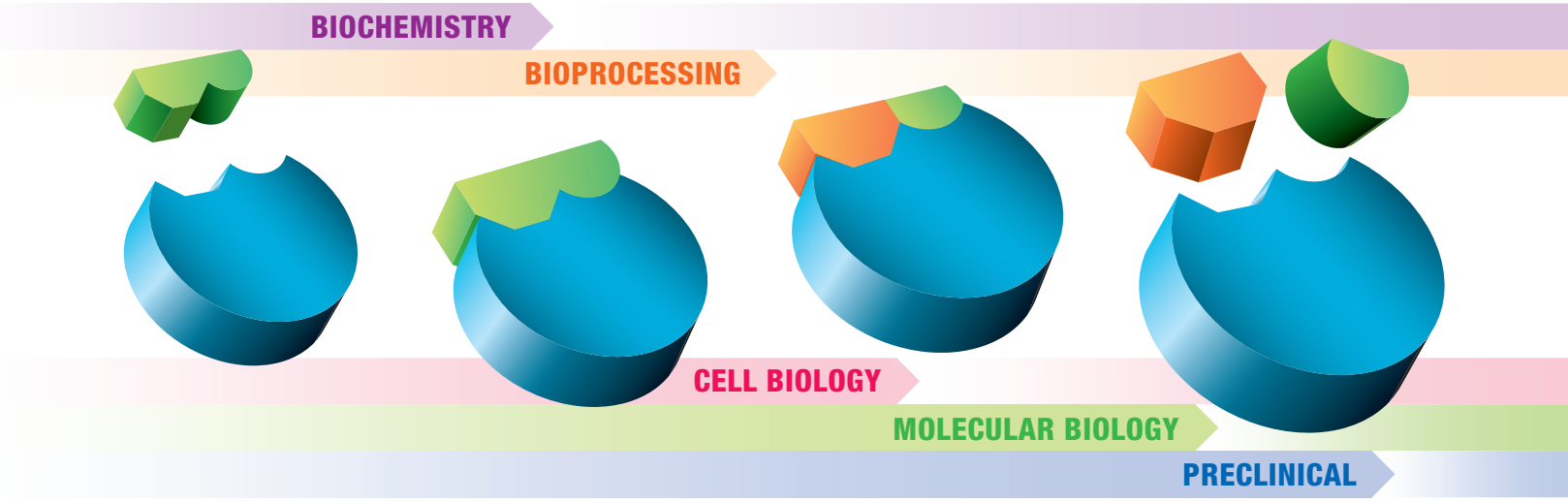
INTRODUCTION TO
ENZYMES



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INTRODUCTION TO ENZYMES

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The use of enzymes in the diagnosis of disease is one of the important benefits derived from the intensive research in biochemistry since the 1940s. Enzymes have provided the basis for the field of clinical chemistry.

Given the dramatic growth of life science research over recent decades, interest in diagnostic enzymology has multiplied. New AI online tools aid researchers in the area of medical research in which the diagnostic potential of enzyme reactions can be found.

Prepared by Worthington Biochemical Corporation, this overview is a practical introduction to enzymology. As a result of close involvement over the years in the theoretical as well as the practical aspects of enzymology, Worthington's knowledge covers a broad spectrum of the subject. This information has been assembled here for the benefit of a new generation of researchers.

Enzymes and Life Processes

The living cell is the site of tremendous biochemical activity called metabolism. This is the process of chemical and physical change which goes on continually in the living organism. These changes include the build-up of new tissue, replacement of old tissue, conversion of food to energy, disposal of waste materials, reproduction, etc. – all the activities that we characterize as "life."

This building up and tearing down takes place in the face of an apparent paradox. The greatest majority of these biochemical reactions do not take place spontaneously. The phenomenon of catalysis makes possible biochemical reactions necessary for all life processes. Catalysis is defined as the acceleration of a chemical reaction by some substance which itself undergoes no permanent chemical change. The catalysts of biochemical reactions are enzymes and are responsible for bringing about almost all of the chemical reactions in living organisms. Without enzymes, these reactions take place at a rate far too slow for the pace of metabolism.

The oxidation of a fatty acid to carbon dioxide and water is not a gentle process in a test tube – extremes of pH, high temperatures and corrosive chemicals are required. Yet in the body, such a reaction takes place smoothly and rapidly within a narrow range of pH and temperature. In the laboratory, the average protein must be boiled for about 24 hours in a 20% HCl solution to achieve a complete breakdown. In the body, the breakdown takes place in four hours or less under conditions of mild physiological temperature and pH.

It is through attempts at understanding more about enzyme catalysts – what they are, what they do, and how they do it – that many advances in medicine and the life sciences have been brought about.

Early Enzyme Discoveries

The existence of enzymes has been known for well over a century. Some of the earliest studies were performed in 1835 by the Swedish chemist, Jon Jakob Berzelius who termed their chemical action *catalytic*. It was not until 1926, however, that the first enzyme was obtained in pure form, a feat accomplished by James B. Sumner of Cornell University. Sumner was able to isolate and crystallize the enzyme urease from the jack bean. His work was to earn him the 1947 Nobel Prize.

John H. Northrop and Wendell M. Stanley of the Rockefeller Institute for Medical Research shared the 1947 Nobel Prize with Sumner. They discovered a complex procedure for isolating and purifying pepsin. This precipitation technique devised by Northrop and Stanley has been used to crystallize several enzymes.

Chemical Nature of Enzymes

Most enzymes are proteins, although a few are catalytic RNA molecules. Catalytic RNA molecules are called ribozymes. Enzymes are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds (**Figure 1**). Enzymes can be denatured and precipitated with salts, solvents and other reagents. They have molecular weights ranging from 10,000 to 2,000,000 Da.

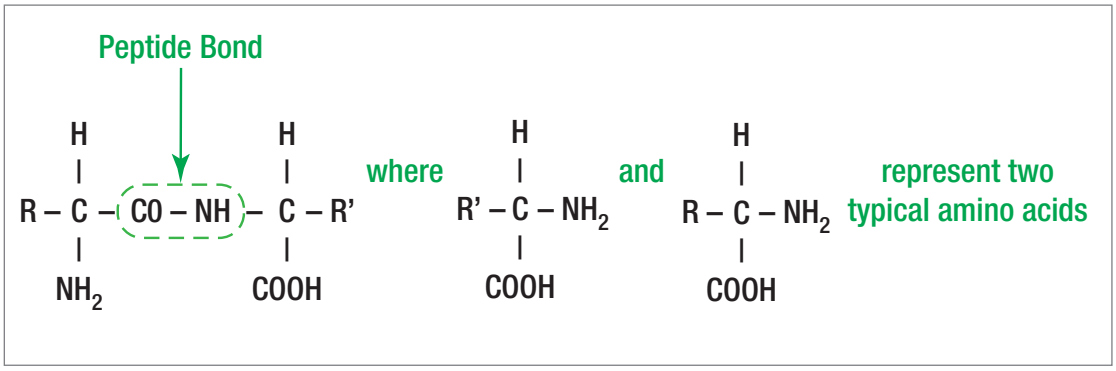

Figure 1: Typical protein structure – two amino acids joined by a peptide bond.

Many enzymes require the presence of other compounds – cofactors – before their catalytic activity can be exerted. This entire active complex is referred to as the holoenzyme; i.e., apoenzyme (protein portion) plus the cofactor(s) (coenzyme, prosthetic group or metal-ion activator) **(Figure 2)**.

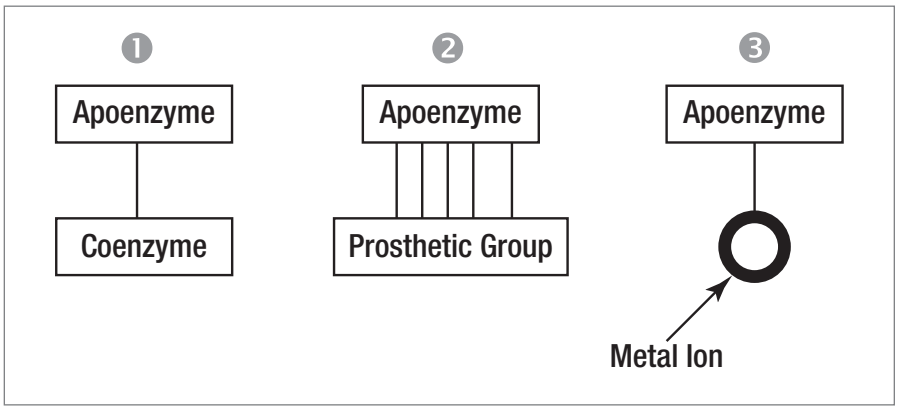

Figure 2: Holoenzymes plus various types of cofactors.

According to Holum, the cofactor may be:

1. **A coenzyme** – a non-protein organic substance which is dialyzable, thermostable and loosely attached to the protein part.
2. **A prosthetic group** – an organic substance which is dialyzable and thermostable which is firmly attached to the protein or apoenzyme portion.
3. **A metal-ion-activator** – these include K^{+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, and Mo^{3+}.

Specificity of Enzymes

One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group.

In general, there are four distinct types of specificity:

1. **Absolute specificity** – the enzyme will catalyze only one reaction.
2. **Group specificity** – the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
3. **Linkage specificity** – the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
4. **Stereochemical specificity** – the enzyme will act on a particular steric or optical isomer.

Though enzymes exhibit great degrees of specificity, cofactors may serve many apoenzymes. For example, nicotinamide adenine dinucleotide (NAD) is a coenzyme for a great number of dehydrogenase reactions in which it acts as a hydrogen acceptor. Among them are the alcohol dehydrogenase, malate dehydrogenase and lactate dehydrogenase reactions.

Naming and Classification

Except for some of the originally studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in “ase”. The International Union of Biochemistry (I.U.B.) initiated standards of enzyme nomenclature which recommended that enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. Under this system, the enzyme uricase is called urate: O_2 oxidoreductase, while the enzyme glutamic oxaloacetic transaminase (GOT) is called L-aspartate: 2-oxoglutarate aminotransferase.

The I.U.B. devised a system of classification and identification of enzymes in terms of the reactions they catalyse. This relies on a numerical system (the EC number) to classify enzymes in groups according to the types of reaction catalysed and systematic naming that describes the chemical reaction involved. This is now in widespread use, and the official list of enzymes classified can be found at ExplorEnz – The Enzyme Database (http://www.enzyme-database.org). The enzyme nomenclature is incorporated into many other resources, including the ExPASy-ENZYME, BRENDA and KEGG bioinformatics databases.

The EC number or Enzyme Commision number is a four-component identifier which classifies an enzyme according to class, subclass, sub-subclass and the final compontent being a serial number within that sub-subclass. The EC classes are currently listed as:

1. **Oxidoreductases** – To this class belong all enzymes catalysing oxidation reduction reactions. The substrate that is oxidized is regarded as hydrogen donor.
2. **Transferases** – Transferases are enzymes transferring a group, e.g. a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor).

3. **Hydrolases** – These enzymes catalyse the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds.
4. **Lyases** – Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds.
5. **Isomerases** – These enzymes catalyse geometric or structural changes within one molecule. According to the type of isomerism, they may be called racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases or cycloisomerases.
6. **Ligases** – Ligases are enzymes catalysing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate
7. **Translocases** – Catalysing the translocation of hydrogen ions, inorganic cations and anions, amino acids, carbohydrates or other compounds. A new EC class was created in 2018.

Basic Enzyme Reactions

Enzymes are catalysts that increase the the speed of a chemical reaction without themselves undergoing any permanent chemical change. They are neither used up in the reaction nor do they appear as reaction products.

The basic enzymatic reaction can be represented as follows:

$$\mathbf{S} + \mathbf{E} \rightarrow \mathbf{P} + \mathbf{E}$$

E represents the enzyme catalyzing the reaction; **S**, the substrate, the substance being modified; and **P**, the product of the reaction.

The Enzyme Substrate Complex

A theory to explain the catalytic action of enzymes was proposed by the Swedish chemist Savante Arrhenius in 1888. He proposed that the substrate and enzyme formed some intermediate substance which is known as the enzyme/substrate complex (**ES**). The reaction can be represented as:

$$\mathbf{S} + \mathbf{E} \rightarrow \mathbf{ES}$$

S = Substrate; **E** = Enzyme; **ES** = Enzyme/Substrate Complex

If this reaction is combined with the original reaction equation, the following results:

$$\mathbf{S} + \mathbf{E} \rightarrow \mathbf{ES} \rightarrow \mathbf{P} + \mathbf{E}$$

S = Substrate; **E** = Enzyme; **ES** = Enzyme/Substrate Complex; **P** = Product

The existence of an intermediate enzyme-substrate complex has been demonstrated in the laboratory, for example, using catalase and a hydrogen peroxide derivative. At Yale University, Kurt G. Stern observed spectral shifts in catalase as the reaction it catalyzed proceeded. This experimental evidence indicates that the enzyme first binds with the substrate and then returns to its original form after the reaction is concluded.

Chemical Equilibrium

The study of a large number of chemical reactions reveals that most do not go to true completion. This is likewise true of enzymatically-catalyzed reactions. This is due to the reversibility of most reactions.

In general:

$$A + B \xrightarrow{K_{+1}} C + D \text{ (Forward Reaction)}$$

$$C + D \xrightarrow{K_{-1}} A + B \text{ (Revese Reaction)}$$

K_{+1} is the forward reaction rate constant and K_{-1} is the rate constant for the reverse reaction.

Combining the two reactions gives:

$$A + B \underset{K_{-1}}{\overset{K_{+1}}{\rightleftarrows}} C + D$$

Applying this general relationship to enzymatic reactions allows the equation:

$$E+S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftarrows}} ES \underset{K_{-2}}{\overset{K_{+2}}{\rightleftarrows}} P + E$$

Equilibrium, a steady state condition, is reached when the forward reaction rates equal the backward rates. This is the basic equation upon which most enzyme activity studies are based.

Energy Levels

Chemists have known for almost a century that for most chemical reactions to proceed, some form of energy is needed. They have termed this quantity of energy, "the energy of activation." It is the magnitude of the activation energy which determines just how fast the reaction will proceed. It is believed that enzymes lower the activation energy for the reaction they are catalyzing **(Figure 3)**. The enzyme is thought to reduce the "path" of the reaction. This shortened path would require less

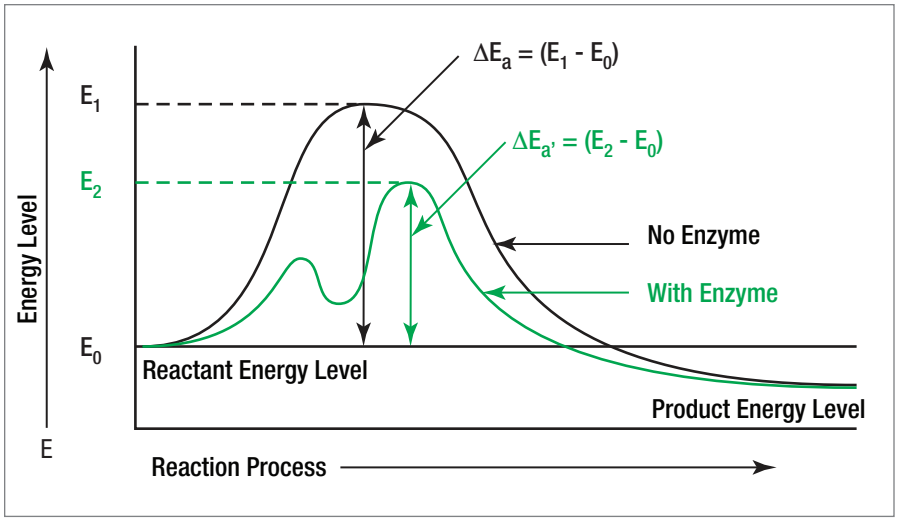

Figure 3: Energy of activation ΔE_a is less than $\Delta E_a'$ due to the effect of the enzyme on the substrate.

energy for each molecule of substrate converted to product. Given a total amount of available energy, more molecules of substrate would be converted when the enzyme is present (the shortened "path") than when it is absent. Hence, the reaction is said to go faster in a given period of time.

Factors Affecting Enzyme Activity

Knowledge of basic enzyme kinetic theory is important for enzyme analysis in order to both understand the basic enzymatic mechanism and select a method for enzyme analysis. The conditions selected to measure the activity of an enzyme would not be the same as those selected to measure the concentration of its substrate. Several factors affect the rate at which enzymatic reactions proceed – enzyme concentration, substrate concentration, the presence of any inhibitors or activators, temperature, and pH.

Enzyme Concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; i.e., the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. Graphically this can be represented as indicated in **Figure 4**. These reactions are said to be "zero order" because the rates are independent of substrate concentration, and are equal to some constant k. The formation of product proceeds at a rate which is linear with time. The addition of more substrate does not serve to increase the rate. In zero order kinetics, allowing the assay to run for double time results in double the amount of product **(Table 1)**.

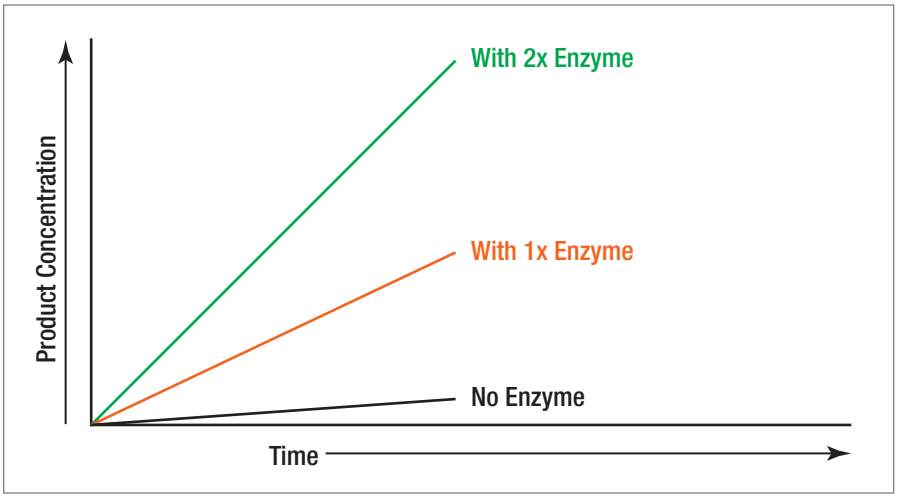

Figure 4: "Zero order" reaction rate is independent of substrate concentration.

Table 1: Reaction orders with respect to substrate concentration.

Order	Rate Equation	Comments
Zero	rate = k	rate is independent of substrate concentration
First	rate = k[S]	rate is proportional to the first power of substrate concentration
Second	rate = k[S][S] = $k[S]^2$	rate is proportional to the square of the substrate concentration
Second	rate = $k[S_1][S_2]$	rate is proportional to the first power of each of two reactants

The amount of enzyme present in a reaction is measured by the activity it catalyzes. The relationship between activity and concentration is affected by many factors such as temperature, pH, etc. An enzyme assay must be designed so that the observed activity is proportional to the amount of enzyme present in order that the enzyme concentration is the only limiting factor. It is satisfied only when the reaction is zero order.

In **Figure 5**, activity is directly proportional to concentration in the area AB, but not in BC. Enzyme activity is generally greatest when substrate concentration is not limiting.

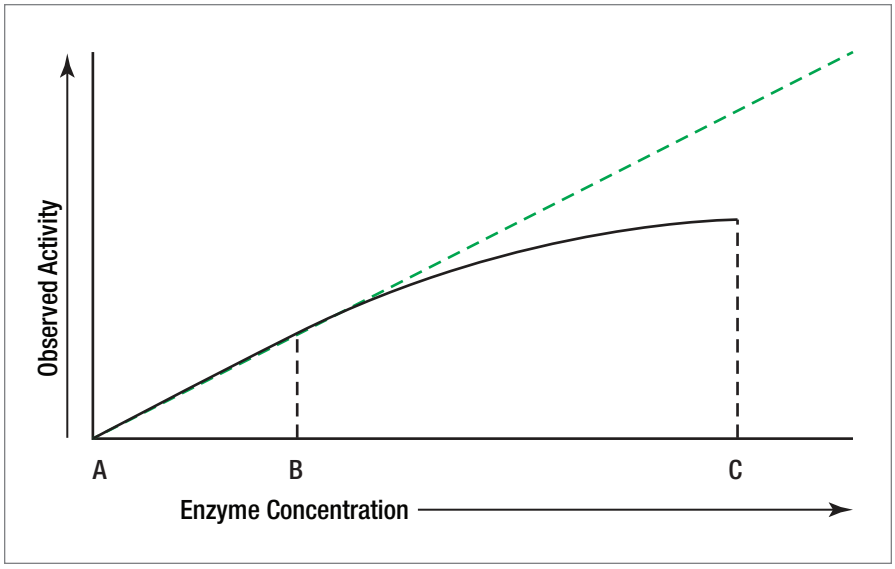

Figure 5: Activity vs. concentration.

When the concentration of the product of an enzymatic reaction is plotted against time, a similar curve results **(Figure 6)**. Between A and B, the curve represents a zero order reaction; that is, one in which the rate is constant with time. A substrate is used up, the enzyme's active sites are no longer saturated, substrate concentration becomes rate limiting, and the reaction becomes first order between B and C.

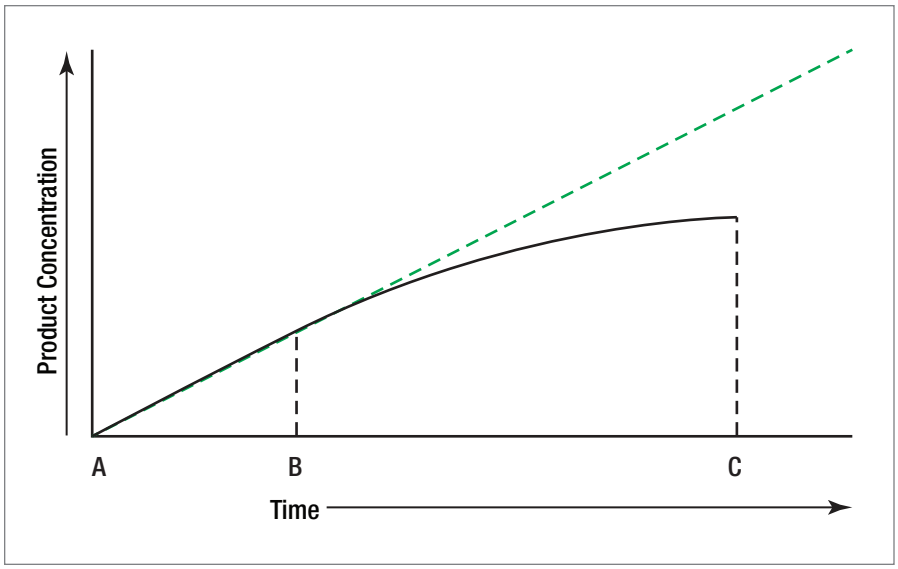

Figure 6: Reaction rate limited by substrate concentration.

To measure enzyme activity ideally, the measurements must be made in that portion of the curve where the reaction is zero order. A reaction is most likely to be zero order initially since substrate concentration is then highest. To be certain that a reaction is zero order, multiple measurements of product (or substrate) concentration must be made.

Figure 7 illustrates three types of reactions which might be encountered in enzyme assays and shows the problems which might be encountered if only single measurements are made.

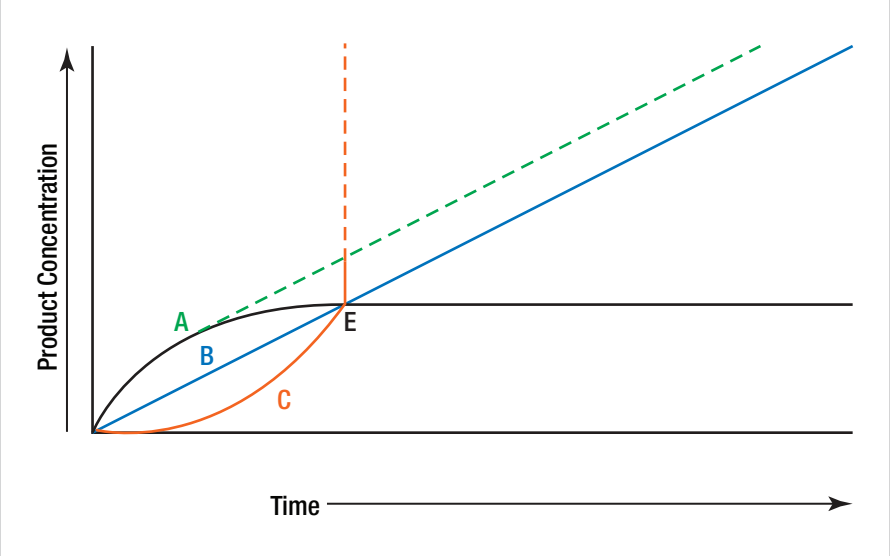

Figure 7: Leading, lagging, and linear reaction.

B is a straight line representing a zero order reaction which permits accurate determination of enzyme activity for part or all of the reaction time. **A** represents the type of reaction that was shown in **Figure 6**. This reaction is zero order initially and then slows, presumably due to substrate exhaustion or product inhibition. This type of reaction is sometimes referred to as a "leading" reaction. True "potential" activity is represented by the dotted line. Curve **C** represents a reaction with an initial "lag" phase. Again the dotted line represents the potentially measurable activity. Multiple determinations of product concentration enable each curve to be plotted and true activity determined. A single end point determination at **E** would lead to the false conclusion that all three samples had identical enzyme concentration.

Substrate Concentration

It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum. After this point, increases in substrate concentration will not increase the velocity (Δ Absorbance/Δ Time). This is represented graphically in **Figure 8**.

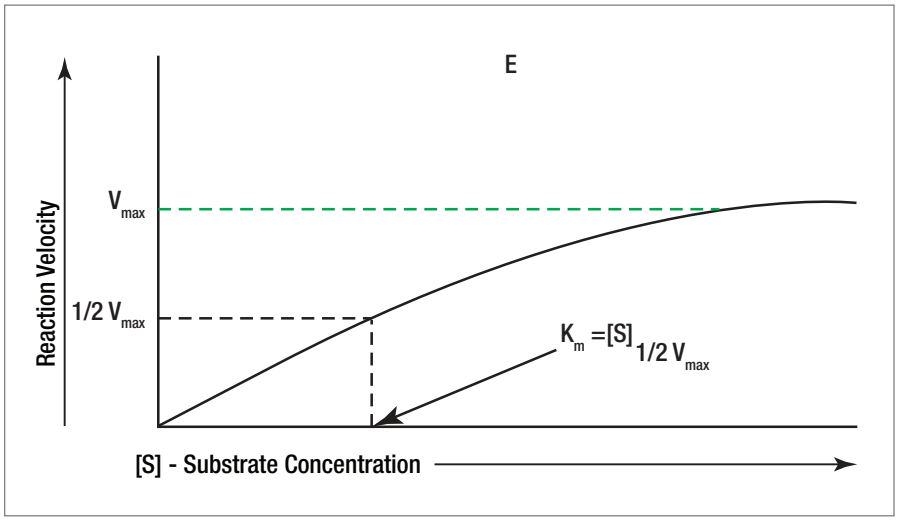

Figure 8: Effect of substrate concetration.

It is theorized that when this maximum velocity had been reached, all of the available enzyme has been converted to **ES**, the enzyme/substrate complex. This point on the graph is designated Vmax. Using this maximum velocity and equation, Michaelis developed a set of mathematical expressions to calculate enzyme activity in terms of reaction speed from measurable laboratory data.

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftarrows}} ES \underset{K_{-2}}{\overset{K_{+2}}{\rightleftarrows}} P + E \qquad K_m = \frac{K_{-1} + K_2}{K_{+1}} = S_{Vmax} \qquad v_t = \frac{V_{max}[S]}{K_m + [S]}$$

v_t = the velocity at any time
[S] = the substrate concentration at this time
V_{max} = the highest velocity under this set of experimental conditions (pH, temperature)
K_m = the Michaelis constant for the particular enzyme being investigated

Michaelis constants have been determined for many of the commonly used enzymes. The size of K_m tells us several things about a particular enzyme.

1. A small K_m indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.
2. A large K_m indicates the need for high substrate concentrations to achieve maximum reaction velocity.
3. The substrate with the lowest K_m upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes.

Effects of Inhibitors on Enzyme Activity

Enzyme inhibitors are substances which alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop catalysis. There are three common types of enzyme inhibition – competitive, non-competitive and substrate inhibition.

Most theories concerning inhibition mechanisms are based on the existence of the enzyme-substrate complex ES. As mentioned earlier, the existence of temporary ES structures has been verified in the laboratory.

Competitive inhibition occurs when the substrate and a substance resembling the substrate are both added to the enzyme. A theory called the "lock-and-key theory" of enzyme catalysts can be used to explain why inhibition occurs **(Figure 9)**.

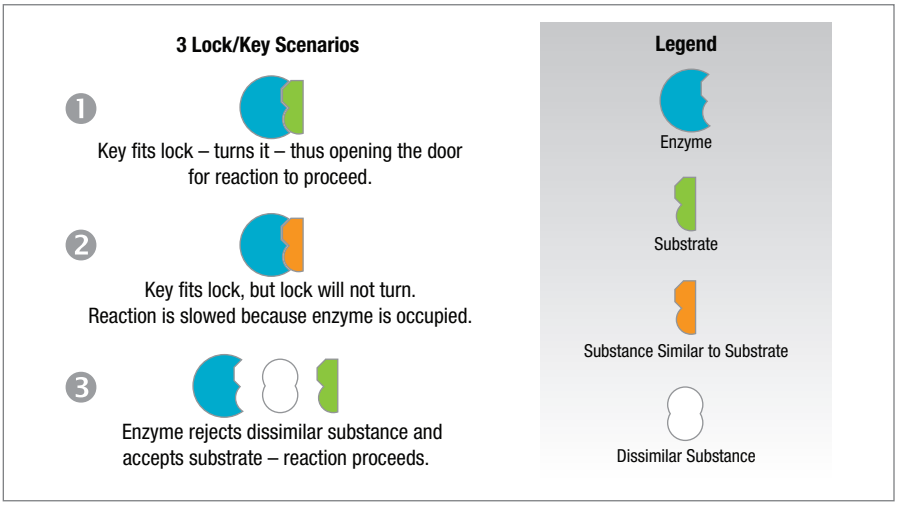

Figure 9: Lock and key theory – competitive analysis.

The lock and key theory utilizes the concept of an "active site." The concept holds that one particular portion of the enzyme surface has a strong affinity for the substrate. The substrate is held in such a way that its conversion to the reaction products is more favorable. We consider the enzyme as the lock and the substrate the key – the key is inserted in the lock, the key is turned, and the door is opened (the reaction proceeds) **(Figure 9)**. However, when an inhibitor which resembles the substrate is present, it will compete with the substrate for the position in the enzyme lock. When the inhibitor wins, it gains the lock position but is unable to open the lock. Hence, the observed reaction is slowed down because some of the available enzyme sites are occupied by the inhibitor. If a dissimilar substance that does not fit the site is present, the enzyme rejects it, accepts the substrate instead, and the reaction proceeds normally.

Non-competitive inhibitors are considered to be substances which when added to the enzyme alter the enzyme in a way that it can no longer accept the substrate **(Figure 10)**. Substrate inhibition will sometimes occur when excessive amounts of substrate are present.

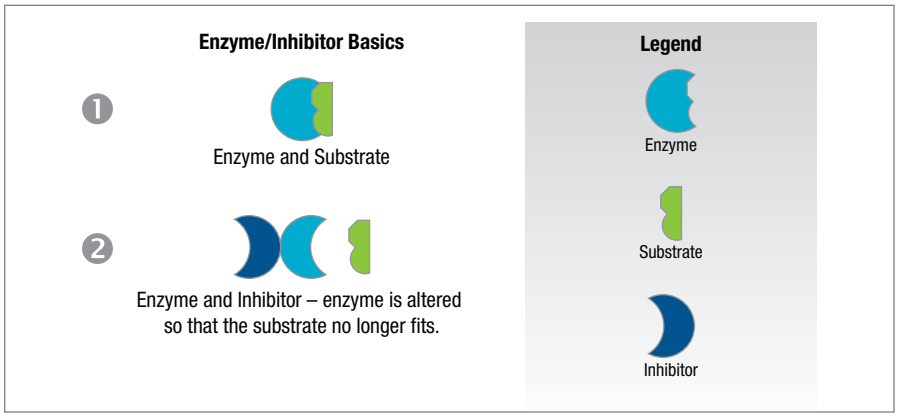

Figure 10: Non-competitive inhibition.

Figure 11 shows the reaction velocity decreasing after the maximum velocity has been reached.

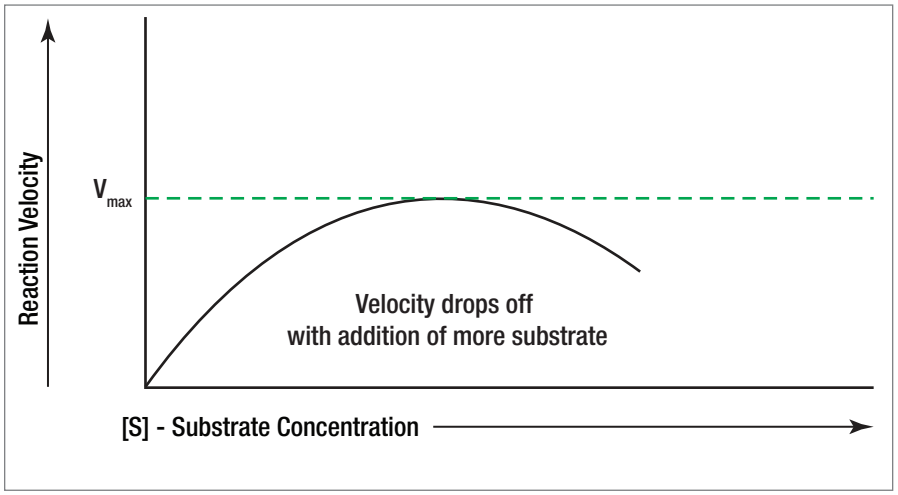

Figure 11: Substrate becoming rate-inhibiting.

Additional amounts of substrate added to the reaction mixture after this point actually decrease the reaction rate. This is thought to be due to the fact that there are so many substrate molecules competing for the active sites on the enzyme surfaces that they block the sites and prevent any other substrate molecules from occupying them **(Figure 12)**.

This causes the reaction rate to drop since all of the enzyme present is not being used.

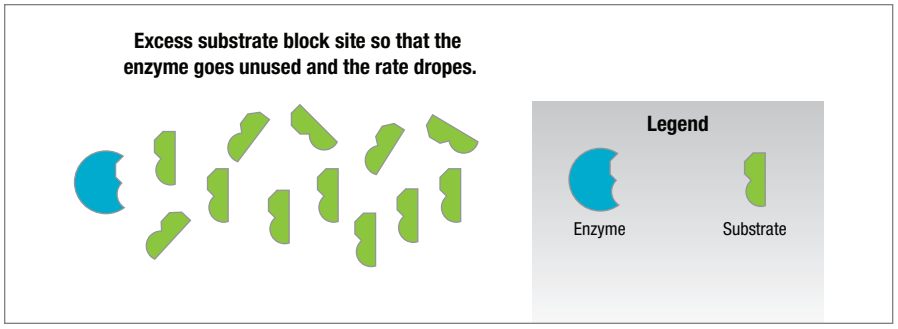

Figure 12: Substrate inhibition.

Temperature Effects

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. As shown in **Figure 13**, the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most animal enzymes rapidly become denatured at temperatures above 40°C, most enzyme determinations are carried out somewhat below that temperature.

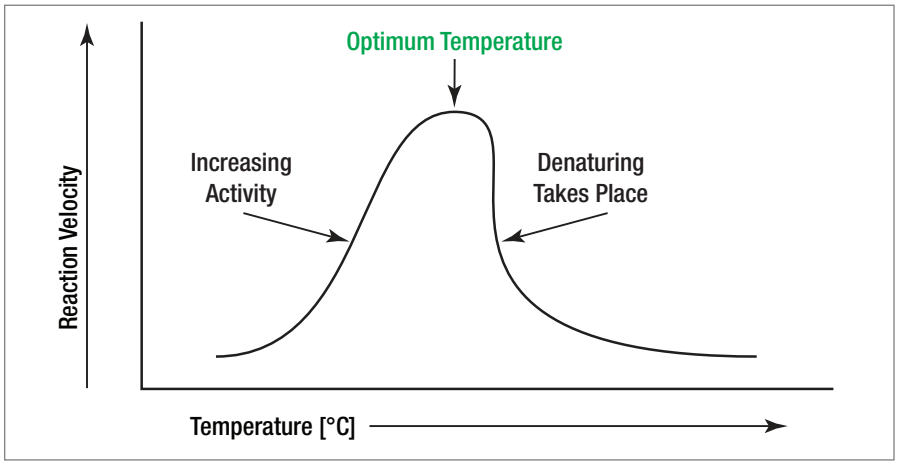

Figure 13: The effect of temperature on the reaction rate.

Over a period of time, enzymes will be deactivated at even moderate temperatures. Storage of enzymes at 5°C or below is generally the most suitable. Some enzymes lose their activity when frozen.

Effects of pH

Enzymes are affected by changes in pH. The most favorable pH value – the point where the enzyme is most active – is known as the optimum pH. This is graphically illustrated in **Figure 14**.

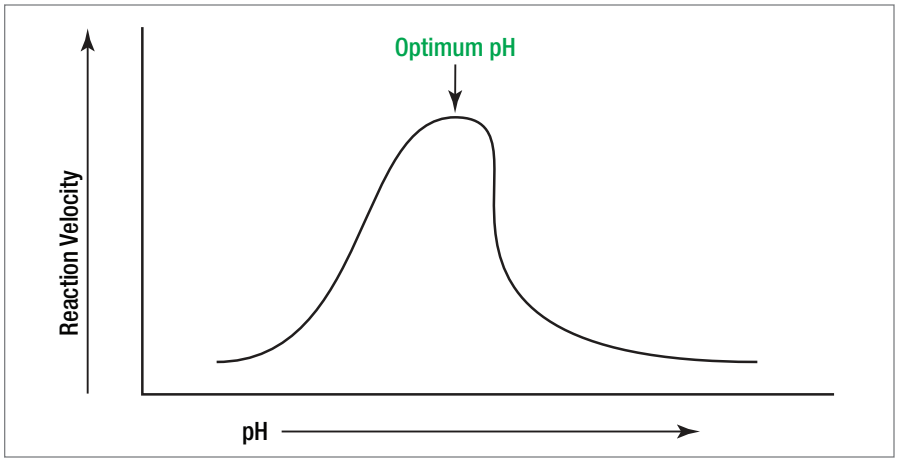

Figure 14: The effect of pH on the reaction rate.

Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme there is also a region of pH optimal stability. The optimum pH value will vary greatly from one enzyme to another, as **Table 2** shows. These physical (temperature) and chemical (pH) parameters must be considered and optimized, in order for an enzymatic reaction to be accurate and reproducible.

Table 2: pH for optimum activity.

Enzyme	Optimum pH
Lipase (pancreas)	8.0
Lipase (stomach)	4.0 – 5.0
Lipase (castor oil)	4.7
Pepsin	1.5 - 1.6
Trypsin	7.8 - 8.7
Urease	7.0
Invertase	4.5
Maltase	6.1 - 6.8
Amylase (pancreas)	6.7 - 7.0
Amylase (malt)	4.6 - 5.2
Catalase	7.0

Concluding Thoughts:

Many of the current challenges facing researchers in the life sciences require an appreciation of the complexities of living organisms. These complexities are driven in large part by the enzymes that work together to make life happen. Many metabolic disorders, cancers, and hereditary diseases involve an enzyme that is not working properly. In addition, new therapeutic treatments for disease often require a thorough understanding of both the enzymes involved in the disease and the biochemical environment in which these enzymes are functioning. Further complexity can also be introduced when there is an infection present and an additional organism's enzymes (e.g., bacterial antibiotic resistance mechanisms) need to be considered.

Decades after the initial discovery of enzymes, the details of how enzymes work individually and how systems of enzymes perform a specific function (e.g., synthesize a complex biochemical) continue to require investigation. Moving forward, as scientists develop new pharmaceutical drugs and unravel the complexities of aging and disease, the basics of enzymology will continue to be invaluable.

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