°C, or if the reaction requires additional substrates or cofactors that are not present in the crude extract.) The v versus $[S]_{add}$ data might be as shown below in columns 1 and 2. The third column represents an attempt to "correct" the observed initial velocity for the contribution of the endogenous substrate. The numbers in the table were chosen for an enzyme with a K_m of 1.0, an $[E]_t$ per assay equivalent to a V_{max} of 1.0, and a level of contaminating endogenous substrate in the preparation sufficient to add $[S]_{endog} = K_m$ to each assay mixture. Figure II-32 shows the reciprocal plots of $1/v_{observed}$ versus $1/[S]_{add}$, $1/v_{\text{“corrected”}}$ versus $1/[S]_{add}$, and the true curve that would be observed if no endogenous substrate were present. The plot of $1/v_{observed}$ versus $1/[S]_{add}$ is curved and intercepts the $1/v$-axis at $1/V_{max}$. This is not surprising since the contribution of the endogenous substrate disappears as the concentration of added substrate approaches infinity. At high values of $1/[S]_{add}$, the plot approaches a horizontal limit. In other words, as the concentration of added substrate approaches zero, the velocity plateaus at the value dictated by the fixed concentration of endogenous substrate. The "corrected" data yield a linear reciprocal plot, but the intercepts do not give the correct values for K_m and V_{max}. The fact that the plot is linear can easily deceive an investigator into believing that the "correction" was valid. The correction would be valid if the "zero added substrate" value represented endogenous noninhibitory product or a compound that behaves like the true product in the final

$[S]_{add}$	Observed v	"Corrected" v
0	0.500	0.000
0.5	0.600	0.100
1	0.667	0.167
2	0.750	0.250
3	0.800	0.300
5	0.858	0.358
8	0.900	0.400
20	0.955	0.455
100	0.990	0.490

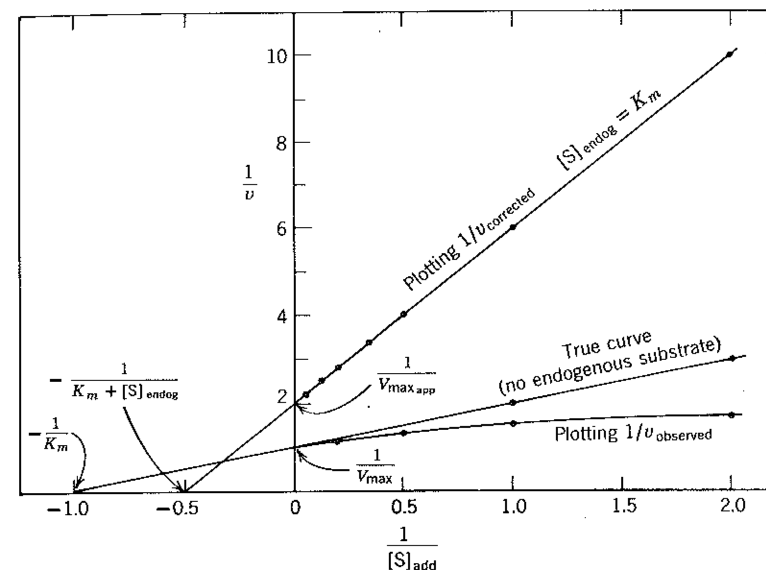


Fig. II-32. $1/v$ versus $1/[S]$ plot in the presence of an endogenous substrate.

analysis. (Non-enzymatic formation of P from endogenous or added S will also complicate the determination of K_m and V_{max}. In this case, a "minus enzyme" control should be run at each added substrate concentration.) Because valid corrections for endogenous substrates or activators cannot be made easily, it is good practce to dialyze or pass a crude extract through a gel filtration column before assaying the enzyme.

If an assay is based on the rate of production of radioactively labeled P from labeled S, endogenous unlabeled substrate will not only affect the assay substrate concentration, but also the specific radioactivity of the substrate. The effect on the calculated reaction rate is illustrated in Table II-3. Column 1 indicates the concentration of labeled substrate in the assay mixture (i.e., the assumed concentration). Column 2 indicates the true substrate concentration assuming that the amount of crude cell-free extract contributes $[S] = K_m$. Column 3 indicates the actual specific radioactivity of the substrate after dilution of the labeled added substrate (S.A. $= 10^5$ cpm/μmole) by endogenous unlabeled substrate. Column 4 indicates the true reaction rate. Column 5 indicates the observed rate in terms of cpm/min. If this rate is converted to μmoles/min using the assumed specific activity of the substrate (10^5 cpm/μmole), we obtain the calculated rate shown in column 6. Surprisingly, a reciprocal plot of $1/v_{calc}$ versus $1/[S]_{add}$ is linear (Fig. II-33). The curve extrapolates to the true $1/V_{max}$, which is not

Table II-3 Effect of Unlabeled Endogenous Substrate on the Calculated Reaction Rates in an Assay Employing a Radioactive Substrate[a]

Assumed Substrate Concentration $[S^*]_{add}$(mM)	Actual Substrate Concentration $[S^*]_{add}+[S]_{endog}$	True Specific Activity (cpm/μmole)	True Rate (μmoles/min)	Observed Rate (cpm/min)	Calculated Rate (μmoles/min)
0.1	1.1	0.91×10^4	5.24	4.76×10^4	0.476
0.2	1.2	1.66×10^4	5.46	9.06×10^4	0.906
0.25	1.25	2.0×10^4	5.56	11.1×10^4	1.11
0.33	1.33	2.5×10^4	5.72	14.3×10^4	1.43
0.50	1.5	3.33×10^4	6.0	20×10^4	2.0
1.0	2.0	5.0×10^4	6.67	33.4×10^4	3.34
2.0	3.0	6.66×10^4	7.5	50×10^4	5.0
3.33	4.33	7.67×10^4	8.13	62.4×10^4	6.24
4.0	5.0	8.0×10^4	8.33	66.7×10^4	6.67
5	6.0	8.33×10^4	8.57	71.5×10^4	7.15
10	11.0	9.11×10^4	9.16	83.6×10^4	8.36

[a] $V_{max}=10\mu$moles/min, $K_m=1$mM, S.A. of $S^*=10^5$ cpm/μmole, $[S]_{endog}=1$mM. Vol = 10 ml.

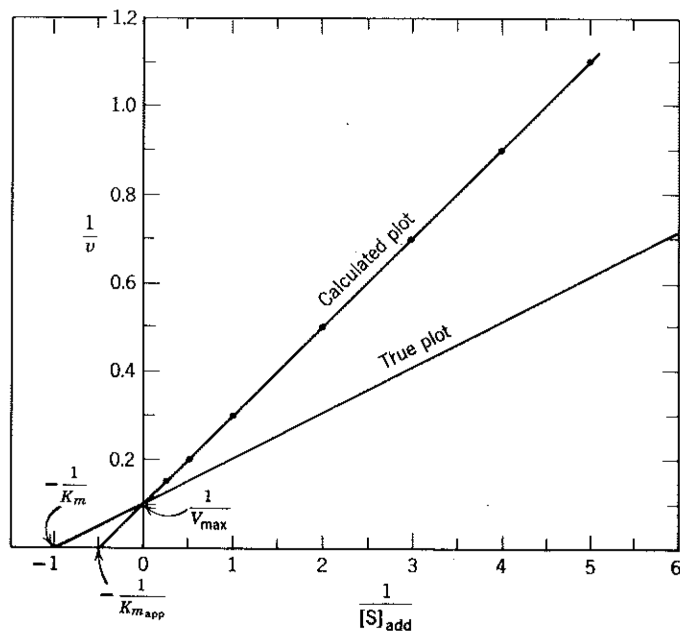
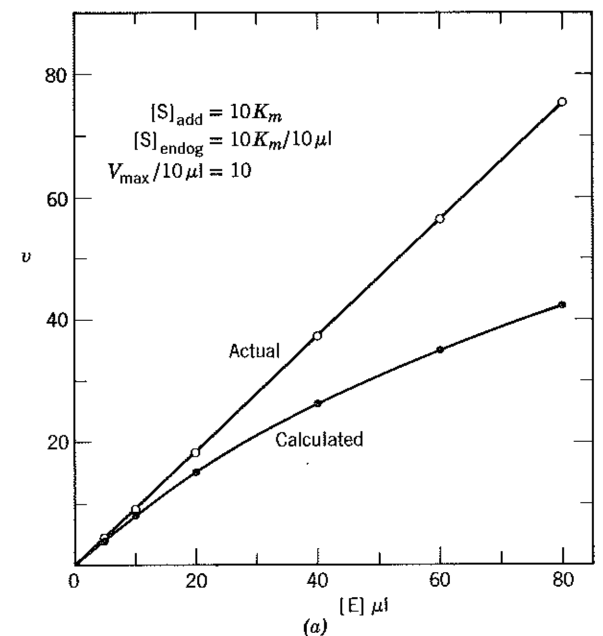


Fig. II-33. Effect of endogenous substrate ($[S]=K_m$) on the $1/v$ versus $1/[S]$ plot when the assay depends on the rate of radioactive P appearance (see data in Table II-3).



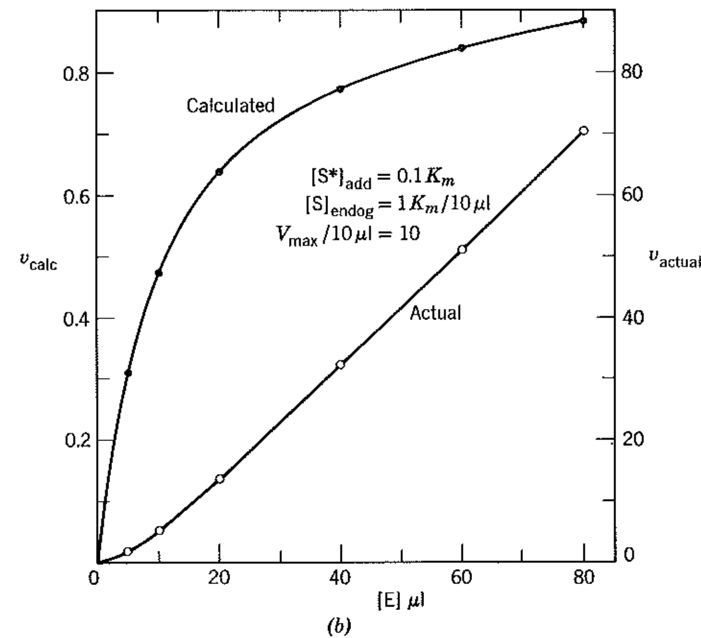


Fig. II-34. Effect of endogenous substrate on the v versus [E] plot when the assay depends on the rate of radioactive P appearance.

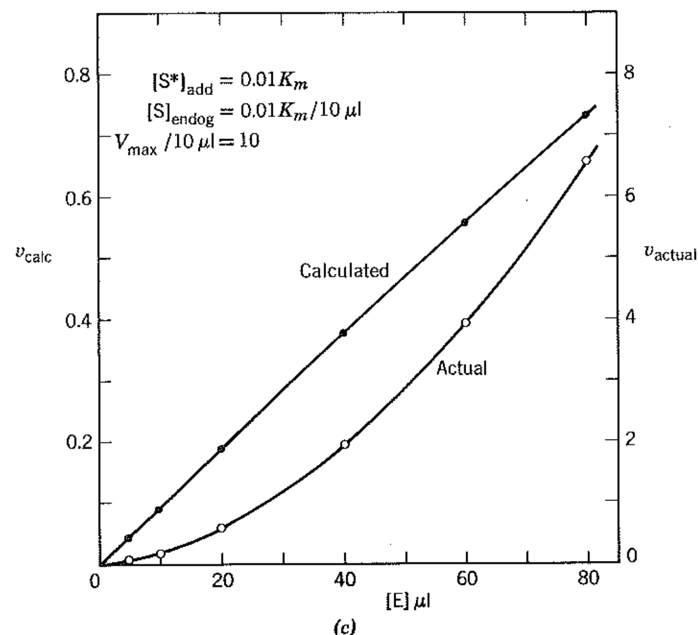


Fig. II-34. (Cont.)

unexpected. (As $[S^*]_{add}$ increases, the difference between the assumed and true substrate concentrations decreases and at the same time the specific activity of the substrate approaches the assumed value.) The extrapolated K_m, however, is incorrect. In fact, the unlabeled endogenous substrate has exactly the same effect on K_m as a competitive inhibitor (Chapter Three).

$$K_{m_{app}} = K_m\left(1 + \frac{[S]_{endog}}{K_m}\right) \tag{II-84}$$

The v versus [E] plots will be nonlinear. The curvature is opposite to that observed when a nonradioactive assay is used. Three examples are shown in Figure II-34*a*, *b*, and *c*. At low $[S^*]_{add}$ and low $[S]_{endog}$, the calculated velocities may yield a near-linear v versus [E] plot (Fig. II-34*c*), yet the calculated velocities (hence the calculation of $[E_t]$) can be significantly in error. The effects of contaminating inhibitors in the enzyme preparation and in the substrate are discussed in Chapter Three.

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