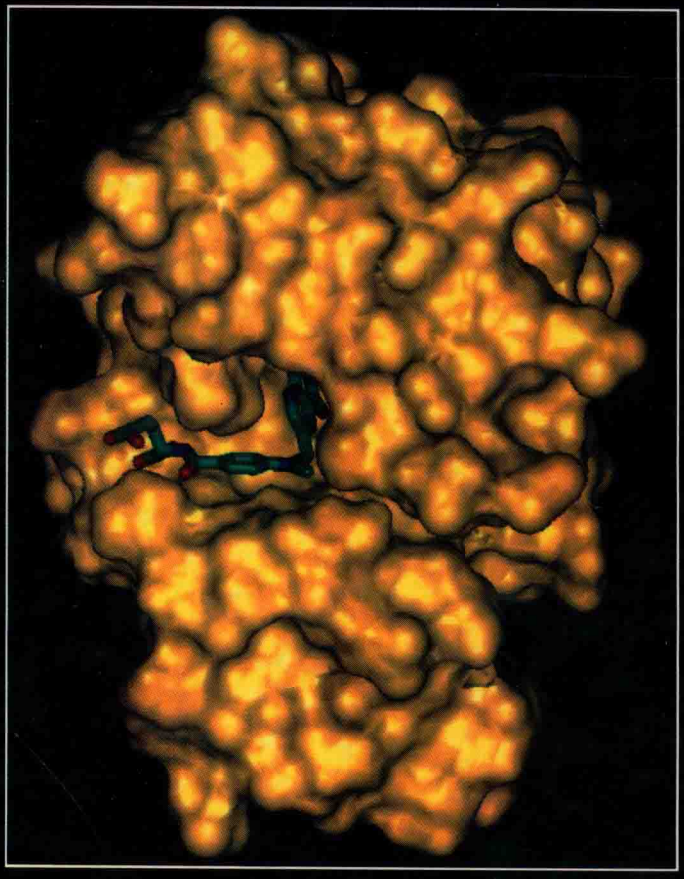
ENZYMES

A Practical Introduction to Structure, Mechanism, and Data Analysis



Robert A. Copeland

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Enzymes

To Clyde Worthen
for teaching me all the important lessons;
arigato sensei.

And to Theodore (Doc) Janner
for stoking the fire.

Preface

The latter half of this century has seen an unprecedented expansion in our knowledge and use of enzymes in a broad range of basic research and industrial applications. Enzymes are the catalytic cornerstones of metabolism, and as such are the focus of intense research within the biomedical community. Indeed enzymes remain the most common targets for therapeutic intervention within the pharmaceutical industry. Since ancient times enzymes also have played central roles in many manufacturing processes, such as in the production of wine, cheese, and breads. During the 1970s and 1980s much of the focus of the biochemical community shifted to the cloning and expression of proteins through the methods of molecular biology. Recently, some attention has shifted back to physicochemical characterization of these proteins, and their interactions with other macromolecules and small molecular weight ligands (e.g., substrates, activators, and inhibitors). Hence, there has been a resurgence of interest in the study of enzyme structures, kinetics, and mechanisms of catalysis.

The availability of up-to-date, introductory-level textbooks, however, has not kept up with the growing demand. I first became aware of this void while teaching introductory courses at the medical and graduate student level at the University of Chicago. I found that there were a number of excellent advanced texts that covered different aspects of enzymology with heavy emphasis on the theoretical basis for much of the science. The more introductory texts that I found were often quite dated and did not offer the blend of theoretical and

practical information that I felt was most appropriate for a broad audience of students. I thus developed my own set of lecture notes for these courses, drawing material from a wide range of textbooks and primary literature.

In 1993, I left Chicago to focus my research on the utilization of basic enzymology and protein science for the development of therapeutic agents to combat human diseases. To pursue this goal I joined the scientific staff of the DuPont Merck Pharmaceutical Company. During my first year with this company, a group of associate scientists expressed to me their frustration at being unable to find a textbook on enzymology that met their needs for guidance in laboratory protocols and data analysis at an appropriate level and at the same time provide them with some relevant background on the scientific basis of their experiments. These dedicated individuals asked if I would prepare and present a course on enzymology at this introductory level.

Using my lecture notes from Chicago as a foundation, I prepared an extensive set of notes and intended to present a year-long course to a small group of associate scientists in an informal, over-brown-bag-lunch fashion. After the lectures had been announced, however, I was shocked and delighted to find that more than 200 people were registered for this course! The makeup of the student body ranged from individuals with associate degrees in medical technology to chemists and molecular biologists who had doctorates. This convinced me that there was indeed a growing interest and need for a new introductory enzymology text that would attempt to balance the theoretical and practical aspects of enzymology in such a way as to fill the needs of graduate and medical students, as well as research scientists and technicians who are actively involved in enzyme studies.

The text that follows is based on the lecture notes for the enzymology course just described. It attempts to fill the practical needs I have articulated, while also giving a reasonable introduction to the theoretical basis for the laboratory methods and data analyses that are covered. I hope that this text will be of use to a broad range of scientists interested in enzymes. The material covered should be of direct use to those actively involved in enzyme research in academic, industrial, and government laboratories. It also should be useful as a primary text for senior undergraduate or first-year graduate course, in introductory enzymology. However, in teaching a subject as broad and dynamic as enzymology, I have never found a single text that would cover all of my students' needs; I doubt that the present text will be an exception. Thus, while I believe this text can serve as a useful foundation, I encourage faculty and students to supplement the material with additional readings from the literature cited at the end of each chapter, and the primary literature that is continuously expanding our view of enzymes and catalysis.

In attempting to provide a balanced introduction to enzymes in a single, readable volume I have had to present some of the material in a rather cursory fashion; it is simply not possible, in a text of this format, to be comprehensive in such an expansive field as enzymology. I hope that the literature citations

will at least pave the way for readers who wish to delve more deeply into particular areas. Overall, the intent of this book is to get people *started* in the laboratory and in their thinking about enzymes. It provides sufficient experimental and data handling methodologies to permit one to begin to design and perform experiments with enzymes, while at the same time providing a theoretical framework in which to understand the basis of the experimental work. Beyond this, if the book functions as a stepping-stone for the reader to move on to more comprehensive and in-depth treatments of enzymology, it will have served its purpose.

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Acknowledgments

It is a great pleasure for me to thank the many friends and coworkers who have helped me in the preparation of this work. Many of the original lecture notes from which this text has developed were generated while I was teaching a course on biochemistry for first-year medical students at the University of Chicago, along with the late Howard S. Tager. Howard contributed greatly to my development as a teacher and writer. His untimely death was a great loss to many of us in the biomedical community; I dearly miss his guidance and friendship.

As described in the preface, the notes on which this text is based were significantly expanded and reorganized to develop a course of enzymology for employees and students at the DuPont Merck Pharmaceutical Company. I am grateful for the many discussions with students during this course, which helped to refine the final presentation. I especially thank Diana Blessington for the original suggestion of a course of this nature. That a graduate-level course of this type could be presented within the structure of a for-profit pharmaceutical company speaks volumes for the insight and progressiveness of the management of DuPont Merck. I particularly thank James M. Trzaskos, Robert C. Newton, Ronald L. Magolda, and Pieter B. Timmermans for not only tolerating, but embracing this endeavor.

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Finally, and most importantly, I wish to thank my wife, Nancy, and our children, Lindsey and Amanda, for their constant love, support, and encouragement, without which this work could not have been completed.

"All the mathematics in the world is no substitute for a reasonable amount of common sense."

W. W. Cleland

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CHAPTER

1

A Brief History of Enzymology

Life depends on a well-orchestrated series of chemical reactions. Many of these reactions, however, proceed too slowly on their own to sustain life. Hence nature has designed catalysts, which we now refer to as *enzymes*, to greatly accelerate the rates of these chemical reactions. The catalytic power of enzymes facilitates life processes in essentially all life-forms from viruses to man. Many enzymes retain their catalytic potential after extraction from the living organism, and it did not take long for mankind to recognize and exploit the catalytic power of enzyme for commercial purposes. In fact, the earliest known references to enzymes are from ancient texts dealing with the manufacture of cheeses, breads, and alcoholic beverages, and for the tenderizing of meats. Today enzymes continue to play key roles in many food and beverage manufacturing processes and are ingredients in numerous consumer products, such as laundry detergents (which dissolve protein-based stains with the help of proteolytic enzymes). Enzymes are also of fundamental interest in the health sciences, since many disease processes can be linked to the aberrant activities of one or a few enzymes. Hence, much of modern pharmaceutical research is based on the search for potent and specific inhibitors of these enzymes. The study of enzymes and the action of enzymes has thus fascinated scientists since the dawn of history, not only to satisfy erudite interest but also because of the utility of such knowledge for many practical needs of society. This brief chapter sets the stage for our studies of these remarkable catalysts by providing a historic background of the development of enzymology as a science. We shall see that while enzymes are today the focus of basic academic research, much of the early history of enzymology is linked to the practical application of enzyme activity in industry.

1.1 Enzymes in Antiquity

The oldest known reference to the commercial use of enzymes comes from a description of wine making in the Codex of Hammurabi (ancient Babylon, circa 2100 B.C.). The use of microorganisms as enzyme sources for fermentation was widespread among ancient people. References to these processes can be found in writings not only from Babylon but also from the early civilizations of Rome, Greece, Egypt, China, India. Ancient texts also contain a number of references to the related process of vinegar production, which is based on the enzymatic conversion of alcohol to acetic acid. Vinegar, it appears, was a common staple of ancient life, being used not only for food storage and preparation but also for medicinal purposes.

Dairy products were another important food source in ancient societies. Because in those days fresh milk could not be stored for any reasonable length of time, the conversion of milk to cheese became a vital part of food production, making it possible for the farmer to bring his product to distant markets in an acceptable form. Cheese is prepared by curdling milk via the action of any of a number of enzymes. The substances most commonly used for this purpose in ancient times were ficin, obtained as an extract from fig trees, and rennin, as rennet, an extract of the lining of the fourth stomach of a multiple-stomach animal, such as a cow. A reference to the enzymatic activity of ficin can, in fact, be found in Homer's classic, the Iliad:

> As the juice of the fig tree curdles milk, and thickens it in a moment though it be liquid, even so instantly did Paeëon cure fierce Mars.

The philosopher Aristotle likewise wrote several times about the process of milk curdling and offered the following hypothesis for the action of rennet:

> Rennet is a sort of milk; it is formed in the stomach of young animals while still being suckled. Rennet is thus milk which contains fire, which comes from the heat of the animal while the milk is undergoing concoction.

Another food staple throughout the ages is bread. The leavening of bread by yeast, which results from the enzymatic production of carbon dioxide, was well known and widely used in ancient times. The importance of this process to ancient society can hardly be overstated.

Meat tenderizing is another enzyme-based process that has been used since antiquity. Inhabitants of many Pacific islands have known for centuries that the juice of the papaya fruit will soften even the toughest meats. The active enzyme in this plant extract is a protease known as papain, which is used even today in commercial meat tenderizers. When the British Navy began exploring the Pacific islands in the 1700s, they encountered the use of the papaya fruit as a meat tenderizer and as a treatment for ringworm. Reports of these native uses of the papaya sparked a great deal of interest in eighteenth-century Europe, and may, in part, have led to some of the more systematic studies of digestive enzymes that ensued soon after.

1.2 Early Enzymology

While the ancients made much practical use of enzymatic activity, these early applications were based purely on empirical observations and folklore, rather than any systematic studies or appreciation for the chemical basis of the processes being utilized. In the eighteenth and nineteenth centuries scientists began to study the actions of enzymes in a more systematic fashion. The process of digestion seems to have been a popular subject of investigation during the years of the enlightenment. Wondering how predatory birds manage to digest meat without a gizzard, the famous French scientist Réaumur (1683–1757) performed some of the earliest studies on the digestion of buzzards. Réaumur designed a metal tube with a wire mesh at one end that would hold a small piece of meat immobilized, to protect it from the physical action of the stomach tissue. He found that when a tube containing meat was inserted into the stomach of a buzzard, the meat was digested within 24 hours. Thus he concluded that digestion must be a chemical rather than a merely physical process, since the meat in the tube had been digested by contact with the gastric juices (or as he referred to them, "a solvent"). He tried the same experiment with a piece of bone and with a piece of a plant. He found that while meat was digested, and the bone was greatly softened by the action of the gastric juices, the plant material was impervious to the "solvent"; this was probably the first experimental demonstration of enzyme specificity.

Réaumur's work was expanded by Spallanzani (1729–1799), who showed that the digestion of meat encased in a metal tube took place in the stomachs of a wide variety of animals, including humans. Using his own gastric juices, Spallanzani was able to perform digestion experiments on pieces of meat in vitro (in the laboratory). These experiments illustrated some critical features of the active ingredient of gastric juices: by means of a control experiment in which meat treated with an equal volume of water did not undergo digestion Spallanzani demonstrated the presence of a specific active ingredient in gastric juices. He also showed that the process of digestion is temperature dependent, and that the time required for digestion is related to the amount of gastric juices applied to the meat. Finally, he demonstrated that the active ingredient in gastric juices is unstable outside the body; that is, its ability to digest meat wanes with storage time.

Today we recognize all the foregoing properties as common features of enzymatic reactions, but in Spallanzani's day these were novel and exciting findings. The same time period saw the discovery of enzyme activities in a large number of other biological systems. For example, a peroxidase from the horseradish was described, and the action of α-amylase in grain was observed. These early observations all pertained to materials—crude extract from plants or animals—that contained enzymatic activity.

During the latter part of the nineteenth century scientists began to attempt fractionations of these extracts to obtain the active ingredients in pure form.

For example, in 1897 Bertrand partially purified the enzyme laccase from tree sap, and Buchner, using the "pressed juice" from rehydrated dried yeast, demonstrated that alcoholic fermentation could be performed in the absence of living yeast cells. Buchner's report contained the interesting observation that the activity of the pressed juice diminished within 5 days of storage at ice temperatures. However, if the juice was supplemented with cane sugar, the activity remained intact for up to 2 weeks in the ice box. This is probably the first report of a now well-known phenomenon—the stabilization of enzymes by substrate. It was also during this period that Kühne, studying catalysis in yeast extracts, first coined the term "enzyme" (the word derives from the medieval Greek word *enzymos*, which relates to the process of leavening bread).

1.3 The Development of Mechanistic Enzymology

As enzymes became available in pure, or partially pure forms, scientists' attention turned to obtaining a better understanding of the details of the reaction mechanisms catalyzed by enzymes. The concept that enzymes form complexes with their substrate molecules was first articulated in the late nineteenth century. It is during this time period that Emil Fischer proposed the "lock and key" model for the stereochemical relationship between enzymes and their substrates; this model emerged as a result of a large body of experimental data on the stereospecificity of enzyme reactions. In the early twentieth century, experimental evidence for the formation of an enzyme–substrate complex as a reaction intermediate was reported. One of the earliest of these studies, reported by Brown in 1902, focused on the velocity of enzyme-catalyzed reactions. Brown made the insightful observation that unlike simple diffusion-limited chemical reactions, in enzyme-catalyzed reactions "it is quite conceivable... that the time elapsing during molecular union and transformation may be sufficiently prolonged to influence the general course of the action." Brown then went on to summarize the available data that supported the concept of formation of an enzyme–substrate complex:

> There is reason to believe that during inversion of cane sugar by invertase the sugar combines with the enzyme previous to inversion. C. O'Sullivan and Tompson... have shown that the activity of invertase in the presence of cane sugar survives a temperature which completely destroys it if cane sugar is not present, and regard this as indicating the existence of a combination of the enzyme and sugar molecules. Wurtz [1880] has shown that papain appears to form an insoluble compound with fibrin previous to hydrolysis. Moreover, the more recent conception of E. Fischer with regard to enzyme configuration and action, also implies some form of combination of enzyme and reacting substrate.

Observations like these set the stage for the derivation of enzyme rate equations, by mathematically modeling enzyme kinetics with the explicit involvement of an intermediate enzyme–substrate complex. In 1903 Victor