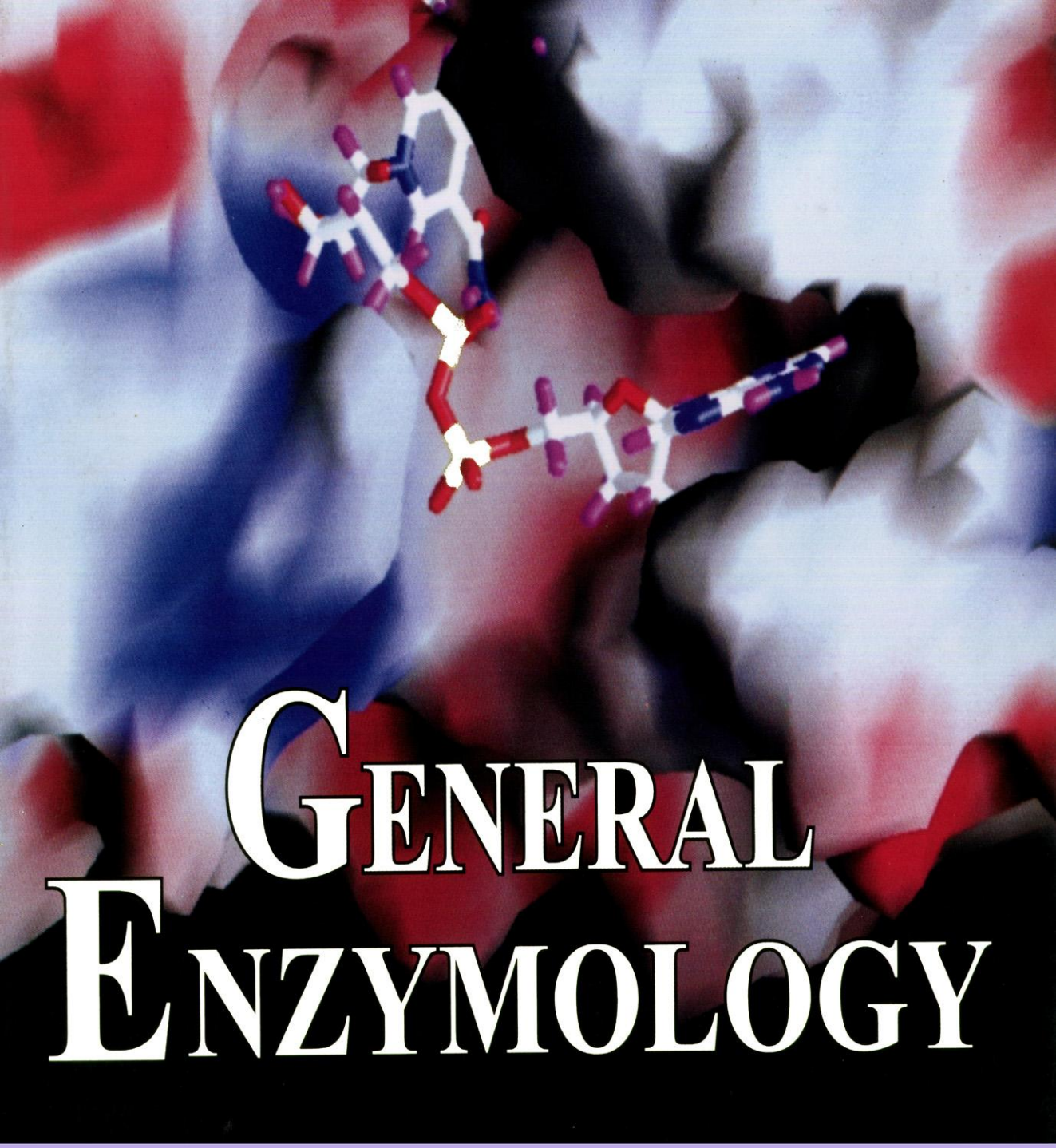

General Enzymology

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Dr. N.S.Kulkarni
M.Sc. Ph.D [Microbiology]
Head,
Department of Microbiology
R.A. Arts, Shri M.K.Commerce
and Shri S. R Rathi Science College,
Washim, (M.S.)
NAAC "A" grade Accredited
College with potential for excellence

M.S.Deshpande
M.Sc.[Biochemistry]
State Eligibility Test qualifier [M.S.]
Department of Biochemistry
R.L.T.College, Akola, (M.S.)

Himalaya Publishing House
MUMBAI • DELHI • NAGPUR • BANGALORE • HYDERABAD • CHENNAI • PUNE • LUCKNOW

ISBN : 978-81-83188-32-6
First Edition : 2007

Published by : Mrs. Meena Pandey
HIMALAYA PUBLISHING HOUSE PVT. LTD.
"Ramdoot", Dr. Bhalerao Marg, Girgaon, **Mumbai - 400 004.**
Phones : 2386 01 70/2386 38 63, Fax : 022 - 2387 71 78
E-mail : himpub @ vsnl.com
Website: www.himpub.com

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Nagpur : Kundanlal Chandak Industrial Estate, Ghat Road, **Nagpur - 440 018.**
Phones : 0712-272 12 16 / 2738731 / 3296733 Fax : 0712-2721215
E-mail : hphngp@hotmail.com

Bangalore : No.16/1, (Old 12/1), First Floor, Next to Hotel Highland, Madhava Nagar, Race course Road, **Bangalore - 560 001.**
Phones : 2228 15 41, 2238 54 61, Fax : 080-2228 66 11.
E-mail: hphblr@vsnl.com

Hyderabad : No 2-2-1 167/2H, 1st Floor, Near Railway Bridge, Tilak Nagar, Main Road, **Hyderabad - 500 044.** Phone : 5550 17 45, Fax: 040-2756 00 41.

Chennai : No. 2 Ramakrishna Street, North Usman Road, T. Nagar, **Chennai - 600 017.** Phones: 044-28144004 / 28144005.

Pune : Lakshya Apts. First floor, 527, Mehunpur, Shaniwarpeth, **Pune - 30**

Lucknow : C-43, Sector - C, Ali Gunj, **Lucknow - 226 024.**
Phone: 0522-4047594.

Printed at : Geetanjali Press Pvt. Ltd., Ghat Road, Nagpur - 440 018. Ph. No. 2721216.

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Introduction

Enzymes are special molecules that catalyze biological transformations that cater for almost all the needs of a living system. They are mostly proteins in nature with a few exceptions of the self-splicing RNA molecules. The word "enzyme" means "in yeast", a term proposed by Kuhn in the beginning to indicate their association with yeast cells. It is now however known that they are present in almost all living systems.

General Properties of Enzymes

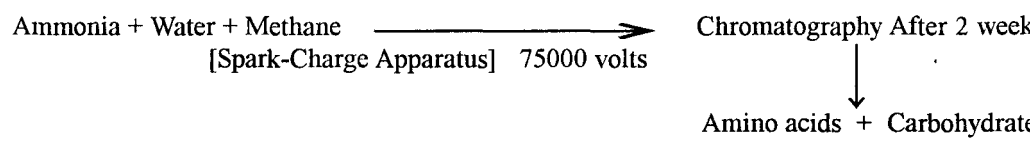
1. Enzymes are recovered unaltered, in their native form after the reaction.
2. Enzymes enhance rate of reaction without any alterations in the chemical equillibrium.
3. Enzymes may catalyse reaction in the reverse direction if the cell needs it.
4. Enzymes are high molecular weight, colloidal state and show slow rate of diffusion.
5. Enzymes are required in minute concentration for catalysis.
6. Enzymes have exceptionally high catalytic efficiency and specificity.
7. Enzymes are susceptible to varios environmental parameters.

It is a matter of great interest to know some facets of how enzymes were evolved during the evolution of species before studying their chemistry, organization and mechanism of action. The present chapter shall try to shed some light on the related issues.

Evolution of Enzymes

The Russian scientist and biochemist A.I.Oparin was the first to suggest that a long evolution of chemicals preceded the origin of life on earth. He proposed that the primordial atmosphere of earth had water, methane and ammonia from which a colloidal body called as coacervate resulted due to the series of changes. The coacervates were not living but behaved much like them directed by the chemicals to natural selection and reproduction by fragmentation.

Miller and Urey devised an apparatus to prove the synthesis of chemicals during the prebiotic atmosphere.



Miller's Experiment

Miller's final solution contained many carbohydrates, amino acids and organic compounds. This supported Oparin's hypothesis.

Many scientists did similar kind of work during 1940s to 1960s. The modern self-assembly theory states, protobiogenesis occurred due to the inherent property of chemicals of self-assembly and organization due to the conversion of micro molecules to macromolecules and probably occurred frequently on earth. Schmitt who synthesized collagen from simple molecules gave the first clear-cut demonstration. The work of

Lehninger, Frankel—Conrat, Ramchandran, Caspar has provided further support to the hypothesis.

Proteins were the first bio macromolecules synthesized during the pre biotic period. Initially they were random and contained some enzymatic activity due to the polymerization of diverse amino acids, by using the thermal energy available in the environment and may have at some stage acted as an informational molecule for synthesis of new proto proteins. These proto proteins upon contact with water underwent self-assembly through inter and intra molecular forces to form protocells. The work of Fox *et.al* on the synthesis of proteinoids and microspheres is an intelligent treatise that clearly explains the evolution of macromolecules. The protenoids formed were found to have some catalytic activity such as degradation of glucose, which could be lost on heating them. Researchers developed many other models during the same period.

Over the period the slow evolution has resulted into the formation of the present day efficient and highly complicated multi cellular, multi organ systems.

Thus it can be said that the evolution of enzymes is concurrent to the evolution of life. They seem to have played a vital role in the process of evolution and development of the cellular systems.

Organization Of Enzymes In Cells

Although system of many enzymes, works perfectly well in simple solution the system of the cell is far more complex and not essentially homogenous. Moreover the cell contains many sub cellular organelles, each of which carries out a specific set of functions, each in turn requiring the role of specific enzymes. Microscopy, especially the electron microscopy has played a great role in our understanding of the cellular organization. Although the detailing of the structure of cell membrane and cytoplasmic inclusions is out of the scope of this book, it is necessary to make a brief mention of some basic facets of the system.

A typical cell is bounded with a cell membrane that is semi permeable and broadly regulates what comes in and goes out of the cell. It is differentiated generally into cytoplasm and nucleus. The cytoplasm is a viscous liquid in which the different cell organelles are suspended. The organelles each in turn are membrane bound structures that include the Golgi apparatus, mitochondria, mesosomes, ribosomes, endoplasmic reticulum, vesicles and in plants, chloroplasts, glyoxysomes, etc. Each organelle generally performs specific functions, for example mitochondria carries out TCA, fatty acid oxidation, ETC and oxidative phosphorylation, ribosomes are involved in protein synthesis, and hence each carries a special mix of enzymes within. It is therefore clear that the cell is a highly organized system, and the study of intracellular localization of the various enzymes is an important tool to know more about the composition and function of the cell. It should be noted here that mere extraction of enzymes from minced tissue does not give a clear-cut idea about the exact location within the particular organelle. Yet another consideration required here is that not all enzymes are restricted to particular organelles or cytoplasm but may be distributed in cytoplasm and specific organelles as well. [For example malate dehydrogenase, or PEPCK]

When a particular enzyme is specifically present in a particular cell fraction only it is called as a *marker enzyme*. [discussed a little later].

Understanding The Localization Of Enzymes: Cellular Fractionation

There have been two approaches employed to study the intracellular localization

1. Micro-histo-chemical methods.
2. Differential centrifugation

1. Micro-Histo-Chemical Methods

The micro histo- chemical techniques depend upon the liberation of a staining substance in the tissue sections after the enzymatic activity, followed by microscopic examination of the stained tissue. They have been very helpful in localizing many reactions and tissue or organ specific distribution of enzymes and biochemicals. Techniques like micro dissection have been used for cells that are large enough. The partition of the enzymes can be then compared. Hotler has presented a classic example of the localization of succinate dehydrogenase, which is located in the mitochondria and di peptidase, which is uniformly distributed.

2. Differential Centrifugation Technique

The most of our present day knowledge about localization of enzymes in cells and organelles has been generated by use of cell fractionation and differential centrifugation. Homogenization and mincing of the tissue is a prerequisite for subjecting it to fractional separation. This allows the subjection of the crude extract to differential centrifugal fields thereby isolating particular organelles, which can be further used for isolating the enzymes contained in them. One of the most striking features, which have presented themselves during such studies, is the indication that most of the times the enzymes and coenzymes related to a particular pathway are localized within the same particle [for example the enzymes of glycolysis are entirely located in the cytoplasm while that of the oxidative phosphorylation are located in the mitochondria]. It is also interesting to note that many of the essential coenzymes are largely concentrated in the mitochondria. Schneider reports that about 50% of the total liver Co A, 65% of FAD, some part of NAD and almost all the cytochromes a, b and c are located in mitochondria.

The lysosomes are related with the hydrolytic processes and so are the microsomes. Microsomes contain about eight esterases. Some other enzymes are also present in them.

Many enzyme systems are present in the ground plasm of the cell. The supernatant also contains systems that bring about glycolysis, synthesis of glutamine etc. a comparative study has indicated the following distribution of enzymes in rodent livers.

Fraction Number	Some Enzymes
1. Nuclear fraction	Glycolytic enzymes, LDH, Nucleotidyl transferases etc.
2. Smooth & Rough ER fraction	Cholesterol biosynthesis, fatty acid elongation, drug metabolizing enzymes
3. Microsomal fraction	Esterase,AlkalinePhosphatase, Sulphatase etc.
4. Mitochondrial fraction	TCA and ETC enzymes etc.
5. Soluble enzymes	LDH,Xanthine oxidase, Deaminase etc.

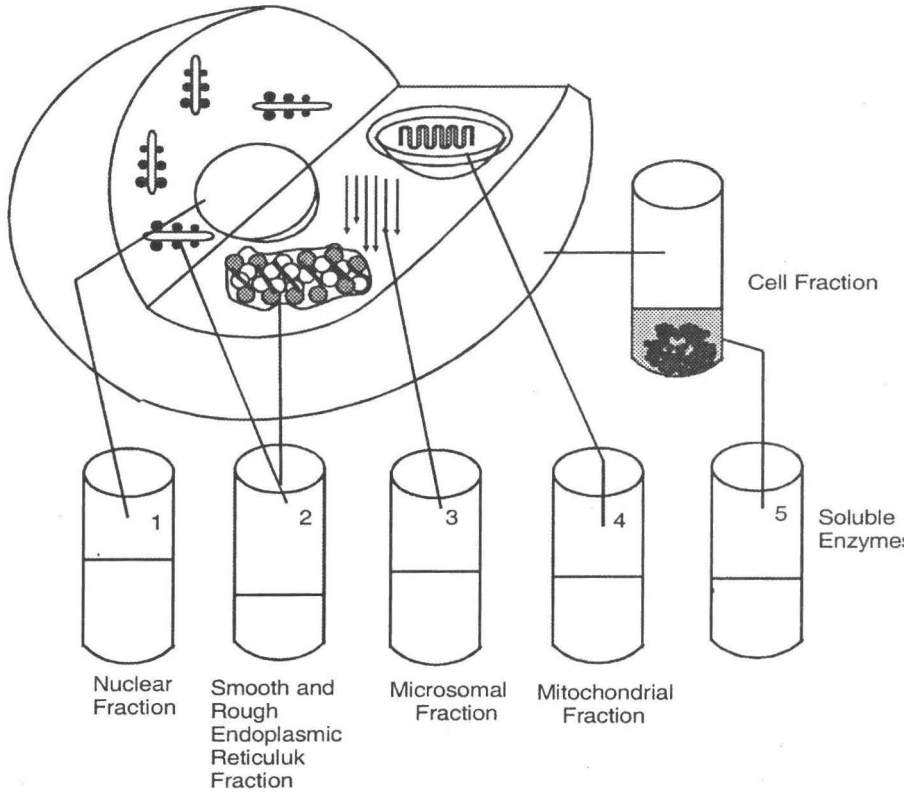


Fig 1.1 Distribution of enzymes in liver extract

Fraction number	Some enzymes
1. Nuclear fraction	Glycolytic enzymes, LDH, Nucleotidyl transferases etc.
2. Smooth & Rough ER fraction	Cholesterol biosynthesis, fatty acid elongation, drug metabolizing enzymes
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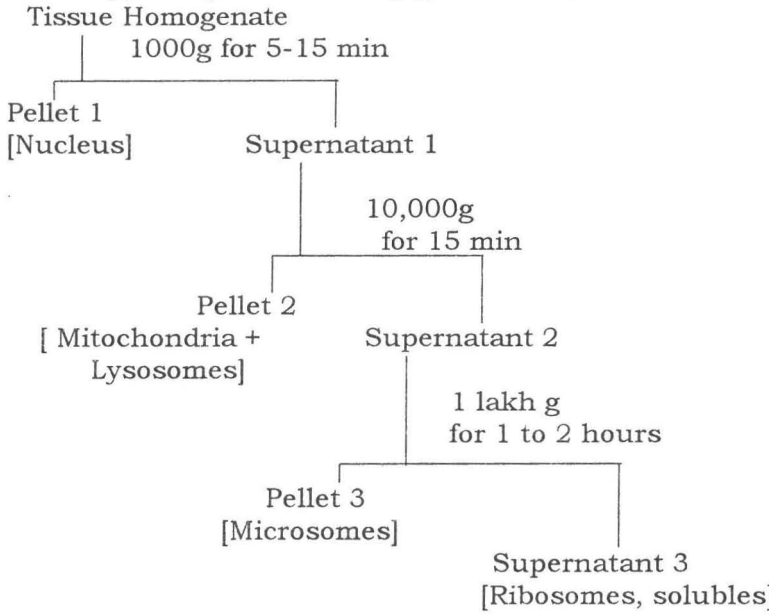
4. Mitochondrial fraction	TCA and ETC enzymes etc.
5. Soluble enzymes	LDH,Xanthine oxidase, Deaminase etc.

Nuclear Fraction

The nucleus is the largest sub cellular organelle. A typical liver cell nucleus has a volume of about 5% of the total cell. The enzymes present in the nucleus are glycolytic enzymes, pentosephosphate enzymes, LDH, malate dehydrogenase, arginase in the soluble space, RNA nucleotidyl transferases, nucleoside triphosphatase, DNA nucleotidyl transferases, NMN adenylyltransferase, bound to chromatin, RNA methyltransferases, ribonucleases concentrated in the nucleolus and glucose-6-phosphatase and acid phosphatase bound to the membranes.

Mitochondrial Fraction

The mitochondrion is the powerhouse of the cell. The liver parenchymal cell carries about 1000 – 1600 mitochondria. It is divided into the outer membrane, inner membrane including the cristae, and the matrix. The enzymes distributed in the mitochondria include D-3-hydroxybutyrate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase, glutamate dehydrogenase, urate oxidase, cytochrome oxidase, acetyl coA acyl transferase, adenylate kinase, ribonuclease, thiosulphate-sulphurtransferase, acid phosphatase, deoxyribonuclease, aryl sulphatases A&B, β galactosidase, β-acetyl amino deoxy glucosidase,



Microsomal Fraction

The microsomal fraction is isolated after the separation of the mitochondria and lysosomes, and subjecting the supernatant to 100,000 g for 3 hrs of centrifugation. The pellet obtained contains the microsomes, which have the enzymes including Carboxylesterase, acetylcholinesterase, cholesterolesterase, alkaline phosphatase, glucose-6-phosphatase, arylsulphatase etc.

Soluble Enzymes Present In Supernatant

The supernatant is obtained after the separation of the microsomal fraction after the homogenate is subjected to the proper g*min combination. It comprises most of the soluble phase from both the cytoplasmic and luminal sides of the endoplasmic reticulum. It will also contain soluble contaminants from any disrupted sub cellular organelles. It is not clear whether there are levels of organization within the cytosol or whether it is a random mixture of proteins, nucleic acids and other molecules. It is possible that at least some form of weak associations may exist within the cytoplasmic enzymatic systems. In some cases definite associations like formation of a multi enzyme system through physico-chemical bondings have been observed. The various enzymes found in this fraction include Lactate dehydrogenase, isocitrate dehydrogenase, xanthine

oxidase, glutathione reductase, aldolase, adenosine deaminase, hexosediphhosphatase, phosphoglucomutase, glucokinase, leucine aminopeptidase, aconitase, purine nucleoside phosphorylase. Etc.

Endoplasmic Reticulum

The endoplasmic reticulum is divided into rough endoplasmic reticulum and the smooth endoplasmic reticulum. The smooth ER seems, to contain groups of enzymes related to cholesterol biosynthesis, steroid hydroxylation, fatty acid elongation, drug-metabolizing enzymes etc.

Compartmentalization of Enzymatic Pathways

In the intact cell there are many connections within the various pathways that occur simultaneously since some of the substrates, coenzymes and cofactors as well as the regulatory molecules and even some enzymes may be common to some of the pathways.

This can be explained with the inter relationships between the pathways of glycolysis, gluconeogenesis, and TCA with the metabolism of fat within the liver cells. The sequence of glycolytic enzymes from phosphorylase to lactate dehydrogenase is located in the cytosol. The reversal of glycolysis, gluconeogenesis uses the same enzymes except for the three steps indicated below:

Step I

$$ATP + \text{pyruvate} + CO_2 + H_2O \xleftarrow{\text{Pyruvate Carboxylase}} ADP + Pi + \text{Oxaloacetate}$$

$$GTP + \text{Oxaloacetate} \xleftarrow{\text{PEPCK}} GDP + CO_2 + \text{Phosphoenolpyruvate}$$

Step II

$$\text{D-fructose 1,6- bisphosphate} + H_2O \xrightarrow{\text{bisphosphatase}} \text{D-fructose 6-phosphate} + Pi$$

Step III

$$\text{Glucose-6-phosphate} + H_2O \xrightarrow{\text{glucose-6-phosphatase}} \text{D-glucose} + Pi$$

Fructose bisphosphatase is located in the cytosol but glucose-6-phosphatase is the enzyme of endoplasmic reticulum [microsomal fraction]. The alternative pathway for conversion of glucose-6-phosphate [pentose phosphate pathway] also occurs in the cytosol.

The TCA cycle and β - oxidation of fatty acids occurs in the mitochondria, all enzymes being present in the mitochondrial matrix except the succinate dehydrogenase which is bound to the inner surface of the inner mitochondrial membrane. The fatty acids are synthesized from acetyl CoA using a specific carboxylase and fatty acid synthetase complex present in the cytosol. The principal connections between the pathways are through the redox cofactors, NAD^+, $NADP^+$, adenine nucleotides, and metabolic Acetyl-CoA. Metabolic intermediates of one pathway may also be regulators of other pathways [for example, citrate and acetyl CoA, are regulators for glycolysis.] Acetyl CoA is the metabolite that serves as a link between these pathways. Now the

acetyl CoA generating pathways exist in the mitochondria, therefore fatty acids for their oxidation have to be activated and transferred into the mitochondrion from the cytosol. This is brought out by the carnitine transferase system. Similarly the pyruvate generated in the cytosol also has to enter the mitochondrion and this occurs by the pyruvate – proton symport located in the mitochondrial membrane. The citrate produced in the TCA can be brought out to the cytosolic compartment by a tricarboxylate transporter. The reducing equivalents are transported across the membranes through special shuttle systems. The malate—oxaloacetate and the malate aspartate shuttles are some such examples.

Membrane Enzymes Play Important Roles

The fluid mosaic model of membranes has been a big leap in understanding the constituent proteins of the membranes and their functions. Studies with these have strongly suggested the vectorial arrangement of the proteins in the membrane, however some proteins span across the membranes. Many cells and cell organelles contain some enzymes associated with them. Examples include aldolase, glyceraldehyde phosphate dehydrogenase. The mitochondrial inner membrane contains the respiratory complexes including the NADH reductase, the cytochrome oxidase, adenylate cyclase.

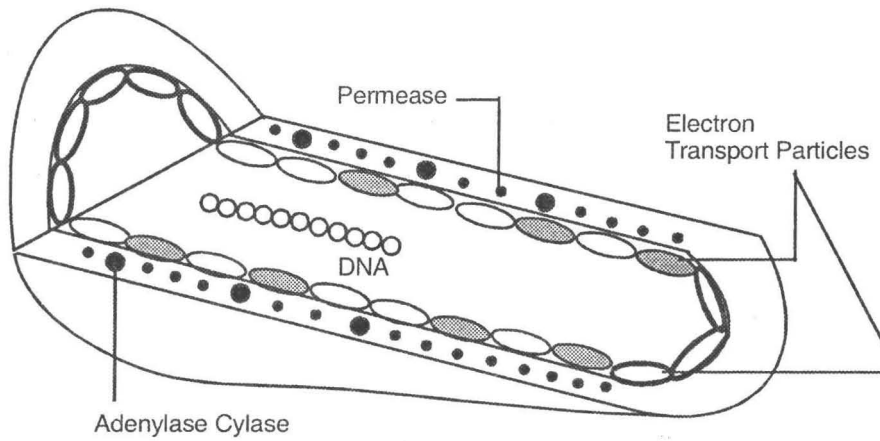


Fig. 1.2 Membrane Bound Enzymes

The glycerol phosphate dehydrogenase of the mitochondrial membrane is a part of the glycerol phosphate shuttle that transports reducing equivalents. The enzyme is located in the inner mitochondrial membrane and contains FAD. The cytoplasmic NADH is used up in converting the dihydroxyacetone phosphate to glycerol 3 phosphate by the cytoplasmic enzyme glycerol –3-phosphate dehydrogenase. The glycerol phosphate then donates the reducing equivalents to the FAD attached to the membrane bound glycerol –3- phosphate dehydrogenase. The $FADH_2$ is then transferred to the respiratory chain.

Adenylate cyclase on the other hand catalyzes the synthesis of cyclic AMP from ATP. The compound acts as a second messenger between the hormone and the ultimate cellular response that is modified by it. It has to be noted here that many other compounds like the cholera toxin manipulate the adenylate cyclases.

The role of the membrane in relation to the enzymes bound to it may be either of the following:

1. The membrane acts as an anchor for example in the case of amino peptidase bound to the brush border of the intestine that enables the increased uptake of amino acids after the hydrolysis of the oligopeptides.
2. For some enzymes the substrates and the products are less soluble in the aqueous environment but they are fairly soluble in the hydrophobic environment, this being especially true about the glyco lipid synthesizing enzymes or the ubiquinone carriers.
3. The membrane serves as a medium for specific spatial arrangement of the multi enzyme complexes, for example the acyl Co A desaturase system of the endoplasmic reticulum.
4. They may act as a separating system that is involved in transport of substrate, product or cofactors.

Table 1.1 Membrane Bound Enzymes

Membrane Bound Enzyme	Location	Function
Glycerol 3 Phosphate dehydrogenase	mitochondrial membrane	Transfer of reducing equivalents [FADH]
Adenylate cyclase	Cell membrane	Synthesis of Camp
Glucose 6 phosphatase	ER membrane	Release of free glucose

Enzymes In Prokaryotes

The prokaryotes are small, mostly unicellular organisms that are usually devoid of a well-developed intracellular membrane and organelle system, and particularly lack a distinct nucleus. It is usually thought that the prokaryotic cells perform all the essential functions with the help of their membranes. It can thus be said that the microbes contain many enzymes bound to their membranes. Yet another important consideration here is that these organisms are devoid of the supra cellular controls such as hormones and neural controls and that makes them evolve a variety of regulatory systems that accommodate the changes occurring in the environment and the need of the cells. The prokaryotes normally can survive if the medium can provide their basic requirements such as minerals, a carbon and nitrogen source, as well as the other growth requirements. The processes are made easy by the use of regulatory mechanisms that modulate the *inducible enzymes & constitutive enzymes.*

It should be remembered here that most enzymes are universal, that is, irrespective of the fact, that they are produced by prokaryotes or eukaryotes most enzymes are similar in composition and functions. A species specific or organ specific types are however encountered, which alters the affinity and activity of enzymes.

Enzymes in Eukaryotes

The eukaryotic cells on the other hand are a more complex system. More so because of the multi organ inter relationships, the complex phenomena that employ neural and hormonal control mechanisms. The eukaryotic cells also contain well-defined and distinct intracellular organelles each having specific functions and therefore have a related milieu of enzymes and modulators.

The eukaryotic cells employ the genetic as well as cellular levels of control of both, the synthesis and activation of enzymes. The synthesis of enzymes is a continuous process in every living organism. An increase in the number of molecules of each essential enzyme must clearly take place whenever the quantity of living matter increases during growth. In animals the intracellular protein is in a state of dynamic equilibrium, in which the proteins are continually being broken and replaced by re synthesis. In the case of certain animal enzymes there is a direct isotopic evidence of fairly rapid turnover.

The biosynthesis of enzymes is therefore an extremely important process, without which life would soon come to a halt. The complete synthesis of an active enzyme involves the building up of both the protein part and the prosthetic group if any. The synthesis of enzymes normally takes place within the living organisms, but it can occur in much simpler systems. It can take place in perfused organs or even in tissue slices in vitro, in disrupted bacterial cells, and even [in certain cases] in cell free extracts from animal tissues. The synthesis of enzymes in the living cell is under two different kinds of control.

In the first place their production, like that of proteins in general, is under genetic control, a given enzyme can only be formed if the corresponding gene is present in the cell and if the gene is absent or damaged by mutation the enzyme will also be absent.

In the second place the production of many enzymes is strongly influenced by the presence of metabolites, usually, but not always, either substrates or products. The mere presence of a gene does not guarantee that the corresponding active enzyme will be produced in significant amounts, the presence of an "inducer" [most frequently, the substrate of the enzyme or a related small molecular substance] may also be required. The phenomenon of enzyme induction is specially marked and easy to demonstrate in microbes but a number of cases of induction in animal tissues have been observed. Two of these are,

1. The ten- fold increase in the alcohol dehydrogenase content of rat kidney as a result of adding alcohol to the diet for a month, and
2. Increase in liver arginase in rats kept on a high protein diet and therefore needing to convert large amount of arginine into urea.

The converse of the induction effect is "enzyme repression" which is a specific inhibition of the formation of a particular enzyme caused by an accumulation of a product of the reaction which it catalyses.

An important phenomenon in eukaryotes is the *activation of enzymes by modification of their inactive precursors, the conversion of the zymogens into active enzymes.* This is true about many proteases, which have a target site away from their site of synthesis. This is usually brought about by elimination of a leader sequence of a few amino acids. This is especially true about the protein digesting enzymes like pepsin, chymotrypsin, trypsin etc.

Multienzyme Complexes

The high specificity of enzymes and the diverse, multi step and multidirectional movements of metabolism through the synchronization of the pathways involved require a proximity and possibly binding of at least some enzymes, and/or coenzymes to bring about effectively the formation of desired product [s]. Such a chemical aggregate of

enzymes and/or coenzymes is termed as a multi enzyme complex. Many multi enzyme complexes are present throughout the living systems and some of them have been isolated and characterized. The best studied include

(a) The pyruvate and a keto glutarate dehydrogenase complexes

(b) The fatty acyl synthetase complex

(c) The "U" and "A" complexes of pyrimidine de Novo synthesis.

A brief description of each of these is given below:

(a) The Pyruvate And Alpha Keto Glutarate Dehydrogenase Complexes

They exist in the mitochondria, and each comprises of three enzymes and five coenzymes. The constituent enzymes differ but the coenzymes are same. [Table 1.2] These multi enzyme complexes catalyze the conversion of the respective keto acid into a Co A derivative, acetyl Co A in case of pyruvate dehydrogenase and succinyl Co A in case of alpha keto-glutarate dehydrogenase complex.

A comparison in between the two complexes is given as:

Table 1.2: The keto Acid Dehydrogenase Complexes Compared

Point of comparison	PDH Complex	KDH Complex
Location	Mitochondrial matrix	Mitochondrial inner membrane (?)
Product formed	Acetyl Co A	Succinyl Co A
Enzymes	Pyruvate dehydrogenase, dihydrolipoyltransacetylase, dihydrolipoyldehydrogenase	α ketoglutarate dehydrogenase, succinyl transacetylase, dihydrolipoyl dehydrogenase
Coenzymes	TPP, Lipoate, Co A, FAD, NADH	TPP, Lipoate, Co A, FAD, NADH

The reactions of the pyruvate dehydrogenase complex are written as:

1. Pyruvate + TPP —E1 → hydroxy ethyl TPP
2. Hydroxyethyl TPP + Lipoate –E2 → acetyl lipoate derivative + TPP
3. Acetyl lipoate derivative + Co A —E2 → acetyl Co A + diydrolipoate
4. Dihydrolipoate + FAD —E3 → $FADH_2$ + Lipoate
5. $FADH_2$ + NAD^+—— E3 → NADH + H^+ + FAD

(b) The Fatty Acyl Synthetase Complex

The fatty acyl synthetase is a multi enzyme complex comprising of seven proteins, six of which are enzymes and a central binding protein called as the acyl carrier protein. The reactions of the fatty acyl synthetase complex occur in the cytoplasm. The complex seems to exist as a dimer having dimensions of about 25 nm X 21 nm. The monomer seems to have three domains; domain 1 has three enzymatic activities, 3- ketoacyl synthase, Malonyl- transacetylase and acetyl transacylase. The domain 2 seems to have four proteins, which include the acyl carrier protein, 3-hydroxy acyl dehydratase, enoyl reductase, 3-ketoacyl reductase, while the third domain contains the thioesterase activity.

The fatty acyl synthetase complex seems to synthesize two palmitate molecules at one time.

Table 1.3: Components Of Fatty Acyl Synthetase Complex

Protein	Function
Acyl carrier protein	Central protein and carrier of acyl groups
Transacetylase	Transfer of acyl groups
Malonyl transferase	Transfer of malonyl group from Co A
Ketoacyl synthase	Condensation of acetyl and malonyl groups
Ketoacyl reductase	Reduction of b keto group
Hydroxyacyl dehydrogenase	Removal of water from beta-hydroxy group
Enoyl reductase	Formation of saturated acyl residue

(c) The multi Enzyme Complexes of The Pyrimidine Synthesis

There are two multi-enzyme complexes employed during the de Novo synthesis of pyrimidines. The "A" complex comprises of the first three enzymes that sequentially catalyze the reactions leading to the synthesis of dihydro orotate from carbon-di-oxide, ammonia and phosphate. They are

(a) Carbamoyl phosphate synthetase

(b) Aspartate transcarbamoylase

(c) Dihydro orotase

The complex has a molecular weight of about 210 Kdal. The second complex, "U" comprises of two enzyme activities,

(A) Orotate phosphoribosyl transferase and

(B) OMP decarboxylase,

It has a molecular weight of 51 Kdal. The uridylate thus synthesized is used for the synthesis of other pyrimidines.

Marker Enzymes

The marker enzymes have been defined earlier as those enzymes are firmly bound to specific organelle membranes. Their localization and occurrence has been a matter of interest for various scientists. They have immensely contributed to the study of metabolism, cellular or organellar localization of pathways, distribution of biochemicals and the understanding of trans-membrane transport of molecules. Some of the important marker enzymes are tabulated as,

Table 1.4: Marker Enzymes

Organelle	Marker Enzyme
Cell membrane	5' nucleotidase
Nucleus	Deoxyribonuclease
Mitochondrion	Succinate dehydrogenase
Microsomal fraction	Glucose-6-phosphatase

A brief discussion of two marker enzymes is given here.

The Succinate Dehydrogenase (Mitochondrial Marker)

This enzyme, tightly bound to the inner mitochondrial membrane is rather difficult to extract from the membrane in soluble form. Many years of research have been required to analyze its composition, properties and mechanism. Its reducible coenzyme FAD functions as hydrogen acceptor in the reaction. The reduced enzyme can donate electrons to various artificial electron acceptors, for example reducible dyes. The beef-heart mitochondrial succinate dehydrogenase has a molecular weight of about 100,000 and contains one molecule of FAD, 8 atoms of iron and 8 acid labile sulfur atoms.

The enzyme appears to have 2 subunits of 30000 and 70000 molecular weight. The larger subunit of succinate dehydrogenase contains the FAD, 4 atoms of iron and 4 acid labile sulfur groups. The smaller subunit is an iron-sulfur protein containing 4 iron atoms and 4 acid labile sulfurs. The FAD is covalently bound and can be released on tryptic digestion on the large sub unit [indicating that it is a prosthetic group firmly bound to the polypeptide]. The FAD is attached via the methyl group of flavin moiety to the imidazole nitrogen of histidine side chain. The iron atoms of both the sub units of succinate dehydrogenase undergo a valency change [Fe II – Fe III] during the electron transfer from succinate to the respiratory chain. The enzyme removes Trans hydrogen atoms from succinate.

It seems to be an allosterically modulated enzyme since ATP, succinate and phosphate and QH_2 act as its positive modulators, while low concentrations of oxaloacetate inhibit the enzyme.

The Glucose – 6 – Phosphatase (microsomal marker)

The enzyme catalyses the most important step of gluconeogenesis, the formation of free glucose from glucose-6-phosphate. It occurs in the liver predominantly however some activity is seen in kidneys and intestinal epithelium. It requires Mg^{++} as an essential; cofactor and brings about the hydrolysis of the C6 phosphate. The reaction is exergonic with a free energy difference of –3.3 Kcal. It is absent in muscles and brain.

It also acts on 2-amino, 2-deoxy glucose-6-phosphate.

Terms Used In Enzymology

1. Enzyme

The most comprehensive definition of enzyme can be written as " a bio catalyst, mostly protein in nature, which brings about biological transformations within a specified range of physicochemical parameters and with specificity of action.

It was initially said that " all enzymes are protein in nature but all proteins are not enzymes", but this was modified with the discovery of Ribozymes [RNA as enzyme] in 1980s by Thomas Cech et.al.

Enzymes are usually large molecules with high molecular weight and take part in the reactions by forming an ES complex.

2. Holoenzyme

A holo enzyme = apo enzyme + coenzyme + cofactor. Where holo enzyme represents a fully active enzyme, example includes glucokinase, which converts glucose into glucose – 6 – phosphate.

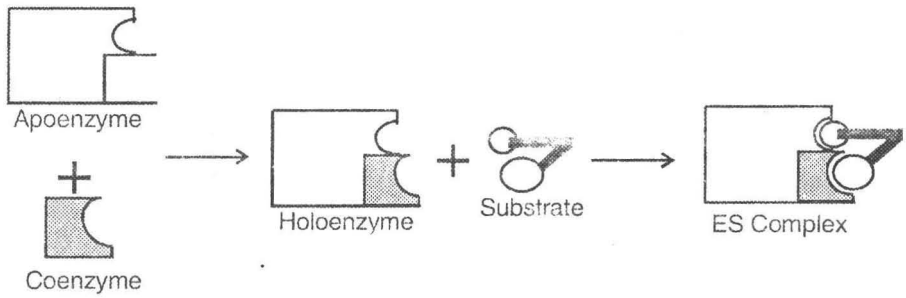


Fig 1.3 Holoenzyme, Coenzyme And ES Complex

3. Apo Enzyme

It is defined as the protein part of the enzyme that can combine with specific coenzymes and/or cofactors for catalytic activity.

4. Substrate

A substrate is defined as a substance upon which the enzyme specifically acts, to convert it into product/[s]. For example, glucose is the substrate for hexokinase or glucokinase.

5. ES Complex

In contrast to the chemical catalysts, enzymes take part in the reaction through formation of a transition complex called as the ES complex. The complex is in equilibrium with free E & S and slowly proceeds in forward direction to produce the products.

6. Product

The substance[s] formed after the ES complex dissociates is called as product[s].

7. Coenzyme

It is defined as a substance that is essential for activity of specific enzymes. It is a small organic compound separable from enzyme by dialysis, in the absence of which the activity of enzyme is either lost or markedly reduced. Examples of coenzymes include ATP, NADH, FAD, TPP etc.

8. Cofactor

A cofactor is a small inorganic molecule or ion that is required in addition to the coenzyme by many enzymes for their action. Many authors use the terms synonymously but they can be differentiated on the basis of their nature and size. Consider the reaction of hexokinase:

Glucose + ATP + Mg++ $\longrightarrow$ glucose 6 phosphate + ADP

Here glucose is substrate, ATP is a coenzyme, and Mg ion is a cofactor.

9. Activator

Certain enzymes require activation prior to their activity and this is brought about by modulators that may be the substrates themselves or other small organic molecules or ions. Such molecules or ions are called as activators. Chloride acts as an activator for the enzyme amylase.

10. Inhibitors

Any substance that negatively affects the enzymatic activity by drastically reducing the rate of catalysis or completely stopping the reaction , is called as an inhibitor. They are broadly of two types viz. Irreversible and reversible inhibitors.

11. Irreversible Inhibitors

They are also called as enzyme poisons. They bind to the active site residues forming stable covalent bonds which completely and irreversibly decrease the rate of reaction. Examples of irreversible inhibition include action of cyanide on Xanthine Oxidase[1.2.3.2] and nerve gas on Cholinesterase[3.1.1.7 3.1.1.8].

12. Reversible inhibitors

They are substances that reversibly bind to free enzyme, ES complex or both thereby temporarily reducing the rate of the enzyme catalysed reaction. This type of inhibition is characterized by the modifications observed in the Lineweaver Burke plots that show characteristic changes according to the type of inhibition.

Reversible inhibitions are of three types viz.: competitive, noncompetitive and uncompetitive inhibition.

13. Competitive Inhibition

It is inhibition caused by a substance that is a substrate analog, which competes with the substrate for the active site of the enzyme. It forms an unproductive, reversible EI complex. It affects the Km alone and its effects can be overcome by increasing the substrate concentration. The example of competitive inhibition is action of malonate on succinate dehydrogenase.

14. Non-Competitive Inhibition

It is a substance that does not compete with the substrate but binds at some other site with free enzyme or ES complex and usually EIS complex is formed which breaks at the rate same as ES complex. The diagnostic feature of this type of inhibitor is that Km does not change however the Vmax is altered [reduced]. Increasing the substrate concentration does not eliminate the effect of the inhibitor. The examples of non-competitive inhibitors include chelating agents that block the metal ions that are required by enzymes for their activity.

[EDTA blocks Me++ and thus those enzymes that require them].

15. Uncompetitive Inhibitor

The uncompetitive inhibitor does not bind to the free enzyme or free substrate but binds to the ES complex only, forming the ESI complex. The diagnostic feature of this type of inhibitor is that it alters the Km and Vmax both by the same factor. In the Lineweaver – Burke plots parallel lines with the same slope are obtained with increasing [I]. A classical example is that of inhibition of acetylcholinesterase by Pi.

16. Michaelis Constant (Km)

From the studies of Michaelis and Menten the curve obtained for the effect of substrate concentration on reaction velocity was found to be a rectangular hyperbola. The mathematical expression is given by the expression,

$$V_0 = \frac{V_{max}[S]}{Km + [S]}$$

Where, V_0 = initial velocity, V_{max} = maximal velocity, [S] = substrate conc. And Km = Michaelis constant

From experimental observation it has been proved that Km represents [S] at half-maximal velocity. In other words Km = Max/ 2 and it is characteristic of a given enzyme – substrate pair and indicates the affinity between them. More the Km, less is the affinity and lesser the Km more is the affinity between enzyme and substrate.

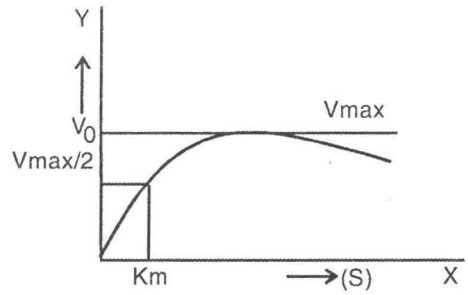


Fig1.4: MM Plot V_0 Versus [S]

A typical Michaelis Menten plot obtained when initial velocity is plotted against the substrate concentration, a rectangular hyperbola is obtained from which Vmax and Km can be determined.

17. V max Or Maximal Velocity

It indicates the maximal velocity of reaction at complete saturation of the enzyme active sites. It is a constant for a given enzyme substrate.

18. Active Site

It is the three dimensional structure where the substrate binds and the transformation takes place. It should be understood that the contributing amino acids might not be sequential as per the primary sequence but those that are far away from each other in the primary sequence. It is also called as *catalytic site* or *substrate binding site.*The actual amino acid side chains within the active site that are involved in creating the substrate binding domain and catalytic activity comprise the active center.

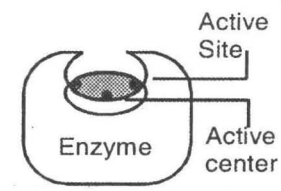


Fig:1.5 Active Site Of An Enzyme

19. Activity of The Enzyme

It is expressed in units/ml or IU [international units] or katals. A unit of enzyme activity is defined as that amount which will catalyze the transformation of one micromole of substrate per minute under defined conditions, [temp = 25^0, pH, [S], [E] etc are optimal. A katal is defined as amount of enzyme that transforms 1 mole of substrate per second.

20. Specific Activity

The specific activity is expressed as units of enzyme /mg protein. The determination of the specific activity of the pure enzyme involves the measurement of the initial velocity and also the amount of enzyme [mg of protein/ml] that produces this reaction velocity.

21. Molecular Activity

It is defined as the number of molecules of substrate transformed per minute per molecule of enzyme. It is also called as turnover number.

22. Enzyme Specificity

The ability of an enzyme to react with one or substrates determines its specificity. Generally three types are defined, viz. absolute, group and broad specificity.

23. Absolute Specificity

The ability of an enzyme to react with only a single substrate is called as absolute specificity. The classical example is of glucokinase, which can react with only glucose as its substrate. It does not react with any other hexose.

24. Group Specificity

Certain enzymes can react with a range of related compounds and this ability is called as group specificity. The best example is of Hexokinase, which can bring about the phosphorylation of hexoses,. Another example is of L – amino acid oxidases with many amino acids of the L- configuration.

25. Broad Specificity

Some enzymes can react with seemingly varied substrates. Such enzymes are said to have broad specificity. Example is of trypsin and chymotrypsin, which can react with proteins, shorter peptides and esters etc.

26. Multi Enzyme Complexes

Certain metabolic pathways are catalyzed by a group of enzymes and coenzymes that are physically linked or bonded to each other to form a complex aggregate. Such a complex is known as a multi enzyme complex. Examples include Pyruvate and alpha ketoglutarate dehydrogenase complexes and fatty acyl synthetase complex.

27. Marker Enzymes

Due to the compartmentalization of cells, certain enzymes are found bound or located in certain cell organelles or fractions during differential centrifugation. These are referred as marker enzymes since their presence and activity is used as a marker to isolate the specific organelle during cell fractionation. Examples include, succinate dehydrogenase for mitochondria and glucose 6 phosphatase for the microsomal fraction.

28. Inducible Enzymes

Certain enzymes are not present within the cells at all times but are synthesized after the appearance of their respective substrate only. Such enzymes are called as inducible enzymes. They usually undergo degradation after their use is over. Example includes the enzymes of lactose degradation in *E.coli*.

29. Inducer

A substance that is responsible for the synthesis of a particular inducible enzyme is called as inducer. For example lactose acts as inducer for an enzyme b- galactosidase.

30. Repressor

It is a substance that prevents the formation of enzyme. It is many times structurally related to the product or the product itself formed at the end of the reaction. Examples include repression of argininosuccinate lyase by arginine, or aspartate kinase by l- lysine.

31. Constitutive Enzymes

Certain enzymes are always present in the cells, i.e. they are not synthesized after the appearance of their substrates but are regulated by activation – deactivation phenomena. Such enzymes are called as constitutive enzymes. Examples include enzymes of the TCA cycle.

32. Enzyme Denaturation

The loss of the three dimensional structure of enzyme due to the action of physical or chemical agents is known as enzyme denaturation. This usually results in partial or complete loss of activity of the enzyme. Some of the agents that cause enzyme denaturation include 8M urea, temperature, pH, and Covalent bond forming modifying agents.

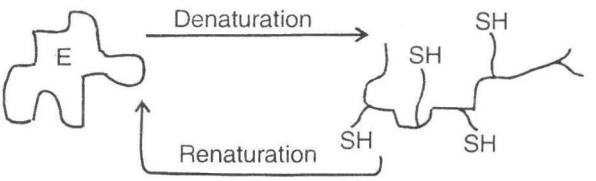


Fig 1.6: Denaturation Of Enzyme

33. Renaturation Of Enzymes

Upon removal of the denaturating conditions, some enzymes can regain their native, active conformation. This phenomenon is called as renaturation of enzymes.

34. Intracellular Enzymes

Enzymes located within the cells are called intracellular enzymes. They are usually secreted in the active form. They are difficult to isolate and purify than extra cellular enzymes. They can be present in the cytosol or intracellular organelles and therefore often called as endo enzymes. Examples include enzymes of glycolysis, HMP shunt FAS complex etc.

35. Extracellular Enzymes

They are secreted outside the cells where they are synthesized and usually their site of action is away from their site of synthesis. They are synthesized and secreted as inactive precursors and are easy to isolate and purify. They are also called as exo enzymes. Examples include digestive enzymes like pepsin, trypsin, etc.

36. Zymogens

They are inactive precursors of enzymes and usually contain many amino acids more than the active form. They are also called as pro enzymes and need to be c

37. Isozymes

Certain enzymes exist in different tissues and differ slightly from each other in amino acid content and activity. Such enzymes are called as isoenzymes. Example includes isozymes of LDH, amylase etc. Isoenzymes are often used as a diagnostic aid for differential diagnosis.

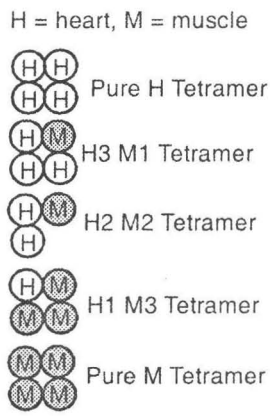


Fig1.7 Different forms of Lactate Dehydrogenase isoenzymes.

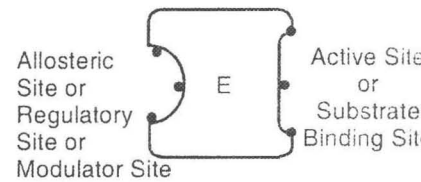


Fig 1.8 Allosteric and Active sites in enzyme.

38. Allosteric Enzymes

Enzymes that contain the other site [allos = other, stereos = site] for positive or negative modulation are called as allosteric enzymes. They are usually of regulatory nature and show sigmoidal kinetics. They comprise of sub units.

39. Oligomeric Enzymes

Enzymes having sub unit structures and that show positive and negative cooperativity are called oligo meric enzymes. Most of the allosteric enzymes are oligomeric in nature i.e. they contain more than one polypeptide in their structure. Examples include aspartate transcarbamoylase[ATCase].

40. IUB EC Number

With the discovery of thousands of enzymes, it became necessary to have a universally acceptable system of classification and nomenclature for enzymes. In 1956 the International Union of Biochemists set up an enzyme commission to formulate the rules for the same. The commission then gave a four digit numbering system for the classification of the enzymes.

Example of a 4-digit number is 1.1.1.1 for alcohol dehydrogenase , [alcohol:NAD oxidoreductase].

41. Prosthetic Group

It is a non-protein part firmly bound and integral constituent, present in some enzymes. It cannot be dialyzed or easily separated from the polypeptide chain it is associated with. Examples include FAD in dihydro lipoyl dehydrogenase, and biotin in pyruvate carboxylase]

42. Anti Enzymes

The antibodies produced after injecting enzymes in the blood of animals are called as anti enzymes. They are important tools to study differences between enzymes obtained from different sources.

43. Metallo Enzymes

They are complexes of metals with enzymes .Many enzymes carry metal ions as integral constituents in their structures and they are either involved in transfer of electrons, reducing equivalents etc. examples include cytochrome oxidase that carries Cu^{++}.

44. Transit Time

In a multiple enzyme system the overall rate of reaction does not depend only on the rates of the individual reactions but also on the time taken for the transfer of the product of the previous reaction which acts as a substrate for the next enzyme. The time taken for this transfer is referred as the transit time. The transit time is more in unorganised enzyme systems than that of the multi enzyme complexes.

45. Immobilized Enzymes

Many times commercially important enzymes are isolated from their natural sources and immobilized into an insoluble matrix or beads for their effective reutilization and commercial exploitation. Such enzymes are called as immobilized enzymes.

46. Bi Substrate Reactions

Some enzymes require more than one substrate for their activity. Such reactions can take place in two ways, viz. single displacement and double displacement. The single displacement reactions are of two types, Random and ordered.

47. Single And Double Reciprocal Plots

The classical MM plots are of the rectangular hyperbola type and at later stages do not satisfactorily explain the relationship of reaction velocity with the substrate concentration. Many scientists have proposed transformations of the MM equation based on which either single reciprocal [ex- Woolf plot] or double reciprocal [ex - Lineweaver Burke plot] are obtained. They are frequently employed for interpretation of kinetic data.

48. Ribozymes

The RNA molecules which exhibit tertiary structure can act as enzymes or show catalytic activity specifically by catalyzing the transesterification and hydrolysis of phosphodiester bonds in RNA molecules as well as also obey Michaelis Menten Kinetics are called as ribozymes.

49. Bifunctional Enzymes

When an enzyme has two different domains that perform specific and different activities, the enzyme is called as a bifunctional enzyme. Example of such enzymes includes the debranching enzyme that catalyzes two types of activities,

[a] Amylo transglucosidase activity.

[b] α - 1,6 glucosidase activity.

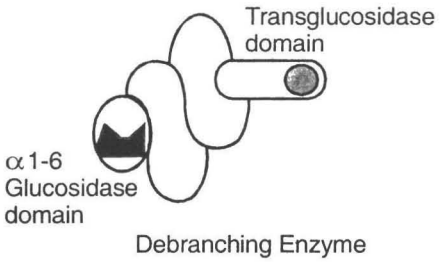


Fig: 1.9 Bifunctional Enzyme

50. Abzymes

Abzymes are defined as antibodies acting as enzymes. Antibodies are mostly proteins or glycoproteins and may show enzymatic activity.

BRIEF HISTORY OF ENZYMOLOGY

The Early Period

Enzymology is a science of comparatively recent origin however the pace at which it is expanding is a bit surprising. Originating from the basic sciences like chemistry, physics and biology, it has now grown in an independent science and has touched almost all the branches including the most recent applied sciences including biotechnology, diagnostics and fermentation technology.

The study of the history and development of enzymology makes an interesting reading. Various scientists from seemingly unrelated areas have developed genius approaches and ideas that have largely contributed to our present day understanding of the subject.

The brief review of the work of different scientists who have helped in understanding the various facets of what enzymes are, how they work and their commercial exploitation is discussed here.

Most modern authors accept that the origin of enzymology dates back to early 19th century. Dubrunfaut in 1830 prepared a malt extract from germinating barley seeds. This extract had the power to form sugar from starch.

A Payen and J.F.Persoz in 1833 found that an alcohol precipitate of the malt extract contained a thermo labile substance that converted starch into sugar. They named it as *diastase* from the word *diastasis,* which means separation. It separated soluble dextrins from the insoluble envelopes of starch grains.

In 1836, while investigating digestive processes, the German physiologist Theodor Schwann isolated a substance responsible for albuminous digestion in the stomach and named it pepsin, the first enzyme prepared from animal tissue.

Theodor Schwann was the founder of the theory of modern histology, defining the cell as the basic unit of animal structure. At the time he was an assistant at the Anatomisch-Zootonischen Institut in Berlin. Three years later he became professor of anatomy at the Catholic University of Louvain, in 1848 professor of physiology and comparative anatomy at Liege, and in 1880 he retired from teaching.

Berzillius J J in 1837 postulated the catalytic nature of fermentation. In 1839 the eminent German chemist Jutus von Liebig developed a mechanistic explanation for the role of yeast in the fermentation process. He viewed the yeast present in the fermentation mixture as a decomposing matter that emitted certain vibrations, the sugar atoms suffer a displacement; they rearrange themselves in such a way as to form alcohol and carbon dioxide.

On the other hand, alcoholic fermentation was considered to a be spontaneous reaction until 1858, when the French chemist and biologist Louis Pasteur proved in a series of publications that fermentation occurs only in the presence of living cells - a phenomenon correlated with life - a physiological act, as he called it. This divergence in the understanding of the nature of yeast in the fermentation process caused heated debate between Liebig and Pasteur.

In the year 1877, F.W.Kuhne coined the term ENZYME and distinguished them from bacteria. While in the year 1893, Ostwald proved that enzymes are catalysts.

Emil Fischer in 1894 demonstrated the specificity of enzymes and proposed the lock and key hypothesis. It was a brilliant attempt to explain the mechanism of enzyme action.

The year 1897 saw the contributions of Bertrand, who coined the term "coenzyme", while H. Buchner & E. Buchner showed that cell free extracts of yeast can ferment sugars, to form Carbon dioxide and ethanol.

During these early years, many scientists found similarity between the action of enzymes and that of yeast in fermentations. Enzymes were thus named as ferments, a name that is still in use in Germany.

Louis Pasteur: (1822-1895) His schooling up to Ph.D took place in Paris after which he became an Assistant Professor in Chemistry at Strasborough in 1849. He developed vaccines against anthrax and rabies. The Pasteur Institute was opened by the French Government in 1888 to treat cases of rabies. He is also known for the Pasteurization process that is now a routinely used in preservation of milk and wines.
He died in the year 1895.

Pasteur during his work on fermentation developed a firm belief that the processes like fermentation were inseparable from cells. Only intact cells had the power to bring about such processes. The views were not accepted by the other school of scientists led by J.Liebig, who held that processes of fermentation and respiration were due to the action of chemicals. The use of the terms like *organized ferments* and *unorganized ferments* was done to denote Cells and Cell free extracts respectively.

Liebig died in 1873 and Pasteur in 1895 without the debate being concluded. Subsequently, however, the German chemists Eduard Buchner and Hans Buchner discovered in 1897 that a cell-free extract of yeast could cause alcoholic fermentation. The ancient puzzle was solved; the yeast cell produces the enzyme, and the enzyme brings about fermentation. The Liebig-Pasteur dispute was thus finally settled, by Hans

and Eduard Buchner, who demonstrated, that cellfree yeast extract could convert glucose into ethanol and carbon dioxide just like viable yeast cells. In other words, the conversion was not attributable to yeast cells as such, but to their nonviable enzymes.

20th Century

In the year 1902, Emil Fischer & Hofmeister demonstrated that proteins are polypeptides.

[Fischer was the first to determine the molecular structures of glucose (or grape sugar) and fructose and to synthesize them from glycerol in 1890.]

The year 1905 saw some more interesting contributions, Knoop deduced Beta oxidation of fatty acids, while Harden & Young showed the requirement of phosphate in alcoholic fermentation and isolated the first coenzyme, cozymase now known as NAD. S. Sorenson in 1909, showed the effect of pH on enzyme action. He devised a pH scale, which is now universally accepted and used.

Batelli & Stern in 1912, discovered dehydrogenases, in the same year, Carl Neuberg proposed a chemical pathway for fermentation while Warburg postulated the respiratory enzyme for the activation of oxygen, showed the requirement of iron in respiration and demonstrated cyanide inhibition.

Wieland (1912-1922) showed the activation of hydrogen in dehydrogenation reactions.

Fundamental enzyme kinetics dates back to 1903. At that time Victor Henri concluded in Paris that an enzyme combines with its substrate to form an enzyme-substrate complex as an essential step in enzyme catalysis. Based on this idea, the general theory of enzyme action was expressed mathematically by Leonor Michaelis of Germany and Maud Lenora Menten of Canada in 1913. They postulated that the enzyme E first combines with its substrate S to form an enzyme-substrate complex ES in a relatively fast reversible reaction: E + S =ES. The latter complex then breaks down in a second, slower reversible reaction to yield the reaction product P and the free enzyme: ES = P + E.

In the year 1922, Otto Warburg devised mano metric methods to study metabolism of living cells. Briggs & Haldane in 1925, made important refinements in the theory of Michaelis and Menten.

Theodore Svedberg [1925-1930] contributed the development of Ultracentrifuge and its use to estimate the molecular weight of Hemoglobin.

James Sumner [1926] first obtained crystals of an enzyme, urease from jackbean and proved it to be a protein.

James Sumner : James Batcheller Sumner was born on 19th Nov.1887 at Canton. He graduated from Harvard College in 1910 in Chemistry. He studied Biochemistry under Prof.Otto Folin[1912-14] during which he obtained his Ph.D. He joined Cornell Medical School in 1914 and remained there till 1929.He crystallized urease, the first enzyme to be crystallized, in the year 1926. He received his Nobel Prize in 1946. He died on 12th August 1955 of cancer.

Sumner's research work at Cornell first centred on analytical methods, but despite his hard work he was unable to obtain any interesting results. He then decided to isolate an enzyme in pure form, an ambitious aim never achieved by anyone up to then,

but a type of research suited to his scanty apparatus and meagre laboratory staff. In particular, he worked with urease. His method for the isolation of enzyme is given further in the book.

For many years his work was unsuccessful, but in spite of the discouragement of colleagues who doubted whether any enzyme could ever be isolated in pure form he continued. In 1921, when his research was still in its early stages, he had been granted an American-Belgian fellowship and decided to go to Brussels to work with Jean Effront, who had written several books on enzymes.

The plan fell through, however, because Effront thought Sumner's idea of isolating urease was ridiculous. Back in Ithaca, he resumed his work until finally, in 1926, he succeeded. His isolation and crystallization of urease met with a mixed response; it was ignored or disbelieved by most biochemists, but it brought him a full professorship in 1929 and the Nobel Prize in Chemistry in 1946.

In the same year, K. Linderstrøm-Lang investigated many important detailed chemical properties of proteins at the Carlsberg Laboratory in Copenhagen. The 1924 publication The Ionization of Proteins laid down a basic formalism for the production of enzymes. The Lang theory is still the first approximation and remains in use for many problems where the molecular structure is not known. Warburg in 1928-33 deduced the iron – porphyrin nature of the respiratory enzyme. Around the same period isolation and crystallization of trypsin and pepsin was done by John Northrop, who also proved their protein nature.

John Northrop: John Howard Northrop was born in New York on 5th July 1891. He entered Columbia University in 1908 to study Zoology and Chemistry, graduated in 1912, Master of Arts in 1913 and received a Ph.D. in Chemistry in 1915. He joined the Rockefeller Institute in 1915 where he remained until 1949, when he was appointed Professor of Bacteriology, University of California, and later as Professor of Biophysics. He isolated crystalline pepsin in 1929 and with the same technique isolated other proteases. He died in 1987.

Warburg & Christian discovered the "yellow enzyme" a flavo protein. 1933 was another year, which saw enzymology growing by leaps and bounds. These were the days when metabolic pathways were worked out, important discoveries and contributions were made by H.A.Krebs & Kurt Hanseleit [discovery of the urea cycle], G.Embden & Otto Meyerhoff [proposed intermediates of glycolysis and fermentation.] In the same year Tiselius introduced electrophoresis for separation of proteins in solution. H.Krebs in 1937 postulated the citric acid cycle while transamination reactions were studied in the year 1938 by Kritzman *et.al*

George Beadle & Edward Tatum in 1940 proposed the one gene – one enzyme hypothesis. C.Cori & F.Cori in 1941 elucidated the Cori cycle for lactic acid. The next year, 1942, D.Mac Clean & I.M.Rowlands discovered hyaluronidase from mammalian sperms. Chance in 1943, applied sensitive spectroscopic techniques, to Enzyme – Substrate interactions. In the same year Leloir & Munoz demonstrated fatty acid oxidation in cell free systems, while Lehninger showed the stoichiometry of fatty acid oxidation and the requirement of ATP for it.

1950-1960: This decade was the beginning of a golden period for the development of enzymology, contributions from various scientists in the fields of coenzymes, metabolic sequences, protein and enzyme sequencing, development and advancements in

biochemical techniques etc. were made by many intelligent workers and worth mentioning are names that include, Linus Pauling, Richard Corey, [a- helix and beta pleated structures of proteins], Zamecnik et al [ribonucleoproteins and protein synthesis] Horecker, Dickens and Racker [elucidated the phoshogluconate pathway] Krebs & Kornberg H L [discovered glyoxalate bypass] while Arthur Kornberg discovered DNA Polymerase.

1960 – 1970: The 3D structure of the protein myoglobin was described by John C Kendrew.from sperm whale, while the amino acid sequence of ribonuclease was determined by W.Moore and S.Stein. The 3-D structure of lysozyme was presented by Phillips et.al while Temin, Dulbecco and Baltimore elucidated reverse transcription in 1970. Werner Arber introduced DNA cutting by enzymes. The first major breakthrough for microbial enzymes in the food industry came in the early 1960s with the launch of a glucoamylase free of transglucosidase. It allowed starch to be broken down into glucose. Since then, almost all glucose production has switched from traditional acid hydrolysis to enzymatic hydrolysis. By way of example, compared to the old acid process the enzymatic liquefaction process cut steam costs by 30%, ash by 50% and by-products by 90%.

Since 1973, when Termamyl was introduced for the continuous starch liquefaction process, the starch processing industry has grown to be the largest market for enzymes after the detergent industry. In a concentrated solution of starch, hydrolysis results in rapid viscosity reduction. Termamyl is consequently often referred to as the liquefying amylase.

Enzymatic hydrolysis is used to form syrups through liquefaction, saccharification and isomerization steps. 1974 saw the launch of the immobilized glucose isomerase Sweetzyme, thereby signalling another successful breakthrough in the starch industry. Sweetzyme is one of the few enzymes to be produced by continuous fermentation.

Yester years: Recombinant DNA technology has brought about a revolution in the development of new enzymes.

Although the recent developments have been more in the field of recombinant DNA technology, or biotechnology, enzymology has been making its contribution in its development. Some of the important topics that have attracted the attention of the scientific community include Restriction enzymes [Hamilton Smith, Daniel Nathans] Ribozymes [Sidney Altman & Thomas Cech in 1982] Polymerase chain reaction [Kary Mullis in 1985].

In 1995,the introduction of more sophisticated techniques allowed the computer modelling scientists at Novozymes to combine the molecular knowledge of enzyme 3D structures with DNA sequence knowledge and use them together as the basis for making new variants through site- directed protein engineering. The first product for which site-directed mutagenesis was used was the bleach-stable enzyme Everlase™. Enzymes have been given other useful properties using this technique, e.g. improved heat stability, higher activity at low temperatures and reduced dependency on cofactors such as calcium. One example is Termamyl SC for the starch and biofuel industry. In the late 90s considerable effort was invested in developing a new fungal expression system for the large-scale production of enzymes. In 2000 Novozymes was given the green light by the regulatory authorities to produce enzymes in Fusarium venenatum, an organism characterized as GRAS (Generally Recognized As Safe). Novozymes now

has Bacillus subtilis and Bacillus licheniformis as its main bacterial hosts and Aspergillus niger and Aspergillus oryzae, together with Fusarium venenatum, as its main fungal enzyme production.

The Future of Enzymology

As one can note, with the increasing interdisciplinary approaches and understanding it can now be said that enzymology has a great future ahead. It will take a center stage in all the areas, as it can solidly contribute in:

* ***Diagnostics:*** Alteration in the enzymatic or iso enzyme activity has been conveniently used for diagnosis and differential diagnosis. Take for example lactate dehydrogenase. There are 5 isoenzymes of LDH, namely H4, M3H, M2H2, H3M & M4. Each of these is present in specific tissues and the alteration of their activity as observed in the graphical pattern is an indication of the possible tissue damaged. Or the acid phosphatase is present as the prostatic and non-prostatic fractions.

Some of the commonly employed clinically important enzyme assays are tabulated as,

Table 1.5: Clinically Important Enzymes

Enzyme	Clinical Significance
Amylase	Pancreatic disease and function
Acid phosphatase	Prostate cancer
Alkaline phosphatase	Hepatobiliary or Bone diseases
Alanine Transaminase [SGPT]	Liver disorder
Aspartate Transaminase [SGOT]	Liver disorders, Myocardial infarction
Lactate dehydrogenase	Myocardial infarction, liver diseases, anemias, renal disorders, muscular dystrophy, malignancy.
Creatine Kinase	Myocardial infarction, muscular dystrophy, hypothyroidism, pulmonary and vascular diseases
Hydroxybutyrate dehydrogenase	Heart muscle damage

Alternately enzymes are often used to diagnose qualitative and quantitative presence of many important biochemicals. Some examples are given in the table,

Table 1.6 Enzymes Used In Diagnosis

Enzymes	Used To Diagnose
Glucose oxidase	Blood glucose level
Urease	Blood urea
Alkaline phosphatase	ELISA tests
Glucose6 phosphate dehydrogenase	Glucometer strips

* ***Industrial fermentations:*** The immobilization of enzymes has opened up a new era in the areas of industrial fermentations. Dairies, bakeries and single cell protein production are some of the target areas where advances in enzymology have revolutionised the processes and products.

Cellulases, glucanases, amylases, proteases	For liquefaction, clarification and malting processes in beer making
Amyloglucosidase	Production of sugar from starch for substrate production in alcohol manufacturing
Pectinases	Clarification of fruit juice in wine industry
Bromelain ficin, papain	Beer haze removal
Naringinase	Removal of bitter taste

* ***Food processing:*** Enzymes are used for an increasing range of applications: bakery, cheese making, starch processing and production of fruit juices and other drinks. Here they can improve texture, appearance and nutritional value and may generate desirable flavors and aromas. Currently used food enzymes sometimes originate in animals and plants [for example, a starch digestive enzyme, amylase, can be obtained from germinating barley seeds] but most come from a range of beneficial microbes.

Table 1.7 Commercial Uses Of Enzymes

Baking Industry	Alpha amylases	Maltose production, production of white bread etc.
	Amyloglycosidases	Saccharification
	Proteases	Protein hydrolysis, Biscuit manufacturing
	Glucose oxidase	Stability of dough
Meat Processing	Trypsin, Pepsin, Papain	Meat tenderizing
Protein and protein products	Proteases, trypsin, aminopeptidases	Breakdown of complex proteins to partial digests and other products
Starch Industry	Alpha amylase, amyloglucosidases, hemicellulases, glucose isomerase.	Modification and conversion to dextrose and fructose syrups, Manufacturing of low scalorie sweetners from starch.
Fats and oil processing	Lipases	Hydrolysis of glycerides and esters
Vinegar production	Alcohol dehydrogenase and acetylating enzymes	Production of white vinegar
Confectionary industry	Invertase	Prevents crystallization of sugars and chocolates
Paper industry	Amylase,	Starch degradation for sizing and coating papers with lower viscosity products
Rubber industry	Catalase	Conversion of latex to foam rubber
Detergent industry	Amylase, proteases, lipases	Washing of clothes and crockery

The advantages of enzymes in food production are multiple. They include:

1. They can replace certain chemicals thereby adding to energy conservation, better health and environmental conditions, easy digestibility and biodegradability.
2. They are more specific in action than synthetic chemicals. Their use lowers

production of wastes and thus pollution at the same time generating products of higher purity and quality

3. They allow some processes to be carried out which would otherwise be impossible. [for example the production of clear apple juice concentrate which uses pectinase to reduce the cloudiness of the final product.]

Some uses of enzymes in commercial processes are given below,

* ***Dairy Industry:*** enzymes have been widely used in dairy industry. Some of the enzymes and their uses are tabulated below

Table1.8 Enzymes In Dairy Industry

Enzyme	Application[s]
Rennet[protease]	Cheese production
Lactase	Lactose free milk and milk products
Lipase	Flavoring of milk products
Protease	Hydrolysis of whey proteins
Catalase	Removal of hydrogen peroxide

* ***Pharmacy:*** Production of many high potency drugs may involve single step manipulation of their precursors. Using enzyme-mediated modifications may bring about many such processes. Terreni et.al have presented a research paper on one-pot synthesis of cefazolin[antibiotic] from cefalosporin-C involving three consecutive bio transformations catalyzed by D- amino acid oxidase, glutaryl acylase and penicillin-G acylase.

* ***Fuel production:*** It is a well known fact that the fossil fuel stores are being rapidly depleted and thus the search for alternative fuels [synthetic hydrocarbons, liquid hydrogen, methane etc] and their production will become an essentiality in the near future. Use of enzymes in such processes cannot be ignored. Researches are underway to synthesize methane and octane from waste materials using immobilized enzyme complexes.

* ***Alternate energy generation:*** Bioenergy has drawn the attention of many scientists worldwide. Bioluminescence and related sectors are employing enzymatic systems. For example the enzyme luciferase in *Panus stypticus luminescence* has been shown to play a role in light emission. It requires the reduced NADH, an acceptor, and possibly molecular oxygen to produce bioluminescence.

* ***Treatment and dISposal of sludge, agricultural wastes:*** Agricultural wastes many times contain cellulose, hemicelluloses, lignin and pectin. They can be treated and converted into useful products by using enzymes.

* ***Bio leaching:*** Originally bioleaching was based upon use of microbial strains that had an ability to digest particular impurities or purify commercially important ores. Nowadays however the immobilized enzymes have made it easier to process the ores.

* ***Forensics:*** Enzymes are used to treat latent fingerprints for sensitivity enhancement.[J.For.Sc:31,825 : (1986)]

* ***Biotechnology:*** The restriction enzymes, ligases and enzymes used in the polymerase chain reaction are the basic fundamental requirements of biotechnology, a field that has a promising future. Plant and animal eugenics shall lead to a better crop, better cattle and better survival abilities.

* ***Therapeutic uses of enzyme inhibitors:*** The understanding of the inhibition of several enzymes by specific chemicals called as inhibitors has given rise to a new possibility of their use as potential drugs to control metabolic diseases. Some such examples include, use of zidovudine as inhibitor of reverse transcriptase of HIV, to control AIDS, allopurinol in cases of gout as inhibitor of xanthine oxidase, sorbimil as inhibitor of aldose reductase in diabetic retinopathy.

And all such processes and branches as our imaginations would permit and limit. It is not very inappropriate to say that the future of mankind shall depend on the intelligent use and reuse of the enzymes.

◆◆◆◆◆

2

ENZYME ISOLATION AND PURIFICATION

Introduction

Need of isolation of enzymes

- Identification of source of enzymes:
- General handling procedures for source and crude extracts:
    - Plant tissue
    - Microbial cells
    - Animal tissue
    - Handling of crude extracts

Methods of isolation

Methods for purification

- Some representative techniques employed initially for enzyme isolation
- Choice of methods of purification
- Terms related to enzyme purification

Development of assays:

- Qualitative assays
- Quantitative assays

Stabilization and crystallization of enzymes

Criteria for purity

Methods of preservation of purified enzymes

- General properties of enzymes

Introduction

The enzymes are found distributed widely in the living systems and are a major factor in the growth, maintenance and propagation of the living system. In fact any alteration in these "little hinges" may lead to pathological condition and even death. The initial days of development of enzymology were full of controversies about them related to their structure, chemical nature, occurrence and functions. The Pasteur – Liebig controversy has been already mentioned earlier.

Once it was understood that the enzymes have an ability to work in cell free systems, serious attempts were being made about their isolation and purification. Willstatter and his colleagues between 1920 and 1928 carried out the early attempts of purification of enzymes. Dixon and Kodama attempted the purification of xanthine oxidase.

The first enzyme to be crystallized was Urease in 1926 by Sumner. This was followed by the purification and crystallization of proteases like trypsin and pepsin by Northrop. The details will be discussed a little later.

Need Of Isolation Of Enzymes

In general isolation and purification of enzymes is required for the following reasons:

1. It is required for the study about the various aspects of that particular enzyme.
2. It is required for the identification of substrates and the substrate specificity of the enzyme
3. It is required to assess the role of various coenzyme/cofactors on the rate of the reaction.
4. It is required for the researches and as diagnostic components.
5. It is required for therapeutic purposes.
6. It is required for development of commercially exploitable immobilized enzyme systems for the industrial applications, fermentations and value addition processes
7. It is required for leaching of commercially important ores.
8. Pure enzymes are also employed in forensics, food processing and for the synthesis of anti enzymes which have a medicinal value.
9. The inhibition of enzyme studies can indicate potential inhibitors, which may be used to treat metabolic and inheritable disorders.

Before the enzymes are isolated and purified certain general considerations are important; some of which are discussed here.

Extra Cellular Enzymes Are Easy to Isolate Than The Intracellular Enzymes

Irrespective of the source, i.e. plant or animal tissue, extra cellular enzymes are easily collected. Their collection usually does not require the destruction of tissues and therefore reduces the expenses and time required for processes such as homogenization or any other such processes.

Many Enzymes are Present in Various Sources But Some Contain Them in Comparatively Large Amounts:

Most of the enzymes are common to many organisms and are basic requirements for the general metabolism. However different tissues contain different amounts of the same enzyme and a comparison should be made to assess the quantitative contents of the enzyme from which an ideal source can be identified.

The Enzyme Source Has to be Pre-Selected Before it is Commercially Exploited:

The early qualitative and quantitative studies are important before an ideal source is finalized. Many times a host of pilot scale experiments are necessary to determine an ideal source for the enzyme of interest. In some cases the seasonal variations in the yields have to be worked out.

Plant and Microbial Enzymes are Generally Cheaper

Animal tissues are a costlier source for the commercial production of an enzyme isolate. This is primarily because many times the complete animal has to be purchased and slaughtered before a specific tissue or organ can be isolated and utilized as a source of the enzyme. For example rennet required for cheese production required procurement and slaughtering of tender calves to obtain the contents of their fourth pouch which had to be obtained and processed immediately.

A Wide Range of Methods is Available for Isolation and Purification of Enzymes

These vary according to many criteria, which include the source of enzyme, its availability, the economics of the processes, the time factor involved and the availability and requirement of the instrumentation.

The topic of enzyme isolation and purification shall be dealt under following points:

(1) Identification of source of enzymes
(2) General handling procedures for source and crude extracts
(3) Methods for isolation
(4) Methods for purification
(5) Development of assays: qualitative and quantitative
(6) Stabilization and crystallization
(7) Criteria for purity
(8) Methods for preservation of isolated enzymes

(1) Identification of Source of Enzymes

The specificity of the action of enzymes is an important characteristic and their localization in specific tissues and cells or cell organelles is based on their necessity and utility there. Thus it is better to have some basic idea about the role of the enzyme before its actual isolation and purification is employed. It should be understood however that there are some differences within the similar enzymes isolated from different tissues. In general an enzyme may be found in enough amounts where its substrate may exist in sumptuous amounts. The amylases, for example are found in the germinating cereal seeds, while the proteases shall be abundant in germinating pulses.

Table 2.1 Some common sources of enzymes

Enzyme	Common source
Salivary amylase	Salivary juice
Proteases	Germinating pulses
β amylases	Sweet potato
Lipases	Germinating groundnut
α amylases	Germinating cereals
Papain	Papaya fruit
bromelain ficin	Pineapple, Fig.

In the past few decades it has become a common practice to use the microorganisms for isolating enzymes. They have the advantages of

- Growing on cheap media reducing cost of production
- Fast growth leading to lesser time in isolation
- Economic processes of recovery in case of extracellular enzymes.

Some of the commonly used microorganisms and enzymes obtained from them are given in the following table.

Table 2.2 Some Microbial Sources of Enzymes

Enzyme	Microbial source
Amylase	Bacillus subtilis
Acid resistant amylase	Aspergillus niger
Amyloglucosidase	Rhizopus niveus
Cytochrome catalse	Saccharomyces boraldi
Cellulase	Trichoderma viride
Diastase	Aspergillus oryzae
Glucose isomerase	Bacillus coagulans
Glucose oxidase	Aspergillus niger
Invertase	Saccharomyces cerevisiae
Keratinase	Streptomyces fradiae
Lipase	Rhizopus nigricans
Laccase	Coriolus versieolor
Microbial rennet	Mucor species
Naringinase	Aspergillus niger
Penicillinase	Bacillus cereus
Pectinase	Sclerotina libertina
Protease	Streptomyces griseus
5' Phosphodiesterase	Penicillium citrionum

In general the criteria for choosing a particular source for the commercial production of enzyme of interest revolve around the following factors:

It Should Be Cheap

The microbial cells and plant materials are comparatively cheap although some microbial cells may require specially formulated broth or medium for growth. But they are ideal sources for extra cellular enzymes like the amylases, cellulases or proteases. The plant materials, unless it is difficult to cultivate, form a major source. The germinating seeds are many times used to produce amylases and proteases. Animal tissue is comparatively costlier however the slaughterhouse wastes can be effectively used as a source for serum enzymes.

It Should Be Readily Available

A locally available source is readily found when required as compared to one that has to be transported from some other place. The transportation increases the cost, and lessens the available time and if a particular source is rapidly perishable then it will accrue losses in case of transportation and processing delays.

It Should Be Easy to Store And Handle

Many times the raw materials have to be procured in amounts more than that can be immediately processed. In such cases the remaining materials have to be stored for some period before it can be processed. The ideal source in such a case should be easy to store and handle.

It Should Have a Comparatively Longer Shelf Life

When the situation of preprocessing storage of raw materials arises, an ideal raw material has a longer shelf life. This reduces the possible losses that may occur due to its perishability.

IT Should Contain The Enzyme Of Interest In Enough Quantity

The source should contain enough amount of the particular enzyme of interest. This is important to make the isolation and purification processes economical. The comparative studies made by research workers are important guidelines while one chooses a particular source for the enzyme of interest.

It Should Not Generate Any Toxic Waste Products During Its Processing

It has been often seen that when animal tissue based sources are used for enzyme isolation, the byproducts or wastes generated produce hazards if disposal is not proper. This is primarily because a large part of the tissues has to be disposed and may be a source of biohazard due to microbial and chemical action on the tissue waste.

2. General Handling Procedures For Source and Crude Extracts

Once the ideal source is identified and procured, it is many times necessary to pre treat or pre process the same before the actual isolation process is implemented. Many times however the raw material is available in the form of a crude extract or solution, which needs to be processed before isolation and purification of the enzyme. In case the source is a tissue it usually undergoes following processing methods:

(i) Plant Tissue

In general the plant tissues used for enzyme isolation are leaves, however sometimes tender whole plants, bark or roots may be used. The apical tissue or germinal tissue often is the ideal source of enzymes of interest.

Plant Tissue → *Harvest* → *Dry* → *Powder* → *Use*

Macerate with ideal solvent → *Filter or Centrifuge* → *Use*

The tissue is harvested, cleaned and often after the removal of unrequired parts may be processed as such or subjected to shade drying. The partially or fully dried tissue may be then powdered and stored for some time before it is used for the actual isolation process. Sometimes dry powders of seeds and other tissues are used (for example jack bean meal is used for isolation of urease) and in such cases the powders are produced and stored in bottles which can be kept at room temperature for a long period.

(ii) Microbial Cells

The microbial cells are often cultured in liquid media (broths) or on solid media (agar plates) before they are used as a source for the enzymes. If the enzyme is extra cellular, then the broth is centrifuged at a proper "g" value for a specified time to allow the sedimentation of the microbial cells and particulate impurities if any, while the enzyme remains in the supernatant. The supernatant is then used as the crude extract.

Microbial cells → *Cultivation in enrichment media* → *Centrifuge* → *Use Supernatant for extracellular enzymes*

Centrifuge ↓ *Sedimented Pellet of cells* → *Physiochemical treatments (Enzyme extraction)* ↓ *Use extracts for intracellular enzymes (crude)*

In case the enzyme is of intracellular localization, the isolated cells of the microorganisms are subjected to homogenization or any feasible physical or chemical method that disrupts its cell wall/ membrane to allow the collection of its cytoplasm as the crude extract.

(iii) Animal Tissue

In case of the animal tissue, which mostly serves as a source of intracellular enzymes, the tissue after isolation has to be kept in cold (icebox) or deep freeze conditions until it is minced and homogenized. The homogenate is often subjected to differential centrifugation before a crude extract is generated. This is mainly to eliminate all sub cellular fractions that may not contain the enzyme and to separate that organelle which contains the enzyme of interest.

(iv) Handling of Crude Extracts

In case of the crude extracts, there are two primary procedures that have to be performed. The first is straining or filtration to remove all particulate matter and

sediments. The second is a primary assay to ensure that the enzyme of interest is present and active. Although a qualitative assay would suffice to determine the presence of the enzyme, many times a pilot quantitative assay is performed and taken as the primary reading on the basis of which the fold purification is determined.

(3) Methods of Isolation

A number of methods have been used for breaking open cells and extracting the cellular contents including the sub cellular organelles. Some of these are briefly described here.

(A) Grinding With Abrasives

Plant tissues or microbial tissue with cell wall is often subjected to grinding with an abrasive material such as alumina or sand. The shear and strain causes the disruption of the cell wall leading to the collection of cell sap as a crude homogenate. Simple pestle- mortar systems or mixers and grinders are used to bring about the desired results.

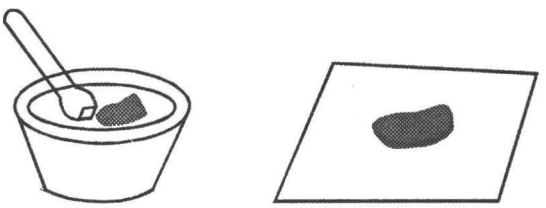


Fig 2.1 Grinding with abrasives

(B) Alternate Freezing And Thawing

This is based on the fact that upon freezing water molecules form ice which expand in size multifold and cause a strain on the cell membrane leading to its disruption. Thawing which causes the melting of ice follows this leading to formation of a crude homogenate.

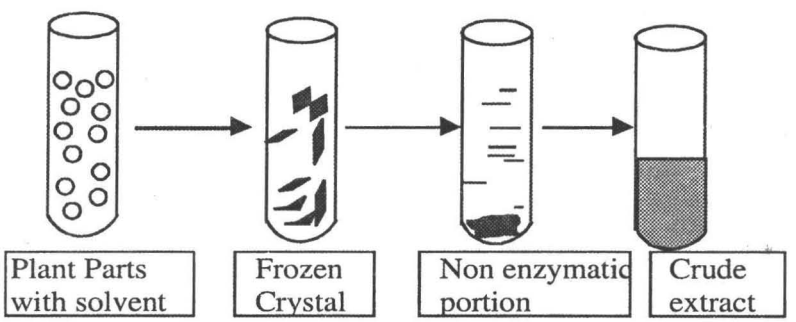


Fig. 2.2 Alternate freezing and thawing

Long periods of Bending

The cells, when subjected to long periods of blending are broken due to the

mechanical forces of collision against the walls of the blender generating a crude homogenate.

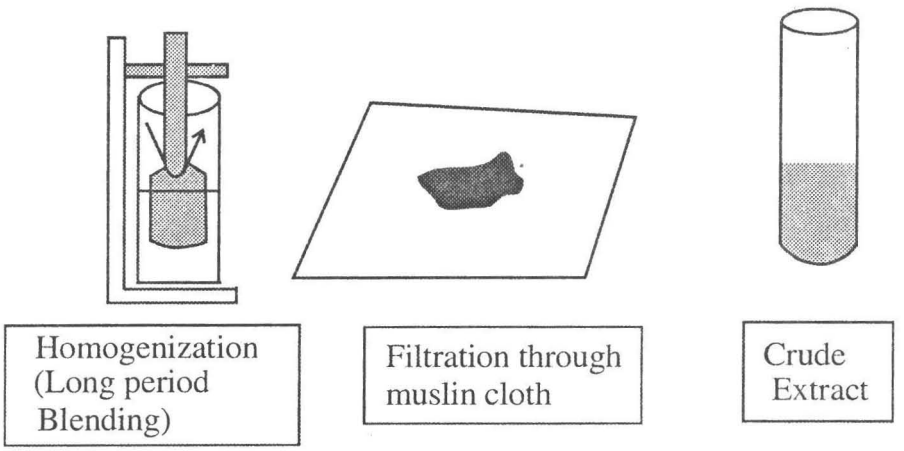


Fig. 2.3 Long periods of blending

(D) Addition Of Glass Beads During Blending

Addition of glass beads or acid washed sand is often employed to increase the mechanical shear which makes the process more effective. Waring blenders are often used in the process. Sometimes instruments with speed adjustments are also employed.

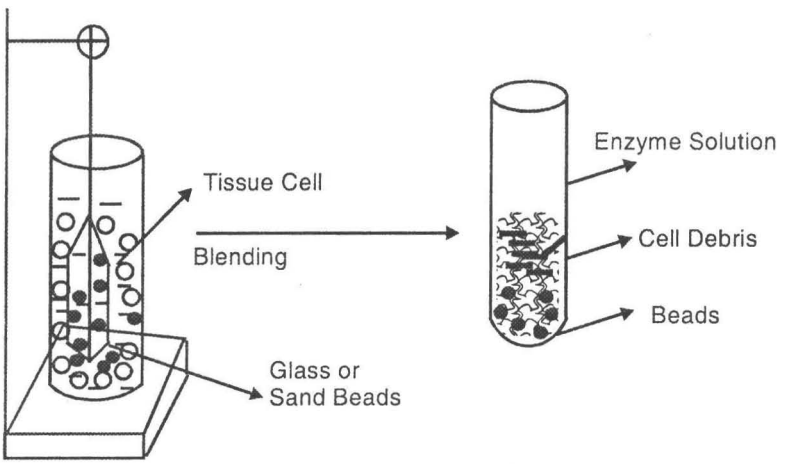


Fig.2.4: Blending with beads

(E) Use Of Appropriate Hydrolytic Enzymes

A different approach has been used with many animal tissues. They are subjected to the action of appropriate enzymes (including lysozyme). Proteases and lipases are conveniently used to cause localized removal of membrane particles leading to formation of holes or pores through which the cell sap and organelles can be brought into solution.

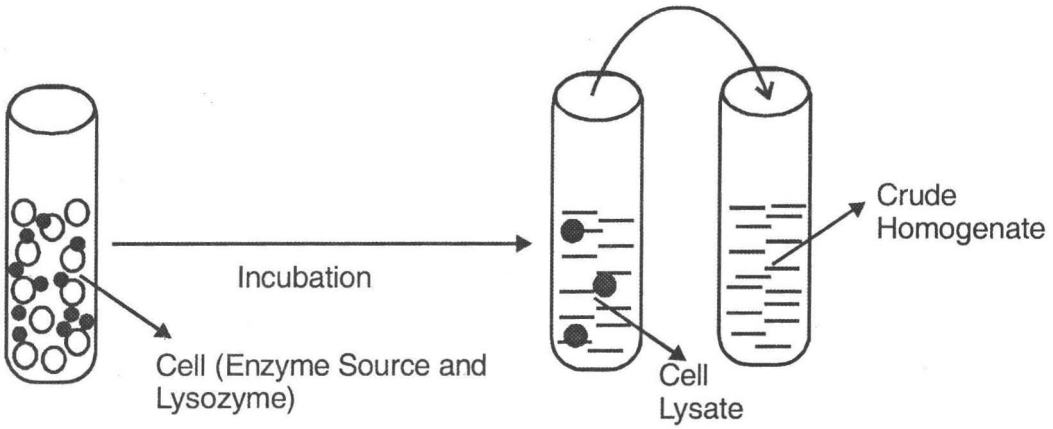


Fig 2.5: Use of hydrolyzing enzymes.

(F) Homogenization by Potter- Elvehjem Homogenizer or a High-Speed Blender

The Potter-Elvehjem homogenizer comprises of a glass tube which perfectly fits on a Teflon pestle which is connected to a high-speed motor. The tissue to be homogenized is minced and mixed with appropriate quantity of isotonic solution and then subjected to homogenization at a very high speed (about 600rpm) for about 5 to 10 minutes.

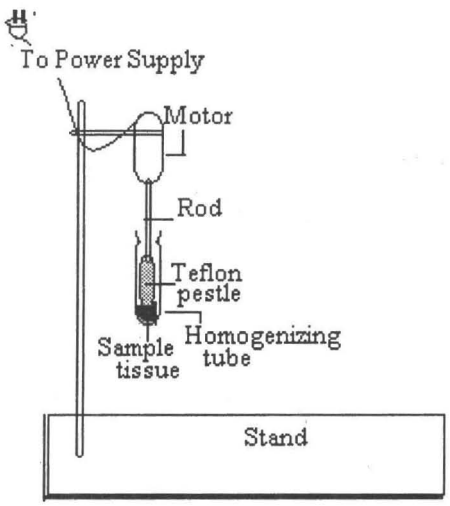


Fig 2.6: Potter Elvehjem Homogenizer.

(G) Use Of Hypotonic Solutions:

Suspension of cells in hypo tonic solutions leads to absorption of water by them and their swelling leading to a turgor pressure that is great enough to cause the cells to burst. Usually sucrose solutions or KCl solutions are employed.

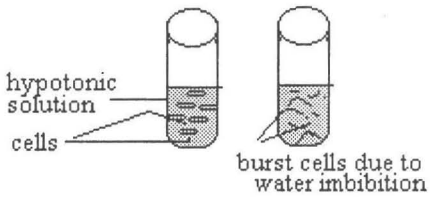


Fig 2.7: Use of hypotonic solution, for lysis of cells during isolation of enzymes.

(H) Ultrasonic Vibrations

The cells are subjected to ultrasonic vibrations that result in their disruption and formation of a homogenate.The cells are subjected to ultrasonic vibrations that result in their disruption and formation of a homogenate.

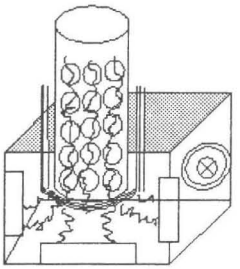


Fig 2.8: Ultrasonicator

(I) Alteration Of pH Of The Solution

Subjecting the cells to an altered pH environment causes the cell membrane proteins to coagulate leading to formation of pores through which the cell sap oozes out as a homogenate.

(J) Organic Solvents

The membranes are lipoprotein bi layers and appropriate organic solvents such as acetone, chloroform, ether, etc. are often employed to dissolve the lipid constituents leading to formation of pores. The cell cytoplasm then flows out as a crude homogenate.

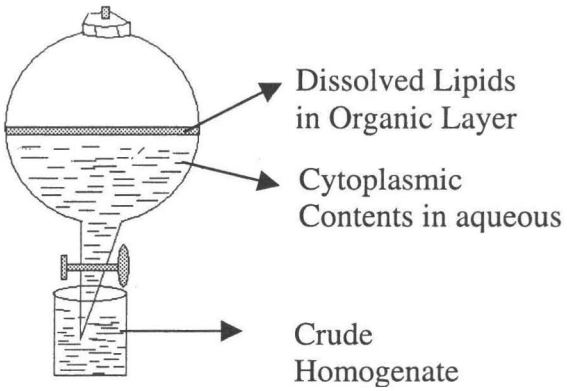


Fig 2.9: Use of Organic Solvents

(4) Methods For Purification

Once the crude homogenate is obtained, the aim of a purification procedure should be to isolate a given enzyme of interest with the maximum possible yield based on fold purification. In addition the preparation should possess the maximum ability of catalysis. In other words it should be free from contamination of other inactive and active proteins.

In the early days of enzyme purification, formation of crystals was taken to be a proof of purity but recent advances have enabled us to determine impurities in crystalline preparations. It is therefore necessary to determine the purity by using other methods and criteria that may or may not include the measurement of catalytic activity.

The general strategy for use of methods of purification and their sequence is dependant upon the source of enzyme, instrumentation available, the time span of the procedures and the economics of the processes. In other words different methods may be chosen by different workers as per their convenience to achieve the purification of a desired enzyme. Some of the methods that are routinely used for enzyme purification are summarized in table 2.3.

Table 2.3 Methods for purification of enzymes

Property	Method	Scale
Size or mass		
	Centrifugation	Large or small
	Gel filtration	Generally small
	Dialysis or ultra filtration	Generally small
Charge		
	Ion exchange chromatography	Large or small
	Electrophoresis	Generally small
	Isoelectric focussing	Generally small
Solubility		
	Change in pH	Generally large
	Change in ionic strength	Large or small
	Decrease in dielectric constant	Generally large
Specific binding sites		
	Affinity chromatography	Generally small
	Affinity elution	Large or small

The term large scale is used to indicate that the amount of protein greater than about 100 mg can be handled at that particular step in the purification procedure. The details of each method are briefly discussed here. It should be remembered however that the choice of methods and sequence is entirely dependent upon the workers using them.

(A) Methods Depending On Size Or Mass

The methods depend upon the general principle that the molecules, sediment at different speeds related to their sizes or masses. Some of the frequently employed methods include:

(1) Centrifugation

Large molecules such as enzymes can be sedimented by the high centrifugal fields (up to 300,000g) generated by an ultra centrifuge. Although the rate at which any particular enzyme will sediment depends on a variety of factors including the size and shape of the molecule and the viscosity of the solution, it is found that in general the

higher the molecular weight, the greater the rate of sedimentation. This method is not widely used in purification procedures to separate one enzyme from another because only small volume (few ml) can be dealt within ultra centrifuges operating at high centrifugal fields. However centrifugation is very widely used to remove precipitated or insoluble material in the course of isolation, for e.g. to remove cell debris after homogenization or to collect enzyme which has been precipitated by the addition of salts.

Density gradient or zonal centrifugation is a widely used and versatile procedure for separating not only proteins and other type of macromolecules but also organelles and viruses. In the most common procedure, a continuous density gradient of sucrose is first prepared in a plastic centrifuge tube by a device that mixes concentrated sucrose solution and water in decreasing ratio as the tube is filled, so that the density of the medium is greatest at the bottom of the tube. The mixture of macromolecules to be resolved is layered on the top of the gradient. Centrifugation of the tube in a horizontal position in a rotor at a high speed causes each type of macromolecule to sediment down the density gradient at its own rate, determined by its particle weight, density and shape in the form of separate bands or zones. Usually centrifugation is stopped before equilibrium is reached. The position of the protein bands can be located optically or by draining of the content of the tube carefully through a pinhole in the bottom and analyzing successive small samples. Alternatively the plastic tube can be frozen and then cut into thin slices for analysis.

It is diagrammatically represented as follows:

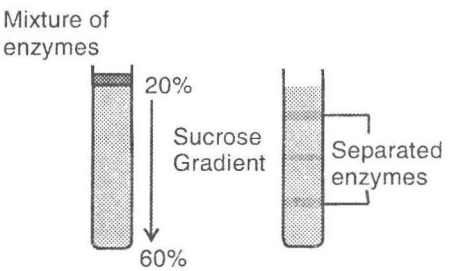


Fig 2.10: Density gradient

(2) Gel Filtration

Gel filtration or gel permeation chromatography is a separation method dependent upon molecular size. The method is also known as molecular sieve, or molecular exclusion chromatography. Its excellent reproducibility, comparatively short time and relatively inexpensive equipment make it far widely used separation method. It is based on the following simple principle,

A column of gel beads or porous glass granules is allowed to attain equilibrium with a solvent suitable for the molecules to be separated. If the mixture of molecules of different size is placed on the top of such an equilibrated column the large molecules pass through the interstitial spaces between the beads. This is because the pores of the gel have smaller diameter than what is needed for the large molecules to enter. Large molecules therefore move down the column with little resistance. The small molecules however can enter the pores and are thereby effectively removed from the stream of the eluting solvent. These molecules are thus retarded. The degree of retardation of a

molecule is proportional to the time it spends inside the gel pore that is a function of the molecule's size and the pore diameter. The molecules having diameter equal to or larger than the pore diameter do not enter the gel and are said to be excluded. The techniques used are column chromatography or thin layer chromatography.

An ideal gel should have the following characteristics:

(i) The gel material should be chemically inert

(ii) It should preferably contain vanishingly small number of ionic groups.

(iii) Gel material should provide a wide choice of pore and particle sizes

(iv) A given gel should have uniform pore and particle size

(v) The gel matrix should have high mechanical rigidity.

Some routinely used gel materials include

(a) Cross- linked dextrans (sephadex):

For proteins and most of the bio molecules, sephadex is by far the most popular of all the gels. Dextrans are cross-linked by epichlorhydrin. A schematic representation of its structure is given in fig 2.11

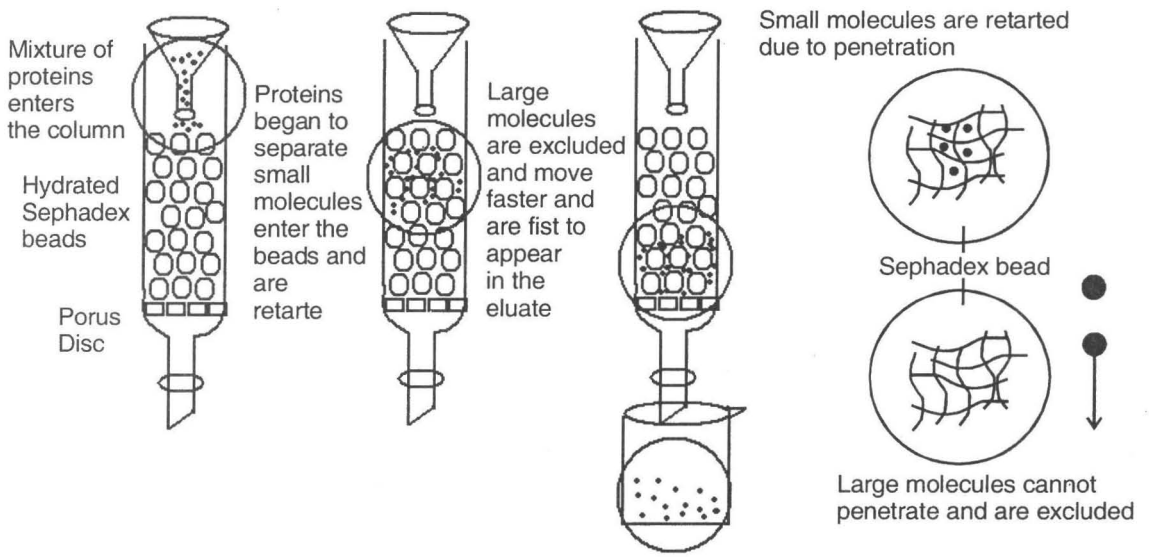


Fig 2.11: Separation by use of sephadex-beads

(b) Polyacrylamide gels:

Polymerization of acrylamide in bead form results in the formation of polyacrylamide. The gels are used to separate molecules having a molecular weight up to 300,000 Dalton. However at such large pore size they lack mechanical rigidity and become compressed in the column. Although they can be used in the pH range of 2-11, they are unstable to bases due to hydrolysis of amide groups. The cross- linking agent is bisacrylamide and the reaction is brought about in the presence of ammonium per sulfate. The gels are insoluble in water and common organic solvents.

(c) Agarose gels:

They are produced from agar. The gels are hydrophilic and almost completely free of charged groups. They have greater pore sizes and can be conveniently used to separate molecules having a molecular weight of a few million Dalton. They are compatible with all aqueous buffers and completely stable within pH range 4-10. Freezing temperatures and temperatures more than 30 degrees alter the gel structure.

(d) Styragel:

The styragel is a cross-linked polymer of polystyrene used for completely non-aqueous separations. It can be prepared in a wide range of porosities and the gel structure is unaffected by temperatures as high as 150 degrees. It can be used with solvents like carbon tetra chloride, trichloro- benzene, cresol, tetrahydrofuran, chloroform and dimethylsulfoxide etc. it is unstable with water, acetone and alcohols.

(e) Controlled pore glass beads:

Borosilicate glass beads are also often used. They give very high flow rates due to their total rigidity. They are treated with hexamethyldisilazane to overcome the problem of protein adsorption. They have a molecular exclusion limit between 3000 to 9 million Dalton.

(3) Dialysis or Ultra filtration:

A dialysis membrane such as cellophane can be used to separate the globular proteins or molecules with a molecular weight of around 20,000 Dalton. The pore size can be changed by various mechanical and chemical treatments. The method is routinely used to separate small organic molecules, salts and organic solvents from the crude extracts. The volume handling capacity is generally low. The process cannot be employed to separate enzymes from each other. Ultra filtration on the other hand is a modification of the dialysis procedure in which small molecules and ions pass through a dialyzing membrane under the influence of applied pressure (usually Nitrogen gas at 4 atm. Pressure). This leads to concentration of enzyme solution, which is useful in reducing the volume of the extract sample during the purification procedure.

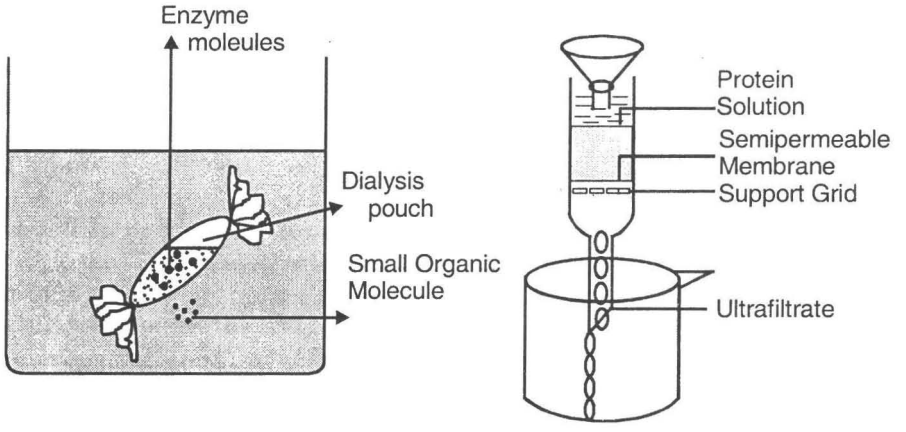


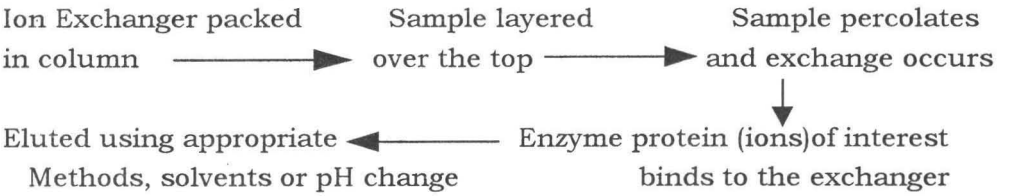
Fig 2.12: Dialysis and Ultrafiltration

(B) Methods Depending Upon Charge:

The methods depend upon the fact that enzymes carry charged side chains which give the molecule a net charge and that the mobility of a charged molecule under an electrical field or in a charged environment depends on the charges carried by it. Some of the routinely used methods include the following:

(1) Ion –Exchange Chromatography:

Ion exchange may be defined as the reversible exchange of ions in solution, with ions, electrostatically bound to inert support medium. The electrostatic force of attraction in turn depends upon the relative charge, the radius of the hydrated ions, and the degree of non-bonding interactions. The technique generally employs ion-exchange columns. There are two types of ion-exchangers, anion exchangers and cation exchangers. The medium is an inert support medium that is covalently bound to a positive(anion exchanger) or negative(cation exchanger) functional groups. The ions electrostatically bound to the exchanger are referred to as counter ions. This technique is extremely useful in the separation of charged compounds and even uncharged compounds that can be tagged with charged group or a variance of pH.



The sample containing the ionic species to be separated is allowed to percolate through the exchanger for such a length of time as will be sufficient for the attainment of equilibrium, E—Y + X ⟵⟹ E—X + Y, where E is an exchanger and X and Y are exchangeable ions. The exchanger and the counter ions are oppositely charged. In case the ion to be exchanged is cation, then the neutral and anionic molecules will not bind at all and shall be washed away out of the column. The exchanged ions (X) bound can then be eluted either by percolating the medium with increasing concentration of Y, or in other case the change in pH, favorable for the elution of X. This principle is also applicable for separation of proteins and nucleic acids which are capable of possessing both positive and negative charges. The amphoteric nature of macromolecules particularly proteins, can be exploited for purification purposes by using ion exchange chromatography. For example the desired protein may be made to behave as a cation by lowering the pH of the protein mixture (the pH is lowered to a limit where most of the other proteins in the mixture behave as anions). This preparation if chromatographed on a cation exchanger will remove many of the anionic protein species. This process will remove many of the unwanted proteins from the starting mixture and resultant mixture is rich for the desired protein. If the pH of this resultant mixture is now increased the desired protein will exist predominantly as an anion (the pH is increased to a limit where most other proteins in the mixture still behave as cations). If the preparation is now chromatographed on an anion exchanger, many cationic proteins will be lost. It should thus be clear that anion and cation exchange chromatography used sequentially can afford a large degree of purification. Routinely used ion exchange resins:

Table 2.2: Ion-exchange resins

Type	Nature	Trade name
Strong cation	Sulfonated polystyrene	Dowex 50 Amberlite IR 120
	Sulfopropyl cellulose	SP- Sephadex
Weak cation	Condensed acrylic acid Carboxymethyl cellulose	Amberlite IRC 50 CM-Sephadex
Strong anion	Polystyrene with –CH2NMe3Cl Diethyl(2 hydroxypropyl) quarternary amino cellulose	Dowex IAmberlite IRA 400QAE Sephadex
Weak anion	Polystyrene with secondary amine	Dowex 3 Amberlite IR 45
	Diethylaminoethyl cellulose	DEAE-Sephadex
	Diethylaminoethyl agarose	DEAE-Sepharose

(2) Electrophoresis:

Electrophoresis is the migration of charged particles or molecules in a medium under the influence of an applied electric field. Upon suspension in an aqueous solvent almost all particles including bio molecules such as enzymes acquire either positive or negative charges. The acquisition of such charges depends upon the nature of the particle / molecule and the solvent. These groups determine the net charge density of the protein molecule, which makes it move in an electric field in a direction, and at a velocity dependant upon the sign and quantity of this net charge density. Even if two molecules have the same charge, they might not migrate together because if there is difference in their molecular weights they will have different charge: mass ratio. The electrophoretic mobility is affected by following factors:

The Sample:

Charge/mass ratio of the sample dictates the electrophoretic mobility. The mass consists of the size and shape of the molecule.

I Charge: The higher the charge, greater is the mobility, the charge however depends on the pH of the medium

II Size: The bigger the molecule the greater are the frictional and electrostatic forces exerted upon it by the medium. Large particles thus move slower than small particles.

III Shape: Rounded contours generate lesser frictional and electrostatic retardation compared to sharp contours.

Other Factors:

These include the electric field, the medium, the buffer etc. For details the readers are advised to go through books on biophysics or physical chemistry.

The routine techniques employed include:

(A) The Moving Boundary Electrophoresis:

In this technique a buffered solution of macromolecules is placed under a layer of pure buffer solution in a U- shaped observation cell. The pH of the buffer is so chosen that all the macromolecules bear a net negative charge. The movements of macromolecules, consequent to generation of electric field between the electrodes will then be towards an anode. As they do so they migrate from the macromolecule solution to the pure buffer or into the zone of macromolecule free buffer and form a boundary or front. As a result of this there is a sharp change in the refractive index of the solution at this boundary. The refractive index changes along the electrophoretic cell, when measured by appropriate optical devices yield electrophoretic patterns that show the direction and relative rate of migration of the major molecules in the sample. In recent years however it has been superceded by techniques collectively known as zone electrophoresis.

(B) Zone Electrophoresis:

Zone electrophoresis is the name given to the separation technique employing stabilizing media most of which are gels such as agar, starch and polyacrylamide. Upon separation the molecules are immobilized by fixation in different zones. The molecules are then detected by staining them on the supporting medium. Other methods to detect the separated molecules are

1. visualization by ultraviolet light.
2. detection by virtue of enzymatic reaction.
3. detection by radioactivity if the molecules are radiolabeled.
4. Elution of separated components from the medium and further detection.

Zone electrophoresis can also be utilized as a large scale or preparative method whereby large amounts of a component can be purified for further characterization.

Based on the support medium, the techniques are classified as

Paper electrophoresis

Gel electrophoresis

Cellulose acetate electrophoresis

Specialized techniques including the disc gel, gradient gel, high voltage, two-dimensional electrophoresis etc.

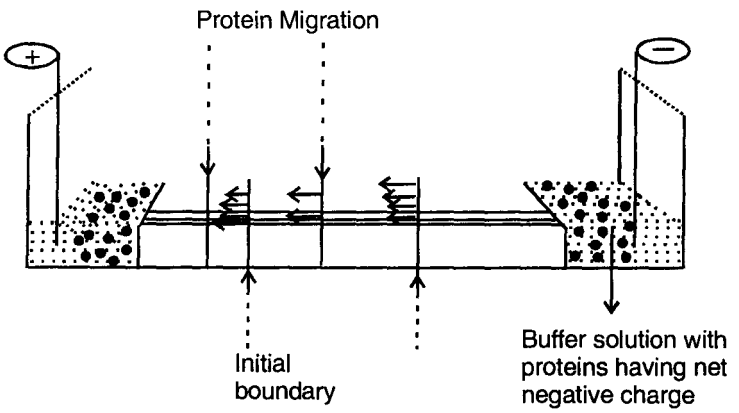


Fig.2.13: Electrophoresis

(3) Isoelectric Focussing:

This technique was discovered by H.Svensson in Sweden and has a high-resolution power. A simple comparison would help establish its superiority. While paper electrophoresis resolves plasma proteins into 6 bands, isoelectric focussing resolves it into at least 40 bands. Protein molecules may have a net positive charge in an acid solution because most amino groups carry a positive net charge and most carboxylic groups are protonated and electrically uncharged. With a gradual increase in pH, the number of carboxyl groups carrying a negative charge increases, while the number of positively charged group decreases. At a certain pH value, the isoionic point, the net charge of the protein molecule is zero. The number and types of protolytic groups and their dissociation constants thus determine the isoionic point of a molecule. Although there is considerable variation in the isoionic point of protein, they are generally in the pH range of 3-11. In conventional electrophoresis, the pH between anode and cathode is constant and the positively charged ions migrate towards the cathode and negative ions migrate to the anode.

In isoelectric focussing, on the other hand, a stable pH gradient is arranged, the pH increases gradually from anode to cathode. A protein introduced into this system at a point where the pH is lower than its isoionic point will possess a net positive charge and will migrate in the direction of the cathode. Due to the presence of the pH gradient, the protein will migrate to an environment of successively higher pH values, in turn, will influence the ionization and net charge of the molecule. Finally the protein will encounter a pH where its net is zero and will stop migrating. This is the isoelectric point of the protein. The consequence of this is that every protein will migrate to and focus at its respective isoelectric point in a stable pH gradient, irrespective of its origin in the apparatus at the time the current was applied. Thus the point of application and volume of the protein solution are not critical. Diffusion, which is an obstacle with every other method of electrophoresis, is not a problem with this technique because focussing effect works against diffusion. Thus once a final stable focussing is reached the resolution will be retained even if the experiment is continued for a long time.

The pH gradient is established with the help of carrier ampholytes. They are special buffer substances that possess following properties:

(i) They should have a certain buffering capacity at their isoelectric point.

(ii) They should have a conductance at their isoelectric point.

(iii) They should have low molecular weight so that macromolecules can be separated from them easily after electrofocussing.

(iv) They should be soluble in water. This hydrophilic character will also prevent their binding to hydrophobic regions of the proteins.

(v) Ideally they should have a low light absorption at 280 nm. This would permit the detection of proteins after electrofocussing by measurements at 280 nm.

Electrofocussing also needs provision for stabilization of separating protein zones against convective flow in the solution. Three ways are in use that include

(a) Density Gradient:

Density gradients suitable for electrofocussing can be made with many uncharged solutes, which can be dissolved in water to a concentration that will increase the density sufficiently. The compounds should not react with proteins and should have a low

content of heavy metals. They should be of high purity. Sucrose is the most preferred compound however other compounds like mannitol, sorbitol, ethylene glycol, dextran and ficoll may be used.

(b) Gels:

In electrofocussing the gel serves only as an anticonvectant and not as a molecular sieve. Obviously the gel concentration should be low to provide large diameter pores. For large proteins, MW exceeding 200 KD, lower concentration of acrylamide might be used in combination with agarose. Acrylamide is the preferred gel for electrofocussing. Agarose starch gels are not preferred as in these gels pH gradient drifts considerably during prolonged experiment.

(C) Zone Convection Electrofocussing

The apparatus is made up of two rectangular boxes, the upper one being the cover. The upper surface of the lower box is corrugated with ridges, separated by depressions corresponding to the ridges of the cover. Thus they fit in with each other leaving a space of few millimeters in between, producing a narrow wave like channel like a series of interconnected broad U tubes. The carrier ampholyte solution is filled in this space with the electrodes situated at the two ends.

A coolant liquid is allowed to flow through the hollow channels built into both the boxes to maintain a constant temperature. When the current is on a density gradient is formed in each depression of the bottom part by the solute. When proteins become immobile at their isoelectric pH, the density increases locally and the proteins settle down in the depressions of the bottom part. They can be separated as fractions after the completion of the experiment without contamination by neighboring fractions.

Separation Of Carrier Ampholytes From Proteins

The average MW of ampholytes is about 800 where as the protein has a MW of 10,000 or more. Dialysis or gel filtration can be effectively used for their separation. Sometimes other methods like ammonium sulfate precipitation; ion exchange chromatography and partition chromatography may be used.

(C) Methods Based On Change In Solubility

The solubility of a compound in a given solvent depends on the balance of the forces between solute and solute and those between solute and solvent. If the former predominate, the compound will be insoluble where as if the latter are predominant, the compound will be soluble. In the course of enzyme purification it is possible to alter the balance between these forces and hence precipitate the enzyme of interest or remove contaminating enzymes. Three of the most important ways of changing the solubility of enzymes are, (1) to change the pH, (2) to change the ionic strength, (3) to decrease the dielectric constant. These methods can be applied on a large scale and are often used in the initial stage of a purification procedure.

(1) Change in pH

An enzyme is least soluble at its isoelectric point since at this pH the repulsive electrostatic forces within the enzyme molecules cease to exist. Under such condition the enzyme molecules tend to precipitate. Adjustment of the pH to the proper value can therefore be used to precipitate the enzyme. It is important to note that the enzyme of

interest should be rendered completely inactive due to this procedure. This process isolates Adenosinetriphosphatase from beef heart.

The method is sometimes employed to remove impurities, as in case of the isolation of adenylate kinase.

Isolation of adenylate kinase from pig muscle: The enzyme, when isolated from pig muscle gives crystals large enough to be used for x-ray crystallography and is exceptionally stable at low pH. The general procedure includes following steps.

1. *Minced muscle is treated with 0.01M KCl and strained through cheesecloth to obtain an extract.*
2. *The pH is adjusted to 3.5 and after 5 min is readjusted to 7.0. The solution is centrifuged.*
3. *Supernatant is loaded on phospho-cellulose column and enzyme is eluted with AMP.*
4. *Fractions are checked for activity and pooled, followed by ammonium sulfate precipitation.*
5. *Precipitate is redissolved in small volume and subjected to gel filtration.*
6. *Crystallization is brought out at 62% ammonium sulfate.All the steps are performed at 0-5 degree.*

(2) Change in Ionic Strength:

The proteins often precipitate on addition of high amount of salts. This is also called as salting out of proteins. The theoretical basis for this is not clearly understood however it is assumed that at high salt concentrations, the concentration of water is greatly reduced leading to a decrease in the solute- solvent interactions and in turn the solubility of proteins. Many salts have been employed for "salt fractionation" or salting out the enzymes. The most widely used and preferred salt is ammonium sulfate due to its following advantages:

- It is cheap
- It is highly soluble in water
- It does not cause any harmful effects on the enzyme to be purified.
- It is a weak acid and the fold purification achieved is about 10 times. It is best used in the initial stages of purification.
- The enzymes glyceraldehyde phosphate dehydrogenase and fructose bis phosphate aldolase have been isolated and purified by using ammonium sulfate fractionation.

Purification of glyceraldehyde phosphate dehydrogenase from rabbit muscle: The enzyme is easy to purify due to its abundant occurrence and contains tightly bound NAD+. It is soluble in high concentrations of ammonium sulfate (up to 70% saturation) which is the basis of the purification procedure given below.

1. The minced muscle is mixed with 0.05 molar EDTA/NH4+ at pH 8.4 and centrifuged at1000g for 20 min.

2. The pH of extract is adjusted to 7.4, solid ammonium sulfate is added up to 50% saturation and centrifuged.
3. The supernatant is added with more ammonium sulfate till saturation is 70% and is centrifuged.
4. Additional ammonium sulfate up to 72% saturation is added and precipitate is collected. It is dissolved in buffer and refractionated with ammonium sulfate
5. The enzyme is allowed to crystallize.

(3) Decrease in Dielectric Constant:

Addition of water –miscible organic solvents will decrease the dielectric constant of a solution and hence increase electrostatic forces. This leads to the alteration in solute-solute and solute-solvent interaction generally leading to precipitation of large molecules such as enzymes. The choice of solvent is crucial, as many enzymes are rendered inactive due to the action of organic solvents. Working at low temperatures is advisable, as many solvents are volatile and hazardous at high temperatures. The commonly used solvents include ethanol, butanol, acetone etc.

(d) Methods Based on Specific Binding Sites:

The characteristic property of specificity associated with enzymes can be employed to purify them. There are two methods based on the stage of use of affinity. (1) Affinity chromatography uses the affinity, at the stage of adsorption of the enzyme of interest, on to an ion exchanger and (2) Affinity elution uses the specificity at the stage of desorption. They are briefly discussed below:

(1) Affinity Chromatography:

The technique exploits the capacity for specific, non-covalent binding of enzymes with other molecules called ligands. Most of the times a substrate analogue (a molecule that resembles the substrate but does not cause fruitful reaction) specific for the enzyme of interest is bound to a solid and inert matrix (like agarose). When the mixture is loaded on the top of the column, only the desired enzyme will bind to the immobilized substrate analogue and shall retard. All other enzymes and proteins will pass down and out of the column.

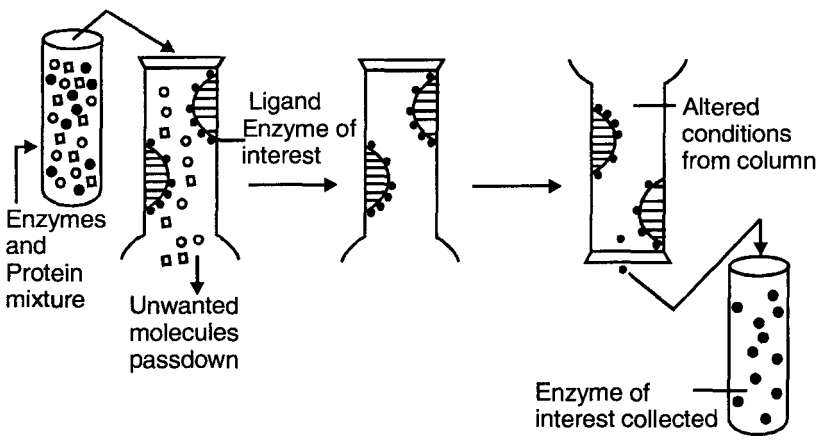


Fig.2.14: Affinity technique, adsorption of enzyme on column.

If the conditions are altered, the enzyme bound to the ligand shall dissociate and be eluted. The fractions can be collected and checked for the enzymatic activity. The enzyme collected by this technique is fairly pure. Some important standard matrix-ligand systems are given below

Table 2.3 Matrix –ligand systems for protein purification.

Matrix- ligand system	Nature of ligand	Used for isolation of
Cibacron – blue –agarose	Dye resembling nucleotides	Kinases, dehydrogenases, DNA polymerases, nitrate reductases, blood coagulation factors
Heparin-agarose	Heparin (natural anticoagulant)	DNA polymerase, bone collagenase etc.
Polynucleotide agarose	Oligo dT	m-RNA, transcription factors, other DNA binding proteins
Lysine agarose	Lysine	Plasminogen, ribosomal RNAs
Protein A agarose	Cell wall protein of *Staphylococcus aureus*	IgG
Lectin- sepharose	Lectins like concanavalin A or wheat germ lectins	Glycoproteins and polysaccharides.

A number of problems are associated with the use of this technique for purification of enzymes. These include:

1. Attaching a suitable substrate analogue to the matrix is a difficult task.
2. Linking of the ligand to the matrix may interfere with the binding property of the enzyme leading to partial or complete loss of specificity. Use of spacer arms may be necessary.
3. The strength of the enzyme-ligand binding is a crucial factor. If it is too weak then retardation of enzyme will not be satisfactory, while too strong interaction will pose the problem of elution.
4. Enzymes catalyzing bi-substrate reactions create special problems. It is necessary to study and pay particular attention to the elution techniques involved. In case of liver alcohol dehydrogenase, it gets bound to AMP analogue bond with the matrix but its elution requires the use of NAD and pyrazole. Pyrazole is a competitive inhibitor of the enzyme.

(2) Affinity Elution:

It is a complimentary technique to affinity chromatography. It is used with partially purified enzyme mixtures and is more advantageous than affinity chromatography as:

a. The complex reactions of binding the substrate analogue are eliminated.

b. The columns are cheaper because ion exchangers are cheaper than affinity matrices.

The best example of use of affinity elution is, the separation of enzymes of the glycolytic sequence in a single multiple operation scheme. A partially fractionated mixture

of the enzymes (formed by saturation by ammonium sulfate up to 45-65 %) is loaded on a CM- cellulose column at pH 6.5 Elution with phosphoenol pyruvate yields pyruvate kinase, while elution with fructose-bis-phosphate yields aldolase. The molarity of the eluting solution is also important.

Variations In Affinity Techniques

The techniques of affinity based enzyme separations are increasingly required however they suffer two major disadvantages:

1. The techniques are costly because of the high cost of affinity matrices, costly ligands and long incubation time along with the complications of process.
2. They could not be used at the initial stages of purification, primarily because a host of contaminants in the extract tend to clog the system rendering it useless.

Variations have however been worked out to counter these problems. Some of them are briefly mentioned below

(a) Affinity Cross-Flow Ultra filtration

The technique uses micro porous membranes with a pore size of 3-5 mm. They are made of cellulose acetate, polytetrafluoroethylene (PTFE) or polyvinylidene. Prepared activated membranes are now commercially available.

When the mixture is incubated with the membrane, it retains the protein-ligand complex while the unwanted material passes through the membrane pores. A change of buffer can be used to release the protein from the complex. The flow rates are usually high and greatly improve the steps such as washing and elution. It is important to note that the ligand used should be very specific and the material of the membrane should have minimum non-specific adsorption.

(b) Affinity Precipitation

The technique uses polymers that are soluble under particular conditions and precipitate on change in any condition like pH, ionic strength, temperature or addition of an agent such as a metal ion.

A proper ligand is bound to the polymer and this ligand – polymer complex is kept in solution. When the mixture of proteins or enzymes is added to this solution the enzyme of interest binds with this and after a proper incubation time a change in any condition mentioned above is brought about causing the precipitation of the complex. The precipitate is separated and subjected to change in buffer leading to easiness in isolation of the desired enzyme. The technique is cheaper as it eliminates the costly matrices and ligand binding reactions; moreover it can directly be used at the first step of purification.

(c) Matrix less Affinity Separations

This is a variation of the earlier technique that does not require any ligand or matrix. This is illustrated from the following example. The wheat germ lectin has an affinity for N acetyl glucosamine. Conventionally to separate the wheat germ lectin one would have to use an insoluble matrix to which N acetyl glucosamine has been covalently bound. But instead of such a matrix, polymer of N acetyl glucosamine, chitin is a natural constituent of the exoskeleton of arthopods. It can be partially deacetylated to form chitosan. Chitosan can act as a polymer that can remain in solution or suspension

based on alteration of conditions. This polymer also has an affinity for wheat germ lectin. The technique is to incubate wheat germ extract with chitosan at pH 5.5where the chitosan exists as a solution. After the incubation time is completed, the pH is changed to 8.5 at which the chitosan- wheat germ complex precipitates. This complex is separated and redissolved by altering the pH to 5.5. After this a simple gel filtration can separate the chitosan and wheat germ lectin.

(E) Some Other Methods Of Enzyme Purification

Although most of the methods mentioned earlier are routinely used for the isolation of enzymes, some other methods have been used. A brief mention of these is given here.

(1) Fractional Precipitation By Heat

This treatment is a valuable tool to eliminate much of the unwanted protein contamination, hence is useful as the primary step in purification. Each enzyme has a characteristic and sharp destruction temperature. By heating the mixture for a definite time to a temperature just below the destruction temperature, it is possible to coagulate much of the unwanted protein that can be centrifuged off and eliminated. The fact that in presence of substrate, an enzyme is able to withstand a higher temperature by about 10 degrees than in its absence can be effectively used to eliminate even more proteins that are contaminants. The system generally comprises of three water baths, one at the desired temperature, other a few degrees higher and one colder. The solution is kept in a round bottom flask, about half filled and is first transferred to the hottest bath, with a continuous swirling to avoid overheating of a particular part. The temperature is monitored with a thermometer and immediately on reaching the desired value, the flask is transferred to the second bath (with the desired temperature) where it is kept for the necessary time period. After this the flask is transferred to cold and the contents are rapidly cooled with the help of swirling. It is then left in cold till precipitation occurs after which centrifugation is done. The process has to be strictly monitored and the time of heating is between 10-15 min.

(2) Fractional Adsorption On Calcium Phosphate Gels

Fractional adsorption is a widely used method and the chief adsorbents include calcium phosphate gel and alumina Cγ gel. The pH is about 5.5 and the electrolyte concentration is low. Usually a mixture treated with dialysis is used for adsorption. This lowers salt concentration and thus reduces interference in adsorption.

The procedure is simple. Small successive portions of the gel are added to the enzyme solution, followed by mixing, spinning down and removal of each portion before adding the next. The procedure is followed by activity tests on small samples withdrawn from the main solution. Calcium phosphate gel has the advantage that initially the other proteins are removed followed by a sudden removal of the enzyme leaving an inactive solution with many proteins.

A slightly alkaline buffer is used for the elution of the adsorbed enzyme from active fractions. Usually phosphate buffer at pH 7.6 is used. If this fails to elute the enzyme it is repeated till further removal of contaminants is done. The final elution is done by using phosphate containing 10% ammonium sulfate.

The volume of the elutant should not be large and high-speed stirrers may be used to increase efficiency.

(3) Additional Methods:

Some other methods, which are not so popular now, include chromatography on hydroxyapatite, hydrophobic chromatography, and precipitation by polyethylene glycol and concentration by freeze-drying. Heavy metal salts can also be used for purification of enzymes. They result in enzyme precipitation and form characteristic crystals. The heavy metals are then carefully removed by using chelating agents. In recent years use of western blotting technique has also become a common tool for enzyme isolation.

In *Western Blotting* the mixture is subjected to SDS-PAGE and then transferred on a membrane from the gel. The membrane is bound with specific antibodies against the desired enzyme protein and therefore after the incubation only the enzyme binds with the antibody. This is then reacted with a second antibody that is tagged with an enzyme that enables a color mediated detection of the complex.

Some representative techniques employed initially for enzyme isolation

These are discussed in brief,

(A) Sumner's Method For Isolation Of Urease

The method which Sumner and collegues have used to obtain the crystals is extremely simple. It consists of the following,

1. Extract finely powdered, fat-free jack bean meal with 31.6 per cent acetone
2. Allow the material to filter by gravity in an ice chest.
3. After standing overnight the filtrate is centrifuged and the precipitate of crystalline urease is obtained.
4. The precipitate is stirred with cold 31.6 per cent acetone and centrifuged again.
5. The crystals can be now dissolved in distilled water and centrifuged free from insoluble and inactive matter that has passed through the filter during the filtration.
6. Of the urease extracted from the meal as much as 47 per cent may be present in the crystals.
7. If one uses coarsely ground jack bean meal that has not been freed from fat the crystals are still obtained, but in traces only.
8. The process described above has been repeated many times since first discovering the crystals and has always had success.

(B) Northrop's Procedure For Isolation Of Pepsin

The procedure primarily uses ammonium sulfate precipitation followed by magnesium sulfate precipitation. In the later stages sodium acetate and sulfuric acid are used. A brief description is given as follows,

1. Contents of the fourth pouch are emptied after slaughter and immidiately filtered at 6degreesC for 48 hours. The filtrate is designated as solution 1.(volume approx. 5 litres)
2. Solution 1 is saturated with ammonium sulfate, decanted and filtered with suction. The precipitate is dissolved in 200 ml of N/500 hydrochloric acid. This is solution 2.

3. Solution 2 is cooled to –10 degrees C. To this 300 ml of cold acetone is added mixed and centrifuged. The supernatant is solution 3.
4. Solution 3 is cooled to –10 degrees C and to this 500 ml of cold acetone is added. The solution is filtered and the ppt is dissolved in 300 ml of N/500 hydrochloric acid. This is solution 4.
5. To the solution 4, 1 volume of saturated magnesium sulfate solution is added and mixed. It is centrifuged and the precipitate is dissolved and 100ml of N/500 hydrochloric acid is added. This gives solution 5.
6. 1 volume of saturated magnesium sulfate is added to solution 5. It is mixed and centrifuged. The precipitate is dissolved and 100 ml of N/500 HCl is added. This gives solution 6.
7. The procedure is repeated again and 70 ml of N/500 HCl is added to obtain solution 7.
8. The procedure is repeatedfor one moretime and 75ml of N/500 HCL is added. This gives solution 8 with a volume of about 80ml.
9. 240 ml of solution 8 equivalent to about 15 liters of gastric juiceis mixed with 1 volume of staurated magnesium sulfate and then centrifuged. The precipitate is about 3 grams.
10. To this precipitate, add 10 ml of N/10 sodium acetate and titrate it to pH 3. Add N/2 sulfuric acid till a dark viscous liquid with a slight ppt is obtained. To this add 1 volume of saturated magnesium sulfate and filter the suspension with suction. The precipitate is dissolved in 8 ml of N/10 sodium acetate solution. The solution is yellow and clear. This is titrated to pH 3 and N/2 sulfuric acid is added. It is allowed to stand for 18 hrs at 6 degrees C. The solution is then filtered with slow suction. The precipitate is dissolved in minimum quantity of water at 45 degrees C. It is slowly cooled and allowed to crystallize. The crystals appear in about an hour as yellow colored crystals.
11. They are kept at 20 degrees C for 24 hours and then filtered. The yield is about 0.1 gm. They may be later dissolved in about 20ml of N/10 sodium acetate and used as enzyme solution.

Choice Of Methods Of Purification

The readers are by now aware that there are many methods that can be used for purification of enzymes. It should be noted that no single procedure is good enough to give 100% pure enzyme isolate. The choice of methods and their sequence of use are dependent on various criteria. They are briefly discussed below:

(A) The Volume Of The Crude Extract

Usually when an enzyme is isolated and purified the initial volume of the homogenate or crude extract is considerably large. It is known that many purification procedures have a volume restriction. Ideally in the initial stages, salt fractionation or solvent fractionation is often employed. For those enzymes that are heat stable the heat precipitation method may be used. Chromatographic techniques in the column mode are sometimes used.

(B) The Instrumentation Required

Many laboratories do not have updated and sophisticated instruments and in such case, the fractionation methods are a practically feasible alternative. These have an advantage of simple instrumentation and cheapness although the purification may not be great. Saturation with ammonium sulfate and precipitation by acetone are the preferred techniques. Dialysis is sometimes used.

(C) The Time Period Available

Some techniques like matrix less affinity separation are rapid and so are the salting out methods. Other methods may be time consuming. The time factor available with the worker is of great importance, based on which he may choose the methods

(D) The Economics Of The Process Involved

Some processes are financially expensive while other are labor intensive. It is necessary to choose methods that balance between the two.

(E) The Purity Required

Sometimes the enzyme isolated need to be extra pure, as in the case of research, diagnostics and therapeutic administrations. Contamination in such preparation may not give expected results. For this more sophisticated techniques based on affinity and specificity of the enzymes are usually employed.

Terms Related To Enzyme Purification

Whether the enzyme has been purified or not is generally determined by checking its activity in the purified preparation. Since most enzymes are proteins in nature the purified preparation is subjected to activity determination and protein quantitation to determine the specific activity and fold purification of the preparation. The terms most often used include,

(A) Yield.

(B) Specific activity

(C) Fold purification

The terms are discussed in brief as,

(A) Yield:

The crude extract is often subjected to fractionation and to check the presence of the desired enzyme in the purified fraction, its activity is compared with that of the crude extract. The yield is then determined using the formula,

$$\text{Yield} = \frac{\text{Units of enzyme activity in fraction}}{\text{Units of enzyme in the crude extract}}$$

(B) Specific Activity

It is given by activity in units per gram of protein in the solution. This is obtained by dividing the activity in units by the total amount of protein in the solution. In terms of the formula used it is written as,

$$\text{Specific activity} = \frac{\text{Units of enzyme activity in solution}}{\text{Total protein content in the fraction.}}$$

(C) Fold Purification

During the purification of the enzymes, different processes are employed. The fold purification is based on comparison between the specific activities of the enzyme in the purified fraction and that of the crude extract. It is written as,

$$\text{Fold purification} = \frac{\text{Specific activity of fraction}}{\text{Specific activity of crude extract}}$$

(5) Development of Assays

While working with the enzyme purification, it is necessary and customary to check the activity of enzymes. In fact enzyme assays are performed with the crude extract as well to determine whether the enzyme of interest is present in the homogenate or not. The assays may use determination of the formation of product or the utilization of substrate and this can change with the enzymes (for example activity of amylase may be followed by disappearance of starch while that of a phosphatase may be followed by measuring the inorganic phosphate generated as a result of enzymatic catalysis.

In general there are two types of assays:

(A) Qualitative Assays

The qualitative assays are not stoichiometric and are only used in the first step to determine that the enzyme of interest is present in the chosen source for isolating it or sometimes as a confirmatory test for the final isolate. These assays are simple to design. A little knowledge of the reaction catalyzed, substrate required and the other requirements (including the proper reaction conditions) is enough for formulating the qualitative assay.

Qualitative assay of amylase: determination of the achromic point.

Amylase can be isolated from saliva, germinating cereal grains or sweet potato(Ipomea batatas). Saliva can be clarified if necessary and directly used as an enzyme solution. The amylase from grains or sweet potato needs fractional precipitation or salting out procedure before it can be used. Readers probably are aware that the amylases act on starch and produce sequentially different products that include various dextrins, reducing oligosaccharides and glucose. The qualitative assay of amylase is based on the fact that with the activity of the enzyme, its substrate starch disappears and while starch has an ability to form a blue color with iodine, the dextrins, oligosaccharides and glucose do not give color reaction with iodine. Thus the determination of achromic point (disappearance of color) can be taken as a proof of amylase activity. The procedure is simple and does not require costly instrumentation.A mixture of buffer, enzyme, NaCl, and starch is made and kept at room temperature or 37 degrees C for incubation. Simultaneously a series of test tubes containing buffered iodine solution are prepared and numbered. Each contains the same amount of iodine. After regular time intervals of 60 seconds a small quantity, say 0.1ml is withdrawn from the reaction mixture and added to a tube containing iodine soluton. It is then

observed for color formation. The first tube shows a distinct blue color due to the reaction of starch and iodine which fades in subsequent tubes. The tube which does not show any change in the iodine color is taken as the achromic point. It should be noted here that NaCl is added as Cl acts as an activator. The iodine solution should be same in concentration and quantity. Alternately a multicavity tile may be used in which periodically the reaction mixture and iodine solution can be added till the achromic point is reached.

Qualitative assay of urease

Urease can be isolated from jackbean meal. The enzyme catalyzes the reaction of splitting urea into ammonia and carbondioxide. The isolation is done by using acetone. The crude enzyme is added to a solution of urea and incubated at 40degrees c for 30 min. the ammonia liberated is qualitatively assayed by addition of Nessler' s reagent. A yellow color is formed.

(B) Quantitative Assays

The quantitative assays are used to measure the activity of the enzyme, within a set of well-defined parameters. The conditions are predefined and all the parameters except the variable are kept constant. These studies generate important kinetic data. The end of the reaction is usually followed by the quantitative estimation of the product (s) formed through colorimetric analysis. Quantitative assays are used to determine the fold purification during the subsequent steps employed for the purification of the enzyme. They are also important for enzymes that are used in research, diagnosis and treatment. The protocols are arranged so that keeping all other requirements constant only the parameter whose effect on the enzyme activity is to be studied is varied. For example in case where the effect of the substrate concentration has to be determined, a set of tubes from blank is taken wherein the substrate concentration is increased by 0.2 ml increments. The volume is made to say 2 ml with an appropriate buffer or distilled water and all other reagents are added. The tubes are then kept at appropriate temperature for the reaction time (usually 15min or 30 min). The color developing agent is added, mixed and the tubes are read at the appropriate wavelength to obtain optical densties from which a graph is constructed. The OD of the unknown solution is similarly worked out and using the graph the amount of product formed is calculated. From this value it is easy to determine the units of enzyme activity.

There are two types of quantitative assays viz. Two point assay and Kinetic assay.

(a) Two Point Assay

It is also called as fixed time assay. The reaction time is fixed in this method and the enzyme activity is expressed as the amount of substrate transformed, product formed or cofactor altered by the measured amount of enzyme solution under controlled conditions. In other words the assay is performed after the end of the specific reaction time.

(b) Kinetic Assay

It is also called continuous monitoring assay. The readings are recorded continuously for the determination of reaction velocity hence multiple readings are obtained. When the substrate concentration decreases the rate of reaction is also decreased. In most clinical enzymological assays this method is adopted.

Quantitative assay of Succinate Dehydrogenase: The enzyme is a marker enzyme for the mitochondrial fraction. It catalyzes the conversion of succinate to fumarate simultaneously reducing FAD to $FADH_2$ If TTZ (triphenyl tetrazolium chloride) is added to the reaction mixture it accepts the hydrogen atoms from $FADH_2$ and forms formazan, a colored compound that is easily estimated at 420nm. Phosphate buffer at pH 7.4 is used.

(6) Stabilization and Crystallization of Enzymes:

The stability of an enzyme poses a major problem since the enzymes may lose their activity under many laboratory conditions. Specific steps are therefore necessary to prevent the denaturation and loss of activity of the enzyme. The general recommendations are on the line of preservation of proteins but the best conditions for each enzyme have to be determined specifically. It can be said that enzymes are optimally stabilized by specific solution conditions of pH, ionic strength, anionic/cationic composition and so on. It should be noted here that the conditions of stability are different than the conditions of optimum activity.

For long-term storage, enzymes should be kept at cryogenic temperatures around –70degrees or under liquid nitrogen. Although most laboratories use conventional freezing techniques that use temperatures between 0 to –20 degrees, the enzymes stocked undergo unintentional freeze-thaw cycles, as higher temperatures are required to keep them frost-free. The stability of the enzyme protein can be greatly enhanced by adding equal volume of glycerol and mixing it properly with the sample. This enables the protein to remain in the liquid phase at low temperatures and prevent their possible denaturation due to freeze-thaw cycles.

Yet another concern is about the microbial contaminants and their degrading activities on the proteins. The samples should be sterile-filtered through micro filters composed of a low protein binding material and placed in sterilized cryogenic tubes to avoid bacterial contamination. A frozen enzyme sample should be thawed only up to the required amounts and used immediately. To avoid wasting of enzyme samples, they should be stored in small amounts. Once thawed the enzyme should be kept at ice temperature (4 degrees) for as long as possible before equilibration to the assay temperature. Certain additives enhance the stability of enzymes for long-term storage at cryogenic temperatures and sometimes for short-term storage in solutions. They include glycerol, sucrose and cyclodextrans. The amounts of required stabilizers are different for different enzymes and have to be worked out specifically. Some enzymes are stabilized in the presence of their cofactors, substrates, or even inhibitors that bind to their active sites to form transient compounds having more stability.

Enzymes may sometimes lose activity due to their non-specific adsorption on the walls of storage devices made of glass or ordinary plastic. It is therefore advisable to use storage containers made of polypropylene or polyethylene. The problem can be countered with the addition of inert carrier proteins at a concentration much higher than the enzyme, which then saturates the protein binding surfaces and leaves the enzyme molecules free and intact. Bovine serum albumin and gelatin have been frequently used with great success by enzymologists world wide for this purpose. Gelatin at a concentration of 1mg/ml satisfies most enzyme stock solutions, as it does not interfere with ultraviolet assays due to its lack of aromatic amino acids.

Crystallization of enzymes was initially taken as a criterion for purity however later it was found that many crystalline enzymes contained up to 50 % impurities. Re crystallization may be often required before a crystal can be said to be pure enough. The most commonly employed method is formation of crystals from ammonium sulfate solutions where the solution is kept standing for long periods up to weeks during which good quality crystals can be obtained. There are two approaches that are commonly employed. The salt is added to a comparatively concentrated enzyme solution until a slight turbidity appears following which the solution is allowed to stand and the concentration of salt is slowly increased by adding a few drops of saturated salt solution after long time intervals. Alternately capillary mediated delivery systems may be used. Dialysis or simple evaporation of the solution may produce similar effects. Another approach is to keep the salt concentration unchanged and modify the pH or temperature so that crystallization is effected. Crystallization is better with enzyme isolates separated with solvent fractionations, probably due to elimination of interfering lipids during such steps. Formation of acetone powders is also used routinely in many laboratories.

Some researchers have used heavy metals to form crystals of enzymes. For example the enzyme, phosphopyruvate hydratase was crystallized as an inactive mercury salt.

In general crystallization of enzymes have been done since 1930s and the enzyme crystals have a number of advantages over solutions. Some of these include:

They can be easily packaged, stored and transported

They have a less chance of microbial attack and spontaneous changes

They can be used conveniently in micro quantities

They can be used for crystallographic studies for getting more information about the enzyme.

(7) Criteria For Purity

The purity of an enzyme preparation is difficult to determine and there is no single method that is good enough, however an enzyme is said to be pure if a combination of tests reveal it to be homogenous. The tests are based on physical properties or catalytic action and specificity of enzymes.

Many of the tests are same as the techniques used for purification. A single band with electrophoresis, or single component sedimentation or with ultracentrifuge. This is because the sedimentation constants of proteins are not uniformly distributed and they lie in close probable values, increasing the chances of contamination by non-enzymatic proteins. As it often occurs many proteins have similar molecular weights or shape, leading to their grouping in a single band or sedimentation coefficient. Even the electrophoretic mobility is not a criterion that might be taken as a 100% proof of purity, as many proteins may have similar electrical charges resulting into same mobility. The sensitivity of the affinity techniques is supposedly the most yet there are many times when contaminants end up in the final preparations. The second disadvantage while working with protein solutions is that no techniques are sensitive enough to detect small amounts of contaminants. The solubility tests are a better alternative. A series of enzyme solutions in regularly increasing increments are mixed with a constant amount of water or a salt solution, shaken and then filtered or centrifuged and the protein in the resultant liquid is estimated. If the amount is plotted against the amount taken then initially the graph is linear (as all the protein added dissolves, a line with unit

slope is obtained) but as further additions are made, the solution becomes saturated and a horizontal line is obtained. If a second, contaminating protein is present, initially the protein that is more soluble reaches a saturation stage after which the less soluble protein continues to dissolve till its saturation point is reached, which is apparent by the bend seen in graph.

A simplified procedure, proposed by Northrop, uses the determination of two points, one at the occurrence of the bend and the other at the far right. Two tubes are taken and in the first amount of protein enough to generate slight turbidity is added. To the second tube 10 times the amount is added, mixed and both the tubes are centrifuged. If the supernatants contain exactly same amounts of protein, then the preparation is considered to be pure. If however there is an increase, it is considered as an impure or contaminated solution.

Re crystallization is often used as it has been observed that the specific activity value reaches a constant after a few re crystallization cycles. It is thus taken that purity is maximal. In case of isoenzymes of a particular enzyme, agar gel separation is often used.

The choice of the type and number of methods used for determination of purity of the enzyme preparation is dependent up on the choice of workers as well as the facilities available and the economical support.

(8) Methods Of Preservation Of Purified Enzymes

Once the enzymes are purified and stabilized, they are preserved till further use. The methods of preservation used depend up on the volume of isolate, the frequency and period of utilization and storage facilities available. By far cryogenic preservation has been the most effective technique. However this facility is not available with most of the laboratories. Some research labs store their isolates at a freezing temperature in deep freezers (-4 to –20degrees C) and such preparations are often added with some stabilizing materials to enhance their keeping quality and time. Many others use acetone powders and crystalline enzymes.

In general some precautions are common to the storage of all isolates. They include:

1. The storage should be in micro aliquots.
2. The materials such as glass, quartz and ordinary plastic should be avoided while choosing containers.
3. The preparation should be micro filtered to remove any possible bacterial contamination before it is preserved.
4. If a part of the micro aliquot is used for an experiment, the remaining solution should not be preserved.
5. Some enzymes, especially the allosteric enzymes are unstable at 0 degree and they lose their sub-unit interactions.
6. Stabilizers are often recommended however the exact amount and the type of stabilizer required needs to be specifically worked out.

General Properties Of Enzymes

Enzymes are remarkable biomolecules that act as catalysts. They are responsible for the biotransformations that include catabolism and biosynthesis of important

compounds in living systems. Although they act as catalysts, they differ in several properties from chemical catalysts.

A comparison between enzymes and chemical catalysts is given in a tabular form as,

Table 2.4 Comparison of enzymes with chemical catalysts.

Property	Enzymes	Chemical catalysts
Chemical nature	Protein or RNA	Inorganic ions,acids &alkali.
Molecular weight	High	Low
Specificity	Specific in action	Not specific in action
Working Environment	Cell or cell free systems	Reaction mixtures / furnaces
High temperature tolerance	Present in rare cases	Present in all cases
Effect of pH	Range restriction	Not influential
Colloidal properties	Present	Absent
Source	Living cells	Extracellular
Coenzyme/cofactor requirements	May be required	Not required
Commercial exploitation	More	Less
Environmental competability	More	Less
Immobilization	Possible	Rare
Exhaustion of source	Impossible	Possible
Utility spectrum	Broad	Limited
Examples	Hexokinase, pepsin, etc.	Mn, Mg, Zn, Cu, Fe , H_2SO_4 HNO_3 etc.

The various properties of enzymes are briefly discussed as below,

- ***They are synthesized in cells:*** Almost all the living systems except viruses contain enzymes. In fact the cells can survive, grow and bring about all the changes, and transformations within them, with the enzymes. However it is interesting to note that they can be synthesized in reaction mixtures that contain all the components required for their synthesis.
- ***They function as biocatalysts:*** They are required to bring about the various biosynthetic and degradation reactions in the cell. They enable the cell to store materials (e.g.: glucose is stored as glycogen in liver and muscle by the action of specific enzymes), or they can use the stored materials (eg. on breakdown of glycogen, the glucose molecules are reformed). They oxidize substrate to produce energy, (eg. glycolysis, HMP shunt, ETC produce ATP and reducing equivalents that generate chemical energy in the cells) and synthesize new materials as and when required (eg. de Novo synthesis of nucleotides amino acids, sugars etc.)
- ***They are mostly universal:*** Except a few differences, enzymes catalyzing similar reactions are common to all living systems. For example the hexokinase isolated from prokaryotes and from eukaryotes shall catalyze similar reactions.
- ***They are mostly proteins in nature:*** Barring a few exceptions almost all the enzymes are protein in nature, and comprise of naturally occurring amino

acids. The exceptions include the ribo zymes or catalytic RNA molecules. It was earlier believed that all enzymes are protein in nature, however the concept was modified with the discovery of the self splicing RNA from *tetrahymena spp.*

- ***They are also active in cell free systems*:** One of the most remarkable properties of enzymes is that they are active even in the cell free systems. During the developmental stages of enzymology, some scientists including Pasteur believed that the enzymes are inseparable from the cells, resulting into the famous Pasteur – Liebig controversy, which lasted till the evidence shown by Buchner that they are able to remain active in the cell free systems. The present day techniques often use immobilized enzymes, enzyme electrodes etc for diagnosis.
- ***They can catalyze reactions in both directions:*** Many enzymes are reversible in nature, i.e. they can catalyze reactions in both the directions, for example the enzyme lactate dehydrogenase can catalyze the conversion of pyruvate to lactate and vice versa.
- ***They catalyze reactions by lowering activation energy:*** Enzymes combine transiently with the reactants to produce a transition state having a lower energy of activation than the transition state of the un catalysed reaction. Thus they accelerate biochemical reactions by lowering the energy of activation. When the reaction products are formed, the free catalyst is regenerated.
- ***They are highly sensitive to environmental parameters:*** Enzymes function within a limited range of conditions and their activity is markedly affected by various parameters. These include the factors like pH, temperature, substrate concentration, enzyme concentration, inhibitors, activators, ions, radiations, etc. for example a drastic alteration in the pH or temperature leads to the denaturation of the "3D" structure of the active site thereby greatly reducing or completely stopping catalysis.
- ***They may be synthesized in precursor forms:*** Certain enzymes, especially those that act away from the site of their synthesis are usually synthesized in the precursor forms. The proteases are the best examples studied. The precursors are referred as the zymogen forms and have to be activated before they are able to act on their substrates. Examples include trypsinogen, chymotrypsinogen, pepsinogen etc. Their activation is usually brought about by removal of a leader sequence comprising of a few amino acids.
- ***They react specifically:*** Enzymes are specific in action, they react with single or limited substrates. Those that react with a single substrate are called as absolutely specific enzymes (example includes Glucokinase that converts glucose to glucose-6-phosphate) while others could be group specific (example: hexokinase) or broadly specific (example is trypsin that can act on proteins, peptides, esters etc). Specificity has been also found in terms of geometrical or spatial arrangements (D amino acid oxidizes do not act on L amino acids) or optical properties of substrates.
- ***They may be coenzyme/ cofactor dependent:*** Certain enzymes are dependent on inorganic or small organic compounds for their activity. A detailed discussion

is given elsewhere in the book. It is enough to remember here that these components serve various functions including enhancement of substrate affinity, lowering of Km, bridging the enzyme and substrate, affecting the proximity and orientation, playing a role in catalysis etc.

- ***Many enzymes are allosteric in nature:*** The allosteric enzymes are those having a site different than the substrate-binding site. They also have a sub unit structure and show sigmoidal kinetics, due to positive or negative co - operativity. They usually serve as regulatory points in the metabolic sequence. The best-studied example is of Aspartate transcarbamoylase. It catalyses the transfer of aspartate to carbamoyl phosphate in the de Novo synthesis of pyrimidines.
- ***They may be localized within the cell:*** Many times the enzymes that are responsible for a particular metabolic pathway are found in the vicinity of each other, localized in specific region of the cell or a particular sub cellular organelle. Such a togetherness may be due to mere aggregation without any physicochemical bonding of the various components amongst themselves in some instances (for example the enzymes of the glycolysis pathway) while in others they form a multi enzyme complex (examples include the fatty acyl synthetase complex, the keto acid dehydrogenase complexes, and the complexes involved in pyrimidine synthesis)
- ***They may show directional preferences while catalyzing reversible reactions:*** They work equally efficiently in vitro and in vivo. Although some reactions show directional preferences when the enzymes are present in the cell but are freely reversible in the cell free systems, this is primarily due to the specific needs and energy status of the cell. This ability has been greatly helpful in the commercial exploitation of industrially important enzymes. Many enzymes are regularly used in diagnosis and differential diagnosis, therapeutic treatments, industrial fermentations etc.
- ***They can be effectively reused in cell free systems:*** Enzymes after immobilization in solid matrices can be re employed to bring about the catalytic conversion of specific substrates for many number of times.
- ***Sometimes they are better tools than using whole microbial cells:*** This is especially true in single step transformations. Where microbial cells require a stringent control of growth and nutrition parameters and still have a chance of undergoing spontaneous mutations thereby affecting the overall yield, isolated and immobilized enzymes do not lose their efficiency for considerable time.
- ***Some enzymes can act on themselves:*** This is the characteristic feature of the tetrahymena RNA, however many enzymes can act on themselves for their activation.

(For example: pepsin can act on pepsinogen and bring about its activation.)

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3

NOMENCLATURE AND CLASSIFICATION OF ENZYMES

The Early period

- Classification based on the root indicating substrate acted upon.
- Classification based on the product formed by the action of the enzyme.
- Classification based on the root indicating the substrate and type of reaction.
- Classification based on the product synthesized.
- Classification based on the general type of reaction catalyzed by the enzymes.

The IUB EC SYSTEM

Advantages of the IUB-EC System:

- Rules of Nomenclature of Enzymes.
- General rules for naming enzymes.
- Rules for particular classes.
- Details of IUB – EC Classification.

The Early Period

The ongoing discovery of enzymes made it essential for a standardized nomenclature and classification.With the discovery of *diastase* by Payen and Persoz, enzymes were often called as diastase. This was continued till about half a century until Duclaux in 1898 suggested the use of "-ase" suffix to be attached to the root indicating the nature of the substance that was acted upon. Some enzymes were named by adding the suffix "-in", and the names are still in use. For example: pepsin, trypsin, papain, chymotrypsin etc.

During the early period many enzymes were named as *ferments after their so-called similarity with the process of fermentation.*[as suggested by Louis Pasteur]

There were many attempts to classify the enzymes in the first half of the twentieth century and many trivial names are still in use.

Some of the criteria for classifying and naming enzymes that were used earlier are briefly given here.

1. Classification Based on the Root Indicating Substrate Acted Upon

As proposed by Duclaux, many enzymes were named by adding the suffix "-ase" to the name of the substrate acted upon. Thus maltase acts on maltose, lipase acts on lipids, acetyl cholinesterase acts on acetylcholine, protease acts on protein, Deoxyribonuclease acts on DNA etc.

2. Classification Based on the Product Formed by the Action of the Enzyme

Some enzymes have been named after the product that is formed by the action of the enzyme. Example includes citrate synthase.

3. Classification Based on the Root Indicating the Substrate and Type of Reaction

It was subsequently discovered that many substrates had an ability to enter different types of reactions. Hence merely using their name and adding a suffix "ase" did not give a clear idea of what the enzyme was actually doing. Take the compound pyruvate, which can be converted, to Acetyl-CoA by oxidative de carboxylation and to oxaloacetate by carboxylation. Thus the enzymes have been named respectively as pyruvate dehydrogenase and pyruvate carboxylase. In the earlier instance it uses the name of the substrate and the reaction that it undergoes that is dehydrogenation, while the later indicates that it undergoes carboxylation. A similar example is that of alcohol dehydrogenase, which catalyzes the dehydrogenation of alcohol.

4. Classification Based on the Product Synthesized

Many enzymes have been named after the product that is formed after the reaction they catalyze. For example, Citrate synthase, which catalyzes the condensation of Acetyl-CoA with oxalo acetate to produce citrate, or malate synthase, which catalyzes the synthesis of malate from glyoxalate and Acetyl-CoA.

5. Classification Based on the General Type of Reaction Catalyzed by the Enzymes:

Around the 1950s much data about the enzymatic catalysis was obtained and a few generalizations about the types of reactions were possible. A serious attempt was

made by Hoffmann and Ostenhof, and also by Dixon and Webb in 1958 to incorporate all the enzymes known till then in some groups. Three groups were recognized viz. a] The hydrolyzing enzymes b] the transferring enzymes and c] the other enzymes including synthetases, stereoisomerases and the enzymes adding groups to the double bonds. A detailed presentation can be read in the first edition of the book enzymes written by Dixon and Webb.[1958].

The IUB EC SYSTEM

Despite the various attempts made earlier, a universally acceptable system was not designed until the first attempt made by the International Union of Biochemists – Enzyme Commission in 1956. The commission presented the report, which was accepted and implemented in 1961. The rules used for classification and nomenclature of the enzymes using the enzyme commission number are explained a little later. However it is to be noted here that the names prescribed by the enzyme commission are lengthy and complicated in some instances and for such, the commission has allowed the use of the trivial names that are satisfactory and unobjectionable.

Advantages Of The IUB-EC System:

The enzyme commission developed the system of classification and nomenclature around a numbering system. It is proposed that each enzyme be assigned a 4-digit number, each separated by points and arranged on the following principles:

(A) The First Digit Shows The Class Of The Enzymes

There are six classes that have been organized on the basis of the general reactions that occur due to the specificity of the enzymes. The classes are as follows:

Table 3.1: Classes of Enzymes [IUB Classification]

Class Name	General Reaction Pattern	Example
1. Oxidoreductases	$A_{(red)} + B_{(oxd)} \rightarrow A_{(oxd)} + B_{(red)}$	Alcohol Dehydrogenase
2. Transferase	$A\text{-}g + B \rightarrow A + B\text{-}g$	Phosphorylase
3. Hydrolase	$A + H_2O \rightarrow B + C$	Lipase
4. Lyase	$A\text{-}C\text{-}C\text{-}B \rightarrow A\text{-}B + C{=}C$	Fumarase
5. Isomerase	$L\text{-}A \rightarrow D\text{-}A$	Alanine isomerase
6. Ligase	$A + B + ATP \rightarrow A\text{-}B + ADP$	Glutamine synthetase

(B) The Second Digit Refers To The Sub Class Within A Given Class.

Usually the subclasses of oxido reductases are based on the group in the donors, which undergo the oxidation and reduction reactions. Those of transferases it is the nature of the group transferred. For hydrolases they are based on the type of bond hydrolyzed, while in case of lyases it denotes the type of link, which is broken between the group, removed and the remainder. For the isomerases it is based on the type of isomerisation involved. The ligases are sub classified on the basis of the bond formed.

(C) The Third Figure Indicates The Sub-sub Class

This is a further specification that enables the differentiation of the enzymes within a subclass, for example the sub-sub classes of oxido reductases are defined on the basis of the type of acceptor involved for each type of donor. [For example, the sub-sub class is 1 when pyridine nucleotides are acceptors, 2 when it is cytochrome, 3 when it is Oxygen.]. For the transferases the sub-sub classes are on the basis of the particulars of group transferred [for example it is 1 when single carbon groups are transferred, 2 when the group is formyl, or hydroxymethyl etc]

(D) The Fourth Figure Is The Serial Number Of The Particular Enzyme In The Sub- Sub Class

This system of naming and numbering not only classifies the enzymes but also avoids the disadvantages of consecutive numbering through the whole list which will not accommodate any newly discovered enzymes without disturbing the sequence of a particular subclass or sub-sub class. Any necessity to create any new sub classes or sub sub classes would not disturb the existing sequence and numbers.

Rules of Nomenclature of Enzymes

While deciding the nomenclature of the enzymes the IUB-EC has recommended a set of rules, some of which are briefly given below:

General rules for naming enzymes

1. The names of substrates forming the part of the enzyme names should be given as per their IUPAC nomenclature (for example: 2-amino-2-deoxy-D-glucose and not glucosamine), and the substituents should be numbered as 1, 2, 3 instead of α, β, γ.
2. Where the substrate is normally an anion, its name should end as "-ate", rather than "ic" (for example: lactate dehydrogenase, and not lactic dehydrogenase).
3. The commonly used and prevailing abbreviations may be used but no new abbreviations should be created. (ATP is allowed but GDH or MDH should be strongly discouraged).
4. The use of enzyme names of descriptions such as "yellow enzyme", "pH 6 enzyme"and such names should be substituted with the names as per recommendations when the reactions are understood.
5. If the systematic name is short enough, use of trivial names should be avoided., similarly, a trivial name should not be based on a substance which is not its true substrate.
6. In general, the name of enzyme shall consist of two parts, the first will consist of the name of substrate[s],separated by a colon and the second part should end with "-ase", indicating the nature of the process.
7. The suffix "ase" should not be directly attached to the name of substrate.
8. In the case of reversible reactions the direction chosen for naming should be as per the general direction of all the enzymes in the class, even if this direction has not been demonstrated at all.

9. When the overall reaction includes two different changes, the second function should be indicated by adding a suitable participle in parenthesis [for example 1.4.3.1 D- aspartate oxidase, is named as D-aspartate:O2 oxidoreductase (deaminating)]
10. When an enzyme catalyses more than one type of reaction or acts on more than one substrate the name will normally refer to only one substrate.

Rules For Particular Classes

Class I

1. For naming enzymes in oxidation- reduction reactions, the names are formed on the pattern,"donor:acceptor oxidoreductase"
2. For oxidoreductases using pyridine nucleotides, the coenzymes will always be named as acceptors[foe example IMP: NAD oxidoreductases]
3. Where the true acceptor is not known, the word, acceptor is written in parentheses [for example, "succinate:(acceptor) oxidoreductase]

Class II

1. Enzymes catalysing group transfer reactions will be named transferases on the pattern, "Donor: acceptor, group transferred – transferase". [for example: ATP: acetate, phosphotransferase]
2. In the case of the kinases, ATP will be always being named as the donor. In case of aminotransferases, involving 2-oxo-glutarate, the latter will be named as acceptor.

Class III

1. Hydrolysing enzymes will be named on the pattern "substrate hydrolase". Where the enzyme is specific for the removal of a particular group, the group may be named as a prefix [for example adenosine amino hydrolase,3.5.4.4]
2. A systematic nomenclature covering all peptide hydrolases is not possible at present owing to their overlapping specificities. The separate identity of some of them seems to be somewhat doubtful.

Class IV

1. Enzymes removing groups from substrate non-hydrolytically, leaving double bonds [or adding groups to double bonds] will be called "lyases" in the systematic nomenclature. Prefixes, such as "hydro-", "ammonia" will be used to denote the type of the reaction[for example " citrate(isocitrate) hydro-lyase, 4.2.1.3]. Decarboxylases will be regarded as carboxylases. A hyphen should always be written before "lyases", to avoid confusion with hydrolases, carboxylases etc.
2. The complete molecule, not either part into which it is separated, is named as substrate.

Class V

1. " Isomerase" will be used as a general name for the enzymes of this class.
2. The types of isomerization will be indicated by prefixes,[for example: Maleate cis-trans- isomerase" or(5.2.1.1) phenolpyruvate-keto-enol isomerase (5.3.2.1)
3. Isomerases catalyzing inversions of asymmetric groups will be termed racemases or epimerases, according to whether the substrate contains one or more than one center of asymmetry, A numerical prefix to the word "epimerase" will be used to show the position of the inversion.[for example: D- ribulose-5-phosphate 3- epimerase catalyses the conversion of ribulose –5- phosphate to xylulose – 5- phosphate]

Class VI

1. The class of enzymes catalyzing the linking together of two molecules coupled with the breaking of a pyrophosphate link in ATP etc. will be known as "ligases". It is based on the product and not on the substrate [for example: DNA ligase].
2. The systematic names will be formed on the pattern "X-Y ligase (ADP)" where X and Y are the two molecules to be joined together. The substance shown in parenthesis (ADP or AMP) is the product formed from the triphosphate. Thus the reaction will be X + Y + ATP = X—Y + ADP + Pi
3. In case where glutamine acts as a donor of ammonia, the name "amido-ligase" will be used.

Details Of IUB – EC Classification:

The key to numbering and classifying the enzymes is given as,

CLASS 1: OXIDOREDUCTASES:

1.1 Acting On The –CHOH Group Of Donors

1.1.1 With NAD or NADP as acceptor

1.1.2 With cytochrome as acceptor

1.1.3 With O_2 as acceptor

1.1.99 With other acceptors

1.2 Acting On The Aldehyde Or Keto Group Of Donors

1.2.1 With NAD or NADP as acceptor

1.2.2 With a cytochrome as an acceptor

1.2.3 With O_2 as an acceptor

1.2.4 With lipoate as an acceptor

1.2.99 With other acceptors

1.3 Acting On The CH—CH Group Of Donors

1.3.1 With NAD or NADP as acceptor

1.3.2 With cytochrome as acceptor

1.3.3 With O_2 as acceptor

1.3.99 With other acceptors

1.4 Acting On The CH—NH2 Group Of Donors

1.4.1 With NAD or NADP as acceptor

1.4.3 With O_2 as acceptor

1.5 Acting On The C—NH Group Of Donors

1.5.1 With NAD or NADP as acceptor

1.5.3 With O_2 as acceptor

1.6 Acting On NADH or NADPH As Acceptor

1.6.1 With NAD or NADP as acceptor

1.6.2 With cytochrome as acceptor

1.6.4 With a disulphide compound as an acceptor

1.6.5 With a quinone or related compound as an acceptor

1.6.6 With nitrogenous group as acceptor

1.6.99 With other acceptors

1.7 Acting On Nitrogenous Compounds As Donors

1.7.3 With O_2 as acceptor

1.7.99 With other acceptors

1.8 Acting On Sulphur Groups Of Donors

1.8.1 With NAD or NADP as acceptor

1.8.3 With O_2 as acceptor

1.8.4 With disulphide compounds as acceptor

1.8.5 With quinone or related compound as acceptor

1.8.6 With nitrogenous group as acceptor

1.9 Acting On Heme Group Of Donors

1.9.3 With O_2 as acceptor

1.9.6 With nitrogenous group as acceptor

1.10 Acting On Diphenols And Related Substances As Donors

1.10.3 With O_2 as acceptor

1.11 Acting On H_2O_2 As Acceptor

1.98 Acting On H_2 As Reductant

1.99 Other Enzymes Using O_2 As Oxidant

1.99.1 Hydroxylases

1.99.2 Oxygenases

CLASS 2: TRANSFERASES

2.1 Transferring One Carbon Group

2.1.1 Methyl transferases

2.1.2 Hydroxymethyl - , formyl- and related transferases

2.1.3 Carboxyl and Carbamoyl transferases

2.2 Transferring Aldehydic Or Ketonic Residues

2.3 Acyl Transferase

2.3.1 Acyltransferases

2.3.2 Aminoacyltransferases

2.4 Glycosyltransferases

2.4.1 Hexosyltransferases

2.4.2 Pentosyltransferases

2.5 Transferring Alkyl Or Related Groups

2.6 Transferring Nitrogenous Groups

2.6.1 Aminotransferases

2.6.2 Amidotransferases

2.6.3 Oximinotransferases

2.7 Transferring Phosphorus Containing Groups

2.7.1 Phosphotransferases with an alcohol group as acceptor

2.7.2 Phosphotransferases with a carboxyl group as acceptor

2.7.3 Phosphotransferases with a nitrogenous group as acceptor

2.7.4 Phosphotransferases with a phospho- group as acceptor

2.7.5 Phosphotransferases, apparently intramolecular

2.7.6 Pyrophosphotransferases

2.7.7 Nucleotidyltransferases

2.7.8 Transferases for other substituted phospho groups

2.8 Transferring Sulphur Containing Groups

2.8.1 Sulphurtransferases

2.8.2 Sulphotransferases

2.8.3 Co-A transferases

CLASS 3: HYDROLASES

3.1 Acting On Ester Bonds

3.1.1 Carboxylic ester hydrolases

3.1.2 Thiol ester hydrolases

3.1.3 Phosphoric monoester hydolases

3.1.4 Phosphoric di ester hydrolases

3.1.5 Triphosphoric mono ester hydrolases

3.1.6 Sulphuric ester hydrolases

3.2 Acting On Glycosyl Compounds

3.2.1 Glycoside hydrolases

3.2.2 Hydrolysing –N- glycosyl compounds

3.2.3 Hydrolysing- S- glycosyl compounds

3.3 Acting On Ether Bonds

3.3.1 Thio ether hydrolases

3.4 Acting On Peptide Bonds (Peptide Hydrolases)

3.4.1 a aminopeptide aminoacidohydrolases

3.4.2 a carboxypeptide aminoacidohydrolases

3.4.3 Dipeptide hydrolases

3.4.4 Peptide peptidohydrolases

3.5 Acting On C—N Bonds Other Than Peptide Bonds

3.5.1 In linear amides

3.5.2 In cyclic amides

3.5.3 In linear amidines

3.5.4 In cyclic amidines

3.5.99 In other compounds

3.6 Acting On Acid—Anhydride Bonds

3.6.1 In phosphoryl – containing anhydrides

3.7 Acting on C—C bonds

3.7.1 In ketonic substances

3.8 Acting On Halide Bonds

3.8.1 In C—halide compounds

3.8.2 In P—halide compounds

3.9 Acting On P—N Bonds

CLASS 4: LYASES

2.1 Carbon—Carbon lyases

2.1.1 Carboxy -lyase

2.1.2 Aldehyde -lyase

2.1.3 Ketoacid -lyase

2.2 Carbon—Oxygen Lyases

2.2.1 Hydro-lyases

5.2.99 Other Carbon—Oxygen lyases

4.3 Carbon—Nitrogen Lyases

4.3.1 Ammonia lyase

4.3.2 Amidine lyase

4.4 Carbon—Sulphur Lyases

4.5 Carbon—Halide Lyases

CLASS 5: ISOMERASES

5.1 Racemases And Epimerases

5.1.1 Acting on aminoacids and derivatives

5.1.2 Acting on hydroxyacids and derivatives

5.1.3 Acting on carbohydrates and derivatives

5.2 Cis-trans Isomerases

5.3 Intramolecular Oxidoreductases

5.3.1 Interconverting aldoses and ketoses

5.3.2 Interconverting keto and enol groups

5.3.3 Transposing C=C bonds

5.4 Intramolecular Transferases

5.4.1 Transferring acyl groups

5.4.2 Transferring phosphoryl groups

5.4.99 Transferring other groups

5.5 Intramolecular lyases

CLASS 6: LIGASES

6.1 Forming C—O bonds

6.1.1 Amino acid RNA ligases

6.2 Forming C—S bonds

6.2.1 Acid thiol ligases

6.3 Forming C—N bonds

6.3.1 Acid ammonia ligases [amide synthetases]

6.3.2 Acid amino acid ligases [peptide synthetases]

6.3.3 Cyclo ligases

6.3.4 Other C—N ligases

6.3.5 C—N ligases with glutamine as N donor

6.4 Forming C—C Bond

List of enzymes

A complete list of enzymes known so far is given in the end.

◆◆◆◆◆

4

STRUCTURAL COMPONENTS OF ENZYMES

Introduction

- Determination of molecular weights
- Determination of composition of enzymes

Short Course on amino acids

Primary structure of enzyme proteins

- Large polypeptides are cleaved prior to their sequencing
- Peptides are purified before analysis
- Determination of composition of amino acids
- Determination of sequence of peptides
- Determination of N terminal amino acid
- Determination of the C-terminal amino acid

Secondary structure of the enzyme

Tertiary structure of proteins

- Physical Interactions
- Chemical interactions

Quaternary structure of proteins

The prosthetic groups

- Determination of number of prosthetic groups per molecule
- Determination of the number of the active sites per molecule
- Use of affinity labels

Introduction:

After obtaining a purified preparation of a particular enzyme, the next part is the study of the various facets of its molecular weight, composition, structure, substrate specificity, mechanism of action etc. this is not a very easy task and requires the use of many advanced techniques and instrumentation. Moreover this needs an integrated approach, an interdisciplinary work which calls for contribution of physicists, organic chemists, analytical chemists.

Many times the data obtained during a particular study are helpful in the interpretation and investigations on other facets related to the enzyme. The present chapter shall deal with the techniques, and routinely used methodologies for the determination of various aspects of enzymes.

(A) DETERMINATION OF MOLECULAR WEIGHTS

Enzymes are mostly polymers of amino acids that differ in the composition with respect to the number and sequence of amino acids. Depending up on the content of amino acids, each enzyme has characteristic molecular weight. The determination of the molecular weight is extensively used in characterization studies of enzymes. The molecular weight of an enzyme is a valuable piece of information since it allows us to convert the concentration of a solution from units of weight per volume to units of molarity. This information can then be used in a whole variety of ways. Such as in consideration of composition of the type of amino acids and their number, catalytic activity, the number of substrate molecules transformed per unit time, and ligand binding, the number of ligand molecules binding per molecule of the enzyme. Measurement of molecular weights made in the absence and presence of denaturing agents will show whether or not the enzyme is composed of sub units and may indicate the number of such sub units.

There are several techniques that are routinely used for the determination of molecular weights, some of which are discussed here.

(1) Ultra Centrifugation

The ultracentrifuge can operate at speeds up to 75000 rpm providing centrifugal force in excess of 500,000g.At such a speed the friction between air and spinning rotor generates significant amount of heat, which is eliminated by employment of vacuum and refrigeration system that maintains the rotor temperatures between 0-4 degrees. There are many techniques, those that are used for isolation of cells and sub cellular organelles come under preparatory ultra centrifugation, while the techniques used in characterization come under analytical centrifugation

The Analytical Ultracentrifuge

It can be used in two main ways to determine molecular weights of enzymes, viz. Sedimentation velocity and sedimentation equilibrium.

Sedimentation Velocity

In this type of experiments, the ultracentrifuge [see fig 4.1] is operated at high speeds to generate centrifugal forces, which are sufficient to sediment, the enzymes. The sedimentation is characteristic and depends upon the sedimentation coefficient, which is monitored by using special optical devices.

The molecular weight of enzymes can be determined by the Svedberg equation:

$$M = \frac{RTs}{D\left(1 - \bar{v}\, r\right)}$$

Where

M = anhydrous molecular weight of enzyme

R = gas constant

T = absolute temperature

s = sedimentation coefficient of the molecule,

D = diffusion coefficient of the molecule.

$\bar{v}$ = Partial specific volume of the enzyme

r = Density of the medium

The partial specific volume of a solute is defined as the volume change upon addition of 1 g of that solute to a large volume of solution. Keeping all other parameters constant, u can be determined from very accurate measurements of the densities of solutions or calculated from the amino acid composition of the enzyme. The diffusion coefficient is worked out separately and used for calculations.

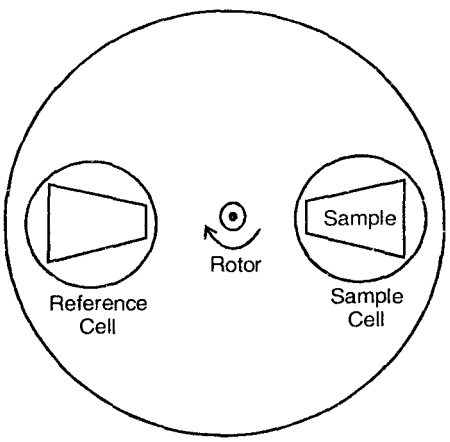


Fig 4.1: Ultracentrifuge

Sedimentation Equilibrium

If the rotor speed is not great enough to cause a complete sedimentation of the enzyme, then, after a while, an equilibrium state is reached. In this, the tendency of the enzyme molecules to be sedimented is balanced by their tendency to diffuse from the region of high concentration at the bottom of the ultracentrifuge cell to the region of low concentration towards the surface layer of the solution. From measurements of the distribution of the concentration, c, of the enzyme as a function of distance, r, along the cell (distance from the axis of rotation), the molecular weight can be calculated provided

that we also know u & r as in sedimentation velocity experiment. The formula used is,

$$M = \frac{2RT}{\left(1 - \bar{v}\, r\right)\omega^2} \times \frac{d \ln c}{dr^2}$$

Where,

M = molecular weight, R is gas constant, T is the temperature and ω is the angular velocity in rad/s. The value of $dlnc/dr^2$ is obtained from the slope of the plot of ln c against r^2

The method eliminates the considerations of shape of the solute or viscosity of the solution. If a short column is used, the time required to reach equilibrium is quite short.

The molecular weights determined by the method are fairly close to the values calculated from primary structure.

Approach to The Equilibrium Method:

Calculation of molecular weight by sedimentation equilibrium is based on the fact that upon reaching equilibrium there is no change in concentration at any point. Archibald pointed out that such a condition could exist at any time, at two points, namely the upper meniscus and the bottom of the cell. However since it depends on the data from the extremities of the solution column, precise measurements are not possible and the method is inferior in accuracy, and thus is not used anymore.

(2) Gel Filtration

Gel filtration is a simple technique, which is able to separate the molecules based on their size. The globular proteins show a linear relationship between the logarithm of their molecular weight and their distribution coefficient Kd, over a range of certain molecular weights.

The value of Kd for a given solute is defined by the relationship,

$$Kd = \frac{Ve - V_0}{Vi - V_0}$$

Where,

Ve = the elution volume of the molecule of interest,

Vo = the elution volume of a molecule, completely excluded by the column,

Vi = the elution volume of a molecule totally included by the column.

Thus, Kd = 0 for solutes that are totally excluded while it is taken 1 for the solutes completely included. The linear range of molecular weights has been found to be between 40,000 to 200,000 Daltons for Sephadex-G 200.

The distribution coefficients of standard proteins of known molecular weights are plotted against the log of molecular weights, the position of the distribution coefficient, of the unknown protein on the plot will lead to the determination of its molecular weight.

While doing, errors are possible if the shapes of the protein differ widely. Using solvent like guanidium chloride, which makes the proteins behave like randomly coiled linear Homo polymers errors can be eliminated.

The correlation of molecular weight and elution volume for proteins from a gel permeation column can be determined using standard calibration curve.

(3) Sodium Dodecyl Sulfate PAGE

The mobility of a charged molecule in an electric field is normally a function of various factors such as the size and shape of the molecule and the charge it carries and therefore it would be expected that electrophoresis would not normally give any reliable estimates of molecular weights.

Molecular weights of enzymes, however, can be determined by using, Sodium Dodecyl Sulfate [SDS] also known as Sodium Lauryl Sulfate. 2- mercapto ethanol is also added to break the disulfide linkages. This has the following effects:

(1) Nearly all proteins bind SDS in a more or less constant ratio, 1.4g SDS per g protein. The protein SDS complex has a constant charge/ mass ratio.

(2) The protein SDS complex is a rod shape with a length proportional to the molecular weight of the protein.

Since the charge and hydrodynamic properties of the protein SDS complex are both simple functions of the molecular weight, the mobility on electrophoresis is a function of molecular weight alone. In practice it is found that a graph of the logarithm of the molecular weight against mobility is linear and the molecular weight of an unknown protein can be determined by reference to the standard straight line. Different ranges of molecular weight can be examined by the use of gels of different polyacrylamide concentrations, thus 10% gel give good separation in the range 10,000 to 70,000 and 5% gels are satisfactory in the range 25000 to 200,000. The accuracy of the molecular weight obtained is estimated to be 10% or better. The molecular weight obtained should be checked by other methods. There are cases known where anomalous behavior is observed on electrophoresis. Histones for example give rise to lower electrophoretic mobility. Similarly many glycoproteins have impaired binding with SDS and result in lower electrophoretic mobility.

Addition of SDS dissociates an enzyme into its sub units and mercaptoethanol disrupts the disulfide links within the sub unit, so it should be remembered that the molecular weight determined by this method is of the individual polypeptide chain.

The graphical representation of relative mobility against log of molecular weight is represented in fig 4.2

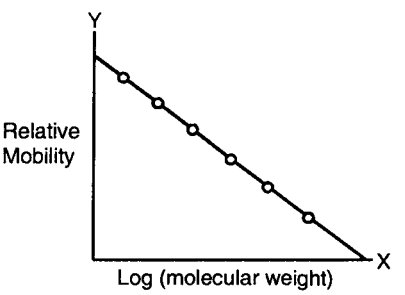


Fig 4.2: Determination of molecular weights from relative mobilities. The gels used are 10% polyacrylamide.

(B) Determination of Composition of Enzymes

Enzymes are mostly proteins. They are polymers of amino acids however sometimes non- protein parts may be present as the integral constituents.

A short course on the amino acids is given here for a better understanding of the further aspects of enzyme structure and activity.

Short Course On Amino Acids:

Naturally occurring amino acids are 20, and except proline, which is an imino acid, they have a general formula,

$$H_3N^+ - CH(R) - COO^-$$

The central carbon, associated with the carboxylic group is called as the a carbon and R represents the side chain. The amino acids encountered in the natural proteins are of the L – type, however in rare cases D stereo isomers have been found to be present in biological compounds.

Classification of Amino Acids

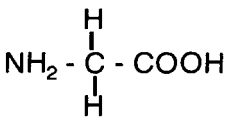
The amino acids are classified into various groups based on different criteria, some of which are briefly mentioned here:

1. Classification Based on Nature of the Side Chain

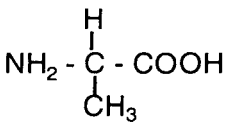
Various authors have tried to categorize amino acids in different groups based on the nature of their side chains. The most comprehensive classification divides the naturally occurring amino acids into seven groups as under,

(A) Aliphatic Amino Acids

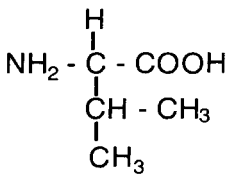
Their side chains contain unbranched or branched aliphatic residues. This group includes Glycine, Alanine, Valine, Leucine, and Isoleucine. Their structures are represented as,



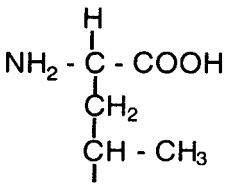
GLYCINE



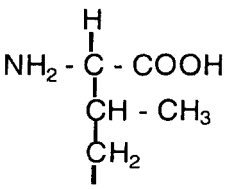
ALANINE



VALINE



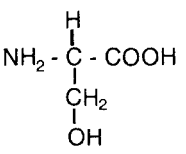
LEUCINE



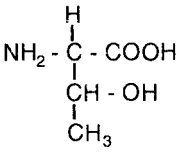
ISOLEUCINE

(B) Hydroxy-Amino Acids

Their side chains contain hydroxyl groups in their side chains. There are two such amino acids namely Serine and Threonine. Their structures are represented as,



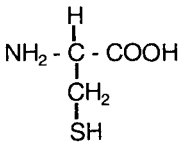
SERINE



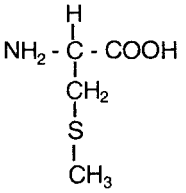
THREONINE

(C) Sulfur Containing Amino Acids

They contain sulfur atom in their side chain. There are two such amino acids namely, Cysteine and Methionine. Their structures are represented as follows:



CYTEINE



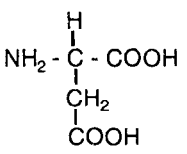
METHIONINE

(D) Acidic Amino Aids and Their Amides

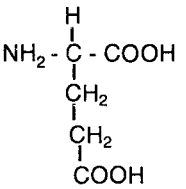
This group comprises of two acidic amino acids and their amides. Their names are Aspartic acid and Asparagine, and Glutamic acid and Glutamine. Their structures are represented as follows,

(E) Basic Amino Acids

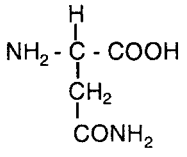
They contain additional amino or guanido groups in their structures. There are two basic amino acids, namely Lysine and Arginine. Lysine contains an e amino group while Arginine contains a guanido group. Their structures are represented as



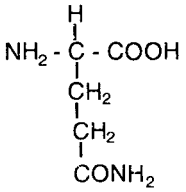
ASPARATE



GLUTAMATE



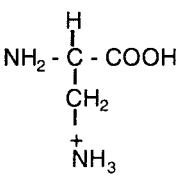
ASPARAGINE



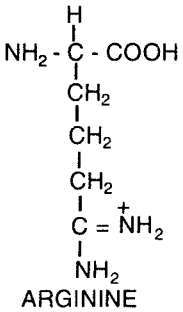
GLUTAMATE

(F) Aromatic Amino Acids

As the name indicates, these amino acids contain aromatic groups in their side chains. There are four such amino acids and they are Histidine, Phenylalanine, Tyrosine, and Tryptophan. Their structures are written as,



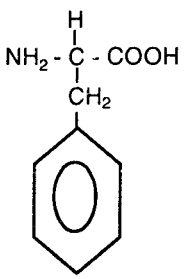
LYSINE



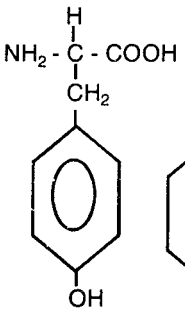
ARGININE

(G) Imino acid

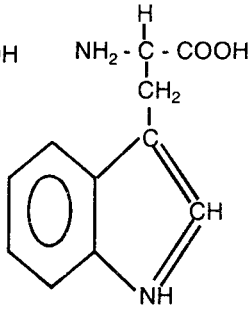
The imino acids do not carry the characteristic amino group. There are two compounds, the imino acid proline and its hydroxy derivative, hydroxy proline. The structure of proline is given as,



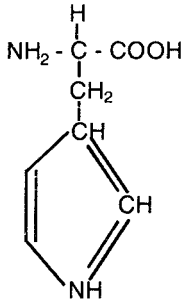
PHENYLALANINE



TYROSINE



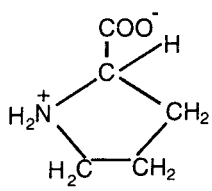
TRYPTOPHAN



HISTIDINE

2. Classification Based on Ability of Humans to Synthesize Them

Alternately amino acids are classified as essential, semi essential and nonessential based on the criterion of the ability of humans to synthesize them. The essential amino acids need to be supplied through the diet and cannot be synthesized by the human beings. There are 9 such amino acids, which include Methionine, Arginine, tryptophan threonine, valine isoleucine, leucine, phenylalanine, and lysine. There is only one semi essential amino acid, histidine, while all other amino acids are nonessential.



PROLINE

3. Based on The Fate of The Carbon Skeletons

Amino acids are classified as glycogenic; those which lead to synthesis of carbohydrates and ketogenic; those forming ketone bodies. Only leucine is purely ketogenic. Most of the amino acids are glucogenic and ketogenic.

Physical Properties of Amino Acids

1. Amino acids are soluble in polar solvents such as water and ethanol but they are insoluble in non- polar solvents such as benzene or ether
2. Their melting point is above 200 deg.C
3. The aromatic amino acids tryptophan, tyrosine, histidine phenylalanine, absorbs ultraviolet light.

Color reactions of Amino Acids

Amino acids react with various chemical reagents to produce color compounds. A general reaction is the Ninhydrin reaction. Ninhydrin is a powerful oxidizing agent, which causes oxidative decarboxylation of a amino acids yielding carbon dioxide, ammonia and an aldehyde. The reduced ninhydrin then reacts with the liberated ammonia forming a blue complex. Proline and hydroxyproline produce a yellow color in this reaction. Other tests which can identify specific amino acids include those given in table 4.1

Table 4.1: Tests for amino acid detection

Amino acid detected	Name of test	Final color
Cysteine	Nitroprusside test	Red
Tryptophan	Hopkins Cole test	Purple
Tyrosine	Millons test	Red
Tyrosine, Trp, Phe,	Xanthoproteic test	Yellow
Arginine	Sakagauchi test	Red
Histidine	Pauly test	Red

Most of the amino acids in a protein have their charged amino and carboxylic group used up in formation of a peptide bond. The identity and composition of the side chain of the amino acids chemically and physically distinguish them from each other. This is because the different side chains confer different chemical properties to the respective amino acids leading to different reactions.

Properties of Amino Acids Contributing To Specific Reactions

Some important properties of the amino acids that enable them to participate in specific reactions are discussed below,

1. ***Hydrophobicity:*** When hydrophobic molecules are dissolved in a polar solvent, such as water they tend to cluster together to minimize the amount of surface areas exposed to the solvent, a property called as hydrophobic interaction. This property of the hydrophobic groups to undergo repulsion from the solvent molecules and gather into a core is strong enough to fold the protein into specific three- dimensional forms. Hydrophobic amino acids also help to stabilize the binding of non-polar substrate and ligands in the binding sites of the enzymes. They are mostly found in the interior regions of the folded proteins, separated from the solvent molecules as well as the polar side chains, and the hydrophilic amino acids remain exposed to the water molecules, or the exterior

of the folded protein.

2. ***Hydrogen bonding:*** Hydrogen bonding of the amino acids side chains and polypeptide backbone groups can greatly stabilize protein structures. Additionally hydrogen bonds can be formed between amino acid side chains and ligand atoms and can contribute to the overall binding energy for the enzymatic reactions. Side chains that are capable of acting as hydrogen bond donors include those in the following table 4.2,

Table 4.2: Amino acid side-chains acting as hydrogen donors

Amino acid	Hydrogen donor group
Tyrosine	—O—H
Serine	—O—H
Threonine	—O—H
Tryptophan	—N—H
Histidine	—N—H
Cysteine	—S—H
Aspartic acid (at low pH)	—COO—H
Glutamic acid(at low pH)	—COO—H

Alternately the dissociated side chains can act as acceptors of hydrogen under suitable conditions. Methionine sulfur also acts as hydrogen acceptor under proper conditions.

3. ***Electrostatic interactions (formation of salt bridges):*** Due to the vicinity, of the dissociated oppositely charged chains, resulting from the folding of protein backbone, noncovalent electrostatic interactions can occur between electropositive and electronegative side chains. For example a carboxylate ion from acidic side chain can react with the amino group of a basic side chain and vice versa. These are often referred as salt bridges and can occur, intra molecularly as well as inter molecularly. At low concentration of salts there is an increase in such interactions leading to enhanced solubility of proteins, a phenomenon called as "salting in" of proteins. A typical electrostatic interaction is represented as,

$$R\text{———}N^{+}H_3 \text{ ———————} O^{-}\text{—CO ———————} R'$$

4. ***Acid base properties of amino acids:*** During the course of catalysis many amino acid side chains can contribute to the rate enhancement through phenomena referred as *general acid-base catalysis or specific acid-base catalysis.* (Details discussed elsewhere in the book) The tendency of the side chain groups to ionize is a function of the pKa values, however the local environment of the side chain often modifies these values.

5. ***Binding with metals and ions:*** Many enzymes have been found to be associated with metal ions, divalent cations and anionic groups. There are many roles that can be assigned to these groups.

They can provide stability to the folded structure

They can serve as coordination points for substrate or ligand binding

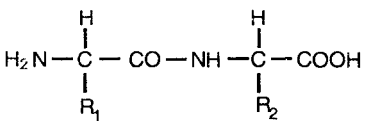
They may be prosthetic components involved in binding and transfer of protons, hydrogen or electrons

They can serve as electrophilic or nucleophilic reactants

6. ***Formation of covalent bonds:*** Amino acid side chains can also form covalent bonds. These are responsible for intramolecular as well as intermolecular interactions, which stabilize the polypeptide chain or the transition complex during the enzymatic reaction. Covalent catalysis is an important mode to enhance the rate of the enzyme-catalyzed reaction and its details will be discussed a little later.
7. ***Ability to form disulfide linkages:*** Disulfide linkages are important interactions, which are used to stabilize intramolecular folding or even sub unit binding. They are formed by the interaction between the side chains of Cysteine residues and are represented as (-S-S-). The interactions can take place among amino acids or a cysteine residue and a thiol containing ligand or small organic molecule. This reaction has been exploited in quantifying the free cysteine residues in many enzymes through their reaction with dithiol pyridine.
8. ***Formation of phosphorylated derivatives:*** Phosphorylation de phosphorylation mechanisms are extensively used by living system to effect mechanisms of control. The enzymes that bring about these reactions include the kinases and the phosphatases. Many covalent catalytic intermediates are formed through the phospho or acyl derivatives of certain side chains, the most commonly seen include, serine, threonine and tyrosine. However many times side chains of histidine and lysine have also been identified to take part in such reactions.
9. ***Glycosylation:*** Amino acid side chains are often employed to form O- glycosyl or N glycosyl derivatives during the formation of glycoproteins. The associations with sugars significantly alter the properties of solubility, folding, and biological activity of a protein.

The amino acids can polymerize through the formation of peptide bonds to give rise to oligopeptides, polypeptides and proteins, terms that are comparative and arbitrarily denote the probable number of amino acid residues present in them.

A peptide bond is formed when a molecule of water is eliminated during condensation of two amino acids. It is denoted by



Where (CO—NH) represents the peptide bond. The terms that are often used in relation to the peptides are defined as:

Dipeptide: They consist of two amino acid residues, linked to each other through a peptide linkage. In other words a dipeptide on hydrolysis yields two amino acids.

Oligopeptide: The oligopeptide by definition, on hydrolysis, yields between 2 and 50 amino acid residues, and are intermediates of protein digestion. There are many peptide hormones, which occur naturally and fall in this category. Oxytocin, vasopressin and glutathione are some of the examples.

Polypeptides: The polymers of amino acids containing more than 50 residues are arbitrarily termed as polypeptides. There is no specific difference between a polypeptide and a protein. Most authors use these terms synonymously.

Primary Structure of Enzyme Proteins

The sequence of amino acids in a polypeptide is referred to as its primary structure. Conventionally the N terminal amino acid is always shown at the left and the C terminal amino acid at the right of the polypeptide chain.

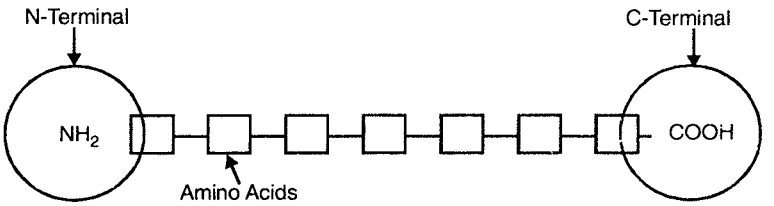


Fig 4.4: Primary structure of protein

The individual amino acids in an enzyme are identified numerically in sequential order, starting with N terminus. The N terminal amino acid is labeled number 1, and the numbering continues till C terminal.

Even small changes in the primary structure of the proteins may produce remarkable physiologic effects. Substitution of single amino acid for another in any polypeptide may alter or completely abolish biological activity. The example of sickle cell hemoglobin is a proof of such alteration. Many metabolically inherited defects have their basis in this phenomenon.

The determination of primary structure of proteins and enzymes would have been a time consuming task but for the advancement and intelligent use of many biophysical techniques that have simplified the process. The general scheme followed nowadays is briefly mentioned here.

Large Polypeptides are Cleaved Prior To Their Sequencing:

The enzymes are large polypeptides and many of them also comprise of sub unit structures each of which is an individual, folded polypeptide that is linked or associated with the other sub units. It is therefore necessary to cut the large chains into smaller fragments that can be easily handled and sequenced. This is because the automated sequencing instruments operate most efficiently on polypeptides containing 20-60 amino acid residues. Specific and complete cleavage at rare sites is required and many reagents are specifically used to achieve this. These include,

Cyanogen bromide, Trypsin, O-iodosobenzene, Hydroxylamine, Protease V8

Mild acid-hydrolysis.

The use of two or more agents, cleave the polypeptide into fragments that can be overlapped and the sequence worked out. The fragments produced are processed further.

Peptides are Purified Before Analysis:

The fragments obtained are first purified and the process may employ salt or solvent fractionation, differential centrifugation, gel filtration and electrophoresis. Alternately selective adsorption and elution of fragments from ion-exchange resins or use of DEAE- cellulose or CM cellulose has also been used successfully. The processes are rapid and sensitive.

Determination of Composition of Amino Acids:

On obtaining the fragments of the enzyme it is a normal procedure to determine the amino acid composition of the fragments before the actual sequence is determined. This is afforded by the use of HPLC in most cases, however limitation of expenses may dictate the use of chromatographic techniques to determine the amino acid composition.

Determination of Sequence of Peptides:

The first successful attempt was probably made by Sanger, who dissociated the two chains of insulin and determined their sequence by using 1 fluoro 2,4 dinitro benzene.

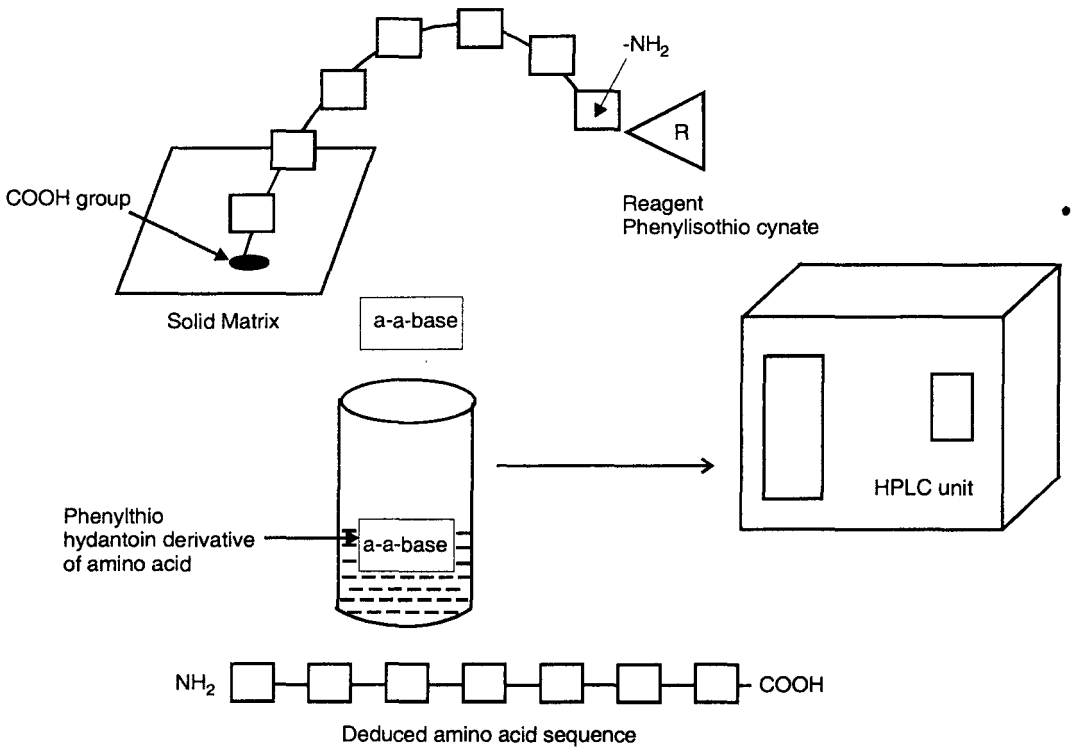


Fig:4.5 Determination of Amino Acid Sequence of Polypeptide.

The method determines the sequence unambiguously however is laborious and time consuming. Recent advances have developed amino acid sequencers, which employ the Edman reaction that utilizes phenylisothiocyanate. The reaction sequence releases the amino terminal amino acid as a phenylthio hydantoin derivative, which is then identified by HPLC. The next amino acid in sequence is then derivatized and removed and this process is repeated till the end. The reaction takes place on a solid matrix to which the carboxyl terminal of the peptide has been covalently coupled.

A brief description of the various methods used in determination of primary structure of peptides is given here.

Determination of N Terminal Amino Acid

The most widely used method involves reaction of the peptides with Dansyl chloride [dimethyl amino phthyl sulfonyl chloride] under alkaline conditions followed by acid hydrolysis to break the peptide bond. The labeled N terminal amino acid can be readily identified by electrophoresis or chromatography. Dansyl chloride has now almost totally replaced the earlier N terminal labeling reagent, 1-fluoro, 2,4 dinitro benzene [FDNB] or Sanger's reagent, since dansylated amino acid are highly fluoroscent and can be detected at concentrations much less than that are required for the FDNB procedure. The structure of dansyl chloride and the general process are given as

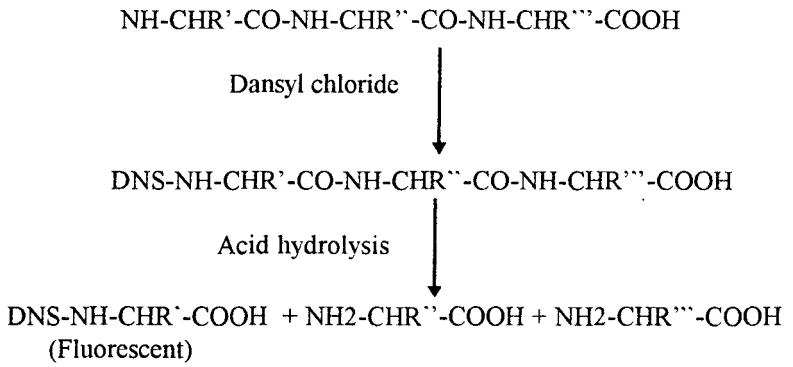


Fig: Labeling of a tri-peptide by reaction with dansyl chloride [DNS- Cl].

The DNS amino acid bond is stable to acid.

Some other methods used for determination of the N terminal amino acid include,

1. **Edman's Reaction:** This reaction is based on the action of phenyl iso thio cyanate with the alpha amino group of the peptides under alkaline conditions to form phenyl thio carbamyl peptides. These on treatment with acids form the phenyl thio hydantoin derivatives of N terminal amino acids and liberate the remainder of the peptide for a second reaction. The derivatives can be identified by the paper chromatographic procedure.
2. **Leucine Aminopeptidase:** This is a catalytic protein, which hydrolyzes the peptide bond formed by the N-terminal amino acid and the next amino acid in the chain. After the hydrolysis of the first N terminal peptide bond, a free amino acid and a peptide containing some other N terminal amino acid is formed. This new N terminal amino acid is then hydrolyzed from the peptide and the

process is continued. The released amino acids can be identified before the repetition of the action of the enzyme on the next N terminal amino acid as it is the only free amino acid.

Determination of the C-terminal amino acid

.Methods for determination of the C-terminal amino acid in peptides use the following procedures,

1. **Hydrazinolysis For the C-Terminal Amino Acid Residue:** When a peptide is treated with hydrazine all the peptide groups are split and carbonyl component is converted to the corresponding hydrazides. Only the C terminal amino acid is free and does not form a hydrazide. Chromatographic techniques can be used to identify the free amino acid, which is the C terminal amino acid.
2. **Treatment With Carboxypeptidase:** The enzyme carboxypeptidase splits the C-terminal amino acid specifically and upon its action the liberated free amino acid can be isolated and identified.

The determination of the primary sequence of the peptides is important as it provides the insight on various aspects of enzyme structure and function. Some of the issues are discussed here.

It Aids in Calculation of Molecular Weight of the Enzyme: The definitive molecular weight is only obtained when the primary structure is established and this calculated molecular weight can be used as a reference on the validity of other experimental methods used to determine the molecular weights. For many enzymes it was found that the molecular weight worked out by ultracentrifugation and other methods were erroneous and 15- 20% higher.

It Helps To Locate a Particular Amino Acid in the Enzyme and To Assign a Specific Role To Such Amino Acid: The primary structure of an enzyme helps to identify the surrounding environment of a particular amino acid, present in the active site of the enzyme, thereby shedding a light on its role and interactions during the course of catalysis. The readers are aware that the dissociated side chain groups contribute to electrostatic and other interactions that are responsible for the particular three-dimensional structure as well as the activity of the residues present in the active site. This in turn enables us to understand the mechanism of action of the enzyme.

It Helps Prediction of Three- Dimensional Structure Of An Enzyme: The primary structure determines the folding of the covalent backbone from which predictions about the three-dimensional structure, composition of active site as well as the possible mechanism of action can be worked out. The localization of polar and non- polar region provides specific binding and action properties to the enzyme.

To Explore Evolutionary Relationships Between Enzymes: Amino acid sequence provides an important data, which can be used to make comparisons between enzymes. This can help understanding the processes involved in enzyme evolution. Usually the change in amino acid arises from the alteration of the DNA coding for that enzyme. Comparison of the sequence of the homologous enzymes i.e. those catalyzing the same reaction in different organisms shows that most changes in sequence can be explained

on the basis of point mutation. Presumably the pattern of amino acid substitutions reflects a combination of two processes. The first is mutation, which can occur randomly in the gene. The second is the survival of such mutant, which is non-random. Replacement of a single amino acid by another with different and possibly opposite side chain character (a polar side chain replaced by a non-polar side chain or an acidic residue replaced by a basic one) would produce a non-functional enzyme. The natural selection process would eliminate such mutated organism.

When enzymes catalyzing similar reactions within an organism are compared, evidence is often seen supporting gene duplication. The two copies of a particular gene produced are followed by independent evolution of these two copies by point mutations. The high degree of sequence homology of various proteinases of different specificity like trypsin, chymotrypsin, elastase, thrombin etc especially around the amino acids involved in the catalytic mechanism suggest very strongly, that, these enzymes evolved from some common precursor proteinase by a process of gene duplication followed by subsequent independent evolution.

Other example of such a divergent evolution from a common precursor is provided by alcohol dehydrogenases in mammalian liver. On the other hand comparison between certain other enzymes suggests that process of convergent evolution [or parallel evolution meaning a similarity of amino acid composition with reference to active site composition only] may have occurred in which a similar type of functional unit has been evolved starting from quite different precursor protein chains. The homology in primary structure of these enzymes or their three-dimensional structure is very little. Bovine trypsin, chymotrypsin, thrombin, porcine elastase, bacterial subtilisin , all differ in the primary sequence around the essential serine, histidine and aspartate residues that are active components of the active site. The placement and mode of action of these residues indicate that convergent evolution has taken place.

To interpret data obtained from X-ray crystallography: The X-ray crystallographs determine a three-dimensional electron density map of the enzyme. Comparing the sequence of amino acids one can formulate the possible orientation and localization of peptide bonds and side chains from which the evidence of the correctness can be had about the structure of the sub-units and the enzyme in general.

Secondary Structure of The Enzyme

Globular proteins indicate a coiled structure in which peptide bonds are folded in a regular manner. The folding results from the linking of the carboxyl and amino groups of the peptide chains by means of hydrogen bonds and disulfide bonds. Such folding are referred to as the secondary structure of the protein. In many proteins the hydrogen bonding produces a regular coiled arrangement called α helix.

This structure was first predicted by Linus Pauling on the basis of stereochemical properties of polypeptides. The structure is stabilized by a network of hydrogen bonds between carbonyl oxygen of residue [i] and the nitrogenous proton of residue [i+ 4]. For most of the residues in the helix, there are thus two hydrogen bonds formed with neighboring peptide bonds. Each of them contributes to the overall stability of the helix. The side chains of the amino acid residues all point away from the axis in this structure thus minimizing steric crowding. The individual peptide bonds are aligned

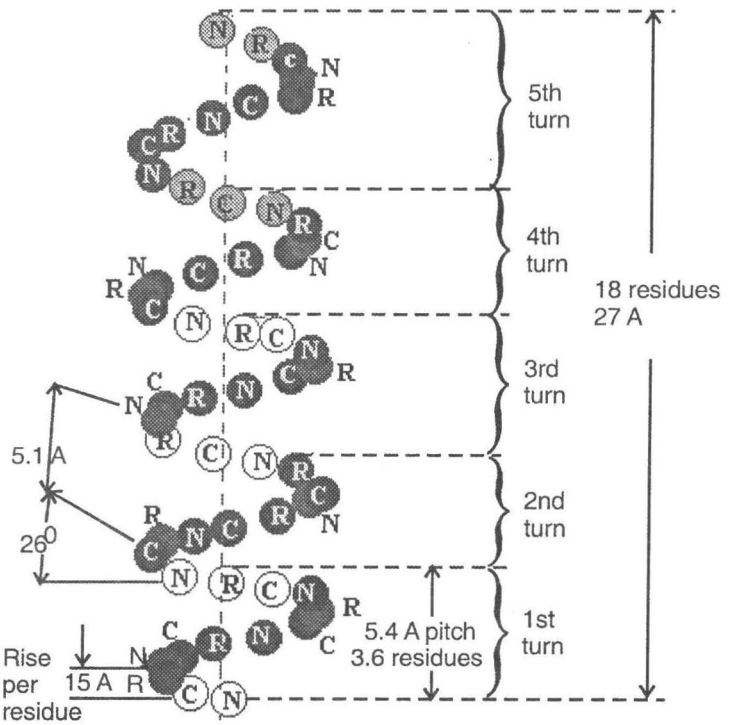
within the α helical structure producing in addition an overall dipole moment associated with the helix. The amino acid residues in an alpha helix conform to a very precise stereochemical arrangement. Each turn of the helix requires 3.6 amino acid residues, with a translation along the helical axis of 1.5 A per residue or 5.4A per turn.

Within the membrane bilayer where hydrogen bonding with solvent is not possible peptides and polypeptides tend to form an alpha helical structure.

Since alpha helix is the lowest energy form, it forms spontaneously. The right handed helix when occurs in proteins is significantly more stable than the left handed helix when the residues are L- amino acids. Certain amino acids like proline tend to disrupt the helix. The favorable and non-favorable forms are worked out with the help of Ramchandran plots.

Pauling and Corey also proposed a second ordered structure, "The β pleated sheet". In an α helix the polypeptide chain is condensed, in "The β pleated sheet" it is almost fully extended. In a β pleated sheet when the adjacent polypeptide chains run in opposite directions, the structure is termed an anti parallel β pleated sheet but when the chains run in the same direction it is termed parallel. Regions of β pleated structure are present in many proteins and both parallel and anti parallel forms occur. The alpha helix is stabilized by hydrogen bonding between peptide bonds, 4 residues apart in a primary structural sense, stabilization of the β pleated sheet results from formation of hydrogen bonds between peptides far removed from one another in a primary structural sense.

The structural representation of the helix and the pleated sheets are given in fig 4.4 and 4.5(A) and (B).



Average dimensions of an alpha-helix

Fig 4.4 Dimensions Of The Alpha Helix

The structure A represents the beta pleated type of secondary level of protein structure, while B represents the extended beta pleated sheet.

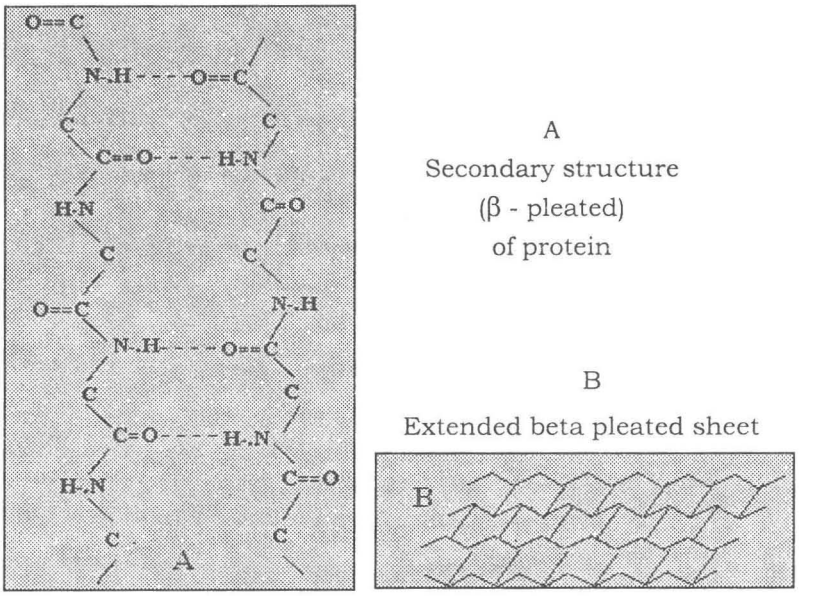


Fig 4.5(A) and (B): Beta Pleated Structure, Diagrammatic Representation.

Tertiary Structure of Proteins

The tertiary structure is a higher level of arrangement or three-dimensional super coiling of the secondary structure. During the coiling of the primary chain to form the secondary structure, some distinctly placed and reactive side chains come in the vicinity of each other, leading to a possibility of interaction between them. These interactions include a number of different physical and chemical interactions. They are briefly discussed here.

Physical Interactions:

They include Van der Waal's forces, electrostatic interactions, hydrogen bonds, and hydrophobic interactions.

Van der Waal's Forces:

Within the molecules, the electrons tend to distribute asymmetrically around the atoms. This generates a dipole moment leading to attractive force between atoms, referred to as van der Waal's force. Although the force is much weaker [about 1Kcal/mole], than the electrostatic interactions or hydrogen bonds and they are non specific in nature, however they are large in number that their collective forces can provide a significant stabilizing energy to protein- protein or protein- ligand interactions.

The attractive force between electron clouds increases as the two atoms approach each other but is counterbalanced by a repulsive force at very short distances. The optimal attraction between atoms occurs when, they are separated by a critical distance, known as the van der Waals contact distance, which depends on the electronic

configuration of the atom. The van der Waal radii for the different atoms found in the proteins are different and this difference causes interplay between the repulsive and attractive forces which play an important role in the specificity of interactions between proteins and ligands.

ElectrostaticInteractions

When two oppositely charged groups come into close proximity, they are attracted towards each other depending upon the distance between them, dielectric constant of the medium, and the charges carried by them. These attractive interactions are referred to as ionic interactions, electrostatic interactions or salt bridges. The net interaction is a result of the balance between the attractive forces and the repulsive forces associated with the interacting species. The average distance for such bridge formation is about 2.8Angstrom.

Hydrogen- Bonding

In many biological molecules, a hydrogen atom is often shared by two electronegative heteroatoms. One which has a covalently bound hydrogen which carries a d positive charge due to which, an electronegative atom in its vicinity, carrying a d negative charge, is attracted towards it causing a condition which is represented as,

$$\begin{array}{l} \delta- \quad \delta+ \quad \delta- \\ N: \text{——} H \ldots O \end{array}$$

The hydrogen bonds are weaker than covalent bond, and vary in bond energy between 2.5 and 8 Kcal/ mole. The amount of such interactions is very great in the proteins and adds to the stability of the protein molecule.

Hydrophobic Interactions

The tendency of the hydrophobic groups to separate themselves from polar medium and cluster in vicinity of each other is known. These interactions are not bonds in the true sense but in case of proteins, or enzymes play a significant role in the three-dimensional structure and activity of the enzyme. The aromatic side chains and the long aliphatic side chains predominantly show this type of reactions due to their bulky structure.

Chemical Interactions

Apart from the physical interactions, enzyme tertiary structure also contains chemical interactions, which play an important role in the three-dimensional structure as well as binding with substrate or ligands. These include the covalent bond, co-ordinate bond and the disulfide linkages. Each of these is briefly discussed here.

Covalent Bond

Redistribution of orbital and sharing of electron pairs within atoms and molecules form a covalent bond. Covalent bonds in the enzymes can be formed in intra chain or inter chain interactions. The bonds have considerable bond energy and are comparatively stable than hydrogen bonds. The energy is between 30-100 Kcal/mole. Covalent bonds may be formed during the course of catalysis, which shall be discussed elsewhere.

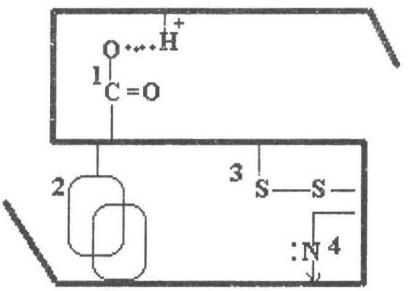
Coordinate Bond

When a basic atom carrying a lone pair of electrons donates its lone pair to an acceptor to form a bond, the bond is called a coordinate bond. Coordinate bonds are important interactions that contribute to the stability of enzymes containing transition metal containing prosthetic groups.

Disulfide Linkages

The cysteine side chains can interact to form disulfide linkages. They can be inter chain or intra chain. In fact many proteins contain both the types.

The following figure shows some of the interactions,



1. Hydrogen Bond
2. Hydrophobic Bond
3. Disulphide Link
4. Co-ordinate Bond

Fig 4.6: Bonds involved in formation and stabilization of higher levels of proteins.

Quaternary Structure of Proteins

The term quaternary structure refers to a higher order of arrangement, represented by the association of sub units. The sub- units are a characteristic feature of oligomeric or regulatory enzymes. Each sub unit is a polypeptide having a three-dimensional secondary and tertiary structure of its own.

The various facets of quaternary structure are related to,

The number of sub units

The arrangement of sub units in the enzyme,

The forces involved in the binding of sub units, and

The significance of sub-units in the enzyme structure and function.

The Number of Sub Units:

The molecular weight studies performed in the absence and presence of denaturing agents such as SDS or guanidine hydrochloride indicate whether an enzyme consists of multiple sub units.

1. The number of sub units can be deduced from the molecular weight data. From the data of SDS polyacrylamide gel electrophoresis, yeast pyruvate kinase was supposed to comprise of eight sub units of 20,000 each. Under milder conditions the enzyme was found to consist of four sub units each of 55,000.
2. Use of cross-linking agent such as dimethylsuberimidate helps to determine the number of sub units. This compound reacts with the lysine residues

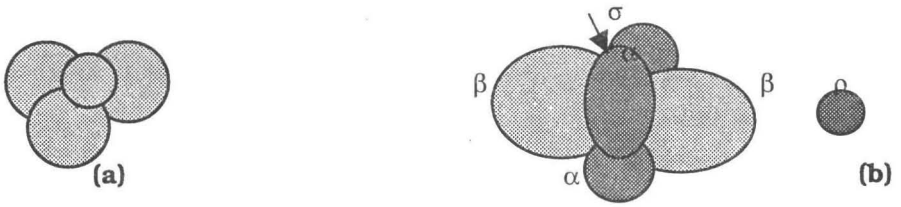
occurring on the surface of the enzymes, to form cross linkages that are stable to the denaturing agents. If an enzyme having four sub units is reacted with the agent different species will be formed depending on the cross-linking of the sub units. These species are separated by SDS polyacrylamide gel electrophoresis. The bands produced indicate the number of sub units. In this case, four bands will be produced. It is necessary to perform the experiment with caution to avoid complete inter sub unit cross-linking and intermolecular cross-linking. In the later case very large aggregates will be formed.

1. Ligand binding studies are also often used to indicate the number of sub-units. The alcohol dehydrogenases from yeast and liver bind to four and two moles of NADH per mole of enzyme respectively in agreement with the tetrameric and dimeric structures of the respective enzymes.
2. X-ray crystallography studies can many times be used to indicate the number of sub units. The sub units of aspartate transcarbamoylase have been worked out with the help of such studies.
3. The identity of the sub units is another issue related to the determination of the number of sub units of an enzyme. This is because many enzymes contain non- identical sub units. Determination of the N and C terminals of sub units can be quantitatively estimated. Although the ultimate proof of sub unit identity is related to the determination of the complete sequence, a reasonable estimate is possible by analyzing the number of fragments produced by digestion of the sub units by a specific protease.
4. X-ray crystallography studies can many times be used to indicate the number of sub units. The sub units of aspartate transcarbamoylase have been worked out with the help of such studies.
5. The identity of the sub units is another issue related to the determination of the number of sub units of an enzyme. This is because many enzymes contain non- identical sub units. Determination of the N and C terminals of sub units can be quantitatively estimated. Although the ultimate proof of sub unit identity is related to the determination of the complete sequence, a reasonable estimate is possible by analyzing the number of fragments produced by digestion of the sub units by a specific protease.

The Arrangement of The Sub Units

X-ray crystallographic studies have been instrumental in our understanding of the three-dimensional arrangement of sub units in oligomeric enzymes. Although there are different arrangements that are encountered with different enzymes, it is generally believed that the sub units are such arranged that the inter- sub unit contacts are maximal. Thus tetrahedral, octahedral symmetric arrangements are more often seen than linear or square arrangements. The possible structure of a tetrameric enzyme would be seen as,

This type of structure allows maximal sub unit interaction (6 bonds). A linear arrangement would allow (3) while a square would allow (4)]. The studies performed on this aspect of sub unit structure of enzyme. RNA polymerase has shown that octahedral symmetry can occur as, shown in the structure given below. The core enzyme consists of 2 alpha chains, 2 beta chains and a sub unit sigma.



TETRAHEDRAL ARRANGEMENT **RNA POLYMERASE**

Fig 4.7: Arrangement of sub-units, A = tetramer, B= RNA polymerase enzyme

The forces involved in the binding of the sub units:

It is known that the sub units of the oligomeric enzymes can be dissociated by using denaturing agent like guanidine chloride. This indicates that the bonds or interactions involved in the binding of sub units are weak and non- covalent type. Studies on various enzymes have indicated that the interactions involved have a relation as follows,

Electrostatic< hydrogen bonds< non-polar contacts.

The non-polar groups contribute the non-polar interactions and most of the times are predominantly the van der Waals interactions and hydrophobic interactions. Hydrogen bonds, due to their considerable number are another important interactions.

The Significance of Multiple Sub Units

It is clearly understood that the sub unit structure is predominant in those enzymes that have a regulatory activity or role within a given metabolic pathway. This ability to bring about regulation is but one reason for sub unit structure. There are other reasons as well. It has been proposed that the sub unit structure permits variations in catalytic properties, a classical example is of RNA polymerase, the sub units sigma and rho carry specific functions. There is also a proposal that the aggregation of sub units provide stability to the molecule. This is drawn from the fact that most of the sub units contain non-polar residues at the surface and thus the individual occurrence of each would lead to their instability due to the hydrophobicity.

The Prosthetic Groups

Many enzymes require the role of accessory factors for their action. These are divided into three groups viz. Coenzymes cofactors and prosthetic groups. Although many authors tend to use these terms synonymously there is a fine distinction and the definitions are mentioned in earlier chapters. The present discussion is restricted to prosthetic groups. As discussed earlier, the prosthetic groups are firmly bound or integral non- protein portions of enzymes. A true prosthetic group undergoes its whole catalytic cycle remaining attached to the same enzyme polypeptide molecule. By convention, haems, flavins, biotin and pyridoxal phosphate are supposed to be prosthetic groups.

Determination of Number of Prosthetic Groups Per Molecule

The number of prosthetic groups may be determined by two methods. These include the spectroscopic analysis and chemical analysis. In case of flavin and heme

prosthetic groups, measurement of the intensity of the characteristic absorption band will give the molar concentration of these groups. For flavin compounds their absorption at 450 mm is helpful. Two spectroscopic methods are commonly used for heme prosthetic group determination.

The absorption of unknown is compared with that of a standard heme protein of known heme content. For example, other cytochromes may be compared with cytochrome c or the heme groups may be detached and converted to common reference compound, the pyridine ferrohemochrome, and compared with the standard solution of the compound. Special methods are also available for the determination of particular prosthetic group when detached from enzyme proteins. These groups can be added to reaction mixtures containing enzymes requiring them and monitoring the activity by analytical methods or they can be added to media as growth factors for the growth of suitable microorganisms. Quantitative emission spectrography or chemical methods of estimation are used to determine the prosthetic groups containing metals. It should be remembered here that proteins have a tendency to take up metals from solution and they may be impurities.

The methods described give the amount of the prosthetic groups in relation to the total protein present. From the studies on molecular weight determination the number of such groups can be worked out.

Determination Of The number Of The Active Sites Per Molecule

Monitoring alteration of physical properties of enzyme upon binding of substrate molecule can lead to determination of maximum number of substrate molecules bound by one molecule of enzyme. The best known example of this is the combination of NAD with alcohol dehydrogenase, which is monitored by change in ultraviolet spectrum. These studies have been used to determine the binding of two moles of coenzyme NAD per mole of the liver enzyme.

Two physical methods namely the ***dialysis equilibrium method & the ultracentrifugation method*** **have also helped understanding of substrate binding with the active site.**

The Dialysis Equilibrium Method

This method was developed by Klotz et.al. This is performed as follows:

1. A known volume of known molar concentration of enzyme solution is placed in the dialysis sac.
2. It is dialyzed against a known volume of substrate [non- reactive under given condition] solution or a competitive inhibitor of known molarity.
3. When equilibrium is reached, the concentration of the substrate [or the competitive inhibitor] in the external fluid is determined and is assumed to be that of the free substrate in the enzyme solution also.
4. From the total amount of substrate added the concentration of substrate bound by the enzyme might be calculated.
5. This procedure is carried out with several different concentrations of substrates and extrapolation of the data gives the total number of binding sites per molecule.

6. From such studies on chymotrypsin it was shown to contain a single active site.

The Ultracentrifugation Method

Hayes and Velick developed this method. It depends upon the same principle. Taking samples of the solution at different levels in the tube after ultracentrifugation and estimating substrate and proteins carries it out. The technique has pointed out that yeast alcohol dehydrogenase has 4 active sites while the liver alcohol dehydrogenase has 2 active sites.

Determination With Specific Reagents

When an enzyme is completely inhibited by combination with a small number of molecules of an inhibitor it is usually assumed that this number is the maximum number of active centers in the molecule. This assumption however is not always correct. In case of urease, which is completely inhibited by 4 Ag+ ions are bound per molecule, this might be taken as indication that the enzyme contains only a small number of active centers per molecule. However this is far from reality. An alternative is the use of specific reagents acting as irreversible inhibitors, which produce complete loss of activity when only one or two molecules combine per molecule of enzyme. One such reagent is DFP [di isopropyl fluoro phosphate].

Using radioactive DFP with a-chymotrypsin, it was shown that one atom of radioactive phosphorus and two isopropyl groups bound per molecule inhibited the enzyme completely. This indicated that the enzyme has one active site. Similar results were obtained with trypsin.

A difficulty when using enzymes that are not sufficiently pure for example acetylcholinesterase is that a part of the DFP may be combining with groups other than active centers of the enzyme. This has been overcome by the method proposed by Cohen and Warringa.

The Method Is As Follows

1. The active center is first masked by a strong competitive inhibitor
2. The enzyme preparation is then treated with excess unlabeled DFP
3. The sites apart from the active center are thus saturated
4. The excess DFP and the inhibitor are completely removed by dialysis
5. This is checked by the activity studies indicating free active centers.
6. The preparation is treated with radioactive DFP, which combines only with the active center.
7. The labeled phosphorus is measured after prolonged dialysis.

Determination Of The Chemical Nature Of The Active Center

The active site or active center is the actual part of an enzyme structure that binds with the substrate and brings about the transformation. It is related to the activity as well as the specificity of the enzyme. It should be noted here that the active center might be associated with more than one polypeptide, if there are many polypeptides within an enzyme. In such a case the activity will depend on the existence of the combining groups as well as the intactness and configuration of the native protein

molecule. The identification of the residues that are involved in catalysis can be helpful in understanding the mechanism of action of enzymes.

The methods of identifying specific groups in the active center employ three types of criteria.

1. Deduction of pK values of substrate binding groups from pH effects
2. Degradation studies
3. Inactivation by specific chemical reagents

The deduction of pK values is done from kinetic measurements and their effects on Km or Vo. There is a fairly wide range of pK values from about 3 to 11. An interesting point is that in the majority of cases a group with a pK between 6 and 7 is present in the active center and as histidine is the only amino acid having a pK in this region many authors have assigned special importance to it.

The degradation studies have been used for many enzymes and various peptidases have been used to remove residues without any change in the activity of the enzymes in question. For example,

1. **Ribonuclease:** it can be completely inactivated by pepsin with the spitting of only one peptide bond and the removal of the tetra peptide Asp-Ala-Ser-Val-COOH from the C terminal end. The removal of valine, serine and alanine with carboxypeptidase does not cause any loss of activity. This means that the aspartate residue is part of the active center.
2. **Papain:** an inactive mercury derivative of the enzyme was prepared and degraded stepwise by leucine aminopeptidase until 109 of the total 185 residues were removed. On removal of the mercury from the remaining polypeptide, activity was restored. The activity center in this case as well appears to be located near the C terminal because aminopeptidase cleaves the residues from the N terminal.
3. **Phosphopyruvate hydratase:** similar studies have indicated that about 150 residues amounting to molecular weight 20,700 out of the total 64000, can be removed without loss of activity.

A number of groups have been identified in active centers of enzymes on the basis of inactivation by chemical reagents. For such studies it is essential that the reagent employed should be really specific for the particular group in question and should not attack any of the other groups present in the proteins. It should be realized that the amino acid residue in a particular position [active center or otherwise] in an enzyme, may behave differently than similar residues in other position, due to its interactions with neighboring residues.

Identification By Use Of Modifiers Of Thiol Groups

Reagents like iodoacetate or bromoacetate were used to identify the thiol groups in enzymes, by inactivation studies. Although they have been helpful in the cases of glyceraldehyde phosphate dehydrogenase, succinate dehydrogenase, yeast alcohol dehydrogenase, papain, and many kinases, their inhibition of ribonuclease is due to their attack on a histidine residue rather than a thiol group.

In many cases the inhibition was produced by an alteration caused in the three-

dimensional structure rather than inactivation of the active site thiol group. Sometimes steric hindrance may also be produced resulting in the apparent loss of activity.

Identification By Use Of DFP

The organophosphorus compounds, especially DFP react only with enzymes having esterase activity. In case of chymotrypsin the phosphoryl group becomes attached to the hydroxyl group of a serine residue. The enzyme contains more than 20 serine residues but the modification of ser195 alone places it in focus. This residue is the part of the active center of the enzyme. Other enzymes containing the serine residue as a part of the active site component include trypsin, pancreopeptidase, thrombin, cholinesterase and carboxylesterase. Subtilopeptidase from B.subtilis also contains serine in its active site.

Chemical modification procedures have also shown many other amino acids to be a part of the active site. A summary of the reagents used and amino acids modified is given below:

Table 4.3: Reagents Used to Modify Amino Acid Aide Chain

Reagents used	Amino acid modified
Mercurials [chloromercury benzoate]	Cysteine
Disulfides	Cysteine
Iodoacetamide	Cysteine
Trinitrobenzene sulfonate	Lysine
Pyridoxal phosphate, Na BH4	Lysine
Diethyl pyrocarbonate	Histidine
Phenyl glycol	Arginine
2,3-butanedione	Arginine
Tetra nitro-methane	Tyrosine
N acetyl imidazole	Tyrosine
Iodine	Tyrosine
N- bromo succinimide	Tryptophan
Water soluble carbodiimide + nucleophile	Aspartic acid
Water soluble carbodiimide + nucleophile	Glutamic acid

Change in pH can alter the specificity of the reagents used. A classic example is of iodoacetate, which reacts with cysteine sulfur around pH 7-8, while it reacts with methionine side chain around the pH 5.6, at which the cysteine side chains are unaffected.

Use of Affinity Labels

Affinity labels have indicated the presence of many active site residues. Compounds like bromo hydroxy acetone phosphate and N-p toluene sulfonyl L- phenylalanine chloromethyl ketone [TPCK] have been used with chymotrypsin and trypsin. The His57 and Ile16 were identified by labeling studies.

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5

ENZYME KINETICS

Introduction

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Chemical reactions and energetics

Classification of chemical reactions on the basis of number of molecules

- Zero order reactions
- First order reactions
- Second order reactions
- Third order reaction

The transition state of chemical reactions

Chemical Reactions in the living systems

The transition state of bio-chemical [enzymatic] reactions

Theories of enzyme kinetics

Theoretical background

- Equilibrium assumption [rapid state]
- The steady state assumption:

Transformations of the Michaelis Menten equations and plots

The significance of Km and Vmax

Effect of different parameters on enzyme catalyzed reactions

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- Competitive inhibition
- Uncompetitive inhibition
- Noncompetitive inhibition

Irreversible inhibition

Kinetics and Diagnosis of Reversible Inhibition types

Kinetics of Bi-substrate reactions

Enzymes catalyzing two reactions simultaneously

Single substrate- two enzyme reactions

Kinetics of allosteric enzymes

Models of action of allosteric enzymes

Introduction

Chemists have been making a serious study of the rates of reactions and the factors that control them, since the middle of the 19th century. Often they found that the rates were proportional to the concentrations of the substances that were reacting together. In 1892, it was found in a study of the rate of fermentation of sucrose in the presence of yeast that the rate seemed to be independent of the amount of sucrose present . Later it was suggested that this result could be explained if the invertase molecules present in the yeast formed an addition complex with the sucrose.

This was the first time that the existence of an enzyme–substrate complex was deduced from the kinetics of an enzyme reaction. It was not the first time the idea had been suggested. It had been found that papain appears to form an insoluble compound with fibrin prior to hydrolysis, and that the activity of invertase in the presence of sucrose survives a temperature that completely destroys it if the sucrose is not present; which was regarded as an indication of the combination of enzyme and sucrose molecules. The specificity of enzyme action is explained in terms of the precise fitting together of enzyme and substrate molecules; this is referred to as Fischer's lock and key hypothesis. Normally a lock can only be operated by a given key; a slight modification to a key usually means that the key no longer works. Fischer's ideas became particularly fruitful when physical methods, such as kinetics and X-ray crystallography, were applied to them. The next important step in enzyme kinetics was made by the finding that "the rate of reaction is independent of the concentration of the substrate" — is only observed at higher concentrations of substrate. At lower concentrations the rate becomes proportional to the concentration of substrate. To explain this it was considered that an enzyme–substrate complex is formed. The process can be represented as

$$E + S = ES = E + \text{products}$$

where E is the enzyme molecule, S the substrate molecule, and ES the complex.

The idea of Michaelis and Menten was that an equilibrium is established between E, S and ES, and that the slow step is the breakdown of ES. Since usually there are many more molecules of substrate present than of enzyme [on account of the high molecular mass of the enzyme], the enzyme becomes saturated with substrate at higher substrate concentrations. The concentration of ES will therefore remain the same, and the rate will remain the same, as the concentration of substrate is varied. At low substrate concentrations, on the other hand, the enzyme will not be saturated, and the concentration of ES, and therefore the rate of reaction, will be proportional to the concentration of S.

This reasoning led to the well-known Michaelis–Menten equation, which contains a constant known as the Michaelis constant.

This constant is defined in such a way that a high value means that there is only weak binding between the enzyme and the substrate; a low value means strong binding.

Some years later a more general formulation of the Michaelis–Menten equation was given. It was pointed out that the Michaelis assumption that an equilibrium exists between E, S, and ES is not always justified, and should be replaced by the assumption that ES is present not necessarily at equilibrium but in a steady state. The resulting equation is of the same form, but the Michaelis constant has a different significance with respect to the different rate constants.

Michaelis and his colleagues also made important contributions to our understanding of the way in which the rate of an enzyme catalysed reaction is affected by the pH of the solution. For some time it had been noticed in many enzyme systems that the rate is low if the pH is high or low, and passes through a maximum at some intermediate value, which is usually not far from neutrality [pH 7]. In 1911, two years before the formulation of the Michaelis–Menten equation, it had been concluded from this pH behaviour that the catalytic centres of enzymes must involve two ionizing groups. For effective catalytic action one of these must be in the form in which it is capable of accepting a proton, while the other must be in a position to donate a proton.

Over the years this basic idea was extended, particularly to take into account the ionization of the enzyme-substrate complex as well as that of the enzyme. A strong body of evidence shows that the powerful catalytic activity of enzymes is due in part to the fact that they function by being simultaneously able to donate a proton to, and accept another proton from, the substrate molecule. This has been referred to as a ***push-pull mechanism.***

The basic idea that an enzyme reaction involves an enzyme–substrate complex as an intermediate is important, but requires much extension and elaboration. There is now a considerable body of evidence suggesting that in many cases there are two or more intermediates.

A reaction scheme involving two intermediates may be written as

$$E + S = ES = ES' = E + \text{products}$$

Where ES´ is the second intermediate.

Good evidence for such a mechanism has been obtained for the hydrolysis of acetylcholine by the enzyme acetylcholinesterase. In this system the rate goes through a maximum with increasing substrate concentration, and the evidence suggests that this is due to the attachment of substrate molecules to the second intermediate ES´

With the isolation and purification of enzymes, there was a spur of experiments to analyze and understand the various facets of enzyme catalyzed reactions. The earlier half of the twentieth century was a golden period for the development and studies related to the kinetics of enzyme catalyzed reactions. Understanding the kinetics of an enzyme -catalyzed reaction is important due to the following,

1. Kinetics in conjunction with other techniques provides valuable information on the mechanism of action of an enzyme.
2. It can give an insight into the role of an enzyme under the conditions which exist in the cell and the response of an enzyme to change in the concentration of metabolites
3. It can help to show how the activity of an enzyme can be controlled which may provide a valuable pointer to mechanism of regulation under physiological conditions
4. When commercial exploitation of a particular enzyme is proposed, the kinetic data obtained serves as a guideline to manipulate the conditions of action resulting into maximal economy of the process, maximal yields and recovery of the commercially valuable product.
5. Designing of drugs based on the inhibition of enzymes can help treatment of

metabolically inherited diseases in which particular enzyme deficiency or hyperactivity is involved.

Obtaining The Kinetic Data

The kinetic data is primarily obtained from monitoring the reaction velocity in response to various parameters. It is usually followed by measuring the rate of formation of product or disappearance of substrate under specific conditions. It is then possible to vary certain parameters such as concentration of substrate, enzyme, pH, temperature or concentration of a modifying agent [activator or inhibitor] and collect data to analyze in terms of theoretical models of enzyme action.

The rate of a particular enzyme catalyzed reaction can often be measured in a number of ways however in each case individually a particular method is better than most of others. When the method of assay is decided upon, it is important to take a few precautions to obtain a reliable data. Some of the important considerations necessary are as follows,

1. The substrate, buffers etc should be of as high purity as possible since contaminants may affect the interaction and activity of the enzyme in question. For example, commercial preparation of NAD sometimes contains inhibitors of dehydrogenases and recently it has been found that certain preparations of ATP contain trace amount of vanadate ions, which act as a powerful inhibitor of ATPase. Alternately same can be said about commercial preparations of enzymes especially the proteases that can carry protease inhibitors resulting into incorrect kinetic data about substrate concentration requirements.
2. It must be confirmed that the enzyme preparation is pure and free from interfering substances, which might alter the assays. Appropriate controls are also necessary to eliminate the effects of spontaneous or non-enzymatic transformations of substrate molecule, which might generate erroneous data. For example inversion of sucrose is also possible in presence of mild acids upon long standing and such condition may alter the data on activity of invertase.
3. The enzyme should be stable during the time taken for assay. It should not lose its catalytic power. Breakdown of substrate other than by the enzyme should not occur either.
4. Since the activity of an enzyme can be markedly affected by changes in pH, temperature etc it is important to ensure that these parameters are stabilized by use of buffers, thermostatically controlled baths etc.
5. It should be checked that once the steady state has been achieved the measured rate of reaction is constant over the time interval of the reaction and is proportional to the amount of enzyme added.
6. The initial rate of reaction should be measured to avoid possible complication arising from product inhibition, occurrence of the reverse reaction and depletion of substrate.

Before elaborating on the background of the kinetics of enzyme catalyzed reactions, it is necessary to go through a basic course on aspects of chemical reactions and energetics.

Chemical Reactions And Energetics

Energy is a fundamental physical concept. It is the equivalent of or the capacity to do work. The physical dimension of energy, ML^2/T^2, a dimensional formula signifying mass X square of length $[L^2]$ divided by square of time $[T^2]$, are the same as that of work. Different kinds of energy may be distinguished for example, the potential and kinetic energies of mechanical system, surface energy, pressure volume energy, chemical energy, electrical energy and radiant energy. These are inter-convertible and the law of conservation of energy states that energy can neither be created nor be destroyed. It is important to realize that under biological conditions only a fraction of the energy released in vital processes is available for work of any kind. This fraction is called as free energy.

Chemical reactions are always associated with energy changes that finally appear as heat changes and are measured as such. Some reactions proceed with heat evolution and these are called as **exothermic reactions.** Other reactions absorb heat from the surroundings and are called as **endothermic reactions.** In general only those reactions liberating energy are capable of taking place spontaneously though a catalyst may be necessary to make them go in actual practice. Reactions that absorb energy proceed only when energy generally as heat, is supplied. Most of the processes concerned with the breakdown of foods in the body are exothermic and are catalyzed by enzymes.

Classification of Chemical Reactions on the Basis of Number of Molecules

Chemical reactions may be classified on the basis of the number of molecules that must ultimately react to form the reaction products. Reactions can be classified into mono molecular, bimolecular and termolecular depending on one, two or three molecules taking part in the reaction. On the basis of kinetics of reaction, they are classified by reaction order.

Zero Order Reactions:

The zero order reactions are those in which the rate of the reaction is independent of the concentration of the reactant. Generally spontaneous reactions such as radioactive radiation fall into this category. However in case of enzymes, when the active sites in the reaction mixture are completely saturated with the substrate, addition of any more amount does not affect the rate of reaction, i.e. the rate of enzymatic reaction becomes independent of the substrate concentration. Such a situation is called as zero order reaction. In the classical MM plots, such a case is observed at the Vmax or the horizontal phase of the hyperbola.

First Order Reactions:

They are those which proceed at a rate exactly proportional to the concentration of one reactant. The simplest example is when the rate of the reaction in which a single substrate is converted to a single product,

$$A \longrightarrow Y,$$

The rate at any time t will be given by the equation,

$$-d[A]/dt = K[A]$$

Where [A] is the molar concentration of A and $-d[A]/dt$ is the rate at which the concentration of the substrate A decreases. The constant K is called rate constant or specific reaction rate. They have the dimensions of reciprocal time, 1/sec.

Integrating the equation, we have,

Log [Ao] / [A] = Kt / 2.303,

Where, [Ao] is the concentration of A at zero time and [A] is the concentration at time t.

The following figure [5.1] illustrates the relation.

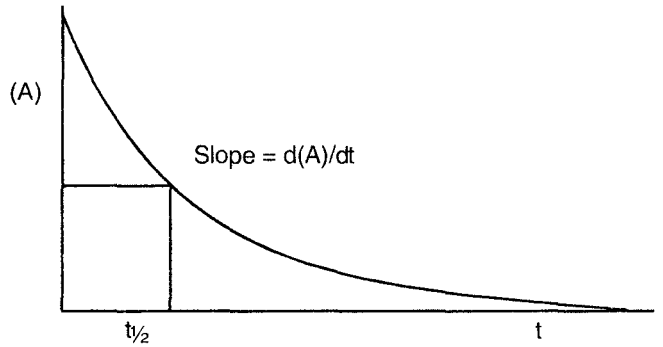


Fig 5.1: First order reaction.

The half time of the reaction is given by, t ½ = 0.693 / Kt.

It is independent of the initial concentration of the substrate.

Second Order Reactions

When the rate of reaction is proportional to the product of the concentration of two reactants or to the second power of a single reactant, the reaction is designated as a second order reaction. Consider a simple reaction,

P + Q = R,

Where P and Q are substrates and R is the product.

In such a reaction,

The rate is designated as +d [R]/ dt, which is given by,

+ d [R]/dt = K [P] [Q]

The square brackets indicate molar concentrations of the substrates; P and Q while k is the second order rate constant. Alternately the reaction might proceed in the form given as,

2P ⟶ R,

In case of which, the rate of reaction will be proportional to the product of the molar concentration of two reacting molecules and will be given by,

+ d[R]/ dt = K [P] [P]

The dimensions of such a reaction are 1/ [concentration x time].

Integrating the second order rate equation we get,

$$t = \frac{2.303}{K(P_0)-(Q_0)} \log \frac{(Q_0)(P)}{(P_0)}$$

Here $[P_0]$ and $[Q_0]$ are the initial concentrations of substrates and [P] and [Q] are the concentrations at time t.

Reactions in which the initial concentrations of the reactants are equal have a half time equal to 1/Cok where Co is the initial concentration of reactants and k is the second order constant.

In case of the apparent or pseudo first order reaction, where the concentration of one of the reactant is very low and that of the second is very high, the rate is nearly proportional to the concentration of only one reactant which is present in low concentration.

Third order reaction

These types of reactions are generally rare. But here the rate of the reaction is proportional to the product of three concentration terms.

It is not necessary that the reaction be purely of a particular order, in fact most reactions are of a mixed order.

Chemical Reactions In The Living Systems

Living systems operate at constant temperature [isothermal condition] and cannot convert heat energy directly into work. There are three terms that are often mentioned in relation to energy,

Free energy: it is the energy of a system capable of doing work.

Enthalpy: it is the heat energy consumed or released in a system at constant pressure.

Entropy: it is the energy associated with disorder or randomness in a system.

All the mechanical and chemical self operating systems, function energetically according to the equation,

$G = H - T * S$,

Where G = free energy or the energy for work, H = the total heat energy of the system, T = temperature and S = entropy per degree.

Since energy for operating physiological processes is derived from chemical changes, the biochemist is specially interested in the application of the equation mentioned above to the chemical reactions, as it is the only the free energy of these reactions that is useful. In general, reactions take place in the body at approximately constant temperature, due to a balance between energy production and heat loss.

In a reaction,

A + B = C + D + energy,

The energy represented by A + B is greater than that of C + D by the amount of energy given out in the reaction. The system as a whole energetically passes isothermally from A+B to C+D of which the energies and entropies are different. We are interested in the energy change in going from A+B to C+D, especially the free energy change. Suppose the energy states before and after the reactions are represented as,

$G_1 = H_1 - T * S_1$ ..... [1]

And

$G_2 = H_2 - T * S_2$ ..... [2]

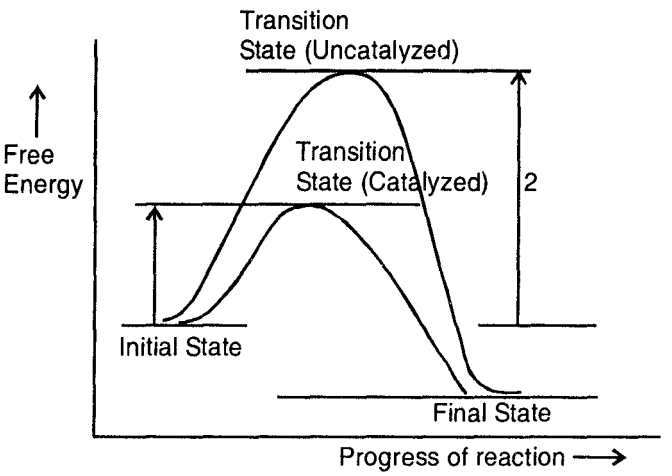
The change in free energy in the reaction will be given by,

$G_2 — G_1 = [H_2 - H_1] - T [S_2 — S_1]$,

Which states that the free energy, given off in a reaction is equal to the heat energy or enthalpy change – the entropy change.

Entropy energy as pointed out is the energy of system, unavailable to do work. Entropy may be considered as a measure of the degree of the disorder of a system, the greater the degree of disorder, the greater the entropy.

The energy given off or absorbed in a chemical reaction is dependent only upon the initial and final state of the reactants and products concerned and is independent of the pathway. Consider the figure,



1. Progress energy of activation (catalyzed)
2. Free energy of activation (uncatalyzed)

Fig 5.2: Diagrammatic representation of energy of activation of a reaction in the presence and absence of a catalyst.

When the reactants in a reaction are present in very low concentration as is often the case in biological reactions, concentrations may be taken as equal to the respective activities and used in the calculations.

It must be remembered that the free energies of reactions vary with concentrations [activities] of the reactants temperature etc., for comparison it is essential to determine free energies under standard conditions. In many biological reactions protons are involved and the free energy of reaction may vary widely with variations in pH. Consequently the pH at which the free energy is determined must be specified if protons are involved.

Reactions in which there is a free energy decrease of the system are called exergonic reactions i.e., free energy flows from the system [capacity to do work decreases]. Conversely reactions in which there is a free energy increase of the system are called endergonic reactions.

One of the chief functions of living organisms is to maintain biochemical reactions in a state removed from equilibrium so that free energy may be provided by the reaction to support the living processes. This is accomplished by the intake and oxidation of food and the excretion of waste products.

Living organisms operate to decrease their entropy meaning that energy generated in the organism is used to control the activity of reactants and products of essential reactions so that entropies represented by the system as a whole are low. [Far removed from equilibrium]. This enables the reaction to proceed with a decrease in free energy and increase in entropy with the performance of work.

The Transition State Of Bio-chemical [Enzymatic] Reactions

A chemical reaction proceeds spontaneously when the free energy of the product-state is lower than that of the reactant- state. The path taken does not affect the free energy of the beginning or ending state but it does affect the rate at which the reaction might occur, depending on the free energy associated with any intermediate state the reactant must go through as it proceeds through the reaction.

Enzymatic reactions involve making and breaking of covalent bonds. In case of a reaction, if new bonds are to be formed and old bonds are broken, a chemical entity exists at an instant in which the old and new bonds might exist at the same instant and such a molecule would have a very large amount of free energy. This molecule would be largely unstable. Such an entity is designated as the transient state. The higher the energy of the transient state in relation to the reactant- state the more it is difficult for the reaction to proceed. The attainment of the transition however makes it effortless to form product or return back to the reactant-state.

The energy required to proceed from the reactant-state to the transition- state is called as the ***activation energy*** or the energy barrier of the reaction. It is equal to the difference in free energy of the two states. The activation energy is an important concept as the height of the energy barrier is can be directly related to the rate of chemical reaction. Consider an enzymatic reaction in which one substrate is transformed into a single product, the reaction might proceed as,

$$E + S \leftrightarrow ES \leftrightarrow ES' \leftrightarrow EP \rightarrow E+P,$$

The formation of ES complex is a result of the collision of free E and S in solution. This is reversible in vivo but under lab conditions, the formation of ES is favored. The high- energy transition ES' once formed, can either revert back to ES or be converted to EP. The formation of product, due to the downhill progress from the free energy point is favored once the ES' is formed, which is similar to the un-catalyzed reaction. Since the energy states of the reactants and products are unaltered, it is clear that the enzymes cannot alter the equilibrium between products and substrates but enzymes and in fact all catalysts speed up the rate at which equilibrium can be established in a chemical system. Or in other words enzymes accelerate the rate of chemical reactions. Given that the substrate is present in ample quantity it is possible to form much more product per unit time in the presence of an enzyme that in its absence. The uncatalyzed reactions proceed at a slower rate, as they have to overcome the energy barrier to reach the transition-state. The reaction proceeds faster if the transition-state can be stabilized and the energy barrier lowered. The enzymes thus accelerate the velocity of chemical reaction by stabilizing the transition-state of the reaction hence lowering the energy barrier that needs to be overcome.

Several mechanisms in the active site of the enzyme are responsible for the stabilization of the transition-state and destabilization of the ground state of the substrate. The actual catalytic process occurring at the active site of the enzyme follows

general organic chemical reaction mechanisms. They involve various factors that include electrophilic and nucleophilic attacks, and general acid-base or concerted acid-base catalysis. This is made possible by the contribution of the side chains of specific amino acid residues anchored in the active site along with the role of coenzymes and the prosthetic groups. The binding of the substrate brings the susceptible groups into close proximity, leading to localized concentration of the groups enhancing the rate of reaction. In addition to the proximity effects, the forces in the active site can align the substrate and reactive groups of enzyme into proper orientation for reaction.

The various physical and chemical interactions like hydrogen bonds, ionic interactions, hydrophobic attraction, van der Waal's forces and covalent or coordinate bonds can lead to distortion within the substrate molecule towards the transition state. This causes a perturbation in the molecular orbitals of the substrate by aligning them with key reactive side chains present in the enzyme active site so that they reach the orbital configurations of the transition-state.

The further steps leading to the transition-state and beyond proceed with less loss of entropy. This also contributes to the rate enhancement by enzymes. To fully understand the rate enhancements and specificity of the chemical reactions catalyzed by the enzymes, consideration of active site structure and its relationship to the structure of the substrate in its ground and transition-state is necessary. Although the active site structure and composition varies with enzymes, some generalizations are possible.

1. The active sites of enzymes are a small part of the total volume of the enzyme molecule.
2. The active site has a particular three-dimensional arrangement, in terms of the amino acid side chains, prosthetic groups and the substrate molecule.
3. In most cases the initial interaction between the enzyme and substrates are non-covalent involving hydrogen bonds or other interactions.
4. The active sites of the enzymes usually occur in clefts in the protein structure. This leads to shielding of the substrate from the bulk solvent, which would reduce the catalytic activity of the enzyme by dilution effects.
5. The specificity of substrate binding depends on well-defined arrangements of atoms in the enzyme active site that compliment the substrate structure.

Theories Of Enzyme Kinetics

[Michaelis Menten Equation]

Various theories have been put forth by scientists to explain the kinetics of enzyme catalyzed reactions. The earliest was put forth by Henri, which was elaborated by Michaelis and Menten. The concept was further modified by Briggs and Haldane.

The mathematical expressions are now derived and explained.

For the sake of simplicity only single substrate- single enzyme and single product system is considered here.

Theoretical Background

It is assumed that catalysis occurs via rapid and reversible formation of a complex [ES] between free enzyme E and substrate S. this complex then breaks down in a slow

step to give the product P and regenerate enzyme E. it is represented as,

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES$$

$$ES \xrightarrow{K_2} E + P$$

Where K_1, K_{-1}, and K_2 represent the rate constants for the individual steps.

The mathematical expression describing the kinetics of this scheme can be derived by making one of the two types of assumptions.

[1] Equilibrium Assumption [rapid state]

The assumptions are based on the Michaelis theory which assumes that,

a. E and S rapidly react to form the ES complex.

b. This ES complex is in equilibrium with free E and S and that this equilibrium is only slightly disturbed by the breakdown of ES to give product.

c. The value of K_2 is lower than K_{-1}

The equilibrium constant K is defined by,

$$K = \frac{[E][S]}{[ES]} \quad \text{or} \quad [ES] = \frac{[E][S]}{K} \qquad \ldots\ldots [1]$$

Where [E] and [S] are the concentrations of free enzyme and free substrate respectively. Since the concentration of S is fairly large essentially all the substrate is free.

At any concentration of S we can evaluate the fraction F of enzyme present as ES as follows,

$$F = \frac{[ES]}{[E] + [ES]} \qquad \ldots\ldots [2]$$

from equations [1] and [2],

$$F = \frac{[E][S]/K}{[E] + \frac{[E][S]}{K}}$$

Therefore, $$F = \frac{[S]}{K + [S]} \qquad \ldots\ldots [3]$$

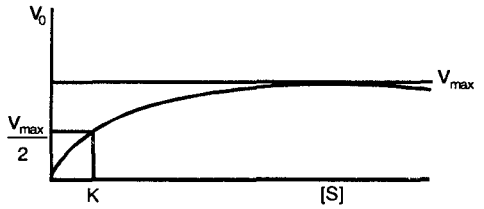


Fig 5.3: Rapid State Equilibrium.

If the total concentration of the enzyme is $[E_0]$, then [ES] = F$[E_0]$,

Thus the equation takes the form,

$$[ES] = \frac{[E_0][S]}{K + [S]}$$

The rate of product formation v is given by, $v = K_2 [ES]$,

Therefore,

$$v = \frac{[K_2][E_0][S]}{K + [S]} \quad \ldots\ldots [4]$$

Sometimes K_2 is replaced by Kcat, which is the first order rate constant.

The graphical representation of the above equation can be shown as,

The maximum rate will be observed when, all the enzyme is in the form of the ES complex. Let the maximum rate be Vmax. This will be equal to K_2 [Eo], then equation can be rewritten as,

$$v = \frac{V\max [S]}{K + [S]} \quad \ldots\ldots [5]$$

The rate of reaction is first order when [S] << K and zero order when [S]>> K

For intermediate values of [S], the reaction is of a fractional order of [S].

[2] The Steady State Assumption

In this approach, it is assumed that ES is in a steady state that is the concentration of ES remains constant because the rate of its formation equals the rate of its breakdown. If the variation of ES with time is examined in a typical experiment a graph of the following type is observed.

After the initial phase [pre-steady state period] the concentration of ES remains fairly constant and thus the steady state assumption can be applied to the equation [1] to evaluate the fraction of enzyme in the form of the ES complex.

This assumption is valid if the rate of change of [ES] is small as compared to the rate of change of [P] and [S]. Under experimental conditions where the [S] is very large as compared to [ES], the value and therefore the change in [ES] is small enough. The steady state assumption states that rate of formation of [ES] is equal to rate of breakdown of [ES], which is mathematically expressed as,

K_1 [E] [S] = K_{-1} [ES] + K_2[ES] ..... [1]

Where, K1 [E] [S] = rate of formation of [ES] while,

K_{-1} [ES] = rate of breakdown of [ES] into [E] and [S], and K_2[ES] = rate of breakdown of [ES] into[E] and [P].

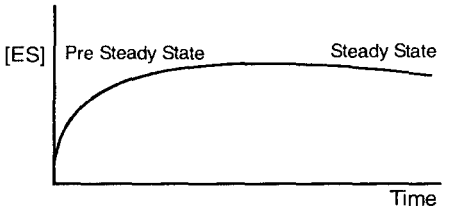


Fig. 5.4: Steady State Equilibrium

The equation [1] can be written as,

$$[ES] = \frac{K_1[E][S]}{K_{-1} + K_2} \quad \text{or} \quad [ES] \text{ X } [K_{-1} + K_2 / K_1] = [E][S] \quad \text{..... [2]}$$

Putting all the constants into a single constant Km, we may write,

Km [ES] = [E] [S] or

$$[ES] = \frac{[E][S]}{Km} \quad \text{..... [3]}$$

If the total concentration of enzyme is [Eo] then,

[Eo] == [E] + [ES] ..... [4]

Therefore, [E] == [Eo] - [ES] ..... [5]

From equations [5] and [3] we can write,

$$[ES] = \frac{\{[E_0] - [ES]\}[S]}{Km} \quad \text{..... [6]}$$

Therefore,

Km [ES] = [Eo][S] – [ES][S]

Or,

Km[ES] + [ES][S] = [Eo][S]

And so,

[ES] [Km +[S]] = [Eo][S]or,

$$[ES] = \frac{[E_0][S]}{Km + [S]} \quad \text{..... [7]}$$

Multiplying both the sides by K_2 we get,

$$K_2[ES] = \frac{K_2[E_0][S]}{Km + [S]} \quad \text{..... [8]}$$

Now the initial velocity, v is given by, $v = K_2[ES]$ while the maximum velocity is given by $Vmax = K_2 [Eo]$. Thus the equation [8] will change to,

$$V = \frac{V\,max[S]}{Km + [S]} \quad \text{.....[9]}$$

This is the Michaelis Menten equation.

The interpretation of the data to calculate the values of Km and Vmax by plotting a graph of v versus [S] is difficult, as exact values cannot be deduced from the hyperbola. Transformation of the data into plots that yield a straight line leads to more accurate determination of the values.

Many attempts were made to transform the data to get a linear graphical representation; three most commonly used methods are briefly discussed here.

Transformations Of The Michaelis Menten Equations And Plots

[1] The Lineweaver Burk equation [double reciprocal plot]:

The Lineweaver Burk equation is obtained by taking the reciprocal of equation [6]. It is written as,

$$1/V = \frac{Km}{V\,max} \times \frac{1}{[S]} + \frac{1}{V\,max} \quad \text{.....[10]}$$

Which represents the equation for a straight line, $y = mx + c$

Where m represents the slope of the line and c is the y intercept. It can be thus drawn that for the straight line graph obtained as per equation 10, the slope will be given by Km/Vmax and the y intercept will be 1/Vmax.[see fig 5.5] From these it is easy to determine the kinetic constants Km and Vmax. The y intercept occurs where the x-intercept is zero. Determination of Km can be done in two ways,

1. Dividing the slope Km/Vmax by the y intercept value, 1/Vmax, directly gives the Km value.
2. Extrapolation of linear fit to the point of intersecting the x-axis gives x-intercept, which is equal to −1/Km, from which Km can be determined by taking the reciprocal of the x intercept.

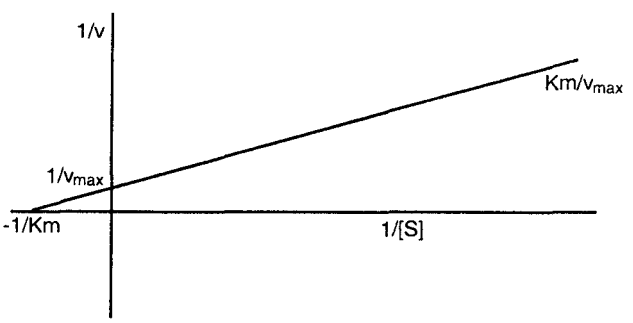


Fig 5.5: Double reciprocal plot.

The data points are not evenly spread on the x-axis because we are taking the reciprocals of the points that were evenly spaced in the un-transformed plots. This makes the points concentrate locally in regions where highest errors of measurement are associated. This is the reason why such plots are criticized. If a system allowing the even spread of x coordinates, is available a better acceptable plot and convenient determination of the kinetic constants would be possible. This is done by generating data points that are evenly spread on the 1/[S] scale.

[2] The Eadie Hofstee Plots

The MM equation can be transformed into the Eadie Hofstee equation as under,

$$v = \frac{V\,max[S]}{Km + [S]}$$

Multiplying both the sides by Km + [S],

v (Km + [S]) = Vmax [S] or,

v Km + V [S] = Vmax [S]

Dividing both the sides by [S], we get,

$$\frac{vKm + v[S]}{[S]} = Vmax,$$

Or v = –Km v/[S] + Vmax ..... [11]

Which is of the form, y = mx + c.

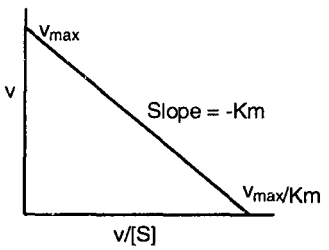


Fig.5.6: Eadie Hofstee plot for determination of Km.

In the Eadie Hofstee plots therefore when v is plotted against v/[S], a straight line is obtained with the slope –m. The y- intercept gives the Vmax.

[3] The Hanes –Woolf Plot

The Hanes Woolf plot utilizes a graph obtained by plotting [S]/v against [S]. The equation for this can be derived as follows,

$$\frac{1}{v} = \frac{Km}{V\,max} \times \frac{1}{[S]} + \frac{1}{V\,max}$$

Multiplying both the sides by [S], we get,

[S]/v = [1/Vmax] × [S] + Km/Vmax ..... [12]

This also represents a straight- line equation y = mx + c, and the plot yields a straight line [fig 5.7] The slope of the line is 1/Vmax and the y intercept yields Km/Vmax while the x intercept is -Km.

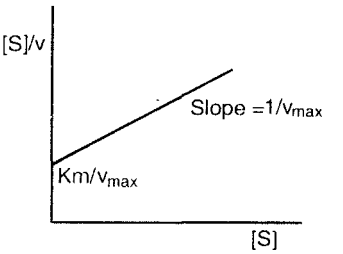


Fig. 5.7 The Hanes - Woolf Plot

[4] Eisenthal And Cornish-Bowden Plot

The Eisenthal and Cornish-Bowden are direct linear plots that enable error-free kinetic estimations. In this method a line represents each observation. The lines are plotted from the substrate concentration values on X axis to the corresponding observed value on Y axis. A series of such lines will intercept at a single point. The coordinates of this point indicate Vmax on Y axis and Km on X axis. In experimental observation however there may be scattering of lines leading to more than one intercepts. In this case the median value of the different intercepts may be used to determine the X and Y coordinates. A typical Eisenthal and Cornish-Bowden plot is represented asin Fig. 5.7 [a].

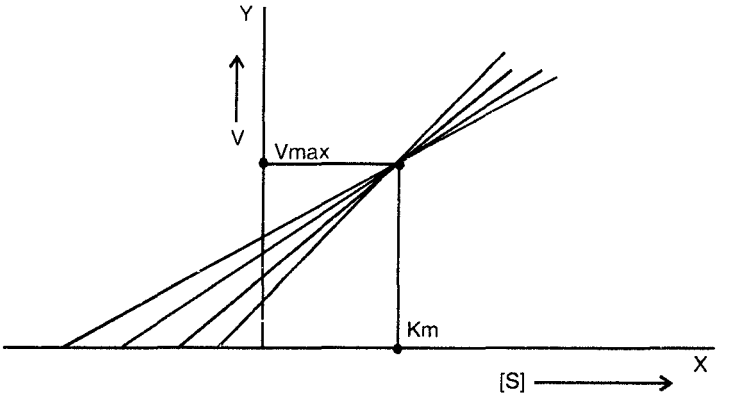


Fig 5.7 a: Eisenthal and Cornish-Bowden plot

[5] Hill Plot

The hill equation is used to determine Km and Vmax of those enzymes that follow sigmoidal kinetics and deviate from the classical Michaelis Menten kinetics. These enzymes show positive and negative cooperativity and therefore the saturation kinetics are expressed by the Hill equation written as,

$$\text{Log } v/\ Vmax - v = n \log [S] - \log K$$

Where, v = velocity of reaction, Vmax = Maximum velocity, [S] = substrate concentration and K = complex constant.

A typical Hill plot can be represented as in Fig. 5.7 [b].

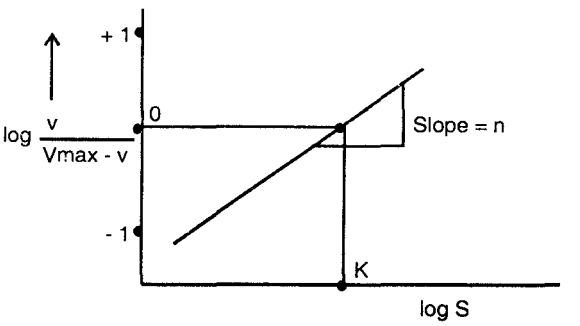


Fig 5.7 b : Hill Plot

The Significance Of Km And Vmax

The Km and Vmax are characteristic for a given enzyme- substrate interaction. The Km is often defined as the concentration of the substrate, required to attain half-maximal velocity. In other words it is the concentration at which half of the active sites present in the solution are saturated. It predicts the affinity between the enzyme and the substrate, the lower the Km higher is the affinity and vice versa. The studies and determination of Km and Vmax enable us to understand the effect of modulators on the enzyme- catalyzed reactions. They are especially useful in the diagnosis of the type of inhibitor and inhibition.

Effect Of Different Parameters On Enzyme Catalyzed Reactions

The enzymatic reactions occur within a limited range of the set of parameters and are greatly affected by alterations in them. Although the effects vary with the enzymes, as well as the parameter concerned, some generalizations are possible. Temperature, pH, concentration of substrate, concentration of enzyme, activators and inhibitors are some of the parameters that drastically alter the rate of reaction. They are briefly described here.

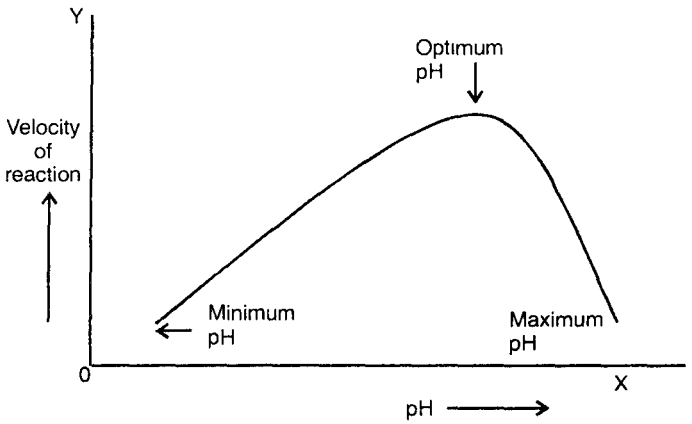


Fig. 5.8: Effect of pH on enzyme catalyzed reaction.

Effect Of pH

The pH of an enzyme-catalyzed reaction can affect the overall catalytic activity in a number of ways. Since most enzymes are proteins having a characteristic native conformation as a result of the interactions of the amino acid side-chains and the peptide bonds, they undergo denaturation at extremely high or low pH values. The range of pH values in which a particular enzyme might remain active shall vary from enzyme to enzyme. It is generally accepted that most enzymes are stable and active at the pH 7.4 but many enzymes show different pH optima in vitro showing much higher or lower values than the physiological pH. An enzyme may remain active over a comparatively broad range of pH values with a difference of around 4 to 5 units of pH, but within this range the velocity of enzymatic reaction varies greatly with pH. A typical pH dependent curve of an enzymatic reaction within a pH range in which denaturation of the enzyme is not a major factor shows that the range of pH values over which the catalytic efficiency is maximized is narrow. Maintaining the pH at optimum levels for catalysis, the reaction mixture is required to be buffered. The choice of buffer should be such that the pK of a component of the buffer should be near the desired pH value. Even within a narrow range of the desired pH there may be a number of different buffers that can be used. A typical pH dependent curve[fig 5.8] can be shown as follows,

The pH effects on the activity of the enzyme are of practical importance in optimizing

the assay conditions. They also give important information about the ionizable and participatory side-chains of amino acids and also about the acid-base relations within the enzyme molecules that are important in catalysis. They shed light on the groups that are involved in the enzyme- substrate complex formation. They might also give information about the dissociation of the ES complex and the acid –base groups involved in this catalytic step for formation and release of product [s].

In many enzymes having a hydrophobic interior the pK of the ionizing amino acid side-chains can be altered, as have been indicated in the case of the enzymes and amino acids listed below.

Table 5.1: Alteration in pK values due to hydrophobic surrounding

Enzyme	Amino acid	pK	pKapp.
Lysozyme	Glutamate	3.9	6.5
Papain	Histidine	6.8	3.4
Ribonuclease	Histidine	6.8	5.2
Papain	Cysteine	8.3	4.0

Three terms are often mentioned in relation to the effect of pH on the enzyme-catalyzed reactions.

1. **Minimum pH:** It is defined as the minimum pH to begin enzymatic activity. In other words there is no enzymatic activity below this pH.
2. **Optimum pH:** It is defined as the pH at which the enzyme shows maximum activity.
3. **Maximum pH:** It is the maximum pH tolerated by the enzyme. The activity is far lower than that of the optimum pH. In other words it may be said as the pH after which any increase in the pH value would completely stop the enzymatic reaction.

It must be remembered that each enzyme has a specific value for all these terms.

Effect Of Temperature

The effect of increase in temperature on chemical reactions is such that normally for every 10 degrees rise in temperature, the rate of reaction nearly doubles. Similar increases have been many times noted with enzyme catalyzed reactions. However enzymes might undergo thermal denaturation if the rise in temperature is drastic and more than the tolerance limit of the enzyme protein. Thus with enzymatic reactions the temperature increase is allowable only in a comparative narrow range. The effect of temperature on enzyme catalyzed reactions is such that for a certain narrow range the catalytic activity significantly increases but after a critical temperature there is a steep decline due to the denaturation of the enzyme protein. The studies on the effect of temperature on the enzymatic reactions therefore have practical importance in designing the assays. The general interest would be in measuring the enzymatic activity at the optimum temperature, with minimum denaturation effects and is experimentally convenient. It is thus apparent that most of the enzyme assays are conducted either at 25 Or 37 degrees c. The studies of the effect of temperature changes within the allowable range are important in understanding the changes occurring within the enzyme conformation related to the effects seen on the activity.

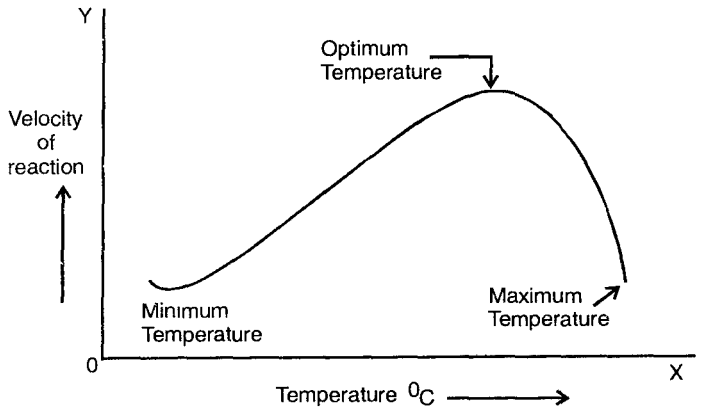


Fig 5.9: Effect of temperature on enzyme catalyzed reactions.

Since the temperature increases can have such dramatic effects on the enzyme catalyzed reactions, it is very important to control temperatures during the experimental measurements of the initial velocity.

This is usually done by equilibrating the reaction mixture and the substrate solution at the temperature near to or at the desired temperature of catalysis. This is done so that the additions do not alter the temperature. In case when the enzyme solution needs to be added to the reaction mixture, it is generally stabilized at 4degrees c. the addition of enzyme is done in such concentrated solution that the desired amount of enzyme is delivered in the minimal volume of the enzyme solution added. The volume is so small as compared to the remaining reaction mixture that it does not alter the temperature of the reaction.

As in case of pH, the three terms are also defined in case of the effect of temperature on the enzyme- catalyzed reactions. They are,

1. **Minimum temperature:** It is the temperature below which there is no enzymatic activity seen.
2. **Optimum temperature:** It is the temperature at which the enzyme shows maximum catalytic activity.
3. **Maximum temperature:** It is the temperature above which a slight increase causes the denaturation and complete stopping of the catalytic activity.

The values for these vary with enzyme to enzyme as also with the source of enzyme. For example, the DNA polymerase of E.coli is active around 37-degree c. But the DNA polymerase of T.aquaticus is active at 98 degrees c.

Effect Of Enzyme Concentration

The effect of enzyme concentration on the enzyme-catalyzed reaction has been studied by various scientists. The results obtained for pepsin are much different than those obtained for trypsin and chymotrypsin. In general it can be said that in the initial stages the rates may be proportional to the concentration of the enzyme added but after a critical concentration there is a steep and sudden decline in the rate of reaction, explained by the fact that the substrate is completely exhausted. A graphical representation shown below might be usually expected to appear as the effect of the enzyme concentration on the enzyme catalyzed reaction.

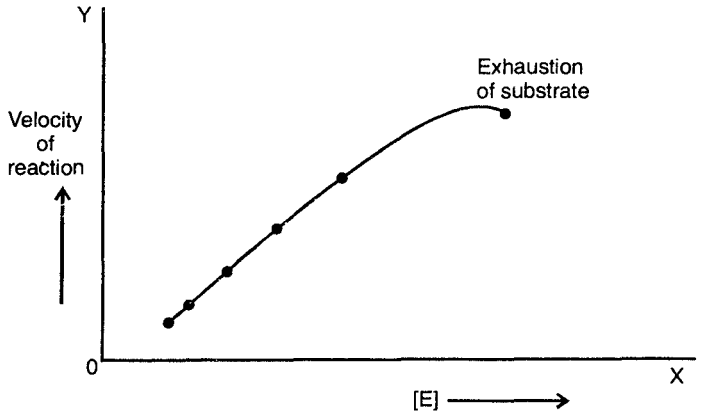


Fig.5.10: Effect of enzyme concentration on rate of enzyme catalyzed reaction.

Effect Of Substrate Concentration

If an enzyme is mixed with its substrate under proper conditions of the reaction to proceed, and the appearance of product and/or disappearance of the substrate are monitored a graphical representation as shown below is seen.

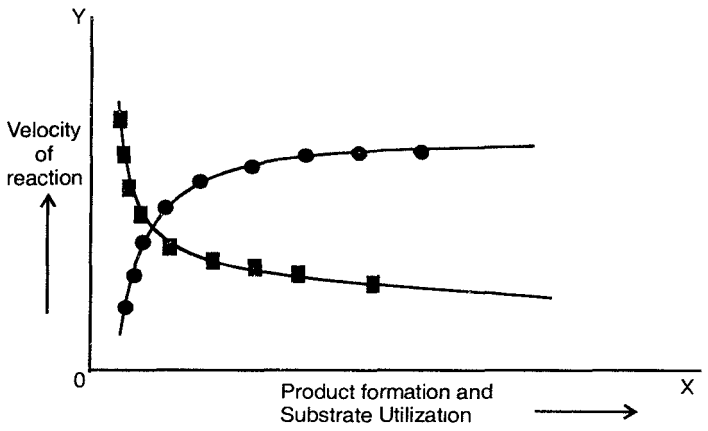


Fig 5.11: Effects of enzymatic reaction, Loss of substrate/ formation of product.

Early in the reaction the loss of substrate and formation of product are rapid but with the passage of time these rates diminish and reach zero when all the substrate is exhausted and converted to product. Such time courses are well explained by the first order kinetic equation,

$$[A] = [Ao]\, e^{-Kt}$$

Where [A] is the substrate concentration remaining at time t, $[A_0]$ is the initial substrate concentration and K is the rate constant for the reaction. In the very early portion of this plot, the increase in production tracks approximately linear with time. For this limited time period the initial velocity $[v_0]$ can be approximated as the slope of the linear plot of concentrations of substrate or product as a function of time.

It is therefore expected the velocity of a first order reaction to depend linearly on the initial substrate concentration. When early studies were performed on the enzyme-catalyzed reactions however, the reactions followed the substrate dependence that resembled a rectangular hyperbola. Three distinct regions of the curve in question can be identified.

1. At low concentration of substrate the rate appears to obey first order kinetics, resulting into a straight line.
2. At intermediate concentrations, it assumes a mixed order form
3. At very high concentrations it shows the kinetics of zero order reactions, or in other words it becomes independent of the substrate concentration.

A qualitative explanation for this behavior was proposed by Brown. He suggested that the enzyme- catalyzed reaction followed the following reaction scheme.

$$E + S \underset{.K_{-1}}{\overset{.K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$

This scheme predicts that the reaction velocity will be proportional to the [ES] and will be given by,

$v = K_2 [ES]$

Under conditions when the enzyme concentration is kept same and the concentration of substrate is varied, at low [S], [ES] is directly proportional to [S]. The rate will follow the first order kinetics. At very high concentrations of [S], all the enzyme shall exist as [ES]. The velocity of reaction under this condition will depend upon the rate of chemical transformation that converts ES to EP and the release of product regenerating free E. addition of any more amount of substrate will not affect this rate and thus a zero order situation shall be obtained. This resembles the rectangular hyperbola as predicted by Brown.

The mathematical expressions have been already derived earlier. The MM equation obtained earlier can be transformed into various form relating to the [S] and the final forms change as follows,

$$v = \frac{V\max[S]}{Km + [S]}$$ MM equation.

Case I: when [S]<< Km,

Km + [S] ~ Km, the equation takes the form, v = Vmax /Km × [S],

Or v is directly proportional to [S].

Case II: When [S] >> Km,

Km + [S] = [S], the equation takes the form, v = Vmax

Case III: When [S] = Km,

The equation takes the form, v = Vmax [S]/2[S], or v = Vmax/ 2.

Thus Km is defined as the substrate concentration at half maximal velocity.

Some enzymes fail to show the classical MM kinetics, that is the rectangular hyperbola, and instead show a sigmoidal curve [fig 5.12] for saturation kinetics. The feature is

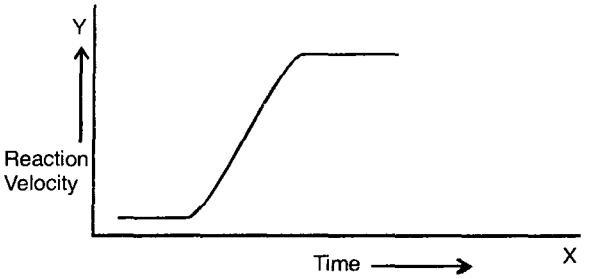


Fig.5.12 Sigmoidal curve shown by oligomeric enzymes.

characteristic of the oligomeric enzymes, that show positive and negative cooperation. However this is not the only cause for deviation from the classical MM kinetics. Some other reasons include.

1. Presence of inhibitors.
2. More than one enzymes acting on the same substrate.
3. Presence of activators.

Quantification of the number of ligand binding sites is possible by use of the Hill equation,

$$\text{Log}\frac{v}{V\,max - v} = h \log [S] - \log K$$

Where h is the number of ligand binding sites on the oligomeric enzyme and is often called as Hill coefficient. K' is the complex constant. The form is similar to the Lineweaver Burk plot.

The transformed plot has the appearance as in fig 5.13.

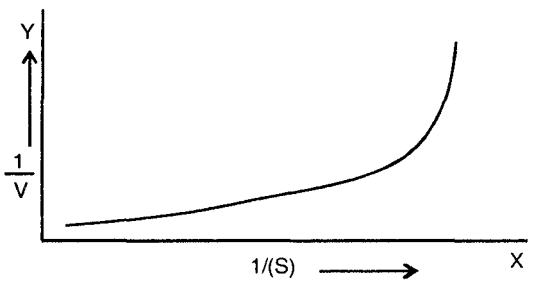


Fig. 5.13: LB transformation of sigmoidal kinetics.

Effect Of Activators

In general, the activity of many enzymes is affected by ionic species. Certain ions are absolutely necessary for the activity of some enzymes, while others are highly toxic to almost all enzymes. Many times an ion that acts as an inhibitor for an enzyme acts as an activator for other. The effects vary even with the concentrations of ion, at some concentration they act as activators and at others they inhibit enzymes. There may be specific requirements by the active sites of certain enzymes and many different mechanisms have been proposed for the role of metals in enzyme catalysis. The topic is discussed in details elsewhere in the book.

Anions in general are unspecific, almost any anion may show similar effects with enzymes. In case of cations however the enzyme is mostly inactive in their absence and the type of cation required by an enzyme is fairly specific. Enzymes may require a single cation or in some cases a multiple cation requirement has been identified. For example, inorganic pyrophosphatase specifically requires Mg++ for its activity, while the enzyme fumarase can be activated by a number of different anions. The cation effects are much more specific than the anion effects. Probably there exists a more fundamental difference between the nature of action of cation and anions, however the picture is not clear today.

About 15 different metal cations are found to work as activators for some or the other enzyme. They include Na+, K+, Rb+, Cs+, Mg++, Ca++, Zn++, Cd++, Cr+++, Cu++, Mn++, Fe++, Co++, Ni++, Al+++ NH4+ and molybdenum. The atomic numbers of these metal components are within a limited range; most of them lie between 19 and 30. The heavy metals having atomic number more than 55 are not known to activate enzymes by themselves.

In case of many oligomeric enzymes, small organic molecules, like cyclic AMP, small biomolecules like citrate, phosphate or even the substrate molecules can act as activators. It has been proposed that most activators help in reducing the activation energy barrier during the formation of the enzyme substrate complex, or in enhancing the binding rate of the substrate with the enzyme active site. In other cases they may enhance the rate of product release from the EP complex.

Effect Of Isotopes

In many enzyme-catalyzed reactions the rate- limiting step is associated with the transfer of a particular group. Using a kinetic isotope containing a heavy atom would slowdown the rate of catalysis, helping in the identification of atoms of the substrate molecule that are undergoing transfer during catalysis by the enzyme. Since proton transfers are most widely occurring reactions, use of heavy isotopes of hydrogen can be easily studied. A possible explanation of the reduction in rate by the use of heavy isotopes is that the heavier isotope, for example deuteron causes an increase in the overall activation energy. In proton transfer reactions it is found that the magnitude of the kinetic isotope effect is influenced by the pKa of the general base group that participates in the transfer step of the proton. The largest kinetic isotope effect occurs when the pKa of the general base is well matched to that of acidic group of the proton donor. If the differences in these values are high, the magnitude, of kinetic isotope effect, decreases.

The studies are very helpful in identification of specific atoms that participate in the rate limiting group transfer step during the catalysis. The approach consists of synthesizing substrate analogs in which specific atoms have been substituted with their heavy isotopes and then comparing the effect on he rate of the reaction till the kinetic isotope effects are observed. The information guides about the groups involved as well as the actual rate limiting step during the mechanism of catalysis by the particular enzyme. Alternately isotopic substitution of the solvent water hydrogen atoms can affect the rate of enzymatic reaction if the solvent itself serves as a proton donor during catalysis. Such effects are called as solvent isotope effects. The equation depends on the number of proton transfers, a linear equation is obtained with a single proton transfer, a quadratic equation is required for two protons while three protons will require a cubic equation.

Effect Of Inhibitors

Enzyme inhibitors have helped in understanding the mechanism and pathways of enzymatic catalysis, their substrate specificity, the composition, and role of specific side chain groups in the active site and the residues responsible for the required three-dimensional structure of the enzymes. They have also helped in understanding the regulatory aspects and the reversible inhibition based mechanisms of control of enzyme activity in vivo.

Inhibition of enzymes is broadly grouped in two types,

Reversible inhibition

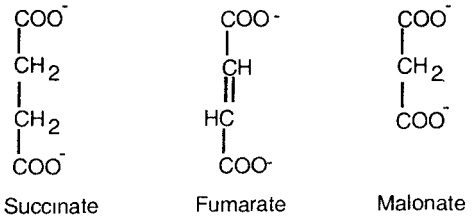
Irreversible inhibition

Reversible Inhibition

Reversible enzyme inhibition is of three types, namely,

Competitive Inhibition

In this type of inhibition the inhibitor is a substrate analog that competes with the normal substrate for binding with the active site. Its effect can be counter balanced by increasing the concentration of the normal substrate thereby increasing the probability of the enzyme active site –substrate interaction. Thus the presence of a competitive inhibitor increases the apparent Km of the enzyme for the substrate. This type of inhibition is easily diagnosed from the LB plots. The classical example of competitive inhibition is the inhibition of succinate dehydrogenase by malonate and other dicarboxylate ions.



Uncompetitive Inhibition

In this type of inhibition the inhibitor does not combine with the free enzyme. It however combines with the ES complex to form an inactive ESI complex. Its effect is not reversed by further addition of the substrate. When diagnosed by the LB plots, a characteristic uncompetitive inhibitor shows parallel lines of the same slope with increase in inhibitor concentration. Such inhibitions are rare in single substrate reactions however are frequently seen in multiple substrate reactions.

Non-Competitive Inhibition

The non-competitive inhibitors bind to a site other than the active site and often change the conformation is such a manner that the formation of ES complex does not occur at normal rates. The EIS complex does not decompose at the normal rate to form products. Increasing substrate concentration does not nullify the effect of this type of inhibitor.

There are two ways, in which this type of inhibition can occur,

a. Binding of reagents with some functional group, outside the active site of the enzyme, which is required to maintain the active three-dimensional conformation of the enzyme molecule.
b. Agents capable of binding essential metal required for enzymatic activity through formation of chelates, for example EDTA can non-competitively inhibit the enzyme.

Irreversible Inhibition

It usually refers to covalent and permanent modification of the enzyme leading to its complete inactivation. The modification occurs at the groups that are actually involved in the catalysis. This type of inhibition is not treated by MM kinetics, as the EI complex formed is irreversible. This type of inhibition is slow and time dependent, in other words it is incomplete at first and increases with time as chemical modification of increasing fraction of the enzyme molecules take place. Such studies have been extensively used to modify and identify the active site residues.

Kinetics And Diagnosis Of Reversible Inhibition Types

1. The general scheme for the competitive inhibitor is,

$$\begin{array}{l} E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P \\ + \\ I \\ \Updownarrow K_i \\ EI \end{array}$$

As seen here the inhibitor can bind only to the free enzyme to form a reversible EI complex and competes with the substrate. From the MM derivation we have,

$$[ES] = \frac{[E][S]}{Km} \quad \ldots\ldots [1]$$

Similarly for the inhibitor we can write,

$$[EI] = \frac{[E][I]}{Ki} \quad \ldots\ldots [2]$$

Let the total enzyme be [Et], which will be given by,

$[Et] = [E] + [ES] + [EI]$ ..... [3]

$$= [E] + \frac{[E][S]}{Km} + \frac{[E][I]}{Ki}$$

$$= [E]\,[\,1 + [S]/Km + [I]/Ki]$$

Therefore,

$$[E] = \frac{[Et]}{1 + [S]/\,Km + [I]/Ki} \quad \ldots\ldots [4]$$

From equation [1] and [4], we have,

$$[ES] = \frac{[Et]}{1 + [S]/Km + [I]/Ki} \times \frac{[S]}{Km}$$

$$= \frac{[Et][S]}{Km + [S] + Km([I]/Ki)}$$

$$[ES] = \frac{[Et][S]}{Km + [S] + Km[I]/Ki} \quad \text{..... [5]}$$

Multiplying both the sides by K_2 we get,

$$K_2[ES] = \frac{K_2[Et][S]}{[S] + Km\left[\frac{1+[I]}{Ki}\right]} \quad \text{..... [6]}$$

But from MM equation derivation we have,

K_2 [ES] = v or initial velocity ..... [7]

And K_2 [Et] = Vmax or maximal velocity ..... [8]

Thus from equations [6], [7] and [8] we have,

$$v = \frac{V\,max[S]}{[S] + Km(1+[I]/Ki)} \quad \text{..... [9]}$$

This is the equation for competitive inhibition. When transformed into LB equation it takes the form,

$$1/v = \frac{Km(1+[I]/Ki}{V\,max} \times \frac{1}{[S]} + \frac{1}{V\,max} \quad \text{..... [10]}$$

The LB plot appears as follows,

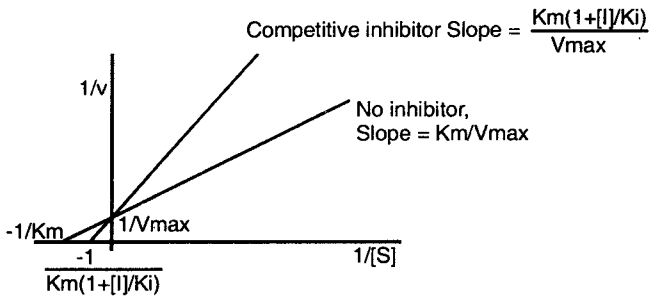


Fig.5.14: LB plots for the competitive inhibitor

-1/Km and −1/Km [1+[I]/Ki] are the intercepts of the straight line in absence and presence of a competitive inhibitor respectively.

2. The general scheme for an un-competitive inhibitor is as,

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$

$$ES + I \rightleftharpoons ESI \quad (K_i)$$

An un-competitive inhibitor binds only with the ES complex. It does not bind to free enzyme. So we can define the quantities [ES] and [ESI] mathematically as,

$$[ES]=\frac{[E][S]}{Km} \quad \text{..... [1]}$$

And,

$$[ESI]=\frac{[ES][I]}{Ki} \quad \text{..... [2]}$$

At any time the total enzyme concentration [Et] can be given by,

$$[Et] = [E] + [ES] + [ESI] \quad \text{..... [3]}$$

$$[Et]=[E]+\frac{[E][S]}{Km}+\frac{[ES][I]}{Ki} \quad \text{..... [4]}$$

From equation [2], [3] and [4], we have,

$$[Et]=[E]+\frac{[E][S]}{Km}+\frac{[E][S]}{Km}\times\frac{[I]}{Ki}$$

$$= [E]\,[1+ [S]/Km + [S]/Km \times [I]/ Ki] \quad \text{..... [5]}$$

$$[E] =\frac{Et}{1+[S]/Km\left[1+[I]/Ki\right]} \quad \text{..... [6]}$$

Putting the value of [E] from equation [5] in equation [1], we get,

$$[ES]=\frac{[Et]}{1+\frac{[S]}{Km}+\frac{[S]}{Km}\times\frac{[I]}{Ki}}\times\frac{[S]}{Km} \quad \text{..... [7]}$$

$$[ES]=\frac{[Et][S]}{\left\{\left(1+\frac{[S]}{Km}\right)\left(1+\frac{[I]}{Ki}\right)\right\}Km}$$

$$[ES]=\frac{[Et][S]}{Km+[S]\left(1+\frac{[I]}{Ki}\right)}$$

Multiplying both sides by K_2, we get,

$$K_2[ES]=\frac{K_2[Et][S]}{Km+[S]\left(1+\frac{[I]}{Ki}\right)} \quad \text{..... [8]}$$

But, $K_2[ES]=V$ and $K_2[Et] = Vmax$,

$$v = \frac{V\,max[S]}{Km + [S]\left[1 + \frac{[I]}{Ki}\right]} \quad \text{.....(9)}$$

Dividing numerator and denominator by [1+[I]/Ki], it becomes,

$$v = \frac{\frac{V\,max[s]}{1+\frac{[I]}{Ki}}}{\frac{Km}{1+\frac{[I]}{Ki}} + [S]} \quad \text{.....[10]}$$

This is the MM equation for uncompetitive inhibitor, transforming it to the LB form we get,

$$1/v = \frac{Km}{V\,max} \times \frac{1}{[S]} + \frac{1}{V\,max}\left(\frac{1+[I]}{Ki}\right)$$

The graphical representation of this is given as,

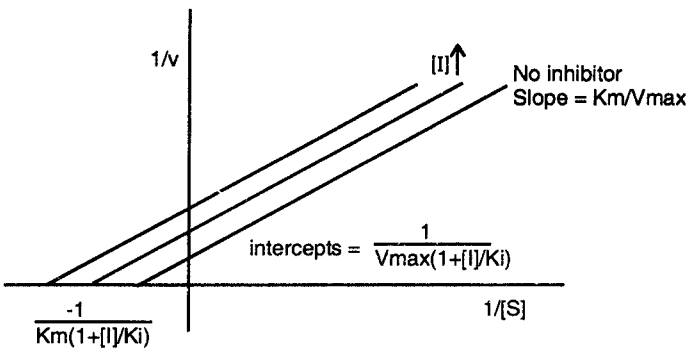
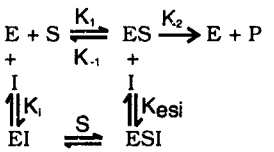


Fig.5.15 LB plots for uncompetitive inhibitors.

The classical example of this type is the inhibition of acetylcholinesterase by Pi.

3. The general scheme for non-competitive inhibitor is as,



A non-competitive inhibitor is a compound that can reversibly bind with the free enzyme as well as the ES complex. We can define three quantities ES, EI and ESI in terms of mathematical expressions as,

$$[ES] = \frac{[E][S]}{Km} \quad \text{..... [1]}$$

$$[EI] = \frac{[E][I]}{Ki} \quad \text{..... [2]}$$

$$[ESI] = \frac{[ES][I]}{Ki} \quad \text{..... [3]}$$

At any instant the total enzyme concentration is given by,

$[Et] = [E] + [ES] + [EI] + [ESI]$ ..... [4]

From equations, [1] to [4], we have,

$$[Et] = [E] + \frac{[E][S]}{Km} + \frac{[E][I]}{Ki} + \frac{[ES][I]}{Ki} \quad \text{..... [5]}$$

From equations, [5] and [3], we have,

$$[Et] = [E] + \frac{[E][S]}{Km} + \frac{[E][I]}{Ki} + \frac{[E][S][I]}{KmKi} \quad \text{..... [6]}$$

$= [E]\,[1 + [S]/Km + [I]/Ki + [S]/Km \times [I]/Ki]$ ..... [7]

$= [E]\,[1 + [I]/Ki + [S]/Km\,[1 + [I]/Ki]$ ..... [8]

Therefore the value of [E] can be written as,

$$[E] = \frac{[Et]}{(1 + [I]/Ki) + [S]/Km(1 + [I]/Ki)} \quad \text{..... [9]}$$

From equations [1] and [9],

$$[ES] = \frac{[Et][S]}{\{(1 + [I]/Ki) + [S]/Km(1 + [I]/Ki)\}Km} \quad \text{..... [10]}$$

$$[ES] = \frac{[Et][S]}{Km(1 + [I]/Ki) + [S]/(1 + [I]/Ki)}$$

Or,

$$[ES] = \frac{[Et][S]}{Km + [S]\left(1 + \frac{[I]}{KI}\right)} \quad \text{..... [11]}$$

Multiplying both the sides by K2,

$$K_2[ES] = \frac{K_2[Et][S]}{Km + [S]1 + \frac{[I]}{K}}$$

Substituting the values of v and Vmax, it takes the form,

$$v = \frac{V\max[S]}{(Km + [S](1 + [I]/Ki)} \quad \ldots\ldots [12]$$

Dividing the numerator and denominator by [1+ [I]/Ki] we get,

$$v = \frac{\frac{V\max}{1 + \frac{[I]}{Ki}}[S]}{Km + [S]}$$

This is the final form of the MM equation for non-competitive inhibition. Transforming it to LB equation we have,

$$1/v = \frac{1}{V\max}\left[1 + [I]/Ki\right] + \frac{Km}{V\max}\left[1 + [I]/Ki\right] \times \frac{1}{[S]}$$

The graphical representation for this is shown as,

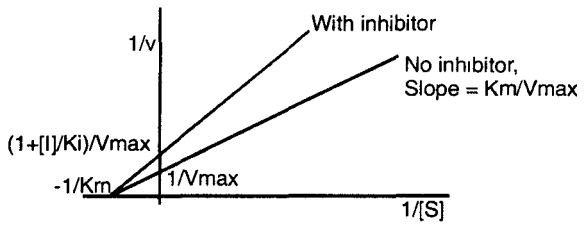


Fig 5.16: LB plot for non-competitive inhibitors.

The summary of the effects of inhibitors on the LB plots can be tabulated as below,

Table 5.2: Comparison between effects of inhibitors as seen in LB plots.

Inhibitor type	Slope	Alteration seen
No inhibitor	Km/Vmax	None
Competitive	Km/Vmax × [1+[I]/Ki]	Km increased
Uncompetitive	Km/Vmax	Both altered by same factor
Non-competitive	Km/Vmax × [1+[I]/Ki]	Vmax reduced

Kinetics Of Bi-substrate Reactions

Many enzymes catalyze the reactions that involve more than one substrate and as expected, these reactions show much more complex behaviors. The most frequently encountered enzymes that catalyze bi-substrate reactions belong to the class *transferases*. These enzymes catalyze the transfer of specific groups, atoms or molecules from one substrate to another. The other class that also involves reactions with two substrates is the *ligases*. They bring about the condensation of two molecules.

The simplest form of a bi-substrate reaction is represented by the equation,

$$A + B \xleftarrow{\text{Enzyme}} P + Q.$$

The transition complexes involved in this type of reaction include binary and ternary complexes such as EA, EB, EP, EQ, EAB, EPQ etc, depending upon the binding and dissociation sequences involved. Under experimental conditions however the approach used to determine the Km and Vmax for these reactions is similar to the single substrate reactions.

The concentration of one substrate, say B is usually fixed at a saturating level while the concentration of the other, say A is varied to determine the effect on the initial velocity. This can determine the KmA [Michaelis constant for substrate A]. Reversing the experimental arrangement can help calculate the Km for the substrate B, or KmB [Michaelis constant for substrate B].

The bisubstrate reactions can be classified into 2 main groups,

1. Single displacement reactions
2. Double displacement reactions

1.Single Displacement Reactions

The characteristic feature of these reactions is the essential presence of both the substrates at the active site of the enzyme to form a ternary complex, for example EAB, where,

E = enzyme, A = substrate 1 and B = substrate 2.

The single displacement reactions are of two types,

a. Random single displacement reactions
b. Ordered single displacement reactions.

These differ from each other in the sequence of binding of the substrates to the enzyme active site.

Random Single Displacement Reactions

In case of the *random single displacement reactions* any one of the substrates can bind initially to the active site and is followed by the remaining substrate. Thus the formation of the ternary complex can take place in either of the ways shown below,

$$E + A \rightleftharpoons EA \qquad \text{OR} \qquad E + B \rightleftharpoons EB$$

$$EA + B \rightleftharpoons EAB \qquad EB + A \rightleftharpoons EAB$$

Where, the reactions of formation of the complexes are reversible. Such types of reactions are shown by many phosphotransferases, for example creatine kinase, that catalyzes the reaction,

$$ATP + \text{Creatine} \xleftarrow{\textit{Creatine kinase}} ADP + \text{Phosphocreatine}$$

In this reaction the binding of both ATP and creatine to the active site is compulsory for the reaction to proceed. The sequence of binding is however random, in the formation of the ternary complex. Even the dissociation of the products is random, they may leave in either sequence. A schematic representation is given as follows,

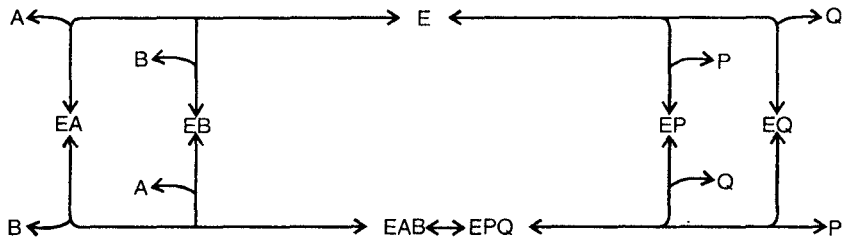


Fig.5.17: Schematic representation of random single displacement reaction.

Ordered Single Displacement Reactions

In the ***ordered single displacement reactions***, there is a compulsory sequence of reaction so that a leading substrate has to bind first followed by the binding of the following substrate at the active site. A schematic representation of the same is shown as,

E + A = EA

EA + B = EAB,

Where, E = enzyme, A = leading substrate, B = following substrate.

A classical example is of the reaction catalyzed by the enzyme malate dehydrogense{NAD dependant} which can be written as,

Malate + NAD+ ═══════ Oxaloacetate + NADH + H+

In this reaction, NAD acts as a leading substrate that forms the E-NAD complex which, then binds to Malate to form the ternary complex, E-NAD-Malate. Kinetic studies, especially on the inhibition of enzymes give important insight to distinguish the random bisubstrate reactions from the ordered bisubstrate reactions. In general, if the last reaction product inhibits the overall reaction by competing with the leading substrate the reaction is an ordered type of reaction. In the reaction given above, excess NADH inhibits the overall reaction by competing with NAD. In some reactions the product appears to result directly from the reaction type EA + B, rather than the formation of the ternary complex. This is due to the rapid vanishing of the same. Such observations are often seen with the NAD-dependant Alcohol dehydrogenase.

A diagrammatic representation of the ordered type of reactions is given as,

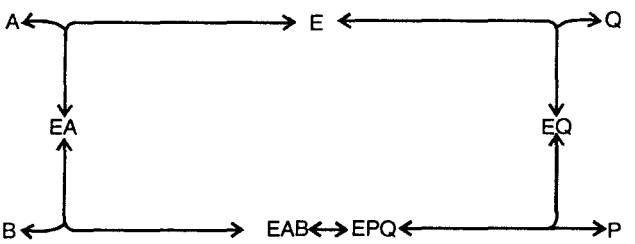


Fig.5.18: Representation of Ordered Single Displacement Reaction.

2. Double Displacement Reactions

The characteristic feature of the reactions of this type is that, **one substrate must bind, get transformed and the product released, before the binding of the**

second substrate, its transformation and release of the second product from the active site of the enzyme. As expected in these reactions, the first substrate binds with the enzyme and covalently modifies it through the donation of a functional group at the active site side-chain residue or the prosthetic group of the enzyme. In the second step, this group is accepted by the second substrate to form the respective product. The transaminases are a wonderful example of this type to illustrate the reaction. There are various transaminases occurring in the liver. We might consider the reaction of alanine transaminase [ALT or SGPT] which, is written as follows,

Glutamate + Pyruvate ⟷ α keto glutarate + Alanine.

In the synthesis of alanine from pyruvate, glutamate acts as a leading substrate and binds to the enzyme transaminase, donating its amino group to the tightly bound prosthetic group, pyridoxal phosphate, converting it into pyridoxamine phosphate. The ketoglutarate formed is then subsequently released before the binding of the second substrate pyruvate. The pyruvate binds to the active site and accepts the amino group to form alanine. The native E-PLP is generated at the end of the reaction.

The schematic representation of this type of mechanism is shown as,

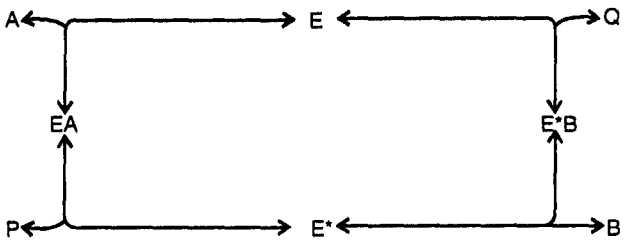


Fig 5.19: Double Displacement Reaction, Schematic Representation.

The mechanism of this type is aptly named as PING-PONG mechanism.

Enzymes Catalyzing Two Reactions Simultaneously

When an enzyme is not absolutely specific, it may act on more than one, related substrates present at the same time. In such conditions, apparently the two substrates compete for the active site due to their partial resemblance. Thus the two reactions can occur simultaneously,

$$E + A \rightleftharpoons EA$$

$$EA \longrightarrow E + P \quad \ldots\ldots [1]$$

$$E + B \rightleftharpoons EB$$

$$EB \longrightarrow E + Q \quad \ldots\ldots [2]$$

Where, E = enzyme, A & B are substrates that are converted into respective products, P & Q.

Considering that the substrates are acting as competitive inhibitors for each other, their rates of breakdown will be given by the equations,

$$va = \frac{Vmax[A]}{[A] + Ka\left(\frac{1+[B]}{Kb}\right)} \quad \ldots\ldots [3]$$

$$vb = \frac{V\max[B]}{[B] + Kb\left(\frac{1+[A]}{Ka}\right)} \quad \text{.....[4]}$$

Where square brackets indicate the concentrations of the substrates, va and vb are the initial velocities when A and B are the substrates, while Ka and Kb are the rates of breakdown constants of A and B respectively.

In any mixture containing concentrations a and b of A and B respectively, the total velocity, vt must lie between the two velocities that would be obtained in case of separate measurements at the same concentrations. Two special cases of interest arise when two substrates are present in equal concentrations. In this case the measurements may be done separately or for the mixture.

Three measurements of the velocity may be used for determining the ratio of affinities of the enzyme for the two substrates. One each for separate substrate and third for the equimolar mixture of both. The mixed substrate method has been more frequently used in semi-qualitative way to determine whether the two reactions are due to the same enzyme. Suppose the rate of transformation of A per hour is 200 micro liters and that of B is 450 micro liters and that for the mixture, an intermediate value is obtained, it is indicative that the reactions are carried out by the same enzyme. Because, if the enzymes would have been different, the rate of transformation would have represented the sum total value of the two independent rates.

A slightly different situation would arise if the substrate contained an impurity, in the form of a related substrate having much higher affinity and at the same time a much lower Vmax than the substrate being tested. Under such conditions a long latent period proportional in duration to the amount of substrate and impurity taken initially is observed. Removal of impurity while re crystallization results in the disappearance of such a lag phase.

Single Substrate- Two Enzyme Reactions

In many reaction mixtures, where the enzyme preparation is not sufficiently pure, a situation may arise in which a single substrate is acted upon by two enzymes. in such a case the disappearance of the substrate is dependent upon the rates of both the enzyme-catalyzed reactions. Theoretical treatments are possible and if we suppose that the Vmax values for these reactions are Vmax1 and Vmax2 respective for enzymes 1 and 2, and that the total initial velocity is vt, it will be given by the equation,

$$vt = \frac{V\max 1}{\frac{1+Km_1}{[S]}} + \frac{V\max 2}{\frac{1+Km_2}{[S]}}$$

The shape of the curve obtained will depend on the relative values of the Km for each reaction. A straight line would indicate that both the Km values are equal. A similar result will be obtained at very low substrate concentration as only the enzyme having a high affinity shall contribute to the reaction. In case the Km values differ and the concentration of the substrate is sufficient, a straight line will not be obtained.

Kinetics Of Allosteric Enzymes

The effects of ligands on the control of activity of enzymes were first observed around 1950s-60s. The discovery and the study of many such enzymes led Monod et.al to formulate certain general principles about feedback inhibition. They are mentioned here,

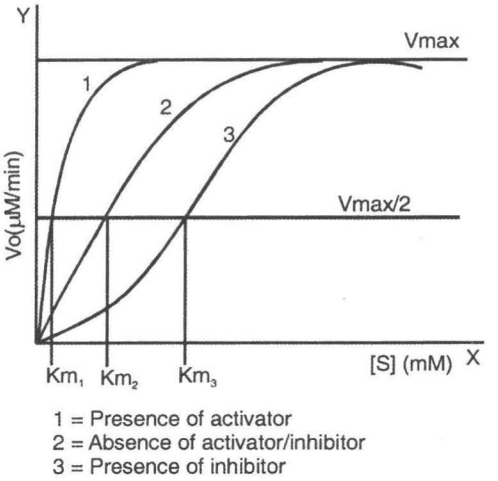


Fig 5.20: Schematic Representation of Activator and Inhibitor on Allosteric Enzyme Activity.

[1] The ligands are usually structurally different from the normal substrate or product of relevant reactions. Thus the effector must bind to a site other than the catalytic site.

[2] Many of these enzymes do not follow simple reversible inhibition shown by competitive, non-competitive or uncompetitive inhibition.

[3] Experimental evidences indicated that desensitizing treatments cause the enzymes to show regular hyperbolic curve. Under normal conditions however these enzymes show a sigmoidal curve. The sigmoidal shape implies that within a certain range of substrate concentration the velocity is more sensitive to [S] than would normally be seen in the case of an enzyme. The plots are schematically represented as,

[4] The binding of the regulator molecule induces a reversible conformational change in the enzyme structure resulting in the alteration of its kinetic properties.

Fig.5.21: Binding of regulator to allosteric enzyme.

Models of Action of Allosteric Enzymes

Several models have been proposed to explain qualitatively the effects and behavior of these enzymes. The models of Hill and Adair are purely mathematical while those of Monod et.al and Koshland et.al are related to the behavior of enzymes or proteins. A brief description of these models is given here.

Symmetry Or Concerted Model

It was proposed by J.Monod and collegues in 1965. The postulates of the hypothesis can be written as,

- The enzymes exist in two states or conformations, active and inactive.
- All the sub-units either exist in the active state or the inactive state.
- The binding of substrate to one of the sub-units increases the probability of transition from inactive to the active state, through conservation of symmetry.

It is diagrammatically represented as,

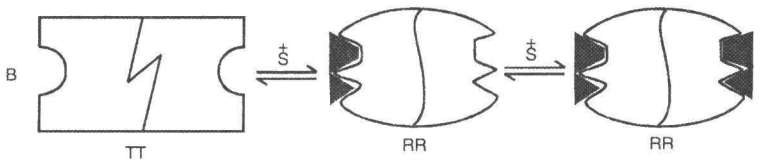


Fig. 5.22: Model of J. Monod et.al

Sequential Model

It was proposed by D.E.Koshland Jr in 1966. The postulates of the hypothesis can be written as,

- The enzyme can exist in only two forms, tense [T] form that has low affinity for the substrate and the relaxed [R] form that has a high affinity for the substrate.
- The binding of a substrate to the first sub-unit induces a T ◊ R transition in the same unit but does not alter the sub-unit.
- However the probability that the binding of the substrate to the substrate to the second sub-unit is increased due to the alteration at the interface of the sub-units.
- There is a possibility of many intermediate transitions in this model than the symmetry model.

It is represented diagrammatically as,

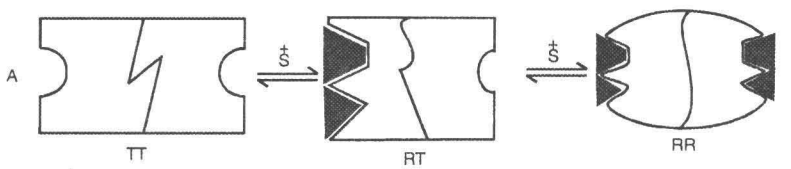


Fig.5.23: Koshland's model for allosteric enzymes

The effects of activators and inhibitors on the allosteric enzymes can be explained as follows,

The allosteric inhibitor preferably binds to the T form and does not allow its conversion to the R form. On the other hand the allosteric activator preferably binds to the R form and increases the binding of the substrate to the enzyme. A diagrammatic representation is shown as follows,

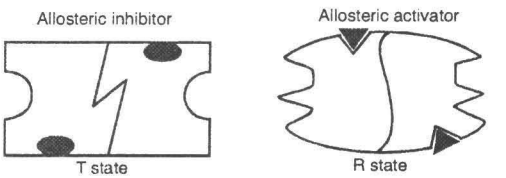


Fig: 5.24: effects of activators and inhibitors [allosteric modulators]

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6

ENZYME ACTION MECHANISMS

Introduction

Factors affecting rate enhancements

- Proximity and Orientation effects
- Acid- Base catalysis
  - Specific acid-base catalysis
  - General acid-base catalysis
    - Covalent catalysis
    - Strain and Distortion effects
    - Changes in environment
    - Proton abstractions
    - Formation of Schiff's base during catalysis

Models of enzyme action

- The three point attachment theory
- The Lock and Key Hypothesis
- The induced fit model
  - Mechanism of action of enzymes
  - Chymotrypsin
  - Fructose-bisphosphate-aldolase
  - Ribonuclease

Introduction

It is now well understood that the enzymes can transform the substrate with very high efficiency and great rate enhancements. In the earlier part of the twentieth century, enzymologists were puzzled by the nature of these transformations and the mechanisms involved in it. Adding to this riddle was the fact that most enzymes are highly specific in nature and bind only with a single substrate or a very limited range of related substrates. The present chapter shall try to elaborate certain attempts made by various scientists to explain the mechanism of action of enzymes.

The mechanism of action of enzymes helps us to understand two fundamental properties of the enzymes, namely, their catalytic power and specificity. In the past few decades, studies on X-ray crystallography and kinetic studies have contributed enormously to our understanding of the mechanisms of enzyme action. The determination of the intermediates as well as chemical modification studies with the amino acid side chains present in the active site have also added to the understanding of enzyme mechanisms.

In solving the problem of mechanism of an enzyme- catalyzed reaction, the determinations of following things are essential,

1. The sequence of the intermediate complexes that are formed during the transformation of substrate to product[s]
2. The rates, at which these complexes are formed, inter-converted or decomposed.
3. The structures and the spatial relationships involved in these complexes.

Factors Affecting Rate Enhancements

Studies of mechanisms in organic chemistry have pointed to various factors that are equally effective in rate enhancements of enzyme catalyzed reactions. These are however not involved in each and every enzymatic reactions. Enzymes may employ different rate enhancement strategies based on the composition of their active sites as well as their interactions with substrates and the overall reaction catalyzed by them.

Some of the factors shall be briefly discussed here. They include

a. Proximity and orientation effects
b. Acid- base catalysis
c. Covalent catalysis
d. Strain and distortion factors
e. Changes in environment
f. Proton abstractions
g. Schiffs base formation

A. Proximity and Orientation Effects

In case of ligases or transferases the enzymes might increase the rates by binding the two substrates so that they come in close proximity of each other resulting into increased possibility of interaction and therefore a fruitful reaction [See Fig 6.1]. Many experiments have been carried out with organic model reactions to assess the effects of proximity on the rate of reactions. T.C. Bruice et.al studied the intra-molecular catalysis of the hydrolysis of mono-phenyl esters of di-carboxylic acids in which the free carboxylate

group functions as a catalyst. The reaction rate was found to be directly proportional to the nearness of the catalyst to the susceptible bond. In one such case the rate enhancement was nearly 53,000 fold. These however cannot be accounted for, by the nearness factor alone. Studies of Storm and Koshland have enabled them to formulate that the active site residues, on binding with the substrates bring about an *orbital steering*, or specific alignment of the catalytic groups towards the susceptible bonds of the substrate. These results in the transformation of the ES binding in catalytically important Transition State that can suitably cross the activation energy barrier.

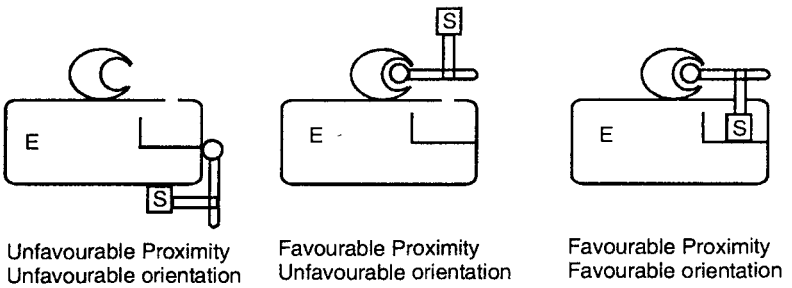


Fig.6.1: Effects of Proximity and Orientation on Rate Enhancements.

Page and Jencks have studied estimates of the effects of orientation and proximity. Their calculations suggest that, each factor can contribute, up to about 10,000-fold increase in the rate of reaction.

B. Acid- Base Catalysis

Enzymes contain a number of amino acid side-chains that can act as proton donors and acceptors during the course of catalysis. Acid-Base catalysis can contribute significantly to rate enhancements during enzymatic catalysis.

There are two broad types of acid base catalysis,

1. specific acid base catalysis
2. general acid base catalysis

1.Specific Acid-Base Catalysis

The specific acid catalysis causes rate enhancement proportional to the proton concentration while the specific base catalysis causes rate enhancement related to concentration of hydroxyl ions. Specific acid base catalysis by concentration of protons and hydroxyl ions is of limited importance in enzymatic reactions.

2. General Acid-Base Catalysis

The general acid catalysis is related to the rate enhancement caused by the role of a general acid as defined by Lowry and Bronsted. Enzyme active sites contain many amino acid side-chains that can act as proton donors within a range of pH values. Similarly the general base catalysis is related to the rate enhancement by the participation of general bases, that can accept protons during the course of catalysis. Many amino acid side chains present in the active site can act as proton acceptors.

In these type of reactions the catalyst at some point in the catalytic mechanism acts as a proton donor or acceptor. Usually the proton transfer involves some carbon of

the transition state complex. Some reactions employ both proton donors and acceptors in the course of catalytic mechanism, a situation termed as *concerted proton transfer.* In such situation the transition-state is related to both, proton donation and acceptance by the respective general acid and general base groups.

Two important factors appear to affect the rate of reaction catalyzed by a general acid or general base.

a. Strength of general acid or base that is its proton dissociation constant.

b. Rate at which the donation or acceptance of proton occurs.

General acid base catalysis provides a means for catalyzing reactions at neutral pH where the concentrations of protons and hydroxyl ions are very low. For example uncatalyzed hydrolysis of peptide bonds requires very high concentrations of protons and hydroxyl ions, high temperatures and long reaction periods. But the same reaction when catalyzed by protease such as chymotrypsin occurs at a neutral pH, physiological temperature and at a rapid rate due to the participation of the general acid-base groups in catalysis.

Some of the general acid base groups in enzymes are tabulated below,

Table 6.1: Amino acid side chains acting as acids and bases.

General acid groups	General base groups
-COOH	-COO
$-NH_3$	$-NH_2$
Phenolic –OH [tyrosine]	Phenolic-O [tyrosine]
Sulfhydril [cysteine]	-S [cysteine]
Imidazole [histidine]	Imidazole [histidine]

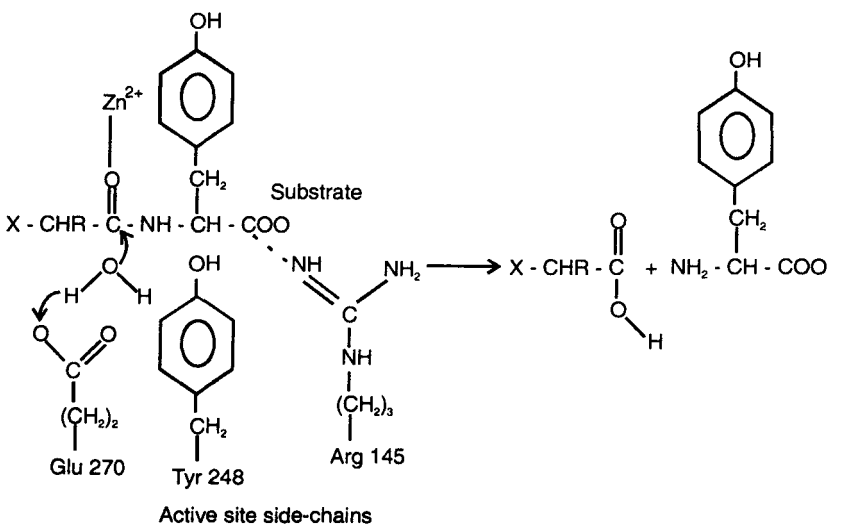


Fig.6.2: Mechanism of acid-base catalysis

The mechanism of general acid-base catalysis can be explained on the basis of action of carboxypeptidase on a synthetic substrate as shown in fig 6.2

The carboxylate of glutamate270 acts as a nucleophile that acts directly or through the activation of a water molecule. The zinc ion acts as a polarizing influence on the carbonyl group and the phenolic hydroxyl group of the side-chain of tyrosine248 acts as a proton donor. The susceptible –NH during the cleavage of the –CO –NH bond in the substrate accepts the proton.

C. Covalent Catalysis

Many enzymes form covalent ES complexes in the course of catalysis. This phenomenon is not restricted to a particular class. Enzymes forming a covalent transient intermediate include, glyceraldehyde phosphate dehydrogenase (1.2.1.12), Acetyl CoA acetyl transferase (2.3.1.9), Glucose-6-phosphatase (3.1.3.9), Fructose bis phosphate aldolase (4.1.2.13), Phosphoglucomutase (5.4.2.2), Succinyl CoA synthetase (6.2.1.4)

Moreover the types of intermediates formed also vary. Some enzymes form acyl-intermediate, while some form phospho-enzymes and yet others form Schiff's base.

Enzymes that form covalent ES intermediates are classified into groups based on the amino acid side chain participating in the formation of the covalent bond. These are mentioned below,

A. Serine class: This includes enzymes like acetylcholinesterase, chymotrypsin, trypsin, phosphoglucomutase, etc. The participating side chain is usually a serine residue located in the active site that may form an acyl derivative as in the case of chymotrypsin, or a phospho derivative as in case of phosphoglucomutase.

B. Cysteine class: This class includes enzymes that form an acyl intermediate through the sulfhydril side chain of cysteine residue located in the active site. The examples include papain, acetyl CoA acetyl transferase.

C. Histidine Class: The class includes enzymes that form intermediates having a phospho-enzyme transition complex. It includes enzymes such as glucose-6-phosphatase and succinyl Co A synthetase.

D. Lysine class: The enzymes belonging to this class include enzymes that form Schiff's base in the course of catalysis. This indicates that a lysine with an active e - amino group is present in the active site constituents. The examples of these enzymes include fructose-bis-phosphate aldolase, transaldolase, and D-amino acid oxidase.

These enzymes show kinetic behavior that resembles bisubstrate reactions i.e. the ping pong mechanism. They contain groups that act as nucleophilic attacking agents on the electrophilic carbon atoms of the substrate. The nucleophilic groups contain electron rich atoms that can donate electrons and are very effective and versatile catalysts. The side chains that probably play an important role in covalent catalysis include histidine imidazole, cysteine sulfhydril, and serine hydroxyl group. In case of some enzymes the prosthetic groups such as CoA, pantothenyl moeity etc. serve the function. A classical example is the transfer of acyl group. The enzyme reacts with the acyl group donor to form an acyl-enzyme intermediate, which in the successive step donates it to an acceptor. The transition complex formation lowers the energy barrier.

Alternately enzymes may use electrophilic catalysis. In this process the intermediates are formed between cationic electrophile of the enzyme and electron rich portion of the substrate molecule. The amino acid side chains are comparatively poor

electrophiles and hence the enzymes use electrodeficient organic coenzymes or ionic entities like cationic metal centers. Some examples are represented in the following table 6.2

Table 6.2: Electrophiles in covalent catalysis

Enzyme	Electrophile
Acetoacetate decarboxylase	Lysine [Schiff's base]
Aldolase	Same as above
Aspartate amino transferase	Pyridoxal phosphate
Carbonic anhydrase	Zn++
L-Malate dehydrogenase	Mn++
Pyruvate decarboxylase	TPP

D. Strain and Distortion Effects

Optical rotation measurements and X-ray crystallographic studies have often indicated that the enzyme structure undergoes considerable alterations during the course of catalysis. Such conformational changes are suspected to contribute in the rate enhancements of enzyme catalyzed reactions by strain and distortion effects. The lyases, hydrolases etc. may involve these effects to at least some extent. The possible mechanism of action can be schematically represented as follows,

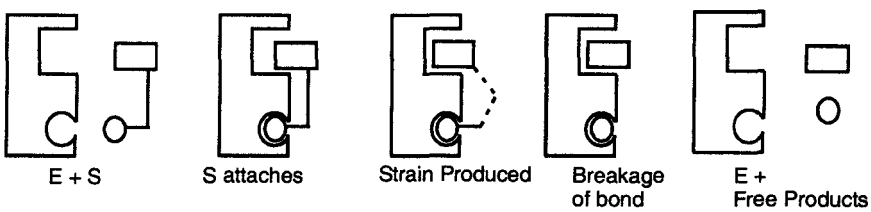


Fig.6.3: Strain and Distortion effects on rate enhancements.

Where, E is free enzyme, ES is the transition state, EP is the complex indicating formation of products by strain and distortion, P1 and P2 are the products. The strain and distortion effects are best explained probably with the induced fit model however the only objection rests with those substrate that have a very high Km values and could be weak comparatively to bring about a change within the enzyme conformation at a very fast rate. The distortion of dihydroxyacetone phosphate after its binding to triose phosphate isomerase as shown by infra red spectroscopy is an excellent example in support of the strain and distortion theory.

A different lookout to the effects of strain and distortion could be the stabilization of the transition ES complex than destabilization of the substrate. It may be thought that the enzyme is suited to form favorable contacts with the transition- state of the substrate than its ground or native form. Experiments are being designed for the use of transition state analogs of the substrate and it has been observed that such a compound binds tightly than the substrate itself with the enzyme.

E. Changes in Environment:

The rates of enzyme catalyzed reactions are affected by the solvents in which they occur. Dipolar aprotic [without proton or hydroxide ions] solvents have been found to be best for nucleophilic displacement reactions.

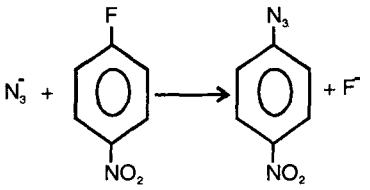


Fig.6.4: Effect of Aprotic Solvent.

The reaction given above [fig 6.4] is best catalyzed when the solvent is aprotic like dimethylsulfoxide or dimethyl formamide. The rate of this reaction in these solvents is about 12000 fold than its rate in water as solvent. The hydrophobic environments present in the cleft of the active site of many enzymes provide a totally different milieu for the reaction to occur and where non-polar substrate or susceptible regions are concerned; such an environment is usually responsible for bringing out the rate enhancements. Usually this is further aided by the properly positioned ionic groups like aspartate side chains that help in stabilization of a positively charged portion to create a fruitful orientation in the active site.

F. Proton Abstractions

During the course of catalysis, especially base catalysis, the reaction proceeds via the phenomenon of proton abstraction. A classical example is of the hydrolysis of the hydrolysis of the phenyl-β-D glucopyranoside.

The catalysis proceeds via abstraction of a proton from the hydroxyl of second carbon followed by the nucleophilic attack of the –O negative group on the first carbon to form an epoxide. This epoxide then undergoes an attack by water to form glucose.

G. Formation of Schiff's Base During Catalysis

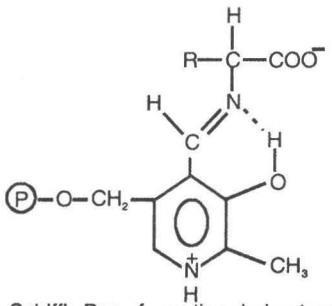
Many enzymes form a Schiff's base during the course of catalysis [see fig 6.5]. The reactions involved in transamination, decarboxylation of amino acids, their racemizations, dehydration of serine, or removal of sulfur from cysteine all involves pyridoxal phosphate as a coenzyme. The binding of amino acids with this coenzyme involves the formation of Schiff's base. The transamination reactions have been studied to a great extent and shall be used here to explain the same.

The pyridoxal phosphate is non-covalently linked and tightly bound to the transaminase protein through the charged ring nitrogen atom. During its catalytic cycle it undergoes reversible transition between its aldehyde form [pyridoxal phosphate] and its amine form [pyridoxamine phosphate].

1. The a amino group of the amino acid binds to the aldehyde carbon of the pyridoxal phosphate moiety by elimination of water molecule to form the aldimine.

2. The aldimine tautomerizes to the corresponding ketimine
3. Both the aldimine and ketimine are Schiff's bases.
4. Hydrolysis liberates the keto acid and leaves behind the amino group attached to the pyridoxal moiety as the pyridoxamine phosphate.
5. The incoming a keto acid binds to the pyridoxamine phosphate forming a ketimine.
6. The ketimine tautomerizes to aldimine.
7. The addition of water molecule liberates the new amino acid and regenerates the pyridoxal phosphate.

A schematic representation of the Schiffs' base formation during the transamination reaction can be given as,



Schiff's Base formation during transamination reaction

Fig. 6.5: Formation of Schiff's base in catalysis.

Models of Enzyme Action

The most intriguing subject in the enzymatic catalysis was the attachment of the substrate to the active site and various scientists have over a considerable period tried to solve this problem. Several hypotheses [models] were put forward out of these, three have gained wide support and these will be briefly discussed here.

A. The Three Point Attachment Theory

During the course of reaction the overall tertiary structure of an enzyme imparts a precise three-dimensional to the enzyme active site. This enables an enzyme to select an exclusive stereo selectivity towards substrate. Consider the example of alcohol dehydrogenase that catalyzes the transfer of hydrogen atoms from alcohol to the coenzyme NAD. [Fig 6.6]

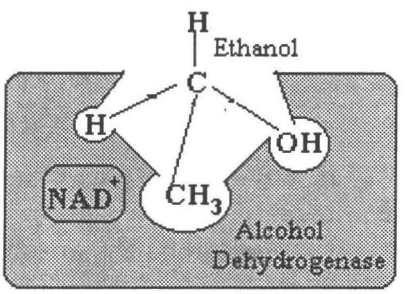


Fig 6.6: Attachment of Ethanol With Enzyme. [The Three Point Attachment Theory].

Since the hydrogen atoms transferred from substrate to the coenzyme are the same in every catalytic cycle, it can be said that the alcohol (substrate) binds with the active site components through specific interactions of its methyl group, hydroxyl group and one of its hydrogen to form a

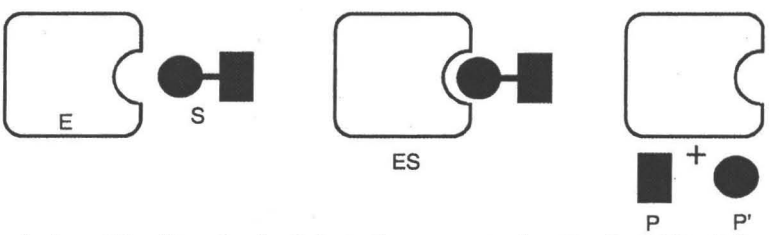
three point attachment with the enzyme active site. (See figure above). The specific hydrogen transferred to NAD is due to the commitment of the enzyme resulting from its proximity to the acceptor coenzyme. The three-point attachment theory is often used to explain the stereo specificity of certain enzymes. A particular case can be mentioned of the L-amino acid oxidases that do not catalyze the oxidation of the D-amino acids.

B. The Lock and Key Hypothesis

The lock and key hypothesis [fig 6.7] was proposed by Emil Fischer in 1898. It is an interesting and important proposition as almost all enzymes act by the formation of the transition ES complex as proposed in the postulates of the model. It is also called as *the template model.* The hypothesis has the following postulates,

a. The enzyme is a large structure possessing a rigid three-dimensional structure that includes a specific cleft in which the substrate can fit. It can be compared to the lock in which the key fits.
b. The substrate is comparatively small rigid structure, which bears a structure that is exactly complementary to the spatial arrangement in the active site of the enzyme. The substrate can be compared with the key.
c. The enzyme and substrate interact with each other to form a transient ES complex, based on the "intermolecular fit concept", which decomposes into free enzyme and product (s).
d. The intermediate is usually unstable and the redistribution of energy is such that it results into lowering of the activation energy barrier.
e. The situation at the active site due to the formation of the ES complex results into rendering certain bonds within the substrate molecule susceptible for breakage leading to its transformation.

A schematic representation of the Lock and Key hypothesis can be shown as follows,



The lock and Key Hypothesis: Schematic representation, E + S → ES → E + P

Fig 6.7: Lock and key Hypothesis.

The hypothesis was successful and greatly accepted due to the following advantages:

1. It explained the absolute specificity of the enzymes
2. The transition complexes mentioned were observed in X ray studies.
3. It explained the orientation effects in rate enhancements of enzyme catalyzed reactions.
4. It explains the stereo specificity of enzymes.

Disadvantages of the Lock and key hypothesis

The Lock and Key hypothesis however failed to explain certain aspects of enzyme catalyzed reactions,

1. Reversibility of an enzyme catalyzed reaction.
2. Reactions involving more than one substrates
3. Group or broad specificity of an enzyme.
4. Difference in Km values of enzymes acting on the same substrate.
5. It does not account for the competitive inhibition by substrate analogs.

C. The Induced Fit Model

The disadvantages of the lock and key hypothesis were overcome by the proposition of the induced fit model. [Fig 6.8] The hypothesis was proposed by Koshland in 1958. While the postulate that the ES complex is formed and results into the formation of product (s) is retained, the concept of rigidity of the active site has been modified.

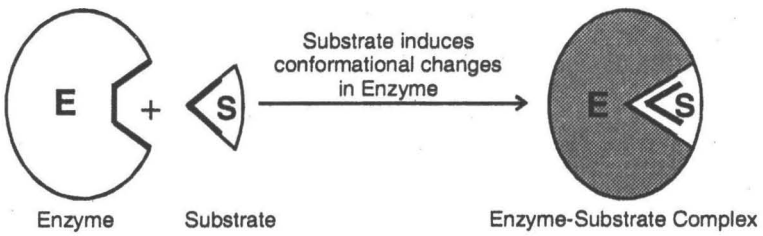


Fig 6.8 Induced Fit Model of Koshland

Koshland postulated that the native enzyme polypeptide has a different conformation that is changed by the nearness of the substrate resulting into an

" Induced-fit" intermolecular interaction. The schematic representation of the model can be shown as above,

The model also helps in understanding of the effect of the competitive inhibitor or substrate analog. When a substrate binds with the enzyme the spatial arrangement is such that the alignment of the concerned groups is most favorable, but when a competitive inhibitor or substrate analog binds, due to its partially different structure the orientation and interactions are not proper. The events taking place in the induced fit mechanism may take the following courses,

1. The substrate may bind to some groups on the polypeptide that cause alteration in the polypeptide chain resulting into proper orientation of the other groups.
2. The nearness of the substrate is sufficient to cause an alteration in the enzyme conformation followed by the binding of the substrate.
3. The binding and the alteration of enzyme conformation may occur simultaneously.

The hypothesis has now gained wide acceptance as it explains almost all phenomena involved in the enzyme-catalyzed reactions, including those interactions that could not be explained on the basis of lock and key hypothesis.

Mechanism of Action of Enzymes
(Chymotrypsin, Fructose bis aldolase and Ribonuclease)

A few representative examples of enzyme mechanisms and how some particular residues participate in enzyme catalysis are considered here.

1. Chymotrypsin

Chymotrypsin is a protease synthesized in the pancreatic acini. The form in which it is synthesized is the zymogen [precursor] called as chymotrypsinogen. Its site of action lies in the small intestine. It has to be activated prior to its action and various proteolytic enzymes such as trypsin and pepsin bring about its activation. The enzyme is broadly specific and can act on proteins, peptides and certain esters and amide compounds containing bulky hydrophobic chains. The enzyme has a molecular weight of about 25000, and can exist in three forms depending upon the composition, namely π, δ & α chymotrypsin.

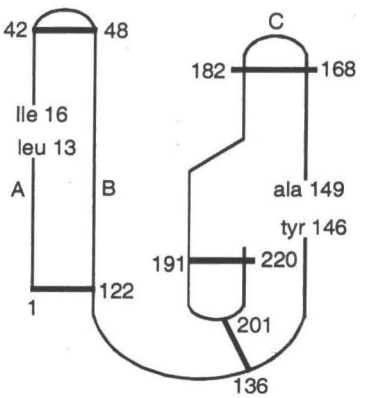
Structure of the Enzyme

There are three chains in the enzyme molecule linked by inter chain disulfide linkages. Intra-chain disulfides also exist. The chain compositions are as follows,

Chain A 1 –13, Chain B 16- 146, Chain C 149- 245.

The five- disulfide bonds are between Cys1 S-S Cys122, Cys42 S-S Cys58, Cys136 S-S Cys201, Cys168 S-S Cys182, and Cys191 S-S Cys220. The molecule exists as an ellipsoid structure with a maximum dimension of 5.1nm. The active site is a depression comprising of three amino acid side-chains, Ser195, His57 and Asp102. They are of crucial importance in the catalytic mechanism. Two short stretches of helix are found between amino acids 164 - 173 and 235 – 245. Both helices are somewhat distorted. The structure also contains considerable amount of anti parallel β sheet. It is schematically represented in fig 6.9.

The N terminal is at amino acid 1 while the C terminal is at amino acid 245. A, B, C represent the chains of the enzyme formed after the activation, while the green color bonds represent the disulfide links.



Structure of α chymotrypsin

Fig. 6.9 Schematic representation of structure of chymotrypsin

Activation of the Zymogen

The activation of the chymotrypsinogen molecule occurs in stages.

1. The enzyme trypsin cleaves the Arg15-Ile16 bond. The product formed is fully active and designated as π chymotrypsin.
2. The activated chymotrypsin can act on the Leu13-Ser14 bond causing its cleavage to form δ chymotrypsin.
3. The δ chymotrypsin forms α chymotrypsin by the cleavage of Tyr146-Thr147 and Asn148-Ala149 bonds.

The alpha chymotrypsin is the most studied form. The comparison between the three-dimensional structures of he active and the inactive forms of the enzyme shows that the Asp102...His57...Ser195 charge relay system is present in both of them and that they occupy almost identical places in the three-dimensional conformation. The zymogen is inactive probably due to the improper formation of the substrate-binding pocket. Cleavage of the residues during activation allows attainment of a structure that enables the proper positioning of the substrate.

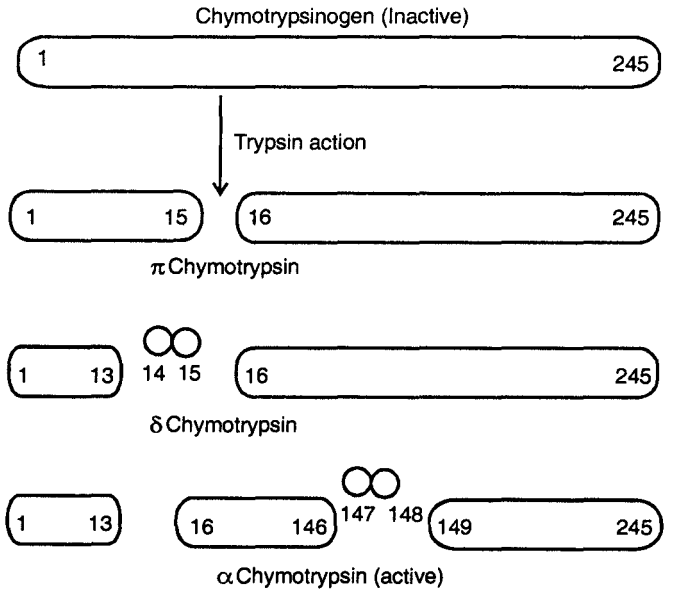
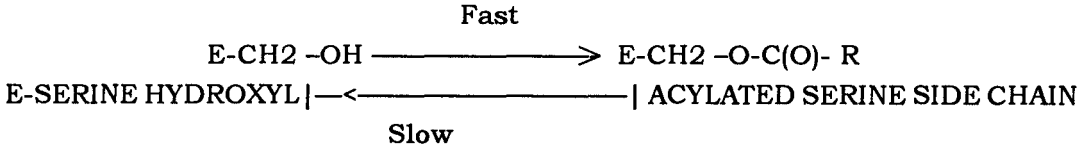


Fig: 6.10 Activation of Chymotrypsin.

Identification of Active Site Residues

Three types of studies were employed in identification of the active site residues involved in the catalytic activity of the enzyme. Evidence generated from the X ray crystallographic studies was compared with the intermediate detection studies and finally the amino acid side-chains were modified by affinity labeling studies that confirmed the roles of the side chains implicated by the X ray studies.

The chymotrypsin catalyzed hydrolysis of esters such as p- nitrophenyl acetate proceeds by formation of an acyl- enzyme intermediate. The rate of deacylation step is pH dependent and can be slowed down to enable the isolation of the intermediate complex. Isolation and characterization showed that the ser195 residue is involved in the transition complex formation.



The formation and decomposition of the acyl enzyme proceeds by the tetrahedral intermediate formation as shown by reactions of organic chemistry. Direct evidences for the existence of tetrahedral intermediates is also available. Studies on related enzymes

like trypsin and papain have shown that such tetrahedral intermediates exist in the course of catalysis.

X- ray crystallographic studies of strong complexes of trypsin with small protein inhibitors from soybean and bovine pancreatic inhibitor indicate that the crucial amide bonds of the inhibitors are distorted to form tetrahedral complexes.

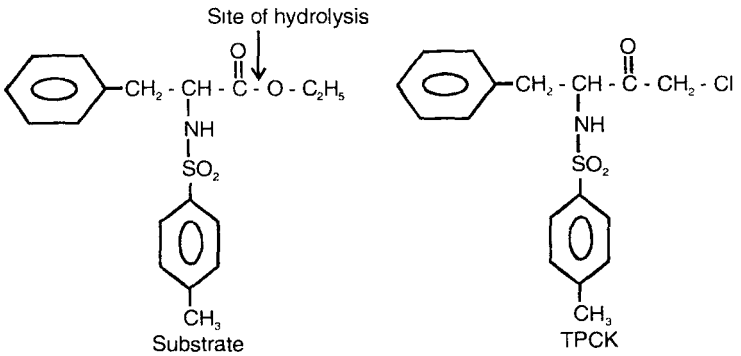


Fig. 6.11 Structure of TPCK

The identification of active site component side chain by covalent modification studies has finally provided the proof about the involvement of the asp102, ser195, his57 and ile16 in the course of catalysis.

The importance of ser195 was deduced from modification of its side chain by using di isopropyl fluoro phosphate. Affinity labeling experiments indicate the involvement of his57 residue in catalysis. The reagent TPCK [N p-Toluene sulfonyl L Phenylalanine Chloromethyl Ketone] used resembles the substrate ester and inactivates the enzyme. [fig 6.11]. Its structural resemblance with the substrate.

TPCK does not inactivate trypsin, which has a substrate-binding pocket that differs from the active site of chymotrypsin. In case of trypsin therefore a different reagent is required. It is designated as TLCK, [N p-Toluene sulfonyl L- Lysine Chloromethyl Ketone] and it does not inactivate chymotrypsin.

The importance of the a amino group of Ile16 in chymotrypsin is suggested by chemical modification studies. This particular amino group is difficult to selectively modify due to the presence of other amino groups in the enzyme. The problem is solved by using a double labeling system. The chymotrypsinogen is first acylated by reacting it with acetic anhydride causing the acylation of all amino groups and then using trypsin to convert it to the delta chymotrypsin. This forms an active enzyme molecule with a free amino group of the Ile16. Labeling it with radioactive acetic anhydride it is found that the activity is lost in proportion to the amount of radioactivity incorporated into the alpha amino group of Ile16. This is indicative of its importance in catalysis.

Binding of the Substrate

The binding of substrate can be recognized by the studies of binding of 'slow substrates' or competitive inhibitors and many such studies have been performed with the serine class proteases. N-formyl tryptophan has been used for chymotrypsin as its amide act as good substrate for the enzyme. The aromatic side chain binds in the hydrophobic cleft. The –N-H of the N formyl group forms a hydrogen bond with the ser214 resulting in the orientation of the position of the susceptible bond near the

ser195 residue. The carbonyl of the peptide may form hydrogen bond with the ser195 or gly193.

It is important to note that the hydrophobic domain is an essential point of recognition that aids in the proper positioning and orientation of the substrate in the active site of the enzyme chymotrypsin. [Fig 6.12] A schematic representation of the binding of the inhibitor is given from which the binding of substrate can be understood.

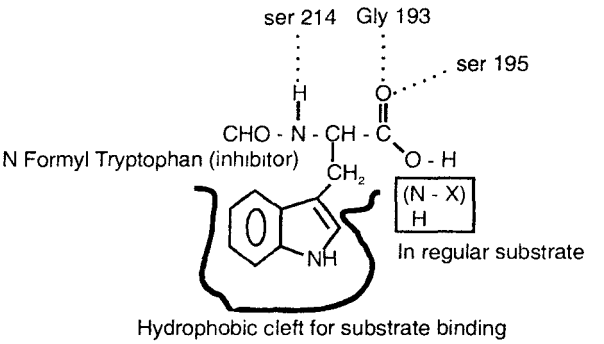


Fig. 6.12 Binding of Substrate/Inhibitor to Chymotrypsin.

Proposed Mechanism of Action of Chymotrypsin

Chymotrypsin catalyses hydrolysis of amides, esters, peptides and proteins. There are two major phases namely acylation and deacylation. [Fig.6.13] The proposed mechanism can be summarized as below,

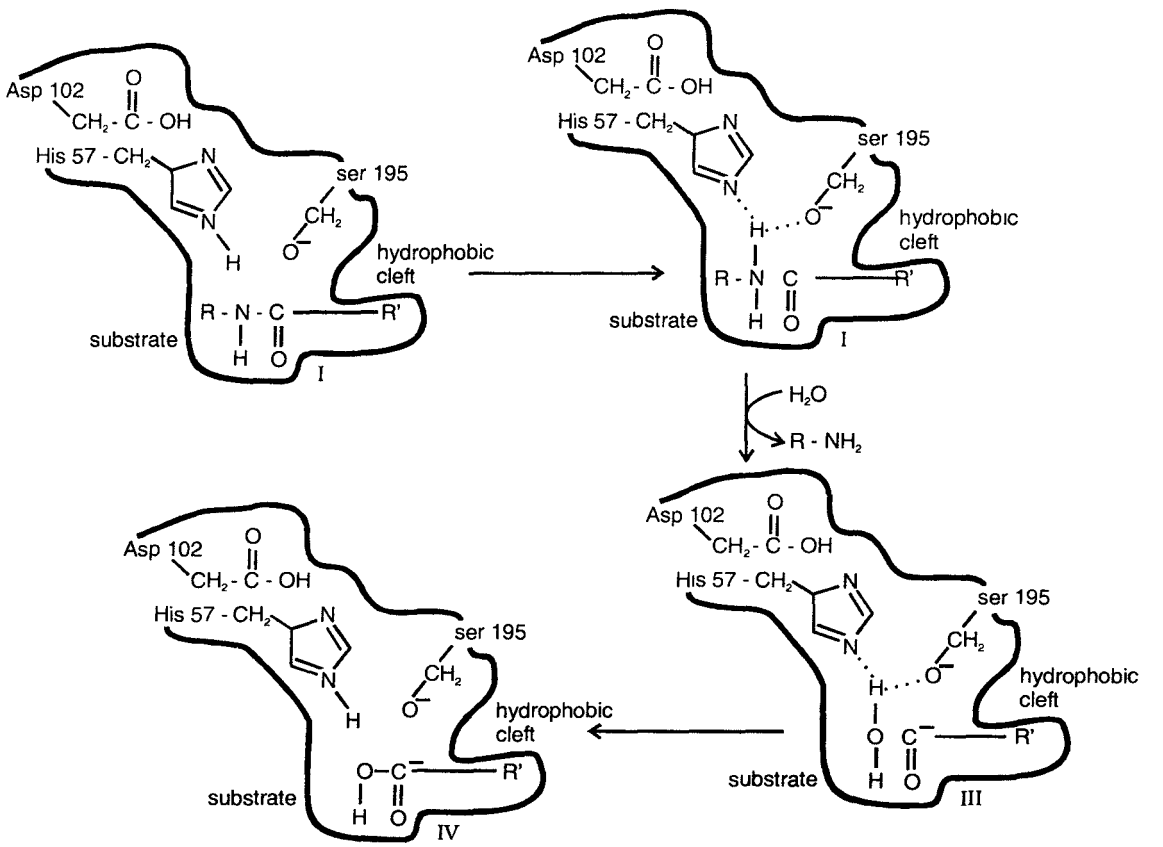


Fig.6.13: Proposed mechanism of action of Chymotrypsin.

1. The catalysis is brought about by a charge relay system involving a catalytic triad comprising of asp102, his57 and ser195.
2. The nucleophile in the acylation phase is the oxygen of the ser195 residue.
3. As the ser195 oxygen attacks, the carbonyl carbon of the susceptible peptide, the hydrogen bonded his57 functions as a general base.
4. It abstracts the serine proton
5. The negatively charged asp102 stabilizes the positive charge that forms on the his57 residue.
6. This prevents the formation of a very unstable positive charge on the ser195 hydroxyl increasing its nucleophilicity.
7. His57 can also act as a proton donor to protonate the amino group in the displaced portion of the substrate.
8. A similar set of proton transfers occurs in the deacylation step.

2. Fructose-bisphosphate-aldolase

The enzyme is located in the cytoplasm and catalyzes the aldol condensation reaction. The reaction is reversible and in vivo; fructose bis phosphate is cleaved into glyceraldehyde-3- phosphate and dihydroxy acetone phosphate. It is an important step in both, glycolysis and gluconeogenesis. There are many forms of the enzyme [table 7.3] depending upon the chain-heterogeneity. There are two broad classes or categories,

Class I: It occurs in higher plants and animals and possesses a lysine side-chain in the active site

Class II: It occurs in fungi and bacteria and has an essential requirement of a metal ion. The enzyme exists in animal tissue in three isoenzyme forms,

Table 6.3: Multiple forms of Fructose bio Phosphate Aldolase

Isoenzyme Occurrence	FBP/F1P affinity ratio
Isoenzyme 1 or C	Brain26
Isoenzyme 2 or A Muscle	50
Isoenzyme 3 or B	Liver 1

The ratio indicates that the isoenzyme 2 or A has a very high affinity for the substrate fructose bis phosphate, the isoenzyme 3 or B has almost equal affinity for both the substrates while the brain isoenzyme has an intermediate affinity ratio. The ability of the liver isoenzyme to use fructose-1-phosphate as a substrate is related to its role in fructose metabolism.

Most of the work on this enzyme has been done on the isolate from rabbit skeletal muscle. There are 5 distinct forms present depending on the association of the sub-units in the tetramer. There are two polypeptide chains namely a & b. these differ in the C-terminal sequence by a single amino acid. a Chain has Asparagine while b chain has Aspartic acid. The enzyme has a molecular weight of about 160,000. The sub-units are arranged in a pair of dimers rather than a tetrahedral complex.

The reaction proceeds via the formation of a ternary complex through an ordered single displacement type and can be represented as,

$$E \overset{+DHAP}{\rightleftharpoons} E^{DHAP} \overset{+G3P}{\rightleftharpoons} E^{DHAP}_{G3P} \rightleftharpoons E^{FBP} \rightleftharpoons E + FBP$$

Identification of Active Site Residues

The intermediates were identified and it was found that the reaction proceeds by the formation of Schiffs' base with Lys227.

The amino acids involved were studied by modification studies. The lys107, lys227 and his359 seem to play an important role in the course of catalysis.

The lys227 was identified by trapping experiments with sodium borohydride and radioactively labeled DHAP mixed with the enzyme. The labeled enzyme showed that the Schiff's base was formed between carbonyl group of DHAP and the amino group of lysine227. Other experiments have suggested that there is a second lysine side chain at the active site of the enzyme. Pyridoxal phosphate inactivates the enzyme by the formation of a Schiff's base. However the residue involved in formation of the Schiff's base is lysine107. It has been proposed that the lys107 side chain is involved in electrostatic interactions with the C6 phosphate of fructose 1,6 bis phosphate. And this results in the positioning of the C2 carbonyl so that it can interact with the lys227 side chain.

Use of N-bromoacetyl ethanolamine phosphate in modification studies has indicated that the reagent inactivates the enzyme by alkylating the his359 residue. This residue may be involved in the proton transfer reactions of E-DHAP complex.

Mechanism of Action of Fructose-bis phosphate Aldolase

From the experimental evidences and x-ray studies, a general outline of the mechanism of the action of the enzyme fructose1, 6 bis aldolase has been proposed as [Fig 6.14],

- The active site environment consists of a positively charged residue X contributed either by arginine55 or lysine146, lys227, his359 and lys107
- The DHAP phosphate is attracted by the X and this orients the carbonyl group of DHAP in the close vicinity of the lys227 side chain amino group.
- The imidazole of his359 accepts a proton and aids in the formation of a Schiff's base in between DHAP and lys227 side chain.
- The carbanion then attacks glyceraldehyde 3 phosphate to form fructose1, 6 bis phosphate. The lys107 residue helps in orientation of the G3P, while the imidazole donates the proton during the formation of the product. The FBP remains bound to the enzyme.
- A mole of water is required to release the FBP and regenerate the ammonium group side chain of lys227.

The hypothesis proposed above has not been proved yet and may require certain modifications due to the following reasons,

1. Chemical modification studies indicate a possible role of cysteine and tyrosine side chains but their exact role is not clear.
2. The mechanism proposed would require a straight, open chain structure of fructose bis phosphate rather than the furanose form, which is the more common form in which it exists in solution.

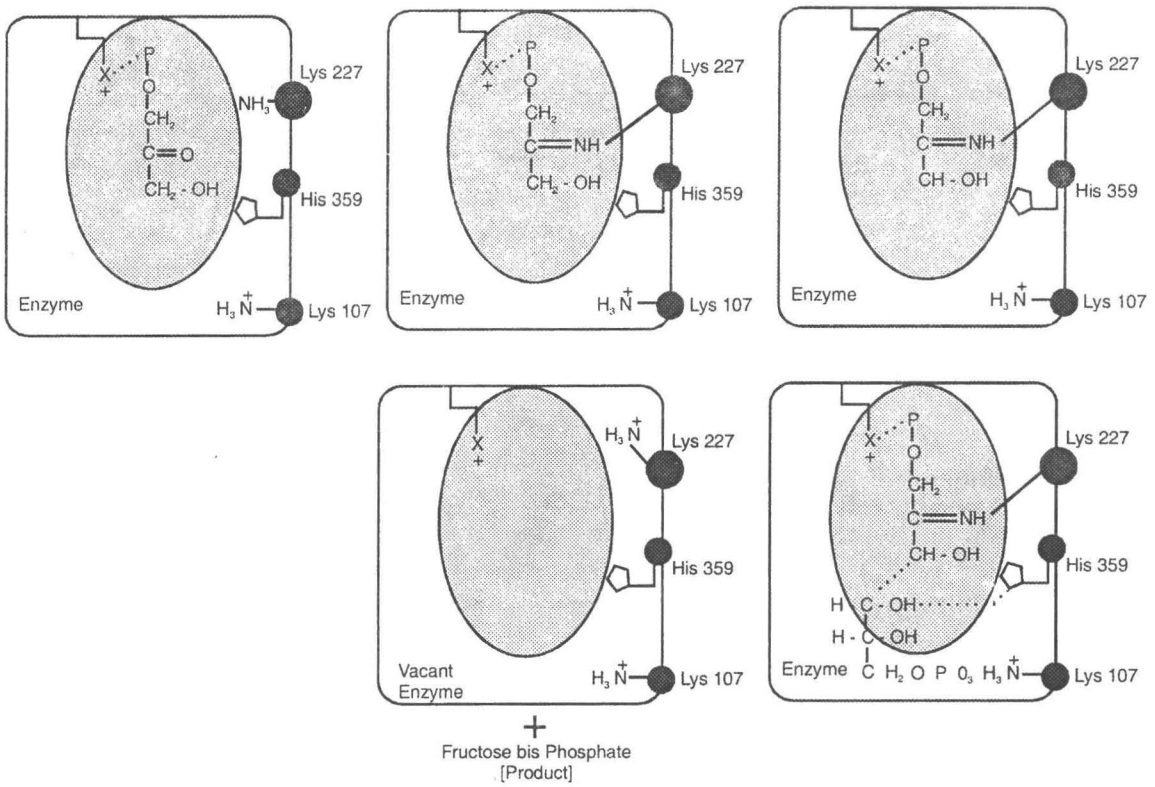


Fig. 6.14: Mechanism of Class I Aldolases.

Mechanism of Class II Fructose bis Aldolase

The class II aldolases are devoid of the lysine chain and exist generally as dimers containing one Zn ion per sub unit. The zinc ion serves to polarize the carbonyl group in a manner similar to the protonated Schiff's base in the class I enzymes. The proton transfer role is performed by Glu or Asp side chains in class II aldolases in a similar manner to the role of the His359 in class I aldolases.

A schematic representation is given as,

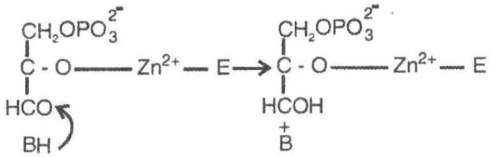


Fig.6.15: Mechanism of class II aldolases.

3. Ribonuclease

The enzyme ribonuclease acts on ribonucleic acid. It is a single polypeptide chain containing 124 amino acid residues, and has a molecular weight of about 13,700. Its structure was worked out by two groups in 1950.

Structure

Most of the work on the enzyme has been done with the bovine pancreatic isolate. It contains 124 amino acids. The N terminal residue is lysine while the C terminal residue is val124. The structure of the bovine ribonuclease can be represented as,

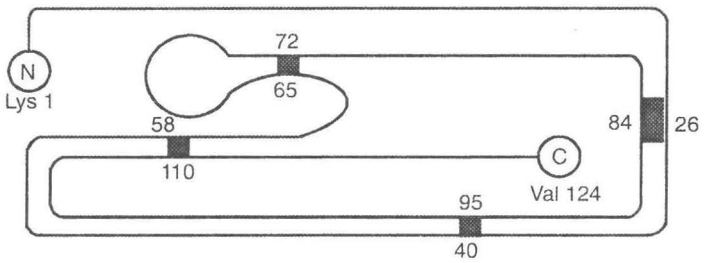


Fig.6.16: Representation of the three dimensional structure of ribonuclease.

Determination of active site residues

The enzyme undergoes hydrolysis by subtilisin under proper conditions to produce a peptide of 20 residues [called as s peptide] by cleavage of the peptide bond between Ala20-Ser21 residues. The peptide remains attached to the larger fragment and this cleaved enzyme remains active. Separation of the s-peptide and s-protein renders both of them inactive.

Replacement of the s- peptide by a synthetic peptide containing 1 to 13 residues same as the s-peptide restores the activity to about 70% on combination with s-protein. But replacement by synthetic peptide containing 1 to 11 residues does not restore any activity. This indicates that his12, met13 or both are involved in catalysis but the residues 14-20 are not directly involved.

Pepsin cleaves the peptide bond between phe120 and asp121 and the enzyme that is devoid of residues 121-124 is inactive. Use of carboxypeptidase to remove val124 does not alter the activity of the enzyme but further cleavage of ser123 reduces the activity to 45%. Further digestion up to removal of phe120 completely inactivates the enzyme.

Carboxymethylation studies have indicated that his12 supports the enzyme activity.

When the enzyme was subjected to carboxymethylation by iodo acetic acid at pH 5.5, the extent of carboxymethylation was proportional to inactivation. The completely inactive molecule contained 1Cm derivative per molecule. Two fractions were obtained; one that constituted 15% of the total carried Cm derivative at his119, while the other carried Cm derivative at the his12. [Cm = carboxymethyl]. Both forms are inactive but neither of them contains 2-Cm residues. Addition of further IAA does not result in the alkylation of the unsubstituted residue. This indicates that both the histidine residues lie in close proximity and act as nucleophiles in the alkylation of each other.

Lyophilization from 50% acetic acid results in the formation of enzyme dimers that have full activity. Mixing of equal proportions of Cm his119 and Cm his12 and lyophilization from acetic acid results in enzyme dimers that show up to 50% of specific activity of unmodified dimers. The activity of the preparation is lost when it is heated at 67deg.C for 10 min.

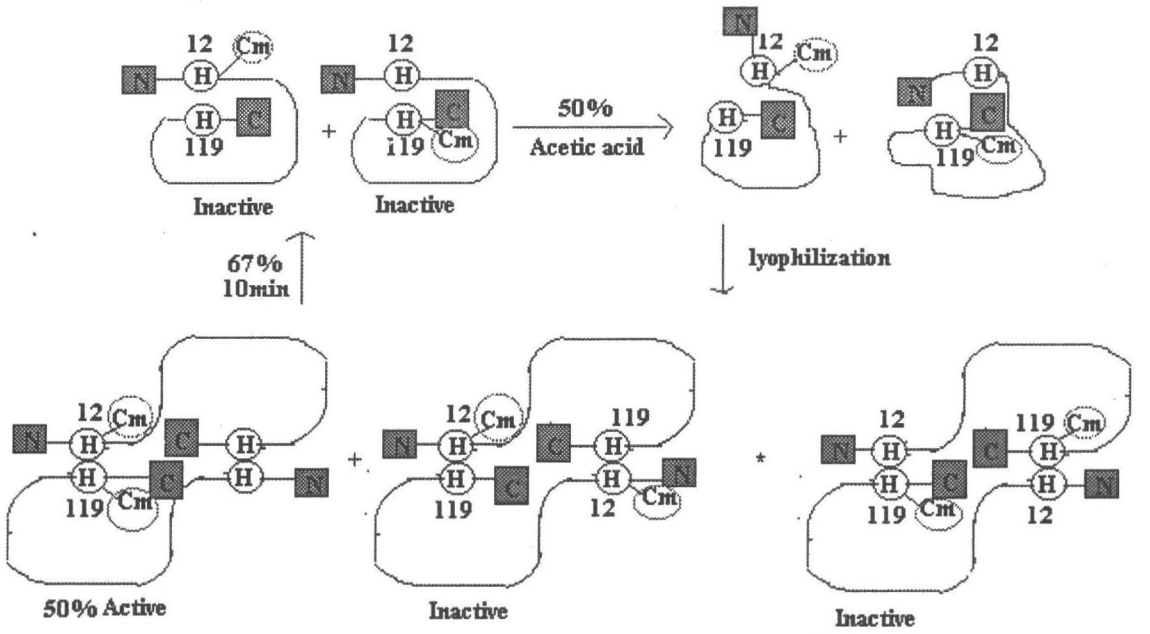


Fig.6.17: Carboxymethylation of Ribonuclease.

The studies with inhibitors have indicated the role of the lys41 residue side chain in the course of catalysis. It is required for proper orientation and positioning of the susceptible group between the his12 and his 119 residues during cleavage of the phosphodiester bond. Examination of the crystal structures in the presence of inhibitors showed that each binds at the same site near the two, histidine residues. The his12 and his119 residues lie in close vicinity and in juxtaposition to the phosphate ester linkage to be hydrolyzed. The purine and pyrimidine rings get specifically localized so that hydrogen bonds are formed between the pyrimidine ring and the hydroxyl and peptide of thr45. The phe120 lies on one side and the val43 on the other and together they form a groove for pyrimidine binding.

Mechanism of action of RNAse:

From the facts and experimental evidences there have been many attempts to propose a mechanism of action of the enzyme but none has attained a full acceptance. It is however clear that

- The his12 and his119 are an essential component of the active site
- Some domains are responsible for the proper orientation of the susceptible bond in the juxtaposition of these residues
- The probable residues involved in the formation of such pocket are phe120-thr45 and val43.
- The his12 and his119 residues act as proton donors and acceptors during the cleavage of the bond.

The lys41 is also required for stabilization of the susceptible bond at the active site. It forms an electrostatic interaction with the phosphate oxygen.

The RNA binds at the active site so that the susceptible phosphodiester bond is so oriented that one oxygen atom of the phosphodiester is attracted towards the imidazole NH of protonated his119, and other towards the lys41. The contributing bases are oriented such that the pyrimidine is placed in the cleft formed by phe120—thr45—val43 and the 2'OH of the sugar of the pyrimidine forms a hydrogen bond with the imidazole N: of his12.

This leads to the protonation of his12 and formation of a 3'-2' cyclic phospho diester bond in the sugar residue with a simultaneous donation of hydrogen by protonated his119 to the $-CH_2-O-$ group of the purine, cleaving the susceptible bond.

Water from the solution then attacks the cyclic phosphodiester leading to re acceptance of donated proton by the his119 and donation of proton by protonated his12 imidazole to form native enzyme and release of products.

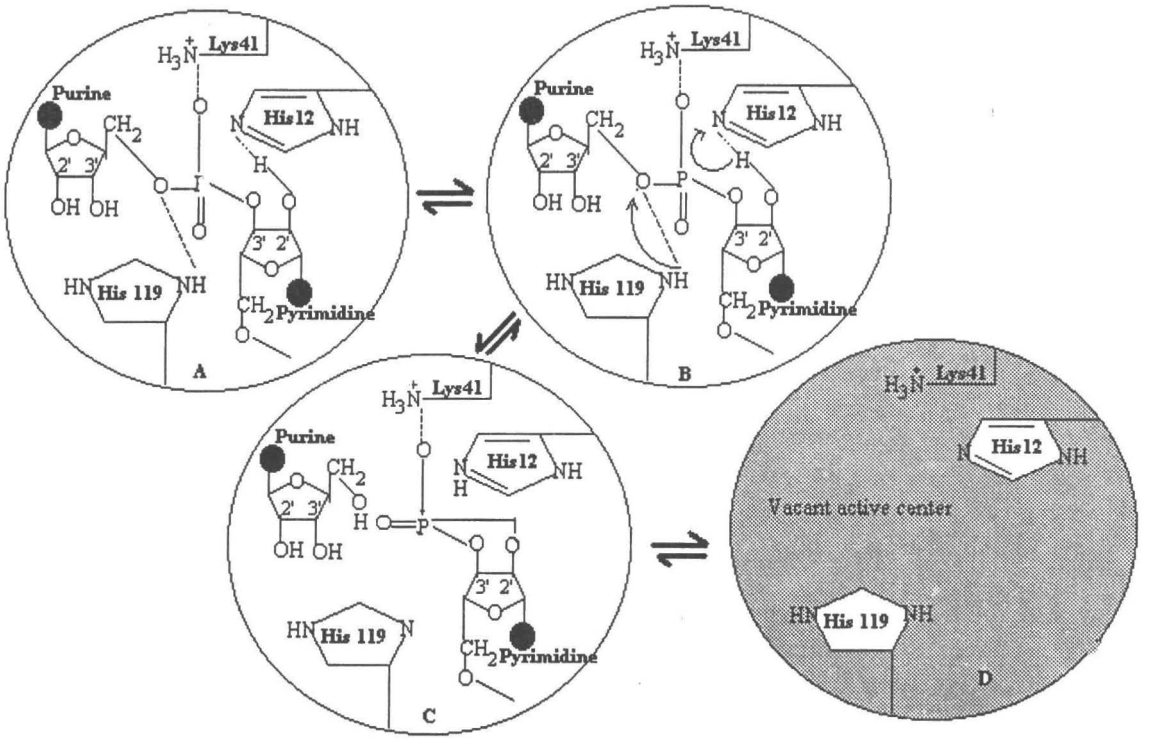


Fig 6.18: Proposed mechanism of action of ribonuclease.

Legend:

A = Binding of RNA substrate at the active center of enzyme ribonuclease

B = Proton rearrangements through electrostatic interactions

C = Formation of 2'—3' cyclic phosphodiester bond

D = End products released and recovery of native enzyme.

◆◆◆◆◆

7

COENZYMES AND COFACTORS

Introduction

Classification of coenzymes

- The reactions in which they participate
- Chemical characteristics
- Their relation with vitamins

Definition of coenzymes

Differentiation between Coenzymes and Prosthetic groups

Detailed classification of coenzymes

The hydrogen and or electron carriers

- The Nicotinamide coenzymes:
- The flavin coenzymes:
- Lipoic acid:
- Glutathione:
- Quinones:
- Cytochromes:

The amino group carriers

- The Pyridoxal and related coenzymes

The phosphate carriers

The acyl group carriers

- The thiamine pyrophosphate
- Coenzyme A

Carriers of the one-carbon groups

- Derivatives of the folic acid.
- Biotin.
- S-Adenosyl Methionine.
- Cobamide coenzymes.

The metal and inorganic cofactors

- Metal activated enzymes.
- Metalloenzymes.
- Role of minerals in Enzyme activity.

Introduction

Many enzymatic reactions have an essential additional requirement of substances other than substrates. These substances are usually small molecular weight organic substances, mostly derivatives of vitamins. Many such compounds act as co-substrates or even as links between two successive reactions. They might exist as integral components of multi-enzyme complexes, for example, the dehydrogenase complexes that act on keto –acids comprise of 5 coenzymes namely TPP, lipoic acid, Co A, NAD and FAD.

1. The non-protein organic and inorganic factors that are essential for the activity of certain enzymes are designated as coenzymes and cofactors.
2. They can freely dissociate from the enzymes as and when required.
3. They may exist in more than one forms, a property that helps them to carry hydrogen and other groups from one metabolic sequence to other.
4. They can be phosphorylated, acylated and reduced in the catalytic process.
5. They have small molecular weights as compared to enzymes.
6. They are mostly heat stable.

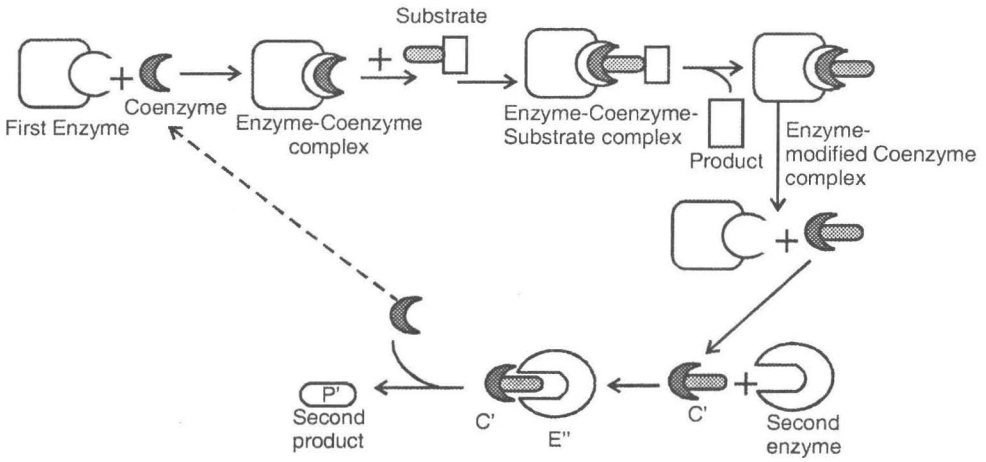


Fig 7.1:A schematic representation of the role of coenzyme in enzyme catalyzed reactions

Classification Of Coenzymes

The coenzymes are classified based on the following properties,

[A] The Reactions In Which They Participate

Based on this criterion there are 6 groups that can be written as,

1. The hydrogen and or electron carriers: this group includes the nicotinamide coenzymes, NAD and NADP, flavin coenzymes, lipoic acid, glutathione, quinones, cytochromes
2. The amino group carriers: they include glutamate, pyridoxamine,
3. The phosphate carriers: nucleoside phosphates[ATP and related compounds],

4. The acyl group carriers: coenzyme A, TPP
5. The carriers of one carbon groups: folate derivatives, biotin,
6. The metal cofactors

[B] Chemical Characteristics

On the basis of this criterion there are 3 groups,

1. Coenzymes containing an aromatic hetero ring: This group includes ATP and related compounds, NAD[P], FMN, TPP, Pyridoxal phosphate.
2. Coenzymes that contain a non-aromatic hetero ring: Biotin, Lipoic acid etc.
3. Coenzymes that do not contain a hetero ring: This group includes sugar phosphates and CoQ.

[C] Their Relation With Vitamins

On this basis there are two groups,

1. Coenzymes that are derivatives of B complex members: These include about 8 compounds that are derivatives of the members of the vitamin B group.
2. Coenzymes that are derivatives of other compounds: These include the ubiquinone, glutathione, cytochromes etc.

A tabular representation of some members of coenzymes other than the B complex vitamin derivatives is summarized with their functions as,

Table 7.1 Coenzymes other than B-complex vitamin derivatives.

Name of the Coenzyme	Function[s]
Ubiquinone	Electron transfer
Adenosine triPhosphate	Donor of phosphates and adenosine derivatives
Glutathione	Reduction reactions
Cytidine diPhosphate	Carrier in phospholipid synthesis
Cytochromes	Electron carriers
Uridine diPhosphate	Sugar carrier in metabolism
S-Adenosyl Methionine	Methyl group donor
Phosphoadenosine phosphosulfate	Sulfate donor in mucopolysaccharide synthesis

Definition of Coenzymes

Some authors separate the prosthetic groups from the coenzymes as, by definition, a coenzyme is a small organic molecule that is ***dialysable,*** while the prosthetic group is a non–protein component ***firmly bound or integral*** to the structure of the enzyme protein.

The coenzymes might play different roles.

1. They may help the enzyme in binding with the substrate.
2. They may stabilize the transition ES complex
3. They may result into specific orientation of the enzyme and substrates

4. They may lower the activation energy barrier
5. They may render certain groups in the substrate susceptible for removal.

Differentiation between Coenzymes and Prosthetic groups

Table 7.2 Differentiation between coenzymes and prosthetic groups

Coenzymes	Prosthetic groups
They act as substrates for many enzymes	They do not act as substrates
They return to native state at the end of reaction	They do not undergo any alterations during reaction
They may bind loosely to the enzyme	They are tightly bound to the enzyme
Examples and functions:	Examples and functions:
Vitamin C – Redox reactions	FAD – Redox
Coenzyme A – Acyl carrier	Pyridoxyl phosphate – Transamination
Tetrahydro Folate – C1 carrier	Biotin – Carboxylation

Many times coenzymes and prosthetic groups are collectively termed as cofactors.

Detailed classification of coenzymes

A discussion about the chemistry, mode of action, properties and analogs of different coenzymes is now given.

1. The hydrogen and or electron carriers

As the name of the group suggests these coenzymes are involved in the transfer of the hydrogen atoms that are rendered labile by the action of the respective enzymes. The coenzymes are generally specific for enzymes; those enzymes that utilize NAD do not generally use NADP as coenzymes. Even the enzymes that use FMN do not use FAD. The coenzymes are briefly discussed here.

(A) The Nicotinamide coenzymes

There are two compounds belonging to this group, both existing as dinucleotides comprising of adenine and pyridine moieties. Their existence has been known since early twentieth century. Harden and Young showed the existence of a thermostable coenzyme involved in fermentation in 1904. Von Euler et.al and Warburg and Christian independently purified the coenzyme in 1936. Its phosphorylated form, NADP was discovered in 1931 by Warburg and Christian, as the coenzyme of glucose-6-phosphate dehydrogenase. They also demonstrated that the active group in both the coenzymes is the pyridine moiety that can undergo reduction and oxidation.

The coenzymes have been recognized by several names that include, cozymase, phosphocozymase, coenzyme I and II, diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) respectively.

Structure of NAD and NADP

The structures of the coenzymes NAD and NADP can be written as,

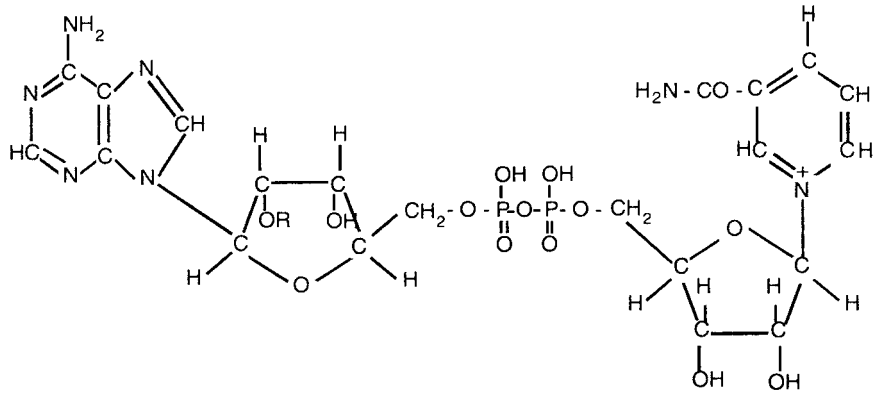


Fig.7.2: Structure of pyridine nucleotides.

Usually some anion accompanies this structure. The ribose sugar moiety is linked to the nicotinamide and adenine through their nitrogen atoms. Both the coenzymes give adenylic acid on hydrolysis by nucleotide pyrophosphatase.

Chemistry Of Nicotinamide Nucleotides

The nicotinamide nucleotides comprise of two parts, an adenine mono- nucleotide consisting of adenine, ribose and phosphate linked to a pseudo nucleotide consisting of an amide of pyridine [nicotinamide], ribose and phosphate. In case of NADP the C2 of the ribose sugar attached to the adenine carries a phosphate residue.

The maximum absorption of the oxidized forms of both the coenzymes is around 260 nm while the reduced forms maximally absorb at 340 nm.

Properties Of The Nicotinamide Coenzymes

1. The coenzymes can undergo reversible reduction- oxidation and act as carriers of hydrogen in many biological redox reactions.
2. Since the kinetic behavior of the Pyridine linked dehydrogenases show that the reactions proceed by ordered bisubstrate reactions, which follow a compulsory sequence of addition of substrates and co-substrates, the pyridine nucleotides act as the leading substrates.
3. The coenzymes can be reduced non-enzymatically by reducing agents such as sodium dithionite or sodium borohydride and the reduced forms may be similarly oxidized by ferricyanide but not molecular oxygen.
4. After the transfer of reducing equivalents from the substrate to coenzyme, the oxidized substrate leaves the active site first. This leaves behind the reduced form of the coenzyme.
5. The redox potential of the NADH-NAD or NADPH-NADP is –0.32Volts
6. They bind non-covalently and relatively loosely to the enzymes and thus serve as dissociable carriers of electron and hydrogen.

Mechanism Of Action Of Nicotinamide Coenzymes

The reduced substrate carries specific hydrogen atoms that are rendered labile by the action of the enzyme and are transferred to the coenzyme. Out of the two hydrogen

atoms, one is accepted by C4 of the pyridine nucleotide while the other H is split into a proton and electron. [Fig 7.3] The electron is utilized to neutralize the positive charge carried by the N1 of nicotinamide. It is therefore technically always written as NADH + proton rather than $NADH_2$.

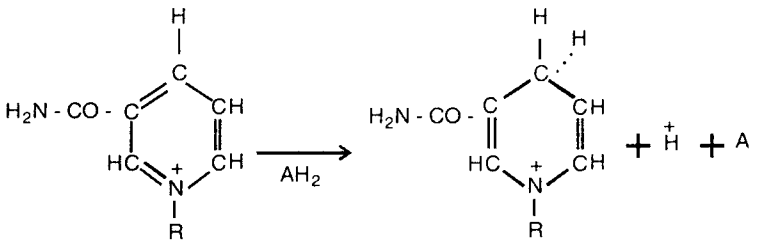


Fig.7.3: Mechanism of action of NAD [P]

Examples of enzymes requiring the coenzymes of nicotinamide

A list of the enzymes using the coenzymes is tabulated as

Table 7.3: Enzymes using pyridine nucleotides.

NAD (H) enzymes	NADP (H) enzymes
Cystine reductase	Glucose-6-P dehydrogenase
Glyceraldehyde 3 P dehydrogenase	6-Phosphogluconate dehydrogenase
Lactate dehydrogenase	Malic enzyme
Pyruvate dehydrogenase	Isocitrate dehydrogenase (cytoplasmic)
α- ketoglutarate dehydrogenase	HMG CoA reductase
Malate dehydrogenase	Squalene synthase
Isocitrate dehydrogenase	Enoyl reductase
UDP glucose dehydrogenase	Aldose reductase
β-hydroxyacyl CoA dehydrogenase	Dihydrofolate reductase

Analogs of Nicotinamide Coenzymes

Modifications of NAD and NADP have been used for study of enzymatic properties. The modification of the molecule excluding the nicotinamide residue (for example methiodide derivative or nicotinamide mononucleotide) produced inactive coenzymes. Modification of adenine to produce hypoxanthine mononucleotide reduces activity in variable proportions with different dehydrogenases. Modification of sugar residue also affects the biological activity.

Some analogs produced by modification of the C3 side chain. The modified substituents include,

-H, $-CH_3$, $-NH_2$, $-NH-CO-CH_3$, $-CS-NH_2$, -CO-NH-OH, - $CO-NH-NH_2$, -CO-H,

$-CO-CH_3$, $-CHOH-CH_3$, $-CO-CH-(CH_3)_2$, $-CO-C_6H_5$, -CH=NOH, $-CH=CH-CO-NH_2$

Many of these analogs have been found to be inactive, and some have far less activity than the native coenzymes.

Biosynthesis of Nicotinamide Nucleotides

Liver tissue can synthesize nicotinamide from amino acid tryptophan; the amount of tryptophan required is about 60mg for synthesizing 1 mg of coenzyme. The pathway occurs as follows,

Tryptophan → N formyl Kynurenine → Kynurenine → 3-OH-Kynurenine →
3 OH anthranilic acid → α amino, β carboxy muconic acid semialdehyde →
Quinolinic acid → nicotinate mono nucleotide → desamido NAD+ → NAD → NADP.

B. The flavin Coenzymes

The flavin coenzymes are a group of 2 compounds namely the flavin mono nucleotide (FMN), and flavin adenine dinucleotide (FAD). The flavin coenzymes are distributed widely in nature. They are often firmly bound to the enzymes and are therefore designated as prosthetic groups.

The first flavoprotein enzyme was isolated from yeast by Warburg and Christian in the year 1932. They separated the flavin nucleotide from the colloid [protein] component and called it as yellow flavin group. The constitution of the flavin group was established by Kuhn et.al and Karrer et.al in 1935. The biological activity of these compounds is related to oxidation-reduction reactions, in which they act as carriers of hydrogen atoms. The reduction is readily achieved by its normal substrate through the enzymatic action. The labile hydrogen atoms are accepted by the flavin ring structure. The formation of semiquinone in this process is proposed. Some spectroscopic studies have proved the assumption to be correct.

Apart from their specific substrates they can react with dyes such as indophenol and methylene blue. Some can react with NAD linked enzymes as well. Such reactions are important in the biological oxidation reduction and ATP synthesis.

Structure of FMN and FAD

The structures of FMN and FAD comprise of a flavin ring structure made up of 6,7 dimethyl-9-D –1 –ribityl isoalloxazine. The R group in FMN consists of ribitol and phosphate, while in case of FAD the R group consists of ribitol phosphate linked to adenine nucleotide through its phosphate.

The rings in the structure are designated as A B and C, as shown in the figure.

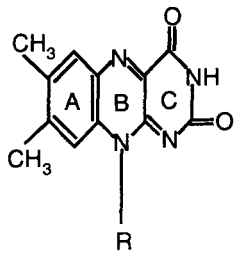


Fig7.4: Isoalloxazine ring structure in flavin coenzymes

Properties Of The Flavin Nucleotides

1. The flavin nucleotides act as carriers of hydrogen atoms as they can undergo reversible reduction and oxidation.
2. They are coenzymes for a group of enzymes designated as flavoproteins or metalloflavoproteins.
3. In contrast to the pyridine moiety that carries the hydride ion and a loosely bound proton the isoalloxazine ring of the flavin accepts both hydrogen atoms forming $FMNH_2$ or $FADH_2$.
4. The redox potential of the oxidized/reduced pair of the coenzymes is about –0.30 volts.

Mechanism of action of the flavin coenzymes

The mechanism of action of the flavin coenzymes is schematically represented as,

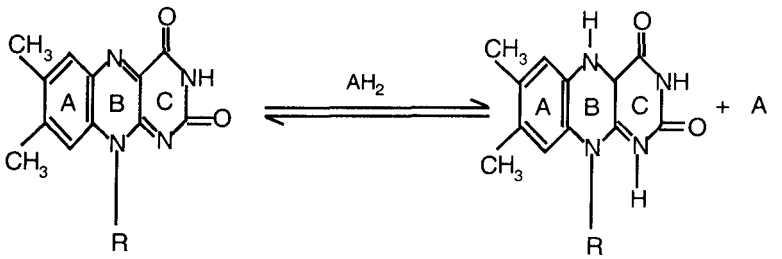


Fig.7.5: Mechanism of flavin nucleotides.

The specific hydrogen atoms on the substrate are rendered labile by the enzyme active site residues and are transferred to the isoalloxazine ring. One hydrogen is accepted by the N^1 in ring A, while the other is accepted by the N^{10} of the ring B. Semiquinones may be formed in the process. The substrate on dehydrogenation usually forms a compound containing a Trans- double bond. The best-studied examples include the reaction of succinate dehydrogenase of the TCA and fatty acyl CoA dehydrogenase of the beta-oxidation of fatty acids.

Enzymes Using Flavin Nucleotides as Coenzymes

Table 7.4: Enzymes using Flavin nucleotides.

FMN enzymes	FAD enzymes
Glycollate oxidase[plants]	Glucose oxidase[moulds]
Lactate oxidase[bacteria]	Aldehyde oxidase[liver]
Oxalate oxidase[mosses]	Xanthine oxidase[milk]
L-amino acid oxidase[animal tissue]	Aspartate oxidse[kidney]
Pyridoxamine phosphate oxidase[liver]	D-amino acid oxidase[kidney]
Quinone reductase[bacteria]	Pyruvate dehydrogenase[bacteria]
Lactate dehydrogenase[yeast]	Nitrite reductase[bacteria]
	Butyryl CoA dehydrogenase[animal tissue]
	Succinate dehydrogenase[liver]
	Glutathione reductase
	Lipoamide dehydrogenase[heart, muscle]

Analogs of Riboflavin

Two analogs have been frequently used in studies on flavin dependent reactions. They are galactoflavin, in which the ribitol residue is replaced by galactose and demethyl riboflavin in which the ring C is demethylated.

Biosynthesis of Flavin Coenzymes

The dietary riboflavin absorbed is converted to FMN by the action of a kinase and the FMN is converted to FAD by FAD synthase.

C. Lipoic Acid:

Lipoate was first discovered as a growth factor for *Tetrahymena* and was referred as factor II. It was later studied and named with different names by many scientists, Reed, De Busk, Gunsalus and Hornberger in 1951. They named it as a- Lipoic acid due to its solubility in organic solvents. It was also called thioctic acid due to the presence of sulfur in its structure. The name lipoic acid is now commonly used. A considerable part of the lipoate in cells appears to exist bound to proteins attached by a peptide bond to a lysine residue and can be removed enzymatically.

Structure of Llipoic Acid

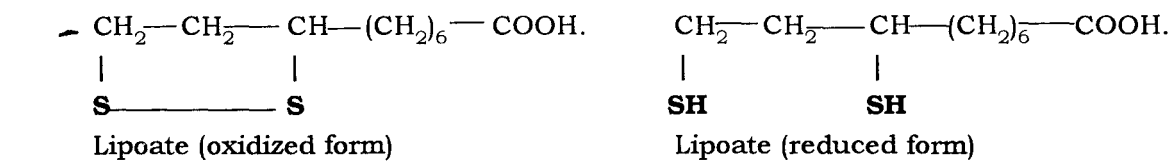


Fig.7.6: Structure of Lipoate

Properties of Lipoate

1. The substance is yellow in color in the oxidized state and has an absorption maximum at about 335 nm due to the ring structure present.
2. Strong reducing agents such as zinc in HCl open the ring by reducing the disulfide bond.
3. Other compounds that can reduce the lipoate include, pyruvate, oxoglutarate, and TPP that can transfer an acyl group
4. The reduced lipoate after donating the acyl group can be re oxidized by NAD dependent dehydrogenase or mild chemical oxidizing agents such as iodine.

Mechanism Of Action Of Lipoate

The best studied reactions where lipoate is an essential coenzyme include the dehydrogenation-decarboxylation reactions of the alpha keto acids. The schematic representation of the reactions of the pyruvate dehydrogenase complex in which lipoate takes part can be written as shown in fig 7.7.

Enzymes Using Lipoate As A Coenzyme

Lipoate is an integral constituent coenzyme of the pyruvate dehydrogenase and the alpha ketoglutarate dehydrogenase complexes. It is a coenzyme for dihydrolipoyl transacetylase and dihydrolipoyl transsuccinylase.

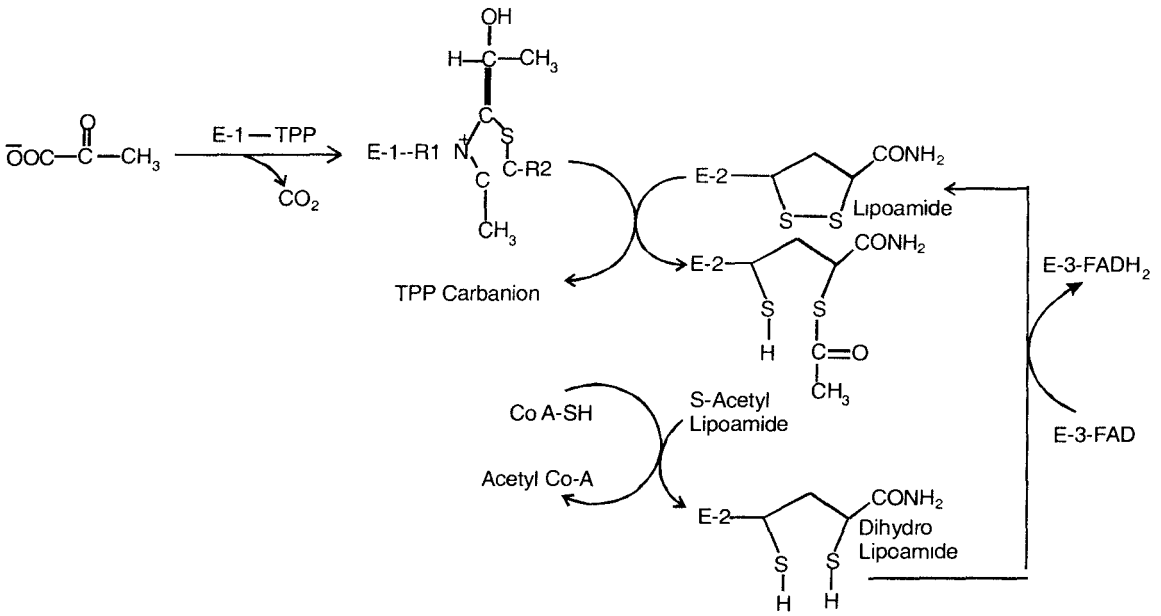


Fig.7.7: Mechanism of action of lipoic acid.

D. Glutathione

Hopkins discovered this tripeptide in 1921. Originally though to be a dipeptide of cysteine and glutamate, Hopkins on its isolation and crystallization showed it to be a tripeptide of glutamate, cysteine and glycine. Kendall also isolated and crystallized it simultaneously. The functional group is the thiol residue of cysteine and the reduced form of the coenzyme is represented as GSH, while the oxidized form is designated as G-S-S-G.

Structure of Glutathione

The structure of the tripeptide was under some controversy for some time but it was finally established by synthesis to be γ-Glutamyl –L-Cysteinyl-Glycine.

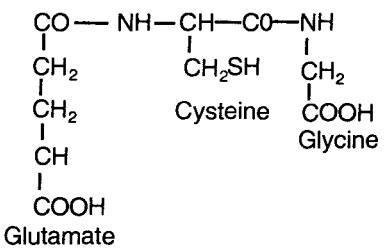


Fig.7.8: Structure of glutathione [γ– glutamyl-cysteinyl-glycine]

Properties of Glutathione

1. Mild oxidizing agents like iodine and ferricyanide can oxidize glutathione. It occurs in a similar manner, analogous to oxidation of cysteine to cystine. It can be schematically represented as,

   2GSH - 2H == GSSG

2. Under suitable conditions, molecular oxygen and cytochrome C can oxidize glutathione.
3. It is oxidized enzymatically by dehydroascorbate in the presence of glutathione dehydrogenase
4. Its reduction can be brought out by powerful reducing agents or enzymatically by NADP linked dehydrogenases.
5. Although its function is relatively obscure it may act as a hydrogen carrier as originally proposed by Hopkins.

Mechanism of Action of Glutathione

Glutathione can provide a path for oxidation of the coenzymes through ascorbate and either ascorbate oxidase in plants or cytochrome oxidase in animals. It can also act as a coenzyme for the glyoxylase system for maleylacetoacetate and maleyl pyruvate isomerases and formaldehyde dehydrogenases. Glutathione keeps many enzymes in the reduced form.

E. Quinones

The quinones are widespread in the living kingdom. They have been identified as carriers of hydrogens before transfer of electrons to the cytochromes. They include two families, viz. the ubiquinone derivatives that contain poly isoprenoid side-chains and the vitamin K group.

Ubiquinone was first discovered by R.A.Morton et.al in 1953 and Krane et.al in 1957. The structure was worked out in 1958 by two different groups simultaneously. The role of UQ was worked out in Institute of enzyme studies in Wisconsin.

It was designated by different names, but the two frequently used names are ubiquinone and coenzyme Q.

Structure of Ubiquinone

The structure of the Co Q and vitamin K group compounds contain substituted aromatic rings. [fig.7.9] The side chain residues are usually polymers of isoprene units, and several related compounds differing in the number of isoprene groups have been identified in both plant and animal kingdom. The vitamin K group consists of vit K1, K2 and menadione, in which the substituent R group differs. K1 contains 4 isoprene units, while K2 may contain 6, 7 or 9 isoprene units. Menadione contains H as the substituent.

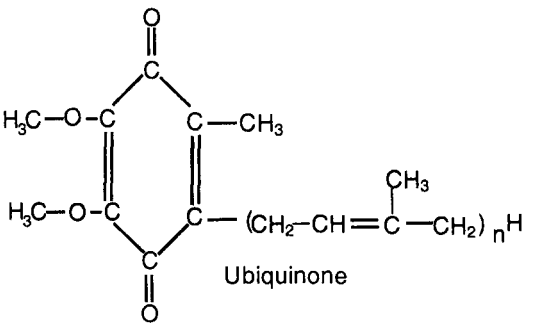


Fig.7.9: Structure of Co Q.

Properties of Ubiquinone

1. The ubiquinone moiety undergoes reduction to form a hydroquinone residue, a property that is fundamental and physiologically important.
2. It occurs in mitochondria and chloroplasts and is an integral component of the electron transport and phosphorylation sequence.
3. It may form semiquinones during the process of its reduction.
4. It is insoluble in water but dissolves in organic solvents.
5. It probably plays a role in mitochondrial oxidation of succinate through the cytochrome c.
6. The absorption maximum of the oxidized form is around 275 nm, while that of the reduced form is near 300 NM.

Mechanism of Action of Ubiquinone

The mechanism of action of CoQ can be represented as in fig 7.10

The quinone oxygen atoms accept the hydrogen atoms and are converted to hydroxyl groups forming the compound ubiquinol. As indicated earlier if single hydrogen is accepted the compound formed is designated as a semi quinone derivative.

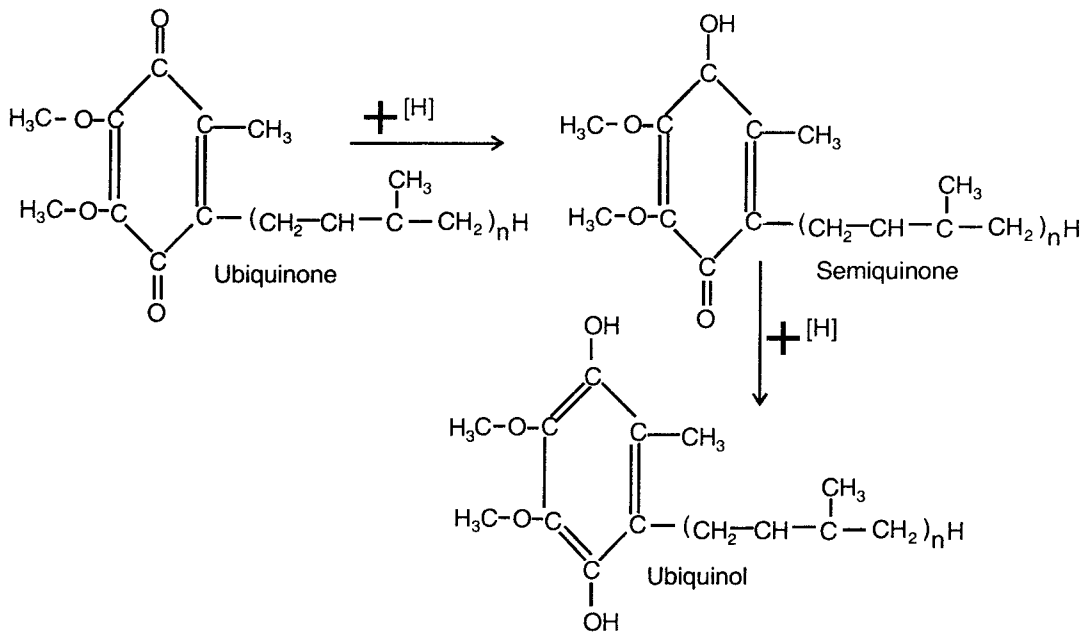


Fig 7.10: Mechanism of action of Co Q.

Enzymes Using Co Q

It is difficult to state the exact number of substrates and in turn their specific enzymes that depend upon the coenzyme Q for their oxidation. However the mitochondrial systems that use the cytochromes do require the role of CoQ in their reactions.

Analogs of Vitamin K /ubiquinone

Dicumarol, an analog of vitamin K has been extensively used in studies.

F. Cytochromes

The cytochromes were discovered in the 1920s and Keilin gave the name to such compounds that are intracellular hemoproteins showing a characteristic absorption in the visible range. The functions of a number of cytochromes are not specifically known, some of them are enzymes while others are carriers of reducing equivalents or electrons. In all most of them can participate in oxidation-reduction reactions. Presently over 20 different cytochromes are known.

The enzyme commission has recommended a 4-group classification of the cytochromes.

A cytochromes with heam group containing formyl porphyrin

B cytochromes containing a protoheam group

C cytochromes containing a mesoheam group linked to the protein

D cytochromes with a heam group containing dihydroporphyrin.

The most frequently encountered cytochrome groups in tissues belong to b and c groups followed by a group cytochromes.

Structure of Cytochromes:

The general structure of cytochromes is represented as,

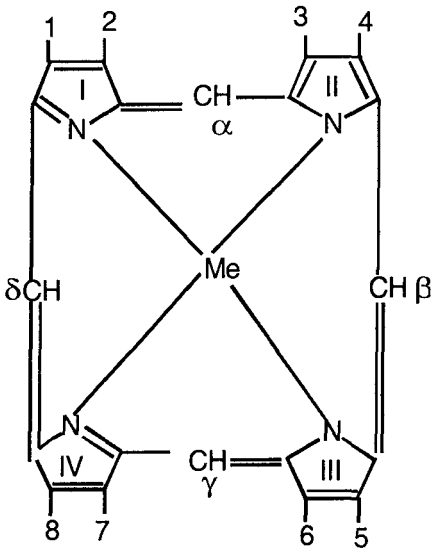


Fig. 7.11: Structure of Cytochromes.

The substituted groups [1 to 8] are different for the various cytochromes and they may be represented in a tabular form as,

Table 7.5: Substituent groups in different cytochromes

Group	1	2	3	4	5	6	7	8
A	Methyl	-CHOH	Methyl	Vinyl	Methyl	Propionyl	Propionyl	Formyl
B	Methyl	Vinyl	Methyl	Viny	Methyl	Propionyl	Propionyl	Methyl
C	Methyl	Acetyl	Methyl	Acetyl	Methyl	Propionyl	Propionyl	Methyl
D	Methyl	-CHOH	Methyl	$CH=CR_2,R_3$	Methyl	Propionyl	Propionyl	Methyl

α, β, γ, δ represent methylene bridges while Me represents metal ion.

The nature of the prosthetic group has a considerable influence on the absorption of the cytochromes. The 'a' type cytochromes absorb around 590nm, b, around 554-563 nm, while c, around, 550-557 nm.

Properties of Cytochromes

1. The cytochromes except the cytochrome oxidase are anaerobic dehydrogenases. They act as carriers of electrons from flavoproteins to cytochrome oxidase.
2. They contain Fe ions that can undergo reduction and oxidation by one electron exchange.
3. They are involved in sequential transfer of electrons. The electron transport involves the roles of the cytochromes b, c and aa3.
4. The cytochromes are also found associated with the endoplasmic reticulum and plant, bacteria and yeast.
5. Cytochrome c is exceptional in being freely soluble due to which it was the first isolated and purified cytochrome.
6. The cytochromes concerned in cell respiration are located in intracellular particles, usually in mitochondria in case of animal tissues. Cyt.b5 is located in microsomes.
7. Reduced cytochrome c is very rapidly oxidized by oxygen in the presence of cytochrome oxidase. Dithionite, cysteine, polyphenols and ascorbate can reduce the oxidized form.

2. The Amino Group Carriers

The amino group carriers are important from the point that the tissue protein metabolism, and in turn the amino acid metabolism is in a very dynamic state. The catabolism and new synthesis of amino acids as well as the conversion of keto acids into their respective amino acids is a continuously occurring process. Most of the transaminases use glutamate in their reactions as donor of the amino group. The amino group is usually carried by the pyridoxal phosphate and it often occurs as a prosthetic group of the respective enzymes. Since glutamate acts as a substrate rather than as a coenzyme, the pyridoxal derivatives shall be discussed here in details.

(A) The Pyridoxal and Related Coenzymes

The pyridoxine [B6] was isolated by five different groups of workers in the year 1938. Its structure was established in 1939 in Germany and USA. The B6 group comprises of three compounds namely pyridoxine, pyridoxal phosphate and

pyridoxamine phosphate. The aldehyde and amine derivatives act as coenzymes for transaminases, deaminases, transsulfurases and desulfurases. They are also related to porphyrin synthesis and synthesis of other important compounds including CoA, serotonin etc.

Structure of B6 Coenzymes:

The structures of these compounds are shown as,

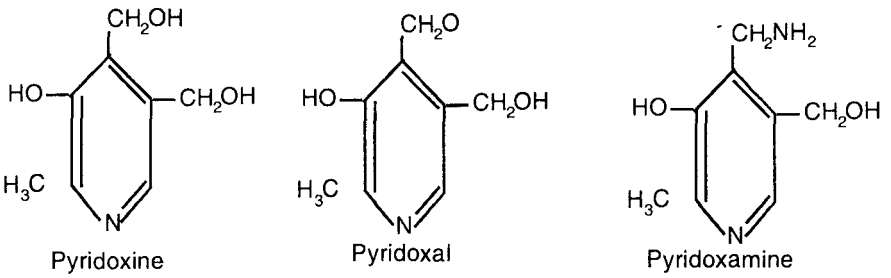


Fig. 7.12: Structure of B6 coenzymes

The pyridoxine derivatives contain an aromatic hetero ring in which the primary hydroxymethyl [-CH_2OH] is converted to an aldehyde or a primary amine.

Chemistry of B6 Coenzymes:

The pyridoxal and pyridoxamine are the most active forms. Pyridoxine is 3 hydroxy, 4,5-di hydroxymethyl, 2-methyl pyridine. The pyridoxal contains an aldehyde group at C4 while the pyridoxamine contains a primary amine at the C4.

They are interchangeable.

Properties of B6 Coenzymes:

1. pyridoxine is readily soluble in water
2. it slowly decomposes on exposure to light in alkaline solution
3. it gives color reactions with phenol reagent or 2,6-dichloroquinone chlorimide.
4. The coenzymes can participate in a variety of biological reactions. Their role in transaminations has been extensively studied.

Mechanism of Action

The coenzymes are usually present as prosthetic groups of the transaminases. The general reaction of transamination can be written as follows,

E-PLP + amino acid 1 ======= E-PMP + corresponding keto acid

E-PMP + keto acid 2 ======== E-PLP + corresponding amino acid.

In these reactions that usually follow double displacement kinetics, the coenzymes are involved in reversible acceptance and donation of the amino group at the C4 substituent group. It is shown as,

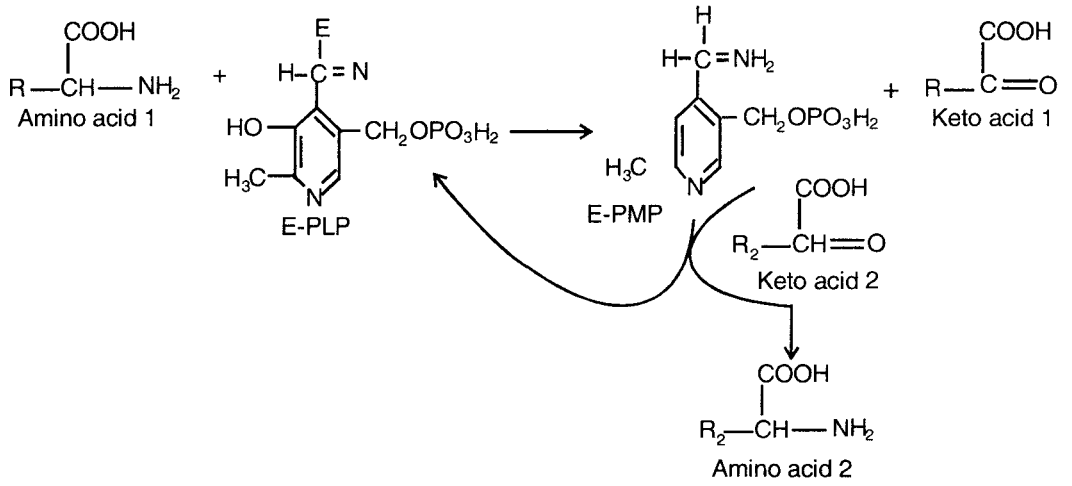


Fig. 7.13: Mechanism of action of B6 coenzyme (transamination reaction).

E-PLP = enzyme bound periodical phosphate, E-PMP = enzyme bound pyridoxamine phosphate.

Enzymes Using B6 Coenzymes

Some of the enzymes and reactions that require the B6 coenzymes are summarized as,

Table 7.6: Enzymes using Coenzymes of B6.

Enzymes	Metabolic role
SGOT	Transamination
DOPA carboxylase	Dopamine synthesis
5-Hydroxy tryptophan decarboxylase	Serotonin synthesis
ALA synthase	Heme synthesis
Kynureninase	Nicotinamide synthesis
Serine dehydratase	Serine catabolism
Cysteine desulfhydrase	Deamination of cysteine
Glycine synthase	Glycine metabolism
Glycogen phosphorylase	Glycogenolysis
3-keto sphinganine synthase	Myelin synthesis
Cystathionase	Methionine metabolism

3. The Phosphate Carriers

The cellular metabolism revolves around the reactions that involve phosphorylation of substrates and they are probably constantly occurring within the cells. The reactions are concerned with, chemical-energy transfers. The compounds are coenzymes for kinases and other phosphotransferases and can be arbitrarily divided into two groups.

1. The nucleoside phosphates:
2. The other phosphate carriers like creatine phosphate

The nucleotide phosphates are further classified into three groups depending upon the number of phosphate groups carried by them. The diphosphates and triphosphates usually serve as coenzymes.

The cyclic phosphates [cAMP, cGMP etc] are also involved in metabolism and it is indicated that they act as second messengers for the regulation of metabolism.

1. The Nucleoside Phosphates:

The nucleoside phosphate group comprises of the derivatives of adenine, [fig.7.14] guanine, uracil, cytosine and thymine. Adenine, guanine and uracil nucleotides are the most frequently encountered coenzymes.

Structure of Nucleotides

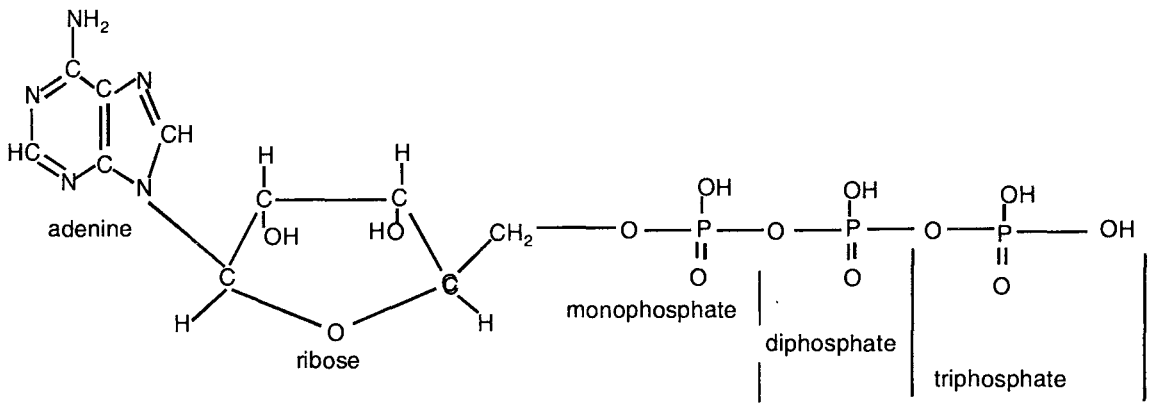


Fig. 7.14: The phosphate carrying nucleotides of adenine.

The replacement of the ***nitrogenous*** base forms respective mono, di and tri phosphate derivatives of the respective purine / pyrimidine. Chemically thus the nucleotides comprise of a nitrogenous base, a pentose sugar and phosphate moieties. The phosphates are attached to the 5' position of the sugars.

Properties of the Phosphate Carriers

Some of the important properties of the phosphate carriers include,

1. They act as coenzymes for kinases.
2. They may act as coenzymes for synthetases.
3. The enzyme coenzyme relationship is generally specific however some kinases may react with more than one nucleotide, although at different rates.
4. Some biological carriers may not contain nucleoside residues.
5. They may usually link two kinases or a kinase-synthetase pair.
6. They may be linked with glycosyl transfers as in the case of UDPG in sucrose synthesis.

Examples of Reactions of Phosphate Carriers

In the muscles, creatine phosphate serves as a donor of phosphate to ADP. This ATP is then utilized for muscular contraction; while in liver phosphorylation of glucose is the primary step in glycolysis.

The reactions are represented as follows,

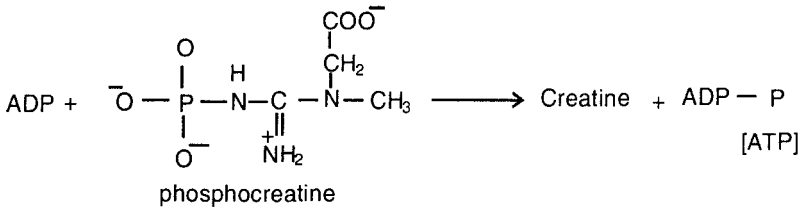


Fig. 7.15: Synthesis of ATP. Donation of phosphate by creatine phosphate.

Some enzymes that use the phosphate carrying coenzymes are tabulated as,

Table 7.7: Enzymes that use phosphate carriers as coenzymes.

Enzyme	Coenzyme
RNA adenylyl transferase	ATP
RNA cytidyl transferase	CTP
GDP mannose phosphorylase	GDP
UDPG Pyrophosphorylase	UTP
Glucosel phosphate thymidylyl transferase	d-TTP
Choline phosphotransferase	CDP- choline
Hexokinase / glucokinase	ATP

The phospho transferases belong to 2.7 class-subclass of the EC classification. There are 8 sub sub classes depending upon the acceptors of phosphate.

4. The Acyl Group Carriers

The acyl group carriers are important coenzymes and participate in metabolic reactions of carbohydrates, lipids and proteins. There are two important coenzymes namely TPP, and coenzyme A. they are briefly explained as follows,

A] The Thiamine Pyrophosphate:

It was first recognized in 1937 as a cofactor for pyruvate decarboxylase. Lohmann and Schuster prepared a crystalline- substance from yeast that could act as a cofactor for pyruvate decarboxylase. Green, Herbert and Subrahmanyan showed it to be thiamine pyrophosphate.

It was given various names that include cocarboxylase, diphosphothiamine [DPT], thiamine pyrophosphate [TPP], aneurin pyrophosphate, or vit B1 pyrophosphate.

The coenzyme is also required for oxidation of keto acids namely the pyruvate and ketoglutarate.

Structure of TPP

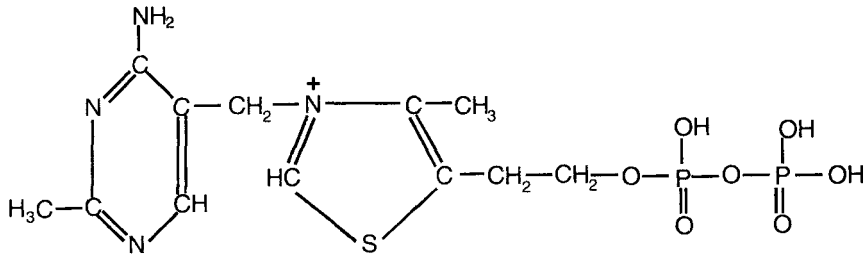


Fig. 7.16. Structure of TPP.

Chemistry of TPP

Thiamin comprises of two components, 4-amino 2,5 di-methyl pyrimidine moiety and 4-methyl 5-hydroxydiethyl-thiazole moiety. [Fig 7.16] The active component in the structure is the thiazole moiety. It can accept active acetyl residues that can be transferred to other acceptors.

Properties of TPP

1. Thiamin hemihydrate forms white needle shaped crystals that are water-soluble.
2. Thiamin is sensitive to heat, reducing agents or oxidizing agents.
3. Its active form is synthesized in the body by the action of ATP dependent thiamin pyrophosphokinase.
4. It acts as a coenzyme for reactions including oxidative decarboxylation of keto acids, transketolase reactions etc.

Mechanism of Action of TPP

The mechanism of action of TPP can be shown as in Fig. 7.17.

The thiazole residue accepts the pyruvate molecule to form pyruvyl TPP, which on loss of carbon-di-oxide is converted to hydroxyethyl TPP. The hydroxyethyl TPP donates its hydroxyethyl group to lipoic acid such that acetyl group is transferred to a S[C6] and H is transferred to another S[C8] of the lipoate residue, regenerating the TPP carbanion.

Enzymes Using TPP

Table 7.8 Enzymes that use TPP.

Enzymes	Role
Pyruvate dehydrogenase complex	Oxidation of pyruvate
Keto glutarate dehydrogenase complex	Oxidation of ketoglutarate
Transketolase	HMP Shunt

Analogs of B1

B1 antagonists like pyrithiamin causes polyneuritis with loss of appetite, poor growth etc.

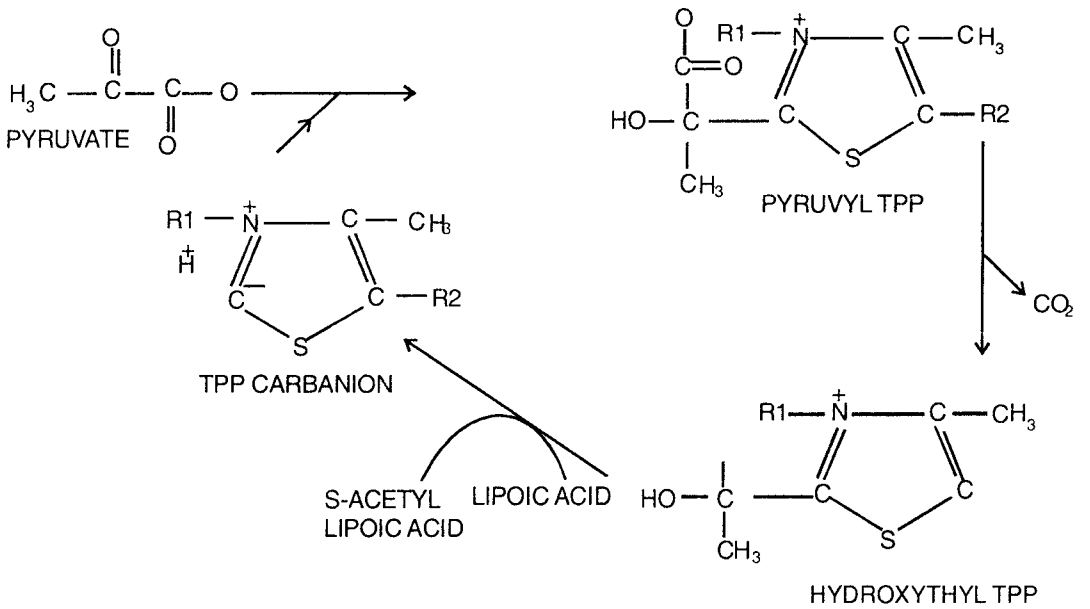


Fig. 7.17: Mechanism of action of TPP (transfer of acetyl residue)

B. Coenzyme A

Lipmann in 1947 showed that a coenzyme was required for acetylations in liver and micro-organisms. Its structure was later deduced by workers in his laboratory. It was indicated to play role in acyl, acetyl transfers and the reactions like fatty acid metabolism, oxidation of keto acids etc.

Structure and Chemistry of Coenzyme A

The coenzyme A is chemically 3' phospho ADP pantoyl β alanyl cysteamine.

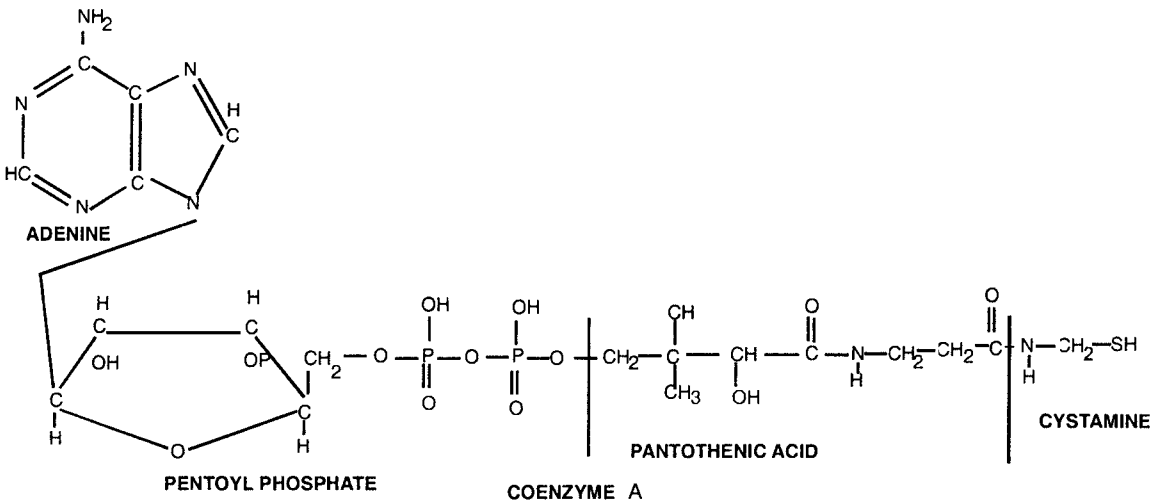


Fig. 7.18: Structure of coenzyme A.

There are three distinguishable components in the structure. The first is a phospho adenosine diphoshate nucleotide carrying an additional phosphate at the 3' of the ribose sugar, linked to a pantothenic acid group [α, γ dihydroxy-β, β dimethyl butyryl-β'alanine] attached to a cysteine amide. The active residue is the thiol group of the cysteamide residue. The structure of the coenzyme can be written as,

Properties of Coenzyme A

1. It is a colorless compound having an absorption maximum in the ultraviolet-range [257 nm].
2. It serves as a coenzyme for the enzymes belonging to 6.2.1 sub group, which are employed for the synthesis Co A thiol esters from free acids.
3. The enzymes belonging to the sub group 2.3.1 also transfer acyl groups to or from the Co A.
4. It is essential for the initiation of the TCA, and its ratio with succinyl Co A is one of the regulatory mechanisms for tissue metabolism.
5. It is also an essential coenzyme for beta oxidation of fatty acids as well as the synthesis of fatty acids and ketone bodies.

Mechanism of Action of Co A

The active group in the Co A is the thiol [-SH] residue that can form S-acetyl or – S acyl esters with active acetyl or fatty acyl molecules. Some of the reactions can be written as,

Acetic acid + Coenzyme A-SH ———→ acetyl –S-Co A + water

S-acetyl lipoate + Coenzyme A-SH ——→ dihydrolipoate + acetyl-S-Co A

Oxalo succinate + coenzyme A-SH —-→ Succinyl-S-Co A + carbon dioxide.

Fatty acid + CoA-SH + ATP ——-→ AMP + PPi + fatty acyl – S- Co A.

Analogs of Co A

Various analogs of the coenzyme are available, some for example N-acetyl cysteamine can act as coenzyme for many enzymes however many other enzymes specifically require Co A.

5. Carriers of the One-Carbon Groups

Single carbon transfers form an array of important reactions that are involved in metabolism of carbohydrates, lipids, amino acids, nitrogenous bases, porphyrins and vitamins.

The groups that are transferred include formyl, methyl, hydroxymethyl, formimino, methylene or carbon-di-oxide. The coenzymes involved in these transfers are derivatives of folate, biotin, S-adenosyl Methionine, and cobamide coenzymes etc.

A. Derivatives of the Folic Acid:

The derivatives of folic acid, especially, the group of tetra hydro folate compounds, have been long recognized. They play important role in metabolic single carbon transfers. These compounds are widespread in living systems, liver, yeast, amaranth, and mint being good sources, while considerable amounts are also present in intestinal and ruminant bacteria.

The parent compound, folic acid and the structure of its derivatives can be shown as,

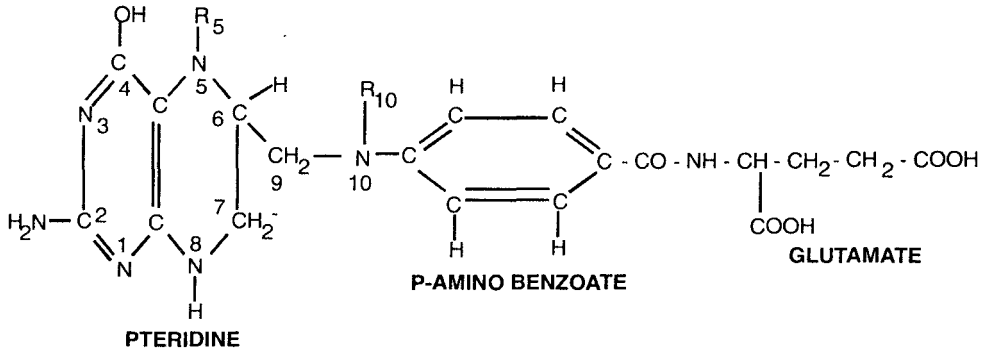


Fig. 7.19: Structure of folate

Table 7.9: Substituent groups of folate to form folate coenzymes.

Derivative of tetra hydrofolate	R5	R10
Tetrahydrofolic acid [THFA]	-H	-H
5-formyl tetrahydrofolic acid	-CO. H	-H
10- formyl tetra hydro folic acid	-H	-CO. H
5,10 methenyl tetra hydro folic acid	>N = CH-N<	
5,10 methylene tetrahydrofolic acid	>N-CH_2-N<	
5, hydroxymethyl tetrahydrofolic acid	-CH_2OH	-H
5, formimino tetra hydro folic acid	-CH=NH	-H

Chemistry of the Folate Derivatives

Pteroyl glutamic acid [PGA] or folic acid comprises of three components, a pigment, called pteridine, one molecule of p-amino benzoic acid, and glutamic acid. [Fig 7.19]

In yeast and plants, conjugates containing seven glutamate molecules (pteroyl heptaglutamate) are seen while liver contains pteroyl pentaglutamate while Lactobacillus casei requires (pteroyl triglutamate)

Properties of Folate Coenzymes

1. Tetra hydro folate is colorless but it along with its derivatives has characteristic absorption spectra in the ultraviolet range.
2. It can undergo auto oxidation and can form dihydro folate by molecular oxygen.
3. Enzymatic oxidation is generally NADP dependent.
4. It can be reduced to dihydro and tetra hydro derivatives by NADPH dependent enzymes.
5. It acts as a carrier for single carbon groups and is involved in the metabolic pathways of carbohydrates, nucleotides, amino acids etc.

6. The carrier positions vary between 5' 10', or 5' and 10' depending on he reactions and the groups transferred.
7. It differs from other carriers in that the group transported may undergo alteration while attached to the carrier so that the group that is donated to the acceptor might not be the same that was taken in the first place. For example, it may accept a formyl group and deliver it as a hydroxy methyl group.

Mechanism of action of THFA and derivatives

The role of the coenzymes in the metabolism of glycine, serine and the de Novo synthesis of purine nucleotides has been clearly understood.

The reactions are written as in fig 7.20

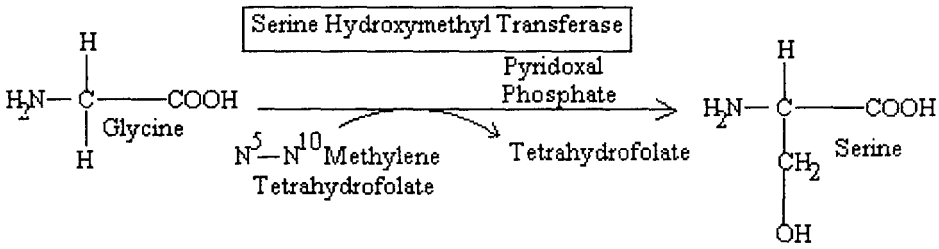


Fig. 7.20 Donation of hydroxymethyl group by THF derivative.

Antagonists of Folate

The antagonists of folate namely aminopterin and amethopterin have been used to create the folate deficiency. Experimental folate deficiency resulted in megaloblastic, macrocytic anemia.

B. Biotin

Biotin was first isolated by Fritz Kogl, a Dutch biochemist in crystalline form in the year 1935. In 1939 Szent Gyorgi et.al proved that biotin was the "anti- egg-white injury factor". Biotin is also called coenzyme R, as it is essential for the growth of Rhizobium. It occurs widely in animal and plant kingdoms. It occurs naturally as biocytin, a conjugate of biotin and amino acid lysine. [Fig.7.21]

Structure of Biotin

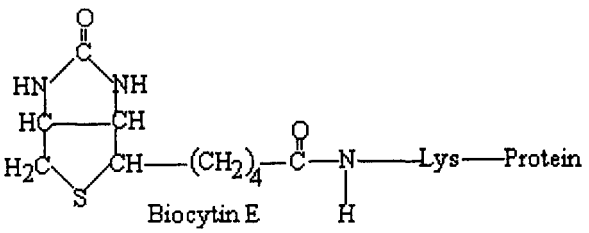


Fig. 7.21: Structure of biocytin.

The structure of biotin is shown above.

Chemistry of Biotin

The structure of biotin was established in 1942. It consists of a fused thiophene and a imidazole moiety with a fatty acid side chain. Chemically it is 2'-keto-3, 4 imidazolido-2-tetrahydrothiophene-n-valeric acid. It is an optically active compound.

Properties of Biotin

1. It forms colorless, needle like crystals.
2. It is slightly soluble in water and ethyl alcohol but it is insoluble in chloroform and ether.
3. It is heat stable and is resistant to both acids and alkalies.
4. It has a melting point of 230 degrees c.

Mechanism of Action of Biotin

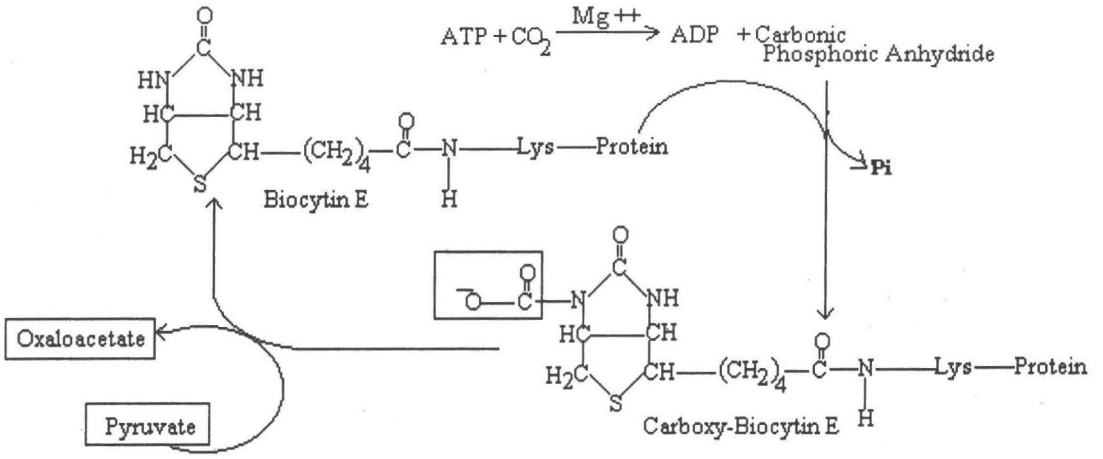


Fig 7.22: Reaction of biotin dependent carboxylation

Biotin is involved in transfer of single carbon and acts as a prosthetic group of carboxylases. A general mechanism can be schematically represented as above.

Enzymes Using Biotin as Prosthetic group

Some of the enzymes and reactions using biotin can be tabulated as,

Table 7.10: Enzymes that use biotin as coenzymes.

Enzymes	Reactions	Metabolic role
Pyruvate carboxylase	Pyruvate + ATP + bicarbonate → oxaloacetate + ADP+Pi	Gluconeogenesis
Acetyl-CoA carboxylase	Acetyl CoA + ATP + Bicarbonate → Malonyl CoA + ADP+ Pi	Fatty acid synthesis

Other enzymes include propionyl CoA carboxylase and b- methyl crotonyl CoA carboxylase.

C. S-Adenosyl Methionine

It is responsible for transfer of methyl groups in biochemical reactions. Due to its destabilizable sulfonium ion, it also acts as a good alkylating agent. Electron dense groups are usually attracted towards the susceptible methyl group. S-Adenosyl methionine is synthesized from methionine in presence of ATP by the catalytic action of methionyl adenosyl transferase. The adenosyl grop is transferred to the sulfur atom of methionine to form a sulfonium derivative with simultaneous release of inorganic phosphate and pyrophosphate groups.

Structure of S-Adenosyl Methionine

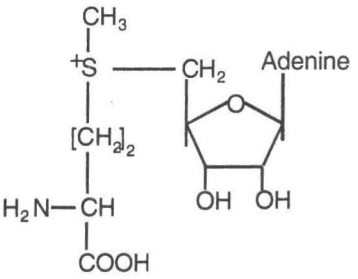


Fig. 7.23: Structure of S-adenosyl Methionine

Mechanism of Action of S-Adenosyl Methionine

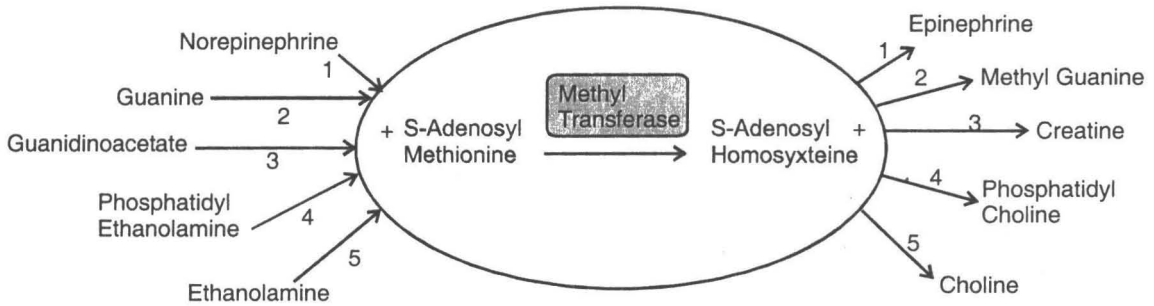


Fig. 7.24 Mechanism of Methyl transfer by S-adenosyl Methionine.

It acts as a coenzyme for enzymes generally called as transmethylases or methyl transferases. There are several biologically important reactions that employ these enzymes. They are summarized as in fig 7.24

D. Cobamide Coenzymes:

The cobamide coenzymes are derivatives of vitamin B12. They contain cobalt and a corrin ring. The vitamin was originally discovered as anti pernicious anemia factor by Minot and Murphy in the year 1926. The factor was isolated and crystallized in 1948 independently by E. Rickes and K. Folkers.

The vitamin was named as factor X, or L.L.D factor. It is found only in animals. It is predominantly synthesized b anaerobic microorganisms.

Structure of B12:

The structure of the B12 vitamin can be written as,

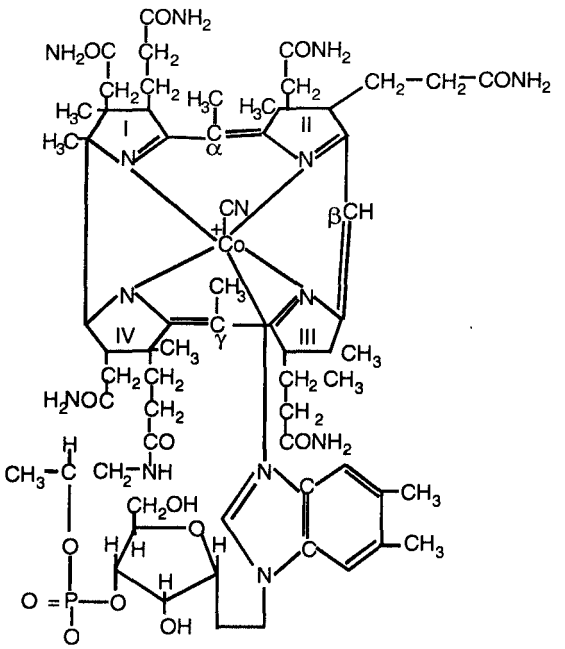


Fig. 7.25: Structure of B12 [Cyanocobalamin]

The coenzyme form differs from the vitamin as they do not contain the cyanide residue. Instead they contain a 5' deoxyadenosyl nucleotide attached to the cobalt or a methyl group as in case of methylcobalamine.

Chemistry of Cobamide Coenzymes:

The group of cobamide coenzymes comprises of a closely related compounds containing a corrin ring comprising of a highly substituted tetrapyrrole, in which the pyrrole rings are reduced and linked to each other through methylene bridges or directly. The molecule contains a central cobalt residue linked directly to a 5, 6 dimethyl benzimidazole nucleotide. Depending on the 6^{th} substituent, the compounds are designated as cyanocobalamin[-CN], methyl cobalamin [$-CH_3$], hydroxy cobalamin [-OH], nitrocobalamin and chlorocobalamin.

Properties of Cobalamine

1. Cobalamine is a deep red crystalline substance.
2. It is soluble in water, alcohol and acetone but insoluble in chloroform.
3. It is stable to heat in neutral solution but is destroyed by heat in acidic or alkaline solutions.

Mechanism of Action

The coenzyme is associated with different biochemical reactions.

They include,

a. Shift of hydrogen and adjacent groups.

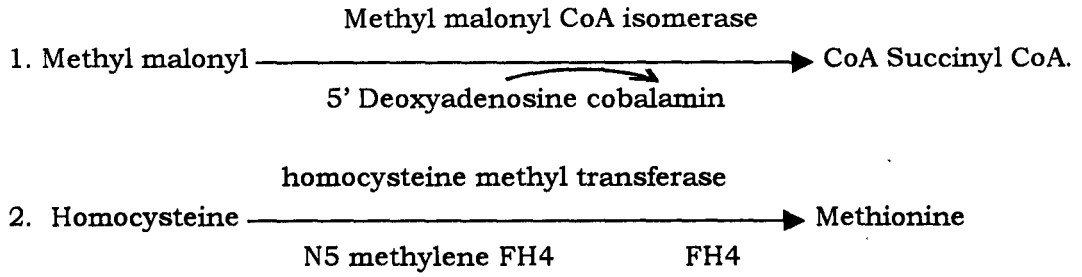
b. Transfer of methyl group

c. Isomerization of dicarboxylic acids

d. Dismutation of vicinal diols.

It is also involved in many other biologically important mechanisms such as myelin synthesis, synthesis of thymidine etc.

Two examples can be quoted here,



The first reaction is an example of intramolecular rearrangement in which the COOH and H are interchanged between C1 and C2.

In the second reaction the methyl group is initially donated by N5' methyl FH4 to the cobalt, forming an enzyme bound methylcobalamin, and later it is transferred to homocysteine forming methionine.

6. The metal and inorganic cofactors

Many enzymes, in addition to the coenzymes require the role of metals in the course of catalysis.

There are many inorganic elements that have been identified to be associated with enzymatic reactions. Some of these include, K+, Cu++, Mg++, Mn++, Ca++, Zn++, Fe+++, Co++, Mo++, Cl-, Pi-, etc. The requirement of these minerals may be specific or they may be interchangeable depending on the enzyme and the reaction catalyzed.

Generally in most cases, if the inorganic elements are removed, chelated or blocked, the activity of the enzyme is either greatly reduced or completely lost.

Depending on the Metal-enzyme associations, Enzymes are generally classified as, Metal activated enzymes and Metalloenzymes.

Table 7.11 Enzymes and their required inorganic elements

Enzymes	Required Inorganic Elements
Cytochrome oxidase, Ascorbate oxidase	Cu^{++}
Carbonic anhydrase, Alcohol dehydrogenase, DNA polymerase	Zn^{++}
Phosphatase, Hexokinase, ATPase,	Mg^{++}
Arginase, Phosphohydrolases, Phosphotransferase	Mn^{++}
Urease,	Ni^{++}
Glutothione peroxidase,	Se
Catalase, Cytochromes, Nitrogenase, Peroxidase, Ferridoxin,	Fe^{++} / Fe^{+++}
Peptidase	Co
Pyruvate Kinase	K^{+}
Nitrogenase	Mo
Nitrate Reductase	Va
Lecithinase, Lipase,	Ca^{++}

Metal Activated Enzymes

Enzymes that form loose complexes with certain metals for the catalytic state are designated as metal activated enzymes. Removal of the metal by dialysis or chelation significantly lowers the enzymatic activity. The ratio of metal to enzyme activity is constant in these reactions and metal is not necessarily unique or in other words it is generally replacable. For example Mg^{++} replaces Mn^{++} and vice versa in many metabolic reactions.

Metalloenzymes

Enzymes that form tight complexes with specific metals for the native and active states are designated as metalloenzymes. Removal of the metal by most physicochemical treatments is not possible. If the metal is however removed, there is complete loss of enzymatic activity. The metal is generally unique, in other words the metal is an integral constituent of the enzymes. The ratio of metal to enzymatic activity is variable.

Role of Minerals in Enzyme activity:

Minerals bring about rate enhancements in the enzyme catalyzed reactions by one or more ways as mentioned below.

Direct Participation in Catalysis as Carriers of Electrons or Groups:

The cofactors are many times integral constituents of active enzymes and are involved in carriage of electrons or groups during reactions, for example, Fe, Cu in the cytochrome oxidase and Co, in case of the homocysteine methyl transferase respectively carry electrons and a methyl group.

Formation of Metal-Substrate Conjugate:

The minerals may bind to the substrate prior to its binding at the active site. This may facilitate the binding or cause an orientation or recognition effect and hasten the formation of the transition complex.

Generally in such reactions a scheme as shown can be observed,

S + M ======== SM

SM + E ======= EMS ——————▶ E + M + P.

Formation of metal- enzyme conjugate

Alternately a metal or inorganic ion may bind to free enzyme before the substrate binds to form the transition complex. This binding may be at the active site or some other site and may have an effect on the enzymatic reaction. The metal may aid in activation of the enzyme, facilitation of substrate binding, ionic interactions within the active site side chains, stabilization effects on the pre transition state etc.

In this case the reaction proceeds by the following sequence,

E + M ======== EM

EM + S ======== EMS ——————▶ E + M + P.

In such cases the transition complex formed may resemble the Grignard reagent type system in which the metal or inorganic factor acts as a bridge between the enzyme and the substrate.

Induction of a Conformational Change Within the Enzyme Polypeptide

The metal may interact at the active site or an allosteric site leading to a conformational change that is effective in substrate binding or aids in product removal.

Alteration of the Equilibrium Constant

Metals may change the apparent equilibrium constants by changing the nature of reactants.

Aid in Crossing the Activation Energy Barrier

Metal binding may cause alterations in localized interactions resulting in the redistribution of energy facilitating in the crossing of the activation energy barrier resulting in rate enhancement of the enzymatic reaction.

Catalytic functions of certain metal ions are tabulated as below,

Table 7.12 Metals and their roles in enzyme catalysis

Metal ion	Catalytic functions
Mo	Integral part of nitrogenase complex
Cu	Electron carrier component of cytochrome oxidase
Mn	Cofactor of enzyme arginase
Zn	Cofactor of enzyme alcohol dehydrogenase
Se	Component of enzyme Glutathione peroxidase
Va	Component of nitrate reductase
Mg	Forms complex with ATP and binds kinases

It should however be noted here that metals, especially the heavy metals might strongly antagonize the enzyme- catalyzed reactions. They act as enzyme poisons and

in most cases irreversibly inhibit the reactions. Ion antagonisms have been studied by many scientists and it has been noted that many times a replacement of a specific ion may result into markedly reduced rate or complete inhibition of an enzyme catalyzed reaction. Some of such antagonistic pairs are tabulated below,

Table 7.13: Metal antagonism in enzymatic catalysis.

Enzyme	Activator ions	Inhibitor ions
Acetyl CoA synthetase	Mg or Mn + K	Na or Li
Pyruvate Kinase	Mg	Ca
Glycerol dehydrogenase	NH4	Zn
Argininosuccinate synthetase	Mg	Mn or Ca
Riboflavin kinase	Mg, Zn or Mn	Hg, Fe, or Cu
Myosin ATPase	Ca	Mg
Glutamine synthetase	Mg or Mn	Ca
Inorganic pyrophosphatase	Mg	Ca or Zn

◆◆◆◆◆

8

ENZYME REGULATION

Introduction

Salient Features of the enzyme biosynthesis process

The central dogma

The formation of amino-acyl t-RNA molecules

The synthesis of proteins on ribosomes

Types of regulatory mechanisms

Regulation of enzyme synthesis at the genetic level

The concept of operons

Inducible operons

Repressible operons

The regulator genes

Repression in multi-path systems

Regulation of enzymatic activity by precursor activation and substrate chanelling:

Some Considerations

Signal amplifications in metabolic regulations:Examples of control of metabolic pathways:

Introduction

The vast number of enzymes and enzymatic reactions within a given living cell is often intriguing. A question that easily arises is how the actions of these enzymes are regulated. In case of prokaryotes that are subjected to constantly altering conditions depending upon the environmental factors and availability of nutrients, accumulation of metabolites in the medium and other stressful conditions, the survival is mainly the result of altered metabolic patterns resulting from the altered enzymatic status of the cells. This is directly related to the ability of these cells to utilize, or degrade certain chemicals that may pose a threat to their existence. Even in case of eukaryotes, especially the animals, the needs of the cells vary greatly with respect to their composition and general functions as well as the environmental conditions and the patho-physiological status of the individual organism.

In man inability to synthesize particular enzymes leads to specific metabolic diseases, and many of these are genetically inherited. The needs and availability is constantly changing and that calls for a stringent control of the activity of specific enzymes within the body. This brings forth the core issue of enzyme regulation.

Salient Features of the Enzyme Biosynthesis Process

Proteins are normally synthesized in the ribosomes. Very small components are synthesized in nuclei and mitochondria, but the major portion is synthesized in the cytoplasm through the protein synthesizing apparatus i.e.ribosomes. Scientists have successfully demonstrated the synthesis of many enzymes with the help of isolated ribosomes *in vitro*. The nature and content of the enzyme or protein synthesized is determined by the specific gene encoding its composition. Recent advances in science especially in the field of molecular biology have made it clear beyond doubt that a gene contains all the information about the specific protein that it synthesizes. The components of this system include,

a. The polynucleotide sequences serve as templates for the synthesis of polypeptides.
b. The complementarity between the template and the polypeptide synthesized on it.
c. The specificity of the ligases and other enzymes involved in the overall process of synthesis of the polypeptides.

A gene that comprises of the code for synthesis of a protein, is composed of polynucleotides, generally deoxy ribonucleotides [DNA]. The information specifying the sequence of amino acids in the protein or enzyme thus resides in the sequence of the nitrogenous bases within the polynucleotide.

The Central Dogma

The central dogma is generally represented as,

DNA======➔ RNA ========➔ Protein [enzyme]

1. The transmission of information is initially through the synthesis of a specific RNA based on the code of nucleotides present in the *template DNA*. This message is exactly complementary to the template sequence.
2. The RNA so formed is aptly called as *Messenger RNA*, as it binds to the ribosome and causes the formation of the particular specific protein and no other protein can be formed.

3. The translation process is achieved by the role of specific t-RNAs, charged with their respective amino acids, that can bind to the protein synthesizing machinery based on the complementarity between the anticodons on the t-RNA and the codon on the m-RNA.
4. The binding of amino acid-t-RNA to the ribosome facilitates the synthesis of a peptide bond and such bonds are successively formed till all the information has been translated [the specific protein has been completely synthesized].
5. This is followed by the release of the newly synthesized polypeptide chain from the complex by an ATP dependent terminator.

The messenger RNA forms only a small part of the total cellular RNA but it undergoes the highest turnover, about one hundred times compared to other RNA forms, during the protein synthesis. Because it is immediately degraded after the translation and since the protein synthesis is almost an ongoing process within the cell. In most cells therefore the flow of m-RNAs from the nucleus to ribosomes exists in the cell. It is also apparent in infection of the cell by a foreign DNA.

The Formation Of Amino-acyl t-RNA Molecules

The charging of t-RNA with specific amino acids is brought about by ligases or synthetases usually belonging to 6.1.1 that catalyze the reaction generally written as,

Amino acid + t-RNA + ATP === amino acyl –t RNA + AMP + Ppi

Many such synthetases are isolated and purified and are indicative of the fact that a specific enzyme may exist for a specific amino acid and its corresponding t-RNA. Thus each amino acid becomes tagged to the specific t-RNA bearing a particular anti-codon [sequence of three nucleotides complementary to the three-nucleotide sequence read on the messenger RNA]. This therefore translates the nucleotide sequence of the template [and that of the original gene] into a polypeptide resulting into the exact sequential incorporation of the amino acids in the protein that is synthesized. The t-RNA molecules are therefore termed as adapters that enable the amino acid to occupy its specific position in the sequence of the protein.

After the charging of the amino acid to the t-RNA, its position in the protein is solely determined by the anticodon carried on the t-RNA. In other words if a charged amino acid is chemically modified, and a different amino acid generated, it will not affect its incorporation at the place where the original amino acid was to be placed. The experiment of Chapeville et.al has demonstrated the fact. They charged cys-t-RNA with cysteine and later modified the amino acid to form alanine. When this alanine containing cys-t-RNA was used, alanine was incorporated in the place of cysteine.

Few of the t-RNAs have been purified so far. They are generally small molecules containing around 150-200 nucleotides in their chains. All the t-RNA isolated so far end with a CCA terminal. The adenosine sugar combines with the amino acid through its 2' or 3' hydroxyl group. The sequences in the t-RNA that guide the binding of specific amino acid to it by the action of the synthetases are not properly understood. It can be said that since the terminal is same for all the AA-t-RNA the probability that some remote nucleotide sequence prompts the specificity of reaction between the enzyme and the RNA cannot be ruled out. However the exact length and location of this recognition sequence is not known.

Experimental evidence shows that the components are not species specific in most cases. In other words, during in vitro experiments, a general interchange of t-RNAs and the synthetases obtained from different species have produced fruitful AA-t-RNA complexes. This confirms the fact that the code is widely common if not hundred percent universal.

The Synthesis of Enzymes [proteins] on Ribosomes:

Ribosomes are sub cellular organelles that act as a site for protein synthesis. A cell may contain many ribosomes, but at a given time only some of them appear to be associated with protein synthesis. More so in the case of eukaryotic m-RNA as these RNA are mono cistronic. It is indicative that there is an apparent insufficiency of messenger RNA to combine with ribosome. Addition of m-RNA in the presence of Mg++, it combines with free ribosome spontaneously. Before the beginning of the synthesis of polypeptide the ribosome undergoes a sequence of events termed as the ribosome cycle.

The ribosome initially undergoes dissociation into its constituent sub units followed by the binding of the small sub unit with the m-RNA. This is followed by the binding of the large sub-unit to the complex. This is followed by the attachment of the first amino acyl carrying t-RNA.

The incorporation of amino acids in the polypeptide is not a spontaneous process but is an enzymatic reaction. The enzymatic reaction involves GTP, per mole of amino acid incorporated. After the amino acid is added to the growing polypeptide chain the t-RNA is discharged in its active form and can be reused for charging with its specific amino acid. The enzyme catalyzing the transfer is unspecific for the t-RNA and m-RNA but seems to be specific for the ribosome. The formation of the peptide bond is such that the carboxyl end of the existing polypeptide is condensed with the amino group of the incoming amino acid, followed by the transfer of the elongated chain to the newly joined AA-t-RNA. The completed chain does not leave the complex spontaneously, but another ATP dependent enzyme is required for the release mechanism. The antibiotic puromycin however can cause the release of incompletely synthesized chains.

Types of Regulatory Mechanisms

Studies on the regulatory aspects of enzyme action have generated a body of evidence that *in vivo,* there are three broad categories under which enzyme regulation can be discussed.

1. Regulation of enzyme biosynthesis [genetic control]
2. Regulation of enzyme activity through precursor activation mechanisms and substrate chanelling.
3. Enzyme inhibition and allosterism.

The basic concepts involved in these mechanisms are now discussed here.

Regulation of Enzyme Synthesis at the Genetic Level (Induction and Repression)

It is now known that the synthesis of the enzymes in the cells is brought about by the transcription and translation. For the formation of a given enzyme in the cell, the presence of the corresponding structural genes is essential. In many cases however the mere presence of the genes does not produce the required enzymes. Some small molecules that may be related to the reaction catalyzed by the enzyme must be present [cause induction] or absent [those causing repression] for the enzyme to be synthesized in

enough amounts. The induction and de-repression mechanisms play an important role in regulating the synthesis of enzymes according to the requirement of the cell.

The repression and induction effects differ from the activation and inhibition effects. Repression and induction affect the actual amount of the enzyme formed while inhibition and activation affect the activity of the enzyme. Under the influence of an inducer, the enzyme protein, that was previously undetectable, is synthesized de Novo and becomes apparent in the cell. Many such enzymes have been identified.

If an inducer is added to a growing bacterial culture, the production of related enzyme begins in a short time interval and continues till the inducer is present. The synthesis ceases in a short interval of two to three minutes after the inducer is removed.

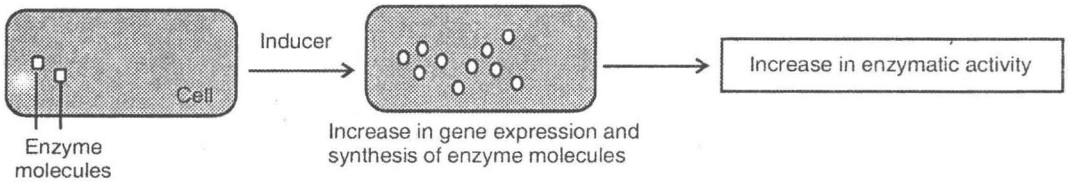


Fig. 8.1 Effect of inducer on enzyme synthesis

Similar effects have been seen with the repressors. Removal of the repressor from the growth medium can increase the synthesis of the enzyme multifold. Re addition of the repressor causes a drop in the level of the enzyme in a short while. In cases where the repressor is the product of the enzymatic reaction, its removal speeds the enzyme synthesis and activity but after some time the enzyme synthesis stops as the reaction product accumulates.

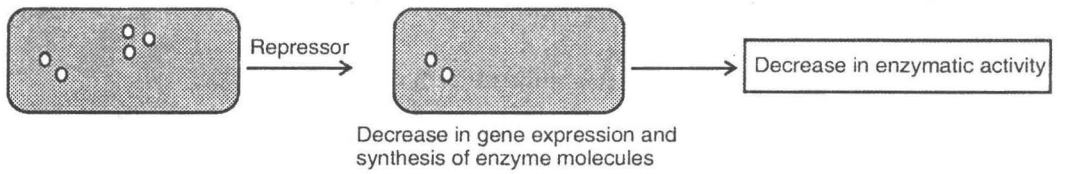


Fig. 8.2 Effect of repressor on enzyme synthesis

Both induction and repression are specific effects. The specificity is related to both the chemical composition as well as spatial arrangement [stereo-specificity]. The natural substrate of an enzyme is not necessarily the best inducer. Even the rate of induction may not be proportional to the concentration of the inducer.

The induction mechanism is not related with the occurrence of the reaction catalyzed by the enzyme. In other words the inducer may not act as a substrate or in other cases the enzyme synthesized in response to the inducer may not be active. i.e. if an enzyme requires an essential contribution of a coenzyme that is rendered unavailable, the enzymatic reaction does not occur however this does not stop the synthesis of the apoenzyme. This is shown from the synthesis of tyrosine decarboxylase by Streptococci in a pyridoxine-deficient condition in response to tyrosine, which acts as an inducer for its synthesis. Addition of pyridoxal phosphate then leads to the activation of enzyme.

It is a general observation that most inducible enzymes are synthesized in response to their substrates or substrate analogs although the occurrence of the reaction is not essential. It is sometimes seen that the addition of a chemical,[substrate or analog] may induce the synthesis of more than one enzymes that are usually components of the pathway that catabolizes it as seen in the lac operon.

The repression differs from induction on two grounds.

1. The repressor acts to prevent the synthesis of enzyme, while the inducer causes its synthesis.
2. The inducer is related to substrate while the repressor is related to the product.

They are thus complementary to each other. In case of an enzyme deficiency, the substrate shall accumulate and alternately cause the induction of its synthesis. This will lead to transformation of substrate into product [s] that on accumulation shall lead to repression. The net result is adjustment of the levels of the enzyme within the cell.

There has been a speculation whether both the mechanisms are used for the regulation of action of an enzyme. In other words is it possible that, the same enzyme is induced by a substance while repressed by the other? In general, evidence indicates that in majority of the cases if the enzyme is formed by induction, it is not subject to repression. It is however possible that an enzyme is inducible in an organism while it is repressible in another species.

A single substance affects the formation of more than one enzyme and this is equally common to both, induction and repression. Usually these enzymes are metabolically related or a part of the same catabolic or anabolic pathway. For example a beta galactoside induces the synthesis of 3 enzymes, viz, galactoside permease, beta galactosidase, and acetyl transferase. In case of induction by D-fucose, the three enzymes of the gal operon, are namely galactokinase, hexose-1 phosphate uridyltransferase and UDP glucose epimerase. Although the latter 3 enzymes are also related to galactose metabolism, they are not induced by methyl-beta-thio galactoside, a strong inducer for the enzymes of the lac operon.

In case of repression, L-histidine represses the formation of four enzymes in Salmonella. They are, imidazole-glycerol phosphate dehydratase, histidinol phosphate amino transferase, histidinol phosphatase, and histidinol dehydrogenase, all involved in the synthesis of L- histidine. Such an effect is termed as coordinate repression. Similar effects are seen with arginine, and uracil and its derivatives on their respective synthetic pathways. In case of uracil the effect is probably due to its conversion to UMP, that acts as the actual repressor, but in case of the amino acids, they do act as repressors themselves. In case of metabolic pathways, if an enzyme is not produced due to a genetic manipulation, it leads to the repression of all the enzymes.

Stanier suggested a sequential model for induction. It hypothesizes that within the sequence of reactions, each product of the enzymatic reaction acts as an inducer for the subsequent reaction in which it acts as a substrate and there may be no resemblance of the latter substances with the primary inducer. For example, if Pseudomonas is grown in presence of tryptophan, there is a formation of many enzymes that catalyze its transformation through the formation of intermediates like kynurenine, anthranillate, muconate etc. and these enzymes are absent in case of the cells grown in the absence of tryptophan.

A 'sequential repression' can be easily explained in similar terms. The repression

of a particular enzyme would result in the accumulation of its substrate and that may repress earlier enzyme and in turn all earlier enzymes would be sequentially repressed. Such a repression would be independent of a genetic block and be sequential in time. In actual conditions, the enzymes are all affected simultaneously and in many cases the products do not act as repressors for their respective enzymes.

The Concept Of Operons

In most of the microorganisms, most of the related genes exist in clusters on the chromosomes. It is comparatively less frequent with higher organisms. The chromosomes thus contain a volume of information about,

a. The content of enzymes in terms of amino acid composition

b. The metabolic pathways that are catalyzed by these enzymes.

A group of consecutive genes functioning as a unit, arranged on a chromosome is known as the "operon". An operon is schematically represented as,

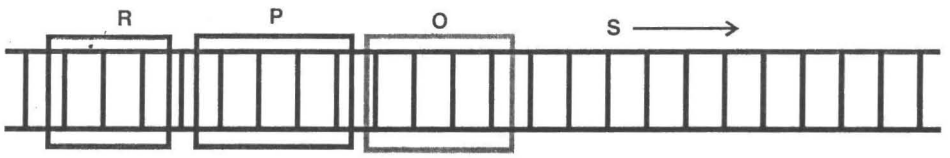


Fig. 8.3. Schematic representation of an operon.

Legend: R= regulator, P = promoter, O = operator, S = structural genes.

If the genes are located within the operon, they are all affected during induction or repression, a phenomenon called as coordinate induction or repression. While in case they are dispersed they are affected in different magnitudes and the phenomenon is called as non- coordinate induction or repression. The transcription of an operon is such that it behaves like a single unit, starting from one end through to the other. The point at which the synthesis of m-RNA begins on the operon is called as operator, (o). Mutations may alter the operator in such manner that there is no m-RNA synthesis from this point. In such a case the complete operon is made inactive and no enzymes are synthesized even in the presence of the inducer. This inactive operon cannot act on other gene clusters on the chromosome and is not affected by the other active operators. It is thus apparent that the operator provides a vehicle for control of synthesis of the enzymes related to a particular pathway.

Although the work on operon indicates their presence in microorganisms, it is possible that they may exist in higher organisms. They are however not the only mechanism of regulation since in many cases the structural genes do not lie in a single cluster and are found to be scattered on the chromosome. In such a case each gene may have its individual operator having a common or related structural feature

The relationships between the repressor protein, operator region, effector molecules and expression of the operon can be generally represented as,

Inducible Operons

Free repressor ⟶ binds to operator ⟶ GENES "OFF" = no transcription

Repressor + inducer ⟶ do not bind to operator ⟶ GENES "ON" = transcription.

Repressible operons

Free repressor ⟶ cannot bind to operon ⟶ GENES "ON" = transcription

Repressor + corepressor ⟶ bind to operator ⟶ GENES "OFF" = no transcription.

The Regulator Genes

The regulator genes exist within chromosomes and control the synthesis of the various enzymes or enzyme groups. They differ from the structural genes in that they do not control the sequence of the enzymes synthesized but regulate the rate at which the enzymes are synthesized. Their location on the chromosomes is not necessarily in the vicinity of the gene clusters they control. On the contrary they may control a cluster located on a different chromosome, indicative of the fact that they may do so with the help of a intracellular substance usually formed as their product. Study on mutation in these genes led to the understanding of the mechanism of induction and repression.

For example, mutations in the E.coli galactosidase operon give rise to i.- constitutive mutants. In case of the normal i.+ cells the enzyme galactosidase is formed only in the presence of the inducer. In case of the constitutive mutants the enzyme is synthesized in both, presence and absence of the inducer. If two chromosomes are present, one containing the i.+z.- and the other, i.- z.+ the enzyme is formed in the presence of the inducer.

Isotopic experiments indicate that this cytoplasmic repressor acts by preventing the formation of m-RNA by the gene it controls. It probably combines with the operator and so prevents the RNA formation.

The inducer may act by combining with the cytoplasmic repressor and thereby prevent its action. In other words it can be said that the free cytoplasmic repressor has an ability to bind to the operator but its conjugate with the inducer cannot bind to the operator. The phenomenon thus becomes of the type, "derepression" rather than induction, and in such a case the enzyme induction and repression, are the variants of the same basic mechanism. The production of the enzyme thus, in both cases is blocked depending on the interaction of the repressor with the operator. In case of the inducible system the free cytoplasmic repressor can combine with the operator while its compound with the inducer cannot. Whereas in repressible system the free cytoplasmic repressor is unable to combine with the operator and it is only a specific conjugate of it, with the corepressor that can bind to the operator. The effect of inducer is to unmask the operator while that of a repressor is to mask the operator. The effect can be diagrammatically shown as,

Legend: R= repressor, C = corepressor, i.= inducer, E = polymerase.

The nature of the cytoplasmic repressor is probably a small molecular RNA. There are several points that support this hypothesis.

1. It is formed on DNA and its structure is determined by the sequence of DNA coding for it.
2. Its structure has a specific relation and affinity towards the operator where it binds.

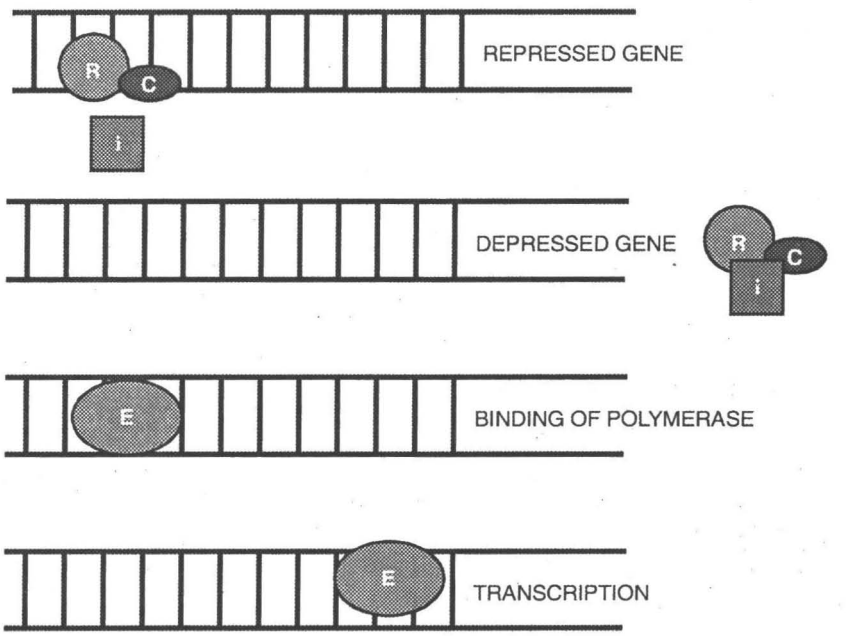


Fig. 8.4: Possible mechanism of induction/repression.

3. Its formation is unaffected by chloramphenicol, a protein synthesis inhibitor.
4. The corepressor is often an amino acid or a nucleotide.
5. There is a high specificity between the binding of the repressor and corepressor.

This may lead us to assume that the repressor-RNA contains a nucleotide sequence that is complementary to the operator. When the same repressor controls genes that are scattered it is possible that all these genes have similar terminal sequences.

The repression of all the enzymes of a single operon is coordinate, while in case of scattered genes it is non-coordinate.

Examples Of Operon Controls

Some examples of control by operons are briefly discussed here,

1. The Lac Operon Of E.coli

There are three strucural genes in the lac operon, viz., lac z, lac y and lac a. The operon is negatively controlled by a repressor protein. The repressor protein is a product of the gene lac i. and its formation is independent of the operon. The structural genes produce specific enzymes and this is tabulated as,

Table 8.1 Structural genes of lac operon

Gene	Enzyme
lac z	β– galactosidase
lac y	β– galactoside permease
lac a	β– galactoside transacetylase

[a] The Repressor Protein

It is a tetramer with each sub unit of about 40 k.dal. It binds to the operator region and prevents the movement of RNA polymerase that binds to the promoter sequence. It contains two types of sites for interaction, one for binding with the inducer and other for binding with the DNA at the operator region. Binding with the inducer causes an allosteric modification resulting into a conformational change that makes it unfit to bind to the operator region of the DNA.

[b] Catabolite Repression And Positive Control Of The lac Operon

When glucose is present, it is preferably used as the source of carbon and therefore all the operons for utilization of all other carbohydrates are switched off. This is accomplished by a process called as catabolite repression. It has been observed that glucose utilization is accompanied by low levels of cAMP. If cAMP and lactose are supplied to the growing cells, the lac operon is preferably induced. The cAMP complexes with a protein called catabolite activator protein (CAP) and releases the repression of the lac operon by glucose metabolites. The complex binds to a region on the DNA called as the catabolite recognition element (CRE) present upstream of the promoter region. The binding of CAP-cAMP to the CRE enhances the transcription of the lac operon. In the absence of the CAP-cAMP-CRE complex, the level of transcription of the operon is very low.

The relation of glucose level and CAP-cAMP and the transcription of the lac operon is summarized as,

(i) Glucose (high) ⟶ cAMP (low) ⟶ no CAP-cAMP complex = no transcription.

(ii) Glucose (low) ⟶ cAMP (high ⟶ CAP-cAMP complex = transcription of lac genes.

2. The trp Operon

The trp operon is under dual control. [a] Repressor control [b] attenuator system.

The operon comprises of 5 structural genes, A, B, C, D, E. The products of these genes catalyze the conversion of chorismic acid to tryptophan.

[a] The Repressor Control

The product of the R gene (regulator gene located outside the operon) needs to complex with the effector (trp itself, as it acts as a corepressor) to bind to the operator and prevent transcription. At low concentrations of tryptophan the complex is not formed, and transcription is turned on.

[b] The Attenuator

The attenuator serves as a double check for the control of trp synthesis. It is located between the promoter and trp E gene. It provides a barrier to transcription into the structural genes. It responds to the level of tryptophan. When tryptophan is present, the operon is repressed, and the polymerase that escapes from the promoter then terminates at the attenuator.

The leader sequence can be written in alternative base-paired structures.

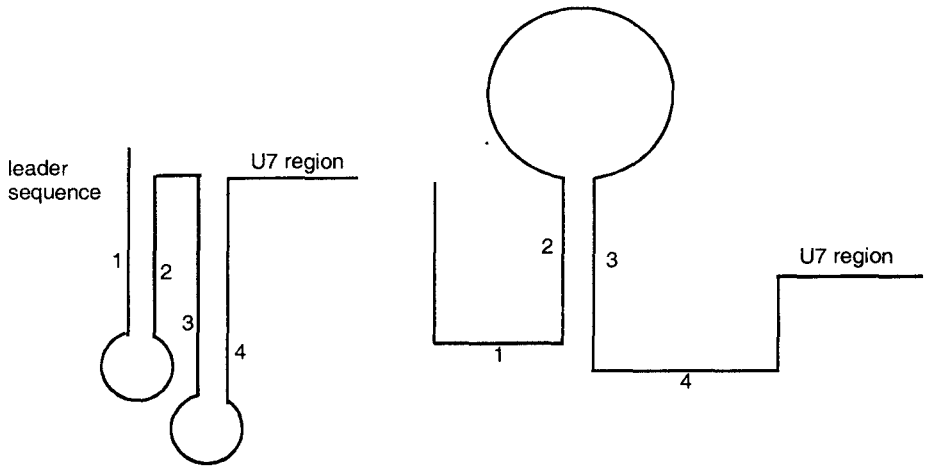


Fig. 8.5 Schematic representation of attenuator in tryptophan operon

The movement of the ribosome through the leader sequence controls the transition between the structures, which in turn decides the termination of the operon at the attenuator. In the first structure, region1 pairs with region 2 and region 3 pairs with region 4. This type of pairing results in the formation of the hairpin preceding the U7 sequence. This serves as an essential signal for intrinsic termination.

When trp is present, the ribosomes are able to synthesize the leader peptide, they continue to the UGA codon lying between regions 1 and 2 and then extend over region 2 preventing its base-pairing with region 3. This results into a base pairing between regions 3 and 4 forming a terminator hairpin. Under these conditions therefore, the polymerase terminates at the attenuator.

In absence of trp, the ribosomes stall at the trp codons located in region 1, which is therefore sequestered in the ribosomes and unavailable to form a base-pairing region with region 2. This means that the regions 2 and 3 are paired before the transcription of region 4 compelling it to remain single stranded. This does not form the required terminator hairpin and polymerase continues transcription past the attenuator.

Repression In Multi-Path Systems

A repressible enzyme may play a part in more than one biosynthetic pathway, leading to the formation of two distinct end products. Take for example the aspartate kinase of E.coli that participates in the synthesis of lysine and threonine. If the enzyme could be repressed independently by both end products it would result in the case that excess of lysine would inhibit the formation of both lysine and threonine, leading to a threonine deficiency and vice versa.

It has been reported that the organism contains two isoenzymes, separable by salt fractionation. Both these enzymes catalyze the same reaction and show identical kinetic behaviors. But the first enzyme is repressed by lysine while the second is unaffected. Lysine also acts as a non-competitive inhibitor for the enzyme. Threonine on the other hand acts as a competitive inhibitor for the second enzyme but does not affect the activity of the first. So even if the enzyme is repressed by lysine excess, the second isoenzyme remains functional eliminating the chances of threonine deficiency.

In an alternative case where the analogous reactions in different pathways are carried by the same enzyme [s] as in the case of synthesis of isoleucine from ketoglutarate and that of valine from pyruvate. The last four steps are common to both pathways and are catalyzed by the same enzymes.

It was found that the enzyme that is involved in the production of one of the amino acids is repressed by the amino acid itself. But the enzyme that is involved in the biosynthesis of all the three amino acids is repressed only by a mixture of all three and not by any single amino acid. Such a type of repression is called as "multivalent repression".

The multivalent repression prevents the deficiency of any one product due to the accumulation of other in pathways that branch for the synthesis of multiple products using partial common pathways.

Regulation Of Enzymatic Activity By Precursor Activation And Substrate Chanelling

[A] Precursor Activations

In the higher living organisms many enzymes are synthesized and secreted in precursor forms. This is especially true about the protein splitting enzymes like pepsin, chymotrypsin, trypsin, rennin and carboxypeptidases as well as those that involved in the blood clotting mechanisms.

Conversion of the precursors into the active forms is brought about by the catalytic removal of specific portions from the inactive precursor or by the activity of the hydrogen ions. For example, pepsinogen and prorennin are activated by hydrogen ions or by pepsin, chymotrypsinogen and procarboxypeptidase A by trypsin while trypsinogen is activated by activated trypsin itself. In case of trypsin thus the activation sequentially becomes rapid as the activated molecules further cause activation of the inactive precursors.

In most of the cases the activation is brought about by breaking specific peptide bonds with or without the removal of peptides and amino acids.

The difference in the amino acid composition of the inactive and active forms of the enzymes as well as the elucidation of the mechanism of activation of these enzymes have led to the understanding of the importance of the structural and chemical composition of the active site. They also reflect on the role of some auxillary groups that are required to maintain the specific three-dimensional structure as well as the ionization pattern of the active site residues. In many cases the activation process seems to unmask the active center or alteration of the microenvironment, so that the substrate binding is more efficient. A better understanding can be had with the illustration of specific examples. Following is a brief discussion on the activation of pepsinogen and trypsinogen.

The Activation Of Pepsinogen

The pepsinogen from pig has a molecular weight of 42,000 while that of pepsin is about 35,000. Under electrical field pepsin migrates as a negative ion and on isoelectric focussing it can migrate up to pH 1.0 whereas the inactive form settles at pI 3.7. It thus appears that pepsin is much more acidic than its precursor.

The activation involves the removal of nearly $1/5^{th}$ of the molecule that is predominantly basic.

The activation begins by the addition of pepsin or by acidification and proceeds auto-catalytically below pH 5.0 the maximum rate being around the pH 2. The formation of active pepsin from pepsinogen is accompanied by removal of six small peptides, the largest of these is termed as the trypsin inhibitor and remains attached to the molecule above pH 5.4, under these conditions the process is not autocatalytic.

The active and the inactive precursor both comprise of a single polypeptide chain. Use of carboxypeptidase to determine the C terminal sequence of both the forms indicates that the sequence is common. It was found to be R-valyl-leucyl-alanine-COOH. It can mean that the pepsin forms the C-terminal part of the pepsinogen molecule. The process of activation of pepsinogen and its conversion to pepsin can be represented as,

$$\text{Pepsinogen} \xrightarrow[\text{.pH < 5.4}]{\text{pepsin}} \text{Pepsin — inhibitor complex} + 5 \text{ peptides}$$

$$\text{Pepsin — inhibitor complex} \;\text{==========}\; \text{pepsin} + \text{inhibitor}$$

$$\text{Inhibitor} \xrightarrow{\text{(pepsin)}} 4 \text{ peptides}$$

The analysis of the amino acid sequence at the N terminal of the inactive enzyme, inhibitor and the active enzyme has been performed and the sequences can be tabulated as,

Table 8.2. N-terminal of active and inactive PEPSIN

Peptide/ polypeptide	N terminal sequence observed
Pepsinogen	N- leucyl-isoleucyl-R
Inhibitor	N- leucyl-glutamyl-R
Pepsin	N- isoleucyl-glycyl-R

It is thus apparent that the N terminal sequence differs in these cases indicating that the inhibitor does not occupy the N-terminal position in the pepsinogen molecule. Pig and Ox pepsins appear to be identical proteins by all ordinary tests like the appearance of crystals, solubility, physical properties and activity on several different substrates. The activation and action of pepsins from different species may show some species specificity. Inhibitor from pig pepsinogen inhibits pig and ox pepsins but does not inhibit fowl pepsin.

A schematic representation of the arrangement of the components in the pepsinogen can be shown as,

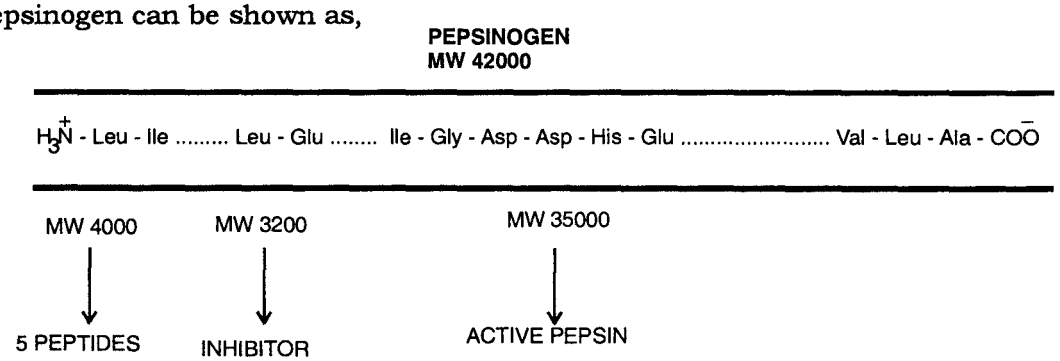


Fig. 8.6. Arrangement of pepsinogen and pepsin

The Activation Of Trypsinogen

The enzyme trypsin is secreted as a precursor in the form of trypsinogen in the pancreatic juice. Crystalline trypsinogen can be activated by trypsin itself, indicating that under proper conditions the process is autocatalytic. However other enzymes isolated from other sources including the enteropeptidases can also activate the zymogen and in all the cases the same trypsin is formed.

During autocatalysis two reactions proceed. One is the conversion of trypsinogen into an inactive protein and the other resulting in the formation of active trypsin. Both these reactions compete and the pH and the ionic mileu dictate the ratio of the inactive and active proteins formed. Of particular importance is the effect of calcium. The calcium ions accelerate the synthesis of inactive protein and supress the synthesis of active trypsin. The effects of autocatalysis and the action of enteropeptidase can be studied by varying the concentration of the inactive precursor.

The molecular weight studies do not indicate any difference in the inactive and active form but this might be an error. Both the forms comprise of a single polypeptide chain in which the N terminal is detectable but free C terminal appears to be absent. This has led to a suggestion that the enzyme may have a cyclic structure at the C end. In case of the ox trypsinogen N terminal analysis proves that there is a loss of a hexa-peptide during the conversion of the inactive form to the active form. This is achieved by a single split between the Lys and Ile residues. The species- specific differences are present here as well. The trypsinogen from pig pancreas loses an octa peptide from the N terminal during its activation. Moreover the C terminal is apparent in this species and the usual C terminal sequence is Asp-Ala-Ile-COOH or Glu-Ala-Ile-COOH.

A schematic representation of activation of trypsinogen is given as,

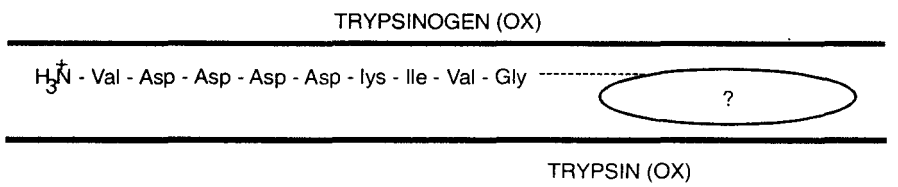


Fig. 8.7. Ox trypsinogen.

The schematic representation of the activation can be written as,

Trypsinogen ———trypsin/kinases/acidic pH—→ Trypsin + hexapeptide.

[B] Substrate Channeling

The regulation of enzymatic activity can also be brought about by the channeling and access of the substrates. Efficient mechanisms are present in terms of compartmentalization of metabolic pathways and enzymes that involve a fine tuning in terms of the supply of the substrates and cofactors across subcellular organelles such as mitochondria, lysosomes, nuclei etc.

In case of the prokaryotes the compartmentalization within the cell prevents the contact between enzyme and its substrate whereas eukaryotic cell organelles often display selective permeability to avoid the contact between enzyme and substrate.

On the other hand multienzyme complexes allow the efficient channeling of intermediates so that they are not lost in the bulk of the cytoplasm and the overall reaction is rapid. Since the product of the earlier reaction serves as a substrate for the next reaction the transit time is greatly reduced and there is a high local concentration of the intermediate in the vicinity of the next enzyme active site. The other advantage is the protection of the chemically labile intermediates and in case if the metabolite can take part in many different reactions, then channeling enables the fruitful completion of a particular pathway without the exit of the intermediate to another pathway.

Enzyme Inhibition and Allosterism

The regulation of enzymes at the cellular level is an efficient method as a lot of different modulation mechanisms are available to bring about the regulation of enzymes. In general, the activity of enzymes is relevant with the concentration of their substrate, and variation of the substrate in the intracellular conditions could reflect on the rate of reaction. Similarly fluctuation in the ionic species may also provide a mechanism for regulation in case of enzymes that require cofactors for activity.

Some enzymes however possess certain properties that make them special in terms of regulation of the metabolic pathways. This is due to their ability to react with certain specific compounds, end products of the particular pathways that they catalyze [feed back inhibition], or modulators that modify their rate of reaction through covalent and non-covalent interactions [activators, reversible inhibitions and covalent modification by phosphorylation-dephosphorylation mechanisms]. Such enzymes are termed as regulatory enzymes and most of them show allosterism.

Allosteric Enzymes

The term "allosteric" originated from the Greek words, allos = other, and stereos = space. It defines enzymes that carry 'other space', a site other than the active site, where the allosteric effector binds. The word effector is used in a broad sense and it could be a positive or a negative modulator of enzyme activity.

Allosteric enzymes are typically oligomeric in nature. They contain many sub units and in turn substrtate binding sites and allosteric sites. Their structure is referred as quaternary structure, a term introduced to denote macromolecular assemblies constructed through non- covalent association of separate polypeptides, each of which may have its own tertiary structure. The subunit is called as a monomer.

The monomers may be identical [example: lactate dehydrogenase] or non identical [NADP: sulfite oxidoreductase]. On the basis of the geometrical arrangement, the association of sub units is homologous or isologous, [binding sets comprising of head to head or tail to tail] and heterologous [binding sets comprise of head to tail and vice versa].

The enzymes show a typical kinetic behavior that differs from the classical MM kinetics. Some of the properties include,

(A) Sigmoidal curves of v against [S].

(B) Lag periods or initial bursts appear

(C) Synergistic or antagonistic effects are seen.

(D) Action of regular metabolites is time dependent.

They also have a much larger molecular size, and are unstable at zero degrees centigrade.

When the rate of the enzymatic reaction is modulated by a single modulator, it is termed as monovalent modulation. When more than modulators are required, it is termed as polyvalent modulation. Depending on the nature of the modulator there are two broad groups of allosteric Enzymes,

They are,

1. **Homotropic allosteric enzymes:** These enzymes are modulated by the action of their substrates. In other words the substrate acts as a positive modulator.
2. **Heterotropic allosteric enzymes:** These enzymes are modulated by substances 'other than' the substrate.

Some enzymes may show a hybrid characteristic and can be modulated by both, its normal substrate and some other molecules.

Some of the commonly employed methods of regulating these enzymes are briefly discussed as,

(a) Regulation by Feed Back Inhibition

In some metabolic pathways the regulatory enzyme that is usually responsible for catalyzing one of the initial reactions, is inhibited by the ultimate end product generated by the pathway, mostly so when the concentration of such a chemical entity increases than the cells immediate requirement. The slowing of the regulatory enzyme in turn decreases the rate of the overall reaction and almost all the individual steps involved in it as the substrates for the respective steps are decreased by mass action. The result is that the concentration of the ultimate is balanced to the need of the cell. The mechanism by which the buildup of a final product causes a decrease in its rate of synthesis by lowering the rate of the overall reaction through the regulation of a regulatory enzyme is called as *feedback inhibition.*

It is a common feature with the synthetic pathways of many bio-molecules. There are at least two ways to bring about the feed back inhibition.

1. The concerted feedback inhibition
2. The sequential feedback inhibition.

Illustration of these mechanisms can be understood through examples of each that are briefly discussed as below,

The concerted feed back inhibition: case of glutamine synthetase of E.coli

The glutamine synthetase in bacteria is one of the most complex regulatory enzymes known. It is subject to allosteric and covalent modification. [Fig 8.8] There are 12 identical sub-units and at least 6 end products of the glutamine metabolism and alanine and glycine are allosteric inhibitors of the enzyme. Each sub-unit has binding sites for all 8 inhibitors as well as an active site for catalysis. Each inhibitor alone can partially inhibit the enzyme. The effects of the different inhibitors are more than additive and all 8 together virtually shut down the enzyme. Along with the allosteric regulation it is inhibited by adenylylation of the tyr397 located near the active site. This covalent modification enhances the sensitivity of the enzyme for the allosteric modulators and the decrease is proportional to the number of sub units carrying the adenylyl group.

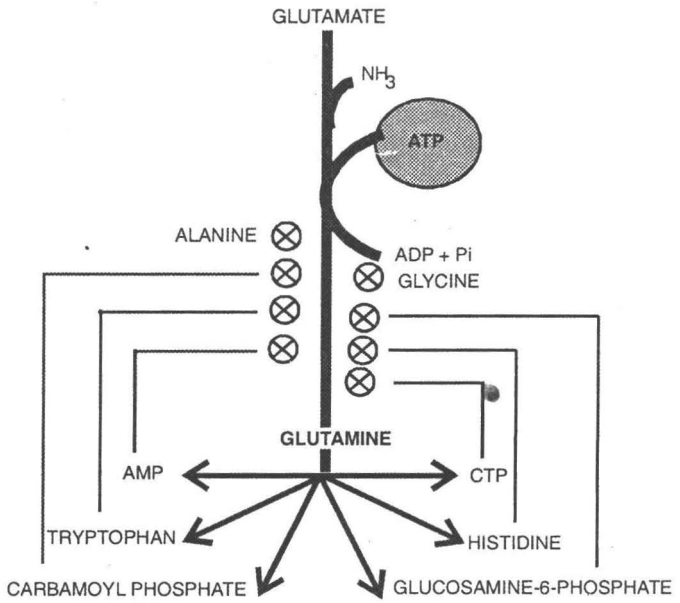


Fig. 8.8. Concerted feed back mechanism: Case of glutamine synthetase

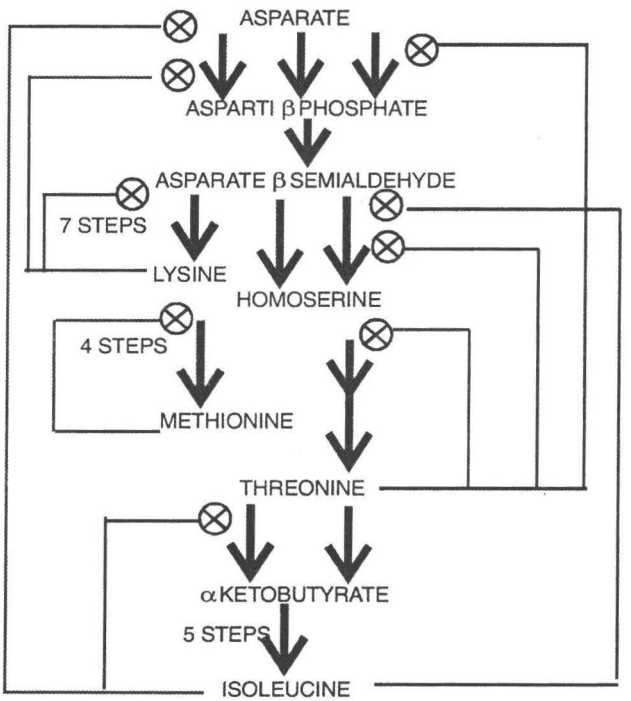


Fig. 8.9. Sequential feed back of isoleucine synthesis

The net result of the mechanisms is a decrease in the glutamine synthetase activity when the glutamine levels are high and increase in activity when glutamine levels are low and the required substrates are available.

The sequential feed back mechanism: case of isoleucine synthesis from aspartate.

The sequence from aspartate to isoleucine is subject to multiple overlapping negative feed back inhibition. [Fig 8.9] Isoleucine inhibits the enzyme that converts threonine to alpha keto butyrate. Threonine on accumulation inhibits conversion of homoserine to phosphohomoserine by homoserine kinase as well as the formation of homoserine by homoserine dehydrogenase. This leads to accumulation of aspartate –β– semialdehyde which then undergoes conversion to lysine. Lysine on accumulation can inhibit the first enzyme, aspartate kinase. A schematic representation is shown as,

Microorganisms use several mechanisms to regulate enzymes through feed back inhibitions. Some of the most commonly found mechanism patterns include,

1. Isofunctional Enzymes

This is characteristic of the branched pathways in which the first step is catalyzed by two or more isozymes. Consider a metabolic pathway that branches at the 4th step to proceed for the production of two different products as shown in the following figure,

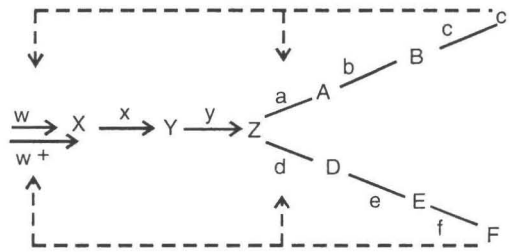


Fig. 8.10 Isofunctional enzymes.

Accumulation of the product C inhibits the enzymes **a** and **w.** However it does not affect the isoenzyme **w*.** this allows the synthesis of the product F. Similarly the accumulated product F inhibits the enzymes **d** and **w*.** However it does not affect the isoenzyme **w.**

The synthesis of amino acids threonine, lysine and methionine from aspartate in *E.coli*, is the best example of this type of mechanism.

2. Sequential Feedback Mechanism

This type of mechanism is seen in case of synthesis of aromatic amino acids in many bacteria. The characteristic feature is that after the branching the products that are formed are capable of inhibiting the first enzyme after the branch point. If the branch point intermediate accumulates, it inhibits the first enzyme in the metabolic sequence. Sequential feedback mechanism is schematically shown as,

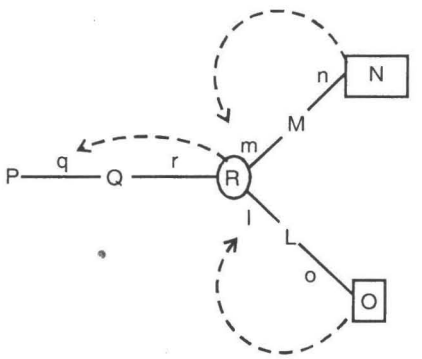


Fig. 8.11 Sequential feedback mechanism

Example of this type of mechanism is the synthesis of isoleucine and threonine in *Rhodopseudomonas spheroides.*

3. Concerted Feedback Mechanism

This mechanism is characteristic of those branched pathways where the first enzyme is not inhibited by either of the products alone, but when both the products

accumulate they act in concert [together] to inhibit the enzyme. A concerted feedback is seen in case of inhibition of aspartate kinase in *R.capsulata.* the schematic representation is shown as,

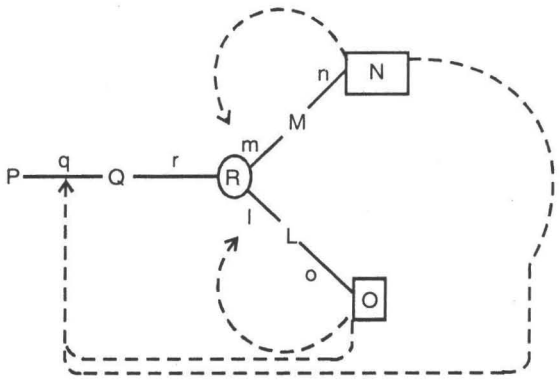


Fig. 8.12. Concerted feedback mechanism

4. Cumulative Feedback Mechanisms

When the first enzyme of the pathway is inhibited by the products independently of each other to a limited extent and the complete inhibition requires the presence of all the end products, the mechanism is termed as cumulative feed back mechanism. A schematic representation of this type of mechanism is shown in the following figure,

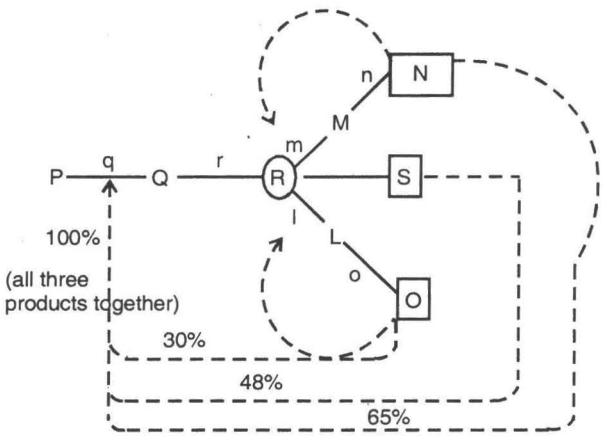


Fig 8.13: Mechanism of cumulative feedback

The example of this type of mechanism is the regulation of glutamine synthetase in *E.coli.*

5. Combined Activation and Inhibitions

In these type of regulations the product formed acts as an inhibitor for its own production but at the same time it enhances the rate of other reactions as an activator. The general scheme of this type of mechanism is represented as,

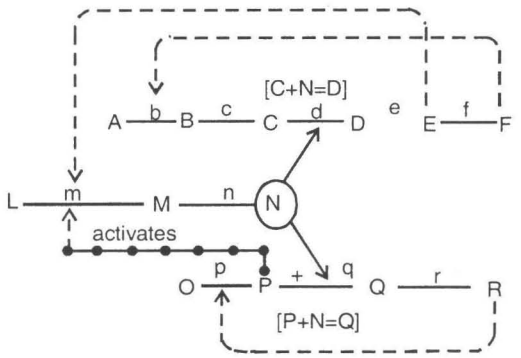


Fig. 8.14: Combined activation and inhibition mechanism

(b) Regulation by cellular metabolites through covalent modulation

The enzymes of this class are inter-converted into active and inactive forms by covalent modifications of their structures usually by other enzymes and proteins. Two such classes are known,

1. Those that use phosphorylation – dephosphorylation mechanisms
2. Those that use adenylation mechanisms.

The classical example of the first type of mechanism is the regulation of glycogen phosphorylase, an enzyme that hydrolyzes glycogen to produce glucose-1-phosphate in animal tissues, especially liver and muscles.

This enzyme occurs in two forms, phosphorylase a, the more active form and phosphorylase b the less active form. The phosphorylase a, comprises of 4 major sub units, each of which contains a phosphorylated serine residue. The phosphate groups are required for maximum catalytic activity. Action of phosphorylase-phosphatase removes the phosphates attached to the serine residues causing the active enzyme [tetramer] to dissociate into inactive enzyme [dimer] or phosphorylase b, which is much less active in cleaving the glycogen molecule. The reaction of the conversion of active enzyme to inactive form can be simply represented as,

Phosphorylase a + 4 H2O ————[phosphatase]—à 2 phosphorylase b + 4 Pi.

The less active form can be converted to more active form by the action of the enzyme phosphorylase kinase, at the expense of 4 moles of ATP.

2 phosphorylase b + 4 Pi —— kinase—→ 4ADP + phosphorylase a

A schematic representation of the conversions can be shown as,

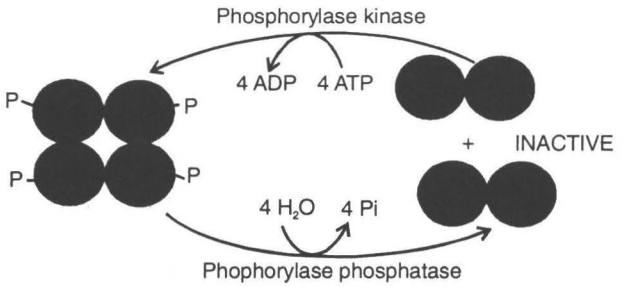


Fig. 8.15. Covalent modifications in regulation of phosphorylase.

Such enzymes usually greatly amplify the signal. This is done by employing cascade mechanisms. One mole of kinase can convert many molecules of less active form into the more active form. This in turn results in the formation of many more moles of glucose-1-phosphate. The kinase is also under a similar regulation and works as a part of the cascade beginning with the binding of epinephrene to membrane receptors.

The adenylation mechanism can be explained with the help of earlier example of glutamine synthetase, from E.coli. The enzyme catalyzes the synthesis of glutamine from glutamate at the expense of 1 mole of ATP. As described earlier, the enzyme comprises of 12 sub units each containing an active tyrosine residue. Addition of the adenylyl group to the phenolic hydroxyl of the amino acid results into conversion of the active form into a less active form. The de- adenylylation of the tyrosine side chains results in the conversion into a more active form. The extent of decrease and increase in the activity of the enzyme is proportional to the adenylylation of the sub units.

[c] Reversible inhibitions in enzymatic regulations

The types and kinetics of the reversible inhibitors are already discussed in previous chapters. These do not essentially fall in the category of feed back or covalent modification based enzyme regulation.

As discussed earlier, reversible inhibitors are of three types, Competitive, Non-competitive and Un-competitive. Each type has characteristic features that appear in the graphical plots that can distinguish them from each other.

The salient features of each can be recollected as,

[I] Competitive Inhibitors

(a) A competitive inhibitor carries structural resemblance with the normal substrate of the enzyme

(b) It therefore competes for the active site, reducing the probability of formation of ES complex.

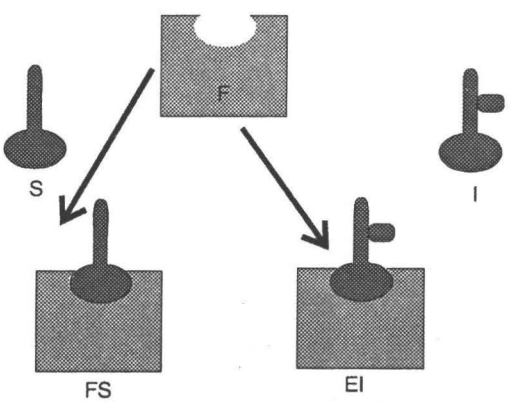
(c) In other words the degree of inhibition depends on the relative concentrations of the substrate or inhibitor. Increase in the concentration of one shall reduce the effect of other.

(d) It can bind to the free enzyme alone and does not interact with the ES complex. The total enzyme is therefore given by,

$$[Et] = [E] + [ES] + [EI],$$

where ES and EI are enzyme-substrate and enzyme- inhibitor complexes.

A schematic representation of action of a competitive inhibitor can be shown as,



Competitive inhibition: substrate and inhibitors are analogs

Fig. 8.16. Schematic representation of competitive inhibition.

[II] Non-Competitive Inhibitors

[a] A non-competitive inhibitor has no structural resemblance with the substrate.

[b] It binds at a site other than the active site.

[c] It can bind with both, free enzyme as well as ES complex.

[d] It does not change the Km but reduces the Vmax.

[e] Its effect cannot be reversed by increasing the substrate concentration.

[f] The total enzyme concentration is given by,

$[Et] = [E] + [ES] + [EI] + [EIS]$.

A schematic representation can be given as,

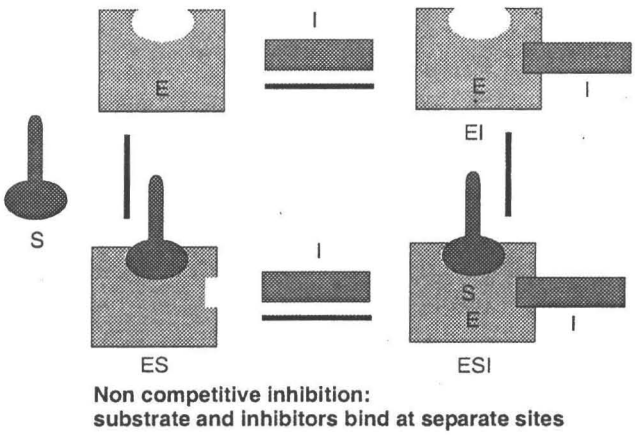


Fig. 8.17. Schematic representation of non-competitive inhibitor.

[III] Un competitive Inhibitors

(a) The un competitive inhibitors are characteristic in the sense that they do not bind to the free enzyme but bind to the ES complex.

(b) The inhibitor does not carry any structural similarity with the substrate

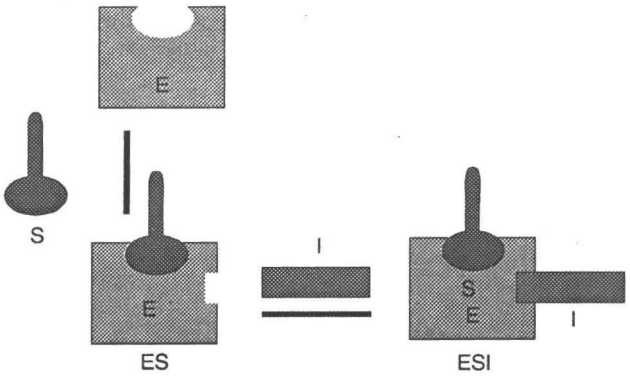
[c] The alterations in the graphs are that both the Km and Vmax are altered by the same factor yielding parallel lines in the LB plots.

(d) The total enzyme concentration is given by,

$[Et] = [E] + [ES] + [EIS]$.

A schematic representation of the un competitive inhibition can be shown as,

After considering the general concepts involved in the regulation of the enzymes, we might consider some specific examples of regulation of metabolic pathways.



Uncompetitive inhibition:
substrate and inhibitors bind at separate sites inhibitor binds after the substrate converting ES to ESI

Fig. 8.18. Schematic representation of uncompetitive inhibition.

Regulation of Metabolic Pathways: Some Considerations

So far the discussion was mostly related to regulation of single enzymes. These considerations can be applied to regulation of metabolic pathways.

Metabolic pathways are broadly of two types,

[a] Linear pathways

Consider a linear pathway,

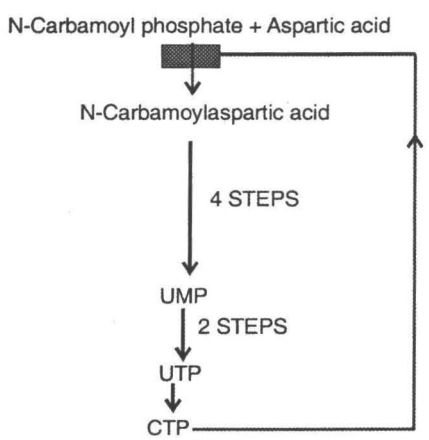
$$\underset{}{A} \overset{a}{-} \underset{}{B} \overset{b}{-} \underset{}{C} \overset{c}{-} \underset{}{D} \overset{d}{-} \underset{}{E} \overset{e}{-} \underset{}{F}$$

Fig. 8.19 A linear pathway

The conversion of A to F is done by its subsequent conversion to the intermediates shown, each step catalyzed by respective enzymes represented by a to e. in such pathways it is much economical to regulate the enzyme a, so that the unwanted intermediates are not synthesized and accumulated.

In case of a feed back mechanism, accumulation or exogenous supply of F would shut off its production. A fall in the concentration of F would resume its production. A classical example of this type of regulation is the synthesis of CTP from carbamoyl phosphate and aspartate catalysed by aspartate transcarbamoylase. The enzyme is inhibited by CTP.

It is diagrammatically shown as,



Regulation of linear pathway: CTP biosythesis

Fig. 8.20. Regulation of CTP biosynthesis

[b] Branched Pathways

Now consider a branched pathway shown as,

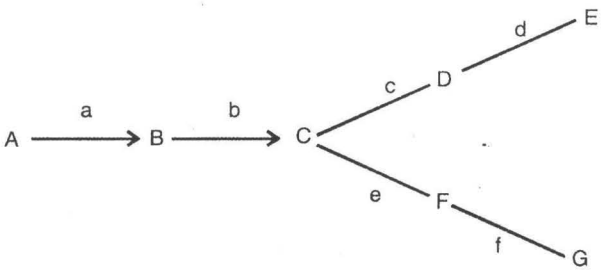
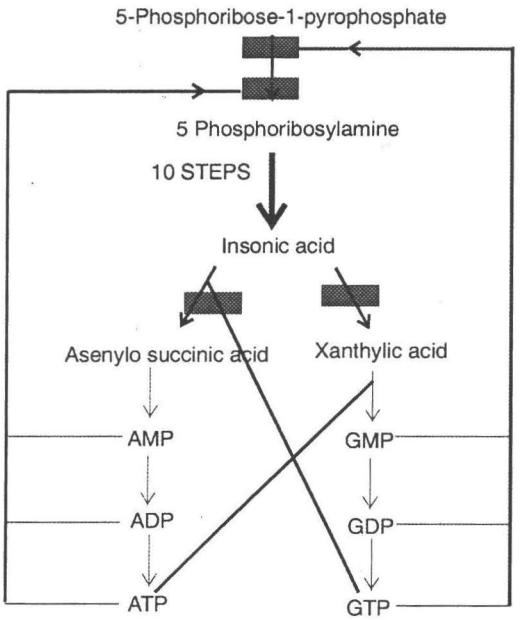


Fig. 8.21. A Branched metabolic pathway

The pathway branches at C. Now in such a case if the feed back were to cause the inhibition of enzyme a, increase in the concentration of any one would lead to the stoppage of the synthesis of both resulting into unwanted effects. In such cases therefore the control would be expected to lie with enzymes c and e.

This type of inhibition is characteristically seen in de novo synthesis of purine mono- nucleotides.



Regulation of branched pathway: de novo purine synthesis

Fig. 8.22. Regulation of a branched pathway, example of purine biosynthesis.

One of the important features of the regulation of metabolic pathways is the amplification of the signal. There are two types of mechanisms involved in such controls.

Signal Amplifications in Metabolic Regulations

[A] Amplification of signals through inter-conversion of enzymes in active and inactive forms

[B] Substrate cycles.

[A] The classical amplification illustration is of the regulation of glycogen metabolism cascade.

It is briefly discussed here,

Regulation Of Glycogen Metabolism:

Glycogen serves as a reserve carbohydrate in the mammalian liver and muscles that serves as an immediate source of glucose for production of energy. It is usually synthesized after a carbohydrate rich diet by assimilation of glucose and its addition to an existing primer of glycogen.

Of prime importance, in glycogen metabolism, are two enzymes, namely glycogen synthase and phosphorylase. Both the enzymes are subject to regulation through allosteric as well as covalent modification. The regulation of these enzymes is given in brief.

(I) Glycogen Synthase:

It occurs in two forms, D or dependent form, so called due to its dependence on glucose-6- phosphate for its allosteric activation. It is the phosphorylated form. When converted into the dephosphorylated form it is active and does not require glucose-6-phosphate . This form is called as the 'I' or independent form. The activation is due to action of phosphoprotein phosphatase.

Glycogen acts as a negative modulator for synthase, by inhibiting the conversion of inactive form to active form. The conversion of the active enzyme to the inactive enzyme is catalyzed by the enzyme protein kinase, an ATP dependent phosphorylating enzyme.

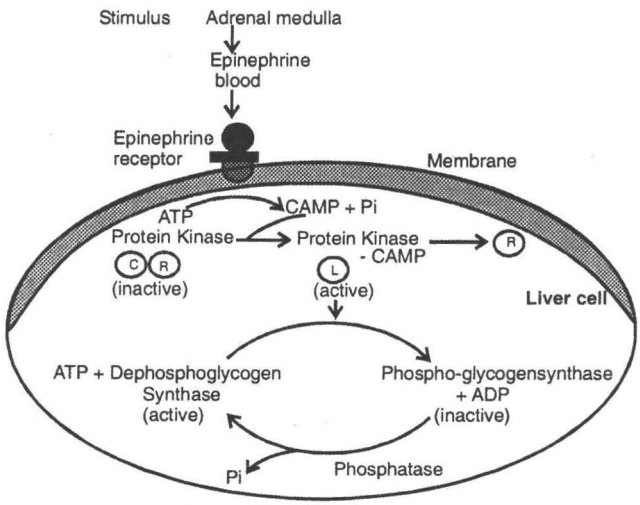


Fig 8.23: Regulation of glycogen synthase.

(ii) Phosphorylase

It also occurs in two forms, phosphorylase a for active and phosphorylase b for inactive. The active form is phosphorylated while the inactive form is dephosphorylated, a system that is exactly opposite to the synthase enzymes. This is an interesting situation as the cell can utilize a common hormone and messenger mediated pathway to regulate both the processes.

The amplification cascade can be schematically represented in the fig 8.24

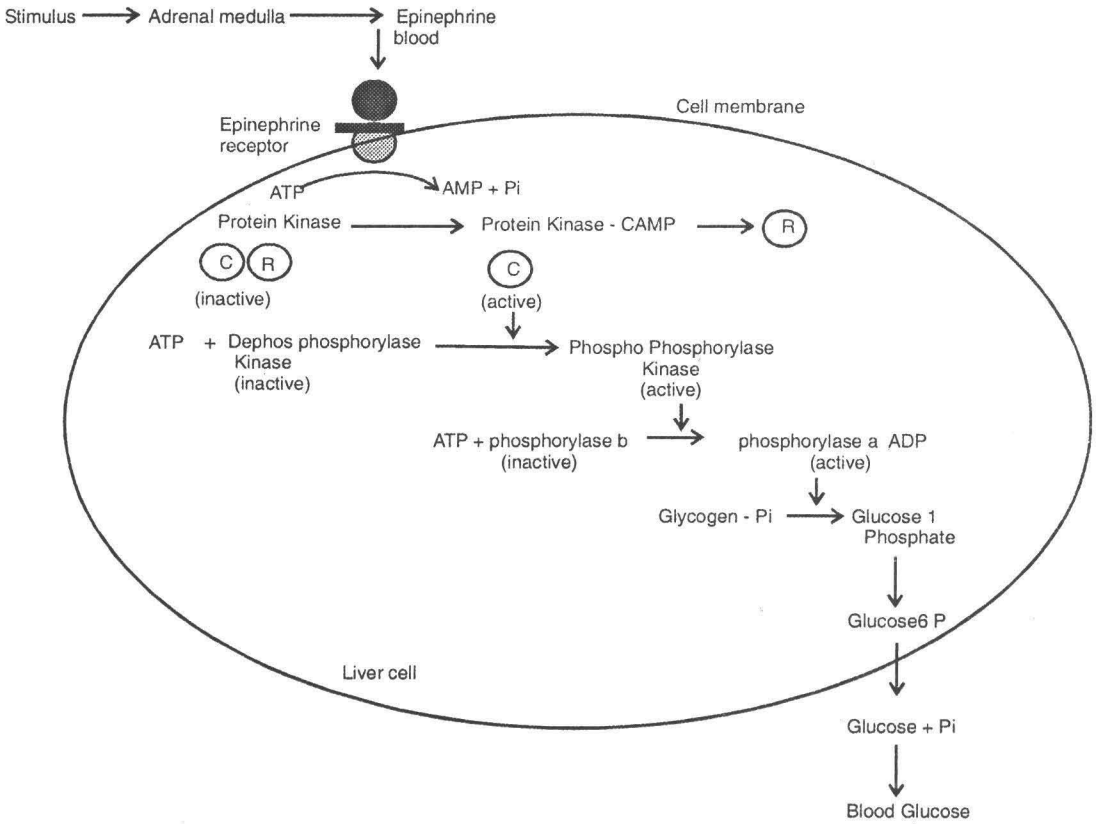


Fig 8.24: Regulation of Glycogen Phosphorylase.

[B] Substrate cycles

Consider a segment of a metabolic pathway that involves inter-conversion of two consecutive intermediates into each other through non- reversible, unidirectional reactions, as shown below, [Fig 8.25]

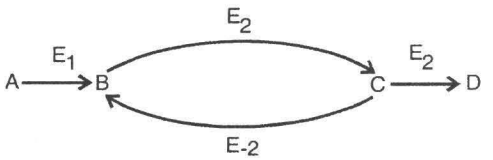


Fig 8.25: Schematic representation of substrate cycles.

The enzymes involved are separate for both the forward and reverse reactions. If they are active simultaneously a 'substrate cycle' occurs. Examples of such reactions include inter-conversions of,

[a] Fructose and fructose-6- phosphate

[b] Glucose and glucose-6-phosphate

In both the cases the forward reaction is catalyzed by a kinase while the reverse reaction is catalyzed by a phosphatase. If both the enzymes are active simultaneously the net result is the hydrolysis of ATP to ADP and Pi. The importance of the substrate cycles is that the enzymes involved can be separately controlled. This in turn leads to a more precise control of the flux than in the case of a single enzyme. Usually the activities of the enzymes are reciprocally controlled. For example, AMP activates the kinase while inhibits the phosphatase and thus even a small change in its concentration can lead to greater amplification on the flux.

A schematic representation of the reaction is given as,

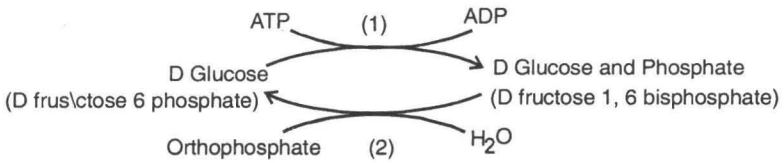


Fig.8.26: Example of substrate cycle.

Examples Of Control Of Metabolic Pathways:

A few examples of metabolic pathways and their control mechanisms are discussed here.

[A] Control of Glycolysis:

Glycolysis appears to be universally important pathway for oxidation of glucose. It is of utmost importance in the muscles as the variations in this tissue are dramatic. It thus appears that the pathway is under a very fine control and tuning mechanism.

The schematic representation of the regulatory steps in the glycolytic sequence is shown in fig 8.27 as under,

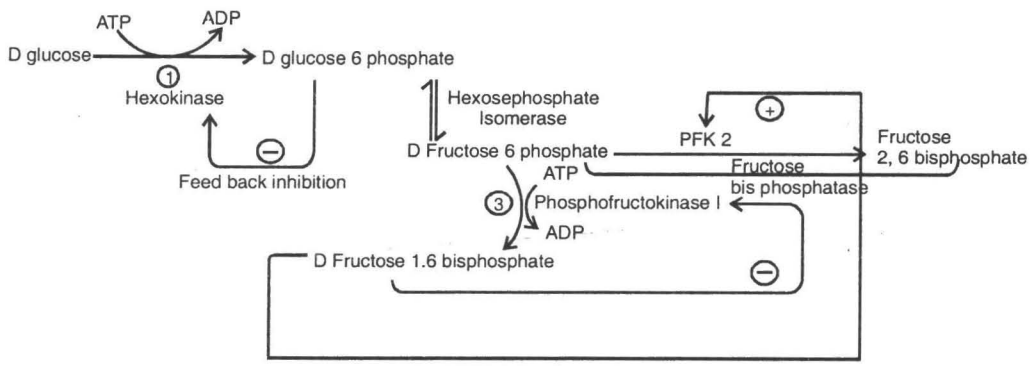


Fig 8.27: Sequence of regulatory reactions of glycolysis.

The likely control points in the mechanism are the steps catalyzed by the enzymes, hexokinase and 6-phosphofructo kinase.

Regulation Of Hexokinase:

The enzyme catalyzes the conversion of glucose to glucose-6-phosphate. Muscle hexokinase has a very great affinity for glucose (Km about 0.1 milli Moles). The glucose entering in the muscle tissue produces an intracellular concentration that is high enough to cause the saturation of hexokinase leading to its maximum activity. The product formed, (glucose-6-phosphate) acts as an allosteric reversible inhibitor for the enzyme. If the concentration of the product greatly increases, its production is temporarily switched off.

In liver the predominant enzyme is glucokinase, an isoenzyme of hexokinase (and hence called as hexokinase IV or hexokinase D). it differs from the muscle hexokinase in two respects.

1. It has a low affinity for glucose (Km about 10 milli Moles). This is value is much higher than the normal content of glucose in blood. Since the glucose level in the hepatocyte is in equilibrium with the concentration of glucose in blood, the enzyme is therefore regulated by the concentration of glucose in blood. Increase in blood glucose due toa carbohydrate rich meal leads to the transport of excess glucose to hepatic cytoplasm where it is converted to glucose-6-phosphate.
2. The enzyme is inhibited by the isomer (fructose-6-phosphate) of its product (glucose-6-phosphate). These two compounds are always in equilibrium due to the action of the enzyme
3. Phosphohexose isomerase. The partial inhibition of glucokinase by fructose-6-phosphate requires an additional regulator protein.

Regulation Of phosphofructokinase:

The enzyme catalyzes the conversion of fructose-6-phosphate to fructose1, 6 bisphosphate at the expense of 1 ATP.

There are two isoenzymes, namely PFK1 and PFK2. PFK1 is the enzyme for the principal rate- limiting step of glycolysis. In resting muscles, the physiological concentration of ATP (~ 5mM) inhibits the enzyme allosterically reducing its affinity for its substrate, fructose-6-phosphate. A decrease in ATP by 20% raises the rate of glycolysis by about 10 times, the activity of the enzyme

Increasing by about 50%. The physiological concentration of AMP opposes the negative allosteric effect of ATP. The ATP/ AMP ratio therefore plays an important role in the regulation of the rate of PFK1. The rise in the ratio inhibits while the fall in the ratio promotes the activity of the enzyme.

In the presence of high concentration of fructose-6-phosphate, some of it is converted to fructose 2,6 bisphosphate by PFK2. The fructose 2,6 bisphosphate allosterically activates PFK1 by decreasing the Km and increasing its affinity for its substrate that is fructose-6-phosphate. It also counteracts the ATP mediated inhibition of the enzyme. The net effect is an increase in the activity of the enzyme and in turn of glycolysis. High level of citrate intensifies the inhibition of the enzyme PFK1.

[B] Control Of TCA:

The tricaboxylic acid cycle (TCA) is probably the most important pathway that links the oxidative and biosynthetic pathways of the carbohydrate, fat, amino acid metabolism. It is often mentioned as the intermediary metabolic sequence. the cycle operates in the mitochondrial matrix and is highly operative in cardiac muscle, liver and red striated muscle. It does not operate in red blood cells.

The sequence of reactions can be written as in fig 8.28,

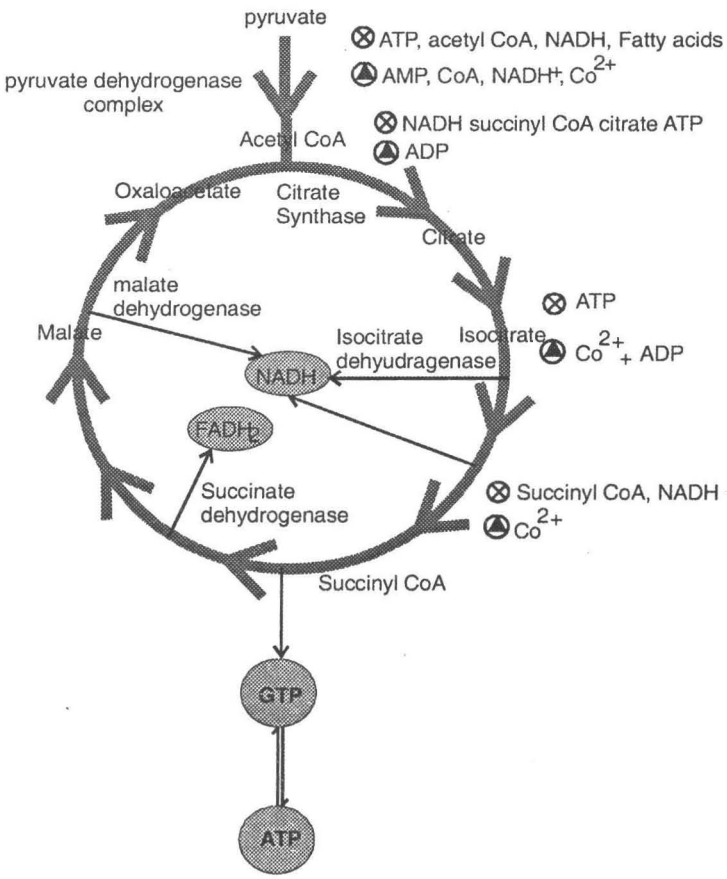


Fig. 8.28: TCA and its regulation

The pathway is a multi-point entry and exit system. There are 3 main points of control of TCA. They are citrate synthase, isocitrate dehydrogenase and ketoglutarate dehydrogenase. The control of each is briefly discussed as,

[a] Citrate synthase:

It is activated by increase in the concentrations of acetyl- Co A and oxaloacetate. It is competitively inhibited by citrate and succinyl Co A, citrate competing with oxaloacetate and succinyl Co A competing with acetyl Co A respectively.

The increase in concentration of ATP, NADH and long chain acyl Co A in the cell also inhibit the enzyme.

[B] Isocitrate Dehydrogenase:

The enzyme is activated by increase in the intracellular concentration of Ca++. The influx of Calcium ions is due to membrane depolarization in case of the muscle tissue. Isocitrate tends to activate the enzyme while ATP and NADH, inhibit it. The

The enzyme is allosterically activated by ADP. The action is probably through lowering the Km and increasing the affinity between the enzyme and its substrate i.e.isocitrate.

[C] α- Keto-glutarate Dehydrogenase:

This enzyme is regulated allosterically. Rise in ATP/ADP, NADH/NAD, and Succinyl Co A/ Co A ratio indicative of a high-energy status, inhibit the enzyme. The result is an overall decline in the TCA rate. The enzyme is activated by calcium ions, ADP or AMP.

The fall of cellular ATP leads to increase in ADP and AMP concentration. This leads to increase in rate of the citric acid cycle. Reversal of the condition declines the rate.

[D] Anaplerotic Reactions:

Many anabolic and catabolic reactions remove TCA intermediates. For example oxaloacetate is used up in transamination and gluconeogenesis. Glutamate synthesis uses alpha keto glutarate while citrate is transferred to the cytoplasm for NADPH synthesis required for fatty acid biosynthesis. Succinate is used for porphyrin synthesis.

The anaplerotic reactions comprise of compensating reactions that enable the smooth completion of the TCA. One of these is the synthesis of oxaloacetate by the action of pyruvate carboxylase, a biotin dependent enzyme that catalyzes the condensation of carbon-di-oxide from bicarbonate to pyruvate at the expense of one mole of ATP.

Replacement of ketoglutarate by glutamate dehydrogenase or transaminase reactions involves other anaplerotic reactions.

[C] Control Of Fatty Acid Synthesis:

The synthesis of fatty acids occurs in mitochondria. An important feature of this mechanism is that it is catalyzed by the multi enzyme complex called as fatty acyl synthase system. [Fig 8.29] It is a complex of seven proteins in E.coli and eight in yeast, mammals and birds. The latter is a ellipsoid dimer of about 25nm X 21nm in yeast. The monomers are arranged in head to tail manner with non -covalent links. Each monomer has three domains that are schematically shown as,

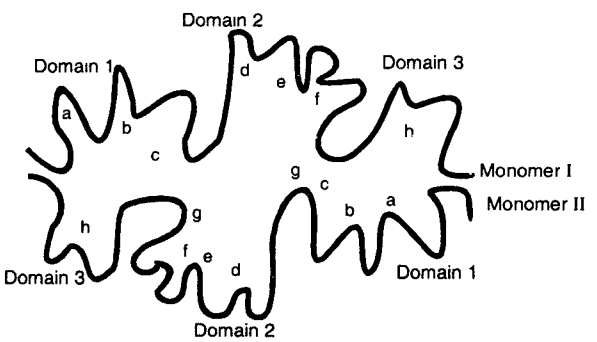


Fig 8.29: The fatty acyl synthase complex.

The regulation of fatty acid synthesis is regulated on short term as well as long term.

The Short-term Regulation

It revolves around the concentration of acetyl Co A, citrate and long chain acyl Co A, all of which affect the activity of the enzyme acetyl Co A carboxylase. This is the rate-limiting enzyme for the fatty acid biosynthesis. Citrate is an allosteric modulator for the enzyme. High rate of glucose oxidation by glycolysis forms large amounts of pyruvate and in turn of acetyl Co A, and citrate. Citrate changes the globular inactive structure of carboxylase to fibrous active polymers with a high Vmax. This increases the rate of malonyl Co A formation resulting into enhanced fatty acid synthesis. Palmitoyl Co A and other long chain acyl Co A molecules antagonize the effect of citrate and de-polymerize the enzyme to its inactive monomers. Palmitoyl Co A also inhibits the tricarboxylate transporter of inner mitochondrial membrane reducing the availability of cytoplasmic acetyl Co A. In a nutshell it reduces fatty acid biosynthesis by altering the concentration of acetyl Co A in the cytoplasm and its carboxylation to malonyl Co A.

The second mechanism is related to the concentration of accumulated fatty acyl residues after a high fat diet. This in combination with non-availability of glycero-phosphate for triacyl glycerol synthesis leads to accumulation of acetyl Co A and reduce fatty acid synthesis.

The covalent modification of acetyl Co A carboxylase by phosphorylation-dephosphorylation respectively inhibits and activates the enzyme. This also helps the short term control.

Since the concentration of acetyl Co A is an important factor, inhibition of PDH results into lowering in its concentration and hence decreases the rate of fatty acid synthesis. In starvation and prolonged diabetes, the PDH activity is inhibited due to high β - oxidation of fatty acids and high NADH formed due to it. PDH is also inactivated due to high ATP/ADP ratio in mitochondria. This results from the inactivation of the ATP/ADP transporter by a high fat diet. Conversely, a high carbohydrate diet enhances glycolysis, formation of pyruvate which in turn inactivates PDH kinase and activates PDH. This forms ample acetyl Co A and increases the rate of fatty acid synthesis.

Fatty acid synthesis is also under hormonal control. Adrenaline and glucagon reduce fatty acid biosynthesis in several ways.

[a] They activate the adenylate cyclase leading to increase in cAMP in adipocytes leading to lipolysis. This results in accumulation of acetyl Co A and lowering the activity of acetyl Co A carboxylase.

[b] There is an increase in the activity of protein kinase that phosphorylates the carboxylase converting it into inactive form.

[c] They may also increase the influx of calcium ions activating the calcium-calmodulin dependent phosphorylation of acetyl Co A carboxylase, decreasing the activity of the enzyme.

An opposite effect is shown by the hormone insulin. It reduces lipolysis, reducing the allosteric inhibition of the carboxylase. It may also activate a phosphatase that activates the enzyme by dephosphorylation mechanism. It increases glucose uptake by adipocytes, promoting glycolysis and in turn acetyl Co A formation. It may also promote dephosphorylation and consequent activation of PDH.

The Long-term Regulation

It is largely mediated by modulating the activity of enzymes that form the raw materials for fatty acid biosynthesis.

Insulin induces the synthesis of fatty acid synthase, acetyl Co A carboxylase thereby enhancing fatty acid biosynthesis. It also induces the synthesis of ATP- Citrate lyase resulting into formation of cytoplasmic acetyl Co A for fatty acid synthesis. It also promotes induction of those enzymes that produce NADPH required for fatty acid biosynthesis.

Starvation causes a long-term decrease in the activity of all the related enzymes leading to a decrease in the fatty acid synthesis.

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9

ISOENZYMES

Introduction

Polymorphism in enzymes

1.Genetic alterations

2.Polymerization

3.Conformational isomerization

4.Presence of charged groups

Isoenzymes in Clinical Diagnosis

The mechanisms that cause appearance of enzymes in serum

(a) Synthesis and secretion by liver

(b) Non-functional plasma enzymes

(c) Passive diffusion of isoenzymes in circulation

The half life or clearing of enzymes from circulation

The environmental conditions, i.e. the physical and physiological conditions of the person

The effects of age, sex, time of sampling, etc.

Diagnostics Principles and procedures:

(a) Isoenzymes of Acid Phosphatase:

(b) Isoenzymes of Alkaline phosphatase:

(c) Isoenzymes of creatine phosphokinase:

(d) Isoenzymes of Cholinesterase:

(e) Isoenzymes of Lactate Dehydrogenase

Non-Diagnostic isoenzymes

Isoenzymes of fructose bis phosphate aldolase

Introduction

The improvements in the electrophoretic techniques, especially the SDS-electrophoresis, zone electrophoresis etc have indicated that separation of human serum showed the distribution of lactate dehydrogenase activity in 5 peaks. Thus there are 5 isoenzymes of lactate dehydrogenase enzyme. Similarly creatine kinase has three isomeric forms, CPK1 is predominant in brain tissue, CPK2 in heart and CPK3 in skeletal muscles.

Different authors have used the terms 'isoenzymes' and 'isozymes', and they mean the same as is intended when the term isotope is used in chemistry.

> *Thus the term isoenzymes can be defined as the forms of a given enzyme that differ in the intracellular distribution or organ distribution and have slight differences in kinetic behavior, and composition. In other words the multiple forms of an enzyme that catalyze the same reaction but differ from each other in their primary structure, substrate affinities, Vmax and regulatory properties are called as isoenzymes.*

They have been demonstrated in higher plants as well as animals. More than hundred enzymes are now known that exist in two or more molecular forms. The different forms occur in different concentrations in different tissues as per the needs of the cells. For example Hexokinase, which has a low Km value and thus more affinity for glucose is predominant in muscle while glucokinase, which has a high Km value and thus less affinity for glucose is predominant in liver.

This property has been commercially exploited in diagnosis and differential diagnosis of diseases.

Polymorphism In Enzymes

Enzymes can show polymorphism [isomeric forms] due to several reasons. Some of the possible reasons are briefly explained here.

1.Genetic Alterations

The oligomeric and polymeric enzymes are formed by associations of different polypeptide chains, which are the products of specific genes. These genes may be located near each other or separated from each other and occur at different loci. Variations in the combinations of polypeptides result in the formation of enzyme molecules that have slightly different kinetic properties and physico-chemical properties although they might be able to catalyze the same reaction. Moreover a mutation in any of the gene may result in the formation of a polypeptide that may greatly alter the kinetic behavior of the final enzyme product. In any case, the association of the different monomers or polypeptides generates a variety of isozyme forms.

LDH has 5 isomers that can be distinctly identified on the basis of properties that include, Electrophoretic mobility, stability to heat, effects of 2M urea solutions, effects of inhibitors like oxalate and oxamate etc. Creatine phosphokinase comprises of 2 sub units and has 3 isomers, muscle type, brain type and a hybrid.

2.Polymerization

The variations in the polymerization of sub units may result in the formation of multiple forms with different activities. The enzyme cholinesterase from human sera separates into 5 bands on starch gel electrophoresis (ChE 1 to 5) with most of the activity seen in the isoenzyme 5. When subjected to concentration the amount and thus activity of the isoenzyme 5 increases and on dilution, it depolymerizes into 4 different smaller molecules designated as ChE 1 to 4. The different polymers have different surface charges causing a difference in their electrophoretic mobility.

3.Conformational Isomerization

Since most of the enzymes are proteins and their activities are greatly dependent on the particular 3-dimensional conformation of the molecule, alterations in their structure that do not greatly affect the activity but alter the electrophoretic and other properties give rise to isoenzymes. The aspartate transaminases from the cytoplasmic and microsomal fractions are an example of such conformational isomerism.

4.Presence of Charged Groups

In mammalian systems there are three predominant forms of the enzyme alkaline phosphatase, namely liver, intestine and bone. A forth form, the placental form is also identified during pregnancy. These forms separate in different bands on agar gel and cellulose acetate electrophoresis corresponding to $\alpha 2$, pre-β and γ regions.

The $\alpha 2$, is of liver origin, the pre-beta is of the bone while the gamma is of the intestine. In case of many hepato-biliary patients an extra band in the alpha1 region has also been obtained. The isoenzymes differ in their sensitivity to the inhibitors. [Table 9.1]

Table 9.1: Isoenzymes of Alkaline Phosphatase

Type of Isoenzyme	Inhibited By
Placental	L-phenylalanine
Intestinal	L-phenylalanine
Bone and Liver	L-homoarginine

The isoenzymes show different sensitivities to heat. The placental isoenzyme is heat stable but the bone isoenzyme is heat labile. These differences have been related with the presence of different number of sialic groups on the molecules. The intestinal isoenzyme is devoid of the sialic residues. The other three contain different numbers of these charged groups.

Isoenzymes in Clinical Diagnosis

From the preceding discussions it is clear that an alteration in the pattern of isoenzymes of any particular enzyme may indicate the tissue/organ affected resulting into the specific change and thus help in differential diagnosis. It has to be remembered that various factors influence the levels of enzymes and isoenzymes in the serum.

- The mechanisms that cause appearance of enzymes in serum
- The half life or clearing of enzymes from circulation

- The environmental conditions, i.e. the physical and physiological conditions of the person
- The effects of age, sex, time of sampling, etc.

The Mechanisms That Cause Appearance Of Enzymes In Serum

There are three different mechanisms, by which enzymes appear in serum,

(a) Synthesis And Secretion By Liver

Enzymes like psuedocholinesterase, fibrinolytic enzymes and enzymes related to blood coagulation comprise the group of 'active serum enzymes' and are present in plasma in much higher amounts than in the cells. Most of these are synthesized in liver and secreted to the circulation. Rise in their levels, do not indicate much, however their decrease is indicative of liver disease or obstruction. Deficiency of enzymes involved in blood coagulation is known to occur in acquired and inherited disorders associated with increased bleeding tendency. The assessment of these enzymes aids in the differential diagnosis of bleeding disorders.

(b) Non-Functional Plasma Enzymes

Some enzymes including transaminases, CPK, LDH etc are found in very high concentrations in tissues with very low values in the plasma. They are therefore designated as non-functional plasma enzymes. Their rise in the serum is indicative of cell damage and moreover since they have isoenzymes with organ specificity, the alteration in isoenzyme pattern can lead to diagnosis of organ relation in the disease. The diagnosis of LDH, CPK, SGOT, SGPT is therefore employed in routine clinical observations.

More and more isoenzymes are now being exploited in clinical and pathological studies.

(c) Passive Diffusion Of Isoenzymes In Circulation

Obstruction many times results in passive diffusion of various isoenzymes in serum leading to an increase in their levels. Such a case is seen in pancreatic enzymes and alkaline phosphatase. The levels are increased in obstruction of pancreatic duct as well as obstructive jaundice respectively.

Obstruction of bile flow results in enhanced synthesis of ALP by hepatic cells.

The Half Life Or Clearing of EnzymesFrom Circulation

Different enzymes have different half- life periods as their rates of clearance from serum differ, the clearance rates of enzymes of CPK, SGOT, SGPT and LDH are given in table 9.2.

Thus in the patients of myocardial infarction, the levels of CPK are normal after 3 days but the levels of the transaminases and LDH remain elevated. It is therefore important to relate the time of sampling to the onset of symptoms more so when the enzymes are secreted in the circulation periodically.

Table 9.2: Clearance Rates of Enzymes

Name Of The Enzyme	Half-life
CPK	1.4 days
SGOT	2.0 days
SGPT	6.3 days
LDH	6.8 days

The environmental conditions, i.e. the physical and physiological conditions of the person:

Since the activity of an enzyme is markedly affected by environmental parameters like pH, temperature, concentration of substrate and products, the conditions of assays need stringent control to eliminate erroneous results obtained due to alteration in these factors. They are also affected by certain drugs that may be present in the serum and act as activators or inhibitors for the enzyme in question. Use of advanced instrumentation may help in the elimination of these problems.

The Effects Of Age, Sex, Time Of Sampling, etc.:

The age, sex and other conditions of the patients are also important while deduction of inferences of the enzyme studies. Many enzymes show considerable variations in their activity with the age of the patient. Activity of enzymes like CPK is altered after a rigorous muscular activity or intramuscular injections. Many enzyme activities may be altered as a result of pregnancy in females.

Diagnostics Principles And Procedures

The principle of use of isoenzyme patterns in diagnosis revolves around the fact that in diseases leading to the leakage of tissue enzymes from the damaged tissue to the plasma, a pattern of isoenzymes characteristic to the damaged tissue would be observed in plasma leading to an altered electrphoretogram. Comparing this with the standard distribution the origin of this alteration and in turn the tissue damaged can be easily identified.

Some of the important isoenzymes routinely used in diagnosis are now briefly considered.

(a) Isoenzymes of Acid Phosphatase

The enzyme hydrolyzes, the esters of phosphoric acid at acidic pH of 4 to 7.

ACP

ParaNitroPhenyl Phosphate + Water ======== ParaNitroPhenol + Orthophosphate.
[Acidic pH]

The enzyme is present in RBC, platelets, leukocytes and prostate but has also been shown to be present in minor amounts in other tissues. The prostate fraction is clinically important in the diagnosis of the prostate cancers. It can be distinguished from the red blood cells and platelet isoenzymes by its stability to formaldehyde and lability to L-Tartarate.

The serum levels are significantly raised in prostatic carcinoma with metastasis to bone. In absence of bone metastasis, levels enhance by 25% only. Benign prostatic hyperplasia or prostitis do not cause any increase in the levels of the enzyme. The enzyme levels return to normal after removal of cancer or 3-4 weeks after estrogen therapy.

(b) Isoenzymes of Alkaline Phosphatase

Alkaline phosphatase commonly known as ALP hydrolyzes the aliphatic, aromatic and heterocyclic esters of phosphoric acid at an alkaline pH. The pH optimum is between 9-10.

It is widely distributed in the human body in the intestinal mucosa, renal tubules, liver, bones, placenta and the lactating mammary glands. It is localized in the cell membrane. It is important in the diagnosis of hepatobiliary diseases and the bone diseases. The level of serum ALP increases 10 to 12 fold its normal upper range in case of liver diseases associated with cholestasis which is either intrahepatic or extrahepatic.

The intrahepatic cholestasis is caused by viral hepatitis, damage by alcohol or drugs or mechanical obstruction by neoplasia. Extrahepatic cholestasis is due to gallstones. In these cases there is possible an enhanced net synthesis of the enzyme rather than the accumulation due to obstruction.

The enzyme levels are mildly elevated in viral hepatitis, infectious mononucleosis and cirrhosis of the liver. The elevation is much higher if there is occlusion of biliary canaliculi.

It is moderately increased in osteomalacia, rickets, and hyperparathyroidism, but the levels are greatly increased in Paget's disease. The levels increase drastically in bone cancers. Moderate increase is seen in Hodgkin's disease, congestive heart failure and ulcerative colitis.

It is normally high in growing children and pregnant women.

The relation of raised isoenzyme levels to the damaged tissue can be stated as in table 9.3,

Table 9.3: Isoenzymes Of ALP And Their Relation To Tissue Damage

Isoenzyme Increased	Tissue Involved
Alpha isoenzyme	Liver damage
Pre-beta	Osteoblastic activity
Gamma	Intestinal lesion
Placental	Pregnancy

The isoenzymes are identified on the basis of heat treatment or inhibitory action of L-phenylalanine.

The principle of clinical diagnosis of ALP is as follows,

Alkaline phosphatase acts on p-Nitro phenyl phosphate to yellow colored

p-Nitrophenol and phosphate. The absorbance is measured at 405 nm is directly proportional to enzyme activity.

The protocol is as,

Table 9.4: protocol for estimation of ALP

Reagents	Blank	Test
Purified water	1.0ml	Nil
Working ALP reagent	Nil	1.0ml
Serum		20 ml

(c) Isoenzymes Of Creatine Phosphokinase

The enzyme catalyzes the reaction of phosphorylation of Creatine at the expense of ATP.

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{CPK}} \text{Creatine Phosphate} + \text{ADP}$$

The highest activity is seen in heart, striated muscle and brain. Liver and lungs have minimal activity and necrosis of liver or pulmonary embolism and infarction do not affect serum levels. The enzyme activity is markedly increased in serum following a myocardial infarction. It exceeds about 6-20 times the upper normal limit in the first 3 hours after the onset of pain. The level also increases greatly in progressive muscular dystrophy and this rise is consistent. It occurs in infancy or early childhood. In muscular atrophy of neurogenic origin, serum CPK values are often within normal range however muscle trauma, fractures and IM injections may raise the enzyme activity to high levels. The enzyme activity in serum is increased even in case of chronic alcoholics, hypothyroidism, acute cerebrovascular disease and after high doses of salicylates.

Three isomers of CPK are known. They are dimers of two sub units, M and B. [M for muscle and B for brain]. The arrangement may be MM, MB, and BB depending on the association of sub units. The MB isoenzyme is present in heart. Skeletal muscle contains MM only, heart muscle contains MM and MB while brain contains BB isoenzymes. The MB isomer forms only 20% activity of the heart muscle but is almost specific for this organ. A rise in excess of 6% of the MB activity is of clinical significance. Peak activity of MB reaches 12 hours before CPK, in patients suffering from myocardial infarction. Total CPK and MB are also raised in myocarditis, severe arrythmia, myocardial trauma, while no elevation in MB is seen following physical exertion, hypothyroidism, muscle damage, hypoxia or intra muscular injections, although the total CPK is generally increased under these conditions.

The principle of diagnosis of CPK [CK-MB] is as follows,

The procedure involves measurement of creatine kinase activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half of CK-MB while not affecting the B sub unit activity of CK-MB and CK-BB. The method is used to quantitatively estimate the activity of CK-BB. CK catalyzes the

reaction between creatine phosphate and ADP, giving creatine and ATP. ATP in presence of glucose and hexokinase forms glucose-6-phoaphate, which undergoes dehydrogenation by NAD dependent glucose-6-P dehydrogenase. The rate of NADH formation is monitored at 340 nm. The CK-MB activity is obtained by multiplying the CK-BB by 2.

The manual procedure is as follows,

Take 1ml reagent in a test tube and add 50 micro liters of sample, mix well and after 5min, read absorbance at a minute's interval for two minutes and calculate the difference in absorption.

(d) Isoenzymes of Cholinesterase

There are two isoenzymes of Cholinesterase, differentiated on the basis of substrate specificity and action of inhibitors.

Acetyl cholinesterase: The enzyme occurs in RBC, nerve endings, lung, spleen and gray matter in the brain. It hydrolyses acetyl choline and acetyl-b-methyl choline and is also known as cholinesterase I, true, specific or red cell cholinesterase.

Acetyl Cholinesterase

Acetyl choline + Water ========== choline + Acetyl ion

Pseudocholinesterase: It occurs in serum, pancreas, liver heart and white matter of brain. It hydrolyses the acyl choline molecules. It does not hydrolyze the acetyl -b-methyl choline.

The serum cholinesterase is synthesized in the liver and is a sensitive indicator of liver function. Viral hepatitis, advanced cirrhosis and metastatic carcinoma of the liver, malnutrition and chronic debilitating diseases decrease the level of enzyme. Comparatively little reduction in enzyme levels is seen in surgical procedures, myocardial infarction and pulmonary embolism, muscular dystrophy and acute infection of liver.

Both the isomers are inhibited by organophospho- insecticide compounds. The inhibition is irreversible and in the case of RBC, complete replacement of old cells by new one is necessary. The isoenzyme of RBC is more important.

(e) Isoenzymes Of Lactate Dehydrogenase

The enzyme catalyzes a reversible reaction of inter conversion of pyruvate and lactate shown as follows,

Lactate Pyruvate

The enzyme occurs in nearly all cells but is predominantly present in liver, myocardium, skeletal muscle, kidney and RBC. The tissue enzyme activity is about 1000 fold of the serum activity and the leakage from a small necrotic portion, changes the serum LDH activity pattern resembling the tissue damaged.

- The serum LDH levels are affected by several factors,
- values in children are greater than adults
- diurnal variations range from 30 to 200%
- in summer months the levels are enhanced by about 20%
- Post- exercise, a rise in the activity is seen.
- Myocardial infarction increases the activity 5-10 times.
- The rise begins after 6-12 hrs of the onset of pain and remains high till about 6-8 days
- The LDH1and 2 predominantly increase in the first 6-12 hrs and remain elevated for 1-2 weeks. A renewed infarction raises the levels further and the increase is proportional to the size of the infarct.
- Myocardial infarction followed by liver damage causes an increase in the LDH5 fraction along with LDH 1 and 2.
- However the pattern is normal in angina pectoris, pericarditis, arrhythmia and pulmonary embolism.
- Diseases associated with the hepato-cellular damages cause an increase in LDH 4 and 5 fractions.
- Progressive muscle dystrophy, renal disease, carcinomas, megaloblastic and hemolytic anemia, leukemia and lymphomas cause increase in LDH.

A summary of changes occuring in the isoenzymes of LDH related to causing conditions can be tabulated as,

Causitive conditions	**LDH isoenzymes altered**
Hepatitis, Acute necrosis, CCl_4 toxicity	LDH 5 increases
Myocardial Infarction	LDH 1 increases
Anemia	LDH 1 increases
Organic nervous disease	LDH 2 and 3 increase
Chronic renal disorder	LDH 5 increases
Rheumatoid arthritis	LDH 5 increases
Prostate cancer	LDH 4 and 5 increase
Pregnancy	LDH 3 and 4 increase

Non-Diagnostic Isoenzymes

Isoenzymes Of Fructose bis Phosphate Aldolase

The enzyme fructose bis phosphatase aldolase occurs in nearly all plants animal tissues and in most of the microorganisms. It is a tetrameric enzyme, in which the subunits occur as dimeric units rather than a regular tetrahedron. It occurs in two

classes FBA class I and FBA class II. The FBA class I occurs in three different isoenzymes namely FBA 1(Brain), FBA 2(Muscle) and FBA 3(Liver).

The isoenzymes from higher plants and animals contain a lysine side chain in the active site while that from fungi and bacteria contain an essential metal ion in their active sites. There are 5 different species of the enzyme in rabbit skeletal muscle, that are differentiaited on the basis of electrophoretic mobility in gels. The reaction can be represented as,

$$\text{Glyceraldehyde 3 phosphate} + \text{Dihydroxyacetone phosphate} \xrightarrow{\text{FBA}} \text{Fructose bis phosphate}$$

◆◆◆◆◆

10

ENZYME TECHNOLOGY

Introduction

Enzyme Technology

Commercial uses of enzymes

- Alcoholic beverages
- Bread Making
- Use of proteases
  - Cheese making and proteases
  - Meat tenderizing and proteases
- Production of sweet syrups
- Enzymes in detergents

Immobilization of enzymes

- Advantages of immobilization of enzymes
- Methods of immobilization of enzymes
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  - Ionic bonding
  - Covalent bonding
  - Cross linking
  - Entrapment
  - Encapsulation

Enzymes in diagnosis

Enzyme electrodes

Enzyme immuno assay

Enzyme therapy:Enzymology of Genetics

Production of enzymes using microorganisms

Introduction

The later part of the twentieth century has seen the advent of new techniques that have greatly contributed to the development of enzymology. The 'enzyme technology' has redefined process like cheese making, brewing, food processing, leather making, detergents; etc. 'Clinical enzymology' has contributed to new techniques for diagnosis and differential diagnosis. 'Enzymology of genetics' has greatly contributed to fields of molecular biology and biotechnology. Some of the important aspects of all these shall be dealt in brief to introduce these concepts to the readers.

Enzyme Technology

Whole cells of fungi and bacteria have been used for fermentation and food processing since early 16th century by mankind. The use of isolated enzymes has been a comparatively recent development. More and more industrial processes are now employing isolated and purified or immobilized enzymes. The advantages of isolated enzymes are listed already elsewhere in the book. A brief recapitulation is given here. The use of isolated enzymes is advantageous as,

(a) It is possible to obtain higher catalytic activity

(b) Undesirable side reactions and diversions are avoided

(c) Increased reproducibility of the procedures involved.

(d) If high temperatures are involved, the enzymes from thermophiles may be easily used to counter the problem of denaturation.

Commercial Uses Of Enzymes

Some of the important enzymes produced on industrial scale and their use in industrial processes are discussed here.

(1) Alcoholic Beverages

The traditional brewing process is brought about by germinating barley so that sufficient quantities of α & β amylases are produced that hydrolyze starches to oligosaccharides. The hydrolytic enzymes cleave the starches to oligosaccharides and reducing sugars so that the hydrolytic steps do not become rate limiting.

Enzyme supplementation has not been used in brewing very frequently, however they have been used when spirits are produced from sorghum, rye, potatoes and wheat. The enzymes used in such cases are given in table 10.1.

Table 10.1: Amylases from microbes used in beverage production.

Enzyme	Source
α amylase	Bacillus subtilis
α&β amylases	Aspergillus niger and A.oryzae
α 1,6 glucosidase	Aspergillus niger

The enzymes from Aspergillus spp.are used as they produce low calorie spirits due to nearly complete hydrolysis of the starch molecules. The amylase from Bacillus spp.has the advantage of heat stability and is therefore used when a little higher temperatures are required by the fermentation processes.

However the amylases are not the only enzymes that are used in industry to metabolize carbohydrates. Some of the other enzymes are given in table 11.2,

Table 10.2 Carbohydrate hydrolysing Enzymes that are used in industrial processes

Enzyme	Reaction type	Microbial source	Industrial Utilization
Exo-1,4 glucosidase	Hydrolysis of 1,4 glycosidic bonds from non-reducing ends	A. niger, A.oryzae Rhizopus spp.	Production of glucose from starch
Cellulase	Hydrolysis of β 1,4 glycosidic bonds in cellulose and glucans	Trichoderma spp A.niger	Cellulose→ cellobiose
Polygalacturonase	Hydrolysis of pectins	Mucor, Penicillium, Aspergillus	Clarification of wines and fruit juices
β–D-galactosidase	Hydrolysis of lactose	Aspergillus spp	Sweeter and more soluble sugars
β–D-fructofurano-sidase	Hydrolysis of sucrose	A.oryzae, Saccharomyces	Sweeter and more soluble sugars
Glucose oxidase	Glucose→ gluconolactone	A.niger, Penicillium	Analytical reagent, desugaring egg products etc.
Xylose isomerase	Glucose←→ fructose	Streptomyces spp, Lactobacillus spp.	Production of high fructose syrups.

Clarification of beer is often done by subjecting it to a protease like papain that hydrolyses the protein turbidity in the final product.

(2) Bread Making

The process of bread making also involves the fermentation of sugars obtained from starch. The activities of amylases result in the formation of sugars that are fermented with simultaneous release of carbon dioxide that is useful for aeration and raising of the dough. In many countries, the fungal amylases are preferably used to enhance starch hydrolysis. The fungal enzymes are advantageous as,

- They can work at lower temperatures
- They are heat labile and are easily destroyed during baking
- They promote a limited starch hydrolysis improving the texture of the product.

The quality of the finished products may be enhanced by the use of proteases from fungi that improve the viscosity and elasticity of the dough.

(3) Use Of Proteases

A variety of proteases find a host of uses in various industries. Some of the important uses of proteases and the processes that use them are given in table number 10.3,

Table 10.3: Some industrially important proteases

Enzyme	Type of reaction	Source	Utilization
Papain	Peptide cleavage	Papaya latex	Clarification of beers, juices, meat tenderizers
Rennin	Milk coagulation	Calves, plants	Cheese making
Trypsin	Protein hydrolysis	Animal pancreas	Meat tenderizer, tryptones and bacterial media components
Chymotrypsin	Hydrolysis of protein	Animal pancreas	Medicinal use, meat tenderizing, digestive aid.
Fungal proteinases	Peptide bond hydrolysis	Aspergillus spp	Leather, meat and beer processing etc, substitutes for chymosin
Subtilisin	Peptide hydrolysis	Bacillus subtilis	Detergents, gelatin manufacturing etc.

(a) Cheese Making And Proteases

The classical method of cheese manufacturing involved the use of rennet obtained from the 4th pouch of stomach, of the tender calves, after their slaughter. It was also called as chymosin. The increased demand of cheese and cheese based products along with the reduced availability of chymosin led to a search for chymosin substitutes. The criterion was the ability of the enzyme to cause a limited hydrolysis of the casein as further hydrolysis impairs the texture and flavor of the finished product. The proteases from Mucor pusillus and Mucor miehei are good substituents of chymosin. Similarly a protease from Edothia parasitica has also given good results. The main fermentation reaction common to all cheeses is the fermentation of lactose to lactate. The microorganisms used mainly included the S.lactis, S.cremoris, S. diacetylactis and Leuconostoc spp. The second stage fermentation were carried by use of specific organisms.

Table 10.4: Cheese type and microorganism used

Type of cheese	Organism used
Swiss cheese	Propionobacteria spp
Camembert cheese	Penicillium camemberti
Roquefort cheese	Penicillium roqueforti

The characteristic flavor in each is due to the variety of the carboxylic acids, alcohol, ester and other volatile compounds formed by the limited proteolysis and fermentation brought out by the organisms. Trials are underway to use combinations of enzymes that may produce similar cheeses.

(b) Meat Tenderizing And Proteases

The toughness of meats is primarily due to the rigidity of collagen, elastin and actomyosin. Enzymes are often used in the USA to tenderize lower cut meats. The proteases are either injected in the vascular system before slaughter or are applied after the slaughter. Usually the proteinases from A.oryzae or trypsin, chymotrypsin, papain and pepsin are used. This is a common practice in case of beef production. The breakdown of muscle proteins and elastin reduces the rigidity of the finished products to yield soft meats.

(4) Production Of Sweet Syrups

The production of glucose and fructose syrups is quantitatively a very important process as these products are extensively used in food and pharmaceutical industries as sweeteners. A glucose-fructose mixture is sweeter than glucose alone. Use of fructose has an advantage, as the sugar does not crystallize at comparative higher concentration. Isomerization of glucose can be brought about by using xylose isomerase or glucose isomerase after the starches have been hydrolyzed by appropriate enzymes. Starches are often hydrolyzed by using the enzyme exo-1, 4-D glucosidase from A.niger. Cornstarch and sucrose from sugar beet oı cane sugar are used as raw materials to produce the reducing sugars. Beta galactosidases are also used to partially hydrolyze lactose to glucose and galactose in the ice cream manufacturing. The advanced versions of these processes use immobilized enzyme columns.

(5) Enzymes In detergents

During the 1960s detergents containing enzymes were extensively manufactured and used. However the indication of possible health hazards drastically reduced the use of enzymes in detergents. After research and alterations enzymes are now selectively used in detergents.

The principle enzymes used in detergents include amylases and neutral or alkaline proteinases that are active over a pH range of 6 to10 and a range of temperatures 30deg.c to 60 degrees C. The partial degradation of carbohydrates and proteins is required while lipids can be removed by ionic reagents at alkaline pH values.

Immobilization Of enzymes

A new dimension was added to the development of enzymology with the advances in techniques that permit the immobilization of enzyme to insoluble matrix without any adverse effect on their activities.

Immobilized enzymes are fast replacing traditional chemical and fermentation processes employing microbes. Table 10.5 lists some of the currently used immobilized enzymes.

Table 10.5: Industrial uses of immobilized enzymes.

Immobilized enzyme	Industrial use
Amino acylase	Separation of amino acids from racemic mixtures
Xylose isomerase	Production of glucose-fructose mixtures
β- galactosidase	Dairy and ice cream products
Urease	Artificial kidneys
Glucose dehydrogenase	Glucose electrode

Advantages Of Immobilization Of Enzymes

There are many advantages of using immobilized enzymes. Some of them are given here in brief,

(a) Reutilization

Once the enzymes are trapped in a column or insoluble matrix, the columns may be repeatedly used to process subsequent batches of substrates with almost the same efficiency, a number of times.

(B) Continuous Use

Alternately, immobilized enzymes may be used in continuous flow fermentation columns.

(C) Saving Of Labor

Use of immobilized enzymes saves labor during fermentation that would otherwise be required to scale up the media, culture, inoculum build up, and other allied efforts.

(D) Economy In Processing

The use of immobilized enzymes greatly reduces initial as well as recurring expenses that are required for isolation and maintenance of cultures, scale up units and purification processes that would be otherwise required.

(E) Better Recovery

The products formed by use of immobilized enzymes are usually free from contaminants that may be produced in the use of whole cells. Moreover since the enzymes are efficient catalysts that are specific in action, chances that the final product is pure are very high. This saves time, money and labor on purification of the product of interest.

(F) Time Efficiency

The rate enhancements in case of enzymatic reactions are dramatic. Up to several thousand times of substrate molecules are transformed per unit time than in the case of un-catalyzed reaction. This saves time and provides for faster and better production.

(G) Even Distribution And Activity

The enzymes in whole cells are localized and so are the cells in fermentation vats. This reduces the activity area to a comparatively small, localized portion in the system. Enzyme immobilization spreads the enzyme molecules evenly along the cross section area and therefore increases the efficiency of the process.

(H) Stability Of The Enzyme

Entrapment or immobilization often adds to the stabilization of the enzyme. The system is devoid of the natural inhibitors present within the cell and therefore the activity can be continued without any hindrance.

(I) Better Control Of Catalysis

In contrast to the use of whole cells, where the process requires large scale manipulations to obtain synchronous or continuous cultures, immobilized enzymes prove easy to control and hence there is easier control of catalysis.

(J) Process Efficiency Over Whole Cells

Whole cells may undergo spontaneous mutations that may result in low efficiency or production of unwanted by-products. This does not occur when immobilized enzymes are used.

(K) Synthetic Multi Enzyme Analogs

The immobilization of more than one enzyme can produce a multi enzyme analog that may be constructed using more efficient, stable enzymes from different cells. There is also a probability of clubbing together two or more processes that do not normally occur within a single cell.

(L) Rapid Diagnosis Systems

The advances in diagnostics that are under trials may use immobilized enzymes, to form enzyme electrodes that may help in rapid detection of glucose, urea and other important bio-molecules.

(M) Enzyme Patches

Immobilizing protein-degrading enzymes on patches can be used to degrade unwanted or dead tissue, from wound surfaces and enhance treatment and therapy options.

Methods Of Immobilization Of Enzymes

During the later part of the last century, many methods have been used for the immobilization of enzymes. Few methods are routinely used. Some important methods are given here.

(1) Adsorption

This is the mildest of all methods. Adsorption is mediated by ionic, hydrophobic or H bonding. Stirring of the enzyme with ion exchange resin can cause its adsorption. It can also be done by change in pH, ionic strength of solution or alteration of concentration.

The process is advantageous as,

It is simple and inexpensive

It requires mildest treatments

It provides easy and unhindered approach of substrate to the bound enzyme.

It can be applied to both; whole cells as well as isolated enzymes

It uses a polymer support.

However there are some disadvantages that include,

The bound enzyme may be leached during change in pH or ionic concentration

The similarly charged substrate may interfere with the binding of enzyme to support.

A schematic representation of the adsorption phenomenon is shown as,

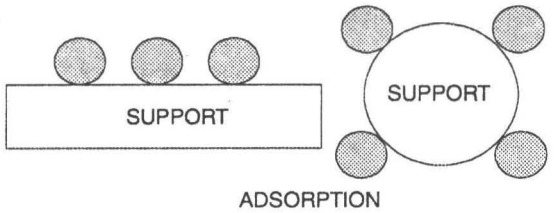


Fig.10.1: Immobilization of enzymes by adsorption.

(2) Ionic Bonding

The ionic bonding utilizes the advantage of ionic interaction of the enzyme. The support matrix is chosen either that has a suitable ionic group or the inert molecules are tagged with a suitable ionic residue that enables the attachment of enzyme with the matrix. (If the enzyme carries a positive charge then matrix should carry negative charges to ensure ionic bonding). This is also a surface technique.

The advantages of the technique include,

Higher specificity

Lesser contamination

Even distribution along the surface or cross section of column

The disadvantages are,

Requirement of specific support materials

High cost of columns

Susceptibility to charged molecules that may interfere with the ionic bonding

A schematic representation may be given as,

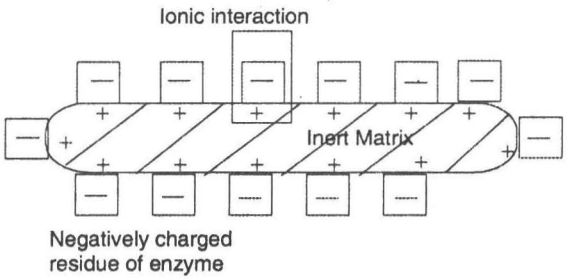


Fig. 10.2: Immobilization of enzymes using ionic bonding.

(3) Covalent Bonding

It is the most widely used technique. The enzyme is bound to the support molecules through covalent linkages either directly by activating the polymer with a reactive group,

The materials used as matrix include, cellulose, agar, agarose, or other polymers. The amino acid side chains containing hydroxy and amino group bind with the support or the reagent used as a bridge.

The technique has the following advantages

The binding is specific

There is little or no elution

There is a wide choice of carriers, matrix materials and bridging agents.

The disadvantages of the technique includes

The procedure is labor intensive.

The costs are high.

The procedures are complicated, require harsh environments and are difficult to regenerate.

A schematic diagram is represented as

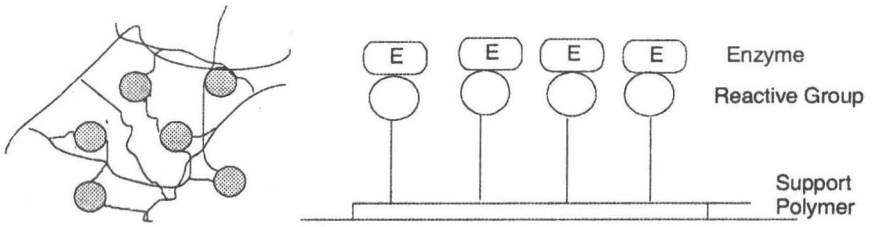


Fig 10.3: Immobilization by covalent bonding

(4) Cross Linking

The enzymes may alternately linked to a support by using a bifunctional reagent that binds to the polymer on one hand and the enzyme of interest on the other. The reagent acts as a bridge between the polymer and the enzyme. The most preferred agent is glutaraldehyde however other reagents like dimethyl adipimate are also used. Alternately copolymerization with inert protein like albumin has also been used.

A schematic representation can be shown as,

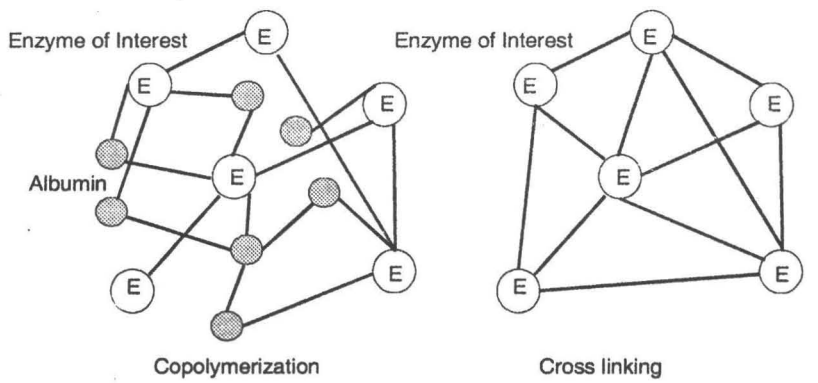


Fig. 10.4: Copolymerization or cross linking in enzyme immobilization.

(5) Entrapment

The method is based on entrapment of the enzymes or whole cells within the polymers of insoluble matrices. The enzyme is embedded inside the matrix, in contrast to the surface attachment in the adsorption technique. The enzyme is initially mixed with the monomer solution and then it is activated for polymerization during which the enzyme molecule gets entrapped within the matrix formed. The polymerization can be effected by chemical reactions, change in pH, temperature or addition of a complexing agent.

The advantages of entrapment include

It is a simple procedure

It requires very less instrumentation

It is based on mild treatments

It does not inactivate the enzyme

It does not create any constraints on the enzymes

The disadvantages of the method are

It may create steric hindrances for large substrates interfering with the rate of reactions

In cases where the gels are compacted it may affect the activity to a minor extent

In some cases where the matrix pores are large enough, the elution of the enzyme may occur

A schematic diagram of entrapment can be shown as,

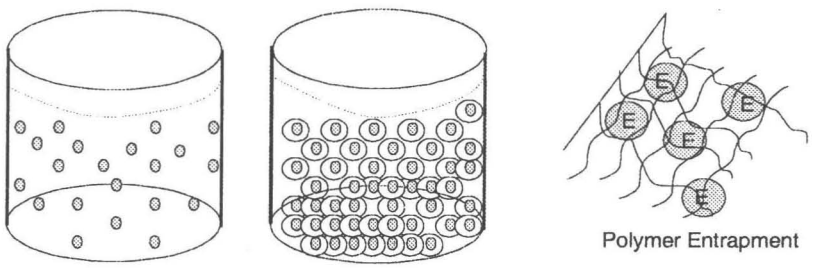


Fig. 10.5: Entrapment of enzymes in polymers and beads

(6) Encapsulation

Enzymes are many times enclosed inside membranous structures that permit the inflow of substrates and outflow of products however the enzymes are not able to pass out through them. Various materials like nylon, silicates, cellulose nitrate, phospholipids or liposomes can be used to manufacture encapsulating materials.

The technique has following advantages

The process is simple

It is cheap

It provides a large surface area to volume ratio

It does not require costly apparatus and instrumentation.

The disadvantages are as follows

The enzymes may not be stable

It cannot be used on large scale.

Schematic representations of encapsulation are shown as,

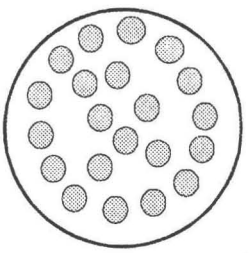


Fig 10.6: Encapsulation in membranes, liposomes etc.

There is an ever-increasing utility of immobilized enzymes in industry. Some times enzymes and cells are co-immobilized to increase the efficiency and productivity of the process. Some examples of immobilized enzymes used in industry are as follows.

Table 10.6: Enzyme immobilization methods and uses of some, commercially important enzymes.

Enzyme	Method of immobilization	Activity
Aminoacylase	Adsorption on DEAE sephadex	Separation of L-amino acids from racemic mixtures
Amyloglucosidase	Adsorption on charcoal	Conversion of starch and dextrins to glucose
Lactase	Covalent bonding, entrapment or adsorption on resin	Degradation of whey lactose, production of galactose from lactose
Glucose isomerase	Cross linking with glutaraldehyde	High glucose-fructose syrups
Penicillin acylase	Covalent bonding to sephadex	Production of 6-amino penicillanic acid from penicillin G
Nitrilase	Entrapment in cationic acrylamide	Conversion of acrylonitrile to acrylamide
Fumarase	Entrapment	Fumarate to malate
Aspartase	Adsorption	Fumarate to aspartate

Enzymes In Diagnosis

The use of enzymes in diagnosis has drastically increased in the past few years. This is primarily due to advancement of diagnostic techniques as well as the enhancement in production of such enzymes due to the contribution of biotechnology. The relation of enzymes with diagnostic tests is in two ways.

(1) Diagnosis of alteration of enzymatic levels in the patient's sample that will provide a clue to differential diagnosis or indicate the root of damage.

(2) Use of enzymes to diagnose and quantify the important bio-molecules that can help in diagnosis of specific diseases, their monitoring and responses of the patient to certain drugs.

In the chapter on isoenzymes the role of several isoenzymes in diagnosis has been discussed. The second aspect, that is the use of enzymes in the diagnosis and quantification of bio-molecules is emphasized in this chapter.

There are two important ways, in which the enzymes aid in quantification.

(a) Enzymes act on substrates to form colored compounds that can be estimated by colorimetry. In such cases the substance to be quantified should serve as the substrate of the enzyme in use.

(b) Enzymes are used as tagging agents in EIA (enzyme-immuno- assay).

Various enzymes from plant, animal or microbial origin are employed for diagnosis. Their stability, specificity and shelf life are more important than their purity. However no interfering substance should be present in the enzyme preparation when it is used as a diagnostic tool.

The dehydrogenases and oxidases are the most frequently employed enzymes. In case of the dehydrogenases, since most are NADH dependant, monitoring of NADH by using spectrophotometer can aid in diagnosis. The oxidases produce hydrogen peroxide that reacts with a different substance that forms a color compound that is monitored by colorimeter.

(a) Many enzymes are routinely used in the diagnosis; some are mentioned in the table given in table 10.7.

Table 10.7: Enzymes in diagnosis

Enzyme	Use
Alcohol dehydrogenase	Assay of alcohol in fluid
Arginase	Assay of L-arginine
Creatine kinase	Diagnosis of cardiac function
Cholesterol esterase	Determination of serum cholesterol
Glucose oxidase – peroxidase	Glucose estimation
Glycerol kinase	Determination of serum triglycerides
Glutamate dehydrogenase/Urease	Assay of BUN
Uricase	Determination of uric acid

Some of the important assays are mentioned here.

(1) Assay of Blood Glucose

The prevalence of diabetes in the world has greatly increased the need of diagnosis of blood glucose as a marker for the diagnosis and monitoring of the disease. While many methods are available for the diagnosis, the enzymatic methods have the advantage of rapidity, simplicity, accuracy, reliability and sensitivity. Two assay systems are commonly used.

(a) GOD-POD Method

The determination of glucose is done by using the enzyme glucose oxidase. The hydrogen peroxide formed reacts with 4 hydroxy benzoic acid and 4-aminophenazone to form a red-violet dye, quinoneimine that is read at 505 nm. The intensity of the dye is proportional to the amount of glucose in the sample. The reactions are represented as follows,

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

(b) Glucose-6-phosphate Dehydrogenase Method

The principle of the technique is that glucose is converted to glucose-6-phosphate by the action of hexokinase. Glucose-6-phosphate is dehydrogenated by NAD dependent dehydrogenase to 6-phospho gluconate and NADH. The NADH is formed in proportion to glucose originally present. Therefore the monitoring of NADH at 340 nm, can lead to quantification of glucose.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} \xrightarrow{\text{G6P-DH}} \text{6-phospho gluconate} + \text{NADH} + \text{H}^+$$

(2) Assay Of Cholesterol

Enhancement of level of cholesterol is indicative of risk of IHD. The diagnosis of cholesterol uses an enzymatic system comprising of cholesterol oxidase and peroxidase. The reactions can be represented as,

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{4-Cholestenone} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-Amino antipyrene} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

Quinoneimine is a red-violet dye that can be estimated at 500 nm. The formation of the dye is proportional to the amount of cholesterol in the sample.

(3) Assay Of Triglycerides

Triglycerides are associated with atherosclerosis and coronary artery diseases. The triglycerides tend to precipitate on the internal walls of coronary arteries. This causes a loss of elasticity of the blood vessels as well as the reduction in the internal diameter of the arteries. The reactions involved include,

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{Glycerol-3- Phosphate} + O_2 \xrightarrow{\text{Glycerol-3-Phosphate Oxidase}} \text{Dihydroxyacetone Phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrene} + \text{chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + HCl + 2\,H_2O$$

(4) Assay Of Blood Urea And Uric Acid

Elevated levels of urea and uric acid are related with various diseases. Chronic and acute renal failure, nephritis etc. are associated with the increase of blood urea and increased levels of uric acid are associated with problems of nucleotide metabolism. Urate crystals are deposited in joints due to elevated levels of uric acid found in gout. Stones may be found in urinary tract, while in chronic hyperuricaemia, tophi are deposited in tendons and cartilage.

The diagnosis of urea uses urease and glutamate dehydrogenase while that of uric acid uses uricase and peroxidase. The reactions are represented as,

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2\overset{+}{N}H_4 + CO_2$$

$$2\alpha\text{Ketoglutarate} + 2\text{NADH} + 2\overset{+}{N}H_4 \xrightarrow{\text{Glutamate dehydrogenase}} 2\ \text{Glutamate} + 2\ \overset{+}{N}AD + 2H_2O$$

The NAD formed is monitored by spectrophotometer. Alternately the diagnosis may involve the reactions of ammonium ions with phenol and hypochlorite in the presence of nitroprusside forming a blue colored complex that absorbs at the wavelength of 640nm.

The reactions involved in diagnosis of uric acid are represented as,

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminophenazone} + \text{3,5-dichloro-2,hydroxybenzene sulfonic acid} \xrightarrow{\text{Peroxidase}}$$

$$\text{Quinoneimine} + 4H_2O$$

(5) Assay Of Creatinine

The prevalent techniques use the creatinine picrate formation for colorimetric analysis. The enzymatic techniques are under trials and Boehringer Mannheim has now formulated a method that is under trial.

(6) Assay Of Phenylalanine

Phenyl ketone urea, a genetically inherited disease is caused by the inability to metabolize phenylalanine and in such patients the amino acid accumulates causing untoward reactions. The amino acid is estimated by an enzyme system that requires NAD, and the NADH produced is reacted with iodonitrotoluene to produce a red colored product. The intensity of the color is directly proportional to the concentration of the amino acid in the sample.

Enzyme Electrodes

The enzyme electrode, are a new type of bio-sensors that can be used for the assay of important bio substrates like glucose, urea, amino acids, alcohol and lactate. They may contain digital display as shown in fig. 10.7.

They consist of an electrochemical sensor in contact with a thin semi-permeable membrane containing bound enzyme molecules, having ability to react with the molecules mentioned.

When the enzyme electrode is immersed in the test sample the substrate diffuses through the outer membrane up to the enzyme molecules and the catalytic reaction occurs. These enzymatic reactions usually produce oxygen, carbon dioxide, protons, ammonium ions or other small molecules that are detected with the help of specific sensors. The magnitude determines the concentration of original substrate. The exact concentration is determined by referring a standard curve.

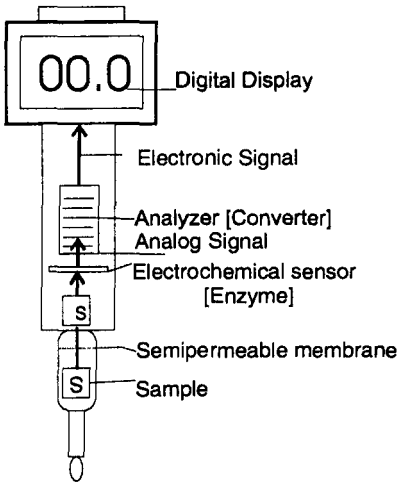


Fig 10.7: Glucose Electrode (Pen model)

An alternative construction the glucose electrode is shown in (Fig 10.8) The electrode is such constructed that when dipped in the solution containing glucose, it allows the passage of glucose and oxygen through the membrane and glucose is converted to gluconic acid and hydrogen peroxide as a result of oxidation reduction reactions. The oxygen concentration in the gel around the electrode is lowered down.

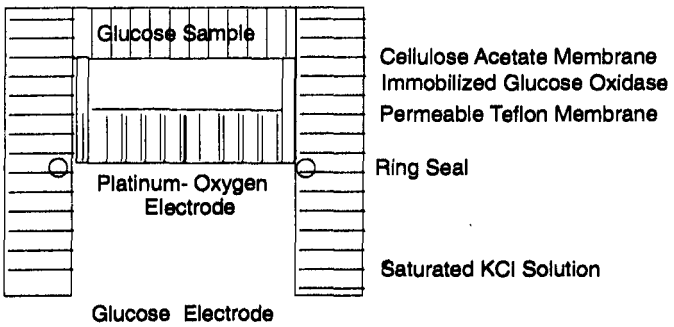


Fig 10.8: Schematic representation of Glucose electrode.

The hydrogen peroxide changes the measurable signal. The rate of diminition of oxygen concentration is proportional to the glucose concentration of the sample. It responds linearly to glucose concentrations over a range of 1/10 - 1/100000 mol/ dm3 with a response time of 1 min.

Alternately strips impregnated with the necessary enzymes are also frequently used. An enzymatic strip for glucose is schematically shown in fig 10.9

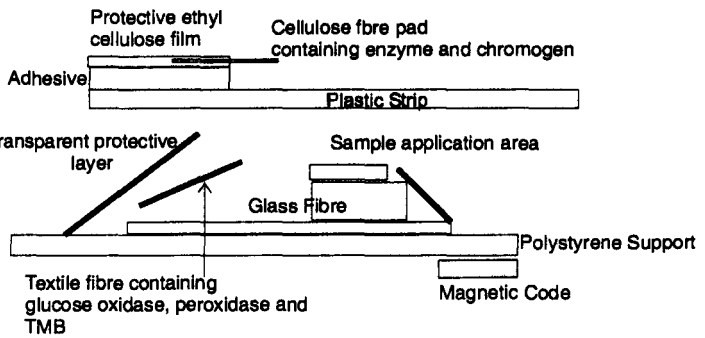


Fig 10.9: Strips for glucose detection.

Enzyme Immuno Assay

The EIA is an indirect method, to measure the concentration of substrates present in the body fluids, that uses enzymes as an amplification system. The EIA methods rely on specificity of antigen-antibody interaction for binding the substrate assayed together with some method of increasing the sensitivity of detection. This is done by using an enzyme-label.

The small molecules such as hormones and drugs so not induce antibody formation when injected into the blood of a different animal. If they are combined to a protein such as albumin, prior to their injection, they produce antibodies on injection into the blood of a different animal. These antibodies specifically combine with the antigen to form insoluble antigen-antibody complex. The antibodies combine with the hapten (original small molecule that was combined with albumin to produce antibodies) however in this case the complexes are soluble and do not precipitate. Thus many small molecules acting as haptens, can be estimated by immuno assays. The interest is to design a method that can assay antibody-hapten complexes especially when they are present in small concentrations. The methods incorporate an enzyme molecule on to the antigen or antibody to amplify the diagnostic system.

The principle can be illustrated with the help of an example as follows. The molecule to be assayed, 'S', is combined with a protein like albumin and the conjugate is injected in the rabbit to raise the antibodies against the conjugate. The rabbit serum is fractionated and these antibodies are separated for use as a reagent. A second conjugate of S with a purified enzyme such as peroxidase is prepared so that the enzyme remains active. The solution containing an unknown concentration of S is mixed with a fixed concentration of the antibodies, so that excess of antibodies are present. A known concentration of enzyme molecules labeled with S, are then added. A portion of the latter combines with the remaining antibodies and becomes inactive, leaving behind some Enz-S molecules that remain active and are assayed by using the specific substrate. From back calculations the concentration of S present in the original solution can be determined.

A diagrammatic representation of the EIA principle explained above is shown in fig 10.10.

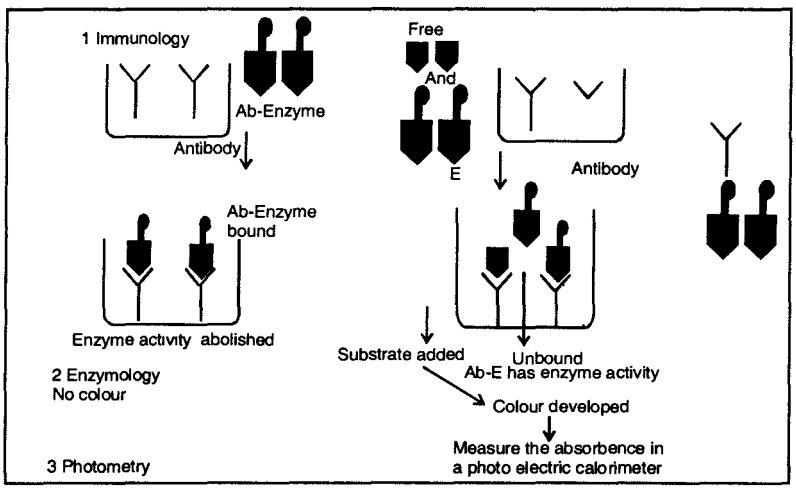


Fig 10.10:
General principle of EIA

Heia (Homogenous enzyme immuno assay)
The three phases of EIA : immunology, enzymology and photometry.

Depending on the technique used there are many types of EIA. Three are diagrammatically shown here,

1. Direct EIA

The antigens are adsorbed on a solid support like microtiter pipettes or nitrocellulose paper or plastic beads. The specific antibodies tagged with enzymes are then reacted with the adsorbed antigens. The preparation is washed and then the substrate is added. The activity of the enzyme is indicative of the presence of the antigens. A diagrammatic representation is shown in fig 10.11

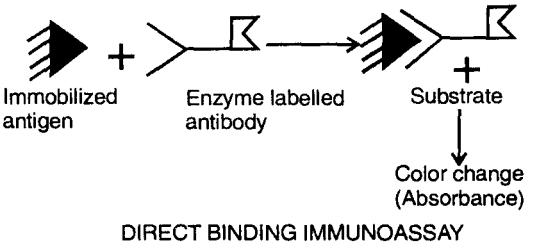


Fig.10.11: Direct EIA

2. Sandwich EIA

The sandwich assay uses antigen specific immobilized antibody for trapping the antigen in the sample. The enzyme is usually attached to a second antibody that is specific for a different determinant. Many times a staphylocccal protein is used in place of the second antibody. It is diagrammatically shown in fig 10.12

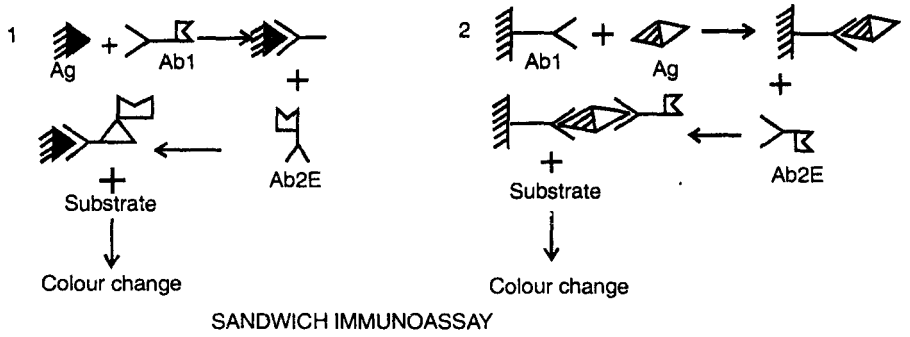


Fig 10.12: Sandwich enzyme immuno assay

3. Competitive EIA

As the name indicates, these assays use the limiting amounts of antigen specific antibodies that are either bound to a support or remain free in solution. Equal amounts of enzyme linked antigen are reacted with the antibody in presence of unlabelled antigen in the sample solution. If the concentration of unlabeled antigen in the sample is more then the probability of the binding of the unlabeled antigen is less. (Fig10.13)

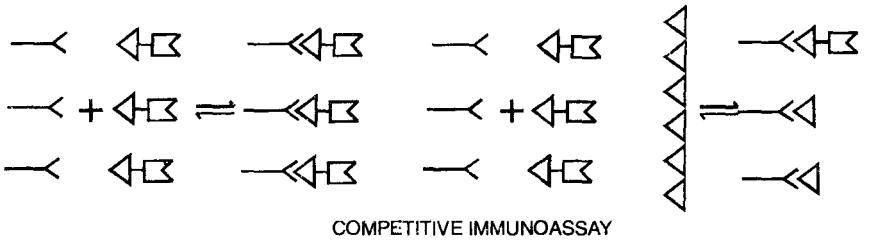


Fig 10.13: Competitive enzyme immunoassay

Using different known concentrations of the antigen, a standard curve can be obtained by plotting enzyme activity against concentration of the antigen. These curves help in the exact determination of the concentration of antigen in the unknown sample.

Different assays have been designed that are now routinely used in clinical diagnosis. Trials are underway to develop EIA assays for detection of parasitic, bacterial and viral infections.

Enzyme Therapy

The recent advances in enzymology have opened a new field called "Enzyme therapy". The genetically inherited diseases area menace in human beings and trials are underway to establish gene therapy treatments but the establishment of an efficient gene therapy may require a few decades and thus enzyme replacement therapies have been used as intermediate solutions to provide some relief to such patients.

The enzymes perform roles that include,

- As a replacement therapy agent in case of enzyme deficiency
- As tissue digestive agents to limit the growth of inflammatory diseases and injuries
- As a detoxification agent in chemical poisoning
- As a digestive aid
- As anticancer agents
- For treatment of blood coagulation disorders etc.

There are however limitations to the use of enzymes that include,

- Slow clearance of enzymes from blood
- Immunological responses
- Availability of pure enzymes at low cost.
- Delivery systems that enable targeted delivery of the enzymes

The use of enzymes for therapeutic purposes, require them to be of highest possible purity and specificity. The kinetic properties preferred are low Km and high Vmax for the maximum efficiency of the enzyme.

Some of the enzymes used in enzyme therapy are as shown in table 10.8.

Table 10.8: Therapeutic uses of enzymes.

Therapeutic purpose	Enzymes
Anticoagulant	Serine protease
Thrombolytic agents	Urokinase, Streptokinase, Fibrinolysin
Anti leukemic treatments	Asparginase, Glutaminase
Debriding agents	Trypsin, Papain, Collagenase, Amine oxidase
Digestive aids	Lactase, Pepsin, Pancrelipase, Papain
Liver failure, jaudice	Tyrosinase Bilirubin oxidase
Renal failure	Urease
Reduction of inflammatory response	Chymotrypsin, bromelain]

The roles of some enzymes used in therapy are detailed as follows,

- **Dnase:** In case of cystic fibrosis, the DNA liberated from infecting bacteria in lungs causes production of viscous mucus. The treatment with Dnase causes degradation of microbial DNA molecules so that the formation of mucus is retarded.
- **Debriding enzymes:** The enzymes clean open wounds by removal of dead and damaged tissues. Most frequently used enzymes are Trypsin, pepsin and collagenase. This results in rapid healing of wounds and trials are underway to use enzymes in post-operative conditions.
- **Digestive aids:** The indigestion can be treated with the enzymes that are involved in degradation of carbohydrates (amylases and lactase), Proteins (pepsin, papain, bromelain, etc) lipids (lipases) amino acids.
- **Asparginase:** The enzyme catalyzes the conversion of asparagine to aspartic acid and ammonia. All cells require asparagine for their normal activity. The enzyme is synthesized in most cells however certain malignant cells are unable to synthesize it. Administration of the enzyme thus reduces the availability of the amino acid and may prevent or retard growth of the cancerous cells. The enzyme is used in treatment of acute lymphoblastic leukaemia (ALL) and acute myeloblastic leukaemia (AML) in children and adults, along with various anti-neoplastic drugs.
- **Superoxide Dismutase:** It catalyzes the conversion of the highly reactive superoxide ion to hydrogen peroxide. The hydrogen peroxide and superoxide ions are extremely reactive and can cause extensive damage to the cells by reacting with the unsaturated fatty acids in the membrane. The two enzymes namely superoxide dismutase and catalase bring about the conversion of these radicals to water. Oxidative damage can occur in heart attacks, heart surgery and organ transplants where the enzyme may be used as a drug.
- **Rennin:** Human rennin has been produced by recombinant DNA technology. This enzyme has been used to treat abnormalities of blood pressure.
- **Streptokinase:** The enzyme can break down the blood clots in the vein and prevent heart attacks and strokes. The immune response generated by the enzyme due to its microbial origin is limiting factor in its use.
- **Lipase:** Many children suffer from accumulation of glucocerebrosides (Gaucher's disease). A glucocerebrosidase has been clinically tried to treat such patients.

Enzymology Of Genetics

Genetic engineering, recombinant DNA technology, biotechnology, are terms that are nowadays touching almost every branch of science. Enzymology is no exception. In fact enzymology has greatly contributed to the development of the science of biotechnology and neogenetics. The discovery of restriction enzymes, Taq polymerase, etc have enabled us to 'tailor' genetic materials from plants, microorganisms and the advent of PCR has added new dimensions to many branches of science including forensics.

Some of the important enzymes that are routinely employed in genetic studies and genetic engineering are discussed here.

(1) Alkaline Phosphatase

The enzyme can act on both nucleic acids. It digests the terminal phosphate group at the 5'end of the DNA. It comprises of two identical sub units and has a molecular weight of 140,000. The enzyme also contains 4 zinc atoms. It is used in genetics for two purposes,

(a) To Remove Terminal Phosphate From Linear Vector To Prevent It's Self Annealing

Vectors are circular extra chromosomal DS-DNA molecules that are used to incorporate foreign DNA to produce recombinant DNA molecules. For this purpose they have to be cut by specific restriction enzymes that form cohesive ends that may re-anneal to reform the vector molecule through the formation of a phosphodiester bond by the free 5'phosphate. The 5'phosphate is therefore cleaved by the enzyme alkaline phosphatase.

(b) For Labeling Of Dna And Rna

When the nucleic acids have to be labeled, it is necessary to remove the 5' phosphate so that the radio- label of p32 can be tagged at the 5'end. The removal of phosphate is brought about by alkaline phosphatase.

(2) DNA Ligase

The DNA ligase is an enzyme that joins the ends of two duplex DNA molecules by the formation of a phosphodiester bond between 3'OH and 5'phosphate at the site of the nick. It does not seal the nick in the absence of a 5'phosphate. There are 2 ligases that are frequently used, one from E.coli, and other from T4 lambda phage. The enzyme has a molecular weight of 68000, and requires ATP. The E.coli enzyme requires NAD in addition to ATP.

Its uses include,

- It joins vector and target DNA molecules to form recombinant DNA.
- It is used to construct vectors and chimeric DNA
- It is used in binding linker DNA to vectors and chromosomes
- It is used in the ligase chain reaction (LCR) for the chemical synthesis of DNA.

The action of DNA ligase occurs in three stages,

(1) Formation of activated enzyme –AMP- complex

(2) Transfer of the activated AMP to 5' phosphate of the nick

(3) Nucleophilic attack of the 3'OH on the activated 5' phosphate.

The diagrammatic representation is given in fig 10.14 as,

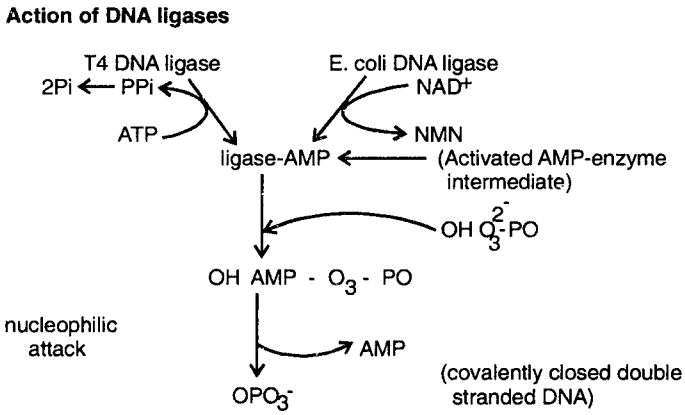


Fig. 10.14: Action of Ligase

(3) DNA Polymerase

The DNA polymerases are involved in the synthesis of a polymer of deoxyribonucleotides from dNTP molecules. There are two basic categories of DNA polymerases, based on the type of template used by them.

Table 10.9: Polymerases

Enzyme	Template
DNA dependant DNA polymerase	DNA
RNA dependant DNA polymerase	RNA

The second enzyme is also called as reverse transcriptase and shall be discussed a little later.

The DNA dependant DNA polymerases in prokaryotes are of three types, a comparison of these is tabulated in table no 10.10,

Table 10.10: Comparison of types of DNA polymerases

Property	DNAP I (Pol I)	DNAP II (Pol II)	DNAP III (Pol III)
Size in Kdal	109 Kdal	90 Kdal	900 Kdal
Number of structural genes	1	1	9
Constitution	Monomer	Monomer	Heterodimer
Molecules per cell	400	120	10-20
5'-3' polymerization	Yes	Yes	Yes
3'-5' exonuclease activity	Yes	Yes	Yes
5'-3' exonuclease activity	Yes	No	No
Polymerization rate	10-20 nts/sec Nts = nucleotides	1 nts/sec	750 nts/sec

* *DNA polymerase I*

It was the first DNA polymerase identified by Arthur Kornberg in 1956. Originally it was thought to be essential for DNA synthesis but later it was shown to have DNA repair and discontinuous synthesis.

The enzyme shows three types of activities,

(a) 5' –3' polymerization: The enzyme can synthesize short segments of DNA and takes part in repair synthesis

(b) 3'-5' exonuclease activity: DNAP I catalyses the breakdown of the nucleotides of a strand in the opposite direction of the polymerization. It functions as a "proofreader" that edits the mismatched nucleotidesat the primer terminus before the synthesis of the strand.

(c) 5'-3' exonuclease activity: This activity is very important in the removal of thymine dimers. The thymine dimers are formed on the exposure of DNA to UV light. The excision is followed by polymerase activity.

The enzyme is a single polypeptide of 109Kdal and contains one zinc atom per chain. There are a number of important sites on the enzyme chain that include, *template site, primer site, primer terminus site, triphosphate site.*

The Klenow fragment is a 68 Kdal fragment obtained by cleavage of trypsin or subtilisin. The Klenow fragment has a synthetic activity (5'-3' polymerization) and an exonuclease activity (3'-5 exonuclease).

* *DNA Polymerase II*

DNAP II is a single polypeptide chain with a molecular weight of 90 Kdal. There are about 40 molecules per cell of E.coli.

The enzyme shows 5'-3' polymerization activity and also an associated nuclease that cleaves the DNA in 5'-3' direction. It does not show the 5'-3' exonuclease activity. The 3'-5' exonuclease activity of the enzyme is indicative of its involvement in repair or editing function.

The enzyme is shown to elongate the Okazaki fragments in the absence of PolI.

* *DNA Polymerase III Or DNA Pol III HE (holoenzyme)*

This holoenzyme consists of a multi sub unit structure made up of 10 subunits and is mainly involved in the chromosomal replication. It is similar in structure and function to the eukaryotic chromosomal replicase. Its assembly occurs through three intermediates, **pol III core, polIII' and pol III***. A tabular representation of the composition and function of these intermediates is given in table no 10.11 as,

Table 10.11: Sub-units of Pol III

Sub unit	M Wt.	Functions
Thetaθ	8.6 kd	Stimulates exonuclease
Epsilon ε	27.5 kd	Proof reading 3'-5' exonuclease
Alpha α	129.9 kd	Polymerization of DNA
Tau τ	71.1 kd	DNA dependant ATPase forms dimer of core
Gamma γ	47.5 kd	Binds ATP

Delta δ	38.7 kd	Binds to β
Delta' δ'	36.9 kd	Stimulates clamp loading
Chi χ	16.6 kd	Binds SSB
Psi ψ	15.2 kd	Acts as bridge between chi and gamma
Beta β	40.6 kd	Sliding clamp on DNA

The composition of assemblies is as follows,

Table 10.12: Assemblies of Pol III

Assembly	Sub unit Composition
Core	θ, ε,α
Pol III'	θ, ε, α, τ.
Gamma complex	γ, δ, δ', χ, ψ
Pol III*	Gamma complex + pol III'
Pol III HE	β + pol III*

The holoenzyme is highly active. It can process several thousand nucleotides without dissociating from the DNA template.

The Taq DNA polymerase is isolated from the thermophile, *Thermus aquaticus* and is extensively used for the polymerase chain reaction. It is a single polypeptide chain having a molecular weight of 95000. It is a thermostable enzyme and can withstand a temperature of 95 degrees centigrade. Although it can withstand such high temperatures, the optimum temperature is around 75 degrees centigrade. It adds deoxynucleotides to the primer based on the template to form a complementary DNA strand. The enzyme has an unusual property of adding an extra adenine base to the 3' end of the growing strand.

The uses of the Taq polymerase include,

- Gene manipulation in genetic engineering
- DNA sequencing by Maxam-Gilbert method
- Producing DNA probes for DNA fingerprinting
- Forensic tests
- Clinical diagnosis

(4) Polynucleotide Kinase

The enzyme is a oligomeric protein comprising of 4 sub units and has a molecular weight of 34,000. It transfers a phosphate from ATP to the 5'OH group of the dephosphorylated DNA or RNA.

The uses of the enzyme include,

- Rephosphorylation of the 5'OH
- Radioactive labeling of the nucleic acids for,

(a) To make hybridization probes

(b) To make diagnostic kits

(c) To analyze base sequence of DNA

(d) To construct restriction maps.

(5) Reverse Transcriptase

The retroviral reverse transcriptase has a molecular weight about 70,000. It shows three types of activities,

RNA dependant DNA polymerase activity: The enzyme synthesizes a complementary DNA copy of the genomic RNA by a process called as RNA directed DNA synthesis requiring a polymerase activity by the enzyme.

Ribonuclease H activity: The enzyme degrades the RNA from the DNA-RNA hybrid leaving behind, a SS DNA.

Formation of a ds-DNA: The enzyme then synthesizes a second strand of DNA on the SS DNA template.

The most commonly used reverse transcriptases, include, those from the avian myoblastosis virus (AMV) or the murine leukemia virus (MuLV).

The mechanism of action of reverse transcriptase is shown as,

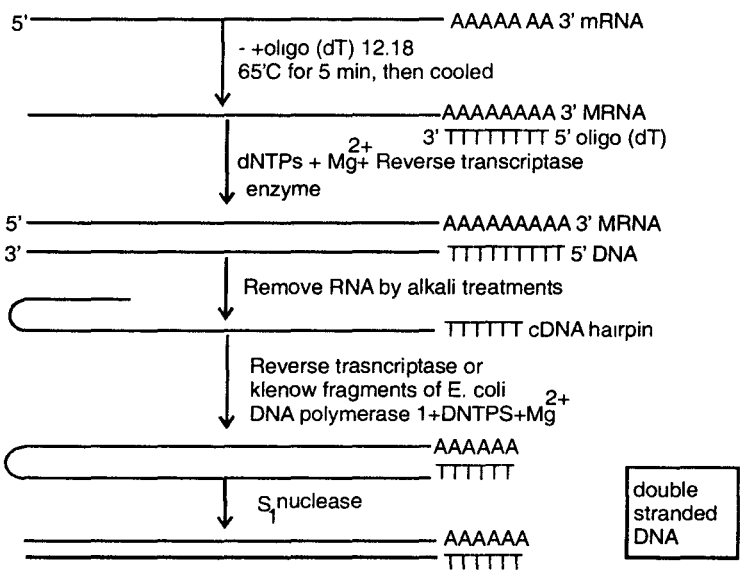


Fig 10.15: Action of Reverse Transcriptase

(6) Ribonuclease H

This enzyme is a nuclease that specifically cleaves and hydrolyses m-RNA present in a RNA-DNA hybrid. The enzyme has been isolated from various retroviruses including the *Rous sarcoma virus, avian myoblastosis virus, murine leukemia virus and MMTV.* The molecular weight of the enzyme is about 90,000. The peculiar character of this enzyme is that it does not cleave free RNA and hydrolyses only those RNA that are bound to the DNA in the hybrid.

The enzyme is used for,

- Hydrolysis of RNA from the hybrid for synthesis of second strand of cDNA
- Reisolation DNA probes from hybrids when RNA is not required.

(7) S1 Nuclease

It is a glyco-protein of molecular weight 38,000. The enzyme cuts selectively the single stranded regions in the DNA molecule. It however does not degrade ds-DNA molecules.

The enzyme is used for,

- It degrades the hairpin loop formed during synthesis of the cDNA from m-RNA.
- It is used to remove unwanted tails from DNA sequences to synthesize blunt ends
- It is used to remove the extra A from DNA prepared during PCR
- It is used to determine the complementarity between DNA strands during hybridization studies

The mechanism of the enzyme is diagrammatically shown in fig 10.16 as,

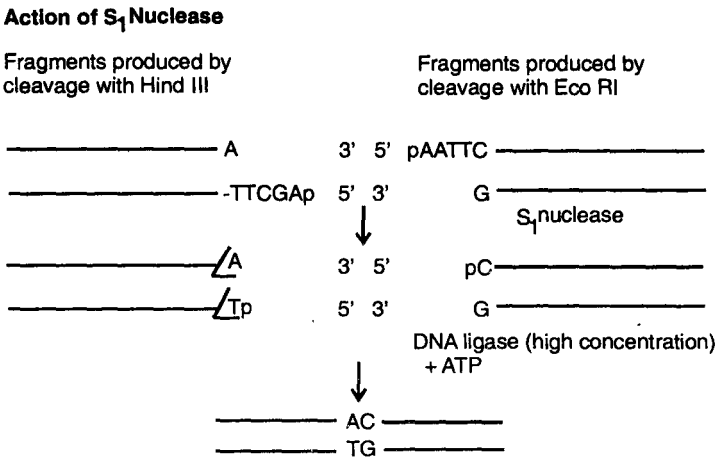


Fig 10.16: Action of S1 Nuclease

(8) Terminal Nucleotidyl Transferase

It adds mononucleotide phosphate to the 3' OH with a simultaneous hydrolysis andrelease of Ppi from the NTP. The enzyme does not require a template strand. The enzyme has two non-identical sub units and has a molecular weight of about 32,000. The uses of enzyme are,

- It makes homo- polymer cohesive tails. This helps in joining of blunt ends.
- It is used to make radioactive DNA probes.

(9) Restriction Enzymes

The enzymes that cleave double stranded- DNA molecules at unique sequences are called restriction endonucleases.

Werner Arber proposed the existence of restriction enzymes in the1960s while studying bacteriophages. He observed that on entry into the bacterial cell, the viral DNA was cut into small fragments and destroyed. He theorized that while the enzymes could cut the infecting DNA, it did not affect the host DNA, moreover the action of the enzyme on the viral DNA was found to be at specific sites. This was followed by the discovery of many restriction enzymes by different scientists', and in each case the enzyme acted on specific sequences of nucleotides present in the degraded DNA. Presently more than 300 restriction enzymes are recognized. Some of the important restriction endonucleases that are frequently used in genetic engineering include:

Table 10.13: Some restriction endonucleases.

Restriction enzyme	Source	Sequence recognized
(a) Generating cohesive /sticky ends		
1. Eco RI	Escherichia coli Ry 13	G*AATTC CTTAA*G
2.Bam HI	Bacillus amyloliquefaciens H	G*GATCC CCTAG*G
3 Hind III	Haemophilus influenzae Rd	A*AGCTT T TC GA*A
4. Taq I	Thermus aquaticus	T*CGA AGC*T
(b) Generating Blun ends		
1. Hind II	Haemophilus influenzae -d	GTY*PuAC CAPu*Y TG
2. Bal I	Brevibacterium albidum	TGG*CCA ACC*GGT
3. Sau 3 A I	Staphylococcus aureus 3A	GA*TC CT*AG

NOTE: * indicates site of cutting, Y indicates pyrimidines, Pu indicates purine.

Types Of Restriction Endonucleases

There are 3 major classes of restriction endonucleases:

TYPE I

These are most complex, bi-functional, (they possess both cutting and modification activities). The enzymes contain three non-identical sub units. The molecular weight is in the range of 400 kdal. The sequence of cutting is non-specific and the site is usually about 1000 nucleotides from the recognition site. The host- DNA are protected by methylation. They require ATP, Mg++ and adenosyl- methionine for their activation.

TYPE II

These are simplest, comprise of two identical sub units. They possess only cutting-activity. The molecular weight is between 200 to 1000kdal. The restriction site (4-6 base pairs) is specific and lies near the recognition site and is often palindromic. They do not require ATP and can function in presence of Mg++ as a cofactor. More than 70 such enzymes are commercially available.

TYPE III

They are moderately complex, bi-functional and have two sub-units. The recognition site is asymmetrical sequence of 5-7 base pairs. The cleavage site is 24-28 base pairs down stream from recognition site.

The type I and type III enzymes are of limited use in genetic manipulation. The use of type II enzymes is more frequent in genetic engineering as,

- They cleave in site specific manner at a site within the recognition site or near it.
- They do not require ATP.
- Mg ions are required as cofactors.
- They do not have modification activity and other modifying enzymes are required.
- They cut in palindrome sequence either at the axis or around it.

Nomenclature

The nomenclature system was proposed by Smith and Nathans in 1973. The names usually carry 4 alphabets and roman numerals.

- The first alphabet indicates the generic name of host
- The second and third letters indicate the species.
- The fourth alphabet indicates the strain.
- The Roman numeral indicates different restriction-modification systems in the same strain when more than one enzyme is obtained from the same organism. They may also denote the order of their discovery.
- The examples in table 10.14 can illustrate the nomenclature system as,

Table 10.14: Nomenclature of restriction enzymes: Examples and illustration

EcoRI	***HindIII***	***Sau 3AI***
E= genus *Escherichia*	H= genus *Haemophilus*	S= genus *Staphylococcus*
co = species *coli*	in = species *influenzae*	au= species *aureus*
R = strain Ry 13 H	d = strain Rd	3A = strain
I = first endonuclease isolated	III = third endonuclease isolated	I = first endonuclease isolated

Mechanism Of Action

Restriction enzymes either cut to form blunt ends and cohesive or sticky ends.

(1) Straight across the DNA to give blunt ends

(2) Straight single strand cuts producing short, single stranded projection at each end of cleaved DNA to produce cohesive or sticky ends.

Plane Of Cutting

Some of the restriction enzymes cut the double stranded DNA along the axis of symmetry of the restriction site. They break the phosphodiester bond, one in either strand of the restriction site at the axis of symmetry to form two blunt ends. (Fig 10.17)

Example of such enzymes include Bal I.

The diagrammatic representation is shown as,

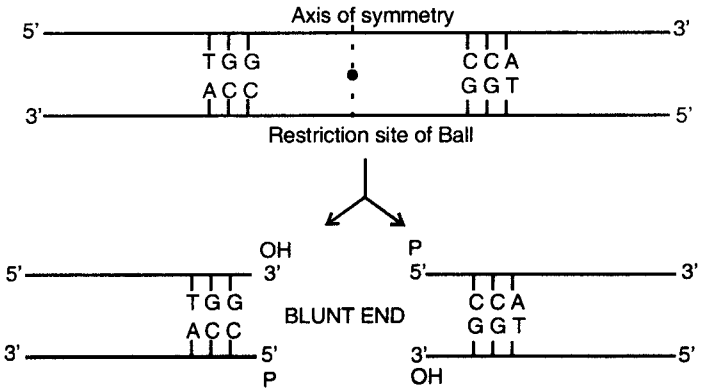


Fig. 10.17: Formation of blunt-ends due to cut along the axis of symmetry.

Several restriction enzymes cut ano strand at the left side of the axis of symmetry and the other strand at the right side of the axis of symmetry. After cutting the hydrogen bonds between the base pairs of the restriction site, two single stranded extensions called as sticky or cohesive ends are formed that are complementary to each other (fig 10.17). The 5' end carries a phosphate while the 3' carries a free OH group. Example of such enzymes includes EcoRI.

The diagrammatic representation is shown as,

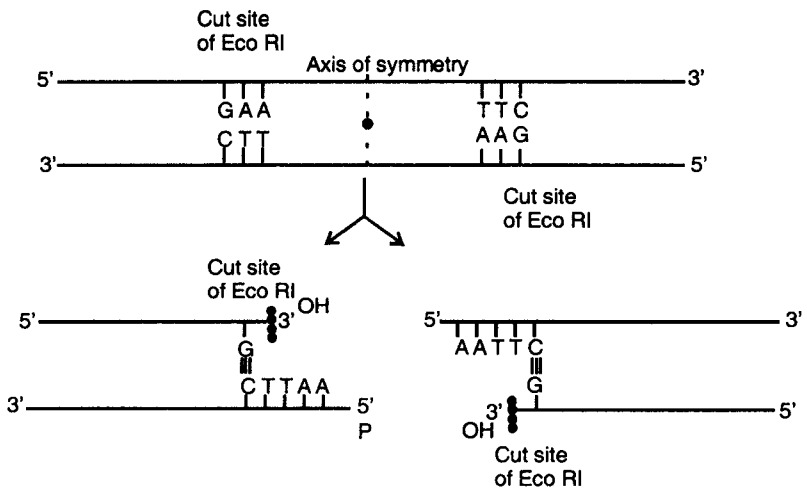


Fig 10.18: Cohesive ends formed due to action of EcoRI.

Uses Of Restriction Endonucleases

The uses of these enzymes are,

- To cut the source DNA for isolation of gene of interest for cloning
- To cut and remove unwanted sequences of natural vectors and construct active vectors

- To cut vectors at specific sites for cloning
- To construct restriction maps of DNAs
- To perform nucleotide sequencing of large DNA

(9) Methyl Transferases

They are also called methylases and are involved in the transfer of methyl group to Cytosine and Adenine of DNA. It is a monomer of molecular weight 62,000. The adenine is converted to 6-methyl adenine and the cytosine forms 5-methyl cytosine.

Only one methyl group is added at a time, after which it immediately dissociates from the DNA. For a second addition it has to re-bind to the DNA. The methylated-bases are shown by astrick (*) mark near the base. The methylation is not sequence specific. Methylation protects the host DNA from the activity of restriction enzymes.

The diagrammatic representation is given as,

The uses of the enzyme include,

- To methylate the desired DNA in r-DNA to protect it from restriction endonucleases.
- To protect some restriction sites from the activity of R.Enzymes when the DNA has many such sites.

Production Of Enzymes Using Microorganisms

The uses of the isolated enzymes are briefly mentioned earlier. Commercial production of enzymes, often use, microbial cells as their sources. The advantages of using microbes are obvious. Some of the important ones are mentioned here,

1. Microbes are easy to isolate and maintain in pure cultures.
2. They use comparatively cheap raw materials for their growth. (they can grow on industrial wastes and yet yield commercially important products)
3. They can be conveniently manipulated to allow the production of the enzyme of interest
4. Their rapid and exponential growth make the process economic
5. Isolation of extra cellular products from the broth is very easy.
6. In case of intracellular enzymes, a single step process that breaks the cell is sufficient. There is no requirement of differential centrifugation or fractional isolation.
7. The microbes from extreme environments possess enzymes that can tolerate related extreme conditions. In case if the industrial process employing such isolated enzymes requires an extreme treatment at some point, such enzymes, can be an asset.

Within a few years more than 1000 enzymes will be regularly used for industrial processes ranging widely from food industry to biotechnology, immunology, pharmacy etc. this actually calls for the identification and manipulation of microbes that may yield commercially exploitable enzymes. Some of the related considerations include,

Selection Of Strain

The selection of a preferred strain is related to its growth and cultural characteristics and its relation to the optimum conditions used in the industrial process. In other words, if an enzyme is to be used at a temperature around 45degrees C, it can be generally accepted that the organism that optimally grows around that temperature shall contain enzymes that will be suitable for the process. An example is of the amylase, from thermophile, *Bacillus coagulans* that can tolerate 10degrees higher temperature than the enzyme of the mesophile, *Bacillus amyloliquefaciens.*

Whether a strain is capable of producing an enzyme is primarily dependent upon the availability of the substrate under its normal growth conditions or habitat. For example, the ligninases and pentosanases are found in microbes growing in forest soils, while the uric acid decomposers can be the best source for uric acid oxidase.

The isolation of such strains often use traditional microbial techniques like enrichment of culture media, use of selective media, and the creative imagination of the workers. For example, uric acid-salt medium was used to cultivate and isolate those microorganisms that are capable of producing the enzyme uric acid oxidase, while a media fortified with 0.1M sodium sesquicarbonate and casein was used to isolate those species that produce alkaline proteases.

The identification and characterization of the strain is essential before the commercial utilization of the strain as biological hazards due to production of toxins and pathogenicity is common to microflora.

Development Of Strain

A commercially important strain must possess following properties,

- It should grow on an inexpensive medium
- It should give a constant and high yield of the enzyme of interest
- It should not produce unwanted and hazardous metabolites
- The recovery of the enzyme should be simple.
- The process should not produce biohazards
- The effluents of the process should be environmentally friendly.

This can result only from the optimization of strain properties and process parameters. The search for a constitutive mutant solves the problem easily while offering a permanent solution. With the advances in genetics and biotechnology, this has now become a regular practice. Mutagenesis, cloning and production of the hybrid strains are often employed. Completely genetically engineered strains are not a very distant possibility.

Once the desired strains are identified or produced, they need to be protected against degeneration, contamination, spontaneous mutation and loss of their viability and alterations in cultural characters. This is usually afforded by lyophilization of the strain or storing it at cryogenic temperatures under liquid nitrogen.

The Optimization Of The Fermentation Process

Until about 50 years back most of the traditional fermentation except the brewing processes were carried out on a surface culture basis. The advances in process control,

lowering of the handling costs and reduced risk of infections have prompted the use of submerged fermentation techniques. The various factors that affect the process are briefly discussed as,

(a) Preparation Of Inoculum

Since the production of specific enzymes may employ highly mutated strains, their stability during the propagation from seed culture to the fermentation vat level is of prime importance. The best method would be to inoculate the seed (lyophilized culture) into a single propagation tank that supplies enough inoculum to be used in the fermentation vat. The medium in the seed tank often resembles the actual fermentation medium. Multiple cultivation steps should be avoided as far as possible.

(b) Formulation And Preparation Of Cultivation Medium

An ideal medium is one that provides,

- An easily assimilable source of carbon and nitrogen
- Salts that are required by the microbe for its optimum growth
- Substances that promote good growth of the strain
- An inducer that promotes the synthesis of the specific enzyme of interest.

However use of glucose in the medium at high concentrations is known to produce catabolite repression. The sources that are generally used for supply of carbon and nitrogen are given in table 10.15

Table 10.15: Sources of nutrients for growth of microorganisms

Nutrient	Sources
Carbon	Partially hydrolyzed starch, mollases, corn, barley, wheat
Nitrogen	Soybean meal, corn steep liquor, whey, yeast extracts etc

Salts are often added and many such compounds may serve as auxiliary sources of nitrogen. The pH is also controlled and many fermentation processes require use of appropriate buffers.

The economics are important in the formulation of medium and since raw materials can amount to more than 60% of the expenses, researches are often targeted to reduce the cost of raw materials used for fermentation. Ground whole grains or flakes are often used.

The Equipment For Fermentation

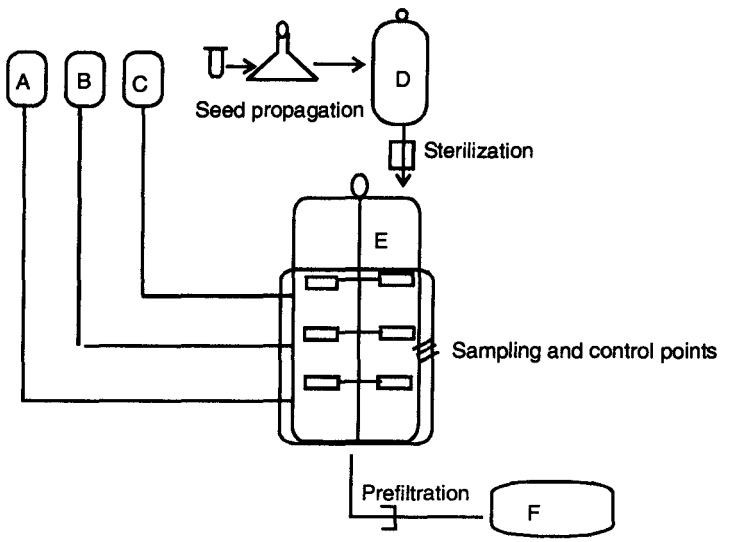
The equipment usually comprises of typical antibiotic fermentation machinery with certain modifications to suit the "low volume" fermentation required for enzyme production. The sampling and controls that are normally recommended include those given in 10.16

Table 10.16: Sampling and controls in fermentation.

Sampling	Controls
Cell counts	Temperature
PH	Pressure
Dissolved solids	Acid base flow
Viscosity	Foaming
Color	Nutrients
Enzyme activity	Carbon dioxide
Oxygen	Air flow

Enzyme production is often susceptible to microbial contamination. Special care is taken to prevent and control contamination. Extra cellular enzymes are usually produced by batch fermentation. The usual time interval is between 30-150 hours. Continuous techniques are applied on laboratory scale but since the growth and enzyme production conditions differ, often two stage processes are used. The first is usually for the maximal growth while the second is for the enzyme production.

A typical assembly can be diagrammatically shown as,



A = antifoam, B = Acid, C = nutrients, D = inoculum, E = fermentation vat, F = recovery

Fig 10.19: Schematic representation of fermentation assembly.

The process of fermentation is terminated at a point that facilitates recovery and in many cases this point may lie well before or after the fermentation optimum. The termination point is usually recognized by a characteristic change in pH, acid value or oxygen tension. Most enzymes are formed in the post-log phase although generalization is not possible. The protein content of the isolate is usually 1-5% of dry weight of starting medium and the residual nutrients are about 15%.

The Process Of Recovery

The recovery process includes,

- Pre cooling of the broth
- Centrifugation of the broth and separation of solids
- Vacuum evaporation
- Precipitation of proteins by suitable agents
- Finishing into crystals, powders or immobilized enzymes.

◆◆◆◆◆

11

ENZYMES FROM EXTREME ENVIRONMENTS

Introduction

Life at low temperatures

Life under pressure

Life at high temperature

- The Obligate thermophiles
- Facultative thermophiles
- The thermo-tolerant bacteria

Life at high salt and solute concentrations

Life at extreme pH values

- Low pH habitats
- High pH habitats

Introduction

Throughout the globe the environmental conditions vary greatly on the basis of geographical locations as well as the cycle of the seasons. The adaptation of the various species and especially the microorganisms has been a topic of interest since a bacillus was isolated that was capable of growing at 0 degrees centigrade. Scientific explorations of natural and man-made, low temperature environments were common in 1960-75. Many organisms isolated grew over a very limited range of temperature (0 degrees to 20 degrees C) however some could grow on a comparatively large range of temperatures.

Extreme environments include drastic temperatures, (low or high), pressures, salt and solute concentrations, pH values, heavy metals, and high irradiation. Since life propagation is related on the organism's ability to adapt itself to the extreme condition expressed through its ability to metabolize, grow and reproduce, it is necessary to understand the possible alterations in the enzyme systems that help the organism in its battle of survival. A brief discussion on life under extreme environments and a possible relation to enzymatic activities now follows.

Life At Low Temperatures

The low temperature zones are equally distributed in aquatic and non-aquatic portions of the land. Interest in the life at low temperature developed after the isolation of a psychrofile that could grow well at zero degrees C. During the period 1960-75 many workers have isolated and characterized life forms able to grow at temperatures below 5 degrees C.

The typical psychrophilic habitats include,

- High altitude habitats of the troposphere
- Caves
- Freshwater environs/lakes
- Rivers and streams
- Soils
- Marine environs
- Snow and ice

In each of the cases, great variations are observed with the seasonal cycles. It is not a compulsion however that only psychrophiles exist in such habitats. For example many water bodies undergo freezing and subfreezing temperatures in the temperate zones and still are natural habitats of the psychrophiles, mesophiles and the thermophiles. The subterranean caves on the other hands usually have low temperatures (below 10 degrees C), absence of organic materials or very low levels, and a relatively high humidity. Most microbes growing in these conditions belong to the species of *Arthobacter, Pseudomonas, & Flavobacterium.*

The low temperature maximum for growth shown by the organisms in these habitats is more often than not related to the thermal-susceptibility of the enzymes found in them. This relation is however established by performing sensitivity tests on enzyme in intact cell as well as in cell free system. In other words some microbes may produce enzymes that are not sensitive to temperature while present in the cells but

show temperature sensitivity in isolated extracts, a phenomenon that may be due to internal stabilization of the enzyme. On the other hand some species may produce enzymes that are equally sensitive in both the cases. Some of the microorganisms and their temperature sensitive enzymes are tabulated as,

Table 11.1: Temperature Sensitive Enzymes And Organisms Producing Them

Organism	*Enzyme(s)*
V. marinus MP1	*Malic dehydrogenase*
B.psychrophilus	*Glucose oxidase*
Basidiomycetes	*β-glucosidase*
C. utilis	*Pyruvate decarboxylase*
C.gelida	*Glucose fermentation.*

Life Under Pressure

The hydrostatic pressure in oceans is drastic. With a depth of 10 mts, the water column pressure increases by 1 atm. The pressure at oceanic floor has been found to be 380atm. The organisms that grow at such high pressures need to be essentially barophilic. Some of these strains have been found to be baroduric or pressure tolerant.

The general rule is that these organisms grow at very slow rate, with the exception of organisms present in the gut of amphipods found at deep sea levels. These organisms grow rapidly at 3degrees C at 750atm.

Another natural environment where the high pressure prevails is the deep oil or sulfur wells. In any case the interest in microbial baro-biology is from the point that pressure has a great potential in modifying the physiology of industrially important microbes, as a sterilizing and disinfecting agent, as a modifying factor for industrial enzymatic processes including those that make use of immobilized enzymes. It is also expected that the substitution of amino acids in the enzymes can render baro-stability to enzymes.

It is generally observed that the activation energy or Vmax are not the factors but the enzyme-substrate, enzyme-cofactor or enzyme modulator affinities affect the enzymatic functions under high pressure environments. Studies on the enzyme Fructose diphosphatase from rainbow trout and rat-tail fish have indicated the adaptation of the enzyme to high pressure environments due to high concentrations of substrate and cofactors.

Life At High Temperature

There are several habitats that have high temperatures. They include erupting volcanoes, boiling springs, compost piles, seaweed piles, coal refuse piles etc. Industrial water heaters, and steam heated buildings, cooling columns also serve as a habitat for certain non-photosynthetic bacteria provided that other conditions are appropriate. The flora comprises of protozoa, algae, fungi, cyanobacteria, photosynthetic bacteria,

chemolithotrophic bacteria and heterotrophic bacteria. The thermophiles are differentiated into three main groups by Farrell and Campbell.

(1) The Obligate Thermophiles

They grow only above 40 degrees and have optimum temperature around 65-70 degrees centigrade.

(2) Facultative Thermophiles

They have a maximum growth temperature around 50-65 degrees centigrade but can reproduce around room temperature.

(3) TheThermo-Tolerant Bacteria

They normally grow at room temperature but can tolerate temperatures around 45-50 degrees centigrade.

The thermostability of enzymes was studied initially around 1949 and demonstrated that the enzyme Malate dehydrogenase from a thermophile was stable at 65 degrees C for 2 hours in contrast to the homologous enzyme from the mesophile that was inactivated at that temperature within few minutes. In 1968 studies were carried out on 11 enzymes and the report suggested that in general all the thermophilic enzymes were resistant to heat inactivation than their counterparts from mesophiles. In particular the enzyme Glyceraldehyde-3-phosphate dehydrogenase was found to be stable at 90 degrees C.

The original idea was that the enzymes from the thermophiles are rigid and inflexible in conformation and therefore cannot undergo allosteric modulations. Later studies indicated that many of the thermophilic enzymes are subject to allosteric control.

The enzymes are divided into two groups,

1. Those enzymes, that show allosteric activity, at both, high and low temperatures. The examples of this group include Aspartokinase, Threonine deaminase, Phosphofructokinase, etc.
2. Those enzymes, that show allosteric activity only at high temperatures. The examples of this group include Lactate dehydrogenase, Homoserine dehydrogenase, Uridine kinase, and Ribonucleotide reductase.etc.
3. The important thermo stable proteins isolated and studied in detail include Glyceraldehyde 3 phosphate dehydrogenase, Ferridoxin, Thermolysin, Formyl tetra hydro folate synthetase, Taq polymerase, etc.

The Taq polymerase has already been used in genetic engineering and PCR.

Life At High Salt And Solute Concentrations

Almost all living-systems contain aqueous system in which the biochemical reactions occur. The solutes dissolved in water and the availability of water are the major control system that regulates the enzyme activity, membrane structure and function and the assembly and dissociation of cellular organelles.

High concentrations of salt and sugar have been therefore used to inhibit the

growth of spoilage microorganisms in food. However the observation that some microorganisms were able to tolerate such high concentrations of salts and solutes and thus cause food spoilage developed an interest in the halophiles. The other reason was the occurrence of natural habitats that allowed the growth of high salt tolerant strains.

These habitats include salt lakes, dead-sea etc that contain very high dissolved solutes including cations.

The halophiles are distributed among the bacteria, fungi, algae, protozoa etc. the distinctive halophiles belong to the genera, *Halobacterium & Halococcus*. The organisms from these genera tolerate very high salt (up to 32%).

The internal machinery of extreme halophiles is well adjusted to high internal salt concentration, in many cases a high content of potassium ions. This is suggested by the high requirement of salt for activity and stability of the enzymes isolated from these organisms. The pattern of response towards salts can be distinguished into 3 types.

Table 11.2: Requirement of salt for halophile enzymes

Above 1M	*Aspartate transcabamoylase, amino acyl tRNA synthases etc*
0.5-1.5 M	*Isocitrate dehydrogenase*
Absence	*RNA polymerase, fatty acid synthetase etc.*

Interesting studies conducted on the enzymes from halophiles have generated a vast data on the responses of the salt concentrations. The best- studied ones include the Aspartate transcarbamoylase and Threonine deaminase from *H. cutirubrum*.

Life at Extreme pH Values

The pH is one of the most fundamental factors that affect life processes and growth of the organisms. This is dependent on two factors,

a. The hydrogen ion concentration affects the ionic state of many metabolites and in turn their utilization by the organism,

b. The pH affects the three- dimensional structure and in turn the activity of the enzymes and life processes as a whole.

Most natural environs have pH values near neutrality and therefore the organisms in these habitats are sensitive to drastic values of pH. The range of pH values tolerated by microorganisms is 4-9. Microorganisms that thrive on pH values around or below 3 are rare and are referred as acidophiles. Those that survive at pH 10 or more are called alkalophiles.

(1) Low pH Habitats

These types of habitats include, pine soils, some lakes and acidic bogs.

They support the growth of many eukaryotic algae, bacteria, plant and animal forms. *Bactoderma, Caulobacter, Mycrocyclus, Planktomyces and Thivobium* are found in pH 3-5.

The acetic acid bacteria also grow in the pH range of 3-5. *A.acidophilus has a minimum pH 2.8 and maximum pH of 4.3 for its growth.*

Similar environs are also found in coal- mines reuse piles, drainage waters and mining effluents. They contain high amounts of dissolved sulfates, iron and hydrogen ions, all of which lead to the formation of sulfuric acid through oxidation of sulfides.

The most studied examples of obligate extreme acidophiles, *Thiobacillus thiooxidans, & Thiobacillus ferrooxidans.* Low pH tolerant species also include the *genera, Bacillus, Micrococcus, Sarcina, Crenothrix, & Microsporium.* Various autotrophs and spore-former heterotrophs including B.acidocaldarius, algae, fungi and yeast have been isolated from such environs.

(2) High pH Habitats

These types of habitats are associated with soils, formed from complete oxidation of organic materials in areas of high aeration and high temperature, those containing high concentrations of alkali minerals, animal excreta and decaying proteins.

The flora includes the ammonifiers, nitrate reducers, sulfate reducers etc. Reports indicate that the microorganisms of the genera *Nitrosomonas*, and *Nitrobacter* can survive at pH values around 13. *Agrobacterium radiobacter, Bacillus pasteuri, Bacillus cereus, Flavobacterium spp.* all have a pH optimum around 9-11.

The enzymes from the organisms that survive drastic conditions can be employed in such industrial catalytic processes that employ conditions where enzymes from normal mesophilic micro-flora are denatured. A classical example is the use of Taq polymerase in the PCR. The cycles of PCR require the reaction mixture to tolerate a temperature of 98 degrees centigrade to allow DNA melting followed by primer annealing around 55 degrees centigrade and polymerization occurring around 72 (65-75) degrees centigrade.

Following the enzyme mediated adaptations in life at extreme environments, it is easy to deduce that with the help of biotechnology and enzyme technology, research can be undertaken for the commercial exploitations of enzymes in processes that may encounter drastic conditions as these would afford specificity and maximization of the productivity.

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Appendix 1
List of Enzymes
ENZYME NOMENCLATURE AND EC NUMBERS

EC 1 Oxidoreductases
EC 1.1 Acting on the CH-OH group of donors
EC 1.1.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.1.1.1 alcohol dehydrogenase
EC 1.1.1.2 alcohol dehydrogenase ($NADP^+$)
EC 1.1.1.3 homoserine dehydrogenase
EC 1.1.1.4 (*R,R*)-butanediol dehydrogenase
EC 1.1.1.5 acetoin dehydrogenase
EC 1.1.1.6 glycerol dehydrogenase
EC 1.1.1.7 propanediol-phosphate dehydrogenase
EC 1.1.1.8 glycerol-3-phosphate dehydrogenase (NAD^+)
EC 1.1.1.9 D-xylulose reductase
EC 1.1.1.10 L-xylulose reductase
EC 1.1.1.11 D-arabinitol 4-dehydrogenase
EC 1.1.1.12 L-arabinitol 4-dehydrogenase
EC 1.1.1.13 L-arabinitol 2-dehydrogenase
EC 1.1.1.14 L-iditol 2-dehydrogenase
EC 1.1.1.15 D-iditol 2-dehydrogenase
EC 1.1.1.16 galactitol 2-dehydrogenase
EC 1.1.1.17 mannitol-1-phosphate 5-dehydrogenase
EC 1.1.1.18 inositol 2-dehydrogenase
EC 1.1.1.19 L-glucuronate reductase
EC 1.1.1.20 glucuronolactone reductase
EC 1.1.1.21 aldehyde reductase
EC 1.1.1.22 UDP-glucose 6-dehydrogenase
EC 1.1.1.23 histidinol dehydrogenase
EC 1.1.1.24 quinate dehydrogenase
EC 1.1.1.25 shikimate dehydrogenase
EC 1.1.1.26 glyoxylate reductase
EC 1.1.1.27 L-lactate dehydrogenase
EC 1.1.1.28 D-lactate dehydrogenase
EC 1.1.1.29 glycerate dehydrogenase
EC 1.1.1.30 3-hydroxybutyrate dehydrogenase
EC 1.1.1.31 3-hydroxyisobutyrate dehydrogenase
EC 1.1.1.32 mevaldate reductase
EC 1.1.1.33 mevaldate reductase (NADPH)
EC 1.1.1.34 hydroxymethylglutaryl-CoA reductase (NADPH)
EC 1.1.1.35 3-hydroxyacyl-CoA dehydrogenase
EC 1.1.1.36 acetoacetyl-CoA reductase
EC 1.1.1.37 malate dehydrogenase
EC 1.1.1.38 malate dehydrogenase (oxaloacetate-decarboxylating)
EC 1.1.1.39 malate dehydrogenase (decarboxylating)
EC 1.1.1.40 malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$)
EC 1.1.1.41 isocitrate dehydrogenase (NAD^+)
EC 1.1.1.42 isocitrate dehydrogenase ($NADP^+$)
EC 1.1.1.43 phosphogluconate 2-dehydrogenase
EC 1.1.1.44 phosphogluconate dehydrogenase (decarboxylating)
EC 1.1.1.45 L-gulonate 3-dehydrogenase
EC 1.1.1.46 L-arabinose 1-dehydrogenase
EC 1.1.1.47 glucose 1-dehydrogenase
EC 1.1.1.48 galactose 1-dehydrogenase
EC 1.1.1.49 glucose-6-phosphate 1-dehydrogenase
EC 1.1.1.50 3α-hydroxysteroid dehydrogenase (B-specific)
EC 1.1.1.51 3(or 17)β-hydroxysteroid dehydrogenase
EC 1.1.1.52 3α-hydroxycholanate dehydrogenase
EC 1.1.1.53 3α(or 20β)-hydroxysteroid dehydrogenase
EC 1.1.1.54 allyl-alcohol dehydrogenase
EC 1.1.1.55 lactaldehyde reductase (NADPH)
EC 1.1.1.56 ribitol 2-dehydrogenase
EC 1.1.1.57 fructuronate reductase
EC 1.1.1.58 tagaturonate reductase
EC 1.1.1.59 3-hydroxypropionate dehydrogenase
EC 1.1.1.60 2-hydroxy-3-oxopropionate reductase
EC 1.1.1.61 4-hydroxybutyrate dehydrogenase
EC 1.1.1.62 estradiol 17β-dehydrogenase
EC 1.1.1.63 testosterone 17β-dehydrogenase
EC 1.1.1.64 testosterone 17β-dehydrogenase ($NADP^+$)
EC 1.1.1.65 pyridoxine 4-dehydrogenase
EC 1.1.1.66 ω-hydroxydecanoate dehydrogenase
EC 1.1.1.67 mannitol 2-dehydrogenase
EC 1.1.1.68 now EC 1.7.99.5
EC 1.1.1.69 gluconate 5-dehydrogenase
EC 1.1.1.70 deleted, included in EC 1.2.1.3
EC 1.1.1.71 alcohol dehydrogenase [$NAD(P)^+$]
EC 1.1.1.72 glycerol dehydrogenase ($NADP^+$)
EC 1.1.1.73 octanol dehydrogenase
EC 1.1.1.74 deleted
EC 1.1.1.75 (*R*)-aminopropanal dehydrogenase

EC 1.1.1.76 (*S,S*)-butanediol dehydrogenase
EC 1.1.1.77 lactaldehyde reductase
EC 1.1.1.78 D-lactaldehyde dehydrogenase
EC 1.1.1.79 glyoxylate reductase ($NADP^+$)
EC 1.1.1.80 isopropanol dehydrogenase ($NADP^+$)
EC 1.1.1.81 hydroxypyruvate reductase
EC 1.1.1.82 malate dehydrogenase ($NADP^+$)
EC 1.1.1.83 D-malate dehydrogenase (decarboxylating)
EC 1.1.1.84 dimethylmalate dehydrogenase
EC 1.1.1.85 3-isopropylmalate dehydrogenase
EC 1.1.1.86 ketol-acid reductoisomerase
EC 1.1.1.87 homoisocitrate dehydrogenase
EC 1.1.1.88 hydroxymethylglutaryl-CoA reductase
EC 1.1.1.89 deleted, included in EC 1.1.1.86
EC 1.1.1.90 aryl-alcohol dehydrogenase
EC 1.1.1.91 aryl-alcohol dehydrogenase ($NADP^+$)
EC 1.1.1.92 oxaloglycolate reductase (decarboxylating)
EC 1.1.1.93 tartrate dehydrogenase
EC 1.1.1.94 glycerol-3-phosphate dehydrogenase [$NAD(P)^+$]
EC 1.1.1.95 phosphoglycerate dehydrogenase
EC 1.1.1.96 diiodophenylpyruvate reductase
EC 1.1.1.97 3-hydroxybenzyl-alcohol dehydrogenase
EC 1.1.1.98 (*R*)-2-hydroxy-fatty-acid dehydrogenase
EC 1.1.1.99 (*S*)-2-hydroxy-fatty-acid dehydrogenase
EC 1.1.1.100 3-oxoacyl-[acyl-carrier-protein] reductase
EC 1.1.1.101 acylglycerone-phosphate reductase
EC 1.1.1.102 3-dehydrosphinganine reductase
EC 1.1.1.103 L-threonine 3-dehydrogenase
EC 1.1.1.104 4-oxoproline reductase
EC 1.1.1.105 retinol dehydrogenase
EC 1.1.1.106 pantoate 4-dehydrogenase
EC 1.1.1.107 pyridoxal 4-dehydrogenase
EC 1.1.1.108 carnitine 3-dehydrogenase
EC 1.1.1.109 now EC 1.3.1.28
EC 1.1.1.110 indolelactate dehydrogenase
EC 1.1.1.111 3-(imidazol-5-yl)lactate dehydrogenase
EC 1.1.1.112 indanol dehydrogenase
EC 1.1.1.113 L-xylose 1-dehydrogenase
EC 1.1.1.114 apiose 1-reductase
EC 1.1.1.115 ribose 1-dehydrogenase ($NADP^+$)
EC 1.1.1.116 D-arabinose 1-dehydrogenase
EC 1.1.1.117 D-arabinose 1-dehydrogenase [$NAD(P)^+$]
EC 1.1.1.118 glucose 1-dehydrogenase (NAD^+)
EC 1.1.1.119 glucose 1-dehydrogenase ($NADP^+$)
EC 1.1.1.120 galactose 1-dehydrogenase ($NADP^+$)
EC 1.1.1.121 aldose 1-dehydrogenase
EC 1.1.1.122 D-*threo*-aldose 1-dehydrogenase
EC 1.1.1.123 sorbose 5-dehydrogenase ($NADP^+$)
EC 1.1.1.124 fructose 5-dehydrogenase ($NADP^+$)
EC 1.1.1.125 2-deoxy-D-gluconate 3-dehydrogenase
EC 1.1.1.126 2-dehydro-3-deoxy-D-gluconate 6-dehydrogenase
EC 1.1.1.127 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase
EC 1.1.1.128 L-idonate 2-dehydrogenase
EC 1.1.1.129 L-threonate 3-dehydrogenase
EC 1.1.1.130 3-dehydro-L-gulonate 2-dehydrogenase
EC 1.1.1.131 mannuronate reductase
EC 1.1.1.132 GDP-mannose 6-dehydrogenase
EC 1.1.1.133 dTDP-4-dehydrorhamnose reductase
EC 1.1.1.134 dTDP-6-deoxy-L-talose 4-dehydrogenase
EC 1.1.1.135 GDP-6-deoxy-D-talose 4-dehydrogenase
EC 1.1.1.136 UDP-*N*-acetylglucosamine 6-dehydrogenase
EC 1.1.1.137 ribitol-5-phosphate 2-dehydrogenase
EC 1.1.1.138 mannitol 2-dehydrogenase ($NADP^+$)
EC 1.1.1.139 deleted, included in EC 1.1.1.21
EC 1.1.1.140 sorbitol-6-phosphate 2-dehydrogenase
EC 1.1.1.141 15-hydroxyprostaglandin dehydrogenase (NAD^+)
EC 1.1.1.142 D-pinitol dehydrogenase
EC 1.1.1.143 sequoyitol dehydrogenase
EC 1.1.1.144 perillyl-alcohol dehydrogenase
EC 1.1.1.145 3β-hydroxy-D^5-steroid dehydrogenase
EC 1.1.1.146 11β-hydroxysteroid dehydrogenase
EC 1.1.1.147 16α-hydroxysteroid dehydrogenase
EC 1.1.1.148 estradiol 17α-dehydrogenase
EC 1.1.1.149 20α-hydroxysteroid dehydrogenase
EC 1.1.1.150 21-hydroxysteroid dehydrogenase (NAD^+)
EC 1.1.1.151 21-hydroxysteroid dehydrogenase ($NADP^+$)
EC 1.1.1.152 3α-hydroxy-5β-androstane-17-one 3α- dehydrogenase
EC 1.1.1.153 sepiapterin reductase
EC 1.1.1.154 ureidoglycolate dehydrogenase

EC 1.1.1.155 identical to EC 1.1.1.87
EC 1.1.1.156 glycerol 2-dehydrogenase ($NADP^+$)
EC 1.1.1.157 3-hydroxybutyryl-CoA dehydrogenase
EC 1.1.1.158 UDP-*N*-acetylmuramate dehydrogenase
EC 1.1.1.159 7α-hydroxysteroid dehydrogenase
EC 1.1.1.160 dihydrobunolol dehydrogenase
EC 1.1.1.161 cholestanetetraol 26-dehydrogenase
EC 1.1.1.162 erythrulose reductase
EC 1.1.1.163 cyclopentanol dehydrogenase
EC 1.1.1.164 hexadecanol dehydrogenase
EC 1.1.1.165 2-alkyn-1-ol dehydrogenase
EC 1.1.1.166 hydroxycyclohexanecarboxylate dehydrogenase
EC 1.1.1.167 hydroxymalonate dehydrogenase
EC 1.1.1.168 2-dehydropantolactone reductase (A-specific)
EC 1.1.1.169 2-dehydropantoate 2-reductase
EC 1.1.1.170 3β-hydroxy - 4α - methylcholestenecarboxylate 3 - dehydrogenase (decarboxylating)
EC 1.1.1.171 now EC 1.5.1.20
EC 1.1.1.172 2-oxoadipate reductase
EC 1.1.1.173 L-rhamnose 1-dehydrogenase
EC 1.1.1.174 cyclohexane-1,2-diol dehydrogenase
EC 1.1.1.175 D-xylose 1-dehydrogenase
EC 1.1.1.176 12α-hydroxysteroid dehydrogenase
EC 1.1.1.177 glycerol-3-phosphate 1-dehydrogenase ($NADP^+$)
EC 1.1.1.178 3-hydroxy-2-methylbutyryl-CoA dehydrogenase
EC 1.1.1.179 D-xylose 1-dehydrogenase ($NADP^+$)
EC 1.1.1.180 deleted, included in EC 1.1.1.131
EC 1.1.1.181 cholest-5-ene-3β,7α-diol 3β-dehydrogenase
EC 1.1.1.182 deleted, included in EC 1.1.1.198, EC 1.1.1.227 and EC 1.1.1.228
EC 1.1.1.183 geraniol dehydrogenase
EC 1.1.1.184 carbonyl reductase (NADPH)
EC 1.1.1.185 L-glycol dehydrogenase
EC 1.1.1.186 dTDP-galactose 6-dehydrogenase
EC 1.1.1.187 GDP-4-dehydro-D-rhamnose reductase
EC 1.1.1.188 prostaglandin-F synthase
EC 1.1.1.189 prostaglandin-E_2 9-reductase
EC 1.1.1.190 indole-3-acetaldehyde reductase (NADH)
EC 1.1.1.191 indole-3-acetaldehyde reductase (NADPH)
EC 1.1.1.192 long-chain-alcohol dehydrogenase
EC 1.1.1.193 5-amino-6-(5-phosphoribosylamino) uracil reductase
EC 1.1.1.194 coniferyl-alcohol dehydrogenase
EC 1.1.1.195 cinnamyl-alcohol dehydrogenase
EC 1.1.1.196 15-hydroxyprostaglandin-D dehydrogenase ($NADP^+$)
EC 1.1.1.197 15-hydroxyprostaglandin dehydrogenase ($NADP^+$)
EC 1.1.1.198 (+)-borneol dehydrogenase
EC 1.1.1.199 (*S*)-usnate reductase
EC 1.1.1.200 aldose-6-phosphate reductase (NADPH)
EC 1.1.1.201 7β-hydroxysteroid dehydrogenase ($NADP^+$)
EC 1.1.1.202 1,3-propanediol dehydrogenase
EC 1.1.1.203 uronate dehydrogenase
EC 1.1.1.204 now EC 1.17.1.4
EC 1.1.1.205 IMP dehydrogenase
EC 1.1.1.206 tropine dehydrogenase
EC 1.1.1.207 (-)-menthol dehydrogenase
EC 1.1.1.208 (+)-neomenthol dehydrogenase
EC 1.1.1.209 3(or 17)α-hydroxysteroid dehydrogenase
EC 1.1.1.210 3β(or 20α)-hydroxysteroid dehydrogenase
EC 1.1.1.211 long-chain-3-hydroxyacyl-CoA dehydrogenase
EC 1.1.1.212 3-oxoacyl-[acyl-carrier-protein] reductase (NADH)
EC 1.1.1.213 3α-hydroxysteroid dehydrogenase (A-specific)
EC 1.1.1.214 2-dehydropantolactone reductase (B-specific)
EC 1.1.1.215 gluconate 2-dehydrogenase
EC 1.1.1.216 farnesol dehydrogenase
EC 1.1.1.217 benzyl-2-methyl-hydroxybutyrate dehydrogenase
EC 1.1.1.218 morphine 6-dehydrogenase
EC 1.1.1.219 dihydrokaempferol 4-reductase
EC 1.1.1.220 6-pyruvoyltetrahydropterin 2'-reductase
EC 1.1.1.221 vomifoliol 4'-dehydrogenase
EC 1.1.1.222 (*R*)-4-hydroxyphenyllactate dehydrogenase
EC 1.1.1.223 isopiperitenol dehydrogenase
EC 1.1.1.224 mannose-6-phosphate 6-reductase
EC 1.1.1.225 chlordecone reductase

EC 1.1.1.226 4-hydroxycyclohexanecarboxylate dehydrogenase
EC 1.1.1.227 (-)-borneol dehydrogenase
EC 1.1.1.228 (+)-sabinol dehydrogenase
EC 1.1.1.229 diethyl 2-methyl-3-oxosuccinate reductase
EC 1.1.1.230 3α-hydroxyglycyrrhetinate dehydrogenase
EC 1.1.1.231 15-hydroxyprostaglandin-I dehydrogenase ($NADP^+$)
EC 1.1.1.232 15-hydroxyicosatetraenoate dehydrogenase
EC 1.1.1.233 *N*-acylmannosamine 1-dehydrogenase
EC 1.1.1.234 flavanone 4-reductase
EC 1.1.1.235 8-oxocoformycin reductase
EC 1.1.1.236 tropinone reductase
EC 1.1.1.237 hydroxyphenylpyruvate reductase
EC 1.1.1.238 12β-hydroxysteroid dehydrogenase
EC 1.1.1.239 3α(17β)-hydroxysteroid dehydrogenase (NAD^+)
EC 1.1.1.240 *N*-acetylhexosamine 1-dehydrogenase
EC 1.1.1.241 6-*endo*-hydroxycineole dehydrogenase
EC 1.1.1.242 zeatin reductase now EC 1.3.1.69
EC 1.1.1.243 carveol dehydrogenase
EC 1.1.1.244 methanol dehydrogenase
EC 1.1.1.245 cyclohexanol dehydrogenase
EC 1.1.1.246 pterocarpin synthase
EC 1.1.1.247 codeinone reductase (NADPH)
EC 1.1.1.248 salutaridine reductase (NADPH)
EC 1.1.1.249 reinstated as EC 2.5.1.46
EC 1.1.1.250 D-arabinitol 2-dehydrogenase
EC 1.1.1.251 galactitol-1-phosphate 5-dehydrogenase
EC 1.1.1.252 tetrahydroxynaphthalene reductase
EC 1.1.1.253 now EC 1.5.1.33
EC 1.1.1.254 (*S*)-carnitine 3-dehydrogenase
EC 1.1.1.255 mannitol dehydrogenase
EC 1.1.1.256 fluoren-9-ol dehydrogenase
EC 1.1.1.257 4-(hydroxymethyl)benzenesulfonate dehydrogenase
EC 1.1.1.258 6-hydroxyhexanoate dehydrogenase
EC 1.1.1.259 3-hydroxypimeloyl-CoA dehydrogenase
EC 1.1.1.260 sulcatone reductase
EC 1.1.1.261 glycerol-1-phosphate dehydrogenase [$NAD(P)^+$]
EC 1.1.1.262 4-hydroxythreonine-4-phosphate dehydrogenase
EC 1.1.1.263 1,5-anhydro-D-fructose reductase
EC 1.1.1.264 L-idonate 5-dehydrogenase
EC 1.1.1.265 3-methylbutanal reductase
EC 1.1.1.266 dTDP-4-dehydro-6-deoxyglucose reductase
EC 1.1.1.267 1-deoxy-D-xylulose-5-phosphate reductoisomerase
EC 1.1.1.268 2-(*R*)-hydroxypropyl-CoM dehydrogenase
EC 1.1.1.269 2-(*S*)-hydroxypropyl-CoM dehydrogenase
EC 1.1.1.270 3-keto-steroid reductase
EC 1.1.1.271 GDP-L-fucose synthase
EC 1.1.1.272 (*R*)-2-hydroxyacid dehydrogenase
EC 1.1.1.273 vellosimine dehydrogenase
EC 1.1.1.274 2,5-didehydrogluconate reductase
EC 1.1.1.275 (+)-*trans*-carveol dehydrogenase
EC 1.1.1.276 serine 3-dehydrogenase
EC 1.1.1.277 3β-hydroxy-5β-steroid dehydrogenase
EC 1.1.1.278 3β-hydroxy-5α-steroid dehydrogenase
EC 1.1.1.279 (*R*)-3-hydroxyacid ester dehydrogenase
EC 1.1.1.280 (*S*)-3-hydroxyacid ester dehydrogenase
EC 1.1.1.281 GDP-4-dehydro-6-deoxy-D-mannose reductase
EC 1.1.1.282 quinate/shikimate dehydrogenase
EC 1.1.2 With a cytochrome as acceptor
EC 1.1.2.1 now EC 1.1.99.5
EC 1.1.2.2 mannitol dehydrogenase (cytochrome)
EC 1.1.2.3 L-lactate dehydrogenase (cytochrome)
EC 1.1.2.4 D-lactate dehydrogenase (cytochrome)
EC 1.1.2.5 D-lactate dehydrogenase (cytochrome *c*-553)
EC 1.1.3 With oxygen as acceptor
EC 1.1.3.1 deleted, included in EC 1.1.3.15
EC 1.1.3.2 now EC 1.13.12.4
EC 1.1.3.3 malate oxidase
EC 1.1.3.4 glucose oxidase
EC 1.1.3.5 hexose oxidase
EC 1.1.3.6 cholesterol oxidase
EC 1.1.3.7 aryl-alcohol oxidase
EC 1.1.3.8 L-gulonolactone oxidase
EC 1.1.3.9 galactose oxidase
EC 1.1.3.10 pyranose oxidase
EC 1.1.3.11 L-sorbose oxidase
EC 1.1.3.12 pyridoxine 4-oxidase
EC 1.1.3.13 alcohol oxidase

EC 1.1.3.14 catechol oxidase (dimerizing)
EC 1.1.3.15 (*S*)-2-hydroxy-acid oxidase
EC 1.1.3.16 ecdysone oxidase
EC 1.1.3.17 choline oxidase
EC 1.1.3.18 secondary-alcohol oxidase
EC 1.1.3.19 4-hydroxymandelate oxidase
EC 1.1.3.20 long-chain-alcohol oxidase
EC 1.1.3.21 glycerol-3-phosphate oxidase
EC 1.1.3.22 now EC 1.17.3.2
EC 1.1.3.23 thiamin oxidase
EC 1.1.3.24 L-galactonolactone oxidase
EC 1.1.3.25 cellobiose oxidase
EC 1.1.3.26 now EC 1.21.3.2
EC 1.1.3.27 hydroxyphytanate oxidase
EC 1.1.3.28 nucleoside oxidase
EC 1.1.3.29 *N*-acylhexosamine oxidase
EC 1.1.3.30 polyvinyl-alcohol oxidase
EC 1.1.3.31 deleted
EC 1.1.3.32 now EC 1.14.21.1
EC 1.1.3.33 now EC 1.14.21.2
EC 1.1.3.34 now EC 1.14.21.3
EC 1.1.3.35 now EC 1.14.21.4
EC 1.1.3.36 now EC 1.14.21.5
EC 1.1.3.37 D-arabinono-1,4-lactone oxidase
EC 1.1.3.38 vanillyl-alcohol oxidase
EC 1.1.3.39 nucleoside oxidase (H_2O_2-forming)
EC 1.1.3.40 D-mannitol oxidase
EC 1.1.3.41 xylitol oxidase
EC 1.1.4 With a disulfide as acceptor
EC 1.1.4.1 vitamin-K-epoxide reductase (warfarin-sensitive)
EC 1.1.4.2 vitamin-K-epoxide reductase (warfarin-insensitive)
EC 1.1.5 With a quinone or similar compound as acceptor
EC 1.1.5.1 Deleted, see EC 1.1.99.18 cellobiose dehydrogenase (acceptor)
EC 1.1.5.2 quinoprotein glucose dehydrogenase
EC 1.1.99 With other acceptors
EC 1.1.99.1 choline dehydrogenase
EC 1.1.99.2 2-hydroxyglutarate dehydrogenase
EC 1.1.99.3 gluconate 2-dehydrogenase (acceptor)
EC 1.1.99.4 dehydrogluconate dehydrogenase
EC 1.1.99.5 glycerol-3-phosphate dehydrogenase
EC 1.1.99.6 D-2-hydroxy-acid dehydrogenase
EC 1.1.99.7 lactate—malate transhydrogenase
EC 1.1.99.8 alcohol dehydrogenase (acceptor)
EC 1.1.99.9 pyridoxine 5-dehydrogenase
EC 1.1.99.10 glucose dehydrogenase (acceptor)
EC 1.1.99.11 fructose 5-dehydrogenase
EC 1.1.99.12 sorbose dehydrogenase
EC 1.1.99.13 glucoside 3-dehydrogenase
EC 1.1.99.14 glycolate dehydrogenase
EC 1.1.99.15 now EC 1.7.99.5
EC 1.1.99.16 malate dehydrogenase (acceptor)
EC 1.1.99.17 now EC 1.1.5.2
EC 1.1.99.18 cellobiose dehydrogenase (acceptor)
EC 1.1.99.19 uracil dehydrogenase
EC 1.1.99.20 alkan-1-ol dehydrogenase (acceptor)
EC 1.1.99.21 D-sorbitol dehydrogenase (acceptor)
EC 1.1.99.22 glycerol dehydrogenase (acceptor)
EC 1.1.99.23 polyvinyl-alcohol dehydrogenase (acceptor)
EC 1.1.99.24 hydroxyacid-oxoacid transhydrogenase
EC 1.1.99.25 quinate dehydrogenase (pyrroloquinoline-quinone)
EC 1.1.99.26 3-hydroxycyclohexanone dehydrogenase
EC 1.1.99.27 (*R*)-pantolactone dehydrogenase (flavin)
EC 1.1.99.28 glucose-fructose oxidoreductase
EC 1.1.99.29 pyranose dehydrogenase (acceptor)
EC 1.1.99.30 2-oxoacid reductase
EC 1.2 Acting on the aldehyde or oxo group of donors
EC 1.2.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.2.1.1 formaldehyde dehydrogenase (glutathione)
EC 1.2.1.2 formate dehydrogenase
EC 1.2.1.3 aldehyde dehydrogenase (NAD^+)
EC 1.2.1.4 aldehyde dehydrogenase ($NADP^+$
EC 1.2.1.5 aldehyde dehydrogenase [$NAD(P)^+$]
EC 1.2.1.6 deleted
EC 1.2.1.7 benzaldehyde dehydrogenase ($NADP^+$)
EC 1.2.1.8 betaine-aldehyde dehydrogenase
EC 1.2.1.9 glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$)
EC 1.2.1.10 acetaldehyde dehydrogenase (acetylating)
EC 1.2.1.11 aspartate-semialdehyde dehydrogenase
EC 1.2.1.12 glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
EC 1.2.1.13 glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$) (phosphorylating)

EC 1.2.1.14 now EC 1.1.1.205
EC 1.2.1.15 malonate-semialdehyde dehydrogenase
EC 1.2.1.16 succinate-semialdehyde dehydrogenase [NAD(P)$^+$]
EC 1.2.1.17 glyoxylate dehydrogenase (acylating)
EC 1.2.1.18 malonate-semialdehyde dehydrogenase (acetylating)
EC 1.2.1.19 aminobutyraldehyde dehydrogenase
EC 1.2.1.20 glutarate-semialdehyde dehydrogenase
EC 1.2.1.21 glycolaldehyde dehydrogenase
EC 1.2.1.22 lactaldehyde dehydrogenase
EC 1.2.1.23 2-oxoaldehyde dehydrogenase (NAD$^+$)
EC 1.2.1.24 succinate-semialdehyde dehydrogenase
EC 1.2.1.25 2-oxoisovalerate dehydrogenase (acylating)
EC 1.2.1.26 2,5-dioxovalerate dehydrogenase
EC 1.2.1.27 methylmalonate-semialdehyde dehydrogenase (acylating)
EC 1.2.1.28 benzaldehyde dehydrogenase (NAD$^+$)
EC 1.2.1.29 aryl-aldehyde dehydrogenase
EC 1.2.1.30 aryl-aldehyde dehydrogenase (NADP$^+$)
EC 1.2.1.31 L-aminoadipate-semialdehyde dehydrogenase
EC 1.2.1.32 aminomuconate-semialdehyde dehydrogenase
EC 1.2.1.33 (*R*)-dehydropantoate dehydrogenase
EC 1.2.1.34 deleted, included in EC 1.1.1.131
EC 1.2.1.35 now EC 1.1.1.203
EC 1.2.1.36 retinal dehydrogenase
EC 1.2.1.37 now EC 1.1.1.204
EC 1.2.1.38 *N*-acetyl-g-glutamyl-phosphate reductase
EC 1.2.1.39 phenylacetaldehyde dehydrogenase
EC 1.2.1.40 3α,7α,12α-trihydroxycholestan-26-al 26-oxidoreductase
EC 1.2.1.41 glutamate-5-semialdehyde dehydrogenase
EC 1.2.1.42 hexadecanal dehydrogenase (acylating)
EC 1.2.1.43 formate dehydrogenase (NADP$^+$)
EC 1.2.1.44 cinnamoyl-CoA reductase
EC 1.2.1.45 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase
EC 1.2.1.46 formaldehyde dehydrogenase
EC 1.2.1.47 4-trimethylammoniobutyraldehyde dehydrogenase
EC 1.2.1.48 long-chain-aldehyde dehydrogenase
EC 1.2.1.49 2-oxoaldehyde dehydrogenase (NADP$^+$)
EC 1.2.1.50 long-chain-fatty-acyl-CoA reductase
EC 1.2.1.51 pyruvate dehydrogenase (NADP$^+$)
EC 1.2.1.52 oxoglutarate dehydrogenase (NADP$^+$)
EC 1.2.1.53 4-hydroxyphenylacetaldehyde dehydrogenase
EC 1.2.1.54 γ-guanidinobutyraldehyde dehydrogenase
EC 1 2.1.55 now EC 1.1.1.279
EC 1.2.1.56 now EC 1.1.1.280
EC 1.2.1.57 butanal dehydrogenase
EC 1.2.1.58 phenylglyoxylate dehydrogenase (acylating)
EC 1.2.1.59 glyceraldehyde-3-phosphate dehydrogenase (NAD(P)$^+$) (phosphorylating)
EC 1.2.1.60 5-carboxymethyl-2-hydroxymuconic-semialdehyde dehydrogenase
EC 1.2.1.61 4-hydroxymuconic semialdehyde dehydrogenase
EC 1.2.1.62 4-formylbenzenesulfonate dehydrogenase
EC 1.2.1.63 6-oxohexanoate dehydrogenase
EC 1.2.1.64 4-hydroxybenzaldehyde dehydrogenase
EC 1.2.1.65 salicylaldehyde dehydrogenase
EC 1.2.1.66 mycothiol-dependent formaldehyde dehydrogenase
EC 1.2.1.67 vanillin dehydrogenase
EC 1.2.1.68 coniferyl-aldehyde dehydrogenase
EC 1.2.1.69 fluoroacetaldehyde dehydrogenase
EC 1.2.2 With a cytochrome as acceptor
EC 1.2.2.1 formate dehydrogenase (cytochrome)
EC 1.2.2.2 pyruvate dehydrogenase (cytochrome)
EC 1.2.2.3 formate dehydrogenase (cytochrome-*c*-553)
EC 1.2.2.4 carbon-monoxide dehydrogenase (cytochrome-b-561)
EC 1.2.3 With oxygen as acceptor
EC 1.2.3.1 aldehyde oxidase
EC 1.2.3.2 now EC 1.1.3.22
EC 1.2.3.3 pyruvate oxidase
EC 1.2.3.4 oxalate oxidase
EC 1.2.3.5 glyoxylate oxidase
EC 1.2.3.6 pyruvate oxidase (CoA-acetylating)
EC 1.2.3.7 indole-3-acetaldehyde oxidase
EC 1.2.3.8 pyridoxal oxidase
EC 1.2.3.9 aryl-aldehyde oxidase

EC 1.2.3.10 deleted
EC 1.2.3.11 retinal oxidase
EC 1.2.3.12 now EC 1.14.13.82
EC 1.2.3.13 4-hydroxyphenylpyruvate oxidase
EC 1.2.4 With a disulfide as acceptor
EC 1.2.4.1 pyruvate dehydrogenase (acetyl-transferring)
EC 1.2.4.2 oxoglutarate dehydrogenase (succinyl-transferring)
EC 1.2.4.3 deleted, included in EC 1.2.4.4
EC 1.2.4.4 3-methyl-2-oxobutanoate dehydrogenase (2-methylpropanoyl-transferring)
EC 1.2.7 With an iron-sulfur protein as acceptor
EC 1.2.7.1 pyruvate synthase
EC 1.2.7.2 2-oxobutyrate synthase
EC 1.2.7.3 2-oxoglutarate synthase
EC 1.2.7.4 carbon-monoxide dehydrogenase (ferredoxin)
EC 1.2.7.5 aldehyde ferredoxin oxidoreductase
EC 1.2.7.6 glyceraldehyde-3-phosphate dehydrogenase (ferredoxin)
EC 1.2.7.7 3-methyl-2-oxobutanoate dehydrogenase (ferredoxin)
EC 1.2.7.8 indolepyruvate ferredoxin oxidoreductase
EC 1.2.7.9 2-oxoglutarate ferredoxin oxidoreductase
EC 1.2.99 With other acceptors
EC 1.2.99.1 now EC 1.1.99.19
EC 1.2.99.2 carbon-monoxide dehydrogenase (acceptor)
EC 1.2.99.3 aldehyde dehydrogenase (pyrroloquinoline-quinone)
EC 1.2.99.4 formaldehyde dismutase
EC 1.2.99.5 formylmethanofuran dehydrogenase
EC 1.2.99.6 carboxylate reductase
EC 1.2.99.7 aldehyde dehydrogenase (FAD-independent)
EC 1.3 Acting on the CH-CH group of donors
EC 1.3.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.3.1.1 dihydrouracil dehydrogenase (NAD^+)
EC 1.3.1.2 dihydropyrimidine dehydrogenase ($NADP^+$)
EC 1.3.1.3 cortisone b-reductase
EC 1.3.1.4 cortisone a-reductase
EC 1.3.1.5 cucurbitacin D^{23}-reductase
EC 1.3.1.6 fumarate reductase (NADH)
EC 1.3.1.7 *meso*-tartrate dehydrogenase
EC 1.3.1.8 acyl-CoA dehydrogenase ($NADP^+$)
EC 1.3.1.9 enoyl-[acyl-carrier-protein] reductase (NADH)
EC 1.3.1.10 enoyl-[acyl-carrier-protein] reductase (NADPH, B-specific)
EC 1.3.1.11 2-coumarate reductase
EC 1.3.1.12 prephenate dehydrogenase
EC 1.3.1.13 prephenate dehydrogenase ($NADP^+$)
EC 1.3.1.14 orotate reductase (NADH)
EC 1.3.1.15 orotate reductase (NADPH)
EC 1.3.1.16 b-nitroacrylate reductase
EC 1.3.1.17 3-methyleneoxindole reductase
EC 1.3.1.18 kynurenate-7, 8-dihydrodiol dehydrogenase
EC 1.3.1.19 *cis*-1,2-dihydrobenzene-1, 2-diol dehydrogenase
EC 1.3.1.20 *trans*-1,2-dihydrobenzene-1, 2-diol dehydrogenase
EC 1.3.1.21 7-dehydrocholesterol reductase
EC 1.3.1.22 cholestenone 5a-reductase
EC 1.3.1.23 cholestenone 5b-reductase
EC 1.3.1.24 biliverdin reductase
EC 1.3.1.25 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase
EC 1.3.1.26 dihydrodipicolinate reductase
EC 1.3.1.27 2-hexadecenal reductase
EC 1.3.1.28 2,3-dihydro-2, 3-dihydroxybenzoate dehydrogenase
EC 1.3.1.29 *cis*-1,2-dihydro-1, 2-dihydroxynaphthalene dehydrogenase
EC 1.3.1.30 progesterone 5a-reductase
EC 1.3.1.31 2-enoate reductase
EC 1.3.1.32 maleylacetate reductase
EC 1.3.1.33 protochlorophyllide reductase
EC 1.3.1.34 2,4-dienoyl-CoA reductase (NADPH)
EC 1.3.1.35 phosphatidylcholine desaturase
EC 1.3.1.36 geissoschizine dehydrogenase
EC 1.3.1.37 *cis*-2-enoyl-CoA reductase (NADPH)
EC 1.3.1.38 *trans*-2-enoyl-CoA reductase (NADPH)
EC 1.3.1.39 enoyl-[acyl-carrier-protein] reductase (NADPH, A-specific)
EC 1.3.1.40 2-hydroxy-6-oxo-6-phenylhexa- 2, 4-dienoate reductase
EC 1.3.1.41 xanthommatin reductase
EC 1.3.1.42 12-oxophytodienoate reductase
EC 1.3.1.43 cyclohexadienyl dehydrogenase
EC 1.3.1.44 *trans*-2-enoyl-CoA reductase (NAD^+)
EC 1.3.1.45 2'-hydroxyisoflavone reductase

EC 1.3.1.46 biochanin-A reductase
EC 1.3.1.47 a-santonin 1,2-reductase
EC 1.3.1.48 15-oxoprostaglandin 13-oxidase
EC 1.3.1.49 *cis*-3,4-dihydrophenanthrene-3, 4-diol dehydrogenase
EC 1.3.1.50 now EC 1.1.1.252
EC 1.3.1.51 2'-hydroxydaidzein reductase
EC 1.3.1.52 2-methyl-branched-chain-enoyl-CoA reductase
EC 1.3.1.53 (3*S*,4*R*)-3,4-dihydroxycyclohexa-1, 5-diene-1,4-dicarboxylate dehydrogenase
EC 1.3.1.54 precorrin-6A reductase
EC 1.3.1.55 now EC 1.3.1.25
EC 1.3.1.56 *cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase
EC 1.3.1.57 phloroglucinol reductase
EC 1.3.1.58 2,3-dihydroxy-2,3-dihydro-*p*-cumate dehydrogenase
EC 1.3.1.59 1,6-dihydroxy-5-methylcyclohexa-2, 4-dienecarboxylate dehydrogenase
EC 1.3.1.60 dibenzothiophene dihydrodiol dehydrogenase
EC 1.3.1.61 terephthalate 1,2-*cis*-dihydrodiol dehydrogenase
EC 1.3.1.62 pimeloyl-CoA dehydrogenase
EC 1.3.1.63 2,4-dichlorobenzoyl-CoA reductase
EC 1.3.1.64 phthalate 4, 5-*cis*-dihydrodiol dehydrogenase
EC 1.3.1.65 5,6-dihydroxy-3-methyl-2-oxo-1,2,5, 6-tetrahydroquinoline dehydrogenase
EC 1.3.1.66 *cis*-dihydroethylcatechol dehydrogenase
EC 1.3.1.67 *cis*-1,2-dihydroxy-4-methylcyclohexa-3, 5-diene-1-carboxylate dehydrogenase
EC 1.3.1.68 1,2-dihydroxy-6-methylcyclohexa-3, 5-dienecarboxylate dehydrogenase
EC 1.3.1.69 zeatin reductase
EC 1.3.1.70 D^{14}-sterol reductase
EC 1.3.1.71 $D^{24(241)}$-sterol reductase
EC 1.3.1.72 D^{24}-sterol reductase
EC 1.3.1.73 1,2-dihydrovomilenine reductase
EC 1.3.1.74 2-alkenal reductase
EC 1.3.1.75 divinyl chlorophyllide *a* 8-vinyl-reductase
EC 1.3.1.76 precorrin-2 dehydrogenase
EC 1.3.2 With a cytochrome as acceptor
EC 1.3.2.1 now EC 1.3.99.2
EC 1.3.2.2 now EC 1.3.99.3
EC 1.3.2.3 galactonolactone dehydrogenase
EC 1.3.3 With oxygen as acceptor
EC 1.3.3.1 dihydroorotate oxidase
EC 1.3.3.2 lathosterol oxidase
EC 1.3.3.3 coproporphyrinogen oxidase
EC 1.3.3.4 protoporphyrinogen oxidase
EC 1.3.3.5 bilirubin oxidase
EC 1.3.3.6 acyl-CoA oxidase
EC 1.3.3.7 dihydrouracil oxidase
EC 1.3.3.8 tetrahydroberberine oxidase
EC 1.3.3.9 secologanin synthase
EC 1.3.3.10 tryptophan a,b-oxidase
EC 1.3.5 With a quinone or related compound as acceptor
EC 1.3.5.1 succinate dehydrogenase (ubiquinone)
EC 1.3.7 With an iron-sulfur protein as acceptor
EC 1.3.7.1 6-hydroxynicotinate reductase
EC 1.3.7.2 15,16-dihydrobiliverdin:ferredoxin oxidoreductase
EC 1.3.7.3 phycoerythrobilin:ferredoxin oxidoreductase
EC 1.3.7.4 phytochromobilin:ferredoxin oxidoreductase
EC 1.3.7.5 phycocyanobilin:ferredoxin oxidoreductase
EC 1.3.99 With other acceptors
EC 1.3.99.1 succinate dehydrogenase
EC 1.3.99.2 butyryl-CoA dehydrogenase
EC 1.3.99.3 acyl-CoA dehydrogenase
EC 1.3.99.4 3-oxosteroid 1-dehydrogenase
EC 1.3.99.5 3-oxo-5a-steroid 4-dehydrogenase
EC 1.3.99.6 3-oxo-5b-steroid 4-dehydrogenase
EC 1.3.99.7 glutaryl-CoA dehydrogenase
EC 1.3.99.8 2-furoyl-CoA dehydrogenase
EC 1.3.99.9 now EC 1.21.99.1
EC 1.3.99.10 isovaleryl-CoA dehydrogenase
EC 1.3.99.11 dihydroorotate dehydrogenase
EC 1.3.99.12 2-methylacyl-CoA dehydrogenase
EC 1.3.99.13 long-chain-acyl-CoA dehydrogenase
EC 1.3.99.14 cyclohexanone dehydrogenase
EC 1.3.99.15 benzoyl-CoA reductase
EC 1.3.99.16 isoquinoline 1-oxidoreductase
EC 1.3.99.17 quinoline 2-oxidoreductase
EC 1.3.99.18 quinaldate 4-oxidoreductase
EC 1.3.99.19 quinoline-4-carboxylate 2-oxidoreductase
EC 1.3.99.20 4-hydroxybenzoyl-CoA reductase

EC 1.3.99.21 (*R*)-benzylsuccinyl-CoA dehydrogenase
EC 1.4 Acting on the CH-NH_2 group of donors
EC 1.4.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.4.1.1 alanine dehydrogenase
EC 1.4.1.2 glutamate dehydrogenase
EC 1.4.1.3 glutamate dehydrogenase [NAD(P)$^+$]
EC 1.4.1.4 glutamate dehydrogenase ($NADP^+$)
EC 1.4.1.5 L-amino-acid dehydrogenase
EC 1.4.1.6 deleted, included in EC 1.4.4.1
EC 1.4.1.7 serine 2-dehydrogenase
EC 1.4.1.8 valine dehydrogenase ($NADP^+$)
EC 1.4.1.9 leucine dehydrogenase
EC 1.4.1.10 glycine dehydrogenase
EC 1.4.1.11 L-*erythro*-3,5-diaminohexanoate dehydrogenase
EC 1.4.1.12 2,4-diaminopentanoate dehydrogenase
EC 1.4 1.13 glutamate synthase (NADPH)
EC 1.4.1.14 glutamate synthase (NADH)
EC 1.4.1.15 lysine dehydrogenase
EC 1.4.1.16 diaminopimelate dehydrogenase
EC 1.4.1.17 *N*-methylalanine dehydrogenase
EC 1.4.1.18 lysine 6-dehydrogenase
EC 1.4.1.19 tryptophan dehydrogenase
EC 1.4.1.20 phenylalanine dehydrogenase
EC 1.4.2 With a cytochrome as acceptor
EC 1.4.2.1 glycine dehydrogenase (cytochrome)
EC 1.4.3 With oxygen as acceptor
EC 1.4.3.1 D-aspartate oxidase
EC 1.4.3.2 L-amino-acid oxidase
EC 1.4.3.3 D-amino-acid oxidase
EC 1.4.3.4 amine oxidase (flavin-containing)
EC 1.4.3.5 pyridoxamine-phosphate oxidase
EC 1.4.3.6 amine oxidase (copper-containing)
EC 1.4.3.7 D-glutamate oxidase
EC 1.4.3.8 ethanolamine oxidase
EC 1.4.3.9 deleted, included in EC 1.4.3.4
EC 1.4.3.10 putrescine oxidase
EC 1.4.3.11 L-glutamate oxidase
EC 1.4.3.12 cyclohexylamine oxidase
EC 1.4.3.13 protein-lysine 6-oxidase
EC 1.4.3.14 L-lysine oxidase
EC 1.4.3.15 D-glutamate(D-aspartate) oxidase
EC 1.4.3.16 L-aspartate oxidase
EC 1.4.3.17 now EC 1.3.3.10
EC 1.4.3.18 deleted, not approved
EC 1.4.3.19 glycine oxidase
EC 1.4.4 With a disulfide as acceptor
EC 1.4.4.1 now EC 1.21.4.1
EC 1.4.4.2 glycine dehydrogenase (decarboxylating)
EC 1.4.7 With an iron-sulfur protein as acceptor
EC 1.4.7.1 glutamate synthase (ferredoxin)
EC 1.4.99 With other acceptors
EC 1.4.99.1 D-amino-acid dehydrogenase
EC 1.4.99.2 taurine dehydrogenase
EC 1.4.99.3 amine dehydrogenase
EC 1.4.99.4 aralkylamine dehydrogenase
EC 1.4.99.5 glycine dehydrogenase (cyanide-forming)
EC 1.5 Acting on the CH-NH group of donors
EC 1.5 1 With NAD^+ or $NADP^+$ as acceptor
EC 1.5.1.1 pyrroline-2-carboxylate reductase
EC 1.5.1.2 pyrroline-5-carboxylate reductase
EC 1.5.1.3 dihydrofolate reductase
EC 1.5.1.4 deleted, included in EC 1.5.1 3
EC 1.5.1.5 methylenetetrahydrofolate dehydrogenase ($NADP^+$)
EC 1.5.1.6 formyltetrahydrofolate dehydrogenase
EC 1.5.1.7 saccharopine dehydrogenase (NAD^+, L-lysine-forming)
EC 1.5.1.8 saccharopine dehydrogenase ($NADP^+$, L-lysine-forming)
EC 1.5.1.9 saccharopine dehydrogenase (NAD^+, L-glutamate-forming)
EC 1.5.1.10 saccharopine dehydrogenase ($NADP^+$, L-glutamate-forming)
EC 1.5.1.11 D-octopine dehydrogenase
EC 1.5.1.12 1-pyrroline-5-carboxylate dehydrogenase
EC 1.5.1.13 now EC 1.17.1.5
EC 1.5.1.14 deleted, included in EC 1.5.1.21
EC 1.5.1.15 methylenetetrahydrofolate dehydrogenase (NAD^+)
EC 1.5.1.16 D-lysopine dehydrogenase
EC 1.5.1.17 alanopine dehydrogenase
EC 1.5.1.18 ephedrine dehydrogenase
EC 1.5.1.19 D-nopaline dehydrogenase
EC 1.5.1.20 methylenetetrahydrofolate reductase (NADPH)
EC 1.5.1.21 D^1-piperideine-2-carboxylate reductase
EC 1.5.1.22 strombine dehydrogenase
EC 1.5.1.23 tauropine dehydrogenase
EC 1.5.1.24 N^6-(carboxyethyl)ornithine synthase
EC 1.5.1.25 thiomorpholine-carboxylate dehydrogenase

EC 1.5.1.26 b-alanopine dehydrogenase
EC 1.5.1.27 1,2-dehydroreticulinium reductase (NADPH)
EC 1.5.1.28 opine dehydrogenase
EC 1.5.1.29 FMN reductase
EC 1.5.1 30 flavin reductase
EC 1.5.1 31 berberine reductase
EC 1.5.1.32 vomilenine reductase
EC 1.5.1.33 pteridine reductase
EC 1.5 1.34 6,7-dihydropteridine reductase
EC 1.5 3 With oxygen as acceptor
EC 1.5 3.1 sarcosine oxidase
EC 1.5 3.2 *N*-methyl-L-amino-acid oxidase
EC 1.5 3.3 deleted
EC 1.5 3.4 N^6-methyl-lysine oxidase
EC 1.5.3.5 (*S*)-6-hydroxynicotine oxidase
EC 1.5 3.6 (*R*)-6-hydroxynicotine oxidase
EC 1.5.3.7 L-pipecolate oxidase
EC 1.5.3.8 deleted, included in EC 1.3 3.8
EC 1.5.3.9 now EC 1.21.3.3
EC 1.5.3.10 dimethylglycine oxidase
EC 1.5.3.11 polyamine oxidase
EC 1.5.3 12 dihydrobenzophenanthridine oxidase
EC 1.5.4 With a disulfide as acceptor
EC 1.5 4.1 pyrimidodiazepine synthase
EC 1 5.5 With a quinone or similar compound as acceptor
EC 1.5.5.1 electron-transferring-flavoprotein dehydrogenase
EC 1.5.8 With a flavin as acceptor
EC 1.5.8.1 dimethylamine dehydrogenase
EC 1.5.8.2 trimethylamine dehydrogenase
EC 1.5.99 With other acceptors
EC 1.5.99 1 sarcosine dehydrogenase
EC 1 5.99.2 dimethylglycine dehydrogenase
EC 1.5.99.3 L-pipecolate dehydrogenase
EC 1.5.99.4 nicotine dehydrogenase
EC 1.5 99.5 methylglutamate dehydrogenase
EC 1.5 99.6 spermidine dehydrogenase
EC 1.5.99.7 now EC 1.5.8 2
EC 1 5.99 8 proline dehydrogenase
EC 1.5.99.9 methylenetetrahydromethanopterin dehydrogenase
EC 1 5 99.10 now EC 1.5.8.1
EC 1.5.99.11 5, 10-methylenetetrahydromethanopterin reductase
EC 1.5.99.12 cytokinin dehydrogenase
EC 1 6 Acting on NADH or NADPH
EC 1 6.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.6.1.1 $NAD(P)^+$ transhydrogenase (B-specific)
EC 1 6.1.2 $NAD(P)^+$ transhydrogenase (AB-specific)
EC 1.6.2 With a heme protein as acceptor
EC 1.6.2.1 now EC 1.6 99.3
EC 1.6 2.2 cytochrome-b_5 reductase
EC 1.6.2.3 deleted
EC 1 6.2.4 NADPH—hemoprotein reductase
EC 1.6.2 5 NADPH—cytochrome-c_2 reductase
EC 1.6.2.6 leghemoglobin reductase
EC 1.6.3 With oxygen as acceptor
EC 1.6 3.1 NAD(P)H oxidase
EC 1 6.4 With a disulfide as acceptor
EC 1.6 4.1 now EC 1.8.1.6
EC 1.6.4.2 now EC 1.8.1.7
EC 1.6 4.3 now EC 1.8 1.4
EC 1 6.4.4 now EC 1.8 1.8
EC 1.6.4 5 now EC 1.8.1 9
EC 1.6 4.6 now EC 1.8.1.10
EC 1 6.4.7 now EC 1.8.1.11
EC 1.6.4 8 now EC 1 8.1.12
EC 1 6.4.9 now EC 1.8.1.13
EC 1.6.4 10 now EC 1.8 1.14
EC 1.6.5 With a quinone or similar compound as acceptor
EC 1.6.5.1 deleted
EC 1 6 5.2 now EC 1.6.99 2
EC 1 6.5 3 NADH dehydrogenase (ubiquinone)
EC 1.6.5 4 monodehydroascorbate reductase (NADH)
EC 1 6.5.5 NADPH quinone reductase
EC 1.6 5 6 *p*-benzoquinone reductase (NADPH)
EC 1.6.5 7 2-hydroxy-1,4-benzoquinone reductase
EC 1 6.6 With a nitrogenous group as acceptor
EC 1 6.6.1 now EC 1.7 1 1
EC 1.6.6 2 now EC 1 7.1.2
EC 1.6.6.3 now EC 1.7 1.3
EC 1.6.6 4 now EC 1.7.1 4
EC 1.6.6.5 now EC 1 7 99.3
EC 1 6.6.6 now EC 1 7.1 5
EC 1.6 6.7 now EC 1.7 1.6
EC 1.6 6.8 now EC 1.7.1.7
EC 1.6 6 9 trimethylamine-*N*-oxide reductase
EC 1.6.6.10 now EC 1.7.1.9
EC 1.6.6.11 now EC 1 7.1.10

EC 1 6.6.12 now EC 1 7 1.11
EC 1 6 6.13 now EC 1 7 1.12
EC 1.6.7 With a iron-sulfur protein as acceptor
EC 1.6.7 1 now EC 1 18.1 2
EC 1 6.7.2 now EC 1.18.1.1
EC 1.6.8 With a flavin as acceptor
EC 1.6.8 1 now EC 1 5 1.29
EC 1.6.8.2 now EC 1.5.1.30
EC 1.6.99 With other acceptors
EC 1.6 99.1 NADPH dehydrogenase
EC 1.6.99.2 NAD(P)H dehydrogenase (quinone)
EC 1.6.99.3 NADH dehydrogenase
EC 1 6.99.4 now EC 1.18.1.2
EC 1 6 99.5 NADH dehydrogenase (quinone)
EC 1 6.99.6 NADPH dehydrogenase (quinone)
EC 1 6.99.7 now EC 1.5.1.34
EC 1.6.99.8 now EC 1.16.1.3
EC 1.6 99.9 now EC 1.16.1.4
EC 1.6.99.10 deleted, included in EC 1 6.99.7
EC 1.6.99.11 now EC 1.16.1.5
EC 1.6.99.12 now EC 1 16.1.6
EC 1.6.99.13 now EC 1.16 1.7
EC 1.7 Acting on other nitrogenous compounds as donors
EC 1.7.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.7.1 1 nitrate reductase (NADH)
EC 1.7.1.2 nitrate reductase [NAD(P)H]
EC 1.7 1.3 nitrate reductase (NADPH)
EC 1.7.1.4 nitrite reductase [NAD(P)H]
EC 1.7.1.5 hyponitrite reductase
EC 1 7.1.6 azobenzene reductase
EC 1.7.1.7 GMP reductase
EC 1.7 1.8 deleted entry
EC 1 7 1.9 nitroquinoline-*N*-oxide reductase
EC 1.7.1.10 hydroxylamine reductase (NADH)
EC 1.7.1.11 4-(dimethylamino)phenylazoxybenzene reductase
EC 1.7.1.12 *N*-hydroxy-2-acetamidofluorene reductase
EC 1.7.2 With a cytochrome as acceptor
EC 1.7.2.1 nitrite reductase (NO-forming)
EC 1.7.2.2 nitrite reductase (cytochrome; ammonia-forming)
EC 1.7.2.3 trimethylamine-*N*-oxide reductase (cytochrome *c*)
EC 1.7.3 With oxygen as acceptor
EC 1.7.3 1 nitroethane oxidase
EC 1.7.3.2 acetylindoxyl oxidase
EC 1.7 3 3 urate oxidase
EC 1.7.3.4 hydroxylamine oxidase
EC 1.7.3.5 3-*aci*-nitropropanoate oxidase
EC 1.7.7 With an iron-sulfur protein as acceptor
EC 1.7.7.1 ferredoxin—nitrite reductase
EC 1.7.7.2 ferredoxin—nitrate reductase
EC 1.7.99 With other acceptors
EC 1 7.99.1 hydroxylamine reductase
EC 1.7.99.2 deleted
EC 1 7.99.3 included with EC 1.7 2.1
EC 1.7.99.4 nitrate reductase
EC 1.7.99.5 5,10-methylenetetrahydrofolate reductase ($FADH_2$)
EC 1 7.99.6 nitrous-oxide reductase
EC 1.7.99 7 nitric-oxide reductase
EC 1.7.99.8 hydroxylamine oxidoreductase
EC 1.8 Acting on a sulfur group of donors
EC 1.8.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.8.1.1 deleted
EC 1.8.1 2 sulfite reductase (NADPH)
EC 1.8.1.3 hypotaurine dehydrogenase
EC 1.8.1 4 dihydrolipoyl dehydrogenase
EC 1.8.1 5 2-oxopropyl-CoM reductase (carboxylating)
EC 1 8.1.6 cystine reductase
EC 1.8 1.7 glutathione-disulfide reductase
EC 1.8.1.8 protein-disulfide reductase
EC 1.8.1 9 thioredoxin-disulfide reductase
EC 1.8.1.10 CoA-glutathione reductase
EC 1.8 1.11 asparagusate reductase
EC 1 8.1.12 trypanothione-disulfide reductase
EC 1.8.1.13 bis-g-glutamylcystine reductase
EC 1 8.1.14 CoA-disulfide reductase
EC 1 8.1.15 mycothione reductase
EC 1.8.2 With a cytochrome as acceptor
EC 1.8.2.1 sulfite dehydrogenase
EC 1.8.2.2 thiosulfate dehydrogenase
EC 1.8.3 With oxygen as acceptor
EC 1.8.3.1 sulfite oxidase
EC 1.8.3.2 thiol oxidase
EC 1 8.3.3 glutathione oxidase
EC 1.8.3 4 methanethiol oxidase
EC 1.8.3.5 prenylcysteine oxidase
EC 1.8.4 With a disulfide as acceptor
EC 1.8.4.1 glutathione—homocystine transhydrogenase
EC 1.8.4 2 protein-disulfide reductase (glutathione)

EC 1.8.4.3 glutathione—CoA-glutathione transhydrogenase
EC 1.8.4.4 glutathione—cystine transhydrogenase
EC 1 8.4.5 methionine-*S*-oxide reductase
EC 1.8.4.6 protein-methionine-*S*-oxide reductase
EC 1.8.4.7 enzyme-thiol transhydrogenase (glutathione-disulfide)
EC 1.8.4.8 phosphoadenylyl-sulfate reductase (thioredoxin)
EC 1.8.4.9 adenylyl-sulfate reductase (glutathione)
EC 1.8.4.10 adenylyl-sulfate reductase (thioredoxin)
EC 1.8.5 With a quinone or similar compound as acceptor
EC 1.8.5.1 glutathione dehydrogenase (ascorbate)
EC 1.8.6 With an nitrogenous group as acceptor
EC 1.8.6.1 deleted, included in EC 2.5.1.18
EC 1.8.7 With an iron-sulfur protein as acceptor
EC 1.8.7.1 sulfite reductase (ferredoxin)
EC 1.8.98 With other, known, acceptors
EC 1.8.98.1 CoB—CoM heterodisulfide reductase
EC 1.8.99.1 sulfite reductase
EC 1.8.99.2 adenylyl-sulfate reductase
EC 1.8.99.3 hydrogensulfite reductase
EC 1.8.99.4 now EC 1.8.4.8
EC 1.9 Acting on a heme group of donors
EC 1.9.3 With oxygen as acceptor
EC 1.9.3.1 cytochrome-*c* oxidase
EC 1.9.3.2 included with EC 1.7.2.1
EC 1.9.6 With a nitrogenous group as acceptor
EC 1.9.6.1 nitrate reductase (cytochrome)
EC 1.9.99 With other acceptors
EC 1.9.99.1 iron—cytochrome-*c* reductase
EC 1.10 Acting on diphenols and related substances as donors
EC 1.10.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.10.1.1 *trans*-acenaphthene-1,2-diol dehydrogenase
EC 1.10.2 With a cytochrome as acceptor
EC 1 10.2.1 L-ascorbate—cytochrome-b_5 reductase
EC 1.10.2.2 ubiquinol—cytochrome-*c* reductase
EC 1.10.3 With oxygen as acceptor
EC 1.10.3.1 catechol oxidase
EC 1.10.3.2 laccase
EC 1.10.3.3 L-ascorbate oxidase
EC 1.10.3.4 *o*-aminophenol oxidase
EC 1.10.3.5 3-hydroxyanthranilate oxidase
EC 1.10.3.6 rifamycin-B oxidase
EC 1.10.3.7 now EC 1.21.3.4
EC 1.10.3.8 now EC 1.21.3.5
EC 1.10.99 With other acceptors
EC 1.10.99.1 plastoquinol—plastocyanin reductase
EC 1.11 Acting on a peroxide as acceptor
EC 1.11.1 Peroxidases
EC 1.11.1.1 NADH peroxidase
EC 1.11.1.2 NADPH peroxidase
EC 1.11.1.3 fatty-acid peroxidase
EC 1.11.1.4 now EC 1.13.11.11
EC 1.11.1.5 cytochrome-*c* peroxidase
EC 1.11.1.6 catalase
EC 1.11.1.7 peroxidase
EC 1.11.1.8 iodide peroxidase
EC 1.11.1.9 glutathione peroxidase
EC 1.11.1.10 chloride peroxidase
EC 1.11.1.11 L-ascorbate peroxidase
EC 1.11.1.12 phospholipid-hydroperoxide glutathione peroxidase
EC 1.11.1.13 manganese peroxidase
EC 1.11.1.14 diarylpropane peroxidase
EC 1.12 Acting on hydrogen as donor
EC 1.12.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.12.1.1 now EC 1.18.99.1
EC 1.12.1.2 hydrogen dehydrogenase
EC 1.12.1.3 hydrogen dehydrogenase ($NADP^+$)
EC 1.12.2 With a cytochrome as acceptor
EC 1.12.2.1 cytochrome-c_3 hydrogenase
EC 1.12.5 With a quinone or similar compound as acceptor
EC 1.12.5.1 hydrogen:quinone oxidoreductase
EC 1.12.7 With an iron-sulfur protein as acceptor
EC 1.12.7.1 now EC 1.18.99.1
EC 1.12.7.2 ferredoxin hydrogenase
EC 1.12.98 With other known acceptors
EC 1.12.98.1 coenzyme F_{420} hydrogenase
EC 1.12.98.2 5,10-methenyltetrahydromethanopterin hydrogenase
EC 1.12.98.3 *Methanosarcina*-phenazine hydrogenase
EC 1.12.99 With other acceptors
EC 1.12.99.1 now EC 1.12.98.1
EC 1.12.99.2 deleted, composed of EC 1.12.98.3 and EC 1.8.98.1.
EC 1.12.99.3 now EC 1.12.5.1
EC 1.12.99.4 now EC 1.12.98.2

EC 1.12.99.5 identical to EC 1.13.11.47
EC 1.12.99.6 hydrogenase (acceptor)
EC 1.13 Acting on single donors with incorporation of molecular oxygen (oxygenases)
EC 1.13.1.1 now EC 1.13.11.1
EC 1.13.1.2 now EC 1.13.11.2
EC 1.13.1.3 now EC 1.13.11.3
EC 1.13.1.4 now EC 1.13.11.4
EC 1.13.1.5 now EC 1.13.11.5
EC 1.13.1.6 now EC 1.13.11.6
EC 1.13.1.7 now EC 1.13.11.7
EC 1.13.1.8 now EC 1.13.11.8
EC 1.13.1.9 now EC 1.13.11.9
EC 1.13.1.10 now EC 1.13.11.10
EC 1.13.1.11 now EC 1.13.99.1
EC 1.13.1.12 now EC 1.13.11.11
EC 1.13.1.13 now EC 1.13.11.12
EC 1.13.11 With incorporation of two atoms of oxygen
EC 1.13.11.1 catechol 1,2-dioxygenase
EC 1.13.11.2 catechol 2,3-dioxygenase
EC 1.13.11.3 protocatechuate 3,4-dioxygenase
EC 1.13.11.4 gentisate 1,2-dioxygenase
EC 1.13.11.5 homogentisate 1,2-dioxygenase
EC 1.13.11.6 3-hydroxyanthranilate 3,4-dioxygenase
EC 1.13.11.7 deleted
EC 1.13.11.8 protocatechuate 4,5-dioxygenase
EC 1.13.11.9 2,5-dihydroxypyridine 5,6-dioxygenase
EC 1.13.11.10 7,8-dihydroxykynurenate 8, 8a-dioxygenase
EC 1.13.11.11 tryptophan 2,3-dioxygenase
EC 1.13.11.12 lipoxygenase
EC 1.13.11.13 ascorbate 2,3-dioxygenase
EC 1.13.11.14 2,3-dihydroxybenzoate 3, 4-dioxygenase
EC 1.13.11.15 3,4-dihydroxyphenylacetate 2, 3-dioxygenase
EC 1.13.11.16 3-carboxyethylcatechol 2, 3-dioxygenase
EC 1.13.11.17 indole 2,3-dioxygenase
EC 1.13.11.18 sulfur dioxygenase
EC 1.13.11.19 cysteamine dioxygenase
EC 1.13.11.20 cysteine dioxygenase
EC 1.13.11.21 now EC 1.14.99.36
EC 1.13.11.22 caffeate 3,4-dioxygenase
EC 1.13.11.23 2,3-dihydroxyindole 2,3-dioxygenase
EC 1.13.11.24 quercetin 2,3-dioxygenase
EC 1.13.11.25 3,4-dihydroxy-9,10-secoandrosta-1, 3, 5(10)-triene-9,17-dione 4, 5-dioxygenase
EC 1.13.11.26 peptide-tryptophan 2,3-dioxygenase
EC 1.13.11.27 4-hydroxyphenylpyruvate dioxygenase
EC 1.13.11.28 2,3-dihydroxybenzoate 2, 3-dioxygenase
EC 1.13.11.29 stizolobate synthase
EC 1.13.11.30 stizolobinate synthase
EC 1.13.11.31 arachidonate 12-lipoxygenase
EC 1.13.11.32 2-nitropropane dioxygenase
EC 1.13.11.33 arachidonate 15-lipoxygenase
EC 1.13.11.34 arachidonate 5-lipoxygenase
EC 1.13.11.35 pyrogallol 1,2-oxygenase
EC 1.13.11.36 chloridazon-catechol dioxygenase
EC 1.13.11.37 hydroxyquinol 1,2-dioxygenase
EC 1.13.11.38 1-hydroxy-2-naphthoate 1, 2-dioxygenase
EC 1.13.11.39 biphenyl-2,3-diol 1,2-dioxygenase
EC 1.13.11.40 arachidonate 8-lipoxygenase
EC 1.13.11.41 2,4'-dihydroxyacetophenone dioxygenase
EC 1.13.11.42 indoleamine-pyrrole 2,3-dioxygenase
EC 1.13.11.43 lignostilbene ab-dioxygenase
EC 1.13.11.44 linoleate diol synthase
EC 1.13.11.45 linoleate 11-lipoxygenase
EC 1.13.11.46 4-hydroxymandelate synthase
EC 1.13.11.47 3-hydroxy-4-oxoquinoline 2, 4-dioxygenase
EC 1.13.11.48 3-hydroxy-2-methyl-quinolin-4-one 2,4-dioxygenase
EC 1.13.11.49 chlorite O_2-lyase
EC 1.13.11.50 acetylacetone-cleaving enzyme
EC 1.13.12 With incorporation of one atom of oxygen (internal monooxygenases or internal mixed function oxidases)
EC 1.13.12.1 arginine 2-monooxygenase
EC 1.13.12.2 lysine 2-monooxygenase
EC 1.13.12.3 tryptophan 2-monooxygenase
EC 1.13.12.4 lactate 2-monooxygenase
EC 1.13.12.5 *Renilla*-luciferin 2-monooxygenase
EC 1.13.12.6 *Cypridina*-luciferin 2-monooxygenase
EC 1.13.12.7 *Photinus*-luciferin 4-monooxygenase (ATP-hydrolysing)
EC 1.13.12.8 *Watasenia*-luciferin 2-monooxygenase
EC 1.13.12.9 phenylalanine 2-monooxygenase
EC 1.13.12.10 covered by EC 1.14.13.59

EC 1.13.12.11 methylphenyltetrahydropyridine *N*-monooxygenase
EC 1.13.12.12 apo-b-carotenoid-14', 13'-dioxygenase
EC 1.13.12.13 *Oplophorus*-luciferin 2-monooxygenase
EC 1.13.99 Miscellaneous
EC 1.13.99.1 inositol oxygenase
EC 1.13.99.2 now EC 1.14.12.10
EC 1.13.99.3 tryptophan 2'-dioxygenase
EC 1.13.99.4 now EC 1.14.12 9
EC 1.13.99.5 now EC 1.13.11.47
EC 1.14 Acting on paired donors, with incorporation or reduction of molecular oxygen
EC 1.14.1.1 now EC 1.14.14.1
EC 1.14.1 2 now EC 1.14.13.9
EC 1.14.1.3 deleted, covered by EC 1.14.99.7, EC 5.4.99.7
EC 1.14.1.4 now EC 1.14.99.2
EC 1.14 1.5 now EC 1.14.13.5
EC 1 14.1.6 now EC 1.14.15.4
EC 1.14.1.7 now EC 1.14.99.9
EC 1.14.1.8 now EC 1 14.99.10
EC 1.14.1 9 deleted
EC 1.14.1.10 now EC 1.14.99.11
EC 1.14.1.11 deleted
EC 1.14.2 With ascorbate as one donor
EC 1.14 2.1 now EC 1.14.17.1
EC 1.14.2.2 now EC 1.13.11.27
EC 1.14.3 With reduced pteridine as one donor
EC 1.14.3.1 now EC 1.14.16.1
EC 1.14.11 With 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors
EC 1.14 11.1 g-butyrobetaine dioxygenase
EC 1.14.11 2 procollagen-proline dioxygenase
EC 1.14.11.3 pyrimidine-deoxynucleoside 2'-dioxygenase
EC 1.14.11.4 procollagen-lysine 5-dioxygenase
EC 1.14.11.5 deleted, included in EC 1.14.11.6
EC 1.14.11.6 thymine dioxygenase
EC 1.14.11.7 procollagen-proline 3-dioxygenase
EC 1.14.11.8 trimethyllysine dioxygenase
EC 1.14.11 9 naringenin 3-dioxygenase
EC 1.14 11.10 pyrimidine-deoxynucleoside 1'-dioxygenase
EC 1.14.11.11 hyoscyamine (6*S*)-dioxygenase
EC 1.14.11.12 gibberellin-44 dioxygenase
EC 1 14.11.13 gibberellin 2b-dioxygenase
EC 1.14.11.14 6b-hydroxyhyoscyamine epoxidase
EC 1.14.11.15 gibberellin 3b-dioxygenase
EC 1.14 11.16 peptide-aspartate b-dioxygenase
EC 1.14.11.17 taurine dioxygenase
EC 1.14.11.18 phytanoyl-CoA dioxygenase
EC 1.14.11.19 leucocyanidin oxygenase
EC 1.14.11.20 desacetoxyvindoline 4-hydroxylase
EC 1.14.11.21 clavaminate synthase
EC 1.14.12 With NADH or NADPH as one donor, and incorporation of two atoms of oxygen into one donor
EC 1.14.12 1 anthranilate 1,2-dioxygenase (deaminating, decarboxylating)
EC 1.14.12.2 now EC 1.14.13 35
EC 1.14.12.3 benzene 1,2-dioxygenase
EC 1.14.12.4 3-hydroxy-2-methylpyridinecarboxylate dioxygenase
EC 1 14.12 5 5-pyridoxate dioxygenase
EC 1.14.12.6 now EC 1.14.13.66
EC 1.14.12.7 phthalate 4,5-dioxygenase
EC 1.14.12.8 4-sulfobenzoate 3,4-dioxygenase
EC 1.14.12.9 4-chlorophenylacetate 3, 4-dioxygenase
EC 1 14.12.10 benzoate 1,2-dioxygenase
EC 1.14.12.11 toluene dioxygenase
EC 1.14.12.12 naphthalene 1,2-dioxygenase
EC 1.14.12.13 2-chlorobenzoate 1,2-dioxygenase
EC 1.14.12.14 2-aminobenzenesulfonate 2, 3-dioxygenase
EC 1.14.12.15 terephthalate 1,2-dioxygenase
EC 1.14.12.16 2-hydroxyquinoline 5,6-dioxygenase
EC 1.14.12.17 nitric oxide dioxygenase
EC 1.14.12.18 biphenyl 2,3-dioxygenase
EC 1.14.13 With NADH or NADPH as one donor, and incorporation of one atom of oxygen
EC 1.14.13.1 salicylate 1-monooxygenase
EC 1.14.13.2 4-hydroxybenzoate 3-monooxygenase
EC 1.14 13.3 4-hydroxyphenylacetate 3-monooxygenase
EC 1.14.13.4 melilotate 3-monooxygenase
EC 1.14.13.5 imidazoleacetate 4-monooxygenase
EC 1.14.13.6 orcinol 2-monooxygenase
EC 1.14.13.7 phenol 2-monooxygenase
EC 1.14.13.8 dimethylaniline monooxygenase (*N*-oxide-forming)

EC 1 14 13.9 kynurenine 3-monooxygenase
EC 1.14.13.10 2,6-dihydroxypyridine 3-monooxygenase
EC 1.14.13.11 *trans*-cinnamate 4-monooxygenase
EC 1.14 13.12 benzoate 4-monooxygenase
EC 1.14.13 13 calcidiol 1-monooxygenase
EC 1.14 13.14 *trans*-cinnamate 2-monooxygenase
EC 1.14.13.15 cholestanetriol 26-monooxygenase
EC 1.14.13 16 cyclopentanone monooxygenase
EC 1.14.13.17 cholesterol 7a-monooxygenase
EC 1.14 13.18 4-hydroxyphenylacetate 1-monooxygenase
EC 1.14.13.19 taxifolin 8-monooxygenase
EC 1.14.13.20 2,4-dichlorophenol 6-monooxygenase
EC 1.14.13.21 flavonoid 3'-monooxygenase
EC 1 14.13.22 cyclohexanone monooxygenase
EC 1 14 13 23 3-hydroxybenzoate 4-monooxygenase
EC 1.14.13.24 3-hydroxybenzoate 6-monooxygenase
EC 1.14 13.25 methane monooxygenase
EC 1.14.13.26 phosphatidylcholine 12-monooxygenase
EC 1.14.13.27 4-aminobenzoate 1-monooxygenase
EC 1 14 13 28 3,9-dihydroxypterocarpan 6a-monooxygenase
EC 1.14.13.29 4-nitrophenol 2-monooxygenase
EC 1.14.13.30 leukotriene-B_4 20-monooxygenase
EC 1.14.13.31 2-nitrophenol 2-monooxygenase
EC 1.14.13 32 albendazole monooxygenase
EC 1 14.13.33 4-hydroxybenzoate 3-monooxygenase [NAD(P)H]
EC 1.14.13.34 leukotriene-E_4 20-monooxygenase
EC 1.14.13.35 anthranilate 3-monooxygenase (deaminating)
EC 1.14 13.36 5-*O*-(4-coumaroyl)-D-quinate 3'-monooxygenase
EC 1.14.13.37 methyltetrahydroprotoberberine 14-monooxygenase
EC 1 14 13.38 anhydrotetracycline monooxygenase
EC 1.14.13 39 nitric-oxide synthase
EC 1.14 13.40 anthraniloyl-CoA monooxygenase
EC 1.14 13.41 tyrosine *N*-monooxygenase
EC 1.14.13.42 hydroxyphenylacetonitrile 2-monooxygenase
EC 1.14.13 43 questin monooxygenase
EC 1 14.13.44 2-hydroxybiphenyl 3-monooxygenase
EC 1.14.13.45 now EC 1.14.18.2
EC 1.14.13.46 (-)-menthol monooxygenase
EC 1.14 13 47 (*S*)-limonene 3-monooxygenase
EC 1 14 13.48 (*S*)-limonene 6-monooxygenase
EC 1.14 13.49 (*S*)-limonene 7-monooxygenase
EC 1.14.13.50 pentachlorophenol monooxygenase
EC 1 14.13 51 6-oxocineole dehydrogenase
EC 1 14 13 52 isoflavone 3'-hydroxylase
EC 1.14 13.53 isoflavone 2'-hydroxylase
EC 1 14 13 54 ketosteroid monooxygenase
EC 1 14 13 55 protopine 6-monooxygenase
EC 1 14.13.56 dihydrosanguinarine 10-monooxygenase
EC 1 14 13 57 dihydrochelirubine 12-monooxygenase
EC 1.14 13 58 benzoyl-CoA 3-monooxygenase
EC 1 14.13.59 L-lysine 6-monooxygenase (NADPH)
EC 1 14 13 60 27-hydroxycholesterol 7a-monooxygenase
EC 1.14 13.61 2-hydroxyquinoline 8-monooxygenase
EC 1 14 13 62 4-hydroxyquinoline 3-monooxygenase
EC 1 14.13.63 3-hydroxyphenylacetate 6-hydroxylase
EC 1.14 13 64 4-hydroxybenzoate 1-hydroxylase
EC 1 14 13.65 2-hydroxyquinoline 8-monooxygenase
EC 1 14 13.66 2-hydroxycyclohexanone 2-monooxygenase
EC 1.14.13.67 quinine 3-monooxygenase
EC 1.14.13 68 4-hydroxyphenylacetaldehyde oxime monooxygenase
EC 1 14.13.69 alkene monooxygenase
EC 1.14.13.70 sterol 14-demethylase
EC 1 14 13 71 *N*-methylcoclaurine 3'-monooxygenase
EC 1.14.13.72 methylsterol monooxygenase
EC 1.14.13.73 tabersonine 16-hydroxylase
EC 1 14.13.74 7-deoxyloganin 7-hydroxylase
EC 1.14.13.75 vinorine hydroxylase
EC 1.14 13 76 taxane 10b-hydroxylase
EC 1.14.13 77 taxane 13a-hydroxylase
EC 1 14.13.78 *ent*-kaurene oxidase
EC 1.14 13.79 *ent*-kaurenoic acid oxidase
EC 1.14.13.80 (*R*)-limonene 6-monooxygenase
EC 1.14.13.81 magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase
EC 1.14.13.82 vanillate monooxygenase

EC 1.14.13.83 precorrin-3B synthase
EC 1.14.13.84 4-hydroxyacetophenone monooxygenase
EC 1.14.14 With reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen
EC 1.14.14.1 unspecific monooxygenase
EC 1.14.14.2 deleted, included in EC 1.14.14.1
EC 1 14.14.3 alkanal monooxygenase (FMN-linked)
EC 1.14.14.4 deleted, identical to EC 1.14.15.7
EC 1.14 14.5 alkanesulfonate monooxygenase
EC 1.14.15 With reduced iron-sulfur protein as one donor, and incorporation of one atom of oxygen
EC 1.14.15.1 camphor 5-monooxygenase
EC 1.14.15.2 camphor 1,2-monooxygenase
EC 1.14.15.3 alkane 1-monooxygenase
EC 1.14.15.4 steroid 11b-monooxygenase
EC 1.14.15.5 corticosterone 18-monooxygenase
EC 1.14.15.6 cholesterol monooxygenase (side-chain-cleaving)
EC 1.14.15.7 choline monooxygenase
EC 1.14.16 With reduced pteridine as one donor, and incorporation of one atom of oxygen
EC 1.14.16.1 phenylalanine 4-monooxygenase
EC 1.14.16.2 tyrosine 3-monooxygenase
EC 1.14.16.3 anthranilate 3-monooxygenase
EC 1.14.16.4 tryptophan 5-monooxygenase
EC 1.14.16.5 glyceryl-ether monooxygenase
EC 1.14.16.6 mandelate 4-monooxygenase
EC 1.14.17 With reduced ascorbate as one donor, and incorporation of one atom of oxygen
EC 1.14.17.1 dopamine b-monooxygenase
EC 1.14.17.2 deleted, included in EC 1.14.18.1
EC 1.14.17.3 peptidylglycine monooxygenase
EC 1.14.17.4 aminocyclopropanecarboxylate oxidase
EC 1.14.18 With another compound as one donor, and incorporation of one atom of oxygen
EC 1.14.18.1 monophenol monooxygenase
EC 1.14.18.2 CMP-*N*-acetylneuraminate monooxygenase
EC 1.14.19 With oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water
EC 1.14.19.1 stearoyl-CoA 9-desaturase
EC 1.14.19.2 acyl-[acyl-carrier-protein] desaturase
EC 1.14.19.3 linoleoyl-CoA desaturase
EC 1.14.20 With 2-oxoglutarate as one donor, and the other dehydrogenated
EC 1.14.20.1 deacetoxycephalosporin-C synthase
EC 1.14.21 With NADH or NADPH as one donor, and the other dehydrogenated
EC 1.14.21.1 (*S*)-stylopine synthase
EC 1.14.21.2 (*S*)-cheilanthifoline synthase
EC 1.14.21.3 berbamunine synthase
EC 1.14.21.4 salutaridine synthase
EC 1.14.21.5 (*S*)-canadine synthase
EC 1.14.99 Miscellaneous
EC 1.14.99.1 prostaglandin-endoperoxide synthase
EC 1.14.99.2 kynurenine 7,8-hydroxylase
EC 1.14.99.3 heme oxygenase (decyclizing)
EC 1.14.99.4 progesterone monooxygenase
EC 1.14.99.5 now EC 1.14.19.1
EC 1.14.99.6 now EC 1.14.19.2
EC 1.14.99.7 squalene monooxygenase
EC 1.14.99.8 deleted, included in EC 1.14.14.1
EC 1.14.99.9 steroid 17a-monooxygenase
EC 1.14.99.10 steroid 21-monooxygenase
EC 1.14.99.11 estradiol 6b-monooxygenase
EC 1.14.99.12 4-androstene-3,17-dione monooxygenase
EC 1.14.99.13 now EC 1.14.13.23
EC 1.14.99.14 progesterone 11a-monooxygenase
EC 1.14.99.15 4-methoxybenzoate monooxygenase (*O*-demethylating)
EC 1.14.99.16 now EC 1.14.13.72
EC 1.14.99.17 now EC 1.14.16.5
EC 1.14.99.18 deleted
EC 1.14.99.19 plasmanylethanolamine desaturase
EC 1.14.99.20 phylloquinone monooxygenase (2,3-epoxidizing)
EC 1.14.99.21 *Latia*-luciferin monooxygenase (demethylating)
EC 1.14.99.22 ecdysone 20-monooxygenase
EC 1.14.99.23 3-hydroxybenzoate 2-monooxygenase
EC 1.14.99.24 steroid 9a-monooxygenase
EC 1.14.99.25 now EC 1.14.19.3
EC 1.14.99.26 2-hydroxypyridine 5-monooxygenase
EC 1.14.99.27 juglone 3-monooxygenase
EC 1.14.99.28 linalool 8-monooxygenase

EC 1.14.99.29 deoxyhypusine monooxygenase
EC 1.14.99.30 carotene 7,8-desaturase
EC 1.14.99.31 myristoyl-CoA 11-(*E*) desaturase
EC 1.14.99.32 myristoyl-CoA 11-(*Z*) desaturase
EC 1.14.99.33 D^{12}-fatty acid dehydrogenase
EC 1.14.99.34 monoprenyl isoflavone epoxidase
EC 1.14.99.35 thiophene-2-carbonyl-CoA monooxygenase
EC 1.14.99.36 b-carotene 15,15'-monooxygenase
EC 1.14.99.37 taxadiene 5a-hydroxylase
EC 1.15 Acting on superoxide as acceptor
EC 1.15.1.1 superoxide dismutase
EC 1.15.1.2 superoxide reductase
EC 1.16 Oxidizing metal ions
EC 1.16.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.16.1.1 mercury(II) reductase
EC 1.16.1.2 diferric-transferrin reductase
EC 1.16.1.3 aquacobalamin reductase
EC 1.16.1.4 cob(II)alamin reductase
EC 1.16.1.5 aquacobalamin reductase (NADPH)
EC 1.16.1.6 cyanocobalamin reductase (cyanide-eliminating)
EC 1.16.1.7 ferric-chelate reductase
EC 1.16.1.8 [methionine synthase] reductase
EC 1.16.3 With oxygen as acceptor
EC 1.16.3.1 ferroxidase
EC 1.16.8 With flavin as acceptor
EC 1.16.8.1 cob(II)yrinic acid *a,c*-diamide reductase
EC 1.17 Acting on CH or CH_2 groups
EC 1.17.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.17.1.1 CDP-4-dehydro-6-deoxyglucose reductase
EC 1.17.1.2 4-hydroxy-3-methylbut-2-enyl diphosphate reductase
EC 1.17.1.3 leucoanthocyanidin reductase
EC 1.17.1.4 xanthine dehydrogenase
EC 1.17.1.5 nicotinate dehydrogenase
EC 1.17.3 With oxygen as acceptor
EC 1.17.3.1 pteridine oxidase
EC 1.17.3.2 xanthine oxidase
EC 1.17.3.3 6-hydroxynicotinate dehydrogenase
EC 1.17.4 With a disulfide as acceptor
EC 1.17.4.1 ribonucleoside-diphosphate reductase
EC 1.17.4.2 ribonucleoside-triphosphate reductase
EC 1.17.4.3 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
EC 1.17.5 With a quinone or similar compound as acceptor
EC 1.17.5.1 phenylacetyl-CoA dehydrogenase
EC 1.17.99 With other acceptors
EC 1.17.99.1 4-cresol dehydrogenase (hydroxylating)
EC 1.17.99.2 ethylbenzene hydroxylase
EC 1.18 Acting on iron-sulfur proteins as donors
EC 1.18.1 With NAD^+ or $NADP^+$ as acceptor
EC 1.18.1.1 rubredoxin—NAD^+ reductase
EC 1.18.1.2 ferredoxin—$NADP^+$ reductase
EC 1.18.1.3 ferredoxin—NAD^+ reductase
EC 1.18.1.4 rubredoxin—$NAD(P)^+$ reductase
EC 1.18.2 With dinitrogen as acceptor now EC 1.18.6)
EC 1.18.2.1 now EC 1.18.6.1
EC 1.18.3 With H^+ as acceptor
EC 1.18.3.1 now EC 1.18.99.1
EC 1.18.6 With dinitrogen as acceptor
EC 1.18.6.1 nitrogenase
EC 1.18.96 With other, known, acceptors
EC 1.18.96.1 now EC 1.15.1.2
EC 1.18.99 With H^+ as acceptors
EC 1.18.99.1 now EC 1.12.7.2
EC 1.19 Acting on reduced flavodoxin as donor
EC 1.19.6 With dinitrogen as acceptor
EC 1.19.6.1 nitrogenase (flavodoxin)
EC 1.20 Acting on phosphorus or arsenic in donors
EC 1.20.1 Acting on phosphorus or arsenic in donors, with $NAD(P)^+$ as acceptor
EC 1.20.1.1 phosphonate dehydrogenase
EC 1.20.4 Acting on phosphorus or arsenic in donors, with disulfide as acceptor
EC 1.20.4.1 arsenate reductase (glutaredoxin)
EC 1.20.4.2 methylarsonate reductase
EC 1.20.98 Acting on phosphorus or arsenic in donors, with other, known acceptors
EC 1.20.98.1 arsenate reductase (azurin)
EC 1.20.99 Acting on phosphorus or arsenic in donors, with other acceptors
EC 1.20.99.1 arsenate reductase (donor)
EC 1.21 Acting on X-H and Y-H to form an X-Y bond
EC 1.21.3 With oxygen as acceptor
EC 1.21.3.1 isopenicillin-N synthase
EC 1.21.3.2 columbamine oxidase
EC 1.21.3.3 reticuline oxidase
EC 1.21.3.4 sulochrin oxidase [(+)-bisdechlorogeodin-forming]
EC 1.21.3.5 sulochrin oxidase [(-)-bisdechlorogeodin-forming]
EC 1.21.3.6 aureusidin synthase

EC 1 21.4 With a disulfide as acceptor
EC 1.21.4.1 D-proline reductase (dithiol)
EC 1.21 4.2 glycine reductase
EC 1.21.4.3 sarcosine reductase
EC 1.21.4.4 betaine reductase
EC 1.21.99 With other acceptors
EC 1.21.99.1 b-cyclopiazonate dehydrogenase
EC 1.97 Other oxidoreductases
EC 1 97.1.1 chlorate reductase
EC 1.97.1.2 pyrogallol hydroxyltransferase
EC 1 97.1.3 sulfur reductase
EC 1.97.1.4 formate acetyltransferase activating enzyme
EC 1.97.1.5 now EC 1 20 4.1
EC 1.97 1.6 now EC 1.20.99 1
EC 1.97.1.7 now EC 1.20.4.2
EC 1.97 1.8 tetrachloroethene reductive dehalogenase
EC 1.97.1.9 selenate reductase
EC 1.97.1.10 thyroxine 5'-deiodinase
EC 1.97.1.11 thyroxine 5-deiodinase
EC 1.98 Enzymes using H_2 as reductant
EC 1 98.1.1 now EC 1.18.99.1
EC 1.99 Other enzymes using O_2 as oxidant
EC 1.99.1 Hydroxylases (now EC 1.14)
EC 1.99.1.1 deleted, now EC 1.14.14 1
EC 1.99.1.2 deleted, now EC 1.14.16.1
EC 1.99.1.3 deleted
EC 1.99.1.4 deleted
EC 1.99.1.5 deleted, now EC 1.14.13.9
EC 1 99.1.6 deleted
EC 1.99.1.7 deleted, now EC 1.14.15.4
EC 1 99.1.8 deleted
EC 1.99.1.9 deleted, now EC 1.14.99.9
EC 1.99.1.10 deleted
EC 1.99 1.11 deleted, now EC 1.14.99.10
EC 1.99.1.12 deleted
EC 1 99 1.13 deleted, covered by EC 1.14.99.7 and EC 5.4.99.7
EC 1.99.1.14 deleted, now EC 1.13 11.27
EC 1.99.2 Oxygenases (now EC 1.13)
EC 1.99.2.1 deleted, now EC 1.13.11.12
EC 1.99.2.2 deleted, now EC 1.13.11.1
EC 1.99.2.3 deleted, now EC 1.13 11.3
EC 1.99.2 4 deleted, now EC 1.13.11.4
EC 1.99.2.5 deleted, now EC 1.13.11.5
EC 1 99.2.6 deleted, now EC 1.13.99.1
EC 2. Transferases
EC 2.1 Transferring One-Carbon Groups
EC 2.1.1 Methyltransferases
EC 2.1.1.1 nicotinamide *N*-methyltransferase
EC 2.1.1.2 guanidinoacetate *N*-methyltransferase
EC 2 1.1.3 thetin—homocysteine *S* - methyltransferase
EC 2.1.1 4 acetylserotonin *O*-methyltransferase
EC 2 1.1.5 betaine—homocysteine *S*-methyltransferase
EC 2.1.1.6 catechol *O*-methyltransferase
EC 2 1.1.7 nicotinate *N*-methyltransferase
EC 2 1 1.8 histamine *N*-methyltransferase
EC 2.1 1 9 thiol *S*-methyltransferase
EC 2.1.1.10 homocysteine *S*-methyltransferase
EC 2.1.1.11 magnesium protoporphyrin IX methyltransferase
EC 2.1 1.12 methionine *S*-methyltransferase
EC 2.1 1 13 methionine synthase
EC 2.1.1.14 5-methyltetrahydropteroyltriglutamate—homocysteine *S*-methyltransferase
EC 2.1.1.15 fatty-acid *O*-methyltransferase
EC 2.1.1.16 methylene-fatty-acyl-phospholipid synthase
EC 2.1.1 17 phosphatidylethanolamine *N*-methyltransferase
EC 2.1.1.18 polysaccharide *O*-methyltransferase
EC 2.1.1.19 trimethylsulfonium—tetrahydrofolate *N*-methyltransferase
EC 2.1.1.20 glycine *N*-methyltransferase
EC 2 1.1.21 methylamine—glutamate *N*-methyltransferase
EC 2.1.1.22 carnosine *N*-methyltransferase
EC 2.1.1.23 now covered by EC 2.1.1.124, EC 2.1.1.125 and EC 2.1.1.126
EC 2.1.1.24 now covered by EC 2.1.1.77, EC 2.1.1.80 and EC 2.1.1.100
EC 2.1.1.25 phenol *O*-methyltransferase
EC 2.1.1.26 iodophenol *O*-methyltransferase
EC 2.1.1.27 tyramine *N*-methyltransferase
EC 2.1.1.28 phenylethanolamine *N*-methyltransferase
EC 2.1.1.29 tRNA (cytosine-5-)-methyltransferase
EC 2.1.1.30 deleted
EC 2 1.1.31 tRNA (guanine-N^1-)-methyltransferase
EC 2.1 1.32 tRNA (guanine-N^2-)-methyltransferase
EC 2.1.1.33 tRNA (guanine-N^7-)-methyltransferase

EC 2.1.1 34 tRNA (guanosine-2'-*O*-)-methyltransferase
EC 2.1 1 35 tRNA (uracil-5-)-methyltransferase
EC 2.1.1 36 tRNA (adenine-N^1-)-methyltransferase
EC 2.1.1.37 DNA (cytosine-5-)-methyltransferase
EC 2.1.1.38 *O*-demethylpuromycin *O*-methyltransferase
EC 2 1.1 39 inositol 3-methyltransferase
EC 2.1 1 40 inositol 1-methyltransferase
EC 2.1.1.41 sterol 24-*C*-methyltransferase
EC 2.1.1.42 luteolin *O*-methyltransferase
EC 2.1.1.43 histone-lysine *N*-methyltransferase
EC 2.1 1 44 dimethylhistidine *N*-methyltransferase
EC 2.1.1.45 thymidylate synthase
EC 2 1 1.46 isoflavone 4'-*O*-methyltransferase
EC 2.1.1.47 indolepyruvate *C*-methyltransferase
EC 2.1.1.48 rRNA (adenine-N^6-)-methyltransferase
EC 2.1.1.49 amine *N*-methyltransferase
EC 2.1.1.50 loganate *O*-methyltransferase
EC 2 1 1 51 rRNA (guanine-N^1-)-methyltransferase
EC 2.1.1.52 rRNA (guanine-N^2-)-methyltransferase
EC 2.1.1.53 putrescine *N*-methyltransferase
EC 2.1 1.54 deoxycytidylate *C*-methyltransferase
EC 2.1 1 55 tRNA (adenine-N^6-)-methyltransferase
EC 2.1.1.56 mRNA (guanine-N^7-)-methyltransferase
EC 2.1 1.57 mRNA (nucleoside-2'-*O*-)-methyltransferase
EC 2.1.1.58 deleted, included in EC 2 1 1 57
EC 2 1.1.59 [cytochrome *c*]-lysine *N*-methyltransferase
EC 2 1.1.60 calmodulin-lysine *N*-methyltransferase
EC 2.1.1.61 tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase
EC 2.1.1 62 mRNA (2'-*O*-methyladenosine-N^6-)-methyltransferase
EC 2.1.1.63 methylated-DNA—[protein]-cysteine *S*-methyltransferase
EC 2.1.1.64 3-demethylubiquinone-9 3-*O*-methyltransferase
EC 2.1.1 65 licodione 2'-*O*-methyltransferase
EC 2.1.1.66 rRNA (adenosine-2'-*O*-)-methyltransferase
EC 2.1.1.67 thiopurine *S*-methyltransferase
EC 2 1.1.68 caffeate *O*-methyltransferase
EC 2 1 1 69 5-hydroxyfuranocoumarin 5-*O*-methyltransferase
EC 2 1 1.70 8-hydroxyfuranocoumarin 8-*O*-methyltransferase
EC 2.1.1.71 phosphatidyl-*N*-methylethanolamine *N*-methyltransferase
EC 2 1.1.72 site-specific DNA-methyltransferase (adenine-specific)
EC 2.1 1.73 deleted
EC 2.1 1.74 methylenetetrahydrofolate—tRNA-(uracil-5-)-methyltransferase ($FADH_2$-oxidizing)
EC 2.1 1.75 apigenin 4'-*O*-methyltransferase
EC 2 1.1.76 quercetin 3-*O*-methyltransferase
EC 2.1 1.77 protein-L-isoaspartate(D-aspartate) *O*-methyltransferase
EC 2 1 1.78 isoorientin 3'-*O*-methyltransferase
EC 2.1.1 79 cyclopropane-fatty-acyl-phospholipid synthase
EC 2.1 1.80 protein-glutamate *O*-methyltransferase
EC 2.1.1 81 deleted, included in EC 2.1 1.49
EC 2.1.1.82 3-methylquercitin 7-*O*-methyltransferase
EC 2.1 1 83 3,7-dimethylquercitin 4'-*O*-methyltransferase
EC 2.1.1.84 methylquercetagetin 6-*O*-methyltransferase
EC 2 1 1.85 protein-histidine *N*-methyltransferase
EC 2.1 1.86 tetrahydromethanopterin *S*-methyltransferase
EC 2 1 1.87 pyridine *N*-methyltransferase
EC 2.1.1.88 8-hydroxyquercitin 8-*O*-methyltransferase
EC 2.1.1.89 tetrahydrocolumbamine 2-*O*-methyltransferase
EC 2.1 1 90 methanol—5- hydroxybenzimidazolylcobamide *Co*-methyltransferase
EC 2 1.1 91 isobutyraldoxime *O*-methyltransferase
EC 2 1.1 92 bergaptol *O*-methyltransferase
EC 2.1.1.93 xanthotoxol *O*-methyltransferase
EC 2.1 1 94 11-*O*-demethyl-17-*O*-deacetylvindoline *O*-methyltransferase
EC 2.1.1 95 tocopherol *O*-methyltransferase
EC 2.1.1.96 thioether *S*-methyltransferase
EC 2.1.1.97 3-hydroxyanthranilate 4-*C*-methyltransferase
EC 2.1 1 98 diphthine synthase
EC 2.1 1 99 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase
EC 2.1 1.100 protein-*S*-isoprenylcysteine *O*-methyltransferase
EC 2 1.1.101 macrocin *O*-methyltransferase

EC 2.1.1.102 demethylmacrocin *O*-methyltransferase
EC 2.1.1.103 phosphoethanolamine *N*-methyltransferase
EC 2.1.1.104 caffeoyl-CoA *O*-methyltransferase
EC 2.1.1.105 *N*-benzoyl-4-hydroxyanthranilate 4-*O*-methyltransferase
EC 2.1.1.106 tryptophan 2-*C*-methyltransferase
EC 2.1.1.107 uroporphyrin-III *C*-methyltransferase
EC 2.1.1.108 6-hydroxymellein *O*-methyltransferase
EC 2.1.1.109 demethylsterigmatocystin 6-*O*-methyltransferase
EC 2.1.1.110 sterigmatocystin 7-*O*-methyltransferase
EC 2.1.1.111 anthranilate *N*-methyltransferase
EC 2.1.1.112 glucuronoxylan 4-*O*-methyltransferase
EC 2.1.1.113 site-specific DNA-methyltransferase (cytosine-N^4-specific)
EC 2.1.1.114 hexaprenyldihydroxybenzoate methyltransferase
EC 2.1.1.115 (*RS*)-1-benzyl-1,2,3,4-tetrahydroisoquinoline *N*-methyltransferase
EC 2.1.1.116 3'-hydroxy-*N*-methyl-(*S*)-coclaurine 4'-*O*-methyltransferase
EC 2.1.1.117 (*S*)-scoulerine 9-*O*-methyltransferase
EC 2.1.1.118 columbamine *O*-methyltransferase
EC 2.1.1.119 10-hydroxydihydrosanguinarine 10-*O*-methyltransferase
EC 2.1.1.120 12-hydroxydihydrochelirubine 12-*O*-methyltransferase
EC 2.1.1.121 6-*O*-methylnorlaudanosoline 5'-*O*-methyltransferase
EC 2.1.1.122 (*S*)-tetrahydroprotoberberine *N*-methyltransferase
EC 2.1.1.123 [cytochrome *c*]-methionine *S*-methyltransferase
EC 2.1.1.124 [cytochrome *c*]-arginine *N*-methyltransferase
EC 2.1.1.125 histone-arginine *N*-methyltransferase
EC 2.1.1.126 [myelin basic protein]-arginine *N*-methyltransferase
EC 2.1.1.127 [ribulose-bisphosphate carboxylase]-lysine *N*-methyltransferase
EC 2.1.1.128 (*RS*)-norcoclaurine 6-*O*-methyltransferase
EC 2.1.1.129 inositol 4-methyltransferase
EC 2.1.1.130 precorrin-2 C^{20}-methyltransferase
EC 2.1.1.131 precorrin-3B C^{17}-methyltransferase
EC 2.1.1.132 precorrin-6Y $C^{5,15}$-methyltransferase (decarboxylating)
EC 2.1.1.133 precorrin-4 C^{11}-methyltransferase
EC 2.1.1.134 now with EC 2.1.1.129
EC 2.1.1.135 now EC 1.16.1.8
EC 2.1.1.136 chlorophenol *O*-methyltransferase
EC 2.1.1.137 arsenite methyltransferase
EC 2.1.1.138 deleted
EC 2.1.1.139 3'-demethylstaurosporine *O*-methyltransferase
EC 2.1.1.140 (*S*)-coclaurine-*N*-methyltransferase
EC 2.1.1.141 jasmonate *O*-methyltransferase
EC 2.1.1.142 cycloartenol 24-*C*-methyltransferase
EC 2.1.1.143 24-methylenesterol *C*-methyltransferase
EC 2.1.1.144 *trans*-aconitate 2-methyltransferase
EC 2.1.1.145 *trans*-aconitate 3-methyltransferase
EC 2.1.1.146 (iso)eugenol *O*-methyltransferase
EC 2.1.1.147 corydaline synthase
EC 2.1.1.148 thymidylate synthase (FAD)
EC 2.1.1.149 myricetin *O*-methyltransferase
EC 2.1.1.150 isoflavone 7-*O*-methyltransferase
EC 2.1.1.151 cobalt-factor II C^{20}-methyltransferase
EC 2.1.1.152 precorrin-6A synthase (deacetylating)
EC 2.1.2 Hydroxymethyl-, Formyl- and Related Transferases
EC 2.1.2.1 glycine hydroxymethyltransferase
EC 2.1.2.2 phosphoribosylglycinamide formyltransferase
EC 2.1.2.3 phosphoribosylaminoimidazolecarboxamide formyltransferase
EC 2.1.2.4 glycine formiminotransferase
EC 2.1.2.5 glutamate formiminotransferase
EC 2.1.2.6 deleted, included in EC 2.1.2.5
EC 2.1.2.7 D-alanine 2-hydroxymethyltransferase
EC 2.1.2.8 deoxycytidylate 5-hydroxymethyltransferase
EC 2.1.2.9 methionyl-tRNA formyltransferase
EC 2.1.2.10 aminomethyltransferase
EC 2.1.2.11 3-methyl-2-oxobutanoate hydroxymethyltransferase
EC 2.1.2.12 now EC 2.1.1.74
EC 2.1.3 Carboxy- and Carbamoyltransferases
EC 2.1.3.1 methylmalonyl-CoA carboxytransferase
EC 2.1.3.2 aspartate carbamoyltransferase
EC 2.1.3.3 ornithine carbamoyltransferase
EC 2.1.3.4 deleted

EC 2.1.3.5 oxamate carbamoyltransferase
EC 2.1.3.6 putrescine carbamoyltransferase
EC 2.1.3.7 3-hydroxymethylcephem carbamoyltransferase
EC 2.1.3.8 lysine carbamoyltransferase
EC 2.1.4 Amidinotransferases
EC 2.1.4.1 glycine amidinotransferase
EC 2.1.4.2 inosamine-phosphate amidinotransferase
EC 2.2 Transferring Aldehyde or Ketonic Groups
EC 2.2.1 Transketolases and Transaldolases
EC 2.2.1.1 transketolase
EC 2.2.1.2 transaldolase
EC 2.2.1.3 formaldehyde transketolase
EC 2.2.1.4 acetoin—ribose-5-phosphate transaldolase
EC 2.2.1.5 2-hydroxy-3-oxoadipate synthase
EC 2.2.1.6 acetolactate synthase
EC 2.2.1.7 1-deoxy-D-xylulose-5-phosphate synthase
EC 2.2.1.8 fluorothreonine transaldolase
EC 2.3 Acyltransferases
EC 2.3.1 Transferring groups other than amino-acyl groups
EC 2.3 1.1 amino-acid *N*-acetyltransferase
EC 2.3.1 2 imidazole *N*-acetyltransferase
EC 2.3.1.3 glucosamine *N*-acetyltransferase
EC 2.3.1.4 glucosamine 6-phosphate *N*-acetyltransferase
EC 2.3.1 5 arylamine *N*-acetyltransferase
EC 2.3.1.6 choline *O*-acetyltransferase
EC 2.3.1.7 carnitine *O*-acetyltransferase
EC 2.3.1.8 phosphate acetyltransferase
EC 2.3.1.9 acetyl-CoA *C*-acetyltransferase
EC 2.3.1.10 hydrogen-sulfide *S*-acetyltransferase
EC 2.3.1.11 thioethanolamine *S*-acetyltransferase
EC 2.3.1.12 dihydrolipoyllysine-residue acetyltransferase
EC 2.3.1.13 glycine *N*-acyltransferase
EC 2.3.1.14 glutamine *N*-phenylacetyltransferase
EC 2.3.1.15 glycerol-3-phosphate *O*-acyltransferase
EC 2.3.1.16 acetyl-CoA *C*-acyltransferase
EC 2.3.1.17 aspartate *N*-acetyltransferase
EC 2.3.1.18 galactoside *O*-acetyltransferase
EC 2.3.1.19 phosphate butyryltransferase
EC 2.3.1.20 diacylglycerol *O*-acyltransferase
EC 2.3.1.21 carnitine *O*-palmitoyltransferase
EC 2.3.1.22 2-acylglycerol *O*-acyltransferase
EC 2.3.1.23 1-acylglycerophosphocholine *O*-acyltransferase
EC 2.3.1.24 sphingosine *N*-acyltransferase
EC 2.3.1.25 plasmalogen synthase
EC 2.3.1.26 sterol *O*-acyltransferase
EC 2.3.1.27 cortisol *O*-acetyltransferase
EC 2.3.1.28 chloramphenicol *O*-acetyltransferase
EC 2.3.1.29 glycine *C*-acetyltransferase
EC 2.3.1.30 serine *O*-acetyltransferase
EC 2.3.1.31 homoserine *O*-acetyltransferase
EC 2.3.1.32 lysine *N*-acetyltransferase
EC 2.3.1.33 histidine *N*-acetyltransferase
EC 2.3.1.34 D-tryptophan *N*-acetyltransferase
EC 2.3.1.35 glutamate *N*-acetyltransferase
EC 2.3.1.36 D-amino-acid *N*-acetyltransferase
EC 2.3.1.37 5-aminolevulinate synthase
EC 2.3.1.38 [acyl-carrier-protein] *S*-acetyltransferase
EC 2.3.1 39 [acyl-carrier-protein] *S*-malonyltransferase
EC 2.3.1.40 acyl-[acyl-carrier-protein]—phospholipid *O*-acyltransferase
EC 2.3.1.41 3-oxoacyl-[acyl-carrier-protein] synthase
EC 2.3.1 42 glycerone-phosphate *O*-acyltransferase
EC 2.3.1.43 phosphatidylcholine—sterol *O*-acyltransferase
EC 2.3.1 44 *N*-acetylneuraminate 4-*O*-acetyltransferase
EC 2.3.1.45 *N*-acetylneuraminate 7-*O*(or 9-*O*)-acetyltransferase
EC 2.3.1.46 homoserine *O*-succinyltransferase
EC 2.3.1.47 8-amino-7-oxononanoate synthase
EC 2.3.1.48 histone acetyltransferase
EC 2.3.1.49 deacetyl-[citrate-(*pro*-3*S*)-lyase] *S* - acetyltransferase
EC 2.3.1.50 serine *C*-palmitoyltransferase
EC 2.3.1.51 1-acylglycerol-3-phosphate *O* - acyltransferase
EC 2.3.1.52 2-acylglycerol-3-phosphate *O* - acyltransferase
EC 2.3.1.53 phenylalanine *N*-acetyltransferase
EC 2.3.1.54 formate *C*-acetyltransferase
EC 2.3.1.55 now EC 2.3.1.82
EC 2.3.1.56 aromatic-hydroxylamine *O* - acetyltransferase
EC 2.3.1.57 diamine *N*-acetyltransferase
EC 2.3.1.58 2,3-diaminopropionate *N* - oxalyltransferase
EC 2 3.1.59 gentamicin 2'-*N*-acetyltransferase
EC 2.3.1.60 gentamicin 3'-*N*-acetyltransferase

EC 2.3.1.61 dihydrolipoyllysine-residue succinyltransferase
EC 2.3.1.62 2-acylglycerophosphocholine *O* - acyltransferase
EC 2.3 1.63 1-alkylglycerophosphocholine *O* - acyltransferase
EC 2.3.1.64 agmatine N^4-coumaroyltransferase
EC 2.3.1 65 glycine *N*-choloyltransferase
EC 2.3.1.66 leucine *N*-acetyltransferase
EC 2.3 1.67 1-alkylglycerophosphocholine *O* - acetyltransferase
EC 2 3 1.68 glutamine *N*-acyltransferase
EC 2.3.1.69 monoterpenol *O*-acetyltransferase
EC 2.3.1.70 CDP-acylglycerol *O* - arachidonoyltransferase
EC 2.3.1.71 glycine *N*-benzoyltransferase
EC 2.3.1.72 indoleacetylglucose—inositol *O* - acyltransferase
EC 2 3.1 73 diacylglycerol—sterol *O*-acyltransferase
EC 2 3.1.74 naringenin-chalcone synthase
EC 2.3.1.75 long-chain-alcohol *O* - fatty-acyltransferase
EC 2.3.1.76 retinol *O*-fatty-acyltransferase
EC 2 3.1.77 triacylglycerol—sterol *O*-acyltransferase
EC 2 3.1 78 heparan-a-glucosaminide *N* - acetyltransferase
EC 2.3.1.79 maltose *O*-acetyltransferase
EC 2.3.1 80 cysteine-*S*-conjugate *N* - acetyltransferase
EC 2 3.1.81 aminoglycoside $N^{3'}$-acetyltransferase
EC 2.3 1.82 aminoglycoside $N^{6'}$-acetyltransferase
EC 2 3.1 83 phosphatidylcholine—dolichol *O* - acyltransferase
EC 2.3 1.84 alcohol *O*-acetyltransferase
EC 2 3.1.85fatty-acid synthase
EC 2 3 1.86 fatty-acyl-CoA synthase
EC 2.3.1.87 aralkylamine *N*-acetyltransferase
EC 2 3.1.88 peptide a-*N*-acetyltransferase
EC 2.3.1.89 tetrahydrodipicolinate *N* - acetyltransferase
EC 2.3 1.90 b-glucogallin *O*-galloyltransferase
EC 2 3.1.91 sinapoylglucose—choline *O* - sinapoyltransferase
EC 2.3.1.92 sinapoylglucose—malate *O* - sinapoyltransferase
EC 2 3.1 93 13-hydroxylupinine *O*-tigloyltransferase
EC 2.3.1.94 erythronolide synthase
EC 2.3.1.95 trihydroxystilbene synthase
EC 2.3.1.96 glycoprotein *N*-palmitoyltransferase
EC 2.3.1.97 glycylpeptide *N* - tetradecanoyltransferase
EC 2.3.1.98 chlorogenate—glucarate *O* - hydroxycinnamoyltransferase
EC 2 3.1.99 quinate *O*-hydroxycinnamoyltransferase
EC 2.3.1.100 [myelin-proteolipid] *O* - palmitoyltransferase
EC 2.3 1.101 formylmethanofuran—tetrahydromethanopterin *N* -f ormyltransferase
EC 2.3.1.102 N^6-hydroxylysine *O*-acetyltransferase
EC 2.3.1.103 sinapoylglucose—sinapoylglucose *O* - sinapoyltransferase
EC 2 3.1 104 1-alkenylglycerophosphocholine *O* - acyltransferase
EC 2.3.1 105 alkylglycerophosphate 2-*O*-acetyltransferase
EC 2.3.1 106 tartronate *O* - hydroxycinnamoyltransferase
EC 2.3 1.107 17-*O*-deacetylvindoline *O* - acetyltransferase
EC 2.3 1 108 a-tubulin *N*-acetyltransferase
EC 2.3.1 109 arginine *N*-succinyltransferase
EC 2.3 1.110 tyramine *N*-feruloyltransferase
EC 2.3 1.111 mycocerosate synthase
EC 2 3.1 112 D-tryptophan *N*-malonyltransferase
EC 2.3 1.113 anthranilate *N*-malonyltransferase
EC 2.3 1.114 3,4-dichloroaniline *N* - malonyltransferase
EC 2.3 1.115 isoflavone-7-*O*-b-glucoside 6" - *O*-malonyltransferase
EC 2.3 1 116 flavonol-3-*O*-b-glucoside *O* - malonyltransferase
EC 2.3.1.117 2,3,4,5-tetrahydropyridine-2, 6-dicarboxylate *N*-succinyltransferase
EC 2 3.1.118 *N*-hydroxyarylamine *O* - acetyltransferase
EC 2.3.1.119 icosanoyl-CoA synthase
EC 2 3.1.120 deleted
EC 2.3.1.121 1-alkenylglycerophosphoethanolamine *O* - acyltransferase
EC 2.3 1.122 trehalose *O*-mycolyltransferase
EC 2.3 1.123 dolichol *O* - acyltransferase
EC 2.3.1.124 deleted
EC 2 3.1.125 1-alkyl-2-acetylglycerol *O* - acyltransferase
EC 2.3.1.126 isocitrate *O* - dihydroxycinnamoyltransferase

EC 2.3.1.127 ornithine *N*-benzoyltransferase
EC 2 3 1.128 ribosomal-protein-alanine *N*-acetyltransferase
EC 2.3.1.129 acyl-[acyl-carrier-protein]—UDP-*N*-acetylglucosamine *O*-acyltransferase
EC 2.3 1.130 galactarate *O* - hydroxycinnamoyltransferase
EC 2.3.1.131 glucarate *O* - hydroxycinnamoyltransferase
EC 2.3 1 132 glucarolactone *O* - hydroxycinnamoyltransferase
EC 2.3 1 133 shikimate *O* - hydroxycinnamoyltransferase
EC 2.3.1.134 galactolipid *O*-acyltransferase
EC 2.3.1.135 phosphatidylcholine—retinol *O* - acyltransferase
EC 2.3.1.136 polysialic-acid *O*-acetyltransferase
EC 2 3.1.137 carnitine *O*-octanoyltransferase
EC 2.3.1.138 putrescine *N* - hydroxycinnamoyltransferase
EC 2.3.1.139 ecdysone *O*-acyltransferase
EC 2.3.1 140 rosmarinate synthase
EC 2.3 1.141 galactosylacylglycerol *O* - acyltransferase
EC 2 3.1.142 glycoprotein *O*-fatty-acyltransferase
EC 2.3.1 143 b-glucogallin—tetrakisgalloylglucose *O*-galloyltransferase
EC 2 3.1.144 anthranilate *N*-benzoyltransferase
EC 2.3.1.145 piperidine *N*-piperoyltransferase
EC 2.3 1.146 pinosylvin synthase
EC 2.3.1.147 glycerophospholipid arachidonoyl-transferase (CoA-independent)
EC 2.3 1 148 glycerophospholipid acyltransferase (CoA-dependent)
EC 2 3.1.149 platelet-activating factor acetyltransferase
EC 2.3.1.150 salutaridinol 7-*O*-acetyltransferase
EC 2.3.1.151 benzophenone synthase
EC 2.3 1.152 alcohol *O*-cinnamoyltransferase
EC 2 3.1.153 anthocyanin 5-aromatic acyltransferase
EC 2.3 1.154 propionyl-CoA C^2-trimethyltridecanoyltransferase
EC 2 3 1 155 acetyl-CoA *C*-myristoyltransferase
EC 2 3.1.156 phloroisovalerophenone synthase
EC 2 3.1.157 glucosamine-1-phosphate *N*-acetyltransferase
EC 2.3.1.158 phospholipid:diacylglycerolacyltransferase
EC 2 3.1 159 acridone synthase
EC 2.3 1.160 vinorine synthase
EC 2.3.1.161 lovastatin nonaketide synthase
EC 2.3.1 162 taxadien-5a-ol *O*-acetyltransferase
EC 2.3.1 163 10-hydroxytaxane *O*-acetyltransferase
EC 2.3 1 164 isopenicillin-N *N*-acyltransferase
EC 2.3.1.165 6-methylsalicylic acid synthase
EC 2.3 1 166 2a-hydroxytaxane 2-*O*-benzoyltransferase
EC 2.3 1 167 10-deacetylbaccatin III 10-*O*-acetyltransferase
EC 2 3.1 168 dihydrolipoyllysine-residue (2-methylpropanoyl)transferase
EC 2.3.1.169 CO-methylating acetyl-CoA synthase
EC 2.3.2 Aminoacyltransferases
EC 2 3.2.1 D-glutamyltransferase
EC 2 3 2 2 g-glutamyltransferase
EC 2.3 2 3 lysyltransferase
EC 2 3.2.4 g-glutamylcyclotransferase
EC 2 3.2.5 glutaminyl-peptide cyclotransferase
EC 2 3.2 6 leucyltransferase
EC 2.3.2.7 aspartyltransferase
EC 2 3.2.8 arginyltransferase
EC 2 3 2.9 agaritine g-glutamyltransferase
EC 2.3 2 10 UDP-*N*-acetylmuramoylpentapeptide-lysine N^6-alanyltransferase
EC 2.3.2.11 alanylphosphatidylglycerol synthase
EC 2 3 2.12 peptidyltransferase
EC 2.3 2.13 protein-glutamine g-glutamyltransferase
EC 2 3 2 14 D-alanine g-glutamyltransferase
EC 2 3 2.15 glutathione g-glutamylcysteinyltransferase
EC 2.3.3 Acyl groups converted into alkyl on transfer
EC 2 3.3.1 citrate (*Si*)-synthase
EC 2 3.3.2 decylcitrate synthase
EC 2.3 3.3 citrate (*Re*)-synthase
EC 2 3.3.4 decylhomocitrate synthase
EC 2.3 3 5 2-methylcitrate synthase
EC 2.3.3.6 2-ethylmalate synthase
EC 2 3 3 7 3-ethylmalate synthase
EC 2.3.3.8 ATP citrate synthase
EC 2.3 3.9 malate synthase
EC 2.3 3.10 hydroxymethylglutaryl-CoA synthase
EC 2.3.3.11 2-hydroxyglutarate synthase
EC 2.3 3.12 3-propylmalate synthase
EC 2.3.3.13 2-isopropylmalate synthase
EC 2.3.3.14 homocitrate synthase
EC 2 3.3.15 sulfoacetaldehyde acetyltransferase

EC 2.4 Glycosyltransferases
EC 2.4.1 Hexosyltransferases
EC 2.4.1.1 phosphorylase
EC 2.4.1.2 dextrin dextranase
EC 2.4.1.3 deleted, included in EC 2.4.1.25
EC 2.4.1.4 amylosucrase
EC 2.4.1.5 dextransucrase
EC 2.4.1.6 deleted
EC 2.4.1.7 sucrose phosphorylase
EC 2.4.1.8 maltose phosphorylase
EC 2.4.1.9 inulosucrase
EC 2.4.1.10 levansucrase
EC 2.4.1.11 glycogen(starch) synthase
EC 2.4.1.12 cellulose synthase (UDP-forming)
EC 2.4.1.13 sucrose synthase
EC 2.4.1.14 sucrose-phosphate synthase
EC 2.4.1.15 a, a-trehalose-phosphate synthase (UDP-forming)
EC 2.4.1.16 chitin synthase
EC 2.4.1.17 glucuronosyltransferase
EC 2.4.1.18 1, 4-a-glucan branching enzyme
EC 2.4.1.19 cyclomaltodextrin glucanotransferase
EC 2.4.1.20 cellobiose phosphorylase
EC 2.4.1.21 starch synthase
EC 2.4.1.22 lactose synthase
EC 2.4.1.23 sphingosine b-galactosyltransferase
EC 2.4.1.24 1,4-a-glucan 6-a-glucosyltransferase
EC 2.4.1.25 4-a-glucanotransferase
EC 2.4.1.26 DNA a-glucosyltransferase
EC 2.4.1.27 DNA b-glucosyltransferase
EC 2.4.1.28 glucosyl-DNA b-glucosyltransferase
EC 2.4.1.29 cellulose synthase (GDP-forming)
EC 2.4.1.30 1, 3-b-oligoglucan phosphorylase
EC 2.4.1.31 laminaribiose phosphorylase
EC 2.4.1.32 glucomannan 4-b-mannosyltransferase
EC 2.4.1.33 alginate synthase
EC 2.4.1.34 1, 3-b-glucan synthase
EC 2.4.1.35 phenol b-glucosyltransferase
EC 2.4.1.36 a,a-trehalose-phosphate synthase (GDP-forming)
EC 2.4.1.37 fucosylgalactoside 3-a-galactosyltransferase
EC 2.4.1.38 b-*N*-acetylglucosaminylglycopeptide b-1, 4-galactosyltransferase
EC 2.4.1.39 steroid *N*-acetylglucosaminyltransferase
EC 2.4.1.40 glycoprotein-fucosylgalactoside a-*N*-acetylgalactosaminyltransferase
EC 2.4.1.41 polypeptide *N*-acetylgalactosaminyltransferase
EC 2.4.1.42 deleted, included in EC 2.4.1.17
EC 2.4.1.43 polygalacturonate 4-a-galacturonosyltransferase
EC 2.4.1.44 lipopolysaccharide 3-a-galactosyltransferase
EC 2.4.1.45 2-hydroxyacylsphingosine 1-b-galactosyltransferase
EC 2.4.1.46 1,2-diacylglycerol 3-b-galactosyltransferase
EC 2.4.1.47 *N*-acylsphingosine galactosyltransferase
EC 2.4.1.48 heteroglycan a-mannosyltransferase
EC 2.4.1.49 cellodextrin phosphorylase
EC 2.4.1.50 procollagen galactosyltransferase
EC 2.4.1.51 now covered by EC 2.4.1.101, EC 2.4.1.143, EC 2.4.1.144 and EC 2.4.1.145
EC 2.4.1.52 poly (glycerol-phosphate) a-glucosyltransferase
EC 2.4.1.53 poly (ribitol-phosphate) b-glucosyltransferase
EC 2.4.1.54 undecaprenyl-phosphate mannosyltransferase
EC 2.4.1.55 now EC 2.7.8.14
EC 2.4.1.56 lipopolysaccharide *N*-acetylglucosaminyltransferase
EC 2.4.1.57 phosphatidylinositol a-mannosyltransferase
EC 2.4.1.58 lipopolysaccharide glucosyltransferasel
EC 2.4.1.59 deleted, included in EC 2.4.1.17
EC 2.4.1.60 abequosyltransferase
EC 2.4.1.61 deleted, included in EC 2.4.1.17
EC 2.4.1.62 ganglioside galactosyltransferase
EC 2.4.1.63 linamarin synthase
EC 2.4.1.64 a,a-trehalose phosphorylase
EC 2.4.1.65 3-galactosyl-*N*-acetylglucosaminide 4-a-L-fucosyltransferase
EC 2.4.1.66 procollagen glucosyltransferase
EC 2.4.1.67 galactinol—raffinose galactosyltransferase
EC 2.4.1.68 glycoprotein 6-a-L-fucosyltransferase
EC 2.4.1.69 galactoside 2-a-L-fucosyltransferase
EC 2.4.1.70 poly(ribitol-phosphate) *N*-acetylglucosaminyl-transferase
EC 2.4.1.71 arylamine glucosyltransferase
EC 2.4.1.72 now EC 2.4.2.24

EC 2 4 1 73 lipopolysaccharide glucosyltransferase -II
EC 2 4 1.74 glycosaminoglycan galactosyltransferase
EC 2 4 1 75 UDP-galacturonosyltransferase
EC 2 4.1 76 deleted, included in EC 2.4.1.17
EC 2.4.1.77 deleted, included in EC 2.4.1 17
EC 2.4.1 78 phosphopolyprenol glucosyltransferase
EC 2.4.1.79 galactosylgalactosylglucosylceramide b-D-acetylgalactosaminyltransferase
EC 2.4.1.80 ceramide glucosyltransferase
EC 2 4 1.81 flavone 7-*O*-b-glucosyltransferase
EC 2 4 1 82 galactinol-sucrose galactosyltransferase
EC 2.4.1.83 dolichyl-phosphate b-D-mannosyltransferase
EC 2.4.1.84 deleted, included in EC 2.4.1.17
EC 2.4.1.85 cyanohydrin b-glucosyltransferase
EC 2.4.1.86 glucosaminylgalactosylglucosylceramide b-galactosyltransferase
EC 2.4.1.87 *N*-acetyllactosaminide 3-a-galactosyltransferase
EC 2 4.1 88 globoside a-*N*-acetylgalactosaminyltransferase
EC 2.4 1.89 deleted, included in EC 2 4.1 69
EC 2.4.1.90 *N*-acetyllactosamine synthase
EC 2.4 1 91 flavonol 3-*O*-glucosyltransferase
EC 2.4.1 92 (*N*-acetylneuraminyl)-galactosylglucosylceramide *N*-acetylgalactosaminyltransferase
EC 2.4 1.93 now EC 4.2.2.18
EC 2.4.1.94 protein *N*-acetylglucosaminyltransferase
EC 2.4.1 95 bilirubin-glucuronoside glucuronosyltransferase
EC 2.4.1.96 *sn*-glycerol-3-phosphate 1-galactosyltransferase
EC 2 4 1.97 1,3-b-D-glucan phosphorylase
EC 2.4.1.98 deleted, included in EC 2.4.1.90
EC 2.4.1.99 sucrose:sucrose fructosyltransferase
EC 2.4.1.100 2,1-fructan:2,1-fructan 1-fructosyltransferase
EC 2.4.1.101 a-1,3-mannosyl-glycoprotein 2-b-*N*-acetylglucosaminyltransferase
EC 2 4.1.102 b-1,3-galactosyl-*O*-glycosyl-glycoprotein b-1,6-*N*-acetylglucosaminyltransferase
EC 2.4.1.103 alizarin 2-b-glucosyltransferase
EC 2 4 1.104 *o*-dihydroxycoumarin 7-*O*-glucosyltransferase
EC 2.4.1 105 vitexin b-glucosyltransferase
EC 2 4 1 106 isovitexin b-glucosyltransferase
EC 2.4.1 107 deleted, included in EC 2.4.1 17
EC 2 4.1 108 deleted, included in EC 2.4.1.17
EC 2.4 1.109 dolichyl-phosphate-mannose-protein mannosyltransferase
EC 2 4 1.110 tRNA-queuosine b-mannosyltransferase
EC 2.4.1.111 coniferyl-alcohol glucosyltransferase
EC 2.4 1.112 a-1,4-glucan-protein synthase (UDP-forming)
EC 2 4.1 113 a-1,4-glucan-protein synthase (ADP-forming)
EC 2.4 1.114 2-coumarate *O*-b-glucosyltransferase
EC 2.4.1.115 anthocyanidin 3-*O*-glucosyltransferase
EC 2.4 1.116 cyanidin-3-rhamnosylglucoside 5-*O*-glucosyltransferase
EC 2 4.1 117 dolichyl-phosphate b-glucosyltransferase
EC 2 4 1 118 cytokinin 7-b-glucosyltransferase
EC 2.4.1.119 dolichyl-diphosphooligosaccharide-protein glycotransferase
EC 2 4 1.120 sinapate 1-glucosyltransferase
EC 2.4 1.121 indole-3-acetate b-glucosyltransferase
EC 2 4.1.122 glycoprotein-*N*-acetylgalactosamine 3-b-galactosyltransferase
EC 2.4.1.123 inositol 3-a-galactosyltransferase
EC 2.4 1.124 now included with EC 2.4.1.87
EC 2.4.1.125 sucrose—1,6-a-glucan 3(6)-a-glucosyltransferase
EC 2.4.1.126 hydroxycinnamate 4-b-glucosyltransferase
EC 2.4 1.127 monoterpenol b-glucosyltransferase
EC 2.4.1.128 scopoletin glucosyltransferase
EC 2.4.1.129 peptidoglycan glycosyltransferase
EC 2.4.1.130 dolichyl-phosphate-mannose-glycolipid a-mannosyltransferase
EC 2 4.1 131 glycolipid 2-a-mannosyltransferase
EC 2 4.1.132 glycolipid 3-a-mannosyltransferase
EC 2.4.1.133 xylosylprotein 4-b-galactosyltransferase
EC 2.4.1.134 galactosylxylosylprotein 3-b-galactosyltransferase
EC 2 4.1.135 galactosylgalactosylxylosylprotein 3-b-glucuronosyltransferase
EC 2.4.1.136 gallate 1-b-glucosyltransferase

EC 2.4.1.137 *sn*-glycerol-3-phosphate 2-a-galactosyltransferase
EC 2.4.1.138 mannotetraose 2-a-*N*-acetylglucosaminyltransferase
EC 2.4.1.139 maltose synthase
EC 2.4 1.140 alternansucrase
EC 2.4.1 141 *N*acetylglucosaminyldiphosphodolichol *N*-acetylglucosaminyltransferase
EC 2.4.1.142 chitobiosyldiphosphodolichol b-mannosyltransferase
EC 2 4.1.143 a-1,6-mannosyl-glycoprotein 2-b-*N*-acetylglucosaminyltransferase
EC 2.4.1.144 b-1,4-mannosyl-glycoprotein 4-b-*N*-acetylglucosaminyltransferase
EC 2.4.1.145 a-1,3-mannosyl-glycoprotein 4-b-*N*-acetylglucosaminyltransferase
EC 2.4.1.146 b-1,3-galactosyl-*O*-glycosyl-glycoprotein b-1,3-*N*-acetylglucosaminyltransferase
EC 2.4.1.147 acetylgalactosaminyl-*O*-glycosyl-glycoprotein b-1,3-*N*-acetylglucosaminyltransferase
EC 2.4.1.148 acetylgalactosaminyl-*O*-glycosyl-glycoprotein b-1,6-*N*-acetylglucosaminyltransferase
EC 2.4.1.149 *N*-acetyllactosaminide b-1,3-*N*-acetylglucosaminyltransferase
EC 2.4.1.150 *N*-acetyllactosaminide b-1,6-*N*-acetylglucosaminyl-transferase
EC 2.4.1.151 included with EC 2.4.1.87
EC 2.4.1.152 galactoside 3-fucosyltransferase
EC 2.4.1.153 dolichyl-phosphate a-*N*-acetylglucosaminyltransferase
EC 2.4.1.154 globotriosylceramide b-1, 6-*N*-acetylgalactosaminyl-transferase
EC 2.4.1.155 a-1,6-mannosyl-glycoprotein 6-b-*N*-acetylglucosaminyltransferase
EC 2.4.1.156 indolylacetyl-*myo*-inositol galactosyltransferase
EC 2.4.1.157 1,2-diacylglycerol 3-glucosyltransferase
EC 2.4.1.158 13-hydroxydocosanoate 13-b-glucosyltransferase
EC 2.4.1.159 flavonol-3-*O*-glucoside L-rhamnosyltransferase
EC 2.4.1.160 pyridoxine 5'-*O*-b-D-glucosyltransferase
EC 2.4.1.161 oligosaccharide 4-a-D-glucosyltransferase
EC 2.4.1.162 aldose b-D-fructosyltransferase
EC 2.4.1.163 b-galactosyl-*N*-acetylglucosaminylgalactosylglucosyl-ceramide b-1,3-acetylglucosaminyltransferase
EC 2.4.1.164 galactosyl-*N*-acetylglucosaminylgalactosylglucosyl-ceramide b-1, 6-*N*- acetylglucosaminyltransferase
EC 2 4.1.165 *N*-acetylneuraminylgalactosylglucosylceramide b-1,4-*N*-acetylgalactosaminyltransferase
EC 2.4.1.166 raffinose—raffinose a-galactotransferase
EC 2.4.1.167 sucrose 6^F-a-galactotransferase
EC 2.4.1.168 xyloglucan 4-glucosyltransferase
EC 2.4.1.169 now EC 2.4.2.39
EC 2.4.1.170 isoflavone 7-*O*-glucosyltransferase
EC 2.4.1.171 methyl-*ONN*-azoxymethanol b-D-glucosyltransferase
EC 2.4.1.172 salicyl-alcohol b-D-glucosyltransferase
EC 2.4.1.173 sterol 3b-glucosyltransferase
EC 2.4.1.174 glucuronylgalactosylproteoglycan 4-b-*N*-acetylgalactosaminyltransferase
EC 2.4.1.175 glucuronosyl-*N*-acetylgalactosaminyl-proteoglycan 4-b-*N*-acetylgalactosaminyltransferase
EC 2.4.1.176 gibberellin b-D-glucosyltransferase
EC 2.4.1.177 cinnamate b-D-glucosyltransferase
EC 2.4.1.178 hydroxymandelonitrile glucosyltransferase
EC 2.4.1.179 lactosylceramide b-1,3-galactosyltransferase
EC 2.4.1.180 lipopolysaccharide *N*-acetylmannosaminouronosyltransferase
EC 2.4.1.181 hydroxyanthraquinone glucosyltransferase
EC 2.4.1.182 lipid-A-disaccharide synthase
EC 2.4.1.183 a-1,3-glucan synthase
EC 2.4.1.184 galactolipid galactosyltransferase
EC 2.4.1.185 flavanone 7-*O*-b-glucosyltransferase
EC 2.4.1.186 glycogenin glucosyltransferase
EC 2.4.1.187 *N*-acetylglucosaminyldiphosphoundecaprenol *N*-acetyl-b-D-mannosaminyltransferase
EC 2.4.1.188 *N*-acetylglucosaminyldiphosphoundecaprenol glucosyltransferase
EC 2.4.1.189 luteolin 7-*O*-glucuronosyltransferase

EC 2.4.1.190 luteolin-7-*O*-glucuronide 2"-*O*-glucuronosyltransferase
EC 2.4.1.191 luteolin-7-*O*-diglucuronide 4'-*O*-glucuronosyltransferase
EC 2.4.1.192 nuatigenin 3b-glucosyltransferase
EC 2.4.1.193 sarsapogenin 3b-glucosyltransferase
EC 2.4.1.194 4-hydroxybenzoate 4-*O*-b-D-glucosyltransferase
EC 2.4.1.195 thiohydroximate b-D-glucosyltransferase
EC 2.4.1.196 nicotinate glucosyltransferase
EC 2.4.1.197 high-mannose-oligosaccharide b-1, 4-*N*-acetylglucosaminyltransferase
EC 2.4.1.198 phosphatidylinositol *N*-acetylglucosaminyltransferase
EC 2.4.1.199 b-mannosylphosphodecaprenol—mannooligosaccharide 6-mannosyltransferase
EC 2.4.1.200 now EC 4.2.2.17
EC 2.4.1.201 a-1,6-mannosyl-glycoprotein 4-b-*N*-acetylglucosaminyltransferase
EC 2.4.1.202 2,4-dihydroxy-7-methoxy-2*H*-1, 4-benzoxazin-3(4*H*)-one 2 -D-glucosyltransferase
EC 2.4.1.203 *trans*-zeatin *O*-b-D-glucosyltransferase
EC 2.4.1.204 now EC 2.4.2.40
EC 2.4.1.205 galactogen 6b-galactosyltransferase
EC 2.4.1.206 lactosylceramide 1,3-*N*-acetyl-b-D-glucosaminyltransferase
EC 2.4.1.207 xyloglucan:xyloglucosyl transferase
EC 2.4.1.208 diglucosyl diacylglycerol synthase
EC 2.4.1.209 *cis-p*-coumarate glucosyltransferase
EC 2.4.1.210 limonoid glucosyltransferase
EC 2.4.1.211 1,3-b-galactosyl-*N*-acetylhexosamine phosphorylase
EC 2.4.1.212 hyaluronan synthase
EC 2.4.1.213 glucosylglycerol-phosphate synthase
EC 2.4.1.214 glycoprotein 3-a-L-fucosyltransferase
EC 2.4.1.215 *cis*-zeatin *O*-b-D-glucosyltransferase
EC 2.4.1.216 trehalose 6-phosphate phosphorylase
EC 2.4.1.217 mannosyl-3-phosphoglycerate synthase
EC 2.4.1.218 hydroquinone glucosyltransferase
EC 2.4.1.219 vomilenine glucosyltransferase
EC 2.4.1.220 indoxyl-UDPG glucosyltransferase
EC 2.4.1.221 peptide-*O*-fucosyltransferase
EC 2.4.1.222 *O*-fucosylpeptide 3-b-*N*-acetylglucosaminyltransferase
EC 2.4.1.223 glucuronyl-galactosyl-proteoglycan 4-a-*N*-acetylglucosaminyltransferase
EC 2.4.1.224 glucuronosyl-*N*-acetylglucosaminyl-proteoglycan 4-a-*N*-acetylglucosaminyltransferase
EC 2.4.1.225 *N*-acetylglucosaminyl-proteoglycan 4-b-glucuronosyltransferase
EC 2.4.1.226 *N*-acetylgalactosaminyl-proteoglycan 3-b-glucuronosyltransferase
EC 2.4.1.227 undecaprenyldiphospho-muramoylpentapeptide b-*N*-acetylglucosaminyltransferase
EC 2.4.1.228 lactosylceramide 4-a-galactosyltransferase
EC 2.4.1.229 [Skp1-protein]-hydroxyproline *N*-acetylglucosaminyltransferase
EC 2.4.1.230 kojibiose phosphorylase
EC 2.4.1.231 a,a-trehalose phosphorylase (configuration-retaining)
EC 2.4.1.232 glycolipid 6-a-mannosyltransferase
EC 2.4 Glycosyltransferases
EC 2.4.2 Pentosyltransferases
EC 2.4.2.1 purine-nucleoside phosphorylase
EC 2.4.2.2 pyrimidine-nucleoside phosphorylase
EC 2.4.2.3 uridine phosphorylase
EC 2.4.2.4 thymidine phosphorylase
EC 2.4.2.5 nucleoside ribosyltransferase
EC 2.4.2.6 nucleoside deoxyribosyltransferase
EC 2.4.2.7 adenine phosphoribosyltransferase
EC 2.4.2.8 hypoxanthine phosphoribosyltransferase
EC 2.4.2.9 uracil phosphoribosyltransferase
EC 2.4.2.10 orotate phosphoribosyltransferase
EC 2.4.2.11 nicotinate phosphoribosyltransferase
EC 2.4.2.12 nicotinamide phosphoribosyltransferase
EC 2.4.2.13 now EC 2.5.1.6
EC 2.4.2.14 amidophosphoribosyltransferase
EC 2.4.2.15 guanosine phosphorylase
EC 2.4.2.16 urate-ribonucleotide phosphorylase
EC 2.4.2.17 ATP phosphoribosyltransferase
EC 2.4.2.18 anthranilate phosphoribosyltransferase
EC 2.4.2.19 nicotinate-nucleotide diphosphorylase (carboxylating)
EC 2.4.2.20 dioxotetrahydropyrimidine phosphoribosyltransferase
EC 2.4.2.21 nicotinate-nucleotide—dimethylbenzimidazole phosphoribosyltransferase
EC 2.4.2.22 xanthine phosphoribosyltransferase
EC 2.4.2.23 deoxyuridine phosphorylase

EC 2.4.2.24 1,4-b-D-xylan synthase
EC 2.4.2.25 flavone apiosyltransferase
EC 2.4 2.26 protein xylosyltransferase
EC 2.4.2.27 dTDP-dihydrostreptose—streptidine-6-phosphate dihydrostreptosyltransferase
EC 2.4 2 28 5'-methylthioadenosine phosphorylase
EC 2.4.2.29 queuine tRNA-ribosyltransferase
EC 2.4 2.30 NAD^+ ADP-ribosyltransferase
EC 2.4 2.31 $NAD(P)^+$—protein-arginine ADP-ribosyltransferase
EC 2.4.2.32 dolichyl-phosphate D-xylosyltransferase
EC 2 4.2.33 dolichyl-xylosyl-phosphate—protein xylosyltransferase
EC 2.4.2.34 indolylacetylinositol arabinosyltransferase
EC 2 4.2 35 flavonol-3-*O*-glycoside xylosyltransferase
EC 2 4.2.36 NAD^+—diphthamide ADP-ribosyltransferase
EC 2.4.2.37 NAD^+—dinitrogen-reductase ADP-D-ribosyltransferase
EC 2.4.2.38 glycoprotein 2-b-D-xylosyltransferase
EC 2.4.2.39 xyloglucan 6-xylosyltransferase
EC 2.4.2 40 zeatin *O*-b-D-xylosyltransferase
EC 2 4.99 Transferring Other Glycosyl Groups
EC 2.4.99 1 b-galactoside a-2,6-sialyltransferase
EC 2.4.99.2 monosialoganglioside sialyltransferase
EC 2 4.99 3 a-*N*-acetylgalactosaminide a-2,6-sialyltransferase
EC 2.4.99.4 b-galactoside a-2,3-sialyltransferase
EC 2.4.99.5 galactosyldiacylglycerol a-2,3-sialyltransferase
EC 2.4.99.6 *N*-acetyllactosaminide a-2,3-sialyltransferase
EC 2.4.99.7 (a-*N*-acetylneuraminyl-2,3-b-galactosyl-1,3)-*N*-acetyl-galactosaminide 6-a-sialyltransferase
EC 2.4.99.8 a-*N*-acetylneuraminate a-2,8-sialyltransferase
EC 2.4.99.9 lactosylceramide a-2,3-sialyltransferase
EC 2.4.99.10 neolactotetraosylceramide a-2,3-sialyltransferase
EC 2.4.99.11 lactosylceramide a-2,6-*N*-sialyltransferase
EC 2.5 Transferring Alkyl or Aryl Groups, Other than Methyl Groups
EC 2.5.1.1 dimethylallyl*trans*transferase
EC 2.5.1.2 thiamine pyridinylase
EC 2.5.1.3 thiamine-phosphate diphosphorylase
EC 2.5.1.4 adenosylmethionine cyclotransferase
EC 2.5.1.5 galactose-6-sulfurylase
EC 2.5.1.6 methionine adenosyltransferase
EC 2.5.1.7 UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase
EC 2.5.1.8 tRNA isopentenyltransferase
EC 2 5.1.9 riboflavin synthase
EC 2.5.1.10 geranyl*trans*transferase
EC 2.5.1 11 *trans*-octaprenyl*trans*transferase
EC 2.5.1.12 deleted, included in EC 2.5.1.18
EC 2.5.1.13 deleted, included in EC 2.5.1.18
EC 2 5.1.14 deleted, included in EC 2.5.1.18
EC 2 5.1.15 dihydropteroate synthase
EC 2.5.1.16 spermidine synthase
EC 2.5.1.17 cob(I)yrinic acid *a,c*-diamide adenosyltransferase
EC 2.5.1 18 glutathione transferase
EC 2 5.1.19 3-phosphoshikimate 1- carboxyvinyltransferase
EC 2.5.1.20 rubber *cis*-polyprenyl*cis*transferase
EC 2.5.1.21 farnesyl-diphosphate farnesyltransferase
EC 2.5.1.22 spermine synthase
EC 2.5 1.23 *sym*-norspermidine synthase
EC 2.5.1 24 discadenine synthase
EC 2.5.1.25 tRNA-uridine aminocarboxypropyltransferase
EC 2 5.1.26 alkylglycerone-phosphate synthase
EC 2.5.1.27 adenylate dimethylallyltransferase
EC 2.5.1.28 dimethylallyl*cis*transferase
EC 2.5.1.29 farnesyl*trans*transferase
EC 2.5.1.30 *trans*-hexaprenyl*trans*transferase
EC 2.5 1.31 *di-trans,poly-cis*-decaprenyl*cis*transferase
EC 2.5.1.32 geranylgeranyl-diphosphate geranylgeranyltransferase
EC 2.5.1.33 *trans*-pentaprenyl*trans*transferase
EC 2.5.1.34 tryptophan dimethylallyltransferase
EC 2.5.1.35 aspulvinone dimethylallyltransferase
EC 2.5.1 36 trihydroxypterocarpan dimethylallyltransferase
EC 2.5.1.37 now EC 4.4.1.20
EC 2.5.1.38 isonocardicin synthase
EC 2.5.1.39 4-hydroxybenzoate nonaprenyltransferase
EC 2.5.1.40 now EC 4.2.3.9

EC 2 5.1.41 phosphoglycerol geranylgeranyltransferase
EC 2.5.1.42 geranylgeranylglycerol-phosphate geranylgeranyltransferase
EC 2.5.1 43 nicotianamine synthase
EC 2.5.1.44 homospermidine synthase
EC 2.5.1.45 homospermidine synthase (spermidine-specific)
EC 2.5.1.46 deoxyhypusine synthase
EC 2.5.1 47 cysteine synthase
EC 2.5.1.48 cystathionine g-synthase
EC 2.5.1.49 *O*-acetylhomoserine aminocarboxypropyltransferase
EC 2 5.1.50 zeatin 9-aminocarboxyethyltransferase
EC 2.5.1.51 b-pyrazolylalanine synthase
EC 2.5.1.52 L-mimosine synthase
EC 2.5.1.53 uracilylalanine synthase
EC 2.5.1.54 3-deoxy-7-phosphoheptulonate synthase
EC 2.5.1.55 3-deoxy-8-phosphooctulonate synthase
EC 2.5.1.56 *N*-acetylneuraminate synthase
EC 2.5.1.57 *N*-acylneuraminate-9-phosphate synthase
EC 2.5.1.58 protein farnesyltransferase
EC 2.5.1.59 protein geranylgeranyltransferase type I
EC 2 5 1.60 protein geranylgeranyltransferase type II
EC 2.5.1.61 hydroxymethylbilane synthase
EC 2.5.1.62 chlorophyll synthase
EC 2.5.1.63 adenosyl-fluoride synthase
EC 2.5.1.64 2-succinyl-6-hydroxycyclohexa-2,4-diene-1-carboxylate synthase
EC 2.6 Transferring Nitrogenous Groups
EC 2.6.1 Transaminases
EC 2.6.1.1 aspartate transaminase
EC 2.6.1.2 alanine transaminase
EC 2.6.1.3 cysteine transaminase
EC 2.6 1.4 glycine transaminase
EC 2.6.1.5 tyrosine transaminase
EC 2.6.1.6 leucine transaminase
EC 2.6.1.7 kynurenine—oxoglutarate transaminase
EC 2.6.1.8 2,5-diaminovalerate transaminase
EC 2.6.1.9 histidinol-phosphate transaminase
EC 2.6.1.10 deleted, included in EC 2.6.1.21
EC 2.6.1.11 acetylornithine transaminase
EC 2.6.1.12 alanine—oxo-acid transaminase
EC 2.6.1.13 ornithine—oxo-acid transaminase
EC 2.6.1.14 asparagine—oxo-acid transaminase
EC 2.6.1.15 glutamine—pyruvate transaminase
EC 2.6 1.16 glutamine—fructose-6-phosphate transaminase (isomerizing)
EC 2.6.1.17 succinyldiaminopimelate transaminase
EC 2 6 1 18 b-alanine—pyruvate transaminase
EC 2.6 1.19 4-aminobutyrate transaminase
EC 2 6.1 20 deleted
EC 2 6.1 21 D-alanine transaminase
EC 2 6 1 22 (*S*)-3-amino-2-methylpropionate transaminase
EC 2.6 1.23 4-hydroxyglutamate transaminase
EC 2.6.1.24 diiodotyrosine transaminase
EC 2.6.1 25 deleted, included in EC 2.6 1.24
EC 2.6 1.26 thyroid-hormone transaminase
EC 2.6.1.27 tryptophan transaminase
EC 2.6.1 28 tryptophan—phenylpyruvate transaminase
EC 2.6.1 29 diamine transaminase
EC 2.6.1.30 pyridoxamine—pyruvate transaminase
EC 2.6.1.31 pyridoxamine—oxaloacetate transaminase
EC 2.6.1.32 valine—3-methyl-2-oxovalerate transaminase
EC 2.6.1.33 dTDP-4-amino-4,6-dideoxy-D-glucose transaminase
EC 2.6.1.34 UDP-2-acetamido-4-amino-2,4,6-trideoxyglucose transaminase
EC 2.6.1.35 glycine—oxaloacetate transaminase
EC 2.6.1.36 L-lysine 6-transaminase
EC 2 6.1 37 (2-aminoethyl)phosphonate—pyruvate transaminase
EC 2.6.1.38 histidine transaminase
EC 2.6 1.39 2-aminoadipate transaminase
EC 2.6 1.40 (*R*)-3-amino-2-methylpropionate—pyruvate transaminase
EC 2.6.1 41 D-methionine—pyruvate transaminase
EC 2 6 1 42 branched-chain-amino-acid transaminase
EC 2.6.1.43 aminolevulinate transaminase
EC 2 6.1.44 alanine—glyoxylate transaminase
EC 2.6.1.45 serine—glyoxylate transaminase
EC 2.6.1.46 diaminobutyrate—pyruvate transaminase
EC 2.6 1 47 alanine—oxomalonate transaminase
EC 2.6.1 48 5-aminovalerate transaminase
EC 2.6.1.49 dihydroxyphenylalanine transaminase
EC 2.6.1.50 glutamine—*scyllo*-inositol transaminase

EC 2.6.1.51 serine—pyruvate transaminase
EC 2.6.1.52 phosphoserine transaminase
EC 2.6.1.53 now EC 1.4.1.13
EC 2.6.1.54 pyridoxamine-phosphate transaminase
EC 2.6.1.55 taurine—2-oxoglutarate transaminase
EC 2.6.1.56 1D-1-guanidino-3-amino-1, 3-dideoxy-*scyllo*-inositol transaminase
EC 2.6.1.57 aromatic-amino-acid transaminase
EC 2.6.1.58 phenylalanine(histidine) transaminase
EC 2.6.1.59 dTDP-4-amino-4,6-dideoxygalactose transaminase
EC 2.6.1.60 aromatic-amino-acid—glyoxylate transaminase
EC 2.6.1.61 identical to EC 2.6.1.40
EC 2.6.1.62 adenosylmethionine—8-amino-7-oxononanoate transaminase
EC 2.6.1.63 kynurenine—glyoxylate transaminase
EC 2.6.1.64 glutamine—phenylpyruvate transaminase
EC 2.6.1.65 N^6-acetyl-b-lysine transaminase
EC 2.6.1.66 valine—pyruvate transaminase
EC 2.6.1.67 2-aminohexanoate transaminase
EC 2.6.1.68 ornithine(lysine) transaminase
EC 2.6.1.69 now EC 2.6.1.11
EC 2.6 1.70 aspartate—phenylpyruvate transaminase
EC 2.6.1.71 lysine—pyruvate 6-transaminase
EC 2.6.1 72 D-4-hydroxyphenylglycine transaminase
EC 2.6.1.73 methionine—glyoxylate transaminase
EC 2.6.1.74 cephalosporin-C transaminase
EC 2.6.1.75 cysteine-conjugate transaminase
EC 2.6.1.76 diaminobutyrate—2-oxoglutarate transaminase
EC 2.6.1.77 taurine—pyruvate aminotransferase
EC 2.6.2 Amidinotransferases
EC 2.6.2.1 now EC 2.1.4.1
EC 2.6.3 Oximinotransferases
EC 2.6.3.1 oximinotransferase
2.6.99 Transferring Other Nitrogenous Groups
EC 2.6.99.1 dATP(dGTP)—DNA purinetransferase
EC 2.7 Transferring Phosphorus-Containing Groups
EC 2.7.1 Phosphotransferases with an Alcohol Group as Acceptor
EC 2.7.1.1 hexokinase
EC 2.7.1.2 glucokinase
EC 2.7.1.3 ketohexokinase
EC 2.7.1.4 fructokinase
EC 2.7.1.5 rhamnulokinase
EC 2.7.1.6 galactokinase
EC 2.7.1.7 mannokinase
EC 2.7.1.8 glucosamine kinase
EC 2.7.1.9 deleted
EC 2.7.1.10 phosphoglucokinase
EC 2.7.1.11 6-phosphofructokinase
EC 2.7.1.12 gluconokinase
EC 2.7.1.13 dehydrogluconokinase
EC 2.7.1.14 sedoheptulokinase
EC 2.7.1.15 ribokinase
EC 2.7.1.16 ribulokinase
EC 2.7.1.17 xylulokinase
EC 2.7.1.18 phosphoribokinase
EC 2.7.1.19 phosphoribulokinase
EC 2.7.1.20 adenosine kinase
EC 2.7.1.21 thymidine kinase
EC 2.7.1.22 ribosylnicotinamide kinase
EC 2.7.1.23 NAD^+ kinase
EC 2.7.1.24 dephospho-CoA kinase
EC 2.7.1.25 adenylyl-sulfate kinase
EC 2.7.1.26 riboflavin kinase
EC 2.7.1.27 erythritol kinase
EC 2.7.1.28 triokinase
EC 2.7.1.29 glycerone kinase
EC 2.7.1.30 glycerol kinase
EC 2.7.1.31 glycerate kinase
EC 2.7.1.32 choline kinase
EC 2.7.1.33 pantothenate kinase
EC 2.7.1.34 pantetheine kinase
EC 2.7.1.35 pyridoxal kinase
EC 2.7.1.36 mevalonate kinase
EC 2.7.1.37 protein kinase
EC 2.7.1.38 phosphorylase kinase
EC 2.7.1.39 homoserine kinase
EC 2.7.1.40 pyruvate kinase
EC 2.7.1.41 glucose-1-phosphate phosphodismutase
EC 2.7.1.42 riboflavin phosphotransferase
EC 2.7.1.43 glucuronokinase
EC 2.7.1.44 galacturonokinase
EC 2.7.1.45 2-dehydro-3-deoxygluconokinase
EC 2.7.1.46 L-arabinokinase
EC 2.7.1.47 D-ribulokinase
EC 2.7.1.48 uridine kinase
EC 2.7.1.49 hydroxymethylpyrimidine kinase
EC 2.7.1.50 hydroxyethylthiazole kinase
EC 2.7.1.51 L-fuculokinase

EC 2.7.1.52 fucokinase
EC 2.7.1.53 L-xylulokinase
EC 2.7.1.54 D-arabinokinase
EC 2.7.1.55 allose kinase
EC 2.7.1.56 1-phosphofructokinase
EC 2.7.1.57 deleted
EC 2.7.1.58 2-dehydro-3-deoxygalactonokinase
EC 2.7.1.59 *N*-acetylglucosamine kinase
EC 2.7.1.60 *N*-acylmannosamine kinase
EC 2.7.1.61 acyl-phosphate—hexose phosphotransferase
EC 2.7.1.62 phosphoramidate—hexose phosphotransferase
EC 2.7.1.63 polyphosphate—glucose phosphotransferase
EC 2.7.1.64 inositol 3-kinase
EC 2.7.1.65 *scyllo*-inosamine 4-kinase
EC 2.7.1.66 undecaprenol kinase
EC 2.7.1.67 1-phosphatidylinositol 4-kinase
EC 2.7.1.68 1-phosphatidylinositol-4-phosphate 5-kinase
EC 2.7.1.69 protein—N^P-phosphohistidine-sugar phosphotransferase
EC 2.7.1.70 identical to EC 2.7.1.37.
EC 2.7.1.71 shikimate kinase
EC 2.7.1.72 streptomycin 6-kinase
EC 2.7.1.73 inosine kinase
EC 2.7.1.74 deoxycytidine kinase
EC 2.7.1.75 now EC 2.7.1.21
EC 2.7.1.76 deoxyadenosine kinase
EC 2.7.1.77 nucleoside phosphotransferase
EC 2.7.1.78 polynucleotide 5'-hydroxyl-kinase
EC 2.7.1.79 diphosphate—glycerol phosphotransferase
EC 2.7.1.80 diphosphate—serine phosphotransferase
EC 2.7.1.81 hydroxylysine kinase
EC 2.7.1.82 ethanolamine kinase
EC 2.7.1.83 pseudouridine kinase
EC 2.7.1.84 alkylglycerone kinase
EC 2.7.1.85 b-glucoside kinase
EC 2.7.1.86 NADH kinase
EC 2.7.1.87 streptomycin 3"-kinase
EC 2.7.1.88 dihydrostreptomycin-6-phosphate 3'a-kinase
EC 2.7.1.89 thiamine kinase
EC 2.7.1.90 diphosphate—fructose-6-phosphate 1-phosphotransferase
EC 2.7.1.91 sphinganine kinase
EC 2.7.1.92 5-dehydro-2-deoxygluconokinase
EC 2.7.1.93 alkylglycerol kinase
EC 2.7.1.94 acylglycerol kinase
EC 2.7.1.95 kanamycin kinase
EC 2.7.1.96 deleted, included in EC 2.7.1.86
EC 2.7.1.97 deleted, identical to EC 2.7.1.125
EC 2.7.1.98 deleted
EC 2.7.1.99 [pyruvate dehydrogenase (lipoamide)] kinase
EC 2.7.1.100 *S*-methyl-5-thioribose kinase
EC 2.7.1.101 tagatose kinase
EC 2.7.1.102 hamamelose kinase
EC 2.7.1.103 viomycin kinase
EC 2.7.1.104 diphosphate—protein phosphotransferase
EC 2.7.1.105 6-phosphofructo-2-kinase
EC 2.7.1.106 glucose-1,6-bisphosphate synthase
EC 2.7.1.107 diacylglycerol kinase
EC 2.7.1.108 dolichol kinase
EC 2.7.1.109 [hydroxymethylglutaryl-CoA reductase (NADPH)] kinase
EC 2.7.1.110 dephospho-[reductase kinase] kinase
EC 2.7.1.111 now EC 2.7.1.128
EC 2.7.1.112 protein-tyrosine kinase
EC 2.7.1.113 deoxyguanosine kinase
EC 2.7.1.114 AMP—thymidine kinase
EC 2.7.1.115 [3-methyl-2-oxobutanoate dehydrogenase (lipoamide)] kinase
EC 2.7.1.116 [isocitrate dehydrogenase ($NADP^+$)] kinase
EC 2.7.1.117 [myosin-light-chain] kinase
EC 2.7.1.118 ADP—thymidine kinase
EC 2.7.1.119 hygromycin-B kinase
EC 2.7.1.120 caldesmon kinase
EC 2.7.1.121 phospho*enol*pyruvate—glycerone phosphotransferase
EC 2.7.1.122 xylitol kinase
EC 2.7.1.123 Ca^{2+}/calmodulin-dependent protein kinase
EC 2.7.1.124 [tyrosine 3-monooxygenase] kinase
EC 2.7.1.125 rhodopsin kinase
EC 2.7.1.126 [b-adrenergic-receptor] kinase
EC 2.7.1.127 inositol-trisphosphate 3-kinase
EC 2.7.1.128 [acetyl-CoA carboxylase] kinase
EC 2.7.1.129 [myosin-heavy-chain] kinase
EC 2.7.1.130 tetraacyldisaccharide 4'-kinase
EC 2.7.1.131 [low-density-lipoprotein] kinase

EC 2.7.1 132 tropomyosin kinase
EC 2.7.1.133 now with EC 2.7.1.134
EC 2.7.1.134 inositol-tetrakisphosphate 1-kinase
EC 2 7.1.135 [*tau*-protein] kinase
EC 2.7.1.136 macrolide 2'-kinase
EC 2.7 1 137 phosphatidylinositol 3-kinase
EC 2.7 1.138 ceramide kinase
EC 2 7.1.139 now with EC 2 7.1.134
EC 2 7.1 140 inositol-tetrakisphosphate 5-kinase
EC 2.7 1 141 [RNA-polymerase]-subunit kinase
EC 2.7.1.142 glycerol—3-phosphate-glucose phosphotransferase
EC 2 7.1.143 diphosphate-purine nucleoside kinase
EC 2 7.1.144 tagatose-6-phosphate kinase
EC 2 7.1 145 deoxynucleoside kinase
EC 2 7 1.146 ADP-dependent phosphofructokinase
EC 2 7.1.147 ADP-dependent glucokinase
EC 2.7.1.148 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase
EC 2 7.1.149 1-phosphatidylinositol-5-phosphate 4-kinase
EC 2.7.1.150 1-phosphatidylinositol-3-phosphate 5-kinase
EC 2.7.1.151 inositol-polyphosphate multikinase
EC 2 7 1 152 now EC 2.7.4 21
EC 2.7 1 153 phosphatidylinositol-4,5-bisphosphate 3-kinase
EC 2 7 1.154 phosphatidylinositol-4-phosphate 3-kinase
EC 2.7.1.155 diphosphoinositol-pentakisphosphate kinase
EC 2.7.1 156 adenosylcobinamide kinase
EC 2.7.2 Phosphotransferases with a carboxy group as acceptor
EC 2.7.2.1 acetate kinase
EC 2.7.2.2 carbamate kinase
EC 2.7 2 3 phosphoglycerate kinase
EC 2 7.2.4 aspartate kinase
EC 2.7.2.5 now EC 6.3.4.16
EC 2 7 2.6 formate kinase
EC 2 7 2 7 butyrate kinase
EC 2.7 2.8 acetylglutamate kinase
EC 2 7.2.9 now EC 6.3.5.5
EC 2.7.2.10 phosphoglycerate kinase (GTP)
EC 2 7.2.11 glutamate 5-kinase
EC 2.7.2.12 acetate kinase (diphosphate)
EC 2.7 2.13 glutamate 1-kinase
EC 2.7.2.14 branched-chain-fatty-acid kinase
EC 2.7.3 Phosphotransferases with a nitrogenous group as acceptor
EC 2.7 3.1 guanidinoacetate kinase
EC 2 7.3.2 creatine kinase
EC 2 7.3 3 arginine kinase
EC 2.7.3 4 taurocyamine kinase
EC 2 7.3.5 lombricine kinase
EC 2 7.3 6 hypotaurocyamine kinase
EC 2.7.3 7 opheline kinase
EC 2.7.3.8 ammonia kinase
EC 2 7.3.9 phospho*enol*pyruvate—protein phosphotransferase
EC 2.7.3.10 agmatine kinase
EC 2.7.3.11 protein-histidine *pros*-kinase
EC 2.7 3.12 protein-histidine *tele*-kinase
EC 2.7 4 Phosphotransferases with a phosphate group as acceptor
EC 2 7.4 1 polyphosphate kinase
EC 2.7.4 2 phosphomevalonate kinase
EC 2.7 4.3 adenylate kinase
EC 2 7.4 4 nucleoside-phosphate kinase
EC 2.7.4.5 deleted, included in EC 2.7.4.14
EC 2.7.4 6 nucleoside-diphosphate kinase
EC 2.7.4.7 phosphomethylpyrimidine kinase
EC 2 7 4.8 guanylate kinase
EC 2 7 4.9 dTMP kinase
EC 2.7.4.10 nucleoside-triphosphate—adenylate kinase
EC 2 7 4.11 (deoxy)adenylate kinase
EC 2.7.4 12 T_2-induced deoxynucleotide kinase
EC 2.7.4.13 (deoxy)nucleoside-phosphate kinase
EC 2.7.4.14 cytidylate kinase
EC 2.7.4.15 thiamine-diphosphate kinase
EC 2.7.4.16 thiamine-phosphate kinase
EC 2.7.4 17 3-phosphoglyceroyl-phosphate—polyphosphate phosphotransferase
EC 2.7.4.18 farnesyl-diphosphate kinase
EC 2 7.4 19 5-methyldeoxycytidine-5'-phosphate kinase
EC 2.7.4 20 dolichyl-diphosphate—polyphosphate phosphotransferase
EC 2.7.4 21 inositol-hexakisphosphate kinase
EC 2 7.5 Phosphotransferases with regeneration of donors, apparently catalysing intramolecular transfers
EC 2 7 5.1 now EC 5 4.2.2
EC 2.7 5.2 now EC 5.4.2.3
EC 2.7.5.3 now EC 5.4 2.1

EC 2.7.5.4 now EC 5.4.2.4
EC 2.7.5.5 now EC 5 4.2.5
EC 2.7 5.6 now EC 5.4.2 7
EC 2.7.5.7 now EC 5.4 2 8
EC 2.7 6 Diphosphotransferases
EC 2.7.6 1 ribose-phosphate diphosphokinase
EC 2.7.6.2 thiamine diphosphokinase
EC 2.7.6 3 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase
EC 2.7 6.4 nucleotide diphosphokinase
EC 2.7 6.5 GTP diphosphokinase
EC 2 7.7 Nucleotidyltransferases
EC 2.7.7.1 nicotinamide-nucleotide adenylyltransferase
EC 2 7.7.2 FMN adenylyltransferase
EC 2.7.7 3 pantetheine-phosphate adenylyltransferase
EC 2.7 7.4 sulfate adenylyltransferase
EC 2.7 7.5 sulfate adenylyltransferase (ADP)
EC 2 7 7.6 DNA-directed RNA polymerase
EC 2 7 7.7 DNA-directed DNA polymerase
EC 2.7.7.8 polyribonucleotide nucleotidyltransferase
EC 2 7 7.9 UTP—glucose-1-phosphate uridylyltransferase
EC 2.7 7.10 UTP—hexose-1-phosphate uridylyltransferase
EC 2.7 7.11 UTP—xylose-1-phosphate uridylyltransferase
EC 2.7 7.12 UDP-glucose—hexose-1-phosphate uridylyltransferase
EC 2.7.7.13 mannose-1-phosphate guanylyltransferase
EC 2.7 7 14 ethanolamine-phosphate cytidylyltransferase
EC 2.7 7.15 choline-phosphate cytidylyltransferase
EC 2.7.7.16 now EC 3.1.27.5
EC 2.7.7.17 now EC 3.1.27.1
EC 2.7.7.18 nicotinate-nucleotide adenylyltransferase
EC 2.7.7.19 polynucleotide adenylyltransferase
EC 2 7.7.20 deleted
EC 2 7.7 21 tRNA cytidylyltransferase
EC 2.7.7.22 mannose-1-phosphate guanylyltransferase (GDP)
EC 2.7 7 23 UDP-*N*-acetylglucosamine diphosphorylase
EC 2.7.7.24 glucose-1-phosphate thymidylyltransferase
EC 2.7.7.25 tRNA adenylyltransferase
EC 2.7.7 26 now EC 3.1.27 3
EC 2 7.7 27 glucose-1-phosphate adenylyltransferase
EC 2.7.7.28 nucleoside-triphosphate-hexose-1-phosphate nucleotidyltransferase
EC 2.7.7.29 identical to EC 2.7 7.28
EC 2.7.7.30 fucose-1-phosphate guanylyltransferase
EC 2.7 7 31 DNA nucleotidylexotransferase
EC 2.7.7 32 galactose-1-phosphate thymidylyltransferase
EC 2 7.7.33 glucose-1-phosphate cytidylyltransferase
EC 2 7.7 34 glucose-1-phosphate guanylyltransferase
EC 2.7.7 35 ribose-5-phosphate adenylyltransferase
EC 2.7.7.36 aldose-1-phosphate adenylyltransferase
EC 2.7 7.37 aldose-1-phosphate nucleotidyltransferase
EC 2.7.7.38 3-deoxy-*manno*-octulosonate cytidylyltransferase
EC 2.7.7 39 glycerol-3-phosphate cytidylyltransferase
EC 2.7.7.40 D-ribitol-5-phosphate cytidylyltransferase
EC 2.7.7 41 phosphatidate cytidylyltransferase
EC 2.7.7.42 [glutamate—ammonia-ligase] adenylyltransferase
EC 2.7.7.43 *N*-acylneuraminate cytidylyltransferase
EC 2.7 7.44 glucuronate-1-phosphate uridylyltransferase
EC 2.7.7.45 guanosine-triphosphate guanylyltransferase
EC 2.7.7.46 gentamicin 2"-nucleotidyltransferase
EC 2.7.7 47 streptomycin 3"-adenylyltransferase
EC 2.7.7 48 RNA-directed RNA polymerase
EC 2.7.7 49 RNA-directed DNA polymerase
EC 2.7.7.50 mRNA guanylyltransferase
EC 2 7.7.51 adenylylsulfate—ammonia adenylyltransferase
EC 2.7.7.52 RNA uridylyltransferase
EC 2.7.7.53 ATP adenylyltransferase
EC 2.7 7.54 phenylalanine adenylyltransferase
EC 2.7.7.55 anthranilate adenylyltransferase
EC 2.7.7.56 tRNA nucleotidyltransferase
EC 2.7.7 57 *N*-methylphosphoethanolamine cytidylyltransferase
EC 2.7.7.58 (2,3-dihydroxybenzoyl)adenylate synthase

EC 2.7.7.59 [protein-PII] uridylyltransferase
EC 2.7.7.60 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase
EC 2.7.7.61 holo-ACP synthase
EC 2.7.7.62 adenosylcobinamide-phosphate guanylyltransferase
EC 2.7.8 Transferases for other substituted phosphate groups
EC 2.7.8.1 ethanolaminephosphotransferase
EC 2.7.8.2 diacylglycerol cholinephosphotransferase
EC 2.7.8.3 ceramide cholinephosphotransferase
EC 2.7.8.4 serine-phosphoethanolamine synthase
EC 2.7.8.5 CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase
EC 2.7.8.6 undecaprenyl-phosphate galactose phosphotransferase
EC 2.7.8.7 holo-[acyl-carrier-protein] synthase
EC 2.7.8.8 CDP-diacylglycerol — serine *O*-phosphatidyltransferase
EC 2.7.8.9 phosphomannan mannosephosphotransferase
EC 2.7.8.10 sphingosine cholinephosphotransferase
EC 2.7.8.11 CDP-diacylglycerol—inositol 3-phosphatidyltransferase
EC 2.7.8.12 CDP-glycerol glycerophosphotransferase
EC 2.7.8.13 phospho-*N*-acetylmuramoyl-pentapeptide-transferase
EC 2.7.8.14 CDP-ribitol ribitolphosphotransferase
EC 2.7.8.15 UDP - *N* - acetylglucosamine — dolichyl-phosphate *N*-acetylglucosaminephospho transferase
EC 2.7.8.16 deleted, included in EC 2.7.8.2
EC 2.7.8.17 UDP-*N*-acetylglucosamine — lysosomal-enzyme - *N* - acetylglucosaminephosphotransferase
EC 2.7.8.18 UDP-galactose—UDP-*N*-acetylglucosamine galactose phosphotransferase
EC 2.7.8.19 UDP-glucose—glycoprotein glucose phosphotransferase
EC 2.7.8.20 phosphatidylglycerol—membrane-oligosaccharide glycerophosphotransferase
EC 2.7.8.21 membrane-oligosaccharide glycerophosphotransferase
EC 2.7.8.22 1-alkenyl-2-acylglycerol choline phosphotransferase
EC 2.7.8.23 carboxyvinyl-carboxyphosphonate phosphorylmutase
EC 2.7.8.24 phosphatidylcholine synthase
EC 2.7.8.25 triphosphoribosyl-dephospho-CoA synthase
EC 2.7.8.26 adenosylcobinamide-GDP ribazoletransferase
EC 2.7.9 Phosphotransferases with paired acceptors
EC 2.7.9.1 pyruvate, phosphate dikinase
EC 2.7.9.2 pyruvate, water dikinase
EC 2.7.9.3 selenide, water dikinase
EC 2.7.9.4 a-glucan, water dikinase
EC 2.8 Transferring Sulfur-Containing Groups
EC 2.8.1 Sulfurtransferases
EC 2.8.1.1 thiosulfate sulfurtransferase
EC 2.8.1.2 3-mercaptopyruvate sulfurtransferase
EC 2.8.1.3 thiosulfate—thiol sulfurtransferase
EC 2.8.1.4 tRNA sulfurtransferase
EC 2.8.1.5 thiosulfate—dithiol sulfurtransferase
EC 2.8.1.6 biotin synthase
EC 2.8.1.7 cysteine desulfurase
EC 2.8.2 Sulfotransferases
EC 2.8.2.1 aryl sulfotransferase
EC 2.8.2.2 alcohol sulfotransferase
EC 2.8.2.3 amine sulfotransferase
EC 2.8.2.4 estrone sulfotransferase
EC 2.8.2.5 chondroitin 4-sulfotransferase
EC 2.8.2.6 choline sulfotransferase
EC 2.8.2.7 UDP-*N*-acetylgalactosamine-4-sulfate sulfotransferase
EC 2.8.2.8 [heparan sulfate]-glucosamine *N*-sulfotransferase
EC 2.8.2.9 tyrosine-ester sulfotransferase
EC 2.8.2.10 *Renilla*-luciferin sulfotransferase
EC 2.8.2.11 galactosylceramide sulfotransferase
EC 2.8.2.12 deleted, identical to EC 2.8.2.8
EC 2.8.2.13 psychosine sulfotransferase
EC 2.8.2.14 bile-salt sulfotransferase
EC 2.8.2.15 steroid sulfotransferase
EC 2.8.2.16 thiol sulfotransferase
EC 2.8.2.17 chondroitin 6-sulfotransferase
EC 2.8.2.18 cortisol sulfotransferase
EC 2.8.2.19 triglucosylalkylacylglycerol sulfotransferase
EC 2.8.2.20 protein-tyrosine sulfotransferase
EC 2.8.2.21 keratan sulfotransferase
EC 2.8.2.22 arylsulfate sulfotransferase

EC 2.8.2.23 [heparan sulfate]-glucosamine 3-sulfotransferase 1
EC 2.8.2.24 desulfoglucosinolate sulfotransferase
EC 2.8.2.25 flavonol 3-sulfotransferase
EC 2.8.2.26 quercetin-3-sulfate 3'-sulfotransferase
EC 2.8.2.27 quercetin-3-sulfate 4'-sulfotransferase
EC 2.8.2.28 quercetin-3,3'-bissulfate 7-sulfotransferase
EC 2.8.2.29 [heparan sulfate]-glucosamine 3-sulfotransferase 2
EC 2.8.2.30 [heparan sulfate]-glucosamine 3-sulfotransferase 3
EC 2.8.3 CoA-transferases
EC 2.8.3.1 propionate CoA-transferase
EC 2.8.3.2 oxalate CoA-transferase
EC 2.8.3.3 malonate CoA-transferase
EC 2.8.3.4 deleted
EC 2.8.3.5 3-oxoacid CoA-transferase
EC 2.8.3.6 3-oxoadipate CoA-transferase
EC 2.8.3.7 succinate—citramalate CoA-transferase
EC 2.8.3.8 acetate CoA-transferase
EC 2.8.3.9 butyrate—acetoacetate CoA-transferase
EC 2.8.3.10 citrate CoA-transferase
EC 2.8.3.11 citramalate CoA-transferase
EC 2.8.3.12 glutaconate CoA-transferase
EC 2.8.3.13 succinate—hydroxymethylglutarate CoA-transferase
EC 2.8.3.14 5-hydroxypentanoate CoA-transferase
EC 2.8.3.15 succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase
EC 2.8.3.16 formyl-CoA transferase
EC 2.8.3.17 cinnamoyl-CoA:phenyllactate CoA-transferase
EC 2.8.4 Transferring alkylthio groups
EC 2.8.4.1 coenzyme-B sulfoethylthiotransferase
EC 2.9 Transferring Selenium-Containing Groups
EC 2.9.1 Selenotransferases
EC 2.9.1.1 L-seryl-tRNA^{Sec} selenium transferase
EC 3. Hydrolases
EC 3.1 Acting on Ester Bonds
EC 3.1.1 Carboxylic Ester Hydrolases
EC 3.1.1.1 carboxylesterase
EC 3.1.1.2 arylesterase
EC 3.1.1.3 triacylglycerol lipase
EC 3.1.1.4 phospholipase A_2
EC 3.1.1.5 lysophospholipase
EC 3.1.1.6 acetylesterase
EC 3.1.1.7 acetylcholinesterase
EC 3.1.1.8 cholinesterase
EC 3.1.1.9 deleted
EC 3.1.1.10 tropinesterase
EC 3.1.1.11 pectinesterase
EC 3.1.1.12 deleted
EC 3.1.1.13 sterol esterase
EC 3.1.1.14 chlorophyllase
EC 3.1.1.15 L-arabinonolactonase
EC 3.1.1.16 deleted, mixture of EC 5.3.3.4 and EC 3.1.1.24
EC 3.1.1.17 gluconolactonase
EC 3.1.1.18 deleted, included in EC 3.1.1.17
EC 3.1.1.19 uronolactonase
EC 3.1.1.20 tannase
EC 3.1.1.21 retinyl-palmitate esterase
EC 3.1.1.22 hydroxybutyrate-dimer hydrolase
EC 3.1.1.23 acylglycerol lipase
EC 3.1.1.24 3-oxoadipate *enol*-lactonase
EC 3.1.1.25 1,4-lactonase
EC 3.1.1.26 galactolipase
EC 3.1.1.27 4-pyridoxolactonase
EC 3.1.1.28 acylcarnitine hydrolase
EC 3.1.1.29 aminoacyl-tRNA hydrolase
EC 3.1.1.30 D-arabinonolactonase
EC 3.1.1.31 6-phosphogluconolactonase
EC 3.1.1.32 phospholipase A_1
EC 3.1.1.33 6-acetylglucose deacetylase
EC 3.1.1.34 lipoprotein lipase
EC 3.1.1.35 dihydrocoumarin hydrolase
EC 3.1.1.36 limonin-D-ring-lactonase
EC 3.1.1.37 steroid-lactonase
EC 3.1.1.38 triacetate-lactonase
EC 3.1.1.39 actinomycin lactonase
EC 3.1.1.40 orsellinate-depside hydrolase
EC 3.1.1.41 cephalosporin-C deacetylase
EC 3.1.1.42 chlorogenate hydrolase
EC 3.1.1.43 a-amino-acid esterase
EC 3.1.1.44 4-methyloxaloacetate esterase
EC 3.1.1.45 carboxymethylenebutenolidase
EC 3.1.1.46 deoxylimonate A-ring-lactonase
EC 3.1.1.47 1-alkyl-2-acetylglycerophosphocholine esterase
EC 3.1.1.48 fusarinine-C ornithinesterase
EC 3.1.1.49 sinapine esterase
EC 3.1.1.50 wax-ester hydrolase
EC 3.1.1.51 phorbol-diester hydrolase

EC 3.1.1.52 phosphatidylinositol deacylase
EC 3.1.1.53 sialate *O*-acetylesterase
EC 3.1.1.54 acetoxybutynylbithiophene deacetylase
EC 3.1.1.55 acetylsalicylate deacetylase
EC 3.1 1.56 methylumbelliferyl-acetate deacetylase
EC 3.1.1.57 2-pyrone-4,6-dicarboxylate lactonase
EC 3.1.1.58 *N*-acetylgalactosaminoglycan deacetylase
EC 3.1 1.59 juvenile-hormone esterase
EC 3 1.1.60 bis(2-ethylhexyl)phthalate esterase
EC 3.1.1.61 protein-glutamate methylesterase
EC 3.1.1.62 now EC 3.5.1.47
EC 3.1.1.63 11-*cis*-retinyl-palmitate hydrolase
EC 3.1.1.64 *all-trans*-retinyl-palmitate hydrolase
EC 3.1.1.65 L-rhamnono-1,4-lactonase
EC 3.1.1.66 5-(3,4-diacetoxybut-1-ynyl)-2, 2'-bithiophene deacetylase
EC 3.1.1.67 fatty-acyl-ethyl-ester synthase
EC 3.1.1.68 xylono-1,4-lactonase
EC 3.1.1.69 now EC 3.5.1.89
EC 3.1.1.70 cetraxate benzylesterase
EC 3.1.1.71 acetylalkylglycerol acetylhydrolase
EC 3.1.1.72 acetylxylan esterase
EC 3.1.1.73 feruloyl esterase
EC 3.1 1.74 cutinase
EC 3.1.1.75 poly(3-hydroxybutyrate) depolymerase
EC 3.1.1.76 poly(3-hydroxyoctanoate) depolymerase
acyloxyacyl hydrolase
EC 3 1.1 77 acyloxyacyl hydrolase
EC 3.1.1.78 polyneuridine-aldehyde esterase
EC 3.1.1.79 hormone-sensitive lipase
EC 3.1.2 Thiolester Hydrolases
EC 3.1.2.1 acetyl-CoA hydrolase
EC 3.1.2.2 palmitoyl-CoA hydrolase
EC 3.1.2.3 succinyl-CoA hydrolase
EC 3.1.2.4 3-hydroxyisobutyryl-CoA hydrolase
EC 3.1.2.5 hydroxymethylglutaryl-CoA hydrolase
EC 3.1.2.6 hydroxyacylglutathione hydrolase
EC 3.1.2.7 glutathione thiolesterase
EC 3.1.2.8 deleted, included in EC 3.1.2.6
EC 3.1.2.9 deleted
EC 3.1.2.10 formyl-CoA hydrolase
EC 3.1.2.11 acetoacetyl-CoA hydrolase
EC 3.1.2.12 *S*-formylglutathione hydrolase
EC 3.1.2 13 *S*-succinylglutathione hydrolase
EC 3.1.2 14 oleoyl-[acyl-carrier-protein] hydrolase
EC 3.1.2.15 ubiquitin thiolesterase
EC 3.1.2.16 [citrate-(*pro*-3*S*)-lyase] thiolesterase
EC 3.1.2.17 (*S*)-methylmalonyl-CoA hydrolase
EC 3.1.2.18 ADP-dependent short-chain-acyl-CoA hydrolase
EC 3.1.2.19 ADP-dependent medium-chain-acyl-CoA hydrolase
EC 3.1.2.20 acyl-CoA hydrolase
EC 3.1.2 21 dodecanoyl-[acyl-carrier protein] hydrolase
EC 3 1.2.22 palmitoyl[protein] hydrolase
EC 3 1.2 23 4-hydroxybenzoyl-CoA thioesterase
EC 3.1 2.24 2-(2-hydroxyphenyl)benzenesulfinate hydrolase
EC 3.1.2.25 phenylacetyl-CoA hydrolase
EC 3.1.3 Phosphoric Monoester Hydrolases
EC 3.1.3.1 alkaline phosphatase
EC 3.1.3.2 acid phosphatase
EC 3.1.3.3 phosphoserine phosphatase
EC 3.1.3 4 phosphatidate phosphatase
EC 3.1.3.5 5'-nucleotidase
EC 3 1.3.6 3'-nucleotidase
EC 3.1.3.7 3'(2'),5'-bisphosphate nucleotidase
EC 3.1.3.8 3-phytase
EC 3.1 3.9 glucose-6-phosphatase
EC 3.1.3.10 glucose-1-phosphatase
EC 3 1.3.11 fructose-bisphosphatase
EC 3.1.3.12 trehalose-phosphatase
EC 3.1.3.13 bisphosphoglycerate phosphatase
EC 3.1.3.14 methylphosphothioglycerate phosphatase
EC 3.1.3.15 histidinol-phosphatase
EC 3.1.3.16 phosphoprotein phosphatase
EC 3 1.3.17 [phosphorylase] phosphatase
EC 3.1.3.18 phosphoglycolate phosphatase
EC 3.1.3.19 glycerol-2-phosphatase
EC 3.1.3.20 phosphoglycerate phosphatase
EC 3.1.3.21 glycerol-1-phosphatase
EC 3.1 3.22 mannitol-1-phosphatase
EC 3.1.3.23 sugar-phosphatase
EC 3.1.3.24 sucrose-phosphatase
EC 3.1.3.25 inositol-1(or 4)-monophosphatase
EC 3.1.3.26 4-phytase
EC 3.1.3.27 phosphatidylglycerophosphatase
EC 3.1.3.28 ADPphosphoglycerate phosphatase
EC 3.1.3.29 *N*-acylneuraminate-9-phosphatase
EC 3.1.3 30 deleted, included in EC 3.1.3.31
EC 3.1.3.31 nucleotidase
EC 3.1.3.32 polynucleotide 3'-phosphatase
EC 3.1.3.33 polynucleotide 5'-phosphatase

EC 3.1.3 34 deoxynucleotide 3'-phosphatase
EC 3.1.3.35 thymidylate 5'-phosphatase
EC 3.1.3 36 phosphoinositide 5-phosphatase
EC 3.1 3.37 sedoheptulose-bisphosphatase
EC 3 1 3 38 3-phosphoglycerate phosphatase
EC 3 1.3 39 streptomycin-6-phosphatase
EC 3.1.3.40 guanidinodeoxy-*scyllo*-inositol-4-phosphatase
EC 3.1 3.41 4-nitrophenylphosphatase
EC 3 1.3.42 [glycogen-synthase-D] phosphatase
EC 3.1.3.43 [pyruvate dehydrogenase (lipoamide)]-phosphatase
EC 3.1.3.44 [acetyl-CoA carboxylase]-phosphatase
EC 3.1 3 45 3-deoxy-*manno*-octulosonate-8-phosphatase
EC 3.1.3.46 fructose-2,6-bisphosphate 2-phosphatase
EC 3 1 3 47 [hydroxymethylglutaryl-CoA reductase (NADPH)]-phosphatase
EC 3.1.3.48 protein-tyrosine-phosphatase
EC 3.1.3.49 [pyruvate kinase]-phosphatase
EC 3 1.3.50 sorbitol-6-phosphatase
EC 3 1.3 51 dolichyl-phosphatase
EC 3.1.3 52 [3-methyl-2-oxobutanoate dehydrogenase (lipoamide)]-phosphatase
EC 3 1.3.53 [myosin-light-chain] phosphatase
EC 3.1.3.54 fructose-2,6-bisphosphate 6-phosphatase
EC 3 1.3.55 caldesmon-phosphatase
EC 3.1.3 56 inositol-polyphosphate 5-phosphatase
EC 3.1.3 57 inositol-1,4-bisphosphate 1-phosphatase
EC 3 1.3.58 sugar-terminal-phosphatase
EC 3 1.3.59 alkylacetylglycerophosphatase
EC 3.1.3.60 phospho*enol*pyruvate phosphatase
EC 3.1.3.61 deleted
EC 3.1.3.62 multiple inositol-polyphosphate phosphatase
EC 3.1.3.63 2-carboxy-D-arabinitol-1-phosphatase
EC 3.1.3.64 phosphatidylinositol-3-phosphatase
EC 3 1 3.65 now with EC 3.1.3.64
EC 3 1.3.66 phosphatidylinositol-3,4-bisphosphate 4-phosphatase
EC 3.1.3.67 phosphatidylinositol-3, 4,5-trisphosphate 3-phosphatase
EC 3.1.3.68 2-deoxyglucose-6-phosphatase
EC 3.1.3.69 glucosylglycerol 3-phosphatase
EC 3.1.3.70 mannosyl-3-phosphoglycerate phosphatase
EC 3 1 3 71 2-phosphosulfolactate phosphatase
EC 3.1.3 72 5-phytase
EC 3.1.3.73 a-ribazole phosphatase
EC 3.1.4 Phosphoric Diester Hydrolases
EC 3.1.4.1 phosphodiesterase I
EC 3.1.4.2 glycerophosphocholine phosphodiesterase
EC 3.1.4.3 phospholipase C
EC 3 1.4.4 phospholipase D
EC 3.1.4.5 now EC 3.1 21.1
EC 3.1.4.6 now EC 3.1.22.1
EC 3.1.4.7 now EC 3.1.31.1
EC 3.1 4 8 now EC 3.1.27.3
EC 3.1.4.9 now EC 3.1.30.2
EC 3 1.4 10 now EC 4.6.1.13
EC 3 1 4.11 phosphoinositide phospholipase C
EC 3.1 4.12 sphingomyelin phosphodiesterase
EC 3.1.4 13 serine-ethanolaminephosphate phosphodiesterase
EC 3.1 4.14 [acyl-carrier-protein] phosphodiesterase
EC 3 1 4.15 adenylyl-[glutamate—ammonia ligase] hydrolase
EC 3.1.4.16 2',3'-cyclic-nucleotide 2'-phosphodiesterase
EC 3.1.4.17 3',5'-cyclic-nucleotide phosphodiesterase
EC 3.1.4.18 now EC 3.1.16.1
EC 3.1.4.19 now EC 3.1 13 3
EC 3.1.4.20 now EC 3.1.13.1
EC 3.1.4.21 now EC 3.1.30.1
EC 3.1.4 22 now EC 3.1.27.5
EC 3.1 4 23 now EC 3.1.27.1
EC 3.1 4.24 deleted
EC 3.1.4.25 now EC 3.1.11.1
EC 3.1.4.26 deleted
EC 3 1.4.27 now EC 3.1.11.2
EC 3.1.4.28 now EC 3.1.11 3
EC 3.1.4.29 deleted
EC 3.1.4.30 now EC 3.1 21.2
EC 3.1.4.31 now EC 3.1.11.4
EC 3.1.4.32 deleted
EC 3.1.4.33 deleted
EC 3.1.4.34 deleted
EC 3.1.4.35 3',5'-cyclic-GMP phosphodiesterase
EC 3.1.4.36 now with EC 3.1.4.43

EC 3.1.4.37 2',3'-cyclic-nucleotide 3'-phosphodiesterase
EC 3.1.4.38 glycerophosphocholine cholinephosphodiesterase
EC 3.1.4.39 alkylglycerophosphoethanolamine phosphodiesterase
EC 3.1.4.40 CMP-*N*-acylneuraminate phosphodiesterase
EC 3.1.4.41 sphingomyelin phosphodiesterase D
EC 3.1.4.42 glycerol-1,2-cyclic-phosphate 2-phosphodiesterase
EC 3.1.4.43 glycerophosphoinositol inositolphosphodiesterase
EC 3.1.4.44 glycerophosphoinositol glycerophosphodiesterase
EC 3.1.4.45 *N*-acetylglucosamine-1-phosphodiester a-*N*-acetylglucosaminidase
EC 3.1.4.46 glycerophosphodiester phosphodiesterase
EC 3.1.4.47 now EC 4.6.1.14
EC 3.1.4.48 dolichylphosphate-glucose phosphodiesterase
EC 3.1.4.49 dolichylphosphate-mannose phosphodiesterase
EC 3.1.4.50 glycosylphosphatidylinositol phospholipase D
EC 3.1.4.51 glucose-1-phospho-D-mannosylglycoprotein phosphodiesterase
EC 3.1.5 Triphosphoric Monoester Hydrolases
EC 3.1.5.1 dGTPase
EC 3.1 6 Sulfuric Ester Hydrolases
EC 3.1.6.1 arylsulfatase
EC 3.1.6.2 steryl-sulfatase
EC 3.1.6.3 glycosulfatase
EC 3.1.6.4 *N*-acetylgalactosamine-6-sulfatase
EC 3.1.6.5 deleted
EC 3.1.6.6 choline-sulfatase
EC 3.1.6.7 cellulose-polysulfatase
EC 3.1.6.8 cerebroside-sulfatase
EC 3.1.6.9 chondro-4-sulfatase
EC 3.1.6.10 chondro-6-sulfatase
EC 3.1.6.11 disulfoglucosamine-6-sulfatase
EC 3.1.6.12 *N*-acetylgalactosamine-4-sulfatase
EC 3.1.6.13 iduronate-2-sulfatase
EC 3.1.6.14 *N*-acetylglucosamine-6-sulfatase
EC 3.1.6.15 *N*-sulfoglucosamine-3-sulfatase
EC 3.1.6.16 monomethyl-sulfatase
EC 3.1.6.17 D-lactate-2-sulfatase
EC 3.1.6.18 glucuronate-2-sulfatase
EC 3.1.7 Diphosphoric Monoester Hydrolases
EC 3.1.7.1 prenyl-diphosphatase
EC 3.1.7.2 guanosine-3',5'-bis(diphosphate) 3'-diphosphatase
EC 3.1.7.3 monoterpenyl-diphosphatase
EC 3.1.8 Phosphoric Triester Hydrolases
EC 3.1.8.1 aryldialkylphosphatase
EC 3.1.8.2 diisopropyl-fluorophosphatase
EC 3.1.11 Exodeoxyribonucleases Producing 5'-Phosphomonoesters
EC 3.1.11.1 exodeoxyribonuclease I
EC 3.1.11.2 exodeoxyribonuclease III
EC 3.1.11.3 exodeoxyribonuclease (lambda-induced)
EC 3.1.11.4 exodeoxyribonuclease (phage SP3-induced)
EC 3.1.11.5 exodeoxyribonuclease V
EC 3.1.11.6 exodeoxyribonuclease VII
EC 3.1.13 Exoribonucleases Producing 5'-Phosphomonoesters
EC 3.1.13.1 exoribonuclease II
EC 3.1.13.2 exoribonuclease H
EC 3.1.13.3 oligonucleotidase
EC 3.1.13.4 poly(A)-specific ribonuclease
EC 3.1.14 Exoribonucleases Producing 3'-Phosphomonoesters
EC 3.1.14.1 yeast ribonuclease
EC 3.1.15 Exonucleases Active with either Ribo- or Deoxyribonucleic Acids and Producing 5'-Phosphomonoesters
EC 3.1.15.1 venom exonuclease
EC 3.1.16 Exonucleases Active with either Ribo- or Deoxyribonucleic Acids and Producing 3'-Phosphomonoesters
EC 3.1.16.1 spleen exonuclease
EC 3.1.21 Endodeoxyribonucleases Producing 5'-Phosphomonoesters
EC 3.1.21.1 deoxyribonuclease I
EC 3.1.21.2 deoxyribonuclease IV (phage-T_4-induced)
EC 3.1.21.3 type I site-specific deoxyribonuclease
EC 3.1.21.4 type II site-specific deoxyribonuclease
EC 3.1.21.5 type III site-specific deoxyribonuclease
EC 3.1.21.6 CC-preferring endodeoxyribonuclease
EC 3.1.21.7 deoxyribonuclease V
EC 3.1.22 Endodeoxyribonucleases Producing 3'-Phosphomonoesters
EC 3.1.22.1 deoxyribonuclease II

EC 3.1.22.2 *Aspergillus* deoxyribonuclease K_1
EC 3.1.22.3 now EC 3.1.21.7
EC 3.1.22.4 crossover junction endodeoxyribonuclease
EC 3.1.22.5 deoxyribonuclease X
EC 3.1.23 and EC 3.1.24 now EC 3.1.21.3, EC 3.1.21.4 and EC 3.1.21.5
EC 3.1.25 Site-Specific Endodeoxyribonucleases Specific for Altered Bases
EC 3.1.25.1 deoxyribonuclease (pyrimidine dimer)
EC 3.1.25.2 now EC 4.2.99.18
EC 3.1.26 Endoribonucleases Producing 5'-Phosphomonoesters
EC 3.1.26.1 *Physarum polycephalum* ribonuclease
EC 3.1.26.2 ribonuclease alpha
EC 3.1.26.3 ribonuclease III
EC 3.1.26.4 calf thymus ribonuclease H
EC 3.1.26.5 ribonuclease P
EC 3.1.26.6 ribonuclease IV
EC 3.1.26.7 ribonuclease P4
EC 3.1.26.8 ribonuclease M5
EC 3.1.26.9 ribonuclease [poly-(U)-specific]
EC 3.1.26.10 ribonuclease IX
EC 3.1.26.11tRNase Z
EC 3.1.27 Endoribonucleases Producing 3'-Phosphomonoesters
EC 3.1.27.1 ribonuclease T_2
EC 3.1.27.2 *Bacillus subtilis* ribonuclease
EC 3.1.27.3 ribonuclease T_1
EC 3.1.27.4 ribonuclease U_2
EC 3.1.27.5 pancreatic ribonuclease
EC 3.1.27.6 *Enterobacter* ribonuclease
EC 3.1.27.7 ribonuclease F
EC 3.1.27.8 ribonuclease V
EC 3.1.27.9 tRNA-intron endonuclease
EC 3.1.27.10 rRNA endonuclease
EC 3.1.30 Endoribonucleases Active with either Ribo- or Deoxyribonucleic Acids and Producing 5'-Phosphomonoesters
EC 3.1.30.1 *Aspergillus* nuclease S_1
EC 3.1.30.2 *Serratia marcescens* nuclease
EC 3.1.31 Endoribonucleases Active with either Ribo- or Deoxyribonucleic Acids and Producing 3'-Phosphomonoesters
EC 3.1.31.1 micrococcal nuclease
EC 3.2 Glycosylases
EC 3.2.1 Glycosidases, i.e. enzymes hydrolysing *O*- and *S*-glycosyl compounds
EC 3.2.1.1 a-amylase
EC 3.2.1.2 b-amylase
EC 3.2.1.3 glucan 1,4-a-glucosidase
EC 3.2.1.4 cellulase
EC 3.2.1.5 deleted
EC 3.2.1.6 endo-1,3(4)-b-glucanase
EC 3.2.1.7 inulinase
EC 3.2.1.8 endo-1,4-b-xylanase
EC 3.2.1.9 deleted
EC 3.2.1.10 oligo-1,6-glucosidase
EC 3.2.1.11 dextranase
EC 3.2.1.12 deleted, included in EC 3.2.1 54
EC 3.2.1.13 deleted, included in EC 3.2.1.54
EC 3.2.1.14 chitinase
EC 3.2.1.15 polygalacturonase
EC 3.2.1.16 deleted
EC 3.2.1.17 lysozyme
EC 3.2.1.18 exo-a-sialidase
EC 3.2.1.19 deleted
EC 3.2.1.20 a-glucosidase
EC 3.2.1.21 b-glucosidase
EC 3.2.1.22 a-galactosidase
EC 3.2.1.23 b-galactosidase
EC 3.2.1.24 a-mannosidase
EC 3.2.1.25 b-mannosidase
EC 3.2.1.26 b-fructofuranosidase
EC 3.2.1.27 deleted
EC 3.2.1.28 a,a-trehalase
EC 3.2.1.29 deleted, included in EC 3.2.1.52
EC 3.2.1.30 deleted, included in EC 3.2.1.52
EC 3.2.1.31 b-glucuronidase
EC 3.2.1.32 xylan endo-1,3-b-xylosidase
EC 3.2.1.33 amylo-1,6-glucosidase
EC 3.2.1.34 deleted, included in EC 3.2.1.35
EC 3.2.1.35 hyaluronoglucosaminidase
EC 3.2.1.36 hyaluronoglucuronidase
EC 3.2.1.37 xylan 1,4-b-xylosidase
EC 3.2.1.38 b-D-fucosidase
EC 3.2.1.39 glucan endo-1,3-b-D-glucosidase
EC 3.2.1.40 a-L-rhamnosidase
EC 3.2.1.41 pullulanase
EC 3.2.1.42 GDP-glucosidase
EC 3.2.1.43 b-L-rhamnosidase
EC 3.2.1.44 fucoidanase
EC 3.2.1.45 glucosylceramidase
EC 3.2.1.46 galactosylceramidase
EC 3.2.1.47 galactosylgalactosylglucosylceramidase

EC 3.2.1.48 sucrose a-glucosidase
EC 3.2.1.49 a-*N*-acetylgalactosaminidase
EC 3.2.1.50 a-*N*-acetylglucosaminidase
EC 3 2.1 51 a-L-fucosidase
EC 3.2.1.52 b-L-*N*-acetylhexosaminidase
EC 3.2.1.53 b-*N*-acetylgalactosaminidase
EC 3.2.1 54 cyclomaltodextrinase
EC 3.2.1.55 a-*N*-arabinofuranosidase
EC 3.2.1.56 glucuronosyl-disulfoglucosamine glucuronidase
EC 3.2.1.57 isopullulanase
EC 3.2.1.58 glucan 1,3-b-glucosidase
EC 3 2 1.59 glucan endo-1,3-a-glucosidase
EC 3.2.1.60 glucan 1,4-a-maltotetraohydrolase
EC 3.2.1.61 mycodextranase
EC 3 2.1.62 glycosylceramidase
EC 3.2 1.63 1,2-a-L-fucosidase
EC 3.2 1.64 2,6-b-fructan 6-levanbiohydrolase
EC 3.2.1.65 levanase
EC 3.2.1.66 quercitrinase
EC 3.2.1.67 galacturan 1,4-a-galacturonidase
EC 3.2.1.68 isoamylase
EC 3.2.1.69 deleted, included in EC 3.2.1 41
EC 3.2.1 70 glucan 1,6-a-glucosidase
EC 3 2.1 71 glucan endo-1,2-b-glucosidase
EC 3.2.1.72 xylan 1,3-b-xylosidase
EC 3.2.1.73 licheninase
EC 3.2.1.74 glucan 1,4-b-glucosidase
EC 3 2.1.75 glucan endo-1,6-b-glucosidase
EC 3 2.1.76 L-iduronidase
EC 3.2.1.77 mannan 1,2-(1,3)-a-mannosidase
EC 3 2.1 78 mannan endo-1,4-b-mannosidase
EC 3 2.1.79 deleted, included in EC 3.2.1.55
EC 3.2 1.80 fructan b-fructosidase
EC 3.2.1.81 agarase
EC 3.2.1.82 exo-poly-a-galacturonosidase
EC 3.2.1.83 k-carrageenase
EC 3 2.1.84 glucan 1,3-a-glucosidase
EC 3.2.1.85 6-phospho-b-galactosidase
EC 3.2.1.86 6-phospho-b-glucosidase
EC 3.2.1.87 capsular-polysaccharide endo-1, 3-a-galactosidase
EC 3.2.1.88 b-L-arabinosidase
EC 3.2.1.89 arabinogalactan endo-1, 4-b-galactosidase
EC 3.2.1.90Deleted, not sufficiently characterised.
EC 3.2.1.91 cellulose 1,4-b-cellobiosidase
EC 3.2 1.92 peptidoglycan b-*N*-acetylmuramidase
EC 3.2.1 93 a,a-phosphotrehalase
EC 3.2.1.94 glucan 1,6-a-isomaltosidase
EC 3 2 1.95 dextran 1,6-a-isomaltotriosidase
EC 3 2.1 96 mannosyl-glycoprotein endo-b-*N*-acetylglucosaminidase
EC 3.2.1.97 glycopeptide a-*N*-acetylgalactosaminidase
EC 3.2 1 98 glucan 1,4-a-maltohexaosidase
EC 3.2.1.99 arabinan endo-1,5-a-L-arabinosidase
EC 3.2.1.100 mannan 1,4-mannobiosidase
EC 3.2.1.101 mannan endo-1,6-a-mannosidase
EC 3.2.1.102 blood-group-substance endo-1, 4-b-galactosidase
EC 3 2.1 103 keratan-sulfate endo-1, 4-b-galactosidase
EC 3.2.1.104 steryl-b-glucosidase
EC 3.2.1.105 strictosidine b-glucosidase
EC 3.2.1.106 mannosyl-oligosaccharide glucosidase
EC 3.2.1.107 protein-glucosylgalactosylhydroxylysine glucosidase
EC 3.2 1.108 lactase
EC 3.2 1.109 endogalactosaminidase
EC 3.2.1.110 mucinaminylserine mucinaminidase
EC 3.2.1 111 1,3-a-L-fucosidase
EC 3 2.1.112 2-deoxyglucosidase
EC 3.2.1.113 mannosyl-oligosaccharide 1, 2-a-mannosidase
EC 3 2.1.114 mannosyl-oligosaccharide 1,3-1, 6-a-mannosidase
EC 3.2.1.115 branched-dextran exo-1, 2-a-glucosidase
EC 3.2.1.116 glucan 1,4-a-maltotriohydrolase
EC 3.2.1.117 amygdalin b-glucosidase
EC 3.2.1.118 prunasin b-glucosidase
EC 3.2.1 119 vicianin b-glucosidase
EC 3.2 1.120 oligoxyloglucan b-glycosidase
EC 3.2.1.121 polymannuronate hydrolase
EC 3.2.1.122 maltose-6'-phosphate glucosidase
EC 3.2.1.123 endoglycosylceramidase
EC 3.2.1.124 3-deoxy-2-octulosonidase
EC 3.2.1.125 raucaffricine b-glucosidase
EC 3.2.1.126 coniferin b-glucosidase
EC 3.2.1.127 1,6-a-L-fucosidase
EC 3.2.1.128 glycyrrhizinate b-glucuronidase
EC 3.2.1.129 endo-a-sialidase
EC 3.2.1.130 glycoprotein endo-a-1,2-mannosidase

EC 3 2.1.131 xylan a-1,2-glucuronosidase
EC 3.2.1.132 chitosanase
EC 3.2.1.133 glucan 1,4-a-maltohydrolase
EC 3.2.1.134 difructose-anhydride synthase
EC 3.2.1.135 neopullulanase
EC 3.2.1.136 glucuronoarabinoxylan endo-1, 4-b-xylanase
EC 3.2.1.137 mannan exo-1,2-1,6-a-mannosidase
EC 3.2.1 138 now EC 4.2.2.15
EC 3.2.1.139 a-glucuronidase
EC 3.2.1.140 lacto-*N*-biosidase
EC 3.2.1.141 4-a-D-{(14)-a-D-glucano}trehalose trehalohydrolase
EC 3.2.1.142 limit dextrinase
EC 3.2 1 143 poly(ADP-ribose) glycohydrolase
EC 3.2.1.144 3-deoxyoctulosonase
EC 3 2.1.145 galactan 1,3-b-galactosidase
EC 3.2.1.146 b-galactofuranosidase
EC 3.2.1.147 thioglucosidase
EC 3.2.1.148 ribosylhomocysteinase
EC 3.2.1.149 b-primeverosidase
EC 3.2.1.150 oligoxyloglucan reducing-end-specific cellobiohydrolase
EC 3.2.1.151 xyloglucan-specific endo-b-1, 4-glucanase
EC 3.2.2 Hydrolysing *N*-Glycosyl Compounds
EC 3.2.2.1 purine nucleosidase
EC 3.2.2.2 inosine nucleosidase
EC 3.2.2.3 uridine nucleosidase
EC 3.2.2.4 AMP nucleosidase
EC 3.2.2.5 NAD^+ nucleosidase
EC 3.2.2.6 $NAD(P)^+$ nucleosidase
EC 3.2.2.7 adenosine nucleosidase
EC 3.2.2.8 ribosylpyrimidine nucleosidase
EC 3.2.2.9 adenosylhomocysteine nucleosidase
EC 3.2.2.10 pyrimidine-5'-nucleotide nucleosidase
EC 3.2.2.11 b-aspartyl-*N*-acetylglucosaminidase
EC 3.2.2.12 inosinate nucleosidase
EC 3.2.2.13 1-methyladenosine nucleosidase
EC 3.2.2.14 NMN nucleosidase
EC 3.2.2.15 DNA-deoxyinosine glycosylase
EC 3.2.2.16 methylthioadenosine nucleosidase
EC 3.2.2.17 deoxyribodipyrimidine endonucleosidase
EC 3.2.2.18 deleted, included in EC 3.5.1.52
EC 3.2.2.19 ADP-ribosylarginine hydrolase
EC 3.2.2.20 DNA-3-methyladenine glycosylase I
EC 3.2 2.21 DNA-3-methyladenine glycosylase II
EC 3.2.2.22 rRNA *N*-glycosylase
EC 3.2.2.23 DNA-formamidopyrimidine glycosylase
EC 3 2.2.24 ADP-ribosyl-[dinitrogen reductase] hydrolase
EC 3.2.3 Hydrolysing *S*-Glycosyl Compounds
EC 3.2.3 1 now EC 3.2.1.147
EC 3.3 Acting on Ether Bonds
EC 3.3.1 Trialkylsulfonium hydrolases
EC 3.3.1.1 adenosylhomocysteinase
EC 3.3.1.2 adenosylmethionine hydrolase
EC 3.3.1.3 now EC 3.2.1.148
EC 3.3.2 Ether Hydrolases
EC 3.3.2.1 isochorismatase
EC 3.3.2.2 alkenylglycerophosphocholine hydrolase
EC 3.3.2.3 epoxide hydrolase
EC 3.3.2.4 *trans*-epoxysuccinate hydrolase
EC 3.3.2.5 alkenylglycerophosphoethanolamine hydrolase
EC 3.3.2.6 leukotriene-A_4 hydrolase
EC 3.3.2.7 hepoxilin-epoxide hydrolase
EC 3.3.2.8 limonene-1,2-epoxide hydrolase
EC 3.4 Acting on peptide bonds (Peptidases)
EC 3.4.1 a-Amino-Acyl-Peptide Hydrolases (discontinued)
EC 3.4.1.1 now EC 3.4.11.1
EC 3.4.1.2 now EC 3.4.11.2
EC 3.4.1.3 now EC 3.4.11.4
EC 3.4.1.4 now EC 3.4.11.5
EC 3.4.2 Peptidyl-Amino-Acid Hydrolases (discontinued)
EC 3.4.2.1 now EC 3.4.17.1
EC 3.4.2.2 now EC 3.4.17.2
EC 3.4.2.3 now EC 3.4.17.4
EC 3.4.3 Dipeptide Hydrolases (discontinued)
EC 3.4.3.1 now EC 3.4.13.18
EC 3.4.3.2 now EC 3.4.13.18
EC 3.4.3.3 now EC 3.4.13.3
EC 3.4.3.4 now EC 3.4.13.5
EC 3.4.3.5 now EC 3.4.13.6
EC 3.4.3.6 now EC 3.4.13.8
EC 3.4.3.7 now EC 3.4.13.9
EC 3.4.4 Peptidyl Peptide Hydrolases (discontinued)
EC 3.4.4.1 now EC 3.4.23.1
EC 3.4.4.2 now EC 3.4.23.2
EC 3.4.4.3 now EC 3.4.23.4
EC 3.4.4.4 now EC 3.4.21.4

EC 3 4.4.5 now EC 3.4.21.1
EC 3.4.4.6 now EC 3.4.21.1
EC 3.4.4.7 now covered by EC 3.4.21.36, EC 3 4.21.37
EC 3.4.4.8 now EC 3.4.21.9
EC 3.4.4.9 now EC 3.4.14.1
EC 3.4.4.10 now EC 3.4.22.2
EC 3.4.4.11 now EC 3.4.22.6
EC 3.4.4.12 now EC 3.4.22.3
EC 3.4.4.13 now EC 3.4.21.5
EC 3.4.4.14 now EC 3.4.21.7
EC 3.4.4.15 now EC 3.4.23.15
EC 3.4.4.16 now covered by EC 3.4.21.62 to EC 3.4.21.67
EC 3.4.4.17 now covered by EC 3.4.23.20 to EC 3.4.23.30
EC 3.4.4.18 now EC 3.4.22.10
EC 3.4.4.19 now EC 3.4.24.3
EC 3.4.4.20 now EC 3.4.22.8
EC 3.4.4.21 now EC 3.4.21.34
EC 3.4.4.22 now EC 3.4.23.3
EC 3.4.4.23 now EC 3.4.23.5
EC 3.4.4.24 now covered by EC 3.4.22.32 and EC 3.4.22.33
EC 3.4.4.25 deleted
EC 3.4.11 Aminopeptidases
EC 3.4.11.1 leucyl aminopeptidase
EC 3.4.11.2 membrane alanyl aminopeptidase
EC 3.4.11.3 cystinyl aminopeptidase
EC 3.4.11.4 tripeptide aminopeptidase
EC 3.4.11.5 prolyl aminopeptidase
EC 3.4.11.6 arginyl aminopeptidase
EC 3.4.11.7 glutamyl aminopeptidase
EC 3.4.11.8 now EC 3.4.19.3
EC 3.4.11.9 Xaa-Pro aminopeptidase
EC 3.4.11.10 bacterial leucyl aminopeptidase
EC 3.4.11.11 deleted
EC 3.4.11.12 deleted (Supplement 4)
EC 3.4.11.13 clostridial aminopeptidase
EC 3.4.11 14 cytosol alanyl aminopeptidase
EC 3.4.11.15 lysyl aminopeptidase
EC 3.4.11.16 Xaa-Trp aminopeptidase
EC 3.4.11.17 tryptophanyl aminopeptidase
EC 3.4.11.18 methionyl aminopeptidase
EC 3.4.11.19 D-stereospecific aminopeptidase
EC 3.4.11.20 aminopeptidase Ey
EC 3.4.11 21 aspartyl aminopeptidase
EC 3.4.11.22 aminopeptidase I
EC 3.4.11.23 PepB aminopeptidase
EC 3.4.12 Peptidylamino-Acid Hydrolases or Acylamino-Acid Hydrolases
EC 3.4.12.1 now EC 3.4.16.1
EC 3.4.12.2 now EC 3 4.17.1
EC 3.4.12.3 now EC 3.4.17.2
EC 3.4.12.4 now EC 3.4.16.2
EC 3.4.12.5 now EC 3 4.19.10
EC 3.4.12.6 now EC 3.4.17.8
EC 3.4.12.7 now EC 3.4.17.3
EC 3.4.12.8 now EC 3.4.17.4
EC 3.4.12.9 deleted
EC 3.4.12.10 now EC 3.4.19.9
EC 3.4.12.11 now EC 3.4.17.6
EC 3.4.12.12 now EC 3.4.16.1
EC 3.4.12.13 deleted
EC 3.4.13 Dipeptidases
EC 3.4.13.1 now EC 3.4.13.18
EC 3.4.13.2 now EC 3.4.13.18
EC 3.4.13.3 Xaa-His dipeptidase
EC 3.4.13.4 Xaa-Arg dipeptidase
EC 3.4.13.5 Xaa-methyl-His dipeptidase
EC 3.4.13.6 now EC 3.4.11.2 (Supplement 4)
EC 3.4.13.7 Glu-Glu dipeptidase
EC 3.4.13.8 now EC 3.4.17.21 (Supplement 6)
EC 3.4.13.9 Xaa-Pro dipeptidase
EC 3.4.13.10 now EC 3.4.19.5
EC 3.4.13.11 deleted, included in EC 3.4.13.18
EC 3.4.13.12 Met-Xaa dipeptidase
EC 3.4.13.13 deleted, included in EC 3.4.13.3
EC 3.4.13.14 deleted
EC 3.4.13.15 deleted, included in EC 3.4.13.18
EC 3.4.13.16 deleted
EC 3.4.13.17 non-stereospecific dipeptidase
EC 3.4.13.18 cytosol nonspecific dipeptidase
EC 3 4.13.19 membrane dipeptidase
EC 3.4.13.20 b-Ala-His dipeptidase
EC 3.4.13.21 dipeptidase E
EC 3.4.14 Dipeptidyl-peptidases and tripeptidyl-peptidases
EC 3.4.14.1 dipeptidyl-peptidase I
EC 3.4.14.2 dipeptidyl-peptidase II
EC 3.4.14.3 now EC 3.4.19.1
EC 3.4.14.4 dipeptidyl-peptidase III
EC 3.4.14.5 dipeptidyl-peptidase IV
EC 3.4.14.6 dipeptidyl-dipeptidase

EC 3.4.14.7 deleted
EC 3.4.14.8 now covered by EC 3.4.14.9, EC 3.4.14.10
EC 3.4.14.9 tripeptidyl-peptidase I
EC 3.4.14.10 tripeptidyl-peptidase II
EC 3.4.14.11 Xaa-Pro dipeptidyl-peptidase
EC 3.4.15 Peptidyl-dipeptidases
EC 3.4.15.1 peptidyl-dipeptidase A
EC 3.4.15.2 now EC 3.4.19.2
EC 3.4.15.3 deleted, included in EC 3.4.15.5 (supplement 2)
EC 3.4.15.4 peptidyl-dipeptidase B
EC 3.4.15.5 peptidyl-dipeptidase Dcp
EC 3.4.16 Serine-type carboxypeptidases
EC 3.4.16.1 deleted, included in EC 3.4.16.5, EC 3.4.16.6 (supplement 1)
EC 3.4.16.2 lysosomal Pro-Xaa carboxypeptidase
EC 3.4.16.3 deleted, included in EC 3.4.16.5 (supplement 1)
EC 3.4.16.4 serine-type D-Ala-D-Ala carboxypeptidase
EC 3.4.16.5 carboxypeptidase C
EC 3.4.16.6 carboxypeptidase D
EC 3.4.17 Metallocarboxypeptidases
EC 3.4.17.1 carboxypeptidase A
EC 3.4.17.2 carboxypeptidase B
EC 3.4.17.3 lysine carboxypeptidase
EC 3.4.17.4 Gly-Xaa carboxypeptidase
EC 3.4.17.5 deleted
EC 3.4.17.6 alanine carboxypeptidase
EC 3.4.17.7 now EC 3.4.19.10
EC 3.4.17.8 muramoylpentapeptide carboxypeptidase
EC 3.4.17.9 deleted, included in EC 3.4.17.4
EC 3.4.17.10 carboxypeptidase E
EC 3.4.17.11 glutamate carboxypeptidase
EC 3.4 17.12 carboxypeptidase M
EC 3.4.17.13 muramoyltetrapeptide carboxypeptidase
EC 3.4.17.14 zinc D-Ala-D-Ala carboxypeptidase
EC 3.4.17.15 carboxypeptidase A2
EC 3.4.17.16 membrane Pro-Xaa carboxypeptidase
EC 3.4.17.17 tubulinyl-Tyr carboxypeptidase
EC 3.4.17.18 carboxypeptidase T
EC 3.4.17.19 carboxypeptidase Taq
EC 3.4.17.20 carboxypeptidase U
EC 3.4.17.21 glutamate carboxypeptidase II
EC 3.4.17.22 metallocarboxypeptidase D
EC 3.4 18 Cysteine-type carboxypeptidases
EC 3.4.18.1 cathepsin X
EC 3.4.19 Omega peptidases
EC 3.4.19.1 acylaminoacyl-peptidase
EC 3.4.19 2 peptidyl-glycinamidase
EC 3.4.19.3 pyroglutamyl-peptidase I
EC 3.4.19.4 deleted
EC 3.4.19.5 b-aspartyl-peptidase
EC 3 4 19.6 pyroglutamyl-peptidase II
EC 3.4.19.7 *N*-formylmethionyl-peptidase
EC 3.4.19.8 now EC 3.4.17.21 (Supplement 6)
EC 3.4.19.9 g-glutamyl hydrolase
EC 3.4.19.10 now EC 3.5.1.28 (Supplement 4)
EC 3.4.19.11 g-D-glutamyl-*meso*-diaminopimelate peptidase I
EC 3.4.19.12 ubiquitinyl hydrolase 1
EC 3.4.21 Serine endopeptidases
EC 3.4.21.1 chymotrypsin
EC 3.4.21.2 chymotrypsin C
EC 3.4.21.3 metridin
EC 3.4.21.4 trypsin
EC 3.4.21.5 thrombin
EC 3.4.21.6 coagulation factor Xa
EC 3.4.21.7 plasmin
EC 3.4.21.8 now covered by EC 3.4.21 34 and EC 3.4.21.35
EC 3.4.21.9 enteropeptidase
EC 3.4.21.10 acrosin
EC 3.4.21.11 now covered by EC 3.4.21.36 and EC 3.4.21.37
EC 3.4.21.12 a-Lytic endopeptidase
EC 3.4.21.13 now EC 3.4.16.1
EC 3.4.21.14 now covered by EC 3.4.21.62 to EC 3.4.21.65 and EC 3.4.21.67
EC 3.4.21.15 now EC 3.4.21.63
EC 3.4.21.16 deleted
EC 3.4.21.17 deleted
EC 3.4.21.18 deleted
EC 3.4.21.19 glutamyl endopeptidase
EC 3.4.21.20 cathepsin G
EC 3.4.21.21 coagulation factor VIIa
EC 3.4.21.22 coagulation factor IXa
EC 3.4.21.23 deleted
EC 3.4.21.24 deleted
EC 3.4.21.25 cucumisin
EC 3.4.21.26 prolyl oligopeptidase
EC 3.4.21.27 coagulation factor XIa

EC 3.4.21.28 deleted, included in EC 3.4.21.74
EC 3 4.21.29 deleted, included in EC 3.4.21.74
EC 3.4.21.30 deleted, included in EC 3.4.21.74
EC 3.4.21 31 now covered by EC 3.4.21.68 and EC 3.4.21.73
EC 3.4.21.32 brachyurin
EC 3.4.21.33 deleted
EC 3.4.21.34 plasma kallikrein
EC 3.4.21.35 tissue kallikrein
EC 3.4 21.36 pancreatic elastase
EC 3.4.21 37 leukocyte elastase
EC 3.4.21.38 coagulation factor XIIa
EC 3.4.21.39 chymase
EC 3.4.21.40 deleted
EC 3.4.21.41 complement subcomponent C
EC 3.4.21.42 complement subcomponent C
EC 3.4.21.43 classical-complement-pathway C3/C5 convertase
EC 3.4.21.44 deleted, included in EC 3.4.21.43
EC 3.4.21.45 complement factor I
EC 3.4.21.46 complement factor D
EC 3 4.21.47 alternative-complement-pathway C3/C5 convertase
EC 3.4.21.48 cerevisin
EC 3.4.21.49 hypodermin C
EC 3.4.21.50 lysyl endopeptidase
EC 3.4.21.51 deleted
EC 3.4.21.52 deleted
EC 3.4.21.53 endopeptidase La
EC 3.4.21 54 g-renin
EC 3.4.21.55 venombin AB
EC 3.4.21.56 deleted
EC 3.4.21.57 leucyl endopeptidase
EC 3.4.21.58 deleted
EC 3.4.21.59 tryptase
EC 3.4.21.60 scutelarin
EC 3.4.21.61 kexin
EC 3.4.21.62 subtilisin
EC 3.4.21.63 oryzin
EC 3.4.21.64 endopeptidase K
EC 3.4.21.65 thermomycolin
EC 3.4.21.66 thermitase
EC 3.4.21.67 endopeptidase So
EC 3.4.21.68 t-plasminogen activator
EC 3.4 21.69 protein C (activated)
EC 3.4.21.70 pancreatic endopeptidase E
EC 3.4.21.71 pancreatic elastase II
EC 3.4.21.72 IgA-specific serine endopeptidase
EC 3.4.21.73 u-plasminogen activator
EC 3.4.21.74 venombin A
EC 3.4 21.75 furin
EC 3.4.21.76 myeloblastin
EC 3.4.21.77 semenogelase
EC 3.4.21.78 granzyme A
EC 3.4.21.79 granzyme B
EC 3.4.21.80 streptogrisin A
EC 3.4.21.81 streptogrisin B
EC 3.4.21.82 glutamyl endopeptidase II
EC 3.4.21.83 oligopeptidase B
EC 3.4.21.84 limulus clotting factor
EC 3.4.21.85 limulus clotting factor
EC 3.4.21.86 limulus clotting enzyme
EC 3.4.21.87 omptin
EC 3.4.21.88 repressor LexA
EC 3.4.21.89 signal peptidase I
EC 3.4.21.90 togavirin
EC 3.4.21.91 flavivirin
EC 3.4.21.92 endopeptidase Clp
EC 3.4.21.93 proprotein convertase 1
EC 3.4.21.94 proprotein convertase 2
EC 3.4.21.95 snake venom factor V activator
EC 3.4.21.96 lactocepin
EC 3.4.21.97 assemblin
EC 3.4.21.98 hepacivirin
EC 3.4.21.99 spermosin
EC 3.4.21.100 pseudomonalisin
EC 3.4.21.101 xanthomonalisin
EC 3.4.21.102 C-terminal processing peptidase
EC 3.4.21.103 physarolisin
EC 3.4.22 Cysteine endopeptidases
EC 3.4.22.1 cathepsin B
EC 3.4.22.2 papain
EC 3.4.22.3 ficain
EC 3.4.22.4 now covered by EC 3.4.22.32 and EC 3.4.22.33
EC 3.4.22.5 now EC 3.4.22.33
EC 3.4.22.6 chymopapain
EC 3.4.22.7 asclepain
EC 3.4.22.8 clostripain
EC 3.4.22.9 now EC 3.4.21.48
EC 3.4.22.10 streptopain
EC 3.4.22.11 now EC 3.4.24.56 (supplement 3)
EC 3.4.22.12 now EC 3.4.19.9
EC 3.4.22.13 deleted

EC 3.4.22.14 actinidain
EC 3.4.22.15 cathepsin L
EC 3.4.22.16 cathepsin H
EC 3.4 22.17 now EC 3.4.22.52 and EC 3.4.22.53
EC 3.4.22.18 deleted, included in EC 3.4.21.26
EC 3.4.22.19 deleted, included in EC 3.4.24.15
EC 3.4.22.20 deleted
EC 3.4.22 21 deleted, included in EC 3.4.99.46
EC 3.4.22.22 now EC 3.4 24.37
EC 3.4.22.23 deleted, included in EC 3.4.21.61
EC 3.4.22.24 cathepsin T
EC 3.4.22.25 glycyl endopeptidase
EC 3.4.22.26 cancer procoagulant
EC 3.4.22.27 cathepsin S
EC 3.4.22.28 picornain 3C
EC 3.4.22.29 picornain 2A
EC 3.4.22.30 caricain
EC 3.4 22.31 ananain
EC 3.4.22.32 stem bromelain
EC 3.4.22.33 fruit bromelain
EC 3.4.22.34 legumain
EC 3.4.22.35 histolysain
EC 3.4.22.36 caspase-1
EC 3.4.22.37 gingipain R
EC 3.4.22.38 cathepsin K
EC 3.4.22.39 adenain
EC 3.4.22.40 bleomycin hydrolase
EC 3.4.22.41 cathepsin F
EC 3.4.22.42 cathepsin O
EC 3.4.22.43 cathepsin V
EC 3.4.22.44 nuclear-inclusion-a endopeptidase
EC 3.4.22.45 helper-component proteinase
EC 3.4.22.46 L-peptidase EC 3.4.22.47 gingipain K
EC 3.4.22.48 staphopain
EC 3.4.22.49 separase
EC 3.4.22.50 V-cath endopeptidase
EC 3.4.22.51 cruzipain
EC 3.4.22.52 calpain-1
EC 3.4.22.53 calpain-2
EC 3.4.23 Aspartic endopeptidases
EC 3.4.23.1 pepsin A
EC 3.4.23.2 pepsin B
EC 3.4.23.3 gastricsin
EC 3.4.23.4 chymosin
EC 3.4.23.5 cathepsin D
EC 3.4.23.6 now covered by EC 3.4.23.18 to EC 3.4.23.28 and EC 3.4.23.30
EC 3.4.23.7 now EC 3.4.23.20
EC 3.4.23.8 now EC 3 4.23.25
EC 3.4.23.9 now EC 3.4.23.21
EC 3.4.23.10 now EC 3.4 23.22
EC 3.4.23.11 deleted
EC 3.4.23.12 nepenthesin
EC 3.4.23.13 deleted
EC 3.4.23.14 deleted
EC 3.4.23 15 renin
EC 3.4.23.16 HIV-1 retropepsin
EC 3.4.23.17 Pro-opiomelanocortin converting enzyme
EC 3.4.23.18 aspergillopepsin I
EC 3.4.23.19 aspergillopepsin II
EC 3.4.23 20 penicillopepsin
EC 3.4.23 21 rhizopuspepsin
EC 3.4.23.22 endothiapepsin
EC 3.4 23.23 mucorpepsin
EC 3.4.23.24 candidapepsin
EC 3.4.23.25 saccharopepsin
EC 3.4.23.26 rhodotorulapepsin
EC 3.4.23.27 now EC 34.21 103
EC 3.4.23.28 acrocylindropepsin
EC 3.4.23.29 polyporopepsin
EC 3.4.23.30 pycnoporopepsin
EC 3.4.23.31 scytalidopepsin A
EC 3.4.23.32 scytalidopepsin B
EC 3.4.23.33 now EC 3.4.21.101
EC 3.4.23.34 cathepsin E
EC 3.4.23.35 barrierpepsin
EC 3.4.23.36 signal peptidase II
EC 3.4.23.37 now EC 3.4.21.100
EC 3.4.23.38 plasmepsin I
EC 3.4.23.39 plasmepsin II
EC 3.4.23.40 phytepsin
EC 3.4.23.41 yapsin 1
EC 3.4.23.42 thermopsin
EC 3.4.23.43 prepilin peptidase
EC 3.4.23.44 nodavirus endopeptidase
EC 3.4.23.45 memapsin 1
EC 3.4.23.46 memapsin 2
EC 3.4.23.47 HIV-2 retropepsin
EC 3.4.23.48 plasminogen activator Pla
EC 3.4.24 Metalloendopeptidases
EC 3.4.24.1 atrolysin A
EC 3.4.24.2 deleted
EC 3.4.24.3 microbial collagenase

EC 3.4.24.4 now covered by EC 3.4.24.25 to EC 3.4.24.32, EC 3.4.24.39 and EC 3.4.24.40
EC 3.4.24.5 now covered by EC 3.4.22.17 and EC 3.4.25.1
EC 3.4.24.6 leucolysin
EC 3.4.24.7 interstitial collagenase
EC 3.4.24.8 deleted, included in EC 3.4.24.3
EC 3.4.24.9 deleted
EC 3.4.24.10 deleted
EC 3.4.24.11 neprilysin
EC 3.4.24.12 envelysin
EC 3.4.24.13 IgA-specific metalloendopeptidase
EC 3.4.24.14 procollagen N-endopeptidase
EC 3.4.24.15 thimet oligopeptidase
EC 3.4.24.16 neurolysin
EC 3.4.24.17 stromelysin 1
EC 3.4.24.18 meprin A
EC 3.4.24.19 procollagen C-endopeptidase
EC 3.4.24.20 peptidyl-Lys metalloendopeptidase
EC 3.4.24.21 astacin
EC 3.4.24.22 stromelysin 2
EC 3.4.24.23 matrilysin
EC 3.4.24.24 gelatinase A
EC 3.4.24.25 vibriolysin
EC 3.4.24.26 pseudolysin
EC 3.4.24.27 thermolysin
EC 3.4.24.28 bacillolysin
EC 3.4.24.29 aureolysin
EC 3.4.24.30 coccolysin
EC 3.4.24.31 mycolysin
EC 3.4.24.32 b-lytic metalloendopeptidase
EC 3.4.24.33 peptidyl-Asp metalloendopeptidase
EC 3.4.24.34 neutrophil collagenase
EC 3.4.24.35 gelatinase B
EC 3.4.24.36 leishmanolysin
EC 3.4.24.37 saccharolysin
EC 3.4.24.38 gametolysin
EC 3.4.24 39 deuterolysin
EC 3.4.24.40 serralysin
EC 3.4.24.41 atrolysin B
EC 3.4.24.42 atrolysin C
EC 3.4.24.43 atroxase
EC 3.4.24.44 atrolysin E
EC 3.4.24.45 atrolysin F
EC 3.4.24.46 adamalysin
EC 3.4.24.47 horrilysin
EC 3.4.24.48 ruberlysin
EC 3.4.24.49 bothropasin
EC 3.4.24.50 bothrolysin
EC 3.4.24.51 ophiolysin
EC 3.4.24.52 trimerelysin I
EC 3.4.24.53 trimerelysin II
EC 3.4.24.54 mucrolysin
EC 3.4.24.55 pitrilysin
EC 3.4.24.56 insulysin
EC 3.4.24.57 O-sialoglycoprotein endopeptidase
EC 3.4.24.58 russellysin
EC 3.4.24.59 mitochondrial intermediate peptidase
EC 3.4.24.60 dactylysin
EC 3.4.24.61 nardilysin
EC 3.4.24.62 magnolysin
EC 3.4.24.63 meprin B
EC 3.4.24.64 mitochondrial processing peptidase
EC 3.4.24.65 macrophage elastase
EC 3.4.24.66 choriolysin L
EC 3.4.24.67 choriolysin H
EC 3.4.24.68 tentoxilysin
EC 3.4.24.69 bontoxilysin
EC 3.4.24.70 oligopeptidase A
EC 3.4.24.71 endothelin-converting enzyme
EC 3.4.24.72 fibrolase
EC 3.4.24.73 jararhagin
EC 3.4.24.74 fragilysin
EC 3.4.24.75 lysostaphin
EC 3.4.24.76 flavastacin
EC 3.4.24.77 snapalysin
EC 3.4.24.78 gpr endopeptidase
EC 3.4.24.79 pappalysin-1
EC 3.4.24.80 membrane-type matrix metalloproteinase-1
EC 3.4.24.81 ADAM10 endopeptidase
EC 3.4.24.82 ADAMTS-4 endopeptidase
EC 3.4.24.83 anthrax lethal factor endopeptidase
EC 3.4.24.84 Ste24 endopeptidase
EC 3.4.24.85 S2P endopeptidase
EC 3.4.24.86 ADAM 17 endopeptidase
EC 3.4.25 Threonine endopeptidases
EC 3.4.25 1 proteasome endopeptidase complex
EC 3.4.99 Endopeptidases of unknown catalytic mechanism
EC 3.4.99.1 now EC 3.4.23.28
EC 3.4.99.2 deleted
EC 3.4.99.3 deleted

EC 3.4.99.4 now EC 3.4.23.12
EC 3.4.99.5 now EC 3.4.24.3
EC 3.4.99.6 now EC 3.4.24.21
EC 3.4.99.7 deleted
EC 3.4.99.8 deleted
EC 3.4.99.9 deleted
EC 3.4.99.10 now EC 3.4.24.56 (supplement 3)
EC 3.4.99.11 deleted
EC 3.4.99.12 deleted
EC 3.4.99.13 now EC 3.4.24.32
EC 3.4.99.14 deleted
EC 3.4.99.15 deleted
EC 3.4.99.16 deleted
EC 3.4.99.17 deleted
EC 3.4.99.18 deleted
EC 3.4.99.19 now EC 3.4.23.15
EC 3.4.99.20 deleted
EC 3.4.99.21 deleted
EC 3.4.99.22 now EC 3.4.24.29
EC 3.4.99.23 deleted
EC 3.4.99.24 deleted
EC 3.4.99.25 deleted, included in EC 3.4.23.21
EC 3.4.99.26 now covered by EC 3.4.21.73 and EC 3.4.21.68
EC 3.4.99.27 deleted
EC 3.4.99.28 now EC 3.4.21.60
EC 3.4.99 29 deleted
EC 3.4.99.30 deleted, included in EC 3.4.24.20
EC 3.4.99.31 deleted, included in EC 3.4.24.15
EC 3.4.99.32 now EC 3.4.24.20
EC 3.4.99.33 deleted
EC 3.4.99.34 deleted
EC 3.4.99.35 now EC 3.4.23.36 (supplement 2)
EC 3.4.99.36 now EC 3.4.21.89 (supplement 2)
EC 3.4.99.37 deleted
EC 3.4.99.38 now EC 3.4.23.17
EC 3.4.99.39 deleted
EC 3.4.99.40 deleted
EC 3.4.99.41 now EC 3.4.24.64 (supplement 2)
EC 3.4.99 42 deleted
EC 3.4.99.43 now EC 3.4.23.42 (supplement 6)
EC 3.4.99.44 now EC 3.4.24.55 (supplement 1)
EC 3.4.99.45 now EC 3.4.24.56 (supplement 1)
EC 3.4.99.46 now EC 3.4.25.1 (supplement 6)

EC 3.5 Acting on Carbon-Nitrogen Bonds, other than Peptide Bonds

EC 3.5.1 In Linear Amides

EC 3.5.1.1 asparaginase
EC 3.5.1.2 glutaminase
EC 3.5.1.3 w-amidase
EC 3.5.1.4 amidase
EC 3.5.1.5 urease
EC 3.5 1.6 b-ureidopropionase
EC 3.5.1.7 ureidosuccinase
EC 3.5.1.8 formylaspartate deformylase
EC 3.5.1.9 arylformamidase
EC 3.5.1.10 formyltetrahydrofolate deformylase
EC 3.5.1.11 penicillin amidase
EC 3.5.1.12 biotinidase
EC 3.5.1.13 aryl-acylamidase
EC 3.5.1.14 aminoacylase
EC 3.5.1.15 aspartoacylase
EC 3.5.1.16 acetylornithine deacetylase
EC 3.5.1.17 acyl-lysine deacylase
EC 3.5.1.18 succinyl-diaminopimelate desuccinylase
EC 3.5.1.19 nicotinamidase
EC 3.5.1.20 citrullinase
EC 3.5.1.21 *N*-acetyl-b-alanine deacetylase
EC 3.5.1.22 pantothenase
EC 3.5.1.23 ceramidase
EC 3.5.1 24 choloylglycine hydrolase
EC 3.5.1.25 *N*-acetylglucosamine-6-phosphate deacetylase
EC 3.5.1.26 N^4-(b-*N*-acetylglucosaminyl)-L-asparaginase
EC 3.5.1.27 *N*-formylmethionylaminoacyl-tRNA deformylase
EC 3.5 1.28 *N*-acetylmuramoyl-L-alanine amidase
EC 3.5.1.29 2-(acetamidomethylene)succinate hydrolase
EC 3.5.1.30 5-aminopentanamidase
EC 3.5.1.31 formylmethionine deformylase
EC 3.5.1.32 hippurate hydrolase
EC 3.5.1.33 *N*-acetylglucosamine deacetylase
EC 3.5.1.34 deleted, same as EC 3.4.13.5
EC 3.5.1.35 D-glutaminase
EC 3.5.1.36 *N*-methyl-2-oxoglutaramate hydrolase
EC 3.5.1.37 deleted, same as EC 3.5.1.26
EC 3.5.1.38 glutamin-(asparagin-)ase
EC 3.5.1.39 alkylamidase
EC 3.5.1.40 acylagmatine amidase
EC 3.5.1.41 chitin deacetylase
EC 3.5.1.42 nicotinamide-nucleotide amidase
EC 3.5.1.43 peptidyl-glutaminase

EC 3.5.1.44 protein-glutamine glutaminase
EC 3.5.1 45 now EC 6.3.4.6
EC 3.5.1.46 6-aminohexanoate-dimer hydrolase
EC 3.5.1.47 *N*-acetyldiaminopimelate deacetylase
EC 3.5.1.48 acetylspermidine deacetylase
EC 3.5.1.49 formamidase
EC 3.5.1.50 pentanamidase
EC 3.5.1.51 4-acetamidobutyryl-CoA deacetylase
EC 3.5.1.52 peptide-N^4-(*N*-acetyl-b-glucosaminyl)asparagine amidase
EC 3.5.1.53 *N*-carbamoylputrescine amidase
EC 3.5.1.54 allophanate hydrolase
EC 3.5.1.55 long-chain-fatty-acyl-glutamate deacylase
EC 3.5.1.56 *N,N*-dimethylformamidase
EC 3.5.1.57 tryptophanamidase
EC 3.5.1.58 *N*-benzyloxycarbonylglycine hydrolase
EC 3.5.1.59 *N*-carbamoylsarcosine amidase
EC 3 5 1.60 *N*-(long-chain-acyl)ethanolamine deacylase
EC 3.5.1 61 mimosinase
EC 3.5.1.62 acetylputrescine deacetylase
EC 3.5.1.63 4-acetamidobutyrate deacetylase
EC 3.5.1.64 N^a-benzyloxycarbonylleucine hydrolase
EC 3.5.1.65 theanine hydrolase
EC 3 5.1.66 2-(hydroxymethyl)-3-(acetamidomethylene)succinate hydrolase
EC 3 5.1.67 4-methyleneglutaminase
EC 3.5.1.68 *N*-formylglutamate deformylase
EC 3.5.1.69 glycosphingolipid deacylase
EC 3.5 1.70 aculeacin-A deacylase
EC 3.5.1.71 *N*-feruloylglycine deacylase
EC 3.5.1.72 D-benzoylarginine-4-nitroanilide amidase
EC 3.5.1.73 carnitinamidase
EC 3.5.1.74 chenodeoxycholoyltaurine hydrolase
EC 3.5.1.75 urethanase
EC 3.5.1.76 arylalkyl acylamidase
EC 3.5.1.77 *N*-carbamoyl-D-amino acid hydrolase
EC 3.5.1.78 glutathionylspermidine amidase
EC 3.5.1.79 phthalyl amidase
EC 3.5.1.80 deleted, identical to EC 3.5.1.25
EC 3.5.1.81 *N*-acyl-D-amino-acid deacylase
EC 3 5.1.82 *N*-acyl-D-glutamate deacylase
EC 3.5.1.83 *N*-acyl-D-aspartate deacylase
EC 3.5.1.84 biuret amidohydrolase
EC 3.5.1.85 (*S*)-*N*-acetyl-1-phenylethylamine hydrolase
EC 3.5.1.86 mandelamide amidase
EC 3.5.1.87 *N*-carbamoyl-L-amino-acid hydrolase
EC 3.5.1.88 peptide deformylase
EC 3.5.1.89 *N*-acetylglucosaminylphosphatidylinositol deacetylase
EC 3.5.1.90 adenosylcobinamide hydrolase

EC 3.5.2 In Cyclic Amides
EC 3.5.2.1 barbiturase
EC 3.5.2.2 dihydropyrimidinase
EC 3.5.2 3 dihydroorotase
EC 3.5.2.4 carboxymethylhydantoinase
EC 3.5.2.5 allantoinase
EC 3.5.2.6 b-lactamase
EC 3.5.2.7 imidazolonepropionase
EC 3.5.2.8 deleted, included in EC 3.5.2.6
EC 3.5.2.9 5-oxoprolinase (ATP-hydrolysing)
EC 3.5.2.10 creatininase
EC 3.5.2.11 L-lysine-lactamase
EC 3.5.2.12 6-aminohexanoate-cyclic-dimer hydrolase
EC 3.5.2.13 2, 5-dioxopiperazine hydrolase
EC 3.5.2.14 *N*-methylhydantoinase (ATP-hydrolysing)
EC 3.5.2 15 cyanuric acid amidohydrolase
EC 3 5 2.16 maleimide hydrolase
EC 3.5.2.17 hydroxyisourate hydrolase
EC 3.5.3 In Linear Amidines
EC 3.5.3.1 arginase
EC 3.5.3.2 guanidinoacetase
EC 3.5.3.3 creatinase
EC 3.5.3.4 allantoicase
EC 3.5.3.5 formiminoaspartate deiminase
EC 3.5.3.6 arginine deiminase
EC 3.5.3.7 guanidinobutyrase
EC 3.5.3.8 formimidoylglutamase
EC 3.5.3.9 allantoate deiminase
EC 3.5.3.10 D-arginase
EC 3.5.3.11 agmatinase
EC 3.5.3.12 agmatine deiminase
EC 3.5.3.13 formiminoglutamate deiminase
EC 3.5.3.14 amidinoaspartase
EC 3.5.3.15 protein-arginine deiminase
EC 3.5.3.16 methylguanidinase

EC 3.5.3.17 guanidinopropionase
EC 3 5.3.18 dimethylargininase
EC 3.5.3.19 ureidoglycolate hydrolase
EC 3.5.3.20 diguanidinobutanase
EC 3.5.3.21 methylenediurea deaminase
EC 3.5.3.22 proclavaminate amidinohydrolase
EC 3.5.4 In Cyclic Amidines
EC 3.5.4.1 cytosine deaminase
EC 3.5.4.2 adenine deaminase
EC 3.5.4.3 guanine deaminase
EC 3.5.4.4 adenosine deaminase
EC 3.5.4.5 cytidine deaminase
EC 3.5.4.6 AMP deaminase
EC 3.5.4.7 ADP deaminase
EC 3.5.4.8 aminoimidazolase
EC 3.5.4.9 methenyltetrahydrofolate cyclohydrolase
EC 3.5.4.10 IMP cyclohydrolase
EC 3.5.4.11 pterin deaminase
EC 3.5.4.12 dCMP deaminase
EC 3.5.4.13 dCTP deaminase
EC 3.5.4.14 deoxycytidine deaminase
EC 3.5.4.15 guanosine deaminase
EC 3.5.4.16 GTP cyclohydrolase I
EC 3.5.4.17 adenosine-phosphate deaminase
EC 3.5.4.18 ATP deaminase
EC 3.5.4.19 phosphoribosyl-AMP cyclohydrolase
EC 3.5.4.20 pyrithiamine deaminase
EC 3.5.4.21 creatinine deaminase
EC 3.5.4.22 1-pyrroline-4-hydroxy-2-carboxylate deaminase
EC 3.5.4.23 blasticidin-S deaminase
EC 3.5.4.24 sepiapterin deaminase
EC 3.5.4.25 GTP cyclohydrolase II
EC 3.5.4.26 diaminohydroxyphosphoribosylamino-pyrimidine deaminase
EC 3.5.4.27 methenyltetrahydromethanopterin cyclohydrolase
EC 3.5.4.28 *S*-adenosylhomocysteine deaminase
EC 3.5.4.29 GTP cyclohydrolase IIa
EC 3.5.4.30 dCTP deaminase (dUMP-forming)
EC 3.5.5 In Nitriles
EC 3.5.5.1 nitrilase
EC 3.5.5.2 ricinine nitrilase
EC 3.5.5.3 now EC 4.3.99.1
EC 3.5.5.4 cyanoalanine nitrilase
EC 3.5.5.5 arylacetonitrilase
EC 3.5.5.6 bromoxynil nitrilase
EC 3.5.5.7 aliphatic nitrilase
EC 3.5.5.8 thiocyanate hydrolase
EC 3.5.99 In Other Compounds
EC 3.5.99.1 riboflavinase
EC 3.5.99.2 thiaminase
EC 3.5.99.3 hydroxydechloroatrazine ethylaminohydrolase
EC 3.5.99.4 *N*-isopropylammelide isopropylaminohydrolase
EC 3 5.99.5 2-aminomuconate deaminase
EC 3.5.99.6 glucosamine-6-phosphate deaminase
EC 3.5.99.7 1-aminocyclopropane-1-carboxylate deaminase
EC 3.6 Acting on Acid Anhydrides
EC 3.6.1 In Phosphorus-Containing Anhydrides
EC 3.6.1.1 inorganic diphosphatase
EC 3.6.1.2 trimetaphosphatase
EC 3.6 1.3 adenosinetriphosphatase
EC 3.6.1.4 deleted, included in EC 3.6.1.3
EC 3.6.1.5 apyrase
EC 3.6.1.6 nucleoside-diphosphatase
EC 3.6.1.7 acylphosphatase
EC 3.6.1.8 ATP diphosphatase
EC 3.6.1.9 nucleotide diphosphatase
EC 3.6.1.10 endopolyphosphatase
EC 3.6.1.11 exopolyphosphatase
EC 3.6.1.12 dCTP diphosphatase
EC 3.6.1.13 ADP-ribose diphosphatase
EC 3.6.1.14 adenosine-tetraphosphatase
EC 3.6.1.15 nucleoside-triphosphatase
EC 3.6.1.16 CDP-glycerol diphosphatase
EC 3.6.1.17 bis(5'-nucleosyl)-tetraphosphatase (asymmetrical)
EC 3.6.1.18 FAD diphosphatase
EC 3.6.1.19 nucleoside-triphosphate diphosphatase
EC 3.6.1.20 5'-acylphosphoadenosine hydrolase
EC 3.6.1.21 ADP-sugar diphosphatase
EC 3.6.1.22 NAD^+ diphosphatase
EC 3.6.1.23 dUTP diphosphatase
EC 3.6.1.24 nucleoside phosphoacylhydrolase
EC 3.6.1.25 triphosphatase
EC 3.6.1.26 CDP-diacylglycerol diphosphatase
EC 3.6.1.27 undecaprenyl-diphosphatase
EC 3.6.1.28 thiamine-triphosphatase
EC 3.6.1.29 bis(5'-adenosyl)-triphosphatase
EC 3.6.1.30 $m^7G(5')pppN$ diphosphatase
EC 3.6.1.31 phosphoribosyl-ATP diphosphatase
EC 3.6.1.32 now EC 3.6.4.1

EC 3.6.1.33 now EC 3.6.4.2
EC 3.6.1.34 now EC 3.6.3.14
EC 3.6.1.35 now EC 3.6.3.6
EC 3.6.1.36 now EC 3.6.3.10
EC 3.6.1.37 now EC 3.6.3.9
EC 3.6.1.38 now EC 3.6.3.8
EC 3.6.1.39 thymidine-triphosphatase
EC 3.6.1.40 guanosine-5'-triphosphate, 3'-diphosphate diphosphatase
EC 3.6.1.41 bis(5'-nucleosyl)-tetraphosphatase (symmetrical)
EC 3.6.1.42 guanosine-diphosphatase
EC 3.6.1.43 dolichyldiphosphatase
EC 3.6.1.44 oligosaccharide-diphosphodolichol diphosphatase
EC 3.6.1.45 UDP-sugar diphosphatase
EC 3.6.1.46 now EC 3.6.5.1
EC 3.6.1.47 now EC 3.6.5.2
EC 3.6.1.48 now EC 3.6.5.3
EC 3.6.1.49 now EC 3.6.5.4
EC 3.6.1.50 now EC 3.6.5.5
EC 3.6.1.51 now EC 3.6.5.6
EC 3.6.1.52 diphosphoinositol-polyphosphate diphosphatase
EC 3.6.2 In Sulfonyl-Containing Anhydrides
EC 3.6.2.1 adenylylsulfatase
EC 3.6.2.2 phosphoadenylylsulfatase
EC 3.6.3 Acting on acid anhydrides; catalysing transmembrane movement of substances
EC 3.6.3.1 Mg^{2+}-ATPase
EC 3.6.3 2 Mg^{2+}-importing ATPase
EC 3.6.3.3 Cd^{2+}-exporting ATPase
EC 3.6.3.4 Cu^{2+}-exporting ATPase
EC 3.6.3.5 Zn^{2+}-exporting ATPase
EC 3.6.3.6 H^+-exporting ATPase
EC 3.6.3.7 Na^+-exporting ATPase
EC 3.6.3.8 Ca^{2+}-transporting ATPase
EC 3.6.3.9 Na^+/K^+-exchanging ATPase
EC 3.6.3.10 H^+/K^+-exchanging ATPase
EC 3.6.3.11 Cl^--transporting ATPase
EC 3.6.3.12 K^+-transporting ATPase
EC 3.6.3.13 deleted, identical to EC 3.6.3.1
EC 3.6.3.14 H^+-transporting two-sector ATPase
EC 3.6.3.15 Na^+-transporting two-sector ATPase
EC 3.6.3.16 arsenite-transporting ATPase
EC 3.6.3.17 monosaccharide-transporting ATPase
EC 3.6.3.18 oligosaccharide-transporting ATPase
EC 3.6.3.19 maltose-transporting ATPase
EC 3.6.3.20 glycerol-3-phosphate-transporting ATPase
EC 3.6.3.21 polar-amino-acid-transporting ATPase
EC 3.6.3.22 nonpolar-amino-acid-transporting ATPase
EC 3.6.3.23 oligopeptide-transporting ATPase
EC 3.6.3.24 nickel-transporting ATPase
EC 3.6.3.25 sulfate-transporting ATPase
EC 3.6.3.26 nitrate-transporting ATPase
EC 3.6.3.27 phosphate-transporting ATPase
EC 3.6.3.28 phosphonate-transporting ATPase
EC 3.6.3.29 molybdate-transporting ATPase
EC 3.6.3.30 Fe^{3+}-transporting ATPase
EC 3.6.3.31 polyamine-transporting ATPase
EC 3.6.3.32 quaternary-amine-transporting ATPase
EC 3.6.3.33 vitamin B_{12}-transporting ATPase
EC 3.6.3.34 iron-chelate-transporting ATPase
EC 3.6.3.35 manganese-transporting ATPase
EC 3.6.3.36 taurine-transporting ATPase
EC 3.6.3.37 guanine-transporting ATPase
EC 3.6.3.38 capsular-polysaccharide-transporting ATPase
EC 3.6.3.39 lipopolysaccharide-transporting ATPase
EC 3.6.3.40 teichoic-acid-transporting ATPase
EC 3.6.3.41 heme-transporting ATPase
EC 3.6.3.42 b-glucan-transporting ATPase
EC 3.6.3.43 peptide-transporting ATPase
EC 3.6.3.44 xenobiotic-transporting ATPase
EC 3.6.3.45 steroid-transporting ATPase
EC 3.6.3.46 cadmium-transporting ATPase
EC 3.6.3.47 fatty-acyl-CoA-transporting ATPase
EC 3.6.3.48 a-factor-transporting ATPase
EC 3.6.3.49 channel-conductance-controlling ATPase
EC 3.6.3.50 protein-secreting ATPase
EC 3.6.3.51 mitochondrial protein-transporting ATPase
EC 3.6.3.52 chloroplast protein-transporting ATPase
EC 3.6.3.53 Ag^+-exporting ATPase
EC 3.6.4 Acting on acid anhydrides; involved in cellular and subcellular movement
EC 3.6.4.1 myosin ATPase
EC 3.6.4.2 dynein ATPase
EC 3.6.4.3 microtubule-severing ATPase
EC 3.6.4.4 plus-end-directed kinesin ATPase
EC 3.6.4.5 minus-end-directed kinesin ATPase
EC 3.6.4.6 vesicle-fusing ATPase

EC 3.6.4.7 peroxisome-assembly ATPase
EC 3.6.4.8 proteasome ATPase
EC 3.6.4.9 chaperonin ATPase
EC 3.6.4.10 non-chaperonin molecular chaperone ATPase
EC 3.6.4.11 nucleoplasmin ATPase
EC 3.6.5 Acting on GTP; involved in cellular and subcellular movement
EC 3.6.5.1 heterotrimeric G-protein GTPase
EC 3.6.5.2 small monomeric GTPase
EC 3.6.5.3 protein-synthesizing GTPase
EC 3.6.5.4 signal-recognition-particle GTPase
EC 3.6.5.5 dynamin GTPase
EC 3.6.5.6 tubulin GTPase
EC 3.7 Acting on Carbon-Carbon Bonds
EC 3.7.1 In Ketonic Substances
EC 3.7.1.1 oxaloacetase
EC 3.7.1.2 fumarylacetoacetase
EC 3.7.1.3 kynureninase
EC 3.7.1.4 phloretin hydrolase
EC 3.7.1.5 acylpyruvate hydrolase
EC 3.7.1.6 acetylpyruvate hydrolase
EC 3.7.1.7 b-diketone hydrolase
EC 3.7.1.8 2,6-dioxo-6-phenylhexa-3-enoate hydrolase
EC 3.7.1.9 2-hydroxymuconate-semialdehyde hydrolase
EC 3.7.1.10 cyclohexane-1,3-dione hydrolase
EC 3.8 Acting on Halide Bonds
EC 3.8.1 In C-Halide Compounds
EC 3.8.1.1 alkylhalidase
EC 3.8.1.2 (*S*)-2-haloacid dehalogenase
EC 3.8.1.3 haloacetate dehalogenase
EC 3.8.1.4 now EC 1.97.1.10
EC 3.8.1.5 haloalkane dehalogenase
EC 3.8.1.6 4-chlorobenzoate dehalogenase
EC 3.8.1.7 4-chlorobenzoyl-CoA dehalogenase
EC 3.8.1.8 atrazine chlorohydrolase
EC 3.8.1.9 (*R*)-2-haloacid dehalogenase
EC 3.8.1.10 2-haloacid dehalogenase (configuration- inverting)
EC 3.8.1.11 2-haloacid dehalogenase (configuration-retaining)
EC 3.8.2 In P-Halide Compounds
EC 3.8.2.1 now EC 3.1.8.2
EC 3.9 Acting on Phosphorus-Nitrogen Bonds
EC 3.9.1.1 phosphoamidase
EC 3.10 Acting on Sulfur-Nitrogen Bonds
EC 3.10.1.1 *N*-sulfoglucosamine sulfohydrolase
EC 3.10.1.2 cyclamate sulfohydrolase
EC 3.11 Acting on Carbon-Phosphorus Bonds
EC 3.11.1.1 phosphonoacetaldehyde hydrolase
EC 3.11.1.2 phosphonoacetate hydrolase
EC 3.12 Acting on Sulfur-Sulfur Bonds
EC 3.12.1.1 trithionate hydrolase
EC 3.13 Acting on Carbon-Sulfur Bonds
EC 3.13.1.1 UDP-sulfoquinovose synthase
EC 4 Lyases
EC 4.1 Carbon-Carbon Lyases
EC 4.1.1 Carboxy-Lyases
EC 4.1.1.1 pyruvate decarboxylase
EC 4.1.1.2 oxalate decarboxylase
EC 4.1.1.3 oxaloacetate decarboxylase
EC 4.1.1.4 acetoacetate decarboxylase
EC 4.1.1.5 acetolactate decarboxylase
EC 4.1.1.6 aconitate decarboxylase
EC 4.1.1.7 benzoylformate decarboxylase
EC 4.1.1.8 oxalyl-CoA decarboxylase
EC 4.1.1.9 malonyl-CoA decarboxylase
EC 4.1.1.10 deleted, included in EC 4.1.1.12
EC 4.1.1.11 aspartate 1-decarboxylase
EC 4.1.1.12 aspartate 4-decarboxylase
EC 4.1.1.13 deleted
EC 4.1.1.14 valine decarboxylase
EC 4.1.1.15 glutamate decarboxylase
EC 4.1.1 16 hydroxyglutamate decarboxylase
EC 4.1.1.17 ornithine decarboxylase
EC 4.1.1.18 lysine decarboxylase
EC 4.1.1.19 arginine decarboxylase
EC 4.1.1.20 diaminopimelate decarboxylase
EC 4.1.1.21 phosphoribosylaminoimidazole carboxylase
EC 4.1.1.21 phosphoribosylaminoimidazole carboxylase
EC 4.1.1.22 histidine decarboxylase
EC 4.1.1.23 orotidine-5'-phosphate decarboxylase
EC 4.1.1.24 aminobenzoate decarboxylase
EC 4.1.1.25 tyrosine decarboxylase
EC 4.1.1.26 deleted, included in EC 4.1.1.28
EC 4.1.1.27 deleted, included in EC 4.1.1.28
EC 4.1.1.28 aromatic-L-amino-acid decarboxylase
EC 4.1.1.29 sulfoalanine decarboxylase
EC 4.1.1.30 pantothenoylcysteine decarboxylase
EC 4.1.1.31 phospho*enol*pyruvate carboxylase

EC 4.1.1.32 phospho*enol*pyruvate carboxykinase (GTP)
EC 4.1.1.33 diphosphomevalonate decarboxylase
EC 4.1.1.34 dehydro-L-gulonate decarboxylase
EC 4.1.1.35 UDP-glucuronate decarboxylase
EC 4.1.1.36 phosphopantothenoylcysteine decarboxylase
EC 4.1.1.37 uroporphyrinogen decarboxylase
EC 4.1.1.38 phospho*enol*pyruvate carboxykinase (diphosphate)
EC 4.1.1.39 ribulose-bisphosphate carboxylase
EC 4.1.1.40 hydroxypyruvate decarboxylase
EC 4.1.1.41 methylmalonyl-CoA decarboxylase
EC 4.1.1.42 carnitine decarboxylase
EC 4.1.1.43 phenylpyruvate decarboxylase
EC 4.1.1.44 4-carboxymuconolactone decarboxylase
EC 4.1.1.45 aminocarboxymuconate-semialdehyde decarboxylase
EC 4.1.1.46 *o*-pyrocatechuate decarboxylase
EC 4.1.1.47 tartronate-semialdehyde synthase
EC 4.1.1.48 indole-3-glycerol-phosphate synthase
EC 4.1.1.49 phospho*enol*pyruvate carboxykinase (ATP)
EC 4.1.1.50 adenosylmethionine decarboxylase
EC 4.1.1.51 3-hydroxy-2-methylpyridine-4, 5-dicarboxylate 4-decarboxylase
EC 4.1.1.52 6-methylsalicylate decarboxylase
EC 4.1.1.53 phenylalanine decarboxylase
EC 4.1.1 54 dihydroxyfumarate decarboxylase
EC 4.1.1.55 4,5-dihydroxyphthalate decarboxylase
EC 4.1.1.56 3-oxolaurate decarboxylase
EC 4.1.1.57 methionine decarboxylase
EC 4.1.1.58 orsellinate decarboxylase
EC 4.1.1.59 gallate decarboxylase
EC 4.1.1.60 stipitatonate decarboxylase
EC 4.1.1.61 4-hydroxybenzoate decarboxylase
EC 4.1.1.62 gentisate decarboxylase
EC 4.1.1.63 protocatechuate decarboxylase
EC 4.1.1.64 2,2-dialkylglycine decarboxylase (pyruvate)
EC 4.1.1.65 phosphatidylserine decarboxylase
EC 4.1.1.66 uracil-5-carboxylate decarboxylase
EC 4.1.1.67 UDP-galacturonate decarboxylase
EC 4.1.1.68 5-oxopent-3-ene-1,2,5-tricarboxylate decarboxylase
EC 4.1.1.69 3,4-dihydroxyphthalate decarboxylase
EC 4.1.1.70 glutaconyl-CoA decarboxylase
EC 4.1.1.71 2-oxoglutarate decarboxylase
EC 4.1.1.72 branched-chain-2-oxoacid decarboxylase
EC 4.1.1.73 tartrate decarboxylase
EC 4.1.1.74 indolepyruvate decarboxylase
EC 4.1.1.75 5-guanidino-2-oxopentanoate decarboxylase
EC 4.1.1.76 arylmalonate decarboxylase
EC 4.1.1.77 4-oxalocrotonate decarboxylase
EC 4.1.1.78 acetylenedicarboxylate decarboxylase
EC 4.1.1.79 sulfopyruvate decarboxylase
EC 4.1.1.80 4-hydroxyphenylpyruvate decarboxylase EC 4.1.1.81 threonine-phosphate decarboxylase
EC 4.1.2 Aldehyde-Lyases
EC 4.1.2.1 deleted, included in EC 4.1.3.16
EC 4.1.2.2 ketotetrose-phosphate aldolase
EC 4.1.2.3 deleted
EC 4.1.2.4 deoxyribose-phosphate aldolase
EC 4.1.2.5 threonine aldolase
EC 4.1.2.6 deleted
EC 4.1.2.7 deleted, included in EC 4.1.2.13
EC 4.1.2.8 deleted
EC 4.1.2.9 phosphoketolase
EC 4.1.2.10 mandelonitrile lyase
EC 4.1.2.11 hydroxymandelonitrile lyase
EC 4.1.2.12 ketopantoaldolase
EC 4.1.2.13 fructose-bisphosphate aldolase
EC 4.1.2.14 2-dehydro-3-deoxy-phosphogluconate aldolase
EC 4.1.2.15 now EC 2.5.1.54
EC 4.1.2.16 now EC 2.5.1.55
EC 4.1 2.17 L-fuculose-phosphate aldolase
EC 4.1.2.18 2-dehydro-3-deoxy-L-pentonate aldolase
EC 4.1.2.19 rhamnulose-1-phosphate aldolase
EC 4.1.2.20 2-dehydro-3-deoxyglucarate aldolase
EC 4.1.2.21 2-dehydro-3-deoxy-6-phosphogalactonate aldolase
EC 4.1.2.22 fructose-6-phosphate phosphoketolase
EC 4.1.2.23 3-deoxy-D-*manno*-octulosonate aldolase
EC 4.1.2.24 dimethylaniline-*N*-oxide aldolase
EC 4.1.2.25 dihydroneopterin aldolase
EC 4.1.2.26 phenylserine aldolase
EC 4.1.2.27 sphinganine-1-phosphate aldolase
EC 4.1.2.28 2-dehydro-3-deoxy-D-pentonate aldolase

EC 4.1.2.29 5-dehydro-2-deoxyphosphogluconate aldolase
EC 4.1 2.30 17a-hydroxyprogesterone aldolase
EC 4.1.2.31 deleted, included in EC 4.1.3.16
EC 4 1.2.32 trimethylamine-oxide aldolase
EC 4.1.2.33 fucosterol-epoxide lyase
EC 4.1.2.34 4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase
EC 4.1.2.35 propioin synthase
EC 4.1.2.36 lactate aldolase
EC 4.1.2.37 acetone-cyanhydrin lyase
EC 4.1.2.38 benzoin aldolase
EC 4.1.2.39 hydroxynitrilase
EC 4.1.2.40 tagatose-bisphosphate aldolase
EC 4.1.2.41 vanillin synthase
EC 4.1.3 Oxo-Acid-Lyases
EC 4.1.3.1 isocitrate lyase
EC 4.1.3.2 now EC 2.3.3.9
EC 4.1.3.3 *N*-acetylneuraminate lyase
EC 4.1.3.4 hydroxymethylglutaryl-CoA lyase
EC 4.1.3.5 now EC 2.3.3.10
EC 4.1.3.6 citrate (*pro*-3*S*)-lyase
EC 4.1.3.7 now EC 2.3.3.1
EC 4.1.3.8 now EC 2.3.3.8
EC 4.1.3.9 now EC 2.3.3.11
EC 4.1.3.10 now EC 2.3.3.7
EC 4.1.3.11 now EC 2.3.3 12
EC 4.1.3.12 now EC 2.3.3.13
EC 4.1.3.13 oxalomalate lyase
EC 4.1.3.14 3-hydroxyaspartate aldolase
EC 4.1.3.15 now EC 2.2.1.5
EC 4.1.3.16 4-hydroxy-2-oxoglutarate aldolase
EC 4.1.3.17 4-hydroxy-4-methyl-2-oxoglutarate aldolase
EC 4.1.3.18 now EC 2.2.1.6
EC 4.1.3.19 now EC 2.5.1.56
EC 4.1.3.20 now EC 2.5.1.57
EC 4.1.3.21 now EC 2.3.3.14
EC 4.1.3.22 citramalate lyase
EC 4.1.3.23 now EC 2.3.3.2
EC 4.1.3.24 malyl-CoA lyase
EC 4.1.3.25 citramalyl-CoA lyase
EC 4.1.3.26 3-hydroxy-3-isohexenylglutaryl-CoA lyase
EC 4.1.3.27 anthranilate synthase
EC 4.1.3.28 now EC 2.3.3.3
EC 4.1.3.29 now EC 2.3.3.4
EC 4.1.3.30 methylisocitrate lyase
EC 4.1.3.31 now EC 2.3.3.5
EC 4.1.3.32 2,3-dimethylmalate lyase
EC 4.1.3.33 now EC 2.3.3.6
EC 4.1.3.34 citryl-CoA lyase
EC 4.1.3.35 (1-hydroxycyclohexan-1-yl) acetyl-CoA lyase
EC 4.1.3.36 naphthoate synthase
EC 4.1.3.37 now EC 2.2.1.7
EC 4.1.3.38 aminodeoxychorismate lyase
EC 4 1.99 Other Carbon-Carbon Lyases
EC 4.1.99.1 tryptophanase
EC 4.1.99.2 tyrosine phenol-lyase
EC 4.1.99.3 deoxyribodipyrimidine photo-lyase
EC 4.1.99.4 now EC 3.5.99.7
EC 4.1.99.5 octadecanal decarbonylase
EC 4 1.99.6 now EC 4.2 3.6
EC 4.1.99.7 now EC 4.2.3.9
EC 4.1.99.8 now EC 4.2.3.14
EC 4.1.99.9 now EC 4.2.3.15
EC 4.1.99.10 now EC 4.2.3.16
EC 4.1.99.11 benzylsuccinate synthase
EC 4.2 Carbon-Oxygen Lyases
EC 4.2.1 Hydro-Lyases
EC 4.2.1.1 carbonate dehydratase
EC 4.2.1.2 fumarate hydratase
EC 4.2.1.3 aconitate hydratase
EC 4.2.1.4 citrate dehydratase
EC 4.2.1.5 arabinonate dehydratase
EC 4.2.1.6 galactonate dehydratase
EC 4.2.1.7 altronate dehydratase
EC 4.2 1.8 mannonate dehydratase
EC 4.2.1.9 dihydroxy-acid dehydratase
EC 4.2.1.10 3-dehydroquinate dehydratase
EC 4.2.1.11 phosphopyruvate hydratase
EC 4.2.1.12 phosphogluconate dehydratase
EC 4.2.1.13 now EC 4.3.1.17
EC 4.2.1.14 now EC 4.3.1.18
EC 4.2.1.15 now EC 4.4.1.1
EC 4.2.1.16 now EC 4.3.1.19
EC 4.2.1.17 enoyl-CoA hydratase
EC 4.2.1.18 methylglutaconyl-CoA hydratase
EC 4.2.1.19 imidazoleglycerol-phosphate dehydratase
EC 4.2.1.20 tryptophan synthase
EC 4.2.1.21 now EC 4.2.1.22
EC 4.2.1.22 cystathionine b-synthase

EC 4.2.1.23 deleted
EC 4.2.1.24 porphobilinogen synthase
EC 4.2.1.25 L-arabinonate dehydratase
EC 4.2.1.26 now EC 4.3.1.21
EC 4.2.1.27 acetylenecarboxylate hydratase
EC 4.2.1.28 propanediol dehydratase
EC 4.2.1.29 now EC 4.99.1.6
EC 4.2.1.30 glycerol dehydratase
EC 4.2.1.31 maleate hydratase
EC 4.2.1.32 L(+)-tartrate dehydratase
EC 4.2.1.33 3-isopropylmalate dehydratase
EC 4.2.1.34 (*S*)-2-methylmalate dehydratase
EC 4.2.1.35 (*R*)-2-methylmalate dehydratase
EC 4.2.1.36 homoaconitate hydratase
EC 4.2.1.37 now EC 3.3.2.4
EC 4.2.1.38 now EC 4.3.1.20
EC 4.2.1.39 gluconate dehydratase
EC 4.2.1.40 glucarate dehydratase
EC 4.2.1.41 5-dehydro-4-deoxyglucarate dehydratase
EC 4.2.1.42 galactarate dehydratase
EC 4.2.1.43 2-dehydro-3-deoxy-L-arabinonate dehydratase
EC 4.2.1.44 *myo*-inosose-2 dehydratase
EC 4.2.1.45 CDP-glucose 4,6-dehydratase
EC 4.2.1.46 dTDP-glucose 4,6-dehydratase
EC 4.2.1.47 GDP-mannose 4,6-dehydratase
EC 4.2.1.48 D-glutamate cyclase
EC 4.2.1.49 urocanate hydratase
EC 4.2.1.50 pyrazolylalanine synthase
EC 4.2.1.51 prephenate dehydratase
EC 4.2.1.52 dihydrodipicolinate synthase
EC 4.2.1.53 oleate hydratase
EC 4.2.1.54 lactoyl-CoA dehydratase
EC 4.2.1.55 3-hydroxybutyryl-CoA dehydratase
EC 4.2.1.56 itaconyl-CoA hydratase
EC 4.2.1.57 isohexenylglutaconyl-CoA hydratase
EC 4.2.1.58 crotonoyl-[acyl-carrier-protein] hydratase
EC 4.2.1.59 3-hydroxyoctanoyl-[acyl-carrier-protein] dehydratase
EC 4.2.1.60 3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase
EC 4.2.1.61 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase
EC 4.2.1.62 5a-hydroxysteroid dehydratase
EC 4.2.1.63 now EC 3.3.2.3
EC 4.2.1.64 now EC 3.3.2.3
EC 4.2.1.65 3-cyanoalanine hydratase
EC 4.2.1.66 cyanide hydratase
EC 4.2.1.67 D-fuconate dehydratase
EC 4.2.1.68 L-fuconate dehydratase
EC 4.2.1.69 cyanamide hydratase
EC 4.2.1.70 pseudouridylate synthase
EC 4.2.1.71 identical to EC 4.2.1.27
EC 4.2.1.72 now EC 4.1.1.78
EC 4.2.1.73 protoaphin-aglucone dehydratase (cyclizing)
EC 4.2.1.74 long-chain-enoyl-CoA hydratase
EC 4.2.1.75 uroporphyrinogen-III synthase
EC 4.2.1.76 UDP-glucose 4,6-dehydratase
EC 4.2.1.77 *trans*-L-3-hydroxyproline dehydratase
EC 4.2.1.78 (*S*)-norcoclaurine synthase
EC 4.2.1.79 2-methylcitrate dehydratase
EC 4.2.1.80 2-oxopent-4-enoate hydratase
EC 4.2.1.81 D(-)-tartrate dehydratase
EC 4.2.1.82 xylonate dehydratase
EC 4.2.1.83 4-oxalmesaconate hydratase
EC 4.2.1.84 nitrile hydratase
EC 4.2.1.85 dimethylmaleate hydratase
EC 4.2.1.86 16-dehydroprogesterone hydratase
EC 4.2.1.87 octopamine dehydratase
EC 4.2.1.88 synephrine dehydratase
EC 4.2.1.89 carnitine dehydratase
EC 4.2.1.90 L-rhamnonate dehydratase
EC 4.2.1.91 carboxycyclohexadienyl dehydratase
EC 4.2.1.92 hydroperoxide dehydratase
EC 4.2.1.93 ATP-dependent NAD(P)H-hydrate dehydratase
EC 4.2.1.94 scytalone dehydratase
EC 4.2.1.95 kievitone hydratase
EC 4.2.1.96 4a-hydroxytetrahydrobiopterin dehydratase
EC 4.2.1.97 phaseollidin hydratase
EC 4.2.1.98 16a-hydroxyprogesterone dehydratase
EC 4.2.1.99 2-methylisocitrate dehydratase
EC 4.2.1.100 cyclohexa-1,5-dienecarbonyl-CoA hydratase
EC 4.2.1.101 *trans*-feruloyl-CoA hydratase
EC 4.2.1.102 now EC 4.2.1.100
EC 4.2.1.103 cyclohexyl-isocyanide hydratase
EC 4.2.1.104 cyanate hydratase
EC 4.2.2 Acting on Polysaccharides
EC 4.2.2.1 hyaluronate lyase
EC 4.2.2.2 pectate lyase

EC 4.2.2.3 poly(b-D-mannuronate) lyase
EC 4.2.2.4 chondroitin ABC lyase
EC 4.2.2.5 chondroitin AC lyase
EC 4.2 2.6 oligogalacturonide lyase
EC 4.2 2.7 heparin lyase
EC 4.2.2.8 heparin-sulfate lyase
EC 4.2.2.9 pectate disaccharide-lyase
EC 4.2.2.10 pectin lyase
EC 4.2.2.11 poly(a-L-guluronate) lyase
EC 4.2.2.12 xanthan lyase
EC 4.2.2.13 exo-(14)-a-D-glucan lyase
EC 4.2.2.14 glucuronan lyase
EC 4.2.2.15 anhydrosialidase
EC 4.2.2.16 levan fructotransferase (DFA-IV-forming)
EC 4.2.2.17 inulin fructotransferase (DFA-I-forming)
EC 4.2.2.18 inulin fructotransferase (DFA-III-forming)
EC 4.2.3 Acting on phosphates
EC 4.2.3.1 threonine synthase
EC 4.2.3.2 ethanolamine-phosphate phospho-lyase
EC 4.2.3.3 methylglyoxal synthase
EC 4.2.3.4 3-dehydroquinate synthase
EC 4.2.3.5 chorismate synthase
EC 4.2.3.6 trichodiene synthase
EC 4.2.3.7 pentalenene synthase
EC 4 2.3.8 casbene synthase
EC 4.2.3.9 aristolochene synthase
EC 4.2.3.10 (-)-*endo*-fenchol synthase
EC 4.2.3.11 sabinene-hydrate synthase
EC 4.2.3.12 6-pyruvoyltetrahydropterin synthase
EC 4.2.3 13 (+)-d-cadinene synthase
EC 4.2.3.14 pinene synthase
EC 4.2.3.15 myrcene synthase
EC 4.2.3.16 (4*S*)-limonene synthase
EC 4.2.3.17 taxadiene synthase
EC 4.2.3.18 abietadiene synthase
EC 4.2.3.19 *ent*-kaurene synthase
EC 4.2.3.20 (*R*)-limonene synthase
EC 4.2.3.21 vetispiradiene synthase
EC 4.2.99 Other Carbon-Oxygen Lyases
EC 4.2.99.1 now EC 4.2.2.2
EC 4.2.99.2 now EC 4.2.3.1
EC 4.2.99.3 now EC 4.2.2.2
EC 4.2.99.4 now EC 4.2.2.3
EC 4.2.99.5 deleted
EC 4.2.99.6 deleted, included in EC 4.2.2.4 and EC 4.2.2.5
EC 4.2.99.7 now EC 4.2.3.2
EC 4.2.99.8 now EC 2.5.1.47
EC 4.2.99.9 now EC 2.5.1.48
EC 4.2.99.10 now EC 2.5.1.49
EC 4.2.99.11 now EC 4.2 3.3
EC 4.2.99.12 carboxymethyloxysuccinate lyase
EC 4.2.99.13 now EC 2.5.1.50
EC 4.2.99.14 now EC 2.5.1.51
EC 4.2.99.15 now EC 2.5.1 52
EC 4 2.99.16 now EC 2.5.1.53
EC 4.2.99.17 now EC 4.2.99.14
EC 4.2.99.18 DNA-(apurinic or apyrimidinic site) lyase
EC 4.2.99.19 2-hydroxypropyl-CoM lyase
EC 4.3 Carbon-Nitrogen Lyases
EC 4.3.1 Ammonia-Lyases
EC 4.3.1.1 aspartate ammonia-lyase
EC 4 3.1.2 methylaspartate ammonia-lyase
EC 4.3.1.3 histidine ammonia-lyase
EC 4.3.1.4 formiminotetrahydrofolate cyclodeaminase
EC 4.3.1.5 phenylalanine ammonia-lyase
EC 4.3.1.6 b-alanyl-CoA ammonia-lyase
EC 4.3.1.7 ethanolamine ammonia-lyase
EC 4.3.1.8 now EC 2.5 1.61
EC 4.3.1.9 glucosaminate ammonia-lyase
EC 4.3.1.10 serine-sulfate ammonia-lyase
EC 4.3.1.11 dihydroxyphenylalanine ammonia-lyase
EC 4.3.1.12 ornithine cyclodeaminase
EC 4.3.1.13 carbamoyl-serine ammonia-lyase
EC 4.3.1.14 3-aminobutyryl-CoA ammonia-lyase
EC 4.3.1.15 diaminopropionate ammonia-lyase
EC 4.3.1.16 *threo*-3-hydroxyaspartate ammonia-lyase
EC 4.3.1.17 L-serine ammonia-lyase
EC 4.3.1.18 D-serine ammonia-lyase
EC 4.3.1.19 threonine ammonia-lyase
EC 4.3.1.20 *erythro*-3-hydroxyaspartate ammonia-lyase EC 4.3.1.21 identical to EC 4.3.1.9
EC 4.3.2 Amidine-Lyases
EC 4.3.2.1 argininosuccinate lyase
EC 4.3.2.2 adenylosuccinate lyase
EC 4.3.2.3 ureidoglycolate lyase
EC 4.3.2.4 purine imidazole-ring cyclase
EC 4.3.2.5 peptidylamidoglycolate lyase
EC 4.3.3 Amine-Lyases
EC 4.3.3.1 3-ketovalidoxylamine C-N-lyase

EC 4.3 3.2 strictosidine synthase
EC 4.3.3.3 deacetylisoipecoside synthase
EC 4.3.3.4 deacetylipecoside synthase
EC 4.3.99 Other Carbon-Nitrogen Lyases
EC 4.3.99.1 now EC 4.2.1.104
EC 4.4 Carbon-Sulfur Lyases
EC 4.4.1.1 cystathionine g-lyase
EC 4.4.1.2 homocysteine desulfhydrase
EC 4.4.1.3 dimethylpropiothetin dethiomethylase
EC 4.4.1.4 alliin lyase
EC 4.4.1.5 lactoylglutathione lyase
EC 4.4.1.6 *S*-alkylcysteine lyase
EC 4.4.1.7 deleted, included in EC 2.5.1.18
EC 4.4.1.8 cystathionine b-lyase
EC 4.4.1.9 L-3-cyanoalanine synthase
EC 4.4.1.10 cysteine lyase
EC 4 4.1.11 methionine g-lyase
EC 4.4.1.12 deleted
EC 4.4.1.13 cysteine-*S*-conjugate b-lyase
EC 4.4.1.14 1-aminocyclopropane-1-carboxylate synthase
EC 4.4.1.15 D-cysteine desulfhydrase
EC 4.4.1.16 selenocysteine lyase
EC 4.4.1.17 holocytochrome-*c* synthase
EC 4.4.1.18 now EC 1.8.3.5
EC 4.4.1.19 phosphosulfolactate synthase
EC 4.4.1.20 leukotriene-C_4 synthase
EC 4.5 Carbon-Halide Lyases
EC 4.5.1.1 DDT-dehydrochlorinase
EC 4.5.1.2 3-chloro-D-alanine dehydrochlorinase
EC 4.5.1.3 dichloromethane dehalogenase
EC 4.5.1.4 L-2-amino-4-chloropent-4-enoate dehydrochlorinase
EC 4.5.1.5 *S*-carboxymethylcysteine synthase
EC 4.6 Phosphorus-Oxygen Lyases
EC 4.6.1.1 adenylate cyclase
EC 4.6.1.2 guanylate cyclase
EC 4.6.1.3 now EC 4.2.3.4
EC 4.6.1.4 now EC 4.2.3.5
EC 4.6.1.5 now EC 4.2.3.7
EC 4.6.1.6 cytidylate cyclase
EC 4.6.1.7 now EC 4.2.3.8
EC 4.6.1.8 now EC 4.2.3.10
EC 4.6.1.9 now EC 4.2.3.11
EC 4.6.1.10 now EC 4.2.3.12
EC 4.6.1.11 now EC 4.2.3.13
EC 4.6.1.12 2-*C*-methyl-D-erythritol 2, 4-cyclodiphosphate synthase
EC 4.6.1.13 phosphatidylinositol diacylglycerol-lyase
EC 4.6.1.14 glycosylphosphatidylinositol diacylglycerol-lyase
EC 4.6.1.15 FAD-AMP lyase (cyclizing)
EC 4.99 Other Lyases
EC 4.99.1.1 ferrochelatase
EC 4.99.1.2 alkylmercury lyase
EC 4.99.1.3 sirohydrochlorin cobaltochelatase
EC 4.99.1.4 sirohydrochlorin ferrochelatase
EC 4.99.1.5 aliphatic aldoxime dehydratase
EC 4.99.1.6 indoleacetaldoxime dehydratase
EC 5. Isomerases
EC 5.1 Racemases and Epimerases
EC 5.1.1 Acting on Amino Acids and Derivatives
EC 5.1.1.1 alanine racemase
EC 5.1.1.2 methionine racemase
EC 5.1.1.3 glutamate racemase
EC 5.1.1.4 proline racemase
EC 5.1.1.5 lysine racemase
EC 5 1.1.6 threonine racemase
EC 5.1.1 7 diaminopimelate epimerase
EC 5.1.1.8 4-hydroxyproline epimerase
EC 5.1.1.9 arginine racemase
EC 5.1.1.10 amino-acid racemase
EC 5.1.1.11 phenylalanine racemase (ATP - hydrolysing)
EC 5.1.1.12 ornithine racemase
EC 5.1.1.13 aspartate racemase
EC 5.1.1.14 nocardicin-A epimerase
EC 5.1.1.15 2-aminohexano-6-lactam racemase
EC 5.1.1.16 protein-serine epimerase
EC 5.1.1.17 isopenicillin-N epimerase
EC 5.1.2 Acting on Hydroxy Acids and Derivatives
EC 5.1.2.1 lactate racemase
EC 5.1.2.2 mandelate racemase
EC 5.1.2.3 3-hydroxybutyryl-CoA epimerase
EC 5.1.2.4 acetoin racemase
EC 5.1.2.5 tartrate epimerase
EC 5.1.2.6 isocitrate epimerase
EC 5.1.3 Acting on Carbohydrates and Derivatives
EC 5.1.3.1 ribulose-phosphate 3-epimerase
EC 5.1.3.2 UDP-glucose 4-epimerase
EC 5.1.3.3 aldose 1-epimerase
EC 5.1.3.4 L-ribulose-phosphate 4-epimerase
EC 5.1.3.5 UDP-arabinose 4-epimerase

EC 5.1.3.6 UDP-glucuronate 4-epimerase
EC 5.1.3.7 UDP-*N*-acetylglucosamine 4-epimerase
EC 5.1.3 8 *N*-acylglucosamine 2-epimerase
EC 5.1.3.9 *N*-acylglucosamine-6-phosphate 2-epimerase
EC 5.1.3.10 CDP-abequose epimerase
EC 5.1.3.11 cellobiose epimerase
EC 5.1.3.12 UDP-glucuronate 5'-epimerase
EC 5.1.3.13 dTDP-4-dehydrorhamnose 3, 5-epimerase
EC 5.1.3.14 UDP-*N*-acetylglucosamine 2-epimerase
EC 5.1.3.15 glucose-6-phosphate 1-epimerase
EC 5.1.3.16 UDP-glucosamine 4-epimerase
EC 5.1.3.17 heparosan-*N*-sulfate-glucuronate 5-epimerase
EC 5.1.3.18 GDP-mannose 3,5-epimerase
EC 5.1.3.19 chondroitin-glucuronate 5-epimerase
EC 5.1.3.20 ADP-*glyceromanno*-heptose 6-epimerase
EC 5.1.3.21 maltose epimerase
EC 5.1.99 Acting on Other Compounds
EC 5.1.99.1 methylmalonyl-CoA epimerase
EC 5.1.99.2 16-hydroxysteroid epimerase
EC 5.1.99.3 allantoin racemase
EC 5.1.99.4 a-methylacyl-CoA racemase
EC 5.2 *cis-trans*-Isomerases
EC 5.2.1.1 maleate isomerase
EC 5.2.1.2 maleylacetoacetate isomerase
EC 5.2.1.3 retinal isomerase
EC 5.2.1.4 maleylpyruvate isomerase
EC 5.2.1.5 linoleate isomerase
EC 5.2.1.6 furylfuramide isomerase
EC 5.2.1.7 retinol isomerase
EC 5.2.1.8 peptidylprolyl isomerase
EC 5.2.1.9 farnesol 2-isomerase
EC 5.2.1.10 2-chloro-4-carboxymethylenebut-2-en-1,4-olide isomerase.
EC 5.2.1.11 4-hydroxyphenylacetaldehyde-oxime isomerase
EC 5.3 Intramolecular Oxidoreductases
EC 5.3.1 Interconverting Aldoses and Ketoses
EC 5.3.1.1 triose-phosphate isomerase
EC 5.3.1.2 deleted
EC 5.3.1.3 arabinose isomerase
EC 5.3.1.4 L-arabinose isomerase
EC 5.3.1.5 xylose isomerase
EC 5.3.1.6 ribose-5-phosphate isomerase
EC 5.3.1.7 mannose isomerase
EC 5.3.1.8 mannose-6-phosphate isomerase
EC 5.3.1.9 glucose-6-phosphate isomerase
EC 5.3.1.10 now EC 3.5.99.6
EC 5.3.1.11 deleted
EC 5.3.1.12 glucuronate isomerase
EC 5.3.1.13 arabinose-5-phosphate isomerase
EC 5.3.1.14 L-rhamnose isomerase
EC 5.3.1.15 D-lyxose ketol-isomerase
EC 5.3.1.16 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase
EC 5.3.1.17 4-deoxy-L-*threo*-5-hexosulose-uronate ketol-isomerase
EC 5.3.1.18 deleted
EC 5.3.1.19 now EC 2.6.1.16
EC 5.3.1.20 ribose isomerase
EC 5.3.1.21 corticosteroid side-chain-isomerase
EC 5.3.1.22 hydroxypyruvate isomerase
EC 5.3.1.23 5-methylthioribose-1-phosphate isomerase
EC 5.3.1.24 phosphoribosylanthranilate isomerase
EC 5.3.1.25 L-fucose isomerase
EC 5.3.1.26 galactose-6-phosphate isomerase
EC 5.3.2 Interconverting Keto- and Enol-Groups
EC 5.3.2.1 phenylpyruvate tautomerase
EC 5.3.2.2 oxaloacetate tautomerase
EC 5.3.3 Transposing C=C Bonds
EC 5.3.3.1 steroid D-isomerase
EC 5.3.3.2 isopentenyl-diphosphate D-isomerase
EC 5.3.3.3 vinylacetyl-CoA D-isomerase
EC 5.3.3.4 muconolactone D-isomerase
EC 5.3.3.5 cholestenol D-isomerase
EC 5.3.3.6 methylitaconate D-isomerase
EC 5.3.3.7 aconitate D-isomerase
EC 5.3.3.8 dodecenoyl-CoA D-isomerase
EC 5.3.3.9 prostaglandin-A_1 D-isomerase
EC 5.3.3.10 5-carboxymethyl-2-hydroxymuconate D-isomerase
EC 5.3.3.11 isopiperitenone D-isomerase
EC 5.3.3.12 dopachrome isomerase
EC 5.3.3.13 polyenoic fatty acid isomerase
EC 5.3.4 Transposing S-S Bonds
EC 5.3.4.1 protein disulfide-isomerase
EC 5.3.99 Other Intramolecular Oxidoreductases
EC 5.3.99.1 deleted
EC 5.3.99.2 prostaglandin-D synthase
EC 5.3.99.3 prostaglandin-E synthase

EC 5.3.99.4 prostaglandin-I synthase
EC 5.3.99.5 thromboxane-A synthase
EC 5.3 99.6 allene-oxide cyclase
EC 5.3.99.7 styrene-oxide isomerase
EC 5.4 Intramolecular Transferases
EC 5.4.1 Transferring Acyl Groups
EC 5.4.1.1 lysolecithin acylmutase
EC 5.4.1.2 precorrin-8X methylmutase
EC 5.4.2 Phosphotransferases (Phosphomutases)
EC 5.4.2.1 phosphoglycerate mutase
EC 5.4.2.2 phosphoglucomutase
EC 5.4.2.3 phosphoacetylglucosamine mutase
EC 5.4.2.4 bisphosphoglycerate mutase
EC 5.4.2.5 phosphoglucomutase (glucose-cofactor)
EC 5.4.2.6 b-phosphoglucomutase
EC 5.4.2.7 phosphopentomutase
EC 5.4.2.8 phosphomannomutase
EC 5.4.2.9 phospho*enol*pyruvate mutase
EC 5.4.2.10 phosphoglucosamine mutase
EC 5.4.3 Transferring Amino Groups
EC 5.4.3.1 deleted
EC 5.4.3.2 lysine 2,3-aminomutase
EC 5.4.3.3 b-lysine 5,6-aminomutase
EC 5.4.3.4 D-lysine 5,6-aminomutase
EC 5.4.3.5 D-ornithine 4,5-aminomutase
EC 5.4.3.6 tyrosine 2,3-aminomutase
EC 5.4.3.7 leucine 2,3-aminomutase
EC 5.4.3.8 glutamate-1-semialdehyde 2, 1-aminomutase
EC 5.4.4 Transferring hydroxy groups
EC 5.4.4.1 (hydroxyamino)benzene mutase
EC 5.4.4.2 isochorismate synthase
EC 5.4.4.3 3-(hydroxyamino)phenol mutase
EC 5.4.99 Transferring Other Groups
EC 5.4.99.1 methylaspartate mutase
EC 5.4.99.2 methylmalonyl-CoA mutase
EC 5.4.99.3 2-acetolactate mutase
EC 5.4.99.4 2-methyleneglutarate mutase
EC 5.4.99.5 chorismate mutase
EC 5.4.99.6 now EC 5.4.4.2
EC 5.4.99.7 lanosterol synthase
EC 5.4.99.8 cycloartenol synthase
EC 5.4.99.9 UDP-galactopyranose mutase
EC 5.4.99.10 deleted, included in EC 5.4.99.11
EC 5.4.99.11 isomaltulose synthase
EC 5.4.99.12 tRNA-pseudouridine synthase I
EC 5.4.99.13 isobutyryl-CoA mutase
EC 5.4.99.14 4-carboxymethyl-4-methylbutenolide mutase
EC 5.4.99.15 (14)-a-D-glucan 1-a-D-glucosylmutase
EC 5.4.99.16 maltose a-D-glucosyltransferase
EC 5.4.99.17 squalene—hopene cyclase
EC 5.5 Intramolecular Lyases
EC 5.5.1.1 muconate cycloisomerase
EC 5.5.1.2 3-carboxy-*cis,cis*-muconate cycloisomerase
EC 5.5.1.3 tetrahydroxypteridine cycloisomerase
EC 5.5.1.4 inositol-3-phosphate synthase
EC 5.5.1.5 carboxy-*cis,cis*-muconate cyclase
EC 5.5.1.6 chalcone isomerase
EC 5.5.1.7 chloromuconate cycloisomerase
EC 5.5.1.8 geranyl-diphosphate cyclase
EC 5.5.1.9 cycloeucalenol cycloisomerase
EC 5.5.1.10 a-pinene-oxide decyclase
EC 5.5.1.11 dichloromuconate cycloisomerase
EC 5.5.1.12 copalyl diphosphate synthase
EC 5.5.1.13 *ent*-copalyl diphosphate synthase
EC 5.99 Other Isomerases
EC 5.99.1.1 thiocyanate isomerase
EC 5.99.1.2 DNA topoisomerase
EC 5.99.1.3 DNA topoisomerase (ATP-hydrolysing)
EC 6. Ligases
EC 6.1 Forming Carbon-Oxygen Bonds
EC 6.1.1 Ligases Forming Aminoacyl-tRNA and Related Compounds
EC 6.1.1.1 tyrosine—tRNA ligase
EC 6.1.1.2 tryptophan—tRNA ligase
EC 6.1.1.3 threonine—tRNA ligase
EC 6.1.1.4 leucine—tRNA ligase
EC 6.1.1.5 isoleucine—tRNA ligase
EC 6.1.1.6 lysine—tRNA ligase
EC 6.1.1.7 alanine—tRNA ligase
EC 6.1.1.8 deleted
EC 6.1.1.9 valine—tRNA ligase
EC 6.1.1.10 methionine—tRNA ligase
EC 6.1.1.11 serine—tRNA ligase
EC 6.1.1.12 aspartate—tRNA ligase
EC 6.1.1.13 D-alanine-poly(phosphoribitol) ligase
EC 6.1.1.14 glycine—tRNA ligase
EC 6.1.1.15 proline—tRNA ligase
EC 6.1.1.16 cysteine—tRNA ligase
EC 6.1.1.17 glutamate—tRNA ligase
EC 6.1.1.18 glutamine—tRNA ligase
EC 6.1.1.19 arginine—tRNA ligase

EC 6.1.1.20 phenylalanine—tRNA ligase
EC 6.1.1.21 histidine—tRNA ligase
EC 6.1.1.22 asparagine—tRNA ligase
EC 6.1.1.23 aspartate—$tRNA^{Asn}$ ligase
EC 6.1.1.24 glutamate—$tRNA^{Gln}$ ligase
EC 6.1.1.25 lysine—$tRNA^{Pyl}$ ligase
EC 6.2 Forming Carbon-Sulfur Bonds
EC 6.2.1 Acid-Thiol Ligases
EC 6.2.1.1 acetate—CoA ligase
EC 6.2.1.2 butyrate—CoA ligase
EC 6.2.1.3 long-chain-fatty-acid—CoA ligase
EC 6.2.1.4 succinate—CoA ligase (GDP-forming)
EC 6.2.1.5 succinate—CoA ligase (ADP-forming)
EC 6.2.1.6 glutarate—CoA ligase
EC 6.2.1.7 cholate—CoA ligase
EC 6.2.1.8 oxalate—CoA ligase
EC 6.2.1.9 malate—CoA ligase
EC 6.2.1.10 acid—CoA ligase (GDP-forming)
EC 6.2.1.11 biotin—CoA ligase
EC 6.2.1.12 4-coumarate—CoA ligase
EC 6.2.1.13 acetate—CoA ligase (ADP-forming)
EC 6.2.1.14 6-carboxyhexanoate—CoA ligase
EC 6.2.1.15 arachidonate—CoA ligase
EC 6.2.1.16 acetoacetate—CoA ligase
EC 6.2.1.17 propionate—CoA ligase
EC 6.2.1.18 citrate—CoA ligase
EC 6.2.1.19 long-chain-fatty-acid—luciferin-component ligase
EC 6.2.1.20 long-chain-fatty-acid—[acyl-carrier-protein] ligase
EC 6.2.1.21 covered by EC 6.2.1.30
EC 6.2.1.22 [citrate (*pro*-3*S*)-lyase] ligase
EC 6.2.1.23 dicarboxylate—CoA ligase
EC 6.2.1.24 phytanate—CoA ligase
EC 6.2.1.25 benzoate—CoA ligase
EC 6.2.1.26 *o*-succinylbenzoate—CoA ligase
EC 6.2.1.27 4-hydroxybenzoate—CoA ligase
EC 6.2.1.28 3a,7a-dihydroxy-5b-cholestanate—CoA ligase
EC 6.2.1.29 3a,7a,12a-trihydroxy-5b-cholestanate—CoA ligase
EC 6.2.1.30 phenylacetate—CoA ligase
EC 6.2.1.31 2-furoate—CoA ligase
EC 6.2.1.32 anthranilate—CoA ligase
EC 6.2.1.33 4-chlorobenzoate—CoA ligase
EC 6.2.1.34 *trans*-feruloyl-CoA synthase
EC 6.3 Forming Carbon-Nitrogen Bonds
EC 6.3.1 Acid-Ammonia (or Amine) Ligases (Amide Synthases)
EC 6.3.1.1 aspartate—ammonia ligase
EC 6.3.1.2 glutamate—ammonia ligase
EC 6.3.1.3 now EC 6.3.4.13
EC 6.3.1.4 aspartate—ammonia ligase (ADP-forming)
EC 6.3.1.5 NAD^+ synthase
EC 6 3.1.6 glutamate—ethylamine ligase
EC 6.3.1.7 4-methyleneglutamate—ammonia ligase
EC 6.3.1.8 glutathionylspermidine synthase
EC 6.3.1.9 trypanothione synthase
EC 6.3.1.10 adenosylcobinamide-phosphate synthase
EC 6.3 2 Acid—Amino-Acid Ligases (Peptide Synthases)
EC 6.3.2.1 pantoate—b-alanine ligase
EC 6.3.2.2 glutamate—cysteine ligase
EC 6.3.2.3 glutathione synthase
EC 6.3.2.4 D-alanine—D-alanine ligase
EC 6.3.2.5 phosphopantothenate—cysteine ligase
EC 6.3.2.6 phosphoribosylaminoimidazolesuccinocarboxamide synthase
EC 6.3.2.7 UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate—L-lysine ligase
EC 6.3.2.8 UDP-*N*-acetylmuramate—L-alanine ligase
EC 6.3.2.9 UDP-*N*-acetylmuramoylalanine—D-glutamate ligase
EC 6.3.2.10 UDP-*N*-acetylmuramoylalanyl-tripeptide—D-alanyl-D-alanine ligase
EC 6.3.2.11 carnosine synthase
EC 6.3.2.12 dihydrofolate synthase
EC 6.3.2.13 UDP-*N*-acetylmuramoylalanyl-D-glutamate—2,6-diamino-pimelate ligase
EC 6.3.2.14 2,3-dihydroxybenzoate—serine ligase
EC 6.3.2.15 deleted, due to EC 6 3.2.10
EC 6.3.2.16 D-alanine—alanyl- poly (glycerolphosphate) ligase
EC 6.3.2.17 tetrahydrofolylpolyglutamate synthase
EC 6.3.2.18 g-glutamylhistamine synthase
EC 6.3.2.19 ubiquitin—protein ligase
EC 6.3.2.20 indoleacetate—lysine synthetase
EC 6.3.2.21 ubiquitin—calmodulin ligase
EC 6.3.2.22 diphthine—ammonia ligase
EC 6.3.2.23 homoglutathione synthase

EC 6.3.2.24 tyrosine—arginine ligase
EC 6.3.2.25 tubulin—tyrosine ligase
EC 6.3.2.26 *N*-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase
EC 6.3.2.27 aerobactin synthase
EC 6.3.3 Cyclo-Ligases
EC 6.3.3.1 phosphoribosylformylglycinamidine cyclo-ligase
EC 6.3.3.2 5-formyltetrahydrofolate cyclo-ligase
EC 6.3.3.3 dethiobiotin synthase
EC 6.3.3.4 (carboxyethyl)arginine b-lactam-synthase
EC 6.3.4 Other Carbon-Nitrogen Ligases
EC 6.3.4.1 GMP synthase
EC 6.3.4.2 CTP synthase
EC 6.3.4.3 formate—tetrahydrofolate ligase
EC 6.3.4.4 adenylosuccinate synthase
EC 6.3.4.5 argininosuccinate synthase
EC 6.3.4.6 urea carboxylase
EC 6.3.4.7 ribose-5-phosphate—ammonia ligase
EC 6.3.4.8 imidazoleacetate—phosphoribosyldiphosphate ligase
EC 6.3.4.9 biotin—[methylmalonyl-CoA-carboxytransferase] ligase
EC 6.3.4.10 biotin—[propionyl-CoA-carboxylase (ATP-hydrolysing)] ligase
EC 6.3.4.11 biotin—[methylcrotonoyl-CoA-carboxylase] ligase
EC 6.3.4.12 glutamate—methylamine ligase
EC 6.3.4.13 phosphoribosylamine—glycine ligase
EC 6.3.4.14 biotin carboxylase
EC 6.3.4.15 biotin—[acetyl-CoA-carboxylase] ligase
EC 6.3.4.16 carbamoyl-phosphate synthase (ammonia)
EC 6.3.4.17 formate—dihydrofolate ligase
EC 6.3.5 Carbon-Nitrogen Ligases with Glutamine as Amido-N-Donor
EC 6.3.5.1 NAD^+ synthase (glutamine-hydrolysing)
EC 6.3.5.2 GMP synthase (glutamine-hydrolysing)
EC 6.3.5.3 phosphoribosylformylglycinamidine synthase
EC 6.3.5.4 asparagine synthase (glutamine-hydrolysing)
EC 6.3.5.5 carbamoyl-phosphate synthase (glutamine-hydrolysing)
EC 6.3.5.6 asparaginyl-tRNA synthase (glutamine-hydrolysing)
EC 6.3.5.7 glutaminyl-tRNA synthase (glutamine-hydrolysing)
EC 6.3.5.8 aminodeoxychorismate synthase
EC 6.3.5.9 hydrogenobyrinic acid *a,c*-diamide synthase (glutamine-hydrolysing)
EC 6.3.5.10 adenosylcobyric acid synthase (glutamine-hydrolysing)
EC 6.4 Forming Carbon-Carbon Bonds
EC 6.4.1.1 pyruvate carboxylase
EC 6.4.1.2 acetyl-CoA carboxylase
EC 6.4.1.3 propionyl-CoA carboxylase
EC 6.4.1.4 methylcrotonoyl-CoA carboxylase
EC 6.4.1.5 geranoyl-CoA carboxylase
EC 6.4.1.6 acetone carboxylase
EC 6.5 Forming Phosphoric Ester Bonds
EC 6.5.1.1 DNA ligase (ATP)
EC 6.5.1.2 DNA ligase (NAD^+)
EC 6.5.1.3 RNA ligase (ATP)
EC 6.5.1.4 RNA-3'-phosphate cyclase
EC 6.6 Forming Nitrogen—Metal Bonds
EC 6.6.1 Forming Coordination Complexes
EC 6.6.1.1 magnesium chelatase
EC 6.6.1.2 cobaltochelatase

The list of enzymes given above is specifically for the reference of students only and is as it appears on the internet. Readers interested in further details can visit

http://www.chem.qmul.ac.uk/iubmb/enzyme

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Appendix - II

List of books recommended for further reading:

1. Enzymes, **3rd edition: Dixon and Webb, Longman group, London**
2. Biochemistry, **2nd edition, Albert Lehninger, Kalyani publishers**
3. Biotechnology **revised edition 2001: V. Kumaresan, Saras publications, Kanyakumari**
4. Genes V, **1994: Benjamin Lewin, Oxford university press**
5. Fundamentals of enzymology, **reprint edition 1984, Nicholas Price, Lewis Stevens, Oxford University Press**
6. Principles of Biochemistry, **6th edition: White, Handler, Smith, Hill, Lehman, McGraw Hill**
7. Biophysical Chemistry: Principles and Techniques, **3rd revised edition Nath, Upadhyay, Upadhyay, Himalaya Publishing House**
8. Outlines of Biochemistry,**5th edition: Conn, Stumpf, Buening, Doi, John Wiley and Sons Inc.**
9. Genetic Engineering: Principles and practice, **reprint 2004: Sandhya Mitra, MacMillan India,**
10. Textbook of Biochemistry and Human Biology,**3rd edition: Talwar, Srivastava, Prentice –Hall India.**
11. Genetics Vol I, **1st edition 2003: Powar.C.B. Himalaya Publishing House**
12. Genetics Vol II, **1st edition 2003: Powar.C.B. Himalaya Publishing House**
13. Microbiology, **5th edition: Pelczar, Chan, Krieg, McGraw Hill.**
14. Textbook of Biochemistry,**4th edition,: West, Todd, Mason, VanBruggen, Oxford and IBH**
15. Fundamentals of Biochemistry,**18th edition, Deb.A.C., New Central Book Agency**
16. Biochemistry, **8th edition, Debajyoti Das, Academic Publishers**
17. Fundamentals of Biochemistry,**5th revised edition, Jain.J.L, S.Chand and Company.**
18. General Microbiology Vol I, **Powar , Daginawala, Himalaya Publishing House**
19. General Microbiology Vol II, **Powar, Daginawala, Himalaya Publishing House**
20. Enzymes: A practical introduction to Structure,Mechanism and Data Analysis,**1996, Robert.A.Copeland, VCH publishers Inc.**
21. A Textbook of Biotechnology,**1st edition 1993, R.C.Dubey, SChand and Company**
22. Advances in Biotechnology,**3rd edition reprint 2003, S.N.Jogdand, Himalaya Publishing House**
23. A Textbook of Biotechnology, **reprint 1994, H.D.Kumar, Affiliated East-West Press**
24. Principles of Biochemistry, **Lehninger, Nelson, Cox.**
25. Clinical Diagnosis and Management,by Laboratory methods **7th edition, John Bernard Henry, W.B.Saunders Company Philadelphia.**