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Enzyme Kinetics

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

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ENZYME KINETICS

To Leigh

For her tireless editorial assistance, expert artwork, keen professional advice, saintly patience, and excellent cooking, all of which made this book possible.

PREFACE

A thorough kinetic analysis is an essential part of the characterization of any enzyme. Yet, too often such studies are either omitted from research publications or are presented in a very superficial manner. This is unfortunate but somewhat understandable. Many biologists study enzymes but are intimidated by the apparent complexity of enzyme kinetics. I wrote *Enzyme Kinetics* for two purposes: to introduce the varieties of enzyme behavior to advanced undergraduates and graduate students in the biological sciences and to serve as a useful, accessible reference work on enzyme kinetics for professional researchers.

Enzyme Kinetics is a large book because my aim is to teach the subject and not just to present a multitude of equations. Thus the book is written in the same highly detailed, step-by-step fashion as a textbook and I have deliberately devoted as much attention to elementary and classical kinetics of unireactant enzymes as I have to modern steady-state kinetics of multireactant enzymes. I have tried to provide readers with a basic understanding of the procedures used to convert an idea for a model into a velocity equation (which can then be tested experimentally) and, conversely, the procedures for developing a model from experimental data. Simple equilibrium concepts are used to explain the basis of cooperative, concerted, and cumulative feedback inhibition, regulation by "energy charge", the major models of allosteric enzymes, and the effects of pH and metal ion activators. After seeing how multiple replot graphical analysis can be used to analyze simple rapid equilibrium systems, the reader should have no difficulty in following the same procedures as applied to more complicated steady-state and isotope exchange systems.

Since my objective is to describe kinetic behavior and analysis *in general*, specific enzymes are not discussed except where they represent a unique example of a mechanism. (Nevertheless, specific examples are given in the references.) Presteady-state transient kinetics is not covered because few biologists have access to the necessary rapid reaction instruments, and, also, books on this aspect of enzyme behavior are already available.

This book could not have been written without the cooperation, encouragement, and contributions of many people. Foremost are the scientists whose published works were the basis for the book. My colleagues in the

Department of Biochemistry and Biophysics and our students and postdoctorals contributed by providing me with an intellectual atmosphere conducive to the task and by helping me when I got stuck. I am grateful to Ms. Carolyn Sherwood, the world's greatest typist, who never missed a deadline. I thank my editor, Dr. Theodore P. Hoffman, of Wiley-Interscience for his confidence in me and for the guidelines that he set, and Ms. Joan Samuels of the Production Division, who calmly handled all the crises.

IRWIN H. SEGEL

Davis, California
1974

THE AUTHOR

Irwin H. Segel was born on Staten Island, New York, in 1935. He attended Brooklyn Technical High School and Rensselaer Polytechnic Institute (B.S. in Chemistry, 1957). His undergraduate research on bacterial glucose metabolism was done under the direction of Professor Henry L. Ehrlich. Two summers were spent studying mammalian niacin metabolism with Dr. Lawrence V. Hankes at Brookhaven National Laboratory. His graduate research on sulfur metabolism of fungi was directed by Professor Marvin J. Johnson at the University of Wisconsin. After receiving his Ph.D. degree in 1962, Dr. Segel spent two years as a National Science Foundation and U.S. Public Health Service Fellow with Professor Jacques C. Senez at the Centre National de la Recherche Scientifique, Marseille, France. In 1964, he joined the faculty of the Department of Biochemistry and Biophysics at the University of California at Davis where he is now Professor of Biochemistry. His current research is on the enzymes of polysaccharide metabolism, inorganic sulfur and nitrogen metabolism, and on membrane transport systems of bacteria and fungi. Dr. Segel teaches introductory biochemistry and a course in enzyme kinetics. His first book *Biochemical Calculations* (John Wiley & Sons, 1968) is used as a supplementary text at many colleges and universities in this country and has been translated into Japanese and Spanish.

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 1898. 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.

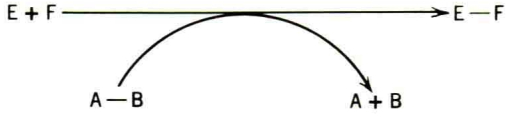
B. LIFE, ENERGY, AND COUPLED REACTIONS

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:

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

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

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:

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

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

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

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

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

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 ΔG° 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:

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

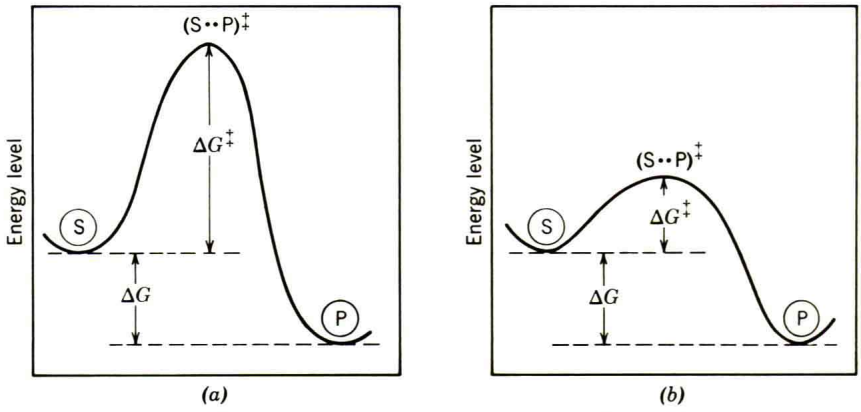


Fig. I-1. ΔG and $\Delta G^{\ddagger}$ of (*a*) nonenzymatic and (*b*) enzymatic reactions.

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 $10\,\text{min}^{-1}$; k_{-1} must also increase 10,000-fold to $10^{-1}\ \text{min}^{-1}$; 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:

$$S \xrightarrow{E} P$$

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:

$$S + E \longrightarrow ES \longrightarrow E + P$$

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

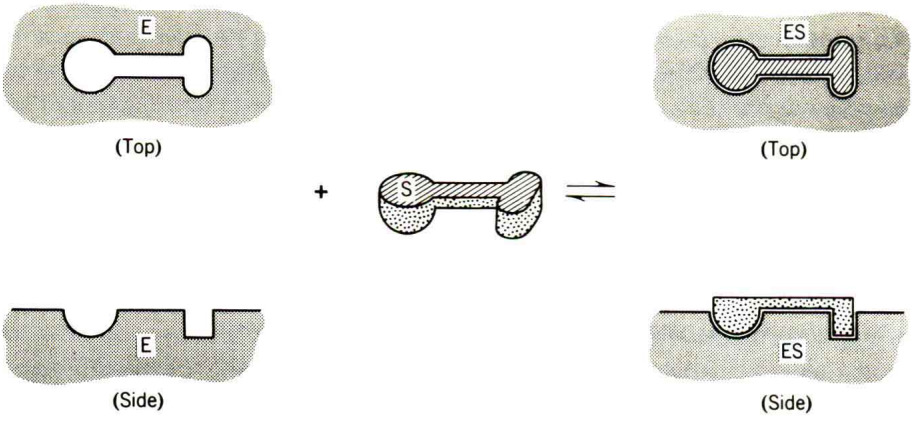


Fig. I-2. Lock and key (template) hypothesis of enzyme specificity.

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).

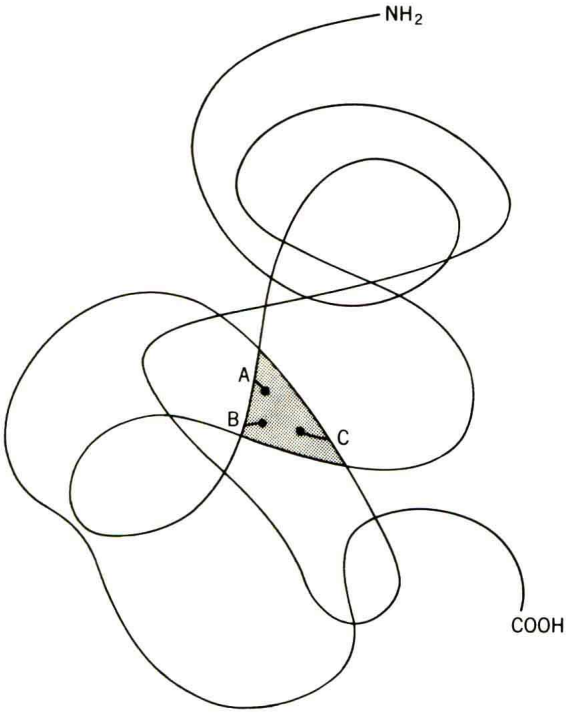
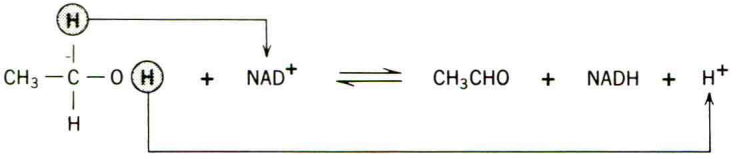


Fig. I-3. The active site (shaded area) occupies only a small region of the enzyme. A, B, and C are the amino acid R-groups responsible for substrate binding and catalytic activity.

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