Enzymes: Historical Aspects

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Historically, the field of enzymology was born out of practical and theoretical considerations. A perusal of early enzyme literature indicates that the field has evolved from fundamental questions about their function, their nature, and their biological role. This chapter outlines the course of historical development of enzymology, and some of these landmarks are listed in Table 2.1.

2.1 Biocatalysis: The Beginnings

Past human industry like cheese making provided insights into some properties of enzymatic processes. The earliest recorded example of cheese making contains reference to extracts of fig tree – a source of the proteolytic enzyme ficin. Only later did rennet (a source of another protease chymosin) become popular in cheese processing. Meat tenderizing is the other application that implicitly used enzymes over the years. Apart from the fig tree extract, the fruit and other parts of papaya (*Carica papaya*; contains the now well-known proteolytic enzyme papain) have found early utility in meat tenderizing.

Indeed the work on gastric digestion of meat – proteases in particular – by Rene Reaumur (1751) and Lazzaro Spallanzani (1780) laid a scientific foundation for the study of enzyme catalysis. Reaumur's experiments with digestion of meat represent the first systematic record of the activity due to an enzyme. However the term enzyme was yet to be coined then! Theodor Schwann used the word pepsin in 1836 for the proteolytic activity of the gastric mucosa. He also conducted careful quantitative experiments, to establish that acid was necessary but not sufficient for this reaction to take place. Among his many other contributions, Schwann also coined the term metabolism.

Parallel to the work on proteolytic enzymes, developments with fermentation and on starch hydrolysis have equally contributed to the initial growth of enzymology.

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Table 2.1 **Landmarks in enzyme studies (enzymology classics)**

Author(s)	Year (Discovery/ Publication)	Contribution
R. Reaumur	1751	Gastric digestion in birds
L. Spallanzani	1780	Digestion of meat by gastric juice
A. Payen & J. Persoz	1833	Amylase (diastase) activity
J. Berzelius	1836	Catalysis as a concept
W. Kuhne	1867	"Enzyme" term defined
J. Takamine	1894	Patent on fungal diastase
E. Fischer	1894	Lock and key concept
G. Bertrand	1897	Co-ferment (coenzyme) conceived
P.E. Duclaux	1898	Enzyme names to end with suffix "ase"
V. Henri	1903	Hyperbolic rate equation
S.P.L. Sorensen	1909	pH scale and buffers
L. Michaelis & M. Menten	1913	Equilibrium treatment for *ES* complex
R.M. Willstatter	1922	Trager theory of enzyme action
G.E. Briggs & J.B.S. Haldane	1925	Steady-state treatment for *ES* complex
J.B. Sumner	1926	Urease – Purification and crystallization
H. Lineweaver & D. Burk	1934	Double reciprocal plot (1/v versus 1/[*S*])
K. Stern	1935	First *ES* complex observed
M. Doudoroff	1947	Radioisotope use in enzyme mechanisms
A.G. Ogston	1948	Asymmetric interaction with substrate
L. Pauling	1948	Enzyme binds *TS* better than *S*
F. Westheimer	1951	Enzymatic hydride transfer (^{2}H, ^{3}H used)
D.E. Koshland Jr.	1958	Induced fit hypothesis
C.H.W. Hirs et al.	1960	First enzyme sequenced – RNase A
Enzyme commission	1961	Enzyme classification and nomenclature
D.C. Phillips et al.	1962	First enzyme structure – lysozyme
W.W. Cleland	1963	Systematization of enzyme kinetic study
J. Monod et al.	1965	Model for allosteric transitions
R.B. Merrifield	1969	Chemical synthesis of RNase A
S. Altman & T.R. Cech	1981	Catalysis by RNA molecules

Gottlieb Kirchhoff discovered plant amylase (later identified as α-amylase) activity while characterizing the hydrolysis of starch to sugar. He demonstrated the acid-facilitated conversion of starch to sugar and clearly recognized that the formation of sugar from starch during germination of grain is akin to chemical hydrolysis (1815). The work of Kirchhoff on starch hydrolysis was extended by Anselme Payen and Jean Persoz (1833). They enriched (first attempts of enzyme purification!) the hydrolytic activity from malt gluten and termed it as diastase. The name diastase (Greek; *diastasis* – to make a breach) has significantly influenced the development of the field of enzymology since then (see below). Yet another source of a starch hydrolyzing activity was identified in saliva by Erhard Leuchs (1931). Remarkably, this report also invoked the possible practical utility of this activity.

The two non-hydrolytic enzyme activities reported early include the peroxidase activity from horseradish and catalase. These two enzymes were recognized much ahead of the study of oxidative enzymes in early twentieth century. Work on catalase by Louis Thenard (1819) is the first quantitative study of an enzymatic reaction. He also anticipated that such activities may be found in other animal and vegetable secretions.

Enzymology finds its roots in some of the greatest names since eighteenth century, both in chemistry and biology. Clearly this subject is a true and sturdy bridge between contemporary chemistry and biology. Among the greats who contributed to its early development include Reaumur, Spallanzani, Thenard, Schwann, Berzelius, Liebig, Berthelot, Pasteur, Buchner, and Fischer. Many fundamental contributions were made to enzymology by chemists of fame like Berzelius, Liebig, and Berthelot. It is however important to note that historically, the idea of catalysis arose because of the study of enzymes and their action. Jons Jacob Berzelius was the first to define the term "catalyst" in 1836. In his view, a catalyst was a substance capable of wakening energies dormant, merely by its presence. He was also the first to recognize the similarity of catalysis in a chemical reaction and inside a living cell. However, Berzelius made no distinction between the catalytic phenomenon occurring in animate and inanimate world. He also used the now famous words isomer, polymer, ammonium, protein, and globulin. Those were the times when a "vital force" was associated with living cells and biocatalysts were part of this explanation. Only much later did the concept take root that ordinary physical and chemical principles apply to enzyme catalysis.

Pierre Berthelot was the first to derive a second-order rate equation which influenced the publication by Guldberg and Waage on law of mass action leading to chemical kinetics.

As a part of their study on catalytic phenomena, Wohler and Liebig discovered "emulsin" (a β-glucosidase) from almonds in 1837. Indeed this enzyme was cleverly used by Fischer subsequently (almost 50 years later!) to define enzyme specificity.

2.2 "Enzyme": Conceptual Origin

Swedish chemist Berzelius (1779–1848) proposed the name catalysis (from the Greek *kata*, wholly, and *lyein*, to loosen) in 1836. When Berzelius first invoked the term "catalysis," he did not make any distinction between the chemical catalysis and catalysis in (or by) biological systems. He used a generic term "contact substance" for a catalyst. The origin of the word "enzyme" dedicated to biological catalysts has a convoluted history. Much of this drama was played out during a vigorous debate on whether there is special force (the "vital force") associated with reactions occurring in living systems. Ever since Payen and Persoz (1833) introduced this name for the starch hydrolytic activity, "diastase" has often been used to generally mean a catalyst of biological origin. In fact Victor Henri in his

1903 book on enzyme kinetics (an early classic on enzyme action) used diastase to mean an "enzyme." Many other French scientists including Pierre Duclaux and Gabriel Bertrand did use diastase to mean what we now call enzymes. The suffix "ase" – arising out of diastase – was subsequently recommended for all enzyme names (by Duclaux in 1898).

Ferment as a term was used to describe both living yeast and the action of its cellular contents. Berthelot's extraction of "ferment" (1860) from yeast cells marks the beginning of action of enzymes outside of a living cell. This also dealt a blow to vitalistic thinking in biochemistry. The analogy between ferment-catalyzed and acid-catalyzed hydrolysis of starch was well-recognized by the successive contributions of Kirchhoff, Payen and Persoz, and Berzelius. Schwann had used a similar analogy for pepsin. Willy Kuhne in 1867 extended this further to pancreatic digestion of proteins and called this activity trypsin in 1877. The essential meaning of "ferment" was consolidated by Kuhne; subsequently the word **enzyme** (*in yeast*) was first used by him in 1877. In fact trypsin was the first candidate "ferment" to be called an enzyme.

The evolution and acceptance of word enzyme have taken its time. Both the descriptions – "diastase" (mostly in French scientific literature) and "ferment" – were used occasionally well into the early twentieth century.

The vitalistic theory was firmly laid to rest with Eduard Buchner's conclusive demonstration that suitable extract from yeast cells could convert sucrose to alcohol. This was revolutionary in 1897 since fermentation was shown to occur "without living yeast" for the first time. The activity was ascribed to a single substance which was named "zymase" (and alcoholase by Emile Roux). It is now history that this activity in fact represents the entire glycolytic sequence of reactions. Out of controversy on the nature of alcoholic fermentation, the word "enzyme" was born. This word reminds us that yeast ("zyme") and its activities were resolved through the prisms of biology and chemistry to create the rich domain of enzymology.

2.3 Key Developments in Enzymology

Protein Nature of Enzymes Early progress on enzymes was impeded because not much was known on the chemical nature of proteins. Much less was known about the chemical nature of enzymes. One approach to understand them was to purify them for detailed analysis. Kuhne and Chittenden extensively used the technique of protein fractionation by ammonium sulfate and also introduced the use of dialysis and dialysis tubing (1883). Powerful methods to purify enzymes were developed by Richard Willstatter – the first introduction of alumina Cγ gel was made. Peroxidase was taken to such high level of purity that the preparation failed in then prevailing tests for protein. This unfortunately led him to wrongly conclude that enzymes are not proteins (1926). The seminal discovery by James Sumner, proving that urease is a protein, therefore assumes great significance (Sumner 1933). This view was further confirmed by purification and crystallization of three more enzymes – pepsin, trypsin, and chymotrypsin – by Northrop and Kunitz (between 1930 and 1935). It must impress anyone to note that all this was accomplished by just two simple purification techniques – fractional precipitation of proteins by ammonium sulfate and pH changes.

Laccase is one of the early examples of an enzyme that was not a hydrolase. Bertrand (1895) described it as an “oxidase” and suggested that this enzyme contained a divalent metal. His description of “co-ferment” – a nonprotein component of laccase – is the first descriptor of an enzyme cofactor.

More powerful yet gentler procedures of protein purification (and dialysis, etc.) hastened the progress of enzymology by providing many pure enzyme preparations. The end of the nineteenth century saw an increase in the number of reports on enzymes. By 1955 the number of enzymes reported was so large that their proper organization into categories became necessary. Under the auspices of the International Union of Biochemists, the International Commission on Enzymes was established to systematize the classification and naming of enzymes. As a result, the Enzyme Commission produced guidelines on enzyme nomenclature and brought out its recommendations in 1961.

Kinetic Foundations Because they are excellent catalysts, enzyme kinetic behavior could be studied regardless of meager knowledge of their composition. Even after their protein nature was established, it has taken long to relate structural basis of enzyme kinetic behavior.

As early as 1898, the reversibility of an enzyme reaction was reported. The enzymatic synthesis of a glucoside (maltose from glucose) by the yeast maltase established some key features: (a) an enzyme being a catalyst speeds up the reaction in both directions of a reversible reaction, (b) at least some steps in metabolism may go in either direction, and (c) enzymes may be involved in the cellular biosynthetic processes.

The reversibility of enzyme catalysis brought it within the ambit of thermodynamic analysis and physical chemistry. The thermodynamic constraints imposed upon catalyzed and uncatalyzed reactions were set forth by J van’t Hoff. This subsequently led JBS Haldane to relate enzyme kinetic parameters with reaction thermodynamics and arrive at the famous Haldane relationship (Enzymes 1930).

Yeast invertase has the singular distinction as the working example for early work on enzyme reaction kinetics and thermodynamics. AJ Brown (1902) deduced the formation of invertase-sucrose complex (the *ES* complex) from initial rate measurements. It was in 1903 that V. Henri for the first time derived the hyperbolic rate equation for a single-substrate enzymatic reaction. He provided the general process used to derive such rate equation – an exercise central to any enzyme kinetic study. Henri also recognized that ‘the validity of a rate equation is necessary but not sufficient to prove the postulated kinetic mechanism’. In fact the now famous Michaelis–Menten equation, based on the equilibrium treatment of the system, was published about 10 years later in 1913. A more general form of the Henri-Michaelis-Menten equation to describe enzyme kinetics was derived by Briggs and Haldane via the steady-state approach in 1925. We continue to use this fundamental equation even today to describe the substrate saturation phenomenon of an enzyme reaction. A very popular linear form of this hyperbolic relation between initial velocity and substrate concentration is attributed to Lineweaver and Burk (1934).

A systematic study of enzyme reaction rates dictated that buffers be used to control hydrogen ion concentrations. This was indeed the impetus to the work published in 1909 by Sorenson on the pH scale and buffers. Subsequently Leonor Michaelis and others emphasized the importance of pH on enzyme activity and routinely controlled it in all their studies.

The *ES* complex formation was a kinetic concept to begin with. First direct observation of an enzyme substrate complex of catalase was made by KG Stern (1935); he monitored the catalase–HOOEt complex using spectroscopy.

Mechanistic Studies Emil Fischer was an unusual organic chemist of highest caliber. He was responsible for establishing the rigor of synthetic and analytical skills of organic chemistry to biological problems. As early as 1894, he observed that substrates for invertin (now the invertase or sucrose hydrolase) are not substrates for emulsin (a β-glucosidase) and *vice versa*. Fischer opined that "enzymes are fussy about the configuration of their object of attack." For example, the enzyme and the glucoside on which it acts must fit each other like a "lock and key" to be able to catalyze the chemical reaction. The future, as we know it, confirmed the genius of Fischer. This laid the foundation for describing fundamental properties of enzyme like specificity, stereoselectivity, and the famous lock-and-key analogy for enzyme–substrate interactions.

In an attempt to explain how enzymes work, the "Trager" or carrier theory was proposed by Willstatter (in 1922). According to him enzymes contained smaller reactive groups that have affinity toward specific groups on the substrate – leading to enzyme specificity. Of course, these reactive groups were thought to be attached to an inert colloidal carrier to form the enzyme. Clearly the fact that enzymes are proteins was not yet established then.

The hypothesis by AG Ogston (1948) attempted to explain how enzymes achieve chemical asymmetry through three point contact with their substrates. This paved the way for further experiments in elucidating enzyme chemical mechanisms. Redox reactions involving pyridine nucleotides and the mechanism of hydride transfer followed shortly thereafter. Frank Westheimer and his colleagues, working with alcohol dehydrogenase and lactate dehydrogenase as examples, showed that the substrate hydrogen was transferred selectively to one side of the nicotinamide ring. This pioneering research in 1953 made use of deuterium- and tritium-labeled substrates to establish the stereospecificity of these hydride transfers.

Work by Michael Duodoroff's group (1947) on bacterial disaccharide phosphorylases forms an early and brilliant example of use of radioisotopes (^{32}P phosphate) in the study of enzyme mechanisms. Two similar reactions involving disaccharide phosphorolysis, namely, sucrose phosphorylase and maltose phosphorylase, were shown to follow completely different mechanisms. This led directly to the notion of single displacement versus double displacement reactions and subsequently the S_N1 and S_N2 reaction pathways.

The theory of kinetic criteria to distinguish enzyme mechanisms was elaborated by WW Cleland in three seminal papers (1963). At the least, this provided a common language to present enzyme kinetic data, for an otherwise confusing variety of notations found in enzyme kinetic literature. The impact of systematizing enzyme kinetics served two useful purposes – (1) it provided a common kinetic notation for presentation and (2) provided a summary of criteria on how to relate kinetic data with reaction mechanisms.

The rigidity of enzyme active site structure became untenable over time. Complementarity of enzyme active site to accommodate the transition state structure (rather than the substrate or the product) by Linus Pauling (1946) was prophetic; this clearly anticipated the need for protein motion, however subtle, at enzyme active sites. The idea of conformational flexibility of protein molecules as a prerequisite for enzyme activity superseded the earlier lock-and-key concept. This theme further matured into the concept of induced fit hypothesis as proposed by Koshland (1958). The conformational flexibility of a protein and ligand binding through induced fit later became key elements of allosteric transitions. The plasticity of protein structure for regulation thus became inescapable (the famous Monod-Wyman-Changeux model to explain cooperative interactions in oligomeric proteins).

Recognition that enzymes bring about enormous rate accelerations quickly led to a search for underlying principles of such catalysis. Attempts to demystify and explain enzyme catalysis in physicochemical terms were made. Different contributory factors were dissected out through model chemical reactions as well as enzymes. The work of TC Bruice, WP Jencks, ML Bender, DE Koshland Jr., and others is significant in this query. A combination of factors – intermolecular/conformational effects, general acid/base catalysis, nucleophilic/electrophilic catalysis, etc. – contributed to accomplish remarkable rate accelerations observed with enzymes. It is now well-recognized that a combination of many factors produces an enzyme. However search for novel catalytic tools evolved by nature continues unabated even today.

Structure and Synthesis The stamp of chemist's contribution to the study of enzymes is obvious from the progression – isolation and structure elucidation followed by total synthesis. Insulin was the first protein whose complete chemical structure was determined. However, among enzymes, this credit goes to bovine pancreatic ribonuclease A (RNase A) – it was the first enzyme whose primary sequence was elucidated. But lysozyme (this was followed later by RNase A) is the first enzyme whose three-dimensional structure was made available through X-ray crystallography. In a typical organic chemist's approach, total synthesis of a molecule completes the structure elucidation process. In this sense, RNase A was the first enzyme whose total synthesis was achieved (RB Merrifield), and it culminated in a catalytically active protein.

In summary, the history of enzymology is a rich source of factual and conceptual discoveries. Developments in this field were accelerated by chemists and biologists in equal measure. Once established, enzymology revolutionized both the parent disciplines – biology and chemistry. This is amply evident from the list of Nobel laureates (Table 2.2) and their work recognized by the two scientific communities that nurtured the study of enzymes.

Table 2.2 Nobel laureates who contributed to the growth of enzymology

Scientist	Year[a]	Enzymology – topic of study
E. Fischer	1902-C	Stereochemistry and lock-and-key concept
S. Arrhenius	1903-C	Activation energy and catalysis
E. Buchner	1907-C	Cell-free extracts and fermentation
A. Harden and H. von Euler	1929-C	Coenzymes and fermentation
C. Eijkman and F.G. Hopkins	1929-M	Vitamins, nutrition, and coenzymes
O. Warburg	1931-M	Respiratory enzymes
A. Szent-Gyorgyi	1937-M	Fumarate catalysis of TCA cycle
R. Kuhn	1938-C	Vitamins and coenzymes
A. Fleming	1945-M	Penicillin and lysozyme
J.B. Sumner, J.H. Northrop, and M. Kunitz	1946-C	Purification and crystallization of enzymes
C. Cori and G. Cori	1947-M	Enzymes of glycogen metabolism
H.A. Krebs and F. Lipmann	1953-M	TCA cycle and coenzyme A
L. Pauling	1954-C	Secondary structure – α helix; concept that enzyme binds the transition state
H. Theorell	1955-M	Oxidative enzyme mechanisms
A.R. Todd	1957-C	Nucleotides and nucleotide coenzymes
F. Sanger	1958-C	Insulin sequence through proteases
S. Ochoa and A. Kornberg	1959-M	Nucleic acid biosynthesis enzymes
M.F. Perutz and J.H. Kendrew	1962-C	Crystal structure of globular proteins
D. Crowfoot Hodgkin	1964-C	Structure of vitamin B12
K. Bloch and F. Lynen	1964-M	Cholesterol and fatty acid enzymes
F. Jacob, A. Lwoff, and J. Monod	1965-M	Genetic control of enzyme synthesis and allostery
L.F. Leloir	1970-C	Sugar nucleotides and carbohydrate biosynthesis
E.W. Sutherland Jr.	1971-M	Enzyme and metabolic regulation by cAMP
C.B. Anfinsen, S. Moore, and W.H. Stein	1972-C	Chemical structure – Catalytic activity of RNase A
J.W. Cornforth	1975-C	Stereochemistry of enzyme reactions
W. Arber, D. Nathans, and H.O. Smith	1978-M	Restriction endonucleases
F. Sanger	1980-C	DNA sequencing (ddNTP method) with enzymes
R.B. Merrifield	1984-C	Chemical synthesis of RNase A
J.W. Black, G.B. Elion, and G.H. Hitchings	1988-M	Inhibitors (enzyme) as drugs
S. Altman and T.R. Cech	1989-C	Catalysis by RNA molecules
E.H. Fischer and E.G. Krebs	1992-M	Protein kinases and protein phosphorylation
K.B. Mullis	1993-C	Polymerase chain reaction

(continued)

Table 2.2 (continued)

Scientist	Year[a]	Enzymology – topic of study
P.D. Boyer, J.E. Walker, and J.C. Skou	1997-C	ATP synthase and Na/K-ATPase
A.H. Zewail	1999-C	Detection/existence of transition state
I. Rose (and others)	2004-C	Ubiquitin-protein degradation and isotope exchanges in enzymology
A. Warshel (and others)	2013-C	Computational enzymology
F.H. Arnold	2018-C	Directed evolution of enzymes

[a] Prize awarded this year for *C* chemistry, *M* physiology and medicine

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